The overall goal of this project is to develop an understanding of tRNA gene structure and transcript processing in the halophilic Archaea. During the second year of these studies we have extended our analysis of the processing of the halobacterial intron containing tRNA Trp precursor. Intron endonuclease has been found to have unique substrate recognition properties and progress has been made in developing a tRNA ligase assay using the products of this reaction. Intron endonuclease activity has also been detected in other Archaea. Continued purification of RNAase P, the enzyme which removes 5' flanking sequences from tRNA primary transcripts, indicates that this enzyme may have a RNA component. Searches for stable intron containing precursor tRNAs in the halophilic Archaea suggest that tRNA may be the only interrupted tRNA gene in these organisms.
RESEARCH OBJECTIVE: To determine the mechanism of tRNA intron processing in the halophilic archaeabacteria; 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 archaeabacteria.

PROGRESS:

A. Intron Processing. We have chosen the \textit{H. volcanii} and closely related \textit{H. mediterranei} intron containing tRNA\textsuperscript{Trp} precursors as model substrates to investigate intron processing the Archaeabacteria. Previously we had established an \textit{in vitro} assay for the tRNA intron endonuclease using transcripts of these genes produced from T7 RNA polymerase transcription vectors. The enzyme was partially purified and shown to be capable of precise and accurate cleavage of the \textit{in vitro} produced precursors. During this past year, we have extended our studies on the mechanism of intron processing. Further characterization of the endonuclease reaction products has indicated that phosphodiester bond cleavage results in the formation of 2',3' cyclic phosphate and 5' hydroxyl termini, similar to the eukaryotic tRNA intron endonuclease. In spite of this similarity, substrate recognition by the halophilic endonuclease was found to be distinctly different from its eukaryotic counterpart. In eukaryotes, recognition of intron containing tRNA precursors requires the presence of intact mature tRNA structure. The enzyme
interacts specifically with the anticodon stem, D stem and extra arm positions of the precursor and may use a measurement mechanism, from the central portion of the molecule to identify cleavage sites. We have prepared a number of deleted substrates lacking either exon or intron sequences and examined their activities as substrates (Fig. 1). These molecules either lacked regions of the exons preventing the formation of mature tRNA structures (Δ13, Δ1311, Δ13115), portions of the intron (Δ167) or both (Δ1311-10, Δ1311-24). From these studies we have concluded that only those exon sequences near the cleavage site are required (anticodon stem and loop); the presence of mature tRNA-like structure, a requirement for recognition by the eukaryotic endonuclease, is not required. The large intron, while not absolutely required, does affect the accuracy and efficiency of the cleavage reaction.

Since the halobacterial endonuclease would not cleave a typical eukaryotic intron containing pre-tRNA, pre-tRNA<sup>Phes</sup>, this enzyme must require a specific sequence or structure. Among potential structural interaction possible in the deleted substrates, those with extended anticodon helices were most energetically favorable. These structures were reminiscent of the RNaseIII cleavage sites found in rRNA operon primary transcripts.
examination of RNaseIII cleavage sites in the halobacteria indicated sequence and structural similarity between tRNA^Trp^ intron cleavage and the RNaseIII processing site of 23S rRNA (Fig. 2). To determine whether the endonuclease would recognize this RNA, a model substrate mimicking the halobacterial 23S rRNA RNaseIII cleavage site was prepared (Fig. 3). When assayed under standard conditions, the intron endonuclease was capable of cleaving this molecule. This suggested that the extended helix structure in which the cleavage sites are localized, the sequence 5'-UCCAG-3', or both are important for recognition.

**Figure 2.** Comparison of cleavage sites for the tRNA^Trp^ intron endonuclease and 23S rRNA RNaseII. A. The exon intron boundaries of the A167 substrate. B. The proposed RNaseIII cleavage site of the *H. cutirubrum* 23S rRNA. Boxed nucleotides are conserved between these RNAs and include the tRNA^Trp^ anticodon sequence CCA. Cleavage sites for intron endonuclease and RNaseIII are indicated by solid and open arrows, respectively.

**Figure 3.** Cleavage of a RNaseIII model substrate. A. Double stranded structure of the self complementary RNA representing the 23S rRNA RNaseIII cleavage site of *H. cutirubrum*. RNA was obtained by T7 RNA polymerase directed in vitro transcription. B. Cleavage of the model substrate labeled with α[32P]ATP with partially purified *H. volcanii* tRNA^Trp^ intron endonuclease. Lane 1, uncut; Lane 2, cleavage at 25°C; and Lane 3 cleavage at 37°C.

Utilizing the products of the tRNA^Trp^ endonuclease reaction, we have begun studies on the ligation of tRNA half molecules, the second step in the processing pathway. Products similar in size to those predicted for combined endonuclease and ligase reactions can be detected in freshly prepared crude
extracts (Fig. 4). However, this activity is rapidly inactivated. The lack of ligase activity is not unique to halobacteria. We have also been unable to detect significant amounts of ligase activity in extracts of Thermoplasma, Methanosarcina and Sulfobolus. Studies are currently underway to examine the structure of this product to determine whether correct ligation has occurred.

![Combined endonuclease and ligase activities. Uniformly labeled Al6 RNA was processed with the partially purified endonuclease preparation (Lane 1) or with a freshly prepared 10% PEG precipitate fraction of a H. volcanii S30 extract (Lane 2). The proposed ligation product (115 nt) is indicated.](image)

**B. Removal of 5' leader sequences by RNaseP.** We have continued our characterization of the H. volcanii RNaseP enzyme. Using an *in vitro* generated substrate derived from the H. volcanii tRNA\(^{\text{Val}}\) gene, we have established an assay for this enzyme and are developing strategies for the purification of this enzyme. While we have been unable to demonstrate a functional requirement for RNA by micrococcal nuclease treatment due to EGTA inhibition, we observe RNA species copurifying with the enzymatic activity. A large RNA of approximately 405 nucleotides and two smaller RNAs (140 and 105 nucleotides) are likely candidates for the RNaseP RNA (Fig. 5). The smaller RNAs have been shown by Southern analysis to be nonribosomal in origin and have been localized to a unique PstI restriction fragment. We have identified several proteins which copurify with these RNAs. Among these is a 17,000 dalton protein which is similar in size to the protein component of the eubacterial RNaseP complex.
C. Identification of intron containing tRNA genes. Using Northern analysis, two approaches have been used to search for additional intron containing precursor tRNA molecules in the *H. volcanii*. The presence of intron containing pretRNA<sup>Trp</sup> molecules in total RNA preparations suggested that other precursors may also be present in detectable amounts in these preparations. Complimentary tDNAs were prepared by first adding poly A tails to the total tRNA population followed by second strand synthesis using reverse transcriptase and an oligo dT primer. These ctDNAs hybridized to mature tRNAs and a species which was similar in size to the predicted intron containing pretRNA<sup>Trp</sup> molecule. A second probe was also used. Based on the sequences of 44 *H. volcanii* tRNAs, a consensus antitRNA oligonucleotide probe was synthesized. Like the ctDNAs, this probe hybridized to mature tRNAs and intron containing pretRNA<sup>Trp</sup> sized molecules. These data do not exclude the presence of other intron-containing pre-tRNAs, however they do suggest the possibility that tRNA<sup>Trp</sup> may be the only interrupted tRNA gene in this organism. A comprehensive sequence analysis of tRNA genes in this organism appears to be the only method to resolve this question.

In an examination of other organisms for interrupted tRNA genes and processing activity, we have detected tRNA<sup>Trp</sup> endonuclease activity in
extracts of *Thermoplasma* (Fig. 6A), suggesting that the tRNA$^{Trp}$ gene or possibly other tRNA genes in this organism are interrupted by an intron. This activity appeared to be specific for the Archaeabacterial substrate. Like the halobacterial enzyme, it did not cleave the yeast tRNA$^{Ph}$*. Using a halobacterial tRNA$^{Trp}$ exon probe, we have identified restriction fragments bearing these sequences (Fig. 6B). Sequences related to the halobacterial tRNA$^{Trp}$ intron did not hybridize, even under low stringency conditions, indicating that the tRNA$^{Trp}$ gene, if interrupted, had a different intron.

![Figure 6](image.png)

**Figure 6.** Endonuclease activity in *Thermoplasma*. A. Cleavage of uniformly labeled yeast pretRNA$^{Ph}$* (Lane 1) and *H. volcanii* pretRNA$^{Trp}$ Δ16 (Lane 2) by a crude lysate of *Thermoplasma*. B. Southern analysis of HindIII (Lane 1, 1') or PstI (Lane 2, 2') cleaved total *Thermoplasma* DNA probed with *H. volcanii* tRNA$^{Trp}$ sequences under high (Lanes 1 & 2) or low (Lanes 1' & 2') stringency conditions.

**WORKPLAN:**

**A. Intron processing.** In keeping with our goal to characterize the mechanism of tRNA processing, we will continue our efforts to develop a tRNA ligase assay. A possible factor which may influence the efficiency of the reaction may be a requirement for mature 5' and 3' termini. To examine this, we will use RNaseP to remove the 5' leader of pretRNA$^{Trp}$ and the yeast pretRNA$^{Ph}$* gene and yeast endonuclease to produce tRNA half molecules. These
latter molecules will have 5' phosphate and 2',3' cyclic phosphate termini on the first exon and a 5' hydroxyl and 3' CCA residues on the second exon, features which are representative of the in vivo substrate and required by the yeast ligase in vitro. In an attempt to isolate stable activity, both high and low salt extracts of *H. volcanii* will be examined. Protease inhibitors and other stabilizing agents will also be employed.

We will continue our characterization of the substrate recognition properties of the endonuclease. Using site specific mutational analysis, we will determine whether this enzyme recognizes the tRNA\(^{\text{Trp}}\) anticodon sequences, a specific structure or both. In parallel, we will use additional chromatographic procedures such as CM-cellulose, dye columns, poly I·C double stranded RNA columns and possibly affinity chromatography to further purify this activity. This is particularly important in view of the possibility that this enzyme may act on both tRNA and rRNA precursors.

B. RNaseP. Using Southern analysis and direct RNA sequencing, the relationship between the three RNAs which copurify with RNaseP activity will be investigated. These RNAs will then be used to identify and clone their corresponding genes. Comparative structural analysis of potential transcripts from these genes with other RNaseP molecules and reconstruction assays using \textit{in vitro} produced RNA, in the presence and absence of added proteins, will be used to verify the role of this RNA in the reaction.

C. Occurrence of introns. Examination of tRNA intron distribution will focus on the tRNA\(^{\text{Trp}}\) genes of *Thermoplasma*. This gene will be cloned using the *H. volcanii* gene probe. Sequence of this gene will indicate whether an intron is present and if the intron has structural or sequence similarity to the halobacterial gene. Additional tRNA genes will be cloned using end labeled *Thermoplasma* tRNA as probe and screened by Northern analysis for precursor forms. We will also continue in our efforts to clone tRNA genes from *H. volcanii* large (>90 kbp) BamH1 tRNA-rich restriction fragments isolated by field inversion electrophoresis. These experiments remain part of the long term goal of this project, but must be considered lower in priority due to the uncertainty of finding additional introns.
PUBLICATIONS AND PRESENTATIONS:


   This work has also been presented at three meetings:


TRAINING ACTIVITIES:

Two graduate students, Leo D. Thompson and Daniel T. Nieuwlandt and an undergraduate student, Jorge L. Acevedo, were supported by this grant. Mr. Acevedo is a minority student from Puerto Rico.

AWARDS:

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