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Review

The Complexity of Mitochondrial tRNA Import

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ABSTRACT

Import of nucleus-encoded, cytoplasmic tRNAs into mitochondria to compensate evolutionary loss of the corresponding mitochondrial genes has been documented in a large number of species. Although the phenomenon has been known for more than 25 years, it was only recently that the mechanism of tRNA import started receiving the sustained attention of workers investigating yeast, protozoal and higher plant systems. The purpose of this review is to summarize recent developments that shed new light on the selectivity of the process, the identity of the import apparatus and the nature of the bioenergetic transactions leading to tRNA translocation, and to build a working model of the import complex suggested by these observations.

Mitochondria are genetic parasites believed to have evolved through the establishment of single or multiple endosymbioses between amitochondriate eukaryotes and ancestral α-proteobacteria.[1] Accumulating data point to a monophyletic origin of mitochondria from the order Rickettsiales.[2] Only about a dozen protein-coding genes, coding for highly hydrophobic components of respiratory complexes, have been retained in the mitochondria of most organisms, though the actual number varies between species, and may include ribosomal protein genes, genes involved in cytochrome c biogenesis and unidentified open reading frames; the remainder of the thousand or so mitochondrial proteins are imported from the cytoplasm. While the mitochondria from all organisms retain the genes for mitochondrial rRNAs, the loss of one or more mitochondrial tRNA genes has been documented in a significant number of species.[3] In such cases the corresponding nucleus-encoded tRNAs are imported in order to sustain mitochondrial translation (for recent reviews of import, see refs. 4 and 5).

The size of mitochondrial genome varies considerably among species; this is probably due to the loss or transfer of many endosymbiont genes to the host genome, duplication with or without concatenation, etc. Presumably, these events occurred independently in different organisms after the initial endosymbiosis. Kinetoplastid protozoa such as *Trypanosoma* and *Leishmania*, have single mitochondria (the kinetoplast-mitochondrion) with two types of DNA, maxicircles and minicircles. Maxicircle- encoded mRNAs are edited extensively by addition or deletion of uridine residues with the aid of maxi- and minicircle-encoded guide RNAs inside the kinetoplast matrix by one or more RNA editing complexes to restore the proper reading frame.[6] The other uniqueness of kinetoplast-mitochondria is the presence of the smallest rRNA known, which is encoded by maxicircle DNA. Interestingly, no tRNA encoding genes has been identified on either maxi- or minicircle DNA.

The present review focuses on recent developments on three aspects of mitochondrial tRNA import: import signals, import factors and bioenergetics.

RANGE AND SCOPE

By Northern hybridization analysis,[7,8] by using mutation-tagged tRNA substrates expressed in vivo[9] and by direct transfection of radiolabeled tRNAs,[10] import of cytosolic tRNAs into kinetoplast-mitochondria of *Leishmania* and *Trypanosoma* has been documented. Mitochondrial tRNA import has also been observed in *Saccharomyces cerevisiae*,[11] in *Tetrahymena*,[12] in different plants,[13-17] and in marsupials.[18] During the last few years, in vitro assay systems have been developed from yeast,[19] *Leishmania*,[20-22] *Trypanosoma*[23,24] and potato.[25] This has made it possible to obtain initial ideas regarding the mechanism of import, enabling the comparison of the kinetoplastid system with those from other species.

tRNALys(CUU) is the only tRNA imported in yeast mitochondria,[11] though a functional RNALys gene is encoded by the mitochondrial genome. Therefore the physiological significance of import in yeast mitochondria is questionable. Recently, the role of tRNA in nuclear export of a translation initiator factor has been documented.[26] It is possible that in yeast cytosolic tRNALys is similarly needed for coimport of some associated protein factor(s).

The situation in higher plants is much more complicated. The number and identities of tRNAs imported varies widely, and are not always consistent with the assigned phylogenetic positions of the plant species;[13-17] possibly, evolutionary tRNA gene loss and invention of import of the lost tRNAs have occurred repeatedly in individual clades.

Table 1 **Import Signals in tRNAs**

Organism	tRNA species	Location of signal	Evidence	Reference
Trypanosoma brucei	tRNAMet-e(UAC)	T arm	Point mutation	40
	tRNALeu(CAA)	5′ flank	Deletion, point mutation	29
Leishmania spp.	tRNATyr(GUA)	D arm	Fragmentation, Point mutation	35
	tRNAIle(UAU)	D arm	Domain swap	34
		V-T arm	Fragmentation	32
	tRNATrp(CCA)	Anticodon	C→U editing	30
	tRNAThr(AGU)	V-loop	Base insertion	39
	tRNAGlu(UUC)	Anticodon	Base modification	38
Saccharomyces	tRNALys(CUU)	Anticodon, Acceptor stem	Point mutation	37
Tetrahymena	tRNAGln(UUG)	Anticodon	Point mutation	31
Arabidopsis	tRNAVal(AAC)	Anticodon + D stem	Point mutation	36

In *Trypanosoma* and *Leishmania*, nucleus- encoded tRNAs are distributed between the cytosol and mitochondrion. So far, only tRNAGln(CUG) and initiator tRNAMet-i are known to be predominantly or exclusively cytosolic in *Leishmania* and *Trypanosoma*, respectively.[27,28] There is some controversy regarding the nature of the import substrate in *Trypanosoma*, in which tRNAs are transcribed as polycistronic units with 5' and 3' extensions. Such precursor tRNAs are imported more efficiently than the mature tRNAs in vitro.[24] Recently, a consensus import signal sequence was shown to be present in the 5' extension region of the precursor tRNA and not in the mature tRNA itself.[29] In contrast, Tan et al. report data with transgenic *T. brucei* cell lines suggesting that mature tRNALeu is the import substrate in vivo.[28] Similarly, tRNA end-processing was shown to precede mitochondrial import in *Leishmania*.[30] These apparently contradictory observations in two kinetoplastid protozoa have yet to be reconciled.

SIGNALS AND SELECTIVITY

Two ideas have been put forward to explain the selectivity of tRNA import: [31] the presence of *determinants* on importable tRNAs, which interact positively with tRNA binding import factors, or of *anti-determinants*, which act to retain nonimportable tRNAs in the cytoplasm. In the former case, the mitochondrion positively selects for importable tRNAs in the cytoplasmic pool; in the latter, there is negative selection against nonimportable tRNAs. The presence of a strong positive selection mechanism on *Leishmania* mitochondria was demonstrated by the rapid in vitro selection (SELEX) of import aptamers from a random sequence pool of high complexity.[32] Sequences homologous to various domains of different tRNAs were recovered by this method, suggesting the presence of multiple determinants, a prediction that is being borne out by more recent experiments (see below).

Mutagenesis studies have revealed the presence of positive determinants on importable tRNAs. Irrespective of base sequence, tRNAs have a largely conserved L-shaped structure with a flexible hinge, held together by secondary and tertiary interactions between the bases.[33] The import machinery may recognize either the primary sequence, or the secondary and/or tertiary structure, or both. A variety of in vivo and in vitro approaches, including site-directed mutagenesis, tRNA fragmentation, and domain swapping have been used to determine the nature of the import signal in different systems (Table 1). From the currently available data, it is apparent that there is no unique, universal signature. Different domains such as the D arm,[34-36] anticodon,[30,31,36-38] acceptor stem,[37] V loop,[39] T stem[10,32,40] and even the 5'-flanking region[29] may contain import determinant bases. In some cases, e.g., *Leishmania* tRNAIle(UAU), there is evidence for the presence of more than one independent signal in the same molecule, one in the D domain and another in the V-T region.[10,32,34] Moreover, in *Arabidopsis* tRNAVal(AAC), both the anticodon and D arm sequences are required, indicating a bipartite signal.[36]

There is scant information on the role of tRNA secondary and/or tertiary structure in import. A nicked tRNA is imported into yeast mitochondria, suggesting that it is imported as a folded molecule.[37] On the other hand, small tRNA-derived hairpins containing an import signal are selectively taken up into *Leishmania tropica* mitochondria both in vitro and in transfected cells,[32,41] indicating that the entire tRNA structure is not necessary in this system. Mutational analysis demonstrated the importance of the primary sequence as well as the stability of the hairpin stem for import.[41] Uptake of small RNAs into *L. tarentolae* mitochondria has also been observed, but in this case there was no apparent correlation between importability and either the presence of an import signal or the secondary structure.[22] Nonspecific, structure-independent uptake in the latter case could be due to the use of very high RNA concentrations (up to 10 μM), as opposed to nanomolar concentrations in the former.

The possible involvement of tRNA nucleotide modifications as anti-determinants is currently being investigated. A cytosol-specific modification (thiolation of the wobble base) of tRNAGlu(UUC) and tRNAGln(UUG) was observed; the modified, native cytosolic form of the latter is imported less efficiently in vitro than the corresponding unmodified transcript, suggesting a role of base modification in retention of part of the tRNA in the cytosol,[38] but whether the thiolation per se is involved is unclear because corresponding data for the mitochondrial form of the same tRNA are not available. Similarly, native tRNAMet-e isolated from *T. brucei* mitochondria is imported in vitro, whereas the cytosolic form of tRNAMet-e is not,[40] but in this case the nature of the cytosol-retaining modification is unknown. The imported tRNAMet-e undergoes N-formylation within the mitochondria prior to its participation in translation initiation.[42] In *L. tarentolae*, cytosolic tRNATrp undergoes C→U editing

at the first position of the anticodon; the edited tRNA is imported less efficiently in vitro than the nonedited species.[30] None of the in vitro import systems used for these studies requires a cytosolic factor for the observed selectivity; thus it is possible that base modification prevents interaction of the tRNA with a mitochondrial receptor, i.e., inhibits a positive determinant, rather than promoting interaction with a cytoplasmically retained protein. It is important to distinguish between cause and effect by ascertaining the site of the tRNA modification, i.e., whether the nucleus, cytosol or mitochondrion.

Fractionation of mitochondria into sub-compartments has allowed comparisons to be made between tRNA translocation through the outer and inner membranes. In potato mitochondria, transfer through the outer membrane is less efficient, but more specific, than through the inner membrane.[25] In sharp contrast, the inner membrane of *Leishmania* mitochondria is a more stringent 'filter' than the outer membrane, and tRNAs which translocate or leak through the outer membrane, such as the nonimported tRNAGln(CUG),[43] or tRNAIle(UAU)[32] are retained at the inner membrane. Moreover, outer membrane transfer is much less sensitive to respiratory inhibitors and uncouplers than inner membrane transfer;[43] as shown by the differential behavior of mutant substrates, the RNA structural requirements for the two processes are distinct;[41] and the inner membrane, unlike the outer membrane, is unable to positively select import aptamers.[32] These results indicate the presence of distinct tRNA receptors at the two membranes.

The regulatory role of the inner membrane of *Leishmania* mitochondria was further highlighted by the observation that aptamers and tRNAs interact cooperatively or antagonistically for transfer into the mitochondrial matrix.[10,32] Two major types of interacting RNAs have been identified: Type I RNAs (such as tRNATyr) and Type II RNAs (such as tRNAIle), that have high and low intrinsic efficiencies of inner membrane transfer, respectively. Moreover, Type I RNAs stimulate the matrix entry of Type II RNAs, whereas Type II RNAs inhibit transfer of Type I RNAs. Based on these observations a "ping-pong" mechanism of tRNA import has been proposed,[10,43] highlighting the conformational flexibility of tRNA receptors in the import machinery that allows allosteric transitions leading ultimately to the generation of a balanced pool of tRNAs in the mitochondrial matrix. Intriguingly, the import signal in the 5'-flanking region of a *Trypanosoma* tRNA precursor has been shown to regulate the import of other endogenous tRNAs in *trans*,[29] recalling the Type I activity of the homologous D arm motif of *Leishmania* tRNATyr. It will be interesting to determine if a similar type of regulatory mechanism occurs at the outer membrane of plant mitochondria.

FACTORS AND FACTORIES

Till now, most studies have focused on the tRNA substrate, and information on the components of the import apparatus remains sketchy, partly because in vitro systems have been only recently developed. There is the requirement for membrane-bound components in all systems, as evidenced by the susceptibility of import to protease treatment of mitochondria.[19-25] The yeast in vitro system was used to show the requirement of cytosolic protein factors for import of yeast tRNALys(CUU).[19] One of these factors has been identified as the precursor form of the nucleus-encoded mitochondrial lysyl tRNA synthetase (preMSK); another is the cytosolic form of the lysyl tRNA synthetase, which charges the tRNA, aminoacylation being a prerequisite for import of the wild-type sequence,[44] although some sequence variants of the tRNA, in spite of being aminoacylated poorly, are nonetheless imported efficiently.[37] These two proteins are necessary but not sufficient; additional factors present in cytosolic extracts have yet to be identified. Interestingly, the yeast preMSK, together with a crude yeast extract, directs the import of the same tRNA into human mitochondria.[45,46] In plants, aminoacylation is not required in vitro,[25] but may play a role in vivo, since alteration of aminoacylation specificity—from valine to methionine—by changing the anticodon abolishes import.[36] In *Trypanosoma*, a nonaminoacylatable mutant tRNA is imported in vivo,[9] while in *Leishmania*, aminoacylation does not influence import in vitro (Bhattacharyya S, Adhya S, unpublished data).

Using mitochondria from the appropriate mutant yeast strains, the apparent involvement of specific components of the protein import machinery has been observed. These include Tom 20, an outer membrane preprotein receptor, and Tim 44, an inner membrane component.[47] The bioenergetic requirements of tRNA import (a membrane potential and internal and external ATP) are similar to those of mitochondrial protein import in yeast.[48] Moreover, agents that block protein import channels of human mitochondria also inhibit preMSK mediated import of tRNALys(CUU).[46] Therefore it has been proposed that tRNALys is coimported with preMSK through protein import channels. An unresolved problem of this mechanism is that proteins have to be unfolded prior to translocation; how the unfolded preMSK remains associated with the tRNA during coimport, or whether it merely delivers the tRNA to the mitochondrial surface before dissociating from it, remains an open question. One would also have to assume that the preMSK:tRNA complex is somehow unique among all the complexes of cytosolic tRNAs with their cognate aminoacyl tRNA synthetases since, whereas synthetases for all tRNAs have to be imported, only a single tRNA is translocated.

In contrast to the situation in yeast, tRNA import in kinetoplastid protozoa does not require soluble factors. Import into *Trypanosoma* mitochondria is not inhibited by substrates of protein import.[23] Moreover, inhibitor studies suggest that whereas protein import requires the electrical ($\Delta\psi$) but not the chemical (ΔpH) component of the proton electrochemical gradient,[48] tRNA import in *Leishmania tropica* mitochondria requires both components.[43] These observations are in keeping with the direct import model,[49] according to which tRNA is recognized by specific receptors associated with channels distinct from that for protein import. The only known import factor associated with the outer membrane of *Leishmania* mitochondria is a 15- kDa protein with RNA binding properties,[21] but the molecular identity of this protein has not been established.

Recently, there has been significant progress towards a molecular definition of the *Leishmania* inner membrane import machinery by the isolation of a large multi-subunit complex (the RNA import Complex, or RIC) that reconstitutes import activity in liposomes.[50] The complex carries out ATP-dependent, allosterically regulated transfer of tRNA across membranes. By immunochemical and photocrosslinking experiments, two tRNA binding components were identified: a 45-kDa receptor for tRNATyr (GUA); a Type I RNA, and a 21-kDa receptor for tRNAIle(UAU), a Type II RNA.[50] More experiments are needed to molecularly characterize these proteins.

PROTONS AND PUMPS

tRNA molecules are polyanionic at physiological pH, while the matrix side of the inner membrane of respiring mitochondria has a negative charge with respect to the intermembrane space due to the selective extrusion of protons, generating the potential problem of

electrostatic repulsion. By analogy with the inner membrane transport of anions such as inorganic phosphate, dicarboxylic acids and ADP/ATP,[51] this problem could be resolved by either the coupling of tRNA import to export of another anion, or by coimport with a counterbalancing cation.

In all systems examined to date, tRNA import uses ATP as the sole energy source. In the *Leishmania*[43] and the *Trypanosoma*[24] systems it has been shown that ATP hydrolysis is necessary both within the matrix and outside the mitochondrion. The role of ATP at the outer membrane is not clear; one possibility is that ATP is hydrolyzed by a helicase-like molecule to partially unfold the tRNA for translocation through the outer membrane channel.

The relationship between tRNA import and the inner membrane potential has been investigated by the use of respiratory inhibitors and uncouplers. In yeast,[47] and potato,[25] import is inhibited by protonophores that dissipate the trans-membrane proton gradient. In *Leishmania* and *Trypanosoma*, contradictory results have been reported. While the *L. tropica* system[41,43,50] is sensitive, the *L.tarentolae* system[22] is not. Similarly, the import of a tandem tRNASer- tRNALeu transcript into *T. brucei* mitochondria is disrupted by uncouplers,[24] whereas that of a synthetic tRNATyr transcript is not.[23] These apparent contradictions could be due to differences in strains/species or protocols, or to the fact that the intramitochondrial location of the internalized tRNA has not been examined in most cases, and it is possible that the tRNA passes through the outer membrane but cannot enter the matrix in the absence of a membrane potential, as has been observed in *L. tropica*.[43] In vitro import is also universally sensitive to oligomycin, an inhibitor of proton flow through the F1-F0 ATPase complex.[52] Recently, it was shown that low external pH can replace ATP as an energy source for the inner membrane import complex (RIC),[53] further highlighting the crucial role of protons in tRNA translocation.

These results have generated the idea that the oligomycin-sensitive F1-F0 ATPase generates a proton gradient through ATP hydrolysis, which subsequently drives import.[43] However, it has been recently observed that RIC itself has a tRNA-dependent ATPase activity and can generate a membrane potential.[53] This indicates that the import complex is a proton pump coupled to tRNA translocation, similar to the F- and V-type ATPases, which are proton pumps on the inner mitochondrial and vacuolar membranes respectively.[54] Moreover, since oligomycin inhibits generation of the membrane potential, but not the initial ATP hydrolysis or the subsequent proton-dependent import,[53] the bioenergetic transactions could be defined as consisting of a number of discrete steps (see below).

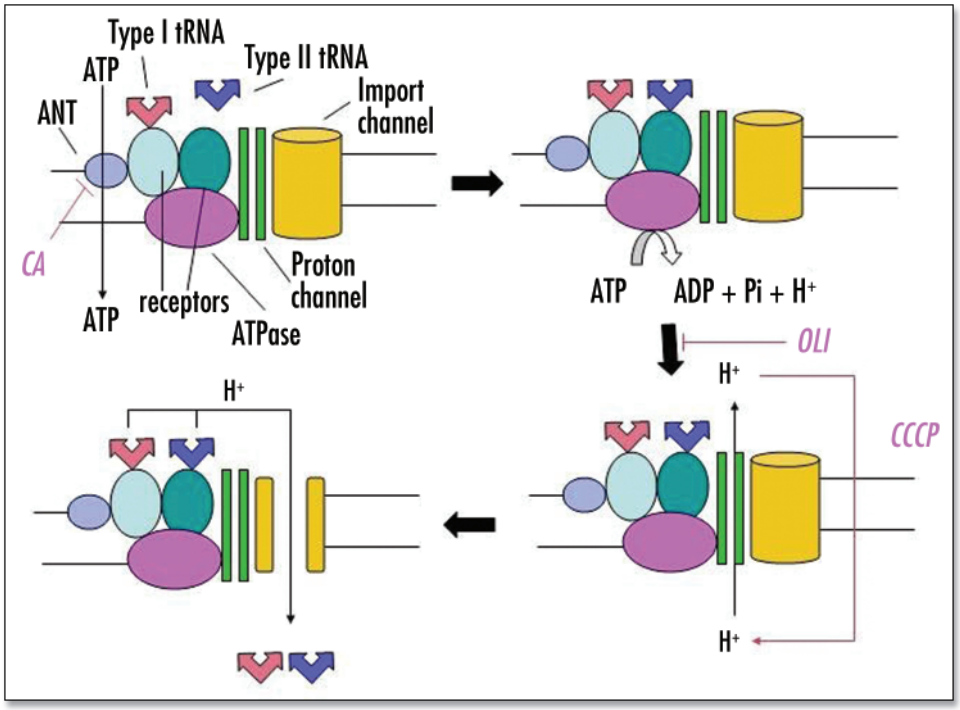

Figure 1. Model for tRNA transport through the inner membrane of *Leishmania* mitochondria. For details, see text. *CA*, carboxyatractyloside, an inhibitor of the adenine nucleotide translocator (*ANT*). *OLI*, oligomycin. *CCCP*, carbonylcyanide *m*-chlorophenylhydrazone (a protonophore uncoupler).

THE MODEL

The information so far available in the *Leishmania* system can be accommodated in a working model of the inner membrane transport complex (Fig. 1). A number of distinct entities, each catalyzing or participating in a discrete step, are envisaged: more than one tRNA receptor, a carboxyatractyloside-sensitive ATP transporter (similar or identical to the Adenine Nucleotide Translocator, or ANT), an ATPase, and distinct proton and RNA channels. The initial binding of tRNA to its appropriate receptor triggers a series of conformational changes in the complex. Allosteric modulation of neighboring receptors alters their binding affinities for other tRNAs, allowing loading or unloading of other import substrates. tRNA binding also results in the activation of an ATPase that hydrolyzes ATP in the inner compartment, i.e., matrix. ATP hydrolysis generates protons that are pumped through discrete oligomycin-sensitive proton channels to the exterior. Finally, RNA channels are opened for the passage of the tRNA.

FOR THE FUTURE

Biochemical and genetic methods have begun to reveal the components of the import apparatus in different systems, and such work will undoubtedly carry on well into the future. It will be interesting to determine if import complexes similar to that found in *Leishmania* are also present in higher plants and other species, if such complexes are related to the electron transport and oxidative phosphorylation complexes on the inner membrane, and if there is general conservation of import factors across the phylogenetic scale. The precise role of protons in tRNA import will have to be defined. The generality of allosteric regulation via inter-tRNA interactions should be investigated. The induction of in vitro tRNA import into human

mitochondria[45] is of great potential interest, offering the possibility of therapeutic intervention in a number of genetic diseases caused by mutations in mitochondrial tRNA genes.[55]

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