Evidence for an RNA-based catalytic mechanism in eukaryotic nuclear ribonuclease P

BRIAN C. THOMAS,1,2,5 JOEL CHAMBERLAIN,3,6 DAVID R. ENGELKE,3,4 and PETER GEGENHEIMER1,2

1Department of Molecular Biosciences, The University of Kansas, 2045 Haworth Hall, Lawrence, Kansas 66045-2106, USA
2Molecular Genetics Program, The University of Kansas, 2045 Haworth Hall, Lawrence, Kansas 66045-2106, USA
3Program in Cellular and Molecular Biology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0606, USA
4Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0606, USA

ABSTRACT

Ribonuclease P is the enzyme responsible for removing the 5′-leader segment of precursor transfer RNAs in all organisms. All eukaryotic nuclear RNase Ps are ribonucleoproteins in which multiple protein components and a single RNA species are required for activity in vitro as well as in vivo. It is not known, however, which subunits participate directly in phosphodiester-bond hydrolysis. The RNA subunit of nuclear RNase P is evolutionarily related to its catalytically active bacterial counterpart, prompting speculation that in eukaryotes the RNA may be the catalytic component. In the bacterial RNase P reaction, Mg(II) is required to coordinate the nonbridging phosphodiester oxygen(s) of the scissile bond. As a consequence, bacterial RNase P cannot cleave pre-tRNA in which the pro-R P component is catalyzed by a protein enzyme whose mechanism does not involve Mg(II) coordinated by the pro-R P oxygen. To determine whether the mechanism of nuclear RNase P resembles more closely an RNA- or a protein-catalyzed reaction, we analyzed the ability of Saccharomyces cerevisiae nuclear RNase P to cleave pre-tRNA containing a sulfur substitution of the pro-R P oxygen at the cleavage site. Sulfur substitution at this position prohibits correct cleavage by eukaryotic RNase P thus depends on the presence of a thio-sensitive ligand to the pro-R P oxygen of the scissile bond, and is consistent with a common, RNA-based mechanism for the bacterial and eukaryal enzymes.

Keywords: catalytic mechanism; enzyme; magnesium ion; ribonuclease P; ribozyme; tRNA processing; thiosubstitution; yeast

INTRODUCTION

Transfer RNA molecules are synthesized in vivo as precursors (pre-tRNA) with extra sequences at their 5′ and 3′ termini. These precursor-specific RNAs must be removed before the tRNA can function. Ribonuclease P (RNase P) is the enzyme responsible for removing the 5′ extension, or leader, of precursor transfer RNAs in all organisms. It hydrolyzes the phosphodiester bond between the 5′ to the first nucleotide of the tRNA domain, releasing a 5′ leader with a 3′ hydroxyl group, and a mature tRNA with a 5′ phosphoryl group. A remarkable feature of this enzyme is its composition: the RNA component of bacterial RNase P is the catalytic moiety (Guerrier-Takada et al., 1983; reviewed in Frank & Pace, 1998), with the protein subunit assisting in maintaining the structure of the RNA subunit and facilitating discrimination between substrate and product (Reich et al., 1988; Talsjo & Kirsebom, 1993; Kurz et al., 1998).

Recently, the complete polypeptide composition of yeast RNase P (Chamberlain et al., 1998) and the nearly complete composition of the human enzyme (Jarrous et al., 1998) have been determined. The yeast enzyme contains at least nine polypeptides, ranging from ~16 kDa to 100–115 kDa. As predicted, only about...
20% of the eukaryotic holoenzyme mass is RNA. In contrast, the bacterial holoenzyme is 80–90% RNA. In Saccharomyces cerevisiae nuclear RNase P, the RNA subunit is essential for activity both in vitro and in vivo (Lee & Engelke, 1989; Lee et al., 1991), but has not been shown to be directly involved in catalysis. The current structural and phylogenetic evidence (Tranguch & Engelke, 1993; Chen & Pace, 1997; Pitulle et al., 1998; reviewed in Frank & Pace, 1998) supports the hypothesis that eukaryotic RNase P RNA possesses much or all of the catalytic machinery and that the role of the protein is to maintain the required tertiary structure of the RNA.

To determine whether the reaction mechanism of a eukaryotic ribonucleoprotein RNase P was similar to that of the bacterial ribozyme RNase P, we analyzed the ability of S. cerevisiae nuclear RNase P to cleave a pre-tRNA containing a sulfur substitution at the pro-R P nonbridging oxygen of the scissile bond. The bacterial RNase P RNA subunit requires divalent metal cations, preferably Mg(II) or Mn(II), for substrate binding and catalysis (Gardiner et al., 1985; Guerrier-Takada et al., 1986; Smith et al., 1992; Beebe et al., 1996). Using the Escherichia coli RNase P RNA subunit and a phosphorothioate-substituted pre-tRNA, previous experiments revealed that the catalytic RNA subunit absolutely requires a Mg$^{2+}$ ion coordinated to the pro-R P nonbridging oxygen of the scissile bond (Chen et al., 1997). As a consequence, bacterial RNase P cannot cleave pre-tRNA in which this oxygen is replaced by sulfur.

One exception to the rule of ribozyme RNase P has been identified. The RNase P reaction in plant chloroplasts is catalyzed by a protein enzyme (Wang et al., 1988; Thomas et al., submitted). This reaction, unlike the ribozyme-catalyzed reaction, does not involve Mg(II) coordinated either directly or indirectly to the pro-R P oxygen, as judged by the ability of the chloroplast enzyme to cleave, accurately and efficiently, an R P-thiosubstituted phosphodiester bond (Thomas et al., 2000).

We can use the phosphorothioate inhibition of cleavage as a benchmark for the reaction chemistry of the E. coli RNase P RNA. If the RNA subunit of yeast nuclear RNase P were the catalytic moiety of this enzyme, we would expect an all-Mg mechanism, requiring Mg(II) bound to the pro-R P oxygen of the pre-tRNA, and hence exhibiting complete inhibition by phosphorothioate-substitution at the scissile bond. On the other hand, if catalysis were performed exclusively by a protein subunit, we predict little or no inhibition of cleavage by an R P-phosphorothioate-substituted scissile bond.

**RESULTS**

**Phosphorothioate inhibition of pre-tRNA processing**

RNase P cleaves the phosphodiester bond between nucleosides −1 and +1 of a pre-tRNA to generate products with a 3′ hydroxyl and a 5′ phosphoryl, as illustrated in Figure 1. To be able to determine whether any

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Schematic representation of precursor tRNA substrates. Nucleotides are numbered with respect to the 5′ end of mature tRNA as +1. The large arrow indicates the RNase P cleavage site 5′ to the a-phosphate of nucleotide +1. A: Yeast pre-tRNA$^{\text{Phe}-\text{G1}}$ contains a 43-nt 5′-leader RNA (shaded box to position −1) and a 73-nt mature-tRNA$^{\text{Phe}}$ domain followed by the 3′-terminal CCA$_{35}$k. B: Yeast pre-tRNA$^{\text{Phe}-\text{A1}}$ is identical to yeast pre-tRNA$^{\text{Phe}-\text{G1}}$, except that G1:C72 was changed to A1:U72.
inhibition of pre-tRNA cleavage arising from phosphorothioate substitution resulted uniquely from alteration at the scissile bond, rather than from nonspecific interactions elsewhere within the pre-tRNA or from a perturbation of pre-tRNA secondary structure, we employed the pair of pre-tRNAs used earlier for similar investigations of metal ion involvement in the reactions catalyzed by the E. coli RNase P RNA subunit (Chen et al., 1997) and the spinach chloroplast RNase P protein (Thomas et al., 2000). These pre-tRNA\textsuperscript{Phe} substrates differ by a single nucleotide at the scissile bond. As shown in Figure 1, the wild-type precursor, designated pre-tRNA\textsuperscript{Phe}\textsubscript{-G}\textsubscript{1}, or pre-G\textsubscript{1}, possesses GMP at position +1, the first nucleotide of the mature tRNA domain. The variant, called pre-tRNA\textsuperscript{Phe}\textsubscript{-A}\textsubscript{1}, or pre-A\textsubscript{1}, has AMP as nucleotide +1.

We tested a substantially purified preparation of RNase P from S. cerevisiae for its ability to process wild-type or variant substrates lacking or containing phosphorothioate substitutions in the presence of MgCl\textsubscript{2}. Reactions were performed for a time sufficient to give complete cleavage of unsubstituted substrates. As seen in Figure 2A, yeast nuclear RNase efficiently processes both substrates containing unmodified nucleotides to give mature tRNA and 5\textsuperscript{9} leader (lanes 1 and 4). Similarly, pre-G\textsubscript{1},Phe[A\textsubscript{α}S] and pre-A,Phe[G\textsubscript{α}S] are cleaved almost as well as unsubstituted precursors (Fig. 2A, lanes 2 and 6). In contrast, both pre-G\textsubscript{1},Phe[G\textsubscript{α}S] and pre-A,Phe[A\textsubscript{α}S] are cleaved poorly, if at all, by nuclear RNase P (Fig. 2A, lanes 3 and 5). These results demonstrate that RNase P cleavage is blocked only when the phosphorothioate is present at the scissile bond itself.

In these latter reactions, as well as in some reactions lacking enzyme, small amounts of aberrant cleavage products, migrating more slowly than tRNA and more quickly than 5\textsuperscript{9} leader (and denoted with an asterisk in Fig. 2A), are formed. The sizes of these products are consistent with their being formed by a single break upstream of the correct tRNA terminus. These products will be described in detail below.

**Failure to restore cleavage with Mn(II)**

Inhibition of phosphodiester-bond hydrolysis by sulfur replacement of a nonbridging oxygen is consistent with direct coordination of hard metal ions, such as Mg(II), to that oxygen. If so, the reaction can often be restored or “rescued” by concomitant addition of a softer, more thiophilic ligand such as Mn(II), Zn(II), or Cd(II) (Peco-
We attempted to restore activity to thio-inhibited reactions by addition of 5 mm MnCl₂ to reactions containing 8 mm MgCl₂, as shown in Figure 2B. Cleavage of pre-G₁Phe and pre-A₁Phe, unsubstituted or substituted with AMPₐS or GMPₐS, was first repeated in the presence of 8 mM MgCl₂ only (Fig. 2B, lanes 1–6). In those reactions in which the thiosubstitution occurs at the scissile bond (Fig. 2B, lanes 3 and 5), the normal RNase P cleavage products do not appear, but are replaced by small amounts of species migrating more slowly than tRNA and more quickly than 5’ leader. The size of the smaller RNA suggests that these species could be generated by a single cleavage 1–2 nt upstream of the normal tRNA 5’ terminus.

When the same reactions are performed in the presence of 5 mM MnCl₂, the extent of processing is substantially reduced, but the appearance of incorrectly processed products (Fig. 2B, lanes 9 and 11) is not altered. Likewise, an identical pattern is seen in reactions containing both 8 mM MgCl₂ and 5 mM MnCl₂ (Fig. 2B, lanes 13–18). The incorrect cleavage of substrates with phosphorothioate scissile bonds is not detectably corrected under these conditions (Fig. 2B, lanes 15 and 17). The extent of cleavage and the sizes of RNAs detected after RNase P reaction of pre-tRNAs containing thiosubstituted scissile bonds (Fig. 2B, lanes 3, 5, 9, 11, 15, 17) are similar to those observed after the incubation of unsubstituted substrates in the absence of enzyme (Fig. 2B, lanes 19 and 20), suggesting that the aberrant RNA species are breakdown products not dependent upon RNase P.

We also attempted to restore activity to reactions containing pre-G₁Phe[ₐGₐS] or pre-A₁Phe[ₐAₐS] by the replacement of MgCl₂ with 0.5–8.0 mM MnCl₂ or an addition of 0.5–8.0 mM MnCl₂ to reactions containing 2.5 mM MgCl₂ (data not shown). With neither of these metal combinations did we detect an increase in the production of mature tRNA.

**Identification of the scissile bond**

The scissile bond in each of these pre-tRNAs was located by the determination of the 5’-terminal nucleotide of [³²P]CMP-labeled tRNA-sized products recovered from preparative reactions like those shown in Figure 3A and digested to completion with ribonuclease T2. Hydrolysis of authentic 5’-mature tRNA with RNase T2 releases ribonucleoside 3’-monophosphates (Np) from internal positions, and a ribonucleoside 3’-5’ bis-phosphate (pNp) from the 5’ terminus. These products can be separated cleanly by anion-exchange thin-layer chromatography (TLC). Figure 4 illustrates the 5’ termini expected from RNase T2 digestion of RNAs resulting from pre-tRNA cleavage at any single site between –2

---

**FIGURE 3.** Preparative-scale processing reactions. Substrates and thiosubstitutions were as in Figure 2. A: [³²P]CMP-labeled pre-tRNAₚₚₚ substrates (30 pmol) were incubated with 5 μL RNase P for 60 min at 30°C. B: [³²P]UMP-labeled substrates were treated with 15 μL RNase P (lanes 1–3) or without enzyme (lane 4) for 130 min at 30°C.
and +2. (In none of the TLC analyses could we have detected 5′ termini of pCp or pUp*, because these nucleotides are not well-separated from nucleoside monophosphates by the solvent system used.)

The results of this analysis are shown in Figure 5. RNase T2 digestion of unsubstituted tRNA^Phe^G1 releases labeled pGp* (Fig. 5, lane 1), and digestion of tRNA^Phe^A1 releases pAp* (Fig. 5, lane 4). Similarly, the tRNA-sized products from pre-G, Phe[αS] and pre-A, Phe[αS] possess 5′-terminal pGp* and pAp*, seen in Figure 5, lanes 2 and 6, respectively, as expected for authentic mature tRNAs. In contrast, the aberrant tRNA-sized species released from substrates pre-G, Phe[αS] (Fig. 5, lane 3) and pre-A, Phe[αS] (Fig. 5, lane 5) did not yield any labeled 5′-specific product from a complete RNase T2 digestion, even after prolonged exposure of the analytical chromatogram. The expected 5′-terminal nucleoside bisphosphates are a mixture of p(S)Gp* and its oxidation product pGp*, or p(S)Ap* plus pAp*. Authentic p(S)Gp migrates close to the origin in the chromatographic system used here (Chen et al., 1997; Thomas et al., 2000), and p(S)Ap is expected to migrate just ahead of p(S)Gp.

**Phosphorothioate-containing pre-tRNAs are not cleaved by RNase P**

As we were unable to detect a normal 5′ end for the tRNA-sized species released from pre-G, Phe[αS], we asked whether yeast RNase P, like the E. coli enzyme (Chen et al., 1997), might shift its cleavage to the first oxygen-containing phosphodiester upstream of the phosphorothioate. If so, the product would possess a 5′-terminal pAp. tRNA-sized material was hence isolated by preparative-scale cleavage of [32P]AMP-labeled pre-G, Phe[αS]. RNase T2 digestion failed to release any detectable p(S)Ap (data not shown).

In further analyses to locate the aberrant hydrolysis site, we determined the oligonucleotide content of 5′-leader-size RNAs, as leader RNAs terminating at different positions will differ in their content of the 3′-most oligonucleotide ApUp. Figure 4 presents the combinations of tRNA 5′ end and leader RNA 3′ end predicted for pre-tRNA cleavage at every possible position between −1 and +2.

Leader RNAs, recovered from preparative-scale reactions like those shown in Figure 3B, were subjected...
FIGURE 5. Determination of the RNase P cleavage site. tRNA-sized products from preparative-scale processing reactions with [32P]CMP-labeled substrate (shown in Fig. 3A) were digested with RNase T2 and spotted onto PEI-cellulose TLC plates. Substrates and thiosubstitutions are as in the legend to Figure 2. The positions of unlabeled nucleotides pAp, pGp, ApG, or Gp, visualized under UV illumination, are indicated by dotted lines.

to a two-dimensional oligonucleotide separation, or mini-fingerprint analysis, after digestion with RNases A plus T1. This treatment generates oligonucleotides of the sequence A_Bp, in which B is G, C, or U. These analyses are shown in Figure 6. The minifingerprints of RNase P-generated 5'-leader from unsubstituted [32P]AMP-labeled pre-G1Phe (Fig. 6A), or [32P]UMP-labeled pre-A1Phe (Fig. 6D), contain the amount of ApUp expected for RNase P cleavage at the correct site. On the other hand, the aberrant 5'-RNA product from pre-G1Phe[GuS] also contains ApUp* (shown in Fig. 6B), consistent only with cleavage at -ApUp-1ApUp+1HOAp(S)+1G-. Further, the leader-sized RNA released from pre-A1Phe[AαS] (Fig. 6E) contains an oligonucleotide, designated X, in a position consistent with ApUOH or a derivative of ApUp(S) and indicative of hydrolysis either 5' (or 3') to phosphorothioate(-1), for example at -ApUp-1ApUp+1Ap- (see Fig. 4B).

Leader-sized RNA was also isolated from preparative-scale incubation of [32P]UMP-labeled pre-A1Phe[AαS] in the absence of enzyme (Fig. 3B, lane 4). The mini-fingerprint of this RNA, shown in Figure 6F, is absolutely indistinguishable from the fingerprint of leader RNA formed in the presence of enzyme (Fig. 6E). This result strongly suggests that the RNAs formed during incubation with RNase P of pre-tRNA thiosubstituted at the normal scissile bond are products of nonenzymatic breakdown.

DISCUSSION

The conservation of secondary structure and active-site sequence between the RNA subunit of eukaryal nuclear RNase P and bacterial RNase P (Tranguch & Engelke, 1993) led us to ask whether the nuclear enzyme also contained an RNA catalyst. Benchmarks existed that can differentiate between pre-tRNA 5' cleavage by a ribozyme or a protein RNase P. Chen et al. (1997) showed that the E. coli RNase P RNA subunit cannot cleave pre-tRNA containing a phosphorothioate substitution at the scissile bond. On the other hand, Thomas et al. (2000) recently demonstrated that the all-protein RNase P from spinach chloroplasts accurately and efficiently cleaves a phosphorothioate-containing scissile bond. Our strategy was to use the chemical differences between these cleavage reactions to assess whether the catalytic mechanism of nuclear RNase P resembles more closely that of an RNA or of a protein enzyme.

Determination of the cleavage site

RNase P processing of substrates containing no thio-substitutions, or thiosubstituted at purines other than the scissile bond, yields leader RNA ending with ApUpA, and tRNA_Phe-G1 initiating with pGpC, or tRNA_Phe-A1 starting with pApC. (The 3' nearest neighbor is italicized.) Inspection of Figure 4 shows that these oligonucleotides could result only from cleavage at the normal RNase P site, -ApUp-1ApUp+1GpC- for pre-G1Phe and -ApUp-1Alp1-GpC- for pre-A1Phe.

In contrast, RNase P treatment of substrates thiosubstituted at the scissile bond produces no products above the background of nonenzymatic breakdown. Together, our analyses indicate that S. cerevisiae nuclear RNase P cannot cleave a pre-tRNA in which the normal scissile phosphodiester is substituted with a non-bridging Rp sulfur atom.

Previous investigation of the reaction catalyzed by the E. coli RNase P RNA subunit showed that phosphodiester bond hydrolysis was likewise prevented by the presence of an Rp-phosphorothioate substitution at the scissile bond. Unlike the yeast nuclear enzyme, however, the bacterial RNA was able to cleave with correct polarity at positions 1 nt upstream or downstream of the correct site when the correct site was blocked. Furthermore, the ability of Mn(II) ion to restore
cleavage to the normal site and at a rate commensurate with the affinity of Mn(II) for sulfur (Chen et al., 1997) provided a direct demonstration of inner-sphere coordination of Mg(II) by the pro-R P oxygen during catalysis by the E. coli ribozyme RNase P. In contrast, we were unable to demonstrate any recovery of activity by the yeast nuclear enzyme upon addition of Mn(II), either in the absence or presence of Mg(II). Indeed, MnCl$_2$ seemed to inhibit the MgCl$_2$-dependent reaction. However, an analogous situation is observed with the E. coli RNase P ribozyme when its action is blocked by phosphorothioate substitution of the pro-S$_P$ oxygen of the scissile bond. This inhibition cannot be reversed by addition of Cd$^{2+}$ (Warnecke et al., 1996) or Mn$^{2+}$ (Li, 1996). In addition, those studies were carried out with the bacterial RNA subunit alone. The accessibility of metal-ion-binding sites to different hydrated metal ions may differ substantially between the free RNA and the ribonucleoprotein holoenzyme.

**CONCLUSION**

The work presented in this study demonstrates that pre-tRNA processing by partially purified *S. cerevisiae* nuclear RNase P is severely inhibited when the precursor tRNA is phosphorothioate-substituted at pro-R$_P$.
positions, and that the inhibition is specific to the oxygen at the scissile bond. Because the yeast and bacterial RNA subunits are derived from a common ancestor, and because the bacterial RNA subunit is the catalytic component, we had hypothesized that the eukaryotic RNA subunit would also be involved in catalysis. Our results support this hypothesis, as the yeast holoenzyme, like the bacterial ribozyme RNase P but unlike the all-protein chloroplast RNase P, is sensitive to Rₚ-thio substitution. The yeast RNase P holoenzyme differs, however, in that Mn²⁺ cannot restore reactivity to an Rₚ-phosphorothioate substrate. Currently, we do not know whether this difference reflects a narrower metal-ion specificity of the yeast enzyme, or whether this discrepancy results from comparing the yeast holoenzyme to the reaction catalyzed by the bacterial RNase P subunit alone. It is not impossible that the large increase in protein content of eukaryotic RNase P has been accompanied by a slight modification in catalytic mechanism. Further mechanistic investigation of both enzymes is likely to clarify these remaining questions.

MATERIALS AND METHODS

Preparation of pre-tRNA substrates

A precursor to wild-type yeast tRNA^{Phe}(G₋₋₋), called pre-\text{G₃Phe}, was prepared by in vitro transcription of the gene YF0 (Sampson & Uhlenbeck, 1988), and a precursor to tRNA^{Phe}(A₋₋₋), denoted pre-\text{A₃Phe}, by transcription of the variant gene YF0-G1A,C72U (Chen et al., 1997). Transcription of BstNI-linearized plasmid DNA by phage T3 RNA polymerase yields 5'−extended, 3'−mature precursor, 119 nt in length, whose 43-nt 5' leader has a sequence of a phage T7 promoter and whose 3' terminus is the mature tRNA CCA\text{OH}_2\text{O} sequence. Reaction transcription reactions were performed according to Thomas et al. (2000) (Chen et al., 1997) to give pre-tRNA^{Phe} with specific activity of 35 pCi/fmol RNA. Alternatively, Ampliscribe in vitro transcription kits (Ambion) were used as recommended by the manufacturer. To synthesize phosphorothioate-substituted RNAs, one of the rNTPs was replaced 100% with the same concentration of the S₉ isomer of nucleoside 5'(α-thio)triphosphates (NEN). Transcripts were purified by denaturing gel electrophoresis (Wang et al., 1988; Thomas et al., 2000).

RNase P preparation and assay

Partially purified RNase P from S. cerevisiae nuclei was prepared as described by Chamberlain et al. (1996). These experiments were performed with fractions from the final Mono-Q H/R column. These fractions contain RNase P, but lack detectable amounts of the related ribosomal RNA-processing enzyme RNase MRP (Chamberlain et al., 1996).

Standard processing assays (20 μL) were done with 1−2 μL partially purified RNase P and 2−10 pmol [²³²P]pre-tRNA^{Phe} in 1× processing buffer [20 mM HEPES-KOH (pH 8.0), 50 mM KCl, 8 mM MgCl₂, 0.2 mM Na-EDTA (pH 8.0), 1% glycerol (introduced from the enzyme preparation), 0.2 mM DTT, 0.1% NP-40] for 5−10 min at 30°C.

Preparative-scale processing reactions (100 μL) were conducted with 5−15 μL of enzyme and 20−30 pmol [²³²P]pre-tRNA^{Phe} for 1 h at 30°C. Reactions were terminated and transcripts purified by denaturing gel electrophoresis as before (Wang et al., 1988; Thomas et al., 2000). RNA species were quantitated by measuring Cerenkov radiation in excised gel slices, with correction for background in each lane.

5’-terminal oligonucleotide determination

Gel-purified tRNA species were digested with RNase T2 (Calbiochem; 0.2−0.5 U/μL in 20−100 mM NH₄OAc) in sealed microcapillary tubes for 2−4 h at 50°C (Chen et al., 1997). Products were separated by polyethyleneimine-cellulose thin-layer chromatography as before (Thomas et al., 2000).

Two-dimensional oligonucleotide fractionation (minifingerprinting)

The 5’−leader RNA was gel purified after preparative-scale processing reactions. The RNA pellet was resuspended in 1−9 μL sterile distilled water containing 20 μg deproteinized carrier yeast RNA and mixed with 5 μL of 0.5 mg RNase A/ml plus 2.5 U RNase T1/ml (both from Calbiochem) in TE buffer [10 mM Tris-Cl (pH 7.4), 1 mM Na-EDTA (pH 8.0)]. Unlabeled marker oligonucleotides were prepared separately by digestion of 60 μg carrier RNA. Samples were incubated at 50°C for 2 h, and product oligonucleotides were fractionated according to Volckaert and Fiers (1977). The digest was spotted onto a 7 × 10-cm sheet of PEI-cellulose [pre-treated by washing for 5 min each in 2 M pyridine formate, pH 2.2, and then in water (Volckaert et al., 1976)]. The long dimension of the plate was developed with water to 1 cm past the origin, dried, and developed to the top with 22% formic acid. The second dimension was developed with 0.1 M pyridine formate (pH 4.3) to 1 cm above the origin, dried, and developed with 1.1 M pyridine formate (pH 4.3) to the top of the plate. Plates were dried overnight, then washed in methanol for 5 min and air-dried. Labeled products were visualized by autoradiography, and unlabeled markers by UV illumination.

Identification of phosphorothioate-containing oligonucleotides was inferred from analyses of thio-containing dinucleotides in the chromatographic system. The presence of an internal phosphorothioate decreases the mobility of the dinucleotide AP₈₉G relative to ApG in the chromatographic system used for this analysis (Li, 1996).

ACKNOWLEDGMENTS

The advice and encouragement of Drs. Norm Pace, Mark Richter, and Dick Himes is gratefully acknowledged. This work was supported in part by National Science Foundation grant OSR 92-55223 (PG) and National Institutes of Health grant RO1-34869 (DRE).

Received July 12, 1999; returned for revision September 20, 1999; revised manuscript received January 27, 2000
REFERENCES

Beebe JA, Kurz JC, Fricke CA. 1996. Magnesium ions are required by Bacillus subtilis ribonuclease P RNA for both binding and cleaving precursor tRNA\textsuperscript{Apt}. Biochemistry 35:10493–10505.


Li L. 1996. The catalytic function of Mg\textsuperscript{2+} ions in the E. coli RNase P reaction. Ph.D. Dissertation. University of Kansas-Lawrence, Lawrence, Kansas.

Pecoraro VL, Hermes JD, Cleland WW. 1984. Stability constants of Mg\textsuperscript{2+} and Cd\textsuperscript{2+} complexes of adenine nucleotides and thionucleotides and rate constants for formation and dissociation of MgATP and MgADP. Biochemistry 23:5262–5271.


Warnecke JM, Furste JP, Hardt WD, Erdmann VA, Hartmann RK. 1996. Ribonuclease P (RNase P) RNA is converted to a Cd\textsuperscript{2+} ribozyme by a single Rp-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site. Proc Natl Acad Sci USA 93:8924–8928.
Evidence for an RNA-based catalytic mechanism in eukaryotic nuclear ribonuclease P.

B C Thomas, J Chamberlain, D R Engelke, et al.

RNA 2000 6: 554-562

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.

To subscribe to RNA go to:
http://rnajournal.cshlp.org/subscriptions