Am. J. Hum. Genet. 50:934–949, 1992

The Mitochondrial tRNA$^{Leu(UUR)}$ Mutation in Mitochondrial Encephalomyopathy, Lactic Acidosis, and Strokelike Episodes (MELAS): Genetic, Biochemical, and Morphological Correlations in Skeletal Muscle

Carlos T. Moraes,*,† Enzo Ricci,‡,[1] Eduardo Bonilla,*,‡ Salvatore DiMauro,*,‡ and Eric A. Schon*,†,‡

*H. Houston Merritt Clinical Research Center for Muscular Dystrophy and Related Disorders, and Departments of †Genetics and Development and ‡Neurology, Columbia University College of Physicians and Surgeons, New York

Summary

Mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS) has recently been associated with an A→G transition at position 3243 within the mitochondrial tRNA$^{Leu(UUR)}$ gene. Besides altering the tRNA$^{Leu(UUR)}$ sequence, this point mutation lies within a DNA segment responsible for transcription termination of the rRNA genes. We have studied the distribution and expression of mutant mtDNAs in muscle biopsies from MELAS patients. Histochemical, immunohistochemical, and single-fiber PCR analysis showed that ragged-red fibers (RRF) are associated both with high levels of mutant mitochondrial genomes (>85% mutant mtDNA) and with a partial cytochrome *c* oxidase deficiency. By quantitative in situ hybridization, the steady-state ratios of mRNAs:rRNAs were found to be similar to controls in six of eight patients studied. In two other patients the relative levels of heavy-strand mRNAs were slightly increased, but a patient with myoclonic epilepsy and RRF also exhibited a similar increase. These results directly correlate the A→G transition at mtDNA position 3243 with muscle mitochondrial proliferation, partial respiratory-chain impairment, decreased mitochondrially synthesized protein content, and no specific alterations in mitochondrial ratios of mRNAs:rRNAs.

Introduction

Several mutations of human mtDNA have been identified in patients with mitochondrial encephalomyopathies, a clinically heterogeneous group of diseases defined by morphologically abnormal mitochondria, defects in mitochondrial metabolism, or maternal mode of inheritance (for review, see Shoffner and Wallace 1990; Moraes et al. 1991*b*). Recently, pathogenic mtDNA mutations have been identified in three major mitochondrial encephalomyopathies. First, heteroplasmic deletions of mtDNA have been associated with Kearns-Sayre syndrome (KSS) and are often seen in patients with paralysis of extraocular muscles (progressive external ophthalmoplegia [PEO]) and ragged-red fibers (RRF) (Holt et al. 1988, 1989; Moraes et al. 1989; Nelson et al. 1989). Second, myoclonic epilepsy and RRF [MERRF] has been associated with an A→G transition in the TψC loop of the tRNA^{Lys} gene (Shoffner et al. 1990; Yoneda et al. 1990). Third, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS) (Pavlakis et al. 1984) has been associated with an A→G transition at position 3243 of the tRNA$^{Leu(UUR)}$ gene (herein called "the MELAS mutation"; Goto et al. 1990*b*; Kobayashi et al. 1990; Tanaka et al. 1991).

While a malfunction of tRNA^{Lys} during protein synthesis can explain the biochemical (and probably the clinical) abnormalities seen in MERRF patients (Cho-

Received October 15, 1991; revision received December 26, 1991.

Address for correspondence and reprints: Dr. E. A. Schon, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.

1. Present address: Department of Neurology, Universita Cattolica del Sacro Cuore, Rome.

0002-9297/92/5005-0007$02.00

myn et al. 1991), the $tRNA^{Leu(UUR)}$ mutation in MELAS offers an alternative pathogenetic mechanism, as the A→G transition at nucleotide 3243 may alter the recognition site for a protein responsible for transcription termination of rRNA genes (Christianson and Clayton 1986; Kruse et al. 1989). Transcription termination at this specific site is considered important in maintaining the appropriate steady-state ratios of mRNAs:rRNAs (approximately 0.1:25–1:25 in HeLa cells, depending on the mRNA species [Gelfand and Attardi 1981]). Hess et al. (1991) have shown that the MELAS mutation has a striking effect on transcription termination in vitro, with the mutant site being approximately 10-fold less effective than the wild type in promoting transcription termination.

In the present study we correlated the presence of the MELAS mutation with abnormalities in muscle morphology. We also studied the role of rRNA transcription termination in the pathogenesis of MELAS in vivo.

Material, Patients, and Methods

Restriction enzymes were from Boehringer Mannheim and New England Biolabs. Klenow fragment of *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. RNAse A and chemicals were from Sigma. [α-^{32}P]- and [α-^{35}S]-dATP (800 Ci/mmol) were from New England Nuclear. Single-stranded oligonucleotide primers were from Genosys.

Patients

Patients MELAS 1–3, 5, and 7 in this paper are patients 6, 18, 9, 5, and 21, respectively, in the study by Ciafaloni et al. (1992). All had typical MELAS syndrome defined by (1) strokelike episodes, (2) lactic acidosis or RRF, and (3) at least two of the following: focal or generalized seizures, dementia, or recurrent headache and vomiting. The clinical features of the patients have been reported elsewhere (Ciafaloni et al. 1992). Patient MELAS 6 (relative 27 in Ciafaloni et al. 1992) is the mother of a typical MELAS patient. She had recurrent migraine headaches and mild lactic acidosis. Patients MELAS 4 and 8 (patients 1 and 5 in table 4 of Ciafaloni et al. 1992) were clinically atypical. In brief, patient MELAS 4 was a 30-year-old man with childhood-onset PEO, exercise intolerance, limb weakness, sensorineural hearing loss, and lactic acidosis. Patient MELAS 8 was a 25-year-old man with developmental delay, short stature, PEO, seizures, ataxia, and incomplete right bundle-branch block; he had no RRF in the muscle biopsy. All the MELAS patients had the $tRNA^{Leu(UUR)}$ mutation described by Goto et al.(1990*b*) and Kobayashi et al. (1990). Patient MERRF 1, a 32-year-old woman, presented at age 29 years with generalized tonic-clonic and partial complex seizures and, a year later, developed myoclonic jerks, ataxia, dysarthria, and limb weakness. She had lactic acidosis and RRF in the muscle biopsy. Family history was compatible with maternal inheritance. Muscle mtDNA studies detected an A→G transition at position 8344 (Shoffner et al. 1990; Yoneda et al. 1990) in 97% of mitochondrial genomes in skeletal muscle. Control patients had no clinical, histological, or biochemical evidence of mitochondrial abnormalities.

Histochemistry, Immunohistochemistry, and In Situ Hybridization

Eight-micrometer-thick muscle biopsy sections from patients and controls were mounted on the same slide and were used in all experiments. Staining for cytochrome *c* oxidase (COX), succinate dehydrogenase (SDH), and myosin ATPase at pH 9.4 were performed according to methods described elsewhere (Seligman et al. 1968; Dubowitz and Brooke 1973). For visualization of nuclei, ethidium bromide staining of frozen sections was accomplished by incubating SDH-stained sections in 0.1% ethidium bromide for 30 min, followed by three washes with distilled water for a total time of 5 min. Indirect immunofluorescence against NADH dehydrogenase subunit 1 (ND1) and against COX subunit II (COX II) were performed according to a method described elsewhere (Tritschler et al. 1991). Pyruvate dehydrogenase complex (PDH) was immunodetected with autoantibodies (diluted 1:200) from patients with primary biliary cirrhosis (Sudoyo et al. 1990). The in situ hybridization procedure with ^{35}S-labeled probes has been described elsewhere (Mita et al. 1989; Moraes et al. 1991*a*). All the hybridizations were performed with 10^6 cpm/100 μl of hybridization solution.

Determination of the Proportion of Normal and Mutant mtDNAs in MELAS Patients

Approximately 1 μg of genomic DNA was submitted to PCR amplification (1 min at 94°C, 1 min at 55°C, and 0.75 min at 72°C; 25 cycles) according to the manufacturer's (Perkin Elmer Cetus) instructions, using the following primers: light-strand positions 3116–3134 and heavy-strand positions 3353–3333 (numbers are according to Anderson et al. 1981). One

extra cycle was then performed (2 min at 94°C, 1 min at 55°C, and 12 min at 72°C) after the addition of 10 μCi of [α-^{32}P]-dATP, 100 pmol of each primer, and 2.5 U of *Taq* polymerase. Adding radioactive dATP only in the last cycle ("last-cycle hot PCR") avoids detection of heteroduplexes between mutant and wild-type strands, which would cause underestimation of the mutant population after restriction-enzyme digestion (Shoffner et al. 1990). The DNA fragment produced by the PCR reaction was purified using the Gene-Clean kit (BIO-101) and was digested with *Hae*III for 1–2 h. The A→G transition at position 3243 creates a new *Hae*III site which is diagnostic for the MELAS mutation (fig. 1; Ciafaloni et al. 1991). The digestion products were electrophoresed through a 12% nondenaturing polyacrylamide gel, and the radioactive fragments were quantitated by scanning the gel in a Betascope 603 Blot Analyzer (Betagen). RFLP analysis of the tRNA^{Lys} mutation in MERRF patients was performed with the aid of a "mispairing primer" (Seibel et al. 1990) that creates a new *Ban*II site in combination with the A→G transition at position 8344 within the tRNA^{Lys} gene.

Single-Fiber PCR

Thirty-micrometer-thick muscle sections from test subjects and controls were placed side by side on the same slide and were stained for SDH activity. Stained slides were then immersed in a petri dish containing 50 ml of 50% ethanol. Single fibers were isolated by aerosol-protected mouth suction with siliconized microcapillaries under an inverted microscope (see fig. 2). Each isolated fiber was placed directly into 20 μl of water in a 250-μl Eppendorf tube, was frozen at −20°C, and was boiled for 10 min. Thirty microliters of a PCR reaction mix (pretreated by exposure to 254- and 300-nm UV for 1 h [Sarkar and Sommer 1990]) were added to the tube, to a final volume of 50 μl. *Taq* polymerase was added immediately before starting the thermo-cycling. The concentrations of *Taq* polymerase, deoxynucleotides, and primers were as suggested by the manufacturer (Perkin Elmer Cetus). After 30 cycles (1 min at 94°C, 1 min at 55°C, and 0.75 min at 72°C), 2 U of *Taq* polymerase were added to each tube, and 10 more cycles were performed. Thirty microliters of the reaction mixture were transferred to another tube with 20 μl of a solution containing 10 μCi of [α-^{32}P]-dATP, 100 pmol of each primer, and 2 U of *Taq* polymerase in 1 × PCR buffer. Last-cycle hot PCR and RFLP analysis were as described above. A control dissection and PCR amplification for carryover of mtDNA from adjacent fibers through the overlying buffer showed no carryover. Blood vessels were isolated from muscle sections by the same procedure.

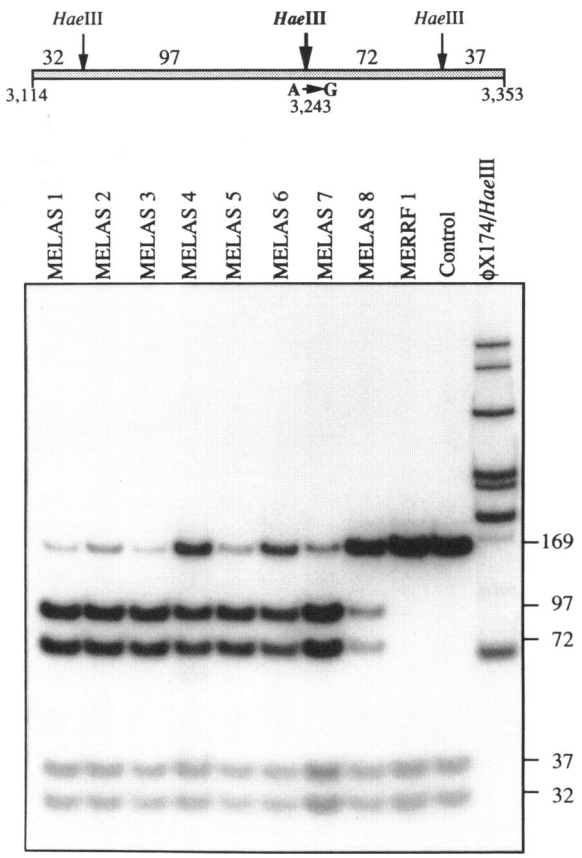


Figure 1 Percentage of mutant mitochondrial genomes in MELAS patients. The percentages of mtDNA with the A→G transition at position 3243 was determined by RFLP analyses of PCR-amplified fragments. The map at the top shows both how the mutation creates a new *Hae*III site (shown in boldface), and the expected sizes of DNA fragments (in bp) after *Hae*III digestion of PCR-amplified mtDNA. The lower panel shows an autoradiograph of PCR fragments from patients and controls obtained after "last-cycle hot PCR" followed by digestion with *Hae*III. The 169-bp fragment is derived from wild-type mtDNAs lacking the *Hae*III site at position 3243. The sizes of *Hae*III fragments (in bp) are at the right. The percentages of mutant mtDNAs are listed in table 1.

Preparation of mtDNA Probes

Specific mtDNA regions contained in M13 clones were used as templates (a gift from M. P. King and G. Attardi). The labeling and purification procedures have been described elsewhere (Moraes et al. 1991*a*). The mtDNA fragments cloned in M13 (+ strand) were clone mp18.XS5.51, L-strand positions 1194–1465

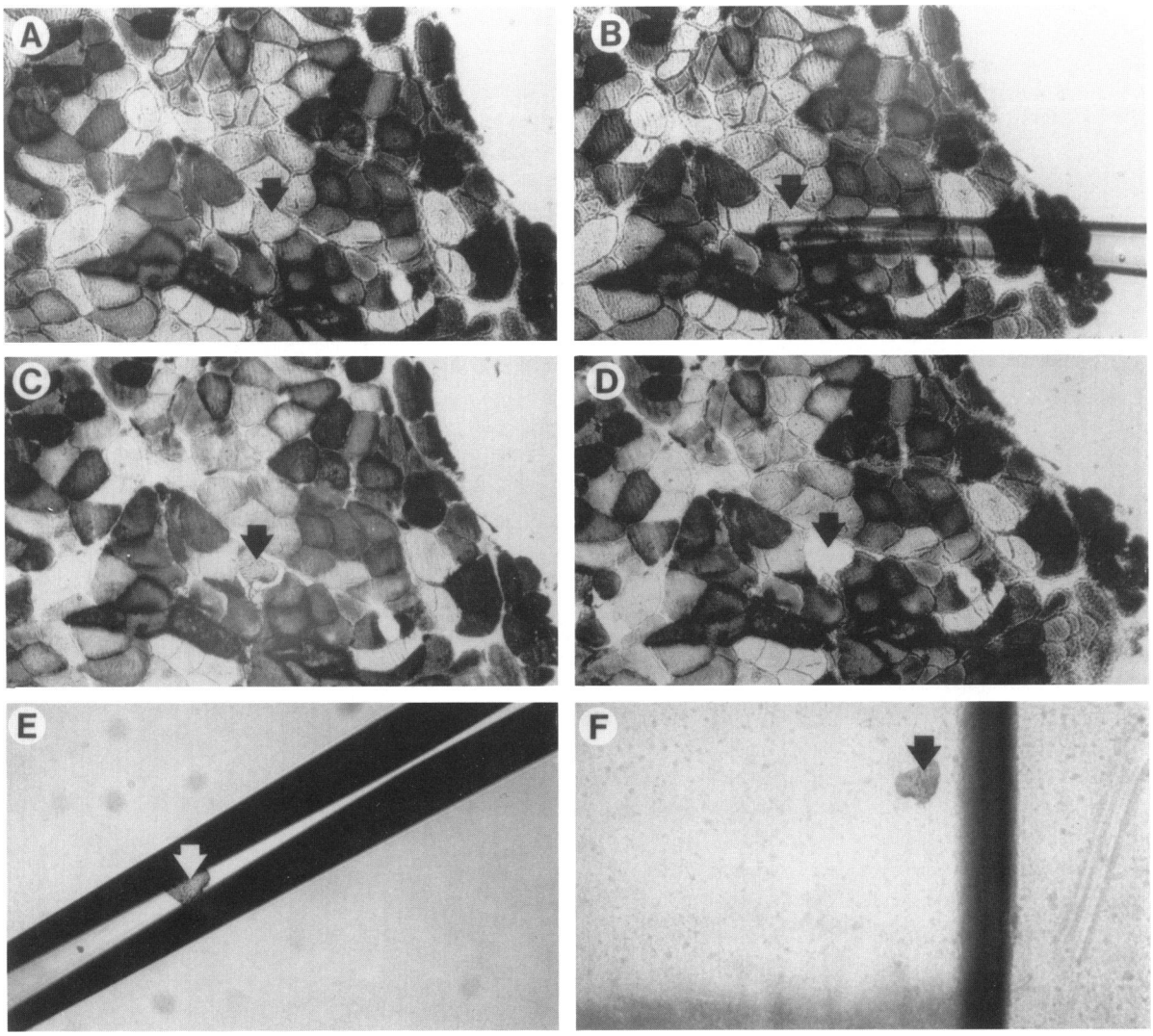


Figure 2 Microdissection of single fibers for PCR and RFLP analyses. The figure illustrates the procedure used for isolating single muscle fibers (for description, see Material, Patients, and Methods section). The specimen was initially stained for SDH activity (*A*). By means of a small glass microcapillary, the desired fiber (arrow) was loosened from its surrounding connective tissue (*B* and *C*). The fiber was removed from the section with the aid of a second capillary pipette and aerosol-protected mouth suction (*D* and *E*). The fiber was finally placed in an Eppendorf tube (*F*) and subjected to PCR as described in the Material, Patients, and Methods section.

within the 12S rRNA gene region; clone mp 19.XS6.2, L-strand positions 2441–2954 within the 16S rRNA region; clone mp18.XB66, L-strand positions 7888–8287 within the COX II region; clone mp19.XB66, H-strand positions 7658–8287 within the COX II region; clone mp19.BR54, L-strand positions 3850–4122 within the ND1 region; and clone mp19.BS51, L-strand positions 9020–9648 within the COXIII/APTase 6 region.

Image Analysis and Quantitation

Slides containing muscle sections probed by in situ hybridization were exposed to X-ray films simultaneously with a set of serially diluted ^{35}S-labeled marker spots. The quantitation was performed using an RAS-DG 1000 autoradiography analysis system (Amersham). Standard curves were constructed with the internal ^{35}S-labeled markers. Second-order correlations

Table 1

Genetic and Morphological Features of Skeletal Muscle Mitochondria from MELAS Patients and a MERRF Patient

Patient	% Mutant mtDNA	% RRF[a]	%RRF±[b]
MELAS 1	95	13.5	15.5
MELAS 2	91	6.8	13.7
MELAS 3	95	17.8	13.2
MELAS 4	63	9.5	9.5
MELAS 5	91	11.4	18.7
MELAS 6	59	.5	.5
MELAS 7	91	7.8	18.9
MELAS 8	25	.0	9.0
MERRF 1	97[c]	9.0	33.0

[a] Determined by analyzing 200–300 adjacent fibers after SDH staining. Only fibers with extensive subsarcolemmal accumulation of mitochondria were considered RRF.

[b] Percentage of fibers with SDH staining that is stronger than that in normal type I fibers but that does not fulfill the strict criteria for RRF (RRF±).

[c] Percentage refers to A→G transition at position 8344 within the mitochondrial $tRNA^{Lys}$ gene. (Patient MERRF 1 does not harbor the MELAS mutation [fig. 1].)

of .97–.99 were obtained for ^{35}S markers spanning 20-fold increments (with sample signals within this range) in all the X-ray films analyzed. After calibration with internal markers, the muscle-section images were digitalized and quantified according to the manufacturer's guidelines. The boundaries of the quantitation were restricted to large but intact areas of the specimen image.

Results

Mitochondrial Genotype and Morphologic Abnormalities in Muscle of MELAS Patients

We studied eight patients with documented A→G transitions at position 3243 (fig. 1). The percentages of mutant mtDNA ranged from 25% in patient MELAS 8 to 95% in patients MELAS 1 and MELAS 3 (table 1). Mitochondrial proliferation, reflected by intense staining for SDH activity (i.e., showing RRF), was detected in all but one patient, MELAS 8. Table 1 shows, for each patient, the percentage of typical RRF (%RRF) as well as the percentage of fibers that were not RRF but which reacted more intensely than normal for SDH activity (%RRF±). There was an apparent exponential correlation between percentage of mutant mtDNA and %RRF.

Distribution of Mutant and Wild-Type mtDNA in Muscle Fibers

Single muscle fibers from patient MELAS 7 were dissected from 30-μm-thick muscle sections previously stained for SDH activity (fig. 2). In this patient (91% mutant mtDNA in muscle and 7.8% RRF) we found that RRF contained 95%–97% mutant mtDNA, while non-RRF had 86%–95% mutant mtDNA (fig. 3*A*). Although this analysis showed that mutant mitochondrial genomes were present in high percentages in RRF, non-RRF also contained a predominance of mutant mtDNAs. The small differences and overlaps in the percentage of mutant mtDNAs observed between RRF and non-RRF made a conclusive association difficult. We therefore performed RFLP analysis in single fibers isolated from a patient with a lower percentage of mutant mitochondrial genomes (MELAS 6, containing 59% mutant mtDNA in muscle and 0.5% RRF). This analysis revealed a striking contrast between mutant mtDNA content in RRF as compared with "normal" fibers: RRF contained 85%–97% mutant mtDNAs while non-RRF had 17%–72% (fig. 3*B*). The results obtained with this patient showed a clear association between percentages of mutant mtDNA and abnormal mitochondrial proliferation.

The presence of high levels of mutant mitochondrial genomes in non-RRF observed in patient MELAS 7 suggested that secondary factors are involved in RRF formation. We have explored two possible factors responsible for this variability: (1) fast- and slow-twitch fibers have different oxidative requirements and may be differentially susceptible to RRF formation, and (2) mitochondrial proliferation, though triggered by a defect in oxidative phosphorylation, is probably regulated by nuclear-encoded factors: therefore, nuclear density in a specific muscle domain could influence RRF formation.

Serial sections stained for SDH and myosin ATPase at pH 9.4 showed a predominance of slow-twitch (type I) fiber involvement in mitochondrial proliferation (the percentage of RRF identified as type I was 82% in MELAS 1, 90% in MELAS 3, and 83% in MELAS 7), implying that fiber type plays an important role in determining RRF formation in MELAS. Nuclear density was investigated by staining 15–20 serial sections for SDH activity, followed by costaining with ethidium bromide. The number of nuclei in a single fiber was counted in successive serial sections (i.e., 100–200 μm of fiber length). This analysis showed no obvious association between RRF and nuclear density. Normal muscle fibers from patient

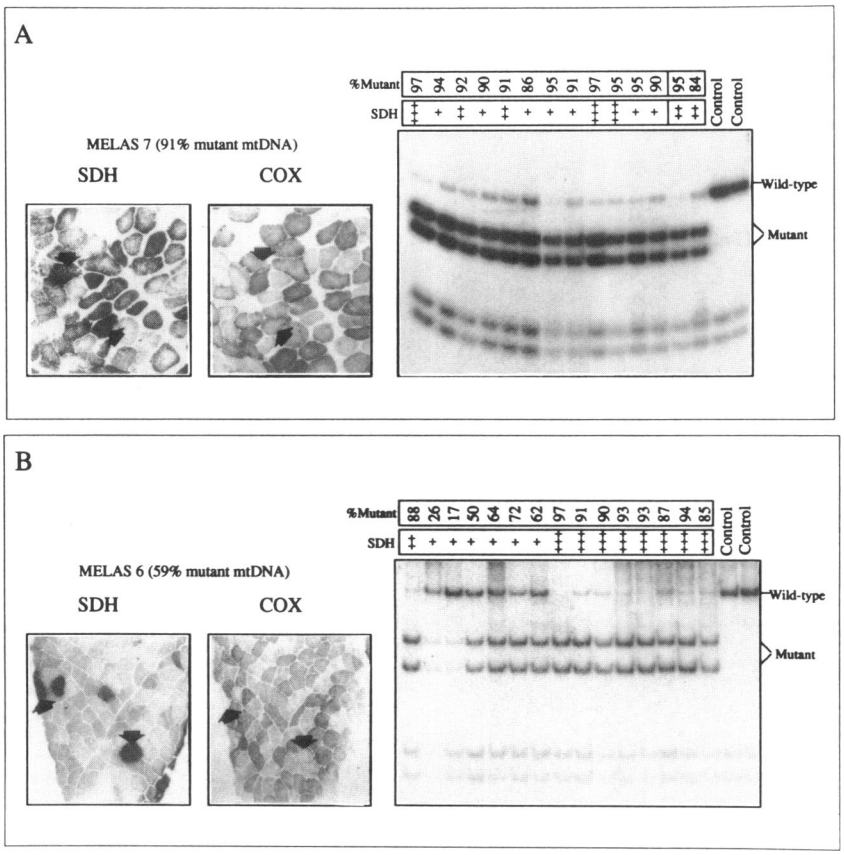


Figure 3 Percentage of mutant mtDNA in single muscle fibers from two MELAS patients. The small panels at the left show the morphological characteristics of each patient's muscle. Arrows show typical RRF. Isolated fibers and blood vessels obtained by the procedure described in fig. 2 were subjected to PCR and RFLP analyses (see Material, Patients, and Methods section). The morphological features of the selected fibers, as well as the percentage of mutant mitochondrial genomes in those fibers, are shown on top of each lane. One plus sign (+) indicates a fiber staining normally with SDH; two plus signs (+ +) indicate a fiber staining strongly with SDH, but not fulfilling the requirements of a RRF (for definition; see table 1); and three plus signs (+ + +) indicate RRF. Two strongly SDH-reactive vessels from patient MELAS 7 were also dissected and analyzed (A, two right-most lanes). *A,* RFLP analysis of single muscle fibers and vessels from patient MELAS 7 (91% mutant mtDNA in muscle). *B,* RFLP analysis of single muscle fibers from patient MELAS 6 (59% mutant mtDNA in muscle). In both *A* and *B,* control lanes correspond to fibers dissected from a control specimen adjacent to the MELAS specimens.

MELAS 1 had a nuclear density of 13 ± 4 nuclei/100 μm, as compared with 15 ± 3 nuclei/100 μm in RRF (three RRF were analyzed). Normal fibers from patient MELAS 7 had 17 ± 5 nuclei/100 μm, as compared with 20 ± 6 nuclei/100 μm in RRF (two RRF were analyzed).

Muscle biopsies of MELAS patients consistently show the presence of blood vessels that react strongly for SDH (strongly SDH-reactive vessels [SSV] [Hasegawa et al. 1991]), suggesting mitochondrial proliferation within smooth muscle cells. Microdissected blood vessels (i.e., SSV) from patient MELAS 7 showed a high percentage of mutant genomes, similar to the proportion observed in muscle cells (84% and 95% in two SSV, as compared with 86%–97% in muscle fibers; fig. 3*A*).

Correlations between Mitochondrial Proliferation, COX Activity, and mtDNA-encoded Polypeptides

Although RRF (observed by strong SDH staining) also showed a positive staining for COX activity in most cases, the contrast between surrounding "normal fibers" and RRF observed by COX staining was far less striking than the contrast observed by SDH staining (e.g., see figs. 3 and 4). This suggested a partial COX deficiency per organelle in RRF. While the partial reduction in COX activity at the single-fiber level was consistently observed in RRF, this decrease was

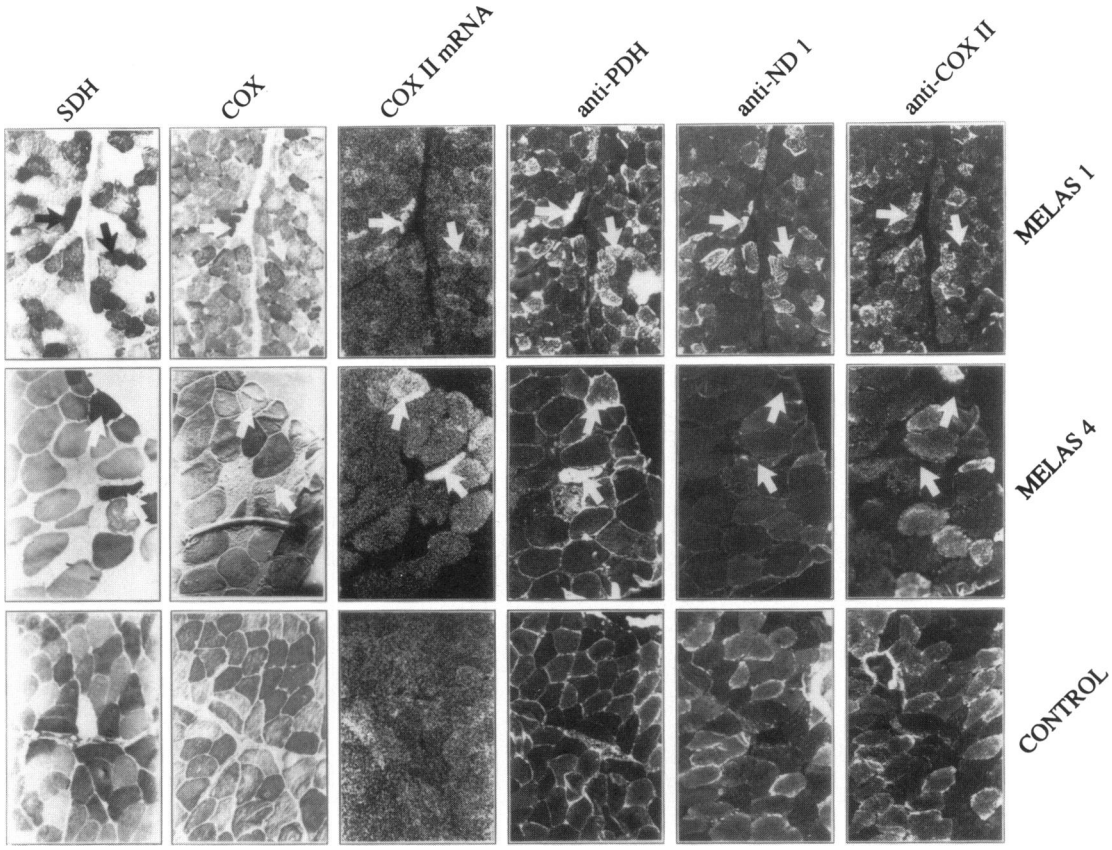
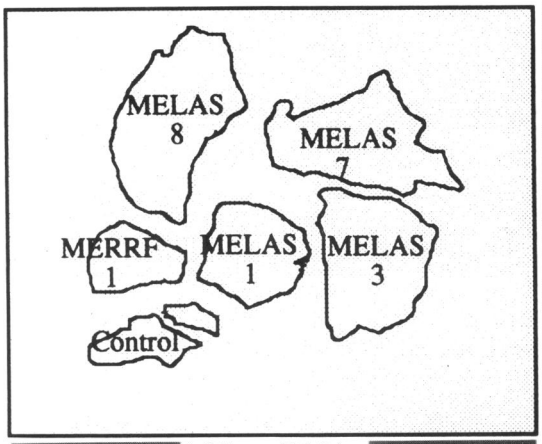


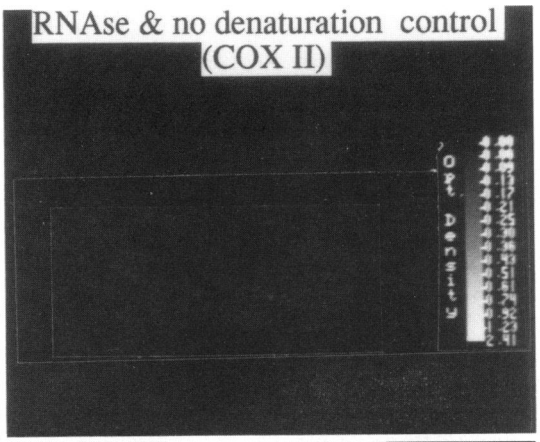
Figure 4 Detection of mitochondrial polypeptides in muscle of MELAS patients. Serial muscle sections were stained for SDH and COX enzyme activity, were probed for COX subunit II mRNA, and were immunostained for PDH, COX II, and ND 1 polypeptides (see Material, Patients, and Methods section). Patients MELAS 1 and MELAS 4 represent the extremes in terms of degree of COX deficiency at the single-fiber level. The arrows show typical RRF. The immunostaining photomicrographs for any one antibody were taken from the same slide and with the same exposure and developing times, thus allowing for direct comparison between patients and controls.

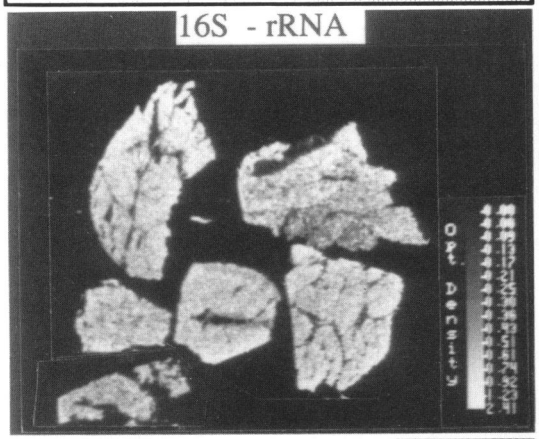
quite variable among patients, with no apparent correlation with the proportion of mutant genomes. Specifically, the reduction was severe in fibers from patients MELAS 2 (91% mutant mtDNA) and MELAS 4 (63%), moderate for patients MELAS 6 (50%), MELAS 5 (91%), and MELAS 7 (91%), and mild for patients MELAS 1 (95%), MELAS 3 (95%), and MELAS 8 (25%).

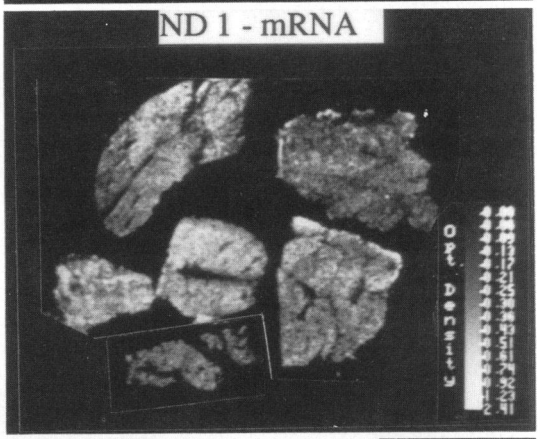
Immunostaining observations were compatible with COX histochemistry, with similar patient-to-patient variability. Patients MELAS 1 and MELAS 4 (fig. 4) represent opposite extremes in terms of COX activity. Immunostaining using antibodies against mitochondrial proteins encoded by the nuclear genome (anti-PDH) showed a strong signal in RRF, with a pattern very similar to that observed for SDH staining, which

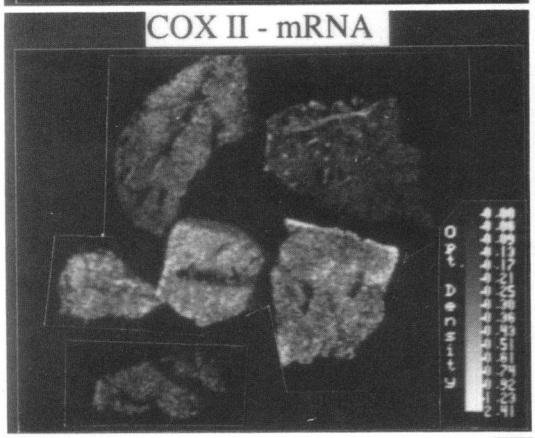
Figure 5 Macroscopic image analyses of in situ hybridization signals. Slides of muscle sections subjected to in situ hybridization were exposed to X-ray films for different times. Each X-ray image was digitalized and quantitated as described in the Material, Patients, and Methods section. The upper-left panel identifies the muscle section shown in the other seven panels. The upper-right panel show background levels obtained after RNAse treatment but no denaturation (only samples MERRF 1 and MELAS 1 were present on this slide). The remaining panels show the digitalized image obtained for the denoted probes used for detection of either RNA (no RNAse and no denaturation) or DNA (RNAse and denaturation). The brightness scale is shown at the right of each panel. The image of 16S rRNA was obtained from an X-ray film exposed for 6 h. The images of the different mRNAs were obtained from an X-ray film exposed for 16 h. DNA images, as well as the RNAse and no-denaturation control images, were obtained from a film exposed for 20 h.

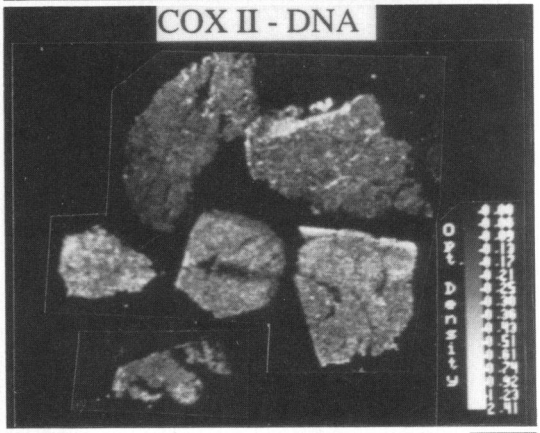

MELAS
8
MELAS
7
MERRF
1
MELAS
1
MELAS
3
Control

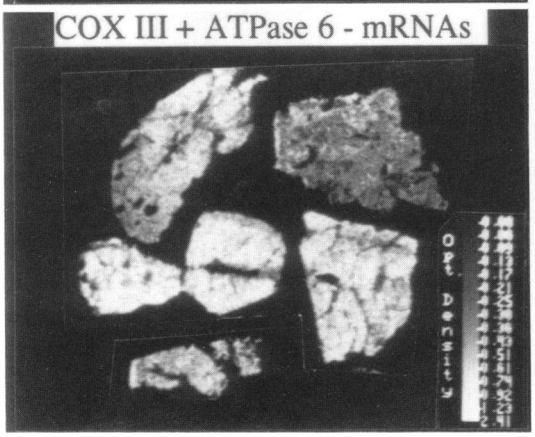

RNAse & no denaturation control
(COX II)

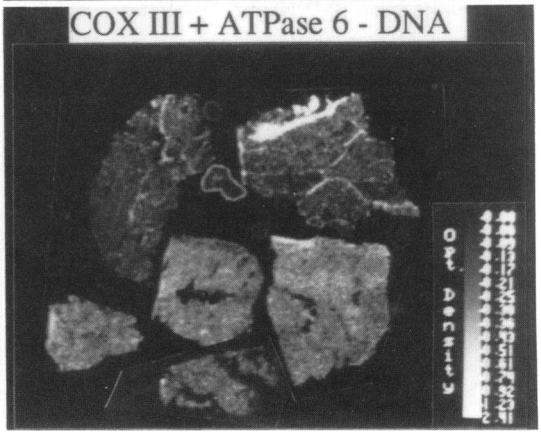

16S - rRNA


ND 1 - mRNA


COX II - mRNA


COX II - DNA


COX III + ATPase 6 - mRNAs


COX III + ATPase 6 - DNA

is also encoded by nuclear genes (fig. 4). Mitochondrially encoded polypeptides (COX II and ND1), though also present in muscle fibers of MELAS patients, showed a less intense staining pattern that resembled the pattern of COX enzyme activity. The reduction in mitochondrially synthesized polypeptides in patient MELAS 4 was far more severe than that in patient MELAS 1, in keeping with the correlation observed for COX activity. Moreover, the overall intensity of the immunostaining with anti-COX II or anti-ND1 in several controls was consistently stronger than that observed in non-RRF of MELAS patients (fig. 4). In situ hybridization against COX II (fig. 4) and ND1 mRNAs (not shown) showed that these mRNA species were abundant in muscle fibers with reduced COX II and ND1 polypeptides, suggesting a defect at the protein-synthesis level.

Muscle Mitochondrial RNA Levels

Because the limited amount of fresh tissue available from MELAS patients precluded the use of classical quantitative RNA assays (e.g., northern blots), we developed a semiquantitative in situ hybridization assay. Serial muscle sections were hybridized with different probes for detection of RNA or DNA. Slides containing muscle sections used for in situ hybridization were then exposed to X-ray films in order to obtain a macroscopic signal reflecting RNA or DNA levels. Figure 5 shows the digitalized image of X-ray films from which cpm values were later obtained by image analysis (fig. 6). Although all hybridizations were performed with 10^6 cpm of probe/100 μl of hybridization solution, differences in size and specific activity among different probes did not allow for the measurement of absolute molar ratios of the different RNA species. However, our approach did allow for comparisons between relative ratios observed in patients and controls.

In order to assess a possible impairment of transcription termination in the $tRNA^{Leu(UUR)}$ region, we performed five similar but independent experiments (one of which is shown in figs. 5 and 6). The levels of different RNA species were then expressed relative to those of 16S rRNA (fig. 7). Patients MELAS 1 and 3, who had the highest proportions of mutant mtDNA (95%) and RRF (13.5% and 17.8%, respectively), showed a slight increase in the ratios of H-strand mRNA relative to 16S rRNA when compared with those in the controls. The other MELAS patients had mRNA:16S rRNA ratios within or close to 1 SD of

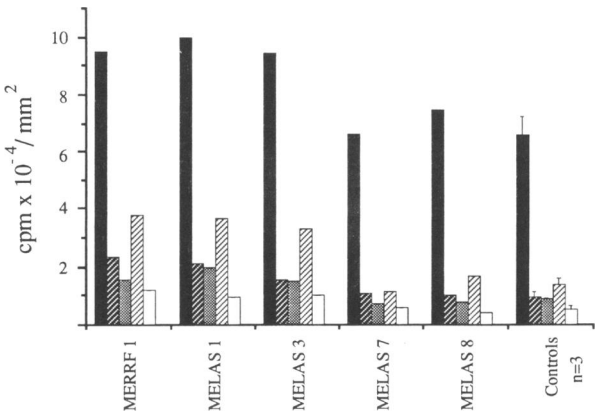


Figure 6 Mitochondrial nucleic acids levels in MELAS patients. The RNA and DNA levels (cpm/mm^2) derived from image analyses of the in situ hybridization experiment shown in fig. 4 are represented in the histograms. DNA levels were detected by the same probe used to detect ND1 mRNA levels, but after subjecting the muscle sections to RNAse and denaturation treatment. Error bars in the controls correspond to 1 SD. ■ = 16S rRNA; ▨ = ND1 mRNA; ▩ = COX II mRNA; ▨ = COX III + ATPase6 mRNAs; and □ = mtDNA.

those in the controls (fig. 7). The increased ratio of mRNA:rRNA observed for some MELAS patients was consistent with the suggested impairment of transcription termination in the $tRNA^{Leu(UUR)}$ gene (Hess et al. 1991). However, patient MERRF 1 (who harbors a $tRNA^{Lys}$ mutation at position 8344 but not an A→G transition at position 3243 within $tRNA^{Leu(UUR)}$ [fig. 1]) also showed a similar increase in relative levels of H-strand transcripts (fig. 6). These observations suggest that the small increase in mRNA:rRNA ratios observed in two patients with MELAS is not specific for the MELAS mutation. The increase in mRNA:rRNA ratios observed in some patients also correlated with increased numbers of mitochondrial genomes (fig. 6).

We also measured the steady-state levels of large L-strand transcripts in serial sections of the samples shown in figures 5 and 6*D*. The steady-state levels of these transcripts are very low (Gelfand and Attardi 1981), and we could not observe a macroscopic signal above background for the controls, or in patients MELAS 7 and 8. However, a detectable macroscopic signal was observed for patients MERRF 1, MELAS 1, and MELAS 3, probably because of the extensive mitochondrial proliferation observed in these patients (not shown). Elevated L-strand signals were also observed microscopically in RRF (see below).

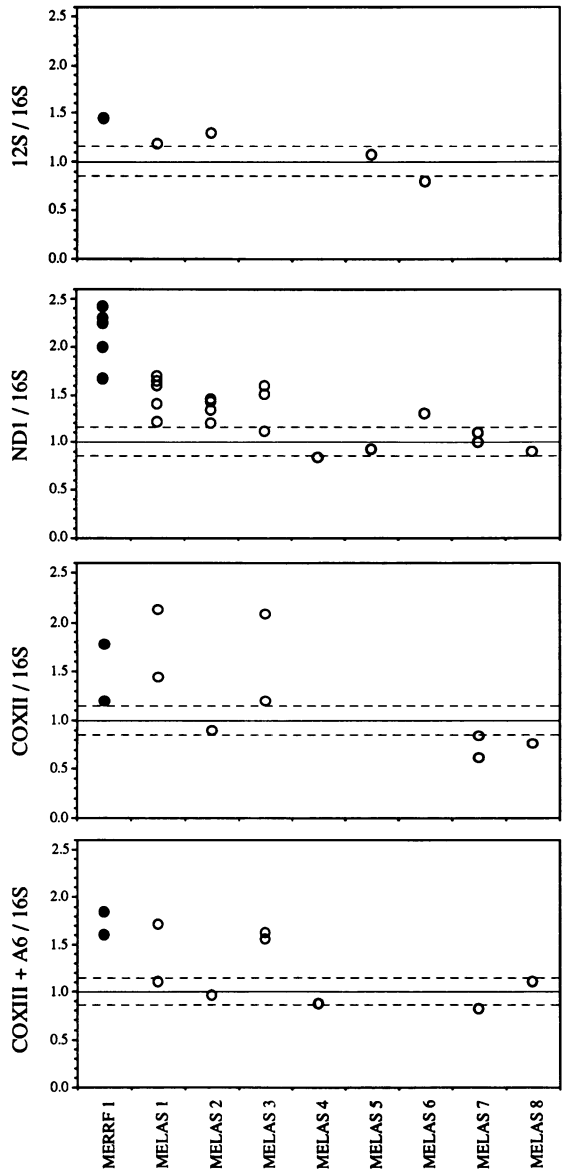


Figure 7 Mitochondrial mRNA:rRNA ratios in MELAS patients. The image analysis results from five independent in situ hybridization experiments (similar to the experiment shown in figs. 5 and 6) were used to calculate the ratios of different RNA species relative to 16S rRNA. The figure shows these ratios after normalization to ratios obtained from control specimens (dotted lines correspond to ± 1 SD of n controls: n = 12 for ND1; n = 2 for 12S; n = 7 for COX II; and n = 6 for COX III/A6). Each circle represents one independent measurement. White circles denote MELAS patients; and black circles denote patient MERRF 1. The number of measurements for each patient was different, and not all RNA species were determined for every patient.

Microscopic Correlations between Steady-State Levels of Mitochondrial Nucleic Acids and Respiratory-Chain Function

All slides used for macroscopic quantitation were analyzed microscopically after exposure to photographic emulsion. Our findings are exemplified by the results obtained with patients MELAS 1 and 7 (fig. 8) and were representative of the analysis in the other MELAS patients. Visual inspection of individual fibers showed that mitochondrial genomes and transcripts (16S rRNA, ND1, COX II, COX III + ATPase6, and large polycistronic L-strand mRNAs) were increased in RRF (fig. 8, arrows), reflecting abnormal mitochondrial proliferation. Both the specificity of the signal and the low background at the single-fiber level (fig. 8) confirmed the specificity of the in situ hybridization signal and validated the macroscopic analysis.

Discussion

Using a combination of histochemistry, immunohistochemistry, in situ hybridization, and PCR techniques, we have shown that the genetic defect in muscle from MELAS patients harboring the A→G transition at mtDNA position 3243 in the $tRNA^{Leu(UUR)}$ gene most likely causes a decrease in levels of mtDNA-encoded polypeptides. This conclusion is supported by the apparent decrease in immunocytochemical staining of ND1 and COX II, whereas the corresponding transcripts were not decreased. The reduction in protein synthesis was most evident in RRF, in which we found the highest levels of mutant genomes. There appears to be a threshold level of a minimum of 85% mutant genomes required for RRF formation. Finally, we found no compelling evidence for a significant role of defective transcription termination in the pathogenesis of this disease.

Morphological and Genetic Characterization of Skeletal Muscle from MELAS Patients

We tried to correlate the mitochondrial $tRNA^{Leu(UUR)}$ mutation with the skeletal muscle pathology seen in MELAS patients. All patients studied had an A→G transition at position 3243 within the $tRNA^{Leu(UUR)}$ gene. The percentage of mutant mitochondrial genomes correlated in an apparently exponential fashion with mitochondrial proliferation in muscle (i.e., with %RRF), suggesting that after a threshold number of mutant mitochondrial genomes (approximately 90%) is attained massive mitochondrial proliferation en-

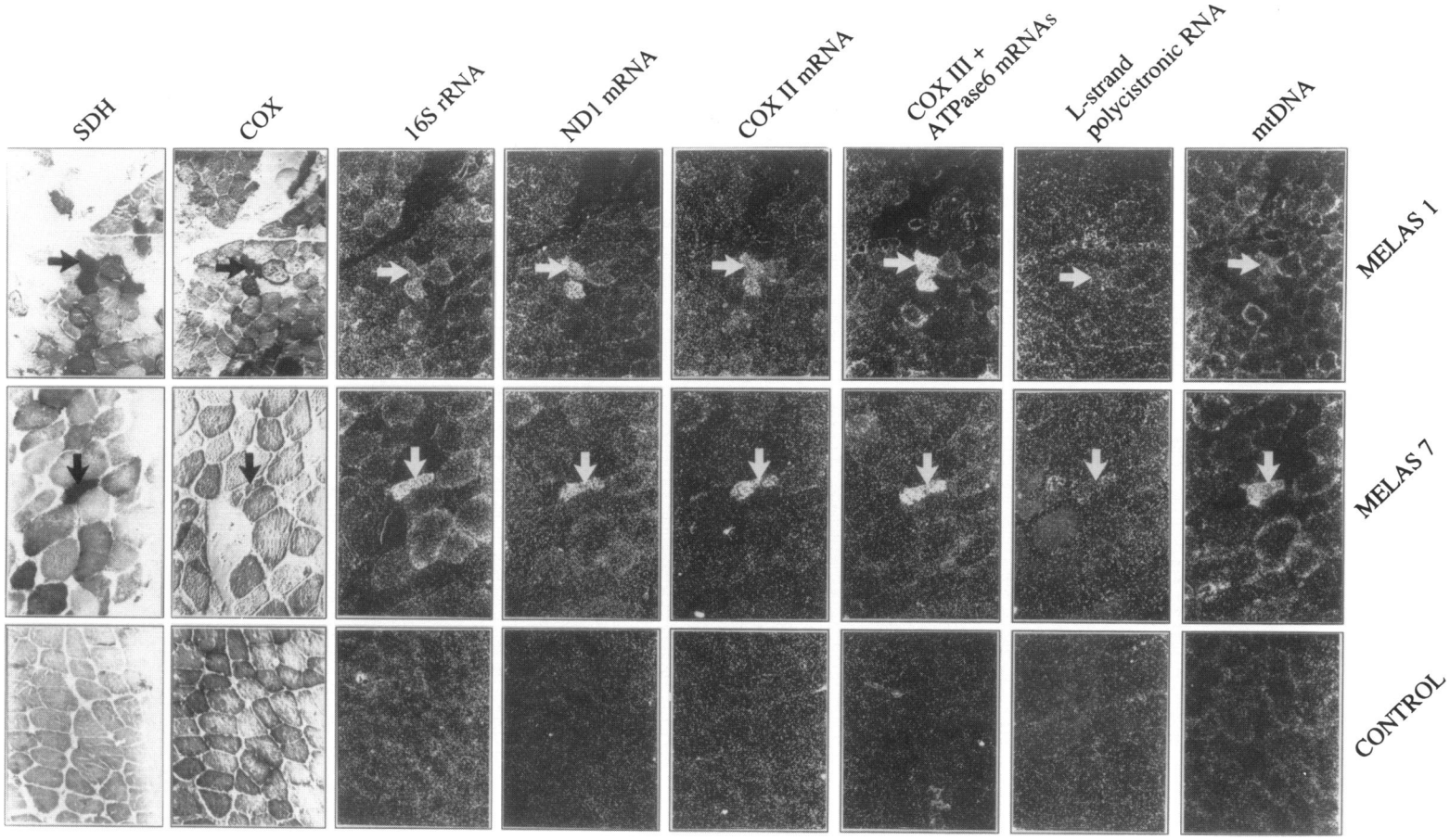


Figure 8 Microscopic correlation between mitochondrial RNA levels and mitochondrial function. Serial muscle sections were stained for SDH and COX enzyme activity and were probed for the indicated mitochondrial transcripts as well as for mitochondrial genomes. In situ hybridization slides were dipped in photographic emulsion and developed at different times, according to the intensity of the initial signal. Therefore, the absolute number of silver grains does not allow for interslide comparison of RNA and DNA levels. Although only patients MELAS 1 and MELAS 7 are depicted, similar results were obtained for all MELAS patients. Muscle sections of controls and of patients were present on the same slide and were treated identically at all steps of the experiment. The arrow shows typical RRF throughout the serial sections of patients MELAS 1 and MELAS 7. Muscle sections flanking the ones used for in situ hybridization were stained for SDH to assure that RRF features were present throughout the length of the sections (not shown).

sues. These numbers are in agreement with our single-fiber analysis, where no RRF with less than 85% mutant genomes were detected (see below).

The reduction in COX activity in muscle fibers of MELAS patients (Koga et al. 1988; present study) appears to be less severe than what has been described in MERRF (Lombes et al. 1989) or KSS/PEO (Mita et al. 1989; Goto et al. 1990*a*; Shoubridge et al. 1990), syndromes caused by different mtDNA mutations. In MERRF and KSS/PEO, RRF are consistently COX negative, while in MELAS patients RRF often show positive COX staining. Because of its qualitative nature, histological staining does not allow for easy recognition of partial COX deficiency in fibers with extensive mitochondrial proliferation. In addition, the partial COX deficiency observed microscopically in MELAS patients varied significantly among different cases, and even patients with similar percentages of mutant mtDNA had different degrees of COX deficiency. For example, patient MELAS 2 (91% mutant DNA) showed a marked COX deficiency, while patients MELAS 5 and MELAS 7 (also with 91% mutant mtDNA) had less severe impairment. Additional mitochondrial or nuclear sequences, in association with the MELAS mutation, may play an important role in determining the respiratory competence of muscle cells.

COX-positive RRF in MELAS demonstrate that mitochondrial proliferation is not always an unsuccessful response to mitochondrial dysfunction. Because the respiratory-chain defect caused by the MELAS mutation is partial, extensive mitochondrial proliferation is able to compensate (at least partially) for the local needs of oxidative phosphorylation in an affected muscle domain, as evinced by positive COX activity in RRF. This compensation seems to be more effective in some patients (e.g., MELAS 1 and MELAS 3) than in others (e.g., MELAS 2 and MELAS 4); the reasons for this variability remain to be determined.

Immunostaining studies showed that fibers (both RRF and non-RRF) with a partial COX deficiency have a reduction of mitochondrially synthesized proteins (COX II and ND1). This partial reduction in cross-reacting material is apparently specific to mtDNA-encoded proteins, as there was a normal immunostaining pattern of a nuclear-encoded mitochondrial protein (i.e., PDH). Serial muscle sections subjected to in situ hybridizations showed that COX II and ND1 mRNAs were abundant in RRF, indicating that the partial COX deficiency is caused at the level of protein synthesis. The reduction in COX II levels appears to be more severe than in ND1, suggesting that mitochondrially synthesized polypeptides are affected differentially.

mtDNA Heteroplasmy and RRF Formation

Single-fiber PCR followed by RFLP analysis showed that RRF have higher proportions of mutant mtDNA than do non-RRF. This correlation was clear in a patient with a relatively small percentage of mutant mitochondrial genomes (patient MELAS 6, who had 59% mutant mtDNA) but was less striking when a patient harboring 91% mutant mitochondrial genomes was analyzed. A minimum of approximately 90% mutant mitochondrial genomes harboring the MELAS mutation seems to be necessary to induce abnormal mitochondrial proliferation. However, many muscle domains containing these percentages of mutant mitochondrial genomes did not undergo mitochondrial proliferation, indicating that additional factors may participate in RRF formation. As observed in other mitochondrial myopathies (Olson et al. 1972; Karpati et al. 1973; Romero et al. 1989), there is a preferential involvement of type I fibers in mitochondrial proliferation. Because of its predominantly glycolytic activity, type II fibers are probably less sensitive to a reduction in oxidative phosphorylation capacity and may require an extremely high percentage of mutant mtDNA before mitochondrial proliferation is triggered. Nuclear density in a particular muscle domain did not show a significant correlation with RRF formation.

The cause and origin of RRF has puzzled investigators for several years. Recently, Mita et al. (1989) and Shoubridge et al. (1990) showed that RRF in patients with KSS are associated with the presence of partially deleted mitochondrial genomes. The most sensible hypothesis emerging from these observations is that a high concentration of defective organelles in a muscle domain triggers a compensatory mechanism (namely, mitochondrial proliferation). Although this hypothesis agrees with our observations in MELAS, there are substantial differences between the two situations. Non-RRF in KSS seem to be devoid of partially deleted mtDNA (Shoubridge et al. 1990), whereas in MELAS patients non-RRF may contain as much as 95% mutant mtDNA. This difference reinforces our view that the MELAS mutation is not a null mutation and that it is probably still able to support a certain degree of mitochondrial protein synthesis, as has been shown in a tissue culture system (King et al. 1992).

When disorders caused by mtDNA mutations are correlated with RRF formation, an interesting pattern

appears. Mitochondrial proliferation seems to be associated with a reduction in mitochondrially synthesized proteins, rather than with an oxidative-phosphorylation defect per se. RRF are commonly observed in MELAS, MERRF, and syndromes caused by mtDNA deletions or depletion (Shoffner et al. 1990; Moraes et al. 1991*b*), all of which cause a reduction in mitochondrially synthesized proteins. In addition, RRF were observed in a recently identified mitochondrial tRNA mutation associated with familial cardiomyopathy (Zeviani et al. 1991). On the other hand, mitochondrial diseases caused by mtDNA mutations in structural genes (leading to amino acid substitutions) do not trigger abnormal mitochondrial proliferation (e.g., Leber hereditary optic neuropathy [Wallace et al. 1988; Howell et al. 1991; Huoponen et al. 1991] and a multisystem disorder caused by a mutation in the ATPase 6 gene [Holt et al. 1990]).

Blood-Vessel Involvement and the Pathogenesis of MELAS

By definition, typical MELAS patients suffer from strokelike episodes, suggesting an important role for blood-vessel involvement in the pathogenesis of MELAS. Hasegawa et al. (1991) have shown that muscle arterioles react strongly for SDH activity (i.e., appear as SSV). Although SSV are sometimes observed in other mitochondrial diseases, such as MERRF and KSS, they are consistently present only in MELAS (Hasegawa et al. 1991; E. Ricci and E. Bonilla, unpublished observations). While this phenomenon may be due to a high proportion of mutant mitochondrial genomes observed in SSV (fig. 3*A*), we found that non-SSV in a MERRF patient also had high proportions of mutant mitochondrial genomes, similar to the proportions observed in muscle fibers (C. T. Moraes and E. Ricci, unpublished observations). Therefore, it remains to be determined whether blood vessels are particularly sensitive to the $tRNA^{Leu(UUR)}$ mutation and why mitochondria proliferate in small blood vessels of MELAS patients.

Transcription Termination in MELAS

In vitro results had suggested than an impairment of transcription termination in the $tRNA^{Leu(UUR)}$ gene could have an important role in the pathogenesis of MELAS (Hess et al. 1991). Lack of termination could cause mitochondrial dysfunction by at least three possible mechanisms: (1) interference with 16S rRNA synthesis, (2) alteration in mRNA:rRNA ratios, and (3) a feedback mechanism leading to overproduction of the termination factor interfering with light-strand transcription (the D-loop region also has possible binding sites for the termination factor [Christianson and Clayton 1986]). We have tested some of these predictions by measuring changes in steady-state ratios of mitochondrial heavy-strand transcripts in vivo.

rRNA synthesis does not seem to be reduced, as judged by the strong signal obtained by in situ hybridization. Although our methods could not distinguish whether the 16S rRNA was processed correctly, work done with cybrids containing exclusively mtDNA with the MELAS mutation showed that the vast majority of 16S rRNA molecules are correctly processed, within the limits of northern blot analysis (King et al. 1992).

MELAS patients with close to 100% mutant mtDNA in muscle showed a small but noticeable increase in the H-strand mRNA:rRNA ratios, as would be expected if transcription termination were impaired. However, we believe that this mild increase was a nonspecific phenomenon, because of the following two observations: (1) increased mRNA:rRNA ratios were not observed in all MELAS patients, including some with more than 90% mutant mitochondrial genomes; and (2) the increase in mRNA:rRNA ratios observed in some MELAS patients was also seen in a patient with MERRF.

Finally, abnormal light-strand transcription termination, caused by a possible overproduction of the termination factor (Hess et al. 1991), also does not seem to take place in patients with MELAS. Premature L-strand transcription termination would probably impair mtDNA replication, because an L-strand transcript is required for initiation of replication (Chang and Clayton 1985). Muscle specimens from some patients with MELAS showed increased mtDNA levels, and although this increase can be explained by mitochondrial proliferation, this would not be expected if an abnormal termination of L-strand transcription were involved in the pathogenesis. In addition, we detected increased levels of L-strand transcription in patients with extensive mitochondrial proliferation (patients MELAS 1, and MELAS 3, and MERRF 1). Although our observations suggest that the MELAS mutation in skeletal muscle causes no specific changes in steady-state ratios of mRNA:rRNA, it is still possible that transcription termination is decreased and that abnormal RNA levels are adjusted posttranscriptionally (Gelfand and Attardi 1981).

The molecular pathogenesis of the MELAS mutation has not been addressed directly in our studies, but our results suggest that reduced mitochondrial protein synthesis is an important factor in the patho-

genesis of MELAS. The 3243 mutation could affect the $tRNA^{Leu(UUR)}$ function in a number of ways (King et al. 1992), and the mechanism(s) involved is currently under investigation.

In agreement with the morphological findings described here, tissue culture studies have shown that the MELAS mutation causes a partial impairment of respiratory-chain activity (Kobayashi et al. 1991; King et al. 1992), a reduction in mitochondrial protein levels, and no apparent alteration of steady-state rRNA:mRNA levels (King et al. 1992). The recent finding that a second point mutation associated with MELAS is also localized in the $tRNA^{Leu(UUR)}$ gene (Goto et al. 1991) suggests the involvement of this specific tRNA in the pathogenesis of MELAS. This new mutation is at position 3271, which is outside the DNA binding domain of the rRNA transcription termination protein (Kruse et al. 1989), supporting further the hypothesis that transcription termination is not a major factor in pathogenesis. On the other hand, our finding of decreased protein levels could be due to a number of other causes, including altered binding of the $tRNA^{Leu(UUR)}$ to either the ribosome or its cognate aminoacyl tRNA synthetase, noncharging or mischarging of some $tRNA^{Leu(UUR)}$ molecules, or altered processing of the primary transcript in the region of this tRNA gene.

Acknowledgments

This work was supported by grants NS11766 and NS28828 from the National Institutes of Health; by grants from the Muscular Dystrophy Association, the Aaron Diamond Foundation (to E.A.S.), and UILDM-Telethon Italia 90; and by a donation from Libero and Graziella Danesi (Milan). C.T.M. was supported by the Brazilian Research Council (CNPq), and E.R. was supported by UILDM, Rome. We thank Dr. Serenella Servidei (Universita Cattolica del Sacro Cuore, Rome) for referring and providing clinical results on patients MELAS 4, MELAS 5, and MELAS 6, and for helpful discussions; Dr. Michael P. King for the M13 clones, advice, and critically reading the manuscript; Drs. Emma Ciafaloni and Gabriella Silvestri for identifying patients; Dr. Kurenai Tanji for assistance with muscle sectioning for nuclei density determination; Dr. Jean-Lud Cadet for making the image-analyses system available; Frank Conlon for help in establishing the microdissection conditions; and James Sadlock and Jeffrey Rogers for expert technical assistance.

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