MOD5 Translation Initiation Sites Determine N⁶-Isopentenyladenosine Modification of Mitochondrial and Cytoplasmic tRNA

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MOD5 is one of several genes that code for enzymes found in mitochondria and another cellular compartment. Like other such genes, it contains two in-frame ATGs that could be used to produce two proteins, differing from each other by an amino-terminal extension. Certain other genes produce heterogeneous mRNAs with some 5' ends falling upstream of the longest open reading frame and some 5' ends falling between the first and second ATGs. In these cases, selection of transcription start sites appears to play a significant role in translation start site selection. *MOD5*, in contrast, produces mRNAs with 5' ends that all fall upstream of both ATGs. To determine how *MOD5* encodes isozymes that are located in different cellular compartments and to determine whether they differ in structure, we constructed *MOD5* and *MOD5-COXIV* fusions with mutations of the first, second, or both ATGs. The effect of these alterations on protein production, tRNA modification, and cellular location was assessed. Both the first and second ATGs are used to produce *MOD5* protein in vivo, but only the long form of the protein is imported into mitochondria. Thus, the first 11 amino acids present on the amino-terminal extended protein are necessary for mitochondrial import. Surprisingly, this extension does not promote complete import of the long form of the protein, but rather a functional pool of the extended protein remains in the cytoplasm. The amino-terminal extension is also unusual because it is probably not proteolytically removed upon import and therefore does not constitute part of a mitochondrial presequence.

Numerous examples of chemical reactions catalyzed in both the mitochondrion and some other cellular compartment have been documented (reviewed in reference 24). The majority of enzymes that catalyze reactions in two cellular compartments are probably the products of separate genes. However, some single genes encode isozymes, which we propose to call sorting isozymes, that are sorted to more than one cellular compartment (5, 9, 11, 16, 18, 40, 41, 47, 49, 52; reviewed in reference 46). These genes encode at least two translation initiation sites and multiple mRNAs with 5' ends falling upstream of as well as in between potential translation initiation sites (6, 9, 16, 40, 41, 49, 52). Thus, the mRNAs have different coding capabilities with respect to amino-terminal sequences.

Amino-terminal sequences play an important role in mitochondrial targeting (reviewed in reference 3), and in several cases the presence or absence of the peptide sequences encoded between the first and second ATGs has been shown to be essential for import of the mitochondrial isozyme (5, 9, 40). However, in at least one case, the amino-terminal extension is not essential for mitochondrial targeting and a single translation product is able to distribute to both mitochondrial and nuclear compartments (14, 36).

The *MOD5* gene of *Saccharomyces cerevisiae* encodes Δ^2 -isopentenyl pyrophosphate:tRNA isopentenyl transferase (IPP transferase). IPP transferase is an enzyme that modifies both mitochondrial and cytoplasmic tRNA. The *MOD5* gene encodes two closely spaced potential translation initiation sites. Utilization of both of the first two AUG codons for translational initiation could generate two pro-

teins, one having 11 more amino acids at the amino terminus than the other. By analogy with other genes that code sorting isozymes, the longer protein would be expected to be targeted specifically to mitochondria. However, in contrast to the other genes, the 5' ends of all detectable *MOD5* mRNAs fall upstream of the first ATG (39). Because the coding capacities of all *MOD5* mRNAs are potentially the same, the question arises as to whether both AUGs are actually used in synthesis of two different proteins and, if so, whether amino-terminal protein variants distribute to different subcellular compartments. Alternatively, one protein may be produced by *MOD5* and may partition between the mitochondrial and cytosol or nuclear compartments.

Our experiments were designed to investigate the role of initiation of translation at the first two AUG codons of *MOD5* in the synthesis of IPP transferase isozymes. We produced initiation codon mutations in *MOD5* through oligonucleotide-directed in vitro mutagenesis and analyzed the effects of these mutations on IPP transferase and modification of mitochondrial and cytoplasmic tRNA. We also studied the effects of these mutations on the mitochondrial import of *MOD5-COXIV* fusion proteins. The results of our experiments indicate that mitochondrial and cytoplasmic tRNA modification and mitochondrial association of IPP transferase are affected by changes in translation initiation codons.

MATERIALS AND METHODS

Strains, media, and growth conditions. *Escherichia coli* HB101 (8) was used for propagation of recombinant DNA molecules. Strain NM522 (21) or RZ1032 (33) was used for propagation of M13 phage, and JM109 (53) was the recipient

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for mutagenesis reactions. *E. coli* cells were maintained on YT or 2XYT media containing the appropriate antibiotic to select for plasmid expression.

S. cerevisiae MD14A has the following genotype: *MAT α SUP7 can1-100 ade2-1 his5-2* or *his4-519* or both *leu2-3,112 lys1-1* or *lys2-1* or both *trp1 ura3-1*. MT-8 is a derivative of MD14A containing a disruption of the *mod5-1* gene produced by gene replacement as described below. WD-1 has the genotype *MAT α ura3 leu2 his3 coxIV::LEU2* (12). Yeast strains were maintained on YEPD medium or synthetic medium (28) lacking the appropriate nutritional ingredient for selection of plasmid expression. Yeast cells harboring pJDB plasmids were grown in synthetic medium lacking leucine and containing galactose as the carbon source. For determination of growth rates on nonfermentable carbon sources, cells were inoculated into liquid culture of defined medium lacking uracil and containing 3% (wt/wt) glycerol and 2% (wt/wt) lactic acid.

Transformation of *E. coli* and *S. cerevisiae*. *E. coli* was transformed with plasmid DNA by the calcium chloride procedure (37). Yeast cells were transformed by a lithium acetate procedure (30).

Plasmids. Centromere-containing plasmids were constructed for expression of single-copy *MOD5* genes in yeast cells. Plasmid YCp50 (34) was modified to include the fl phage origin of replication from pUC-f1 (Pharmacia). The 541-bp *EcoRI-HindIII* fragment of pUC-f1 was ligated to *EcoRI-HindIII*-digested YCp50 for production of YCf50. YCf50 was digested with *Bam*HI, and the 1.8-kb *MOD5 Bgl*II fragment from YEpMOD5-1F (11) was added to generate YCfMOD5.

Plasmids for overexpression of *MOD5* genes are derivatives of pJDB207 (4). The 1.8-kb *Bgl*II fragment of YEpMOD5 (11) was ligated into the *Bam*HI site of pJDB207 to generate pJDBMOD5.

The parent vector used for construction of *MOD5-COXIV* gene fusions was G18, which contains sequences coding for mature COXIV protein preceded by a polylinker and the yeast *ADH1* promoter (50). YCpMOD5-COXIV was generated by a two-step procedure. First, a 641-bp *Sau*3A fragment containing the *MOD5* promoter and codons 1 to 156 was ligated into the *Bam*HI site of the polylinker region of plasmid G18. This created a 1-bp out-of-frame fusion of *MOD5* and *COXIV*. The reading frame was then adjusted by digestion of the resulting plasmid at the *Xba*I site spanning codons 20 and 21 of *COXIV*, filling in of the 4-bp overhang by the Klenow fragment of DNA polymerase, and ligation. pUCMOD5-COXIV contains the 1.7-kb *EcoRI-HindIII* fragment from YCpMOD5-COXIV extending from upstream of the *MOD5* promoter to downstream of the *COXIV* sequences inserted into pUC19. pMC4 encodes precursor cytochrome oxidase subunit IV under the transcription regulation of the *ADH1* promoter (29). pMSS encodes the pseudomature subunit IV of cytochrome oxidase lacking the first 19 amino acids of the precursor protein (29).

The plasmid used for in vitro transcription of *MOD5* was a derivative of pT7/T3-18 (Bethesda Research Laboratories). pT7/T3-18 was digested with *Sma*I and *Bam*HI and ligated to the 1.6-kb *Sna*BI-*Bgl*II fragment of YEpMOD5-1F.

Construction of *mod5::TRP1*. The 2.9-kb *Cla*I fragment from YEpMOD5(9.0) (11) was added at the unique *Cla*I site of vector pBR322 (no *Eco*RI) (obtained from R. Keil). The resulting plasmid, pBR-MOD5(2.9), was digested with *Eco*RI and *Sna*BI, thereby removing the first 751 bp of the *MOD5* protein-coding region and 35 bp of 5' untranslated region. *Eco*RI-*Sna*BI-digested DNA was treated with the

Klenow fragment of DNA polymerase and ligated to a 1.3-kb *Hinc*II fragment from plasmid pUC-T containing the *TRP1* gene (17). The plasmid containing the *mod5::TRP1* disruption, pBR-mod5::TRP1, was digested with *Cla*I, and the 3.3-kb fragment was isolated for transformation of MD14A (44). The correct chromosomal replacement of *mod5-1* by *mod5::TRP1* in tryptophan prototrophic transformants was confirmed by DNA blotting.

Oligonucleotide-directed mutagenesis. Oligonucleotides were purchased from Hershey Medical Center Macromolecular Core Facility, Genetic Designs, Inc., or Penn State University Biotechnology Institute. Deblocked oligonucleotides were purified by gel filtration (31). Phage M13-4W was used for production of *MOD5* initiation codon mutations. M13-4W contains the 641-bp *Sau*3A fragment of YEpMOD5 (1.8) (11) inserted into phage M13mp8 at the *Bam*HI site. Single-stranded phage DNA was isolated (33), and mutagenesis reactions were carried out as previously described (19). To produce the double mutant *mod5-M1,2*, *MOD5* sequences were mutated to alter the first ATG and then M2 oligonucleotide was the primer for a second mutagenesis.

Mutant sequences were subcloned into plasmids YCfMOD5 and pJDBMOD5 by first transferring a 308-bp *Sna*BI-*Nsi*I fragment from M13-4W to a pUC plasmid (pUC-f1-mod5-E) containing the 1.5-kb *Eco*RI fragment from YCfMOD5. The replacement of wild-type sequences with mutant sequences was confirmed by DNA sequencing. The 1.5-kb *Eco*RI fragment of pUC-f1-mod5-E derivatives was transferred to *Eco*RI fragment-deleted YCfMOD5 for reconstruction of *MOD5* genes containing ATG codon mutations. pJDBMOD5 derivatives were constructed from YCfmod5-M1, YCfmod5-M2, and YCfmod5-M1,2 by ligation of the 1.8-kb *Bst*YI fragment containing *MOD5* sequences into *Bam*HI-digested pJDB207.

A multistep procedure was used to generate mutant and wild-type *MOD5-COXIV* gene fusions. The *Sna*BI-*Nsi*I fragments from the replicative form of the mutant and wild-type versions of M13-4W were used to replace the comparable sequences in the plasmid pUCMOD5-COXIV. The *Eco*RI-*Hind*III fragment containing the entire *MOD5-COXIV* region from each of the four plasmids was used to replace the comparable region in YCpMOD5-COXIV. Digestion of each of the four YCp plasmids with *Eco*RI and *Sna*BI removed the *MOD5* promoter sequences and placed the wild-type and mutant *MOD5-COXIV* sequences under the control of the *ADH1* promoter. The resulting plasmids are YCpADH1MOD5-COXIV, YCpADH1mod5-M1-COXIV, YCpADH1mod5-M2-COXIV, and YCpADH1mod5-M1,2-COXIV.

Radioimmunoassay for i⁶A tRNA. Affinity-purified anti-N⁶-isopentenyladenosine (i⁶A) antibody (11) was used for the detection of i⁶A-modified tRNA. tRNA was isolated (26) and aminoacylated with 5 μ Ci of [³H]tyrosine (Amersham) and aminoacyl-tRNA synthetases from either *S. cerevisiae* or *E. coli* (Sigma Chemical Co.). For assessing modification of cytosolic tRNA, aliquots containing 100 ng of tRNA aminoacylated with yeast tRNA synthetases were precipitated with 10% trichloroacetic acid or incubated for 10 min with 50 μ l of affinity-purified anti-i⁶A antibody. The trichloroacetic acid-precipitated samples were collected on Whatman GF/C glass filters. The samples incubated with anti-i⁶A antibody were collected onto nitrocellulose filters (Schleicher & Schuell) that had been preincubated in phosphate-buffered saline containing 1 mM tyrosine. *E. coli* aminoacyl-tRNA synthetases were used to selectively label mitochondrial tRNA^{Tyr} in samples of total tRNA as described above except that reaction mixtures contained 10 μ g of aminoacy-

FIG. 1. Mutations introduced into the *MOD5* gene and oligonucleotides used in mutagenesis. The nucleotide sequence of the *MOD5* gene is shown from two nucleotides 5' of the *SnaBI* site upstream of the open reading frame to codon 14, followed by the sequence in the area of the first *NsiI* site. The DNA sequences of oligonucleotides M1 and M2 are shown below the corresponding wild-type (WT) sequence. Restriction endonuclease recognition sites are underlined and indicated above the sites. Encoded amino acids are in three-letter code below codons. The first two methionine codons are indicated by capital letters. The M2 oligonucleotide encodes the only amino acid change in *MOD5* (Met→Ile); all other changes become part of the 5' noncoding portion of the gene. Arrows show positions of 5' ends of *MOD5* transcripts.

lated tRNA. The level of i⁶A modification in tRNA^{Tyr} was determined by calculating the ratio of radioactivity in anti-i⁶A-treated samples to the radioactivity in trichloroacetic acid-precipitated samples. These ratios were then compared with the value for the positive control (MT-8 expressing YCfMOD5) and expressed as percentages of the wild-type value.

Preparation of cell protein and mitochondrial extracts. Protein extracts from yeast cells were prepared by a modification of the procedure described by Schultz et al. (45) as detailed elsewhere (20). Mitochondria were isolated from 500-ml cultures of cells (A_{600} of 0.7 to 1.1) by the procedure of Daum et al. (10) with the homogenization buffer described by Hase et al. (23). For concentration of dilute solutions, samples of postmitochondrial supernatant and total homogenate were lyophilized and resuspended in sample buffer.

Protein concentration was estimated by the method of Peterson (42), using bovine serum albumin as a standard and without deoxycholic acid in the precipitation step.

Production of *MOD5* antipeptide antibody. A potentially antigenic peptide was chosen by analysis of the hydrophilicity of the predicted *MOD5* protein sequence, using the computer program DNA Strider 1.0. The peptide sequence NH₂-Lys-Thr-Asp-Asp-Asn-Thr-Val-Lys-Leu-Glu-Asp-Cys-Ile-Glu-Arg-Met-Lys-Thr-Arg-Thr-COOH (positions 270 to 289) (P20), predicted to be hydrophilic (25), was chosen for use as an antigen and was synthesized by Applied Biosystems. P20 was conjugated to keyhole limpet hemocyanin (KLH; Calbiochem) by reaction with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS; Aldrich Chemical Co.) (22).

Female New Zealand White rabbits were immunized with KLH-MBS-P20 conjugate. Preimmune serum was collected from each rabbit prior to initial subcutaneous injection with 300 μ g of antigen (500 μ l) and Freund's complete adjuvant (500 μ l; GIBCO Laboratories). Rabbits were given injections of 100 μ g of KLH-MBS-P20 conjugate with Freund's incomplete adjuvant at 2- to 3-week intervals for 4 months. After 4 months, injections were given every 8 weeks. Antibodies were checked for reaction with *MOD5*-specific proteins by protein blotting and indirect immunodetection.

Antipeptide antibodies were purified from serum by affinity chromatography on CNBr-Sepharose 4B (Pharmacia) or Affi-Gel 15 (Bio-Rad) resins conjugated to KLH-MBS-P20 antigen. Coupling KLH-MBS-P20 to CNBr-Sepharose was done as described by the manufacturer. KLH-MBS-P20 was

coupled to Affi-Gel 15 as described by Bio-Rad. Affinity purification of antibody was done as described by Kilmartin and Adams (32), using glycine for elution.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-(SDS)-polyacrylamide gel electrophoresis (PAGE) and blotting to nitrocellulose were performed essentially as described by Towbin et al. (48). SDS-polyacrylamide gels were 0.75 mm thick and contained 5% acrylamide (30:0.8, acrylamide/bisacrylamide) in the stacking and 8% acrylamide in the separating gel. Proteins were transferred electrophoretically to nitrocellulose sheets and were incubated with NET blocking buffer (27) overnight at room temperature. Affinity-purified antipeptide antibody was added to NET blocking buffer at a final dilution of 1:500, and blots were incubated for 4 to 8 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories/Organon Teknika) was incubated with the washed blot at a 1:1,000 dilution in NET blocking buffer for 2 h. Alkaline phosphatase-conjugated antibody was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) (7). Dried blots were scanned on a Beckman DU-8B spectrophotometer, using standard blot scanning software.

In vitro transcription and translation. For in vitro synthesis of ³⁵S-labeled IPP transferase, plasmid pT7-MOD5-1F was linearized by digestion with *SaII* and added to a T7 RNA polymerase reaction mix according to the protocol of the supplier (Promega). In vitro-synthesized mRNA was added to rabbit reticulocyte lysate in vitro translation reaction mix with [³⁵S]methionine as recommended by Promega. A 10- μ l aliquot of the 100- μ l translation reaction mix was resolved by SDS-PAGE for comparison to immunologically detected IPP transferase. Translation product was detected by autoradiography.

RESULTS

IPP transferase is synthesized from multiple translation initiation sites. To study the role of the first two AUGs in expression of *MOD5*, we used site-directed oligonucleotide mutagenesis to mutate the first, second, or both ATGs of the *MOD5* gene. The oligonucleotides used for construction of the mutations and the resulting changes are diagrammed in Fig. 1. Changing the first ATG codon to AAG (*mod5-M1*) should result in an IPP transferase truncated at the amino terminus. A change of the second ATG codon to ATT

FIG. 2. Detection of IPP transferase isozymes expressed from wild-type and AUG mutant *MOD5* genes. Total cellular proteins or mitochondria were prepared as described in Materials and Methods, separated by electrophoresis, and electroblotted, and *MOD5* proteins were detected with affinity-purified *MOD5* antipeptide antibody. The approximate positions of the IPP transferase isozymes IPPT-I, IPPT-II, and IPPT-III are indicated. (A) Lanes 1 to 5 contain 40 μ g of total cellular protein from the *mod5::TRP1* strain MT-8 expressing the indicated *MOD5* gene on high-copy-number plasmids derived from pJDB207. (B) Composite photograph of an immunoblot and an autoradiograph. Lanes: 1, aliquot of an in vitro translation reaction mixture programmed with in vitro-transcribed *MOD5* mRNA; 2 to 5, 20 μ g of mitochondrial protein from cells expressing the indicated genes on high-copy-number plasmids; 6, 50 μ g of total cellular protein from the *mod5::TRP1* strain MT-8.

(*mod5-M2*) produces a methionine-to-isoleucine change in the full-length IPP transferase protein while preventing synthesis of amino-terminal-truncated IPP transferase. Mutation of both the first and second ATG codons (*mod5-M1,2*) should eliminate translation from either of the predicted start positions.

To facilitate the detection of low-abundance IPP transferase, the *mod5::TRP1* strain MT-8 was transformed with the high-copy-number plasmid pJDB207 carrying the various *MOD5* alleles. Protein extracts from the transformed cells were fractionated by SDS-PAGE, and the *MOD5* antigen was detected with antipeptide antibodies (Fig. 2). Cells containing no plasmid show no cross-reactivity with the antipeptide antibody (Fig. 2B, lane 6). Cells harboring the wild-type *MOD5* gene have three cross-reacting peptides (Fig. 2A, lane 2). The slowest migrating, IPPT-I, is in low abundance and is poorly resolved from a slightly faster migrating abundant species, IPPT-II. The fastest-migrating protein, IPPT-III, is clearly resolved but is in low abundance in cells harboring a wild-type *MOD5* gene. These data show that the antipeptide antibody is specific for IPP transferase and that *MOD5* likely encodes three protein products.

Cells harboring a plasmid with a mutation of the first ATG (pJDBmod5-M1) contain the most abundant IPP transferase isozyme, IPPT-II, and the fastest-migrating isozyme, IPPT-III, but lack the slowest-migrating protein, IPPT-I (Fig. 2A; compare lanes 2 and 3). Extracts from cells containing a plasmid with the first ATG intact and the second ATG mutated, pJDBmod5-M2, lack IPPT-II but contain isozymes IPPT-I and III (Fig. 2A; compare lanes 2 and 3 with lanes 1 and 4). The third, fastest-migrating form of IPP transferase, IPPT-III, is present in extracts of cells expressing all *MOD5*

genes tested. IPPT-III is most prominent in cells harboring a plasmid with a mutation at the second ATG or both the first and second ATGs (pJDBmod5-M2 and pJDBmod5-M1,2; Fig. 2A, lanes 1, 4, and 5).

In summary, the first AUG must be present for the production of IPPT-I and the second AUG must be present for the production of IPPT-II. Therefore, these species most likely result from translation initiation at the first and second AUGs, respectively. Since neither the first nor the second AUG is needed for IPPT-III, this protein may be the product of translation from an internal methionine codon of *MOD5*. It is also possible that IPPT-III is produced by translation initiation at a non-AUG codon or by proteolysis.

Mitochondrial IPP transferase is produced by initiation of translation at the first AUG codon of *MOD5* and apparently is not processed. Most nuclear-encoded mitochondrial proteins have an amino-terminal sequence that serves as a mitochondrial targeting signal (reviewed in reference 3). Therefore, translation from the first AUG codon of the *MOD5* open reading frame is expected to be necessary for mitochondrial localization. Figure 2B shows an immunoblot of mitochondrion-enriched protein fractions from cells containing wild-type and mutant *MOD5* genes on multicopy plasmids. IPPT-I is detected in mitochondrial fractions (Fig. 2B, lanes 2 and 4). In other experiments (data not shown), we have shown that IPPT-I cofractionating with mitochondria is inaccessible to added protease. Therefore, IPPT-I is not simply associated with mitochondria but is imported into this organelle. Cells harboring multicopy plasmids lacking the first ATG (M1 or M1,2) are deficient in mitochondrial IPP transferase (Fig. 2B, lanes 3 and 5), although small amounts of the fastest-migrating IPP transferase isozyme, IPPT-III, are present in the mitochondrion-enriched fractions. However, as shown below, IPPT-III does not function in mitochondria. Since IPPT-I but not IPPT-II is imported into mitochondria, the first 11 amino acids of *MOD5* appear to be necessary mitochondria targeting.

If the first 11 amino acids of *MOD5* are necessary for mitochondrial import, then a construct containing the codons for these amino acids would be expected to encode passenger proteins imported into mitochondria and constructs not coding these amino acids might fail to do so. To test this prediction, we produced four different plasmids carrying *MOD5-COXIV* gene fusions. YCpADH1MOD5-COXIV contains codons 1 to 156 of *MOD5* fused in frame to *COXIV*, and expression of these sequences is under the control of the yeast *ADH1* promoter. YCpADH1mod5-M1-COXIV, YCpADH1mod5-M2-COXIV, and YCpADH1mod5-M1,2-COXIV are the same except for having mutations at the first, second, or both ATGs of *MOD5*, respectively. Each of the four plasmids was tested for the ability to complement the deletion-disruption allele of *COXIV* in strain WD-1 (Table 1). Cells containing YCpADH1-MOD5-COXIV and YCpADH1-mod5-M2-COXIV complement *coxIV::LEU2*. That is, they have nearly the same generation time in nonfermentable carbon source media as cells containing pMC4, which encodes the authentic precursor form of COXIV protein. In contrast, cells containing YCpADH1-mod5-M1-COXIV and YCpADH1-mod5-M1,2-COXIV fail to complement *coxIV::LEU2*, as evidenced by little or no growth in such media. This lack of growth is also demonstrated by cells containing pMSS, which produces a form of COXIV lacking mitochondrial targeting signals. Thus, constructs coding for amino acids 1 to 11 produce proteins able to target COXIV protein to mitochondria, whereas constructs lacking these codons fail to do so, thereby confirming

TABLE 1. Influence of initiation codon mutations on mitochondrial import of MOD5-COXIV fusion proteins

Plasmid	Generation time (h) ^a	
	–Uracil +glucose	–Uracil +glycerol +lactate
YCpADH1-MOD5-COXIV	2.4	7.2
YCpADH1-mod5-M1-COXIV	2.4	>120 ^b
YCpADH1-mod5-M2-COXIV	2.3	5.6
YCpADH-mod5-M1,2-COXIV	2.1	>166 ^c
pMC4	2.6	5.5
pMSS	2.8	>166 ^c

^a Determined by monitoring growth at 30°C by spectroscopy at A₆₀₀.^b Cultures grew suddenly after 5 days.^c No growth at the last time point of the experiment.

the prediction. Data from experiments testing the behavior of in vitro-generated MOD5-COXIV chimeric proteins in a cell-free mitochondrial import system are consistent with the interpretation that the in vivo differences in the complementation of *coxIV::LEU2* are due to differences in uptake of proteins by mitochondria (7a).

The targeting sequence of most nuclear-encoded mitochondrial proteins is usually part of a transient presequence and generally constitutes the most amino-terminal segment of the presequence. A slight molecular mass difference of 1.1 kDa between IPPT-I and IPPT-II is expected for the initial translation products because these protein species are predicted to differ by 11 amino acids. Since most presequences are more than 11 amino acids long (reviewed in reference 3), a processed mitochondrial isozyme produced by translation from the first AUG of *MOD5* is expected to have a lower molecular weight than a product of initiation of translation from the second AUG. However, the mobility of mitochondrial IPP transferase on protein blots suggests this isozyme is of higher molecular weight than the nonmitochondrial isozyme. This suggests that IPPT-I is not processed.

To investigate whether mitochondrial IPPT-I has had a presequence removed, we compared its mobility with that of IPP transferase made in vitro. Figure 2B is a composite photograph of an immunoblot and an autoradiograph of the same blot. ³⁵S-labeled IPP transferase protein synthesized in vitro and detected by autoradiography in lane 1 migrates the same as IPPT-I produced by pJDBMOD5 and pJDBmod5-M2 harboring the wild-type and mutant second ATG alleles, respectively. Since the protein made in vitro comigrated with mitochondrial IPPT-I, IPPT-I does not appear to be modified upon in vivo import into the organelle. We have also determined that translation initiates preferentially at the first AUG in vitro and that the N-terminal sequences of *MOD5* are not removed upon mitochondrial in vitro import (7a).

Translation initiation site influences mitochondrial and cytoplasmic tRNA modification. The effects of *MOD5* initiation codon mutations on mitochondrial and cytoplasmic tRNA modification were determined for single-copy *MOD5* genes in the *mod5::TRP1* strain MT-8. This provides a functional test for the compartmentalization of IPP transferase isozymes, since the tRNA substrates of both compartments are known not to mix. The quantity of i⁶A in tRNA from MT-8 cells harboring a centromere-containing plasmid encoding the wild-type *MOD5* gene was compared with the amount of i⁶A-modified tRNA from cells harboring the same plasmids encoding various *MOD5* alleles (Table 2).

The assay used for detection of i⁶A-modified tRNA is

TABLE 2. Effects of translational start sites on i⁶ modification of tRNA

Plasmid	% tRNA with i ⁶ A modification ^a	
	Mitochondrial	Cytoplasmic
YCfMOD5	100	100
YCf50	0.3 ± 5.3	1.4 ± 6.9
YCfmod5-M1	0.4 ± 1.9	103.6 ± 18.5
YCfmod5-M2	117.0 ± 1.2	59.3 ± 6.8
YCfmod5-M1,2	0.0 ± 7.6	12.7 ± 1.4

^a Expressed as a mean percentage ± standard deviation of the i⁶A modification determined for YCfMOD5. All means were derived from at least four independent determinations.

based on differential aminoacylation of mitochondrial and cytoplasmic tRNAs (38) and detection of labeled aminoacylated molecules by radioimmunoassay (11). Mitochondrial and cytoplasmic tyrosine-isoaccepting tRNAs in total yeast tRNA preparations were specifically labeled with [³H]tyrosine by *E. coli* and yeast aminoacyl-tRNA synthetases, respectively. Labeled molecules were incubated with an anti-i⁶A antibody, immunoreactive tRNA molecules were collected, and the amount of radioactivity was determined.

Cells containing the wild-type *MOD5* gene on plasmid YCfMOD5 are the reference for 100% i⁶A modification of mitochondrial tRNA. MT-8 containing vector (YCf50) alone shows very little i⁶A modification of mitochondrial tRNA (0.3% ± 5.3% of wild-type levels). Similarly, cells harboring plasmids with the first ATG of *MOD5* destroyed (YCfmod5-M1) contain negligible levels of i⁶A on mitochondrial tRNA (0.4% ± 1.9%). Cells transformed with YCfmod5-M1,2 (both ATG codons altered) have negligible levels of mitochondrial i⁶A tRNA (0.0% ± 7.6%) even though some IPPT-III fractionates with mitochondria. In contrast, cells harboring plasmids with the first ATG intact but the second ATG mutant (YCfmod5-M2) have wild-type or greater than wild-type levels of i⁶A on mitochondrial tRNA (117% ± 1.2%). Therefore, the first ATG of *MOD5* is necessary and sufficient for wild-type levels of i⁶A modification of mitochondrial tRNA. These results are consistent with the fractionation of IPPT-I and not IPPT-II with mitochondria.

Cells transformed with vector alone have negligible levels of cytoplasmic i⁶A modification. Mutation of the first ATG has no significant effect on cytoplasmic i⁶A tRNA (YCfmod5-M1; 103.6% ± 18.5%). Therefore, modification of cytoplasmic tRNA is not dependent on an intact first ATG codon as is modification of mitochondrial tRNA.

Mutation of the first and second ATGs in YCfmod5-M1,2 causes a decrease in cytoplasmic i⁶A modification to 12.7% of wild-type levels. It seems likely that this partial modification is due to the low amount of IPPT-III present in cells. Alternatively, this isozyme may have low catalytic activity. Mutation of the second ATG alone (YCfmod5-M2) produced an unexpected result. If IPPT-I was efficiently targeted to mitochondria, then little of this isozyme should be available to modify cytosolic tRNA, yet in cells containing YCfmod5-M2, 59.3% of substrate cytosolic tRNA contains i⁶A.

The amount of i⁶A tRNA in the cytoplasm can also be measured by monitoring *SUP7*-mediated suppression of *ade2-1* and *can1-100* (Table 3). The i⁶A modification affects the efficiency of suppression by the nuclear-encoded suppressor tRNA *SUP7* (35). Inactivation of *MOD5* prevents i⁶A modification of *SUP7* and other cytoplasmic tRNAs. Cells unable to suppress the *ade2-1* nonsense allele accumu-

TABLE 3. Effects of translation start sites on nonsense suppression

Plasmid	Suppression of <i>ade2-1</i> (color)	Suppression of <i>can1-100</i>
YCfMOD5	+ (white)	+
YCf50	– (red)	–
YCfmod5-M1	+ (white)	+
YCfmod5-M2	+/- (pink)	+/-
YCfmod5-M1,2	– (red)	–

late a red pigment, causing red colony color on rich media, and are unable to grow on media lacking adenine. Cells unable to suppress *can1-100* are deficient in arginine permease and are therefore resistant to canavanine; efficient suppression causes sensitivity to this amino acid analog.

We found that MT-8 cells harboring vector alone (YCf50) showed no suppression of *ade2-1* and *can1-100*. Cells containing YCfmod5-M1,2 were indistinguishable from cells containing vector alone in color and resistance to canavanine. Therefore, the level of i^6A measured by the direct assay (12.7%) severely limits suppression of the *ade2-1* and *can1-100* alleles. Consistent with the direct measurements of i^6A on cytoplasmic tRNA, cells harboring YCfmod5-M1 were indistinguishable in suppression from cells containing the wild-type allele. Also consistent with the direct assay, mutation of the second ATG (YCfmod5-M2) resulted in partial suppression of *ade2-1* and *can1-100*. Thus, by both direct assay of i^6A and in vivo suppression, we show that IPPT-I is functional in modification of cytosolic tRNA.

IPP transferase produced by initiation of translation at the first ATG is not exclusively a mitochondrial isozyme. Two explanations can account for the modification of cytoplasmic tRNA by IPPT-I. Either this form of IPP transferase is predominantly localized in the mitochondria but can act on cytoplasmic tRNA during transport into mitochondria or at least some of this isozyme remains in the cytoplasm. To differentiate between these possibilities, we compared the distribution of IPP transferase isozymes in mitochondria and postmitochondrial supernatant (mostly cytoplasm) fractions.

Cells harboring multicopy plasmids containing the various *MOD5* alleles (pJDBMOD5, pJDBmod5-M1, pJDBmod5-M2, and pJDBmod5-M1,2) were fractionated. Samples of total homogenate, postmitochondrial supernatant, and mitochondria from each strain were analyzed for *MOD5*-specific antigen (Fig. 3). The protein blot in Fig. 3 was incubated with affinity-purified antibody to the β subunit of F_1 -ATPase after detection of IPP transferase with *MOD5* antipeptide antibody. The β subunit of F_1 -ATPase (54 kDa) serves as an indicator of the distribution of mitochondrial proteins in samples and as a reference for the predicted molecular masses of IPPT-I (50.2 kDa) and IPPT-II (49.1 kDa). The only immunoreactive protein detected among proteins from the *mod5::TRP1* strain MT-8 is the β subunit of F_1 -ATPase (Fig. 3, lane 1). This mitochondrial protein is enriched in all mitochondrial fractions (Fig. 3, lanes 2, 5, 8, and 11) and is detected in total homogenate but not in postmitochondrial supernatants.

The majority of IPPT-II from extracts of cells containing pJDBmod5-M1 is found in the postmitochondrial supernatant (Fig. 3, lane 9). However, $\sim 1\%$ of IPPT-II cofractionates with mitochondria; this may represent cytosolic contamination of mitochondria. The fastest-migrating IPP transferase isozyme, IPPT-III, is present in all samples from *mod5-M1,2* cells (Fig. 3, lanes 2 to 4). IPPT-III is also

FIG. 3. Immunodetection of IPP transferase isozymes in mitochondrion-enriched and mitochondrion-depleted fractions. Experiments were carried out as for Fig. 2 except that blots were incubated a second time with affinity-purified rabbit anti- F_1 -ATPase β -subunit antibody, and mitochondrial F_1 -ATPase β -subunit-specific bands were detected by a second incubation of the blot with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The positions of F_1 -ATPase β subunit (F_1 - β) and IPP transferase isozymes IPPT-I, IPPT-II, and IPPT-III are indicated. Lanes: 1, 50 μ g of total cellular protein from the *mod5::TRP1* strain MT-8 as a negative control for IPP transferase (the indicated *MOD5* genes were expressed on multicopy plasmids in the cells subjected to fractionation); 2, 5, 8, and 11, 20 μ g of protein from mitochondrion-enriched fractions; 3, 6, 9, and 12, 50 μ g of protein from postmitochondrial supernatant; 4, 7, 10, and 13, 50 μ g of total homogenate protein.

present in mitochondria and postmitochondrial supernatant fractions of *mod5-M2* cells (Fig. 3, lanes 5 and 6) but is not detected on this blot in cells expressing *mod5-M1* and *MOD5*.

The slowest-migrating form of IPP transferase (IPPT-I) is found in mitochondrial fractions from cells expressing *MOD5* and *mod5-M2* (Fig. 3, lanes 5 and 11). This form is also detected in the postmitochondrial supernatant of cells expressing *mod5-M2* (Fig. 3, lane 6). We have quantitated the amount of IPP transferase isozymes by densitometric scanning of the blots and normalized these data for the total amount of protein in the various fractions. This analysis indicates that about 9% of the total IPP transferase (IPPT-I plus IPPT-II) encoded by *MOD5* is associated with mitochondria. Approximately 40% of the total IPP transferase encoded by *mod5-M2* (IPPT-I) fractionates with mitochondria. Thus, in contrast to the mitochondrial marker, the β subunit of F_1 -ATPase, a significant fraction of IPPT-I does not fractionate with mitochondria.

By overexpression of *MOD5* genes, the natural distribution of IPP transferase isozymes may be altered. However, these data concerning the distribution of isozymes in cells harboring *MOD5* alleles on multicopy pJDB plasmids are consistent with the measurements of i^6A in tRNA and with the assessment of suppression in cells expressing *MOD5* alleles on the single- or low-copy-number YCf vector. These data support the hypothesis that a portion of IPPT-I is not localized to mitochondria.

DISCUSSION

A general mechanism for production of sorting isozymes has emerged, although not all members of this group have been studied sufficiently to show adherence to this scenario (see, for example, reference 18). In general, these genes code for two sets of transcripts, one initiating upstream of the first ATG and producing an amino-terminal extended mitochondrial isozyme and the other initiating upstream of a second in-frame ATG and encoding a protein localized in a different subcellular compartment. The *MOD5* gene is the only reported exception to this general scheme in that all detectable transcripts initiate upstream of the *MOD5* open reading frame (39). Despite this difference, our data provide strong evidence that *MOD5* encodes multiple proteins with a mitochondrial protein sorting signal arranged similarly to mitochondrial isozymes encoded by other single genes.

The results of our studies of *MOD5* wild type and variants with mutations of the first, second, or both ATGs support a model that *MOD5* encodes for at least two major proteins, one by initiation of translation at the first AUG and the other by initiation at the second AUG of *MOD5* transcripts. Formally, these studies do not eliminate an alternative model that IPPT-II is derived by proteolytic processing of IPPT-I at the second methionine. According to this model, IPPT-II is a product of proteolytic processing at the second methionine and *mod5-M2* produces only IPPT-I because this methionine has been altered. However, in other studies we have generated a construct that synthesizes solely wild-type IPPT-I, thereby negating the possibility of proteolytic processing at the second methionine (45a).

The product of initiation at the first AUG (IPPT-I) is imported into mitochondria, whereas the product of initiation from the second AUG (IPPT-II) is not. Therefore, the first 11 amino acids encoded by the *MOD5* gene are necessary for mitochondrial targeting of IPP transferase. Whether this peptide sequence comprises the entire mitochondrial targeting signal of IPP transferase and is sufficient for import of the protein is currently under investigation.

We also detect a third species of protein (IPPT-III) encoded by *MOD5*. Our data indicate that this protein arises from initiation at a third AUG located at codon 48. First, the electrophoretic mobility of this protein is consistent with initiation at this site. Second, the electrophoretic mobility of this protein is not altered by mutation of the first or second ATG. Third, the quantity of this protein increases when either the second or both of the first two ATGs are mutated, as if loss of an AUG on mRNAs allows ribosomes to scan to the third AUG for initiation of translation. Interestingly, IPPT-III possesses some enzyme activity. When coded from a single-copy *MOD5* gene, IPPT-III causes a low level of i^6A modification but no restoration of nonsense suppression. However, when it is expressed on a multicopy plasmid, nonsense suppression is efficient (data not shown).

The *MOD5* gene is unusual because it appears that the largest form of this protein is only partially targeted to mitochondria. Even though nuclear-encoded mitochondrial proteins can be imported posttranslationally both in vivo and in vitro (reviewed in reference 3), the processes of translation and import are closely coupled. Cytoplasmic polysomes translating mitochondrial proteins are found bound to mitochondria (1), and no or very low levels of cytoplasmic pools of mitochondrial proteins are found in *S. cerevisiae* (2, 43). However, our data support the existence of significant cytoplasmic pools of IPPT-I. With cell fractionation procedures in which most F_1 -ATPase β subunit fractionates with

mitochondria, only about 40% of IPPT-I is found in this fraction. It is possible the amount of IPP transferase found in the cytosol is artifactually high. First, since F_1 -ATPase β subunit associates with mitochondrial membranes, it might fractionate more efficiently than would a matrix-localized protein. Second, to conduct our studies we used vectors resulting in high expression of IPP transferase. Although overexpression could be the cause of cytoplasmic pools of mitochondrial IPP transferase, it seems unreasonable that overproduction of one of hundreds of imported proteins would saturate the import system in vivo. Even for single-copy *MOD5* genes, some IPPT-I must reside in the cytosol since cells harboring YCfmod5-M2 show ~60% wild-type levels of i^6A modification of cytoplasmic tRNA and intermediate levels of SUP7-mediated nonsense suppression. Therefore, our results indicate that IPPT-I is shared by two cellular compartments. A similar phenomenon has been reported for *TRM1*. However, in this case the form of protein produced by initiation of translation at the second AUG is found in two compartments, mitochondria and nuclei (14, 36).

We have considered two explanations for the incomplete mitochondrial import of IPPT-I. The first concerns the structure of the mitochondrial targeting signal. The predicted amino terminus of IPPT-I shows some similarities to and some differences from the majority of mitochondrial targeting sequences. Like other amino-terminal targeting sequences, the predicted amino terminus of IPPT-I is basic and amphiphilic. However, in contrast to other mitochondrial targeting sequences with helical hydrophobic moments averaging a value of 10 (51), the IPPT-I helical hydrophobic moment is 5.27. We are currently testing the hypothesis that this low helical hydrophobic moment results in inefficient mitochondrial import. A second explanation concerns the substrate of IPPT-I. It has been shown that secondary structure of proteins affects mitochondrial import. For example, a fusion protein consisting of cytochrome oxidase subunit IV and dihydrofolate reductase cannot be imported into mitochondria if a dihydrofolate reductase ligand is present (13). It is possible IPPT-I encounters tRNA substrate in the cytosol, rendering import inefficient.

Most proteins targeted to the interior of mitochondria possess presequences that are proteolytically removed by a matrix-localized protease (reviewed in reference 3). Our data suggest that the amino terminus of IPPT-I is not removed upon import into mitochondria. It is interesting that two other mitochondrial sorting isozymes, *TRM1* (15) and *LEU4* (6), also do not appear to possess cleavable presequences. In fact, only two members of this class of proteins, neither in yeast cells, have been reported to have a cleaved amino terminus (47, 49). It will be interesting to learn whether genes encoding other sorting isozymes are similar to *MOD5*, *TRM1*, and *LEU4* in this respect.

By studying *MOD5*, we have uncovered some aspects of gene expression and mitochondrial import that appear unique and other aspects that are similar to other members of this newly described category of genes. Further studies of *MOD5* and the other genes encoding sorting isozymes should broaden the understanding of gene expression and protein sorting in eukaryotes.

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