Coordinate Induction of Energy Gene Expression in Tissues of Mitochondrial Disease Patients*

(Received for publication, March 2, 1999, and in revised form, May 20, 1999)

Abdelaziz Heddir, Georges Stepień, Paul J. Benke**, and Douglas C. Wallace†

From the Department of Genetics and Molecular Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, the Laboratoire de Biologie Appliquée, INSERM-U203, INSERM bâtiment 406, 20 avenue Albert Einstein, 69621 Villeurbanne cedex, France, the Division of Medical Genetics, University of Miami School of Medicine, Mailman Center, Miami, Florida 33101, and the Laboratoire de Biochimie et Biologie Moléculaire A, CHU d'Angers, 4 rue Larrey, 49000 Angers, France

We have examined the transcript levels of a variety of oxidative phosphorylation (OXPHOS) and associated bioenergetic genes in tissues of a patient carrying the myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) A3243G mitochondrial DNA (mtDNA) mutation and the skeletal muscles of 14 patients harboring other pathogenic mtDNA mutations. The patients' tissues, which harbored 85% or more mutant mtDNA, had increased levels of mtDNA transcripts, increased nuclear OXPHOS gene transcripts including the ATP synthase β subunit and the heart-muscle isoform of the adenine nucleotide translocator, and increased ancillary gene transcripts including muscle mitochondrial creatine phosphokinase, muscle glycogen phosphorylase, hexokinase I, muscle phosphofructokinase, the E1α subunit of pyruvate dehydrogenase, and the ubiquinone oxidoreductase. A similar coordinate induction of bioenergetic genes was observed in the muscle biopsies of severe pathologic mtDNA mutations. The more significant coordinated expression was found in muscle from patients with the MELAS, myoclonic epilepsy with ragged red fibers, and chronic progressive external ophthalmoplegia with ragged red muscle fibers and mitochondrial paracrystalline inclusions. High levels of mutant mtDNAs were linked to a high induction of the mtDNA and nuclear OXPHOS genes and of several associated bioenergetic genes. These observations suggest that human tissues attempt to compensate for OXPHOS defects associated with mtDNA mutations by stimulating mitochondrial biogenesis, possibly mediated through redox-sensitive transcription factors.

Over the past 10 years, multiple mitochondrial DNA (mtDNA)1 mutations have been associated with degenerative diseases of muscle and nervous system (1–5). Mitochondrial DNA diseases can have a wide spectrum of clinical presentations, but they frequently have a delayed onset and a progressive course, with the severity of the mutation and the percentage of mutant mtDNAs in heteroplasmic individuals correlating with the time of onset (5–8).

Mitochondrial DNA mutations that affect energy metabolism fall into two major classes, base substitution and insertion-deletion (rearrangement) mutations. Pathogenic base substitution mutations can alter the amino acid sequence of mtDNA-encoded proteins (missense mutations) or alter the structure and function of the tRNAs or rRNAs (protein synthesis mutations). Two well characterized missense mutations of the electron transport chain are the np 11778 G to A transition in the ND4 gene associated with the Leber's hereditary optic neuropathy (LHON) (MTND4*LHON11778G) (9) and the np 8893 T to G transition in ATP6 associated with neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and Leigh's syndrome (MTATP6*NARP8993G) (10–12). Well defined protein synthesis mutations include the tRNA^{Leu(UUR)} np 3243 A to G mutation associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (MTTL1*MELAS3243G) (13–15) and the tRNA^{Leu(UUR)} np 8344 A to G mutation associated with myoclonic epilepsy and ragged red muscle fibers (MERRF) (MTTK*MERRFS8993G) (9, 16). Affected individuals harboring the MELAS and MERRF mutations frequently reveal a characteristic muscle histology that includes ragged red muscle fibers (RRFs) caused by the proliferation of abnormal mitochondria containing paracrystalline inclusions (PCI) (17).

Rearrangement mutations can result in an array of symptoms. The mildest presentation is maternally transmitted diabetes mellitus and deafness (MDMD). This disease has been associated with the transmission of a trimolecular heteroplasmic including normal mtDNAs, mtDNAs with a 6.1-kb insertion and mtDNAs with the reciprocal 10.4-kb deletion (18). Deleted molecules were primarily responsible for the pathological phenotype in such patients (19). More severe deletion syndromes due to deleted mtDNA include the spontaneously occurring chronic progressive external ophthalmoplegia (CPEO) and the Kearns-Sayre syndrome (KSS) (20). CPEO and KSS

fibers; mGP, muscle glycogen phosphorylase; mMCK, muscle mitochondrial creatine phosphokinase; mPFK, muscle phosphofructokinase; mPK, muscle pyruvate kinase; NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa; ND, NADH dehydrogenase; nDNA, nuclear DNA; OXPHOS, oxidative phosphorylation; PCI, paracrystalline inclusions; PDH, pyruvate dehydrogenase; EloPDH, Elo subunit of pyruvate dehydrogenase; RRF, ragged red muscle fiber; kb, kilobase(s); np, nucleotide pair; Ub, ubiquitin; cyt b, cytochrome b; ANT1, adenine nucleotide translocator isoform 1.
are associated with progressive muscle weakness including ophthalmoplegia and ptosis, RRFs, and abnormal mitochondria, as well as multisystem degeneration (21).

Analysis of skeletal muscle biopsies of patients with RRFs because of the MTTL1*MELAS3243G and MTPK*MERRF-8344G mutations (22) and a 7.4-kb CPEO and KSS deletion (22, 23) revealed a coordinate increase on the mRNA levels of mtDNA and nuclear DNA (nDNA) encoded OXPHOS genes. A similar OXPHOS gene induction was observed in cardiac tissue from patients with ischemic heart disease associated with increased somatic mtDNA rearrangements (24). Several nuclear OXPHOS genes have been shown to be induced in these patients. The ATPαnψγδ gene, which is expressed in all tissues but is more prevalent in heart and muscle (25); the ANT1 gene, which is expressed in heart and muscle but not in other tissues; and ANT2, which is generally not expressed unless the cells are undergoing a glycolytic metabolism (26, 27).

The increase in nDNA and mtDNA OXPHOS transcript levels in skeletal muscle of patients with mitochondrial protein synthesis defects suggests that the affected tissues attempt to compensate for the energy deficiency by induction of mitochondrial biogenesis. This suggestion raises several questions. Is the OXPHOS gene induction specific for muscle tissue, or is it a common feature of tissues with respiratory defects? Is the induction restricted to OXPHOS genes or does it extend to other genes involved in energy metabolism?

To address these questions, we performed two sets of experiments. First, we examined the mRNA levels of a variety of OXPHOS and glycolysis genes in the autopsy tissues of a young woman who died of hypertrophic cardiomyopathy secondary to the MTTL1*MELAS3243G mutation. Second, we analyzed the expression of the same set of genes in muscle biopsies of patients that harbored various mtDNA mutations including base substitutions and rearrangements.

**EXPERIMENTAL PROCEDURES**

**Autopsy and Biopsy Tissues**

**Patient Samples**—The MELAS proband patient died at 16 years old of cardiac failure, secondary to hypertrophic cardiomyopathy. A complete autopsy was performed, and all tissues were flash frozen in liquid nitrogen. Quadriceps muscle biopsies from patients harboring different pathologic mtDNA mutations were collected with informed consent as a component of routine diagnostic analysis (8). The mitochondria were isolated, and the OXPHOS enzymes were assayed as described previously (9). The clinical, pathological, and molecular diagnostic data for the patients of this study are summarized in Table I.

**Control Samples**—Control heart 1 was an autopsy from a 7-year-old male who died of asphyxiation. Control heart 2 was an autopsy from a 15-year-old male who died of a gunshot wound. Control muscle 1 was a deltoid biopsy from a 33-year-old male who had a malignant fibrous histiocytoma. Control muscle 2 was a pectoralis biopsy from a 38-year-old female presenting a breast carcinoma. Control kidney 1 was a biopsy of the healthy part of a kidney from a 71-year-old female who had a renal carcinoma. Control kidney 2 was a biopsy of the healthy part of a kidney from a 54-year-old female who had a renal oncocytoma. Control liver 1 was an autopsy from a 38-year-old male who died of asthma. Control liver 2 was a biopsy of the normal liver region from a 56-year-old female who had a metastatic hepatocellular carcinoma. Control brain 1 was a biopsy from temporal lobe from a 26-year-old female seizure patient. Control brain 2 was an autopsy from a 48-year-old female who died of lymphoma. Three independent control skeletal muscle samples were used in our study of gene expression in patients harboring different mtDNA mutations. Control 1 was a muscle biopsy from a 33-year-old male, control 2 was a muscle biopsy from a 21-year-old female, and control 3 was a muscle biopsy from a 23-year-old male. All control and patient tissues were provided either by Emory University Hospital or by the cooperative human tissue network (Birmingham, AL).

**Nuclear Probes**

The human ANT1 probe was a 1152-base pair HindIII/HincII restriction fragment of the cDNA from pHMANT (28). The human ANT2 probe was a 1200-base pair Xhol/HindIII restriction fragment of the cDNA from pSKHANT21 derived from hp21 (kindly provided by Dr. R. Baserga, Temple University Medical School, Philadelphia, PA) by subcloning into pBluescript SK+ (Stratagene). The ATPαnψγδ probe was a 995-base pair EcoRI restriction fragment of a cDNA encompassing the COOH-terminal two-thirds of the coding sequence (29). Human muscle glycogen phosphorylase (mGPP), muscle cytosol creatine kinase (mCK), muscle pyruvate kinase (mPK), and human E1α pyruvate dehydrogenase subunit (E1αPDH) were purchased from American Type Culture Collection. The mGP probe was a 2.3-kb BamHI/HindIII restriction fragment of the cDNA from the pMCMP1 clone (30). mCK was a 1.5-kb HindIII/BamHI restriction fragment of the cDNA from the pJN2CM clone (31), mPK was a 1.8-kb EcoRI restriction fragment from the HHHUD25 clone, and E1αPDH was a 0.9-kb EcoRI/EcoRI fragment from HFBE57 clone (32). Human hexokinase I (H1K), human sarcomeric mitochondrial creatine kinase (mMtCK), and muscle phosphofructokinase (mPFK) were kindly provided by Dr. Graeme I. Bell (Howard Hughes Medical Institute, Departments of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, IL), Dr. Arnold W. Strauss (Department of Biological Chemistry, Medicine and Pediatrics, Washington University School of Medicine, St. Louis, MO), and Dr. Alan McLachlan (Department of Molecular and Experimental Medicine, Scripps Clinic and Research Foundation/BCR-7, La Jolla, CA), respectively. HK1 was a 3.3-kb EcoRI restriction fragment from the cDNA from pHK15–2 clone (33). mMtCK was the full-length cDNA from phMtCK3 (34), and mPFK was the entire cDNA from HPFK-M (35). The ubiquitin probe was generously provided by Dr. Russ Price (Emory University School of Medicine).

**Mitochondrial Probes**

Mitochondrial probes were generated by polymerase chain reaction amplification from 5 ng of HeLa DNA. The 12 S rRNA probe encompassed mtDNA np 534–1896 and was amplified using primers 5'-CC-CCATCCCCGACCCAC and 5'-GGATGGGGTTGTTTGCTAGG. The 7.7-kb mtDNA deletion localized between np 7,669 and 15,437 (36). The resulting 0.386-kb probe was homologous to 51 bases of the 1.52-kb COI mRNA, 83 bases of the 0.708-kb COII mRNA, 112 bases of the 1.14-kb cytb mRNA, and the encompassed tRNAser and tRNAAsp.

**RNA Isolation and Northern Blot Analysis**

Total cellular RNA was isolated by pulverizing 50–200 mg of frozen tissues in liquid nitrogen (Omni 5000, Bioquip, Inc.), and homogenization in guanidinium isothiocyanate. RNA samples (10–20 μg) were denatured by incubation at 55 °C for 20 min in 1 m deionized glyoxal, 50% (v/v) dimethyl sulfoxide, and 10 mM NaH2PO4, pH 7.0, electrophoresed on 1.3% agarose gels (Ultrapure, Life Technologies, Inc.) containing 10 mM NaH2PO4, pH 7.0, and blotted overnight onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) in 20× SSC (standard sodium citrate). The blots were baked 2 h at 80°C, prehybridized overnight at 50 °C (Hybrid oven in 5× SSPE (standard sodium phosphate EDTA), 5× Denhardt’s solution, 50% deionized formamide (Fisher, molecular biology grade), 0.2% SDS, and 0.1% denatured salmon sperm DNA, and hybridized overnight at 42 °C in the same buffer following the addition of 6 × 106 cpm [32P]dCTP-labeled probe and 100 μg/ml sheared denatured salmon sperm DNA. Blots were washed three times for 20 min in 1× SSC and 50°C at 55°C and once for 20 min at 5°C. The filters were obtained by exposing Hyperfilm-MP (Amersham Pharmacia Biotech) with intensifying screens at −80°C for 2 h to 3 days. Autoradiographic exposure intensities were compared using an Ultroscan XL enhanced laser densitometer (two-dimensional gel scan program, Amersham Pharmacia Biotech). The amount of RNA blotted for each sample was normalized by hybridization of human β2 microglobulin RNA probe (provided by the late Dr. R. D. Schmickel, University of Pennsylvania School of Medicine, Philadelphia, PA) with an exposure time of 1 h at room temperature.

**Total Cellular DNA Purification and Southern Blot**

Total cellular DNA was extracted from 50–100 mg of tissue pulverized in liquid nitrogen. Tissue powder was then homogenized in 2 ml of 1× STE (100 mM NaCl, 25 mM Na2EDTA, 10 mM Tris-HCl, pH 8.0), the proteins were digested overnight at 55°C in presence of 0.5% of SDS
Clinical and histological data from the muscle biopsies used in energy gene expression

| Subject | Sex/Age | Mutation | Mutation | RRF | PCI |
|---------|---------|----------|----------|-----|-----|
| Control 1 | M/38 | – | – | – | – |
| Control 2 | F/21 | – | – | – | – |
| Control 3 | M/23 | – | – | – | – |
| LHON 1 | M/63 | 11778 | 100 | – | – |
| NARP | M/11 | 8993 | 95 | – | – |
| MELAS | F/MA | 3243 | 50 | – | – |
| 1 | MELAS | M/16 | 3243 | 91 | + |
| 2 | MELAS | H/45 | 3243 | 72 | – |
| 3 | MERRF | F/45 | 8344 | 97 | + |
| 1 | MERRF | F/70 | 8344 | 94 | + |
| 2 | MERRF | M/MA | 9344 | 78 | ND |
| 3 | MERRF | F/24 | 8344 | 94 | + |
| 4 | MERRF | F/25 | 8344 | 94 | + |
| 5 | MDM 1 | F/49 | 4398:14,822 DL | 67 | – |
| 6 | MDM 1 | F/49 | 8282:13,851 DP | 9 | – |
| 7 | MDM 2 | F/25 | 4398:14,822 DL | 31 | – |
| 8 | MDM 2 | F/25 | 8282:13,851 DP | 19 | – |
| 9 | CPEO | F/42 | 4-kb deletion | 50 | ND |
| 10 | FSMD | M/49 | Chr 4 | nuclear | – |

a Subsarcolemmal accumulation of mitochondria were noted on Gomori trichrome analysis. However, ragged rod fibers were not present.

b The MELAS2 sample was from a fresh muscle biopsy distinct from the autopsy muscle sample used in the tissue expression study.

and 15 μg/ml of proteinase K (Boehringer), and the cellular RNAs were digested with 5 μg/ml of ribonuclease A (Sigma) for 1 h at 37 °C. Two phenol/chloroform extractions were performed, followed by DNA precipitation. 1–3 μg of DNA were digested with 40 units of ApaI, and the fragments were separated on a 0.8% agarose/TAE gel. The gel was treated 30 min with 0.25 M HCl, 30 min with 0.4 M NaOH and 1 M NaCl, neutralized 30 min in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5, and transferred to nylon membrane (Hybond N, Amersham Pharmacia Biotech). The blot was prehybridized and hybridized as described above, first with the 18S ribosomal RNA probe, exposed to Hyperfilm-MP (Amersham Pharmacia Biotech), and exposed again. The bands in the autoradiographic film were analyzed in the tissues of MELAS 2 proband by comparing the relative intensities of 1.8-kb (mutant) and 3.0-kb (normal) bands in ApaI digests. The percentage of mutant mtDNAs was variable in the proband tissues, from 73% in kidney to 95% in heart and brain (Table II), and there was no increase in the proportion of total mtDNAs as compared with control tissues.

Levels of Nuclear OXPHOS Transcripts—The expression of the nuclear OXPHOS genes for ATPsγβ, ANT1, and ANT2 were also elevated in MELAS tissues relative to controls when the RNA levels were normalized to the cytosolic 18S rRNA level (Fig. 2). The ATPsγβ and ANT1 mRNA levels were increased in all of the MELAS tissues from 1.4- to 8.7-fold. The ANT2 mRNA levels were very low in all control tissues except in kidney but were dramatically increased in MELAS tissues from 6.4-fold in heart to 27-fold in skeletal muscle.

Levels of Glycolytic and Pyruvate Dehydrogenase Transcripts—The amount of transcript for key muscle glycolytic genes, mGP, HKI, phosphofructokinase, and pyruvate kinase (mPK), as well as for the E1α subunit of pyruvate dehydrogenase (E1αPDH) were examined in the MELAS tissues and controls. When normalized to the 18S rRNA (Fig. 3), all of these gene transcripts except mPK were increased between 1.5- and 6-fold, with the greatest increases found in the heart. The E1αPDH gene was represented by two transcripts, 1.65 and 3.3 kb in size (not shown), as previously reported (37). Both transcripts were increased in the MELAS tissues, especially in heart where the 3.3-kb transcript was increased more than 25-fold.

Levels of Cytosolic and Mitochondrial Muscle Creatine Kinase Transcripts—Four creatine kinase isoenzymes are known: brain cytosolic creatine kinase, ubiquitous mitochondrial creatine kinase, mCCk, and mMtCK. The mRNA levels of the mMtCK gene, but not the mCCk gene, were substantially increased in MELAS heart and skeletal muscles (Fig. 4). The mMtCK was increased 2.2-fold in MELAS skeletal muscle and 1.9-fold in MELAS heart. By contrast, the expression of the mCCk was slightly or drastically (10-fold) decreased in the MELAS skeletal muscle.

Levels of the Ubiquitin Transcripts—The 76-amino acid ubiquitin (Ub) (38) is a cofactor in the ATP-dependent, nonlysosomal, protein degradation pathway of abnormal proteins (39). In all the organisms examined, multiple Ub genes are present, and their size differences reflect variable numbers of randomly arranged Ub coding repeats. The translation products are polyubiquitins that are processed into the monomeric form. In
In human tissues, three sizes of mRNA have been reported: 650 (UbA), 1100 (UbB), and 2500 (UbC) nucleotides (40). In the MELAS patient, the level of the Ub transcripts was increased in skeletal muscle, kidney, liver, and brain but not in the heart. Relative to 18 S rRNA, the mRNA level was increased 3–23-fold, with skeletal muscle and brain being highest at 23.6- and 8.1-fold, respectively (Fig. 5).

**Energy Gene Induction in Skeletal Muscle from Patients with Mitochondrial DNA Diseases**

To determine whether induction of energy gene transcripts is a general response to pathologic mtDNA mutations, we examined the mtDNA and nDNA transcripts from skeletal muscle biopsies of patients harboring the mtDNA base substitutions MTTL1*MELAS3243G, MTTK*MERRF8344G, MTATP6*NARP8993G, and MTND4*LHON11778A (7), the diabetes mellitus and deafness insertion-deletion mutation (18, 41), and a CPEO deletion. We also examined a facio-scaphulo-humeral muscular dystrophy (FSHMD) muscle sample, because this mutation maps close to ANT1 on the long arm of chromosome 4 (42, 43). A summary of the patients and their percentages of heteroplasmy are provided in Table I.

**Levels of mtDNA Transcripts**—The mtDNA ND5/6, cytb (Fig. 6), 12 S rRNA, COI, and COII (not shown) gene transcript levels were examined in three control muscle biopsies, ages 21–33, and found to be similar. This confirms the results of our previous study of 10 muscle biopsies from controls aged 6–69 years, in which both mtDNA and nDNA transcript levels remained at similar levels with age (22). For the LHON muscle, the mtDNA transcript level for the ND5/6 genes were partially increased. Among the MELAS muscles, the ND5/6 and cytb transcript levels were increased in MELAS2, which harbored 91% mutant mtDNAs (Fig. 3), but not in MELAS 1 and 3, which

**TABLE II Mitochondrial DNA heteroplasmy and mitochondrial/nuclear DNA ratios in MELAS tissues**

| Percentage of mutant mtDNA | Heart | Skeletal muscle | Kidney | Liver | Brain |
|---------------------------|-------|----------------|--------|-------|-------|
| Patient mtDNA/nDNA        | 7.5   | 8.0            | 2.3    | 2.2   | 5.2   |
| Control mtDNA/nDNA        | 6.8   | 12.0           | 2.3    | 3.3   | 5.5   |

**Fig. 1. Quantitation of mitochondrial gene transcripts from control and MELAS tissues.** The histograms represent the values determined by quantitative scanning densitometry of the Northern blot (not shown). Data are expressed in arbitrary units normalized by the relative intensity of the 18 S nuclear rRNA band. C, control; M, MELAS.
harbored 50 and 72% mutant mtDNAs, respectively. The MERRF muscle samples, with 78 and 94% mutant mtDNAs, showed variable increases in the ND5/6 transcript, although all showed increased cyt b transcript. Among the rearrangement patient muscles (MDMD1, MDMD2, and CPEO), no increase or a slight decrease of increase was seen in the mtDNA transcripts for genes encompassed within the deletion (ND5/6 and COII, see Fig. 2; COI, data not shown), but high transcript levels were found for the cyt b gene, which was outside the deletion. Finally, both mtDNA transcripts were increased in the FSHMD skeletal muscle.

Comparing these results with muscle pathology revealed an overall trend. Skeletal muscle samples that exhibited ragged red fibers (Table I) were the most likely to show increased mtDNA transcript levels. This observation is consistent with other reports using in situ hybridization (44).

**Levels of Nuclear OXPHOS Gene Transcripts**—The transcript levels for the nuclear OXPHOS ATPsyn β, ANT1 (Fig. 7), and ANT2 (not shown) genes also differed depending on the type of the mtDNA mutation. Both of the missense mutation patients (LHON and NARP) muscles showed a slight to substantial increase in these transcripts. The tRNA mutation patient (MELAS and MERRF) muscles also exhibited increased transcript levels, with the highest increases seen in patients with the greatest proportion of mutant mtDNAs, MELAS 2 with 91% mutant (Figs. 2 and 7) and MERRF 4 and 5 with 94% mutant mtDNAs. The transcript levels for MELAS 1 with 50% mutant mtDNAs and MERRF 3 with 78% mutant mtDNAs were not particularly elevated. Therefore, it appears that nuclear OXPHOS genes are induced in the muscle of tRNA mutation patients where the percentage of mutant mtDNAs is greater than 75%.

For the rearrangement mutation patients, the two MDMD patients harboring the combined insertion-deletion mutation showed no increase in the ATPsyn β transcript level and variable increases in ANT1 expression. By contrast, the CPEO patient with the deletion showed a consistent increases in both ATPsyn β and ANT1 transcripts. This pattern also correlates with the presence or absence of ragged red muscle fibers (Table I).

The FSHMD specimen showed a mild increase in the ANT1 and ATPsyn β levels.

**Levels of Glycolytic Gene Transcripts**—The transcript levels for muscle mGP, HKI (Fig. 8), mPFK, mPK, and E1αPDH (not shown) were also examined in the mtDNA disease and FSHMD patients. mGP, HKI, E1αPDH, and to a lesser extent mPFK showed increases in transcript levels similar to those of ATPsyn β and ANT1.

![Fig. 2. Quantitation of OXPHOS nuclear transcripts from controls and MELAS tissues.](image)

![Fig. 3. Quantitation of muscle-specific gene transcripts from control and MELAS tissues.](image)
For the mtDNA missense mutation muscles, the LHON samples showed an increase in expression of HKI but normal or reduced transcript levels for the other glycolytic genes. The NARP muscle showed increases in mGP, HKI (Fig. 8) and mPFK but not mPK.

For the muscle biopsies of the MELAS and MERRF tRNA mutations, all patients with greater than 70% mutant mtDNA showed an increase of mGP transcripts. The mGP transcript levels correlated with the other nuclear OXPHOS transcript levels, giving correlation coefficients ($r^2$) for ANT1 of 0.88 and ATP5yβ of 0.86. The E1αPDH 1.65-kb transcript showed a similar pattern to mGP. HKI and the E1αPDH 3.3-kb transcript showed high increases for the MELAS 2 specimen with 91% mutant mtDNAs but not for MELAS 1 with 50% mutant or for any of the MERRF specimens. mPFK was not increased in any of the tRNA mutant muscles, and mPK showed the opposite trend to the HKI and E1αPDH 3.3-kb transcripts, being lowest for MELAS 2 with 91% mutant mtDNAs (not shown).

For the mtDNA rearrangement syndromes, the MDMD insertion-deletion patients exhibited some increase for HKI (Fig. 8), and possibly E1αPDH 3.3-kb transcripts (not shown). However, the response was variable for mGP (Fig. 8) and mPFK, unaltered for the E1αPDH 1.65-kb transcript, and slightly reduced for mPK (not shown). As in the case of the nuclear OXPHOS genes, the CPEO deletion muscle exhibited an increase in the mGP, HKI, mPFK, and E1αPDH 3.3-kb and 1.65-kb transcripts. Finally, these transcripts levels were not markedly elevated in the FSHMD muscle.

Levels of Muscle Cytosolic and Mitochondrial Creatine Kinase Transcripts—The transcript levels of mCCK showed considerable variability in both control and patient muscle biopsies (not shown). The highest patient transcript levels were seen for the NARP and CPEO patient muscles.

For the muscle biopsies of the MELAS and MERRF tRNA mutations, all patients with greater than 70% mutant mtDNA showed an increase of mGP transcripts. The mGP transcript levels correlated with the other nuclear OXPHOS transcript levels, giving correlation coefficients ($r^2$) for ANT1 of 0.88 and ATP5yβ of 0.86. The E1αPDH 1.65-kb transcript showed a similar pattern to mGP. HKI and the E1αPDH 3.3-kb transcript showed high increases for the MELAS 2 specimen with 91% mutant mtDNAs but not for MELAS 1 with 50% mutant or for any of the MERRF specimens. mPFK was not increased in any of the tRNA mutant muscles, and mPK showed the opposite trend to the HKI and E1αPDH 3.3-kb transcripts, being lowest for MELAS 2 with 91% mutant mtDNAs (not shown).

For the mtDNA rearrangement syndromes, the MDMD insertion-deletion patients exhibited some increase for HKI (Fig. 8), and possibly E1αPDH 3.3-kb transcripts (not shown). However, the response was variable for mGP (Fig. 8) and mPFK, unaltered for the E1αPDH 1.65-kb transcript, and slightly reduced for mPK (not shown). As in the case of the nuclear OXPHOS genes, the CPEO deletion muscle exhibited an increase in the mGP, HKI, mPFK, and E1αPDH 3.3-kb and 1.65-kb transcripts. Finally, these transcripts levels were not markedly elevated in the FSHMD muscle.

FIG. 4. Quantitation of creatine kinase gene expression. Levels of mCCK and mMtkCK gene transcripts from control and MELAS tissues. The histograms represent the values determined by quantitative scanning densitometry of the Northern blot (not shown). C, control; M, MELAS.

FIG. 5. Quantitation of ubiquitin gene transcripts. Levels of the ubiquitin gene (UbC) transcript from control and MELAS tissues. The histograms represent the values determined by quantitative scanning densitometry of the Northern blot (not shown). C, control; M, MELAS.

DISCUSSION

Analysis of the transcript levels for a variety of energy metabolism genes in patients harboring known pathologic mtDNA
mutations revealed that the expression of many of these genes is increased in response to respiratory deficiency. This induction appears to be common to all tissues and probably represents a compensatory response for the inherited OXPHOS deficiency.

For the MELAS patient autopsy tissues, the induction of transcript levels was generally correlated with the percentage of mutant mtDNAs. Messenger RNA levels were consistently increased in heart, skeletal muscle, liver, and brain, all of which had more than 88% mutant mtDNAs, but were not increased in kidney with 73% mutant mtDNAs. Therefore, it appears that a certain degree of bioenergetic inhibition is required before induction occurs. Overall, both nDNA and mtDNA OXPHOS genes were induced. Coordinate induction was observed in heart and muscle for the nuclear ATPsynβ and ANT1 transcripts. Similarly, increased mRNA levels were seen for the mtDNA OXPHOS genes including the 12 S rRNA, ND5/6, COI, COII, cytb, and tRNAser and tRNA Asp genes. A parallel induction was seen for the muscle-specific mMtCK, but not for the mCCK, and a coordinate induction was also observed for mGP, HKI, phosphofructokinase, and E1αPDH. Thus, OXPHOS deficiency not only induces a compensatory induction of OXPHOS genes but also a variety of genes whose products interface with OXPHOS to maintain cellular energy levels. The high induction of the ubiquitin mRNAs in the MELAS autopsy skeletal muscle, brain, liver, and kidney suggests a high rate of turnover in MELAS tissues as compared with normal. The absence of induction in the hypertrophic heart suggests either that the heart is protected from protein damage or that damaged proteins accumulate in the heart.

The coordinate induction in bioenergetic genes was also observed when analyzing the transcript levels from muscle biopsies of patients harboring a variety of pathologic mtDNA mutations. Partial bioenergetic gene induction was observed in the skeletal muscle of the more severe missense mutation causing NARP and Leigh’s syndrome but was less apparent for the milder LHON patient muscles. Coordinate induction of bioenergetic gene expression was more prominent in patients harboring mtDNA protein synthesis defects that were sufficiently severe to cause RRFs and PCIs. OXPHOS gene induction was a common finding in a MELAS muscle with 72% mutant mtDNAs and in MERRF muscles with 94% mutant mtDNAs. However, induction was not seen in a MELAS muscle with 50% mutant mtDNAs or in a MERRF muscle with 78% mutant mtDNAs. Similarly, strong induction of bioenergetic gene expression was observed in a CPEO-KSS patient muscle with 50% deleted mtDNAs and RRFs but not in two MDMD patients with a combined deletion-insertion mutation and no RRFs. As for the MELAS autopsy patient, coordinate induction was seen for both the nDNA (ANT1 and ATPsynβ) and the mtDNA encoded OXPHOS genes. Moreover, OXPHOS gene induction was generally associated with induction of the mMtCK, but not mCCK, and of the glycolytic enzymes mGP, E1αPDH, and to a lesser extent HK1 and mPFK. By contrast, mPK expression seemed to be depressed. Thus bioenergetic gene induction appears to be proportional to the severity of the mitochondrial defect, with mutations that inhibit protein synthesis and give RRFs and with PCI having the highest induction.

To obtain a more integrated interpretation of the changes that occur in bioenergetic gene expression among the different classes of mtDNA mutations, we used factorial discriminant analysis (45). This analysis looks at the associations between
the changes in multiple variables, rather than the absolute values of each variable. Consequently, the differing levels of response of MERRF and MELAS samples with different percentages of mutant mtDNAs all contribute to the associative relationship. The factorial analysis was performed using the data from 14 independent muscle biopsies, comparing 7 variables: the transcript levels for ANT1, ATPsynβ, mMtCK, E1αPDH, mGP, mPFK, and HKI. Plotting the associations on a two-dimensional plane revealed that changes in ANT1, ATPsynβ, mMtCK, E1αPDH, and mGP were strongly associated. By contrast, changes in mPFK were less associated, and changes in HKI were significantly different (Fig. 9B). Plotting the specimens that showed similar associated changes revealed four groups of mtDNA genotypes. The first group was the controls 1, 2, and 3 (Fig. 9A). The second group was the missense mutations that showed a wide spectrum of changes, primarily associated with HKI induction in the LHON specimen 9 and a more general induction in the NARP specimen 13. The third group of specimens was the rearrangements, the CPEO deletion, and two MDMD specimens 10, 11, and 12. The last group encompassed the MELAS and MERRF tRNA mutations, numbers 5, 6, 7, and 8; patient 4 (MELAS 1) is intermediate between controls and tRNA mutations because of its low percentage of mutant mtDNAs (50% heteroplasmy). In both the mtDNA deletion and tRNA mutation patients, there was a strong association in the change of mRNA levels for the bioenergetic genes: ANT1, ATPsynβ, mMtCK, E1αPDH, and mGP.

Several of these associations lead to a metabolic perspective. The expression of the ATPsynβ subunit that synthesizes ATP and ANT1, which exchanges ATP and ADP across the membrane, should be coupled to assure balanced synthesis and flux of ATP. The ANT2 should be induced, because this isoform has been hypothesized to permit cytosolic ATP to flow back into the mitochondria when ATP generated by OXPHOS is limited (26, 27). The induction of the mMtCK logically follows the induction of the ANT. MtCK is localized in the intermembrane space of the mitochondrion where it collects mitochondrial ATP from the ANT and converts it into the stable energy storage molecule CrP (46). Ultrastructural studies have localized MtCK along the outer surface of the mitochondrial inner membrane, as well as at the contact sites between inner and outer mitochondrial membranes (46, 47) where it is functionally coupled to the inner membrane ANT and the outer membrane porin (46, 48). An increase in mMtCK mRNA would increase MtCK protein, which is consistent with the finding that the PCI of RRFs contain crystallized MtCK (17). MtCK crystalline inclusions can be induced in rat cardiomyocytes by growth in creatine-deficient medium (49).

A similar logic can be applied to the induction of HKI. HKI is most active when associated with porin on the cytosolic side of the mitochondrial outer membrane (50). In this position, the capture of mitochondrial ATP to phosphorylate glucose and drive glycolysis has been shown to be important in the energy metabolism of tumor cells (51, 52) and severe diabetes (53). Similarly, PDH is at the interface between cytosolic glycolysis (pyruvate) and the matrix tricarboxylic acid cycle. Moreover, the E1α subunit contains the phosphorylation sites central to the regulation of the enzyme complex. PFK uses ATP to phosphorylate fructose-6-phosphate to fructose-1,6-biphosphate and is one of the regulatory steps in glycolysis, and GP degrades glycogen to glucose-1-phosphate, thus mobilizing stored glucose in muscle and liver. Therefore, the induction of all of these enzymes would be important in fueling the mitochondrial OXPHOS system.

Although the compensatory induction of mtDNA and nDNA OXPHOS genes as well as associated enzymes in tissues of patients with mitochondrial protein synthesis defects has now been confirmed (22–24), the mechanism for this induction still needs clarification. Several transcription factors that influence OXPHOS gene expression have been already discovered. These include NRF1 and NRF2 (54, 55), the “Mt” element (56, 57), the CREB element (58), and the OXBOX-REBOX elements (42, 59, 60). Some of these elements might be important in compensatory regulation of transcription. For example, the Mt element is localized in both the ATPsynβ and E1αPDH promoters (56) and the OXBOX element in both the ATPsynβ and ANT1 promoters.
In conclusion, severe mtDNA defects, especially those associated with RRFs and PCI, are associated with the coordinate induction of nDNA and mtDNA OXPHOS genes as well as directly linked enzymes of the intermediary metabolism. The coordinated nature of this induction is mediated through the action of unknown transcription factors. If the induction of nDNA and mtDNA bioenergetic gene expression is a prerequisite to the formation of RRFs and PCI, then study of this induction may be very important in understanding the pathological basis for the progression of mtDNA diseases.

**Acknowledgments**—We recognize the assistance of Drs. John Shoffner, Stephen Voljavcev, and Debra Koontz in these studies.

**REFERENCES**

1. Schon, E. A., Bonilla, E., and DiMauro, S. (1997) *Bioenerg. Biomembr.* **29**, 131–149
2. Chomyn, A. (1998) *Am. J. Hum. Genet.* **62**, 745–751
3. Graeber, M. B., and Muller, U. (1998) *J. Neurosci.* **18**, 251–263
4. Schapira, A. H. (1998) *Biochim. Biophys. Acta* **1364**, 261–270
5. Wallace, D. C., Lott, M. T., and Brown, M. D. (1996) in *Human Gene Mapping 1995: A Compendium* (Cuticchia, A. J., Chipperfield, M. A., and Foster, P. A., eds) pp. 1280–1331, The Johns Hopkins University Press, Baltimore
6. Wallace, D. C. (1992) *Ann. Rev. Biochem.* **61**, 1175–1212
7. Lestienne, P., and Ponsot, G. (1988) *J. Biol. Chem.* **263**, 20585–20588
8. Haraguchi, Y., Chung, A. B., Torroni, A., Stepien, G., Shoffner, J. M., Wasmuth, J. J., Costigan, D. A., Polak, M., Alltherr, M. R., Winokur, S. T., Winokur, S., and Wallace, D. C. (1993) *Genomics* **16**, 479–485
9. Wijmenga, C., Winokur, S. T., Padberg, G. W., Shaaraastad, M. I., Alltherr, M. R., Wasmuth, J. J., Murray, J. C., Hofker, M. H., and Frants, R. R. (1993) *Hum. Genet.* **92**, 196–203
10. Schmierer, E. A. (1994) *Bioenerg. Biomembr.* **26**, 301–310
11. Lefèvre, J. (1983) *Introduction aux Analyses Statistiques Multidimensionnelles*, Masson Paris
12. Benz, R., Kotlik, M., and Erdicke, D. (1990) *Biochim. Biophys. Acta* **1022**, 19–30
13. Biermans, W., Bernaert, I., De Bie, C., Nijssen, B., and Jacob, W. (1994) *Biochim. Biophys. Acta* **1285**, 256–258
14. Eckert, A., Lestienne, P., and Wallimann, T. (1993) *J. Theor. Biol.* **165**, 129–132
15. Eppenberger-Eberhardt, M., Riesinger, L., Messeri, S., Schwab, P., Muller, M., Eppenberger, H. M., and Wallimann, T. (1991) *J. Cell Biol.* **113**, 269–282
16. Stockman, K., and Pedersen, P. L. (1991) *J. Biol. Chem.* **266**, 4641–4648
17. Weinhouse, S. (1972) *Cancer Res.* **32**, 2097–2101
18. Bristamante, E., Morris, H. P., and Pedersen, P. L. (1981) *J. Biol. Chem.* **256**, 8699–8704
19. Schroeder, R. E., Devaskar, U. P., Traver, S. E., Demello, D. E., Cole, D. P., and Devaskar, S. U. (1993) *Diabetes* **42**, 1487–1496
20. Evans, M. J., and Scarpulla, R. C. (1989) *J. Biol. Chem.* **264**, 14361–14368
21. Virbasius, J. V., and Scarpulla, R. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1309–1313
22. Tomura, H., Endo, H., Kaga, Y., and Ohba, S. (1990) *J. Biol. Chem.* **265**, 6525–6527
23. Suzuki, H., Hosokawa, Y., Nishikimi, M., and Ozawa, T. (1991) *J. Biol. Chem.* **266**, 2335–2338
24. Gopalakrishnan, L., and Scarpulla, R. C. (1994) *J. Biol. Chem.* **269**, 105–113
25. Li, K., Hodge, J. A., and Wallace, D. C. (1990) *J. Biol. Chem.* **265**, 20585–20588
26. Chung, A. B., Stepien, G., Haraguchi, Y., Li, K., and Wallace, D. C. (1992) *J. Biol. Chem.* **267**, 21154–21161
27. Liao X., and Butow R. A. (1993) *Cell* **72**, 61–71
28. Rothermel B. A., Thornton J. L., and Butow R. A. (1997) *J. Biol. Chem.* **272**, 19801–19807