

ABSTRACT

BARNES, JEFFREY PAUL. Is Mth1483p a subunit of *Methanothermobacter thermoautotrophicus* RNase P? (Under the direction of James W. Brown)

The buoyant density of *M. thermoautotrophicus* RNase P was recently determined to be 1.42 g/mL. This corresponds to RNA to protein ratio of 0.96, indicating that ~93 kDa of protein is present in the holoenzyme. With only 70 kDa of protein identified thus far, additional protein subunits may exist. Recent PSI-Blast searches identified ORF 1483 of *M. thermoautotrophicus* as a homolog of Rpp25, a human RNase P subunit. Mth1483 and Rpp25 were also identified as members of the Alba family of proteins whose members participate in both DNA packaging and RNA interactions. This suggested the possibility that Mth1483p may be a subunit of RNase P in *M. thermoautotrophicus*. To evaluate this, polyclonal antisera was generated against Mth1483p. Western blot analysis of glycerol gradient purified *M. thermoautotrophicus* RNase P showed that Mth1483p did not co-purify with RNase P activity. Also, protein-A agarose beads cross-linked with Mth1483 antibody did not immunoprecipitate RNase P activity. There is no evidence, then, that Mth1483p is an RNase P subunit.

Is Mth1483p a subunit of *Methanothermobacter thermoautotrophicus* RNase P?

by
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Biography

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Ribonuclease P Literature Review

Introduction

In the precursor form, transfer RNAs (tRNAs) are part of contiguous rRNA (ribosomal RNA) transcripts, polycistronic clusters, or in operons with mRNA. In order to reach functional maturity, precursor tRNAs (pre-tRNAs) must undergo cleavage at the 3' and 5' ends. Ribonuclease P (RNase P) is the endoribonuclease that removes the 5' leader sequences from pre-tRNAs (11). RNase P also participates in maturation of other RNAs including 4.5S and 10Sa RNA(30,82) Present in mitochondria, photosynthetic organelles, and all organisms that process tRNAs, the RNase P holoenzyme is comprised of a single catalytic RNA subunit plus a number of protein cofactors that vary in quantity among the bacterial, archaeal, and eukaryotic Domains (39). In all cases studied, the RNA, not the protein, catalyzes the site-specific cleavage reaction (61). In Bacteria and some Archaea, it has been shown that the RNA alone is capable of catalysis *in vitro* (32,62).

Bacterial RNase P

The bacterial RNase P complex consists of a ca. 350-450 nucleotide RNA plus one protein subunit (100-150 amino acids) which is required for activity *in vivo* but is not needed *in vitro* when assayed at higher ionic strength (2,32,37). The protein accounts for only 10% of the mass of the holoenzyme (13). All bacterial RNAs are categorized as either type A, exemplified by that of *Escherichia coli*, or type B, for example that of *Bacillus subtilis* (**Figure 1**) (33). Type A, the ancestral type, includes the RNase P RNAs of Gram-negative

bacteria, high G+C Gram-positive bacteria, and some low G+C Gram-positive bacteria; type B is found in the majority of low G+C Gram-positive bacteria (69).

The RNA subunit has two principal functions: pre-tRNA recognition, and cleavage of the 5' leader sequence. For substrate recognition, contacts with the pre-tRNA are mediated by P8 and L15 loop of the RNA (59,60) as well as the catalytic center. Association of L15 with the 3' NCCA sequence of the pre-tRNA makes the cleavage site accessible and allows the formation of a tighter enzyme-substrate complex (10,60). P8 has been shown to associate with the T-stem loop of the pre-tRNA (59). P1-P4, P7 and domain V of the RNA are integral in catalysis (14,16,26,36,44,45,72). Both P7 and domain V have been shown to bind divalent cations (Mg^{2+} or Mn^{2+}) and recruitment of Mg^{2+} by P4 enables deprotonation of H_2O to hydroxide ions that perform the site specific nucleophilic attack of the pre-tRNA scissile bond to remove the 5' leader sequence (**Figure 2**) (15,61,73,77,85).

Several roles for the function of the protein subunit have been suggested. The protein has been hypothesized to shield the RNA:pre-tRNA complex from electrostatic interference, allowing the complex to fold into the required catalytic conformation (65). The *E. coli* protein subunit has been shown to assist the RNA fold into specific conformations needed to identify non-pre-tRNA substrates (46,47,63). Studies in *B. subtilis* show that addition of the protein relieves product inhibition and enhances RNA:pre-tRNA affinity by a magnitude of 10^4 , thereby greatly improving catalysis (19,50,63,69). This has been attributed to the proteins coordinated contact with the pre-tRNA leader and domain 2 of the RNA (19,50).

Organelar RNase P

RNase P from mitochondria and chloroplasts has proven more difficult to characterize than that of Bacteria, Archaea, and the eukaryotic nucleus. The endosymbiotic

events that initially lead to the evolution of the mitochondria and chloroplast have resulted in a concurrent, continuous transfer of genetic information to the nucleus (34). Mitochondrial RNase P from *Saccharomyces cerevisiae* contains a 490 nucleotide RNA encoded on the mitochondrial chromosome and a 105 kDa protein encoded on the nuclear chromosome that is indispensable for catalysis (7,20,56,58,78). Damage to the RNA during isolation has no effect on catalysis which may indicate the protein is the catalytic unit (57). In other yeast species, the RNA can vary from 140 to 490 nucleotides in size (70,81). It is unclear whether or not the human mitochondrial enzyme contains an RNA subunit (42). The RNA of the mitochondrial enzyme may be identical to the nuclear RNase P RNA (64). This along with glycerol sedimentation values suggests the presence of an RNA (64). However, no mitochondrial encoded RNA gene has been found, and previous findings showing holoenzyme insensitivity to RNA nuclease treatment suggest the absence of an RNA subunit (3,66,67).

Chloroplasts and other photosynthetic organelles originated from cyanobacteria (31). Unlike the RNase P from their bacterial progenitor, the presence of an RNA subunit appears to change from algae to vascular plants (69). In *Cyanophora paradoxa*, a probable intermediate in photosynthetic organelle development, the cyanelle was found to contain a bacterial-like RNA subunit that copurifies with activity (7). Holoenzyme activity is removed with nuclease treatment suggesting that the RNA is needed for catalytic activity (7). In contrast, RNase P from spinach chloroplasts possesses properties suggestive of a complex lacking RNA (27,29). RNA does not copurify with activity, enzyme activity is not altered by nuclease digestion, and a CsCl density of 1.28 g/mL all suggest a protein- only composition (27,29). However, the components of this enzyme have yet to be identified.

Nuclear RNase P

RNase P from the eukaryotic nucleus consists of numerous protein subunits and a single RNA that is not catalytically active *in vitro* (28,41,82). The eukaryotic holoenzyme content is roughly 50-70% protein and Cs₂SO₄ buoyant densities range from 1.28 g/mL-1.4 g/mL (6,13,24,43,48,75). The eukaryotic RNA contains many of the important conserved regions found in the bacterial consensus model (14). The RNase P is inactivated when treated with nucleases demonstrating that the RNA is required for activity (1,24,43,48,75)

The variability in eukaryotic RNase P RNA structure is most evident in the RNAs from the yeast *Saccharomyces cerevisiae* and *Homo sapiens* (**Figure 3**). In humans, the RNase P holoenzyme consists of ten protein subunits: hPop1, Rpp29, hPop5, Rpp20, Rpp30, Rpp21, Rpp38, Rpp40, Rpp25, and Rpp14 (82). *S. cerevisiae* RNase P contains nine protein subunits: Pop1, Pop3, Pop4, Pop5, Pop6, Pop7, Pop8, Rpp1, and Rpr2 (40). Genetic experiments have shown that the RNA and all nine protein subunits are required for RNase P activity (13,18,23,51,54,76). Only Pop1p and Pop4p interact directly with the RNA, but their function and those of the remaining subunits have yet to be determined (40). Pop1p, Pop4p, Pop5p, Pop7p, Rpp1p, and Rpr2p are homologous to human nuclear subunits hPop1p, Rpp29p, hPop5p, Rpp20p, Rpp30p, and Rpp21p respectively (82). Moreover, all yeast protein subunits except Rpr2 are also subunits of RNase MRP (RNase mitochondrial RNA processing), an enzyme that cleaves rRNA precursors in nucleoli (13,17,55,68,71).

Archaeal RNase P

Early data on the RNase Ps of *Sulfolobus solfataricus* (a thermoacidophile) and *Haloferax volcanii* (a halophile) suggested a very different holoenzyme composition (22). The RNase P from *S. solfataricus* was unaffected by nuclease treatment and found to have a buoyant density in Cs_2SO_4 of 1.27 g/mL, characteristics of a composition mostly of protein and lacking an essential RNA (21). In contrast, the *H. volcanii* RNase P buoyant density of 1.61 g/mL and susceptibility to nuclease treatment implied a composition largely of RNA (22). It is now known that these two enzymes are very similar, consisting of an RNA and at least four associated proteins. The RNase Ps from two methanogens, *Methanothermobacter thermoautotrophicus* and *Methanocaldococcus jannaschii*, were recently found to have buoyant densities of 1.42 g/mL and 1.39 g/mL respectively (4).

RNase P from Archaea has features of both the bacterial and eukaryotic nuclear enzymes. Archaeal RNase P RNAs fall into two very different structural classes, type A and type M (**Figure 4**). Type A, for example that of *M. thermoautotrophicus*, resembles the bacterial type A RNA but lacks P13, P14 (also absent in some bacterial type A RNAs), and most notably P18 (38). Type M, found only in the methanococci and *Archaeoglobus*, is in addition missing P6, P8, L15, P16, and P17, the key structural parts needed for tRNA interactions outside of the catalytic center (38). Also evident is a longer P7, P9, and P10/P11. Type A RNAs are the only archaeal RNAs that are catalytically active, and even in the absence of protein this requires much higher concentrations of ammonium acetate and Mg^{2+} than that of the bacterial RNA (62). The apparent reduced structure and lack of compensatory features of type M RNAs do not permit catalysis in the absence of protein (62).

Despite similarities between the archaeal and bacterial RNase P RNAs no archaeal protein with homology to a bacterial protein has been found. However, four proteins from *M.*

thermoautotrophicus, Mth11p, Mth687p, Mth688p, and Mth1618p were identified that are apparently homologous to *S. cerevisiae* proteins Pop4p (Rpp29), Pop5p (hPop5), Rpp1p (Rpp30), and Rpr2p (Rpp21) respectively (35). Western Blots showed that each archaeal protein co-purified with activity and antisera to each protein immunoprecipitated RNase P activity (35). This established that Mth11p, Mth687p, Mth688p, and Mth1618p are RNase P subunits (35). However, it was unclear which subunits were essential for activity. Recently, RNase P activity from two Archaea, *Pyrococcus horikoshii* and *M. thermoautotrophicus*, was reconstituted *in vitro* from the RNA and these four proteins (9,49). In *P. horikoshii*, reconstitution trials using the RNase P RNA and proteins Ph1481p (hPop5), Ph1601p (Rpp21), Ph1771p (Rpp29), and Ph1877p (Rpp30) revealed that only Ph1481p, Ph1601p, and Ph1771p were essential for activity (49). However, RNase P activity dramatically increased upon inclusion of Ph1877p (49). A similar experiment using *in vitro* transcribed *M. thermoautotrophicus* RNase P RNA showed that all four proteins Mth11p (Rpp29), Mth687p (hPop5), Mth688p (Rpp30), and Mth1618p (Rpp21) were required for activity (9).

The deduced molecular masses of Mth11p, Mth687p, Mth688p and Mth1618p are 10.7, 14.6, 27.7, and 17.0 kDa respectively. Given a buoyant density of 1.42 g/mL corresponding to an RNA:protein ratio of 0.96, and a 98 kDa RNA, there must be ca. 93 kDa of protein in the RNA:protein complex. With only 70 total kDa of protein accounted for, either the ratio of one of protein subunits to the RNA is not 1:1 (e.g. two Mth11 proteins required per single RNase P RNA to form a functional holoenzyme) or there is 23 kDa of protein yet to be identified in *M. thermoautotrophicus*.

Mth1483/Alba

Alba is a chromatin binding protein present in a number of Eukaryotes and thermophilic and hyperthermophilic Archaea (8,25,80). Alba is best characterized in the crenarchaeon *Sulfolobus*, where it is known to densely coat DNA and induce negative supercoiling(12,25,53,83). This activity has been shown to protect the DNA from nuclease digestion but no significant compaction of the DNA has been observed (53). *In vitro*, it was also demonstrated that Alba can function as a transcriptional repressor upon deacetylation by ssSir2, a homolog of the eukaryotic Sir2 chromatin protein (8).

Alba is a member of a large family of proteins whose members carry out two very different roles: one in chromosomal binding and the other in RNA functioning (5). Interestingly, Alba was shown to contain the IF3 carboxy terminal domain, an RNA binding motif present in the YhbY group of RNA binding peptides (**Figure 5**), DNase I, ribosomal protein S8, RNA 3' terminal phosphate cyclase, and prolyl tRNA synthetases (52,79,84) On this basis, it is hypothesized that Alba was initially an RNA binding protein that retained this function in some evolutionary lines but was adapted as a DNA binding protein in other lines (5).

In eukaryotes, PSI-Blast searches identified the human RNase P subunit Rpp25 and the yeast RNase P/MRP subunit Pop7 as Alba homologs (5). This suggests that archaeal Alba, in addition to its well established role in DNA packaging, be a subunit of RNase P (5). ORF (open reading frame) 1483 of *M. thermoautotrophicus*, an Alba homolog, was shown to be ~30% similar to Rpp25 (**Figure 6**). However, unlike Mth11p, Mth687p, Mth688p, and Mth1618p, no homolog of Mth1483p has been identified in yeast. Taken collectively with the prospect of unidentified RNase P subunits in Archaea, it seemed likely that Mth1483p may be a subunit (although not an essential one given the previously discussed reconstitution

data) in the RNase P of *M. thermoautotrophicus*. In this study we show that Mth1483p neither copurifies with RNase P activity, nor does anti-Mth1483p serum immunoprecipitate RNase P activity. We conclude that there is no evidence that Mth1483p is a subunit of RNase P or plays a role other than the DNA packaging attributed to other Alba proteins.

A. *Escherichia coli*: Type A Bacterial RNase P RNA

B. *Bacillus subtilis*: Type B Bacterial RNase P RNA

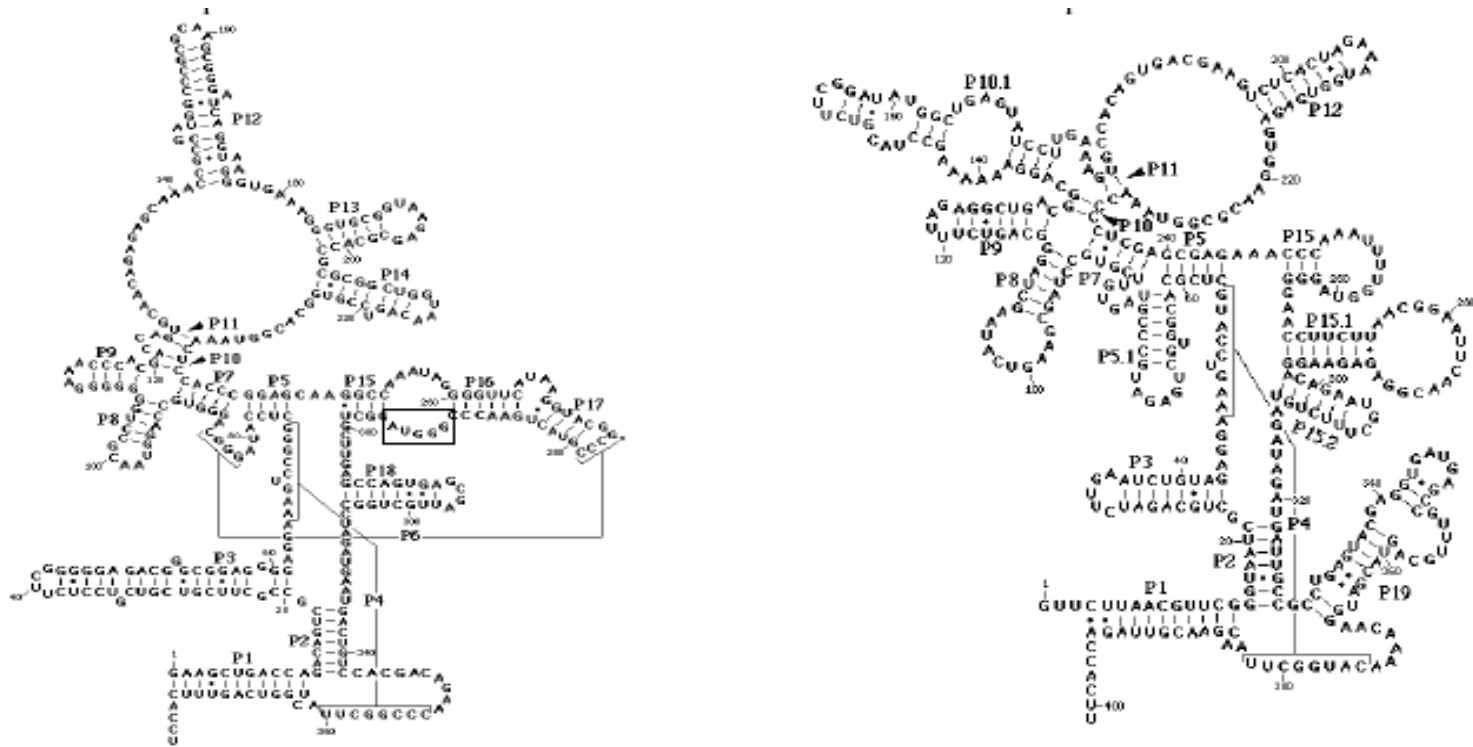


Figure 1. Type A and type B bacterial RNase P RNA

(A) Type A bacterial RNA represented by *E. coli* and **(B)** Type B bacterial RNA represented by *B. subtilis*.

Figure 10 Reference:

a. Pace, N.R. and Brown, J.W. (1995) *J. Bacteriol*, **177**, 1919-1928.

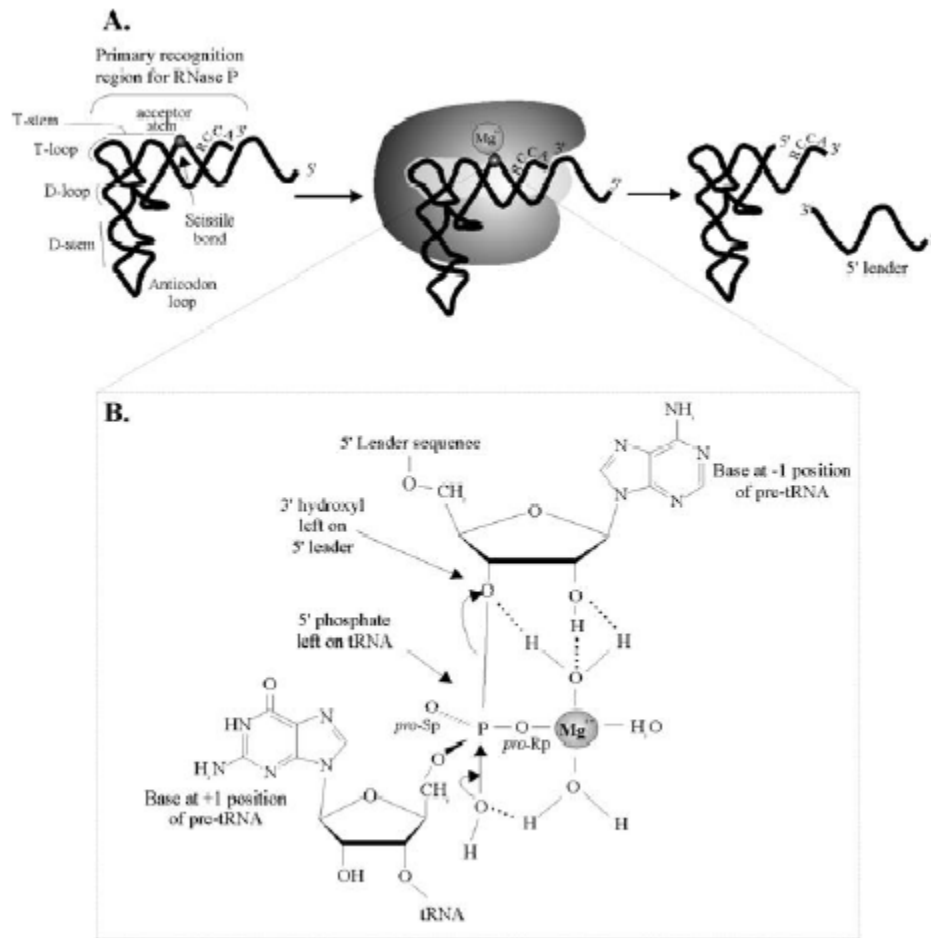


Figure 2. RNase P Mechanism of Cleavage

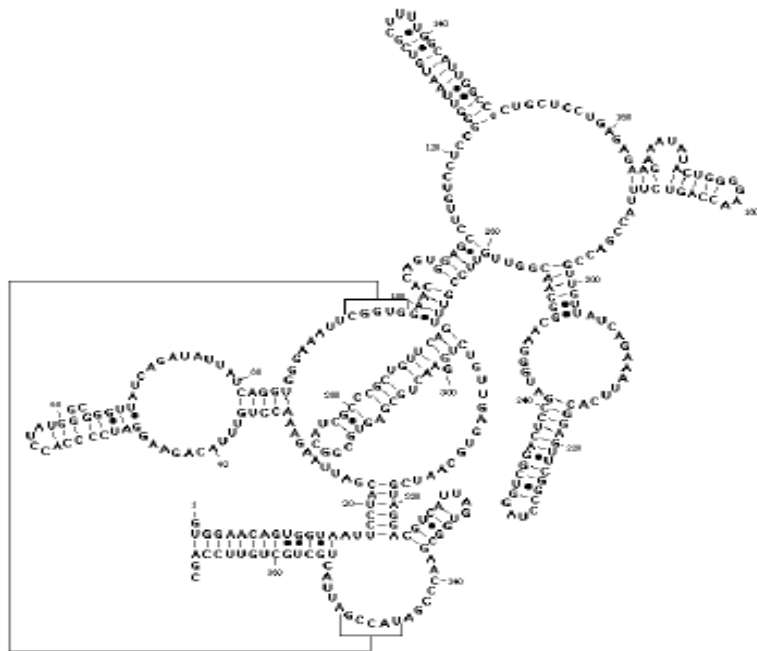
A. RNase P cleavage occurs in three steps. The RNase P holoenzyme recognizes the pre-tRNA substrate by association of P8 with the pre-tRNA stem loop and L15 with the 3' CCA sequence of the pre-tRNA. Next, the coordination of Mg²⁺ ions for site specific cleavage, and lastly dissociation of the mature tRNA and the 5' leader sequence.

B. Illustration of the nucleophilic attack of the pre-tRNA scissile bond. Shown is the use of Mg²⁺ to deprotonate water to hydroxide ions needed to cleave the pre-tRNA scissile bond.

Figure 2 References:

a. Smith, D., and Pace, N.R. (1993) *Biochem.*, **32**, 5273-5281.

A. *Saccharomyces cerevisiae* RNase P RNA



B. *Homo sapiens* RNase P RNA

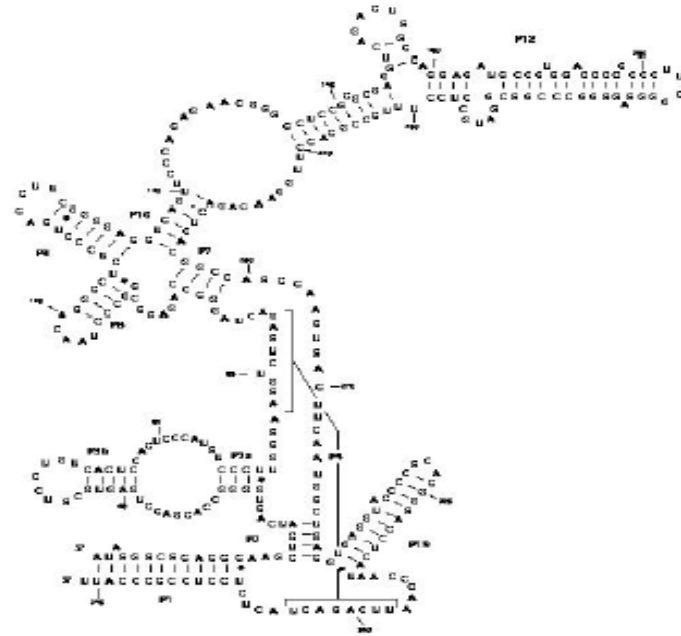
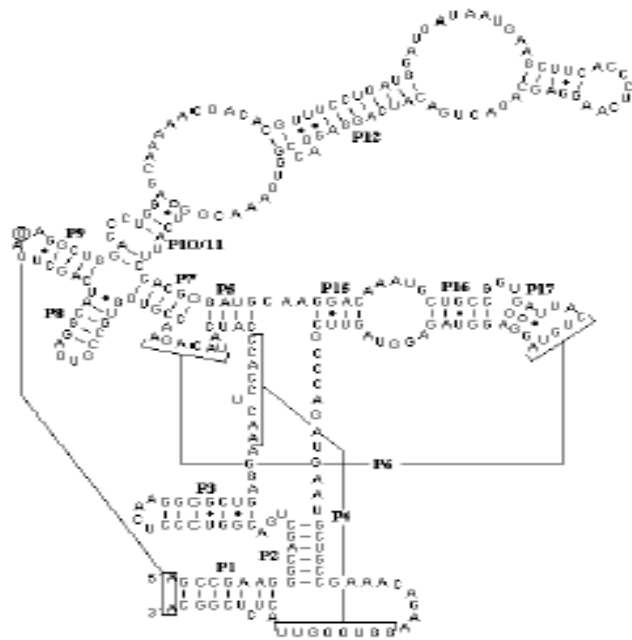


Figure 3. RNase P RNA from **(A)** *S. cerevisiae* and **(B)** *Homo sapiens*

Figure 3. References

a. Frank, D.N., Adamidi, C., Ehringer, M.A., Pitulle, C., and Pace, N.R. (2000) *RNA*, **6**, 1895-1904.

A. : Type A Archaeal RNA
Methanothermobacter thermoautotrophicus RNA



B. Type M Archaeal RNA
Methanocaldococcus jannaschii

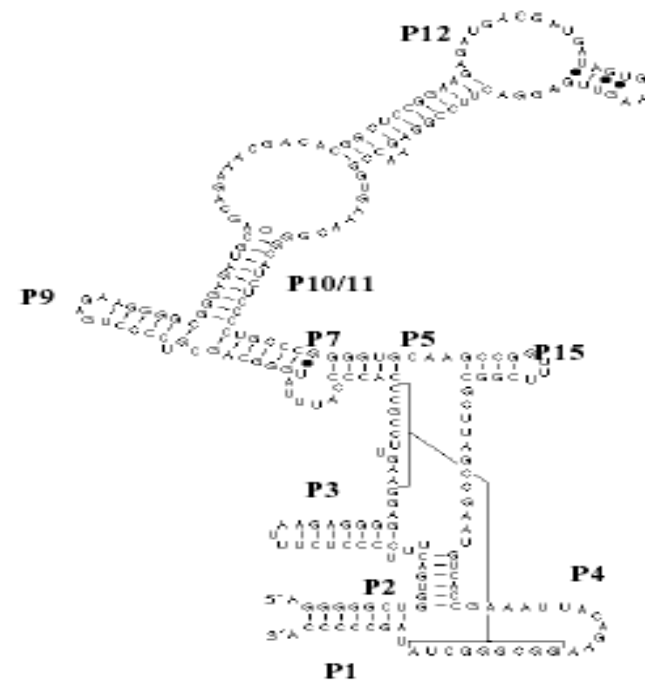


Figure 4. Type A and type M archaeal RNase P RNA

(A) Type A archaeal RNA represented by *M. thermoautotrophicus* and **(B)** Type M archaeal RNA represented by *M. jannaschii*.

Figure 4. References:

Harris, J.K., Haas, E.S., Williams, D., Frank, D.N. and Brown, J.W. (2001) *RNA*, 7, 220-232.

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>gi|15679480|ref|NP_276597.1| conserved protein [Methanothermobacter thermautotrophicus]
gi|7482138|pir||F69064 conserved hypothetical protein MTH1483 - Methanobacterium
thermoautotrophicum (strain Delta H)
gi|2622599|gb|AAB85958.1| conserved protein [Methanothermobacter thermautotrophicus str.
Delta H]
Length = 92

Score = 44.0 bits (103), Expect = 0.002
Identities = 19/95 (20%), Positives = 32/95 (33%), Gaps = 19/95 (20%)

Query: 48 NLMAFATASMAQPATRAIVFSGCGRATTKTVTCAEILKRRLAGLHQVTRLRYRSVREVWQ 107
          N +      M   T  ++   G A ++ V AEI++ R           ++   +
Sbjct: 17 NYVLAVVTQMN-GGTSEVILKARGIAISRAVDVAEIVRNRFP---DIQIENIDI----- 67

Query: 108 SLPPGPTQGQTPGEPAAASLSVLKNVPLAILLSKD 142
          T+      E A+      NV + I L KD
Sbjct: 68 -----CTEEIIGNEGTAT-----NVSAIEIQLRKD 92

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Figure 5. Results of PSI-Blast Search using Rpp25 (AAK5443.1) showing similarity of Mth1483 to Rpp25.

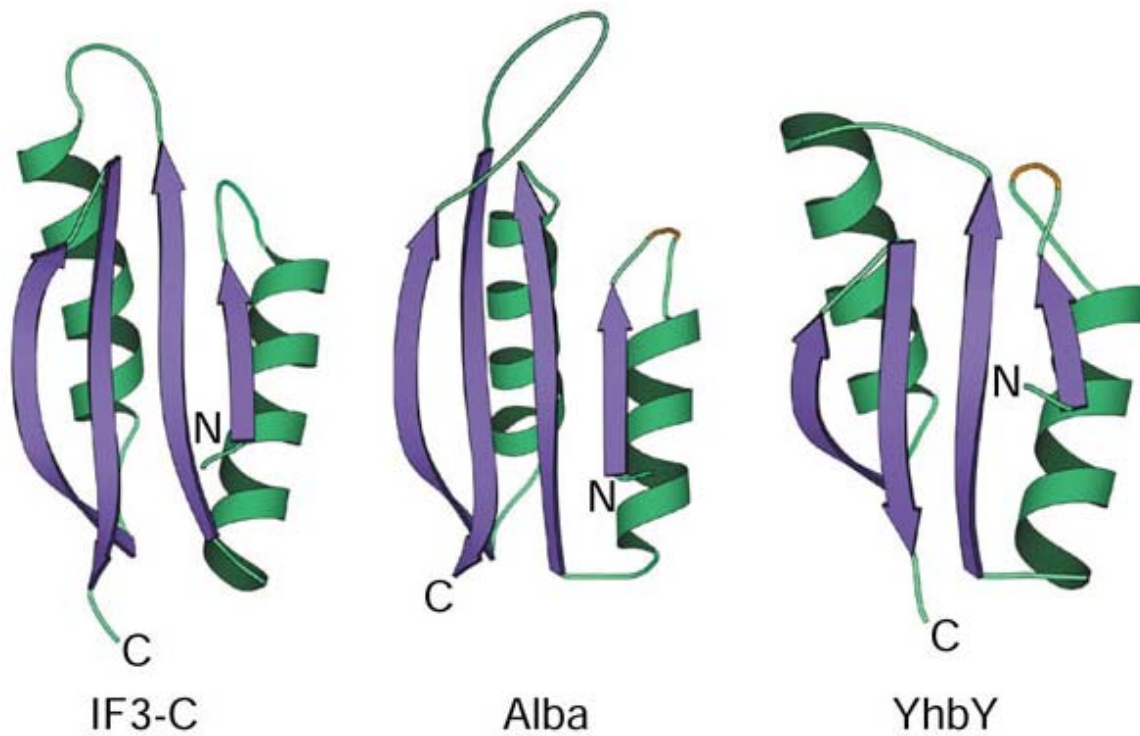


Figure 6. Presence of IF3-C fold in Alba

Shown here is the presence of the IF-3 fold, an RNA binding motif, in Alba and the YhbY group of RNA binding peptides.

Figure 6 References:

Aravind, L., Lakshminarayan, M.I. and Anantharaman, V. (2003) *Genome Bio.*, 4, R64-R64.9.

Materials and Methods

***In vitro* transcription of ^{32}P -GTP labeled *Bacillus subtilis* pre-tRNA^{Asp}**

Plasmid pDW128 containing the gene encoding *B. subtilis* pre-tRNA^{Asp} driven by the T7 promoter was linearized by digestion with *Bst*NI (Promega). The linearized product was purified using the Qiagen QIAquick PCR clean up kit and eluted in 30 μL . ^{32}P -labeled pre-tRNA^{Asp} was generated *in vitro* by run off transcription in a reaction consisting of $\sim 1\mu\text{g}$ of linearized pDW128, 10 μL of 5X Promega Optimized Transcription Buffer, 5 μL of 5mM mix of rATP, rCTP, rUTP (Promega), 1 μL of 5mM rGTP (Promega), 20 μL of α ^{32}P -GTP (ICN), and 2.5 μL of Promega T7 RNA polymerase (20 units). Reagents were thoroughly mixed and incubated at 37°C for 3 hours. The reaction products were separated by electrophoresis on an 8% urea-polyacrylamide gel and visualized by autoradiography. Labeled pre-tRNA substrate migrated slightly under the bromocresol green dye, and was excised from the gel, the gel slice was diced into pieces with a clean razor blade, and the RNA was eluted in 6-10 volumes of 10mM Tris (pH 8.0), 1mM EDTA and 0.1% SDS with shaking overnight at room temperature. The eluted ^{32}P -GTP pre-tRNA was quantitated using the Beckman LS 6500 scintillation counter.

RNase P cleavage assays

Enzyme samples were assayed for RNase P activity in 10 μL reactions consisting of 0.5 μL of ^{32}P -labeled pre-tRNA (ca. 13 kcpm), 1 μL 10X RNase P buffer (500mM Tris-Cl pH 8.0, 100mM MgCl₂), 2 μL 5M ammonium acetate, and DEPC treated water (Fisher) up to 10 μL . Reactions were incubated at 50°C for 30 minutes (unless otherwise specified), and products

were separated by electrophoresis in 8% urea-polyacrylamide gels and visualized using autoradiography.

Cloning and expression of Mth1483p

Cloning and expression of Mth1483p was done by Mark Foster's group at Ohio State University. The plasmid carrying the Mth1483 gene was obtained from Univ. of Toronto (Arrowsmith group). This plasmid (Amp⁺) was introduced into *E. coli* cells (BL21 DE 3) along with a helper plasmid, ArgU (Kan⁺) in order to boost protein expression. These doubly-transformed cells were then inoculated in an LB starter culture which was grown at 37°C overnight. A fresh 1L LB media was inoculated with 10 ml of the overnight culture and was grown at 37°C to an OD of 0.6 at which time protein expression was initiated by the addition of IPTG (final conc. 1mM). The culture was allowed to grow for another 4-6 hours at 37°C and then pelleted by centrifugation at 4°C. The cells were stored frozen (-20 C).

Frozen cells were thawed on ice for 30 min and then resuspended in the lysis buffer (50 mM Phosphate pH 7.4, 500 mM NaCl, 10 mM imidazole). The cells were sonicated for 10 min on ice with intermittent short breaks. The soluble fraction was separated from the insoluble fraction by centrifuging the lysate for 30 min at 12000 rpm in a SS-34 rotor. The soluble fraction was incubated at 50°C for 20 min. Mth1483p remains soluble under this condition whereas the host proteins were denatured. After removing the precipitate by centrifugation, the supernatant was loaded onto a 5 mL Chelating-HiTrap column charged with nickel and equilibrated with lysis buffer. Weakly bound proteins were washed off with lysis buffer containing 120 mM imidazole. The column was then washed with lysis buffer to remove excess imidazole from the previous wash. Mth1483p was eluted by cleavage of the His-tag. Thrombin was dissolved in 5 ml of the lysis buffer to a final concentration of 3

units/ml and then loaded onto the Hitrap column (5ml). The column was incubated at the room temperature overnight and then washed with 5ml of the lysis buffer. This final wash contained ~95% pure Mth1483p with no His-tag.

Production of antisera to Mth1483p

1 mL of a 1mg/mL solution of Mth1483p was sent to CoCalico Biologicals for polyclonal antisera production. 500µg of peptide was mixed with Complete Freud's Adjuvant and injected into rabbits. Three booster injections were administered two, three, and seven weeks after initial inoculation. A pre-bleed was taken before the first inoculation then one and two months after inoculation.

Crude purification of *M. thermoautotrophicus* RNase P

Lysis and preparation of cellular material

-According to Andrews et al. (4)

Approximately 7 g of *M. thermoautotrophicus* cell paste was provided by The Ohio State University fermenting facility. 1.8 g of frozen *M. thermoautotrophicus* cell paste was ground in dry ice for 30 minutes using a mortar and pestle. The ground material was resuspended in 3-5 mLs of TMGN-100 (50mM Tris (pH 7.5), 10mM MgCl₂, 5% glycerol, 0.1mM DTT, 0.1mM PMSF, and N-100 denotes 100mM NH₄Cl) plus 10 µg/mL DNase I (Promega) per 3g of cell material. The material was passed three times through a French Press at 20,000 psi internal pressure and centrifuged at 16,000 X g for 30 minutes at 4°C. The supernatant was transferred into 3-5 inches of Snakeskin dialysis tubing (Pierce, 3500 MWCO) and dialyzed overnight in 2L of TMGN-20 with one replacement of buffer.

DEAE-Trisacryl Chromatography

-According to Andrews et al. (4)

5 mLs of DEAE-Trisacryl Plus M (Sigma D-2540) was loaded into a Pharmacia Econo-column and packed with 10 mL of TMGN-20. The crude cellular material was loaded onto the column and washed with 10 column volumes of TMGN-20. Samples were eluted using a SG-15 gradient maker (Hoefer) that produced a 20 mL gradient of 20mM to 1000mM NH₄Cl. Ca. 0.75 mL fractions were collected into 1.5 mL microcentrifuge tubes and 5 µL from each fraction was assayed 60 minutes for RNase P activity.

Glycerol Gradient Centrifugation

-According to Andrews et al. (4)

The 10% and 40% glycerol was prepared in TMGN-500 containing 0.025% Nonidet P-40 (Sigma). 4.8 mL of 10-40% glycerol gradient was prepared using a SG-15 gradient maker (Hoefer Pharmacia Biotech) in ½ x 2 inch polyallomer centrifuge tubes (Beckman, 326819) and overlaid with 200µL of the active DEAE fraction. Samples were centrifuged in a Sorvall AH-60 swinging bucket rotor at 95,000 X g for 7.5 hours at 4°C. Ca.150 µL fractions were collected and assayed 60 minutes for RNase P activity.

Western Blot for detection of Mth1483p

Five microliters of each active glycerol fraction and 50ng of Mth1483p were loaded onto a 12.5% separating/5% stacking SDS polyacrylamide gel and electrophoresed at 100 volts. The stacking gel was removed and samples were transferred to a nitrocellulose membrane (Micron Separations Inc.) under a constant 23 volts for 1 hour using the X-cell II blot module (Invitrogen). Membranes were blocked overnight with shaking at 4°C in 100 mL of 3% dry milk made in TBS (10mM Tris, 150mM NaCl, pH 6.5). Nitrocellulose membranes were probed with 10 mLs of a 1:1000 dilution of primary anti-Mth1483 serum made in antibody

incubation buffer (TBS, 30 μ L Tween-20) for 2 hours with shaking (320 rpm) at room temperature. The blots were washed in three exchanges of 100 mL of TBS for 10 minutes each and probed with 10 mLs of a 1:100,000 dilution of HRP-conjugated goat anti-rabbit IgG (Supersignal West Pico Rabbit IgG Detection Kit, Pierce #34083) made in antibody incubation buffer for 2 hours with shaking (320 rpm) at room temperature. Blots were washed three times with 100 mL of TBS-T (TBS, 1 mL Tween-20) for 10 minutes each and once with TBS for 10 minutes. Membranes were incubated for 5 minutes in 3 mL of each SuperSignal reagent (Pierce) and exposed to chemiluminescent film (Biomax ML) for 5 minutes.

Immunoprecipitation of RNase P using antisera to Mth1483p

-According to Hall, T and Brown, J.W. (35)

Each immunoprecipitation consisted of an independent immune and pre-immune reaction. For each reaction, 20 mg of protein-A agarose beads (Sigma P-3391) were swollen by incubation in Buffer A (0.02 M NaH_2PO_4 , 0.15 M NaCl, pH 8.0) for 45 minutes on ice. The beads were pelleted for 1 minute in a table-top microcentrifuge and the supernatant discarded. For specific immune reactions, 500 μ L antisera was added to the swollen beads and incubated overnight at 4°C with shaking. For the pre-immune reactions, 400 μ L of pre-immune sera was added to the swollen beads and incubated overnight at 4°C with shaking. Immune and pre-immune beads were pelleted, washed twice with 1 mL of 200mM sodium borate, and resuspended in 1 mL of sodium borate. Ca. 5.1 mg of dimethyl pimelimidate (Sigma D-8388) was added to each reaction and mixed for 30 minutes at room temperature. Beads were pelleted, washed with 1 mL of 200mM ethanolamine pH 8.0, and resuspended in 1 mL of the same buffer for 2 hours with shaking. The beads were washed three times with 1

mL of protein-A buffer (10 mM Tris, 500mM NaCl) and then washed three times with 1 mL of TMGN-100 containing 0.025% NP-40. Beads were resuspended in 400 μ L of the same buffer and 30 μ L of glycerol gradient purified RNase P was added to each reaction. The slurries were mixed overnight at 4°C with shaking. The beads were pelleted and the supernatant or flow-through was collected. The beads were washed four times with 1 mL of TMGN-100. 20 μ L of TMGN-100 heated to 72°C was added to the reactions and each was incubated at 72°C for 30 seconds. Beads were pelleted and the supernatant collected as elution 1. This was repeated two more times for elutions 2 and 3. Three microliters of the bead slurry, flow-through, and elutions were tested for RNase P activity.

Results

Purification of *M. thermoautotrophicus* RNase P

1.8 grams of frozen cell paste was lysed and the cellular debris was removed by centrifugation. The supernatant was dialyzed and bound to a 5 mL DEAE-Trisacryl matrix. The material eluted at ~200mM to 920mM NH₄Cl and thirty-two fractions were collected and assayed for RNase P activity (**Figure 7**). The peak of RNase P activity was eluted at 200mM - 440mM NH₄Cl. Fifty microliters each from DEAE fractions 9-12 were pooled for glycerol gradient centrifugation. Thirty-nine fractions were collected and assayed for RNase P activity (**Figure 8**). Activity was detected at the end of the gradient in fractions 25, 27, 29, 31, 33, and 35 with the peak of activity occurring in fraction 29.

Mth1483p does not co-purify with RNase P activity (Figure 9)

Mth1483p was identified in glycerol gradient fractions 29, 31, 33, 35, 37, and 39, with the highest level of Mth1483p detected in fraction 37. Mth1483p was not found in fractions 25 and 27. Comparison with the respective levels of RNase P activity of each fraction showed that the peak of RNase P activity did not correspond with the peak of Mth1483p. In fraction 29, where the peak of RNase P activity was identified, almost no Mth1483p was found. Conversely, in fraction 37, where the highest amount of Mth1483p was identified, no RNase P activity was detected.

Antibodies to Mth1483p did not immunoprecipitate RNase P activity

Protein-A beads bound with antibody against Mth11p, a known RNase P protein, immunoprecipitated RNase P activity from glycerol gradient purified RNase P (**Figure 10**).

In the immune sera reactions, the majority of RNase P activity was detected in the beads and the elutions. Practically no RNase P activity was evident in the flow-through. In the pre-immune sera reactions, the majority of RNase P activity was found in the flow-through with negligible amounts of activity detected from the beads and the heated elutions.

Protein A beads bound with antibody to Mth1483p did not immunoprecipitate RNase P activity (**Figure 11**). The bulk of RNase P activity was found in the flow-through and only minute amounts of activity were present in the eluted fractions. This was similar to the pre-immune sera reactions in which the bulk of RNase P was likewise retained in the flow-through with very little RNase P activity detected in the elutions.

Conclusions

Using western blot and immunoprecipitation methods, we find no evidence that Mth1483p is physically associated with RNase P in *M. thermoautotrophicus*. Western blot results using polyclonal antisera show that Mth1483p is not present in all active glycerol gradient purified RNase P fractions. The peak of detected Mth1483 protein levels do not correspond with the peak of RNase P activity, demonstrating Mth1483p does not co-purify with enzymatic activity. We have shown that protein-A agarose beads bound with antibody against Mth11p, an essential associated RNase P subunit, immunoprecipitates RNase P activity from glycerol gradient purified samples as demonstrated in a previous study (35). In contrast, protein-A agarose beads bound with anti-Mth1483p antibody did not retrieve the RNase P from glycerol gradient purified samples, indicating Mth1483p does not interact with the RNase P holoenzyme. Collectively, the western blot and immunoprecipitation data show that Mth1483p is not physically associated with the *M. thermoautotrophicus* RNase P. These findings support reconstitution data of *Pyrococcus horikoshii* RNase P in which addition of the Mth1483p homolog did not increase activity beyond that observed with the four required proteins alone (personal communication, Venkat Gopalan).

Although Mth1483 is clearly an Alba homolog, it is not an Alba RNA binding protein. While present eukaryotic Alba homologs appear to have retained their original RNA binding functions, archaeal homologs seem to have lost this RNA function, having been adapted largely for DNA binding and chromatin packaging (5). This is supported by the large number of distinct chromatin binding proteins identified across the Euryarchaea and Crenarchaea lineages, indicating multiple independent recruitments among the different Archaea (5).

On this basis it is probable that Mth1483 is participating in some aspect of DNA binding in *M. thermoautotrophicus*. The small degree of similarity to Rpp25 suggests that Mth1483 has retained a small aspect of this ancestral RNA binding function.

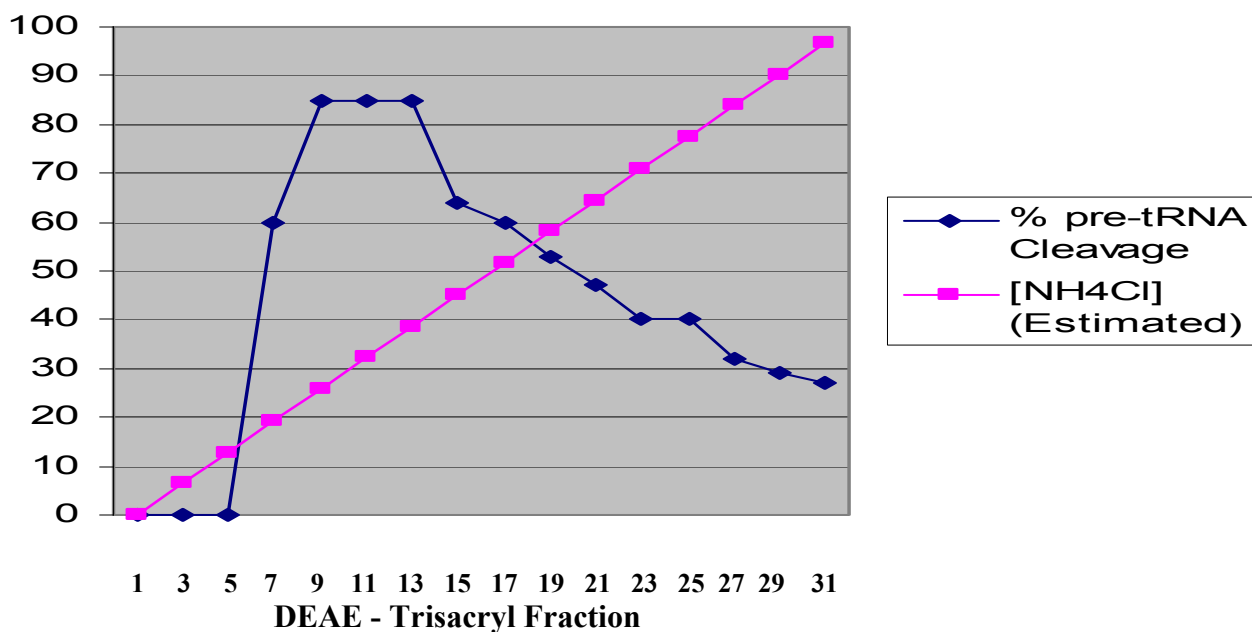


Figure 7. RNase P activity of DEAE – Trisacryl fractions

M. thermoautotrophicus cell lysate was bound to a 5 mL DEAE – Trisacryl column, washed, and fractions eluted with a linear gradient of 20mM – 1000mM NH₄Cl. Fractions were assayed for RNase P activity at 50°C for 60 minutes. Fractions 9-12 were used for further purification.

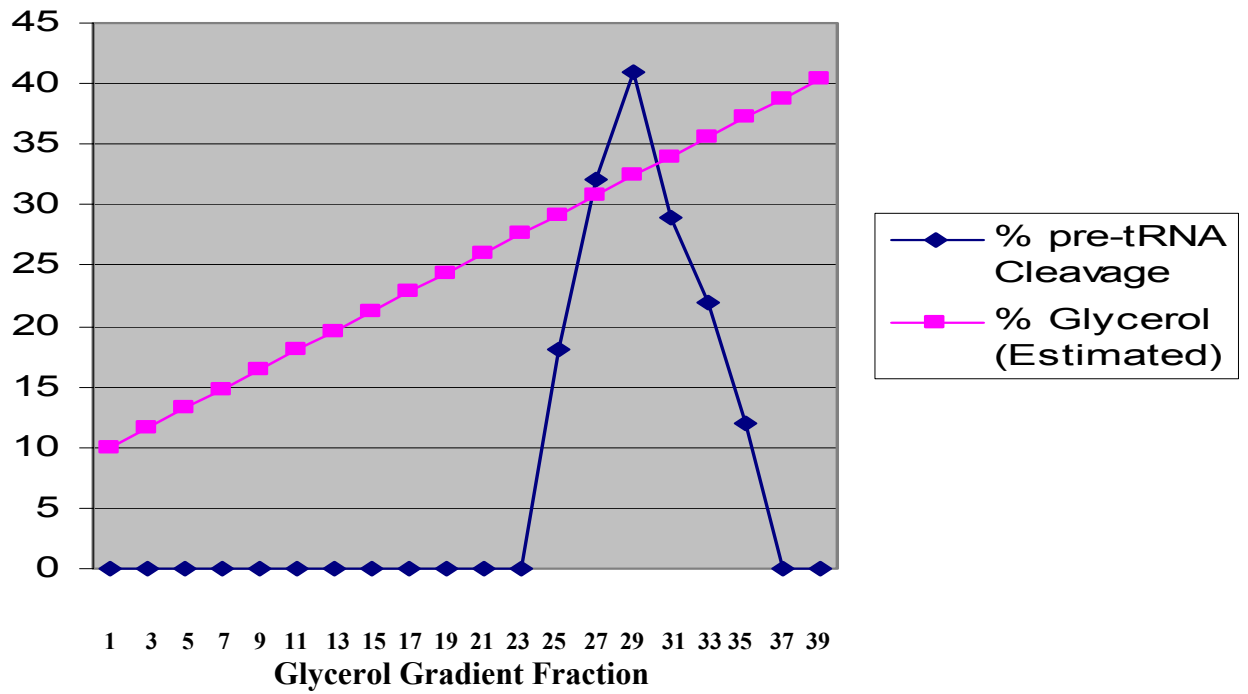


Figure 8. RNase P activity of glycerol gradient fractions

Aliquots of active DEAE fractions were layered onto a 10-40% glycerol gradient and centrifuged at 95,000 x g for 7.5 hours. Fractions were collected and assayed for RNase P activity at 50°C for 60 minutes.

Mth1483p

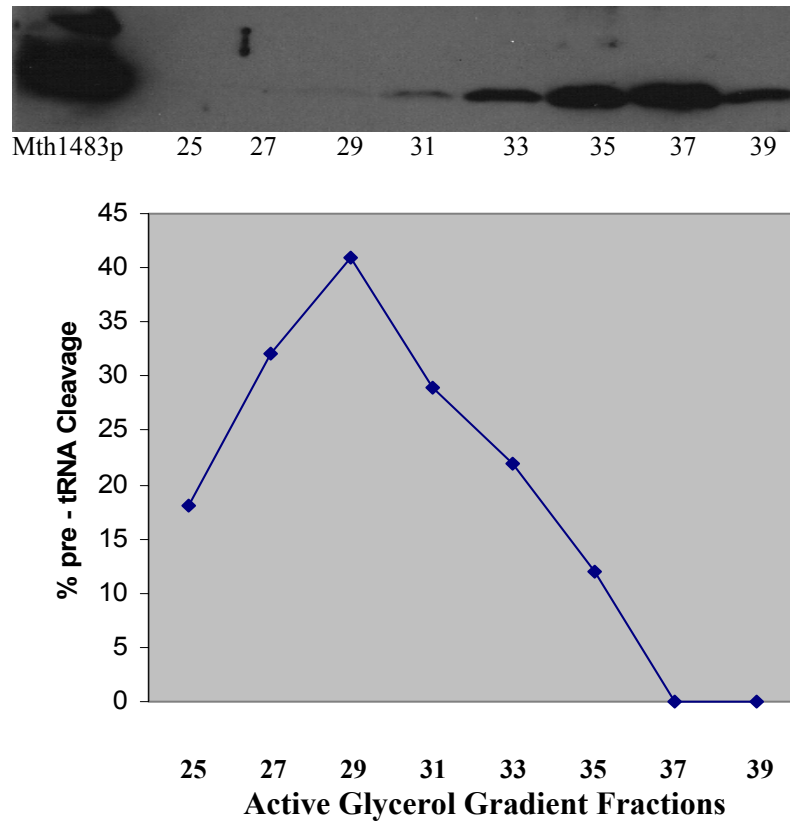
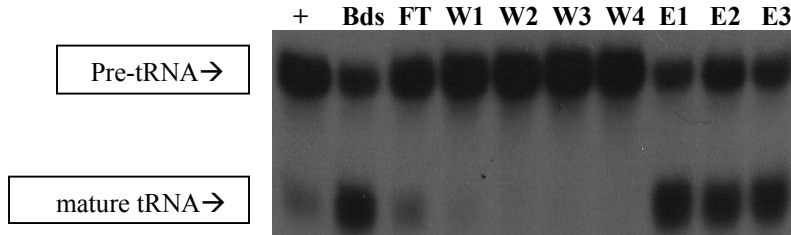


Figure 9. Mth1483p does not copurify with RNase P activity

5 μ L of specified active glycerol gradient fractions were probed with a 1:1000 of antisera to Mth1483p. 50 ng of purified Mth1483p was run as a control. The level of RNase P activity in each fraction is shown with the corresponding level of Mth1483p detected in the western blot.

A. Immune Sera



B. Pre-immune Sera

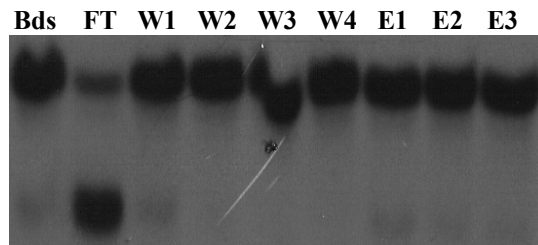


Figure 10. Immunoprecipitation of RNase P activity with antibody to Mth11p

Immune and pre-immune sera to Mth11p was bound to protein-A agarose beads and mixed with glycerol gradient purified RNase P. Beads were washed four times and eluted three times using TMGN-100 heated to 72°C. Beads, washes, and elutions were assayed for 30 minutes at 50°C for RNase P activity. Abbreviations: + = assay of 1µl glycerol gradient fraction; Bds = beads; W1, W2 W3, W4 = washes 1-4; E1, E2, E3 = elutions 1-3.

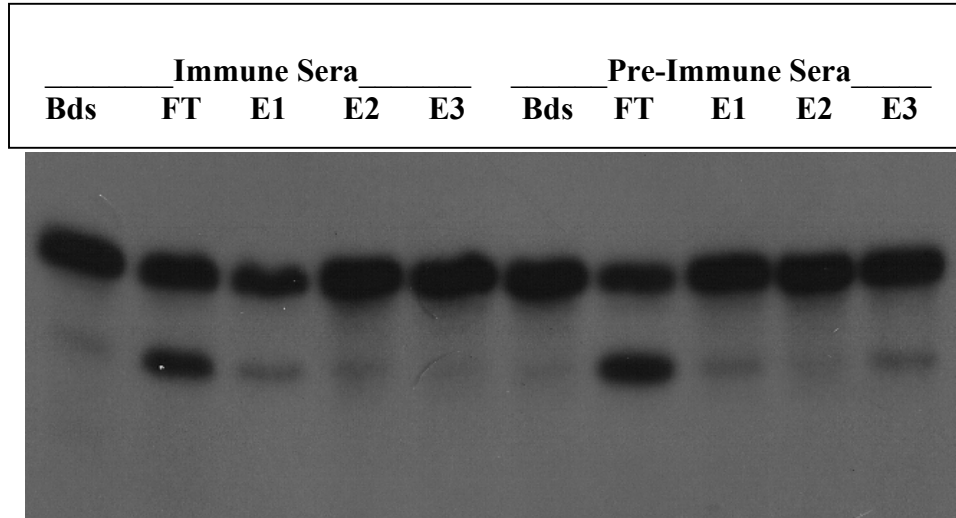


Figure 11. Antibody to Mth1483p does not immunoprecipitate RNase P activity

Immune and pre-immune sera to Mth1483p was bound to protein-A agarose beads and mixed with glycerol gradient purified RNase P. Reactions were washed four times and eluted with three times with TMGN-100 heated to 72°C. Beads and elutions were assayed for RNase P activity at 50°C for 30 minutes.

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