Loss of the mRNA-like region in mitochondrial tmRNAs of jakobids

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ABSTRACT

It has been postulated that a highly reduced form of transfer messenger RNA (tmRNA), a bacterial molecule involved in the rescue of stalled ribosomes during translation, is expressed in the mitochondrion of the jakobid *Reclinomonas americana*. Here we show that genes encoding both one-piece and two-piece tmRNAs are present in six different jakobid mitochondrial DNAs. Mitochondrial tmRNAs have retained the highly conserved tRNA^{Ala}-like domain, but they apparently lack the mRNA-like region present in all bacterial tmRNAs. Comparative analysis of jakobid mitochondrial genomes shows that a potential mRNA-like region in *R. americana* (*orf64*) is located at distant genomic positions in other jakobids. Our results strongly suggest that *orf64* is a *tatA* homolog. Through Northern hybridization we confirm the postulated reduced size of both a one-piece tmRNA in *Jakoba libera* and a two-piece tmRNA in *Seculamonas ecuadoriensis*. The *J. libera* tmRNA is post-transcriptionally modified by addition of a 3' CCA tail, processed in vitro by RNase P RNA, and specifically charged with alanine in vitro by alanyl-tRNA synthetase. Our results strongly support the functionality of these reduced mitochondrial tmRNAs.

Keywords: mitochondrial tmRNAs; jakobids; standard and permuted *ssrA* gene; *orf64*

INTRODUCTION

Genes encoding transfer-messenger RNAs (*ssrA*) are found in bacterial and chloroplast genomes (Williams 2002a). Two functions are associated to tmRNAs: reactivation of ribosomal translation complexes that are blocked by mRNAs without in-frame stop codons (e.g., partially degraded mRNAs), and signalization of the polypeptides for proteolysis (for review, see Karzai et al. 2000). Accordingly, bacterial tmRNAs are composed of two distinct structural elements: (1) a tRNA^{Ala}-like domain responsible for the entry of the alanine-charged tmRNA to the A site of a stalled ribosome; and (2) an mRNA-like region, which codes for a hydrophobic signal peptide that is added to the end of the polypeptide once the stalled ribosome has switched from the original mRNA to the tmRNA reading frame (Keiler et al. 1996). When the ribosome reaches the stop codon of the tmRNA reading frame, translation is terminated, the polypeptide is released,

and subsequently degraded by specific proteases that recognize the C-terminal signal peptide (referred to as “tag” in the following).

It was previously thought that α -Proteobacteria was the only group of bacteria lacking a *ssrA* gene (Felden et al. 1999). However, the universality of tmRNAs in bacteria has been established with the identification of circularly permuted *ssrA* genes that code for two-piece tmRNAs in α -Proteobacteria (Keiler et al. 2000). These variant tmRNAs, also present through an independent gene permutation event in a specific group of Cyanobacteria (Keiler et al. 2000; Gaudin et al. 2002), have been given the attribute “two-piece” (as opposed to standard or “one-piece” tmRNAs) because they are composed of two distinct RNA molecules: (1) the “coding RNA”, which contains the tag reading frame and one part of the tRNA-like sequence, and (2) the acceptor RNA, which carries the other part of the tRNA-like structure that will have the alanine moiety attached at its 3' end (see Fig. 2A below, *Rickettsia prowazekii* tmRNA).

The discovery of two-piece *ssrA* genes in α -Proteobacteria, the closest known bacterial relatives of mitochondria, was immediately followed by the identification of a likewise circularly permuted *ssrA* gene in the mitochondrial genome of the jakobid-like flagellate *Reclinomonas americana* (strain ATCC 50394; Keiler et al. 2000). Jakobid-like flagellates are

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a group of free-living, bacterivorous protists (O'Kelly 1993). In taxonomic terms, they are subdivided into two families based on morphological and ultrastructural data: the Malawimonadidae (malawimonads), and the Jakobidae (jakobids; also known as "core jakobids"; O'Kelly et al. 1999). Jakobid cells have a single mitochondrion, and the most gene-rich and bacterial-like mitochondrial DNA of all eukaryotic cells (Lang et al. 1997; B.F. Lang and G. Burger, unpubl.). As we will show, mitochondrial *ssrA* genes are conserved in the jakobid family, which includes the genera *Jakoba*, *Reclinomonas*, *Histiona*, and *Seculamonas*.

The tmRNA homolog encoded by the mitochondrial *ssrA* gene of *R. americana* has the typical tRNA^{Ala}-like domain, but it appears to have lost the mRNA-like region because a typical tag reading frame has not been found (Keiler et al. 2000). Loss of structural elements characteristic of bacterial tmRNAs has been observed in other endosymbiont tmRNAs (Gueneau de Novoa and Williams 2004), but *R. americana* tmRNA would be the only one to lack a tag reading frame. In the mitochondrial genome of *R. americana*, the sequence coding for the tRNA^{Ala}-like domain is immediately followed by an open reading frame encoding a putative, currently unidentified, protein of 64 amino acids preceded by a canonical Shine-Dalgarno sequence. By comparison with the genetic structure of two-piece bacterial tmRNA genes, the mRNA-like region of *R. americana* tmRNA should be encoded in the region of the mtDNA sequence corresponding to *orf64*.

Comparative analysis with other jakobid mitochondrial tmRNAs (mt-tmRNAs) would greatly help to define the conserved structural elements that might be important for the cellular role of these molecules, and to verify the possible genetic link between *ssrA* and *orf64*. To address these issues, we have identified five new jakobid mt-tmRNAs (in two additional *R. americana* strains, *Histiona aroides*, *Seculamonas ecuadoriensis* and *Jakoba libera*). Comparative and Northern blot analyses confirm that all jakobid mitochondrial tmRNAs have lost the mRNA-like region. We further show that *J. libera* tmRNA is expressed at the RNA level, processed, and post-transcriptionally modified with the addition of the CCA tail. Recognition and modification of this tmRNA in vitro by enzymes that interact with bacterial tmRNAs is also demonstrated. The function of these highly derived tmRNAs is discussed.

RESULTS

One-piece and two-piece tmRNA genes in jakobid mitochondrial genomes

Sequencing of jakobid mitochondrial DNAs (mtDNAs) has permitted the identification of five new mitochondrial *ssrA* genes (Fig. 1). Like in *R. americana* (strain ATCC 50394), these *ssrA* genes encode two-piece mt-tmRNAs, except for *J. libera*, which has a one-piece conformation.

Figure 1 shows the predicted secondary structures of all known mt-tmRNAs. The one-piece conformation of *J. libera* tmRNA allows a precise prediction of both the processing sites and the size of this RNA (102 nucleotides, including the post-transcriptionally added 3' CCA tail; Fig. 1). The two-piece mt-tmRNAs are expected to require at least four RNA processing steps; two, likely mediated by RNase P and a 3' tRNA-like processing activities, to remove the intervening sequence (thus generating the two distinct RNAs of the two-piece tmRNAs); and two mechanistically undefined steps at the two opposite RNA termini. Prediction of the size of these mt-tmRNAs will require additional data from RNA mapping experiments.

The tRNA^{Ala}-like domains of all mt-tmRNAs display the canonical features of the corresponding domains of eubacterial tmRNAs; most notably, a series of G residues at the 5' end, a G–U base pair at the third position of the acceptor stem, and an A residue at the discriminator position (Williams 2002a). Only the mt-tmRNA of *S. ecuadoriensis* lacks the G–U base pair at the third position of the acceptor stem. Other features shared between the tRNA^{Ala}-like domains of bacterial and mitochondrial tmRNAs are the absence of a D stem, and the presence of a short (3 nt) variable loop. Like in the tmRNAs of their α -proteobacterial relatives (Fig. 2A, *R. prowazekii* tmRNA), the mt-tmRNA sequences deviate in the T Ψ C loop from the conserved UUCRANY motif that is otherwise common in tRNAs and tmRNAs (Felden et al. 1998). In addition, the residues G₁₈ and A₁₉ of mt-tmRNAs (referring to the *J. libera* tmRNA numbering, Fig. 1) are universally conserved in bacterial tmRNAs (Williams 2002a). A mutation in this motif has been shown to inhibit the binding in vitro of the protein SmpB to *Escherichia coli* tmRNA (Hanawa-Suetsugu et al. 2002). SmpB binding is essential for the activity of bacterial tmRNAs (Karzai et al. 1999).

Jakobid mt-tmRNAs have lost the mRNA-like region of eubacterial tmRNAs

The small one-piece *J. libera* mt-tmRNA clearly does not include the mRNA-like region that is present in all eubacterial and plastid tmRNAs. For two-piece mt-tmRNAs, it remains to be shown whether or not an mRNA-like region is encoded downstream of the tRNA^{Ala}-like domain.

In the mitochondrial genome of all three *R. americana* strains, a functionally unassigned ORF (*orf64*) follows the 3' end of the tRNA^{Ala}-like domain. A short AT-rich segment (~50 nt in *R. americana* 50394 tmRNA) separates the predicted Shine-Dalgarno sequence preceding *orf64* and the tRNA^{Ala}-like domain (Fig. 2A, *R. americana* 50394 tmRNA). For *R. americana* tmRNAs to have an mRNA-like domain, *orf64* would have to be genetically included, either partially or completely, into the two-piece tmRNA genes based on the genetic structure of two-piece bacterial tmRNAs. Our analyses of the mitochondrial gene organi-

FIGURE 1. Predicted secondary structure of the tRNA^{Ala}-like domains of jakobid mitochondrial tmRNAs. All of the tmRNAs presented, except *J. libera* tmRNA, are in the two-piece conformation. The conservation of the primary structure between all mitochondrial tmRNAs (blue), and between the three *R. americana* tmRNAs (blue and red), is shown. Straight lines separate the sequences (known as intervening sequences in two-piece tmRNAs) that are expected to be cut during the processing of mt-tmRNAs. The mature 5' and 3' ends located below the tRNA^{Ala}-like domain are still unknown for two-piece mt-tmRNAs. Therefore, this region is represented by curved lines. The 5' end of *S. ecuadoriensis* tmRNA shown here corresponds to the first nucleotide after the stop codon of the gene (*rps2*) located immediately upstream of the *ssrA* gene. The 3' CCA tail is not encoded in the mitochondrial tmRNA genes and is therefore predicted to be post-transcriptionally added.

zation of jakobid species with two-piece tmRNAs show that *H. aroides* has an *orf64* homolog located immediately downstream of its *ssrA* gene (Fig. 2B). However, the *orf64* homolog in *S. ecuadoriensis* is located upstream of the *ssrA* gene, and a *rps2* gene (ribosomal small subunit protein 2) is located between the *orf64* homolog and the *ssrA* gene. These observations make it highly unlikely that *orf64* encodes a derived mRNA-like region. To further demonstrate the genetic independence of *ssrA* and *orf64*, we have searched the Orf64 amino acids sequence against public databases. Based on the results from this search, we conclude that *orf64* codes for a TatA homolog. Tat proteins are implicated in the twin-arginine protein translocation pathway that requires both the TatA and TatC proteins for activity (Wu et al. 2000). *tatC* genes (also known as *mttB*) have been identified in all jakobid mtDNAs (B.F. Lang and G. Burger, unpubl.), which is in line with our finding that *orf64* is a *tatA* homolog.

A comparison of all mt-tmRNA sequences shows that the conservation of the primary structure is restricted to the tRNA^{Ala}-like domain (see color coding in Fig. 1). This could be an indication that the 5' and 3' termini of the mature two-piece mt-tmRNAs are adjacent to the regions of primary sequence similarity. Results from Northern blot analyses for *S. ecuadoriensis* mt-tmRNA validate this assumption, through identification of two small RNAs of ~30 nt and ~40 nt, corresponding to the coding RNA and the acceptor RNA, respectively (data not shown). These results confirm the small size of the two-piece mt-tmRNA of *S. ecuadoriensis*, and the absence of an mRNA-like domain.

J. libera mt-tmRNA is processed at the expected nucleotide positions

To determine if mt-tmRNAs are processed at the expected nucleotide positions (see straight lines in Fig. 1), we have chosen the experimentally simplest one-piece mt-tmRNA of *J. libera* as a model. A stable mt-tmRNA transcript of ~100 nt was identified in *J. libera* by Northern

FIGURE 2. Verification of the potential genetic link between *ssrA* and *orf64*. (A) Based on the structure of bacterial two-piece tmRNAs (e.g., *R. prowazekii* tmRNA), the mRNA-like region of *R. americana* mt-tmRNAs (e.g., *R. americana* 50394) should be present in the mtDNA sequence assigned to the functionally uncharacterized *orf64*. SD: predicted Shine-Dalgarno sequence of *orf64*. (B) The presence of an *orf64* homolog immediately downstream of the *ssrA* gene is conserved between *R. americana* and *H. aroides*. However, this mitochondrial gene organization is different in *S. ecuadoriensis* because *orf64* is upstream of *ssrA* and separated from it by *rps2* (ribosomal small subunit protein 2). The mitochondrial gene organization in *S. ecuadoriensis* does not support the hypothesis that the *orf64* mtDNA sequences encode the mRNA-like domains of the two-piece mt-tmRNA genes of jakobids.

blot analysis (Fig. 3A). This is consistent with the expected size of the mt-tmRNA if it processed at the 5' and 3' ends to generate a mature tRNA^{Ala}-like domain. A more precise primer extension assay shows the exact 5' processing site of *J. libera* mt-tmRNA (Fig. 3B).

Additional evidences that mt-tmRNAs are functional in mitochondria come from the analysis of the 3' CCA tail, which is essential for the aminoacylation of tRNAs and tmRNAs. Although it is encoded in the tRNA genes of some bacteria, it is post-transcriptionally added by a specific nucleotidyl transferase (CCase) to most bacterial, and to all mitochondrial and eukaryotic cytoplasmic tRNAs (e.g., Chen and Martin 1988; Joyce and Gray 1989; Tomita et al. 1996; Laforest et al. 1997; Schurer et al. 2001). As expected, the tRNA and tmRNA genes in the jakobid mitochondrial genomes lack the 3' CCA tail. However, ligation of the 5' and 3' ends of the mt-tmRNA of *J. libera*, followed by RT-PCR and sequencing, reveal the addition of the CCA tail on this mt-tmRNA (Fig. 3C). This result also confirms the 5' end processing of *J. libera* mt-tmRNA, precisely at the

predicted nucleotide position, and the recognition of this RNA by a CCase-like enzyme.

RNase P RNA processes precursor tmRNAs of *J. libera* and *R. americana* 50394

One-piece bacterial precursor tmRNAs fold into tRNA-like structures that are processed by the ribonucleoprotein RNase P to generate the mature 5' end in the tRNA^{Ala}-like domain (Komine et al. 1994). To verify if RNase P can process precursor mt-tmRNAs, we have performed an in vitro RNA processing assay using the RNase P RNA of *E. coli*, which is known to cleave pre-tRNAs in vitro in the absence of a protein component (Guerrier-Takada and Altman, 1984). For the assay, precursors of the one-piece *J. libera* tmRNA and two-piece *R. americana* 50394 were synthesized in vitro. Because the importance for efficient processing of base-pairing between leader and trailer sequences is unknown for mt-tmRNAs, two different *J. libera* pre-tmRNAs were synthesized. One has a 5' wild-type sequence

FIGURE 3. Characterization of the mature structure of *J. libera* tmRNA. (A) A *J. libera* tmRNA transcript has been detected by Northern blot analysis. The size of this transcript estimated from the RNA ladder (Ambion) is ~100 nucleotides, which is consistent with its expected size (102 nt) if it is processed like the bacterial tmRNAs. (B) A primer extension assay shows the 5' end of the mature mitochondrial tmRNA. The result obtained in the next experiment confirms that the upper band is an artifact caused by the reverse transcriptase. (C) Circularization of *J. libera* mt-tmRNA, followed by RT-PCR and sequencing, demonstrate the presence of the nonencoded CCA tail, and confirms the position of the mature 5' end identified with the primer extension assay.

extension and a CCA tail (Fig. 4A), while the other one contains 5' and 3' wild-type sequence extensions (Fig. 4B).

The experimental results indicate that the enzyme cleaves precisely all three synthetic pre-tmRNAs. As expected for the activity of an *E. coli* RNase P RNA (Guerrier-Takada and Altman, 1984; Guerrier-Takada et al. 1984), the *J. libera* pre-tmRNA with the CCA tail was processed more efficiently than the one lacking this element (Fig. 4; cf. lanes 3 and 6, and cf. lanes 4 and 7). These results strongly suggest that the mt-tmRNAs of jakobids fold in vivo into structures that are cleaved by RNase P. In the mitochondria of jakobids, they are probably processed at the 5' end by the mitochondrial RNase P, whose RNA subunit is mtDNA-encoded in all jakobids.

J. libera tmRNA is aminoacylated with alanine in vitro

Another enzyme known to modify eubacterial tmRNAs is alanyl-tRNA synthetase. This enzyme is responsible for the alanylation of the tmRNAs (Komine et al. 1994). This process depends on the presence in all known eubacterial tmRNAs (Williams 2002b) of the tRNA^{Ala} identity elements (a G–U base pair at the third position of the acceptor arm and A as the unpaired discriminator base preceding the CCA tail). Because these identity elements are also present in the mt-tmRNAs of jakobids (except *S. ecuadoriensis* tmRNA, which lacks the G–U base pair), an aminoacylation assay was performed for *J. libera* tmRNA to verify its alanine-charging ability in vitro. For the assay, we used the partially purified *E. coli* alanyl-tRNA synthetase.

The mt-tmRNA of *J. libera* is indeed specifically aminoacylated with alanine in vitro (Fig. 5). However, the efficiency of aminoacylation, as determined by comparison of the aminoacylation plateau, is significantly lower for the mt-tmRNA compared to *E. coli* tRNA^{Ala} (20–30% of the tRNA^{Ala} level, depending on the preparation).

DISCUSSION

The initial suggestion that the mt-tmRNA of *R. americana* might not contain an mRNA-like region was based on the lack of similarity to other tmRNAs outside of the tRNA^{Ala}-like domain (Keiler et al. 2000). An open reading frame (*orf64*) located immediately downstream of the tRNA^{Ala}-like domain was tentatively excluded as a possible mRNA-like region in part because a potential Shine-Dalgarno sequence is found upstream of the presumed initiation codon. Yet, in the absence of more information on the function of *orf64*, and the lack of evidence that the initiation codon was correctly inferred, the possibility that this open reading frame encodes a highly derived variant of an mRNA-like region could not be ruled out. Here we provide several lines of evidence to show that structural similarity between mt-tmRNAs and bacterial tmRNAs is indeed restricted to the tRNA^{Ala}-like domain as suggested by Keiler et al. (2000).

Structural characterization of the mt-tmRNA of *J. libera* has confirmed that it is composed in vivo of a single RNA molecule. The existence of a one-piece mt-tmRNA in *J. libera* likely results from a secondary, circular

FIGURE 4. In vitro RNase P processing assay. Two different *J. libera* pre-tmRNAs and one *R. americana* 50394 pre-tmRNA were produced by in vitro transcription. The two tmRNAs of *J. libera* have a wild-type 5' sequence extension (red) and either the 3' CCA tail (A: lanes 2–4) or a wild-type 3' sequence extension (B: lanes 5–7). 10 nM (lanes 1, 3, 6, and 8), 20 nM (lanes 4 and 7), or 100 nM (lane 9) of *E. coli* RNase P RNA were used. *R. americana* tRNA^{Pro} (lane 1) and the three pre-tmRNAs incubated in absence of enzyme (lanes 2, 5, and 10) served as positive and negative controls, respectively. The three G residues at the 5' end of all three pre-tmRNAs are not encoded in the mtDNA genomes, but serve to increase the efficiency of in vitro transcription. The expected sites of cleavage are at the junctions of the red and blue nucleotides (black arrows). In each lane, the *upper* band (red-blue arrow) is the unprocessed RNA. When a RNA is processed by the enzyme, there is a band (blue arrow) *below* the upper band, and a band (red arrow) closer to the *bottom* of the gel.

permutation event of an originally permuted *ssrA* gene, because all known α -Proteobacteria (and by inference, the mitochondrial ancestor) have two-piece tmRNAs. A

secondary rearrangement of a *ssrA* gene, from a permuted to a standard conformation, has never been observed before.

FIGURE 5. In vitro aminoacylation with alanine; 30 pmole of each RNA produced by in vitro transcription were used for the aminoacylation assay at 37°C. (●) tRNA^{Ala} *E. coli*, (■) tmRNA *J. libera*, (▲) tRNA^{Cys} *E. coli*.

J. libera tmRNA is modified post-transcriptionally by the addition of a CCA tail. This strongly suggests that this, and by inference all mitochondrial tmRNAs, are aminoacylated with alanine in vivo. *S. equadoriensis* mt-tmRNA lacks the distinctive G–U base pair at the third position of the acceptor arm. This element serves as a major molecular determinant for the alanylation of all known tmRNAs (Komine et al. 1994). Because two mtDNA-encoded tRNAs are edited at their 3' end in *S. equadoriensis* (Leigh and Lang 2004), it is possible that the G–C base pair present at the third position of the acceptor stem is edited to G–U, although this type of editing usually restores Watson–Crick base pairs rather than eliminating them.

The functionality of mt-tmRNAs is supported by the in vitro aminoacylation of *J. libera* tmRNA. In our assay, the efficiency of aminoacylation of the mt-tmRNA is significantly lower compared to *E. coli* tRNA^{Ala}. However, similar results have been obtained in other in vitro aminoacylation assays of bacterial tmRNAs, when compared to tRNA^{Ala} (Komine et al. 1994; Ushida et al. 1994; Barends et al. 2000). It is known that binding of the protein SmpB specifically increases the aminoacylation of *E. coli* tmRNA in vitro (Felden et al. 1998; Barends et al. 2001). The crystal structure of the tRNA^{Ala}-like domain of *E. coli* tmRNA in complex with SmpB shows that this protein makes contact with highly conserved nucleotides located in the variable loop and 3' end of the D-loop (Gutmann et al. 2003). The conservation of these nucleotides in the mt-tmRNAs (i.e., G₁₈, A₁₉, and A₇₃, referring to *J. libera* numbering) could be an indication that SmpB is also needed for their cellular role. We have failed to identify a gene encoding a SmpB homolog in completely sequenced jakobid mitochondrial genomes.

The results from the Protist EST Program (http://megasun.bch.umontreal.ca/pepdb/pep_main.html) will help to determine if such a gene is present in jakobid nuclear genomes.

The in vitro alanylation of the *J. libera* mt-tmRNA suggests that jakobid tmRNAs interact in vivo with mitochondrial ribosomes. The lack of an mRNA-like region implies that they cannot tag a protein for degradation by switching to an internal reading frame. However, they might have conserved, with some mechanistic modifications, the ability to liberate stalled ribosomes. In bacteria, this activity has been proposed to be more important than peptide tagging for degradation (Withey and Friedman 1999). Recruitment of release factors by mitochondrial tmRNAs has been suggested as a possible mechanistic alternative to switching to the tmRNA reading frame in order to free stalled ribosomes (Keiler et al. 2000).

We have attempted to verify if *J. libera* mt-tmRNA liberates stalled ribosomes by expressing it from a low-copy plasmid in *E. coli* X90 (*ssrA::cat*). This *E. coli* strain was also transformed with a high-copy plasmid producing a specific mRNA lacking an in-frame stop codon (Keiler et al. 1996). If *J. libera* mt-tmRNA liberates stalled ribosomes through a transpeptidation reaction in *E. coli*, we might expect to detect a single-alanine tagged reporter protein. Mass spectrometry of the purified protein encoded by the nonstop mRNA has not revealed an alanine tagging (data not shown). Because it has been shown that this reporter protein tagged with alanine is quite stable upon overproduction in *E. coli* X90 (Williams et al. 1999), we can eliminate the possibility that addition of a single alanine by the mt-tmRNA has resulted in protein degradation. One explanation for the lack of an alanine addition in our reporter system is a somewhat impaired processing of the precursor mt-tmRNA that was expressed in *E. coli*, as determined by Northern blot analysis (data not shown). An alternative explanation is that mt-tmRNAs cannot add an alanine, either in jakobid mitochondria or in the heterologous *E. coli* system. If mt-tmRNAs liberate stalled mitochondrial ribosomes, they might be able to do so without being involved in a transpeptidation reaction. Why then do we find the tRNA^{Ala} identity elements in mt-tmRNAs? Alanylation might be important to help protect mt-tmRNAs from degradation and to deliver them to the mitochondrial ribosomes.

If mt-tmRNAs do participate in transpeptidation, could the addition of a C-terminal alanine to incomplete proteins mimic the addition of the hydrophobic peptide tag by bacterial tmRNAs? Alanylation as a mechanism of targeting proteins for degradation can probably be excluded because some jakobid mitochondrial genes are predicted to code for proteins with alanine as their last C-terminal amino acid residue. For example, from the 61 protein-coding genes in the *J. libera* mitochondrial genome, three (*cox3*, *nad1*, and *atp1*) are predicted to code for proteins with a C-terminal alanine residue. This observation virtually excludes that a

single C-terminal alanine, added to a polypeptide by a mt-tmRNA, would serve as a signal for its degradation. In mitochondria of other eukaryotes, there is no known sequence motif that can trigger protein degradation (Kaser and Langer 2000). Quality control of mitochondrial proteins seems to be dependent only on the folding state of the proteins. Nonassembled or incorrectly folded polypeptides are selectively removed by ATP-dependent proteases in the various mitochondrial subcompartments (Wagner et al. 1994; Pearce and Sherman 1995; Savel'ev et al. 1998). An efficient sensor system for such misfolded proteins in jakobid mitochondria might be sufficient to degrade all partially synthesized proteins freed from stalled ribosomes by the action of mt-tmRNAs. In support of this hypothesis, it has been shown in *S. cerevisiae* that the mitochondrial inner membrane protease *m*-AAA can carry out proteolysis of partially synthesized mitochondrial translation products (Pajic et al. 1994). Efficient proteolysis of incomplete proteins without the need for a peptide tag might explain the absence of the mRNA-like region in mt-tmRNAs of jakobids.

At the moment, there is no data that demonstrates the assumed functional role of mt-tmRNAs, that is, that these highly derived RNA molecules have at least retained part of the functions of bacterial tmRNAs. The identification of a SmpB protein homolog in jakobid mitochondria, and biochemical studies of the purified, native tmRNA–protein complex will be required to address this question.

MATERIALS AND METHODS

Sequences

Mitochondrial tmRNA sequences were identified in ongoing jakobid sequencing projects of the Organelle Genome Mega-sequencing Project. *J. libera* cells were grown at 25°C in F/2 medium, all other jakobids were grown at 25°C in WCL medium (<http://megasun.bch.umontreal.ca/People/lang/FMGP/methods/>).

Purification of small mitochondrial RNAs

Mitochondrial DNA and RNAs from jakobids were extracted from partially purified mitochondria obtained after breaking the cells with glass beads. Mitochondria were separated from the cell debris by differential centrifugations and lysed by incubation with 1% SDS and 100 µg/mL of Proteinase K for 1 h at 25°C. The mitochondrial fraction was placed over a 30% glycerol solution and the small mitochondrial RNAs (including tRNAs, tmRNA, and 5S rRNA) were separated from the large RNAs and DNA by ultracentrifugation (240,000 × g, 3 h, 10°C). Small mitochondrial RNAs were collected from the upper part of the glycerol solution and precipitated twice with 95% ethanol/0.5 M ammonium acetate, washed with 80% ethanol, and stored at –70°C in DEPC-treated water.

Northern blot analysis and primer extension assay

The oligonucleotide (5'-TACTGCCCATGGTTTATCTA) used for the Northern blot analyses and primer extension assays was

5'-labeled with (γ-³²P)-ATP (Perkin-Elmer) and T4 Polynucleotide Kinase (USB). For Northern blot analyses, 500 ng of small mitochondrial RNAs of *J. libera* were separated on a 10%–12% polyacrylamide/8M urea gel. The separated RNAs were electroblotted onto a Hybond-N membrane (Amersham Pharmacia Biotech). Prehybridization was performed in 1 mM EDTA, 6× SSPE, 0.2% SDS, 7.5× Denhardt's reagent for 2 h, and hybridization in 6× SSPE overnight. The membrane was washed twice for 10 min at room temperature in 6× SSPE, 15 min at hybridization temperature in 6× SSPE, and 10 min at hybridization temperature in 6× SSPE. The membrane was exposed overnight at –70°C. For the primer extension assay, cDNA synthesis and preparation of the DNA ladder was performed as described (Sambrook et al. 1989). The cDNAs and the DNA ladder were migrated on a 5% polyacrylamide/8M urea gel.

RNA circularization and RT-PCR

Circularization of mitochondrial RNAs, and cDNAs synthesis was based on the protocol of Yokobori and Pääbo (Yokobori and Pääbo 1995). The oligonucleotide used for cDNA synthesis of *J. libera* RNAs was 5'-GGATTACTTAAATATCTAACTAA, and cDNAs were PCR-amplified with the additional primer 5'-GCTTAGATAAAACCATGGGCAGTA. The resulting PCR fragment of appropriate size was purified on an agarose gel, cloned into the EcoRV restriction site of the phagemid pFBS (B.F. Lang, unpubl.), and sequenced by the dideoxy chain-termination method.

In vitro RNase P RNA processing assay

The *ssrA* genes of *J. libera* and *R. americana* 50394 was PCR-amplified with oligonucleotides to produce DNA fragments that were used to generate precursor mt-tmRNAs. For *J. libera*, two different pre-tmRNAs were synthesized (for details, see the RNA structures in Fig. 4). They both have a wild-type 5' sequence extension, and (1) a wild-type 3' sequence extension or (2) a 3' CCA tail that is required for more efficient processing by *E. coli* RNase P (Liu and Altman 1996). The run-off transcription reaction was performed as described (Yuan et al. 1992). The precursor tmRNAs were purified on a 10% polyacrylamide/8M urea gel and quantified in a liquid scintillation counter.

The expression plasmid carrying the *E. coli* RNase P RNA gene (generously provided by Sidney Altman) was used for run-off transcription of the ribozyme. The resulting RNA was purified on a G50 column, precipitated twice with 95% ethanol/0.5 M ammonium acetate, washed with 80% ethanol, and stored in DEPC-treated water at –80°C. For the assays, the RNA was first activated by heating the sample to 65°C for 5 min, and cooling it slowly to room temperature in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NH₄Cl. tmRNA substrates (1000 cpm) were incubated at 37°C in 10 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 100 mM NH₄Cl, 4% PEG, and in the presence of different RNase P RNA concentrations (10 nM, 20 nM or 100 nM). The total volume of the reaction was 10 µL. After 1 h, 10 µL of loading buffer was added, and the sample was heated at 75°C for 2 min before loading on a 8% polyacrylamide/8M urea gel.

In vitro aminoacylation assay

Oligonucleotides were designed to PCR-amplify the mitochondrial *ssrA* gene of *J. libera*, and tRNA^{Ala} and tRNA^{Cys} genes from *E. coli*.

For amplification of the *J. libera* *ssrA* sequence, the oligonucleotide was designed such that the amplified sequence would include a (nonencoded) 3' CCA tail. The PCR fragments were cloned into the EcoR5 restriction site of pFBS and sequenced. The plasmids were digested with MvaI, and subsequently used in run-off transcription. The resulting RNAs were purified on a denaturing 15% polyacrylamide/8M urea gel, and quantified by spectrophotometry.

The partial purification of *E. coli* alanyl-tRNA synthase, and the aminoacylation reactions were performed as described (Komine et al. 1994), with slight modifications: 1.5 g of wet weight *E. coli* cells were collected from an overnight culture and sonicated. The enzyme was partially purified from an S100 extract by ion-exchange chromatography (on DEAE-Amersham Pharmacia Biotech). The fraction containing the enzyme was desalted, concentrated to 200 μ L, and partially purified from smaller proteins by using Amicon Ultra-15 Centrifugal Filter Units, Membrane NMWL 100,000 (Millipore). Glycerol was added to the enzyme solution to reach a final concentration of 50%, and stored at -20°C . For the aminoacylation reactions, the RNAs were activated in water by heating for 5 min at 90°C , followed by rapid cooling on ice-cold water.

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REFERENCES

- Barends, S., Wower, J., and Kraal, B. 2000. Kinetic parameters for tmRNA binding to alanyl-tRNA synthetase and elongation factor Tu from *Escherichia coli*. *Biochemistry* **39**: 2652–2658.
- Barends, S., Karzai, A.W., Sauer, R.T., Wower, J., and Kraal, B. 2001. Simultaneous and functional binding of SmpB and EF-Tu-TP to the alanyl acceptor arm of tmRNA. *J. Mol. Biol.* **314**: 9–21.
- Chen, J.Y. and Martin, N.C. 1988. Biosynthesis of tRNA in yeast mitochondria. An endonuclease is responsible for the 3'-processing of tRNA precursors. *J. Biol. Chem.* **263**: 13677–13682.
- Felden, B., Hanawa, K., Atkins, J.F., Himeno, H., Muto, A., Gesteland, R.F., McCloskey, J.A., and Crain, P.F. 1998. Presence and location of modified nucleotides in *Escherichia coli* tmRNA: Structural mimicry with tRNA acceptor branches. *EMBO J.* **17**: 3188–3196.
- Felden, B., Gesteland, R.F., and Atkins, J.F. 1999. Eubacterial tmRNAs: Everywhere except the alpha-proteobacteria? *Biochim. Biophys. Acta* **1446**: 145–148.
- Gaudin, C., Zhou, X., Williams, K.P., and Felden, B. 2002. Two-piece tmRNA in cyanobacteria and its structural analysis. *Nucleic Acids Res.* **30**: 2018–2024.
- Gueneau de Nova, P. and Williams, K.P. 2004. The tmRNA website: Reductive evolution of tmRNA in plastids and other endosymbionts. *Nucleic Acids Res.* **32**: D104–D108.
- Guerrier-Takada, C. and Altman, S. 1984. Catalytic activity of an RNA molecule prepared by transcription *in vitro*. *Science* **223**: 285–286.
- Guerrier-Takada, C., McClain, W.H., and Altman, S. 1984. Cleavage of tRNA precursors by the RNA subunit of *E. coli* ribonuclease P (M1 RNA) is influenced by 3'-proximal CCA in the substrates. *Cell* **38**: 219–224.
- Gutmann, S., Haebel, P.W., Metzinger, L., Sutter, M., Felden, B., and Ban, N. 2003. Crystal structure of the transfer-RNA domain of transfer-messenger RNA in complex with SmpB. *Nature* **424**: 699–703.
- Hanawa-Suetsugu, K., Takagi, M., Inokuchi, H., Himeno, H., and Muto, A. 2002. SmpB functions in various steps of trans-translation. *Nucleic Acids Res.* **30**: 1620–1629.
- Joyce, P.B. and Gray, M.W. 1989. Chloroplast-like transfer RNA genes expressed in wheat mitochondria. *Nucleic Acids Res.* **17**: 5461–5476.
- Karzai, A.W., Susskind, M.M., and Sauer, R.T. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J.* **18**: 3793–3799.
- Karzai, A.W., Roche, E.D., and Sauer, R.T. 2000. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat. Struct. Biol.* **7**: 449–455.
- Kaser, M. and Langer, T. 2000. Protein degradation in mitochondria. *Semin. Cell Dev. Biol.* **11**: 181–190.
- Keiler, K.C., Waller, P.R., and Sauer, R.T. 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271**: 990–993.
- Keiler, K.C., Shapiro, L., and Williams, K.P. 2000. tmRNAs that encode proteolysis-inducing tags are found in all known bacterial genomes: A two-piece tmRNA functions in *Caulobacter*. *Proc. Natl. Acad. Sci.* **97**: 7778–7783.
- Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K., and Inokuchi, H. 1994. A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*. *Proc. Natl. Acad. Sci.* **91**: 9223–9227.
- Laforest, M.J., Roewer, I., and Lang, B.F. 1997. Mitochondrial tRNAs in the lower fungus *Spizellomyces punctatus*: tRNA editing and UAG "stop" codons recognized as leucine. *Nucleic Acids Res.* **25**: 626–632.
- Lang, B.F., Burger, G., O'Kelly, C.J., Cedergren, R., Golding, G.B., Lemieux, C., Sankoff, D., Turmel, M., and Gray, M.W. 1997. An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* **387**: 493–497.
- Leigh, J. and Lang, B.F. 2004. Mitochondrial 3' tRNA editing in the jakobid *Seculamonas ecuadoriensis*: A novel mechanism and implications for tRNA processing. *RNA* (this issue).
- Liu, F. and Altman, S. 1996. Requirements for cleavage by a modified RNase P of a small model substrate. *Nucleic Acids Res.* **24**: 2690–2696.
- O'Kelly, C.J. 1993. The jakobid flagellates: Structural features of *Jakoba*, *Reclinomonas* and *Histiona* and implications for the early diversification of eukaryotes. *J. Eukaryot. Microbiol.* **40**: 627–636.
- O'Kelly, C.J., Farmer, M.A., and Nerad, T.A. 1999. Ultrastructure of *Trimastix pyriformis* (Klebs) Bernard et al.: Similarities of *Trimastix* species with retortamonad and jakobid flagellates. *Protist* **150**: 149–162.
- Pajic, A., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. 1994. Yta10p is required for the ATP-dependent degradation of polypeptides in the inner membrane of mitochondria. *FEBS Lett.* **353**: 201–206.
- Pearce, D.A. and Sherman, F. 1995. Degradation of cytochrome oxidase subunits in mutants of yeast lacking cytochrome *c* and suppression of the degradation by mutation of *yme1*. *J. Biol. Chem.* **270**: 20879–20882.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A*

- laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Save'ev, A.S., Novikova, L.A., Kovaleva, I.E., Luzikov, V.N., Neupert, W., and Langer, T. 1998. ATP-dependent proteolysis in mitochondria. m-AAA protease and PIM1 protease exert overlapping substrate specificities and cooperate with the mtHsp70 system. *J. Biol. Chem.* **273**: 20596–20602.
- Schurer, H., Schiffer, S., Marchfelder, A., and Morl, M. 2001. This is the end: Processing, editing and repair at the tRNA 3'-terminus. *Biol. Chem.* **382**: 1147–1156.
- Tomita, K., Ueda, T., and Watanabe, K. 1996. RNA editing in the acceptor stem of squid mitochondrial tRNA(Tyr). *Nucleic Acids Res.* **24**: 4987–4991.
- Ushida, C., Himeno, H., Watanabe, T., and Muto, A. 1994. tRNA-like structures in 10Sa RNAs of *Mycoplasma capricolum* and *Bacillus subtilis*. *Nucleic Acids Res.* **22**: 3392–3396.
- Wagner, I., Arlt, H., van Dyck, L., Langer, T., and Neupert, W. 1994. Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *EMBO J.* **13**: 5135–5145.
- Williams, K.P. 2002a. Descent of a split RNA. *Nucleic Acids Res.* **30**: 2025–2030.
- . 2002b. The tmRNA Website: Invasion by an intron. *Nucleic Acids Res.* **30**: 179–182.
- Williams, K.P., Martindale, K.A., and Bartel, D.P. 1999. Resuming translation on tmRNA: A unique mode of determining a reading frame. *EMBO J.* **18**: 5423–5433.
- Withey, J. and Friedman, D. 1999. Analysis of the role of *trans*-translation in the requirement of tmRNA for lambdaimmP22 growth in *Escherichia coli*. *J. Bacteriol.* **181**: 2148–2157.
- Wu, L.F., Ize, B., Chanal, A., Quentin, Y., and Fichant, G. 2000. Bacterial twin-arginine signal peptide-dependent protein translocation pathway: evolution and mechanism. *J. Mol. Microbiol. Biotechnol.* **2**: 179–189.
- Yokobori, S. and Pääbo, S. 1995. Transfer RNA editing in land snail mitochondria. *Proc. Natl. Acad. Sci.* **92**: 10432–10435.
- Yuan, Y., Hwang, E.S., and Altman, S. 1992. Targeted cleavage of mRNA by human RNase P. *Proc. Natl. Acad. Sci.* **89**: 8006–8010.

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