756–763 *Nucleic Acids Research, 1999, Vol. 27, No. 3*

Search for differences in post-transcriptional modification patterns of mitochondrial DNA-encoded wild-type and mutant human tRNA^{Lys} and tRNA$^{Leu(UUR)}$

Mark Helm[1,2], Catherine Florentz[1,2,*], Anne Chomyn[1] and Giuseppe Attardi[1]

[1]Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA and [2]UPR 9002 du CNRS, Institut de Biologie Moléculaire et Cellulaire, 15, rue René Descartes, 67084 Strasbourg Cedex, France

Received October 12, 1998; Revised and Accepted November 26, 1998

ABSTRACT

Post-transcriptional modifications are characteristic features of tRNAs and have been shown in a number of cases to influence both their structural and functional properties, including structure stabilization, amino-acylation and codon recognition. We have developed an approach which allows the investigation of the post-transcriptional modification patterns of human mitochondrial wild-type and mutant tRNAs at both the qualitative and the quantitative levels. Specific tRNA species are long-term labeled *in vivo* with [^{32}P]orthophosphate, isolated in a highly selective way, enzymatically digested to mononucleotides and then subjected to two-dimensional thin layer chromatographic analysis. The wild-type tRNA^{Lys} and the corresponding tRNA^{Lys} carrying the A8344G mutation associated with the MERRF (Myoclonic Epilepsy with Ragged Red Fibers) syndrome exhibit the same modified nucleotides at the same molar concentrations. By contrast, a quantitatively different modification pattern was observed between the wild-type tRNA$^{Leu(UUR)}$ and its counterpart carrying the A3243G mutation associated with the MELAS (Mitochondrial Myopathy, Encephalopathy with Lactic Acidosis and Stroke-like episodes) syndrome, the latter exhibiting a 50% decrease in m^{2}G content. Complementary sequencing of tRNA$^{Leu(UUR)}$ has allowed the localization of this modification at position 10 within the D-stem of the tRNA. The decreased level of this modification may have important implications for understanding the molecular mechanism underlying the MELAS-associated mitochondrial dysfunction.

INTRODUCTION

Transfer RNAs (tRNAs), the key molecules in protein synthesis, have been the object of extensive investigations over the years, so that a deep understanding of both their structural and multiple functional properties is available (1). A typical and fascinating feature of these small RNAs is their high content of modified nucleotides. About one in 8–10 nucleotides in cytosolic tRNAs is post-transcriptionally modified in a specific way and >90 different modifications have been reported (2,3). However, the detailed function of each of these modifications is not yet known. By contrast, their overall importance in determining the structural properties of some tRNAs and the role of a limited number of modifications in controlling the functional properties, in particular aminoacylation and codon recognition, are well established (3,4). The relatively large part of the genome coding for enzymes involved in modification of tRNAs, up to 1% of the *Escherichia coli* genome, confirms the importance of this process. Analysis of the tRNA database (5) showed that in mitochondrial (mt) tRNAs only one nucleotide in 13–16 is post-transcriptionally modified and in mammalian mt tRNAs only one in 17–18 is modified (5), suggesting that the modifications retained by evolution are of even higher importance.

Information on the individual 22 human mt tRNAs is limited. Only tRNA$^{Ser(AGY)}$ (6), tRNA^{Lys} (7) and tRNA^{Pro} (8) have been sequenced as RNAs and the global content of methylated nucleotides in the 22 tRNAs has been estimated to be only 4.6% (9). [Although the general tRNA nomenclature refers to different isoacceptors by indicating the sequence of the anticodon triplet, the human mitochondrial isoaccepting tRNAs are commonly distinguished by the potentially decoded codon triplet(s).] Interestingly, both structural and functional analyses have demonstrated the importance of post-transcriptional modifications in human mitochondria. Indeed, folding of tRNA^{Lys} into a cloverleaf structure (7) and aminoacylation of tRNA^{Ile} (10) are both dependent on the presence of modified bases within the tRNAs.

Human mt tRNAs are of particular interest due to their frequent involvement in mitochondrial diseases (11–13). In order to enlarge our knowledge of the post-transcriptional modification patterns in human mt tRNAs, we have developed an approach aimed at investigating both the qualitative and quantitative aspects of the modifications. We present here comparative data on tRNA^{Lys} and tRNA$^{Leu(UUR)}$ from wild-type cell lines as well as from cell lines carrying either the MERRF-associated mutation A8344G or the MELAS-associated mutation A3243G. The approach is based on *in vivo* labeling of nucleic acids, highly specific purification of individual tRNA species by oligonucleotide trapping, followed by two-dimensional thin layer chromatography analysis of the nucleotide content. Our data show that tRNA^{Lys} is globally undermodified (0.1–0.9 mol/mol tRNA for most of the individual modifications) and that no significant difference exists between wild-type and mutant tRNA^{Lys}. In contrast, in addition to the establishment of the tRNA$^{Leu(UUR)}$ post-transcriptional modification pattern, i.e. seven modifications present at 1 mol/mol tRNA, one modification

*To whom correspondence should be addressed at: UPR 9002 du CNRS, Institut de Biologie Moleculaire et Cellulaire, 15, rue René Descartes, 67084 Strasbourg Cedex, France. Tel: +33 3 88 41 70 59; Fax: +33 3 88 60 22 18; Email: florentz@ibmc.u-strasbg.fr

present at 0.6 mol/mol and one modification present in very low amount, we present evidence for a quantitative decrease by 50% of m^2G10 in the $tRNA^{Leu(UUR)}$ bearing the mutation A3243G, as compared with the wild-type tRNA. This change in modification level may affect the functional properties of this tRNA and must be considered when attempting to understand the pathogenetic mechanism of this mutation.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was from Irvine Scientific (Santa Anna, CA), [^{32}P]phosphoric acid (10 mCi/20 µl, carrier free) from ICN (Costa Mesa, CA), RNAzol B from BiotexC (Houston, TX), Streptavidin Magnesphere R PRP for nucleic acids from Promega (Madison, WI), ribonuclease P1 from Boehringer Mannheim (Mannheim, Germany), Tip-20 columns were from Qiagen (Chatsworth, CA) and Cellulose Avicel thin-layer chromatography plates from Schleicher & Schuell (Dassel, Germany). 5′-Biotinylated oligonucleotides were either from Eurogentec (Seraing, Belgium) or from the Caltech microchemical facility.

Sequencing of human mt $tRNA^{Leu(UUR)}$

The tRNA was isolated from both placenta and HeLa cells and independently sequenced. Total tRNA was prepared from human placenta as described (8) and from HeLa cells as described below for cybrid cell lines, except that cells were grown in the absence of [^{32}P]. Human mt $tRNA^{Leu(UUR)}$ was isolated from total mt tRNA by hybridization to a specific complementary oligonucleotide as described below. Sequencing was performed by a post-labeling technique adapted from Stanley and Vassilenko (14), as described (7,8), and complemented by electrophoresis/homochromatography (15) for the 3′-end nucleotides.

Cell lines and media

The human cell line pT4, carrying in predominant form (93%) the A→G transition at position 8344 in the mt $tRNA^{Lys}$ gene, associated with MERRF syndrome (16), and the cell line pT3, carrying in homoplasmic form the wild-type version of the $tRNA^{Lys}$ gene, were previously isolated by transfer into human mtDNA-less (ρ°) 206 cells of mitochondria from myoblasts of a MERRF patient (17). The cell line R1C3 was a homoplasmic mutant subclone isolated from pT1, a sibling clone of pT3 and pT4 (J.Cabezas, J.A.Enriquez and G.Attardi, unpublished results). The cell line 43B, carrying in nearly homoplasmic form (99%) the A→G transition at position 3243 in the mt $tRNA^{Leu(UUR)}$ gene, associated with the MELAS syndrome, and the 94I cell line, carrying in homoplasmic form the wild-type version of the $tRNA^{Leu(UUR)}$ gene, were obtained in a similar way from myoblasts of a MELAS patient and his maternal aunt (18).

The transformants were grown in DMEM, supplemented with 10% dialyzed fetal calf serum (FBS) and 100 µg/ml bromodeoxyuridine (BrdU). The parental line of ρ° 206, 143B.TK$^-$, was grown in DMEM with 5% FBS and 100 µg/ml BrdU. For *in vivo* labeling of tRNAs, the cells were plated in 100 10 cm Petri dishes, each containing 10 ml of reconstituted DMEM medium with low phosphate (0.3 instead of 0.9 mM as in normal DMEM) and supplemented with 5% dialyzed FBS. The initial concentrations of cells were as follows: 3.5×10^4/ml for 143B, pT3 and 94I, which have similar doubling times of 17–20 h; 8×10^4/ml for pT4, R1C3 and 43B, with longer doubling times (~30 h). After initial growth for 4 h at 37°C, the medium was removed and replaced with the same medium containing 10 mCi carrier-free [^{32}P]phosphoric acid/l. Cells were grown further for 68 h at 37°C under 5% CO_2. All media contained 70 µM streptomycin sulfate, 70 µM kanamycin monosulfate and 500 U penicillin G/ml.

Isolation of mitochondria

^{32}P-labeled mitochondria were isolated as described (19). Cells were collected by trypsinization followed by addition to the cell suspension of 1/10 vol calf serum and centrifugation at 250 g_{av} for 9 min. After two washes in 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, the cells were incubated for 3 min in 10 mM Tris–HCl, pH 6.7, 10 mM KCl, 1.5×10^{-4} M $MgCl_2$ and broken in a Potter–Elvejem homogenizer with a rotating pestle up to ~80% cell breakage. The homogenate was then brought to 0.25 M sucrose, centrifuged for 5 min at ~1200 g_{av} to remove large debris and nuclei. The mitochondria were collected by centrifugation for 10 min at 8000 g_{av} and washed in 0.25 M sucrose, 10 mM Tris–HCl, pH 6.7 (25°C), 1.5×10^{-4} M $MgCl_2$. A major part of the ribosomes were removed by incubation of the mitochondrial fraction in 0.25 M sucrose, 10 mM Tris–HCl, pH 6.7, 10 mM EDTA for 10 min at 4°C, followed by centrifugation at 8000 g_{av} for 10 min.

Isolation of pure, labeled tRNA species

Total ^{32}P-labeled RNA was extracted from mitochondria by treatment with 7 ml of RNAzol B according to the manufacturer's instructions. RNA was precipitated with 3 vol of ethanol. After incubation at –20°C for 1 h and centrifugation at 14 000 *g* for 30 min, the RNA pellet was washed with 75% ethanol and dissolved in 1 ml 50 mM MOPS, pH 7.0, 300 mM NaCl, 15% ethanol (buffer A). Total tRNA was isolated by chromatography of total mitochondrial RNA on a Qiagen Tip-20. The tip was first equilibrated in buffer A, loaded with 1 ml total RNA and washed twice with 1 ml buffer A. Total tRNA was recovered by elution with 1.5 ml 50 mM MOPS, 800 mM NaCl, 15% ethanol, concentration on Centricon-10 (Amicon, Beverly, MA) and precipitation with 2.5 vol of ethanol.

Pure tRNA species were isolated from the total tRNA by hybridization to specific biotinylated oligonucleotides, complementary to their 3′-ends over 30 nucleotides (except the CCA end) and coupled to streptavidin-coated magnetic beads, following a protocol adapted from established procedures (7,20). Total tRNA (~15 µg in 40 µl H_2O) and magnetic beads complexed to the oligonucleotide (17 µl of beads in 20× SSC) were first independently incubated for 5 min at 65 ($tRNA^{Lys}$) or 75°C ($tRNA^{Leu(UUR)}$), then mixed and maintained at the same temperature for an additional 5 min before being cooled to 25°C over a period of 40 min in a thermal cycler. Beads were collected, carefully washed twice with 6× SSC and resuspended in 5 µl 50% sucrose, 7 M urea, 10 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue. After 2 min at 65°C and chilling on ice, the beads were loaded on an 8% polyacrylamide gel. After electrophoresis for 1 h at 150 V and autoradiography, the pure tRNA was extracted from the gel slice by two successive elutions in 150 µl 0.5 M ammonium acetate, 10 mM EDTA, 0.1% SDS and precipitated in the presence of 0.3 M sodium acetate, 40 µg carrier total yeast tRNA and 2.5 vol ethanol. In one experiment aimed at

investigating the sensitivity of post-transcriptional modifications to high temperatures, $tRNA^{Lys}$ was extracted by hybridization for 1 h at 37°C instead of the above mentioned treatment at 65°C and the beads were not heated before loading on the gel.

Nucleotide analysis

Pure labeled tRNA (2000–6000 Cerenkov counts), mixed with carrier yeast tRNA, was dissolved in 20 µl of 50 mM ammonium acetate, pH 5.3, containing 0.25 µg nuclease P1, and incubated for at least 3 h at 37°C. This enzyme digests the tRNAs completely, releasing 5′-phosphate–3′-OH mononucleotides. The digest was spotted onto a 20 × 20 cm^2 Cellulose Avicel thin layer chromatographic plate (by sequential superimposition of 1.5 µl aliquots) and mononucleotides were separated by two-dimensional chromatography. The first dimension was run in isobutyric acid:25% NH_4OH:H_2O (50:1.1:28.9) and the second dimension in 0.1 M sodium phosphate buffer (pH 6.8):ammonium sulfate:*n*-propanol (100:60:2 v/w/v) (21,22). Radioactive spots were detected by exposure of a PhosphorImager screen to the chromatogram (Molecular Dynamics, Sunnyvale, CA). Assignment of spots was done according to established tables (22). Quantitative analysis was performed with ImageQuant software. Molar amounts of each type of nucleotide were calculated as Nt × counts per spot/total counts, Nt representing the total number of nucleotides of the tRNA, 73 for $tRNA^{Lys}$ and 78 for $tRNA^{Leu(UUR)}$.

Sequencing of tRNA genes

Regions encompassing the $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ genes were amplified from mtDNA by PCR. The primers were used at 0.2 µM. Their sequences spanned nucleotides 3010–3031 (22mer) and 3420–3400 (21mer) to amplify the $tRNA^{Leu(UUR)}$ gene, and nucleotides 8237–8256 and 8484–8465 (20mers) to amplify the $tRNA^{Lys}$ gene (23). The PCR products were gel purified and subjected to cycle sequencing with reagents from an Ampli Taq Kit, according to the manufacturer's instructions. Reaction products were analyzed on an Applied Genetics automated sequencer.

RESULTS

Establishment of the experimental procedure

Comparative analysis of the post-transcriptional modification content of wild-type and disease-causing mutant human mt tRNAs is technically limited for two basic reasons. First, mt tRNAs are present only in limited amounts in cells (24) and, second, mutated versions of human mt tRNAs, which for obvious reasons can only be extracted from cultured cell lines, are usually contaminated by wild-type versions of the tRNA due to the heteroplasmy of mtDNA (presence of both wild-type and mutant copies of mtDNA within a single cell). Thus, an approach based on *in vivo* labeling of nucleic acids within cybrid cell lines has been used. The cybrids referred to here are clones isolated from the fusion of a mtDNA-less cell line with enucleated cells from a patient. The cybrid cell lines, possessing either 100% wild-type mtDNA or ~100% of the same mtDNA with a single mutation in the $tRNA^{Lys}$ or $tRNA^{Leu(UUR)}$ gene, have been described (17,18).

The different cell lines were grown in the presence of [^{32}P]orthophosphate for 68 h, which allowed approximately two doublings of the mutant cells and approximately three doublings of the wild-type cells, and were collected while still in exponential growth. Mitochondria were isolated by differential centrifugation, followed by an incubation in 10 mM EDTA to remove a major part of contaminating ribosomes (24,25). Total RNA was prepared by phenol extraction with RNAzol and total tRNA was recovered after chromatography through a disposable Qiagen Tip-20 column. This procedure allowed a rapid separation of the small from the large RNAs and contaminating DNA, yielding an enriched total tRNA fraction still containing 5S RNA. $tRNA^{Leu(UUR)}$ or $tRNA^{Lys}$ were isolated by trapping them on 30mer oligonucleotides, complementary to the 3′-portion of the respective tRNAs, covalently linked to biotin at their 5′-ends, and coupled to paramagnetic streptavidin-coated beads. After gel purification, the pure tRNA species were digested to completion with ribonuclease P1, which generates 5′-phosphate mononucleosides. These were in turn separated by conventional thin layer chromatography (21). The best separation of mononucleotides was obtained in the isobutyric acid-NH_4OH/ammonium sulfate-phosphate solvent system, therefore, this system was used throughout.

The labeling conditions used in the present work yielded 2000–6000 Cerenkov counts corresponding to ~5 pmol of pure tRNA. This amount of radioactivity was sufficient for analysis by PhosphorImager of the thin layer chromatograms. The slower growth rate of cybrids carrying mutant DNA, and thus exhibiting a respiration-deficient phenotype (17,18), was compensated by seeding the cells at higher concentrations. However, they were grown for the same length of time as the corresponding wild-type cells. On average, 3×10^8 cells were collected after the 72 h growth. Typically, the specific activity of mutant tRNAs was 2- to 3-fold lower than that of wild-type, reflecting the lower number of doublings of the mutant cells in radioactive medium. It was not possible to recover $tRNA^{Leu(UUR)}$ from total tRNA from which $tRNA^{Lys}$ had already been extracted and *vice versa*, so that each analysis had to be done in an independent experiment.

Quantitative analysis of the nucleotide content of individual tRNAs was done using ImageQuant software. Each chromatographic plate was analyzed twice, revealing variations of ~2% for non-modified nucleotides and 10–30% for modified nucleotides. The final data presented correspond to the mean of at least two independent (usually three to four) experiments ± 2 SE (standard error of the mean). Any variation in a given nucleotide content >±2 SE was considered to be significant. Since all calculations were done assuming that all the radioactivity present on a single chromatographic plate corresponds to 1 mol tRNA, they were affected neither by the total number of cells collected, nor by the yield of the pure tRNA preparation in individual experiments, nor by the specific activity of the tRNA.

As judged from their nucleotide content (Table 1), both tRNAs investigated were highly pure, a prerequisite for the present investigation. Indeed, within the limits of experimental errors, the observed content of nucleotides corresponded well to the theoretical values, as deduced from the gene sequences (23) completed by the non-encoded CCA termini. Appearance of any spontaneous mutation in the $tRNA^{Leu(UUR)}$ or $tRNA^{Lys}$ genes during cell culture was ruled out for all cell lines investigated in the present work, by direct re-sequencing of the corresponding genes. Human mitochondria possess only one $tRNA^{Lys}$; in contrast, they contain, besides $tRNA^{Leu(UUR)}$, another $tRNA^{Leu}$ isoacceptor, $tRNA^{Leu(CUN)}$, which has a nucleotide content very different from $tRNA^{Leu(UUR)}$, so that contamination by this species could be excluded. Contamination by cytosolic tRNAs could also be ruled out on the basis of the diverging nucleotide composition of these species (5).

Table 1. Evidence for purity of isolated human mt tRNA$^{Leu(UUR)}$ and tRNA^{Lys}

	tRNA$^{Leu(UUR)}$			tRNA^{Lys}		
	94I (wt, *n* = 3)	43B (mut, *n* = 2)	R1C3 (wt, *n* = 2)	pT3 (wt, *n* = 3)	R1C3 (mut, *n* = 4)	143B (wt, *n* = 3)
A	**26** 25.33 ± 1.02	**25** 24.95 ± 1.02	**26** 24.01 ± 2.00	**28** 28.20 ± 0.82	**27** 27.15 ± 0.64	**28** 28.44 ± 0.98
G	**13** 12.59 ± 1.48	**14** 13.79 ± 0.46	**13** 14.18 ± 1.47	**9** 8.64 ± 0.68	**10** 9.31 ± 0.10	**9** 8.73 ± 0.46
U	**21** 21.29 ± 1.84	**21** 21.48 ± 1.64	**21** 22.07 ± 1.06	**20** 20.08 ± 0.36	**20** 20.69 ± 0.58	**20** 19.90 ± 0.34
C	**18** 17.97 ± 2.14	**18** 17.54 ± 0.56	**18** 16.90 ± 0.92	**16** 15.87 ± 0.24	**16** 15.75 ± 0.88	**16** 15.65 ± 0.26

Theoretical versus observed nucleotide composition in various cell lines are given. Cell lines are indicated at the top of the columns. wt stands for wild-type, mut for mutant and *n* corresponds to the number of independent experiments. Theoretical values as derived from the gene sequences are in underlined bold characters. Experimental values, expressed as mol nucleotides/mol tRNA, correspond to the sum of the non-modified parental nucleotides and the related modified nucleotides. As an example, the U content in tRNA$^{Leu(UUR)}$ takes into account unmodified U as well as T, D and Ψ. Values correspond to the mean of *n* experiments and are listed ± 2 SE (standard error of the mean). Detailed values are given in Tables 2 and 3, respectively.

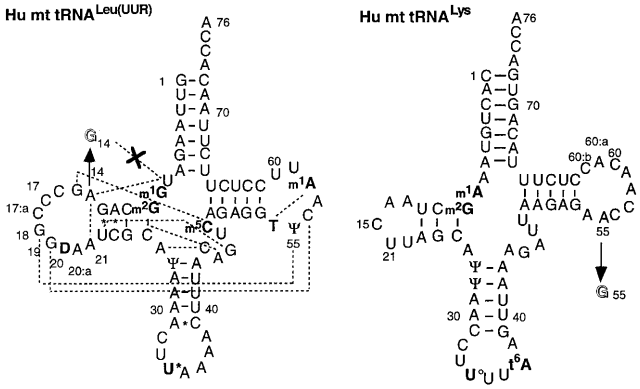

Figure 1. Secondary structures of human mitochondrial tRNA$^{Leu(UUR)}$ (**A**) and tRNA^{Lys} (**B**) from HeLa cells. Sequencing of tRNA$^{Leu(UUR)}$ has been performed according to Stanley and Vassilenko (14). The sequence of tRNA^{Lys} extracted from placental mitochondria has been established previously (7) and extended herein to HeLa cell mitochondria. The numbering of nucleotides in the tRNAs as well as the nomenclature of the modified nucleotides are according to tRNA rules (5). D, dihydrouridine; Ψ, pseudouridine; T, thymine. The positions of the point mutations investigated in the present work are indicated. The MELAS disease-related mutation 3243 in tRNA$^{Leu(UUR)}$ leads to an A→G transition at position 14, whereas the MERRF disease-related mutation 8344 in tRNA^{Lys} leads to an A→G transition at position 55. Potential tertiary interactions in tRNA^{Leu} are represented by dotted lines. A likely structural effect of mutation A14→G14 in this tRNA is schematized by a cross.

Modified nucleotide content of human mt tRNA$^{Leu(UUR)}$

Sequencing of mt tRNA$^{Leu(UUR)}$, from both placenta and HeLa cells, by the Stanley–Vassilenko approach revealed the presence of eight well-assigned modified nucleotides, namely m^{1}G9, m^{2}G10, D20, Ψ27, m^{5}C48, T54, Ψ55 and m^{1}A58 (Fig. 1). In addition, there is a modification at position U34, whose chromatographic behavior is clearly distinct from that of any known hypermodification of uridine (22). Further investigation is needed to identify its specific nature. A difference between tRNA$^{Leu(UUR)}$ from the two sources was observed at the level of position 40. U40 is partially (~50%) modified to Ψ40 in placenta and not modified in HeLa cells. In what follows, we assume that the tRNA$^{Leu(UUR)}$ extracted from cybrid cell lines is modified at the same positions as it is in HeLa cells.

An accurate quantitative analysis of the modification pattern of tRNA$^{Leu(UUR)}$ has been carried out with the approach described above on cybrid cell line 94I, homoplasmic for the wild-type tRNA$^{Leu(UUR)}$ gene. Figure 2A presents the typical pattern of a two-dimensional thin layer chromatogram of the different types of nucleotides present in this tRNA. In addition to the four major spots corresponding to the non-modified mononucleotides pA, pG, pU and pC, eight well-separated spots corresponding to the different types of modified nucleotides are seen. The quantitative analysis of the spot corresponding to the unknown modification of residue 34, named pU*34, led to non-reproducible results, therefore, data on this particular modification are not given. Quantitative data on all other nucleotides are given in Table 2 and schematized in Figure 3. They show the presence of ~2 mol Ψ/mol tRNA (substituting uridines at positions 27 and 55), 1 mol each of m^{1}A, m^{1}G, m^{5}C, T and D per mol tRNA and 0.6 mol m^{2}G/mol. Experiments performed on the unrelated cell line R1C3, also expressing wild-type tRNA$^{Leu(UUR)}$, for the most part confirmed the data observed in cell line 94I (Figs 2A and 3 and Table 2), in particular, a content in m^{2}G of ~0.6 mol/mol. However, in this second cell line, wild-type for tRNA$^{Leu(UUR)}$, but possessing a point mutation in the tRNA^{Lys} gene and thus exhibiting a mutant phenotype, the level of m^{5}C is somewhat lower than in 94I, with only ~0.6 mol/mol. As for wild-type cell line 94I, no reproducible data could be obtained for nucleotide 34.

Table 2. Nucleotide composition of human mt tRNA$^{Leu(UUR)}$ in various cell lines

Nucleotide	Cell line		
	94I (wt) (*n* = 3)	43B (mut) (*n* = 2)	R1C3 (wt) (*n* = 2)
A	24.27 ± 0.72	24.0.8 ± 0.44	23.13 ± 1.82
m^{1}A	1.06 ± 0.30	0.87 ± 0.04	0.88 ± 0.18
G	10.93 ± 0.56	12.55 ± 0.18	12.48 ± 1.06
m^{1}G	1.04 ± 0.10	0.96 ±0.17	0.98 ± 0.36
m^{2}G	0.62 ± 0.08	0.28 ± 0.11	0.73 ± 0.05
U	17.50 ± 0.48	17.85 ± 0.96	18.46 ± 0.48
T	0.91 ± 0.22	0.84 ± 0.18	0.80 ± 0.32
D	0.90 ± 0.14	0.96 ± 0.12	0.88 ± 0.22
Ψ	1.98 ± 0.08	1.83 ± 0.38	1.93 ± 0.04
C	16.95 ± 0.72	16.85 ± 0.46	16.24 ± 0.72
m^{5}C	1.09 ± 0.26	0.69 ± 0.10	0.66 ± 0.20

Data are expressed as mol nucleotide/mol tRNA. They correspond to the mean of *n* experiments and are listed ±2 SE.

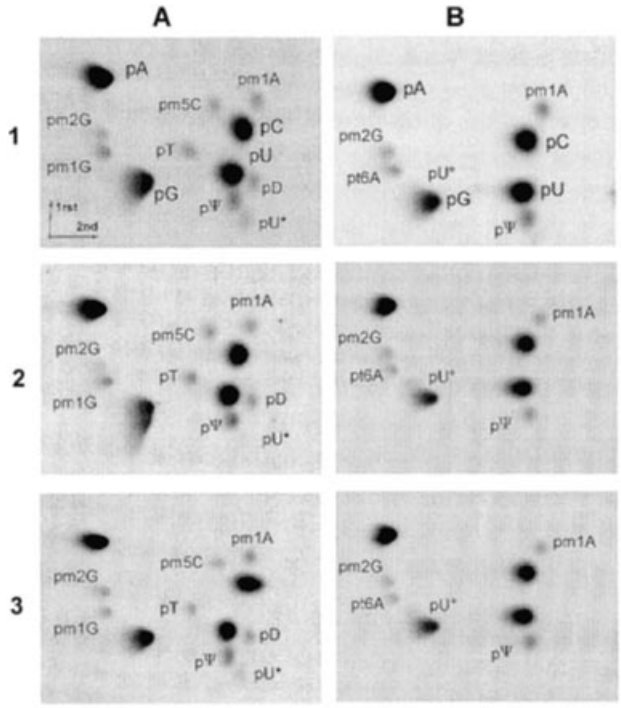

Figure 2. Autoradiograms of thin layer chromatograms after two-dimensional fractionation of total nuclease P1 digests of human mt tRNA$^{Leu(UUR)}$ (**A**) and tRNA^{Lys} (**B**). tRNAs labeled *in vivo* were isolated from total mt tRNA by specific hybridization to complementary oligonucleotides, as described in Materials and Methods. The modification pattern of tRNA$^{Leu(UUR)}$ was investigated in the homoplasmic or nearly homoplasmic cell lines 94I (**A1**) and R1C3 (**A3**) for wild-type tRNA and 43B (**A2**) for tRNA$^{Leu(UUR)}$ carrying the MELAS disease-related mutation A3243G. Notice that in the three panels (A1)–(A3), the spots corresponding to pG vary in size, a fact probably linked both to the poor migration properties of pG and to the amount of non-labeled carrier pG present, rather than to contamination by other spots. The modification pattern of tRNA^{Lys} was investigated in the homoplasmic cell lines pT3 (**B1**) for the wild-type species and R1C3 (**B2**) for the tRNA^{Lys} carrying the MERRF disease-related mutation A8344G; this tRNA was also analyzed in the parental cell line 143B (**B3**). Arrows indicate the first and second dimensions of the chromatographies.

The post-transcriptional modification pattern of tRNA$^{Leu(UUR)}$ bearing the MELAS disease-related single point mutation was established on the tRNA extracted from cybrid cell line 43B (18). This cell line is identical to cell line 94I, except that it carries in homoplasmic form the A3243G mutation in the mt tRNA$^{Leu(UUR)}$ gene. This mutation corresponds to position 14 according to the standard numbering of tRNAs (see Fig. 1). A typical chromatogram is displayed in Figure 2A and quantitative data are given in Table 2 and Figure 3. The modification pattern of the mutated tRNA is mostly identical to that of the wild-type tRNA both qualitatively and quantitatively, except for m^2G and m^5C. Nucleotide m^2G is present only at 0.28 ± 0.11 mol/mol tRNA, which corresponds to a 50% drop relative to the wild-type tRNA. The level of m^5C is the same as that in cell line R1C3, ~0.6 mol/mol, and different from the level in wild-type cell line 94I (1 mol/mol).

Modified nucleotide content of human mt tRNA^{Lys}

The RNA sequence of tRNA^{Lys} extracted from placenta was established previously (7) and this tRNA was shown to contain post-transcriptional modifications at six positions, namely m^1A9, m^2G10, Ψ27, Ψ28, an unassigned modification at U34 (U34°, different from that found in tRNA$^{Leu(UUR)}$), and t^6A37 partially hypermodified to ms^2t^6A37. The same modifications were found in tRNA^{Lys} extracted from HeLa cells, except that t^6A37 is not converted to ms^2t^6A37 (Fig. 1B). Analysis of this tRNA, extracted from cell line pT3, homoplasmic for wild-type tRNA-^{Lys}, confirmed these qualitative data. The chromatogram in Figure 2B displays the expected modifications including a faint but reproducible spot next to the pG spot, corresponding to the unassigned modification at U34. Quantitative analysis (Table 3 and Fig. 3) reveals partial modification of several nucleotides within the tRNA. Whereas the m^1 modification of A9 reaches 1 mol/mol tRNA, t^6A37 is present at only ~0.9 mol/mol, m^2G10 at ~0.7 mol/mol and the two Ψ at positions 27 and 28 total 1.7 mol/mol. The level of U°34 is very low, reaching only 0.1–0.15 mol/mol. It was verified that this modification of tRNA^{Lys} is not temperature sensitive, since the same quantitative data were obtained after extraction of tRNA^{Lys} at 37°C (not shown). Very similar data, including a very low but constant content in modified U34, were also obtained for tRNA^{Lys} purified from additional cell lines, namely pT4, containing 93% mtDNA mutated at position 8344, and 94I, wild-type for tRNA^{Lys} (data not shown).

Table 3. Nucleotide composition of human mt tRNA^{Lys} in various cell lines

Nucleotide	Cell line		
	pT3 (wt) (*n* = 3)	R1C3 (mut) (*n* = 4)	143B (wt) (*n* = 3)
A	26.23 ± 0.62	25.51 ± 0.58	26.56 ± 0.86
m^1A	1.05 ± 0.16	0.85 ± 0.04	1.03 ± 0.10
t^6A	0.92 ± 0.04	0.79 ± 0.08	0.85 ± 0.02
G	7.98 ± 0.56	8.58 ± 0.09	7.96 ± 0.36
m^2G	0.66 ± 0.12	0.73 ± 0.02	0.77 ± 0.10
U	18.24 ± 0.20	19.02 ± 0.32	17.76 ± 0.12
U°	0.13 ± 0.02	0.12 ± 0.06	0.39 ± 0.06
Ψ	1.71 ± 0.14	1.55 ± 0.20	1.75 ± 0.16
C	15.87 ± 0.24	15.75 ± 0.44	15.65 ± 0.26

Data are expressed as mol nucleotide/mol tRNA. They correspond to the mean of *n* experiments and are listed ± 2 SE.

The effect of the MERRF disease-related mutation A8344G in the tRNA^{Lys} gene on the modification pattern of tRNA^{Lys} was investigated on the cybrid cell line R1C3. This mutation corresponds to an A→G transition at position 55 of the tRNA (Fig. 1B). As seen in the autoradiogram of Figure 2B and by the values displayed in Table 3 and Figure 3, no qualitative nor quantitative difference from the corresponding wild-type-cell line pT3 could be observed.

Interestingly, analysis of wild-type tRNA^{Lys} from the parental cell line 143B revealed similar levels of modifications at positions 9, 10, 27, 28 and 37, but a significantly different level of U°34 (Figs 2B and 3 and Table 3). In this case, U°34 reached 0.4 mol/mol. The same data were obtained for tRNA^{Lys} extracted from 143B cells grown to stationary phase (data not shown). Direct sequencing of mtDNA excluded the absence of any mutation in the mt tRNA^{Lys} gene in this cell line which may have been introduced during growth. It is most likely that this variation

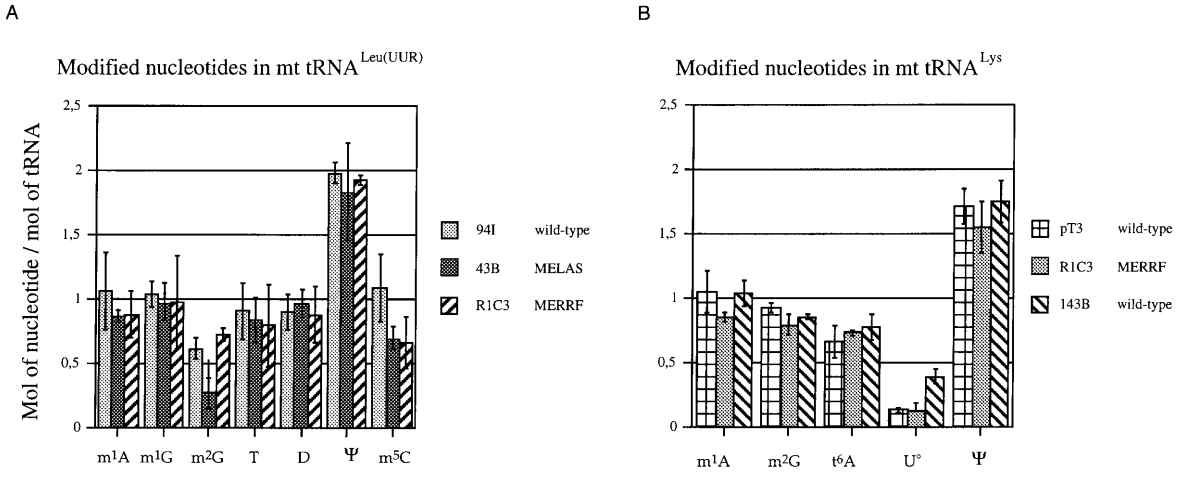

Figure 3. Graphical representation of the quantitative analysis of modified nucleotides in human mitochondrial tRNA$^{Leu(UUR)}$ (**A**) and tRNA^{Lys} (**B**) in different cell lines. Data for tRNA$^{Leu(UUR)}$ are from Table 2 and those for tRNA^{Lys} are from Table 3. Bars for different cell lines are as indicated at the right side of the panels.

in level of modification is correlated to the nuclear background or to the physiology of this special cell line (see Discussion).

DISCUSSION

Modification pattern of wild-type human mt tRNA$^{Leu(UUR)}$

tRNA$^{Leu(UUR)}$ is the fourth human mt tRNA to be sequenced at the RNA level (6–8) and only the third to be analyzed for its modified nucleotide pattern (following tRNA^{Pro} and tRNA^{Lys}). This tRNA from HeLa cells contains eight different modifications at nine positions all along its sequence which represents a very high proportion, 11.5%, of the total number of nucleotides. The same tRNA from placenta contains eight types of modifications at 10 positions which leads to an even higher proportion of modified nucleotides (12.6%). The average modification level of mammalian mt tRNAs corresponds to 5.6%. In human tRNA^{Pro} and tRNA^{Lys}, this level is also high, 11 and 8.2%, respectively. The level of methylation of HeLa cell mt tRNAs was determined to be 4.6% (9), which fits well with the average of methyl groups found in tRNA^{Lys} (2.7%), tRNA^{Pro} (4.2%) and tRNA$^{Leu(UUR)}$ (6.4%).

All modifications present in tRNA$^{Leu(UUR)}$ are simple and well identified, except a U modification present at position 34 in the anticodon, whose chromatographic behavior diverges from that of any of the modified nucleotides reported in the established tables. An unassigned modification was also reported in the case of bovine mt tRNA$^{Leu(UUR)}$, the only other mammalian mt tRNA^{Leu} sequenced so far (5). As could be anticipated from their very similar primary sequences, the human tRNA shares a number of modifications with the bovine tRNA, i.e. m^{1}G9, m^{2}G10, D20, Ψ27, a Ψ to the 3′-side of the anticodon at positions 39 and 40 in the human and bovine tRNA, respectively, and m^{5}C48. However, three additional modifications, T54, Ψ55 and m^{1}A58, are found in the T-loop of the human tRNA, whereas the T-loop of the bovine species is not modified at all. This is probably linked to the presence and absence, respectively, of signature motifs required by the corresponding modification enzymes. These motifs have been defined for the *E.coli* (26,27) and yeast (28) enzymes. Although hitherto only found in a single mammalian mt tRNA other than tRNA$^{Leu(UUR)}$, namely human tRNA^{Pro}, methylation of U54 to T54 is likely to occur also in human mt tRNA$^{Ser(UCN)}$ and tRNA^{Gln}, as well as in other mammalian tRNAs $^{Leu(UUR)}$, since the required signature motifs are conserved in these species (5). Bovine tRNA$^{Leu(UUR)}$ can be considered an exception.

The secondary structures of mt tRNAs often diverge form the canonical cloverleaf (23,29–31). However, the primary sequence of tRNA$^{Leu(UUR)}$ allows a canonical secondary folding (Fig. 1A), i.e. with stems and loops of classical sizes and a number of conserved or semi-conserved nucleotides typically involved in the tertiary folding of classical tRNAs (32). This net of nucleotides includes U8-A14-A21, G15-m^{5}C48, A26-C44, m^{1}A58-T54, G18-Ψ55, G19-C56, (m^{2}G10-C25)-A45 and (G13-U22)-G46. Only the combination 9-(12-23) is non-classical, since residues A12 and C23 do not form a Watson–Crick base pair. In combination with the unusually high number of modified nucleotides reported here, the structural features present in tRNA$^{Leu(UUR)}$ and absent to different extents in any of the other 21 human mt tRNAs make tRNA$^{Leu(UUR)}$ the most canonical mt tRNA from a structural point of view. Interestingly, the MELAS disease-related mutation A3243G, i.e. A14G transition in the tRNA, disrupts the potential tertiary interaction U8-A14-A21. Since the U8-A14 interaction is of reverse Hogsteen type and involves nitrogen 7 and the exocyclic amino group at position 6 of the adenine (33,34), replacement of A14 by G14 will not allow the formation of a similar tertiary interaction. Thus, mutation A3243G probably has structural consequences.

Quantitative alteration of the modification pattern of the MELAS disease-related mutant tRNA$^{Leu(UUR)}$

The quantitative analysis reported in the present work shows that the wild-type tRNA$^{Leu(UUR)}$ extracted from homoplasmic cybrid cell lines is not modified in a uniform manner. Indeed, whereas most modifications reach levels of 1 mol/mol tRNA, the methylation at position G10 reaches a level of only 0.6 mol/mol. Thus, there appear to be at least two populations of tRNA$^{Leu(UUR)}$ isoacceptors, one with G10, representing 40% of the tRNA molecules, and the other with m^{2}G10, corresponding to 60% of the tRNA molecules.

The effect of the MELAS disease-related mutation A3243G on the post-transcriptional modification pattern of $tRNA^{Leu(UUR)}$ was measured in a sibling cell line of the wild-type cell line. Both cell lines have the same nuclear background and differ presumably only in the identity of nucleotide 3243 in the mitochondrial genome. This mutation does not affect the variety of post-transcriptional modifications detected in the investigated tRNA, but is accompanied by a 50% drop in the level of m^2G10 and to a 45% drop in that of m^5C48. To distinguish between the possibility that the lower levels of these modifications are truly the consequence of the point mutation in the $tRNA^{Leu}$ gene or the possibility that they are linked to the pathological state of the cell line (lower growth rate, defective respiration, altered metabolism,…), an additional cell line has been investigated. The cell line R1C3 has the same nuclear background as 43B and a wild-type gene for $tRNA^{Leu(UUR)}$, but has a pathological phenotype due to the presence of the A8344G mutation in the $tRNA^{Lys}$ gene. Its growth rate is about the same as that of 43B cells. The quantitative modification pattern of $tRNA^{Leu(UUR)}$ appears to be affected only by a 40% drop of m^5C48. We thus conclude that only the 50% drop in m^2G10 observed in 43B cells may be directly linked to the presence of the A3243G MELAS mutation. This is the first report of a direct correlation between a defect in post-transcriptional modification and a human disease-causing mtDNA mutation-related pathology. Arguments in favor of such a correlation have been previously presented for the negative effect of mutation C15990T in $tRNA^{Pro}$ on methylation by an *E.coli* enzyme (8).

Methylation of nucleotide G10 may be affected by the replacement of A14 by G14 in two different ways. Either A14 is an important direct recognition element for the methyltransferase and its absence is deleterious for methylation, or the structural effect of the mutation, as discussed above, hampers proper methylation. Since neither the human mitochondrial enzyme nor any of its homologs have been characterized to date, these possibilities remain as yet unanswered.

Even though the A3243G mutation appears to be responsible for the undermethylation of G10, it is not clear whether the undermethylation is in turn responsible for the disease phenotype of the cell or if it is an unrelated event. However, it should be mentioned that the absence of a single methyl group may affect the binding properties of the tRNA to any of its partners of the mitochondrial protein synthesis machinery, in particular the leucyl-tRNA synthetase and/or the elongation factor, and thus contribute to the pathology. A number of examples of involvement of modified nucleotides in tRNA aminoacylation specificity have been reported (35–38). In yeast $tRNA^{Asp}$, a single methyl group is the key element for restricting the aminoacylation to aspartate. Absence of this group relaxes the specificity and allows additional efficient aminoacylation by arginyl-tRNA synthetase (38,39). In marsupial mitochondria, tRNAs specific for either aspartate or glycine are generated from the same precursor, depending on a post-transcriptional modification (40). Initiator $tRNA^{Met}$ in *E.coli* is rejected by the elongation factor due to a post-transcriptional modification at position 62 (41). In contrast to the possible effect of the methylation on the binding properties of $tRNA^{Leu(UUR)}$, it is unlikely that the absence of a methyl group affects the structural properties of the $tRNA^{Leu(UUR)}$, since methylation of nitrogen 1 of G10 is not involved in secondary or tertiary interactions of this nucleotide.

Interestingly, in cybrid cell lines carrying the A3243G MELAS-related mutation, protein synthesis is decreased to a varying extent (18). Furthermore, and similarly to what has been reported for the disease-related A8344G MERRF mutation (42), both the steady-state level of the mt $tRNA^{Leu(UUR)}$ and its aminoacylation efficiency are decreased in the 43B cell line with a resulting drastic decrease in available aminoacyl-tRNA for protein synthesis (43).

The diminished steady-state level of the $tRNA^{Leu(UUR)}$ can be explained by a slowed processing rate of the precursor transcript, as has been found *in vivo* (44) and *in vitro* (45). Since it is well known that post-transcriptional modifications contribute to tRNA stability (46), it is tempting to propose that the diminution of methylation reported in the present work may accelerate the rate of degradation of the tRNA and thus contribute to its lowered steady-state level.

The present data raise also the question of whether a 50% drop in the modification of one of 10 nucleotides in the tRNA, a nucleotide already undermodified in the wild-type tRNA, can cause a pathology of the severity of MELAS. It is well established that the phenotype of mitochondrial mutation-related diseases is very sensitive to the ratio between mutant and wild-type mtDNA in patient cells. A similar threshold effect may also exist at the tRNA level, where a shift in the $tRNA^{Leu(UUR)}$ population from a 60 to 40% ratio of tRNAs with and without m^2G10 in wild-type cells to a 30 to 70% ratio tRNA population in disease-affected cells, combined with a lower steady-state level of $tRNA^{Leu(UUR)}$, would be sufficient to trigger the pathology. Further work will be aimed at confirming on other MELAS mutation-carrying cell lines the undermethylation of $tRNA^{Leu(UUR)}$ observed in the 43B cell line and at understanding its significance from the pathogenetic point of view.

Non-pathology-related variations in post-transcriptional modifications of $tRNA^{Lys}$

Analysis of sibling cell lines carrying either the wild-type or the MERRF disease-related A8344G mutant $tRNA^{Lys}$ did not reveal any qualitative or quantitative difference in the modification patterns of both tRNAs. Thus, the mutation A8344G does not affect the post-transcriptional modification process of $tRNA^{Lys}$. These data rule out the possibility of a connection between the disease-causing mutation and the methylation of residue A9, which is of dramatic importance for the correct folding of the tRNA (7). Indeed, we have shown that an *in vitro* transcript of $tRNA^{Lys}$ does fold into an extended bulged hairpin, whereas the native fully modified tRNA folds into a cloverleaf structure. A mutational analysis has identified the methylation of residue A9 as a dominant factor for correct folding (7).

Measurements of the human mt $tRNA^{Lys}$ modifications have revealed that this tRNA is undermodified to a larger extent than the wild-type $tRNA^{Leu(UUR)}$. This may be linked to the particular structural characteristics of this tRNA (7) which would make it a worse substrate than $tRNA^{Leu(UUR)}$. This undermodification is interpreted to indicate the presence of a family of isoaccepting $tRNA^{Lys}$, some members of which are modified at a given position, while others are not. The biological significance of this situation remains unclear.

Analysis of the $tRNA^{Lys}$ modification pattern in the cell line 143B, the parental cell line of all the cybrids used in the present work, pointed to an unexpected variation in the level of modification at position U34. This modification in 143B cells reaches a level of 0.4 mol/mol, whereas in the cybrid cell lines

pT3, pT4, R1C3 and 94I, it reaches a level of only 0.15 mol/mol. The large difference in U34 modification level between 143B cells, on the one hand, and pT3 and 94I cells, on the other, all wild-type for the $tRNA^{Lys}$ gene, may have several explanations. Thus, this difference may reflect some variation in the nuclear background of the cell lines investigated, having arisen either stochastically during the long growth *in vitro* or as a result of the different conditions to which they were exposed. Indeed, the cybrid cell lines were constructed with ρ° (mtDNA-less) cells, derived from 143B cells which had been treated for a long time with ethidium bromide in order to deplete them of their native mtDNA (47). This treatment may have affected the nuclear DNA and possibly the gene encoding the corresponding modification enzyme or another factor involved in the modification of U34. The 143B cells used in the present work as wild-type controls for mt $tRNA^{Lys}$ had not been exposed to ethidium bromide. It is also possible that metabolic differences between the 143B cells and the ρ° 206-derived cybrids underlie the observed phenomenon.

Variations in post-transcriptional modification levels of tRNAs have been reported in eukaryotes in a number of situations, including cell differentiation, aging, starvation, hormonal responses, drug and carcinogen responses, tumoral growth and virus infection (48). Our data extend this list of factors to differences among exponentially growing human cell lines and presence of point mutations in mt tRNA genes related to neuromuscular diseases. Modification levels are considered to act as regulatory devices, since they have been found to affect the activity of tRNAs in translation and influence the regulation of intermediary metabolism, gene expression, cell division, cell cycle control, UV sensitivity, mutation frequency, antibiotics production, etc. (reviewed in 49–51). It is hoped that the elucidation of the molecular dysfunctions operating in the MELAS disease may lead to an understanding of the significance of the observed difference in modification level in the 43B cell line.

ACKNOWLEDGEMENTS

We are grateful to A. Drew and B. Keeley for their excellent technical assistance, J. A. Enriquez and Y. Michikawa for help in preliminary experiments and sequencing of mtDNA, H. Grosjean and H. Brulé for helpful comments and to R. Giegé for constant support and critical reading of the manuscript. This work was supported by NIH grant GM 11726 to G.A., by the Association Française contre la Myopathie (AFM), a NATO Grant for senior scientists to C.F. on sabbatical at Caltech and a Marie Curie fellowship from the EC to M.H.

REFERENCES

1 Söll,D. and RajBhandary,U.L. (eds) (1995) *tRNA: Structure, Biosynthesis and Function*. American Society for Microbiology Press, Washington, DC.
2 Crain,P.F. and McCloskey,J.A. (1997) *Nucleic Acids Res.*, **25**, 126–127.
3 Grosjean,H. and Benne,R. (eds) (1998) *Modification and Editing of RNA*. American Society for Microbiology Press, Washington, DC.
4 Björk,G.R. (1995) In Söll,D. and RajBhandary,U.L. (eds) *tRNA: Structure, Biosynthesis and Function*. American Society for Microbiology Press, Washington, DC, pp. 165–205.
5 Sprinzl,M., Horn,C., Brown,M., Ioudovitch,A. and Steinberg,S. (1998) *Nucleic Acids Res.*, **26**, 148–153.
6 de Bruijn,M.H.L. and Klug,A. (1983) *EMBO J.*, **2**, 1309–1321.
7 Helm,M., Brulé,H., Degoul,F., Cepanec,C., Leroux,J.-P., Giegé,R. and Florentz,C. (1998) *Nucleic Acids Res.*, **26**, 1636–1643.
8 Brulé,H., Holmes,W.M., Keith,G., Giegé,R. and Florentz,C. (1998) *Nucleic Acids Res.*, **26**, 537–543.
9 Attardi,B. and Attardi,G. (1971) *J. Mol. Biol.*, **55**, 231–249.
10 Degoul,F., Brulé,H., Cepanec,C., Helm,M., Marsac,C., Leroux,J.-P., Giegé,R. and Florentz,C. (1998) *Hum. Mol. Genet.*, **7**, 347–354.
11 Wallace,D.C. (1992) *Annu. Rev. Biochem.*, **61**, 1175–1212.
12 Larsson,N.-G. and Clayton,D.A. (1995) *Annu. Rev. Genet.*, **29**, 151–178.
13 Schon,E.A., Bonilla,E. and DiMauro,S. (1997) *J. Bioenerg. Biomembr.*, **29**, 131–149.
14 Stanley,J. and Vassilenko,S. (1978) *Nature*, **274**, 87–89.
15 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.
16 Villani,G. and Attardi,G. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 1166–1171.
17 Chomyn,A., Meola,G., Bresolin,N., Lai,S.T., Scarlato,G. and Attardi,G. (1991) *Mol. Cell. Biol.*, **11**, 2236–2244.
18 Chomyn,A., Martinuzzi,A., Yoneda,M., Daga,A., Hurko,O., Johns,D., Lai,S.T., Nonaka,I., Angelini,C. and Attardi,G. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 4221–4225.
19 Attardi B., Cravioto,B. and Attardi,G. (1969) *J. Mol. Biol.*, **44**, 47–70.
20 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, pp. 107–111.
21 Nishimura,S. (1972) *Prog. Nucleic Acid Res. Mol. Biol.*, **12**, 50–85.
22 Keith,G. (1995) *Biochimie*, **77**, 142–144.
23 Anderson,S., Bankier,A.T., Barrel,B.G., de Bruijn,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.
24 King,M.P. and Attardi,G. (1993) *J. Biol. Chem.*, **268**, 10228–10237.
25 Lynch,D.C. and Attardi,G. (1976) *J. Mol. Biol.*, **102**, 125–141.
26 Gu,X. and Santi,D.V. (1991) *Biochemistry*, **30**, 2999–3002.
27 Gu,X., Ivanetich,K.M. and Santi,D.V. (1996) *Biochemistry*, **35**, 11652–11659.
28 Becker,H.F., Motorin,Y., Florentz,C., Giegé,R. and Grosjean,H. (1998) *Nucleic Acids Res.*, **26**, 3991–3998.
29 Dirheimer,G. and Martin,R.P. (1990) *J. Chromatogr. Libr.*, **45B**, B197–B264.
30 Steinberg,S., Gautheret,D. and Cedergren,R. (1994) *J. Mol. Biol.*, **236**, 982–989.
31 Steinberg,S., Leclerc,F. and Cedergren,R. (1997) *J. Mol. Biol.*, **266**, 269–282.
32 Goddard,J.P. (1977) *Prog. Biophys. Mol. Biol.*, **32**, 233–308.
33 Rich,A. and RajBhandary,U.L. (1976) *Annu. Rev. Biochem.*, **45**, 805–860.
34 Moras,D., Comarmond,M.B., Fischer,J., Weiss,R., Thierry,J.-C., Ebel,J.-P. and Giegé,R. (1980) *Nature*, **288**, 669–674.
35 Sylvers,L.A., Rogers,K.C., Shimizu,M., Ohtsuka,E. and Söll,D. (1993) *Biochemistry*, **32**, 3836–3841.
36 Muramatsu,T., Yokoyama,S., Horie,N., Matsuda,A., Ueda,T., Yamaizumi,Z., Kuchino,Y., Nishimura,S. and Miyazawa,T. (1988) *J. Biol. Chem.*, **263**, 9261–9267.
37 Nureki,O., Niimi,T., Muramatsu,T., Kanno,H., Kohno,T., Florentz,C., Giegé,R. and Yokoyama,S. (1994) *J. Mol. Biol.*, **236**, 710–724.
38 Perret,V., Garcia,A., Grosjean,H., Ebel,J.-P., Florentz,C. and Giegé,R. (1990) *Nature*, **344**, 787–789.
39 Pütz,J., Florentz,C., Benseler,F. and Giegé,R. (1994) *Nature Struct. Biol.*, **1**, 580–582.
40 Börner,G.V., Mörl,M., Janke,A. and Pääbo,S. (1996) *EMBO J.*, **15**, 5949–5957.
41 Kiesewetter,S., Ott,G. and Sprinzl,M. (1990) *Nucleic Acids Res.*, **18**, 4677–4682.
42 Enriquez,J.A., Chomyn,A. and Attardi,G. (1995) *Nature Genet.*, **10**, 47–55.
43 Chomyn,A., Enriquez,J.A., Micol,V., Fernandez-Silva,P. and Attardi,G. (1997) *Am. J. Hum. Genet.*, **61** (suppl. S), **1787**.
44 Kaufmann,P., Koga,Y., Shanske,S., Hirano,M., DiMauro,S., King,M.P. and Schon,E.A. (1996) *Ann. Neurol.*, **40**, 172–180.
45 Rossmanith,W. and Karwan,R. (1998) *FEBS Lett.*, **433**, 269–274.
46 Agris,P.F. (1996) *Prog. Nucleic Acid Res. Mol. Biol.*, **53**, 79–129.
47 King,M.P. and Attardi,G. (1989) *Science*, **246**, 500–503.
48 Dirheimer,G., Baranowski,W. and Keith,G. (1995) *Biochimie*, **77**, 99–103.
49 Persson,B.C. (1993) *Mol. Microbiol.*, **8**, 1011–1016.
50 Winkler,M.E. (1998) In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. American Society for Microbiology Press, Washington, DC, pp. 441–469.
51 Björk,G.R. and Rasmuson,T. (1998) In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. American Society for Microbiology Press, Washington, DC, pp. 471–491.