Mitochondrial tRNA Import and Its Consequences for Mitochondrial Translation

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aminoacyl-tRNA synthetase, mitochondrial biogenesis, mitochondrial protein import, organellar translation, 5S rRNA

Abstract

The mitochondrial genomes of most eukaryotes lack a variable number of tRNA genes. This lack is compensated for by import of a small fraction of the corresponding cytosolic tRNAs. There are two broad mechanisms for the import of tRNAs into mitochondria. In the first one, the tRNA is coimported together with a mitochondrial precursor protein along the protein import pathway. It applies to the yeast $tRNA^{Lys}$ and has been elucidated in great detail. In the second more vaguely defined mechanism, which is mainly found in plants and protozoa, tRNAs are directly imported independent of cytosolic factors. However, results in plants indicate that direct import of tRNAs may nevertheless require some components of the protein import machinery. All imported tRNAs in all systems are of the eukaryotic type but need to be functionally integrated into the mitochondrial translation system of bacterial descent. For some tRNAs, this is not trivial and requires unique evolutionary adaptations.

Contents

INTRODUCTION

Mitochondria are double-membrane-bounded organelles of endosymbiontic evolutionary origin. Their main function is oxidative phosphorylation. All mitochondria have their own genome reflecting their bacterial ancestry. Although there is a great diversity in the size and structure of mitochondrial genomes, the number of proteins encoded on the mitochondrial DNA is always very small, indicating that more than 95% of all mitochondrial proteins are nuclear encoded, synthesized in the cytosol, and subsequently imported into mitochondria. The mechanism of mitochondrial protein import has been analyzed in great detail (see **Figure 1** to see how proteins are imported into the mitochondrial matrix) (reviewed in References 1–3).

Mitochondrial matrix: a soluble compartment that is surrounded by the mitochondrial inner membrane

Unlike what many textbooks state, mitochondrial biogenesis in most organisms not only requires the import of a large number of proteins but also requires the import of at least a few cytosolic tRNAs (reviewed in References 4–6). More than 40 years ago, in 1967, Suyama reported that a large fraction of isolated mitochondrial tRNAs from the protozoan *Tetrahymena* hybridizes to nuclear rather than mitochondrial DNA (7). This prompted him to suggest that these molecules are imported from the cytosol. The conclusion of his study was far ahead of its time and therefore was greeted with skcepticism. It took 10 years before the next example of mitochondrial tRNA import was described in 1977. Isolating mitochondrial tRNAs from the yeast *Saccharomyces cerevisiae*, Martin et al. (8) showed that one of the two mitochondrial $tRNAs^{Lys}$ hybridizes exclusively to nuclear DNA. But again, these results where largely ignored. This was very different for mitochondrial protein import, discovered in the years 1977–1979 by Neupert (9) and by Schatz & Blobel with their coworkers (10), as it immediately attracted a lot of interest. We then had to wait another 10 years to learn that also in the plant *Phaseolus vulgaris* many mitochondrial tRNAs are of nuclear origin (11) and that the same is true for all mitochondrial tRNAs in the trypanosomatids, i.e., *Leishmania tarentolae* (12) and *Trypanosoma brucei* (13). The first direct evidence for mitochondrial tRNA import was finally provided in 1992 by Small et al. (14), who showed that in transgenic plants the transcript of a mutationally tagged nuclear tRNA gene is found in both the cytosol and the mitochondria. It was only after these experiments that the existence of mitochondrial tRNA import was generally accepted.

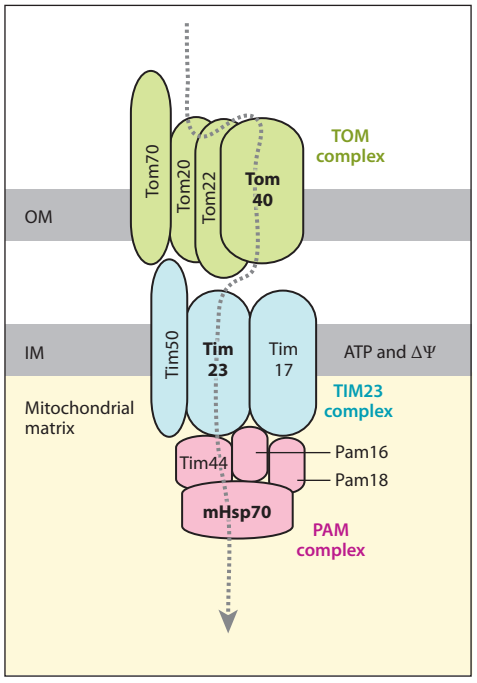

Figure 1

Protein import into the mitochondrial matrix requires three heterooligomeric protein complexes. One is the translocase of the outer mitochondrial membrane, the TOM complex (*green*). It represents the general entry gate for essentially all imported proteins and consists of the receptors Tom20, Tom22, and Tom70; the conserved pore-forming core subunit Tom40, and a number of smaller subunits (not shown). Translocation of matrix proteins across the mitochondrial inner membrane is mediated by the main protein translocase of the inner membrane, the TIM23 complex (*blue*); the core component of TIM23 is the conserved channel-forming Tim23. The TIM23 complex cooperates with the presequence translocase-associated motor, called the PAM complex (*purple*). Its main subunit, the mitochondrial Hsp70 (mHsp70), drives ATP-dependent protein translocation across the mitochondrial inner membrane. The arrow indicates the path along which matrix proteins are imported. Translocation requires unfolding of the proteins. Moreover, transport across the inner membrane depends on the membrane potential ($\Delta\Psi$). The matrix is shown in yellow. OM, mitochondrial outer membrane; IM, mitochondrial inner membrane.

However, compared to other aspects of mitochondrial biogenesis, mitochondrial tRNA import has remained a rather neglected area of research. This is unfortunate because it is a very widespread process, and studying it has not only much to offer for basic research but may also have implications for combating mitochondrial diseases and parasitic infections. It is the aim of this review to summarize the progress that has been made in understanding the mitochondrial tRNA import process as well as the consequences it has for mitochondrial translation.

HOW WIDESPREAD IS MITOCHONDRIAL tRNA IMPORT?

Bioinformatics is a powerful tool to obtain a global view of the extent and the occurrence of mitochondrial tRNA import. It relies on the analysis of the complete mitochondrial DNA sequences, which are available for more than 2,000 species, and allows predictions of the number of mitochondrial tRNA genes in these organisms (15). The predicted tRNAs can then be matched to the codons that are used in the corresponding mitochondrial translation systems. The most plausible explanation for the many incomplete sets of mitochondrial tRNA genes found in such analyses is that the missing genes are compensated for by import of cytosolic tRNAs (16).

Table 1 shows that mitochondrial tRNA import is very widespread and that the number of mitochondrial tRNA genes that have been retained in different species is highly variable. Interestingly, organisms having a complete set of mitochondrial tRNA genes are essentially restricted to the eukaryotic supergroup of the Opisthokonta. However, also among this group there are many examples of species that have lost mitochondrial tRNA genes.

Mitochondria are derived from free-living bacteria. Having a complete set of mitochondrial tRNA genes and not requiring import of cytosolic tRNAs therefore represents the

Table 1 Occurrence and extent of mitochondrial tRNA import

Class[a]	Species/taxon	Eukaryotic supergroup[b]	Amino acids specified by mitochondrially encoded tRNA genes[c]	Amino acids specified by imported tRNAs[d]	Redundant tRNA import[e,f]
I	*Homo sapiens*	Opisthokonta (Metazoa)	20	0	$tRNA^{Gln}$
	Saccharomyces cerevisiae	Opisthokonta (Fungi)	20	0	$tRNA^{Gln}$ $tRNA^{Lys}$
	Allomyces macrogynus	Opisthokonta (Fungi)	20	0	n.d.
	Oscarella carmella	Opisthokonta (Porifera)	20	0	n.d.
II	*Didelphis virginiana*	Opisthokonta (Metazoa)	19 (1 $tRNA^{Lys}$ pseudogene)	1 ($tRNA^{Lys}_{CUU}$)	n.d.
	Reclinomonas americana	Excavata	19	1 ($tRNA^{Thr}$)	n.d.
	Phytophthora infestans	Chromalveolata	19	1 ($tRNA^{Thr}$)	n.d.
	Chondrus crispus	Archeaplastida	19	1 ($tRNA^{Thr}$)	n.d.
III	*Plakortis angulospiculatus*	Opisthokonta (Porifera)	6	14	n.d.
	Hyaloraphidium curvatum	Opisthokonta (Fungi)	7	13	n.d.
	Steganacarus magnus	Opisthokonta (Metazoa)	6	14	n.d.
	Tetrahymena thermophila	Chromalveolata	7	13	–
	Arapidopsis thaliana	Archeaplastida	14	6	–
	Chlamydomonas reinhardtii	Archeaplastida	3	17	–
	Dictyostelium discoideum	Amoebozoa	14	6	n.d.
IV	Cnidaria	Opisthokonta (Metazoa)	1 or 2 ($tRNA^{Met}$ or $tRNA^{Met}$ and $tRNA^{Trp}$)	19 or 18	n.d.
	Igernella notabilis	Opisthokonta (Porifera)	2 ($tRNA^{Met}$ and $tRNA^{Trp}$)	19 or 18	n.d.
	Chaetognatha	Opisthokonta (Metazoa)	1 ($tRNA^{Met}$)	19	n.d.
	Trypanosomatidae (e.g., *Trypanosoma brucei*, *Leishmania*)	Excavata	0	20	–
	Apicomplexa (e.g., *Plasmodium*, *Toxoplasma*)	Chromalveolata	0	20	–
	Polytomella capuana	Archeaplastida	1 ($tRNA^{Met}$)	19	–

[a]Based on the completeness of the mitochondrial set of tRNA genes, eukaryotes can be divided into four classes. Organisms of class I and II have a complete or nearly complete set of mitochondrial tRNA genes, respectively. Class III organisms lack a large fraction of their mitochondrial tRNA genes, and species in class IV have lost all or nearly all mitochondrial tRNA genes.

[b]For the Opisthokonts, the lower taxonomic rank is indicated in parentheses.

[c]For organisms encoding only one or two tRNA genes, the identity of these genes is indicated in parentheses.

[d]For organisms importing only one or two cytosolic tRNAs, the identity of the tRNAs is indicated in parentheses.

[e]Organisms whose mitochondria encode an apparently complete set of tRNAs genes and were nevertheless shown to import the indicated cytosolic tRNAs.

[f]Definitions: n.d., not determined; –, no redundant tRNA import has been detected experimentally.

ancestral situation. Thus, the punctuated phylogenetic distribution of Opisthokont species that lack mitochondrial tRNA genes within a majority of Opisthokonts having a complete set of mitochondrial tRNA genes suggests that mitochondrial tRNA import may have evolved multiple times independently (16). Should this be the case, it must have happened—in evolutionary terms—in a very short time because in some cases [e.g., within the Porifera (17) and the fungi (18)] we find species that import tRNAs that are closely related to species that are predicted to have a complete set of mitochondrial tRNA genes.

However, it should be considered that bioinformatic analysis assumes that, for any given mitochondrially encoded tRNA, the corresponding cytosolic tRNA that is able to read the same codons is not imported. This is not true in all cases: Yeast and humans are predicted to have a complete set of mitochondrial tRNA genes, but experimental evidence shows that they nevertheless import a few tRNAs (19, 20). Only few species have been analyzed to the extent that this type of "redundant" tRNA import would have been detected. Furthermore, it is clear that bioinformatic analysis has its own limitations: Some mitochondrially encoded tRNAs have noncanonical structures or are edited, and therefore, their genes can not be recognized easily by the currently used algorithms.

Thus, an alternative scenario to the polyphyletic origin of mitochondrial tRNA import, consistent with the prevalent occurrence of the process across all eukaryotic supergroups, would be that import of tRNAs is a universal trait of mitochondria that already existed in the ancestor of all eukaryotes. This would mean also that organisms, which show no evidence for import of tRNAs, would have a—possibly cryptic—tRNA import system. In this case, the evolution of mitochondrial tRNA import would not occur through repeated de novo formation of distinct and complex tRNA import machineries but would rely on the activation or adaptation of a preexisting universal, conserved pathway.

Finally, it should be mentioned that there are reports that in a few cases other RNAs than tRNAs are also imported into mitochondria. The best-documented case concerns import of the 5S rRNA into human mitochondria and is discussed below (21, 22). Other more controversial examples are the import of the RNA subunits of RNase P and MRP RNase into human mitochondria (23–27). Moreover, indirect evidence suggests that tRNAs are not only imported into mitochondria but also into plastids (28–31).

MECHANISM OF MITOCHONDRIAL tRNA IMPORT

Before tRNAs are imported into mitochondria, they are subject to a complex series of obligatory processing and transport steps, which are summarized in **Figure 2** (32–34). During these steps, tRNAs are directly handed over from one protein to the next without ever being dissociated into the cell fluid (35–37). Thus, before a tRNA can be imported into mitochondria it needs to be diverted from the protein synthesis pathway, indicating that the decision to commit a tRNA to import is taken in the cytosol before the tRNA actually interacts with mitochondria. The next sections discuss what is known about the tRNA import pathways in the different systems and try to derive general principles for all of them. Only physiological examples of mitochondrial RNA import are given.

Mitochondrial tRNA Import in *Saccharomyces cerevisiae*

Mitochondria of *S. cerevisiae* encode an apparently complete set of tRNA genes. Nevertheless, it has long been known that a small fraction of the cytosolic $tRNA^{Lys}$(CUU) is imported into mitochondria, whereas the other isoacceptor $tRNA^{Lys}$(UUU) remains in the cytosol (19). More recently, it was suggested that, in addition to the $tRNA^{Lys}$(CUU), two cytosolic $tRNAs^{Gln}$ might also be imported into yeast mitochondria (38).

Opisthokonta: one of six presumably monophyletic eukaryotic supergroups which includes metazoans and fungi

RNase P: a ribonuclease generally consisting of RNA and protein subunits that catalyzes the endonucleolytic 5′ processing of tRNA precursors

MRP RNase: a ribonuclease related to RNase P, consisting of RNA and protein subunits, that catalyzes the endonucleolytic processing of rRNA precursors

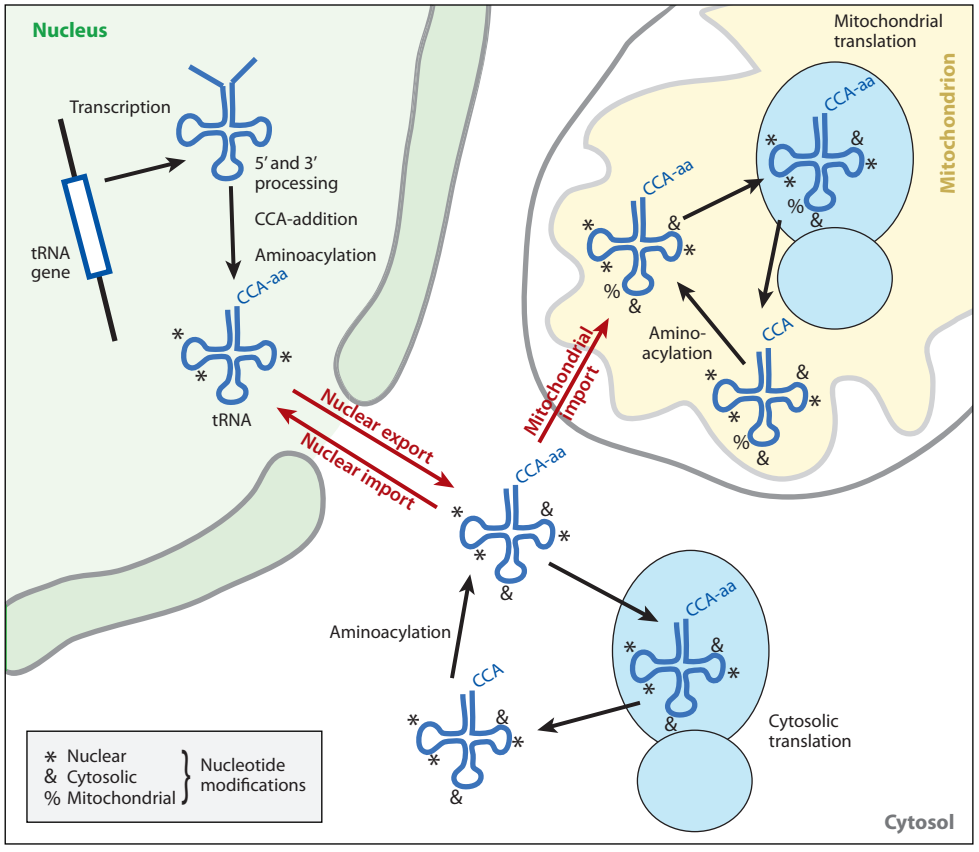

Figure 2

Schematic view of the biogenesis of a nuclear-encoded tRNA. The nuclear, cytosolic, and mitochondrial matrix compartments are shown in green, white, and yellow, respectively. Ribosomes are shown in blue. tRNA transport pathways connecting different organelles are depicted by red arrows. Black arrows indicate processing steps and binding to, or release from, the ribosome. Nuclear, cytosolic, and mitochondrial nucleotide modifications are indicated by *, &, and %, respectively. tRNA splicing, which affects a subset of tRNAs, is not considered in the picture. Depending on the organism, it may occur in the nucleus or the cytosol. aa, amino acid; CCA, posttranscriptionally added 3′-terminal CCA.

Import of $tRNA^{Lys}$. The pathway by which the $tRNA^{Lys}$(CUU) is imported into mitochondria has been analyzed in great detail. The fraction of the $tRNA^{Lys}$(CUU) that is destined to be imported is diverted from cytosolic translation to the mitochondrial import pathway by specific binding to the glycolytic enzyme enolase (39). In yeast, there are two isoforms of enolase, Eno1p and Eno2p. Of these, only Eno2p is able to bind tRNA, and it only recognizes the $tRNA^{Lys}$(CUU) but not the $tRNA^{Lys}$(UUU). The tRNA-Eno2p complex then transits to the surface of mitochondria, where the tRNA is handed over to the precursor of mitochondrial lysyl-tRNA synthetase (pre-LysRS), which is synthesized in the vicinity of mitochondria (**Figure 3*a***) (39). The resulting tRNA- pre-LysRS complex, finally, is coimported into the mitochondrial matrix using the protein import pathway (40, 41), whereas the free enolase is integrated into a glycolytic multiprotein complex that is associated with the mitochondrial outer membrane (**Figure 3*a***) (42).

pre-LysRS: precursor of the mitochondrial lysyl-tRNA syntethase

The suggested model in **Figure 3*a*** is supported by results of many different experiments. Both in vitro import of the $tRNA^{Lys}$ as well as in vitro import of matrix proteins require the membrane potential, ATP, as well as an intact protein import machinery (40, 41). Import of $tRNA^{Lys}$(CUU) depends on sequential interactions with both Eno2p and pre-LysRS. Mature LysRS lacking the presequence cannot substitute for pre-LysRS. Binding of $tRNA^{Lys}$(CUU) to pre-LysRS has a much higher affinity than to Eno2p and the presence of Eno2p facilitates complex formation between $tRNA^{Lys}$(CUU) and pre-LysRS.

More recent experiments using fluorescently labeled $tRNA^{Lys}$(CUU) and Förster resonance energy transfer (FRET) analysis investigated the conformation of the $tRNA^{Lys}$(CUU) when bound to Eno2p or pre-LysRS, respectively (43). The results suggest that when bound to Eno2p the tRNA adopts a conformation in which, unlike the classical L shape, the 3′ end of the tRNA is close to the T-loop region. Upon transfer of the tRNA to pre-LysRS, this structure is converted to a conformation similar, but not identical, to the classical L shape. Thus, the specificity of tRNA import in *S. cerevisiae* might be governed by the selective ability of a $tRNA^{Lys}$(CUU) to adopt an alternative structure upon binding to Eno2p. Knowing the structural changes that occur in the tRNA along the import pathway can provide much insight. One important consideration is that the proposed model is mainly based on in vitro experiments using in vitro transcribed unmodified $tRNA^{Lys}$(CUU) as a substrate.

Interestingly, pre-LysRS can only charge the mitochondrial-encoded $tRNA^{Lys}$(UUU) but not $tRNA^{Lys}$(CUU) with which it is coimported into mitochondria. This suggests that the interaction between the imported $tRNA^{Lys}$(CUU) and pre-LysRS is different than the interaction with its cognate substrate, the mitochondrially encoded $tRNA^{Lys}$(UUU). In line with this is the fact that $tRNA^{Lys}$(CUU) needs to be aminoacylated to bind to pre-LysRS, although certain mutant versions of the tRNA can bypass this requirement (44).

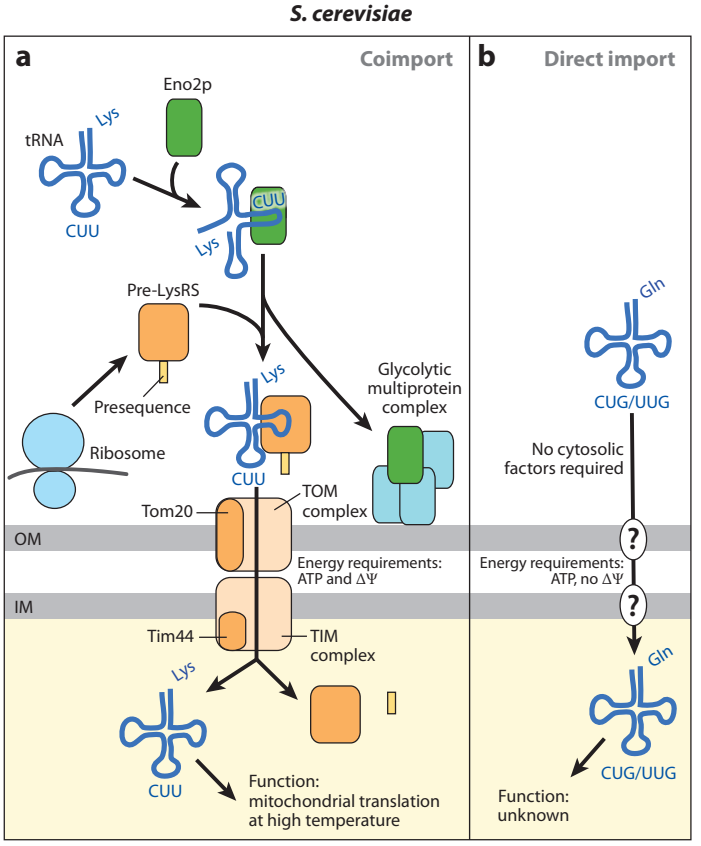

Figure 3

Mitochondrial tRNA import in *Saccharomyces cerevisiae*. (*a*) Model of the way the $tRNA^{Lys}$(CUU) is imported into yeast mitochondria. tRNA targeting factor, Eno2p, is indicated in green. The tRNA changes its structure upon binding to Eno2p (see text for details). Components whose involvement in membrane translocation of the $tRNA^{Lys}$ has directly been shown are depicted in orange. Components whose involvement has been inferred indirectly are shown in light orange. Other factors not linked to tRNA import are shown in blue. pre-LysRS is synthesized on ribosomes that are localized in the vicinity of mitochondria. After release of the $tRNA^{Lys}$, Eno2p is targeted to a glycolytic multiprotein complex associated with the outer membrane of mitochondria. (*b*) Model of the way $tRNAs^{Gln}$ are imported into yeast mitochondria. The two broad mechanisms of mitochondrial RNA import, coimport and direct import, are indicated above the system to which they apply. The function of the imported tRNAs is indicated at the bottom when known. Import systems of unknown nature are depicted by question marks. Δψ, mitochondrial membrane potential; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane.

LysRS belongs to the aminoacyl-tRNA synthetase (aaRS) IIb class that consists of two domains linked by a hinge region. In vitro and in vivo analyses of truncated versions of pre-LysRS have shown that the import-directing

activity is confined to the N-terminal domain and the hinge region, whereas aminoacylation of $tRNA^{Lys}$(UUU) requires the full-length protein (44). Moreover, it was possible to find variants of the C-terminally truncated pre-LysRS versions that had dramatically increased import directing activity when compared to wild-type pre-LysRS.

The coimport model implies that pre-LysRS and the $tRNA^{Lys}$(CUU) are imported into mitochondria as a complex using the protein translocase of the outer membrane (TOM complex) and the main protein translocase of the inner membrane (TIM23 complex). Mitochondrial import of proteins generally requires full unfolding of the transported proteins (45, 46), which raises the question of how the interaction between $tRNA^{Lys}$(CUU) and pre-LysRS can be maintained during import. This problem is exacerbated by the fact that the tRNA itself does not appear to be unfolded during import (47). However, in vitro import experiments have shown that the protein import machinery permits the passage of branched polypeptides (48) and precursors carrying a single- or even a double-stranded oligonucleotide at their C termini (49). The mitochondrial protein import machinery may therefore accommodate proteins that have retained some residual structure that is sufficient to maintain pre-LysRS-$tRNA^{Lys}$(CUU) interaction.

Mitochondria of *S. cerevisiae* encode a $tRNA^{Lys}$(UUU), which in principle can decode both lysine codons. Thus, for a long time, the import of the cytosolic $tRNA^{Lys}$(CUU) seemed redundant. Moreover, even though the $tRNA^{Lys}$(CUU) is imported in its aminoacylated state, it could only be used for a single round of elongation because it cannot be recharged inside mitochondria. However, an elegant series of experiments recently demonstrated that, although the imported $tRNA^{Lys}$(CUU) is dispensable for growth of yeast at the permissive temperature of 30°C, it becomes essential at 37°C (50). This can be explained by the fact that at 37°C the thiomodified wobble uridine of mitochondrially encoded $tRNA^{Lys}$(UUU) becomes hypomodified, preventing it from decoding the AAG lysine codon. For this reason, decoding of the AAG codons at 37°C requires the imported cytosolic $tRNA^{Lys}$(CUU).

In summary, the import pathway of the yeast $tRNA^{Lys}$(CUU) has been characterized in great detail (**Figure 3*a***). The strength of these studies is that they are based on a combination of in vitro and in vivo experiments as well as on yeast genetics. Such a combination is missing for most of the other systems and thus makes $tRNA^{Lys}$(CUU) import in *S. cerevisiae* a point of reference for the less-advanced studies in other organisms.

Import of $tRNA^{Gln}$. Import of cytosolic $tRNA^{Gln}$(CUG) and $tRNA^{Gln}$(UUG) into yeast mitochondria has not been studied in detail, but it appears to follow a different pathway than is used by the $tRNA^{Lys}$(CUU) (38). The evidence for import of the $tRNAs^{Gln}$ included an elegant genetic experiment showing that a suppressor version of the cytosolic $tRNA^{Gln}$(CUG) was able to partially complement an artificially introduced nonsense mutation in the mitochondrially encoded cytochrome oxidase subunit II gene. Moreover, it was shown that the cytosolic $tRNA^{Gln}$(CUG) could be imported into isolated yeast mitochondria and that this import, unlike the one of the $tRNA^{Lys}$(CUU), requires neither a membrane potential nor the addition of cytosolic factors (38). This led to the surprising conclusion that yeast mitochondria may have two independent, mechanistically distinct tRNA import systems (**Figure 3**).

What could be the function of the imported $tRNA^{Gln}$ (6)? Glutaminyl-$tRNA^{Gln}$ can be formed either by direct aminoacylation using a glutaminyl-tRNA synthetase (GlnRS) or by indirect transamidation (51). In this case, the $tRNA^{Gln}$ is first misaminoacylated by a nondiscriminating glutamyl-tRNA synthetase (GluRS), and the resulting glutamate-$tRNA^{Gln}$ is then converted into the correctly charged glutamine-$tRNA^{Gln}$ in a reaction catalyzed by a tRNA-dependent amidotransferase. It has been

Presequence: an N-terminal targeting sequence that directs proteins to mitochondria and that generally is cleaved off in the matrix

Förster resonance energy transfer (FRET): energy transfer from an excited fluorophore to another fluorophore in close proximity

T-loop region: a specialized region on the tRNA molecule that contains thymidine and pseudouridine residues

aaRS: aminoacyl-tRNA synthetase

Translocase: a membrane-embedded protein complex that mediates translocation of polypeptides across a membrane

TOM complex: the translocase of the outer mitochondrial membrane that functions as general entry gate for all imported proteins

TIM23 complex: the translocase of the inner mitochondrial membrane that mediates translocation of proteins across and into the inner membrane

thought that organelles, like their bacterial ancestors, exclusively employ the transamidation pathway (52). However, a tRNA-dependent amidotransferase initially could not be identified in yeast. It was therefore suggested that mitochondria of *S. cerevisiae* import the cytosolic glutaminyl-tRNA^{Gln} formation pathway, which would have explained why a fraction of the two cytosolic tRNAs^{Gln} was recovered in mitochondria. Recently, however, a heterotrimeric tRNA-dependent amidotransferase and a nondiscriminating GluRS, which surprisingly is derived from the cytosolic GluRS, have been characterized in mitochondria of *S. cerevisiae* (53). Moreover, although the authors detected the imported tRNA^{Lys}(CUU) in their mitochondrial fractions, they did not find any trace of the cytosolic tRNA^{Gln}.

Mitochondrially encoded tRNA^{Gln}(UUG) contains the modified nucleotide 5-carboxymethylaminomethyl-2-thiouridine (mcm^5S^2U) at the first position of the anticodon (54). It has been speculated that this modification may restrict base pairing of the tRNA^{Gln}(UUG) to the CAA glutamine codon (54). Should this be the case, mitochondrial import of the cytosolic tRNA^{Glu}(CUG) might be necessary to decode the CAG glutamine codon. However, there is no consensus on the in vivo effects of mcm^5S^2U on base pairing. In fact, in the analogous case of the mitochondrially encoded tRNA^{Lys}(UUU) of yeast, which is modified in essentially the same way, we have the converse situation, meaning that only the modified tRNA can decode both the AAA and the AAG lysine codons (50).

In summary, at present, there is no clear reason why the cytosolic tRNA^{Gln} of yeast should be imported. The question whether yeast mitochondria indeed have two mechanistically distinct tRNA import pathways operating side by side, however, is of great importance and should be studied in more detail.

Mitochondrial tRNA Import in Plants

Although mitochondria of all plants rely on import of cytosolic tRNAs, both the number as well as the identity of the imported tRNAs can differ dramatically between closely related species, illustrating that import specificity can evolve very rapidly (55–58).

In vivo studies with transgenic plants identified mutations in a tRNA that prevented both its import and its aminoacylation. These results suggest that aaRSs may be required for tRNA import (59). However, aminoacylation is known to be a prerequisite for nuclear export of tRNAs (60). It is therefore possible that the lack of mitochondrial import of aminoacylation-deficient tRNA variants is due to nuclear retention.

In some plants, the extent to which a given tRNA is imported may be tailored to the needs of mitochondria. In *Chlamydomonas reinhardtii*, it was found that the mitochondrial abundance of at least a subset of imported tRNAs is correlated with the codon usage of mitochondrial genes (55). These results indicate that the mechanism by which the extent of tRNA import is regulated must include a feedback control from mitochondria.

In vitro transcribed tRNA can be imported into isolated mitochondria of a potato in an ATP-dependent and membrane potential-dependent manner. In vitro import did not require cytosolic factors and could be saturated by an excess of substrate tRNA. However, whereas import into isolated mitochondria showed some specificity, it only partially matched the in vivo situation (61).

In vitro import of tRNAs could be inhibited by the addition of monospecific antisera directed against the voltage-dependent anion channel (VDAC), which is the metabolite transporter and the most abundant protein of the mitochondrial outer membrane (62). Import of tRNA was also inhibited when using antibodies against Tom20 and Tom40, two components of the TOM complex (**Figure 1** and **Figure 4*a***) (62). Further experiments showed that plant VDAC directly binds tRNAs. The fact that anti-Tom20 and anti-Tom40 antisera, in contrast to the anti-VDAC antibodies, already inhibit binding of the tRNA to mitochondria suggests that the two proteins may act as tRNA import receptors, whereas VDAC

Voltage-dependent anion channel (VDAC): an abundant protein of the mitochondrial outer membrane that builds a nonselective channel for metabolites

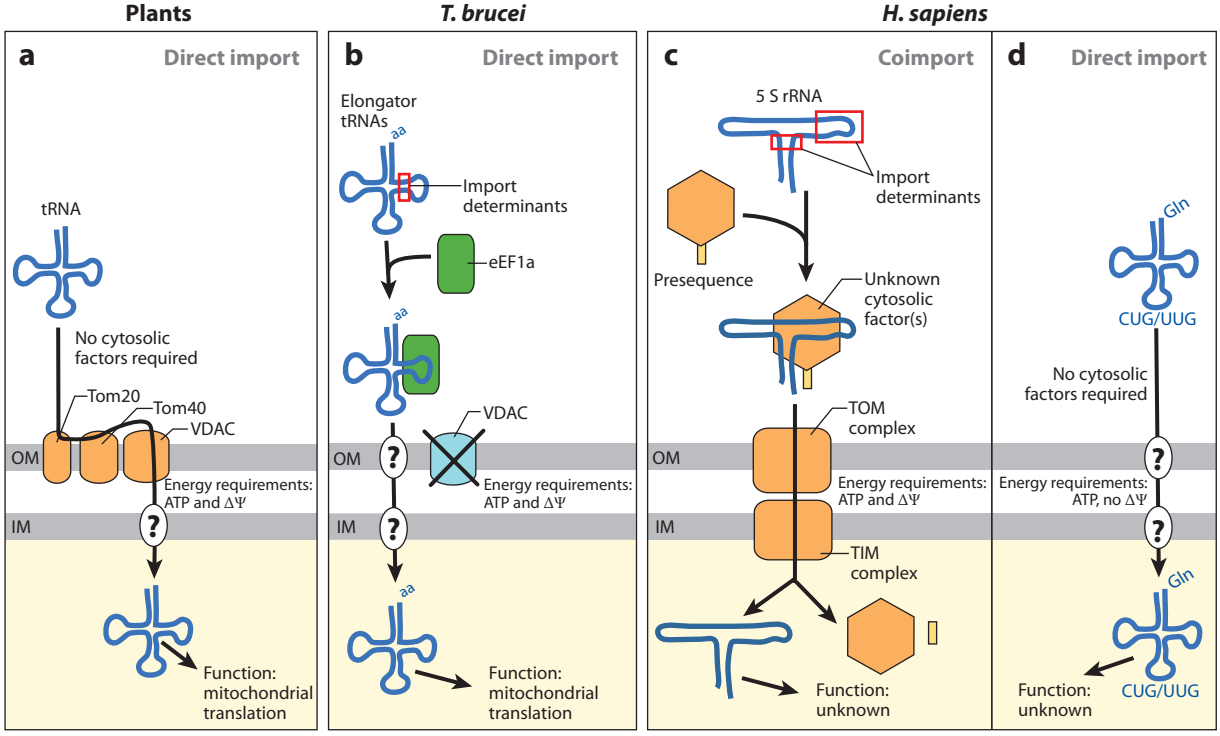

Figure 4

Mitochondrial RNA import in plants, *Trypanosoma brucei*, and *Homo sapiens*. The two broad mechanisms of mitochondrial RNA import, direct import and coimport, are indicated at the top of the systems to which they apply. The colors have the following meanings: green, tRNA targeting factor; orange, components whose involvement in membrane translocation of RNA has been shown directly; and red rectangles, regions in the imported RNAs that function as import determinants. The function of the imported RNA is indicated when known. (*a*) Model of the way tRNAs are imported into plant mitochondria. Tom20 and Tom40 act before the voltage-dependent anion channel (VDAC), but whether Tom20 acts before Tom40 is not known. (*b*) Model of the way tRNAs are imported into mitochondria of *Trypanosoma brucei*. In contrast to plants, VDAC is not required for tRNA import in trypanosomes. (*c*) Model of the way 5S rRNA is imported into *Homo sapiens* mitochondria. Cytosol is required for import, but the cytosolic proteins that mediate importare not yet known. (*d*) Model of the way $tRNA^{Gln}$ is imported into human mitochondria. Import systems of unknown nature are depicted by question marks. Δψ, mitochondrial membrane potential; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane.

may form the actual translocation channel (**Figure 4*a***). Finally, it was shown that addition of a synthetic presequence interferes with import of proteins but did not affect import of tRNAs.

In summary, these results show that in plant mitochondria there are some overlaps between tRNA and protein import. However, the fact that tRNA import does not require cytosolic factors and does not compete with import of a presequence suggests that plant tRNAs, unlike the yeast $tRNA^{Lys}$(CUU), are not coimported with proteins (**Figure 4*a***).

Selenoproteins: proteins containing the genetically inserted amino acid selenocysteine

Mitochondrial tRNA Import in Protozoa

The mitochondrial genomes of trypanosomatids such as *T. brucei* and *Leishmania* spp. are devoid of tRNA genes. Thus, all of their mitochondrial tRNAs are imported from the cytosol (12, 13). The initiator $tRNA^{Met}$ and the $tRNA^{Sec}$, however, have an exclusive cytosolic localization (63, 64). This is explained by the fact that, owing to the bacterial-type mitochondrial translation initiation and the absence of mitochondrially encoded selenoproteins, these two tRNAs cannot function inside

mitochondria. The extent of mitochondrial localization of different imported tRNAs varies by at least one order of magnitude (63, 65, 66). However, unlike in *Chlamydomaonas rheinhardtii*, the abundance of imported tRNAs does not appear to reflect an adaptation to the mitochondrial codon usage (63).

Recent studies have identified the features within a tRNA that specify import and have suggested how they may function. Using the closely related but differentially localized initiator and elongator $tRNAs^{Met}$, it was possible to show that a subregion of the T stems of the two tRNAs is both necessary and sufficient to specify their localization (67). Further studies have shown that the same T-stem localization signal is likely to act on all trypanosomal tRNAs. Eukaryotic initiator $tRNA^{Met}$ needs to bind to eukaryotic translation initiation factor 2, but it also needs to be prevented from binding to eukaryotic translation elongation factor 1a (eEF1a). Interestingly, the cytosolic localization signal in the T stem of trypanosomal initiator $tRNA^{Met}$ is identical to the region that acts as an antideterminant for eEF1a binding (68). Consistent with this finding, it could be shown that binding to eEF1a in the cytosol is a prerequisite for a tRNA to be imported into mitochondria of *T. brucei* (**Figure 4**) (69).

It has been proposed that in *L. tarentolae* the cytosol-specific thiomodifications of the wobble uridine in the $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) might be antideterminants for mitochondrial import (70). However, RNAi-mediated ablation of the thiomodifications in trypanosomal $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) did not increase their import, indicating that at least in *T. brucei* this is not the case (71, 72).

How tRNAs are translocated across the two mitochondrial membranes of trypanosomatids is unknown. However, it is clear that the single trypanosomal VDAC is not required for import (73), suggesting that plants and trypanosomatids use a different tRNA import mechanism.

A number of studies in *Leishmania* spp. and in *T. brucei* were using in vitro import systems to investigate mitochondrial tRNA import (69, 74, 75). Although most in vitro import systems show some specificity regarding the RNAs that are imported, there is no clear connection to the specificity observed in vivo. This discrepancy might be because none of the in vitro systems contains cytosolic factors. Regarding the energetics of mitochondrial tRNA import in trypanosomatids, there is a consensus that it requires ATP (69, 74, 75). However, whether it also requires a membrane potential is controversial. Much of the inconsistencies we observe in different in vitro studies might be caused by methodological shortcomings owing to the difficulty of isolating highly active mitochondria.

There are numerous publications by Adhya and coworkers regarding the tRNA import pathway in *Leishmania tropica* (reviewed in References 76 and 77). However, a closer analysis of the published data raises a number of questions; some of these have been discussed in previous reviews (3, 4). Moreover, the *Proceedings of the National Academy of Sciences of the United States of America* has recently published an editorial concern (78) regarding the results in one of the key publications of the Adhya group (79). Without knowing the outcome of the pending investigation regarding this paper, I decided to not discuss the issue here.

tRNA import has also been studied in the apicomplexan *Toxoplasma gondii*, which as trypanosomatids lacks mitochondrial tRNA genes and therefore imports all of its mitochondrial tRNAs (80). Although aminoacylated mitochondrial tRNAs can be detected in *T. gondii*, no aaRSs are localized to its mitochondrion (81). This suggests that tRNAs are imported in their aminoacylated state (discussed below).

One of the best-characterized in vivo signals for mitochondrial tRNA import has been identifed in *Tetrahymena*, the system where mitochondrial tRNA import was orginally discovered (7). *Tetrahymena* has three very similar $tRNAs^{Gln}$, but only one of these is in part imported into mitochondria (82). Using transgenic cells, it was shown that the anticodon UGG of the imported $tRNA^{Gln}$ is both necessary and sufficient to induce import of any of three $tRNAs^{Gln}$ (83).

eEF1a: eukaryotic translation elongation factor 1a

Mitochondrial RNA Import in Mammals

It has been demonstrated that a small fraction of cytosolic 5S rRNA is imported into mitochondria of mammals (21, 22). The region of the 5S rRNA needed for mitochondrial targeting appears to be confined to two distinct structural regions of the molecule (84). Using an in vitro assay, it was shown that import of 5S rRNA requires ATP hydrolysis, the membrane potential, as well as a cytosolic protein fraction. Blocking the mitochondrial protein import channel inhibited in vitro import of 5S rRNA, indicating that a functional protein import machinery is required for import (**Figure 4*c***) (85). In summary, these conditions are reminiscent to the import of $tRNA^{Lys}$ into yeast mitochondria (**Figure 3*a***) (40) and suggest that in mammals RNA can be coimported with mitochondrial precursor proteins.

5S rRNA is an universal component of bacterial, archeal, eukaryotic, and plastid ribosomes, but in mitochondria, its gene has only been found in plants and a few protists (15, 86). In *S. cerevisiae*, cytosolic 5S rRNA is neither encoded on the mitochondrial genome nor imported from the cytosol, illustrating that mitochondrial ribosomes can, in principle, function without a 5S rRNA. Even though the imported 5S rRNA is one of the most abundant organellar RNAs in mammals, it has not yet been shown to be physically associated with mitochondrial ribosomes; thus, its functional significance remains unclear.

Mammalian mitochondria, with the exception of a few marsupisals (87), encode a complete set of mitochondrial tRNA genes. However, recent experiments showed that in humans the cytosolic $tRNAs^{Gln}$ are, in part, recovered in the mitochondrial fraction (20). Moreover, in vitro transcribed $tRNA^{Gln}$ could be imported into isolated mitochondria in a process that requires ATP but that is independent of cytosolic factors and the membrane potential (**Figure 4*d***).

Thus, it is possible that mammalian mitochondria have two distinct RNA import machineries: One requires cytosolic factors and an intact protein import system, reminiscent of $tRNA^{Lys}$(CUU) import in yeast (**Figure 3*a***), and one is independent of soluble factors, similar to the proposed import of the $tRNAs^{Gln}$ into yeast mitochondria (**Figure 3*b***).

Common Features of Mitochondrial RNA Import

Experimental analysis suggests that different tRNA import machineries and mechanisms are operational in the different systems. However, a closer view reveals some common concepts and principles.

A targeting step that diverts tRNAs to the mitochondrial import pathway and that determines the specificity of the process appears to be essential in vivo. In yeast (in the case of the $tRNA^{Lys}$) and in *T. brucei*, this step is mediated by cytosolic factors (39, 69). The same is likely to be the case for 5S rRNA import into mammalian mitochondria (85). Because of different import specificities, the targeting factors—enolase in yeast and eEF1a in *T. brucei*—are different. However, both are housekeeping proteins that moonlight as tRNA targeting factors. In vitro import systems in most organisms do not include cytosolic factors. Thus, unlike in vivo systems, tRNAs are present as free molecules, meaning that no tRNA channeling can occur. This may explain why the import specificity observed in vitro does not match the one that is observed in vivo.

In vitro studies show that there is a universal ATP requirement for a tRNA to be translocated across the mitochondrial membranes. A survey of the different systems suggests that there are two different mechanisms by which this can be achieved (6).

The first one implies that the tRNA is imported in a complex with a mitochondrial precursor protein along the protein import pathway. It was first described in yeast, where the $tRNA^{Lys}$ is coimported with pre-LysRS (**Figure 3*a***) (40), but much evidence suggests that it also applies for the import of 5S rRNA into mammalian mitochondria (**Figure 4*c***) (85).

The defining feature of the second mechanism is that tRNAs are directly imported into isolated mitochondria without the need of cytosolic factors. This seems to be the case in plants and trypanosomatids (**Figure 4*b***). Moreover, it also applies to the import of $tRNA^{Gln}$ into yeast and human mitochondria (**Figures 3*b*** and **4*d***). Whether this type of import requires a membrane potential is not clear. Studies in plants (61), *L. tropica* (89), and *T. brucei* (75) suggest so, whereas experiments in *L. tarentolae* (74) and studies of $tRNA^{Gln}$ import into human and yeast mitochondria (20, 38) do not find such a requirement. It was shown that import of tRNAs into isolated plant mitochondria requires VDAC, Tom20, and Tom40 (61). In all the other systems, the membrane proteins that mediate cytosol-independent tRNA import are unknown, although it could be shown in *T. brucei* that VDAC is dispensable for the process (73).

It is important to point out that, whereas the coimport model is based on a powerful combination of in vitro and in vivo studies as well as on yeast genetics, the direct import model is based on only a limited number of in vitro import results. It is therefore not as well defined as the coimport model and may include mechanistically different import systems, whose only shared feature is that they do not require cytosolic proteins. Alternatively, it may turn out that some of the discussed differences between the two import mechanisms simply reflect how little we know about the direct import model. In fact, there are some unexpected similarities between the two models. The coimport of $tRNA^{Lys}$ with pre-LysRS into yeast mitochondria and also direct import of tRNAs into isolated plant mitochondria require components of the protein import system. In yeast and human mitochondria, we find the intriguing situation that both types of import mechanisms appear to operate side by side.

Except in plants, the components responsible for cytosol-independent membrane translocation of tRNAs have not been identified. However, knowing these facts is a prerequisite to determining if the connection to the protein import system is a general feature of mitochondrial tRNA import in all organisms.

MITOCHONDRIAL TRANSLATION WITH EUKARYOTIC-TYPE tRNAS

An often overlooked aspect of mitochondrial tRNA import is that all imported tRNAs are of the eukaryotic type but have to function in the context of the bacterial-type translation system of the mitochondrion (3). Many tRNAs might, in principle, be interchangeable between the cytosol and mitochondria. However, for some tRNAs, this is not the case. This is best illustrated by the fact that the ultimate and penultimate mitochondrial tRNA genes that were lost during evolution always code for the $tRNA^{Met}$ and $tRNA^{Trp}$ (**Table 1**). The retention of a mitochondrial $tRNA^{Met}$ gene in most species is no surprise. Because of the distinct translation initiation mechanisms of bacterial- and eukaryotic-type systems, the two protein synthesis machineries require specialized initiator $tRNAs^{Met}$ that are not functionally interchangeable. Moreover, a mitochondria-specific $tRNA^{Trp}$ is required as in most mitochondria the UGA stop codon has been reassigned to tryptophane. Surprisingly, there are a number of organisms—the best studied are the evolutionarily unrelated trypanosomatids and the apicomplexans—that lack mitochondrial tRNA genes altogether. Their mitochondrial translation systems depend exclusively on cytosolic eukaryotic-type tRNAs and therefore require unique evolutionary adaptations.

Mitochondrial Translation Initiation with Eukaryotic-Type tRNAs

Even though trypanosomes lack a bacterial-type initiator $tRNA^{Met}$, mitochondrial translation initiation uses a formylated $tRNA^{Met}$. The tRNA that becomes formylated is a fraction of the imported eukaryotic-type elongator $tRNA^{Met}$ (89). Formylation is catalyzed by an unusual $tRNA^{Met}$-formyltransferase that, unlike conventional formyltransferases,

TrpRS: tryptophanyl-tRNA synthetase

AspRS: aspartyl-tRNA synthetase

selectively formylates elongator-type $tRNA^{Met}$. The formylated methionine on the imported elongator $tRNA^{Met}$ is the main determinant that is recognized by an apparently conventional bacterial-type translation initiation factor 2 (90).

The apicomplexan *T. gondii* uses a different strategy to deal with the absence of a bacterial-type initiator $tRNA^{Met}$. As in trypanosomes, the only $tRNA^{Met}$ that is imported into its mitochondrion is the cytosolic elongator $tRNA^{Met}$. However, *T. gondii* lacks a mitochondrial $tRNA^{Met}$-formyltransferase, indicating that, unlike in *T. brucei*, the requirement for a specialized formylmethionyl-$tRNA^{Met}$ for translation initiation has been bypassed (81). The absence of a mitochondrial $tRNA^{Met}$-formyltransferase is in line with absence of mitochondrial aaRSs discussed below and might be linked to the fact that apicomplexan mitochondria only synthesize three proteins (91).

Reassignment of UGA to Tryptophane

T. brucei has only a single $tRNA^{Trp}$. In contrast to its cytosolic counterpart, the imported fraction of the $tRNA^{Trp}$ has to decode UGA in addition to the normal tryptophane codon UGG. Experimental analysis has shown that this can be achieved by a mitochondria-specific RNA editing event that converts the CCA anticodon of the imported $tRNA^{Trp}$ to UCA, which is then able to decode both UGG and UGA codons (92, 93). However, the CCA anticodon is an identity determinant for the cytosolic tryptophanyl-tRNA synthetase (TrpRS). As a consequence, the edited $tRNA^{Trp}$ in mitochondria cannot be charged by a cytosolic TrpRS that is also targeted to the mitochondrion. To adapt to this situation, trypanosomatids evolved a highly divergent eukaryotic-type TrpRS that is specific for mitochondria and that, unlike its cytosolic counterpart, can aminoacylate both edited and unedited $tRNA^{Trp}$ (93). Apicomplexans mitochondria use the standard genetic code and therefore are not faced with the same problem.

Trypanosome-Specific Mitochondrial Aspartyl- and Lysyl-tRNA Synthetases

Essentially all trypanosomal aaRSs are dually targeted to the cytosol and the mitochondrion. However, in addition to the two TrpRSs discussed above, we also find two distinct genes for the aspartyl-tRNA synthetase (AspRS) and LysRS (93). Both AspRSs are of the eukaryotic type, but the cytosolic one—analogous to the TrpRS—cannot aminoacylate the imported $tRNA^{Asp}$ (94). This indicates that cytosolic and mitochondrial $tRNA^{Asp}$, although derived from the same nuclear gene, are physically different, most likely owing to an as yet unknown mitochondria-specific nucleotide modification. Trypanosomes also have a mitochondria-specific LysRS. Its substrate specificity has not been analyzed, but it is speculated that the imported $tRNA^{Lys}$ may have a unique nucleotide modification that interferes with aminoacylation by the cytosolic enzyme. Interestingly, the enzyme needs to be activated after import into mitochondria by cleavage of a C-terminal extension, suggesting that it has to be prevented from being active when it crosses the cytosol (95).

T. brucei is the causative agent of human sleeping sickness, a devastating disease that occurs in sub-Saharan Africa. Both prevention and treatment of sleeping sickness are still in a very unsatisfactory state (96). The mitochondrial TrpRS, AspRS, and LysRS define a trypanosomatid-specific class of mitochondrial aaRSs that might be attractive novel drug targets. The enzymes are significantly divergent from the corresponding human counterparts and show—at least in the case of the TrpRS and AspRS—a different substrate specificity. Moreover, they are likely to be essential in the disease-causing bloodstream stage of the parasite.

Coevolution of Mitochondrial tRNA Import and aaRSs

The previous paragraphs discussed examples where mitochondrial tRNA import in all

likelihood was the driving force for the evolution of a new class of mitochondria-specific aaRSs. However, coevolution of mitochondrial tRNA import with aaRSs is a more general phenomenon, which in most cases has led to a reduction in the number of aaRSs.

The mitochondrion of apicomplexans is an extreme case in this respect. Its genome only encodes three proteins and a number of short transcripts corresponding to the rRNAs. A global study has shown that, although aminoacylated tRNAs can be detected in the mitochondrion of *T. gondii*, this is not the case for aaRSs (81), which suggests that tRNAs are imported in their aminoacylated state. Thus, mitochondrial import of aminoacylated tRNAs allowed the cell to lose all mitochondrial aaRSs. As a consequence, the imported aminoacylated tRNAs can only be used for a single round of translation elongation.

The more frequent case is that the emergence of mitochondrial tRNA import may have been favored by dual targeting of aaRSs or vice versa. Essentially, all imported tRNAs in trypanosomatids, as well as the single imported $tRNA^{Lys}$ in marsupials, are aminoacylated by a dually targeted aaRSs (87, 93). Bioinformatic evidence suggests that the same is the case in Cnidaria (97). Moreover, all aaRSs of *A. thaliana* that are dually targeted to the mitochondria and the cytosol aminoacylate imported tRNAs (98, 99). However, the fact that there are dually targeted aaRSs that aminoacylate mitochondrially encoded tRNAs, as well as imported tRNAs that are aminoacylated by organelle-specific aaRSs, shows that there is no preset order of events. Evidence for coevolution of aaRSs with mitochondrial tRNA import is also provided by a bioinformatic analysis showing that the pattern of disappearance of distinct mitochondrial tRNA genes across eukaryotes might be caused by the different capabilities of mitochondrial aaRSs to charge imported eukaryotic-type tRNAs (100).

tRNA IMPORT: A NOVEL STRATEGY TO TREAT MITOCHONDRIAL DISEASES

Many human diseases are associated with mutations in mitochondrial DNA, and half of these mutations affect mitochondrial tRNA (101). The mitochondrial dysfunctions caused by these diseases are often life threatening, and treatment is purely symptomatic. Mitochondrial tRNA import offers a novel concept for a therapy. If it is possible to induce import of a cytosolic tRNA that is able to functionally replace the mutated mitochondrial tRNA, the mitochondrial dysfunction caused by the impairment of organellar translation might be alleviated. Recent experiments have tested this new treatment concept and have been amazingly successful both in vitro and in cell culture (102–105). A detailed discussion of these studies is beyond the scope of this review. For recent expert discussions of the biomedical applications of mitochondrial tRNA import, see References 4 and 106.

The successful induction of mitochondrial tRNA import in systems that under physiological conditions do not import tRNAs might be surprising, but it is in line with the postulated existence of a ubiquitous but in part cryptic tRNA import system. Further progress in this area requires the characterization of the tRNA import mechanisms and machineries in the different organisms to reveal how they are related to the enigmatic cryptic tRNA import capability that appears to be universally present in all eukaryotes.

SUMMARY POINTS

1. Mitochondrial tRNA import is a quasi-universal process. The number of tRNAs that are imported in different species is highly variable and can evolve rapidly. All imported tRNAs derive from cytosolic tRNAs, meaning that the same nuclear gene provides tRNAs for cytosolic and mitochondrial translation.

2. The specificity of mitochondrial tRNA import is in many cases mediated by soluble targeting factors. The membrane translocation step can be divided into two broad mechanisms: a well-defined one that is mediated by coimport with a mitochondrial precursor protein across the protein import pathway and a more vaguely defined one that does not rely on soluble factors.
3. All known factors required for mitochondrial tRNA import are housekeeping proteins that have a dual function.
4. Functional integration of imported eukaryotic-type tRNAs into the bacterial-type mitochondrial translation system may require unique evolutionary adaptations.
5. Mitochondrial tRNA import may offer novel approaches to combat mitochondrial diseases as well as infections caused by parasitic protozoa.

FUTURE ISSUES

1. How well do the different in vitro import systems reflect the in vivo situation?
2. What is the composition of the (t)RNA import systems in the different organisms?
3. What determines how much of a given tRNA is imported into mitochondria?
4. Is the connection to the mitochondrial protein import system a general feature of mitochondrial (t)RNA import in all organisms?
5. What are the similarities and differences of the two distinct mitochondrial (t)RNA import systems postulated to work side by side in yeast and humans?

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