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Correction of the consequences of mitochondrial 3243A>G mutation in the *MT-TL1* gene causing the MELAS syndrome by tRNA import into mitochondria

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ABSTRACT

Mutations in human mitochondrial DNA are often associated with incurable human neuromuscular diseases. Among these mutations, an important number have been identified in tRNA genes, including 29 in the gene *MT-TL1* coding for the $tRNA^{Leu(UUR)}$. The m.3243A>G mutation was described as the major cause of the MELAS syndrome (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes). This mutation was reported to reduce $tRNA^{Leu(UUR)}$ aminoacylation and modification of its anti-codon wobble position, which results in a defective mitochondrial protein synthesis and reduced activities of respiratory chain complexes. In the present study, we have tested whether the mitochondrial targeting of recombinant tRNAs bearing the identity elements for human mitochondrial leucyl-tRNA synthetase can rescue the phenotype caused by MELAS mutation in human *trans*mitochondrial cybrid cells. We demonstrate that nuclear expression and mitochondrial targeting of specifically designed transgenic tRNAs results in an improvement of mitochondrial translation, increased levels of mitochondrial DNA-encoded respiratory complexes subunits, and significant rescue of respiration. These findings prove the possibility to direct tRNAs with changed aminoacylation specificities into mitochondria, thus extending the potential therapeutic strategy of allotopic expression to address mitochondrial disorders.

INTRODUCTION

The m.3243A>G mutation in the mitochondrial DNA (mtDNA) *MT-TL1* gene coding for mitochondrial $tRNA^{Leu(UUR)}$ ($mt\text{-}tRNA^{Leu(UUR)}$) was first identified as a genetic cause of mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS, MIM 5400 000) (1). It is one of the most common mitochondrial pathogenic mutations with a carrier frequency estimated in the range between 0.95 and 18.4/100 000 in northern European populations (2–4). Like many mutations affecting mitochondrial respiratory chain, the m.3243A>G mutation is associated not only with MELAS, but also with other clinical phenotypes, including CPEO (Chronic Progressive External Ophthalmoplegia), DMDF (Diabetes Mellitus and DeaFness), etc. (5). In all cases, the m.3243A>G mutation was present in a heteroplasmic state, which means the co-existence of mutant and wild-type mtDNA molecules in one cell. The proportion of mutant mtDNA molecules that leads to the manifestation of the disease varied strongly in different tissues (6). Patients with m.3243A>G mutation often show severe respiratory chain deficiency with complexes I and IV affected in a first place (7,8), but the precise mechanism connecting the mutation with clinical phenotypes is still

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not fully understood. Accumulated data, mostly obtained on *trans*mitochondrial cybrid cells, suggest a deficiency of aminoacylation of mutant mt-tRNA$^{Leu(UUR)}$ (9–13) and hypomodification of its anti-codon wobble position affecting recognition of UUG codons (14–17) to be the origin of a mitochondrial translation defect. This leads to a decrease of respiratory chain complexes steady-state levels (7,18) and affected respiration rate (11,19,20). The mitochondrial translation defect observed varied from moderate in some cell lines to severe in others. Moreover, different groups reported qualitatively different patterns of mitochondrial translation in cells bearing the m.3243A>G mutation. In association with the mutation, it has been observed that there was a specific decrease of polypeptides rich in UUG codons (for instance, ND6) and appearance of abortive translation products (18,20). In other reports, no qualitative differences or specific correlation between number of leucine UUR codons and level of synthesis of particular mitochondrial proteins were found (11,21). The data on amino acid misincorporation at UUR codons are also somewhat controversial (18,22,23).

Up to now, no efficient therapy for MELAS and other mitochondrial diseases has been demonstrated. Antioxidants and vitamins have been used, but there have been no consistent successes reported (24). Spindle transfer, where the nuclear DNA is transferred to another healthy egg cell leaving the defective mtDNA behind, is a potential treatment procedure that has been successfully carried out on monkeys (25). Using a similar pronuclear transfer technique, healthy DNA in human eggs from women with mitochondrial disease was successfully transplanted into the eggs of women donors who were unaffected (26). Embryonic mitochondrial transplant and protofection have been proposed as a possible treatment for inherited mitochondrial disease. Allotopic expression of mitochondrial proteins (i.e. expression of mtDNA-encoded mitochondrial proteins in the nucleus) was also tested as a radical treatment for mtDNA mutation load. Promising results were obtained with MELAS cybrid cells overexpressing the mitochondrial leucyl-tRNA synthetase (mt-LeuRS) (27,28). Authors observed an increase in steady-state level of aminoacylated mt-tRNA$^{Leu(UUR)}$, partial restoration of COX1, COX2 and ND1 steady-state levels and increase of respiration rate. Interestingly, the rate of mitochondrial protein synthesis was almost the same as that in parental cells bearing MELAS mutation. Authors suggested that mutation suppression occurred via a mechanism that increased protein stability rather than translation rate. In an independent study, overexpression of mitochondrial translation factors EFTu and EFG2 in myoblasts derived from a MELAS patient partially restored mitochondrial translation, steady-state levels of certain respiratory chain subunits, assembly and activity of the OXPHOS complexes (18). A similar approach was also formerly and successfully modelled in yeast (29).

In addition to proteins, human mitochondria also import from the cytosol small RNAs such as 5S rRNA (30–32,34), tRNA^{Gln} (33), or RNase P and MRP RNA components (35,36). Moreover, we previously demonstrated that yeast importable tRNA^{Lys} derivatives and some other small artificial RNA substrates could be imported into mitochondria after their expression in human cells (37,38). We have shown that yeast tRNA^{Lys} derivatives targeted to mitochondria of cybrid cells or patient fibroblasts with the m.8344A>G mutation in the mtDNA *MT-TK* gene coding for mt-tRNA^{Lys} (commonly associated with the MERRF syndrome) partially restored their mitochondrial translation, activity of respiratory complexes, electrochemical potential across the mitochondrial inner membrane and respiration rate (39). In order to enlarge the spectrum of mtDNA mutations addressed we investigated here the possibility to rescue the MELAS mutation by allotopic expression of recombinant and importable tRNAs whose aminoacylation identity had been changed from lysine to leucine.

MATERIALS AND METHODS

Cell culture

The MELAS cybrid cell line used in this study was kindly provided by E. A. Shoubridge (Montreal Neurologic Institute, Quebec, Canada). It carried 90 ± 5% of m.3243A>G mutation and was functionally characterized previously (40). They were generated by fusing rho^0 cells from osteosarcoma cell line 143B.TK$^-$ with cytoplasts from clonal primary myoblasts established from a patient carrying the m.3243A>G point mutation in *MT-TL1* gene (MELAS mutation) as described elsewhere (41). Cybrid cells were cultivated in DMEM medium with high glucose (4.5 g/l), sodium pyruvate (110 mg/l) and L-glutamine (2 mM) from Sigma, supplemented with 10% (w:v) fetal calf serum (FCS), 50 mg/ml uridine, standard concentrations of antibiotics (penicillin, streptomycin and fungizone) and, for stable transfectants, 2 μg/ml of puromycin. 143Brho$^+$ cells were used as healthy cell control and were cultivated in the same conditions as MELAS cybrid cells. HEK-293T cells were used for production of lentiviral particles and were cultivated in standard DMEM medium with 1 g/l glucose. All cell lines were cultivated at 37°C and 5% of CO_2.

Cell transfection

Transfection of MELAS cybrid cells with tRNA transcripts was performed using Lipofectamine2000 (Invitrogen) as described previously (32) with minor modifications: 1 μg of transcript and 12.5 μl Lipofectamine2000 were used per 2×10^6 cells. Transient transfection was performed with a mix of 4 μg of pBK-CMV-tRK plasmid and 12 μl of Lipofectamine2000 per 600×10^3 cells according to manufacturer protocol. Efficiency of transfection was estimated by FACS analysis of GFP expression from pmax-GFP plasmid transfected in parallel. MELAS cybrid cells stably expressing recombinant tRNAs were obtained by lentiviral transfection. Production of lentiviral particles was performed in HEK-293T cells using FuGENE6 transfection reagent (Roche Applied Sciences), 3 μg of pLKO.1-tRK (Addgene), 1.5 μg of pLP1, 0.75 μg of pLP2 and 0.75 μg pLP-VSGV packaging plasmids (Invitrogen) according to manufacturer protocol. Infection of MELAS cybrid cells was performed with

virus-containing medium from HEK-293T cells during 2–3 days. Cells containing transgenes were selected in the presence of 2μg/ml of puromycin during 2–3 days.

Construction of recombinant tRNA genes and plasmids

The hmtLeuRS gene without mitochondria-targeting sequence (186–302 nucleotides coding for the first 39 amino acids), was PCR-amplified from cDNA purchased from the RIKEN collection and cloned in the expression pET3a (Amp^r) vector.

Cloning of yeast tRK1, tRK2 (G1-C72; G73; U34) and tRK3 genes was performed previously (42). tRNA genes coding sequences were placed under control of T7 promoter in pUC19 (Amp^r) (Invitrogen), *Bst*NI site was introduced at its 3′-terminus to further generate the CCA-3′ sequence in the T7 transcript. Mutations aimed to change tRNA aminoacylation identity (Lys > Leu) were introduced by several steps of PCR-mutagenesis. Discriminator base A73—using oligonucleotides: tRK1-T7 GGGATCCATAATACGACTCACTATA GCCTT GTTGGCG, tRK1-A73-BstNI GGGATCCTGGTGC CCTGTAGGGGGCTCG, tRK2-G1-T7 GGGATCCA TAATACGACTCACTATAGCCTTGTTAGCTCAG, tRK2-C72A73-BstNI GGGATCCTGGTGCCTCATAG GGGGCTCG. Anti-codon substitution was performed by Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer protocol. The following pairs of oligonucleotides were used ('As' for forward and 'Br' for reverse): tRK1UAAAs GACTTAA AATCATAAGG, tRK1UAABr TATGATTTTAAGTC ATACGC, tRK1CAAAs GACTCAAAATCATAAGG, tRK1CAABr TATGATTTTGAGTCATACGC, tRK2UAAAs GTTCGGCTTAAAACCG, tRK2UAABr CATTTCGGTTTTAAGCCG, tRK2CAAAs GTTCGG CTCAAAACCG, tRK2CAABr CATTTCGGTTTTG AGCCG, tRK3UAAAs GTCTTAAAAGCAACCC, tRK3UAABr GCTTTTAAGACAAC, tRK3CAAAs CA GTTGTCTCAAAAGCAACCC, tRK3CAABr GGGTT GCTTTTGAGACAACTG.

For transient expression in MELAS cybrid cells, tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA genes we cloned in pBK-CMV (Kan^r) vector (Stratagene) in *Bgl*II/*Bam*HI (*Bgl*II in the fragment and cohesive site *Bam*HI - in the vector) sites using oligonucleotides: TRK1/F1-Aviv GGCAAGATCTGGTCAGATTTCCA ATAACAGAATATCCTTGTTAGCCTTGTTGGCG, TRK1/F1-A73-Bviv GGCAAGATCTGTCATCGTGTT TTAAAAAAAAAAAGAATGCCCTGTAGGGGGCT C, TRK2/F1-G1-Aviv GGCAAGATCTGGTCAGATTT CCAATAACAGAATATCCTTGTTAGCCTTGTTAG CTCAG, TRK2/F1-C72A73-Bviv GGCAAGATCTGTC AT CGTGTTTTAAAAAAAAAAAGAATGCCTCATA GGGGGCTCG, TRK3/F1-Aviv CCCAAGAGATCTG GTCAGATTTCCAATAACAGAATAGAGAATATTG TTTAATG, TRK3/F1-Bviv CCCAAGAGATCTGTCA TCGTGTTTTAAAAAAAAAAAGAATGAGAATAG CTGGAGTTG. tRNA genes were flanked by non-coding flanking regions of one of the well expressed tRK1 copies and were cloned in an opposite direction with respect to the CMV promoter in order to favour their transcription from internal promoter by RNA polymerase III (RpoIII) and further correct maturation.

For stable transfection tRK1UAA/CAA, tRK2UAA/CAA genes were cloned in plKO.1 (Amp^r) lentiviral vector (Addgene) (43) in *Age*I/*Eco*RI sites under the control of external U6-promoter without any flanking regions using oligonucleotides: trk1plkoAs GGCAACCGGTGC CTTGTTGGCG, trk1plkoBr GGCAGAATTCAAAAA TGCCCTGTAGGG, trk2plkoAs GGCAACCGGTGCC TTGTTAGCTCAG, trk2plkoBr GGCAGAATTCAAA AATGCCTCATAGGGGG.

Purification of hmtLeuRS and *in vitro* aminoacylation assay

His-tagged hmtLeuRS and hmtLysRS were purified to homogeneity from BL21 CodonPlus (DE3)-RIL *Escherichia coli* strain through nickel affinity chromatography, followed by protein concentration through Nanosep 30 K (Pall) columns, and stored as 40% glycerol solution at −20°C. Activities of different enzyme fractions were tested on commercially available preparation of *E. coli* tRNA. Aminoacylation of tRNA T7-transcripts was done according to the described procedure (44). Final conditions were: 50 mM HEPES–NaOH (pH 7.6), 25 mM NaCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/ml BSA, 0.8 μM [^{3}H]-Leu or 0.8 μM [^{3}H]-Lys (>400 Ci/mmole, NEC) and adapted concentrations of tRNA and enzyme. Aminoacylation rates and Km were measured as described elsewhere (45,46). Aminoacylation efficiencies of recombinant tRNA transcripts were compared to that of wild-type human mt-tRNA$^{Leu(UUR)}$ and mt-tRNA^{Lys} T7-transcripts.

Isolation and analysis of DNA

Total cellular DNA was isolated by standard procedures and the m.3243A>G mutation level was tested systematically by *Apa*I restriction analysis, as described elsewhere (47). Briefly, mutation containing mtDNA region was PCR-amplified using oligonucleotide primers: hp3081 G TAATCCAGGTCGGTTTCT and hp3380 CGTTCGGT AAGCATTAGG, PCR-products were digested by *Apa*I for 2 h and analyzed by gel-electrophoresis.

Isolation and analysis of total and mitochondrial RNAs

Total and mitochondrial RNAs were isolated by standard TRIzol-extraction (Invitrogen) from cells and purified mitochondria, respectively. Mitochondria were isolated from cells as described previously, using the differential centrifugation protocol (48). RNA preparations were analyzed by Northern-hybridization with [^{32}P]-5′-end-labelled oligonucleotide probes. To detect tRK1 versions we used oligonucleotide probe anti-tRK1 (1–34): GAGTC ATACGCGCTACCGATTGCGCCAACAAGGC, for tRK2 versions, the probe anti-tRK2 (2–32): GCCGAACG CTCTACCAACTCAGCTAACAAGG, for tRK3 versions, the probe anti-tRK3 (1–39): CTTAAAAGACAACTGTT TTACCATTAAACAAATATTCTC. The probes anti-mt-tRNA^{Leu}: GAACCTCTGACTCTAAAG and anti-mt-tRNA^{Thr}: CATCTCCGGTTTACAAG were used to control the quality of mitochondrial RNA. The probes anti-cy-tRNA^{Lys}: CTTGAACCCTGGACC and

anti-cy-5.8SrRNA: AAGTGACGCTCAGACAGGCA to control the absence of contamination of mitochondrial RNA by cytosolic RNA.

Analysis of aminoacylation *in vivo*

Analysis of aminoacylation levels of recombinant tRNAs *in vivo* was performed through PAGE in acid conditions and subsequent Northern hybridization analysis as described elsewhere (49). Briefly, RNAs from cells were isolated with TRIzol-reagent (Invitrogen), precipitated on ice with 50% isopropanol and dissolved in 10 mM Sodium acetate pH4.5, 1 mM EDTA. Deacylated controls were prepared by 30 min incubation at 37°C in 0.25 M Tris-HCl pH8.5, 0.25 M $MgCl_2$ followed by RNA precipitation. RNAs in a loading buffer with 0.1 M Sodium acetate pH5.0 were run in a cold room through 35 cm long denaturing acid 6.5% PAAG with 0.1 M Sodium acetate pH5.0 and analyzed by Northern hybridization with [^{32}P]-5′-end-labelled oligonucleotide probes.

Immunoblotting

For immunoblotting, whole cells were solubilized in a Laemmli's buffer (50) in the way to have SDS: protein ratio ~25–30 (w/w), sonicated for 5 s to break cellular DNA; incubated for 10 min at 60°C, and 30 μg of protein were run on a 12.5% SDS–PAGE, and subsequently transferred to a nitrocellulose membrane. When several cell lines were compared, we either used the same filter cut at the levels of different proteins of interest or separate runs of equal aliquots of the same protein extract with simultaneous electrotransfer, to ensure the comparability of the results. In all cases the amount of analyzed proteins was in the linear area of signal detection, as verified by in-gel titration with following scanning in the Typhoon-Trio. In no case the signal was saturated in the experiments presented. For immunodetection following antibodies were used: polyclonal antibodies against COX2, ND1 and commercially available monoclonal antibodies against porin (Calbiochem 529538), α-tubulin (Sigma T6074). Detection was done either using ECL^{TM} horseradish peroxidase linked secondary antibodies and 'ECL Plus Western Blotting Detection Reagent', or using ECL Plex^{TM} Cy3 and Cy5-conjugated secondary antibodies, on Typhoon-Trio (all from GE Healthcare). Signal quantification was performed in ImageQuantTL programme from the same manufacturer.

In vivo mitochondrial translation

The analysis of mitochondrial protein synthesis was performed as previously described (39) with minor modifications. Briefly, 600×10^3 cells were incubated for 10 min in DMEM w/o methionine (Sigma) in the presence of 100 μg/ml of emetine to inhibit cytoplasmic translation, followed by 30 min with 200 μCi/ml [^{35}S]-methionine (>1000 Ci/mmole, GE Healthcare), and, finally, 10 min chase in the normal growth medium. Cells were solubilized in a Laemmli's buffer (50), sonicated for 5 s to fragmentize chromosomal DNA; incubated for 10 min at 37°C, and 100 μg of protein were run on a 10–20% gradient SDS–PAGE. Protein amounts loaded were before normalized by anti-porin immunoblotting of the same preparations. Visualization and quantification were performed using Typhoon-Trio and ImageQuantTL software from GE Healthcare.

Measurement of oxygen consumption

The rates of oxygen consumption were measured using Hansatech Oxygraph. Respiration of intact (non-permeabilized) cells was measured using $1–2 \times 10^6$ cells/ml in PBS in the presence of 5 mM glucose. A 1 μg/ml of oligomycin, FCCP in the range of 10–500 nM and 1 mM of KCN were sequentially added to measure coupled, uncoupled and non-mitochondrial oxygen consumption, respectively.

The rates of substrate dependent oxygen consumption were measured on $1–2 \times 10^6$ cells/ml in a respiration buffer containing 20 mM HEPES–KOH (pH 7.4), 200 mM sucrose, 3 mM $MgCl_2$, 10 mM KH_2PO_4, 0.5 mM EGTA and 1 g/l of BSA. Cells were permeabilized by digitonin [100 μg of digitonin per 1 mg of total cell protein (~1×10^6 cells)]. Succinate was used to donate electrons at Complex II level, while Complex I was inhibited by 0.5 μM rotenone. Ascorbate was used to donate electrons at Complex IV level, while both Complex I and Complex II (by 5 mM malonic acid) were inhibited. Maximal possible respiration rates were measured in the presence of the uncoupler FCCP (50–500 nM) and oligomycin (1 μg/ml). Non-mitochondrial oxygen consumption was measured at the end of each experiment upon addition of 1 mM KCN.

RESULTS

Construction of importable tRNAs with leucine aminoacylation identity

The major identity elements required for recognition of $tRNA^{Leu(UUR)}$ by mitochondrial LeuRS are 'discriminator' base A73 and the A14 base (affected by m.3243A>G mutation) (Figure 1) (44,45). Among yeast tRNA derivatives mitochondrially importable *in vivo* in human cells we have previously characterized three lysine isoacceptor tRNAs (tRKs). Two of them are cytosolic type tRNAs: wild-type tRK1 and recombinant tRK2 (G1-C72; G73; U34). The third—a mitochondrial-type : tRK3, which can be nuclearly expressed and then targeted into mitochondria (Figure 1) (39). We introduced in these three tRNAs the discriminator base A73 and leucine anticodons, either UAA or CAA. The first one with the expectation that the U in the wobble position would be correctly modified, the last one with the purpose to decode UUG codons even if the anticodon will be not modified. Therefore, six different versions of potentially therapeutic tRNAs: tRK1UAA, tRK1CAA, tRK2UAA, tRK2CAA, tRK3UAA and tRK3CAA were designed and further used for *in vitro* and *in vivo* assays (Figure 1). All these versions were compared for their capacity to be aminoacylated by recombinant human mitochondrial leucyl-tRNA- and lysyl-tRNA synthetases (Table 1). As expected, all versions gained the capacity to be leucinylated with efficienies comparable with that of human mt-

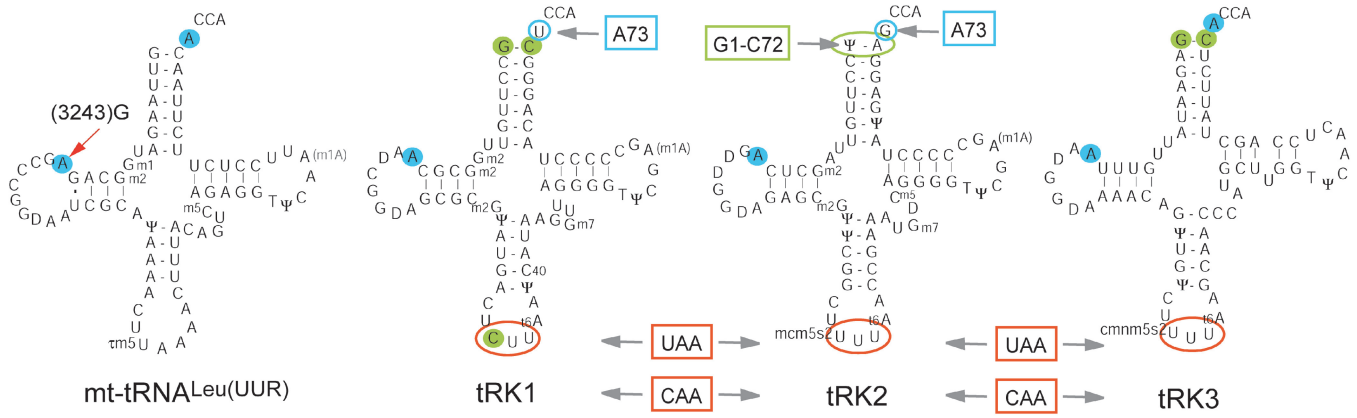

Figure 1. Cloverleaf structures of tRNAs used in this study. From left to right: native human mitochondrial $tRNA^{Leu(UUR)}$, major identity elements of recognition by mtLeuRS are in blue filled circles and MELAS m.3243A>G mutation is indicated by the red arrow; three yeast lysine tRNAs, tRK1, tRK2; tRK3 and their recombinant versions, tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA, with determinants of mitochondrial import indicated in green filled circles, identity elements for human mtLeuRS are in blue filled circles, mutations and regions where they were introduced are indicated by arrows and enclosed in blue for leucine aminoacylation identity elements, in green—for import determinants and in red—for leucine anticodons. Post-transcriptional modifications: 5-taurinomethyluridine (τm^5U), 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), 7-methylguanosine (m^7G), 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), pseudouridine (Ψ), dihydrouridine (D), 5-methyluridine (T) and N6-threonylcarbamoyladenosine (t^6A).

$tRNA^{Leu}$ (the tRK2CAA version having a lowest efficiency, but still at the level of 37% in comparison with the cognate tRNA). On the other hand, we observed that most of the version also retained a low capacity to be lysinylated. The best version from the point of view of aminoacylation properties was tRK1CAA, which was as well leucinylated as the cognate mt-$tRNA^{Leu}$ and retained a lowest lysinylation capacity (6% of mt-$tRNA^{Lys}$).

We tested then whether new recombinant tRNAs preserved their ability to be imported into mitochondria of human cells. To this end, MELAS cybrid cells were transfected with corresponding T7-transcripts, total and mitochondrial RNAs from cells were isolated 24 h after transfection and analyzed by Northern hybridization with ^{32}P-5′-end labelled oligonucleotide probes (Figure 2A). The import capacity of different recombinant tRNAs was evaluated as a ratio of the hybridization signal corresponding to the recombinant RNA in purified mitochondrial RNA fraction to that of human mt-$tRNA^{Leu}$ in the same isolate. It was then expressed as relative value in comparison with the import of tRK1 or tRK3 transcripts taken as 1.0 in each series. All synthetic tRNAs tested were found to be imported into mitochondria of MELAS cybrid cells *in vivo* with various efficiencies (Figure 2B). Mutations introduced in the anti-codon region of tRK3UAA (U35:A35, U36:A36) and tRK3CAA (U34:C34, U35:A35, U36:A36) did not significantly reduce efficiency of their import, while substitutions made in tRK1UUA (U73:A73, C34:U34, U35:A35, U36:A36) and tRK1CAA (U73:A73, U35:A35, U36:A36) decreased their import 3- and 5-fold, respectively, compared to tRK1 without mutations. Import efficiency of tRK2CAA was estimated comparing to tRK1 transcript, since wild-type tRK2 is not imported into mitochondria (42,51–53), and its import was approximately seven times lower when compared to tRK1.

Table 1. Aminoacylation of tRK mutant versions by recombinant human mitochondrial LeuRS et LysRS

tRNA	Km (μM)	V (pmoles × 10^{-3}/min)	V/Km	Relative efficiency of aminoacylation (%)
LysRS				
mt-$tRNA^{Lys}$	1	50	50.0	100
tRK3	0.3	25	83.3	167
tRK2CAA	0.9	27.5	30.6	61
tRK2UAA	2.2	50	22.7	45
tRK3CAA	3.7	21	5.7	11
tRK3UAA	8	75	9.4	19
tRK1CAA	4.2	12.5	3.0	6
tRK1UAA	1.9	31	16.4	33
LeuRS				
mt-$tRNA^{Leu}$	0.5	62.5	125.0	100
tRK2CAA	0.8	37	46.3	37
tRK2UAA	0.6	80	133.3	107
tRK3CAA	0.65	59	90.8	73
tRK3UAA	0.6	60	100.0	80
tRK1CAA	0.7	81	115.5	93
tRK1UAA	0.55	58	105.5	84

T7-transcripts were used as substrates. The mean values presented are the result of several ($n > 3$) independent measures with the error <10%.

Using the same approach, we found that T7-transcripts were stable in transfected cells at least 48 h after transfection. Despite efficient targeting into mitochondria of all synthetic recombinant tRNAs, we were not able to observe any effect on mitochondrial translation (data not shown). Moreover, the analysis of aminoacylation state of these transcripts using the acid gel method (49) showed that they were mostly present in deacylated form, a finding which could be explained by the absence of posttranscriptional modifications in T7-transcripts. To overcome this problem, the experiments were axed at *in vivo* expression of the recombinant tRNAs.

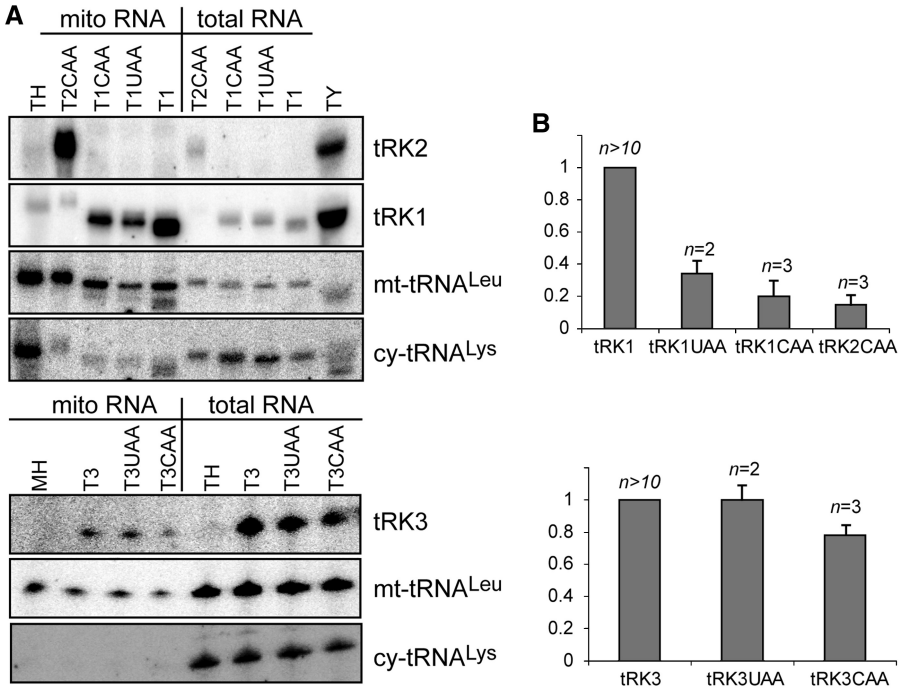

Figure 2. Analysis of *in vivo* import of synthetic recombinant tRNAs in mitochondria of MELAS cybrid cells. (**A**) Northern hybridization of total and mitochondrial RNA isolated from MELAS cybrid cells transfected with T7-transcripts. Specific [^{32}P]-oligonucleotide probes to tRK1, tRK2 and tRK3 were used to check for mitochondrial import, mt-tRNA-Leu probe—to control the absence of degradation of mitochondrial tRNAs and cy-tRNA-Lys probe to control the absence of its contamination by cytosolic tRNAs (the probes are indicated at the right of the panels). Minor bands visible with tRK1 probe in tRK2-transfectants and with cy-tRNA-Lys probe in tRK1 and tRK2 ones represent the unwashed traces of previous hybridizations of the same membrane and are unspecific (they do not migrate as the cognate tRNAs and therefore do not alter any interpretation of the specific signals). TH and MH are total and mitochondrial RNAs from non-transfected MELAS cells, TY stands for total yeast RNA used as the control of hybridization specificity. T1 and T3 are the transcripts of yeast tRK1 and tRK3 without mutations used to estimate the import efficiency of recombinant tRNAs (**B**) Relative amount of imported recombinant tRNAs calculated as a ratio of hybridization signals for recombinant tRNAs in mitochondrial RNA fraction to that of mt-tRNA^{Leu} in the same preparation. The import efficiencies presented on the graph represent relative values in comparison to these of tRK1 or tRK3 taken as 1.0 in each series. The presented error bars are issued from several independent experiments, as indicated above the diagrams.

Analysis of transient expression of recombinant tRNAs in MELAS cybrid cells

In order to express functional recombinant tRNAs in cybrid cells, we performed transfection with DNA constructs containing corresponding mutated genes. To this end, we first used mammalian expression vector pBK-CMV and cloned recombinant tRNA genes in an opposite direction with respect to the CMV promoter in order to favour their transcription from their internal promoter for RNA polymerase III (RpoIII) and their further correct maturation. MELAS cybrid cells were transiently transfected with pBK-CMV-tRK plasmids with the efficiency close to 90% (as revealed by FACS analysis of the transfection control with a GFP-expressing plasmid). Total cellular RNA was then analyzed by Northern hybridization 24 h, 48 h and 72 h after transfection to check expression and stability of recombinant tRNAs. tRK1 and tRK2-based versions were stable for 24 h, while their amount strongly decreased at day 2 (Figure 3A). Expression of tRK3 versions was not detected, which can be explained by non-optimal sequence of internal promoter for RpoIII in these transgenes as compared to tRK1/tRK2 versions. In order to increase the period during which transgenic tRNAs are present in transfected cells, we performed successive transfections, with a second transfection on the 3rd day after the first one. MELAS cybrid cell line used has defective steady-state level of the mtDNA-encoded COX2 subunit and decreased cytochrome *c* oxidase (COX) enzymatic activity, which is in agreement with previously published data (40). We analyzed steady-state level of COX2 protein during 6 days after transfection by immunoblotting of total cell protein extracts (Figure 3B). For several tRNA versions, reproducible increase of COX2 was observed with the most pronounced effect detected after transfection with tRK2CAA, where COX2 level was increased approximately three times as compared to non-transfected cybrid cells with its maximum being at the fourth day (1 day after the second transfection). tRK1UAA version also caused a two-fold increase of COX2 on the fifth day (2 days after second transfection), while tRK1CAA induced only a slight increase (1.5 times) 1 day after first transfection. The increase of COX2 level was however temporary in all assays, which is in agreement with the transient presence of recombinant RNAs in transfected cells.

The presence of recombinant tRNA versions in mitochondria of transfected cells was checked by Northern hybridisation (Figure 3C, left panel). All three

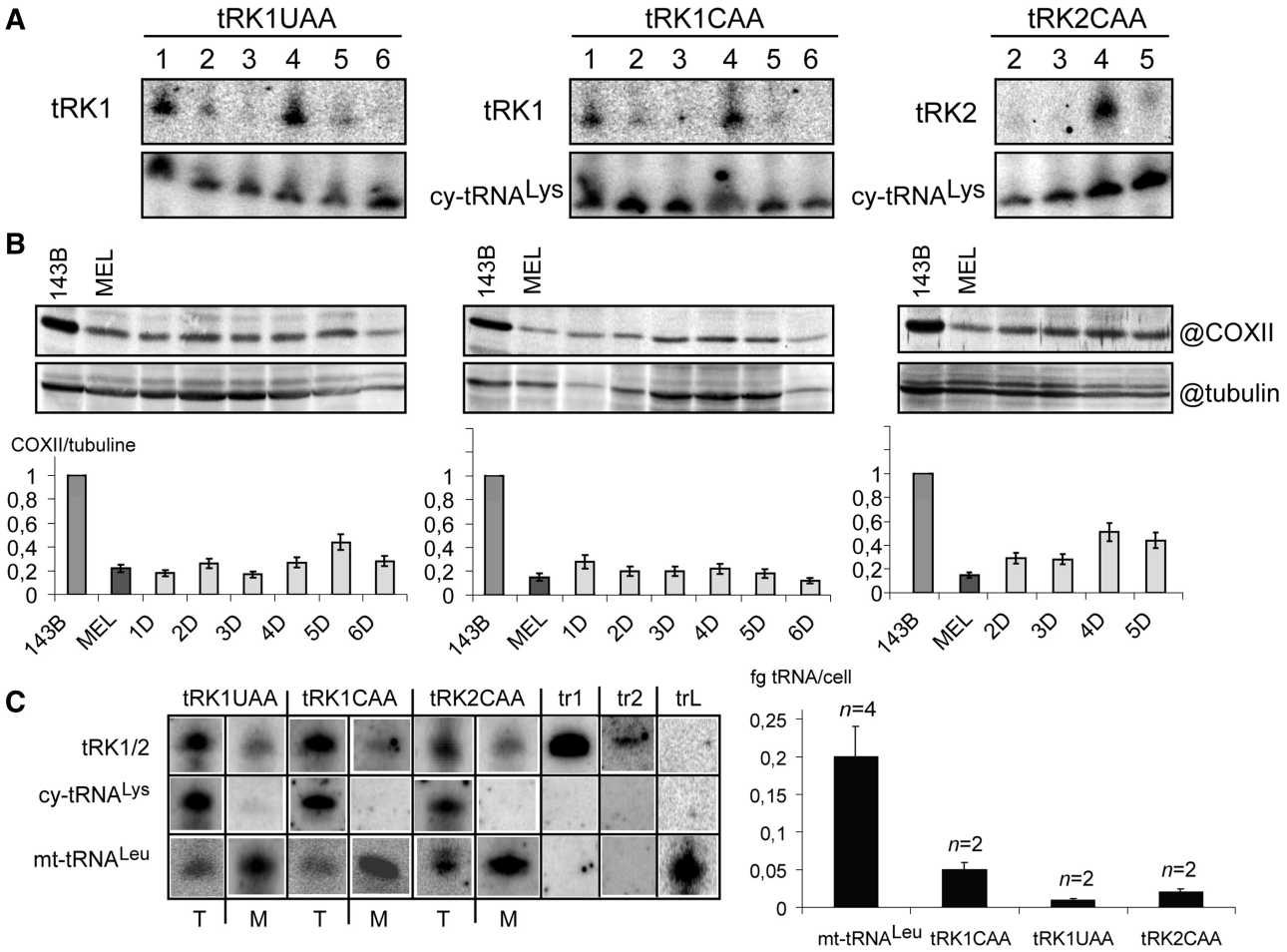

Figure 3. Effect of transient expression of recombinant tRNAs on COXII level in MELAS cybrid cells. (**A**) Northern hybridization analysis of total RNAs isolated from pBK-CMV-tRK transfected MELAS cybrid cells. Cells were transfected twice, second transfection was performed on the third day after the first one, and RNAs were isolated 1–6 days after the first transfection. Specific [^{32}P]-oligonucleotide probes are indicated on the left of the autoradiographs (tRK1, tRK2 or cy-tRNA^{Lys}—for the control cytoplasmic tRNA^{Lys} used as the quantification reference). (**B**) Western analysis of protein extracts from the transfected cells with anti-COX2 antibodies. Anti-tubulin antibodies were used as the quantification reference. Two different MW zones of the same blot were analyzed for COX2 and tubulin. The steady-state levels of COX2 before and after transfection, normalized to tubulin, are shown as diagrams for each tRNA-version in the lower panel of the figure. Values are presented relative to COX2 level in 143B parental cells, taken as 1. Error bars correspond to the results of 3–5 independent assays. (**C**) Northern hybridization of total (T) and mitochondrial (M) RNA isolated from MELAS cybrid cells 24 h after transient transfection with recombinant tRNAs. Hybridisation probes are indicated at the left. Transfected RNAs (tRK1UAA; tRK1CAA, tRK2CAA) and quantitative controls (2 ng of T7-transcritpts of tRK1, tr1, tRK2, tr2 and mt-tRNA^{Leu}, trL) are indicated at the top. The diagram at the right is a result of at least two independent experiments, as indicated.

recombinant versions were present in mitochondria which did not contain any detectable cytosolic contamination. tRK1 versions were analysed along with T7 transcripts of tRK1, tRK2 and mt-tRNA^{Leu}, which permitted quantification of the import (Figure 3C, right panel). The amount of all three versions was significantly lower than that of the reference host mt-tRNA^{Leu}, which can explain the limited effect of their expression on COX2 level, so far this recovery effect was observed in all the cases in a reproducible manner.

Generation of MELAS cybrid cells stably expressing leucine recombinant tRNA genes

In an attempt to stabilize the curative effect of mitochondrial import of recombinant tRNAs, we used the lentiviral transfection system. tRNA genes were cloned in the vector pLKO.1 under the control of an external U6 RpoIII promoter. Before infection, pLKO.1-tRK constructs were first verified in the transient transfection system, in which expression of tRK1UAA/CAA and tRK2UAA/CAA was observed. For stable lentiviral transfection, MELAS cybrid cells were analyzed 72 h or 96 h after infection for transgene expression, and tRK1CAA was found to be the only one stably and efficiently expressed (Figure 4A). Other constructs, giving no sufficient levels of expression (although the presence of the transgene was confirmed by PCR) were not analyzed further. The MELAS-pLKO.1-tRK1CAA cell line was therefore analyzed in depth. Furthermore, this very version proved to be the most prospective one, taking into account its best aminoacylation properties (Table 1) and import capacity comparable with other versions.

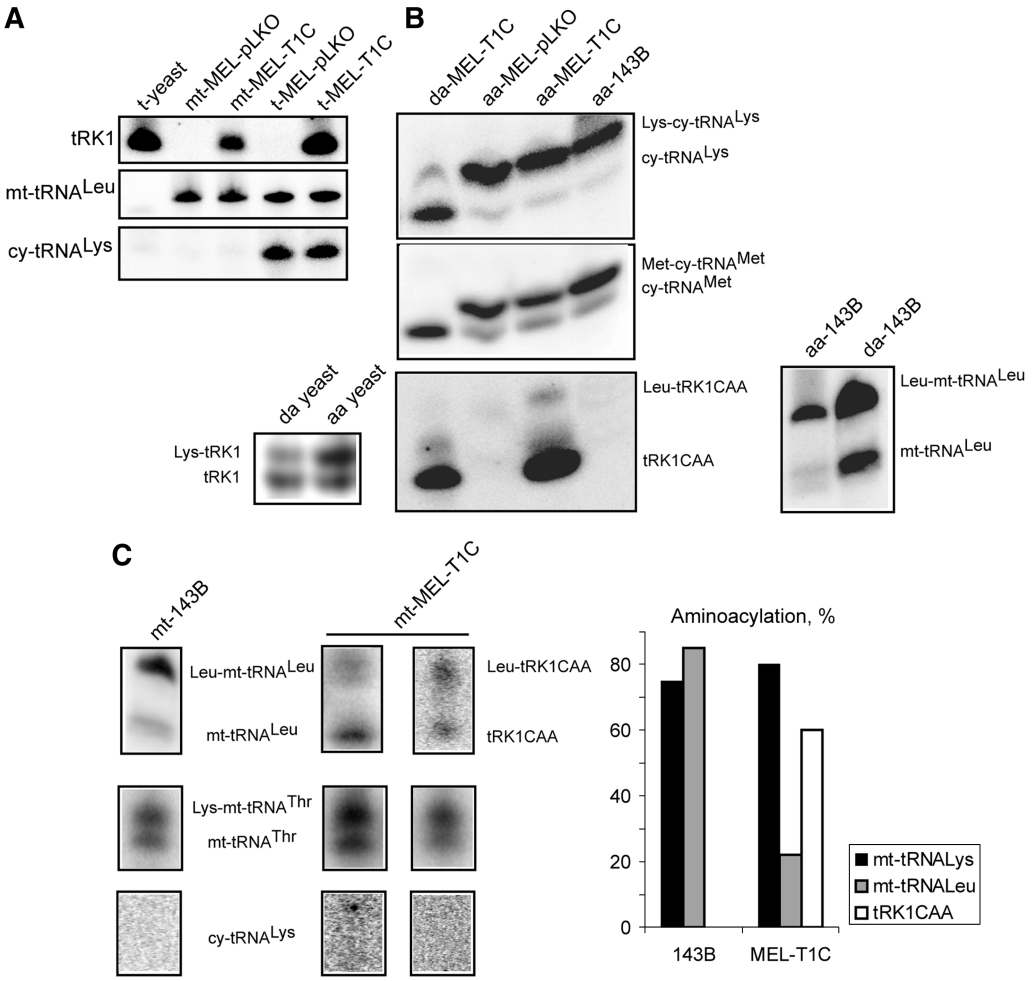

Figure 4. Import and aminoacylation of tRK1CAA stably expressed in MELAS cells. (**A**) Northern hybridization of total (t) and mitochondrial RNA (mt) from MEL-pLKO.1-T1CAA (MEL-T1C), MEL-pLKO.1 (MEL-pLKO) human cells and yeast with tRK1 and cy-tRNALys-specific [^{32}P]-labelled oligonucleotide probes. (**B**) Acid gel and northern analysis of total RNAs isolated from MEL-T1C, MEL-pLKO, 143B human cells and yeast with tRK1, mt-tRNA^{Leu}, cy-tRNA^{Lys} and cy-RNA^{Met}-specific [^{32}P]-labelled oligonucleotide probes (aa, aminoacylated RNA; da, RNA partially deacylated in basic conditions before loading). (**C**) Acid gel and northern analysis of mitochondrial RNA isolated from parental 143B and MEL-T1C cells. Aminoacylated and deacylted forms of mt-tRNA^{Leu}, mt-tRNA^{Thr} and tRK1CAA are indicated. The diagram at the right shows the quantification of aminoacylation levels for all these tRNAs (tRK1CAA was not analysed in the parental line since this RNA is absent therein).

By Northern hybridization analysis of purified mitochondrial RNA, we have shown that fraction of tRK1CAA was imported into mitochondria of MELAS-pLKO.1-tRK1CAA cells (Figure 4A), which confirmed our results obtained with T7-transcripts. RNA acid gel analysis demonstrated that at least a part of recombinant tRK1CAA in MELAS cells was aminoacylated (Figure 4B). Comparing the distance between aminoacylated and not aminoacylated forms of tRK1CAA and of other cellular tRNAs, we concluded, that the upper band corresponded to tRK1CAA aminoacylated with leucine. The middle band could correspond to tRK1CAA aminoacylated with lysine, so far, taking into account its low lysinylation level *in vitro* (Table 1) and the fact that the same band is detectable in both native and deacylated preparations, we suggest rather a non-specific signal. Furthermore, a possible unspecific aminoacylation is most likely done by cytoplasmic LysRS rather than by its mitochondrial counterpart taken into account that in mitochondria the amount of tRK1CAA is lower and mitochondrial LeuRS is present as a competitor. Therefore we hope that the minor portion of tRK1CAA possibly charged with lysine, if any, would have no considerable effects either on mitochondrial translation, or on cytosolic protein synthesis. Since leucine identity elements for human mitochondrial LeuRS we introduced in tRK1CAA were distinct from those for cytosolic LeuRS (44), and taking into account the possibility to leucinylate the recombinant tRNA *in vitro*, we conclude that tRK1CAA was aminoacylated with the leucine by mitochondrial LeuRS after its mitochondrial import. The portion of leucinylated tRK1CAA is modest probably because of non-optimal aminoacylation efficiency of recombinant tRNAs *in vivo*, but also because of naturally low amounts of tRNAs imported into mitochondria.

The above conclusions were verified by measuring the extent of tRK1CAA leucinylation in mitochondrial RNA preparations (Figure 4C). We compared the stable

tRK1CAA expressor with the MELAS cybrid line and observed, as expected, a very low aminoacyaltion level for the host mt-tRNA^{Leu} affected by the mutation (10–20%). At the same time the overall level of tRNA aminoacylation (as judged by the reference mt-tRNA^{Thr}) was high (>70%). The recombinant tRK1CAA was found partially aminoacylated (50–60%), which is significantly more pronounced than in the total RNA. This result is in agreement with the speculations above, and is likely to be explained by the efficient aminoacylation by the LeuRS in the mitochondria, while the major portion of deacylated tRK1CAA resides in the cytosol of the transfectant. Therefore, one can consider that if the level of import may be low (if comparable with the transient expression experiment), the aminoacylation by the mitochondrial enzyme was rather efficient and therefore one could expect to detect the curative effect.

Improvement of mitochondrial functions in MELAS-pLKO.1-tRK1CAA cells

In agreement with previous reports (11,21), the original MELAS cybrid cells showed strongly decreased mitochondrial protein synthesis, with large polypeptides ND2, COX1, COX2 and COX3 being particularly affected, but the qualitative pattern of mitochondrial translation products was unchanged. In MELAS-pLKO.1-tRK1CAA cells amounts of mitochondrial translation products were found to be increased to 30–50% in a generalized manner, compared to control cells transfected with an empty vector (MELAS-pLKO.1) (Figure 5). Further western analysis of mitochondrial DNA-encoded respiratory subunits revealed a reproducible increase of COX2 (+22%) and ND1 (+10%) proteins in MELAS cells stably expressing tRK1CAA transgene (Figure 6).

Oxygen consumption levels in MELAS-pLKO.1-tRK1CAA and MELAS cells transfected with the empty plasmid were then compared (Figure 7A and B). The reference MELAS-pLKO.1 cells demonstrated strongly decreased respiration with the ratio of uncoupled to coupled respiration (respiratory control) approximately two times lower than for 143B cells, which was in agreement with previously reported data (19). In MELAS-pLKO.1-tRK1CAA cells the rates of coupled and uncoupled oxygen consumption were increased about two times in comparison with the respective rates in MELAS-pLKO.1 cells, while respiration control was also improved.

To decipher this positive effect on cellular respiration, substrate-dependent oxygen consumption on digitonin permeabilized cells was analyzed. MELAS and 143B cells revealed similar succinate-supported respiration rates through complex II entirely encoded by nuclear DNA (Figure 7C). This succinate-supported respiration was not affected by tRK1CAA expression, as expected. In contrast, there was a substantial decrease in respiration rate through complex IV in MELAS-pLKO.1 cells comparing to 143B cells (Figure 7D). Importantly, this ascorbate-supported respiration was strongly increased in the MELAS cells with tRK1CAA, which confirmed previously observed rise of COX2 steady-state level and general improvement of mitochondrial translation.

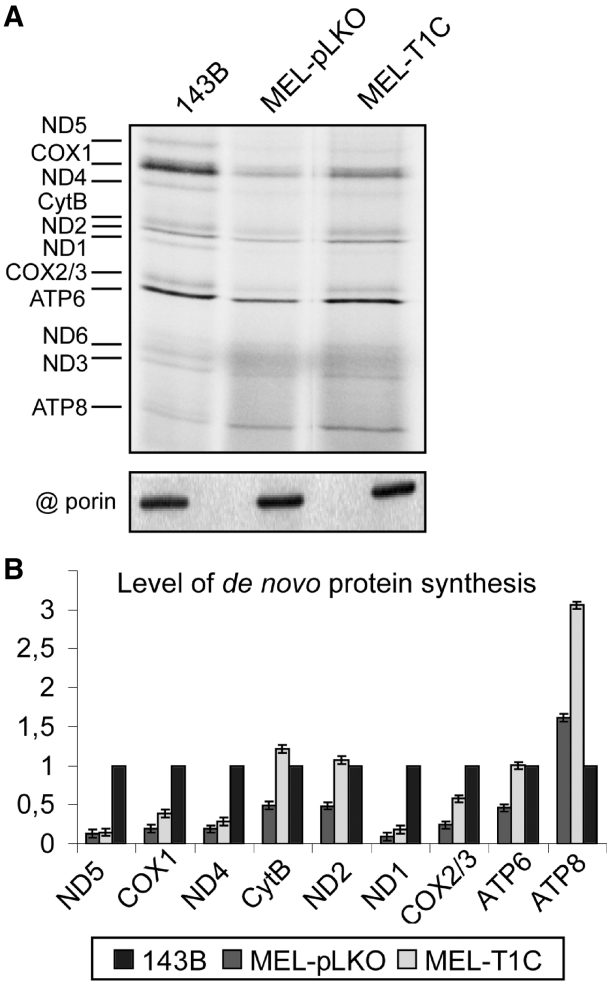

Figure 5. Mitochondrial translation in MELAS-pLKO-tRK1CAA cybrid cells. (**A**) Pulse-chase analysis of mitochondrial translation. Radioautograph of [^{35}S]-labelled mitochondrial translation products separated by SDS–PAGE is presented. The lines used are indicated at the top: 143B, MEL-pLKO and MEL-T1C. The bands corresponding to individual translation products are indicated according to standard pattern (56). Equal amounts of proteins were loaded in each case, which was controlled by Western analysis of porin in the same samples performed in parallel (below the main panel). (**B**) The diagram shows the levels of individual translation products in each cell line relative to those in 143B cells (taken as 1).

Since the high heteroplasmy level in MELAS-pLKO.1-tRK1CAA was unchanged upon transfection (~90%), one can conclude that the observed functional improvement of mitochondrial functions was due to expression and mitochondrial import of the recombinant tRNA.

DISCUSSION

The results of this study clearly demonstrate that one can alter aminoacylation identity of a tRNA preserving its ability to be imported *in vivo* into human mitochondria and to participate in the organellar translation. Indeed, we

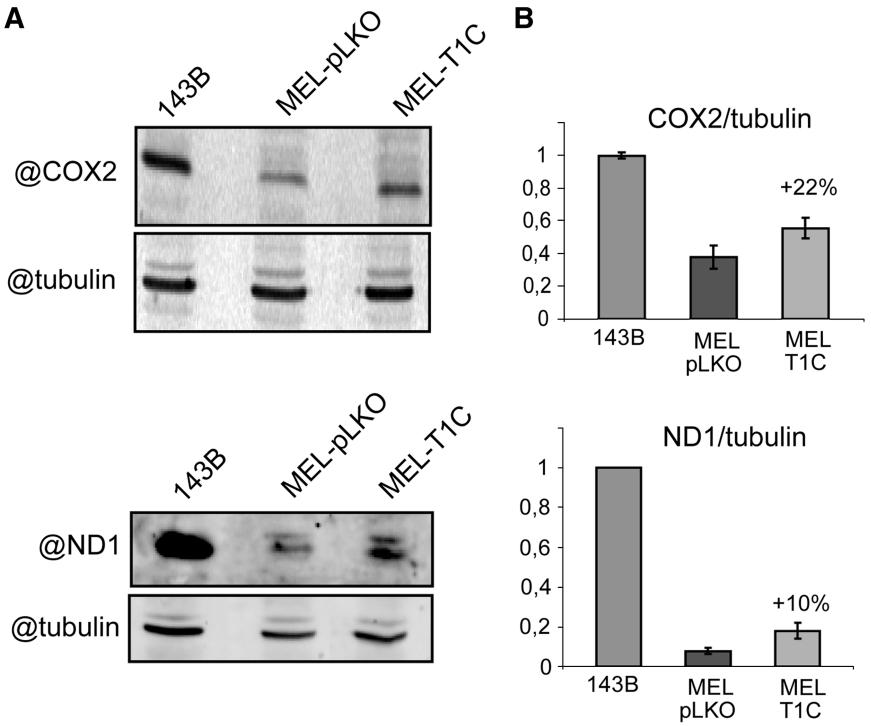

Figure 6. Steady-state level of mitochondrial proteins in MELAS-pLKO-tRK1CAA cybrid cells. (**A**) Western analysis of protein extracts from 143B, MEL-pLKO and MEL-T1C cell lines with antibodies against mitochondrial proteins COX2, ND1 and tubulin. (**B**) COX2 and ND1 steady-state levels in each cell line, normalized to tubulin and relative to the steady-state level of the corresponding protein in parental 143B cells (taken as 1).

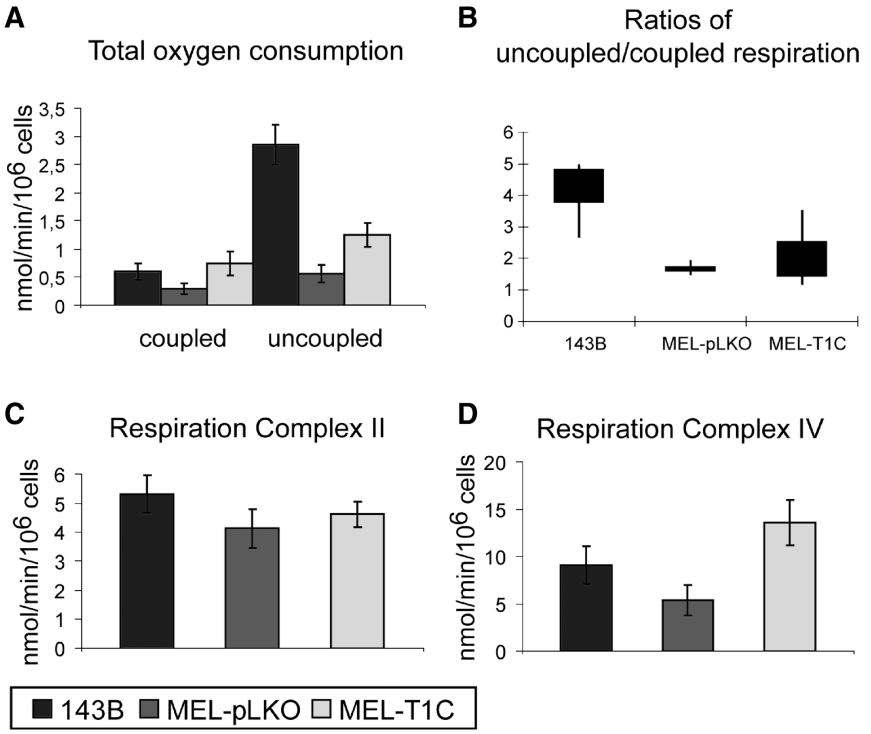

Figure 7. Oxygen consumption in MELAS-pLKO-tRK1CAA cybrid cells. (**A**) The rates of coupled and uncoupled oxygen consumption (the mean values ± SD, $n = 3$) in 143B, MEL-pLKO and MEL-T1C cell lines in nmol $O_2/10^6$ cell*min. (**B**) Box and whisker plots (57) represent the ratios of uncoupled to coupled respiration rates in 143B, MEL-pLKO and MEL-T1C cell lines. The overall data range is represented as a vertical line, while boxes represent the interquartile range containing central 50% data. (**C**) The rates of the respiration on succinate in permeabilized 143B, MEL-pLKO and MEL-T1C cells. (**D**) The rates of the respiration on ascorbate in permeabilized 143B, MEL-pLKO and MEL-T1C cells.

show that nuclear expression and subsequent mitochondrial import of specifically designed tRNAs of yeast origin with artificially changed identity (cytoplasmic lysine to mitochondrial leucine) partially rescues various negative effects of the mutation m.3243A>G in the mitochondrial $tRNA^{Leu}$ gene underlying the MELAS syndrome in cultured human cells. This rescue was observed at the levels of mitochondrial translation, steady state of mitochondrial DNA-encoded subunits of the respiratory chain and cellular respiration.

The increase of COX2 steady-state level that we observed in MELAS cells in transient transfection system varied in strength and duration depending on tRNA version expressed (Figure 3). This could indicate on possible differences in behaviour and action of different recombinant tRNAs in the cell. Thus, lower import efficiency of tRK1CAA comparing to tRK1UAA (Figure 2) can partially explain its moderate effect on COX2 level. On the other hand, the less efficient import substrate tRK2CAA lead to the best improvement of COX2 level, probably taking advantage of its CAA anti-codon, which did not need post-transcriptional modification to decode leucine UUG codons. This experimental fact is in frame of a suggestion that the MELAS mutation might affect essentially decoding UUG, but not UUA. On the other hand, it is to be outlined that the improvement of mitochondrial function depended not only on the efficiency of a particular tRNA to participate in mitochondrial translation, but also on the time it was present in cell. For example, the effect of tRK1CAA on COX2 steady-state level was more pronounced once it has been expressed in cybrid cells in a stable manner. The importance of the effect depends on a multitude of linked or non-linked factors influencing each step of the experiment: efficiency of transfection, efficiency of expression of the tRNA gene, efficiency of import, mitochondrial activity state that may vary significantly even in continuously cultivated line, modification and aminoacylation of the transgenic tRNAs, the stability of the given transgenic tRNA in the cell and in the mitochondria, misaminoacylation of the tRNA, which can give negative effects on mitochondrial or cytoplasmic translation, etc. On the other hand, if the efficiency of different versions varies, all of them provided a detectable and reproducible curative effect, thus validating the strategy, whose efficiency certainly needs to be improved to make it applicable in therapy.

Meanwhile, MELAS-pLKO.1-tRK1CAA cells clearly demonstrated a significant rescue of mitochondrial functions at all levels analyzed (Figures 5–7). Thus, synthesis of certain polypeptides reached or even exceeded (for ND2, CytB and ATP6) its level in 143B wild type cells, while synthesis of others was improved up to 30% (for COX1)—50% (for COX2/3) of its wild-type level, indicating that tRK1CAA participated in mitochondrial translation. However, no clear correlation between number of leucine codons (neither UUG, nor UUR) and translation level of particular mitochondrial proteins was observed in MELAS cybrid cell before or after transfection, which is corroborated by previously reported data (11,21). The partial nature of the effect could be explained by insufficient level of expression of the recombinant tRNA gene(s), defective post-transcriptional modifications of recombinant tRNAs, ineffective export of tRNAs from nucleus and targeting into mitochondria or, finally, low efficiency of re-aminoacylation inside the organelle. Furthermore, one could also imagine that the growth conditions used (high glucose and pyruvate) do not favour the switch from glycolysis to oxidative phosphorylation in transfected cybrid cells energy production. In any case, the observed increase of mitochondrial translation level and subsequent augmentation of COX2 and ND1 steady states was sufficient to improve the rate of oxygen consumption in MELAS-pLKO.1-tRK1CAA cells about 2-fold. Such an increase may prove to be sufficient for further therapeutic application. The question that might emerge, is the validity of the strategy described here for various cell/tissue types. The efficiency of the approach relies to the RNA import mechanism, which is far to be understood in details. Indeed, our studies of the human RNA import (both natural, for 5S rRNA, and artificial, for tRNA derivatives) indicate on the importance of the membrane charge and ATP hydrolysis for import (31,54). It was reported that isolated and discharged mitochondria from cybrid cells bearing a pathogenic mutation fail to incorporate tRNAs (33). So far, these data are in contradiction with our previously published report of the *in vivo* import of $tRNA^{Lys}$ derivatives which, in spite of the lower energized state of mitochondria in MERRF cybrids can import tRNAs enough to complement mitochondrial deficiency (39). So, we believe that, since no mtDNA mutation described led to a complete discharging of the mitochondrial membrane potential, one could expect the possibility to target RNAs into mitochondria. As a confirmation of our former study, the results obtained in the current work show that in the strongly affected cybrid cell line tRNA import may be achieved and the phenotypic effect is measurable, if even the cells were affected by another mutation (MELAS versus MERRF) and the phenotypic alteration caused by the mutation were different. On the other hand, it is evident that the efficiency of the approach will not only depend on the efficiency of import *per se*, but also on the amount of the 'therapeutic' RNA in a given type of cell, which will depend on the delivery procedure, as well as on the functionality of the tRNA itself, in terms of aminoacylation and decoding properties.

In conclusion, the recombinant leucine tRNAs validated in the present work could be tested in modelling therapeutic approaches to a number of other disorders caused by dysfunction of $mt\text{-}tRNA^{Leu(UUR)}$. As an important issue of the study, it clearly appears that the mitochondrial translation system in human cells possesses a significant extent of flexibility, accepting not only cytosolic-type tRNAs, but also tRNAs of yeast origin, which are evolutionary very distinct from the human ones. This makes us believe that the strategy developed in this study can be applied to all tRNA mutations which are not affecting the overall expression of mtDNA, however for each case the importable and functionally active molecule must be designed. Construction of these new importable versions may be helped by a

comprehensive view of the mechanisms governing the selectivity of tRNA import, taking into account the identified import determinants (42,51,55) and the possibility of their structural rearrangements (52). The new observations, together with previously published data (38,39), strongly support the approach of allotopic expression of mitochondrially targeted RNAs as a powerful tool for different kinds of disorders caused by mutations in mitochondrial tRNA-coding genes.

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