Human Molecular Genetics, 2004, Vol. 13, No. 20 **2519–2534**
doi:10.1093/hmg/ddh267
Advance Access published on August 18, 2004

Nuclear DNA-encoded tRNAs targeted into mitochondria can rescue a mitochondrial DNA mutation associated with the MERRF syndrome in cultured human cells

Olga A. Kolesnikova[1,2,3,†], Nina S. Entelis[1,†], Clarisse Jacquin-Becker[1], Francine Goltzene[1], Zofia M. Chrzanowska-Lightowlers[3], Robert N. Lightowlers[3], Robert P. Martin[1] and Ivan Tarassov[1,*]

[1]CNRS-FRE 2375, Institute of Physiology and Biological Chemistry, Université Louis Pasteur, 21 René Descartes, 67084 Strasbourg, France, [2]Department of Molecular Biology, Biology Faculty, Moscow State University, 119992 Moscow, Russia and [3]Mitochondrial Research Group, Medical School, Neurology Department, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

Received June 28, 2004; Revised July 29, 2004; Accepted August 4, 2004

Mitochondrial DNA (mtDNA) mutations are an important cause of human disease for which there is no efficient treatment. Our aim was to determine whether the A8344G mitochondrial tRNA^{Lys} mutation, which can cause the MERRF (myoclonic epilepsy with ragged-red fibers) syndrome, could be complemented by targeting tRNAs into mitochondria from the cytosol. Import of small RNAs into mitochondria has been demonstrated in many organisms, including protozoans, plants, fungi and animals. Although human mitochondria do not import tRNAs *in vivo*, we previously demonstrated that some yeast tRNA derivatives can be imported into isolated human mitochondria. We show here that yeast tRNA^{Lys} derivatives expressed in immortalized human cells and in primary human fibroblasts are partially imported into mitochondria. Imported tRNAs are correctly aminoacylated and are able to participate in mitochondrial translation. In transmitochondrial cybrid cells and in patient-derived fibroblasts bearing the MERRF mutation, import of tRNA^{Lys} is accompanied by a partial rescue of mitochondrial functions affected by the mutation such as mitochondrial translation, activity of respiratory complexes, electrochemical potential across the mitochondrial membrane and respiration rate. Import of a tRNA^{Lys} with a mutation in the anticodon preventing recognition of the lysine codons does not lead to any rescue, whereas downregulation of the transgenic tRNAs by small interfering RNA (siRNA) transiently abolishes the functional rescue, showing that this rescue is due to the import. These findings prove for the first time the functionality of imported tRNAs in human mitochondria *in vivo* and highlight the potential for exploiting the RNA import pathway to treat patients with mtDNA diseases.

INTRODUCTION

Mitochondrial and nucleo-cytosolic compartments of eukaryotic cells are in a permanent cross-talk (1). Mitochondrial DNA (mtDNA) codes for only a small subset of macromolecules residing in the organelle, whereas the majority of proteins and some small RNAs are expressed from nuclear genes and targeted into mitochondria (2,3). Import of nuclear-encoded RNAs into the mitochondrial matrix can now be considered as an universal process. Indeed, found in a variety of biological systems, it appears to be a fundamental mechanism of intercompartmental targeting of macromolecules, along with other pathways better characterized, like protein delivery to mitochondria, chloroplasts or peroxisomes. The mechanism of RNA mitochondrial import has been largely addressed in the past few years in four main models: yeast and human

*To whom correspondence should be addressed: Tel: +33 390241481; Fax: +33 388417070; Email: i.tarassov@ibmc.u-strasbg.fr
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

cells (in our group, reviewed in 3), trypanosomatids (4–10) and plants (11–13). The mechanisms have some similarity, especially with respect to energetic and physico-chemical requirements in all different organisms. On the other hand, the requirement of targeting factors is different: in yeast and human cells aminoacyl-tRNA synthetases were shown to be involved as essential import factors (14,15), in plants these enzymes may also be involved but rather as specificity factors (11,12), whereas in trypanosomatids no soluble factors seem to be involved (5,6). Mitochondrial receptors are also distinct: in yeast and human cells the pre-protein import apparatus is clearly involved (15,16), whereas in trypanosomes protein and RNA import seem to be independent (7,17).

In the yeast *Saccharomyces cerevisiae*, a subpopulation of the cytosolic $tRNA^{Lys}_{CUU}$ (hereafter, tK1) is imported into mitochondria, the second yeast cytosolic $tRNA^{Lys}_{UUU}$ (tK2) is not imported, whereas mtDNA codes for the third $tRNA^{Lys}_{UUU}$ (tK3) (18). In human cells, tRNAs are normally not imported (as all mt-tRNAs required for mitochondrial translation are encoded in the mitochondrial genome), whereas other small RNA species (5S rRNA, MRP RNase and RNase P RNA components) have been reported to be mitochondrially targeted (19,20). We previously found that yeast tK1, mutant versions of tK2 and the synthetic transcript of tK3 could be imported into isolated human mitochondria (15).

In yeast, two aminoacyl-tRNA synthetases are involved in tK1 targeting into mitochondria: the cytosolic lysyl-tRNA synthetase KRS1p aminoacylates the tRNA *prior* to the import, whereas the cytosolic precursor of the mitochondrial enzyme, pre-MSK1p, serves as the carrier/targeting factor to deliver the tRNA to the organelle (3,14,21). Import of tRNAs into isolated human mitochondria was found to be dependent on soluble proteins (22) and yeast protein extracts can be replaced by human cell extract (15), suggesting that human cells possess a cryptic tRNA import mechanism. When yeast $tRNA^{Lys}$ derivatives were imported into isolated human mitochondria, it was suggested (on the basis of immunodepletion experiments) that the human counterpart of the pre-MSK1p was able to replace the yeast protein without loss of import specificity (15).

RNA import pathways revealed a high degree of flexibility, permitting the RNAs from one organism to be imported into mitochondria of another (for example, yeast tRNAs in human mitochondria, yeast tRNAs in trypanosomatid ones, tRNAs from one plant species into the mitochondria of another plant, where they are not normally imported) (3,23). We have shown previously that one can import into yeast and human mitochondria mutant versions of cytosolic tRNAs that are normally not imported (tK2-variants), tRNAs that are normally encoded in mtDNA (tK3), as well as tRNAs that had altered aminoacylation and decoding properties [tK1cau, aminoacylated with methionine; tK1/2(G3:U70) versions aminoacylated with alanine; tK1/2cua versions able to decode *amber* stop codons] (3,24). Such flexibility leads us to propose that the RNA import pathway could be exploited to rescue dysfunctions associated with mt-tRNA mutations in humans. We previously proved the possibility of such complementation in yeasts (22), and in the present study aimed to demonstrate that such rescue may be achieved in human cells.

More than 200 mutations in human mtDNA have been associated with neurodegenerative and muscular pathologies (25–27). As a model, we have chosen the A8344G MERRF (myoclonic epilepsy with ragged-red fibres) mutation affecting the mt-$tRNA^{Lys}$ gene and localized in the T-arm of the tRNA, which is one of the best-studied and relatively common pathological mutations in human mtDNA (28,29). The A8344G mutation has been reported to cause poor aminoacylation of the mutant tRNA (30), hypomodification of its anticodon wobble-position (31) and premature termination of translation at some lysine codons (30), all of which result in decreased activities of respiratory complexes I and IV, low respiration rate and decreased potential across the mitochondrial inner membrane (32). We demonstrate here for transmitochondrial cybrid cells and primary fibroblasts that import $tRNA^{Lys}$ derivatives from cytosol into mitochondria may partially rescue all the mitochondrial functions affected by the MERRF mutation.

RESULTS

Expression of yeast $tRNA^{Lys}$ versions in immortalized human cells

We previously found that tK1, several of its mutant versions, mutant versions of tK2 and the synthetic transcript of tK3 could be imported into isolated human mitochondria (15,24). We tested whether the import could be reproduced in cultured human cells for three tK derivatives shown to be imported *in vitro*. Genes encoding a mutant version of tK1 (tK1cau), tK3 and a mutant version of tK2 (tK93) (Fig. 1A) were cloned into a mammalian expression vector pBK-CMV. Following stable transfection of human HepG2 cells, tK1cau, tK93 and tK3 expression was detected by northern hybridization (Fig. 1B–D). pBK-CMV vector was uniquely used for introducing the tRNA genes in the cell, but not their expression directed from the CMV promotor, since tRNA genes are transcribed by RNA polymerase III (RpoIII) in the nucleus, and by mitochondrial RNA polymerase (mtRpo) in mitochondria (33). The genes encoding both human and yeast cytosolic tRNAs possess conserved blocks (T- and D-motifs) which constitute the internal promoter for RpoIII (34,35). Therefore, tK1cau and tK93 versions of cytosolic yeast tRNA genes may be transcribed by the host RpoIII in human cells. In contrast, tK3 is coded for by mtDNA in yeast. Fortunately, tK3 sequence revealed the presence of sequence motifs very similar to the conserved blocks of RpoIII internal promoter. To be more affirmative, one can suggest using specific inhibitors of RpoII and III to distinguish between the two polymerases. However, taking into account that the tRNA genes were cloned in an opposite direction when compared with the CMV promoter, one can suggest that tK93, tK1cau and tK3 were expressed by the human RpoIII, and not by the host RpoII. Comparison of gel migration of tK1cau, tK93 and tK3 isolated from yeast and human cells revealed no detectable difference (data not shown), therefore, we suggest that yeast tRNA derivatives were correctly processed in human cells.

In agreement with the *in vitro* data, a fraction of the sucessfully expressed transgenic tRNAs were localized in highly

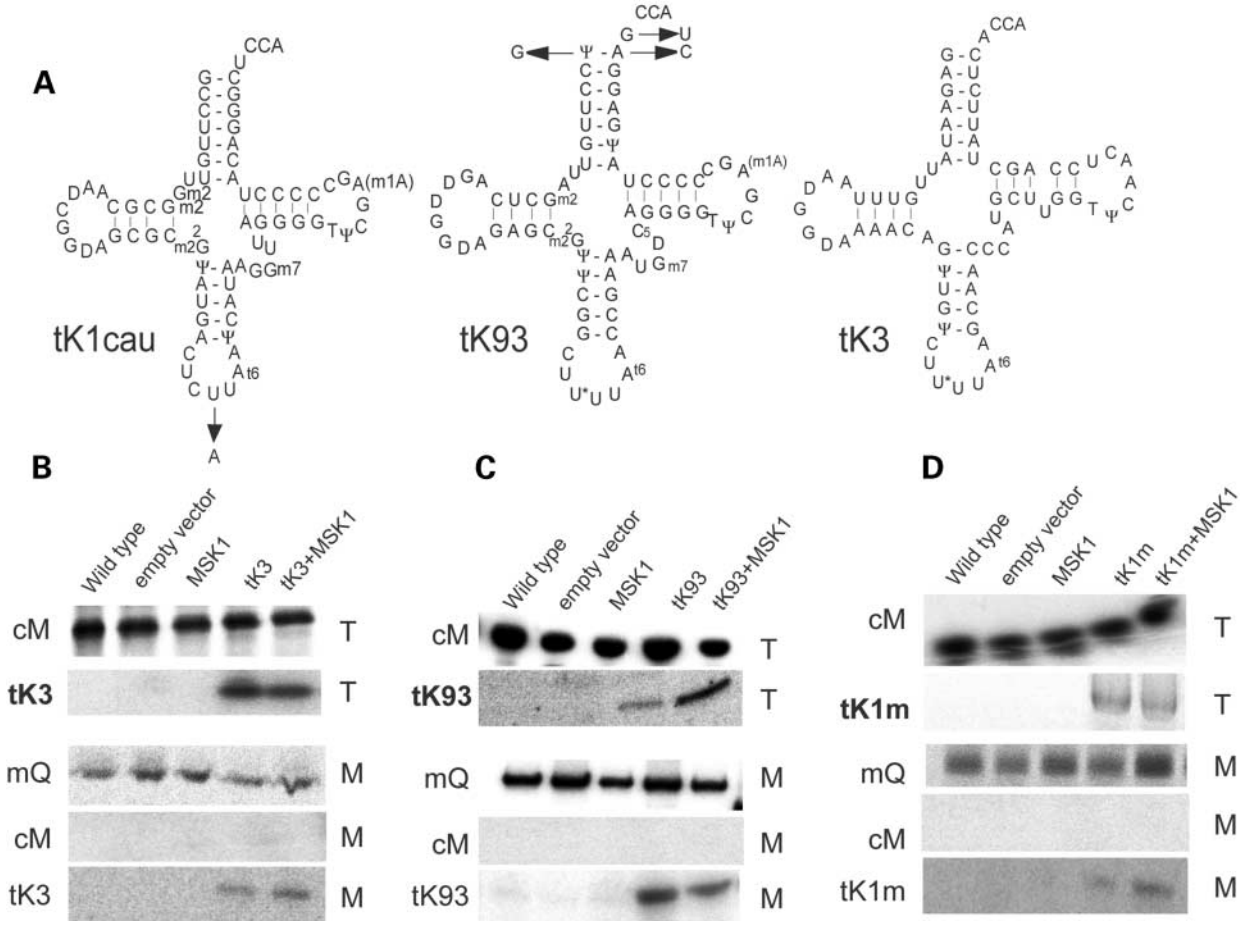

Figure 1. Mitochondrial import of yeast cytoplasmic tRNAs expressed in stably transfected human HepG2 cells. (**A**) Cloverleaf structures of yeast $tRNA^{Lys}$ derivatives. The arrows show mutations introduced into tK1 (U34A) to confer methionine aminoacylation identity (version tK1cau) and into tK2 (Ψ1G, A72C and G73U) to induce its import (version tK93). (**B**–**D**) Northern hybridization results are shown for total and mitochondrial RNA isolated from tK3 (B), tK93 (C) and tK1cau (D)—expressing HepG2 cells and controls (wild-type, non-transfected cells; empty vector, cells transfected with pBK-CMV alone; MSK1, cells transfected with pBK-CMV containing only the *MSK1* gene). On the right, the source of RNA: T—total cellular RNA; M—RNA from purified mitochondria. On the left, the hybridization probes are indicated: cM ($tRNA_i^{Met}$) is present only in the cytosol and mQ ($tRNA^{Gln}$) only in mitochondria. The amounts of RNAs loaded in each lane were equal, but the blots with positive controls (mQ with mitochondrial RNA isolates and cM with total RNA) were underexposed, since the foreign yeast tRNAs are expressed at lower levels than the endogenous ones.

purified mitochondria. Since purification included the step of mitoplast generation (removal of the outer membrane), the most probable localization of the tK version was the mitochondrial matrix, where host mtRNAs normally reside. Real-time RT–PCR revealed that ~2–5% of the total cellular pool of tK versions was associated with the mitochondrial compartment of the cell. Taking into account the importance of the carrier protein, pre-MSK1p, for tK1 import into yeast mitochondria, all three versions were tested in human cells either alone, or in the presence of the pre-MSK1p-coding gene on the same plasmid. For versions tK3 and tK1cau, the import efficiency was reproducibly 30–40% higher in the presence of the *MSK1* gene, whereas there was no reproducible effect of *MSK1* coexpression on tK93 import (Fig. 1B–D). This result is in agreement with our previous *in vitro* data and suggests that pre-MSK1p may be dispensable for import in human mitochondria. However, taking into account a clear positive effect of *MSK1* coexpression on import efficiency of at least some of the versions, for all further experiments, *MSK1*-containing constructs were used. These results are in agreement with our previous *in vitro* data and demonstrate that a cryptic tRNA import mechanism can be activated in human cells by a simple expression of a suitable tRNA version. It is clear, however, that the detailed mechanism remains to be clarified and using pre-MSK1p or its mutant version may permit to improve import efficiency for at least some of the constructs.

Expression of tK versions in transmitochondrial cybrid cells bearing the MERRF mutation

The A8344G MERRF mutation affects the $tRNA^{Lys}$ gene encoded in human mtDNA. We wished to determine the effect of expressing importable yeast $tRNA^{Lys}$ on mitochondrial functions in two genetically independent transmitochondrial cybrid MERRF cell lines, M90 and M100, both

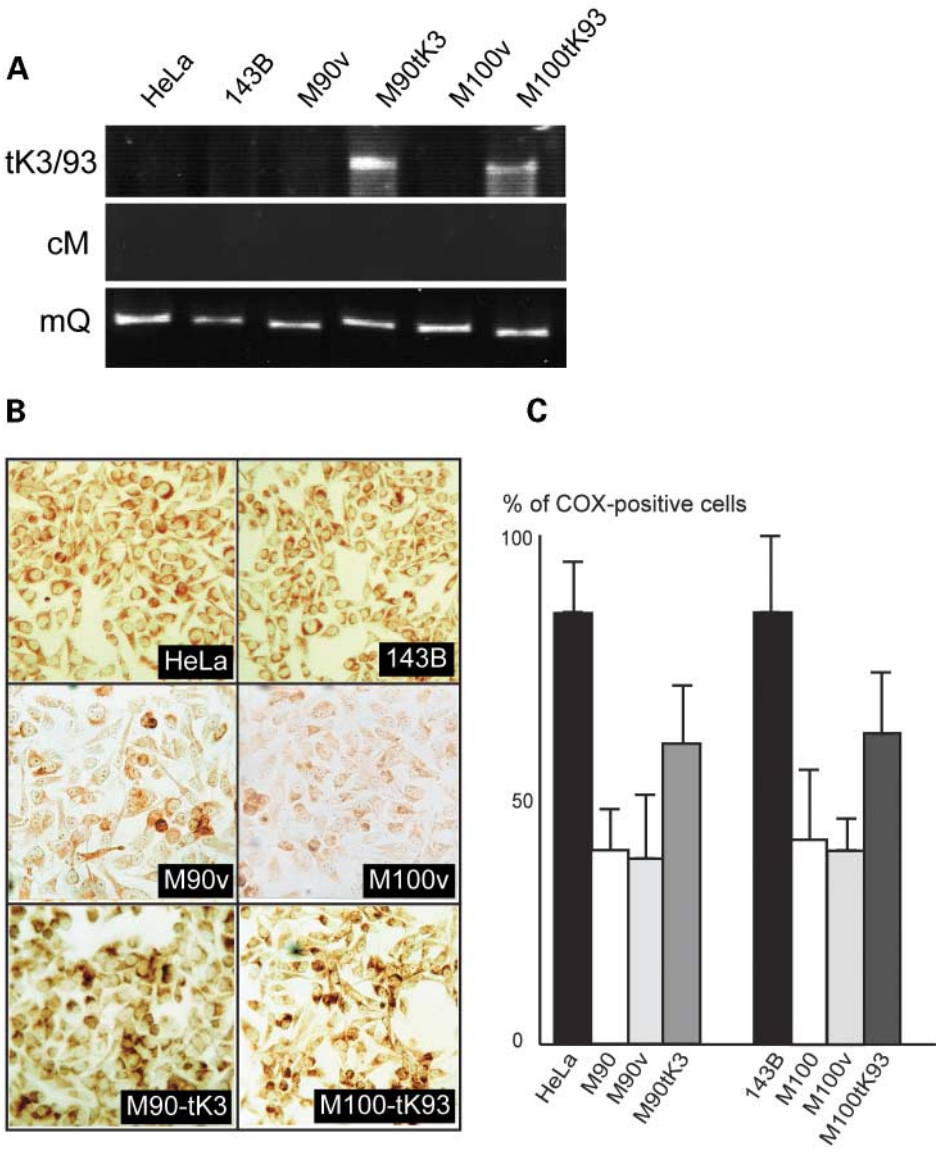

Figure 2. Mitochondrial import of yeast cytoplasmic tRNAs expressed in stably transfected M90 and M100 transmitochodrial cybrid cells. (**A**) RT–PCR detection of imported tK3 ans tK93 in mitochondrial RNA of transfectants. Cell lines are indicated above the photographs of ethidium bromide-colored gels. RT–PCR was done with tK3-specific primers for M90tK3, tK93-specific primers for M100tK93 and with both pairs of primers for control lines (parental HeLa, 143B and empty-vector-transfected M90v and M100v). The absence of contamination with cytosolic RNA was confirmed by RT–PCR with primers specific to the cytosolic $tRNA^{Met}$ (cM), the intactness of mitochondrial tRNAs was confirmed by RT–PCR with primers specific to the mitochodrial $tRNA^{Gln}$ (mQ). (**B**) Cytochemical detection of cytochrome *c* oxidase (COX) activity in tK3 and tK93-expressing cybrid cells. Light microscopy pictures of a representative population of cells following staining for COX activity (brown) are shown. HeLa and 143B, non-transfected positive controls of parental cell lines; M90v and M100v, two lines of cybrids transfected with the *MSK1* gene containing pBK-CMV, without tRNA genes; M90t3 and M100t93, cybrid cells transfected with tRK3 and tRK93, respectively. (**C**) Quantification of COX-positive cells in the population. At least five independent anonymized counts of >500 cells were done for each type of cells as detailed in Materials and Methods.

almost homoplasmic for the mtDNA mutation, and in the relevant wild-type (*rho*+) parental lines (HeLa and 143B, respectively).

Expression of tK3 in M90 and tK93 in M100 cybrids leads to an association of a portion of yeast tRNA derivatives with purified human mitochondria (Fig. 2A), similar to the results shown above for the 'healthy' HepG2 cells. Wild-type parental lines and stable transfectants were cytochemically analyzed for cytochrome *c* oxidase activity (complex IV). We found that populations of the non-transfected MERRF cybrids, as well as cybrids transfected with the vector alone, demonstrated strongly decreased number of COX-positive cells (30–40% in comparison with their respective parental cell lines), whereas populations of cybrid transfectants expressing either tK93 or tK3 contained a greater number of cytochrome *c* oxidase (COX)-positive cells, 60–70% in

comparison with the parental cells (Fig. 2B and C). Although tK93/3 expression appeared to increase the COX activity, rescue was heterogeneous and an important fraction of the cells in the transfectant pool still retained low levels of enzymatic activity. Since this effect may have been due to differences of levels of expression of transgenes in different clones, we performed further experiment on individual clones of transfectants expressing tK versions at reproducibly stable levels.

Analysis of mitochondrial functions in individual clones of transfected cybrid cells

We selected individual clones of M90 and M100 cells stably expressing and importing tK93 or tK3 with different efficiencies (Fig. 3A). These clones were still shown to be nearly homoplasmic for the MERRF mutation (>95% of mutant genomes), similar to the original cybrids and individual clones expressing the vector alone (Fig. 3B). Selected clones were then subjected to a detailed analysis of mitochondrial functions and compared with a series of clonal transfectants containing the empty vector (or the vector containing the pre-*MSK1* gene without tRNA genes) and with parental *rho*+ cell lines.

Among 50 initially characterized clones, several were analyzed in detail. Three clonal M90 transfectants expressing tK3, three M100 transfectants expressing tK93 and three clones with the vector alone in each line were first analyzed for their substrate-dependent respiration (Fig. 4A and B). No detectable effect was found in any clone transfected with the vector alone. In contrast, a clear correlation was found between the amount of imported tRNA and the increased respiration rate for both M90 and M100 transfectants. For clones expressing the highest levels of the yeast tRNAs (M90tK3c2 and M100tK93c7), respiration reached 60–80% of the wild-type parental cells, which represents a gain of 80–120% in comparison with the non-transfected cells.

To measure the membrane potential, we used the lipophilic cation methyltriphenyl-phosphonium (TPMP), which is imported into mitochondria as a function of the electrochemical potential across the inner mitochondrial membrane. The loss of inner membrane charge results in a decrease of TPMP binding by digitonin-permeabilized cells (32). Again, we found that the level of tRNA import coincided with the rescue in membrane potential reaching 70% of the parental lines for the highest tK93 and tK3 expressors (Fig. 4C).

Respiratory enzymes activity in mitochondrial fractions from various cell lines was also determined. In agreement with previous reports (32), the original cybrid lines showed decreased activities of complexes I and IV, but not markedly for II and III. Expressing tK3 and tK93 in M90 and M100 cells partially rescued activities of complexes I and IV, whereas the same lines transfected with the vector alone exhibited activities similar to those of non-transfected cells (Table 1).

The MERRF cybrids were reported to be unable to grow on media containing galactose instead of glucose, conditions under which oxidative phosphorylation is required for growth (36). Therefore we compared the growth rate of transfected and non-transfected cybrid cells on galactose-containing media (Fig. 5). As expected, M90 and M100 cybrids were unable to grow in galactose medium. In contrast, tK3-expressing M90 and tK93-expressing M100 clones were able to propagate, although more slowly than parental cells.

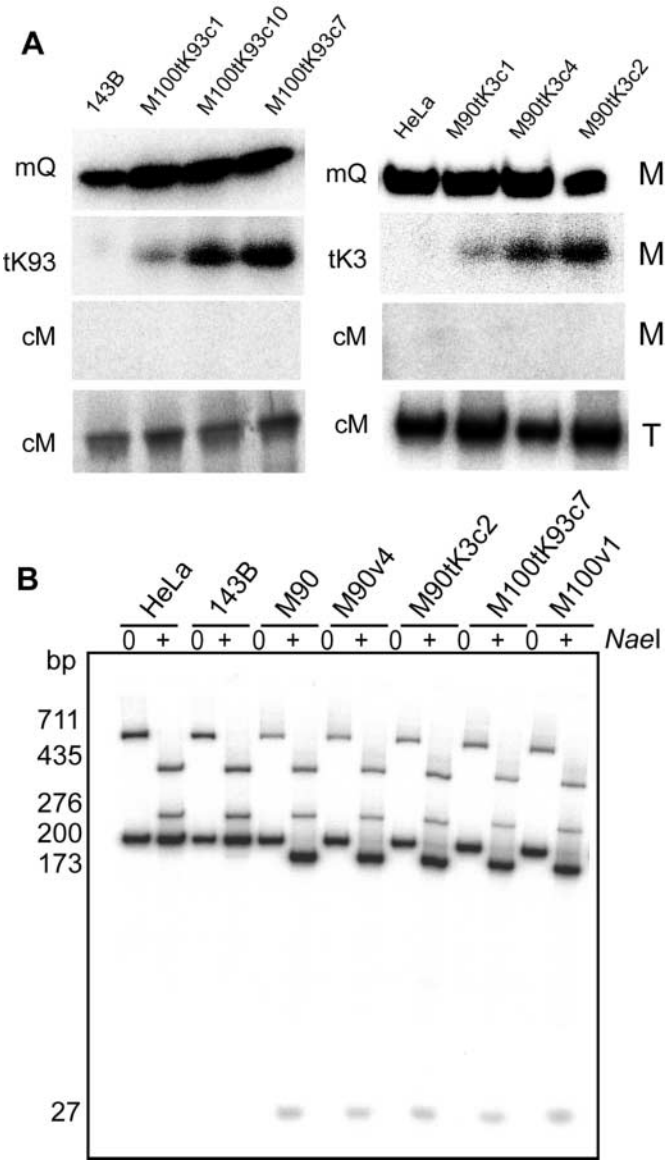

Figure 3. Analysis of individual cybrid clones expressing tK3 and tK93. (**A**) Import of tK93 and tK3 in individual clones of transfected cybrids: clones 1, 10 and 7 of M100tK93, and clones 1, 4 and 2 of M90tK3 transfectants. Mitochondrial (M) and total (T) cellular RNAs were isolated from individual clones and northen hybridizations performed. The absence of cytosolic contaminations in mitochondrial RNA isolates and the absence of degradation of mitochondrial tRNAs was confirmed by hybridization with the corresponding probes (mQ and cM). The amount of RNA loaded was equal in each case, but the positive control blots (mQ) were underexposed. (**B**) Hot-last cycle PCR verification of heteroplasmy levels in individual clones of M90 and M100 transfectants. Digestion with *Nae*I only cleaves the amplicon containing the A8344G mutation, resulting in two products of 173 and 27 bp. As a cleavage control, all digests were spiked with a 711 bp amplicon carrying a single *Nae*I site. The cell lines used to isolate DNA are indicated at the top. + and 0 indicate *Nae*I-cleaved and non-cleaved PCR products, respectively.

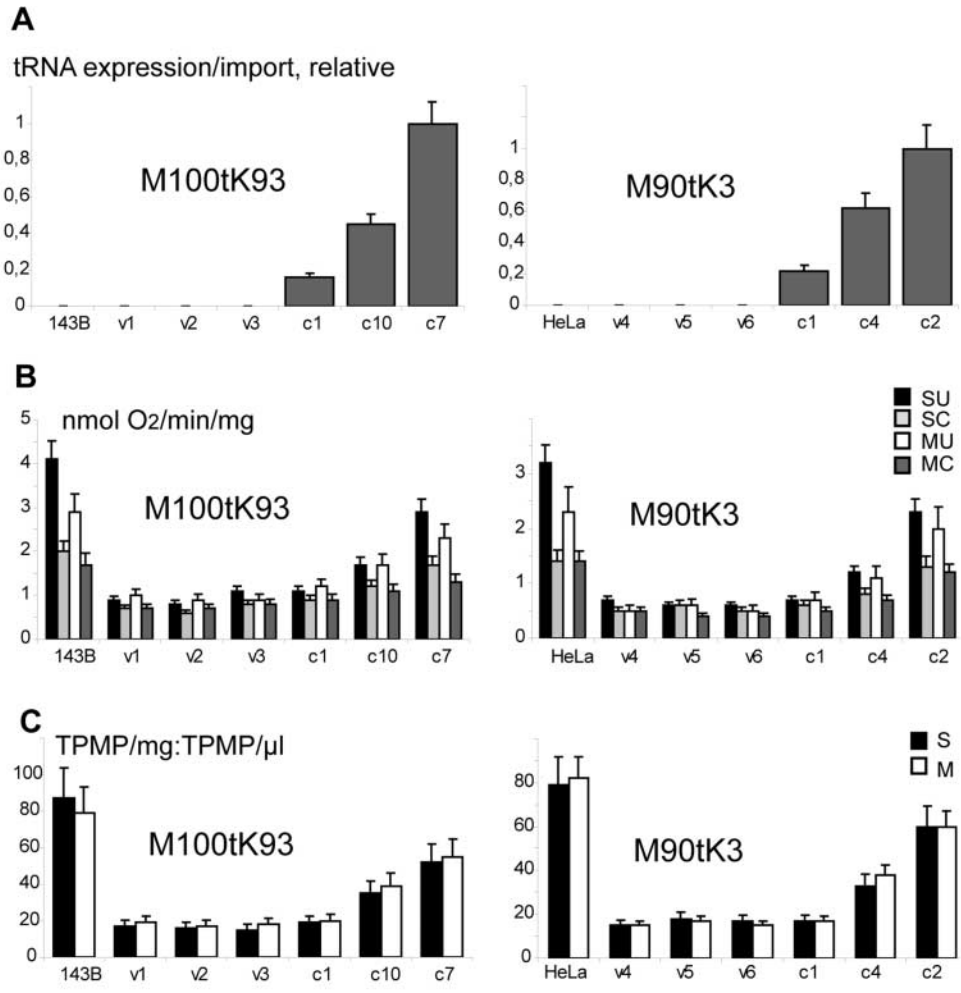

Figure 4. Effect of tRNA import on mitochondrial respiration and membrane potential in stable cybrid transfectants. (**A**) Relative values of tRNAs expression as deduced from quantitative northern hybridization (presented in Fig. 3A). The level of tK93 and tK3 expression was normalized to host mitochondrial $tRNA^{Gln}$. The expression level of the best clone was taken as 1.0 in each series (M90tK3c2 and M100tK93c7, respectively). The quantification is a result of three independent experiments with $\pm$SEM $<$10%. (**B**) Respiration rate of various cell lines in digitonin-permeabilized cells. Values are expressed as nmoles of oxygen consumed per min and per mg of protein (*Y*-axis). The $\pm$SEM values are the result of at least five independent measurements. The statistical significance *P* was between 0.01 and 0.05 in all the cases. SC and SU, respectively, coupled and uncoupled respiration with succinate and rotenone; MC and MU, respectively, coupled and uncoupled respiration with glutamate and malate. (**C**) Measurement of membrane charge as TPMP accumulation in digitonin-permeabilized cells. S, with succinate and rotenone; M, with glutamate and malate. Values were expressed as (TPMP/mg protein)/(TPMP/µl) (*Y*-axis).

This behaviour of the transfectants suggests a rescue of mitochondrial oxidative metabolism due to the import of complementing tRNAs.

Analysis of the translational functionality of imported tRNAs

The MERRF mutation has been reported to lead to a decrease in mitochondrial translation (37). We also detected a 40–50% drop of mitochondrial translation rate in M90 and M100 cells (Fig. 6A). In contrast, expression of tK3 and tK93 partially restored mitochondrial translation (70–80% of parental lines for the best expressor clones M90tK3c2 and M100tK93c7). This result may indicate that imported tRNAs of yeast origin can pariticipate in human mitochondrial translation.

Depending on the MERRF cell line used, several truncated mitochondrially synthesized polypeptides have been reported (30,38,40). The most abundant product of such abortive translation detected in all cell lines expressing the A8344G mutation was related to Cox1p (38). When analyzing increased (with respect to control parental cells) amounts of labelled mitochondrially sysnthesized polypeptides on protein gels, we also detected this polypeptide among the products of *in vivo* mitochondrial translation in M90 and M100 cells (Fig. 6B). This polypeptide was not detected in parental cell lines, was severely decreased in M100tK93c7 clone (by 80%) and was virtually undetectable in M90tK3c2. This effect was directly related to the levels of yeast $tRNA^{Lys}$

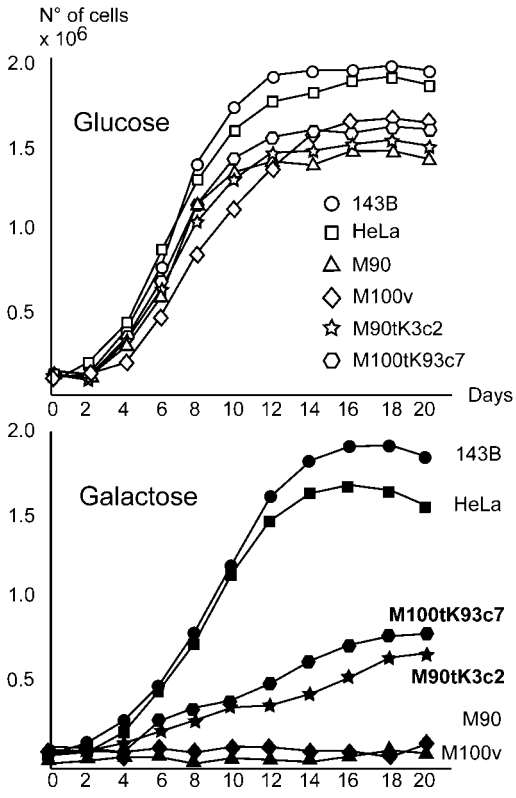

Figure 5. Growth curves of control and cybrid cells on glucose and galactose-containing media. In 9.5 cm^2 wells 10^5 cells were grown. Each value represents a total number of cells per well calculated from the average number of cells in 10 individual counts in 1 mm^2. At 8, 12 and 20 days, these counts were validated by detaching the totality of cells from the wells (in parallel cultivations) and subjecting them to a haemocytometer counts.

import, since it was not detected either in empty-vector expressing clones, or in clones expressing tK3 or tK93 to low levels (data not shown). This result also is in agreement with our hypothesis that imported tRNAs of cytosolic type may be active in mitochondrial translation.

The rescue of translational defects suggests that yeast tRNA derivatives must be aminoacylated in human mitochondria. To confirm that imported tK versions were aminoacylated, we analyzed mitochondrial tRNA of the transfectants isolated and separated under acidic conditions, to conserve the aminoacyl-tRNAs intacts (41), by northern hybridization (Fig. 6C). The difference in migration of the aminoacylated and deacylated forms of tK3 and tK93 extracted from human and yeast mitochondria was similar, suggesting that the yeast tRNAs expressed in human cells may be aminoacylated with lysine, as expected. tK3 was present in the mitochondria essentially in its aminoacylated form; poor resolution of acid–gel analysis for this low expressed version does not permit to be too affirmative, but approximately, >80% of labelled probe was associated with the upper (aminoacylated) form. tK93 version was partially deacylated, only 30% of the tRNA being aminoacylated (Fig. 6C). If normalized against the host mitochondrial $tRNA^{Gln}$, it appears that the level of expression of tK3 in the clone M90tK3c2 was lower than that of tK93 in the clone M100tK93c7. Nevertheless, the amounts of aminoacylated tK3 and tK93 in mitochondria were similar for both clones, in agreement with a comparable rescue of mitochondrial functions.

These experiments permit us to hypothesize that yeast $tRNA^{Lys}$ versions were correctly aminoacylated in human cells, and may function in mitochondrial translation, thus permitting the rescue of defects in mito-translation due to the MERRF mutation.

Table 1. Mitochondrial complexes activity in cybrid clones

Cells	Citrate synthase	Complex I	Complexes II and III	Complex IV
HeLa	285 ± 15 (3)	25 ± 4 100%	62 ± 8 100%	66 ± 18 100%
M90	174 ± 21 (4), $k = 0.61$	3 ± 1 20%**	33 ± 4 88%**	15 ± 2 37%**
M90v4	180 ± 14 (4), $k = 0.63$	3 ± 1 22%*	36 ± 5 93%*	18 ± 2 43%**
M90t3c1	197 ± 23 (3), $k = 0.68$	5 ± 2 28%**	36 ± 3 86%*	20 ± 4 45%**
M90t3c2	223 ± 62 (6), $k = 0.79$	11 ± 3 58%**	44 ± 6 89%*	36 ± 3 69%**
143B	410 ± 31 (3)	31 ± 5 100%	70 ± 6 100%	69 ± 10 100%
M100v1	232 ± 35 (4), $k = 0.56$	3 ± 1 17%*	27 ± 3 69%*	15 ± 3 38%*
M100t93c1	264 ± 22 (4), $k = 0.65$	4 ± 1 21%**	32 ± 5 71%*	19 ± 3 42%*
M100t93c7	286 ± 47 (6), $k = 0.68$	11 ± 2 52%**	42 ± 6 89%*	29 ± 4 61%*

The activities were expressed as nmol/min per mg of mitochondrial protein. To eliminate any changes due to general depletion of mitochondria, the activities of the MERRF cybrids, normalized against citrate synthase activity, were also expressed as a percentage of the normalized activity of the parental cells (HeLa for M90 and 143B for M100 cybrids; k, the coefficient used for correction of all values in relation to citrate synthase activity). The number of independent measures is indicated in parentheses. The statistical significance of all measurements was calculated relative to control cells (HeLa) using Student's unpaired two-tailed t-test.
*$P < 0.05$.
**$P < 0.01$.

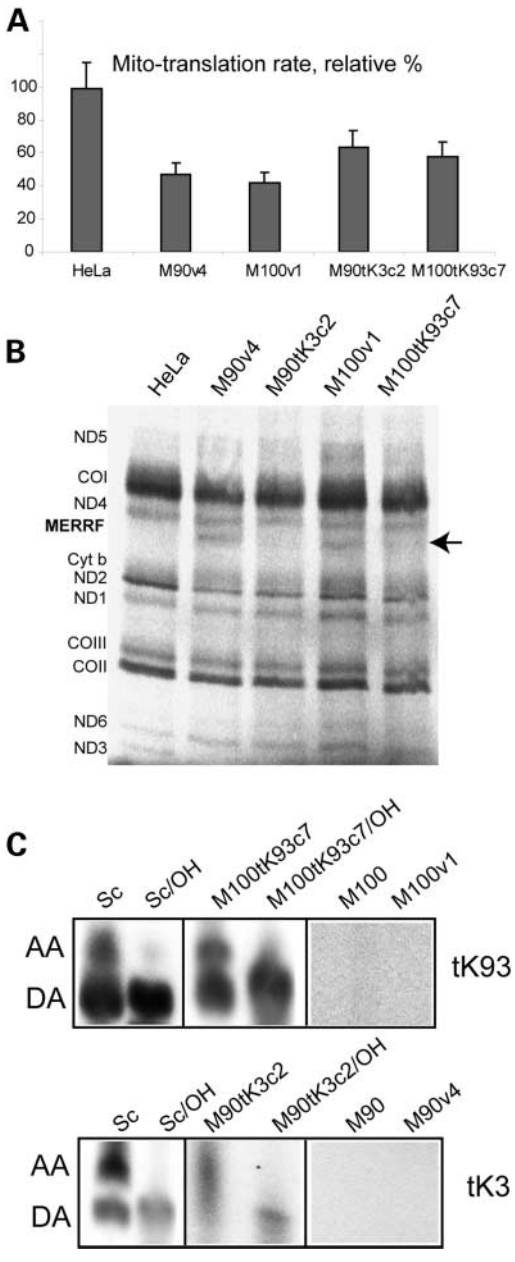

Figure 6. Effect of tK3 and tK93 expression on mitochondrial translation activity in stable cybrid transfectants. (**A**) Quantification of *in vivo* mitochondrial translation activity in cybrid cells and their transfected clones. ^{35}S-labelled translation products were TCA-precipitated and aliquots corresponding to an equal number of cells were scintillation counted. The activity corresponding to the control cells (HeLa) was taken as 100%. The values presented are the result of two independent measures with three different numbers of cells. The standard deviation was less than 10% in each case. (**B**) The autoradiograph of PAGE-separated ^{35}S-labelled mitochondrial translation products in cybrid cells and their transfected clones. Cell lines analyzed are indicated at the top. Mitochondrial translation products are indicated on the left, accordingly to the standard human mitochondrial translation pattern (38). ND1–6, NADH dehydrogenase subunits 1–6; COI–III, cytochrome *c* oxidase subunits; Cyt *b*, cytochrome *b*. The ATP synthase component, ATPase 6, migrates identically on this gel system to either COII or III. The product of premature termination of COI translation characteristic for the MERRF mutation is shown in bold and indicated with an arrow. To visualize more easily the MERRF characteristic translation product, equal radioactivity amounts were loaded on the gel (approximatively twice more for MERRF cells than for the controls). As controls, only M90v4 and M100v1 are presented, but four other clones transfected with the vector alone were analyzed and gave a similar result. (**C**) *In vivo* analysis of tK3 and tK93 aminoacylation in mitochondria of transfected cybrid cells. The autoradiographs of northern hybridizations after acid gel migration are presented. 'Sc', RNA isolated from either wild-type yeast mitochondria (for tK3 detection) or tK93 expressing and importing yeast strain (39). On the right panels, RNA analyzed was isolated form M100 or M90 transfected clones as indicated. '/OH', RNA was deacylated by incubation in Tris–HCl (pH 9.0) before loading on gel. 'AA', aminoacylated form; 'DA' deacylated form of the tRNA. An aliquot of 1 μg of yeast mitochondrial RNA and 10 μg of human mitochondrial RNA were analyzed.

Studying the effect of siRNA-mediated downregulation of tK versions onto mitochondrial functions in transfected cybrid clones

A corellation of tK3/93 import efficiency with mitochondrial functions rescue is consistent with the hypothesis that the rescue is due to the import of tK versions. To rule out the possibility that the rescue of mitochondrial functions was due to an inadvertent selection of respiratory proficient clones from the original population of transfected cells, downregulation of the expressed tRNAs was attempted with siRNAs in clonal transfectants of cybrid cells. The RNA interference (RNAi) pathway is being extensively exploited for silencing a number of genes in insect, plant and mammalian cells. RNAi is based on an ancient anti-viral defence mechanism found in eukaryotes. It is induced by double-stranded RNAs, which are processed to 21–23 nt small interfering RNAs (siRNAs), that promote the recognition and subsequent degradation of homologous endogenous mRNA (42–45). On the other hand, small non-coding RNA molecules may be mediators of a post-transcriptional gene silencing mechanism that regulates the expression of developmental genes, although in this pathway, the translation of mRNAs is affected (46). It is clear that to affect tRNAs by RNAi only the degradation pathway should be effective.

Having confirmed the efficacy of the siRNA inhibition with the luciferase reporter system (Fig. 7A), we transiently transfected the clones M90tK3c2 and M100tK93c7 with siRNAs against either tK3 or tK93. Real-time RT–PCR quantification indicated that expression of tK3 and tK93 was specifically inhibited since siRNA against tK3 did not alter the expression of tK93 and vice versa (Fig. 7B). Our experiments clearly demonstrated that small RNA duplexes can indeed induce specific degradation of tRNAs. We cannot, however, pretend that this approach is efficient enough to achieve silencing of any tRNA, since our targets were transgenic species, which were under-represented in human cultured cells. Nor can we be sure that the exact molecular mechanism of mRNA or tRNA degradation is the same. Nevertheless, we demonstrate for the first time that non-coding RNAs may be affected by dsRNA-induced downregulation as well as messenger RNAs.

Contrary to the impressive downregulation of luciferase activity, mitochondrial parameters were only slightly affected by inhibtion of tK3 and tK93 expression at the day 3 post-transfection (10–20%). At the day 6, however, we observed a strong specific inhibition of respiration and of the membrane potential, resulting in levels similar to the non-transfected cybrid cells (Fig. 7C and D). Consistent with reported data

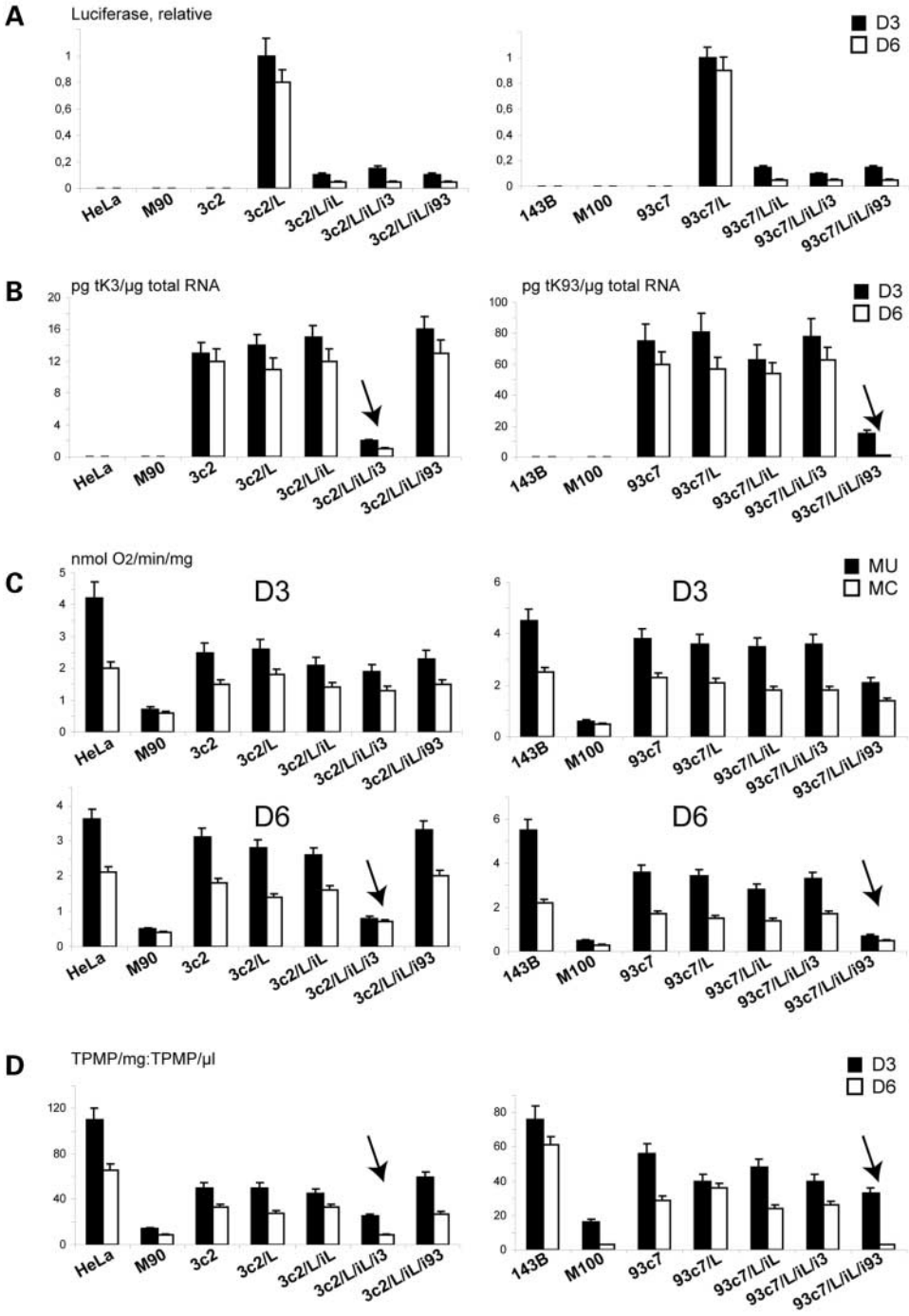

Figure 7. Knock-down of tK3 and tK93 expression in transfected cybrid cells by RNAi. (**A**) Control RNAi assay for luciferase activity. We transiently co-transfected the clones M90tK3c2 and M100tK93c7 with the reporter system (pGL3 coding for the luciferase and the siRNA duplex against luciferase mRNA) and with siRNA duplexes against either tK3 or tK93. The presence of the anti-luciferase siRNA strongly inhibited (by 85–90%) the expression of the reporter gene already at day 3 after transfection and this inhibition became almost complete at day 6. HeLa, 143B, M90, M100, M90tK3c2 (3c2) and M100tK93c7 (93c7) without pGL3; 3c2/L and 93c7/L, the clones were transfected with pGL3; 3c2/L/iL and 93c7/L/iL, the clones were co-tansfected with pGL3 and the siRNA against the luciferase mRNA; 3c2/L/iL/i3 and 93c7/L/iL/i93, the clones were co-transfected with pGL3, the siRNA against the luciferase mRNA and either the siRNA against tK3 or that against tK93, respectively. D3 and D6 indicate the number of days after the first transfection (as detailed in Materials and Methods). Luciferase activity was normalized to protein amount and was expressed relative to the positive controls (3c2/L and 93c7/L). (**B**) Quantification of the tK3 and tK93 by RT–qPCR. Quantification was done as detailed in Materials and Methods and expressed in pg per µg of total or mitochondrial RNA. To quantify, synthetic T7-transcripts were used as controls. The cytosolic $tRNA_i^{Met}$ and mitochondrial $tRNA^{Gln}$ served for normalization in each series. (**C**) Respiration rates of various cell lines in digitonin-permeabilized cells. Values are expressed as nmoles of oxygen consumed per min and per mg of protein (*Y*-axis). The ± SEM values are the result of at least two independent measurements and were < 10%. MC and MU, coupled and uncoupled respiration with glutamate and malate, respectively. (**D**) Measurement of membrane charge as TPMP accumulation in digitonin-permeabilized cells with glutamate and malate. Values were expressed as (TPMP/mg protein)/(TPMP/µl) (*Y*-axis). The ± SEM values are the result of two independent measurements and were < 10%.

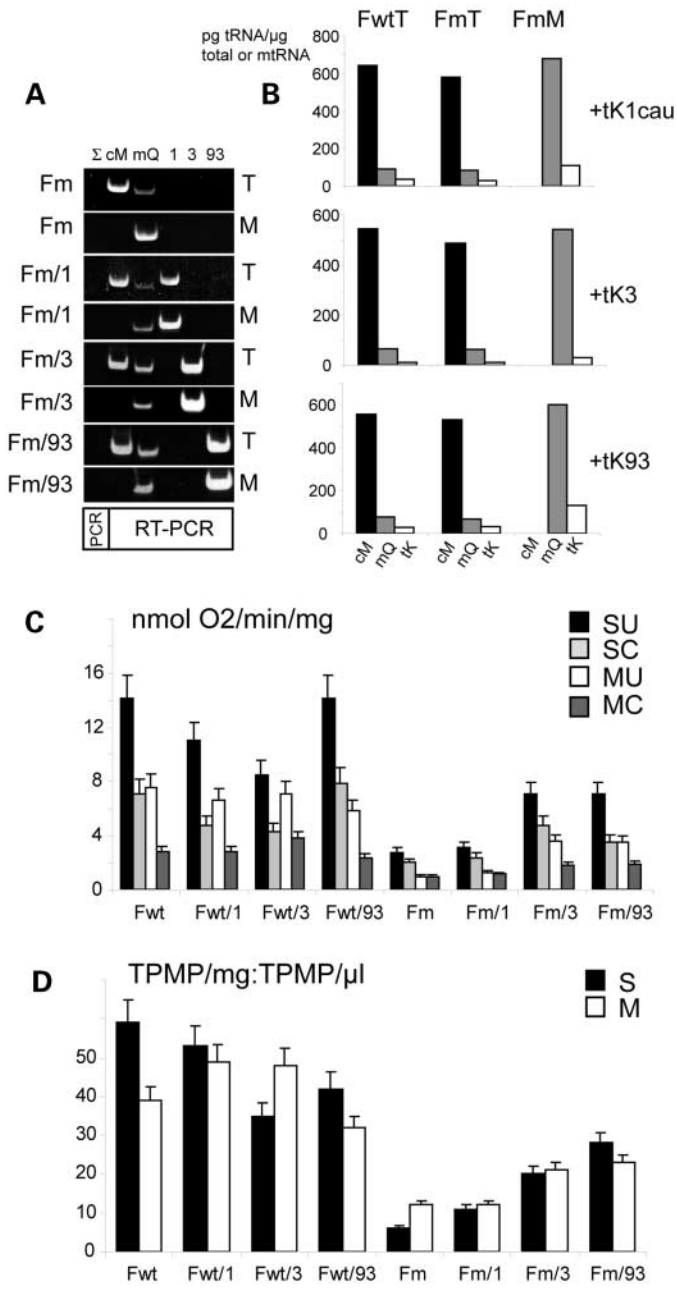

Figure 8. The effect of tK3 and tK93 transient expression in primary human healthy and MERRF-mutation-bearing fibroblasts. (**A**) Detection of transient expression and mitochondrial import of tK1cau (1), tK3 (3) and tK93 (93) by RT–PCR. The conditions used permit specific detection but not quantification of the RNAs, and the lower amount of the amplicon corresponding to the control mitochondrial tRNA (mQ) is due to a decreased efficiency of the primers used in the given conditions. Only RT–PCRs with the MERRF-patient fibroblasts are presented. Analysis of healthy fibroblasts gave the same results (data not shown). Fm, fibroblasts from the MERRF patient; Fm/1, fibroblasts transfected with tRK1cau bearing plasmid; Fm/3 and Fm/93, fibroblasts transfected with either tK3 or tK93 bearing plasmids. T, total RNA; M, mitochondrial RNA. The primers used are indicated above the gels: Σ, all five pairs of primers; cM, cyto-tRNA$_i^{Met}$; mQ, mito-tRNA^{Gln}; 1, tK1; 3, tK3 and 93, tK93. The first lane corresponds to the reaction without reverse transcriptase and in the presence of the five pairs of primers (PCR, as indicated at the bottom) to confirm that the RNA isolates were DNA-free. (**B**) Quantitation of mitochondrial tRNAs in the transfected MERRF fibroblasts by real-time RT–PCR. Quantitation was performed in serial dilutions of the RNA (1–1000 pg) and calculated in comparison with the T7-transcripts of tRK1, tRK93 and tRK3 used as standards for each series (Fm, fibroblasts with MERRF mutation). The values presented are picograms of the given tRNA per microgram of total or mitochondrial RNA. Each sample was analyzed in triplicate ($\pm$SEM value was $<10\%$) and the average value is presented. In wild-type transfectants only total RNA was analyzed. (**C**) Respiration rate of control and transfected fibroblasts in digitonin-permeabilized cells. Values are expressed as nmoles of oxygen consumed per min and per mg of protein (*Y*-axis). The $\pm$SEM values are the result of at least two independent measurements and were $<10\%$. (**D**) Measurement of membrane charge as TPMP accumulation in digitonin-permeabilized cells. The values $\pm$SEM are the result of two independent measurements and were $<10\%$.

(47), this delayed effect is likely to be due to a low turnover of the mitochondrial respiratory complexes. These experiments provide very strong evidence that the mitochondrial import of tK3 and tK93 is indeed the cause of the rescued mitochondrial functions.

Validation of the tRNA rescue approach in primary patient-derived fibroblasts

Having shown that tRNA import could rescue mitochondrial function in cybrid cells, we wished to determine whether a similar effect could be achieved in a more physiologically relevant cell line. For that purpose we used primary fibroblasts derived from a patient harbouring the A8344G MERRF mutation. As demonstrated by hot-last cycle PCR, these fibroblasts contained $70 \pm 5\%$ of mutant mtDNA (data not shown). MERRF fibroblasts demonstrated decreased respiration (by 60–70%) and mitochondrial membrane potential (by 70–80%) in comparison with the fibroblasts of a healthy individual, which is in agreement with previously reported data (32). As in cybrid cells, transient expression of tK3 and tK93 in human fibroblasts resulted in their partial import into mitochondria (Fig. 8A). To show the specificity of the rescue effect, we used a tRNA^{Lys} derivative tK1cau as a negative control. This derivative carries a point mutation in the anticodon (Fig. 1A) that prevents recognition of the lysine codons and, therefore, cannot substitute for the mutant human tRNA^{Lys} in mitochondrial protein synthesis. As expected from the experiments of stable expression described above (Fig. 1), we observed that tK1cau was indeed imported into fibroblast mitochondria (Fig. 8A). Expression and import of all transgenic tRNAs were quantified by real-time RT–PCR (Fig. 8B). As in cybrid cells, tK3 had the lowest expression level, whereas tK93 and tK1cau were expressed and imported with a similar efficiency. The fact that tK3 is expressed at a lower level than tK1cau and tK93 may reflect that its internal promoter is weaker than that in the genes coding for cytosolic tRNAs (see earlier). Transgenic tK versions were significantly under-represented in the cell, in comparison with the host cytosolic and mitochondrial tRNAs. Nevertheless, 10 days after transfection, fibroblasts expressing tK3 and tK93 demonstrated a partial rescue of substrate-dependent respiration and mitochondrial membrane potential (Fig. 8C and D), contrary to tK1cau, whose expression and mitochondrial import were not accompanied with any detectable rescue. Transfection of healthy fibroblasts with each of the three

constructs did not cause significant fluctuation of mitochondrial function, although for some transfectants we did observe a minor decrease (15–25%) of respiration and membrane potential. We suggest that this minor inhibition may reflect the negative effect of local concentrations of lipofectamine, which appeared to be more toxic to fibroblasts than to the cybrid cells. In spite of this effect, expression of the tK3 and tK93 versions in the MERRF-mutation-harbouring fibroblasts had a significant positive effect on both mitochondrial parameters analyzed.

These data show that tK3 and tK93 import can rescue mitochondrial dysfunctions in primary human cells bearing the MERRF mutation, consistent with the results obtained with immortalized transmitochondrial cybrids.

DISCUSSION

Versatility of RNA import

RNA import into mitochondria, first suggested almost twenty years ago (48), is now considered as quasi-universal. Found in most important groups of organisms (fungi, protozoans, animals and plants) this pathway seems, however, to share only partial similarity among different species. The differences of RNA import mechanisms among trypanosomatid, plant, human and yeast cells (3,23) suggest to propose that this pathway may have appeared more than one time during the evolution. It had previously been considered that the absence of some tRNA genes on the mitochondrial genome that were essential for mitochondrial translation indicated that the corresponding tRNA species must be imported from the cytosol (for example, for the trypanosomes lacking any mtDNA-encoded tRNA genes) (49). However, it has become clear that this criterion is not exhaustive. Indeed, in yeasts a $tRNA^{Lys}$ is imported (tK1), in spite of the presence of an additional $tRNA^{Lys}$ gene in mitochondria (tK3). In plants, in some cases, several isoacceptor tRNAs were shown to be imported, although only a subset may be considered as essential (50,51). In this work, we demonstrate that some yeast tRNAs expressed in human cells are partially targeted and imported into mitochondria. Under physiological conditions, tRNA import has not been described in human cells (52,53). We therefore suggest that this is a cryptic mechanism that can be activated. The existence of such a mechanism is not so surprising, as other small non-coding RNAs are believed to be mitochondrially imported in human cells (MRP RNA, RNase P RNA and 5S rRNA) (19,20,54,55), and mitochondrial tRNA import has been suggested for other mammalian species (56). Conversely, preservation of such a mechanism in the cells which have no apparent need of it, may reflect that mitochondrial RNA import has a fundamental importance for the cell. One may propose that such a mechanism may be activated in the case of specific need, either metabolic or genetic. Therefore, the flexibility of the RNA import pathway may provide the cell with one additional adaptive mechanism. It would be of a significant interest to search for possible regulation of RNA import under different conditions of cell growth and in association with different mitochondrial genotypes. Another potentially exploitable aspect of mitochondrial RNA import, more related to the current work, is the possibility to induce tRNA import in human cells, which seems to give a unique opportunity to specifically address 'therapeutic' molecules of nucleic acids into mitochondria.

Transient and stable effects and validity of the model

Two types of cultured human cells were used in this study: transmitochondrial cybrids and primary fibroblasts. Cybrids, being produced by fusion between patient's cytoplasts and immortalized *rho zero* cells, are genetically aneuploid (57). On the other hand, cybrids are a convenient model to study the effects of mtDNA mutations on cellular functions in long-term culture, as would be required for producing stably transfected cells. It should also be noted that the vast majority of data exploring the molecular pathogenecity of mitochondrial mutations were obtained using the cybrid technology (58). Our results clearly show that in cybrids, the various negative effects of the MERFF mutation in the $tRNA^{Lys}$ gene are partially rescued by imported tRNAs of yeast origin, both of cytosolic or mitochondrial type (tK93 and tK3, respectively). This conclusion is based on the following facts: (1) transgene expression and tRNA import correlated with the extent of rescue; (2) two genetically independent cybrid lines showed similar patterns of rescue; (3) an imported mutant version of tK1 that is not able complement the MERRF mutation (tK1cau) did not rescue the mitochondria-deficient phenotype; and (4) downregulation of tK3 and tK93 expression resulted in a specific loss of phenotypic rescue.

Nevertheless, one can argue that various functions (including mitochondrial ones) may be differently affected in cybrids when compared with primary cell lines. To rule out this possibility, we performed similar transfection experiments but with primary cells from a patient harbouring the A8344G transition associated with the MERRF syndrome. Taking into account that primary cells have a limited lifespan, the transfection procedure was performed in conditions favouring transient expression of the transgenes. The conditions used and the time period taken to measure the effects of transient expression was optimized to obtain: (1) a rescue of growth of the transfectants; and (2) a maximal level of expression of the transgene. Transfection procedures for primary cells are known to be rather inefficient and to have strong toxic effects on the cells. Even in our optimized conditions, only 25–30% of cells survived after the transfection procedure. A period of 8–10 days was shown to be the minimal time period to allow the cells to divide still retaining robust expression of transgenes. At this time, the expression of tK3 and tK93 clearly had a positive effect on mitochondrial function, whereas tK1cau did not. Taking into account that tK93 and tK1cau were expressed and imported with similar efficiencies, and tK3 with a lesser one (Fig. 8B), one can conclude that the rescue was specific and due to the import of the yeast $tRNA^{Lys}$ derivatives. These results are in full agreement with the data obtained with the cybrids and therefore suggest that cybrids can be used as a relevant model for developing a gene therapy approach.

Modelling gene therapy by exploiting RNA import pathway

Mutations in the mitochondrial genome are associated with a large spectrum of human diseases (25–27). The incidence of each disorder is in the order of 1/10 000, so they can be considered as 'rare'. However, their total number and their chronic progressive nature makes their overall medico-economical significance much more impressive. These mutations cause respiratory deficiencies and are associated with various severe and, for a majority of cases, incurable syndromes often with muscular and neurodegenerative and muscular dystrophy manifestations. Point mutations in of the mitochondrial genome are commonly associated with these human pathologies (Wallace and Lott, http://www.mitomap.org). For the vast majority of cases, the molecular consequences of these mutations are unclear and there is no efficient therapy for these disorders. Most deleterious mitochondrial tRNA mutations are heteroplasmic, i.e. mutated mtDNA molecules coexist with wild-type mtDNA molecules. The clinical status of human disorders associated with heteroplasmic mtDNA mutations is greatly dependent on the residual amount of wild-type mtDNA molecules. Clinical symptoms or respiratory chain defects are detected only above a relatively high threshold of mutant mtDNA proportion. As possible tools for therapeutic purposes, we propose to exploit the RNA mitochondrial import pathway, on the basis of artificially developed tRNA targeting into mitochondria of human cells.

Our results demonstrate that specifically designed tRNAs can be addressed into human mitochondria *in vivo* and, most probably, may be active in mitochondrial translation, thus curing the effect of mutation in a host mitochondrial gene. Such a flexibility of RNA import leads us to propose several modes for complementing mtDNA mutations: to design importable tRNAs with a variety of aminoacylation identities (more than 70 pathogenic point mutations are localized in tRNA genes) (Wallace and Lott, http://www.mitomap.org); to direct into mitochondria suppressor tRNAs to complement missense or nonsense mutations (more than 30 pathogenic cases described); to use imported RNAs as vectors to deliver into mitochondria RNAs with 'therapeutic' activity (for example, interfering with the replication of mutant mtDNA molecules). This last possibility will ideally use the 5S rRNA import pathway, since the efficiency of import (in terms of the number of imported molecules) is much higher than in the case of the artificial tRNA import (15).

Data described here clearly illustrate the existence of a cryptic tRNA mitochondrial import mechanism in human cells and its potential for alleviating mtDNA mutation-induced mitochondrial defects. This finding may lead to the development of further models of complementation that exploit the RNA import pathway, and may help to provide, in future, a new rationale for therapy of these disorders.

MATERIALS AND METHODS

Plasmid construction

tK1cau, *tK93* and *tK3* genes were PCR-cloned into *Hind* III and *Eco* RI sites of pBK-CMV vector (Stratagene). For expression in *S. cerevisiae*, the *tK93* gene was also PCR-cloned into the *Bam* HI site of pRS416 vector. All tRNA genes included the sequences found 35 bp upstream and 20 bp downstream of one of the well-expressed *tK1* gene copies (59). Additionally, constructs carried the gene of the yeast mitochondrial lysyl-tRNA synthetase. The full-length *MSK1* gene (YNL073w) was PCR-amplified with additional flanking *Bcl* I site and cloned under control of the CMV promoter in the same vector into *Bam* HI. The pGL3-control vector (Promega) contained a functional modified *luciferase* gene (*luc* +) under control of SV40 promoter. Before stable transfection, the plasmids were cleaved by *Apa* L1. Plasmids were not cleaved for transient transfection.

Cell lines, cultivation and transfection

HeLa cells (Euromedex), HepG2 and 143Brho+ cells were used as healthy cell controls. Two lines of the MERRF transmitochondrial cybrids used were: M90, derived from HeLa cells and kindly provided by J.-I. Hayashi (University of Tsakuba), which originally contained 90% of mutant genomes, but upon cultivation this line became nearly homoplasmic (>95% of mutant genomes); and the osteosarcoma 143B.206-derived line, M100, kindly provided by E. Schon (Columbia University), original examination of which also revealed nearly total homoplasmy for the MERRF mutation (>95%). Healthy and MERRF mutation containing human fibroblasts were obtained from D. Turnbull, University of Newcastle upon Tyne. Patient fibroblasts harboured 70 ± 5% A8344G MERRF mtDNA and, as expected (32), were strongly affected in mitochondrial functions. 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 μg/ml uridine, standard concentrations of antibiotics (penicillin, streptomycin and fungizone) and, for transfectants, 400 μg/ml of G418. To analyze transfectants for respiration rescue, we used the same medium with 0.9 mg/ml of galactose, which was substituted for glucose. Fibroblasts were cultivated in EMEM medium with non-essential amino acids supplemented with 50 μg/ml of uridine, 1 mM pyruvate and 10% (w : v) FCS. For translation experiments and for testing the growth on galactose, FCS was dialyzed before use. All cell lines were cultivated at 37°C and 5% of CO_2.

Cells were counted in a Neubauer haemocytometer and protein concentrations measured with the Bradford reagent (Bio-Rad). Light microscopy of cell monolayers suggested that non-transfected transmitochondrial MERRF cells and transfectants had similar cell volumes but were smaller than the parental rho+ cells. However, since the percentage of mitochondrial protein in non-transfected cybrids and in transfectants were comparable (17.4 ± 1.5% of total protein in parental cells, 16.3 ± 0.9% in M90 cells and 13.8 ± 1.9% in M100 cells), we considered that change in size of cells did not affect protein levels of our cellular preparations and that comparison between cell lines was valid.

Stable transfection of cybrid cells was with Superfect reagent (Qiagen) as described by the manufacturer. Stable

expression of yeast tRNAs and their mitochondrial import in HepG2 cells had no detectable effect on growth and respiration of transfectants. Transient transfection of cybrids (for RNAi experiment) and of fibroblasts was with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. For cybrid cell transfection we used 70% confluent cells and a 1:2.5 ratio of DNA (μg)/Lipofectamine (μl). For fibroblast transfection, 90% confluent cells were used and Lipofectamine concentration was reduced to the ratio 1:1 to decrease cytotoxic effects, higher for this type of cells.

Isolation of mitochondria

Mitochondria were isolated as described elsewhere (60) with minor modifications (24). The integrity of mitochondria was checked by comparison of citrate synthase activities in isotonic medium (0.6 M sorbitol) and hypotonic media (10 mM HEPES-OH, pH 6.8) in the presence of 0.5% (v:v) Triton X-100 as described elsewhere (61). In all experiments, more than 85% of mitochondria were intact. The absence of contamination of mitochondria with cytosolic RNAs and proteins was confirmed by RT–PCR, quantitative northern hybridization and western analyses as described previously (15,24).

Measurement of enzymatic activities, respiration rate and membrane potential

Complex I activity was measured in mitochondria following several cycles of freezing–thawing by the standard procedure and calculated as the rotenone-sensitive NADH:ubiquinone oxidoreductase activity (32). Complexes II and III (succinate:cytochrome *c* oxidoreductase) activity was assayed in 50 mM KH_2PO_4 buffer (pH 7.4) with 1 mM EDTA, 20 mM K-succinate, 2 mM KCN and 10 μM rotenone. The rate of reduction of cytochrome *c* (50 μM) was measured at 550 nm and the background was measured after addition of myxothiazol to 400 nM. Complex IV activity was measured in 200 mM Tris–HCl (pH 7.5), 10 μM EDTA, 0.3% (v:v) Tween-80, 10 μM rotenone, 300 nM antimycin A_1, 100 μM ferrocytochrome *c* at 520 nm, with the background rate measured after addition of KCN (200 μM) (32).

To measure respiration rate, the cells (0.5–1 mg of protein) were suspended in 1 ml of 10 mM HEPES–KOH (pH 7.4), 120 mM KCl, 1 mM EGTA, 100 μg/ml of digitonin and either 10 mM succinate with 50 μM rotenone, or 5 mM glutamate with 5 mM malate. O_2 uptake was measured by using a Clark-type oxygen electrode (Strathkelvin Instruments) during 10 min at 37°C with sequential addition of oligomycin (25 μM), FCCP (200 nM) and myxothiazol (800 nM) to measure coupled, uncoupled and non-mitochondrial oxygen consumption, respectively. Non-mitochondrial O_2 uptake (ranging between 0.5 and 1.2 nmol/min/mg protein) was subtracted from all values.

Mitochondrial membrane potential was measured in digitonin-permeabilized cells essentially as described (32). An aliquot of 50–200 μg of phosphate-buffered saline (PBS)-washed cells were suspended in 200 μl of 10 mM HEPES–KOH (pH 7.4), 120 mM KCl, 1 mM EGTA, 1 μM of (TPMP) iodide, 100 nCi/ml ^{3}H-TPMP (from NEN, 76 Ci/mmol), 20 μg/ml of digitonin and either 10 mM succinate with 50 μM rotenone, or 5 mM glutamate with 5 mM malate. After 10 min of incubation at 37°C, the cells were pelleted and 10 μl aliquots of supernatants and pellets resuspended in 20 μl of 0.5% (v:v) Triton X-100 were counted in a liquid scintillator. Mitochondrial-membrane-independent TPMP accumulation was measured in parallel incubations in the presence of 400 nM of FCCP and subtracted from all the values. The TPMP uptake was expressed as the ratio between the number of counts (cpm) of TPMP per mg of cellular protein of the pellet and cpm of TPMP per μl of supernatant.

Luciferase activity was determined by using the Luciferase Assay System of Promega as described by the supplier. Measurement was done in a scintillation counter and normalized against a positive control (cells transiently transfected with the control pGL3 vector) and expressed in relative units per mg of protein.

Cytochrome *c* oxidase cytochemistry

For cytochemical analysis, 5×10^5 cells were plated on poly-lysine treated coverslip, grown for 24 h, washed with PBS and air-dried. Preincubation was performed at room temperature for 15 min in 50 mM Tris–Cl (pH 7.6), 0.28 mg/ml of $CoCl_2 \cdot 6H_2O$, 0.5% (v:v) DMSO, 1% sucrose (w:v). After a wash with NPS buffer [0.1 M Na-phosphate (pH 7.6), 10% (w:v) sucrose], the cells were incubated in NPS containing 1 mg/ml of cytochrome *c* (type IV, Sigma), 1 mg/ml DAB-hydrochloride, 0.25% (w:v) DMSO, 20 μg/ml catalase for 6 h in the dark. After washing with NPS and PBS, the cells were mounted in DPX and analyzed by optic microscopy. COX-positive cells were counted on anonymized samples. Background was established with a mock colouration of the same cell lines without cytochrome *c* addition. For each line, we carried out at least five independent counts of more than 500 cells and a mean percentage of ±SEM. value was established.

In vivo mitochondrial translation

To analyze mitochondrial translation, 0.5×10^6 of semi-confluent cells were preincubated in RPMI1640 with 0.5% (v:v) of dialyzed FCS lacking methionine and supplemented with 0.2 mg/ml of emetine for 5 min at 37°C, followed by 30 min with 200 μCi/ml [^{35}S]-methionine (>1000 Ci/mmol, Amersham), and, finally, with 0.1 mM of non-labelled methionine for a 10 min chase (62). The cells were PBS-washed, released by PBS–EDTA treatment, disrupted by sonication in 0.1% (v:v) Triton X-100, 10 U/ml of DNAse I, diluted in Laemmli's sample buffer and separated in gradient 10–20% denaturing PAGE. Following visualization with a PhosphorImager (Fuji), quantification was done using the MacBAS v2.5 software. To measure the overall translation activity in mitochondria, the cells suspended in PBS were dissolved by addition of NaOH to 2%, proteins were precipitated with TCA (20%), collected onto millipore filters, washed with 10% of TCA at 70°C for 5 min, several times at 0°C, once with ethanol, dried and scintillation counted.

Isolation and analysis of mtDNA and RNA

Total cellular DNA was isolated by standard procedures (63) and the heteroplasmy level was determined by PCR as described (64) by generating an amplicon with the two oligonucleotides: R8372–8345: GGGGCATTTCACTGTAAAGAGGTGCCGG and F8191–8210: TGTAAAACGACGGCCAGTAAACCAC AGTTTCATGCCCA, a 38-mer carrying 20 residues common to mtDNA 3′ to sequence from bacteriophage M13 routinely used to prime DNA sequence analysis. Two mismatches (underlined) of the reverse primer contribute to a *Nae* I site generated with mutant but not wild-type mtDNA. A second pair of primers was used to generate an amplicon to control for *Nae* I digestion: F516–534: CACACACACCGCTGCTAAC and R1190–1172: GATATGAAGCACCGCCAGG. PCR-amplified fragments were ^{32}P-labelled during an additional cycle of polymerization, cut with *Nae* I and analyzed on 10% non-denaturing polyacrylamide gels and PhosphorImaging.

Mitochondrial RNA was isolated with Trizol-reagent (Invitrogen) by standard procedures and analyzed by northern hybridization as previously described (24). To analyze aminoacylation *in vivo*, mtRNA was isolated in acid conditions, separated in 0.1 M sodium acetate (pH 4.8, 6.5% PAGE as described elsewhere (41) and analyzed by hybridization. To detect tK3, we used the oligonucleotide probe antiK3 (1–39): CTTAAAAGACAACTGTTTTACCATTAAACAAT ATTCTC; for tK93, the probe antiK2 (2–32): GCCGAAC GCTCTACCAACTCAGCTAACAAGG, for tK1cau, the probe antitK1(met): CTTATGATTATGAGTCAT; for human cytosolic $tRNA_i^{Met}$, the probe anti-cM: TGGTAGCAGAGGATGG TTTCG, for human mitochondrial $tRNA^{Gln}$, anti-mQ: CTA GGACTATGAGAATCG.

For RT–PCR detection of tRNAs, we used the following pairs of oligonucleotides ('f' for forward and 'r' for reverse). For tK93, t93f: CTTGTTAGCTCAGTTGGT and t93r: TGGA GCCTCATAGGGGGC; for tK3, t3f: GAGAATATTGTTT AATGGTAAAAC and t3r: GGTGAGAATAGCTGGAGTT G; for tK1cau, t1f: GCCTTGTTGGCGCAATCGG and t1r: GGAGCCCTGTAGGGGGCTCG; for mitochondrial $tRNA^{Gln}$, hMQr: TAGGACTATGAGAATCG and hMQf: AGGAT GGGGTGTGATAG; for cytoplasmic $tRNA_i^{Met}$, hCMr: GGT AGCAGAGGATGGTTTCG and hCMf: CAGAGTGGCGC AGCGGAAG. Amplification was done by using the single-step RT–PCR kit of Qiagen in the following conditions: 50°C, 30 min; 95°C, 15 min, 15 cycles at 95–55–72°C, 10 cycles at 95–58–72°C and 5 cycles at 95–62–72° (each step for 1 min), with the final step at 72°C for 10 min. In all cases, a control PCR was included, performed in similar conditions as RT–PCR by *Taq* DNA polymerase, to confirm the absence of DNA contamination in RNA isolates. Quantitative (real-time) RT–PCR was done using a BioRad i-Cycler with the One-Step RT–qPCR Mastermix for SYBR green I (Eurogentec). For RT–qPCR, conditions were similar as above, but the number of the cycles at 58°C was increased to 25. For quantitation, serial RNA dilutions were done and compared with the calibration curve obtained in parallel reactions with a series of diluted gel-purified T7-transcripts (tK1, tK3 or tK93), ranging from 0.1 to 10 ng per reaction. All qPCR samples were done in three parallels and the ±SEM values were less than 5% in each case.

RNA interference

siRNA duplexes, 21 and 22 nt long, with symmetric 2 nt 3′ overhangs directed against the reporter gene (*luciferase*), tK3 and tK93 were purchased from Genset. The following oligoribonucleotides were used. LucSiF: GGAUUCUAAAA CGGAUUACCdTdT and LucSiR: GGUAAUCCGUUUUA GAAUCCdTdT for luciferase (forward and reverse, respectively, correspond to the region 456–476 of the luciferase coding ORF in pGL3); t93SiF: GGCUUUUAACCGAAAUG UCdTdT and t93SiR: GACAUUUCGGUUAAAAGCCdTdT for tK93 (the region 30–48 of the tRNA); t3SiF: GUUGU CUUUUAAGCAACCCdTdT and t3SiR: GGGUUGCUU AAAAGACAACdTdT (the region 26–44 of the tRNA). The 2 nt 3′ ovehangs represented two 2′-deoxythymidine residues (dT). Upon transfection with Lipofectamine 2000 reagent (Invitrogen), we used siRNA duplexes at 50 nM. An aliquot of 5 μg of uncut pGL3 was used in co-transfection experiments per one 10 cm^2 well. This ratio DNA:siRNA was sufficient for a nearly full inhibition of luciferase activity. The day after transfection, the medium without antibiotics was replaced by the same madium with G418. At day 3 after transfection, one half of the samples were subjected to a second round of transfection in the same conditions as initially. At the days 3 and 6, the cells were detached and analyzed for RNA concentrations (by RT–qPCR), luciferase activity, mitochondrial membrane potential and respiration.

ACKNOWLEDGEMENTS

We thank Professor E. Schon (Columbia University, USA) and Professor J. Hayashi (Tsukuba University, Japan) for providing the MERRF cybrid cell lines, Professor D. Turnbull (Newcastle University, UK) for MERRF primary fibroblasts and J. Enriquez (University Zaragoza, Spain) for helpful discussions on the phenotypic analysis of cybrid cells. O.A.K. had an FEBS fellowship and an AFM PhD training fellowship, C.J.B. had an AFM postdoctoral fellowship. This work was partially supported by the CNRS, ULP, AFM, EU (MitEURO concerted action QLRT-2000-00966 and INTAS grant 96-1515), a grant from GIS Institut de Maladies Rares, RFBR (grant no. 00-04-48488) and HFSP (grant no. RG0349/1999M). RNL would like to acknowledge support from The Wellcome Trust (programme grant no. 056605).

REFERENCES

1. Scheffler, I.E. (2001) Mitochondria make a come back. *Adv. Drug Deliv. Rev.*, **49**, 3–26.
2. Neupert, W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.*, **66**, 863–917.
3. Entelis, N.S., Kolesnikova, O.A., Martin, R.P. and Tarassov, I.A. (2001) RNA delivery into mitochondria. *Adv. Drug Deliv. Rev.*, **49**, 199–215.
4. Adhya, S., Ghosh, T., Das, A., Bera, S.K. and Mahapatra, S. (1997) Role of an RNA-binding protein in import of tRNA into *Leishmania* mitochondria. *J. Biol. Chem.*, **272**, 21396–21402.
5. Mahapatra, S. and Adhya, S. (1996) Import of RNA into *Leishmania* mitochondria occurs through direct interaction with membrane-bound receptors. *J. Biol. Chem.*, **271**, 20432–20437.
6. Rubio, M.A., Liu, X., Yuzawa, H., Alfonzo, J.D. and Simpson, L. (2000) Selective importation of RNA into isolated mitochondria from *Leishmania tarentolae*. *RNA*, **6**, 988–1003.

7. Mukherjee, S., Bhattacharyya, S.N. and Adhya, S. (1999) Stepwise transfer of tRNA through the double membrane of *Leishmania* mitochondria. *J. Biol. Chem.*, **274**, 31249–31255.
8. Bhattacharyya, S.N., Chatterjee, S., Goswami, S., Tripathi, G., Dey, S.N. and Adhya, S. (2003) 'Ping-pong' interactions between mitochondrial tRNA import receptors within a multiprotein complex. *Mol. Cell. Biol.*, **23**, 5217–5224.
9. LeBlanc, A.J., Yermovsky-Kammerer, A.E. and Hajduk, S.L. (1999) A nuclear encoded and mitochondrial imported dicistronic tRNA precursor in *Trypanosoma brucei. J. Biol. Chem.*, **274**, 21071–21077.
10. Sbicego, S., Nabholz, C.E., Hauser, R., Blum, B. and Schneider, A. (1998) *In vivo* import of unspliced tRNATyr containing synthetic introns of variable length into mitochondria of *Leishmania tarentolae*. *Nucl. Acids Res.*, **26**, 5251–5255.
11. Small, I., Marechal-Drouard, L., Masson, J., Pelletier, G., Cosset, A., Weil, J.H. and Dietrich, A. (1992) *In vivo* import of a normal or mutagenized heterologous transfer RNA into the mitochondria of transgenic plants: towards novel ways of influencing mitochondrial gene expression? *EMBO J.*, **11**, 1291–1296.
12. Delage, L., Dietrich, A., Cosset, A. and Marechal-Drouard, L. (2003) *In vitro* import of a nuclearly encoded tRNA into mitochondria of *Solanum tuberosum*. *Mol. Cell. Biol.*, **23**, 4000–4012.
13. Delage, L., Duchene, A.M., Zaepfel, M. and Marechal-Drouard, L. (2003) The anticodon and the D-domain sequences are essential determinants for plant cytosolic tRNAVal import into mitochondria. *Plant J.*, **34**, 623–633.
14. Tarassov, I., Entelis, N. and Martin, R.P. (1995) Mitochondrial import of a cytoplasmic lysine-tRNA in yeast is mediated by cooperation of cytoplasmic and mitochondrial lysyl-tRNA synthetases. *EMBO J.*, **14**, 3461–3471.
15. Entelis, N.S., Kolesnikova, O.A., Dogan, S., Martin, R.P. and Tarassov, I.A. (2001) 5 S rRNA and tRNA import into human mitochondria. Comparison of *in vitro* requirements. *J. Biol. Chem.*, **276**, 45642–45653.
16. Tarassov, I., Entelis, N. and Martin, R.P. (1995) An intact protein translocating machinery is required for mitochondrial import of a yeast cytoplasmic tRNA. *J. Mol. Biol.*, **245**, 315–323.
17. Nabholz, C.E., Horn, E.K. and Schneider, A. (1999) tRNAs and proteins are imported into mitochondria of *Trypanosoma brucei* by two distinct mechanisms. *Mol. Biol. Cell*, **10**, 2547–2557.
18. Martin, R.P., Schneller, J.M., Stahl, A.J. and Dirheimer, G. (1979) Import of nuclear deoxyribonucleic acid coded lysine-accepting transfer ribonucleic acid (anticodon C–U–U) into yeast mitochondria. *Biochemistry*, **18**, 4600–4605.
19. Magalhaes, P.J., Andreu, A.L. and Schon, E.A. (1998) Evidence for the presence of 5S rRNA in mammalian mitochondria. *Mol. Biol. Cell*, **9**, 2375–2382.
20. Puranam, R.S. and Attardi, G. (2001) The RNase P associated with HeLa cell mitochondria contains an essential RNA component identical in sequence to that of the nuclear RNase P. *Mol. Cell. Biol.*, **21**, 548–561.
21. Entelis, N., Kieffer, S., Kolesnikova, O., Martin, R. and Tarassov, I. (1998) Structural requirement of $tRNA^{Lys}$ for its import into yeast mitochondria. *Proc. Natl Acad. Sci. USA*, **95**, 2838–2843.
22. Kolesnikova, O.A., Entelis, N.S., Mireau, H., Fox, T.D., Martin, R.P. and Tarassov, I.A. (2000) Suppression of mutations in mitochondrial DNA by tRNAs imported from the cytoplasm. *Science*, **289**, 1931–1933.
23. Schneider, A. and Marechal-Drouard, L. (2000) Mitochondrial tRNA import: are there distinct mechanisms? *Trends Cell Biol.*, **10**, 509–513.
24. Entelis, N., Kolesnikova, O., Kazakova, H., Brandina, I., Kamenski, P., Martin, R.P. and Tarassov, I. (2002) Import of nuclear encoded RNAs into yeast and human mitochondria: experimental approaches and possible biomedical applications. *Genet. Eng.*, **24**, 191–213.
25. Wallace, D.C. (2001) A mitochondrial paradigm for degenerative diseases and ageing. *Novartis Found. Symp.*, **235**, 247–263.
26. Wallace, D.C. (1999) Mitochondrial diseases in man and mouse. *Science*, **283**, 1482–1488.
27. Larsson, N.G. and Clayton, D.A. (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.*, **29**, 151–178.
28. Hammans, S.R., Sweeney, M.G., Brockington, M., Lennox, G.G., Lawton, N.F., Kennedy, C.R., Morgan-Hughes, J.A. and Harding, A.E. (1993) The mitochondrial DNA transfer RNA(Lys)A→G(8344) mutation and the syndrome of myoclonic epilepsy with ragged red fibres (MERRF). Relationship of clinical phenotype to proportion of mutant mitochondrial DNA. *Brain*, **116**, 617–632.
29. Shoffner, J.M., Lott, M.T., Lezza, A.M., Seibel, P., Ballinger, S.W. and Wallace, D.C. (1990) Myoclonic epilepsy and ragged-red fibre disease (MERRF) is associated with a mitochondrial DNA tRNA (Lys) mutation. *Cell*, **61**, 931–937.
30. Enriquez, J.A., Chomyn, A. and Attardi, G. (1995) MtDNA mutation in MERRF syndrome causes defective aminoacylation of tRNA (Lys) and premature translation termination. *Nat. Genet.*, **10**, 47–55.
31. Yasukawa, T., Suzuki, T., Ishii, N., Ueda, T., Ohta, S. and Watanabe, K. (2000) Defect in modification at the anticodon wobble nucleotide of mitochondrial tRNA (Lys) with the MERRF encephalomyopathy pathogenic mutation. *FEBS Lett.*, **467**, 175–178.
32. James, A.M., Wei, Y.H., Pang, C.Y. and Murphy, M.P. (1996) Altered mitochondrial function in fibroblasts containing MELAS or MERRF mitochondrial DNA mutations. *Biochem. J.*, **318** (Pt 2), 401–407.
33. Clayton, D.A. (1992) Transcription and replication of animal mitochondrial DNAs. *Int. Rev. Cytol.*, **141**, 217–232.
34. Huang, Y. and Maraia, R.J. (2001) Comparison of the RNA polymerase III transcription machinery in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and human. *Nucl. Acids Res.*, **29**, 2675–2690.
35. Paule, M.R. and White, R.J. (2000) Survey and summary: transcription by RNA polymerases I and III. *Nucl. Acids Res.*, **28**, 1283–1298.
36. Hayashi, J., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y. and Nonaka, I. (1991) Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc. Natl Acad. Sci. USA*, **88**, 10614–10618.
37. Masucci, J.P., Davidson, M., Koga, Y., Schon, E.A. and King, M.P. (1995) *In vitro* analysis of mutations causing myoclonus epilepsy with ragged-red fibres in the mitochondrial tRNA (Lys) gene: two genotypes produce similar phenotypes. *Mol. Cell. Biol.*, **15**, 2872–2881.
38. Enriquez, J.A., Cabezas-Herrera, J., Bayona-Bafaluy, M.P. and Attardi, G. (2000) Very rare complementation between mitochondria carrying different mitochondrial DNA mutations points to intrinsic genetic autonomy of the organelles in cultured human cells. *J. Biol. Chem.*, **275**, 11207–11215.
39. Kazakova, H.A., Entelis, N.S., Martin, R.P. and Tarassov, I.A. (1999) The aminoacceptor stem of the yeast tRNA (Lys) contains determinants of mitochondrial import selectivity. *FEBS Lett.*, **442**, 193–197.
40. Yasukawa, T., Suzuki, T., Ishii, N., Ohta, S. and Watanabe, K. (2001) Wobble modification defect in tRNA disturbs codon–anticodon interaction in a mitochondrial disease. *EMBO J.*, **20**, 4794–4802.
41. Varshney, U., Lee, C.P. and RajBhandary, U.L. (1991) Direct analysis of aminoacylation levels of tRNAs *in vivo*. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutaminyl-tRNA synthetase. *J. Biol. Chem.*, **266**, 24712–24718.
42. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.*, **20**, 6877–6888.
43. Arenz, C. and Schepers, U. (2003) RNA interference: from an ancient mechanism to a state of the art therapeutic application? *Naturwissenschaften*, **90**, 345–359.
44. Lavery, K.S. and King, T.H. (2003) Antisense and RNAi: powerful tools in drug target discovery and validation. *Curr. Opin. Drug Discov. Dev.*, **6**, 561–569.
45. Matzke, M. and Matzke, A.J. (2003) RNAi extends its reach. *Science*, **301**, 1060–1061.
46. Pickford, A.S. and Cogoni, C. (2003) RNA-mediated gene silencing. *Cell. Mol. Life Sci.*, **60**, 871–882.
47. Chrzanowska-Lightowlers, Z.M., Turnbull, D.M., Bindoff, L.A. and Lightowlers, R.N. (1993) An antisense oligodeoxynucleotide approach to investigate the function of the nuclear-encoded subunits of human cytochrome c oxidase. *Biochem. Biophys. Res. Commun.*, **196**, 328–335.
48. Suyama, Y. (1986) Two dimensional polyacrylamide gel electrophoresis analysis of tetrahymena mitochondrial tRNA. *Curr. Genet.*, **10**, 411–420.
49. Simpson, A.M., Suyama, Y., Dewes, H., Campbell, D.A. and Simpson, L. (1989) Kinetoplastid mitochondria contain functional tRNAs which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function. *Nucl. Acids Res.*, **17**, 5427–5445.
50. Kumar, R., Marechal-Drouard, L., Akama, K. and Small, I. (1996) Striking differences in mitochondrial tRNA import between different plant species. *Mol. Gen. Genet.*, **252**, 404–411.

51. Schneider, A. (1994) Import of RNA into mitochondria. *Trends Cell Biol.*, **4**, 282–286.
52. Amalric, F., Merkel, C., Gelfand, R. and Attardi, G. (1978) Fractionation of mitochondrial RNA from HeLa cells by high-resolution electrophoresis under strongly denaturing conditions. *J. Mol. Biol.*, **118**, 1–25.
53. Lynch, D.C. and Attardi, G. (1976) Amino acid specificity of the transfer RNA species coded for by HeLa cell mitochondrial DNA. *J. Mol. Biol.*, **102**, 125–141.
54. Yoshionari, S., Koike, T., Yokogawa, T., Nishikawa, K., Ueda, T., Miura, K. and Watanabe, K. (1994) Existence of nuclear-encoded 5S-rRNA in bovine mitochondria. *FEBS Lett.*, **338**, 137–142.
55. Doersen, C.J., Guerrier-Takada, C., Altman, S. and Attardi, G. (1985) Characterization of an RNase P activity from HeLa cell mitochondria. Comparison with the cytosol RNase P activity. *J. Biol. Chem.*, **260**, 5942–5949.
56. Dorner, M., Altmann, M., Paabo, S. and Morl, M. (2001) Evidence for import of a Lysyl-tRNA into Marsupial mitochondria. *Mol. Biol. Cell*, **12**, 2688–2698.
57. King, M.P. and Attardi, G. (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science*, **246**, 500–503.
58. Turnbull, D.M. and Lightowlers, R.N. (2002) A roundabout route to gene therapy. *Nat. Genet.*, **30**, 345–346.
59. Entelis, N.S., Kieffer, S., Kolesnikova, O.A., Martin, R.P. and Tarassov, I.A. (1998) Structural requirements of tRNALys for its import into yeast mitochondria. *Proc. Natl Acad. Sci. USA*, **95**, 2838–2843.
60. Pallotti, F. and Lenaz, G. (2001) Isolation and subfractionation of mitochondrial preparations from animal cells and tissue culture lines. In Pon, L.A. and Schon, E.A. (eds), *Mitochondria.* Academic Press, San Diego, CA, Vol. 65, pp. 1–35.
61. Rickwood, D., Wilson, M.T. and Darley-Usmar, V.M. (1987) Isolation and characteristics of intact mitochondria. In Darley-Usmar, V.M., Rickwood, D. and Wilson, M.T. (eds), *Mitochondria, A Practical Approach.* IRL Press, Oxford, pp. 1–16.
62. Chomyn, A., Meola, G., Bresolin, N., Lai, S.T., Scarlato, G. and Attardi, G. (1991) *In vitro* genetic transfer of protein synthesis and respiration defects to mitochondrial DNA-less cells with myopathy-patient mitochondria. *Mol. Cell. Biol.*, **11**, 2236–2244.
63. Kawasaki, E.S. (1990) Sample preparation from blood, cells, and other fluids. In Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds), *PCR Protocols: A Guide to Methods and Applications.* Academic Press, San Diego, CA, pp. 146–152.
64. Muratovska, A., Lightowlers, R.N., Taylor, R.W., Turnbull, D.M., Smith, R.A., Wilce, J.A., Martin, S.W. and Murphy, M.P. (2001) Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expression and disease. *Nucl. Acids Res.*, **29**, 1852–1863.