Overexpressed mitochondrial leucyl-tRNA synthetase suppresses the A3243G mutation in the mitochondrial tRNA_{Leu(UUR)} gene

HYEJEONG PARK, EDGAR DAVIDSON, and MICHAEL P. KING
Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

ABSTRACT
The A3243G mutation in the human mitochondrial tRNA_{Leu(UUR)} gene causes a number of human diseases. This mutation reduces the level and fraction of aminoacylated tRNA_{Leu(UUR)} and eliminates nucleotide modification at the wobble position of the anticodon. These deficiencies are associated with mitochondrial translation defects that result in decreased levels of mitochondrial translation products and respiratory chain enzyme activities. We have suppressed the respiratory chain defects in A3243G mutant cells by overexpressing human mitochondrial leucyl-tRNA synthetase. The rates of oxygen consumption in suppressed cells were directly proportional to the levels of leucyl-tRNA synthetase. Fifteenfold higher levels of leucyl-tRNA synthetase resulted in wild-type respiratory chain function. The suppressed cells had increased steady-state levels of tRNA_{Leu(UUR)} and up to threefold higher steady-state levels of mitochondrial translation products, but did not have rates of protein synthesis above those in parental mutant cells. These data suggest that suppression of the A3243G mutation occurred by increasing protein stability. This suppression of a tRNA gene mutation by increasing the steady-state levels of its cognate aminoacyl-tRNA synthetase is a model for potential therapies for human pathogenic tRNA mutations.

Keywords: mitochondria; mtDNA; tRNA; suppression; A3243G mutation; tRNA synthetase

INTRODUCTION
More than 130 pathogenic mutations have been identified in the human mtDNA-encoded tRNA genes, including 24 in the tRNA_{Leu(UUR)} gene (MTTL1) (MITOMAP: A Human Mitochondrial Genome Database, http://www.mitomap.org, 2008). The A3243G mutation in the tRNA_{Leu(UUR)} gene was first identified as the genetic cause of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome (Goto et al. 1990) and is one of the most common mitochondrial pathogenic mutations. The A3243G mutation is associated with numerous other clinical outcomes including cardiomyopathy, chronic progressive external ophthalmoplegia, diabetes, and diabetes with deafness (Finsterer 2007). Estimates of the population prevalence of the A3243G mutation range from 1 to 236 per 100,000 individuals (Majamaa et al. 1998; Chinnery et al. 2000; Manwaring et al. 2007; Uusimaa et al. 2007; Schaefer et al. 2008).

Cells with >94% A3243G mutated mtDNA have defects in mitochondrial protein synthesis and severe reductions in respiratory chain activity (Chomyn et al. 1992; King et al. 1992; Dunbar et al. 1996; Janssen et al. 1999). The A3243G mutation changes the adenosine at position 14 of tRNA_{Leu(UUR)} to guanosine. The mutation does not alter the accuracy of processing of the mtDNA polycistronic RNA transcript at tRNA_{Leu(UUR)} (King et al. 1992; Koga et al. 1993; Kaufmann et al. 1996). A3243G mutant cells have decreased steady-state levels of tRNA_{Leu(UUR)} (Chomyn et al. 1992; Janssen et al. 1999; Chomyn et al. 2000; Yasukawa et al. 2000; Park et al. 2003) and can also have reductions in the fraction of tRNA_{Leu(UUR)} that is aminoacylated (El Meziane et al. 1998; Janssen et al. 1999; Borner et al. 2000; Chomyn et al. 2000; Park et al. 2003). The reduction in the fraction of aminoacylated tRNA_{Leu(UUR)} is likely due to a 25-fold decrease in aminoacylation efficiency of A3243G mutant tRNA_{Leu(UUR)} as compared with wild-type tRNA_{Leu(UUR)} (Park et al. 2003). The combined reductions in steady-state tRNA_{Leu(UUR)} levels and the fraction of aminoacylated tRNA_{Leu(UUR)} in mutant cells result in large...
decreases in the levels of aminoaoylated tRNA\textsuperscript{Leu(UUR)} relative to wild-type cells. The A3243G tRNA\textsuperscript{Leu(UUR)} is deficient in post-transcriptional modifications of nucleotides G10 and U34 (Helm et al. 1999; Yasukawa et al. 2000). An unmodified U34, the wobble position of the tRNA anticodon, may affect codon–anticodon interactions and contribute to a reduction in the rate of mitochondrial translation (Yasukawa et al. 2001; Kirino et al. 2004).

There are currently no curative treatments for mitochondrial disorders. While direct manipulation of the mtDNA is presently not possible, advances are being made in genetic therapies for human diseases based upon manipulation of the nuclear genome and its expression. In Saccharomyces cerevisiae, growth defects due to mitochondrial tRNA mutations can be suppressed by extra copies of genes encoding proteins that interact with tRNAs, for example, aminoaacyl-tRNA synthetases or elongation factors, although the mechanisms of suppression have not been investigated (Rinaldi et al. 1997, 2003; Francisci et al. 1998; Feuermann et al. 2003; De Luca et al. 2006). We found previously that A3243G mutant tRNA\textsuperscript{Leu(UUR)} is metabolically unstable and defective in its aminoaacylation capability by mitochondrial leucyl-tRNA synthetase (LeuRS) (Park et al. 2003). We investigated whether overexpression of human mitochondrial LeuRS suppressed translation and respiratory chain defects associated with the pathogenic A3243G mutation in human cells.

RESULTS

Overexpressed human mitochondrial LeuRS suppresses respiratory chain defects

We investigated whether or not human mitochondrial LeuRS can suppress the respiratory chain deficiency associated with the A3243G mtDNA mutation. The transmitochondrial cell line WS227.546 that contains 99.6% A3243G mutated mtDNA (Park et al. 2003) was transfected with an expression construct containing the human mitochondrial LeuRS cDNA. Twenty-three stable transformants with the LeuRS expression construct (LeuRS transformants) were isolated and analyzed, along with 12 control transformants obtained from transfection with the vector alone.

We measured the rates of oxygen consumption of the LeuRS transformants, parental mutant cells, and isogenic wild-type cells. The parental mutant cells had 32 ± 3% (mean±1 SD) of the wild-type rate of oxygen consumption. The rates of oxygen consumption of the 23 LeuRS transformants ranged from 20% to 113% of the wild-type level (Fig. 1A), with 12 having rates of oxygen consumption that were significantly higher than that of parental mutant cells ($P < 0.02$). Only one of 12 control transformants showed a significant increase in the rate of oxygen consumption over that of the parental mutant cells (data not shown). The rates of oxygen consumption of LeuRS transformants were significantly different from those transfected with empty vector ($P < 0.01$).

We correlated the rates of oxygen consumption of LeuRS transformants with the relative amounts of mitochondrial LeuRS in these cells, obtained from quantitative Western analyses using antisera directed against LeuRS. The rates of oxygen consumption in LeuRS transformants were directly proportional to the steady-state levels of mitochondrial LeuRS (Fig. 1A, $r^2 = 0.78$). The two LeuRS transformants with the highest rates of oxygen consumption also had the highest levels of LeuRS. A101, whose rate of oxygen consumption was 96 ± 14% of the wild-type rate, had 15-fold higher steady-state levels of LeuRS than the parental mutant cells (Fig. 1A). Similarly, A104 consumed oxygen at 113 ± 19% of the wild-type rate and had 17-fold higher levels of LeuRS than the parental mutant cells. The LeuRS transformants A101 and A104 were selected for further studies to investigate the mechanism by which increased levels of LeuRS suppressed the A3243G mutation.

Suppression in LeuRS transformants was not due to alterations of mtDNA

Before performing additional studies, we confirmed that quantitative or qualitative alterations in the mtDNA were not responsible for the suppression in LeuRS transformants A101 and A104. We determined the levels of the A3243G mutation in the mtDNA from parental mutant cells and A101 and A104 (Fig. 2). The fraction of A3243G mutated mtDNA in transformants A101 (99.8 ± 0.1%) and A104 (99.8 ± 0.1%) was not decreased from that in parental mutant cells (99.6 ± 0.1%). These mutation levels did not change over the course of subsequent experiments (data not shown). The relative levels of mtDNA were also similar in parental mutant cells (defined as 1), LeuRS transformants (0.90 ± 0.16 for A101; 0.83 ± 0.29 for A104), and isogenic wild-type cells (1.06 ± 0.32).

Since a suppressor mutation for the A3243G mutation was discovered previously in the mitochondrial tRNA\textsuperscript{Leu(CUN)} gene (El Meziane et al. 1998), we sequenced the two mtDNA-encoded tRNA\textsuperscript{Leu} genes in A101 and A104 and parental mutant cells. The sequences of tRNA\textsuperscript{Leu(UUR)} and the tRNA\textsuperscript{Leu(CUN)} genes for each cell line were identical, and the sequence profiles showed no indication of low levels of an altered sequence.

These experiments demonstrated that increased respiratory chain function in LeuRS transformants was not due to increased proportions of wild-type mtDNA, increases in mtDNA copy number, or to a suppressor mutation in an mtDNA-encoded tRNA\textsuperscript{Leu} gene.

Suppression in A101 is reversed by decreasing the level of LeuRS

To confirm the correlation between rates of oxygen consumption and levels of LeuRS in LeuRS transformants
Suppression of a human mitochondrial tRNA mutation

Fig. 1A, LeuRS expression in A101 cells was reduced by siRNA. Preliminary experiments showed a 30%–80% decrease in mitochondrial LeuRS mRNA 48 h after transfection with 0.1–1 nM siRNA directed against mitochondrial LeuRS. These mRNA levels did not increase significantly up to 5 d after transfection (not shown). We transfected A101 cells with 0.1, 0.5, or 1.0 nM siRNA or 1.0 nM nontarget control siRNA and determined the effects on LeuRS protein levels and rates of oxygen consumption. Cells were analyzed five days after transfection to minimize the contributions of LeuRS and mtDNA-encoded proteins synthesized prior to siRNA treatment, since some mtDNA-encoded proteins have a half-life of over 100 h (Hare and Hodges 1982; Grisolia et al. 1985).

In A101 cells transfected with anti-LeuRS siRNA, steady-state LeuRS protein levels were decreased by 45%–95% depending on the amounts of siRNA used. The decreases in LeuRS were accompanied by proportional decreases in the rates of oxygen consumption (Fig. 1B, 25%–75%; r² = 0.98). When the LeuRS level was similar to that of mutant cells, suppression was completely reversed. The LeuRS knockdown experiments confirmed that it was the increased levels of LeuRS protein in A3243G mutant cells that resulted in increased rates of oxygen consumption.

High levels of LeuRS increased the steady-state levels, but not the fraction of aminoacylated tRNA_{Leu(UUR)}

To investigate the mechanism of suppression, we examined the effect of high levels of LeuRS on the steady-state levels of tRNA_{Leu(UUR)} and the proportion that is aminoacylated. The fraction of tRNA_{Leu(UUR)} that was aminoacylated in the LeuRS transfectants A101 (31 ± 4%) and A104 (34 ± 6%) was not significantly different (P > 0.05) from that in the parental mutant cells (30 ± 6%) (Fig. 3A,C). Although these values are lower than that for wild-type cells (55 ± 5%), this was not due to deacylation during the RNA isolation or gel electrophoresis, since all cells contained similar fractions of aminoacylated mitochondrial trna^{Lys} (69 ± 3% of trna^{Lys} aminoacylated in wild-type cells, 69 ± 2% in parental mutant cells, 67 ± 3% in A101, and 68 ± 4% in A104) (Fig. 3B).

We next quantitated the steady-state levels of mitochondrial tRNA_{Leu(UUR)} from Northern blots of deacylated...
Overexpressed LeuRS increased the amount of mitochondrial tRNA<sub>Leu(UUR)</sub> but not the proportion that is aminoacylated. (A,B) Shown are representative Northern blot analyses of the levels of aminoacylated tRNAs in wild-type WS241 (WT), the parental mutant WS227,546 (MT), and LeuRS transformants A101 and A104. Mitochondrial RNAs were isolated under acidic conditions and aminoacylated tRNAs were separated from nonaminoacylated tRNA by acid gel electrophoresis. The aminoacylated and nonaminoacylated species of tRNA<sub>Leu(UUR)</sub> (A) and tRNA<sub>Lys</sub> (B) were detected by Northern blotting. The top band in each phosphorimage corresponds to the indicated aminoacyl-tRNA species and the bottom band to the nonaminoacylated tRNA species. (C) Shown is a histogram of the fraction of aminoacylated tRNA<sub>Leu(UUR)</sub> for the indicated cell lines. The values for transformants A101 and A104 are not significantly different than that for the parental mutant cells (P > 0.05). Error bars indicate 1 SD. (D-E) Shown are representative Northern blot analyses of the steady-state levels of tRNA<sub>Leu(UUR)</sub> and tRNA<sub>Lys</sub> in wild-type (WT), mutant (MT), and LeuRS transformants A101 and A104. tRNA<sub>Leu(UUR)</sub> and tRNA<sub>Lys</sub> were detected by Northern blotting after total mitochondrial RNA was electrophoresed through 20% polyacrylamide-7M urea gels. (F) Shown is a histogram of the quantitation of the steady-state levels of tRNA<sub>Leu(UUR)</sub>. The levels of tRNA<sub>Leu(UUR)</sub> were normalized to the levels of tRNA<sub>Lys</sub> for the indicated cell lines. The steady-state levels of tRNA in cell lines A101 and A104 were increased over those in the parental mutant cell line (*P < 0.01). Error bars indicate 1 SD.

tRNAs and normalized these to the levels of tRNA<sub>Lys</sub> determined from the same blots (Fig. 3D,E). The steady-state level of tRNA<sub>Leu(UUR)</sub> in the parental mutant cells was 53 ± 2% of the wild-type level, while the steady-state levels of tRNA<sub>Leu(UUR)</sub> were 73 ± 7% of the wild-type level in A101 and 67 ± 5% in A104 (Fig. 3F, P < 0.01). Although the high levels of LeuRS in A101 and A104 did not alter the proportion of aminoacylated tRNA<sub>Leu(UUR)</sub>, the increases in steady-state levels of tRNA<sub>Leu(UUR)</sub> resulted in a 44% increase in levels of aminoacylated tRNA<sub>Leu(UUR)</sub> in each transformant above that found in the parental mutant cells.

Rates of mitochondrial translation were not increased in suppressed cells

We examined the effect of overexpression of LeuRS on mitochondrial protein synthesis by analyzing mitochondrial translation during 30-min or 60-min pulse labelings (Fig. 4). For each cell line, the amounts of [35S] that were incorporated into all mtDNA-encoded proteins exhibited a linear increase for at least 60 min, demonstrating that the amounts of [35S] incorporation at 30 min and 60 min represented the rates of mitochondrial protein synthesis. A3243G mutant cells had a rate of mitochondrial translation that was 53 ± 9% (30 min) and 61 ± 12% (60 min) of the rate in wild-type cells. The rates of mitochondrial translation in A101 and A104 cells, which have high levels of LeuRS and wild-type rates of oxygen consumption, were not significantly different from that of the parental A3243G mutant cells (Fig. 4B, P > 0.05). For the 30-min labelings, the rate of translation for A101 was 70 ± 15% of the wild-type rate, and the rate for A104 was 53 ± 9% of the wild-type rate. For the 60-min labelings, the rate in A101 was 70 ± 7% of the wild-type rate and the rate for A104 was 64 ± 20% of wild type.

Steady-state levels of mtDNA-encoded proteins were increased in suppressed cells

Suppression did not result from increased rates of translation; therefore, the effects of LeuRS overexpression on the steady-state levels of mitochondrial translation products were investigated by Western analyses. Although the mtDNA-encoded proteins in A101 and A104 had rates of
translation similar to those in parental A3243G mutant cells, the steady-state levels of several mtDNA-encoded proteins were increased up to threefold (Fig. 5). The steady-state level of COX I in the mutant cells was 39 ± 7% of that in wild-type cells. In suppressing cells this increased to 81 ± 6% (A101) and 52 ± 2% (A104) of wild type. The level of COX II in mutant cells was decreased to 24 ± 2% of the wild-type level, but in suppressing cells COX II was increased to 79 ± 10% (A101) and 63 ± 6% (A104) of wild type. For ND1, the level in mutant cells was 17 ± 5% of the wild-type level but increased to 54 ± 14% (A101) and 56 ± 11% (A104) in suppressing cells.

These data demonstrated a correlation between levels of LeuRS and steady-state levels of mtDNA-encoded proteins. This was further shown in A101 cells transfected with siRNA directed against LeuRS. The siRNA-mediated reductions in steady-state levels of LeuRS (Fig. 1B) were accompanied by decreased steady-state levels of COX I and COX II (Fig. 6). These results verify that the increased levels of these proteins resulted from the increased steady-state levels of LeuRS.

DISCUSSION

We have shown that the respiratory chain deficiency caused by the A3243G mutation in mitochondrial tRNALeu(UUR) was suppressed by increased levels of human mitochondrial LeuRS. The rates of oxygen consumption in suppressed cells were directly proportional to the steady-state levels of LeuRS in mitochondria. Mutant cells with 15-fold increases in mitochondrial LeuRS levels displayed a full recovery of respiratory chain function to wild-type levels, while siRNA-mediated reductions in LeuRS in these cells were accompanied by proportional reductions in respiratory chain function and in steady-state levels of mtDNA-encoded proteins (Figs. 1, 6). In mutant cells over-expressing LeuRS, there were no quantitative or qualitative alterations to the

FIGURE 4. Rates of mitochondrial translation were not increased in suppressed cells. (A) Shown are representative phosphorimages of mitochondrial translation products in wild-type WS241 (WT), mutant WS227.546 (MT), and LeuRS transformants A101 and A104. Mitochondrial translation products were labeled with [35S]-methionine and [35S]-cysteine for 30 min or 60 min and mitochondrial lysates were electrophoresed through Tricine-SDS 10% polyacrylamide gels. (B) Shown are histograms of the rates of mitochondrial translation. The rates of mitochondrial translation in cell lines A101 and A104 were not significantly different from the rates of translation of the parental mutant cell line (P > 0.05). Rates of mitochondrial translation were determined by measuring the total amounts of [35S]methionine and [35S]cysteine incorporated into mitochondrial proteins. Error bars indicate 1 SD.

FIGURE 5. Steady-state levels of mtDNA-encoded proteins increased in cells overexpressing LeuRS. (A) mtDNA-encoded COX I and COX II and nucleus-encoded porin were detected in Western analyses of mitochondrial fractions isolated from the indicated wild-type WS241 cells (WT), mutant WS227.546 cells (MT), and LeuRS transformants A101 and A104. (B) mtDNA-encoded ND1 and nucleus-encoded porin were detected in Western analyses of mitochondrial fractions isolated from the indicated wild-type WS241 cells (WT), mutant WS227.546 cells (MT), and LeuRS transformants A101 and A104. (C) Shown are the steady-state levels of COX I, COX II, and ND1 in the indicated cells normalized to the levels in wild-type cells. A101 and A104 cells showed increases in the steady-state levels of COX I, COX II, and ND1 over the levels in the parental mutant cells (*P < 0.02; **: P < 0.05). Error bars indicate 1 SD.
tRNALeu(UUR) lacks the 5-taurinomethyl uridine (m5U) normally present at the anticodon wobble base, U34 (Yasukawa et al. 2000, 2005; Suzuki et al. 2002), and displays decreased in vitro translation of poly(UUA) and greatly decreased translation of poly(UUG) mRNAs (Kirino et al. 2004). Mutant cells have decreased steady-state levels of tRNA\textsubscript{Leu(UUR)} (Chomyn et al. 1992, 2000; Janssen et al. 1999; Yasukawa et al. 2000; Park et al. 2003) and frequently reductions in the fraction of tRNA\textsuperscript{Aeu(UUR)} that is aminoacylated (El Meziane et al. 1998; Janssen et al. 1999; Borner et al. 2000; Chomyn et al. 2000; Park et al. 2003). The combined reductions in steady-state tRNA\textsubscript{Leu(UUR)} levels and the fraction of aminoacylated tRNA\textsubscript{Leu(UUR)} in mutant cells result in large decreases in the levels of aminoacylated tRNA\textsuperscript{Leu(UUR)} relative to wild-type cells. These reductions could contribute to the decreased rates of mitochondrial translation. Thus, the A3243G mutation may result in decreased rates of translation by decreasing both the amount of tRNA\textsuperscript{Leu(UUR)} and its ability to function in translation. Additionally, there is evidence that misincorporation of amino acids may contribute to defective translation in A3243G mutant cells (Flierl et al. 1997; Janssen et al. 1999).

In this study, the LeuRS-mediated suppression in transformants correlated with increased steady-state levels of tRNA\textsuperscript{Leu(UUR)}. High levels of LeuRS may stabilize metabolically unstable mutant tRNA\textsuperscript{Leu(UUR)} by binding the tRNA, as was hypothesized for the suppression of S. cerevisiae mitochondrial tRNA gene mutations by tRNA-interacting proteins (Rinaldi et al. 1997, 2003; Francisci et al. 1998). Elevated levels of human mitochondrial valyl-tRNA synthetase increase the steady-state levels of mutant tRNA\textsuperscript{Val} (Rorbach et al. 2008). We previously found that LeuRS induces the correct folding of tRNA\textsuperscript{Leu(UUR)} transcripts that lack nucleotide modifications (Sohm et al. 2004). Increased levels of LeuRS may therefore facilitate the correct folding and stabilization of the hypomodified tRNA\textsuperscript{Leu(UUR)}.

Although there was no increase in the proportion of aminoacylated tRNA in A101 and A104, the increased levels of tRNA\textsuperscript{Leu(UUR)} did result in 44% increases in the steady-state levels of aminoacylated tRNA\textsuperscript{Leu(UUR)}. Despite these increases, the rates of mitochondrial protein synthesis were not greater than those in parental mutant cells (Fig. 4). However, the steady-state levels of mitochondrial translation products were up to threefold higher than the levels in the parental mutant cells, an increase sufficient for the full restoration of respiratory chain function (Figs. 1, 5). This suggests that suppression of the A3243G mutation in LeuRS transformants occurred via a mechanism that increases protein stability, rather than by increasing the rates of translation. Increased stability could result from reductions in mistranslation in A3243G mutant cells. Alternatively, increased stability could result through direct stabilization of mtDNA-encoded proteins by LeuRS.

Misincorporation of amino acids resulting from translational errors would likely reduce the stability of mitochondrial proteins, leading to decreases in their steady-state levels and in the levels of respiratory chain enzymes. Inaccurate mitochondrial translation could result from misaminoacylation of mutant tRNA\textsuperscript{Leu(UUR)}, recognition of noncognate codons by mutant tRNA\textsuperscript{Leu(UUR)}, or recognition of UUR codons by noncognate tRNAs. In the cytoplasm, amino acid misincorporation resulting from defective editing by a cytoplasmic tRNA synthetase disrupts protein metabolism and increases the levels of misfolded proteins (Lee et al. 2006; Nangle et al. 2006).

Misaminoacylation of A3243G mutant tRNA\textsuperscript{Leu(UUR)} was not detected in tRNA isolated from mutant cells (Yasukawa et al. 2000). The human mitochondrial LeuRS is more specific than bacterial enzymes even though the human enzyme lacks the editing domain characteristic of bacterial LeuRS (Lue and Kelley 2005). Decreased leucine incorporation into mitochondrial proteins in A3243G mutant cells suggests that misincorporation of leucine at noncognate codons does not occur (Flierl et al. 1997; Janssen et al. 1999). Additionally, mutant tRNA\textsuperscript{Leu(UUR)} does not recognize the UUC Phe codon in an in vitro translation system (Kirino et al. 2004). Therefore it is unlikely that the mutant tRNA\textsuperscript{Leu(UUR)} recognizes noncognate codons.

Low levels of an aminoacyl-tRNA can increase translational errors by allowing misreading of codons by noncognate tRNAs (Parker et al. 1978; Kramer and Farabaugh 2007). In A3243G mutant cells, decreased levels

**FIGURE 6.** SiRNA knockdown of overexpressed LeuRS results in decreased levels of mtDNA-encoded proteins. Shown are the steady-state levels of LeuRS, COX I, COX II, and porin in the A101 transformants transfected with 0.1, 0.5, or 1 nM siRNA directed to LeuRS, and 1 nM nontargeting control (NTC) siRNA.
of aminoacyl-tRNA<sub>Leu(UUR)</sub> combined with the decreased ability of the hypomodified mutant tRNA to decode UUA and UUG codons may therefore allow increased amino acid misincorporation by noncognate tRNAs competing successfully for UUR codons.

Some cell models of the A3243G mutation exhibit wild-type rates of mitochondrial translation but display severe reductions in mitochondrial respiratory chain activity (Flierl et al. 1997; Janssen et al. 1999), suggesting that the quality of proteins synthesized in mitochondria is compromised. Mistranslation is supported by experiments that showed decreased leucine incorporation into mitochondrial proteins and aberrant proteolytic fragments obtained from mtDNA-encoded proteins in A3243G mutant cells (Flierl et al. 1997; Janssen et al. 1999). However, a mass spectrometry analysis did not detect amino acid misincorporation in a tryptic peptide of COX I and a tryptic peptide of COX II isolated from mutant cells (Janssen et al. 2007). The absence of definitive evidence for amino acid misincorporation may reflect the likelihood that affected proteins would be more rapidly turned over than unaffected proteins and would therefore be under-represented in the steady-state population. This could explain why mass spectrometric analysis of mitochondrial proteins, which consisted primarily of long-lived proteins, did not detect amino acid misincorporation (Janssen et al. 2007). In contrast, experiments that showed the presence of aberrant proteolytic digestion products used short-term labelings with [35S]-labeled amino acids to enable the detection of products that may be rapidly degraded (Flierl et al. 1997).

Suppression of mistranslation by increased steady-state levels of LeuRS could occur by several mechanisms and may provide insights into the pathogenic mechanisms of the A3243G mutation. The increased steady-state levels of aminoacylated tRNA<sub>Leu(UUR)</sub> in A101 and A104 may decrease the ability of other tRNAs to successfully compete for UUR codons and thus would diminish amino acid misincorporation. Also, stabilization of mutant tRNA by LeuRS may induce correct folding of mutant tRNA<sub>Leu(UUR)</sub> and enhance the levels of post-transcriptional nucleotide modifications, leading to more accurate mitochondrial protein synthesis. Further investigations to elucidate the precise mechanism by which overexpression of LeuRS increases the steady-state levels of mtDNA-encoded proteins will advance our understanding of the molecular mechanisms of pathogenesis of the A3243G mutation in tRNA<sub>Leu(UUR)</sub>.

MATERIALS AND METHODS

Expression of human mitochondrial LeuRS in mutant cells

Transmitochondrial cell line WS227.546 containing the A3243G mutation, the isogenic wild-type cell line (WS241), and the p<sup>+</sup> 143B206 cell line were described previously (King and Attardi 1989; King et al. 1992; Park et al. 2003). Cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) containing 4.5 mg/mL glucose and 110 μg/mL sodium pyruvate, supplemented with 5% fetal bovine serum. The 143B206 cells were supplemented with 50 μg/mL uridine.

The mammalian expression vector pEE/myc/cyt (Invitrogen) containing the human mitochondrial LeuRS cDNA was described previously (Park et al. 2003). The LeuRS expression vector or the empty vector was introduced into the mutant cells using Effectene (Qiagen). After selection for G418 resistance (400 μg/mL, Invitrogen), stably transformed cells were isolated using cloning rings. After initial selection, LeuRS transformants were maintained in DMEM supplemented with 5% fetal bovine serum and 100 μg/mL G418. G418 was omitted from the medium for molecular genetic and biochemical analyses.

Measurement of rate of oxygen consumption

The rates of oxygen consumption of 5 × 10<sup>5</sup> cells were measured by polarography as described (King et al. 1992). The rates were measured at least twice for each cell line, and Student’s t-test with two-tailed distribution with two-sample equal variance was used for the analyses of LeuRS transformed cells. For the comparison of LeuRS transformants with control transformants, Student’s t-test with two-tailed distribution with two-sample unequal variance was used.

Analyses of mtDNA

The fractions of A3243G mutated and wild-type mtDNAs were quantitated from DNA isolated from transformants, parental mutant, and isogenic wild-type cells as described previously (King et al. 1992). Steady-state levels of mtDNA were quantitated as described (King and Attardi 1989) using four or five cell samples for each cell line obtained on at least two different days. The mtDNA probe was synthesized by primer extension of the M13 universal primer on single-stranded template isolated from M13 clone mp8.M11, which contains nucleotides 3063–3659 of mtDNA (King and Attardi 1989, 1993). Hybridization signals were quantitated using a Typhoon 8600 PhosphorImager with ImageQuant software (GE Healthcare).

The tRNA<sub>Leu(UUR)</sub> and tRNA<sub>Leu(CUN)</sub> genes of LeuRS transformants and parental mutant cells were amplified by PCR (King et al. 1992; El Meziane et al. 1998). Isolated PCR fragments were sequenced at the Kimmel Cancer Center Nucleic Acid Facility, Thomas Jefferson University, using the PCR primers as sequencing primers.

Knockdown of LeuRS by siRNA

A101 cells overexpressing LeuRS were transfected with 0.1–1 nM siRNA pool directed against human mitochondrial LeuRS (ON-TARGETplus siRNA for LARS2: Dharmacon) using Lipofectamine 2000 (Invitrogen). ON-TARGETplus siCONTROL from Dharmacon was used at 1 nM as nontargeting control. Total RNA was prepared from 5 × 10<sup>5</sup> cells using RNeasy miniprep kit (Qiagen) including treatment with RNase-free DNase (Qiagen). The levels of mitochondrial LeuRS mRNA were quantitated by real-time qPCR (SYBR GreenER Two-Step qRT-PCR Kit from Invitrogen) using primer pair LCF (TCTCAGGTGACCACCCCATTTCCA)
and LCR (AGGGCACAAAAAGCATCTCAAAC). TATA-binding protein mRNA was used as a PCR reference [primer pair TBPR (GCTCTCTGATCCCTTGGCCT) and TBPR (GACCTACAGAAGGGCATCA)]. Real-time qPCR analyses were performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

**Determination of in vivo steady-state levels of tRNA\(^{\text{Leu(UUR)}}\)** and aminoacylated tRNA\(^{\text{Leu(UUR)}}\)

Mitochondria were prepared from 5 \( \times 10^7 \) cells, and mitochondrial RNA was isolated at pH 5.0 (Enriquez and Attardi 1996). Five micrograms of each RNA sample was electrophoresed at 4\(^\circ\)C through 6.5% polyacrylamide-7M urea gels, pH 5.0, with circulating buffer. RNA was electropherstated onto Zeta-probe membrane (Bio-Rad). In vitro-transcribed anti-sense tRNA\(^{\text{Leu(UUR)}}\) or tRNA\(^{\text{lys}}\) were used as probes (Park et al. 2003). Hybridization signals for aminoacylated and nonaminoacylated tRNA\(^{\text{Leu(UUR)}}\) were quantitated with a PhosphorImager. The Northern blots were reprobed to determine the fraction of aminoacylated mitochondrial tRNA\(^{\text{lys}}\). Levels of aminoacylated tRNA were determined from three independent RNA isolations with at least two Northern analyses of each RNA preparation. Data were analyzed using Student’s t-test with two-tailed distribution with two-sample equal variance.

To determine the steady-state levels of tRNA\(^{\text{Leu(UUR)}}\), total mitochondrial RNA samples were decacylated by heating at pH 8.5 at 80\(^\circ\)C for 5 min. Samples were electrophoresed through 20% polyacrylamide-7M urea gels at room temperature and were transferred onto Zeta-probe membrane. Quantitation of tRNA\(^{\text{Leu(UUR)}}\) and tRNA\(^{\text{lys}}\) was performed as described above. Student’s t-test with two-tailed distribution with two-sample equal variance was used for statistical analyses.

**Analyses of mitochondrial protein synthesis**

Metabolic labeling of mtDNA-encoded proteins was performed as described (Hoffbuhr et al. 2000). Cells were incubated in DMEM lacking methionine and cysteine and containing 100 \( \mu \)g/mL emetine (Sigma) to inhibit cytoplasmic protein synthesis and labeled for 30 min or 60 min in the presence of 250 \( \mu \)Ci of \[^{35}S\] Easy Tag EXPRE\(^{\text{SPO}}\) Protein Labeling Mix (>1000 Ci/m mole, Perkin-Elmer). Mitochondria were isolated from labeled cells by homogenization and differential centrifugation (Hoffbuhr et al. 2000). Protein concentrations were determined using the DC protein assay (Bio-Rad). Forty micrograms of protein was electrophoresed at 4\(^\circ\)C for 5 min. Samples were electrophoresed through 20% polyacrylamide-7M urea gels (Schagger and von Jagow 1987). The amounts of \[^{35}S\]-labeled mitochondrial proteins were quantitated using a PhosphorImager. The rates of mitochondrial translation were determined from two independent protein labeling experiments using at least two gel analyses of each labeling.

**Western analyses of mitochondrial proteins**

Mitochondria were prepared from exponentially growing cells using a Qproteome Mitochondrial Isolation Kit (Qiagen). For LeuRS knockdown studies, mitochondria were prepared from cells recovered after measuring their rates of oxygen consumption. Mitochondria from 2 \( \times 10^8 \) cells were electrophoresed through a 4%–12% gradient NuPAGE Novex Bis-Tris gel (Invitrogen), electrotransferred to Immobilon FL (Millipore) (Towbin et al. 1979), and incubated with the following antibodies. A polyclonal antiserum directed against human mitochondrial LeuRS was obtained from a rabbit using recombinant LeuRS expressed in and purified from *Escherichia coli* (Park et al. 2003) as antigen, according to the standard protocol at Pocono Rabbit Farm & Laboratory. Mouse monoclonal antibodies against mtDNA-encoded subunits of cytochrome c oxidase, COX I (1D6-E1-A8) and COX II (12C4-F12), were from Invitrogen. The mouse monoclonal antibody against the mtDNA-encoded NADH dehydrogenase subunit ND1 (209) was from Santa Cruz Biotechnology. The rabbit polyclonal antibody directed against porin (ab15895) was from abcam. Anti-rabbit IgG H\&L, Fluor 647 conjugate and FAM labeled anti-mouse IgG (Anaspec) were used as the secondary antibodies. The signal was quantitated with a Typhoon 9400 (GE Healthcare). The amounts of LeuRS, COX I, COX II, and ND1 were normalized to the amount of porin for each sample. The steady-state levels were determined from two independent mitochondrial preparations with at least two Western analyses of each mitochondrial preparation. Intensities of fluorescent signals were within the linear dynamic range as determined by Western blots of serial dilutions of mitochondrial extracts from A101. Student’s t-test with two-tailed distribution with two-sample equal variance was used for statistical analyses.

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Suppression of a human mitochondrial tRNA mutation


Overexpressed mitochondrial leucyl-tRNA synthetase suppresses the A3243G mutation in the mitochondrial tRNA\text{Leu(UUR)} gene

Hyejeong Park, Edgar Davidson and Michael P. King

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