N^2,N^2-Dimethylguanosine-specific tRNA Methyltransferase Contains Both Nuclear and Mitochondrial Targeting Signals in *Saccharomyces Cerevisiae*

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Abstract. The *TRM1* gene of *Saccharomyces cerevisiae* encodes a tRNA modification enzyme, N^2,N^2-dimethylguanosine-specific tRNA methyltransferase, which modifies both mitochondrial and cytoplasmic tRNAs. The enzyme is targeted to mitochondria for the modification of mitochondrial tRNAs. Cellular fractionation and indirect immunofluorescence studies reported here demonstrate that this enzyme is also localized to the nucleus. Further, immunofluorescence experiments using strains that overproduce the enzyme show a staining at the periphery of the nucleus suggesting that the enzyme is found in a subnuclear destination near or at the nuclear membrane. There is no obvious cytoplasmic staining in these overproducing strains. Fusion protein technology was used to begin to localize sequences involved in the nuclear targeting of this enzyme. Indirect immunofluorescence studies indicate that sequences between the first 70 and 213 NH$_2$-terminal amino acids of the methyltransferase are sufficient to target *Escherichia coli* β-galactosidase to nuclei.

PRIMARY transcripts of tRNA genes undergo diverse posttranscriptional reactions during their maturation. A large proportion of these processing steps result in the modification of specific nucleoside residues with the majority of the modifications being methylation reactions. Although the biological roles of these methylations are not clearly defined, the methylated nucleosides account for 30–70% of the modified nucleosides in tRNA (Nau, 1976). Mitochondrial and cytoplasmic tRNAs have different but overlapping populations of methylated bases (Martin et al., 1976). Therefore, enzymes that are required for the synthesis of the methylations unique to each type of cellular RNA are likely specifically targeted to either mitochondria or the nucleus/cytoplasm to function. Enzymes responsible for the synthesis of the same modified bases in both mitochondrial and cytoplasmic tRNAs could either be functionally related but structurally distinct isozymes or they could be structurally identical but found in multiple cellular locations.

trm1 is a nuclear mutation that affects N^2,N^2-dimethylguanosine (m$_2^2$G)[1] biosynthesis of both mitochondrial and cytoplasmic tRNAs in *Saccharomyces cerevisiae* (Hopper et al., 1982). Strains carrying the mutation and thus unable to make m$_2^2$G, do not show any noticeable phenotype. We have previously reported the isolation of the *TRM1* gene and demonstrated that it encodes m$_2^2$G-specific tRNA methyltransferase (Ellis et al., 1986). Two forms of the enzyme can be made from this gene because some mRNAs transcribed from this gene include two AUGs separated by 15 codons while other, shorter mRNAs have only the second AUG from which to initiate protein synthesis (Ellis et al., 1987). Enzyme synthesized from either the first or second AUG can be imported into mitochondria for m$_2^2$G base modification. The m$_2^2$G base modification of cytoplasmic tRNAs appears to be carried out largely by enzyme synthesized from the second AUG (Ellis et al., 1987).

Methylation of nucleosides represents a major modification step in tRNA maturation. Although at least eight distinct methylation enzymes have been identified in *S. cerevisiae*, very little is known about the intracellular locations of these enzymes (Bjork and Svensson, 1969). The fact that some mitochondrial tRNAs contain m$_2^2$G suggests that the enzyme must be found in mitochondria, a supposition supported by the identification of NH$_2$-terminal sequences that are sufficient to target passenger proteins to mitochondria (Ellis et al., 1989). Biochemical evidence indicates that nuclear precursor tRNAs contain m$_2^2$G, and they are believed to be nuclear restricted (Peebles et al., 1979; Nishikura and De Robertis, 1981). This suggests that the enzyme responsible for this modification must be imported into the nucleus. It was not known whether the enzyme is also located in the cytoplasm.

Cell fractionation and indirect immunofluorescence experiments demonstrate that m$_2^2$G-specific tRNA methyltransferase is a nuclear enzyme in yeast. Specific nuclear rim staining is observed in strains that overproduce the enzyme, suggesting that the enzyme is at special subnuclear locations, possibly on or close to the nuclear membrane. Gene fusion

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1. *Abbreviations used in this paper*: β-gal, β-galactosidase; DAPI, 4',6'-diamidino-2-phenylindole; m$_2^2$G, N^2,N^2-dimethylguanosine.

technology has shown that the NH₂-terminal 213 amino acids of the enzyme contain sufficient information to target β-galactosidase (β-gal) to both the nucleus and mitochondria. Since a fusion protein containing only the NH₂-terminal 70 amino acids of the modification enzyme are not targeted to nuclei, we infer that the region between amino acid 70 and 213 of this enzyme contains sufficient information to target β-gal to the yeast nucleus and have identified a stretch of amino acids in this region that are similar to other known nuclear targeting signals.

Materials and Methods

Strains and Media

Strains used in these experiments were MH41-7B (*MATa, ade2, his1*), W303-1b (*MATa, ade2-1, his3-11/15, leu2-3/112, ura3-1, trp1-1, can1-100*), SN1015-2a (*MATα, SUP4, trm1, trm2, gal1, gal7, ade2-1, leu1, ura3, met, lys2-1, trp, ura3-1*), W303-1bTHT (a strain carrying a disrupted allele of the *TRM1* gene), DB745 (*his4, leu2-3, ura3, ade2-1*), and SEY2101 (*MATa, ura3-52, leu2-3/112, suc2-9, his4-519, gal2*).

Plasmids

HC-3 is a *Yep24-TRM1* recombinant plasmid which supports the synthesis of two forms of m₂²G-specific tRNA methyltransferase differing by 16 NH₂-terminal amino acids because two in-frame ATGs are present in the gene and both are used (Ellis et al., 1986). A *TRM1* gene, altered by site-directed mutagenesis to introduce a Hind III site at position 8, was cloned into the Hind III site of pBM272 which contains the Gal1-Gal10 promoter region (Johnston and Davis, 1984). The first ATG is upstream of the Hind III site so that synthesis of m₂²G-specific tRNA methyltransferase is from only the second ATG of the wild-type *TRM1* gene in the resulting plasmid, pGT554.

TRM1-LacZ fusion genes were produced by cloning an Eco RI/Bam HI fragment containing the *TRM1* promoter, both in-frame ATGs, and 213 codons counted from the first ATG (Ellis et al., 1987) into the Eco RI/Bam HI sites of the vector, pSEY101 (Emr et al., 1986). The resulting plasmid, pTRMZ213, generates chimeric proteins 1-213 TRM1 and 17-213 TRM1 derived from the first or second in-frame ATG, respectively. Both have β-gal activity. Plasmid pTRMZ70 was derived from pTRMZ213 by deleting a Bgl II/Bam HI fragment. This deleted *TRM1* sequence codes for amino acids 71 to 213 and thus the plasmid generates chimeric proteins 1-70 TRM1 and 17-70 TRM1 with β-gal activity but containing fewer amino acids derived from the *TRM1* sequence than those proteins derived from pTRMZ213. An identical strategy was used to construct plasmids that would synthesize only 17-213 TRM1 and 17-70 TRM1 proteins by using a *TRM1* gene in which the first ATG had been altered by site-directed mutagenesis (Ellis et al., 1987). The plasmid pLG669Z produces authentic β-gal (Guarente et al., 1982). All plasmids were introduced into yeast by the method of Ito et al. (1983). DNAs from yeast transformants were prepared using the procedure developed by Zakian and Scott (1982) and confirmed either by restriction analysis or by DNA sequencing (Sanger et al., 1977).

Preparation of Crude Extracts for the m₂²G-specific tRNA Methyltransferase Assay and Western Blot

Yeast strain DB745 carrying pGT554 was grown in either YPD(glucose) or YPGD(galactose) medium. Other strains were grown in media minus uracil (Hopper et al., 1982) and extracts prepared as described by Silver et al. (1984). Briefly, when the optical density of the culture reached 1.0 OD₆₀₀, cells were harvested by centrifugation, washed with NET-NP(150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.5% NP-40), and suspended in NET-NP buffer containing 1 mM PMSF. Glass beads were added to the suspension and mixed on a vortex mixer at maximum speed for five 30-s intervals separated by cooling in ice. After centrifugation at 15,000 g for 1 min, the supernatant was saved. Protein concentrations were determined using the method of Bradford (1976). m₂²G-specific tRNA methyltransferase activity was measured as described previously (Ellis et al., 1986). Western blots were done according to the procedure described by Tobin et al. (1979).

Antigen Preparation

An oligopeptide of 15 amino acids, CNPIKNWGPKARPNTS, predicted from the COOH terminus of *TRM1* was ordered from the Biochemistry Department, University of Texas Southwestern Medical Center (Dallas, TX). A Cys residue was added to the NH₂-terminus for cross-linking of the peptide to keyhole limpet hemocyanin. The coupling reaction of the peptide to keyhole limpet hemocyanin was done with chemical cross-linkers (Lerner et al., 1981) by Immuno-Dynamics, Inc. (La Jolla, CA).

Antibody Production

New Zealand white male rabbits were immunized according to the procedure of Lerner et al. (1981). Two rabbits were injected subcutaneously on day 0, 14, 28, 42, and 49 with 0.8 mg of peptide-keyhole limpet hemocyanin conjugate. 0.5 ml of 1.6 mg/ml peptide in PBS was emulsified with 0.5 ml Freund's complete adjuvant (day 0) or incomplete adjuvant (day 14, 28, 42, and 49). Rabbits were bled first on day 35 and then 6-9 d after the booster injections on day 42 and 49.

10-15 ml of sera were collected each time from each rabbit. Blood was set at room temperature for 1 h. The blood clot was rimmed and placed in the cold for 1 h and then centrifuged at 7,700 g for 5 min. The serum was transferred into a clean centrifuge tube and again centrifuged for 5 min at 7,700 g. The clear supernatant was then incubated at 56°C for 30 min to denature the complement component of the serum.

Affinity Purification of the Antipeptide Antibody

Antibodies were purified by affinity chromatography. The peptide was coupled to CNBr-activated Sepharose 4B according to Livingston (1974). 1 ml of the peptide-Sepharose resin was placed in a column and equilibrated with 50 ml buffer A containing 20 mM Tris-HCl, pH 7.5, and 100 mM NaCl. Antiserum was diluted 1:3 with buffer A and loaded on the column by gravity. The flow-through was recycled twice. After loading the serum, the column was washed extensively with 1 liter buffer A. The peptide-specific antibody was eluted with 10 ml of 0.2 M glycine, pH 2.2. 1-ml fractions of eluate were collected into an Eppendorf tube (Brinkmann Instruments Co., Westbury, NY) containing 100 μl of 1 M K₂HPO₄ to neutralize the pH immediately. Protein concentrations were determined by the method of Bradford (1976) and the antibody quality was assayed by Western blots (Tobin et al., 1979).

Preparation of Enzyme Extracts for Inhibition Assays

185 g of MH41-7B were suspended in 300 ml of buffer A containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EDTA, 25% ethylene glycol, 0.5 mM benzamidine, 0.5 mM PMSF, and 10 mM β-mercaptoethanol. The solution was decanted, mixed with (NH₄)₂SO₄ to a final concentration of 1 M and Triton X-100 to a final 1% and stirred for 1 h at 4°C. Cells debris was removed by centrifugation at 35,000 g for 15 min and the supernatant centrifuged at 100,000 g for 1 h. After centrifugation, the supernatant was dialyzed with several changes of buffer B overnight. Buffer B is the same as Buffer A except that the pH of the Tris-HCl is 8.0 instead of 7.5. More than 80% of the enzyme activity was recovered in the supernatant.

A 1.5 × 10-cm DE53 column was packed and equilibrated with buffer B. The S100 extract was loaded on the column at a flow rate of 5 ml/h. After loading, the column was washed with buffer B until the optical density of the eluate was <0.01 OD₂₈₀. The enzyme was eluted from the column with a KCl gradient between 0 and 500 mM in buffer B. Fractions containing enzyme activity were pooled and quickly frozen at −70°C. The enzyme was enriched 50-fold by this procedure.

Enzyme Inhibition Assay

1.25 μg of the enzyme extract prepared as described above was preincubated with purified antipeptide antibody on ice for 2 h. Total IgG protein was kept constant, but the ratio of immune IgG to preimmune IgG protein increased. The incubation mixture was then added to a premixed enzyme reaction solution. All the remaining procedures were the same as described previously (Ellis et al., 1986).

Nuclear Fractionation

The fractionation of extracts into nuclear and nonnuclear components was done according to Silver (1984). 300 ml (5 × 10⁷ cells/ml) were harvested

and suspended in 8 ml of 1 M sorbitol, 20 mM K_2HPO_4-KH_2PO_4, pH 6.5, 0.5 mM $CaCl_2$, 0.5% 2-mercaptoethanol, 0.5 mM PMSF. Zymolyase 60,000 was added to a final concentration of 0.25 mg/ml, and incubated at room temperature until spheroplast formation occurred. Spheroplasts were spun down at 3,500 g for 3 min, and suspended in 2 ml lysis buffer containing 0.2% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM PMSF. 15 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) were used to lyse the spheroplasts.

Crude nuclei were collected by centrifugation at 3,000 g for 5 min and suspended in 30 ml of 50% (wt/vol) percoll, 40 mM Pipes, pH 6.8, 10 mM $MgCl_2$, 0.5 mM PMSF, 0.05% Triton X-100. The suspension was then centrifuged at 21,000 g for 35 min in a Ti-30 rotor. The nuclear band was harvested and the nuclei pelleted at 5,000 g after diluting the percoll solution 10-fold with 0.01 M K_2HPO_4-KH_2PO_4, pH 6.5.

β-Galactosidase Assay

Assays for β-galactosidase were done according to Miller's procedure (Miller, 1972) with some modifications specific for yeast. 0.1–1 ml of yeast culture with an optical density of 1 OD_{600} was mixed with 2 vol of Z buffer containing 0.1 M K_2HPO_4-KH_2PO_4, pH 7.0, 0.01 M KCl, 1 mM $MgSO_4$, and 0.05 M 2-mercaptoethanol. The mixture was centrifuged in a clinical centrifuge for 1 min. The cell pellet was suspended in 1 ml Z buffer. 50 μl $CHCl_3$ and 20 μl 0.1% SDS were added. The mixture was mixed for 10 s at maximum speed on a vortex mixer to break the cells. The cell suspension was then incubated at 28°C for 2 min. The enzyme substrate o-nitrophenyl-β-D-galactoside was prewarmed at 28°C. The enzyme reaction was started by adding 0.2 ml o-nitrophenyl-β-D-galactoside and continued till the reaction solution turned to light yellow. 0.5 ml of 1 M Na_2CO_3 was then added to stop the reaction. Cell debris and $CHCl_3$ were separated from the reaction solution by centrifugation. The top clear yellow solution was transferred to a new tube and the optical density (OD_{420}) was measured with a spectrophotometer.

Antimycin A-insensitive NADH–Cytochrome C Reductase Assay

The enzyme assay was done according to Sottocassa's procedure (Sottocassa et al., 1967). The blank was a mixture of 0.4 ml cytochrome C (0.1 mM), 0.1 ml KCN (100 mM), 0.1 ml antimycin A (0.5 mg/50 ml ethanol), 0.5 ml buffer (0.1 M K_2HPO_4-KH_2PO_4, 10^{-4} M EDTA, pH 7.6) and either 10 μl mitochondrial suspension or 100 μl cytosol fraction. The blank was monitored for 1 min for the absorption change. In the assay, everything was the same except that 0.1 ml NADH (1 mM) was used and 0.4 ml, instead of 0.5 ml, buffer was added. The kinetic reaction was measured for 1 min.

Alcohol Dehydrogenase Assay

The assay was used as an assay for cytoplasmic contamination. This assay is based on the increase in absorbance at 340 nm which results from the reduction of NAD in the presence of alcohol and alcohol dehydrogenase (Vallee and Hoch, 1955). The 10× reaction mixture contains 160 mM sodium pyrophosphate, pH 8.8, 3.3 M ethanol, and 83.3 mM NAD. Cell extracts and water were added to the reaction mixture to a final volume of 3 ml. The reaction was carried out for 5 min at room temperature and the optical density (OD_{340}) was measured with a spectrophotometer.

RNA Polymerase Assay

RNA polymerase was used as a nuclear marker. The assay was done according to the procedure of Winkley et al. (1985). The reaction mixture contains 60 mM Tris-HCl, pH 7.9, 2 mM $MnCl_2$, 500 μM UTP, CTP, and GTP, 10 μl α-[^{32}P]ATP (800 Ci/mmol), 50 mM $(NH_4)_2SO_4$, 150 μg/ml calf thymus DNA and cell extracts to final volume of 40 μl. The reaction was carried out at 30°C for 10 min, and stopped by spotting 20 μl of the reaction mixture onto DE81 paper. The remaining steps were based on Roeder's procedure (Roeder, 1974). DE81 filters were immersed in 0.5 M Na_2HPO_4, washed six times with the same buffer, then washed twice in water, twice in 95% ethanol, and once in ether. The filters were dried and radioactivity was measured in a scintillation counter.

Indirect Immunofluorescence

Indirect immunofluorescence experiments were carried out according to

Kilmartin's procedure with some modifications (Kilmartin et al., 1982). 10 ml of a culture in early log phase were harvested in a clinical table centrifuge for 5 min at maximum speed. Cell pellets were suspended in 5 ml of solution A containing 40 mM K_2HPO_4-KH_2PO_4, pH 6.5, and 80 mM $MgCl_2$. 0.6 ml 37% formaldehyde was added to the suspension to fix cells at room temperature for 2 h without shaking. The fixed cells were spun down and washed three times with solution B containing 40 mM K_2HPO_4-KH_2PO_4, pH 6.5, 80 mM $MgCl_2$, and 1.2 M sorbitol. Cells were suspended in 1 ml solution B. 55 μl Glusulase, 10 μl β-mercaptoethanol, and 20 μl Zymolyase 100T (1 μg/μl in water) were added and the mixture was incubated at 37°C for 1 h to make spheroplasts.

Wells on slides were coated with 0.1% polylysine 400,000 by adding 10 μl of polylysine into each well and removing it by aspiration 10 s later. After the wells dried, they were washed three times with water. Spheroplasts were washed once with solution B and then suspended in 1 ml solution B. 10-μl spheroplasts were applied into each well and incubated for 20 s. The cell suspension was aspirated and the wells were air dried. 10 μl affinity purified antipeptide antibody diluted 1:10 to 1:50 was applied to each well, and incubated for 1.5 h at room temperature in a moist environment. The primary antibody was washed off with eight changes of solution F containing 0.73 mM KH_2PO_4, 145 mM NaCl, and 0.1% BSA. 10 μl of FITC-conjugated second antibody from 1:600 to 1:1,000 dilution was added to each well and incubated for another 1.5 h at room temperature in a dark and moist environment. Solution F was again changed eight times to remove the secondary antibody. For double staining, 20 μl 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/ml) was applied to each well and incubated for 1 min. The solution was removed and wells were washed once with PBS. Wells were mounted with mounting medium and the coverslip was sealed with nail polish. The results were analyzed with a Nikon Inc. Biological Microscope OPTIPHOT (Garden City, NY).

The primary antibody was the affinity-purified antipeptide antibody. The secondary antibody was goat anti-rabbit IgG, conjugated with FITC (Cappel Laboratories, Cochranville, PA). Nuclear and mitochondrial DNAs were stained by DAPI to serve as location markers for both the nucleus and the mitochondria. Anti-β-galactosidase antibody was purchased from Worthington Biochemical Corp. (Freehold, NJ) and purified through a β-gal-Sepharose 4B affinity column.

Results

The TRM1 Gene Encodes the m²₂G-specific tRNA Methyltransferase

We have shown previously that introduction of the *TRM1* gene into *trm1* mutant yeast cells restores the ability of the mutant to make m²₂G. Furthermore, expression of the *TRM1* gene in *Escherichia coli* converts that organism into one that is able to make this particular base modification (Ellis et al., 1986). These experiments led us to propose that *TRM1* encodes m²₂G-specific tRNA methyltransferase. Here we present two additional experiments that strengthen that conclusion. First, manipulations which alter the expression of the *TRM1* gene in yeast alter the level of m²₂G-specific tRNA methyltransferase. Specifically, the *TRM1* gene was introduced into a multicopy plasmid and placed under control of the inducible *GAL1* promoter. This promoter drives increased transcription when yeast cells are grown in galactose. Cells containing the *TRM1* gene under *GAL1* control show a 30-fold induction of this specific methyltransferase enzyme activity above the noninduced level found in cells grown in glucose (Fig. 1).

A more direct demonstration that the *TRM1* gene codes for the enzyme was obtained by using an antibody raised against a synthetic antigen whose sequence was predicted from the *TRM1* open reading frame (Ellis et al., 1987). The *TRM1* open reading frame predicts a protein of 63 kD and the antipeptide antibody recognizes a protein of 63 kD (Fig. 2 *A*, lane *1*). The smaller protein seen at 60 kD in this particular
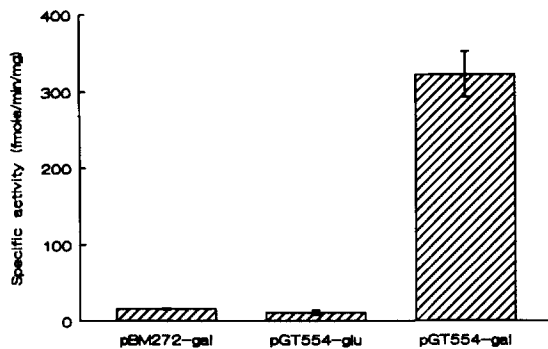
Figure 1. Alteration of *TRM1* gene expression alters m$_2^2$G-specific tRNA methyltransferase activity. Methytransferase activity was measured in extracts prepared from cells containing the vector alone and grown in galactose medium (pBM272-gal), or from cells containing the pGT554 plasmid derived from pBM272 by cloning the *TRM1* gene under control of the *Gal1* promoter and grown in glucose (*pGT554-glu*) or galactose (*pGT554-gal*).

Figure 3. The antipeptide antibody inhibits m$_2^2$G-specific tRNA methyltransferase. Enzyme extracts were incubated for 2 h on ice with an invariant amount of total immunoglobin protein. The ratio of preimmune to immune IgG in each sample varied. Enzyme activity was measured by the incorporation of [^{3}H]methyl groups from [^{3}H]methyl-*S*-adenosyl-L-methionine into m$_2^2$G-deficient tRNAs as described in Materials and Methods.

sample is presumably a proteolytic fragment of the 63 kD protein as it is not observed in most other preparations. For example, it is absent from Fig. 2 *A*, lane 2, and *B*, lane *1*. The protein in Fig. 2 *A*, lane 2, was isolated from the original *trml* mutant while that in Fig. 2 *A*, lane 3, was from a strain of yeast carrying a disrupted allele of the *TRM1* gene. No 63-kD protein was detected in this strain. The protein in lane *1* of Fig. 2 *B* was isolated from cells that produce the *TRM1* product under the control of the *GAL1* promoter. Wild-type cells transformed with the *GAL1* promoter plasmid without the *TRM1* gene make only wild-type levels of m$_2^2$G-specific tRNA methyltransferase (detected only on longer exposures of the Western blot) compared to cells carrying the *TRM1* gene on the same vector. This result is consistent with the
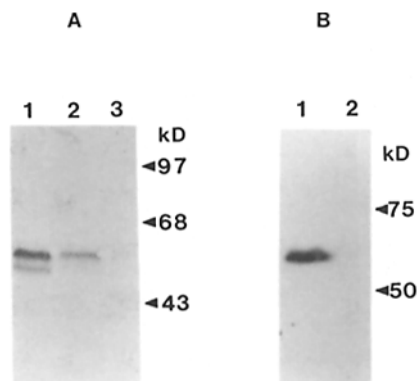
data on the enzyme activity present in Fig. 1. We conclude from these experiments that this antibody is specific to the *TRM1* gene product.

To determine if the protein coded by the *TRM1* gene is the m$_2^2$G-specific tRNA methyltransferase, enzyme assays were performed in the presence and absence of the antibody. When enzyme extracts were incubated with the antipeptide antibody before assaying for enzyme activity, enzyme activity was inhibited (Fig. 3). The total amount of protein was kept constant in each assay, but the ratio of immune to preimmune IgG was increased. Therefore, the increasing inhibition of the enzyme activity is specifically caused by an increasing amount of the antipeptide antibody used in each assay mixture. The assay was an inhibition assay, not a precipitation assay so the fact that the inhibition never reached 100% may be explained if interaction of the antibody with the enzyme did not cause 100% inhibition of activity.

The m$_2^2$G-specific tRNA Methyltransferase Is Located in the Nucleus

The m$_2^2$G-specific tRNA methyltransferase is a mitochondrial enzyme responsible for the modification of tRNAs encoded by mitochondrial DNA (Ellis et al., 1987). However, the cellular location for the addition of m$_2^2$G to cytoplasmic tRNAs was not known. To determine whether the methyltransferase is located in nuclei, cytoplasm, or both, cell fractionation

Figure 2. Identification of the *TRM1* protein with immunoblots using the antipeptide antibody (*A*). Lane *1*, the wild-type strain W303-1b; lane 2, the *trml* mutant strain SN1015-2a; and lane 3, the strain W303-1bTHT which carries a disrupted allele of *TRM1*. Overproduction of the *TRM1* product from the GAL1 promoter (*B*). Lane *1*, cells carrying *TRM1* on a multicopy plasmid under the control of the *GAL1* promoter; lane 2, wild-type cells transformed with the *GAL1* promoter plasmid without the *TRM1* gene. Upon longer exposure, TRM1 product is observed in *B*, lane 2. 100 μg protein was separated on a 10% SDS-PAGE and transferred to nitrocellulose. The primary antibody was affinity-purified antipeptide antibody and the secondary antibody was affinity-purified goat anti-rabbit antibody conjugated with alkaline phosphatase.
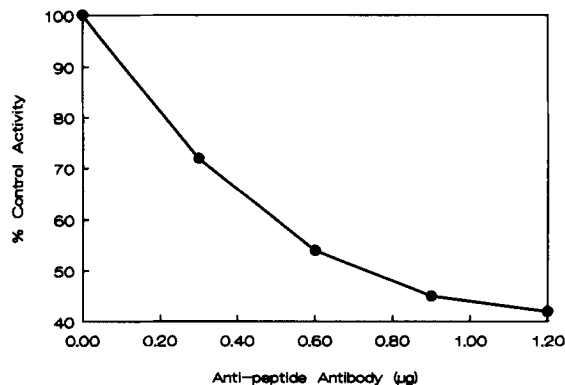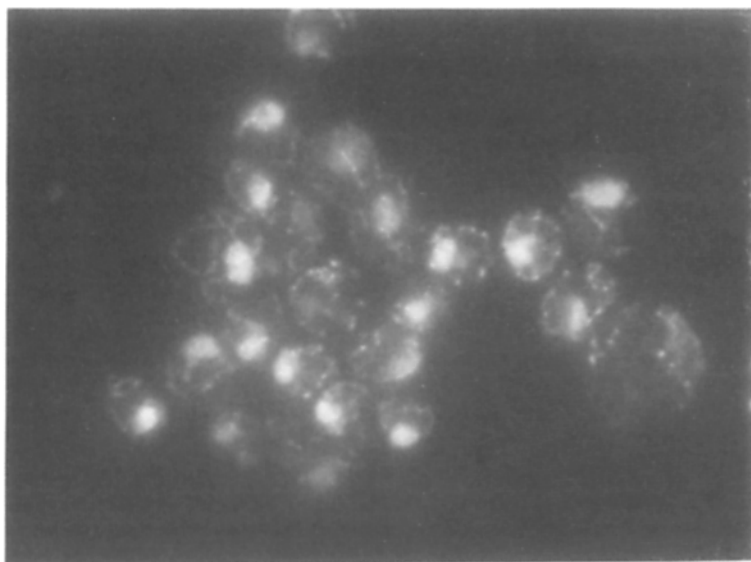
Table I. Enzyme Distribution between the Nucleus and Cytoplasm

Enzyme	Nucleus	Cytoplasm
	%	%
β-galactosidase	3	97
ADHase*	11	89
RNA polymerase	86	14
m$_2^2$Gtase‡	84	16

* Alcohol dehydrogenase.
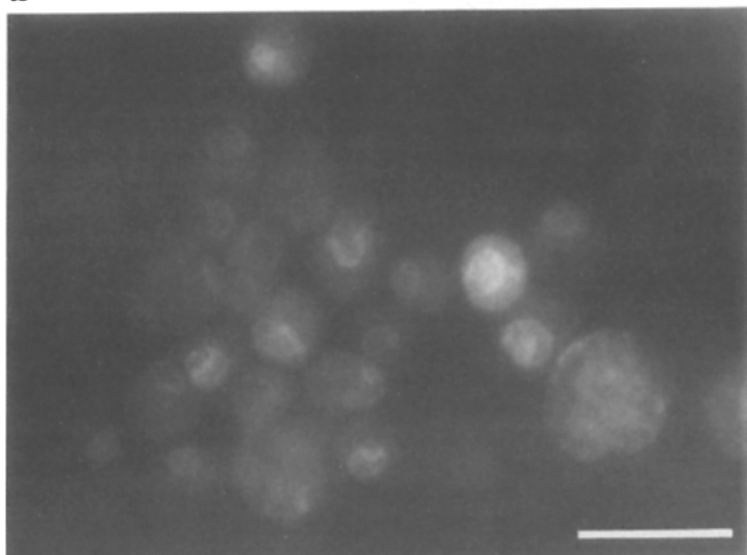‡ m$_2^2$G-specific tRNA methyltransferase.

a

b

Figure 4. Localization of the *TRM1* gene product in cells carrying the multicopy plasmid HC-3 by indirect immunofluorescence. (*a*) The fluorescent pattern from the DNA-specific stain DAPI; and (*b*) the fluorescence pattern due to the FITC-conjugated goat anti-rabbit IgG interacting with the antipeptide antibody. Bar, 10 μm.

studies were carried out. Yeast nuclei were isolated from wild-type cells by differential centrifugation and purified on a 50% percoll gradient. As shown in Table I, 86% of the RNA polymerase activity was associated with the isolated nuclei and 14% of this marker enzyme was not nuclear associated. Some of the activity in the nonnuclear fractions may arise from the mitochondrial-specific polymerase and some from leakage from nuclei. Cytoplasmic contamination to the nuclear fraction was assessed by assaying two nonnuclear activities. One is endogenous alcohol dehydrogenase activity, the other bacterial β-gal activity produced from an *E. coli* gene. This latter protein has been shown to be cytoplasmic (Douglas et al., 1984). Only 3% of the authentic β-gal and 11% of the alcohol dehydrogenase were associated with the isolated nuclei. In the same fractions, 84% of the m$_2^2$G-specific tRNA methyltransferase activity from wild-type cells was associated with the nuclear fraction.

In addition to the cell fractionation studies, we carried out indirect immunofluorescence experiments using the antipeptide antibody. We found that the amount of enzyme in wild-type cells was below the limit of detection.

Fig. 4 shows micrographs of cells carrying the plasmid HC-3 which carries the wild-type *TRM1* gene and thus produces protein from both ATGs. Ellis et al. (1986) showed that these cells produce 10 times more methyltransferase than do wild-type cells and that the vast majority of the protein is made from the second ATG. Fig. 4 *a* shows a micrograph of these cells stained with DAPI, a DNA-specific fluorescent dye. Fig. 4 *b* shows the staining pattern observed when these cells were treated with the antipeptide antibody followed by FITC-conjugated goat anti-rabbit antibodies. A comparison of Fig. 4, *a* and *b*, shows that the FITC fluorescence outlines the nuclei detected with the DAPI stain. No clear mitochondrial staining was detected in these experi-
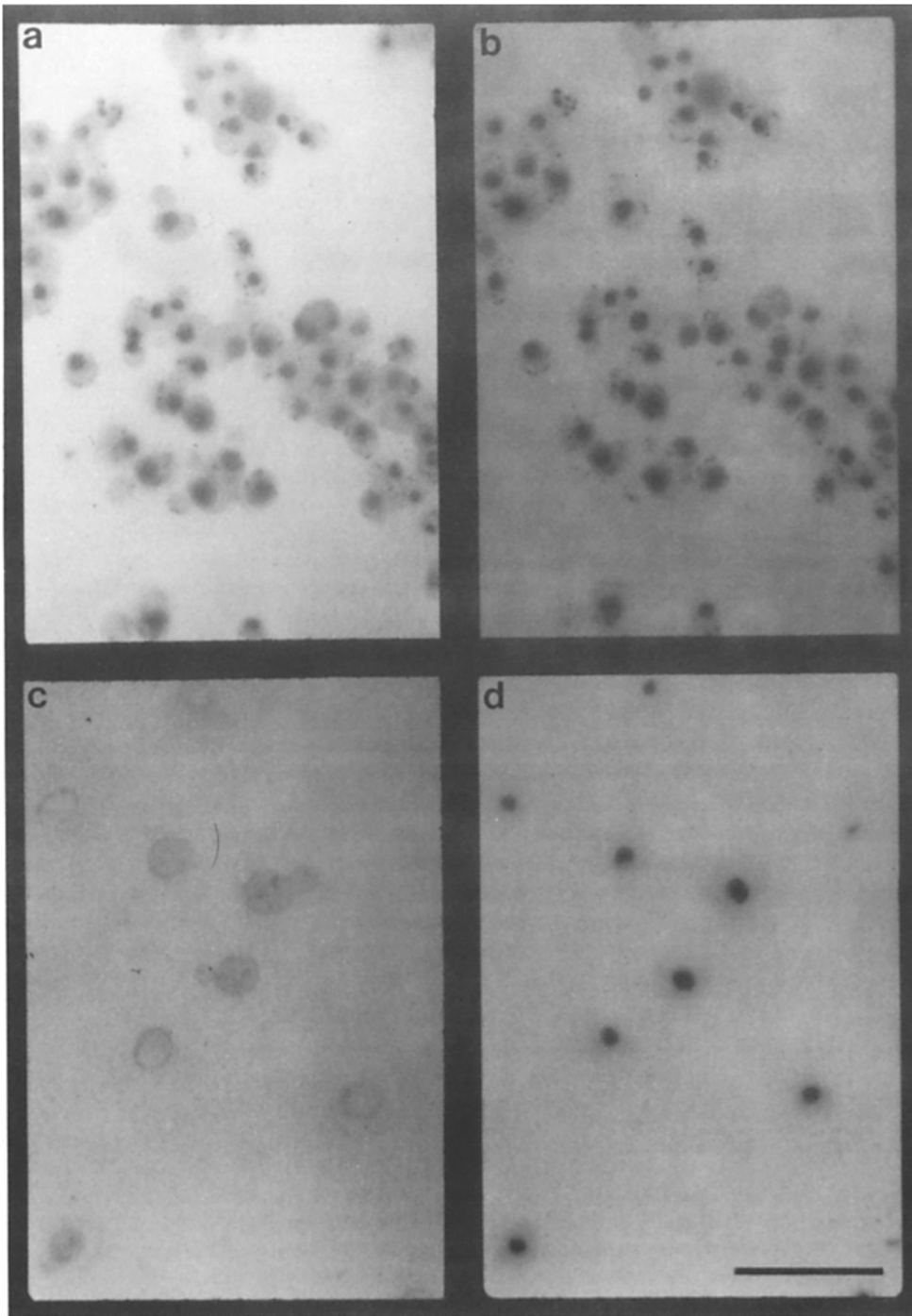
Figure 5. Localization of the *TRM1*-β-gal fusion proteins by indirect immunofluorescence. Cells containing either pTRMZ213 (*a* and *b*) or pTRMZ70 (*c* and *d*) were prepared for indirect immunofluorescence and treated with affinity-purified rabbit anti-β-gal antibody, followed by FITC-conjugated anti–rabbit IgG to identify the fusion proteins (*a* and *c*). DAPI was used to stain both nuclear and mitochondrial DNAs (*b* and *d*). Note the absence of mitochondrial DNA in cells carrying pTRMZ70. Bar, 20 μm.

ments. Cells transformed with Yep24, the vector without the *TRM1* gene, gave no staining (data not shown). The same experiment was done with cells carrying pGT554, a plasmid with the *TRM1* gene under control of the *GAL1* promoter. When grown in glucose there is no staining but when grown in galactose the nuclear targeting is evident (data not shown). Both overproducing strains have a staining pattern which is most intense around the nuclear periphery resulting in the appearance of a ring-shaped staining pattern. Further analyses will be necessary to confirm this observation and to begin to understand how it may occur.

The NH₂-terminal 213 Amino Acids of the Enzyme Are Sufficient to Target β-Gal to Both Nuclei and Mitochondria

The *TRM1* gene has two in-frame ATGs that are both used for the initiation of protein synthesis. The vast majority of the *TRM1* gene products start from the second ATG and are used to modify both the mitochondrial and cytoplasmic tRNAs in wild-type cells (Ellis et al., 1987). To begin to determine what sequences within the enzyme can promote the proper intracellular localization of the protein, we created

several *TRM1-LacZ* fusion genes. The *TRM1* gene with its normal promoter was fused to *LacZ* so proteins starting from the first ([1–213 TRM1]β-gal) or the second ([17–213 TRM1]-β-gal) ATG of the gene can be made. A *TRM1* gene that had the first ATG removed was also fused to *LacZ* so that only the [17–213 TRM1]β-gal fusion protein would be made. The results obtained with both constructions were the same, indicating that the vast majority of the staining is due to proteins beginning at the second ATG. Fig. 5 *b* shows the DNA staining pattern and Fig. 5 *a* shows the FITC staining pattern observed in cells containing the fusion gene with both ATGs. In this figure, the DAPI and FITC staining appear as dark signals against a light background. The protein produced by this gene is clearly targeted to both nuclei and mitochondria.

Genes that produce fusion proteins with fewer amino acids derived from the *TRM1* gene were derived from those described above. These genes both produced a protein, [17–70 TRM1]β-gal, from the second ATG of the *TRM1* gene and the gene that retained the first ATG also produces a longer protein, [1–70 TRM1]β-gal. β-Gal activity in these cells is twofold more than those carrying the 213 amino acid fusion. As can be seen from comparing Fig. 5, *c* and *d*, these proteins fail to localize β-gal to the nucleus. Instead, what β-gal can be seen appears to be in a few discrete cytoplasmic regions. Since cells carrying these fusions have lost their mitochondrial DNA, and we have not been successful in identifying another mitochondrial marker in our studies, we cannot determine whether these regions of staining colocalize with mitochondria. On the other hand, the fact that the fusion, but not β-gal alone, causes loss of mitochondrial DNA suggests that the protein is being delivered to the mitochondria where it interferes in some way with the biogenesis of a functional organelle.

Discussion

Both mitochondrial and cytoplasmic tRNAs in *S. cerevisiae* contain m$_2^2$G and a mutation in the *TRM1* gene abolishes this modification in both tRNA populations. Evidence from expression studies of *TRM1* as well as antibody inhibition experiments demonstrate that *TRM1* encodes m$_2^2$G-specific tRNA methyltransferase, the enzyme responsible for the m$_2^2$G modification. The original *trm1* mutant makes a 63-kD protein so that a missense mutation or small in-frame deletion must be responsible for the lack of enzyme activity in this strain. Cell fractionation and indirect immunofluorescence studies demonstrate that m$_2^2$G-specific tRNA methyltransferase is a nuclear, not a cytoplasmic, enzyme. A nuclear location for this enzyme is consistent with the previous report that precursor tRNAs have m$_2^2$G and are presumably modified inside the nucleus (Peebles et al., 1979; Nishikura and De Robertis, 1981). These previous experiments did not give insight as to whether some enzymes would also be found in the cytoplasm. In fact, our results suggest that the enzyme is predominantly in the nuclei and mitochondria.

How a protein with two different destinations is sorted in the cell is unknown. The observation that the fusion protein 17–213 TRM1 β-gal is localized to mitochondria and nuclei suggests, but does not prove, that sequences important for the targeting of the native *TRM1* product to dual compartments may be located in this region of the protein. Ellis et al. (1989) have constructed fusion proteins between the amino-terminal

sequences of *TRM1* and cytochrome oxidase subunit IV or dihydrofolate reductase. They found that amino acids 1–48 and 17–48 coded by the *TRM1* open reading frame were sufficient to target these passenger proteins to the mitochondria and that the longer *TRM1* sequence resulted in more efficient import of the fusion proteins. We have not attempted to measure the relative efficiency of mitochondrial import of the TRM1-β-gal fusions and, indeed, have not even demonstrated convincingly that the [1–70 TRM1]/[17–70 TRM1]β-gal fusions are imported in vivo. Immunofluorescence staining appears concentrated in a few distinct regions of the cytoplasm but since we have never recovered cells that contain both the plasmid which directs the synthesis of these fusions and mitochondrial DNA, we can no longer use DAPI staining as a measure of colocalization. Results of cell fractionation studies show that these fusions do not accumulate in the mitochondria (data not shown). The loss of mitochondrial DNA caused by the fusions may be due to a secondary consequence of incomplete translocation of TRM1-LacZ fusion proteins such that the import of other nuclear-coded mitochondrial proteins needed for replication, stability, or segregation of mitochondrial DNA would be blocked.

The shorter fusions do not localize to nuclei. If there is not a dramatic difference in the stability of the different fusions then the region between amino acids 70 and 213 of *TRM1* should contain information sufficient to target β-gal to nuclei. When comparing this region with known nuclear targeting signals of other nuclear proteins, we find a similarity between an amino acid cluster of nine amino acids within this region and one of the nuclear targeting signals of the glucocorticoid receptor and the nuclear targeting signal of SV-40 large T antigen (Picard and Yamamoto, 1987; Kalderon et al., 1984*a,b*) as well as histone H$_2$B (Moreland et al., 1987). In fact, seven out of nine amino acids are conserved between the m$_2^2$G-specific tRNA methyltransferase and part of the nuclear targeting signal from the glucocorticoid receptor (Table II). We have initiated site-directed mutagenesis experiments to test the hypothesis that this sequence is important to the nuclear localization of native methyltransferase.

The m$_2^2$G-specific tRNA methyltransferase produced in cells carrying the *TRM1* gene on multicopy plasmids appears to be concentrated near or at the nuclear membrane. The indirect immunofluorescent staining pattern we observe is different from the patch-like pore staining and diffuse nucleoplasm staining reported for yeast tRNA ligase (Clark and Abelson, 1987) and the nucleoplasm staining of the yeast *RNA11* gene product (Chang et al., 1988). A concentration of staining is observed ∼100–300 nm from the nuclear envelope for these proteins by EM techniques. The staining pattern of the yeast *NSP1* gene, at or near the nuclear membrane

Table II. A Sequence in the Methyltransferase Similar to Other Nuclear Targeting Signals

Nuclear Targeting Signal	Sequence
SV-40 large T antigen*	[125]Pro Pro Lys Lys Lys Arg Lys[132]
Glucocorticoid receptor*	[510]Arg Lys Thr Lys Lys Lys Ile Lys[517]
Histone H2B1*	[30]Lys Lys Arg Lys Ser Lys Ala Lys[38]
Methyltransferase	[95]Lys Lys Ser Lys Lys Lys Arg Cys[102]

* Known nuclear targeting signals.

(Hurt, 1988), is dot-like while that observed with *TRMl* is uniform. Recently, Adachi and Yanagida (1989) have observed a staining pattern quite similar to that we see with TRM1 antibodies using antibodies to the *crml⁺* (chromosomal region maintenance) gene product of *S. pombe*. We do not know, of course, whether the m$_2^2$G-specific tRNA methyltransferase in wild-type cells localizes to the periphery of the nucleus because the level of protein has, so far, precluded our detection by the immunofluorescent technique. The question of the role that overproduction may play in the subnuclear localization we observe cannot be ignored. However, it seems less likely to us that overproduction of a protein would convert it from a nonperipheral to a peripheral protein. Overproduction of a known peripheral protein, the tRNA ligase in yeast, does result in an increase in nuclear staining away from the periphery as if sites near the membrane become saturated (Clark and Abelson, 1987). The fact that the [17–213 TRM1]β-gal fusion protein has a diffuse location throughout the nucleus and its synthesis is driven by the wild-type *TRMl* promoter might argue against these ideas on the effects of overproduction. On the other hand, if sequences to the carboxy-terminal side of amino acid 213 are needed for subnuclear localization of native TRM1, they would be missing from this fusion. That sequences direct the subnuclear location of nuclear proteins has been demonstrated by Siomi et al. (1988). The first 20 amino acids of human T cell leukemia virus type I p27ˣⁱⁱⁱ are sufficient to localize β-gal in the nucleolus. This region must, by itself, constitute a structure that is recognized by the nuclear transport machinery and that once imported interacts with nucleolar components. Longer *TRMl–LacZ* fusions that could be useful in locating a subnuclear localization signal are currently being constructed.

Although there are quite a few proteins now identified that are shared by the mitochondria and the cytoplasm (Beltzer et al., 1986; Natsoulis et al., 1986; Wu and Tzagoloff, 1987; Chatton et al., 1988; Gillman, E. C., L. Slusher, N. C. Martin, and A. K. Hopper, unpublished observations), m$_2^2$G-specific tRNA methyltransferase is the only one to our knowledge that is shared by the mitochondria and the nucleus. As such, it presents a unique opportunity to ask how a single protein is sorted to both compartments. In addition, while the *TRMl* gene is now well characterized, little is known about the function of m$_2^2$G modification on tRNAs and why the enzyme responsible for this base modification is located in the nucleus. Further analyses promise to increase our knowledge about both of these questions.

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References

Adachi, Y., and M. Yanagida. 1989. Higher order chromosome structure is affected by cold-sensitive mutations in a *Schizosaccharomyces pombe* gene crm1⁺ which encodes a 115-kD protein preferentially localized in the nucleus and at its periphery. *J. Cell Biol.* 108:1195-1207.

Beltzer, J. P., L.-F. L. Chang, A. G. Hinkkanen, and G. B. Kohlhaw. 1986. Structure of yeast *LEU4*: the 5' flanking region contains features that predict two modes of control and two productive translation starts. *J. Biol. Chem.* 261:160-167.

Bjork, G. R., and I. Svensson. 1969. Studies on microbial RNA. *Eur. J. Biochem.* 9:207-215.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye bind-ing. *Anal. Biochem.* 72:248-254.

Chang, T. H., M. W. Clark, A. J. Lustig, M. E. Cusick, and J. Abelson. 1988. RNA11 protein is associated with the yeast spliceosome and is localized in the periphery of the cell nucleus. *Mol. Cell. Biol.* 8:2379-2393.

Chatton, B., P. Walter, J. P. Ebel, F. Lacroute, and F. Fasiolo. 1988. The yeast *VAS1* gene encodes both mitochondrial and cytoplasmic valyl-tRNA synthetases. *J. Biol. Chem.* 263:52-57.

Clark, M. W., and J. Abelson. 1987. The subnuclear localization of tRNA ligase in yeast. *J. Cell Biol.* 105:1515-1526.

Douglas, M. G., B. L. Geller, and S. D. Emr. 1984. Intracellular targeting and import of an F1-ATPase β-subunit-β-galactosidase hybrid protein into yeast mitochondria. *Proc. Natl. Acad. Sci. USA.* 81:3983-3987.

Ellis, S. R., A. K. Hopper, and N. C. Martin. 1987. Amino-terminal extension generated from an upstream AUG codon is not required for mitochondrial import of yeast N^2,N^2-dimethylguanosine-specific tRNA methyltransferase. *Proc. Natl. Acad. Sci. USA.* 84:5172-5176.

Ellis, S. R., A. K. Hopper, and N. C. Martin. 1989. 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. *Mol. Cell. Biol.* 9:1013-1028.

Ellis, S. R., M. J. Morales, J. M. Li, A. K. Hopper, and N. C. Martin. 1986. Isolation and characterization of the *TRMl* locus, a gene essential for the N^2,N^2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in Saccharomyces cerevisiae. *J. Biol. Chem.* 261:9703-9709.

Emr, S. D., A. Vassarotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas. 1986. The amino terminus of the yeast F1-ATPase β-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* 102:523-533.

Guarente, L., R. Yocum, and P. Gifford. 1982. A *GAL10-CYC1* hybrid yeast promoter identifies the *GAL4* regulatory region as an upstream site. *Proc. Natl. Acad. Sci. USA.* 79:7410-7414.

Hopper, A. K., A. H. Furukawa, H. D. Pham, and N. C. Martin. 1982. Defects in modification of cytoplasmic and mitochondrial transfer RNAs are caused by single nuclear mutations. *Cell.* 28:543-550.

Hurt, E. C. 1988. A novel nucleoskeletal-like protein located at the nuclear periphery is required for the life cycle of *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4323-4334.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168.

Johnston, M., and R. W. Davis. 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4:1440-1448.

Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith. 1984a. Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature (Lond.).* 311:33-38.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984b. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499-509.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.

Lerner, R. A., N. Green, H. Alexander, F.-T. Liu, J. G. Sutcliffe, and T. M. Shinnick. 1981. Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proc. Natl. Acad. Sci. USA.* 78:3403-3407.

Livingston, D. M. 1974. Immunoaffinity chromatography of proteins. *Methods Enzymol.* 34:723-731.

Martin, R. P., J. M. Schneller, A. J. C. Stahl, and G. Dirheimer. 1976. Studies of odd bases in yeast mitochondrial tRNA. II. Characterization of rate nucleosides. *Biochem. Biophys. Res. Commun.* 70:997-1002.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 352-353.

Moreland, R. B., G. L. Langevin, R. H. Singer, R. L. Garcea, and L. M. Hereford. 1987. Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol. Cell. Biol.* 7:4048-4057.

Natsoulis, G., F. Hilger, and G. R. Fink. 1986. the *HTS1* gene encodes both the cytoplasmic and mitochondrial histidine tRNA synthetase of *S. cerevisiae*. *Cell.* 46:235-243.

Nau, F. 1976. The methylation of tRNA. *Biochimie (Paris).* 58:629-645.

Nishikura, K., and E. M. De Robertis. 1981. RNA processing in microinjected *Xenopus* oocytes, sequential addition of base modifications in a spliced tRNA. *J. Mol. Biol.* 145:405-420.

Peebles, C. L., R. C. Ogden, G. Knapp, and J. Abelson. 1979. Splicing of yeast tRNA precursors: a two-stage reaction. *Cell.* 18:27-35.

Picard, D., and K. R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3333-3340.

Roeder, R. G. 1974. Multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in *Xenopus laevis*. *J Biol. Chem.* 249:241-248.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.

Silver, P. A., L. P. Keegan, and M. Ptashne. 1984. Amino terminus of the yeast *GAL4* gene product is sufficient for nuclear localization. *Proc. Natl. Acad. Sci. USA.* 81:5951-5955.

Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatamaka. 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. Cell. 55:197–209.

Sottocassa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. J. Cell Biol. 32:415–438.

Tobin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.

Vallee, B. L., and F. L. Hoch. 1955. Zinc, a component of yeast alcohol dehydrogenase. Proc. Natl. Acad. Sci. USA. 41:327–328.

Winkley, C. S., M. J. Keller, and J. A. Jaehning. 1985. A multicomponent mitochondrial RNA polymerase from Saccharomyces cerevisiae. J. Biol. Chem. 260:14214–14223.

Wu, M., and A. Tzagoloff. 1987. Mitochondrial and cytoplasmic fumerases in Saccharomyces cerevisiae are encoded by a single nuclear gene FUM1. J. Biol. Chem. 262:12275–12282.

Zakian, V. A., and J. F. Scott. 1982. Construction, replication, and chromatin structure of TRP1 R1 circle, a multiple-copy synthetic plasmid derived from Saccharomyces cerevisiae chromosomal DNA. Mol. Cell. Biol. 2:221–232.