Suppression of Mutations in Mitochondrial DNA by tRNAs Imported from the Cytoplasm

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Mitochondrial import of a cytoplasmic transfer RNA (tRNA) in yeast requires the preprotein import machinery and cytosolic factors. We investigated whether the tRNA import pathway can be used to correct respiratory deficiencies due to mutations in the mitochondrial DNA and whether this system can be transferred into human cells. We show that cytoplasmic tRNAs with altered aminoacylation identity can be specifically targeted to the mitochondria and participate in mitochondrial translation. We also show that human mitochondria, which do not normally import tRNAs, are able to internalize yeast tRNA derivatives in vitro and that this import requires an essential yeast import factor.

Mitochondrial import of nuclear encoded tRNAs has been described in yeast, plants, and protozoans. The complexity of the imported tRNA pool varies among organisms, from a complete set required for reading all the codons of the mitochondrial genetic code in trypanosomatids to a single tRNA in the yeast *Saccharomyces cerevisiae* (*1, 2*). In no case has it been shown directly that imported cytoplasmic tRNAs are functional on mitochondrial ribosomes. The mechanisms by which negatively charged RNAs can cross the mitochondrial membranes are poorly understood, and different mechanisms may operate in different organisms. In *S. cerevisiae*, one of the two cytoplasmic lysine isoacceptors, $tRNA^{Lys}_{CUU}$ (tRK1, Fig. 1A), is partially associated with the mitochondria, whereas the second, $tRNA^{Lys}_{SUU}$ (tRK2, Fig. 2A), is localized only in the cytoplasm (*3*). Mitochondrial targeting of tRK1 is dependent upon the preprotein import machinery and requires cytosolic factors including pre-MSK, the precursor of the mitochondrial lysyl-tRNA synthetase (LysRS), which likely serves as a carrier for mitochondrial transport of the tRNA (*4, 5*). Binding of tRK1 to pre-MSK requires aminoacylation by the cytosolic LysRS, and aminoacylation is thought to induce a conformational change in the tRNA that allows interaction with pre-MSK (*6–8*). It is hypothesized that in plants, mitochondrial aminoacyl-tRNA synthetases are also involved in tRNA import, although they alone are not sufficient for import (*9, 10*). In trypanosomatids, import in vitro of tRNAs and tRNA precursors can proceed in the absence of cytosolic factors, and an RNA-specific mitochondrial membrane receptor is involved (*11–13*).

We first investigated whether the yeast tRNA import system can be manipulated to import cytosolic tRNAs with altered aminoacylation identities. The identity of tRK1 was switched by replacing the second anticodon base, U, by an A ($tRK1_{CAU}$, Fig. 1A). The anticodon CAU is the main identity element of the yeast methionyl-tRNA synthetase (MetRS) (*14*). Accordingly, we observed that the methionylation level of $tRK1_{CAU}$ was comparable to that of $tRNA^{Met}i$. Methionylated $tRK1_{CAU}$ was found to be efficiently imported into isolated yeast mitochondria (Fig. 1B). Therefore, whereas aminoacylation is a prerequisite for import (*5–7*), the nature of the amino acid charged on the tRNA is less important. This allowed us to test whether a cytosolic tRNA is functional in mitochondrial translation. The $tRK1_{CAU}$ was aminoacylated with [^{35}S]methionine and imported into isolated mitochondria. After incubation of mitochondria in intraorganelle protein synthesis conditions (*15*), chloramphenicol-sensitive incorporation of [^{35}S]Met into proteins was detected within 30 min after import of [^{35}S]Met-$tRK1_{CAU}$ (*16*). The labeled polypeptides gave an electrophoretic pattern expected for mitochondrial DNA (mtDNA)–coded proteins (Fig. 1C). Moreover, immunoprecipitation experiments demonstrated incorporation of [^{35}S]Met into the three mtDNA-coded subunits of cytochrome c oxidase.

To study whether an imported cytoplasmic tRNA can function in mitochondrial protein synthesis in vivo, we designed a system involving suppression of a nonsense mutation (Ala-to-*amber*) introduced in the mitochondrial *COX2* gene in strain HM4 (*17–19*). Several versions of tRK1 and tRK2 carrying an anticodon CUA complementary to the *amber* UAG stop codon were tested for their expression and import in vivo. The best import efficiency, comparable to that of tRK1, was found for $tRK2_{CUA}$(G3:U70) (Fig. 2A). This tRNA harbors the base C34, which was previously shown to confer import to the normally nonimported tRK2 (*7*), and a strong determinant for aminoacylation by alanyl-tRNA synthetase (AlaRS), the G3:U70 base pair (*20*). Indeed, in vitro–synthesized $tRK2_{CUA}$ (G3:U70) was aminoacylated with alanine as efficiently as a $tRNA^{Ala}$ transcript. Determination of the aminoacylation level in vivo (*21*) showed that the mutant tRNA was almost fully charged, most likely with alanine (Fig. 2B). Expression of $tRK2_{CUA}$(G3:U70) in HM4 cells conferred

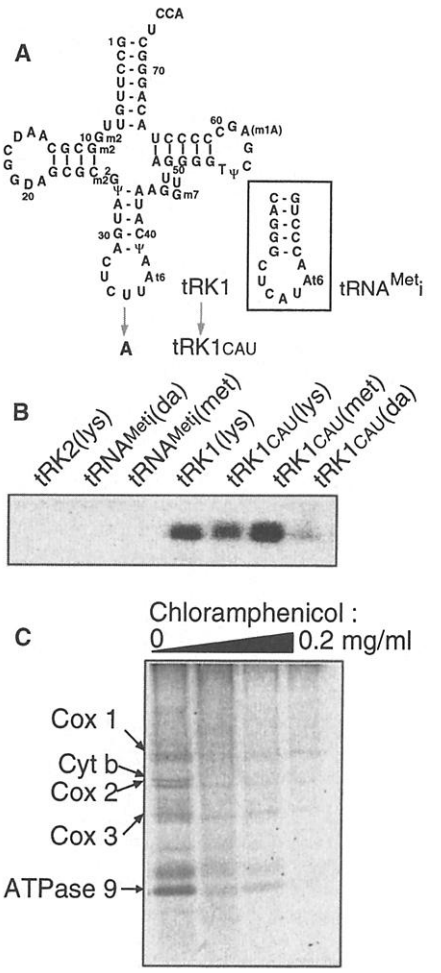

Fig. 1. Imported $tRK1_{CAU}$ is active in yeast mitochondrial translation in vitro. (**A**) Cloverleaf structure of tRK1. The arrow indicates the introduced mutation. The anticodon loop of the yeast $tRNA^{Met}i$ (inset) is identical to that of $tRK1_{CAU}$. (**B**) In vitro import assay, as in (*6–8*). T7-synthesized tRNAs were preincubated with either purified cytoplasmic LysRS (lys) or MetRS (met); da, deacylated. (**C**) Autoradiography of [^{35}S]-labeled translation products separated by SDS-PAGE. Intraorganelle protein synthesis, as in (*15*), was carried out for 30 min after achievement of [^{35}S]Met-$tRK1_{CAU}$ import.

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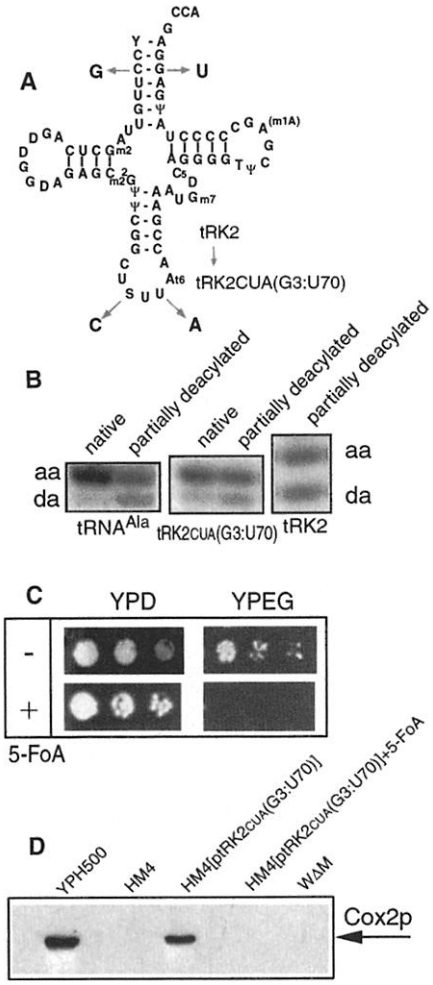

Fig. 2. Imported $tRK2_{CAU}$(G3:U70) is active as an *amber* suppressor in yeast mitochondria. (**A**) Cloverleaf structure of tRK2. Arrows indicate introduced mutations. S, 5-[(methylamino)-methyl]-2-thiouridine. (**B**) Analysis of the in vivo aminoacylation level of $tRK2_{CUA}$(G3:U70) by electrophoresis in acidic conditions (*21*). Hybridization probes: tRK2, GACATTTCGGTTAAAAG; $tRK2_{CUA}$ (G3:U70), CAAGCATGGGTTGCTTAAAAG; $tRNA^{Ala}$, ATGACCTCTTCCTTGCAAG. For partial deacylation, tRNAs were incubated in 0.2 M tris-HCl (pH 9.0) for 10 min at 50°C. "aa" and "da" indicate aminoacylated and deacylated forms, respectively. (**C**) Suppression of the *cox2-114*^{amber} mutation (strain HM4) by $tRK2_{CUA}$(G3:U70) expressed from plasmid pRS416. YPD and YPEG, rich media containing either glucose or glycerol plus ethanol, respectively, were used as a carbon source. "+" or "−" 5-FoA, counterselection of the *URA3*-containing plasmid with 5-fluoro-orotate. (**D**) Western analysis of mitochondrial polypeptides with anti-Cox2 antibodies. WΔM was the control ρ° strain.

plasmid-dependent growth on glycerol-containing media (Fig. 2C), indicating that the imported tRNA is active as a suppressor of the mitochondrial *cox2-114*^{amber} mutation. Moreover, although the HM4 strain lacked immunologically detectable Cox2p, the $ptRK2_{CUA}$(G3:U70)-transformed clones contained the full-length Cox2p (Fig. 2D).

These results demonstrate that cytosolic

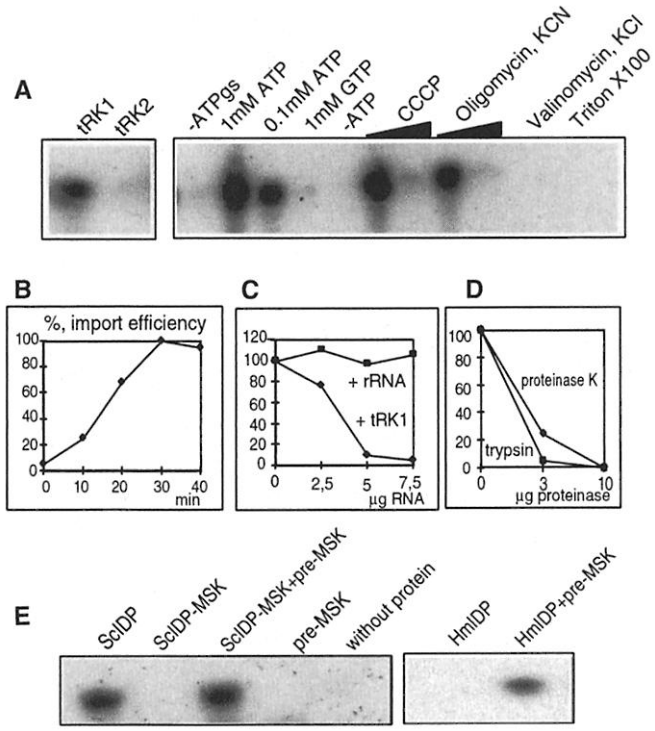

Fig. 3. tRNA import requirements of isolated human mitochondria. Mitochondria were isolated from cultured HepG2 cells (*27*). In vitro import conditions were similar to those described for yeast mitochondria (*4–6*). (**A**) tRK1 and tRK2 import assays (left panel). Dependence of tRK1 import upon ATP and $\Delta\Psi$ (right panel). "–ATPgs", without ATP-generation system. The membrane potential was dissipated with 5 and 50 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), with 1 and 10 µM oligomycin in 0.5 mM KCN, or with 5 µM valinomycin in 50 mM KCl. In the last lane, the mitochondria were treated with 0.1% Triton X-100 before RNase treatment. (**B**) Kinetics of tRK1 import. (**C**) Competition assay measured [^{32}P]tRK1 import in the presence of increasing amounts of nonlabeled RNA, either tRK1 or *Escherichia coli* 16*S* + 23*S* rRNAs. (**D**) Inhibition of tRK1 import by pretreatment of mitochondria with proteinases. (**E**) Dependence of tRK1 import upon soluble proteins. "ScIDP-MSK," ScIDPs lacking pre-MSK were isolated from strain WΔM, which is a deletant of the *MSK1* gene in strain W303; "ScIDP-MSK + preMSK," ScIDPs lacking pre-MSK supplemented with 20 ng of recombinant pre-MSK. HmIDPs were isolated as in (*28*). "HmIDP + pre-MSK," HmIDPs supplemented with 20 ng of pre-MSK.

tRNAs with altered aminoacylation identities can be selectively targeted into yeast mitochondria, where they are functional in mitochondrial translation and can cure respiratory defects due to nonsense mutations in mtDNA-coded protein genes. This finding may be of interest with respect to human cells, because a number of mitochondrial diseases are associated with mtDNA mutations in either protein-coding or tRNA genes (*22*). It would be desirable to suppress such mutations by engineered tRNAs expressed in the nucleus and targeted to the mitochondria. However, no tRNA import has yet been found in mammalian mitochondria, although other small RNAs are thought to be imported, that is, 5*S* ribosomal RNA (rRNA), and RNA components of ribonuclease (RNase) P and mitochondrial RNA processing (MRP) endonuclease (*23–25*).

We investigated whether the yeast tRNA import system can be transferred into human cells. To this end, we tested if tRNAs can be imported into isolated human mitochondria. Mitochondria isolated from HepG2 cells were incubated in a standard import mixture (*4–6*) containing labeled tRK1 or tRK2; tRK1 was protected from RNase digestion whereas tRK2 was not (Fig. 3, A and B). Addition of increasing amounts of nonlabeled tRK1 specifically inhibited tRK1 internalization (Fig. 3B). Internalized tRK1 becomes accessible to RNases after disruption of the mitochondrial membranes by a detergent. Furthermore, tRK1 import requires external and internal adenosine triphosphate (ATP) and an electrochemical potential across the mitochondrial inner membrane (Fig. 3A). Finally, mitochondria pretreated with proteinases fail to internalize tRK1 (Fig. 3D), suggesting that receptor(s) at the mitochondrial surface are essential. All these requirements are comparable to those observed for tRNA import into yeast mitochondria (*4*), and also to those described for mitochondrial preprotein import (*26*).

Import of tRNA into yeast mitochondria requires cytosolic import-directing factors (ScIDPs) (*5*). We found that tRK1 import into human mitochondria is also dependent upon ScIDPs, and human cytosolic extracts (HmIDPs) could not substitute for ScIDPs (Fig. 3E). Pre-MSK is an essential factor, because ScIDPs devoid of pre-MSK failed to direct the import of tRK1 whereas addition of pure pre-MSK restored import. However, pre-MSK alone was ineffective, which suggests that other factor(s) are also required. Interestingly, when HmIDPs were supplemented with pre-MSK, tRK1 import was observed (Fig. 3E). It will be interesting to determine whether expression of yeast pre-MSK in human cells is sufficient to provide tRK1 mitochondrial import in vivo.

We next tested a panel of tRK1 and tRK2 mutant versions (*7, 8*) for their abilities to be imported into isolated human mitochondria,

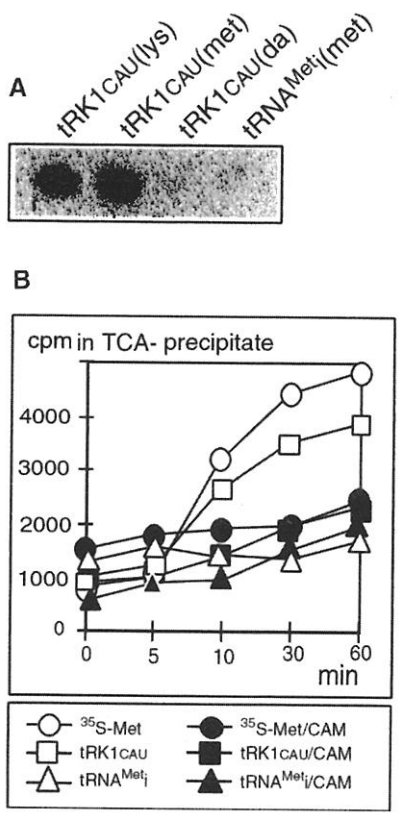

Fig. 4. Imported $tRK1_{CAU}$ is active in human mitochondrial translation. **(A)** In vitro import of labeled tRNAs into isolated HepG2 mitochondria. **(B)** Kinetics of incorporation of [^{35}S]Met into mitochondrial polypeptides. $tRK1_{CAU}$ and $tRNA^{Met}i$ were aminoacylated by the yeast MetRS. Intraorganelle protein synthesis was as in (*29*). Free [^{35}S]Met was used as a control. Equal molar amounts of [^{35}S]Met were used in each case. "/CAM" indicates addition of chloramphenicol at 100 μg/ml.

and found that tRNA import selectivity is similar in human and yeast mitochondria. As expected, $tRK1_{CAU}$ was efficiently imported in its methionylated form (Fig. 4A). This finding was exploited to test whether $tRK1_{CAU}$ is active in human mitochondrial translation. We observed chloramphenicol-sensitive incorporation of [^{35}S]Met into mitochondrial protein after import of [^{35}S]Met-$tRK1_{CAU}$ (Fig. 4B), and the labeled polypeptides were shown to contain antigenic determinants for human Cox1p. Therefore, the imported yeast cytosolic tRNA is functional on the human mitochondrial translation apparatus.

These results suggest the possibility of developing a tRNA mitochondrial import system in human cells. Such a system could be useful for either suppressing nonsense mutations in protein-coding genes or to replace nonfunctional mutant tRNAs.

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16. The absence of incorporation of [^{35}S]Met into proteins after incubation of mitochondria with $tRNA^{Met}i$, which is not imported, indicates that deacylation and reuse of the free-labeled amino acid by mtMetRS does not occur.
17. To construct the HM4 strain, an *amber* codon was introduced at alanine codon 114 of the *COX2* gene by oligonucleotide-directed mutagenesis of pJM2 (*18*). The mutagenized plasmid was introduced into the mitochondria of MCC123ρ^0 cells by high-velocity microprojectile bombardment (*19*). Transformed cells harboring the mutant plasmid were selected by screening for marker-rescue of the *cox2-103* allele after mating to strain HMD3. The *cox2^{amber}* mutation was then integrated into mtDNA by cytoduction of the ρ^- mitochondria into strain NB40-16B. Recombinant ρ^+ progeny were identified by their ability to marker-rescue the *cox2-103* mutation after crossing to HMD13. The presence of the *cox2-114^{amber}* mutation in the ρ^+ mtDNA was verified by sequencing the relevant region of the *COX2* gene. Genotype of HM4 is *MATα leu2-3,112 lys2 ura3-52 arg8::hisG* [ρ^+ *cox2-114^{amber}*].
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28. HmIDPs were prepared as ScIDPs (*3*), except for cell lysis conditions. The cell culture flasks were washed with phosphate-buffered saline (PBS) and incubated for 5 min at 37°C in PBS containing 1 mM EDTA. The cells were collected, washed with PBS without EDTA, resuspended in HKM [10 mM Hepes-KOH (pH 6.5), 50 mM KCl, 2 mM $MgCl_2$] with 10% glycerol in the presence of a cocktail of protease inhibitors, and disrupted by sonication. After removal of nucleic acids by polyethylenimine treatment, proteins were collected from the supernatant by ammonium sulfate precipitation (70%) and dialyzed against HKM with 50% glycerol.
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The Productive Conformation of Arachidonic Acid Bound to Prostaglandin Synthase

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Prostaglandin H synthase-1 and -2 (PGHS-1 and -2) catalyze the committed step in prostaglandin synthesis and are targets for nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin. We have determined the structure of PGHS-1 at 3 angstrom resolution with arachidonic acid (AA) bound in a chemically productive conformation. The fatty acid adopts an extended L-shaped conformation that positions the 13pro*S* hydrogen of AA for abstraction by tyrosine-385, the likely radical donor. A space also exists for oxygen addition on the antarafacial surface of the carbon in the 11-position (C-11). While this conformation allows endoperoxide formation between C-11 and C-9, it also implies that a subsequent conformational rearrangement must occur to allow formation of the C-8/C-12 bond and to position C-15 for attack by a second molecule of oxygen.

PGHSs convert AA, O_2, and two electrons to prostaglandin H_2 (PGH_2) in the committed step in prostaglandin synthesis. PGHSs catalyze two semi-independent reactions (*1*, *2*)—a *bis* oxygenase [cyclooxygenase (COX)] reaction that uses AA and two O_2 molecules to form PGG_2 and a peroxidase (POX) reaction in which PGG_2 undergoes a two-electron re-