The Pathogenic A3243G Mutation in Human Mitochondrial tRNA$^{Leu(UUR)}$ Decreases the Efficiency of Aminoacylation†

Hyejeong Park, Edgar Davidson, and Michael P. King*

Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 233 South 10th Street, BLSB 308, Philadelphia, Pennsylvania 19107

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ABSTRACT: Mutations of mtDNA, particularly those in mtDNA-encoded tRNA genes, are emerging as a significant cause of human disease. We examined the effects of the pathogenic A3243G and T3271C mutations in the mitochondrial tRNA$^{Leu(UUR)}$ gene on the aminoacylation of tRNA$^{Leu(UUR)}$. Transmitochondrial cells carrying these mutations have decreased steady-state levels of mitochondrial tRNA$^{Leu(UUR)}$. The A3243G mutation also results in a decrease in the fraction of aminoacylated tRNA$^{Leu(UUR)}$. To determine if the decreased fraction of aminoacylated tRNA$^{Leu(UUR)}$ in A3243G mutant cells was due to a defect in the ability of mutant tRNA to be aminoacylated by the human mitochondrial leucyl-tRNA synthetase, we examined the aminoacylation kinetics of wild-type and mutant tRNA$^{Leu(UUR)}$, using both native and in vitro transcribed tRNA$^{Leu(UUR)}$. Native A3243G mutant tRNA$^{Leu(UUR)}$ was 25-fold less efficiently aminoacylated in vitro, compared to native wild-type tRNA$^{Leu(UUR)}$. The T3271C mutation in tRNA$^{Leu(UUR)}$ did not affect the efficiency of aminoacylation of the native tRNA. There were no differences in aminoacylation efficiencies among wild-type and mutant tRNA$^{Leu(UUR)}$ transcripts. The combined effects of the reductions in the steady-state levels and the aminoacylated fraction of tRNA$^{Leu(UUR)}$ are likely to contribute to the decreases in the rates of mitochondrial translation observed in mutant cells. These results also suggest that the A3243G and T3271C mutations may have distinct mechanisms of pathogenesis.

Pathogenic mutations in human mitochondrial tRNA genes are associated with diverse clinical features. Most frequently, they result in multisystem disorders, including the well-characterized MELAS[1] syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes). MELAS is associated primarily with mutations A3243G [A14G in the tRNA numbering system (*1*)] and T3271C (T39C) in the mitochondrial tRNA$^{Leu(UUR)}$ gene (Figure 1) (*2*, *3*). The A3243G mutation has been associated with numerous clinical phenotypes since its initial description as the molecular genetic cause of MELAS. Among the most frequent and clinically important are cardiomyopathy, chronic progressive external ophthalmoplegia, diabetes, and diabetes with deafness (*4*−*6*).

The A3243G mutation is one of the most extensively studied of the human mitochondrial tRNA mutations; however, the findings have not been consistent (*7*). This mutation usually results in decreases in the rates of mitochondrial translation in transmitochondrial cells (*8*−*11*). Transmitochondrial cells containing the A3243G mutation showed a decreased steady-state level of tRNA$^{Leu(UUR)}$ in most studies whether the parental mtDNA-less (ρ^0) cells were HeLa cells (*12*), osteosarcoma cells (*13*, *14*), or lung sarcoma cells (*11*). Most laboratories found large decreases in the fraction of aminoacylated tRNA$^{Leu(UUR)}$ with the A3243G mutation in transmitochondrial cell lines derived from

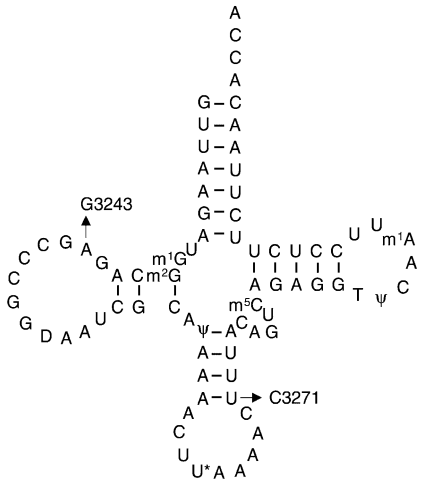

FIGURE 1: Secondary structure of the human mitochondrial tRNA$^{Leu(UUR)}$. Shown is the human mitochondrial tRNA$^{Leu(UUR)}$ folded to show its predicted secondary structure. The mutations A3243G (A14G) and T3271C (T39C) associated with MELAS are indicated with arrows. The posttranscriptional nucleotide modifications present in this tRNA are m^{1}A, 1-methyladenosine; m^{1}G, 1-methylguanosine; m^{2}G, 2-methylguanosine; m^{5}C, 5-methylcytosine; T, ribothymidine; U*, 5-taurinomethyluridine; ψ, pseudouridine; D, dihydrouridine (*12*, *31*, *46*).

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* To whom correspondence should be addressed: Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 233 South 10th Street, BLSB 308, Philadelphia, PA 19107. Telephone: 215-503-4845. Fax: 215-503-5393. E-mail: michael.king@mail.tju.edu.

[1] Abbreviations: MELAS, mitochondrial myopathy; encephalopathy, lactic acidosis and stroke-like episodes; mtDNA, mitochondrial DNA; LeuRS, leucyl-tRNA synthetase.

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osteosarcoma cells (*13*, *14*) or lung sarcoma cells (*15*). A decrease in the fraction of aminoacylated tRNA$^{Leu(UUR)}$ was also observed in tissue samples from patients carrying the A3243G mutation (*16*). However, there was only a small decrease in the fraction of aminoacylated tRNA$^{Leu(UUR)}$ in transmitochondrial HeLa cells with the A3243G or T3271C mutations (*12*).

We previously showed that transmitochondrial osteosarcoma cells containing the A3243G or T3271C mutations have defects in the rates of mitochondrial translation as compared to those containing isogenic wild-type mtDNAs (*9*, *17*). To investigate further the translational defects associated with the A3243G and T3271C mutations in the tRNA$^{Leu(UUR)}$ gene, we analyzed the effects of these mutations on aminoacylation both in vivo and in vitro. We confirmed reductions in the in vivo steady-state levels of tRNA$^{Leu(UUR)}$ and fraction of aminoacylated tRNA$^{Leu(UUR)}$ in mutant transmitochondrial cells. We then examined the effects of the mutations on the efficiency of in vitro aminoacylation of tRNA$^{Leu(UUR)}$ by human mitochondrial leucyl-tRNA synthetase (LeuRS) to determine if decreases in the fraction of aminoacylated tRNA$^{Leu(UUR)}$ in vivo were a consequence of altered kinetics of aminoacylation.

EXPERIMENTAL PROCEDURES

Cell Culture. Transmitochondrial cell line WS227.546, in which at least 99.7% of the mtDNA contains the A3243G mutation, was derived from WS227 (*9*) by treatment of the cells with ethidium bromide (*18*). The amount of the A3243G mutation was quantitated by PCR as described previously (*9*). The isogenic wild-type cell line (WS241) was described previously (*9*). Transmitochondrial cell lines homoplasmic for the T3271C mutation (AF145) or isogenic wild-type mtDNA (AF126.111) (Y. Koga, L. L. Zhang, and M. P. King, unpublished observations) were derived as described previously (*18*, *19*). Both wild-type tRNA$^{Leu(UUR)}$ gene sequences are identical to the Cambridge sequence (*20*). All cells were grown in Dulbecco's Modified Eagle medium containing 4.5 mg/mL glucose and 110 μg/mL sodium pyruvate, supplemented with 5% fetal bovine serum.

Determination of In Vivo Steady-State Levels of Aminoacylated tRNA$^{Leu(UUR)}$. Mitochondria were prepared from 5×10^7 cells and mitochondrial RNA was isolated at pH 5.0 (*21*). Five micrograms of each RNA sample was electrophoresed at 4 °C through 6.5% polyacrylamide−7 M urea gels, pH 5.0, with circulating buffer. RNA was electrotransferred onto Zeta-probe membrane (Bio-Rad). An EcoRI/HindIII fragment from pUC19 containing tRNA$^{Leu(UUR)}$ (described below) or tRNA^{Lys} (*22*) was subcloned into pBluescriptII KS (Stratagene) to facilitate synthesis of antisense tRNA probes for Northern analyses. tRNA$^{Leu(UUR)}$ and tRNA^{Lys} hybridization probes were prepared by in vitro transcription with T3 RNA polymerase (Ambion) in the presence of [α-^{32}P]CTP (Perkin-Elmer). Aminoacylated and non-aminoacylated tRNA$^{Leu(UUR)}$ were quantitated with a phosphorimager (Typhoon 8600, Molecular Dynamics). The steady-state levels of tRNA$^{Leu(UUR)}$ were determined by electrophoresis of deacylated RNA samples through 20% polyacrylamide−7 M urea gels. RNA samples were deacylated by heating at pH 8.5 at 80 °C for 5 min. RNA quantitation was performed as described above. Levels of aminoacylated and total tRNA were determined from a minimum of three independent RNA isolations with at least two Northern analyses of each RNA preparation.

Characterization and Purification of Human Mitochondrial LeuRS. A human cDNA clone (KIAA0028) that encoded a putative human mitochondrial LeuRS was obtained from Kazusa DNA Research Institute (Chiba, Japan; *23*). The protein encoded by this cDNA was shown to be a LeuRS (*24*). The putative mitochondrial LeuRS cDNA was subcloned into pEF/myc/cyto (Invitrogen) so that a *myc* tag was encoded at the 3′ end of the gene. The mitochondrial localization of the recombinant LeuRS was confirmed by transfecting human 143B osteosarcoma cells with the LeuRS mammalian expression plasmid construct using Effectene (Qiagen). The LeuRS, identified with an antibody directed against the *myc* epitope (Babco), co-localized with MitoTracker RedCM-H_2XRos (Molecular Probes) (data not shown). The mitochondrial targeting sequence was predicted to consist of amino acids 1−39, using principles outlined by von Heijne (*25*). The coding region of the predicted mature form of LeuRS, starting at Ile40, was amplified using a forward primer containing an NdeI restriction site (5′-CATATGATCTACAGTGCCACGGGAAAGTG-3′) and a reverse primer containing an XhoI restriction site (5′-CTCGAGATCTTGCACCAGGAAGTTGATGAGGGCAG-3′) and cloned into the *Escherichia coli* expression vector pET24d(+) (Novagen) which adds a His·Tag at the C-terminus of the protein. All plasmid constructs were confirmed by sequencing at the Kimmel Cancer Center DNA Sequencing Facility, Thomas Jefferson University. Recombinant human mitochondrial LeuRS was expressed in BL21-(DE3)pLysS (Novagen) by induction with 1 mM IPTG when the OD_{600} of the *E. coli* culture reached 0.6, and cells were then grown at 15 °C overnight. LeuRS with a His·Tag was purified using Talon affinity resin according to the protocol provided by the manufacturer (Clontech). Protein concentration was determined by DC protein assay (Bio-Rad) and densitometry of the Coomassie Brilliant Blue R250 stained SDS−polyacrylamide gel using Alpha Image software (Alpha Innotech Co.). One milligram of LeuRS with >90% purity was obtained from 1 L of *E. coli* culture.

Transcription and Purification of tRNA Transcripts. Wild-type and mutant human mitochondrial tRNA$^{Leu(UUR)}$ genes were amplified by PCR using DNA isolated from wild-type and mutant transmitochondrial cells. The forward primer, 5′-TCCAGAATTCTAATACGACTCACTATAGTTAAGAT-GGCAGAGCCCGG-3′ for wild-type and T3271C tRNA$^{Leu(UUR)}$ or 5′-TCCAGAATTCTAATACGACTCAC-TATAGTTAAGATGGCAGGGCCCGG -3′ for A3243G tRNA$^{Leu(UUR)}$, contained an EcoRI restriction site and a T7 RNA polymerase promoter sequence. The reverse primer, 5′-CTAAAGCTTCCTGGTGTTAAGAAGAGGAATTG -3′, contained BstNI and HindIII restriction sites. The PCR amplified fragments were cloned into pUC19 and the plasmid constructs were confirmed by sequencing. In vitro transcription of tRNA$^{Leu(UUR)}$ using T7 RNA polymerase (*26*) was performed at 37 °C for 4 h in a reaction consisting of 40 mM Tris-HCl, pH 8.0, 24 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 4 mM each ATP, CTP, GTP, and UTP, 16 mM GMP, 40 units/μL T7 RNA polymerase, and 0.1 μg/μL DNA template (linearized by BstNI digestion). The transcripts were purified by electrophoresis through 12% polyacrylamide−7 M urea gels.

Purification of Native Mitochondrial tRNA$^{Leu(UUR)}$. Mitochondria were prepared from 1.5×10^9 wild-type or mutant transmitochondrial cells (*27*). Mitochondrial tRNAs were isolated using a DNA/RNA purification kit (Qiagen). Mitochondrial tRNA$^{Leu(UUR)}$ was isolated from total mitochondrial tRNA by hybridization to a biotinylated oligonucleotide (*28*), complementary to 33 nucleotides at the 3′ end of tRNA$^{Leu(UUR)}$ (5′-TGTTAAGAAGAGGAATTGAACCTCTGACTGTAA-biotin-3′), bound to Dynabeads M280 coated with streptavidin (Dynal). The tRNA$^{Leu(UUR)}$ eluted from the oligonucleotide was further purified by electrophoresis through 20% polyacrylamide−7 M urea gels. Typically, 13 pmol of native tRNA$^{Leu(UUR)}$ was isolated from wild-type cells, and 3−5 pmol from mutant cells carrying the A3243G or T3271C mutations.

Aminoacylation of tRNA Transcripts Using [^{3}H]Leucine. Transcripts were heated at 60 °C for 2 min in the presence of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$, and slowly cooled to 37 °C prior to use in aminoacylation analyses. The aminoacylation assay was conducted at 37 °C as described previously (*24*) with minor modifications (50 mM HEPES−KOH, pH 7.5, 25 mM KCl, 3 mM $MgCl_2$, 25 mM DTT, 0.2 mg/mL BSA, 2.5 mM ATP, 50 μM leucine, 0.1 μCi/μL [^{3}H]leucine (44 Ci/mmol, Perkin-Elmer)). The maximum proportion of transcript that could be aminoacylated (plateau) was determined by a time course of aminoacylation using 0.8 μM tRNA transcripts and 9.25 μM LeuRS. At plateau, 15.4 ± 2.5% (mean ± 1 standard deviation) of the wild-type transcript and 16.5 ± 2.2% of the transcript containing T3271C mutation was aminoacylated while 7.67 ± 0.01% of the transcript containing A3243G mutation was aminoacylated. The tRNA transcript concentrations used for the initial rate analyses were derived from the plateau aminoacylation values. Initial rates of aminoacylation were determined in a volume of 20−24 μL with 0.15 μM LeuRS and transcript concentrations ranging from 2 to 23 μM. At each time point, 4 μL aliquots were spotted onto Whatman 3MM filter paper which was immediately immersed in 5% trichloroacetic acid at 0 °C. Filter papers were washed twice with 5% trichloroacetic acid, twice with 95% ethanol, and dried before scintillation counting. The counting efficiency of [^{3}H]leucine-tRNA$^{Leu(UUR)}$ on 3MM filter paper was determined by comparing a direct count of aminoacylated tRNA (purified through a G25 Quick Spin Column (Roche)) with that obtained after spotting the tRNA on 3MM filter paper.

Aminoacylation of [^{32}P]-Labeled Native or Transcript tRNA. A second method to assay for aminoacylation was performed according to Wolfson and Uhlenbeck (*29*). The 3′ terminal adenosine of native or transcript mitochondrial tRNA$^{Leu(UUR)}$ was labeled with [α-^{32}P]ATP (3000 Ci/mmol, Perkin-Elmer) by *E. coli* tRNA nucleotidyl transferase-catalyzed pyrophosphorolysis and nucleotide exchange. *E. coli* tRNA nucleotidyl transferase was a generous gift from Dr. Y.-M. Hou, Thomas Jefferson University. The labeled tRNA was separated from unincorporated nucleotides with a G25 Quick Spin Column (Roche). The conditions used for aminoacylation were as described above except that [^{3}H]-leucine was omitted. After aminoacylation, reaction mixtures were digested with nuclease P1 (Roche), and the products were separated by chromatography on polyethyleneimine cellulose TLC plates (Aldrich) using 0.2 M ammonium acetate, pH 5.0, as the mobile phase. The amounts of AMP and leucyl-AMP were quantitated by phosphorimager. The maximum charging ability of the native tRNA was determined using 2.5 nM substrate tRNA and 50 nM LeuRS. At plateau, 24 ± 2% of A3243G tRNA$^{Leu(UUR)}$ and 56 ± 3% of the isogenic wild-type tRNA$^{Leu(UUR)}$ was aminoacylated. For T3271C tRNA$^{Leu(UUR)}$, 42 ± 2% of the mutant tRNA, and 56 ± 6% of isogenic wild-type tRNA$^{Leu(UUR)}$ was aminoacylated at plateau. The tRNA concentrations used for the initial rate analysis were calculated from these values. Initial rates were measured with 0.6−12 nM tRNA and 0.25 nM LeuRS. k_{cat}/K_m was determined using 5 nM of native tRNA and 0.25 nM LeuRS. The maximum charging ability of the transcript tRNA was determined using 9 nM substrate and 500 nM LeuRS. At plateau, 12.5 ± 1.7% of wild-type tRNA$^{Leu(UUR)}$, 2.77 ± 1.02% of A3243G, and 13.7 ± 3.0% T3271C tRNA$^{Leu(UUR)}$ was aminoacylated. k_{cat}/K_m was determined with 25 nM tRNA transcript and 3 nM LeuRS.

RESULTS

In Vivo Levels of Aminoacylated Mitochondrial tRNA$^{Leu(UUR)}$. We examined the effects of the A3243G and T3271C mutations, in the mitochondrial tRNA$^{Leu(UUR)}$ gene, on the levels of aminoacylated tRNA$^{Leu(UUR)}$ in mutant and isogenic wild-type transmitochondrial cells in vivo. The cells containing wild-type mtDNA were isogenic with their respective mutant cells since wild-type and mutated mtDNA were derived from the same heteroplasmic patient cells. Total mitochondrial RNA was isolated from cells under acidic conditions to preserve the aminoacyl-tRNA linkage. Aminoacylated tRNAs were separated from non-aminoacylated tRNAs on acidic denaturing polyacrylamide gels and quantitated after Northern analyses (Figure 2A). In cells with the A3243G mutation, 30 ± 6% of tRNA$^{Leu(UUR)}$ was aminoacylated, while 55 ± 5% of tRNA$^{Leu(UUR)}$ was aminoacylated in isogenic wild-type cells, a 45% decrease in mutant cells. For the T3271C mutation, the fraction of aminoacylated tRNA$^{Leu(UUR)}$ was similar in isogenic wild-type and mutant cells (50 ± 9% and 47 ± 8%, respectively). The Northern blots used in these analyses were reprobed to determine the levels of aminoacylated mitochondrial tRNA^{Lys}. For tRNA^{Lys}, 69 ± 2% was aminoacylated in the A3243G mutant cells, and 69 ± 3% was aminoacylated in isogenic wild-type cells. In the T3271C mutant cells, 65 ± 3% of tRNA^{Lys} was aminoacylated, and 66 ± 2% was aminoacylated in isogenic wild-type cells. These results confirmed that differences in aminoacylation between mutant and wild-type tRNA$^{Leu(UUR)}$ were not due to variations among the different RNA preparations.

The steady-state levels of mitochondrial tRNA$^{Leu(UUR)}$ and tRNA^{Lys} were quantitated from the Northern blots of tRNAs deacylated prior to electrophoresis (Figure 2B). The relative steady-state levels for tRNA$^{Leu(UUR)}$ were obtained by normalizing the values of tRNA$^{Leu(UUR)}$ to those of tRNA^{Lys}. In A3243G mutant cells, the steady-state level of tRNA$^{Leu(UUR)}$ was reduced to 50 ± 12% of the wild-type level, and for T3271C mutant cells, tRNA$^{Leu(UUR)}$ was 62 ± 23% of the wild-type level.

In Vitro Aminoacylation of tRNA$^{Leu(UUR)}$. To determine whether the decrease in the fraction of aminoacylated tRNA$^{Leu(UUR)}$ in A3243G cells was due to a defect in the

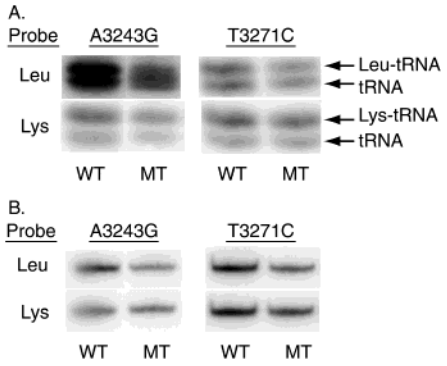

FIGURE 2: In vivo levels of mitochondrial tRNA$^{Leu(UUR)}$ and aminoacyl-tRNA$^{Leu(UUR)}$ in mutant and isogenic wild-type cells. (A) Shown is a representative Northern blot analysis of the levels of aminoacylated tRNAs in wild-type (WT) and mutant (MT) transmitochondrial cells. Mitochondrial RNAs were isolated under acidic conditions from mutant and wild-type cells. Aminoacylated tRNA was separated from non-aminoacylated tRNA by acid gel electrophoresis. The aminoacylated and non-aminoacylated species of tRNA$^{Leu(UUR)}$ and tRNA^{Lys} were detected by Northern blotting. The upper band in each phosphorimage corresponds to the indicated aminoacyl-tRNA species and the lower band corresponds to the non-aminoacylated tRNA species. The identifications of the aminoacylated and non-aminoacylated species of tRNA were confirmed by comparison with deacylated tRNA samples electrophoresed in parallel (data not shown). (B) A representative Northern blot of the steady-state levels of tRNA$^{Leu(UUR)}$ and tRNA^{Lys} in wild-type and mutant transmitochondrial cells is shown. tRNA$^{Leu(UUR)}$ and tRNA^{Lys} were detected by Northern blotting after total mitochondrial RNA was electrophoresed through 20% polyacrylamide−7M urea gels.

Table 1: Kinetic Parameters of in Vitro Aminoacylation of Wild-type and Mutant tRNA$^{Leu(UUR)}$ Transcripts by Human Mitochondrial LeuRS[a]

substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min$^{-1}\mu$M^{-1})	relative k_{cat}/K_m
wild-type	2.14 ± 0.33	3.82 ± 0.22	0.56 ± 0.12	1.00
A3243G	0.95 ± 0.25	4.13 ± 1.96	0.24 ± 0.05	0.43
T3271C	1.61 ± 0.67	8.05 ± 4.46	0.21 ± 0.03	0.38

[a] Initial rates of aminoacylation with [^{3}H]leucine were determined using 2 to 23 μM tRNA transcripts and 0.15 μM LeuRS as described in Experimental Procedures. k_{cat} and K_m values were determined from Eadie-Hofstee plots. Shown are means ± 1 standard deviation derived from three independent determinations.

ability of the mutant tRNA to be aminoacylated by its cognate tRNA synthetase, we performed in vitro aminoacylation studies using wild-type and mutant tRNA$^{Leu(UUR)}$ with recombinant human mitochondrial LeuRS. Both native and in vitro synthesized tRNA$^{Leu(UUR)}$ were utilized as substrates.

Initially, we measured the kinetics of aminoacylation of mutant and wild-type tRNA$^{Leu(UUR)}$ transcripts with [^{3}H]-leucine. K_m and k_{cat} were derived from Eadie-Hofstee plots of initial rates of aminoacylation (Table 1). k_{cat} was decreased by 2.2-fold with the A3243G tRNA$^{Leu(UUR)}$ transcripts and 1.3-fold with the T3271C tRNA$^{Leu(UUR)}$ transcripts, as compared to k_{cat} for the wild-type tRNA$^{Leu(UUR)}$ transcript. The K_m was unchanged for the A3243G tRNA$^{Leu(UUR)}$ transcript and decreased by 2-fold for the T3271C tRNA$^{Leu(UUR)}$ transcript as compared to the K_m for the wild-type tRNA$^{Leu(UUR)}$ transcript. The A3243G and T3271C mutations resulted in a 2−2.5-fold decrease in the aminoacylation efficiency (k_{cat}/K_m) of the mutant transcripts.

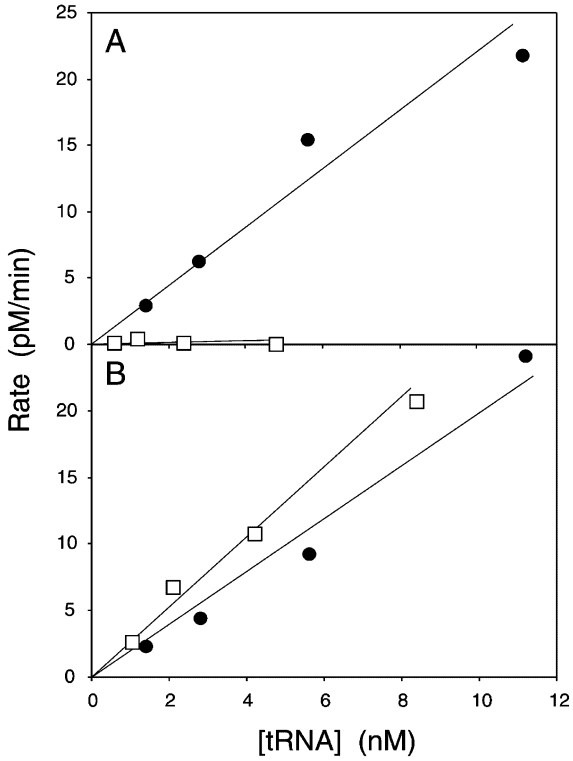

FIGURE 3: Initial rates of aminoacylation of native tRNA$^{Leu(UUR)}$. Shown are representative analyses of the initial rates of aminoacylation for tRNA$^{Leu(UUR)}$ isolated from wild-type (solid circles) and mutant (open rectangles) cells. Panel A is the analysis of A3243G and isogenic wild-type tRNA$^{Leu(UUR)}$. Panel B is the analysis of T3271C and isogenic wild-type tRNA$^{Leu(UUR)}$. Initial rates were measured at 0.6 to 12 nM native tRNA with 0.25 nM LeuRS for 8 min.

Aminoacylation studies were performed next with native tRNA$^{Leu(UUR)}$ isolated from wild-type and mutant transmitochondrial cells. The amounts of native tRNA$^{Leu(UUR)}$ that we could isolate were insufficient for the aminoacylation assay utilized for the tRNA$^{Leu(UUR)}$ transcripts. Therefore, a more sensitive aminoacylation assay was used (*29*). The initial rates of aminoacylation of native A3243G mutant tRNA$^{Leu(UUR)}$ were severely impaired as compared to those for the isogenic native wild-type tRNA$^{Leu(UUR)}$ (Figure 3A). In contrast, the initial rates of aminoacylation of native T3271C mutant tRNA$^{Leu(UUR)}$ and its isogenic wild-type native tRNA$^{Leu(UUR)}$ were similar (Figure 3B).

The limiting amounts of native tRNA$^{Leu(UUR)}$ did not permit direct determinations of K_m or k_{cat}. Therefore, k_{cat}/K_m was determined at 5 nM tRNA, below a plausible value for K_m [K_m for human native mitochondrial tRNAs: tRNA^{Ile}, 0.13 μM (*30*); tRNA^{Lys}, 0.71 μM (*31*); tRNA$^{Ser(GCU)}$, 0.37 μM (*32*); tRNA$^{Ser(UGA)}$, 0.22 μM (*32*)]. The k_{cat}/K_m of the native A3243G mutant tRNA$^{Leu(UUR)}$ was 25-fold lower than that for its isogenic wild-type native tRNA$^{Leu(UUR)}$ (Table 2). Native T3271C mutant tRNA had a k_{cat}/K_m that was similar to that of isogenic wild-type native tRNA$^{Leu(UUR)}$ (Table 2).

The efficiency of aminoacylation of the A3243G mutant native tRNA$^{Leu(UUR)}$ was severely impaired, a finding that was not revealed in the analysis of tRNA$^{Leu(UUR)}$ transcripts. To ensure that the differences in k_{cat}/K_m between equivalent native and transcript tRNAs were not due to the different assays employed, we repeated the kinetic analyses of aminoacylation of wild-type and mutant tRNA$^{Leu(UUR)}$ tran-

Table 2: Efficiencies (k_{cat}/K_m) of in Vitro Aminoacylation of Native and Transcript $tRNA^{Leu(UUR)}$ by Human Mitochondrial LeuRS[a]

		native		transcript	
substrate		k_{cat}/K_m	relative	k_{cat}/K_m	relative
A3243G	wild-type	8.24 ± 1.83	1.00	0.18 ± 0.08	1.00
	mutant	0.32 ± 0.34	0.04	0.16 ± 0.05	0.89
T3271C	wild-type	5.54 ± 0.42	1.00	0.18 ± 0.08	1.00
	mutant	7.52 ± 2.06	1.35	0.11 ± 0.03	0.61

[a] k_{cat}/K_m ($min^{-1}\mu M^{-1}$) for wild-type and mutant native $tRNA^{Leu(UUR)}$ was determined at 5 nM native tRNA and 0.25 nM LeuRS. k_{cat}/K_m for $tRNA^{Leu(UUR)}$ transcripts were determined at 25 nM tRNA and 3 nM LeuRS. For both determinations, the tRNA aminoacylation assay of Wolfson and Uhlenbeck (*29*) was used. Means $\pm$ 1 standard deviation were derived from three independent experiments.

scripts in the manner used for native tRNAs. At $tRNA^{Leu(UUR)}$ concentrations below K_m, the k_{cat}/K_m values for all tRNA transcripts (Table 2) were similar to those values obtained from separate determinations of k_{cat} and K_m using the less sensitive aminoacylation assay (Table 1).

DISCUSSION

We have demonstrated that the pathogenic A3243G mtDNA mutation causes a 25-fold decrease in the aminoacylation efficiency of native mitochondrial $tRNA^{Leu(UUR)}$. This is the likely cause of the 45% decrease in the fraction of aminoacylated $tRNA^{Leu(UUR)}$ in cells carrying the A3243G mutation, as compared to isogenic wild-type cells. In contrast, native $tRNA^{Leu(UUR)}$ with the T3271C mutation was aminoacylated with a similar efficiency as native wild-type $tRNA^{Leu(UUR)}$, and consequently the fraction of aminoacylated $tRNA^{Leu(UUR)}$ in T3271C mutant cells in vivo was similar to that in isogenic wild-type cells. The T3271C mutation may have a different pathogenic mechanism than the A3243G mutation because it has little effect on the efficiency of aminoacylation in vitro and the fraction of aminoacylated tRNA in vivo.

The A3243G and T3271C mutations resulted in large decreases in the steady-state levels of $tRNA^{Leu(UUR)}$, consistent with other studies (*11*−*14*). This is likely due to the decreased stabilities of the mutant tRNAs (*12*). In cells containing the A3243G mutation, the reductions in the steady-state levels and the aminoacylated fraction of $tRNA^{Leu(UUR)}$ combined to decrease the amount of the aminoacylated $tRNA^{Leu(UUR)}$ to 27% of that present in wild-type cells. In cells containing the T3271C mutation, the levels of aminoacylated $tRNA^{Leu(UUR)}$ were decreased to 58% of the levels present in wild-type cells. This decrease was due almost entirely to the reduced steady-state levels of $tRNA^{Leu(UUR)}$.

Generally, decreases in the fraction of aminoacylated tRNA in cells result in reductions in the rates of protein translation (*33*−*35*). The 73% reduction in the steady-state levels of A3243G leucyl-$tRNA^{Leu(UUR)}$ is likely to contribute to the 45% decrease in the rates of mitochondrial protein synthesis seen in the A3243G mutant cells used here (H. Park, E. Davidson, and M. P. King, unpublished observations). For the T3271C mutation, the levels of leucyl-$tRNA^{Leu(UUR)}$ were 42% lower than wild-type levels and consequently the rates of mitochondrial protein synthesis were 32% lower than wild-type rates (H. Park, E. Davidson, and M. P. King, unpublished observations). The reductions in the levels of leucyl-$tRNA^{Leu(UUR)}$ may not be the only factor contributing to decreases in the rates of mitochondrial protein synthesis. A defect in modification at the anticodon wobble position of $tRNA^{Leu(UUR)}$ may also contribute to the observed decreases in rates of mitochondrial translation (*12*, *31*, *36*).

Although mitochondrial tRNAs are generally considered to have structures that differ from those of conventional tRNAs (*37*), $tRNA^{Leu(UUR)}$ is one of the few mitochondrial tRNAs predicted to fold as a classical tRNA, utilizing all conserved tertiary interactions found in canonical tRNAs (*38*). The A3243G mutation in mitochondrial $tRNA^{Leu(UUR)}$ changes the A at position 14 of the tRNA (Figure 1), eliminating a reverse Hoogsteen pairing, between A14 and U8, that is thought to have a role in maintaining the L-shape of the tRNA (*39*). The A14:U8 base pair is highly conserved among tRNAs (*37*, *38*). For human mitochondrial $tRNA^{Leu(UUR)}$, the A14G mutation decreased the in vivo stability of human mitochondrial $tRNA^{Leu(UUR)}$ (*12*). This mutation also allowed dimer formation between mutant $tRNA^{Leu(UUR)}$ transcripts in vitro (*40*).

Mutations at position 14 in tRNA transcripts affect the efficiency of aminoacylation differently, depending upon the tRNA species. An A14C substitution in *E. coli* $tRNA^{Ala(CUA)}$ reduced the efficiency of aminoacylation (k_{cat}/K_m) less than 2-fold (*41*). However, an A14G substitution in *E. coli* $tRNA^{Leu(CUG)}$ transcripts decreased aminoacylation efficiency by 100-fold (*42*). Mutational analyses of the interactions between positions 8 and 14 suggested that A14 is a determinant for recognition of $tRNA^{Leu(CUG)}$ by the *E. coli* LeuRS (*42*). For native human mitochondrial $tRNA^{Leu(UUR)}$, we found that A14G mutation decreases the aminoacylation efficiency of the tRNA by 25-fold. It was suggested that the formation of dimers by the A14G $tRNA^{Leu(UUR)}$ transcripts affected the tRNA's ability to be aminoacylated (*40*). However, the tRNA concentrations required for dimer formation were far higher than those utilized in our aminoacylation studies with native $tRNA^{Leu(UUR)}$, and thus dimers are not likely to contribute to the decreased aminoacylation efficiency described here. Our results indicate that A14 is important for the aminoacylation of human mitochondrial $tRNA^{Leu(UUR)}$, but further experiments are required to determine if A14 is a determinant for recognition by human mitochondrial LeuRS.

We found that the wild-type human mitochondrial $tRNA^{Leu(UUR)}$ transcript was 30−45-fold less efficiently aminoacylated than the native $tRNA^{Leu(UUR)}$. Further, we were unable to detect major effects of the A3243G mutation on aminoacylation of transcripts despite the 25-fold difference in aminoacylation efficiency between wild-type and A3243G mutant native tRNAs. These differences between native and transcript tRNAs were likely due to the presence of post-transcriptionally modified nucleotides in the native tRNAs. These modifications contribute to stabilization of tRNA tertiary structure and to the efficiency and accuracy of translation (reviewed in ref *43*). Their presence in mitochondrial tRNAs can be crucial for tRNA structure and function (*36*, *44*).

The absence of modifications may not affect transcript structure consistently, but may depend on the individual tRNA species. We have found that some human mitochondrial tRNA transcripts cannot be aminoacylated by their

cognate aminoacyl-tRNA synthetases, suggesting that some unmodified mitochondrial tRNAs are incapable of folding into a functional structure (E. Davidson, H. Park, and M. P. King, unpublished observations). The lack of modifications in mitochondrial tRNA^{Ile} transcripts has not prevented the detection of deleterious effects of mutations on tRNA structure and function (*45*), although the transcripts were 50-fold less efficiently aminoacylated than native tRNA^{Ile} (*30*). However, our results suggest that it is prudent to compare transcripts to native tRNAs when analyzing the effects of nucleotide substitutions on the structure and function of mitochondrial tRNAs.

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