

Reduction of mitochondrial tRNA^{Leu}(UUR) aminoacylation by some MELAS-associated mutations

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Abstract The mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) is a rare congenital disorder of mitochondrial DNA. Five single nucleotide substitutions within the human mitochondrial tRNA^{Leu}(UUR) gene have been reported to be associated with MELAS. Here, we provide *in vitro* evidence that the aminoacylation capacities of these five hmtRNA^{Leu}(UUR) transcripts are reduced to different extents relative to the wild-type hmtRNA^{Leu}(UUR) transcript. A thermal denaturation experiment showed that the A3243G and T3291C mutants, which were the least charged by LeuRS, have fragile structures. In addition, the T3291C mutant can inhibit aminoacylation of the wild-type hmtRNA^{Leu}(UUR), indicating that it may act as an inhibitor in the mitochondrial heteroplasmic environment.

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Keywords: Human mitochondrion; MELAS-related mutation; tRNA^{Leu}(UUR); Aminoacylation

1. Introduction

The aminoacylation of tRNAs with cognate amino acids by aminoacyl-tRNA synthetases (aaRS) is a key step in the decoding of genetic information [1]. The efficiency and fidelity of protein synthesis are dependent on the presence of functional tRNAs.

In the last two decades, an increasing number of single nucleotide substitutions within the human mitochondrial tRNA (hmtRNA) genes have been linked to a variety of multisystem disorders [2]. More than 90 mutations have been reported in 21 hmtRNA genes [3]. The human mitochondrial tRNA^{Leu}(UUR) [hmtRNA^{Leu}(UUR)] gene (human mitochondrial genome 3231–3305) is a hotspot for pathology-related mutations. Over 20 mutations have been found within this

region (see <http://www.mitomap.org>). Investigation of their detailed molecular influences will help to elucidate their pathogenic mechanism and will provide information on the mechanisms of mitochondrial protein synthesis.

The mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) is a rare congenital disorder of human mitochondrial DNA (hmDNA) [4]. It is reported to be associated with five single nucleotide substitutions (A3243G, A3252G, C3256T, T3271C, and T3291C) [5–9] within the hmtRNA^{Leu}(UUR) gene (Fig. 1). Mutation at A3243 of hmtRNA^{Leu}(UUR) is reported to decrease the efficiency of aminoacylation *in vivo* and *in vitro* [10–12]. The T3271C variant also shows attenuated aminoacylation *in vitro* despite to less degree [12]. The effects of the substitutions at other three positions, A3252G, C3256T, and T3291C, have not yet been reported.

We investigated the processing of human mitochondrial leucyl-tRNA synthetase (hmLeuRS) in insect cell and found that the precursor protein is cleaved between residues 39 and 40 [13]. The gene encoding the mature hmLeuRS was constructed in a plasmid and was overexpressed in *Escherichia coli* (*E. coli*) [14]. The protein, which we designated hmLeuRS-40, was purified from the *E. coli* transformant by one step Ni-NTA affinity chromatography. The purified protein had a specific activity of 125 unit/mg ($k_{\text{cat}} = 2.1 \times 10^{-1} \text{ s}^{-1}$) when *E. coli* tRNA^{Leu} was used as the substrate. This high specific activity allows the purified protein to be used for analyzing the aminoacylation of hmtRNA^{Leu} mutants, information that will help to clarify the pathogenic mechanisms of hmtRNA^{Leu} mutation-associated diseases.

In the present study, we constructed the other three MELAS-associated point mutations (A3252G, C3256T, and T3291C) in hmtRNA^{Leu}(UUR). Comparison of the aminoacylation capacities of the five MELAS-associated variants *in vitro* showed that their aminoacylation levels are reduced compared to wild-type hmtRNA^{Leu}(UUR), but to varying extents. Thermal denaturation experiments showed that the severely attenuated charging of A3243G and T3291C mutant tRNAs corresponds with significantly decreased melting temperatures (T_m), indicating that they have more fragile structures than the wild-type tRNA. Furthermore, the T3291C variant competitively inhibited aminoacylation of the wild-type hmtRNA^{Leu}(UUR), indicating that the mutant may inhibit charging of the substrate in the potentially heteroplasmic environment of the mitochondria with mixtures of wild-type and mutant tRNAs [15,16].

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Abbreviations: MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome; hmLeuRS, human mitochondrial leucyl-tRNA synthetase; hmtRNA, human mitochondrial tRNA; hmDNA, human mitochondrial DNA; *E. coli*, *Escherichia coli*

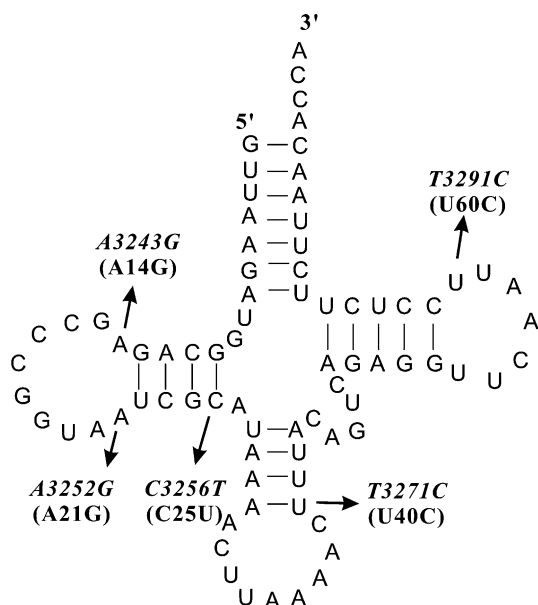


Fig. 1. Location of MELAS-associated mutations within the cloverleaf secondary structure of human mitochondrial tRNA^{Leu}(UUR). The mutations are indicated with arrows. The numbers corresponding to the genomic locations are shown in italics and the corresponding secondary structure locations are presented in brackets.

2. Materials and methods

2.1. Materials

Except where noted, all chemicals were purchased from Sigma (St. Louis, MO, USA). [¹⁴C]Leucine was purchased from Amersham Bioscience (Buckinghamshire, UK). Kinase, ligase, restriction endonucleases, and RNasin ribonuclease inhibitor were obtained from Promega (Madison, WI, USA). T7 RNA polymerase and the hmLeuRS-40 protein were purified from overexpressing *E. coli* [14,17].

2.2. Preparation of hmtRNAs^{Leu}(UUR) by *in vitro* transcription

Plasmids encoding the tRNA transcripts were constructed by ligating overlapping oligonucleotides into a pUC19 plasmid between the *Eco*RI and *Bam*HI cleavage sites. The tRNA constructs were placed between a T7 RNA polymerase promoter sequence and a *Bst*OI cleavage site and were connected to the 3' end of a hammerhead ribozyme sequence to increase the transcription yields [18]. The clones for the wild-type hmtRNA^{Leu}(UUR) and five variants, A3243G, A3252G, C3256T, T3271C, and T3291C (numbering according to their location in the hmDNA), were obtained. The identities of the clones were confirmed by DNA sequencing.

In vitro transcription and subsequent ribozyme cleavage were performed as described by Sohm et al. [12] and Fechter et al. [18], respectively. The transcripts were purified to single nucleotide resolution on polyacrylamide-urea gels (15%), excised, and extracted for 4 h at 25 °C with a solution of 125 mM NH₄Ac, 0.5 μM EDTA, and 0.025% SDS. After ethanol precipitation from this extract, the transcripts were resolved in diethyl pyrocarbonate-treated deionized distilled water containing 12 mM MgCl₂. All hmtRNA^{Leu}(UUR) transcripts were annealed by heating for 3 min at 80 °C and cooled on ice for 30 min. As the denaturation–renaturation process may affect the folding of transcript, we attempted to optimize the folding condition for each tRNA variant. However, no obvious increase in charging level was detected for each transcript. The tRNA concentrations were determined by UV absorbance at 260 nm. Extinction coefficients were calculated from the sequence of each tRNA [19].

2.3. Assay of tRNA charging capacity

The time course of aminoacylation was assessed at 37 °C in a 70 μl reaction mixture containing 50 mM HEPES, pH 7.6, 25 mM KCl, 10 mM MgCl₂, 2.5 mM ATP, 1 mM spermidine, 0.5 U/μl ribonuclease

inhibitor, 100 μg/ml bovine serum albumin, 20 μM [¹⁴C]leucine (402 cpm/pmol), and 1.5 μM hmtRNA^{Leu}(UUR) transcript. The reactions were initiated by the addition of 1 μM hmLeuRS-40. At various time points, a 10 μl aliquot was removed and spotted onto Whatman 3MM filter paper, precipitated with 5% trichloroacetic acid, and washed with 5% trichloroacetic acid and ethanol. Adsorbed radioactivity on the filter papers was measured by scintillation counting. The maximum proportion of transcript that could be aminoacylated (plateau) was determined in 40-min incubation times.

The aminoacylation kinetic constants of hmLeuRS-40 for tRNA^{Leu}(UUR) and its mutants were measured at 37 °C as described above, except that the concentration ranges of tRNAs and hmLeuRS-40 were adjusted according to the accepting activities of tRNA (1–20 μM for tRNAs and 0.3–1 μM for hmLeuRS-40). The apparent kinetic parameters, *k*_{cat} and *K*_m, of hmLeuRS-40 for hmtRNA^{Leu}(UUR) and its mutants were derived from Eadie-Hofstee plots and were the average of three independent determinations.

2.4. Assay the effect of mutant tRNA to wild-type tRNA^{Leu}(UUR) aminoacylation

The effect of the A3243G or T3291C mutants on wild-type hmtRNA^{Leu}(UUR) aminoacylation was determined by addition of either tRNA variant to a wild-type hmtRNA^{Leu}(UUR) aminoacylation mixture. The pH and buffer were the same as in the aminoacylation assay, but 1.5 μM wild-type hmtRNA^{Leu}(UUR) and 0.3 μM hmLeuRS-40 were used. Different concentrations (0, 1.5, 3, or 4.5 μM) of the A3243G or T3291C variant were added prior to initiating the reaction. The reactions were carried out at 37 °C for 5 min and 15 μl aliquots were removed for monitoring the generation of Leu-tRNAs.

The pattern of inhibition of wild-type tRNA^{Leu}(UUR) aminoacylation by the T3291C variant was determined from Lineweaver–Burk plots. In these experiments, the concentrations of wild-type tRNA^{Leu}(UUR) were 1, 1.25, 2, and 5 μM, and the concentrations of the T3291C variant were 0, 2, and 5 μM. Except for the use of 0.3 μM hmLeuRS-40, the other reaction conditions were the same as described for the standard aminoacylation assay.

2.5. Determination of the *T*_m of hmtRNA^{Leu}(UUR) mutants

The tRNA variants (0.5 μM) were prepared in 50 mM HEPES buffer, pH 7.6, containing 25 mM KCl and 10 mM MgCl₂. All tRNAs were annealed before the thermal denaturation experiment. Denaturation of the samples was achieved by increasing the temperature from 35 to 89 °C and was followed at 260 nm. The *T*_m value was determined as the maximum of the first derivative of the melting curves measured at 260 nm.

3. Results

3.1. Construction of hmtRNA^{Leu}(UUR) mutants

The aminoacylation activity for a set of hmtRNAs^{Leu}(UUR) mutants, including A3243G, A3243T, and T3271C, has been investigated to elucidate the role of hmtRNA^{Leu}(UUR) in some pathological disorders [12]. In the present work, we constructed and studied the activities of five MELAS-related mutant hmtRNA^{Leu}(UUR) transcripts. These mutations are located at different regions of the tRNA cloverleaf secondary structure (Fig. 1). Mutations A3243G and A3252G are in the D-loop of the tRNA at positions 14 and 21, respectively; mutation C3256T replaces a GC base pair with a GU mismatch at position 25 in the canonical D-stem sequence; mutation T3271C gives rise to an AC mismatch at position 40 in the anticodon stem of hmtRNA^{Leu}(UUR); and the T3291C substitution changes the U residue at position 60 in the theoretical TΨC loop to a C. Although the locations of these mutations are known, the effects of A3252G, C3256T, and T3291C on the leucylation of hmtRNA^{Leu}(UUR) have not been previously reported.

3.2. MELAS-related hmtRNA^{Leu}(UUR) mutations decrease the efficiency of leucylation

We next examined the effects of the five MELAS-associated mutations on the ability of hmtRNA^{Leu}(UUR) to be leucylated by hmLeuRS-40. As shown in Fig. 2, all of the mutants had decreased abilities to be aminoacylated compared to the wild-type hmtRNA^{Leu}(UUR). Under the experimental conditions, the plateau value of the wild-type tRNA^{Leu}(UUR) aminoacylated by hmLeuRS-40 is 70%. However, for the C3256T, A3252G, and T3291C variants only 24%, 17%, and less than 4%, respectively, can be aminoacylated. Similar to the findings of Soh et al. [12], the A3243G and T3271C variants were 7% and 30% charged, respectively. Thus, all MELAS-associated point mutations decreased the efficiency of leucylation and the accepting activity of T3291C was the lowest among the five mutants.

The aminoacylation assay of hmtRNA^{Leu}(UUR) showed that these single nucleotide substitutions mainly decrease the catalytic rate of hmLeuRS instead of the apparent binding affinity (Table 1). In fact, K_m values of hmLeuRS-40 for all hmtRNA^{Leu}(UUR) mutants were lower than that for the wild-type hmtRNA^{Leu}(UUR), indicating stronger binding of all hmtRNA^{Leu}(UUR) mutants to hmLeuRS-40. The K_m value for T3291C was the smallest (4.5-fold reduction), and those for T3271C and C3256T were similar to the wild-type. Although the change in K_m of hmLeuRS-40 for hmtRNA^{Leu}(UUR) mutants was not significant, the k_{cat} values of hmLeuRS-40 for hmtRNA^{Leu}(UUR) mutants clearly decreased. Compared to the wild-type hmtRNA^{Leu}(UUR), the k_{cat} value for T3291C decreased 345-fold; even for the T3271C variant there was

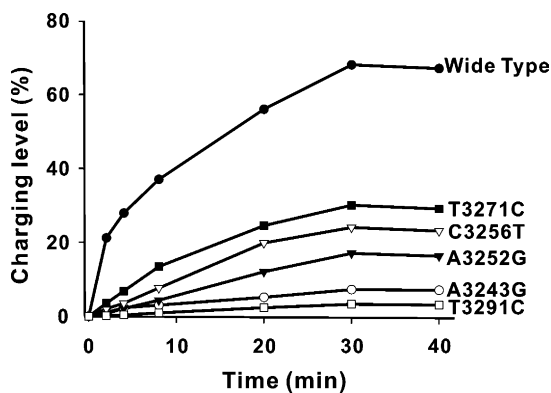


Fig. 2. Effects of MELAS-related mutations on the aminoacylation of human mitochondrial tRNA^{Leu}(UUR). Assays contained 1.5 μ M tRNA transcript and 1 μ M hmLeuRS-40 and were performed at 37 °C. Charging levels are expressed as percentages of charged tRNA vs. the total amount of specific tRNA.

Table 1

Apparent kinetic parameters of in vitro aminoacylation of wild-type and MELAS-related mutant hmtRNA^{Leu}(UUR) transcripts by hmLeuRS

HmtRNA ^{Leu} (UUR) transcripts	k_{cat} (10^{-3} s ⁻¹)	K_m (μ M)	k_{cat}/K_m (10^{-3} s ⁻¹ μ M ⁻¹)	K_{cat}/K_m relative	Loss (L)
Wild type	20.2	13.6	1.49	1	1
A3243G	0.61	5.2	0.12	0.081	12.3
A3252G	1.8	4.5	0.40	0.268	3.73
C3256T	4.3	11.9	0.36	0.242	4.13
T3271C	5.2	12.4	0.42	0.288	3.47
T3291C	0.058	3.0	0.02	0.013	74.5

Aminoacylation reactions were as described in Section 2. The L values in this table were the average values with a variation of <15% from three independent determinations.

an approximately 4-fold reduction in the k_{cat} . For these hmtRNA^{Leu}(UUR) mutants, the aminoacylation efficiency (k_{cat}/K_m) of hmLeuRS-40 decreased 3.7- to 74.5-fold as compared to that for the wild-type. Specifically, the decrease in efficiency was the largest for the T3291C mutant (74.5-fold); for the T3271C, A3252G, and C3256T variants, the efficiency was reduced by approximately 4-fold; and for A3243G, the efficiency was reduced 12.3-fold.

3.3. Determination of T_m for hmtRNA^{Leu}(UUR) mutants

We suspected that the effect of single substitutions on the aminoacylation of hmtRNA^{Leu}(UUR) is due to altered folding. This possibility was investigated by comparing the T_m of the mutants (Table 2). In a thermal denaturation experiment, the T_m value of the wild-type tRNA^{Leu}(UUR) transcript was 74.5 °C. There were obvious decreases in the T_m of the A3243G (65.5 °C) and T3291C (67 °C) variants. In contrast, the T_m values of A3252G, C3256T, and T3271C variants were very similar to that of wild-type tRNA.

3.4. T3291C mutant transcript inhibits the leucylation of wild-type hmtRNA^{Leu}(UUR)

Kinetic assays show that the T3291C mutation severely decreases aminoacylation efficiency of hmtRNA^{Leu}(UUR). A further experiment was carried out to monitor aminoacylation in heterogeneous mixtures of wild-type hmtRNA^{Leu}(UUR) and the T3291C variant. As shown in Fig. 3A, the charging of wild-type hmtRNA^{Leu}(UUR) was decreased by the addition of the T3291C mutant. This result is consistent with the idea that the T3291C variant binds hmLeuRS, but cannot be efficiently charged. We performed the same experiment with a mixture of wild-type hmtRNA^{Leu}(UUR) and the A3243G variant, but did not observe inhibition.

Inhibition of the wild-type tRNA aminoacylation by the T3291C variant was analyzed by Lineweaver–Burk double reciprocal plots. As revealed in Fig. 3B, as the concentration of T3291C was lowered, the K_m increased, but there was no significant decrease in V_{max} . This shows that the T3291C variant is a competitive inhibitor of the aminoacylation of wild-type tRNA^{Leu}(UUR). In other words, the T3291C variant competes with wild-type tRNA at least at some putative tRNA binding sites on hmLeuRS.

4. Discussion

There is a great deal of interest and excitement recently in understanding the pathological mechanisms of mitochondrial diseases. A given mutation in a tRNA gene may have impacts

Table 2
The melting temperature of hmtRNA^{Leu}(UUR) transcripts

HmtRNA ^{Leu} (UUR) transcripts	Melting temperature
wild type	74.5 ± 1.5
A3243G	65.5 ± 0.5
A3252G	74.0 ± 0.5
C3256T	75.5 ± 1.5
T2371C	75.5 ± 1.5
T3291C	67.0 ± 1.0

The melting temperature was measured as described in Section 2. All data presented here were the average values from two independent determinations.

on the tRNA at several stages, such as transcription, processing of the termini, aminoacylation, and, thus, ultimately induce diseases [10,11,20,21]. In vitro transcription is an efficient approach to systematically study the effects of different hmtRNA mutations. Although the tRNAs produced through in vitro transcription are devoid of base modification and sometimes do not achieve the classical cloverleaf structure [22,23], they have been successfully used in studying the effects of pathology-related mutations in the genes of hmtRNA^{Ile} [24,25], hmtRNA^{Lys} [26] or hmtRNA^{Leu} [12,27]. Our work is attempted to reveal the mechanism of MELAS based on the related tRNA aminoacylation.

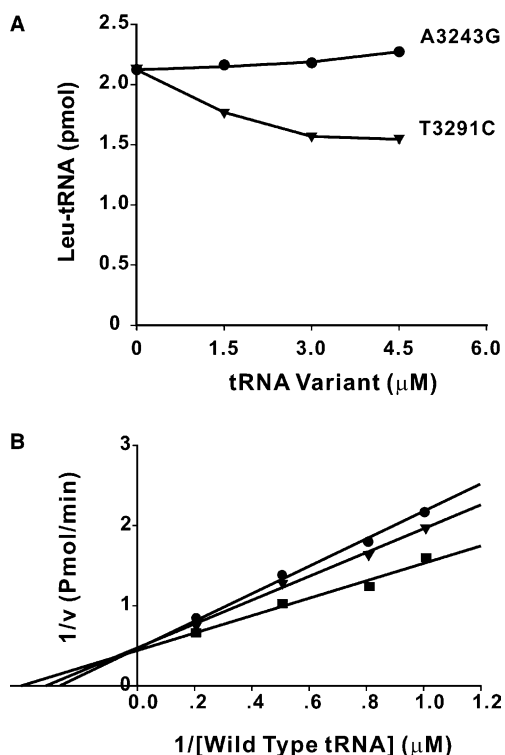


Fig. 3. Mutant T3291C is an inhibitor of wild-type hmtRNA^{Leu}(UUR). (A) Effect of the A3243G or T3291C variant on wild-type hmtRNA^{Leu}(UUR) aminoacylation. Different concentrations of the A3243G or T3291C variants were mixed with 1.5 μl wild-type tRNA, and the reaction was carried out with 0.3 μM hmLeuRS-40 for 5 min at 37 °C. (B) Lineweaver–Burk plot of the kinetic data for the T3291C variant. The reciprocal of reaction velocity is plotted vs. the reciprocal of the wild-type tRNA^{Leu}(UUR) concentration at 0 (■), 2 (▼), and 5 (●) μM T3291C.

To gain insight into the pathogenic mechanisms of the MELAS-associated substitutions in hmtRNA^{Leu}(UUR), we constructed five MELAS-associated tRNA variants and characterized their aminoacylation in vitro. We found that the accepting activity of the tRNA was decreased by each mutation, although to different extent. The impaired aminoacylation was consistent with the defect in mitochondrial protein synthesis found in trans-mitochondrial yeast [28]. The substitutions of 3291T to C and 3243A to G dramatically decreased the ability of tRNA to be aminoacylated. Subsequent measurements of T_m indicated that the structures of A3243G and T3291C mutants were far more fragile than those of other tRNA variants. Therefore, the aminoacylation deficiency appears to be associated with instability in the tRNA structure. The A3243G mutation corresponds to an A14G substitution in the D-loop of the tRNA. It eliminates a reverse Hoogsteen pairing between A14 and U8 and is thought to impair the tertiary structure of tRNA [29]. The T3291C mutation corresponds to a U60C substitution in the TΨC loop. Nucleotide 60, which is not involved in the canonical tRNA tertiary interactions, has not been found as an aminoacylation identity element in prokaryotic or eukaryotic tRNA^{Leu} investigated so far [22]. Thus, U60 might contribute to the hmtRNA^{Leu}(UUR) structure and identity in an indirect way.

We also found that the T3291C variant can competitively inhibit the aminoacylation of the wild-type hmtRNA^{Leu}(UUR). Although this variant is the least leucylated by hmLeuRS, it appears to retain some structural elements necessary for binding to hmLeuRS. The data herein suggested that, besides the low accepting activity, the T3291C variant might disturb the aminoacylation of the wild-type tRNA in the potentially heteroplasmic environment of the mitochondria. This result provided an explanation for the positive correlation between the relative amount of T3291C mutation and the severity of the pathological phenotype [30].

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