The RNA Component of RNase P from the Archaebacterium
*Haloferax volcanii*

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RNase P, an endoribonuclease responsible for generating the mature 5' termini of tRNA precursors, is composed of both RNA and protein. It has been demonstrated that the eubacterial RNase P RNA will, under the appropriate reaction conditions, exhibit catalytic activity in vitro. Evidence has not been obtained for catalytic activity by the RNAs of eukaryotic RNase P enzymes. Using a cDNA probe prepared from RNA copurifying with RNase P activity from the archaebacterium *Haloferax volcanii*, we have characterized the gene encoding the RNase P RNA. The proposed transcript from this gene can assume a structure resembling the eubacterial RNase P RNA and includes many of the highly conserved sequences of these RNAs. This RNA was incapable of cleaving pre-tRNA substrates in the absence of protein under a variety of in vitro conditions. Catalytic activity was observed when this RNA was combined with the protein subunit of the *Bacillus subtilis* RNase P complex. Similarities among the archaebacterial, eubacterial, and eukaryotic RNase P RNA sequences and structures are discussed.

Archaebacteria, like the eubacteria and eukaryotes, possess an activity that removes the 5' leader sequences from tRNA primary transcripts. In eubacterial and eukaryotic cells, this reaction is carried out by the enzyme RNase P, which is composed of both protein and RNA (for reviews, see Refs. 1–3). The role of the RNA component in the eubacterial RNase P complex is now clear; RNA functions as the catalytic RNA that copurifies with RNase P activity from the archaebacterium *Haloferax volcanii*. The transcript from this gene can assume a folded structure resembling the eubacterial RNase P RNA and includes many of the highly conserved sequences of these RNAs. This RNA was incapable of cleaving pre-tRNA substrates in the absence of protein under a variety of in vitro conditions. Catalytic activity was observed when this RNA was combined with the protein subunit of the *Bacillus subtilis* RNase P complex. Similarities among the archaebacterial, eubacterial, and eukaryotic RNase P RNA sequences and structures are discussed.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) M61003.

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\(^*\) Portions of this paper (including "Materials and Methods" and Figs. 1, 2, and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
low salt extracts (16). This activity is sensitive to micrococcal nuclease and has a high buoyant density (1.61 g/cm³). We have observed that fractionation of extracts from these cells by gel filtration on Sepharose 4B followed by Sephadex G-200 in 2 M KCl (physiological salt conditions) gave an enzyme that remained active over a broad range of salt conditions (0.05-3 M KCl; data not shown). Consistent with the earlier observation that this enzyme contains an RNA component, we also noted that an approximately 435-nucleotide RNA copurified with this activity.

To determine if this RNA was a component of the RNase P activity of these cells, the gene encoding this RNA was cloned. cDNAs were prepared from a narrow window of RNAs, containing the 435-nucleotide RNA, which had been isolated from RNase P-active Sephadex G-200 fractions. These cDNAs hybridized to a number of restriction fragments, including several containing the genes for rRNAs (Fig. 2). Based on previous hybridization information for the rRNA operons of H. volcanii (18, 19) and by blocking Southern blots with a single non-rRNA gene-containing MluI (2.6 kb)3 and SalI (1.0 kb) restriction fragments that hybridized to the cDNAs were identified (Fig. 2). In parallel, a cosmid bank from H. volcanii was screened for hybridization with the cDNAs. Again, after eliminating rRNA-containing cosmids, a single cosmid was identified that hybridized to the cDNAs. This cosmid, cos282, was found to contain both the 2.6-kb MluIand 1.0-kb SalI fragments. A 985-bp MluI-SalI fragment containing the hybridizing region was subcloned and subjected to Northern analysis. The sequence of this fragment is presented in Fig. 3.

To verify that this region encoded an RNA, Northern analysis was performed, using as probes the 2.6-kb MluI-and 985-bp MluI-SalI fragments from the cosmid. The oligonucleotide, 5'-NNGGACTTCTCTCCNCNCC-3' (where N is any nucleotide), which contains sequences complementary to the highly conserved eubacterial RNA P RNA sequence 5'-GAGGAAAAGUCC-3' present within the 985-bp MluI-SalI fragment was also used as a probe (see Fig. 3). In each case, the DNAs hybridized to a single RNA species of 435 nucleotides. Fig. 4 illustrates the hybridization obtained with the oligonucleotide probe. The 5' end of this RNA was localized by primer extension and S1 mapping to one of 2 G residues located immediately downstream from a region containing four archaeabacterial promoter-like sequences (Figs. 3 and 4). Although the 3' terminus of this RNA was not mapped, Northern data and localization of the 5' terminus suggest that the transcript ends in a short stretch of U residues, similar to other archaeabacterial transcripts.

Using the criteria established from phylogenetic comparisons of eubacterial RNase P RNAs, this RNA can be folded into a structure that is similar to the proposed eubacterial structure (Fig. 5). The H. volcanii RNA can assume a three-loop core structure with base-pairing interactions between the 5' and 3' ends; it also has several of the conserved helical structures that protrude from these loops. This similarity extends beyond structural features. The halobacterial RNA contains many of the universally conserved sequence elements of the eubacterial RNase P RNAs, including the longest conserved block, 5'-GAGGAAAAGUCC-3' (Fig. 5).

There is little sequence and structure similarity between eubacterial and eukaryotic RNase P RNAs. A small set of nucleotides have been identified in several eukaryotic RNase P RNAs (26); many of these sequences are also present in the halophilic RNase P RNA (Fig. 5). One structural feature that may be conserved between the eubacterial and eukaryotic RNase P RNAs is the formation of a pseudoknot involving two regions of high sequence conservation (13, 27). The halobacterial RNase P RNA also retains the ability to form this pseudoknot structure (Fig. 5).

A fundamental difference between the eubacterial and eukaryotic RNase P RNAs is the ability of the eubacterial RNA to catalyze the cleavage of pre-rRNAs in the absence of protein (4, 5). To examine whether the halophilic RNA could act as a catalytic RNA, a Mael-BstBI fragment containing the gene region was subcloned into the T7 RNA polymerase expression vector pBl31. Transcripts from this clone produce an RNA that, based on the proposed gene structure, has 20 and 9 nucleotides of 5' and 3'-flanking sequence, respectively. This RNA was assayed with both the halophilic tRNA5' substrate and the CCA-containing B. subtilis tRNA3' substrate under a variety of solution conditions. Ionic conditions optimal for catalysis by E. coli RNase P RNA (100 mM MgCl2 and 100 mM NH4Cl) (28) and B. subtilis RNase P RNA (300 mM MgCl2 and 1.2 M NH4Cl) (29) were tested. Also examined were NH4Cl concentrations from 0.1 to 3.0 M in the presence of 30 mM MgCl2, MgCl2 concentrations from 10 to 250 mM in the presence of 600 mM NH4Cl, and KCl concentrations up to 2 M. Other solution conditions tested were polyethylene glycol 8000, ethanol, and glycerol from 2.5 to 10% and temperatures from 16 to 70°C in buffer containing 30 mM MgCl2 and 1.2 M NH4Cl. The halophilic RNA lacked catalytic activity under all conditions tested. As a further test for catalytic activity, the halophilic RNA was
combined with the protein component of *B. subtilis* RNase P in a heterologous reconstitution experiment. Incubation conditions were optimized for the activity of the *B. subtilis* RNase P RNA plus its protein. Under these conditions, the halophilic RNA exhibited cleavage activity with the tRNA$_{\text{Val}}$ substrate (Fig. 6). Full recovery of the activity was not possible with the heterologous complex. Approximately 10% of the cleavage activity was recovered when compared to the halophilic holoenzyme. Similar results were obtained with the *B. subtilis* tRNA$_{\text{Asp}}$ substrate (Fig. 7).

**DISCUSSION**

Using cDNAs prepared against RNAs that copurified with RNase P activity from *H. volcanii*, we have isolated the gene that encodes the RNA component of this enzyme. Identification of this RNA as a component of the RNase P complex stems from three observations. Previous studies on the physical properties and nuclelease sensitivity of this enzyme strongly suggested that this activity had a required RNA component (16). Second, sequence data indicate that this RNA can assume a structure similar to the core structure proposed for eubacterial RNase P RNAs, and it contains many of the conserved sequence elements. And finally, in vitro transcripts from this gene, when combined with the RNase P protein from *B. subtilis*, exhibit catalytic activity.

A comparison of the structure and sequence features of the halophilic, eubacterial, and eukaryotic RNase P RNAs indicates that the halophilic RNA is most similar to the eubacterial RNase P RNA. Its overall structure closely resembles the eubacterial core structure, and it contains 46 of the 60 conserved eubacterial RNase P nucleotides. Further correlations between these RNAs with respect to catalytic centers and protein-binding sites are tentative due to the lack of understanding of these issues in the eubacterial enzyme. Some structural regions and specific nucleotides of the RNase P RNA, which have been ascribed to a particular function, have correlates in the halophilic RNA. For example, the protein components of *E. coli* and *B. subtilis* RNase P RNAs are thought to interact with sequences in the uppermost loop and the helical region that separates this loop from the central loop (see Fig. 5). In *E. coli*, the C5 protein protects regions 82–96 and 170–185 (2, 30). A point mutation in this region, G$^{89}$ to A$^{89}$, leads to a defect in protein association (31). Insertions into the analogous helical region separating the central and uppermost loops in the *B. subtilis* RNase P RNA also affect protein interaction (32). The halophilic RNA contains both similar helical structures and related sequences in this region. Therefore, this region of the halophilic RNA may play a role in protein interaction. Other mutations that affect cleavage efficiency in the eubacterial RNase P RNAs have been described. These mutations are located throughout the molecule, and it has been difficult to distinguish "true" catalytic mutations from those that have an indirect effect on the activity due to changes in RNA folding (1, 2). The diversity of structures among the eukaryotic nuclear and organelar RNase P RNAs makes comparisons between these and the other RNAs difficult. One common structural feature that may be shared between the halobacterial, eubacterial, and eukaryotic RNase P RNAs and the mitochondrial RNA processing RNase P is the formation of a pseudoknot (13, 27). Interestingly, these interactions bring together most of the universally conserved sequence elements that are present in distant regions of the molecule. Located in this region of the *H. volcanii* RNase P RNA are 12 of the 19 nucleotides conserved between this RNA and the eukaryotic RNase P RNAs (26) (see Fig. 5). Sequence homology between the halobacteria and eukaryotic RNase P RNAs can be extended further if sequences conserved in the pseudoknot interaction are also included. Each eukaryotic RNase P RNA contains the conserved sequence element 5'-GNAANNUCNGNG3'-3, which pairs with the sequences of the 3'-terminal loop (27) (see Fig. 5). Five of these nucleotides are conserved in the *H. volcanii* RNase P RNA. If all of these nucleotides are considered, then 13 nucleotides are conserved in the RNase P RNAs from all three kingdoms. The pseudoknot structure model is also consistent with earlier mutagenesis data for *B. subtilis* RNase P RNA, which indicated that active site formation involves the interaction between distant portions of the molecule (33).

The inability of the halobacterial RNase P RNA to act catalytically in the absence of protein is puzzling. In the eubacterial system, the protein component appears to act as a cofactor that shields the ionic repulsion forces between the substrate and catalytic RNAs (28, 29). Its presence affects the $V_{\text{max}}$ of the reaction, but not the binding of substrate. It is unlikely that the protein component of the halophilic RNase P plays this role since the internal monovalent ion concentra-
E. coli

Hf. volcanii

Helofexx volcanii

Fig. 5. Structure of the E. coli and H. volcanii RNase P RNAs. In the upper panel are structures of the RNase P RNAs derived from phylogenetic comparisons of eubacterial RNase P RNAs (Ref. 13, J. W. Brown, E. S. Haas, and N. R. Pace, personal communications). Circled nucleotides represent nucleotides that are present in similar locations in all eubacterial RNase P RNAs. Arrows in the H. volcanii structure indicate nucleotides that have been identified as conserved in eukaryotic RNase P RNAs (Ref. 26; see text). Arced lines and boxes indicate sequences that can participate in pseudoknot interactions. Lower panel, potential structures resulting from pseudoknot formation. Helix designations are those described by Forster and Altman (27). Nucleotides indicated by circles and arrows are as indicated above; boxed nucleotides are sequences that are conserved in several eukaryotic RNase P RNAs (Ref. 26; see text).
in that it has a much higher protein:RNA ratio (17). We have noted in the purification of the RNase P enzyme from Thermoplasma acidophilum, a related archaeabacterium, that the RNA associated with this activity is approximately 340 nucleotides. This is significantly smaller than the H. volcanii RNase P RNA. Thus, the archaeabacteria as a group may contain a variety of RNase P enzyme complexes. This apparent diversity, coupled with the demands of their unusual environments (high salt and high temperatures) on the structure of RNA and protein, make these organisms an interesting system for the study of RNase P enzymes.

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SUPPLEMENTARY MATERIAL

The RNA Component of RNase P from the Archaeabacterium

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Culture conditions and materials. **E. coli** strain E30 (ATCC 29393) was grown in a medium containing 12 g NaCl, 1 g MgSO4·6H2O, 10 g KH2PO4, 0.5 g KCl, 1.5 g CaCl2·2H2O, 3 g yeast extract, and 5 g glucose per liter of distilled water (10). Cultures were grown at 37°C with vigorous shaking, 100 rpm. The **E. coli** and the **H. marismortui** ribosomal RNA (rRNA) P preparations were generous gifts from the laboratories of Fred Doudna and Myron Pace, respectively. **Saccharomyces cerevisiae**, **Deinococcus** and **E. coli** RNA were obtained from Pharmacia (Piscataway, NJ). The vector pJIB1 and pM2298 and pJIB2 were purchased from Invitrogen (San Diego, CA). T4 DNA ligase, T4 RNA ligase, and [γ-32P]ATP were purchased from Amersham (Buckinghamshire, UK). All other reagents and enzymes used were from Pharmacia. All cultures were grown on an orbital shaker at 26°C. 

Identification of the **H. marismortui** RNase P RNA. In separate experiments, RNase P activity in **H. marismortui** was detected using the previously described colony filter assay (25, 26). 

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by substituting a Hpa I-Hae III restriction fragment containing the first of two tandemly repeated H. volcanii RNAV41 genes from pPY2 (23). This fragment was subsequently subcloned into the expression vector pT72 under the transcriptional control of a T7 RNA polymerase promoter. Transcripts from this gene product at RNA with 3' and 5' nucleotides of 5' and 3' flanking sequences, respectively. This RNA also lacks the 3' terminal CCA residues. Unlike [16] UTP labeled and non-labeled transcripts were synthesized as described previously (24). In vivo synthesized preRNAV41 RNA was electrophoresed through a 6 % denaturing acrylamide gel and detected by autoradiography or by UV shadowing (26). The RNAs were recovered from gel slices by elution at 35°C in elution buffer (100 mM NaCl, 10 mM MgOAc, 0.1 mM EDTA, and 0.1 % SDS) followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). RNA concentrations were estimated from their absorbance at 260 nm. To assay for activity in cellular fractions, the substrate was added directly to 100 μl of each fraction in buffer A and incubated at 37°C for 45 min. The reaction was then terminated with phenol, ethanol precipitated, and electrophoresed through a 6 % denaturing polyacrylamide gel. Reaction products were detected by autoradiography.

For RNA analysis and heteronucleus nucleotidation the gene encoding the H. volcanii RNase P RNA was subcloned as a Max I-Hae III restriction fragment into the Hind III site of the vector pRSV to give the plasmid pRSV. This plasmid the RNase P gene under the transcriptional control of a T7 RNA polymerase promoter. The Pae I Hind III digests left complementary 5' overhang sequences, whereas digestion of the Max I site in the Hind III site required filling in of the 5' overhangs with Klenow fragment prior to blot out digestion (this results in restriction of the Hind III site). T7 RNA polymerase run-off transcripts were prepared from the 1917 inserted pRSV DNA, as described above. The resulting 463 nucleotide transcript, containing a 20-base 5' flanking sequence and a 9-base 3' flanking sequence, was purified on a 5 % denaturing polyacrylamide gel and recovered as described above. Assays of H. volcanii RNase P RNA were performed with the preRNAV41 and the B. subtilis preRNAVAP (25) substrates. RNA analysis standard reactions contained 50 mM Tris-HCl pH 8.0, 0.1% SDS, 0.066 M NaOH, approximately 2.5 ng of [α-32P] ATP-labeled preRNAV41 or preRNAVAP, 20 ng B. subtilis RNase P RNA (from pRSV transcription) and varying concentrations of MgCl2 and NaClO4 in a total volume of 15 μl. Heteronucleus nucleotidation reactions with H. volcanii RNase P RNA and B. subtilis RNase P protein in 10 μl of the following buffer: 50 mM Tris-HCl, pH 8.0, 30 mM MgCl2, 0.05 μM NTP, and 100 mM NaClO4. These reactions were also performed with preRNAVAP. All reactions were incubated at 37°C for 45 min, phenol-extracted, ethanol precipitated, and analyzed on a 6 % denaturing polyacrylamide gel.

Figure 1. Construction of the H. volcanii preRNAV41 substrate clone. Left panel: subcloning the H. volcanii RNAV41 gene from pPY2 into the T7 RNA polymerase expression vector pT72. Restriction sites are: A, Acc I; H, Hind III; B, BamH I; R, Bgl II; Bst, Bst X; E, Eco RI; S, Sal I. The preRNAV41 transcription and the expressed RNase P cleavage products are indicated. Right panel: sequence of the transcribed strand for the gene region of the pPY2 RNAV41 construct. Underlined RNA sequence regions are undefined.

Figure 2. Southern analysis of H. volcanii genomic DNA using RNase P RNA derived cDNA as probe. Lane 1: Max I and lane 2: Max I digested H. volcanii DNA. Hybridizing fragments identified as being of non-chromosomal origin are indicated on the right margin. Fragments are given in kilobases (kb).

Figure 7. Cleavage of B. subtilis preRNAVAP by H. volcanii RNase P RNA. B. subtilis RNase P protein complex. Reactions were performed at 37°C for 10 min in buffer containing 50 mM Tris-HCl pH 8.0, 30 mM MgCl2, 0.05% NP-40 and 100 mM NaClO4. Lane 1, preRNAVAP only. Lane 2, preRNAVAP plus H. volcanii RNase P homology. Lane 3, preRNAVAP, 20 ng (12 μg) H. volcanii RNase P RNA and 2 μg (12 μg) B. subtilis RNase P protein. Each reaction contained approximately 2.5 ng (7000 cpm) of preRNAVAP. Products are indicated. RNAs designated by solid dots are degradation products present in the preRNAVAP preparation.