NUCLEOTIDE SEQUENCE OF A YEAST MITOCHONDRIAL THREONINE-tRNA ABLE TO DECODE THE C—U—N LEUCINE CODONS

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1. Introduction

Sequencing studies of mitochondrial (mt) tRNAs and tRNA genes during the past two years led to the finding of unusual features in the codon reading patterns and in the genetic code in mitochondria [1–3]. The most striking example is that the U—G—A codon, instead of acting as a termination signal, is used to specify tryptophan in mitochondria [4,5]. Whereas this exception to the standard genetic code is found in mitochondria from various sources, there are others which differ from one organism to another [1–3], so there is not even a common code in different mitochondria. In the yeast *Saccharomyces cerevisiae*, such an exception has been deduced from the observation that in the ATPase proteolipid amino acid sequence, the threonine residue at position 46 is encoded by the C—U—A codon for leucine [6,7]. This unexpected amino acid assignment for the C—U—A codon has been explained in [7] where the gene for a tRNA^{Thr} bearing an unorthodox anticodon loop with a presumed anticodon U—A—G was sequenced.

To determine whether this unique codon assignment is also found at the level of the corresponding functional threonine-tRNA and whether this tRNA is able to translate all the four codons of the leucine family C—U—N (N = A, G, U or C) into threonine, we have purified and sequenced the mt tRNA$_1^{Thr}$.

2. Materials and methods

Crude mt tRNA, extracted from yeast mitochondria (*S. cerevisiae* IL8-8C) using standard procedures [8], was fractionated by RPC-5 column chromatog-

raphy [9]. The fractions showing threonine accepting activity ([^{3}H]threonine, 2–10 Ci mmol, New England Nuclear) were detected with a mitochondrial enzyme extract [10]. mt tRNA$_1^{Thr}$ was further purified using two-dimensional polyacrylamide gel electrophoresis [8,11].

For the sequence determination of mt tRNA$_1^{Thr}$, two post-labeling methods were utilized:
(i) The technique developed in [12] with the modifications reported in [13];
(ii) Read-off sequencing gels [14] using either 5'- or 3'-end-labeled tRNA.
The experimental conditions were those in [13]. [γ-^{32}P]ATP (2000 Ci mmol) and [α-^{32}P]ATP (400–600 Ci mmol) were from Amersham/Searle.

3. Results

3.1. *Threonine-tRNA isoacceptors in yeast mitochondria*

Yeast mt DNA contains two distinct tRNA^{Thr} genes which have been localized on the genetic map between the oxi-1 and oxi-2 markers, for tRNA$_1^{Thr}$ and near the cap locus, for tRNA$_2^{Thr}$ [15–17]. Preparative column fractionation of crude mt tRNA in the RPC-5 system showed resolution of three threonine-tRNA isoacceptor species: tRNA$_1^{Thr}$, tRNA$_{2a}^{Thr}$ and tRNA$_{2b}^{Thr}$ (fig.1A). The material recovered from fraction Thr-1 was separated by two-dimensional polyacrylamide gel electrophoresis. Fig.1B shows the resulting fractionation pattern and the identification of the resolved tRNA species. It should be noted that tRNA$_1^{Thr}$ does not show any leucyl acceptor activity, but only charges with threonine in the standard aminoacylation conditions used. Hybridization of end-
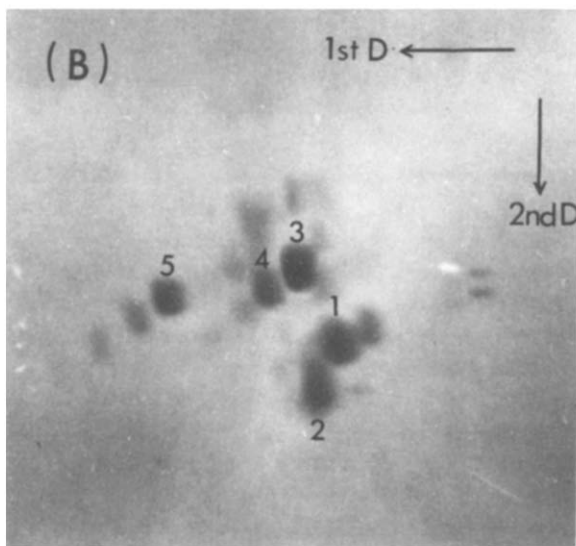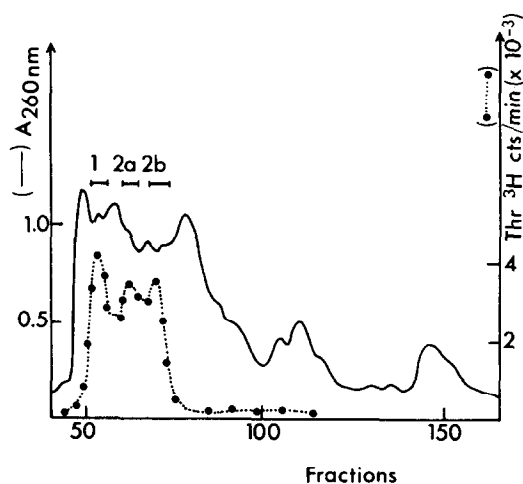
(A)

(B)

Fig.1. Purification of mt $tRNA_1^{Thr}$. (A) RPC-5 column chromatography of crude mt tRNA (500 A_{260} units) in 10 mM sodium acetate buffer (pH 4.5), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol. Elution was done in the same buffer with a linear gradient (2 × 1 litre) from 0.35–0.70 M NaCl: (——) A_{260} nm; (●– – –●) [³H]threonyl accepting activity detected using a yeast mt enzymatic extract. (B) Two-dimensional polyacrylamide gel separation pattern of the Thr-1 fraction (40 μg) from (A). Aliquots of the material eluted from spots 1–5 were assayed for acceptor activity using different ³H-amino acids. The following mt tRNAs were identified: $tRNA_m^{Met}$ (spot 1); $tRNA_1^{Thr}$ (spot 2); $tRNA_1^{Arg}$ (spot 3); $tRNA^{Ala}$ (spot 4); and $tRNA^{Gly}$ (spot 5).

labeled $tRNA_1^{Thr}$ to Sau 3A restriction fragments of mt DNA (strain MH41-7B) allowed us to localize its gene on a 4.1 kilobases fragment which also carries the gene for $tRNA^{Phe}$ and $tRNA^{Val}$ [18]. This fragment has been shown to map in the oxi-1–oxi-2 region of mt DNA [19]. From these results we infer that mt $tRNA_1^{Thr}$ is transcribed from the unusual threonine-tRNA gene sequenced in [7].

The $tRNA_{2a}^{Thr}$ and $tRNA_{2b}^{Thr}$ isoacceptors we have detected (fig.1A) were not resolved in [16]; they correspond to the $tRNA_2^{Thr}$ species, the gene of which is located near the cap locus of mt DNA [16,17]. The two isoacceptors differ probably by only post-transcriptional modification(s) since:

(i) Both migrate in the same spot when analysed by two-dimensional gel electrophoresis (not shown);

(ii) Only one threonine-tRNA gene was found in the cap region of mt DNA by sequencing [3].

3.2. Primary structure of mt $tRNA_1^{Thr}$

The nucleotide sequence of mt $tRNA_1^{Thr}$ was determined primarily using the technique in [12]. The results obtained gave the sequence of nucleotides 2–69. In fig.2, the one-dimensional separations by thin-layer chromatography of the ³²P-labeled 5′-termini corresponding to sequences 13–43 and 49–64 are shown. In addition, all the modified nucleotides were identified using two-dimensional thin-layer chromatography. This is illustrated in fig.3, where the characterization of residue m^2G in position 25 and m^1G in position 37 is shown. By contrast, none of the residues corresponding to the central part of the anticodon loop (positions 33–36) has been found to be modified.

The results obtained with the above sequencing technique were confirmed and completed by analyses on sequencing gels of either 5′- or 3′-labeled mt $tRNA_1^{Thr}$ (not shown).

4. Discussion

The cloverleaf structure of yeast mt $tRNA_1^{Thr}$ shown in fig.4 is in total agreement with the structure derived from the DNA sequence in [7]. It has an unusual anticodon loop composed of 8 nucleotides instead of the 7 present in all tRNAs, except in the frameshift suppressor $tRNA^{Gly}$ from Salmonella typhimurium sufD which also contains 8 nucleotides [20]. Besides this important exception, mt $tRNA_1^{Thr}$
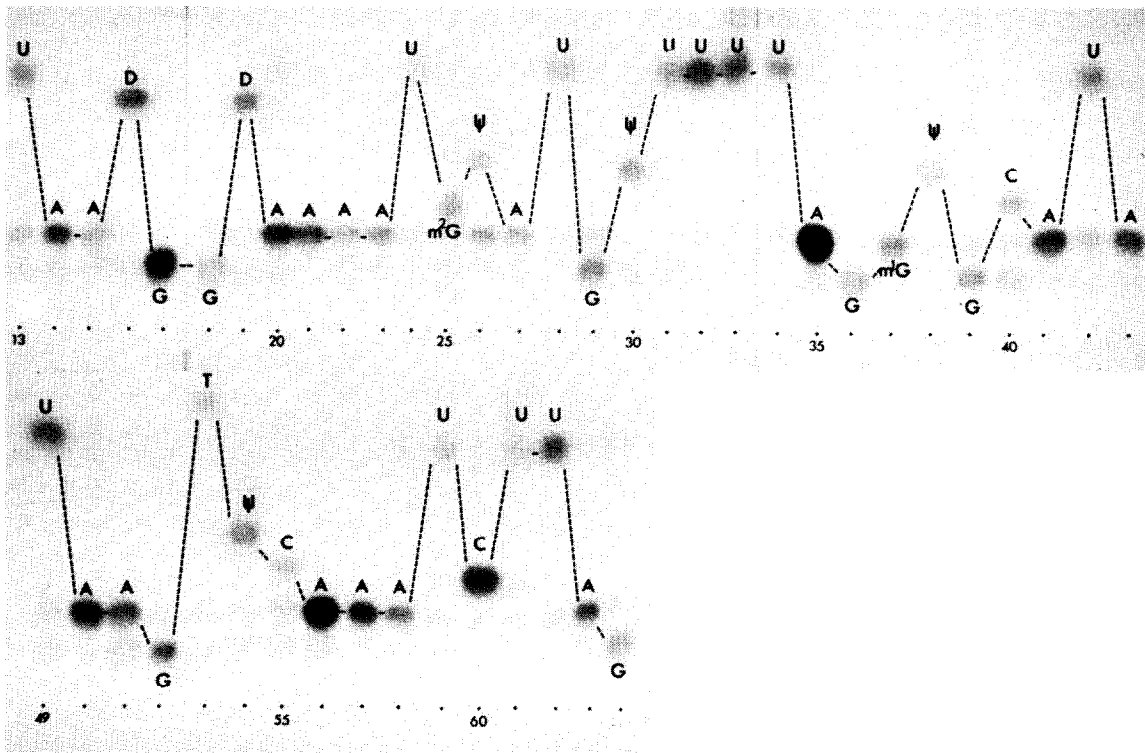
Fig.2. Thin-layer chromatography of the 5'-^{32}P-labeled termini corresponding to residues 13–43 and 49–64 in the sequence of mt tRNA$_1^{Thr}$. 2.5 μg tRNA was incubated at 80°C for 3 min in 10 μl bidistilled water. The digestion products were 5'-end-labeled and separated by polyacrylamide gel electrophoresis. Fragments were eluted from the gel and totally digested with P$_1$ nuclease. The resulting mononucleosides [5'-^{32}P]phosphate were separated on cellulose plates using solvent B (see fig.3).

contains all the other structural features (including the invariant and semi-invariant residues) which are constant in the standard cloverleaf [21].

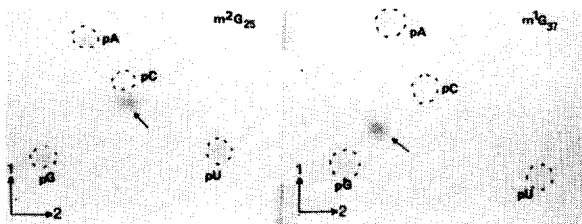mt tRNA$_1^{Thr}$ contains 75 nucleotides, 9 of which are modified: D in positions 16 and 19, ψ in positions

Fig.3. Two-dimensional separation on cellulose plates of the 5'-^{32}P-termini corresponding to residues 25 and 37 in the mt tRNA$_1^{Thr}$. First dimension: isobutyric acid/H$_2$O/25% NH$_4$OH, 66/33/1 (by vol.) (solvent A); second dimension: 2-propanol/conc. HCl/H$_2$O, 68/17.6/14.4 (by vol.) (solvent B). Circles show the non-radioactive marker nucleotides.
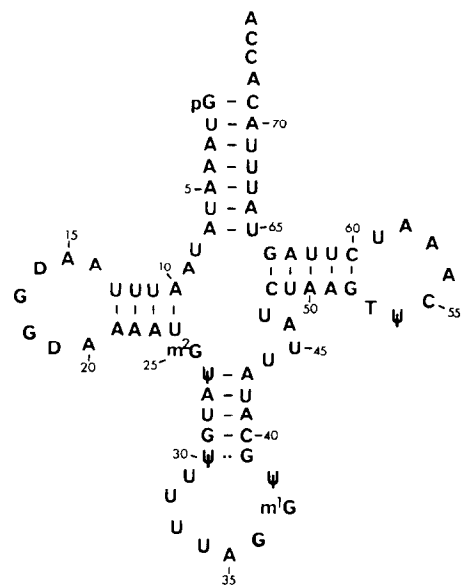

Fig.4. Cloverleaf structure of yeast mt tRNA$_1^{Thr}$.

Table 1
Percentages of sequence homology between yeast mt $tRNA_1^{Thr}$ and other sequenced threonine tRNAs [20]

E. coli	B. subtilis	Phage T$_4$	Spinach chloroplast	N. crassa mitochondria	Yeast mito-chondria-2[a]	Yeast cytoplasm
49	44	39	40	51	51	39

[a] As deduced from gene sequence [Berlani, R. et al. (1980) J. Bacteriol. 141, 1086–1097]

26, 30, 38 and 54, rT in position 53 and two methyl-ated guanosines, m^2G and m^1G. The m^2G residue located between the D-stem and the anticodon stem has not yet been found in any other mt tRNA, though it is present in this position in some non-mitochon-drial tRNAs [20]. Like several other mt tRNAs, mt $tRNA_1^{Thr}$ contains a m^1G residue in the anticodon loop (position 37). Since the location of this modified nucleoside is restricted to the position 3′ of the anti-codon in the known mt tRNAs [20], the anticodon U–A–G (positions 34–36) can be assigned to mt $tRNA_1^{Thr}$. This was not evident since the U–U–A sequence (positions 33–35) which is the other triplet near the center of this unusual anticodon loop of 8 nucleotides, could also have been a possible anti-codon. Furthermore, the presence of the U–A–G anticodon in this tRNA explains why the C–U–A codon for leucine is translated into threonine in yeast mitochondria [6], unlike in any other mitochondrial or non-mitochondrial genetic systems.

In [2] it was shown from determinations of the nucleotide sequences of 6 mt tRNAs that *Neurospora crassa* mitochondria show unique features in the codon reading patterns, i.e., mt tRNAs containing an unmodified uridine in the first position of the anti-codon are capable of reading all four codons of an unmixed codon family, whereas those containing a modified uridine derivative in the same position are restricted to reading mixed codon families ending in A or G. Our previous sequencing of leucine-, arginine 1-, tryptophan-, serine 2- and glycine-mt tRNAs from yeast ([22,23], R. P. M., in preparation) showed that the rules governing the specificity of the codon–anti-codon interaction which have been described for *N. crassa* mitochondria [2] are also operative in yeast mitochondria. Our finding that the uridine residue in the U–A–G anticodon of mt $tRNA_1^{Thr}$ is unmodified suggests that this tRNA not only translates the C–U–A codon into threonine, but also the 3 other triplets of the C–U–N leucine family.

From an evolutionary point of view, the yeast mt

$tRNA_1^{Thr}$ does not seem to be closely related to any other threonine-tRNA, neither from procaryotes nor from eucaryotes or even from mitochondria, since it shows rather poor sequence homology with known $tRNAs^{Thr}$ from either class (see table 1). Interestingly, the highest homology (59%) is observed with the mt $tRNA_2^{Leu}$ (anticodon U–A–G) from *N. crassa* [2]. On the basis of this resemblance, we favor the hypothesis that yeast mt $tRNA_1^{Thr}$ originates from an ancestral mt leucine-tRNA gene having an anticodon U–A–G and a normal size for the corresponding loop. A uri-dine-inserting mutation in the anticodon loop of this tRNA would have resulted into a change in its amino-acylation specificity. This would explain how mt $tRNA_1^{Thr}$ became a threonine-inserting species in response to C–U–N codons in the present-day yeast mitochondria. Such an hypothesis is consistent with earlier findings [24] showing that a mutational change within the C–C–A anticodon of *Escherichia coli* $tRNA^{Trp}$ (su^+7 mutation) not only alters the tRNA to a U–A–G suppressor but also provokes the loss of tryptophan accepting specificity and the acquisition of glutamine acceptor activity. The mutation giving rise to a leucine-to-threonine change in mitochondrial proteins from yeast would only have arisen at a late stage in mitochondria evolution since the C–U–N codons specify leucine and not threonine in other mitochondrial systems [1,2]. This change might have survived since:

(i)   The C–U–N codons are infrequently used in yeast mitochondrial genes [25]; and

(ii) The mt DNA-coded proteins would not have been affected functionally by this change.

Acknowledgements

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