RNA (1995), *1*:210–218. Cambridge University Press. Printed in the USA.

Identification of phosphates involved in catalysis by the ribozyme RNase P RNA

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ABSTRACT

The RNA subunit of ribonuclease P (RNase P RNA) is a catalytic RNA that cleaves precursor tRNAs to generate mature tRNA 5′ ends. Little is known concerning the identity and arrangement of functional groups that constitute the active site of this ribozyme. We have used an RNase P RNA–substrate conjugate that undergoes rapid, accurate, and efficient self-cleavage in vitro to probe, by phosphorothioate modification-interference, functional groups required for catalysis. We identify four phosphate oxygens where substitution by sulfur significantly reduces the catalytic rate (50–200-fold). Interference at one site was partially rescued in the presence of manganese, suggesting a direct involvement in binding divalent metal ion cofactors required for catalysis. All sites are located in conserved sequence and secondary structure, and positioned adjacent to the substrate phosphate in a tertiary structure model of the ribozyme–substrate complex. The spatial arrangement of phosphorothioate-sensitive sites in RNase P RNA was found to resemble the distribution of analogous positions in the secondary and potential tertiary structures of other large catalytic RNAs.

Keywords: circularly permuted RNA; modification-interference; phosphorothioate; ribozyme; RNA catalysis; RNase P; RNA structure

INTRODUCTION

Bacterial ribonuclease P (RNase P) is a ribonucleoprotein enzyme with a catalytic RNA component (see Darr et al., 1992; Altman et al., 1993; Pace & Brown, 1995, for reviews). In vitro, in the presence of divalent metal ions (optimally magnesium), RNase P RNA catalyzes the hydrolysis of a specific phosphodiester bond in pre-tRNA to form the mature tRNA 5′ end. The secondary structure of the ribozyme is well established by phylogenetic covariation analysis (James et al., 1988; Haas et al., 1994) and models of its tertiary structure have been proposed (Harris et al., 1994; Westhof & Altman, 1994). Phylogenetic conservation (Haas et al., 1994), mutational (Shiraishi & Shimura, 1986; Baer et al., 1988; Kirsebom & Svård, 1993; Schlegl et al., 1994), and crosslinking (Burgin & Pace, 1990) studies have indicated some regions of RNase P RNA that likely are involved in catalysis. However, the identity and arrangement of functional groups in the active site of the ribozyme remain undefined.

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All ribozymes require divalent metal ion cofactors for catalysis (for reviews, see Pan & Uhlenbeck, 1993; Pyle, 1994). Multiple magnesium ions are required for catalysis mediated by RNase P RNA (Guerrier-Takada et al., 1983, 1986; Gardiner et al., 1985; Nichols & Schmidt, 1988; Smith & Pace, 1993). Effects of modifications of specific substrate functional groups on RNase P RNA activity suggest the 2′-hydroxyl at the cleavage site is important for divalent metal ion interactions (Perreault & Altman, 1993; Smith & Pace, 1993). A pre-transition state model consistent with the available substrate-modification and kinetic data has been proposed and makes predictions about the arrangement of magnesium ions relative to the substrate phosphate (Smith & Pace, 1993); however, little is known about the elements of RNase P RNA that are involved in metal ion binding. Specific cleavage of RNase P RNA by Mg^{2+} and Pb^{2+} suggests that specific metal ion binding sites exist (Kazakov & Altman, 1991; Zito et al., 1993), but their precise identity and relationship to catalysis remain in question.

Modification-interference experiments have proven to be useful for the identification of specific functional groups involved in nucleic acid function (e.g., Sieben-

list & Gilbert, 1980; Conway & Wickens, 1987). In this approach, important functional groups are identified by comparing the patterns of chemically modified residues in active and inactive molecules drawn from a randomly modified population. Experiments involving phosphorothioate-substitution have been used to probe phosphate oxygens involved in catalysis mediated by ribozymes (Waring, 1989; Ruffner & Uhlenbeck, 1990; Christian & Yarus, 1992; Chanfreau & Jacquier, 1994). Sites in pre-tRNA where phosphorothioate-substitution has modest effects on cleavage by RNase P RNA have also been observed (Gaur & Krupp, 1993). Phosphorothioate-modified RNAs produced by transcription in vitro contain sulfur in place of nonbridging phosphate oxygens, in the pro-Rp configuration (Griffiths et al., 1987). Substitution by sulfur at these sites is thought to inhibit the binding of magnesium ions without significant disruption of secondary structure and perhaps to perturb tertiary contacts (Pecoraro et al., 1984; Eckstein, 1985; Frey & Sammons, 1985; Jaffe & Cohn, 1987).

Modification-interference experiments rely on the ability to separate active from inactive molecules in mixed populations of modified RNAs. Native RNase P RNA, unlike other ribozymes, reacts in an intermolecular fashion and undergoes no change during the reaction. Therefore, in order to separate active from inactive RNase P RNAs for phosphorothioate modification-interference analysis of RNase P RNA-mediated catalysis, we have exploited a recently developed conjugate between RNase P RNA and its substrate that reacts intramolecularly (Frank et al., 1994). This allowed selection of active from inactive RNase P RNAs based on the self-cleavage reaction. By using this strategy for phosphorothioate modification-interference, we identify four phosphate oxygens in RNase P RNA where substitution by sulfur dramatically reduces the catalytic rate (50–200-fold). One site exhibits a partial rescue of interference in the presence of manganese, suggesting a direct involvement of that phosphate in binding divalent metal ion cofactors required for catalysis. The locations of the phosphorothioate-sensitive sites in the secondary and potential tertiary structure of RNase P RNA suggest a cooperative role in catalysis.

RESULTS

In order to separate active from inactive RNase P RNAs, we exploited the ability of the RNase P RNA–tRNA conjugate PT332 RNA to undergo rapid ($t_{1/2}$ = 0.2 s), accurate, and efficient self-cleavage in vitro (Frank et al., 1994). The secondary structures of native *Escherichia coli* RNase P RNA and PT332 RNA are compared in Figure 1. PT332 RNA contains a *Bacillus subtilis* pre-$tRNA_{asp}$ sequence appended to a circularly permuted *E. coli* RNase P RNA at nucleotide 331. Chemical protection (LeGrandeur et al., 1994) and crosslinking (Burgin & Pace, 1990; Harris et al., 1994) data indicate that the 5′ leader sequence is in close proximity to nucleotide 332 in the *E. coli* ribozyme–substrate complex. Therefore, attachment of tRNA sequences at RNase P RNA nucleotide 331 positions the substrate at the active site of the ribozyme. Self-cleavage excises the tRNA moiety of the conjugate and consequently allows separation of active (cleaved) ribozymes from less active RNAs based on molecular weight.

We first examined the effect of phosphorothioate nucleotide substitution on the PT332 RNA self-cleavage reaction (Fig. 2). Unmodified PT332 RNA self-cleaves to yield mature tRNA and a larger 5′ fragment containing RNase P RNA sequences (Fig. 2A). RNAs were allowed to react for a comparatively long period of time ($20 \times t_{1/2}$) to assess the effect of substitution on the extent of reaction. Complete substitution of PT332 RNA with either thio-G or thio-A essentially abolished self-cleavage, whereas molecules completely substituted with either thio-C or thio-U retained some self-cleavage activity. Comparison of the extent of cleavage of partially substituted PT332 RNAs showed that the reaction was most sensitive to thio-G, thio-A, and thio-U substitution, whereas substitution with equivalent levels of thio-C was less detrimental to catalysis (Fig. 2B). The rate of cleavage of PT332 RNA was not significantly affected by low levels of substitution (5–10%) with thio-A, G, or U (data not shown). Determination of the cleavage rate of PT332 RNA substituted with greater than 10% thio-A and G was not feasible due to the low degree of conversion to cleaved products. Partial substitution with thio-C had only a moderate effect on the extent of cleavage. In addition, the cleavage rate of PT332 RNA completely substituted with thio-C was still 50% of the native cleavage rate (data not shown). These data show that modification of nonbridging phosphate oxygens with sulfur disrupts some aspect of RNase P RNA catalysis. Such results could be due to cumulative nonspecific structural effects, or could indicate the involvement of particular phosphate oxygens in catalysis. The sensitivity of the activity of RNase P RNA to thio-G, thio-A, and thio-U incorporation suggests that modification of specific sites perturbs catalysis.

Specific sites of sensitivity to phosphorothioate-substitution were mapped by modification-interference. End-labeled RNAs containing approximately two A, G, U, or C phosphorothioates per molecule were allowed to undergo reaction. Then, sites of modification in gel-purified products and residual precursors were determined by cleavage with I_2. Initially, RNAs were allowed to react for approximately 50 half-lives (10 s) in order to map sites where modification results in a significant (>100-fold) effect on catalysis. The cleavage patterns of PT332 RNAs modified with thio-A and thio-G are shown in Figure 3. Little or no product RNAs modified at A67, G68, and A352 were detected in the product

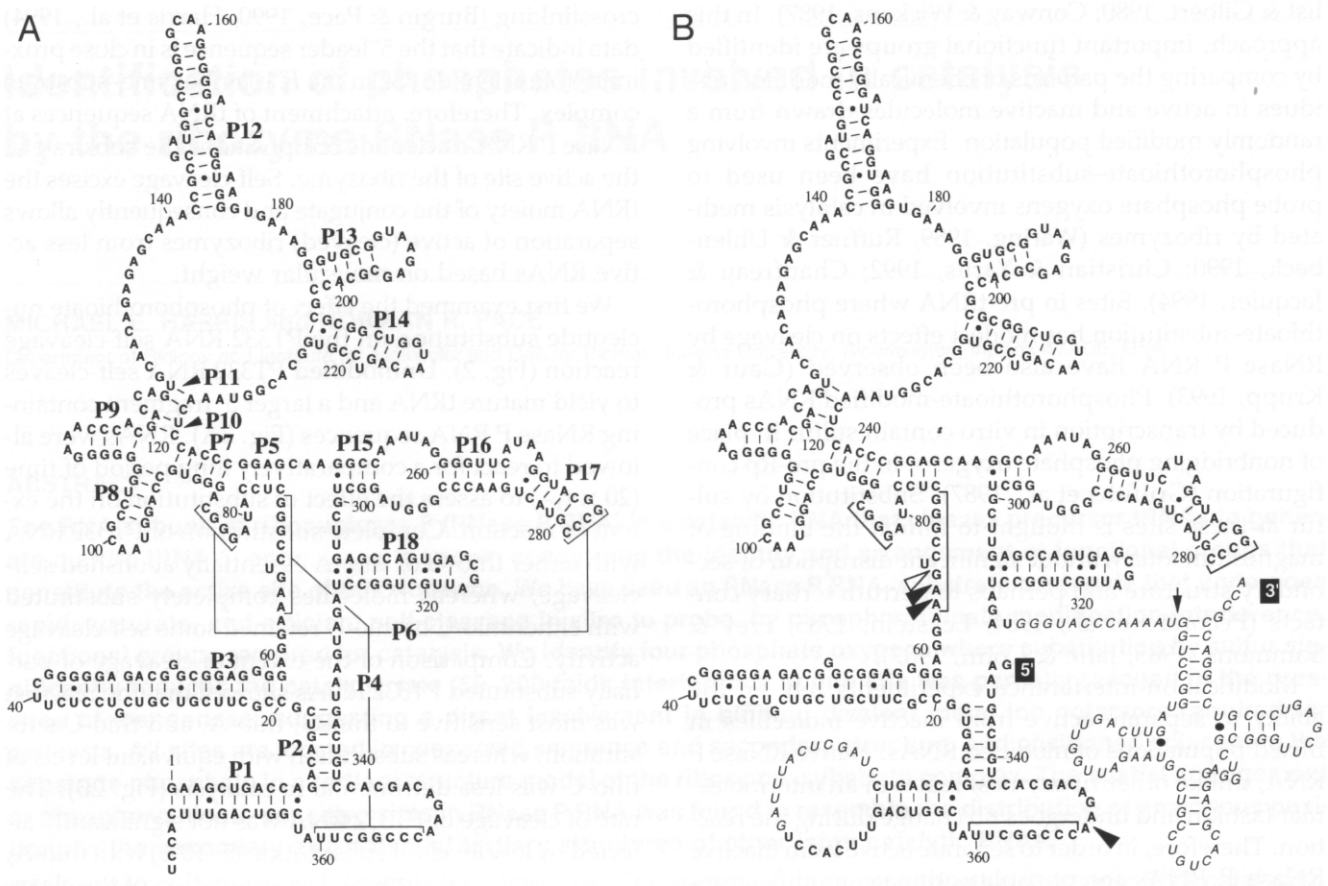

FIGURE 1. A: Secondary structure of native *E. coli* RNase P RNA. Paired regions indicated by covariation analysis are indicated as P1 through P18 (Haas et al., 1994). Two pairings interrupted in the two-dimensional representation (P4 and P6) are indicated by brackets connected by lines. **B:** Secondary structure of PT332 RNA. Native RNase P RNA sequences are shown in bold; tRNA and linker sequences are italicized. RNase P RNA sequences are numbered according to the native *E. coli* RNase P RNA sequence. Sites of phosphorothioate sensitivity determined in this analysis are shown by large arrowheads. A less sensitive, but significant, interference effect at U69 is indicated by an open arrowhead. The pre-tRNA cleavage site is indicated by a smaller arrow.

populations, consistent with the greater sensitivity of the cleavage reaction to thio-A and thio-G incorporation. No convincing interference effects at C residues were observed and, under these conditions, only a slight effect at a single U residue was seen (below).

We estimate that substitution of phosphate oxygens 5′ to nucleotides A67, G68, and A352 causes a decrease of approximately 200-fold in the PT332 RNA reaction rate. The amount of RNAs modified at positions A67, G68, and A352 in the product population was less than 10% of the level detected in the remaining precursor population. The assumption of first-order kinetics and the observation of no more than 10% conversion of RNAs modified at these positions after 10 s corresponds to an estimated k_{app} of 0.63 min^{-1} (ln 0.9 = k_{app} [10 s]) for the modified RNAs compared to the 130 min^{-1} native rate (Frank et al., 1994). Such dominantly sensitive sites are the best candidates for nonbridging phosphate oxygens directly involved in catalysis.

In addition to strong interference effects at A67, G68, and A352, a minor effect was also detected at U69 (Fig. 4). In order to amplify less dramatic phosphorothioate-substitution effects, the analysis was repeated with RNAs allowed to react for only a comparatively short period of time, approximately 5 half-lives. This required slowing the native catalytic rate by reducing the reaction pH from 8.0 to 6.0, which has been shown to reduce specifically the rate of the chemical step of the RNase P reaction by approximately 100-fold (Smith & Pace, 1993). Under this altered reaction condition, a more marked effect at U69 is observed (Fig. 4). Considering the approximately 10% conversion of RNAs modified at U69 after 4 half-lives, the decrease in cleavage rate due to sulfur substitution is estimated to be about 50-fold (ln 0.9 = k_{app} [5 min] corresponds to a rate of 0.02 min^{-1}, compared with the native rate of 1.3 min^{-1} at pH 6.0). Interferences at A67, G68, and A352 were still detected, but no additional strong interference effects were observed at low pH (data not shown). We conclude that substitutions at only four pro-Rp phosphate oxygens have significant effects on catalysis by RNase P RNA.

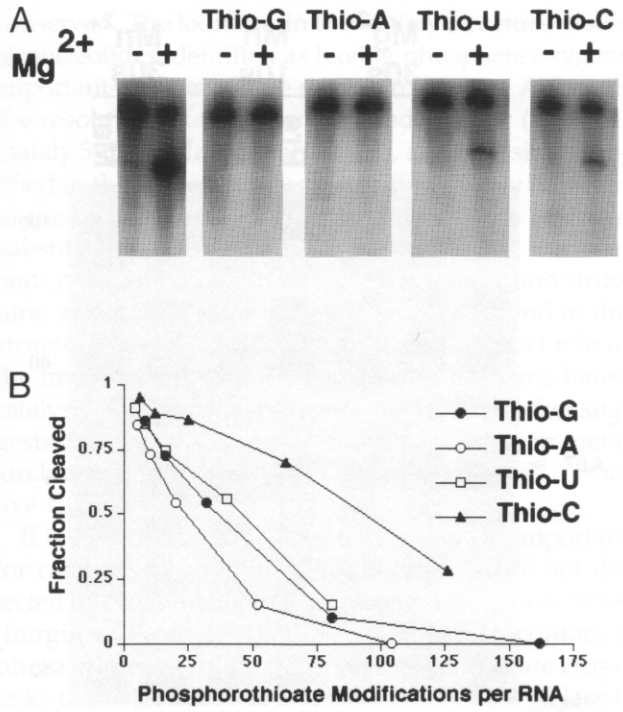

FIGURE 2. Effect of phosphorothioate-substitution on the PT332 RNA self-cleavage reaction. **A:** Reaction of unsubstituted PT332, and PT332 RNA completely substituted with phosphorothioate G, A, C, or U nucleotides. Two nanomolar unsubstituted PT332 RNA, or RNAs completely substituted with a particular phosphorothioate nucleotide were allowed to react for 10 min at 50 °C in the presence of 10 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.0, 3 M ammonium acetate and products were resolved by polyacrylamide gel electrophoresis. Mature tRNA fragments running well below the 5′ cleavage fragment are not shown. **B:** Effect of increasing phosphorothioate incorporation on PT332 cleavage. Filled circles indicated the extent of reactions of thio-G substituted RNAs; open circles, thio-A; open squares, thio-U; and filled triangles, thio-C. The greatest extent of substitution represents complete modification of the appropriate nucleotide as shown in A.

Magnesium can coordinate with nonbridging phosphate oxygens in RNA, as seen in tRNA crystal structure (Holbrook et al., 1977; Jack et al., 1977; Quigley et al., 1978), and disruption of such interactions could explain these specific phosphorothioate effects on catalysis by RNA. Manganese has a more relaxed ligand specificity than magnesium, discriminating less well between oxygen and sulfur (Jaffe & Cohn, 1978; Pecoraro et al., 1984). Modification-interference reactions performed in parallel in the presence of magnesium or alternatively manganese were compared in order to determine if any of the specific phosphorothioate effects observed in the presence of magnesium were alleviated in the presence of manganese. In only one case, A67, a modest manganese-rescue was observed. Substitution of magnesium by manganese resulted in a significant increase in the amount of PT332 RNA modified at position A67 in the product population, while the interferences at positions G68, U69, and A352 were unaffected (Fig. 5, data not shown). This suggests that at

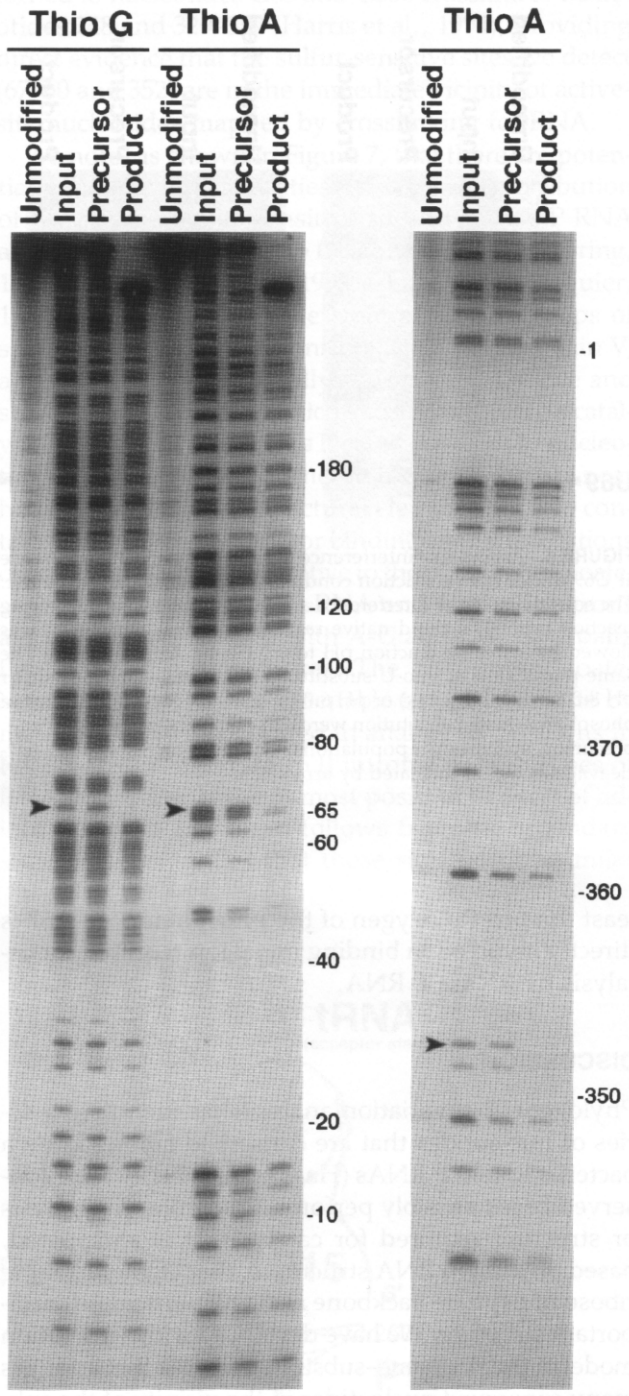

FIGURE 3. Identification of phosphorothioate-sensitive sites affecting RNase P RNA activity. 5′ End-labeled PT332 RNAs that were modified at approximately two substitutions per molecule were allowed to react for 10 s ($50 \times t_{1/2}$). The input RNA, precursor, and product populations were purified by gel electrophoresis; sites of phosphorothioate-modification were mapped by cleavage with I_2. Unmodified RNA was also analyzed as a control for nonspecific degradation. Sites displaying clear modification-interference are indicated by arrows. Thio-G and thio-A modified RNA populations are shown. No convincing effects at C residues were observed (data not shown) and only a moderate effect at a single U residue was seen (see text, Fig. 4).

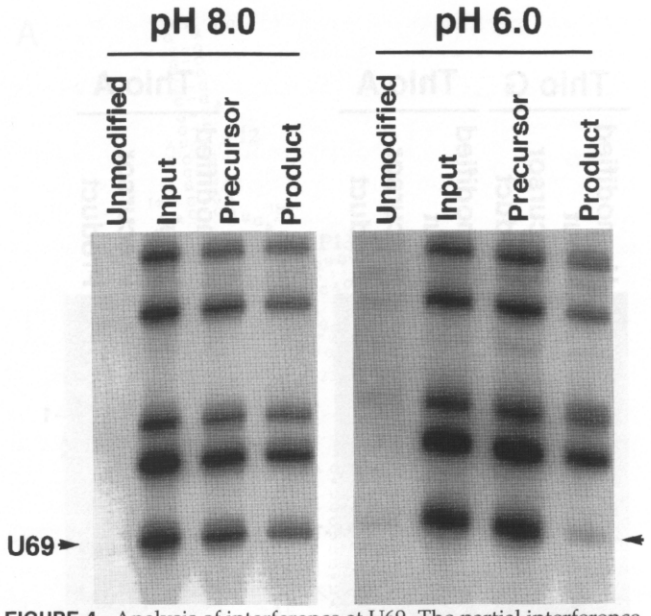

FIGURE 4. Analysis of interference at U69. The partial interference at U69 under native reaction conditions (pH 8.0) is shown at left. The sensitivity of the interference assay was increased by reducing reaction time. The rapid native reaction rate (k_{app} = 0.2 s^{-1}) was slowed by lowering reaction pH to 6.0 (Smith & Pace, 1992). The same population of thio-U substituted RNA was reacted at either pH 8.0 for 10 s (50 × $t_{1/2}$) or pH 6.0 for 2 min (4 × $t_{1/2}$), and sites of phosphorothioate-substitution were mapped in the respective input, precursor, and product populations. The band corresponding to I_2 cleavage at U69 is indicated by arrows.

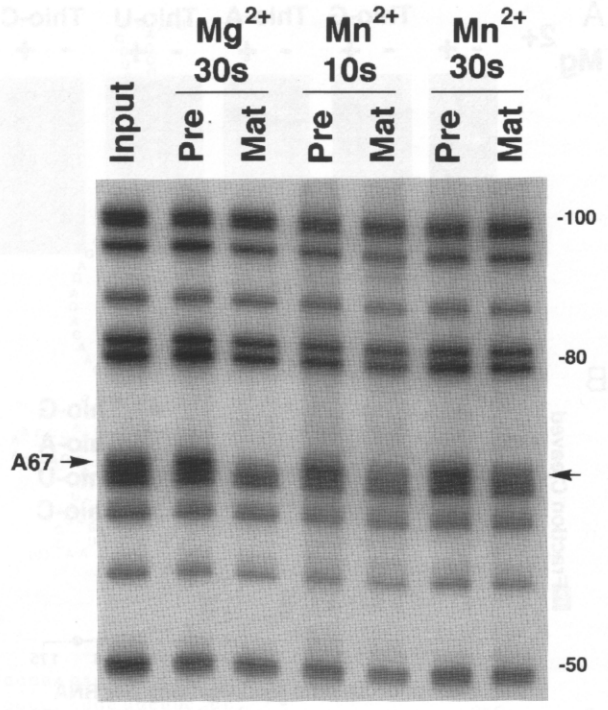

FIGURE 5. Manganese-rescue of interference at A67. Modification-interference effects observed in the presence of either magnesium or manganese are compared. End-labeled RNAs modified with thio-A were reacted in the presence of Mg^{2+} or Mn^{2+} and sites of phosphorothioate modification were determined. Reactions contained 100 mM divalent metal ion (either magnesium or manganese as indicated), 40 mM Tris-HCl, pH 8.0, and 3 M ammonium acetate. Cleavage reactions were incubated for the length of time indicated. Input, precursor (Pre) and mature (Mat) RNA populations are indicated. The band corresponding to I_2 cleavage at A67 is shown by arrows.

least the pro-Rp oxygen of the phosphate 5′ to A67 is directly involved in binding metal ion required for catalysis by RNase P RNA.

DISCUSSION

Phylogenetic covariation analysis has identified a series of nucleotides that are conserved among known bacterial RNase P RNAs (Haas et al., 1994). These conserved bases possibly perform direct roles in catalysis or structure required for catalysis. It is anticipated, based on known RNA structures, that elements of the ribose phosphate backbone also will participate in important structure. We have developed a low-resolution model of the ribozyme–substrate complex that arranges many conserved nucleotides in the vicinity of the substrate phosphate (Harris et al., 1994). However, a detailed description of the active site of the enzyme is not yet possible. In the present analysis we have begun to survey functional groups required for RNase P RNA-mediated catalysis. Until recently, the intermolecular nature of the RNase P RNA reaction precluded application of methods relying on selection of RNAs from mixed populations. We show here that rationally designed enzyme–substrate conjugates are effective tools for probing RNase P RNA catalysis, in this case by modification-interference.

We have identified for the first time specific functional groups in RNase P RNA that are involved in catalytic function. Only a small subset of assayed positions displayed marked sensitivity to sulfur-substitution (4 out of 476). The stringent conditions of the selection, dictated by the rapid self-cleavage rate of PT332 RNA, likely preclude the identification of sites that are only moderately sensitive to phosphorothioate-modification. In addition, the high monovalent salt concentrations used to obtain optimal levels of PT332 RNA self-cleavage would tend to suppress moderate structural perturbation that might result from incorporation of analogs as seen in similar analysis of Group II introns (Chanfreau & Jacquier, 1994). The tethering of the substrate close to the active site likely overcomes effects of modifications that only weaken substrate binding.

The distribution of the phosphorothioate-sensitive sites in the secondary structure of the RNase P RNA is shown in Figure 1B. All of the sites identified in this study are located in or adjacent to helix P4, which is present in all known bacterial RNase P RNAs (Haas et al., 1994). In each case, the phosphates identified are positioned between nucleotides that are universally

conserved. The locations in the tertiary structure model of nucleotides identified as having phosphate oxygens important for catalysis are shown in Figure 6. Although the resolution of the structure model is low (approximately 5–10 Å [Harris et al., 1994]), all of the sites identified in the current analysis are immediately adjacent to the cleavage site in pre-tRNA. The large effect of substitution of single atoms on catalytic rate, the locations of sensitive sites in conserved sequence and structure, and their proximity to the substrate bond in the structure model are all consistent with a direct role of the implicated phosphate oxygens in RNA-mediated catalysis. The proximity of these sites to each other suggests a cooperative role in catalysis, possibly in metal ion binding or in docking of the substrate into the active site.

It is curious that the sites we determine as important for catalysis by modification-interference are not detected by crosslinking to 5′ photoagent-modified tRNA (Burgin & Pace, 1991). tRNA modified to contain a photoaffinity agent on the substrate phosphate crosslinks to nucleotides 248 and 330–334 in *E. coli* RNase P RNA, and at the corresponding positions in two different RNase P RNAs, from *Chromatium vinosum* and *B. subtilis*. It is possible that the range of the photoagent is restricted, perhaps by steric occlusion in the ribozyme–substrate complex, or that the sites detected in the modification-interference analysis are indirectly (though by definition critically) involved in positioning other moieties in the active site. However, photoagents attached to nucleotides G63 and G350 crosslink to nucleotides 248 and 329–330 (Harris et al., 1994), providing direct evidence that the sulfur-sensitive sites we detect (67–60 and 352) are in the immediate vicinity of active-site nucleotides mapped by crosslinking to tRNA.

We note, as shown in Figure 7, that there are potentially interesting similarities between the distribution of phosphorothioate-sensitive sites in RNase P RNA and in Group I and Group II catalytic introns (Waring, 1989; Christian & Yarus, 1992; Chanfreau & Jacquier, 1994). The majority of sites that affect both steps of splicing by Group II introns are located in Domain V, a region of phylogenetically conserved sequence and structure. Most sites at which substitution affects catalysis by Group I introns are located adjacent to nucleotides that are drawn together in tertiary structure near helix P7, a conserved structural element that also contains the guanosine-cofactor binding site. The locations of conserved sequences relative to the phosphorothioate-sensitive sites in RNase P RNA and Group II introns are particularly similar, with A-U and G-C base pairs flanking two adjacent sites. The phosphorothioate-sensitive sites that are amenable to partial rescue by manganese are also located in analogous positions in RNase P RNA and Group II introns: in both types of RNA they occur at the 5′-most position in a trio of adjacent sites in a helix. It follows from the secondary structural similarities that these sites occupy similar

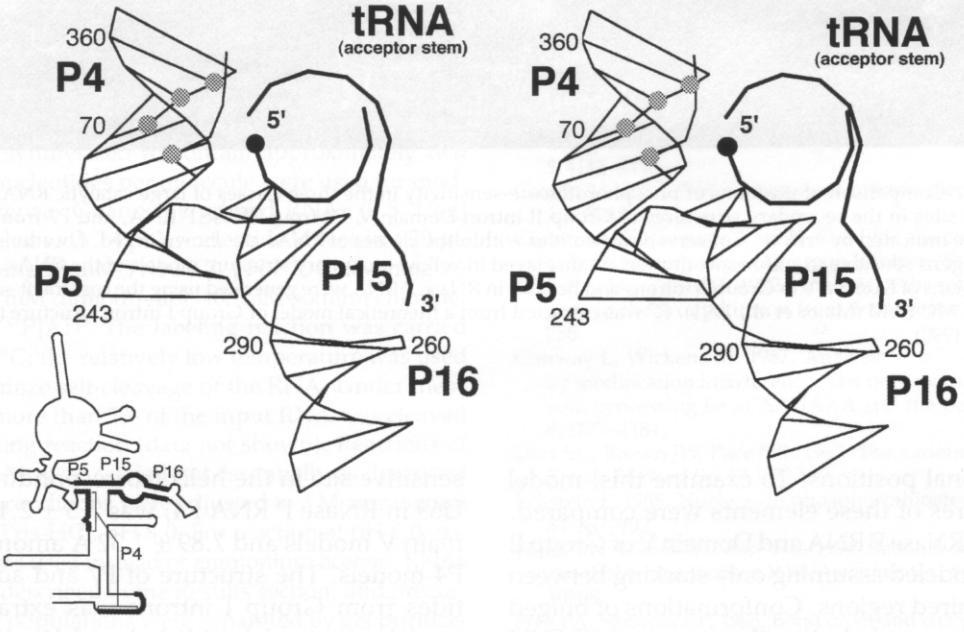

FIGURE 6. Position of phosphorothioate-sensitive sites in RNase P RNA relative to the tRNA substrate phosphate in a model of the ribozyme–substrate complex. A portion of a low-resolution model of the RNase P RNA–pre-tRNA complex (Harris et al., 1994) is shown in crosseye stereo. The region of RNase P RNA included is shown on the secondary structure diagram at left. Only the tRNA acceptor stem is shown, for clarity. The position of the substrate phosphate in pre-tRNA is shown as a filled sphere. The positions of phosphates sensitive to phosphorothioate-substitution in RNase P RNA are shown as gray spheres. The RNase P sequence is numbered according to Figure 1A.

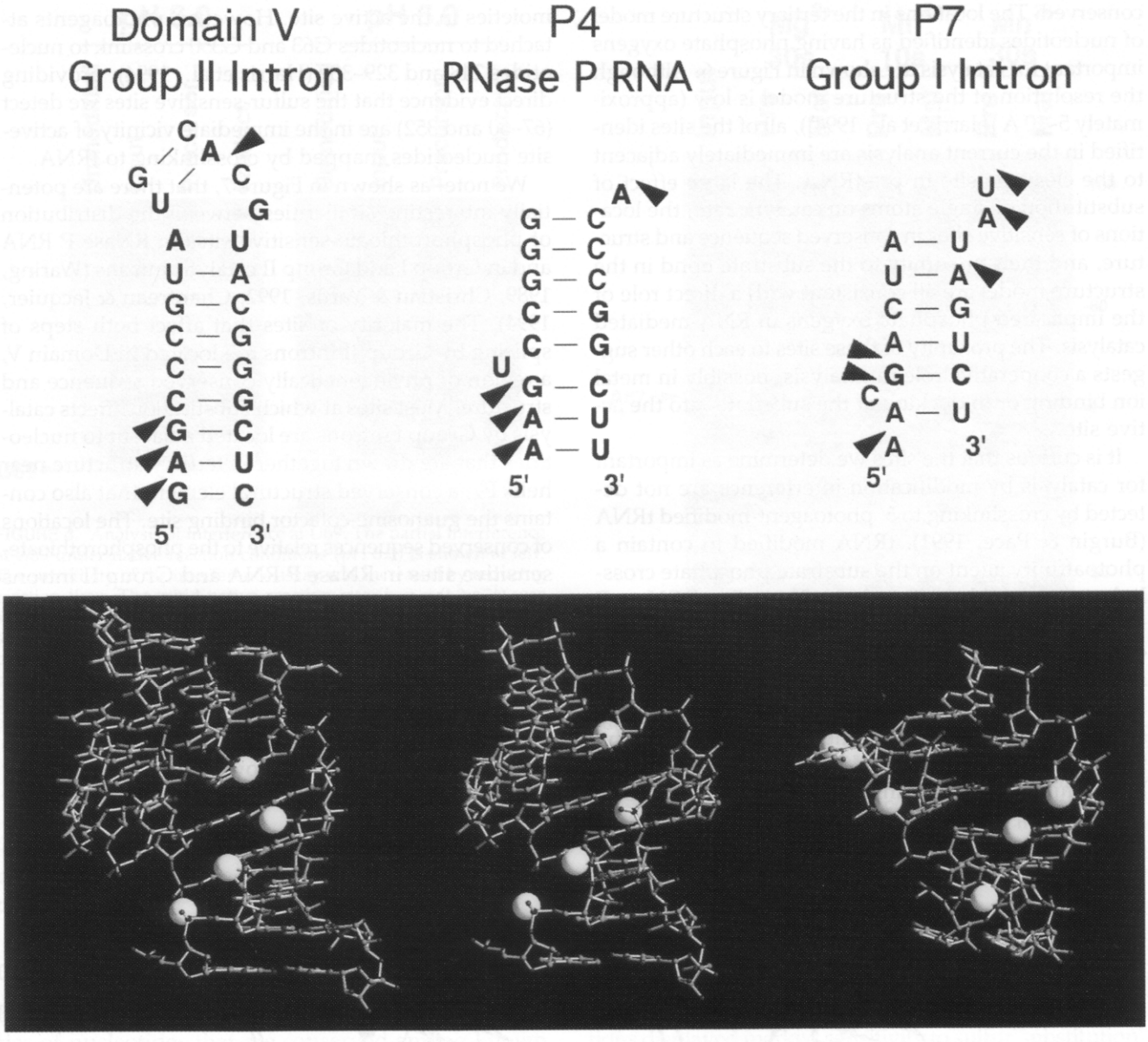

FIGURE 7. Comparison of positions of phosphorothioate-sensitivity in the three classes of large catalytic RNAs. The locations of sites in the secondary structures of Group II intron Domain V, P4 from RNase P RNA, and P7 from Group I introns are indicated by arrows. Conserved nucleotides within the classes of RNAs are shown in red. Locations of phosphate oxygens sensitive to sulfur-substitution are displayed in yellow on tertiary structure models of the RNAs. Potential conformations of Domain V in Group II introns and helix P4 in RNase P RNA were generated using the constraint-satisfaction algorithm MC-SYM (Major et al., 1991). P7 was extracted from a theoretical model of Group I intron structure (Michel & Westhof, 1990).

three-dimensional positions. To examine this, model tertiary structures of these elements were compared. Both helix P4 in RNase P RNA and Domain V of Group II introns were modeled assuming only stacking between the two base-paired regions. Conformations of bulged nucleotides were sampled using the program MC-SYM (Major et al., 1992). Nineteen Domain V structures and 17 P4 structures were considered. The average distance between the single, phosphorothioate-sensitive phosphate oxygen in bulged nucleotides and the 3′-most sensitive site in the helix (corresponding to A352 and U69 in RNase P RNA P4) was 7.3 ± 2.18 Å in the Domain V models and 7.89 ± 1.32 Å among the different P4 models. The structure of P7 and adjacent nucleotides from Group I introns was extracted from the model proposed by Michel and Westhof (1990). As shown in the three-dimensional portrayals of the structures (Fig. 7), the sites that display sensitivity to sulfur-substitution occur juxtaposed across the major groove of the respective helices in each type of RNA. All three

of these RNAs are similar in that they are able to catalyze reactions at RNA phosphates in the presence of magnesium, and they must bind substrate helices in order to position the substrate phosphate at the active site. It is possible that these functional commonalities are reflected in structural similarities, as suggested by the similar positions of phosphorothioate sensitivity. Additional perspective on the role of these elements in RNA-mediated catalysis will be required to test their possible functional analogies.

MATERIALS AND METHODS

The RNAs used in this study were prepared by in vitro transcription using phage T7 RNA polymerase essentially as described by Frank et al. (1994). NTP:αS-NTP ratios were adjusted to affect the level of αS-NMP incorporation using the values of Christian and Yarus (1992) for relative efficiencies of incorporation of phosphorothioate and normal nucleotides. For 5′ end-labeling, RNAs were primed with guanosine. This was accomplished by addition of guanosine (at a ratio of 10:1 guanosine:GTP) in the transcription reactions. For analysis of cleavage rate and extent, RNAs were internally labeled with [α^{32}P]GTP. Cleavage reactions contained 10 nM RNA, 3 M sodium chloride, 40 mM Tris-HCl, pH 6.0, in a volume of 20 μL. Reactions were preincubated for 5 min at 65 °C, then transferred to 50 °C and preincubated for an additional 5 min. To initiate self-cleavage, magnesium was added to 10 mM. To assess cleavage rate, reactions were incubated a sufficient amount of time in order to effect no more than 10–20% conversion (10–20 s). For measurements of the extent of reaction, incubation times were lengthened to 20 min. Reactions were terminated by addition of 180 μL of ice-cold 2 mM EDTA and the reaction products precipitated by the addition of 600 μL ethanol. Separation of precursor from cleaved products was accomplished by electrophoresis though 6% polyacrylamide/8 M urea gels. Gels were dried, and conversion to cleaved products was analyzed using a Molecular Dynamics Phosphorimaging system.

PT332 RNAs synthesized to contain approximately two thiophosphate nucleotides per molecule were used for modification-interference. Guanosine-primed RNAs (0.5–1 μg) were 5′ end-labeled with T4 polynucleotide kinase in a 20-μL reaction containing 20 mM Tris-HCl, pH 7.5, 5 mM magnesium chloride, 1 mM dithiothreitol, 100 mM sodium chloride, and 250 μCi [γ-^{32}P]ATP. The labeling reaction was carried out for 1 h at 25 °C; the relatively low temperature was used in order to minimize self-cleavage of the RNA. Under these conditions, no more than 5% of the input RNA was cleaved during the labeling reaction (data not shown). Reactions of end-labeled RNAs were carried out essentially as described above. Reaction conditions were adjusted to 3 M ammonium acetate, 40 mM Tris-HCl, pH 8.0. For reaction at pH 6.0, sodium chloride was used instead of ammonium acetate. RNAs were reacted as described in the Results section, and precursor and product populations were separated by gel purification. Labeled RNAs were located by autoradiography and eluted into 400 μL 0.2 M sodium acetate, 40 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1% SDS.

I_2 was used to cleave end-labeled RNAs at sites of phosphorothioate modification. Labeled RNA (0.5–1 × 10^6 cpm) in 2 μL water was combined with 2 μL of a 1:500 dilution in water of 0.1 M iodine in ethanol. The reaction was incubated at 95 °C for 2 min. Four microliters of 80% formamide, 0.05% bromphenol blue, and 0.05% xylene cyanol was added, and the 95 °C incubation was continued for an additional 2 min. The sample (1–2 μL) was loaded on 6% or 10% denaturing acrylamide gels.

Structures of RNase P RNA P4 and Group II intron Domain V were generated using MC-SYM (Major et al., 1992) on a Silicon Graphics Indigo II computer. Potential structures were compared by superimposition using the program Insight II. Representative structures were refined by steepest decent energy minimization using the AMBER forcefield (Discover). Graphical representations of structures shown in Figure 7 were generated using the program RIBBONS (Carson, 1990). PDB coordinates of the P4 and domain V models and TIFF images are available by anonymous ftp at IUBio.indiana.edu in the directory molbiol/rnase-p/threeD.

ACKNOWLEDGMENTS

We thank Dan Frank for advice during the course of this study. We also thank Pat Maroney and Tim Nilsen for advice on phosphorothioate modification-interference experiments, and Jim Nolan, Marty Pagel, and Steve Harvey for helpful comments and assistance with computer modeling. This work was supported by a grant from the NIH to N.R.P. (GM 34527) and an NIH postdoctoral fellowship to M.E.H. (GM 15979).

Received March 9, 1995; returned for revision March 29, 1995; revised manuscript received March 31, 1995.

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