The pathogenic U3271C human mitochondrial tRNA$^{\text{Leu(UUR)}}$ mutation disrupts a fragile anticodon stem

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Received October 8, 2002; Revised and Accepted November 4, 2002

ABSTRACT

The U3271C mutation affecting the human mitochondrial transfer RNA$^{\text{Leu(UUR)}}$ (hs mt tRNA) is correlated with diabetes and mitochondrial encephalopathies. We have explored the relationship between the structural effects of this mutation and its impact on function using chemical probing experiments and in vitro aminoacylation assays to investigate a series of tRNA constructs. Chemical probing experiments indicate that the U3271C substitution, which replaces an AU pair with a CA mispair, significantly destabilizes the anticodon stem. The introduction of a compensatory A3261G mutation reintroduces base pairing at this site and restores the structure of this domain. In fact, the anticodon stem of the A3261G/U3271C mutant appears more structured than wild-type (WT) hs mt tRNA$^{\text{Leu(UUR)}}$, indicating that the entirely AU stem of the native tRNA is intrinsically weak. The results of the chemical probing experiments are mirrored in the aminoacylation activities of the mutants. The U3271C substitution decreases aminoacylation reactivity relative to the WT tRNA due to an increase in $K_m$ for the pathogenic mutant. The binding defect is a direct result of the structural disruption caused by the pathogenic mutation, as the introduction of the stabilizing compensatory mutation restores aminoacylation activity. Other examples of functional defects associated with the disruption of weak domains in hs mt tRNAs have been reported, indicating that the effects of pathogenic mutations may be amplified by the fragile structures that are characteristic of this class of tRNAs.

INTRODUCTION

The human mitochondrial (hs mt) genome encodes 13 proteins, two ribosomal RNAs and 22 transfer RNAs (tRNAs) (1,2). The hs mt tRNAs have sequences and structures that differ significantly from canonical bacterial and cytoplasmic tRNAs (3). Moreover, the tRNAs functioning in human mitochondria are less thermodynamically stable, as they generally contain higher numbers of mismatched and AU base pairs (4).

The structural instability of the hs mt tRNAs may contribute to their involvement in mitochondrial diseases. Almost 50% of the 145 pathology-associated mutations currently identified within the hs mt genome affect tRNAs (http://www.mitomap.org), despite the fact that the regions coding for these molecules comprise only ~10% of the genome. Indeed, molecular-level studies of hs mt tRNA$^{\text{Ile(UUR)}}$ and tRNA$^{\text{Ile}}$ have revealed that pathogenic mutations produce severe structural perturbations causing functional defects (5–7). In particular, hs mt tRNA$^{\text{Ile}}$ mutants containing mismatch-producing substitutions in regions of secondary structure displayed markedly reduced aminoacylation (6,7). The fact that these functional defects were structural in origin was confirmed with compensatory mutations, which completely restored activity by reintroducing Watson–Crick base pairs (6).

To date, 18 pathogenic mutations have been identified within the gene encoding hs mt tRNA$^{\text{Leu(UUR)}}$ (http://www.mitomap.org). Based on the canonical tRNA folding pattern, three of these mutations are predicted to alter the tertiary structure of hs mt tRNA$^{\text{Leu(UUR)}}$, eight mutations would affect its secondary structure, and seven mutations do not have an obvious structural impact on hs mt tRNA$^{\text{Leu(UUR)}}$. One mutation predicted to disrupt tertiary structure, the A3243G substitution associated with maternally inherited diabetes and deafness (MIDD) and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), was recently shown to attenuate aminoacylation (5). The loss of function was linked to the formation of an inactive dimeric complex in addition to the disruption of a tertiary contact (5).

Studies of pathogenic hs mt tRNA mutants correlated with disease have provided insight into potential functional defects that could impede cellular function (5–15). Many different aspects of tRNA function can be impaired by point mutations, including aminoacylation, processing, post-transcriptional modification and participation in ribosomal protein synthesis. Understanding how disruptions in these activities are related to the structural effects of pathogenic mutations presents a means to understand the molecular-level defects that may contribute to disease.

To evaluate the relationship of structural defects caused by disease-related tRNA mutations to function, we have
investigated the MELAS- and MIDD-related U3271C (U40C) substitution in hs mt tRNA_{Leu(UUR)} that introduces a CA mispair into the anticodon stem (Fig. 1) (16,17). This mutation has been the subject of a variety of studies that have elucidated losses in processing (8), modification levels (9) and cellular levels of tRNA_{Leu(UUR)} (9). However, none of these studies investigated whether a conformational disruption induced by the U3271C mutation was responsible for the loss in function. Given the high AU content of the anticodon stem of hs mt tRNA function more highly dependent on the anticodon stem may be even more significantly affected.

MATERIALS AND METHODS

Cloning and preparation of tRNA constructs

DNA templates for in vitro transcription were created by ligating overlapping oligonucleotides into pUC18 digested with BamHI and PstI. The hs mt tRNA_{Leu(UUR)} genes were flanked by a T7 polymerase promoter sequence and a BstNI cleavage site. Plasmids were harvested in milligram quantities from Escherichia coli DH5α and cleaved with BstNI to generate the 3′-CCA end. In vitro transcription reactions contained template DNA (100–200 µg in 1–2 ml), T7 RNA polymerase (overexpressed in E.coli), 40 mM Tris–HCl (pH 8), 10 mM NaCl, 2 mM spermidine, 20 mM MgCl₂, 4 mM NTPs and 5 mM dithiothreitol. Transcription reactions were incubated for 3 h at 37°C. Plasmid DNA was digested by the addition of DNase I (Takara). Reactions were extracted with 5:1 phenol (pH 4.7)/chloroform, ethanol precipitated, and purified by 12% denaturing polyacrylamide gel electrophoresis (PAGE) using a 0.5× TBE buffer (45 mM Tris base/45 mM boric acid/1 mM EDTA) on a 16.5 cm (w) × 26 cm (h) × 3 mm (d) gel for 12 h. tRNA samples were recovered by electroelution, ethanol precipitated and resuspended in 0.5× TE [5 mM Tris–HCl (pH 8), 0.5 mM EDTA].

Concentrations of tRNA solutions were determined by quantitating the absorbance at 260 nm and applying an extinction coefficient of 895 000 M⁻¹ (mononucleotide) cm⁻¹ (http://www.scripps.edu/mb/gottesfeld/ExtCoeff.html). Charging plateaus performed with an excess of hs mt leucyl-tRNA synthetase (LeuRS) confirmed the correspondence of concentrations determined spectrophotometrically with levels of chargeable tRNA (5), but absorbance-based measurements were used to determine tRNA concentrations, as these values yielded more reproducible and accurate results. tRNA samples were annealed by incubation at 70°C for 5 min in 0.5× TE, addition of MgCl₂ (10 mM), and immediate cooling on ice.

Preparation of hs mt LeuRS

Hs mt LeuRS was cloned and purified as described (5,18). SDS–PAGE was used to confirm the purity of the protein, and a Bradford assay was used to determine protein concentration. The activity of the enzyme was confirmed using a pyrophosphate exchange assay.

Preparation of 5′-³²P-labeled hs mt tRNA_{Leu(UUR)}

Prior to labeling, tRNA samples were dephosphorylated using calf intestinal alkaline phosphatase (Takara). Dephosphorylated tRNA (100 pmol) was 5′ radiolabeled in a reaction containing 100 pmols of [γ-³²P]ATP (ICN Biomedicals), 100 U of T4 polynucleotide kinase (New England Biolabs), 70 mM Tris–HCl (pH 7.6), 10 mM MgCl₂ and 5 mM dithiothreitol. The samples were incubated at 37°C for 30 min and purified using G-25 columns (Amersham Pharmacia). Further purification of labeled samples was performed using a 12% denaturing PAGE with a 0.5× TBE buffer. Labeled tRNAs were isolated, electroeluted and ethanol precipitated. Samples were resuspended in 0.5× TE unless otherwise noted.

Aminoacylation assays

Prior to the aminoacylation assay, tRNA samples were annealed as described above. Assays were performed at 37°C in reaction mixtures containing 50 mM HEPES (pH 7.6), 100 µM spermine, 25 mM KCl, 0.2 mg/ml bovine serum albumin, 2.5 mM ATP, 100 µM leucine, 4.6 µM [3,4,5-³H]leucine, 7 mM MgCl₂, 40 or 50 nM enzyme, and 400 nM to 20 µM tRNA. Assays were otherwise executed and analyzed as described (19). K_m and k_cat values were extracted from a non-linear fit of initial rate values (measured with at least six different tRNA concentrations) to the following adaptation of the Michaelis–Menten equation: \( v_o = \frac{V_{max}[S]}{K_m + [S]} \).

Chemical probing

Experiments conducted under non-denaturing conditions [approximating the native environment of hs mt tRNA_{Leu(UUR)}] (20) were performed with tRNAs prepared in 50 mM sodium cacodylate (pH 7), 25 mM NaCl and 10 mM...
MgCl₂. Reaction solutions were heated at 70°C for 5 min and cooled on ice for 20 min. Neat diethylpyrocarbonate (DEPC) (4 µl) was added and the samples were incubated for 1 h at 37°C. Reactions were halted by precipitation with 0.3 M sodium acetate (pH 5) and 2.5 vol of ethanol followed by incubation at −80°C. For comparison (data not shown), chemical probing was also carried out under denaturing conditions, which used buffer conditions that differed in the omission of 25 mM NaCl and 10 mM MgCl₂ and inclusion of 1 mM EDTA.

Strand scission of carbethoxylated hs mt tRNAs
To achieve strand scission at modified positions each sample was resuspended in 20 µl of 1 M aniline (pH 4.5 in concentrated glacial acetic acid) (21). Samples were incubated in the dark for 20 min at 60°C. To halt the reaction, samples were flash frozen in liquid nitrogen and dried. The samples were resuspended in 20 µl of H₂O, flash frozen and dried. After final drying, each sample was resuspended in 10 µl of denaturing PAGE loading buffer (containing 8 M urea).

Sequencing reactions
RNase T1 digestion was performed to create a G ladder. Wild-type (WT) hs mt tRNA⁶⁰(UUR) (4 µM) in 50 mM Tris base and 2 mM EDTA was incubated at 70°C for 5 min and cooled on ice (22). The reaction solution (5 µl) was incubated at 37°C for 20 min with 0.02 U of RNase T1 (Fermentas). WT hs mt tRNA⁶⁰(UUR) in 10 mM NaHCO₃ and 2 mM EDTA (5 µl) was heated for 8 min at 90°C to form an alkaline ladder (22). Both reactions were stopped through the addition of 5 µl of denaturing PAGE loading dye and cooling on ice.

Gel electrophoresis of DEPC-treated tRNAs
Samples were loaded onto a 21 cm (w) × 40 cm (h) × 0.4 mm (d) 15% polyacrylamide gel (19:1) containing 8 M urea buffered with 0.5× TBE. Gels were typically electrophoresed for 1.25 h at 50 mA and then exposed to a phosphorimager plate overnight.

RESULTS AND DISCUSSION
To investigate how the U3271C mutation affected the structure of hs mt tRNA⁶⁰(UUR), we monitored the reactivity of anticodon stem nucleotides with the chemical probe DEPC under non-denaturing conditions. Three tRNAs were used in these studies: the WT hs mt tRNA⁶⁰(UUR), a tRNA construct containing the U3271C mutation, and a tRNA construct containing the U3271C mutation and a compensatory A3261G mutation reintroducing a Watson–Crick pair at the site of the pathogenic substitution (Fig. 1). DEPC was used for the chemical probing because this reagent reacts preferentially with adenines and the region of interest was the AU-rich anticodon stem (20,21).

Figure 2. (A) PAGE autoradiogram for DEPC chemical probing experiments with WT, U3271C and A3261G/U3271C hs mt tRNA⁶⁰(UUR). Lanes 1–3 (labeled WT, U3271C and A3261G/U3271C) are control lanes for the 5'–radiolabeled hs mt tRNA⁶⁰(UUR) samples. Lane G is a T1 digest of denatured WT hs mt tRNA⁶⁰(UUR). Lanes 5–7 are chemical probing reactions performed under native conditions. Samples were analyzed by 15% PAGE. The numbering of the bands corresponds to the positions of adenosine within the hs mt tRNA⁶⁰(UUR) sequence. Increased nuclease susceptibility was observed reproducibly for the U3271C mutant, as evidenced by the larger intensities of the bands in the control lane for this sample. (B) Analysis of chemical probing. The relative percent cleavage at the various adenine positions was quantitated (Image Quant) by first normalizing the values against the uncleaved tRNA within each lane to adjust the values for loading variations. The cleavage intensities at each adenine position for the mutant tRNAs were compared with those of the WT sample. Position 30 within the A3261G/U3271C mutant sequence is not an adenine. Data were obtained from greater than three independent trials; protection values from different trials varied ±0.02.
PAGE analysis of WT, U3271C and A3261G/U3271C hs mt tRNA\(^{\text{Leu(UUR)}}\) treated with DEPC illustrated striking structural differences among these constructs (Fig. 2A). The cleavage ratios at the various adenine positions were quantitated and normalized, yielding protection values for each adenine position within the folded structures of the mutants in comparison with WT hs mt tRNA\(^{\text{Leu(UUR)}}\) (Fig. 2B). The protection values clearly establish that the structural integrity of the anticodon stem is lost in the presence of the U3271C substitution, as A28, A29, A30 and A31 are heavily reduced. The protection values further confirm the intermediacy level of cleavage at these positions, and the A3261G/U3271C mutant exhibits strong protection from DEPC. It appears that the stabilizing mutation significantly strengthens the anticodon stem by introducing a GC pair into this domain that is otherwise composed of AU pairs in WT hs mt tRNA\(^{\text{Leu(UUR)}}\). It is noteworthy that the native tRNA\(^{\text{Leu(UUR)}}\) contains a pseudouridine residue at position 27 which may impart a slight increase in the stability of this stem compared with the unmodified constructs studied here.

The same protection trends are observed for A26 (Fig. 2A and B). This nucleotide is typically part of the anticodon domain (23), but is more distant from the site of the pathogenic mutation than the nucleotides discussed above. Interestingly, this position is less protected for the U3271C mutant than WT hs mt tRNA\(^{\text{Leu(UUR)}}\), but appears less exposed (as judged from protection values) than the anticodon stem nucleotides. This indicates that the structural disruption may be less severe in the upper portion of this domain.

Different levels of cleavage at anticodon loop nucleotides are also observed for the different tRNA constructs. A35, A36, A37 and A38 exhibit similar reactivities for the WT and A3261G/U3271C tRNAs, but appear significantly more accessible in the U3271C mutant (Fig. 2A). Nucleotides within the anticodon loop, despite not being involved directly in interactions stabilizing secondary or tertiary structure, commonly exhibit less reactivity with chemical probes in folded tRNAs where stacking may occur (20). The pronounced accessibility of these residues in the pathogenic mutant relative to the other constructs studied [and the fact that the intensities of cleavage at these positions are comparable under non-denaturing and denaturing conditions (data not shown)] offers another piece of evidence that the anticodon domain is completely unstructured. The effects of the U3271C mutation on more distal domains of the structure of hs mt tRNA\(^{\text{Leu(UUR)}}\) can also be evaluated from the DEPC cleavage data. A number of D-stem adenines (e.g. A12 and A14) are cleaved with higher frequency when the U3271C mutation is present, indicating that the conformational perturbation introduced by the mutation in the anticodon stem does affect the adjacent domain. However, the adenines of the acceptor stem display only slight increases in cleavage intensities for the U3271C construct, suggesting that the mutant tRNA is not completely denatured.

To explore how the structural effects of the U3271C mutation impacted an aspect of the function of hs mt tRNA\(^{\text{Leu(UUR)}}\), we monitored aminoacylation, an essential step in protein synthesis. Recombinant hs mt LeuRS expressed in E.coli was used to test the reactivity of the transcripts of both WT hs mt tRNA\(^{\text{Leu(UUR)}}\) and the U3271C mutant [previous studies indicated that hs mt tRNA\(^{\text{Leu(UUR)}}\) transcripts are suitable substrates for this enzyme (5)]. This experiment revealed that the rate of aminoacylation for the U3271C mutant was reduced (Fig. 3) relative to WT hs mt tRNA\(^{\text{Leu(UUR)}}\).

The presence of a binding defect for U3271C hs mt tRNA\(^{\text{Leu(UUR)}}\) was elucidated through the measurement of kinetic parameters for the aminoacylation reaction. The U3271C substitution increased the apparent binding affinity (\(K_m\)), but did not affect the catalytic rate constant (\(k_{\text{cat}}\)) for aminoacylation (Table 1). The apparent \(K_m\) of the U3271C hs mt tRNA\(^{\text{Leu(UUR)}}\) mutant was almost 5-fold higher (9.7 \(\mu\)M) than WT (2.3 \(\mu\)M). The \(k_{\text{cat}}\) values for WT hs mt tRNA\(^{\text{Leu(UUR)}}\) and the U3271C mutant were comparable (0.020 and 0.024 s\(^{-1}\), respectively).

To determine whether the structural perturbation detected in chemical probing experiments was the source of the \(K_m\) defect, we also investigated the aminoacylation of the construct containing both the pathogenic U3271C and compensatory A3261G mutations. Indeed, the A3261G/U3271C mutant exhibited markedly increased aminoacylation activity compared with the U3271C mutant, and was even more efficiently aminoacylated than the WT substrate (Fig. 3). The restored activity of the A3261G/U3271C mutant indicates that a loss of secondary structure in the anticodon stem causes the reduced aminoacylation activity for the U3271C mutant. Measurement of the kinetic parameters revealed that \(K_m\) for the A3261G/U3271C mutant was identical to WT hs mt tRNA\(^{\text{Leu(UUR)}}\) (Table 1). Interestingly, the increase in the rate of aminoacylation reflected a 2-fold enhancement in \(k_{\text{cat}}\) (0.047 s\(^{-1}\)) above WT.

**Table 1.** Kinetic parameters for the aminoacylation of hs mt tRNA\(^{\text{Leu(UUR)}}\) constructs

<table>
<thead>
<tr>
<th></th>
<th>(K_m) ((\mu)M)</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(k_{\text{cat}} / K_m) (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.3 (\pm) 0.5</td>
<td>0.020 (\pm) 0.002</td>
<td>1</td>
</tr>
<tr>
<td>U3271C</td>
<td>9.7 (\pm) 0.9</td>
<td>0.024 (\pm) 0.001</td>
<td>0.3</td>
</tr>
<tr>
<td>A3261G/U3271C</td>
<td>2.3 (\pm) 0.4</td>
<td>0.047 (\pm) 0.004</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Figure 3.** Aminoacylation of WT (circles), U3271C (squares) and A3261G/U3271C (diamonds) hs mt tRNA\(^{\text{Leu(UUR)}}\) by hs mt LeuRS. Assays were performed at pH 7.6 at 37°C with 5 \(\mu\)M tRNA and 40 nM hs mt LeuRS.
enzymes rely on the anticodon domain of their cognate tRNAs. Studies of bacterial IleRS and LeuRS indicate that these two enzymes may have a terminal G30/C40 base pair in the anticodon stem; however, other species besides Homo sapiens and Mus musculus have a different base pair in the anticodon stem. For Homo sapiens mt tRNA Leu(UUR), the aminoacylation activity was affected by a mutation in the anticodon stem and loop (25, 26). This behavior is mirrored in the behavior of the E. coli tRNALeu substrate completely lacking the anticodon stem and loop, which is aminoacylated, albeit with 10-fold lower efficiency than for the intact tRNA (27). However, E. coli tRNAleu substrates lacking the anticodon, or with point mutations in the anticodon stem, show very poor substrates for IleRS, with activities reduced by ~10^6 (25, 26). These observations, made during studies exploring the bacterial analogs of tRNAleu and tRNAlen, are mirrored in the behavior of mt tRNAs containing pathogenic mutations in the anticodon stem. Although the structural disruptions that disease-related mutations introduce into mt tRNAs may be similar in different molecules, their effects on tRNA-specific enzymes, such as the aminoacyl-tRNA synthetases, likely differ because of the idiosyncrasies of tRNA recognition.

ACKNOWLEDGEMENTS
This work was supported in part by the Research Corporation, the Dreyfus Foundation NIH (GM63890) and Boston College.

REFERENCES


