Functional and Molecular Mitochondrial Abnormalities Associated with a C → T Transition at Position 3256 of the Human Mitochondrial Genome

THE EFFECTS OF A PATHOGENIC MITOCHONDRIAL tRNA POINT MUTATION IN ORGANELLE TRANSLATION AND RNA PROCESSING*

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We have previously identified a mitochondrial DNA polymorphism (a C → T transition at position 3256, within the mitochondrial tRNALeu(UUR) gene) in a patient with a multisystem disorder. Although there were several indicators suggesting a pathogenic role for this mtDNA polymorphism, its heteroplasmic nature made functional and molecular studies difficult to interpret. We have now fused enucleated fibroblasts from the patient with a mtDNA-less cell line to generate transmi- tochondrial cybrids harboring different proportions of mutated and wild-type mtDNA. Individual clones harboring essentially 100% wild-type or >99% mutated mtDNAs were characterized and studied for respiratory capacity, respiratory chain enzymes activity, mitochondrial protein synthesis, and RNA steady-state levels and processing. Our results showed that cell lines containing exclusively mutated mtDNAs respire poorly, over- produce lactic acid, and have significantly impaired ac- tivity of respiratory complexes I and IV. Molecular studies showed that mutant clones have a decrease in steady-state levels of mitochondrial tRNALeu(UUR), and a partial impairment of mitochondrial protein synthesis and steady-state levels, suggesting that these molecular abnormalities are involved in the pathogenetic mechanism of the mtDNA 3256 mutation.

Mutations in the mtDNA have been implicated in the pathogenesis of different clinical syndromes (1, 2). In the past 7 years, pathogenic large-scale rearrangements as well as point mutations in the human mtDNA have been described, most of them heteroplasmic (i.e. mutated mtDNA co-existing with the wild-type mtDNA). Point mutations in mitochondrial tRNA genes seem to be particularly frequent in neuromuscular disorders, possibly because of their generalized effect on mitochondrial protein synthesis, and consequent impairment of multiple oxidative phosphorylation enzyme complexes (3). Several patho- genic mutations in the mitochondrial tRNALeu(UUR) gene have been described (3–10). One of these, an A → G transition at nucleotide 3243 (numbers according to Anderson et al. (11)), is seen most frequently in patients with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS).1 The functional consequences of the 3243 mutation have been extensively analyzed (12–14), though no final conclusion could be drawn from these observations. The 3243 mutation segregates with a respiration dysfunction and a partial impairment in mitochondrial protein synthesis, but the molecular mechanisms associated with these abnormalities are not understood. Because cells with essentially 100% mutated mtDNAs have higher levels of an intermediate transcript (termed RNA1 Env(UUR)) and two adjacent RNAs, it was suggested that this processing abnormality would either affect the RNA from adequate levels of mature transcripts (12) or that RNA 19 would interfere with translation by “stalling” mitochondrial ribosomes (15). Similar processing abnormalities have been observed in association with two other mutations in the mitochondrial tRNALeu(UUR) gene (7, 16), suggesting that abnormal processing could be a common pathogenetic mechanism associated with mutations in this particular mitochondrial gene.

We have described a patient with a multisystem mitochon- drial disorder including: progressive external ophthalmoplegia, seizures, diabetes, cardiomyopathy, and retinopathy, harboring yet another mutation in the mitochondrial tRNALeu(UUR) gene at position 3256. More recently, a second family with MELAS, harboring the same mtDNA mutation was identified in Japan (17). In the present report we establish the association between this mutation and an oxidative phosphorylation dys- function, and explore potential pathogenetic mechanisms.

EXPERIMENTAL PROCEDURES

Cell Lines—The human osteosarcoma-derived cell line 143B(TK−) and its mtDNA-less derivative 143B/206 were a kind gift of Dr. Michael P. King (Columbia University, New York). Growth conditions and the characterization of the 143B/206 line were described elsewhere (18). Transmitchondrial cybrids were grown in the absence of uridine, except when functional or molecular studies were to be performed. In these cases, uridine (50 μg/ml) was added to the medium 48 h before the experiment. A fibroblast line from a patient harboring an heteroplasmic (48%) mtDNA mutation at position 3256 was obtained and character- 

1 MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; COX, cytochrome c oxidase; RFLP, restriction fragment polymorphism; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase pair(s).
from the initial foci, exposing their DNA by a micro alkaline lysis procedure (19), and genotyping the mtDNA for the 3256 position by restriction fragment polymorphism (RFLP) of PCR-amplified fragments (3) (Fig. 1, A and B). To determine the sensitivity of the RFLP assay, we PCR-amplified a DNA fragment encompassing the mtDNA 3256 position (between nucleotides 3316 and 3353) from a wild-type and a mutant cell line of a mitochondrial cybrid line. These PCR fragments were then cloned into a plasmid vector (TA cloning PCR I kit from Invitrogen). Individual bacterial clones were isolated, and their plasmid was tested for the presence or absence of the 3256 mutation. We mixed known amounts of purified wild-type and mutant plasmid corresponding to 10, 5, 2, 1, 0.5, and 0.1% wild-type mtDNA (for a final concentration of 0.7–1 µg) and incubated with a commercial testing kit (Sigma) and normalized by the number of total DNA extracted from exponentially growing cells (21). The probe used was the COX II polyclonal antibody (both individually and together), for 14 h at 37°C, and subsequently incubated with anti-mouse IgG conjugated to alkaline phosphatase, anti-rabbit IgG conjugated to alkaline phosphatase, or both (Sigma). Bands were developed with 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and nitro blue tetrazo- lium chloride (Life Technologies, Inc.) for 10 s.

Northern Blotting—Total RNA of each cybrid line was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (22). Twenty µg of total RNA was electrophoresed on 1.2% agarose, 5% formaldehyde gels and transferred to nylon membrane. The filters were hybridized overnight at 42°C with specific mtDNA probes (see below). For detection of the RNA

\[ \text{RNA}^{\text{AUCUUC}} \]

and the mtDNA 3256 mutation, we used a synthetic 48-nucleotide oligonucleotide (between mtDNA positions 3304 and 3327) end-labeled with \[ \gamma^{\text{32P}} \] ATP. The probe for the detection of ND1 mRNA was obtained by gel purification of a 786-bp fragment (corresponding to mtDNA positions 3337–4121) produced by digestion of a 1426-bp PCR fragment (primers spanning nucleotide positions 3116–3125 and 4542–4546) with RsaI and EcoRI. The 16 S rRNA probe was a 1555-bp PCR fragment corresponding to mtDNA positions 1690–3246, obtained by PCR amplification. The \[ \text{RNA}^{\text{AUCUUC}} \] and \[ \text{RNA}^{\text{GAGAGG}} \] probe was a 204-bp PCR fragment obtained by digestion of a 382-bp PCR fragment (spanning mtDNA positions 4260–4542) with RsaI. The 204-bp fragment corresponding to positions 4260–4464 was gel-purified and used as template. A 1.9-kb \[ \gamma \text{-actin} \] mRNA probe was obtained by EcoRI digestion of a cloned insert (23). All double-stranded DNA templates were labeled by the random primer method (Boehringer Mannheim). Northern hybridizations were performed in 50% formamide, 5 \% Denhardt’s solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, 25 µM sodium phosphate pH 6.8, 5 × SSC, and washed twice in 1 × SSC, 0.1% SDS at room temperature for 1 h and subsequently in 0.25 × SSC, 0.1% SDS at 55°C for 1 h, and finally in 0.1 × SSC at 55°C for 1 h. The blot in Fig. 6 was subsequently hybridized and stripped with different probes in the following order. \[ \text{RNA}^{\text{AUCUUC}} \], ND1, \[ \text{RNA}^{\text{GAGAGG}} \], 16 S rRNA, and finally \[ \gamma \text{-actin} \].

High resolution Northern blotting was performed essentially as previously described (24). For detection of the \[ \text{RNA}^{\text{AUCUUC}} \] we use the same probe described above. The mitochondrial \[ \text{RNA}^{\text{GAGAGG}} \] was detected with a 13P-labeled PCR fragment corresponding to mtDNA positions 1460–1479 (Fig. 5B). The same blot was first hybridized with a \[ \text{RNA}^{\text{AUCUUC}} \] probe, stripped, and hybridized with a \[ \text{RNA}^{\text{GAGAGG}} \] probe.

Statistical Analyses—Comparisons between different cell line groups were done by Student’s t test using Mac Statworks software package (Cricket Software Inc.).

RESULTS

Isolation and Characterization of Transmitochondrial Cybrids—Thirty-three individual cybrid clones were isolated from two independent fusions of enucleated fibroblasts from the patient (containing 48% mutated mtDNA) with mtDNA-less 143B/206 cells. The vast majority of the transmitochondrial cybrids were essentially homoplasmic at position 3256. Only a few clones were heteroplasmic (heteroplasmy from 50 to 80% mutated mtDNA). Mitochondrial genotype analysis was performed after no longer than 10 population doubling (<10^3 cells) by collecting and analyzing mtDNA from approximately 100 cells during the transfer of initial cell foci from cloning rings to larger wells (see “Materials and Methods”). Three homoplasmic mutant and three homoplasmic wild-type lines were used in the subsequent studies. The homoplasmic nature of the mtDNA 3256 nucleotide was confirmed in expanded cultures (Fig. 1, A and B). Our RFLP assay for the detection of wild-type sequences was sensitive to 1% wild-type mtDNA (see “Materials and Methods”), indicating that all three mutant clones had >99% mutated mtDNA. Mitochondrial DNA levels were also measured by Southern analysis using a multiprobe nuclear gene (18 S rRNA) as internal reference (Fig. 1C). Mutant clones had slightly elevated mtDNA levels (mtDNA signal/nDNA signal = 12.8 ± 4.1 (mean ± S.D.) when compared to wild-type clones (8.1 ± 1.1) and 143B (6.9) levels. The patient-derived mtDNA identity of transmitochondrial cybrids was confirmed by RFLP analysis of an unrelated mtDNA polymorphism previously identified in the patient (3) (Fig. 1D).

Functional Mitochondrial Assays—Functional mitochondrial assays showed a clear deficiency in the respiratory chain ac-
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High molecular weight polypeptides appeared to be particularly reduced in mutant clones, a trend that was better observed in a 12.5% SDS-PAGE (Fig. 3, right panel). We quantitated the intensity of the 35S signal for the individual polypeptides present in the 12.5% gel (i.e. only the higher molecular weight polypeptides). Mutant clones had mean values that ranged from 20% (ND1) to 85% (ATP6) of the of wild-type mean values (Fig. 4).

Steady-state levels of two subunits of COX were measured by Western blot. Both the mtDNA-encoded subunit COX II, and in a lesser extend, the nuclear-encoded subunit COX IV were reduced in the mutant clones (Fig. 5). In mutant clones, COX II mean value was only 25% of the wild-type, while COX IV mean value was 42% of wild-type values. The ratio of COX IV/COX II was 1.6 for 143B, 1.9 ± 0.4 (mean ± S.D.) for wild-type clones, 4.0 ± 2.5 for mutant clones. The mtDNA-less cell line 143B/206 had normal levels of COX IV but lacked COX II (Fig. 5).

Mitochondrial RNAs Steady-state Levels and Processing—Because of previously reported RNA processing abnormalities associated with mitochondrial tRNA^{Leu(UUR)} mutations, we analyzed RNA species surrounding the tRNA^{Leu(UUR)} by Northern blot hybridization (Fig. 6). ND1 levels were significantly reduced in mutant clones (mean value was 44% of values observed for wild-type clones; p = 0.005, and 47% when normalized to γ-actin; p = 0.003). An intermediate unprocessed transcript encompassing the 16 S rRNA, tRNA^{Leu(UUR)}, and ND1 (termed RNA 19) (12) was slightly increased in mutant clones (mean value was 109% of wild-type and 116% when normalized to γ-actin), but these values were not significantly different from the wild-type values (p = 0.68, and p = 0.24, respectively). RNA 19 could also be detected with the tRNA^{Leu(UUR)} and 16 S rRNA (Fig. 6, B and C). A downstream probe, specific to the three tRNAs cluster (Ile, Gin, and Met) also showed intermediate transcripts, but no major differences between wild-type and mutant clones (Fig. 6D). The integrity and amount of RNA samples was determined by hybridizing the membrane with a γ-actin probe (Fig. 6E).

The relative levels of tRNA^{Leu(UUR)} were also measured by high resolution Northern blots (Fig. 7). Mutant clones had a tRNA^{Leu(UUR)}:tRNA^{Val} ratio that was approximately 30% lower than the ratio observed for wild-type or 143B clones.

DISCUSSION

The increasing number of reports on mtDNA mutations associated with human diseases has created the need for functional studies to corroborate the genetic data. Although features such as: heteroplasmy, evolutionary conservation, and clinical-genetic correlations are strong indicators of etiologic mtDNA mutations, they cannot replace functional studies in providing prove for pathogenicity. Several potentially pathogenic mutations in the mitochondrial tRNA^{Leu(UUR)} gene have been described, many of which may share similar pathogenetic mechanisms. These include mutations at mtDNA positions: 3243 (MELAS, ocular myopathy) (4); 3251 (myopathy) (8); 3252 (encephalopathy) (9); 3256 (myoclonus epilepsy with ragged red fibers/ocular myopathy and MELAS) (3, 17); 3260 (myopathy and cardiomyopathy) (6); 3271 (MELAS) (5); 3291 (MELAS) (25); 3302 (myopathy) (7); 3303 (myopathy and cardiomyopathy) (10); and a single base pair deletion between positions 3271 and 3273 (encephalopathy) (24).

The present study tried to establish a correlation between a C → T transition at mtDNA position 3256 and a mitochondrial dysfunction, and to correlate these findings with results obtained with other tRNA^{Leu(UUR)} mutations. We used a trans-mitochondrial cybrid system (12, 14, 26, 27) to segment wild-type mtDNAs from 3256 mutated mtDNAs in different cell lines. Most trans-mitochondrial cybrid lines were homoplasmic
soon after fusion (after approximately 10 population doublings). Other investigators have also observed this tendency to generate homoplasmic transmitochondrial clones (28). The fast segregation of these mtDNA molecules, and the paucity of heteroplasmic clones obtained, suggest that either: 1) The fibroblast culture was a mixture of essentially homoplasmic wild-type or homoplasmic mutant cell lines, or 2) mtDNA heteroplasmy is unstable in the 143B/206 transmitochondrial system.

Homoplasmic mutant clones (>99% mutated mtDNA) had a severe deficiency in respiratory chain function, as shown by oxygen consumption, lactate production and enzymes activity. They also showed a 75% reduction in the steady-state levels of a mitochondrially synthesized polypeptide (COX II), and a 15–80% deficiency in synthesizing different mtDNA encoded polypeptides when compared to wild-type clones. The partial reduction in the nuclear-encoded subunit IV of COX could be explained by the primary deficiency of COX II, which would limit the number of properly assembled holoenzymes. However, this explanation may not be satisfactory because COX IV was present at normal levels in mitochondria isolated from the mtDNA-less 143B/206 line, even though COX II was completely absent.

The observations described above suggest that the mitochondrial dysfunction associated with the C → T transition at mtDNA position 3256 is caused by an impairment in mitochondrial protein synthesis and steady-state levels. Similar observations have been made for other pathogenic mitochondrial tRNA mutations (12, 14, 27). Although different mechanisms could account for the translation impairment, our results suggest that, at least partially, it is caused by a reduction in the steady-state levels of tRNA<sup>Leu(UUR)</sup> and possibly of other tran-
scripts, such as ND1. It is not clear why ND1 levels were reduced beyond what could be accounted by the increase in RNA 19 levels, but it may be related to the location of the mutation within a transcriptional regulatory site (see below). Bindoff et al. (7) described a different pathogenic mutation in the mitochondrial tRNALeu(UUR) gene (an A→G transition at position 3302) associated with reduced steady-state levels of tRNALeu(UUR). In their patient, however, the decrease in free tRNALeu(UUR) was accompanied by a marked accumulation of RNA 19. Although we found only a mild increase in RNA 19 levels, it is possible (as in the case of Bindoff et al. (7)) that patient tissues such as muscle or CNS would accumulate higher levels of RNA 19. RNA 19 was also increased in trans-mitochondrial cybrids harboring an A→G transition at mtDNA position 3243 and a T→C transition at position 3271, both within the same tRNA gene (12, 30).

Besides the role of the 3256 mutation in tRNA function and RNA processing, some of our results are also compatible with alternative pathogenetic mechanisms. The 3256 mutation is located within the last base pair footprinted by a mitochondrial transcription termination factor (33), potentially altering the binding of this trans-acting factor, that could lead to an unbalance in the levels of transcripts located upstream and downstream of the termination site (34). In vitro, the 3243 mutation (located in the middle of the termination factor binding site) reduces transcription termination, leading to an increase in the levels of downstream transcripts (34). It is possible that the 3256 mutation has an opposite effect (i.e. it strengthens transcription termination), therefore reducing transcription of downstream genes. This hypothesis would be compatible with the observed reduction in ND1 and tRNALeu(UUR) transcripts.

**FIG. 4. Densitometric analysis of high molecular weight mitochondrial translation products.** Fluoro-grams of mitochondrial translation products labeled with [35S]methionine after electrophoresis through a 12.5% SDS-PAGE were scanned and quantitated by densitometry. The histogram represents the different band intensities. Note the different level of impairment of specific polypeptides in the mutant cell lines.

**FIG. 5. Steady-state levels of two COX subunits.** The figure shows a Western blot of isolated mitochondria from different cell lines incubated simultaneously with two antibodies specific to COX subunits II and IV. Color development was stopped before previously determined half-maximum band intensities.

**FIG. 6. Northern blot hybridization analyses.** Autoradiograms of total RNA, extracted from the cell lines listed on top of each lane, and hybridized to different probes are shown. Probes are specific for the following mitochondrial transcripts: A, ND1; B, tRNALeu(UUR); C, 16S rRNA; D, tRNAsIle,Gln,Met; E, nuclear-coded γ-actin. The ethidium bromide-determined positions of 28 S and 18 S rRNAs are shown on the left of each panel. Band assignments were based on molecular weight and probe specificity. RNA 19 corresponds to an intermediary transcript composed of 16 S rRNA + tRNALeu(UUR) + ND1. Transfer RNA-specific probes cannot detect the small molecular weight tRNAs because they run out of the agarose gel.

However, these two transcripts seem to be preferentially decreased in comparison to other transcripts downstream of ND1 (see Fig. 6D).
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As in previous reports (12, 18), we found some phenotypic heterogeneity among mutant clones. Part of this variation may be explained by the aneuploid character of these transformed cell lines. Although we found partial quantitative abnormalities in transcripts and polypeptides produced by mutant cell lines, we do not know if alone, they can account for the severe oxygen consumption impairment observed. Therefore, we cannot exclude that the 3256, as well as other tRNA\textsubscript{Leu(UUR)} mutations affect cellular respiration by an yet unidentified mechanism.

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REFERENCES


FIG. 7. High resolution Northern hybridization. Total RNA extracted from different cell lines was electrophoresed through a 20% polyacrylamide gel, electrotransferred to a nylon membrane, and hybridized to tRNA-specific probes. Panel A shows hybridization to a tRNA\textsubscript{Leu(UUR)} probe, while Panel B shows a hybridization to a tRNA\textsubscript{Val} specific probe.
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