

The D arm of tRNA^{Tyr} is necessary and sufficient for import into *Leishmania* mitochondria *in vitro*

Sridam Mahapatra, Subhagata Ghosh, Saphal Kanti Bera, Trina Ghosh, Anish Das and Samit Adhya*

Genetic Engineering Laboratory, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Calcutta 700032, India

Received February 9, 1998; Revised and Accepted March 13, 1998

DDBJ/EMBL/GenBank accession nos X51821, X98595

ABSTRACT

Transfer RNAs are selectively imported from the cytoplasm into mitochondria of kinetoplastid protozoa such as *Leishmania*. The specific structural features of tRNA which determine selectivity are largely unknown. Using an *in organello* system from *Leishmania*, the import signals on tRNA^{Tyr} and on a synthetic transcript which binds to the same receptor, were studied by deletion and reconstruction analyses. In both cases, short oligoribonucleotides (minihelices) containing the sequence UGGYAGAG were imported with high efficiency in the presence of ATP. This motif is present in the D arm of tRNA^{Tyr}, as well as in the majority of imported *Leishmania* tRNAs. Deletion of the D arm, or a point mutation in the conserved motif, reduces importability. The import signal coincides with the binding site for the mitochondrial receptor TAB. tRNA^{Gln}, which is not imported, forms non-productive, TAB-independent complexes with the mitochondrial surface. However, the observation that the imported:bound ratio of the D arm minihelix is higher than that of the entire molecule suggests that the post-binding translocation step is constrained in terms of size or structural flexibility. Kinetic studies of minihelix import indicate stepwise insertion of the molecule into import channels.

INTRODUCTION

The mitochondrial genomes of many species of protozoa, fungi and plants lack the minimal complement of the 20 or so tRNA genes required for translation of organellar mRNA. Evidence has accumulated that in these cases the corresponding nuclear-encoded tRNAs are imported from the cytoplasm (1–9). However, the number and identities of the imported tRNAs vary between species. For example, only a single tRNA^{Lys} in yeast (1), up to 11 tRNAs in plants (2), and most or all of the cytoplasmic tRNAs in kinetoplastid protozoa such as *Leishmania* and *Trypanosoma* (4–9), are imported. Moreover, in *Leishmania* different tRNA species may be partitioned to different extents between the cytosol and mitochondria (8). Presumably, the rate and extent of import of a particular tRNA depends on its interaction with

mitochondrial receptors; this, in turn, would depend on the presence of a specific import signal or determinant on the tRNA. Conversely, an anti-determinant (10) may prevent such an interaction, thereby limiting the tRNA to the cytosolic compartment.

A clearcut definition of the nature of the import signal(s) in *Leishmania* and *Trypanosoma* tRNAs is lacking. Transfection experiments indicate that the presence of an intron adjacent to, or mutations within, the anticodon of tRNA^{Tyr} does not affect its import into *Trypanosoma* mitochondria (9). When the D loops of tRNA^{Ile} (imported) and tRNA^{Gln} (not imported) were exchanged, both hybrid tRNAs were imported (11), suggesting that other regions of the molecule besides the D arm may also play a role. Our previous experiments with an *in organello* system from *Leishmania* had shown that the import pathway for tRNA^{Tyr} is used by synthetic antisense transcripts derived from the 5'-untranslated region of the β -tubulin gene (12–14). Thus, the two RNAs cross-compete for import (13), bind to the 15 kDa outer membrane-associated protein TAB (13,14), and antibody against TAB specifically inhibits their import (14), suggesting that tRNA^{Tyr} and antisense transcripts share a common import signal. In this study, the import signals on both RNAs were independently defined by mutagenesis and reconstruction experiments. The results indicate that the D arm of tRNA^{Tyr} contains a necessary and sufficient signal for import *in vitro*.

MATERIALS AND METHODS

Cell culture and isolation of mitochondria

Promastigotes of *Leishmania tropica* strain UR6 were cultured on solid blood agar medium, and mitochondria were isolated by Percoll gradient centrifugation, as previously described (12).

Preparation of import substrates

Clone pSG3S contains the region between positions –20 and +25 of the *Leishmania* β -tubulin gene inserted in vector pSPT19 (15). Clones pSG3 β and pSG3 δ were derived from pSG3S by limited exonuclease III digestion from the upstream side, followed by *EcoRI* linker ligation, restriction and vector circularization, following standard procedures (16). Deletion endpoints were confirmed by DNA sequencing. To obtain clone pSG3e, a synthetic double-stranded oligonucleotide spanning the region –15 to +5 was inserted between the *HindIII* and *EcoRI* sites of

*To whom correspondence should be addressed. Tel: +91 33 473 0492; Fax: +91 33 473 5197; Email: iichbio@giasc101.vsnl.nct.in

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

vector pGEM4Z (Promega). Clones pSKB-1, pSKB-1(Δ -1) and pSKB-2, containing, respectively, the entire tRNA^{Tyr}(GUA) gene (including intron), the 5'-terminal 39 nt of the tRNA^{Tyr}(GUA) gene and the tRNA^{Gln}(CUG) gene, have been described (14). To prepare D-arm minihelix templates, the promoter primer GGAATTCTAATACGACTCACTATAGGGGACTGTAGCTC, containing an *Eco*RI linker, a T7 RNA polymerase promoter sequence and nucleotides 5–13 of tRNA^{Tyr}(GUA) (7), was annealed to the template oligonucleotide of either wild-type sequence: ATGCTCTACCAATTGAGCTACAGTC, or mutant sequence: ATGCTCACGAATTGAGCTACAGTC, each containing sequences complementary to positions 5–27 of the tRNA^{Tyr}(GUA) gene and an 11 bp complementarity with the promoter primer. The resulting partially double-stranded molecule was end-filled with MMLV reverse transcriptase (16). High specific activity ³²P-labelled RNAs were prepared by runoff transcription of linearised plasmid clones or oligonucleotide templates with T7 RNA polymerase, as previously described (15). Full-length minihelix transcripts were purified from a 10% polyacrylamide sequencing gel, and their sequences verified by 2-dimensional polyethyleneimine cellulose thin layer chromatography (17). Oligonucleotide-directed RNase H cleavage of tRNA^{Tyr} was carried out by annealing ³²P-labelled tRNA^{Tyr} (0.6 pmol) with the wild-type oligonucleotide complementary to positions 5–27 (10 pmol) in presence of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 M KCl in 10 μ l volume, by heating at 65 °C for 5 min followed by slow cooling to room temperature. The annealed mixture was diluted with 40 μ l of 20 mM Tris–HCl, pH 7.5, 10 mM MgAc₂ and 0.1 M DTT, then 3 U of RNase H (US Biochemicals) were added and the reaction incubated for 1 h at 37 °C. After phenol–chloroform extraction, the RNA was ethanol precipitated.

Import assays

Unless otherwise indicated, ³²P-labelled RNA of specified concentration was incubated with purified mitochondria (80 μ g protein) in 20 μ l reactions containing 10 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM ATP, 6.7 mM creatine phosphate and 60 μ g/ml creatine phosphokinase for 45 min at 25 °C. Then 0.1 mg/ml RNase A and 2000 U/ml RNase T1 were added, and incubation continued for 15 min at 25 °C. Mitochondria were diluted into 0.5 ml of isotonic sucrose buffer STE-B (12), reisolated by centrifugation, resuspended in 20 μ l STE-B, and treated with 0.1 mg/ml proteinase K for 5 min at 25 °C to inactivate residual RNase. The mitochondria were disrupted by the addition of 200 μ l of ice-cold guanidinium buffer (4 M guanidinium isothiocyanate, 25 mM Na citrate, pH 7.0, 0.5% sarkosyl and 0.1 M β -mercaptoethanol), then the following were added successively with mixing: 40 μ l of 1 M Na acetate, pH 4.2; 0.2 ml of water-saturated phenol; and 40 μ l of chloroform–isoamyl alcohol (49:1). After incubation on ice for 15 min followed by centrifugal phase separation, the RNA was recovered by isopropanol precipitation and analyzed by denaturing polyacrylamide gel electrophoresis. The amount of RNA imported was quantified by scintillation counting of excised dried gel bands. Antibody inhibition experiments were performed with mitochondria preincubated with normal or anti-TAB IgG as previously described (14).

Binding assays

³²P-labelled RNA was incubated with purified mitochondria (40 μ g protein in 10 μ l reaction) in import buffer containing, additionally,

0.1 M KCl (to ensure specificity), for 15 min at 25 °C. Mitochondria were washed with STE-B, deproteinized, and the bound RNA analyzed by gel electrophoresis.

Gel-shift assays

Dialyzed, heat-treated S-100 extracts enriched for TAB were prepared as previously described (15). ³²P-labelled RNA (2 fmol) was incubated with indicated amounts of the extract in 10 μ l reactions containing 10 mM Tris–HCl, pH 7.5, 5 mM Mg acetate, 2 mM DTT, 5 mg/ml heparin for 30 min at 0 °C, then electrophoresed on a native 5% polyacrylamide gel and autoradiographed.

RESULTS

The import signal in antisense RNA

We first constructed deletions in the β -tubulin antisense transcript (Fig. 1A) in order to define its import signal. Deletion mutants mapping up to –12 from the upstream side of the β -tubulin gene from position –20 were imported but deletion of an additional 11 nt (endpoint –1) resulted in complete loss of importability (Fig. 1B). ATP-dependent import was restored in a transcript spanning positions +5 to –15 (Fig. 1B). From these data it could be concluded that the region between –12 and –1 is necessary and sufficient for import.

It was shown previously that RNA interacts rapidly with mitochondrial surface receptors to form a stable complex; this is followed by a slow ATP-dependent internalization step (13). To determine whether importability of the mutants correlates with their ability to interact with mitochondrial surface receptors, RNA binding assays were performed with purified mitochondria under sequence-specific conditions, i.e., in presence of 0.1 M KCl which eliminates non-specific binding (13). Receptor-binding was normal for deletions extending up to –12, was abolished in a mutant mapping to –1, and restored in the +5/–15 transcript (Fig. 1C). In all cases, binding was sensitive to anti-TAB antibody (ref. 14 and data not shown). Binding of the mutants to detergent-solubilized TAB was further checked by a gel-shift assay. As shown in Figure 1D, the mutant mapping to –12 was able to bind TAB, but not the mutant mapping to –1, and binding was restored in the +5/–15 RNA. The correlation between TAB binding (Fig. 1C and D) and importability (Fig. 1B) demonstrates that the binding site for TAB in antisense RNA coincides with its import signal, and includes the purine-rich sequence GAUGG-CAGAG (Fig. 1A).

The time course of import of the +5/–15 transcript (Fig. 1B) showed that at 15 min of incubation, the major RNase-resistant species is a few nucleotides shorter than the input RNA. With time, this species disappears and is replaced by the full-length molecule. This result is consistent with the formation of an import intermediate with the RNA partly inside the import channel, followed by transfer of the remainder of the molecule. Alternative possibilities, such as transient nucleotide modifications or altered secondary structure, cannot be excluded until this species is sequenced.

An import signal in the D-arm of tRNA^{Tyr}

The D-arm of tRNA^{Tyr} contains the sequence UGGUAGAG (ref. 7; see also Fig. 4) which is nearly identical to the import signal on antisense RNA (see above). To determine its role in import, the

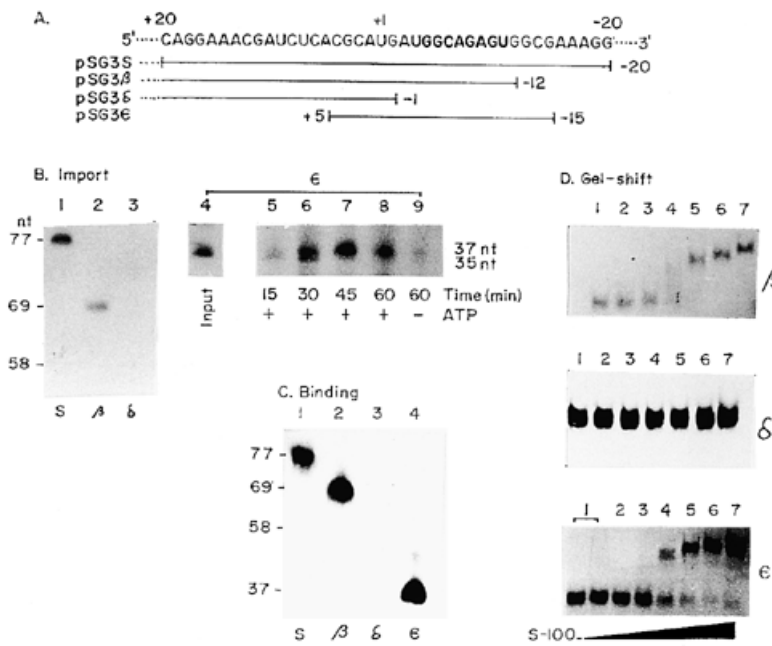


Figure 1. Deletion analysis of the import signal in antisense RNA. (A) Sequence of β -tubulin antisense RNA (15) showing endpoints of deletions from the 5'-upstream side of the β -tubulin gene. pSG3S, pSG3 β and pSG3 δ RNAs each contain 33 additional nucleotides upstream (derived from the T7 polymerase start site, polylinker and nucleotides +25 to +21 of the β -tubulin gene) and four additional nucleotides downstream (the *EcoRI* runoff sequence). pSG3 ϵ RNA contains a 13 nt 5'-leader and a 4 nt downstream sequence. The conserved purine-rich motif is shown in bold. (B) Import assays of 32 P-labelled runoff transcripts (5 nM) from clones pSG3S, pSG3 β , and pSG3 δ (lanes 1–3) and pSG3 ϵ incubated with mitochondria in presence of ATP for 15, 30, 45 and 60 min (lanes 5–8), or in absence of ATP for 60 min (lane 9). Lane 4, input pSG3 ϵ RNA (2 fmol). (C) Binding of RNA (5 nM) from clones pSG3S, pSG3 β , pSG3 δ and pSG3 ϵ to intact mitochondria (lanes 1–4, respectively). (D) Gel-shift assays of RNA (2 fmol) from clones pSG3 β (upper), pSG3 δ (middle) and pSG3 ϵ (lower) incubated with no protein (lanes 1), or with 0.125, 0.25, 0.5, 1, 2 and 4 μ g (lanes 2–7, respectively) of 55°C treated, TAB-enriched fraction.

D-arm (positions 5–27 of tRNA^{Tyr}) was selectively removed by oligonucleotide-directed RNase H cleavage. The resulting molecule, tRNA^{Tyr}[28–73], containing the anticodon, intron, variable loop, T arm and the acceptor end, was not imported *in vitro* (Fig. 2). The second RNase H cleavage product, containing a 14 nt 5'-leader and nucleotides 1–5 of tRNA^{Tyr}, was also not imported (data not shown). These results indicate the requirement of the D-arm sequence, with or without the 5'-part of the acceptor stem, for import.

If the D-arm contains sufficient information to signal import, it should be possible to isolate it from the remainder of the molecule without sacrificing importability. Partial tRNA molecules (minihelices) have been successfully employed to determine the specificity of RNase P processing, aminoacylation and codon-anticodon interaction (18). Although minihelices by definition do not reflect all possible interactions involving the native molecule, they allow the specific structural requirements of import to be studied in the absence of complications arising from other reactions of tRNA such as aminoacylation and translation. A deletion mutant of tRNA^{Tyr} containing the 5' 39 nt of the gene (tRNA^{Tyr}[1–39]), including the entire D-arm and parts of the acceptor and anticodon arms, was imported 6–8 times more efficiently than tRNA^{Tyr} itself (Fig. 3A). While the import of tRNA^{Tyr} was saturated at 2.5 nM RNA, that of tRNA^{Tyr}[1–39] continued to be proportional to the RNA concentration up to at least 5 nM (Fig. 3A). Import of tRNA^{Tyr}[1–39] was ATP-dependent and specifically inhibited by antibody against the mitochondrial receptor TAB (Fig. 3B). Import of tRNA^{Tyr} was competitively inhibited by tRNA^{Tyr}[1–39], and vice versa (data not shown).

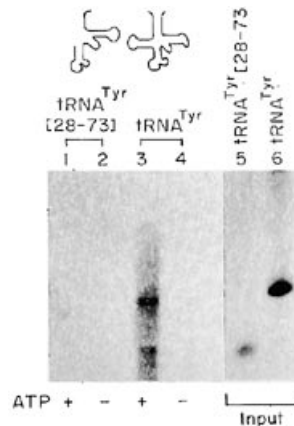


Figure 2. Effect of removal of the D arm and 5'-part of the acceptor stem on import of tRNA^{Tyr}. Import assays were carried out using 5 nM of tRNA^{Tyr}[28–73] (lanes 1 and 2), or intact tRNA^{Tyr} (lanes 3 and 4), in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 1 mM ATP. Lanes 5 and 6 show the corresponding input RNAs.

These results indicate that the import signal of tRNA^{Tyr} is localized within the 5' 39 nt and that interaction of this region with TAB is necessary for import.

To examine whether the enhanced import efficiency of tRNA^{Tyr}[1–39] can be accounted for in terms of receptor binding efficiency, quantitative binding assays were performed. Binding of tRNA^{Tyr}[1–39] to mitochondrial receptors was about the same

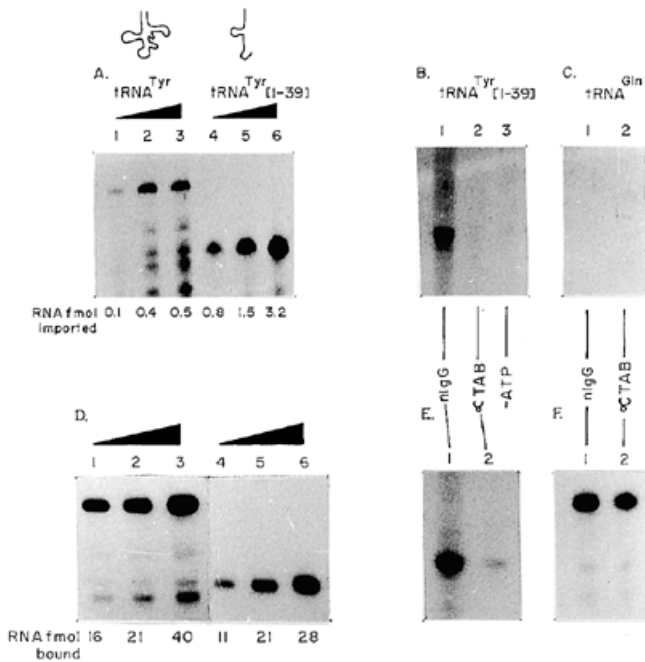


Figure 3. Import of tRNA^{Tyr}[1-39]. (A) Import assay of tRNA^{Tyr} (lanes 1-3) and tRNA^{Tyr}[1-39] (lanes 4-6). RNA concentrations present in the reactions were 1 nM (lanes 1 and 4), 2.5 nM (lanes 2 and 5) and 5 nM (lanes 3 and 6). (B) Effect of anti-TAB antibody and ATP on import of tRNA^{Tyr}[1-39] (5 nM). Mitochondria were preincubated with normal IgG (lane 1), or anti-TAB IgG (lane 2). Lane 3, import assay lacking ATP and ATP-regenerating system. (C) Import of tRNA^{Gln}(CUG) into mitochondria preincubated with normal IgG (lane 1) or anti-TAB IgG (lane 2). (D) Binding of tRNA^{Tyr} (lanes 1-3), or tRNA^{Tyr}[1-39] (lanes 4-6) on intact mitochondria. RNA concentrations in binding reactions were 1.25 nM (lanes 1 and 4), 2.5 nM (lanes 2 and 5) and 5 nM (lanes 3-6). Effect of anti-TAB antibody on binding of tRNA^{Tyr}[1-39] (E) or tRNA^{Gln}(CUG) (F). Binding reactions were carried out with 5 nM RNA and mitochondria preincubated with normal IgG (lanes 1) or anti-TAB IgG (lanes 2).

(within a factor of 2) as that of tRNA^{Tyr} itself at all RNA concentrations tested (Fig. 3D), and specifically inhibited by anti-TAB antibody (Fig. 3E). Therefore, the higher import

efficiency of tRNA^{Tyr}[1-39] is due to facilitation of a step subsequent to the initial receptor-binding, e.g., transfer through import pores, possibly as a result of its smaller size or removal of inhibitory sequences elsewhere in the molecule.

By using the appropriate oligonucleotide templates for T7 RNA polymerase transcription, shorter minihelices containing nucleotides 5-27 of tRNA^{Tyr}, i.e., the entire D-arm, were synthesized (see Materials and Methods; Fig. 4A). The tRNA^{Tyr}[5-27] with wild-type sequence is imported as efficiently as the larger minihelix (Fig. 4B). A number of apparently shorter RNase-resistant species were also produced. Since they are not observed in absence of ATP or if the mitochondria are lysed with detergent after import (data not shown), these shorter RNAs presumably represent translocation intermediates. Similar species were observed during import of the antisense +5/-15 transcript (Fig. 1).

A single G¹⁸-to-C point mutation in tRNA^{Tyr}[5-27] reduces the rate and extent of import by 2-3-fold (Fig. 4B). Since G¹⁸ is a part of the conserved consensus motif (Fig. 4A), we conclude that interactions involving individual nucleotides of this motif are critical for the translocation process.

As a negative control, binding and import of tRNA^{Gln}(CUG), which is not imported *in vivo* (7), was examined. As shown previously (15), internalization of this molecule *in vitro* was barely or not detectable (Fig. 3C). tRNA^{Gln}(CUG) did bind to the mitochondrial surface, with ~50% the efficiency of tRNA^{Tyr}, but this binding was inhibited by anti-TAB antibody by <20% (Fig. 3F), indicating the formation of non-productive complexes with some other surface protein.

DISCUSSION

The results presented in this paper support the notion that a short purine rich sequence containing the conserved motif UGGYA-GAG in the D-arm of tRNA^{Tyr} acts as an import signal *in vitro*. This region directly binds to TAB on the mitochondrial surface to initiate translocation, but the precise contact sites remain to be determined. No other region of the tRNA molecule appears to be necessary; indeed, other domains or larger structures than the D arm may actually hinder import. Finally, transfer of RNA through

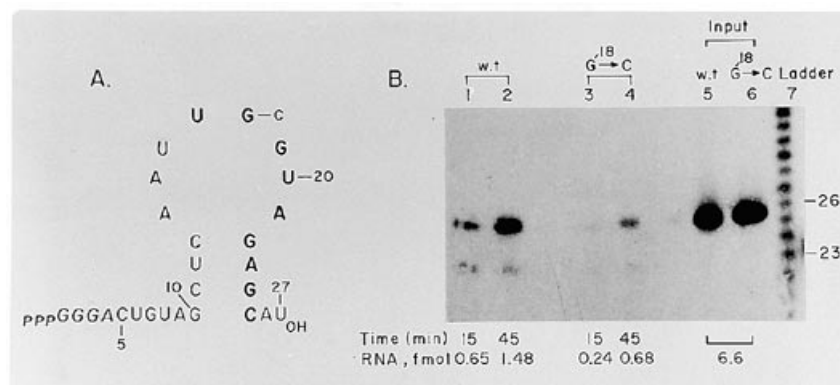


Figure 4. Effect of a point mutation on import of the D-arm minihelix of tRNA^{Tyr}. (A) The sequence of the transcript. The conserved motif is shown in bold, and the position of the mutation indicated. Four additional bases at the 5' end (italics) constitute the start signal for T7 RNA polymerase. (B) Import assays. Gel-purified wild-type (lanes 1 and 2) or G¹⁸-to-C mutant (lanes 3 and 4) RNA (2.5 nM) was incubated with mitochondria for 15 min (lanes 1 and 3) or 45 min (lanes 2 and 4) and the imported RNA was analyzed by 10% polyacrylamide sequencing gel electrophoresis. Lanes 5 and 6, input wild-type or mutant RNA (6 fmol each). Lane 7, ladder of DNA oligonucleotides. Note the displacement of the RNA bands relative to those of DNA, due to presence of 5'-triphosphate in the former.

import pores may be a multistep process with defined kinetic intermediates.

How general is this D-arm signal for tRNA import in kinetoplastid protozoa? A survey of tRNA sequences known to be imported in *Leishmania* showed that 8 out of 15 species (i.e., 53%) contain similar (differing by one base) or identical motifs in the D arm. In contrast, of 36 yeast tRNAs, none contain an identical D-arm motif and only two (i.e., 5%) deviate by a single base (data not shown). This explains why *Leishmania* but not yeast tRNA competes effectively for import of tRNA^{Tyr} (14) or of the β -tubulin antisense transcript (13). However, some tRNA species may contain multiple import signals. For example, a hybrid tRNA^{Ile} (UAU) in which the D arm was replaced by the inactive D arm of tRNA^{Gln}, was still imported *in vivo* (11). It is possible that in this case some other region of the tRNA, e.g., the anticodon, contains an additional determinant.

A few tRNAs from other species are imported into *Trypanosoma brucei* mitochondria, leading to the idea that tRNA structure as a whole, rather than specific motifs, determines importability (19). We note, however, that one of these tRNAs, human tRNA^{Lys}, contains the sequence CGGUAGAG, while the other, yeast tRNA^{His}, contains the related sequence UGGUAGUA, in the D arm (19). It is possible that the observed import of these two tRNAs is due to the adventitious presence of the conserved D-arm motif recognized by the protozoal import machinery.

Quantitative comparisons between tRNA^{Tyr} and tRNA^{Tyr}[1–39] revealed that, whereas binding to TAB on the mitochondrial surface was indistinguishable for the two RNAs, internalization of tRNA is less efficient and is saturated at lower RNA concentration (Fig. 3). This indicates the presence of a limiting component in the mitochondria with which the native molecule must interact before it is transferred through import pores. tRNAs have a characteristically conserved L-shaped three-dimensional structure of considerable flexibility (20). Within this structure, the D arm occupies the 'hinge' of the L, engaged in a number of tertiary interactions with the T arm and the V loop. The distance between the anticodon loop and the acceptor end is ~8 nm (20), whereas the general import pores for protein can accommodate the DNA double helix of 2 nm diameter (21). Assuming that RNA import pores are similarly wide, it may be difficult for the native tRNA molecule to translocate unless its conformation is altered. One possibility is that the binding of TAB to the D arm hinge results in recruitment of a limiting component with consequent disruption of the hinge and 'straightening' or unfolding of the molecule for easier passage.

Through the use of RNA oligonucleotides and high resolution gel analysis we are beginning to define specific steps in the translocation process. The kinetics of import of the +5/-15 transcripts (Fig. 1) clearly indicates multistep insertion with

discrete intermediates. Partially inserted molecules were also observed with the D-arm minihelix (Fig. 4). It will be important to characterize these intermediates further in order to determine the nature of the kinetic barriers. It may also be feasible by mutagenesis to construct derivatives which are permanently arrested the import pores. The synthesis of small membrane-permeable RNA oligonucleotides would open up new possibilities of selective inhibition of RNA import *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Science and Technology, Government of India. S.M. and S.K.B. received Pool Officership and Junior Research Fellowship, respectively, from the Council of Scientific and Industrial Research. A.D. was a Senior Research Fellow of the University Grants Commission. We thank Swadesh Sahu and H.N. Dutta for the artwork.

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