Archaeal RNase P has multiple protein subunits homologous to eukaryotic nuclear RNase P proteins

THOMAS A. HALL1,2 and JAMES W. BROWN†
1Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615, USA

ABSTRACT
Although archaean RNase P RNAs are similar in both sequence and structure to those of Bacteria rather than eukaryotes, and heterologous reconstitution between the Bacillus subtilis RNase P protein and some archaean RNase P RNAs has been demonstrated, no archaean protein sequences with similarity to any known bacterial RNase P protein subunit have been identified, and the density of Methanothermobacter thermoautotrophicus RNase P in Cs2SO4 (1.42 g/mL) is inconsistent with a single small bacterial-like protein subunit. Four hypothetical open reading frames (MTH11, MTH687, MTH688, and MTH1618) were identified in the genome of M. thermoautotrophicus that have sequence similarity to four of the nine tRNA (pre-tRNA) (for review, see Pace & Brown, 1995; Frank & Pace, 1998; Schón, 1999). The enzyme is composed of a single RNA and a protein content that varies among organisms (Pace & Brown, 1995). Under elevated ionic conditions in vitro, the RNAs from Bacteria are catalytic in the absence of protein (Guerrier-Takada et al., 1983). However, in Eukarya, the proteins are indispensable for catalysis, and in all organisms studied both the RNA and protein are essential in vivo (Pace & Brown, 1995). Although catalytic activity has not been demonstrated for some archaean RNase P RNAs, the RNAs from many show a low level of catalytic activity in vitro under extremely high ionic conditions (Pannucci et al., 1999).

INTRODUCTION
Ribonuclease P (RNase P) is the ribonucleoprotein enzyme responsible for removing the 5’ leader sequence in the maturation of the acceptor stem of all precursor tRNA (pre-tRNA) (for review, see Pace & Brown, 1995; Frank & Pace, 1998; Schón, 1999). The enzyme is composed of a single RNA and a protein content that varies among organisms (Pace & Brown, 1995). Despite the availability of ~435 bacterial, 40 archaean, and 60 eukaryal RNase P RNA sequences, as well as many bacterial and eukaryotic protein subunits (Brown, 1999) and extensive biochemical investigations, RNase P structure and catalysis are not well understood. The secondary structure of bacterial and archaean RNAs is known in detail from phylogenetic comparative analyses (Brown et al., 1996; Haas et al., 1996a, 1996b; Haas & Brown, 1998; Brown, 1999; Harris et al., 2001). However, the three-dimensional structure of the RNA has not been determined and the structure of the holoenzyme is largely unknown. Only recently has significant information been obtained about the protein composition of eukaryotic nuclear RNase P (Eder et al., 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Jarrous et al., 1999; van Eenennaam et al., 1999). The nuclear RNase P holoenzyme has recently been purified from Saccharomyces cerevisiae and been shown to contain nine protein subunits, eight of which are shared with a related enzyme, RNase MRP (Chamberlain et al., 1998).

Methanothermobacter thermoautotrophicus (formerly Methanobacterium thermoautotrophicum strain ΔH; Wasserfallen et al., 2000), a moderately thermophilic
methanogenic archaean, has an RNase P RNA component that closely resembles its bacterial counterparts in both sequence and secondary structure (Brown, 1999; Pannucci et al., 1999; Harris et al., 2001). Indeed, this RNA is among those of the archaean RNase P RNAs that are catalytically proficient in vitro, although the RNA requires extreme ionic conditions (3 M ammonium acetate, 300 mM MgCl₂) and exhibits only a fraction of the activity of bacterial RNase P RNA (Pannucci et al., 1999). M. thermoautotrophicus RNase P RNA has been functionally reconstituted with the Bacillus subtilis RNase P protein in vitro (Pannucci et al., 1999).

Only two archaean RNase P holoenzymes have been characterized previously. The enzyme from Sulfolobus acidocaldarius, a thermoacidophilic Crenarchaeote, has a 315-nt RNA that remains after micrococcal nuclease treatment (LaGrandeur et al., 1993), and has a buoyant density of 1.27 g/mL in Cs₂SO₄, similar to that of protein alone (Darr et al., 1990). Haloferax volcanii, an extremely halophilic Euryarchaeote, has an RNase P that is sensitive to micrococcal nuclease and has a buoyant density of 1.61 g/mL in Cs₂SO₄, similar to DNA alone (Lawrence et al., 1987). The H. volcanii enzyme contains a 435-nt RNA (Nieuwlandt et al., 1999). Recently, it has been shown that H. volcanii RNase P RNA transcribed in vitro can be rendered catalytically active in 4 M ammonium acetate and 300 mM MgCl₂ (Pannucci et al., 1999). Catalytic proficiency of the S. acidocaldarius RNA has not been demonstrated. To date, no protein subunit of an archaean RNase P has been confirmed, despite the availability of the complete genome sequences of several archaean species including M. thermoautotrophicus (Bult et al., 1996; Klenk et al., 1997; Smith et al., 1997; Kawarabayasi et al., 1998, 1999; Kawashima et al., 2000; Ng et al., 2000; Ruepp et al., 2000; Lecompte et al., 2001). The Methanobacteria represent a group roughly intermediate in evolutionary distance between Sulfolobus and Halofexerax based on 16S rRNA phylogenetic trees. Because of the similarity between methanobacterial RNase P RNAs and those of Bacteria, and the availability of the full genome sequence from M. thermoautotrophicus, a computational search utilizing all available RNase P protein subunit sequences was performed in an attempt to identify likely RNase P protein subunit genes in the genome of M. thermoautotrophicus.

There is no open reading frame (ORF) in any currently completed archaean genome with obvious similarity to a known bacterial RNase P protein subunit. However, four ORFs were identified in the genome of M. thermoautotrophicus that encode proteins with similarity to the yeast nuclear RNase P protein subunits Pop4p (the carboxyl half of the protein), Pop5p, Rpp1p, and Rpr2p. These four ORFs are MTH11, MTH687, MTH688, and MTH1618, respectively. All four ORFs have obvious homologs in other archaean genomes and all four have been recently assigned to clusters of orthologous groups of proteins (COGs) with their probable eukaryal homologs (Koonin et al., 2001; Tatusov et al., 2001). In this investigation, we demonstrate that all four of these proteins are subunits of archaean RNase P.

RESULTS

The buoyant density of M. thermoautotrophicus RNase P in cesium sulfate is 1.42 g/mL

M. thermoautotrophicus cell lysate was passed over Sepharose CL-4B and peak active fractions were combined and brought to 1.39 g/mL with Cs₂SO₄. Peak RNase P activity banded in density gradients (120,000 × g, 42 h) at 1.42 g/mL (Fig. 1). This density is roughly intermediate between protein alone (about 1.255 g/mL) and RNA alone (about 1.645 g/mL; Hamilton, 1971). This suggests a greater protein content than the single small protein subunit found in bacterial RNase P, but less than eukaryotic RNase P (see Discussion).

The Archaea possess apparent homologs of S. cerevisiae Pop4p, Pop5p, Rpp1p, and Rpr2p

Although standard BLAST searches with known RNase P proteins revealed no significant similarity with any archaean proteins, a PSI Blast search (Altschul et al., 1997) using the sequence of the S. cerevisiae RNase P subunit Pop5p identified M. thermoautotrophicus ORF MTH687 (E = 4e-30, iteration 2), and Standard BLAST searches with this sequence revealed obvious homologs in several other Archaea (Fig. 2). Similarity between these archaean MTH687-like proteins and Pop5p ranged from 38.8% (Aeropyrum pernix) and 49.7% (Methanococcus jannaschii), whereas identity ranged from 16.7% (A. pernix) and 25.7% (Pyrococcus abyssi). Similarity of the archaean homologs to MTH687 ranged from 41.1% to 64.8% (identity from 21.1% to 39.1%). This observation motivated production of antibodies to rMth687p and the biochemical investigation of its presence in the RNase P holoenzyme. A second ORF, MTH688, was identified in the M. thermoautotrophicus genome that has similarity to the yeast RNase P subunit Rpp1p (E = 8e-52, PSI-BLAST iteration 2), is found adjacent to MTH687 in an operon, and also has conserved homologs in the other Archaea (Fig. 3). The proximity of MTH688 to MTH687, their location together in a likely operon, and the strong PSI-BLAST score with Rpp1p, coupled with biochemical evidence for the presence of Mth687p in the RNase P enzyme led us to examine MTH688 as a second possible RNase P protein gene.
PSI-BLAST searches also revealed possible homologs in the *M. thermoautotrophicus* genome of yeast RNase P subunits Pop4p (MTH11, E = 2e-21, iteration 3) and Rpr2p (MTH1618, E = 3e-19, iteration 2). Both sequences have apparent homologs in other archaeal genomes as well (Figs. 4 and 5). The conservation of these proteins in the Archaea motivated the production of antiserum to the recombinant versions of these proteins.

Mth11p, Mth687p, Mth688p, and Mth1618p are physically associated with purified *M. thermoautotrophicus* RNase P

His-tagged recombinant Mth11p, Mth687p, Mth688p, and Mth1618p proteins were expressed in *Escherichia coli*, purified, and used to generate polyclonal rabbit antisera. In western blots, anti-his-rMTH11, anti-his-rMTH687, anti-his-rMTH688, and anti-his-rMTH1618 antisera specifically recognized bands of ~13.5, 16, 29.5, and 21.5 kDa, respectively, from glycerol gradient fractions that demonstrated RNase P activity (Fig. 6). RNase P activity was 2400-fold purified prior to loading material onto glycerol gradients. The level of antiserum signal correlated directly with the level of RNase P activity in the glycerol gradient fractions (Fig. 6), except in the case of anti-his-rMth1618p (the least reactive antiserum), in which a protein signal is seen only in the single most active fraction. The calculated molecular masses of Mth11p, Mth687p, Mth688p, and Mth1618p are 10.7, 14.6, 27.7, and 17.0 kDa, respectively. However, the recombinant proteins run in 12% Tris-glycine SDS-PAGE gels 15.5, 18, 31.5, and 23.5 kDa, respectively. The size of the histidine tag in the cloned constructs was 24 amino acids (~2.9 kDa). Each immune-serum recognized a protein approximately 2 kDa below the apparent molecular mass of its associated recombinant positive control, according to comparisons to molecular weight standards, suggesting that this histidine tag adds about 2 kDa when fused to another protein, and each protein migrates roughly 1.5 to 3.5 kDa higher than its predicted molecular weight. Pre-immune sera recognized neither these bands nor the recombinant positive controls (not shown). Mth687p could be detected in cleared lysate from *M. thermoautotrophicus* only by loading so much material into a gel that clear resolution was not possible (data not shown).
No attempt was made to detect Mth11p, Mth687p, and Mth1618p proteins in crude cleared lysates.

Affinity-purified anti-rMth11p, rMth687p, rMth688p, and rMth1618p antibodies immunoprecipitated RNase P activity from partially purified *M. thermoautotrophicus* RNase P

The association of Mth11p, Mth687p, Mth688p, and Mth1618p with active RNase P was also assessed by immunoprecipitating RNase P activity with antibodies affinity purified against each recombinant antigen and crosslinked to protein A beads. Antibodies against rMth687p, rMth688p, and rMth1618p efficiently depleted enzyme activity compared to preimmune serum-coated beads (Fig. 7, lane f). Anti-rMth11p antibody partially depleted enzyme activity, but not as efficiently as the other antibodies (Fig. 7B, ~21% lower than for preimmune serum-coated beads).

Substantial RNase P activity eluted from each specific antibody/bead preparation in 72°C buffer compared to preimmune serum-coated beads (Fig. 7, lanes e1, e2, and e3). RNase P activity was associated directly with the antigen-purified antibody/protein-A bead fraction of each immunoprecipitation reaction (Fig. 7, lane B). Either the physical association of the enzyme with the bead-bound antibody did not fully disrupt enzyme activity, or the assay conditions (60°C) disrupted antibody–antigen interactions sufficiently to allow substrate cleavage.

**DISCUSSION**

Four hypothetical conserved proteins with sequence similarity to the yeast RNase P protein subunits Pop4p, Pop5p, Rpp1p, and Rpr2p were identified in genomes of Archaea. In *M. thermoautotrophicus*, these proteins (encoded by ORFs MTH11, MTH687, MTH688, and MTH1618, respectively) were shown by western analysis and immunoprecipitation to be associated with the RNase P holoenzyme. None of the four RNase P proteins has apparent similarity to any known bacterial protein. Although the RNA component of archaeal RNase P is closely related to those of Bacteria, rather than Eukarya (Harris et al., 2001), it is not particularly surprising to find eukaryotic-like proteins associated with an archaeal pre-tRNA-processing enzyme (Smith et al., 1997).

This is the first biochemical demonstration of RNase P-associated proteins in the Archaea. However, a re-
cent computational investigation predicted independently that MTH687 and MTH688 and their homologs in the other Archaea might encode RNase P subunits. It also suggested a functional (and possibly physical) association of RNase P with components of a complex homologous to the eukaryotic exosome, as well as two ribosomal proteins (Koonin et al., 2001). MTH687 and MTH688 were not found adjacent to each other in other archaeal genomes, and their archaeal homologs appeared to be maintained in a similar operon only in M. thermoautotrophicus. However, large parts of this gene cluster appear to be maintained throughout the archaea, similar to the conservation of the operon containing MTH687 and MTH688 (Koonin et al., 2001).

MTH11 and MTH1618, and their archaeal homologs, have more recently been assigned by the NCBI with the exception of S. solfataricus and Thermoplasma volcanium, in which a homolog of this ORF has not been identified. MTH1618 is located in a likely operon with 11 other open reading frames that include ORFs similar to four ribosomal proteins, the signal recognition particle GTPase, a possible ATPase-containing subunit of tRNA methyltransferase, a KH-domain-containing RNA binding protein, and an unidentified conserved protein. MTH1618 is homologous to the gene encoding the only known RNase P protein subunit in S. cerevisiae and P. abyssi, that is not also found in RNase MRP (Chamberlain et al., 1998). The homolog of MTH1618 is contained in a similar operon only in A. pernix. However, large parts of this gene cluster appear to be maintained throughout the archaea, similar to the conservation of the operon containing MTH687 and MTH688 (Koonin et al., 2001).

The C₅SO₄ density of 1.42 g/mL (Fig. 1) suggests that M. thermoautotrophicus RNase P is approximately 50% protein. Using the specific volumes in C₅SO₄ for RNA and protein of 0.608 and 0.797 ml/g, respectively (Hamilton, 1971), the M. thermoautotrophicus RNase P holoenzyme should contain approximately 98 kDa of protein. A similar calculation based on the known small

### FIGURE 3.

Alignment of eukaryotic nuclear RNase P protein subunit Rpp1p with Mth688p and other potential homologs. Sequences were aligned with ClustalW (Thompson et al., 1994). Identities (black) and similarities (gray) were shaded with a 60% threshold using the PAM250 scoring matrix. Abbreviations (accession numbers are given in parentheses): Hsa: Homo sapiens Rpp30p (NP_006404); Mmu: Mus musculus RNase P protein p30 (NP_062301) (eukaryotic sequence returned by a PSI-BLAST search with Rpp1p, but not demonstrated to be an RNase P subunit); Spo: S. pombe YDL4_SCHPO (P87120) (eukaryotic sequence returned by a PSI-BLAST search with Rpp1p, but not demonstrated to be an RNase P subunit); Dme: Drosophila melanogaster Cg11606p (AAF51526) (eukaryotic sequence returned by a PSI-BLAST search with Rpp1p, but not demonstrated to be an RNase P subunit); Sce: S. cerevisiae Rpp1p (NP_011929); Dme: Drosophila melanogaster Cg11606p (AAF51526) (eukaryotic sequence returned by a PSI-BLAST search with Rpp1p, but not demonstrated to be an RNase P subunit); Mth: M. thermoautotrophicus MTH688p (F69191); Mja: M. jannaschii Af2317p (NP_071142); Pab: P. abyssi Pab1136p (F75024); Ape: A. pernix Ape1450p (BA680448); Atl: A. fulgidus Atl2317p (NP_071142).
ribosomal subunit components in *M. thermoautotrophicus* predicts the density in Cs₂SO₄ should be 1.44 g/mL, in very good agreement with the observed density of bulk ribosomal material of 1.47 g/mL (Fig. 1). The calculated molecular weights for the four RNase P proteins are 44 kDa, suggesting that there may be additional unidentified subunits, or that the stoichiometry of all subunits is not one per holoenzyme. In addition, it is not known whether all of the four identified RNase P proteins are essential for catalytic activity; the technology is not yet available to test this genetically in this organism, and reconstitution of activity from recombinant proteins and RNA has not yet been accomplished (data not shown). We are currently attempting to purify this enzyme to homogeneity to identify any additional RNase P subunits (see Andrews et al., 2001). The bacterial RNase P protein facilitates substrate binding and product release (Reich et al., 1988) and alters the substrate specificity of the enzyme (Peck-Miller & Altman, 1991; Liu & Altman, 1994). It has been shown that the protein component of *B. subtilis* RNase P increases the affinity of the holoenzyme for substrate compared to product by making direct contacts to the 5′ leader of pre-tRNA (Crary et al., 1998; Kurz et al., 1998; Loria et al., 1998; Niranjanakumari et al., 1998). Although the RNA subunit of *M. thermoautotrophicus* RNase P is capable of some catalysis in vitro in high ionic conditions, the protein complement of this enzyme enhances the affinity for substrate by at least 1000-fold; the Km of the RNA alone is at least 40 μM (Pannucci et al., 1999), whereas the holoenzyme has a Km of 34.5 ± 3.4 nM (Andrews et al., 2001). This

---

**FIGURE 4.** Alignment of eukaryotic nuclear RNase P protein subunit Pop4p with Mth11p and other potential homologs. Sequences were aligned with ClustalW (Thompson et al., 1994). Identities (black) and similarities (gray) were shaded with a 60% threshold using the PAM250 scoring matrix. Abbreviations (accession numbers are given in parentheses): Hsa: *H. sapiens* Pop4p (CAZ39167); Dme: *D. melanogaster* Cg0383p (AA50498) (eukaryotic sequence returned by a PSI-BLAST search with Pop4p, but not demonstrated to be an RNase P subunit); Cel: *Caenorhabditis elegans* C1584 (CAZ02730) (eukaryotic sequence predicted, but not proven, to be associated with tRNA processing); Mth: *M. thermoautotrophicus* Mth11p (AA884512); Mja: *M. janulifera* Mj0645p (H64357); Mva: *M. vagans* YRP2_METVA (P14022); Ape: *A. pernix* Ape0636p (BAA79317); Hsp: *Haloarcula marismortui* YROP_HALMA (P22527); Mha: *Halobacterium halobium* YROP_HALHA (O24785).
FIGURE 5. Alignment of eukaryotic nuclear RNase P protein subunit Rpr2p with Mth1618p and other potential homologs. Sequences were aligned with ClustalW (Thompson et al., 1994). Identities (black) and similarities (gray) were shaded with a 60% threshold using the PAM250 scoring matrix. Abbreviations (accession numbers are given in parentheses): Hsa: H. sapiens unnamed protein product (BAB15433) (eukaryotic sequence returned by a PSI-BLAST search with Rpr2p, but not demonstrated to be an RNase P subunit; a human homolog of yeast Rpr2p has not been identified to date); Mmu: M. musculus putative (BAB22353) (eukaryotic sequence returned by a PSI-BLAST search with Rpr2p, but not demonstrated to be an RNase P subunit); Sce: S. cerevisiae Rpr2p (NP_012280); Spo: S. pombe SPBC1105c (T39293) (eukaryotic sequence returned by a PSI-BLAST search with Rpr2p, but not demonstrated to be an RNase P subunit); Mth: M. thermoautotrophicus Mth1618p (B69083); Mja: M. jannaschii MJ0962p (Q58372); Pho: P. horikoshii Ph1601p (A71039); Pab: P. abyssi Pab0385p (E75175); Afu: A. fulgidus Af0109p (NP_068950); Hsp: Halobacterium sp. NRC-1 Vng0599c (AAG19111).

FIGURE 6. Mth11p, Mth687p, and Mth1618p copurify with RNase P activity. Aliquots of the indicated fractions of glycerol gradients loaded with 2,400-fold purified M. thermoautotrophicus RNase P were probed with a 1:1000 dilution of the appropriate specific antiserum. In each blot, recombinant his10-tagged recombinant protein (~50–150 ng) was run as a positive control. For each, another blot was probed with a 1:1,000 dilution of preimmune serum, which did not detect any proteins (not shown). The level of RNase P activity in each gradient fraction is shown in the graph below the western blot. A: Anti-his-rMth687p detected a band of ~16 kDa. B: top: Anti-his-rMth1618p detected a band of ~21.5 kDa. bottom: Anti-his-rMth11p detected a band of ~13.5 kDa. C: Anti-his-Mth688p detected a band of ~29.5 kDa. Each native band detected in fractions displaying enzyme activity migrated approximately 2 kDa smaller than its associated positive control. The predicted molecular weight of the his-tag is 2.9 kDa. In A (anti-Mth687p), a faint doublet running near the top of the gel was also seen to correlate with RNase P activity. The nature of this band has not been determined, although it is not detected by preimmune serum (not shown).
Archaeal RNase P proteins

FIGURE 7. Immunoprecipitation of RNase P activity with affinity-purified antibody against recombinant M. thermoautotrophicus proteins. Antigen-specific affinity-purified antibodies against recombinant his-rMth11p, Mth687p, Mth688p, and Mth1618p were used to immunoprecipitate RNase P activity from a partially purified M. thermoautotrophicus RNase P preparation. Partially purified RNase P was subjected to protein-A agarose beads coated with preimmune serum or affinity-purified antibodies against his-rMth11p (A), his-rMth687p (B), his-rMth688p (C), or his-rMth1618p (D). Columns were washed three times, then eluted three times in preheated 72°C buffer. Fractions were assayed for RNase P activity in 2-min (flow-through) or 6.5-min (beads and elutions) RNase P activity assays with B. subtilis pre-tRNA<sup>Asp</sup>. All assays were performed in triplicate. The first lane of each triplicate is shown. Abbreviations: f.t.: flow-through (supernatant from the binding reaction); B: beads; e1, e2, e3: heat elutions 1, 2, and 3, respectively.

The conservation of the four archaeal proteins in the nuclear enzyme implies that they are particularly important in the function of RNase P in these organisms. In yeast, the homolog of Mth11p (Pop4p) has been shown in three-hybrid analysis to interact with the nuclear RNase P RNA (F. Houser-Scott & D.R. Engelke, pers. comm.). The human homologs of Mth11p (Rpp29p) and Mth1618p (Rpp21p) have been implicated in substrate binding (Jarrous et al., 2001), and these two proteins as well as the homolog of Mth688p (Rpp30p) apparently bind to the human nuclear RNase P RNA (Jiang et al., 2001). On the other hand, it seems unlikely that these proteins, in the nucleus, function primarily in nuclear or nucleolar localization. (Although the amino-terminal sequences of Pop4p that are not present in the archaeal proteins are not excluded on this account.) It also seems unlikely that these proteins are involved in recognition of the substrate T-loop (carried out by P8/9 in Bacteria, and so presumably in Archaea) or 3′-NCCA (carried out by L15 in Bacteria, and so presumably in Archaea). Instead, these proteins are probably involved in leader recognition and stabilization of the RNase P RNA or enzyme:substrate superstructure (by analogy to the bacterial protein), or other functions required in Archaea and the nucleus but not in Bacteria.

The sequences and secondary structures of archaeal RNase P RNAs are similar to those of Bacteria (Harris et al., 2001). With the exceptions of the RNase P RNAs of Bacillus and relatives (type B), Thermomicrobium (type C), and Methanococcus and relatives (type M), RNase P RNAs from both domains fall into a common structure class, type A (Haas et al., 1994). The RNA subunit of most of the type A archaeal RNase P RNAs tested are, like those of Bacteria, capable of catalysis in vitro in the absence of protein, and can be reconstituted with the B. subtilis protein to create catalytically proficient chimeric holoenzymes (Pannucci et al., 1999). Eukaryal RNase P RNAs are distinct (type E) from those of Bacteria and Archaea. This implies that the type A RNA is the ancestral form of the RNA (Fig. 8).

FIGURE 8. Placement of the large evolutionary changes in RNase P RNA structure and protein type in evolutionary history. The phylogenetic tree is a representation based on small-subunit ribosomal RNA phylogenetic analysis from Olsen (1987). A large star is placed in the midpoint of branch in which the large evolutionary change has taken place in the RNA subunit (above) and the protein composition (below). The branches including the apparently primitive forms of each are thin; branches distal to large evolutionary shift are heavy, indicating the “innovative” form of the subunit. Small stars in the RNA tree indicate the smaller changes that resulted, independently, in the generation of the types B, C, and M RNase P RNAs.
that underwent a substantial innovative change in the common specific ancestry of the Eukarya. We have shown here that protein subunits of archaeal RNase P are homologous to eukaryal RNase P subunits, rather than those of Bacteria. This implies that the eukaryal/archaeal type (POP-type) proteins may be the ancestral type of RNase P proteins, and the bacterial (rnpA type) proteins are the result of an innovative evolutionary change. This is counter to the commonly held belief that the bacterial RNase P, with its minimal “accessory” protein, represents a primitive state, not far removed from the “RNA world.” This belief is not entirely inconsistent with our observations, but it demands that the root of the evolutionary tree be more distal along the bacterial-specific branch than is the location of the evolutionary change in protein composition along this same branch, a highly constrained scenario. If the three phylogenetic domains emerged more or less independently from a last common ancestral population (Woese, 1998), our data suggest that the RNA-centric nature of bacterial RNase P is a recent innovation, not entirely a remnant of an “RNA World.”

MATERIALS AND METHODS

RNase P cleavage assays

Two-nanomolar substrate (~2 nCi 32P-GTP-labeled Bacillus subtilis pre-tRNAAsp) was incubated with enzyme samples in 10-µL reactions of 50 mM Tris-Cl, pH 8, 10 mM MgCl2, 500 mM ammonium acetate at 60 °C (optimal conditions for M. thermoautotrophicus RNase P with this substrate). Reaction products were analyzed by electrophoresis in 12% acrylamide, 8 M urea gels, exposing the gels to a phosphorimager screen, and quantitating band volumes with ImageQuant 1.0 (Molecular Dynamics).

Determination of buoyant density in Cs2SO4

M. thermoautotrophicus cell paste (~7 g) was ground with a mortar and pestle in liquid nitrogen, suspended in 20 mL TMGN-100 with 10 µg/mL DNase I (TMG is composed of 50 mM Tris-Cl, pH 7.5, 10 mM MgCl2, 5% glycerol, 0.1 mM DTT, 0.1 mM PMSF, and N-100 specifies 100 mM NH4Cl) and centrifuged 16 h at 230,000 × g. The pellet was resuspended in 1 mL of TMGN-60 and passed over Sepharose CL-4B in the same buffer. Peak RNase P fractions were combined and an aliquot containing ~1 mg total protein (BCA assay, Pierce) was adjusted to 3 mL TMGN-60, 1.39 g/mL Cs2SO4 (final density), centrifuged at 120,000 × g for 42 h at 4 °C, and fractions were assayed for RNase P activity. Density was determined by weighing 100 µL of each fraction.

Cloning and expression of recombinant Mth11p, Mth687p, Mth688p, and Mth1618p

The MTH11, MTH687, MTH688, and MTH1618 open reading frames were amplified from genomic DNA and cloned as in-frame fusions into pET16b (Novagen) to create amino terminal his10-tagged constructs. The recombinant proteins were expressed in E. coli/BL21 (DE3) CodonPlus RIL (Stratagene) and purified with Ni-NTA resin (Qiaegen) under denaturing conditions according to the recommendations of the manufacturer. The proteins were further purified to apparent homogeneity in 12% SDS-PAGE gels.

Generation of antiserum to recombinant proteins

Approximately 1 mg of rMth687p (in 50 mM Tris, pH 8) was injected into rabbits in a 50% emulsion of TiterMax Gold adjuvant (CytRx Corporation). Two booster injections (1 mg) were spaced 1 and 2 months after the first challenge. Bleeds were taken prior to the initial injection and at 1, 2, and 3 months. Approximately 1 mg each of rMth11p, rMth1618p, and rMth688p were used to generate polyclonal antisera commercially (CoCalico Biologicals, Inc.).

Partial purification of M. thermoautotrophicus RNase P

M. thermoautotrophicus cell paste (115 g wet weight) was ground in liquid nitrogen, suspended in 245 mL of TMGN-20 with 10 µg/mL DNase I, and passed twice through a French press at 20,000 psi (internal pressure), then cleared at 25,000 × g for 30 min at 4 °C. The cleared lysate was dialyzed overnight in 4 L TMGN-20 at 4 °C, then loaded onto a 50-mL DEAE-trisacyl (Sigma) column and washed with 600 mL TMGN-20. Peak RNase P activity eluted at ~270 mM NH4Cl in a 20–500-mM linear gradient. Active fractions were pooled and dialyzed overnight in 4 L TMGN-20. The sample was brought to a density of 1.4 g/mL with Cs2SO4. Samples were centrifuged at 120,000 × g for 45 h at 8 °C (Beckman 70.Ti rotor). Peak active fractions were combined and centrifuged as before. Active fractions were combined, dialyzed overnight in TMGN-20 with 0.025% NP-40, bound to 20 mL of Q-Sepharose (Sigma), and washed with 250 mL TMGN-20 + 0.025% NP-40. RNase P eluted at ~635 mM NH4Cl in a 250-mL linear gradient of 20–1000 mM NH4Cl in TMG + 0.025% NP-40. Active fractions were combined. Specific RNase P activity was approximately 2400-fold purified compared to cleared lysate (enzyme activity/total protein) after Q-Sepharose chromatography. Pooled active fractions were dialyzed overnight in TMGN-20 + 0.025% NP-40 and aliquots were concentrated ~15-fold using Amicon Microcon 10-kDa MWCO concentrators (Millipore). Concentrated aliquots (100 µL) were layered onto 1.9 mL 10–40% glycerol gradients prepared in TMGN-500, 0.025% NP-40, 0.02% SB-12 (Sigma), and centrifuged 7.5 h at 95,000 × g in a Beckman TLS-55 swinging bucket rotor (20 °C). Gradient fractions were western blotted for detection of the MTH11, MTH687, MTH688, and MTH1618 proteins (below).

Western blots for Mth11p, Mth687p, Mth688p, and Mth1618p

Glycerol gradient fractions (25 µL) were precipitated with acetone and samples were separated on SDS-PAGE gels and transferred to nitrocellulose. One of each blot was probed with a 1:1000 dilution of the appropriate antiserum and an-
other with preimmune serum from the same rabbit for 2 h at room temperature. Blots were washed, probed for 2 h at room temperature with a 1:15,000 dilution of HRP-conjugated goat anti-rabbit IgG (Sigma A6154), washed, developed in Supersignal chemiluminescent reagent (Pierce), and exposed to X-ray film.

Affinity purification of antibodies from crude antisera

Recombinant Mth11p, Mth687p, Mth688p, and Mth1618p were expressed in E. coli and purified to apparent homogeneity as described above. Purified proteins were dialyzed overnight at 4 °C in 100 mM NaHCO₃, pH 8.3, 500 mM NaCl (a buffer suitable for coupling to a cyanogen bromide (CNBr)-activated agarose matrix). Proteins were conjugated to CNBr-activated agarose (Sigma C-9142) according to the manufacturer’s recommendations, with the exception that 0.1% SDS was added to help solubilize the proteins.

Immune serum (700 µL each) was added to 40–50 µL of recombinant protein column matrix in 500 µL 10 mM Tris, pH 7.5, 500 mM NaCl, and mixed overnight at 4 °C. Bead/supernatant was collected, and then the beads were pelleted in a tabletop microcentrifuge and 20 µL bead slurry was added to 20 mg protein A beads and slurries were mixed overnight at 4 °C. The equivalent of 13 mg of protein-A agarose with specific antibody or preimmune serum antibody were equilibrated from the bead suspensions and 20 µL bead slurry was added to 20 mg protein A beads and slurries were mixed overnight at 4 °C. Each elution was collected directly into 0.1 mL vol (10 µL) 1 M Tris, pH 8, to neutralize the acidic elution buffer.

Immunoprecipitation of RNase P activity with antigen-specific antibodies

M. thermoautotrophicus RNase P was partially purified as described above. Eight aliquots of 20 mg each of protein-A agarose beads (Sigma P-3391) were hydrated in 10 mM Tris, pH 7.5, 500 mM NaCl. For affinity-purified antibodies, combined elution fractions (described above, 500 µL each antibody) were added to 20 mg protein A beads and slurries were mixed overnight at 4 °C. For preimmune sera, 400 µL preimmune serum was added to 20 mg swollen protein-A agarose and mixed overnight at 4 °C.

Antibody-bound protein-A beads were washed twice with 1 mL of 200 mM sodium borate, pH 9, then resuspended in 1 mL of the same buffer. To crosslink antibody to the protein-A, 5.1 mg dimethyl pimelimidate (Sigma D-8388, 20 mM) were added to each slurry and mixed for 30 min. Each preparation was washed with 1 mL 200 mM ethanolamine, pH 8, then suspended in 1 mL 200 mM ethanolamine, pH 8 for 2 h to deactivate the crosslinking reaction. The beads were equilibrated in 10 mM Tris, pH 8, 500 mM NaCl with three 1.5 mL washes.

The equivalent of 13 mg of protein-A agarose with specific antibody or preimmune serum antibody were equilibrated in TMGN-100 and brought up to 400 µL in TMGN-100. Q-sepharose-purified RNase P (30 µL) was added to each bead slurry. Slurries were mixed gently overnight at 4 °C. The beads were pelleted in a table-top microcentrifuge and the supernatant was removed as elution 1. This heat elution process was repeated twice more. The binding supernatants (1:20 dilution) were tested for RNase P activity as described below in 2-min assays to test for immunodepletion of RNase P activity. Bead fractions and heat elutions (1:20 dilutions) were assayed as described above for 6.5 min to test for specific immunoprecipitation and recovery of RNase P activity. All assays were performed in triplicate.

ACKNOWLEDGMENTS

We thank Steve Libby for supplying the pET16b vector and assistance in antisera production, and Barbara Jean Welker for her assistance with antisera production. We would also like to express thanks to Felicia Scott and David Engelke for helpful discussions. This work was supported by National Institutes of Health Grant GM52894, and a GAANN fellowship to T.A. Hall.

Received November 15, 2001; returned for revision December 10, 2001; revised manuscript received December 18, 2001

REFERENCES


Nieuwlant DT, Haas ES, Daniels CJ, 1991. The RNA component of

Rhine-RNA from the archaeabacteria Halofexus volcanii. J Biol

Chem 266:5689–5695.

Niranjanakumari S, Stams T, Crarya SM, Christianson DW, Fierce KA,

1998. Protein component of the ribosome ribonucleoprotein P alters

substrate recognition by directly contacting precursor RNA. Proc

Natl Acad Sci USA 95:15212–15217.

Olsen GJ. 1987. Earliest phylogenetic branchings: Comparing rRNA-
based evolutionary trees inferred with various techniques. Cold


Pace NR, Brown JW. 1995. Evolutionary perspective on the

structure and function of ribonuclease P, a ribozyme. J Bacteriol 177:

1919–1928.


P RNAs from some Archaea are catalytically active. Proc Natl

Acad Sci USA 96:7803–7808.

Peck-Miller KA, Altman S. 1991. Kinetics of the processing of the

precursor to 4.5 S RNA, a naturally occurring substrate for RNase


Reich C, Olsen GJ, Pace B, Pace NR. 1988. Role of the protein

moiety of ribonuclease P, a ribonucleoprotein enzyme. Science


Ruepp A, Graml W, Santos-Martinez M-L, Koretki KK, Volker C,


The genome sequence of the thermophilic scavenger Thermoplasma


Schön A. 1999. Ribonuclease P: The diversity of a ubiquitous RNA


Smith DR, Doucette-Stamm LA, Deloughery C, Lee H, Dubois J,

Alport R, Bashirzadeh B, Smolyk D, Cook F, Gilbert K, Harri-

son D, Hoang L, Kegel P, Lumm W, Pothier B, Oiu D, Scapadora R,


A, Bush D, Reeve JN. 1997. Complete genome sequence of

Methanobacterium thermoautotrophicum ΔH: functional analysis


RNase P required for processing of precursor RNA and 3′S

 precursor rRNA in Saccharomyces cerevisiae. Genes & Dev

11:2926–2937.

Tatusov RL, Natale DA, Garkavtsev IV, Shankavaram UT, Rao BS,

Kiryult B, Galperin MY, Fedorova ND, Koonin EV. 2001. The

COG database: New developments in phylogenetic classification


Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improv-

ing the sensitivity of progressive multiple sequence alignment

through sequence weighting, position-specific gap penalties and


protein subunit of the human RNase MRP and RNase P ribonu-


Wasserfallen A, Nolling J, Pfister P, Reeve J, Conway de Macaro E,

2000. Phylogenetic analysis of 18 thermophilic Methanobacte-

riae isolates supports the proposals to create a new genus, Metha-

nothermobacter gen. nov., and to reclassify several isolates in

three species. Methanothermobacter thermoautotrophicus comb. nov.,

Methanothermobacter wolfei comb. nov. and Methanother-


95:6854–6859.