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The goal of these studies was to establish model systems to investigate the interaction of proteins with nucleic acids in the Archaebacteria. The maturation of tRNA molecules in halophilic and thermophilic organisms was chosen for study. An assay was developed for the tRNA intron endonuclease from *Halobacterium volcanii*. This enzyme differed from its eukaryotic counterparts in substrate recognition and may participate in other RNA maturation reactions. Endonuclease was detected in a variety of Archaebacteria, including extreme thermophiles. Removal of 5' leader sequences from tRNA primary transcripts was also investigated. RNaseP activity was detected in *H. volcanii* and *Thermoplasma*. Preliminary findings indicate that this activity has an RNA component and may therefore be a ribozyme. The structure of stable RNA genes was examined in the extreme thermophile *Methanothermus fervidus*. In general, these RNAs have a greater degree of G-C base paring than mesophilic organisms suggesting that this property contributes to thermostability.

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RESEARCH OBJECTIVES.

To determine the mechanism of tRNA intron processing in the halophilic Archaebacteria; characterize the enzyme responsible for the removal of 5'-flanking sequences from tRNA gene primary transcripts; examine the structure and distribution of tRNA introns in the halophilic Archaebacteria.

Development of an in vitro Assay for tRNA Intron Endonuclease. In analyzing tRNA genes from Halobacterium volcanii we found that one of the genes, $tRNA^{Trp}$, was was interrupted by a 106 base pair intron located between nucleotides 37 and 38 of the anticodon loop. The occurrence of this intron was not unique to H. volcanii, it was detected in ten other halophilic archaebacterial strains. When sequences related to the intron were characterized in H. cutirubrum and H. mediterranei, they were found in the genes for $tRNA^{Trp}$ between nucleotides 37 and 38 of the tRNA, encoding introns of 105 and 104 nucleotides, respectively. Each exhibited a high degree of similarity to the H. volcanii intron (89% and 94%, respectively). The occurrence of introns in stable RNA genes in the Archaebacteria raised interesting questions concerning the distribution of interrupted genes in these organisms and the mechanism(s) of their of removal.

Although the intron containing $tRNA^{Trp}$ precursor is detectable in vivo by Northern analysis, it was not practical to use in vivo preRNA as a substrate for the development of an in vitro assay. As an alternative, a subclone of the H. mediterranei $tRNA^{Trp}$ gene was cloned into a T7 expression vector and modified by deletion such that a transcript resembling the in vivo precursor could be made in vitro. This RNA,Δ16, contained a 5' leader (36 nucleotides), a 3' trailing sequence (5 nucleotides) and lacked base modifications and the 3' CCA residues. Despite these difference from the in vivo precursor, this molecule was readily cleaved by crude extracts to give three distinct products: 5' leader+exon I, intron and exon II+3' trailer. Using this assay the endonuclease was partially purified by polyethylene glycol precipitation, DEAE chromatography and hydroxylapatite chromatography; this led to a 240 fold purification. The accuracy of this reaction was verified by direct RNA sequence analysis of the products and nearest neighbor transfer. Analysis of the terminal nucleotides of the reaction products demonstrated that phosphodiester bond cleavage generated 5' hydroxyl and 2'3' cyclic phosphate termini, similar to the eukaryotic endonuclease. The properties of this enzyme, and a comparison with the yeast nuclear endonuclease are given in Table 1.

Table 1. Comparison of yeast and halophilic tRNA intron endonucleases

Property	Yeast Nuclear	Halophilic
pH optima	pH 7-8	pH 6-9
Ion requirement	Spermidine >Mg^{++} Monovalent inhibitory at 120 mM	Spermidine >Ca^{++} >Mg^{++} Monovalent inhibitory at 50 mM
Cleavage termini	5' hydroxyl 2',3' cyclic phosphate	5' hydroxyl 2',3' cyclic phosphate
Substrate recognition regions	Mature tRNA structure in precursor	Exon-intron boundary
Substrate range	All nuclear-encoded intron-containing precursor tRNAs	Halophilic $tRNA^{Trp}$ intron-containing precursors
Subcellular location	Membrane	Soluble (and membrane)
Associated ligase activity	*Yes, in crude preparation*	Not detectable

H. volcanii endonuclease enzyme preparation were devoid of ligase activity, the second step in the processing reaction. This did not appear to be the result of separation of these two activities during endonuclease purification, since only minor amounts of ligase were detectable using this substrate in crude extracts prepared in high or low salts. Moreover, ligase activity was not detected in other archaebacterial extracts active in endonuclease cleavage (see below). Unlike endonuclease, ligase may require completion of other maturation events in the precursor, such as base modifications and processing of the 5' and 3' termini, before it will recognize the substrate.

Substrate Recognition by the Endonuclease. Mechanistically the halophilic endonuclease enzyme appeared to to be similar to the eukaryotic endonuclease. To determine whether this similarity extended to substrate recognition properties, we obtained an intron containing yeast $tRNA^{Phe}$ clone (John Abelson, Cal Tech) in which this gene was under the control of a T7 RNA polymerase promoter. Runoff transcripts of this clone produced a precursor tRNA with a 19 nucleotide intron and a 3' CCA terminus. When this RNA was used as substrate in the H. volcanii endonuclease assay it was not cleaved, either specifically or nonspecifically. This indicated that the $tRNA^{Phe}$ precursor lacked the required structure or sequence necessary for recognition or cleavage by the halophilic enzyme.

To examine the recognition properties of the halophilic endonuclease a number of modified derivatives of the parental substrate, Δ16, were constructed. Analysis of

substrates with exon deletions indicated that the acceptor stem, DHU stem and loop and the TψC stem and loop could be removed without affect on endonuclease cleavage. Deletions within the intron indicated that the presence of the large intron influenced the efficiency of the reaction but the complete intron was not required for cleavage. One such intron deletion substrate, Δ167, which contained intact exons and a 22 nucleotide intron (obtained by deleting an internal HinP1 fragment from the intron; see Fig. 1A) was cleaved accurately by the endonuclease. Therefore, this substrate retained the necessary intron sequence or structure required for cleavage. It was apparent from these results that the halophilic endonuclease did not require mature-like structure in the substrate and that recognition must be directed at sequences or structures in the anticodon stem and loop and/or intron-exon boundaries. These features must also differ from those present in the yeast pretRNA^{Phe} molecule since the halophilic enzyme can distinguish between these molecules despite the fact that their introns are located in the same relative position and are similar in size.

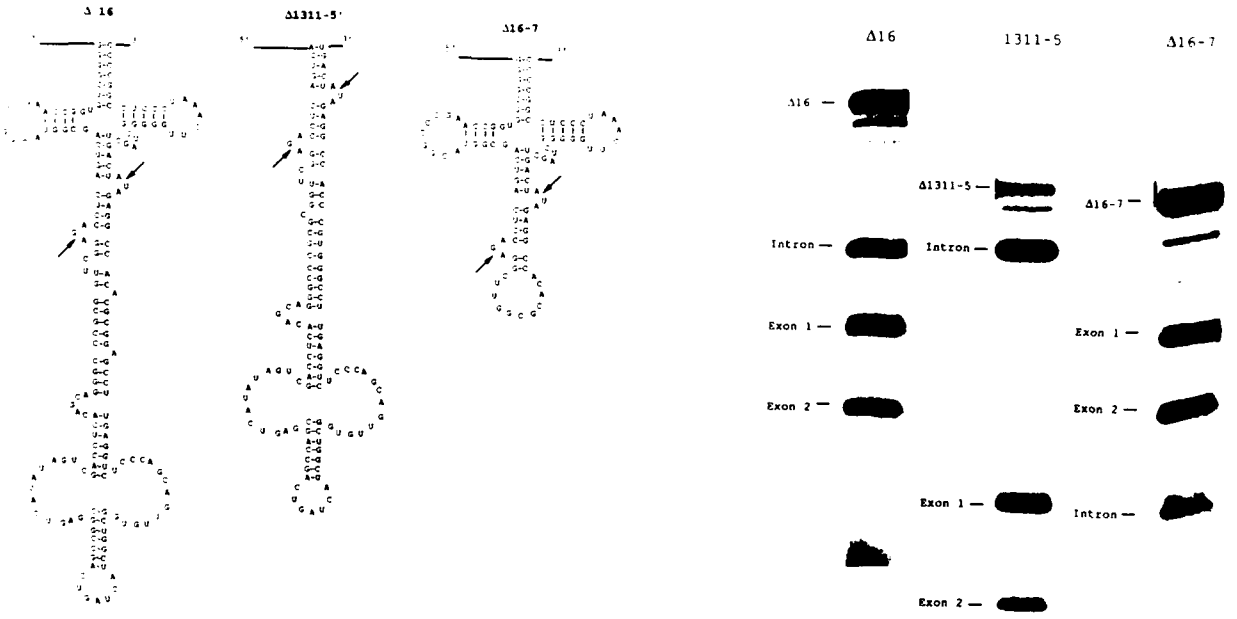


Figure 1. Cleavage of the minimal exon and minimal intron Δ16 substrates. The substrates Δ16, Δ13$^{11-5}$ (Δ16 lacking all exon sequences except those associated with the anticodon stem), and Δ16^{7} (Δ16 lacking all but 22 nucleotides of the intron) were assayed with the halophilic endonuclease under standard reaction conditions. Arrows indicate the cleavage sites. Left panel: secondary structure for the full length (Δ16), minimal exon (Δ13$^{11-5}$), and minimal intron (Δ16^{7}) primary T7 RNA polymerase transcripts. Right panel: cleavage assay of Δ16, Δ13$^{11-5}$, and Δ16^{7}, respectively. The identities of the cleavage products are identified.

Using the Δ167 substrate as a model minimum substrate, energy calculations indicated that one of the most energetically favorable structures for the anticodon stem, loop and intron regions was a structure in which the the two cleavage sites were located in two, three nucleotide loops separated by four base pairs (Fig.1). An examination of other archaebacterial intron containing tRNA precursors revealed that this was a common structural feature and was not limited to those introns located between nucleotides 37 and 38 (Fig. 2). The T. tenax pretRNAs in which the introns are located in the anticodon and in the anticodon stem position, can also assume this structure through an alternative base pairing scheme. A reevaluation of the possible structures at the intron exon boundaries of

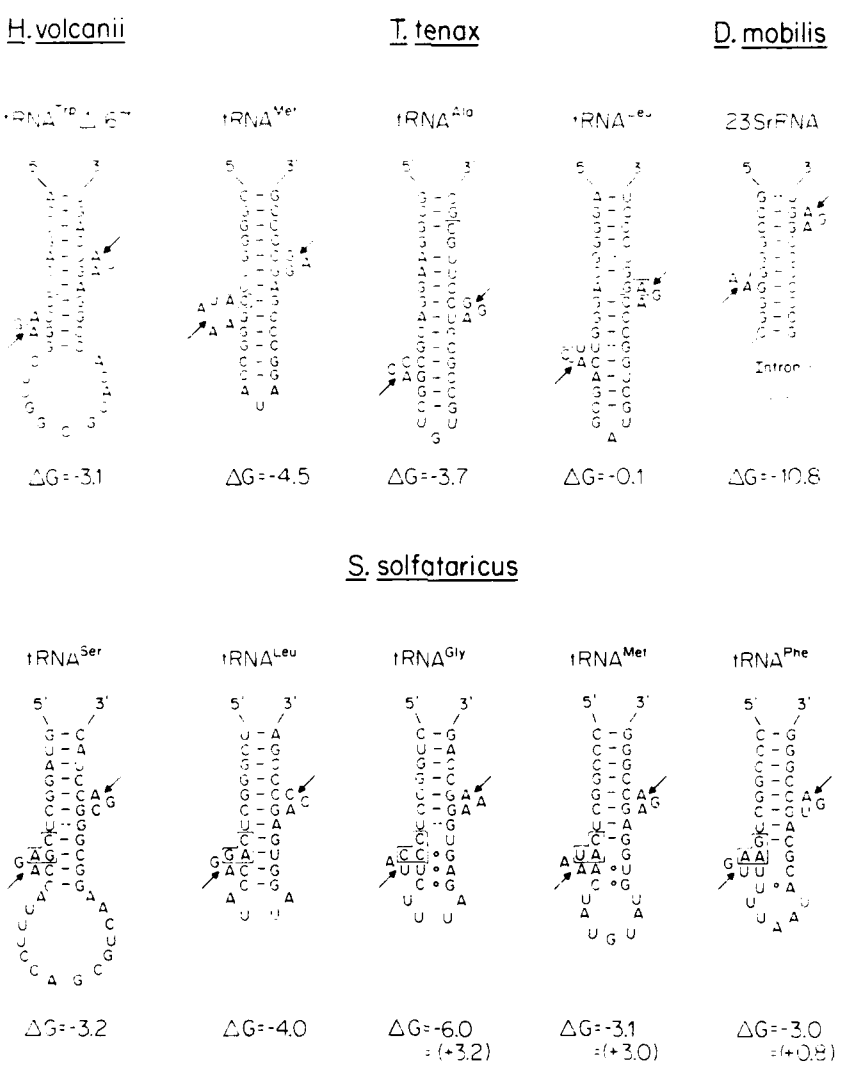


Figure 2. Intron-exon boundaries for Archaebacterial intron-containing transcripts. Endonuclease cleavage sites are indicated by arrows. Free energy values for some *Sulfolobus solfataricus* tRNAs indicate free energy values for structures which include base pairing interactions designated by dots.

the D. mobilis 23S rRNA intron, indicate that this intron can also assume this structure (Fig. 2). A comparison of the sequences in this region for all precursors indicated there is a strong preference for purines in the loops. It appears that there may be a common recognition signal for all intron containing preRNAs in the Archaebacteria. This proposal is consistent with the inability of the halobacterial enzyme to cleave the yeast $tRNA^{Phe}$ precursor, which lacks these features, and supports a possible relationship between 23S rRNA intron processing and tRNA intron processing in D. mobilis.

To test the hypothesis for a conserved recognition mechanism, a number of Archaebacterial extracts were examined for their ability to cleave the halophilic $tRNA^{Trp}$ precursor. Extracts from Methanosarcina barkeri, Thermoplasma acidophilum, Sulfolobus sulfataricus, and Methanothermus fervidus were found to cleave the precursor (Fig. 3). Furthermore, these activities are optimal at the physiological growth temperature for each organism. However, as observed for the halophilic endonuclease, these extracts did not exhibit ligase activity in the presence or absence of ATP.

Relationship to rRNA Processing. The indications that the halophilic intron endonuclease recognizes substrates through a limited interaction involving short helical regions

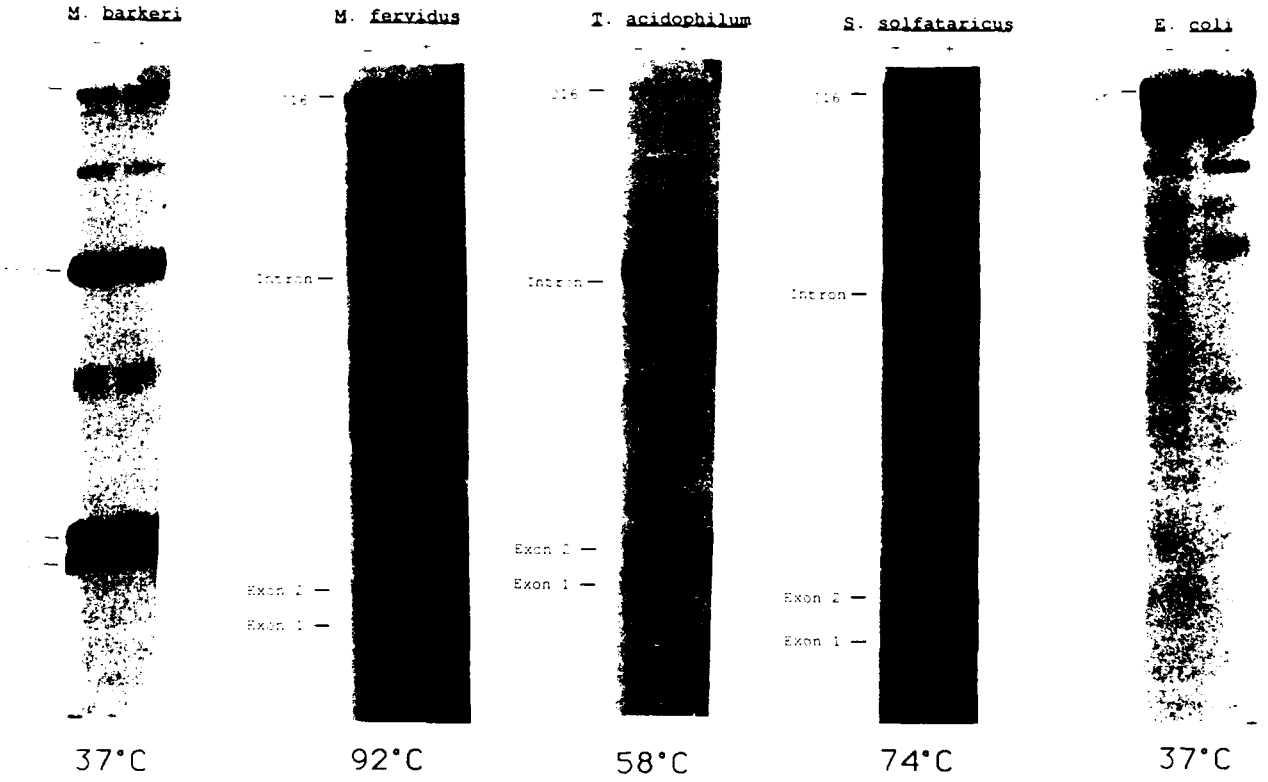


Figure 3. Survey of intron endonuclease activity present in other Archaebacteria. Cellular extracts representing methanogens (M. barkeri, M. fervidus), thermoacidophiles (T. acidophilum, S. solfataricus) and E. coli were assayed with o16 RNA ($tRNA^{Trp}$ lacking flanking regions) under standard assay conditions in the presence (+) and absence (-) of ATP and at physiological temperature. Identities of the cleavage products are indicated; no cleavage of o16 with the E. coli extract was observed.

with small loops suggested that this enzyme may act on other non-tRNA molecules. Likely targets might be the helical structures formed by the inverted repeats flanking the 16S and 23S rRNAs, the RNase III cleavages sites. Comparison of the available sequences revealed that the H. cutirubrum 16S and 23S rRNAs and the Halococcus morrhuae 23S rRNA proposed RNase III sites were similar to the tRNA endonuclease recognition sites. To test the ability of the intron endonuclease to cleave these molecules, model substrates representing the H. cutirubrum 16S (a gift from P. Dennis) and 23S RNaseIII cleavage sites were constructed . Taking advantage of T7 RNA polymerase's ability to transcribe partially double stranded DNA, oligonucleotides were synthesized which contained the noncoding strand of the T7 RNA polymerase promoter fused to DNA sequences corresponding to the 5' and 3' portions of the noncoding regions of H. cutirubrum 16S and 23S RNase III cleavage sites. When combined with an oligonucleotide complementary to the T7 promoter region, T7 RNA polymerase produced a runoff transcript which formed a hairpin containing the RNase III sites (Fig. 4). Incubation of 5' end-labeled 16S and 23S RNase III RNAs with the H. volcanii endonuclease resulted in the production of single specific products similar in size to those predicted for RNase III cleavage (Fig. 4). This supported the proposal that these RNAs are possible substrates for this enzyme.

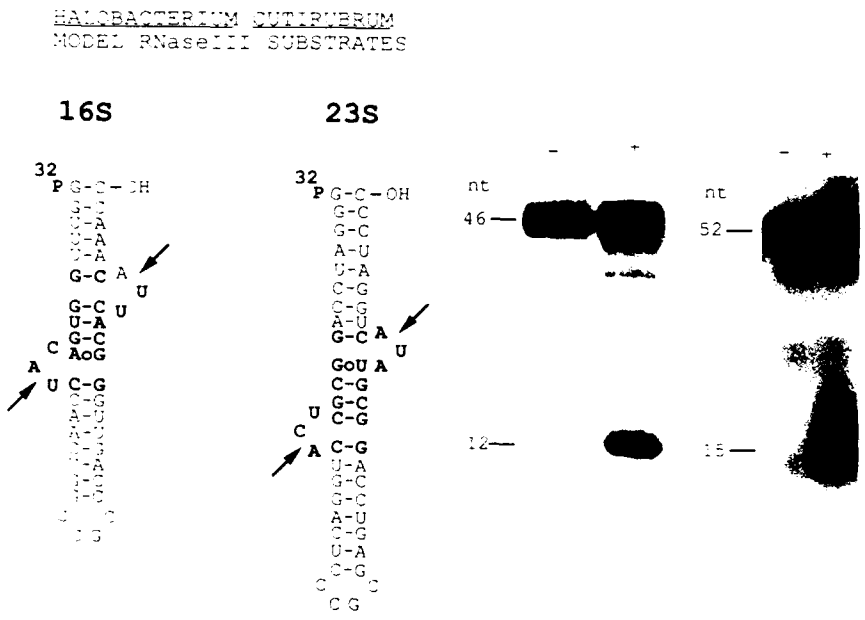
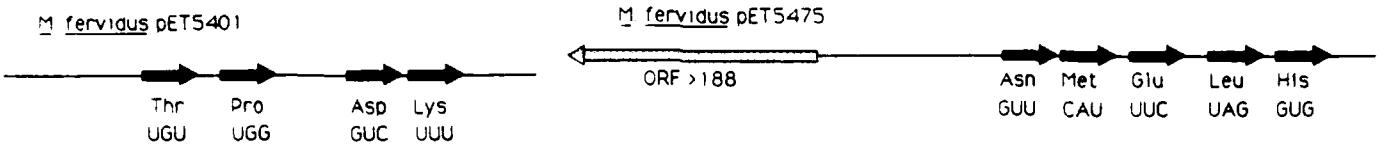


Figure 4. Cleavage of model RNaseIII substrates. Structures of the 16S and 23S rRNA RNaseIII model substrates generated from T7 in vitro transcription from partially doubled stranded DNAs are shown on the left. Darkened sequences represent structural similarity to the exon-intron boundaries of $tRNA^{Trp}$. Cleavage products formed from 5' end labelled RNAs following incubation in the absence (-) or the presence (+) of the H. volcanii intron endonuclease are shown on the right.

Removal of tRNA 5' leader sequences. We again utilized the T7 RNA polymerase transcription system to generate substrate molecules for the development of an RNaseP assay. The H. volcanii $tRNA^{Val}$ gene with a short 5' leader sequence was placed under the transcriptional control of the T7 RNA polymerase promoter. In preliminary studies during years one and two, this assay was used to partially purify an activity from low salt H. volcanii extracts. A small RNA of approximately 450 nucleotides copurified with this activity suggesting that this activity was a ribozyme like its eubacterial and eukaryotic counterparts. Formal demonstration of an RNA requirement could not be shown by sensitivity of the activity to micrococcal nuclease since the enzyme was inhibited by EGTA, which is necessary to block micrococcal nuclease activity during the assay. More recently, we have partially purified this activity from high salt extracts and tentatively identified a 450 nucleotide RNA as a component of this complex. This activity remains active at 3M salt and exhibits a higher degree of specificity at the physiological incubation conditions than at lower salt concentrations. cDNAs prepared from this RNA component have been used to identify and clone restriction fragments containing the gene encoding the RNA. A 1.0 kbp Sal1 fragment which hybridizes to a 450 nucleotide RNA in Northern Analysis is currently being sequenced. Analysis to date suggests that the RNA components of the low salt and high salt activities are similar and that activity at high salt may be affected by additional protein components present in the high salt enzyme preparation. We have also detected a similar activity in extracts from Thermoplasma which exhibit maximum activity at 58°C. Possible involvement of an RNA in this activity has not yet been evaluated.

Identification of intron containing tRNA genes. Our primary goal in these studies was to evaluated whether the occurrence of introns in halophilic tRNA genes was limited to the genes encoding $tRNA^{Trp}$. Two approaches, based on the detection of precursor forms of $tRNA^{Trp}$ in Northern Analysis were employed; ctDNA prepared from total tRNAs and a universal tRNA probe were used to probe for precursor forms in bulk RNA populations. Neither approach produced reproducible hybridization signals consistent with the presence of precursor forms. It appeared that this approach was not sufficiently sensitive to detect precursors since a recent report described the occurrence of an intron containing $tRNA^{Met}$ precursor in H. volcanii (Data et. al. Can J. Micro. 35: 189, 1989). In year three we have broadened our focus on tRNA gene structure to include an analysis of stable RNA genes in thermophilic Archaebacteria, in particular Thermoplasma and M. fervidus, an extremely thermophilic methanogen. For Thermoplasma we have established a lambda library and identified 12 clones encoding tRNA genes. One clone currently being analyzed contains sequences related to the $tRNA^{Trp}$ gene of Halobacterium volcanii. Since Thermoplasma has tRNA intron endonuclease activity, this gene is a likely candidate for the presence of an intron. Analysis of M. fervidus stable RNA genes has been done in collaboration with John Reeve's laboratory (OSU). Genes encoding several tRNAs, the 16S, portions of the 23S and 5S rRNAs have been cloned and sequenced. Two M. fervidus tRNA clusters were identified and characterized; clone pET5401 contained genes encoding tRNAs threonine(UGU), proline (UGG), aspartic acid (GUC), and lysine (UUU) and clone pET5475 containing genes encoding asparagine (GUU), methionine (CAU), glutamic acid (UUC), leucine(UAG) and histidine (GUG) (Fig. 4). None of these genes were interrupted by introns. The rRNA genes were localized in two operons having the typical halophilic/methanogen organization, 16S-$tRNA^{Ala}$-23S-5S. In each case these RNAs exhibited a higher degree of G-C base pairing in the helical regions when compared to their mesophilic counterparts, suggesting that this is one feature that imparts thermostability.



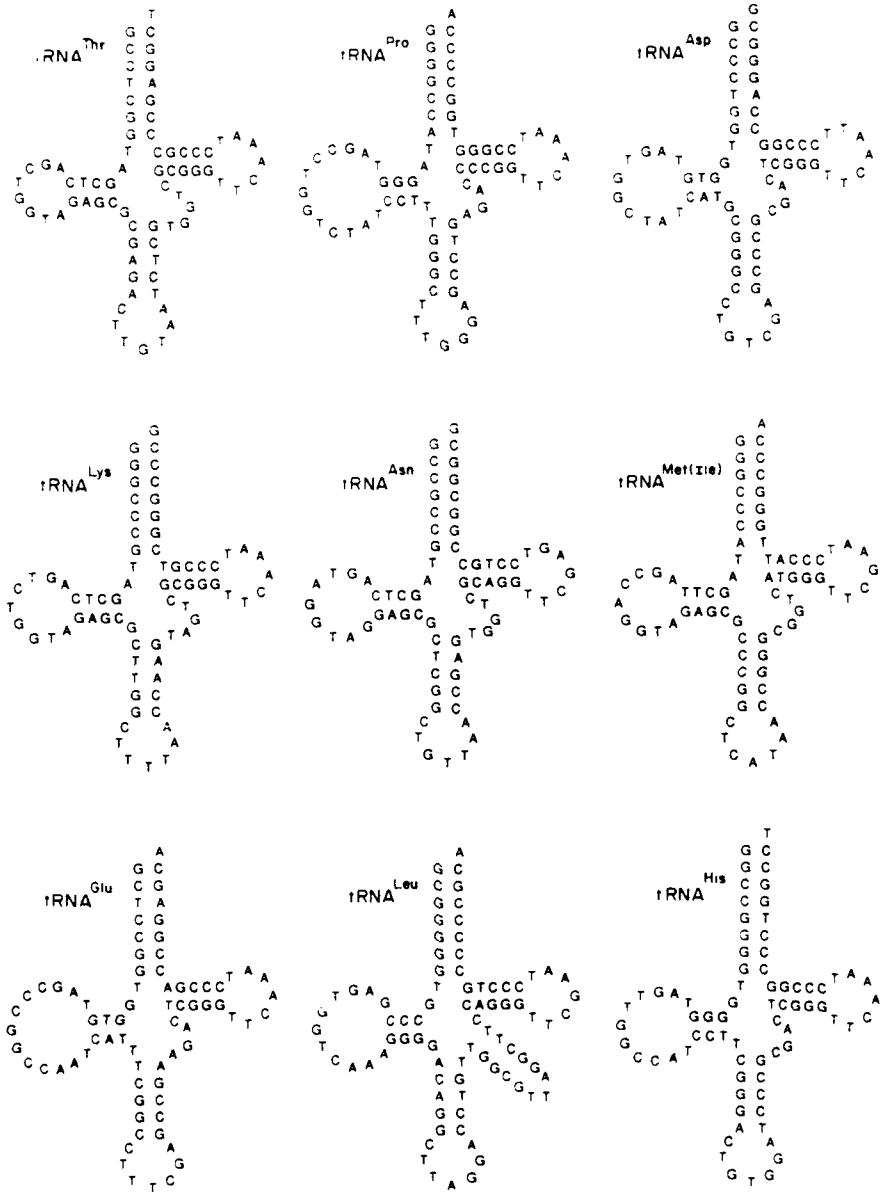


Figure 5. Gene organizations for the M. fervidus tRNA containing clones pET5401 and pET5475. Cloverleaf structures of the tDNAs predicted from these genes are shown below.

In Vivo Studies. The availability of a selectable marker in halobacteria, resistance to the HMGCoA reductase inhibitor mevinolin, has led us to investigate the possibility of developing an in vivo system for RNA processing. Using a plasmid containing the mevinolin resistance gene provided to us by Ford Doolittle and promoter regions from halobacterial tRNA genes, we are constructing an expression vector for H. volcanii. Constructions have been isolated in which altered $tRNA^{Trp}$ genes have been placed under the transcriptional control of the H. volcanii $tRNA^{Lys}$ promoter region. Using "marked" $tRNA^{Trp}$ exons the fate of intron and exons from these molecules can be monitored by Northern analysis. In parallel, we are isolating auxotrophic strains for use in the development of a suppression assay for tRNA processing and temperature sensitive lethal mutants which affect endonuclease activity. More than 100 auxotrophs and three cold sensitive lethal mutants have been isolated and are being characterized.

PROSPECTS.

The development of an in vitro assay system for intron endonuclease in the Archaebacteria has provided a well defined system for studying the interaction of proteins and nucleic acids in these unusual organisms. Of immediate interest is the definition of substrate recognition by this enzyme; which structures and sequences are necessary for binding and identification of cleavage sites. In an attempt to define the minimum requirement for activity we have begun to analyze the cleavage of model substrates by the halophilic enzyme. Extension of these studies to include the extreme thermophiles should provide information on the requirements for RNA-protein interaction at high temperature. A relationship between intron endonuclease and general rRNA maturation has not been reported for other organisms containing interrupted tRNA genes. It is possible that this is a unique feature found only in the Archaebacteria.

Despite the extensive characterization of RNaseP RNAs in eubacterial and eukaryotic organisms, the mechanism of catalysis and the structure of the catalytic center of this enzyme have not been defined. The prospect of identifying a RNA component in the activity for 5' end maturation of tRNAs in the Archaebacteria is exciting. In addition to providing a larger data base for comparative structure analysis, these RNAs may provide insight into the characteristic and requirements for RNA catalysis at extreme temperatures. The converse situation, the absence of a RNA component in this activity, is also interesting since it has important implications about the evolution of RNA maturation enzymes.

The definition of intron distribution and intron type within archaebacterial genes remains an important question. As member of a distinct evolutionary group, genomes of the Archaebacteria may harbor new intron types or introns which are evolving new processing mechanisms. For example, the 23S rRNA intron of D. mobilis has some structure and sequence features in common with self-cleaving introns however, its processing most closely resembles tRNA intron processing. The utilization of Archaebacterial as expression hosts or as sources of biotechnologically important macromolecules will require an understanding of basic gene structure and expression. This will in part be aided by the growing analysis of Archaebacterial genes, but more importantly by the recent developments in the establishment of genetic exchange systems in these organisms.

PUBLICATIONS.

1. Thompson, L.D. and Daniels, C.J. 1988. A $tRNA^{Trp}$ intron endonuclease from the archaebacterium Halobacterium volcanii: Unique substrate recognition properties. J. Biol. Chem. 263: 17951-17957.

2. Brown, J.W., Daniels, C.J. and Reeve. J.N. 1989. Gene structure, organization and expression in archaebacteria. Critical Reviews in Microbiology, 16: 287-338.

3. Thompson, L.D., Brandon, L., Nieuwlandt, D.T. and Daniels, C.J. 1989. Transfer RNA intron processing in the halophilic archaebacteria. Can. J. Microbiol. 35: 36-42.

4. Haas, E.S., Daniels, C.J. and Reeve, J.N. 1989. Stable RNA genes from the extremely thermophilic archaebacterium Methanothermus fervidus. Gene 77: 253-263.

ABSTRACTS.

1. Genes with Introns. American Society for Microbiology. May 1989

2. Unusual Substrate Recognition Properties of the Archaebacterial Intron Endonuclease. 13th International tRNA Workshop. June, 1989

3. RNA Processing in the Archaebacteria. Canadian Institute for Advanced Research: Genome Evolution. October, 1988.

4. RNA Processing in the Archaebacteria. Third International Symposium on the Molecular Biology of the Archaebacteria. August 1988.

5. Unique Substrate recognition Properties of an Archaebacterial tRNA endonuclease. RNA Processing: Cold Spring Harbor Laboratory. May 1988.

6. Intron Processing in the Halophilic Archaebacteria. Canadian Institute for Advanced Research: Genome Evolution. October. 1987.

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AWARDS. Dr. C.J. Daniels was appointed as an associate of the Canadian Institute for Advanced Research, April 1988.