Identification by modification-interference of purine N-7 and ribose 2’-OH groups critical for catalysis by bacterial ribonuclease P

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ABSTRACT

The RNA subunit of bacterial ribonuclease P is a catalytic RNA that cleaves precursor tRNAs to generate mature tRNA 5’ ends. A self-cleaving RNase P RNA–substrate conjugate was used in modification-interference analysis to identify purine N-7 and ribose 2’-hydroxyl functional groups that are critical to catalysis. We identify six adenine N-7 groups and only one 2’-hydroxyl that, when substituted with 7-deazaadenine or 2’-deoxy analogues, respectively, reduce the RNase P catalytic rate approximately 10-fold at pH 8 and limiting concentration of magnesium. Two sites of low-level interference by phosphorothioate modification were detected in addition to the four sites of strong interference documented previously. These modification-interference results, the absolute phylogenetic conservation of these functional groups in bacterial RNase P RNA, their proximity to the substrate-phosphate in the tertiary structure of the ribozyme–substrate complex, and the importance of some of the sites for binding of catalytic magnesium all implicate these functional groups as components of the RNase P active site. Five of the 7-deazaadenine interferences are suppressed at pH 6, where the hydrolytic step is rate-limiting, or at saturating concentrations of magnesium. We propose, therefore, that these base functional groups are specifically engaged in the catalytic center of RNase P RNA, possibly by involvement in magnesium-dependent folding. One 7-deazaadenine interference and one 2’-deoxy-interference, although partially suppressed at pH 6, are not suppressed at saturating magnesium concentrations. This implicates these groups in magnesium-independent folding of the catalytic substructure of the ribozyme.

Keywords: phosphorothioate; nucleoside triphosphate analogues; ribozyme; RNA structure; tRNA processing

INTRODUCTION

Ribonuclease P is the ubiquitous endonuclease that catalyzes the hydrolysis of a specific phosphodiester bond in pre-tRNA to release the 5’ precursor sequence and produce the mature 5’ end of tRNA (Gopalan et al., 1994; Pace & Brown, 1995; Harris et al., 1998 for reviews). All species of cellular RNase P are ribonucleoproteins composed of one RNA molecule and at least one protein component. In the case of the bacterial RNase P, the RNA component alone is capable of processing pre-tRNA in vitro (Guerrier-Takada et al., 1983). Thus, bacterial RNase P RNA is a ribozyme; it is the only known natural RNA enzyme that catalyzes multiple-turnover reactions. The secondary structure of bacterial RNase P RNA has been determined by phylogenetic-comparative analysis (Pace & Brown, 1995), and increasingly reliable structure models of the ribozyme–substrate complex have been proposed (Harris et al., 1994, 1997; Westhof & Altman, 1994; Chen et al., 1998). There is relatively little information, however, on the identity and arrangement of the functional groups that constitute the active site of RNase P RNA.

Modification-interference has proven to be an effective method to identify functional groups important for the activity of large RNAs (Conway & Wickens, 1989; Cate et al., 1997). This approach relies on the modification, in a random manner, of particular functional groups in a pool of RNA, for instance, by incorporation of nucleotide analogues. A selection step is then applied to the pool of modified RNAs to separate molecules impaired in some property, for instance, substrate-binding or catalysis, from those unaffected by modification. Comparison of modified sites in reactive and unreacted populations identifies residues at which the modification of the functional group is detrimental to
the reaction. Modification-interference experiments with phosphorothioates and 2'-deoxy phosphorothioates have been used to locate pro-Rp phosphate oxygens and 2'-hydroxy groups in RNase P that, when modified, interfere with substrate binding (Hardt et al., 1995, 1996).

Modification-interference relies on the separation of fully active molecules from less active ones, which restricts its application to cis-acting ribozymes. RNase P RNA normally acts in trans and undergoes no detectable change upon reaction, so the native reaction is not subject to modification-interference experiments that address activity. Separation of active and less-active RNase P RNAs can be achieved, however, by the use of self-cleaving conjugates of pre-tRNA tethered to RNase P RNA so that the substrate phosphodiester is positioned at the active site. Such conjugates can undergo rapid and precise self-cleavage in vitro with rates similar to the single-turnover rate of the native RNase P RNA reaction (Frank et al., 1994; Oh, 1996). The results of phosphorothioate modification-interference experiments with an RNase P RNA–pre-tRNA conjugate have revealed four pro-Rp phosphate oxygens in the ribozyme that, upon substitution with sulfur, interfere with catalysis (Harris & Pace, 1995).

Other functional groups that could be important for catalysis by RNase P are purine N-7 and 2'-ribose hydroxy-groups. Apart from their involvement in a variety of noncanonical hydrogen bonding interactions crucial for RNA structural stability (Quigley & Rich, 1976; Jucker & Pardi, 1995; Cate et al., 1996), purine N-7 and 2'-hydroxy groups have been implicated in specific magnesium binding (Jack et al., 1977; Scott et al., 1995; Cate & Doudna, 1996). Unlike nonbridging phosphorothioates, which can be cleaved by oxidation (Gish & Eckstein, 1988), base and ribose modifications incorporated into RNA are not readily detectable. If, however, nucleotide analogues bearing base and ribose modifications also are tagged with phosphorothioates, their distribution can be monitored (Conrad et al., 1995; Strobel & Shetty, 1997).

We report here the use of phosphorothioate analogues of 7-deazapurine- and 2'-deoxy-containing nucleotides in modification-interference experiments to locate purine N-7 and ribose 2'-hydroxyl groups that are critical for catalysis by bacterial RNase P RNA. 7-Deazaadenosine and 7-deazaguanosine nucleotide analogues are isosteric to the corresponding purine nucleotides in the sense that they retain the geometry of the bases and the arrangement of exocyclic functional groups, but they lack the hydrogen bonding and metal-coordinating properties of the purine 7-position due to the replacement of nitrogen by carbon. 2'-Deoxynucleotide analogues lack those properties at the 2' position of ribose due to deoxy-substitution. We identify six adenine N-7 groups and one 2'-hydroxyl group which, upon modification, interfere with the action of RNase P. These results and other properties of these functional groups implicate them as components of the RNase P active site.

**RESULTS**

**Incorporation of nucleotide analogues into a cis-acting RNase P**

In order to separate active RNA species from those rendered less active by modification, we exploited the ability of the RNase P-pre-tRNA conjugate PT332(−5) (Fig. 1) to undergo precise and efficient self-cleavage in vitro (Frank et al., 1994; Oh, 1996). Cleavage of the backbone at the RNase P processing site releases mature tRNA from the conjugate RNA, so fully active and less active populations of the ribozyme can be sorted on the basis of size (Harris & Pace, 1995). PT332(−5) differs from the previously used conjugate PT332 in that the linker that separates the ribozyme from the substrate is shortened from 14 to 5 nt, resulting in somewhat improved catalytic properties (Oh, 1996), possibly by reducing structural distortions at the site of tethering. We find that the self-cleavage rate of PT332(−5) under optimal conditions is approximately the same as the single-turnover rate of Escherichia coli RNase P RNA (Smith & Pace, 1993 and data not shown).

In order to incorporate 7-deazapurine and 2'-deoxy modifications randomly, PT332(−5) RNA was prepared by transcription in vitro in the presence of 5'-O-(1-thiotriphosphate) analogues (NTP(αS)) of the corresponding modified nucleosides. 7-Deazapurine nucleotide analogues were synthesized as described in Materials and Methods; 2'-dTTP(αS) was used instead of the commercially unavailable 2'-deoxyuridine analogue to introduce 2'-deoxy modifications at uridine nucleotides of PT332(−5). Analogues were incorporated as phosphorothioates to render the RNA backbone labile to iodine-cleavage at the site of incorporation of the analogues. Ribonucleoside phosphorothioates and their 7-deaza-analogues were used as pure Sp-stereoisomers, whereas 2'-deoxynucleoside phosphorothioates were used as commercially available mixtures of Sp and Rp-stereoisomers. Enzymatic incorporation of Sp-stereoisomers of modified nucleotides into RNA during transcription results in inversion of the configuration at the α-phosphorous atom, thus resulting in Rp-phosphorothioate substitution at the 5' side of the modified nucleotides (Eckstein, 1985). Rp-stereoisomers of phosphorothioates neither are enzymatically incorporated into RNA, nor noticeably inhibit incorporation of corresponding Sp-phosphorothioates (Griffiths et al., 1987). Because excessive incorporation of any modifications into functional RNA could result in a loss of activity due to the cooperative action of nonspecific effects, we optimized the nucleotide concentrations in transcription reactions so as to yield, on average, five
modified nucleotides per molecule (see Materials and Methods for details). Such extent of modification (approximately 5%) results in good signal-to-noise ratio upon autoradiography of gel-resolved, end-labeled products of iodine-cleaved, phosphorothioate-modified RNAs. The cooperative contribution of nonspecific effects at this level of modification is negligible (Christian & Yarus, 1992; Hardt et al., 1996; Strobel & Shetty, 1997).

Cleavage-interference experiments

RNase P RNA requires multiple magnesium ions for optimal activity (Smith & Pace, 1993; Beebe et al., 1996). Additionally, the pH dependence of the cleavage step suggests that hydroxide ion, thought to be magnesium-coordinated, acts as the nucleophile in an in-line attack on the scissile phosphodiester bond (Guerrier-Takada...
et al., 1986; Smith & Pace, 1993). In an attempt to resolve functional groups that might be critical for either the binding of catalytic magnesium or the hydrolytic step, modification-interference experiments were performed at different concentrations of magnesium and at different pH values.

Several modes of binding of catalytic magnesium ions by RNase P RNA have been reported (Smith & Pace, 1993; Beebe et al., 1996), with dissociation constants ranging from micromolar to millimolar. Because modification of functional groups involved in binding of catalytic magnesium is expected to have the most significant effect on ribozyme activity under low, unsaturating, concentrations of Mg2+, we performed a series of modification-interference experiments at a low (2.5 mM) concentration of magnesium chloride (optimal 10–15 mM at saturating monovalent salt concentration) and cleavage products were resolved by electrophoresis through a preparative polyacrylamide gel. Precursor and product RNAs were eluted from the gel, cleaved with iodine at sites of modification (Harris & Pace, 1995), and cleavage products were resolved by electrophoresis through denaturing polyacrylamide sequencing gels. Sites of cleavage were identified by comparison to RNA sequencing ladders.

We find that 2′-dA, 2′-dT, 2′-dG, and C-7G phosphorothioate modifications interfere only at sites that previously were shown to exhibit strong phosphorothioate-interference (A67, G68, U69, and A352; Harris & Pace, 1995). Because the effects of phosphorothioate modification at these positions are strong (rate reduction 50–200 times even at high concentrations of magnesium), we are unable to assess the importance of the purine N-7 and ribose 2′-OH groups of these nucleotides for catalysis.

New sites of interference, however, were observed in the case of 2′-dC and C-7A phosphorothioate modifications. Apart from the previously described sites of strong interference by phosphorothioate, 7-deazadenine phosphorothioate modifications of A62, A65, A66, A249, A334, and A351, and 2′-deoxy phosphorothioate modifications of C70, C71, and C353 interfere with PT332(−5) self-cleavage (Fig. 2). Kinetics of self-cleavage were first-order during the incubation period (Harris & Pace, 1995 and data not shown), so we could estimate from band-intensities in gels that each of the modifications reduces the rate of self-cleavage of the ribozyme roughly 10-fold. Because phosphorothioate modification per se at A62, A65, A66, A249, A334, or A351 does not interfere with activity of the ribozyme, interference can be ascribed to the 7-deazaadenine-modification of these nucleotides (Fig. 2A,C,D). Similarly, the interference at C353 apparently results from modification by 2′-deoxy (Fig. 2E), rather than the phosphorothioate substitution. The interferences at C70 and C71, in contrast, result from modification by phosphorothioate only, because the magnitudes of interference are approximately the same for both phosphorothioate and double modifications (Fig. 2B). Surprisingly, we are unable to detect any interference by 7-deazaguanosine (see Discussion).

**Rescue of interferences by magnesium**

If the observed interferences were caused by modification of a functional group involved in binding of catalytically important magnesium, then increase in magnesium concentration would be expected to suppress the effect of the modification. We find that interferences by 7-deazaadenine at A62, A66, A249, A334, and A351 are almost completely rescued upon increasing magnesium chloride concentration from 2.5 to 20 mM (Fig. 2), whereas the weak interferences by phosphorothioate modifications at C70 and C71 are suppressed only partially (not shown). The strong interferences by phosphorothioate at A67, A352, by 7-deazaadenine at A65, and by 2′-deoxy at C353 are not suppressed by the elevated concentration of magnesium chloride (Fig. 2), so the interactions in which these functional groups participate are likely to be structural in nature.

**Effects of pH on interferences**

Reduction of pH from 8.0 to 6.0 reduces the rate of the RNase P hydrolytic step 100-fold and thus renders the hydrolytic step rate-limiting when the substrate is bound to the ribozyme (Smith & Pace, 1993). In an attempt to identify purine N-7 and ribose 2′-hydroxy groups that might be important in the true chemical step of the reaction, we therefore performed modification-interference experiments at pH 6.0. We find that reduction of pH from 8.0 to 6.0 rescues interferences by 7-deazaadenine at A62, A65, A66, A249, A334, and A351. The interference by 2′-deoxy at C353 also is partially rescued (Fig. 2E). On the other hand, interferences by phosphorothioate at A67, G68, U69, C70, C71, and A352 are not suppressed at lower pH. No novel interferences were observed at pH 6.0 in the case of modifications by 2′-dT, 2′-dG, or C-7G phosphorothioate. Although these findings confirm that lowering of pH changes the rate-limiting step of PT332(−5) self-cleavage (presumably to the hydrolytic step), they suggest that no ribose 2′-OH or purine N-7 in RNase P RNA is directly involved in the catalytic step of the reaction.

**DISCUSSION**

The modification of a particular chemical group in a ribozyme could interfere with several different func-
tional aspects of the RNA, such as, general folding, binding of substrate or metal cofactor, folding of the active site, or stabilization of the transition versus ground states of the reaction. Use of a tethered construct such as PT332(−5), in which the substrate pre-tRNA is covalently attached near the active site of RNase P RNA, presumably bypasses modifications that are detrimental to the binding of the substrate (Harris & Pace, 1995). Hence, modification-interference experiments employing tethered RNAs are expected to monitor steps in the catalytic pathway subsequent to substrate binding, including the hydrolytic step.

The magnitudes of the observed interferences by 7-deazaadenine and 2′-deoxy, as well as the interferences by Rp-phosphorothioate at C70 and C71 (approximately 10-fold rate-reduction), correspond to free energy losses of approximately 1 kcal/mol at 50°C, consistent with the loss of a single hydrogen bond with influence on the function of the ribozyme. Structural distortions caused by phosphorothioate modification, change of sugar pucker (2′-deoxy modifications), or alteration of the base-stacking pattern (7-deaza modifications) also could contribute to the observed interferences. The Rp-phosphorothioate modifications at C70 and C71 have significantly less effect on the activity of the ribozyme than the previously reported phosphorothioate modifications at A67, G68, U69, and A352 (ca. 5-fold versus 50–200-fold). These relatively small effects suggest that pro-Rp phosphate oxygens of C70 and C71 are not directly involved in coordination of catalytically important magnesium ions (Harris & Pace, 1995), or alternatively that the free energy contribution to catalysis by these sites is negligible. We find, however, that these interferences tend to be rescued by increase in the concentration of magnesium, suggesting that the Rp-phosphorothioate modifications at C70 and C71 interfere with the binding of catalytic magnesium. Replacement of oxygen by sulfur could affect the binding of magnesium by, for instance, changing the strength of a putative hydrogen bond that could contribute to the structure of the magnesium-binding pocket, or by distortion of the catalytic structure due to the larger size of the sulfur atom. Of course, it is possible that the enhancement of activity by magnesium is due to global stabilization of the bulk structure by the divalent cation.

Increase in concentration of the magnesium cofactor also suppresses interferences by 7-deazaadenine at residues A62, A66, A249, A334, and A351, indicating that the N-7 groups of these nucleotides may participate in the binding of catalytically important magnesium. Magnesium ions are not known to coordinate directly to pyrimidic nitrogens of purines, the more probable mode being outer-sphere coordination through magnesium-bound water (Jack et al., 1977; Cate & Doudna, 1996). Alternatively, these adenine N-7 atoms could interact with other functional groups in the active site and, thereby, contribute to the magnesium-binding pocket. Because RNase P absolutely requires magnesium, the folding of the active site into the catalytically competent conformation is likely to depend upon the presence of magnesium ions. We propose, therefore, that the N-7 atoms of A62, A66, A249, A334, and A351 and the pro-Rp phosphate oxygens of C70 and C71 all participate in magnesium-dependent folding of the active site (Harris & Pace, 1995; Harris et al., 1998). Suppression of the interference at these residues by low pH supports this conclusion by ruling out the participation of these modification-sensitive groups in the hydrolytic step. The observation that these interferences are rescued by low pH also provides additional evidence that the rate-limiting step of the reaction at pH 6.0 is different from that at pH 8.0. Although the overall effects of the 7-deaza substitution at A65 and 2′-deoxy at C353 are small compared to strong phosphorothioate-effects (ca. 10-fold versus 50–200-fold rate-reduction), the insensitivity of their interferences to concentration of magnesium suggests that these residues participate in magnesium-independent folding of the active site.

Available structural, functional, and phylogenetic-comparative information are all consistent with the involvement of the implicated residues in the active site of RNase P. All of the modification-sensitive sites that we observe are absolutely conserved among bacteria (Fig. 3), consistent with their importance to the structure or function of the ribozyme. The identity of the nucleotide is not conserved in two cases, A67 and C353; however, the modification-sensitive parts of the backbone (phosphate or 2′-hydroxy) are still universally present. Most of the modification-sensitive sites are located in helix P4 and its flanking nonhelical sequences, the portion of this ribozyme that is thought to comprise much of the active site (Harris et al., 1998). Additionally, as shown in Figure 4, all the regions containing modification-sensitive sites are close to each other and to the 5′ end of the mature tRNA in the low-resolution structure of the enzyme–substrate complex (Harris et al., 1994, 1997; Chen et al., 1998). Moreover, in vitro selection experiments have identified the base C70 as critical for catalytic metal specificity of bacterial RNase P RNA (Frank & Pace, 1998), and the pro-Rp phosphate oxygen of A67 has been implicated in direct coordination of catalytic magnesium ion (Harris & Pace, 1995). Thus, the functional groups that are susceptible to modification-interference in catalysis are likely to be constituents of the active site of RNase P.

Although we are unable to detect any modification-interference by 7-deazaguanine, the nature of the experiments does not allow us to assess the possible importance of N-7 functional groups at G68 and G332 for catalysis. In these experiments, G332 has a native guanine residue due to guanosine-priming of the transcript RNAs (Materials and Methods) and G68 exhibits...
a strong interference due to the phosphorothioate modification that could obscure any effect of the guanine 7-deaza modification. Both of these nucleotides are absolutely conserved among bacteria, emphasizing their importance for the function of the ribozyme. Moreover, a photoagent attached to the 5′ end of the product tRNA crosslinks with high efficiency to G332 (Burgin & Pace, 1990), revealing a juxtaposition of G332 to the substrate phosphate in the RNase P–tRNA complex. Thus, G68 and G332 remain interesting targets for modification-interference experiments directed at the N-7 group.

It is noteworthy that most of the modification-sensitive functional groups that we identify in this study of a bacterial ribozyme also are constituents of the eucaryotic and archaeal RNase P RNAs (Chen & Pace, 1997).
These universally conserved elements include the N-7 atoms of A65, A66, A334, and purine-351; and backbone elements of nt 67–71, 352, and 353. Such profound conservation of these functional groups indicates that the eucaryal and archaeal RNase P RNAs, although inactive without protein components, still remain the catalytic centers of the corresponding holoenzymes.

**MATERIALS AND METHODS**

**General**

Except where noted, reagents were obtained commercially and used without further purification. Dimethylformamide (DMF) was distilled over CaH₂ under reduced pressure and stored over 4A molecular sieves. Pyridine was distilled over P₂O₅ and stored over 4A molecular sieves. Tri-n-butylamine was distilled twice over NaOH under reduced pressure. Triethylphosphite was distilled over CaH₂ under reduced pressure and stored over 4A molecular sieves. Thiophosphoryl chloride (PSCl₃) was freshly distilled before every use in thiophosphorylation. Anhydrous of bis(tri-n-butylammonium)-pyrophosphate in DMF was prepared as described elsewhere (Ludwig & Eckstein, 1989). 7-Deazaadenosine (Tubercidin) was purchased from Sigma. 7-Deazaguanosine was synthesized according to Seela et al. (1990) from commercially available 7-deazaguanine (Sigma) and 2,3-O-isopropylidened-D-ribo-1,4-lactone (ICN Biomedicals). Satisfactory TLC, UV, and ¹H NMR data were obtained for every intermediate compound. UV and ¹H NMR spectra of the product nucleoside were identical to that of β-anomer of 7-deazaguanosine (Seela et al., 1990). 2′-Deoxy and ribonucleotide-5′-O-(1-thiotriphosphates) (G, A, T, and C) and all radioactive nucleotides were purchased from Amersham.

³¹P NMR spectra (161.978 MHz) were recorded using a Bruker AM 400 spectrometer. For proton decoupling, a WALTZ-16 CPD pulse-sequence was applied. Sodium phosphate, pH 7.0, was added to the samples to 30 mM concentration as an internal reference. UV spectra were recorded using a Shimadzu Biospec-1601 spectrophotometer.

**Synthesis of nucleoside-5′-O-(1-thiotriphosphates)**

Phosphorylations were performed as generally described by Bogachev (1987) as follows: 30 μmol of nucleoside (7-
deazaadenosine or 7-deazaguanosine) was evaporated once with anhydrous pyridine (1 mL) and once with anhydrous DMF (0.5 mL) at room temperature; evaporation then was continued in vacuo (200 mtorr) at 90°C. After 1 h, the reaction flask was filled with argon and sealed with a rubber septum. During the following operations, low positive pressure of argon was maintained by connecting the flask to an argon-containing tank. To the dry nucleoside, 300 μL of triethylphosphate was injected, and the flask was heated at 90°C until the nucleoside was dissolved completely. The solution was then cooled to 0°C with stirring, and 100 μL of 0.66 M anhydrous pyridine (66 μmol) in triethylphosphate was injected, followed by addition of 50 μL of 0.66 M PSCl3 (33 μmol) in the same solvent. After stirring for 30 min (7-deazaadenosine) or 2 h (7-deazaguanosine) at 0°C, a mixture of 24 μL of tri-n-butylamine (166 μmol) and 500 μL of 0.4 M bis-(tri-n-butylammonium) pyrophosphate (200 μmol) in anhydrous DMF was injected. After stirring for 10 min at room temperature, the septum was removed and 2 mL of 25% pyridine in water was added. Stirring was continued for another 45 min, then the reaction mixture was diluted to 11 mL with water and filtered. Reaction products were separated by ion-exchange chromatography on a 1-mL HiTrap Q column (Pharmacia Biotech) upon elution with 40 mL of linear gradient (0.03–1 M) of ammonium bicarbonate, pH 7.0; fractions of 1 mL were collected. Product NTP[aS] were eluted as a mixture of Sp and Rp stereoisomers between 0.43 and 0.51 M buffer (C-7GTP[aS]) and between 0.48 and 0.57 M (C-7ATP[aS]). Fractions containing products were combined and evaporated to dryness on a rotary evaporator, and the residue was coevaporated five times with 50% aqueous ethanol to remove traces of the buffer. Yields were 63% for C-7ATP[aS] and 23% for C-7GTP[aS]. Separation of the stereoisomers was achieved by reverse-phase HPLC on a Sephasil C18 column (Pharmacia Biotech) with isocratic elution using 50 mM ammonium bicarbonate, pH 7.0. The configurations of the nucleoside-5′-O-(1-thiotriphosphates) at the anomeric α-phosphorous atom were identified by the ability to support in vitro transcription by T7 RNA polymerase (Griffiths et al., 1987). Spectral data for Sp stereoisomers were as follows.

**FIGURE 3.** Modification-sensitive sites in a context of bacterial minimum consensus structure (Pace & Brown, 1995). Nucleotides that are absolutely conserved among bacterial RNase P RNAs are represented by capital letters. Nucleotides that are invariant in 80% of known bacterial RNase P RNAs are represented by lowercase letters. Closed circles indicate nucleotides present in all bacterial RNase P RNAs and open circles indicate nucleotides present in 80% of known bacterial RNase P RNAs. Sites of interference are identified according to Figure 1.
7-Deazaadenine-5′-O-(1-thiotriphosphate): UV (aqueous solution, pH 6.5) λ_{max} = 271 nm, 1H-decoupled 31P spectrum: \( \delta = 41.02 \) (d, \( J_{AP} = 26.4 \) Hz, \( P_o \)), -8.34 (d, \( J_{PY} = 19.6 \) Hz, \( P_y \)), -24.70 (dd, \( P_o \)). 7-Deazaguanine-5′-O-(1-thiotriphosphate): UV (aqueous solution, pH 6.5) λ_{max} = 259 nm, \( \varepsilon_{259}/\varepsilon_{280} = 5 \). 1H-decoupled 31P spectrum: \( \delta = 40.89 \) (d, \( J_{AP} = 26.5 \) Hz, \( P_o \)), -8.52 (d, \( J_{PY} = 21.1 \) Hz, \( P_y \)), -24.90 (dd, \( P_o \)).

In proton-coupled 31P spectra of the products, each line of \( P_o \)-doublets was split into a triplet with \( J_{PA} \) of ca. 6 Hz, confirming that 5′-OCH\(_2\) group was the site of 1-thiotriphosphate attachment.

### Preparation of modified RNAs

PT332(−5) RNA was prepared by in vitro transcription with T7 RNA polymerase in a buffer containing 40 mM Tris-HCl, pH 8.0, 2 mM spermidine, 5 mM dithiothreitol, 0.05% NP-40 (Fluka), 6 mM magnesium chloride, 0.05 \( \mu \)g/\( \mu \)L linearized template DNA, and 1 mM nonmodified NTPs for 4–5 h. Guanosine (10 mM) was included to facilitate subsequent 5′ end-labeling using T4 polynucleotide kinase. For random incorporation of ribonucleoside-phosphorothioates and their 7-deazapurine analogues, the corresponding NTP[\( \alpha^32P \)] were added to 50 \( \mu \)M and transcription was performed at 25 °C to minimize the self-cleavage of PT332(−5) RNA (Harris & Pace, 1995). For incorporation of 2′-deoxy nucleoside-phosphorothioates, 1 mM manganese chloride was added (Gaur & Krupp, 1993), and transcription was performed in the presence of dNTP[\( \alpha^32P \)]. To increase the yield of 2′-deoxy-modified transcripts, reactions were performed at 37 °C and a 27-mer antisense oligonucleotide complementary to nt 68–95 of the PT332(−5) RNA was added to 50 \( \mu \)M to suppress the self-cleavage at this temperature (J.-L. Chen & N.R. Pace, unpubl. data). Ratios of dNTP[\( \alpha^32P \)] to nonmodified NTPs were optimized to achieve approximately five modifications per molecule (below).

Transcript RNAs were purified by denaturing polyacrylamide gel electrophoresis and stored under 70% ethanol at −20 °C.

### Optimization of 2′-deoxy(5′-phosphorothioate) incorporation

In a series of experiments, internally \( ^{32}P \)-labeled PT332(−5) RNA was prepared by transcription (see above) in the pres-
ence of different ratios of dNTPs to the corresponding NTPs. Transcripts were gel-purified, digested with RNase T2 (Conrad et al., 1995), and the products were resolved by thin-layer chromatography on PEI-cellulose (elucent: 80% 50 mM ammonium sulfate, 18% 1 M sodium acetate, pH 7.0, 2% isopropanol). Radioactivity in spots corresponding to nucleoside 3'-phosphates and to mixed ribo-/deoxy dimers and oligomers was quantitated by Phosphorimager (Molecular Dynamics).

A double-labeling technique also was used to quantify 2'-deoxycytidine and 2'-deoxyadenosine phosphorothioate modifications. [α-32P]GTP and [α-35S]dCTP or [α-35S]dATP were added to transcription reactions containing different ratios of CTP:dCTP[α-S] or ATP:dATP[α-S], respectively, and the relative amounts of the two isotopes in the modified RNAs were determined by a liquid scintillation counter.

With both methods, the extents of modification were plotted against the corresponding ratio of modified/unmodified nucleotide in the transcription reactions. The plots obtained by different methods did not differ significantly for the same nucleotide. The ratios of modified/nonmodified nucleotide that result in approximately five modifications per PT332 (–) molecule were interpolated from the plots and were as follows: dGTP[α-S]/GTP = 0.5; dATP[α-S]/ATP = 1.0; dCTP[α-S]/U TP = 0.3; dCTP[α-S]/CTP = 0.5.

Modification-interference
Guanosine-primed PT332 (–) RNAs (0.5–1.0 μg) were 5' end-labeled with T4 polynucleotide kinase in 10 μL 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol, and 300 μCi of [γ-32P]ATP. To minimize self-cleavage during preparation, end-labeling reactions were performed at room temperature (Harris & Pace, 1995), stopped by addition of 1 μL of 50 mM EDTA and immediately used in self-cleaving experiments without further purification.

PT332 (–) RNA self-cleavage reactions were performed at 50°C in 100 μL 16.5 mM PIPES, 44 mM Tris buffer, pH 6.0 or 8.0 at 50°C, and 3 M ammonium acetate, pH 8.0, or NaCl, pH 6.0 (Smith & Pace, 1993). Prior to cleavage, RNAs were preincubated at 75°C for 5 min, then at 50°C for 7 min. Self-cleavage was initiated by addition of a preheated solution of MgCl2 in the same buffer to achieve the specified concentrations. Cleavage was allowed to proceed for approximately one half-life at the conditions of interest (determined independently; data not shown), then stopped by addition of 50 μL of 50 mM EDTA. RNA was precipitated with isopropanol and catalytically active (cleaved) and less active (uncleaved) populations were isolated by electrophoresis in a 4% denaturing polyacrylamide gel. Iodine cleavage was performed as described elsewhere (Harris & Pace, 1995), and products were resolved by electrophoresis in 6, 8, or 12% denaturing polyacrylamide gels as appropriate.

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Functional groups in RNase P


Identification by modification-interference of purine N-7 and ribose 2'-OH groups critical for catalysis by bacterial ribonuclease P.

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