

## BRIEF REPORT

## Mutant Mitochondrial Elongation Factor G1 and Combined Oxidative Phosphorylation Deficiency

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## SUMMARY

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Although most components of the mitochondrial translation apparatus are encoded by nuclear genes, all known molecular defects associated with impaired mitochondrial translation are due to mutations in mitochondrial DNA. We investigated two siblings with a severe defect in mitochondrial translation, reduced levels of oxidative phosphorylation complexes containing mitochondrial DNA (mtDNA)-encoded subunits, and progressive hepatooencephalopathy. We mapped the defective gene to a region on chromosome 3q containing elongation factor G1 (EFG1), which encodes a mitochondrial translation factor. Sequencing of *EFG1* revealed a mutation affecting a conserved residue of the guanosine triphosphate (GTP)-binding domain. These results define a new class of gene defects underlying disorders of oxidative phosphorylation.

**O**XIDATIVE PHOSPHORYLATION DRIVES THE SYNTHESIS OF ATP AND takes place through a series of reactions mediated by complexes I, II, III, IV, and V, which are integral to the inner mitochondrial membrane. Each complex is made up of several or many proteins.<sup>1,2</sup> For example, complex I is made up of 7 proteins encoded by mitochondrial genes and at least 39 proteins encoded by nuclear genes. Some diseases result from a deficit in a single complex. For example, Leigh's syndrome is caused by a single mutation in one of several mitochondrial and nuclear genes encoding proteins that make up, assemble, or maintain complex I, II, IV, or V. The cause of diseases associated with a deficit in more than one complex and of nuclear origin has been unclear, and the prevalence of such mitochondrial disorders is similar to that of the aggregate of disorders caused by the deficiency of a single complex. In this report, we describe an association between the mutation of a nuclear gene encoding a protein that mediates translation of mitochondrial DNA (mtDNA) and the occurrence of early fatal hepatooencephalopathy in two siblings, each with a deficit in more than one complex of the respiratory chain.

## CASE REPORTS

The index patient, a female, was born of consanguineous Lebanese parents (first cousins) by cesarean section with intrauterine growth retardation and mild microcephaly. She was very stiff and had few spontaneous movements. From day 10 of life, profound metabolic acidosis was evident (blood lactate level, 17.1 mmol per liter [normal, <2.1 mmol per liter]; lactate:pyruvate ratio, 38 [normal, 12 to 18]). Liver dysfunction, as measured by an elevation of direct bilirubin, was observed from day 7 and progressed to full liver failure on day 12 and death on day 27. Postmortem examination of the liver showed cholestasis and extensive necrosis. There was hypoplasia of the corpus callosum, and several symmetrical cystic lesions were present in the white matter in the area of the basal ganglia. The heart was normal, and a histologic examination of the skeletal muscle showed normal mitochondrial morphology and no ragged-red fibers. The activities of the oxidative phosphorylation complexes in fibroblasts were 40 percent, 69 percent, and 18 percent of the lowest control value for complex I, complex III, and complex IV, respectively. In muscle, complex I activity was 52 percent and complex IV activity was 54 percent of the lowest control value.

A male sibling of the index patient was born at 41 weeks of gestation. The maximal blood lactate level was 9.3 mmol per liter, with a lactate:pyruvate ratio of 84. An ultrasonographic examination of the brain showed generalized atrophy and a small corpus callosum. The child had extremely delayed growth and development, as well as increased muscle tone in his upper extremities. Signs of liver failure were present at week 7, leading to death at five months of age. The heart was normal. The activities of complex I and complex IV in fibroblasts were 13 percent and 31 percent of the lowest control value, respectively. Analysis of mtDNA by Southern blotting showed no rearrangements or reduction in mtDNA levels.

## METHODS

**CONTROLS**

Samples from 100 ethnically matched controls were analyzed for restriction-fragment-length polymorphisms and showed a pattern that was homozygous for the wild-type allele.

**CELL CULTURE**

To extend the life span of cells, we immortalized primary human skin fibroblasts with a retrovirus containing the *E6* and *E7* genes of human papillomavirus type 16, or with *E7* alone and a retroviral vector expressing the protein component of human telomerase reverse transcriptase.<sup>3</sup> Skin fibroblasts from the siblings and controls were cultured in Dulbecco's modified Eagle medium supplemented with 10 percent fetal-calf serum, penicillin, and streptomycin. Samples were collected after informed consent was given.

**ENZYME MEASUREMENTS**

Complex I, complex III, complex IV, and citrate synthase activities were measured in fibroblast-cell extracts as described previously.<sup>4,5</sup> The rho<sup>0</sup> test was performed, whereby 143B.TK<sup>-</sup> rho<sup>0</sup> cells (cells without mtDNA that were derived from 143B.TK<sup>-</sup> cells) were fused with enucleated skin fibroblasts from the index patient and then selected in Dulbecco's modified Eagle medium containing 10 percent fetal-calf serum without pyruvate or uridine. Colonies were harvested for immunoblot analysis.

**CHROMOSOME TRANSFER**

The E6-and-E7-immortalized fibroblasts from the siblings were fused with human chromosomes isolated by microcell-mediated chromosome transfer<sup>4,6</sup> from a panel of mouse cell lines containing one extra human chromosome with a hygromycin-resistant tag.<sup>7</sup>

**MICROSATELLITE MAPPING**

The short tandem-repeat microsatellite markers used for homozygosity and deletion mapping of chromosome 3 after chromosome transfer were derived from the Applied Biosystems ABI PRISM Linkage Mapping Set (version 2.5 MD-10). The intermarker distance was about 10 cM.

**CDNA SEQUENCING**

RNA was isolated from skin fibroblasts from the siblings and controls with the use of RNazol. Primers were designed for the amplification of complementary DNA (cDNA) from *MRPS22* and *EFG1*. The polymerase-chain-reaction (PCR) fragments were used for direct sequencing with the ABI PRISM 377 DNA Sequencer (Applied Biosystems).

**ANALYSIS OF RESTRICTION-FRAGMENT-LENGTH POLYMORPHISMS**

Genomic DNA was isolated from skin fibroblasts from the index patient and from blood of the parents, the sibling, and 100 controls. The DNA was amplified and digested with the restriction enzyme *Mse*I.

**BLUE-NATIVE POLYACRYLAMIDE-GEL ELECTROPHORESIS AND IMMUNOBLOTTING**

Blue-native polyacrylamide-gel electrophoresis<sup>8</sup> was used for separation of oxidative phosphorylation complexes on 5 percent to 15 percent polyacrylamide-gradient gels. Mitoplasts, which were prepared from fibroblasts by treatment with 0.8 mg of digitonin per milligram of protein, were solubilized with 1 percent lauryl maltoside, and 10 to 20  $\mu$ g of the solubilized protein was used for electrophoresis. Complexes I through V were detected by immunoblot analysis with the use of monoclonal antibodies against complex III core protein 1, the  $\alpha$  subunit of complex V, the 70-kD subunit of complex II (Molecular Probes), monoclonal antibodies against complex IV subunit IV, and a polyclonal anti-ND1 antibody (an antibody acting against the mitochondrial-encoded ND1 subunit of complex I). For immunoblotting, fibroblasts were solubilized with 1.5 percent lauryl maltoside in phosphate-buffered saline, and 30  $\mu$ g of protein was separated by TRIS-Tricine sodium dodecyl sulfate-polyacrylamide-gel electrophoresis. The proteins were transferred to nitrocellulose, and antibodies directed against complex IV subunits II and IV and complex III core protein 2 were used for detection.

**PULSE-LABELING OF MITOCHONDRIAL TRANSLATION PRODUCTS**

In vitro labeling of mitochondrial translation was performed as previously described.<sup>9</sup> Briefly, cells were labeled for 60 minutes at 37°C in methionine-free Dulbecco's modified Eagle medium containing 200  $\mu$ Ci per milliliter of [<sup>35</sup>S]methionine and 100  $\mu$ g per milliliter of emetine followed by 10 minutes in regular Dulbecco's modified Eagle medium. Total cellular protein (50  $\mu$ g) was resuspended in loading buffer containing 93 mM TRIS-hydrogen chloride (pH 6.7), 7.5 percent glycerol, 3.5 percent sodium dodecyl sulfate, 0.25 mg bromophenol blue per milliliter, and 3 percent mercaptoethanol, was sonicated for 3 to 8 seconds, and was loaded and run on 12 percent to 20 percent polyacrylamide-gradient gels.

**cDNA CONSTRUCTS**

Retroviral vectors containing the cDNA sequence of three mitochondrial translation factors (*EFG1*, *EFG2*, and *IF3*) were created with the Gateway cloning system (Invitrogen). We used the reverse-transcriptase PCR (OneStep RT-PCR kit, Qiagen) to amplify cDNA from *EFG2* and *IF3* genes with the use of specific primers modified for cloning into Gateway vectors. The cDNA from *EFG1* was amplified from IMAGE (Integrated Molecular Analysis of Genomes and Their Expression) clone 5574223 with use of specific primers modified for cloning into Gateway vectors. The PCR constructs were cloned into a Gateway-modified retroviral-expression vector, pLXSH. The fidelity of the cDNA clones was confirmed by automated DNA sequencing.

**INFECTION WITH VIRUSES**

Virus-producing cell lines were generated according to procedures described previously.<sup>10</sup> Briefly, a human 293-derived retroviral packaging cell line (293GPG) was transfected with a retroviral construct containing one of the mitochondrial translation factors. Tetracycline was removed from the medium two days after transfection to induce virus production. Fibroblasts from the index patient and the controls were infected 72 to 96 hours later by exposure to virus-containing medium in the presence of 4  $\mu$ g per milliliter of polybrene as previously described.<sup>3</sup>

**GENBANK ACCESSION NUMBERS**

The GenBank accession numbers for *EFG1*, *EFG2*, *IF3*, and *MRPS22* are NM\_024996, NM\_032380, AF410851, and NM\_020191, respectively.

**RESULTS**

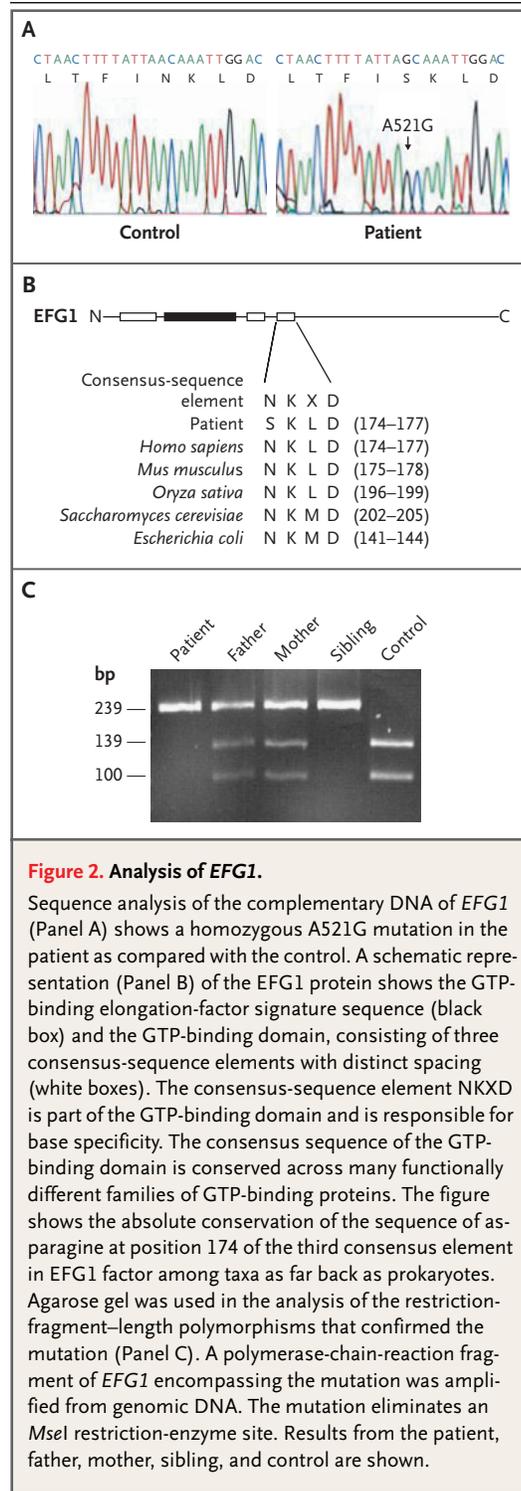
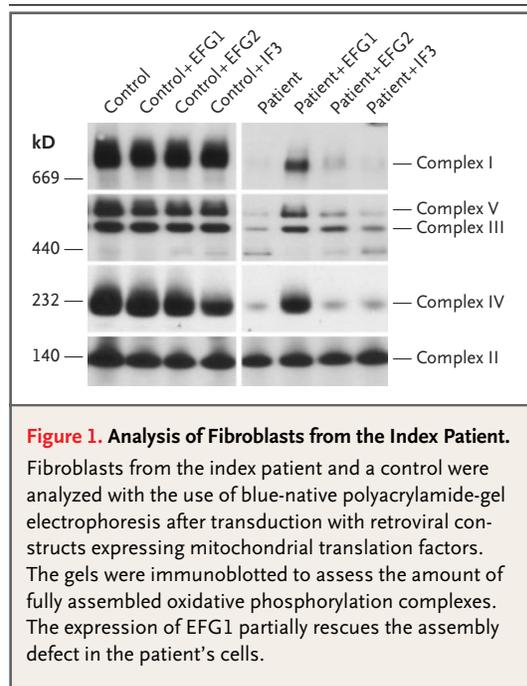
Fibroblasts from the index patient showed low levels of fully assembled complexes I, III, IV, and V but normal levels of complex II (Fig. 1). To determine whether this assembly defect was of nuclear or mitochondrial origin, we prepared trans-mitochondrial cytoplasmic hybrid (cybrid) cells by fusing enucleated fibroblasts from the index patient with rho<sup>0</sup> cells, which are devoid of mtDNA but contain a normal nuclear genome. We observed normal steady-state levels of complex IV subunit II, as well as complex III core protein 2 and complex IV subunit IV in the cybrid cells (data not shown), confirming that the oxidative phosphorylation deficiency was recessive and of nuclear origin.

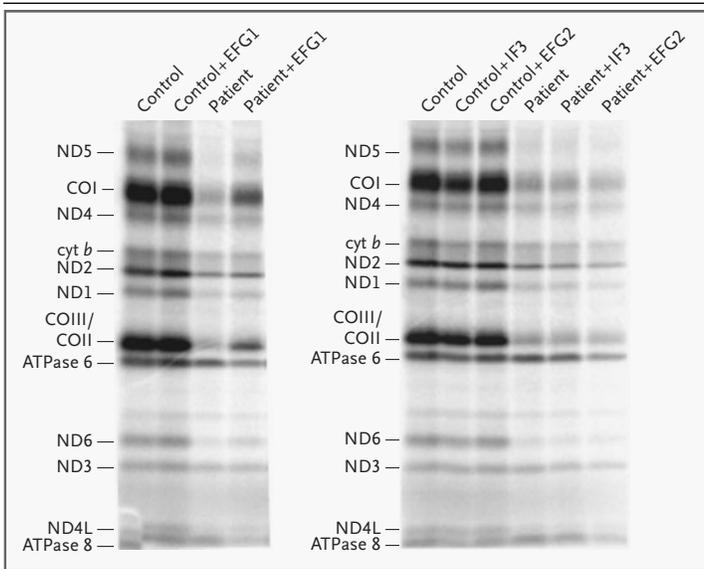
To map the defective gene, we used microcell-mediated chromosome transfer to identify a normal human chromosome that could functionally complement the biochemical defect in the fibroblasts from the index patient.<sup>4,6</sup> We transferred all human autosomes and the X chromosome, one at a time, into an immortalized fibroblast line from the patient and used immunoblot analysis of complex IV subunit II as an assay for complementation. The protein levels of complex IV subunit II were restored only after the transfer of chromosome 3 (in 18 of 19 clones; data not shown).

Chromosomes incorporated into the genome of recipient cells by microcell-mediated chromosome transfer often undergo rearrangements and deletions. Deletion mapping of the donor chromosome in isolated clones can thus be used for fine mapping of the genomic region containing the complementing gene. Microsatellite mapping with a panel of polymorphic markers for chromosome 3 showed that one of the complementing clones contained only marker D3S1279, and that the fibroblast line from the patient was homozygous for markers D3S1279 and D3S1285. These data suggested that the candidate gene maps to the region located about 20 cM from marker D3S1279 at 3q22–26.2 and flanked by markers D3S1569 and D3S1614.

A database search for candidate genes in this region identified two genes, *EFG1* and *MRPS22*, that

code for proteins that are part of the mitochondrial translation machinery.<sup>11,12</sup> Sequence analysis of cDNA showed a homozygous mutation (A521G) in the *EFG1* cDNA isolated from the patient's immor-





**Figure 3. Analysis of Mitochondrial Translation Products.**

Fibroblasts from the index patient that were overexpressing the mitochondrial translation factors EFG1, EFG2, and IF3 were pulse-labeled with [<sup>35</sup>S]methionine in the presence of an inhibitor of cytoplasmic protein synthesis (emetine) to assess the rate of mitochondrial translation. The patient's fibroblasts showed a severe defect in mitochondrial translation that overexpression of EFG1, as shown by Northern blotting, partially rectified (to about 40 percent of the expression observed in the controls) but that overexpression of the other translation factors did not affect. The proteins include seven subunits of complex I (ND), three subunits of complex IV (CO), the cytochrome *b* subunit of complex III (*cyt b*), and two subunits of adenosine triphosphate (ATP) synthase (ATPase 6 and ATPase 8).

talized fibroblasts (Fig. 2A), which predicts an amino acid substitution of serine for asparagine (N174S) at a position that has been absolutely conserved during evolution (Fig. 2B). The presence of the mutation was confirmed in genomic DNA from the index patient and the sibling by analysis of restriction-fragment-length polymorphisms (Fig. 2C). The same analysis showed that both parents were heterozygous for the mutation. The mutation could not be detected in genomic DNA of 100 controls of Lebanese origin who were found to be homozygous for the wild-type allele by analysis of restriction-fragment-length polymorphisms. The index patient's cDNA sequence of *MRPS22* was normal.

Pulse labeling of the mitochondrial translation products with [<sup>35</sup>S]methionine showed a decrease in overall mitochondrial translation to about 20 percent of the overall translation in the controls. The deficit in translation varied from protein to protein. For instance, the expression of complex IV

subunits I, II, and III was reduced to about 15 percent of the expression of the complex IV subunits in the controls, and expression of ND5 and ND6 to about 10 percent of control equivalents, whereas the other ND subunits were less affected (30 to 60 percent of control equivalents). Overexpression of EFG1 cDNA in fibroblasts from the index patient increased the overall mitochondrial translation to about 40 percent of the expression observed in the controls (Fig. 3); overexpression of two other mitochondrial translation factors (*EFG2* or *IF3*) had no effect on mitochondrial translation.

Rescue of mitochondrial translation in fibroblasts from the index patient was accompanied by a rescue of complex IV activity from 20 percent of the activity in the controls to 72 percent. Furthermore, we observed an increase in the amount of fully assembled complexes I, III, IV, and V (Fig. 1). Overexpression of EFG2 slightly increased the amount of fully assembled complexes I, III, and V but did not result in a substantial increase in complex IV activity.

## DISCUSSION

Synthesis of mitochondrial proteins requires a number of initiation, elongation, and termination (or release) factors, all of which are encoded by nuclear genes. The cDNA for two initiation factors (IF2, IF3),<sup>13,14</sup> four elongation factors (EFTu,<sup>15</sup> EFTs,<sup>16</sup> EFG1,<sup>17</sup> and EFG2<sup>3</sup>), and a release factor (RF1)<sup>18</sup> have been cloned and sequenced in human and other mammalian species. IF2 promotes the binding of formyl methionyl-transfer RNA (fMet-tRNA) to the small ribosomal subunit in the presence of GTP and a template,<sup>19</sup> whereas IF3 promotes the dissociation of the two ribosomal subunits, which produces free small subunits for the initiation of translation.<sup>14</sup> The mammalian elongation factor Tu participates in the formation of the ternary complex that includes EFTu, GTP, and aminoacyl-tRNA, which delivers the aminoacyl-tRNA to the acceptor site of the ribosome.<sup>20</sup> The energy required for this process is supplied by the hydrolysis of GTP, which is followed by the release of EFTu from the ribosome as an EFTu-guanosine diphosphate (GDP) complex. The exchange of GDP for GTP, which regenerates EFTu-GTP, is accomplished by EFTs.<sup>20</sup>

Although the roles of the mammalian elongation factors G1 and G2 have not been studied, elongation factor G in prokaryotes hydrolyzes GTP<sup>21</sup>

and, in so doing, catalyzes the translocation of peptidyl-tRNA from the ribosomal-acceptor site to the peptidyl site after peptide-bond formation. Concomitant movement of mRNA exposes the next codon in the acceptor site.<sup>22</sup> RF1 recognizes stop codons and promotes the release of the completed protein chain.

It is not clear why there are two mammalian homologues of the prokaryotic elongation factor G. Perhaps each catalyzes translation from a specific set of mRNA, or perhaps they are redundant, to ensure survival when one fails. Studies of yeast lend support to the latter hypothesis; deletion mutations of either *EFG1* or *EFG2* are viable, although *EFG1* mutations have a phenotype of respiratory deficiency.<sup>23</sup> Our observations suggest that *EFG1* is an essential gene and that *EFG1* and *EFG2* have different but overlapping roles in mitochondrial translation (Fig. 1). Although the expression of both proteins is ubiquitous,<sup>11,17</sup> the proteins may have tissue-specific functions, consistent with the severe effect of mutant *EFG1* in the liver and brain and the mild effect in the heart and muscles.

Many GTP-binding proteins contain three highly conserved consensus elements (Fig. 2B).<sup>24</sup> The A521G mutation effects the substitution of a highly conserved asparagine residue present in the consensus-sequence element NKXD (where X indicates any amino acid), which is part of the GTP-

binding domain and is responsible for base specificity.<sup>24</sup> A mutant variant of another GTP-binding protein, Ras p21, with replacement of the equivalent asparagine by lysine or tyrosine, is unable to bind guanine nucleotides or to transform NIH 3T3 cells.<sup>25</sup> The mutant residue in *EFG1* may also blunt GTP-binding activity, with consequent loss of efficiency of mitochondrial translation.

We report a mutation affecting a nuclear-encoded component of the mitochondrial translation system in an oxidative phosphorylation disorder. Patients with combined deficiencies of oxidative phosphorylation enzymes are at least as common as those with isolated deficiencies of single-enzyme complexes. Nuclear genes encoding the mitochondrial translation factors are good candidates as causative genes in these cases.

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