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A Stop-Codon Mutation in the Human mtDNA Cytochrome *c* Oxidase I Gene Disrupts the Functional Structure of Complex IV

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Summary

We have identified a novel stop-codon mutation in the mtDNA of a young woman with a multisystem mitochondrial disorder. Histochemical analysis of a muscle-biopsy sample showed virtually absent cytochrome *c* oxidase (COX) stain, and biochemical studies confirmed an isolated reduction of COX activity. Sequence analysis of the mitochondrial-encoded COX-subunit genes identified a heteroplasmic G→A transition at nucleotide position 6930 in the gene for subunit I (COX I). The mutation changes a glycine codon to a stop codon, resulting in a predicted loss of the last 170 amino acids (33%) of the polypeptide. The mutation was present in the patient's muscle, myoblasts, and blood and was not detected in normal or disease controls. It was not detected in mtDNA from leukocytes of the patient's mother, sister, and four maternal aunts. We studied the genetic, biochemical, and morphological characteristics of transmitochondrial cybrid cell lines, obtained by fusing of platelets from the patient with human cells lacking endogenous mtDNA (ρ^0 cells). There was a direct relationship between the proportion of mutant mtDNA and the biochemical defect. We also observed that the threshold for the phenotypic expression of this mutation was lower than that reported in mutations involving tRNA genes. We suggest that the G6930A mutation causes a disruption in the assembly of the respiratory-chain complex IV.

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Introduction

The mitochondrial diseases are a heterogeneous group of disorders in which a primary mitochondrial dysfunction is suspected or proved on the basis of morphological, genetic, biochemical, or molecular criteria. Clinically, most of these disorders affect muscle, either alone or in combination with other systems, most often brain (DiMauro and Bonilla 1997). Since 1988, when the first mutations in mtDNA were described (Holt et al. 1988; Wallace et al. 1988), >50 specific pathogenic mtDNA point mutations have been reported (Servidei 1999). These mutations can be divided into two groups: those that affect general mitochondrial protein synthesis (e.g., point mutations or deletions involving rRNA or tRNA genes) and those that affect specific respiratory-chain complexes (e.g., point mutations in the polypeptide-coding genes) (Schon et al. 1997). Although most of the mtDNA pathogenic mutations described so far are located in the tRNA genes, mutations in polypeptide-coding genes are being reported in increasing number (Servidei 1999).

Cytochrome *c* oxidase (COX) is embedded in the mitochondrial inner membrane and forms the terminal component (complex IV) of the respiratory chain. The enzyme is a complex metalloprotein that catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen and has a proton-pumping activity (Taanman 1997). Human COX is a dimer composed of 13 subunits, the 3 largest of which (i.e., I–III) are encoded by mtDNA. Combined defects of COX and other respiratory-chain enzymes are often observed in tRNA point mutations or large-scale deletions, which result in generalized impairment of intramitochondrial protein synthesis (Schon et al. 1997). Isolated COX deficiencies have been reported in a number of tissue-specific or generalized Mendelian disorders, including Leigh syndrome (Tiranti et al. 1998; Zhu et al. 1998). During the past 4 years, several mtDNA mutations in COX genes have been identified in patients with different clinical mani-

festations, most of them presenting as sporadic cases (Manfredi et al. 1995; Keightley et al. 1996; Gattermann et al. 1997; Comi et al. 1998; Hanna et al. 1998; Polyak et al. 1998). Here, we report a stop-codon mutation in the subunit I gene of COX (COX I), resulting in premature termination of translation, in a young woman with a multisystem mitochondrial disorder.

Patient and Methods

Clinical History

The proposita, a 21-year old Italian woman, was the product of a full-term pregnancy and a normal delivery and had normal early-motor and early-cognitive development. At age 3 years, she suffered from bilateral cataract, which required surgical treatment. At age 7 years, she developed progressive sensorineural hearing loss. During the following years, the patient developed myoclonic epilepsy with electroencephalographic evidence of slow waves and isolated spikes, cerebellar ataxia, mild muscle weakness, and progressive visual loss. Serum lactic acid at age 12 years was 5.8 mM (normal value 0.1–2.2), and serum creatine kinase was 1,000 UI/liter (normal value <150). Clinical examination at age 21 years showed diffuse muscle atrophy, severe generalized muscle weakness, limb ataxia, severe visual defect (1/30 OD, 3/10 OS) with optic atrophy, and complete deafness. Electromyography showed a myopathic pattern. Nerve conduction studies showed severe sensorimotor neuropathy in the legs. Magnetic-resonance imaging of the brain revealed diffuse cerebellar atrophy and bilateral small symmetrical nodular hyperintensities in the basal ganglia (head of the caudatus and putamen) in T2-weighted images. A muscle biopsy was performed at age 21 years.

The family history is negative for neuromuscular disorders. There is no parental consanguinity, and a younger sister of the patient is asymptomatic.

Muscle Histochemistry and Biochemistry

For morphological studies, 8-μm-thick sections of frozen muscle were stained to determine the activities of COX and succinate dehydrogenase (SDH) (Tritschler et al. 1991). Mitochondrial enzyme activities on total muscle homogenate were determined as described elsewhere (DiMauro et al. 1987).

mtDNA Analysis

Total DNA was extracted from blood, muscle, myoblasts, and cybrid-cell cultures, by standard techniques. DNA extracted from muscle was used for DNA sequencing. All mitochondrial tRNA genes and all three mitochondrially encoded COX subunits were amplified by use of the set of primers previously reported for sequencing (Rieder et al. 1998). Direct sequencing of PCR products was performed by use of the same set of primers (forward and reverse) in an automatic sequencer (ABI 310 Automatic Sequencer; Perkin-Elmer). To screen for the G6930A mutation and to quantitate the proportion of mutant mtDNA, we performed PCR/RFLP analysis. A 217-bp fragment was amplified by PCR using the following set of primers: a 28-mer forward primer (nucleotide positions 6744–6771) and a 30-mer mismatch reverse primer (GGCCACCTACGGTGAAAAGAAAGATGAAGC). In the presence of the mutation, the mismatched reverse primer creates a restriction site for *Alu*I, which is absent in the wild-type cells. Conditions for the mismatch PCR amplification were as follows: initial denaturating step at 94°C for 2 min; 30 cycles each of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 10 min. The last cycle was performed in the presence of $\alpha[^{32}P]$-dATP. After digestion with *Alu*I, the fragments were separated on a 12% polyacrylamide gel and were quantified by scanning the gel in a GS-363 Molecular Imager System (BioRad, Hercules).

Cell Culture and Growth Curves

Myoblast cultures were obtained from a fresh muscle biopsy, by procedures described elsewhere (Shoubridge et al. 1996). Cybrids were generated by fusion of platelets, which were isolated from the patient's blood and contained 27% G6930A mutant mtDNA, with human osteosarcoma 143B cells lacking mtDNA (ρ^0 cells), as described elsewhere (King and Attardi 1989). In brief, platelets were isolated from plasma, by centrifugation at 2,250 *g* for 10 min, and the platelet pellet was washed three times in Dulbecco's modified essential medium (DMEM); 1×10^6 ρ^0 cells were added; cells and platelets were pelleted together, and all traces of medium were removed; 0.5 ml of a 50% solution of polyethylene glycol in DMEM was added for 40 s; and the cells were diluted in 40 ml of DMEM supplemented with 10% FBS, 50 μg of uridine/ml, and 100 μg of bromodeoxyuridine/ml. After 24 h, the cells were grown in uridine-lacking medium, to select those which had been repopulated with platelet mtDNA. The surviving colonies were trypsinized in a cloning ring when they reached ~1 mm in diameter and were expanded in a 100-mm plate. DNA was extracted from each clone, and the mtDNA was analyzed as described above.

Growth curves of cybrid lines were determined by seeding, in triplicate, 30-mm plastic petri dishes with either 2×10^4 cells in 3 ml of DMEM supplemented with 5% dialyzed FBS, 2 mmol of L-glutamine/liter, 4.5 mg of glucose/ml, and 110 mg sodium pyruvate/liter or the same medium without glucose but supplemented with 5 mM galactose. Cells from individual plates were trypsinized and were counted at 24-h intervals for 6 d.

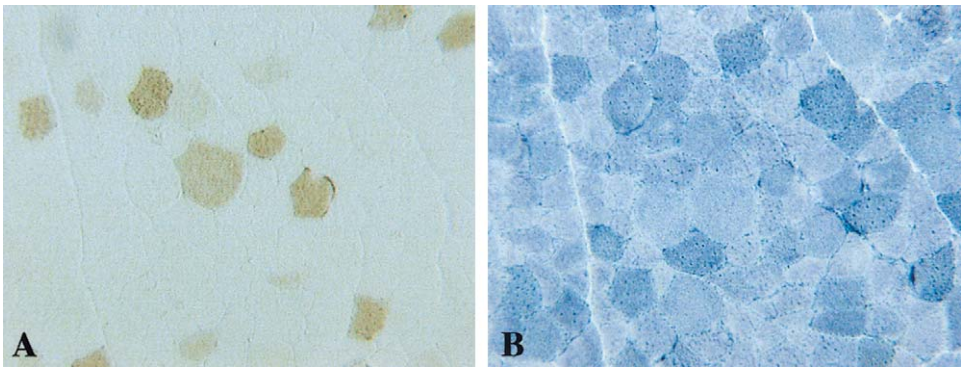

Figure 1 Histochemical reactions, on serial muscle sections, for COX (*A*) and SDH (*B*). COX activity was markedly reduced or absent in the majority of fibers. SDH activity was normal.

Biochemistry and Immunocytochemistry of Cybrid Cell Lines

COX and citrate synthase activities were measured on mitochondria isolated from cybrid cells, as described elsewhere (DiMauro et al. 1987). Measurement of oxygen consumption by intact cells was performed as described elsewhere (King et al. 1992).

For immunocytochemical analyses, cells grown on glass coverslips were immunostained with antibodies against mtDNA-encoded COX I, COX II, and COX III and against nuclear-encoded COX IV, COX V, and COX VIc, as described elsewhere (Moraes et al. 1989). Monoclonal antibodies against COX I, COX III, COX IV, and COX V (kind gift of Dr. R. A. Capaldi) and against COX VIc (Molecular Probes) were used at a dilution of 5 μg/ml. The polyclonal antibody to COX II was a kind gift from Dr. A. Lombes. The cells were photographed by a Zeiss II photomicroscope equipped with epi-illumination.

RNA Analyses

Total RNA was isolated from exponentially growing cells by use of a total-RNA isolation kit (Ambion) and was electrophoresed through 1% agarose-formaldehyde gel, for mRNA analysis, was transferred to Zeta-Probe membranes (BioRad, Hercules), and was hybridized according to the manufacturer's suggested protocol. Probes for mRNAs were PCR fragments corresponding to portions of selected mtDNA genes (COX I and ND2), labeled by random priming as suggested by the manufacturer (Roche Molecular Biochemicals). A DNA fragment corresponding to the β-actin gene was used as an internal control for the quantitation.

Analyses of Mitochondrial Translation Products

Exponentially growing transmitochondrial lines plus 143B parental cells, as well as 143Bρ^0 cells, were labeled with [^{35}S]methionine (>1,000 Ci/mmol, 100–200 μCi/ml; Dupont NEN) for 30 min in 4 ml of methionine- and cysteine-free DMEM supplemented with 100 μg of emetine/ml and 5% dialyzed FBS, as described elsewhere (Chomyn et al. 1991). The translation products were analyzed by loading equal amounts of total protein (Lowry et al. 1951) from each cell line onto a 10% tricine SDS-PAGE gel and subjecting it to fluorography on Kodak XAR film (Schägger and Von Jagow 1987). Quantitation of mtDNA-encoded polypeptides was performed with a GS-363 Molecular Imager System (BioRad, Hercules).

Immunoblot Analysis

lmmunoblot analysis of equal amounts of total cell protein (Lowry et al. 1951) from cybrid cells and from ρ^0 cells was performed as described elsewhere (Marusich et al. 1997), by use of rabbit polyclonal antibodies directed against COX II.

Results

Muscle Histochemistry and Biochemistry

Histochemical analysis of the patient's muscle-biopsy sample revealed a severe reduction of COX activity. Only 10% of the muscle fibers showed normal COX staining (fig. 1*A*). SDH was normal, and no ragged red fibers (RRF) were identified (fig. 1*B*). Biochemical analysis of muscle homogenate revealed a 90% decrease of COX activity, compared with that in normal controls (0.22 μmol/min/g tissue; normal value 2.80 $\pm$ 0.52), whereas all other respiratory chain–complex activities were normal.

mtDNA Analysis

Sequencing of all 22 mitochondrial tRNA genes and of the genes for subunit I–III of COX showed three nucleotide substitutions, compared with the reference sequence (Anderson et al. 1981): a C7476T transition in

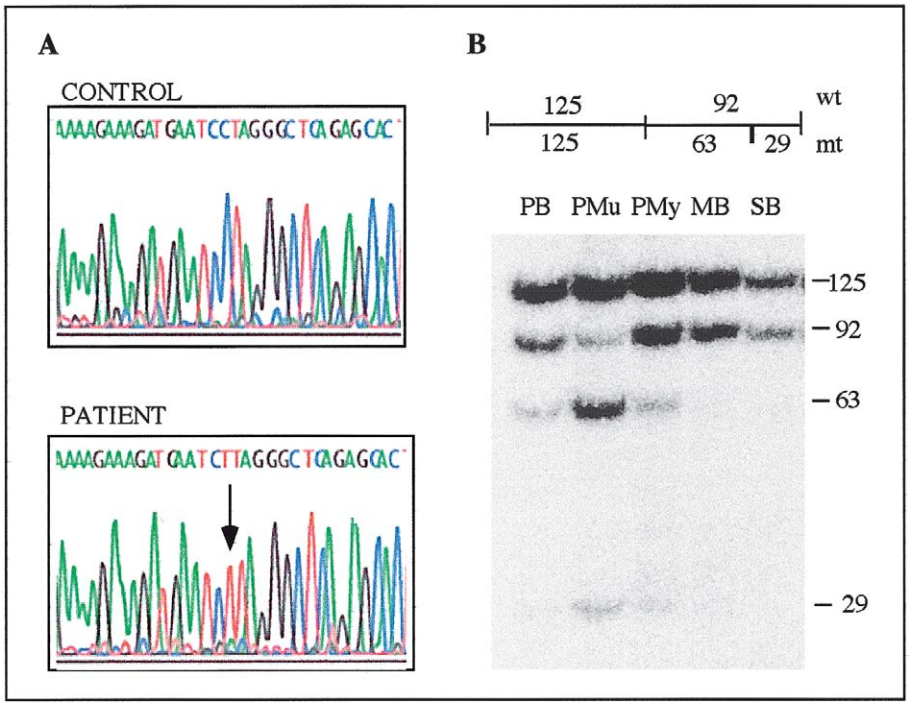

Figure 2 *A*, Electropherogram of the COX I gene, showing the sequence of muscle DNA from a control and from the patient. Sequencing was performed by means of a reverse primer; the G→A transition at nucleotide position 6930 (C→T transition in the reverse sequence) is indicated by the arrow. *B*, PCR/RFLP analysis using *Alu*I digestion to detect the G6930A mutation. *Top*, Map showing 228-bp region of the mtDNA amplified by PCR. Digestion with *Alu*I produces fragments having the sizes (in bp) indicated. *Bottom*, Autoradiogram of the *Alu*I-digested PCR products throughout a 12% nondenaturing polyacrylamide gel. Molecular sizes are indicated to the right of the gel. PB = patient's blood; PMu = patient's muscle; PMy = patient's myoblasts; MB = mother's blood; SB = sister's blood.

$tRNA^{Ser(UCN)}$, a G9559C transversion in the gene for subunit III of COX, and a G6930A transition in the gene for subunit I of COX (fig. 2*A*). The first two changes had been described, in previous reports (Marzuki et al. 1991; Houshmand et al. 1994), as neutral polymorphisms. The G→A transition at nucleotide position 6930, which changes a glycine (GGA) to a stop codon (AGA), had not been described elsewhere. This premature stop codon is predicted to result in loss of the last 170 amino acids of the C-terminal region of COX I.

PCR-RFLP analysis showed that the mutation was heteroplasmic in the patient's blood (27%), muscle (75%), and myoblasts (33%); but it was not detectable in blood from her mother, her sister (fig. 2*B*), or her four maternal aunts (data not shown). The mutation was not present in 105 normal controls or in 18 disease controls with other pathogenic mtDNA mutations. By PCR-RFLP analysis of cybrid cells, we selected three clones harboring 65% (CA65), 35% (CA35), or 0% (CAWT) mutant mtDNA.

Growth Curves and Biochemical Analyses of Cybrid Cells

In glucose-containing medium, there was no difference in the growth rates of mutant and wild-type cells (data not shown). Growth rates of mutant cybrid cells in medium containing galactose as the sole fermentable carbon source were markedly slower than those in wild-type cells (fig. 3). The growth rate of CA35 cells, containing only 35% mutant mtDNA, was almost as slow as that of the CA65 cells (65% mutant mtDNA).

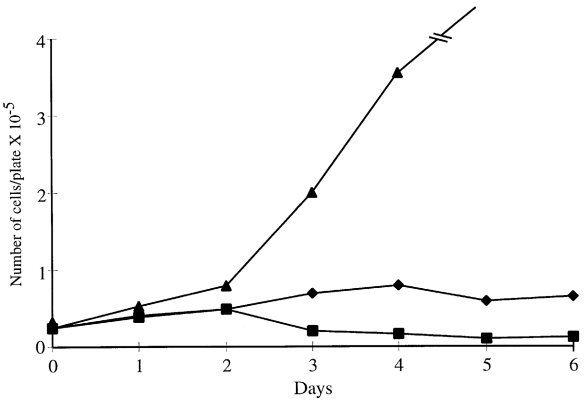

Figure 3 Growth curves of wild-type cells (*triangles*) and of CA35 (*diamonds*) and CA65 (*squares*) mutant cybrids. Cells were grown in medium containing galactose in the absence of glucose. The counts shown are the average of three independent determinations.

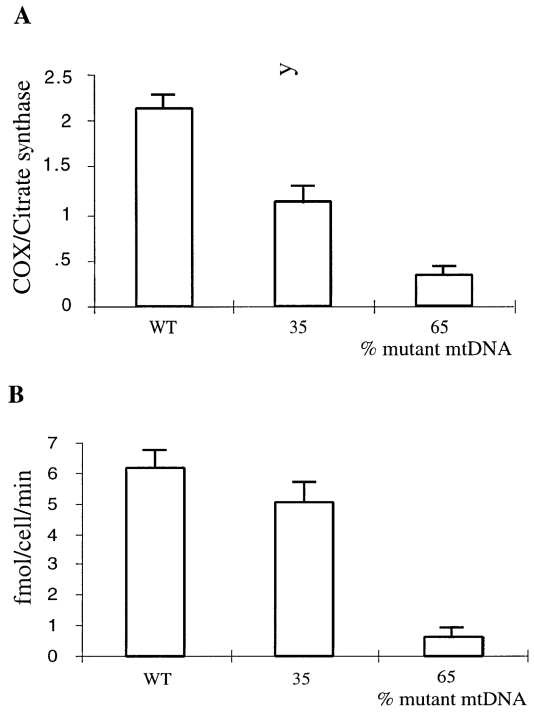

Figure 4 *A,* COX activity measured on isolated mitochondria from wild-type cells (0% mutant mtDNA) and from mutant cybrids CA35 (35%) and CA65 (65%). COX is normalized to the activity of citrate synthase. Error bars represent 2 standard errors. *B,* Oxygen consumption. Rates of oxygen consumption of the intact wild-type cells (0%) and of mutant cybrids CA35 (35%) and CA65 (65%) are shown, with error bars representing 2 standard errors.

COX activity in mitochondria isolated from cybrid cells, normalized to citrate synthase (a nuclear-encoded–matrix enzyme), revealed a decrease in activity, to ~25% in CA65 cells and to ~50% in CA35 cells, compared with that in CAWT cells (fig. 4*A*). The rate of oxygen consumption by intact cells was reduced to ~10% in CA65 cells and to ~75% in CA35 cells, compared with that in CAWT cells (fig. 4*B*).

RNA Analyses

To analyze the mtDNA-encoded mRNA, we first hybridized the northern blot with a probe for the nuclear-encoded cytoplasmic β-actin gene (to estimate the amount of total RNA loaded) and then probed it sequentially with labeled PCR fragments corresponding to COX I and ND2 genes, after stripping each previously hybridized probe. When β-actin expression was used as a normalizing factor, there was a 30% decrease in the level of COX I mRNA in CA65 cells, compared with that in CAWT cells. No difference was found in the steady-state level of COX I mRNA in CA35 cells, compared with that in CAWT cells. The ratio of ND2 transcripts to β-actin was unchanged in both mutant cell lines, compared with that in CAWT cells (fig. 5).

Analyses of Mitochondrial Translation Products

We examined steady-state mitochondrial protein synthesis in all three cybrid lines, as well as in the 143B parental and 143Bρ^0 cells, by labeling cells with [^{35}S]methionine in the presence of emetine, an inhibitor of cytoplasmic protein synthesis. After the intensity of the COX I was normalized to that of ATPase subunit 6 (A6), COX I in CA65 and CA35 cells was lower than that in wild-type or 143B parental cells (fig. 6). In CA65 cells the COX I/A6 ratio was 25% of that in wild-type cells, and in CA35 it was 50% of that in wild-type cells. As expected, no mitochondrial protein synthesis was observed in 143Bρ^0 cells. The intensities of all other mtDNA-encoded polypeptides normalized to A6 were unchanged in mutants, compared with that in wild-type cells.

Immunodetection of COX Subunits

Immunostaining of CAWT, CA65, and 143Bρ^0 cells was performed with primary antibodies directed against different COX subunits. Staining with antibodies against COX I was less intense in CA65 cells than in CAWT cells (fig. 7). A similar pattern was observed with

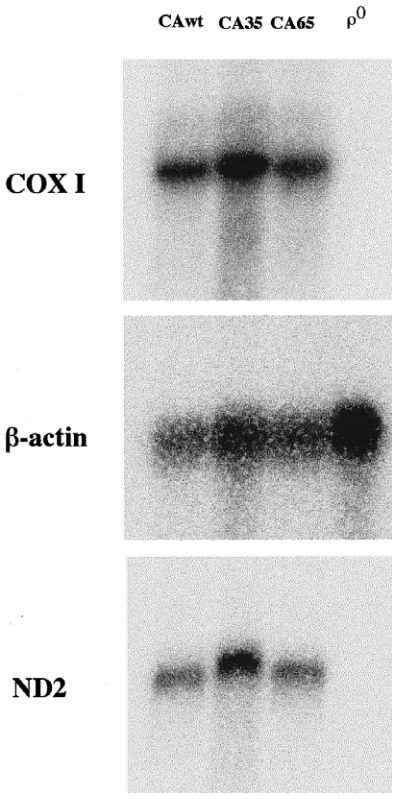

Figure 5 Northern blot–hybridization analyses. Total RNA was isolated from the 143B parental cells and from cybrid cell lines and ρ^0 cells and hybridized with probes for COX I, β-actin, and ND2, as indicated to the left of each autoradiogram.

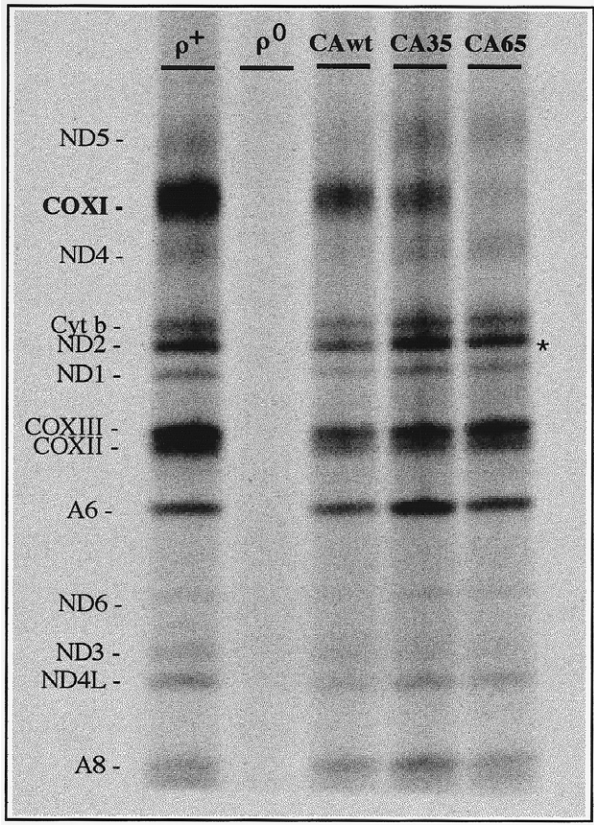

Figure 6 Fluorogram of mitochondrial translation products in cybrid cells. Mitochondrial translation products were labeled with [^{35}S]methionine for 20–30 min, in the presence of 100 μCi [^{35}S]methionine/ml and 100 μg emetine/ml, and electrophoresed through 10% gradient SDS-polyacrylamide gel. An equal amount of cellular proteins was loaded in each lane of the gel. COX I, COX II, and COX III are subunits of COX; ND1, ND2, ND3, ND4, ND4L, and ND5 are subunits of NADH dehydrogenase; A8 and A6 are subunits of ATP synthase; and cyt *b* is apocytochrome *b*. The asterisk (*) denotes the predicted size of the truncated COX I protein (38 kD).

anti–COX II antibodies. By immunoblot analysis, COX II was estimated to be reduced by ~70% (fig. 8). A lesser decrease in immunostaining was also observed with anti–COX VIc antibodies. All other antibodies used (i.e., anti–COX III, anti–COX IV, and anti–COX V) did not show any significant difference in staining. In 143Bρ^0 cells, staining for all three mtDNA-encoded subunits and for nuclear-encoded COX VIc was absent, as had been reported elsewhere (Marusich et al. 1997), whereas staining for nuclear-encoded COX IV and COX V was normal (fig. 7).

Discussion

During the past few years, increasing numbers of pathogenic mutations in mtDNA polypeptide-coding genes have been reported (Servidei 1999). In particular, mutations in COX subunits have been described in association with diverse clinical phenotypes. In 1995, we described a missense mutation in the COX III gene (Manfredi et al. 1995) in a patient with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (i.e., MELAS syndrome). Two other mutations were reported in the COX III gene: an in-frame microdeletion in a patient with exercise intolerance and myoglobinuria (Keightley et al. 1996) and a stop-codon mutation in a patient with myopathy (Hanna et al. 1998). In addition, three pathogenic mutations were reported in the COX I gene: two missense mutations in patients with acquired idiopathic sideroblastic anemia (Gattermann et al. 1997) and an out-of-frame microdeletion in one patient with motor neuron disease (Comi et al. 1998). Moreover, homoplasmic somatic mtDNA mutations in COX genes have been described in human colorectal-cancer cell lines, one of which was a stop-codon in COX I (Polyak et al. 1998).

We have now identified a novel stop-codon mutation at nt 6930 in the COX I gene in a young woman with a multisystem progressive mitochondrial disorder. We believe that the G6930A mutation is responsible for the mitochondrial disorder in our patient, for the following reasons: (1) it created a stop codon that causes premature termination of translation, with a predicted loss of 170 amino acids (approximately one-third of the total polypeptide) at the C-terminus of COX I; (2) it was consistent with the biochemical defect (i.e., isolated COX deficiency); (3) it was heteroplasmic in multiple tissues of the patient, a feature commonly associated with pathogenic mtDNA mutations; and (4) it was absent in 123 individuals (normal and disease controls) of various ethnic backgrounds, indicating that it is unlikely to occur either as a population polymorphism or in association with other pathogenic mtDNA mutations.

In the patient's muscle, which contained 75% mutant mtDNA, COX activity was virtually absent in most of the fibers. Some COX reactivity was present in a few scattered fibers, in which the mutation content probably was below the threshold for the expression of COX deficiency. We did not see any RRF in the patient's muscle. A similar apparent lack of mitochondrial proliferation has been reported in other polypeptide-coding gene mutations, such as T8993G in ATPase 6 (neuropathy, ataxia, and retinitis pigmentosa [or NARP]) (Holt et al. 1990), T9957C in COX III (MELAS) (Manfredi et al. 1995), G9952A in COX III (myopathy) (Hanna et al. 1998), and G15762A in cytochrome *b* (myopathy) (Andreu at al. 1998). However, both a patient with myopathy and an in-frame COX III microdeletion (Keightley et al. 1996) and another patient with motor neuron disease and an out-of-frame microdeletion in COX I (Comi et al. 1998) had RRF. The muscle-morphology difference among these cases is unexplained, especially considering

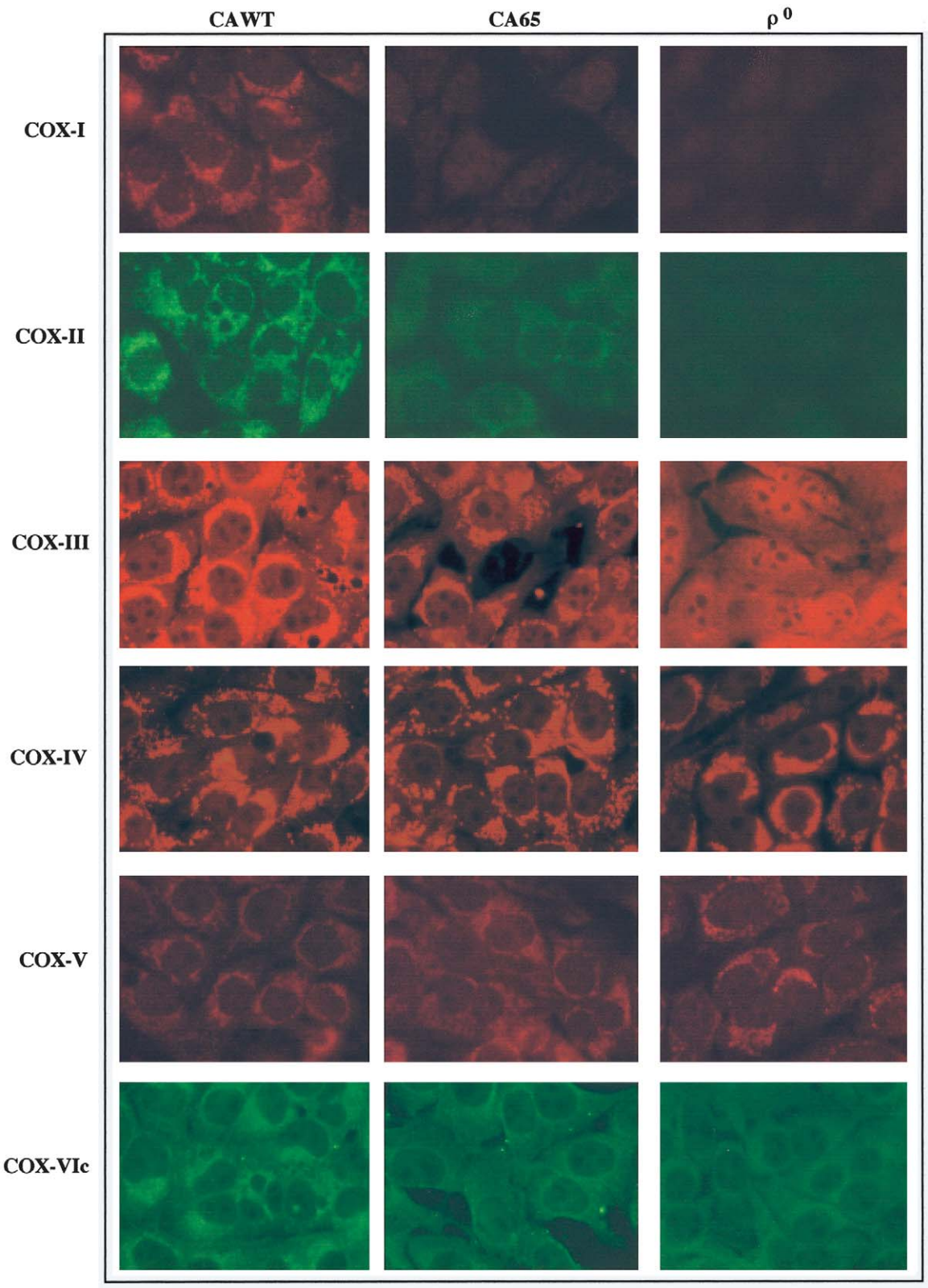

Figure 7 Immunocytochemistry of the cybrid cell lines. Wild-type (CAWT), mutant cybrid (CA65), and ρ^0 cells were grown on coverslips and were analyzed by immunocytochemistry with antibodies directed against COX I, COX II, COX III, COX IV, COX V, and COX VIc. Each set of photographs was shot under identical conditions of exposure and contrast.

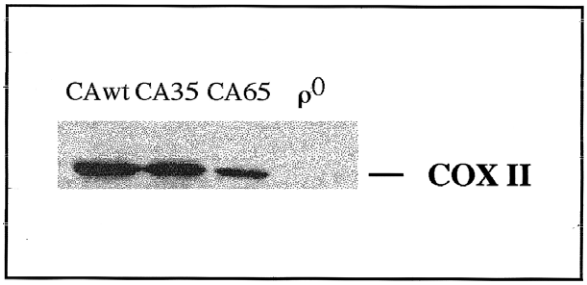

Figure 8 Immunoblot of whole-cell lysates from cybrids and ρ^0 cells, with use of a polyclonal anti–COX II antibody.

that both the patient described by Comi et al. (1998) and our patient harbored COX I mutations causing early termination of translation.

To better understand the relationship between mutation load and biochemical phenotype, we generated cybrid cell lines containing different levels of mutant and wild-type mtDNA. We obtained clones with 0% (CAWT), 35% (CA35), and 65% (CA65) mutant mtDNA. We attempted to create a cell line containing 100% mutation, by exposing heteroplasmic clones to ethidium bromide treatment (King and Attardi 1989) and, after removal of ethidium bromide, growing them in uridine-lacking medium. We obtained only clones containing ⩽65% mutation, suggesting that perhaps our method of selection did not allow for the survival of cells containing higher mutational loads. When we analyzed the biochemical features of the cybrids, we observed a decrease of COX activity, which was approximately directly proportional to the mutation load in both CA35 and CA65 mutant cells. Oxygen consumption was only mildly decreased in the CA35 cells but was much more decreased (90% reduction) in CA65 cells. Growth rates of both the CA35 and CA65 mutant cell lines in medium containing galactose as the sole fermentable carbon source were markedly slower than the growth rate of wild-type cells. Because the ATP derived from galactose metabolism is produced mainly in mitochondria, cells with defects of the respiratory chain do not grow well in galactose medium (Robinson 1996). These results indicate that even relatively low levels of mutant mtDNA (i.e., 35%–65%) cause a biochemical dysfunction that is approximately correlated with the amount of mutant mtDNA. This is in contrast to the high and sharp threshold described for mitochondrial tRNA point mutations: these affect overall mitochondrial protein synthesis, and even small percentages of wild-type mtDNA are sufficient to prevent respiratory-chain dysfunction (Schon et al. 1997). A likely explanation for this difference is that mitochondrial tRNAs are synthesized in excess, whereas the synthesis of respiratory-chain polypeptides is tightly regulated, either at the postrancriptional level or at the translational level. Alternatively, those polypeptides that are translated in excess but that are not promptly assembled are rapidly degraded. An additional interpretation is that, since COX functions as a dimer, a truncated COX I might act as a dominant negative. In this case the expected proportion of complexes that only contain wild-type COX I in CA65 cells would be 12.25% (i.e., $[0.35]^2$), consistent with the residual 10% oxygen consumption in this clone.

On the basis of x-ray crystallography studies of the structure of bovine heart complex IV (Tsukihara et al. 1996), we hypothesized that the COX I loss caused by the G6930A stop-codon mutation could affect the assembly of other subunits of complex IV. To test this hypothesis, we immunostained the CA65 cells with antibodies directed against different COX subunits. As expected, we observed a marked decrease of COX I, but, interestingly, COX II and COX VIc also were less abundant in mutant than in wild-type cells. The reduction of COX II was also confirmed by immunoblotting (fig. 8). The remaining subunits tested—COX III, COX IV, and COX V—were normal (fig. 7). The interpretation of these findings, on the basis of the available crystal structure of complex IV (Tsukihara et al. 1996), could be the following: our mutation eliminates α-helices IX–XII. This portion of COX I contacts the two helices (I and II) of COX II. Furthermore, subunit VIc is in contact solely with helix I of subunit II (Tsukihara et al. 1996). Therefore, either loss of the C-terminal third of COX I or loss of COX I in toto could result in the parallel loss COX II and COX VIc. Because we did not know the epitope recognized by the anti–COX I monoclonal antibody utilized for immunostaining, we could not exclude the presence of a truncated polypeptide. To ascertain whether the predicted truncated polypeptide was present in mitochondria, we studied mitochondrial translation products in cybrid cells. Full-length COX I was reduced, in mutant cells, proportionally to the mutation load (fig. 6). However, we did not detect any aberrant, lower-migrating band corresponding to the predicted truncated protein (38 kD). These data suggest that the truncated COX I protein, if translated at all, is immediately degraded by mitochondrial proteases. Although COX II was translated normally, interaction with COX I might be required for its correct assembly in the complex, explaining why immunostaining and immunoblotting showed a reduced abundance of COX II in mutant mitochondria. In turn, nuclear-encoded COX VIc, which is presumably synthesized normally in the cytoplasm, needs COX II for assembly, thus explaining why it too was reduced in mutant mitochondria.

A similar pattern of COX-subunit involvement has been described in a patient with a COX I out-of-frame

deletion (Comi et al. 1998). Also in that case, there was a reduction of the full-length COX I, the predicted truncated polypeptide was not detectable, and COX II was markedly decreased. Those findings and ours confirm the importance of COX I, not just as a catalytic subunit but also as a transmembrane "scaffold" for complex IV assembly (Taanman 1997).

Two additional missense mutations in COX I have been identified in patients with acquired idiopathic sideroblastic anemia (Gattermann et al. 1997), and both have been studied in cybrid systems (Broker et al. 1998). In those cases, however, cybrid cells containing homoplasmic mutant mtDNA displayed only subtle decreases in COX activity and oxygen consumption. The authors of those reports pointed out that those mutations did not impair energy metabolism in cultured cells. Polyak et al. (1998) have reported a homoplasmic somatic stop-codon mutation in COX I in human colorectal-cancer cells. However, it is difficult to define the role of this mutation, since it was studied only in homoplasmic tumor cells. Therefore, our study represents the first cybrid analysis of a clearly pathogenic mutation in a COX gene.

Although we could analyze only muscle and blood from our patient, the multisystemic nature of her disease suggests that the G6930A mutation is also present in other tissues, including both the CNS and the peripheral nervous system. The mutation appeared to be sporadic in our patient, since it was not found in blood from any maternal relative examined. Therefore, it is likely that the mutation arose as a sporadic event, during either oogenesis or the early phases of embryogenesis, as suggested by the fact that it was present in multiple tissues with different embryological derivation.

In conclusion, we have identified a novel pathogenic stop-codon mtDNA mutation in the COX I gene in a patient with a multisystem mitochondrial disorder characterized clinically by myopathy, encephalopathy, blindness, hearing loss, and peripheral neuropathy. We have shown that the mutation impairs both translation of the COX I polypeptide and assembly of complex IV, thus resulting in severe COX deficiency.

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