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Stepwise Transfer of tRNA through the Double Membrane of *Leishmania* Mitochondria*

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Import of tRNA into *Leishmania* mitochondria involves transfer through a double membrane barrier. To examine whether specific sorting mechanisms for individual tRNAs direct them to different mitochondrial compartments, the distribution of tRNA transcripts, internalized *in vitro*, was examined by suborganellar fractionation. Significant amounts of $tRNA^{Tyr}$ were localized in the matrix and on the outer face of the inner mitochondrial membrane. With time, the matrix:membrane ratio increased. Translocation through the inner membrane apparently required the presence of a specific signal in the D arm of $tRNA^{Tyr}$, and $tRNA^{Gln}$(CUG), lacking this sequence, was excluded. Hydrolysis of ATP was necessary at both the outer and inner membranes. However, the protonophores carbonylcyanide *m*-chlorophenylhydrazone and nigericin, the K^+ ionophore valinomycin, and the F_1F_0 ATPase inhibitor oligomycin had only marginal effects on uptake through the outer membrane but severely inhibited inner membrane translocation, indicating the unusual requirement of both the electrical and chemical components of the electromotive force generated across the inner membrane. The results are consistent with a mechanism involving stepwise transfer of tRNA through distinct outer and inner membrane channels.

The mitochondrial genomes of many protozoal species, including leishmania, trypanosomes, and plasmodium, are unusual for their apparently total lack of tRNA genes (1–3). To sustain the translation of organellar mRNAs, a large number of nuclear-encoded tRNAs are imported from the cytoplasm (4–10). Mitochondrial import of one or more tRNAs has also been documented in tetrahymena (11), yeast (12), and several species of higher plants (13, 14).

To understand the mechanism of import, we have developed an *in organello* system from leishmania (15). It was shown that import is ATP-dependent and specific for RNA sequence (15, 16). A purine-rich oligonucleotide motif in the D arm of tRNA was identified as an import signal *in vivo* (17) as well as *in vitro* (18). A 15-kDa protein associated with the outer mitochondrial membrane acts as carrier or receptor for direct import of tRNA, without the mediation of soluble factors (19).

Analysis of intact mitochondria or mitoplasts for tRNA internalized *in vivo* or *in vitro* does not address the question of the distribution of the RNA in the various intramitochondrial compartments, *viz.* the outer membrane, intermembrane space, inner membrane, or matrix. We have previously reported UTP labeling of *in vitro* imported small RNAs (15), presumably by the matrix-localized terminal uridylyl transferase (20). A fraction of $tRNA^{Ile}$ associated with the mitochondrial fraction *in vivo* was found to be resistant to micrococcal nuclease in the presence of digitonin, which selectively solubilizes the outer mitochondrial membrane, suggesting the matrix location of this tRNA (17). It is not known whether different tRNAs are sorted to different locations once inside the mitochondrion, in a manner analogous to the sorting of imported proteins, and if so, whether separate matrix localization and sorting signals exist within the tRNA structure. The study of intramitochondrial distribution is important as it may enable a distinction to be made between 1) a *concerted* mechanism of import through a single transport channel spanning both the outer and inner membranes, and 2) a *stepwise* mechanism involving separate outer and inner membrane translocation channels.

In this study, mitochondria were fractionated into various compartments for the study of the distribution of imported RNA, specifically, the sequence specificity and energy requirements of translocation through the inner membrane. The results support the notion of stepwise insertion of RNA through the outer and inner mitochondrial membranes.

MATERIALS AND METHODS

Cell Culture and Preparation of Mitochondria—Promastigotes of *Leishmania tropica* strain UR6 were cultured on solid blood agar medium (21) supplemented with 150 μg/ml biopterin and 50 μg/ml adenine. Mitochondria were purified from DNase I-treated lysates by Percoll gradient centrifugation and stored in 50% glycerol storage buffer, as described (15). Before use, mitochondria were diluted with a 10-fold excess of ice-cold isotonic sucrose-Tris-EDTA (STE buffer)[1] (15), washed by centrifugation, and resuspended in STE at a final protein concentration of 8–10 mg/ml.

Import Substrates—^{32}P-labeled $tRNA^{Tyr}$(GUA) transcript was prepared by runoff transcription of a genomic copy of the corresponding gene in plasmid pSKB-1 (19), using T7 RNA polymerase and [α-^{32}P]UTP, as described (22). $tRNA^{Tyr}$(1–39) was obtained similarly from plasmid pSKB-1(Δ-1) (19) and $tRNA^{Gln}$(CUG) from plasmid pSKB-2 (19). δ RNA, a runoff transcript of plasmid pSG3δ (18), contains the antisense sequence of the −12 to +25 region of the leishmania β-tubulin gene.

Enzymes and Inhibitors—Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), oligomycin, valinomycin, and nigericin (all from Sigma) were dissolved in ethanol and diluted 100-fold into import reactions. The sodium salt of carboxyatractyloside (Sigma) was dissolved in water

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Dedicated to the memory of Dr. Jyotirmoy Das, the late Director of the Indian Institute of Chemical Biology.

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[1] The abbreviations used are: STE, sucrose-Tris-EDTA; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PAGE, polyacrylamide gel electrophoresis; AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate.

and similarly diluted. Mitochondria (80–100 μg of protein) were preincubated with inhibitor (plus 0.1 M KCl in the case of valinomycin) for 15 min on ice before dilution with an equal volume of import buffer, as indicated. Rabbit muscle myokinase (Roche Molecular Biochemicals), an ammonium sulfate suspension, was recovered by microcentrifugation and suspended in 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 50% glycerol before use.

Import Incubation—Unless otherwise stated, washed mitochondria (80–100 μg of protein) were incubated at 37 °C for 15 min with 100 fmol of ^{32}P-labeled import substrate in 10 mM Tris-HCl, pH 7.5, 10 mM $MgAc_2$, 2 mM dithiothreitol, and 4 mM ATP in a total volume of 20 μl. Then RNase A (2.5 μg/ml) and RNase T1 (50 units/ml) were added and incubation continued for an additional 15 min at the same temperature. Mitochondria were washed in cold STE and recovered by centrifugation.

Submitochondrial Fractionation—RNase-treated mitochondria containing internalized radiolabeled RNA were incubated with 320 μM digitonin (Roche Molecular Biochemicals) in 10 μl of STE for 15 min on ice (23). Alternatively, mitochondria were subjected to hypotonic shock in 20 μl of 1 mM Tris-HCl, pH 8.0, 1 mM EDTA (15) for 15 min on ice, followed by addition of 2.5 μl of 2 M sucrose. Mitoplasts were separated from the intermembrane space fraction by microcentrifugation at 3450 × *g* for 3 min at 4 °C. The mitoplasts were suspended in 10 μl of freeze-thaw buffer (0.6 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA; Ref. 24) and subjected to three freeze-thaw cycles, each consisting of freezing at −70 °C followed by rapid thawing at 37 °C. The inner membrane and matrix fractions were separated by centrifugation at 7000 × *g* for 5 min at 4 °C. RNA was recovered from each fraction by guanidinium isothiocyanate extraction and isopropanol precipitation (18). To quantify the amount of RNA, an aliquot was spotted on DEAE anion exchange paper (DE 81, Whatman) and counted. Alternatively, the RNA was resolved by urea-PAGE (15), followed by counting of the dried gel band.

Enzyme Assays—Kynurenine hydroxylase and succinate dehydrogenase were spectrophotometrically assayed as described previously (16, 19). To assay malate dehydrogenase, reactions (1 ml) containing 96 mM potassium phosphate, pH 7.4, 150 μM NAD^+, and mitochondrial extract were initiated by the addition of 260 μM sodium malate and the reduction of NAD^+ followed by the increase in absorbance at 340 nm (ϵ = 6.22 mM^{-1} cm^{-1}).

Measurement of Membrane Potential—The uptake of rhodamine 123 was used as a measure of the mitochondrial membrane potential (25, 26). Reactions (1 ml) containing 10 mM Tris-HCl, pH 8.0, 10 mM $MgAc_2$, 1 mM dithiothreitol, 220 mM sucrose, 1 mM EDTA, and 2.63 μM rhodamine 123 (Sigma) were placed in a cuvette, and the absorbances at 516 and 495 nm were measured. Mitoplasts (derived from 200 μg of mitochondria), 4 mM ATP, and 50 μM CCCP were sequentially added, and the absorbances at the above wavelengths continuously recorded over time.

RESULTS

Optimization of the Ribonuclease Protection Assay—Previous import assays (15, 16, 18, 19) had employed relatively high concentrations of RNase and a suboptimal incubation temperature (25 °C) to digest excess (unimported) RNA. Under these conditions we sometimes observed partial degradation of imported RNA, possibly by residual RNase during the postimport washing and mitochondrial lysis steps.[2] In order to make the assay more reliable in terms of RNA intactness and yield, the RNase concentration was lowered, whereas the incubation temperature was raised to 37 °C to increase the rate of degradation. A RNase titration experiment (Fig. 1*A*) showed that at 37 °C, protection of intact RNA was observed using only 2.5% of the original enzyme concentration. At lower concentrations, there was evidence of incomplete endonucleolytic cleavage leading to smearing in the lane, whereas at higher concentrations, the amount of protected RNA was reduced and finally eliminated. These results emphasize the importance of controlling the RNase step for successful observation of import.

The amount of protected RNA increased in a time-dependent manner up to 20 min of incubation of the mitochondria with radiolabeled $tRNA^{Tyr}$ and was completely sensitive to RNase if the mitochondria were lysed with Triton X-100 (Fig. 1*B*), as expected for uptake into the membrane-bound organelle.

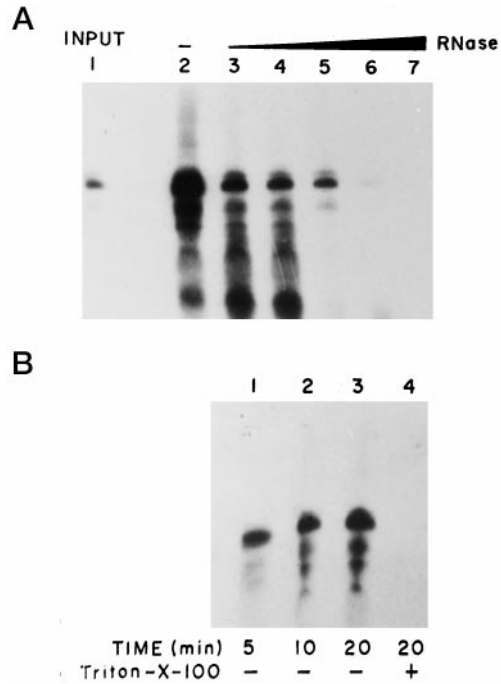

FIG. 1. **Effect of ribonuclease on the quality and yield of imported RNA.** *A,* mitochondria (100 μg of protein) were incubated with ^{32}P-labeled $tRNA^{Tyr}$ (100 fmol) and 4 mM ATP for 15 min at 37 °C, and then 1 μl of the following dilutions of a mixture of RNase A (100 μg/ml) and RNase T1 (2000 units/ml) were added and incubation continued for a further 15 min at 37 °C: 1:10, 1:20, 1:40, 1:80, and 1:160 (*lanes 7–3,* respectively). *Lane 2,* no RNase control; *lane 1,* input RNA (2 fmol). *B,* time course of RNase protection. Mitochondria were incubated with radiolabeled $tRNA^{Tyr}$ and ATP for 5 (*lane 1*), 10 (*lane 2*), and 20 min (*lanes 3* and *4*) at 37 °C. Then, Triton X-100 (0.5%) was added to reaction shown in *lane 4*, and 1 μl of the 1:40 dilution of RNase was added to all reactions; these were incubated for 15 min at 37 °C. Mitochondria were lysed, and the total internalized RNA was analyzed by urea-PAGE.

Separation of Mitochondrial Compartments—In order to assess the intramitochodrial distribution of tRNAs imported *in vitro*, a simple scheme for separating the various intramitochondrial compartments was developed (Fig. 2*A*). Mitochondria were treated with digitonin, a detergent that selectively solubilizes the outer membrane (23). Under the conditions employed, the outer membrane marker kynurenine hydroxylase (19), but not the inner membrane marker succinate dehydrogenase (19), was solubilized (Table I), attesting to the selectivity of the digitonin treatment.

After separation of the digitonin-soluble fraction (outer membrane plus intermembrane space) by centrifugation, the particulate fraction (mitoplasts), containing membrane vesicles of more or less uniform size,[2] was subjected to freeze-thaw cycles (24) to liberate the matrix contents, leaving behind an insoluble fraction enriched with the inner membrane. Light microscopy of this fraction revealed the presence of large, presumably fused, membrane lamellae, with no evidence of intact mitoplasts.[2] As expected, malate dehydrogenase was predominantly located in the soluble fraction and succinate dehydrogenase in the particulate fraction (Table I). (Some malate dehydrogenase activity was also detected in the digitonin-soluble fraction; a similar result has been obtained with mammalian mitochondrial preparations (23) and probably represents contamination with the cytosolic form of the enzyme.) The mitochondrial enzyme terminal uridylyl transferase (TUTase), implicated in RNA editing mechanisms (20), was also released by

[2] S. Mukherjee, S. N. Bhattacharyya, and S. Adhya, unpublished data.

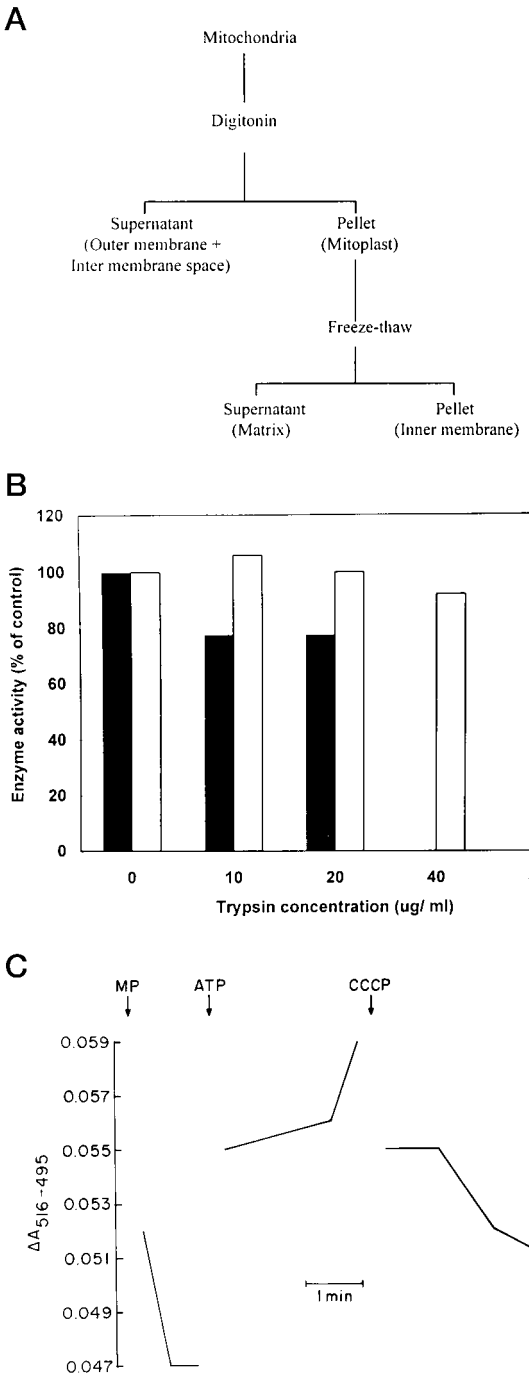

FIG. 2. **Separation of submitochondrial compartments.** *A,* fractionation scheme. *B,* effect of trypsinization of mitoplasts on marker enzyme activities. Digitonin-extracted mitochondria were treated with the indicated concentrations of trypsin for 20 min on ice, then soybean trypsin inhibitor (180 μg/ml) and 0.5 mM phenylmethylsulfonyl fluoride were added and incubated on ice for 10 min more. The mitoplasts were washed and extracted with 0.5% Triton X-100 in STE, and the soluble extracts were assayed for malate dehydrogenase (*open bars*) and succinate dehydrogenase (*filled bars*). Activities obtained in the absence of trypsin (taken as 100%) were 0.225 and 0.04 nmol min^{-1}, respectively. *C,* red shift of rhodamine 123 in the presence of digitonin-extracted mitochondria. The difference in absorbance of rhodamine 123 at 516 nm and 495 nm ($\Delta A_{516-495}$) upon addition of mitoplasts (*MP*), 4 mM ATP, and 50 μM CCCP as a function of time is shown. *Scale bar,* 1 min.

TABLE I
Marker enzyme activities in submitochondrial fractions

Fraction	Specific activity[a]		
	Kynurenine hydroxylase	Succinate dehydrogenase	Malate dehydrogenase
	nmol min^{-1} mg^{-1}		
Digitonin-soluble (OM + IMS)[b]	300	0.0017	13.4
Digitonin-insoluble (MP)	15	2.5	11.9
Freeze-thaw pellet (IM)	<0.001	1.23	1.27
Freeze-thaw supernatant (MX)	<0.001	<0.0001	51.7

[a] Substrates were kynurenine, 2,6-dichlorophenolindophenol, and NAD^+ for kynurenine hydroxylase, succinate dehydrogenase, and malate dehydrogenase, respectively.
[b] OM, outer membrane; IMS, intermembrane space; MP, mitoplast; IM, inner membrane; MX, matrix.

freeze-thaw lysis, confirming its presence in the matrix.[2]

Treatment of mitochondria with digitonin resulted in enhanced sensitivity of inner membrane proteins, such as succinate dehydrogenase, to protease treatment, whereas the matrix marker malate dehydrogenase remained unaffected (Fig. 2*B*), indicating the intactness of the inner membrane.

To determine whether the mitoplasts obtained by this procedure can develop a membrane potential ($\Delta\psi$), digitonin-treated preparations were incubated with rhodamine 123. The uptake of this lipophilic cation into energized mitochondria is strictly dependent upon the presence of $\Delta\psi$ across the inner membrane and is accompanied by a red shift in the absorption spectrum (25, 26). When mitoplasts were added to rhodamine 123, a rapid and transient red shift was observed (Fig. 2*C*), due to incomplete energization by endogenous substrates (26). Addition of ATP resulted in a more sustained red shift (Fig. 2*C*), indicating dye uptake into the mitoplasts. Further addition of CCCP, a protonophore that dissipates $\Delta\psi$, reversed the red shift (Fig. 2*C*), confirming the development of an ATP-induced proton gradient across the mitoplast membrane.

Intramitochondrial Distribution of $tRNA^{Tyr}$—Mitochondria were incubated with radiolabeled $tRNA^{Tyr}$ transcript, then digested with RNase. After washing, submitochondrial fractions were separated as above and analyzed for the presence of RNA.

In the presence of ATP, mitochondria accumulated internalized $tRNA^{Tyr}$ in different submitochondrial compartments (Fig. 3*A*). The majority of the RNA (68% in this experiment) was in the matrix, 31% was bound to the inner membrane, and less than 1% was in the intermembrane space. RNase treatment of the digitonin-treated mitoplasts resulted in nearly complete digestion of the membrane-bound RNA, whereas the RNA in the matrix was resistant (Fig. 3*A*), as expected. Thus, the RNA bound to the inner membrane is exposed to the intermembrane space. Essentially identical results were obtained when mitoplasts were prepared by hypotonic shock instead of digitonin.[2]

The intramitochondrial distribution of RNA changed with time (Fig. 3*B*). At early times of incubation, the majority of the RNA was in the inner membrane fraction, whereas after 10 min at 37 °C, increasing amounts appeared in the matrix, with a concomitant reduction in the membrane-bound fraction, whereas the total amount internalized increased only marginally. These results indicate that translocation through the inner membrane is slower than that through the outer membrane.

The effect of temperature on the intramitochondrial distribution of $tRNA^{Tyr}$ was examined (Fig. 3*C*). Total uptake increased sharply between 5 and 15 °C, but rose only marginally thereafter. In contrast, transfer into the matrix was favored at 35 and 45 °C. Near the optimum growth temperature of the promastigote form of *Leishmania* (25 °C), the RNA was equally distributed between the inner membrane and matrix compartments (Fig. 3*C*), reflecting a reduced rate of matrix transfer at the lower temperature.[2]

Sequence Discrimination at the Inner Membrane—It was pre-

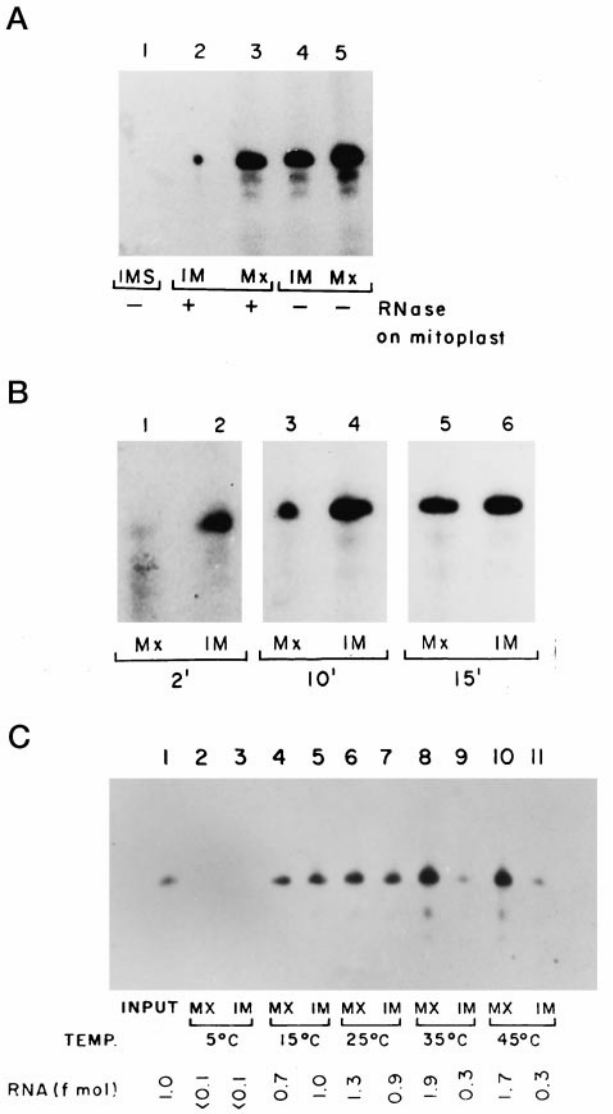

FIG. 3. **Intramitochondrial distribution of tRNA^{Tyr}.** *A,* ^{32}P-labeled tRNA^{Tyr} (100 fmol) was incubated with mitochondria (200 μg of protein) in the presence of 4 mM ATP for 15 min at 37 °C. After RNase treatment, the mitochondria were fractionated with digitonin to separate the intermembrane space fraction (*IMS*) (*lane 1*) from mitoplasts. The mitoplasts were then incubated with (*lanes 2* and *3*) or without (*lanes 4* and *5*) 2.5 μg/ml RNase A and 50 units/ml RNase T1 for 15 min at 37 °C, washed, and separated into inner membrane (*IM*) (*lanes 2* and *4*) and matrix (*MX*) (*lanes 3* and *5*) fractions by freeze-thaw lysis. RNA in each fraction was analyzed by urea-PAGE. The amount of RNA recovered in *lanes 1–5* was estimated by gel band counting to be 0.02, 0.15, 1.26, 1.21, and 2.64 fmol, respectively. *B,* mitochondria (100 μg of protein) were incubated with 100 fmol of ^{32}P-labeled tRNA^{Tyr} in the presence of 4 mM ATP for 2, 10, or 15 min, treated with RNase, and fractionated as before. The RNA in matrix (*lanes 1, 3,* and *5*) and inner membrane (*lanes 2, 4,* and *6*) fractions was analyzed by urea-PAGE. *C,* effect of temperature on intramitochondrial distribution. Import incubations were carried out with ^{32}P-labeled tRNA^{Tyr} and 4 mM ATP for 15 min at 5, 15, 25, 35, and 45 °C, respectively. After RNase treatment, the RNA contents of the matrix (*MX*) and inner membrane (*IM*) fractions were determined.

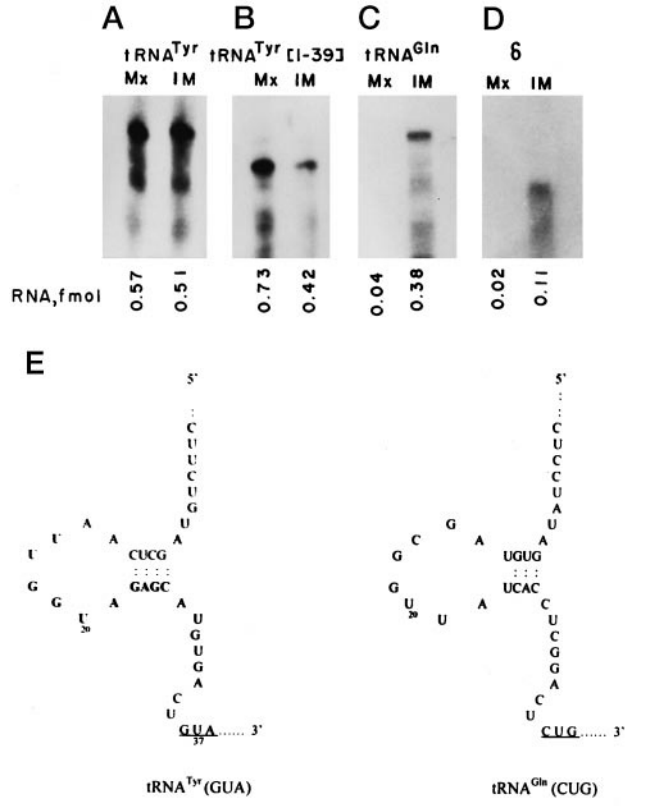

FIG. 4. **Sequence specificity of transfer through the inner membrane.** Mitochondria (100 μg of protein) were incubated with 100 fmol of ^{32}P-labeled tRNA^{Tyr} (*A*), tRNA^{Tyr}(1–39) (*B*), tRNA^{Gln}(CUG) (*C*), or δ RNA (*D*). After incubation at 37 °C for 15 min, followed by RNase treatment, the RNA contents of the matrix (*MX*) and inner membrane (*IM*) fractions were analyzed by urea-PAGE and quantified by gel band counting. *E,* structures of the D arm of tRNA^{Tyr}(GUA) (*left*) and tRNA^{Gln}(CUG) (*right*) (from Ref. 7). The conserved import signal sequence (18) in the former is shown in *boldface*.

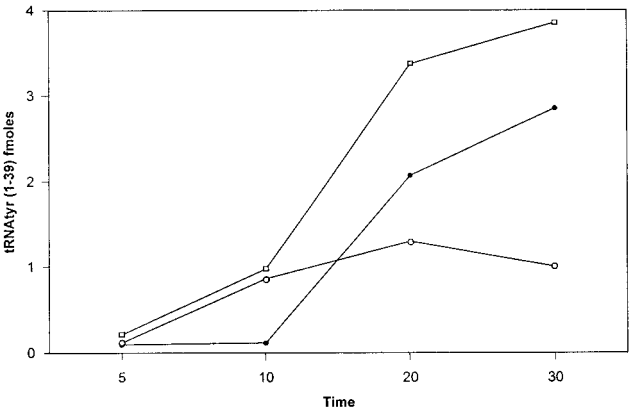

FIG. 5. **Time course of transfer of tRNA^{Tyr}(1–39) through the inner membrane.** Mitochondria (100 μg of protein) were incubated with ^{32}P-labeled tRNA^{Tyr}(1–39) in the presence of 4 mM ATP at 37 °C for the indicated times (min), treated with RNase, and subfractionated. The matrix (*filled circles*), inner membrane (*open circles*), and total (*squares*) RNA contents are plotted.

viously shown that uptake of RNA through the outer membrane (as measured by RNase protection assays of intact mitochondria) requires recognition of a conserved motif with the consensus sequence UGGYAGRRY in the D arm of tRNA (see Fig. 4) by the outer membrane receptor, TAB (18, 19). To examine whether such a sequence is sufficient for transfer into the matrix, a deletion derivative of tRNA^{Tyr}, containing the 5′-terminal 39 nucleotides, including the D arm, was tested for its intramitochondrial distribution. Entry of tRNA^{Tyr}(1–39) into the matrix was better than that of the parental molecule (Fig. 4, *A* ad *B*). The time course of intramitochondrial distribution was also similar (Fig. 5). At early times of incubation, almost all of the RNA was bound to the inner membrane, whereas after 10 min at 37 °C, the matrix content increased and the amount of membrane-associated RNA decreased. This kinetic pattern is consistent the presence of an intermediate, inner membrane-bound state. Recently,

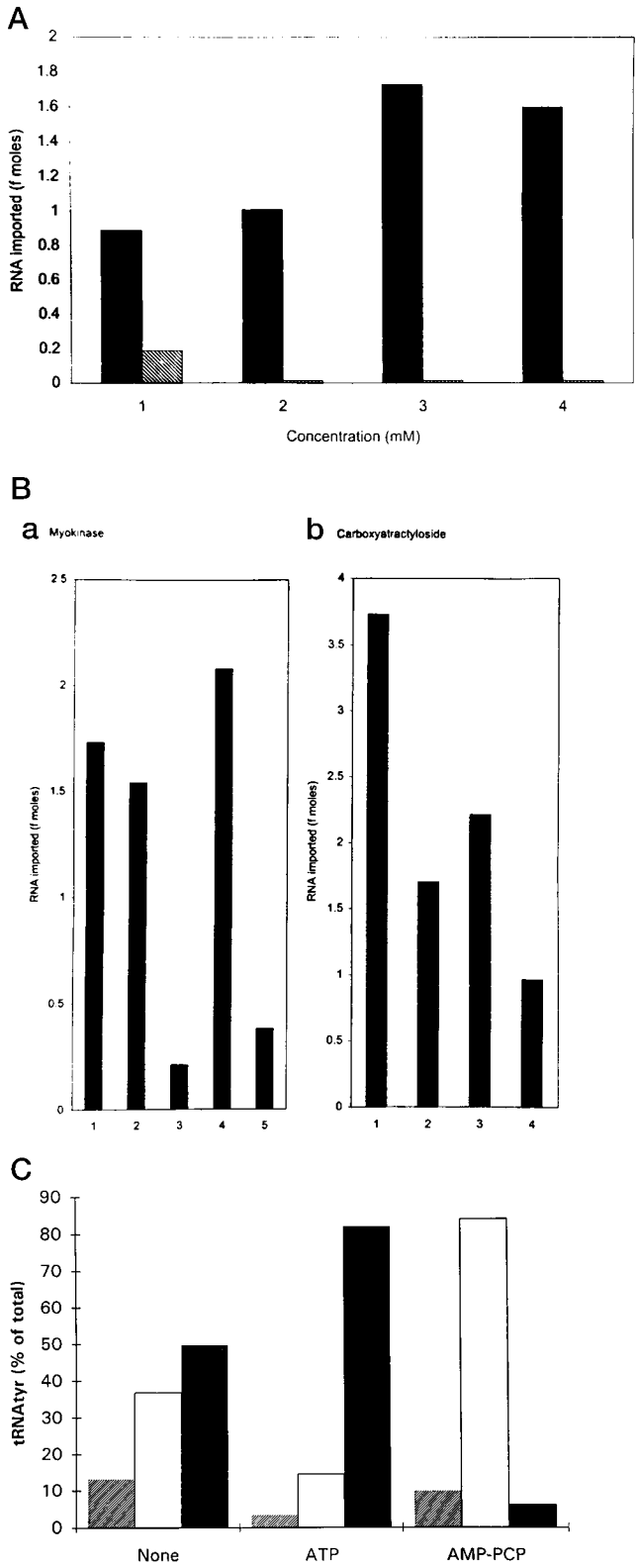

FIG. 6. **Requirement of ATP at the inner and outer membranes.** *A,* effect of ATP hydrolysis on total RNA uptake. Mitochondria were incubated with 5 nM ^{32}P-labeled tRNA^{Tyr} in the presence of the indicated concentrations of ATP (*filled bars*) or of AMPPCP (*hatched bars*) for 45 min at 25 °C. After RNase treatment, the total RNA imported was quantified by DEAE paper adsorption. A baseline value of 0.35 fmol obtained in the absence of ATP was subtracted from each point. *B,* role of internal and external ATP. In the *left panel, bars 1–3,* import incubations (45 min, 25 °C) were carried out in the presence of 4 mM ATP alone (*bar 1*), 4 mM AMP (*bar 2*), or 4 mM AMP plus 1.5 units of myokinase (*bar 3*). In the remaining reactions, mitochondria were preloaded with 4 mM ATP in STE buffer for 15 min at 25 °C and then washed and incubated with RNA and import buffer (no ATP) for 45 min at 25 °C in the absence (*bar 4*) or presence (*bar 5*) of AMP and myokinase. In the *right panel,* mitochondria were preincubated without (*bar 1*) or with (*bar 2*) 100 μM carboxyatractyloside and then diluted to twice the volume with RNA, import buffer, and 4 mM ATP for import incubation. The other reactions were performed with ATP-preloaded mitochondria in the absence (*bar 3*) or presence (*bar 4*) of 50 μM carboxyatractyloside. In each case, total RNA uptake was measured by the DEAE paper method. *C,* the role of ATP hydrolysis at the inner membrane. Mitochondria were incubated with 100 fmol of ^{32}P-labeled tRNA^{Tyr} for 15 min at 37 °C in the absence or presence of 4 mM ATP or 4 mM AMPPCP. After RNase treatment, mitochondria were fractionated into intermembrane space (*hatched bars*), inner membrane (*open bars*), and matrix (*filled bars*) fractions. RNA in each fraction was determined by urea-PAGE followed by gel band counting and expressed as a percentage of the total amount internalized. The total uptake values obtained in the absence of ATP, in the presence of ATP, and in the presence of AMPPCP, were 0.39, 1.62, and 0.34 fmol, respectively.

we have observed[2] efficient matrix localization of an oligoribonucleotide containing only nucleotides 5–27 of tRNA^{Tyr}, *i.e.* the D arm hairpin loop (18).

The outer membrane system discriminates between tRNA^{Tyr}, which is imported *in vivo* (7) and contains the conserved D arm sequence (18), and tRNA^{Gln}(CUG), which is not detectable in mitochondrial RNA (7) and lacks the conserved D arm signal (16) (see Fig. 4). However, low levels (about 25% of that of tRNA^{Tyr}) of transfer of tRNA^{Gln}(CUG) through the outer membrane could still be observed *in vitro* (19), indicating that this discrimination is not absolute. When the intramitochondrial distribution of the internalized tRNA^{Gln} was examined, 90–95% was found to be confined to the inner membrane; little or no RNA was located in the matrix (Fig. 4*C*). Similarly, very low levels of δ RNA, a synthetic transcript that lacks the import signal and is transferred to an insignificant extent through the outer membrane (18), were found to be exclusively on the inner membrane (Fig. 4*D*).

These results indicate that 1) the D arm signal is sufficient to penetrate the inner membrane, and 2) the inner membrane has an even greater selectivity for RNA sequence than the outer membrane.

Requirement of ATP Hydrolysis at the Inner and Outer Membranes—Total uptake of tRNA^{Tyr} increased with the concentration of added ATP until saturation was reached at 3–4 mM (Fig. 6*A*). The nonhydrolyzable analogs AMPPCP (Fig. 6*A*) and AMPPNP[2] were unable to replace ATP, illustrating the requirement of cleavage of the β-γ pyrophosphate bond.

To determine the sites of ATP action, mitochondria were incubated with RNA and ATP in the presence of AMP and the nonpenetrant enzyme myokinase, to scavenge the external ATP via the reaction ATP + AMP → 2ADP. This treatment resulted in inhibition of outer membrane transfer (Fig. 6*B, left panel*). When mitochondria were preloaded with ATP and then incubated with RNA in the absence of further ATP addition, normal levels of outer membrane transfer were observed, but myokinase inhibited this process, indicating that some or all of the ATP must be exported from the matrix or intermembrane space in order to sustain transfer (Fig. 6*B, left*).

When mitochondria were incubated with carboxyatractyloside, a specific inhibitor of the adenine nucleotide translocator on the inner membrane (27), and then ATP and RNA were added, import was inhibited (Fig. 6*B, right*). This suggests that accumulation of ATP in the matrix via the adenine nucleotide translocator is important. Mitochondria preloaded with ATP, washed, and subsequently challenged with RNA in the presence of carboxyatractyloside were also inhibited (Fig. 6*B, right*). Because external ATP is absent and matrix ATP is prevented from translocating to the intermembrane space by carboxyatractyloside, this result is consistent with the requirement of ATP in the

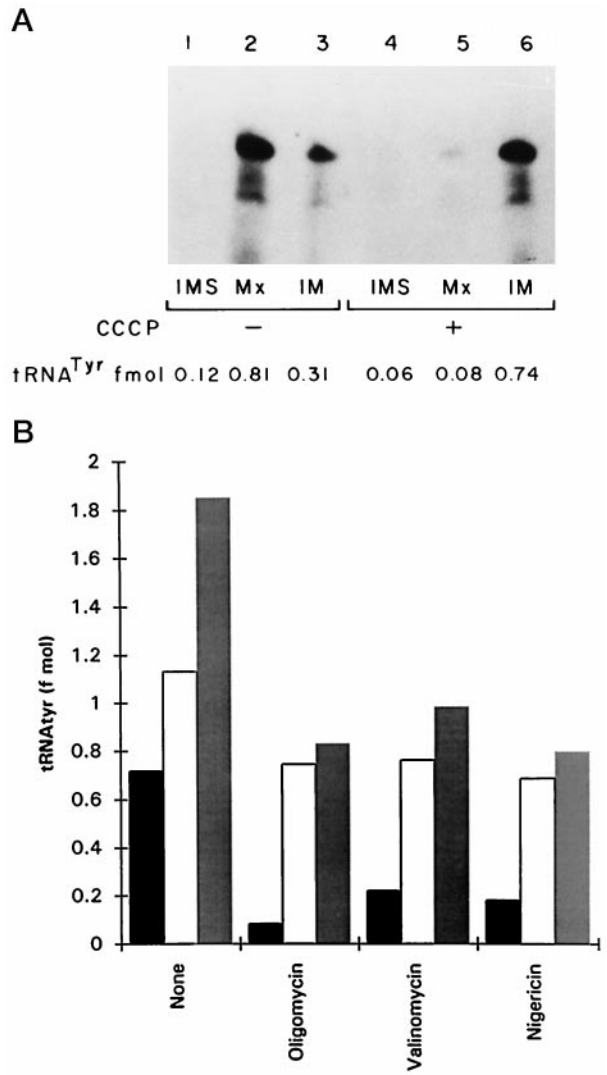

FIG. 7. **Effect of inhibitors on RNA transfer through the outer and inner membranes.** *A,* effect of CCCP. Mitochondria were preincubated without (*lanes 1–3*) or with (*lanes 4–6*) 100 μM CCCP for 15 min on ice and then at 37 °C for 15 min with RNA (100 fmol) and ATP (4 mM) (final CCCP concentration, 50 μM). After RNase treatment, the distribution of RNA between intermembrane space (*IMS*) (*lanes 1* and *4*), matrix (*Mx*) (*lanes 2* and *5*), and inner membrane (*IM*) (*lanes 3* and *6*) was determined. *B,* effect of oligomycin, valinomycin, and nigericin. In each case, mitochondria were preincubated with inhibitor (50 μM), and in the case of valinomycin, they were also preincubated with 50 mM KCl. Submitochondrial distribution was determined as before. The total (*hatched bars*), matrix (*filled bars*), and inner membrane (*open bars*) RNA contents are shown.

intermembrane space or at the outer membrane.

The effect of ATP hydrolysis on the intramitochondrial distribution of $tRNA^{Tyr}$ was examined (Fig. 6*C*). Addition of ATP caused a 7-fold increase in the level of RNA in the matrix but only a 1.6-fold increase in the level of inner membrane-bound RNA. In the presence of AMPPCP, translocation into the matrix was reduced to negligible levels, whereas 84% remained associated with the inner membrane. Taken together, these results demonstrate the requirement of ATP hydrolysis for transfer of RNA through both the outer and inner membranes.

Role of the Electromotive Force across the Inner Membrane—Many mitochondrial transport processes are energetically driven by the electromotive force across the inner membrane generated by vectorial proton movement coupled to respiratory electron transport. These include translocation of proteins (28, 29) and of various low molecular weight metabolites (27). A conceivable role of ATP in RNA import is to generate a proton gradient by the hydrolytic activity of the oligomycin-sensitive F_1F_0 ATPase, which subsequently drives transfer through the membrane.

This possibility was tested by observing the effect of various respiratory inhibitors on ATP-dependent total uptake, as well as the intramitochondrial distribution of $tRNA^{Tyr}$. Initial titrations with the protonophore CCCP, which dissipates the proton gradient (30) and hence the electromotive force, revealed only insignificant inhibition (less than 2-fold) of total uptake even at high inhibitor concentrations (up to 200 μM),[2] suggesting that outer membrane transfer does not require an electromotive force. However, more than 90% of the internalized RNA was restricted to the inner membrane, very little being found in the matrix (Fig. 7*A*); the membrane-bound form was located on the outer surface, exposed to the intermembrane space.[2] Clearly, insertion through the inner membrane requires a proton gradient.

Oligomycin, at a concentration of up to 50 μM, which is 50–100 times the dose for 50% inhibition of leishmania mitochondrial ATPase (31, 32), could inhibit total uptake about 2-fold, but it caused a 10-fold reduction in inner membrane translocation (Fig. 7*B*), indicating a role of the F_1F_0 ATPase in transfer to the matrix.

The electromotive force consists of two components: the membrane potential ($\Delta\psi$), caused by charge separation across the membrane, and the pH gradient (ΔpH), due to the difference in proton concentration on the two sides (30). In the presence of valinomycin, a K^+ ionophore that reduces $\Delta\psi$ but not ΔpH (30), transfer of $tRNA^{Tyr}$ into the matrix was inhibited (Fig. 7*B*). Nigericin, which carries out an electroneutral K^+-H^+ exchange, thereby reducing ΔpH without any effect on $\Delta\psi$ (30), had a similar inhibitory effect (Fig. 7*B*). The results suggest that both the electrical and chemical components of the electromotive force are necessary for translocation of tRNA through the inner membrane.

DISCUSSION

We describe here a modified import assay that monitors the distribution of internalized RNA in different submitochondrial compartments. Several modifications of previous assay methods (15, 16, 18, 19) were introduced. The import incubation was carried out at 37 instead of 25 °C. At the higher temperature, the rate but not the yield of import *in vitro* was higher, and entry into the matrix was facilitated (Fig. 3). The broad temperature range over which import is active may be a reflection of the adaptability of the parasite to grow at different temperatures as it passes through invertebrate and mammalian hosts. The postimport RNase treatment was also carried out at 37 °C using only 2.5% of the concentration of RNase previously employed (Fig. 1); these conditions were sufficient to digest excess RNA and at the same time minimized the risk of RNA degradation during subsequent fractionation steps. Third, the combination of digitonin and freeze-thaw lysis of mitoplasts allows the intramitochondrial compartments to be rapidly and easily separated (Fig. 2) without the need for prolonged ultracentrifugation steps.

Selective permeabilization of the outer membrane with digitonin or by hypotonic shock revealed the presence of a significant fraction of internalized tRNA associated with the outer side of the inner membrane, *i.e.* facing the intermembrane space (Fig. 3). The kinetics of accumulation of this membrane-bound form (Figs. 3 and 5) are consistent with it being an intermediate in matrix translocation and not merely a nonfunctional bye-product. Moreover, there was no consistent evidence of any membrane-associated RNA of less than full-length size, as would be expected of a membrane-spanning intermediate. Full length and complete accessibility to RNase of the membrane-bound RNA are incompat-

ible with concerted models of import that predict translocation intermediates spanning both membranes and enclosed in a transport pore consisting of inner and outer membrane proteins. This type of general insertion pore located at inner membrane-outer membrane contact sites conducts cytoplasmic proteins into the mitochondrial matrix (33).

In concerted models of import, the responsibility for selection of the correct import substrate lies with receptors located at the outer membrane, which recognize appropriate import signals. Thus, misrecognition at this step should lead to entry of the inappropriate substrate into the matrix. Our previous studies showed that the outer membrane system can discriminate between $tRNA^{Tyr}$ and $tRNA^{Gln}$(CUG), but not absolutely (19). However, the residual level of $tRNA^{Gln}$ passing through the outer membrane is almost exclusively restricted to the inner membrane (Fig. 4). In contrast, derivatives of $tRNA^{Tyr}$ retaining the conserved D arm import signal (18) pass into the matrix at least as effectively as the entire molecule (Fig. 4). Because this matrix targeting sequence interacts with TAB, a protein located on the outer membrane (18, 19), one possibility is that the TAB-D arm complex passes through the outer membrane and subsequently interacts with the inner membrane channel for matrix entry. According to this scenario, RNAs, such as $tRNA^{Gln}$, that do not interact with TAB on the mitochondrial surface (18) would be effectively excluded from the matrix.

Although ATP hydrolysis is required for translocation of RNA through both the outer and inner membranes (Fig. 6), the roles of ATP at these two steps are different. Notably, inhibition of the proton-pumping F_1F_0 ATPase with oligomycin, or dissipation of the proton gradient with CCCP or nigericin, results in severe inhibition of inner membrane translocation but only a marginal effect on transfer through the outer membrane (Fig. 7). Because nigericin, through electroneutral K^+-H^+ exchange, affects only the chemical component (ΔpH) of the electromotive force (30), it is likely that transfer of RNA through the inner membrane is driven by the excess proton concentration in the intermembrane space. Symport of polyanionic RNA with protons would be an effective charge-balancing mechanism necessary to pump the RNA against the membrane potential (negative inside) of respiring mitochondria. An analogous example is that of the inner membrane phosphate transporter, which is coupled to the proton gradient by P_i-H^+ symport (27).

The inhibition of inner membrane translocation by valinomycin (Fig. 7), which neutralizes the K^+ gradient and thus reduces membrane potential ($\Delta\psi$), exposes the paradoxical situation that a negatively charged membrane is required for transfer of negatively charged RNA. Several possibilities may be considered to explain this. 1) Import of RNA is coupled to export of some other anion such that the process is electrogenic, with the matrix losing net negative charge. An example of this type of transport is the $\Delta\psi$-requiring exchange of matrix ATP for cytoplasmic ADP catalyzed by the adenine nucleotide translocator (27). 2) RNA is complexed with a positively charged protein factor that moves electrophoretically into the negatively charged matrix. This mechanism has been invoked to explain the transfer of the amphipathic helix constituting the import signal of matrix-targeted proteins (33).

Whereas most inner membrane transport processes, including that of proteins, require either the $\Delta\psi$ or ΔpH component of the electromotive force, transport of tRNA may be unusual in requiring both. This is probably a consequence of the high negative charge of the molecule as well as of its sequence-specific interaction with a positively charged species. According to this hypothesis, $\Delta\psi$ would be required for initiation, and ΔpH for completion of transfer of the phosphodiester backbone through the inner membrane.

The co-import model of mitochondrial tRNA uptake (34) is a concerted mechanism involving the passive transfer of RNA bound to a matrix-targeted carrier protein through the general insertion pore. However, the results presented here support a stepwise mechanism of import of tRNA through the two mitochondrial membranes. The first step consists of TAB-D arm recognition at the outer membrane followed by ATP-dependent transfer to the intermembrane space. The RNA then rapidly associates with the outer surface of the inner membrane. We assume that inner membrane binding is fast, because significant accumulation of RNA in the intermembrane space fraction was not observed, even at early times of incubation (Figs. 3 and 5). Subsequently, the RNA (possibly as an RNP complex) is transferred to the matrix by a process requiring the D arm signal as well as both the chemical and electrical components of the electromotive force. Attempts to define the biochemical components of the membrane-bound machinery are currently in progress.

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