The 2-thiouridylase function of the human MTU1 (TRMU) enzyme is dispensable for mitochondrial translation

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MTU1 (TRMU) is a mitochondrial enzyme responsible for the 2-thiolation of the wobble U in tRNA⁹⁸, tRNA⁹¹ and tRNA⁹²⁸, a post-transcriptional modification believed to be important for accurate and efficient synthesis of the 13 respiratory chain subunits encoded by mtDNA. Mutations in MTU1 are associated with acute infantile liver failure, and this has been ascribed to a transient lack of cysteine, the sulfur donor for the thiouridylation reaction, resulting in a mitochondrial translation defect during early development. A mutation in tRNA⁹¹ that causes myoclonic epilepsy with ragged-red fibers (MERRF) is also reported to prevent modification of the wobble U. Here we show that mitochondrial translation is unaffected in fibroblasts from an MTU1 patient, in which MTU1 is undetectable by immunoblotting, despite the severe reduction in the 2-thiolation of mitochondrial tRNA⁹⁸, tRNA⁹¹ and tRNA⁹²⁸. The only respiratory chain abnormality that we could observe in these cells was an accumulation of a Complex II assembly intermediate, which, however, did not affect the level of the fully assembled enzyme. The identical phenotype was observed by siRNA-mediated knockdown of MTU1 in HEK 293 cells. Further, the mitochondrial translation deficiencies present in myoblasts from mitochondrial encephalomyopathy, lactic acidosis and stroke-like episode and MERRF patients, which are associated with defects in post-transcriptional modification of mitochondrial tRNAs, did not worsen following knockdown of MTU1 in these cells. This study demonstrates that MTU1 is not required for mitochondrial translation at normal steady-state levels of tRNAs, and that it may possess an as yet uncharacterized function in another sulfur-trafficking pathway.

INTRODUCTION

The 2008 update of MODOMICS, a database of RNA modification pathways, identified 119 different posttranscriptional modifications in all types of RNA, the largest number of which are present in tRNAs (1). The uridine at position 34 (U34), the first (wobble) position of the anticodon in the tRNAs for Lys, Glu and Gln, is almost universally modified at carbons 2 and 5. While carbon 2 is modified exclusively through thiolation (s²), various methyl derivatives can be found at carbon 5 (xm⁵), examples of which include methylaminomethyl (mm⁵) and carboxymethylaminomethyl (cmnm⁵) in the bacterial tRNAs, methoxycarbonylmethyl (mcm⁵) in the cytoplasmic tRNAs of eukaryotes (2) and taurinomethyl (tm⁵) in the mammalian mitochondrial tRNAs (3). The tRNAs for Lys, Glu and Gln recognize each a set of two codons which are part of split codon boxes (coding for two amino acids) and which end in a purine. The xm⁵ s² modification was postulated to confer conformational rigidity to the U34 wobble base of these tRNAs, leading to preferential pairing with purines, and preventing misreading of the near-cognate codons ending in pyrimidines (4). In contrast, a study in E. coli demonstrated increased misreading by wild-type (fully modified), when compared with hypomodified, tRNA³⁸ of the near-cognate codons which end in a pyrimidine and which code for asparagine (5). In this case, the hypomodified forms of tRNA³⁸ were carrying either the mm⁵U34 or the s²U34 modification. An alternative role for the U34 wobble base modifications in increasing the efficiency, rather than the accuracy of translation, was suggested by

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several studies, including the one in which the lethal phenotype of a yeast strain containing a double deletion for enzymes involved in the modifications of the cytoplasmic tRNAs at carbons 2 and 5 was rescued by overexpression of the unmodified forms of tRNA^{1-ys} and tRNA^{6Gln} (6).

Aside from directly modulating the accuracy or efficiency of translation, the 2-thio group has been shown to be important for the recognition of tRNA^{Glu} by the E. coli glutamyl-tRNA synthetase (7), for ribosome binding of E. coli tRNA^{Glu} at the appropriate codons (8), for promoting bacterial growth at higher temperatures through thermal stabilization of tRNAs (9) and for improving reading frame maintenance (10).

The biosynthesis of s^{2}U34 in E. coli requires the 2-thiouridylase MnmA and the cysteine desulfurase IscS (11), as well as a complex sulfur-relay system composed of the products of five genes: tusA, tusB, tusC, tusD and tusE (12). Recently, the sulfur-relay system required for 2-thiouridine biosynthesis at the wobble position of cytoplasmic tRNAs in Saccharomyces cerevisiae was described (13). Given the lack of conservation of the Tus proteins even across bacteria (12), it is not surprising that the sulfur mediators and reaction mechanism are distinct in yeast and bacteria.

The mitochondrial tRNA-specific 2-thiouridylase, MTU1 (also known as TRMU or MTO2) is the homolog of the bacterial MnmA (14). Although the official symbol for the human gene is TRMU, for ‘tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase’, we selected for the purpose of this work the symbol MTU1, which describes accurately the function of this enzyme, namely thiouridylase, rather than methyltransferase. The enzyme responsible for 2-thiouridylation of tRNAs in the eukaryotic cytoplasm, TUC1/NCS6, is the homolog of the E. coli TucA enzyme, which is required for the synthesis of 2-thiocytidine at position 32 in a few bacterial tRNA species, a modification absent in yeast (6). Studies in yeast have demonstrated that deletion mutants of MTU1 display impaired 2-thio modification of the mitochondrial tRNAs for Lys, Glu and Gln (14) and a decrease in steady-state levels of these and other mitochondrial tRNAs (15), resulting in a severe defect in mitochondrial translation and impaired growth on non-fermentable carbon sources (14,16). Mutations in MTU1 have also recently been reported in acute infantile liver failure resulting from deficiencies in multiple respiratory chain enzymes (17,18).

Lack of post-transcriptional modifications at the wobble positions of mitochondrial tRNAs for Leu and Lys has been associated with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged-red fibers (MERRF), respectively (19). In MELAS, lack of the taurine modification at the wobble position of tRNA^{Leu} harboring either the A3243G or the T3271C mutation leads to severely reduced translation of UUG (but not UUA) codons (20). In MERRF, both s^{2} and m^{5} modifications are absent in the tRNA^{Lys} bearing the A8344G mutation (21), leading to impaired decoding of both codons for lysine (22). Based on studies in E. coli showing the pivotal role of 2-thiolation at the wobble position of tRNA^{Lys} in efficient decoding of lysine codons (8), it has been suggested that the lack of the s^{2} modification in the mutant A8344G tRNA^{Lys} might be central to the decoding deficiency in MERRF. However, this has not been tested directly. One study performed in human cells showed that HeLa cells in which MTU1 was knocked down display reduced 2-thiolation of the mitochondrial tRNA^{Lys}, decreased oxygen consumption and defective membrane potential (14).

In the present study, we knocked down MTU1 in different human cell types and assessed the effect on 2-thiolation of the three s^{2}-modified mitochondrial tRNAs and on mitochondrial translation in these cells and in fibroblasts from a patient carrying a pathogenic mutation in the MTU1 gene.

RESULTS

MTU1 is not essential for mitochondrial translation in HEK 293 cells

To investigate directly the involvement of human MTU1 in mitochondrial protein synthesis, we knocked down MTU1 in HEK 293 cells using three different siRNA constructs, and then pulse-labeled the mitochondrial translation products in these cells with 35[S]-(methionine/cysteine). All siRNA constructs tested were similar in efficiency and effectiveness in knocking down MTU1 below the detectable levels, as shown by immunoblot analysis with a specific anti-human MTU1 antibody (Fig. 1B). Surprisingly, this had no measureable effect on mitochondrial translation, which was similar in control and MTU1-knocked down cells (Fig. 1A).

Next, we assessed the steady-state levels and the extent of 2-thiouridylation of the mitochondrial tRNAs for Lys, Glu and Gln in the cells in which MTU1 had been knocked down, using tRNA\textsuperscript{Trp} for normalization of the signal. To this end, we performed [(N-acryloylaminophenyl)mercuric chloride (APM)-northern blot analysis, in which the organometallic compound APM (23) is polymerized in the gel, resulting in specific retardation of thio-modified tRNAs through its binding to the sulfur in the tRNA. While the tRNAs for Lys, Glu and Gln are almost completely modified (>90%) in HEK 293 cells, knocking down MTU1 resulted in a severe reduction in the 2-thiouridylation of all three mitochondrial tRNAs, with residual levels of <10% of total for tRNA\textsuperscript{Glu}, 20–25% of total for tRNA\textsuperscript{Gln} and 30–40% for tRNA\textsuperscript{Glu} (Figs 2B and 3B). As confirmed by analysis of gels from which APM was excluded, the steady-state levels of tRNA\textsuperscript{Glu} were normal, while those of tRNA\textsuperscript{Lys} and tRNA\textsuperscript{Gln} were decreased to 70–80% of control (Figs 2A and 3A). We conclude that MTU1 is dispensable for mitochondrial translation in HEK 293 cells, although it is clearly involved in the synthesis of 2-thiouridine at the wobble position of the mitochondrial tRNAs for Lys, Glu and Gln.

Blue native polyacrylamide gel electrophoresis analysis of oxidative phosphorylation assembly in MTU1-depleted HEK 293 cells

Given the role of MTU1 in sulfur trafficking, we tested whether the human enzyme might be further involved in the biogenesis of the oxidative phosphorylation (OXPHOS) complexes containing iron-sulfur clusters by blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis. On first inspection, the overall assembly of all five OXPHOS complexes appeared to be normal in HEK 293 cells in which MTU1 was knocked down. Longer exposure of the blot...
showed a subcomplex of Complex II, an enzyme complex containing two Fe–S clusters (Fig. 4), using an antibody against the 70 kDa subunit. When Complex II was detected with an antibody against its 30 kDa subunit, the specific absence of a different subcomplex was revealed in the MTU1-knocked down cells. This, however, did not result in a reduced amount of fully assembled Complex II.

**MTU1 is not essential for mitochondrial translation in human fibroblasts**

To test the validity of our findings in other cell types, we analyzed patient fibroblasts in which a compound heterozygous mutation in the *MTU1* gene was described previously (17,24). We confirmed that, of the two mutations described in this case, only the 9 bp allele in-frame insertion is expressed. The other, a 1 bp insertion causing a frameshift and premature stop probably leads to nonsense-mediated mRNA decay. Mitochondrial translation in patient fibroblasts appeared to be normal (Fig. 5A), despite the complete absence of immunodetectable MTU1 protein (Fig. 5B). BN-PAGE analysis revealed normal assembly of the five OXPHOS complexes in patient fibroblasts, as well as the presence of a subcomplex of Complex II of the same appearance and size as that described above in HEK 293 cells (Fig. 5C).

Next, we assessed the steady-state levels and the extent of 2-thiouridylation of the mitochondrial tRNAs for Lys, Glu and Gln. Total RNA was extracted from HEK 293 cells transiently transfected with the siRNA constructs used in Figure 1, and 5 μg total RNA/sample were run on a 10% polyacrylamide gradient gel in the absence (A) or the presence (B) of 1 μg/ml APM. After transfer to a nitrocellulose membrane, hybridization was performed with oligonucleotide probes complementary to mitochondrial tRNAs for Lys, Glu and Gln, using tRNA^Trp^ as a loading control. The thiolated and unmodified forms are indicated at the right of the figure.
and Gln in the patient fibroblasts, as done in the HEK 293 cells (Fig. 5D). Similar to the observations made in HEK 293 cells, 90% of the tRNAs for Lys, Glu and Gln are 2-thio modified in control fibroblasts, while in patient fibroblasts, the modification is either completely (tRNALys) or almost completely (tRNAGlu and tRNAGln) abolished (Fig. 5D and E). The total levels of these tRNAs are similar in patient and control fibroblasts (Fig. 5E). Thus, a lack of immunodetectable MTU1 protein in patient fibroblasts, which essentially prevents the 2-thio modification, has no effect on mitochondrial protein synthesis.

Mitochondrial translation levels are unchanged following knockdown of MTU1 in myoblasts derived from patients with MELAS and MERRF

We next tested whether knocking down MTU1 in cells derived from patients with an existing defect in mitochondrial protein synthesis would lead to a further decrease in their levels of mitochondrial translation products. For this purpose, we used homoplasmic mutant myoblast clones derived from a MERRF patient with the A8344G mutation and a MELAS patient with the A3243G mutation, both of which are known to be deficient in post-transcriptional modifications of their mitochondrial tRNAs. At the same time, this analysis allowed us to assess the consequences of knocking down MTU1 in additional human cell types and compare them to the effects observed in the HEK 293 cells and fibroblasts.

Unexpectedly, the steady-state levels of MTU1 protein in MERRF and MELAS myoblasts were decreased when compared with wild-type cells on immunoblot analysis (Fig. 6B). A further decrease in the levels of MTU1 could be achieved using siRNA knockdown in mutant (especially MELAS) myoblasts, while in the wild-type cells, the knockdown lowered MTU1 levels to the levels observed in mutant cells (Fig. 6B). Despite this reduction in parental levels of MTU1, mitochondrial translation appeared to be normal in the wild-type myoblasts, and there was no evidence of a further decrease in the levels of mitochondrial translation products synthesized in the mutant cells (Fig. 6A and D). The assembly of all OXPHOS complexes was equally unaffected by the knockdown of MTU1 in wild-type and mutant myoblasts, as shown by BN-PAGE analysis (Fig. 6C). Longer exposure of the blots did not reveal a subcomplex of Complex II, perhaps because of the less-efficient knockdown of MTU1 in myoblasts when compared with HEK 293 cells or the MTU1 patient fibroblasts.

Next, we used APM-northern blotting analysis to assess the 2-thiouridylation status and the levels of mitochondrial tRNAs for Lys, Glu and Gln in wild-type and mutant myoblasts (Figs 7 and 8). Similar to HEK 293 cells and fibroblasts, about 90% of the mitochondrial tRNAGln is 2-thio modified in myoblasts; however, only 70% of the tRNALys and 80% of the tRNAGlu appear to be thiouridylated at the wobble position in myoblasts. As expected, both the total levels and the percentage of thiouridylated tRNALys are decreased in the MERRF myoblasts: the total amount in the mutant is between 20 and 50% of the amount in wild-type cells, of which only 30–40% is thio modified.

Knockdown of MTU1 in wild-type and MELAS myoblasts resulted in a decrease in the thiouridylation fraction of tRNALys from 70 to 30–40% of total, which is similar to the percentage observed in the MERRF myoblasts (Figs 7 and 8B). The total levels of tRNALys were unaffected by the knockdown in the wild-type cells and decreased to 70% of the transfection control in the MELAS myoblasts (Figs 7 and 8A). No further decrease in total levels or the thyouridylated fraction could be observed for tRNALys in MERRF myoblasts following knockdown of MTU1. Although the effects of the knockdown on the 2-thio modification of the tRNAGlu and tRNAGln were more subtle than for tRNALys, they were, nevertheless, observed consistently in all the cells tested: the 2-thio-modified fraction decreased from 80 to, on average, 60% for tRNAGlu, and from 90 to, on average, 80% of total for tRNAGln (Figs 7 and 8B). The total levels of the tRNAGlu and tRNAGln were, depending on the line tested, either unaffected or slightly decreased by the knockdown of MTU1.

DISCUSSION

In this study, we demonstrate that mitochondrial translation in three different human cell types is unaffected by the lack of
MTU1 protein, despite a resulting defect in the 2-thiouridylation of the wobble position of the mitochondrial tRNAs for Lys, Glu and Gln. In contrast, yeast ΔMTU1 deletion strains, which also show impaired 2-thiouridylation of the three mitochondrial tRNAs, have a severe defect in mitochondrial protein synthesis (14,16). One likely explanation for this discrepancy is that ΔMTU1 mutant yeast have an additional, significant reduction in the steady-state levels of the total amount of the three 2-thio-modified mitochondrial tRNAs, with residual levels between 19 and 37% of control (15), whereas the total steady-state tRNA levels (thiolated and unmodified) in human cells are only slightly reduced. This logic supports a role for the 2-thio modification in increasing tRNA-decoding efficiency.

Several studies suggest a central role for post-transcriptional modifications, in particular 2-thiouridylation of tRNAs, in increasing the efficiency of translation. Viability of a yeast strain lacking the mcm5 and s2 modifications at the wobble positions of their cytoplasmic tRNAs can be restored by the overexpression of the unmodified tRNALys and tRNAGln (6). Indeed, in this context, if post-transcriptional modifications were important in preventing misreading, over-expression of the unmodified forms of the tRNAs might have been detrimental, and not beneficial to the cell. In another study, deletion of any one of the three genes required for the formation of either the mcm5 or the s2 modifications in the S. cerevisiae cytoplasmic tRNAs resulted in similar phenotypes, including defects in transcription, chromatin remodeling and exocytosis, all of which could be suppressed by elevated levels of hypomodified tRNA^{Lys} and tRNA^{Gln} (containing either only the s2 or only the mcm5 modification, respectively) (25). Another report demonstrated the presence of the cmnm5s2U modification at the wobble position of the tRNAs for Leu and Trp and the absence of 2-thiolation at the wobble base of tRNAs for Lys, Glu and Gln in nematode mitochondria (26). The mitochondrial genes of nematodes contain an unusually high percentage of leucine codons, and infrequent codons for Lys, Glu and Gln (27,28), suggesting a role for post-transcriptional modification in increasing the efficiency of tRNAs decoding high usage codons.

Other cellular functions apart from mitochondrial translation might be affected by the knockdown of MTU1. We hypothesized that MTU1 might be involved in the assembly of enzyme complexes containing iron–sulfur clusters. We identified a subcomplex of Complex II of the respiratory chain in HEK 293 cells and in fibroblasts lacking immunodetectable MTU1 protein as a result of either knockdown or the pathogenic mutation. The subcomplex appeared as a doublet at ~70 kDa. Since the detection was performed with an antibody against the 70 kDa subunit of Complex II, the doublet might indicate an early assembly intermediate containing this particular subunit. When Complex II was detected with an antibody against its 30 kDa subunit, an assembly intermediate of ~70 kDa normally found in HEK 293 cells was absent upon knockdown of MTU1. Since the 30 kDa subunit contains the Fe–S clusters of Complex II, it is conceivable that the lack of MTU1 interferes with the addition of the clusters onto this subunit. Fully assembled Complex II (140 kDa) likely results from the assembly of the subcomplexes detected with the antibodies against the 30 kDa subunit of Complex II, the doublet might indicate an early assembly intermediate containing this particular subunit. When Complex II was detected with an antibody against its 30 kDa subunit, an assembly intermediate of ~70 kDa normally found in HEK 293 cells was absent upon knockdown of MTU1. Since the 30 kDa subunit contains the Fe–S clusters of Complex II, it is conceivable that the lack of MTU1 interferes with the addition of the clusters onto this subunit. Fully assembled Complex II (140 kDa) likely results from the assembly of the subcomplexes detected with the antibodies against the 30 kDa and the 70 kDa subunits, as each is ~70 kDa. Thus, in cells in which MTU1 is knocked down, the absence of the subcomplex containing the 30 kDa subunit would result in unassembled subcomplex containing the 70 kDa subunit. Although fully assembled Complex II was present in normal amounts in these cells, the presence of the subcomplex containing the 70 kDa subunit was specifically
and consistently observed in patient fibroblasts and with each of the three siRNA constructs in HEK 293 cells, suggesting that MTU1 could play some other role in sulfur trafficking.

Bifunctional enzymes involved in post-transcriptional modifications of tRNAs have already been described; for instance, the \textit{E. coli} methyltransferase MnmC was shown to catalyze two reactions in the same pathway through two distinct domains with independent enzymatic functions (29,30). Another bacterial methyltransferase, the \textit{trmA} gene product, which is involved in the synthesis of 5-methyluridine, was...
shown to be essential for viability in *E. coli*; however, a strain containing a mutation in this gene is viable despite the lack of the 5-methyluridine modification in its tRNA, suggesting that the enzyme has a second function essential for viability (31). Similarly, deletion of the *truB* gene encoding pseudouridine synthase in *E. coli* results in growth defects that can be rescued by a *truB* gene containing a mutation in the active side of the enzyme (32), again pointing to an additional function of this particular enzyme. In the latter two examples, the authors speculate that this additional function could be that of RNA chaperone, either for RNA components of the ribosome or for tRNAs.

Another way in which cellular functions apart from mitochondrial translation could be affected by the knockdown of MTU1 is if the 2-thiolated mitochondrial tRNAs serve...
other functions in addition to their role in protein synthesis. A few such examples have, in fact, been described: aminoacyl-tRNAs have been shown to participate in N-terminal protein modification (33) which signals protein degradation (34,35), in porphyrin biosynthesis (36,37), in the aminoacylation of phospholipids in the cell membrane (38) and in the crosslinking of peptidoglycan in the cell walls of Gram-positive bacteria (39). Chloroplast tRNA\textsubscript{Glu} containing the mnm\textsubscript{5}s\textsuperscript{2} modification at the wobble position is required in the first step of chlorophyl biosynthesis (40), and it is possible that post-transcriptional modifications of tRNAs are relevant to such processes.

Mutations in \textit{MTU1} were recently shown (17,18) to be associated with acute infantile liver failure with a reversible outcome. In one study (18), the authors demonstrated a reduction in the extent of 2-thiolation of mitochondrial tRNAs for Lys, Glu and Gln to 20–30% of control and modest, nonspecific decreases in the total levels of mitochondrial tRNAs in patient fibroblasts. Symptomatic individuals had multiple OXPHOS enzyme deficiencies in liver and muscle, which were, however, reversible. The limited availability of cysteine, the sulfur donor for MTU1, during the neonatal period, was suggested as a possible pathogenic mechanism. The gradual increase in the enzymes involved in the endogenous synthesis of cysteine over the first months of life would then explain the reversibility of the biochemical phenotype. The present study shows that lack of MTU1 does not impair mitochondrial translation in three different cell types, including myoblasts, but we cannot of course exclude such a defect in the neonatal liver. It seems unlikely that a

Figure 8. Quantification of the relative, total levels (A) and the percentage of thioerydilation (B) of the mitochondrial tRNAs for Lys, Glu and Gln in wild-type (WT 1 and WT 2) and mutant (MELAS and MERRF) myoblasts in which MTU1 was knocked down (Fig. 7).
defect in Fe–S biosynthesis is responsible for the enzyme deficiencies in the patients as Complex IV, which does not contain an Fe–S center, is affected in the patients. What then could be the mechanism? One possible explanation would be limiting steady-state levels of total mitochondrial tRNAs during early development. If, as we have argued above, the primary role of wobble U thiolation is to increase tRNA-decoding efficiency, a translation defect would only be observed in the absence of MTU1 when the tRNAs that are modified become limiting. This idea could be tested by measuring total tRNA levels in affected tissues from MTU1 patients, or by reducing the amount of charged tRNAs in cell culture models of MTU1 deficiency.

MATERIALS AND METHODS

Cell culture and siRNA transfection

Fibroblasts and myoblasts were immortalized by transduction with a retroviral vector expressing the HPV-16 E6 and E7 genes and with another one expressing the catalytic component of human telomerase (hTERT) (41). Cells were grown at 37°C and 5% CO2, either in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (fibroblasts and HEK 293 cells) or, for myoblasts, in supplement grown medium as described previously (42).

Stealth RNAi duplexes 118697H06–118697H11 (Invitrogen) or the fluorescent oligo control Block-iT Alexa Fluor® Red (Invitrogen) were transiently transfected at a final concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s specifications. Transfection was repeated on Day 3, and cells were harvested and analyzed on Day 6.

Anti-MTU1 antibody production, purification and immunoblot analysis

The synthetic peptide CESPSDSPED was conjugated to ImmunoGold™ Maleimide Activated mCKLH (Pierce) as specified by the manufacturer, and then injected into rabbits. Rabbits were boosted three times at intervals of 3–4 weeks, bled periodically, and screened for antibody response by immunoblot analysis. Anti-MTU1 polyclonal antibodies were affinity-purified from serum of immunized animals with SulfoLink Imject™ Kit (Pierce), then used for immunoblotting at a dilution of 1:1000.

For immunoblotting, protein extracts were prepared in 1.5% n-dodecyl maltoside/PBS from the following: whole HEK 293 cells, a mitochondria-enriched fraction of fibroblasts prepared by differential centrifugation or digitonized myoblasts [1.2 mg digitonin/mg protein (43)]. Ten to twenty micrograms of protein/sample were used for Tris–glycine SDS–PAGE, then transferred to a nitrocellulose membrane and used for detection of MTU1; of the 70 kDa subunit of Complex II, with a monoclonal antibody from Molecular Probes; or of porin, with a monoclonal antibody from Sigma (St. Louis, MO, USA).

Pulse-labeling of mitochondrial translation products and BN-PAGE

Pulse-labeling of mitochondrial translation products and BN-PAGE were carried out as described in detail elsewhere (44).