Defective kinetics of cytochrome *c* oxidase and alteration of mitochondrial membrane potential in fibroblasts and cytoplasmic hybrid cells with the mutation for myoclonus epilepsy with ragged-red fibres ('MERRF') at position 8344 nt

Hana ANTONICKÁ*†, Daniel FLORYK*†, Petr KLEMENT*†, Leona STRATILOVÁ†, Jana HEŘMANSKÁ*, Hana HOUŠŤKOVÁ†, Martin KALOUS*, Zdeněk DRAHOTA*, Jiří ZEMAN† and Josef HOUŠTĚK*[1]

*Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic, and †Department of Pediatrics, 1st Medical Faculty, Charles University, Ke Karlovu 2, CZ-120 00 Prague, Czech Republic

We have investigated pathogenic effects of the tRNA^{Lys} A8344G mutation associated with the syndrome myoclonus epilepsy with ragged-red fibres (MERRF) by using fibroblasts and fibroblast-derived cytoplasmic hybrid cells harbouring different percentages of mutated mitochondrial DNA (mtDNA). The activity of cytochrome *c* oxidase (COX) in patient fibroblasts with 89% mutated mtDNA was decreased to 20% of the control levels. COX exhibited altered kinetics, with a decreased V_{max} for both the low-affinity and high-affinity phases; however, the K_m values were not significantly changed. The substrate-dependent synthesis of ATP was decreased to 50% of the control. Analysis of the mitochondrial membrane potential, $\Delta\Psi$, in digitonin-treated cells with tetramethylrhodamine methyl ester (TMRM) with the use of flow cytometry showed a 80% decrease in $\Delta\Psi$ at state 4 and an increased sensitivity of $\Delta\Psi$ to an uncoupler in fibroblasts from the patient. The investigation of transmitochondrial cytoplasmic hybrid clones derived from the patient's fibroblasts enabled us to characterize the relationship between heteroplasmy of the MERRF mutation, COX activity and $\Delta\Psi$. Within the range of 87–73% mutated mtDNA, COX activity was decreased to 5–35% and $\Delta\Psi$ was decreased to 6–78%. These results demonstrate that the MERRF mutation affects COX activity and $\Delta\Psi$ in different proportions with regard to mutation heteroplasmy and indicate that the biochemical manifestation of the MERRF mutation exerts a very steep threshold of $\Delta\Psi$ inhibition.

Key words: cytofluorimetry, mitochondrial diseases, OXPHOS (oxidative phosphorylation), TMRM (tetramethylrhodamine methyl ester).

INTRODUCTION

Myoclonus epilepsy with ragged-red fibres (MERRF) is a maternally inherited mitochondrial encephalomyopathy characterized by myoclonus epilepsy, generalized seizures, ataxia and myopathy. Recent work has associated MERRF with four different point mutations. Three of these are connected with the mitochondrial DNA (mtDNA)-encoded tRNA^{Lys} gene (an A → G transition at nt 8344 [1,2], a T → C transition at nt 8356 [3,4] and a G → A transition at nt 8363 [5]) and one is in the mitochondrial tRNA^{Leu} gene, an A → G transition at nt 3243, which is usually linked with mitochondrial encephalopathy, lactic acidosis and strokes ('MELAS'), another mitochondrial disease [6].

The most common mutation, at nt 8344, has been analysed previously in muscle tissue [1,3,7–9], in blood [10–15], and in cytoplasmic hybrid (cybrid) cells [16,17] and has been associated with severe defects in protein synthesis. Impaired mitochondrial protein synthesis leads to a general decrease in respiration rate and oxygen consumption in cells and tissue mitochondria [15,18–20]. A distinct decrease in the activities of complex I and cytochrome *c* oxidase (COX) has been observed, whereas F_1F_o-ATPase and complex III are usually less affected [7,15,18,19].

The nt 8344 mutation is heteroplasmic; the severity of the defect is correlated with the percentage of mutated mtDNA found in different tissues [12,21]. However, the relative amount of mutated mtDNA in blood samples does not seem to be indicative of clinical severity [13,21]. In MERRF there is a decrease in tRNA^{Lys} aminoacylation capacity that leads to the premature termination of translation, resulting in an impairment of mitochondrial protein synthesis and an accumulation of truncated mitochondrial peptides [22]. Pedigree patterns and transmitochondrial cybrids have been used to define the threshold level for the restoration of respiratory function. Larsson et al. [19] have shown that above a threshold level of 35–40% mutated mtDNA in the mother, it is very likely that transmission of the mutation to all children will occur. In transformants carrying a heteroplasmic form of the MERRF nt 8344 mutation, full protection of the cells against the defects in protein synthesis and respiration caused by the mutation has been observed when the wild-type mtDNA exceeded 10–15% of the total complement [7,23,24].

Here we describe an atypical biochemical pattern of a COX deficiency, caused by an nt 8344 MERRF mutation, by using a combination of functional and structural methods, including the measurement of ATP synthesis and the fluorescence measure-

Abbreviations used: COX, cytochrome *c* oxidase; CS, citrate synthase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MERRF, myoclonus epilepsy with ragged-red fibres; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; TMRM, tetramethylrhodamine methyl ester.

[1] To whom correspondence should be addressed (e-mail houstek@biomed.cas.cz).

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ments of $\Delta\Psi$ based on flow cytometry analysis of cells stained with TMRM to characterize the functional consequences of the MERRF mutation.

MATERIALS AND METHODS

Materials

Serva Blue G, acrylamide, bisacrylamide and SDS were obtained from Serva-Feinbiochemica (Heidelberg, Germany); tetramethylrhodamine methyl ester (TMRM) and MitoTracker Green FM were from Molecular Probes (Eugene, OR, U.S.A.). Other chemicals were purchased from Sigma–Aldrich.

Ethics

This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the committees of medical ethics at all collaborating institutions. Informed consent was obtained as appropriate.

Tissues

Muscle autopsy material obtained within 2 h of death was frozen at −70 °C. Muscle homogenate [5 % (w/v), glass–glass Dounce homogenizer] prepared in 150 mM KCl/50 mM Tris/HCl/2 mM EDTA (pH 7.4) was filtered through 250 μm pore-size nylon screen and centrifuged for 10 min at 600 ***g*** to obtain the post-nuclear supernatant. Mitochondria were pelleted by centrifugation for 10 min at 10000 ***g***.

Cell cultures and preparation of cybrids

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (Sevac, Prague, Czech Republic) with 10 % (v/v) foetal calf serum. Transmitochondrial cybrids were prepared by the method of Tiranti et al. [25]. Fibroblasts from the COX-deficient patient and from controls were enucleated by centrifugation in Dulbecco's modified Eagle's medium containing 10 μg/ml cytochalasin B and then fused with mtDNA-less (rho^0) tumour cells (derived from $143B.TK^-$ osteosarcoma cells). After 24 h, cells were treated with trypsin, and replated; selection was performed by cultivation of cells for 2 weeks in Dulbecco's modified Eagle's medium containing 100 μg/ml 5-bromodeoxyuridine and lacking uridine. The cells were grown to approx. 90 % confluence and harvested with 0.05 % trypsin and 0.02 % EDTA. For the selection of cell clones, cybrids were plated at low density in the same medium as that used for rho^0 cells. For enzyme activity measurements, cells were resuspended in 0.25 M sucrose/10 mM Tris/HCl/1 mM EDTA (pH 7.2) and homogenized with a glass/Teflon homogenizer. For electrophoretic analysis, mitoplasts were prepared by treatment with 0.8 mg of digitonin/mg of protein, as described previously [26].

Electrophoretic methods

Blue native PAGE [27] was used for the separation of samples in the first dimension on a non-linear 6–15 % (w/v) polyacrylamide gradient, as described previously [26]. Mitoplasts prepared from cultured cells or mitochondria isolated from post-nuclear supernatant by centrifugation for 10 min at 10000 ***g*** were solubilized with 1 % (w/v) lauryl maltoside at a protein concentration of 1 mg/ml, and 5–10 μg of the solubilized proteins was used in each slot for electrophoresis. For two-dimensional analysis, stripes of the first-dimension gel were incubated for 45 min with 1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol, then subjected to SDS/PAGE [10 % (w/v) gel] for separation in the second dimension [28]. COX and F_1F_o-ATPase were detected by Western blotting with semi-dry transfer of proteins [29] to Hybond™ C-extra membrane (Amersham). Previously characterized [30–33] specific antisera against ATPase and COX were used at titres of 1:1000–1:10000. Immunocomplexes were detected by the peroxidase-conjugated secondary antibody (FAB fragment 1:3000; Bio-Rad) and enhanced chemiluminescence (ECL®; Amersham). Luminograms exposed on X-ray films and gels stained with Coomassie Blue or silver [34] were quantified on a Molecular Dynamics Computing Densitometer with IMAGEQUANT software (Molecular Dynamics).

ATP synthesis

The rate of ATP synthesis by digitonin-treated cultured skin fibroblasts or cybrids was measured as described by Wanders and van Roermund [35]. Cells resuspended at a protein concentration of 0.16 mg/ml were subjected to 40 μg/ml digitonin and incubated with different respiratory substrates (10 mM) for 30 min at 37 °C. The ATP produced was measured fluorimetrically [36] and the rate of ATP production was expressed as nmol/30 min per mg of protein; values were corrected for the production of ATP in the presence of 2 μg/ml antimycin.

Measurements of mitochondrial membrane potential

Mitochondrial membrane potential, $\Delta\Psi$, was measured in digitonin-treated cells with TMRM by using FACS. Cells harvested by treatment with trypsin were washed three times in cold PBS and resuspended in KCl medium [80 mM KCl/10 mM Tris/HCl/3 mM $MgCl_2$/1 mM EDTA/5 mM KH_2PO_4 (pH 7.4)] containing 10 mM succinate and 1 μM rotenone at a protein concentration of 1 mg/ml. Cells were incubated with digitonin (0.03 mg of digitonin/mg of protein for cybrids; 0.1 mg of digitonin/mg of protein for fibroblasts) for 5 min on ice, washed once and resuspended in KCl medium at 0.2 mg/ml protein. Aliquots of cells were incubated with 20 nM TMRM at room temperature for 10 min and, where indicated, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5–50 nM) was added to TMRM-stained cells 1 min before measurements of fluorescence. To estimate the cellular content of mitochondria, the cells were incubated with 20 nM MitoTracker Green FM for 10 min at room temperature. Cytofluorimetric analysis was done on the FACSort flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) equipped with a 488 nm argon laser. The TMRM signal was analysed in the FL2 channel, which was equipped with a bandpass filter at 580 ± 30 nm; the photomultiplier value of the detector was 631 V. The MitoTracker Green FM signal was analysed in the FL1 channel at 705 V. Data were acquired on a logarithmic scale by using CellQuest (Becton Dickinson) and analysed with WinMDI 2.7 software (J. Trotter, The Scripp Research Institute, La Jolla, CA, U.S.A.). Arithmetic mean values of the fluorescence signal in arbitrary units were determined for each sample for subsequent graphical representation. All experiments were repeated three times.

DNA analysis

The isolation of total DNA, digestion with restriction enzymes, electrophoresis of DNA fragments on agarose gels and single-strand conformation polymorphism were performed by using standard methods [37]. PCR analysis of the MERRF nt 8344 mutation was performed by the method of Zeviani et al. [9]. The origins of mtDNA and nuclear DNA in prepared cybrids were verified by analysis of the mtDNA 500–16130 region of the D-

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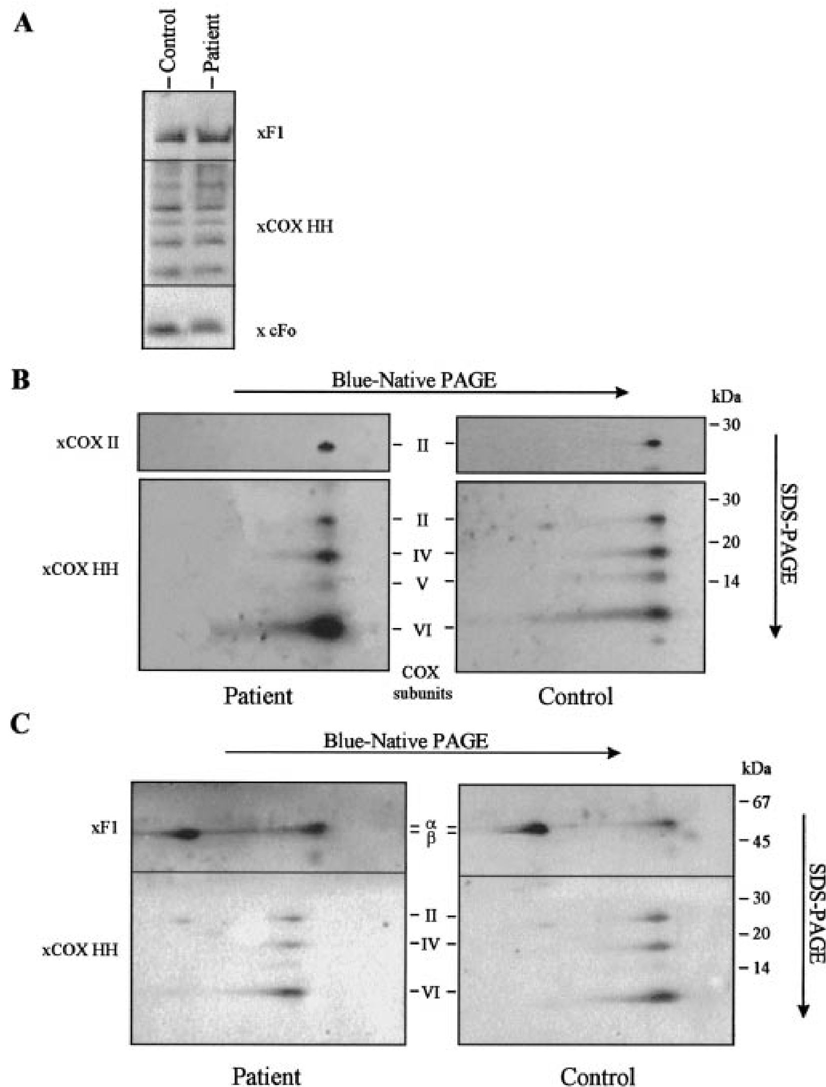

Figure 1 Western blot analysis of oxidative phosphorylation complexes in muscle and fibroblast of the MERRF patient

(**A**) Protein aliquots (5 μg) of muscle homogenate from patient and control were separated by SDS/PAGE; (**B**) protein aliquots (5 μg) of muscle mitochondria from patient and control or (**C**) protein aliquots (10 μg) of patient and control fibroblasts were separated by using blue native PAGE in the first dimension and SDS/PAGE in the second dimension. Immunodetection was performed as indicated with antibodies against F_1-ATPase (xF1, 1:10000), subunit *c* of F_o-ATPase (x cFo, 1:1000), human COX holoenzyme (xCOX HH, 1:3000) and bovine COX II subunit (xCOX II, 1:10000). The migration of molecular mass standards is indicated at the right.

loop and of the highly polymorphic repetitive sequence at the D11S533 locus on chromosome 11q respectively [25]. Radioactivity was detected with a PhosphorImager (Molecular Dynamics).

Enzyme assays

The activities of COX [38], NADH:Q_{10} oxidoreductase, succinate cytochrome *c* reductase, succinate dehydrogenase [39], F_1F_o-ATPase [40], citrate synthase (CS) [41] and pyruvate dehydrogenase [42,43] were measured in accordance with standard procedures. The kinetic parameters of COX were measured in fibroblasts after solubilization with 1.5 % (w/v) lauryl maltoside. Concentrations of cytochrome *c* were in the range 0.21–230 μM. Results were analysed by reversed Eadie–Hofstee plots. The protein concentration was measured in lauryl maltoside-solubilized proteins by the method of Bradford [44] and that in other samples by the method of Lowry et al. [45], with BSA as a standard.

Histochemistry

The COX activity of the cells grown on glass coverslips was detected cytochemically with the 3,3′-diaminobenzidine tetrahydrochloride method described by Tiranti et al. [46].

Case report

Failure to thrive, muscle hypotonia, cardiomyopathy and psychomotor retardation developed in a 5-month-old boy. His

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father was healthy, whereas his mother had muscle weakness and weight loss from the age of 25 years. Blood and cerebrospinal fluid lactate (8.1 and 4.1 mM, control range 0.6–1.9 and 0.6–2 mM respectively; $n = 25$) and the ratio of lactate to pyruvate were elevated. The boy died 7 months later. At autopsy, hypertrophic cardiomyopathy, neuronal degeneration and gliosis in the ventral horns of the spinal cord were found. An analysis of muscle and fibroblasts of the patient revealed a selective decrease in COX activity and the presence of heteroplasmic MERRF mutation at position 8344 nt of mtDNA, which was also found in fibroblasts of the mother. The specific activity of COX was decreased to 5 % in the muscle (less than 3.0 nmol/min per mg of protein; controls 58.5 ± 33.5) containing 92 % of the mutated mtDNA and to approx. 20 % in fibroblasts (6.0 nmol-/min per mg of protein; controls 29.6 ± 14.8) harbouring 89 % MERRF mutation. Activities in patient fibroblasts of other oxidative phosphorylation (OXPHOS) complexes [activities in nmol/min per mg of protein, means ± S.D. of controls ($n = 6$): NADH:Q_{10} oxidoreductase 7.7, controls 5.6 ± 1.6; succinate cytochrome *c* reductase 5.0, controls 6.7 ± 1.9; succinate dehydrogenase 4.1, controls 4.2 ± 0.4; oligomycin-sensitive ATPase 10.4, controls 16.8 ± 3.3] and of pyruvate dehydrogenase (1.0 nmol/min per mg of protein; controls 1.7 ± 1.2) were within the control range but their ratio to CS was about half of normal owing to a higher activity of CS (113 nmol/min per mg of protein; controls 53.5 ± 32.1). Fibroblasts from the mother contained 35 % mutated mtDNA; COX activity was 15.5 nmol/min per mg of protein. Clinically the boy's condition belonged to the fatal infantile form of COX deficiency and his mother's to the myopathic form with delayed onset.

RESULTS

Characteristics of the COX deficiency

Western blot analysis of the muscle homogenate subjected to SDS/page (Figure 1A) showed a normal content of immunodetectable COX enzyme (95–109 % of control levels) and ATPase (97–112 % of control) in the muscle of patient and control. The unchanged proportion between COX and other OXPHOS complexes was confirmed by silver-staining of two-dimensional gels (blue native PAGE followed by SDS/PAGE). In fibroblasts from the patient, an approx. 35 % lower content of all OXPHOS complexes was found but the proportion between the complexes was unchanged. Blue native PAGE and two-dimensional electrophoresis also showed a normal mobility of the native COX enzyme as well as an unchanged profile of immunodetectable COX subunits, in both muscle (Figure 1B) and cultured fibroblasts (Figure 1C) from the patient. These results show clearly that a pronounced defect in COX activity is not caused by selective changes in content of the COX enzyme or by a detectable structural defect in the enzyme.

When the kinetics of the COX reaction was measured in fibroblasts from the patient (Figure 2 and Table 1), we observed for the high-affinity phase a normal Michaelis constant (K_m) of 1.49 μM, but a decrease in the V_{max} to 25 % (3.8 compared with 15.3 nmol/min per mg of protein in the control). In fibroblasts from the mother, the high-affinity K_m was also normal but V_{max} was 4.7 nmol/min per mg of protein. The low-affinity-phase values of the COX reaction showed a slight increase in K_m but decreases to 29 % and 54 % in the V_{max} in fibroblasts from the patient and mother respectively.

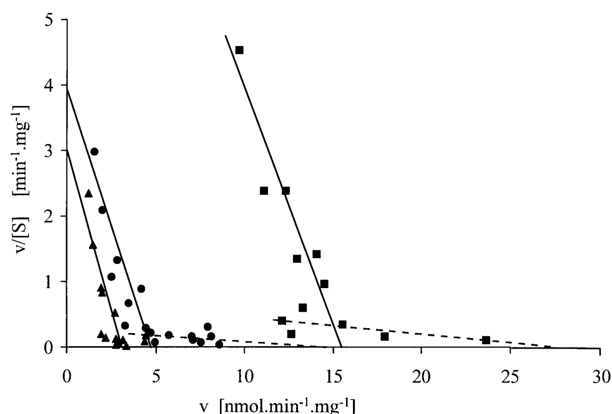

Figure 2 Eadie–Hofstee plot of COX kinetics in cultured fibroblasts

COX activity was determined spectrophotometrically in cultured fibroblasts from patient (▲), mother (●) and control (■) at various cytochrome *c* concentrations (0.21–230 μM). Solid lines show the high-affinity phase of the COX kinetics; broken lines show the low-affinity phase.

Table 1 Calculated values of V_{max} and K_m for the high-affinity and low-affinity phases of COX reaction

The results shown in Figure 2 were used to calculate these values.

	High-affinity phase		Low-affinity phase	
Source	V_{max} (nmol/min per mg)	K_m (μM)	V_{max} (nmol/min per mg)	K_m (μM)
Patient	3.8	1.49	7.8	50.6
Mother	4.7	1.35	14.7	49.3
Control	15.3	1.41	27.1	37.1

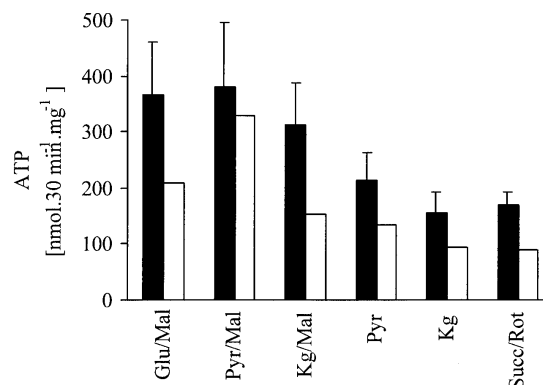

Figure 3 Activity of ATP synthesis in digitonin-treated fibroblasts

ATP synthesis was measured in cultured fibroblasts from patient (open bars) and control (filled bars) with the indicated substrates and inhibitors at 37 °C. Abbreviations: Glu, glutamate; Mal, malate; Kg, α-oxoglutarate; Pyr, pyruvate; Succ, succinate; Rot, rotenone. The activity of ATP synthesis is expressed in nmol/30 min per mg of protein. Control results are means ± S.D. for 15 independent measurements in duplicate.

Changes in activity of ATP synthesis and mitochondrial membrane potential

To analyse the effect of the COX deficiency on mitochondrial energetics we measured the ability of digitonin-treated fibroblasts to synthesize ATP when supplied with different respiratory substrates (Figure 3). ATP synthesis in patient fibroblasts was

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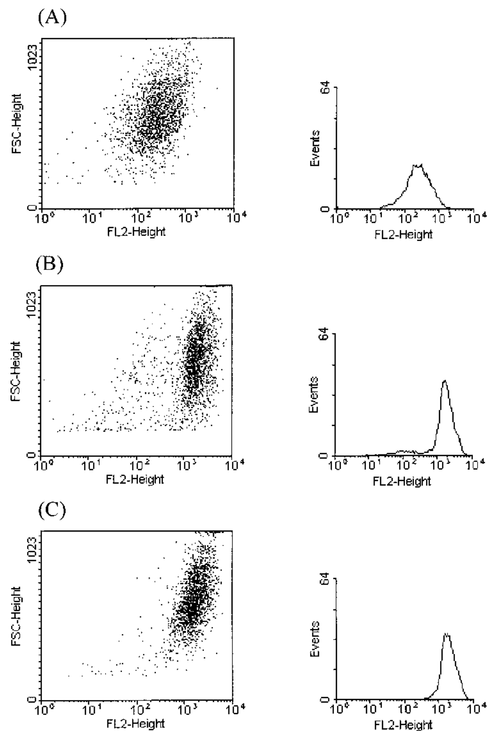

Figure 4 Detection by FACS of mitochondrial $\Delta\Psi$ with TMRM fluorophore

Fibroblasts from patient (**A**), mother (**B**) and control (**C**) were treated with digitonin and stained with 20 nM TMRM in KCl medium [10 mM Tris/Cl (pH 7.4)/80 mM KCl/3 mM $MgCl_2$/1 mM EDTA/5 mM KH_2PO_4/10 mM succinate/1 μM rotenone] to assess $\Delta\Psi$ as described in the Materials and methods section. The abscissa is the intensity of fluorescence; the ordinate is the side scatter height (FSC-Height) (dot-plots) or the relative cell number (histograms).

decreased to 50–70 % of the control with NADH-dependent substrates (glutamate plus malate, pyruvate plus malate, α-oxoglutarate plus malate) as well as with succinate.

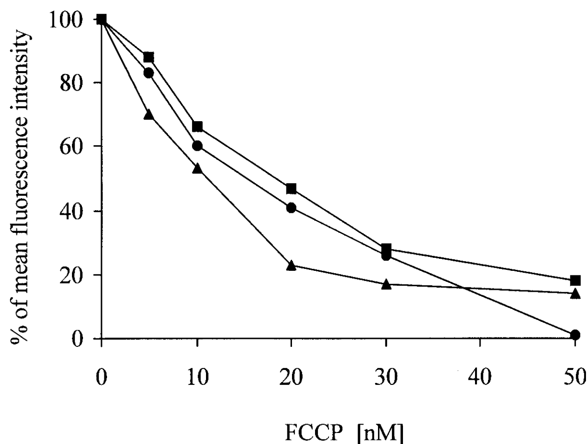

Figure 5 Dose response of TMRM fluorescence to FCCP in fibroblasts from patient, mother and control

Digitonin-treated fibroblasts from patient (▲), mother (●) and control (■) were stained with 20 nM TMRM as described in the legend to Figure 4 in the presence of various concentrations of FCCP (5–50 nM). TMRM fluorescence is expressed as a percentage of the mean fluorescence signal in the absence of FCCP.

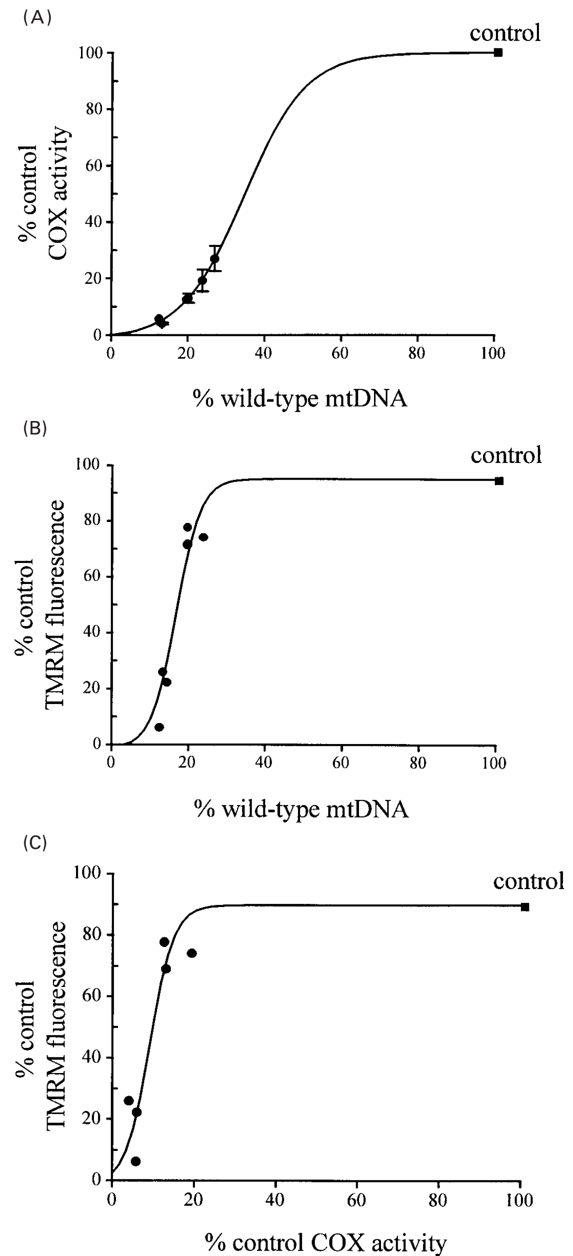

Figure 6 Relation between the amount of wild type mtDNA, COX activity and inner-membrane potential

(**A**, **B**) COX activity (**A**) and TMRM fluorescence (**B**) were analysed in cybrid clones derived from fibroblasts from the patient with different proportions of mutated and wild-type mtDNA. COX activity was measured as described in the Materials and methods section; the conditions for TMRM fluorescence measurement were as described in the legend to Figure 4. (**C**) Relationship between COX activity and TMRM fluorescence in patient cybrids. The sigmoidal fit was calculated with the Microcal™ Origin™ program.

In further experiments we measured $\Delta\Psi$ in cultured fibroblasts with the fluorescent probe TMRM by using FACS analysis. This approach is based on the use of digitonin-treated cells; it is sensitive enough to detect small changes in $\Delta\Psi$ within a broad range. An analysis of the cells supplied with 10 mM succinate in the absence of ADP shows a significantly lower value of the TMRM signal in fibroblasts from the patient (Figure 4A) in comparison with the control cells (Figure 4C), indicating a lower

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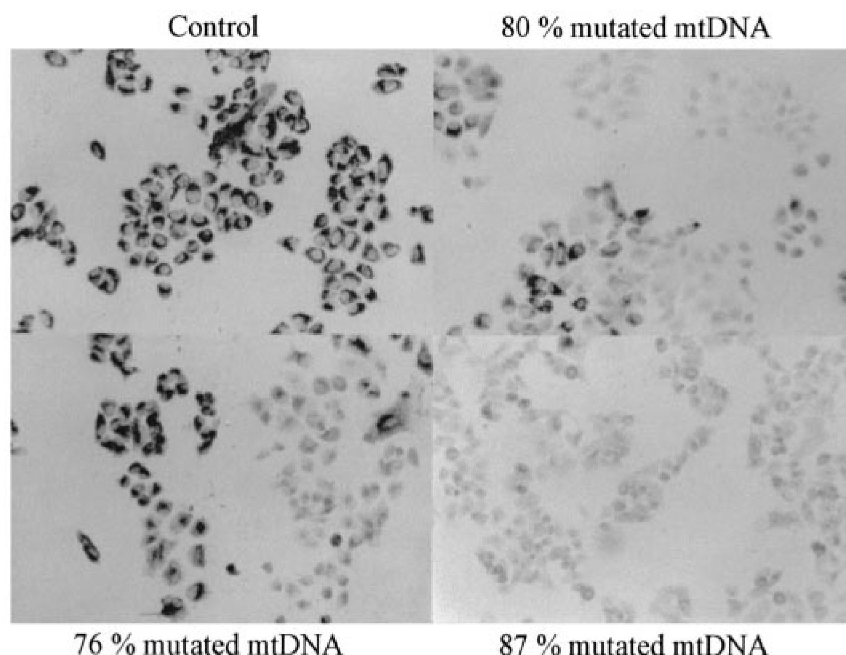

Figure 7 Histochemical staining of COX in cybrid clones with different proportions of MERRF mutation

Histochemical staining of COX in control and patient cybrid clones was performed for 2 h with the 3,3′-diaminobenzidine tetrahydrochloride method, as described in the Materials and methods section.

$\Delta\Psi$ in the former. A dot-plot of the patient cells (Figure 4A) shows a disperse population of the cells, exhibiting a broader range of TMRM fluorescence with a lower intensity in comparison with the control cells. In the cells from the mother (Figure 4B) there were two distinct populations of the cells: one with a high TMRM fluorescence intensity (82 %), similar to control values, and a less numerous population (18 %) with a lower TMRM signal. In all cell types the TMRM fluorescence was completely prevented by 2 μM FCCP (results not shown). However, when the cells were incubated with different concentrations of FCCP (Figure 5), the TMRM signal in patient fibroblasts became inhibited at a significantly lower FCCP concentration than in the control cells, resulting in a 50 % lower K_i value. Cytofluorometric analysis with MitoTracker Green FM revealed the same cellular content of mitochondria in the fibroblasts of the patient, his mother and in the controls. These results indicate that the MERRF mutation affected both the maximal value of $\Delta\Psi$ and its stability, which reflects the equilibrium between generation and dissipation of the potential.

COX activity and $\Delta\Psi$ in cybrid transformants harbouring different percentages of mutated mtDNA

With the aim of studying the functional consequences of the MERRF mutation, we prepared cybrid transformants from control fibroblasts and fibroblasts from the patient. Cybrids were cloned and tested for MERRF mutation heteroplasmy. From the original patient cybrids we obtained six final clones with 13–27 % of the wild-type mtDNA. The activity of COX in cybrid clones measured spectrophotometrically varied between 4 % and 30 % of the control values (Figure 6A). COX histochemistry (Figure 7) showed an analogous decrease in COX activity. Interestingly, the COX staining was not completely uniform in individual clones and regions: clusters with higher and lower COX activities were found despite the fact that cloning lasted for 6–8 months and represented 20–30 cell passages.

When $\Delta\Psi$ was measured in cybrid clones, the TMRM fluorescence values were also decreased with an increasing percentage of mtDNA mutation (Figure 6A). The relationship between the TMRM fluorescence, the COX activity and the percentage of mtDNA mutation shown in Figure 6 demonstrates that both the COX activity and $\Delta\Psi$ values change in proportion to the heteroplasmy of the mutation. However, the response of the two biochemical parameters to changing amounts of mutated mtDNA was clearly different, indicating that the COX activity needs to be decreased to 30 % to affect $\Delta\Psi$ (Figure 6B).

DISCUSSION

The nt 8344 MERRF mutation is associated with a decreased aminoacylation capacity of the $tRNA^{Lys}$ gene [22]; this is accompanied by a decreased synthesis of mitochondrially encoded proteins, which results in deficient respiratory activity. Villani and Attardi [47] show that in cells carrying the MERRF mutation, the activity of complex I is the rate-limiting step. In contrast, we describe an MERRF patient with a generalized defect in COX, which presents in fibroblasts as a selective deficiency of COX activity.

Decreased concentrations of COX or its subunits are frequently found in patients with a deficiency of COX activity [48,49]. However, in our patient the low activity of COX was not due to a decreased amount of enzyme. The content of COX was normal in muscle mitochondria; the size of the native complex and its subunit composition analysed by two-dimensional electrophoresis were normal in muscle and fibroblasts. The observed normal electrophoretic pattern of the OXPHOS enzymes is in agreement with the mechanism of the nt 8344 MERRF mutation [22]. Enriquez et al. [22] showed that the nt 8344 mutation causes

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the premature termination of mitochondrial translation and the accumulation of truncated polypeptides of nearly the same size as predicted. This probably reflects the fact that the termination points are near the C-terminus, allowing the polypeptides to take a nearly normal secondary structure and therefore enable them to assemble into a complex. The resulting low activity of oxidative phosphorylation and cell growth at a permanent energetic deprivation might be responsible for the observed 35 % decrease in the content of all the inner-mitochondrial-membrane enzymes in MERRF fibroblasts and the lower ratio of their activities to CS activity (see the Case report section).

For a more detailed characterization of the defect, the kinetics of COX reaction in fibroblasts was studied at a low ionic strength. The analysis of the high-affinity-phase kinetics of cytochrome *c* oxidation showed that V_{max} was 25 % of the control but the K_m was normal. Similarly, the V_{max} of the low-affinity phase was changed but not the K_m. This indicates that the affinity of COX (subunit II) for cytochrome *c* was not altered, in contrast with the patient with COX deficiency described by Nijtmans et al. [50].

The biochemical manifestation of the MERRF mutation was further apparent as a decreased ability of the cells to synthesize ATP and as a decrease in $\Delta\Psi$ under steady-state conditions. Although the method used did not permit the exact evaluation of $\Delta\Psi$ in mV, a more than 80 % decrease in TMRM fluorescence indicated a significant decrease in $\Delta\Psi$ in the patient's cells. Moreover, titration with FCCP clearly revealed a disturbed balance between the $\Delta\Psi$-forming and $\Delta\Psi$-dissipating processes.

The use of transmitochondrial cybrids derived from fibroblasts [25,51,52], myoblasts [16,20] and blood platelets [53] has previously been described as a very useful approach to the study of mtDNA-linked diseases. We have prepared clones of cybrids, derived from fibroblasts from the patient, with the proportion of mutated mtDNA ranging from 73 % to 87 %. Experiments with cybrid cells showed that both the COX activity and $\Delta\Psi$ are significantly, but differently, affected by an increase in the amount of mutated mtDNA. The estimated threshold level for the full restoration of the COX activity was approx. 60 % wild-type mtDNA, which is much higher than a previously described genetic protective threshold of 15 % wild-type mtDNA for the MERRF mutation [7]. This discrepancy could have been caused by the different biochemical manifestation of the MERRF mutation in our patient, where COX was selectively affected. It has been previously noted that the MERRF mutation can evoke different outcomes at the biochemical level: whereas in some cases 90 % mutated mtDNA does not cause any decrease in COX activity [7,19], other patients with 64–74 % mutation show decreased COX activity [15,18].

The threshold level for the recovery of a normal $\Delta\Psi$ was estimated in our experiments to be 25 % wild-type mtDNA; this threshold level is also higher than the previously described threshold level of 10 % wild-type mtDNA for the full restoration of oxygen consumption [20]. In contrast, Larsson et al. [19] have shown that the respiration of muscle mitochondria from MERRF patients on succinate/rotenone is affected when the COX activity is decreased to less than 30 %. This is in agreement with our observation that $\Delta\Psi$ is affected when the activity of COX is decreased to less than 30 % (Figure 6C). A significantly low COX activity affects $\Delta\Psi$ and the synthesis of ATP. The decreased ATP synthesis in patient fibroblasts to 50 % of control is in agreement with the 'lethal level' of ATP synthesis discussed by Schon et al. [54].

Histochemical analysis of the patient clones showed that even after a high number of passages clusters of the cells could be found with detectable COX activity, next to cells without COX activity. Therefore the spectrophotometrically measured COX activity and the percentage of mutated mtDNA are averages of positive and negative COX activities in the cells within one clone and of higher and lower amounts of mutated mtDNA respectively. Such a pattern would imply a segregation of the mutation in distinct organelles within one cell. Attardi et al. [23] suggested that the protective effect of a small amount of wild-type mtDNA depends in major part on the intermixing and co-operation of the wild-type and mutant gene products within the same organelle. The observations presented here show that the same genetic background can imply different biochemical outcomes.

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