Nucleic Acids Research, 1998, Vol. 26, No. 2 537–543

Effect of a mutation in the anticodon of human mitochondrial tRNA^{Pro} on its post-transcriptional modification pattern

Hervé Brulé, W. Michael Holmes+, Gérard Keith, Richard Giegé and Catherine Florentz*

Unité Propre de Recherche 9002, 'Structure des Macromolcules Biologiques et Mécanismes de Reconnaissance', Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, 15 rue René Descartes, F-67084 Strasbourg Cedex, France

Received September 11, 1997; Revised and Accepted November 7, 1997

ABSTRACT

Although the gene sequences of all 22 tRNAs encoded in the human mitochondrial genome are known, little information exists about their sequences at the RNA level. This becomes a crucial limitation when searching for a molecular understanding of the growing number of maternally inherited human diseases correlated with point mutations in tRNA genes. Here we describe the sequence of human mt-tRNA^{Pro} purified from placenta. It shows absence of editing events in this tRNA and highlights the presence of eight post-transcriptional modifications. These include T54, never found so far in an animal mt-tRNA, and m^{1}G37, a modification known to have fundamental functional properties in a number of canonical tRNAs. Occurrence of m^{1}G37 was further investigated in an analysis of the substrate properties of *in vitro* transcripts of human mt-tRNA^{Pro} towards pure *Escherichia coli* methylguanosine transferase. This enzyme properly methylates G37 in mt-tRNA and is sensitive to the presence of a second G at position 36, neighboring the target nucleotide for methylation. Since mutation of nt 36 was shown to be correlated with myopathy, the potential consequences of non-modification or under-modification of mt-tRNA nucleotides in expression of the particular myopathy and of mitochondrial diseases in general are discussed.

INTRODUCTION

Knowledge on human mitochondrial (mt) transfer RNAs (tRNAs) is very limited. Although the gene sequences of its 22 tRNAs are known (1), so far only tRNA$^{Ser(AGY)}$ has been sequenced and analyzed with respect to structural features at the RNA level (2). This delay is a serious drawback when a correlation between a growing number of maternally inherited neuromuscular diseases and mutations in human mt-DNA and especially in genes encoding tRNAs has been reported (3–5). Direct sequencing of the gene products will not only allow detection of potential editing in human mitochondria but also establishment of the post-transcriptional modification patterns of tRNAs.

Although all the roles that the numerous modified nucleotides found in tRNAs (~80) might play have not yet been deciphered, some have been demonstrated. Thus their contribution to structural stabilization was confirmed by a variety of approaches, including chemical and enzymatic probing in solution, NMR and crystallography (reviewed in 6). As to their functional properties, post-transcriptional modifications were reported as main factors in both aminoacylation and codon binding (reviewed in 7). Base modifications have been reported as crucial identity elements for several specific aminoacylations (8–11) and as elements hindering mischarging by non-specific aminoacyl-tRNA synthetases (8,12,13). They are also involved in correct codon–anti-codon interactions (14,15) and preventing frameshifting during mRNA translation (16). Clearly, mutations in tRNA genes may affect the post-transcriptional modification pattern of gene products and thus affect their structural and/or functional properties.

Here we describe the primary structure and post-transcriptional modification pattern of human mt-tRNA^{Pro}. Our attention has focused particularly on methylation of residue G37 within the anticodon loop of the tRNA. This position is often methylated in tRNAs. In *Escherichia coli*, for example, methylation of G37 occurs in several tRNAs, catalyzed by a specific tRNA methyl-1-guanosine transferase (1MGT) that has been cloned, overexpressed, and well characterized (17,18). This enzyme requires not only the presence of G37 but also of a neighboring guanosine at position 36 (19,20), a sequence combination that is present in the human mt-tRNA^{Pro} investigated here. In addition to residues G36 and G37, which can be transplanted into several heterologous tRNA species which are not natural substrates, converting them to excellent substrates (20), the enzyme requires a proper conformation of its substrate for maximum catalytic

*To whom correspondence should be addressed. Tel: +33 388 41 7058; Fax: +33 388 60 2218; Email: florentz@ibmc.u-strasbg.fr. Present address: Division of Biology, California Institute of Technology, Pasadena, CA, 91125, USA. Tel: +1 626 395 8434; Fax: +1 626 449 0746; Email: florentz@seqaxp.bio.caltech.edu

+Permanent address: Institute of Structural Biology and Drug Design, Virginia Biotechnology Research Park, 800 East Leigh Street, Suite 212B, Richmond, VA 23219, USA

efficiency. Deletion of any of the loops of tRNAs with a long variable arm or disruption of D loop–T loop or core interactions in classical tRNAs dramatically impair methylation efficiency (20). Proper base stacking in the anticodon stem is also important (19). Given these observations we believe that 1MGT can serve as a probe in tRNA conformation studies, provided that the crucial G36G37 couplet is present. Furthermore, a human myopathy has been reported to be correlated with the single mutation C15990T in the mt-tRNA^{Pro} gene (21), a mutation which corresponds to a G36→A36 transition at the tRNA level. Thus we have investigated the methylation properties of an *in vitro* transcript of human mt-tRNA^{Pro} by *E.coli* 1MGT and the effects of a mutation at position 36 on these properties. Assuming that the methylase performing post-transcriptional modification of tRNA^{Pro} in human mitochondria follows the same rules as the *E.coli* enzyme, we discuss the potential impact of alteration of the modified nucleotide pattern of tRNAs in mitochondrial disease on the basis of our present knowledge of the role of modified nucleotides in tRNA structure–function relationships.

MATERIALS AND METHODS

Chemicals, oligonucleotides and enzymes

[^{3}H]S-adenosyl L-methionine (AdoMet) (78 Ci/mmol, 1 µCi/µl) was from Amersham and non-labeled AdoMet from Sigma. Non-labeled 1 mM AdoMet stock solution as well as isotopic dilutions were prepared in 5 mM sulfuric acid solution, pH 2.0, and kept frozen at –20°C. [γ-^{32}P]ATP and 5′-[^{32}P]pCp were from NEN Dupont.

Digitonin was from Fluka, toluidine blue O from Sigma, Sephadex G25 (medium size) from Pharmacia Biotech and Rotiphorese gel 40 from Roth-Sochiel. 3MM paper was from Whatman. Streptavidin-coated magnetic beads (Streptavidin MagneSphere paramagnetic particles) were from Promega, Microcon 10 centrifugation units from Amicon (Millipore) and the non-aqueous scintillation cocktail Ecoscint O™ from National Diagnostics (Prolabo). Cellulose avicel F 1440 plates for TLC and cellulose acetate CA 250/0 strips for electrophoresis were from Schleicher & Schuell. Polygram CEL300 DEAE/HR-2/15 (20 × 40 cm^2) plates for homochromatography were from Macherey-Nagel. Oligonucleotides used for cloning were generated on an Applied Biosystems 381A DNA synthesizer using the phosphoramidite method and purified by HPLC on Nucleosil 100-5-C18 columns (Macherey-Nagel). The 5′-biotinylated deoxyoligonucleotide 5′-CAGAGAAAAAGTCTT-TAACTCCACCATTAG-3′, complementary to the 3′-end of human mt-tRNA^{Pro}, was obtained from Eurogentec (Seraing, Belgium). All other chemicals were of the highest purity available.

Nuclease P1 was from Boehringer-Mannheim, RNase T2 and yeast hexokinase from Sigma, bacterial alkaline phosphatase from Amersham Life Science, T4 polynucleotide kinase, and T4 RNA ligase from New England Biolabs (Ozyme). Phage T7 RNA polymerase was prepared from the overproducing strain pBL221 (provided by Dr Studier, Brookhaven) according to Becker *et al.* (22). *Escherichia coli* 1MGT was purified to homogeneity from an overproducing *E.coli* strain (20). Human placentas were recovered from a local hospital within 1 h after delivery and transported to the laboratory on ice.

Purification of native human mitochondrial tRNA^{Pro}

Human mitochondria were isolated from placenta by differential centrifugation (23) and mitochondria converted into mitoplasts by digitonin treatment (24). Mitochondrial tRNAs were extracted according to Roe (25) and further purified from contaminating RNAs by chromatography on Qiagen-tip 500 (elution buffer 50 mM MOPS–NaOH, pH 7.0, 800 mM NaCl, 15% ethanol).

Native human mt-tRNA^{Pro} was isolated from total mt-tRNAs by hybridization to a complementary biotinylated oligonucleotide bound to streptavidin-coated magnetic particles according to Mörl *et al.* (26), with minor modifications. Hybridization in 6× SSC started at 70°C and release of bound tRNA was performed by heating to 80°C. The supernatant was concentrated on a Microcon 10 unit. The tRNAs were then loaded on a thin (0.4 mm) 12% polyacrylamide (19/1) denaturing gel (7 M urea, 1× TBE, pH 8.3) of 80 cm length and run for ~12 h at 3000 V, allowing the xylene cyanol dye to reach the bottom of the gel. This long migration allows both elimination of contaminating biotinylated oligonucleotide from the tRNA and separation of tRNAs at one nucleotide resolution (necessary for the subsequent sequencing procedures). The tRNA bands were located by staining with toluidine blue solution (0.03% in water). In the case of tRNA^{Pro} two bands were observed. They were excised and eluted overnight in RNA elution solution (500 mM ammonium acetate, 10 mM magnesium acetate, 0.1 mM EDTA, 0.1% SDS) at room temperature. The eluates were concentrated to 10 µl using Microcon 10 (toluidine blue does not pass through the filter), precipitated with 0.1 vol. 3 M sodium acetate, pH 4.8, 10 vol. cold $LiClO_4$/acetone (2% w/v) and recovered by immediate centrifugation. This precipitation method is particularly suited for small amounts or small fragments of RNA (27). Pellets were washed once with 80% ethanol and dried under vacuum.

Sequencing of native tRNA^{Pro}

The primary structure of tRNA^{Pro} was determined using a post-labeling method (28), adapted from Stanley and Vassilenko (29). For partial degradation tRNA^{Pro} was heated for 3 min at 95°C in 2.2 M imidazole, pH 7.0, and the hydrolysate precipitated with $LiClO_4$/acetone. Post-labeled fragments were separated on denaturing polyacrylamide gel or, for the shortest fragments, by electrophoresis–homochromatography (28,30). After detection of the labeled fragments by autoradiography the radioactive material was eluted and digested with nuclease P1 to yield the terminal labeled 5′-monophosphate nucleotides (the reaction proceeded for 3 h at 37°C in 50 mM ammonium acetate, pH 5.3, buffer with 0.15 U nuclease P1/10 µg carrier tRNA). Each sample was analyzed on a 10 × 10 cm^2 TLC plate using the isobutyrate/phosphate bidimensional solvent system (31). The nature of the modified nucleotides was determined by comparison with reference maps (32) and confirmed using another solvent system (33).

The identities of the 5′- and the 3′-end nucleotides of the tRNA were determined by TLC analysis of the nuclease P1 digest of 5′-end labeled and of the RNase T2 digest of 3′-end labeled tRNA^{Pro} respectively. For 5′-end labeling 0.2 µg tRNA^{Pro} was dephosphorylated using 1 U bacterial alkaline phosphatase and rephosphorylated according to standard procedures using [γ-^{32}P]ATP and T4 polynucleotide kinase. For 3′-end labeling 0.2 µg tRNA^{Pro} was incubated with 5′-[^{32}P]pCp and T4 RNA ligase according to England *et al.* (34) and Keith (35). Both

labeled tRNAs were purified on polyacrylamide denaturing gels at one nucleotide resolution, as described above.

The total content in modified nucleotides of human mt-tRNA^{Pro} was established by 2D-TLC (20 × 20 cm^2 plates) analysis of 5′-monophosphate nucleotides obtained by two different methods: (i) complete RNase T2 hydrolysis of tRNA^{Pro}, 5′-labeling of the mononucleotides, consumption of excess labeled ATP by glucose kination (36) and total nuclease P1 digestion; (ii) random fragmentation and post-labeling (as described above), consumption of excess labeled ATP, separation of labeled fragments from glucose 6-phosphate on 22% polyacrylamide denaturing gel, electroelution of RNA fragments and total nuclease P1 digestion.

Cloning of synthetic genes and *in vitro* transcription

Plasmid pUTL$_4$ allowing *in vitro* transcription of *E.coli* tRNA$^{Leu}_1$ has already been described (19). Plasmids containing the synthetic genes for wild-type and mutant human mt-tRNA^{Pro} to be used for *in vitro* transcription were prepared according to Perret *et al.* (37). For wild-type mt-tRNA^{Pro} the sequence was as in the mitochondrial genome (1) and for the myopathy-related variant according to Moraes *et al.* (21). Briefly, for each clone a double-stranded construct, containing the T7 RNA polymerase promoter followed by the tRNA gene sequence plus a CCA triplet and two G residues to create a *Bst*N1 restriction site, was generated using four overlapping oligonucleotides inserted into pUC 119. Plasmids were amplified in *E.coli* TG2 cells and purified by standard procedures (38).

Plasmids were digested with *Bst*N1 prior to run-off transcription with T7 RNA polymerase in the presence of the four classical ribonucleoside triphosphates (37). Transcripts were purified from contaminating DNA and excess XTP on preparative 12% polyacrylamide–urea gels (40 × 20 × 0.22 cm^3). Electrophoresis was at 600 V for 6 H. The transcripts were visualized by UV shadowing, excised, electroeluted using a Schleicher & Schuell Biotrap apparatus and ethanol precipitated. They were desalted by filtration on a 1 ml Sephadex G25 spin column. Quantification was by spectroscopy assuming A_{260} = 1 corresponds to a tRNA concentration of 40 µg/ml. Based on the nucleotide composition, 1 µg transcript corresponds to 36 pmol tRNA$^{Leu}_1$ and 44 pmol tRNA^{Pro}. In what follows transcripts corresponding to *E.coli* tRNA$^{Leu}_1$, to human wild-type mt-tRNA^{Pro} and to its disease-related variant will be referred to as Leu1, ProWT and Pro(G36A) respectively.

Methylation of transcripts by 1MGT

The standard methylation reaction was carried out in a final volume of 50 µl containing 100 mM Tris–HCl, pH 8.0 at 37°C, 1 mM DTE, 0.1 mM EDTA, 6 mM magnesium chloride, 24 mM ammonium chloride, 100 µM AdoMet (specific activity 0.2 Ci/mmol), variable concentrations of tRNA transcripts (0.5–56 µM) and an adequate amount of 1MGT (diluted in buffer containing 50 mM Tris–HCl, 10% glycerol and BSA to obtain a final concentration in the reaction mixture of 0.1 mg/ml). Reactions were initiated by addition of AdoMet, which was kept on ice until use. Aliquots were removed at different time points, spotted onto Whatman 3MM paper squares and precipitated in cold 5% TCA for 10 min. The paper squares were washed twice with cold TCA (2 × 10 min), once with ethanol (5 min) and dried. The radioactivity retained on the papers was counted by liquid scintillation. Specific activity of the methyl group bound to tRNA was estimated from total methylation of the Leu1 transcript and found to be on average 11.5-fold higher than specific activity of free AdoMet. This coefficient was used for subsequent determinations.

For plateau measurements each transcript at 8 and 16 µM was methylated by 80 µg/ml 1MGT for times up to 20 min. For kinetic parameter determination by Lineweaver–Burk analysis the amount of enzyme was optimized to ensure that the velocity was not close to V_{max} when using the highest concentration of substrate and that methylation was linear up to 8 min when using the lowest concentration. For substrate concentrations around the K_m these conditions were achieved with 2 and 4 µg/ml 1MGT for Leu1 and ProWT respectively. For inhibition tests of ProWT methylation by Pro(G36A) a constant concentration of 4.5 µM ProWT and increasing concentrations (0–35 µM) of Pro(G36A) were used. The enzyme concentration was 1 µg/ml and samples were collected after 4 min incubation.

Search for methylated nucleotides within *in vitro* methylated tRNA^{Pro} transcript

ProWT transcript was methylated *in vitro* using non-labeled AdoMet (10 µg ProWT and 2.5 nmol AdoMet were incubated with 1 µg 1MGT in 50 µl for 10 min at 37°C). After purification at one nucleotide resolution on a denaturing gel the search for methylated nucleotides was by the post-labeling method described above.

RESULTS

Primary structure of human mitochondrial tRNA^{Pro}

Human mt-tRNA^{Pro} is known by its gene sequence (Fig. 1a). This gene is located between the tRNA^{Thr} gene and the regulatory D loop region and is encoded by nt 15955–16023 of the light strand of mt-DNA. The canonical CCA 3′-end is not encoded. In order to determine the tRNA nucleotide sequence human mt-tRN^{Pro} was isolated from placental total mt-tRNA by hybridization to a specific biotinylated 30mer oligonucleotide. One single binding cycle to streptavidin-coated magnetic beads yielded ~2 µg tRNA^{Pro} starting from 300 µg total mt-tRNA. This tRNA^{Pro} preparation displayed two bands on a 12% denaturing polyacrylamide gel, differing by only 1 nt. Both bands have the same content of modified nucleotides and differ only in the 3′-end sequences (-CC and -CCA respectively) (data not shown). The total pattern of modified nucleotides after TLC is displayed in Figure 2. The full nucleotide sequence of the natural tRNA^{Pro} is presented in Figure 1b. It corresponds to that expected from the gene sequence and shows in addition the presence of eight modified nucleotides. These are m^{1}A9, Ψ27, Ψ28, Ψ32, m^{1}G37, Ψ38, T54 and Ψ55. Nucleotides 27, 28 and 38 were modified to only 10–30%. Numbering of the nucleotides is according to canonical rules (39). In the D loop the absence of conserved residues G18 and G19 and the presence of conserved or semi-conserved residues A14, R15, U17, U20 and A21 lead to an arbitrary gap in numbering between residues 17 and 20. Nucleotide 47 in the variable domain is absent, as is one base pair in the T stem (see Discussion below).

Methylation by *E.coli* 1MGT of *in vitro* transcribed human mitochondrial tRNA^{Pro}

As shown in Figure 3, ProWT is efficiently methylated in the presence of Ado-Met and pure *E.coli* 1MGT. The level of methylation is as high as in *E.coli* Leu1, which corresponds to one of the natural substrates of the enzyme. Indeed, the methylation reaction reaches a plateau within 20 min and the level of

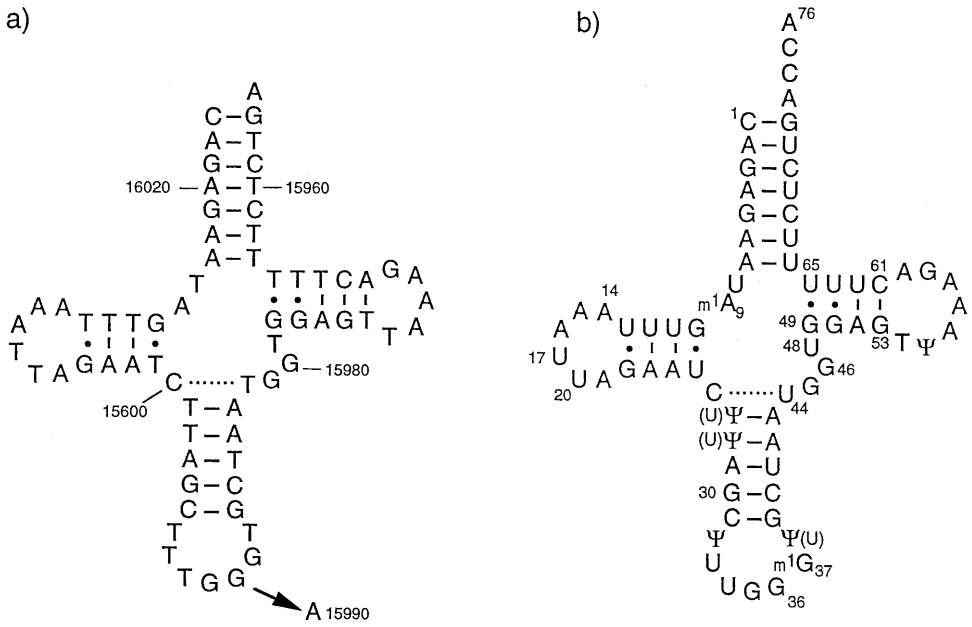

Figure 1. Secondary structure of human mt-tRNA^{Pro} according to its gene sequence (**a**) and to direct sequencing after extraction of native tRNA from human placental mitochondria (**b**). In (a) numbering is that of the mitochondrial genome (1). Position of the myopathy-inducing mutation at nt 15990 is indicated. Note that the CCA 3′-end of the tRNA is not encoded. In (b) numbering is according to Sprinzl *et al*. (39). T stands for ribothymidine, Ψ for pseudouridine. At positions 27, 28 and 38 U residues are under-modified: only 10–30% are converted into Ψ.

methylation of ProWT relative to Leu1 is 92% (Fig. 3a). Moreover, the kinetic parameters of methylation of both molecules are similar. For Leu1 the K_m is 2.2 µM and the V_{max} 215 pmol/min/µg enzyme, whereas for ProWT the K_m is 52 µM and the V_{max} 212 pmol/min/µg enzyme (Fig. 3b). Thus the V_{max}/K_m ratio for ProWT drops by a factor of 24 only relative to Leu1. This effect is linked to differences in K_m only. As expected, methylation of ProWT occurred only at nitrogen 1 of G37, as checked by sequencing of the *in vitro* methylated transcript (not shown).

Effect of mutation G36A in tRNA^{Pro} on *in vitro* methylation by *E.coli* 1MGT

The role of the nucleotide at position 36 in methylation of human mt-tRNA^{Pro} was checked by studying the substrate properties of an *in vitro* transcript with a mutation G36→A36. This mutation is reminiscent of that described in the case of a human pathology (21) and is referred to as transition C15990T in the human mitochondrial genome. Since tRNA^{Pro} is encoded by the light strand of mt-DNA, this mutation corresponds to replacement of G36 by A36 in the tRNA sequence. When assayed under the same conditions as described above the corresponding *in vitro* transcript Pro(G36A) could not be methylated significantly. Indeed, only a low level of radioactivity could be incorporated into Pro(G36A) after 20 min incubation (Fig. 3a), thus preventing more elaborate kinetic measurements. We conclude that Pro(G36A) is an extremely poor substrate for *E.coli* 1MGT. In addition, Pro(G36A) is neither an inhibitor of the enzyme nor a competitor of ProWT methylation, as shown by competition assays (Fig. 3c). Indeed, no decrease in ProWT methylation was observed with increasing amounts of Pro(G36A). The slight increase in radioactivity incorporation found with an 8-fold excess of Pro(G36A) could reflect moderate methylation of the Pro(G36A) mutant. In conclusion, mutation G36A disables efficient interaction of human mt-tRNA^{Pro} *in vitro* transcript with the methylase and strongly impairs susceptibility to methylation of the tRNA.

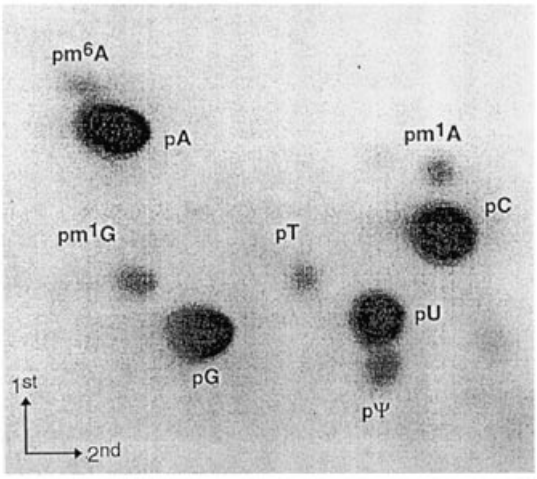

Figure 2. 2D-TLC showing the presence of m^{1}A9, T54, m^{1}G37 and Ψ besides the four standard nucleotides and a rearrangement product of m^{1}A, m^{6}A. This pattern was obtained by P1 digestion of post-labeled fragments produced by random hydrolysis of tRNA^{Pro} (see Material and Methods) and run in the 2D solvent system described in Silberklang *et al*. (31). Other slight spots (for instance to the right of pU) do not correspond to known nucleotides, even when analyzed with other solvent systems, and furthermore were not observed in the band-by-band analysis of the post-labeling sequencing procedure, therefore they may represent artifacts.

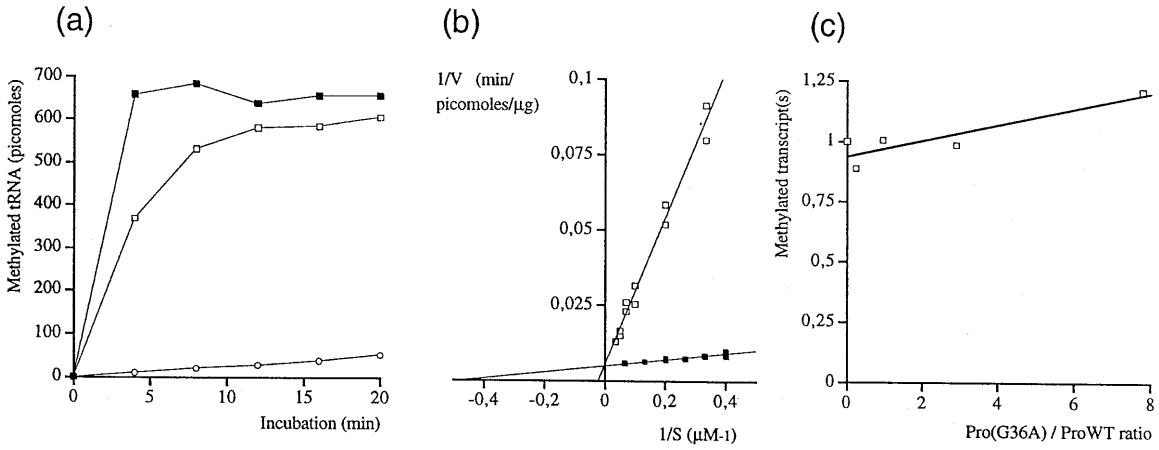

Figure 3. *In vitro* methylation by *E.coli* 1MGT of *in vitro* produced transcripts. (**a**) Methylation of 800 pmol Leu1 (solid squares), ProWT (open squares) and Pro(G36A) (open circles). (**b**) Lineweaver–Burk representation of methylation of Leu1 (solid squares) and ProWT (open squares) transcripts, allowing calculation of kinetic parameters. (**c**) Inhibition experiments of methylation of 4.5 µM ProWT by increasing concentrations of Pro(G36A). All values are relative to the methylation level of ProWT alone.

DISCUSSION

Nucleotide sequence of human mt-tRNA^{Pro}

Here we describe the first nucleotide sequence of an animal proline-specific tRNA, namely that from human mitochondria. The primary structure of this tRNA^{Pro} corresponds to that predicted from its gene sequence (1). Thus no editing is involved in maturation of this tRNA, although editing of nucleotides is frequent in mt-tRNAs, in particular within the anticodon (40) and at the discriminator position (41,42). Editing processes have been observed in birds and non-placental mammals, but not yet in human mitochondria.

Human mt-tRNA^{Pro} contains eight modified nucleotides among its 71 residues, i.e. 11% of the nucleotides are modified. This content of modified nucleotides is higher than usually found in animal mt-tRNAs (6%). For comparison, the average level of modification is 8–11% for eubacterial and non-animal mt-tRNAs and 17% for animal cytosolic tRNAs (39,43,44). These eight modified nucleotides include five pseudouridines (Ψ) and three types of methylated residues (one each of m^{1}A, m^{1}G and m^{5}U).

Pseudouridines are canonical modifications found in all kingdoms and Ψ55, present in human mt-tRNA^{Pro}, is a highly conserved nucleotide within tRNAs. The four other pseudouridine residues found in human mt-tRNA^{Pro} (Ψ27, 28, 32 and 38) are located at positions where U residues, when present, are often pseudouridylated in other mt-tRNAs (44). Three of them (Ψ27, 28 and 38) are modified to only 10–30%. The reasons for this hypomodification remain unknown, although it can be speculated that they are due either to differential catalytic efficiencies of the Ψ synthases for mt-tRNA^{Pro} or to the particular metabolic state of the placenta after delivery. In agreement with this view is the unusual deficiency of queuosine in human placental tRNA (45).

T54 (ribothymidine or m^{5}U) is a modified nucleotide typical of eubacterial, cytosolic, chloroplastic and non-animal mt-tRNAs (44). However, up to now it has never been found in animal mt-tRNAs (39) and human mt-tRNA^{Pro} is the first example of such a tRNA having a T in its T loop. The absence of T in the animal mt-tRNA compilation is probably explained by the fact that few of them have a U in position 54 or, if this U is present, they do not possess the recognition elements required by the tRNA (m^{5}U54)-methyltransferase. These elements were investigated in *E.coli* and yeast and pointed to the particular importance of a seven member T loop (46–48). The T stem and loop of human mt-tRNA^{Pro} is formed by 15 nt, instead of 17 as in canonical cloverleaves. These nucleotides can be arranged into a classical 5 bp stem and a reduced 5 nt loop (Fig. 1a). In that case U54 would pair with A60. However, the presence of a T at position 54 strongly suggests that instead the loop is of normal size (7 nt) with a stem shorter (4 bp) than usual in tRNAs (Fig. 1b). Furthermore, this loop probably has the canonical T loop conformation, as suggested by the presence of the conserved G53–C61 base pair and the possibility of a T54–A58 Hoogsteen interaction. Indeed, in canonical tRNAs where these elements are always present the T54–A58 Hoogsteen base pair is stacked onto the T stem, thanks to the peculiar hydrogen bonding pattern of phosphate 60, which interacts with N3 of C61 and O2′ of A58 (49–51).

An m^{1}A at position 9 is a hallmark of animal mt-tRNAs. Indeed, m^{1}A9 is absent in all other known tRNAs and when an animal mt-tRNA gene carries an adenine at position 9 (which is the case in ~75% of them), sequencing the tRNA always reveals the presence of m^{1}A9 (39). The frequency of this modified nucleotide suggests a unique function(s) in tRNAs from animal mitochondria. It has been shown that this modification is of particular structural importance in human mt-tRNA^{Lys} (52).

Nucleotide G37 is totally converted into m^{1}G37 in native human mt-tRNA^{Pro}. This modified nucleotide seems important for tRNA^{Pro} function since, with only one exception, it is present in all proline-specific tRNAs sequenced so far (39). The importance of methylation of nitrogen 1 of guanine at position 37 in tRNAs, already described in several cases (see below), as well as the particular interest of nucleotide 36 in the context of disease-related mutations in human mt-tRNA genes, prompted us to further investigate methylation of tRNA^{Pro}.

Recognition of human mt-tRNA^{Pro} transcripts by the *E.coli* 1MGT

tRNA m^{1}G37 methyltransferases from both eubacteria and eukaryotes are tools to probe the overall conformation of tRNAs. Indeed, methylation experiments using enzymes from *E.coli* and *Xenopus* and large sets of tRNA mutants have shown that these enzymes are sensitive to the global architecture of the substrate tRNA (19,20,53). Since the human mt-tRNA^{Pro} wild-type transcript is efficiently methylated by *E.coli* 1MGT, it presumably adopts a three-dimensional structure close to that of a regular tRNA. A canonical three-dimensional conformation for ProWT was, however, not obvious because some nucleotides involved in tertiary interactions and conserved in canonical tRNAs (reviewed in 54) are lacking in this tRNA. In particular, the absence of G18, G19 and C56 and shortening of the T stem make the T loop–D loop interaction elusive. The fact that tRNA^{Pro} transcript harboring the nucleotide substitution G36→A36 was extremely poorly methylated at position 37 by the *E.coli* methylase *in vitro* and did not compete with the wild-type molecule for the methylation reaction (Fig. 3) confirms the requirement for two neighboring G residues to confer methylation potential to the tRNA.

Human mitochondrial and prokaryotic enzymes

The 13 protein genes encoded by the human mitochondrial genome are all components of the oxidative phosphorylation system (1,55). Thus mt-tRNA modifying enzymes are encoded in the nucleus and imported from the cytosol. However, mitochondria are believed to have arisen from a prokaryotic ancestor that was engulfed by the ancestral eukaryotic cell, a theory known as the endosymbiotic hypothesis of the origin of mitochondria. A corollary of the theory postulates that many of the endosymbiont's genes were transferred to the nucleus of the host cell but their products are still addressed to mitochondria (reviewed for example in 56). Accordingly, it seems possible that human mt-tRNA modifying enzymes share similarities with their eubacterial equivalents. In other words, post-transcriptional modification of tRNAs in mitochondria may be governed by the same rules as in prokaryotes. Thus the presence of G36 may be required for methylation of G37 in human mitochondria and its mutation to A36 may drastically impair m^{1}G37 formation *in vivo*. If so, the anticodon loop of the myopathy-related variant of tRNA^{Pro} will differ not only by 1 but by 2 nt from its wild-type counterpart: the sequence G36m^{1}G37 will be replaced by the sequence A36G37.

Biological perspectives

In the initial paper describing the relationship between the presence of mutation C15990T in the tRNA^{Pro} gene and a myopathy (21) Moraes and co-workers carefully discussed the theoretical implications of this mutation which converts the proline anticodon to a serine-specific anticodon. A likely effect is that the mutation affects the aminoacylation properties of the tRNA and provokes either non-charging or mischarging, in addition to more obvious effects on mRNA decoding. Such effects, however, remain to be demonstrated explicitly. Unfortunately, although we were able to prepare human mitochondrial enzymatic extracts possessing a number of active aminoacyl-tRNA synthetases, no activity for prolylation has been detected so far (Brulé *et al.*, in preparation). Preliminary aminoacylation assays of ProWT *in vitro* synthesized transcript with pure *E.coli* and human cytosolic prolyl-tRNA synthetase were also unsuccessful (F.Yang and K.Musier-Forsyth, personal communication). Thus information about the effect of mutation at position 36 of human tRNA^{Pro} on the aminoacylation properties of the tRNA still awaits experimental support. An interesting way to answer the question would be a comparison of *in vivo* aminoacylation properties of both wild-type and mutated tRNAs, but this approach relies on the availability of cybrid cell lines expressing 100% mutated mt-DNA.

Our present data suggest that in addition to the above-mentioned effects, mutation C15990T would potentially lead to hypo-methylation or no methylation at all of residue 37 in human mt-tRNA^{Pro}. An additional effect, linked to the deficit in modified bases, may contribute to expression of the disease. Indeed, hypomodification may either enhance or alternatively attenuate the severity of dysfunction of the tRNA. The large contribution of anticodon nucleotides to the function of tRNAs (57,58), especially to the aminoacylation properties of tRNA^{Pro} (59), suggests that mutations in this domain of the tRNA have much stronger effects than mutations elsewhere in the tRNA structure. Recessive anticodon alterations may, however, be compatible with life when heteroplasmic (presence in a given mitochondria of wild-type DNA molecules in addition to mutated species) or when the functional properties of the mutated tRNA are controlled by a combination of molecular effects. Interestingly, particular roles have been ascribed to m^{1}G37 in both translational fidelity and tRNA identity. Thus m^{1}G37 in *Salmonella typhimurium* tRNA^{Pro} and, possibly, in other kingdoms prevents frameshifting (16). As to tRNA identity, m^{1}G37 was identified as a negative determinant hindering recognition of a tRNA by a non-cognate aminoacyl-tRNA synthetase,whose absence leads to misaminoacylation of the tRNA (13). Similar properties may occur for the mutated tRNA^{Pro}, which might become mischarged rather than non-charged. Considerations along these lines suggest that the particular mutation C15990T in the tRNA^{Pro} gene could be detected in patients because the dramatic effects on prolylation, due to replacement of residue 36, may be compensated for to some extent by new properties acquired by that tRNA in response to the absence of methylation at position 37. This analysis might also explain why no other mutations in the anticodon loop of tRNAs correlated with a mitochondrial disease have been described so far. Indeed, within the 32 different mutations reported (5,60) all occur at nucleotide positions scattered over the different tRNA domains except in the anticodon. Mutations at this strategic domain might be so strong that they are lethal.

Our data emphasize that a mutation of one particular nucleotide can have remote effects on other nucleotides (mutation of residue 36 affects modification of nt 37). Indirect effects of mutations on maturation and function of tRNAs are of more general occurrence and mutations are known to trigger long range effects. For example, an insertion in the anticodon loop hinders modification at position 13 in the D stem located ~20 Å away (53) and mutations in the anticodon loop of many tRNAs strongly impair their aminoacylation at the other extremity of the molecule (see for example 57). In the case of human mitochondrial diseases related to mutations in tRNA genes such long range effects might explain why most of the mutated positions in tRNAs are located at apparently 'insignificant' positions, i.e. positions not involved in structural or functional properties when referred to canonical tRNAs (see for example 5,60). On the other hand, some mutations may directly affect nucleotides that are normally modified,

leading to direct effects on tRNA modification. Both indirect and direct effects of mutations on tRNA modification can be considered only if the primary structures of the natural molecules are known. At present, among the 22 human mt-tRNAs only $tRNA^{Ser(AGY)}$ and $tRNA^{Pro}$ have been sequenced. Deeper understanding of these tRNA-dependent diseases awaits further sequencing of human mt-tRNAs.

ACKNOWLEDGEMENTS

We thank Françoise Degoul for advice and help in preparation of placental mitochondria and Joseph D.Ng for 1MGT preparation. This work was partly supported by grants from the Centre National de la Recherche Scientifique (CNRS), Université Louis Pasteur (Strasbourg) and Association Française contre les Myopathies (AFM). H.B. was supported by an AFM grant and W.M.H. was an associate research fellow from CNRS while on sabbatical leave in Strasbourg.

REFERENCES

1 Anderson,S., Bankier,A.T., Barrel,B.G., deBruijn,M.H.L., Coulson,A.R., Drouin,J., Eperon,J.C., Nierlich,D.P., Roe,B.A., Sanger,F., Schreier,P.H., Smith,A.J.H., Staden,R. and Young,I.G. (1981) *Nature*, **290**, 457–465.
2 de Bruijn,M.H.L. and Klug,A. (1983) *EMBO J.*, **2**, 1309–1321.
3 Wallace,D.C. (1992) *Annu. Rev. Biochem.*, **61**, 1175–1212.
4 Lestienne,P. and Bataillé,N. (1994) *Biomed. Pharmacother.*, **48**, 199–214.
5 Larsson,N.-G. and Clayton,D.A. (1995) *Annu. Rev. Genet.*, **29**, 151–178.
6 Agris,P.F. (1996) *Prog. Nucleic Acid Res. Mol. Biol.*, **53**, 79–129.
7 Björk,G.R. (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology, Washington, DC, pp. 165–205.
8 Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokoyama,S. (1988) *Nature*, **336**, 179–181.
9 Tamura,K., Himeno,H., Asahara,H., Hasegawa,T. and Shimizu,M. (1992) *Nucleic Acids Res.*, **20**, 2335–2339.
10 Sylvers,L.A., Rogers,K.C., Shimizu,M., Ohtsuka,E. and Söll,D. (1993) *Biochemistry*, **32**, 3836–3841.
11 Ohtsuki,T., Kawai,G., Watanabe,Y.I, Kita,K., Nishikawa,K. and Watanabe,K. (1996) *Nucleic Acids Res.*, **24**, 662–667.
12 Perret,V., Garcia,A., Grosjean,H., Ebel,J.-P., Florentz,C. and Giegé,R. (1990) *Nature*, **344**, 787–789.
13 Pütz,J., Florentz,C., Benseler,F. and Giegé,R. (1994) *Nature Struct. Biol.*, **1**, 580–582.
14 Nishimura,S. (1979) In Schimmel,P.R., Söll,D. and Abelson,J.N. (eds), *Transfer RNA: Structure, Properties and Recognition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 59–79.
15 Yokoyama,S. and Nishimura,S. (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology, Washington, DC, pp. 207–223.
16 Björk,G.R., Wikström,P.M. and Byström,A.S. (1989) *Science*, **244**, 986–989.
17 Byström,A.S. and Björk,G.R. (1982) *Mol. Gen. Genet.*, **188**, 440–446.
18 Hjalmarsson,K.J., Byström,A.S. and Björk,G.R. (1983) *J. Biol. Chem.*, **258**, 1343–1351.
19 Holmes,W.M., Andraos-Selim,C., Roberts,I. and Wahab,S.Z. (1992) *J. Biol. Chem.*, **267**, 13440–13445.
20 Redlak,M., Andraos-Selim,C., Giegé,R., Florentz,C. and Holmes,W.M. (1997) *Biochemistry*, **36**, 8699–8709.
21 Moraes,C.T., Ciacci,F., Bonilla,E., Ionasescu,V., Schon,E.A. and DiMauro,S. (1993) *Nature Genet.*, **4**, 284–288.
22 Becker,H.D., Giegé,R. and Kern,D. (1996) *Biochemistry*, **35**, 7447–7458.
23 Gasnier,F., Rousson,R., Lermé,F., Vaganay,E., Louisot,P. and Gateau-Roesch,O. (1993) *Anal. Biochem.*, **212**, 173–178.
24 Schnaitman,C. and Greenawalt,J.W. (1968) *J. Cell Biol.*, **38**, 158–175.
25 Roe,B.A. (1975) *Nucleic Acids Res.*, **2**, 21–42.
26 Mörl,M., Dorner,M. and Pääbo,S. (1994) In Uhlén,M., Hornes,E. and Olsvik,O. (eds), *Advances in Biomagnetic Separation*. Eaton Publishing, Natik, MA, USA. pp. 107–111.
27 Vlassov,V.V., Zuber,G., Felden,B., Behr,J.-P. and Giegé,R. (1995) *Nucleic Acids Res.*, **23**, 3161–3167.
28 Keith,G. (1990) In Gehrke,C.W. and Kuo,K.C.T. (eds), *Chromatography and Modification of Nucleosides*. Elsevier, Amsterdam, The Netherlands, Vol. 45A, pp. A103–A141.
29 Stanley,J. and Vassilenko,S. (1978) *Nature*, **274**, 87–89.
30 Barrell,B.G. (1971) In Cantoni,G.L. and Davies,D.R. (eds), *Procedures in Nucleic Acid Research*. Harper and Row Publishers, New York, NY, Vol. 2, pp. 751–779.
31 Silberklang,F., Gillum,A.M. and RajBhandary,U.L. (1979) *Methods Enzymol.*, **59**, 58–109.
32 Keith,G. (1995) *Biochimie*, **77**, 142–144.
33 Nishimura,S. (1979) In Schimmel,P.R., Söll,D. and Abelson,J.N. (eds), *Transfer RNA: Structure, Properties and Recognition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix III, pp. 551–552.
34 England,T.E., Bruce,A.G. and Uhlenbeck,O.C. (1980) *Methods Enzymol.*, **65**, 65–74.
35 Keith,G. (1983) *Biochimie*, **65**, 367–370.
36 Silberklang,M., Prochiantz,A., Haenni,A.L. and RajBhandary,U.L. (1977) *Eur. J. Biochem.*, **72**, 465–478.
37 Perret,V., Garcia,A., Puglisi,J.D., Grosjean,H., Ebel,J.-P., Florentz,C. and Giegé,R. (1990) *Biochimie*, **72**, 735–744.
38 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39 Sprinzl,M., Steegborn,C., Hübel,F. and Steinberg,S. (1996) *Nucleic Acids Res.*, **24**, 68–72.
40 Mörl,M., Dörner,M. and Pääbo,S. (1995) *Nucleic Acids Res.*, **23**, 3380–3384.
41 Yokobori,S.-I. and Pääbo,S. (1995) *Nature*, **377**, 490.
42 Yokobori,S.-I. and Pääbo,S. (1997) *J. Mol. Biol.*, **265**, 95–99.
43 Keith,G., Desgrès,J., Pochart,P., Heyman,T., Kuo,K.C. and Gehrke,C.W. (1990) *Biochim. Biophys. Acta*, **1049**, 255–260.
44 Grosjean,H., Sprinzl,M. and Steinberg,S. (1995) *Biochimie*, **77**, 139–141.
45 Baranowski,W., Tomaszewski,J. and Keith,G. (1993) *Am. J. Obstet. Gynecol.*, **169**, 581–582.
46 Gu,X. and Santi,D.V. (1991) *Biochemistry*, **30**, 2999–3002.
47 Gu,X., Ivanetich,K.M. and Santi,D.V. (1996) *Biochemistry*, **35**, 11652–11659.
48 Becker,H.F., Motorin,Y., Sissler,M., Florentz,C. and Grosjean,H. (1997) *J. Mol. Biol.*, **274**, in press.
49 Ladner,J.E., Jack,A., Robertus,J.D., Brown,R.S., Rhodes,D., Clark,B.F.C. and Klug,A. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4414–4418.
50 Quigley,G.J., Seeman,N.C., Wang,A.H., Suddath,F.L. and Rich,A. (1975) *Nucleic Acids Res.*, **2**, 2329–2341.
51 Romby,P., Carbon,P., Westhof,E., Ehresmann,C., Ebel,J.-P., Ehresmann,B. and Giegé,R. (1987) *J. Biomol. Struct. Dyn.*, **5**, 669–687.
52 Helm,M., Brulé,H., Degoul,F., Cepanec,C., Leroux,J.-P., Giegé,R. and Florentz,C. (1997) *Abstract Book, 17th International tRNA Workshop*. Kazusa Akademia Center, Chiba, Japan, pp. 3–13.
53 Grosjean,H., Edqvist,J., Straby,K.B. and Giegé,R. (1996) *J. Mol. Biol.*, **255**, 67–85.
54 Giegé,R., Puglisi,J.D. and Florentz,C. (1993) *Prog. Nucleic Acid Res. Mol. Biol.* **45**, 129–206.
55 Attardi,G. and Schatz,G. (1988) *Annu. Rev. Cell Biol.*, **4**, 289–333.
56 Cavalier-Smith,T. (1987) *Annls NY Acad. Sci.*, **503**, 55–71.
57 Pallank,L., Pak,M. and L. Schulman (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology, Washington, DC, pp. 371–394.
58 Watanabe,K. and Osawa,S. (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology, Washington, DC, pp. 225–250.
59 Liu,H., Peterson,R., Kessler,J. and Musier-Forsyth,K. (1995) *Nucleic Acids Res.*, **23**, 165–169.
60 Masucci,J.P. and Schon,E.A. (1996) *Mol. Biol. Rep.*, **22**, 187–193.