Selective importation of RNA into isolated mitochondria from *Leishmania tarentolae*

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ABSTRACT

All mitochondrial tRNAs in kinetoplastid protozoa are encoded in the nucleus and imported from the cytosol. Incubation of two in vitro-transcribed tRNAs, tRNA^{Ile}(UAU) and tRNA^{Gln}(CUG), with isolated mitochondria from *Leishmania tarentolae*, in the absence of any added cytosolic fraction, resulted in a protease-sensitive, ATP-dependent importation, as measured by nuclease protection. Evidence that nuclease protection represents importation was obtained by the finding that *Bacillus subtilis* pre-tRNA^{Asp} was protected from nuclease digestion and was also cleaved by an intramitochondrial RNase P-like activity to produce the mature tRNA. The presence of a membrane potential is not required for in vitro importation. A variety of small synthetic RNAs were also found to be efficiently imported in vitro. The data suggest that there is a structural requirement for importation of RNAs greater than ~17 nt, and that smaller RNAs are apparently nonspecifically imported. The signals for importation of folded RNAs have not been determined, but the specificity of the process was illustrated by the higher saturation level of importation of the mainly mitochondria-localized tRNA^{Ile} as compared to the level of importation of the mainly cytosol-localized tRNA^{Gln}. Furthermore, exchanging the D-arm between the tRNA^{Ile} and the tRNA^{Gln} resulted in a reversal of the in vitro importation behavior and this could also be interpreted in terms of tertiary structure specificity.

Keywords: *Leishmania tarentolae*; mitochondria; RNA import; tRNA

INTRODUCTION

Mitochondrial import of cytoplasmic tRNAs has been demonstrated in plants, fungi, protozoa, and mammals (Schneider et al., 1994; Hauser & Schneider, 1995; Dietrich et al., 1996; Entelis et al., 1996; Marechal-Drouard et al., 1996; Tarassov & Martin, 1996). The number of imported tRNAs varies from a complete set necessary for translation in kinetoplastid protozoa (Simpson et al., 1989; Hancock & Hajduk, 1990; Lye et al., 1993; Chen et al., 1994; Shi et al., 1994) to 19–26 tRNAs in the ciliate protozoa (Suyama, 1986; Pritchard et al., 1990), to one of three lysine isoacceptors in Sac-charomyces cerevisiae (Entelis et al., 1996). Importation of tRNA in yeast and *Tetrahymena* (Rusconi & Cech, 1996a, 1996b) is a very specific process capable of discriminating one particular tRNA out of a set of isoacceptor tRNAs. In the case of the trypanosomatids, the importation system may have a broader specificity due to the fact that all mitochondrial tRNAs are imported.

The mechanism by which negatively charged RNAs cross the hydrophobic environment of the organelle's membranes remains a complete mystery, with apparent differences between species. Importation of tRNA^{Lys} (CUU) into the yeast mitochondrion is energy dependent and requires the preprotein import machinery as well as the presence of the precursor form of the mitochondrial lysyl-tRNA synthetase and also requires the tRNA to be aminoacylated (Tarassov et al., 1995a; Entelis et al., 1996, 1998). In trypanosomatids, mutated tRNAs that cannot be aminoacylated can still be imported in vivo (Schneider et al., 1994). Mahapatra et al. (1998) have shown that no cytosolic factors are required for importation of synthetic tRNA into isolated

Leishmania tropica mitochondria. Two *Leishmania* mitochondrial surface proteins have been reported to bind RNA in the absence of ATP, but their precise role and identity remain to be determined (Adhya et al., 1997). Mahapatra et al. (1998) have also proposed that the sequence motif AUGGCAGAG in the D-loop region of *Leishmania* tRNAs serves as the signal for import. However, a recent analysis (Suyama et al., 1998) of all available trypanosomatid tRNA sequences found no apparent consensus sequences within the D-loop that could account for regulation of tRNA importation.

It has become apparent that the tRNA molecule itself bears structural features required and sufficient for mitochondrial localization. However, the precise features required appear to vary among different organisms: the anticodon in *Tetrahymena* (Rusconi & Cech, 1996a, 1996b), the D-arm in *Leishmania* (Lima & Simpson, 1996; Mahapatra et al., 1998), and the anticodon arm and acceptor stem in yeast (Entelis et al., 1998; Kazakova et al., 1999).

In this article, we demonstrate a selective ATP-dependent importation of in vitro-transcribed tRNAs and several small synthetic RNAs into isolated mitochondria from *L. tarentolae*.

RESULTS

In vitro-transcribed tRNA^{Ile}(UAU) and tRNA^{Gln}(CUG) are protected from micrococcal nuclease digestion upon incubation with isolated mitochondria

Incubation of radioactively labeled in vitro transcribed tRNA^{Ile} and tRNA^{Gln} (Fig. 1) with Percoll-isolated mitochondria in the presence of ATP resulted in protection from micrococcal nuclease digestion (Fig. 2A, lanes 1 and 6). The maximum level of nuclease protection was reached within 10–15 min under these conditions (data not shown). There was an optimum nuclease protection of RNA at pH 8.0 (data not shown). Variation in the concentration of dithiothreitol (DTT) up to final concentrations of 100 mM had little apparent effect. The time of incubation chosen for the standard nuclease protection assay was 5 min, which is within the linear range of the reaction. All nuclease protection experiments in this article used mitochondria obtained from cells broken in hypotonic medium, but mitochondria from cells broken in isotonic media exhibited a similar activity. In control reactions using no mitochondria, all RNAs were completely sensitive to digestion by micrococcal nuclease (Fig. 2A, lanes 4 and 9).

The amount of each tRNA protected from nuclease digestion in the presence of isolated mitochondria reached a plateau at micromolar concentrations of RNA input: 25 μM for tRNA^{Ile} and 3.8 μM for tRNA^{Gln}. Approximately 9% of the input tRNA^{Ile} and 1% of the input tRNA^{Gln} was protected at saturation under these
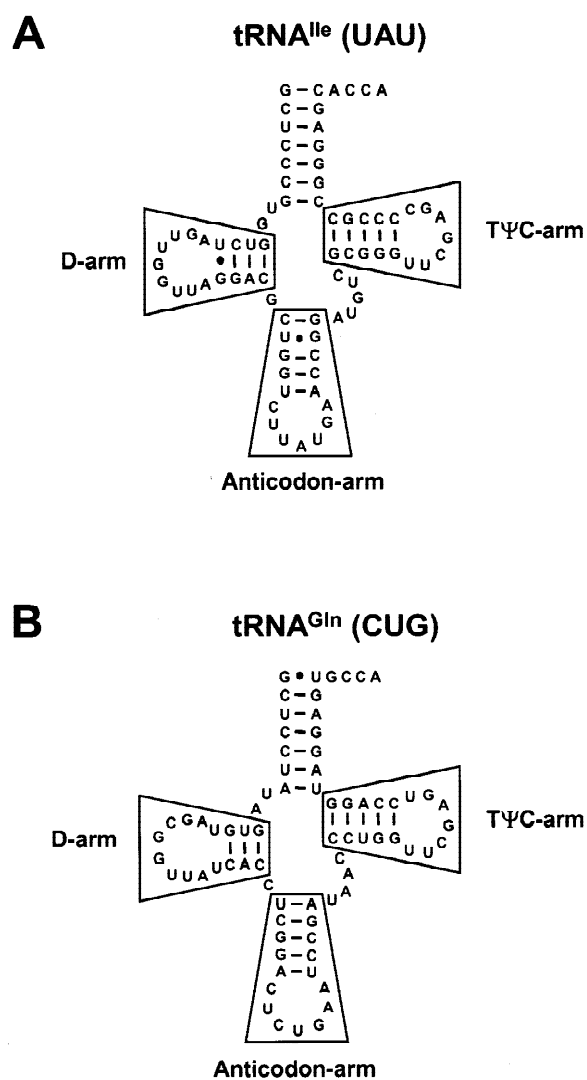

FIGURE 1. Sequences of the *L. tarentolae* tRNAs used in this study. **A**: tRNA^{Ile}(UAU). **B**: tRNA^{Gln}(CUG). The synthetic mini-helix RNAs corresponding to the D-arm, anticodon-arm, and TψC-arm of the respective tRNAs are indicated by boxes.

conditions (Fig. 2B). This difference in the in vitro saturation levels of nuclease protection is consistent with the in vivo localization of these tRNAs, where tRNA^{Ile} is mainly mitochondrial and tRNA^{Gln} is mainly cytosolic (Lye et al., 1993; S. Kapushoc, M.A.T. Rubio, J.D. Alfonzo, L. Simpson, unpubl. results).

Predigestion of the isolated mitochondria with proteinase K (Fig. 2A) or low amounts of trypsin (data not shown) eliminated the ability of the mitochondria to protect the labeled tRNA^{Ile} from nuclease digestion. This suggests the presence of protein membrane receptor(s). Treatment of the mitochondria with Triton X-100 (data not shown) or SDS (Fig. 2A, lanes 5 and 10) also destroyed the ability to protect the tRNA from nuclease digestion, suggesting a requirement for an intact mitochondrial membrane.
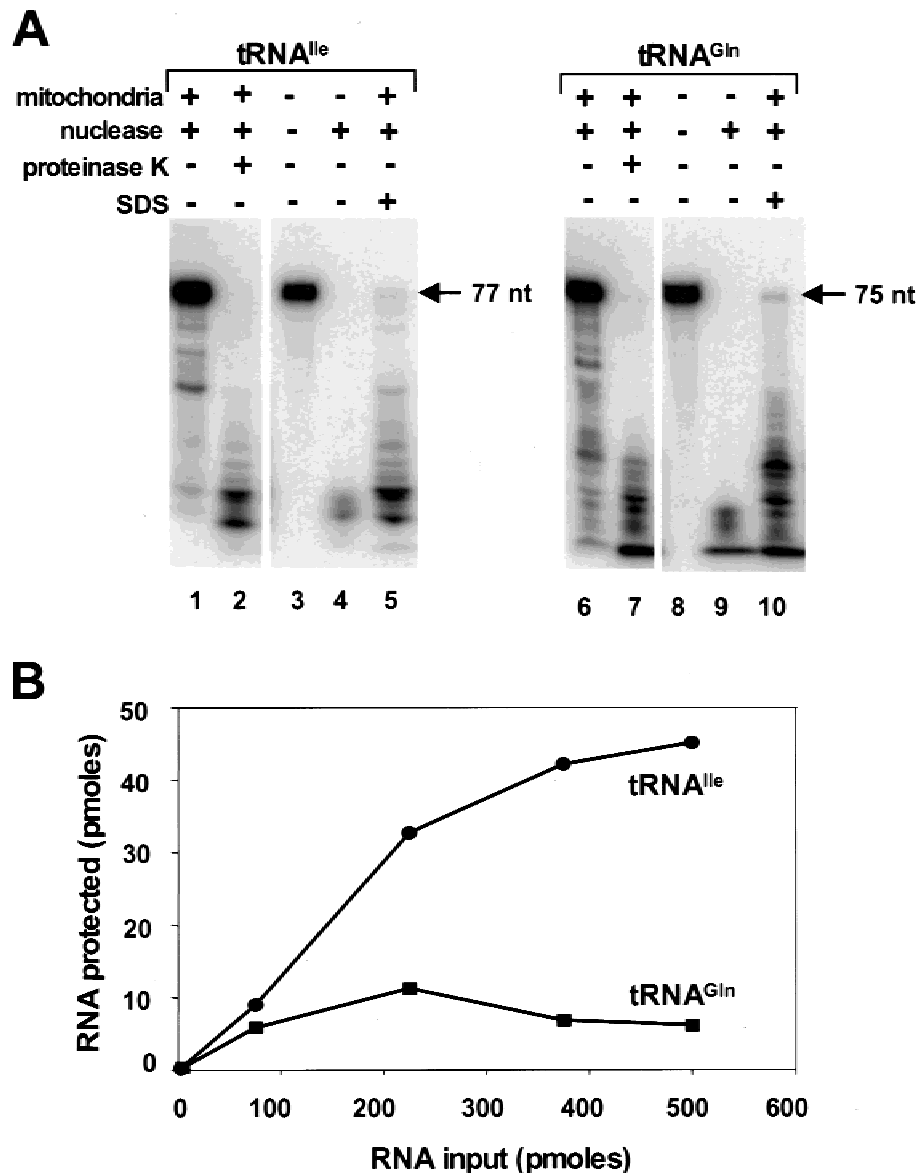
FIGURE 2. Nuclease protection of in vitro-transcribed tRNA[Ile] and tRNA[Gln] incubated with isolated *L. tarentolae* mitochondria. **A:** Uniformly labeled tRNA[Ile] (6.77 pmol, 5×10^5 cpm) (lanes 1–5) and tRNA[Gln] (8.95 pmol, 5×10^5 cpm) (lanes 6–10) were incubated with mitochondria and digested with microccocal nuclease (MN) as indicated. Lanes 2 and 7: mitochondria digested with Proteinase K prior to the addition of the labeled RNA. Lanes 5 and 10: mitochondria lysed with SDS prior to the addition of the labeled RNA. Lanes 3 and 8: 1/10 input RNA. The RNAs were resolved by electrophoresis in 7 M urea/8% acrylamide gels. The migration of the full-length input RNA is indicated by the arrow. Lanes 1–5 were derived from the same gel, lanes 6–10 were from a second gel. Both gels were exposed to a PhosphorImager screen simultaneously. **B:** Increasing concentrations of labeled tRNA[Ile] (circles) and tRNA[Gln] (squares) were incubated with mitochondria, digested with MN, and resolved by denaturing polyacrylamide gel electrophoresis. The calculated amounts of RNA protected per milligram mitochondrial protein at 500 pmol input RNA are as follows: tRNA[Ile], 1,125 pmol/mg; tRNA[Gln], 125 pmol/mg.

The tRNA[Ile](D-tag) and tRNA[Gln](D-tag), each tagged by three mutations in the D-loop, that were used previously for in vivo transfection experiments (Lima & Simpson, 1996) showed behavior in this system identical to wild-type tRNAs (data not shown).

The absence of the 3′-CCA in the in vitro-transcribed tRNAs had no effect on nuclease protection (data not shown).

Complete competition of nuclease protection of the tRNAs was observed when increasing amounts of homologous unlabeled tRNA were added to the reaction, as shown in Figure 3A,B. In each case, the heterologous unlabeled tRNA did not completely compete the nuclease protection of the labeled tRNA. In contrast, the homologous unlabeled tRNA completely competed at 10- to 30-fold molar excess over the labeled input tRNA.
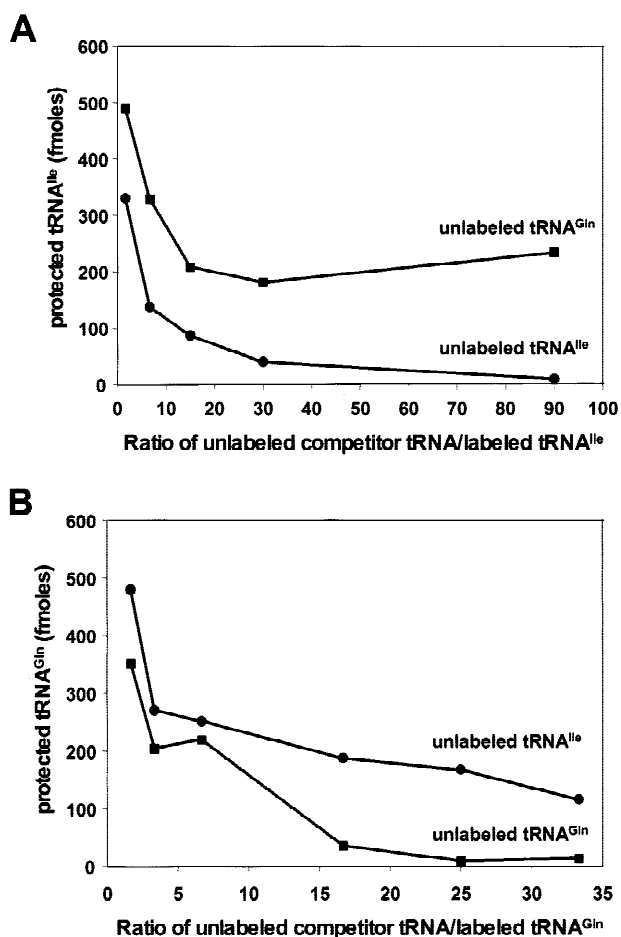
A

B

FIGURE 3. Competition by unlabeled tRNAs. **A**: Labeled tRNA^{Ile} (3 pmol, 2.7×10^4 cpm) was incubated with mitochondria in the presence of increasing amounts of unlabeled tRNA^{Ile} (circles) or tRNA^{Gln} (squares). **B**: Labeled tRNA^{Gln} (3 pmol, 2.4×10^4 cpm) was incubated with mitochondria in the presence of increasing amounts of unlabeled tRNA^{Ile} (circles) or tRNA^{Gln} (squares). The extent of nuclease protection was analyzed as described in Materials and methods.

Evidence that the nuclease protection of tRNA represents importation into the mitochondrion: Cleavage of a heterologous precursor tRNA by an intramitochondrial RNase P-like activity

Pre-tRNA^{Asp} from *Bacillus subtilis* is a precursor tRNA that is cleaved by RNase P into the mature tRNA and a 5′ leader. We found that incubation of T7-transcribed pre-tRNA^{Asp} with *L. tarentolae* mitochondria in the presence of ATP produced a cleavage of the precursor into a species that comigrated with the mature tRNA and that was protected from nuclease digestion (Fig. 4A). Another fragment appeared even at low ATP levels that comigrated with the 33-nt 5′ leader, but as this fragment also appeared with 3′ end-labeled pre-tRNA^{Asp} (Fig. 4B) and not with 5′ end-labeled pre-tRNA^{Asp} (Fig. 4C), it does not represent the 5′ leader and is

probably a degradation product derived from the 3′ end of the molecule.

A primer extension analysis of the nuclease-protected mature tRNA^{Asp} showed that the major cleavage site was approximately 2 nt upstream of the *Escherichia coli* RNase P cleavage site (Fig. 5).

These data indicate that the nuclease protection of RNA represents importation into the matrix of the organelle, because there is processing of the pre-tRNA into a mature tRNA that is carried out by an RNase P-like cleavage activity localized within the mitochondrial matrix. A similar activity was previously detected in mitochondria from *Trypanosoma brucei* (Hancock et al., 1992).

Additional evidence that the nuclease protection of tRNA represents importation into the mitochondrion: Titration with digitonin

Digitonin selectively solubilizes the outer mitochondrial membrane at low concentrations due to the high levels of cholesterol in that membrane (Schnaitman & Greenawalt, 1968). RNAs attached to the outer mitochondrial membrane or the outer side of the inner mitochondrial membrane should be nuclease sensitive at low digitonin concentrations that should only break down the outer membrane. However, RNAs within the matrix should become nuclease sensitive at higher digitonin concentrations that also break down the inner membrane. To provide a matrix marker, endogenous matrix-localized tRNAs were 3′-end labeled with [α-^{32}P]CTP by an intramitochondrial ATP(CTP) nucleotidyltransferase activity previously shown to exist in *T. brucei* mitochondria (Hancock et al., 1992). In a parallel experiment, mitochondria were incubated with uniformly labeled tRNA^{Ile} in the presence of ATP. The nuclease protection of the tRNA^{Ile} at increasing digitonin concentrations was compared to that of the labeled endogenous mitochondrial tRNAs. As shown in Figure 6, the latency of the tRNA^{Ile} with digitonin was identical within experimental error to that of the labeled endogenous tRNAs. This suggests that the tRNA^{Ile} was imported into the mitochondrial matrix and not sequestered on the surface in a nuclease-resistant manner. This method of digitonin solubilization has been used previously to show that tagged tRNAs transcribed from plasmids in vivo are targeted to the mitochondrial matrix (Lima & Simpson, 1996).

Lack of requirement for a membrane potential

L. tarentolae mitochondria isolated in Percoll gradients after hypotonic (Fig. 7A) or isotonic (Fig. 7B) cell breakage have measurable membrane potentials, as determined using the potential-sensitive fluorescent dye, diS-C$_3$-(5) (Sims et al., 1974; Hwang et al., 1989; Hauser et al., 1996). Furthermore, freezing the mitochondria at
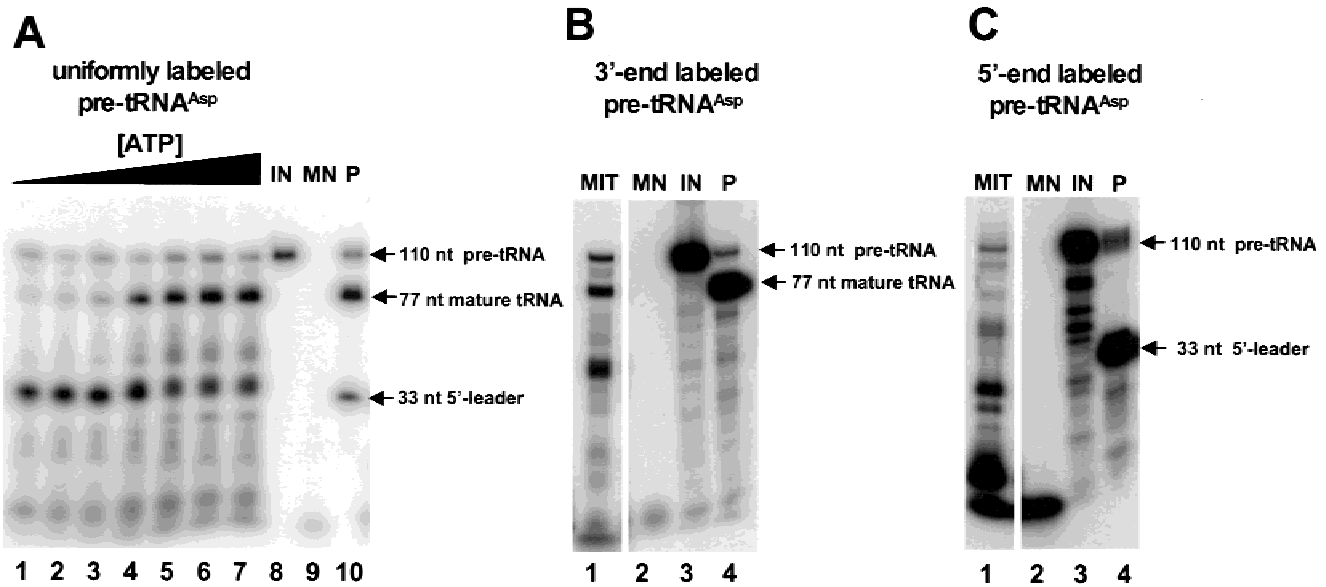
FIGURE 4. Nuclease protection and cleavage of *B. subtilis* pre-tRNA^{Asp} incubated with isolated mitochondria as evidence for importation. **A**: Uniformly radiolabeled, in vitro-transcribed pre-tRNA^{Asp} (5.2 pmol, 5×10^4 cpm) was incubated with isolated mitochondria in the presence of increasing concentrations of ATP. The extent of nuclease-protected RNA was resolved by denaturing gel electrophoresis. Lanes 1–7: ATP concentrations were 0, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 mM, respectively. Lane 8: 0.5 pmol input RNA, no incubation with mitochondria. Lane 9: MN digestion of 5.2 pmol RNA. Lane 10: input RNA digested with *E. coli* RNase P RNA. The pre-tRNA (110 nt), mature tRNA (77 nt) and the 5′ leader RNA (33 nt) are indicated by arrows, respectively. **B**: 3′-end labeled pre-tRNA^{Asp}. Lane 1: incubation of 0.056 pmol (1.5×10^5 cpm) RNA with isolated mitochondria and digestion with MN. Lane 2: MN digestion of 0.0056 pmol RNA. Lane 3: 0.056 pmol input RNA, no incubation with mitochondria. Lane 4: input RNA digested with *E. coli* RNase P RNA. **C**: 5′-end labeled pre-tRNA^{Asp}. Lane 1: incubation of 0.23 pmol (1.5×10^5 cpm) RNA with isolated mitochondria followed by MN digestion. Lane 2: MN digestion of 0.23 pmol RNA. Lane 3: 0.23 pmol input RNA, no incubation with mitochondria. Lane 4: input RNA digested with *E. coli* RNAse P RNA.

−80 °C in glycerol had little effect on either the membrane potential or the nuclease protection assay. However the use of Percoll in the final mitochondrial isolation step was essential, as Renografin-isolated mitochondria were inactive in the nuclease protection assay.

The addition of the potassium ionophore valinomycin to Percoll-isolated mitochondria at a concentration shown to completely discharge the membrane potential (Fig. 7A) had no significant effect on the nuclease protection of the synthetic tRNA^{Ile} (Fig. 7C). Addition of 10 mM dinitrophenol, an uncoupling agent that makes the mitochondrial inner membrane permeable to protons, also had no effect on the development of nuclease protection of tRNA^{Ile} (data not shown).

ATP requirement for nuclease protection

The nuclease protection of synthetic tRNA^{Ile} and tRNA^{Gln} upon incubation with isolated mitochondria requires the presence of ATP. As shown in Figure 8, there is a correlation between the concentration of ATP and the amount of RNA protected. Pretreatment of the mitochondria with apyrase, which depletes the ATP outside the mitochondria and may decrease the endogenous ATP pool, enhanced the ATP-dependent importation of RNAs as detected by protection from nuclease digestion (data not shown).

Replacement of ATP with the poorly hydrolyzable ATP analog, ATP-γ-S, and the nonhydrolyzable analog, AMP-PNP, both produced a decrease in the extent of nuclease protection (Fig. 8D), suggesting a possible requirement for β-γ bond hydrolysis of ATP. The nonhydrolyzable analogs, AMP-PCP and AMP-CPP, both could apparently substitute for ATP in the protection assay and even enhance the reaction (Fig. 8D). However, the nuclease protection with these analogs was not importation, because there was no cleavage of the precursor to yield the mature tRNA^{Asp} in either case (data not shown). We speculate that these analogs may produce conformational changes in the mitochondria that shield the tRNAs adsorbed to the outer surface from the nuclease. Aberrant results with these two analogs were also observed previously in an in vitro U-insertion editing system using a mitochondrial extract from *L. tarentolae* (Frech et al., 1995).

The residual amount of nuclease protection observed using the poorly hydrolyzable analog ATP-γ-S and the nonhydrolyzable analog AMP-PNP does represent importation, because the precursor was cleaved to the mature tRNA (data not shown). We interpret these results to indicate a possible requirement for external ATP hydrolysis at the β-γ bond, but this must be confirmed by further work.
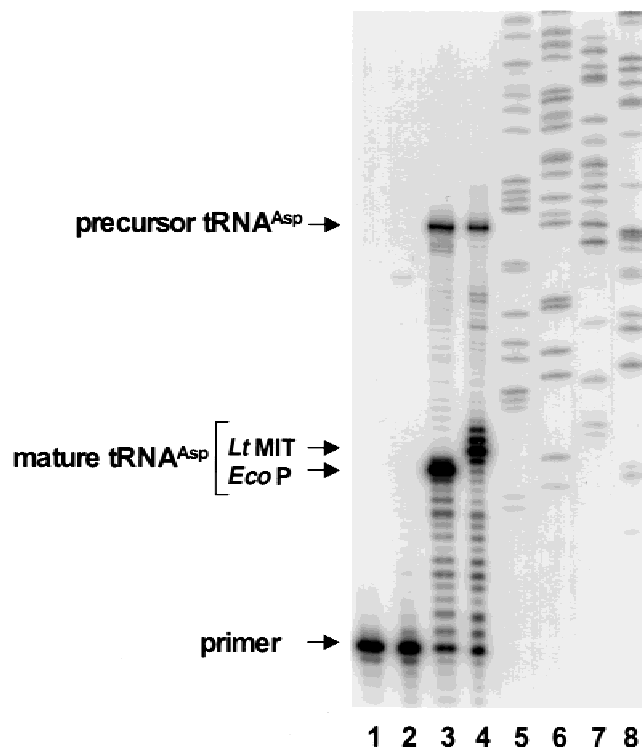
FIGURE 5. Primer extension analysis of cleavage products of precursor tRNA^Asp produced by digestion with *E. coli* RNase P or by importation into *L. tarentolae* mitochondria. Total *L. tarentolae* (*Lt*) mitochondrial RNA without reverse transcriptase (lane 1) and with reverse transcriptase added, (lane 2). Lane 3: *B. subtilis* pre-tRNA^Asp cleaved by *E. coli* (*Eco*) RNase P RNA prior to the reverse transcription reaction. Lane 4: *B. subtilis* pre-tRNA^Asp subjected to the in vitro importation assay into *L. tarentolae* mitochondria prior to reverse transcription. Lanes 5, 6, 7, and 8 are DNA sequencing lanes corresponding to G, A, T, and C, respectively.
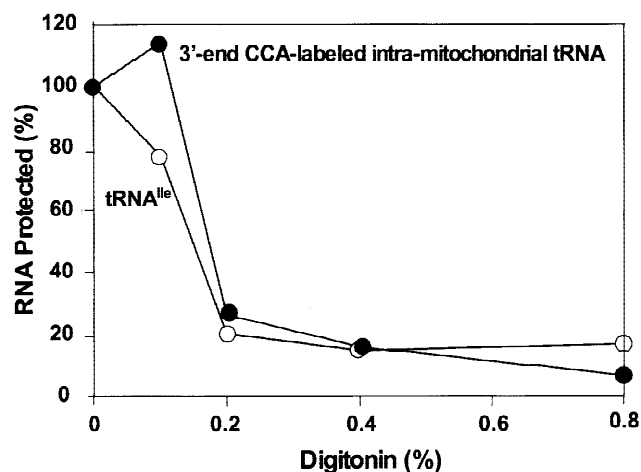

FIGURE 6. Effect of increasing concentrations of digitonin on nuclease protection of uniformly labeled tRNA^Ile and endogenous mitochondrial tRNAs labeled at the 3′-CCA end. Isolated mitochondria were incubated with uniformly radiolabeled, in vitro-transcribed tRNA^Ile (open circles) under importation conditions. In separate reactions, endogenous mitochondrial tRNAs were labeled at the 3′ end with the addition of $[\alpha^{-32}P]$-CTP to the mitochondria under conditions described previously (Hancock et al., 1992). The mitochondria were further treated using increasing concentrations of digitonin followed by digestion with micrococcal nuclease. The digitonin concentration used is plotted versus the percentage of nuclease-protected RNA. This graph depicts a representative experiment. The difference seen between the two curves is within experimental error.

The nuclease protection of tRNA^Ile was also inhibited by oligomycin (Fig. 9), an inhibitor of ATP synthesis that binds to F_0 and blocks proton transfer. This suggests that there is also a requirement for ATP inside the mitochondrion. The possibility that this inhibition is due to the discharge of the proton gradient and not to a decrease in internal ATP is unlikely because, as shown above, complete discharge of the membrane potential with valinomycin has no effect.

Importation of chimeric tRNAs

Previous in vivo transfection experiments had indicated that the *mainly* cytosol-localized tRNA^Gln could be converted into a mainly mitochondria-localized tRNA by exchanging the D-arm with that from the tRNA^Ile (Lima & Simpson, 1996). As shown in Figure 10, exchange of the D-arm between the tRNA^Ile and tRNA^Gln also produced a reversal of the in vitro importation behavior. The chimeric tRNA^Gln(D-Ile) showed a level of importation in vitro equivalent to that observed using tRNA^Ile (Fig. 10B,C), whereas the chimeric tRNA^Ile(D-Gln) showed little in vitro importation (Fig. 10A,C). This dramatic reversal of the importation competence of these chimeric tRNAs in vitro is consistent with the previous in vivo results.

To determine whether the D-arm from tRNA^Ile has a positive determinant that could allow the in vitro importation of an RNA that is not normally imported, the tRNA^Ile D-arm was fused to the 3′ end of the import-incompetent spliced leader RNA (see Fig. 13). This chimeric RNA was not imported in vitro (data not shown), indicating that the presence of only the tRNA^Ile D-arm is not sufficient for the importation of this RNA.

Specificity of importation of RNA into isolated mitochondria

Synthetic RNA mini-helices corresponding to the D-arm, the anticodon arm, and the TψC-arm from both the tRNA^Ile and tRNA^Gln (Fig. 1) were tested for in vitro importation into isolated *L. tarentolae* mitochondria. As shown in Figure 11, all of the RNA mini-helices from both tRNAs showed significant levels of importation in vitro.

The lack of specificity for importation observed with these mini-helix RNAs suggested a size and structure dependence of the importation specificity of RNAs in general. This was tested using several synthetic RNAs unrelated to tRNAs, differing in size and degree of pre-
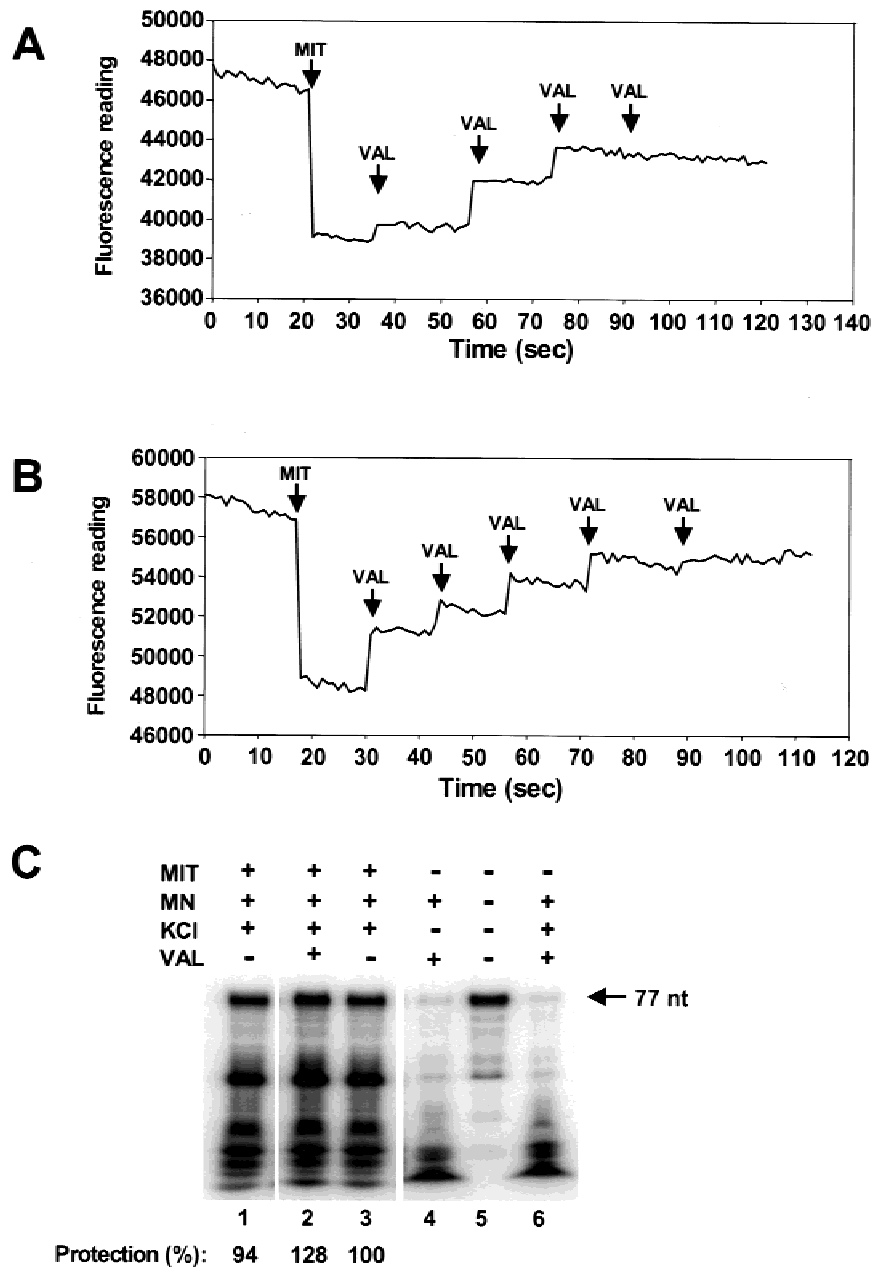
FIGURE 7. Mitochondrial membrane potential is not required for nuclease protection of RNAs. Fluorescence measurements of mitochondria isolated upon cell breakage in hypotonic (**A**) and isotonic (**B**) medium. The membrane potential-sensitive fluorescent dye diS-C_3-(5) was used to measure fluorescence, given in arbitrary units. The addition of mitochondria and detection of membrane potential is depicted by the downward deflection in the fluorescence of the dye. The upward deflection in fluorescence of the dye after addition of 20 μM aliquots of valinomycin (VAL) indicates a discharge of the mitochondrial membrane potential. Valinomycin was added in aliquots until no further increase in fluorescence was observed. The residual fluorescence shift is probably due to light scattering and not membrane potential. **C**: Labeled tRNA^{lle} (2.6 pmol) was incubated with hypotonic mitochondria in the absence (−, lane 1) or presence (+, lane 2) of 100 μM valinomycin, digested with microccocal nuclease (MN), and the amount of nuclease-protected RNA quantitated. Lane 3: same as lane 1, with ethanol at a final concentration of 5% in the reaction as a control for the amount of solvent used to dissolve valinomycin. Lane 4: RNA digested with MN, no mitochondria. Lane 5: 1/10 input RNA, 0.26 pmol. Lane 6: RNA digested with MN in the presence of 100 μM valinomycin and KCl, no mitochondria. 0.07 M KCl was present in the reactions in lanes 1–3. The migration of the full-length input tRNA is indicated by the arrow. The percent of nuclease protection relative to the ethanol control is shown below lanes 1–3.

dicted secondary structure. As shown in Figure 12, two 17-nt RNAs (TAR and GAC) that could fold into hairpin structures were efficiently imported, as was a 170-nt RNA (NF) that could not fold into a predicted secondary structure. However, a 19-nt RNA (ΔND7) and two 24-nt RNAs (6C, 6U), which were also predicted not to have any significant secondary structure, were not imported.

The apparent requirement for secondary structure for RNAs above a certain size appears to be a necessary but not sufficient requirement. For example, the 33-nt 5′ leader RNA, which could form a structure with a free energy of −9.7 kcal/mol, was not imported. In addition, the 107-nt spliced leader RNA (Campbell et al., 1984), and the 169-nt ND7.2x mRNA (Byrne et al.,

1996), both of which have extensive predicted secondary structure, were also not imported in vitro (Fig. 13).

DISCUSSION

We have established an in vitro assay for the importation of in vitro-transcribed tRNAs into isolated *L. tarentolae* mitochondria. The assay involves the protection of imported RNA from digestion by microccocal nuclease. This importation reaction requires external ATP and possibly requires hydrolysis of ATP at the β-γ bond. Internal ATP is apparently required, because the reaction is inhibited by oligomycin. Surprisingly, a membrane potential is not required in this in vitro importation
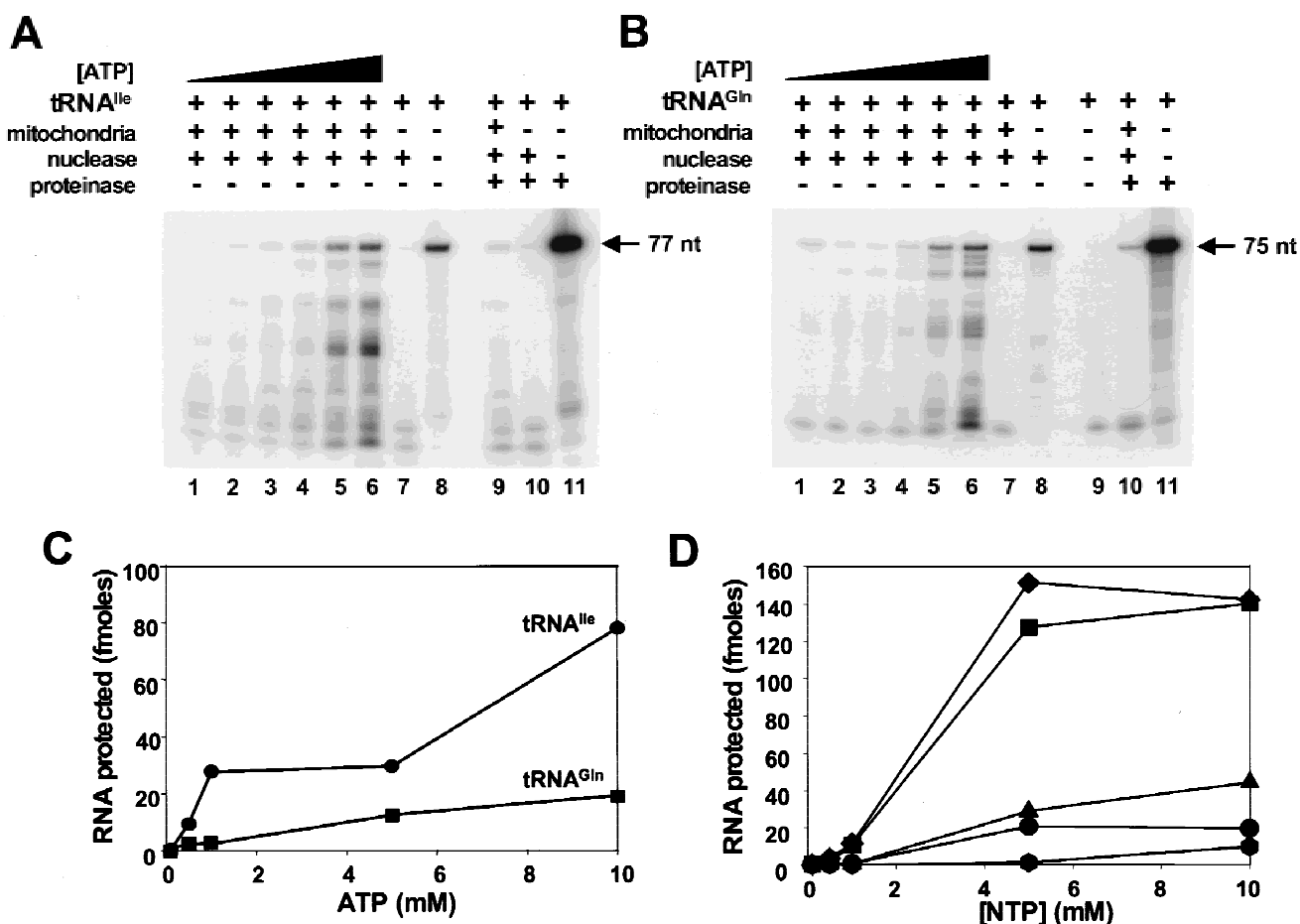
A

[ATP]											
tRNA^Ile	+	+	+	+	+	+	+	+	+	+	+
mitochondria	+	+	+	+	+	+	−	−	+	−	−
nuclease	+	+	+	+	+	+	+	−	+	+	−
proteinase	−	−	−	−	−	−	−	−	+	+	+

← 77 nt

1 2 3 4 5 6 7 8 9 10 11

B

[ATP]											
tRNA^Gln	+	+	+	+	+	+	+	+	+	+	+
mitochondria	+	+	+	+	+	+	+	−	−	+	−
nuclease	+	+	+	+	+	+	+	+	−	+	−
proteinase	−	−	−	−	−	−	−	−	−	+	+

← 75 nt

1 2 3 4 5 6 7 8 9 10 11

C

D

FIGURE 8. Nuclease protection of tRNAs requires ATP. Increasing amounts of uniformly radiolabeled, in vitro-transcribed tRNA were incubated with isolated mitochondria in the presence of increasing concentrations of ATP, and nuclease protection was assayed. **A**: tRNA^Ile (0.6 pmol, 5.0×10^4 cpm) in lanes 1–7 and 9–11. ATP concentrations in lanes 1–11 were 0, 0.1, 0.5, 1, 5, 10, 1, 1, 1, 1, and 1 mM, respectively. Lane 8: 0.06 pmol RNA. Lanes 9–11: 0.6 pmol input RNA. The migration of the full-length input RNA is indicated by the arrow. **B**: tRNA^Gln (0.6 pmol, 5.0×10^4 cpm) used in lanes 1–7 and 9–10. Lanes 1–7: same ATP concentrations as in **A**. Lane 8: 0.02 pmol RNA. Lanes 9 and 10: 0.2 pmol RNA. The migration of the full-length input RNA is indicated by the arrow. **C**: Graph of the RNA protected at each ATP concentration, taken from the gels in (**A**) and (**B**). **D**: Graph of RNA protected versus NTP concentration, for several nonhydrolyzable ATP analogs: ▲: ATP; ◆: ATP-γ-S; ●: AMP-PNP; ■: AMP-PCP; and ◆: AMP-CPP.

system, because there is no apparent effect of valinomycin at a concentration that completely discharges the mitochondrial membrane potential. This is a major difference from the importation of proteins into *Leishmania* mitochondria, which, as in all other protein importation systems studied, has an absolute requirement for a membrane potential (Hauser et al., 1996). Our results also differ from Mukherjee et al. (1999), who reported that importation of tRNAs into isolated *L. tropica* mitochondria occurs in a two-step process, the first of which across the outer membrane does not require a membrane potential, and the second of which across the inner membrane, requires a membrane potential. Yermovsky and Hajduk (1999) also found that in vitro importation of a tRNA into isolated mitochondria from *T. brucei* requires a membrane potential. The difference may be a function either of the trypanosomatid species or the mitochondria isolation procedure.

Unlike the in vitro importation of tRNA into *S. cerevisiae* mitochondria (Tarassov et al., 1995a, 1995b), importation into isolated *L. tarentolae* mitochondria does not require the presence of cytosolic factors. RNA importation into *L. tarentolae* mitochondria in vitro also does not require aminoacylation of the tRNA, as shown previously in the in vivo mitochondrial targeting of a splicing and acylation-defective tRNA in *T. brucei* (Schneider et al., 1994; Sbicego et al., 1998). This does not, of course, rule out the possibility that cytosolic factors play a role in vivo.

The saturation kinetics and the protease sensitivity of uptake for both the tRNA^Ile and the tRNA^Gln suggest the presence of membrane protein receptors. The results show that the uptake of each tRNA could not be completely competed by the unlabeled heterologous tRNA. However, there was complete competition by the homologous tRNA. This indicates that tRNA^Ile and tRNA^Gln
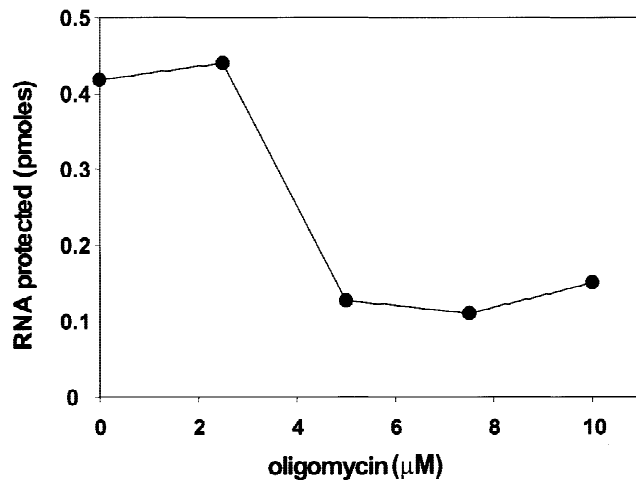
FIGURE 9. Oligomycin inhibits nuclease protection of tRNA^{Ile}. Labeled tRNA^{Ile} (26 pmol, 1.0×10^5 cpm) was incubated with isolated mitochondria, the mitochondria preincubated for 5 min at 27 °C with increasing amounts of oligomycin, and the extent of nuclease-protected RNA assayed by denaturing gel electrophoresis.

are either imported through different receptors or the same receptors with variable affinities.

The maturation of the bacterial precursor tRNA^{Asp} and the titration of the tRNA^{Ile} protection from nuclease digestion upon treatment of mitochondria with increasing digitonin concentrations provide evidence that the nuclease protection of tRNA^{Ile} represents importation into the mitochondrial matrix. The cleavage of the heterologous pre-tRNA^{Asp} was interpreted to indicate the presence of an RNase P-like activity within the mitochondrial matrix, which has been previously reported in extracts of *T. brucei* mitochondria (Hancock et al., 1992). The fact that the cleavage site was localized approximately 2 nt upstream of the *E. coli* RNase P cleavage site is consistent with the observed lack of precision of cleavage of a bacterial precursor tRNA by a mammalian mitochondrial RNase P activity (Rossmanith et al., 1995).

Hancock et al. (1992) suggested that this RNase P-like activity was involved in maturation of the mitochondria-imported 5′-extended precursor tRNAs, which they claimed represented the natural substrate for importation. However, Lima and Simpson (1996) demonstrated that there is no requirement for a 5′-flanking genomic sequence for the in vivo targeting of tRNAs into *L. tarentolae* mitochondria. In addition, Aphasizhev et al. (1998) could obtain no evidence for the existence of 5′-extended tRNAs in *L. tarentolae* or *T. brucei* mitochondrial fractions by Northern blot or primer extension analysis. We speculate that the in vivo role of the mitochondrial RNase P-like activity in *T. brucei* and *L. tarentolae* may be in mitochondrial mRNA processing (Topper et al., 1992; Morrissey & Tollervey, 1995; Reddy & Shimba, 1995).

In other eukaryotic systems, maturation of tRNAs, which includes processing of the 5′ and 3′ ends, post-transcriptional addition of the 3′ CCA end, and most nucleoside modifications, occurs in the nucleus prior to export. It was shown recently (Arts et al., 1998; Lipowsky et al., 1999) that exportin-t, the tRNA nuclear transport receptor in *Xenopus* (Kutay et al., 1998), has a preference for mature tRNA. S. Kapushoc, M.A.T. Rubio, J.D. Alfonzo, and L. Simpson (unpubl. results) have recently analyzed two *L. tarentolae* tRNAs, one that is shared between the cytosol and the mitochondrion and one that is localized mainly in the mitochondrion, of which 5′- and 3′-extended precursors can only be detected in the nucleus. This suggests that the substrates for importation into the mitochondrion, in these cases at least, are the mature tRNAs. It is of course possible that there is an alternative pathway for nuclear export and mitochondrial importation in *Leishmania* that involves precursor tRNAs, but the existence of such a pathway has not yet been established by direct evidence. There is a recent report (Yermovsky-Kammerer & Hajduk, 1999) that a dicistronic tRNA transcript in *T. brucei* (LeBlanc et al., 1999) is preferentially imported into isolated mitochondria, but in vivo evidence for this pathway is lacking.

The efficient importation of the chimeric tRNA^{Gln} with the D-arm from tRNA^{Ile} into isolated mitochondria is consistent with previous in vivo importation results (Lima & Simpson, 1996). This observation not only argues for the specificity of this process, but also raises the possibility that the known functional interaction between the D-arm and the TψC-arm in the tRNA may provide one type of discrimination for importation. In this regard, Nabholz et al. (1997) demonstrated that the chimeric tRNA^{Gln}(D-Ile) is sufficiently folded for aminoacylation to occur by the cytosolic glutaminyl aminoacyl tRNA synthetase.

The possibility that there is a positive determinant on the isoleucyl tRNA D-arm that by itself confers importation was eliminated by the lack of importation of the chimeric RNA in which the D-arm from tRNA^{Ile}(UAU) was fused to the importation-incompetent spliced leader RNA. This context-dependent behavior of the D-arm from the tRNA^{Ile} again suggests that RNA structure is a primary signal for importation.

The finding that five different 16–17-nt mini-helix RNAs, some of a non-tRNA origin, and one 17-nt unstructured RNA were efficiently imported in vitro, and that one essentially unstructured 19-nt and several 24-nt and 33-nt RNAs were not imported, suggests a non-specific importation of any RNA less than approximately 17 nt in size and a requirement for a folded structure for RNAs greater than this size. Additional evidence relevant to this problem includes preliminary in vitro selection experiments showing efficient importation of a pool of 120-nt random sequence RNA molecules into isolated *L. tarentolae* mitochondria. This
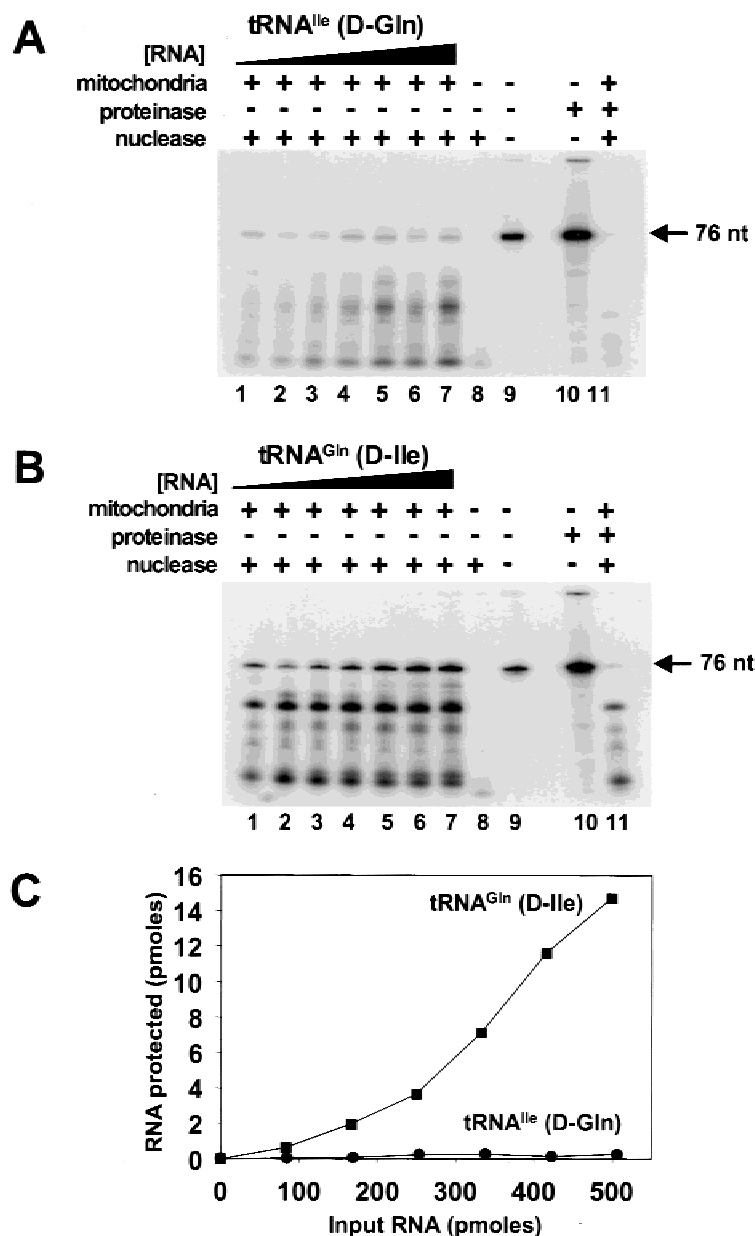
FIGURE 10. Importation of chimeric tRNAs with swapped D-arms into isolated mitochondria. Uniformly labeled tRNAs were incubated with isolated mitochondria under importation conditions and the extent of nuclease-protected RNA resolved by denaturing gel electrophoresis. **A**: Input tRNA^{Ile}(D-Gln) lanes 1–7: 0.19 pmol (5.0×10^4 cpm), 84 pmol (1.0×10^5 cpm), 169 pmol (1.5×10^5 cpm), 253 pmol (2.0×10^5 cpm), 337 pmol (2.5×10^5 cpm), 422 pmol (3.0×10^5 cpm), 506 pmol (3.5×10^5 cpm) input RNA. Lane 8: 0.19 pmol (5.0×10^4 cpm) input RNA incubated with MN. Lane 9: 0.019 pmol (5.0×10^3 cpm) input RNA. Lane 10: 0.19 pmol (5.0×10^4 cpm) input RNA incubated with RNase-free proteinase K, as a control. Lane 11: 0.19 pmol (5.0×10^4 cpm) input RNA incubated with proteinase K-treated mitochondria followed by digestion with MN. The migration of the full-length input RNA is indicated by the arrow. **B**: Input tRNA^{Gln}(D-Ile) lanes 1–7: 0.17 pmol (5.0×10^4 cpm), 83 pmol (1.0×10^5 cpm), 166 pmol (1.5×10^5 cpm), 249 pmol (2.0×10^5 cpm), 332 pmol (2.5×10^5 cpm), 416 pmol (3.0×10^5 cpm), 499 pmol (3.5×10^5 cpm) input RNA. Lane 8: 0.17 pmol (5.0×10^4 cpm) input RNA incubated with MN. Lane 9: 0.017 pmol (5.0×10^3 cpm) input RNA. Lane 10: 0.17 pmol (5.0×10^4 cpm) input RNA incubated with RNase-free proteinase K, as a control. Lane 11: 0.17 pmol (5.0×10^4 cpm) input RNA with proteinase K-treated mitochondria followed by digestion with MN. The migration of the full-length input RNA is indicated by the arrow. **C**: Graph of input RNA protected from nuclease digestion versus input RNA for tRNA^{Ile}(D-Gln) (circles) and tRNA^{Gln}(D-Ile) (squares).

selection yielded multiple aptamers that exhibit extensive predicted secondary structures and which were imported in vitro at levels comparable to tRNA^{Ile} (M.A.T. Rubio and L. Simpson, unpubl. results).

The structural requirements for in vitro importation of RNAs into isolated mitochondria remain to be determined. It is clear that specificity exists, as different tRNAs are imported in vitro to different extents, and because some RNAs (not related to tRNA) are imported efficiently whereas others are not. Many other questions also remain to be addressed, such as the nature of the membrane receptors, the structural state of the RNA during the importation process, and the role of ATP hydrolysis both external and internal. The availability of an in vitro importation system should facilitate the bio-

chemical dissection of the mitochondrial machinery involved in RNA transport.

MATERIALS AND METHODS

Strains and culture conditions

L. tarentolae (UC strain) cells were grown in brain heart infusion (Difco) supplemented with 1.6 μM hemin (Sigma). For small-scale mitochondria preparations (from 1–2 L of culture), cells were grown with rotation in a Cell Production Roller Apparatus (Bellco) at 27 °C. For large-scale mitochondria preparations (15 L), cells were grown in a BioFlo IV fermentor (New Brunswick). The cells were harvested with a Masterflex tangential filter apparatus (Millipore). For purification of mito-
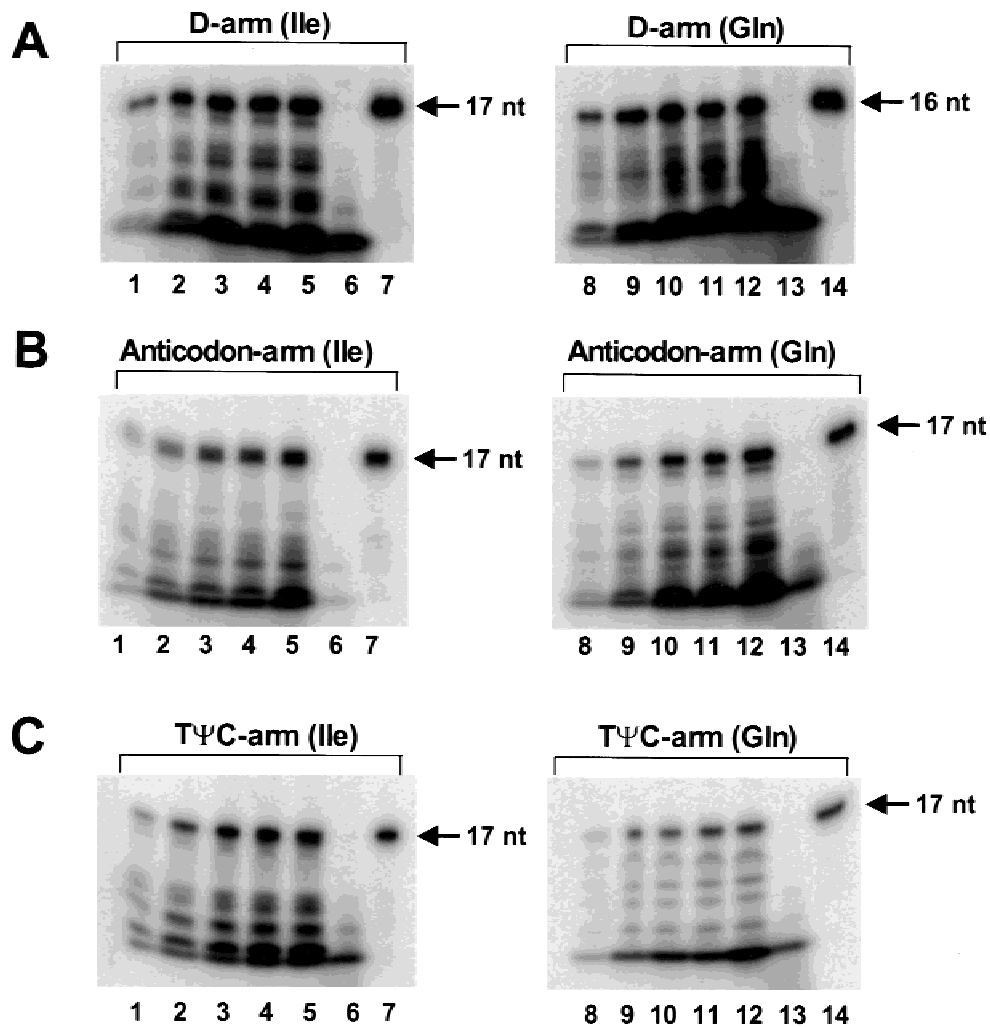
A D-arm (Ile)   D-arm (Gln)

← 17 nt

1 2 3 4 5 6 7

← 16 nt

8 9 10 11 12 13 14

B Anticodon-arm (Ile)   Anticodon-arm (Gln)

← 17 nt

1 2 3 4 5 6 7

← 17 nt

8 9 10 11 12 13 14

C TΨC-arm (Ile)   TΨC-arm (Gln)

← 17 nt

1 2 3 4 5 6 7

← 17 nt

8 9 10 11 12 13 14

FIGURE 11. Importation of synthetic mini-helix RNAs derived from tRNA[Ile] and tRNA[Gln] into isolated mitochondria. (**A**), (**B**) and (**C**) represent importation experiments with D-arm, anticodon-arm, and TΨC minihelices, respectively. The RNAs are shown in boxes in Figure 1. Increasing amounts of $[\alpha\text{-}^{32}P]pCp$-3′-end-labeled RNAs were incubated with isolated mitochondria and the extent of nuclease protection assayed by gel electrophoresis. Lanes 1–5 and 8–12: 10, 50, 100, 150, and 200 pmol RNA. Lane 6 and 13: 50 pmol digested with MN. Lane 7 and 14: 5 pmol input RNA. The full-length input RNA is indicated in each case by an arrow. The calculated amounts of RNA protected per milligram mitochondrial protein at 200 pmol input RNA are as follows: **A**: lane 5: 94 pmol/mg; lane 12: 98 pmol/mg. **B**: lane 5: 147 pmol/mg; lane 12: 177 pmol/mg. **C**: lane 5: 213 pmol/mg; lane 12: 113 pmol/mg.

chondria using the hypotonic cell breakage procedure, cells $(1.5\text{--}1.8 \times 10^8$ cells/mL) were washed in SET buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0, 10 mM Tris-HCl, pH 8) and harvested by centrifugation at $5,000 \times g$ for 10 min at 4 °C. Cells were suspended in DTE buffer (1 mM Tris-HCl, pH 7.9, 1 mM EDTA, pH 8.0) and broken by passage through a 27 gauge needle at 0.689 MPa pressure (Braly et al., 1974). To obtain mitochondria not exposed to hypotonic conditions, cells were broken in 0.6 M sorbitol, 20 mM HEPES (pH 7.5), and 2 mM EDTA by passage through a Stansted Cell Disrupter at 8.274 MPa. The mitochondria were purified by flotation in 20–35% Percoll density gradients (Harris et al., 1990; Simpson et al., 1993, 1996) and were collected and washed with SucTE buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.9, 2 mM EDTA, pH 8.0). The final mitochondrial pellet was suspended in mitochondrial storage buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 50% glycerol) at a con-

centration of 100 mg (wet weight)/mL and stored in aliquots at −80 °C. Protein concentrations were measured using the Micro-BCA Assay (Pierce). The mitochondrial purity was assessed microscopically after staining with 4′, 6-diamidino-2-phenylindole (DAPI), and by measuring the specific activity of succinate dehydrogenase.

Measurement of membrane potential

Mitochondrial membrane potentials were measured at 25 °C using a membrane potential-sensitive dye 3, 3′-dipropylthiadicarbocyanine iodide, diS-C_3-(5) (Molecular Probes, Inc.) (Hauser et al., 1996). An SLM-Aminco 8000 spectrofluorometer was set at 620 nm for excitation and 670 nm for emission to detect fluorescence of the dye. Initially, fluorescence measurement of diS-C_3-(5) was taken (Hauser et al., 1996), added at a final concentration of 5 μM
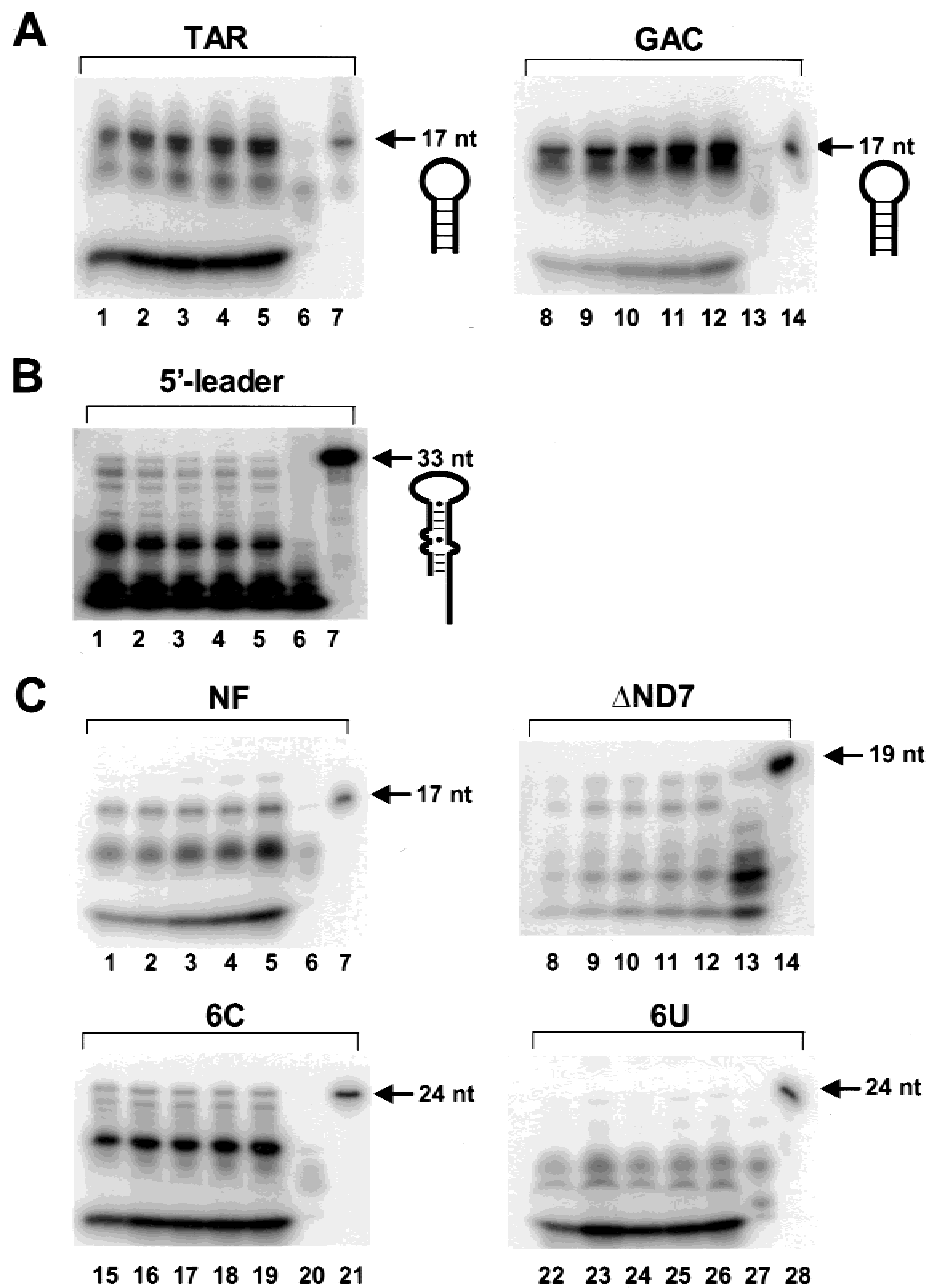
FIGURE 12. Importation of small synthetic RNAs into isolated mitochondria. The predicted foldings of the **A:** TAR and GAC and **B:** 5'leader RNAs are indicated graphically. **C:** The NF, ΔND7, 6C and 6U RNAs have no significant predicted secondary structure (see Materials and Methods). Increasing amounts of each RNA (**A:** Lanes 1–7 and lanes 8–14: 40, 80, 120, 160, 200, 40 and 4 pmol [50, 100, 150, 200, 250, 50 and 5×10^3 cpm], respectively; **B:** Lanes 1–7: 10, 110, 210, 310, 410, 10 and 1 pmol [100, 200, 300, 400, 500, 100 and 10×10^3 cpm], respectively; **C:** Lanes 1–7, 15–21 and 22–28: 40, 80, 120, 160, 200, 40 and 4 pmol [50, 100, 150, 200, 250, 50 and 5×10^3 cpm], respectively; Lanes 8–14: 10, 50, 100, 150, 200, 100 and 5 pmol [30, 150, 300, 450, 600, 300 and 15×10^3 cpm], respectively) were incubated with isolated mitochondria and the extent of nuclease protection assayed by gel electrophoresis. Lanes 6, 13, 20 and 27 represent RNAs digested with nuclease in the absence of mitochondria which were used as controls for digestion. Lanes 7, 14, 21 and 28 are the 1/10 input controls, where the arrows denote the migration of the full-length RNAs and sizes are given in nucleotides (nt). The calculated amounts of RNA protected per mg mitochondrial protein at 200 pmol input RNA for all RNAs, except 410 pmol for the 5'-leader RNA, are as follows: **A:** lane 5, 650 pmol/mg; lane 12, 730 pmol/mg. **B:** lane 5, 8 pmol/mg. **C:** lane 5, 130 pmol/mg; lane 12, 2.5 pmol/mg; lane 19, 19 pmol/mg; lane 26, 1 pmol/mg.

in buffer (20 mM HEPES-KOH, pH 7.4, 0.6 M sorbitol, 25 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM KH_2PO_4, 1 mg/mL fatty acid-free bovine serum albumin, 5 mM NADH, 5 mM succinate, and 1 mM ATP) at 25 °C. Addition of mitochon-

dria was used to initiate the measurement of membrane potential. Presence of a membrane potential is detected by a decrease of fluorescence, measured in arbitrary units, depicted as a downward deflection in the graph. Some of
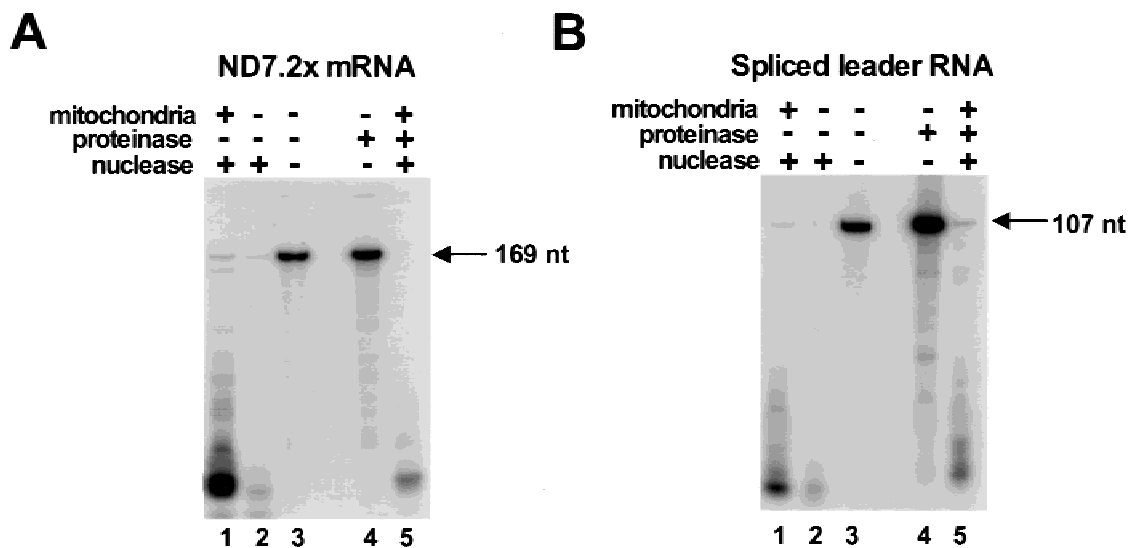
A

ND7.2x mRNA

mitochondria	+	−	−		−	+
proteinase	−	−	−		+	+
nuclease	+	+	−		−	+

← 169 nt

1 2 3 4 5

B

Spliced leader RNA

mitochondria	+	−	−		−	+
proteinase	−	−	−		+	+
nuclease	+	+	−		−	+

← 107 nt

1 2 3 4 5

FIGURE 13. The 169 nt ND7.2x mRNA and the 107 nt spliced leader RNA fragment are not imported in vitro. **A:** Uniformly labeled in vitro transcribed ND7.2x mRNA (5.0×10^4 cpm) were incubated with isolated mitochondria, and the extent of nuclease protection assayed by gel electrophoresis. Lanes 1, 2, and 5: 175 pmol RNA. Lane 3: 0.02 pmol RNA. Lane 4, 0.17 pmol RNA. The full-length input RNA is indicated by the arrow. **B:** Uniformly labeled in vitro transcribed spliced leader RNA (5.0×10^4 cpm) were incubated with isolated mitochondria. Lanes 1, 2 and 5: 192 pmol RNA. Lane 3, 0.01 pmol RNA. Lane 4, 0.1 pmol RNA. The full-length input RNA is indicated by the arrow.

this decrease in fluorescence is due to light scattering. Aliquots of 20 μM valinomycin, a potassium ionophore, were added at 10–20-s consecutive time intervals until there was no further fluorescence change, indicating a complete discharge of the membrane potential.

In vitro transcription and labeling of synthetic RNAs

The various RNAs used in the assays include *L. tarentolae* tRNA^{Ile}(UAU) and tRNA^{Gln}(CUG) with and without the 3′-CCA sequence. The isoleucyl and glutaminyl tRNAs with the swapped D-arms (shown in Fig. 1 in the boxed region) are tRNA^{Ile}(D-Gln) and tRNA^{Gln}(D-Ile). The six mini-helix RNAs indicated by boxes in Figure 1 were chemically synthesized (Oligos Etc.). Other RNAs also used in the importation assays include the 107-nt spliced leader RNA (Sturm et al., 1999), the 169-nt ND7.2x RNA, which is a portion of the NADH subunit 7 mRNA with a mutation in the anchor region (Byrne et al., 1996), *B. subtilis* pre-tRNA^{Asp} (Waugh et al., 1989), and several synthetic RNAs (Xeragon Oligoribonucleotides): TAR RNA (Ippolito & Steitz, 1998), 5′-AGAGCA CUUGGAGCUCU-3′; GAC RNA, 5′-GGGGGAAAAAAA CCCCC-3′; NF RNA, 5′-CUCUCCCUCCUUACCAC-3′; 5′ leader RNA, 5′-GGGAGACCGGAAUUCGAGCUCGGUA CCCAAAAU-3′; ΔND7 RNA (Blanc et al., 1999), 5′-GAGCAGUGUUUACCGAUGA-3′; 6C RNA, 5′-GCUAUG UCUGCUAACUUGCCCCCC-3′; 6U RNA, 5′-GCUAUGU CUGCUAACUUGUUUUUU-3′. RNA folding was performed using MFOLD 3.0 on the M. Zuker website at http://mfold2.wustl.edu/~mfold/rna/form1.cgi. The free energies of the most stable RNA folds in kilocalories per mole are as follows: 5′ leader, −9.4; TAR, −5.1; GAC, −8.4; 6C, −0.3; 6U, −0.7; ΔND7, −1.5; NF, no folding. Plasmids were linear-

ized by restriction digestion and used as templates in T7 in vitro run-off transcription reactions containing T7 RNA polymerase, ribonucleotides and the appropriate buffer (Milligan et al., 1987; Cunningham & Ofengand, 1990). Uniformly labeled RNAs were transcribed in vitro in the presence of [α-^{32}P]UTP (NEN) in the reaction. The 5′-end labeled RNAs were incubated with [γ-^{32}P]ATP (NEN) and T4 polynucleotide kinase for 1 h at 37 °C in T4 polynucleotide kinase buffer (GIBCO-BRL). The synthetic RNAs were 3′-end labeled by ligation of [α-^{32}P]pCp (NEN) using T4 RNA ligase in the buffer provided (GIBCO-BRL). All RNAs were gel purified by electrophoresis through a denaturing 7 M urea/10% acrylamide gel. The RNAs were visualized by either exposure of the gel to X-ray film or by UV shadowing using a 254 nm UV lamp and a K6F Silica Gel 60 Å thin layer chromatography plate (Whatman). The RNAs were eluted in 200−400 μL 300 mM sodium acetate, 0.1 mM EDTA at room temperature overnight. RNAs were precipitated with 3 vol of ethanol at −20 °C, followed by centrifugation at 12,000 × *g* for 30 min. The pellets were suspended in either TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) or water. The concentration of RNA in solution was measured in a GeneQuant spectrophotometer (Pharmacia).

Assay of in vitro importation of RNA into isolated mitochondria

The in vitro RNA importation assay was performed in a 20 μL reaction volume containing 50,000 cpm radioactively labeled RNA, 1 mg (wet weight) mitochondria (approximately 40 μg mitochondrial protein), SucT buffer, 1 mM ATP, 2 mM DTT, 10 mM MgCl$_2$, 0.63 mM creatine phosphate, and 22.5 mg/mL creatine phosphokinase (ATP regeneration system) (Ghosh et al., 1994; Mahapatra et al., 1994; Lima & Simpson, 1996;

Mahapatra & Adhya, 1996). After incubation at 27 °C for 5–30 min, 100 units of microccocal nuclease (MN) (Boehringer Mannheim) and 5 mM CaCl$_2$ were added to digest the RNAs that were not imported into the mitochondria. Microccocal nuclease (MN) was then inhibited by the addition of 10 mM EGTA (pH 8). To isolate protected RNAs, the mitochondrial pellets were washed with SucTE buffer, suspended in 150 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% SDS and extracted with 150 μL water-saturated phenol (pH 4.5). The RNAs were precipitated with 2.5 vol of ethanol, 300 mM sodium acetate (pH 5.2), and 10 μg glycogen. The radioactively labeled RNAs were separated by electrophoresis through a 7 M urea/8% acrylamide gel. After electrophoresis, the gels were dried onto Whatman 3MM chromatography paper and exposed to a PhosphorImager screen (Molecular Dynamics). The nuclease-protected, radioactively labeled RNAs resolved in the denaturing polyacrylamide gels were visualized and quantitated using the ImageQuant program (Molecular Dynamics).

Measurement of mitochondrial importation kinetics

To determine the time course of importation, 150 μL importation reactions were incubated at 27 °C and 4 °C, using either uniformly [α-^{32}P]UTP-labeled tRNA^{Ile} or tRNA^{Gln}. Aliquots of 20 μL were removed at 0, 5, 10, 15, 30, and 60 min and nuclease treated as described above. The protected RNA was quantitated and the data plotted as picomoles of RNA protected versus time of incubation. The radioactively labeled RNAs were quantitated as PhosphorImager units (PI units). The percentage of protected RNA was calculated by dividing the amount of PI units of the full-length RNA by the total PI units of the input RNA and multiplying by 100. The percentage of protected RNA was used to calculate the amount of RNA protected, in picomoles, because the amount of RNA input is known. Control reactions included input RNA, usually 10% of what was used in the import reactions, in the absence of mitochondria and without microccocal nuclease treatment, and 100% of input RNA with microccocal nuclease treatment in the absence of mitochondria. Additional control reactions included input RNA with mitochondria predigested with RNase A-free proteinase K (Ambion) for 5 min at 27 °C, and input RNA incubated with RNase A-free proteinase K in the absence of mitochondria and without microccocal nuclease treatment.

Reverse transcription and DNA sequencing

Reverse transcription reactions were carried out using oligonucleotide 3381R, 5′-GTGACAGGCAGGCATTCTAACCAAC-3′, complementary to the anticodon and D-stems and loops of the pre-tRNA^{Asp} (nt 39–15). The reverse transcription control reactions included 5 μg of total mitochondrial RNA either without or with SuperScript II reverse transcriptase (Gibco-BRL). *B. subtilis* pre-tRNA^{Asp} (40 pmol) was incubated with *E. coli* RNase P RNA (120 pmol), as described below, phenol extracted, then precipitated with 0.3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol prior to the reverse transcription reaction. In parallel, *B. subtilis* pre-tRNA^{Asp} (40 pmol) was incubated with *L. tarentolae* mitochondria under importation conditions as described above, prior to the

reverse transcription reaction. The reverse transcription reaction was carried out as described in the SuperScript II reverse transcriptase first strand synthesis protocol (Gibco-BRL).

Plasmid DNA containing the *B. subtilis* pre-tRNA^{Asp} was sequenced with oligonucleotide 3381R and Sequenase 2.1 following the manufacturer's instructions (USB).

Digitonin treatment of mitochondria

The endogenous mitochondrial tRNAs were 3′-end labeled with [α-^{32}P]-CTP within intact mitochondria as a mitochondrial matrix marker (Hancock et al., 1992). Various concentrations (0, 0.1, 0.2, 0.4, and 0.8%) digitonin were then added to the mitochondria and the amount of RNA protected assayed by microccocal nuclease digestion and analyzed after denaturing polyacrylamide gel electrophoresis. An identical procedure was utilized previously for an investigation of the in vivo mitochondrial and cytosolic targeting of specific tRNAs in *L. tarentolae* (Lima & Simpson, 1996). In parallel, a uniformly labeled, in vitro-transcribed tRNA^{Ile} was imported into mitochondria. After the importation reaction, the fraction was divided into five separate tubes containing various concentrations of digitonin. After incubation with digitonin, microccocal nuclease was added to digest the released and nonimported tRNAs, and the protected RNA assayed by denaturing polyacrylamide gel electrophoresis.

Importation of heterologous RNAs

Uniformly [α-^{32}P]-labeled, in vitro-transcribed NADH dehydrogenase subunit VII (ND7.2x) RNA is a 169-nt portion of the full-length pre-edited maxicircle transcript with a mutated gRNA anchor sequence. The 107-nt spliced leader RNA was also synthesized by T7 in vitro transcription. 50,000 cpm of uniformly [α-^{32}P]UTP-labeled spliced leader RNA was subjected to importation reactions, as described above. In addition, 50,000 cpm of uniformly [α-^{32}P]UTP-labeled ND7.2x RNA was subjected to importation reactions as described above. In another experiment, 5.2 pmol of unlabeled *B. subtilis* pre-tRNA^{Asp}, together with 50,000 cpm uniformly [α-^{32}P]UTP-labeled pre-tRNA^{Asp}, were incubated under importation conditions as described above.

RNase P RNA cleavage assay

E. coli RNase P RNA (5 nM) was incubated with 1 nM *B. subtilis* pre-tRNA^{Asp} in 1 M NH$_4$OAc, 25 mM MgCl$_2$, and 50 mM HEPES, pH 8.0, at 37 °C for 30 min (Waugh et al., 1989). The products were resolved by denaturing acrylamide gel electrophoresis and quantitated as described above.

ACKNOWLEDGMENTS

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