Structural requirements of tRNA_Lys for its import into yeast mitochondria

NINA S. ENTELIS*†, SANDRINE KIEFFER*, OLGA A. KOLESNIKOVA†, ROBERT P. MARTIN*, AND IVAN A. TARASSOV*‡

*Unité Propre de Recherche 9005 du Centre National de la Recherche Scientifique, Mécanismes Moléculaires de la Division Cellulaire et du Développement, and Université Louis Pasteur, Institut de Physiologie et de Chimie Biologique, 21, rue René Descartes, 67084 Strasbourg Cedex, France; and ‡Department of Molecular Biology, Biology Faculty of the Moscow State University, Moscow 119899, Russia

Communicated by Giuseppe Attardi, California Institute of Technology, Pasadena, CA, January 20, 1998 (received for review August 18, 1997)

ABSTRACT In the yeast Saccharomyces cerevisiae, one of the two cytoplasmic lysine tRNAs, tRNA_LysCUU, is partially associated with the mitochondrial matrix. Mitochondrial import of this tRNA requires binding to the precursor of the mitochondrial lysyl-tRNA synthetase, pre-MSK, and aminoacylation by the cytoplasmic lysyl-tRNA synthetase, KRS, appears to be a prerequisite for this binding. The second lysine isoacceptor tRNA_LysCUU (where 5-[(methylamino)-methyl]-2-thiouridine is mmn's2U) is exclusively localized in the cytoplasm. To study import determinants within the tRNA_LysCUU molecule, we introduced a panel of replacements in the original sequences of the imported and nonimported lysine tRNAs that correspond to domains or individual residues that differ between these two isoacceptors. The mutant transcripts were tested for import, aminoacylation, and binding to pre-MSK. Import and aminoacylation efficiencies correlate well for the majority of mutant transcripts. However, some poorly aminoacylated transcripts were rather efficiently imported. Surprisingly, these transcripts retained binding capacity to pre-MSK. In fact, all imported transcripts retained pre-MSK binding capacity but nonimported versions did not, suggesting that this binding, rather than aminoacylation, is essential for import. Substitution of the anticodon arm of tRNA_LysCUU with that of tRNA_LysCUU abolished import without affecting aminoacylation. A version of tRNA_LysCUU with an anticodon CUU was efficiently imported in vitro and was also found to be imported in vivo. This implies that the anticodon arm, especially position 34, is important for recognition by the import machinery. A nicked tRNA_LysCUU transcript is still imported but its import requires reannealing of the two tRNA moieties, which implies that tRNA_LysCUU is imported as a folded molecule.

Mitochondrial import of cytoplasmic tRNAs has been found in a wide range of organisms: plants, fungi, protozoa, and mammals (1–3). The yeast Saccharomyces cerevisiae imports a single nucleoside-tRNA, tRNA_LysCUU (tRK1). This is one of two cytoplasmic lysine isoacceptors, the second one, tRNA_Lysnmn's2UU (tRK2) being localized exclusively in the cytoplasm (4, 5). A third yeast lysine tRNA, tRK3, is encoded by the mitochondrial DNA and its localization is restricted to the mitochondrial compartment. tRK1 is unequally distributed by the mitochondrial DNA and its localization is restricted to the mitochondrial nucleus-made tRNA, tRNA_LysCUU, particularly position 34, and the acceptor stem contain determinants for its import selectivity. We also show that import requires binding of the mutant tRNAs to pre-MSK. In most cases, aminoacylation was found to be necessary for efficient pre-MSK recognition, but some mutations enable tRNA_Lys variants to bind pre-MSK in their decylated form. Finally, we present data implying that tRK1 is translocated across mitochondrial membranes as a folded molecule.

MATERIAL AND METHODS

Strains, Cloning, and Mutagenesis. S. cerevisiae YPH499 (a, ura3–52, lys2–801amber, ade2–101ochre, trp1–Δ63, his3–Δ200, leu2–Δ1) (14) was used for isolation of mitochondria and in vivo expression of tRK2CUU and tRK1CUU–7. Strain ΔMOM195 (a, mom19::URA3, ade2–101ochre, his3–Δ200, leu2–Δ1, ura3–52, trp1–Δ63, lys2–801amber) (15) harbors plasmid pG1176 containing the MSKI gene (16). This strain, which is deficient in mitochondrial preprotein import and accumulates cytoplasmic precursors, was used for preparation of import-directing proteins and a pre-MSK-enriched fraction. Escherichia coli DH5αF and CJ236 were used for cloning and Kunkel mutagenesis, respectively (17, 18).

Cloning, isolation, analysis, and sequencing of recombinant DNA were performed by standard methods (19). The tRK1 gene from plasmid pY109 (20) provided by H. Feldmann, was subcloned by PCR into the BamHI site of M13mp19 by using two oligonucleotides. The 5’ oligonucleotide contained a T7 RNA polymerase promoter for in vitro transcription and the 3’

Abbreviations: MSK, mitochondrial lysyl-tRNA synthetase; KRS, cytoplasmic lysyl-tRNA synthetase.

†To whom reprint requests should be addressed. e-mail: vania@ibm.c.u-strasbg.fr.
oligonucleotide contained a BstNI site to recreate the 3′ terminal CCA sequence upon in vitro transcription. The tRK2 gene from plasmid pFD17 (21), provided by G. Fink, was subcloned in a similar way but the first residue of the tRNA coding sequence T1 was changed into G to allow efficient in vitro transcription and, accordingly, residue A72 was changed into C72 to restore base pairing at the end of the acceptor stem. In addition, the 23-bp intron of the tRK2 gene was removed by PCR deletion mutagenesis. For in vitro expression of tRK2CUU, the entire coding sequence with its intron and 50 bp of 5′ and 3′ flanking sequences was PCR-cloned into the BamHI site of plasmid pRS416 (22) and matugenized to yield pTRK2-CUU. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (18) on single-stranded DNAs. For in vitro expression of the tRK1CUU-7 variant, a 300-bp fragment containing the tRK1 gene and flanking sequences was PCR-cloned from the pY109 plasmid (20) into the BamHI site of pRS416 and oligonucleotide-mutagenized to yield pTRK1-7.

**tRNAs, in Vitro Transcription and Aminoacylation.** Total yeast tRNA was prepared by phenol extraction. tRK1 and tRK2 were purified by reverse-phase chromatography followed by preparative gel electrophoresis and verified by partial sequencing and oligonucleotide hybridization. For isolation of mitochondrial tRNA, partially purified organelles were treated with a mixture of RNases (5) followed by sucrose gradient centrifugation, and mitochondrial RNA was extracted by a hot procedure (25). Transcripts were gel-purified, dephosphorylated by a heat denaturation–renaturation cycle in the presence of polynucleotide kinase. Gel-repurified transcripts were treated with T7 RNA polymerase (Promega) by a standard replication form DNAs (or transcripts were transcribed with T7 RNA polymerase (Promega) by a standard transcription and, accordingly, residue A72 was changed into C72 to restore base pairing at the end of the acceptor stem.

**RESULTS**

*In Vitro Import of Mutated tRNA395 Transcripts.* We have previously shown that an in vitro transcript of tRK1, once aminoacylated, is a good substrate for mitochondrial import in vitro (9). The transcript (which is not expected to carry modified nucleosides) is an even better import substrate than the corresponding fully modified natural tRNA. To identify import determinants within the tRK1 sequence, we have used oligonucleotide mutagenesis coupled with in vitro transcription to generate a set of mutant transcripts and studied their in vitro import efficiencies after aminoacylation with KRS. The sequences of tRK1 and tRK2 differ in 21 positions located in the acceptor arm (including the “discriminator” base at position 73), D arm, anticodon arm, and variable loop (Fig. 1). Because tRK1 is imported but tRK2 is not (4, 5, 7–9), we reasoned that determinants for import selectivity should lie within regions of sequence divergence between the two isoacceptors. We have

![Cloverleaf structures of tRK1 and tRK2 (based on refs. 31 and 32).](image-url)
therefore introduced a panel of replacements in the original tRK1 and tRK2 sequences that correspond to domains (or individual residues) differing between the two isoacceptors.

Import properties of the mutant tRK1 and tRK2 transcripts are summarized in Table 1. For tRK2, it was not possible to obtain a transcript with the wild-type sequence by in vitro transcription with T7 RNA polymerase because the original coding sequence begins with a thymidine. Therefore, the pair tRK2CUU and wild-type (tRK2CUU) was not (see Fig. 2 and Table 1, transcript 30). Import of tRK2CUU may be due to either the replacement of the first base pair in the acceptor stem or the absence of modified nucleosides in the transcript that may have a negative effect on import of the natural tRK2. A change of the tRK2CUU anticodon from UUU to CUU (transcript 2) resulted in a significant increase in the import efficiency (Fig. 2 and Table 1). In fact, transcript 2 was even a better import substrate than the tRK1 transcript. This suggests that residue C34 is at least one of the determinants for import selectivity of tRK1. However, the sequence context around this position appears also to be important because a version of tRK1 with the anticodon UUU (transcript 8) was still imported, though with a lesser efficiency than tRK1, but replacement of the entire anticodon arm of tRK1 with that of tRK2 (transcript 7) resulted in a total loss of import capacity (Fig. 2 and Table 1).

Another region of tRK1 that might also be relevant for its import efficiency is the acceptor stem. Indeed, replacement of bases 67–69 of tRK1 by the corresponding residues of tRK2 (transcript 40), though not disturbing helix formation, significantly inhibits the import (Table 1). However, the same replacement completed by a change of the discriminator base U71 to G and unpairing of the first acceptor helix pair (C72 to A) restored import (Table 1, transcript 4). The importance of the first base pair of the acceptor stem seems to depend on the sequence context, because replacement of the strong G–C pair in the well-imported transcript 2 by a weak G–U pair and a change of the discriminator base (G73 to U) dramatically reduces the import efficiency (Table 1, transcript 33). It is unlikely that this effect is due to the change of the discriminator base, because such a change alone conferred efficient import capacity to a poorly imported variant of tRK2 (Table 1, compare transcript 93 versus transcript 30).

Transcript 25, in which there is only a single m1G9 (of tRK1) → A replacement, is still imported but with a 2–3 times lower efficiency than the nonmutated transcript. However, a m1G9 → A change and replacement of the complete D arm of tRK1 with that of tRK2 (transcript 26) had little or no effect on import efficiency. These results suggest that the D arm of tRK1 does not contain determinants for its import selectivity. The same may be said about the variable loop whose replacement (in transcript 5) does not affect import.

### Table 1. Mutant tRK1 and tRK2 transcripts and their import efficiencies

<table>
<thead>
<tr>
<th>Transcript</th>
<th>tRNA</th>
<th>Original bases</th>
<th>New bases</th>
<th>Import efficiency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tRK1</td>
<td>C67, A68, G69</td>
<td>No change</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>tRK1</td>
<td>C67, A68, G69</td>
<td>U67, G68, A69</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>tRK1</td>
<td>C67, A68, G69, C72, U73</td>
<td>U67, G68, A69, A72, G73</td>
<td>50–75</td>
</tr>
<tr>
<td>29</td>
<td>tRK1</td>
<td>U71</td>
<td>G73</td>
<td>75–100</td>
</tr>
<tr>
<td>25</td>
<td>tRK1</td>
<td>C12, G9</td>
<td>A9</td>
<td>20–30</td>
</tr>
<tr>
<td>26</td>
<td>tRK1</td>
<td>G12, A15, C17, C23</td>
<td>A9, U12, G15, U17, A23</td>
<td>50–75</td>
</tr>
<tr>
<td>8</td>
<td>tRK1</td>
<td>C34</td>
<td>U34</td>
<td>50–75</td>
</tr>
<tr>
<td>6</td>
<td>tRK1</td>
<td>A28, U29, A31, U39, A41, U42</td>
<td>U28, C28, G31, C30, G41, A42</td>
<td>75–100</td>
</tr>
<tr>
<td>7</td>
<td>tRK1</td>
<td>A28, U29, A31, C34, U39, A41, U42</td>
<td>U28, C28, G31, U34, C39, G41, A42</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5</td>
<td>tRK1</td>
<td>G45, U48</td>
<td>U45, C48</td>
<td>75–100</td>
</tr>
<tr>
<td>30</td>
<td>tRK2</td>
<td>33, A72</td>
<td>G1, C72</td>
<td>5–10</td>
</tr>
<tr>
<td>2</td>
<td>tRK2</td>
<td>33, A72</td>
<td>G1, C34, C72</td>
<td>150–200</td>
</tr>
<tr>
<td>93</td>
<td>tRK2</td>
<td>33, A72, G73</td>
<td>G1, C72, U73</td>
<td>75–100</td>
</tr>
<tr>
<td>33</td>
<td>tRK2</td>
<td>33, A72, G73</td>
<td>G1, C34, U72, U73</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Import of transcript 1 was taken as 100%
detect the import. The level of expression of tRK2 CUU was tRK2, whereas at 45°C, only tRK2CUU is detected. Arrows indicate the 40°C, the anti-CUU probe recognizes both mutant and wild-type tRK2CUU (CUU) and tRK2mnm C replacement in tRK2CUU; the anticodon is boxed. (See Table 2).

A tRK1 gene variant, referred to as tRK1UUU-7, containing the tRK1 anticodon arm in the tRK1 backbone. Although well expressed in vitro, tRK1UUU-7 was not found to be imported into mitochondria (Fig. 3d). This result is in perfect agreement with our in vitro import results (see Table 1, transcript 7) and confirms that the anticodon arm of tRK1 contains residues critical for import selectivity.

Aminoacylation and Pre-MSK Binding Properties of Mutant Transcripts. We have previously shown that aminoacylation of tRK1 by the cytoplasmic lysyl-tRNA synthetase (KRS) is a prerequisite for its mitochondrial import (8, 9). To see whether the same holds true for in vitro-synthesized mutant transcripts, we have determined their aminoacylation parameters by using purified KRS. Table 2 shows that among the 13 mutant transcripts studied, transcripts 2, 5, 6, 7, 8, and 29 showed aminoacylation efficiencies comparable to the non-mutated tRK1 transcript, whereas 6 other transcripts, transcripts 4, 40, 33, 93, 25, and 26, showed efficiencies decreased by 30–50 times. All the transcripts with low aminoacylation levels contained mutations in either the acceptor stem (transcripts 4, 40, 33, and 93) or the D-arm region (transcripts 25 and 26) and in all cases the Km remained almost unchanged and the Vmax was strongly decreased (Table 2).

In the majority of cases, import efficiencies of the mutant transcripts can be correlated to their respective aminoacylation efficiencies. For example, transcripts 2, 5, 6, 8, and 29 show both efficient import and high aminoacylation levels. On the other hand, transcripts 33 and 40, which are poorly aminoacylated, are not imported, and transcript 30, which has an intermediate aminoacylation rate, also has an intermediate import efficiency. Surprisingly, among the poorly aminoacylated transcripts at least four (transcripts 4, 25, 26, and 93) are imported rather efficiently. It is therefore possible that these transcripts can be imported without being aminoacylated by KRS.

Our previous results indicated that aminoacylation of tRK1 might be necessary to allow interaction with pre-MSK, which is likely to act as a carrier for mitochondrial translocation of the tRNA (8). We therefore tested all our in vitro constructs for their abilities to bind pre-MSK in an immunoassay (Table 2). A clear correlation was found between pre-MSK binding properties and import efficiencies; i.e., all transcripts that efficiently bind to pre-MSK are imported, whereas those that do not bind are not imported, irrespective of their aminoacylation abilities by KRS. For example, the capacities of transcripts 4, 26, and 93 to bind pre-MSK in their nonacylated form (i.e., in absence of KRS) could explain their ability to be imported. This implies that, contrary to the natural tRK1, some in vitro-synthesized mutant transcripts can be recognized by pre-MSK and imported without previous aminoacylation by KRS. Conversely, the poor binding to pre-MSK of transcript 7 (in which the anticodon arm of tRK1 was replaced by that of tRK2) in both absence or presence of KRS explains its lack of import. This implies that some residues in the anticodon region of tRK1 (including position 34) are directly involved in interaction with pre-MSK.

In Vitro Import of a Nicked tRK1 Transcript. The mechanism of translocation of a tRNA across mitochondrial mem-
branes, though shown in yeast to depend on the mitochondrial preprotein translocation machinery and on cytoplasmic import-directing factors (7, 8), remains unclear. For example, it is not known whether the tRNA molecule is unfolded during translocation across mitochondrial membranes or is imported as a folded molecule. To test these possibilities, we constructed two truncated forms of tRK1 (Fig. 4d). Fragment A represents the first 52 bases of the tRNA and fragment B represents its last 24 residues including the 3′ terminal CCA. When annealed to one another by a cycle of heat denaturation and renaturation in the presence of MgCl2, the A and B moieties form a duplex with a mobility on a nondenaturing gel comparable to that of the noninterrupted tRK1 transcript (Fig. 4b).

*In vitro* import of fragments A and B into isolated mitochondria were performed after preaaminoacylation. The 5′ fragment alone was not detectable within mitochondria but, surprisingly, the 3′ fragment was slightly imported (Fig. 4c, lanes 3 and 4). Import of fragment B strongly decreased in the presence of a 33-fold molar excess of unlabeled tRK1 and was no longer detectable in the presence of a 66-fold excess (Fig. 4c, lanes 5 and 6). We have previously shown that a similar excess of unlabeled tRK1 totally blocks the import of labeled tRK1 (9). This suggests that import of the B fragment and of tRK1 proceed by a similar mechanism. Furthermore, aaminoacylated fragment B showed low but detectable binding to pre-MSK, but fragment A lacked pre-MSK binding capacity (Table 3). This suggests that, as for tRK1, the import of the B fragment is mediated by pre-MSK. Transcript B could not be aaminoacylated by KRS and lysine but was charged by a mixture of labeled amino acids in the presence of a mixture of synthetases (Table 3), suggesting that it is imported in a misacylated form. Misacylation of fragment B may be due to formation of a potentially stable stem-loop structure with a 3′ terminal CCA (Fig. 4d). This structure partially resembles the valine minihelix that can be aaminoacylated by the yeast ValRS.

**FIG. 4.** *In vitro* import of a nicked tRK1 transcript. (a) Secondary structures of tRK1 and of the truncated A and B fragments. (b) Nondenaturing gel electrophoresis of fragments A and B: simple mixture of the two fragments (lane A+B), reannealed A+B fragments [lane A+B(hybrid)], renatured tRK1 transcript (lane tr.N°1). (c) Autoradiographic detection on a denaturing polyacrylamide gel of imported A and B fragments (lanes 3–6) and of the nicked tRK1 transcript (lanes 7 and 8). Before the import reaction, the truncated fragments were aaminoacylated with a mixture of yeast synthetases and a mixture of amino acids and the reannealed fragments with KRS and lysine. The amount of labeled A and B fragment used per assay was 6 pmol of fragment A (lanes 1–8) and either 12 pmol (lanes 1–6 and 8) or 3 pmol (lane 7) of fragment B. Control, labeled fragments A (lane 1) and B (lane 2) used as markers; imported RNAs, *in vitro* import assays of fragment A (lane 3), fragment B (lane 4), fragment B in the presence of either 400 pmol (lane 5) or 800 pmol (lane 6) of unlabeled tRK1 and of the hybridized A+B fragments (lanes 7 and 8). (d) Possible secondary structure of the B fragment.

**Table 3.** Import, aaminoacylation, and pre-MSK binding properties of truncated and nicked tRK1 transcripts

<table>
<thead>
<tr>
<th>RNA</th>
<th>Import, %</th>
<th>Aaminoacylation, %</th>
<th>Pre-MSK binding, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With aa mix</td>
<td>With lysine</td>
<td></td>
</tr>
<tr>
<td>tRK1 transcript</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A fragment</td>
<td>&lt;5</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>B fragment</td>
<td>24</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>A + B</td>
<td>28*</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>A + B (hybrid)</td>
<td>75</td>
<td>35</td>
<td>25</td>
</tr>
</tbody>
</table>

Data for import are percent of transcript 1 import efficiency (average of three experiments). Data for aaminoacylation are percent transcript 1 maximal aaminoacylation. The pre-MSK binding of the aaminoacylated tRK1 transcript was taken as 100% (values of nonspecific binding detected with preimmune antibodies have been subtracted); the tRK1 transcript was aaminoacylated with lysine and fragments A and B with a mixture of amino acids (aa mix). ND, not determined.

*Only fragment B is imported if fragments A and B are mixed without annealing. (33). It is possible that the B fragment can better mimic a substrate for pre-MSK binding than the A fragment, which although it contains the anticodon arm, cannot adopt a tRNA-like structure.

After annealing of fragments A and B, the duplex can be aaminoacylated by KRS and lysine (Table 3) and becomes imported with a significantly higher efficiency than the B fragment alone (Fig. 4c and Table 3). The amount of imported A fragment is dependent upon the amount of added B fragment and the two fragments are imported in an equimolar ratio, implying that they are coinported as a duplex molecule. This result demonstrates that a nicked tRK1 transcript can be imported *in vitro*, which suggests that in *vivo* tRK1 is likely to be translocated across mitochondrial membranes as a folded molecule.

**DISCUSSION**

We have studied, in *in vitro* and *in vivo* experiments, structural features of tRK1 that are relevant to its mitochondrial import. The data presented indicate that the anticodon region of tRK1 contains determinants for its import selectivity. In fact, replacement of the entire anticodon arm of tRK1 by the one of tRK2 totally blocked the import, both *in vitro* and *in vivo*. The most important position in this region appears to be the wobble position of the anticodon (C34), because introduction of C34 into a tRK2 transcript directs its efficient import *in vitro*. This conclusion is strengthened by the fact that this single-base change in the tRK2 anticodon conferred import *in vivo* to this normally nonimported tRNA. However, *in vivo* import of the tRK2CUU version is low when compared with tRK1. In fact, *in vitro* experiments revealed that position 34 is not only important by itself but also in its context, because no significant loss of import efficiency was found with a tRK1CUU transcript (transcript 8). We suggest that in tRK2, the uridine modification 5′-[(methylamino)-methyl]-2-thiouridine, mnm5s2U in position 34 may act as an antideterminant for import, because a tRK2 transcript is weakly imported but natural (fully modified) tRK2 is not. The mitochondrial import apparatus seems to be able to discriminate between mmn5s2U and cytidine in position 34 of the tRNA molecule but not (or, at least, with a much lesser efficiency) between unmodified uridine and cytidine in this position.

A second domain of the tRK1 molecule which might be important for its import selectivity is the acceptor stem, because replacement of residues 67–69 of tRK1 with those of tRK2 (transcript 40) blocks the import. However, this effect can be reversed by additional changes in positions 72 and 73 (transcript 4), which restore the import. Such a differential
effect of mutations in the acceptor stem onto import can be explained by differences in pre-MSK-binding properties of the two transcripts, due to conformational changes in the acceptor helix (see below).

Our previous results indicated that pre-MSK (which is essential for tRK1 import) can form a stable complex with tRK1 but only with its aminoacylated form. We therefore hypothesized that aminoacylation is essential for binding to pre-MSK, which in turn would serve as a carrier for mitochondrial transport of the tRNA (3, 8, 9). We show herein that pre-MSK binding is indeed of critical importance for the import of mutant tRNA transcripts, because all transcripts with high pre-MSK binding capacities are imported well (i.e., transcripts 1, 2, 4, 5, 6, 8, 26, 29, and 93) but those with low binding are poorly imported (i.e., transcripts 7, 30, 33, and 40). However, comparison of aminoacylation capacities of mutant tRNAs showed that aminoacylation is needed for pre-MSK binding and mitochondrial import of only a portion of the mutant tRNAs. The well-imported transcripts 1, 2, 5, 6, and 29 show higher pre-MSK binding in their aminoacylated form. The poorly imported transcripts 30, 33, and 40 (which are also poorly aminoacylated) show low pre-MSK binding capacities. In contrast, transcripts 4, 26, and 93 (which are poorly aminoacylated) retain high pre-MSK binding capacities in their deacylated form and are well imported.

We suggest that the role of aminoacylation in import is to induce a conformational change in tRK1 to facilitate its binding to pre-MSK. As a matter of fact, the role of aminoacylation as a factor of conformational change in tRNA was recently described for the tRNA-like domain of several viral RNAs (34). It was shown that the role of aminoacylation is to induce productive interaction of the tRNA-like domain with replication factors, although the nature of the amino acid at the 3' terminus of the viral RNA is not crucial. If we accept the idea that aminoacylation of tRK1 facilitates its binding to pre-MSK, how then can some tRNA\textsuperscript{A\textsubscript{2\textprime}}\textsubscript{V} variants (i.e., transcripts 4, 26, and 93) bind to pre-MSK in their deacylated form? It is worth noting that these transcripts either contain mutations in the acceptor stem or have an altered tertiary interaction. Transcript 26, for example, has a disrupted A\textsubscript{15}U\textsubscript{48} interaction replaced by a potential G\textsubscript{15}U\textsubscript{48} pair, which is not found in any known tRNA (35). On the other hand, transcripts 4 and 40, which differ by residues 72 and 73 in the acceptor stem, have low aminoacylation rates but differ greatly in their pre-MSK binding and import capacities. It is therefore possible that some particular alterations in the core of the tRK1 L shape structure or in the acceptor helix might induce conformational changes that can mimic the effect of aminoacylation on tRK1 and allow pre-MSK binding.

We have used artificial import substrates to study the mechanism of translocation of tRK1 across mitochondrial membranes in vitro. It has been shown previously that polynucleotides cross-linked to a signal peptide for preprotein targeting into mitochondria can be imported into isolated mitochondria (36, 37). Likewise, if we assume that tRK1 import involves formation of a covalent link with its carrier protein (presumably pre-MSK), then folding of the tRNA should not be required for its mitochondrial translocation. However, our results with a nicked tRK1 transcript are clearly in favor of the importance of the tRNA cloverleaf structure, because the imported 3' moiety of tRNA can direct mitochondrial transport of the 5' portion of the tRNA, which alone is not imported. This suggests translocation of the tRNA as a folded molecule.

We thank H. Kazakova for obtaining mutant 93; A. Tzagoloff, N. Pfanner, G. Fink, H. Feldmann, and M. Mirande for providing strains, plasmids, enzymes, and antibodies; and L. Brubacher for technical assistance. This work was supported by the Centre National de la Recherche Scientifique, the Université Louis Pasteur, the Association Française contre les Myopathies, the Moscow State University, and the Russian Foundation for Basic Research (Grant 96.04.48027). N.S.E. was supported by an European Molecular Biology Organization fellowship for Eastern molecular biologists and by the Université Louis Pasteur.