A Pathogenic 15-Base Pair Deletion in Mitochondrial DNA-encoded Cytochrome c Oxidase Subunit III Results in the Absence of Functional Cytochrome c Oxidase*

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A 15-base pair, in-frame, deletion (9480del15) in the mitochondrial DNA (mtDNA)-encoded cytochrome c oxidase subunit III (COX III) gene was identified previously in a patient with recurrent episodes of myoglobinuria and an isolated COX deficiency. Transiently transfected COS cells harboring 0, 97, and 100% of the 9480del15 deletion were created by fusing human cells lacking mtDNA (ρo cells) with platelet and lymphocyte fractions isolated from the patient. The COX III gene mutation resulted in a severe respiratory chain defect in all mutant cell lines. Cells homoplasmic for the mutation had no detectable COX activity or respiratory ATP synthesis, and required uridine and pyruvate supplementation for growth, a phenotype similar to ρo cells. The cells with 97% mutated mtDNA exhibited severe reductions in both COX activity (6% of wild-type levels) and rates of ATP synthesis (9% of wild-type). The COX III polypeptide in the mutant cells, although translated at rates similar to wild-type, had reduced stability. There was no evidence for assembly of COX I, COX II, or COX III subunits in a multi-subunit complex in cells homoplasmic for the mutation, thus indicating that there was no stable assembly of COX I with COX II in the absence of wild-type COX III. In contrast, the COX I and COX II subunits were assembled in cells with 97% mutated mtDNA.

Cytochrome c oxidase (COX),† an essential component of the respiratory chain, transfers electrons from reduced cytochrome c to molecular oxygen, conserving the energy in the form of an electrochemical gradient. The human enzyme is composed of 13 subunits. The three largest are encoded by mtDNA (COX I, COX II, and COX III) and the remainder by the nuclear DNA. The enzyme complex contains four redox-active metal centers, two coppers, CuA and CuB, and two hemes, a and a3 (1, 2). Three of these are located in COX I including the bimetallic heme a3-CuB center which is responsible for the reduction of oxygen to water coupled to the pumping of protons across the membrane (3, 4). The polar domain of COX II contains the CuA metal center and serves as the cytochrome c docking site. Together, mtDNA-encoded COX I and COX II form the catalytic core of the enzyme (3, 4). The function of COX III, the only mtDNA-encoded COX subunit without prosthetic groups, is unknown but it is hypothesized to play a structural or regulatory role (5, 6). A V-shaped cleft formed by the interface of COX III and COX II is one of several channels for oxygen proposed from the structures of COX (3, 4, 7). The structure of the bovine enzyme (4) has revealed that two nuclear subunits, VIa and VIb, are involved in the dimerization of the enzyme. The functional significance of the other nuclear-encoded COX subunits is unclear, but they are hypothesized to play structural or regulatory roles (1, 8–10).

COX deficiencies comprise a heterogeneous group of disorders that present a broad spectrum of clinical phenotypes of variable severity. COX deficiency has been documented in disorders caused by mutations in mtDNA-encoded rRNA genes, such as mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS; tRNA(Lys)UUR) and mtDNA disorders caused by large deletions of mtDNA such as Kearns-Sayre syndrome. Usually these disorders present with multiple respiratory chain enzyme deficiencies including COX deficiency (11–13). Several types of isolated COX deficiency have been described including Leigh disease and a fatal infantile form presenting as a myopathy or cardiomyopathy (14, 15).

The first documented COX gene mutation (encoded by mtDNA or nuclear DNA) associated with COX deficiency was a 15-bp deletion (9480del15) in the mtDNA-encoded COX III gene (16). This in-frame deletion removed 5 amino acids (92del-Phe-Phe-Phe-Ala-Gly) from a conserved transmembrane region of the COX III polypeptide, one of two regions that contact COX I in the holoenzyme (4). The patient had high levels of mutation in skeletal muscle (92%) and low levels (<1%) in leukocytes. Analysis of single muscle fibers revealed a correlation between levels of mutation and COX activity in muscle fibers, with COX negative fibers having a higher level of mutation (98.6 ± 0.8%) than COX positive fibers (25 ± 25%). Western analyses revealed a reduction in the steady state levels of COX subunits II, IV, Va, Vb, and VIc, indicating that assembly or stability of the holoenzyme was compromised by the 5-amino acid deletion in COX III.

To investigate the consequences of the 9480del15 for COX assembly and enzymatic function, the mutation was transferred to a human cell line devoid of mtDNA (ρo cell line) developed by King and Attardi (17). This cell system allows the study of mtDNA mutations without the contribution of the
patient's nuclear background and has been used successfully to investigate mutations in mitochondrial tRNA (18–21) and protein coding genes (22), and rearrangements of mtDNA (23).

EXPERIMENTAL PROCEDURES

Cell Culture—Human osteosarcoma cell lines 143B and 143B206 (17), and transfection and cell lines C52 154.4, 151.11, 151.12, 51.112, 51.112.50, and 51.112.28 were grown in Dulbecco's modified Eagle's medium containing 4.5 mg of glucose/ml and 110 mg of pyru- vate/ml (DMEM) supplemented with 5% fetal bovine serum (FBS). The medium was changed every 2–3 days. Isolated myoblasts were grown in DMEM supplemented with 100 µg of 5-bromodeoxyuridine/ml and 5% dialyzed FBS (24) 24 h after fusion and were grown for approximately 2–3 weeks. Single colonies were isolated with glass cloning cylinders, and cells were subsequently cultured in DMEM containing 5% FBS.

Transformation of the 143B206 cell line with mitochondria from cultured myoblasts was performed by cytoplasm fusion of enucleated myoblasts (25). Cybrids were plated in selective medium (DMEM supplemented with 100 µg of 5-bromodeoxyuridine/ml and 5% dialyzed FBS) 24 h after fusion and were grown for approximately 2–3 weeks. Single colonies were isolated with glass cloning cylinders, and cells were subsequently cultured in DMEM containing 5% FBS.

For platelet-leukocyte mediated transformation of 143B206 cells, a platelet-leukocyte mixture was incubated with a 1:1 ratio of platelet and leukocyte fraction that were isolated from 20 ml of whole blood by differential centrifugation (26). The platelet and leukocyte fractions were mixed, washed with DMEM, and pelleted by centrifugation. The cell pellet was mixed with 1.5 × 106 143B206 ρ0 cells and membrane fusion was promoted by the addition of polye- nylmethylamine. Cybrids were plated in selective medium 24 h after fusion. Mass cultures were isolated after 2 weeks of growth in selective medium and were replated at low density, in non-selective medium (DMEM supplemented with 5% FBS and 50 µg of uridine/ml) for isolation of cell clones.

Ethidium bromide was used to manipulate the mtDNA content in two cell lines, 51.129 and 51.112 (27). Exponentially growing cells were grown in DMEM supplemented with 5% FBS, 50 mg of ethidium bromide/ml, and 50 µg of uridine/ml for 14 h. After treatment, cells were replated in non-selective medium and grown for 3 days, then replated at low density in either the same medium or DMEM supplemented with 5% dialyzed FBS to isolate individual clones for analysis.

Mutation Analysis—Total DNA was isolated from exponentially growing cells (28). PCR analysis of mutation levels was performed as described previously (16). Amplified DNA fragments of the wild-type (85 bp) or the mutated mtDNA (70 bp) were differentiated by size after electrophoresis through 10% polyacrylamide gels. Gels were dried and Autoradiography of a Mutation in the mtDNA-encoded COX III Gene

ATP Synthesis Assay—Mitochondria were prepared as described pre- viously (35) after scraping monolayer cultures from five 15-cm culture dishes (approximately 3 × 106 cells) into 30 ml of 0.27 mM mannitol, 0.1 mM EDTA, 0.05% bovine serum albumin, 10 mM-Tris-HCl, pH 7.3. After a brief treatment with protease XIV (Sigma), cells were disrupted with four strokes in a Teflon-glass homogenizer and the mitochondrial frac- tion was isolated by differential centrifugation.

The ability of isolated mitochondria to synthesize ATP by oxidative phosphorylation was assayed using succinate as a substrate. ATP synthesis was measured indirectly by the incorporation of 32P into glucose 6-phosphate, following phosphorylation of ADP by inorganic phosphate in the presence of the myokinase inhibitor adenosine pentaphosphate (33). COX activity of isolated mitochondria (100–200 µg of protein) was assayed at 30 °C in 0.3 ml of 30 mM Tris acetate, pH 7.4, 10 mM MgCl2, 10 mM potassium phosphate, 0.5 µCi of [32P]orthophosphate (8,500–9,120 Ci/mmol; NEN Life Science Products Inc.), 6 mM succinate, 20 mM glucose, 20 units of hexokinase, 1 mM ADP, and 50 mM adenosine pentaphosphate in a shaking water bath at 30 °C. Control reactions included 5 µg of F0 ATPase inhibitor oligomycin. The reaction was terminated after 35 min by adding 20% trichloroacetic acid to a final concentration of 10%. The reaction mixture was then centrifuged, and a 0.3-ml aliquot was removed, to which was sequentially added 1 ml of H2O, 1 ml of 3.3% ammonium molybdate in 3.75 N H2SO4, and 200 µl of acetic. Unin- corporated phosphate was removed by extracting 6 times with 1 ml of benzene:isobutanol mixture (50:50, v/v, H2O saturated), after vortexing vigorously for 30 s. Aliquots of the supernatant were determined by scintillation counting, was taken to represent incorporation of radioactive phosphate.

RNA Isolation and Northern Analysis—Total RNA was isolated from approximately 1.5 × 106 exponentially growing cells using the com- mercial RNA isolation reagent, RNazol B (Tel-Test Inc.). RNA was resus- pended in formamid (Tel-test Inc.), and quantitated by UV absorbance.

Total RNA (10–15 µg) was electrophoresed through a 1% agarose gel containing 0.66 µm formaldehyde (37), transferred to Zeta Probe (Bio- Rad), and hybridized according to the manufacturer’s directions. The following probes were labeled by random priming (38) following the manufacturer’s directions (Random prime labeling kit, Roche Molecular Biochemicals Co.; the insert of pHFBA-1, a plasmid containing the human cytoplasmic β-actin gene (39); a PCR amplified fragment corre- sponding to nt 9268–9645 of the mtDNA-encoded COX III gene; a PCR amplified fragment corresponding to nt 7640–8051 of the mtDNA- encoded COX II gene; a PCR amplified fragment corresponding to nt 8540–9202 of the mtDNA-encoded ATP synthetase subunit 6 (A6) gene. A strand-specific probe for the 12 S RNA was obtained by extension of the universal M13 primer on single stranded template (30) isolated from M13 clone mp9.M9 (mtDNA 1–739; Ref. 29). RNA hybridization signals were quantitated with a Bio-Rad PhosphoImager (Molecular Imager) and visualized by autoradiography.

Western Analysis—Equal amounts of total mitochondrial protein were separated by electrophoresis through Tricine-SDS 10% polyacryl- amide gels (40) and transferred to nitrocellulose by electroblotting, following standard protocols, except for the last wash before the transfer buffer. Blots were incubated with a 1:1000 dilution of the appropriate primary antibody in 1 × phosphate-buffered saline and 0.3% Tween. The bound primary antibody was visualized by chemilu- minescent detection (CDP Star Kit, Tropix). Mouse monoclonal antibodies against subunits I, II, and IV of COX were generously provided by R. Capaldi, University of Oregon.

Analysis of Mitochondrial Protein Synthesis—For pulse labelings, 6–10 106 cells of exponentially growing cells were incubated for 60–120 min in methionine-free DMEM containing 250–500 µCi of [35S]methionine (>1,000 Ci/mmol; NEN Life Science Products Inc.), 5% dialyzed FBS, and 100 µg of emetine/ml (31). A modified labeling protocol was used for the short pulse labelings. Cells were labeled for 15 min, 1 h, or 2 h. Immediately after labeling, dishes were washed with ice-cold DMEM containing 2 mM unlabeled methionine, 100 µg of emetine/ml, and five protease inhibitors: 174 µg of phenylmethylsulfonyl fluoride/ml, 10 µg of leupeptin/ml, 3.4 µg of papain/ml, 3.6 µg of E-64/ml, 56 µg of benzamidine HCl/ml. Cells were washed once with ice-cold 5 mM KCl, 130 mM NaCl, 7.5 mM MgCl2, three protease inhibitors, then resuspended in 1 ml of 10 mM-Tris-HCl, pH 6.7, 10 mM KCl, 0.15 mM MgCl2, plus protease inhibitors and homoge- nized; mitochondria were isolated by differential centrifugation.

For pulse-chase labelings, 106 cells of exponentially growing cells were labeled for 2 h in methionine-free DMEM containing 250 µCi of [35S]methionine (>1,000 Ci/mmol; NEN Life Science Products Inc.), 5% dialyzed FBS, and 100 µg of emetine/ml. After labeling, cells were washed twice with DMEM containing 100 µg of emetine/ml and 2 mM

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unlabeled methionine, washed once with DMEM containing 2 mm unlabeled methionine, and then incubated in DMEM containing 2 mm unlabeled methionine and 5% dialyzed FBS at 37 °C. After the chase incubation, mitochondria were isolated from 3–4 10-cm dishes of cells by differential centrifugation (31) and resuspended in 200 μl of 0.25 M sucrose, 0.5% Triton X-100, pH 6.7, 150 μM MgCl₂. For experiments with multiple chase incubations, cells from one 10-cm dish were lysed by the addition of 1% SDS, 10 mm Tris-HCl, pH 7.4.

Labeled polypeptides from cell lysates or an isolated mitochondrial fraction were analyzed by Tricine-SDS 10% polyacrylamide gel electrophoresis (PAGE; Ref. 40) and either subjected to fluorography (NEN Intenstify, Dupont) with Kodak Bio-Max MS film or analyzed with a Bio-Rad PhosphorImager (Molecular Imager). Levels of [35S]methionine incorporation into translated polypeptides were quantitated using Molecular Analyst software (Molecular Imager, Bio-Rad).

Immunoprecipitation—Immunoprecipitation of COX was performed as described previously (42). A mitochondrial fraction (90–150 μg) isolated from pulse labeled or pulse-chase labeled cells was lysed in 0.5% Triton X-100 for 30 min at 4 °C in a total volume of 80 μl. Samples were then diluted 5-fold with IB buffer (5 mm Tris-HCl, pH 6.7, 1 mm phenylmethylsulfonyl fluoride, 0.5% Triton X-100, I mg of bovine serum albumin/ml, and 2 mm methionine) and pre-absorbed for 1 h at 4 °C with 125 μl of 10% (v/v) zysorbin (formaldehyde-fixed protein A-positive Staphylococcus aureus, Zymed Laboratories Inc.) which was resuspended in IB buffer. After centrifugation, supernatants were removed and incubated for 1.5–2 h at 4 °C with 6–10 μl of antisera or normal rabbit serum. Samples were then incubated for 30–120 min at 4 °C with 125 μl of 10% (v/v) zysorbin and 300–500 μg of a mitochondrial fraction which was isolated from unlabeled cells and lysed in 0.5% Triton X-100. Samples were centrifuged and pellets were washed once with IB buffer containing 0.1% SDS, once with IB buffer, and once with 10 mm Tris-HCl, pH 6.7. The immunocomplexes were released by incubation at 37 °C in 30 μl of sample buffer (50 mm Tris-HCl, pH 6.8, 4% SDS, 12% glycerol, 0.02% Serva Blue G, 4% β-mercaptoethanol) and analyzed by Tricine-SDS 10% PAGE as described above. Two controls were performed in all immunoprecipitations, one using normal rabbit serum and another using a protein A-negative, formaldehyde-fixed Staphylococcus aureus.

RESULTS

Creation and Characterization of Transmitochondrial Cell Lines—To investigate the consequences of the 9480del15 COX III mutation on COX function, mitochondria from the patient with the COX III mutation were introduced into ρ0 cells. Two types of fusions were performed. In one, cytoplasts derived from enucleated cultured myoblasts and in another, platelet-leukocyte fractions isolated from blood, were fused with cells from the ρ0 cell line 143B206. Following fusions, cells were plated in selective medium, containing 5-bromodeoxyuridine and lacking uridine. Since 143B206 cells are auxotrophic for pyrimidines due to their respiratory chain deficiency and are also thymidine kinase deficient, only 143B206 cells which received mitochondria from cytoplasts or platelets would be expected to grow. Individual clones were isolated from both myoblast and platelet-leukocyte fusions and were screened by PCR for the presence of the COX III mutation (Fig. 1). Transmission of mitochondrial DNA to the ρ0 cells was confirmed by hybridization in all transmitochondrial cell lines and 143B (parental cell line of ρ0 cell line 143B206). The mtDNA content in wild-type cell lines was 95% (52.144) and 65% (51.118) of the mtDNA level in 143B (9,100 molecules per cell; Ref. 26). In 97% mutant cell lines 51.112 and 51.129, the mtDNA levels were 70% and 93%, respectively, of that of 143B. In 100% mutant cell lines, the mtDNA content was 78% (51.112.50 and 51.112.28) of the level in 143B (9,100 molecules per cell; Ref. 26).

To obtain cell lines homoplastic for the COX III deletion, the ratio of wild-type to mutated mtDNA was manipulated using ethidium bromide to inhibit mtDNA replication (27). Two cell lines containing high levels of the mutation (97–98% mutation; 51.112 and 51.129) were grown in the presence of ethidium bromide and uridine for 14 days. After removal of ethidium bromide and repopulation of mtDNA to normal levels within the cells, cells were plated to isolate colonies. Sixty-two colonies were chosen for expansion and analysis. To avoid selection against cells with a severe respiratory chain deficiency, a portion of the cells plated for colony isolation were grown in medium supplemented with uridine. Five clonal cell lines homoplastic for the mutation were identified by PCR. The remaining cell lines contained either 100% wild-type mtDNA (31 clones) or were ρ0 (26 clones).

The cell lines chosen for further studies included two homoplastic for the COX III mutation (51.112.50 and 51.112.28), two with high levels of mutation (97–98% mutated mtDNA; 51.112 and 51.129), and two homoplastic for the patient’s wild-type mtDNA (52.144 and 51.118) (Fig. 1). In this paper, cells homoplastic for the mutation will be referred to as 100% mutant cells, cells with mutation levels of 97–98% will be referred to as 97% mutant cells, and cells with 0% mutation (100% patient wild-type mtDNA) will be referred to as wild-type cells. Mutation levels in all cell lines were checked periodically and were stable over a period of several months of continuous culture.

The mtDNA content per cell was quantitated by dot blot hybridization in all transmitochondrial cell lines and 143B (parental cell line of ρ0 cell line 143B206). The mtDNA content in wild-type cell lines was 95% (52.144) and 65% (51.118) of the mtDNA level in 143B (9,100 molecules per cell; Ref. 26). In 97% mutant cell lines 51.112 and 51.129, the mtDNA levels were 70% and 93%, respectively, of that of 143B. In 100% mutant cell lines, the mtDNA content was 78% (51.112.50 and 51.112.28) of the level in 143B.

Mutant Transmitochondrial Cells Exhibited a Severe Respiratory Chain Defect—A severe deficiency of COX activity was observed in all mutant cells (Fig. 2A). COX activity was not detectable in 100% mutant cells and was decreased by approximately 94% (0.62 ± 0.07 rate constant (k)/min/mg; mean ± 1 S.E.) in 97% mutant cells compared with wild-type cells (10.0 ± 1.1 k/min/mg).

The ability of isolated mitochondria to synthesize ATP, using succinate as a substrate, was measured in mitochondria isolated from wild-type and mutant cells. ATP synthesis in mutant mitochondria was severely reduced compared with wild-
was 1.1 cells (20). The rate of oxygen consumption in 97% mutant cells was approximately 0.1% of that for wild-type cells (4.4 ± 0.9 μmol of ATP/h/mg of protein). The rate of ATP synthesis for the 97% mutant cells (0.006 ± 0.004 μmol of ATP/h/mg of protein) was less than 4% of wild-type cells (5.5 ± 0.9 μmol of ATP/h/mg of protein). The capacity of the entire respiratory chain in transmitochondrial cells was evaluated by measuring the rates of oxygen consumption of intact cells (Fig. 2B). The rate of oxygen consumption in 100% mutant cells (0.21 ± 0.02 fmol/cell/min (mean ± 1 S.E.)) was less than 4% of wild-type cells (5.5 ± 0.26 fmol/cell/min) and similar to rates determined for ρ° 143B206 cells (20). The rate of oxygen consumption in 97% mutant cells was 1.1 ± 0.12 fmol/cell/min, 20% of the wild-type rate.

The activity of citrate synthase, a mitochondrial matrix enzyme, was measured in wild-type and mutant cells to approximate mitochondrial volume. Citrate synthase levels were similar among mutant and wild-type cell lines (Fig. 2E). The activity of complex III (Fig. 2D), the respiratory chain enzyme responsible for the reduction of cytochrome c, was reduced 50% in 100% mutant cells (9.9 ± 0.75 k/min/mg) and 32% in 97% mutant cells (13.0 ± 1.4 k/min/mg) as compared with wild-type (19.0 ± 2.9 k/min/mg).

**Growth Properties of Transmitochondrial Cells**—A requirement for pyruvate and uridine for growth is a phenotypic characteristic of respiratory deficient cells, such as ρ° cell line 143B206 (17). Wild-type and mutant cells were tested for growth in four types of media (DMEM + pyruvate + uridine; DMEM + pyruvate – uridine; DMEM – pyruvate + uridine; DMEM – pyruvate – uridine). As expected, the growth properties of the wild-type cells were similar in all media tested (Fig. 3A). In contrast, 100% mutant cells exhibited little or no growth in medium lacking either pyruvate or uridine, or lacking both (Fig. 3B). The 100% mutant cells grew in medium containing both pyruvate and uridine, although at a rate lower than that observed for wild-type cells. The 97% mutant cells grew in all media (Fig. 3C). In the absence of pyruvate (either with or without uridine), growth was characterized by an initial lag with only one population doubling occurring by 72 h. In later stages of growth (72–120 h), the growth rate approached that observed in media containing pyruvate and uridine.

The growth characteristics of the 97% mutant cell line suggested that it may be composed of a heterogeneous population of cells with varying levels of mutated mtDNA. To investigate this possibility, the 97% mutant cell line was plated at a low density for isolation of individual cell clones. Fifty-four clonal cell populations were analyzed for levels of mutated mtDNA. The average mutation level in these fifty-four clones was 96%, with the majority of the clonal populations having 95–99% mutated mtDNA. A small proportion of the clonal populations (4 clones of 54 analyzed) had levels of mutated mtDNA significantly lower than the mean (>2 S.D. from the mean; mutation levels <90% mutated mtDNA). Although these results indicated that the 97% mutant cell line was a heterogeneous cell population, repeated mutation analysis of this cell line during the course of experiments presented here consistently ranged from 97 to 98% levels of mutated mtDNA, suggesting that the cells with lower levels of mutation never became a significant proportion of the cell population.

**RNA Analysis**—To determine if the COX III mutation had an effect on the steady-state levels of COX III mRNA, we performed Northern analysis on four RNA transcripts: three derived from mtDNA-encoded genes (COX III, ATP synthetase subunits 8 and 6 (A8/A6), and 12 S rRNA) and one from a nuclear encoded gene (cytoplasmic β-actin). Total RNA was isolated from exponentially growing wild-type, 100% mutant, 143B206 and 143B cells for these analyses (Fig. 4).

The COX III probe detected a band of approximately 1 kb, representing the COX III transcript, and a higher molecular weight band of 1.7 kb in both mutant and wild-type cells. The latter was consistent with a partially processed RNA species encompassing the A8/A6 + COX III genes. Hybridization with an A8/A6 probe detected the fully processed 840-bp A8/A6 mRNA as well as the 1.7-kb transcript, confirming our identi-
Analysis of a Mutation in the mtDNA-encoded COX III Gene

Probes specific for mtDNA encoded COX III and 12 S rRNA and nuclear encoded β-actin. The mRNA species representing the partially processed A8/A6 + COX III transcript is indicated on the right by an asterisk.

The steady-state levels of mtDNA-encoded transcripts were normalized to those of nuclear DNA-encoded β-actin and mtDNA-encoded 12 S rRNAs. These ratios are relative levels and do not reflect the absolute numbers of each species. The steady-state levels (mean ± 1 S.D.) of COX III mRNA (the sum of processed and partially processed mRNA species) were similar in the 100% mutant cells (β-actin, 0.15 ± 0.01; 12 S rRNA, 0.90 ± 0.06) and wild-type cells (β-actin, 0.18 ± 0.02; 12 S rRNA, 0.81 ± 0.05).

Steady-state Levels of COX Polypeptides—The effect of the COX III mutation on the steady-state levels of the COX III polypeptide could not be determined directly because an antisera specific for human COX III was not available. Steady-state levels of mtDNA-encoded COX I and COX II polypeptides and nuclear-encoded COX IV were investigated by Western analysis of mitochondria isolated from wild-type, 100% mutant, 97% mutant, 143B206, and 143B cells. The steady-state levels of both COX I and COX II polypeptides were reduced in mutant cells compared with wild-type and 143B cells (Fig. 5). The Western signal for COX II in 100% mutant cells was approximately 15% of that for COX II in wild-type cells. For the 97% mutant cells, this signal was approximately 45% of that for COX II in wild-type cells. In 97 and 100% mutant cells, the signal for COX I was approximately 55% of that found in wild-type cells. Steady-state levels of nuclear encoded subunit COX IV in mutant cells were similar to levels in wild-type, 143B and ρ0 cells.

Translation and Stability of mtDNA-encoded COX Subunits—Mitochondrial protein synthesis was examined in wild-type and mutant cells by labeling cells with [35S]methionine for 1.5–2 h (pulse) in the presence of emetine, a cytoplasmic protein synthesis inhibitor. Translation products in isolated mitochondria were analyzed by Tricine-SDS-PAGE. To compare levels of translated polypeptides among cell lines, levels of labeled polypeptides were normalized to mtDNA-encoded A6. Wild-type and mutant cells had similar levels of labeled A6 polypeptide after pulsing cells for 1–2 h with [35S]methionine (ratio of mutant to wild-type, 1.3 ± 0.13 (1 S.E.)). The stability of the labeled A6 polypeptide in cells was examined by labeling the cells for 1–2 h and then chasing cells in emetine-free medium containing an excess of unlabeled methionine. The ratio of labeled A6 polypeptide present after a 15–17 h chase to that present at the end of the 1–2-h pulse labeling was similar in wild-type (52.144, 1.1 ± 0.12 (1 S.E.)) and mutant (51.112.50, 0.93 ± 0.09 (1 S.E.)) cells, indicating that A6 is metabolically stable. Since A6 is an abundant polypeptide that is metabolically stable, well resolved by Tricine-SDS-PAGE, and has high levels of [35S]methionine incorporation, levels of labeled A6 were appropriate for normalizing the levels of labeled mitochondrial translation products in these mutant and wild-type cell lines.

Pulse labelings of 97 and 100% mutant cells revealed a COX III polypeptide with a slightly greater electrophoretic mobility than that of wild-type. There were reduced levels of [35S]methionine labeling of COX III in both 97 and 100% mutant cells as compared with the level in wild-type cells (Fig. 6, A and B, pulse lanes). Levels of labeled COX I and COX II polypeptides also were decreased in mutant cells as compared with wild-type. No other qualitative or quantitative alterations of mitochondrial translation products were observed.

To determine if the reduced levels of labeling of COX polypeptides in mutant cells resulted from reduced rates of synthesis, the levels of [35S]methionine-labeled polypeptides in wild-type (52.144) and 100% mutant (51.112.50) cells were quantitated after short periods of labeling (15 min, 1 h, and 2 h; Fig. 7). After a 15-min labeling, both mutant and wild-type cells had similar levels of labeled COX I, II, and III polypeptides, indicating that the COX polypeptides have similar rates of synthesis in mutant and wild-type cells (Fig. 7A). Reductions in the amounts of labeled COX II and COX III polypeptides were observed in mutant cells only for the 1- and 2-h labelings (56–62% of wild-type levels after a 2-h pulse). Levels of labeled COX I polypeptide were reduced after a 1- or 2-h pulse labeling in the 100% mutant cells, but to a lesser extent (80–82% of wild-type). These results suggested that in mutant cells, mtDNA-encoded COX polypeptides were less metabolically stable than in wild-type cells.

The levels of [35S]methionine incorporated into three other mtDNA-encoded polypeptides were examined. The levels of labeled cytochrome b ( cyt b) and NADH dehydrogenase subunits 2 and 3 (ND2 and ND3) were similar in the 100% mutant cells and wild-type cells after a 15-min labeling (90–105% of...
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pulselabeling were performed for each cell line. No labeled polypeptides were detected when mitochondria were immunoprecipitated with normal rabbit serum (Fig. 9, NS lanes) or with a protein A-negative S. aureus strain (not shown).

In most experiments from wild-type cells after a 1-h pulse labeling, only COX II was immunoprecipitated by antisera to the C terminus of COX II (Fig. 9A). On some occasions low levels of COX I or COX I and COX III were also immunoprecipitated with COX II (Fig. 9B). We attribute this difference to minor variations among the pulse labeling experiments. After a 1-h pulse and a 2-h chase, COX I and COX III were always immunoprecipitated with COX II in wild-type cells (Fig. 9, 0% mutation, C lanes). The amounts of immunoprecipitated COX I and COX III were higher than the levels of these proteins immunoprecipitated after a 1-h pulse. These results suggested that after a 1-h labeling in wild-type cells, some COX II polypeptide could be associated with COX I or COX III, or assembled into a multisubunit enzyme complex. The assembly of the newly synthesized COX subunits continued during the 2-h chase. However, a portion of the labeled COX II remained unassembled after the 2-h chase as shown by immunoprecipitations with the antiserum to the N terminus of COX II (Fig. 9, 0% mutation, N lanes).

In experiments with 100% mutant cells, only COX II was immunoprecipitated by either antiserum, after both a 1-h pulse or a 1-h pulse followed by a 2-h chase (Fig. 9A, 100% mutation, N and C lanes). There was no evidence for any association, even transient, of COX II with COX I or COX III. In both 97% and wild-type cells after a 1-h pulse, the antiserum to the C terminus of COX II occasionally immunoprecipitated low levels of COX I, or COX I and COX III, with COX II (Fig. 9B). In most experiments, however, only COX II was immunoprecipitated after a 1-h pulse. After a 2-h chase, COX I and COX III were always immunoprecipitated with COX II in wild-type and 97% mutation, C lanes). The amounts of immunoprecipitated COX I and COX III were higher than the levels of these proteins immunoprecipitated after a 1-h pulse. These results suggested that after a 1-h labeling in wild-type cells, some COX II polypeptide could be associated with COX I or COX III, or assembled into a multisubunit enzyme complex. The assembly of the newly synthesized COX subunits continued during the 2-h chase. However, a portion of the labeled COX II remained unassembled after the 2-h chase as shown by immunoprecipitations with the antiserum to the N terminus of COX II (Fig. 9, 97% mutation, N lanes).
and COX II are stably assembled in a multisubunit complex in 97% mutant cells with kinetics of assembly similar to those of wild-type cells. Although assembly of COX I with COX II depends upon the presence of wild-type COX III, COX III does not necessarily remain associated with this complex.

**DISCUSSION**

The results presented here demonstrate that the 15-bp deletion, 9480del15, within the mtDNA-encoded COX III gene resulted in a specific and severe deficiency in COX activity in transmitochondrial cell harboring this mutation. The stability of the translated COX III polypeptide was severely reduced by this mutation, presumably resulting in reduced steady-state levels of the COX III polypeptide. The mutated COX III polypeptide was not incorporated into the COX enzyme complex and prevented the stable assembly of COX I and COX II subunits in cells homoplasmic for the mutation.

We isolated transmitochondrial cell lines containing high levels of mutated mtDNA (97–98%) from fusions of ρ0 cells with platelet and leukocyte fractions from the patient, and successfully created two cell lines homoplasmic for the mutation by manipulating the mtDNA content in two 97% mutant cell lines. Fusions with cultured myoblasts yielded only transmitochondrial cell lines containing wild-type mtDNA. Individual myoblast clones and pools of myoblasts had no detectable levels of mutation after 3–4 weeks of culture. Since the patient’s skeletal muscle harbored high levels of mutated mtDNA (92%; Ref. 16), these results suggested that the COX III mutation was absent or present at very low levels in muscle satellite cells (undifferentiated muscle precursor cells), in contrast to the skeletal muscle. Alternatively, it is possible that satellite cells and myoblasts containing high levels of mutation were selected against during culture because of a growth disadvantage. Others have reported difficulty establishing clonal cell lines with deletions or point mutations of mtDNA from muscle satellite cells (43–46).

The most striking finding of our studies was the severity of the respiratory defect in transmitochondrial cell lines homoplasmic for the mutation. These cells had no detectable COX activity and no detectable respiratory ATP synthesis. The rate of oxygen consumption, 4% of wild-type, was comparable to the rate observed for the ρ0 cell line 143B206 (20). Similar to 143B206, these cells have an absolute requirement for uridine and pyruvate for growth. These growth properties indicate a complete absence of respiratory chain activity in cells with 100% mutated mtDNA.

The respiratory chain deficiency was less severe in cells harboring 97% mutated mtDNA. COX activity in these cells was 6% of that in wild-type cells and the rate of ATP synthesis was 9% of wild-type. These cells grew in all media, but were characterized by reduced rates of growth and altered growth properties in media lacking pyruvate.

The rates of oxygen consumption in the 97% mutant cells (20% of wild-type) were markedly higher than the levels of COX activity (6% of wild-type). Inhibitor titrations of COX activity have shown that in some cell types a 50–60% reduction in COX activity does not significantly inhibit metabolic respiratory function or ATP synthesis, but larger reductions in COX activity are associated with a sharp decline in respiratory function (47, 48). The amount of control that COX exerts on respiratory function is dependent on the type of cell lines and tissues (47, 49, 50). The relatively high rates of oxygen consumption in the 97% mutant cells suggest that, in these cell lines, COX may exert a low control strength on respiratory function.

To determine if the severity of the COX defect in mutant cells had an indirect effect on other components of the respiratory chain, complex III activities were measured in both wild-type and mutant cell lines. Complex III activities were reduced in both the 97% mutant cells (68% of wild-type levels) and the 100% mutant cells (50% of wild-type levels). The lower complex III activities in the mutant cells implied that a severe COX defect could affect the stability or activity of complex III. Our metabolic labeling studies showed no differences in either the rate of translation or metabolic stability of mtDNA-encoded cyt b in mutant and wild-type cells, suggesting that the activities of both COX and complex III may be intrinsically linked. It is possible that COX and complex III may physically interact within the membrane and this interaction may be important for maintaining some degree of stability for both enzyme complexes. Alternatively, the mutation may alter the structure or amount of the inner mitochondrial membrane, or generally increase the rates of turnover of some of the respiratory chain proteins. Ultrastructural analysis of mutant cells indicated alterations in the organization of cristae in mutant cells.2

To understand the mechanisms resulting in COX deficiency with this COX III mutation, we examined the consequences of the mutation for the function and stability of COX III within the enzyme complex. The mutation had little or no effect on the steady-state levels of COX III mRNA in transmitochondrial cell lines with 100% mutated mtDNA (Fig. 4). Our studies detected two types of COX III transcripts, a fully processed COX III mRNA and a partially processed A8/A6 + COX III mRNA. Partially processed A8/A6 + COX III transcripts have been observed previously, ranging as high as 70% of the total COX III transcript levels (21).

To examine translation and stability of COX III in mutant cell lines, mitochondrial translation products were analyzed by labeling cells with [35S]methionine in the presence of emetine, a cytoplasmic protein synthesis inhibitor. The COX III polypeptide was translated in 100% mutant cells at rates similar to wild-type, although the stability of the newly synthesized COX III in mutant cells was reduced. After metabolic labeling periods of 2 h, levels of COX III in both the 97 and 100% mutant cells were 56–62% of wild-type levels. Additional pulse-chase studies confirmed the increased rate of turnover of the labeled COX III polypeptide in mutant cells (Figs. 6 and 8). For all chase time points, the 100% mutant cells had lower levels of the COX III polypeptide compared with those in wild-type cells, and the labeled COX III polypeptide was barely detectable after a 12–15-h chase. Presumably, the increased rate of turnover resulted in severely reduced steady-state levels of COX III.

Metabolic labeling experiments and Western analysis revealed that levels of labeled COX I and COX II polypeptides were reduced in mutant cells as compared with wild-type cells. The reduction in the steady-state levels of COX II in the 100% mutant cells was particularly striking, the signal being only 15% of wild-type levels. The steady-state levels of COX II in the 97% mutant cells and levels of COX I in both the 97 and 100% mutant cells also were reduced, but to a lesser extent. Presumably, the decreased stabilities of COX I and COX II subunits in mutant cells were a direct consequence of reduced assembly or stability of the COX enzyme complex.

In this study, a proportion of the newly synthesized COX I, COX II, and COX III translation products was unstable even in wild-type cell lines. These results suggested that only a fraction of translation products was assembled into the enzyme complex. There is some evidence that mtDNA-encoded COX subunits and other respiratory chain components are produced in non-stoichiometric levels compared with the nuclear encoded subunits. A subset of mitochondrial translation products was found to be unstable in HeLa cells and rat hepatoma cells after

2 Y. Lu and M. King, unpublished observations.
short labeling periods, suggesting that only a certain fraction of newly synthesized mitochondrial translation products was assembled with nuclear COX subunits (51, 52). Other studies specifically examining levels of mtDNA-encoded COX subunits have shown that mRNA and translated polypeptides of mtDNA-encoded COX subunits may be produced in excess compared with nuclear subunits (53, 54). These studies are consistent with our observations that indicate that only 30–60% of newly synthesized mtDNA-encoded COX polypeptides are incorporated into the enzyme complex.

The 9480del15 in the COX III gene results in the loss of 5 amino acids (92delPhe-Phe-Ala-Gly) from the third transmembrane domain of the polypeptide. Analysis of the crystal structure of bovine COX indicated that this domain of COX III is one of two transmembrane domains which contact the COX I subunit in the enzyme complex (4). The loss of 5 amino acids in this domain may prevent the association of COX III with COX I within the enzyme complex, presumably leading to decreased stability of the COX III polypeptide and decreased assembly or stability of the COX holoenzyme. Alternatively, the 5-amino acid deletion may affect the insertion of COX III into the membrane, altering its topology, and preventing its association with other COX subunits.

There was no evidence of assembly of COX in 100% mutant cells. This suggests that the mutated COX III polypeptide was not incorporated into the enzyme complex and thus prevented the association of mtDNA-encoded COX I with COX II.

The immunoprecipitation experiments with the 97% mutant cell line revealed that COX I and COX II polypeptides were assembled in the 97% mutant cells. In both wild-type and 97% mutant cells, similar amounts of COX I and COX II polypeptides were present in complexes after a 2- or 12-h chase. The amounts of COX I polypeptide associated with COX II were higher than would be expected in a cell line with 97% levels of the COX III mutation, suggesting that a high proportion of COX I and COX II was assembled in the 97% mutant cells. In experiments where complexes were detected after a 1-h pulse labeling, the level of immunoprecipitated COX III was similar to that of wild-type. This raises the possibility that some of the COX III incorporated into the complex is the mutated COX III. Since the complex exists as a dimer, once a wild-type COX III is incorporated into a monomer, a mutated COX III could therefore be assembled as the second COX III of the dimer. Unfortunately, it was not possible to distinguish the wild-type and mutated COX III polypeptides and therefore we were not able to determine if the mutated COX III was incorporated into the complex. The amounts of COX III present in immunoprecipitated complexes after a 2- and 12-h chase, however, were lower in the 97% mutant cells compared with wild-type cells and sometimes were not detectable. This suggests that the COX III polypeptide is not a stable component of the COX I and COX II complex and that COX I and COX II may remain assembled in the absence of COX III. Alternatively, the 97% mutant cells may have more efficiently incorporated newly translated wild-type COX III into the enzyme complex, which would not have been detected because of the absence of label. Taken together, these results indicate that COX III is required for the initial association of COX I with COX II. Additional studies are needed to confirm that COX III is required for the assembly of COX I and COX II polypeptides in the enzyme complex, but is not necessarily required for maintaining COX I and COX II subunit interactions. Furthermore, it needs to be determined if an enzyme complex lacking COX III retains any catalytic activity. Likewise, it would be interesting to determine if, in the 97% mutant cells, mtDNA-encoded COX I and COX II are assembled with nuclear DNA-encoded COX subunits.

Ultimately, one of the goals of these studies is to further our understanding of the pathological consequences of the COX III 9480del15 deletion for the patient's clinical phenotype. Our findings have demonstrated that this mutation acts in a recessive manner. Even the small amount of wild-type COX III present in 97% mutant cells supported low levels of COX activity (6% of wild-type), ATP synthesis (9% of wild-type), and assembly of COX I and COX II subunits. The COX deficiency in the patient was characterized by an 85% reduction in activity (compared with normal controls) in skeletal muscle that harbored 92% mutated mtDNA. Despite this significant reduction in COX activity, the energy production in the patient's muscle was not limiting under normal circumstances (16). As in the case with several other mtDNA mutations, the COX III 9480del15 may exhibit a threshold for expression in which only very high levels of mutation have a significant effect on ATP synthesis. The mutation level in the patient's skeletal muscle may be below this threshold for expression of a more severe phenotype. In the future, studies with cells containing intermediate levels of mutation will determine the threshold levels for the COX III 9480del15 mutation.

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A Pathogenic 15-Base Pair Deletion in Mitochondrial DNA-encoded Cytochrome c Oxidase Subunit III Results in the Absence of Functional Cytochrome c Oxidase

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