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Docteur de l'Université Louis Pasteur, Strasbourg I
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Discipline: Aspects moléculaires et cellulaires de la Biologie

par

Petr KAMENSKIY

Etude du rôle du précurseur de la lysyl-ARNt synthétase mitochondriale dans l'adressage d'ARNt lysine cytoplasmique dans les mitochondries de levure *S. cerevisiae*

Soutenue le 26 Janvier 2007 à l'Université de Moscou devant la commission d'examen :

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CONTENT

ABBREVIATIONS

aaRS – aminoacyl-tRNA synthetase

AspRS – aspartyl-tRNA synthetase

Cox2p - Mitochondrial cytochrome *c* oxidase, subunit 2

Eno2p - enolase-2

IDP - Import Directing Proteins

Krs1p - yeast cytoplasmic lysyl-tRNA synthetase

LysRS – lysyl-tRNA synthetase

MIM – mitochondrial inner membrane

MOM – mitochondrial outer membrane

mtDNA –mitochondrial DNA

MTS - mitochondrial targeting signal

PCR- polymerase chain reaction

preMsk1p – precursor of the mitochondrial lysyl-tRNA synthetase

PYK - pyruvate kinase

RT-PCR – Reverse-Transcription PCR

SAM – sorting and assembly machinery of the mitochondrial outer membrane

TIM – translocase of the mitochondrial inner membrane

TOM – translocase of the mitochondrial outer membrane

tRNA – transfert RNA

tRK1 – $tRNA^{Lys}CUU$, tRNA imported into yeast mitochondria

tRK2 - $tRNA^{Lys}_{mnm5S2UUU}$, non-imported cytoplasmic tRNA

tRK3 - $tRNA^{Lys}_{cmnm5S2UUU}$, mtDNA encoded mitochondrial tRNA

Var1p - yeast mitochondrial ribosomal protein

OBJECTIVES

Mitochondria are known to contain their own genome and to be able to transcribe and to translate their genetic material. However, the vast majority of the macromolecules that functions in the mitochondria is imported there from the cytoplasm. They include, mainly, proteins and RNAs. Mitochondrial genomes from different organisms code for 7-12 proteins while the total amount of mitochondrial proteins varies from 800 to 1500 (Da Cruz et al., 2005). Thus, >95% of all mitochondrial proteins are nuclear-encoded and are imported from the cytoplasm. Protein import into mitochondria is a well-understood process and occurs by more or less universal mechanisms in all eukaryotic cells. In contrast, little is known about RNA import into mitochondria. The commonly imported RNA species are transfer RNAs. tRNA import is observed in protozoan, fungi, plant and marsupial. Besides tRNAs, 5S rRNA and RNA components of RNases P and MRP are also imported in mammalian cells. The pools of imported tRNAs are very different for different organisms: from three in the case of *Saccharomyces cerevisiae* or *Marchantia polyporpha* to the totality of mitochondrial tRNA species *Trypanosomatidae*. The mechanisms of tRNA import into mitochondria are poorly understood. It is clear, however, that they are not universal among all the species.

Our team (in Moscow University and in Strasbourg) worked several years on tRNA import into yeast mitochondria. There are two lysine tRNA species encoded in the yeast nuclear genome, and one of them (tRK1) is partially addressed into mitochondria while the second (tRK2) is located exclusively in the cytosol. The yeast mitochondrial genome codes for the third one (tRK3) involved in mitochondrial translation. The fact that tRK3 might be able to decode both AAA and AAG codons, rises **the question about the function of tRK1 in the organelle.** The previous studies in our team have shown that several nucleotides of tRK1 called “import determinants” provide the specificity of import. They include the first base pair of the acceptor stem (G1-C72), the discriminator base (U73) and the first nucleotide of the anticodon (C34). It was also demonstrated that, to be imported, tRK1 must be folded into a correct ternary structure. tRK1 import was shown to be ATP- and mitochondrial membrane potential-dependent. Three soluble cytosolic proteins were also found to participate in tRK1 mitochondrial targeting. The first is the cytosolic lysyl-tRNA-synthetase which aminoacylates tRK1. Aminoacylation is a prerequisite for tRK1 importation. The second one is the glycolytic enzyme enolase that serves as a transporter of aminoacylated tRK1 to the vicinity of mitochondria where it binds the third protein, the precursor of mitochondrial lysyl-tRNA-synthetase (preMsk1p). It is the typical mitochondrial precursor with N-terminal signal

presequence for mitochondrial targeting. PreMsk1p is translated by mitochondria-associated ribosomes, and is imported into mitochondria. There, in its mature form, this protein aminoacylates tRK3. Binding with preMsk1 is necessary for tRK1 import. It was hypothesized that **tRK1 is imported into mitochondria in preMsk1p-bound form. It was presumed that the complex tRK1•preMsk1p may differ from these known for tRNA•aminoacyl-tRNA synthetase**, since tRK1 was imported in its aminoacylated form.

This thesis work was focused on the role of preMsk1p in the process of tRNA import into yeast mitochondria. **The first aim of the study was to get informations about the structure of the complex tRK1•preMsk1p and to localise the regions of preMsk1p that are required for tRK1 import. The second aim of the study was to exploit this knowledge to develop a preMsk1 version where tRK3 aminoacylation and tRK1 import functions were separated. Such version would permit to study *in vivo* the function of tRK1.**

In the ***1st chapter*** of the thesis, the data concerning the various mechanisms directing macromolecules into mitochondria are compared. Taking into account that, at least in yeasts, the protein import apparatus is involved in RNA import as well, we analysed this pathway in details. Data concerning the different RNA import events are discussed thereafter, followed by recent data describing the possibility of DNA uptake by mitochondria. The chapter is followed by the ***publication 1*** that represents the bibliographic analysis published by Molecular Biology (Moscow). The ***2nd chapter*** is dedicated to show the main results obtained. Most of the results are presented in the form of publications (***publication 2*** already published in Genes & Development and ***publications 3 and 4*** are submitted for publication).

The ***3rd chapter*** of the thesis represents the description of the methods used. The methods are mostly presented in ***publications 5 and 6*** (at the end of the chapter), the methods not present in these two publications are described in the text.

CHAPTER 1. INTRODUCTION

1.1. PROTEIN IMPORT INTO MITOCHONDRIA

Protein import into mitochondria plays a critical role in the function of these organelles since the vast majority of the mitochondrial proteins are imported there from the cytoplasm. For example, mitochondrial DNA of the yeast *S .cerevisiae* codes for only 8 proteins (Foury et al., 1998) while the total amount of mitochondrial proteins is estimated as 750-800 (Sickmann et al., 2003). There is a multisubunit and still not fully studied system of protein transport into mitochondria (Koehler, 2004; Neupert, 1997). This system targets each mitochondrial protein, depending on its function, either to the mitochondrial matrix, or to the intermembrane space, or to one of the mitochondrial membranes. The basic components of this system are:

- cytosolic factors providing the unfolding of the imported proteins and their transport to the vicinity of mitochondria;

- translocases of the outer and inner membranes (consisting of the receptors and the channels);

- outer membrane complex which inserts the integral proteins into the outer membrane;

- mitochondrial peptidases that cleave the targeting sequences of the imported proteins;

- mitochondrial chaperones that fold the imported proteins in the mitochondrial matrix after their import;

- mitochondrial proteins that export several proteins from the mitochondrial matrix to the inner membrane.

We must also point out that the commonly used model organisms for studying protein import are *Saccharomyces cerevisiae* and *Neurospora crassa*. At the same time, homologues of all components of the protein import apparatus were found in nearly all eukaryotic organisms. This fact allowed to conclude that the mechanisms of this process are universal among all species.

The schematic representation of protein import into mitochondria is given on Fig.1. The components of protein import machinery are listed in Table 1.

Table 1. Translocation and assembly complexes of the mitochondrial outer and inner membranes.

Component	Essential for viability	Membrane association	General function	References
Translocase of the outer membrane (TOM)				
Tom70p	No	Integral	Receptor	(Neupert, 1997; Pfanner et al., 1997)
Tom40p	Yes	Integral	Import pore	(Neupert, 1997; Pfanner et al., 1997)
Tom22p	Yes	Integral	Receptor	(Neupert, 1997; Pfanner et al., 1997)
Tom20p	No	Integral	Receptor	(Neupert, 1997; Pfanner et al., 1997)
Tom7p	No	Integral	Translocon dissociation	(Honlinger et al., 1996)
Tom6p	No	Integral	Translocon assembly	(Honlinger et al., 1996)
Tom5p	No	Integral	Receptor	(Dietmeier et al., 1997)
Sorting and assembly machinery (SAM)				
Tob55p/ Sam50p	Yes	Integral	Assembly/channel	(Paschen et al., 2003); (Gentle et al., 2004; Kozjak et al., 2003)
Tom37p/ Mas37p	No	Integral	Assembly/receptor?	(Wiedemann et al., 2003)
TIM23 translocase of the inner membrane				
Tim50p	Yes	Integral	Receptor	(Geissler et al., 2002); (Mokranjac et al., 2003; Yamamoto et al., 2002)
Tim23p	Yes	Integral	Subunit of import channel	(Neupert, 1997; Pfanner et al., 1997)
Tim17p	Yes	Integral	Subunit of import channel	(Neupert, 1997; Pfanner et al., 1997)
Tim21p		Integral	Regulator of TIM23 functional states	(Chacinska et al., 2005; van der Laan et al., 2006).
Import motor/presequence translocase-associated motor (PAM)				
mtHsp70	Yes	Peripheral	Import motor	(Neupert, 1997; Pfanner et al., 1997)
Tim44p	Yes	Peripheral	Membrane anchor	(Neupert, 1997; Pfanner et al., 1997)
Mge1p	Yes	Soluble	Nucleotide exchange	(Neupert, 1997; Pfanner et al., 1997)
Pam18p	Yes	Integral	ATPase stimulation Co-chaperone	(D'Silva et al., 2003); (Mokranjac et al., 2003); (Truscott et al., 2003)
Pam16p		Soluble	Pam18p activity regulation	(Frazier et al., 2004)
Pam17p		Peripheral	Pam18p-Pam16p complex regulation	(van der Laan et al., 2005)
TIM22 translocase of the inner membrane				
Tim54p	Yes	Integral	Translocon assembly	(Kerscher et al., 1997)
Tim22p	Yes	Integral	Translocon pore	(Sirrenberg et al., 1996)

Tim18p	No	Integral	Translocon assembly	(Kerscher et al., 2000); (Koehler et al., 2000)
Tim12p	Yes	Peripheral	Chaperone	(Koehler et al., 1999; Sirrenberg et al., 1998)
Small TIM proteins of the intermembrane space				
Tim13p	No	Soluble	Chaperone	(Davis et al., 2000)
Tim10p	Yes	Soluble	Chaperone	(Koehler et al., 1999; Sirrenberg et al., 1998)
Tim9p	Yes	Soluble	Chaperone	(Adam et al., 1999; Koehler et al., 1998b)
Tim8p	No	Soluble	Chaperone	(Davis et al., 2000; Koehler et al., 1999)
Protein export components				
Oxalp	No	Integral	Export/assembly factor	(Bonnefoy et al., 1994; Hell et al., 1997; Hell et al., 1998)
Mbalp	No	Peripheral	Assembly factor	(Rep and Grivell, 1996)
Pntlp	No	Integral	Cox2p export	(Ludewig and Staben,
Cox18p	No	Integral	Cox2p export	(Souza et al., 2000)
Mss2p	No	Peripheral	Cox2p export	(Broadley et al., 2001)

1.1.1. THE SIGNAL SEQUENCES OF THE IMPORTED PROTEINS.

The signal sequence is defined as the part of the protein which is necessary and sufficient for its targeting into mitochondria. For matrix-targeted proteins, the signal sequence is located on the N-terminus and is called MTS (**M**atrix **T**argeting **S**ignal, (Roise and Schatz, 1988)). In most cases, MTSs are proteolytically cleaved in the matrix. The signal sequences of the proteins that are targeted to other mitochondrial subcompartments are organized in a different way and are located in the internal regions of the proteins.

Most often, the MTSs are located at the very N-terminus of the pre-proteins. Due to this, the MTS-containing proteins are typically imported in the N→C direction (meaning that N-proximal part of the preprotein enters the mitochondria first). But there is at least one exclusion: MTS were found at the C-terminus of the precursor of mitochondrial helicase Hmi1p (Lee et al., 1999). The mechanism of Hmi1p import is absolutely the same as that for any other pre-protein. The unusual position of MTS is explained by the exsistence of the functionally important blocks at the very N-terminus of this protein (Lee et al., 1999).

Typically, MTSs are 20-60 amino acids long. These amino acids, when interacting with the lipid bilayer, form the amphiphilic α-helix (von Heijne, 1986);(Roise et al., 1988). The specific primary structure motifs that are typical for MTSs are not found (Roise, 1997). However, it is known that the majority of the residues are hydrophobic or positively charged.

About 30% of all mitochondrially-targeted protein do not possess MTS (Schatz and Dobberstein, 1996). They are almost all outer membrane proteins, many are intermembrane space proteins and also inner membrane proteins. They all contain so-called internal targeting sequences.

The outer membrane proteins are subdivided into two groups – membrane-anchored and integral membrane proteins. The signal sequences of the representatives of these two groups differ one from another (Rapaport, 2003). In case of membrane-anchored proteins the signal sequence consists of the transmembrane segment itself and several amino acids flanking it from both sides (Shore et al., 1995). The primary structure of these part of the protein does not influence the targeting to the outer membrane. Speaking about the integral β-barrel proteins, their signal sequences have not yet been identified. It is known, however, that they consist of several motifs located at the different parts of the protein (Rapaport, 2003).

The proteins imported to the inner membrane or intermembrane space also contain the internal targeting sequences; however, in some of them, the MTSs are also presented (Brix et al., 1999). Little is known about the internal targeting sequences of these proteins. It has been shown that in some cases they are either hydrophobic transmembrane parts or positively charged loops (Davis et al., 1998).

1.1.2. PRE-TRANSLOCATIONAL UNFOLDING OF THE IMPORTED PROTEINS AND THEIR TRANSPORT TO THE MITOCHONDRIAL SURFACE

The mechanisms of the spontaneous protein unfolding in solution and pre-translocational unfolding are different (Matouschek et al., 2000). The latter is assisted by several cytosolic protein factors that include the chaperone proteins (Beddoe and Lithgow, 2002; Mihara and Omura, 1996). There are some data about the binding of the Hsp70 chaperone to the precursors of the mitochondrial proteins; it maintains their unfolded state. It was also shown that purified Hsp70 binds the N-terminal presequences of synthetic polypeptides, and this binding depends on the amphiphility of the signal helix (Komiya et al., 1996).

Maintenance of the unfolded state is especially important for the proteins inclined to spontaneous folding or aggregation. The other member of the chaperones family, namely Hsc70, is believed to be necessary for such proteins import. It was shown that in the sequences of the imported proteins there are several sites for Hsc70 co-translational binding; this binding prevents the protein aggregation (Terada et al., 1995; Terada et al., 1996).

The Msf1p protein participates in the transport of the precursor proteins to the mitochondrial surface (Mihara and Omura, 1996). It recognizes and binds the N-terminal presequences; on the other hand, it is also the unfolding factor for several proteins (Iwahashi et al., 1992).

The yeast homologue of the bacterial co-chaperone DnaJ, namely Ydj1p, is also involved in the process of protein import into mitochondria (Beddoe and Lithgow, 2002). It is believed to be the transporter of these proteins to the vicinity of mitochondria (Caplan et al., 1992).

The requirement of the pre-translocational unfolding is explained by the fact that most of the proteins are thought to be imported in a post-translational way being already folded. However, a number of proteins are imported co-translationally; in this case, they begin to be translocated before the end of the translation and the correct folding of the molecule. Such co-translational import was directly shown for the precursors of mitochondrial malatedihydrogenase (Funfschilling and Rospert, 1999) and fumarase (Karniely et al., 2006; Knox et al., 1998). It is also well known that in the cytoplasm of the yeast cells there are mitochondria-associated ribosomes that carry mRNAs of the mitochondrial precursor proteins and that are functionally active (Ades and Butow, 1980; Kellems et al., 1975). It was shown that the mRNA localization on the mitochondrial surface is required for the import of the translation product to mitochondria (Kaltimbacher et al., 2006). This finding is in a good agreement with the possibility of co-translational import. In some cases, 3'-UTR of mRNA is a prerequisite for its association with the mitochondria (Sylvestre et al., 2003). On the other hand, removal of the N-terminal presequence-coded mRNA part also results in weaker association of this mRNA with the outer membrane (Ahmed et al., 2006).

1.1.3. TRANSLOCASE OF THE OUTER MITOCHONDRIAL MEMBRANE (TOM)

The TOM-complex (Fig.2) takes part in the transport of all proteins imported to mitochondria through the outer membrane (Pfanner and Geissler, 2001). It consists of seven subunits. Three proteins – Tom20p, Tom22p and Tom70p – are receptors while other four – Tom40p, Tom5p, Tom6p and Tom7p – are involved in the channel function of the complex.

Tom20p recognizes and binds the N-terminal presequences. This protein consists of small transmembrane (residues 5-25) and large cytosolic domains; the latter specifically interacts with MTSs (Brix et al., 1999). Four α-helices of Tom20p (residues 60-145) form a stable structure with a hydrophobic groove. The amphiphilic signal presequence interacts with

this structure so that the hydrophobic surface is inside the groove while the hydrophilic surface faces the cytosol (Abe et al., 2000).

Tom70p interacts with the targeted proteins that do not posses N-terminal presequences (Brix et al., 2000); these interactions are hydrophobic (Brix et al., 1999). Like Tom20p, Tom70p consists of two domains – anchored in the membrane and exposed to the cytosol. During the translocation, several molecules of Tom70p bind one molecule of the targeted protein, and then the latter is bound to Tom22p (Wiedemann et al., 2001).

Tom22p is a multifunctional subunit of TOM translocon and plays an important role in its structural organization (van Wilpe et al., 1999). This protein contains small intermembrane module and large cytosolic domain that are linked with the short transmembrane segment. Tom22p is a functional mediator between the receptors and the channel of TOM-complex (Ryan et al., 1999). Also, the transmembrane segment of this protein participates in the interactions between Tom40p individual molecules, moreover, it regulates the channel activity of the TOM translocon (van Wilpe et al., 1999).

Tom40p is a protein that forms the cation-selective channel in the mitochondrial outer membrane for protein importation (Hill et al., 1998). It forms the typical β-barrel structure with 8 transmembrane domains. One functional channel is formed by two Tom40p molecules; one TOM translocon contains two channels formed by Tom40p (Model et al., 2002). Using negative contrasting electron microscopy it was shown that the diameter of Tom40p-formed channel is about 20Å (Hill et al., 1998). This diameter is sufficient to allow the α-helix and even compactly folded loop to pass through the channel (Model et al., 2002). Tom40p is not a passive channel; it plays an active role in the translocation of the imported proteins. It contains the signal sequences binding sites and assists the directed movement of the proteins through the membrane (Rapaport et al., 1997). Moreover, it can distinguish between the imported proteins with different types of signal sequences and, thus, assist in the protein sorting to the mitochondrial subcompartments (Sherman et al., 2006).

Besides Tom40p, three other proteins are the parts of the channel: Tom5p, Tom6p and Tom7p. All these proteins are anchored in the membrane and exposed to the intermembrane space; Tom5p also contains a cytoplasmic domain (Dietmeier et al., 1997). Tom5p is a functional linker between the TOM translocon and small Tim-proteins of the intermembrane space (see below) (Kurz et al., 1999). It was also shown that in *N.crassa* (but not in yeast) Tom5p plays a critical role in TOM complex structural organization (Sherman et al., 2005). Tom6p mediates the interaction between Tom22p and Tom40 (Dekker et al., 1998) while Tom7p promotes the dissociation of the imported proteins from TOM-complex in the

intermembrane space (Honlinger et al., 1996). Both these two proteins can take part in the targeting of the imported polypeptides to the different mitochondrial subcompartments (Sherman et al., 2005).

1.1.4. PROTEIN INSERTION INTO THE OUTER MEMBRANE BY THE SAM COMPLEX

The TOM-complex is unable to insert integral proteins into the mitochondrial outer membrane and to assemble itself from the newly synthesized proteins. Recently, one more multisubunit complex was identified in the outer membrane (Wiedemann et al., 2003) that is competent in these functions. It is called SAM (**S**orting and **A**ssembly **M**achinery, see Fig.2). For the moment, four components of this complex are known.

Sam50p is believed to be the core component of SAM complex. It is an integral membrane protein that forms a channel of diameter sufficient for the β-barrel proteins to enter it (Paschen et al., 2003). Two other components of this complex, Sam37p and Sam35p, are anchored in the membrane and are exposed to the cytoplasm; they probably carry the receptor function (Waizenegger et al., 2004). One more protein, Mdm10p, is required for the insertion of not all β-barrel proteins, and its activity is regulated by Tom7p (Meisinger et al., 2006). The integral outer membrane protein Mim1p is a part of neither TOM-, nor SAM-complex, but is required for the assembly of the TOM translocon (Waizenegger et al., 2005). The exact functions of all these proteins are still unknown.

Little is known about the mechanisms of SAM complex action. The integral proteins are at first transported to the intermembrane space through the TOM-complex and then, by means of the small Tim-proteins, are transferred to the SAM-complex and are inserted to the outer mitochondrial membrane (Wiedemann et al., 2004).

1.1.5. PROTEIN IMPORT INTO THE MITOCHONDRIAL INTERMEMBRANE SPACE

So then, the protein transport through the mitochondrial outer membrane always occurs by the same mechanism. On the contrary, the mechanism of protein further translocation depend on the mitochondrial subcompartment in which this specific protein should be addressed. We begin to describe these mechanisms starting from the protein import into the intermembrane space. Several proteins that take part in the Fe-S clusters formation as

well as small Tim proteins are localized in this subcompartment. They all do not possess N-terminal presequence. It was shown that, to be imported via the TOM-complex and further folding, the intermembrane space protein Mia40p is required (Chacinska et al., 2004). The same protein participates in the assembly of the oligomeric complexes of small Tim proteins (see below). To be functionally active, Mia40p must contain six oxydized cysteine residues that participate in the formation of disulfide bonds with the corresponding residues of the imported proteins (Mesecke et al., 2005). One more protein, required for the protein import into the intermembrane space, was identified as Erv1p. Its function is the maintenance of the six cysteine residues of Mia40p in oxydized state (Mesecke et al., 2005). Recent studies revealed that all the proteins imported via this pathway are small in their size (not more than 20 kDa) (Gabriel et al., 2007)).

1.1.6. TIM23 TRANSLOCASE AND THE IMPORT MOTOR

There are two translocons in the mitochondrial inner membrane that are responsible for the import of proteins with different signal sequences. TIM23 complex transports mainly the protein precursors with N-terminal presequences (Fig.3) (Mokranjac and Neupert, 2005).

TIM23 translocon may be functionally divided into two parts: the translocon itself which acts in the inner membrane, and the so-called “import motor” localized in the mitochondrial matrix and providing the energy for the translocation process. For the moment, 4 components of the translocon (Tim23p, Tim17p, Tim50p and Tim21p) and 6 components of the import motor (Tim44p, mtHsp70, Mge1p, Pam18p, Pam16p and Pam17p) are described.

Tim23p forms the channel in the inner membrane of mitochondria. Tim17p, probably, is also involved in the channel assembly (Meier et al., 2005). Both these two proteins contain transmembrane segments and the domains exposed to the intermembrane space. Moreover, they were shown to cross the outer membrane (Donzeau et al., 2000). The intermembrane space domains of Tim23p and Tim17p are believed to carry the receptor function (Meier et al., 2005). The channel formed by these proteins consists of 6 subunits of Tim23p and six subunits of Tim17p, it is cation-selective and presequence-specific (Truscott et al., 2001). Using artificial protein precursors of different fixed sizes, the internal diameter of the pore was estimated as around 22 Å (Schwartz and Matouschek, 1999). Tim17p, besides its channel function, is also required for the functional link between the membrane translocon and the import motor (Chacinska et al., 2005).

Tim50p consists of small transmembrane segment and large intermembrane domain. This protein also carries the receptor function (Geissler et al., 2002). Besides this, Tim50p plays a critical role in the functioning of the TIM23 translocon: it keeps the channel formed by Tim23p and Tim27p in a closed state when the protein precursor is absent while the interaction of the presequence with the pore induces the opening of the latter (Meinecke et al., 2006). Thus, the channel is not all the time transport-competent. This makes the unspecific import impossible.

Tim21p is organized in the same manner that Tim50p and consists of transmembrane and intermembrane parts (Chacinska et al., 2005). This protein switches the functional states of the TIM23 translocon (see below). Recently, one more important role of Tim21p has been shown: it promotes functional coupling between the protein translocase and the mitochondrial respiratory chain (van der Laan et al., 2006).

Now we will describe the mechanism of protein translocation via the TIM23 complex. As soon as the imported polypeptide chain enters the intermembrane space, its signal presequence is bound by Tim50p (Geissler et al., 2002). Then the transported protein interacts with Tim23p and Tim17p and enters the channel formed by these two proteins. Crossing both mitochondrial membranes by Tim23p and Tim17p facilitates this process making the membranes closer one to another. Recently, the direct *in organello* contact between TOM and TIM23 translocons was demonstrated: intermambrane space domains of Tim21p and Tom22p specifically interact with each other (Chacinska et al., 2005).

After entering the channel, the polypeptide chain can be imported in two different ways (Mokranjac and Neupert, 2005). The precursors targeted to the mitochondrial matrix are transported through the inner membrane with the help of the import motor (see below). However, if the preprotein contains a special hydrophobic segment directly after the signal presequence, the import of this preprotein is arrested, and it is inserted into the inner membrane by TIM23 translocon. Such proteins do not fully insert into the membrane, they are just anchored in it and are exposed into the mitochondrial matrix. For this process, the membrane potential but not the energy of the import motor is required (Frazier et al., 2004). The details of this process are still unclear. However, recently it was shown that TIM23 translocon providing the insertion of proteins into the inner membrane do contain Tim21p and is not associated to the import motor. Contrary, when transporting the preprotein into the mitochondrial matrix, TIM23 translocon does not contain Tim21p and is associated to the import motor. Thus, TIM23 translocon may act in two functional states, depending on presence or absence of Tim21p (Chacinska et al., 2005).

Translocation motor (or PAM, **P**resequence-**A**ssociated **I**mport **M**otor, see Fig.3) is a complex of matrix proteins providing the energy of ATP hydrolysis for the process of translocation of proteins into the matrix via TIM23 translocon (Neupert and Brunner, 2002). PAM is only required for those proteins that are imported exactly into the matrix. It should be pointed out that the signal presequence of the imported protein is transported through the inner membrane electrophoretically (Geissler et al., 2000), and the energy provided by PAM is required for the translocation of the mature part of the protein. For the moment, six PAM components are known. Among them, the most important are Tim44p and mtHsp70. Tim44p is anchored in the inner membrane and exposed into the matrix (Strub et al., 2002). This protein is a functional organizer of PAM. It attracts the other PAM components to the preprotein entering the matrix (Strub et al., 2002). It also interacts with Tim23p and Tim17p but only in case when they both interact with each other forming the active channel (Mokranjac et al., 2003). Tim44p, in ATP-dependent manner, forms a stable complex with mtHsp70 (that belongs to the Hsp70chaperones family and accomplishes the protein folding in the matrix). mtHsp70 oligomers, one after another, bind the imported polypeptide chain and, thus, catalyze its transport. Mge1p, being the nucleotides exchange factor, assists mtHsp70 as a cofactor (Neupert and Brunner, 2002).

Recently, three more PAM components were described. Pam18p (or Tim14p), being the member of J-proteins family, acts as a co-chaperone for mtHsp70 during polypeptide chain translocation, but not during protein folding (Mokranjac et al., 2003). This protein is anchored in the inner membrane and contains large matrix-exposed domain and small intermembrane part. The latter is in contact with Tim17p (Chacinska et al., 2005). Pam16p (or Tim16p) regulates the Pam18p activity preventing the ATPase activity stimulation when the imported polypeptide chain is absent (Frazier et al., 2004). It is a soluble matrix protein that contains J-like domain but does not belong to J-protein family. The last known protein, Pam17p, is necessary for the Pam18p-Pam16p complex formation and for the association of this complex with TIM23 translocon (van der Laan et al., 2005).

The exact mechanism of mtHsp70 action during the translocation of the preproteins is still unclear. Two hypothesises exist each suggesting an alternative model of mtHsp70 action. According to one of them, the protein precursor, being inside the import channel, is able to move in statistical way either in direction to the cytoplasm, or in direction to the matrix. As soon as the preprotein becomes exposed a little to the matrix, a molecule of mtHsp70 quickly binds it. Thus, mtHsp70 plays a passive role in the process of translocation, binding the imported polypeptide chain and preventing its reverse movement (Liu et al., 2003). The other

model proposes that mtHsp70 binds to the precursor that is entering the matrix and, in ATP-dependent manner, generates an active driving force that pulls down the preprotein to the matrix (Huang et al., 2002).

The mitochondrial peptidases that, in a post-translocational way, cleave the signal sequences also play an important role in the process of the import of MTS-containing proteins (Gakh et al., 2002). There are three types of such peptidases. MPP peptidase (**M**atrix **P**rocessing **P**eptidase) is localized in the mitochondrial matrix and cleaves the presequences of the vast majority of the imported proteins. About 70% of all MPP substrates contain the specific cleavage motifs (Gakh et al., 2002) while in other preproteins such motifs have not been identified. MIP peptidase (**M**atrix **I**ntermediate **P**eptidase) also functions in the matrix of mitochondria. The presence of arginine residue in position –10 relative to the cleavage site is a prerequisite for its action. Interestingly, before the cleavage by MIP, the part of the presequence must be cut by MPP that cleaves in position –8 relative to the MIP site. Only after MPP action, the rest of the presequence is cut by MIP. IMP peptidase (**I**nner **M**embrane **P**eptidase) is localized in the intermembrane space and consists of two subunits, Imp1 and Imp2. They both have peptidase activity and cleave the signal sequences of the intermembrane space proteins. In some cases, IMP action is preceded by cleavage of the part of signal sequence by MPP. The specific motifs that are recognized by IMP are not yet identified.

1.1.7. TIM22 TRANSLOCASE AND THE SMALL TIM PROTEINS OF THE INTERMEMBRANE SPACE

The second translocon of the mitochondrial inner membrane is called TIM22. It is competent for the insertion of the integral proteins containing the internal targeting sequences into the inner membrane (Fig.4) (Rehling et al., 2004). This process works without the energy of ATP hydrolysis but requires the mitochondrial membrane potential. The translocon consists of two parts – the membrane complex and the soluble intermembrane space proteins (small Tim proteins) that assist the hydrophobic imported polypeptides to cross the aqueous intermembrane space.

For the moment, 4 proteins are known to be the parts of the TIM23 membrane complex. They are Tim22p, Tim18p, Tim54p and Tim12p. Tim22p forms the channel in the inner membrane. Its diameter measured by electron microscopy is 18Å. One functional translocon consists of two channels (Kovermann et al., 2002). It should be pointed out that the channel formed by Tim22 has two functional states – opened and partially opened. In the last

case, its diameter is 11Å. The switch between the two states depends on the membrane potential and the presence or absence of the internal signal sequence; thus, Tim22p carries also the receptor function (Kovermann et al., 2002). Little is known about Tim18p and Tim54p. Using co-immunoprecipitation in was shown that they interact one with another. From the other hand, they are not essential components of TIM22 translocon. They are thought to be involved in the maintaining proper conformation of Tim22p (Kovermann et al., 2002). There is almost no data about Tim12p. It is hypothesized that it participates in the transfer of the imported polypeptides from the small Tim proteins to the membrane complex; however, it is even not clear whether Tim12p is a part of the membrane translocon or if it belongs to the small Tim family.

Four small Tim proteins (Tim13p, Tim10p, Tim9p and Tim8p) act like co-chaperones assisting the hydrophobic proteins to cross the aqueous intermembrane space. Each of these proteins contains so-called "twin CX_3C" motif in which two cysteine residues are separated by any three amino acids. Tim8p and Tim13p form a hexameric complex consisting of three molecules of each protein. A complex organized in the same way is formed by Tim9p and Tim10p. Twin CX_3C motifs are important for the formation of these complexes and for their functional activity (Koehler et al., 1999). The Tim8p-Tim13p and Tim9p-Tim10p complexes have different substrate specificity. The only known protein that interacts with both these complexes during its import into mitochondria is Tim23p. However, two complexes interact with the different domains of this protein (Davis et al., 2000). In all the cases, the described complexes bind the hydrophobic parts of the imported proteins, thus screening them from the contact with the aqueous intermembrane space (Curran et al., 2002).

The mechanism of protein insertion in the inner membrane by TIM22 translocon is known in details (Fig.4). The integral inner membrane proteins are transported via TOM translocon as loops (Curran et al., 2002). As soon as the part of the imported protein enters the intermembrane space, it is bound by small Tim proteins (Wiedemann et al., 2001). The resulting oligomeric complexes are transported to the inner membrane. In the absence of the membrane potential the internal signals can bind Tim22p but are unable to enter the channel (Kovermann et al., 2002). When the membrane potential is about 60 mV, the partial translocation through TIM22 complex takes place (Rehling et al., 2003). On this stage, the positively charged internal targeting sequences of the imported proteins play the main role. They enter the channel in electrophoretic manner and finally become exposed to the matrix. Two signal sequences of the same protein molecule enter two channels of the same translocon (Rehling et al., 2004). One of the integral inner membrane proteins, ATP/ADP carrier,

consists of three functional modules each of which contains two internal signal sequences. At first, one of the modules enters the channel, and two other modules cannot cross the inner membrane without the first one (Endres et al., 1999; Smagula and Douglas, 1988). Finally, when the membrane potential is about 120 kV (which is the normal physiological value), the protein is fully inserted into the inner membrane. It is believed that during this process the fusion of two channels of the same translocon and its lateral opening take place (Rehling et al., 2004).

1.1.8. PROTEIN EXPORT FROM THE MITOCHONDRIAL MATRIX

The majority of mitochondrially-synthesized proteins and some imported proteins are exported from the matrix to the inner membrane (Herrmann and Neupert, 2003). Little is known about this process. The most fully characterized component of the protein export machinery is Oxa1p. It is imported from the cytosol via the standard pathway for MTS-containing precursors. Oxa1p contains five transmembrane segments and one large matrix domain. This protein acts as a homotetramer (Herrmann and Neupert, 2003). Some data indicate that in the absence of Oxa1p its function can be fulfilled by other inner membrane translocons (Saint-Georges et al., 2001). The mechanism of Oxa1p action is unknown.

There is also other export system in the inner membrane of mitochondria that provides the transport of the C-terminal domain of Cox2p. This system includes at least three components – Pnt1p, Cox18p and Mss2p (Saracco and Fox, 2002). The mechanism of this system's action is also unclear.

1.2. RNA IMPORT INTO MITOCHONDRIA

While the mechanisms of protein import into mitochondrial are universal in all eukaryotes and are known in details, the situation is almost opposite with RNA import. The experimentally proven RNA import is shown for only several groups of organisms. The vast majority of the imported RNAs are tRNAs that are necessary for the mitochondrial translation since the mitochondrial genome does not code for the proper tRNAs. The mammalians is the only group where the import of other small RNAs was shown. Different pools of RNAs are imported into the mitochondria of different species. Moreover, the mechanisms of RNA import are also different. Below we summarize the information available today about RNA import.

1.2.1. tRNA IMPORT INTO PROTOZOANS MITOCHONDRIA

The mitochondrial genome of Kinetoplastida protozoans does not contain tRNA genes. All tRNAs that are required for the mitochondrial translation are imported to mitochondria from the cytoplasm (Hancock and Hajduk, 1990; Simpson et al., 1989). In *Trypanosomatida*, one nuclear gene codes for both cytosolic and mitochondrial isoforms of tRNA (Schneider et al., 1994a; Schneider et al., 1994b). The cytosolic and mitochondrial tRNA pools of these organisms are equal excluding the initiator $tRNA^{Met}$ that is localized only in the cytoplasm (Tan et al., 2002b). The elongator $tRNA^{Met}$ is imported to mitochondria and is used both as elongator and as initiator. For the latter, the methionine residue bound to this tRNA must be formylated in the mitochondrial matrix (Tan et al., 2002a). Comparing the elongator and initiator $tRNAs_{Met}$, one can found 26 nucleotides that are different in these two species. The nucleotides determined the localization of these tRNAs are located in the T-stem of the tRNA molecule (Crausaz Esseiva et al., 2004). This is the only case of existence of nucleotide determinants of the import. In all other case of trypanosomal tRNAs, the tRNA structure is thought to be the signal of importation. This conclusion was made basing on the fact that some heterological tRNAs are able to be imported in Trypanosome mitochondria (Schneider, 1996). Moreover, the mutant form of $tRNA^{Tyr}$ containing 11 nucleotides long unspliced intron was shown to be imported, and the secondary structure of this mutant tRNA form resembles the right tRNA secondary structure (Schneider et al., 1994a).

It was also shown that mitochondrial isoforms of $tRNA^{Leu}$, $tRNA^{Lys}$ and $tRNA^{Tyr}$ differ from their cytoplasmic isoforms only by the modification of the nucleotide in position 32 (Schneider et al., 1994b). However, in case of $tRNA_{Tyr}$, this modification is not necessary for its import. May be, it is needed for the tRNA adaptation to the mitochondrial translation apparatus (Schneider et al., 1994b).

It is still not clear whether tRNAs are imported in Trypanosome mitochondria as processed molecules or as precursors. Hajduk and colleagues have shown that 5'-flanking regions of tRNA genes are important for tRNA import (Hancock et al., 1992). It is known that some tRNAs are synthesized as bicistronic transcripts in which the tRNAs sequences are separated by 20-30 nucleotides. It was shown that only these transcripts were imported into isolated Trypanosome mitochondria while mature tRNAs were not able to enter the mitochondria (Yermovsky-Kammerer and Hajduk, 1999). On the other hand, Schneider and colleagues have established the analogous experiments *in vivo* and have shown that tRNAs (and, in particular, $tRNA_{Leu}$(CAA) that belongs to one of biistronic transcripts) are imported

independently of their genomic context (Schneider, 1996; Tan et al., 2002b). Based on this finding, it was proposed that tRNAs are imported into Trypanosome mitochondria as mature processed molecules.

In apicomplexal parasite *Toxoplasma gondii*, the tRNA import is organized in the same manner as in Trypanosome (Esseiva et al., 2004). Again, all cytoplasmic tRNA species except for the initiator $tRNA_{Met}$ are imported into the mitochondria of this protozoan. Moreover, $tRNAi_{Met}$ from *T.gongii* expressed in *T.brucei* was also shown to localize exclusively in the cytosol. Unfortunately, this is the only available information about the tRNA import process in this organism.

In *Leishmania*, the exclusive cytoplasmic organization was shown for the only tRNA – $tRNA^{Gln}$(CUG). Isoacceptor $tRNA^{Gln}$(UUG) that has very similar primary structure, however, is imported into mitochondria (Shi et al., 1994). It was shown that the tRNA import ability is determined by tRNA structure (Lima and Simpson, 1996) and that the import determination elements are localized in the D-loop. This conclusion is based on the fact that exchange of this part of $tRNA^{Gln}$(CUG) by the analogous part from $tRNA_{Ile}$(UAU) results in the ability of the mutant tRNA to be imported (Shi et al., 1994). This is confirmed by the finding of the conserved motif in D-loop of *Leishmania* tRNAs which is absent in $tRNA^{Gln}$(CUG) and is required for tRNA importation (Bhattacharyya et al., 2000; Mahapatra et al., 1998). Some researchers think that the main role of the D-loop is the participation of the proper tRNA ternary structure that determines the import ability (Lima and Simpson, 1996; Rubio et al., 2000). However, the additional element determining the possibility of a tRNA to be imported may be found in the other tRNA parts: it was shown that the four-nucleotide insertion into the variable loop of $tRNA^{Thr}$(AGU) inhibits its import (Chen et al., 1994).

Using the *in vitro* selection approach, the motif YGGYAGAGC in the D-loop was shown to be a prerequisite for the tRNA mitochondrial localization (Bhattacharyya et al., 2002). Also, several consensus motifs important for tRNA targeting were revealed in the variable loop, T-arm and anticodon arm. This confirms the data obtained before by tRNA genes mutagenesis (Chen et al., 1994). Also, it was demonstrated that all imported tRNAs from *L.tarentolae* may be subdivided into two groups. The tRNAs of a first group are imported with a high efficiency and stimulate the import of tRNAs of a second group that, in their turn, are imported with a low efficiency and inhibit the import of tRNAs of a first group (Bhattacharyya et al., 2002). As a result, the efficiency of all tRNAs is nearly equal. It was shown that tRNAs of the different groups directly interact with each other on the

mitochondrial inner membrane. This interaction is thought to be the reason of such kind of allosteric import regulation.

The studies of the mechanism of tRNA import into *L.tarentolae* mitochondria (Fig.5) have revealed that the kinetics of the translocation through the outer and inner membrane is different. The crossing of the outer membrane by tRNA requires ATP hydrolysis while the transport across the inner membrane needs both ATP and membrane potential (Mukherjee et al., 1999). Moreover, some mutant tRNAs were able to be transported through the outer membrane but were not detectable in the mitochondrial matrix. Basing on this fact, it was hypothesized that there should be receptors for tRNAs in both mitochondrial membranes (Mahapatra and Adhya, 1996). The outer membrane receptor was identified as 15 kDa protein called TAB (**T**ubulin **A**ntisense-**B**inding Protein). Only imported tRNAs can be specifically bound by this protein (Adhya et al., 1997). However, the outer membrane channel for tRNA transport is not identified yet, as well as the mechanism of tRNA translocation through the outer membrane remains unclear.

In the mitochondrial inner membrane, the multisubunit complex responsible for the tRNA binding and translocation has been identified. It is called RIC (t**R**NA **I**mport **C**omplex) and consists of 9 subunits with molecular weights ranging from 19 up to 62 kDa (Goswami et al., 2006). It was shown that the interaction of tRNA with the components of this complex induces RIC energetic activation through the stimulating of ATP hydrolysis and induction of the membrane potential (Bhattacharyya and Adhya, 2004). This probably leads to the opening of the inner membrane channel.

Two components of RIC are now identified. They are both the receptors for tRNAs of the different groups. Allosteric regulation of tRNA import was demonstrated to occur on the level of binding to these receptors. The receptor for tRNAs of a first group (that are imported with the high efficiency) is α-subunit of F1 ATP synthase. This protein is a component of RIC as well as of the respiratory complex V (Goswami et al., 2006). It hydrolyses ATP in presence of the imported tRNA (Goswami and Adhya, 2006). The receptor for the tRNAs of a second group is also bifunctional protein, subunit 6b of ubiquinol-cytochrome c reductase (Chatterjee et al., 2006). Again, it is a component of two inner membrane complexes – RIC and the respiratory complex V. The inner membrane channel for tRNA translocation is not yet identified. Interestingly, RIC can be inserted into the human mitochondria and stimulates the tRNA import into the mitochondria of human cells. As a result, a set of human cytoplasmic tRNAs become imported, and the mutations in the mitochondrial tRNA genes are suppressed (Mahata et al., 2005; Mahata et al., 2006).

tRNA import into mitochondria also occurs in *Tetrahymena* protozoans. From total 36 mitochondrial tRNAs, 10 are encoded in the mitochondrial genome and all the rest are transported from the cytoplasm. In particular, one out of three cytosolic $tRNAs^{Gln}$, with anticodon UUG, is imported into mitochondria (Rusconi and Cech, 1996a). Only this tRNA is required for the reading of glutamine codons in course of mitochondrial translation while the anticodons of two other tRNAs (non-imported) correspond to the stop-codons in the mitochondrial genetic code of *Tetrahymena*. It was shown that the import of $tRNA^{Gln}$(UUG) does not depend on the genomic context of its gene. The comparison of three $tRNAs_{Gln}$ sequences has revealed that the nucleotide determinants of $tRNA^{Gln}$(UUG) are probably localized in the acceptor stem, D-arm and the anticodon arm (Rusconi and Cech, 1996a). Indeed, the anticodon nucleotides determine the ability of tRNA to be imported (Rusconi and Cech, 1996b). The mechanism of tRNA import in *Tetrahymena* are unknown. Earlier it was proposed that tRNAs are imported in *Tetrahymena* mitochondria being bound to corresponding aminoacyl-tRNA synthetases (Chiu et al., 1975; Suyama, 1967). However, it was demonstrated that all three $tRNAs^{Gln}$ are recognized by both cytosolic and mitochondrial GlnRS (Rusconi and Cech, 1996b). Thus, some other proteins should take part in the process of tRNA import.

The results of *in sillico* prediction of tRNA genes in the mitochondrial genomes show that tRNA import must take place in nearly all protozoans (Schneider and Marechal-Drouard, 2000). However, there are no experimental proof for this hypothesis for the moment.

1.2.2. tRNA IMPORT INTO PLANT MITOCHONDRIA

Even though the mitochondrial genomes of higher plant typically have huge sizes (200-2000 kbp), they never contain the full set of tRNA genes required for the translation inside the organelle (Dietrich et al., 1992). Normally, there are 10-12 tRNA genes in the plant mitochondrial genomes (Dietrich et al., 1996). The only known exeption is *Marchantia polymorpha* where only 2 cytosolic tRNAs are needed for the translation. It was shown that, besides these two ($tRNA^{Ile}$(AAU) and $tRNA^{Thr}$(AGU), (Akashi et al., 1997; Akashi et al., 1996)), the cytoplasmic $tRNA^{Val}$(AAC) is also internalized by *Marchantia* mitochondria. At the same time, mitochondrial tRN^{Val}, theoretically, must efficiently recognize all valine codons in course of mitochondrial translation.

In all other cases, the plant mitochondria import much more tRNA species, and their number and the amino acid specificity are very different (Dietrich et al., 1992). For instance,

14 tRNA species are imported into wheat mitochondria (Glover et al., 2001), 11 – into potato mitochondria (Marechal-Drouard et al., 1990), and 8 – into pea ones (Ramamonjisoa et al., 1998). Most often, tRNAs are partially imported into mitochondria, in other words, each tRNA species is distributed between the cytosol and the mitochondria. In addition, the number and the specificity of the imported tRNA species may differ even in closely-related organisms (Kumar et al., 1996).

Many tRNAs, after being imported into plant mitochondria, contain the methylated G in position 19. However, the same tRNAs, acting in the cytosol, do not possess such modification (Dietrich et al., 1992). It is still unclear whether this modification is the signal for tRNA importation or if it occurs inside the mitochondria after the transport because the enzyme making the methylation is found both in the cytosol and in the mitochondrial matrix (Ramamonjisoa et al., 1998).

Little is known about the mechanism of tRNA import into plant mitochondria. Most likely, each tRNA species is recognized by the specific protein factor (Dietrich et al., 1996). These factors are believed to be aminoacyl-tRNA synthetases. This hypothesis is supported by the fact that the mutated $tRNA^{Ala}$ from *Arabidopsis thialania*, when not able to bind the corresponding aaRs, was not imported into mitochondria while wild-type tRNA was successfully transported through mitochondrial membranes (Dietrich et al., 1996). However, the mutant $tRNA^{Val}$ was efficiently bound by ValRS but was not import-competent (Delage et al., 2003a). Moreover, $tRNA^{Val}$ and ValRS from *A.thialania*, being expressed in *S.cerevisiae* cells, showed different subcellular organization: the protein was found in mitochondria while tRNA was not (Mireau et al., 2000). These findings indicate the presence of at least one more protein factor for tRNA import. Interestingly, studying the tRNA import into isolated pea mitochondria, it was demonstrated that no soluble protein takes part in this process, but some proteins of the outer membrane, as well as ATP hydrolysis and membrane potential, are involved (Delage et al., 2003b). Recently, an outer membrane protein factor was unexpectedly identified as voltage-dependent anion channel (Salinas et al., 2006). Regarding the nucleotide determinants of tRNA transport, it was shown that for *A.thialania* $tRNA^{Val}$ importation its T-arm is critical (Laforest et al., 2005), whereas for $tRNA^{Gly}$ from tobacco – its anticodon and D-arm (Salinas et al., 2005).

1.2.3. tRNA IMPORT INTO YEAST MITOCHONDRIA

The mitochondrial genome of *S.cerevisiae* codes for all the tRNAs needed for the mitochondrial translation (Foury et al., 1998). However, two tRNA species were shown to be partially internalized by mitochondria. They are $tRNA^{Lys}$(CUU) (Martin et al., 1979) and $tRNA^{Gln}$ (Rinehart et al., 2005).

There are three lysine tRNAs in yeast. The first (tRK1, anticodon CUU) is encoded in the nuclear genome and is imported into mitochondria with an efficiency of 5-10% (Tarassov and Entelis, 1992). The second one (tRK2, anticodon UUU) is also nuclear-encoded but is localized exclusively in the cytosol. Finally, the third one (tRK3, anticodon $cmnm^5s^2UUU$) is encoded in the mitochondrial genome and participates in mitochondrial translation.

The *in vitro* and *in vivo* tRNA import systems were established. It was shown that the energetic parameters of tRK1 import are very similar to that of protein import – it requires ATP and membrane potential (Tarassov and Entelis, 1992). Both soluble and membrane proteins were proven to be necessary for tRK1 import process. Further studies revealed that the components of the protein import apparatus, Tom20p and Tim44p, take part in tRK1 targeting (Tarassov et al., 1995a). Moreover, blocking the protein import machinery resulted in tRK1 import inhibition. This suggests that tRK1 is probably imported through the protein import pathways while binding to some imported protein. This protein must be MTS-containing since in was demonstrated that Tom70 (that binds the internal targeting sequences of the imported proteins) does not participate in tRK1 targeting (Tarassov et al., 1995a).

For the present, three soluble cytosolic proteins are known to be so-called “tRK1 import factors”. The first is cytosolic lysyl-tRNA synthetase (Krs1p) that aminoacylates tRK1. The deacylated tRK1 cannot be imported (Tarassov et al., 1995b). However, several mutant transcripts of tRK1 gene are able to enter the mitochondria in their deacylated state (Kolesnikova et al., 2002). It is logical to propose that aminoacylation itself is not a prerequisite for tRK1 import and that the lysylation of this tRNA reinforces it the proper conformation that is needed for the importation. The same conformation is probably given to tRK1 by the mutations that give the tRNA molecule an opportunity to be imported in deacylated state.

The second known protein factor of tRK1 import is the precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p). It is the cytosol-translated protein containing the predicted N-terminal presequence. preMsk1p is imported into mitochondria where, in its mature form, it aminoacylates tRK3. This protein is thought to be the carrier or tRK1 during its importation

because interaction with preMsk1p is a prerequisite for the import of tRK1 as well as for all its mutated forms that are able to be imported (Kolesnikova et al., 2002; Tarassov et al., 1995b). Interestingly, preMsk1p cannot aminoacylate tRK1 but it can bind this tRNA with the K_D of about 180 nM (Entelis et al., 2006) which is comparable to the K_D of the complex $tRNA^{Lys}$-LysRS whose formation leads to aminoacylation of tRNA (Francin et al., 2002).

The last known protein factor necessary for tRK1 import was unexpectedly identified as a glycolytic enzyme enolase (isoform 2, Eno2p). This protein has specific affinity for aminoacylated tRK1 (but neither for its deacylated form nor for tRK2). Eno2p assists the tRK1-preMsk1p complex formation: in presence of this protein the K_D of this complex is decreased (40 instead of 180 nM). Eno2p itself is also able to specifically bind aminoacylated tRK1 with the K_D of about 2500 nM. Furthermore, Eno2p was found to be partially associated with the mitochondrial outer membrane (Entelis et al., 2006). The *ENO2* gene deletion leads to the partial inhibition of tRK1 import. The presence of recombinant preMsk1p and Eno2p is sufficient to direct aminoacylated tRK1 import into isolated mitochondria, although this import is less efficient than the import directed by the crude yeast protein extract. It was also demonstrated that for the tRK1 import, the soluble, but not the mitochondria-associated, Eno2p fraction is needed (Entelis et al., 2006). Taken together, these results lead to the hypothesis that Eno2p is the transporter of tRK1 to the mitochondrial surface where tRNA binds preMsk1p. This is confirmed by the fact that preMsk1p is translated by the mitochondria-associated polysomes (Entelis et al., 2006). Once being free of tRK1, Eno2p is included into the multisubunit complex that contains almost all other glycolytic enzymes and is associated to the surface of mitochondria (Brandina et al., 2006). The scheme of tRK1 import into mitochondria is presented in Fig.6.

As already mentioned, only one out of two lysine tRNAs is imported into mitochondria. Consequently, this process is highly specific. The nucleotide sequences of tRK1 and tRK2 differ one from another by 21 positions. It was proposed that some of the different nucleotides might play the role of import determinants. Indeed, in tRK1, the first base pair of the acceptor stem (G1-C72), discriminatory base (U73) and the first nucleotide of the anticodon (C34) are required for the efficient import (Entelis et al., 1998; Kolesnikova et al., 2002). Other nucleotides of tRK1 that are different from the corresponding nucleotides of tRK2 do not influence the ability of import (Entelis et al., 1998). It should be also pointed out that the native ternary structure of tRK1 is a prerequisite for its importation: two truncated tRK1 versions (which, together, form the full-sized molecule) could be imported only

together, but not separately. Also, mutations affecting the tRNA ternary structure make tRK1 unable to enter the mitochondria (Entelis et al., 1998).

The function of tRK1 inside the mitochondria was still unclear. By implication, the participation of tRK1 in the mitochondrial translation was confirmed. In these experiments, the mutant forms of tRK1 were used in which the amino acid specificity was changed but the nucleotide import determinants were kept. It was shown that these tRNAs take part in the mitochondrial translation both in vitro and in vivo and even are able to suppress nonsence-mutations in the genes of mitochondria proteins (Kolesnikova et al., 2000).

Recently, the $tRNAs_{Gln}$ were also shown to enter the yeast mitochondria (Rinehart et al., 2005). The import of these tRNA does not require any cytosolic proteins, although GlnRS that recognizes it is also imported into mitochondria. The imported tRNAs are believed to participate to the mitochondrial translation. The mechanism of these tRNAs import is unknown. However, it is clear that it strongly differs from tRK1 import mechanism. Earlier, it was proposed that in yeast mitochondria the glutamyl-tRNA formation occurs via the prokaryotic pathway when at first $tRNA_{Gln}$ is recognized by GluRS and is aminoacylated with glutamate, and thereafter Glu-$tRNA_{Gln}$ is aminated resulting in Gln-$tRNA_{Gln}$ formation. Soll and colleagues have shown that, in yeast mitochondria, Gln-$tRNA_{Gln}$ is synthesized directly, and both tRNA and aaRS required for this are imported from the cytoplasm (Rinehart et al., 2005). However, the fact of $tRNA_{Gln}$ import into yeast mitochondria is in contradiction with the earlier obtained results of 2D separation of yeast mitochondrial tRNA species (Martin et al., 1977).

1.2.4. RNA IMPORT INTO MAMMALIAN MITOCHONDRIA

The mammalian mitochondrial genomes code for a full set of tRNA species required for the translation inside the organelle (Anderson et al., 1981). Thus, the cytoplasmic tRNAs are not imported into mammalian mitochondria *in vivo*. The only exeption are the marsupials where the mitochondrial $tRNA^{Lys}$ gene is a pseudogene (Hou and Schimmel, 1988), and the cytoplasmic tRNA is imported into the mitochondria (Dorner et al., 2001).

Other types of RNA are imported into mammalian mitochondria. They are the RNA component of RNAse MRP that cuts RNA-primers in course of mitochondrial DNA replication (Li et al., 1994) as well as RNA component of RNAse P that takes part in 5'-processing of mitochondrial tRNA precursors (Doersen et al., 1985). For the present, the mechanism of these RNAs import is not known.

The third type of RNA imported into mammalian mitochondria is 5S rRNA (Entelis et al., 2001). Normally, this RNA is a part of large ribosomal subunit but it was not found in the human mitochondrial ribosomes. It should be pointed out, however, that these ribosomes were not fully functional (Magalhaes et al., 1998). The participation of 5S rRNA in the mitochondrial translation is supported by the observation that the amount of imported 5S rRNA molecules is comparable with the amount of mitochondrial ribosomes (Entelis et al., 2001).

The mechanism of 5S rRNA import resembles that for tRK1 import into yeast mitochondria: in both cases, ATP hydrolysis, membrane potential and the protein import apparatus are required. However, the mammalian homologue of preMsk1p does not participate in 5S rRNA targeting (Entelis et al., 2001).

Despite the absence of *in vivo* tRNA import into mammalian mitochondria, tRK1 was shown to enter the isolated human mitochondria, and this process can be directed either by yeast or by human protein extract (Entelis et al., 2001). Thus, there are all factors required for tRNA import in human cells. The reason why human lysine tRNA is not imported is, most likely, the big structural difference from tRK1. The human homologue of preMsk1p takes part in tRK1 targeting into human mitochondria. Interestingly, besides tRK1, several mutant versions of this tRNA and other yeast tRNAs were shown to be imported into human mitochondria (Entelis et al., 2001). In particular, the import was demonstrated for the *in vitro* transcript of tRK3 gene and for the mutant version of tRK2 with U1→G, A72→C and G73→U exchanges. These tRNAs, once expressed in human cells, are aminoacylated and imported into mitochondria. Moreover, the ability to use this artificial import for molecular medicine approaches was demonstrated (Kolesnikova et al., 2004). It is known that the single point mutation in human mitochondrial $tRNA^{Lys}$ gene (A8344→G in its T-stem) leads to the significant decrease of its aminoacylation which is accompanied by the critical defects in mitochondrial respiration and translation. It was shown that the presence of tRNAs described above in the human mitochondria possessing this mutation partially restores the normal phenotype (Kolesnikova et al., 2004). Thus, these tRNAs take part in the mitochondrial translation and rescue an efficient protein synthesis.

1. 3. DNA IMPORT INTO MITOCHONDRIA

Import of DNA into isolated mitochondria was shown for plant (Koulintchenko et al., 2003) and humans (Koulintchenko et al., 2006). The details of this process remain unclear. In the case of plant, it is known that DNA import does not depend on its sequence but DNA must be double-stranded. The inner membrane potential is required for this process. Also, it was demonstrated that the mitochondrial porin and adenine nucleotide transporter take part in this process. The imported DNA can be transcribed in the mitochondrial matrix (Koulintchenko et al., 2003). In case of human mitochondria, the imported DNA can be either single- or double-stranded. The DNA size does not significantly influence its import. In contrast to the situation with plant, the DNA import into human mitochondria does not depend on the inner membrane potential. The imported DNA can be replicated and transcribed in the human mitochondrial matrix. Moreover, the resulting polycistronic RNA is efficiently processed and matured. The details of the mechanism of DNA import into mitochondria remain unclear. Also, it is not known whether this process also takes place *in vivo* or not.

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GENERAL PROBLEMS OF MOLECULAR BIOLOGY

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Directed Import of Macromolecules into Mitochondria

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Abstract—Mitochondria are multifunctional eukaryotic organelles that provide cells with energy via oxidative phosphorylation. They participate in the formation of Fe-S clusters, oxidation of fatty acids, and synthesis of certain amino acids and play an important role in apoptosis. Mitochondria have their own genome and are able to transcribe and translate it. However, most macromolecules functioning in mitochondria, such as proteins and some small RNAs, are imported from the cytoplasm. Protein import into mitochondria is a universal process, and its mechanism is very similar in all eukaryotic cells. Today this mechanism is known in detail. At the same time, the RNA import was discovered only in several eukaryotic groups. Nevertheless, it is proposed that this process is typical for most species. A set of imported RNA molecules varies in different organisms. Although the knowledge about the mechanisms of RNA import is less extensive than that of protein import, it becomes clear that these mechanisms greatly differ between different species. The review summarizes information about the import of such macromolecules into mitochondria.

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Key words: mitochondria, protein import, RNA import, outer mitochondrial membrane, inner mitochondrial membrane, mitochondrial matrix

PROTEIN IMPORT INTO MITOCHONDRIA

Protein import into mitochondria plays an important role in the biogenesis of these organelles, since most mitochondrial proteins are imported. For example, the mitochondrial genome of *Saccharomyces cerevisiae* encodes only eight proteins, whereas the total number of proteins in yeast mitochondria is about 750–800. The system of protein transport into mitochondria is complex and is still incompletely understood [1, 2]. Depending on its targeted location, each protein is directed to the matrix, intermembrane space, or one of the mitochondrial membranes. The basic components of the transport system are targeting signals contained in the proteins to be transported into mitochondria, cytoplasmic factors providing for the unfolding of imported proteins and an access of mitochondrial receptors to their targeting signals, translocating complexes of the inner and outer mitochondrial membranes, an outer membrane complex providing for the incorporation of integral proteins into this membrane, mitochondrial peptidases cleaving the targeting signals of imported proteins within the matrix, mitochondrial chaperones participating in the folding of imported proteins within the mitochondrial matrix, and mitochondrial proteins ensuring protein export from the matrix to the inner mitochondrial membrane.

The main model subjects to study the mechanism of protein import into mitochondria are *S. cerevisiae* and *Neurospora crassa*. Proteins homologous to all components of the protein import system were discovered in many different eukaryotes, suggesting the universality of this process.

A general scheme of protein import into mitochondria is shown in Fig. 1.

TARGETING SIGNALS OF IMPORTED PROTEINS

The targeting signal is a part of a mitochondrial protein precursor that is necessary and sufficient for its transportation to the surface of mitochondria and translocation across the mitochondrial membranes. If a precursor should be imported into the mitochondrial matrix, its targeting signal is called a matrix targeting signal (MTS). Within the mitochondrial matrix, most MTSs undergo proteolytic cleavage. The targeting signals of many proteins imported into other submitochondrial compartments have a different organization, are mostly in the inner segments of the protein, and are not subject to proteolysis.

In the majority of cases, MTS is at the N terminus of a precursor molecule. As a rule, the penetration of

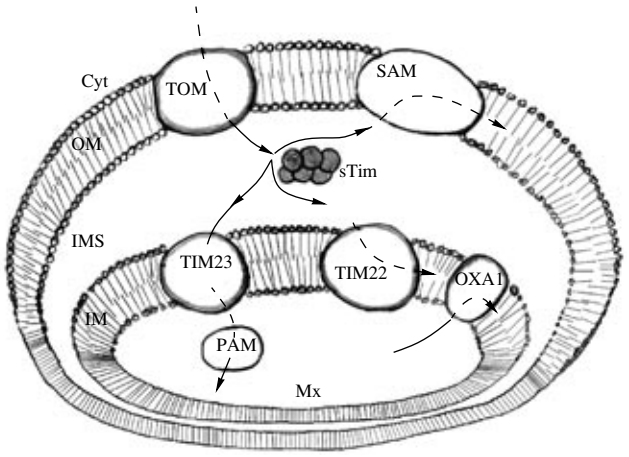

Fig. 1. General scheme of protein import into mitochondria. The pathways of the import of protein precursors are shown with arrows. All precursors are imported from the cytoplasm (Cyt) across the outer mitochondrial membrane (OM) with the help of the TOM complex. Then, three main scenarios are possible. (1) Integral proteins of the outer membrane are built into this membrane with the help of the SAM complex. (2) Precursors of mitochondrial proteins with an N-terminal targeting signal are imported across the inner mitochondrial membrane (IM) with the help of the TIM23 complex; the translocation motor (PAM) is also involved into this process. As a result, the precursors enter the mitochondrial matrix (Mx). (3) Proteins containing internal signal sequences are inserted into the inner mitochondrial membrane with the help of the TIM22 complex. The small Tim proteins (sTim) of the intermembrane space (IMS) are involved into all three scenarios. The OXA1 complex integrates the mitochondrial matrix proteins into the inner membrane. (All figures in this article are original).

a protein precursor into a mitochondrion begins from its N terminus, which is determined namely by MTS. However, there are some exceptions. For example, the C terminus of the precursor of yeast mitochondrial DNA helicase (Hmi1p) has a typical MTS [3], and the mechanism of its import is the same as for proteins with N-terminal MTSs, excepting a reverse orientation of the protein (C $\longrightarrow$ N).

Typical MTSs contain 20–60 amino acid residues, which, interacting with the lipid bilayer, form an amphiphilic α-helix [4]. These MTSs do not have any specific motifs [5], but it is known that hydrophobic or positively charged residues account for an essential part of their amino acids.

About 30% of mitochondrial protein precursors do not contain any specific MTS on their N ends. This group includes practically all proteins of the outer mitochondrial membrane, many intermembrane proteins, and proteins of the inner mitochondrial membrane. Segments responsible for the penetration through the mitochondrial membranes were revealed within some of these proteins.

Proteins of the outer mitochondrial membrane are divided into two main classes. Proteins from one of the classes have only a transmembrane segment and are not fully embedded into the membrane. Targeting signals of proteins from different classes differ from each other [6].

If a protein is just anchored in the membrane, then its transmembrane segment and several flanking amino acid residues serve as a targeting signal. The primary structure of such sequences is not of great importance for their signal function. As regards the proteins fully embedded into the outer membrane and forming a β-barrel within the membrane, their targeting signals are still unknown; it is known only that they include several segments separated by long spacer sequences [6].

Intermembrane proteins and proteins of the inner mitochondrial membrane also contain internal signal sequences and, in some cases, MTSs [7]. Many of these proteins utilize hydrophobic transmembrane segments and positively charged loops as targeting signals.

PRETRANSLOCATION UNFOLDING OF MITOCHONDRIAL PROTEIN PRECURSORS AND THEIR TRANSPORTATION TO THE SURFACE OF MITOCHONDRIA

The pretranslocation unfolding of proteins is promoted by several cytoplasmic factors, including proteins belonging to the chaperone family [8, 9]. For example, chaperone Hsp70 binds to precursors of mitochondrial proteins and renders them unfolded. It was shown that purified Hsp70 binds to the N-terminal targeting signals of synthetic polypeptides, depending on the amphiphilicity of the signal helix [10].

The maintenance of the unfolded configuration is especially important in the case of precursors with a tendency to a spontaneous folding or aggregation. Most likely, Hsc70, another member of the Hsp70 family, is especially important for the import of such proteins. Precursors of mitochondrial proteins have several sites for a cotranslational binding of Hsc70; such binding prevents aggregation of the protein [8].

Msf1p is involved in transporting protein precursors to the surface of mitochondria [8]. This protein forms stable complexes with the targeting signals and mature parts of these precursors.

Ydj1p, a yeast homolog of the bacterial DnaJ cochaperone, is also involved in the import of protein precursors into mitochondria [9]. Most likely, this protein provides for the transportation of Hsp70–precursor complexes to the mitochondrial surface.

The necessity to unfold protein globules before their import into mitochondria is determined by the fact that an essential part of proteins is imported into mitochondria posttranslationally, i.e., after their folding. However, many proteins are imported cotransla-

tionally; in this case, proteins begin to enter mitochondria before they assume their tertiary structure. Such cotranslational import has been experimentally confirmed for the precursors of malate dehydrogenase [11] and fumarase [12]. It is also known that yeast cells have functional ribosomes associated with the surface of mitochondria and carrying mRNAs for the precursors of many mitochondrial proteins. It is assumed that the location of mRNA on the mitochondrial surface provides for an efficient import of the translation products [13], suggesting again a cotranslational import. In some cases, the 3'-untranslated mRNA region is very important for mRNA localization on the mitochondrial surface [14]. On the other hand, a removal of the mRNA part encoding the targeting signal of a protein precursor weakens the mRNA association with mitochondria [15].

TRANSLOCATION COMPLEX OF THE OUTER MITOCHONDRIAL MEMBRANE

The translocation complex of the outer mitochondrial membrane (TOM, translocase of the outer membrane; Fig. 2) transfers all proteins imported into mitochondria across the outer mitochondrial membrane [16]. TOM consists of seven subunits. Three of them—Tom20p, Tom22p, and Tom70p—function as receptors, while the four other proteins—Tom40p (channel proper), Tom7p, Tom6p, and Tom5p—function as a transport channel.

Tom20p interacts with the N-terminal targeting signals of protein precursors. This protein has transmembrane and cytoplasmic domains; the latter is capable of a specific interaction with mitochondrial targeting signals [7]. Four α-helixes of Tom20p form a stable structure with a hydrophobic groove. A targeting signal, which is an amphiphilic α-helix, interacts with this structure in such a way that its hydrophobic surface is within the groove and the hydrophilic surface is exposed to the solution [17].

Tom70p hydrophobically interacts with the proteins that do not contain any N-terminal targeting signals [7]. Like Tom20p, this subunit consists of two domains. One of them is fixed in the outer membrane and the other is exposed to the cytoplasm. During translocation, one imported protein molecule is bound by several Tom70p molecules and then is transferred onto Tom22p [18].

Tom22p is a multifunctional component of TOM and plays an important structural role in its organization [19]. This protein consists of large cytoplasmic and small intermembrane domains, joined by a short transmembrane segment. Tom22p acts as a functional mediator between the receptors and the channel of TOM [20]. In addition, its transmembrane segment is involved into the interaction between single Tom40p

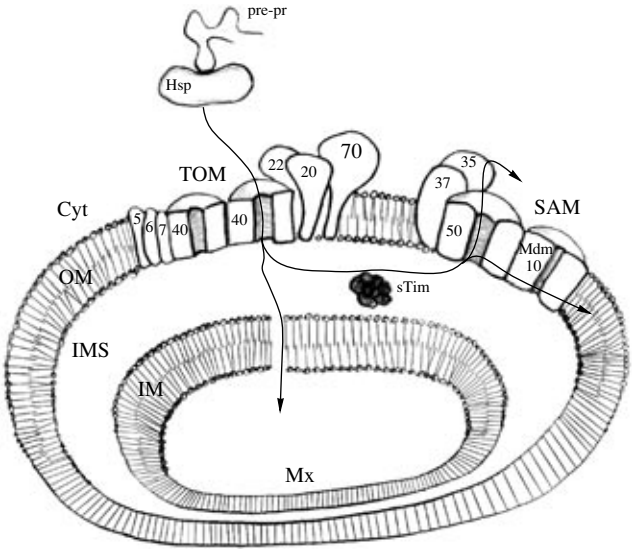

Fig. 2. Translocation complexes of the outer mitochondrial membrane. The TOM complex is responsible for translocation proper, and the SAM complex inserts integral proteins into the outer membrane (see text for details). Here and in Figs. 3–5: pre-pr, precursor of a mitochondrial protein; Hsp, cytoplasmic chaperones; Cyt, cytoplasm; OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane; Mx, mitochondrial matrix.

molecules and regulates the capacity of the protein import channel [19].

Tom40p forms a cation-selective channel for protein import across the outer mitochondrial membrane [21]. The protein has a typical β-barrel structure with eight transmembrane domains. Two Tom40p molecules form one membrane channel and the full-size TOM has two such channels [22]. Electron microscopy with negative staining made it possible to determine that the inner diameter of the channel formed by Tom40p is about 20 Å [21]. This pore size is sufficient for the penetration of an α-helix or even a compact loop into the channel [22]. Tom40p plays an active role in the translocation of proteins across the outer mitochondrial membrane. Tom40p contains binding sites for targeting signals and facilitates a directed translocation of proteins through the channel [23].

In addition to Tom40p, the pore contains three small TOM proteins: Tom5p, Tom6p, and Tom7p. All these proteins are fixed in the outer membrane and exposed to the intermembrane space; Tom5p has an additional cytoplasmic domain. Tom5p is necessary for a functional coupling of TOM with the small Tim proteins of the intermembrane space (see below) [24]. It was shown that this protein plays an important structural role in the organization of TOM in *N. crassa*; however, such function has not been observed in yeast cells [25]. Tom6p mediates the interaction between Tom22p and Tom40p [26], while Tom7p promotes dissociation of imported proteins in

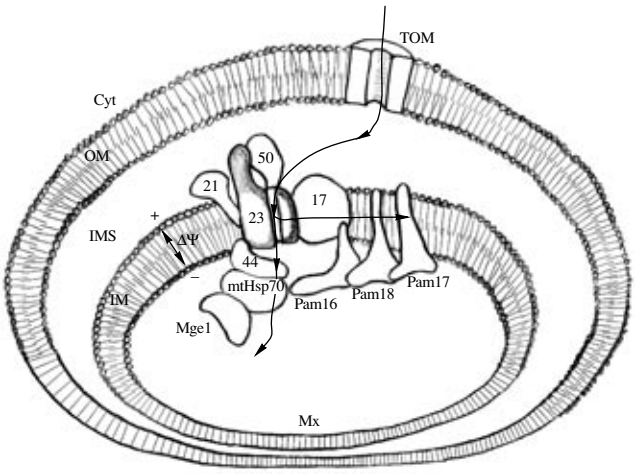

Fig. 3. TIM23 translocation complex of the inner mitochondrial membrane and the translocation motor (PAM). TIM23 transfers protein precursors into the mitochondrial matrix (with the help of PAM) or anchors the imported proteins on the inner membrane (without the participation of PAM). See text for details.

the intermembrane space [27]. Both of these proteins can be involved in targeting proteins into different mitochondrial compartments [25].

INCORPORATION OF PROTEINS INTO THE OUTER MITOCHONDRIAL MEMBRANE VIA OF THE SAM COMPLEX

TOM is incapable of inserting integral membrane proteins into the outer mitochondrial membrane and forming functional translocation complexes from the newly synthesized TOM proteins. A complex responsible for the incorporation of integral membrane proteins having the β-barrel structure was discovered in the outer mitochondrial membrane; however, this complex is not involved in the import of proteins into the inner membrane and mitochondrial matrix [28] (Fig. 2). The complex is called SAM (**S**orting and **A**ssembly **M**achinery). To date, four components of this complex have been identified.

The Sam50p subunit is probably the core component of the SAM complex. This protein is embedded into the outer membrane and forms a channel, whose diameter is sufficient for the penetration of integral proteins of the outer membrane [29]. Two other components of the SAM complex, Sam37p and Sam35p, are fixed in the outer membrane and exposed to the cytoplasm; most probably, they function as receptors [30]. Mdm10p is necessary for the integration of only several membrane proteins; its activity is regulated by Tom7p [31]. Mim1p, an integral protein of the outer mitochondrial membrane, does not belong to the TOM or SAM complexes, but provides for the assembly of the translocation complex of the outer membrane [32]. However, the exact functions of all these proteins are still unknown.

The mechanism of action of the SAM complex is also poorly understood. First, the precursors of integral proteins of the outer membrane penetrate through the TOM complex into the intermembrane space and then, with the help of the small Tim proteins, are transported to the SAM complex, which builds them into the outer mitochondrial membrane [33].

PROTEIN IMPORT INTO THE INTERMEMBRANE SPACE OF MITOCHONDRIA

Although protein transport across the outer mitochondrial membrane follows the same mechanism in all cases, further translocation of imported proteins can proceed by different ways depending on the subcompartment where the protein should be delivered. We start our description with the mechanism of protein import into the intermembrane space, which contains mostly small proteins synthesized in the cytoplasm and lacking N-terminal targeting signals. Their import through the channel of the TOM complex and subsequent folding require Mia40p, a soluble protein of the intermembrane space [34]. In addition, this protein takes part in the assembly of oligomeric complexes of the small Tim proteins (see below). To provide for a proper functioning of Mia40p, six Cys residues of this protein should be oxidized; these residues form intermolecular disulfide bridges with the corresponding residues of imported proteins to ensure their translocation into the intermembrane space [35]. More recently, Erv1p was identified as another factor necessary for protein import into the intermembrane space [35]; its function is to maintain the Cys residues of Mia40p in the oxidized state.

TIM23 TRANSLOCATION COMPLEX OF THE INNER MITOCHONDRIAL MEMBRANE AND TRANSLOCATION MOTOR

Unlike the outer mitochondrial membrane, the inner membrane has two translocation complexes responsible for the import of protein precursors. One, TIM23, provides mainly for translocation of MTS-containing precursors of mitochondrial proteins (Fig. 3) [36]. The other complex, TIM22 (see the next section), provides for the incorporation of integral proteins into the inner mitochondrial membrane.

TIM23 can be spatially and functionally divided into two parts: translocon proper, localized within the inner membrane, and a so-called translocation motor, localized within the mitochondrial matrix and providing energy necessary for the import of protein precursors. Today there are known four components of the TIM23 translocon (Tim23p, Tim17p, Tim50p, and Tim21p) and six components of the translocation

motor (Tim44p, mtHsp70, Mge1p, Pam18p, Pam16p, and Pam17p).

Tim23p forms a channel in the inner membrane to transport protein precursors into the mitochondrial matrix; Tim17p is probably also involved in the channel formation [37]. Both proteins contain transmembrane domains and domains exposed to the intermembrane space; moreover, they traverse the outer mitochondrial membrane [38]. It seems that the intermembrane domains of both proteins function as receptors [37]. The channel formed by the proteins consists of six Tim23p and six Tim17p molecules. This channel is cation-selective and MTS-specific [39]. The use of artificially synthesized protein precursors containing additional elements of different sizes made it possible to determine the internal pore diameter, which is 22 Å [40]. In addition, Tim17p ensures the functional coupling of the TIM23 translocon and translocation motor [41].

Tim50p consists of small transmembrane and large hydrophilic intermembrane domains. This protein also functions as a receptor [42]. In addition, Tim50p plays an important role in the functioning of the TIM23 complex. In the absence of protein precursors, this protein maintains the channel, formed by Tim23p and Tim17p, in the closed state; an interaction between the channel and MTS induces its opening [43]. Therefore, there is no permanent opening of the channel, which prevents nonspecific transport.

Similarly, Tim21p has small transmembrane and large hydrophilic intermembrane domains [41]. This protein acts as a switch between the functional states of the TIM23 complex (see below).

Today the following is known about the transport of protein precursors through the TIM23 complex. As soon as the targeting signal of a protein imported through the TOM complex enters the intermembrane space, it interacts with Tim50p [42]. Then, the imported protein interacts with the intermembrane domains of Tim23p and Tim17p and enters the channel formed by these proteins. This process is facilitated by the fact that both Tim23p and Tim17p traverse two mitochondrial membranes, which brings the translocons together [38]. Recently, it was shown that TOM and TIM23 directly interact *in organello*: the intermembrane domains of Tim21p and Tom22p specifically bind with each other [41].

After the targeting signal of an imported protein interacts with the channel, two variants of the following events are possible [36]. Precursors targeted to the mitochondrial matrix are transferred across the inner membrane with the help of the translocation motor (see below). However, when a protein has a special hydrophobic region just after the targeting signal, its translocation stops and the TIM23 complex builds it into the inner membrane. Such proteins are not fully integrated into the membrane, but are fixed in the inner membrane and exposed to the matrix. This process requires only a membrane potential and does not involve the translocation motor [44]; the mechanism of this phenomenon is still unclear. However, it was shown recently that the TIM23 complex, which builds proteins into the inner mitochondrial membrane, contains Tim21p and is not associated with the translocation motor. On the contrary, TIM23 does not contain Tim21p during the translocation of precursors into the mitochondrial matrix, but acts in an association with the translocation motor. Therefore, the complex can exist in two functional states depending on the presence or absence of Tim21p [41].

The translocation motor, or PAM (**P**resequence-**A**ssociated import **M**otor, Fig. 3), is a complex of mitochondrial matrix proteins that provides ATP hydrolysis energy for translocation of protein precursors across the inner mitochondrial membrane via the TIM23 complex [45]. PAM is necessary only for the proteins transported directly into the mitochondrial matrix. Note that the targeting signal of a protein precursor is transferred across the inner membrane electrophoretically [46] and that PAM is necessary for the transport of the mature protein part. Six components of this complex are known today. The most important are Tim44p and mtHsp70. The former is associated with the inner membrane and exposed to the mitochondrial matrix [47]. This protein is a functional organizer of the translocation motor, recruiting its other components to an imported protein penetrating into the matrix [47]. In addition, Tim44p interacts with Tim23p and Tim17p, but only when these proteins interact with each other to form a functional channel [48]. Tim44p forms a stable complex with mtHsp70, which is a typical member of the Hsp70 chaperone family and provides for protein folding in the mitochondrial matrix; the formation of this complex is ATP-dependent. Oligomers of mtHsp70 bind, one after the other, to a polypeptide chain penetrating into the matrix and thereby catalyze its import. This binding is coupled with ATP hydrolysis. Being a nucleotide exchange factor, Mge1p acts as a cofactor of mtHsp70 in this reaction [45].

Three other PAM components have been discovered recently. Pam18p (or Tim14p), belonging to the family of J-proteins, acts as a cochaperone of mtHsp70 and is required for the import of proteins into the mitochondrial matrix, but not for the folding of mitochondrial proteins [48]. This protein is fixed in the inner membrane and consists of two domains, a large matrix domain (J-domain proper) and a small intermembrane domain, contacting Tim17p [41]. Pam16p (or Tim16p) regulates the activity of Pam18p and prevents it from stimulating the ATPase activity of mtHsp70 in the absence of an imported polypeptide chain [44]. Pam16p is a soluble matrix protein that

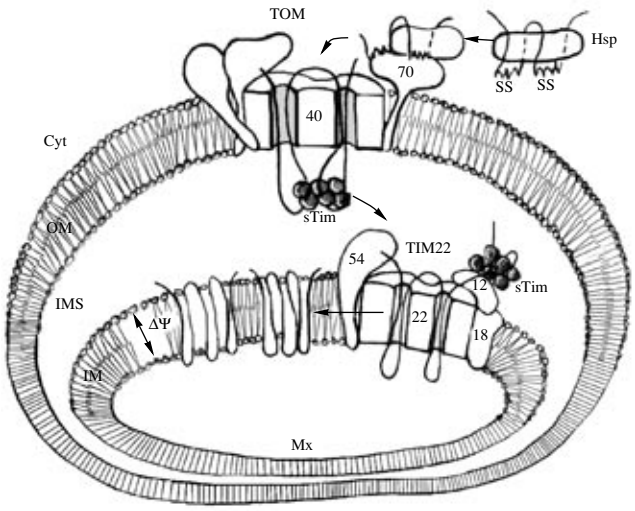

Fig. 4. TIM22 translocation complex of the inner mitochondrial membrane and the import of integral proteins of the inner membrane into mitochondria. Protein precursors carrying internal signal sequences are transported across the outer membrane in the form of a loop, transferred to the TIM22 complex with the help of the small Tim proteins, and then integrated into the inner membrane (see text for details). SS, internal signal sequence of an imported protein.

contains a J-like domain, but it is not a J-protein. The last known PAM component is Pam17p, which is fixed in the inner membrane and exposed to the mitochondrial matrix. This protein is necessary for the formation of the Pam18p · Pam16p complex and the association of these proteins with the TIM23 complex [49].

The mechanism of the Hsp70p action during the translocation of protein precursors into the mitochondrial matrix is still unclear. Two models were proposed on the basis of alternative explanations of the role of this protein. According to one model, when a protein precursor is within the channel, it can slightly move toward the intermembrane space or the matrix. As soon as a new precursor segment enters the matrix as a result of such movements, it is immediately bound by mtHsp70. Thus, mtHsp70 plays a passive role in translocation, binding to the imported polypeptide chain in the ATP-dependent manner and, thereby, preventing it from reverse movements [50]. Another model presumes that, binding to the imported protein entering the mitochondrial matrix, mtHsp70 generates an active "drawing" force, which forces the protein to enter the mitochondrion and is ATP-dependent [51].

To complete the description of the import of precursors with N-terminal targeting sequences, we should mention proteases of the mitochondrial matrix and intermembrane space, providing for posttranslational cleavage of the targeting signals [52]. There are three such peptidases. Mitochondrial processing peptidase (MPP) is in the mitochondrial matrix and cleaves MTSs of most imported precursors. About 70% of MPP substrates have specific motifs that contain Arg near the cleavage site and are recognized by MPP; in the other cases, such specific motifs were not revealed. Mitochondrial intermediate peptidase (MIP) is also in the mitochondrial matrix. To function properly, MIP requires Arg at a distance of 10 amino acid residues before the cleavage site. It is interesting that, before MIP-mediated cleavage, a precursor must be cleaved by MPP, whose cleavage site is two residues farther than the conserved Arg. It is only after this that MIP cleaves the remainder of the targeting signal. Inner membrane peptidase (IMP) is in the intermembrane space and consists of two subunits, Imp1 and Imp2, which both have peptidase activity and cleave targeting signals of intermembrane proteins. In some cases, a removal of part of the targeting sequence with MPP foregoes its cleavage with IMP. There is no information about the existence of any motifs recognized by IMP.

TIM22 TRANSLOCATION COMPLEX OF THE INNER MITOCHONDRIAL MEMBRANE AND SMALL TIM PROTEINS OF THE INTERMEMBRANE SPACE

TIM22, another translocation complex of the inner mitochondrial membrane, inserts integral proteins containing internal signal sequences into the inner membrane (Fig. 4) [53]. This process is ATP-independent, but requires a membrane potential. The translocon consists of two parts, a membrane complex and soluble proteins of the intermembrane space (small Tim proteins), promoting the transport of hydrophobic precursors of integral membrane proteins across the intermembrane space.

Four proteins are known to form the TIM22 membrane complex: Tim22p, Tim18p, Tim24p, and Tim12p. Tim22p forms a channel in the inner membrane; according to electron microscopy, the diameter of this channel is 18 Å. One functional translocon includes two such channels [54]. It should be noted that the channel formed by Tim22 has two functional states, being fully or partly open (in the last case, its diameter is 11 Å). The switching between these states is regulated by the membrane potential and the presence or absence of an internal signal sequence; therefore, Tim22p also has a receptor function [54]. The role of Tim18p and Tim54p proteins is practically unknown. Using coimmunoprecipitation, it was shown that these proteins interact with each other; at the same time, they are not essential components of the translocation complex. Probably, the proteins are necessary for a proper conformation of Tim22p [54]. There is almost no information about Tim12p. It is supposed that Tim12p promotes the transfer of imported proteins from the small Tim proteins to the membrane complex. However, only a few studies

focused on this protein, and it is as yet unclear whether this protein is a component of the membrane complex or belongs to the small Tim proteins.

Four so-called small Tim proteins (Tim8p, Tim9p, Tim10p, and Tim13p) function like chaperones and transport hydrophobic precursors through the hydrophilic intermembrane space. These proteins contain two so-called CX_3C motifs, where Cys residues are separated by any three amino acids. Tim8p and Tim13p form a hexameric complex with a stoichiometric ratio of 3 : 3; Tim9p and Tim10p form a similar complex. The CX_3C motifs are important for both the formation of these complexes and their functional activity [55]. The Tim8p–Tim13p and Tim9p–Tim10p complexes differ in substrate specificity. The only protein that is simultaneously bound by these two complexes during its import is Tim23p; however, the complexes interact with different domains of this protein [56]. It was shown that the complexes interact with hydrophobic segments of imported proteins [57] to protect them from contact with the hydrophilic intermembrane space.

The mechanism of the integration of proteins with internal signal sequences into the inner mitochondrial membrane with the help of the TIM22 complex is well known (Fig. 4). Such proteins take the form of a loop during their translocation through the channel of the outer membrane, which is formed by the Tom40p protein [57]. As soon as a part of a protein enters the intermembrane space, it is immediately bound by the complexes of the small Tim proteins [18] and, then, is transported to the inner mitochondrial membrane. In the absence of a membrane potential, internal signal sequences are able to interact with Tim22p but cannot enter the channel [54]. When the membrane potential rises up to a half of its physiological value (about 60 mV), a protein is partly transferred through the inner membrane channel. Most likely, the major role at this translocation stage is played by positively charged internal signal sequences of the imported protein, which enter the channel under the influence of the electrophoretic force and are then exposed to the matrix. It is necessary to note that two signal sequences simultaneously enter the two channels of the same translocon. One of the proteins undergoing such integration into the inner membrane is the ADP/ATP transporter, which consists of three functional modules, each containing two signal sequences. At first, one of the modules enters the channel; only after this, the other two modules also enter there. When the first module is absent, the other two are not inserted into the membrane. Finally, when the membrane potential reaches its normal value (120 mV or more), the protein fully integrates into the inner membrane; it seems that the two channels of the same translocon are joined at this stage, which is followed by a lateral opening of the complex [53].

PROTEIN EXPORT FROM THE MITOCHONDRIAL MATRIX

Many proteins are synthesized in mitochondria and some proteins imported from the cytoplasm are exported from the mitochondrial matrix and integrated into the inner membrane [58]. Today, little is known about this process.

The best-studied participant of this export process is Oxa1p, imported from the cytoplasm via the standard pathway for MTS-containing precursors. Its function is to integrate several matrix proteins, including itself, into the inner membrane. Oxa1p contains five transmembrane segments and a large domain exposed into the matrix and functions as a homotetramer [58]. According to some data, in the absence of Oxa1p, its function passes to other translocases of the inner membrane [59]. The mechanism of the Oxa1p functioning is still unknown.

There is another export system, which transports the C-terminal domain of Cox2p across the inner membrane and consists of at least three components: Pnt1, Cox18p, and Mss2p [60]; the mechanism of action of this system is still unknown.

RNA IMPORT INTO MITOCHONDRIA

While the mechanisms of protein import into mitochondria are studied quite well, we cannot state the same about RNA import. Unlike protein import, RNA import was experimentally studied in several groups of organisms. Nearly all imported RNAs are tRNAs, which are necessary for mitochondrial translation because the mitochondrial genome lacks their genes. The import of some other small RNAs was confirmed only for mammalian cells. Different RNA sets are imported into mitochondria of different organisms; moreover, their imports probably have different mechanisms. Below we summarize available information about RNA import into mitochondria.

IMPORT OF tRNA INTO MITOCHONDRIA OF PROTOZOA

The mitochondrial genome of protozoa belonging to the order Kinetoplastida does not contain tRNA genes; all tRNAs necessary for mitochondrial translation are imported from the cytoplasm. In the genus *Trypanosoma*, one nuclear gene encodes both cytoplasmic and mitochondrial forms of tRNA [61, 62]. These organisms have the same tRNA set in the cytoplasm and mitochondria, except that the initiator $tRNA^{Met}$ occurs only in the cytoplasm [63]. At the same time, the elongator $tRNA^{Met}$ is imported into mitochondria and used in the mitochondrial matrix as an initiator tRNA as well; in this case, formylation of the methionine residue bound with this tRNA is required. The elongator $tRNA^{Met}$ differs from the ini-

tiator one in 26 nucleotides. The nucleotides determining the localization of the two $tRNA^{Met}$ forms are in the T-stem of the molecule [64]. This is the only case of a nucleotide antideterminant for import; most likely, the structure of a tRNA molecule serves as a mitochondrial targeting signal in other tRNAs. Such conclusion is based on the fact that some heterologous tRNAs are imported into mitochondria of *T. brucei* [65]. Moreover, mutant $tRNA^{Tyr}$ containing a nonexcised intron (11 nt) is imported into mitochondria, and its secondary structure is practically the same as the typical tRNA structure [61].

It is known that mitochondrial $tRNA^{Leu}$, $tRNA^{Lys}$, and $tRNA^{Tyr}$ differ from their cytoplasmic isoforms only by modification of nucleotide 32 [62]. However, in the case of $tRNA^{Tyr}$, its modification is not essential for the import but, probably, is necessary for the adaptation of this tRNA to the mitochondrial translation system [62].

It is still unclear, whether tRNAs are imported into *T. brucei* mitochondria in the mature truncated form or in the precursor form. It was shown that the 5'-flanking sequences of tRNA genes are important for tRNA import into *T. brucei* mitochondria [66]. Moreover, some tRNAs are synthesized in the form of bicistronic transcripts, in which the tRNA sequences are separated by several tens of nucleotides; only such transcripts can penetrate into isolated mitochondria, whereas single mature tRNA molecules are not imported [66]. However, similar experiments in vivo showed that the transport of tRNAs (particularly, $tRNA^{Leu}$(CAA), which is included into a bicistronic transcript) into mitochondria does not depend on the genomic surrounding of the tRNA genes [63, 65]. Based on this fact, an alternative assumption was made about the import of mature tRNAs into mitochondria.

A similar picture of tRNA transfer was revealed in *Toxoplasma gondii*, an apicomplexan parasite [67]. Mitochondria of this organism also import all cytoplasmic tRNAs, excepting the initiator $tRNA^{Met}$. Expressed in *T. brucei*, this *T. gondii* tRNA is not imported into mitochondria. We do not have any other information concerning the tRNA import in *T. gondii*.

Protozoa of the genus *Leishmania* have only one tRNA, $tRNA^{Gln}$(CUG), that is located only in the cytoplasm; at the same time, $tRNA^{Gln}$(UUG), which has a similar primary structure, is partly imported into mitochondria [68]. An import-prohibiting signal is contained in the tRNA structure and is most probably localized in the D-loop, since substitution of this segment of $tRNA^{Gln}$(CUG) with an analogous sequence from the imported $tRNA^{Ile}$(UAU) allows the mutant tRNA to enter mitochondria [68]. These data are confirmed by the fact that the D-loops of *L. tropica* tRNAs contain a conserved motif, which is absent in $tRNA^{Gln}$(CUD) and is necessary for tRNA import into mitochondria [69]. Some researchers suppose that this effect is determined by the important role of D-loop nucleotides in the formation of the tertiary tRNA structure, which can be the main import determinant. However, additional elements determining the ability of tRNA to enter mitochondria can be located in other segments of the molecule. For example, insertion of four nucleotides into the variable loop of $tRNA^{Thr}$(AGU) prevents its penetration into mitochondria [70].

In vitro selection of tRNA-like molecules capable of penetrating into isolated mitochondria made it possible to discover that the YGGYAGAGC motif of the D-loop is necessary for tRNA import [71]. Consensus sequences were revealed in the variable loop, T-loop, and the anticodon loop; these findings confirmed the earlier data on site-directed mutagenesis of tRNA genes [70]. In addition, it was found that all imported tRNAs from *L. tropica* can be divided into two groups; tRNAs from one group are efficiently imported into mitochondria and stimulate the import of tRNAs from the other group, which, in turn, are imported with a low efficiency and inhibit the import of the first group [71]. As a result, the efficiency of import is about the same for tRNAs of both groups. It was shown that tRNAs of different groups directly interact with each other on the inner mitochondrial membrane; these interactions presumably ensure the same import efficiency for all tRNAs.

Studies of the mechanism of tRNA import into *L. tropica* mitochondria (Fig. 5) showed that the translocation kinetics differs between the outer and inner membranes. Translocation across the outer membrane is ATP-dependent, whereas, in the case of the inner membrane, this process depends on both ATP and the membrane potential [72]. Moreover, some mutant tRNAs are transferred across the outer membrane, but do not enter the matrix. Based on these data, a hypothesis about the presence of tRNA import receptors on both mitochondrial membranes was proposed. The outer membrane receptor, a 15-kDa protein, was called a tubulin antisense-binding protein (TAB). An interaction with TAB determines the possibility of tRNA import, since only imported tRNAs specifically bind with this protein [69]. However, the mechanism of tRNA transport across the outer mitochondrial membrane is still unknown.

A 640-kDa complex responsible for tRNA binding and translocation across the membrane was revealed in the inner mitochondrial membrane. It is called tRNA import complex (RIC) and includes nine proteins with molecular masses varying from 19 to 62 kDa [73]. A binding of tRNA with components of this complex induces its energy activation by stimulating ATP hydrolysis and generating a membrane potential; probably, this causes the opening of the inner membrane channel [74]. RIC includes at least two

receptors, interacting with tRNAs of the two different groups. It was shown that tRNA import is allosterically regulated at the level of binding with these receptors. The α-subunit of F1 ATP synthase acts as a receptor for tRNAs of the first group (highly efficient import); this protein is a part of both RIC and respiratory complex V [73]. A receptor for the second tRNA group is also a bifunctional protein, which is a subunit of 6b-ubiquinol-cytochrome *c* reductase. This protein is also involved in both RIC and respiratory complex III [75]. A tRNA import channel of the inner membrane was not identified. It is interesting that RIC is capable of stimulating the transfer of human cytoplasmic $tRNA^{Lys}$ into isolated human mitochondria, although this tRNA is not imported into mitochondria in vivo. The presence of this tRNA in the mitochondrial matrix suppresses a mutation in the gene encoding human mitochondrial $tRNA^{Lys}$ [76].

The import of tRNA is also known for protozoa of the genus *Tetrahymena*. Only 10 of 36 mitochondrial tRNAs are encoded in the mitochondrial genome; the other tRNAs should be imported from the cytoplasm, as, for instance, one of the three cytoplasmic Gln tRNAs (with the UUG anticodon) [77]. Only this tRNA is necessary for translation of Gln codons of the mitochondrial genome, whereas the anticodons of the two other (nonimported) cytoplasmic tRNAs correspond to the stop codons in the *Tetrahymena* mitochondrial translation system. The import of $tRNA^{Gln}$(UUG) does not depend on the surrounding of its coding sequence. A comparison of the three Gln tRNAs made it possible to assume that the nucleotide determinants of the import are in the aminoacceptor stem, D-loop, and anticodon loop [77]. The anticodon nucleotides proved, indeed, to determine the possibility of tRNA import into mitochondria [78]. There is no information about the import mechanisms in *Tetrahymena*. Earlier, a hypothesis was proposed that *Tetrahymena* tRNAs are transported into mitochondria in complex with the corresponding aminoacyl-tRNA synthases. However, all three Gln tRNAs are recognized by both cytoplasmic and mitochondrial glutamyl-tRNA synthases, indicating that other proteins determine the specificity of import [78].

As assumed from in silico analysis of the tRNA genes in different mitochondrial genomes, the import of tRNAs into mitochondria exists in most other protozoa [79]. However, direct experimental studies in this field have not still been carried out.

IMPORT OF tRNA INTO PLANT MITOCHONDRIA

In spite of the large size of the mitochondrial genomes in higher plants (from 200 to 2000 kb), none of them encodes a full set of tRNAs necessary for translation [80]. A plant mitochondrial genome encodes 10–12 tRNAs on average. The only exception is liverwort *Marchantia polymorpha*, whose mitochondrial genome encodes 27 tRNAs; i.e., only two tRNAs are additionally required to ensure translation. It was revealed that, in addition to the missing $tRNA^{Ile}$(AAU) and $tRNA^{Thr}$(AGU), one more tRNA, $tRNA^{Val}$(AAC), also penetrates into mitochondria [81], although $tRNA^{Val}$ encoded by the mitochondrial genome is capable of translating all Val codons.

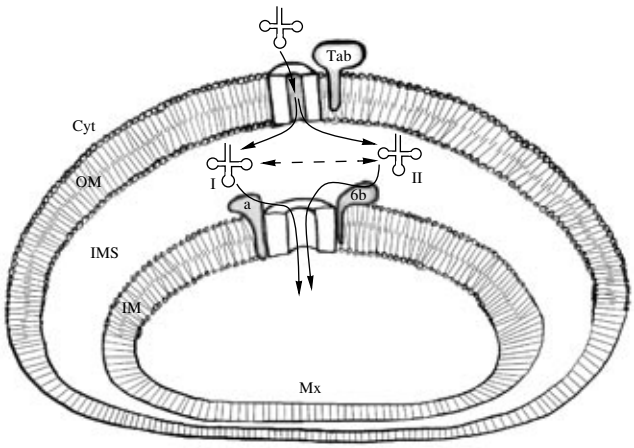

Fig. 5. Scheme of the tRNA import into *Leishmania tropica* mitochondria. First, tRNAs penetrate through the outer membrane via a general mechanism and are then divided into two groups, each having its own receptor on the inner membrane; tRNAs from different groups interact on the inner membrane, reciprocally regulating the import efficiency (see text for details). Designations: Tab, tRNA receptor on the outer membrane; I, tRNA from the first group; II, tRNA from the second group; a, receptor for the first tRNA group (α-subunit of ATP synthase); 6b, receptor for the second tRNA group (6b subunit of ubiquinol-cytochrome *c* reductase).

In the other known cases, plant mitochondria import many tRNAs, whose number and amino acid specificity greatly vary: e.g., mitochondria import 14 tRNAs in wheat, 11 in potato, and 8 in pea. The set of these tRNAs can differ even in closely related species. Most often, tRNAs are only partly transported into plant mitochondria; i.e. they can be detected in both the cytoplasm and mitochondria.

Many plant tRNAs contain methylated G19 when imported into mitochondria and remain nonmodified in the cytoplasm [80]. However, since the corresponding methylating enzyme was detected in both the cytoplasm and mitochondria, it is unclear whether such methylation provides a signal determining the mitochondrial localization of tRNA or takes place when tRNA enters the matrix.

There is only scarce information about the mechanisms of tRNA transport into plant mitochondria. Most likely, each tRNA is recognized by a specific protein factor [82]. Their role can be played by aminoacyl-tRNA synthases. For example, in contrast to

the wild-type analog, an *Arabidopsis thaliana* $tRNA^{Ala}$ mutant unable to bind with alanyl-tRNA synthase does not enter tobacco mitochondria [83]. However, although recognized by valyl-tRNA synthase, $tRNA^{Val}$ with a mutant D-loop does not enter mitochondria [84], suggesting the existence of at least one additional import factor. Another argument for this conclusion is the fact that three Gly tRNAs from potato are recognized by the same glycyl-tRNA synthase, but only two of them are imported into mitochondria. However, a study of the tRNA import into isolated pea mitochondria showed that translocation does not require any soluble cytoplasmic factor, but at least one protein component of the outer mitochondrial membrane, ATP, and the electrochemical membrane potential are involved [85]. As for nucleotide determinants of the import into mitochondria, a critical role is played by the T-loop in the case of $tRNA^{Val}$ from *A. thaliana* [86] or the anticodon and D-loop in the case of $tRNA^{Gly}$ from tobacco [87].

IMPORT OF tRNA INTO YEAST MITOCHONDRIA

The *S. cerevisiae* mitochondrial genome encodes a full set of tRNAs required for translation. Nevertheless, one tRNA is partly imported from the cytoplasm. This tRNA is one of the two cytoplasmic Lys tRNAs, namely, one with the CUU anticodon (tRL1). About 95% of the total tRL1 amount are in the cytoplasm, and only 5% are in mitochondria. The other $tRNA^{Lys}$ (tRL2), carrying the mcm^5s^2UUU anticodon, functions only in the cytoplasm. The yeast mitochondrial genome encodes one more $tRNA^{Lys}$ (tRL3), which carries the $cmnm^5UUU$ anticodon and is theoretically capable of recognizing both Lys codons.

Systems of tRL1 import into mitochondria in vitro and in vivo were developed. The energy parameters of tRNA import proved to resemble those of protein import: ATP hydrolysis and an electrochemical membrane potential are necessary in both cases. It was shown that soluble cytoplasmic proteins and proteins associated with the outer mitochondrial membrane are necessary for tRNA import. Tom20p and Tim44p, components of the mitochondrial protein import system, participate in tRNA import as well [88]. A block of the protein import apparatus prevents tRNA import. This fact testifies that tRL1 is most likely transported into mitochondria via the protein import pathways, being bound with an imported protein. Most probably, the protein is imported via the standard pathway for MTS-containing precursors, since Tom70p, which binds proteins that carry internal signal sequences, does not participate in tRNA import [88].

Three soluble cytoplasmic yeast proteins participating in tRL1 import were identified. One is cytoplasmic lysyl-tRNA synthase (Krs1p), which aminoacylates tRL1. A transfer of deacylated natural tRL1 is impossible [89]. However, some mutant transcripts of the tRL1 gene can enter isolated mitochondria without aminoacylation [90]. Most likely, the role of Krs1p in import does not consist in mere aminoacylation, but is to promote a specific conformation of tRL1 via its aminoacylation; some mutations confer the same conformation on tRL1.

The second known protein factor necessary for tRL1 import is the mitochondrial lysyl-tRNA synthetase precursor (preMsk1p). This protein is synthesized in the cytoplasm, contains an N-terminal targeting signal predicted in silico, and is imported into mitochondria. Most likely, MTS of this protein undergoes proteolytic cleavage in the mitochondrial matrix, and the mature protein aminoacylates tRL3. It is thought that preMsk1p transfers tRNA across the mitochondrial membranes, since the binding with this protein is absolutely necessary for the import of tRL1 and its mutant forms [89, 90]. Interestingly, preMsk1p is incapable of tRL1 aminoacylation, but the dissociation constant of the tRL1–preMsk1p complex is about 180 nM [91], comparable with that of the complex whose formation leads to aminoacylation.

Finally, the last known soluble protein participating in the tRNA import was recently identified as the glycolytic enzyme enolase, or, to be more exact, one of the two of its isoforms, enolase 2 (Eno2p). This protein has a specific affinity for aminoacylated tRL1 (but not for tRL2) and promotes the formation of the tRL1–preMsk1p complex, reducing its dissociation constant to 40 nM. In addition, Eno2p is partly associated with the mitochondrial surface [91]. A deletion of *ENO2* stops the tRL1 import. The presence of recombinant preMsk1p and Eno2p suffices to direct the tRL1 import into isolated mitochondria, although the efficiency of such transfer is lower than with a total cell protein extract. It was also shown that the tRL1 import requires soluble Eno2p, rather than the Eno2p fraction associated with the outer mitochondrial membrane [91]. Most likely, Eno2p acts as a tRL1 carrier to the mitochondrial surface, where tRL1 interacts with preMsk1p and can be transferred. This hypothesis is confirmed by the fact that the preMSk1p mRNA is translated by ribosomes associated with mitochondria. Eno2p free of tRL1 is built into a macromolecular complex containing several other glycolytic enzymes and associated with the mitochondrial surface. The proposed mechanism of the tRL1 import into yeast mitochondria is shown in Fig. 6.

We have already mentioned that only one of the two Lys tRNAs is imported into yeast mitochondria; therefore, this process is highly specific. The nucleotide sequences of tRL1 and tRL2 differ in 21 positions, suggesting that some these nucleotides function as import determinants. Further investigations showed that the first nucleotide pair of the aminoacceptor stem

(G1–C72) and the first anticodon nucleotide (C34) determine the tRL1 import into mitochondria [90, 92]. The other differing nucleotides, located in the D- and T-loops, do not significantly influence this process [92]. Note that the native tertiary structure of tRL1 is necessary for its import into mitochondria; two truncated tRL1 versions (forming the whole molecule together) can be transported only together; in addition, mutations altering the tertiary structure of tRL1 inhibit its import into mitochondria [92].

The tRL1 function in the mitochondrial matrix is still an open question. As already mentioned, tRL3 is capable of recognizing both Lys codons during translation (owing to modification of U34). However, since tRL1 is imported in the aminoacylated form, its participation in biosynthesis of mitochondrial proteins cannot be excluded. This supposition is indirectly confirmed by experiments with mutant tRL1 forms having a modified amino acid specificity and the intact import determinants. The mutant forms participate in mitochondrial biosynthesis of proteins both in vitro and in vivo and even suppress nonsense mutations in the genes encoding mitochondrial proteins [93]. However, a participation of wild-type tRL1 in mitochondrial translation is still not confirmed.

Recent data confirm the transfer of cytoplasmic $tRNA^{Gln}$ into yeast mitochondria [94]. The transfer does not require soluble proteins, although glutamyl-tRNA synthase recognizing this tRNA is also imported into mitochondria. It seems that the imported tRNA participates in mitochondrial translation. The mechanism of the transfer is unclear but, obviously, differs from the mechanism of the tRL1 import. Earlier, a prokaryotic pathway was proposed for the glutamyl-tRNA formation in yeast mitochondria; i.e., $tRNA^{Gln}$ is first recognized by glutaminyl-tRNA synthase and aminoacylated with glutamate, and then glutamate is aminated to yield glutamine. However, the enzyme necessary for this process was not found in yeast mitochondria. Recently, a direct formation of glutaminyl-tRNA was shown, both tRNA and aminoacyl-tRNA synthase being imported into mitochondria from the cytoplasm [94].

RNA IMPORT INTO MAMMALIAN MITOCHONDRIA

The mitochondrial genome of mammals contains genes for all tRNAs necessary for translation within these organelles, suggesting no transfer of cytoplasmic tRNAs into mammalian mitochondria. The only exception concerns marsupials. In silico analysis showed that the marsupial mitochondrial gene for $tRNA^{Lys}$ is a pseudogene. Further investigations showed that cytoplasmic $tRNA^{Lys}$ is actually imported into opossum mitochondria [95].

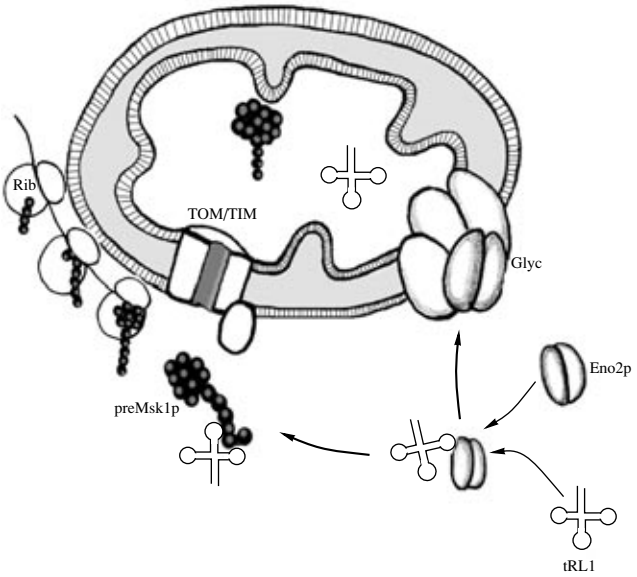

Fig. 6. Scheme of tRNA import into yeast mitochondria. The tRL1–Eno2p complex is transported to the mitochondrial surface and interacts with preMsk1p, which is synthesized by ribosomes associated with mitochondria. The resulting complex is imported into mitochondria (see text for details). Designations: Rib, ribosomes associated with the mitochondrial surface; Glyc, glycolytic complex; TOM/TIM, apparatus of protein import into mitochondria.

Mammal mitochondria import other RNAs, such as the RNA component of MRP RNAse, which cleaves RNA primers during replication of mitochondrial DNA, and the RNA component of P RNAse, which participates in 5'-processing of the precursors of mitochondrial tRNAs. The mechanism of the import of these RNAs is still unknown.

The third type of RNAs imported into the mammalian mitochondria is the 5S rRNA [96]. This RNA is usually a structural component of the large ribosome subunit; however, it was not found in mitochondrial ribosomes of mammals. Note, however, that these mitochondrial ribosomes were not functionally active on natural templates and were capable only of polyphenylalanine synthesis on a poly-U template. The fact that the number of imported 5S rRNA molecules is comparable with the number of mitochondrial ribosomes is evidence for the participation of the 5S rRNA in mitochondrial translation in mammalian cells [96].

Most probably, the mechanism of 5S rRNA import is similar to that of tRNA import into yeast mitochondria: in both cases, the process requires ATP, a membrane potential, and the protein import apparatus [96]. However, a protein analogous to yeast preMsk1p is not involved in this process.

In spite of the fact that tRNAs do not penetrate into mammal mitochondria in vivo, yeast tRL1 is capable

of entering isolated human mitochondria; this process takes place in both human and yeast cell protein extracts [96]. Therefore, human cells contain all factors necessary for tRNA import. The fact that human $tRNA^{Lys}$ is not transferred into mitochondria can be explained by a significant difference between its structure and the structure of tRL1. A human homolog of yeast preMsk1p is involved into tRL1 import into human mitochondria. It is interesting that, like tRL1, its mutant versions and some other yeast tRNAs are also imported into human mitochondria [96]. In particular, this is true for tRL3 and a tRL2 mutant (tRL93) containing U1 $\longrightarrow$ G, A72 $\longrightarrow$ C, and G73 $\longrightarrow$ U nucleotide substitutions. Being expressed in human cells, these tRNAs are efficiently aminoacylated with Lys and enter mitochondria. Moreover, it is possible to use the import of these tRNAs for biomedical purposes [97]. It is known that point substitution A8344 $\longrightarrow$ G in the T-stem of human mitochondrial $tRNA^{Lys}$ significantly decreases the efficiency of its aminoacylation, which disturbs mitochondrial translation and mitochondrial respiration. The resulting human hereditary disease is called the MERRF syndrome (**M**yoclonic **E**pylepsy with **R**ed-**R**agged **F**ibers). The import of tRL3 or tRL93 into mutant human mitochondria partly recovers the wild-type phenotype [97]. Therefore, these tRNAs are involved in mitochondrial translation and provide for effective protein synthesis.

DNA IMPORT INTO MITOCHONDRIA

Recently, it was shown that DNA can also be imported into isolated mitochondria of plants [98] and mammals [99]. In the case of plant mitochondria, it is known only that DNA transfer does not depend on the DNA primary structure, but DNA should be double-stranded. DNA import requires a potential on the inner mitochondrial membrane and involves mitochondrial porin and the adenine nucleotide transporter; imported DNA molecules can be transcribed in the mitochondrial matrix [98]. In the case of mammalian mitochondria, both single- and double-stranded DNAs can be imported and the efficiency of their import is practically independent on their size. Unlike with plant mitochondria, DNA import into mammalian mitochondria does not depend on the membrane potential. Imported DNA can be replicated and transcribed in the mitochondrial matrix; moreover, polycistronic RNAs resulting from transcription undergo processing and maturation [99]. The detailed mechanism of DNA import into mitochondria and the question of whether this process occurs in vivo are still unknown.

CONCLUSIONS

The transfer of macromolecules into mitochondria is observed in all eukaryotes and plays the key role in mitochondrial biogenesis. Proteins account for the majority of the imported macromolecules. The functioning of mitochondria is impossible without such proteins, whose content reaches 98% of the total mitochondrial protein. In general, studies of the mechanisms of protein import into mitochondria will soon be completed. However, even now, researchers discover new components of the protein import apparatus and refine the mechanisms of action and interrelations of its individual parts. Recent investigations clarified the general mechanisms of the assembly of translocation complexes; however, this issue is far from the final solution. Moreover, some mitochondrial membrane complexes involved into protein import are probably unidentified.

Nucleic acid import into mitochondria is very widespread, though not universal. Its mechanisms are understood much more poorly than the mechanisms of protein import. Ample experimental data testify that these mechanisms differ in different groups of organisms. The question of how a negatively charged nucleic acid penetrates the mitochondrial membranes is still unclear. Studies of the nucleic acid transfer is of both fundamental and applied importance, since it proved possible to suppress mutations of the human mitochondrial genome via targeted tRNA import into mitochondria. Further investigations can yield effective strategies of gene therapy for mitochondrial dysfunctions. One of the most promising ways to achieve this aim is to construct special vectors for a targeted delivery of genetic material into mitochondria. In this respect, studies of the 5S rRNA and DNA import into mammalian mitochondria are of great applied importance.

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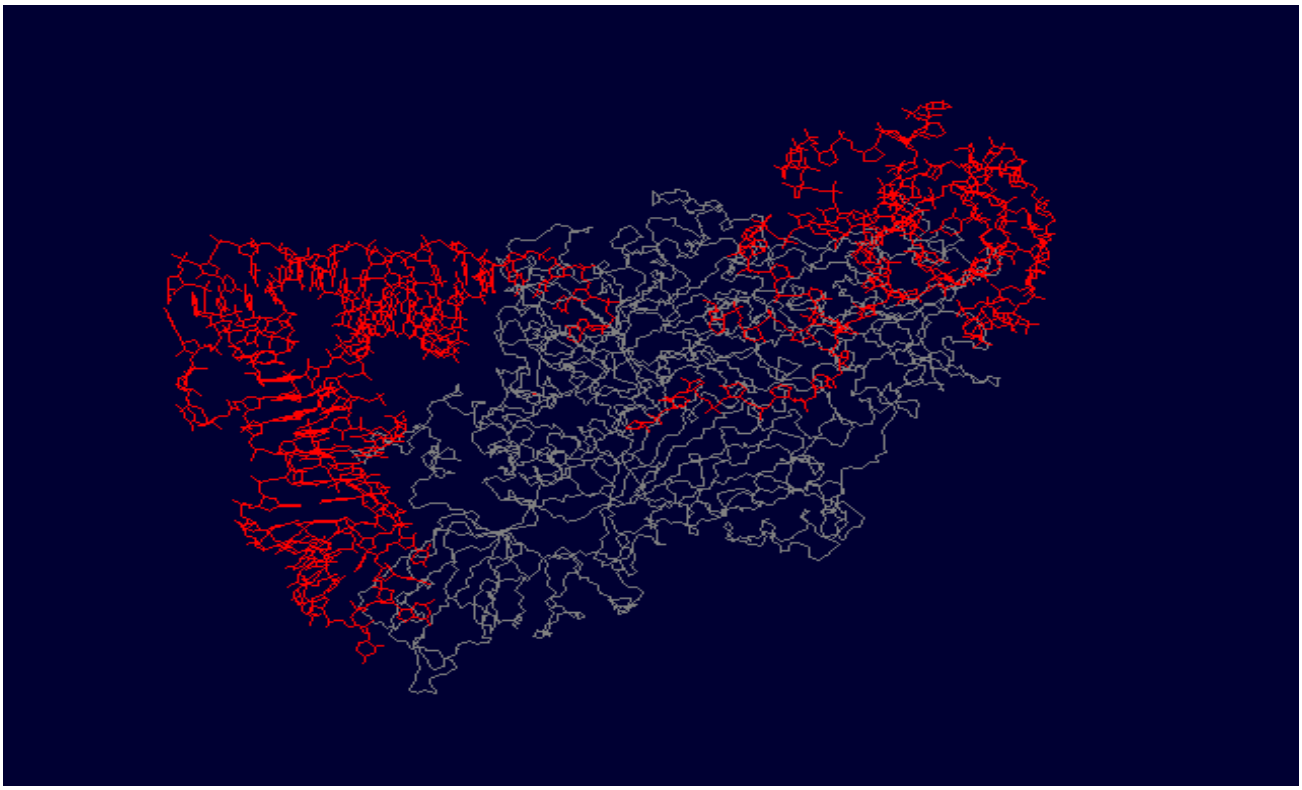
CHAPTER 2. RESULTS & DISCUSSION

2.1. ANALYSIS OF tRK1 INTERACTION WITH preMsk1p

In yeast, there are at least two types of tRK1 complexes with aaRSs. They are tRK1•Krs1p and tRK1•preMsk1p complexes (Tarassov et al., 1995b). Formation of these two complexes leads to the different things: Krs1p provides tRK1 aminoacylation while preMsk1p supports tRK1 transport through the mitochondrial membranes. Importantly, Krs1p cannot stimulate tRK1 import and preMsk1p, either as precursor or in mature form, is not able to aminoacylate tRK1 (Tarassov et al., 1995b). Based on this fact, we have hypotethized that the tRK1 should interact with these two proteins in different ways.

Krs1p is supposed to be a subclass IIb aminoacyl-tRNA synthetase. AaRSs of this subclass contain two spatially and functionally separated domains – N-terminal (that binds tRNA anticodon in course of aminoacylation) and C-terminal (that contains the active center of the enzyme) (Eriani et al., 1990; Moras, 1992). Several aaRSs from subclass IIb were cocrystallized together with their cognate tRNAs (Cavarelli et al., 1993; Rould et al., 1989; Ruff et al., 1991). The structure of yeast AspRS complexed with $tRNA^{Asp}$ is presented in Fig. 1.



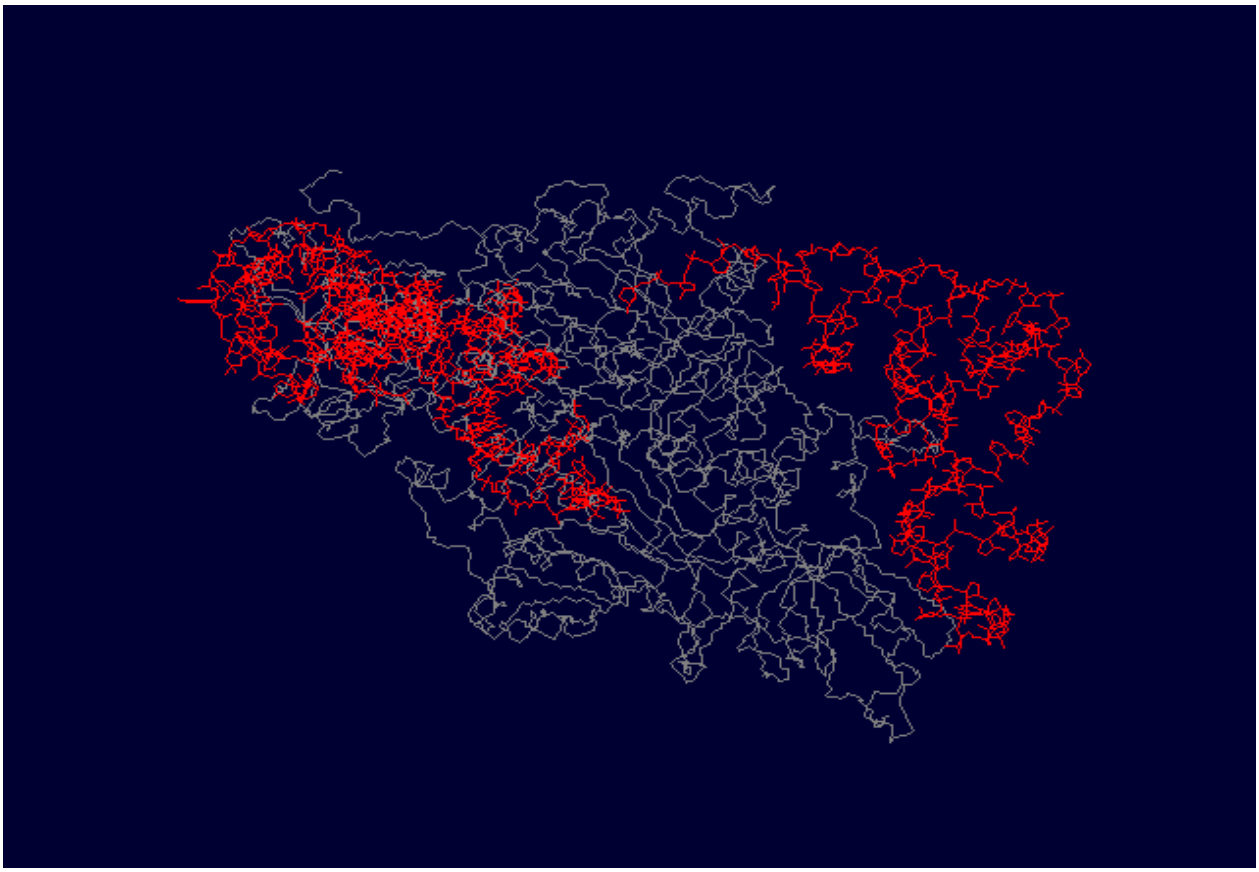


Fig.1. Crystal structure of yeast AspRS dimer complexed with two molecules of $tRNA^{Asp}$ (marked with red) (Ruff et al., 1991). The left tRNA molecule is shown without hydrogene bonds between the nucleotides.

Besides the crystal structure analysis, the footprinting assays were performed in order to study this complex (Rudinger et al., 1992). They have shown that nucleotide determinants of $tRNA^{Asp}$ (three anticodon bases, G73 and the pair G10-U25, (Putz et al., 1991)) are directly involved in the interaction with the protein. The bases of position 11, 12, 13, 29, 40 and 41 are also in direct contact with AspRS. For the complex of human LysRS with the cognate $tRNA^{Lys}$, the K_D was estimated as 30 nM (Francin et al., 2002). These findings provide a lot of information about the organization of subclass IIb aaRS complex with the corresponding tRNA that leads to aminoacylation. However, nothing is known about such a complex that results in tRNA import into mitochondria, tRK1•preMsk1p complex. We decided to perform the K_D measurement and to study the possible role of enolase 2 in the formation of this complex, since, from previously obtained data, it was known that enolase 2 somehow participates in the process of tRK1 targeting into mitochondria. We also did footprinting studies on both tRK1•Krs1p and tRK1•preMsk1p complexes in order to compare the profiles of tRK1 base protection in them.

One more interesting question would be to localize the regions of preMsk1p that provide the protein a unique property of tRNA import directing. It is known that in aminoacyl-tRNA synthetases of the IIb class are organized in a two-domain manner (Eriani et al., 1990), where the N-terminal domain is supposed to have strong tRNA binding properties (Giege et al., 1993), either non-specific, or specific (Frugier et al., 2000). Mammalian LysRS, when lacking its C-terminal domain, shows tRNA binding activity on the level that is comparable to that of full-sized enzyme (Francin et al., 2002). Probably, N-terminal domain of preMsk1p would also keep the tRNA-binding properties of the whole protein. In this case, it should be competent in tRNA import directing. We performed the mutagenesis of *MSK1* gene in order to verify our proposition and to find out the regions and/or structural elements of preMsk1p that are involved in tRNA import into mitochondria.

2.1.1 GEL-RETARDATION ANALYSIS OF tRK1-preMsk1p AND tRK1-Eno2p COMPLEXES

We used the approach of gel-retardation to primary analyze formation of complexes between tRK1 and preMsK1p. For this purpose, the recombinant *E. coli* - expressed proteins (Krs1p, for aminoacylaton of tRK1, and preMsk1p, for complex formation) and 5'-end labeled tRNAs were used. We demonstrated that preMsk1p forms a stable complex with the aminoacylated form of tRK1, but not with either deacylated tRK1 or aminoacylated or deacylated tRK2 (non-imported tRNA species). The K_D of the tRK1-preMsk1p complex was 180±20 nM, which is comparable with the K_D found for the complexes between tRNAs and non-cognate aminoacyl-tRNA synthetases. Enolase-2 (Eno2p), the second tRK1 import factor, is also capable to link tRK1, but with a significantly lower efficiency (K_D=2.5±0.2 μM). No ternary complex involving the tRNA and both proteins were detected, but the presence of Eno2p resulted in a dramatic drop of the K_D for the tRK1-preMsk1p complex (40±10 μM), which suggest Eno2p may have an RNA-chaperone function inducing the correct conformation of tRK1, which, in turn, facilitates its interaction with preMsk1p.

This work is presented in details in the attached **publication 2.**

Publication 2.

Entelis N, Brandina I, Kamenski P, Krasheninnikov IA, Martin RP, Tarassov I.

"A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in Saccharomyces cerevisiae."

Genes Dev. 2006, 20(12): 1609-20.

A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in *Saccharomyces cerevisiae*

Nina Entelis, Irina Brandina, **Piotr Kamenski,** Igor A. Krasheninnikov, Robert P. Martin et Ivan Tarassov

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Pages 1609 à 1620 :

2.1.2. ANALYSIS OF tRK1•preMsk1p COMPLEXES *in vitro*

Previous studies performed in our team permitted to characterize dozens of tRK1 and tRK2 version with very different capacities of import into isolated mitochondria (up to the efficiencies higher than that of the naturally imported tRK1) (Entelis et al., 1998; Entelis et al., 1998; Kolesnikova et al., 2002). A selection of these RNAs was tested by gel-retardation assay. We found that there exists a direct correlation between the ability of the RNA to form the complex with preMsk1p and its ability to be imported into mitochondria, while all the RNA versions that were not able to form such a complex, were not imported.

To further study the mechanism of formation of the "import directing" ribonucleic complex, we used the "footprinting" approach. FeEDTA-treatment was used to determine the sites of contact between the RNA and protein, while S1 and V1 nucleases were used to check if formation of the complex induced conformational changes in the RNA. It was demonstrated that tRK1 interacts with preMsk1p in a significantly different manner than does the cognate cytosolic lysyl-tRNA synthetase, Krs1p (Fig.2). Indeed, preMsk1p protects larger surfaces of the tRK1 molecules. This interaction appears not to change significantly the standard tRNA structure, with one notable exception: the anticodon helix becomes sligtly unpaired in the complex.

Taking into account that Msk1p is a IIb class synthetase, we tested the importance of the N-terminal domain for its tRK1 import direction activity. Surprisingly, we found that the N-terminal domain alone was able to direct tRK1 import *in vitro* with the efficiency lower but comparable to that of the full-sized protein. Moreover, the pattern of protection was globally similar when the whole preMsk1p or its N-terminal domain was used. Based on these results, we performed systematic deletions of the predicted structural elements (helices or β-sheets) in the N-terminal domain to search for the precise element controlling the tRK1 import. It was demonstrated that the shorter versions of preMsk1p N-terminal domain were also capable to direct the tRK1 import, but have lost any specificity and could promote import of all tested small-sized RNAs (Fig.3). Comparative analysis of import directing capacities of the recombinant versions of preMsk1p and of its N-terminal domain permitted to propose that the proximal part of the latter, especially the region H1-H3, was responsible for non-specific recognition of the RNA and may perform its import into isolated mitochondria, while the distal region H5-H6 were responsible for the specificity of interaction and may determine the discrimination between imported and non-imported tRNAs (Fig. 2 and 3B).

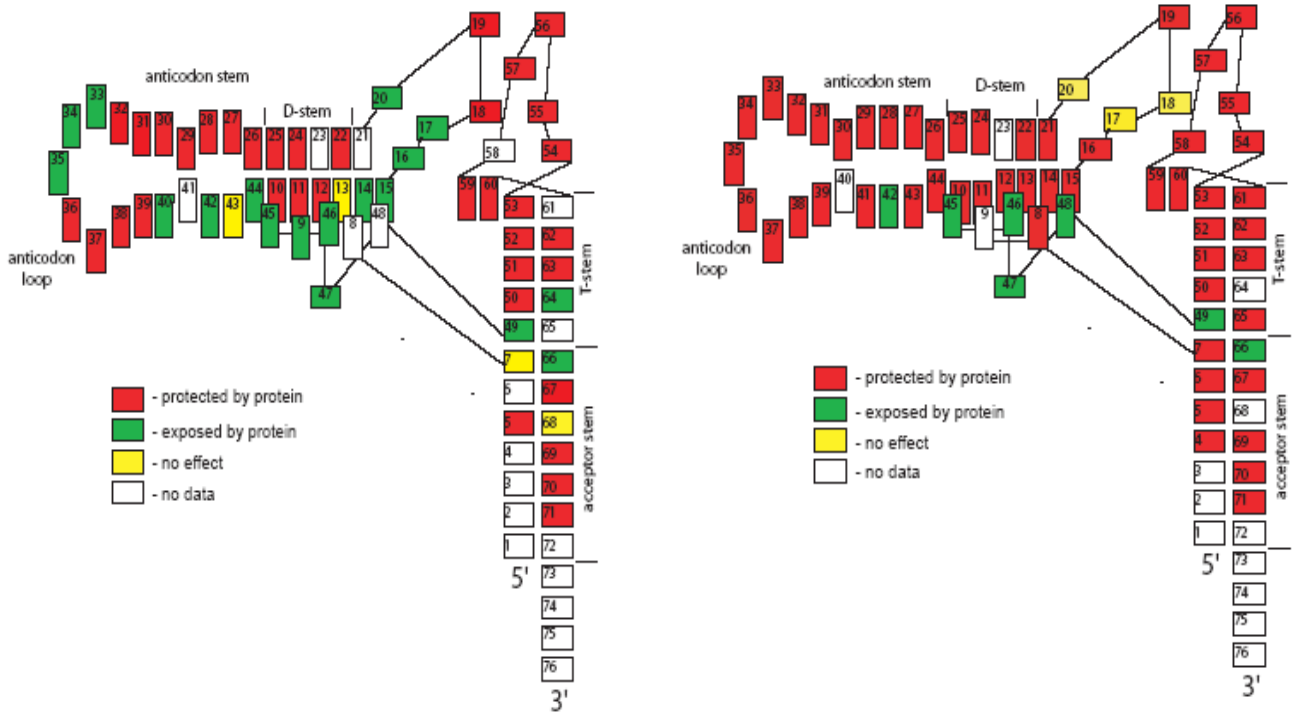

Fig. 2. The schematic representation of interaction of tRK1 with Krs1p (A) and preMsk1p (B).

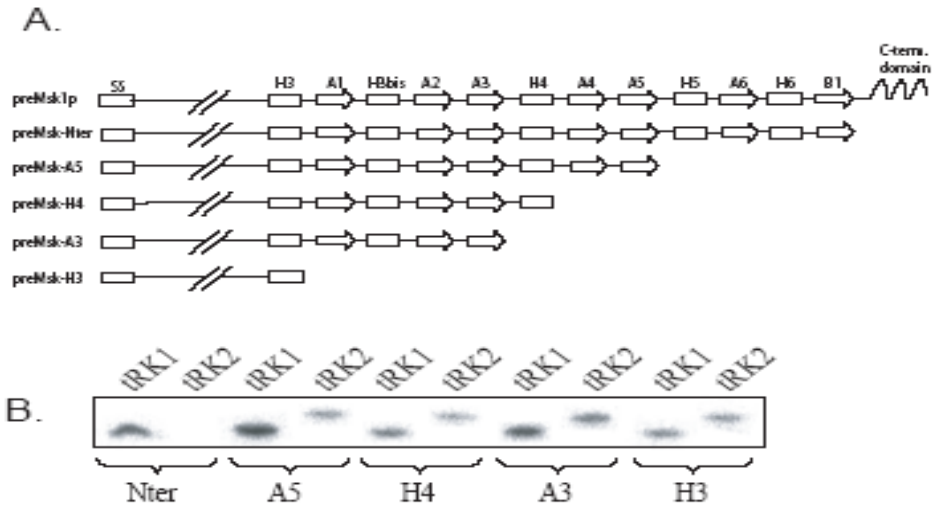

Fig.3. (A) The schematic view of mutant preMsk1p versions. (B and C) N-terminal domain of preMsk1p directs specific tRNA import while four shorter versions do it unspecifically.

2.1.3. ANALYSIS OF tRK1•preMsk1p INTERACTION *in vivo*

To validate the results of *in vitro* tests, several mutant versions of *MSK1* gene containing deletions in the region coding for N-terminal domain were expressed in the yeast strians were the original *MSK1* gene was deleted. Thereafter, we tested the presence of tRK1 in mitochondria or promitochondria (mitochondria that have lost their DNA). Deletions in the region H1-H3 did not affect tRK1 import, while deletion of the H5-H6 region inhibited it (Fig.4). Deletion of the whole C-terminal domain coding sequence of *MSK1* gene did not prevent the tRK1 import, while deletion of the whole N-terminal domain completely blocked it. These *in vivo* results are, therefore, in a perfect agreement with the *in vitro* assays. It is also to be noted that mitochondria of all these recombinant strains were not functional (promitochondria state, or rho° phenotype), presumably due to the absense of tRK3 aminoacylation in the organelle.

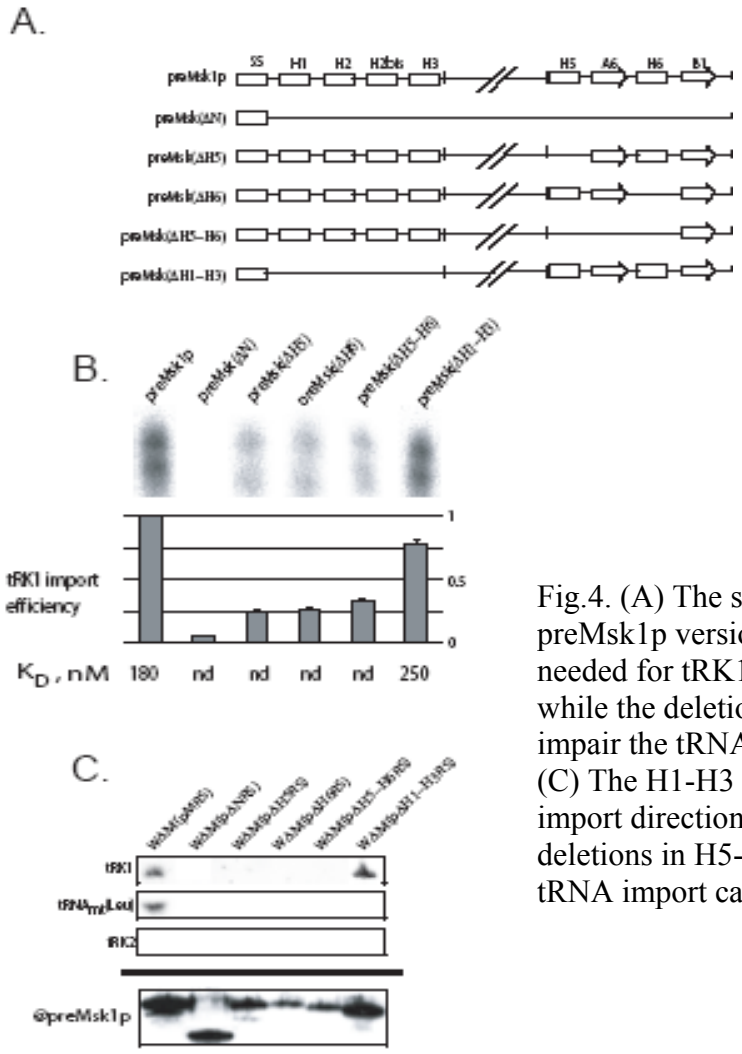

Fig.4. (A) The schematic view of mutant preMsk1p versions. (B) The H1-H3 region is not needed for tRK1 import direction *in vitro* (6th lane) while the deletions in H5-H6 region strongly impair the tRNA import capability (3rd-5th lanes). (C) The H1-H3 region is not needed for tRK1 import direction *in vivo* (6th lane) while the deletions in H5-H6 region strongly impair the tRNA import capability (3rd-5th lanes).

These results are described in details in the **publication 3** (attached below).

Publication 3.

Kamenski P., Entelis N., Dogan S., Krasheninnikov I.A., Martin R. P., Tarassov I.

"*Interaction of cytosolic lysine tRNA with the precursor of mitochondrial LysRS during tRNA import into yeast mitochondria*."

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The N-terminal domain of the mitochondrial lysyl-tRNA synthetase precursor is sufficient for $tRNA^{Lys}$ targeting into yeast mitochondria

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ABSTRACT

In many species, nuclear DNA-encoded tRNAs are targeted into mitochondria. In *Saccharomyces cerevisiae*, one out of two cytosolic $tRNAs^{Lys}$ is partially internalized by mitochondria. Previous studies have shown that several components of protein import machinery are necessary for tRNA importation. Binding of tRNA to its putative protein carrier, the precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p) was found to be a pre-requisite of import. This work was aimed to the study of the complex imported tRNA - preMsk1p by biochemical and genetic methods. We show that preMsk1p strongly protects large surfaces of the imported tRNA in the tRNA-preMsk1p complex. Although preMsk1 presents all classical features of a IIb class aminoacyl-tRNA sythetase, the complex leading to mitochondrial import of the tRNA differs from the complex between the same tRNA and its cognate cytosolic aminoacyl-tRNA synthetase, leading to aminoacylation. We found that the N-terminal domain of preMsk1p with the adjacent hinge region was capable to direct the tRNA import. The hinge region between N- and C-terminal domains (including helices H5 and H7) was shown to be responsible for the discrimination between imported and non-imported RNAs, while shorter versions of the N-terminal domain were also able to direct RNA import but without any specificity.

INTRODUCTION

Mitochondria are essential organelles of almost all eukaryotic cells taking part in several critical cellular processes (1). They contain their own genome and the machinery for its transcription and translation. However, the vast majority of biological macromolecules functioning in the mitochondria are transported there from the cytoplasm. For instance, the total amount of mitochondrial protein species is about 850-900 while yeast mitochondrial genome codes for only 8 proteins (2). All other proteins are imported from the cytoplasm. The

mechanisms of protein import into mitochondria are described in detail and are more or less ubiquitous for all eukaryotes (3). The situation is different for RNAs: the very different pools of RNAs are imported into mitochondria of different organisms, and the mechanisms of this process are believed to be different in each case (4,5). Mitochondria of protozoan, fungi, plants and marsupials internalize tRNAs while in mammalians, 5S rRNA, RNA components of RNases P and MRP are imported. In *Saccharomyces cerevisiae*, only three cytoplasmic tRNAs were reported as targeted into mitochondria: one $tRNA^{Lys}$ (6) and two $tRNAs^{Gln}$ isoacceptors (7). The imported $tRNA^{Lys}_{CUU}$ (further, tRK1) is one out of three lysine tRNAs present in baker's yeast. The second one (tRK2) is located exclusively in cytoplasm, while the third one (tRK3) is encoded in the mitochondrial genome and takes part in mitochondrial translation. tRK1 import into yeast mitochondria is thought to be a very specific process since other isoacceptor $tRNA^{Lys}$ is not imported. The previous studies revealed that several nucleotides in tRK1 sequence, as well as its correct ternary structure, are necessary for its import (8-10). tRK1 import seems to be coupled with the process of protein import into mitochondria since at least two components of protein import apparatus, Tom20p and Tim44p, have been shown to participate in tRK1 importation (11). Finally, three cytosolic proteins were identified as "tRK1 import factors". The first is cytoplasmic lysyl-tRNA-synthetase (Krs1p) that aminoacylates tRK1, the latter being imported only in its aminoacylated form (12). The second is glycolytic enzyme enolase 2 (Eno2p), which is believed to serve as a transporter of tRK1 to the vicinity of mitochondria and facilitates the binding of tRK1 to the third protein factor, the precursor of mitochondrial lysyl-tRNA-synthetase (preMsk1p) (12,13). It is to be noted that interaction with the cytosolic precursor of a mitochondrial enzyme, preMsk1p, does not lead to aminoacylation of tRK1, which should be performed by Krs1p. Therefore, we expected a particular way of interaction between tRK1 and preMsk1p.

In the present study, we show that there is a direct correlation between the ability of mutant tRNAs to be imported into yeast mitochondria and the stability of complexes of these tRNAs with preMsk1p. We found that preMsk1p protects large surfaces of imported tRNAs from nucleases. Our results also indicate that ribonucleoproteic complexes whose formation leads either to tRNA aminoacylation or to its mitochondrial import are not organized in the same manner. N-terminal domain of preMsk1p with its adjacent hinge region (amino acids 92-245) was shown to be essential and sufficient to provide tRK1 import and the region responsible for the specificity of this import was localized. Taken together, these results give new insights on the mechanisms of RNA-protein interactions leading to tRNA import into yeast mitochondria.

MATERIALS AND METHODS

Plasmids and oligonucleotides.

Plasmids used and their brief description are presented in the Table 1. pMRS416 plasmid represents the full functional *MSK1* gene with its own promoter and 1kb flanks in pRS416 shuttle vector (*URA3*-marker) (14). pMRS313 plasmid represents the full functional *MSK1* gene with its own promoter and 1kb flanks in pRS313 shuttle vector (*HIS3*-marker). This plasmid was used to mutagenize *MSK1* gene using QuikChange mutagenesis kit (Stratagene). pET-Msk1 plasmid contained the *MSK1* ORF in frame with 6 His-coding codons at the C-terminus in the pET3a vector. This plasmid was also used for mutagenesis of the *MSK1* gene in order to create truncated recombinant proteins. pET-Msk1-Nter plasmid contained the same construct but with the C-terminal domain (from N245 to the stop codon) of Msk1p deleted.

The following oligonucleotides were used: for Northern analysis – anti-tRK1 TGGAGCCCTGTAGGGGG, anti-tRK2 – TGGCTCCTCATAGGGGG, anti-$tRNA_{Leu}$(mt) – CCCGAATTCCCTGGTTGCTATTTAAAGGAC, for creation of ΔN deletion – CGATGACCGACGTCCAGAGAATACGGCCTGCTCC and CGATGACCGACGTCGAGGTTGAAACGCCTATACTG, for creation of ΔH5 deletion – GTAATAAGCTACCTATAGTATTGTCTGTTTCGAATAGAGTTGTTGACTACCAATTA AACGGC and fully complementary oligonucleotide, for creation of ΔH7 deletion – CAAATCTAATAGAGTTGTTGACTACCAATTAAACTTCGTTGAGGTTGAAACGCCT ATACTG and fully complementary oligonucleotide, for creation of ΔH5-H7 deletion – GTAATAAGCTACCTATAGTATTGTCTGTTTCGAACTTCGTTGAGGTTGAAACGCCT ATACTG and fully complementary oligonucleotide, for creation of ΔH1-H3 deletion – CGAAGTAGCAGGAGCAGGCCGTATTCTCTGGGCAACCCAGCCACATTACACCATG AGG and fully complementary oligonucleotide, for cloning of preMskA5 – TACTACCTCGAGCGGCAGTTGCGAAACAGACAA, for cloning of preMskH4 – TACTACCTCGAGTCCTTTCTTCAAAAAGTTCAT, for cloning of preMskA3 – TACTACCTCGAGCCCACCAATTTTATTGTAGTT, for cloning of preMskH3 – TACTACCTCGAGATTAGGATTATCCTCATGGTG.

Strains and media

As an initial yeast strain, we used the haploid strain WΔM(pMRS) (W303 MATa, *msk1*::KanMX4 (pMRS416)) (14). It was transfected with the plasmids carrying one of the mutant *MSK1* gene versions in pRS313 vector as in (15). The pMRS plasmid was then removed by counter-selection. The resulting strains are presented in Table 2.

Epicurean coli strain BL21 RIL3 Codon Plus (Stratagene) was used for over-expression of recombinant proteins. *Escherichia coli* strain XL10-Gold (Stratagene) was used

for site-mutagenesis. *Escherichia coli* strain XL1blue (Stratagene) was used for cloning. Cultivation was performed in standard LB medium with appropriate antibiotics. for over-expression of proteins, the medium also contained 0.4% of D-glucose. For cultivation of yeast, we used YPD (contained 2% of D-glucose) or YPEG (contained 3% of glycerol and 2% of ethanol) media, as well as synthetic media (Bio101) contained the mixture of amino acids and nucleotides without one or two components (for the selection of the plasmid(s) contained the corresponding marker gene(s)). For exclusion of *URA3*-containing plasmid we used 5-FoA medium (yeast nitrogen base 6.7g/l, CSM-ura 0.77mg/l, uracile 30mg/l, glucose 20g/l, 5-fluoroorotate 1g/l). To characterise respiratory capacities of the yeast strains, the media contained glycerol, ethanol, or lactate as carbon source instead of glucose or galactose (16).

Recombinant proteins

For the induction of expression, 1mM of IPTG was added to the medium, than cells were cultivated 2-4 hours at 25-30°C. After cell lysis in presence of 8M urea, proteins were purified to 90-95% of purity by the affinity chromatography on Ni-NTA agarose (Qiagen) using the manufacturers' protocol and dyalized. Protein fractions were analysed by PAAG-electrophoresis using the Laemmli system (17).

Analysis of RNA-protein complexes

Gel-purified RNAs were 5'-end labelled with γ-^{32}P-ATP by T4 polynucleotide kinase (New England Biolabs). For footprinting, 3'-labelling was also performed: 8-10 μg of tRNA were treated with 0.02 U of phosphodiesterase in presence of 50mM Tris-HCl pH 8 and 10mM $MgCl_2$ for 15 minutes at room temperature. tRNAs were then 3'-labelled with 200-300 μCi of α-^{32}P-ATP in presence of 5 mM Tris-Glycine pH 8.9, 8 mM DTT, 7.2 mM $MgCl_2$,

0.05 mM CTP and 10 U of CCA-transferase (gift form L. Maréchal-Drouard, Strasbourg) for 1 hour at 37°C. Labelled tRNA were gel-purified.

Gel-retardation assay and K_D measurement were done as described in (13).

Treatment of RNA-protein complexes with T1 nuclease and alkaline treatment (for obtaining "alkaline ladder") were done as described in (18). S1 and V1 nucleases treatment was done as described in (19). For the treatment with Fe^{2+}/EDTA/H_2O_2, 20 cpm (0.1-0.5 μg) of tRNA were mixed with 0.5 μg of *E. coli* tRNAs and heated for 1 minute on 90°C. Then the proper protein was added (with molar ratio tRNA:protein being 1:1 or 1:5) and complex formation was done in the buffer containing 50 mM Na-cacodylate pH 7, 270 mM KCl, 20 mM $MgCl_2$ and 0.02% BSA for 10 min at 30°C. After this, 1 μl of each of the solutions listed below was added: 50 mM $(NH_4)_2Fe(SO_4)_2$, freshly prepared 2.5% H_2O_2, 0.25 M DTT and 0.1 M EDTA pH 4. The mixture was incubated for 5 min at room temperature, and then 10 μl of 0.1 M thiourea and 1 μg of *E.coli* tRNAs were added and RNAs were ethanol precipitated. For separation of footprinting products, 15% or 20% PAAG was used. The products were visualized with Fuji Imager and computer program MacBas2000.

Mitochondria isolation, *in vitro* and *in vivo* tRNA import assays

Isolation of mitochondria and import tests were done as described in (20). Isolation of promitochondria (non-functional mitochondria of *rho*° yeast strains) was performed as described in (21) with modifications. For that, the cells were cultivated on glucose-containing medium, washed twice with 10 mM Tris-HCl pH 7, 250 mM KCl and resuspended in 0.2 M sodium-phosphate buffer pH 6.2, 1.35 M sorbitol, 1 mM EDTA (10 ml per gram of cells). Zymolyase 20T was added to the suspension (20 mg per gram of cells), and the yeast were incubated for 1 hour at 37°C with periodical shaking. The resulting spheroplasts were washed three times with large volumes of 10 mM Tris-HCl pH 6.8, 0.75 M sorbitol, 0.4 M mannitol,

0.1% BSA. The spheroplasts were disrupted with a Potter homogenizer at 4°C going forward and backward 30-40 times. All further manipulations (differential centrifugation, RNAses treatment, purification on sucrose gradient and mitochondrial RNA extraction) were done according to (20).

RESULTS

Correlation between tRNA import and the stability of its complex with preMsk1p

We previously have shown that prior the import, the aminoacylated form of tRK1 forms a stable complex with preMsk1p (22) with the apparent K_D of 180 nM (13). By tRNA-binding assay, it was demonstrated that all imported tRK1 versions capable to form this complex, while all versions that have no affinity to preMsk1p are not imported (22). Here, we measured K_D of complexes between tRK1 versions and preMsk1p by a gel-shift assay (Fig. 1). One can see that the imported aminoacylated form of tr1 (T7-transcript of tRK1 gene) forms a complex with recombinant preMsk1p with a K_D=180 nM, while its non-imported deacylated form does not (Fig. 1A,B,D). The mutant version of tRK2, tr93, previously shown to be importable in its deacylated form (8,23), also generates a complex with preMsk1p being deacylated (Fig. 1C,D). Similar assays were performed for several other tRNAs and transcripts (summarized in Table 3). As expected, only those tRNAs that were capable to form a stable complex with preMsk1p are imported into isolated yeast mitochondria. The K_D of these complexes (180-280 nM) were 5-7 times higher than that described for mammalian Lysyl-tRNA synthetases (LysRS) interacting with cognate deacylated $tRNA^{Lys}$. On the other hand, these K_D were comparable to that described for the complexes between the mammalian enzyme with separate domains of the cognate tRNA (mini-helices) (24). The K_D for non-imported mutant RNAs were >1 μM. These results suggested that the mechanism of

interaction tRK1 - preMsk1p and tRK1 - cognate Krs1p are different. On the other hand, on could suggest that interaction between tRK1 and preMsk1p may be similar to that between the mini-helices of the RNA^{Lys} with the LysRS.

Probing the tRNA-protein complexes

Chemical and enzymatic probing was used to characterize tRNA - preMsk1p complexes (Fig. 2). Probing of labelled tRK1 complexed with preMsk1p by Fe^{2+}/EDTA/H_2O_2 and with nucleases S1 and V1 was used in order to identify tRK1 regions protected by protein. S1 nuclease is known to cleave only single-stranded RNA regions, while V1 nuclease recognizes double-stranded or stacked nucleotides. In parallel, to compare the tRK1-preMsk1p complex with the "normal" aaRS-tRNA ones, we probed the complex of deacylated tRK1 with the cognate cytoplasmic lysyl-tRNA synthetase, Krs1p. Finally, the deacylated transcript tr93 was also analysed. An example of the gel separation of probing products is presented on Fig. 2. One can clearly see the difference between complexes of tRK1 with Krs1p (Fig.2, second and third lanes on each set of samples) and preMsk1p (Fig.2, fourth and fifth lanes on each set of samples), especially, in case of Fe^{2+}/EDTA treatment which is not specific to any secondary structure elements and indicates clearly the protection of tRNA nucleotides by protein. The resulting models representing the interaction of tRK1 with Krs1p, tRK1 with preMsk1p and tr93 with preMsk1p, are shown on the Fig. 3. The main difference concerns interaction of LysRS with the anticodon loop of tRK1, which is protected by preMsk1p and exposed upon interaction with Krs1p. Krs1p protects the 5'-part of the anticodon - D-stem region. This is in agreeement with the published data on the tRNA complex with another IIb class aaRS, the yeast $tRNA^{Asp}$-AspRS couple (25,26). The effect "exposure" of the nucleotides 33-36 is also in agreement with the crystallographic model demonstrating that interaction with the aaRS alters the anticodon conformation (27-30).

In contrast with Krs1p, preMsk1p protects the most of the tRK1 molecule, including nucleotides that were not protected by Krs1p (D-loop and anticodon loop). Interaction with preMsk1p also induces alteration of the secondary structure of tRK1: the stacking of the anticodon and D-stem nucleotides became weaker. Probing experiments with tr93 showed that, despite the fact that tr93 is a mutant imported version of tRK2 and its nucleotide sequence strongly differs from that of tRK1, the manner of preMsk1p interaction with the deacylated tr93 was almost the same as with aminoacylated tRK1 (Fig. 3). The only notable differences detected were: nucleotides 7, 17 and 20 are “exposed” in tr93, while nucleotide 42 is protected in tr93 and exposed in tRK1. These results indicate that the imported tRNAs with a different nucleotide sequence form the similar complexes with preMsk1p, which differ from the cognate aaRS-tRNA ones.

Effects of deletions in preMsk1p on tRNA import *in vitro*

PreMsk1p 3D structure was *in sillico* modelised by analogy with the known crystallographic structure of *E. coli* LysRS (31,32) and by analysing multiple alignments of preMsk1p primary structure with all known LysRS (33). The preMsk1p predicted structure revealed to be approximately similar to that of the bacterial enzyme. As all IIb class aaRS, preMsk1p possesses two distinct structural domains, anticodon-binding N-terminal one and the conserved C-terminal catalytic one, linked by the "hinge" region (Fig. 4).

We found that the purified recombinant N-terminal domain with additional structural elements of the hinge region (from the N-terminal presequence to the H7 helix, aminoacids 1-245 termed thereafter as preMsk-N) conserves the capacity to direct tRK1 into mitochondria *in vitro* with an efficiency comparable to that of a whole protein (Table 4, Fig. 5A,B). In order to localize the parts of preMsk1p molecule that are responsible for tRNA import direction, four truncated versions of preMsk-N, each lacking several structural elements in the C-

terminal part of the N-terminal domain, were analysed by *in vitro* import assays (Fig. 5A). The recombinant tagged proteins were expressed in *E. coli* cells, purified and tested for their ability to direct ^{32}P-labeled tRNAs into isolated mitochondria in the presence of protein extracts from the yeast strain lacking *MSK1* gene (WΔM). preMsk-N directed the import of aminoacylated tRK1, but not tRK2, conserving therefore the specificity observed *in vivo*. In contrast, all four truncated versions were capable to direct import of tRK1, but were not capable to discriminate between versions imported or non-imported *in vivo*, *i. e.* tRK1 versus tRK2 (Fig. 5B). Indeed, all other tested small-sized transcripts were also imported into isolated mitochondria in presence of the truncated versions of preMsk1p (Fig. 5C). Comparison of import directing capacities of the preMsk-N recombinant protein and its shorter versions (Table 4) clearly shows that if preMsk-N preserves the preMsl1p specificity in tRNA import diection, its truncated versions have lost it. One can suggest that the truncated versions have lost structural elements responsible for specific tRNA discrimination leading to the import. Since the main difference of the largest of the truncated versions and the preMsk-N is the presence of the hinge region, one can deduce that the latter (elements H5, A6 and H7, see Fig. 4) is responsible for the specificity of tRK1 import.

Interaction of the preMsk1-N protein with tRK1 was then analyzed by the probing (Fig. 3). Sirprizingly, preMsk1-N is capable to protect all the anticodon-D part of the tRNA including D- and anticodon-loops. We observed that the manner by which preMsk1-N interacts with aminoacylated tRK1 is similar to that of the full-sized protein. In the same way, tRNA import leading complex formation does not induce the exposure of the anticodon loop nucleotides, as it ws shown for interaction with the cognate aaRS leading to aminoacylation.

To check whether the deletions of different structural elements of the N-terminal domain and of the hinge region have the same effect on tRNA import in the context of the whole protein, several recombinant versions of preMsk1p with the deletions either in H5-H7

region or in H1-H3 region were constructed (Fig. 6A) and tested for their capability to form stable complexes with aminoacylated tRK1. The mutant version of preMsk1p with the whole N-terminal domain deleted (preMskΔN) was not able either to bind tRK1 or to direct its import *in vitro*. For shorter deletions, we observed that only preMsk(ΔH1-H3) could form stable complex with the K_D=250nM (Fig. 6B, Table 3), whereas all other proteins did not form any detectable complex with tRK1 (within the protein concentration range up to 1 μM). When tested for their capability to direct tRK1 import *in vitro*, all deletant versions proved to be poor importers, with one notable exception. Indeed, deletion of the first four N-terminal helices (amino acids 32 - 85, See Fig. 4) only slightly affected the import directing capacity of the version preMsk(ΔH1-H3) (Fig. 6C). In contrast, any deletion in H5-H7 linker region of preMsk1p strongly affected the ability of protein to interact with tRK1 and to direct its import.

Effects of deletions in preMsk1p on tRNA import *in vivo*

To validate the *in vitro* data described above, tRK1 import was measured *in vivo*, in the cells expressing preMsk1p containing the same deletions instead the wild type protein (Fig. 6A). Recombinant yeast strains were first analyzed for mitochondrial respiration by comparison of growth on the media containing non-fermentable carbon sources. We found that no one of the recombinant strains could grow either on glycerol-containing medium (Fig. 7A) or other media with non-fermentable carbon sources (ethanol, lactate, data not shown). At the same time, the growth on glucose-containing medium was not affected by the deletions. On the other hand, non-respiring cells depleted of mitochondrial DNA (*rho*° phenotype) normally still contain non-functional mitochondria (21). Indeed, Northern analyses performed with RNA isolated from promitochondria of recombinant yeast strains reveal that the only mutant version of preMsk1p still able to direct tRK1 import *in vivo* is preMsk(ΔH1-H3) (Fig.

7B), which is the same version that was capable to direct tRK1 import *in vitro*. All other deletant versions did not direct tRNA import *in vivo* (Fig. 7B), in spite of the fact that they were successfully expressed (Fig. 7C). Taken together, these results indicate on the importance of H5-H7 region of preMsk1p for the process of tRK1 mitochondrial import.

DISCUSSION

In yeast, only a very limited tRNA set (three species) are imported into mitochondria, which suggested existence of a stringent mechanism of discrimination. Previous data on $tRNA^{Lys}_{CUU}$ (tRK1) import strongly suggested that cytosolic proteins are responsible for such a discrimination (8,9,23,34). The case of the two imported $tRNA^{Gln}$ isoacceptors is less evident, since it was reported that they can be internalized by isolated mitochondria in a protein-independent manner (7). So far, even for this last case, there was no evidence that some targeting protein factors could not be involved in import *in vivo*. The essential import factor for tRK1 was previously identified as preMsk1p, which is a mitochondrially-targeted pre-protein and the precursor of lysyl-tRNA synthetase, whose primary function is to aminoacylate the mitochondrially transcribed tRK3. preMsk1p, though able to form stable complexes with tRK1, was not able to aminoacylate it (12). The second tRK1 import factor, Eno2p, was hypothesized to serve as an RNA-chaperone, providing both mitochondrial targeting of tRK1 towards the peri-mitochondrially synthesized preMsk1p and facilitating formation of the preMsk1-tRK1 complex (13). The fact that to target tRK1 into mitochondria, the cell uses a non-cognate aaRS and an RNA-chaperone, lead to the idea that the interaction between tRK1 and preMsk1p might be different from that previously described for cognate couples tRNA-aaRS, but still very specific, to provide discrimination between imported and non-imported tRNA species. Results described here are in a conformity with this hypothesis.

By probing RNA-protein complexes, we observed that large surfaces of the tRK1 molecules are involved in the interaction with preMsk1p. Protection was more pronounced than even by the cognate aaRS, Krs1p. This looks logical taking into account the negative charge of tRNA molecule which should be somehow screened from the mitochondrial membranes that are also negatively charged. Thus, preMsk1p may serve as a shield to protect tRK1 nucleotides from the contact with mitochondrial membranes. Furthermore, it is important to note that interaction with preMsk1p was possible only when tRK1 was aminoacylated, while with Krs1p - only when it was deacylated. This fact reflects different mechanisms of RNA-protein recognition, since the classical aaRS-tRNA complex dissociates following aminoacylation (35). As expected, we observed the difference in protection patterns between Krs1p and preMsk1p, especially in the region of the anticodon. In this context, two sets of data are to be discussed. In this study, we show that (i) the anticodon region is protected by preMsk1p and not protected by Krs1p; and (ii) the hinge region of preMsk1p is essential to provide the import specificity. On the other hand, we have previously shown that introduction of a single C34 instead of U in tRK2 makes this normally non-imported tRNA species capable of mitochondrial import, while replacement of the anticodon arm of tRK1 by that of tRK2 abort the import (22), thus the anticodon contains main import specificity determinants. If the hinge region is required for discrimination between tRK1 and tRK2 in terms of import, one could hypothesize that it may interact directly with the anticodon. Such interaction would be indeed strongly different from the classical aaRS-tRNA one, where the hinge region of the protein is localized in the vicinity of the variable loop of the tRNA (25,30).

The N-terminal domain of preMsk1p with the adjacent hinge region was shown not only to interact with aminoacylated tRK1, but also to be able to direct tRK1 import even in absence of the C-terminal domain. The fact that the N-terminal domain with the adjacent

hinge element of preMsk1p (amino acids 1-245) is essential to direct tRK1 import rises the question about the mechanism of specificity. Msk1p, as class IIb aaRS, has a bi-domain organization. In this family of aaRS, the N-terminal domain structure though less sequence-conserved than that of the catalytic C-terminal catalytic domain, possesses characteristic "oligonucleotide-binding" fold (OB-fold) (25,36). The functions of N-terminal domains were proposed as primary recognition of the tRNA and correct docking of the tRNA by interacting with the anticodon region (35,37,38). In eukaryotic aaRS, the N-terminal domain contains additional extensions, supposed to participate either in subcellular compartmentalization (39), or in specific or non-specific tRNA binding (40). As a matter of fact, mammalian LysRS has been shown to possess an N-terminal extension termed a tRNA-interacting factor (tIF) (41). The tIF element possesses an RNA-binding motif KxxxKK/RxxK that binds specifically to the TΨC stem/loop of the tRNA (42). Finally, our previous *in sillico* studies demonstrated that although preMsk1 is a typical mitochondrial/prokaryotic enzyme, potential structure of its N-terminal domain are even closer to the eukaryotic LysRS (14). Therefore, *a priori*, both specific as well as non non-specific tRNA-aaRS interactions may be relevant the tRNA import. Indeed, our data on tRK1 import directed by deletant forms of preMsk1p confirmed this hypothesis. Looking for the motifs of preMsk1p N-terminal domain responsible for specific tRNA import direction, we showed that the distal region limited by α-helices H5 and H7 (H5-H7 region), comprising the end of the N-terminal domain and the beginning of the hinge region is required for this process *in vitro* as well as *in vivo*. On the other hand, we also found a minimal preMsk1p sequence sufficient for tRNA import direction. This "minimal" import carrier is a part of molecule starting from its beginning (N-terminal signal presequence) and ending with the helix H3. Since in this case the import was non-specific, we suggest that this region of preMsk1p has unspecific RNA-binding activity. On the other hand, H1-H3 region is not needed for the specific tRNA import direction as it can be deleted in the

contest of the whole protein. It is possible that this region facilitates formation of an "active" complex but is not obligatory for it. We believe therefore that RNA-protein interactions leading to tRNA import might involve both non-specific interactions with the proximal part of the N-terminal domain, and sequence-specific interactions with the distal part of the same domain and the adjacent part of the hinge linker.

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Table 1. Plasmids used in the study.

Name	**Vector, genetic marker**	**Description of insertions**	**Reference**
pMRS416	pRS416, *URA3*	3 kpb *Xho*I-fragment with the *MSK1* ORF plus 1-kbp 5'- and 3'-flanks, saver plasmid	(14)
pMRS313	pRS313, *HIS3*	3 kpb *Xho*I-fragment with the full *MSK1* gene	this work
pMRSΔN	pRS313, *HIS3*	*MSK1* gene with the region coding for the N-terminal domain (aminoacids 2-245) deleted	this work
pMRSΔH5	pRS313, *HIS3*	*MSK1* gene with the region coding for the H5 α-helix (aminoacids 198-213) deleted	this work
pMRSΔH7	pRS313, *HIS3*	*MSK1* gene with the region coding for the H7 α-helix (aminoacids 223-244) deleted	this work
pMRSΔ(H5-H7)	pRS313, *HIS3*	*MSK1* gene with the region coding for region between helices H5 and H7 (aminoacids 198-244) deleted	this work
pMRSΔ(H1-H3)	pRS313, *HIS3*	*MSK1* gene with the region coding for the protein part between helices H1 and H3 (aminoacids 32-85) deleted	this work
pET-Msk1	pET3a, Amp^R	pre-Msk1p coding ORF under control of the T7 promoter and with the C-terminal 6-His tag	(14)
pET-Msk1-N	pET3a, Amp^R	The gene coding for N-terminal domain of preMsk1p (aminoacids 1-245) under control of the T7 promoter and with C-terminal 6-His tag	this work
pET-MskΔN	pET3a, Amp^R	pET-Msk1 with the region coding for the N-terminal domain of preMsk1p (aminoacids 2-245) deleted	this work
pET-MskΔH5	pET3a, Amp^R	pET-Msk1 with the region coding for the H5 α-helix (aminoacids 198-213) deleted	this work
pET-MskΔH7	pET3a, Amp^R	pET-Msk1 with the region coding for the H7 α-helix (aminoacids 223-244) deleted	this work
pET-MskΔ(H5-H7)	pET3a, Amp^R	pET-Msk1 with the region coding for the region between helices H5 and H7 (aminoacids 198-244) deleted	this work
pET-MskΔ(H1-	pET3a, Amp^R	pET-Msk1 with the region coding	this work

H3)		for the region between helices H1 and H3 (aminoacids 32-85) deleted	
pET-Msk1-A5	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and A5 β-element (aminoacids 1-194)	this work
pET-Msk1-H4	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and H4 α-helix (aminoacids 1-158)	this work
pET-Msk1-A3	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and A5 β-element (aminoacids 1-140)	this work
pET-Msk1-H3	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and H3 α-helix (aminoacids 1-92)	this work

Table 2. Strains used in the study.

Strain name	**Genotype, plasmid, genetic marker**	**Reference**
WΔM(pMRS)	W303 *msk1*::KanMX4 (pMRS416, *URA3*)	(4)
WΔM(pMRS313)	W303 *msk1*::KanMX4 (pMRS313, *HIS3*)	this work
WΔM(pΔNRS)	W303 *msk1*::KanMX4 (pMRSΔN, *HIS3*)	this work
WΔM(pΔH5RS)	W303 *msk1*::KanMX4 (pMRSΔH5, *HIS3*)	this work
WΔM(pΔH6RS)	W303 *msk1*::KanMX4 (pMRSΔH6, *HIS3*)	this work
WΔM(pΔH5-H6RS)	W303 *msk1*::KanMX4 (pMRSΔH5-H6, *HIS3*)	this work
WΔM(pΔH1-H3RS)	W303 *msk1*::KanMX4(pMRSΔH1-H3, *HIS3*)	this work

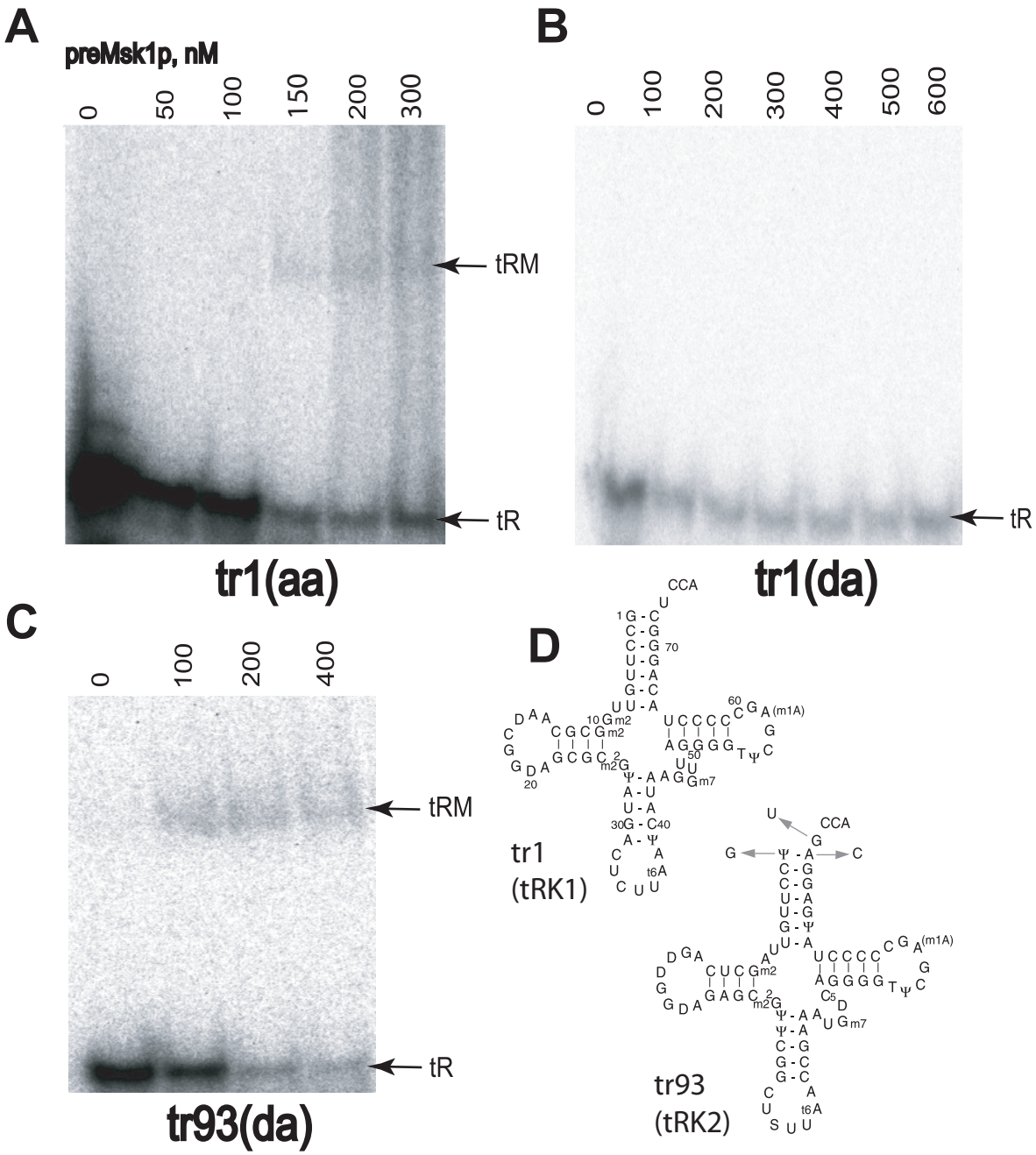
Table 3. Apparent K_D of the complexes between different natural and synthetic tRNAs and preMsk1p and their import efficiencies. (aa), aminoacylated form, (da), deacylated form.

RNA	Description	Import efficiency, % of that for tRK1aa from (10,22,23)	K_D, μM
tRK1	Cytoplasmic imported $tRNA^{Lys}_{CUU}$	100 (aa) 10 (da)	0.18 ± 0.04 (aa) >1 (da) (13)
tr1	T7-transcript of tRK1 gene	110 (aa) 10 (da)	0.28 ± 0.06 (aa) >1 (da)
tRK2	Cytoplasmic non-imported $tRNA^{Lys}_{UUU}$	<5 (aa or da)	>1 (aa or da)
tr7	T7-transcript of tRK1 gene where the anticodon arm (nucleotides 28-42) was replaced by the same region from tRK2	<5 (aa or da)	>1 (aa or da)
tr9	T7-transcript of tRK1 gene, C34G	<5 (aa or da)	>1 (aa or da)
tr93	T7-transcript of tRK2 gene with mutations: Ψ1G, A72C, G73U (cannot be aminoacylated by Krs1p)	85 (da)	0.25 ± 0.06 (da)

Table 4. The efficiencies of *in vitro* import of different RNAs directed by the truncated versions of preMsk1p (see Fig. 6). The efficiency of tRK1 import directed by the full-size preMsk1p was taken as 1. The standard deviation in all experiments (at least 3 independent) is ±0.1-0.2.

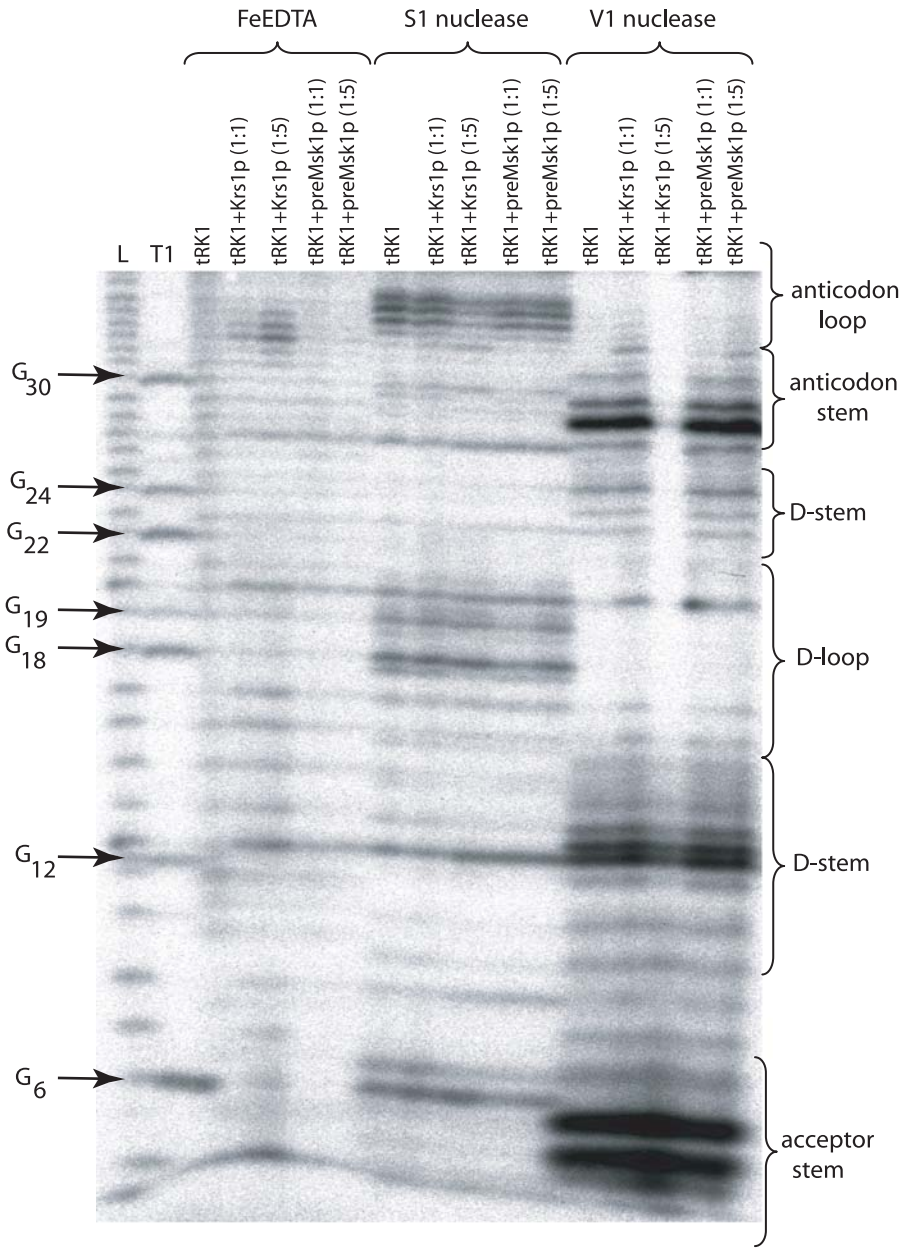
preMsk1p version / RNA species	N-ter	A5	H4	A3	H3
tr1	0,9	0,8	0,9	0,8	1,1
tr93	0,8	0,7	0,8	0,7	0,9
tRK2	-	0,6	0,7	0,7	0,9
tr7	-	0,9	0,7	0,7	1,1
tr9	-	0,9	0,9	0,9	0,8
Human 5S rRNA (transcript)	-	0,6	0,6	0,7	1
E.coli $tRNA_{Asp}$	-	0,8	1	0,6	0,9

Figure 1. The interaction of imported tRNAs with preMsk1p. (A) Gel-retardation of the T7-transcript of tRK1 gene (tr1) in its aminoacylated form (aa) complexed to preMsk1p. (B) Gel-retardation assays of the same transcript in its deacylated form (da). (C) Gel-retardation of the mutant version of tRK2 (tr93, see Table 1) in its deacylated form bound to preMsk1p. The arrows indicate: tR, free tRNA, tRM, tRNA-preMsk1p complex. The concentrations of preMsk1p dimer (shown above the autoradiograph). (D) Cloverleaf structures of tRK1 (imported) and tRK2 (non-imported). The T7-transcript of tRK1 gene (tr1) and the mutant tRK2 T7-transcript with three mutations indicated by the arrows (tr93) were used for gel-retardation assays.



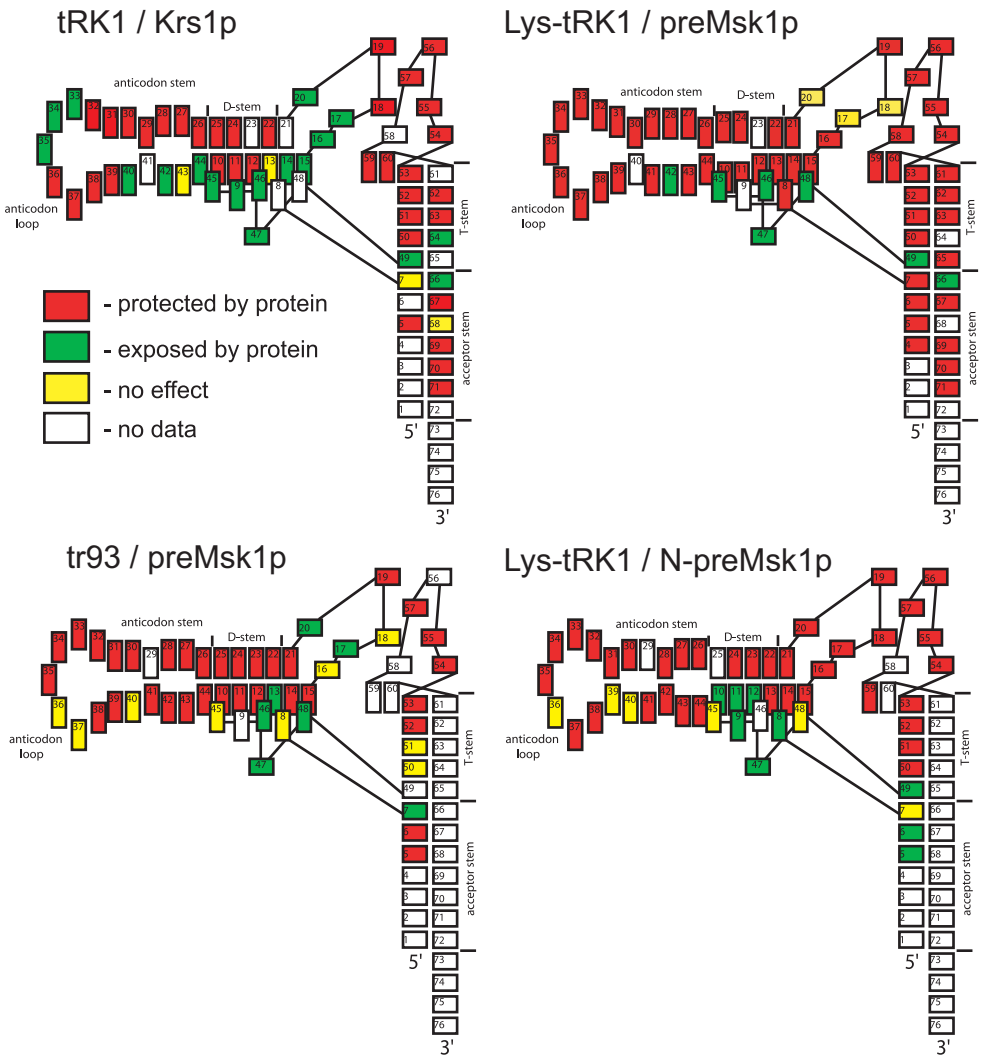
Kamenski et al.
Fig. 1

Figure 2. Enzymatic and chemical probing of RNA-protein complexes. Autoradiograph of 15% denaturing PAAG - separation of the products of tRK1 cleavage by Fe^{2+}/EDTA/H_2O_2, by nuclease S1 or by nuclease V1 (indicated above). tRK1 was either free, or bound to Krs1p, or to preMsk1p. To analyse its interaction with preMsk1p, tRK1 was aminoacylated by Krs1p and Krs11p removed thereafter, while to analyze its interaction with Krs1p, it was deacylated. L, alkaline ladder; T1, the products of cleavage by nuclease T1. 1:1 and 1:5, RNA:protein molar ratios. The tRNA secondary structure elements are indicated on the right. The arrows on the left indicate the guanosine residiues in tRK1 sequence.



Kamenski et al.
Fig. 2

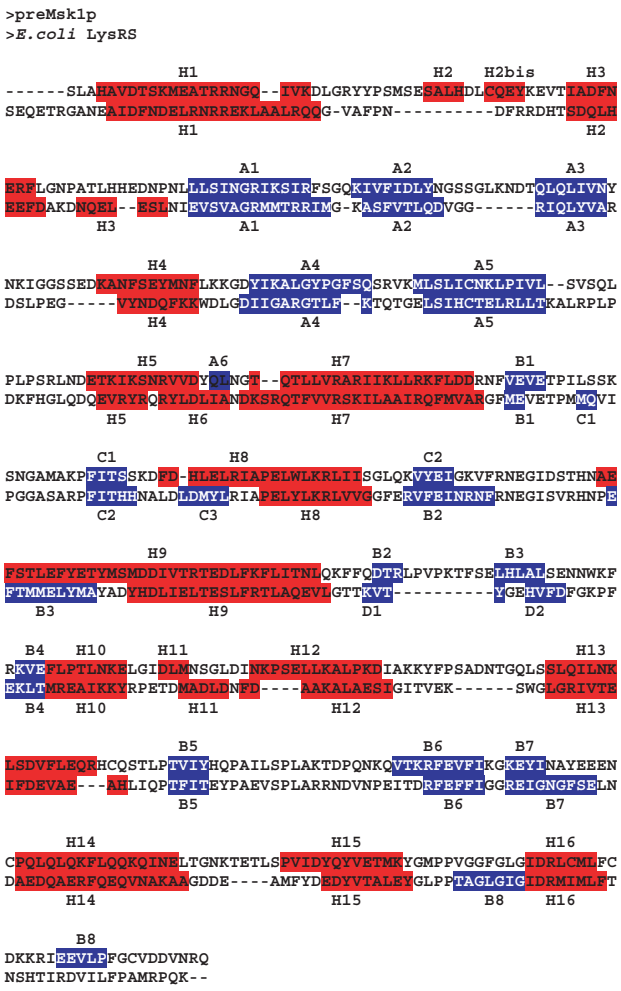
Figure 3. The schematic representation of the patterns of tRNA protection by recombinant proteins. The L-shape representation of the tRNA is according to (43). Structural elements of the tRNA molecules are indicated and the nucleotides numbered in a standard way (44). Patterns of protection presented correspond to aminoacylated tRK1 with preMsk1p, deacylated tRK1 with Krs1p, deacylated tr93 with preMsk1p and aminoacylated tRK1 with preMsk1-N protein. The efficiency of protection is shown by colors, as indicated.



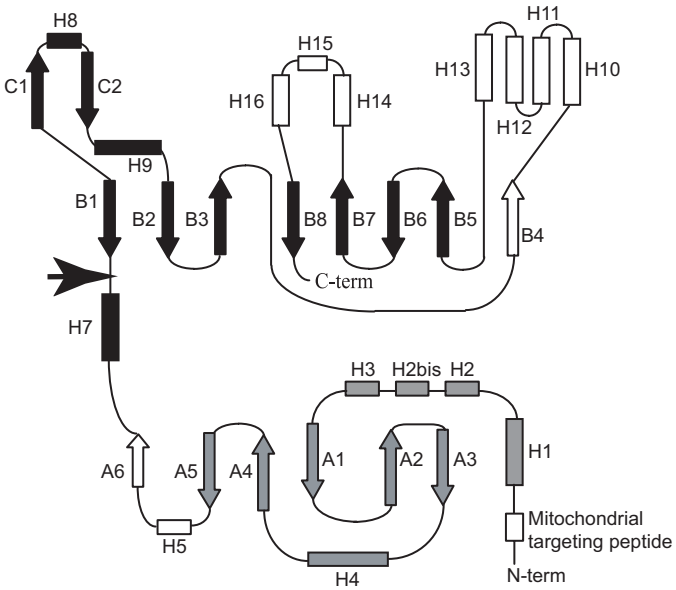
Kamenski et al.
Fig. 3

Figure 4. The topological diagram of preMsk1p molecule organization predicted *in silico*. Prediction was performed using the known *E.coli* LysRS structure and the 3D-PSSM platform (45). (A) Alignment of mature Msk1p primary sequence with *E. coli* LysRS. Structural elements, as determined by crystallography for the *E. coli* enzyme (32), and predicted for Msk1p are outlined by color: helices in red, β-structures - in blue. (B) Topological diagram of the preMsk1p structure. C-terminal active site elements, conserved in all class II aaRS are in black; the N-terminal anticodon-binding domain with characteristic OB-fold are in grey; hinge region and insertions sequences are in white. Rectangles indicate the helices (H), the arrows – β-elements, according to (31,32). The arrow between H7 and B1 indicates the boarder of the truncated N-terminal version of preMsk1p.

A

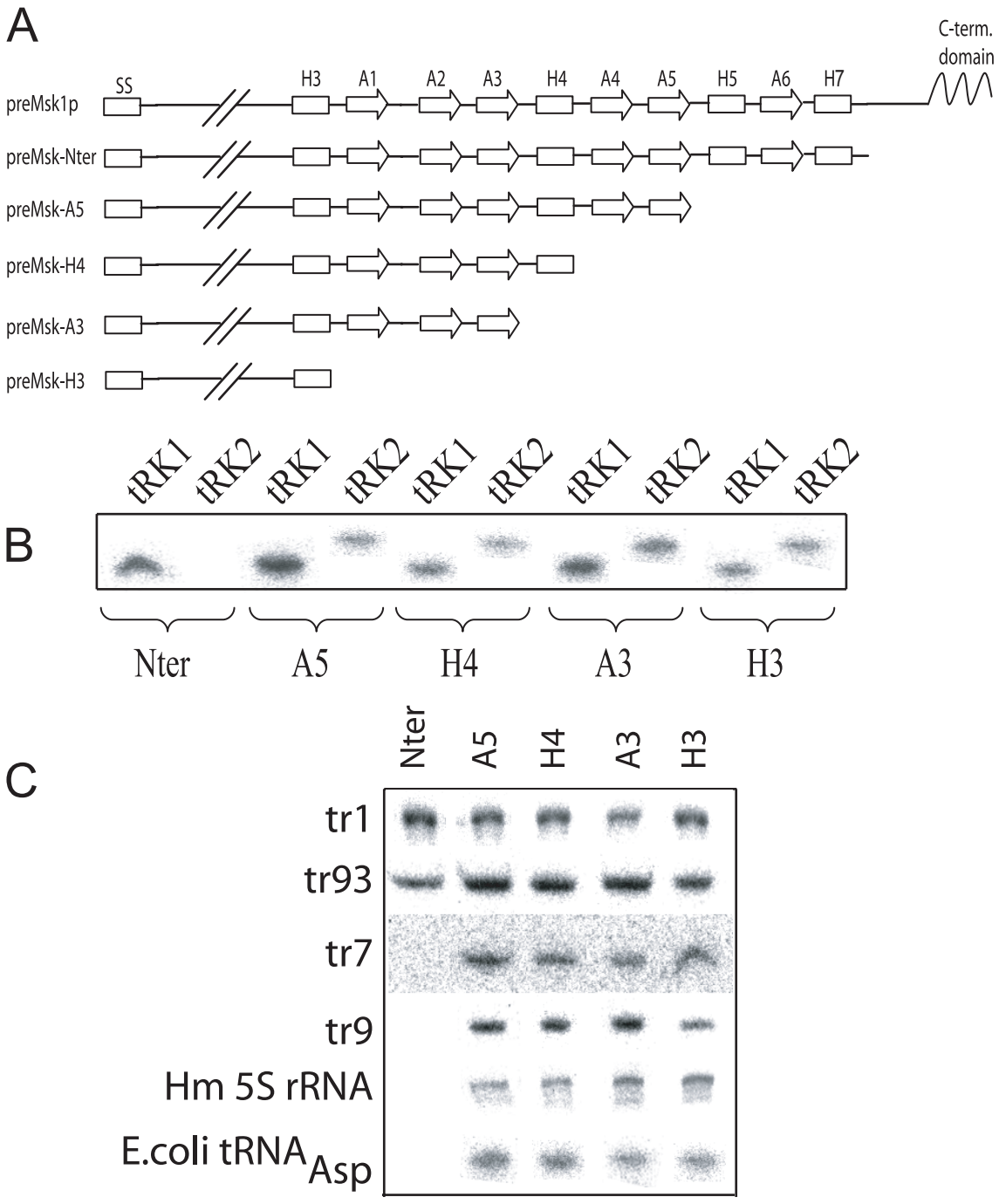


B



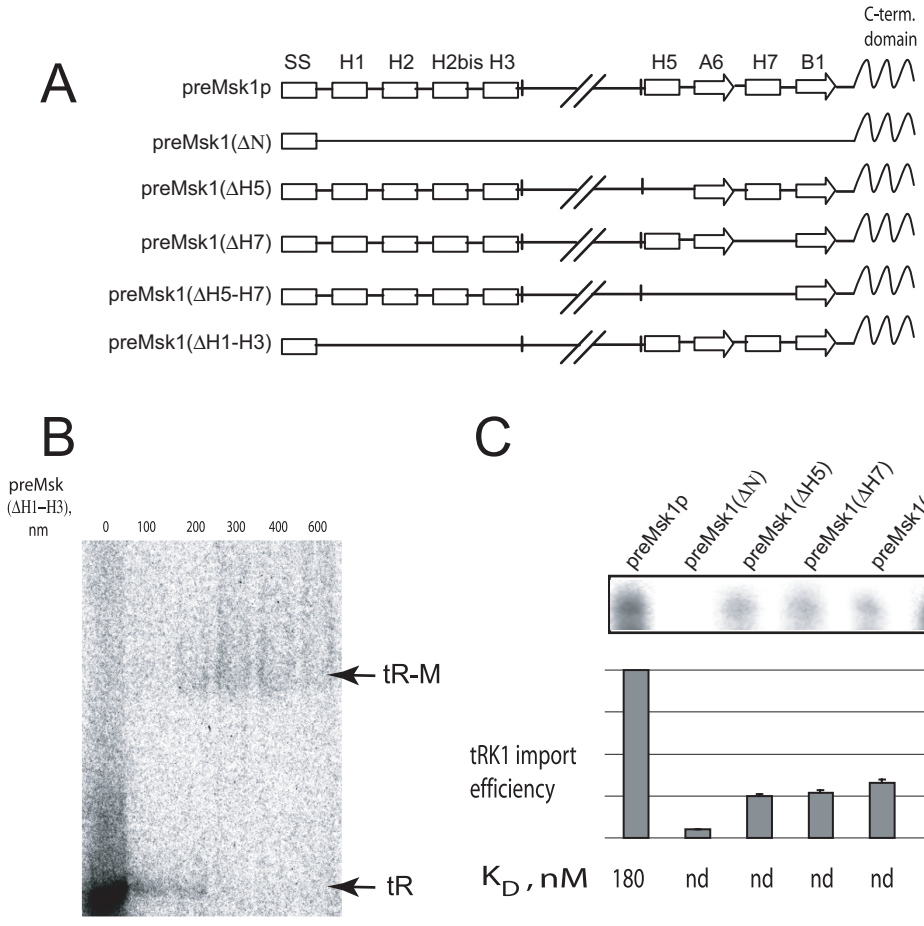
Kamenski et al.
Fig. 4

Figure 5. tRNA mitochondrial import directed by truncated versions of preMsk1p. (A) The schematic representation of the truncated versions of preMsk1p. SS, the N-terminal signal sequence directing the import of the protein into mitochondria. The region between SS and H3 (see Fig. 4) is not shown. (B) The autoradiograph of the gel separation of mitochondrial RNA after the import of labeled tRNA (tRK1 or tRK2, both in aminoacylated form) into isolated mitochondria. The import was performed in presence of protein extracts lacking preMsk1p and one of five truncated versions of preMsk1p (as indicated under the autoradiograph). (C) The results of *in vitro* import assays with different RNA species (the RNA is indicated on the left, the recombinant protein - above).



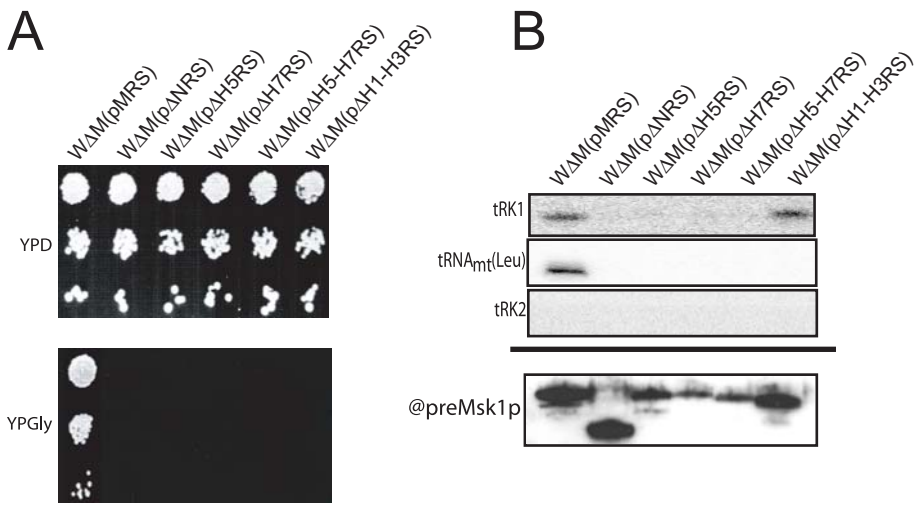
Kamenski et al.
Fig. 5

Figure 6. The effect of deletions in the N-terminal domain of preMsk1p on its capacity to direct tRK1 import *in vitro*. (A) Schematic representation of the mutant versions of preMsk1p (as in Fig. 5). The region between H3 and H5 (see Fig. 4) is not shown. (B) Gel-retardation of aminoacylated tRK1 bound to preMsk(ΔH1-H3). The arrows indicate: tR – free tRNA, tRM – tRNA complexed with the mutated protein. The concentrations of the protein in nanomoles are indicated above the autoradiograph. (C) The autoradiograph of the PAAG-separated mitochondrial RNA after the import of aminoacylated tRK1 into isolated mitochondria. The import was directed by the wild-type preMsk1p or by one of five truncated versions of the protein (as indicated above). The quantified efficiencies of the *in vitro* tRK1 import and K_D of mutant proteins complexes with tRK1 are shown under the autoradiograph.



Kamenski et al.
Fig. 6

Figure 7. The effect of deletions in the N-terminal domain of preMsk1p on its capability to direct tRK1 import *in vivo*. (A) Growth of the yeast strains expressing mutated versions of preMsk1p instead of the wild-type protein on glucose- and glycerol-containing media (YPD and YPGly, correspondingly). (B) Northern-blot hybridization of promitochondrial RNA isolated from the mutant yeast strains. The names of the strains are given above the autoradiograph. The probes used are indicated at the left. The bottom panel represents the Western-blot of the cell proteins extracted form the mutant strains with the antibodies against preMsk1p (@preMsk1p).



Kamenski et al.
Fig. 7

2.2. ANALYSIS OF PHENOTYPIC AND MOLECULAR EFFECTS OF REPLACEMENT OF *MSK1* GENE BY ITS HOMOLOGUE FROM *Ashbya gossypii*

In previously described experiments, all the mutations in *MSK1* gene resulted in loss of mitochondrial function in mutant yeast strains. There are atleast two possible reasons of this effect: either mutant preMsk1p is not able to aminoacylate tRK3 in mitochondria, or it is not competent in tRK1 import directing. Unfortunately, we could not distinguish between these two possibilities. Our next aim was to construct a protein that would be certainly able to aminoacylate tRK3 but not able to direct tRK1 import into mitochodnria. In such a case, it would become possible to develop a genetic system suitable to test mitochondrial functions of tRK1 that are not clear yet. Indeed, tRK3 has an uridine in position 34 and it is agreed that even non-modified U in wobble positions can decode all four nucleotides in the third position of the codons (Lagerkvist, 1986; Yokobori et al., 2001). Moreover, U34 of tRK3 is modified by 5-carboxymethylaminomethyl-2-thio-group. It was demonstrated, that, in some cases, the wobble U modifications of this class are required for correct decoding of not fully complementary codons (Kirino et al., 2005; Kirino et al., 2004; Yasukawa et al., 2001). Thus, tRK3 should be able to recognize both lysine codons, AAA and AAG, in course of mitochondrial translation, and, in principal, tRK1 is not needed for this process.

The results described above demonstrate the importance of the N-terminal domain of preMsk1p for tRK1 import. We have performed multiple alignments of primary sequences of all known mitochondrial lysyl-tRNA synthetases and discovered that in a filmentous fungus, *Ashbya gossypii*, the ortholog of preMsk1p (AshLysRS) is characterized by a strongly shorter predicted N-terminal domain. On the other hand, the mitochondrial $tRNA^{Lys}$ of *A. gossypii* was similar to tRK3. We expressed AshLysRS instead of preMsk1p in the *S. cerevisiae* and demonstrated that, in normal conditions, the recombinant cells had functional mitochondria, correctly aminoacylated tRK3 but no tRK1 in the mitochondria. On the other hand, at elevated temperature (37°C), the mitochondrial functions of the recombinant strain were decreased (Fig. 5). Analysis of mitochondrial translation pattern revealed that the mitochondrial defect is most probably due to the translation inhibition (Fig.6). We found that at 37°C, translation of two mRNAs were specifically affected, Var1p (ribosomal protein) and Cox2p (2nd subunit of cytochrome *c* oxidase). So far, among major products of mitochondrial translation, these two proteins are the only ones two possess AAG codons coding for the lysines (all the rest of

lysines being coded by AAA). We hypothesize, therfore, that imported tRK1 is needed to read these AAG codons at temperature stress conditions.

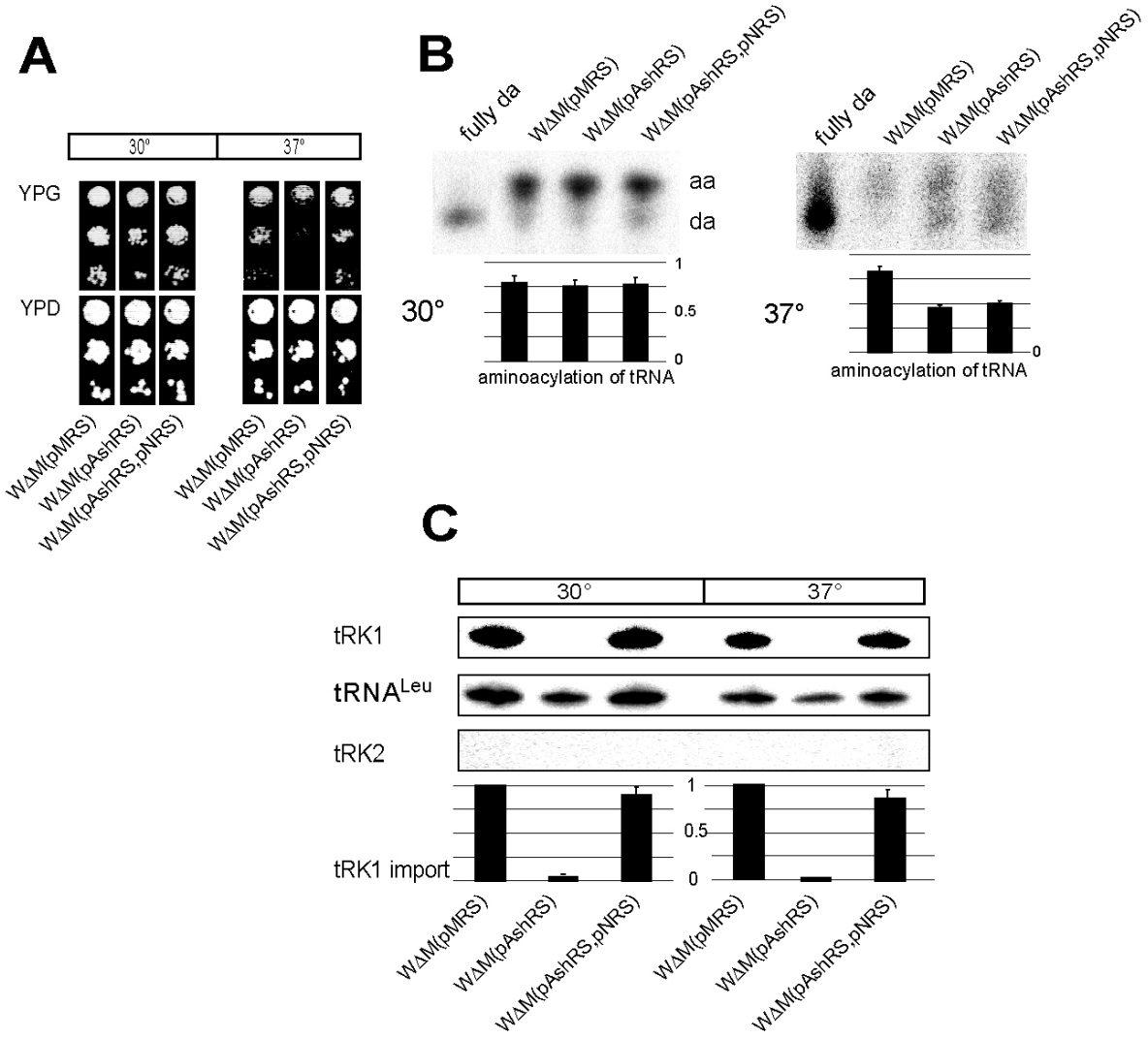


Fig. 5. (A) The growth of the mutany yeast strains on glycerol- and glucose-containing media. The growth of *A.gossypii* LysRS-containing strain is slower at 37°C only. (B) tRK3 aminoacylation level is normal in the *A.gossypii* LysRS-containing strain at 30°C and does not depend on the presence of N-terminal domain of preMsk1p at 37°C. (C) tRK1 is absent in the mitochondria of the *A.gossypii* LysRS-containing strain both at 30 and 37°C.

Which can be the reason of such a regulation? Further experiments demonstrate that at higher temperature, tRK3 becomes under-modified at its wobble position of the anticodon, which causes the translational defect. Our results show therefore, that the cell elaborated a mechanism of complementation of the conditional mitochondrial translation deficiency by a tRNA expressed from a nuclear gene and targeted into the mitochondria (Fig. 6). This finding

is the first description of such a control mechanism and indicates on the potential of RNA mitochondrial import pathway in regulation of the organellar translation.

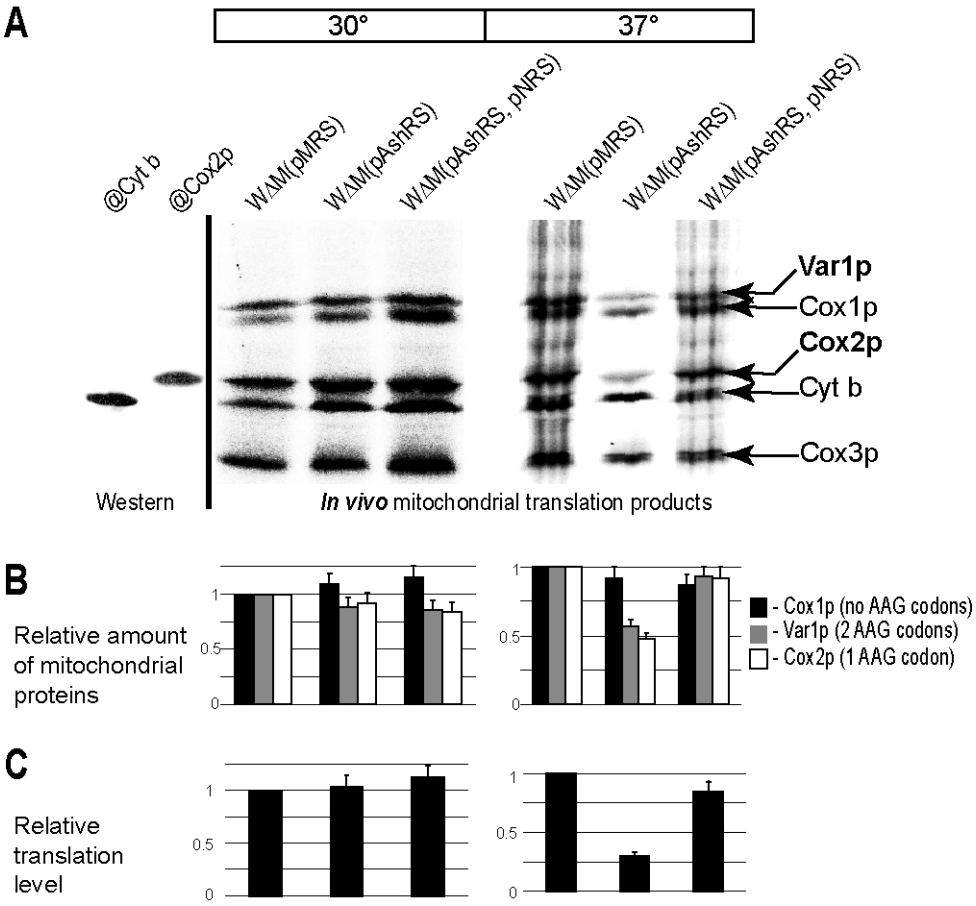


Fig.6. There are global mitochondrial translation defects in *A.gossypii* LysRS-containing strain an 37°C. They are characterised by an overall decrease of the translation efficiency, and, secondly, a specific decrease of the amount of two mitochondrially-encoded proteins, Var1p and Cox2p. These defects are fully restored by expression of the N-terminal domain of preMsk1p showing that they are due to the tRK1 absence in the mitochondria.

These results are described in details in the **publication 4** (attached below).

Publication 4.

Kamenski P., Kolesnikova O., Jubenot V., Entelis N., Krasheninnikov I., Martin R.P., Tarassov I.

"*Evidence for a novel adaptation mechanism of mitochondrial translation via tRNA import in yeast*"

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Evidence for a novel adaptation mechanism of mitochondrial translation *via* tRNA import from the cytosol

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Running head: tRNA mitochondrial import and translational adaptation

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Summary

Although mitochondrial import of nuclear DNA-encoded RNAs is widely occurring, their functions in the organelles are not always understood. Mitochondrial function(s) of $tRNA^{Lys}_{CUU}$, tRK1, targeted into *Saccharomyces cerevisiae* mitochondria was mysterious, since mitochondrial DNA-encoded $tRNA^{Lys}_{UUU}$, tRK3, was hypothesized to decode both lysine codons, AAA and AAG. Mitochondrial targeting of tRK1 depends on the precursor of mitochondrial lysyl-tRNA synthetase, pre-Msk1p. Here we show that substitution of pre-Msk1p by its *Ashbya gossypii* ortholog results in a strain where tRK3 is aminoacylated, while tRK1 is not imported. At elevated temperature, impairment of tRK1 import inhibits mitochondrial translation of mRNAs containing AAG codons, which coincides with the hypomodification of tRK3 anticodon wobble nucleotide. Restoration of tRK1 import cures the translational defect, suggesting the role of tRK1 in conditional adaptation of mitochondrial protein synthesis. This is a novel mechanism of the organellar translation control, which exploits the RNA import pathway.

Introduction

Targeting of small nuclear-encoded RNAs into mitochondria has been described in animal, fungi, plants and protozoans (Entelis et al., 2001b; Schneider and Marechal-Drouard, 2000). The main RNA species to be imported are transfer RNAs, but other small non-coding RNA (5S rRNA, MRP- or RNase P - RNA components) may also be imported (Magalhaes et al., 1998; Puranam and Attardi, 2001). Although the mechanisms of specific delivery of the given RNA towards the organelle and into its matrix appear to differ from one biological system to another (Mahapatra and Adhya, 1996; Salinas et al., 2006; Tarassov et al., 1995a;

Tarassov et al., 1995b), such a wide presence of RNA mitochondrial targeting pathway clearly indicates its functional importance.

In spite of the fact that RNA import into mitochondria concerns essentially the RNAs with normally well-defined functions (transfer RNAs, ribosomal RNA), in numerous cases the function of the imported RNA species is not evident. Indeed, 5S rRNA found in mammalian mitochondria (Magalhaes et al., 1998) was not detected yet in the mitochondrial ribosomes (Sharma et al., 2003). In several organisms, mitochondrially-imported tRNAs seem to be redundant with mitochondrial DNA-coded ones (Entelis et al., 2001b). A striking case concerns the yeast *Saccharomyces cerevisiae* (Martin et al., 1979), where the cytosolic $tRNAs^{Lys}$ ($tRNA^{Lys}_{CUU}$, further referred to as tRK1) is partially addressed into the mitochondria. Yeast cells possess three isoacceptor lysine tRNAs, referred to as tRK1 (partially imported), tRK2 ($tRNA^{Lys}_{UUU}$, cytosolic) and tRK3 ($tRNA^{Lys}_{UUU}$, mitochondrial DNA-encoded) (Fig. 1). In mitochondria, tRK1 co-exists with its mitochondrial isoacceptor tRK3. Due to the modified uridine at the wobble position of the anticodon (5-carboxymethylaminomethyl-2-thiouridine, $cmnm^5s^2U$), tRK3 was supposed to read both AAA and AAG lysine codons (Martin et al., 1990; Umeda et al., 2005). Requirement of the imported tRK1 for mitochondrial translation was therefore not evident. However, it was recently hypothesized that other yeast tRNA species that are mitochondrially imported (two $tRNA^{Gln}$ isoacceptors) were required for mitochondrial translation (Rinehart et al., 2005).

We previously demonstrated that mutant versions of tRK1 can suppress mutations in mitochondrial DNA *in vivo* and participate in mitochondrial translation in isolated organelles (Kolesnikova et al., 2000). This finding was also in agreement with the fact that heterologous expression of yeast tRK1 variants in cultured human cells allowed to complement a pathogenic mutation in the mitochondrial $tRNA^{Lys}$ gene (Kolesnikova et al., 2004). Although

these data suggest that the imported tRK1 may be used for mitochondrial protein synthesis, no direct evidence existed that it really occurs *in vivo*.

Here, we exploit our knowledge of tRK1 import mechanism to develop a genetic system to analyse mitochondrial functions of tRK1 *in vivo*. To be imported, tRK1 must be aminoacylated and must interact with the putative carrier protein, the cytosolic precursor of mitochondrial lysyl-tRNA synthetase (pre-Msk1p), and other cytosolic co-factors (Entelis et al., 2006; Entelis et al., 1998; Entelis et al., 1996; Tarassov et al., 1995a; Tarassov et al., 1995b). We show here that the N-terminal part of pre-Msk1p is essential for tRK1 import and that substitution of *MSK1* gene by its ortholog from a filamentous fungus *Ashbya gossypii* whose characteristic is a shorter N-terminal domain (with respect to Msk1p) leads to a correct aminoacylation of tRK3 but abolishes tRK1 import. Analysis of yeast strains lacking mitochondrial tRK1 allowed us to demonstrate that tRK1 is active in mitochondrial translation. Unexpectedly, this activity was linked to the conditional loss of function of tRK3 due to a defect of base modification. These results demonstrate therefore the existence of a novel mechanism of conditional adaptation of mitochondrial translation based on tRNA import.

Results

The N-terminal domain of pre-Msk1p can direct tRK1 import

Pre-Msk1p, the essential component of tRK1 mitochondrial targeting, is a typical mitochondrial pre-protein possessing a predicted 29 amino acid long targeting and cleavable N-terminal signal. As most of lysyl-tRNA synthetases (LysRS), it belongs to the class IIb of aminoacyl-tRNA synthetases (aaRS) (Eriani et al., 1990). Comparison of primary sequences of pre-Msk1p and other aaRS with known structures permits to propose its spatial organisation similar to the previously characterized bacterial LysRS (Fig. 2A). As other class

IIb aaRS, it is partitioned into two domains, a catalytic C-terminal one, with the aminoacylation active centre, and the N-terminal one, responsible for primary tRNA binding and specific interaction with the anticodon loop, the two domains being linked by a "hinge" region (Cavarelli et al., 1993; Cusack et al., 1996).

We purified the recombinant version of pre-Msk1p lacking the predicted C-terminal domain, and unexpectedly found that it was able to direct the import of tRK1 into isolated mitochondria with efficiency comparable (50%) with that of the full-length recombinant pre-Msk1p (Fig. 2B). To verify if this observation stands true *in vivo*, we replaced the endogenous *MSK1* gene with its truncated form expressing only the N-terminal domain with the hinge region (amino acids 1-245, further termed pre-Msk1Np). This substitution resulted in the loss of mtDNA (*rho*° phenotype) and the absence of mitochondrial respiration (WΔM(pNRS) strain, Fig. 2C). This result was expected, since the truncated protein is not able to aminoacylate tRK3 in the organelle. Nevertheless, as all *rho*° cells, WΔM(pNRS) strain still contained non-respirating organelles, commonly called promitochondria. Promitochondria purified from WΔM(pNRS) cells still contained tRK1, contrary to promitochondria from *rho*° cells, in which the whole *MSK1* gene was deleted (WΔM strain, Fig. 2C). This result confirms that the N-terminal part of pre-Msk1p is essential and sufficient to direct tRK1 import, both *in vitro* and *in vivo*.

Ashbya gossypii mitochondrial LysRS can substitute Msk1p for tRK3 aminoacylation but not for tRK1 import

Multiple alignment of Msk1p amino acid sequence with those of mature forms of all known mitochondrial LysRS revealed that its ortholog from the filamentous fungus *Ashbya gossypii* (AshRS) had a shorter N-terminal domain, whereas its C-terminal domain is highly homologous to that of Msk1p (Fig. 3A). Taking into account the importance of the N-terminal

domain of pre-Msk1p for tRK1 import, one could suggest that AshRS will not be able to target tRK1 into yeast mitochondria. On the other hand, since this protein is a mitochondrial enzyme in a species phylogenetically related to *S. cerevisiae*, one also could expect that AshRS will be able to correctly aminoacylate tRK3.

Indeed, expression of AshRS instead of Msk1p permits *S. cerevisiae* cells to grow on respiratory media (Fig. 3B). This result suggests that the recombinant protein is expressed, mitochondrially imported and supports mitochondrial translation in standard conditions of cultivation. In contrast, at higher temperature (37°C), the growth rate of the AshRS-expressing cells on respiratory media was significantly reduced, while co-expression of pre-Msk1Np and AshRS restored it (Fig. 3B,C). Mitochondrially-dependent oxygen uptake in recombinant strains was is a perfect agreement with their growth on non-fermentable carbon sources (Fig. 3D). At 30°C, the oxygen uptake in WΔM-derived strains expressing pre-Msk1p, AshRS or the N-terminal domain of pre-Msk1p with AshRS, was similar to that of the wild type cells. In contrast, at 37°C their respiration rate was different: in WΔM expressing pre-Msk1p it was similar to that of the wild type control; expression of pre-AshRS instead of pre-Msk1p resulted in a 60±8% drop of oxygen uptake, while co-expression the N-terminal domain of pre-Msk1p with pre-AshRS restored the respiration to the level of the pre-Msk1p expressors. These results suggest that AshRS is able to functionally replace Msk1p at 30°C, but is not sufficient for mitochondrial function at 37°C, and that this conditional negative effect can be cured by the N-terminal domain of pre-Msk1p.

tRK1 import was then analysed by Northern hybridisation in the recombinant yeast strains grown either at 30 or 37°C (Fig. 3E). In AshRS-expressors grown either at 30° or 37°C no tRK1 import was detected, while additional expression of pre-Msk1Np restored tRK1 import. This suggests that, as expected, AshRS was not able to target tRK1 into mitochondria. Surprisingly, the phenotypic effect was detectable only at elevated temperature. This could be

explained either by the need of tRK1 in stress conditions, or by the conditionally deficient aminoacylation of tRK3 by AshRS. To distinguish between these two possibilities, we analysed *in vivo* mitochondrial tRNAs levels and aminoacylation at 30 and 37°C.

Quantitative analysis of three independent mitochondrial tRNAs , tRK3, $tRNA^{Glu}$ (tRE) and $tRNA^{Gln}$ (tRQ) demonstrated that higher temperature had no specific effect on tRK3 concentration/stability, since the ratios tRK3/tRE and tRK3/tRQ do not change with the temperature shift (Fig. 4A). Furthermore, at 37°, tRK3 is also normally processed, since it migrates in a same way as the corresponding T7-transcript on denaturing gel. In contrast, the temperature shift appeared to decrease the extent of tRK3 aminoacylation in AshRS - expressors (Fig. 4B). This means that either AshRS is less efficient than Msk1p in terms of tRK3 aminoacylation, or that all mitochondrial tRNAs are underaminoacylated at elevated temperature. To rule out the latter possibility, we analysed mitochondrial $tRNA^{Leu}$ (tRL) and found that its aminoacylation was only slightly reduced upon the temperature shift, but not affected by the replacement of Msk1p by AshRS. Migration of the aminoacylated form of tRK3 was similar at 30 and 37°C in all strains, suggesting that the charging was correct. Finally, in all strains analysed, and at both 30 and 37°C, reproducible detectable amounts of aminoacylated tRK3 were present. In this context, it is important to outline that no difference was detected between AshRS-expressing cells whether pre-Msk1Np was present or absent at 37°C. Therefore, the rescue of mitochondrial functions observed in the presence of pre-Msk1Np versus the cells expressing only AshRS can be explained exclusively by the rescue of tRK1 import and not by the difference in tRK3 aminoacylation by the foreign enzyme.

To participate in mitochondrial translation, imported tRK1 also must be aminoacylated. As previously demonstrated, this reaction is performed by the cytosolic enzyme, Krs1p, and tRK1 is imported in aminoacylated form (Tarassov et al., 1995a). Aminoacylation levels of mitochondrial pools of tRK1 at 30 and 37°C were similar (Fig. 4C),

which proves that at the non-permissive temperature an important part (at least 50%) of the imported tRK1 molecules remain aminoacylated and therefore may be translationally active.

Abolition of tRK1 import leads to an inhibition of AAG-codons translation at elevated temperature

To verify if the phenotypic effect of tRK1 import inhibition was due to alterations in mitochondrial translation, we compared mitochondrially-synthesized polypeptides in the above recombinant strains grown at two temperatures by pulse-chase incorporation of ^{35}S-methionine in the presence of cytosolic ribosomes inhibitor (Fig. 5). The patterns of mitochondrial proteins were similar in all the recombinant strains at 30°C, but differed at 37°C (Fig. 5A). Two effects are obvious: (a) AshRS expressing cells showed an overall decrease of mitochondrial translation (by 60±7%, Fig. 5C); (b) two polypeptides, Var1p and Cox2p, are decreased in a specific manner (by 50±10%) versus other mitochondrial polypeptides (Fig. 5B).

Analysis of codon usage in yeast mtDNA genes shows that less than 10% of the lysine codons are AAG, while the majority are AAA (Foury et al., 1998). From 39 AAG-codons in open reading frames, 36 are located in intronic ORFs, whose products are synthesized in tiny amounts and are never detected by pulse-chase assays. On the other hand, from the well expressed mitochondrial genes whose products are commonly detected in pulse-chase experiments, the AAG-codons are found only in two cases: in *VAR1* (two codons) and in *COX2* (one codon). We can therefore suggest that the absence of imported tRK1, which possesses a CUU anticodon, affects specifically translation of AAG-containing ORFs, however, this effect is detected only at elevated temperature. If the decrease of aminoacylation of tRK3 in AshRS expressing cells may well be the explanation of a non-

specific decrease of mitochondrial translation, the specific defect of codon-decoding might be caused by another reasons.

tRK3 is hypomodified at elevated temperature

Why the effect of withdrawing of mitochondrial tRK1 on AAG decoding becomes detectable only at elevated temperature? One possible explanation would be that tRK3 becomes a poor decoder of AAG-codons in these conditions. As a matter of fact, pathogenic mutations in human mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu}$ were found to cause hypomodification of the U34 in wobble positions, which, in turn, affected decoding of G in the 3rd position of the corresponding codons (Kirino et al., 2005; Kirino et al., 2004; Yasukawa et al., 2001). To test if in tRK3 the U34 modification was affected at the elevated temperature, we used the method of primer extension by reverse transcriptase (Fig. 6A-B). We expected that the presence of the modification at the wobble-U of tRK3 would cause the arrest of polymerisation by the reverse transcriptase, as it was described previously for other uridine modifications (Kirino et al., 2005). Indeed, at 30°C, a clear arrest of extension at the 2nd base of the anticodon (base U35) was detected (Fig. 6A-C). In contrast, at 37°C, this arrest was strongly reduced, meaning that the modification of the wobble base of tRK3 was significantly decreased.

It was previously shown that the arrest of reverse transcription may be due to the presence of the homologue of carboxymethylaminomethyl-group, the taurinomethyl-, at position 5 of the wobble uridine in human mitochondrial tRNAs (Kirino et al., 2005). However, the arrest in this case was observed at nucleotide 33, while in our case it was at position 35. On the other hand, the thio-group present at the position 2 of the tRK3 wobble uridine (Umeda et al., 2005) may also influence reverse transcription provoking its arrest. We analysed mitochondrial tRNAs isolated from the cells grown at either 30 or 37°C by

Northern-hybridisation after separation on polyacrylamide gels containing APM ((N-Acroylamino)phenyl-mercuric chloride) (Igloi, 1988) (Fig. 6D-E). In this system, the thiolated tRNAs are covalently retained by Hg-groups incorporated in the polyacrylamide gel and have lower mobility than non-thiolated ones. It appears clearly that at 30°C, most of tRK3 molecules are almost fully thiolated. In contrast, at 37°C, the lower band, corresponding to the non-thiolated version, becomes comparable with the upper one, corresponding to the modified tRNA. This means that the modification defect in tRK3 observed at 37°C concerns the thio-group at position 2 of U34. On the other hand, we cannot be affirmative about carboxymethylaminomethylation of the position 5, since no arrest was observed at nucleotide 33 in any case.

It was previously shown that the $cmnm^5s^2U$ in the wobble position of the anticodon occurs in two other yeast mitochondrial tRNAs: $tRNA^{Glu}$ and $tRNA^{Gln}$ (Umeda et al., 2005). The question might rise if these tRNAs are also affected at elevated temperature. APM-gel assays done with $tRNA^{Glu}$ and $tRNA^{Gln}$ show no thiolation defect at 37°C (Fig. 6D-E). This result indicates that the conditional defect of modification is tRK3-specific.

Discussion

Up to now, the main criterion to affirm that a tRNA is imported into mitochondria was the absence of the corresponding gene in mitochondrial DNA (Schneider and Marechal-Drouard, 2000), suggesting that the imported tRNA must be essential for mitochondrial translation. If, as it was in the case of tRK1 in yeast, the mitochondrial isoacceptor tRNA was encoded in the organelle, non-translational functions were searched for (Soidla and Golovanov, 1984). The current study gives new insights on this problem, showing that the function of the imported RNA may be directly related to the organellar translation but used

for its conditional control. We show that the nuclear-encoded tRNA is partially delivered into mitochondria to help mitochondrial translation in stress conditions causing base-modification defects in host mitochondrial isoacceptor tRNA. Our hypothesis is that at elevated temperature, the anticodon first base of the mitochondrial $tRNA^{Lys}_{UUU}$ is undermodified with respect to normal conditions. The absence of a 2-thio-group of the U34 perturbs translation of AAG-codons, while the imported $tRNA^{Lys}_{CUU}$ cures this deficiency by reading minor AAG-codons in mitochondrial mRNAs (Fig. 7).

One issue of this work is that the hypomodification of the uridine at the wobble position of the anticodon may affect AAG-decoding. Normally, a non-modified U in wobble position recognizes all four nucleotides in the third position of the four-codon family (Lagerkvist, 1986; Yokobori et al., 2001). Modified uridines in the wobble positions were therefore suggested to prevent recognition of the neighbouring two-codons families. On the other hand, it was demonstrated that the absence of modification at this position affects decoding of the G's in the 3rd position of the codons in mammalian mitochondria (Kirino et al., 2005; Kirino et al., 2004; Yasukawa et al., 2005; Yasukawa et al., 2001; Yasukawa et al., 2000). It was proposed that wobble-base modification might plays a role in stabilization of the G:U* pairing (Kirino et al., 2004). This concerns especially modifications where a methylene carbon is directly bound to the C5 position of uracyl (xm5U), like 5-taurinometyluridine in human mitochondrial $tRNA^{Leu}$, or 5-carboxymethylaminomethyl-uridine in yeast mitochondrial $tRNA^{Lys}$. Other modifications may affect decoding properties of the tRNAs. For example, the absence of the 5-taurinomethyl-2-s-modification on the wobble-uridine in human mitochondrial $tRNA^{Lys}$ was reported to prevent correct decoding of both lysine codons (Yasukawa et al., 2001), suggesting that the presence of the 2-thio modification is important for decoding AAR codons (Ashraf et al., 1999). Our data, together with those reported for the human system, strongly indicate that alterations of wobble modifications are the important

molecular cause of mitochondrial translation deficiencies in so distant species as yeast and humans.

Another question emerging is what could be the reason for such a conditional defect in one particular mitochondrial tRNA? It can be suggested that some structural or physico-chemical properties of tRK3 are at the origin of its hypomodification at elevated temperature. These may be related to a lower structural stability or formation of alternative structures at 37°C, which, in turn, would affect recognition by the modification enzyme(s). One may only speculate on the molecular reason of such properties. tRK3, contrary to the $tRNA^{Leu}$, possesses a bulged U in the TΨC arm, destabilising this part of molecule (see Fig 1). Indeed, destabilisation of the 3D structure due to bulged bases was often reported before (Ciesiolka et al., 1998; Sarzynska et al., 2000). As a matter of fact, in *Ashbya gossypii*, where we suppose the absence of $tRNA^{Lys}$ mitochondrial import (a speculation based on the fact that tRK1 ortholog in *A. gossypii* is quasi-identical to its *S. cerevisiae* counterpart, while AshRS cannot direct tRK1 into yeast mitochondria), no bulged U was found in the tRK3 homologue. Furthermore, in human mitochondrial tRNAs, pathogenic mutations localized in the TΨC arm (A8344G in $tRNA^{Lys}$ or T3291C in $tRNA^{Leu}$ causing the syndromes MERRF and MELAS, respectively) were also associated with the hypomodification of the uridine in the wobble position of the anticodon (Kirino et al., 2005; Yasukawa et al., 2005; Yasukawa et al., 2001). One can suggest that the TΨC arm may be important for recognition by the modifying enzymes. This or any other tRK3 properties could make its 3D structure destabilized or altered at 37°C thus masking the recognition site.

Abnormality of tRK3 at elevated temperature may have various effects on mitochondrial translation: direct - if the amount of this tRNA is specifically reduced at non-permissive temperature, and indirect - if it causes hypomodification, which, in turn, results in functional defects of the tRNA affecting translation. Modification defects were previously

shown to cause either instability (Alexandrov et al., 2006; Kadaba et al., 2004) or aminoacylation identity (Astrom and Bystrom, 1994; Senger et al., 1997) of tRNAs. Nevertheless, we demonstrated that neither decrease of amount of tRK3 nor misaminoacylation were observed at 37°C in all strains analysed. Aminoacylation of tRK3 was reduced at 37°C, and this effect was stronger in AshRS-expressing cells. This fact explains the overall decrease of translation at 37°C, accentuated in AshRS-expressing strains, while the specific defect of reading AAG codons might be explained by the hypomodification of the tRK3 anticodon wobble position.

Our data reveal a new adaptation mechanism of mitochondrial translation, which exploit the RNA import pathway. The results described can also have biomedical prospects. In this context, one can mention that, firstly, hypomodification of mitochondrial tRNAs was proved to be at the basis of several grave neuromuscular pathologies (syndromes MERRF and MELAS)(Kirino et al., 2005; Kirino et al., 2004), and, secondly, that tRNA import pathway can be introduced in an artificial way in human cells (Kolesnikova et al., 2004; Kolesnikova et al., 2000; Mahata et al., 2006). The pathway of mitochondrial RNA import may, therefore, become a specific tool aimed to correct such translational defects in human mitochondria.

Experimental procedures

Strains and media

Epicurian coli strain BL21 RIL3 codon plus (Stratagene) was used for expression of recombinant proteins. *Escherichia coli* strain XL10-Gold (Stratagene) was used for site-mutagenesis and strain XL1blue (Stratagene) for cloning purposes. Cultivation was performed in standard LB medium with appropriate antibiotics. In case of the strain BL21 RIL3 codon plus the medium also contained 0.4% of D-glucose.

Saccharomyces cerevisiae W303 (MAT a/α) was used as a reference strain. The strain lacking the *MSK1* gene, WΔM, was the W303 (MATα) where the *MSK1* gene was deleted (W303, *msk1*::*KanMX4*) (Entelis et al., 2001a). Cultivation was performed in the media: YPD, YPEG (contains 2% of ethanol and 3% of glycerol as carbon source), YPGal (contains 2% of galactose) (Rose, 1990). Transfectants were sustained at minimal selective media.

Ashbya gossypii strain DSM 3499 was purchased from DSZM Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and was maintained on Ashbya Full Medium (AFM): yeast extract (10g/l), peptone (10g/l), glucose (20g/l), myo-inositol(0.1%) (Altmann-Johl and Philippsen, 1996).

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Plasmid construction

pMRS416 plasmid (Entelis et al., 2001a), contains the full-size functional *MSK1* gene with its own promoter and 1kb flanks in pRS416 shuttle vector (*URA3*-marker).

Plasmid pMRS313 represents the full functional *MSK1* gene with its own promoter and 1kb flanks in pRS313 vector (Sikorski and Hieter, 1989). This plasmid was used for cloning of the *Ashbya gossypii* mitochondrial lysRS gene (AshRS). For this purpose, the full protein coding sequence of *MSK1* in the pMRS313 plasmid was replaced by the *Nco*I site by site-directed mutagenesis, to obtain pMRS313Δ. The full ORF coding for AshRS was PCR-amplified using two following oligonucleotides:
GGGATACTTTTATAATGGTGTTATGTTACATGATTGCCGATGTGGTGCGGAGCCA
GAGCGATGATAGC and
TCTGATTTATTTACAAAAGCATTGGCAGGCGTCGCAAAATCTACTGGCGGTTGAC
GTCGTCTAGACATCC, which are complementary both to the AshRS-coding gene and the *MSK1* gene flanks. The final construction, pAshRS, obtained by co-transfection of yeast cells with *Nco*I-linearized pMRS313Δ and the PCR-product with subsequent homologous

recombination, contains the AshRS coding sequence under control of the *MSK1* gene promoter and in the endogenous context of *MSK1* gene flanks.

To express the N-terminal domain of pre-Msk1p in yeast, we deleted the C-terminal domain of the *MSK1* ORF (from F246 to the stop codon) from the plasmid pG11T6 (Gatti and Tzagoloff, 1991), by using site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene).

pET-Msk1 plasmid contained the *MSK1* ORF in frame with 6 His-coding codons at the C-terminus in the pET3a vector. pET-Msk1-Nter plasmid contained the same construct but with the C-terminal domain of Msk1p deleted. These two constructs were used to express in *E. coli* and to purify the recombinant pre-Msk1p and its N-terminal domain.

Recombinant proteins

To purify the recombinant C-terminus His-tagged pre-Msk1p, IPTG induction was performed for 2h at 25°C and proteins were solubilized in denaturating conditions. The recombinant protein was then affinity-purified to >95% purity on Ni-agarose beads (Qiagen) and refolded as described by manufacturer. To purify the N-terminal domain of pre-Msk1p, induction was performed for 4h at 30°C and the recombinant protein was solubilized in native conditions. For this version, the C-terminal tag was not efficient for purification, therefore, the protein was purified by gel-filtration on Superdex 200 column on AKTA-FPLC system (Amersham) to >90% of purity.

Yeast genetics and phenotypic analysis

S. cerevisiae strain WΔM was not suitable for genetic experiments, since, due to the deletion of the *MSK1* gene (ORF YNL073W), it becomes rapidly *rho*° (loss of mitochondrial DNA). Therefore, the diploid strain was used W303 MAT a/α, *msk1*::KanMX4 / *MSK1*. It

was transfected with the pMRS416 plasmid and then the saved haploid strain W303 MAT a, *msk1*::KanMX4 (pMRS416) was generated and selected. Phenotypic and biochemical analyses showed that all the functions affected by the deletion of the *MSK1* gene were fully complemented by the pMRS416 saver plasmid (respiration, growth on glycerol, ethanol, or lactate containing media, mitochondrial morphology, mitochondrial translation, aminoacylation of tRK3 and tRK1import). The latter strain was transfected by pG11T6ΔC, or pAshRS, or by both and the selection was performed according to the plasmid markers. Thereafter, the saver pMRS416 plasmid was removed by growing on 5-fluoroorotate containing media. The following three strains were selected: W303 *msk1*::KanMX4 (pAshRS, *HIS3*); W303 *msk1*::KanMX4 (pAshRS, *HIS3*; pG11T6ΔC, *LEU2*) and W303 *msk1*::KanMX4 (pG11T6ΔC, *LEU2*). The phenotypic analysis was performed by growing the strains on "respiratory media": YPG, YPEG, YPLac and compared to the growth on YPD or YPGal media, both on agar plated and in liquid.

Isolation of mitochondria, in vitro import assays, oxymetry

Mitochondria were isolated from logarithmic YPEG or YPGal-cultures as described previously (Entelis et al., 2002). Pro-mitochondria were isolated as described elsewhere (Rosenfeld et al., 2004). *In vitro* tRNA import assays were done as described in (Entelis et al., 2002). Each assay contained 5μg of crude import directing proteins (IDP) isolated from the WΔM strain and 0.05-0.1 μg of recombinant proteins. Import efficiency was quantified, after separation of mitochondrial RNA on denaturing polyacrylamide gels, by scanning autoradiography using Phosphor-Imager (Fuji, MacBAS2000 software).

The rate of oxygen consumption by yeast cells (A_{600}=0.3) cultured in 1 ml of YPGal medium was measured in thermostated chamber at 30°C or 37°C using a Clark type electrode

and the SI oxygen meter for 10 min followed by addition of myxothiasol (750 nM) to measure non-mitochondrial oxygen uptake, which was then subtracted from all values.

Isolation and analysis of RNA

RNA was extracted either by hot-phenol treatment, or by TRIzol reagent (Invitrogen). Northern hybridisations were performed as described previously (Entelis et al., 2006; Entelis et al., 2002) with the following oligonucleotide probes: anti-tRK1: CCTAACCTTATGATTAAGAGT; anti-tRK3 : CAAGCATGGGTTGCTTAAAAG, or, to verify the eventual degradation in the anticodon region, the longer one, CTTGCATGGGTTGCTTAAAAGACAACTGTTTTAC; anti-mt-tRNA^{Leu} : TGGTTGCTATTTAAAGGACTTG; anti-tRK2 : GCCGAACGCTCTACCAACTCAGC. Isolation, gel-separation and hybridisation of aminoacylated tRNAs were performed as described in (Varshney et al., 1991).

The primer extension method was as in (Kirino et al., 2005) with modifications. The polynucleotide kinase 5' ^{32}P-labeled primer (0.1 pmol) was incubated with 5 µg of the total RNAs in a 10-µl solution containing 10 mM Tris·HCl (pH 8.0), 1 mM EDTA at 90°C for 2 min and then allowed to stand at room temperature for 1 h. Subsequently, 4 µl of 5x reaction buffer for reverse transcription (Roche), 0.5 µl of d/ddNTP mix containing 1.5 mM of each of the three dNTP and one ddNTP (Amersham), 3 µl of 25 mM $MgCl_2$, and 1 µl of Moloney murine leukemia virus reverse transcriptase (RNase H-minus) (40 units/µl, Roche) were added, and the mixture was incubated at 42°C for 1 h. Nucleic acids were ethanol-precipitated and subjected to 20% PAGE containing 8 M urea (20 cm). The radiolabeled bands were visualized by a BAS5100 bioimaging analyzer (Fuji). The primers used: ACCAAGCATGGGTTGC.

The presence of 2-thiolation at the wobble nucleotide in mitochondrial tRNAs was tested by retardation in an electrophoretic system consisting of a 10% PAAG (20x20x0.1 cm) with 7M urea, tris-borate buffer that was polymerized in the presence of 50µg/ml of (N-Acroylamino)phenyl-mercuric chloride) (APM), which was synthesized following the procedure in (Igloi, 1988). Hybridisation was then done as described in (Shigi et al., 2002). with the following probes: tRK3 - CTTGCATGGGTTGCTTAAAAGACAACTGTTTTAC; $tRNA^{Glu}$ - TGGTAACCTTAATCGGAATCGAAC; $tRNA^{Gln}$ - TGGTTGAATCGGTTTGATTCGAAC.

Mitochondrial translation and western analysis

Mitochondrial translation was analysed as described elseqhere (Fox et al., 1991) with modifications: cells were grown in minimal media with galactose to A600=1.0, transferred to synthetic complete medium lacking Met (0.67% yeast nitrogen base, 0.08% CSM-Met (BIO101), 2% raffinose), with addition of 200 µg/ml of cycloheximide and incubated for 10 min at 30°C with shaking. Cells were then labeled with 10 µCi of [^{35}S]-methionine (1.4 Ci/mmol, Amersham) for 20 min and chased with 2 mM unlabeled methionine. The proteins were TCA-precipitated, water-washed and analyzed on 15% SDS-PAGE.

In silico sequence analysis

AshRS full-length peptide sequence was retrieved from the Ashbya Genome Database (http://agd.unibas.ch/). Peptide sequences of aaRS were analysed by the ClustalW package (http://www.ebi.ac.uk/clustalw/). Potential 3D-structures of pre-Msk1p and AshRS were analysed by analogy with *E. coli* LysRS using the package 3D-pssm (Kelley et al., 2000). tRNA structures were retrieved from the tRNA compilation database (Sprinzl et al., 1998).

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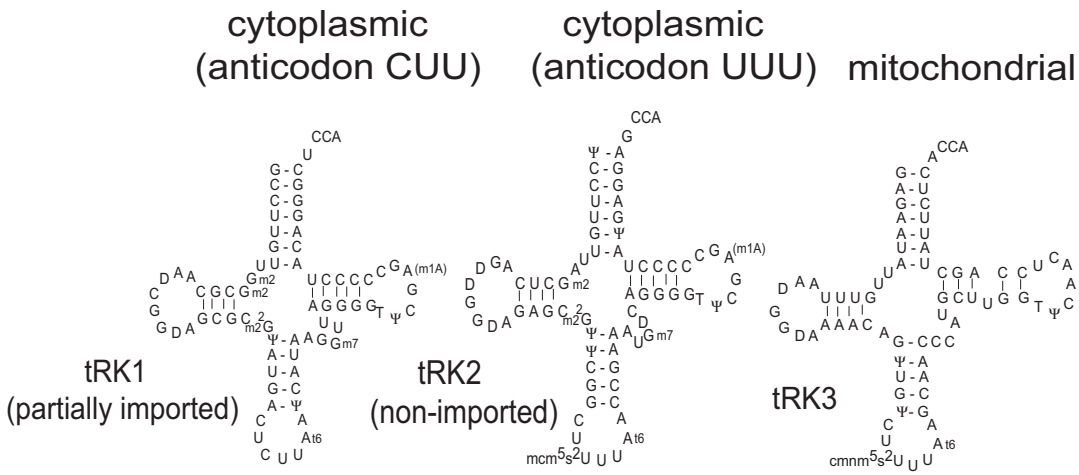
Figure 1. Cloverleaf structures of cytosolic and mitochondrial tRNAs^{Lys} of *S. cerevisiae*. Cloverleaf structures of cytosolic and mitochondrial tRNAs^{Lys} were retrieved from databases and drawn as described in Experimental procedures.

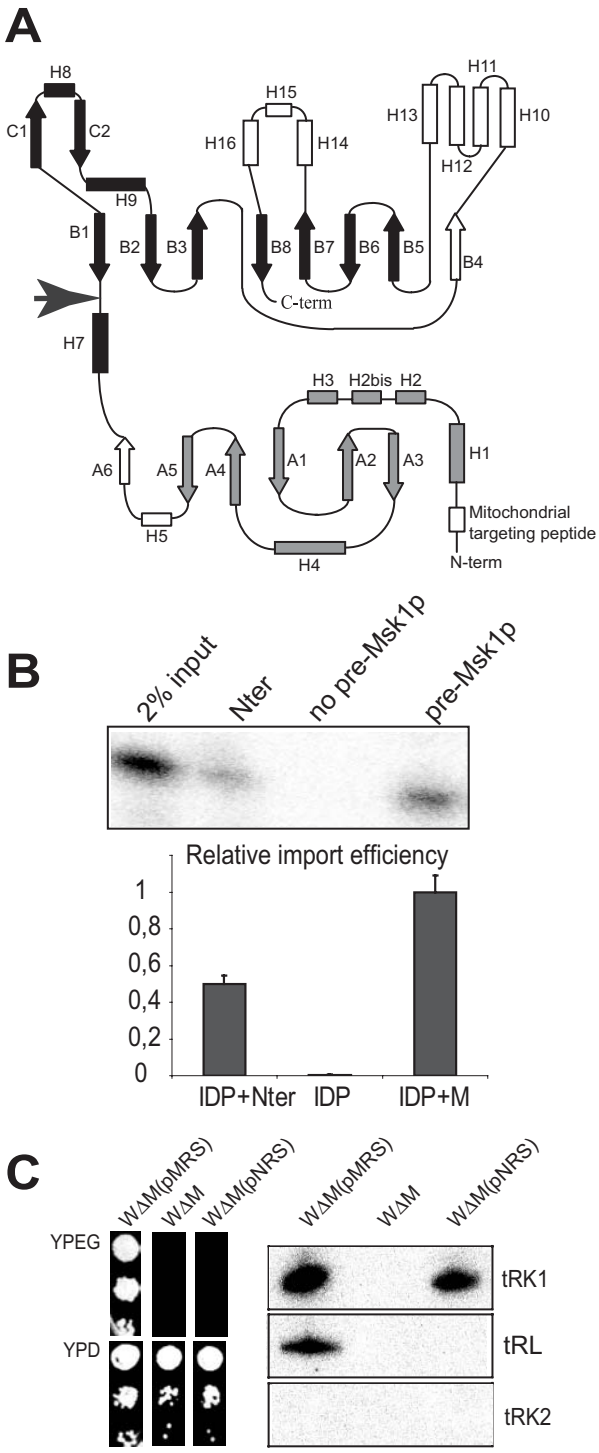
E) Figure 1



Kamenski et al., Fig 1

Figure 2. tRK1 directing activity of the N-terminal region of pre-Msk1p. (**A**) *In silico* predicted spatial organisation (topological diagram) of pre-Msk1p. Structural elements are annotated according to the *E. coli* enzyme (Onesti et al., 2000; Onesti et al., 1995). Active site elements of the C-terminal domain, which are conserved in all class II aaRS are in black; the N-terminal anticodon-binding domain with characteristic OB-fold is in grey; the hinge region and "insertions sequences" are in white. Rectangles indicate the helices (H), the arrows – β-elements. The arrow indicates the boarder of the truncated N-terminal version of pre-Msk1p. (**B**) RNA import into isolated yeast mitochondria. The upper panel represents the autoradiograph of the denaturating PAGE-separated RNAs protected in the *in vitro* import assay. The left path corresponds to 2% of the input . All reactions were performed in the presence of IDPs isolated from the WΔM strain. Recombinant proteins added (50ng) are indicated above: Nter, the N-terminal domain of pre-Msk1p (aminoacids 1-245); pre-Msk1p - full-sized pre-Msk1p. The quantification diagram results from three independent experiments, the import directing efficiency of the IDP (from WΔM) + pre-Msk1p mixture was taken as 1. (**C**) *In vivo* import of tRK1 driven by the N-terminal part of pre-Msk1p. Left panel: growth of the recombinant strain expressing the N-terminal domain of pre-Msk1p instead of the full-size molecule on glucose- (YPD) and glycerol-(YPEG) containing media. WΔM does not express any pre-Msk1p, WΔM(pNRS) express only the N-terminal domain of pre-Msk1p. WΔM(pMRS) is expressing the full-sized pre-Msk1p. Cultivation was at 30°C for 3 days. Serial dilutions (1:10) for each strain are presented. Right panel: Northern-hybridisation of promitochondrial (WΔM and WΔM(pNRS)) or mitochondrial (WΔM(pMRS)) RNAs with tRK1-, tRK2- and mitochondrial $tRNA^{Leu}$ (tRL) - specific probes. The absence of signal with the tRK2-probe demonstrates that mitochondrial preparations were not contaminated with cytosolic tRNAs. The absence of the signal with the tRL probe reflects the loss of mtDNA.

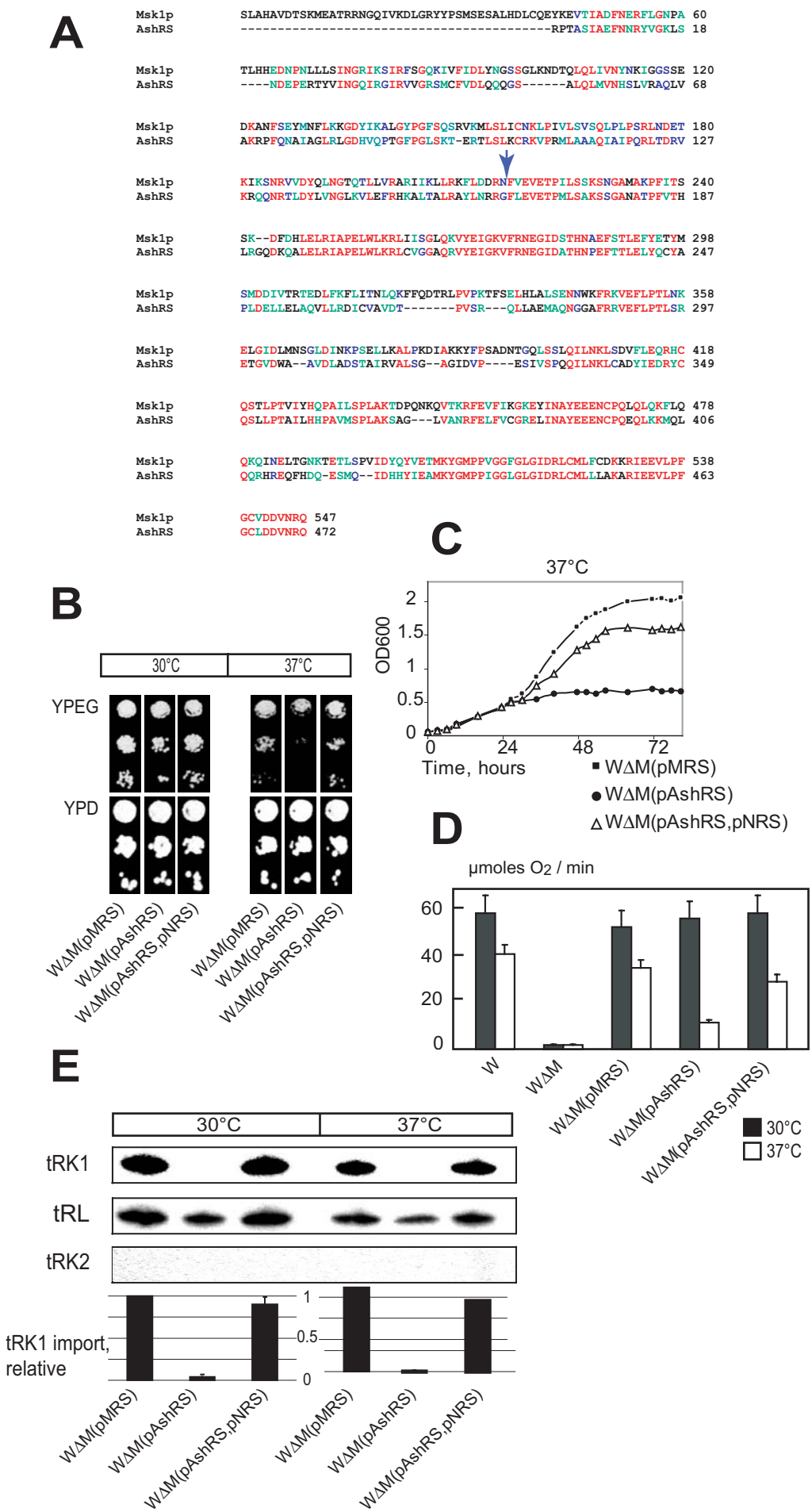
E) Figure 2



Kamenski et al., Fig 2

Figure 3. Functional replacement of the *MSK1* gene by its *A. gossypii* ortholog. (**A**) Alignment of mitochondrial lysyl-tRNA synthetases of *S. cerevisiae* (Msk1p) and *A. gossypii* (AshRS). Conserved residues are in red, semi-conserved - in green, those with similar hydrophobicity - in blue, non-aligned - in black. The arrow indicates the boarder of the truncated N-terminal version of pre-Msk1p. (**B**) Respiratory phenotypes of AshRS expressing WΔM strains WΔM(pAshRS) and WΔM(pAshRS,pNRS) at 30 or 37°C on YPEG medium. (**C**) Growth curves of the recombinant strains in liquid YPEG. (**D**) Oxygen consumption of the AshRS expressing WΔM strains at 30 or 37°C on YPGal medium. (**E**) Northern hybridisation of mitochondrial RNA from AshRS expressing strains (as in (Fig. 2A)). At the bottom, tRK1 import quantification diagram, resulting from at least two independent experiments. The ratio between the signals corresponding to tRK1 and the mtDNA-expressed tRL served to evaluate tRK1 import efficiency. The import level in WΔM(pMRS) was taken as 1.

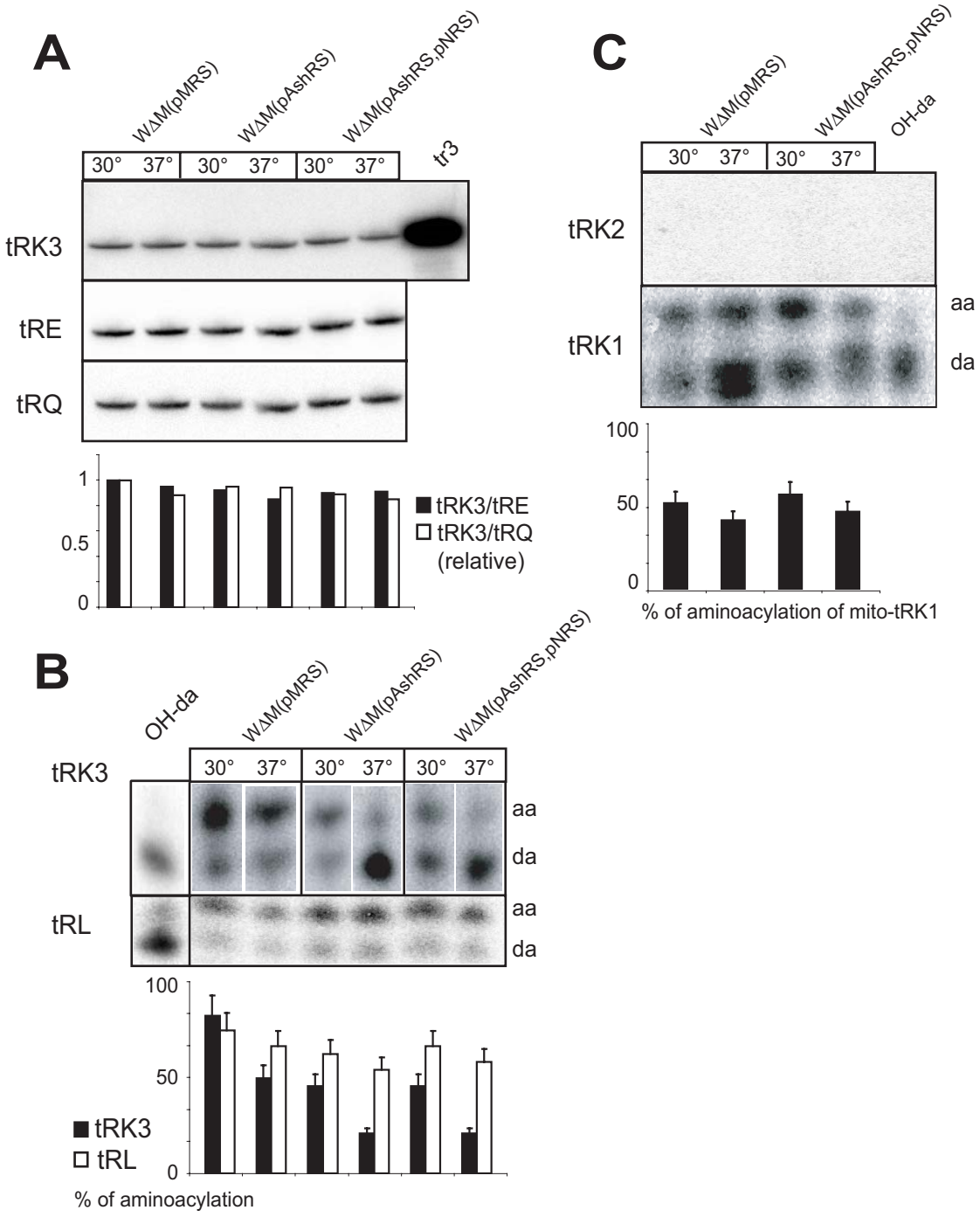
E) Figure 3



Kamenski et al., Fig 3

Figure 4. Stability and aminoacyltion of tRK3 in AshRS - expressing strains. (**A**) Northern-hybridisation. The strains and temperature of cultivation are indicated at the top, the hybridisation probes - at the left: tRK3, $tRNA^{Glu}$ (tRE) and $tRNA^{Gln}$ (tRQ). tr3, 20ng of T7-transcript of the cloned tRK3 gene possessing the same sequence as the mature tRK3. The graph at the bottom shows the ratios between the tRK3 and tRE/Q signals, the ratios in WΔM(pMRS) strain are taken as 1. (**B**) Analysis of tRK3 aminoacylation by Northern-hybridisation of RNA isolated and separated in acid conditions. tRL, hybridisation with mitochondrial $tRNA^{Leu}$-specific probe is used as a reference. OH-da, tRNA from WΔM(pMRS) strain deacylated in basic conditions. Positions of aminoacylated (aa) and deacylated (da) forms are indicated. Quantification diagrams result from at least two independent experiments. 100% corresponds to fully aminoacylated tRNAs. (**C**) Analysis of tRK1 aminoacylation in the mitochondria. tRK2 probe was used to prove the absence of cytosolic contamination.

E) Figure 4



Kamenski et al., Fig 4

Figure 5. Mitochondrial translation in recombinant strains. (**A**) Autoradiograph of the pulse-chase labelled mitochondrial proteins separated on the 15% denaturing PAGE. Equal number of cells was taken for translation reactions in each series. Mitochondrial proteins are indicated according to a standard mitochondrial translation pattern (Fox et al., 1991). To confirm that the observed pattern corresponded to the expected polypeptides, Western analysis was performed with antibodies against yeast cytochrome *b* and Cox2p (@Cytb and @Cox2p), at the left. (**B**) Quantification of the relative amounts of three mitochondrially-synthesized proteins: Cox1p, Cox2p and Var1p (as indicated at the right). Results of at least two independent experiments are presented. The ratio between the signal corresponding to a given protein and the total amount of radioactive polypeptides served for evaluation. The values for the WΔM(pMRS) was taken as 1. (**C**) Quantification of the overall mitochondrial translation activity.

E) Figure 5

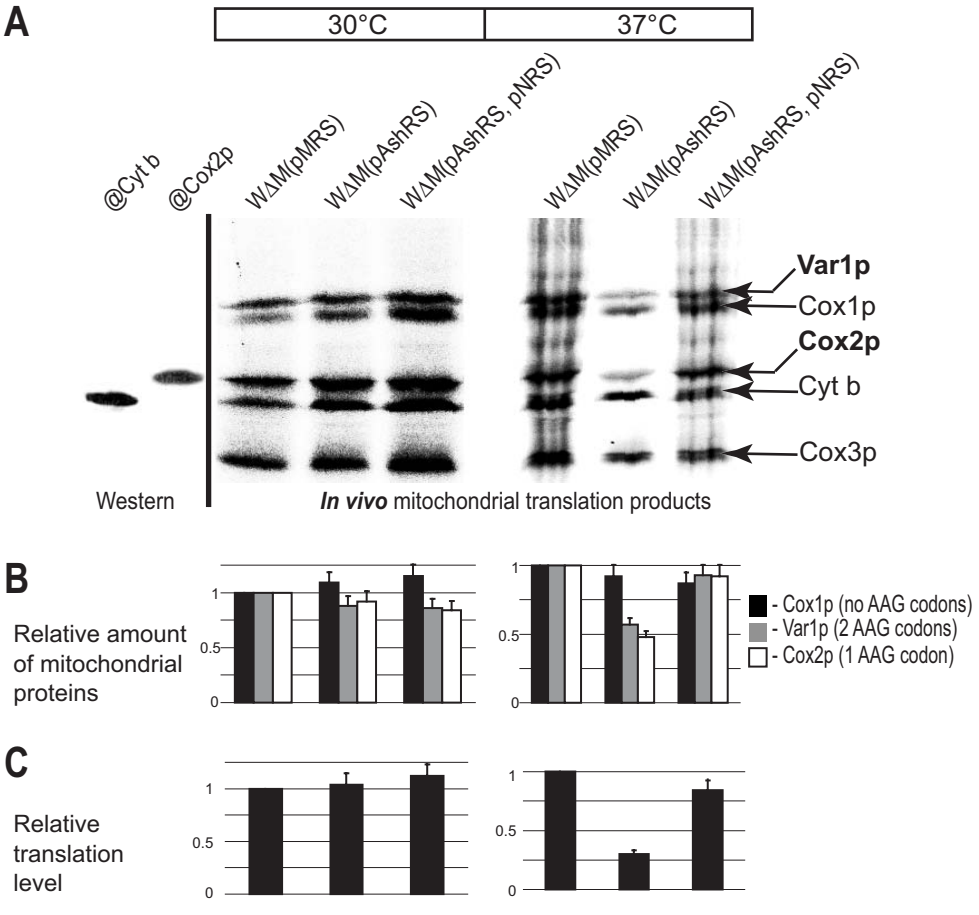
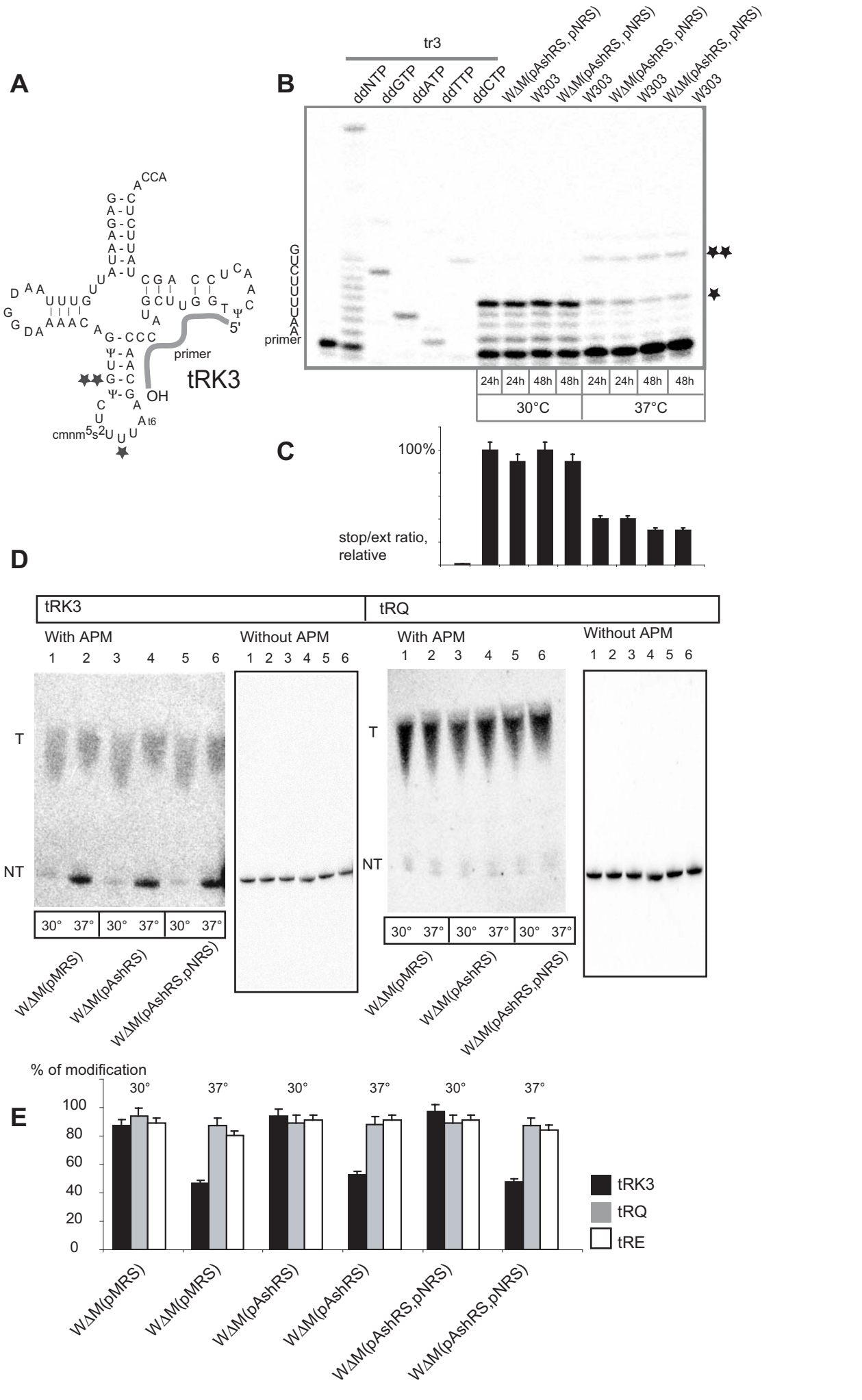

A
30°C
37°C
@Cyt b
@Cox2p
WΔM(pMRS)
WΔM(pAshRS)
WΔM(pAshRS, pNRS)
WΔM(pMRS)
WΔM(pAshRS)
WΔM(pAshRS, pNRS)
Var1p
Cox1p
Cox2p
Cyt b
Cox3p
Western
In vivo mitochondrial translation products
B
Relative amount of mitochondrial proteins
1
0.5
0
- Cox1p (no AAG codons)
- Var1p (2 AAG codons)
- Cox2p (1 AAG codon)
C
Relative translation level
1
0.5
0

Figure 6. The effect of the elevated temperature on mitochondrial tRNAs modification. (**A-C**) Primer extension experiment. (**A**) The cloverleaf structure of the yeast mitochondrial $tRNA^{Lys}$ (tRK3). The primer used is indicated by a grey line, * indicates the anticipated arrest of extension at the modified U34, ** indicates the position of the first G after the start of extension and corresponds to the arrest of extension in the presence of ddC. (**B**) Autoradiograph of the extension products. At the left, the sequence of the RNA is indicated, from the primer to the 5'-end; at the right, asterisks show positions of the arrests. tr3, the T7 transcript of the tRK3 gene used as the control template. "ddNTP", elongation was performed in the presence of all four ddNTPs. Extensions with yeast RNA were performed in presence of ddC. (**C**) Quantification of the extension assay. The ratio between the signals corresponding to the product of reverse transcription arrest at the modified base of the anticodon and at the first G was taken as the indication of the modification extent. (**D**) Analysis of thio-modification at the position 2 of the wobble uridine by Northern-hybridisation of mitochondrial tRNAs separated in APM-containing gels. In parallel, RNA separations were performed in gels without APM. The retarded diffused zone corresponds to the thiolated (T) version of the tRNA, the band at the bottom corresponds to the non-thiolated version (NT). Longer gels without APM are presented to demonstrate the absence of degradation. The numbers above the autoradiographs correspond to the same samples. (**E**) Quantification of the APM-gels. Similar hybridisations were performed with the probes for tRK3, tRQ (presented in **D**) and tRE (included only in the graph). Percentage of modification was evaluated as a % of the M signal with respect to M+MN ones.

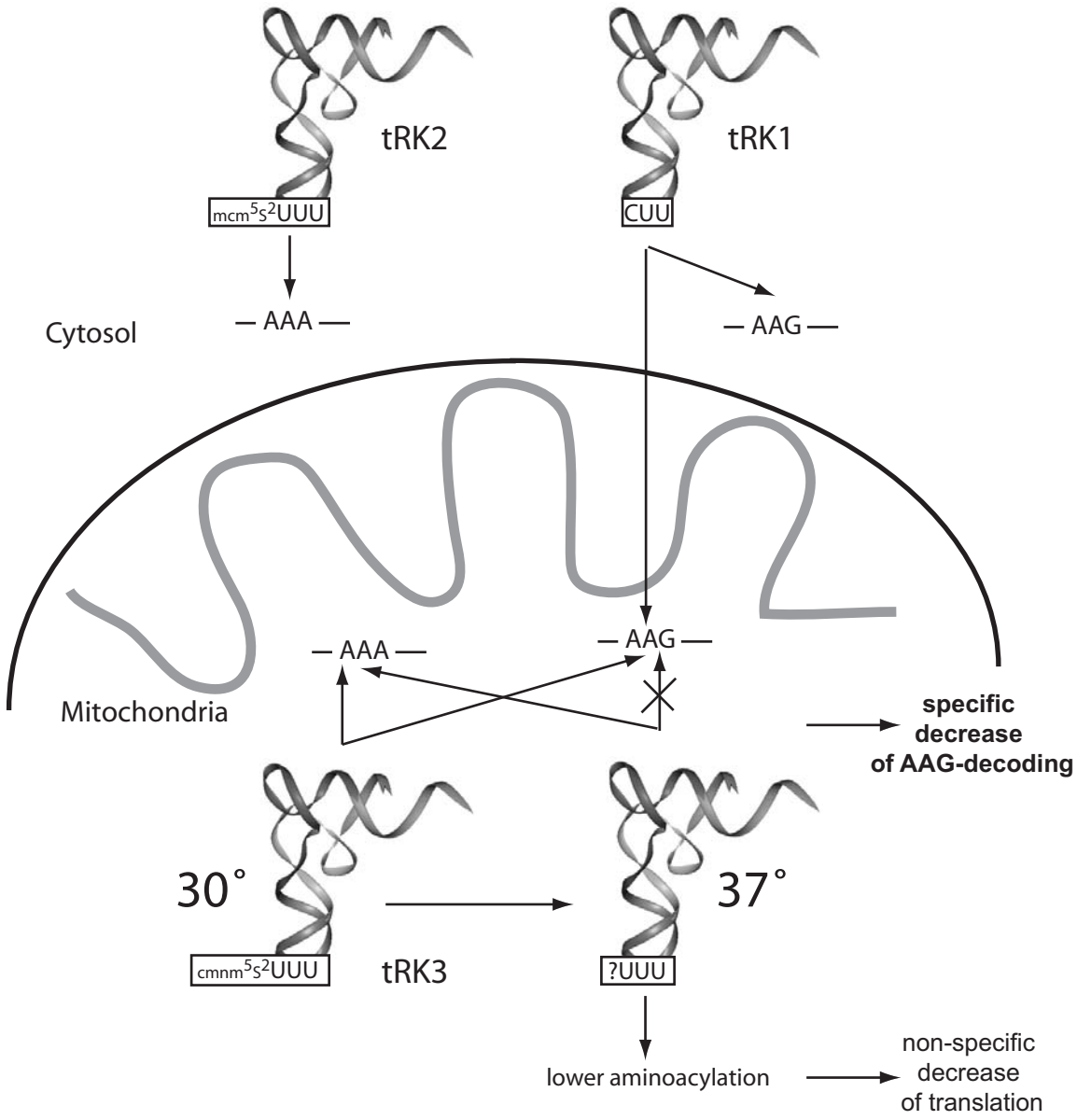
E) Figure 6



Kamenski et al., Fig 6

Figure 7. Hypothetic mechanism of tRK1 involvement in mitochondrial translation. Both tRK2, decoding the AAA- and tRK1, decoding exclusively AAG- lysine codons are required for cytosolic translation. tRK3, encoded in the mitochondrial DNA, can decode both AAA- and AAG-codons in mitochondrial mRNAs at 30°. At 37°C, the $cmnm^5s^2U34$ in the wobble position of tRK3 becomes hypomodified, which makes less efficient AAG-decoding, without affecting AAA-decoding. In these stress conditions, imported tRK1 can cure the deficiency by decoding the AAG-codons.

E) Figure 7



Kamenski et al., Fig 7

CHAPTER 4. CONCLUSIONS & PERSPECTIVES

Based on the results obtained in this work, we can make the following conclusions:

1. Only those tRNAs and transcripts which form a stable complex with the precursor of mitochondrial LysRS (preMsk1p) are able to enter the isolated yeast mitochondria. K_Ds of these complexes are of 180-250 nM.

2. Enolase 2 forms a complex with the imported tRNA with a K_D of 2500 nM. The presence of enolase 2 decreases the K_D of imported tRNA-preMsk1p complex down to 40 nM.

3. The complex of imported tRNA (tRK1) with the cytosolic LysRS is organized in the same manner as the known complexes of other class 2b aminoacyl-tRNA synthetases with their cognate tRNAs. tRK1-preMsk1p complex is organized in a different way: preMsk1p protects larger surfaces of the tRK1 molecule.

4. The N-terminal domain of preMsk1p can specifically direct tRNA import into yeast mitochondria both *in vitro* and *in vivo*. The region of preMsk1p flanked by α-helices H5 and H6 is necessary for specific tRNA import direction. Both this region and the region flanked by α-helices H1 and H3 are required for the mitochondrial $tRNA^{Lys}$ aminoacylation.

5. tRK1 cannot functionally replace the mitochondrial $tRNA^{Lys}$ in the mitochondrial translation.

6. tRK1 may be required for mitochondrial translation in a conditional manner. At 30°C, mitochondrial $tRNA^{Lys}$ (anticodon $cmnm^5UUU$) is sufficient for mitocondrial translation. In contrast, at 37°C, mitochondrial $tRNA^{Lys}$ becomes hypomodified at U34 and reads AAG codons inefficiently.We hypothesize that at elevated temperature, tRK1 is required for conditioanl correction/adaptation of mitochondrial translation.

Despite many questions were answered in this study, there are still many of them remaining. First of all, it would be very interesting to obtain the crystal structure of the tRK1-preMsk1p complex. Indeed, this work was already planned. This will allow understanding of how can a so unusual complex be formed, in which almost all the tRNA molecule is protected by the protein. Unfortunately, although several alternative systems of overexpression were tested, it was impossible to obtain large amounts of recombinant preMsk1p in the native state. Additionally, it would be useful to know the tRNA:protein molar ratio in this complex since normally class 2b aminoacyl-tRNA synthetase is a dimer. Indeed, the N-terminal domain

shown to be active in tRK1 import contains the C-terminal portion which, in the full-sized enzyme, is presumed to serve as a dimerization platform. It is important to understand if the RNA-protein interactions leading to tRNA import includes dimerization of the protein moiety. Even though crystallization of the complex might be difficult, this question might be adressed by gel-filtration analysys.

In this work, the region of preMsk1p involved in tRNA targeting to mitochondria was identified. A very important thing is now to determine the exact amino acids and/or structural elements of this protein that are responsible for this function. The amino acids that directly interact with the impored tRNA can be found by complex crystallization. However, some of them may be not in direct contact with tRNA but still be required for tRNA import. This question can be solved by the mutagenesis of *MSK1* gene. Another possible approach will be cross-linking experiments followed by identification of the interacting protein motifs and tRNA nucleotides.

Some other protein factors might still be necessary for tRK1 import. Indeed, it was shown that tRNA import occurs through the protein import pathway, and preMsk1p is believed to be tRNA carrier, while enolase plays the role of RNA-chaperone. On the other hand, additional experiments performed in our team led us to suggest that other proteins are implicated in this process and might serve as regulatory factors. Implication of the proteasomal proteins (UPS system) was hypothesized (I. Brandina et al., *in preparation*). To further study this aspect, cross-linking approach, as well as the disruption of the genes that code for the components of protein import apparatus, seems to be suitable.

Finally, understanding the mechanism of tRNA import into mitochondria will allow us to use it for gene therapy of the human diseases more efficiently. Also, it will be possible to create vectors for directed delivery of the genetic material into mitochondria. This seems to be also very effective approach to cure the human pathologies associated with the mutations in the mitochondrial genome.

CHAPTER 4. MATERIALS & METHODS

3.1. Strains and media

Epicurean coli strain BL21 RIL3 Codon Plus (Stratagene) was used for expression of recombinant proteins. *Escherichia coli* strain XL10-Gold (Stratagene) was used for site-directed mutagenesis. *Escherichia coli* strain XL1blue (Stratagene) was used for cloning purposes. Cultivation was performed in standard LB medium with appropriate antibiotics. In case of *Epicurian coli* strain BL21 RIL3 Codon Plus the medium also contained 0.4% of D-glucose (to prevent the "promotor leakage"). For cultivation of yeast, we used YPD (contained 2% of D-glucose), or YPEG (contained 3% of glycerol and 2% of ethanol), or YPG (3% of glycerol), or YPLac (2% of lactate), or YPGal (2% of galactose) media, as well as synthetic media (CSM, Bio 101 Inc.) containing the mixture of amino acids and nucleotides without one or two components (for the selection of the plasmid(s) containing the corresponding marker gene(s)). For exclusion of *URA3*-containing plasmid we used 5-FoA medium (yeast nitrogen base 6.7g/l, CSM-ura 0.77mg/l, uracile 30mg/l, glucose 20g/l, 5-fluoorotate 1g/l).

Using methods of yeast genetics we constructed several mutant yeast strains. In each of them *MSK1* gene was substituted with the mutant variant of this gene. As an initial yeast strain, we used the haploid strain WΔM(pMRS) (W303 MAT a, *msk1*::KanMX4 (pMRS416)). The strains used in this study are briefly described in the **Table 1.**

Table 1. Yeast strains used in the study.

Strain name	Genotype, plasmid, genetic marker	Reference
WΔM(pMRS)	W303 *msk1*::KanMX4 (pMRS416, *URA3*)	(Entelis et al., 2001)
WΔM	W303 *msk1*::KanMX4	(Tarassov et al., 1995b)
WΔM(pMRS313)	W303 *msk1*::KanMX4 (pMRS313, *HIS3*)	this work
WΔM(pAshRS)	W303 *msk1*::KanMX4 (pAshRS, *HIS3*)	this work
WΔM(pNRS)	W303 *msk1*::KanMX4 (pNRS, *LEU2*)	this work
WΔM(pMRS313, pAshRS)	W303 *msk1*::KanMX4 (pAshRS, pNRS, *HIS3, LEU2*)	this work
WΔM(pΔNRS)	W303 *msk1*::KanMX4 (pMRSΔN, *HIS3*)	this work
WΔM(pΔH5RS)	W303 *msk1*::KanMX4 (pMRSΔH5, *HIS3*)	this work
WΔM(pΔH6RS)	W303 *msk1*::KanMX4 (pMRSΔH6, *HIS3*)	this work
WΔM(pΔH5-H6RS)	W303 *msk1*::KanMX4 (pMRSΔH5-H6, *HIS3*)	this work
WΔM(pΔH1-H3RS)	W303 *msk1*::KanMX4(pMRSΔH1-H3, *HIS3*)	this work

3.2. Oligonucleotides

In this work we used following oligonucleotides:

for Northern analysis – anti-tRK1 tggagccctgtaggggg, anti-tRK2 – tggctcctcataggggg, anti-tRK3 – tggtgagaataagctgg, anti-$tRNA_{Leu}$(mt) – cccgaattccctggttgctatttaaaggac,

for creation of ΔORF deletion – ggtgttatgttacatgattgccatggattttgcgacgcctgccaatgcttttg and fully complementary oligonucleotide,

for cloning of AshLysRS gene – gggatacttttataatggtgttatgttacatgattgccgatgtggtgcggagccagagcgatgatagc and tctgatttatttacaaaagcattggcaggcgtcgcaaaatctactggcggttgacgtcgtctagacatcc,

for creation of ΔC deletion – cgttgaggttgaaacgcctatactgtcatcaaagtcctaaattttgcgacgcctgccaatgcttttg and fully complementary oligonucleotide,

for creation of ΔN deletion – cgatgaccgacgtccagagaatacggcctgctcc and cgatgaccgacgtcgaggttgaaacgcctatactg,

for creation of ΔH5 deletion – gtaataagctacctatagtattgtctgtttcgaatagagttgttgactaccaattaaacggc and fully complementary oligonucleotide,

for creation of ΔH7 deletion – caaatctaatagagttgttgactaccaattaaacttcgttgaggttgaaacgcctatactg and fully complementary oligonucleotide,

for creation of ΔH5-H7 deletion – gtaataagctacctatagtattgtctgtttcgaacttcgttgaggttgaaacgcctatactg and fully complementary oligonucleotide,

for creation of ΔH1-H3 deletion – cgaagtagcaggagcaggccgtattctctgggcaacccagccacattacaccatgagg and fully complementary oligonucleotide,

for cloning of preMsk(N) for protein expression - gggaattccatatgaatgtgctgttaaaaagacgc and gggttaggtgatcattgtgatggtgatggtgatgactgcctgtttacatcatccac,

for cloning of preMskA5 – gggaattccatatgaatgtgctgttaaaaagacgc and tactacctcgagcggcagttgcgaaacagacaa,

for cloning of preMskH4 – gggaattccatatgaatgtgctgttaaaaagacgc and tactacctcgagtcctttcttcaaaaagttcat,

for cloning of preMskA3 –
gggaattccatatgaatgtgctgttaaaaagacgc and tactacctcgagcccaccaattttattgtagtt,

for cloning of preMskH3 –
gggaattccatatgaatgtgctgttaaaaagacgc and tactacctcgagattaggattatcctcatggtg,

for primer extension on tRK3 – accaagcatgggttgc,

for primer extension on mitochondrial leucine tRNA – actgtaaagtaatacatc,

for automatic sequencing of *MSK1* gene – atgaatgtgctgttaaaaag, gccagagaatacggcctg and caataagccgagcgaact.

3.3. Plasmids

Plasmids and their brief description are presented in the **Table 2**.

pMRS416 plasmid represents the fully functional *MSK1* gene with its own promoter and 1kb flanks in pRS416 shuttle vector (*URA3*-marker).

pMRS313 plasmid represents the full functional *MSK1* gene with its own promoter and 1kb flanks in pRS313 shuttle vector (*HIS3*-marker). This plasmid was used to mutagenize *MSK1* gene using the QuikChange mutagenesis kit (Stratagene) as well as for cloning of the *Ashbya gossypii* mitochondrial lysRS gene (AshRS). For this purpose, the full protein coding sequence of *MSK1* from the ATG to the stop codon in the pMRS313 plasmid was replaced by the *Nco*I site by site-directed mutagenesis, to obtain pMRS313Δ. Then the AshRS gene was inserted into this plasmid (see Genetic Engineering Manipulations).

To construct the N-terminal domain *in vivo* expressing plasmid, we deleted the C-terminal domain of the *MSK1* ORF (from N258 to the stop codon) from the plasmid pG11T6 which contained the *LEU2*-marker, by using site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene).

pET-Msk1 plasmid contained the *MSK1* ORF in frame with 6 His-coding codons at the C-terminus in the pET3a vector. This plasmid was also used for mutagenesis of *MSK1* gene in order to create truncated recombinant proteins. pET-Msk1-Nter plasmid contained the same construct but with the C-terminal domain of Msk1p deleted.

Table 2. Plasmids used in the study.

Name	Vector, genetic marker	Description	Reference
pMRS416	pRS416, *URA3*	3 kpb *Xho*I-fragment with the full *MSK1* gene, saver plasmid	(Entelis et al., 2001)
pMRS313	pRS313, *HIS3*	3 kpb *Xho*I-fragment with the full *MSK1* gene	this work
pG11T6	pYE13, *LEU2*	16 kbp genomic fragment with the full *MSK1* gene	(Gatti and Tzagoloff, 1991)
pNRS	pG11T6, *LEU2*	*MSK1* gene with the region coding for the C-terminal domain deleted	this work
pMRS313Δ	pRS313, *HIS3*	pMRS313 with the full *MSK1* ORF deleted	this work
pAshRS	pRS313, *HIS3*	AshRS gene with the genomic flanks of *MSK1* gene	this work
pMRSΔN	pRS313, *HIS3*	*MSK1* gene with the region coding for the N-terminal domain deleted	this work
pMRSΔH5	pRS313, *HIS3*	*MSK1* gene with the region coding for the H5 α-helix deleted	this work
pMRSΔH6	pRS313, *HIS3*	*MSK1* gene with the region coding for the H6α-helix deleted	this work
pMRSΔ(H5-H6)	pRS313, *HIS3*	*MSK1* gene with the region coding for the protein part between helices H5 and H6 deleted	this work
pMRSΔ(H1-H3)	pRS313, *HIS3*	*MSK1* gene with the region coding for the protein part between helices H1 and H3 deleted	this work
pET-Msk1	pET3a, Amp^R	pre-Msk1p coding ORF under control of the T7 promoter and with C-terminal 6-His tag	(Entelis et al., 2001)
pET-Msk1-Nter	pET3a, Amp^R	The gene coding for N-terminal domain of preMsk1p under control of the T7 promoter and with C-terminal 6-His tag	this work
pET-MskΔN	pET3a, Amp^R	pET-Msk1 with the region coding for the N-terminal domain of preMsk1p deleted	this work
pET-MskΔH5	pET3a, Amp^R	pET-Msk1 with the region coding for the H5 α-helix deleted	this work
pET-MskΔH6	pET3a, Amp^R	pET-Msk1 with the region coding for the H6 α-helix deleted	this work
pET-MskΔ(H5-H6)	pET3a, Amp^R	pET-Msk1 with the region coding for the protein part between helices H5 and H6 deleted	this work
pET-MskΔ(H1-H3)	pET3a, Amp^R	pET-Msk1 with the region coding for the protein part between helices H1 and H3 deleted	this work
pET-Msk1-A5	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and A5 β-element	this work
pET-Msk1-H4	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and H4 α-helix	this work
pET-Msk1-A3	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and A5 β-element	this work
pET-Msk1-H3	pET30a, Kan^R	The gene coding for the preMsk1p part between the signal presequence and H3 α-helix	this work

3.4. Genetic engineering methods

3.4.1. PCR

The polymerase chain reaction was done in 10X buffer from Promega (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 1% Triton X100) with addition of 2.5 mM $MgCl_2$, 10 pmoles of each of two proper oligonucleotides, 0.2 mM of each of four desoxyribonucleotides, 0.1-0.5 µg of template DNA and 0.5-1 unit of Taq DNA polymerase. The melting temperature depended on the oligonucleotides used: T_m (°C) = 59.9 +41*(%GC)-(675/Lentgh), where %GC is the portion of guanosines and cytosines in the oligonucleotide sequence. The PCR products were separated on the 1% agarose gel or on 8% PAAG.

3.4.2. Mutagenesis

The mutagenesis was done using QuikChange Mutagenesis Kit (Stratagene) according to the manufacturer protocol (http://www.stratagene.com/manuals/200518.pdf).

3.4.3. *E.coli* electroporation

The commercial competent cells were used for the transformation. The electroporation was done in 1mm cuvettes (Biorad) with voltage 1.8 kV. After electroporation, the cells were saved by shaking for 1 hour at 37°C with LB liquid medium and plated on the appropriate solid medium.

3.4.4. Yeast transformation

The yeast transformation with the plasmid or linearized DNA was done as described in (Gietz and Woods, 2006).

3.5. Yeast DNA isolation

The method described in (Rose, 1990) was used. To purify yeast genomic DNA for further PCR, 5 ml of yeast culture were grown overnight in a liquid medium, harvested and resuspended in 0.5 ml of 1M sorbitol and 0.1M EDTA pH 7.5. After that, 50 µg of zymolyase 100 000 were added, and cells were incubated at 37°C for 1 hour. Then, cells were pelleted down and resuspended in 0.5 ml of 50 mM Tris-HCl pH 7.4, 20 mM EDTA. 50 µl of 10% SDS were added, and the suspension was well mixed and incubated at 65°C for 30 minutes. Then, 0.2 ml of 5 M potassium acetate pH 5 were added, and the mixture was incubated on ice for 1 minute and centrifuged for 5 minutes. The genomic DNA was precipitated from the supernatant with one volume of isopropanol and dissolved in 0.3 ml of TE. After this, 30 µl of 3M sodium acetate pH 5 was added, and DNA was again precipitated with isopropanol and dissolved in water.

To purify plasmid DNA from yeast, yeast were grown overnight in 2 ml of a liquid medium, harvested and resuspended in the residual liquid. Then, 0.2 ml of 2% Triton X100, 1% SDS, 0.1 M NaCl, 10 mM Tris-HCl pH 8 and 1 mM EDTA was added, followed by the addition of 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1 V/V) and 0.3 ml of glass beads. The mixture was shaked for 5 minutes on ice and centrifuged for 5 minutes. The plasmid DNA from the aqueous phase was precipitated twice with three volumes of ethanol and dissolved in water.

3.6. Expression and purification of the recombinant proteins.

The E.coli cells were transformed with the plasmid containing the gene of the protein of interest cloned into the one of pET family vectors. The transformed cells were always grown on LB medium with 0.4% of D-glucose. The protein expression was inducted by adding of 0,5-1 mM of IPTG to the medium. The temperature and the time of expression were selected experimentally. After expression, cells were frozen and thawed 3 times in buffer containing 50 mM sodium phosphate buffer pH 8.0, 0.3 M NaCl, 10 mM imidazol, 0.1% Triton X100, 20 mM β-mercaptoethanol, 2 mM $MgCl_2$ and proteases inhibitors. After that, the lysates were sonicated 4 times (15 seconds each) on ice and centrifuged for 30 minutes. The pellets were dissolved in 100 mM sodium phosphate buffer pH 8.0, 10 mM Tris-HCl pH 8.0, 8 M urea, shaked for 2 hours and centrifuged for 30 minutes. The presence of the recombinant proteins in "native" and urea-containing lysates was checked by PAGE. The affinity purifications of the recombinant proteins were done using slurry Ni-agarose (Qiagene). In case of "native" lysate the column was washed with the buffers containing 25-150 mM imidazol, and the protein eluted with the buffers containing 200-750 mM imidazol. In case of urea-containing lysate the column was washed with the buffers with pH 6.5-5, and the protein was eluted with the buffers with pH 4.5-3.

3.7. Gel-retardation assay

Gel-retardation assay was done as described in (Entelis et al., 2006). 5-10 cpm of gel-purified labeled tRNA (or labeled transcript of tRNA gene) were incubated with the recombinant protein(s) in the buffer containing 50 mM Na-cacodylate pH 7.5, 270 mM KCl, 20 mM $MgCl_2$ and 0.02% BSA, at 30°C for 10 minutes. Then the RNA-protein complexes were separated on 6.5% PAAG containing 0.5x TBE and 5% glycerol, at 4°C. The gels were fixed, dried and incubated with Fuji-Imager screen.

3.8. Footprinting

Before all the procedures, gel-purified labeled tRNA (or labeled transcript of tRNA gene) was mixed with 0.5 μg of *E.coli* tRNAs, heated for 1 minute on 90°C and quickly put on ice.

To obtain an "alkaline ladder", 20 cpm (0.1-0.5 μg) of tRNA in 0.1 M sodium carbonate buffer pH 8.9 were incubated at 90°C for 5 minutes.

To obtain T1 ladder, 20 cpm (0.1-0.5 μg) of tRNA were treated with 1 unit of T1 nuclease in the buffer containing 20 mM sodium citrate pH 5.0, 10 M urea, 0.02% xylene cyanol and 0,02% bromphenol blue, at 50°C for 5 minutes. The reaction was stopped by adding of 3.33X TBE.

To treat tRNA-protein complexes with S1 or V1 nuclease, the proper protein was added (with a molar ratio tRNA:protein being 1:1 or 1:5) and complex formation was done in the buffer containing 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM NaCl (in case of nuclease S1, the buffer also contained 1 mM $ZnCl_2$). Then the proper nuclease was added (S1 – 40 units, V1 – 10-20 units), and the mixture was incubated at 37°C for 5 minutes. The reactions were stopped by adding of 0.6 M sodium acetate pH 5.0, 10 mM EDTA pH 4.0 and 1 μg of *E.coli* tRNAs. Then tRNAs were phenol-extracted, precipitated with ethanol and dissolved in water.

To treat tRNA-protein complexes with Fe^{2+}/EDTA/H_2O_2, the proper protein was added (with a molar ratio tRNA:protein being 1:1 or 1:5) and complex formation was done in the buffer containing 50 mM Na-cacodylate pH 7.0, 270 mM KCl, 20 mM $MgCl_2$ and 0.02% BSA for 10 min at 30°C. After this, 1 μl of each of the solutions listed below was added: 50 mM $(NH_4)_2Fe(SO_4)_2$, freshly prepared 2.5% H_2O_2, 0.25 M DTT and 0.1 M EDTA pH 4. The mixture was incubated for 5 min at room temperature, and then 10 μl of 0.1 M thiourea and 1 μg of *E.coli* tRNAs were added and RNAs were precipitated with ethanol, dissolved in water and treated with hot phenol pH 5 and 0.1% SDS. Aqueous phase was again precipitated with ethanol and the pellet was dissolved in water.

For separation of footprinting products, 15% or 20% PAAG was used. The products were visualized with Fuji Imager and analysed with the computer program MacBas2000.

3.9. Yeast genetics and phenotypic analysis

For *in vivo* selection of plasmid, yeast cells were always grown on the selective media that do not contain one component (amino acid or nucleotide) which can be synthesized by means of the marker gene of the plasmid.

For plasmid shuffling (Rose, 1990), yeast cells containing the plasmid with *URA3* marker gene were grown on 5-fluoorotate containing media.

The phenotypic analysis was performed by growing the strains on "respiratory media": YPG, YPEG, YPLac and compared to the growth on YPD and YPGal media, both on agar plated and in liquid (Rose, 1990).

3.10. Mitochondrial translation

Mitochondrial translation was analysed essentially as described in (Barrientos et al., 2002) with minor modifications: cells were grown in CSM-His Gal or CSM-His-Leu Gal to A600=1.0, transferred to synthetic complete medium lacking Met (0.67% yeast nitrogen base, 0.08% CSM-Met (BIO101), 2% galactose), with addition of 200 µg/ml of cycloheximide and incubated for 10 min at 30°C with shaking. Cells were then labelled with 10 µCi of [^{35}S]-methionine (1.4 Ci/mmol, Amersham) for 20 min and chased with 2 mM unlabeled methionine. The proteins were TCA-precipitated, water-washed and analyzed on 15% SDS-PAGE.

3.11. Mitochondrial respiration

The rate of oxygen consumption by yeast cells (A_{600}=0.3) cultured in 1 ml of YPGal medium was measured in thermostated chamber at 30°C or 37°C using a Clark type electrode and the SI oxygen meter for 10 min followed by addition of myxothiasol (750 nM) to measure non-mitochondrial oxygen uptake, which was then subtracted from all values.

3.12. Primer extension

The primer extension method was based on the procedure described in (Kirino et al., 2005) with several modifications. The polynucleotide kinase 5' ^{32}P-labeled primer (0.1 pmol) was incubated with 2 µg of the total RNAs in a 10-µl solution containing 10 mM Tris·HCl (pH 8.0), 1 mM EDTA at 90°C for 2 min and then allowed to stand at room temperature for 1 h. Subsequently, 4 µl of 5x reaction buffer for reverse transcription (Roche), 0.5 µl of d/ddNTP mix containing 1.5 mM each of the three dNTP and one ddNTP (Amersham), 3 µl of 25 mM $MgCl_2$, and 1 µl of Moloney murine leukemia virus reverse transcriptase (40 units/µl, Roche)

were added, and the mixture was incubated at 42°C for 1 h. Nucleic acids were the ethanol-precipitated and then subjected to 20% PAAG containing 8 M urea (20 cm). The radiolabeled bands were visualized by a BAS5100 bioimaging analyzer (Fuji).

3.13. Aminoacylated tRNA isolation and separation

To obtain total yeast aminoacylated tRNA, yeast were grown overnight in the liquid galactose-containing selective medium, harvested, washed once with water and resuspended in 0.5 ml of the buffer consisting of 0.3 M sodium acetate pH 5.0, 10 mM EDTA and 0.1% Triton X100. The equal volume of phenol saturated with 0.1 M sodium acetate pH 5 and 0.3 ml of glass-beads were added, and the mixture was shaked for 5 minutes on ice. The small RNAs were extracted from the aqueous phase by differential isopropanol precipitation (20% and 60%) and were dissolved in 10 mM sodium acetate pH 5.0. Separation of deacylated and aminpacylated forms of tRNAs was performed on 4°C using the 6.5% PAAG containing 100 mM sodium acetate pH 5.0 and 8 M urea, as described in (Varshney et al., 1991).

Isolation and characterization of yeast mitochondria, RNA isolation, labeling and analysis, tRNA import assays and other methods not presented above are described in details in the Publication 5 (pp. 196-202) and Publication 6 (pp. 241-247) (attached below).

Publication 5.

Entelis N, Kolesnikova O, Kazakova H, Brandina I, Kamenski P, Martin RP, Tarassov I.

"Import of nuclear-encoded RNAs into yeast and human mitochondria: experimental approaches and possible biomedical applications."

Genet Eng (NY). 2002, 24: 191-213.

Publication 6.

Mager-Heckel AM, Entelis N, Brandina I, Kamenski P, Krasheninnikov IA, Martin RP, Tarassov I.

"*The analysis of tRNA import into mammalian mitochondria."*

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17

The Analysis of tRNA Import Into Mammalian Mitochondria

AU: please provide first name for all author.

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Summary

Ribonucleic acid (RNA) import into mitochondria occurs in a variety of organisms. In mammalian cells, several small RNAs are imported in a natural manner; transfer RNAs (tRNAs) can be imported in an artificial way, following expression of corresponding genes from another organism (yeast) in the nucleus. We describe how to establish and to analyze such import mechanisms in cultured human cells. In detail, we describe (1) the construction of plasmids expressing importable yeast tRNA derivatives in human cells, (2) the procedure of transfection of either immortalized cybrid cell lines or primary patient's fibroblasts and downregulation of tRNA expression directed by small interfering RNA (siRNA) as a way to demonstrate the effect of import in vivo, (3) the methods of mitochondrial RNA isolation from the transfectants, and (4) approaches for quantification of RNA mitochondrial import.

Key Words: Aminoacylation; mitochondrial import; real-time quantification; siRNA downregulation; tRNA.

1. Introduction

Mitochondrial import of small noncoding ribonucleic acids (RNAs) is now considered a quasi-universal pathway. Found in a variety of species (fungi, protozoans, animals, and plants), it differs dramatically from one system to another in a dramatic manner ***(1,2)***. Nuclear-encoded transfer RNAs (tRNAs; ranging from one species to the complete set needed for the organellar translation), but also 5S ribosomal RNA, MRP, or ribonuclease (RNase) P RNA components have been shown to be encoded in nuclear DNA and targeted into the mitochondrial matrix. In animal cells, no tRNA import was found in vivo, although other small RNAs are imported. Nevertheless, we found that tRNA import may be established in human cells in an artificial

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way: by expressing in cultured human cells "importable" versions of yeast tRNAs ***(3,4)***. This artificial import permitted importing functionally active tRNAs that could participate in mitochondrial translation and complement, at least partially, defects caused by mutations of mitochondrial tRNA genes encoded in the mitochondrial genome (mitochondrial deoxyribonucleic acid, mtDNA) ***(5)***. This approach may potentially be exploited to develop new gene therapy strategies for mtDNA diseases for which, up to now, no efficient therapy has been possible ***(6)***.

To establish and characterize quantitatively the artificial tRNA import into human mitochondria in vivo, we optimized several approaches described here. This chapter also describes how to obtain transgenic lines that import tRNAs into mitochondria, how to downregulate artificial tRNA import in vivo, how to isolate mitochondrial RNA (mtRNA), and how to detect and to quantify the import efficiency.

Various versions of all three yeast $tRNAs^{Lys}$ (cytosolic $tRNA^{Lys}_{CUU}$ or tRK1, cytosolic $tRNA^{Lys}_{UUU}$ or tRK2, and mitochondrially encoded $tRNA^{Lys}_{UUU}$ or tRK3) were shown to be internalized by human mitochondria ***(3,4)***. From nearly 40 in vitro importable versions ***(7)***, only the versions mentioned in this work were tested in vivo. However, taking into account high flexibility of the import pathway, one can predict that the methods described are exploitable in a wider way (i.e., to import other tRNAs into mammalian mitochondria). Constructs for in vivo expression were based either on the pBK-CMV vector or on pcDNA-3.1, both bearing the gene of resistance to G418 (neomycin derivative) (**Fig. 1**). tRNA genes were polymerase chain reaction (PCR) subcloned using total yeast DNA as the template for amplification and mutagenized by standard procedures (*see* **Note 1**).

AU: In the paragraph "Various versions of," define pBK-CMV and pcDNA-3.1.

Fig. 1

The transfection procedure depends on the cell line used. Normal immortalized cell lines (like 143B, HepG2, or HeLa cells) are robust, and transfection can be performed with most of commercial transfection reagents. However, cybrid cells seem to be much more fragile and are sensitive to treatment with lipophilic agents. These reagents proved most toxic for primary patients' cells (fibroblasts or myoblasts). To optimize conditions of transfection, one can assess on dilution of the reagent, on the concentration of DNA, on the confluence of cells, on the concentration of the antibiotic, and finally on the exact composition of the growth medium (*see* **Note 2**).

AU: In the paragraph "The assessment of transgene," define MERRF.

The assessment of transgene expression in human fibroblasts derived from patients with the MERRF syndrome has been complicated by two major problems: (1) a high degree of lethality was caused by the transfection procedure, and (2) the effect of transfection on mitochondrial functions was not observed for several days, being significantly delayed. We tried therefore to optimize the procedure by extending the time for transgene expression and to reduce

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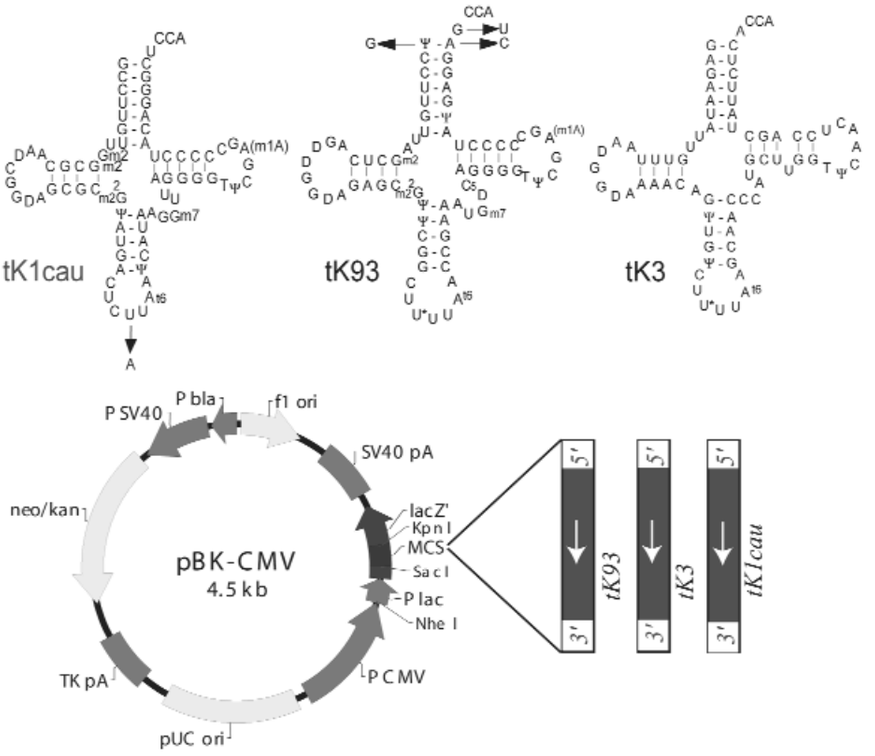

Fig. 1. Schematic drawing of importable tRNAs cloverleaf structures and expressing constructs in pBK-CMV vector. The black arrows indicate base substitutions enabling the import of tK93 version and the mutation in the anticodon of tRK1 (*see* the text for details). (Adapted from ref. ***5***.) The pBK-CMV vector map is adapted from Stratagene. The white arrows indicate the orientation 5′-3′ of the transgenes.

the cytotoxic effect of the transfection reagents. **Figure 2** shows that the Fig. 2 expression of the control luciferase gene was highest 12–24 h after transfection and then decreased because of elimination of the plasmid; the number of cells drops after each transfection procedure because of the cytotoxic effect. These two restrictions necessitated the use of several consecutive transfections before observing the phenotypic effect of transgene expression (*see* **Note 3**).

RNA interference is commonly used to knock down expression of RNA polymerase II-transcribed genes ***(8–10)***. We need to downregulate expression of transgenic tRNAs, which are normally transcribed by RNA polymerase III, to show that the rescued phenotype observed in stable cybrid cell transfectants was caused by the expression of imported tRNAs ***(5)***. We designed several

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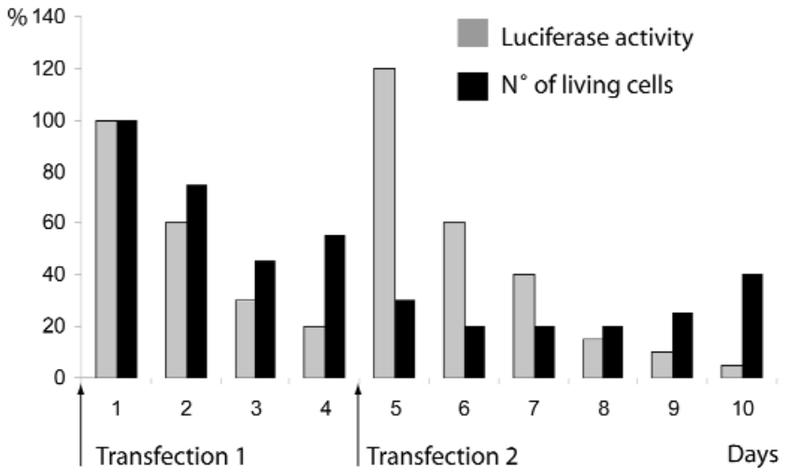


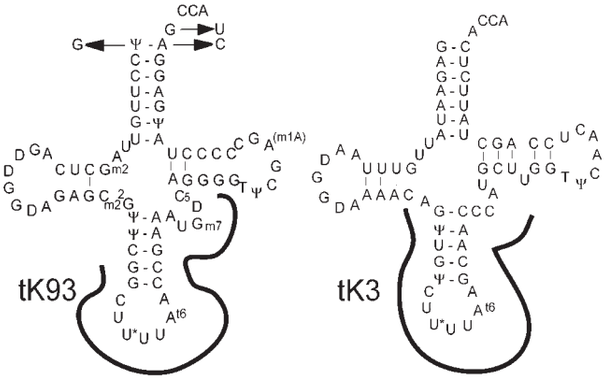
Fig. 2. Assays of transgene expression and cytotoxicity after serial transfection. Here, MERRF cybrid cells (line based on 143B *rho*° line) were transfected twice with the tK3-expressing plasmid, as indicated by the arrows below the graph. The values are presented in percentage of living cells and luciferase (reporter) activity with respect to d 1 after transfection.

Fig. 3

RNA duplexes that efficiently inhibited expression of yeast tRNA derivatives in transfected human cells (**Fig. 3**). All these duplexes were 19–21 bases long and, to provide more resistance to nucleases, contained two protruding thymidines at both termini. To follow knockdown experiments, the transfection has to be performed with the reporter gene of luciferase (pGL3 control plasmid) together with the corresponding small interfering RNA (siRNA) duplex.

Mitochondrially imported RNAs are, as a rule, present in low amounts in the cell. MRP RNA and RNase P RNA components seem to be present at 1–2 molecules per mitochondrion ***(11–13)***; imported tRNAs also are underrepresented in the organellar pool of RNAs ***(1,5)***. This means that to detect import and to quantify it one needs to use either extremely sensitive methods or large amounts of mtRNA. We describe two alternative approaches adapted to these possibilities (*see* **Note 4**).

The choice of oligonucleotide probe is crucial for detection of a given RNA. Before beginning large-scale experiments, one needs to optimize oligonucleotide design for each RNA and to test in which conditions the probe reveals a unique band. Then, all mtRNA samples must be checked for cytosolic contamination. It is possible, indeed, to measure this contamination using cytosol-specific probes (*see* **Note 5**) and then to calculate this contamination



anti-LUC

GGAUUCUAAAACGGAUUACCdTdT
dTdTCCTAAGATTTTGCCTAATGG

anti-tRK93

GGCUUUUAACCGAAAUGUCdTdT
dTdTCCGAAAATTGGCTTTACAG

anti-tRK3

GUUGUCUUUUAAGCAACCCdTdT
dTdTCAACAGAAAATTCGTTGGG

Fig. 3. Design of siRNAs for downregulation assays. Localization of complementary regions on the cloverleaf tRNA structure are indicated in the upper portion (for tK93 and tK3 versions). Sequences of the siRNAs used are indicated at the bottom: anti-LUC is used to control RNA interference by downregulating the reported gene. The presented siRNAs efficiently inhibited (more than 90%) expression of both reporter (luciferase) and tRNA genes in 2–4 d after transfection.

Fig. 4

as a percentage of the value obtained for the RNA of interest (**Fig. 4**). However, it is possible that contamination is completely nonspecific, and different RNA species can contaminate mitochondrial preparation in the same way (which is not necessarily true).

Fig. 5

The most credible results are obtained when two or more cytosolic RNAs give a negative result; the probe against the imported RNA gives a positive signal (**Fig. 5**). The real-time PCR is an extremely powerful tool to measure absolute amounts of a given nucleic acid in solution. However, the application of this approach to tRNAs raises additional problems that may be resolved using the appropriate controls (*see* **Note 6**).

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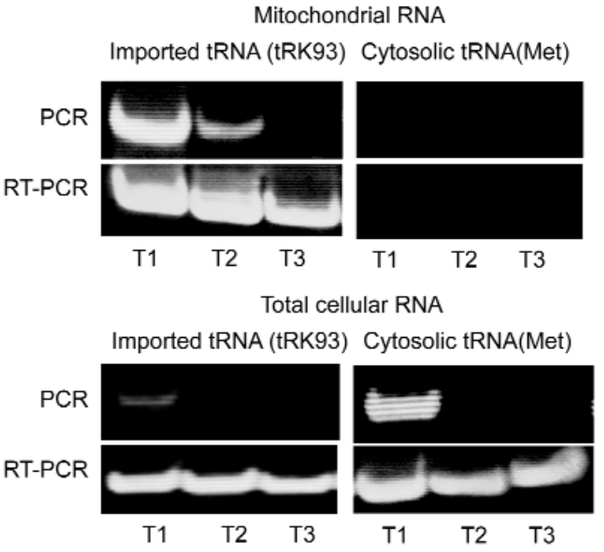

AU: **Figures 5** and **6** are mentioned in the text before **Fig. 4**, so Figs. Were renumbered in the order cited: 5→4, 6→5, 4→6.

Fig. 4. Serial TRIzol extraction of RNA. Total or mitochondrial RNA isolated from tK93-transfectants was used for PCR and RT-PCR reactions, in parallel. T1, T2, and T3 labels indicate the number of TRIzol extractions. Cytosolic $tRNA^{Met}$-specific primers were used to control the absence of cytosolic contamination in mitochondrial isolate. 10% nondenaturating polyacrylamide gels stained by ethydium bromide are presented.

2. Materials

2.1. Transfection of Human Cultured Cells and siRNA Downregulation Assays

1. pGL3-control vector, luciferase reporter plasmid (Promega), luciferase detection kit (Promega).
2. Transfection reagents SuperFect (Qiagen), LipofectAMINE™ 2000 (Invitrogen) or LyoVec6™ (InvivoGen), OptiMEM Reduced Serum medium for transfection (Invitrogen).
3. Cultured human cells: for these experiences, we used cybrid cells containing the MERRF mutation (A8344G) at greater than 95%; they were based on either HeLa or 143B genetic background.
4. Primary human cells: for the experiments described, we used cultured fibroblasts bearing mtDNA that contained 70% levels of the MERRF mutation.
5. Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, sodium pyruvate (110 mg/L), and L-glutamine (Sigma).
6. Uridine (Sigma-Aldrich).
7. EMEM with nonessential amino acids and 1 m*M* pyruvate (Sigma) and 5 µg/mL uridine (Sigma-Aldrich).

AU: In **item 7**, define EMEM.

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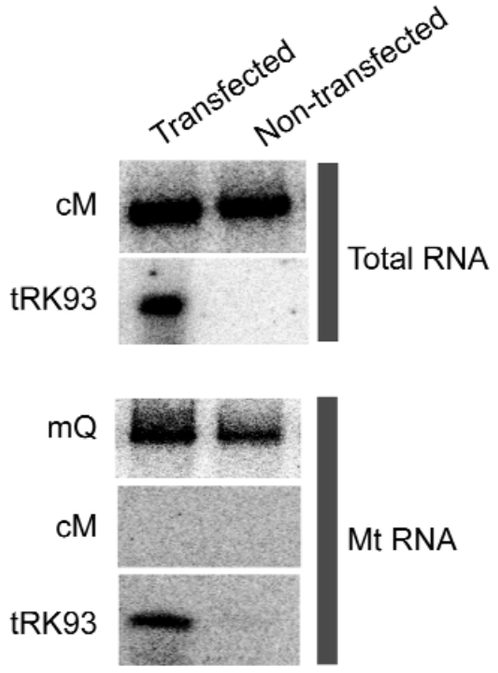

Fig. 5. Typical result of Northern quantitation analysis of import. MERRF cybrid cells and their tK93-stable transfectants were used to isolate mitochondrial RNA (hot phenol protocol). Total and mitochondrial RNA were analyzed for the amount of the transgenic tRNA (tK93) and reference host RNAs: cytosolic $tRNA^{Met}$ (cM) and mitochondrial $tRNA^{Gln}$ (mQ). Equal amounts of cytosolic RNAs (20 µg per slot) and of mitochondrial RNAs (50 µg per slot) were taken for analysis. Autoradiographs of washed membranes are presented.

8. Ham's F14 medium with 6 mg/mL glucose and 1 mg/mL adenosine triphosphate (Vitromex).
9. G418, streptomycin, penicillin, Fungizone (antibiotics) (Sigma or Invitrogen).
10. Synthetic RNA-RNA duplexes 20–21 bases long (siRNAs).

2.2. Isolation of mtRNA for Analysis of Import

AU: In **item 1**, define PIPES.

1. Mito buffer ± bovine serum albumin (BSA): 0.6 *M* mannitol (or 0.44 *M* sorbitol), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* Na-PIPES, pH 6.7, 0.3% (w/v) BSA (to add before use).
2. Bradford protein measurement reactif (Bio-Rad).
3. 2X RNases solution: micrococcale nuclease: 10 U/mL, RNase A: 100 µg/mL, 8 m*M* $MgCl_2$, 2 m*M* $CaCl_2$.

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AU: In **item 4**, define EGTA. In **item 5**, define MOPS.

4. RNase stop buffer: Mito buffer without BSA with 2 m*M* EDTA, 2 m*M* EGTA.
5. Mito-gradient buffer: 0.3 *M* sucrose, 10 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.2, 0.1% BSA.
6. TRIzol reagent (Invitrogen).
7. Phenol saturated with water or 0.1 *M* sodium acetate, pH 5.0 (Roth).
8. Diethyl pyrocarbonate (DEPC) (Sigma).

2.3. Quantitative Analysis of Import

AU: Defined TBE corectly?.

1. Standard equipment and reagents for polyacrylamide gel electrophoresis and blotting.
2. 10X Tris borate EDTA (TBE) electrode buffer: 0.89 *M* Tris base, 0.89 *M* boric acid, pH 8.4.
3. Hybond-N membranes (Amersham-Pharmacia).
4. T4-polynucleotide kinase (New England Biolabs).
5. γ-[^{32}P]-Adenosine triphosphate (>4000 Ci/mmol) (Amersham).
6. Equipment for hybridization: rotating oven, ultraviolet (UV) crosslinking chamber.
7. 20X SSC solution: 3 *M* NaCl, 0.3 *M* sodium citrate, 1 m*M* EDTA.
8. 100X Denhardt solution: 2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone.
9. Equipment for phosphoimaging and corresponding software (here: Fuji-2000, MacBas).
10. Taq polymerase and its corresponding buffers (reaction buffer, deoxynucleotide 5′-triphosphate solution).
11. One-step reverse transcriptase PCR (RT-PCR) kit (e.g., from Qiagen or Bio-Rad).
12. One-step RT-PCR master mix with SYBR Green (e.g., from Eurogentec or Bio-Rad).
13. Real-time PCR apparatus and corresponding software (here: MyiQ apparatus, Bio-Rad).
14. Synthetic oligonucleotides (hybridization probes corresponding to the studied tRNAs and RT-PCR primers).

3. Methods

The methods described outline procedures of transfection of cultured human cells to establish tRNA import and to downregulate it by siRNAs (**Subheading 3.1.**); methods for isolation of mtRNA from the transfected cells expressing yeast tRNAs (**Subheading 3.2.**); and approaches to quantify RNA mitochondrial import efficiency (**Subheading 3.3.**).

3.1. Transfection of Cultured Human Cells

3.1.1. Establishing Stable Expression and Import of tRNAs in Cybrid Cells

AU: In **step 1**, define SVF.

1. Grow the cybrid cells in DMEM or F14 Ham's medium with 20% SVF with penicillin, streptomycin, and Fungizone to the confluence of 30–90%, depending on the line, on Petri dishes for cell culture in a CO_2 incubator (at 37°C, 5% CO_2).
2. At 1 d before transfection, replace the medium with the same medium without antibiotics.

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3. Mix 10 µg linearized plasmid DNA expressing the tRNA gene (*Apa*I or any other appropriate restriction enzyme) and 1 µg intact pGL3 in 20 µL of water with 145 µL DMEM without antibiotics and SVF (or, alternatively, OptiMEM medium) and 20 µL SuperFect reagent; incubate 10 min at 20°C.
4. Add 1 mL full DMEM (with antibiotics and serum) and add to cells washed in phosphate-buffered saline (PBS) on Petri dishes; incubate for 12 h in the incubator.
5. Remove the medium with complexes and replace with fresh DMEM medium; incubate for 24 h.
6. Remove the medium and replace with fresh DMEM supplemented by G418 (200–500 µg/mL, depending on your cell line; we routinely used 350 µg/mL for our cybrid MERRF cells); continue growing until clones are visible by eye (0.5–1.0 mm). At this step (36 h after transfection), one can remove a control portion of cells to measure luciferase activity (by standard methods described by the producer of the corresponding kit).
7. Remove the medium and use cloning rings to remove individual clones. To each ring, add 25 µL PBS with 0.1 m*M* EDTA in each tip. Incubate for 5 min at 37°C, save detached cells in a fresh tube, dilute five times with fresh DMEM (with G418), and place in a well of a 24-well plate; continue to incubate.
8. Expand transfected cells for subcellular fractionation. One needs at least one confluent 225-cm^2 flask (10^6 cells) to isolate mitochondria for RNA preparation.

3.1.2. Establishing Transient Expression and Import of tRNA Into Primary Fibroblast Mitochondria

1. Cultivate primary cells in EMEM medium with standard antibiotics until confluence (70–90%) in 225-cm^2 flasks pretreated to increase cell adherence (we suggest using the yellow series of plasticware from Sarstedt, which was optimized for cells with decreased adhesion capacity). One confluent flask may contain $1–3 \times 10^6$ cells.
2. At 1 d before transfection, replace the medium with the same medium without antibiotics.
3. Mix 50 µg nonlinearized plasmid DNA (expression plasmid) and 5 µg control pGL3 plasmid in 50 µL with 500 µL of OptiMEM medium, incubate for 5 min at 20°C, and combine with 500 µL diluted LipofectAMINE (depending on the cells, the dilution may differ). For MERRF fibroblasts, we used 2 µL LipofectAMINE concentrated solution per 10^6 cells; for control healthy fibroblasts 5 µL, the amount proposed by the manufacturer (Invitrogen), was found too toxic for primary cells. Incubate at 20°C for 10–15 min.
4. Add the DNA-LipofectAMINE mixture to the cells in the flask; gently mix with the medium by rocking flasks back and forth.
5. Incubate at 37°C in a CO_2 incubator for no more than 3 h, remove the medium with DNA-LipofectAMINE complexes, wash with prewarmed PBS, and add a new aliquot of prewarmed fresh medium; continue incubation, changing the medium each 24 h. Each day, take out an aliquot of cells to measure luciferase activity.
6. At d 4, retransfect the cells in the same manner as before, taking into account the decreased number of living cells (with respect to the initially transfected culture; *see* **Fig. 2**).

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3.1.3. siRNA Downregulation of Expression and Import of Transgenic tRNAs

1. Cultivate transgenic cybrid cells in F14 Ham's medium containing G418 (or another appropriate antibiotic) to 70–80% of confluence. The day before transfection, replace by the same medium without antibiotic. To be able to perform RNA quantitative analysis and measure respiration or mitochondrial membrane charge, we started with at least four 75-cm^2 flasks for each assay (approx $1–2 \times 10^6$ cells). When mitochondria are to be isolated, we advise using more cells.
2. For each 75-cm^2 flask, mix 20 µg nonlinearized control pGL3 plasmid, 100 pmol antiluciferase siRNA duplex, and 100 pmol appropriate siRNA duplex (*see* **Fig. 3**) in 50 µL water with 500 µL OptiMEM medium; incubate for 10 min at 20°C and combine with 500 µL diluted LipofectAMINE (for MERRF cybrid transfectants, we used 5 µL LipofectAMINE concentrated solution per 10^6 cells). Incubate at 20°C for 30 min.
3. Add LipofectAMINE-DNA complexes to the cell culture and incubate 24 h in the CO_2 incubator. Remove the medium containing complexes and add a new portion of fresh medium (F14 with G418); continue incubation for another 24 h.
4. At d 4, replace the G418-containing medium with antibiotic-free medium for at least 6 h and repeat the transfection procedure, taking into account the number of living cells, then continue incubation. At regular periods (once every 2 d), take out an aliquot of cells to test luciferase activity.

3.2. Isolation of mtRNA for Analysis of Import

3.2.1. Isolation and Purification of Mitochondria

1. Before starting, refrigerate the Waring blender and the centrifuge.
2. Rinse the cultures in the dishes with 1X PBS.
3. Detach the cells with 1X PBS and 1 m*M* EDTA for 5 min at 37°C.
4. Spin down the cells (10 min at 600*g*).
5. Discard the supernatant and resuspend the pellet in 1X PBS and spin down again.
6. Discard the supernatant, resuspend the cells in 10 mL Mito plus BSA buffer, and keep them on ice.
7. Take 1/10 volume to do a total RNA preparation if needed (1 mL in a 2-mL Eppendorf tube) and transfer the rest to the Waring blender.
8. Break the cells in the blender three times for 10 s at highest speed.
9. Transfer into a 50-mL tube and rinse the blender with 5 mL of Mito plus BSA buffer.
10. Centrifuge for 3 min at 4°C, 1500*g*, and transfer the supernatant into a new tube.
11. Centrifuge the supernatant for 3 min at 4°C and 1500*g*.
12. Centrifuge the supernatant for 20 min or longer at 4°C and 20,000*g*.
13. Discard the supernatant and keep the pellet that contains the mitochondria.
14. Resuspend in 1 mL Mito without BSA buffer, then calculate protein concentration with the Bradford reagent: take 1–5 µL of the mitochondrial suspension, add 50 µL 6% NaOH, add water up to 800 µL total volume, and add 200 µL Bradford reagent (Bio-Rad or another); wait 5 min and measure OD 595 nm (1 OD = 15 µg protein).

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15. Add 1 volume 2X RNases and let stand for 5 min at room temperature.
16. Dilute with 2 volumes RNase stop solution to stop the reaction.
17. Centrifuge for 20 min or longer at 4°C and 12,000*g* and resuspend the pellet of mitochondria in 0.2–0.5 mL Mito buffer without BSA.
18. Resuspend pellet of mitochondria in Mito-gradient buffer until a final volume of 0.2–0.5 mL is reached for 5–10 mg mitochondrial protein.
19. Prepare 28% Percoll (sterile) solution in Mito-gradient solution. Put 1–1.2 mL of it in the centrifuge tubes for Beckman 110 TLA ultracentrifuge rotor; keep on ice.
20. Lay solution of mitochondria over the gradient; centrifuge at 30,000*g* for 45 min at 2°C.
21. Take out mitochondria from the gradient: they are visible as the buff-colored band below colorless membranes; avoid taking out the pellet; wash twice with 500 µL Mito buffer.
22. Prepare a concentrated solution of digitonine in Mito buffer without BSA (1–5 mg/mL). Just before use, add 0.2 mg digitonine/mg mitochondrial proteins; let stand for 20 min at room temperature to generate mitoplasts.
23. Dilute with 2–3 volumes of Mito buffer without BSA and centrifuge for 10 min at 4°C and 10,000*g* (in a tabletop microcentrifuge).
24. Wash the pellet of mitoplasts with Mito buffer without BSA and centrifuge for 10 min at 4°C and 10,000*g* (in a tabletop microcentrifuge); rinse and centrifuge again. Freeze 100- to 250-µg aliquots of mitochondrial suspension in Mito buffer in liquid nitrogen, then place at −80°C. Mitoplasts obtained by the procedure described were not contaminated by any visible nuclear small RNAs or cytosolic tRNAs as judged by Northern analysis and RT-PCR (*see* **Note 7**). On average, the treatment described results in 5–10 mg mitochondrial protein from four 225-cm^2 flasks of confluent cells.

3.2.2. Hot Phenol RNA Extraction Protocol

1. Suspend isolated mitoplasts (freshly prepared or frozen in liquid nitrogen and kept at −80°C) in 0.1 *M* sodium acetate, pH 5.0–5.2, and 10 m*M* $MgCl_2$ at 0°C.
2. Add sodium dodecyl sulfate (SDS) to 1%, vigorously mix, and place from ice to 100°C (boiling water or dry incubator) for 2–3 min.
3. Add an equal volume of water-saturated phenol prewarmed at 60°C, mix, and put the mixture at 60°; incubate for 5 min with occasional shaking.
4. Rapidly place the mixtures in ice and incubate for another 5 min at 0°C.
5. Centrifuge for 10 min at 12,500*g* and 0°C and save the upper aqueous phase.
6. Repeat the extraction of the phenol phase with an equal volume of 0.1 *M* sodium acetate, pH 5.0–5.2 and 10 m*M* $MgCl_2$ at room temperature, centrifuge, and combine both aqueous phases.
7. Add 0.1 volume 3 *M* potassium acetate, pH 5.0, and precipitate with 3 volumes of ethanol (2 h at −80°C).
8. To enrich the RNAs with small-size molecules (small ribosomal RNAs, tRNAs), RNA prepared as described is dissolved in DEPC-treated water, 0.1 volume of 3 *M* sodium acetate, pH 5.0, is added, and large RNAs are precipitated by addition

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of isopropanol to 20% (10 min at 20°C); after centrifugation, isopropanol is added to the supernatant to reach 60%, and the precipitation step is repeated. This procedure may be repeated twice to eliminate large RNA molecules (**Fig. 6**).

Fig. 6

9. RNAs may be stored either under ethanol at −20°C or aliquoted in water at −80°C.

3.2.3. Modified TRIzol Extraction Protocol

1. Detach the cultured cells from plates by treatment with PBS containing 1m*M* EDTA (at 37°C, for 5 min), wash once or twice with PBS, withdraw all liquid, and suspend in TRIzol at a ratio of 1 mL per 10 cm^2 of confluent culture (this ratio must be respected; otherwise, contaminations with DNA can arise).
2. Incubate the homogenate at 30°C for 5 min and add 0.2 mL chloroform per 1 mL TRIzol; mix and incubate at room temperature for 10 min.
3. Centrifuge at 12,000*g* for 10 min at 4°C.
4. Precipitate RNA from the upper phase by adding 0.5 mL isopropanol (*see* **Note 8**) per 1 mL TRIzol used at room temperature (15–20°C) for 10 min, centrifuge, wash the pellet with 80% ethanol (do not use lower concentration of ethanol for washing because it can eliminate small-size RNAs), dry, and dissolve in DEPC-treated water.
5. Add 1 mL of a new portion of TRIzol reagent to 100 µL aqueous solution of RNA; thoroughly mix and repeat all the extraction procedure. This second extraction eliminates traces of DNA in the sample. Sometimes, up to three cycles of extraction are needed to completely remove DNA, which becomes undetectable by PCR (**Fig. 4**; *see* **Note 8**).

3.3. Quantitative Analysis of Import

3.3.1. Quantitative Northern Hybridization

1. Separate RNAs in a 1-mm thick and 20-cm long standard denaturing gel: 13% acrylamide (methylene-bisacrylamide:acrylamide 1:19), 1X TBE buffer, 8 *M* urea at 10 V/cm until the xylene cyanol reaches 3/4 of the gel. One can load up to 50 µg of mtRNA per one 10-mm large well, which may be sufficient to detect underrepresented transgenic tRNAs.
2. Soak the gel in the transfer buffer (25 m*M* phosphate, pH 6.5) for 15 min at room temperature.
3. Electrotransfer RNAs onto Hybond-N membrane in a wet transfer camera in the same buffer at 4°C, 200 mA, 10V for 6–12 h.
4. Fix RNAs on the membrane by irradiation in a crosslinking UV chamber for 3 min.
5. Prehybridize the membrane by rotating in a hybridization oven in 6X SSC, 0.1% SDS, 10X Denhardt solution for 1–4 h at 60°C.

AU: In **step 6**, define DEAE and Cpm.

6. Discard the prehybridization solution; add the hybridization solution, consisting of 1 volume of prehybridization buffer and 1 volume of 5′-end [^{32}P]-labeled oligonucleotide probe in 1 *M* NaCl (purified before use on a small DEAE-cellulose column). We suggest using at least 10^4 Cpm of labeled probe per 1 cm^2 of membrane.
7. Hybridize overnight at appropriate temperature (depending on the probe, the hybridization temperature normally used is 5°C below the melting point).

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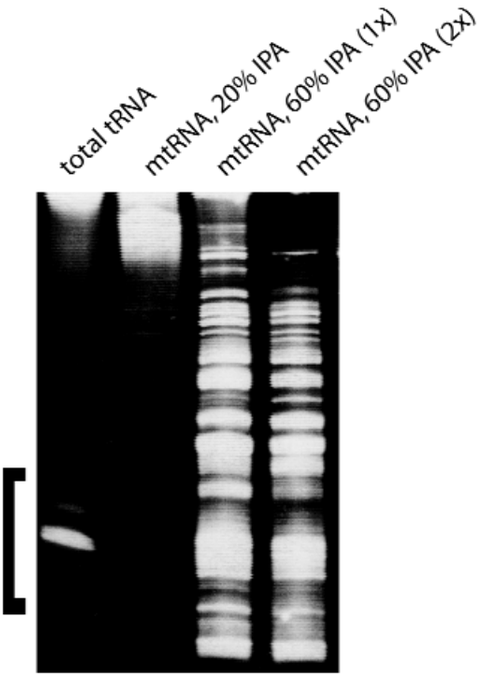

Fig. 6. Isopropanol differential precipitation of small-size mitochondrial RNA. The RNAs were separated on 10-cm 13% denaturating polyacrylamide gel and ethidium bromide stained. Total yeast tRNA (commercial) was used as the reference; the percentage of isopropanol (IPA) used to precipitate RNA is indicated above the gel. The precipitation procedure was done once or twice (as indicated). The bracket indicates location of the transfer RNAs.

8. Remove hybridization solution and wash the membrane three times for 5 min in 2X SSC and 0.1% SDS at the desired temperature (to be optimized for each probe); seal the wet membrane between two thin polyethylene sheets and expose on the phosphoimager.
9. Quantify the signals detecting the individual RNAs and compare ratios between the signal of the RNA of interest and of reference RNAs (cytosolic or mitochondrial) for cellular lines analyzed (**Fig. 5**). Compare also the ratio between mitochondrial and cytosolic reference tRNAs in these lines to be sure that it remains independent of the line. The RNA import efficiency may be calculated as a percentage of the total tRNA species localized in the mitochondria.

3.3.2. Real-Time RT-PCR Analysis

1. Perform preliminary amplification assays by using the Qiagen single-step RT-PCR kit in the following conditions: 50°C, 30 min; 95°C, 15 min; 15 cycles at 95, 55,

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AU: In **step 1**, 95-55, 95-58, 95-62 all are centigrade temperatures and distinct steps? Uses commas instead of hyphens. OK?

72°C; 10 cycles at 95, 58, 72°C; 5 cycles at 95, 62, 72°C (each step for 1 min), final step at 72°C for 10 min. In all cases, include a control PCR reaction performed in similar conditions as RT-PCR by Taq DNA-polymerase (without reverse transcriptase activity) to confirm the absence of DNA contamination in RNA isolates. We advise using, if possible, synthetic T7 transcripts with the same sequence as tRNAs as standards for quantification (*see* **Note 9**).

2. Analyze aliquots on nondenaturing 10% polyacrylamide gel (typical results are presented on **Fig. 4**).
3. Perform quantitative (real-time) RT-PCR using a Bio-Rad i-Cycler with the One-Step RT-qPCR Mastermix for SYBR green following the manufacturer's protocol. For RT-qPCR, conditions used are different from those indicated above: annealing steps are performed at 58°C; the number of cycles is 40–45.
4. For quantitation, serial RNA dilutions are to be done and compared to the calibration curve obtained in parallel reactions with a series of diluted gel-purified T7 transcripts (tK1, tK3, or tK93), ranging from 1 pg to 10 ng per reaction. All qPCR samples have to be done in triplicate. In each series, the corresponding reference (T7 transcript) has to be included, and the same holds for serial dilutions. Blank controls without RNA or oligonucleotides are also to be included in each series.

4. Notes

1. To choose primers, we checked the sequences of the tRNAs available in the Munich Information Center for Protein Sequences database (http://mips.gsf.de/), and the amplicons included the complete tRNA sequence (76 bases). To optimize expression, all three versions of genes cloned (tRK1cau, tRK3, and tRK93) were flanked by short sequences homologous to the flanks of one of the expressed tRK1 copies in yeast ***(14)***, although expression of the tRNA genes is normally driven by the internal promoter for RNA polymerase III. The following sequences were used: 5′ACATATTAAACCTGAGAGGTCAGATTTCCAATAACAGAATA (-1) … and … TTCTTTTTTTTTTTAAAACACGATGACATAAATTTCC-3′. The presence of these flanking sequences enhances the expression. tRK1cau version corresponds to the tRK1 with one-base substitution in the anticodon (U35A), which does not inhibit its import but prevents recognition of the lysine codons. tRK93 corresponds to the mutant version of tRK2. tRK2 is normally not imported into yeast or human mitochondria. However, introducing Ψ1G, A72C, and G73U mutations makes this version importable (tRK93). In addition, all seven corresponding gene copies contain a 28-base intron, which was removed by standard PCR-cloning methods ***(14)*** Yeast tRK3 is normally encoded by mtDNA and resides exclusively in the mitochondria; however, human mitochondria were found to import it as well. This tRNA, because it is mitochondrially encoded, has no need of internal RNA polymerase III promoter, but it possesses the required sequence motifs ***(15)*** and therefore may be expressed without additional mutations. PCR and cloning procedures were performed in a standard way. To obtain plasmid constructions based on pBK-CMV vector in *Escherichia coli*, we used selection on media containing kanamycin. Using pBK-CMV and pcDNA3.1/Neo(±) vectors gives

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similar expression/transfection results. When, for one or another reason, the G418 marker cannot be used (e.g., the line to be transfected is already G418 resistant), one can exploit the vector pcDNA3.1/Zeo(±) (Invitrogen). This vector contains the Zeocine resistance gene, for selection in human cells (to be used at 5–50 µg/mL, depending on the cell line), and the ampicillin resistance gene, to select clones in *E. coli*. When selecting in *E. coli* for kanamycin resistance, it is important to grow cells after the transfection procedure for at least 1.5 h in a rich medium, preferably with low salt (2X YT or SOB).

AU: In **Note 1**, define YT and SOB.

2. For cybrid lines based on 143B or HeLa rho° cells, we found optimal efficiency with the rich Ham's F14 medium and LipofectAMINE 2000 or LyoVec transfection reagents. However, for those cybrids that are more robust, SuperFect reagent and DMEM medium may be used as well. For primary fibroblasts, most reproducible results were obtained with EMEM medium and LipofectAMINE 2000. In all cases, transfection was effective, but the cytotoxic effect was important. To check for the efficiency of transfection, an internal control is to be included. We used the commercially available pGL3 plasmid bearing the genes coding for luciferase. For the control experiment, an aliquot of transfected cells was always plated in one 10-cm^2 well; 24–48 h after the transfection procedure, the luciferase activity was measured using the protocol for the commercialized luciferase detection kit. With the large number of transfection procedures now available, one is confronted with the choice of deciding the most appropriate method. The most easy to use are lipophilic agents, proposed by most leading manufacturers. However, when abnormal cells are to be transformed, it becomes a more complicated task because of different cytotoxic effects of the same product on two different cell lines. For example, for a similar pair of cybrid lines both bearing the MERRF mutation at 95–100% of heteroplasmy but one based on 143B cells and the second on HeLa cells, we had to use different transfection protocols: for the first one, the best results were obtained with 50% confluent cells and the use of OptiMEM I Reduced Serum Medium (Invitrogen) with DNA:LipofectAMINE ratio proposed by Invitrogen; for the second, with 90% confluent cells, the method was optimal with a LipofectAMINE/DNA ratio three times reduced and normal DMEM medium but supplemented with serum. The best expression results with the third independent cybrid cell line were with LyoVec reagent (used as follows: 8 µg linearized expressing plasmid, 2 µg pGL3 with 600 µL LyoVec reagent for one 6-well plate of 70% confluent cells), and LipofectAMINE did not give any detectable expression. Finally, for several lines, one can obtain very different results in stable and transient expression experiments: for MERRF cybrids, we always obtained best stable transfections with the SuperFect reagent and the best transient expression with LipofectAMINE. Unfortunately, there is no way to predict *a priori* the best way to transfect your cell line, and the optimization procedure is a necessary step before any set of stable or transient expression experiments.
3. The main problem of consecutive transfections when working with primary cells is the amount of material needed to purify mitochondria at the final step. For example, to show that imported tRK3/93 versions lead to an increase

of mitochondrial respiration and membrane charge along with detection of import into mitochondria, we initially used four 225-cm^2 flasks of confluent culture for transfection. With this initial amount and after three rounds of transfection, we obtained the equivalent of the amount in only one flask (10^6 cells), which was roughly the minimum needed to isolate mitochondria and to perform respiration, membrane charge, and RNA import analysis.

4. Both Northern and RT-qPCR methods have advantages and disadvantages. For Northern analysis, one needs a significant amount of pure mtRNA, which is complicated when transient expression experiments are performed. We suggest using it for analysis of stable transfectants, which can be expanded to obtain more cells. In addition, Northern analysis is extremely important when one needs to quantify the aminoacylation level of a given tRNA. In this case, the RNA isolation and separation system is different from that described: all manipulations are performed in acid conditions to avoid deacylation ***(16)***. On the other hand, when quantifying Northern experiments, we mainly obtain relative values, that is, ratios between the tRNA of interest (e.g., the imported one) and another, present either in cytosol or in the mitochondrion. It should be assumed, therefore, that the concentrations of these reference RNAs are similar in different cell lines analyzed, which is not always the case. To make data more representative, one needs to quantify several different reference tRNAs and to compare ratios, which may vary if the balance between different tRNAs in the total pool varies from line to line. Real-time PCR seems to offer a good alternative to Northern analysis because it gives absolute values of RNA concentration, which can be normalized to the number of cells, mitochondrial protein, and total or mtRNA. Second, the amount of RNA needed to perform quantification is at least one order less than that needed for Northern analysis. On the other hand, RT-PCR is extremely sensitive, which is not only an advantage, but also any trace of DNA in the reaction completely negates the result, and isolation of mtRNA needs supplementary efforts to eliminate all DNA contamination. In our experiments, we preferred using hot phenol-extracted mtRNA for Northern experiments and the modified TRIzol extraction protocol to isolate templates for RT-qPCR.
5. In our experiments with transgenic human cells, we used the following: to detect tK3, the oligonucleotide probe antiK3(1–39): CTTAAAAGACAACTGTTTTACCATTAAACAATATTCTC; for tK93, the probe antiK2(2–32): GCCGAACGCTCTACCAACTCAGCTAACAAGG; for tK1cau, the probe antitK1(met): CTTATGATTATGAGTCAT; for human cytosolic $tRNA^{Met}_i$, the probe anti-cM: TGGTA GCAGAGGATGGTTTCG, for human mitochondrial $tRNA^{Gln}$, anti-mQ: CTAGGACTATGAGAATCG. For RT-PCR detection of tRNAs, we used the following pairs of oligonucleotides: for tK93, CTTGTTAGCTCAGTTGGT and TGGAGCCTCATAGGGGGC; for tK3, GAGAATATTGTTTAATGGTAAAAC and GGTGAGAATAGCTGGAGTTG; for tK1cau, GCCTTGTTGGCGCAATCGG and GGAGCCCTGTAGGGGGCTCG; for mitochondrial $tRNA^{Gln}$, TAGGACTATGAGAATCG and AGGATGGGGTGTGATAG; for cytoplasmic $tRNA^{Met}_i$, GGTAGCAGAGGATGGTTTCG and CAGAGTGGCGCAGCGGAAG.

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6. One of the problems of using RT-qPCR for tRNA amplification is the limited amplifiable sequence (the tRNA is 76 nucleotides long, and the oligonucleotide probes are at least 15 nucleotides; the polymerized region is less than 50 nucleotides). The second problem is that, contrary to the case of messenger RNAs, the choice of the region of the tRNA to hybridize with the oligonucleotide probes is restricted and may differ only by several bases; otherwise, there would not be any sequence to amplify. These restrictions suggest thorough preliminary optimization of primers in ordinary RT-PCR assays (like experiments presented on **Fig. 4**). The fact that different pairs of probes may require different conditions of PCR results in the need to perform sequential real-time quantifications for different tRNAs present in the same isolate. The current market proposes a number of reagents for real-time PCR and RT-PCR. We found that, when the preliminary optimization work was performed, the more economical and reproducible results were obtained with the single-step RT-PCR procedure, nonlabeled oligonucleotide probes, and CYBR green labeling.
7. The purification procedure depends on the way that the RNA isolated from the mitochondria will be analyzed. When Northern hybridization was used, the main objective was to eliminate contaminations with cytosolic tRNAs; for real-time RT-PCR, contamination with DNA has to be avoided. In the case of Northern analysis, the presence of small amounts of DNA is not harmful.
8. The TRIzol reagent is sold by Invitrogen and is based on extraction with a monophasic solution of phenol and guanidinisothiocyanate according to the protocol described elsewhere ***(17)***. This method gives excellent results on whole cells, but not as good results (with respect to yield and purity) on mitochondria, probably because of lipid contaminations. Another problem when using the manufacturer's protocol is that it usually gives less RNA than the hot phenol extraction, and often the RNA contains trace amounts of DNA, which becomes an important problem when RT-PCR is used to detect (or to quantify) a given RNA species. As a rule, deoxyribonuclease treatment does not permit full elimination of these contaminants. Proposed modifications aim to avoid this problem. The TRIzol extraction is also compatible with the differential precipitation of RNA by isopropanol. Precipitation of high molecular weight RNAs at 20% isopropanol may be performed at the first extraction step of TRIzol treatment. Small-size RNAs are then precipitated by 60% isopropanol as described above.

AU: In the last sentence of **Note 8**, indicate specific subheading number instead of "above."

9. To synthesize a tRNA transcript in vitro, we suggest amplifying the target gene by PCR, including in the primers the promoter for T7 RNA polymerase upstream and a site for *Bst*NI downstream, which gives, on cleavage, the 3′-terminal CCA sequence. The tRNA transcript is then obtained by T7 transcription in vitro followed by gel purification of the RNA. This approach, used in a number of tRNA studies, is complicated when the first 5′-nucleotide of the tRNA is a U or C, which makes the T7 RNA polymerase nonefficient. For the studies we describe here, this is not a problem because the first nucleotide may be replaced in the primer by the optimal G; however, when the tRNA transcript is studied *per se* and not as a reference for quantification, one can use the alternative approach ***(18)***: to synthesize a longer T7 transcript including a ribozyme, which can, on self-cleavage in the presence of Mg^{2+}, release the desired transcript.

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Resumé développé en français

Introduction et objectifs

Chez *S. cerevisiae*, un des deux ARNt^{Lys} codé par l'ADN nucléaire, l'ARNt$^{Lys}_{CUU}$ (ou tRK1), est partiellement adressé dans la matrice mitochondriale. Bien que sa fonction mitochondriale soit inconnue, son abondance dans la mitochondrie est comparable à celle des ARNt codés par le génome mitochondrial. La cellule de levure contient deux autres isoaccepteurs de la lysine: l'ARNt$^{Lys}_{UUU}$ (tRK2), codé dans le noyau et localisé exclusivement dans le cytoplasme et l'ARNt$^{Lys}_{UUU}$ (tRK3), codé par le génome mitochondrial et exclusivement localisé dans la mitochondrie. La position 34 (wobble) de l'anticodon étant modifié, tRK3 devrait suffire à la traduction mitochondriale de codons AAA et AAG, ce qui pose la question sur la fonction de tRK1 importé dans la traduction organellaire.

L'import de tRK1 est un processus actif dépendant de l'hydrolyse de l'ATP et de la charge électrochimique membranaire qui implique la participation du pore d'import des pré-protéines mitochondriales et des facteurs protéiques solubles. Pour le moment trois acteurs de ce cette voie on été identifiés agissant en concert: 1. tRK1 est aminoacylé par la lysyl-ARNt synthétase cytosolique, Krs1p; 2. il devient ensuite la cible de compétition entre les facteurs de traduction cytoplasmiques et une protéine de glycolyse, Eno2p, qui transporte un partie de l'ARN vers la surface mitochondriale; 3. enfin, tRK1 interagit avec le précurseur cytoplasmique de lysyl-ARNt synthétase mitochondriale, preMsk1p, qui permet l'internalisation de l'ARN dans la matrice. Une fois dans la martice, preMsk1p subit un clivage endopeptidique pour former sa forme mature qui peut aminoacyler tRK3. D'autre part, ni preMsk1p, ni MSK1p ne sont pas capables d'aminoacyler tRK1.

Les travaux de la thèse avaient deux objectifs majeurs: (a) étudier le méchanisme d'interaction entre l'ARN importé (tRK1) et le facteur essentiel de son adressage dans la mitochondrie, preMsk1p; (b) exploiter ces connaissances pour mettre au point un test *in vivo* permettant de vérifier si tRK1 serait impliqué dans la traduction mitochondriale.

Travaux effectués

I. Analyse d'interaction preMsk1p / tRK1 *in vitro*

L'approche du retard sur gel a été utilisée pour démontrer que preMSK1p forme un complexe stable avec tRK1 aminoacylé mais n'est pas capable de lier sa forme déacylée ou tRK2. La Kd de ce complexe est de 180±20 nM, ce qui est comparable avec les Kd que l'on trouve pour les complexes entre les lysyl-ARNt synthétases et les ARNt hétérologues. Enolase, le deuxième facteur d'import de tRK1, s'est avérée également capable de lier tRK1 aminoacylé, mais la Kd de ce complexe est d'un ordre plus élevé (2.5±0.2 μM). Il n'a pas été possible de détecter le complexe ternaire entre tRK1 et les deux protéines, mais en présence de enolase, la Kd du complexe tRK1/preMsk1p baissait d'une manière significative (40±10 nM). Ce résultat laisse supposer le rôle de chapérone pour l'enolase.

Plusieurs versions importées ou non de tRK1 et tRK2 on été ensuite analysé par le même test. Nous avions démontré que seuls les transcrits capables de former le complexe stable avec preMsk1p étaient importés dans les mitochondries isolées. L'affinité pour la preMsk1p était en corrélation directe avec l'efficacité de l'import.

Pour mieux comprendre le mécanisme d'interaction entre tRK1 et preMsk1p, l'approche d'empreintes ("footprint") a été utilisée. Un traitement FeEDTA a été utilisé pour définir les site de liaison entre la protéine et l'ARN, tandis que les nucléases S1 et V1 ont été utilisées pour évaluer les changement conformationnels de l'ARN dans le complexe

nucléoprotéique. Il a été démontré que tRK1 interagit avec preMsk1p d'une manière qui diverge sensiblement de celle avec sa propre aminoacyl-tRNA synthétase, Krs1p. En effet, preMsk1p protège de plus large surfaces de la molécule d'ARN. Durant l'interaction, la structure secondaire ne change pas beaucoup, excepté un léger désapariement de bases de l'hélice de l'anticodon.

Il est bien connu, que les aminoacyl-tRNA synthétases de classe IIb, dont Msk1p fait partie, sont organisées en deux domaines, dont le domaine N-terminal possède une forte affinité pour les ARNt. Nous avions démontré que seul le domaine N-terminal de la preMsk1p (preMsk1p-N) peut diriger l'import de tRK1 *in vitro* avec une efficacité semblable à celle de toute la protéine. En plus, les expériences de footprint démontrent que sa manière d'interaction avec l'ARN est semblable à celle de la protéine entière. Les versions recombinantes tronqués de preMsk1p-N se sont également avérées capables de diriger l'import, mais ont perdu toute spécificité. Les expériences systématique avec les versions tronquées nous permettent d'affirmer que cet import aspécifique serait dû au propriétés d'affinité non-spécifique aux ARN de la région proximale de la preMsk1p (région H1-H3), tandis que la partie distale de preMsk1p-N (région H5-H6) serait responsable de l'interaction spécifique avec tRK1.

II. Analyse d'interaction preMsk1p / tRK1 *in vivo*

Pour valider les divers résultats d'expériences *in vitro*, nous avions exprimé plusieurs versions mutantes du gène *MSK1* contenant des délétion dans le domaine N-terminal et avons exprimé ces gènes dans les souches ou le gène *MSK1* a été délété. Ensuite, nous avons testé la présence de tRK1 dans les mitochondries de transféctants. Les délétions dans la région H5-H6 résultaient en inhibition d'import de tRK1, tandis que les délétions dans la région H1-H3 ne l'affectaient pas. La délétion du domaine C-terminal n'empêchait pas l'import, tandis que la délétion du domaine N-terminal le bloquait. Ces résultats étaient donc en parfait accord avec les ceux obtenu *in vitro*. D'autre part, les mitochondries de ces souches recombinantes étaient non-fonctionnelles (états de "pro-mitochondries"), probablement grâce à l'absence d'aminoacylation de tRK3 dans l'organite.

III. Etude des effets phénotypiques et moléculaires du remplacement *in vivo* de preMsk1p par son homologue de *A. gossipii*

Les résultats décrit ci-dessus et démontrant l'importance du domaine N-terminal de lpreMsk1p pour l'import mitochondrial de tRK1 nous ont permis d'approcher le problème de la fonction mitochondriale de tRK1. En effet, si, par mutagenèse du domaine N-terminal il était possible d'obtenir une protéine capable d'aminoacyler tRK3 mais incapable de diriger l'import de tRK1, il serait facile de mettre au point un système génétique pour tester la fonction de l'ARNt importé. Les alignements de séquences des lysyl-ARNt synthétases mitochondriales et la comparaison de séquences d'ARNt^{Lys} mitochondriaux avait permi de choisir un organisme apparenté a *S. cerevisiae*, *Ashbya gossypii*, dont l'orthologue de la preMsk1p (AshLysRS) avait le domaine N-terminal très réduit mais dont l'ARNt^{Lys} mitochondrial était très proche au tRK3. Nous avons exprimé AshLysRS dans les souches de *S. cerevisiae* ou le gène *MSK1* a été délété et avons démontré que la protéine récombinante était exprimée, importé dans les mitochondries et pouvait aminoacyler tRK3. Par contre, l'import de tRK1 était gravement affecté. Le fonctionnement mitochondrial des souches transgéniques s'est avéré normal à 30°C, mais déficient à 37°C. En plus, l'analyse de polypeptides synthétisés dans les mitochondries avait démontré qu'à 37°C les protéines les plus affectées était Var1p et Cox2p, les deux seules protéines mitochondriales détectables qui contiennent les codons AAG (tous les autre Lys de gènes mitochondriaux sont codés par les codons AAA). Nous proposons donc une hypothèse que tRK1 importé serait nécessaire pour lire les codons AAG mitochondriaux à la température élevé.

Conclusions et perspectives

Compte tenu des résultats décrits ci-dessus, les conclusions suivantes s'imposent :

1. Interaction de l'ARNt-Lys (tRK1) avec le précurseur de la lysyl-ARNt synthétase mitochondriale preMsk1p est indispensable pour l'import de l'ARN dans mitochondrie. La formation du complexe tRK1/preMsk1p est facilité par la présence de enolase (Eno2p).
2. L'interaction de l'ARNt importé avec preMsk1p est différente de celle avec la lysyl-ARNt synthétase cytosolique, Krs1p.
3. Seul le domaine N-terminal de la pre-MSK1 est capable de diriger l'adressage de tRK1, la région H5-H6 étant la plus importante.
4. L'homologue de la preMsk1p de Ashbya gossipii est capable d'aminoacyler l'ARNt-Lys mitochondrial mais n'est pas capable de diriger l'import de tRK1.
5. L'inhibition de l'import de tRK1 qui résulte du remplacement du gène *MSK1* par celui de son orthologue de *A. gossypii* résulte en inhibition de traduction de codons AAG à la température élevée. Ceci est la première démonstration directe du rôle de tRK1 importé dans la traduction mitochondriale.

Pour mieux comprendre le mécanisme d'importation d'ARNt, plusieurs questions restent à résoudre. Il serait important de comprendre la raison moléculaire de l'effet conditionnel de l'inhibition d'import sur la traduction. Il serait également intéressant de résoudre la structure tri-dimensionnelle du complexe nucléoprotéique qui dirige l'import de l'ARN. Enfin, il serait possible de greffer le domaine N-terminal de la preMsk1p sur d'autre aminoacyl-tRNA synthétases afin de créer des facteurs d'import au spécificités modifiés, ce qui pourrait être exploité pour mettre au point des modèles de thérapie génique basés sur la voie d'import des ARN.

Liste de Publications et communications

Publications

1. **Kamenski P**, Entelis N, Krasheninnikov IA, Martin RP, Tarassov I. Interaction of cytosolic lysine tRNA with the precursor of mitochondrial LysRS during tRNA import into yeast mitochondria. Manuscript in preparation.

2. **Kamenski P**, Kolesnikova O, Entelis N, Jubenot V, Krasheninnikov IA, Martin RP, Tarassov I.Evidence for a novel mechanism regulating mitochondrial translation *via* tRNA import in *Saccharomyces cerevisiae*. Manuscript in preparation.

3. Mager-Heckel AM, Entelis N, Brandina I, **Kamenski P**, Krasheninnikov IA, Martin RP, Tarassov I. The analysis of tRNA import into mammalian mitochondria. Methods Mol Biol, (Humana Press, NY) 2007, 372: 235-253.

4. **Kamenski P**, Vinogradova E, Krasheninnikov IA, Tarassov I. Import of macromolecules into mitochondria. Mol. Biol. (Russia), 2007, in press.

5. Entelis N, Brandina I, **Kamenski P**, Krasheninnikov IA, Martin RP, Tarassov I. A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in Saccharomyces cerevisiae. Genes Dev. 2006, 20(12): 1609-20.

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Communications

1. **Kamenski P**, Entelis N, Jubenot V, Krasheninnikov IA, Martin RP, Tarassov IA. The role of the precursor of mitochondrial lysyl-tRNA synthetase in tRNA import into yeast mitochondria. Abstracts of 7th Conference “Lévures: Modéles et Outils”, Paris, France, 2006, p.88.

2. **Kamenski P**, Entelis N, Krasheninnikov IA, Martin RP, Tarassov IA. Study of the mechanism of tRNA-LysRS interaction leading to tRNA import into yeast mitochondria. Abstracts of the 21th Iternational tRNA Workshop, Bangalore, India, 2005, p. 128.

3. **Kamenski P**, Krasheninnikov IA. Non-canonical interaction of the precursor of yeast mitochondrial LysRS with the cytosolic lysine tRNA imported to mitochondria. Abstracts of Young Scientists Conference on Fundamental Sciences "Lomonosov”, Moscow, Russia, 2004, p. 57.

4. **Kamenski P**, Entelis N, Krasheninnikov IA, Martin RP, Tarassov IA. Studying RNA-protein interactions leading to import of a cytoplasmic tRNA into yeast mitochondria. Abstracts of the 8th Annual Meeting of the RNA Society, Vienna, Austria, 2003, p. 215.

5. **Kamenski P**. Study of the precursor of yeast mitochondrial lysyl-tRNA synthetase as a factor of tRNA import into yeast mitochondria. Abstracts of Young Scientists Conference on Fundamental Sciences "Lomonosov”, Moscow, Russia, 2001, p. 22.

Resumé

Chez *S. cerevisiae*, l'ARNt$^{Lys}_{CUU}$ (tRK1) codé par l'ADN nucléaire, est adressé dans la mitochondrie. La mitochondrie contient également tRK3, codé par le génome mitochondrial supposé de suffire à la traduction mitochondriale. Ceci pose la question sur la fonction de tRK1 importé dans la traduction organellaire. L'import de tRK1 implique la participation des facteurs protéiques dont le précurseur de lysyl-ARNt synthétase mitochondriale (preMsk1p) et l'enolase (Eno2p). Les travaux de thèse avaient deux objectifs: (a) étudier le mécanisme d'interaction entre tRK1 et preMsk1p; (b) exploiter ces connaissances pour mettre au point un test *in vivo* permettant de vérifier si tRK1 serait impliqué dans la traduction mitochondriale. Il a été démontré que: (i) interaction de l'ARNt-Lys (tRK1) avec le précurseur de la lysyl-ARNt synthétase mitochondriale preMsk1p est indispensable pour l'import de l'ARN dans mitochondrie; (ii) formation du complexe tRK1/preMsk1p est facilité par la présence de enolase (Eno2p). (iii) interaction de l'ARNt importé avec preMsk1p est différente de celle avec la lysyl-ARNt synthétase cytosolique. Nous avions ensuite démontré que seul le domaine N-terminal de la pre-MSK1 serait capable de diriger l'import de tRK1. Cette découverte a été exploité pour mettre au point le système génétique permettant étudier la fonction de l'ARNt importé. L'inhibition de l'import de tRK1 qui résulte du remplacement du gène *MSK1* par celui de son orthologue ayant le domaine N-terminal réduit, a pour effet une inhibition de traduction de codons AAG à la température élevée. Ceci est la première démonstration directe du rôle de tRK1 importé dans la traduction mitochondriale.

Abstract

In *S. cerevisiae*, cytosolic tRNA$^{Lys}_{CUU}$ (tRK1) is targeted into mitochondria. Yeast mitochodnria also possess another lysine isoacceptors, tRK3, coded for by mitochondrial DNA and supposed to be sufficient for mitochondrial translation. Therefore, the mitochondrial function of the imported tRK1 is unclear. tRK1 import requires the presence of the precursor of the mitochondrial lysyl-tRNA synthetase (preMsk1p) and the enolase (Eno2p). The thesis has two objectives: (a) to study the mechanism of tRK1-preMsk1p interaction; (b) to exploit this knowledge for developing an *in vivo* assay suitable to verify the involvement of tRK1 in mitochondrial translation. It was shown that: (i) formation of the complex between tRK1 and preMsk1p is in direct correlation with the tRK1 import efficiency; (ii) formation of the tRK1-preMsk1p complex is facilitated by Enolase-2 (Eno2p); (iii) the mechanism of interaction between tRK1 and preMsk1p differs form that between tRK1 and the cognate cytosolic aminoacyl-tRNA synthetase. Mutagenesis of *MSK1* gene followed by *in vitro* and *in vivo* import assays have shown that the N-terminal domain of preMsk1p is capable to direct tRK1 import. An ortholog of preMsk1 from *Ashbya gossypii* that possesses a sorter N-terminal domain than preMsk1p was shown to aminoacylate tRK3 but not to import tRK1. Replacement of preMsk1p by its ortholog resulted in inhibition of mitochondrial translation of AAG-containing mitochondrial genes at elevated temperature. This is the first direct demonstration of participation of the imported tRNA species in the organellar protein synthesis.

Mots clés: ARNt, traduction, mitochondrie, adressage, aminoacylation, interaction ARN-protéine, aminoacyl-tRNA synthétase, levure

Key words: tRNA, translation, mitochondria, targeting, aminoacylation, RNA-protein interaction, aminoacyl-tRNA synthetase, yeast