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Separate Information Required for Nuclear and Subnuclear Localization: Additional Complexity in Localizing an Enzyme Shared by Mitochondria and Nuclei

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The *TRM1* gene of *Saccharomyces cerevisiae* codes for a tRNA modification enzyme, N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (m_2^2Gtase), shared by mitochondria and nuclei. Immunofluorescent staining at the nuclear periphery demonstrates that m_2^2Gtase localizes at or near the nuclear membrane. In determining sequences necessary for targeting the enzyme to nuclei and mitochondria, we found that information required to deliver the enzyme to the nucleus is not sufficient for its correct subnuclear localization. We also determined that mislocalizing the enzyme from the nucleus to the cytoplasm does not destroy its biological function. This change in location was caused by altering a sequence similar to other known nuclear targeting signals (KKSKKKRC), suggesting that shared enzymes are likely to use the same import pathway as proteins that localize only to the nucleus. As with other well-characterized mitochondrial proteins, the mitochondrial import of the shared methyltransferase depends on amino-terminal amino acids, and removal of the first 48 amino acids prevents its import into mitochondria. While this truncated protein is still imported into nuclei, the immunofluorescent staining is uniform throughout rather than at the nuclear periphery, a staining pattern identical to that described for a fusion protein consisting of the first 213 amino acids of m_2^2Gtase in frame with β-galactosidase. As both of these proteins together contain the entire m_2^2Gtase coding region, the information necessary for association with the nuclear periphery must be more complex than the short linear sequence necessary for nuclear localization.

Proteins that must be routed to specific subcellular compartments contain targeting information ensuring their correct localization. Absence of a correct targeting signal results in mislocalization of sorted proteins, and often an inability to reach the correct subcellular destination can result in deficiencies in enzymatic activity and/or structural defects. In some cases, the consequences of mislocation are so severe that they lead to cell death (12, 40, 43).

While the majority of proteins localizing to subcellular organelles have only one cellular destination, there is a class of enzymes called "sorting isozymes" (24) that have multiple destinations. Enzymes produced by the same gene but localized to more than one compartment include invertase, which has a cytoplasmic and secreted form in yeasts (7, 38), and serine:pyruvate aminotransferase, which has a mitochondrial and peroxisomal form in rat liver (36). Enzymes shared by the mitochondria and cytoplasm include the leucyl-tRNA synthetase of *Neurospora crassa*, rat (44), mouse (16), and yeast (48) fumarase, the histidyl- and valyl-tRNA synthetases of *Saccharomyces cerevisiae* (8, 34), and yeast isopropyl malate synthase (4). Δ^2-isopentenyl pyrophosphate transferase of *S. cerevisiae* is found in mitochondria, cytoplasm, and nuclei (24, 26a, 32), as is the ATP (CTP) tRNA-specific nucleotidyltransferase (2, 10, 37). Finally, the *TRM1* gene product, N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (m_2^2Gtase), appears to be largely excluded from the cytoplasm but is localized in the mitochondria and nuclei and is, as far as we know, the only protein with this particular distribution described to date.

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The original evidence placing m_2^2Gtase in the nucleus was the observation that precursor tRNAs thought to be nuclearly restricted already contain the m_2^2G modification (22, 26, 37). Subsequent cell fractionation and indirect immunofluorescence studies indicated that the majority of the enzyme responsible for the modification of cytoplasmic tRNAs is nuclear and that little of it is found in the cytoplasm (31). Although the sequences necessary to target m_2^2Gtase to the nucleus were not defined by Li et al. (31), sequences sufficient to target a passenger protein to the nucleus were found to be located between amino acids 70 and 213 of the *TRM1* open reading frame.

Evidence supporting a mitochondrial location for m_2^2Gtase includes the fact that in yeast cells, mitochondrial tRNAs are made in the organelle (46) and the organelle contains m_2^2Gtase activity (19). The role of amino-terminal sequences in mitochondrial protein import in general is well established (27), and sequences sufficient to target passenger proteins to mitochondria are found at the amino terminus of m_2^2Gtase (18). The *TRM1* gene contains two in-frame ATGs and produces a long and short form of the enzyme which differ by 17 amino acids depending on which ATG is used as a start site for translation (17). Both forms can be imported into mitochondria in vivo. In vitro studies done by Ellis et al. (18) showed that amino acids 1 through 48 could target both cytosolic dihydrofolate reductase to mitochondria and substitute for the natural targeting signal of the mitochondrial protein COXIV. Amino acids 1 through 16 could not support the import of passenger proteins, but they did increase the efficiency of passenger protein import into mitochondria over that supported by amino acids 17 to 48 alone.

We have now extended our analysis of the sequences

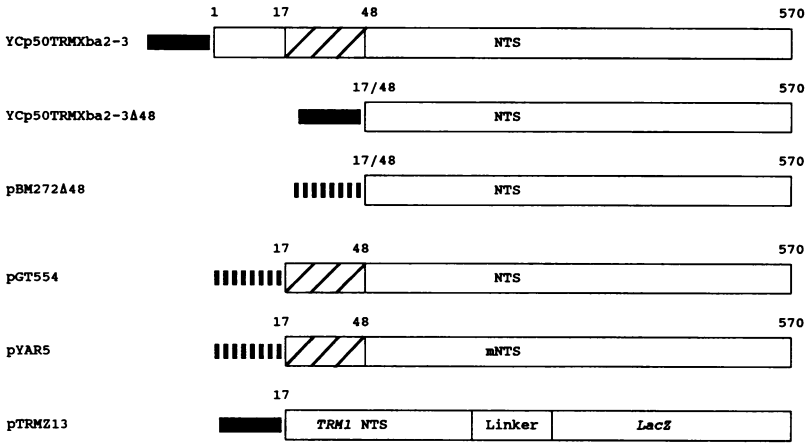

FIG. 1. Schematic representation of *TRM1* alleles and a *TRM1-lacZ* fusion. Open boxes represent the coding regions. The numbers above the boxes represent amino acid positions beginning from amino acid 1 of the *TRM1* reading frame. Methionines at positions 1 and 17 are both used to initiate translation. Filled horizontal lines represent constructions under control of the *TRM1* promoter, and vertical dashed lines represent constructions under control of the *GAL1* promoter. Slashed boxes represent those constructions containing the information necessary for mitochondrial targeting. NTS denotes the *TRM1* NTS. mNTS denotes the nucleotides coding for the mutant NTS which result in KKSKKKRC being changed to EESEEERC. *TRM1* NTS represents the nucleotides coding for the *TRM1* NTS in the *TRM1-lacZ* fusion.

necessary and sufficient for the dual localization of m_2^2Gtase. We constructed a truncated form of the protein lacking the first 48 amino acids of m_2^2Gtase and found that it no longer localizes to the mitochondria. Thus, these sequences are necessary as well as sufficient for mitochondrial protein import in vivo. An examination of amino acids 70 to 213 disclosed a short stretch of amino acids (95 to 102) containing a proposed four-residue consensus sequence for nuclear localization signals (NLS), Lys-Arg/Lys-X-Arg/Lys (9). In addition, amino acids 95 to 102 (KKSKKKRC) showed sequence similarity to the nuclear targeting signals (NTS) for the glucocorticoid receptor (RKTKKKIK) (39) and simian virus 40 large T antigen (PKKKRKV) (29). We used site-directed mutagenesis to alter this sequence and found a dramatic effect on the subcellular location of the enzyme, changing it from a predominantly nuclear peripheral location to a predominantly cytoplasmic location. Thus, two distinct classes of targeting signals must interact with mitochondrial and nuclear targeting pathways to partition the enzyme between these two organelles.

MATERIALS AND METHODS

Strains and media. Yeast strains used in these experiments were DBY745 (5) (*MATα leu2-3 ura3-52 ade1-101*); DBY745Δtrm1 (*trm1*::*LEU2*), W303-1B (R. Rothstein, Columbia University, New York) (*MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*), W303-1B$_{THT}$ (*trm1*::*HIS3*), and SEY2101 (20) (*MATa leu2-3,112 ura3-52 suc2-9 his4-519 gal2*). Bacterial strains used were *Escherichia coli* JM101 (33) [Δ(*lac-pro*) *supE thi-1* F′ *proAB*$^+$ *laci*$^+$ *lacZ*Δ15 *traD36*] and RZ1032 (30) (HfrKL16 PO/45 [*lysA*(61-62)] *dut-1 ung-1 thi-1 relA1 zbd-279*::Tn*10 supE44*). *E. coli* strains were maintained on LB medium (Luria Broth Base; GIBCO) containing ampicillin to select for plasmid expression. Yeast strains were maintained on YPD (1% yeast extract, 1% peptone, 2% dextrose) or selective *TRM* medium (19) minus the appropriate nutritional ingredient for selection of plasmid expression. Strains harboring plasmids under control of the *GAL1* promoter were grown in selective *TRM* medium containing galactose as a carbon source.

Plasmids. The mutagenesis protocol of Geisselsoder et al. (23) was used for introducing mutations into the *TRM1* gene. The various mutations in *TRM1* that were used are shown in Fig. 1. A *TRM1* gene coding for a protein missing the first 48 amino acids was constructed in two steps. First, the most upstream in-frame ATG of the *TRM1* gene in YCp50TRMXba2-3, which differs from the wild-type *TRM1* gene at position −6, creating an *Xba*I site (17), was altered to an ATC by using the oligonucleotide 5′CCTTCGATATCTAGATTGT. Second, a deletion of nucleotides 52 through 143 of *TRM1* was made with the oligonucleotide 5′GAATTTC TGCTTTTGCTAGCATTCCGTGTAAATTTGC, which hybridizes to nucleotides 34 to 51 and 144 to 157. The resulting plasmid, YCp50TRMXba2-3Δ48, retains the *TRM1* promoter but produces a truncated *TRM1* gene product initiating with an ATG at what was amino acid 46 in the wild-type protein and containing a glycine rather than an alanine at position 48. To produce the truncated protein at elevated levels, the truncated *TRM1* gene was cut from the plasmid with *Xba*I at the site previously introduced by Ellis et al. (17) and transferred into the *Bam*HI site of pBM272 (28) by using *Xba*I-*Bam*HI linkers to create pBM272TRMΔ48. This construction retains the coding sequences for *TRM1*, but transcription proceeds under control of the inducible *GAL1* promoter.

To provide a substrate for altering the putative NTS, we cloned the *Hin*dIII fragment containing the *TRM1* gene from pGT554 (31) into pBluescript M13+, and the oligonucleotide 5′AATGAAGAAAGTGAGGAGGAAAGGTGC was used to introduce the desired changes. The *Hin*dIII-*Sal*I fragment containing the mutated *TRM1* gene was cloned into pBM272 to create pYAR5.

The construction of a gene with the *TRM1* promoter and the NTS fused to *lacZ* required several steps. A Mp10 bacteriophage construct containing a *TRM1* fragment from the *Hpa*I site at position −536 to the *Hpa*II site at 738 (17) was cut with *Eco*RI and *Bam*HI (the *Eco*RI site is from the polycloning site in the phage Mp10 and the *Bam*HI site is at position 637 of *TRM1*). The resulting fragment which contains the *TRM1* promoter and sequences coding for amino acids 1 to 213 was cloned into Bluescript KS+. The most

upstream in-frame ATG of the *TRM1* fragment was altered to an ATC by using the oligonucleotide 5'CCTTCGATAT CTAGATTGT. To remove sequences coding for the *TRM1* protein upstream of the NLS this plasmid was mutagenized with the oligonucleotide 5'ATTTCTCTTCAACATTCCGTG, which is complementary to nucleotides 43 to 54 and 271 to 279 of the *TRM1* gene. After mutagenesis, an *Eco*RI-*Hha*I fragment that contained the *TRM1* promoter, second start site for translation, and the NLS was removed and ligated in frame to the *lacZ* gene in pSEY101 (21), using a *Hha*I-*Bam*HI linker with the sequence 5'CGGAAACTAACGATG ATTCG, 5'GATCCGAATCATCGTTAGTTTCCGCG. The resulting plasmid, pTRMZ13, codes for a fusion protein consisting of amino acids 17, 18, and 91 to 102 from *TRM1* in frame with the *lacZ* gene.

Preparation of crude extracts for enzyme assays and Western blot (immunoblot). Crude extracts were prepared as described previously (31). Western blots were done by the method of Towbin et al. (45). The *TRM1* antipeptide antibody is described by Li et al. (31). The second antibody was goat anti-rabbit antibody linked to either alkaline phosphatase (Bio-Rad) or iodinated protein A (ICN).

Enzyme assays. m_2^2G-specific tRNA methyltransferase activity was measured as previously described (19) with some modifications. Briefly, 150-μl reaction mixtures were incubated for 20 min at 37°C. A 130-μl sample of the reaction mix was transferred to 870 μl of ice-cold bovine serum albumin (BSA) (fraction V; Sigma) (100 μg) to which 1 ml of ice-cold 2 N HCl was added. The tubes remained on ice at least 5 min; this was followed by collection of precipitated material on Whatman GF/F filters previously soaked in 1 N HCl. The filters were washed with 1 N HCl (three times with 3 ml) and 95% ethanol (once with 1 ml). The filters were dried, and the amount of tritium incorporated into acid-insoluble material was determined by liquid scintillation counting with a Beckman LS-7000 liquid scintillation counter. The β-galactosidase assay was as described previously (31).

Indirect immunofluorescence. Indirect immunofluorescence was done as described by Li et al. (31) with the following modifications. The cells were fixed as described previously (31) for 20 min in solution A containing 40 mM K_2HPO_4-KH_2PO_4 (pH 6.5) and 80 mM $MgCl_2$ to which was added 0.6 ml of 37% formaldehyde (Fisher). After three 5-ml washes in solution B containing 40 mM K_2HPO_4-KH_2PO_4 (pH 6.5), 80 mM $MgCl_2$, and 1.2 M sorbitol, the cells were converted to spheroplasts in 1 ml of solution B plus 55 μl of glusulase (Dupont), 10 μl of β-mercaptoethanol (Sigma), and 20 μl of yeast lytic enzyme (ICN) (4 mg/ml). Spheroplasts were washed once in 5 ml of solution B and then resuspended in 500 μl of solution B. Spheroplasts (10 μl) were applied to slide wells previously coated with 0.025 to 0.05% poly-L-lysine per well, and excess cells were removed after 20 s. Slides were kept in a moist environment, and 10 μl of antipeptide antibody described by Li et al. (31) diluted 1:10 in solution F or 10 μl of anti-β-galactosidase antibody (Promega) diluted 1:50 was applied per well. The first antibody was allowed to incubate for 1.5 h at room temperature, and then the wells were washed eight times with a drop of solution F containing 0.73 mM KH_2PO_4 (pH 7.4), 145 mM NaCl, and 0.1% BSA. Second antibody (10 μl) (see Fig. 3 legend) was applied and incubated for 1.5 h in the dark, followed by eight washes with a drop of solution F. The slides were then placed in 1× phosphate-buffered saline (PBS) in a slide holder with a metal stir bar and bathed for 1 to 4 h at 4°C with gentle agitation to reduce background. The slides were air dried, and then 20 μl of DAPI (4',6'-diamidino-2-phenylindole) (1 μg/ml) was added, which was aspirated from the wells after 30 s. The slides were rinsed eight times in 1× PBS and air dried, and a coverslip was applied after the mounting medium had been added. Results were analyzed with a Nikon Inc. OPTIPHOT Biological Microscope.

Mitochondrial fractionation. Mitochondria were prepared by the procedure of Daum et al. (13). Mitochondria were treated by proteinase K as described by Ellis et al. (18).

TABLE 1. Methyltransferase activity[a]

Strain and plasmid	Sp act (pmol/min/mg)
W303-1b$_{THT}$/pBM272	ND[b]
W303-1b$_{THT}$/YCp50	ND
W303-1b$_{THT}$/pGT554	305 ± 22
W303-1b$_{THT}$/pYAR5	391 ± 10
W303-1b$_{THT}$/YCp50TRMXba2-3Δ48	ND
W303-1b$_{THT}$/pBM272Δ48	ND

[a] Crude extract was prepared from cells grown in selective media lacking the appropriate nutritional ingredient. Those constructions under control of the inducible *GAL1* promoter (see Fig. 1) were grown in selective media with galactose as the carbon source.

[b] ND, not detected above background by scintillation counting.

RESULTS

Nucleotide changes and plasmid constructions used for the experiments reported here are presented in Fig. 1. YCp50TRMXba2-3 differs from the wild-type gene only at position −6. This nucleotide change created an *Xba*I site upstream of the first ATG. YCp50TRMXba2-3Δ48 and pBM272TRMΔ48 have had the nucleotides coding for the first 48 amino acids altered or removed. YCp50TRMXba2-3Δ48 retains the *TRM1* promoter. pBM272TRMΔ48 retains the coding sequences for *TRM1*, but transcription proceeds under control of the inducible *GAL1* promoter. pGT554 contains all the coding sequences for *TRM1*, with translation proceeding from the second ATG and transcription under control of the inducible *GAL1* promoter. pYAR5 differs from pGT554 in that five nucleotides were changed such that the amino acids necessary for nuclear localization were altered. pTRMZ13 consists of a fusion gene containing the *TRM1* promoter and the *TRM1* nucleotides coding for amino acids 17 (ATG), 18, and 91 to 102 in frame with the *lacZ* gene.

m_2^2Gtase activity in mitochondrial and nuclear targeting mutants. To prepare a gene for determining whether the region of *TRM1* with similarity to other known NTS provides the nuclear targeting information for *TRM1*, we altered the gene such that the protein would have five glutamic acids instead of five lysines at positions 95, 96, 98, 99, and 100. The five nucleotide changes were confirmed by sequence analysis (data not shown), and the wild-type and mutant genes were introduced into a strain without a functional *TRM1* gene. Extract was prepared for the measurement of m_2^2G-specific tRNA methyltransferase (19). Table 1 shows that the mutant and wild-type genes produce comparable activity.

A fusion gene consisting of the nucleotides coding for the putative NTS for *TRM1* in frame with the *lacZ* gene was constructed for use in determining whether this region is sufficient to target a passenger protein to the nucleus. The fusion gene was confirmed by sequence analysis (data not shown). β-Galactosidase activity assays as well as Western analysis confirmed that the plasmid-coded fusion protein

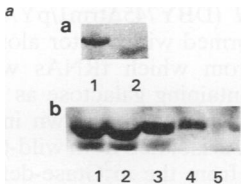

FIG. 2. (a) Amino-terminal-truncated m_2^2Gtase accumulates in yeast cells. Equal amounts of cellular protein were loaded on a 10% gel and analyzed by Western blotting with *TRM1* antipeptide antibody. Cells were grown in selective media minus uracil with glucose as the carbon source. Lanes: 1, wild-type m_2^2Gtase; 2, truncated m_2^2Gtase. (b) Amino-terminal-truncated m_2^2Gtase is not imported into mitochondria. Lanes: 1, total cellular protein; 2, supernatant; 3, mitochondrial pellet; 4, mitochondrial pellet after proteinase treatment; 5, mitochondrial pellet treated with proteinase K and Triton X-100.

was expressed and active (data not shown). The upstream region of the fusion gene contains *TRM1* sequences; therefore, transcription proceeds under control of the *TRM1* promoter.

To extend our previous studies which demonstrated that the amino-terminal sequences of m_2^2Gtase are sufficient for mitochondrial import of passenger proteins, we asked whether the sequences were also necessary. Plasmids with and without the nucleotides coding for this region were introduced into W303-$1B_{THT}$, a strain unable to produce endogenous m_2^2Gtase. Western blot analysis demonstrated that protein of the predicted size accumulates in yeast cells (Fig. 2a). The mutant protein is less abundant than is the wild type, and this suggests that the truncation has an effect on synthesis or stability of the protein. Enzyme extracts from this strain containing plasmids without the nucleotides coding for the first 48 amino acids under control of either the *TRM1* promoter or the inducible promoter for *GAL1* (discussed later) were unable to methylate m_2^2G-negative tRNAs, indicating that the truncated protein is inactive (Table 1).

Effect of alterations on protein localization. Indirect immunofluorescence was used to compare the subcellular locations of wild-type m_2^2Gtase and m_2^2Gtase altered at amino acids 95, 96, 98, 99, and 100. Because Li et al. (31) were unable to detect m_2^2Gtase at single-copy levels, the mutant and wild-type genes were cloned on a multicopy plasmid under control of the inducible *GAL1* promoter. Immunofluorescent staining of the wild-type protein gave a distinct nuclear rim pattern as reported previously (31) (Fig. 3a and b). In contrast, mutant m_2^2Gtase was largely if not totally confined to the cytoplasm. Some cells showed distinct regions of more intense cytoplasmic staining, and most cells had a "black hole" in the region identified as nuclear by DAPI staining (Fig. 3c and d). This result strongly suggests that amino acids 95 to 102 are necessary for the nuclear localization of the *TRM1* protein product.

Having shown that amino acids 95 to 102 are necessary for the nuclear localization of m_2^2Gtase, we wished to determine whether this region was sufficient to target a nonnuclear passenger protein to the nucleus. The immunofluorescent staining pattern observed for the TRM1–NTS–β-galactosidase fusion protein is both nuclear and cytoplasmic (Fig. 3e and f). While the region we identified as necessary for the nuclear localization of m_2^2Gtase appears to be sufficient for targeting β-galactosidase to the nucleus, this region may not contain all the information required to efficiently target the protein (see Discussion).

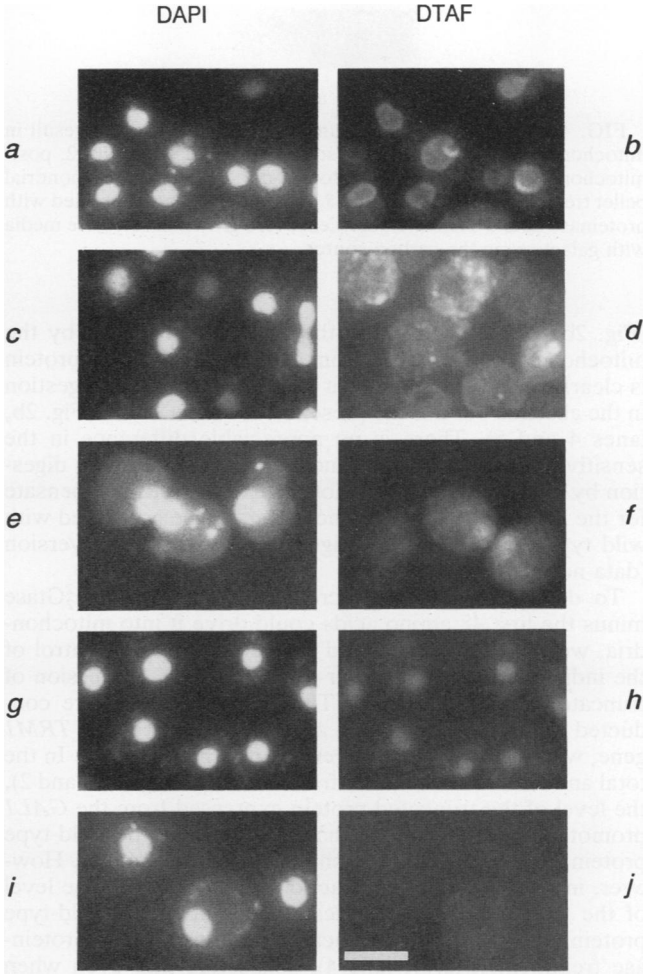

FIG. 3. Subcellular distribution of wild-type and altered TRM1 proteins by indirect immunofluorescence. All TRM1 proteins were plasmid encoded and expressed in W303-$1b_{THT}$. Cells harboring plasmids were grown in selective media minus the appropriate nutritional ingredient. Those cells containing plasmids under control of the inducible *GAL1* promoter (Fig. 1) were grown in selective media with galactose as the carbon source. (a, c, e, g, i). Fluorescence pattern from the DNA-specific stain DAPI. Staining pattern obtained for pGT554 (wild type) (a and b), pYAR5 (mutant NLS) (c and d), pTRMZ13 (TRM1–NTS–β-galactosidase) (e and f), pBM272Δ48 (m_2^2Gtase minus first 48 amino acids) (g and h), W303-$1B_{THT}$ alone (i and j). First antibody in panels b, d, h, and j was *TRM1* antipeptide antibody (1:10 to 1:100); second antibody was DTAF (dichlorotriazinylaminofluorescein)-conjugated goat anti-rabbit immunoglobulin G (Jackson) (1:200). First antibody in panel f was anti-β-galactosidase (Promega) (1:50); second antibody was DTAF-conjugated goat anti-mouse immunoglobulin G (Jackson) (1:200). Bar, 4 μm.

To determine whether m_2^2Gtase minus the first 48 amino acids is imported into mitochondria, we did cell fractionation experiments because we were unable to detect the protein by indirect immunofluorescence. Equal numbers of cells carrying wild-type or truncated genes were mixed and used to isolate mitochondria. Figure 2b (lanes 1 and 2) shows that both wild-type and truncated m_2^2Gtase are present in the homogenate and supernatant, respectively. Both proteins are associated with isolated mitochondria (Fig. 2b, lane 3), but none of the shorter protein survives protease treatment

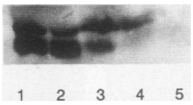

FIG. 4. Overproduction of truncated m_2^2Gtase does not result in mitochondrial localization. Lanes: 1, total cellular protein; 2, post-mitochondrial supernatant; 3, mitochondrial pellet; 4, mitochondrial pellet treated with proteinase K; 5, mitochondrial pellet treated with proteinase K and Triton X-100. Cells were grown in selective media with galactose as the carbon source.

(Fig. 2b, lane 4), indicating that it is not protected by the mitochondrial membranes from digestion. Wild-type protein is clearly in the organelle as it survives proteinase digestion in the absence but not the presence of Triton X-100 (Fig. 2b, lanes 4 and 5). There is no appreciable difference in the sensitivity of the wild-type and truncated proteins to digestion by proteinase K, and a longer exposure to compensate for the lower level of the truncated m_2^2Gtase compared with wild type did not reveal a signal for the truncated version (data not shown).

To determine whether increasing the level of m_2^2Gtase minus the first 48 amino acids could drive it into mitochondria, we placed the truncated *TRM1* gene under control of the inducible *GAL1* promoter to increase the expression of truncated m_2^2Gtase protein. These experiments were conducted in a strain containing an intact chromosomal *TRM1* gene, which served as a convenient internal standard. In the total and extramitochondrial fractions (Fig. 4, lanes 1 and 2), the level of the truncated protein expressed from the *GAL1* promoter is much greater than the level of the wild-type protein expressed from the chromosomal gene copy. However, in the mitochondrial fraction (Fig. 4, lane 3), the level of the truncated protein is less than that of the wild-type protein, and none of the truncated protein survives proteinase treatment (Fig. 4, lane 4), suggesting that even when overexpressed, m_2^2Gtase minus the first 48 amino acids is not imported into mitochondria. This result, when considered with results showing that the first 48 amino acids can promote import of a passenger protein (18), suggests that the first 48 amino acids are both necessary and sufficient for import. We cannot rule out, however, the possibility that no truncated protein is detected because it is imported but rapidly degraded.

Although the truncated protein is not imported into mitochondria, one would predict that it might be imported into nuclei as it retains the nuclear targeting information. The subcellular location of the truncated protein expressed from the *GAL1* promoter in the strain lacking a functional endogenous copy of *TRM1* was determined by indirect immunofluorescence. Using the *TRM1* antipeptide antibody, which specifically recognizes 15 amino acids at the carboxy terminus of m_2^2Gtase, we were able to detect a diffuse staining pattern which appears to be wholly confined to the nucleus (Fig. 3g and h). Interestingly, this protein does not localize to the nuclear periphery.

Mislocalization of m_2^2Gtase does not result in loss of activity in vivo. Changing five lysines to five glutamic acids altered the location of the *TRM1* protein product but did not change the activity measured in vitro. To determine whether the altered localization had an effect on tRNA modification in vivo, we isolated tRNAs and assessed their ability to serve as substrates in our in vitro assay. tRNAs were isolated from wild-type cells (DBY745), an m_2^2Gtase-deficient strain (DBY745Δtrm1), the m_2^2Gtase-deficient strain transformed with mutant *TRM1* (DBY745Δtrm1/pYAR5), and the deficient strain transformed with vector alone (DBY745Δtrm1/pBM272). Cells from which tRNAs were isolated were grown in media containing galactose as the carbon source. Cells harboring plasmids were grown in selective medium lacking uracil. tRNAs isolated from wild-type cells (4.6 ± 1.1 pmol/min/mg) and from the m_2^2Gtase-deficient strain transformed with mutant *TRM1* (10.3 ± 0.7 pmol/min/mg) do not accept methyl groups, while tRNAs from the m_2^2Gtase-deficient strain (131.5 ± 12.5 pmol/min/mg) and the deficient strain transformed with vector alone (117.5 ± 2.5 pmol/min/mg) do. Thus, the caveat of overproduction aside, modification occurs despite the altered location of the protein.

DISCUSSION

Enzymes that are shared by two subcellular compartments must contain the information necessary for their dual localization. We have shown previously that the *TRM1* gene of *S. cerevisiae* codes for a protein that modifies both cytoplasmic and mitochondrial tRNAs and that the enzyme is located in mitochondria and nuclei (19, 31). The work reported here coupled with the previous results shows that these shared isozymes have signals similar to those in proteins destined to go only to mitochondria or only to nuclei. The characteristics of the amino-terminal end of m_2^2Gtase are shared with other mitochondrial targeting signals (18). Thus, we predicted that the first 48 amino acids would be necessary for the import of m_2^2Gtase into mitochondria. The observation that they are complements our previous work showing that these amino acids are sufficient to target passenger proteins to mitochondria (18). Overexpression of the truncated form does not compensate for its defect in mitochondrial protein import. Removing the first 48 amino acids does have an effect on the synthesis or stability of the protein as it is less abundant when compared with the product of the wild-type gene. The protein is also inactive, indicating a structural perturbation that, in addition to the deletion, could affect its ability to interact with components of the mitochondrial import pathway. It is still imported into nuclei; therefore, the change is not sufficient to preclude interactions with the nuclear import pathway.

Amino acids 95 to 102 of m_2^2Gtase, which are similar to other known NTS, are necessary for the nuclear localization of the enzyme. Although changing five lysines to five glutamic acids is not conservative, we reasoned that changing five basic amino acids to five acidic amino acids would be less likely to affect the conformation of the protein than, for example, replacing them with hydrophobic amino acids. The fact that the five-amino-acid change did not affect the activity of the protein suggests that the native conformation is not affected.

The patterns of immunofluorescent staining of the wild-type protein and the mutant are very different. The former localizes at or near the nuclear envelope, while the latter is predominantly cytoplasmic. These results were obtained with proteins produced from the *GAL1* promoter, and thus the proteins are overexpressed relative to wild-type levels. Even overexpression of the mutant protein does not appear to drive it into the nucleus.

Wild-type m_2^2Gtase gives an immunofluorescent staining pattern different from those of other proteins reported to localize to the nuclear periphery in yeast cells (1, 14). The immunofluorescent staining pattern observed for both the *crm1*$^+$ and *NUP1* protein products is more punctate than that observed for m_2^2Gtase. We have recently verified that

the peripheral staining pattern observed for m_2^2Gtase is not an artifact of overproduction as we have succeeded in detecting the protein at wild-type levels by altering the protocol (42a) used by Li et al. (31). Perinuclear staining is also seen with proteins that localize to the endoplasmic reticulum (6, 15, 25). This perinuclear staining, however, appears to occupy a region outside that identified as nuclear by DAPI, in contrast to m_2^2Gtase staining, which appears to overlay the perimeter of the region stained by DAPI. In addition, proteins associated with the endoplasmic reticulum often show linear staining radiating perpendicular to the nucleus and close to the plasma membrane (25). This staining pattern is not observed with m_2^2Gtase. We are unaware of any other descriptions in yeasts of continuous peripheral staining such as we observed here. The most similar pattern is that observed in turkey erythrocytes with anti-lamin B receptor antibodies, in which the staining is continuous around the nucleus (47).

Not only do our experiments implicate KKSKKKRC as being necessary for targeting m_2^2Gtase to nuclei, it appears to be sufficient because it targets β-galactosidase to the nucleus. There are several explanations for why this targeting is not as efficient as is the 1-213 fusion. First, while the amino acid sequence of m_2^2Gtase does not indicate that the NLS might be bipartite as has been determined for many other nuclear proteins (42), we cannot rule out the possibility that additional sequences are required for efficient nuclear targeting. Second, it is well known that the passenger protein can have an effect; for example, the GAL4 NLS was found to more efficiently target invertase than β-galactosidase (35). However, the TRM1–NTS–β-galactosidase fusion protein does localize to the nucleus, indicating that amino acids 95 to 102 are sufficient for the nuclear localization of a normally nonnuclear protein. We have now analyzed two fusion proteins and one truncated protein that are localized to the nucleus, and none of them displays the wild-type rim-staining pattern. We do not yet know what accounts for the subnuclear location of m_2^2Gtase, but it is not likely that it is a short stretch of amino acids. Both the protein lacking 48 amino-terminal amino acids and the 1-213 fusion protein are efficiently transported to the nucleus as judged by the lack of staining in the cytoplasm. Both contain the KKSKKKRC sequence, so while it is required to take the protein to the nucleus, it does not appear to play a role in the subnuclear location we see. Further, all sequences missing from Δ48 are present in 1-213, and all missing in 1-213 are present in Δ48, and neither shows the peripheral staining characteristic of the native protein. Most likely, the native conformation of the protein is required for the association. It is clearly not present in any of the mutants we studied except for the NLS mutation, and that protein simply cannot get in. The diffuse nuclear staining pattern further establishes m_2^2Gtase as a nuclear protein, because if the wild-type protein localized to the endoplasmic reticulum (as do some other proteins with a perinuclear staining pattern) and not the nucleus, disruption of the information required for interaction with the endoplasmic reticulum should have rendered the staining cytoplasmic, not diffuse throughout the nucleus.

The functional significance of TRM1 being located in the periphery of the nucleus is not known. The idea that tRNA biosynthetic enzymes might be localized together in the nucleus is an attractive one and has been suggested previously (11). The tRNA-splicing endonuclease is reported to behave like an integral membrane protein (37, 41), and the tRNA-splicing ligase has been localized near the nuclear envelope (11). On the other hand, one could argue that since 30-fold overproduction of *TRM1* does not change the immunofluorescent staining pattern, saturation of sites does not occur, and it is clear that the enzyme can still provide wild-type levels of m_2^2G even though it is not located in the nucleus. Thus, while m_2^2Gtase may be arranged in a complex with other enzymes, the arrangement is not obligatory for function.

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