Splicing of Intron-containing tRNATrp by the Archaeon *Haloferax volcanii* Occurs Independent of Mature tRNA Structure*

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We have investigated the requirements for mature tRNA structure in the *in vivo* splicing of the *Haloferax volcanii* intron-containing tRNATrp RNA. A partial tRNATrp gene, which contained only the anticodon stem-loop region of the mature tRNA, was fused to a carrier yeast tRNA gene for expression in *H. volcanii*. Transcripts from this hybrid gene were found to be processed by endonuclease and ligase at the tRNATrp exon-intron boundaries. These results verify that the substrate recognition properties of the halobacterial endonuclease observed *in vitro* reflect the properties of this enzyme *in vivo*, namely that mature tRNA structure is not essential for recognition by the endonuclease. The independence of these reactions on mature tRNA provides further support for a relationship between archaeal tRNA and rRNA intron-processing systems and highlight a difference in the substrate recognition properties between the archaeal and eucaryal processing systems. The significance of these differences is discussed in light of the observation that the tRNA endonucleases of these organisms are related.

While intron-containing tRNAs are present in the Archaea (formerly the Archaeabacteria), Eucarya, and Bacteria, these sequences do not represent a single homogeneous class of introns. Bacterial and chloroplast tRNA introns are either group I or group II introns, whereas the eucaryal nuclear and archaeal tRNA introns lack any identifiable sequence or structural relationship to the group I, group II, group III, or mRNA introns (1). In the absence of defining sequence or structural characteristics in the archaeal and eucaryal tRNA introns, speculation on the relatedness of these introns has been based primarily on comparisons of their splicing systems. Until recently, it was thought that the archaeal and eucaryal splicing enzymes were distinct systems that were related in function only, and consequently that eucaryal and archaeal introns potentially represented two separate classes of introns. This argument was based on the observations that the archaeal and eucaryal tRNA intron endonucleases differed in subunit composition and substrate recognition mechanisms. The eucaryal endonuclease was observed to be a heterotrimer (2), which has recently been shown to be a tetramer (3), whereas the archaeal enzyme is a homodimer (4). Recognition of the exon-intron boundaries by the eucaryal enzyme involves a complex mechanism that is dependent on the presence of mature tRNA structure. All eucaryal tRNA introns are located in the anticodon loop between positions 37 and 38 of the mature tRNA, extending the anticodon helix, while maintaining the overall mature tRNA structure (1). The eucaryal enzyme senses the distance from the top of the anticodon stem to the 5' and 3' cleavage sites (5, 6) and requires the formation of a three-nucleotide bulge loop at the intron-exon 2 cleavage site, the A-I interaction (7). This mechanism is well suited for the identification of eucaryal tRNA introns where all introns are located in the same relative position. In contrast, *in vitro* studies with the *Haloferax volcanii* intron endonuclease showed that this enzyme does not require complete mature tRNA structure in its substrate (8, 9). This enzyme requires a defined structural element at the exon-intron boundaries, the bulge-helix-bulge motif (9). In this structure each cleavage site is located in a three-nucleotide bulge loop, and the two loops are separated by 4 base pairs. The enzyme senses the distance between the bulge loops, rather than the length of the anticodon stem (9). This mechanism is well suited for the archaeal tRNA intron since these introns are not restricted to a single location in the mature domain of the tRNA. In the Archaea, tRNA introns have been observed in the anticodon loop, the anticodon stem, and the extra arm (10–16). Despite their variability in location, all archaeal intron-containing tRNAs can assume the bulge-helix-bulge structure at their intron-exon boundaries.

With such fundamental differences in subunit composition and recognition mechanisms, the proposal that the archaeal and eucaryal tRNA processing systems were different appeared justified. However, the recent characterization of the genes encoding the *H. volcanii* and Saccharomyces cerevisiae tRNA intron endonucleases has unexpectedly revealed that these two enzymes are related (3, 4). A comparison of the amino acid sequences of the halobacterial endonuclease monomer and the yeast endonuclease complex revealed that the halobacterial protein shared sequence similarity with two subunits of the yeast tetramer. This similarity extended over an approximately 115-amino acid region, and in each case, this sequence was located in the carboxyl terminus of the protein (4). Knowing that the archaeal and eucaryal endonucleases are related underscored the need to verify that the substrate recognition properties of the archaeal endonuclease defined *in vitro* are the same as those used *in vivo*. In this report we describe experiments to test the proposal that the halobacterial tRNA intron endonuclease can process a tRNA intron from a RNA molecule that lacks full tRNA structure. As an *in vitro* test for this model, we have constructed an *H. volcanii* expression module that is capable of producing a hybrid RNA that encodes a partial *H. volcanii* tRNATrp RNA fused to the 5' leader region of the non-processing yeast tRNAProM RNA (17). The tRNATrpA13115'–tRNAProM hybrid RNA encodes the tRNATrp anticodon stem-loop region and intact intron fused to the carrier RNA. This represents the minimum exon sequences required for *in vitro* cleavage. Analysis of RNA from cells carrying this
hybrid gene demonstrate that this partial tRNATrp RNA is processed by both endonuclease and ligase enzymes in the absence of a complete mature tRNA structure. We discuss the implications of this observation in defining the relationships between archael and eucaryal tRNA processing systems and the roles of these archael enzymes in cellular RNA processing.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions and Materials—** *H. volcanii* strain WFD1 (18) was grown aerobically at 37 °C in complex medium (19), and when necessary to ensure maintenance of pWL-based expression plasmids, this strain was supplemented with 20 μg/ml neomycin and 10 μg/ml carbenicillin (Merck). *Escherichia coli* strains DH5α-F and JM110 were cultured in Luria Broth (LB) medium or LB medium supplemented with 100 μg/ml ampicillin when cells carried pUC- or pWL-based plasmids.

T4 polynucleotide kinase, T4 DNA ligase, Klenow DNA polymerase, SuperScriptTM II, Moloney murine leukemia virus reverse transcriptase, and all restriction enzymes were purchased from Life Technologies, Inc.; SequenaseTM, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside were obtained from U. S. Biochemical Corp.; AmpliTaqTM DNA polymerase and GeneAmp® core reagents were purchased from Perkin-Elmer, and Zeta-Probe nylon membrane was obtained from Bio-Rad Laboratories. Oligonucleotides used in this study were synthesized by The Ohio State University Biochemical Instrument Center or Ransome Hill Biosciences, Inc.

Cloning of the *H. volcanii* tRNATrpA13115′ Derivatives into the *H. volcanii* Expression Vector—The intron-containing *H. volcanii* tRNATrpA13115′ gene, which contains a complete intron and only the anticodon stem and loop regions of the mature tRNA, was isolated from plasmid pTV22-A13115′ (20) as a 150-base pair EcoRI-HindIII restriction fragment. Prutting 5′ and 3′ ends of the tRNATrpA13115′ fragment were filled in using Klenow DNA polymerase, and this fragment was cloned into the HindIII restriction site of the vector pUC1318 (21). The tRNATrpA13115′ gene was recovered from the pUC1318 vector as a Xbal-XbaI restriction fragment, which was then subcloned into the XbaI site of the *H. volcanii* expression vector pWS302A (22) to yield the plasmid pWS302A-A13115′. The A3 to T3 mutation of the tRNATrpA13115′ gene (Fig. 1B) was prepared using the polymerase chain reaction (PCR).3 The PCR reaction contained 30 μl Tricine, pH 8.4, 2 mM MgCl2, 5 μM β-mercaptoethanol, 0.1% gelatin, 0.1% Triton X-100 (Merck), 200 μM each dNTP, 1 μM of the mutagenic primers TT0168A3-T (5′-AGCTCTAGATAATACGACTCACTATAGGGACGACGACTGACTGACTGACGCAC-CCA-3′) and 1 μM of the primer O16809-3′ (5′-GGATTGATCLGCAGGCCGTGCAGCTG-3′), and 1 unit of AmpliTaq DNA polymerase. The reaction mixture was incubated at 95 °C for 2 min, and polymerization was carried out for 30 cycles. Each cycle consisted of incubation at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The resulting fragment was cloned into pUC1318 and subcloned into pWS302A as described for the wild-type tRNATrpA13115′ gene, yielding the plasmid pWS302A-A13115′. These plasmids were introduced into *E. coli* strain JM110, and the plasmids isolated from these strains were then used to transform *H. volcanii* strain WFD1 (21). Passage through strain *E. coli* JY110 (dam−) reduces restriction during transformation of *H. volcanii* (23).

In vitro Endonuclease Assay—Radiolabeled substrate RNAs were generated by T7 RNA polymerase transcription of PCR-amplified DNA and gel-purified as described previously (9). The tRNATrpA13115′ fragment was amplified using the primers T70168A3-T (5′-AGCTCTAGATAATACGACTCACTATAGGGACGACGACTGACTGACGCAC-CCA-3′) and T70168A3-T (5′-AGCTCTAGATAATACGACTCACTATAGGGACGACGACTGACTGACGCAC-CCA-3′) as forward primers, respectively, and O1683′ (5′-AACCCCATCGATCGATCG-3′) as the reverse primer. The reaction mixture was carried out as described above. A typical 3′ ends were introduced into *E. coli* strain JM110, and the plasmids isolated from these strains were then used to transform *H. volcanii* strain WFD1 (21). Passage through strain *E. coli* JY110 (dam−) reduces restriction during transformation of *H. volcanii* (23).

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In Vivo Expression of the *H. volcanii* tRNATrpA13115′-tRNAProM Hybrid RNA—Based on in vitro processing studies, the tRNATrpA13115′ variant of the *H. volcanii* tRNATrp gene was chosen as a model RNA to investigate the requirements for mature tRNA structure in in vivo RNA splicing. This gene encodes an RNA having the complete tRNATrp intron and only the anticodon stem-loop of the mature tRNA. This RNA is accurately and efficiently cleaved by a partially purified *H. volcanii* tRNA intron endonuclease (8). To test whether these

1 The abbreviations used are: PCR, polymerase chain reaction; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
sequences and structures were sufficient for cleavage in vivo we needed a carrier RNA to express this potentially unstable form of the tRNATrp RNA. Previous in vivo expression studies showed that a modified version of the yeast tRNAPro(UGG) RNA, tRNAProM, could be expressed in H. volcanii on the expression plasmid pWL302A1 (22). This gene encoded a single stable transcript that represented the primary transcript from this gene. The production of a single RNA species was the result of two processing defects in this RNA, a U6-U72 pair preventing 5' and 3' termini processing and an intron that is not recognized by the H. volcanii endonuclease (24). The yeast tRNAProM DNA fragment also carried a RNA polymerase III termination element that functioned as a strong terminator in H. volcanii. We reasoned that introduction of the tRNAProM gene into the 5' leader region of the yeast tRNAProM construct would lead to the production of a stable RNA hybrid. This hybrid gene and its expected transcript are shown in Fig. 1.

Analysis of tRNAProM RNA transcripts—Processing of the tRNAProM hybrid transcript was followed by Northern analysis. When an oligonucleotide specific to the yeast tRNAProM RNA exon 2 was used as a probe, three RNA species were detected (Fig. 2A). The approximated sizes of these RNAs were 285, 180, and 140 nucleotides. The largest RNA species corresponded in size to the expected primary transcript, and the smallest RNA species, 140 nucleotides, corresponded in size to the predicted RNA resulting from cleavage at the tRNAProM RNA intron-3' exon boundary. The intermediate species, 180 nucleotides, was similar in size to the product predicted for cleavage at both exon-intron boundaries, followed by exon ligation (Fig. 2A, left panel). The other expected intermediate of the reaction, exon 1-intron RNA, was also detected when an exon 1-specific probe was used (data not shown).

Since correct cleavage and ligation of tRNAProM RNA would generate a structure having sequences identical to the mature tRNAProM anticodon loop, these RNAs were also probed with an oligonucleotide that corresponds to the mature anticodon stem and loop sequence (Fig. 2A, right panel). This probe hybridized to both the mature, chromosome-encoded tRNAProM RNA and the 180-nucleotide species, suggesting that correct cleavage and ligation had occurred with the hybrid RNA. As an independent test that the cleavage reactions observed in vivo were the result of the tRNA intron endonuclease activity, and not a general ribonuclease, the processing pattern of a cleavage-defective form of the tRNAProM RNA was also examined (data not shown).
RNA, tRNATrpΔ13115'T3-ProM, was also examined. This RNA contains a single point mutation at the exon 1-intron boundary, A3 to T3 at position 41 (see Fig. 1B), which leads to an 80% decrease in in vitro cleavage when compared with the wild-type RNA (Fig. 2B, left panel). Northern analysis of RNAs from cells carrying the tRNATrpΔ13115'T3-ProM gene show that this RNA remains predominately as the primary transcript (Fig. 2B, right panel). A minor species (<10% of the total), which corresponded in size to an RNA resulting from cleavage at the exon 1-intron boundary, was also detected. The inability of this RNA to process in vivo is consistent with its cleavage properties in vitro and suggests that the in vivo processing of the tRNATrpΔ13115'T-ProM RNA was the result of endonuclease activity.

To further verify that the 180-nucleotide RNA species produced from the tRNATrpΔ13115'ProM gene was the product of both cleavage and ligation, cDNAs were synthesized from this RNA and used as template for PCR amplification. The cDNA was synthesized using a primer specific for the yeast tRNAProM RNA, and PCR amplification was carried out with oligonucleotides specific for sequences 5’ of the tRNATrpΔ13115’ encoding region and a sequence internal to the yeast tRNAProM RNA. This prevented cDNA synthesis and amplification of chromosome-encoded tRNATrp RNA. Five DNA products were sequenced, and all had the predicted sequence for accurate cleavage and ligation of the tRNATrpΔ13115’ RNA (Fig. 3).

### DISCUSSION

**Fig. 2. Processing of the hybrid H. volcanii tRNATrpΔ13115'-ProM and tRNATrpΔ13115'T3-ProM RNAs.** A: left panel, Northern hybridization of RNAs isolated from H. volcanii cells carrying the gene tRNATrpΔ13115'-ProM with a probe specific for the tRNAProM portion of the hybrid RNA (ProEx1); right panel, Northern hybridization pattern for the same membrane probed with an oligonucleotide specific for the mature tRNATrp anticodon loop (IntDel). Lanes 1 and 3 contain RNAs from cells carrying the control gene, tRNAProM; lanes 2 and 4 contain RNAs from cells carrying the hybrid gene, tRNATrpΔ13115'-ProM. B: left panel, in vitro processing of the wild-type (lane 1) and A3 to T3 mutant (lane 2) of tRNATrpΔ13115’ RNA. Positions of the preRNA, intron, and exons (E1 and E2) are indicated; right panel, Northern hybridization of RNAs from H. volcanii cells carrying the tRNATrpΔ13115'T3-ProM gene using ProEx1 as a probe (lane 3). Positions of the preRNA and a potential intermediate (IE2ProM) are indicated.

Based on in vitro processing studies of the H. volcanii tRNATrp RNA we predicted that in vivo cleavage of the intron from this preRNA would be dependent on exon-intron boundary sequences and structures and independent of mature tRNA structure and sequences beyond those of the anticodon stem and loop (8, 9). To determine if the requirements observed in vitro reflected the requirements for in vivo processing we introduced the H. volcanii tRNATrpΔ13115’ gene into the 5’ unprocessed leader region of the yeast tRNAProM RNA. As anticipated, Northern analysis confirmed that the predicted hybrid RNA was produced and that this RNA underwent cleavage in the absence of a complete mature tRNA structure (Fig. 2A). RNA species consistent with cleavage at both 5’ and 3’ exon-intron boundaries were detected indicating that both cleavage sites were recognized. The inability of the in vitro processing-defective tRNATrpΔ13115’T3-ProM RNA to undergo processing in vivo supported the proposal that the hybrid RNA was cleaved by endonuclease rather than a general ribonuclease. Unexpectedly, an additional 180 nucleotide species was detected in cells that carried the tRNATrpΔ13115'ProM gene. This RNA corresponded in size to an RNA that had undergone cleavage at both sites and exon ligation (Fig. 2B). Sequence analysis of cDNAs derived from this 180-nucleotide RNA by reverse transcription and PCR amplification verified that this RNA resulted from both accurate cleavage and exon ligation (Fig. 3). These data confirm earlier in vitro observations that the halobacterial tRNA processing system is capable of acting on non-tRNA substrates (8) and show for the first time that the tRNA ligation reaction is independent of mature tRNA structure. These results also provide an explanation for how a single endonuclease could act on a population of intron-containing pretRNAs where all introns are not located in the same relative position in the mature tRNA. In this case the primary criteria for recognition would be the presence of the bulge-helix-bulge motif. Indeed, most archaean intron-containing tRNAs possess this or a closely related structure at their exon-intron boundaries (8, 25, 26).

A processing system that is directed toward sequences and structures at the exon-intron boundaries could in theory cleave any RNA, regardless of its origin or final structure. Structural analysis of archaean intron-containing 16 S and 23 S rRNA precursors has shown that these RNAs have the tRNA bulge-helix-bulge motif or closely related structures at their exon-intron boundaries (27). Some rRNA introns also encode homing endonucleases (28–31) similar to those found in some group I introns; however, the characteristic core group I RNA struc-
introns and sequences are absent in these introns. This has led to the proposal that these intron-containing mRNAs are processed by the same enzyme system as the pre-tRNAs (8, 15, 25, 26). In support of this proposal a partially purified Desulfovibrio vulgaris Hildenborough 23 S rRNA intron endonuclease was found to cleave an intron-containing tRNA from this same organism (15). The ability of this endonuclease to act on tRNA and rRNA substrates raises the question of whether this enzyme could act as a general RNA endonuclease. One possible candidate is the yeast tRNA ligase.

Sequence analysis of archaeal rRNA operons has shown that the 16 S and 23 S rRNA coding regions are flanked by large inverted repeats. As in bacterial cells, these helices are though to be sites for RNaseIII cleavage. However, we and others (25, 33) have noted that these structures are recognized by the tRNA endonuclease rather than a RNaseIII-like enzyme. Interestingly, a survey of other subunits in the yeast endonuclease may also influence the interaction of this enzyme with its substrate. Finally, no similarities in the exon-intron boundaries of archaeal rRNAs and tRNAs and the finding that the holobacterial tRNA intron endonuclease and ligase enzymes act on non-tRNA RNAs provide further support for the proposal that the archaeal tRNA and rRNA introns are processed by the same enzyme system. In addition, the recently discovered similarities between the archaeal and eucaryal tRNA intron endonucleases (3, 4) strongly suggests that the tRNA introns of these two domains and the rRNA introns of the Archaea represent a single class of introns. The molecular mechanisms that have led to changes in the substrate recognition properties of the archaeal and eucaryal endonucleases are not yet understood. It is likely that these differences are in part due to the divergence in the two yeast endonuclease subunits that are related to the holobacterial endonuclease protein. These two subunits are not identical as they are in the archaeal enzyme, and so it can be observed that they may have different RNA binding characteristics. The presence of other subunits in the yeast endonuclease may also influence the interaction of this enzyme with its substrate. Finally, no intron-containing mRNAs have been detected in the Archaea to date; however, the properties of the archaeal intron processing system described in this report indicate that a tRNA-like intron could exist in a mRNA.

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