A Watson−Crick Base-Pair-Disrupting Methyl Group (m^1A9) Is Sufficient for Cloverleaf Folding of Human Mitochondrial $tRNA^{Lys}$ †

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ABSTRACT: We have previously shown by chemical and enzymatic structure probing that, opposite to the native human mitochondrial $tRNA^{Lys}$, the corresponding in vitro transcript does not fold into the expected tRNA-specific cloverleaf structure. This RNA folds into a bulged hairpin, including an extended amino acid acceptor stem, an extra large loop instead of the T-stem and loop, and an anticodon-like domain. Hence, one or several of the six modified nucleotides present in the native tRNA are required and responsible for its cloverleaf structure. Phylogenetic comparisons as well as structural analysis of variant transcripts had pointed to m^1A9 as the most likely important modified nucleotide in the folding process. Here we describe the synthesis of a chimeric $tRNA^{Lys}$ with m^1A9 as the sole modified base and its structural analysis by chemical and enzymatic probing. Comparison of this structure to that of the unmodified RNA, the fully modified native tRNA, and a variant designed to mimic the effect of m^1A9 demonstrates that the chimeric RNA folds indeed into a cloverleaf structure that resembles that of the native tRNA. Thus, due to Watson−Crick base-pair disruption, a single methyl group is sufficient to induce the cloverleaf folding of this unusual tRNA. This is the first direct evidence of the role of a modified nucleotide in RNA folding.

Animal mitochondrial transfer RNAs (mt-tRNAs)[1] are well-known for a number of particular structural features which differentiate them from the defined canonical tRNAs (tRNAs from eukaryotic cytosols, eubacteria, archaebacteria, plant mitochondria, and chloroplasts as reviewed in ref *1*). The most dramatic differences concern mt-tRNAs from nematodes all of which lack a complete structural domain, either the D-stem and loop or the T-stem and loop *(2, 3)*. Along the same line, all mammalian $tRNA^{Ser(AGY)}$ are missing the D-stem and loop, which is replaced by a short connecting strand between the amino acid acceptor stem and the anticodon domain *(4, 5)*. This holds true for $tRNA^{Cys}$ of some reptiles *(6)*. Whereas all other animal mitochondrial tRNAs present a cloverleaf secondary structure, these structures, however, deviate from canonical tRNAs by changes in the size of individual stems and/or loops (for example, in the T-loop which is highly conserved in canonical tRNAs) and by the lack of conserved or semiconserved residues known as essential in the establishment of three-dimensional interactions (e.g., *1, 7)*. Thus, for example, many mt-tRNAs have A8 instead of U8, which hinders tertiary interaction with A14, and they miss the G18G19 sequence in the D-loop and the conserved TΨC sequence in the T-loop. Despite these structural abnormalities, mitochondrial tRNAs adopt three-dimensional structures allowing proper function as supported by experimental structural probing in the case of $tRNA^{Ser}$ *(8−11)* and sequence analysis combined with computer-aided model building *(12−14)*.

Animal mitochondrial tRNAs are further surprising at the level of their posttranscriptional modifications, probably as a consequence of their sequence and structure abnormalities. They are not only poorly but also differently modified as compared to canonical tRNAs. Whereas about 17% of nucleotides within cytosolic eukaryotic tRNAs are modified and 11% within eubacterial tRNAs, only about 6% are found modified in animal mitochondrial tRNAs *(15)*. Moreover, the diversity of the modifications is much more restricted *(15, 16)*. This suggests that these modifications are likely of higher importance than in the other tRNAs as they are retained by evolution. Interestingly, the greater importance of modified bases for efficient aminoacylation has already been observed in some mt-tRNAs as opposed to their cytosolic counterparts (e.g., *17−19*, our unpublished results). Indeed, in vitro transcribed tRNAs are much poorer substrates for the aminoacyl-tRNA synthetases than the native modified tRNAs (e.g., *9)*.

A structural analysis of human mitochondrial $tRNA^{Lys}$ lead us to the discovery of another particular structural feature never reported so far for canonical tRNAs. It was observed that the unmodified human mitochondrial $tRNA^{Lys}$, corresponding in sequence to the mitochondrial gene, does not adopt the cloverleaf structure of the fully modified tRNA. Hence, it can be assumed that modified bases are required to reach its appropriate secondary structure *(20)*. This unexpected requirement is opposite to what is found in all canonical tRNAs for which in vitro transcribed versions,

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate; mt, mitochondrial; tRNA, transfer RNA.

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deprived of modified bases, adopt the same fold as the corresponding fully modified native molecules (Figure 1). Analysis of the unusual base-pairings observed in the hairpin-like structure of the in vitro transcribed $tRNA^{Lys}$, combined with phylogenetic comparisons as well as structural analysis of in vitro transcribed variants of the tRNA, allowed us to accumulate indirect evidence for a major involvement of the methyl group at position 1 of adenosine 9 in the correct structuration of this tRNA *(20)*.

In what follows, we present an explicit demonstration of the role of a single methyl group in the cloverleaf folding of human mitochondrial $tRNA^{Lys}$ in constructing a chimeric RNA possessing m^1A9 as the sole modified nucleotide and analyzing its structural properties by chemical and enzymatic probing. Due to the particular localization of residue 9 within the tRNA and due to the positive charge of m^1A, a procedure combining chemical synthesis and ligation with T4 RNA ligase had to be optimized for the construction of the chimera. Those methodological difficulties give opportunity to pinpoint advantages and limitations of the synthetic methods available to construct the engineered $tRNA^{Lys}$. In a more biological perspective, the possibility of a higher importance of epigenetic information in mitochondria as a solution to the apparent necessity to economize genome space in animal mitochondria is discussed.

MATERIALS AND METHODS

Chemicals and Enzymes. Dimethyl sulfate (DMS), tetrabutylammonium fluoride (TBAF), tetrahydrofuran (THF), and ammonium hydroxide were from Aldrich-Chimie (St. Quentin-Fallavier, France). Imidazole (buffer grade), lead-(II) acetate, and aniline were from Merck (Darmstadt, Germany), 1-methyladenosine (m^1A), diethyl pyrocarbonate (DEPC), and hydrazine were from Sigma (St. Louis), and kethoxal was from USB (Cleveland). Radioactive [γ-^{32}P]-ATP (3000 Ci/mmol), [5′-^{32}P]pCp (3000 Ci/mmol), and T4 polynucleotide kinase were from Amersham (Les Ulis). Nuclease S1 and RNases T1 and V1 were from Pharmacia (Paris, France), RNase T2 was from Sigma (St. Louis), and bacterial alkaline phosphatase was from Appligène (Strasbourg). Restriction enzymes and T4 RNA ligase were from New England Biolabs (Beverly, MA), and T4 DNA ligase was from Boehringer-Mannheim. Phage T7 RNA polymerase was prepared according to *(21)*. Streptavidin paramagnetic particles were from Promega (Madison, WI) and Sephadex-G25 (medium) and NAP-25 pre-packed columns from Pharmacia (Paris). Oligodeoxynucleotides for cloning were from NAPS (Göttingen, Germany). A 5′-biotin-labeled oligonucleotide was from Eurogentec (Searing, Belgium). Phenoxyacetyl-adenosine-, benzoyl-cytosine-, isopropyl-phenoxyacetyl-guanosine-, and uracil-cyanoethyl-phosphoramidites were from Glen Research (Cambridge, U.K.). Oligonucleotide synthesis reagents (anhydrous acetonitrile, tetrazole/acetonitrile, acetic anhydride/lutidine/THF, *N*-methylimidazole/THF, iodine/water/pyridine, and TCA/dichloromethane) were from Perkin-Elmer (Buckinghampshire, U.K.).

Purification of Native Human Mitochondrial $tRNA^{Lys}$. Mitochondria were isolated from human placenta according to *(22)* and processed into mitoplasts by treatment with digitonin *(23)*, and total mt-tRNA was obtained by subsequent phenol extraction *(24)*. Human mt-$tRNA^{Lys}$ was further purified by hybridization to a 5′-biotin-labeled 30-mer, complementary to its 3′-end as already described *(20)*, except that the temperature gradient was replaced by incubation at 37 °C for 30 min. The supernatant was removed, and the beads were washed twice with 6× SSC. The beads were then resuspended in 4 M urea, 0.25% bromophenol blue, and 0.25% xylene cyanol (loading buffer), heated to 60 °C, chilled on ice, and loaded on a 15% denaturing sequencing gel. After electrophoresis, the tRNA was visualized by staining with toluidine blue, excised, and passively eluted in 500 mM ammonium acetate, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1% SDS.

Preparation of Full-Length tRNAs by in Vitro Transcription. A synthetic gene corresponding to the T7 RNA polymerase promoter connected to the downstream sequence of a hammerhead ribozyme followed by the human mt-$tRNA^{Lys}$ sequence (clone Kwt-Rz) and terminating at a BstN1 site was constructed from overlapping and complementary oligonucleotides as described *(25, 26)*. Clone Krw-Rz was constructed in the same way, using oligonucleotides allowing an A9→C9 mutation within the tRNA gene. All sequences were cloned into pTFMA (derived from pUC 18; ref *27*). Amplification was done with transformed *Escherichia coli* TG2 cells. Upon transcription, a hammerhead ribozyme containing four nucleotides complementary to the first four nucleotides of the 5′-end of the tRNA cleaves the phosphodiester linkage directly upstream of C1, and liberates an RNA beginning with a 5′-OH-C1. In vitro transcription of 0.1 mg/mL BstN1-linearized plasmid was performed for 3 h at 37 °C in 40 mM Tris-HCl, pH 8.1 (at 37 °C), 22 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% Triton X-100, 1 mM spermidine, 4 mM each nucleoside triphosphate, and T7 RNA polymerase. After phenol extraction, the transcripts were purified on a denaturing 12% polyacrylamide gel, electroeluted, ethanol-precipitated, and desalted on Sephadex-G25.

Preparation of $tRNA^{Lys}$ Subfragments. Oligonucleotides (8- and 9-mers) were prepared by automated RNA synthesis on a Perkin-Elmer ABI 392 DNA/RNA synthesizer using the standard 1 μmol (trityl-off) RNA synthesis program. Deprotection was done by treatment with 1.5 mL of a 3:1 ammonium hydroxide/methanol solution at 55 °C for 16 h, followed by evaporation to dryness and desilylation in 1.5 mL of 1 M TBAF/THF *(28)* at room temperature for 24 h. Preparative purification of the fragments was performed by HPLC on a biocompatible Waters 625 LC system with a Dionex Nucleopac PA-100 column (9 × 250 mm^2) at 75 °C and a flow rate of 1 mL/min. Buffer was 20 mM MES, pH 6.2, 4 M urea. After injection and an isocratic wash at 40 mM $NaClO_4$ for 20 min, RNA was eluted by a linear gradient to 280 mM $NaClO_4$ during 48 min. Fractions containing the desired RNA were pooled, desalted with a NAP-25 Pharmacia prepacked column, and then dried in a Speed-Vac Concentrator.

The G10−A76 sequence (64-mer) of $tRNA^{Lys}$ was cloned downstream of the T7 promoter into pTFMA. Transcription was as above, except that GMP was added to a final concentration of 16 mM to ensure the presence of a 5′-monophosphate. The synthesized RNA was purified on the above-mentioned HPLC column at 75 °C. Before its use in the ligation steps, the 64-mer (400 μg or 10^6 cpm of 5′-labeled fragment) was oxidized at its 3′-end by incubation

in 100 μL of 50 mM NaOAc, pH 4.5, 4 mM $NaIO_4$, for 1 h at 37 °C. The reaction was stopped by desalting the RNA on a NAP-25 prepacked column.

Synthesis of N1-Methyladenosine Bisphosphate. Phosphorylation of m^1A to *N*1-methyladenosine bisphosphate was done according to *(29)* by reaction of pyrophosphoryl chloride (612 μL, 4.4 mmol) with m^1A (123.2 mg, 0.443 mmol) under an argon atmosphere. Purification was done on a DEAE-Sephacel column (2.5 × 30 cm^2) as described. The fractions containing the nucleoside bisphosphate were pooled and evaporated to dryness.

The presence a of 5′-3′ bisphosphate derivative was verified by complete digestion of an aliquot with nuclease P1 (10 μg in 20 μL of 50 mM NH_4OAc, 0.3 unit of nuclease P1, 4 h at 37 °C). Out of the obtained mixture of bisphosphates only the 5′-3′ and not the 5′-2′ isomer is digested to 5′-pm^1A by nuclease P1. This was evidenced by subsequent 2D-TLC analysis, that revealed two spots by UV shadowing, one corresponding in migration behavior to 5′-pm^1A *(30)*. Under certain conditions, m^1A is transformed into m^6A, a reaction known as the Dimroth rearrangement *(31)*. No spot corresponding to 5′-pm^6A could be detected. No further purification was performed since the presence of the 5′-2′ isomer does not interfere with the ligation step of the 5′-3′ isomer to an RNA fragment by T4 RNA ligase *(29)*.

Ligation of the 8-mer C1−A8 to pm¹Ap and Subsequent 3′-End Dephosphorylation. Ligation was done by incubating a 1 mL mixture containing 70 μM 8-mer (C1−A8) and 0.625 mM pm^1Ap in 50 mM HEPES, pH 7.0, 20 mM $MgCl_2$, 225 μM ATP, 3.3 mM dithioerythritol, 0.1 mg/mL BSA, and 1.6 units/μL T4 RNA ligase for 6 h at 37 °C. The reaction was stopped by phenol/ether extraction and the RNA precipitated by 10 volumes of $LiClO_4$/acetone (2% w/v) and immediate centrifugation. The pellet was dissolved in 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$. The 3′-phosphate of the 9-mer was removed by digestion with 0.04 unit/μL BAP for 4 h at 37 °C. The reaction was stopped by phenol/ether extraction and the RNA purified by HPLC chromatography on a Dionex Nucleopac PA-100 column as described above except that the column was run at 65 °C.

Construction of Km¹A-Lg, Kwt-Lg, and Krw-Lg by Ligation of tRNA Subfragments. The different 9-mers (180 μM) were hybridized to the in vitro transcribed 3′-end 64-mer (30 μM) by heating in water to 70 °C for 5 min and slow cooling to 4 °C by a linear temperature gradient over 40 min in a Perkin-Elmer PCR device. Premixed buffer and enzyme were then added to final concentrations of 10 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 5 mM DTT, 2 μg/μL BSA, 1 mM ATP, 10% DMSO, 108 μM 9-mer, 18 μM 64-mer, and 2 units/μL T4 RNA ligase. After incubation of the 30 μL reaction mix at 37 °C for 6 h, 10 volumes of $LiClO_4$/acetone (2% w/v) were added, and the mixture was immediately centrifuged. The pellet was dissolved in loading buffer and purified by 15% PAGE as described above for native tRNA.

Accuracy of the ligation at position 9 was verified with internally labeled constructs obtained from 5′-end-labeled 64-mer (see below for labeling conditions). Constructs were digested to 3′-phosphate nucleosides by incubation with 0.3 unit of RNase T2 in the presence of 30 μg of total tRNA from *E. coli* in 10 μL of 50 mM NH_4OAc, pH 4.6, at 37 °C for 12 h. The digests were spotted onto 10 × 10 cm^2 cellulose plates and submitted to two-dimensional thin-layer chromatography *(32)*. Autoradiographies allowed revelation of radioactive spots corresponding to the nearest upstream neighbor to nucleotide 10.

Structural Probing. Probing was done as described *(20)*, except that buffer conditions were 40 mM sodium cacodylate, pH 7.5, 40 mM NaCl, 10 mM $MgCl_2$ for all probes. Concentrations of probes and incubation times differing from (*20*) are given in the figure legends. RNA molecules were submitted to a denaturation/renaturation procedure ensuring formation of the thermodynamically most stable conformer as described previously *(20)*. Labeling at the 3′-end was done by incubation of 18 μM RNA with 20 units/μL T4 RNA ligase overnight at 4 °C in the presence of 20 μCi of [5′-^{32}P]pCp in 20 μL of 10 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 5 mM DTT, 2 μg/mL BSA, 1 mM ATP, 10% DMSO. Labeling at the 5′-end of RNA was done by incubation of 1 μg of RNA (2.67 μM) with 10 units of T4 polynucleotide kinase for 30 min at 37 °C in the presence of 20 μCi of [γ-^{32}P]ATP in 15 μL of 50 mM Tris-HCl, pH 8.1, 5 mM $MgCl_2$, 2.5 mM DTE, 0.05 mM spermidine.

RESULTS

Approach for the Construction of $tRNA^{Lys}$ Derivatives. A chimeric human mt-$tRNA^{Lys}$ (Km^1A-Lg) containing a single modified nucleotide, namely, m^1A at position 9, has been assembled by an approach combining chemical synthesis, in vitro transcription, and enzymatic ligations. In the same way, the unmodified version of $tRNA^{Lys}$ (Kwt-Lg) as well as a variant of the $tRNA^{Lys}$ presenting the single point mutation A9→C9 (Krw-Lg) have been prepared. Sequences as well as predicted secondary structures of these molecules according to *(20)* are presented in Figure 1. Introduction of the modified base was done by ligation of its 3′-5′-mononucleoside bisphosphate derivative between a synthetic 8-mer corresponding to the 5′-end of the tRNA and an in vitro transcribed 64-mer corresponding to the 3′-part of the tRNA (Figure 2). Both chimeras presenting nonmodified nucleotides at position 9 were prepared from chemically synthesized 9-mers (Figure 2). Their preparation was aimed not only for direct structural comparisons with the molecule possessing the single methyl group but also for a comparative investigation of T4 RNA ligase substrate requirements during the ligation steps. In particular, this allows investigation of the impact of a methyl group and of its positive charge on the phosphate donor and acceptor capacities of substrates. Both the wild-type and the C9 variant have also been produced by in vitro transcription as "transzymes" *(26)*, as additional controls.

Ligation of pm¹Ap to a Synthetic 8-mer Oligonucleotide. An 8-mer oligonucleotide, C1−A8, corresponding to the very 5′-end of Km^1A-Lg, as well as two 9-mers, C1−A9 and C1−C9, corresponding, respectively, to the 5′-end of Kwt-Lg and Krw-Lg, were synthesized by the phosphoamidite method. The 5′,3′-bisphosphate *N*1-methyladenosine was obtained by pyrophosphoryl chloride treatment of *N*1-methyladenosine. The 8-mer and pm^1Ap were ligated by T4 RNA ligase as published *(33)*, with a large excess of pm^1Ap. The reaction product, C1−m^1A9-p, was then 3′-dephosphorylated with bacterial alkaline phosphatase to yield C1−m^1A9-OH. HPLC chromatography on an anion-exchange resin allowed elution

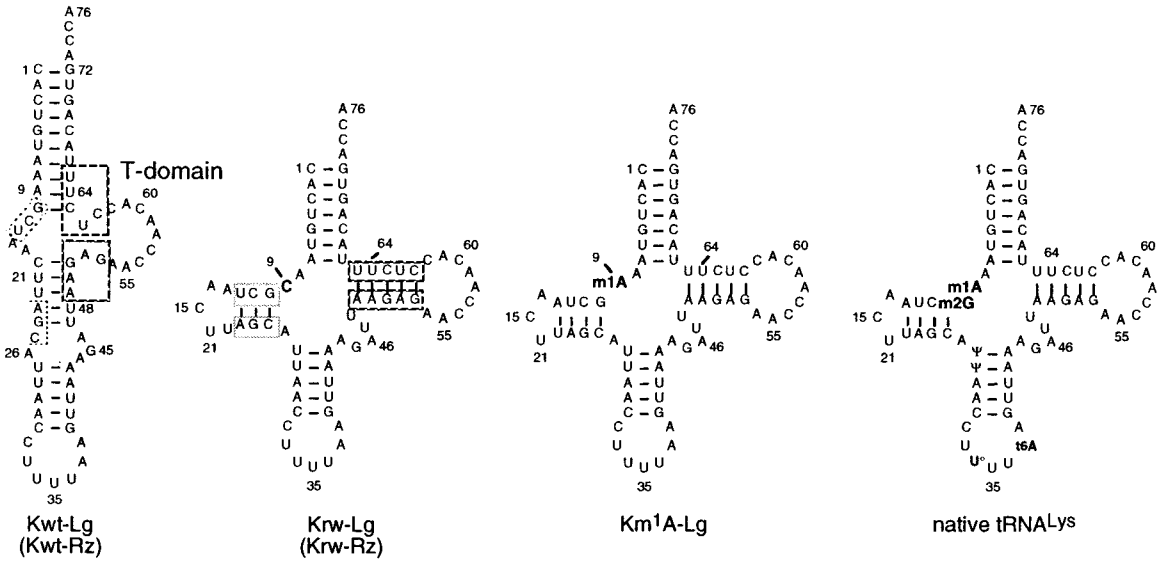

FIGURE 1: Synthetic and native human mitochondrial $tRNA^{Lys}$ investigated in the present work. Kwt-Lg, Krw-Lg, and Km^1A-Lg have been prepared by ligation of RNA fragments according to the procedure summarized in Figure 2. Native $tRNA^{Lys}$ has been extracted from human placental mitochondria. Kwt-Rz and Krw-Rz have been prepared as control molecules by in vitro transcription as transzymes, i.e., transcripts containing a ribozyme at their 5′-ends the catalytic activity of which releases the expected tRNA. Their sequences are the same as Kwt-Lg and Krw-Lg, respectively. According to the working hypothesis of this study, the structure of Km^1A-Lg has been drawn as a cloverleaf. Numbering of nucleotides is as in *(20)*. Notice the gap between residues 15 and 21 and the presence of residues 60a and 60b between residues 60 and 61.

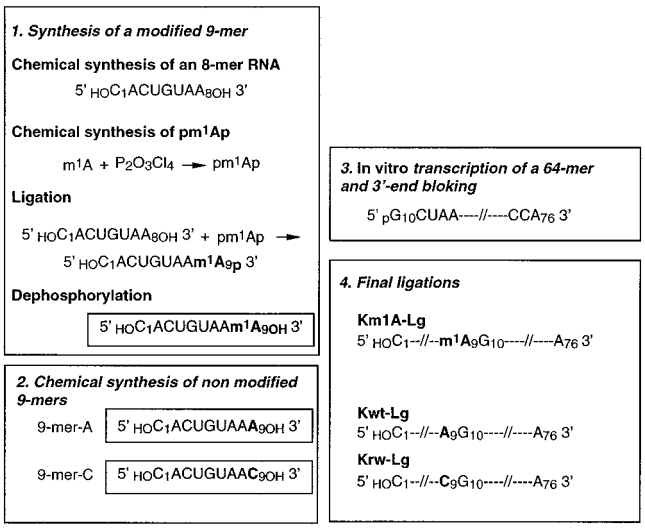

FIGURE 2: Scheme of the construction of three chimeric human mitochondrial $tRNA^{Lys}$. Construction of Km^1A-Lg includes chemical synthesis of an 8-mer oligoribonucleotide, chemical phosphorylation of modified nucleoside m^1A, ligation of the bis-phosphorylated derivative to the 8-mer, and dephosphorylation and ligation to an in vitro transcribed 64-mer corresponding to nucleotides 10−76 of the tRNA *(48)* with T4 RNA ligase. Construction of Kwt-Lg and Krw-Lg includes a single ligation step of synthetic 9-mers to the 64-mer.

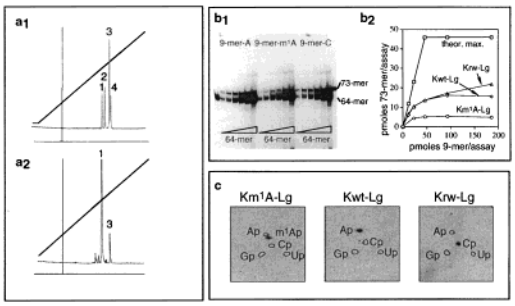

FIGURE 3: Synthesis of chimeric tRNAs. (a) Efficiency of ligation of pm^1Ap to an 8-mer oligonucleotide as verified by HPLC on a Dionex Nucleopac PA-100 column. (a_1) Elution profile of a mixture of oligoribonucleotides. (a_2) Elution profile of the reaction products obtained after both ligation of the 8-mer 5′-HOCACUGUAAOH-3′ to pm^1Ap and dephosphorylation of the outcoming 9-mer. Peak numbers correspond to (1) 9-mer−m^1AOH, (2) 8-mer, (3) 9-mer−m^1Ap or 9-mer−C, and (4) 9-mer−A. Experimental conditions are as described under Materials and Methods. (b) Optimization of the efficiency of ligation of three different 9-mers to a 64-mer for preparation of full-length tRNAs. The 5′-end-labeled 64-mer and either of three 9-mers were incubated in 30 μL reaction mixtures in the presence of T4 RNA ligase as described under Materials and Methods. The reaction products were analyzed by 12% denaturing polyacrylamide gel electrophoresis and autoradiography. (b_1) Variation of the 64-mer concentration (from 19 to 304 pmol/assay) at a constant 9-mer concentration (0.14 μg to 46 pmol/assay). (b_2) Variation of the 9-mer concentration (11.6−186 pmol/assay) at a constant 64-mer concentration (46 pmol/assay). The distribution of ^{32}P over the 64-mer and the full-length 73-mer has been calculated after phosphorimaging of the gels. (c) Next-neighbor analysis of nucleotide 10. After ligation of 5′-end-labeled 64-mer to the various 9-mers, purification of the full-length tRNAs on gels, and complete digestion with RNase T2, the 3′-monophosphate nucleosides have been analyzed on 2D thin-layer chromatography *(32)*.

of each of the RNAs (8-mer, 9-mers, phosphorylated versus unphosphorylated 9-mers) at different, characteristic ionic strengths, with the exception of C1−C9-OH and C1−m^1-A9-p which coelute (Figure $3a_1$). Interestingly, due to the positive charge of m^1A, C1−m^1A9-OH is bound less tightly to the positively charged resin and elutes at significantly lower ionic strength than C1−A9-OH and C1−A8-OH. These properties allow assessment of the reaction efficiencies of ligation of the 8-mer to pm^1Ap and of the dephosphorylation of the 9-mer C1−m^1A9-p into 9-mer C1−m^1A9-OH. Figure $3a_2$ shows a typical HPLC elution profile of a preparation of 9-mer-m^1A-OH. No residual 8-mer educt is present, and only very low amounts of 3′-end-phosphorylated 9-mer-m^1Ap are eluted. We conclude that both ligation and dephosphorylation reactions are quantitative and that pm^1Ap

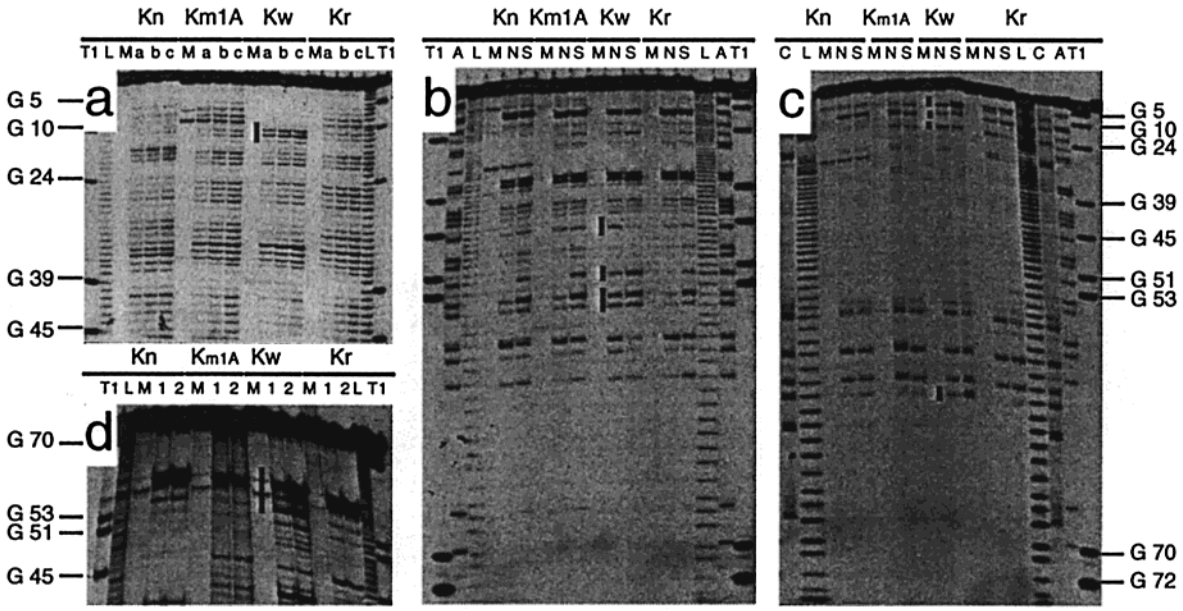

FIGURE 4: Structural probing of various $tRNA^{Lys}$ with chemical and enzymatic probes. Partial autoradiographies of experiments performed with 3′-end-labeled (a−c) and 5′-end-labeled (d) tRNAs. Probing with $Pb(OAc)_2$ (a), DEPC (b), DMS (c), and nuclease S1 (d). T1, G-ladder; C, C-ladder; A, A-ladder; L, alkaline ladder; M, control incubation in the absence of probe; a−c, 5, 10, or 15 min incubation; N, native conditions; S, semidenaturing conditions; 1, 2, 50 and 150 units of S1 nuclease. Numbering of nucleotides is given along both sides of Figure.

is an excellent phosphate donor for ligation by T4 RNA ligase despite its positive charge. Any amount of contamination by 9-mer−m^6A-OH which might have accumulated by Dimroth rearrangement *(31)* would be separated in this purification step due to the loss of the positive charge.

Synthesis of the Full-Length tRNAs Km^1A-Lg, Kwt-Lg, and Krw-Lg by Ligation of 9-mers to a 64-mer. The three different 9-mers (C1−A9, C1−C9, and C1−m^1A9-OH) have been individually ligated to the 64-mer corresponding to the 3′-part of $tRNA^{Lys}$, namely, residues G10 to A76, with T4 RNA polymerase. The 64-mer was obtained by conventional in vitro transcription of the corresponding synthetic gene in the presence of GMP, ensuring that the majority of the transcripts commences with a 5′-monophosphate, as required for the ligation reaction step. Preliminary ligation experiments showed that the 64-mer presenting a hydroxyl group at its 3′-end and a phosphate group at its 5′-end forms undesired oligomeric and cyclic byproducts which affect the yield of desired full-length tRNA. Therefore, the 3′-end ribose of the 64-mer was blocked by periodate oxidation.

To provide both RNA substrates to T4 RNA ligase in a reproducible well-defined structured state, the fragments were mixed and temperature-denatured, and annealed under defined optimized conditions. Only then was the ligation started by addition of buffer and enzyme.

Search for optimal conditions of the ligation step by T4 RNA ligase of the three different 9-mers to the 64-mer was performed with a 5′-end-labeled 64-mer and estimation of the percentage of radioactivity incorporated into full-length molecules. Optimal time and temperature for ligation were found to be 37 °C for 6 h. Incubation at lower temperatures for longer times (e.g., 4 °C, 24 h) leads to 50% lower yields whereas incubation at higher temperatures or during longer times resulted in significant degradation or did not yield any product (not shown). Buffer conditions *(33)* with 10% DMSO were found optimal (not shown). To test the effect of the base modification of the acceptor substrate (9-mer) on T4 RNA ligase activity, ligation efficiencies have been performed (i) under various ratios of 9-mer (phosphate acceptor) versus 64-mer (phosphate donor) substrates and (ii) with each of the three different 9-mers. Figure $3b_1$ illustrates the variation in the amount of full-length tRNA (73-mer) obtained at increasing concentrations of the 64-mer. A 4-fold excess of 64-mer leads to an incorporation of ∼55−70% of both nonmodified 9-mers into full-length tRNAs and of ∼40% of modified 9-mer. Figure $3b_2$ illustrates that an excess of 9-mer over 64-mer leads at most to incorporation of ∼30% of 64-mer when the nonmodified 9-mers C1−A9 or C1−C9 were used, and to ∼15% incorporation with the 9-mer possessing the methyl group (C1−m^1A9).

Next-Neighbor Analysis Confirms Correct Ligation. Proof for ligation between residues 9 and 10 of the three synthesized tRNAs is provided by next-neighbor analysis of residue G10. Ligations with 5′-labeled 64-mer, as discussed above, result in the presence of ^{32}P on the 3′-side of residue 9. As seen in autoradiograms of 2D-TLC plates of these digests presented in Figure 3c, complete digestion by RNase T2 of the ligated tRNAs resulted in 3′-monophosphate nucleotides including m^1A-3′[^{32}P] for Km^1A-Lg, and A-3′[^{32}P] and C-3′-[^{32}P] for Kwt-Lg and Krw-Lg, respectively. Rearrangement of m^1A to m^6A during the preparation of Km^1A-Lg has also been ruled out since no trace of radioactive m^6A-3′[^{32}P] could be detected on the chromatogram.

Solution Structure of Chimeric Human mt-$tRNA^{Lys}$. The basic aspects of structural chemical and enzymatic probing on human mitochondrial $tRNA^{Lys}$ and several of its in vitro transcribed derivatives have been previously described in much detail, with special emphasis on how the unusual secondary structure of transcript Kwt (directly transcribed from the corresponding synthetic gene by T7 RNA polymerase) was deduced *(20)*. Evidence pointing to m^1A9 as the modified base responsible for the rearrangement from

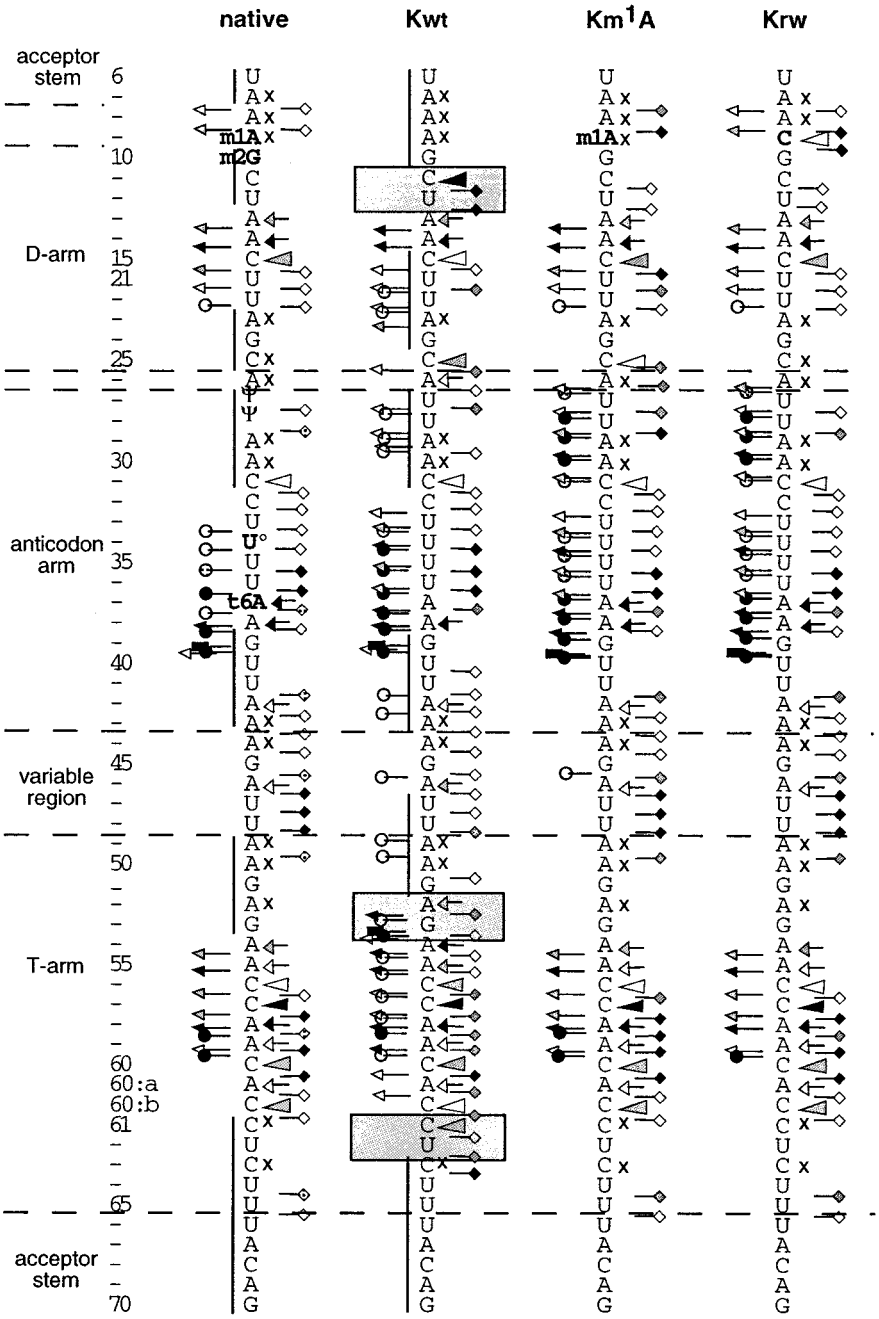

FIGURE 5: Reactivities of the various $tRNA^{Lys}$ derivatives toward chemical and enzymatic probes. For comparison, the RNA backbones are represented as straight lines. Numbering of nucleotides and also the structural domains of the cloverleaf-folded native tRNA are given at the left side of the figure. Only nucleotides 6−70 have been probed. Reactivities toward enzymatic probes (●, nuclease S1; ←, RNase T2; ■, RNase T1) are given on the left sides and reactivities toward chemical probes [→, DEPC; ▲, DMS; ◆, $Pb(OAc)_2$] on the right sides. The intensity of the shading (either white, gray, or black) is proportional to the intensity of cleavage. The crosses (x) delineate the absence of reactivity of adenines or cytidines toward DEPC and DMS, respectively. Major differences in reactivities within Kwt as compared to the other three molecules are squared. Heavy lines along the sequences of the native tRNA and Kwt correspond to base-paired regions in the already defined structures *(20)*.

the elongated hairpin structure of Kwt to the cloverleaf structure of native $tRNA^{Lys}$ came both from phylogenetic comparisons and from structural investigation of in vitro transcribed variants. These approaches suggested that the methyl group at nitrogen N^1 in A9 is crucial in hindering the formation of Watson−Crick interaction A9−U64, and as a consequence also the neighboring base-pairs A8−U65 and G10−C63, characteristic of the unusual structure of the transcript (Figure 1).

Here, structural probing experiments were conducted in parallel on four molecules, namely, (i) Km^1A possessing as single modified nucleotide the target methyl group on adenine at position 9; (ii) Kwt, the "naked" version of the tRNA, completely deprived of modified nucleotides; (iii) Krw, a variant of $tRNA^{Lys}$ deprived of modified nucleotides but mutated at position 9 (A9→C9) as to mimic the Watson−Crick base-pair-disrupting effect of m^1A9 *(20)*; and (iv) native fully modified $tRNA^{Lys}$ extracted from human mitochondria. As an internal control, it has been verified that the solution structures of nonmodified tRNA prepared either by ligation with T4 RNA ligase (Kwt-Lg) or by direct in vitro transcription (Kwt, Krw) or in vitro transcription as

transzymes (Kwt-Rz, Krw-Rz) are the same (results not shown). Accordingly, in what follows, names of the molecules have been shortcut to Kwt and Krw. Typical partial autoradiograms of probing experiments performed with lead acetate, DEPC, DMS, and nuclease S1 are shown in Figure 4. Reactivities toward all probes are summarized in Figure 5.

Since our previous analysis had shown that the most visible differences between a cloverleaf structure and the extended hairpin structure of the wild-type transcript are localized in the central domains of the tRNAs, comparison of the four different tRNAs analyzed will be mainly focused on the D- and T-domains and adjacent nucleotide stretches.

Comparison of Km^{1}A with Kwt Indicates an Important Structural Rearrangement Induced by a Single Methyl Group. The reactivities found for Km^{1}A allow us to clearly distinguish its structure from that of the unmodified RNA Kwt. The most striking differences are observed along nucleotides 52−62. Cleavages by single-strand-specific nucleases (S1, T1, T2) are restricted to a much shorter stretch of nucleotides (A54−C60) in Km^{1}A than in Kwt (A52−C60b). Moreover, G53, which is very sensitive to RNase T1 in Kwt, is totally protected in Km^{1}A. Reactivities toward chemical probes are also instructive. Thus, A52 and C61 are reactive toward DEPC and DMS, respectively, in Kwt but become substantially less reactive or unreactive in Km^{1}A. Finally, whereas all nucleotides of the domain (52−62) are reactive to lead acetate in Kwt, only a few central residues remain reactive in Km1A. In summary, the T-loop is of much shorter size in Km^{1}A than in Kwt, comprising only nucleotides 54−60b. Nucleotides 52, 53 and 61, 62 are involved in base-pairing.

Along the 5′-part of the molecules, probing of N3C with DMS shows a highly reactive C11 in Kwt and a marked decrease in Km^{1}A. Lead ion induced cleavages are found in Kwt at the level of phosphates 11 and 12, but not among phosphates 1 through 10. By contrast, Km^{1}A shows strong cleavage at position 8 (positions 7 and 9 are not assessed due to degradation in the control lane), but significantly less at positions 10, 11, and 12. These data are in good agreement with C1−G10 forming a continuous helix, followed by the single-stranded stretch C11−A14 in Kwt. In Km^{1}A, A8 and m^{1}A9 would be the single-stranded connector region and G10−U12 part of the D-stem helix. Finally, C25 and A26 are much less reactive in Km^{1}A than in Kwt. It can be concluded that the methyl group has greatly rearranged the structure, and that the reactivities are indicative of the existence of T and D stems and loops in Km^{1}A. Further structural interpretation is given below, in comparison with the native tRNA.

Comparison of Km^{1}A with Native tRNA^{Lys} Confirms Its Cloverleaf Structure. The reactivities observed for native tRNA^{Lys} represent a standard that permits evaluation of how far the structure of Km^{1}A approaches its functional state. The reactivities of both molecules in the T-domain and the variable loop are very similar. Both RNAs show virtually identical behaviors in the T-domain toward all applied structural probes. A stabilization of the T-domain by magnesium ions is suggested by the reduced reactivities of A52, -54, and -55 toward DEPC under native conditions as compared to semidenaturing conditions in both molecules.

Two outstanding differences are obvious, however. First, the native tRNA is more resistant to RNases, and higher concentrations of the latter have to be employed in the probing experiments. This indicates, as observed in numerous other cases, an overall stabilization of the tRNA structure by the ensemble of its modified bases (reviewed in ref *34)*. Second, RNase cleavages of nucleotides 28−30, found in Kwt, Krw, and Km^{1}A, are absent in the native tRNA. Knowledge of the full set of modified nucleotides present in native tRNA^{Lys} *(20)* obviously points to a structural influence of two pseudouridines, namely, Ψ27 and Ψ28 in this domain. Pseudouridines are indeed known to stabilize RNA structure (reviewed in refs *16* and *35*). On their 5′-ends, the reactivities of both molecules are mostly the same for all tested nucleotides, especially for residue C11 which is nonreactive toward DMS. It is concluded that Km^{1}A has the same structure as the native tRNA.

Comparison of Km^{1}A with the Unmodified Variant Krw, Mimicking the Effect of m^{1}A9. As shown previously *(20)*, Krw (the homologue of Krw-Rz prepared by direct in vitro transcription), carrying mutation A9→C, folds into a cloverleaf. Indeed, the fact that a tRNA^{Lys} with a m^{1}A9 would not have the possibility to form a Watson−Crick pair with residue 64 had inspired us to design variants neither of which could possibly form the Watson−Crick interaction 9−64.

Nucleases, which map global domains rather than fine structural details, cleave both RNAs in virtually the same manner. To be mentioned are cleavages at G45 by RNase T1 of equally weak intensity, which are absent in the native tRNA. Thus, the variable loop structure in native tRNA seems to be stabilized by modified bases other than m^{1}A9, likely m^{2}G10 and ψ27 and ψ28. Probing with DMS and DEPC did not show any differences between Km^{1}A and Krw either, apart from N3 in C9 weakly reactive only in Krw (where a C is present). The moderate methylation of this residue by the chemical probe indicates that, while buried in the core of the molecule, it is not involved in any (undesired) Watson−Crick interaction, for example, with G45. Slight differences are seen in the lead ion-induced hydrolysis patterns between both tRNAs. However, these are restricted to the vicinity of position 9 and are difficult to interpret, due to the particular migration properties of RNA cleavage fragments containing m^{1}A and to signals in the control lane in this region. In conclusion, our structural probing approach shows that Km^{1}A and Krw have the same structural characteristics, demonstrating that the methyl group hinders base-pairing. However, instead of merely destabilizing the extended structure of Kwt, m^{1}A9 might also stabilize the cloverleaf by additional interaction, e.g., with phosphates in the variable loop, possibly enabled by its positive charge. If any residues elsewhere but near position 9 were found less reactive in Km^{1}A than in Krw, that would be indicative of an additional interaction/stabilization of the cloverleaf by m^{1}A9. The probing data did not allow us to identify any structural reinforcement by m^{1}A. Nucleotide 9 in tRNAs is usually involved in a tertiary interaction with residue 23 which is itself base-paired with residue 12. Interestingly, in a structural model of bovine mt-tRNA^{Phe}, m^{1}A9 is shown in tertiary interaction with A23 and U12 *(36)*. This is likely also the case in tRNA^{Lys}.

DISCUSSION

Strength and Limitations of Synthetic Methods for tRNA Production. Different approaches developed for synthesis of site selectively modified RNAs (reviewed in ref *37*) are derived either from "cut-and-paste" techniques or from phosphoamidite chemistry, or a combination of both. Whereas chemical RNA synthesis is straightforward and whereas a number of synthons allowing incorporation of modified nucleotides have already been prepared and are even commercially available, the special properties of m^1A render the synthesis of the corresponding phosphoramidite derivative rather difficult. Indeed, m^1A has not only a methyl group at position N1 but also a positive charge. The positive charge renders this modified base susceptible to rearrangement to m^6A under alkaline conditions (Dimroth rearrangement). This property prevents the use of conventional protective groups in phosphoramidite synthesis which have to be released by ammonia treatment.

Introduction of the modified base via ligation of its 3′-5′-mononucleoside bisphosphate derivative between a synthetic 8-mer corresponding to the 5′-end of the tRNA and an in vitro transcribed 64-mer corresponding to the 3′-part of the tRNA comprises some potential limitations as well. The approach, introduced by the Uhlenbeck group *(29, 33)*, has already been successfully employed for the incorporation of N^1-methylguanosine into the anticodon of a chimeric tRNA *(38)*, synthesis of mt-tRNAs *(39)*, and also synthesis of tRNAs based on ligation of fragments within the D-loop *(40)* or in the T-loop *(41)*. Limitations of this approach, in the special case of interest here, were expected (i) from the presence of a positive charge in the mononucleoside bisphosphate, pm^1Ap, which may interfere or hinder recognition by T4 RNA ligase, and (ii) from the location of the target-modified nucleoside in the core of the tRNA (position 9), a domain sterically encumbered and thus unfavorable for bringing close together the phosphate and hydroxyl groups involved in the final ligation step. This is at variance with the so-far performed ligations which took place in tRNA anticodon loops where the reactive extremities are easily brought close to each other and readily accessible to the ligase. The use of a DNA oligomer complementary to both RNA molecules to be ligated *(42)* in order to approach both reaction partners and melt their secondary structures was expected to be rather inefficient here due to the mismatch to be introduced by the presence of a methyl group at a Watson−Crick position. Our data show that T4 RNA ligase is able to efficiently use m^1A derivatives as substrates. Not only did the phosphorylated pm^1Ap derivative prove to be an excellent phosphate donor, but also did the 9-mer possessing a m^1A at its 5′-end act as a phosphate acceptor. However, this acceptor activity is substantially diminished (by about 50%) when compared to that of 9-mers with adenine or cytidine at their 5′-ends. Thus, the range of tested substrates for T4 RNA ligase, already including modified nucleotides *(29)*, can be extended to positively charged adenosine. The likely difficulties in catalyzing the formation of a phosphodiester bond in an encumbered position within a tRNA, namely, between residues 9 and 10, could also be overcome by adequate denaturing of both the short and the long RNA fragments.

Role of m^1A9 in mt-$tRNA^{Lys}$. Comparative structural analysis of the chimeric $tRNA^{Lys}$ Km^1A, Krw, and native $tRNA^{Lys}$ revealed no conformational differences at the level of the reactivities of residues 1−26 and 45−76. However, whereas these molecules share common reactivities, and thus common folding of their T, D, and acceptor stem domains, Kwt behaves differently. Some differences in reactivity of nucleotides of the anticodon stem in the fully modified native tRNA, relative to the other tRNAs, are likely not related to drastic structural changes in this part of the tRNA but to a stabilizing effect of Ψ27 and Ψ28 within the anticodon stem. In summary, our data demonstrate that m^1A9 is responsible for the correct structure, i.e., the cloverleaf structure, of human mt-$tRNA^{Lys}$. This argues in favor of a "chaperone" role for the posttranscriptional modification enzyme methyl-1 adenosine transferase. Chaperones are well-known as proteins which trigger correct folding of other proteins (e.g., *43*). This concept already found its significance for RNAs where a protein can help the establishment of a correct RNA folding pathway *(44, 45)*. Whereas it is not known if the m^1A methylase recognizes the transcript before its folding into the hairpin structure, or if it possesses an unwinding function that permits the enzymatic methylation to occur once the full-length primary transcript is synthesized, the chemical group introduced by an epigenetic mechanism would fix the induced structure.

Roles of m^1A in mt-tRNAs. Methyl-1 adenosine is frequently found in canonical tRNAs at position 58. A reverse Hoogsteen base-pair of residue 58 is formed with T54, as found in the crystal structures of yeast $tRNA^{Asp}$ (unmodified A58) and $tRNA^{Phe}$ (m^1A58). The positive charge induced by the methylation is thought to reinforce the hydrogen bonding, hence its frequent occurrence in thermophilic bacteria *(15)*. With a single exception, m^1A at position 9 is exclusively found in mitochondrial tRNAs, where it is very frequent *(15)*. In the particular case of human mitochondrial $tRNA^{Lys}$, m^1A9 prevents base-pairing between A9 and U64 and, as a consequence, also prevents pairing between residues 8 and 65 and between residues 10 and 63. Base-pairing between residues 8−10 and 65−63 is also possible in the corresponding unmodified $tRNA^{Lys}$ from humanoids *(15)*, so that a similar structural role can be anticipated for m^1A9, if present.

All other mammalian mt-$tRNA^{Lys}$ do not have a sequence complementarity between residues 8−10 and 65−63. However, those which have been sequenced at the tRNA level and have an A at position 9 present an m^1A9 residue. This in turn means that in *these* tRNAs, the main function of m^1A9 is not to guide the secondary structure toward a cloverleaf. The actual general function of this posttranscriptional modification is not known but is expected to be of importance due to its high conservation and to the limited number of modified nucleotides in mt-tRNAs, which thus may be rather important.

The tRNA gene sequences are the products of special evolutionary pressure in animal mitochondria and their high genomic drift. In particular, the lack of recombination has been held responsible for peculiarities of both functional and structural nature (reviewed in ref *17*). Thus likely, any information, i.e., any nucleotide of the gene kept by evolution, is of importance. In some instances, the remaining information is so much condensed that it is partially shared

by two adjacent genes *(46)* or by two overlapping genes *(47)*. The functional state of the corresponding transcription products can only be reached through editing mechanisms. We suggest that the particular role of m^1A9 in mitochondrial $tRNA^{Lys}$ is a further example suggesting that posttranscriptional modifications are important epigenetic events necessary to complete the tight genetic information of the mitochondrial genome.

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