Comparative analysis of the pathogenic mechanisms associated with the G8363A and A8296G mutations in the mitochondrial tRNALys gene

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Two mutations (G8363A and A8296G) in the mtDNA (mitochondrial DNA) tRNALys gene have been associated with severe mitochondrial diseases in a number of reports. Their functional significance, however, remains unknown. We have already shown that homoplasmic cybrids harbouring the A8296G mutation display normal oxidative phosphorylation, although the possibility of a subtle change in mitochondrial respiratory capacity remains an open issue. We have now investigated the pathogenic mechanism of another mutation in the tRNALys gene (G8363A) by repopulating an mtDNA-less human osteosarcoma cell line with mitochondrial DNA (mtDNA) tRNALys gene have been associated with severe mitochondrial diseases with defective respiratory-chain enzyme activities and low oxygen consumption, indicating a severe impairment of the oxidative phosphorylation system. Generation of G8363A cybrids within a wild-type or the A8296G mtDNA genetic backgrounds resulted in an important alteration in the conformation of the tRNALys, not affecting tRNA steady-state levels. Moreover, mutant cybrids have an important decrease in the proportion of aminoacylated tRNALys and, consequently, mitochondrial protein synthesis is greatly decreased. Our results demonstrate that the pathogenicity of the G8363A mutation is due to a change in the conformation of the tRNA that severely impairs aminoacylation in the absence of changes in tRNA stability. The only effect detected in the A8296G mutation is a moderate decrease in the aminoacylation capacity, which does not affect mitochondrial protein biosynthesis.

Key words: aminoacylation, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibres (MERRF), mitochondrial DNA (mtDNA) mutation, mitochondrial tRNALys gene.

INTRODUCTION

Mitochondria generate most of the cellular ATP by OXPHOS (oxidative phosphorylation). The OXPHOS system is composed of five different complexes embedded in the inner mitochondrial membrane (complexes I–V) and two small electron carriers, namely ubiquinone and cytochrome c [1,2]. Mammalian mitochondria are endowed with their own semi-autonomous genetic system [mtDNA (mitochondrial DNA)] that encodes a limited number of essential genes for OXPHOS biogenesis: 13 polypeptides of complex I [ND1–ND6 and ND4L (subunits 1–6 and 4L of NADH:ubiquinone oxidoreductase)], complex III (cytochrome b), complex IV (cytochrome c oxidase subunits I–III) and complex V (ATPases 6–8), as well as the RNA components of the translational apparatus, two rRNAs (12 and 16 S) and 22 tRNAs. The rest of the structural subunits of the OXPHOS system and all the factors involved in OXPHOS assembly and regulation, mtDNA expression and mtDNA maintenance are encoded in the nucleus, translated into cytoplasmic ribosomes and imported to their final mitochondrial location [3]. Therefore OXPHOS defects can be produced by mutations in mitochondrially encoded genes, nuclear genes encoding OXPHOS subunits or in nuclear genes encoding factors directly or indirectly involved in OXPHOS regulation [4–6].

To date, more than 200 mtDNA mutations have been implicated in the pathogenesis of mitochondrial diseases with defective OXPHOS, including large mtDNA rearrangements and point mutations in tRNA, rRNA and protein-coding genes [7]. Mitochondrial diseases are usually multisystemic disorders and produce devastating encephalomyopathies since they affect predominantly high energy-demanding tissues such as the nervous system and skeletal and cardiac muscles [8]. Mutations in protein-coding genes affect a single complex of the OXPHOS system. In contrast, mutations in tRNA genes impair the mitochondrial translation system and therefore affect four of the five OXPHOS complexes, producing combined enzyme deficits of the respiratory chain [9]. Two point mutations, A3243G in the tRNALeu(UUR) gene and A8344G in the tRNA13S gene and A8344G in the tRNA13S gene, associated with two well-defined clinical syndromes, MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged-red fibres) respectively, are relatively frequent and have been extensively characterized [10–13]. However, the relationship between genotype (mutation) and phenotype (clinical symptoms) has not been understood so far [8]. Heterogeneity is paramount: the same mutation can be associated with diverse clinical manifestations and different mutations can produce the same symptoms. Dosage and distribution of the altered tRNAs in the different tissues of the organism cannot simply account for these phenomena. In addition, the pathogenic mechanisms of the different mtDNA mutations are not fully understood and have not been studied in detail for most of the mutations described so far.

Abbreviations used: FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged-red fibres; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation.

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We were the first to report on a family segregating the MERRF syndrome in association with a double mutation, A8296G and G8363A, in the tRNA\textsuperscript{\text{Lys}} gene [14]. The A8296G mutation was practically homoplasmic in all the investigated family members, whereas the proportion of the G8363A mutation correlated with the severity of the phenotype. Both mutations disrupt highly conserved base pairs in the aminoacyl stem of the tRNA\textsuperscript{\text{Lys}}. The A8296G mutation has also been independently described in association with Type II (non-insulin-dependent) diabetes mellitus [15,16], hypertrophic cardiomyopathy [17] and the MELAS syndrome [18]. On the other hand, the G8363A mutation had already been associated with MERRF [19], maternally inherited cardiomyopathy and hearing loss in two pedigrees [20], and with spinocerebellar ataxia and multiple symmetric lipomatosis [21]. More recently, we reported that transmitochondrial hybrids homoplasmic for the A8296G mutation had a normal OXPHOS function [22], a finding which challenges a clear-cut pathogenic role. In the present study, we have generated and extensively characterized transmitochondrial hybrids homoplasmic for the single G8363A mutation and the A8296G/G8363A double mutation. We have found that the A8296G mutation decreases moderately the aminoacylation capacity of the tRNA\textsuperscript{\text{Lys}}, although it does not have consequences for mitochondrial protein biosynthesis. In contrast, biochemical and molecular results demonstrate that the G8363A mutation impairs mitochondrial protein synthesis and compromises severely the function of OXPHOS by altering the conformation of the tRNA\textsuperscript{\text{Lys}} and decreasing drastically its aminoacylation capacity. This phenotype is not modified by the presence of the A8296G mutation.

**MATERIALS AND METHODS**

**Cell lines and media**

Two fibroblast cell lines were established from diagnostic skin biopsies obtained from the mother of an MERRF patient harbouring the A8296G-G8363A double mutation [21] and from a patient suffering from cerebellar ataxia and lipomatosis harbouring approx. 80% of the G8363A mutation in the mtDNA tRNA\textsuperscript{\text{Lys}} gene [21]. Cells were grown in DMEM (Dulbecco’s modified Eagle’s medium), supplemented with 10% FBS (fetal bovine serum) and 50 \(\mu\)g/ml uridine. The human osteosarcoma cell line 143 B (TK-), was grown in DMEM supplemented with 10% FBS, 100 \(\mu\)g/ml bromodeoxyuridine and 50 \(\mu\)g/ml uridine. The human osteosarcoma cell line 143 B (TK-) was grown in DMEM supplemented with 10% FBS, 100 \(\mu\)g/ml bromodeoxyuridine and 50 \(\mu\)g/ml uridine. Its mtDNA-less derived 206 cell line (\(\rho^0\)) was grown in DMEM supplemented with 5% FBS and 50 \(\mu\)g/ml uridine.

**Establishment of transmitochondrial hybrids**

The patients’ derived enucleated fibroblasts (0.5 \(\times\) 10\(^6\) cells) were fused to 10\(^6\) \(\rho^0\)-206 cells as described previously [23]. Transmitochondrial hybrid clones (cybrids) were grown in DMEM supplemented with 10% dialysed FBS and 100 \(\mu\)g/ml bromodeoxyuridine. Individual clones were isolated and the mtDNA was analysed for both A8296G and G8363A transitions by the AciI/Asp700 RFLP (restriction-fragment-length polymorphism) method as described in [14].

**Respiratory function assays**

The rate of oxygen consumption was measured as described previously [22] at 37\(^\circ\)C using a Clark-type platinum polarographic electrode using 3–5 \(\times\) 10\(^6\) exponentially intact cells growing in 2 ml of complete DMEM without glucose. To assess respiratory chain enzymes in cells, approx. 5 \(\times\) 10\(^6\) cells were harvested by trypsinization, washed twice with phosphate buffer and resuspended in 2 ml of Mops/sucrose buffer. Digitonin (200 \(\mu\)g) was added and incubation was performed on ice for 5 min. After centrifugation at 5000 \(g\) for 3 min, the pellet was resuspended in 0.5 ml of 10 mM potassium phosphate buffer (pH 7.4) and freeze-thawed twice just before evaluating the enzyme complexes. The activities of rotenone-sensitive NADH-coenzyme Q1 reductase (complex I), succinate dehydrogenase (part of complex II), antimycin-sensitive ubiquinol cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and citrate synthase were measured in duplicate in each assay, and expressed in terms of nmol of substrate \(\cdot\) min\(^{-1}\) \(\cdot\) (mg of protein\(^{-1}\)). We did not normalize the respiratory chain activities for the activity of citrate synthase since this matrix enzyme is also expressed at high levels in the \(\rho^0\) mitochondria of the host cells. Protein concentration was measured by the method of Lowry as described in [22].

**Quantification of mitochondrial DNA**

To extract total DNA, cells were pooled, washed with PBS, resuspended in 1 ml of lysis buffer (100 mM NaCl, 0.5% SDS, 10 mM Tris and 25 mM EDTA, pH 8.0) and incubated in the presence of 0.2 mg/ml proteinase K at 42\(^\circ\)C for 24 h. DNA was extracted by standard methods [24]. Quantification of mtDNA in fibroblasts, cybrid cell lines and \(\rho^0\) cells was performed by slot-blot and Southern-blot analyses. As a probe, we used an \([\alpha-\text{\textsuperscript{32P}}]\text{dCTP}\)-labelled long mtDNA PCR-amplified fragment (using an oligonucleotide from nucleotide positions 13420–13443 as forward primer and an oligonucleotide from nt 8200–8225 as reverse primer). As loading control, the same membranes were incubated with an \([\alpha-\text{\textsuperscript{32P}}]\text{dCTP}\)-labelled 18S rRNA probe. Quantitative analysis was performed by phosphomager detection.

**Quantification of mitochondrial tRNAs**

Total RNA (5 \(\mu\)g) extracted from cybrid cell lines was incubated at 90\(^\circ\)C for 5 min, electrophoresed through a 12% polyacrylamide–7 M urea gel in 1 \(\times\) TB (Tris/borate/EDTA) buffer and electroblotted on to a Zeta-probe membrane at 300 mA for 30 min in 1 \(\times\) TAE (Tris/acetate/EDTA) buffer. Hybridization was performed at 42\(^\circ\)C for 24 h in 6 \(\times\) SSC (1 \(\times\) SSC = 0.15 M NaCl/0.015 M sodium citrate), 1 \(\times\) Denhardt’s solution, 0.5% SDS, 0.05% sodium pyrophosphate and 100 \(\mu\)g/ml denatured salmon sperm DNA.

The tRNA\textsuperscript{\text{Lys}} was detected using a synthetic 20-mer oligonucleotide (spanning mtDNA nucleotide positions 8361–8342), which had been labelled with \([\gamma\text{-\textsuperscript{32P}}]\text{ATP}\) at its 5’-end. After hybridization, the blot was washed twice for 30 min with 6 \(\times\) SSC, 0.1% SDS and 0.05% sodium pyrophosphate at 42\(^\circ\)C. The membrane was stripped out and rehybridized under the same conditions with a \([\gamma\text{-\textsuperscript{32P}}]\text{P}\)-5’-end-labelled 21-mer oligonucleotide corresponding to mtDNA positions 597–577 of the tRNA\textsuperscript{\text{Phe}} gene. The ratio of tRNA\textsuperscript{\text{Lys}} to tRNA\textsuperscript{\text{Phe}} was calculated by quantification of the respective signals either by densitometry or phosphomager analysis.

**Single-strand conformational analysis of mitochondrial tRNAs**

Total RNA (8 \(\mu\)g) extracted from cybrid cell lines was electrophoresed through a 6% non-denaturing polyacrylamide (60:1) gel in 1 \(\times\) TBE buffer as described previously [25]. To detect the tRNA\textsuperscript{\text{Lys}} and tRNA\textsuperscript{\text{Phe}}, we used the same labelled probes and hybridization conditions described above.

**Analysis of the aminoacylation of mitochondrial tRNAs**

Total RNA from cells was extracted under acidic conditions to preserve aminoacylated tRNAs using a previously described
method [26] with minor modifications. Total RNA (3 µg) was electrophoresed through an 8% non-denaturing polyacrylamide (29:1) gel in 0.1 M sodium acetate (pH 5.0) buffer at 100–200 V for 18–20 h, with the buffer being continuously recirculated at 4°C. To deacetyl tRNAs, an aliquot of total RNA was boiled for 10 min (pH 8.0) before electrophoresis. Then, the gel was electroblotted on to a Zeta-probe membrane and hybridized with specific tRNA<sub>lys</sub> and tRNA<sub>phe</sub> probes.

Mitochondrial protein synthesis

To analyse the translation products of mitochondrial genes in the different cybrid cell lines, subconfluent cultures were labelled with [35S]methionine in the presence of 100 µg of emetine · (ml of medium)<sup>−1</sup> by the method of Chomyn [27] with minor modifications. Cells were labelled with 125 µCi of [35S]methionine for 2 h, washed with phosphate buffer and harvested. After centrifugation at 400 g, cells were resuspended in lysis buffer [20 mM Tris/HCl (pH 7.5), 0.1 M SDS, 5 mM MgCl<sub>2</sub>, and 1 mM PMSF]. Total protein (100 µg) was electrophoresed and detected by autoradiography.

RESULTS

Generation of transmitochondrial cybrids

In a previous study, we generated transmitochondrial cybrids by fusing the human mtDNA-less ρ<sup>−</sup>-206 cell line with fibroblasts obtained from an MERRF patient containing >95% of the A8296G mtDNA mutations (the present study). Since the A8296G mutation is virtually homoplasmic in the patient’s derived skin fibroblasts, it was impractical to continue the isolation of clones to obtain cybrids homoplasmic for the G8363A mutation alone. To achieve this, we generated additional transmitochondrial cybrids fusing the human mtDNA-less ρ<sup>−</sup>-206 cell line with fibroblasts obtained from a patient suffering from spinocerebellar ataxia and multiple lipomas, containing approx. 80% of the G8363A mutation. We isolated several independent clones homoplasmic for the G8363A mutation. We used as control a cybrid cell line obtained by the fusion of the ρ<sup>−</sup>-206 cell line with fibroblasts homoplasmic for wild-type mtDNA. The genetic characterization of the different cybrid cell lines was performed by PCR–RFLP as described previously [14] (results not shown). All the clones contained similar amounts of mtDNA as clones containing wild-type mtDNA, suggesting that there is no negative selection against the mutation.

The G8363A mutation produces severe biochemical defects of the respiratory chain in cybrid cell lines

We investigated the respiratory capacity of at least two independent clones harbouring either wild-type mtDNA or the specific kind of mutations by measuring the specific activities of the mitochondrial respiratory chain complexes I–IV and the rate of oxygen consumption in intact cells. We have reported previously that homoplasmic single A8296G mutant clones showed normal values in the respiratory chain activities and oxygen consumption, comparable with those of homoplasmic wild-type mtDNA clones [22]. In contrast, cybrid clones homoplasmic for the G8363A single mutant or A8296G-G8363A double mutant showed a clear defect of the mitochondrial respiratory chain complexes I, III, and IV, with a mean residual activity of 36% (complex I) and 10% (complexes III and IV) in mutant clones when compared with wild-type lines or homoplasmic >95% A8296G clones (Table 1). Oxygen consumption was severely impaired in these clones (Table 1; mean decrease was 95%, P < 0.001).

Mitochondrial tRNA<sub>lys</sub> steady-state levels and conformation analysis

The potential effect of the G8363A mutation on the steady-state level of the tRNA<sub>lys</sub> was investigated by Northern-blot analysis. Total RNA was extracted from mutants and wild-type cybrids, electrophoresed on agarose–formaldehyde gels and probed with a DNA fragment containing the tRNA<sub>lys</sub> gene labelled with [α-<sup>32</sup>P]-dCTP. As control, we have quantified the steady-state level of the tRNA<sub>phe</sub> using a specific tRNA<sub>phe</sub> probe (Figure 1). The ratios between the signals obtained with a tRNA<sub>lys</sub> gene probe and a tRNA<sub>phe</sub> gene probe used as control were similar in all cases, indicating that neither the G8363A mutation alone nor the A8296G-G8363A double mutations significantly decrease the stability of the tRNA<sub>lys</sub>. To find out whether the mutations introduce changes in the conformation of the tRNA, we performed non-denaturing single-strand analysis as described in the Materials and methods section. In contrast with the lack of effect observed in tRNA stability, the G8363A mutation produces a drastic shift in the mobility of the tRNA<sub>lys</sub>, and practically the entire tRNA shows a delayed migration (Figure 2). Interestingly, the tRNA<sub>lys</sub> harbouring the A8296G-G8363A double mutations showed a slightly increased delay in the electrophoretic migration compared to 100% A8296G cell lines, by the two-tailed, Mann–Whitney U test for unpaired samples. Oxygen consumption is expressed in terms of μmol · min<sup>−1</sup> · cell<sup>−1</sup> and mean specific enzyme activity is expressed in terms of (nmol of substrate) · min<sup>−1</sup> · (mg of protein)<sup>−1</sup>.

Table 1 Respiratory function assays in cybrid cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Oxygen consumption</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex III</th>
<th>Complex IV</th>
<th>Citrate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (n = 6)</td>
<td>4.6 ± 0.9</td>
<td>14.3 ± 1.2</td>
<td>10.8 ± 3.5</td>
<td>104.0 ± 25</td>
<td>54 ± 8</td>
<td>200 ± 26</td>
</tr>
<tr>
<td>8363 (n = 2)</td>
<td>0</td>
<td>5.6 ± 0.8</td>
<td>10.2 ± 1.6</td>
<td>150.0 ± 4.5</td>
<td>51.2 ± 2.8</td>
<td>188 ± 22</td>
</tr>
<tr>
<td>8296 (n = 9)</td>
<td>4.5 ± 0.8</td>
<td>11.8 ± 3.4</td>
<td>9.1 ± 4.0</td>
<td>93.1 ± 24</td>
<td>57 ± 3</td>
<td>183 ± 22</td>
</tr>
<tr>
<td>8296/8363 (n = 6)</td>
<td>0.1 ± 0.1*</td>
<td>3.9 ± 1.0*</td>
<td>11.1 ± 3.0</td>
<td>5.5 ± 4.5*</td>
<td>6.4 ± 2.6*</td>
<td>188 ± 22</td>
</tr>
<tr>
<td>143B (n = 3)</td>
<td>4.4 ± 0.5</td>
<td>18.1 ± 3.5</td>
<td>14.1 ± 5.7</td>
<td>102 ± 23</td>
<td>70 ± 20</td>
<td>189 ± 30</td>
</tr>
<tr>
<td>143B/206 (r&lt;sup&gt;−&lt;/sup&gt;) (n = 3)</td>
<td>0</td>
<td>1.5 ± 0.4</td>
<td>11.1 ± 0.8</td>
<td>2 ± 1.8</td>
<td>1 ± 0.8</td>
<td>167 ± 15</td>
</tr>
</tbody>
</table>
hybridized with the tRNALys radiolabelled probe and autoradiographed. The same filter was
polyacrylamide gel under non-denaturing conditions, transferred on to a nylon membrane,
result clearly demonstrated that the conformation of the tRNALys
analyses. No statistically significant differences were detected (the Kruskal–Wallis method).

experiments from each cybrid cell line were quantified by densitometric and/or phosphoimager
characterized tRNALys mutations is the presence of abnormal
mitochondrially encoded polypeptides. A landmark of previously
premature termination of COI (subunit 1 of cytochrome
In particular, one of them, designated pMERRF, is derived from
harbouring the A8296G mutation is similar to wild-type and
peptides. These results confirm that the behaviour of the mtDNA
长寿(411-431) in transmitochondrial wild-type and
mitochondria), total RNA under conditions that preserved the amino-
aclylation capacity of the tRNALys, we isolated, from purified mi-
tochondrial protein synthesis.
indicate that the G8363A mutation has a profound effect on mito-

tRNALys aminoacylation

To determine whether the G8363A mutation impairs the amino-
acylation capacity of the tRNA^{Lys}, we isolated, from purified mi-
contrast with homoplasmic A8296G cybrids [22], homoplasmic
G8363A and double mutant A8296G-G8363A cybrids showed a
marked decrease in the incorporation of [35S]methionine into the
mitochondrially encoded polypeptides. A landmark of previously
characterized tRNA^{Lys} mutations is the presence of abnormal
polypeptides generated by premature termination of translation.
In particular, one of them, designated pMERRF, is derived from
premature termination of COI (subunit 1 of cytochrome c oxidase) and
migrates below the ND4 polypeptide [10]. Although we have
not detected a prominent truncated product in mutant cells, in
the mitochondrial translation pattern of G8363A and A8296G-
G8363A cybrids there are relative increases in the radioactive
signal in the region that migrates below the ND4 polypeptide (indicated by an asterisk in Figure 3), suggesting that, in the
mutant cybrids, there are some accumulations of truncated polypeptides. These results confirm that the behaviour of the mtDNA
harbouring the A8296G mutation is similar to wild-type and
did not detect a prominent truncated product in mutant cells, in

with the tRNA^{Phe} harbouring the single G8363A mutation. This
result clearly demonstrated that the conformation of the tRNA^{Lys}
is not changed by the A8296G mutation and its alteration is due
to the G8363A mutation. Furthermore, the A8296G mutation
has some synergistic role in the change induced by the G8363A mutation.

Synthesis of mitochondrial proteins

Figure 3 shows a representative experiment to investigate the
effect of the mutations on the mitochondrial translation capacity. In contrast with homoplasmic A8296G cybrids [22], homoplasmic
G8363A and double mutant A8296G-G8363A cybrids showed a
marked decrease in the incorporation of [35S]methionine into the
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DISCUSSION

To date, 12 different mutations in the mtDNA tRNA^{Lys} gene have
been described in association with an array of mitochondrial
diseases [7]. We first documented a A8296G-G8363A double
mutant in the mtDNA tRNA^{Lys} gene in a family with MERRF [14].
Both mutations have been reported in isolation in independent
gen consumption and the mitochondrial synthetic capacity in plasmic G8363A and A8296G-G8363A mutations, whereas oxy-
tently, we observed a marked decrease in oxygen consumption and only nuclear DNA-encoded subunits) remained normal. Consis-
tery-chain complexes I, III and IV, which contain mtDNA-
muts caused a drastic decrease in the activities of the respira-
tory function. In this regard, it has been 
addition, the steady-state levels of the flanking mRNAs (COII and ATPases 6–8) are similar in wild-type and mutant cybrids (results 
not shown), suggesting that the processing of the heavy-strand pre-mRNA is correct at the tRNA<sup>Lys</sup> site.

If our results demonstrate unequivocally that the G8363A mutation is pathogenic, the potential pathogenicity of the A8296G mutation is still puzzling. The high degree of conservation of this specific nucleotide in the tRNA<sup>Lys</sup> gene during evolution, together with the fact that several pedigrees are found in which the mutation segregates with the affected phenotype and the absence of the mutation in a large number of ethnically matched normal controls contrast with the apparent lack of a disease-causative role in a commonly used experimental cell system. The modest decrease in the aminoacylation capacity of the tRNA<sup>Lys</sup> that we observed does not suffice to affect the respiratory function. It is possible that the mutation achieves its pathogenic effects when present within a specific nuclear background or, alternatively, that it impairs mitochondrial physiology by a mechanism not related to the OXPHOS function. In this regard, it has been observed that cells with the A3243G mutation, a well-known pathogenic mutation in the mitochondrial tRNA<sub>Lys(UUR)</sub> gene, produced an impaired mitochondrial translation machinery that depends heavily on the specific nuclear background (reviewed in [29]). The A3243G mutation is related to a profound decrease in the steady-state level and aminoacylation capacity of the tRNA<sub>Lys(UUR)</sub> in some cases [11,30], whereas it decreases the association of mRNA with mitoribosomes in other cases [31]. However, aminoacylation is practically unaffected in some other cell lines and impaired mitochondrial protein synthesis occurs because of misreading of the phenylalanine codon due to a decrease in the modification of the U present in the wobble position of the anti-codon [32]. Moreover, it has been described in a different cell system that a defect in processing of the pre-mRNA at the tRNA<sub>Lys(UUR)</sub> site leads to the accumulation of an inter-
mediary transcript that might contribute to the impairment of mitochondrial protein synthesis [13,33]. Finally, and most interest-
ingly, an RNA circularization technique has demonstrated that the decrease in total and/or aminoacylated tRNA<sub>Lys(UUR)</sub> levels also varies considerably in vivo when muscle biopsied samples
from MELAS patients are used [34]. This does not apply to biopsied material from MERRF patients harbouring mutations in the tRNA^{Lys} gene [34]. These results suggest that trans-acting factors and/or compensatory mechanisms that depend on the nuclear background modulate the expression of the mtDNA mutations. In this regard, established cybrid cell lines with defined molecular pathogenic mechanisms may be critical for identifying these factors in the near future [29].

This work was supported by grants from Ministerio de Educaci´on y Ciencia of Spain, Comunidad de Madrid and Instituto de Salud Carlos III, Redes de centros RCMN (C03/08) and Temalicas (G03/011). The work in F. M. S.’s laboratory was partially supported by the Italian Ministry of Health. We thank M. Zeviani for his kind collaboration during the first stage of this work.

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Received 7 June 2004/28 October 2004; accepted 22 November 2004 Published as BJ Immediate Publication 22 November 2004, DOI 10.1042/BJ20040949

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