Mitochondrial tRNA import: are there distinct mechanisms?

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Sequence information from an increasing number of complete mitochondrial genomes indicates that a large number of evolutionary distinct organisms import nucleus-encoded tRNAs. In the past five years, much research has been initiated on the features of imported tRNAs, the mechanism and the energetics of the process as well as on the components of the import machinery. In summary, these studies show that the import systems of different species exhibit some unique features, suggesting that more than one mechanism might exist to import tRNAs.

Mitochondria have their own genome, which reflects their bacterial ancestry. The size of mitochondrial DNA varies considerably (e.g. 17 kb in human and 367 kb in *Arabidopsis thaliana*), but generally encodes only a limited set of proteins (e.g. 13 in human and 32 in *A. thaliana*). Mitochondria, however, are complex structures consisting of up to 1000 different proteins. Import of nucleus-encoded proteins is therefore a process of fundamental importance for mitochondrial biogenesis. Nevertheless synthesis of the limited set of mitochondria-encoded proteins is essential for organelle function[1]. Translation requires rRNAs and a complete set of tRNAs, which, according to most textbooks, are encoded by the mitochondrial genome. However, as early as 1967, the import of nucleus-encoded tRNAs into the mitochondria was suggested to occur in *Tetrahymena*[2]. But it has taken 25 years for the process to be demonstrated directly using transgenic plants[3]. Today, it is well established that mitochondrial tRNA import is a process occurring in a number of evolutionary distinct organisms such as plants, the yeast *Saccharomyces cerevisiae* and many protozoans. In all organisms, for any given tRNA that is imported, most of the total tRNA synthesized in the nucleus remains in the cytosol and functions in cytosolic translation. The specificity and the extent to which individual tRNAs are imported, however, differs greatly between organisms and might reflect fundamental differences in the mechanisms underlying tRNA import. In this report we summarize the recent developments in the field of mitochondrial tRNA import and emphasize the basic problems that need to be solved.

Which organisms import tRNAs?

Mitochondrial import of a variable number of nucleus-encoded tRNAs is predicted to occur in different eukaryotic microorganisms (protozoa, fungi, algae), in some plants and in a few animals belonging to the Cnidaria and the Mollusca (Fig. 1). In most cases, evidence for tRNA import is based on the lack of a complete set of genes encoding tRNAs in the sequence of mitochondrial genomes. This prediction is fairly accurate in organisms that lack a significant number of essential mitochondrial tRNA genes. However, if the set of mitochondrial tRNA genes is nearly complete, the situation is more difficult because tRNA editing and variations in the codon recognition mechanisms provide alternative ways to compensate for the putative missing tRNA genes. Furthermore, there are examples such as *S. cerevisiae*[4] and the liverwort *Marchantia polymorpha*[5] where tRNAs overlapping in function to organelle-encoded ones are imported. Yeast is of special interest in this context because it contains a full set of functional mitochondrial tRNA genes but nevertheless imports a single $tRNA^{Lys}$. Despite this reservation, it is clear that import of tRNA into the mitochondria is a common occurrence among eukaryotes and occurred early in evolution. Interestingly, for some species where tRNA import has been predicted, closely related organisms can be found that most likely do not import tRNAs (Fig. 1). This might suggest that, since the loss of mitochondrial tRNA genes is likely to be irreversible, mitochondrial tRNA import has a polyphyletic origin.

Mammalian mitochondria lack nucleus-encoded tRNAs, but there are some studies claiming that other cytosolic RNAs, such as the RNA subunit of RNase P[6] and MRP RNase[7] as well as cytosolic 5S rRNA[8] are imported. However, in most of these studies, it is difficult to definitively exclude cytosolic contamination. Furthermore, more recent studies have found that human mitochondria might have a distinct RNase-P-like activity that is devoid of an RNA subunit[9]. It is important to determine what types of RNAs are imported as well as to obtain a more accurate picture of the phylogenetic distribution of (t)RNA import. Information gained from such studies might help to answer the enigmatic question of why tRNA import into mitochondria has been 'invented' during evolution.

What are the features of imported tRNAs?

All tRNAs that are imported into mitochondria are of the eukaryotic cytosolic type, meaning that the same gene codes for a tRNA involved in cytosolic as well as in mitochondrial translation. Some tRNAs, however, might acquire additional nucleotide modifications after import[10,11]. In quantitative terms, the imported tRNAs always represent only a small fraction (~5%) of the total cellular amount. This includes the

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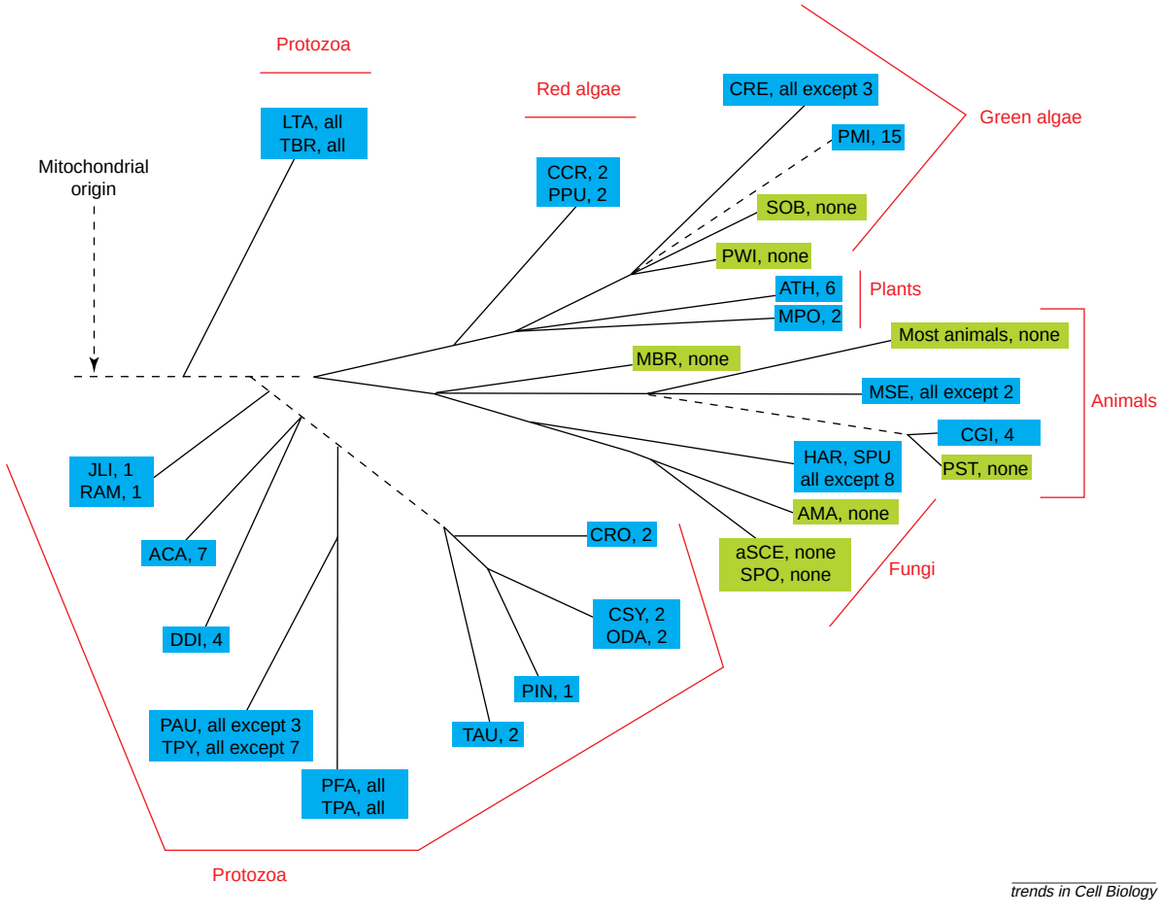

FIGURE 1

Predicted phylogenetic distribution of mitochondrial tRNA import. The phylogenetic tree is according to Gray *et al.*[37] and based on ultrastructural and molecular data. It includes a representative selection of organisms whose mitochondrial genomes have been completely sequenced (URL http://megasun.bch.umontreal.ca/ogmpproj.html). Unbroken lines indicate firmly supported phylogenetic relationships; broken lines indicate preliminary placements. Organisms predicted to import or to not import tRNAs are shown on a blue or green background, respectively. The predicted number of imported tRNAs needed to complement the mitochondria-encoded ones to reach the minimal set required for mitochondrial translation is indicated. The commonly used names for the systematic groupings of the indicated organisms are shown in red. Abbreviations of the different species is according to Gray *et al.*[37] : Euglenozoa: LTA (*Leishmania tarentolae*), TBR (*Trypanosoma brucei*); Histionid: JLI (*Jakoba libera*), RAM (*Reclinomonas americana*); Rhizopod: ACA (*Acanthamoeba castellanii*); Slime mold: DDI (*Dictyostelium discoideum*); Ciliate: PAU (*Paramecium aurelia*), TPY (*Tetrahymena pyriformis*); Apicomplexan: PFA (*Plasmodium falciparum*), TPA (*Theileria parva*); Labyrinthulomycete: TAU (*Thraustochytrium aureum*); Stramenopile: PIN (*Phytophthora infectans*); Chrysophyte: CSY (*Chrysodidymus synuroideus*), ODA (*Ochromonas danica*); Bicosoecid: CRO (*Cafeteria roenbergensis*); Rhodophyte: CCR (*Chondrus crispus*), PPU (*Porphyra purpurea*); Chlorophyte: CRE (*Chlamydomonas reinhardtii*), PMI (*Pedinomonas minor*), SOB (*Scenedesmus obliquus*), PWI (*Prototheca wickerhamii*); Streptophyte: ATH (*Arabidopsis thaliana*); Charophyte: MPO (*Marchantia polymorpha*); Choanoflagellate: MBR (*Monosiga brevicollis*); Cnidarian: MSE (*Metridium senile*); Mollusca: CGI (*Crassostrea gigas*), PST (*Pupa strigosa*); Chytridiomycete: SPU (*Spizellomyces punctatus*), HAR (*Harpochytrium sp.*), AMA (*Allomyces macrogynus*); Ascomycete: SCE (*Saccharomyces cerevisiae*), SPO (*Schizosaccharomyces pombe*). A complete set of mitochondrial tRNA genes is found in SCE, but experimental evidence shows that, despite this, the cytosolic tRNALys (CUU) is in part imported (see text).

$tRNA^{Trp}$ of *Leishmania tarentolae*, which in mitochondria, owing to reassignment of the stop codon to tryptophan in the organellar translation, has to decode UGA in addition to the normal tryptophan codon UGG. Suppression of UGA stop codons in the cytosol is expected to be harmful. In order to solve this problem, *L. tarentolae* imports the normal cytosolic $tRNA^{Trp}$, which is unable to decode the stop codon. Once inside the mitochondria, however, the CCA anticodon of the $tRNA^{Trp}$ gets converted to UCA by RNA editing, allowing the tRNA to read both UGG and UGA codons[12]. The number of imported tRNA species varies to a great extent, and overall this does not correlate with the phylogenetic position of the organism (Fig. 1). For example, *S. cerevisiae* imports a single tRNA, and a number of parasitic protozoa import all mitochondrial tRNAs. Interestingly, independent of the extent of tRNA import, one always finds cytosol-specific tRNAs even in systems that import all mitochondrial tRNAs[13].

What are the distinguishing features of imported and purely cytosolic tRNAs? Despite the importance of this question, there are only two examples where the precise import determinants have been identified, the $tRNA^{Gln}$ in *Tetrahymena* and the $tRNA^{Lys}$ in *S. cerevisiae* (Fig. 2).

Tetrahymena contains three homologous tRNA^{Gln} molecules. Two of them with the anticodons UUA and CUA are cytosol specific and recognize the stop codon UCA, which has been reassigned to glutamine in the nucleus of *Tetrahymena*. The third tRNA^{Gln} with the anticodon UUG recognizes the standard glutamine codons, however, and is found in both the cytosol and the mitochondria. In a quantitative *in vivo* analysis it was shown that the anticodon UUG of the imported tRNA^{Gln} is both necessary and sufficient to induce import of any of the three tRNA^{Gln} molecules[14].

S. cerevisiae contains two different nucleus-encoded tRNA^{Lys}, only one of which is imported into mitochondria[4]. Quantitative *in vivo* and *in vitro* analysis identified the first base pair of the acceptor stem and the anticodon of the imported tRNA^{Lys} in yeast as the main import determinants. It was further shown that import competence correlated with binding to a soluble import factor[15,16] (see below).

The identity of the import determinants in trypanosomatid (*T. brucei, Leishmania*) tRNA remains controversial. The D-stem loop appears to contribute to the signal as shown in an *in vitro* import study for tRNA^{Tyr} of *L. tropica*[19] (Fig. 2). These results are supported by *in vivo* and *in vitro* experiments performed in *L. tarentolae* that show that swapping the D-loop stem from the exclusively cytosolic tRNA^{Gln} with that from the imported tRNA^{Ile} produced a partial mitochondrial localization of the resulting tRNA[17,18]. However, the converse experiment did not work – tRNA^{Ile} remained cytosolic even when carrying the D-stem loop of the cytosolic tRNA^{Gln} (Ref. 17). Furthermore, no clear sequence element within the D-stem loop could be found that is consistently present in imported but not in purely cytosolic tRNAs[20]. In addition, it was shown that even cytosolic tRNAs from yeast or human are imported into mitochondria when expressed in *T. brucei*[21]. It has also been claimed that, in *T. brucei*, actual import substrates are precursor tRNAs having long 5′-extension or dimeric tRNA transcripts[22] (Fig. 2). Using primer extension and northern analysis, high-molecular-weight forms of tRNAs can be detected in mitochondrial RNA. However, some of these appear to be caused by artifactual circularization of tRNAs due to mitochondrial ligase activity[23]. Nevertheless, using RT-PCR a dicistronic precursor transcript consisting of a tRNA^{Ser} and a tRNA^{Leu} separated by a short intergenic sequence has recently been shown to exist *in vivo*[22]. Furthermore, in an *in vitro* import system, only the dicistronic precursor but not the derived mature tRNA^{Leu} was imported[24]. However, *in vivo* studies in *L. tarentolae* and *T. brucei* have shown that tRNAs are imported into mitochondria independently of their genomic context[17,21]. In the latter case, even heterologous tRNAs flanked by non-trypanosomal sequences were imported. *In vivo* evidence for the role of precursors in tRNA import therefore remains to be demonstrated.

In summary, it is clear that more complete and quantitative studies are needed to identify the import determinants in plants and trypanosomatid tRNAs. Such studies are important as they offer the possibility to characterize protein factors interacting with these elements. Furthermore, it is unresolved why it is always only a fraction of a given tRNA that is imported.

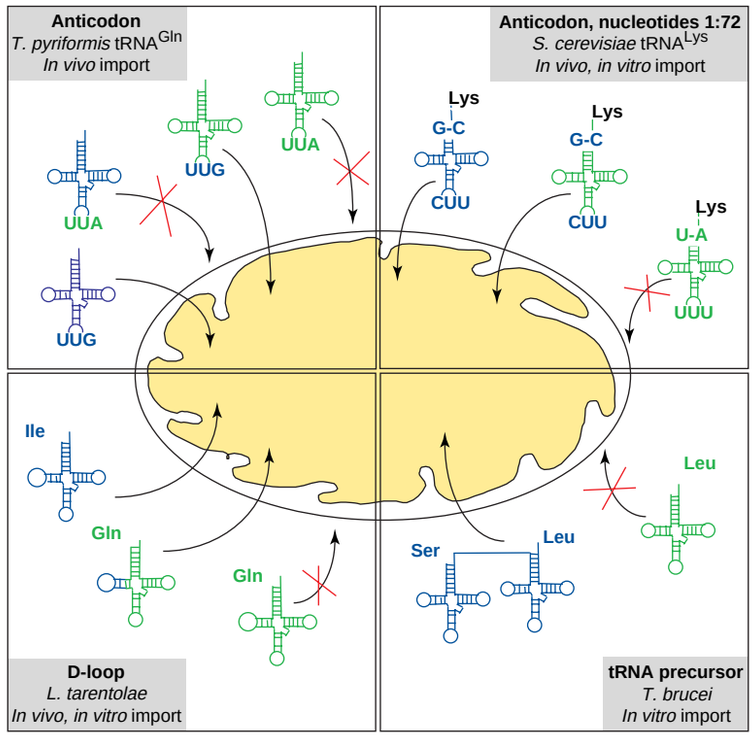

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FIGURE 2

Mitochondrial import determinants of nucleus-encoded tRNAs. Schematic representation of the key import determinants of nucleus-encoded tRNAs of *Tetrahymena pyriformis*[14], *Saccharomyces cerevisiae*[15,16], *Leishmania tarentolae*[17,18] and *Trypanosoma brucei*[24] obtained from *in vivo* and/or *in vitro* analyses. Naturally occurring cytosol-specific tRNAs or imported tRNAs are shown in green or blue, respectively. tRNA variants with domains or nucleotides of their differently localized counterparts are depicted in mixed colours. For *T. brucei*, the dimeric precursor tRNA is shown in blue, whereas the mature tRNA^{Leu} suggested to remain in the cytosol is shown in green. When contributing to the import signal, anticodons and the 1:72 nucleotide pairs are indicated. In *S. cerevisiae*, the aminoacylated tRNA^{Lys} is shown because the lysine residue was shown to be required for import.

How are tRNAs imported into mitochondria?

Although the role of the single imported tRNA^{Lys} in *S. cerevisiae* is unclear as it cannot be aminoacylated inside mitochondria and as a mitochondria-encoded tRNA^{Lys} exists, its import pathway has been elucidated in detail[4]. A combination of *in vitro* and *in vivo* studies showed that the charged tRNA is co-imported across the protein import pore using the mitochondrial precursor of lysyl-tRNA synthetase (preMSK) as a carrier, even though that protein cannot aminoacylate the tRNA it transports. Import of the tRNA – like protein import – requires internal ATP and the membrane potential. Binding of tRNA^{Lys} to preMSK depends on specific regions in the tRNA (as discussed above) as well as on aminoacylation by the cytosolic lysyl-tRNA synthetase. Furthermore, *in vitro* import studies showed that, besides preMSK, at least one other as-yet-unidentified factor is required for import of the tRNA protein

complex[25]. Numerous studies have established that proteins need to be unfolded during passage across the mitochondrial membranes[26]. It is therefore a challenge to explain how the interaction between unfolded preMSK and the imported $tRNA^{Lys}$ can be maintained during transport. Although unfolding of the protein is essential for import, this might not apply for the tRNA since a nicked $tRNA^{Lys}$ could still be imported into mitochondria as long as the two tRNA moieties were reannealed[15]. This is in agreement with earlier *in vitro* studies using artificial protein–DNA chimeras that demonstrated that the protein-import channel is wide enough to accommodate double-stranded nucleic acids[27].

Although preMSK is the crucial import factor in yeast, there is circumstantial evidence that aminoacyl-tRNA synthetases might not play the same role in other systems. Selective import of only some isoacceptors was observed in plants (for the $tRNA^{Gly}$ isoacceptors)[28] and in *Tetrahymena* (for the $tRNA^{Gln}$ isoacceptors)[14]. Furthermore, expression of plant mitochondrial alanyl-tRNA synthetase in yeast did not induce import of the coexpressed plant or the endogenous yeast $tRNA^{Ala}$ (Ref. 29). Finally, in *T. brucei*, a mutant $tRNA^{Tyr}$ that cannot be charged could still be imported[30]. The described experiments argue against a crucial role for aminoacyl-tRNA synthetases in tRNA import – but they cannot definitively exclude it.

Recently, *in vitro* tRNA import systems have been established for *T. brucei*[24,31] and two *Leishmania* species[18,32]. One *T. brucei* system, although initially thought to import full-size tRNAs, was later shown to be competent only for the import of small RNA fragments[31]. Its physiological significance is therefore questionable and it will not be discussed further. All the other systems show some common features: pretreatment of mitochondria with protease abolished import, indicating the need for proteinaceous receptors on the surface of mitochondria. In *L. tropica*, it was shown that antibodies against a protease-sensitive RNA-binding protein of 15 kDa inhibited import. Surprisingly, however, the putative import receptor was found both associated with mitochondria but also localized throughout the cell[32]. In all systems, import required external and probably internal ATP as well as one or both components of the electrochemical proton gradient[18,24,33]. Import substrates between *Leishmania* and *Trypanosoma* appear to be quite different. In *Leishmania*, mature tRNAs were imported and a cytosol-specific tRNA was not[18,32]. In *T. brucei*, on the other hand, only dimeric precursor tRNA was imported – but not its mature derivative[24]. Surprisingly, however, even though an RNase-P-like activity has been detected in mitochondrial extracts[22], no processing of the imported transcript was observed. Furthermore, it remains to be demonstrated that the substrate tRNAs have indeed crossed both membranes and are localized in the matrix.

None of the assays in *Leishmania* or *T. brucei* requires the addition of cytosolic factors, which argues that the import mechanism is different from that in yeast. However, it cannot be excluded that contaminating cytosolic factors are present in the crude mitochondrial fractions used for the import assays. The identity of the 15-kDa putative tRNA import receptor in *L. tropica* is unclear, so it could in principle be a component of the protein-import machinery. Furthermore, the established energy requirement for tRNA import does not allow us to distinguish between yeast-like co-import or any other mechanism. In the light of the evolutionary distribution of tRNA import (Fig. 1), it is unlikely that only one mechanism exists. Identification of import factors in the different organisms should be a priority of future research as it will allow this question to be definitively settled. It would be especially interesting in this regard to learn more about the plant system, the import mechanism of which has remained obscure owing to the lack of an *in vitro* system.

Practical applications of mitochondrial tRNA import

Mitochondrial tRNA import might have some exciting practical applications. At present, only very few systems are amenable to direct mitochondrial transformation. tRNA import offers an alternative tool to study mitochondrial gene expression since it might allow import of synthetic sequences (e.g. antisense RNAs or ribozymes) that would potentially interfere with intramitochondrial functions (e.g. translation or RNA editing). Indeed, it was shown in *L. tarentolae* that a splicing-deficient $tRNA^{Tyr}$ can be used to import synthetic introns of up to 40 nucleotides in length[34]. More recently, an elegant study in *S. cerevisiae* showed that it is possible to complement a nonsense mutation in the mitochondrial *COX2* gene by nuclear transformation of a suppressor variant of the imported $tRNA^{Lys}$ (Ref. 35). Even though the imported $tRNA^{Lys}$ cannot be charged inside mitochondria, it is imported in its aminoacylated form and therefore might still participate in mitochondrial translation. Furthermore, the experiment demonstrates for the first time that tRNA import can be used to cure respiratory defects caused by mutations in the mitochondrial DNA. This is of great medical interest since a number of human mitochondrial cytopathies (e.g. MELAS, mitochondrial myopathy encephalopathy with acid lactosis and stroke-like episodes; MERF, myoclonic epilepsy and ragged-red fibres syndrome) are caused by point mutations in mitochondria-encoded tRNAs[36]. Many of these diseases might theoretically be treated by nuclear transfection with the corresponding wild-type tRNA gene – but only if the tRNA can be imported into mitochondria. Although human mitochondria do not normally import tRNAs, a recent study showed that it is possible to import the yeast $tRNA^{Lys}$ into isolated human mitochondria as long as soluble yeast import-directing factors are present[35]. It should therefore in principle be possible to transplant the tRNA-import system of yeast into human cells. Finally, tRNA import is extensive in trypanosomatids and apicomplexans, many of which are important clinical pathogens. The hosts of these parasites, however, do not import tRNAs. The process offers therefore a novel potential target for a chemotherapeutic attack on these organisms.

Future research on the various tRNA import systems will therefore not only reveal novel insights into an as yet poorly understood basic biological process but may also have impact on the treatment of clinically important human diseases.

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