Aminoacylation properties of pathology-related human mitochondrial tRNA^{Lys} variants

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ABSTRACT

In vitro transcription has proven to be a successful tool for preparation of functional RNAs, especially in the tRNA field, in which, despite the absence of post-transcriptional modifications, transcripts are correctly folded and functionally active. Human mitochondrial (mt) tRNA^{Lys} deviates from this principle and folds into various inactive conformations, due to the absence of the post-transcriptional modification m^{1}A9 which hinders base-pairing with U64 in the native tRNA. Unavailability of a functional transcript is a serious drawback for structure/function investigations as well as in deciphering the molecular mechanisms by which point mutations in the mt tRNA^{Lys} gene cause severe human disorders. Here, we show that an engineered in vitro transcribed "pseudo-WT" tRNA^{Lys} variant is efficiently recognized by lysyl-tRNA synthetase and can substitute for the WT tRNA as a valuable reference molecule. This has been exploited in a systematic analysis of the effects on aminoacylation of nine pathology-related mutations described so far. The sole mutation located in a loop of the tRNA secondary structure, A8344G, does not affect aminoacylation efficiency. Out of eight mutations located in helical domains converting canonical Watson–Crick pairs into G–U pairs or C•A mismatches, six have no effect on aminoacylation (A8296G, U8316C, G8342A, U8356C, U8362G, G8363A), and two lead to drastic decreases (5000- to 7000-fold) in lysylation efficiencies (G8313A and G8328A). This screening, allowing for analysis of the primary impact level of all mutations affecting one tRNA under comparable conditions, indicates distinct molecular origins for different disorders.

Keywords: in vitro transcripts; tRNA; aminoacylation; human mitochondrial; MERRF; identity; post-transcriptional modification

INTRODUCTION

Specific aminoacylation of transfer RNAs (tRNA) by their cognate aminoacyl-tRNA synthetases (aaRS) is a key step in translation of genetic information. Rules governing the recognition process between the partner macromolecules have been well studied for a large number of prokaryotic and cytosolic eukaryotic systems (for review, see Giegé et al. 1998b; McClain 1993a, 1995; Saks et al. 1994; Giegé and Frugier 2003). On the other hand, far less is known for mitochondrial (mt) tRNAs and aaRSs, especially from human and mammals in general (Florentz and Sissler 2003). This is a drawback not only in the frame of fundamental knowledge about these mt aminoacylation systems but also in regard to understanding molecular mechanisms underlying numerous human disorders. Over the past 15 years, an increasing number of point mutations within human mt tRNA genes have been correlated with severe neurodegenerative and/or muscular disorders (e.g., Larsson and Clayton 1995; Schon et al. 1997; Wallace 1999; DiMauro and Andreu 2000; Florentz and Sissler 2001). More than 90 point mutations have been reported so far, located in 21 of the 22 mt tRNA genes (e.g., Kogelnik et al. 1998; Servidei 2002). A large majority of these mutations lead to a decrease in the rate of mitochondrial protein synthesis. However, the detailed molecular level at which each mutation interferes with the tRNA lifecycle remains mostly unknown. tRNA aminoacylation being the key step of protein biosynthesis, a systematic investigation of the effects of point mutations in tRNAs on this process would allow for a straightforward comparison.

As opposed to studies on classical tRNAs, investigation of human mt tRNA aminoacylation systems suffers from a series of limitations linked to intrinsic properties of mitochondria. Their heteroplasmic status, that is, the presence of

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Abbreviations: tRNA, transfer RNA; aaRS, aminoacyl-tRNA synthetase; mt, mitochondrial; DEPC, diethylpyrocarbonate; WT, wild-type.

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both WT and mutant DNA in the same cell, requires the creation of individual *trans*-mitochondrial cell-lines for in vivo investigation of each mutation (King and Attardi 1989). Although such cells allowed for evaluation of steady-state levels of aminoacylation (for review, see by Enriquez and Attardi 1996; Florentz and Sissler 2003), their creation is difficult so that only a handful of mutations could be analyzed in such a way so far. Moreover, the variety of cell types used as a basis for creation of cybrids (e.g., HeLa, osteosarcoma, lymphoblasts) does not allow for straightforward comparisons, due to variations in nuclear backgrounds (Jacobs and Holt 2000). Ex vivo analyses, based on extraction of a specific tRNA from cultured cells or from biopsies, are limited by the quantities of accessible material. This has only been reported in two cases so far (Yasukawa et al. 2001; Park et al. 2003). The alternative approach consists of producing tRNA through in vitro transcription of cloned WT and mutated human mt tRNA genes as has been the case for cohorts of canonical tRNAs (Giegé et al. 1998b; Giegé and Frugier 2003). Such an approach, based on tRNAs deprived of post-transcriptional modifications, has been validated over the past 20 years for a vast majority of aminoacylation systems from various organisms and allowed, for example, the deciphering of aminoacylation identity elements in specific tRNAs. Interestingly, the in vitro approach leads to similar results to those obtained by in vivo methods (for review, see McClain 1993b; Giegé et al. 1998b).

In regard of human mt tRNAs, the approach based on the in vitro synthesis of transcripts was found to be efficient and has already been explored in the case of isoleucine-specific (Degoul et al. 1998; Kelley et al. 2000, 2001) and serine-specific (Shimada et al. 2001) tRNAs but lead to a number of difficulties for other mt tRNAs. Indeed, at least those transcripts specific for lysine (Helm et al. 1998; Tolkunova et al. 2000) and phenylalanine (Bullard et al. 1999) turned out to be unsatisfactory substrates for aaRSs due to unfavorable folding of the RNAs. In the case of $tRNA^{Leu(UUR)}$, aminoacylation is possible despite structural flexibility of the transcript compared with native tRNA (Park et al. 2003; Sohm et al. 2003; Wittenhagen et al. 2003). Because in vitro transcription remains the sole conceivable straightforward approach to access human mt tRNAs (wild type and mutants) and to perform rigorous systematic comparative analysis of tRNA variants for a given function (e.g., maturation, aminoacylation), functional in vitro transcripts need to be engineered.

Here, we consider the specific case of human mt $tRNA^{Lys}$. Previous work showed that an in vitro transcript of this tRNA does not fold into a functional cloverleaf but into alternative extended bulged hairpins (Fig. 1A; Helm et al. 1998), and is poorly aminoacylated in the presence of a human mt crude enzymatic extract (Helm et al. 1998) or of an overexpressed and purified mt LysRS (Tolkunova et al. 2000). The requirement of a single post-transcriptional modification for cloverleaf folding of $tRNA^{Lys}$ was confirmed by synthesis and structural analysis of a chimeric $tRNA^{Lys}$, which displays a unique modified base, a methylated adenosine at position 9 (Fig. 1B; Helm et al. 1999b). This modification impairs the formation of a Watson–Crick base pair between nucleotides 9 and 64 responsible for the extension of the amino acid acceptor stem within the transcript. Interestingly, the structural chaperone effect of m^1A9 modification can be simulated by mutating either nucleotide 9 or nucleotide 64 (provided base-pairing between residues 64–50 is conserved; Helm et al. 1998). Such variants, carrying an altered base pair at position 50–64 replacing A50–U64 by U50–A64 (***Ki***) or by G50–C64 (***Ke***), adopt the expected cloverleaf folding, as demonstrated by enzymatic and chemical probing (Fig. 1C; Helm et al. 1998).

Here, it is shown that these engineered variants are active substrates for LysRS and that they represent valuable reference molecules for further investigation of $tRNA^{Lys}$ nucleotides important for aminoacylation. The lysylation system has further been explored in a systematic investigation of the full set of mutations described so far for the human mt $tRNA^{Lys}$ gene, a hot spot for pathology-related mutations. Results are discussed both in the frame of molecular mechanisms involved in the expression of the diseases, and in the frame of understanding lysylation rules in human mitochondria.

RESULTS

Setting up an efficient lysylation system based on in vitro transcripts

The in vitro transcribed structural variants ***Ke*** and ***Ki*** of human mt $tRNA^{Lys}$ (Fig. 1C) were previously shown to adopt the expected cloverleaf secondary structure (Helm et al. 1998). As the presence of a C-residue at the first position of $tRNA^{Lys}$ highly limits yields of in vitro transcription, a G being most favorable for T7 RNA polymerase efficiency (Sampson and Uhlenbeck 1988), new synthetic genes have been cloned. They consist of the T7 promoter followed by a hammerhead ribozyme (Rz; Fechter et al. 1998) and the tRNA gene, leading to wild-type (***Rz-Kwt***) and "pseudo-WT" (***Rz-Ke*** and ***Rz-Ki***) transcripts. These transcripts were tested for their lysylation properties compared with those of human mt native WT $tRNA^{Lys}$ (extracted from cybrid cell lines, see Experimental Procedures). Because it is laborious to obtain native tRNAs in the large amount required for determination of kinetic parameters, only charging levels have been established for this initial investigation (Fig. 2). The aminoacylation assays were performed in the presence of human cytosolic lysyl-tRNA synthetase. Both cytosolic and mt enzymes are encoded by the same nuclear gene and differ in their mature versions only by 21 to 49 (over 601) amino acids at their N terminus (Tolkunova et al. 2000).

Although ***Rz-Kwt***, the transcript which folds into ex-

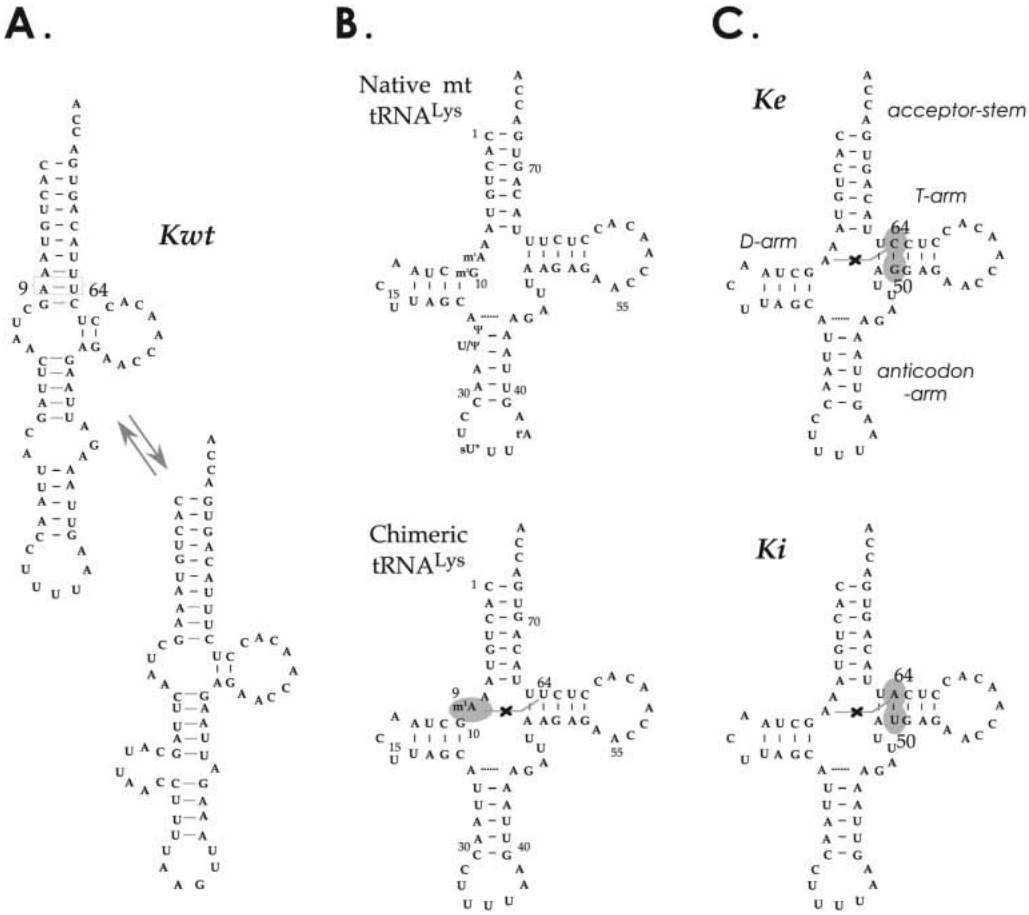

FIGURE 1. Human mitochondrial wild-type and pseudo-WT tRNA^{Lys} transcripts. (*A*) Determined secondary structures of human mt wild-type tRNA^{Lys} transcript (***Kwt***; Helm et al. 1998). (*B*) Experimental secondary cloverleaf structures of native mt tRNA^{Lys} (displaying the six post-transcriptional modifications, abbreviated in the conventional way; Sprinzl et al. 1998) and of a chimeric tRNA^{Lys} methylated at position N1 of A9 as sole post-transcriptional modification (Helm et al. 1999b). (*C*) Pseudo-WT in vitro transcribed tRNA^{Lys} derivatives ***Ke*** and ***Ki***. tRNA domains are labeled for ***Ke***. Structural mutations introduced at positions 50 and 64 (indicated by gray circles) prevent alternate folding into extended hairpin (black crosses; Helm et al. 1998).

tended hairpin structures, is poorly aminoacylated, ***Rz-Ke*** (with A50–U64 replaced by G50–C64) and ***Rz-Ki*** (with A50–U64 replaced by U50–A64) become active substrates for human LysRS with 42% ± 2% charging levels, an activity close to the 57 ± 2% measured for native mt WT tRNA^{Lys}. ***Rz-Ke*** and ***Rz-Ki*** display identical functional properties, demonstrating that the residues paired at positions 50 and 64 do not influence recognition by LysRS.

Effect of MERRF mutation A8344G on aminoacylation levels and on tRNA^{Lys} structure

Among the 13 pathology-related mutations described within the mt tRNA^{Lys} gene, transition A8344G,[2] correlated with the MERRF syndrome (Shoffner et al. 1990; Servidei 2002), is the most prevalent mutation. This mutation, located in the T-loop of the cloverleaf-folded tRNA at position 55 (conventional tRNA numbering, see Fig. 5 below), has been introduced into ***Rz-Ke*** and ***Rz-Ki***, leading to ***Rz-Kem*** and ***Rz-Kim***. Aminoacylation properties as well as structural properties have been investigated for these variants. Interestingly, both ***Rz-Kem*** and ***Rz-Kim*** are aminoacylated to levels similar to the corresponding pseudo-WT transcripts (Fig. 3). Under optimal LysRS concentrations, all four transcripts are charged up to 42 ± 2% (Fig. 3).

The aminoacylation properties of native human mt tRNA^{Lys}, either wild type or with the MERRF mutation, have been established. tRNAs have been extracted from a specific couple of cybrid cell lines (R2–1A and R1–C3, respectively) by hybridization to a complementary biotinylated oligonucleotide. The yield of pure native tRNA^{Lys} species is extremely low (0.3 μg of pure wild type or MERRF-mutated tRNAs were extracted from ~1 or ~1.3 L of corresponding adherent cell cultures, respectively), so that only charging

[2]Numbering is according to location in the mitochondrial genome; Anderson et al. 1981.

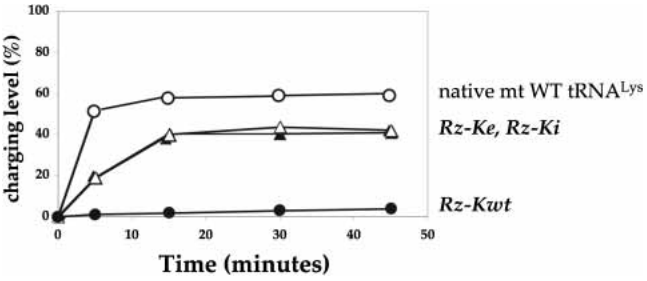

FIGURE 2. From inactive to active in vitro transcribed human mt $tRNA^{Lys}$. Charging levels, determined with human LysRS at 37°C, are expressed as percentages of charged tRNA versus total amount of specific tRNA per experiment. ***Rz-Kwt***, ***Rz-Ke***, and ***Rz-Ki*** correspond to in vitro transcribed wild-type and structural variants of $tRNA^{Lys}$, respectively, purified to single nucleotide resolution on polyacrylamide/urea gels. Purified native wild-type mt $tRNA^{Lys}$ (by hybridization to a specific biotinylated oligonucleotide) form the positive control.

levels could be established. Figure 3 shows that both native $tRNA^{Lys}$s are lysylated to about the same level (57 ± 2%). Thus, as is the case for transcripts, no substantial effect of the MERRF mutation is observed on aminoacylation of native tRNAs.

Possible structural effects of MERRF mutation have been sought. End-labeled transcripts were submitted to chemical probing with DEPC and $Pb(OAc)_2$, as previously performed for ***Kwt***, ***Ke***, and ***Ki*** (Helm et al. 1998). Autoradiographies of comparative structural probing experiments on ***Ke***, ***Ki***, ***Kem***, and ***Kim*** are displayed in Figure 4A. Cleavages were categorized weak (white), moderate (gray), or strong (black), and the results for individual nucleotides are summarized in Figure 4B. The reactivities to probes of all four transcripts were virtually identical. The probes failed to detect any significant difference between the pseudo-WT analogs of mt $tRNA^{Lys}$ on one hand (***Ki*** and ***Ke***), and the "pseudo-MERRF" analogs on the other hand (***Kim*** and ***Kem***).

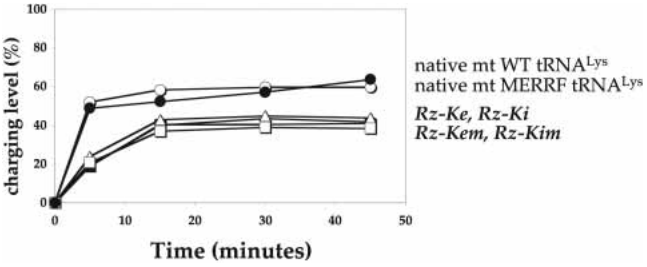

FIGURE 3. Effect of MERRF (A8344G) mutation on aminoacylation. Charging levels of wild type or pseudo-WT (***Rz-Ke*** and ***Rz-Ki***) versus MERRF or pseudo-MERRF (***Rz-Kem*** and ***Rz-Kim***) tRNAs. Native wild-type and MERRF-mutated mt $tRNA^{Lys}$ were extracted from mitochondria of R2–1A and R1–C3 cybrid cells (see Experimental Procedures) using biotinylated oligonucleotides complementary to the 3′ part of the tRNA. ***Rz***- stands for self-cleaved in vitro transcribed tRNAs.

Systematic mutagenic analysis

So far, 10 mutations in the human mt $tRNA^{Lys}$ gene have been reported to be related to pathologies such as muscular disorders, encephalopathies, deafness, ophthalmoplegia, or diabetes (Kogelnik et al. 1998; Servidei 2002). A8348G, a polymorphism unrelated to disorders, was also described (Ingman et al. 2000). These 11 mutations have been individually introduced into the pseudo-wild-type ***Rz-Ki*** sequence (Fig. 5). Only the variant with transition T8355C could not be prepared because the mutation overlaps with the ***Rz-Ki*** structural mutation. An additional transcript has been constructed with a sequence modification in the anticodon central position (T8324A, or U35A in classical tRNA nomenclature). This mutation was hypothesized to have a strongly deleterious effect on the lysylation reaction because nucleotides U35 and U36 from the anticodon triplet are known as major lysine specific identity elements in canonical lysylation systems (e.g., in *Escherichia coli* [McClain et al. 1990; Normanly et al. 1990; Tamura et al. 1992] and human cytosolic $tRNA^{Lys}$ [Stello et al. 1999]). Three mutations are located in loops (MERRF mutation A8344G, polymorphism A8348G, and the rationally designed mutation in the anticodon triplet). All other mutations are located in stems.

All transcripts have been tested for their ability to be lysylated. Kinetic parameters are presented in Table 1 (right part). As anticipated, the polymorphic mutation (A8348G) in the T-loop has no significant effect on aminoacylation, with a charging level of 60% (data not shown) and a negligible loss in aminoacylation efficiency. Mutation within the anticodon triplet, replacing U35 by A35, lead to a drastic decrease in lysylation efficiency (~6000-fold). These two variants can thus be considered as positive and negative controls in the lysylation reaction. The nine pathology-related mutants (including the MERRF-related A8344G mutation discussed above) have aminoacylation properties ranking between these substrates. For six of them (A8296G, T8316C, G8342A, A8344G, T8356C, and G8363A), aminoacylation is not significantly impaired by the mutation, with charging levels between 20% and 50% (data not shown) and, at most, a fourfold loss of catalytic efficiency. A small (2.8-fold) gain in efficiency is observed for the MERRF-related mutant A8344G (gain = 1/L). Variant T8362G shows a 10-fold decrease in efficiency. The two remaining variants, G8313A and G8328A, drastically impair lysylation. Charging levels are <1% (data not shown), and losses in catalytic efficiencies reach 4900- and 7000-fold. This poor charging did not allow for direct establishment of individual kinetic parameters k_{cat} and K_M for these two variants.

Mutations with low effects on aminoacylation are mainly located in the acceptor- and T-stems of the tRNA and at the upper part of the anticodon stem (Table 1, left). These mutations can be segregated into two categories from a mechanistic point of view because some have unaffected

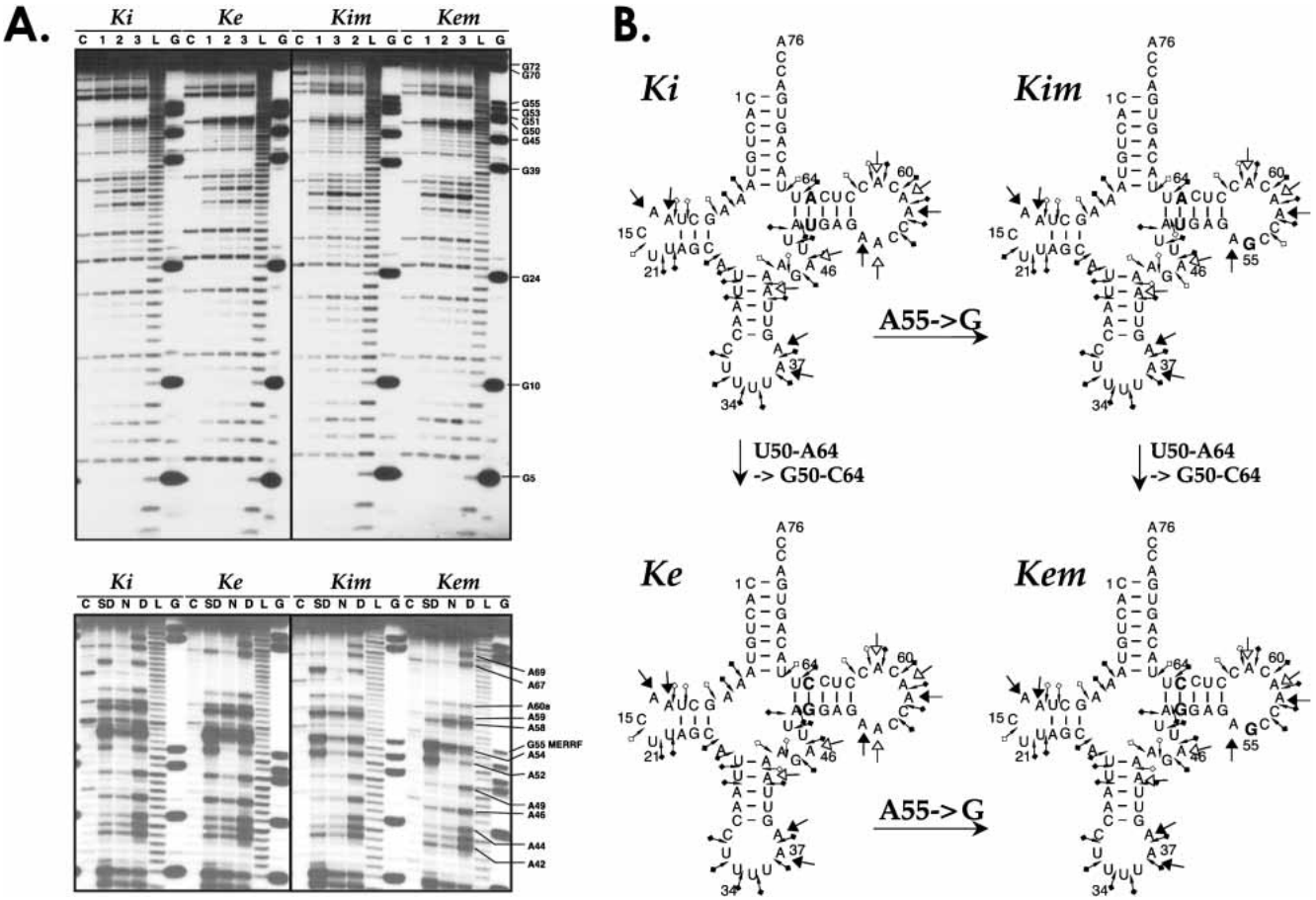

FIGURE 4. Effect of MERRF mutation on the structure of in vitro transcribed tRNAs. (*A*) Autoradiographs of 15% denaturing polyacrylamide gels of chemical probing experiments. G indicates RNase T1 ladder; L, alkaline ladder; and C, control incubation without probe. (*Top*) Structural probing experiments with lead acetate ($Pb(OAc)_2$). 5′-Labeled tRNAs are incubated at 25°C in 20 µL of 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 40 mM NaCl, and 0.1 µg/µL unlabeled tRNA without (C) or with 0.15 mM $Pb(OAc)_2$ for 10 min (1), 20 min (2), or 30 min (3). (*Bottom*) Chemical probing with diethylpyrocarbonate (DEPC). N, SD, and D stand for native, semi-denaturating, and denaturating conditions, respectively. 5′-Labeled tRNAs are incubated for 40 min without (C) or with 5% (v/v) DEPC (N, SD, D) in 50 mM sodium cacodylate with 10 mM $MgCl_2$ and 300 mM KCl (C, N) or with 1 mM EDTA (SD, D). Incubation was performed for 4 min at 60°C for denaturating condition (**D**). *(B)* Chemical probing patterns on cloverleaf structures of pseudo-WT (***Ke*** and ***Ki***) and pseudo-MERRF (***Kem*** and ***Kim***) transcripts. Structural mutations (at position 50–64) and MERRF mutation (at position 55) are highlighted in bold. *Arrowheads* correspond to positions cleaved by probes ($Pb(OAc)_2$ and DEPC). Cleavages are categorized weak (white), modest (gray), or strong (black).

kinetic parameters k_{cat} and K_M (A8296G, G8342A, A8344G, G8363A), whereas others have both a decrease in k_{cat} and in K_M (fourfold to 10-fold), leading also to unaffected aminoacylation efficiencies (T8316C, T8356C). Interestingly, both mutations A8296G and T8362G concern the same base pair but lead to low but significant differences in effects on aminoacylation. Mutation A8296G, converting the A2–U71 pair into G2–U71 has no effect, whereas mutation T8362G, leading to an A2•G71 mismatch, leads to a 10-fold loss in lysylation efficiency. This shows that LysRS is sensitive to some extent to the structural integrity of base pair 2–71 rather than to the nature of the nucleotides present at these positions. Mutations with large negative effects are located within the anticodon domain and within the D-stem (Table 1, left).

DISCUSSION

From inactive to active in vitro transcribed human mitochondrial tRNA^{Lys}

In the present work, in vitro transcription was used to produce active molecules despite the absence of post-transcriptional modification required to produce the canonical secondary structure. In the specific case of tRNA^{Lys}, the extremely low aminoacylation capacity of the wild-type in vitro transcript has been overcome by molecular engineering, leading to an efficient substrate for human LysRS. Exchange of a single Watson–Crick pair by an alternative Watson–Crick pair within the tRNA T-stem simulates the structural chaperone effect of post-transcriptional modification m^1A9, favoring the cloverleaf structure (Helm et al. 1999b).

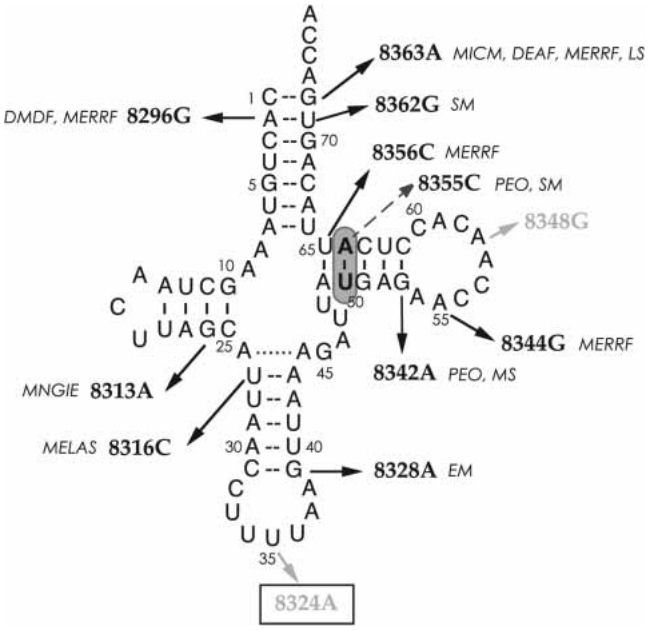

FIGURE 5. Investigated mutations in human mitochondrial $tRNA^{Lys}$. Individual variants were prepared as derivatives of ***Rz-Ki***, the pseudo-WT $tRNA^{Lys}$ with U50–A64 pair instead of A50–U64 pair. The 10 reported pathological mutations (http://www.mitomap.org/) are indicated in black; the dashed arrow represents the one not investigated because it overlaps the structural mutations. Control molecules, indicated in gray, are either the polymorphic A8348G mutation (positive control) or the U8324A sequence variation (which alters a major lysine identity element, negative control). Numbering is either according to the mt genome location (bold characters, for mutations; Anderson et al. 1981) or according to the conventional tRNA annotation (small numbers; Sprinzl et al. 1998). Related pathologies are abbreviated as follows: DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DMDF, diabetes mellitus and deafness; EM, encephalomyopathy; LS, Leigh syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes; MERRF, myoclonic epilepsy and ragged red fibers; MICM, maternal inherited cardiomyopathy; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; MS, myoclonic seizures; PEO, progressive external ophthalmoplegia; and SM, skeletal myopathy.

Two pseudo-WT ribozyme-derived transcripts, ***Rz-Ki*** and ***Rz-Ke***, with structural mutations A50–U64 → U50–A64 or A50–U64 → G50–C64, respectively, become lysylated with aminoacylation capacities significantly increased compared with WT $tRNA^{Lys}$ transcript ***Rz-Kwt***, close to those of native $tRNA^{Lys}$ extracted from mitochondria. As the two base pair exchanges lead to identical functional effects, and the corresponding nucleotide positions have not been shown in $tRNA^{Lys}$ from other species to be involved in LysRS recognition and aminoacylation efficiency, both ***Rz-Ki*** and ***Rz-Ke*** can be used as reference molecules for further functional exploration of $tRNA^{Lys}$.

This has been further confirmed in the case of ***Rz-Ki*** by the aminoacylation properties of four variants, namely, those with the polymorphism A8348G, the rationally designed mutation in the anticodon triplet, and the two pathology-related mutations A8344G and G8313A. Polymorphic mutations have been discovered by comparing mt DNA sequences among large human populations and have been explored to reconstruct human prehistory and population movements (e.g., Allen and Raven 1996; Ingman et al. 2000). Such mutations are assumed to be neutral in molecular and phenotype effects. We show here that this is indeed the case for mutation A8348G in regard to aminoacylation properties of the corresponding ***Rz-Ki*** derivative. Mutating the anticodon triplet was expected to have a strong negative effect on lysylation. Although aminoacylation identity elements for the human mt lysine system were not explored so far, it could be foreseen by analogy with known lysylation systems (McClain et al. 1990; Tamura et al. 1992; Stello et al. 1999) and on the basis of the general concept that major identity elements are conserved among species (Giegé et al. 1998b; Giegé and Frugier 2003), that mutating the central residue of the anticodon triplet would be detrimental to aminoacylation. Indeed, replacing U35 by A35 in the ***Rz-Ki*** framework leads to a ~6000-fold loss in aminoacylation efficiency, demonstrating that the initial replacement of A50–U64 by U50–A64 does not interfere with the expression of identity elements in the tRNA scaffold. Finally, two pathology-related mutations within $tRNA^{Lys}$ have already been investigated for their impact on lysylation by approaches not including in vitro transcribed tRNAs. The impact of MERRF mutation A8344G has been tested by an in vitro approach based on purification of low amounts of native WT $tRNA^{Lys}$ and $tRNA^{Lys}$ with mutation A8344G from large-scale cultures of HeLa-derived cybrid cells (Yasukawa et al. 2001). Kinetic parameters for lysylation of these natural, post-transcriptionally modified molecules revealed a threefold increase in aminoacylation efficiency for the mutant tRNA compared with wild type. This is in line with a 2.8-fold increase observed in the present work on the ***Rz-Ki*** derivative with mutation A8344G compared with ***Rz-Ki*** and with unchanged aminoacylation plateau levels for native $tRNA^{Lys}$ species extracted from osteosarcoma-derived cybrid cell lines. Further, in vivo measurements of aminoacylation levels of mutated $tRNA^{Lys}$ have been performed both for mutation A8344G (Enriquez et al. 1995; Börner et al. 2000) and G8313A (Bacman et al. 2003). Although in the first case, variable results were obtained according to the cell type used (decrease in aminoacylation in osteosarcoma-derived cybrid cell-lines, no effect in biopsies), a strikingly negative effect of mutation G8313A has been reported in cybrid cell-lines. This result is in line with the 7000-fold loss in lysylation observed in the present work for ***Rz-Ki*** derivative bearing second site mutation G8313A. Based on these results, we conclude that the in vitro transcribed derivative of $tRNA^{Lys}$ ***Rz-Ki*** can indeed be considered as a pseudo-wild-type molecule, relevant for further mutagenic analyses.

Variable impacts of pathology-related mutations on lysylation

Ten mt $tRNA^{Lys}$ gene mutations have been reported to be associated with phenotypically wide-ranging human disor-

TABLE 1. Aminoacylation properties of in vitro transcribed pseudo-WT and variant human mt tRNA^{Lys} in the presence of human LysRS

In vitro transcripts	tRNA position	Wild-type sequence	Mutated sequence	k_{cat} (10^{-3} sec^{-1})	K_M (μM)	k_{cat}/K_M (10^{-6} $sec^{-1} \cdot nM^{-1}$)	Loss
Reference molecule							
Rz-Ki	50–64 (TS)	A–U	U·A	12	4.0	3.0	1
Polymorphism							
A8348G	59 (AccS)	A	G	11.8	2.4	4.9	0.6
Anticodon triplet mutation							
T8324A	35 (ACL)	U	A	nd	nd	*0.49 10^{-3}	6100
Disease-related mutations							
A8296G	2 (AccS)	A–U	G·U	10.2	4.4	2.3	1.3
G8313A	24 (DS)	C–G	C·A	nd	nd	*0.43 10^{-3}	7000
T8316C	27 (ACS)	U–A	C·A	2.4	1.5	1.8	2.0
G8328A	39 (ACS)	C–G	C·A	nd	nd	*0.61 10^{-3}	4900
G8342A	53 (TL)	G–C	A·C	8.5	4.1	2.1	1.4
A8344G	55 (TS)	A	G	14.3	1.7	8.4	0.3
T8356C	65 (TS)	A–U	A·C	0.9	1.2	0.75	4.0
T8362G	71 (AccS)	A–U	A·G	nd	nd	*0.3	10
G8363A	72 (AccS)	C–G	C·A	22.9	10.3	2.2	1.4

Names of the tRNA variants, affected tRNA positions (conventional tRNA numbering, according to Sprinzl et al. 1998), and sequence changes are given in the left part of the table. tRNA structural domains are abbreviated as follows: AccS, acceptor stem; TS, T-stem; DS, D-stem; ACS, anticodon stem; and TL, T-loop. Aminoacylation parameters measured at 25°C in the presence of human cytosolic LysRS are presented on the right. Loss-values (L) are defined as ratio $(k_{cat}/K_M)_{Rz\text{-}Ki}/(k_{cat}/K_M)_{variant}$. L-values varied by <10%.
*Ratios k_{cat}/K_M were determined directly as described (Schulman and Pelka 1988).

ders. Nine could be comparatively analyzed in regard to their effect on aminoacylation of in vitro transcripts. They segregate into two categories, with seven leading to little effect on aminoacylation (<10-fold loss in aminoacylation efficiency) and two (mutations G8313A and G8328A) leading to severe decreases (4900- to 7000-fold losses in efficiency). These large differences suggest that a defect in aminoacylation efficiency would not be a common primary molecular impact of pathology-related mutations in tRNA^{Lys}. However, it cannot be ruled out that even a weak effect on aminoacylation, amplified by additional molecular events, might be sufficient to contribute to the disease status. Similar conclusions have been drawn from aminoacylation studies performed on tRNA^{Ile} mutations (Kelley et al. 2000, 2001) and tRNA$^{Leu(UUR)}$ mutations (Sohm et al. 2003). As alternatives to aminoacylation, the impact of individual mutations in various tRNAs were found at other functional levels, including transcription, maturation, post-transcriptional modification, stability, binding to elongation factor EF-Tu, and codon reading (for review, see Florentz et al. 2003). In the specific case of tRNA^{Lys} mutations, only MERRF mutation A8344G has been investigated at different levels. This mutation does not affect mitochondrial transcription (Hanna et al. 1995; Masucci et al. 1995). It does not affect the global post-transcriptional modification pattern of the tRNA (Helm et al. 1999a), except modification at nucleotide 34, which is completely abolished (Yasukawa et al. 2000). The detailed chemical probing analysis in solution performed herein shows that MERRF-mutation A8344G does not alter the tRNA structure. However, the stability of the mutated tRNA is reduced (Enriquez et al. 1995; Yasukawa et al. 2000). Further, its interaction with EF-Tu is decreased (Yasukawa et al. 2001), and most important, its capacity to read the lysine codons is strongly affected (Yasukawa et al. 2001). For most other mutations affecting the human mt tRNA^{Lys} gene, no information is available as to primary molecular impact. Access to in vitro transcripts has allowed a systematic investigation of the effect of individual mutations on aminoacylation and should allow a number of other possibilities, including maturation, stability, structure, and recognition by elongation factor, to be investigated in a systematic way.

The comparative analysis of mutations within the same tRNA also shows only partial correlation between the primary impact of the mutation and the phenotype of the disorder. None of the four mutations correlated with MERRF syndrome (G8363A, T8356C, A8344G, A8296G) or either of the two mutations correlated with deafness (G8363A, A8296G) affect tRNA^{Lys} aminoacylation. On the other hand, two mutations linked to encephalopathies do affect this reaction (G8313A and G8328A), whereas a third one (T8316C, linked to MELAS) does not. MELAS syndrome correlates with mutation A3243G in tRNA$^{Leu(UUR)}$, in which it leads to a significant decrease in leucylation (see Börner et al. 2000; Chomyn et al. 2000; Park et al. 2003; Sohm et al. 2003). Thus, primary mechanisms leading to the same disorder can be different according to the tRNA in which the deleterious mutation occurs.

Rules governing lysylation of human mitochondrial tRNA^{Lys}

Several observations from the present work allow one to distinguish features within human mt tRNA^{Lys} regarding their importance for lysylation. Comparison of aminoacylation properties of native tRNA extracted from human mitochondria with in vitro transcripts defines the contribution of post-transcriptional base modifications to the aminoacylation process. The natural tRNA was shown to possess six modifications: m^1A9, m^2G10, Ψ27, Ψ28, sU*34, and t^6A37 (Helm et al. 1998; Yasukawa et al. 2001). As already discussed, m^1A9 plays an essential role as a structural chaperone, promoting cloverleaf folding. The other modifications, especially those in helical domains, probably also contribute in a limited way to structure stabilization, as was shown for other tRNAs (Agris 1996), and in consequence improve aminoacylation efficiency. Such considerations may explain the slight difference in aminoacylation plateaus observed for the tRNA^{Lys} transcripts ***Rz-Ke*** and ***Rz-Ki*** compared with native tRNA^{Lys}. Modifications of anticodon loop nucleotides of tRNAs can contribute directly to aminoacylation identity (Giegé et al. 1998b; Giegé and Frugier 2003). This is probably not the case for human mt tRNA^{Lys}, as can be deduced from aminoacylation properties of native wild type and native MERRF mutated tRNA^{Lys} (Yasukawa et al. 2001). Both tRNAs are lysylated to similar levels (this work) with very similar efficiencies (Yasukawa et al. 2001) despite the complete absence of modification sU*34 in the MERRF derivative (Yasukawa et al. 2000). In the crystal structures available of lysyl-tRNA synthetase from *T. thermophilus* complexed either to fully modified *E. coli* tRNA^{Lys} or to in vitro transcribed *T. thermophilus* tRNA^{Lys} (Cusack et al. 1996), the mode of binding of the anticodon loops is the same, and both mnm^{5}s^{2}U or a nonmodified C-residue can be accommodated in the anticodon binding site of the enzyme. Although access to comparative kinetic parameters of transcripts and native tRNA^{Lys} are not available (due to the difficulty in purifying sufficient quantities of native tRNAs), the above data lead to the conclusion that, beyond the major structural role of m^1A9, post-transcriptional modifications make no major contribution to the lysylation properties of human mt tRNA^{Lys}. This is in line with the low decrease (10-fold) in k_{cat}/K_M observed for human cytosolic tRNA^{Lys}III transcribed in vitro compared with native tRNA (Stello et al. 1999). In *E. coli*, post-transcriptional modifications make a more pronounced contribution because they improve aminoacylation efficiency 140-fold (Tamura et al. 1992).

Mutagenesis of in vitro transcribed tRNAs has been explored intensively in a large number of aminoacylation systems with the goal of deciphering aminoacylation identity elements (Giegé et al. 1998b; Giegé and Frugier 2003). As an outcome of the investigation of the 11 tRNA^{Lys} variants investigated here, it can be concluded that the central U-residue from the anticodon loop (U35) is a major lysylation identity nucleotide. This was anticipated based on results from all lysylation systems explored so far both in vitro (Tamura et al. 1992; Shiba et al. 1997) and in vivo (McClain et al. 1990; Normanly et al. 1990). It is also clear that nucleotides 24 and 39 (and/or the base pairs in which they are involved) in the D-stem and anticodon-stem respectively, contribute in an essential way to lysylation. On the other hand, nucleotides or base pairs involving nucleotides 2, 71, and 72 in the acceptor stem; nucleotide 27 in the anticodon stem; nucleotides 50, 53, 64, 65 in the T-stem; and nucleotides 53, 55, and 59 in the T-loop are only of limited importance.

Interestingly, the mutations investigated here are distributed all over the secondary cloverleaf structure of the tRNA, with only three mutations in loops and all others in stems. Within stems, each mutation converts a classical Watson–Crick pair into a weaker base pair. Eight mutations lead to C•A, A•C, or A•G mismatches and one to a G•U weak pair. Some drastically reduce aminoacylation; others do not affect this process (Fig. 6A). Occurrence of mismatches of the above type is not specific to tRNA^{Lys} mutations, or to pathology-related mutations, but is a general observation for mt tRNA gene mutations (Florentz and Sissler 2001). These mismatches are expected to destabilize helical domains to some extent (Varani and McClain 2000). Because aminoacylation of tRNAs by their cognate aminoacyl-tRNA synthetase is sensitive to structural properties of the tRNA (Giegé and Frugier 2003), some mismatches can be expected to interfere with the lysylation properties of the mt tRNA. In an attempt to understand these distinct effects, mutated nucleotides have been located within a three-dimensional tRNA structure (Fig. 6C–E). In absence of crystallographic data for human mt tRNA^{Lys}, the structure of yeast tRNA^{Asp} complexed to its cognate synthetase (Ruff et al. 1991; Cavarelli et al. 1993) has been chosen as a model. This structure is not expected to correspond exactly to that of tRNA^{Lys}, especially in regard of the D/T-loops interactions, which obviously are different (the mt tRNA has a very short D-loop of three nucleotides and an enlarged T-loop of nine nucleotides; further, classical nucleotides G18, G19, U56, and C57 allowing for tertiary interactions between the two loops are missing). Both tRNA^{Lys} and tRNA^{Asp} are, however, recognized by aminoacyl-tRNA synthetases belonging to the same subclass IIb of synthetases (Cusack et al. 1990; Eriani et al. 1990). Figure 6, C through E, highlights that mutations affecting aminoacylation are all in the anticodon branch of the L-shaped three-dimensional structure, along the same side of the molecule. These mutations are in line with the single acceptor branch mutation leading to a weak loss in aminoacylation efficiency. All other mutations not affecting aminoacylation either are in the acceptor branch or, if present in the anticodon branch, are located on the opposite side.

The mechanisms by which both deleterious mutations,

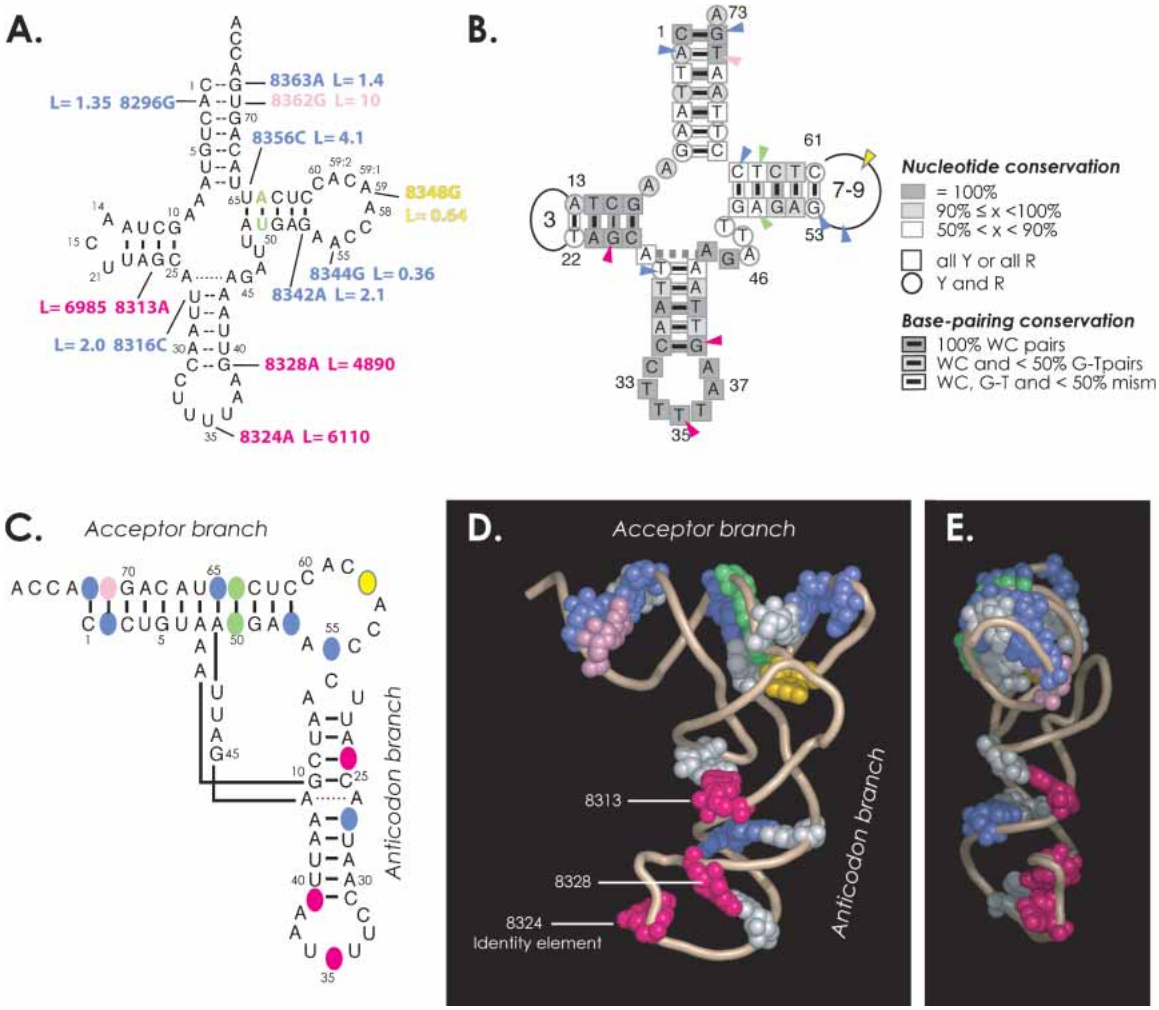

FIGURE 6. Structural insight on pathology-related mutations. (*A*) Summary of the effects of mutations on tRNA^{Lys} aminoacylation efficiencies. Values are from Table 1 and the color code is as follows: structural mutations introduced at positions 50 and 64, which prevent alternate folding into extended hairpin (Helm et al. 1998), are indicated in green. Polymorphic mutation A8348G is in yellow. Neutral, mild, and highly deleterious mutations are, respectively, in blue, pink, and magenta. (*B*) Sequence conservation of mammalian mt tRNA^{Lys} genes (Helm et al. 2000) and location of pathology-related mutations studied in the present work (arrowheads). The color code for arrowheads is as in *A*. (*C–E*) Location of mutations in three-dimensional representations of the tRNA. The color code is the same as in parts *A* and *B*. Nucleotides complementary to mutated positions and thus involved in base-pairing are in white. The model tRNA is a ribbon representation of the crystal structure of yeast tRNA^{Asp} (in its complexed form with AspRS; Ruff et al. 1991; Cavarelli et al. 1993). (*E*) Profile representation emphasizing the location of harmful mutations within a same face of the tRNA. The CCA end is pointed toward the reader.

affecting highly conserved base pairs in mammalian mt tRNA^{Lys} sequences (Fig. 6B; Helm et al. 2000), interfere with lysylation remain to be established. Mutations may either remove identity elements or introduce antideterminants, hindering recognition by the cognate synthetase. In both situations, direct contacts between the enzyme and its substrate would be hindered. Alternatively, if the affected nucleotides are not in direct contact with the synthetase, their sequence variations may lead to perturbations of the tRNA architectural frame, thus hindering signal transmission from the anticodon loop identity elements (in particular, the central anticodon triplet nucleotide) to the 3′-end of the tRNA, where aminoacylation takes place. Finally, mutations with negative effects on aminoacylation may simply remove essential permissive elements. These concepts, already described and illustrated for a number of canonical tRNA aminoacylation systems (see Frugier et al. 1998; Giegé et al. 1998a,b; Choi et al. 2003), now need to be tested experimentally for mt tRNA^{Lys}.

Conclusion and outlook

The present work demonstrates that despite the crucial requirement of a post-transcriptional modification in human mt tRNA^{Lys} folding, it is possible to create well-folded in vitro transcripts, efficient in aminoacylation, and thus to explore the contribution of individual nucleotides on tRNA structural and functional properties by second-site muta-

genesis. Systematic exploration of aminoacylation properties of the full set of pathology-related and polymorphic mutations in $tRNA^{Lys}$ represents an initial application of such engineered substrates. It enlarges both our understanding of lysylation properties of a mt tRNA and of potential molecular mechanisms underlying various human disorders. It also offers new perspectives for exploring further structural and functional properties of this tRNA and of the related variants, including mischarging properties or, formulated differently, the gain of function hypothesis in mitochondrial disorders (Jacobs and Holt 2000). Experiments are underway to fully decipher the lysine identity elements and their mechanism of action.

From a theoretical point of view, most human mt tRNAs have peculiar structural features (Helm et al. 2000; Florentz et al. 2003), including weak base pairs; the presence of mismatches, unusual D-loop, and T-loop sizes; and the absence of classical tertiary interactions. Engineered structural stabilization of in vitro transcripts might represent a successful approach to enable a systematic investigation of structural and functional properties of any member of this tRNA family and to screen for the effect of pathology-related mutations on a simple basis, before undertaking indispensable but laborious in vivo analyses.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were from New England Biolabs and T4 DNA ligase from Boehringer-Mannheim. PCR-purified oligonucleotides were from NAPS or Interactiva. GoldStar Taq DNA polymerase was purchased from Eurogentech. Phage T7 RNA polymerase was prepared from an overexpression clone according to the method of Becker et al. (1996). Plasmid pM368 Hs-LysRS-HisTag encoding the full-length human cytosolic LysRS (LysRS) was kindly provided by Karin Musier-Forsyth (University of Minnesota, Minneapolis, MN). Initial cybrid cell lines R2–1A and R1–C3, displaying, respectively, wild-type and MERRF-mutated human mt DNA, were kindly provided by Guiseppe Attardi and Anne Chomyn (Caltech, Pasadena, CA).

Production of mitochondrial tRNAs by in vitro transcription

Synthetic genes, corresponding to the T7 RNA polymerase promoter followed by the human mt wild-type $tRNA^{Lys}$ gene (***Kwt***) or variants ***Ke*** (G50–C64) and ***Ki*** (U50–A64), and terminating at a *Bst*N1 site, were previously constructed from overlapping and complementary oligonucleotides and cloned into pTFMA as described in (Helm et al. 1998). The synthetic gene corresponding to the T7 promoter connected to the downstream sequence of a hammerhead ribozyme (Fechter et al. 1998) followed by the human mt $tRNA^{Lys}$ sequence (***Rz-Kwt***) was also previously cloned (Helm et al. 1999b). Mutated tRNAs, bearing in all cases the A50U/U64A structural mutations and, respectively, mutation A8296G, G8313A, T8316C, G8328A, G8342A, T8356C, T8362G, G8363A (pathology-related mutations; numbering according to the location in the mitochondrial DNA), A8348G (polymorphic mutation), or T8324A (altering the anticodon triplet), were cloned following the same protocol (Helm et al. 1999b). Clones ***Rz-Ke***, ***Rz-Ki***, ***Rz-Kem***, and ***Rz-Kim*** were derived from ***Rz-Kwt*** by PCR amplification, using in all cases oligonucleotide 659 (5′-CAC CAAGCTTAATACGACTCACTATA-3′) and, respectively, oligonucleotide 660 (5′-CGGGATCCCCTGGTCACTGTAAGGAGGT GTTGGTTCTCCTAATCTTTAA-3′ for mutations A50G and U64C in ***Rz-Ke***), oligonucleotide 662 (5′-CGGGATCCCCTGGT CACTGTAATGAGGTGTTGGTTCTCATAATCTTTAA-3′ for mutations A50U and U64A in ***Rz-Ki***), oligonucleotide 663 (5′-CGG GATCCCCTGGTCACTGTAAGGAGGTGTTGGCTCTCCTAATC TTTAA-3′ for mutations A50G, U64C, and A55G in ***Rz-Kem***), and oligonucleotide 661 (5′-CGGGATCCCCTGGTCACTGTAA TGAGGTGTTGGCTCTCATAATCTTTAA-3′ for mutations A50U, U64A, and A55G in ***Rz-Kim***). Oligonucleotide 659 introduces a HindIII restriction site, and oligonucleotides 660 to 663 introduce BstN1 and BamHI restriction sites. Prior to ligation into pTFMA, PCR products were purified on low-melting 1% agarose gel, extracted with the QIAquick Gel Extraction kit (Qiagen S.A.) and double-digested with HindIII and BamHI (according to manufacturer's instructions). Plasmids were isolated from transformed *E. coli* **TG1** cells. In vitro transcription of 0.1 mg/mL BstN1 linearized plasmids was performed for 4 h at 37°C in 40 mM Tris-HCl (pH 8.1 at 37°C), 30 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% Triton-X100, 1 mM spermidine, and 4 mM each nucleoside triphosphate and T7 RNA polymerase. The hammerhead ribozyme, which contains four nucleotides complementary to the first four nucleotides of the 5′-end of the tRNA, cleaves the phosphodiester linkage directly upstream of residue C_1 of the tRNA. After phenol/chloroform extraction, transcripts were purified to single nucleotide resolution on denaturing 12% polyacrylamide/urea gels, electroeluted, and ethanol precipitated.

Preparation of native $tRNA^{Lys}$

Human cybrid cells (homoplasmic for either wild-type, R2–1A, or MERRF-mutated, R1-C3, human mt $tRNA^{Lys}$ gene) were grown, mitochondria were isolated, and specific tRNAs were extracted by hybridization to solid-phase oligonucleotide as previously described (Chomyn et al. 1991; Helm et al. 1999a). Briefly, mitochondria were isolated from cybrid cells by differential centrifugation and total mt tRNAs were obtained by subsequent extraction using guanidium isothiocyanate. Human mt $tRNA^{Lys}$ was further purified by hybridization to a 5′-biotinylated labeled 30-mer, complementary to its 3′-end as described (Helm et al. 1998). tRNA was recovered from the streptavidin-coated beads by heating for 5 min at 80°C in 2 mM EDTA and immediately chilling on ice. About 0.3 μg of native $tRNA^{Lys}$ were recovered from 3.3 g of pelleted cells (corresponding to ~1 L adherent cell-cultures).

Lysyl-tRNA synthetase

The human cytosolic histidine-tagged LysRS was purified as previously described (Shiba et al. 1997), using a nickel-nitrilotriacetic acid resin (Qiagen) according to the manufacturer's instructions. Enzymatic activity for LysRS was 51 UE/mg (nanomoles of lysine

incorporated per mg of protein per min), in aminoacylation conditions as described below using *E. coli* total tRNAs as substrate.

Aminoacylation reactions

Aminoacylation reactions have been performed as described (Perret et al. 1990; Shiba et al. 1997) in a medium containing 50 mM HEPES-KOH (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 μg/μL BSA, 20 μM [^{3}H]-labeled lysine (specific activity of ~1400 cpm/pmole), and adequate amounts of tRNA (0.1 to 10 μM) and LysRS (1 to 600 nM) at 37°C or 25°C. Before aminoacylation, transcripts were renatured by heating for 2 min at 60°C and slow cooling to room temperature. Kinetic parameters were derived from Lineweaver-Burk plots. Because the concentration of amino acid was subsaturating, only apparent kinetic parameters are given. In all cases, two independent preparations of transcripts have been done and at least two sets of experiments performed to define kinetic parameters. If the variability in functional results varied >10%–20%, a third preparation of molecules was done and analyzed. Doing so, kinetic parameters differing by <10% were obtained for each tRNA. The charging levels (optimal lysylation plateaus) are expressed as percentages of charged tRNA versus total amount of specific tRNA per experiment. For poor tRNA substrates, direct determination of the ratio k_{cat}/K_M was established as the slope of the linear plot of initial rate versus high tRNA concentrations (Schulman and Pelka 1988).

Structural mapping in solution

5′-Labeling and subsequent purification of tRNA transcripts were carried out as previously described (Helm et al. 1998). Cleavage with lead acetate was performed in 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 40 mM NaCl, and 0.1 μg/μl carrier tRNA in the presence of 0.15 mM $Pb(OAc)_2$ for 10 to 30 min at 25°C. Reactions were stopped by addition of 1 volume RNA loading buffer (8 M urea, 100 mM Tris-borate at pH 8.3, 10 mM EDTA). Modification with diethylpyrocarbonate (DEPC) was performed in 50 mM sodium cacodylate (pH 8.0), 10 mM $MgCl_2$, 300 mM KCl (control, C, and native, N, conditions), or 50 mM sodium cacodylate (pH 8.0), 1 mM EDTA (semi-denaturing, SD, and denaturing, D, conditions), respectively. Aliquots of 50 000 cpm 5′-labeled RNA supplemented with 0.05 to 0.1 μg/μL carrier tRNA were incubated with 5% (v/v) DEPC for 40 min at 25°C (N, SD) or 4 min at 60°C (D), respectively. Reactions were stopped by addition of 1 volume 0.6 M NaOAc (pH 7.0) and 20 μg carrier tRNA and subsequent ethanol precipitation. Cleavage of RNA at DEPC-modified adenine residues was done according to the method of Peattie and Gilbert (1980). Cleavage products were analyzed by denaturing separation on 12% PAGE as described (Helm et al. 1998).

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