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Expression of a Yeast Intron-Containing tRNA in the Archaeon *Haloferax volcanii*

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Expression of the yeast tRNAPro(UGG) gene in *Haloferax volcanii* resulted in the production of a single stable transcript that had not undergone intron processing or processing of 5′ and 3′ flanking sequences. Mutation of the exon-intron boundary region of this RNA to produce a precursor RNA with the preferred halobacterial consensus exon-intron boundary structure did not restore intron processing. Processing of 5′ and 3′ flanking sequences was restored when the acceptor stem U6-U67 pair was changed to A6-U67. The significance of these results in defining the recognition requirements of tRNA maturation enzymes in the halophilic domain *Archaea* is discussed.

In vitro studies suggest that the archaeal and eukaryal tRNA intron endonucleases use different mechanisms to identify intron-containing precursor RNAs and their cleavage sites (2, 3, 9, 11, 12, 16, 17, 19, 20). To further explore the relationship between these enzyme systems we have utilized a *Haloferax volcanii* plasmid expression vector (13) to express the *Saccharomyces cerevisiae* intron-containing tRNAPro(UGG) (hereafter referred to as tRNAPro) gene in *H. volcanii* (Fig. 1A). The yeast tRNAPro gene was chosen since it encodes a precursor RNA that contains an intron located at the same relative position in the mature tRNA as halophilic pre-tRNAs (15) but lacks the preferred halobacterial exon-intron boundary structure. In vitro studies show that *H. volcanii* tRNA intron endonuclease requires that each exon-intron cleavage site be located in a 3-nucleotide bulge loop and that these loops be separated by 4 bp (20). While the intron-containing yeast tRNAPro RNA can assume a structure in which the two cleavage sites are located in loops separated by 4 bp, neither loop is the preferred 3-nucleotide bulge loop (Fig. 1A). By examining processing of the wild-type tRNAPro preRNA and a mutant, which has the preferred halophile exon-intron structure, it is possible to determine if exon-intron boundary structure alone is sufficient to direct cleavage in vivo. Expression of the nonhalophilic tRNA in these cells also provides a means to monitor 5′ and 3′ processing reactions by Northern (RNA) analysis, without interference from chromosomal encoded tRNAs.

In this report we describe the processing in *H. volcanii* of RNAs arising from the wild-type yeast tRNAPro gene and a mutant tRNAPro gene in which the exon-intron boundary regions are converted to the preferred halophilic structure. We also describe how changing the U6-U67 pair of the yeast tRNAPro acceptor stem to a Watson-Crick pair, A6-U67, effects RNase P cleavage in vivo.

Construction of *H. volcanii* expression plasmids carrying modified yeast tRNAPro genes. A 148-bp *Rsa*I fragment, containing the yeast tRNAPro gene, was subcloned from plasmid pGKN1 (15) into the *Sma*I site of the phagemid vector pSelect-1 (Promega Corp., Madison, Wis.) to give the recombinant plasmid pSelect-Pro. Prior to the mutagenesis reactions, the *Bam*HI site located 5′ of the tRNAPro gene (within the multiple cloning region of pSelect-1) was destroyed by cleaving the plasmid with *Bam*HI, removing the single-strand sequences with S1 nuclease, and religating the blunt ends. This DNA functioned as a source of the wild-type yeast tRNAPro gene. On the basis of in vitro studies with the *H. volcanii* intron endonuclease (19, 20), we predicted that the tRNAPro preRNA, with its nonhalophilic exon-intron boundary structure, would not be a substrate for in vivo intron processing. In an attempt to make the yeast tRNAPro preRNA an active substrate for the halophilic endonuclease, 4 nucleotides were removed from the intron (Fig. 1A). These changes converted the exon-intron structure to the structure preferred by the halophilic endonuclease, in which the two exon-intron cleavage sites are localized to 3-nucleotide bulge loops separated by 4 bp (20). The deletion of intron nucleotides C58, G59, A81, and G86 was directed by the mutagenic oligonucleotide PROMUT (5′-GGGCCTCTCGCATGTTTGCTTCCTGTTTAATCAGG AAGTCCCAAAGCGAGAATC-3′) by using the Altered Sites in vitro mutagenesis system (Promega Corp.). The mutagenic reaction was carried out as described by the manufacturer. Plasmids containing the mutant tRNAProM gene were identified by DNA sequence analysis. The tRNAPro and tRNAProM genes were subcloned as *Xba*I-*Eco*RI fragments into the *H. volcanii* expression vector pWL205 (13) to yield plasmids pWL221 and pWL222, respectively. In a separate PCR-directed mutagenesis procedure, which was designed to create mutations in the *H. volcanii* tRNALys promoter region of plasmid pWL222 (15a), we obtained a spontaneous transversion mutation, U6 to A6, in the tRNAProM gene. This mutation changed the non-Watson-Crick pair, U6-U67, of the acceptor stem to a Watson-Crick pair, A6-U67 (Fig. 1A). This gene was cloned into the *H. volcanii* expression plasmid pWL205, as an *Xba*I-*Eco*RI fragment, to give plasmid pWL222A6. Each of the tRNAPro-containing expression plasmids was introduced into *H. volcanii* WFD11 as previously described (13).

Expression of yeast tRNAPro and tRNAProM genes in *H. volcanii*. Northern analysis (13) was used to detect transcripts arising from the wild-type and mutant yeast tRNAPro genes. Five oligonucleotide probes were used to monitor expression of the tRNAPro genes: PFLANK (5′-CTCTAGAGTCCCCT TGC-3′), specific for the 5′ leader region of the transcript; PROEXI (5′-CCCAAAGCGAGAATCATACCAC-3′), spe-

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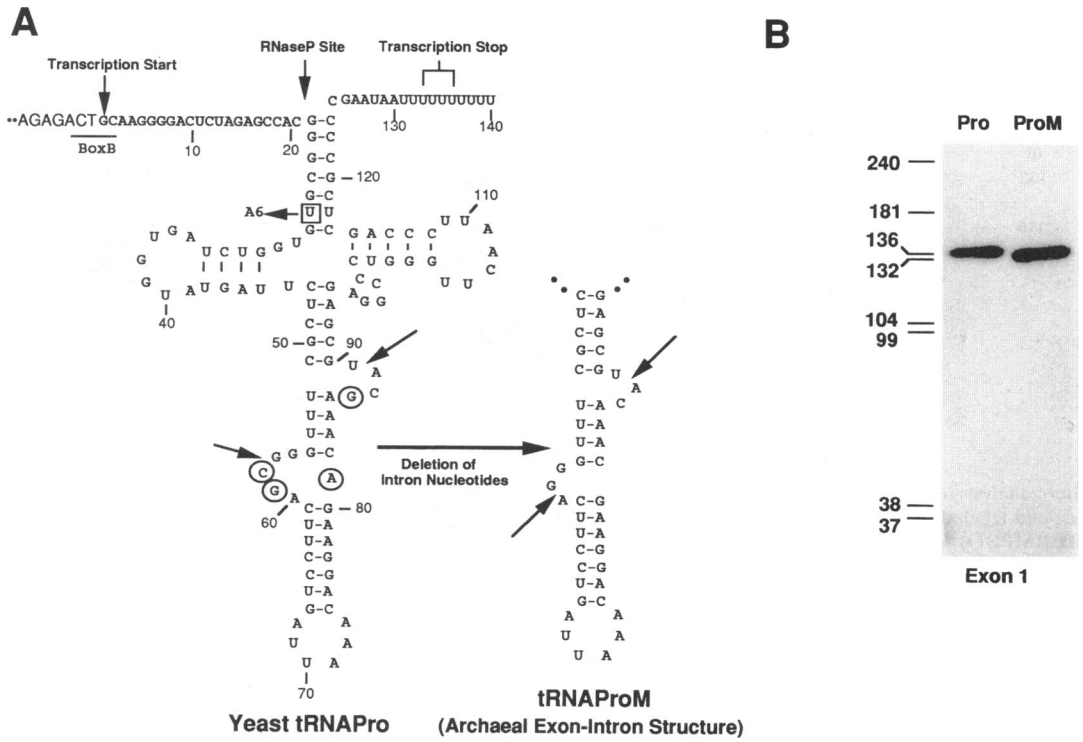

FIG. 1. Expression of the intron-containing yeast tRNAPro gene in *H. volcanii*. (A) Sequence of the predicted transcript for the tRNAPro gene. Circled nucleotides were deleted from the intron to give the tRNAProM gene (indicated to the right). The single nucleotide change in the acceptor stem (U6 to A6), leading to the tRNAProMA6 mutation, is shown on the tRNAPro transcript. Also shown are the sites for transcription initiation and termination as determined by primer extension and S1 nuclease mapping, respectively. (B) Northern analysis of RNAs isolated from *H. volcanii* cells carrying the yeast tRNAPro or tRNAProM gene. Hybridizations were performed with a 5′-end-labeled exon 1-specific oligonucleotide, PROEXI, as the probe.

cific for exon 1; PROINT (5′-GCTTTGTCTTCCTGTTTA ATCAGG-3′) or PMUTINT (5′-GTTTGCTTCCTGTTTA ATC-3′), specific for the wild-type or tRNAProM intron, respectively; and TERMPRO (5′-AAAAAAAAATTATTC GGGGCGA-3′), specific for the 3′ trailing sequence. The 5′ termini of tRNAProM and tRNAProMA6 were determined by primer extension analysis with PROEXI labeled with ^{32}P at the 5′ end as the primer (14).

Results of Northern analysis of RNAs isolated from cells carrying the tRNAPro or tRNAProM gene using the exon 1-specific PROEXI oligonucleotide as the probe are shown in Fig. 1B. The hybridization pattern indicates that each gene produces a single transcript of 136 or 132 nucleotides for tRNAPro and tRNAProM, respectively. Similar hybridization patterns were obtained when PFLANK, PROINT, or PMUTINT, and TERMPRO oligonucleotides were used as the probes (data not shown). It appeared that neither RNA was fully processed, since a 75-nucleotide species, the expected mature tRNAPro, was not detected. The results of 5′ and 3′ terminus mapping provided further support for the notion that these RNAs were unprocessed. The 5′ termini of the 132- and 136-nucleotide species were mapped by primer extension analysis and found to be located at the expected *H. volcanii* tRNALys initiation site (BoxB) (data not shown for tRNAPro; see Fig. 3 for tRNAProM RNA). This indicated that these transcripts were not processed by RNase P. The 3′ terminus of tRNAProM was determined by S1 nuclease mapping (data not shown) and found to lie in the poly-T region of the yeast polymerase III terminator, which accompanied the tRNAPro gene during cloning. These data show that tRNAPro and tRNAProM gene transcripts did not enter into intron, 5′, or 3′ processing pathways. Both RNAs remained as stable primary transcripts.

The inability of the halophilic tRNA intron processing enzymes to act on tRNAPro was predicted from the in vitro model (19, 20), since this RNA lacked the exon-intron boundary structure preferred by the *H. volcanii* tRNA intron endonuclease. However, tRNAProM, which had the preferred exon-intron structure, was also not processed. The inability of this RNA to undergo processing indicates that exon-intron boundary structure itself is insufficient to direct processing and suggests that sequences in this region may also play a role in in vivo processing. A comparison of the archaeal exon-intron boundary sequences (4, 8, 18) with the tRNAProM RNA reveals some notable differences. In the archaeal pre-tRNAs, the 3-nucleotide loop containing the intron-exon 2 cleavage site is usually formed by a C-G pair 5′ to the loop. When the intron is located between positions 37 and 38 of the anticodon loop, as in the *H. volcanii* tRNAs (5, 6), this interaction involves the highly conserved C32 residue, the first nucleotide of the anticodon loop, and the nucleotide three positions 5′ of the intron-exon 2 cleavage site. In the case of yeast tRNAProM the intron-exon 2 cleavage site loop is formed by a U-A base pair. The nucleotide present at the 3′ side of the tRNAProM intron-exon 2 cleavage site also differs from the archaeal consensus. In tRNAPro and tRNAProM this position is occupied by a U residue. In all archaeal intron-containing preRNAs, both tRNA and rRNA precursors, this position is occupied by a purine residue; U is excluded from this position (4, 8, 18). If either or both of these sequences are required by

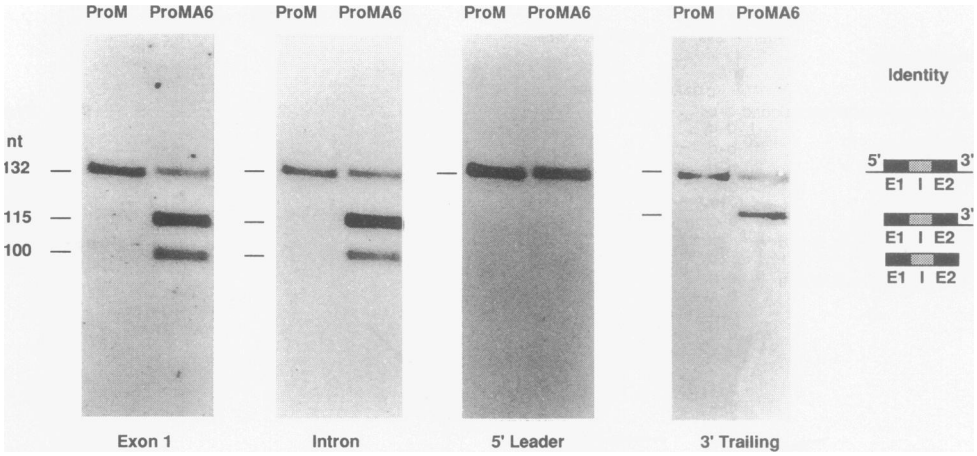

FIG. 2. Northern analysis of RNAs isolated from *H. volcanii* cells carrying the yeast tRNAProM and tRNAProMA6 genes. Hybridizations were performed with 5′-end-labeled oligonucleotides complementary to exon 1 (PROEXI), intron (PMUTINT), 5′ leader region (PFLANK), and 3′ trailing region (TERMPRO) sequences. The identities of the RNAs, based on their hybridization patterns, are shown to the right. RNA sizes are based on the migration marker RNAs that were visualized on the stained gels prior to transfer. nt, nucleotide.

the halophilic endonuclease, this could account for the failure of tRNAProM to act as a substrate. We are currently investigating this possibility.

5′ and 3′ processing of yeast tRNAProMA6 RNA in *H. volcanii*. The expression and processing of a second mutant of the yeast tRNAPro gene were also examined. The tRNAProMA6 RNA contains a U6-to-A6 transversion mutation that changes a U6-U67 pair of the acceptor stem to a A6-U67 pair (Fig. 1A). Three RNA products (132, 115, and 100 nucleotides) were detected when an exon 1-specific probe was used in Northern analysis of RNAs isolated from cells carrying this gene (Fig. 2). A detailed analysis, using probes specific for exon 1 (PROEXI), intron (PMUTINT), 5′ leader (PFLANK), and 3′ trailing sequences (TERMPRO), showed that all RNAs contained exon and intron sequences (Fig. 2). The hybridization patterns also showed that the 115-nucleotide species lacked only the 5′ leader region and that the 100-nucleotide species lacked both 5′ and 3′ sequences. Absent from these products was an RNA species that contained the 5′ leader region without the 3′ trailing sequence. The absence of this RNA indicates that removal of the 5′ sequence precedes processing of the 3′ region. The 5′ terminus of the 115-nucleotide species was determined by primer extension analysis and found to be located at the G residue corresponding to the expected RNase P cleavage site (Fig. 3).

The severity of the U6-U67 effect on RNase P cleavage in *H. volcanii* was unexpected. Although it is well established that recognition by bacterial RNase P involves both the nucleotide at the cleavage site and the length of the acceptor stem (for a review, see reference 1), base pairing between positions 6 and 67 has not been shown to be a major determinant in processing efficiency. In the case of an *Escherichia coli* tRNAHis-*Schizosaccharomyces pombe* tRNASer RNA hybrid tRNA, changing U6-A67 to U6-G67 had little effect on cleavage by *S. pombe* and *E. coli* RNase P's or by the M1 RNA alone (7). On the basis of the observation that the *H. volcanii* RNase P holoenzyme complex can cleave halobacterial and bacterial pre-tRNAs, which have different acceptor stem sequences (10, 14), the requirement for an A-U pair in tRNAProMA6 most likely reflects the need for base pairing in the acceptor helix rather than recognition of specific sequences.

The use of a eukaryal intron-containing tRNA in the development of in vivo tRNA processing assays in *H. volcanii* has uncovered several important features of the tRNA maturation pathways in this organism. The inability of tRNAProM to undergo intron processing indicates that features beyond the exon-intron boundary structure, possibly sequence, are required for in vivo intron processing. Recovery of tRNAProM RNase P cleavage by changing a U6-U67 pair to an A6-U67 pair indicates that *H. volcanii* RNase P is sensitive to mis-

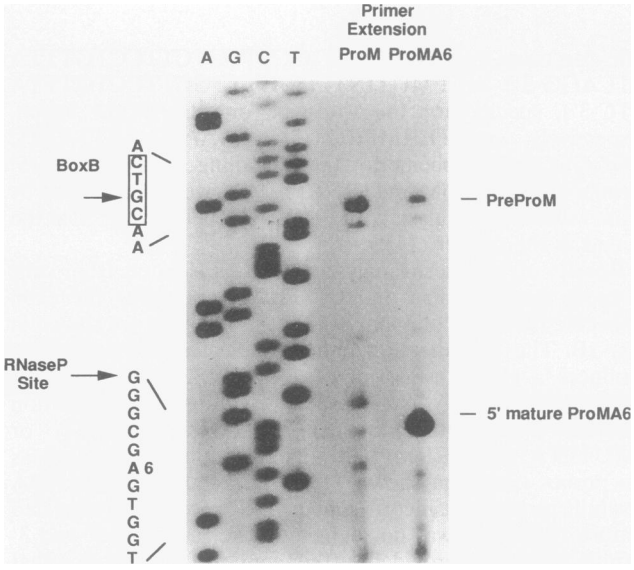

FIG. 3. RNase P cleavage of tRNAProMA6 gene transcripts in *H. volcanii* visualized by primer extension analysis. RNAs isolated from *H. volcanii* cells carrying the tRNAProM and tRNAProMA6 genes were used as templates for cDNA synthesis with a primer complementary to the exon 1 sequence (PROEXI). Migration of the cDNAs, in a denaturing 6% polyacrylamide gel, was compared with the sequence ladder obtained from pUC222A6 DNA (tRNAProMA6). The predicted 5′ terminus of RNase P-processed tRNAProMA6 RNA and the transcription initiation sites (within the tRNALys BoxB) of tRNAProM and tRNAProMA6 are shown adjacent to the sequence ladder.

matches in the acceptor stem. The Northern hybridization pattern observed for cells carrying the tRNAProMA6 gene indicates that removal of 5′ flanking sequences precedes 3′ end maturation and that both 5′ end maturation and 3′ end maturation can occur before intron removal. Finally, these results provide additional support for the hypothesis that archaeal tRNA intron processing enzymes are distinct from their eukaryal counterparts.

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REFERENCES

1. **Altman, S., L. Kirsebom, and S. Talbot.** 1993. Recent studies of ribonuclease P. FASEB J. **7**:7–14.
2. **Baldi, M. I., E. Mattoccia, E. Bufardeci, S. Fabbri, and G. P. Tocchini-Valentini.** 1992. Participation of the intron in the reaction catalyzed by the Xenopus tRNA splicing endonuclease. Science **255**:1404–1408.
3. **Bufardeci, E., S. Fabbri, M. I. Baldi, E. Mattoccia, and G. P. Tocchini-Valentini.** 1993. In vitro genetic analysis of the structural features of the pre-tRNA required for determination of the 3′ splice site in the intron excision reaction. EMBO J. **12**:4697–4704.
4. **Dalgaard, J. Z., and R. A. Garrett.** 1992. Protein-coding introns from the 23S rRNA-encoding gene form stable circles in the hyperthermophilic archaeon *Pyrobaculum organotrophum*. Gene **121**:103–110.
5. **Daniels, C. J., R. Gupta, and W. F. Doolittle.** 1985. Transcription and excision of a large intron in the tRNATrp gene of an archaebacterium, *Halobacterium volcanii*. J. Biol. Chem. **260**: 3132–3134.
6. **Datta, P. K., L. K. Hawkins, and R. Gupta.** 1989. Presence of an intron in elongator methionine-tRNA of *Halobacterium volcanii*. Can. J. Microbiol. **35**:189–194.
7. **Holm, P. S., and G. Krupp.** 1992. The acceptor stem in pre-tRNAs determines the cleavage specificity of RNase P. Nucleic Acids Res. **20**:421–423.
8. **Kjems, J., and R. A. Garrett.** 1991. Ribosomal RNA introns in archaea and evidence for RNA conformational changes associated with splicing. J. Mol. Biol. **218**:293–311.
9. **Kjems, J., J. Jensen, T. Olesen, and R. A. Garrett.** 1989. Comparison of transfer RNA and ribosomal RNA intron splicing in the extreme thermophile and archaebacterium *Desulfurococcus mobilis*. Can. J. Microbiol. **35**:210–214.
10. **Lawrence, N., D. Wesolowski, H. Gold, M. Bartkiewicz, C. Guerrier-Takada, W. H. McClain, and S. Altman.** 1987. Characteristics of ribonuclease P from various organisms. Cold Spring Harbor Symp. Quant. Biol. **52**:233–238.
11. **Mattoccia, E., I. M. Baldi, A. D. Gandini, S. Ciafre, and G. P. Tocchini-Valentini.** 1988. Site selection by the tRNA splicing endonuclease of *Xenopus laevis*. Cell **55**:731–738.
12. **Nieuwlandt, D. T., M. B. Carr, and C. J. Daniels.** 1993. In vivo processing of an intron-containing archaeal tRNA. Mol. Microbiol. **8**:93–99.
13. **Nieuwlandt, D. T., and C. J. Daniels.** 1990. An expression vector for the archaebacterium *Haloferax volcanii*. J. Bacteriol. **172**:7104–7110.
14. **Nieuwlandt, D. T., E. S. Haas, and C. J. Daniels.** 1991. The RNA component of RNase P from the archaebacterium *Haloferax volcanii*. J. Biol. Chem. **266**:5689–5695.
15. **Ogden, R. C., M. C. Lee, and G. Knapp.** 1984. Transfer RNA splicing in *Saccharomyces cerevisiae*: defining the substrates. Nucleic Acids Res. **12**:9367–9382.

15a. **Palmer, J. R., and C. J. Daniels.** Unpublished data.

16. **Reyes, V. M., and J. Abelson.** 1988. Substrate recognition and splice site determination in yeast tRNA splicing. Cell **55**:719–730.
17. **Shapero, M. H., and C. L. Greer.** 1992. Exon sequence and structure requirements for tRNA splicing in *Saccharomyces cerevisiae*. Biochemistry **31**:2359–2367.
18. **Thompson, L. D., L. D. Brandon, D. T. Nieuwlandt, and C. J. Daniels.** 1989. Transfer RNA intron processing in the halophilic archaebacteria. Can. J. Microbiol. **35**:36–42.
19. **Thompson, L. D., and C. J. Daniels.** 1988. A tRNA(Trp) intron endonuclease from *Halobacterium volcanii*. Unique substrate recognition properties. J. Biol. Chem. **263**:17951–17959.
20. **Thompson, L. D., and C. J. Daniels.** 1990. Recognition of exon-intron boundaries by the *Halobacterium volcanii* tRNA intron endonuclease. J. Biol. Chem. **265**:18104–18111.