Nuclear genetic control of mitochondrial translation in skeletal muscle revealed in patients with mitochondrial myopathy

Florin Sasarman¹, George Karpati¹ and Eric A. Shoubridge¹,²,*

¹Department of Neurology and Neurosurgery and ²Department of Human Genetics, McGill University, Montreal, Quebec, Canada, H3A 2B4

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Oxidative phosphorylation deficiencies can be caused by mutations in either the nuclear genome or the mitochondrial genome (mtDNA); however, most pathogenic mutations reported in adults occur in mtDNA. Such mutations often impair mitochondrial translation, and are associated with a characteristic muscle pathology consisting of a mosaic pattern of normal fibres interspersed with fibres that show mitochondrial proliferation (ragged-red fibres) and little or no complex IV (COX) activity. We investigated two adult patients with a severe mitochondrial myopathy in whom all muscle fibres showed mitochondrial proliferation with barely detectable COX activity—a pattern never before reported. Biochemical studies of the respiratory chain in muscle showed decreased activities of complexes I and IV (5% of control) and complex II + III (41% of control). Immunoblot analysis of nuclear and mitochondrial subunits of complexes I, III and IV showed a greater than 90% decrease in the steady-state level of these subunits in mature muscle, but no change in nuclear-encoded subunits of complexes II and V. A generalized mitochondrial translation defect was identified in pulse-label experiments in myotubes, but not in myoblasts cultured from both patients. This defect moved with the nucleus in patient cybrid cells. Myoblasts from one patient transplanted into the muscle bed of SCID mice differentiated into mature human muscle fibres that displayed a defect similar to that seen in the patient muscle. These results suggest a defect in a developmentally regulated nuclear factor important for mitochondrial translation in skeletal muscle.

INTRODUCTION

The enzymatic system responsible for the generation of ATP by oxidative phosphorylation of ADP, located in the inner mitochondrial membrane, is composed of the four complexes of the respiratory chain (complexes I–IV) and ATP synthase (complex V). These five enzymatic complexes consist of subunits that are encoded by both the mitochondrial (mt) and the nuclear genomes. In addition to coding for 13 structural proteins, mtDNA also codes for 2 rRNA genes and 22 tRNA genes (1), which are required for mitochondrial translation. The nuclear genome encodes the vast majority of mitochondrial proteins, and controls many mitochondrial functions such as mitochondrial gene expression, import, export and the assembly pathways required for the biogenesis of functional mitochondria. Mitochondrial diseases caused by oxidative phosphorylation defects can result from mutations in either the nuclear or mtDNA. Over 50 pathogenic base substitution mutations and approximately 100 mtDNA rearrangements (deletions and insertions) have been described in mtDNA (2), and more than 20 different nuclear gene defects have now been reported (3).

Oxidative phosphorylation disorders are generally multisystemic diseases that affect predominantly highly aerobic, post-mitotic tissues, such as muscle and nerve. One of the features of the skeletal muscle of patients with mtDNA deletions or tRNA point mutations is the presence of a variable number of fibres that appear ragged-red with the modified Gomori trichrome stain or ragged-blue with a stain for succinate dehydrogenase (SDH, complex II) activity, reflecting the accumulation of morphologically and biochemically abnormal mitochondria. These fibres usually stain negative for cytochrome c oxidase (COX) activity and are interspersed with apparently normal muscle fibre segments, generating a characteristic mosaic pattern (4). The presence of such features in skeletal muscle fibres of patients usually correlates with impaired mitochondrial protein translation, but is occasionally observed in patients with mutations in protein-coding genes.
A severe, generalized COX deficiency in all muscle fibres, on the other hand, is characteristic of fatal infantile syndromes such as Leigh syndrome or hypertrophic cardiomyopathy, due to nuclear gene defects (5,6).

Here we have investigated two adult, unrelated patients with mitochondrial proliferation and severe COX deficiency in all muscle fibres. The steady-state levels of several mitochondrial as well as nuclear subunits of three of the five oxidative phosphorylation complexes were severely reduced in the mature muscle of both patients. A generalized mitochondrial translation defect was demonstrated in patient myotubes, but not myoblasts, cultured in vitro. We demonstrate that the molecular defect in the two patients is of nuclear origin and developmentally regulated in skeletal muscle. These results suggest a defect in a novel nuclear factor that is important in regulating mitochondrial translation in skeletal muscle.

RESULTS

Muscle histochemistry and immunofluorescence

Muscle sections from both patients were stained for COX and SDH activity. There was a severe reduction in COX activity and a marked increase in SDH activity in all muscle fibres in both patients (Fig. 1A–F). A small amount of COX activity could be detected in the subsarcolemmal region of some muscle fibres, an area where the mitochondrial accumulation was greatest, as demonstrated by SDH staining and electron microscopy (data not shown).

The COII subunit of COX was detected by immunofluorescence in muscle sections from the two patients (Fig. 1G–I). The level of this protein was reduced in all muscle fibres in both patients, with the exception of the subsarcolemmal region of some fibres, suggesting that the lack of COX activity was due to the presence of reduced amounts of protein subunits of COX. Consistent with the observed SDH hyper-reactivity in the patients’ muscle, the 70 kDa subunit of SDH was present at levels higher than control (Fig. 1J–L).

No pathogenic mutations were identified in the mtDNA of the patients

The uniform staining pattern for COX suggested a defect of nuclear, rather than mitochondrial, origin, and the lack of family history in both patients suggested a recessive disorder. However, to rule out the involvement of mtDNA in the observed deficiency, we analyzed mtDNA extracted from the muscle of both patients for mutations. There was no evidence of any large-scale mtDNA rearrangements or mtDNA depletion in either patient by Southern blot analysis (data not shown). Sequencing of all of the tRNA genes revealed only two differences as compared with the Cambridge consensus sequence: in patient L, a homoplasmic T12308C in the variable loop of tRNAVal and in patient E, a homoplasmic A15929G in tRNAThr. Neither of these mutations occurs at an evolutionarily conserved site, and they likely represent neutral polymorphisms. In situ hybridization with probes for 16S rRNA and six different mitochondrial mRNAs (COX I, COX III, ND2, ND1, ND6 and cyt b) showed a similar pattern in serial muscle sections of the patients. All seven probes showed elevated steady-state RNA levels, commensurate with the increase in mitochondrial volume (data not shown) suggesting normal mitochondrial transcription.

Generalized respiratory chain deficiency in patient muscle biopsies

To investigate whether the biochemical deficiency was confined to COX, biochemical analysis of the respiratory chain was performed on mitochondria isolated from the muscle of patient E (insufficient amounts of muscle from patient L precluded this analysis). As seen in Table 1, the activity of COX (complex IV) in the muscle of patient E was 4% of control values. The enzymatic activities of complex I and complex II + III were also reduced, with complex I being less than 6% and complex II + III 41% of control values, demonstrating a severe, generalized respiratory chain deficiency in mature muscle.

Immunoblot analysis of patient muscle

We carried out immunoblotting experiments to test whether the respiratory chain deficiency resulted from the absence of subunits of the respiratory chain complexes. Total protein was extracted from muscle sections cut from frozen muscle of the two patients and one control, and the steady-state levels of several subunits were measured. Three subunits of COX were measured: COI and COII, which are encoded by mtDNA, and COIV, a nuclear subunit (Fig. 2A, lanes 7–9; Fig. 2B, lanes 4–6). The levels of all three subunits were decreased by more than 90% compared to control. We observed a similar decrease for ND1 (mitochondrial, complex I), C-III-Core1 (nuclear, complex III) and cyt b (mitochondrial, complex III) (Fig. 2A, lanes 7–9; Fig. 2B, lanes 4–6). The decrease in the steady-state level of all subunits was greater in patient L than in patient E, and interestingly, the levels of porin, an outer mitochondrial membrane protein, were also substantially reduced (around 90%) in this patient (Fig. 2A, lanes 7–9). In contrast to the above results, no significant differences were observed in the levels of the nuclear subunits of complexes II and V (C-II-70kD and C-V-Alpha) (Fig. 2A, lanes 7–9).

A mitochondrial translation defect in patient myotubes

The presence of ragged-red fibres in the muscle fibres in both patients, and the marked reduction in the steady-state levels of mtDNA-encoded proteins on immunoblot analysis, suggested the possibility of a defect in mitochondrial translation as the molecular basis for the mitochondrial myopathy. Primary myoblasts were cultured from biopsies of patients L and E, and transduced with a retroviral vector expressing HPV16-E6/E7 to increase their lifespan in vitro (7). Myotubes were obtained by culturing myoblasts in low-serum (differentiation) medium. Mitochondrial translation was assessed in myoblasts and myotubes of the two patients and two different controls by pulse-label experiments, in which mitochondrial translation products were specifically labeled with [35S]methionine after selective inhibition of the cytosolic translation with emetine. No differences were observed in the level of mitochondrial translation between
Figure 1. Immunofluorescence and histochemical analysis of patient muscle. (A–C) COX staining shows a severe reduction of COX activity in the muscle fibres of patient L (B) and patient E (C) compared with control (A). Some COX activity is observed in the subsarcolemmal regions of some fibres, where mitochondrial proliferation occurs. (D–F) SDH staining reveals hyper-reactivity of SDH in the muscle fibres of patient L (E) and patient E (F) compared with control (D). (G–I) Immunoreactivity for the anti-COII antibody is decreased in fibres of patient L (H) and patient E (I) compared with control fibres (G). Similar to the staining pattern for COX, some immunoreactivity for this antibody is present in the subsarcolemmal regions of some fibres. (J–L) Immunoreactivity for the anti-C-II-70 kDa antibody shows higher levels of this subunit in the muscle of patient L (K) and patient E (L) compared with control (J), in accordance with the observed SDH hyper-reactivity in patient muscle.
Table 1. Biochemical analysis of the respiratory chain in patient muscle and cultured muscle progenitor cells: a substantial decrease in the activity of the respiratory chain is observed in the muscle of patient E, but not in myoblasts and myotubes of the two patients

<table>
<thead>
<tr>
<th></th>
<th>Complex I</th>
<th>Complex II + III</th>
<th>Complex IV (COX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myoblasts</td>
<td>Myotubes</td>
<td>Muscle</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>0.038 (100%)</td>
<td>0.21 (100%)</td>
</tr>
<tr>
<td>Patient L</td>
<td>ND</td>
<td>0.043 (113%)</td>
<td>ND</td>
</tr>
<tr>
<td>Patient E</td>
<td>ND</td>
<td>0.041 (108%)</td>
<td>&lt; 0.012 (&lt; 5.7%)</td>
</tr>
</tbody>
</table>

Enzymatic activities of the various respiratory chain complexes are expressed relative to the activity of citrate synthase. The values in parentheses represent the percentage of activity relative to control, which was set arbitrarily at 100%. ND, no data.

Figure 2. Steady-state levels of nuclear and mitochondrial subunits of the oxidative phosphorylation enzyme complexes in patient muscle and cultured muscle progenitor cells. (A) Immunoblot analysis was carried out using total protein extracted from myoblasts (lanes 1–3) of one control (1), patient L (2) and patient E (3), from myotubes (lanes 4–6) of one control (4), patient L (5) and patient E (6), and from the muscle (lanes 7–9) of one control (7), patient L (8) and patient E (9). Equal amounts of protein were loaded in each lane (30 μg for myoblasts and myotubes and 15 μg for muscle). The blots were reacted with a mixture of monoclonal antibodies monospecific for the subunits indicated at the left of the figure. While normal levels of all of these subunits are present in myoblasts and myotubes of the two patients, a severe decrease in the level of several of the detected subunits is observed in the muscle of both patients. (B) Immunoblot analysis was carried out as in (A), using either mitochondrial protein isolated from myotubes (lanes 1–3) of one control (1), patient L (2) and patient E (3), or total protein extracted from the muscle (lanes 4–6) of one control (4), patient L (5) and patient E (6). Equal amounts of protein were loaded in each lane (25 μg for myotubes and 30 μg for muscle). The blots were reacted with a mixture of monoclonal and polyclonal antibodies specific for the subunits indicated at the left of the figure. While normal levels of these subunits are present in myotubes of the two patients, a substantial decrease in the level of all of the detected subunits is evident in the muscle of both patients.
patient myoblasts and controls (Fig. 3, lanes 1–4), however, a
generalized defect in mitochondrial translation was observed
in the myotubes of both patients (Fig. 3, lanes 5–8). Transla-
tion of the three subunits of COX encoded by mtDNA (COI, COII and COIII) was approximately 30–40% of
control levels, and translation of several subunits of complex I, such as ND4, ND5 and ND6, was approximately
20% of control levels. These results demonstrate a defect in
mitochondrial translation in the muscle progenitor cells of
both patients, which only develops as differentiation
progresses.

Nuclear origin of the mitochondrial translation defect
The analysis of the mature muscle in both patients suggested
that the defect responsible for the mitochondrial myopathy was
of nuclear origin. To test this, we generated cybrid cells
containing nuclei from the patient myoblasts and mitochondria
from normal myoblasts as follows. Immortalized patient
myoblasts were treated for several days with rhodamine-6-G,
a toxic dye that accumulates selectively in mitochondria but
does not interfere with nuclear function (8). Patient myoblasts
were then rescued by fusion with enucleated primary myoblasts
from normal controls. Following the fusion, the cells were
selected in G-418 to eliminate primary myoblasts that did not
enucleate. The immortalized myoblasts used as the nucleus
donor are resistant to G-418; however, the treatment with
rhodamine-6-G destroys all mitochondrial function and event-
ually kills the cells, unless they are fused with myoblasts
containing functional mitochondria. Hence, only the cybrids
containing a patient nucleus and normal mitochondria could
survive selection. The resulting cybrid cells were allowed to
differentiate into myotubes. Mitochondrial translation in the
myotubes derived from these cells displayed a defect similar to
that observed in the myotubes of the two patients (Fig. 4). Re-
verse cybrids were generated by using nuclei from immor-
talized normal myoblasts and mitochondria from primary myoblasts of patient E. Control levels of mitochondrial
translation products were observed in the myotubes resulting
from these cells (Fig. 5A). These results demonstrate that the
defect in mitochondrial translation is of nuclear origin, since it
moves with the nucleus of the two patients.

To confirm that all the mitochondria from the nuclear donors
were eliminated by the rhodamine-6-G treatment, we took
advantage of a polymorphism present in the tRNAThr of patient E
that introduces a new NciI restriction site. The tRNAThr gene was
amplified by PCR from genomic DNA extracted from the cybrids
and reverse cybrids generated from patient E, and the product
was digested with the NciI restriction enzyme (Fig. 5B). All of
the tRNAThr DNA was cut by the NciI enzyme in patient E, and in
the reverse cybrids (which contain mitochondria from patient E),
while none of the tRNAThr DNA was cut in normal myoblasts or
in the cybrids (which contain mitochondria from control
myoblasts). Thus, the mitochondria of the cybrids and reverse
cybrids originate exclusively from the mitochondrial donor.

Respiratory chain function in patient myoblasts and
myotubes
To test whether the same respiratory chain deficiency observed
in the muscle of the two patients was also present in their
cultured cells, we measured COX and complex I activities in

![Figure 3](image-url) - Mitochondrial translation in patient myoblasts and myotubes. Mitochondrial translation was measured by pulse-labeling myoblasts or myotubes with [35S]methionine for 1 hour in the presence of emetine. The translation products were separated on 12–20% linear-gradient polyacrylamide gel: myoblasts (lanes 1–4) from two controls (lanes 1 and 2), patient L (lane 3) and patient E (lane 4); myotubes (lanes 5–8) from two controls (lanes 5 and 6), patient L (lane 7) and patient E (lane 8). Equal amounts of total protein (50 µg) were loaded in each lane. The 13 mitochondrial translation products are identified at the left of the figure. Mitochondrial protein synthesis in the myoblasts of the two patients is similar to control; however, a generalized mitochondrial translation deficiency is present in the myotubes of both patients.

![Figure 4](image-url) - Mitochondrial translation in differentiated patient cybrids, containing patient nuclei and control mitochondria. Mitochondrial translation was measured as in Figure 3 in myotubes from a control (lane 1), patient L (lane 2) and patient E (lane 3), and in differentiated cybrids from patient L (lane 4) and patient E (lane 5). Equal amounts of total protein (50 µg) were loaded in each lane. Mitochondrial translation products are identified at the left of the figure. A similar mitochondrial translation defect is observed in myotubes and in differentiated cybrids of the two patients, indicating that this defect is of nuclear origin.
The molecular defect is developmentally regulated in skeletal muscle

The above results suggested a nuclear gene defect in a developmentally regulated muscle gene. To test this hypothesis, myoblasts from patient L were allowed to differentiate in vivo to mature muscle fibres, in the muscle bed of severe combined immunodeficient (SCID) mice. While the patient myoblasts have normal COX activity and control levels of COX subunits, they were predicted to differentiate into muscle fibres severely deficient in COX activity and containing substantially reduced levels of COX subunits if the hypothesis were correct. The tibialis anterior muscle of SCID mice was first irradiated, to prevent regeneration by host satellite cells, and then injected with primary myoblasts from patient L or an age-matched control. The injected myoblasts were resuspended in notexin, a snake venom toxin that selectively destroys mature muscle fibres. Thus, in this experiment, only the injected human myoblasts are available to reconstitute the muscle. Since these mice are immunodeficient, there is a high probability that the injected myoblasts survive the transplant. One month later, the mice were euthanized, and the injected muscles removed.

Sections cut from the muscles injected with either control or patient L myoblasts were reacted with an antibody specific to human dystrophin to identify fibres of human origin, and serial sections were then stained for COX and SDH. The fibres formed from patient L myoblasts displayed a severe COX deficiency and were SDH-hyper-reactive (Fig. 6), similar to the pattern seen in the muscle of patient L. To eliminate the possibility that the lack of COX activity in the fibres from patient L was simply due to incomplete maturation of these muscle fibres, sections from the muscle containing patient L fibres were stained for motor endplates with α-bungarotoxin (Fig. 6G). The figure shows a field containing several motor endplates, demonstrating that these fibres are innervated, and thus mature.

We carried out an immunoblot analysis on total protein extracted from sections of the injected muscles using human-specific antibodies against COII and COIV. To estimate the total amount of human protein that was loaded for each sample, the blot was also probed with an antibody specific to human δ-sarcoglycan. We confirmed on separate blots that all three antibodies were specific for the human proteins, and do not recognize their mouse counterparts (data not shown). In patient L fibres, COII was present at 12–15% and COIV at 35–43% of control levels (Fig. 7; the range is from two independent experiments). These results are similar to those obtained from the muscle biopsies of patient L, especially for COII, and demonstrate that the deficiency present in the muscle of patient L is developmentally regulated. We did not observe any dystrophin-positive fibres following the transplantation of myoblasts from patient E. This is possibly due to the greater age of patient E, since the transplantation efficiency appears to decrease with the age of the donor.

Partial complementation of the mitochondrial translation defect following fusion between myoblasts from the two patients

The analysis of cultured cells and of the muscle biopsies from the two patients revealed many similarities, at the molecular

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Figure 5. Mitochondrial translation in differentiated patient reverse cybrids, containing control nuclei and patient mitochondria. (A) Mitochondrial translation was measured as in Figure 3 in myotubes from a control (lane 1) and patient E (lane 2), and in differentiated reverse cybrids from patient E, containing normal nuclei and patient mitochondria (lane 3). Equal amounts of total protein (60 μg) were loaded in each lane. Three of the 13 mitochondrial translation products are indicated at the left of the figure. Mitochondrial translation is normal in the reverse cybrids of patient E, confirming the nuclear origin of the mitochondrial translation defect observed in patient myotubes. (B) Analysis of mtDNA in cybrids and reverse cybrids. RFLP analysis was carried out by enzymatic digestion (using the NciI restriction enzyme) of an mtDNA rRNA\text{Thr} fragment amplified from genomic DNA of the following cells: control myoblasts used as the mitochondrial donor for the generation of patient cybrids (lane 1), control myoblasts used as the nucleus donor for the generation of patient reverse cybrids (lane 2), myoblasts of patient E (lane 3), cybrids of patient E (lane 4) and reverse cybrids of patient E (lane 5). The sizes of the molecular size markers are indicated at the left of the figure. The undigested fragment is found exclusively in the control myoblasts and in the patient cybrids (which contain control mitochondria), while a completely digested fragment is found in patient myoblasts and in patient reverse cybrids (which contain patient mitochondria).
level, between patient L and patient E. However, certain differences have been noted between the two patients, such as the more marked decrease in the steady-state levels of subunits of the respiratory chain complexes and porin in the muscle of patient L, compared with patient E. This prompted us to test whether the two patients belong to the same genetic complementation group. In order to accomplish this, approximately equal numbers of myoblasts from each patient were mixed, and allowed to fuse. Mitochondrial translation was then assessed in the resulting myotubes. As a control, myoblasts from each patient were also fused with myoblasts from a MERRF (myoclonic epilepsy with ragged-red fibres) patient with a tRNA<sup>Lys</sup> mutation (A8344G) in mtDNA. Although the prominent abnormal translation product (COI) (9)

Figure 6. Histochemical and immunohistochemical analysis of the transplanted patient muscle fibres. The presence of human dystrophin was verified with a human-specific anti-dystrophin antibody in the mouse TA muscles that were transplanted with myoblasts from a control (A), or with myoblasts from patient L (D). The COX staining reveals no detectable enzymatic activity in patient L fibres (E), compared with control fibres (B). SDH hyper-reactivity is observed in the fibres of patient L (F), when compared with the activity of SDH in control fibres (C). (A–C) and (D–F) are serial sections. The same muscle fiber is indicated by asterisks in (A–C) and in (D–F), respectively. (G) Double staining for human dystrophin (red) and for motor endplates (green) shows that the fibres generated by the transplantation of myoblasts from patient L are innervated.
in myotubes from the MERRF patient (Fig. 8, lane 4) was eliminated by the fusion with myoblasts from patient E (Fig. 8, lane 6) the overall level of mitochondrial translation in these mixed myotubes was still lower than normal. We observed partial complementation of the mitochondrial translation defect in the myotubes resulting from the fusion of myoblasts from patients L and E (Fig. 8, lane 7). In the myotubes of each patient, the COX subunits encoded by mtDNA were synthesized at about 30% of control levels, while in the myotubes resulting from the fusion of myoblasts from the two patients, the level of these subunits was approximately 50% of control levels (results of two independent experiments).

**DISCUSSION**

The primary clinical symptoms of the two patients involved skeletal muscle weakness and fatigability, although patient E also later developed sideroblastic anemia. There was no evidence for the involvement of other tissues in either patient, particularly the central nervous system, which might be expected to be affected were the defect globally expressed. Cytochemical analysis of the patient muscle biopsies revealed a striking reduction in COX activity associated with mitochondrial proliferation, which was rather uniform across all fibres. Since a mutation in mtDNA would be expected to generate a mosaic pattern of normal and COX-negative fibres, we suspected the presence of a mutation in a nuclear gene. However, in order to rule out the involvement of mtDNA in the observed deficiency, we analyzed the mtDNA of both patients for the presence of known mutations. No pathogenic mutations were identified in any of the 22 mitochondrial tRNAs, and there was no evidence of any large-scale mtDNA rearrangements in either patient by Southern blot analysis. Mitochondrial transcription also appeared to be normal in both patients. These initial results suggested a specific deficiency in COX, which seemed muscle-specific and of nuclear origin. In humans, COX is the only respiratory chain complex that contains muscle-specific, developmentally regulated subunits: VIa-H and VIIa-H (10). During skeletal muscle development, the VIa-H and VIIa-H subunits gradually replace their VIa-L and VIIa-L isoforms, which are initially present in myoblasts, and which are ubiquitous in all other tissues (11,12). Northern blot analysis showed control mRNA levels for both isoforms, and DNA sequence analysis showed no mutations in these genes in either patient.

Subsequently, we demonstrated the involvement of additional enzyme complexes of the respiratory chain in both patients. Biochemical and immunoblot analysis revealed a generalized respiratory chain dysfunction involving complexes I, III and IV (COX), resulting from a marked reduction in the steady-state levels of several nuclear and mtDNA-encoded protein subunits. The levels of the nuclear-encoded α subunit of complex V were normal, presumably because assembly intermediates of the F1 portion of this enzyme complex can form independently of the F0 portion, which contains the mtDNA-encoded subunits (13). The activity of complex V was not measured. Although no pathogenic mutations were identified in the mitochondrial tRNAs of either patient, because of the presence of ragged-red fibres in the muscle of both patients we assessed mitochondrial translation. Normal levels of mitochondrial translation products were present in the myoblasts; however, myotubes of both patients showed a generalized mitochondrial translation defect, which moved with the nucleus in cybrid cells. Over 30 mtDNA protein synthesis mutations have been described so far (14); however, nuclear gene defects that impair mitochondrial translation have not been reported in patients with mitochondrial disorders. A mutant allele of a subunit of the nuclear-encoded mitochondrial RNase-P has been shown to produce reduced translation of the COX subunits encoded in the yeast mitochondrial genome (15).

Except for the tRNAs and rRNAs encoded in mtDNA, all of the elements necessary for mitochondrial translation are encoded by nuclear genes. Relatively little, however, is known
about the mechanism and control of mitochondrial translation. Much of the existing information about mitochondrial translation comes from in vivo studies carried out in the yeast Saccharomyces cerevisiae. Yeast mitochondrial mRNAs are uncapped, and display 5′-untranslated regions (5′-UTRs) ranging from 50 to over 900 bases, which lack typical Shine–Dalgarno sequences (16,17). Translation initiation of yeast mitochondrial mRNAs requires membrane-bound, mRNA-specific activator proteins (16–18). These activators recognize specific sequences in the 5′-UTRs of individual mRNAs, and subsequently mediate the interactions between the mitochondrial mRNA, the small subunit of the mitochondrial ribosome, and the inner mitochondrial membrane. This could allow for cotranslational insertion of the highly hydrophobic mitochondrial gene products in the inner mitochondrial membrane. Recent evidence (16) suggests that yeast mitochondrial ribosomes recognize a common feature of all mRNA 5′-UTRs, which, in conjunction with mRNA-specific translational activation, is required for the initiation of mitochondrial translation. It is not known how either initiation of translation or selection of the initiation codon takes place in mammalian mitochondria. As in yeast, mammalian mitochondrial mRNAs are uncapped; however, unlike yeast mitochondrial mRNAs, mammalian mitochondrial mRNAs lack 5′-UTRs altogether. If translational activators exist in mammals, they must operate through other parts of the mRNAs. Sequences downstream from the initiation codon could be involved in ribosome binding, as is seen in Escherichia coli for some leaderless mRNAs (17).

Although mitochondrial protein synthesis was impaired in myotubes from the patients we investigated, the steady-state levels of mitochondrial and nuclear subunits of complexes I, III and IV, as well as the respiratory chain activity, were normal in both patients. This could be explained by an increased stability of the mRNA coding for these subunits, or by a longer half-life of the corresponding protein subunits, or both. An increase in the stability of nuclear transcripts encoding subunits of the respiratory chain enzymes has been demonstrated following inhibition of mitochondrial protein synthesis in HepG2 cells (19). Control levels of nine transcripts encoding different subunits of complexes II (SDH), IV (COX) and V (ATP synthase) were present 11 days after inhibition of mitochondrial translation. At the protein level, it was demonstrated that the half-life of heme-containing proteins in the respiratory chain, such as cytochrome c, is about 1 week or more (20–22). The myotubes analyzed in this study were generated by fusion of patient myoblasts for a maximum of 6 days, which may not have been long enough for the effect of the translation defect to manifest itself at the protein level.

The contrasting results from the patient muscle biopsy and cultured muscle cells prompted us to test whether the deficiency responsible for the observed mitochondrial myopathy was developmentally regulated. Myoblasts from patient L, displaying normal mitochondrial protein synthesis and control levels of subunits of the respiratory chain enzymes, as well as normal respiratory chain function, were allowed to differentiate in vivo in the muscle bed of SCID mice to mature muscle fibres. One month later, the resulting fibres displayed a severe COX deficiency and SDH hyper-reactivity, similar to that observed in the patient's muscle. The amount of the mtDNA-encoded COII subunit of COX was decreased to the same level in the transplanted fibres and in patient muscle. However, the level of the nuclear-encoded COIV COX subunit was about 40% of control in the transplanted fibres, compared with less than 10% of control in the patient muscle. This result is compatible with a primary defect of mitochondrial translation, and suggests that more than a month might be required for the nuclear subunits to decrease to the levels present in patient muscle.

There are numerous examples of muscle-specific genes that are developmentally regulated, and display isoform switching. Many of these genes encode contractile proteins or enzymes involved in energy metabolism. There are several developmentally regulated isoforms of myosin heavy chain, a major component of the contractile apparatus. An embryonic isoform, is replaced with neonatal isoforms during development, and these are later replaced by fibre-type specific adult isoforms (23,24). It has been proposed that the functional significance of these complex changes might lie in the different physiological needs of fetal, neonatal and adult muscles (25). Troponin T, another contractile protein, also undergoes complex changes in isoform type during development (26). Several enzymes of energy metabolism switch from their fetal, or ubiquitous, non-muscle isoforms to the corresponding muscle isoforms as development progresses. Examples of such enzymes include glycogen phosphorylase (27–29), aldolase A (29), creatine kinase (28,29), phosphoglycerate mutase (27), lactate dehydrogenase (27) and COX VIa (12). In all of the above cases, the isoform switch can be elicited in vitro in human muscle progenitor cells by withdrawing growth factors and promoting differentiation into myotubes, although the switch is not usually complete in these cells.

The complementation experiment that we performed by fusing myoblasts from the two patients showed a partial rescue of the mitochondrial translation defect, suggesting that the two patients might belong to different genetic complementation groups. The rate of translation of the three mtDNA-encoded COX subunits increased from about 30% of control to 50% of control rates following fusion and differentiation of myoblasts from the two patients. There are several possible explanations for this partial rescue. First, it is possible that the time between myoblast fusion and the analysis of mitochondrial translation was insufficient to allow complete complementation. Full complementation between different mutations in mtDNA following PEG-induced cell fusion has been shown to require 10–14 days in some systems (30,31) and in our case, the myotubes could not be kept on the plates for longer than 5–6 days. Second, although equal numbers of cells from each patient were mixed, we do not know if nuclei from the two patients contributed equally to the myotubes. If myoblasts from one patient contributed disproportionately, then partial complementation could result. Finally, it is possible that different mutations are present in the same gene in the two patients. Intragenic complementation can occur when, for instance, a multimeric protein is generated from subunits produced by different mutant alleles of the same gene. The resulting hybrid protein exhibits an activity greater than that found in the protein generated from each mutated allele alone, but less than the activity of the protein produced by the wild-type allele. Such a phenomenon has been reported in human argininosuccinate lyase (ASL) deficiency (32). When two mutant alleles
with 5% and less than 0.01% of wild-type activity were co-
expressed in the same cell, the resulting ASL hybrid exhibited 30% of wild-type activity.

Additional evidence for different molecular defects under-
lying the mitochondrial myopathy in the two patients is the
porin (also calledVDAC) deficiency in the skeletal muscle of
patient L, but not patient E. There are three ubiquitously
expressed isoforms of porin in mammals (VDAC1–3), and an
alternatively spliced variant of isoform 3, whose expression is
limited to brain, heart and skeletal muscle (33,34). The
physiological significance of these isoforms is not known.

The anti-porin antibody that we used for immunoblotting is
specific for isoform 1 of porin (VDAC1) (35). We do not know
if the deficiency in porin in patient L is compensated by
expression of other isoforms of the protein. A case of
mitochondrial encephalomyopathy associated with decreased
rates of pyruvate oxidation and ATP production and porin
deficiency has previously been reported (36,37). VDAC1 was
undetectable in the skeletal muscle of this patient; however, no
mutation could be detected in the VDAC cDNA, either in the
coding or in the untranslated regions (38). These and our
observations raise the possibility of a deficiency in the porin 1
isoform as a contributor to mitochondrial dysfunction in some
cases of mitochondrial myopathy.

The results that we present here suggest the existence of at
least one developmentally regulated nuclear factor involved in
mitochondrial translation in skeletal muscle, which is mutated
in the two patients. One possibility is that such a factor
switches between a fetal, ubiquitously expressed, isoform and
an adult isoform of the protein in skeletal muscle. During
muscle differentiation, the adult form of the protein would
become more important, leading to a translation defect and
ultimately to impaired oxidative phosphorylation. Since defects
in oxidative phosphorylation appear to play an important role
in the pathogenesis of sideroblastic anemia (39,40), it is very
likely that the sideroblastic anemia in patient E is caused by the
same genetic defect responsible for the myopathy in this
patient, so the importance of the translation factor may not be
strictly limited to skeletal muscle. We are currently attempting
to map the nuclear gene involved in the mitochondrial
myopathy observed in patient L through microcell-mediated
chromosome transfer, by following complementation of the
mitochondrial translation defect.

**MATERIALS AND METHODS**

**Patients**

Patient L is a French Canadian woman, 24 years old at the time
of the biopsy, with gradual onset of limb muscle weakness from
age 5 years. Fatigability was a problem, with dyspnea on mild
exertion. Physical examination was impressive for short stature,
markedly diminished muscle bulk and diffuse hypotonia.
Strength testing showed both proximal and distal weakness,
although more significant proximally. The rest of the
neurological examination was normal, including reflexes and
sensation, although gait was impaired owing to leg muscle
weakness. Magnetic resonance spectroscopy showed an
abnormal phosphorus spectrum at rest and during recovery.

Resting venous lactate was elevated (4.6 mM). Electron
microscopy of the left biceps brachii muscle showed sub-
sarcomemmal collections of mitochondria with numerous
paracrystalline inclusions. There is no apparent family history
of this disorder; both parents and the only sibling are healthy.

Patient E is a man of Lebanese descent, 34 years old at the
time of the biopsy. Early development was normal; however, he
was never able to run as fast as others, nor could he lift heavy
weights. He developed occasional muscle stiffness and cramps
after running. Although his muscles remained small, there was
no significant change in his condition until age 31 years. At
that time, he developed exertional dyspnea and chest
discomfort on two separate occasions and was diagnosed with
sideroblastic anemia. His examination was impressive for
asymmetric ptosis and limitation of lateral gaze bilaterally.
Muscular atrophy was diffuse and symmetrical. Weakness was
present proximally in the upper extremities and distally in the
lower extremities. Reflexes were absent. The rest of the
neurological examination was normal. Magnetic resonance
spectroscopy of the gastrocnemius muscle showed an abnormal
phosphorus spectrum at rest and extremely slow recovery of
ADP and phosphocreatine after exercise. Magnetic resonance
imaging of the brain showed small external eye muscles but no
other abnormality. Resting venous lactate was elevated
(3.8 mM). Electron microscopy of the left biceps brachii muscle
revealed a subsarcomemmal accumulation of morphologically
abnormal mitochondria, many containing paracrystalline in-
clusions. The patient is originally from a small village, and
although there is no evidence of consanguinity, it cannot be
ruled out. Family history is negative.

**Immunofluorescence, immunohistochemistry and
histochemistry**

Cryostat sections (5 μm) were fixed in ice-cold acetone for
2 min and subsequently reacted with antibodies raised against
the mtDNA-encoded COII subunit of COX and the 70 kDa
nuclear C-II-70kD subunit of SDH. The anti-human COII
rabbit polyclonal antibody (a kind gift from Dr A. Lombes) was
used at 1:3000. The anti-human C-II-70kD mouse monoclonal
antibody (a kind gift from Dr R. Capaldi) was used at 1:100.
Fluorophore-conjugated Alexa 488 goat anti-rabbit and goat
anti-mouse antibodies from Molecular Probes were used as
secondary antibodies as appropriate at 1:200. For
immunohistochemistry and histochemistry

To detect human dystrophin in fixed, 8 μm cryostat sections of
transplanted mouse muscle, an anti-human dystrophin,
NCL-Dys 3, monoclonal antibody (Novocastra) was used at
1:10. A horse anti-mouse biotinylated antibody from Vector
Laboratories was used as a secondary antibody, at 1:200. For
amplification of the signal, a streptavidin-conjugated horse-
radish peroxidase from Vector Laboratories was used at
1:300. Immunoreactivity was visualized using Sigma Fast with
the metal enhancer set from Sigma-Aldrich. In double-labeling
experiments, fluorophore-conjugated Alexa 594 goat anti-
mouse antibody from Molecular Probes was used as the
secondary antibody, at 1:100.

COX and SDH activities were assessed as described in (41).
Biochemical studies – preparation of mitochondria

Mitochondria were isolated from cultured cells and from frozen muscle as described in (42) and (43), respectively. Complex I activity was assessed as presented in (44). The activity of complex II + III was monitored at 550 nm, at 30°C. To disrupt the mitochondrial membrane, mitochondrial fractions were freeze–thawed three times, and 30 μg of mitochondrial protein were used per assay. The 1 ml reaction mixture also included 50 mM potassium phosphate buffer (pH 7.5), 25 mM sodium succinate, 0.1 mM cytochrome c, 0.3 mM KCN and 3 μg/ml rotenone. The reaction was started by the addition of cytochrome c, and the activity was calculated by using the extinction coefficient of 19.6 mM⁻¹ cm⁻¹ for cytochrome c. The activities of COX and SDH were measured as described in (5).

Immunoblotting

Total protein was extracted from cultured cells and cryostat sections of frozen muscle using the procedure outlined in (45). Western blotting was carried out as described in (45), except that precast 10–20% Tris–Tricine gradient gels (BioRad) were used (run as suggested by the manufacturer). The following primary mouse monoclonal antibodies were a kind gift from Dr R. Capaldi: anti-C-II-70kD, used at 1 : 18000; anti-C-III-Core 1, used at 1 : 200; anti-C-IV-COII, used at 1 : 900; anti-C-IV-COIV, used at 1 : 4400, and anti-C-V-Alpha, used at 1 : 2000. An anti-porin 31HL mouse monoclonal antibody from Calbiochem was used at 1 : 1800. Rabbit polyclonal antibodies – d-SARC, anti-δ-sarcoglycan, mouse monoclonal antibody from Novocastra, used at 1 : 25, and an anti-COII rabbit polyclonal antibody (also used in the immunofluorescence experiments), used at 1 : 800. For amplification and detection of the signal, we used the ECF western blotting kit from Amersham Life Science.

Muscle cell culture

Primary myoblast cultures were established from the biopsy specimens of the two patients and ultimately purified by fluorescence-activated cell sorting (FACS) (46). Immortalization of myoblasts was performed as described in (7). Myotubes were generated by culturing the myoblasts for 3–6 days in differentiation medium, containing DMEM, 2% horse serum and 0.4 μg/ml dexamethasone.

Pulse labeling of mitochondrial translation

Labeling of mitochondrial translation in myoblasts and myotubes was performed as described in (47). Briefly, cells were labeled for 60 min at 37°C in methionine-free DMEM containing 200 μCi/ml [35S]methionine and 100 μg/ml emetine and chased for 10 min in regular DMEM. Total cellular protein (50–60 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 s and loaded and run on 12–20% polyacrylamide gradient gels.

Generation of cybrids

Immortalized myoblasts that were chosen to serve as the nucleus donor were grown for 7–12 days in growth medium containing 4–8 μg/ml rhodamine-6-G. The rhodamine-containing medium was changed every 2–3 days. As demonstrated in (8), after growing for a week in medium containing 4 μg/ml rhodamine-6-G, myoblasts contain compromised mitochondria only, and eventually die, unless rescued by fusion with cells containing healthy mitochondria. The enucleation of the primary myoblasts chosen to serve as the mitochondrial donor, as well as the fusion with the nucleus donor, were carried out as described in (48). The resulting cybrids were selected in 400 μg/ml G-418 for 3 weeks.

Restriction fragment length polymorphism (RFLP) analysis

Genomic DNA was extracted from cells, using the standard, phenol–chloroform method. The mitochondrial Thr, Pro tRNA was amplified through PCR from genomic DNA using primers described in (49). The PCR was carried out in a total volume of 50 μl, containing 0.5 μl genomic DNA, 8 μl dNTPs, 2.5 μl MgCl2, 5 μl 10× buffer, 1 μl of each of the primers, 0.25 μl Taq enzyme and 31.75 μl water. PCR conditions were 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, for 30 cycles. Fifteen microlitres of the PCR product was incubated at 37°C, overnight, with 2 μl of NEB4 buffer, 1 μl of NaeI (from NEB) and 2 μl water. Ten microlitres of the total 20 μl digest were run on a 1% agarose gel.

Transplantation experiments

Four-week-old CB-17 Fox–Chase SCID mice were purchased from Charles River Canada. Transplantation experiments were carried out as described in (50). Briefly, the left posterior leg of each mouse was irradiated with 20 Gy of gamma radiation. Seven to ten days later, the tibialis anterior muscle of the irradiated leg was injected with 2–2.3 × 106 primary myoblasts, resuspended in 10 μl of notexin (10 μg/ml). Seven to ten days after the first myoblast injection, the muscle was re-injected with approximately the same number of cells, resuspended in 10 μl HBSS. One month after the initial myoblast injection, the animals were euthanized, and the injected muscles were removed. The muscles were frozen in liquid-nitrogen-cooled isopentane, and stored at –80°C.

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