

# The molecular basis for tissue specificity of the oxidative phosphorylation deficiencies in patients with mutations in the mitochondrial translation factor EFG1

Hana Antonicka<sup>1</sup>, Florin Sasarman<sup>1</sup>, Nancy G. Kennaway<sup>3</sup> and Eric A. Shoubridge<sup>1,2,\*</sup>

<sup>1</sup>Montreal Neurological Institute and <sup>2</sup>Department of Human Genetics, McGill University, 3801 University Street, Montreal, Quebec, Canada H3A 2B4 and <sup>3</sup>Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR 97239, USA

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Defects in mitochondrial translation are associated with a remarkable, but unexplained diversity of clinical phenotypes. Here we have investigated the molecular basis for tissue specificity in patients with a fatal hepatopathy due to mutations in the mitochondrial translation elongation factor *EFG1*. Blue-native gel electrophoresis revealed unique, tissue-specific patterns in the nature and severity of the defect. Liver was the most severely affected tissue, with less than 10% residual assembly of complexes I and IV, and a 50% decrease in complex V. Skeletal muscle showed a 50% reduction in complex I, and complexes IV and V were 20% of control. In fibroblasts, complexes I and IV were 20% of control, and there was a 40–60% reduction in complexes III and V. In contrast, except for a 50% decrease in complex IV, all complexes were near normal in heart. The severity of the defect paralleled the steady-state level of the mutant EFG1 protein, which varied from 60% of control in heart to undetectable in liver. The ratio of translation elongation factors EFTu:EFTs increased from 1:6 to 1:2 in patient heart, whereas in liver it decreased from 1:1 to 1:4. Over-expression of either EFTu or EFTs in control and patient fibroblasts produced dominant negative effects, indicating that the relative abundance of these factors is an important determinant of translation efficiency. Our results demonstrate marked differences among tissues in the organization of the mitochondrial translation system and its response to dysfunction, and explain the severe hepatopathy, but normal cardiac function in *EFG1* patients.

## INTRODUCTION

The mitochondrial oxidative phosphorylation (OXPHOS) system is responsible for the majority of energy production in the cell. The respiratory chain, which comprises complexes I–IV, functions to generate the proton electrochemical gradient across the inner mitochondrial membrane used by complex V to synthesize ATP. The mitochondrial genome (mtDNA) is a small circular molecule encoding 22 tRNAs, two rRNAs and 13 proteins that code for at least one structural subunit of each OXPHOS complex except for complex II (1). The rest of the OXPHOS sub-

units (more than 70), as well as all the proteins responsible for mtDNA maintenance, mitochondrial transcription and translation and the assembly of the OXPHOS complexes are encoded by nuclear genes.

Mitochondrial diseases due to deficiencies in OXPHOS have an estimated birth incidence of 1:5000 (2). The majority of these diseases are associated with mutations in genes involved with some aspect of the biogenesis of the OXPHOS complexes, most commonly mitochondrial translation [reviewed in (3–5)]. Mitochondrial translation is carried out in the mitochondrial matrix on a dedicated protein translation

\*To whom correspondence should be addressed. Tel: +1 5143981997; Fax: +1 5143981509; Email: eric@ericpc.mni.mcgill.ca

†*EFG1* (NM\_024996), *EFG2* (NM\_032380), *EFTu* (NM\_003321), *EFTs* (NM\_005726).

apparatus that resembles the system in prokaryotes, reflecting the  $\alpha$ -proteobacterial origins of mitochondria. In addition to the tRNAs and rRNAs encoded in mtDNA, translation requires a number of initiation, elongation and termination factors. The cDNAs for two initiation factors, IF2 (6) and IF3 (7); four elongation factors, EFTu (8,9), EFTs (10), EFG1 (11) and EFG2 (12); one release factor, RF1, and one ribosomal recycling factor, RRF (13) have been cloned and sequenced in several mammalian species, including human. Mitochondrial ribosomes (mitoribosomes) are distinct from both prokaryotic and eukaryotic cytosolic ribosomes. Although their antibiotic sensitivity is generally similar to that of prokaryotic ribosomes (14), mitoribosomes are 55S particles composed of a small (28S) and a large (39S) subunit, and contain a much higher protein/RNA ratio than the bacterial 70S ribosome (15). The protein constituents of both mammalian ribosomal subunits have been identified in their entirety: the small subunit contains 29 proteins, 14 of which have homologues in *Escherichia coli* (16,17), and the large subunit has 48 distinct proteins, 28 of which have homologues in *E. coli* (18,19). A cryo-EM structure of the bovine mitochondrial ribosome reveals that the newly evolved eukaryotic proteins by and large occupy new positions, such as protein-protein bridges between the subunits, and peripheral shielding of the rRNA components (20).

Although most components of the mitochondrial translation system are nuclear-encoded, the majority of mutations associated with mitochondrial protein synthesis defects have been reported in mtDNA. These consist of point mutations in tRNAs and the 16S rRNA, as well as large-scale deletions that usually remove several tRNA genes [reviewed in (4,5)]. These gene defects are associated with a broad spectrum of clinical phenotypes that predominantly involve the nervous system, cardiac and skeletal muscle. As nearly all of these mutations are heteroplasmic, the involvement of different cells or tissues in the clinical phenotype can in part be explained by the segregation pattern of mtDNAs carrying the pathogenic mutation. However, this phenomenon cannot explain why particular mutations are usually associated with unique clinical phenotypes.

Recently, mutations in two nuclear factors of the mitochondrial translation machinery have been described: a homozygous mutation in MRPS16 (21), a protein of the small ribosomal subunit, in a patient with fatal neonatal lactic acidosis, and a homozygous mutation in *EFG1* (22), a mitochondrial translation elongation factor, in a patient with lactic acidosis and a fatal hepatopathy. These disorders are inherited as recessive Mendelian traits, and, as they involve housekeeping genes, one might have predicted a global translation defect that would affect all cells and tissues in a similar fashion. The observation that some tissues are severely affected in these cases, whereas others are relatively spared, suggests a different organization or regulation of the mitochondrial translation apparatus in different tissues.

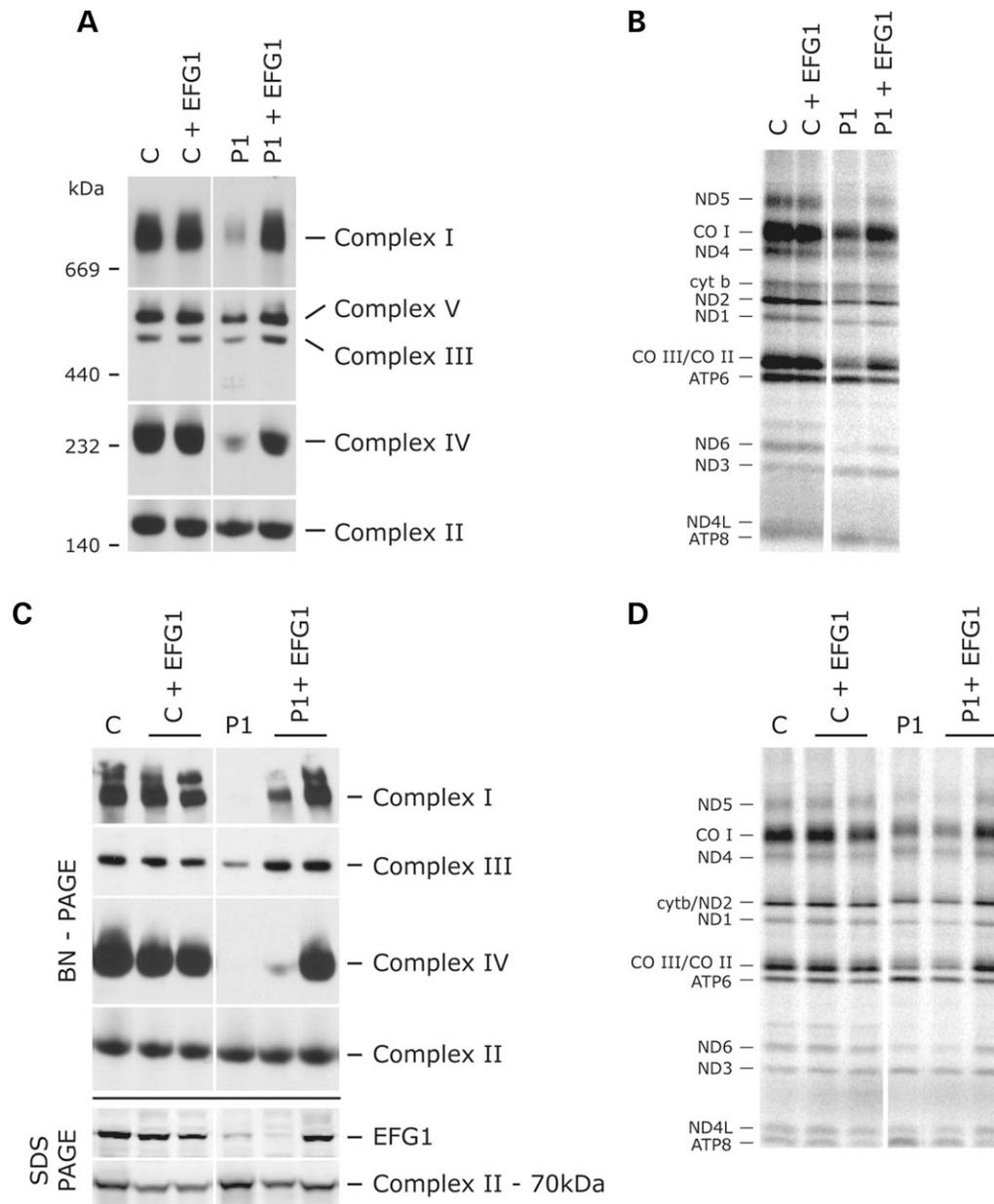
Here we have investigated the molecular basis for tissue specificity in two siblings with novel mutations in *EFG1* (MIM 606639). We show that the translation defect resulting from these mutations is associated with unique, tissue-specific patterns of OXPHOS deficiency. The severity of the defect correlates with the stability of the mutant protein in different tissues, and explains the selective tissue involvement in these patients.

## RESULTS

Biochemical analysis of fibroblasts from Patient 1 showed a mitochondrial translation defect associated with a combined deficiency of complexes I, III, IV and V (Fig. 1A and B). These enzyme deficiencies were rescued by fusion with human 143B rho<sup>0</sup> cells, demonstrating that the defect was of nuclear origin (data not shown). The pattern of the translation defect and the clinical phenotype (early onset fatal hepatopathy) were nearly identical to that reported in a patient with mutations in the mitochondrial translation elongation factor *EFG1* (22) suggesting the possible involvement of this gene. To test this hypothesis we transduced fibroblasts from both patients with a retroviral vector expressing the *EFG1* cDNA. This restored the activity of complex IV (a marker of the combined enzyme deficiency) to 50–60% of control levels, rescued the assembly of all affected OXPHOS complexes (Fig. 1A), and increased the rate of mitochondrial translation (Fig. 1B). Immunoblot analysis showed that the steady-state level of EFG1 was severely reduced in patient fibroblasts, and clonal analysis of patient fibroblasts transduced with the *EFG1* cDNA revealed that the OXPHOS phenotype could be completely rescued in clones expressing control levels of EFG1 (about half of the clones analyzed) (Fig. 1C and D). These results strongly suggested a mutation in *EFG1* as the cause of disease.

RT-PCR analysis of the *EFG1* cDNA from Patient 1 fibroblasts demonstrated the presence of two distinct products: one of the expected length, and another, shorter (data not shown). Subsequent DNA sequencing showed that the shorter cDNA was missing exon 15, and that the full-length cDNA carried a T1068C mutation in exon 7, predicting a serine to proline substitution at position 321 (Fig. 2A and C). Sequence analysis of the exon-intron boundaries of exon 15 revealed a heterozygous 2-bp deletion at the -2 position (1872-2delAG) (Fig. 2B). This results in the deletion of an exon acceptor site, skipping of exon 15, and ultimately in a premature stop codon at amino acid 607 (Fig. 2C). Restriction enzyme analysis of the T1068C mutation verified the mutation in both patients, and showed that the father was heterozygous for this mutation, whereas the mother was homozygous wild-type (data not shown). Sequencing of the exon-intron boundaries of exon 15 showed that both patients and the mother were heterozygous for the 1872-2delAG deletion, whereas the father had a wild-type sequence (data not shown).

To investigate the basis for the tissue specificity of the clinical phenotype, we first used blue-native gel electrophoresis (BN-PAGE) to examine the levels of the five OXPHOS complexes in mitochondria isolated from all available tissues and cells: skeletal muscle, heart, liver and fibroblasts from Patient 1 and controls, and fibroblasts from Patient 2. Considerable differences exist in the relative levels of the individual OXPHOS complexes in mitochondria from control tissues (Fig. 3). Although control skeletal muscle and heart mitochondria have similar amounts of all fully-assembled OXPHOS complexes, liver mitochondria contains less than 20% of complexes I, III, IV and less than 30% of complex V by comparison. The relative levels of all OXPHOS complexes in control fibroblasts are substantially lower than in any of the tissues studied. Despite these large quantitative differences,

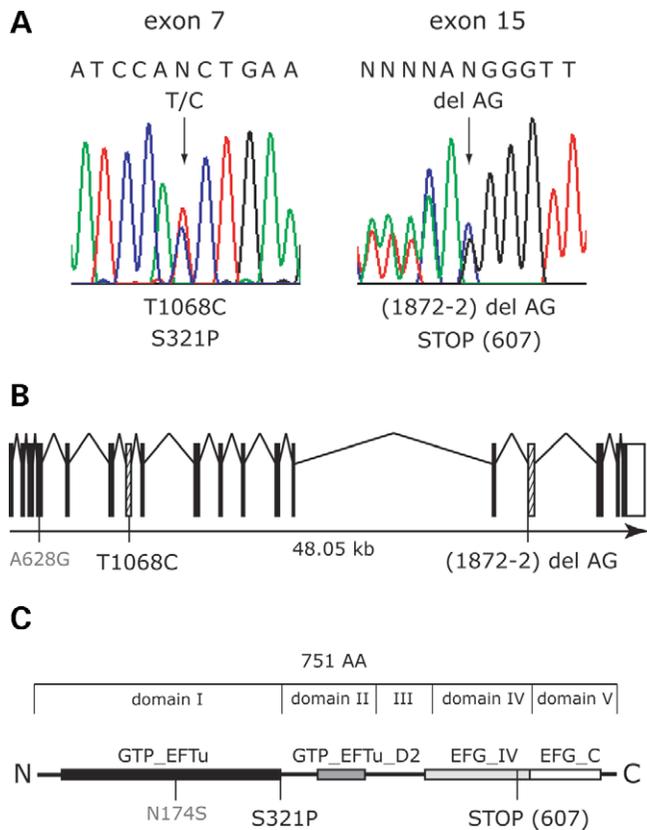


**Figure 1.** Rescue of the mitochondrial translation defect and combined OXPHOS deficiency in patient fibroblasts by expression of *EFG1*. Control (C) and Patient 1 (P1) fibroblasts were transduced with a retroviral vector expressing the wild-type *EFG1* cDNA and analyzed by BN-PAGE (A) and by a mitochondrial translation assay (B). (A) The BN-PAGE blot was incubated with antibodies against subunits of all five OXPHOS complexes. Migration of the molecular weight markers (kDa) is indicated on the left. (B) Fibroblasts were pulse-labeled with [<sup>35</sup>S]methionine and the mitochondrial proteins were separated on a 12–20% gradient gel. The seven subunits of complex I (ND), one subunit of complex III (cyt *b*), three subunits of complex IV (CO) and two subunits of complex V (ATP) are indicated on the left. (C) and (D) Clonal analysis of *EFG1*-expressing cells. Control (C) and Patient 1 (P1) fibroblasts were transduced with a retroviral vector expressing the wild-type *EFG1* cDNA and seeded at clonal density. Clones were picked and analyzed by BN-PAGE and SDS-PAGE (C) and by a mitochondrial translation assay (D). The lanes labeled C and P1 represent untransduced cells. The other lanes represent control or patient clones transduced with the retroviral expression vector as indicated. Samples were loaded in the same order in (C) and (D). The expression of *EFG1* protein was analyzed by immunoblotting with a polyclonal anti-*EFG1* antibody. The level of the 70 kDa subunit of complex II was used as a loading control.

the relative ratios of the individual complexes are generally similar in mitochondria within the cell types we investigated (Fig. 3C). The only exception to this is complex II, which is present at nearly the same level in skeletal muscle, heart and liver. These results are consistent with a study of the specific activities of the OXPHOS complexes in five different human tissues, which showed similar relative ratios in lymphocytes,

fibroblasts, skeletal muscle, heart and liver, except for a higher activity of complex II in liver (23).

BN-PAGE analysis of patient mitochondria revealed striking, tissue-specific differences in the nature and severity of the OXPHOS defect (Fig. 3). The most severely affected tissue was liver, with less than 10% residual assembly of complex I and IV, and a 50% decrease in the assembly of complex V



**Figure 2.** Mutational analysis of *EFG1*. (A) Sequence analysis of the two pathogenic mutations identified in Patient 1. The mutations shown are on the sense strand. Sequencing of exon 15 was done with the reverse primer. (B) Schematic representation of the *EFG1* gene structure consisting of 18 exons (black boxes). The positions of the two mutations identified in the patient are shown, and the affected exons are depicted by hatched boxes. (C) *EFG1* protein structure with the corresponding functional domains and the amino acid substitutions predicted by the mutations. The previously described *EFG1* mutation (A628G; N174S) (22) is shown in grey.

(Fig. 3A and D). Skeletal muscle showed a 50% reduction in complex I, and an 80% reduction in complexes IV and V. A similar 80% reduction in complexes I and IV was observed in fibroblasts, and, in addition, there was a 40–60% reduction in complexes III and V. In marked contrast to these results, except for a 50% decrease in complex IV, all complexes were near normal in heart. Consistent with these results, activity of complex IV [normalized to citrate synthase (CS)] was 29, 54, 15, 20 and 26% of control mean in skeletal muscle, heart, liver, Patient 1 and Patient 2 fibroblasts, respectively. Immunoblot analyses of the individual subunits of the OXPHOS complexes after SDS–PAGE demonstrated decreased levels of mitochondrial-encoded subunits in patient liver, skeletal muscle and fibroblasts in agreement with the very low assembly levels of the complexes I and IV as detected by BN–PAGE (data not shown). Thus, despite the fact that *EFG1* is ubiquitously expressed, the pattern of the combined OXPHOS defect was unique to every tissue and cell type examined.

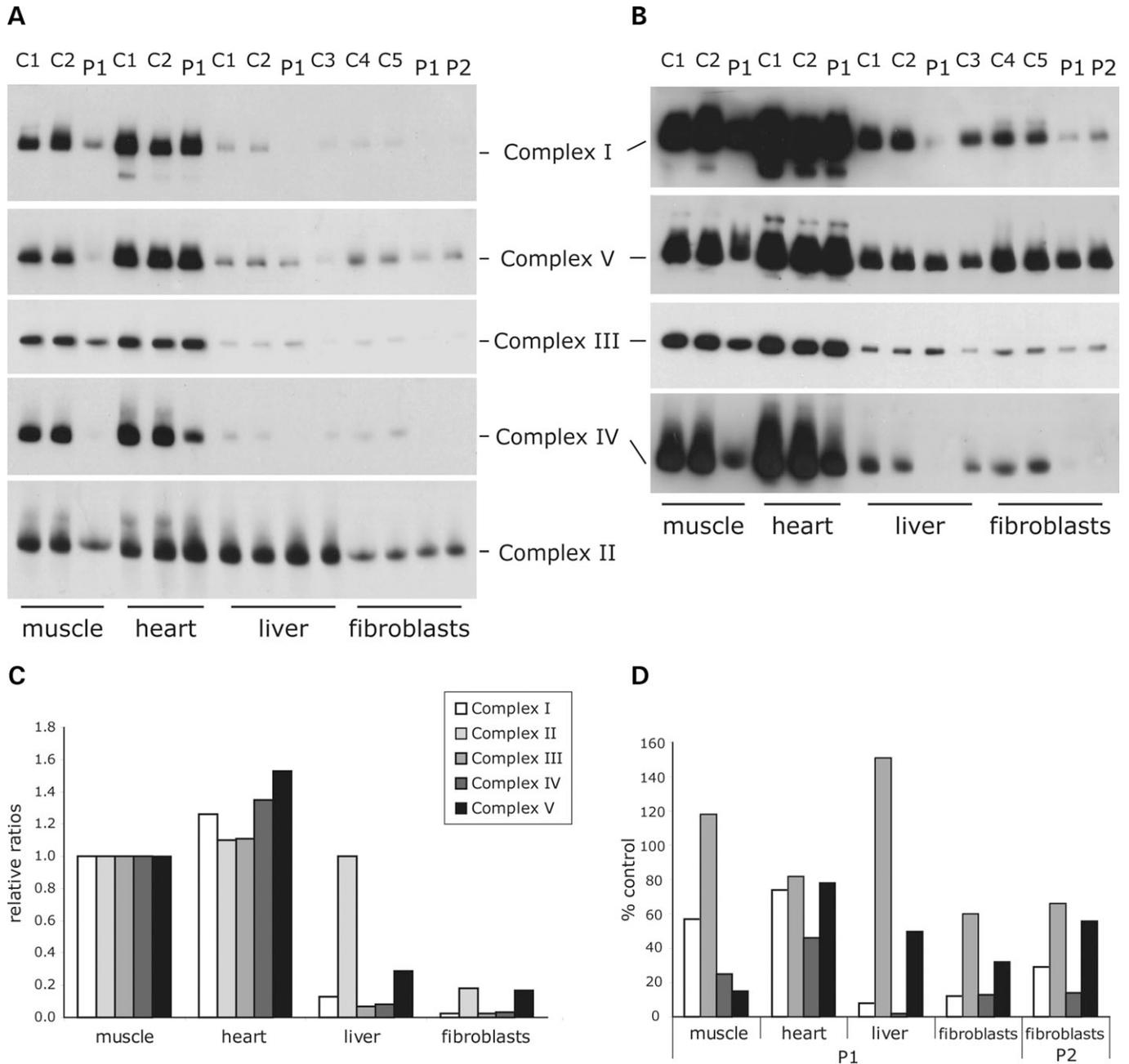
The different response of the various tissues to the presence of the *EFG1* mutation suggested that the stability of the mutant

protein might be different across tissues, or that compensatory responses might have occurred in the other mitochondrial translation elongation factors (*EFG2*, *EFTu*, *EFTs*). To investigate these possibilities we examined the relative steady-state levels of the elongation factors (for which antisera were available) by immunoblot analysis of control and patient tissues (Fig. 4A). In control heart, the levels of *EFG1* and *EFTs* were similar to those in skeletal muscle; however, the level of *EFTu* was less than 30% of that in muscle (Fig. 4B). *EFG1* content was 3-fold higher in liver mitochondria when compared with muscle, whereas the levels of both *EFTu* and *EFTs* were ~15%. In fibroblasts, the *EFG1* protein levels were four times higher than in muscle, and *EFTu* and *EFTs* were ~70%. *EFTu* and *EFTs* have been shown to form a complex and our data show that muscle, liver and fibroblasts contain the same relative ratio of approximately 1:1 between the *EFTu* and *EFTs* proteins; however, in heart this ratio is 1:6.

In mitochondria from patient tissues the residual levels of mutant *EFG1* paralleled the severity of the OXPHOS defect (Fig. 4A and C). *EFG1* protein was undetectable in liver, barely detectable in skeletal muscle and fibroblasts, but was present at ~60% of control levels in heart. Interestingly, *EFTu* levels were increased 4-fold in patient heart mitochondria, slightly decreased in patient muscle and liver, and relatively unchanged in fibroblasts. *EFTs* levels were within the range of controls in patient muscle, increased about 2-fold in liver, and slightly decreased in heart. The net result of these changes is to increase the *EFTu*:*EFTs* ratio in heart to about 1:2, similar to that observed in the other control tissues, and to decrease the ratio to 1:4 in liver.

To determine whether these changes in the relative ratios of translational elongation factors were the result of transcriptional regulation, the steady-state levels of the transcripts for all four mitochondrial elongation factors were measured by quantitative RT–PCR (Fig. 4D). In patient heart, *EFTu* mRNA was increased 3-fold over control, whereas the levels of the other transcripts remained relatively unchanged. The mRNAs for all translation elongation factors in patient liver were decreased to ~50% of control, whereas in muscle and fibroblasts there were no significant changes.

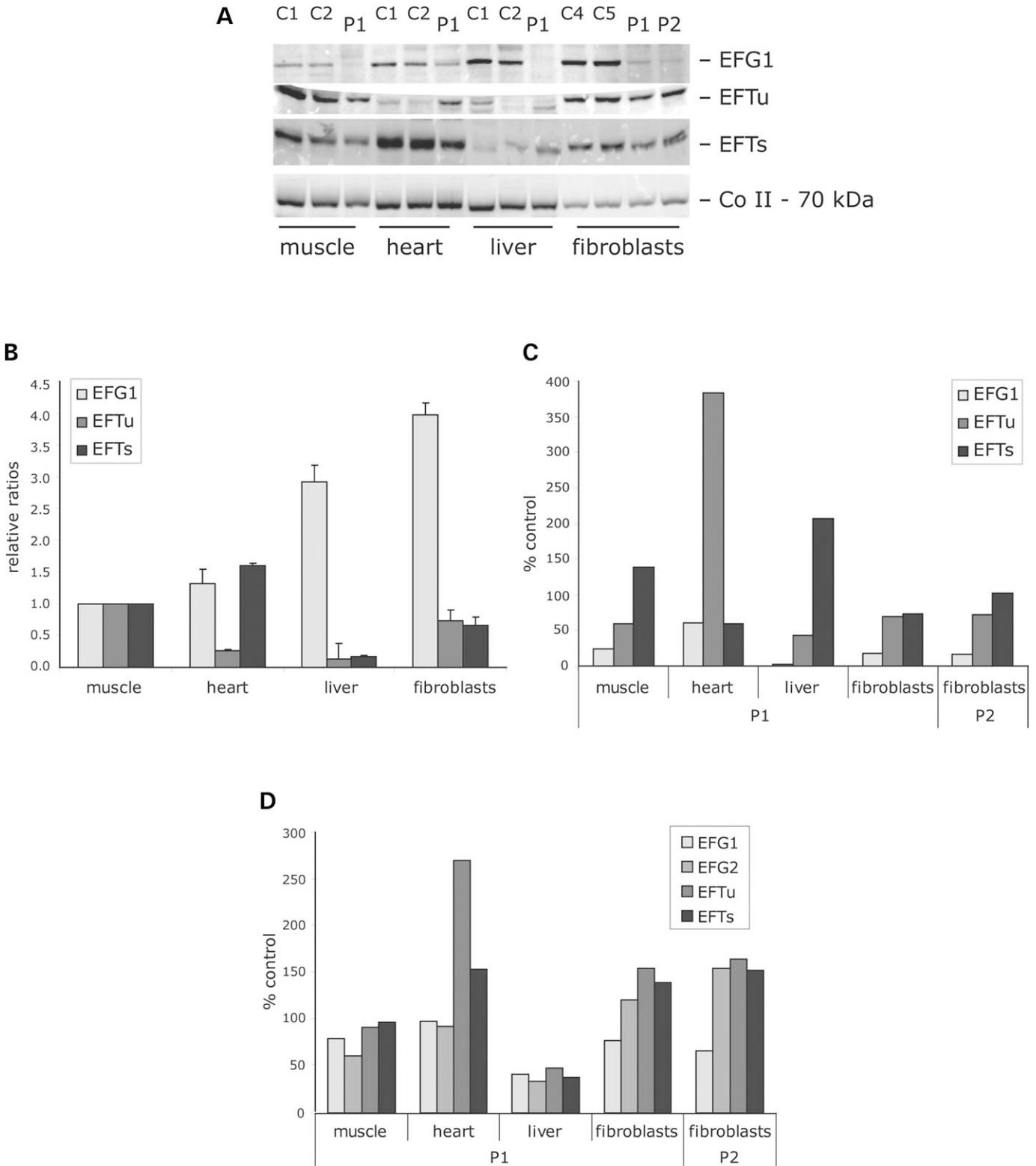
The above results suggested that altered expression of *EFTu* might be part of an adaptive response to the effects of the *EFG1* mutation in the heart. To test whether altered expression of the elongation factors could suppress the translation defect, we over-expressed the four translation elongation factors in control and patient fibroblasts. Expression of the cDNAs from a retroviral vector resulted in variable increases in the steady-state levels of the corresponding mRNAs ranging from 2–3-fold for *EFG1* and *EFG2* in patient cells to 30–50-fold for *EFTu* and *EFTs* in control cell lines (data not shown). Expression of *EFG1* increased the steady-state level of *EFG1* protein 10-fold in patient cells and 4-fold in control cells (Fig. 5C). This resulted in partial rescue of the phenotype in patient cells as expected, and a small (10%) but reproducible decrease in COX/CS activity in control cells (Fig. 5A). In addition, both *EFTu* and *EFTs* increased 2–3-fold in the patient lines. Over-expression of *EFTu*, and *EFTs*, in control fibroblasts resulted in a significant decrease in COX/CS activity (Fig. 5A) by 40 and 18%, respectively, and a similar decrease



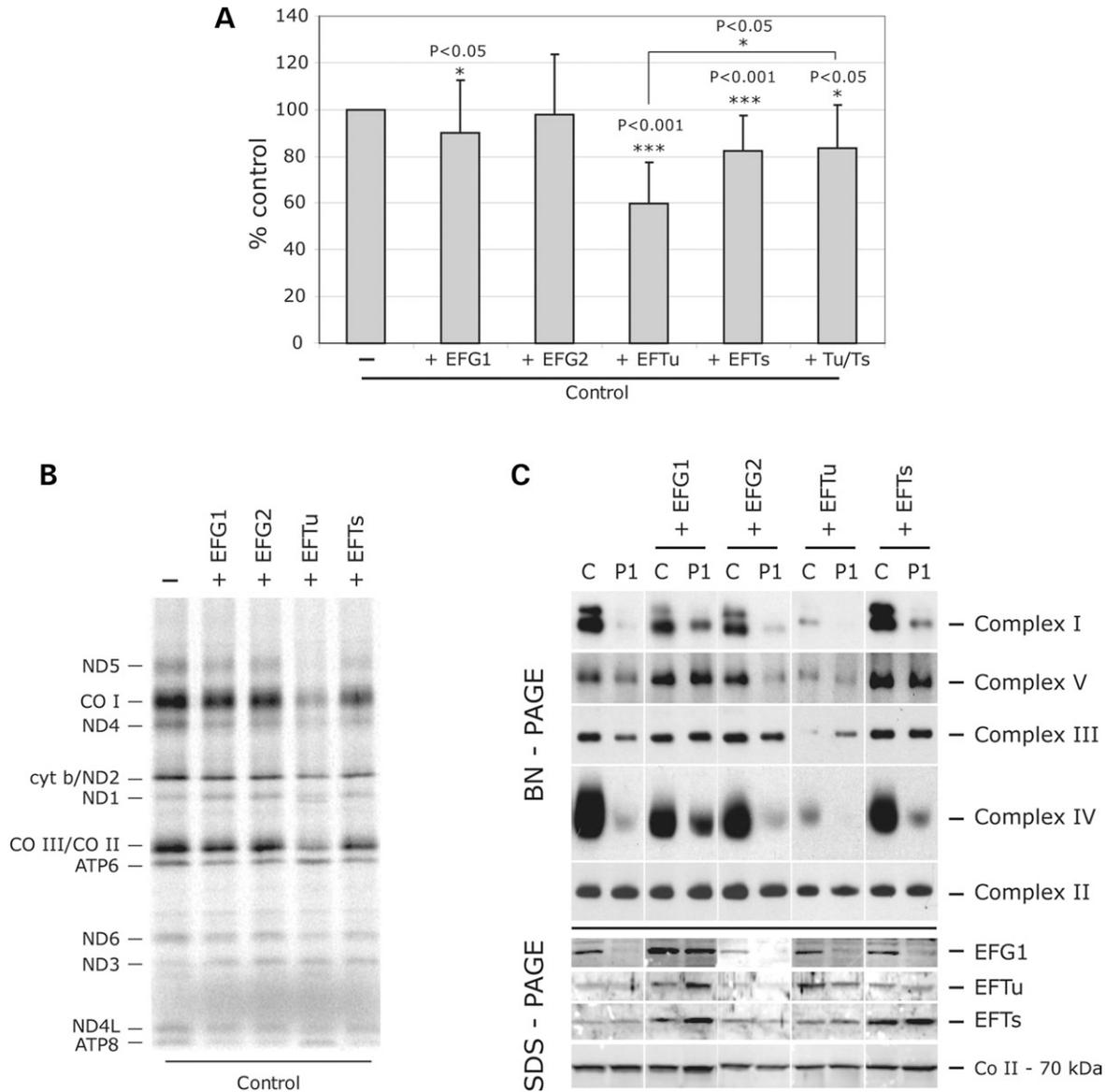
**Figure 3.** Analysis of the assembly of the OXPHOS complexes in control and patient tissues and cells by BN-PAGE. Mitochondria isolated from muscle, heart, liver and fibroblasts from controls (C) and the Patients 1 and 2 (P1 and P2) were separated on a BN-PAGE gel and blotted with antibodies that detect the native forms of the OXPHOS complexes. A long exposure (B) of the immunoblot shown in (A) accentuates complexes I, and III-V in liver and fibroblasts. (C) Relative ratios of the assembly of OXPHOS complexes in different control tissues. The relative levels of OXPHOS complexes in the different tissues shown in (A) were quantified using ImageQuant (Molecular Dynamics), arbitrarily setting the values to 1.0 in control muscle. (D) The relative levels of OXPHOS complexes in patient tissues were normalized to the levels of complex II and are expressed as percent of controls for each tissue.

in mitochondrial translation (Fig. 5B). The diminished mitochondrial translation in these cells led to a reduction of the steady-state levels of mitochondrial-encoded proteins (data not shown) and to a compromised assembly of complexes I, III, IV and V (Fig. 5C), most evident in the cells over-expressing EFTu. As EFTu and EFTs are present in several tissues in a relative ratio of 1:1, we attempted to over-express

these two factors simultaneously. Over-expression of *EFTu/EFTs* together resulted in a small but significant decrease (16%) in COX/CS activity in control fibroblasts (Fig. 5A), and a similar decrease in mitochondrial translation (data not shown). These results suggest that the efficiency of mitochondrial translation is critically dependent on the relative ratios of the translation elongation factors.



**Figure 4.** Analysis of the expression of the mitochondrial translation elongation factors by immunoblot and quantitative RT-PCR. (A) Mitochondria isolated from muscle, heart, liver and fibroblasts from controls (C) and Patients 1 and 2 (P1 and P2) were solubilized with 1.5% lauryl maltoside and separated on a 12% SDS gel. Antibodies against the elongation factors EFG1, EFTu and EFTs were used to visualize the individual proteins. Complex II-70 kDa subunit was used as a loading control. (B) The relative steady-state levels of mitochondrial translation elongation factors in control tissues were quantified using ImageQuant (Molecular Dynamics), arbitrarily setting these values to 1.0 in control muscle. (C) Steady-state levels of the mitochondrial translation elongation factors in patient tissues were normalized to complex II-70 kDa protein and expressed as percent of control. (D) Transcript levels in patient fibroblasts were determined by quantitative RT-PCR, normalized to the expression level of 18S rRNA, and expressed as percent of appropriate controls.



**Figure 5.** Over-expression of mitochondrial translation elongation factors in control and patient fibroblasts. Control (C) and Patient 1 (P1) fibroblasts were transduced with retroviruses expressing the cDNA of the translation elongation factors (*EFG1*, *EFG2*, *EFTu* or *EFTs*) either alone or in combination. (A) COX/CS activity in control cells. (B) Representative mitochondrial translation experiments from control cells transduced with the vectors used in (A). (C) BN-PAGE and SDS-PAGE analysis of control and patient fibroblasts over-expressing the mitochondrial translation factors.

## DISCUSSION

This study clearly demonstrates that mutations in the ubiquitously expressed translation factor *EFG1* produce unique, tissue-specific patterns of OXPHOS deficiency. The severity of the OXPHOS defect correlates with the residual levels of the mutant *EFG1* protein, and this explains the selective involvement of different tissues in the clinical phenotype. In addition, at least in heart, there appears to be an adaptive response that involves transcriptional upregulation of *EFTu*, another translation elongation factor. These results argue that far from being a routine housekeeping process, mitochondrial translation is in fact tailored to meet tissue-specific demands for the mtDNA-encoded structural subunits of the OXPHOS complexes.

*EFG1* is a GTPase that catalyzes the translocation of peptidyl-tRNA from the ribosomal A site to the P site following peptide bond formation, with the concomitant movement of the mRNA and exposure of the next codon in the A site. Mitochondria contain another EFG isoform, *EFG2* (35% identical to *EFG1*); however, it is not clear what role it plays in mitochondrial translation. It has essentially no functional overlap with *EFG1* at least in fibroblasts (22), and deletion of the homologue in yeast (*MEF2*) does not produce a respiratory phenotype (24). Prokaryotic (*E. coli*) EFG is 42% identical to human *EFG1* and consists of five recognizable domains (shown for human *EFG1* in Fig. 2C). It interacts with the 50S large ribosomal subunit near the L7/L12 stalk, and with the sarcin-ricin region of 23S rRNA [reviewed in (25)]. All of the mutations identified

in *EFG1* patients are clear loss of function alleles that can be rescued by expression of the wild-type cDNA. The patient previously described had a homozygous mutation in the GTP-binding domain I (22), and the patients investigated in the present study were compound heterozygotes for a missense and a nonsense allele. The nonsense allele is predicted to produce a premature stop codon at amino acid 607, which would eliminate domain 5 of the protein, an essential region for the interaction with the L7/L12 stalk of the ribosome. The missense mutation results in the S321P amino acid change at the boundary between domains 1 and 2, and likely produces a conformational change that could affect either nucleotide binding or hydrolysis.

The mitochondrial translation defect resulting from these mutations, although global in nature, was not uniform in fibroblasts, the only cell type in which we could make this measurement. For instance, the synthesis of all three subunits of complex IV, and some subunits of complex I (ND5 and ND6) was the most severely decreased (10–15% of control levels), whereas that of both subunits of complex V (ATP6,8) was similar to or greater than in controls (Fig. 1D). We have not seen this overall pattern of mitochondrial translation in approximately 10 other patients with combined OXPHOS deficiencies, all of whom showed global mitochondrial translation deficiencies due to as yet unidentified gene defects. One would expect *a priori* that translation of all polypeptides would be affected to a similar degree by *EFG1* dysfunction, and there is no obvious explanation for this pattern. The positive effect of the mutation on the synthesis of the two ATP subunits could reflect the fact that they are relatively small mRNAs that are translated from a bicistronic mRNA. Consistent with this notion, the rate of synthesis of the other polypeptides that are translated from a bicistronic mRNA, ND4 and ND4L, is similar in both patients and controls. The initiation of translation of these transcripts would presumably be more complex than for monocistronic mRNAs as both have overlapping reading frames; however, the control of translation initiation in mitochondria is poorly understood, and it is not possible to predict what effect this might have on translational efficiency. In any case, it seems likely that the pattern observed in fibroblasts is not the same in all tissues, otherwise it would be difficult to explain the marked deficiency in complex V assembly in skeletal muscle.

*EFG1* is ubiquitously expressed, with highest steady-state levels of the mRNA in skeletal muscle and heart as measured by both northern blot analysis (11) and quantitative RT-PCR (data not shown). The steady-state level of *EFG1* mRNA, however, does not correlate with the pattern of expression of EFG1 protein, which we show is three to four times higher in liver and fibroblasts than in skeletal muscle or heart (Fig. 4A), suggesting that post-transcriptional regulation is important for *EFG1* biosynthesis. Curiously, the steady-state level of EFG1 is inversely correlated with the steady-state levels of the OXPHOS complexes containing mtDNA-encoded subunits. Fibroblast mitochondria, for instance, have four times more *EFG1* than heart, but only ~10% of the content of the OXPHOS complexes containing mtDNA-encoded subunits. Although the reason for this is not clear, one possibility is that it reflects a higher rate of turnover of OXPHOS enzymes in mitotically active tissues.

Alternatively, there may be tissue-specific differences in the efficiency of mitochondrial translation.

The levels of *EFG1* in patient tissues further support the idea that post-transcriptional mechanisms are the most important determinants of *EFG1* stability. Although *EFG1* transcript levels in patient tissues were only slightly decreased, the protein was undetectable in liver, and severely reduced in muscle and fibroblasts (15–25% of control). Strikingly, however, the amount of *EFG1* in patient heart was ~60% of control. If protein from only the missense allele is actually expressed in this patient, this result indicates that it is completely stabilized in heart. The residual level of the mutant protein correlates perfectly with the severity of the OXPHOS defect, leading us to conclude that it is the major determinant of tissue specificity.

Why is the heart able to handle the *EFG1* mutation with a limited effect on mitochondrial translation efficiency, whereas in the liver translation is severely disrupted? The marked difference in the relative ratios of the mitochondrial elongation factors in these tissues indicate that the composition of the mitochondrial translation machinery is at least quantitatively, and possibly qualitatively different. Although we do not know why mutant *EFG1* is stable in heart but not in liver, it is possible that it results from changes in the translation apparatus that compensate for decreased translocase efficiency of the mutant EFG1 protein. Investigation of the other translation elongation factors in patient heart showed a 4-fold increase in the level of EFTu, and a slight decrease in EFTs, resulting in an approximately 1:2 ratio of EFTu:EFTs. This ratio changed in the opposite direction in liver from 1:1 to about 1:4.

EFTu is a GTPase that plays a central role in protein synthesis, upstream of EFG1 by delivering aminoacyl-tRNAs to the acceptor (A) site of the ribosome. Like its prokaryotic counterpart, mammalian EFTu participates in the formation of the (EFTu:GTP:aminoacyl-tRNA) ternary complex, which promotes binding of the aminoacyl-tRNA to the ribosomal A site (26,27). The switch between an active (GTP-bound) and inactive (GDP-bound) form requires the recycling factor EFTs. Both EFTu and EFTs are ubiquitously expressed, with highest mRNA levels present in skeletal muscle, heart, kidney and liver (8,10) (data not shown). Immunoblotting with antibodies against mitochondrial elongation factors EFTu and EFTs revealed significant differences in the levels of these factors that do not correlate in any obvious way with the steady-state levels of OXPHOS complexes in different tissues. Previous analysis of bovine liver mitochondria and cultured mammalian cells demonstrated a 1:1 ratio of EFTu and EFTs in mitochondria (26), and although we have not directly measured the absolute quantities of EFTu and EFTs, our results are consistent with this in skeletal muscle, liver and fibroblasts. In contrast, control heart mitochondria contained much higher levels of EFTs, and a relative ratio of EFTu:EFTs of 1:6. Although we have not been able to assess the functional consequences of the change in the EFTu:EFTs ratio in patient heart (1:2), one would predict that it might slow the recycling rate of EFTu, perhaps bringing it in line with a slower translocation rate of the mutant *EFG1*.

We attempted to mimic the different relative ratios of the elongation factors observed in control and patient tissues by over-expressing them in fibroblasts. Cells over-expressing

EFTu showed a markedly decreased rate of mitochondrial translation, diminished assembly of OXPHOS complexes and low COX activity. Thus, increasing the relative EFTu:EFTs ratio in these cells to 4:1, similar to that found in prokaryotic organisms (28), had a clear negative effect on mitochondrial translation efficiency. On the other hand, cells over-expressing only EFTs (at a relative ratio of 1:5, similar to that observed in control heart), and EFTu/EFTs together (ratio 1:1, found in liver and muscle) showed a small decrease in the rate of mitochondrial translation and in COX activity when compared with untransduced cells. These data indicate that translational efficiency is determined in part by appropriate ratios of the translation elongation factors, and suggest that changes in these ratios, such as those observed in patient heart, are part of a coordinated response to dysfunction of one of the components of the mitochondrial translation machinery. It is unclear why similar responses do not occur in other tissues.

Similar to the previously described *EFG1* patient (22), the patients we investigated here presented with severe growth retardation, lactic acidosis and liver dysfunction. There are a number of similarities between these patients and the recently characterized patient with a mutation in the mitochondrial ribosomal protein MRPS16 (21), including a small or undeveloped corpus callosum, and severe respiratory deficiency in skeletal muscle and liver. Although no biochemical analyses were reported on heart tissue in the MRPS16 patient, there was no evidence of clinical cardiac dysfunction. Thus, it is possible that the heart uniquely possesses mechanisms to circumvent the disruption of mitochondrial translation caused by mutations in the nuclear-encoded components of the translation machinery.

In summary, we have identified striking tissue-specific differences in the mitochondrial translation machinery and its response to dysfunction. Although it is probable that the basic mechanism of translation is the same in all tissues, this study shows that there are significant quantitative differences in the ratios of the translation elongation factors, and it is reasonable to assume that this reflects different tissue-specific demands for the mitochondrially encoded polypeptides. We have shown that unique patterns of OXPHOS deficiency result when one of the nuclear-encoded components of the translation system fails. The tissue-specific differences in the organization of the mitochondrial translation apparatus may also underlie some of the clinical heterogeneity associated with mutations in mtDNA-encoded tRNAs and rRNAs.

## METHODS

### Case Reports

Patient 1 was born at term to a healthy 28-year-old gravida 1 female. At birth, her weight, length and head circumference were all below the second percentile. The pregnancy was complicated in the first trimester by poor weight gain secondary to nausea and vomiting, urinary tract and upper respiratory infections (treated with antibiotics) and mild hypertension in the final two weeks. Fetal ultrasound at 22 weeks gestation was normal but, at 26 weeks, revealed intrauterine growth retardation. The MSAFP triple screen was very abnormal with human chorionic gonadotrophin (HCG) level 6.8 multiples of mean (MOM),  $\alpha$ -fetoprotein 2.2 MOM and estriol 0.4

MOM. Amniocentesis showed a 46, XX karyotype and no molecular evidence of chromosome 22 deletion. The parents are Caucasian and unrelated.

Soon after birth, the patient developed mild hyperbilirubinemia, hypoalbuminemia, severe metabolic acidosis (pH 7.10) (treated with bicarbonate) and greatly elevated lactate (22 mM; normal 1.0–1.8 mM). Mild facial dysmorphism with simple ears and broad nasal bridge were noted. Echocardiogram showed only a large patent ductus arteriosus. CBC with differential and electrolytes was unremarkable. She was transferred to Oregon Health and Science University at 2 days of age. She was alert with normal reflexes and tone. Mild direct hyperbilirubinemia, hypoalbuminemia and severe lactic acidosis persisted. There was mild hyperammonemia and elevated plasma glutamine, alanine, proline and tyrosine. Urine lactate and tyrosine metabolites were greatly elevated but absence of succinylacetone ruled out tyrosinemia type I. Plasma-free carnitine was low (7.7  $\mu$ M; normal 10.1–21.0  $\mu$ M) with a normal acylcarnitine profile. She was treated with bicarbonate, carnitine and a 'vitamin cocktail'. Viral and bacterial cultures were negative. Abdominal ultrasound was unremarkable. Echocardiogram confirmed a patent ductus arteriosus with bi-directional flow; left ventricular function was normal. Blood lactate rose to 34 mM mid-hospitalization. Brain MRI was normal on day 5 but MRS showed marked elevation of lactate. She developed increasingly severe abdominal ascites, coagulopathy requiring multiple transfusions and increasing direct hyperbilirubinemia. Muscle and skin biopsies were performed on day 8. On day 9, she developed respiratory failure requiring intubation. After further deterioration, respiratory support was withdrawn and she died of a pulmonary hemorrhage later that day.

At autopsy, the cardiovascular system showed focal narrowing of the aorta proximal to patent ductus arteriosus. Myocardium was histologically normal. The lungs showed bilateral pulmonary hemorrhage and early hyaline membrane formation. The liver was markedly abnormal with microvesicular steatosis, cholestasis, pseudoacinar formation, bile duct proliferation and marked deposition of iron (hepatocytes and Kupffer cells). Kidneys also showed lipid accumulation. Neuropathology showed bilateral porencephaly, microcephaly, and dysgenesis of cingulate gyri.

Muscle histochemistry showed no ragged red fibers or inclusions, normal succinate dehydrogenase (SDH) and NADH tetrazolium reductase staining but deficient COX (cytochrome *c* oxidase: complex IV) staining in many fibers. EM was unremarkable. Respiratory chain activities in muscle 600g supernatant, expressed relative to SDH, showed marked deficiency of COX and milder deficiency of complex I (10 and 38% of control mean, respectively). Complexes II, II+III and III were 51, 60 and 50% of control mean, respectively.

Enzyme activities in cultured skin fibroblasts showed normal pyruvate dehydrogenase, both native and dichloroacetate activated pyruvate carboxylase and succinate:cytochrome *c* reductase but greatly elevated lactate to pyruvate ratio (104  $\pm$  42; controls 19  $\pm$  3; mean  $\pm$  SE). COX activity was low (20% of control mean) and cytochemistry, immunocytochemistry and western blot analyses demonstrated reduced levels of COX subunits. Southern blot analysis showed a normal ratio of mtDNA:nuclear DNA, ruling out mtDNA depletion.

Patient 2 was the second child born to these parents. Early pregnancy was unremarkable and maternal serum triple screen at 15 weeks gestation was normal. Amniocentesis at this time revealed a 45X (4)/46XX (16) karyotype in cultured cells. Analysis of lactate:pyruvate ratio, COX activity, immunocytochemistry and western blots were inconclusive, and the pregnancy was continued. At 22 weeks gestation, fetal ultrasound showed possible early stages of growth retardation and a slightly echogenic bowel. By 24 weeks, fetal head and abdomen measured 3 weeks behind expected, there was significant oligohydramnios, and a decision was made to induce delivery. The baby died 45 min after birth.

Autopsy on Patient 2 showed no dysmorphic features and internal organs showed normal gross morphology. Placenta and cord appeared unremarkable. Liver histology showed normal architecture but excess stainable iron within hepatocytes. All other organs showed no diagnostic abnormalities. Neuropathology of brain and muscle was unremarkable.

Cytogenetic analysis of fetal cultured skin fibroblasts confirmed low level 45X mosaicism. COX activity was low (26% of control mean) and immunocytochemistry and western blot analysis showed reduced levels of COX subunits. BN-PAGE analysis and *in vitro* mitochondrial translation assay indicated that Patient 2 had a combined OXPHOS defect because of decreased mitochondrial translation with the same pattern as her sister.

### Human studies

Informed consent was obtained and research studies approved by the Oregon Health and Science University and the Montreal Neurological Institute Institutional Review Boards.

### Cell culture and enzyme measurements

Primary human skin fibroblasts were immortalized with a retroviral vector expressing the E7 gene of type-16 human papilloma virus, and another expressing the protein component (hert) of human telomerase (29). Patient and control skin fibroblasts were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. COX and CS activities were measured in fibroblast cell extracts as described previously (30).

### Preparation of mitochondria

Post-mortem heart, muscle and liver tissue specimens from Patient 1 and controls were removed and frozen less than 30 min after death. Heart, liver and fibroblast homogenates (5%) prepared in 250 mM sucrose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) and muscle homogenate (5%) prepared in 150 mM KCl/50 mM Tris-HCl/2 mM EDTA (pH 7.5) were centrifuged twice for 10 min at 600g to obtain post-nuclear supernatant. Mitochondria were pelleted by centrifugation for 20 min at 10 000g.

### BN-PAGE and immunoblotting

BN-PAGE (31) was used for separation of the OXPHOS complexes on 6–15% polyacrylamide gradient gels. Mitoplasts

were prepared from fibroblasts by treatment with 0.8 mg of digitonin/mg of protein, as described previously (32). Mitoplasts or mitochondria were solubilized with 1% lauryl maltoside, and 4–20 µg of the solubilized protein was used for electrophoresis. Complexes I–V were detected by immunoblot analysis using monoclonal antibodies (Molecular Probes) except for the anti-ND1 antibody, a kind gift of A. Lombes (Paris). For immunoblotting, mitochondria or fibroblasts were solubilized with 1.5% lauryl maltoside in PBS and 6–20 µg of protein were run on a Tris-Tricine SDS PAGE gel. The proteins were transferred to nitrocellulose and a monoclonal antibody directed against complex II (Molecular Probes), and polyclonal antibodies directed against EFTu (a kind gift of N. Takeuchi, Japan), EFTu/Ts (a kind gift of L. Spemulli, Chapel Hill, NC, USA), and EFG1 were used for detection.

### EFG1 antibody production

A polyclonal antibody against a peptide (CEATGQLPVKKG-KAKN) from the human EFG1 protein was prepared by New England Peptide Inc. (Gardner, MA, USA). Crude serum was tested on cell lines over-expressing EFG1 protein and detected a band of ~85 kDa. In mitochondria from some tissues several weak non-specific bands of 40–60 kDa could also be detected.

### Pulse labeling of mitochondrial translation products

*In vitro* labeling of mitochondrial translation was performed as previously described (33). Briefly, cells were labeled for 60 min at 37°C in methionine-free DMEM containing 200 µCi/ml [<sup>35</sup>S]methionine and 100 µg/ml emetine and chased for 10 min in regular DMEM. Total cellular protein (50 µg) was resuspended in loading buffer containing 93 mM Tris-HCl, pH 6.7, 7.5% glycerol, 3.5% SDS, 0.25 mg bromophenol blue/ml and 3% mercaptoethanol, sonicated for 3–8 s, loaded and run on 12–20% polyacrylamide gradient gels. In some experiments, the concentration of SDS in the loading buffer was reduced to 1%, resulting in better resolution of the ND4L and ATP8 polypeptides.

### cDNA sequencing and restriction fragment analysis

RNA was isolated from patient and control skin fibroblasts using RNeasy Isolation Kit (Qiagen) and from tissues using Trizol (Invitrogen). Primers were designed for the amplification of *EFG1* cDNA and the PCR fragments were used for direct sequencing. Genomic DNA was isolated (Qiagen DNAeasy Isolation Kit) from patient skin fibroblasts and blood from parents and controls. Primers specific for exons 7 and 15 were used to amplify the DNA followed by either the digestion with restriction enzyme *BclI* (exon 7) or direct sequencing (exon 15).

### Quantitative RT-PCR

cDNAs were synthesized from 2 µg of total RNAs using M-MLV Reverse Transcriptase (Promega). The quantitative PCR was performed using TaqMan PCR Universal Mix (Applied Biosystems) on the Smart Cycler System (Cepheid).

TaqMan Gene Expression Assays primers and PCR probes for human genes *EFG1* (Hs00227997\_m1), *EFG2* (Hs00260735\_m1), *EFTu* (Hs00607042\_gH) and *EFTs* (Hs00245862\_m1) were used. Gene expression was normalized to the levels of 18S rRNA, which was measured using Certified LUX-primers (Invitrogen).

### cDNA constructs and virus production and infection

Retroviral vectors containing the cDNA sequence of four mitochondrial translation elongation factors (*EFG1*, *EFG2*, *EFTu*, *EFTs*) were created with the Gateway™ Cloning system (Invitrogen). cDNAs from the *EFG2* and *EFTs* genes were amplified by OneStep RT-PCR™ (Qiagen) using specific primers modified for cloning into Gateway vectors. *EFG1* and *EFTu* cDNAs were amplified from I.M.A.G.E. clones No. 5574223 and 3629164 (ATCC) respectively, using specific primers. The PCR constructs were cloned into a Gateway-modified retroviral expression vector, pLXSH (34) or pBABE. The fidelity of cDNA clones was confirmed by automated DNA sequencing. Retroviral constructs were transiently transfected into Phoenix packaging cell line using the HBS/Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> method ([http://www.stanford.edu/group/nolan/protocols/pro\\_helper\\_dep.html](http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html)). Patient and control fibroblasts were infected 48 h later by exposure to virus-containing medium in the presence of 4 µg/ml of polybrene as described (29).

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*Conflict of Interest statement.* The authors declare that there is no conflict of interest.

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