Amino-Terminal Extension Generated from an Upstream AUG Codon Increases the Efficiency of Mitochondrial Import of Yeast N^2,N^2-Dimethylguanosine-Specific tRNA Methyltransferases

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Fusions between the *TRM1* gene of *Saccharomyces cerevisiae* and *COXIV* or *DHFR* were made to examine the mitochondrial targeting signals of N^2,N^2-dimethylguanosine-specific tRNA methyltransferase [tRNA (m_2^2G)dimethyltransferase]. This enzyme is responsible for the modification of both mitochondrial and cytoplasmic tRNAs. We have previously shown that two forms of the enzyme are translated from two in-frame ATGs in this gene, that they differ by a 16-amino-acid amino-terminal extension, and that both the long and short forms are imported into mitochondria. Results of studies to test the ability of various *TRM1* sequences to serve as surrogate mitochondrial targeting signals for passenger protein import in vitro and in vivo showed that the most efficient signal derived from tRNA (m_2^2G)dimethyltransferase included a combination of sequences from both the amino-terminal extension and the amino terminus of the shorter form of the enzyme. The amino-terminal extension itself did not serve as an independent mitochondrial targeting signal, whereas the amino terminus of the shorter form of tRNA (m_2^2G)dimethyltransferase did function in this regard, albeit inefficiently. We analyzed the first 48 amino acids of tRNA (m_2^2G)dimethyltransferase for elements of primary and secondary structure shared with other known mitochondrial targeting signals. The results lead us to propose that the most efficient signal spans the area around the second ATG of *TRM1* and is consistent with the idea that there is a mitochondrial targeting signal present at the amino terminus of the shorter form of the enzyme and that the amino-terminal extension augments this signal by extending it to form a larger, more efficient mitochondrial targeting signal.

The *TRM1* gene of *Saccharomyces cerevisiae* codes for an enzyme that catalyzes the modification of a specific guanosine to N^2,N^2-dimethylguanosine in both cytoplasmic and mitochondrial tRNAs (17). In previous reports, we described the isolation and characterization of the *TRM1* gene from *S. cerevisiae* (14, 15). These studies demonstrated that *TRM1* is the structural gene for N^2,N^2-dimethylguanosine-specific tRNA methyltransferase [tRNA (m_2^2G)dimethyltransferase] (15) and that it codes for two forms of the enzyme, which differ by an amino-terminal extension of 16 amino acids (14). The two forms of tRNA (m_2^2G)dimethyltransferase are produced by initiating translation from either of two in-frame AUGs coded by the *TRM1* open reading frame. The AUG used to initiate translation is determined by the locations of the 5' ends of the *TRM1* mRNAs.

The fact that *TRM1* codes for two forms of tRNA (m_2^2G) dimethyltransferase differing by an amino-terminal extension suggested that the amino-terminal extension might function to direct tRNA (m_2^2G)dimethyltransferase into mitochondria for the modification of tRNAs synthesized within the organelle. The shorter form of the enzyme, lacking a putative mitochondrial targeting signal, would be located in the nuclear-cytoplasmic compartment, where it would modify cytoplasmic tRNAs. Our previous studies tested this hypothesis and demonstrated that the amino-terminal extension of tRNA (m_2^2G)dimethyltransferase was not essential for the modification of either mitochondrial or cytoplasmic tRNA (14). Consequently, the shorter form of tRNA (m_2^2G) dimethyltransferase must partition between both mitochondrial and nuclear-cytoplasmic compartments. This implies

that a region common to both forms of the enzyme contains a mitochondrial targeting signal. In this respect, *TRM1* contrasts with other characterized yeast genes that use a single gene to produce functionally analogous enzymes that are targeted to different intra- or extracellular compartments. The *SUC2* (5), *HTS1* (24), *FUM1* (38), *LEU4* (3), and *VAS1* (6) loci of *S. cerevisiae* each code for two enzymes that differ by an amino-terminal extension, and in each case it has been shown that the amino-terminal extension functions to direct the enzyme to an intra- or extracellular location different from that of the enzyme lacking the amino-terminal extension.

Since the amino-terminal extension of tRNA (m_2^2G)dimethyltransferase is not essential for directing this enzyme to mitochondria for subsequent modification of mitochondrial tRNAs, what is its function? Could the amino-terminal extension function by affecting the efficiency with which tRNA (m_2^2G)dimethyltransferase is imported into mitochondria? Very little is known about factors that influence the efficiency with which proteins are targeted to mitochondria. Determining how the amino-terminal extension of tRNA (m_2^2G)dimethyltransferase functions in this regard might provide insight into this aspect of the mitochondrial import process. The amino-terminal extension may function to increase the efficiency with which tRNA (m_2^2G)dimethyltransferase is imported into mitochondria by providing an additional, redundant mitochondrial targeting signal. If so, targeting information would be expected to be present in both the amino-terminal extension of tRNA (m_2^2G)dimethyltransferase and the shorter form of the enzyme. Redundant targeting signals have been shown to affect the rate at which the β subunit of F_1-ATPase is imported into mitochondria

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(2). An alternative mechanism through which the amino-terminal extension could alter the efficiency with which tRNA (m_2^2G)dimethyltransferase is imported into mitochondria is by forming a larger, more efficient targeting signal than that which is encoded downstream of the second ATG of the *TRM1* open reading frame. Finally, it is possible that the amino-terminal extension has no effect on the import of tRNA (m_2^2G)dimethyltransferase into mitochondria.

To examine the function of the amino-terminal extension in the compartmentalization of tRNA (m_2^2G)dimethyltransferase, amino-terminal sequences from this enzyme were tested for the ability to function as surrogate mitochondrial targeting signals when fused to passenger proteins that by themselves are not imported into mitochondria. Results from these experiments indicate that the amino-terminal extension of tRNA (m_2^2G)dimethyltransferase functions poorly as an independent mitochondrial targeting signal. The amino terminus of the shorter form of tRNA (m_2^2G)dimethyltransferase directs passenger proteins into mitochondria to a greater extent than does the amino-terminal extension but to a level substantially lower than that observed when the amino-terminal extension is linked to the amino terminus of the shorter form of the enzyme in the original in vivo configuration. The amino-terminal extension of tRNA (m_2^2G) dimethyltransferase must therefore function in conjunction with sequences derived from the shorter form of the enzyme to create a more efficient mitochondrial targeting signal. The two forms of tRNA (m_2^2G)dimethyltransferase likely partition between mitochondrial and nuclear-cytoplasmic compartments, with the equilibrium of the longer form of the enzyme shifted toward import into mitochondria and the equilibrium of the shorter form shifted toward a nuclear-cytoplasmic localization.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used were D273-10B (*MATα met6*) and WD1 (*MATα leu2 his3 ura3 coxIV::LEU2*). WD1 was transformed by using the alkali metal protocol of Ito et al. (20). Transformants were selected for uracil prototrophy on TRM medium (15). Transformants were tested for respiratory growth on YEPG medium (1% yeast extract, 3% ethanol, 2% peptone, 2% glycerol). The *Escherichia coli* strains used were JM101 (23) and HB101 (4). *E. coli* strains were transformed as described by Maniatis et al. (22).

Plasmid constructions. Hybrid genes were created between *TRM1* and sequences coding for either mouse dihydrofolate reductase (DHFR) or a pseudomature form of yeast cytochrome oxidase subunit IV (pm-COXIV) from *S. cerevisiae*. Coding sequences for DHFR and pm-COXIV were obtained from plasmids pDS5/2 (32) and G18 (33), respectively. Plasmids G18 and pDS5/2 and *S. cerevisiae* WD1 were generous gifts of the laboratory of Geoffrey Schatz, University of Basel, Basel, Switzerland. Several mutations introduced previously into the *TRM1* gene (14) by oligonucleotide-directed mutagenesis (39) facilitated construction of hybrid genes between *TRM1* and either *DHFR* or pm-*COXIV*. An additional mutation was introduced into *TRM1* by the same technique, using the mutagenic oligonucleotide TTCTGCGGATCCTTCCTTGAC. This mutation creates a *Bam*HI site at position +145 of *TRM1* by changing bases +145 to 147 from an AAA to a TCC. All mutations were introduced into a fragment of the *TRM1* gene containing sequences from –536 to +210. Complete *TRM1* genes carrying the desired mutations were reconstructed in pUC19 as described previously (14).

The various mutations in *TRM1* that were used are shown in Fig. 1A. The two *Bam*HI sites introduced at either position +49 or position +145 of *TRM1* were used to create in-frame fusions between the 5' coding regions of *TRM1* and *DHRF*. *TRM1* coding sequences downstream of the mutant *Bam*HI sites were removed by digesting the pUC19-derived plasmids with *Bam*HI and *Hin*dIII, followed by agarose gel electrophoresis. The *Hin*dIII site is from pUC19 and is 3' to the *TRM1* coding sequences. DNA fragments containing the vector and 5' coding sequences of *TRM1* were isolated by electroelution into dialysis tubing (22), followed by NACS (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) chromatography. A DNA fragment containing coding sequences of *DHFR* was isolated after digestion of plasmid pDS5/2 with *Bam*HI and *Hin*dIII. The *Bam*HI-*Hin*dIII fragment containing *DHFR* was ligated downstream of the 5' coding sequences derived from *TRM1* in the pUC19 plasmids. All fusion junctions were confirmed by DNA sequence analysis. Three codons, coding for serine, glycine, and isoleucine, respectively, were inserted at the site of fusion between *TRM1* and *DHFR*.

Two vectors, T3/T7-18 (Bethesda Research Laboratories) and G18 (33), were used to express hybrid genes in vitro and in vivo, respectively. The *Xba*I site introduced at position –7 of the *TRM1* sequence served to clone the *TRM1* open reading frame downstream of the T7 promoter in T3/T7-18. An *Xba*I-*Hin*dIII fragment containing the *TRM1-DHFR* hybrid genes was isolated from the pUC19 plasmids described above and inserted into T3/T7-18 that had been digested with the same restriction enzymes. Fusion genes were created between *TRM1* and pm-*COXIV* by isolating a *Bam*HI fragment from the T3/T7-18 constructions containing the *TRM1-DHFR* hybrid genes. One of the *Bam*HI sites was derived from the polycloning site of the vector, and the other was at the *TRM1-DHFR* fusion junction. The sizes of these fragments were either 58 or 154 base pairs, depending on which mutant *Bam*HI site was used to form the *TRM1-DHFR* hybrid. The *Bam*HI fragments were inserted into the unique *Bam*HI site of G18. Before insertion of either of the *Bam*HI fragments, G18 was digested with *Bam*HI and treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Insertion of either of the *Bam*HI fragments into G18 in the correct orientation created an in-frame fusion between the 5' coding region of *TRM1* and regions of *COXIV* coding for amino acids 19 through 155. In these constructions, the hybrid genes were located downstream of the *ADH1* promoter. The *TRM1-COXIV* hybrid gene was removed intact from the G18 constructions by digestion with *Eco*RI and *Hin*dIII. The purified fragment was inserted into T3/T7-18 that had been digested with the same restriction enzymes. Insertion of the *TRM1-COXIV* hybrids into T3/T7-18 placed the hybrid genes downstream of the T7 promoter.

In vitro transcription, translation, and mitochondrial import. Plasmids were purified for use in in vitro transcription reactions as described by Krieg and Melton (21). Plasmids were digested with *Hin*dIII, extracted with phenol, and precipitated with ethanol before in vitro transcription. Conditions used for in vitro transcription were those described by Chen and Douglas (7). After transcription, the RNA was passed through a Quik-Spin column (Boehringer Mannheim) to remove low-molecular-weight inhibitors of translation. Conditions used for translation, preparation of energized mitochondria, and mitochondrial import were those described by Daum et al. (9) as modified by Chen and Douglas (7), except that the reticulocyte lysate was not centrifuged or
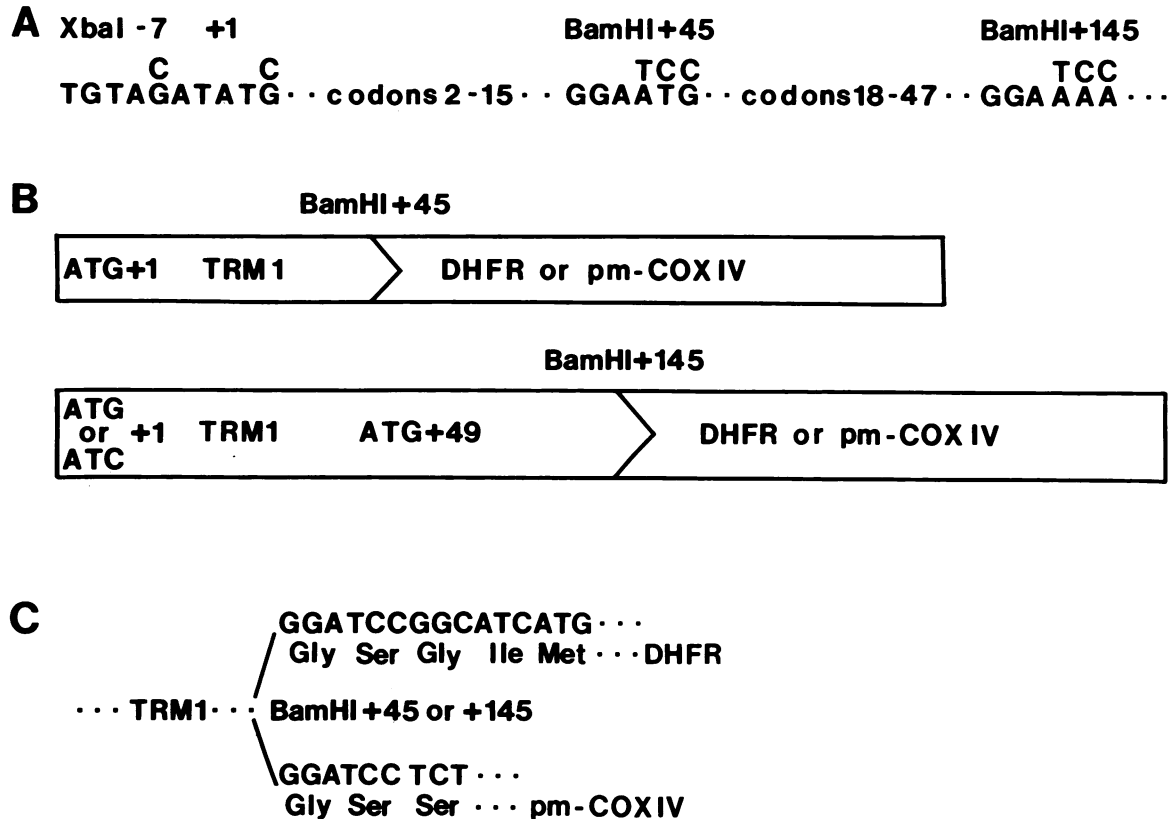
A XbaI -7   +1   BamHI+45   BamHI+145

                 C   C   TCC   TCC
        TGTAGATATG· ·codons2-15· · GGAATG· ·codons18-47· ·GGA AAA · · ·

B   BamHI+45

| ATG+1   TRM1   >   DHFR or pm-COXIV |

                           BamHI+145

| ATG
| or   +1   TRM1   ATG+49   >   DHFR or pm-COXIV
| ATC

C   GGATCCGGCATCATG· · ·
                 / Gly Ser Gly Ile Met · · ·DHFR

         · · ·TRM1· · · BamHI+45 or +145

                 \GGATCC TCT · · ·
                  Gly Ser Ser · · · pm-COXIV

FIG. 1. Construction of hybrid genes between *TRM1* and the gene coding for either mouse DHFR or pm-COXIV from *S. cerevisiae*. (A) Mutations introduced into *TRM1* that facilitated the construction of hybrid genes or were necessary for addressing specific issues pertinent to the function of the amino-terminal extension of tRNA (m$_2^2$G)dimethyltransferase in the compartmentalization of this enzyme. (B) Schematic representation of the hybrid genes. Genes joined at the *Bam*HI site at position +45 of *TRM1* code for amino acids 1 through 16 of tRNA (m$_2^2$G) dimethyltransferase, joined to the entire coding region of *DHFR* or to the region encoding amino acids 19 through 155 of COXIV. Hybrid genes joined at the *Bam*HI site at position +145 of *TRM1* code for either amino acids 1 through 48 or amino acids 17 through 48 of tRNA (m$_2^2$G) dimethyltransferase, depending on whether the first ATG was altered to an ATC, linked to the entire coding region of *DHFR* or to the region encoding amino acids 19 through 155 of COXIV. (C) DNA sequence of fusion junctions. In cases where *TRM1* was fused to *DHFR*, three amino acids, serine, glycine, and isoleucine, were inserted in frame between the two coding regions. When *TRM1* was fused to *COXIV*, there were no amino acids other than those found in the two reading frames inserted at the junction site.

passed through a Sephadex G-25 column. A typical import reaction was carried out in a volume of 100 μl. After a 30-min incubation at 30°C, three 30-μl portions were removed from the import reaction mixture. One portion was untreated, the second was made 0.4 mg/ml in proteinase K, and the third was made 0.4 mg/ml in proteinase K and 0.5% Triton X-100. These samples were incubated on ice for 30 min, made 3 mM in phenylmethylsulfonyl fluoride, and incubated on ice for 5 min. Mitochondrial pellets were collected by centrifugation at 13,000 × *g* for 5 min. Proteins synthesized in vitro were resolved by denaturing polyacrylamide gel electrophoresis. Radiolabeled proteins were detected by autoradiography after the gels were dried. The autoradiograph was used as a template to guide excision of gel pieces containing individual proteins from the gel. These gel fragments were rehydrated, dissolved in 30% hydrogen peroxide at 80°C, mixed with 10 ml of Budget Solve (Research Products, Inc.), and counted in a scintillation counter to determine the amount of radioactivity associated with each protein of interest.

RESULTS

Gene fusions and subsequent import of hybrid proteins into mitochondria were used to characterize the mitochondrial import signals of tRNA (m$_2^2$G)dimethyltransferase and

to determine the function of the amino-terminal extension in the compartmentalization of this enzyme. Nucleotide changes introduced into *TRM1* to facilitate the construction of gene fusions and to address issues relevant to the mitochondrial targeting signal(s) of tRNA (m$_2^2$G)dimethyltransferase are shown in Fig. 1A. Figures 1B and C show the fusions created between *TRM1* and the coding sequences of either mouse *DHFR* or pm-*COXIV* from yeast cells and the sequences across the fusion junctions, respectively. The *TRM1* sequences in these hybrid genes code for amino acids 1 through 16, 17 through 48, or 1 through 48 of tRNA (m$_2^2$G)-dimethyltransferase. Amino acids 1 through 16 are the amino-terminal extension of tRNA (m$_2^2$G)dimethyltransferase, amino acids 17 through 48 are the amino terminus of the shorter form of the enzyme, and amino acids 1 through 48 are a combination of these sequences that form the amino terminus of the longer form of the enzyme. The proteins that they form when fused with DHFR (and COXIV) are designated (1-16)-DHFR (-COXIV), (17-48)-DHFR (-COXIV), and (1-48)-DHFR (-COXIV), respectively.

Import of tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions into mitochondria in vitro. To determine whether the different amino-terminal sequences derived from tRNA (m$_2^2$G) dimethyltransferase could function as surrogate mitochon-
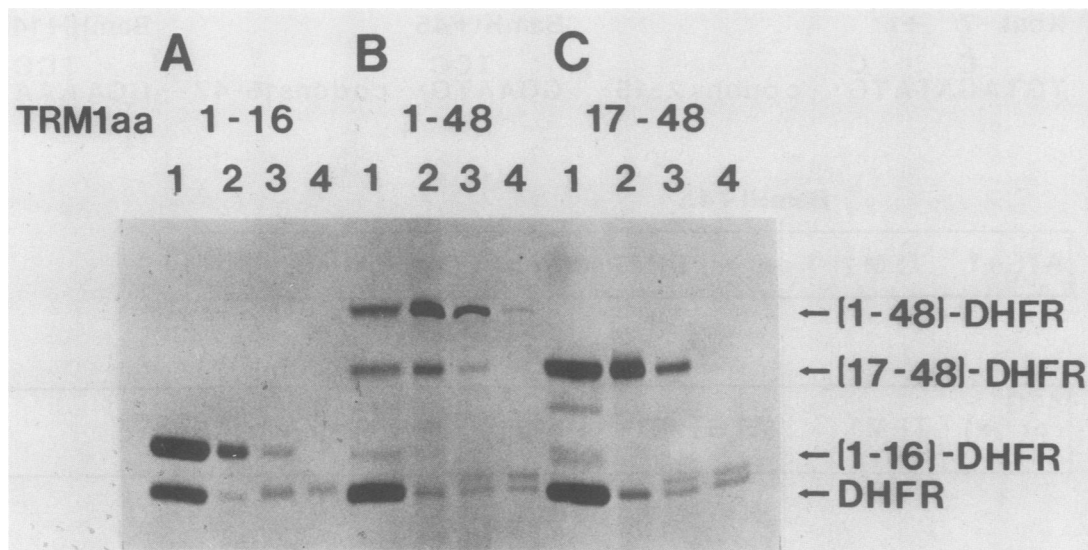
FIG. 2. Import of tRNA (m$_2^2$G)dimethyltransferase-DHFR fusion proteins into mitochondria in vitro. Import reactions were carried out as described in Materials and Methods. (A) (1-16)-DHFR; (B) (1-48)-DHFR; (C) (17-48)-DHFR. Lanes: 1, supernatants from untreated import reaction mixtures; 2, mitochondrial pellets from untreated import reaction mixtures; 3, mitochondrial pellets from import reaction mixtures treated with proteinase K; 4, mitochondrial pellets from import reaction mixtures treated with proteinase K in the presence of Triton X-100.

drial targeting signals for cytosolic DHFR, fusion proteins were tested for import into mitochondria in vitro. Fusion proteins were synthesized by the transcription-translation system of Stueber et al. (32). More than one protein product was synthesized when the hybrid mRNAs were translated in a reticulocyte lysate (Fig. 2). In each case, the uppermost band was the size expected from initiation of translation at the first AUG encountered downstream of the 5' end of the mRNA, and the lower bands can be explained by initiation at internal AUGs. The mRNA coding for the fusion protein with amino acids 1 through 48 of tRNA (m$_2^2$G)dimethyltransferase appeared to produce three prominent proteins in vitro (Fig. 2B, lanes 1 and 2). The sizes of the proteins indicated that translation initiated at sites corresponding to both in-frame AUGs coded by the *TRM1* portion of the hybrid gene and the first AUG of the *DHFR* coding sequence. Translation of mRNAs coding for (1-16)-DHFR and (17-48)-DHFR also generated proteins corresponding to the size of mature DHFR in addition to the expected fusion protein. Why the ribosome chooses certain codons for the initiation of translation in vitro and ignores other AUGs within the *DHFR* coding sequence is unknown.

Radiolabeled proteins were tested for import into yeast mitochondria under the conditions described by Daum et al. (9) as modified by Chen and Douglas (7). Import of proteins into mitochondria is often accompanied by proteolytic processing and is typically assayed by the resistance of the processed form of the protein to externally added protease. In the absence of proteolytic processing, import is assayed by mitochondrion-dependent conversion of a protein from protease sensitivity to protease resistance. Furthermore, if a protein is imported into the mitochondrial matrix, as is expected for the tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions, this conversion should be dependent upon a membrane potential across the mitochondrial inner membrane. In the absence of mitochondria, the full-length form of each of the tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions is sensitive to proteinase K. We did observe fragments that approached the size of mature DHFR but were partially resistant to proteinase K (data not shown). It has been

shown that DHFR is partially resistant to both proteinase K and trypsin, apparently a consequence of its tightly folded structure (11, 36). Therefore, even as a fusion protein, DHFR likely retains its tightly folded structure, whereas the amino acids derived from tRNA (m$_2^2$G)dimethyltransferase are presumably in a protease-sensitive domain independent of the folded conformation of DHFR. The tRNA (m$_2^2$G) dimethyltransferase-DHFR fusions were not proteolytically processed by isolated mitochondria (Fig. 2); therefore, import of these fusions was assayed by the mitochondrion-dependent conversion of the full-length fusion proteins from proteinase K sensitivity to proteinase K resistance.

The various tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions were associated with, and imported into, mitochondria to different extents (Fig. 2). The amounts of (1-48)- and (17-48)-DHFR that were resistant to proteinase K and presumably imported into mitochondria were substantially above the amount of DHFR that was proteinase K resistant (Fig. 2B and C, lanes 2 and 3). Consequently, amino acids 17 through 48 and 1 through 48 of tRNA (m$_2^2$G)dimethyltransferase appeared to function as surrogate signals for targeting cytosolic DHFR to mitochondria. Comparison of the relative amounts of (1-48)- and (17-48)-DHFR that were resistant to proteinase K (Table 1) indicates that amino acids 1 through 48 functioned more efficiently. The association of (1-16)-DHFR with mitochondria was less than that observed with the other two fusion proteins and only about twofold greater than the association seen with similar concentrations of DHFR (Fig. 2A and C, lanes 2). Whether this association represents productive binding is questionable, since the amount of (1-16)-DHFR that was imported into mitochondria as judged by resistance to proteinase K was similar to the amount of DHFR that was protease resistant (Fig. 2A and C, lanes 3). Because DHFR is intrinsically resistant to proteinase K, the background level at which DHFR entered mitochondria was not determined. Since DHFR has been shown to contain a cryptic mitochondrial targeting signal (19), it is possible that a portion of the DHFR that is resistant to proteinase K may have been imported into mitochondria. If DHFR is actually imported into mitochondria to a limited

TABLE 1. Import of tRNA (m$_2^2$G)dimethyltransferase-DHFR or -COXIV fusions into mitochondria in vitro[a]

Protein	% Bound[b]	% Imported[c]
(1-16)-DHFR	13 ± 3	3 ± 1
(17-48)-DHFR	45 ± 1	12 ± 2
(1-48)-DHFR	86 ± 3	36 ± 2
pm-COXIV	9 ± 5	ND[d]
(1-16)COXIV	29 ± 5	ND
(17-34)-COXIV	40 ± 2	<3[e]
(1-48)-COXIV	80 ± 4	43 ± 9

[a] Import reactions are described in Materials and Methods. Values except those for pm-COXIV, which was assayed only twice, and standard deviations are means of three independent experiments.

[b] Percent counts per minute of protein associated with the mitochondrial pellet divided by total radioactivity of the same protein (supernatant plus pellet) in the untreated import reaction mixtures.

[c] Percent counts per minute of protein associated with the mitochondrial pellet that was resistant to proteinase K divided by total radioactivity of the same protein (supernatant plus pellet) in the untreated import reaction mixtures.

[d] ND, Not detected by either autoradiography or scintillation counting.

[e] Detected by autoradiography but not significantly enough above background for quantitation by scintillation counting.

extent, the small amount of (1-16)-DHFR that is imported may in fact be attributed to the DHFR sequences rather than to amino acids 1 through 16 of tRNA (m$_2^2$G)dimethyltransferase. We conclude from these data that amino acids 1 through 16 of tRNA (m$_2^2$G)dimethyltransferase function marginally, if at all, as a surrogate mitochondrial targeting signal for cytosolic DHFR.

To demonstrate that resistance to externally added protease is a true measure of import into mitochondria, the sensitivity of the mitochondrion-associated tRNA (m$_2^2$G) dimethyltransferase-DHFR fusions to proteinase K was measured in the presence of valinomycin and potassium.

Valinomycin is a potassium ionophore that breaks down the membrane potential component of the electrochemical gradient of protons across the mitochondrial inner membrane, thereby inhibiting protein import into the organelle (27). The F$_1$-ATPase β subunit precursor was used as a control in these experiments. Import of the β subunit into mitochondria in vitro has been well characterized (7); upon entry into mitochondria, this protein is processed by the chelator-sensitive matrix protease. The F$_1$-ATPase β subunit was mixed with each of the tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions and incubated under standard import conditions in the presence or absence of valinomycin. In the absence of valinomycin, the β subunit of F$_1$-ATPase was processed to the mature form that is resistant to proteinase K (Fig. 3D). In the same reaction, (1-48)-DHFR was also resistant to proteinase K, which indicated that it too was imported. In the presence of valinomycin (Fig. 3A, B, and C), each of the fusion proteins and the F$_1$-ATPase β-subunit precursor associated with mitochondria but were sensitive to added proteinase K. The small amount of (1-48)-DHFR that was resistant to proteinase K (Fig. 3C, lane 3) represents an incomplete block of import, because a small amount of mature F$_1$-ATPase β subunit was also detected after longer exposures (data not shown). These results demonstrate that the mitochondrion-dependent conversion of each of the tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions from proteinase K sensitivity to resistance is dependent on a membrane potential and likely reflects the import of these proteins through the mitochondrial inner membrane.

Import of tRNA (m$_2^2$G)dimethyltransferase-COXIV into mitochondria in vitro. The differences observed in the amounts of the three tRNA (m$_2^2$G)dimethyltransferase-DHFR fusion proteins that were imported into mitochondria appeared to depend on the sequences derived from tRNA (m$_2^2$G)dimethyltransferase. However, effects of passenger proteins have
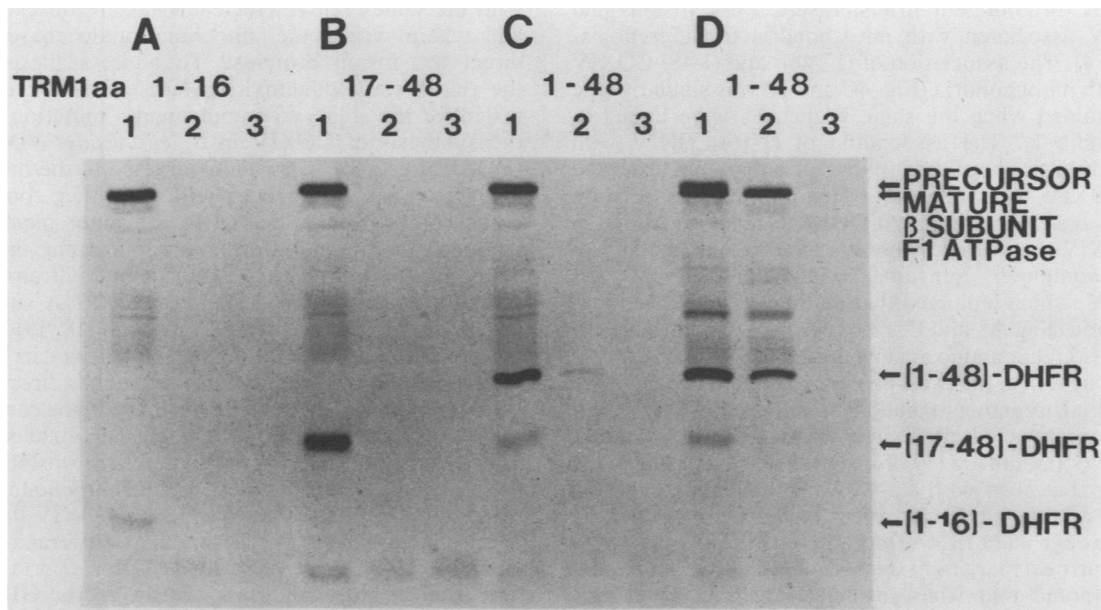

FIG. 3. Requirement of a membrane potential for import of tRNA (m$_2^2$G)dimethyltransferase-DHFR fusions. Import reactions were carried out as described in Materials and Methods. (A through C) 40 μM valinomycin added to import reaction mixtures; (D) no valinomycin addition. (A) (1-16)-DHFR; (B) (17-48)-DHFR; (C) (1-48)-DHFR; (D) (1-48)-DHFR. In addition to containing the tRNA (m$_2^2$G)dimethyltransferase-DHRF fusions, each lane also contained the precursor for the β subunit of F$_1$-ATPase. Lanes: 1, mitochondrial pellets from untreated import reaction mixtures; 2, mitochondrial pellets from import reaction mixtures treated with proteinase K; 3, mitochondrial pellets from import reaction mixtures treated with proteinase K in the presence of Triton X-100.
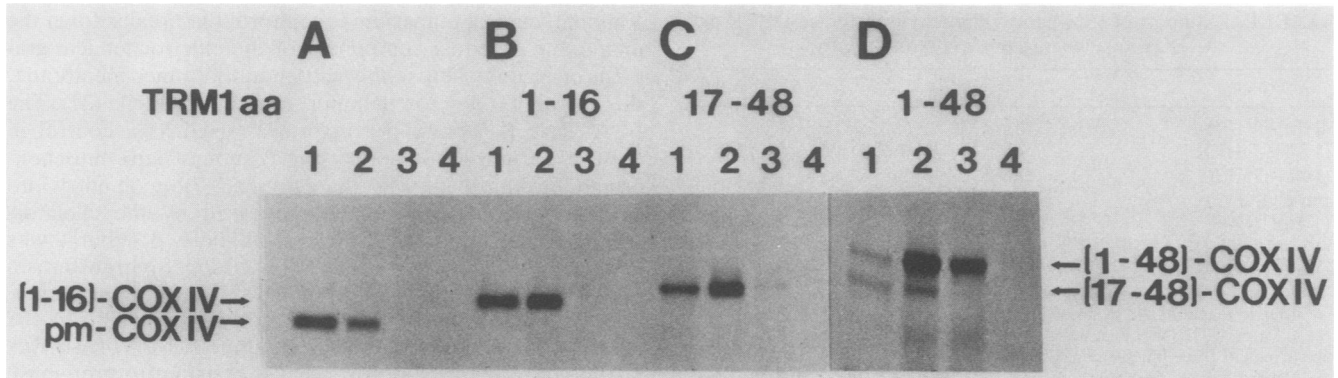
FIG. 4. Import of tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions into mitochondria in vitro. Import reactions were carried out as described in Materials and Methods. (A) pm-COXIV; B, (1-16)-COXIV; (C) (17-48)-COXIV; (D) (1-48)-COXIV. Lanes: 1, supernatants from untreated import reaction mixtures; 2, mitochondrial pellets from untreated import reaction mixtures; 3, mitochondrial pellets from import reaction mixtures treated with proteinase K; 4, mitochondrial pellets from import reaction mixtures treated with proteinase K in the presence of Triton X-100.

been observed in similar in vitro import studies (31, 34). van Steeg et al. (34) showed that the presequence of superoxide dismutase was unable to function effectively as a mitochondrial import signal for invertase but could perform this function for DHFR. The passenger effect was apparently due to a masking of the superoxide dismutase import signal by the invertase molecule. To assess possible effects of the passenger in the interpretation of our results, the sequences derived from *TRM1* that were fused to *DHFR* were also fused to a gene coding for pm-COXIV (Fig. 1B and C). The pm-COXIV used here lacks most of its presequence and is not imported into mitochondria (18, 19).

The fusions between tRNA (m$_2^2$G)dimethyltransferase and pm-COXIV were assayed for import into mitochondria in vitro, using the conditions described previously for fusions between tRNA (m$_2^2$G)dimethyltransferase and DHFR. The three tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions and pm-COXIV associated with mitochondria to different extents (Fig. 4). The association of (17-48)- and (1-48)-COXIV fusions with mitochondria (Fig. 4C and D) was similar to the results obtained when the same sequences were linked to DHFR (Table 1). The association of (1-16)-COXIV with mitochondria was about threefold greater than that observed for pm-COXIV (Fig. 4A and B) and for the association previously observed for (1-16)-DHFR (Table 1). Although (1-16)-COXIV associated with mitochondria, the fusion protein was completely sensitive to proteinase K, as was pm-COXIV, which indicated that neither was imported into mitochondria (Fig. 4A and B, lanes 3). These results support the conclusion that amino acids 1 through 16 of tRNA (m$_2^2$G) dimethyltransferase function poorly, if at all, as a surrogate mitochondrial targeting signal. A small amount of (17-48)-COXIV was imported into mitochondria (Fig. 4C, lane 3). The relative amount of (17-48)-COXIV that was imported was below that seen with (17-48)-DHFR, which suggested that there was no passenger effect on import when amino acids 17 through 48 of tRNA (m$_2^2$G)dimethyltransferase were used as a surrogate targeting signal (Table 1). Roise et al. (31) have also noted that when artificial targeting signals are fused to either DHFR or COXIV, the DHFR fusions are in some cases imported more efficiently than are the COXIV fusions. The nature of this passenger effect is unclear. The relative amount of (1-48)-COXIV that was imported into mitochondria was substantially above that seen for the other two tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions and

was equal to or greater than that observed for (1-48)-DHFR. These results are consistent with the conclusion that amino acids 1 through 48 of tRNA (m$_2^2$G)dimethyltransferase contain a mitochondrial targeting signal that functions much more efficiently as a surrogate signal than do its two component parts, amino acids 1 through 16 and 17 through 48. Furthermore, this signal does not appear to be affected by either of the passenger proteins used here.

Import of tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions into mitochondria in vivo. Amino-terminal mitochondrial targeting signals have at least two functions: they direct proteins into the organelle, and they are involved in sorting the proteins to their correct intramitochondrial locations (33). We were interested in two questions: do the amino-terminal sequences derived from tRNA (m$_2^2$G)dimethyltransferase direct passenger proteins into mitochondria in vivo with the same relative efficiencies as are observed in vitro, and where within the mitochondria do these sequences direct the fusion proteins? To address these questions, the tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions were tested for the ability to complement a mutation that blocks the synthesis of COXIV in *S. cerevisiae* WD1 (18). The COXIV precursor is normally targeted to the mitochondrial matrix, where it associates with the other components of cytochrome oxidase located in the inner membrane (18). Although there is no information concerning the intramitochondrial location of tRNA (m$_2^2$G)dimethyltransferase, it is probably a matrix enzyme, since the tRNA substrates are located in this compartment. Hurt et al. (18, 19) have shown that a variety of amino-terminal sequences can be linked to mature COXIV and that if these sequences direct COXIV to the mitochondrial matrix, the fusion proteins can function in association with the other cytochrome oxidase subunits. Therefore, if the tRNA (m$_2^2$G)dimethyltransferase-COXIV fusion proteins are directed to the mitochondrial matrix, they should be able to complement a COXIV deficiency.

To express tRNA (m$_2^2$G)dimethyltransferase-COXIV fusion proteins in vivo, the *TRM1-COXIV* hybrid genes were first inserted into the yeast shuttle vector G18. Plasmids coding for (1-16)-, (1-48)-, and (17-48)-COXIV were each transformed into WD1 cells (18). To confirm that pm-COXIV was unable to complement the COXIV deficiency in this strain, we used linker insertion to provide an initiation codon for the pm-*COXIV* gene originally present on G18 and transformed WD1 cells with this plasmid as well. Transfor-
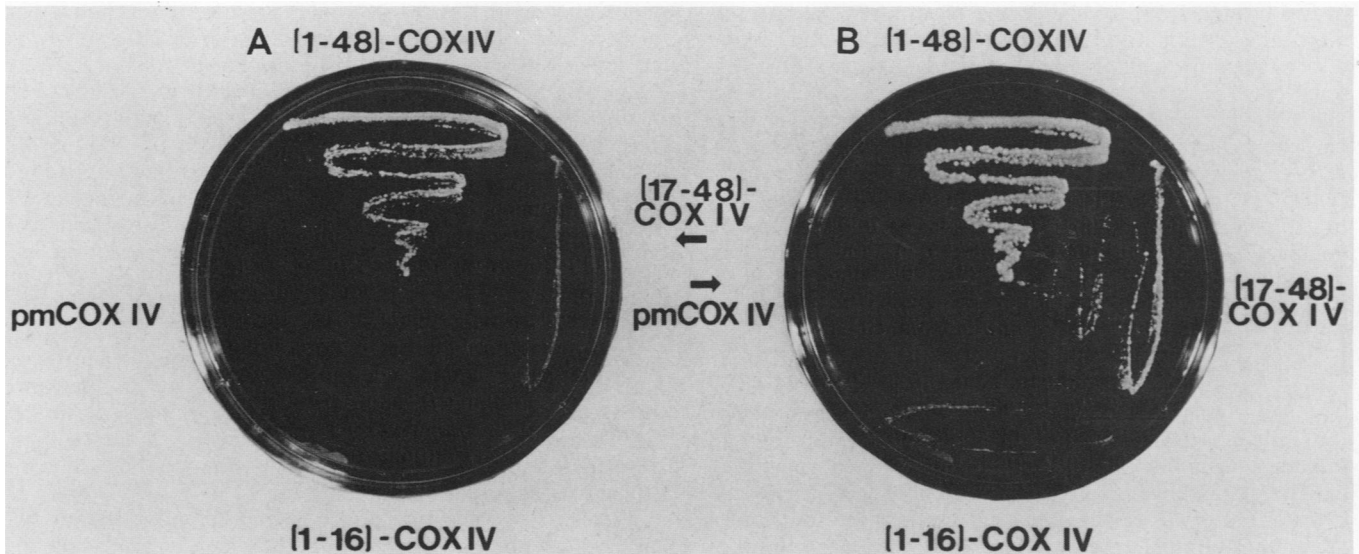
FIG. 5. Import of tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions into mitochondria in vivo. Hybrid genes coding for fusion proteins composed of amino acids 1 through 16, 17 through 48, or 1 through 48 of tRNA (m$_2^2$G)dimethyltransferase linked to amino acids 19 through 155 of COXIV were inserted into the yeast shuttle vector G18 under control of the *ADH1* promoter as described in Materials and Methods. The plasmids were transformed into *S. cerevisiae* WD1. WD1 cells are deficient in COXIV and are incapable of respiratory growth. Complementation of the COXIV deficiency by plasmids expressing the tRNA (m$_2^2$G)dimethyltransferase-COXIV fusion proteins was determined by growth on the nonfermentable carbon sources glycerol and ethanol.

mants were selected for uracil prototrophy on minimal medium plates containing glucose and subsequently streaked onto YEPG plates. Growth of the transformants on YEPG plates that contained the nonfermentable carbon sources ethanol and glycerol would indicate that cells have a functional respiratory chain and that the COXIV defect had been complemented. Comparisons of growth of WD1 cells expressing either (1-16)- or (1-48)-COXIV with growth of cells expressing (17-48)-COXIV from the *TRM1* promoter cannot be readily made because the corresponding mRNAs that code for either the long or short forms of tRNA (m$_2^2$G) dimethyltransferase differ greatly in abundance (14). Only a small fraction of 5' ends extend upstream of the ATG used to initiate translation of (1-16)- or (1-48)-COXIV. The majority of the transcripts (>90%) are capable of producing only (17-48)-COXIV. Therefore, using the *TRM1* promoter, one cannot normalize transcription of mRNAs coding for (1-16)- and (1-48)-COXIV with transcription of mRNAs coding for (17-48)-COXIV. The *ADH1* promoter in the constructs used here should initiate transcription upstream of the *TRM1* open reading frame and was used in an effort to normalize transcription from the *TRM1-COXIV* hybrid genes lacking the 5'-flanking region of *TRM1*.

Figure 5 shows the growth on YEPG plates of strains carrying plasmids expressing the tRNA (m$_2^2$G)dimethyltransferase-COXIV fusions and pm-COXIV. Strains expressing (1-48)-COXIV grew well, with a doubling time in liquid YEPG culture of approximately 7 h (data not shown), compared with 3.5 h reported for the same cells expressing the authentic COXIV precursor (18). Whether the decrease in doubling time of cells expressing (1-48)-COXIV versus wild-type COXIV corresponded to less efficient import of the fusion protein or a reduction in cytochrome oxidase activity as a consequence of the unprocessed tRNA (m$_2^2$G) dimethyltransferase sequences in the enzyme complex was not determined. Strains expressing (17-48)-COXIV grew slowly in YEPG and had an initial doubling time in liquid culture of approximately 30 h, which decreased to approxi-

mately 7 h after about 3 days of growth (data not shown). Biphasic growth was observed with certain fusions in previous studies (19), but the reasons for these growth characteristics are unclear. One possibility is that the acquisition of respiratory competence increases the ability of the mitochondria to import proteins with less efficient targeting signals (2). Strains expressing (1-16)-COXIV take about 7 days for barely detectable growth on YEPG plates and have doubling times of more than 80 h in liquid YEPG culture. The significance of this growth with respect to the function of amino acids 1 through 16 of tRNA (m$_2^2$G)dimethyltransferase in directing COXIV into mitochondria in vivo is marginal considering that under long-term stress, mutations in the 16-amino-acid leader or the mitochondrial import system may be selected (35). No growth was observed with the strains expressing pm-COXIV. We have assumed that the different amino-terminal *TRM1* sequences do not have different effects on COXIV activity, synthesis, or turnover. Therefore, these experiments can give only a qualitative assessment of targeting on the basis of growth, not COXIV protein delivery. Nonetheless, these results closely parallel those of the in vitro studies in that the three amino-terminal sequences derived from tRNA (m$_2^2$G)dimethyltransferase appear to function at different efficiencies as surrogate mitochondrial targeting signals. In addition, the signals present in the tRNA (m$_2^2$G)dimethyltransferase appear to direct the fusion proteins to the mitochondrial matrix.

One potential limitation of the in vivo targeting studies presented here is that in the constructions used, the fusion proteins are expressed from the *ADH1* promoter and may represent a much higher intracellular concentration of the fusion proteins than might be expected if the proteins were expressed from the *TRM1* promoter. Centromere-containing plasmids expressing (17-48)-COXIV from the *TRM1* promoter complemented the COXIV deficiency of WD1 cells (data not shown), which suggested that import of this fusion protein was not simply due to a bypass import pathway dependent on the expression of extremely high levels of the

fusion protein from a very strong promoter (29). Furthermore, the observation that a mitochondrial targeting signal resides in amino acids 17 through 48 of tRNA (m_2^2G)dimethyltransferase is consistent with our previous results showing that mitochondrial tRNAs were modified with m_2^2G even if only the shorter form of tRNA (m_2^2G)dimethyltransferase was expressed in vivo from the *TRM1* promoter on a centromere-containing plasmid (14). Therefore, it is unlikely that the interpretation of our in vivo results depends on nonphysiologically high concentrations of tRNA (m_2^2G)dimethyltransferase-COXIV fusion proteins expressed from the *ADH1* promoter.

DISCUSSION

We have examined the amino-terminal sequences of tRNA (m_2^2G)dimethyltransferase for the presence of mitochondrial targeting signals by determining whether these sequences can direct passenger proteins into mitochondria in vitro and in vivo. Our goal was to obtain insight into the function of the amino-terminal extension located on the longer form of tRNA (m_2^2G)dimethyltransferase in the compartmentalization of this enzyme. Amino acids 1 through 48 and 17 through 48 of tRNA (m_2^2G)dimethyltransferase could function as surrogate mitochondrial targeting signals for both cytosolic DHFR and pm-COXIV. In each case, amino acids 1 through 48 functioned more efficiently as a surrogate targeting signal than did amino acids 17 through 48. The amino-terminal extension, amino acids 1 through 16 of tRNA (m_2^2G)dimethyltransferase, did not contain sufficient information to function effectively as an independent mitochondrial targeting signal. This was evidenced by the inability of the extension to direct pm-COXIV into mitochondria in vitro or in vivo at high enough levels to complement a COXIV deficiency and the borderline ability to promote import of DHFR into mitochondria in vitro. Our results suggest that a mitochondrial targeting signal resides in amino acids 17 through 48 of tRNA (m_2^2G)dimethyltransferase and that the amino-terminal extension functions in conjunction with this signal to increase the efficiency with which mitochondria recognize or translocate the longer form of the enzyme.

Several alternatives could explain how the amino-terminal extension affects the efficiency of import of tRNA (m_2^2G) dimethyltransferase into mitochondria. If the long form of tRNA (m_2^2G)dimethyltransferase contains functionally redundant targeting signals such as those described for the β subunit of F_1-ATPase (2), one located in the amino-terminal extension and the other located at the amino terminus of the shorter form of the enzyme, the combination of the two signals may be more efficient than either one alone. However, since the amino-terminal extension does not serve as a surrogate mitochondrial targeting signal, functionally redundant signals do not appear to offer a satisfactory explanation for how the amino-terminal extension increases the import efficiency of tRNA (m_2^2G)dimethyltransferase.

The mitochondrial targeting signal may be composed of discrete functional units with distinct properties. Amino acids 17 through 48 of tRNA (m_2^2G)dimethyltransferase appear to contain sufficient information for recognition by mitochondria and for translocation into the organelle. However, these sequences function relatively inefficiently and may represent a minimal sequence necessary for import. The amino-terminal extension, since it is not necessary for import and does not serve as a surrogate targeting signal, may be involved strictly in modulating the efficiency of targeting signal usage. In this regard, the amino-terminal extension

might contain sequences that interact with a cytosolic factor not essential for import per se but somehow able to enhance the ability of mitochondria to recognize or translocate the longer form of tRNA (m_2^2G)dimethyltransferase. Cytosolic factors are important for the import of certain mitochondrial proteins (25, 26) while having no effect on the import of others (10). In those cases where cytosolic factors affect the import of mitochondrial proteins, it is unclear whether they are absolutely essential for import or if they participate by enhancing the efficiency of the import process. It seems unlikely that the cytosolic factors would supersede the function of proteinaceous import receptors on the mitochondrial surface (28), so the sequences important for interaction with cytosolic factors may not function in targeting a protein into mitochondria in the absence of other signals that are necessary for receptor binding or translocation. Since nothing is known about sequence requirements for interaction with these cytosolic factors, at present there is no evidence to support or refute this possibility.

Finally, the efficient mitochondrial targeting signal of tRNA (m_2^2G)dimethyltransferase may consist of a single functional entity that is partially found in amino acids 17 through 48 and is completed when these amino acids are found downstream of the amino-terminal extension. The amino-terminal extension may augment the partial signal by altering its context in terms of primary or secondary structure. If so, the sequences that serve as the most efficient targeting signal would span the boundary between the amino-terminal extension and the amino terminus of the shorter form of tRNA (m_2^2G)dimethyltransferase. The partial signal present in amino acids 17 through 48 would be enough to function as an independent targeting signal, but the same would not be true for the partial signal in the amino-terminal extension. Further considerations outlined below lead us to favor this last possibility as an explanation of how the amino-terminal extension functions to increase the efficiency with which tRNA (m_2^2G)dimethyltransferase is imported into mitochondria.

We have analyzed the sequences found at the amino termini of tRNA (m_2^2G)dimethyltransferase for elements of primary and secondary structure shared with other known mitochondrial targeting signals. Comparative analysis of mitochondrial targeting signals has shown that most carry a net positive charge, have a bias toward hydroxylated and hydrophobic amino acid residues, and have a bias against acidic residues (37). Statistical (37) and experimental (30, 31) data support the notion that secondary structure plays an important role in defining a mitochondrial targeting signal. In particular, it has been proposed that the ability of mitochondrial targeting signals to fold into amphiphilic structures is the key to their function. Initially, proposals stated that amphiphilic helices may be the important functional components of mitochondrial targeting signals (30, 37). Later, by using artificial targeting signals, it was shown that other amphiphilic structures can also function in this regard (1, 31). Therefore, it may be an amphiphilic structure per se and not a particular amphiphilic structure that defines a mitochondrial targeting signal. We have examined both the predicted secondary structure and the amphiphilicity of sequences that form the amino termini of tRNA (m_2^2G) dimethyltransferase, using the protein sequence analysis programs available in PC/Gene (IntelliGenetics). Two programs based on the parameters of either Chou and Fasman (8) or Garnier et al. (16) were used for secondary-structure predictions. Both programs indicated that the sequence spanning the junction between the amino-terminal extension

and the amino terminus of the shorter form of tRNA (m$_2^2$G) dimethyltransferase has the propensity to form an α helix. Using the principles of Eisenberg et al. (12, 13), we have examined the hydrophobic moment of sequences through the first 48 amino acids of the longer form of tRNA (m$_2^2$G) dimethyltransferase to predict their degree of amphiphilicity when folded into an α helix. This analysis shows a broad peak of relatively high hydrophobic moment stretching from amino acids 12 through 35, thereby spanning the junction between the amino-terminal extension and the amino terminus of the shorter form of tRNA (m$_2^2$G)dimethyltransferase. The sequence exhibiting the highest hydrophobic moment when an 18-amino-acid window was used consisted of amino acids 13 through 30. The hydrophobic moment calculated for this sequence falls within the values reported for other mitochondrial targeting signals (37), although it is found toward the low end of the spectrum. In contrast, the maximum hydrophobicity of the hydrophobic face of the helix created by this sequence is quite high and falls at the high end of the spectrum reported for mitochondrial targeting signals (37). These results support the view that a mitochondrial targeting signal spans the junction between the amino-terminal extension and the amino terminus of the shorter form of tRNA (m$_2^2$G)dimethyltransferase.

The same analysis on amino acids 17 through 34, which correspond to the amino terminus of the shorter form of tRNA (m$_2^2$G)dimethyltransferase, shows a lower hydrophobic moment which is below values reported for all but one mitochondrial targeting signal (37). However, an 18-amino-acid window in the analysis of amino acids 17 through 48 of tRNA (m$_2^2$G)dimethyltransferase includes amino acids beyond the predicted α-helical region. Including these nonhelical amino acids in the hydrophobic moment calculations decreases the mean hydrophobic moment below that determined by using a smaller window that includes only those amino acids that have the propensity to form an α helix. Repeating the analysis with smaller windows indicates that the mean hydrophobic moment of sequences 17 through 29 falls well within the values for other mitochondrial targeting signals when the values are calculated by using the same scale (data not shown). The relatively small amphiphilic helix predicted in amino acids 17 through 48 of tRNA (m$_2^2$G) dimethyltransferase may explain why this sequence functions as a mitochondrial targeting signal but does so relatively inefficiently.

We have also examined the predicted structure of the fusions made between the amino-terminal extension of tRNA (m$_2^2$G)dimethyltransferase and either pm-COXIV or DHFR. In both cases, the junction site between the two sequences has a high propensity to form a reverse turn. As such, it seems unlikely that fusing the amino-terminal extension to either COXIV or DHFR could potentiate an amphiphilic α-helical structure through the amino terminus of either passenger protein into the amino-terminal extension, as predicted for fusions with the shorter form of tRNA (m$_2^2$G) dimethyltransferase. This analysis may explain why these fusions were not imported or were imported very poorly into isolated mitochondria. In conclusion, these studies suggest that the amino-terminal extension functions to increase the efficiency with which tRNA (m$_2^2$G)dimethyltransferase is imported into mitochondria by extending an amphiphilic helix present at the amino terminus of the shorter form of the enzyme into the amino-terminal extension, thereby forming a larger, more efficient mitochondrial targeting signal.

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