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# Functional and morphological abnormalities of mitochondria harbouring the tRNA$^{Leu(UUR)}$ mutation in mitochondrial DNA derived from patients with maternally inherited diabetes and deafness (MIDD) and progressive kidney disease

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## Abstract

*Aims/hypothesis*. An A to G transition at nucleotide position 3243 in the mitochondrial *tRNA$^{Leu(UUR)}$* gene has been identified in patients with maternally inherited diabetes and deafness, as well as in patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, chronic progressive external ophpthalmoplegia, cardiomyopathy and progressive kidney disease. Variations in the mitochondrial DNA haplotype as well as differences in the degree and distribution of heteroplasmy in a certain tissue are factors that may contribute to the variety in phenotypical expression of the 3243 *tRNA$^{Leu(UUR)}$* mutation. We have done morphological and functional experiments on mitochondria carrying the 3243 mutation derived from patients with either maternally inherited diabetes and deafness or progressive kidney disease to prove the pathogenicity of the 3243 mutation and to examine whether the mtDNA haplotype modulates the pathobiochemistry of this mutation.
*Methods*. We constructed clonal cell lines that contain predominantly mutated or exclusively wild-type mtDNA with a distinct mtDNA haplotype by the methodology of mitochondria-mediated transformation. Cells lacking mitochondrial DNA ($\varrho^\circ$) were used as recipients and donor mitochondria were derived from fibroblasts of a patient with either maternally inherited diabetes and deafness or progressive kidney disease. The fibroblasts from these clinically distinct patients carry different mitochondrial DNA haplotypes with the 3243 mutation in heteroplasmic form.
*Results*. Heteroplasmy in the clonal cybrid cells ranged from 0 to 100%, reflecting the heterogeneity of the mitochondrial donor cell. Cybrid cells containing predominantly mutant mitochondrial DNA showed lactic acidosis, poor respiration and marked defects in mitochondrial morphology and respiratory chain complex I and IV activities. No differences were observed in the extent of the mitochondrial dysfunction between the mutant cells derived from the two donors.
*Conclusion/interpretation*. These results provide evidence for a pathogenic effect of the *tRNA$^{Leu(UUR)}$* mutation in maternally inherited diabetes and deafness and progressive kidney disease, and show no evidence of a contribution of the mitochondrial DNA haplotype as a modulating the biochemical expression of the mutation. [Diabetologia (1999) 42: 485–492]

**Keywords** Mitochondrial DNA, diabetes mellitus, deafness, haplotype, mutation.

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*Abbreviations*: mtDNA, Mitochondrial DNA; MIDD, maternally inherited diabetes and deafness; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; CS, citrate synthase; BrdU, 5-bromodeoxyuridine; L/P, lactate to pyruvate ratio.

Maternally inherited diabetes and deafness (MIDD) is a subtype of diabetes which accounts for 1–2% of people with diabetes world-wide [1–3]. Clinical characteristics related to MIDD are early onset of diabetes and sensorineural hearing loss, a progressive insulin secretory defect, absence of islet-cell antibodies and absence of obesity [2]. Molecular investigation of mitochondrial DNA (mtDNA) has identified an A to G transition at np 3243 in the mitochondrial *tRNA*$^{Leu(UUR)}$ gene in association with MIDD [4]. Despite the clear association of the 3243 mutation with MIDD, no biochemical evidence yet exists for a direct relation between the mutation and an impairment of mitochondrial function, at least in MIDD.

To study the pathobiochemistry of the 3243 mutation, it is desirable to isolate clonal cell lines that contain exclusively mutated or exclusively wild-type mtDNA with the same mtDNA haplotype within the same nuclear background. By use of the methodology of mitochondria transfer from cells of patients with a mitochondrial disease to a human cell line devoid of mtDNA ($\varrho^\circ$) [5], we have isolated stable cell lines that contain various proportions of mutant mtDNA and even cell lines in which nearly all the mtDNA is mutated. The donor mitochondria originated from two patients carrying the *tRNA*$^{Leu(UUR)}$ mutation at nucleotide position 3243 in heteroplasmic form (i.e. coexistence of wild-type and mutant mtDNA), each displaying a different clinical phenotype. One patient exhibits MIDD [4], whereas the second patient suffers from progressive non-diabetic kidney disease and sensorineural hearing loss (Alport-like syndrome) [6]. We investigated mitochondrial function in a panel of clonal cell lines containing either wild-type or mutant mtDNA, derived from each donor, to gain insight into the effect of the *tRNA*$^{Leu(UUR)}$ mutation on mitochondrial function. The results provide evidence for a direct relation between the mutation and an impairment of mitochondrial function in MIDD and progressive kidney disease. Furthermore, we have found no evidence for the contribution of the mtDNA haplotype as a modulating factor in the biochemical expression of the *tRNA*$^{Leu(UUR)}$ mutation.

## Materials and methods

*Patients.* Diabetic members of two pedigrees harbouring the mtDNA *tRNA*$^{Leu(UUR)}$ mutation at position 3243 were investigated. One patient (V) is the proband of a large family with the phenotype of maternally inherited diabetes and deafness (MIDD) whose clinical details and molecular genetic analysis have been reported previously [4]. The second patient (A), the proband of family A as described in [6], suffers from progressive non-diabetic kidney disease and sensorineural hearing loss (Alport-like syndrome). After renal transplantation and during treatment with high doses of prednisone, she developed diabetes mellitus. Fibroblasts were taken at the age of 54 years for patient V and 53 years for patient A.

*Cells and cell culture.* Primary skin fibroblasts were grown on Dulbecco's modified Eagle medium containing 4.5 mg/ml of glucose and 110 μg/ml of pyruvate (DMEM) supplemented with 10% FBS. The osteosarcoma cell line 143B.TK$^-$ (143B) was grown in DMEM supplemented with 100 μg/ml 5-bromodeoxyuridine (BrdU) and 5% FBS. The cell line B$\varrho^\circ$-3, a mtDNA-depleted derivative of 143B cells, was obtained by long-term exposure (more than 20 weeks) to ethidium bromide (50 ng/ml). Southern blot analysis and PCR amplification of mtDNA target sequences confirmed the absence of any residual mtDNA.

The cell line B$\varrho^\circ$-3 was grown in medium of 143B cells supplemented with 50 μg/ml of uridine.

*Construction of transmitochondrial cybrids.* Mitochondria-mediated transformation of B$\varrho^\circ$-3 cells by cytoplast fusion was carried out as previously described [7]. In brief, a 3.5 cm culture dish with $1 \times 10^4$ fibroblasts was placed upside down in a sterile GSA bottle in 30 ml of DMEM with 10% FBS and 10 μg/ml cytochalasin B. Fibroblasts were enucleated by centrifugation at 7,000 rpm (8000 *g*) for 20 min in a prewarmed GSA rotor at 37 °C. Enucleated fibroblasts (cytoplasts) were fused as a monolayer with an excess ($\sim 1 \times 10^6$) of B$\varrho^\circ$-3 cells in $Ca^{2+}$ free DMEM in the presence of 40% (w/w) polyethylene glycol 1500. After 1 min incubation, the cells were carefully washed and subsequently incubated in B$\varrho^\circ$-3 medium. The cells were replated 2 days after fusion in 100 mm dishes and in 96-well plates at a cell density of $1 \times 10^3$ B$\varrho^\circ$-3 cells per well, which finally yielded 0.1 to 0.5 cybrids per well, in DMEM supplemented with 100 μg/ml BrdU and 5% dialysed FBS (selective medium). Single cybrids were isolated 10 to 15 days after fusion. Analysis of mtDNA copy number 3 weeks after cybrid selection showed low copy numbers of mtDNA in all mutant cybrids, but only in one wild-type cybrid, as determined by comparison with 143B cells, which is the parental line from which the B$\varrho^\circ$-3 cells were derived. Initially, after picking single cybrid clones, the culture medium was changed from selective medium (DMEM supplemented with 100 μg/ml BrdU and 5% dialysed FBS) to non-selective (B$\varrho^\circ$-3) medium, to facilitate the growth of cybrids with a fully deficient respiratory chain function. For an unknown reason, propagation of cybrids in B$\varrho^\circ$-3 medium prevented rapid restoration of the mtDNA copy number. When changing the medium back to selective medium, rapid restoration of mtDNA copy numbers was observed (within 1 month).

*DNA analysis.* Total DNA was isolated from 143B cells, B$\varrho^\circ$-3 cells and cybrids by standard procedures [8]. The ratio of mtDNA to nuclear DNA (mtDNA copy number) was determined by RFLP analysis. From the total DNA 5 μg were digested with Pvu II overnight at 37 °C and fragments were resolved on a 0.8% agarose gel, followed by Southern blotting [8]. The blot was hybridised with a mtDNA probe (6 kb PCR fragment spanning the mtDNA region between nt 15788 and 5547 [9]) or with a nuclear 18S rDNA probe. Fragments of DNA were labelled by nick translation, using kits from Amersham Pharmacia Biotech (Hertogenbosch, The Netherlands). Hybridization signals were quantified with a PhosphoImager (Molecular Dynamics, Sunnyville, Calif., USA) and visualised by autoradiography.

The presence and proportion of mutated mtDNAs in total DNA or in cell lysates of the cybrids were determined by testing for the presence of the Apa I site, created by the *tRNA*$^{Leu(UUR)}$ mutation at position 3243, in a $^{32}$P-labelled fragment amplified by PCR using oligonucleotides corresponding to positions 3029–3048 and 3591–3610 [9]. Cell lysates were prepared by incubation of $1 \times 10^5$ cells in PCR buffer with

0.5 % Tween-20 and proteinase K (100 μg/ml) for 2 h at 55 °C, followed by 10 min at 90 °C for proteinase K inactivation. Digested DNA fragments were resolved on a 5 % non-denaturing polyacrylamide gel. Bands were made visible by autoradiography and the proportion of digested and undigested mtDNA was quantified with a PhosphoImager. The ratio of Apa 1 cleaved and uncut fragment was used to calculate the degree of heteroplasmy. Values were subsequently corrected for heteroduplex formation [10]. Re-examination of the heteroplasmy in the selected cybrids after 3 months culturing showed no change in the proportion of wild-type and mutant mtDNAs.

Haplotypes of mtDNA from patients V and A were shown to be different by identifying several polymorphic sites in mtDNA by sequence analysis [6, 11].

*Mitochondrial morphology.* Cells were plated in 35-mm dishes at a density of approximately 100 000 cells per 2 ml on polyornithine-treated glass coverslips and cultured for 2 days. Mitochondria were stained for 30 min at 37 °C with 100 nmol/l of MitoTracker (Molecular Probes, Eugene, Ore., USA). Cells were then washed with PBS, fixed in 4 % paraformaldehyde and washed again extensively with PBS and finally with cold ethanol before mounting on glass slides. Cells were viewed using a Zeiss laserscan confocal 410 microscope (Zurich, Switzerland).

*Measurements of lactate and pyruvate.* The methods for lactate and pyruvate measurement were adapted from a previous study [12]. Briefly, pyruvate production was determined by measuring the oxidation of NADH, in the presence of lactate dehydrogenase (LDH), in RPMI 1640 medium that has been removed from the cultured cells after 24- and 48-h incubation periods. Lactate was measured similarly, except that the reduction of NAD was determined in the presence of hydrazine hydrate 0.1 % (v/v).

*Respiratory chain activity measurements.* The activities of the rotenone-sensitive NADH-ubiquinone-1 oxidoreductase (complex I) [13], succinate-dehydrogenase (complex II), succinate-cytochrome-*c* oxidoreductase (complex II-III) [14], decylubiquinol-cytochrome-*c* oxidoreductase (complex III]) [15], cytochrome-*c* oxidase (complex IV) and citrate synthase [16] were measured in the mitochondria-enriched fraction of fibroblasts or cybrid cell lines. All biochemical values were expressed per mU citrate synthase (CS) to correct for mitochondrial recovery.

*Oxygen consumption assay.* Rates of oxygen consumption were measured in a 2 ml reaction chamber at 37 °C using a Clark-type polarographic electrode. Measurements were made with $5 \times 10^6$ exponentially growing cells in DMEM lacking glucose, supplemented with 5 % dialysed FBS [5].

*Statistical analysis.* Comparisons between different cell line groups were done by the unpaired Student's *t* test using the SPSS (version 7 Chicago, USA) statistics package. The criterion for significance was set at $p$ less than 0.05.

## Results

*Patients.* Skin fibroblast cultures were established from two patients both carrying the *tRNA*$^{Leu(UUR)}$ mutation at position 3243 in heteroplasmic form; one patient (V) is the proband of the family described previously [4] with the phenotype of maternally inherited diabetes and deafness (MIDD); the second patient (A) is the proband of family A with the phenotype of progressive kidney disease and hearing loss (Alport-like syndrome) as reported recently [6]. Haplotypes of mtDNA from patients V and A were shown to be different by examining several polymorphic sites in mtDNA by sequence analysis [6, 11]. Figure 1 A shows the degree of heteroplasmy of the 3243 mutation in fibroblasts from patients V and A. The mtDNA of patient (V) shows a relatively low degree of heteroplasmy (15 %). No functional abnormalities of mitochondria, as detected by oxygen consumption and respiratory chain activity measurements, were found in these primary fibroblasts, a result which is most probably due to the relatively low degrees of heteroplasmy (below threshold). In mitochondria isolated from skeletal muscle, with 38 % mutant mtDNA, the NADH:$O_2$ oxidoreductase (complex I-III-IV) activity was shown to be slightly decreased (8.0 mU/mg protein; controls 16–46) ($n$ = 9) [4]. In fibroblasts of patient A 75 % of mutant mtDNA was detected (Fig. 1 A). Decreased activities

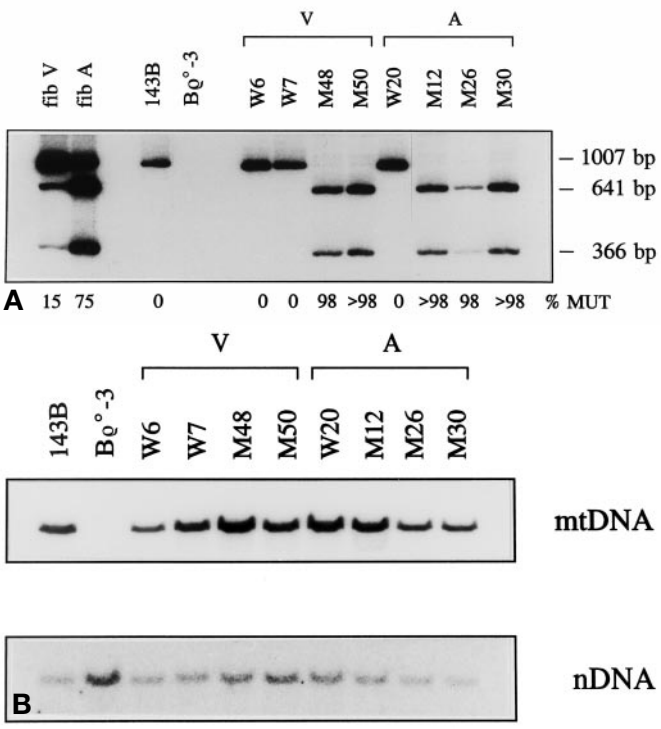


**Fig. 1 A, B.** Genetic characterisation of fibroblasts and cybrid cells from patients with MIDD (V) and progressive kidney disease (A). **A** Analysis for the presence and quantity of the mtDNA 3243 mutation in total DNA isolated from patients' (V) and (A) fibroblasts and derived cybrid cells using Apa I digestion of a PCR-amplified fragment containing the *tRNA*$^{Leu(UUR)}$ gene. Size of the fragments are shown to the right. The proportion of the 3243 mutation is indicated below each lane. **B** The ratio of mtDNA to nuclear DNA (mtDNA copy number) was determined by Southern blot analysis using a mtDNA probe and a nuclear 18S rDNA probe

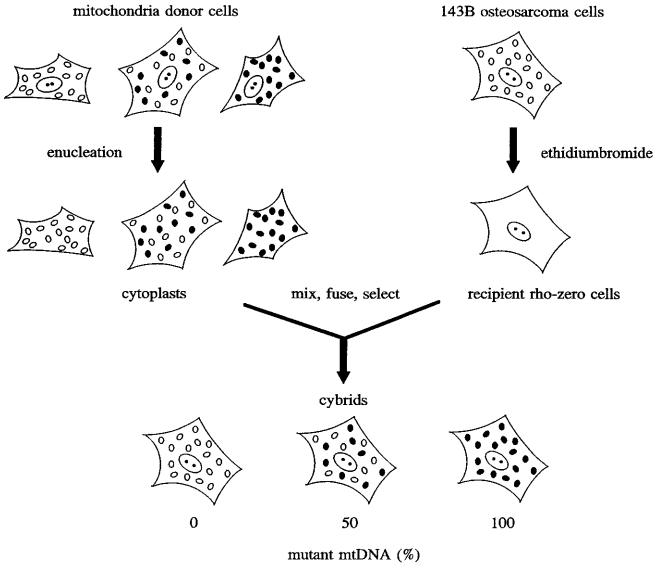


**Fig. 2.** Schematic representation of the methodology of mitochondria-mediated transformation utilising $\varrho^\circ$ cells as recipients and cytoplasts from patients as donors. Cytoplasmically located small ovoidal bodies represent mtDNA molecules (non-filled = wild-type mtDNA; black-filled = mutant mtDNA)

of both NADH:$Q_1$ oxidoreductase [0.02 mU/mU CS; controls $0.18 \pm 0.07$ mU/mU CS ($n = 13$); range 0.10–0.31] and cytochrome-*c* oxidase [0.12 mU/mU CS; controls $0.95 \pm 0.17$ mU/mU CS ($n = 13$); range 0.68–1.19] were shown in the mitochondrial fraction of these fibroblasts.

*Construction and characterisation of cybrids.* Mitochondria from both fibroblast cell lines were transferred into human cells lacking endogenous mtDNA (B$\varrho^\circ$-3) (Fig. 2). Fibroblasts were enucleated and the resulting cytoplasts were fused with an excess of B$\varrho^\circ$-3 cells. After fusion, the cells were replated in medium lacking pyrimidines and containing bromodeoxyuridine. Since B$\varrho^\circ$-3 cells are thymidine kinase deficient and auxothrophic for pyrimidines because of the lack of a functional respiratory chain, only B$\varrho^\circ$-3 cells that had fused with cytoplasts would be expected to grow in this medium. Independent cell clones (cybrids) were selected and grown for further analysis.

The proportion of wild-type and mutant mtDNAs was determined in cybrids using a PCR-based test in which the mutant allele is detected by the presence of an Apa I site in the PCR product. The degree of heteroplasmy of mutant mtDNAs in (V)-derived cybrid clones ($n = 62$) ranged from 0 to 98% (Fig. 3 A). Approximately half of the cybrids were homoplasmic wild-type, the remaining ones contained various proportions of mutant mtDNA. In only two cybrids did the percentages of mutant mtDNA exceed 95%. The percentages of mutant mtDNAs in patient (A)-derived cybrid clones ($n = 41$) ranged from 0 to 99%

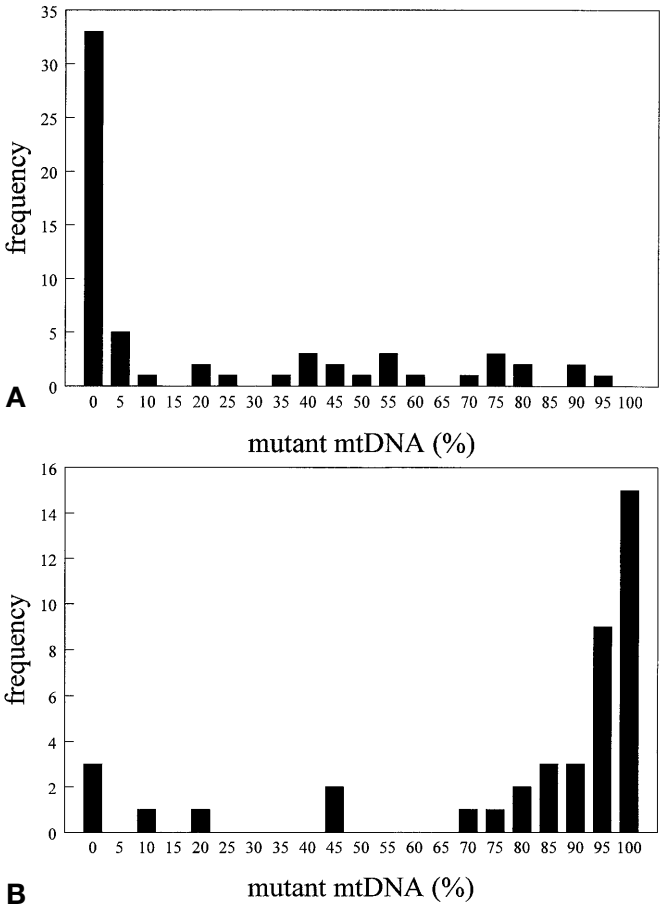


**Fig. 3 A, B.** Frequency analysis of the proportion of the mtDNA 3243 mutation in cybrid cells from patients with MIDD (**A**) and progressive kidney disease (**B**). The proportion of mutated mtDNA in cell lysates of the cybrids was determined by Apa I digestion of a PCR-amplified fragment containing the *tRNA*$^{Leu(UUR)}$ gene. Analysis was done on 62 cybrids from MIDD (**A**) and on 41 cybrids from progressive kidney disease (**B**)

(Fig. 3 B). The majority of cybrids contained high percentages of mutant mtDNAs, with only a few cybrids being homoplasmic wild-type or carrying less than 50% of mutant mtDNAs (Fig. 3 B). The mean percentage of mutant mtDNA in (V)-derived cybrids was 21%, and in (A)-derived cybrids was 80%. These values closely matched those found in the parental fibroblasts (15% and 75%, respectively). These observations agree with a situation where the individual fibroblasts have a distribution of mtDNA genotypes which reflects that observed in the cybrids. Among the products of each fusion, homoplasmic wild-type cybrids and near homoplasmic mutant cybrids (Fig. 1 A) were used for subsequent studies.

The ratio of mtDNA to nuclear DNA (reflecting the mtDNA copy number) was determined in the eight selected cybrids by RFLP analysis using a mtDNA probe and an 18S rDNA probe, respectively. The mtDNA copy numbers in the selected cybrids were all similar to those in 143B cells (Fig. 1 B).

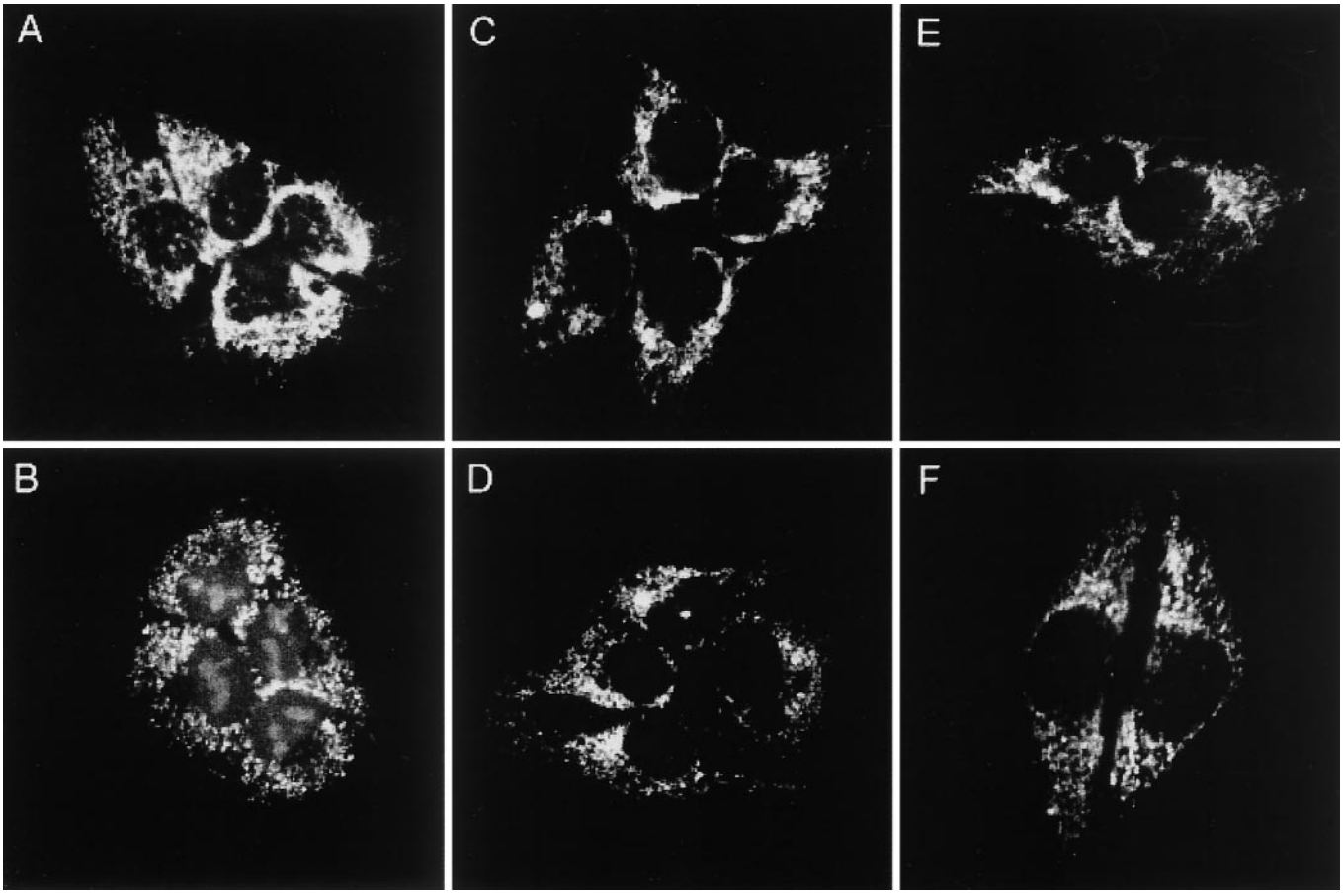


**Fig. 4 A–F.** Mitochondrial morphology. Mitochondria staining with MitoTracker in 143B control cells (**A**) B$\varrho^{\circ}$-3 mtDNA-depleted cells (**B**) W20 (**C**) cybrids M12 (**D**) wild-type W7 (**E**) and mutant M50 (**F**) cybrids. Images were taken with a Zeiss laserscan confocal 410 microscope

*Mitochondrial morphology.* Figure 4 shows confocal images obtained from 143B cells, B$\varrho^{\circ}$-3 cells, and the indicated cybrids, in which the mitochondria have been stained with MitoTracker. Mitochondria from 143B control cells, as well as from cybrids W7 and W20, both carrying wild-type mtDNA, showed a normal rod-shaped or filamentous structure on Mitotracker staining. Mitochondria from cybrids M50 and M12, both carrying mutant mtDNA, showed, however, a less elongated, more rounded shape. Structural changes were even more pronounced in B$\varrho^{\circ}$-3 cells, which totally lack mtDNA; here mitochondria had a dotted appearance.

*Lactate to pyruvate ratio.* Enhanced acidification of the culture medium was observed in mutant cybrids compared with wild-type cybrids. Mutant cybrids are expected to become dependent on glycolysis for energy production, a fact which should result in enhanced pyruvate to lactate conversion. The production of lactate and pyruvate in both wild-type and mutant cybrids was measured 24 and 48 h after start of incubation. Mutant cybrids (M50 and M12) showed approximately fourfold higher lactate to pyruvate (LP) ratio, when compared with wild-type cybrids (W7 and W20) after 24-h incubation ($p < 0.002$) (Fig. 5). The 48-h L/P was slightly higher (1.5-fold) than the 24-h L/P ratio in all cybrids and showed a similar fourfold difference between mutant and wild-type cybrids ($p < 0.005$) (Fig. 5).

*Respiratory chain activity measurements.* The activities of the individual complexes of the respiratory chain (the rotenone-sensitive NADH-ubiquinone-1 oxidoreductase (complex I), succinate-dehydrogenase (complex II), succinate-cytochrome-*c* oxidoreductase (complex II-III), decylubiquinol-cytochrome-*c* oxidoreductase (complex III) and cytochrome-*c* oxidase (complex IV)) were measured in the mitochondria-enriched fraction of the cybrid cell lines. Marked defects in complex I (mean 23% of wild-type values, $p < 0.01$), complex III (mean 32% of wild-type values, $p < 0.01$), complex II + III (mean 41% of wild-type values, $p < 0.01$), and complex IV (mean 2% of wild-type values, $p < 0.001$) activities, when normalised to citrate synthase activity, were observed in mutant cybrids compared with wild-type cybrids (Table 1). Wild-type cybrids showed slightly higher val-

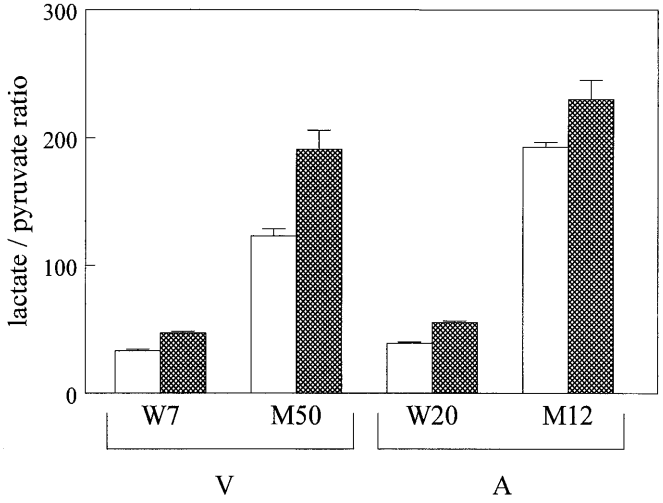


**Fig. 5.** Lactate to pyruvate ratio. Ratio of lactate to pyruvate production in cell culture of wild-type (W7 and W20) cybrids and mutant (M50 and M12) cybrids. The ratios were obtained after 24-h (open bars) and 48-h (shaded bars) incubation. Error bars are standard deviations of four determinations

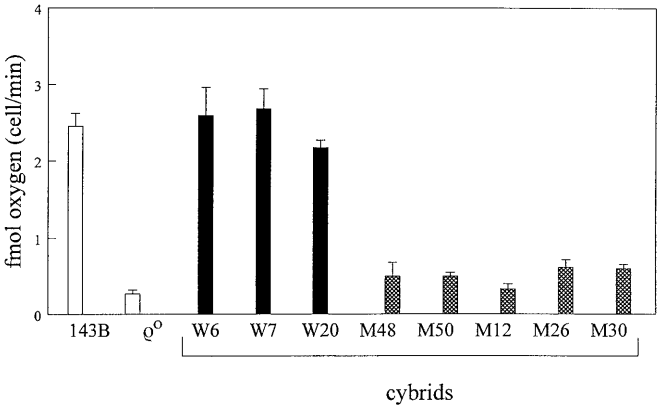


**Fig. 6.** Oxygen consumption. Rates of oxygen consumption per cell of control 143B, mtDNA-depleted Bρ°-3 and the indicated cybrids are shown with error bars representing standard deviations of three to six determinations

ues when compared to the parental 143B cell line. Complex II values did not differ significantly between wild-type and mutant cybrids. This is to be expected, as no mtDNA-encoded subunits are part of this multi-subunit complex.

*Oxygen consumption.* Rates of oxygen consumption were measured in a 2-ml reaction chamber at 37 °C using a Clark-type polarographic electrode on $5 \times 10^6$ exponentially growing cells in DMEM lacking glucose, supplemented with 5 % dialysed FBS [5]. Intact cell respiration measurements showed a statistically significant fivefold decrease in the rate of oxygen consumption (means ± SD) in mutant cybrids ($0.51 \pm 0.11$) compared with wild-type cybrids ($2.48 \pm 0.27$) ($p < 0.001$) and 143B cells ($2.45 \pm 0.17$); they exhibited a rate that approximated Bρ°-3 cells ($0.27 \pm 0.05$) (Fig. 6).

## Discussion

A wide variety of mtDNA alterations (deletions, point mutations and depletion) have been identified in degenerative diseases of the brain, heart, skeletal muscle, kidney and endocrine system [17]. When point mutations are considered, the *tRNA*$^{Leu(UUR)}$ gene appears to be most frequently mutated [18]. The most common mutation in this gene is at np 3243 and it associates with a remarkable spectrum of clinically distinct syndromes, including MIDD, progressive kidney disease, chronic progressive external opthalmoplegia (CPEO), cardiomyopathy, and mitrochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [1, 6, 19–22]. Several factors may account for this difference in phenotypical expression. The severity of a defect in oxidative phosphorylation in a specific tissue depends on the proportion of mutant mtDNAs in these cells, and differences in degree of heteroplasmy in organs possibly account for this phenotypic diversity. Other factors that could account for differences in phenotypical expression are variations in the distribution of mutant mtDNA among individual cells in a tissue due to mosaicism, the involvement of nuclear-encoded factors, and differences in the mtDNA background, which show a large number of haplotypes in a population.

In this study, we have used the methodology of mitochondria-mediated transformation, with Bρ°-3 cells as recipients and donor mitochondria from patients with MIDD and progressive kidney disease, carrying the *tRNA*$^{Leu(UUR)}$ mutation at nucleotide position 3243 in heteroplasmic form, to investigate the patho-

**Table 1.** See text

| | Respiratory chain complex activities (mU/mU CS) | | | | |
|---|---|---|---|---|---|
| | complex I | complex II | complex III | complex II + III | complex IV |
| WT cybrids | 0.073 ± 0.015 | 0.090 ± 0.010 | 1.383 ± 0.122 | 0.210 ± 0.015 | 0.540 ± 0.069 |
| MUT cybrids | 0.017 ± 0.024 | 0.084 ± 0.033 | 0.444 ± 0.430 | 0.086 ± 0.051 | 0.014 ± 0.006 |

Wild-type (WT) cybrid values represent the mean ± SD from single measurements on three different wild-type cybrids (W6, W7, W20). Mutant (MUT) cybrid values represent the mean ± SD from single measurements on five different mutant cybrids (M48, M50, M12, M26, M30)

genicity of this mutation. This methodology allows the analysis whether mutations in mtDNA have functional consequences or are merely polymorphisms. In addition, the distribution of heteroplasmy in the individual $\varrho^\circ$ cell-derived clones after fusion with patients fibroblasts is likely to represent the heteroplasmy in the individual donor cells as heteroplasmy in the repopulated cells is fairly stable during clonal expansion for at least 12 cell divisions. This assumption is underscored by the average degree of heteroplasmy of the repopulated clones being similar to that seen in the collection of donor fibroblasts. Though heteroplasmy in leucocytes originating from rapidly and continuously dividing cells, declines gradually upon ageing, [10] this situation is hardly observed during the continuous culture and expansion of repopulated $\varrho^\circ$ cells for a couple of weeks.

The use of mitochondria from two clinically-unrelated patients, each having a different mtDNA haplotype, enabled us to examine whether the mtDNA haplotype affects the biochemical expression of the 3243 mutation. The results presented here provide evidence for a direct relation between the mutation and morphological and functional abnormalities of mitochondria carrying this mutation. Mitochondria of cybrids carrying predominantly mutant mtDNA had a swollen, less filamentous, appearance than 143B cells. A similar mitochondrial morphology has been observed in HeLa cells with mutations in mtDNA (3243tRNALeu and 4269tRNAIle) [23]. Furthermore, our mutant cybrid showed pronounced defects in respiratory chain complex I, III and IV activities, lactic acid overproduction, and poor cellular respiration. With this technology, we have excluded the possibility that a nuclear gene is solely responsible for the mitochondrial dysfunction as all cybrids carry the same nuclear background, which is also genetically unrelated to that of each of the donor cells. Furthermore, no differences in the extent of mitochondrial dysfunction was observed between mutant cybrids carrying a distinct mtDNA haplotype. This finding argues against a major contribution of other nucleotide differences in the mtDNA (mtDNA background) as a modulating factor in the biochemical expression of the 3243 $tRNA^{Leu(UUR)}$ mutation, at least at the level of mitochondrial functionality in cultured cells. Additional support for this last conclusion comes from results obtained by studying mutant cybrids derived from fusion of $\varrho^\circ$ cells with cytoplasts from patients with MELAS [24, 25]. In these 3243 mutation carrying cybrids a similar impairment of mitochondrial function (e. g. as judged by measurements of cell respiration rate) was observed compared with the results presented in this study. Based on these findings, we conclude that it is improbable that the spectrum of clinical entities associating with the 3243 mutation is the result of modulating contributions by the mtDNA haplotype, suggesting a role of other factors in determining the clinical phenotype.

Differences in mutation load within cells or variation in the distribution of mutant mtDNA among cells in different tissues might be factors involved. We have observed a different pattern of heteroplasmy in cybrid clones derived from fibroblasts of a patient with MIDD and one with progressive kidney disease. This finding suggests that fibroblasts from the patients with MIDD and from the patient with progressive kidney disease have a different distribution of wild-type and mutant mtDNA in individual fibroblasts. In the case of deletions in mtDNA it has been shown that large differences in heteroplasmy values are seen in individual cells within a population of fibroblasts [26]. Such a situation of mozaisism could also exist for the 3243 mutation. In that case the pattern of mozaisism may affect the functional expression of the mutation. For example if none of the cells have heteroplasmy values above the threshold for reduced oxygen consumption [25] it is expected that all cells behave like normal cells. If a fraction of the cells have, however, high heteroplasmy values above the threshold and the other cells have low ones, this can result in the same average heteroplasmy value as in the first example; one expects a situation in which only a fraction of the cells will behave like unaffected cells. We observed a striking asymmetry in the distribution of heteroplasmy for the 3243 mutation in both donor-derived cybrid cell populations. Approximately half of the MIDD-derived cybrids were homoplasmic wild-type, the remaining ones containing varying proportions of mutant mtDNA, with only three cybrids exhibiting percentages of mutant mtDNA exceeding 90 % (3 of 62 cybrids = 5 %). Previously, it has been shown that the 3243 mutation is functionally recessive, with a steep threshold for expression of the pathogenic phenotype [25]; cells containing up to 90 % mutant mtDNA appear phenotypically normal, whereas only in cells with levels of mutant mtDNA exceeding 90 %, does mitochondrial function becomes impaired [25]. In contrast to the MIDD-derived cybrids, 27 of (A)-derived cybrids from a total of 41 (66 %) contained percentages of mutant mtDNA above threshold level ( > 90 %).

Thus, the donor fibroblasts from patients with MIDD and Alport-like syndrome differ not only in mutation load but probably also in the distribution of the mutant mtDNA among the individual cells. Our results closely agree with a previous report [27] in which it was concluded that the differential distribution of fibres with extremely high concentrations of mutant mtDNAs characterises, and probably distinguishes, the skeletal muscle of PEO and MELAS patients harbouring the same 3243 mutation [27].

In conclusion, we have shown here that the $tRNA^{Leu(UUR)}$ mutation, as found in patients with mito-

chondrial diabetes and deafness by itself is sufficient to lead to impairment of mitochondrial function. Furthermore, we have found no evidence for the contribution of the mtDNA haplotype as a modulating factor in the biochemical expression of the *tRNA*$^{Leu(UUR)}$ mutation. Indeed, the distribution of heteroplasmy of the 3243 mutation among individual cells could be a factor involved.

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