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Mitochondrial tRNA import – the challenge to understand has just begun

Juan D. Alfonzo[1,*] and **Dieter Söll**[2,3,*]

[1]Department of Microbiology, Ohio State University Biochemistry Program and The Center for RNA Biology, The Ohio State University, Columbus, OH 43210-1292, USA

[2]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520, USA

[3]Department of Chemistry, Yale University, New Haven, CT 06520, USA

Abstract

Mitochondrial translation is important for the synthesis of proteins involved in oxidative phosphorylation, which yields the bulk of the ATP made in cells. During evolution most mitochondria-containing organisms have lost tRNA genes from their mitochondrial genomes. Thus, to support the essential process of nuanced mitochondrial translation, mechanisms to actively transport tRNAs from the cytoplasm across the mitochondrial membranes into the mitochondrion have evolved. Here, we review the currently known tRNA import mechanisms, comment on recent discoveries of various import factors, and suggest a rationale for forces that lie behind the evolution of mitochondrial tRNA import.

Keywords

import; localization; mitochondria; protein complex; tRNA

Introduction

Eukaryotic cells contain extensive intracellular compartmentalization that allows sub-cellular organelles to keep distinct metabolic processes separate, often as a means to prevent unwanted reactions. In kinetoplastid flagellates (*Trypanosoma* and *Leishmania*) for example, recent studies have shown that compartmentalization of glycolytic enzymes prevents accumulation of toxic glycolytic intermediates in the cytoplasm (Haanstra et al., 2008). Likewise, in the case of tRNAs, given that the mitochondrial translation system is bacteria-like in nature, compartmentalization could prevent unwanted side reactions due to miscoding, during protein synthesis, caused by differences in ribosome specificity. What leads to compartmentalization is an extensive network of membrane systems, which are the foundations of intracellular architecture. Mitochondria and various types of plastids are unique membrane-bound intracellular compartments that usually contain their own genome,

*Corresponding authors Alfonzo.1@osu.edu; dieter.soll@yale.edu.

which encodes a number of protein-coding genes, ribosomal RNA genes, and a variable number of tRNAs (Gray et al., 2004).

Mitochondrial genomes, owing to their bacterial ancestry, still maintain characteristics of their α-proteobacterial progenitor. Mitochondrial protein synthesis utilizes a set of tRNAs encoded in the mitochondrial genome that bear resemblance to their bacterial counterparts. For example, as in *Mycoplasma*, the genetic code in most mitochondria (with the exception of plants) employs UGA as a tryptophan codon which is read by a mitochondria-encoded tryptophan tRNA ($tRNA^{Trp}$) containing a UCA anticodon. As in bacteria, tRNAs that contain G34 in the first anticodon position are used to decode the C-ending codons for the amino acids Ala, Arg, Ile, Leu, Pro, Ser, Thr, and Val. In fact, neither inosine 34-containing tRNAs nor the enzymes necessary to generate inosine are found in mitochondria. Inosine, however, is still widely used for cytoplasmic translation. Despite evolutionary conservation, mitochondrial genomes are dynamic and throughout evolution have relegated a number of functions to the nucleus via gene transfer and gene loss. To date, a wide majority of mitochondrial proteins are indeed encoded in the nucleus, synthesized in the cytoplasm and subsequently imported into the organelle where together with a few mitochondria-encoded subunits constitute the mature, fully functional respiratory complexes, which in turn yield the lion's share of the ATP made in mitochondria-containing eukaryotes.

Remarkably, compartmentalization and the loss of mitochondrial genetic material have also led to the disappearance of tRNA genes from mitochondrial genomes. This has in turn driven the evolution of mechanisms for the import of tRNAs from the cytoplasm, to enable translation of the few mRNAs still encoded in mitochondria (Salinas et al., 2008). However, the factors and/or mechanisms that control tRNA import are not fully understood, but recent advances in the field have started to shed light on the nuances of the various import pathways. In the present review, we will focus on our current knowledge of the diverse import systems, examine some experimental discrepancies, and highlight future challenges.

A number of studies have recently highlighted differences between import systems leading to the claim of the existence of various different import mechanisms. However in our view, the mitochondrial tRNA import field is still too young to support such claims; more rigorous biochemical and genetic data are needed. For the sake of clarity we will refrain from using the term mechanism too loosely in this review, and will strictly divide import systems into broadly defined types: (i) those utilizing the known protein import pathway and (ii) those in which import is shown to occur independent of the protein import machinery. The present review will describe (1) the prevalence of tRNA import systems among various organisms, (2) possible factors, key determinants, and bioenergetics involved in tRNA import, and (3) possible driving forces that led to the evolution of tRNA import systems. There are certain similarities between tRNA import systems and the protein import pathways; yet protein import will not be the subject of this review.

Many organisms… not so many mechanisms

Although mitochondrial protein synthesis is invariably essential, many mitochondrial genomes retained only an incomplete set of tRNAs, which despite being fully functional are

insufficient for translation (Salinas et al., 2008). Since the original description of mitochondrial tRNA import in 1967 (Suyama, 1967), examples of tRNA import have increased steadily. To date, tRNA import systems have been described in diverse organisms including the ciliate *Tetrahymena* (Suyama, 1967, 1986; Rusconi and Cech, 1996a,b), the apicomplexan *Toxoplasma* (Esseiva et al., 2004), the kinetoplastid flagellates *Trypanosoma* and *Leishmania* (Simpson et al., 1989; Mottram et al., 1991; Hancock et al., 1992), plants (Marechal-Drouard et al., 1988), yeast (Martin et al., 1979; Rinehart et al., 2005), and most recently mammals (Rubio et al., 2008). The number of imported tRNAs varies greatly from as few as two tRNAs in the case of yeast to a full set of tRNAs in kinetoplastids and apicomplexans (Salinas et al., 2008).

Two main general mechanisms have been described for tRNA import. The first mechanism was originally described for import of yeast $tRNA^{Lys}$ and is similar to protein import (Tarassov et al., 1995a). This system requires an electrochemical potential across the mitochondrial inner membrane and with the exception of the mitochondrial outer membrane protein MOM72, all other protein import components play a role in $tRNA^{Lys}$ import (Martin et al., 1979; Tarassov et al., 1995a; Tarassov and Martin, 1996). Import also requires the *in vivo* association of the tRNA with the precursor mitochondrial tRNA synthetase and aminoacylation of the tRNA by the cognate cytosolic synthetase (Schmitz and Lonsdale, 1989; Tarassov et al., 1995b). In addition, the metabolic enzyme enolase functions as a shuttle helping the delivery of the imported tRNA/synthetase complex to the import machinery on the mitochondrial surface (Entelis et al., 2006; see Figure 1).

The second, and possibly the most common tRNA import mechanism, was first described for *Leishmania* but has now been found in many other organisms ranging from kinetoplastids to humans (Schneider and Marechal-Drouard, 2000; Salinas et al., 2008). This mechanism is independent of the protein import pathway and can be efficiently reproduced *in vitro* in the absence of cytoplasmic factors (Mahapatra et al., 1994; Yermovsky-Kammerer and Hajduk, 1999; Rubio et al., 2000, 2008); however, it may still be influenced by their presence. For example, in *Trypanosoma brucei*, cytosolic translation elongation factor 1a (eEF1a) plays a key role as a specificity determinant for some imported tRNAs (Bouzaidi-Tiali et al., 2007). Recently, the import of $tRNA^{Gln}$ into yeast mitochondria was shown to occur *in vitro* in the absence of added cytosolic factors (Rinehart et al., 2005). Therefore, *Saccharomyces cerevisiae* contains two pathways for tRNA import: one utilizes the protein import machinery, the second is independent of protein import, a feature that is thus far unique to this organism (Rinehart et al., 2005).

Factors and determinants of tRNA import

To delineate different import mechanisms, one has to first define what specifically constitutes a conserved tRNA determinant or recognition element for organellar import. The presence of universal import determinants has been under much debate. Among the most studied tRNA import pathways are those of kinetoplastids, where a number of *in vitro* and/or *in vivo* determinants for import have been described (Salinas et al., 2008). In *L. tarentolae*, we have adopted an operational definition for tRNA distribution, where tRNAs can be divided into three groups: mostly cytoplasmic, mostly mitochondrial and equally

partitioning between the two compartments, where assessment of distribution is based on comparisons of normalized levels of a specific tRNA to a non-imported control (like ribosomal RNA) and quantitation of this signal between mitochondrial vs. cytoplasmic RNA fractions as determined by Northern blots (Kapushoc et al., 2000, 2002). Earlier studies in *L. tarentolae* showed that generating chimeric tRNAs where the D-arm of a mostly mitochondrial tRNA was placed in the context of a mostly cytoplasmic one could in fact reverse its import distribution *in vivo* (Lima and Simpson, 1996). Nonetheless, different tRNA sequence determinants for import have since been reported even within similar systems. For example, specific sequences in the D-arm and/or the TΨC-arm of tRNAs in *Leishmania tropica* represent motifs determining *in vivo* localization (Mahapatra et al., 1994). However, the presence of these sequences and their evolutionary conservation do not correlate with tRNA localization in *Leishmania tarentolae* (Suyama et al., 1998). In addition, in *L. tarentolae*, mature tRNAs are the *in vivo* substrate for import (Kapushoc et al., 2000) and a full-length tRNA is also the preferred substrate *in vitro* (Rubio et al., 2000). However, smaller substrates representing regions of the full-length tRNA could be imported albeit at much lower efficiencies. Contrary to this, in *T. brucei*, evidence was provided for pre-tRNAs as the substrate for *in vivo* import, in a mechanism akin to pre-protein import (LeBlanc et al., 1999). However, experiments in which tRNA-containing cassettes were integrated at various locations in the *T. brucei* genome, effectively generating different precursor sequences, suggested that *in vivo* import occurs independent of genomic context and thus the pre-sequence cannot contain specific import information (Tan et al., 2002).

Recently, the involvement of modifications as import determinants has been suggested. It was reported that thiolation of tRNAs in the cytoplasm of *L. tarentolae* acted as a negative determinant for import of $tRNA^{Glu}$ (Kaneko et al., 2003); however, the degree of phylogenetic conservation of this mechanism has not been explored further. In their system, only the unmodified tRNA (not containing s^2U) was the substrate for import *in vivo* and *in vitro*, while the modified version was retained in the cytoplasm (Kaneko et al., 2003). We have investigated the possibility of s^2U as a more general determinant for tRNA distribution. In *T. brucei* we knocked down the expression of iscS, the conserved master desulfurase essential for thiolation. We found that, in *T. brucei*, a close relative of *Leishmania*, s^2U formation at the wobble position of $tRNA^{Glu}$ is not a negative determinant for import *in vivo* and/or *in vitro* (Paris et al., 2009), again suggesting that at least this modification is not universally conserved as an import determinant. This of course does not rule out the possibility that other modifications may have a conserved role in import, but most likely RNA modifications will play roles as identity elements in a similar manner as they do for tRNA aminoacylation, where their general role can be more system specific.

One feature common to all tRNA import systems described so far is the requirement for ATP, which has led to a foray into the bioenergetic requirements of import in various organisms (Salinas et al., 2008). Yet different groups have reported different bioenergetic requirements. While an absolute requirement of mitochondrial membrane potential for tRNA import has been postulated in kinetoplastids (Yermovsky-Kammerer and Hajduk, 1999; Bhattacharyya and Adhya, 2004), such a requirement has been questioned (Rubio et al., 2000). Membrane potential is definitely essential for import of $tRNA^{Lys}$ into the yeast mitochondria, where several features of the protein import pathway are required (Tarassov et

al., 1995a). Yet membrane potential is dispensable *in vitro* for a tRNA import pathway described in yeast for $tRNA^{Gln}$ (Rinehart et al., 2005). Likewise, in the mammalian system, a membrane potential does not play a major role in $tRNA^{Gln}$ import. Coincidentally, inhibition of iscS activity in *T. brucei* also leads to ablation of the membrane potential, but no effects on tRNA import were observed (Paris et al., 2009). Therefore, we can conclude that lack of requirement for a membrane potential is a conserved feature of all organisms that import tRNAs by a protein-import independent mechanism. The sole outlier being the case of *L. tropica* where we argue that a requirement for membrane potential may really reflect more on technical nuances of the experimental system rather than on actual realities of *in vivo* import requirements. In recent years, numerous protein factors have been implicated as additional requirements for import of tRNAs into mitochondria. In plants, for instance, the voltage-dependent anion channel (VDAC) in the outer membrane was shown to play a crucial role for import *in vitro* (Salinas et al., 2006). However, the homologous protein is dispensable for import in *T. brucei* (Pusnik et al., 2008).

To date, one of the most studied import systems is that of *Leishmania*, where a putative RNA import complex (RIC) in *L. tropica* has been identified (Mukherjee et al., 2007). This complex contains a number of proteins known to serve alternative functions in respiration, introducing the concept of a `moonlighting' function for these proteins. The core import complex contains six essential factors required for both *in vivo* and *in vitro* import: α subunit of the F_1-ATP synthase, subunit 6b and the Rieske protein of the respiratory complex III, two proteins with no similarities to any other proteins in the database and lastly subunit 6 of the respiratory complex IV (Mukherjee et al., 2007). As part of our studies we have also explored the possibility of the RIC as a conserved feature of import in *T. brucei*. Similar to the case of iscS, we knocked down expression of the Rieske protein, an essential subunit of the RIC. We again found that this protein plays no role in tRNA import distribution in these cells *in vivo* or *in vitro* (Lukes and Alfonzo, unpublished data), bringing into question the universal conservation of this complex. Taken together, recent data illuminated various facets of tRNA import in different organisms, but much more information is needed to define the universal rules, and truly answer the question of how many different mechanisms exist for tRNA import into mitochondria.

Evolutionary basis for the appearance of tRNA import mechanisms

Mammalian and yeast mitochondria encode many tRNA species; according to our current understanding of decoding by the wobble rules, their nature and number should suffice for normal protein synthesis (Agris, 2004; Agris et al., 2007; Gustilo et al., 2008). Furthermore, recent studies solved the last question of mitochondrial aminoacyl-tRNA synthesis by demonstrating that mammalian and yeast mitochondria use the indirect amino acid transformation route for Gln-tRNA formation (T. Suzuki, personal communication). This then raises the question of why tRNA import – and sometimes aminoacyl-tRNA synthetase import – exists, how general it is, and what purpose it serves.

Considering the facts discussed above, it is reasonable to believe that protein import into mitochondria is evolutionarily older than tRNA import. Once systems to transport proteins developed, then these systems were exploited to also import tRNAs. A case in point is yeast

tRNA^{Lys} whose import is coupled to that of the mitochondrial lysyl-tRNA synthetase (LysRS). Alternatively, pre-existing proteins can be recruited to transport RNAs in a manner that is independent of the protein import pathway. In this realm, the system may have to modify pre-existing proteins with the ability to make channels or gates in the membrane, such as the case of the plant VDAC (Salinas et al., 2006) to adapt them to tRNA transport. The system may have also recruited other factors that utilize ATP hydrolysis as the energy fuel for import, while bypassing any requirements for other membrane bioenergetics that play key roles in protein transport. Uncoupling mitochondrial translation from protein import may in the long term effectively add one more layer of regulation of mitochondrial functions at the level of tRNA availability. Supporting this view is a recent report establishing a correlation between tRNA partitioning and codon usage in *Chlamydomonas* (Vinogradova et al., 2009). Clearly, recruitment of additional factors for import does not necessarily imply that the components of modern import machines need resemble pre-existing proteins in sequence, such as the case of the *Leishmania* RIC, many years of evolution could have led to significant sequence divergence.

Despite an ever-increasing number of tRNA import examples, we posit that the most conserved system for tRNA import is that of tRNA^{Gln}. We have suggested that this tRNA (specifically tRNA$^{Gln}_{CUG}$) is universally imported into mitochondria, especially since a tRNA$^{Gln}_{CUG}$ gene is absent from any sequenced mitochondrial genome. Furthermore, in terms of bioenergetics, what is true of tRNA^{Gln} import in yeast is also true for import of this tRNA species into *Leishmania*, *T. brucei*, rat and human mitochondria, where import requires ATP but not a membrane potential (Alfonzo et al., 1999; Nabholz et al., 1999; Rubio et al., 2000, 2008; Rinehart et al., 2005).

A final question concerns the role of the imported cytoplasmic tRNAs in the mitochondrion. An unusual feature of aminoacyl-tRNA synthesis is the fact that Gln-tRNA formation is least conserved and is generated by kingdom-specific pathways that guarantee proper glutamine incorporation during protein synthesis (Tumbula et al., 2000). What roles are then played by the imported cytoplasmic tRNA$^{Gln}_{CUG}$ (cyto-tRNA$^{Gln}_{CUG}$) and tRNA$^{Gln}_{UUG}$? The yeast and mammalian mitochondrial genomes already encode a mitochondrial tRNA^{Gln} with anticodon UUG (mt-tRNA$^{Gln}_{UUG}$). Two mitochondrial tRNA species, t-RNA$^{Gln}_{UUG}$ and tRNA$^{Lys}_{UUU}$, were shown to contain 5-carboxymethylaminomethyl-2-thiouridine (mcm^{5}s^{2}U) in position 34, the first anticodon nucleotide (Nakai et al., 2004). Wobble base pairing would be restricted by mcm5s^{2}U at position 34; thus, mt-tRNA$^{Gln}_{UUG}$ would only decode CAA codons (Okumura et al., 1995). This should necessitate the import of a cyto-tRNA$^{Gln}_{CUG}$ isoacceptor to decode the CAG codons in mitochondrial mRNA. Similarly, mcm^{5}s^{2}U34 in mito-tRNA$^{Lys}_{UUU}$ would also explain the need for the imported cyto-tRNA$^{Lys}_{CUU}$ (Kolesnikova et al., 2000). The function of the imported cyto-tRNA$^{Gln}_{UUG}$ is less clear. The *S. cerevisiae* and mammalian nuclear genomes encode multiple different tRNA$^{Gln}_{UUG}$ species, and the redundancy in this tRNA population may have some important and as of yet unknown function common to both the cytoplasm and the mitochondria. Clearly, the mammalian or yeast cytoplasm contains a much more diverse tRNA population than do the corresponding mitochondria. However, the biosynthesis of certain – possibly non-housekeeping – proteins under changing environmental conditions may demand a more varied codon complement than that found in the mitochondrially encoded tRNAs. In such a

case, tRNA import from the cytoplasm may be the solution for increasing decoding diversity. As the cytoplasmic tRNAs are eukaryotic and therefore not ideal substrates for the mitochondrial aminoacyl-tRNA synthetases, import of the cognate cytoplasmic enzyme may also be beneficial. Thus, a driving force for the evolution of tRNA import may have been the requirement of expanding decoding ability by increasing the diversity of the pool of decoding tRNAs far and beyond those species already present in mitochondria. This in turn could support synthesis of a more varied protein population by altering codon recognition under certain conditions, e.g., environmental stress (Kamenski et al., 2007).

Future prospects

Despite differences in the number and types of factors shown to be associated with tRNA import in various organisms, tRNAs can mechanistically be imported in two different ways: either via the protein import pathway (e.g., co-import with a cytoplasmic aminoacyl-tRNA synthetase) (Tarassov et al., 1995b) or via a pathway independent from protein import that does not require cytosolic factors (Rubio et al., 2000). Future studies may reveal additional pathways. However, the disparate nature of the import mechanisms suggests that tRNA import evolved independently in different systems (Schneider and Marechal-Drouard, 2000). Differences among various systems may also suggest import as a dynamic process that has adapted to intracellular environmental changes provided by a particular organism.

Our discovery that $tRNA^{Gln}$ import may be present in all mitochondria-containing eukaryotes raises the possibility that a thorough understanding of this import system will unveil some truly evolutionarily conserved mechanistic features. Clearly, this would not rule out organism-specific nuances of different import pathways; it may just be a reflection of the long evolutionary road of different eukaryotes from the original appearance of multiple import systems. The challenge to answer many of these standing questions will still require rigorous genetic, biochemical, and structural approaches.

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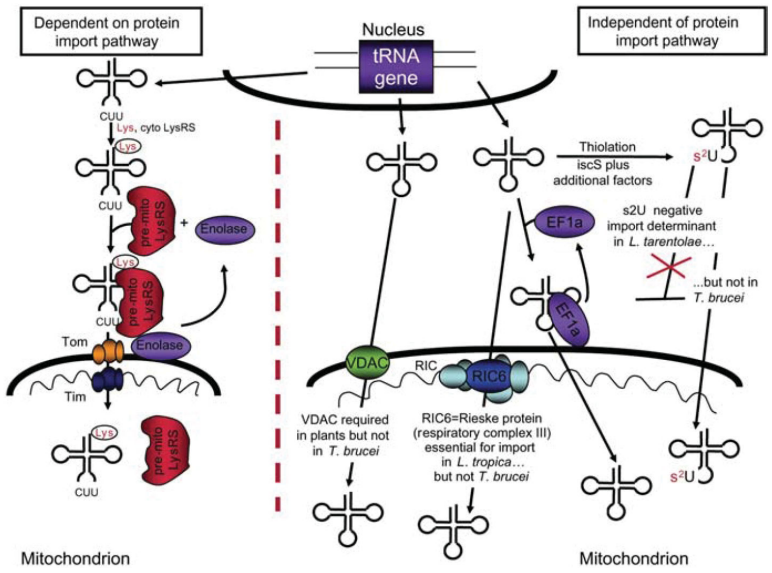

Figure 1.

Two major pathways for tRNA import into mitochondria.

One pathway utilizes the protein import machinery including the translocases of the outer membrane (TOMs) and inner membrane (TIMs). This pathway has thus far only been described in the import of $tRNA^{Lys}_{CUU}$ in yeast and is depicted in the left side of the Figure. In this pathway, the imported tRNA is first aminoacylated by its cognate cytoplasmic synthetase (cyto-LysRS), bound by the pre-mitochondrial lysyl tRNA synthetase (pre-mitoLysRS) and with the aid of enolase is delivered to the mitochondrial surface. The complex is then translocated via the protein import machinery, while enolase remains in the cytoplasm where it can start a new import cycle. The second and perhaps phylogenetically the most widely distributed pathway does not require cytoplasmic factors and it is not directly dependent on the protein import machinery. However, various factors may play a role in various organisms including the VDAC (plants), tRNA thiolation (*Leishmania* but not in *T. brucei*), etc. In the specific case of *T. brucei*, the translation elongation factor eEF1a plays a role in delivering the tRNA to the mitochondrial import machinery. This role is similar to that of enolase in yeast, but has yet to be demonstrated in other organisms. In *L. tropica*, the RNA import complex (RIC) was recently described and is formed by six essential subunits, all are identical to subunits of the respiratory complex. In *T. brucei*, however, at least one of the essential RIC subunits is not involved in tRNA import; therefore, the pathway may differ between the two organisms despite being evolutionarily closely related.