Primary sequence and post-transcriptional modification pattern of an unusual mitochondrial tRNA^{Met} from *Tetrahymena pyriformis*

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Received 3 February 1995

Abstract In a previous investigation of the rDNA region in *Tetrahymena pyriformis* mitochondrial DNA, we identified a putative tRNA^{Met} gene [Heinonen et al. (1987) J. Biol. Chem. 262, 2879–2887]. On the basis of Northern hybridization analyses, we suggested that this gene is expressed, even though the resulting tRNA would be unusually small and have an atypical dihydrouridine stem–loop domain. We report here the complete nucleotide sequence and post-transcriptional modification pattern of this tRNA^{Met}, confirming its predicted primary structure and supporting the view that this structurally aberrant species functions in translation in *T. pyriformis* mitochondria.

Key words: Mitochondrion; *Tetrahymena pyriformis*; Transfer RNA; Modified nucleoside

1. Introduction

We have previously reported the sequence of a putative tRNA^{Met} gene (*trnM*) in *Tetrahymena pyriformis* mitochondrial DNA (mtDNA), and our Northern hybridization analysis indicated that this gene gives rise to a transcript that co-migrates with mitochondrial tRNA in an agarose gel [1]. We therefore proposed that this *trnM* encodes a functional tRNA, even though the corresponding transcript would be unusually short with an atypical dihydrouridine (D) stem–loop domain [1]. Suyama et al. [2] independently determined the sequence of the same region of *T. pyriformis* mtDNA and also argued that this putative *trnM* gives rise to a functional transcript. This conclusion was based on several considerations, including the fact that the smallest mtDNA-encoded tRNA in *T. pyriformis* can be aminoacylated with methionine by a mitochondrial aminoacyl-tRNA synthetase preparation [3,4]. Morin and Cech [5] obtained phylogenetic support for a functional role of the putative tRNA^{Met} by determining the corresponding gene sequence from several *Tetrahymena* species and finding compensating base changes that supported our proposed [1] secondary structure.

Seilhamer et al. [6] detected the homologous tRNA^{Met} sequence in the *Paramecium* mitochondrial genome; however, they argued that the unusual D stem–loop region made it unlikely that this gene encoded a functional tRNA. Instead, these investigators concluded that this sequence represents the 5′-terminal region of the 20 S rRNA [7,8].

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We have now isolated and characterized the smallest *T. pyriformis* mitochondrial tRNA and we show here that it has the primary sequence that we had predicted from the *trnM* sequence. The transcript also contains a 3′-terminal CCA sequence, not encoded in the mtDNA, as well as several modified nucleosides characteristic of known functional tRNAs.

2. Materials and methods

T. pyriformis, amicronucleate strain ST (obtained from Y. Suyama, Department of Biology, University of Pennsylvania), was grown at 28°C with constant shaking in 500 ml Neff's medium [9]. The culture was chilled on ice for 10–15 min, following which cells were collected by centrifugation at 2000 rpm for 5 min, resuspended, and centrifuged at 2000 rpm for 5 min through a layer of ice-cold homogenizing medium (0.35 M sucrose, 10 mM Tris-HCl, pH 7.2, 2 mM EDTA) [10]. Resuspended cells in 50 ml homogenizing medium were disrupted by three passages through a hand emulsifier (nozzle unscrewed 2.5 turns) [11]. Unbroken cells and debris were removed by centrifugation at 3000 rpm (IEC) for 5 min. A mitochondrial pellet was recovered from the resulting supernatant by centrifugation at 8000 rpm for 5 min and washed twice by resuspension in 50 ml homogenizing medium followed by centrifugation (5 min, 8000 rpm). The final mitochondrial pellet was resuspended in 10 ml 0.15 M NaCl, 0.1 M EDTA (pH 9.0) [12] at room temperature. Ten ml of 4% SDS in the same buffer were added and the mitochondrial lysate was then extracted three times with phenol/cresol [13] at room temperature. Nucleic acids were precipitated by addition of 2 vols. of 95% ethanol. For preparation of a post-ribosomal supernatant [14], purified mitochondria were resuspended in buffer (100 mM KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and lysed at a final concentration of 2% Triton X-100. After removal of membrane fragments by centrifugation at 10,000 rpm for 10 min (IEC), the clarified mitochondrial lysate was centrifuged at 42,000 rpm for 90 min in a Ti50 rotor to sediment ribosomes. Transfer RNA was isolated from the supernatant by detergent phenol/cresol extraction.

Nucleic acid samples were either used directly for 3′-end-labeling or, alternatively, the RNA samples (5 μg post-ribosomal supernatant RNA or 20 μg total nucleic acid) were dissolved in 7.5 μl NMF/urea loading buffer [15] containing 0.1% (w/v) xylene cyanol or Bromophenol blue and heated to 65°C for 5 min. After electrophoresis in a thin (0.5 mm) 6% polyacrylamide gel containing 7 M urea, the smallest tRNA was visualized by ethidium bromide staining and eluted [16] in the presence of 20 μg linear polyacrylamide carrier (prepared as in [17], extracted with phenol, precipitated with ethanol and re-dissolved at 2.5 μg/μl). The unlabeled tRNA was either re-purified by gel-electrophoresis and then subjected to modified nucleotide analysis [16] or used directly for end-labeling. 3′-End-labeled tRNA [18] was purified by gel-electrophoresis and used for chemical sequence analysis [18]; both 3′- and 5′-end-labeled tRNAs were subjected to enzymatic sequence analysis [19,20] with alkali ladders generated as described [16].

3. Results

Electrophoresis of 3′-end-labeled *T. pyriformis* mitochondrial tRNA in a sequencing gel yielded a fast-migrating band that was well separated from the rest of the mitochondrial tRNA population (Fig. 1). It was established by chemical

SSDI 0014-5793(95)00179-4

sequence analysis (Fig. 2) that this tRNA is the expected transcript from an unusually short *trnM* that had been identified previously [1,2,5]. Except for the 5′-terminal nucleotide, the entire sequence, including a post-transcriptionally added 3′-CCA, can be read from the autoradiograms presented in Fig. 2; the 5′-terminus reproducibly appeared as a G in enzymatic sequence analysis of 5′-end-labeled RNA. Position 14 gave a band in both the C and U lanes of chemical sequencing gels, which is diagnostic for D residues (unpublished results). Residues at four other positions (31, 32, 48 and 49; predicted to be U from the gene sequence) were not cleaved by any of the chemical sequencing reactions. This result indicates that these positions are occupied by modified U residues. The enzymatic sequencing results indicated that position 48 was completely resistant to cleavage, while the other four modified U residues were cleaved weakly, as compared to normal U's, by RNase PhyM. All other positions were cleaved as expected for unmodified residues by the chemical and enzymatic sequencing reactions. Isolated $tRNA^{Met}$ from a total nucleic acid preparation and $tRNA^{Met}$ from a post-ribosomal supernatant appeared identical, as judged by chemical sequencing. We also attempted chemical sequence analysis of the next-smallest band of 3′-end-labeled tRNA (see Fig. 1). In this experiment we obtained an unreadable gel, containing superimposed sequence ladders, indicating that this band contained more than one tRNA species. Nevertheless, it was clear that this band did not contain an isoaccepting species of the $tRNA^{Met}$ analyzed in this study.

In order to characterize further the modified nucleosides present in the $tRNA^{Met}$, we 5′-end-labeled the products of partial alkaline hydrolysis and separated these tRNA fragments in a 10% gel containing 7 M urea. In one experiment, all of the products that migrated slower than Bromophenol blue (~12 nt) were pooled, eluted, and subjected to snake venom phosphodiesterase treatment followed by thin-layer chromatography. This experiment verified the presence of the dihydrouridine residue and also indicated the presence of pT and pΨ. No other modified nucleotides were detected. In another experiment, 5′-end-labeled partial alkaline digestion products corresponding to positions of interest, including the anticodon, were individually isolated from the gel and analyzed. In this experiment, pT was localized to position 48, while pΨ was found at positions 31, 32 and 49. The final deduced sequence of *T. pyriformis* mitochondrial $tRNA^{Met}$ is presented in Fig. 3.

4. Discussion

Previous work by Suyama [3,4] had established that the smallest tRNA found in *T. pyriformis* mitochondria can be specifically aminoacylated with methionine using a mitochondrial aminoacyl-tRNA synthetase preparation, and that aminoacyl- and/or peptidyl-$tRNA^{Met}$ is found associated with mitoribosomes and therefore presumably functions in protein synthesis. We have now established that this unusually short $tRNA^{Met}$ has the structure that was previously predicted from the sequence of a putative *trnM* ([1,2,5]; see Fig. 3).

The $tRNA^{Met}$ has a 3′-terminal CCA sequence that is not encoded in the mtDNA [5] and therefore must be added post-transcriptionally [21]. The 3′-CCA sequence is a universal feature of all tRNAs and is important in many aspects of tRNA function [22], including aminoacylation [23,24] and interaction with large subunit rRNA [25]. This tRNA also contains several

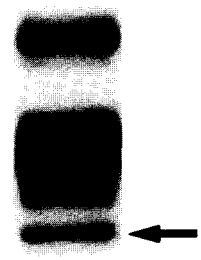

Fig. 1. Autoradiogram of 3′-end-labeled *T. pyriformis* mitochondrial tRNA. The sample was electrophoresed in a 6% sequencing gel. The arrow shows the position of $tRNA^{Met}$.

modified nucleosides that are highly conserved among tRNAs and are thought to be important for tRNA structure and function [26–33].

The $tRNA^{Met}$ (Fig. 3) has three adjacent G–C base pairs at the base of the anticodon stem that are known to be important for binding of eubacterial initiator tRNA to the P site of the ribosome [34]. In eubacteria, a mismatch in the distal pair in the acceptor stem (involving the 5′-terminal nucleotide) is one of the determinants that precludes initiator tRNA from also being used in elongation. Mutant eubacterial tRNAs that have restored base pairing at the distal end of the acceptor stem are able to function in both initiation and elongation [34]. Because the *T. pyriformis* mitochondrial $tRNA^{Met}$ also has a normal base pair at the distal end of the acceptor stem (Fig. 3), a second methionine-accepting tRNA is probably not required in this system. This is consistent with evidence that cytoplasmic $tRNA^{Met}$ is not imported into *T. pyriformis* mitochondria [4] and with the fact that no other *trnM* has been found in the completely sequenced *T. pyriformis* mitochondrial genome ([2]; G. Burger, personal communication).

Suyama [3,4] reproducibly detected two isoaccepting species corresponding to the mitochondrially encoded $tRNA^{Met}$. This result can not be explained by partial modification because our data indicate that modification is complete at all sites where modified nucleotides are present. It is possible that the presence of these modifications allows formation of two different stable conformers of this tRNA (see [31]) in the gel system used by Suyama.

Protein-coding genes in *T. pyriformis* mitochondria do not all encode normal AUG start codons ([35]; G. Burger, personal communication), leading to the proposal [35] that the unusual mitochondrial $tRNA^{Met}$ can translate three potential start codons (AUG, AUA and AUU). In at least some animal mitochondria, it is thought that post-transcriptional modification of the first position of the anticodon to 5-formylcytidine may allow the $tRNA^{Met}$ to read both AUG and AUA codons [36,37]. Our data clearly establish that there are no modified residues in the anticodon of *T. pyriformis* mitochondrial $tRNA^{Met}$; however, the two Ψ residues close to the 3′-end of the anticodon are expected to have considerable effect on the structure of this region of the tRNA [32] and therefore may influence codon reading [27,38]. Another possibility is that *T. pyriformis* mitochondrial mRNAs undergo RNA editing as in some other mitochondrial systems [39,40], thereby acquiring the necessary AUG start codons in the appropriate positions. So far, how-

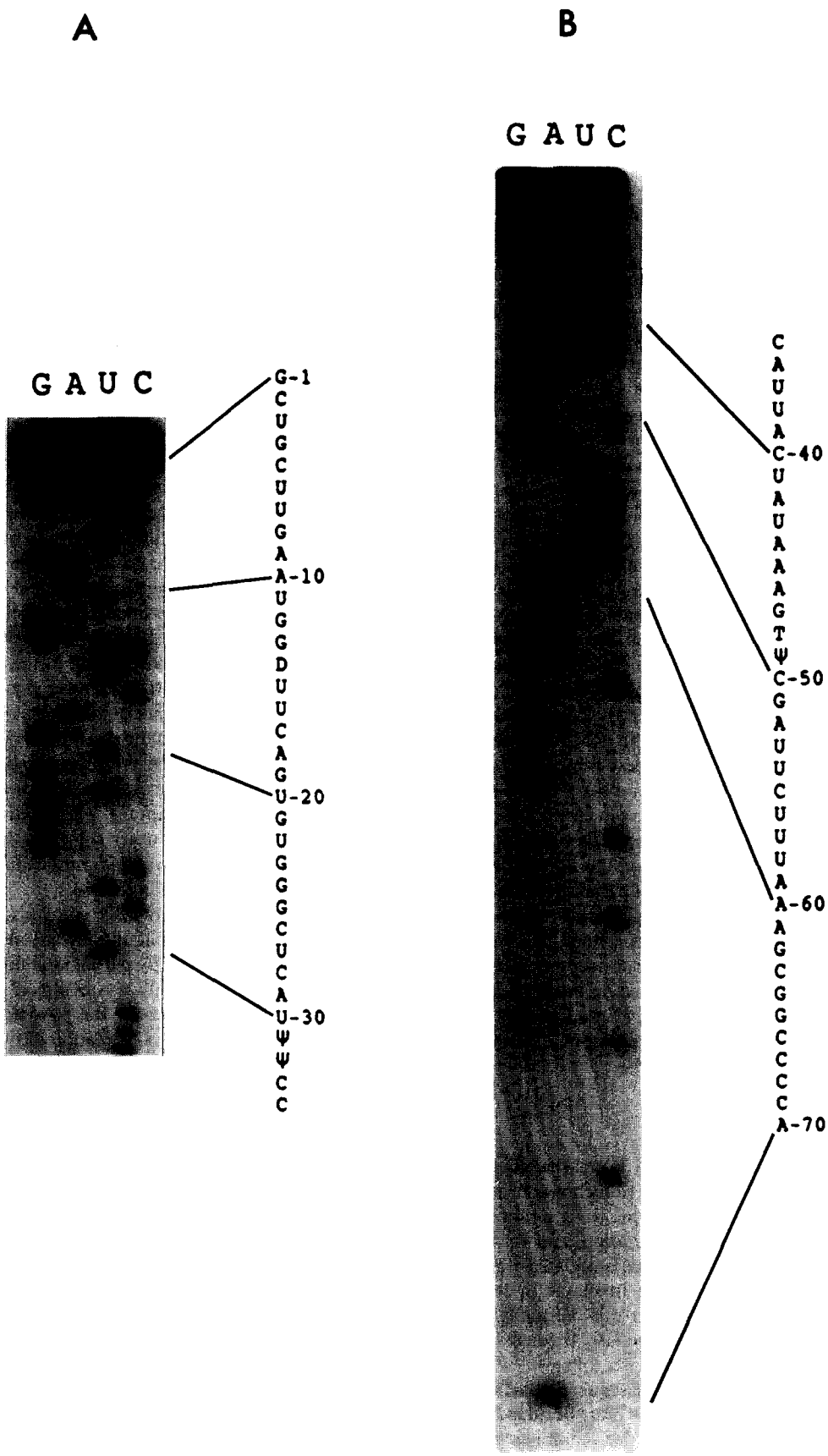

Fig. 2. Autoradiogram of (A) 10% and (B) 20% chemical sequencing gels of *T. pyriformis* mitochondrial $tRNA^{Met}$. The 5′-terminal nucleoside and modified nucleosides were identified in other experiments (see text).

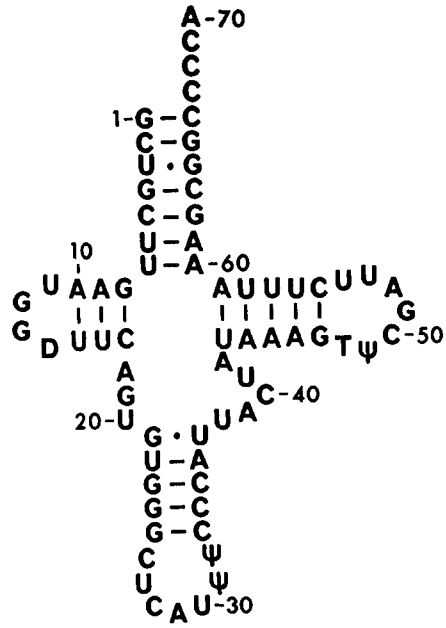

Fig. 3. Primary sequence and potential secondary structure (the latter adapted from [41]) of *T. pyriformis* mitochondrial $tRNA^{Met}$.

ever, such editing has not been detected in analyses designed to investigate this possibility (J. Edqvist, unpublished results).

In summary, we have isolated and fully characterized a $tRNA^{Met}$ from *T. pyriformis* mitochondria. Although this tRNA has an unusually short D stem–loop region, it displays other features characteristic of normal tRNAs, including the presence of modified nucleosides and a 3′-terminal CCA sequence. These results, taken together with the work of Suyama [3,4], validate our previous proposal [1] that an unusual *trnM* in the *T. pyriformis* mitochondrial genome gives rise to a functional transcript. This conclusion forces a re-evaluation of the data obtained for the homologous sequence from the *Paramecium* mitochondrial genome [7]. In that study an S1 nuclease experiment mapped the 5′-terminus of a *Paramecium* mitochondrial transcript to within a few nucleotides of the 5′-end that we have determined here for *Tetrahymena* mitochondrial $tRNA^{Met}$. We previously pointed out [1] that the data of Seilhamer et al. [7] are consistent with the presence of a $tRNA^{Met}$ in *Paramecium* mitochondria that is homologous to the one found in *Tetrahymena*. However, those authors [7,8] contend that their S1 mapping data actually localize the 5′-end of the 20 S rRNA and that the putative *trnM* is unlikely to give rise to a functional tRNA. In view of the data presented here, it would be very surprising indeed if the homologous $tRNA^{Met}$ were not present in *Paramecium* mitochondria. If further work does confirm the existence of the homologous $tRNA^{Met}$ in *Paramecium* mitochondria, this has some implications for a recently revised secondary structure model of *T. pyriformis* mitochondrial $tRNA^{Met}$ [41]. Base substitutions in the corresponding *Paramecium* structure [1] are non-compensating with respect to the additional base pairings proposed in the Steinberg–Cedergren model [41].

Acknowledgements: We thank Y. Suyama for kindly providing the *T. pyriformis* strain used in this study, and T.Y.K. Heinonen for advice on isolation of mitochondria. This work was supported by an operating grant (MT-4124) from the Medical Research Council of Canada (M.W.G.), a Walter C. Sumner Memorial Fellowship (S.J.G.), and a Fellowship (M.W.G.) from the Canadian Institute for Advanced Research.

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