Import of Yeast Mitochondrial Transcription Factor (Mtf1p) via a Nonconventional Pathway*

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The yeast mitochondrial (mt) transcription factor Mtf1p is imported into the mitochondria from the cytoplasm without a conventional mt-targeting presequence. To understand its import the mt translocation of wild type and mutant Mtf1p constructs was investigated in vitro under various assay conditions. We report here that Mtf1p, unlike most mt matrix proteins hitherto studied, is translocated into the mitochondria independent of membrane potential, ATP hydrolysis, and membrane receptor. This unusual import of Mtf1p was also observed on ice (3 °C). Sub-mitochondrial fractionation demonstrated that Mtf1p was translocated in vitro to one or more of the same mt sites as the endogenous protein that includes the matrix. To identify the mt-targeting sequence of Mtf1p, various N-terminal, C-terminal, or internally deleted Mtf1p derivatives were generated. The full-length and C-terminal deletions but not the N-terminal truncated Mtf1p were imported into mitochondria, indicating the importance of its N-terminal sequence for mt targeting. However, the internal deletion of Mtf1p revealed that the first 150-amino acid N-terminal sequence alone was not sufficient for mt targeting of Mtf1p, suggesting that an extended rather than a short N-terminal sequence is required for import. We favor a model in which Mtf1p adopts an import-competent conformation during translation. Consistent with this model are three findings: most of the protein sequence appears to be required for optimal import, urea denaturation eliminates its import competence, and the import-competent form of the protein is more resistant to tryptic hydrolysis than is the denatured protein. This represents a novel mechanism for mitochondrial protein import.

Mitochondria and chloroplasts are unique among eukaryotic organelles in possessing their own genomes. These extra-chromosomal DNAs are expressed by the organelle-specific transcription and translational machinery (1–5). The yeast mt genome encodes seven protein subunits of the energy-transducing enzyme complexes imbedded in the inner membrane, one ribosomal protein (i.e. Var1) of the small mitoribosomal subunit, two rRNAs (i.e. 21 S and 15 S rRNAs for the large and small subunits, respectively), a complete set of tRNAs, and an RNA subunit of mt RNase P (1, 5). The rest of the mt proteins (i.e. ~97%) are encoded by the nuclear genome, translated in the cytoplasm, and then imported into different compartments of mitochondria using protein-specific import mechanisms (6–10). Because the vast majority of mt proteins are the products of nuclear genes, mt protein import represents a fundamental subject of investigation in the cellular protein trafficking and the biogenesis of the organelle.

In recent years a great deal of experimental effort has unveiled some distinct features of the mt protein import pathway. Most of the nuclear-encoded mt proteins so far studied are synthesized with an N-terminal presequence that is positively charged with a notable absence of negatively charged residues and has the potential to form an amphipathic α-helix (6, 7, 10). The mt matrix proteins are imported into mitochondria by a general import pathway composed of cytoplasmic chaperones, two hetero-oligomeric membrane complexes (a TOM complex in the outer membrane and a TIM complex in the inner membrane), and the mt chaperones. In the cytosol the translocation competent conformation of the precursor is maintained by one or more cytoplasmic ATP-dependent chaperone proteins (6–8). The N-terminal presequence of the precursor interacts with a receptor on the cytosolic surface of the mt outer membrane (11–13). This interaction then catalyzes the inward movement of preprotein through the membrane-integrated TOM/TIM channel complexes at the contact sites of the mt outer and inner membranes. Protein import across both mt membranes into the matrix is facilitated by dynamic interaction between the TOM and TIM complexes (14), which provide an aqueous channel in the hydrophobic lipid bilayer for protein transport (15). Further translocation of preprotein into the mt matrix requires a membrane potential across the inner membrane, ATP hydrolysis, and mt chaperones (6–10). In the final event of the import process the N-terminal presequence of the imported protein is often proteolytically removed by the mt processing peptidase to generate a mature protein (16, 17).

We have been investigating mt transcription in budding yeast, Saccharomyces cerevisiae. It was found that a non-aneucleotide (TATAAGTAAA (+2)) promoter (18, 19) and a single mt RNA polymerase consisting of two protein subunits (a 145-kDa core polymerase and a 43-kDa transcription factor Mtf1p) (20–25) are necessary for mt gene-specific transcription. Both subunits of the mt RNA polymerase are nuclear gene products and imported into the mt matrix after synthesis in the cytosol. Interestingly, the initiating methionine is the only amino acid missing from the mature Mtf1p purified from the isolated mitochondria (25) suggesting that mt import of Mtf1p occurs...
EXPERIMENTAL PROCEDURES

Materials—The vector pGEM3 and the rabbit reticulocyte lysate (RRL)-based transcription-translation system were purchased from Promega, Inc., Madison, WI. Redivue T7 (Takara, Japan), 10 mM TCEP, and 10 mM ATP was generously provided by Drs. Nikolaus Pfanner (Germany) and Carla M. Koehler (Switzerland). The oligonucleotide primers were made at the University of Chicago core facilities or from Integrated DNA Technologies, Inc. (Coralville, IA). The oligonucleotide primers were made at the University of Chicago core facilities or from Integrated DNA Technologies, Inc. (Coralville, IA).

Generation of Mutated Mtf1p—The full-length and deletion derivatives of Mtf1p were generated in vitro using various truncated MTF1 templates in the T7 expression system. Different MTF1 regions were subcloned into the pGEM3 vector by PCR. For full-length Mtf1p expression, the whole coding sequence of Mtf1p was copied from the parent plasmid by PCR using a 5′ primer (MTF1–5′: AGGAATTCAGTAAGA-ACCAGAGTGCTCTGTTTGATG) and a 3′ primer (MTF1–3′: GATTCGTCGACATCAACGAGTGTCTCAGTGTAC). The boldfaced triplets, ATG in the 5′ and the stop signal in the 3′ primer, represent translational start and stop signals for MTF1 expression. The underlined sequences represent the restriction sites (e.g. EcoRI and SalI) used in the subsequent subcloning into the pGEM3 vector. Similarly, the 5′ deletion clones of the MTF1 (i.e. Δ(2–30) and Δ(2–52)) were constructed with different 5′ primers corresponding to various regions of the MTF1 coding sequence and the above-described 3′ primer (i.e. MTF1–3′). The 3′ deletion of MTF1 (i.e. Δ(226–341), Δ(299–341), and Δ(325–341)) was also generated using three different 3′ primers carrying a stop codon and the above-described 5′ primer (i.e. MTF1–5′). These PCR DNA products were cloned into the EcoRI/SalI sites of pGEM3 vector and then used for in vitro protein expression.

The internal deletion clones Δ(100–144) and Δ(153–196) were each generated by two separate PCRs followed by two subsequent clonings. For Δ(100-144) construction, the PCR MTF1 product encoding amino acids 1-99 of the N-terminal region of Mtf1p was subcloned first into the EcoRI/KpnI sites of the vector pGEM3, and then another PCR product encoding amino acids 145-341 of the C-terminal sequence of Mtf1p was ligated in-frame into the KpnI/HindIII sites of the same vector. This new construct carries a 45-amino acid internal deletion (i.e. Δ(100–144)) of Mtf1p sequence. Similarly, for the Δ(153–196) clone the PCR product corresponding to amino acids 1-152 of Mtf1p was subcloned into the EcoRI/KpnI sites of vector pGEM3, and the DHFR sequence was placed at the 3′-end of MTF1 in-frame by ligation into the SalI/HindIII sites of the same vector. At the fusion site two additional residues (Val and Asp) were introduced due to the presence of the SalI site between the MTF1 and DHFR sequences. Similarly, the DHFR-MTF1 fusion was con-
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biochemical steps of protein import into the mitochondria. We have used this in vitro assay system to understand more thoroughly the import strategy of Mtf1p using isolated yeast mitochondria. Import of another matrix-targeted mt preprotein F1-ATPase subunit β (F1β) was followed as a control. Mt translocation of F1β carrying a 33-amino-acid presequence, involves the classic import pathway (29). Equal counts of the 35S-labeled F1β or Mtf1p, generated in the RRL-coupled transcription-translation reactions, were used in the import studies. In the absence of proteinase K treatment, both mature and precursor forms of F1β were co-precipitated with mitochondria (Fig. 1, lane 1). The large F1β (designated by “P”), which appears to be the same size as the in vitro synthesized protein, disappeared upon proteinase K treatment (Fig. 1, lane 2). Thus, this represents the precursor F1β that was associated with mitochondria but not imported into the mt inner membrane/matrix. On the other hand, the short form of F1β designated by “M” was an imported product (Fig. 1, lanes 1 and 2) whose leader sequence was removed by an mt matrix protease. In contrast, the apparent molecular size of Mtf1p did not change after import (Fig. 1, compare lane 4 versus lane 5). Furthermore, a higher proportion of the protein associated with the mitochondria was found in a proteinase-resistant compartment in the case of Mtf1p than for F1β (Fig. 1, lane 2 versus lane 5). As described in more detail in a later section, these proteinase K-resistant proteins were deemed to be inside the mitochondria, because total F1β or Mtf1p became sensitive to proteinase K when mitochondria following import were lysed with Triton X-100 and then treated with proteinase K (Fig. 1, lanes 3 and 6). This result indicates that, whether normalized to total import or mt association, Mtf1p appears to be imported at least as efficiently as does F1β.

Mtf1p Import in the Simultaneous Absence of Membrane Potential, ATP, and Physiological Temperature—We have previously shown that the import of Mtf1p occurs in the absence of either membrane potential or ATP (26). It is possible that for Mtf1p import the membrane potential or exogenous ATP may serve as alternate energy sources. To investigate this further, Mtf1p import into the mitochondria was carried out under minimal conditions, i.e. in the simultaneous absence of ATP, membrane potential, and physiological temperature. In the control experiments, externally added ATP or/and NADH was not required for the import of either F1β (Fig. 2A, lanes 1–3) or Mtf1p (Fig. 2A, lanes 8–10) and thus did not discriminate between their import pathways. However, when both exogenous ATP and NADH were not provided, and the electrochemical gradient across the mt inner membrane was discharged with valinomycin (K+ ionophore) or/and CCCP (H+ ionophore), import of F1β (Fig. 2A, lanes 4–6) but not Mtf1p (Fig. 2A, lanes 11–13) was greatly reduced. Similarly, Mtf1p (Fig. 2A, lane 14) but not F1β (Fig. 2A, lane 7) was imported in the simultaneous absence of electrochemical gradient (CCCP/valinomycin-treated mitochondria), an exogenous energy (minus ATP) and the endogenous source of energy (apprerase-treated import mix). A comparable level of Mtf1p import but not F1β import was also observed under these conditions when import was performed on ice (Fig. 2B). Because there are no other examples of protein import into mitochondria occurring in the simultaneous absence of these import requirements, the Mtf1p import pathway appears to be unique.

We have also confirmed an earlier observation (26) that Mtf1p import can occur with trypsin-treated mitochondria but that was not the case for F1β, which requires a protease-sensitive mt receptor (data not shown). This suggests that mt association or import of Mtf1p does not involve a specific interaction with the receptor implicated in mt import. An alternative model is that Mtf1p gains access to the mt matrix by a quite different pathway employing an mt surface receptor that is protease-resistant. Negatively charged phospholipid of mt membrane could be such a recognition molecule for the positively charged internal sequence of Mtf1p (see below) as has been proposed for translocation of yeast mt apocytochrome c (30, 31) and Drosophila antennapedia transcription factor (32). To explore whether the mt membrane phospholipids rather than a proteinaceous receptor is involved in the mt translocation of Mtf1p, the in vitro import reaction was carried out with mitochondria pretreated with one or more phospholipases (i.e. bovine pancreatic phospholipase A2, Clostridium phospholipase C, Streptomyces phospholipase D, and/or human plasma sphingomyelinase) at 37 °C for 2 h. None of these phospholipase treatments of mitochondria influenced the uptake of Mtf1p (data not shown).

Mtf1p Import Is Completed within 5 min—As we described above, the mt import of Mtf1p occurs without the general

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**Fig. 1. In vitro import of Mtf1p and F1β.** The import assays were carried out at 28 °C for 30 min as described under “Experimental Procedures.” After incubation, mitochondria were isolated by centrifugation and then gently re-suspended in the import buffer. One third of the mt suspension remained untreated for mt-assocation assay, one third of the mix was treated with proteinase K to identify the imported Mtf1p, and the remaining one-third mix was treated with 0.1% Triton X-100 followed by proteinase K digestion. After the addition of protease inhibitor PMSF, each mt suspension was lyophilized, suspended in gel buffer, and run on a SDS-PAGE. The radiolabeled protein was visualized by fluorography. The “P” and “M” letters at the left side indicate precursor (511-amino-acid peptide) and mature (478-amino-acid peptide) forms of F1β, respectively.

**Fig. 2. Import of Mtf1p or F1β in the absence of general import factors.** The import assay was carried out at 28 °C (A) or on ice (B) for 30 min in the absence of membrane potential and ATP. Before the addition to the import mix, the isolated mitochondria and in vitro translation products were separately treated with apprerase (5 units) at 25 °C for 20 min to remove endogenous ATP, whereas the membrane potential was destroyed by pretreatment of mitochondria with 40 μM CCCP and 5 μM valinomycin to destroy the mt membrane potential. After a 30-min import reaction, the in vitro imported proteins were detected by proteinase K digestion of intact mitochondria followed by SDS-PAGE and fluorography.
For a zero time control, Mtf1p, proteinase K, and mitochondria were added simultaneously and incubated for variable times under these specific conditions. To identify the intra-mitochondrial location of the imported proteins, we have performed sub-mitochondrial fractionation by selective opening of the mt outer and inner membranes by hypotonic swelling, and freeze-thaw and sonication, respectively (28). The specificity and authenticity of this sub-fractionation procedure was determined by monitoring the distribution of endogenous mt protein markers (i.e. cyt b2 for the inter membrane space, AAC for the inner membrane, and Mtf1p for the matrix) in the mt sub-fractions as well as in the whole mitochondria by immunoblotting with specific antibodies (Fig. 5A). Cyt b2 was detected in the whole mitochondria (Mt) and in the inter membrane space (IMS) (Fig. 5A, lanes 1 and 2) but not in the inner membrane (IM) or matrix (MTX) fraction (Fig. 5A, lanes 3 and 4). The inner membrane protein AAC was detected in the mitochondria and in the inner membrane (Fig. 5A, lanes 1 and 3) but not in the inter membrane space or matrix fraction (Fig. 5A, lanes 2 and 4). The endogenous Mtf1p was detected in the whole mitochondria, matrix and inner membrane fractions (Fig. 5A, lanes 1, 3, 4).

Using the same fractionation procedure the sub-mitochondrial location of the in vitro imported Mtf1p was determined. As before, precursor and mature forms of F1β were seen to be associated with incubated mitochondria (Fig. 5B, top panel, lane 1), and the majority of the mature form but not the precursor was proteinase K-resistant (Fig. 5B, top panel, lane 2) indicating its translocation to the mitochondria. Most of the mt-associated Mtf1p was proteinase K-resistant (Fig. 5B, bottom panel, lanes 1 and 2) indicating its efficient import. After import, when the mt outer membrane was disrupted by osmotic shock, neither Mtf1p nor F1β was released in the solution (Fig. 5B, lane 3). This suggests that these proteinase K-resistant proteins were present within the mitoplasts rather than in the inter membrane space (IMS). The mitoplasts were further separated into matrix (MTX) and inner membrane (IM) by disruption of the inner membrane as described under “Experimental Procedures”. Mtf1p and F1β both were recovered in the matrix and inner membrane fractions, although in different proportions (Fig. 5B, lanes 4 and 5). The majority of the imported F1β was found with the inner membrane fraction, whereas the Mtf1p was equally distributed between the inner membrane fraction and the matrix. A similar distribution of both proteins was also observed when mitoplasts were pretreated with proteinase K before sonication. This suggests that a fraction of imported Mtf1p or F1β was associated with the matrix side of the mt inner membrane. Because F1β is a subunit of the hetero-oligomeric ATPase complex of the mt inner membrane, we did not anticipate F1β in the matrix fraction. It is possible that F1β might be displaced into the MTX upon disruption of the mt inner membrane in the presence of salt. The distribution of Mtf1p among sub-mitochondrial fractions was similar when import was carried out in the absence of ATP and membrane potential (see below). This unexpected distribution of Mtf1p between the soluble matrix and the inner membrane could be

**Fig. 3. Time course for Mtf1p import.** Import was performed at 28 °C (○—○), on ice (3 °C) (■—■), or on ice with mitochondria pretreated with CCCP/valinomycin/apyrase (□—□). Mtf1p was incubated with mitochondria for variable times under these specific conditions followed by proteinase K digestion on ice for 30 min. After re-isolation of mitochondria, Mtf1p was detected by SDS-PAGE and fluorography. For a zero time control, Mtf1p, proteinase K, and mitochondria were added simultaneously and incubated for 30 min. Under these conditions Mtf1p was completely digested by each of these concentrations of proteinase K (Fig. 4, lanes 1, 3, 4). However, the incubated Mtf1p became sensitive to proteinase K when mitochondria were lysed with Triton X-100 after the import reaction (bottom panel), as anticipated.

**Sub-mitochondrial Location of Endogenous Mtf1p and of Mtf1p Imported in Vitro**—To identify the intra-mitochondrial site of the imported proteins, we have performed sub-mitochondrial fractionation by selective opening of the mt outer and inner membranes. It is possible that the efficiency of Mtf1p import might be slower when import is performed at low temperature or/and in the absence of import factors than that under the standard conditions. To explore this possibility we compared the time course for Mtf1p import at 28 °C, on ice (3 °C), or on ice without membrane potential and ATP (Fig. 3). Import was carried out for different time periods as above. Because Mtf1p import is not inhibited at low temperature or under import-poisoning conditions, and proteinase K requires several minutes for complete digestion of Mtf1p, a 0-min import control was also performed. In the 0-min control, Mtf1p, proteinase K, and mitochondria were added simultaneously and incubated on ice for 30 min. The 0-min value was subtracted from the other import measures and then plotted against import time (Fig. 3). It appears that 5-min import of Mtf1p is essentially completed under any of these conditions. Between zero time and 5 min there appears to be a linear increase of Mtf1p import. This suggests that the membrane potential, ATP, or the import temperature did not influence the import capacity of Mtf1p.

**Is Mtf1p Resistant to Proteinase K?**—One possibility for this unusual import behavior of Mtf1p could be due to high resistance of Mtf1p to protease K degradation. To pursue this issue a series of proteinase K concentrations in the range of 0.1 to 1.0 mg/ml was utilized for the digestion of pre-import or imported Mtf1p (Fig. 4). The in vitro translation Mtf1p products were incubated with these concentrations of proteinase K on ice for 30 min. Under these conditions Mtf1p was completely digested by each of these concentrations of proteinase K (Fig. 4, top panel). On the other hand, when Mtf1p was incubated with isolated mitochondria, the imported Mtf1p became resistant to proteinase K digestion using proteinase concentrations that effectively digest pre-import Mtf1p (middle panel). However, the incubated Mtf1p became sensitive to proteinase K when mitochondria were lysed with Triton X-100 after the import reaction (bottom panel), as anticipated.

**Proteinase K (mg/ml)** - 0.1 0.25 0.5 1.0

**Mtf1p alone**

**Mtf1p imported**

**Mtf1p imported and mt lysis with 0.1% Triton X-100**

**Fig. 4. Digestion of Mtf1p with different concentrations of proteinase K.** The proteinase K digestion was carried out on ice for 30 min. The upper panel exhibits proteinase K digestion of Mtf1p without mitochondria. The middle panel represents proteinase K digestion of Mtf1p after incubation with mitochondria, and the bottom panel exhibits proteinase K digestion of Mtf1p following import and lysis of mitochondria with 0.1% Triton X-100. The latter two experiments were performed as described in the legend to Fig. 1. These results are identical whether or not mitochondria were re-isolated prior to proteinase K digestion and Triton X-100 treatment for the results illustrated here.
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Fig. 5. Sub-mitochondrial localization of Mtf1p or F1β following import. A, Mtf1p or F1β was imported into the mitochondria for 30 min and then treated with proteinase K. Mitochondria were re-isolated by centrifugation and then sub-fractionated as described under "Experimental Procedures." The presence of radiolabeled Mtf1p or F1β in these mitochondrial fractions was examined by SDS-PAGE and fluorography. The symbols used: P, precursor F1β; M, matured F1β; Mt, the intact mitochondria; IMS, inter membrane space; IM, inner membrane; MTX, matrix; PK, proteinase K. B, detection of endogenous mitochondrial proteins by immunodecoration with rabbit antibodies raised against these proteins. C, sub-mitochondrial distribution of Mtf1p and F1β under the minimal import conditions. After import and proteinase K digestion, mitochondria were re-isolated and then fractionated into matrix (MTX) and membrane (IM). The labeled protein was detected by SDS-PAGE and fluorography.

Fig. 6. A, schematic presentation of the predominant negatively and positively charged regions in Mtf1p. B and C, mt association and import of Mtf1p carrying N-terminal, C-terminal, and internal deletions, respectively.

In each of the N-terminal truncations the initiator methionine was preserved. The C-terminal truncated Δ299–341 derivative was very similar to the earlier Mtf1p fragment generated from the BglII-digested MTF1 template (26). The internal deletions included a region of predominant positive charge (i.e. Δ153–196), the regions of predominant negative charge (i.e. Δ100–144 and Δ216–260), and two neutral areas (i.e. Δ50–90 and Δ272–291). Each of the MTF1 clones produced one predominant Mtf1p product of the predicted size after incubation with the coupled transcription-translation RRL system. The ability of the mitochondria to import each of the Mtf1p products was examined. Most Mtf1p products exhibited a similar level of mt association (i.e. –15–20% of the input protein) (Fig. 6, B and C, top panels). However, proteinase K digestion revealed that the import capacity of these Mtf1p products varied significantly depending on the sequence they carried. The full-length and the C-terminal truncated Mtf1p products (Δ226–341, Δ299–341, and Δ325–341) retained import capacity (Fig. 6B, bottom panel). Maximum import was observed with the full-length protein, whereas the C-terminal truncated Mtf1p products exhibited weaker import capacity. The mutant Δ325–341 lacking the last 16 amino acid residues, exhibited the greatest import capacity of the C-terminal truncated mutants. On the other hand, the N-terminal deletion derivatives of Mtf1p (i.e. Δ2–30 or Δ2–52), whose association with mitochondria was comparable to the wild type level, did not have any import activity. This result suggests that the N-terminal sequence of Mtf1p is important for its mt translocation. To determine whether one or more of the other sequences had an
impact on Mtf1p import, we also studied a group of internal sequences. In these deletions we focused on regions of Mtf1p that were predominantly negatively charged (i.e. $\Delta$(100–144) and $\Delta$(216–260)), positively charged (i.e. $\Delta$(153–196)), or neutral (i.e. $\Delta$(50–90) and $\Delta$(272–291)) (Fig. 6A). With most of these internal deletions there was a drastic reduction of Mtf1p import (Fig. 6C, bottom panel). The import of $\Delta$(50–90) or $\Delta$(100–144) was profoundly reduced, whereas the $\Delta$(216–260) derivative was least affected (Fig. 6C). These results indicate that at least the first 150 amino acids are required for targeting to the mitochondria.

Does Mtf1p Adsorbed onto the mt Membrane Become Proteinase K-resistant—All Mtf1p constructs described above associate with the isolated mitochondria. However, only some of these membrane-associated Mtf1p products exhibited proteinase K resistance. For example, most N-terminal deletion mutants of Mtf1p were susceptible to proteinase K digestion despite their normal association/adsorption on mitochondria. This argues that the membrane association/adsorption of Mtf1p is not the major factor for its sensitivity or resistance to proteinase K digestion. To address this issue further, we have selected the import-competent full-length Mtf1p, the import-incompetent $\Delta$(2–30)Mtf1p, as well as the control protein $F_1\beta$. The $\Delta$(2–30), which is import defective, was also used as a negative control. After import and with or without proteinase K treatment, the presence of Mtf1p in the mt membrane and matrix fractions was determined by sub-mitochondrial fractionation. Without proteinase K treatment, Mtf1p was detected in both membrane and matrix fractions, but predominately in the membrane portion (Fig. 7, lane 2 versus lane 1). However, the proteinase K treatment of mitochondria following import reduced the amount of Mtf1p on the mt membrane fraction (Fig. 7, lane 4) so that the residual amount in this fraction was similar to the amount in the matrix fraction (Fig. 7, lanes 1 or 3). This suggests that the membrane-associated Mtf1p could be classified into two distinct populations: proteinase K-sensitive and proteinase K-resistant. The protease-sensitive Mtf1p might be on the cytoplasmic surface of the mt outer membrane and be readily accessible to the external proteinase K for degradation. Conversely, the protease-resistant Mtf1p is in an inaccessible site of intact mitochondria, probably on the inner face of the inner membrane. As anticipated, Mtf1p levels in the matrix fraction remained the same whether mitochondria were pre-treated with proteinase K or not (Fig. 7, lane 1 versus lane 3). On the other hand, in the absence of proteinase K treatment, the $\Delta$(2–30) mutant was recovered mainly in the membrane fraction (Fig. 7, lane 6) corroborating our earlier observation that this mutant does associate with the mt membrane. However, the membrane-associated $\Delta$(2–30) mutant on intact mitochondria was fully susceptible to the external proteinase K (Fig. 7, lanes 7 and 8) indicating that it cannot be translocated into the mt matrix or even the inner surface of the inner membrane. In another control experiment, $F_1\beta$ was recovered in both matrix and membrane fractions (Fig. 7, lanes 9 and 10).

Together, this finding clearly indicates that Mtf1p binds to the mt outer membrane (protease-sensitive complex), proceeds through the mt double membrane (protease-resistant?), and then reaches the mt matrix (fully protease-resistant).

**Import of Urea-denatured Mtf1p**—The mutational analysis described above suggests that Mtf1p might use an import-competent secondary structure rather than a short N-terminal presequence. If this is the case, unfolding of the putative Mtf1p structure should inhibit Mtf1p import. To pursue this issue the in vitro synthesized protein was precipitated with ammonium sulfate and then resuspended either in the 10 mM Tris-HCl buffer, pH 7.5, containing no urea or 8 M urea. Import was carried out at 28 °C for different time periods. In the first and third panels from the top, lanes 1–5 represent import of ammonium sulfate-precipitated proteins suspended in Tris buffer, and 80 mM urea (final concentration) was added in an import reaction. In the second and fourth panels from the top, lanes 1–5 represent import of ammonium sulfate-precipitated protein suspended in 8 M urea. These suspended proteins were diluted so that the final urea concentration was 80 mM. C, graphical presentations of mt import of native Mtf1p (●), urea-denatured Mtf1p (○), urea-denatured Mtf1p plus RRL (△–△), or renatured Mtf1p (▲–▲). 10 μl of RRL were directly added to the 100-μl import mix of urea-denatured Mtf1p as a potential source of import factor in the lysate. In the last case, the urea-denatured Mtf1p was diluted 100-fold (i.e. 80 mM urea), final) in the import reaction, incubated for 30 min at room temperature (i.e. 25 °C) to allow renaturation, and then mitochondria were added to monitor import.

**Fig. 7. Sub-mitochondrial fractionation of Mtf1p following import incubation with mitochondria.** After import reaction at 28 °C for 30 min, one half of the import mix was treated with proteinase K, whereas the other half remained untreated. Mitochondria were re-isolated by centrifugation and fractionated into matrix (MTX) and membrane (MM) as described earlier. Mtf1p or $F_1\beta$ in these mt fractions was detected by SDS-PAGE and fluorography.

**Fig. 8. Import of urea-denatured Mtf1p or $F_1\beta$.** A, lanes 1 and 2 represent import of the in vitro translated native protein at 28 °C for 30 min in the absence or presence of 80 mM urea added during import reaction. B, the in vitro translated Mtf1p or $F_1\beta$ product was precipitated with ammonium sulfate (66% saturation). After centrifugation, protein pellet was suspended in 10 mM Tris-HCl buffer, pH 7.5, containing no urea or 8 M urea. Import was carried out at 28 °C for different time periods. In the first and third panels from the top, lanes 1–5 represent import of ammonium sulfate-precipitated proteins suspended in Tris buffer, and 80 mM urea (final concentration) was added in an import reaction. In the second and fourth panels from the top, lanes 1–5 represent import of ammonium sulfate-precipitated protein suspended in 8 M urea. These suspended proteins were diluted so that the final urea concentration was 80 mM. C, graphical presentations of mt import of native Mtf1p (●), urea-denatured Mtf1p (○), urea-denatured Mtf1p plus RRL (△–△), or renatured Mtf1p (▲–▲). 10 μl of RRL were directly added to the 100-μl import mix of urea-denatured Mtf1p as a potential source of import factor in the lysate. In the last case, the urea-denatured Mtf1p was diluted 100-fold (i.e. 80 mM urea, final) in the import reaction, incubated for 30 min at room temperature (i.e. 25 °C) to allow renaturation, and then mitochondria were added to monitor import.
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shown). However, between 15 and 30 min some import of urea-denatured Mtf1p appears to occur (Fig. 8B, second panel from top, lanes 3–5) probably due to renaturation of Mtf1p to its import-competent conformation during import incubation. On the other hand, urea denaturation did not interfere with the mt import of F1β (Fig. 8B, compare the third and fourth panels).

The cell-free RRL translation system carries Hsp70 and Hsp90 chaperone-like import factors (36, 37). There is also a report that the cytoplasmic Hsp70 chaperone binds mt precur-

In Vitro Import of DHFR-Mtf1p Fusion Protein—A chimera of the cytosolic protein dihydrofolate reductase (DHFR) and an mt presequence has been frequently used for functional analysis of mt targeting sequences. To determine whether Mtf1p is capable of targeting a heterologous protein into mitochondria, mouse cytoplasmic protein DHFR was fused to the C terminus of MTF1 clone produced two products: a

FIG. 9. Trypsin digestion of the native, 8 M urea-denatured and renatured Mtf1p. Mtf1p was denatured with 8 M urea as described in Fig. 7. For renaturation the urea-treated Mtf1p was diluted 100-fold in 10 mM Tris-HCl, pH 8.0, buffer, and incubated at room temperature for 30 min. Mtf1p digestion with trypsin was carried out in 10 mM Tris-HCl, pH 8.0, containing 80 mM urea. A, each of these Mtf1p products was digested with various concentrations of trypsin at 25 °C for 30 min. After incubation, trypsin was inactivated by adding 5-fold excess soybean trypsin inhibitor and 1 mM PMSF. The digested products were lyophilized, dissolved in sample buffer and run on SDS-PAGE (3–20% polyacrylamide gel). The protein bands were visualized by phosphorimaging. B, time course for Mtf1p digestion with 100 μg/ml trypsin at 25 °C.

FIG. 10. Mt import of Mtf1p/DHFR chimeras. A, schematic presentation of chimeric proteins. B, the top and bottom panels represent the mt association (+PK, without proteinase K treatment) and import (+PK, proteinase K treatment) of Mtf1p or its fusion products, respectively.

66-kDa major and a 43-kDa minor polypeptide. The 66-kDa polypeptide corresponds to the expected size of full-length Mtf1p-DHFR fusion protein, whereas the 43-kDa polypeptide was most probably Mtf1p alone. On the other hand, the DHFR-MTF1 (DHFR fused to the N terminus of MTF1) construct generated mainly the 66-kDa full-length DHFR-Mtf1p fusion protein.

Under the in vitro import conditions the mt association of these products (i.e. without proteinase K treatment) seems to be similar (Fig. 10B, upper panel), whereas their import capac-
subjected to Western blotting with anti-Mtf1p antibody. The mitochondria were lysed, electrophoresed on SDS-PAGE, and then treated with proteinase K. The full-length Mtf1p was efficiently imported into mitochondria as anticipated. On the other hand, following proteinase K treatment the full-length Mtf1p-DHFR fusion protein disappeared with the enhanced appearance of Mtf1p (Fig. 10B, compare upper and lower panels, lane 2). The simplest interpretation of this result is that the Mtf1p portion of the fusion protein was translocated into the mitochondria while its C-terminal DHFR half got stacked outside of mitochondria and became susceptible to proteinase K digestion. Conversely, most of the DHFR-Mtf1p fusion protein was imported into mitochondria as an intact protein while a small fraction of this fusion protein exhibited import of Mtf1p without its fusion partner DHFR (Fig. 10B, lower panel, lane 3). This result suggests that Mtf1p can translocate a heterologous protein into a protease-resistant compartment only when the protein is attached to the N-terminal end of Mtf1p. We have also found that the DHFR-Mtf1p fusion protein, like Mtf1p itself, is imported into mitochondria without an mt receptor protein, mt membrane potential, or ATP (data not shown). However, unlike Mtf1p the translocation of DHFR-Mtf1p was sensitive to the import temperature with import being significantly reduced at low temperature.

In Vivo Import of Mtf1p Is Not Influenced by the mt Genetic Background—Because the in vitro mt translocation of Mtf1p occurs without a membrane potential and ATP, we have measured the steady-state level of Mtf1p accumulation inside the mitochondria of living cells in which mt ATP synthesis and membrane potential are expected to be substantially reduced due to defective mt DNA. The mt accumulation of imported proteins using the conventional import pathway could be differentially influenced by these genetic (and physiological) variations. Furthermore, mt import and accumulation of Mtf1p, which is a component of mt transcription complex, could also be influenced by its interacting molecules such as mt DNA or the mt core RNA polymerase subunit. To explore these possibilities, we have assessed Mtf1p levels in mitochondria with different genetic backgrounds (Fig. 11). Mitochondria were isolated from the wild type and mutant yeast lacking Mtf1p (i.e., MTF1), the mt RNA polymerase (i.e., RPO41) or mt DNA (i.e., ρ0), and lysed in the sample buffer. Equal amounts of mt protein were electrophoresed on a SDS-PAGE, transferred onto an Immobilon-P nitrocellulose membrane, and then immunodecorated with rabbit polyclonal anti-Mtf1p antibody.

FIG. 11. Western blotting of mt proteins isolated from different yeast strains measured with anti-Mtf1p antibody. A, mitochondria were isolated from the wild type and mutant yeast lacking Mtf1p (i.e., MTF1), the mt RNA polymerase (i.e., RPO41) or mt DNA (i.e., ρ0), and lysed in the sample buffer. Equal amounts of mt protein were electrophoresed on a SDS-PAGE, transferred onto an Immobilon-P nitrocellulose membrane, and then immunodecorated with rabbit polyclonal anti-Mtf1p antibody. B, the steady-state levels of Mtf1p and Mna6p (an mt r-protein) in different mt background, which was determined by Western blotting with anti-Mtf1p and anti-Mna6p antibodies, respectively. The petites P2 and O,P2 carry nonfunctional mt genome, whereas the ρ0 strain lacks the whole mt DNA.

It is possible to construct a genetic model for Mtf1p import. The data suggest that the in vivo import and stability of Mtf1p are not influenced by mt ATP synthesis, mt respiration, or its interacting mt matrix molecules like mt DNA, or mt core RNA polymerase. We have also assessed the relative levels of Mtf1p and an mt small subunit r-protein in the wild type, respiratory-defective petites (P2 and O,P2) (40) as well as ρ0 yeast strains. Interestingly, the steady-state level of mt r-protein was strongly influenced by the mt genotype, whereas the Mtf1p levels remained almost the same (Fig. 11B).

DISCUSSION

We have reported here the conditions under which Mtf1p is imported and localized within isolated yeast mitochondria. Taken together from the results presented here and those from our earlier experiments, it appears that the translocation of Mtf1p into the mt matrix is carried out without a cleavable N-terminal presequence, a proteinaceous surface receptor, an mt membrane potential or ATP hydrolysis. This unusual import of Mtf1p is also observed when import is performed on ice (i.e., 3 °C). To our knowledge, this is the only known case of protein import into the mt matrix that does not involve any of the previously described requisites of the classic import pathway. Mtf1p is also capable of carrying a heterologous protein into the mitochondria by the same pathway. The sub-mitochondrial fractions demonstrated that the in vitro imported protein is translocated to the same sites as the endogenous protein. This suggests that Mtf1p under the in vitro conditions probably follows an import pathway similar to that for the endogenous protein of living cells. This is consistent with observations of intact yeast of various mt petite genotypes. The mt accumulation of Mtflp is unimpaired in yeasts that have functionally deficient mitochondria (petites) or that lack mt DNA (ρ0). This is not the case for an mt matrix protein (mt r-protein Mna6p) that appears to follow the canonical pathway into the mitochondria. Although Mtflp is regarded as a matrix protein, we find a significant portion of the protein associated with the mt inner membrane. We do not know the physiological significance of this distribution.

We favor an import model for Mtf1p that relies on a favorable import-competent conformation for several reasons. First, compared with other mt proteins hitherto studied, Mtf1p carries quite an extended targeting sequence. Indeed almost all of the protein sequence seems to be required for optimal import. Second, in contrast to Fβ (and other proteins using the canonical pathway), Mtflp import is dramatically reduced by urea denaturation. The unfolding induced by urea denaturation is thought, if anything, to facilitate the import of conventionally imported proteins (12, 34, 35). Urea-induced Mtflp unfolding
appears to disrupt the postulated import-competent conformation. The folding of Mtf1p into this conformation probably takes place during translation in the rabbit reticulocyte lysate, where ATP is available. The apparent energy independence of Mtf1p import could relate to the notion that the energy is expended during the synthesis and folding of the protein into an energy-dependent import-competent conformation. This could be facilitated by the charge segregation in the linear sequence of the protein, a segregation that could facilitate charge-charge interaction in the folding of the protein. The productive folding of this protein probably relies on the chaperones present in the reticulocyte lysate. We have evidence that the import of protein synthesized in the wheat germ extract depends upon different sequence domains, perhaps because of the differences in the chaperones of the wheat germ extract. Third, the newly translated and import-competent Mtf1p is more resistant to tryptic hydrolysis than is the urea-denatured protein. This argues that the two forms of the protein differ conformationally.

There are several other mt proteins that enter the mitochondria independent of one or more features that characterize the usual protein import pathway, but none of them is as fully independent of the features upon which mt import depends, as is the case for Mtf1p. Among these proteins, the translocation of subunit Va of cytochrome c oxidase into the mt inner membrane exhibits features that are apparently related to those implied for Mtf1p. Like Mtf1p, the mt import of subunit Va does not require a surface receptor and is insensitive to the temperature of import, and it requires a very low level of ATP (41), although it is dependent on the mt membrane potential. The mt inner membrane protein ADP/ATP carrier (AOC) does not have a cleavable presequence and is translocated into the mt inner membrane independent of ATP hydrolysis and mt-Hsp60 but requires a membrane potential across the inner membrane (42). The mt import of murine CLK-1 protein is guided by its leader sequence but does not need the membrane potential (43). A receptor or/and temperature-independent cellular protein translocation pathway has also been noticed with other non-mt proteins. The human HIV Tat protein or a recombining chimera carrying the 11-residue (YGRKRRQRRRR) highly positively charged transduction domain of Tat protein, moves into the cell in an energy-independent fashion and without any receptor or transporter involvement (44). The Drosophila antennapedia homeoprotein is also internalized into cells in the absence of a specific receptor and even at a low temperature (i.e. 10 °C) (45). Interestingly, yeast mt Mtf1p, HIV Tat, and Drosophila antennapedia homeoprotein are all transcription factors.

The other thoroughly studied exception to the classic mt import pathway is apocytochrome c, which is a 108-amino acid soluble protein of the mt inner membrane space. Apocytochrome c import shares some import features with Mtf1p, although apocytochrome c, unlike Mtf1p, does not cross the inner mt membrane. This apoprotein is synthesized without a cleavable N-terminal presequence, but the initiating methionine is missing from the mature form. The mt translocation of apocytochrome c does not require a receptor, ATP hydrolysis, or an electrochemical potential across the inner membrane of mitochondria (30). Mt targeting of apocytochrome c is mediated through two functionally independent structural domains located at the N and C termini of the protein, respectively (31). Apocytochrome c probably makes initial contacts with the outer membrane surface via an electrostatic association with the phospholipid headgroups (30, 31). This membrane association allows further interaction of this apoprotein with a protease-resistant inter membrane component(s). The apoprotein is then translocated across the outer membrane by diffusion, a reaction that appears to be reversible. Once in the inter membrane space, apocytochrome c interacts with cytochrome c heme lyase (CCHL) on the outer face of the inner membrane, which traps apocytochrome c in the inter membrane space of mitochondria (30, 31). In the final step apocytochrome c is fixed in the inter membrane space by covalent attachment of heme, a reaction catalyzed by CCHL.

What is the structure of Mtf1p that allows for its robust import? The amino acid sequence analysis of several dozens of the cleavable leader peptides of mt precursor proteins of yeast, Neurospora, and mammals reveals that these presequences usually contain several basic and hydroxylated residues but no acidic amino acids (7). These targeting sequences are also predicted to form an amphipathic positively charged α-helix in the membrane-like environments (46, 47), which seems to be required for preprotein recognition by the mt surface receptors (6–10) as well as for preprotein transport across the inner membrane (47, 48). A surplus of positively charged residues in the presequence also greatly facilitates preprotein transport across the inner membrane (i.e. transfer of a precursor protein from the TOM complex to the TIM complex is facilitated by stepwise interactions of the positively charged presequence with the negative charged regions on these two complexes) (47, 48). In contrast, Mtf1p carries neither a cleavable presequence (25) nor does its N-terminal sequence exhibit the usual presequence characteristics (i.e. the first 50-amino acid sequence of Mtf1p is not rich in basic and hydroxylated residues and has little capacity to form a positively charged amphipathic α-helix). This suggests that Mtf1p probably carries a nonconventional mt-targeting signal. To ascertain whether an internal region having some of the characteristics of the targeting sequence may exist, we inspected the sequence of Mtf1p for charge segregation. Between residues 100 to 260 in the middle of the protein there is a 44-amino acid region that contains 10 acidic and 2 basic amino acids, followed by a region between residues 157 and 197 that contains 10 basic but no acidic residues, and a third region between residues 207 and 260 that contains 14 acidic and 3 basic residues. Thus, it appears that an internal positively charged region of Mtf1p is surrounded by more negatively charged flanking sequences (Fig. 6A). We have also noticed a small patch of negatively charged sequence at the very end of Mtf1p. Because the charged amino acid residues are important for sub-cellular translocation of mt precursor proteins (6–10) as well as the human HIV Tat protein (44) we have explored by deletion analysis what role these charged or other noncharged regions may play in Mtf1p translocation. The deletion of N-terminal sequences of various lengths and of several internal sequences revealed that the first 50-amino acid N-terminal sequence of Mtf1p is essential but not sufficient for its mt targeting, because the internal sequences from residues 50–144 are also required. This strongly suggests that an extended sequence from 2 to 144 at least is absolutely required for Mtf1p import. Mtf1p import is also influenced by other sequences between 144 and 341 residues. It is not clear whether this is representative of several separated subdomains in this region of the protein that are required for its translocation.

Like Mtf1p, mt protein import without a canonical cleavable presequence has also been noticed with other mt proteins (e.g. AAC (42), apocytochrome c (31), Tom22 (49), Bcl-2 (50), CCHL (51), and BCS1 (52)). On the contrary, the signal sequence of some mt proteins is encoded by an N-terminal segment that is not removed on import (e.g. 70-kDa mt outer membrane protein (53), inter membrane space protein adenylate kinase (54), mt matrix proteins 3-oxoacyl-CoA thiolase (55) and GTP-AMP

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phosphotransferase (56)). Yet another class of mt or chloroplast proteins carries a cleavable presequence that is not necessary for organelle targeting (e.g. subunit 6 of yeast mt cytochrome bc₁ complex (57), mammalian mt phosphate carrier (58), or outer envelope receptor protein Tom68p of chloroplast (59)).

The recent protein data base from the yeast genome sequencing project reveals that almost one-third of nuclear-coded mt proteins indeed lack cleavable N-terminal presequences. According to the size of cleavable presequences yeast mt proteins can be categorized into three different classes. Class 1 consists of major mt proteins with a sizable presequence, which is often cleaved following import. This class includes the mt core RNA polymerase. Class II proteins do not have a cleavable presequence; however, their initiating methionine residue is missing from the mature form. MtFlp belongs to this class. Proteins belonging to class III remain intact even after their import into mitochondria (i.e. the N-terminal sequences of the preprotein and mature protein are identical) (i.e. Tom6p, r-protein Mrpl24, Met-tRNA synthetase). The proposed mtFlp conformation, unlike the N-terminal sequence of the mature protein, is also possible that MtFlp might use a completely unknown translocation mechanism. As explained above we favor a mechanism that derives from the generation of an appropriate import defective mutants (66). We believe that further understanding of the translocation mechanism of the remarkable proteins like MtFlp, TAT, and Vp22 (67) may open new possibilities for direct and efficient protein delivery systems in the context of protein therapy, as well as for epigenetic experimentation with model organisms. MtFlp may be useful for directing therapeutic targets to the mitochondria in vivo.

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