Copyright

by

Changkeun Lee

2008

The Dissertation Committee for Changkeun Lee Certifies that this is the approved version of the following dissertation:

Biochemical and Genetic Studies of Mitochondrial Protein Synthesis in *Saccharomyces cerevisiae*: Characterization of the AEP3 and TRM5 Gene Products

Committee:

Dean R. Appling, Supervisor

Arlen W. Johnson

Karen S. Browning

Vishwanath R. Iyer

Yuhui W. Yin

Biochemical and Genetic Studies of Mitochondrial Protein Synthesis in *Saccharomyces cerevisiae*: Characterization of the AEP3 and TRM5 Gene Products

by

Changkeun Lee, B.Sc., M.Sc.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August, 2008

Dedication

To my beloved wife, Suna, and children, Grace and Michael

And to my parents & parents-in-law

Acknowledgements

I thank my supervisor, Dr. Dean Appling, for his kind guidance during my research and during the preparation of this dissertation and manuscript for publication. He is one of the best mentors I have ever had in my life. I really appreciate his encouragement and support. I gratefully acknowledge the helpful and thoughtful advice of my committee members, Drs. Arlen Johnson, Karen Browning, Whitney Yin, and Vishwanath Iyer. I also thank Drs. Gisela Kramer and David Graham who worked with me on the TRM5 project for their support and good advice. I also acknowledge all the nice people in Dr. Appling lab: Dr. Anne Tibbetts, Dr. Priya Prasannan, Sky Pike, Swetha Bolusani, Peter Ruymgaart, Nicola Cole, and Richard Lu. I am very grateful to my parents, Soyoung Lee and Sangtae Cho, and my sister, Sujin Lee, and my parents-in-law, Jong-Ryul Woo and Junghye Moon, whose consistent encouragement, support and prayers never failed me. Last but not least, to my wife, Suna, and my children, Grace and Michael, who are my life and reasons to live, I give all my love and gratefulness for their sacrifices and support throughout my years at UT Austin. Above all, I thank God Almighty for staying with me all the time.

Biochemical and Genetic Studies of Mitochondrial Protein Synthesis in *Saccharomyces cerevisiae*: Characterization of the AEP3 and TRM5 Gene Products

Publication No._____

Changkeun Lee, Ph.D.

The University of Texas at Austin, 2008

Supervisor: Dean R. Appling

Protein synthesis in archaebacteria and the cytoplasm of eukaryotes is initiated using the initiator methionyl-tRNA (Met-$tRNA_i^{Met}$). In contrast, formylated methionyl-tRNA (fMet-$tRNA_i^{Met}{}_f$) is found in eubacteria, and in chloroplasts and mitochondria of eukaryotes, and this formylated initiator tRNA was widely believed to be required for initiation of protein synthesis in those systems. However, the fact that initiation of protein synthesis in yeast mitochondria can occur with unformylated initiator tRNA has changed our perspective about the initiation of mitochondrial protein synthesis. This dissertation is composed of two parts. Part I describes an investigation of the yeast *AEP3* gene which was isolated by a genetic screening system in *Saccharomyces cerevisiae*. The main goal of this part was to discover new accessory factor(s) that might be involved in initiation of protein syntheis of yeast mitochondria when there is no formylation of initiator tRNA and determine how they support the initiation process in *Saccharomyces cerevisiae*. The

synthetic petite genetic screen identified the *AEP3* gene. Protein-protein binding assays as well as protein-initiator tRNA binding assays indicate that Aep3p is associated with the initiation process in yeast mitochondrial protein synthesis. This discovery is important because it suggests the possible mechanism by which initiation of protein synthesis in yeast mitochondria occur under conditions where there is no formylation of initiator tRNA. Part II describes a study of the *TRM5* gene encoding a tRNA methyltransferase in *S. cerevisiae*. The *TRM5* gene encodes a tRNA (guanine-N1-)-methyltransferase (Trm5p) previously known to methylate guanosine at position 37 (m^1G37) in certain cytoplasmic tRNAs in *S. cerevisiae*. The main goal of this part was to investigate whether Trm5p is also responsible for m^1G37 modification of mitochondrial tRNAs. Full-length Trm5p, purified as a fusion protein with maltose-binding protein, exhibited robust methyltransferase activity with tRNA isolated from a Δ*trm5* mutant strain, as well as with a synthetic mitochondrial $tRNA^{Met}_f$ and $tRNA^{Phe}$. High pressure liquid chromatography analysis showed the methylated product to be m^1G. Analysis of subcellular fractionation and immunoblotting revealed that the enzyme was localized to both cytoplasm and mitochondria. Our data including the analysis of N-terminal truncation mutants suggest that this tRNA modification plays an important role in reading frame maintenance in mitochondrial protein synthesis.

Table of Contents

List of Tables

List of Figures

PART I

Chapter 1: Introduction

Protein synthesis is carried out by ribosomes, which act as RNA-based enzymes (ribozymes) to catalyze the formation of peptide bonds. The ribosome consists of a small and a large subunit; 30S and 50S in prokaryotes or 40S and 60S in eukaryotes, respectively. The two subunits are assembled into a functional ribosome. Ribosomes can initiate translation on the messenger RNA (mRNA) that contains the genetic information. Translation is usually divided into three stages: Initiation, elongation and termination. Translation initiation is the rate-determining step and requires several initiation factors to assemble together with ribosomes and initiator-tRNA (Met-$tRNA_i^{Met}$). The ribosome possesses three tRNA binding sites: the exit (E), peptidyl (P), and amino-acyl (A) sites.

During the initiation stage, two ribosome subunits are assembled together, along with several different initiation factors (IF1, IF2 and IF3) and cofactors (initiator-tRNA, GTP), to search for the initiation recognition site. In the elongation stage, an incoming aminoacyl-tRNA is delivered to the A site of the ribosome with elongation factor EF-Tu-GTP. Along with several other elongation factors (EF-Tu and EF-G), the ribosome reads the messenger RNA (mRNA) to generate polypeptide chains. Elongation ends when a stop codon on the mRNA is reached. Then, the process goes into the termination stage of protein synthesis. Finally, the newly synthesized protein is released from the ribosome.

Among three stages in protein synthesis, the initiation stage is the rate-limiting step and most highly regulated. Moreover, the mechanism by which the initiation of protein synthesis occurs is best understood in bacteria. The bacterial 70S ribosome comprises two subunits: a large 50S and a small 30S subunit. The aminoacyl charged

tRNA containing an anticodon that is complementary to a codon for that amino acid on mRNA is brought to the ribosome. The initiation process in bacteria is depicted in Figure 1.1. The initiation factor 3 (IF3) binds to the 30S ribosomal subunit to promote dissociation of the 70S ribosome as well as to prevent the association with 50S ribosomal subunit. Initiation factor 1 (IF1) binds to the 30S small subunit to prevent the premature binding of an amino-acyl tRNA corresponding to the A site of 30S ribosome (Moazed et al., 1995), also directing the initiator tRNA to the ribosomal P site (Carter et al., 2001; Dahlquist and Puglisi, 2000). A GTP-bound initiation factor 2 (IF2) forms a complex with an initiator tRNA (fMet-$tRNA_i^{Met_f}$), bringing it to the P site of 70S ribosome (Wu and RajBhandary, 1997). IF2 is one of the largest initiation factors, which can be divided into several domains: the N domain, G domain and C domain (Figure 1.2). The N domain contains a less highly conserved region (Sorensen et al., 2001; Steffensen et al., 1997). The G domain is the site where GTP binding occurs, and it is highly conserved across archaea, bacteria, and eukaryotes. The C domain contains the binding site for fMet-$tRNA_i^{Met_f}$ (Spurio et al., 2000). The 30S preinitiation complex resulting from assembly of 30S small subunit, three initiation factors, and mRNA is relatively unstable. Afterward a conformational change occurs to promote the interaction between codon and anticodon, and then the pre-initiation complex becomes stable (Gualerzi and Pon, 1990; McCarthy and Brimacombe, 1994). IF1 and IF3 are released from the complex, while IF2 promotes the association of the large 50S subunit to the complex. Once initiator fMet-$tRNA_i^{Met_f}$ is bound in the correct position in the P site, IF2 is released from the complex by the hydrolysis of GTP. Finally, the 50S subunit binds to the 30S subunit to form the 70S ribosome.

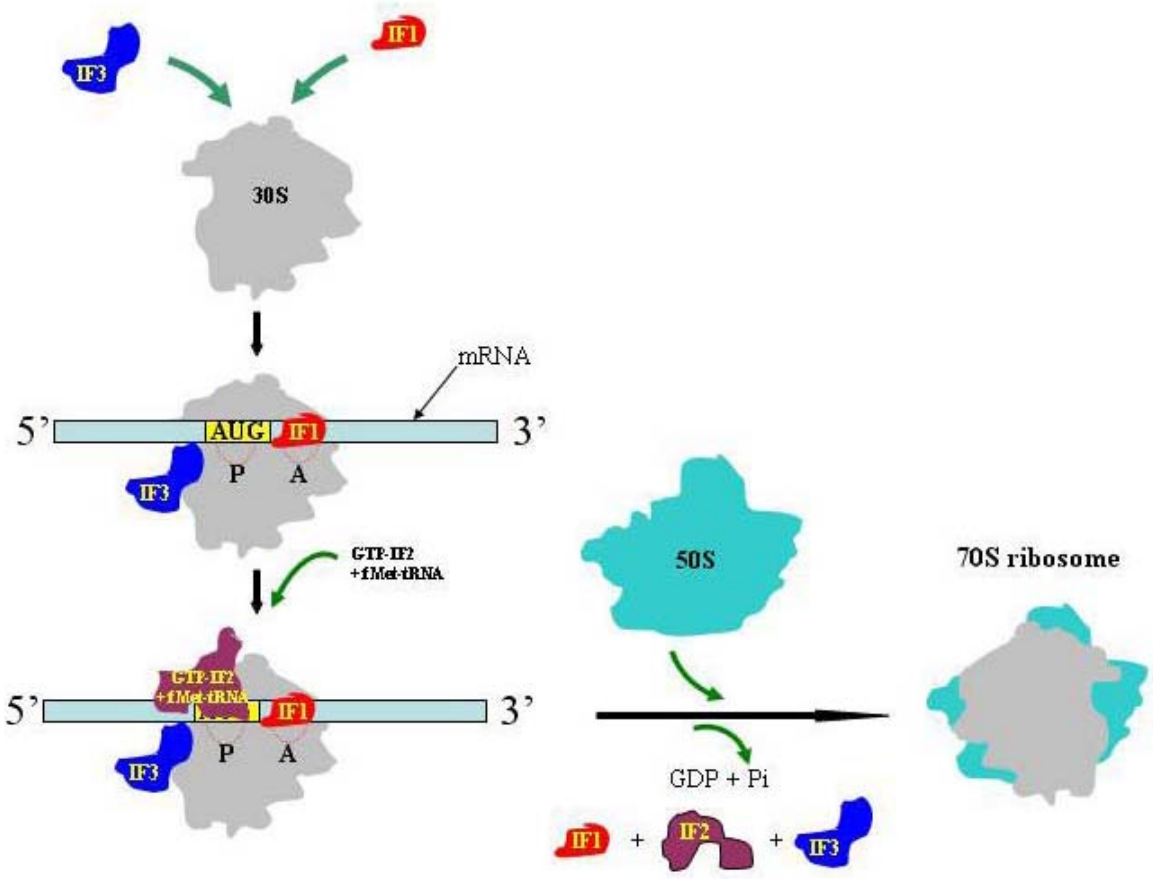

Figure 1.1: Overview of initiation of protein synthesis in prokaryotes. The initiation factor 3 (IF3) and initiation factor 1 (IF1) bind to the 30S small subunit. Once the preinitiation complex formed with GTP-bound IF2 along with fMet-tRNA$_i^{Met}{}_f$ becomes stable, IF3 and IF1 are released from the complex, followed by the release of IF2 with the hydrolysis of GTP. Finally, the 50S large subunit binds to the 30S subunit, forming the 70S ribosome. Blue-shaped: IF3, Red-shaped: IF1, Purple-shaped: IF2.

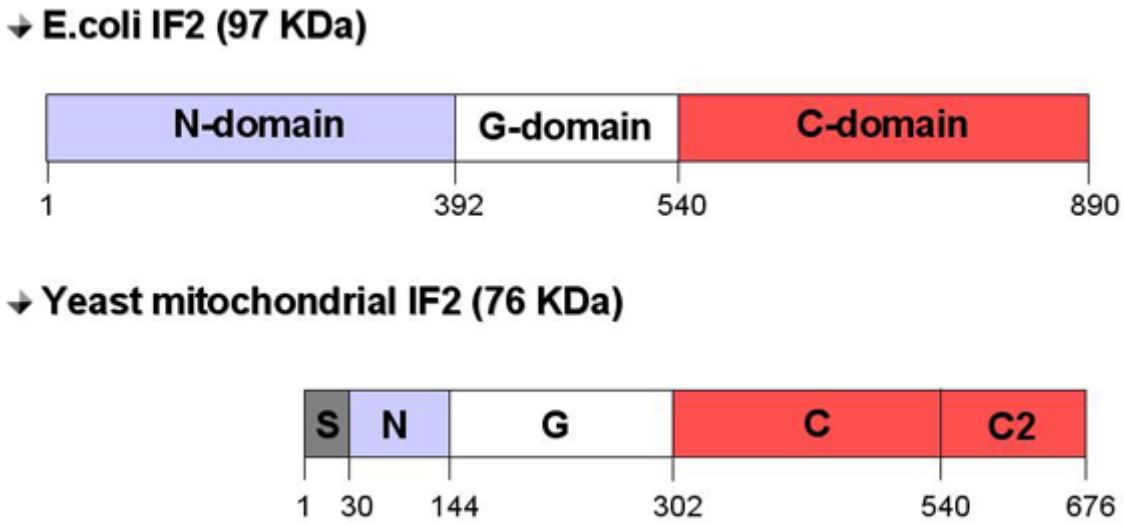

Figure 1.2: Map of domain structure of initiation factor 2. C domain is highlighted in red color. Numbers indicate amino acid positions.

The mechanism of initiation of protein synthesis in eukaryotic cytoplasm is quite complicated compared with that in prokaryotes. Briefly, to initiate translation, the small ribosomal subunit (40S) is assembled with a ternary complex containing eukaryotic initiation factor 2 (eIF2), Met-tRNA$_i^{Met}$, and GTP (Sonenberg, et al., 2000). The resulting 43S preinitiation complex is recruited to the mRNA through interactions with eIF4 factors which bind the cap structure of the mRNA (Marcotrigiano et al., 1997; Matsuo et al., 1997; Tomoo et al., 2002). The 43S complex then is thought to scan along the 5'UTR of the mRNA until it encounters the first start AUG codon (Kozak, 1999, 2002). Following the recognition of AUG, the 60S subunit joins to form the complete 80S ribosome.

Mitochondria of eukaryotes have their own DNA which is expressed by specific transcription and translation systems (Attardi, 1985). Mammalian mitochondria contain two ribosomal subunits; 28S for the small subunit and 39S for the large subunit (O'Brien, et al., 1976). In addition, three elongation factors were purified and characterized (Chung and Spremulli, 1990; Schwartzbach and Spremulli, 1989). In mammalian mitochondria, the mitochondrial mRNAs lack a 5' leader or cap structure, suggesting that the mitochondrial ribosome does follow the mechanism of a cap binding and scanning for initiation of protein synthesis found in the eukaryotic cytoplasm. Mitochondrial initiation factor 2 and initiation factor 3 in bovine mitochondria were purified and characterized (Koc and Spremulli, 2002; Liao and Spremulli, 1991). Based on these experimental data, initiation of protein synthesis in mitochondria of eukaryotes has been thought to be much more similar to the initiation of protein synthesis in prokaryotes than to that of in eukaryotic cytoplasm. Therefore, the model for initiation in bacteria is used to understand the mechanism by which the initiation of protein synthesis in mitochondria is processed (Gualerzi and Pon, 1990).

The following literature review focuses on the formylation of initiator tRNA (fMet-$tRNA_i^{Met}{}_f$) and the effect of unformylated initiator tRNA (Met-$tRNA_i^{Met}$) in *Saccharomyces cerevisiae* mitochondria. For your convenience, the introduction part is divided into four sections. First, general information about the formylation of Met-$tRNA_i^{Met}{}_f$ is described. Second, the discovery of the fact that fMet-$tRNA_i^{Met}{}_f$ is not necessary for initiation of protein synthesis in *S.cerevisiae* is discussed. Third, characterization of yeast mitochondrial initiation factor 2 (ymIF2) is described. Finally, the purpose of this study is summarized.

1.1 Formylation of initiator tRNA in mitochondria

The discovery and subsequent characterization of formylation of initiator tRNA gave an insight into the mechanism by which initiation of protein synthesis is processed. In all organisms, protein synthesis is initiated with methionine or formylmethionine (Kozak, 1983; RajBhandary, 1994). Two types of methionine tRNA have been found in all organisms. The cytoplasmic protein synthesis systems of eukaryotes initiate with methionine. In eubacteria such as *E.coli*, it was found that N-formylmethionine (Figure 1.3) is used to initiate protein synthesis (Marcker and Sanger, 1964). The authors found that N-formylmethionyl-tRNA (fMet-$tRNA_f^{Met}$) was generated from methionyl-tRNA (Met-$tRNA^{Met}$) by enzymatic reaction. Also, they found an unknown compound when they isolated tRNAs from *E.coli* cells, that was identical with N-formylmethionine by chemical hydrolysis and chromatography analyses. As a result, they concluded that the eubacterial protein synthesis is initiated with formylmethionine. Several other experimental data also supported that protein synthesis is initiated with formylmethionine. Eukaryotic organelles such as mitochondria in *Neurospora crassa* contained a formylated methionine (Epler et al., 1970). The authors investigated both

mitochondrial and cytoplasmic extracts for fMet-tRNA. What they found was that a Met-tRNA isolated from mitochondria was formylated with an *E.coli* extract, but the corresponding cytoplasmic Met-tRNA could not be. Using chromatographic analysis, mitochondrial fMet-tRNA is separated from Met-tRNA. Therefore, they concluded that mitochondrial protein synthesis involves a formylated methionine. In addition, N-formylmethionyl-tRNA was identified in Hela cells (Galper and Darnell, 1969). Total RNA obtained from Hela cells was incubated with [^{14}C] methionine, then subjected to RNase digestion and electrophoresis analysis. They found that formylmethionine was attached to terminal adenosine in RNA of Hela cells. The tRNA was the only known form of RNA which attached amino acids to terminal adenosine. The mitochondrial fractions obtained from *Saccharomyces cerevisiae* contained fMet-tRNA (Halbreich and Rabinowitz, 1971). This fraction showed a formyltetrahydrofolic acid: Met-tRNA formylase activity (Figure 1.4). No such activity was detected in cytoplasmic supernatant in yeast. This formylase was most active with mitochondrial Met-tRNA, but not active with yeast cytoplasmic tRNAs. The authors suggested that fMet-tRNA is exclusively localized to the mitochondria in yeast. Schwartz et al. (1967) isolated N-formylmethionine from proteins synthesized in *Euglena* chloroplasts (Schwartz et al., 1967). They showed that the amino-terminal peptide which was digested by chymotrypsin appeared to be identical to the peptide isolated from *E.coli* extracts. Therefore, they concluded that the initiation of protein synthesis in chloroplasts is very similar to that which occurs at *E.coli*. fMet-tRNA was also detected in yeast and rat mitochondria, but not in the corresponding cytoplasmic systems (Smith and Marcker, 1968).

There is additional evidence that protein synthesis in eukaryotic mitochondria and chloroplasts is initiated with formylmethionine in experiments using puromycin. Puromycin is an inhibitor of protein translation causing the premature chain termination during translation (Nathans, 1964). Bianchetti et al. (1971 and 1977) investigated whether yeast mitochondria can synthesize peptide chains with formylmethionine at the N-terminus *in vitro*. What they found was that isolated mitochondria of yeast contained formylmethionyl-puromycin derivatives in the presence of system containing amino acids, formyl group donors, and puromycin. Their data indicated that polypeptide chains start with formylmethionine (Bianchetti et al., 1977; Bianchetti et al., 1971). In addition, using radioactive formate, Feldman et al. (1974) tested whether the mitochondrial protein synthesis utilizes fMet-tRNA in initiation. They found that formate labeled nascent polypeptide chains on mitochondrial ribosomes in yeast, and by pretreatment with puromycin, N-formylmethionyl-puromycin was formed. Also, when yeast cells were labeled with formate, they isolated mitochondrial membranes and did a complete proteolysis. Analysis of amino acids showed that label was found in N-formylmethionine (Feldman and Mahler, 1974; Mahler et al., 1972). Galper et al. (1971) also used puromycin to investigate whether fMet-tRNA is present in HeLa cells. They speculated that fMet-tRNA might function as an initiator for mitochondrial protein synthesis, and they used puromycin to test whether protein synthesis was initiated with fMet-tRNA. Cycloheximide is an inhibitor of cytoplasmic protein synthesis, but mitochondrial protein synthesis is resistant to cycloheximide (Hawley and Greenawalt, 1970). They found that treatment with puromycin resulted in fMet-puromycin, and the production of fMet-puromycin was insensitive to cycloheximide (Galper and Darnell, 1971). Sala et al. (1970) used an assay to test whether the mitochondrial ribosomes in *N. crassa* contained

fMet-puromycin, and they also observed the formation of fMet-puromycin (Sala and Kuntzel, 1970).

Other work showed that several mitochondrially synthesized proteins in *S. cerevisiae*, *N. crassa*, and beef heart mitochondria contained N-formylmethionine at their N-terminus (Beechey et al., 1967; Chomyn et al., 1981; Sebald et al., 1979; Steffens and Buse, 1979; Tzagoloff et al., 1979; Walker et al., 1991).

Taken together, these results led to the dogma that initiation of protein synthesis in mitochondria requires formylated initiator tRNA as in eubacteria. Studies of bovine mitochondria initiation factor 2 (bmIF2) further supported this dogma. Liao et al. (1991) showed that the bmIF2 promoted the binding of fMet-$tRNA_i^{Met}{}_f$ to the mitochondrial ribosomes in an *in vitro* assay (Liao and Spremulli, 1991), and it showed a 50-fold binding preference for fMet-$tRNA_i^{Met}{}_f$ over Met-$tRNA_i^{Met}$ (Spencer and Spremulli, 2004).

The enzyme that is responsible for the formylation of initiator tRNA is a methionyl-tRNA formyltransferase (Figure 1.3). It is specific for the initiator tRNA (Lee et al., 1991; Schmitt et al., 1998). This enzyme has been reported in the mitochondria of several species including *S. cerevisiae*, *N. crassa*, and Hela cells (Epler et al., 1970; Galper and Darnell, 1969; Halbreich and Rabinowitz, 1971). In *S. cerevisiae*, the *FMT1* gene encodes this enzyme. The yeast enzyme is composed of 393 amino acids and has approximately 30% amino acid sequence identity to the eubacterial and bovine mitochondrial homologs (Takeuchi et al., 1998).

The formyl group for the methionyl-tRNA formyltransferase reaction is donated by 10-formyl THF (Dickerman et al., 1967) (Figure 1.3). Yeast mitochondria can synthesize 10-formyl-THF via the activities of the mitochondrial trifunctional C_1-THF synthase encoded by *MIS1* gene (Shannon and Rabinowitz, 1988).

The effect of formylation of initiator tRNA on protein synthesis has shown different results depending on species. In *E.coli*, a strain deleted for the gene encoding methionyl-tRNA formyltransferase showed severe growth defects (Guillon et al., 1992; Meinnel et al., 1993; Varshney and RajBhandary, 1992). On the other hand, initiation of protein synthesis in *Pseudomonas aeruginosa* does not require the formylation of Met-$tRNA_i^{Met}$, indicating that formylation is not necessary for all bacteria (Newton et al., 1999).

1.2 Unformylation of initiator tRNA in yeast mitochondria

The first hint that mitochondrial protein synthesis might not absolutely require formylated initiator tRNA came from studies of the *MIS1* gene, encoding the mitochondrial C_1-THF synthase in yeast (Figure 1.4). Disruption of the *MIS1* gene had no effect on growth on non-fermentable carbon sources (Shannon and Rabinowitz, 1988). This was surprising since growth on non-fermentable carbon sources such as glycerol and ethanol requires respiration and functional mitochondria. Mitochondrial protein synthesis is required for functional mitochondria (Tzagoloff and Dieckmann, 1990), since several of the respiratory chain components are synthesized on mitochondrial ribosomes. In yeast, respiratory-incompetent mutants are easily detected, as they form small colonies (petites) on glucose (growth by fermentation only), and are unable to grow at all on non-fermentable carbon sources.

A second clue came from a report that disruption of the *FMT1* gene, encoding a methionyl-tRNA formyltransferase, also had no effect on growth. Deletion of the *FMT1* gene had no effect on viability (Skala et al., 1992), suggesting that *FMT1* is not essential. Li et al. (2000) directly tested the hypothesis that these mutant yeast strains lacked formylated initiator tRNA in their mitochondria. Using a sensitive gel assay to distinguish formylated from unformylated initiator tRNA, fMet-$tRNA_i^{Met_f}$ was undetectable in Δ*fmt1* or Δ*mis1* or Δ*fmt1mis1* double deletion strains. However, all of these mutant strains grew on non-fermentable carbon sources, suggesting mitochondrial protein synthesis was initiating with unformylated initiator tRNA. Tibbetts et al. (2003) then showed that mitochondria from these strains lacking formylated initiator tRNA did indeed carry out protein synthesis *in vivo*.

1.3 Characterization of yeast mitochondria initiation factor 2 (ymIF2)

The discovery that protein synthesis can be initiated with unformylated initiator tRNA (Met-$tRNA_i^{Met}$) in yeast mitochondria led the Appling lab to explore the initiation process more closely. Therefore, yeast mitochondrial initiation factor 2 (ymIF2) was cloned, purified, and characterized (Garofalo et al., 2003). IF2 is a central factor in the initiation process and is highly conserved across bacteria, archaeabacteria, and eukaryotes (Roll-Mecak et al., 2001). In *S. cerevisiae*, the *IFM1* gene encodes the mitochondrial IF2 consisting of 676 amino acids and a mitochondrial presequence (Figure 1.2). Using biochemical analyses, purified ymIF2 showed functional characteristics quite similar to bacterial IF2s (Garofalo et al., 2003). YmIF2 bound fMet-$tRNA_i^{Met}{}_f$, and delivered it to the ribosome. It also bound to Met-$tRNA_i^{Met}{}_f$, but showed a 6 times higher binding preference for fMet-$tRNA_i^{Met}{}_f$ versus unformylated initiator tRNA. YmIF2 contains ribosome-dependent GTPase activity. Overall, ymIF2 did not appear to function differently from other IF2s in those *in vitro* assays. However, genetic analyses with ymIF2 showed unexpected results. Tibbetts et al. (2003) showed that mitochondrial translation products in *S. cerevisiae* were synthesized normally in the absence of formylated initiator tRNA. The yeast strain containing the deletion of *IFM1* gene (Δ*ifm1*) which encodes the ymIF2 was unable to grow on non-fermentable carbon source, indicating that the strain has defective mitochondrial protein synthesis. However, when a construct either harboring the yeast *IFM1* gene or a cDNA encoding bovine mitochondrial IF2 (bmIF2) was transformed into the Δ*ifm1* strain, each construct complemented the respiratory defect, suggesting that bmIF2 is able to replace ymIF2 and support the mitochondrial function in yeast *in vivo*. They then tested whether the bmIF2 can support mitochondrial function under conditions where there is no formylation of initiator tRNA. They used the strain lacking the *FMT1* gene which encodes the

methionyl-tRNA formyltransferase, and confirmed that this strain did not contain fMet-$tRNA_f^{Met}$. The plasmid expressing bmIF2 was transformed into the double mutant strain (Δ*ifm1*Δ*fmt1*), followed by sporulation and tetrad dissection to isolate a spore carrying the plasmid. This transformant grew on YPEG medium, indicating that bmIF2 can replace ymIF2 *in vivo* even when there is no formylation of initiator tRNA (Tibbetts et al., 2003). This result was a surprise because purified bmIF2 showed a 50-fold higher binding preference for fMet-$tRNA_i^{Met}{}_f$ over Met-$tRNA_i^{Met}{}_f$ in binding assay *in vitro* (Liao and Spremulli, 1991).

1.4 The purpose of present study

The discrepancy between these *in vitro* and *in vivo* results led us to hypothesize that there are accessory factors that become essential for mitochondrial protein synthesis under conditions when fMet-$tRNA_i^{Met}{}_f$ is low or absent.

To search for these factors, the genetic screening system called synthetic petite screen was designed (Bender and Pringle, 1991). The basic principle of this screen is that mutations in two genes, which by themselves do not cause a mutant phenotype, in combination do cause a mutant phenotype (Appling, 1999). We used this screen to search for accessory factors that support the initiation of protein synthesis with unformylated Met-$tRNA_i^{Met}{}_f$. If these factors exist in yeast, we can look to see whether they exist in higher organisms as well. YmIF2 is the only "factor" involved in the initiation of yeast mitochondrial protein synthesis that has been identified and characterized. Other factors involved in mitochondrial protein synthesis have been characterized from mammals (such as the bovine mitochondrial IF2 and IF3) (Koc and Spremulli, 2002; Ma and Spremulli, 1996), but no homologs of IF1 and IF3 exist in the yeast genome. Therefore, the genetic screen might also identify mitochondrial proteins with IF1- or IF3-like initiators.

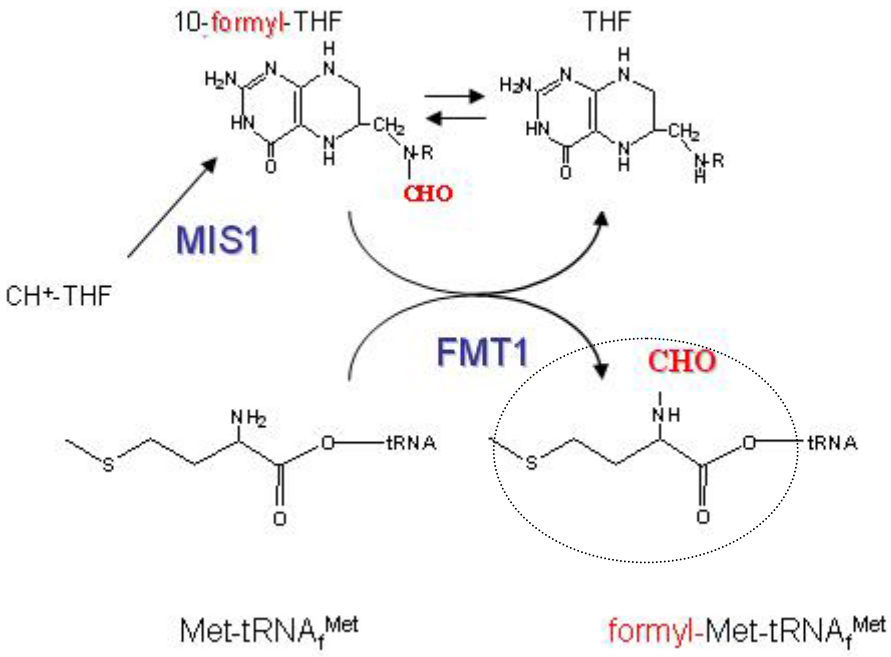

Figure 1.3: Transfer of formyl group to initiator tRNA in *S. cerevisiae* mitochondria. The C_1-THF synthase encoded by the *MIS1* gene converts 5,10-methenyl THF to 10-formyl THF, and the methionyl-tRNA formyltransferase encoded by *FMT1* gene catalyzes the transfer of formyl group to Met-tRNA$_i^{Met}{}_f$. Formylmethionine is indicated as a dotted-circle.

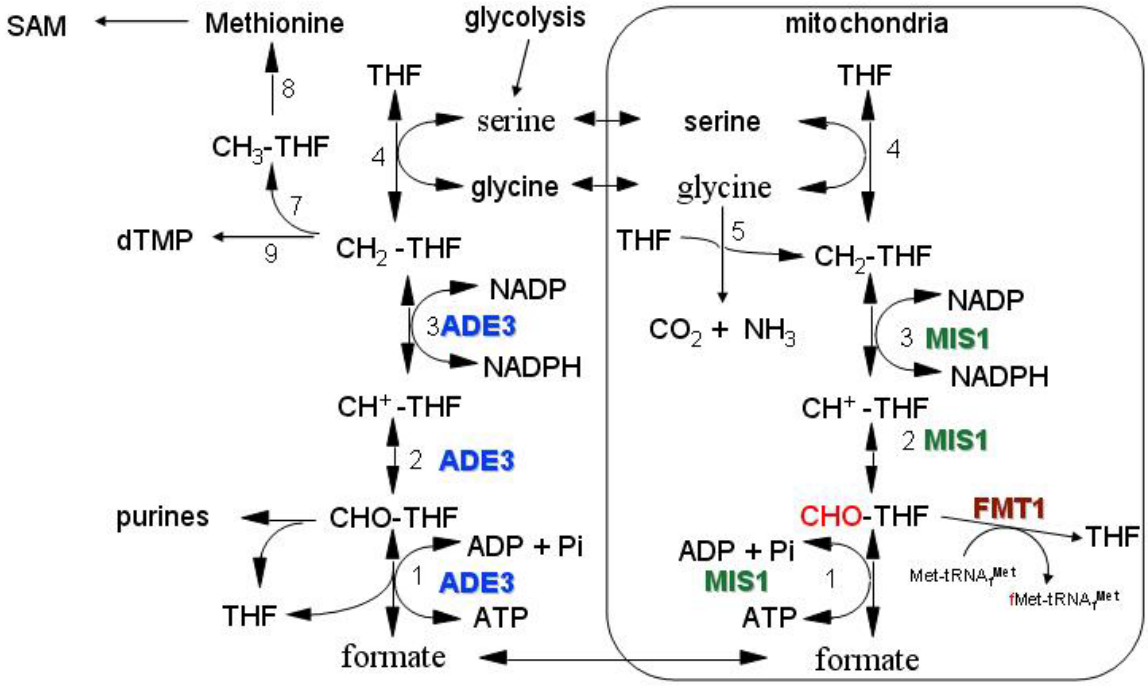

Figure 1.4: Overview of One-carbon cycle pathway in *S. cerevisiae*. Two parallel enzymes, C_1-THF synthase, encoded by *ADE3* gene in cytoplasm and by *MIS1* gene in mitochondria in yeast. A formyl group is highlighted by red color.

Chapter 2: Experimental Procedures

The experimental methods used for the yeast genetic screening system and for analysis of the *AEP3* gene are described in the following sections. Yeast strains and plasmids used in this study were summarized in Appendix C and D.

2.1 Yeast strains, materials, and media

The *Saccharomyces cerevisiae* strains used in this part are summarized in Appendix C. Strain Y03039 was obtained from the European Saccharomyces Cerevisiae Archive for Functional Analysis (EUROSCARF, Frankfurt, Germany). The following materials and reagents were used: yeast media (Difco laboratories), 5-fluoorotic acid (5-FOA, BioVectra DCL, Charlottetown, CA), KOD Hot Start DNA polymerase (Novagen, Gibbstown, NJ), restriction enzymes (New England Biolabs, Ipswich, MA), [^{35}S]methionine (PerkinElmer Life Sciences, Boston, MA), Geneticin (G-418 sulfate, American Bioanalytical, Natick, MA). General chemicals, of the highest grade commercially available, were obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), or VWR (West Chester, PA) and used without further purification.

Media contained 1% yeast extract, 2% Bacto-peptone, and either 2% glucose (YPD) or 2% ethanol and 3% glycerol as carbon sources (YPEG). Synthetic minimal media (YMD) contained 0.7% yeast nitrogen base without amino acids, 2% glucose as carbon sources and was supplemented for necessary growth as follows (final concentration): L-leucine (30 mg/liter), L-lysine (30 mg/liter), L-methionine (20 mg/liter), L-histidine (20 mg/liter), and uracil (20 mg/liter). For solid media plates, 2% Bacto-agar was added (Chan and Appling, 2003).

The radioactive initiator tRNAs ([^{35}S]fMet-tRNA$_f^{Met}$ and [^{35}S]Met-tRNA$_i^{Met}$) and [^{14}C]Lys-tRNA^{Lys} were generous gifts from Dr. Gisela Kramer (the University of Texas at Austin).

2.2 Screen for mutations creating a synthetic petite phenotype with Δ*fmt1*

The wild-type *FMT1* gene containing both 300 base pairs of the 5'UTR (upstream of the start codon for the *FMT1* gene) and 100 base pair 3'UTR (downstream of the stop codon) was amplified by PCR using primers: 5'-CAGTGGATCCACACCCAATTGCGAGCCTAA-3' (FMT1f2; BamHI site is underlined) and 5'-GTAGATCGATATGTAGAGCCGGGTTACAGG-3' (FMT1r2; ClaI site is underlined). The resulting fragment was digested with restriction enzymes, BamHI and ClaI, and inserted into enzyme-cleaved pRS416, a low-copy CEN4 *URA3* plasmid (Sikorski and Hieter, 1989) obtained from the American Type Culture Collection (ATCC, Manassas, VA) to generate pRS416-FMT1. Strain Y03039 was transformed with pRS416-FMT1, and the resulting transformants were exposed to UV radiation (Protocol 8 in Appendix E; performed by Dr. Anne Tibbetts at the University of Texas at Austin) to generate random mutations on the strain (Figure 2.1). Mutagenized cells were spread onto 200 complete nonfermentable carbon source medium plates (YPEG: No.1, Figure 2.1) to isolate single colonies and incubated at 30^{o}C for 6 days. These plates were replica-plated onto 200 5-flouroorotic acid (5-FOA) plates containing glucose as a carbon source to evict the *URA3* plasmids from the cells. The cells lacking the pRS416-FMT1 plasmid were replica-plated onto another 200 complete nonfermentable carbon source medium plates (YPEG: No.2, Figure 2.1). Potential synthetic petite mutants grew on YPEG (No.1), before the loss of pRS416-FMT1 plasmid, but not on YPEG (No.2), after the loss of pRS416-FMT1 plasmid. As a result, eight synthetic petite mutants were isolated from

this screen and tested whether the phenotype of the synthetic petite (s.p.) mutants was caused by a recessive nuclear mutation.

2.3 Isolation and identification of a gene interacting with Δ*fmt1*

Synthetic petite mutants were individually transformed with yeast genomic DNA libraries of *S.cerevisiae* (ATCC) to find a functional gene that corresponds to the mutation causing the synthetic petite phenotype. Cells were spread onto 70 minimal medium plates containing histidine and methionine, and then replica-plated onto another 70 plates containing 5-FOA as well as both ethanol and glycerol as nonfermentable carbon sources. The respiratory competent transformants were isolated, and then restreaked onto 5-FOA/YPEG plates to confirm whether transformants can complement the phenotype of petite mutants. Complementary plasmids were rescued from synthetic mutant strains (Protocol 7 in Appendix E). Identification of the genomic fragment inserts was determined by sequencing using plasmid specific primers flanking the insert: AJO194 (5'-GCTACTTGGAGCCACTATCGACTAC-3') and AJO195 (5'-CAGCAACCGCACCTGTGG-3') (obtained from Dr. Arlen Johnson, the University of Texas at Austin). The obtained DNA sequence was BLASTed against the *Saccharomyces cerevisiae* Genome Database (SGD) to identify the chromosomal locus.

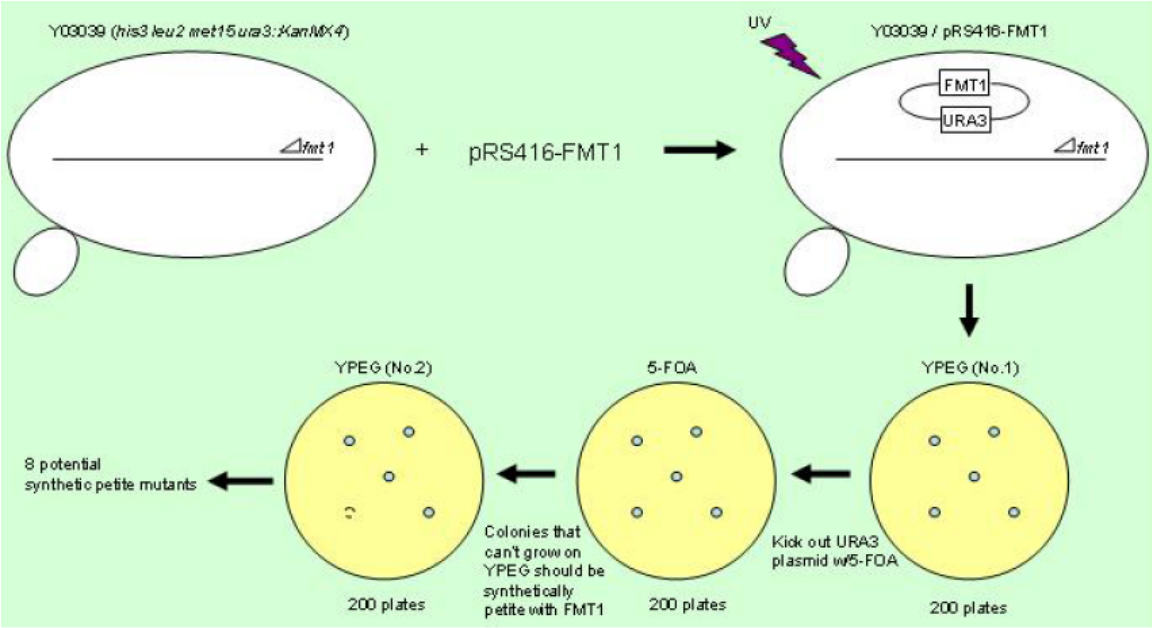

Figure 2.1: Overall scheme for synthetic petite screen.

2.3.1 Tetrazolium chloride overlay assay (TTC) to analyze respiration deficiency of the synthetic petite mutants

For the TTC assay (Ogur et al., 1957), each synthetic petite mutant strain was transformed with a plasmid containing the identified gene, and was spread onto minimal medium plates, and incubated at 30^{o}C for 3 days to generate the transformants. About 2 ml of sodium phosphate buffer (pH 7.0) containing 1.5% Bacto-Agar and 0.1% tetrazolium chloride was poured onto a transformants-contained plate, and then incubated at room temperature for 3 hours.

2.3.2 Construction of a pRS415-AEP3 vector and a pRS415-AEP3 mutant vector

To clone the wild-type *AEP3* gene into a plasmid, yeast genomic DNA obtained from strain DAY4 was amplified by PCR using KOD Hot Start DNA polymerase (Novagen, CA) and two primers; a forward primer 5'-AGCCTCGAGCCATAGCAGATTATACTA-3' (A3_FOR; the XhoI site is underlined) and a reverse primer 5'-AATGGATCCTTCCTGTCCAAATGC-3' (A3_REV; the BamHI site is underlined).

For the mutant AEP3 construct, yeast genomic DNA obtained from the synthetic petite strain, s.p.175, was isolated by PCR amplification using the same primers above. Each construct contained the same 300bp of 5'-UTR and 100bp of 3'-UTR in the *AEP3* genomic DNA, and its sequence was confirmed by DNA sequencing.

2.4 Plasmid constructs for the purification of the AEP3 and the mutant gene products as MBP fusion proteins

To generate a MBP-AEP3 fusion protein, the wild-type *AEP3* gene was isolated by PCR amplification from yeast genomic DNA of DAY4 strain using primers: 5'-CCGGGATCC**ATG**AATACATTAAGG-3' (AP3-pMAL-For; BamHI site is underlined and start codon is bold) and 5'-CCGAAGCTT**TCA**AACCTCCCCAAC-3' (AP3-pMAL-Rev; HindIII site is underlined and stop codon is bold). PCR was carried out using KOD Hot Start DNA polymerase (CalBiochem, Gibbstown, NJ). The resulting PCR fragment was digested with BamHI and HindIII, then gel-purified, and inserted into a pRSET A vector (Invitrogen, CA) which was pre-cleaved with same enzymes. The *AEP3* gene was also cloned into the pMALc2H$_{10}$T vector (a gift from Dr. John Tesmer, University of Michigan) (Kristelly et al., 2004) to generate MBP-Aep3p, following the same procedures described above. The insert was verified at the DNA Core Facilities of the Institute for Cellular and Molecular Biology at the University of Texas at Austin. For the mutant constructs, each procedure was the same as for a wild-type construct except for isolation of a mutant gene from the synthetic petite mutant strain.

To express MBP-Aep3 protein, the MBP-Aep3 construct was transformed into *E.coli* Rosetta 2 (DE3) cells (Novagen, Gibbstown, NJ). The resulting transformants were placed into Luria Broth containing 50 μg/ml ampicillin, grown to OD_{600} of 0.4, and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 15^{o}C overnight. The cells were harvested by centrifugation at 5,000 rpm for 10 min, washed in 1 x binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tri-HCl, pH 7.9), centrifugated under the same condition, and then kept at -80^{o}C. Cells were resuspended in 50 ml of 1x binding buffer, followed by adding a tablet of protease inhibitor (Roche Applied Science, Indianapolis, IN). Cells were lysed by sonication on ice 15 sec with 15 sec intervals for

six cycles. The supernatant was collected after centrifugation at 17,000 rpm for 30 min, and applied to a column filled with 5 ml His·Bind Resin (Novagen, Gibbstown, NJ) that had been charged with Ni^{2+} and equilibrated in 1 x binding buffer containing 5 mM imidazole and 0.5 M NaCl in 20 mM Tris-HCl buffer, pH 7.9. After washing with 50 ml binding buffer, loosely bound protein was eluted with wash buffer containing 60 mM imidazole and 500 mM NaCl in Tris buffer. Tightly bound protein was eluted by 250 mM imidazole in the same Tris/NaCl buffer. Fractions of four drops were collected into 1.5 ml eppendorf tubes, and 1 µl of each fraction was applied to nitrocellulose, followed by staining with a solution of Coomassie brilliant R and destaining to identify fractions containing eluted protein. The pooled protein fractions eluted with 250 mM imidazole were dialyzed against 500 ml of 20 mM Tris-HCl, pH 7.5 for four hours and stored at -80^{o}C in small aliquots. For the expression of mutant protein, the same procedure was used. The concentration of purified proteins was determined by the Bradford protein assay (Bradford, 1976), and analyzed by 10% SDS-PAGE (Laemmli, 1970).

Pure full-length ymIF2 as well as the C2-subdomain of ymIF2 were a generous gift from Dr. Gisela Kramer (The University of Texas at Austin) (Garofalo et al., 2005; Garofalo et al., 2003).

2.5 *In vitro* filter binding assay

The filter binding assay for formation of the binary complex between the wild-type AEP3 protein or yeast mitochondrial initiation factor 2 (ymIF2) or the AEP3 mutant protein and either fMet-tRNA$^{Met}_{f}$ or Met-tRNA$^{Met}_{f}$ or Lys-tRNA^{Lys} was performed as described (Sundari et al., 1976). Different amounts of each protein in 50 µl total reaction mixture containing 50 mM Tris-HCl, pH 7.0, 2 mM DTT, and 10 µg BSA was incubated with [^{35}S] initiator tRNA (4 pmol) for 20 min on ice. Reactions were stopped with 2 ml of

ice-cold solution containing 50 mM Tris-HCl, pH 7.0 and 2 mM DTT, and then filtered through nitrocellulose filters, followed by washing 5 times with the same buffer. Filters were dried at 150^{o}C of for 4 min, and radioactivity on filters was determined by liquid scintillation counting. Each assay was performed in duplicate.

2.6 *In vitro* Maltose binding protein (MBP) Pull-down assay

For MBP-Aep3 pull downs, 5 µl of MBP-Aep3 protein (20 pmol) was incubated with ymIF2 in the indicated concentrations at room temperature for 2 hours in 200 µl of buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM DTT with gentle rotation. About 30 µl of a slurry containing amylose affinity beads (New England Biolabs; diluted 50% with 1 x Phosphate Buffer Saline (PBS)) were added to the mixture and incubate for an additional 1 hour while rotating. To decrease nonspecific binding, the affinity beads were washed six times using same buffer. Bound proteins were eluted by boiling for 5 min in SDS-PAGE sample buffer, and a fraction was analyzed by 10% SDS-PAGE. Immunoblotting was performed using antibodies against ymIF2 (a generous gift from Dr. Gisela Kramer, the University of Texas at Austin) and commercially available anti-MBP (New England Biolabs). The western blots were visualized with enhanced chemiluminescence detection system (GE Healthcare).

2.7 Plasmid constructs for Co-immunoprecipitation (Co-IP) assay

To generate a pRS415-AEP3-HA construct, the wild-type *AEP3* gene was isolated by PCR amplification using two primers: a forward primer 5'-AGCCTCGAGCATAGCAGATTATACTA-3' (A3-FOR; XhoI site is underlined) and 5'- GGCGGATCC**TCA***AGCGTAGTCTGGGACGTCGTATGGGTA*AACCTCCCCAAC-

TATTCTCCTCTTTAC-3' (AP3-HA-Rev; BamHI site is underlined, stop codon is in bold. Italic letters are the HA epitope tag) (Figure 2.2). The resulting fragment from the PCR was purified (PCR purification kit, Qiagen), then digested with both XhoI and BamHI, gel-purified, and ligated into a pRS415 vector which was pre-cleaved by both restriction enzymes. The plasmid construct was isolated from *E.coli* XL1-B cells, and the sequence of the insert was verified at the DNA Core Facilities at the University of Texas at Austin.

To generate the pRS415-AEP3-FLAG (Figure 2.3) or the c-*Myc* construct (Figure 2.4), genomic AEP3 DNA was amplified by PCR using primers: A3-FOR (described above) and a reverse primer 5'-TTTCTAGAGTAATGGATCCAACCTCCCCAAC-3' (XbaI and BamHI sites are underlined, respectively). The PCR product was purified (PCR purification kit, Qiagen), and digested with XhoI and XbaI. The gel-purified fragment was cloned into a pre-cleaved pRS415 vector to generate pRS415-AEP3-XbaI (Figure 2.5). After isolation of the plasmid construct from *E.coli* XL1-B cells, the construct was digested with BamHI and XbaI to make sticky ends at each side of the insert, which will allow the annealed primers to ligate into a pre-cleaved pRS415 vector. For the FLAG tagged construct, two different primers were used; 5'-GATCC*GACTACAAAGATGACGATGACAAG***TAG**T-3' (FLAG-A3-BamHI; BamHI site is underlined. Stop codon is in bold. Italic letters are FLAG epitope tags) and 5'-CTAGA**CTA***CTTGTCATCGTCATCTTTGTAGTC*G -3' (FLAG-A3-XbaI; XbaI site is underlined. Stop codon is in bold. Italic letters are complementary to FLAG epitope tags). Five µl of each primer (10 µM) was mixed with restriction enzyme buffers to give a total volume in 50 µl. The primer mixtures were boiled for 5 min, and cooled down at room temperature for 1 hour to anneal each other. Two µl of the annealed primer mixture was incubated with T4 polynucleotide kinase (Invitrogen, CA) to add an N-terminal

phosphate at 37^{o}C for 1 hour, followed by another incubation at 65^{o}C for 30 min. The annealed primers were diluted 1:20 in ligation buffer, and ligated into a pRS415-AEP3-XbaI construct which was pre-cleaved with BamHI and XbaI. The sequence of the insert was verified by DNA sequencing.

For the pRS415-AEP3-c-Myc construct, the same procedure was carried out described above except for using two different primers. 5'-GATCC*GAACAAAAACTCATCTCAGAAGAGGATCTG***TGA**T-3' (cMyc-A3-BamHI; BamHI site is underlined. Stop codon is in bold. Italic letters are c-Myc epitope tags) and 5'-CTAGA**TCA***CAGATCCTCTTCTGAGATGAGTTTTTGTTC*G-3' (cMyc-A3-XbaI; XbaI site is underlined. Stop codon is in bold. Italic letters are complementary to c-Myc epitope tags).

❑ **HA sequence: 5'- YPYDVPDYA - 3'**

A3-FOR ; 5' – AGC CTCGAG CCA TAG CAG ATT ATA CTA – 3'

AP3-HA-Rev: 5' – GGC GGATCC TCA AGC GTA GTC TGG GAC GTC GTA
A Y D P V D Y
TGG GTA AAC CTC CCC AAC TAT TCT CCT CTT TAC – 3'
P Y

Figure 2.2: Primers and amino acid sequence of the HA epitope-tagged AEP3 construct. The stop codon is highlighted by red color. The HA tag is designated by pale blue color.

❑ **FLAG sequence: 5'-DYKDDDDK-3'**

```
5'-GATCC/GAC TAC AAA GAT GAC GAT GAC AAG/TAG/T-3'
          D   Y   K   D   D   D   D   K

   3'-G/CTG ATG TTT CTA CTG CTA CTG TTC/ATC/AGATC-5'
          D   Y   K   D   D   D   D   K
```

Figure 2.3: Primers and amino acid sequence of the FLAG epitope-tagged AEP3 construct

❑ **c-Myc sequence: 5'-EQKLISEEDL-3'**

```
5'-GATCC/GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG/TGA/T-3'
         E   Q   K   L   I   S   E   E   D   L
   3'-G/CTT GTT TTT GAG TAG AGT CTT CTC CTA GAC/ACT/AGATC-5'
         E   Q   K   L   I   S   E   E   D   L
```

Figure 2.4: Primers and amino acid sequence of the c-*Myc* epitope-tagged AEP3 construct

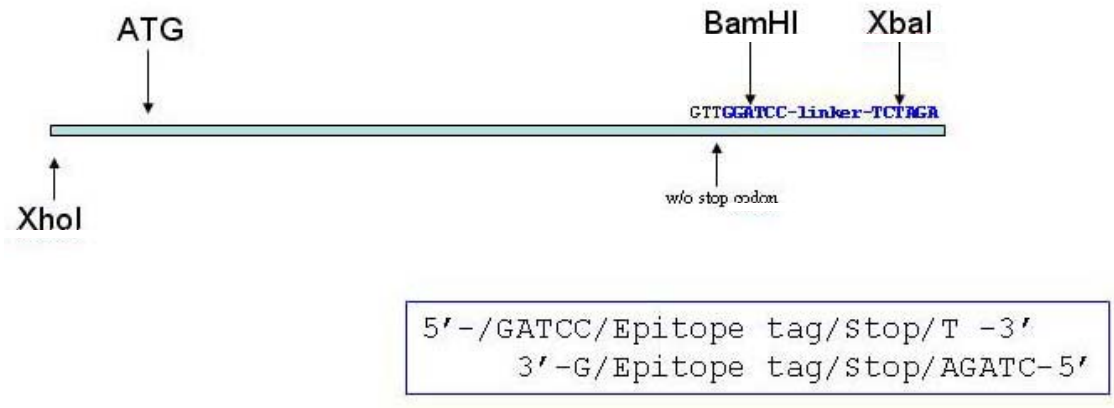

Figure 2.5: Overall scheme for construction of epitope-tagged AEP3 plasmids. The box represents how two primers containing the epitope-tag are designed to be annealed.

2.8 *In vivo* Co-immunoprecipitation (Co-IP) assay

Cells containing HA-tagged *AEP3* gene was used for co-immunoprecipitation (Co-IP) experiments. Yeast cells in 50 ml YPEG culture was harvested and washed twice with ice-cold water. Cells were resuspended in 500 µl of a co-ip buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 10 mM $MgCl_2$, and an aliquot of a cocktail of protease inhibitors (Hiley et al.) and were disrupted with glass beads using FastPrep FP120 cell disrupter (Qbiogene, CA). A cleared soluble lysate was obtained by centrifugation at 16,000 rpm for 30 min. Glass beads were washed in 500 µl of same buffer and centrifuged at 16,000 rpm for 30 min. Each pre-cleared soluble lysate was combined together. Pre-cleared lysate (1 ml) was then incubated with 10 µl of antibodies against HA epitope tags at 4^{o}C for 4 hours while gently rotating. The mixture with anti-HA was incubated with 50 µl of Protein A sepharose beads (Invitrogen, CA) at 4^{o}C for overnight. Antibody-linked immunocomplexes were collected by centrifugation at 1,500 rpm for 2 min, washed five times in 500 µl of the co-ip buffer, and eluted from matrix with a SDS-PAGE sample buffer. Western blotting was performed to probe for HA-tagged AEP3 or ymIF2 using monoclonal HA anti-mouse antibody (Covance, CA) or ymIF2 polyclonal antibody (a generous gift from Dr. Gisela Kramer, the University of Texas at Austin)

Two Co-IP controls were used: Δ*aep3* yeast cells (strain DLY2) expressing on untagged *AEP3* gene from a plasmid, and Δ*ifm1* yeast cells (strain LOY1) expressing HA-tagged *AEP3* gene from a plasmid.

Chapter 3: Results and Discussion

3.1 Identification of a mutation in a gene that causes the synthetic petite phenotype with the deletion of *fmt1*

Deletion of the *FMT1* gene is not essential for respiratory growth (Li et al., 2000). Therefore, we used this fact to design a genetic screening system to isolate mutations in the yeast genome that cause a synthetic petite phenotype (respiratory-deficient) with the deletion of the *FMT1* gene. The strain with the deletion of the *FMT1* gene was transformed with pRS416-FMT1, and then exposed to UV radiation to generate mutations (Protocol 8 in Appendix E). Cells were spread onto 200 complete nonfermentable carbon source medium plates (YPEG). This step eliminates mutations or petites which are not related to *fmt1*. By replica-plating onto 5-fluoroorotic acid contained plates (5-FOA), the *URA3* borne pRS416-FMT1 plasmid was removed from the cells. Missing colonies on 200 new YPEG plates obtained by another replica-plating indicated that a mutation had occurred somewhere in the yeast genome and is synthetically petite with the deletion of *fmt1*. In other words, a gene containing a mutation is genetically interacting with the *FMT1* gene. The genetic screen produced 8 independent recessive nuclear mutations that caused synthetic petite phenotypes only when there is no formylation of initiator tRNA (only in the *fmt1* deletion background).

Among 8 synthetic petite mutant strains, one strain, synthetic petite (s.p.) mutant 79-2, was chosen to search for the mutation by transformation with yeast genomic DNA library plasmids containing fragments of the complete genome of the wild-type yeast (Figure 3.1). The transformants were spread onto 100 minimal media-contained plates without uracil and leucine. By replica-plating onto 100 5FOA/YPEG plates, we isolated 12 colonies that grew on the nonfermentable carbon source, indicating that each colony

has a gene that complemented the synthetic petite mutant phenotype. After rescuing 12 DNA library plasmids from each colony, the DNA regions on the plasmids were sequenced (Experimental procedures 2.3), and analyzed by NCBI BLAST.

3.1.1 Isolation of unknown ORF (open reading frame) on Chromosome XV

The result of BLAST showed that one of the complementing plasmids contained the region in chromosome II where the *FMT1* gene is located. All other eleven complementing plasmids contained common DNA regions on chromosome XV that overlapped with each other. The overlapping region was around 5kbp long, and included three verified genes and one unverified gene (Figure 3.2). The three genes are *PET56* (currently changed to *MRM1*), *HIS3*, and *DED1* gene. To test which gene is responsible for the mutation causing the petite phenotype of s.p.79-2, each gene was isolated by restriction digestion of the plasmid using different enzymes, gel-purified, and inserted into a pre-cleaved pRS415 vector. Each construct was transformed into the s.p.79-2 strain, and the resulting transformants were streaked onto a 5-FOA/YPEG plate (Figure 3.3). The construct pRS415-HIS3, showing a respiratory-competent phenotype, contains both the *HIS3* gene and the unknown ORF (unORF; YOR203W) that is downstream of the *HIS3* gene (Figure 3.4). We realized that approximately 200bp in the middle of the *HIS3* gene was already missing in the original yeast strain (Brachmann et al., 1998). Therefore, we deduced that the *HIS3* gene could not be the candidate, and we focused on the unORF to test whether it is the gene complementing the phenotype of the s.p.79-2 strain.

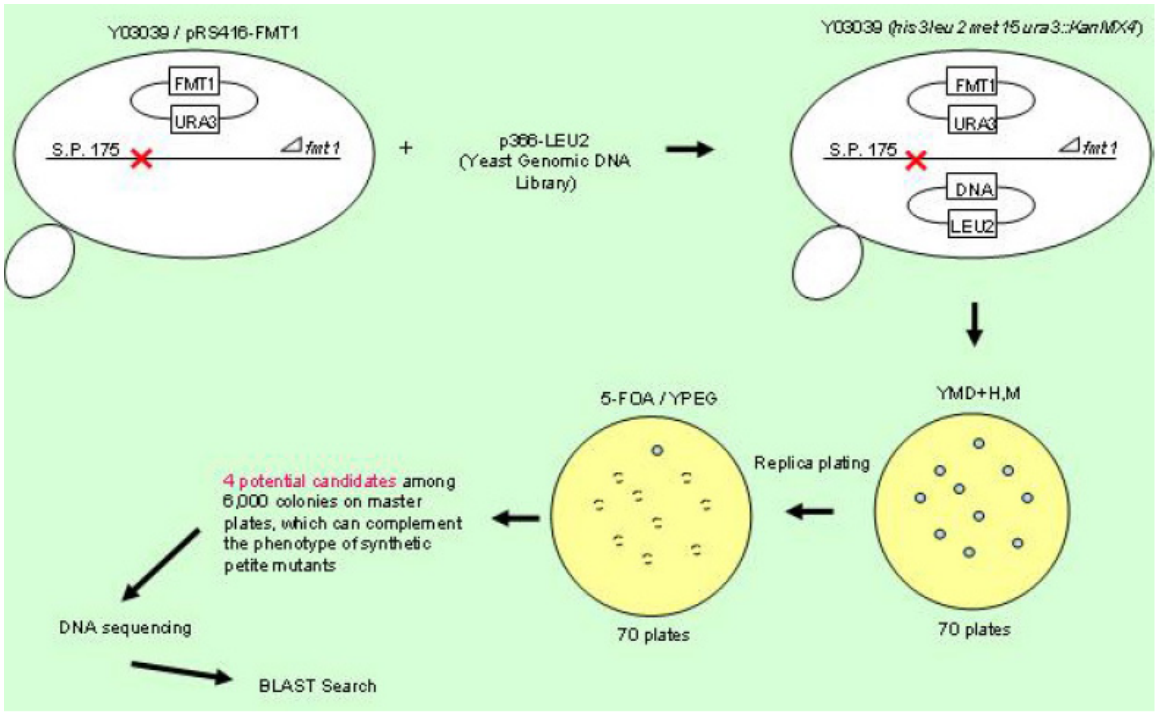

Figure 3.1: Isolation of a mutant gene interacting with Δ*fmt1*. A red X mark represents the potential mutation site created by UV radiation

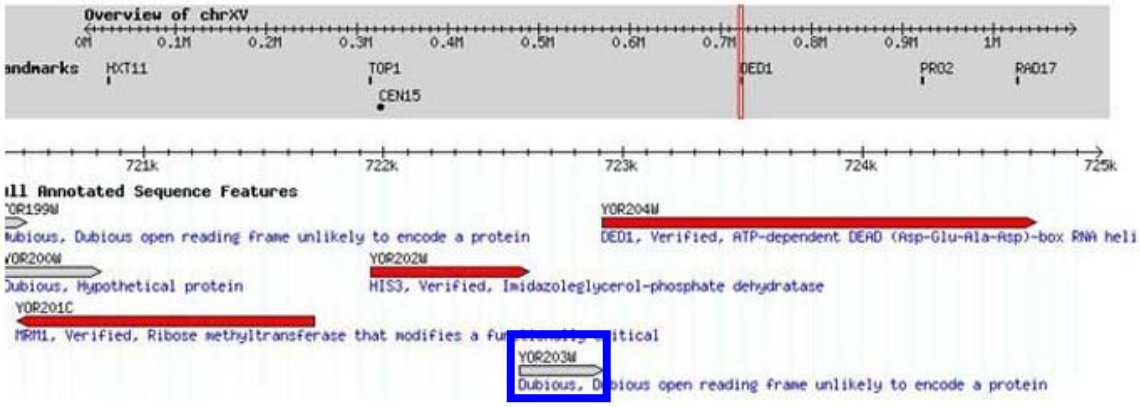

Figure 3.2: Partial map of Chromosome XV containing overlapping DNA region from 11 potential candidates. The blue open box represents an unknown open reading frame (unORF). Red arrows are verified genes.

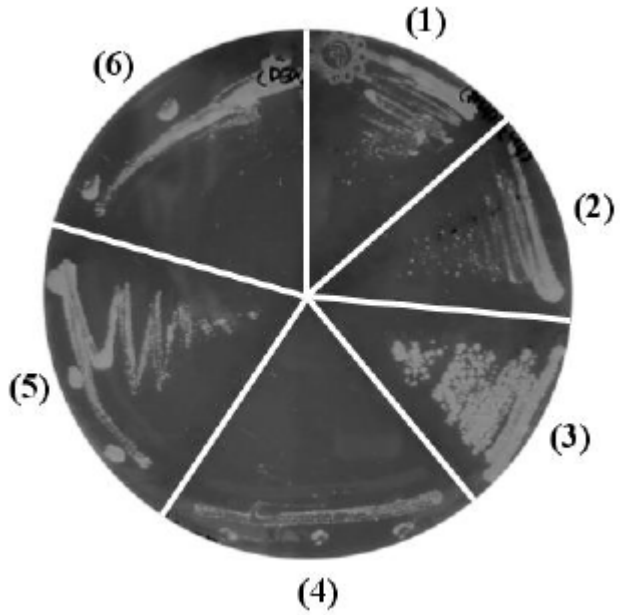

Figure 3.3: Complementation analysis on a 5-FOA/YPEG plate. Cells were grown at 30^{o}C for 6 days. Sectors (1), (2), and (3) were used as positive controls: (1) and (2) are from two of the 11 potential candidates. (3) Cells containing wild-type *FMT1* plasmid. Sector (4) Cells containing a PET56 construct. Sector (5) Cells with the HIS3 construct. Sector (6) Cells with the DED1 construct.

3.1.2 Characterization of unknown ORF on Chromosome XV

The pRS415-HIS3 construct was digested with BamHI and HindIII to generate fragment which contains just the unORF region (Figure 3.4). It was gel-purified, and ligated into a pre-cleaved pRS415 vector to produce the pRS415-unORF construct. This construct was transformed into the s.p.79-2 strain to see whether it can complement the petite phenotype of this mutant strain. The pRS415-unORF construct complemented the petite phenotype of s.p.79-2 just as well as the pRS415-HIS3 construct in a spot dilution assay (Figure 3.5).

Another method, the tetrazolium chloride (TTC) assay (Experimental procedures 2.3.1), was used to test whether the pRS415-unORF construct is responsible for the complementation of the petite phenotype. The principle of the TTC assay is that the colorless chemical tetrazolium chloride diffuses into actively respiring cells, and it accepts electrons from the mitochondrial electron transport chain, reducing TTC to a pink compound, called formazan (Altman, 1976). The accumulation of this compound stains cells with a red color. S.p.79-2 cells expressing pRS415 empty vector (used as a negative control) were shown to have a white color. Cells expressing either the pRS415-HIS3 construct or the pRS415-unORF construct were shown with red color, indicating that both constructs complemented the petite phenotype of s.p 79-2 strain (Figure 3.6).

Table 3.1 summarizes the results of additional tests to confirm whether unORF is the gene that complements the petite phenotype of the s.p.79-2 strain. Further characterizations were hampered by inconsistent results that were obtained in different experiments, except that s.p.79-2 cells expressing pRS415-unORF always showed a positive result. At that point, we speculated whether the unORF is biologically active. One possibility was that unORF might express a functional protein. To test this possibility, site-directed mutagenesis was used to generate pRS415-SDM containing in

frame stop codons downstream of the AUG codon of unORF. If unORF encodes a functional protein, the pRS415-SDM construct could not complement the petite phenotype of s.p.79-2. However, this construct complemented the s.p.79-2 strain in both assays (Table 3.1). Also, we generated a construct containing the disruption of unORF region to investigate whether it is functional. The PstI restriction site is in the middle of unORF region. Therefore, the piece of unORF cut by both PstI and BamHI was cloned into a pRS415 vector to generate the pRS415-PstI construct (Figure 3.4). As shown in Table 3.1, this construct did not show reproducible results in both assays. Another possibility was that a high copy yeast expression vector containing unORF (PVT103L-unORF) could complement s.p.79-2. The PVT103L-unORF construct also did not show reproducible results in two assays.

Finally, we directly sequenced the region of Chromosome XV from s.p.79-2 and found no mutations in 5kbp long including *PET56*, *HIS3*, unORF and *DED1* gene.

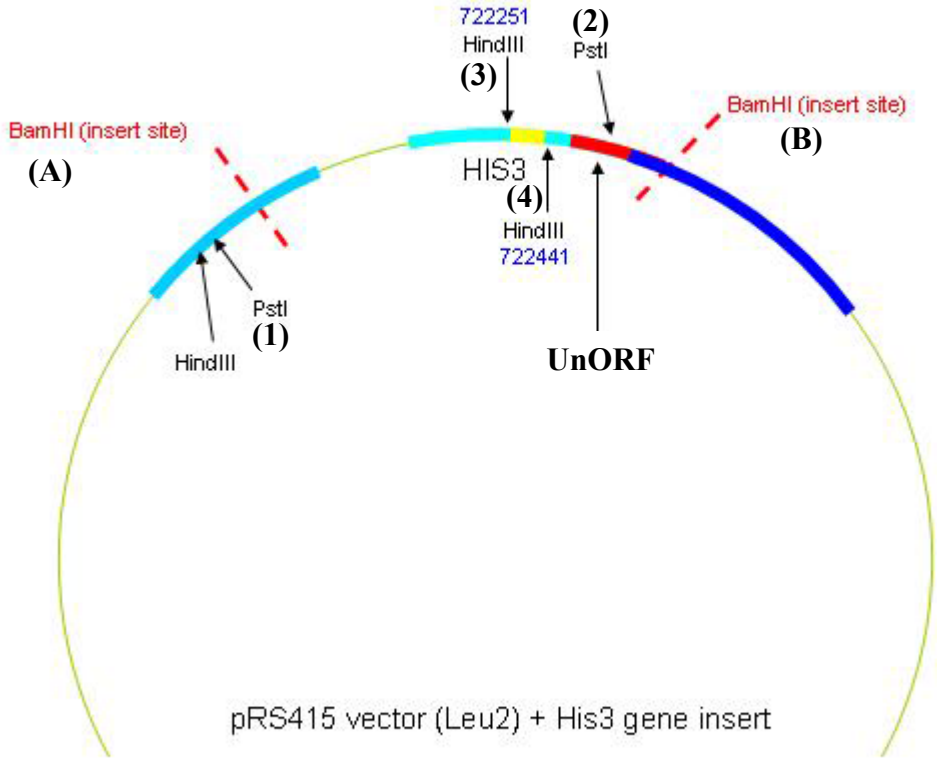

Figure 3.4: Restriction sites map of pRS415-HIS3 construct. Yellow bar: original deletion part of the *HIS3* gene. Red bar: the region of unORF. Pale blue bar: the *HIS3* gene. Blue bar: the *DED1* gene. pRS415-PstI contains the piece digested by PstI (2) and BamHI (B) enzymes. pRS415-unORF contains the piece digested by HindIII (4) and BamHI (B).

Table 3.1: Summary of Inconsistent Results. Highlighted with yellow are inconsistent results. Y/N represents that the result was not reproducible.

Methods / Constructs	Growth on YPEG	TTC assay	Expected results
Sp79-2/pRS416-FMT1	Yes	Red (Yes)	Yes
Sp79-2 w/o any plasmids	No	White (No)	No
Sp79-2/pRS415-PET56	No	White (No)	No
Sp79-2/pRS415-HIS3	Yes	Red (Yes)	Yes
Sp79-2/pRS415-DED1	No	White (No)	No
Sp79-2/pRS415(empty)	Y/N	Y/N	No
Sp79-2/pRS415-unORF	Yes	Red (Yes)	Yes
Sp79-2/pRS415-PstI	Y/N	Y/N	No
Sp79-2/pRS415-SDM	Yes	Red (Yes)	No
Sp79-2/PVT103L	No	White (No)	No
Sp79-2/PVT103L-unORF	Y/N	Y/N	Yes

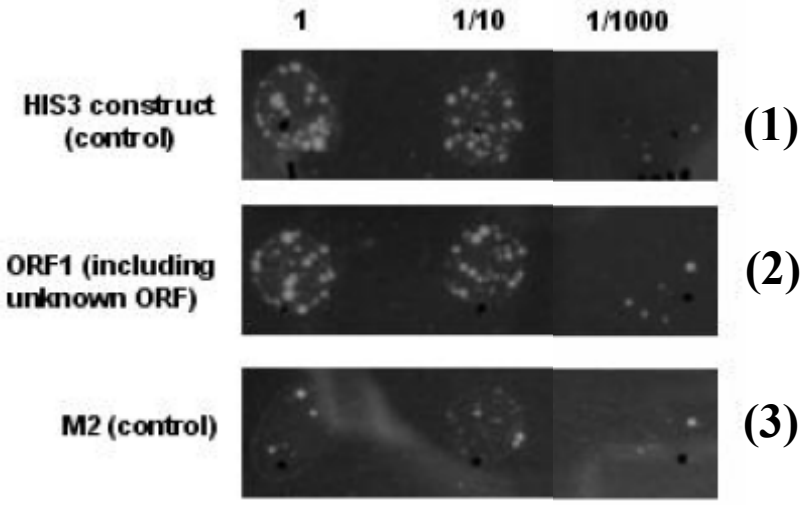

Figure 3.5: Spot dilution assay on a 5-FOA/YPEG plate. (1) Positive control, cells containing both the *HIS3* gene and unORF. (2) Cells expressing the pRS415-unORF. (3) Cells containing a construct with a disruption of the unORF. The dilution is indicated at on the top of the figure.

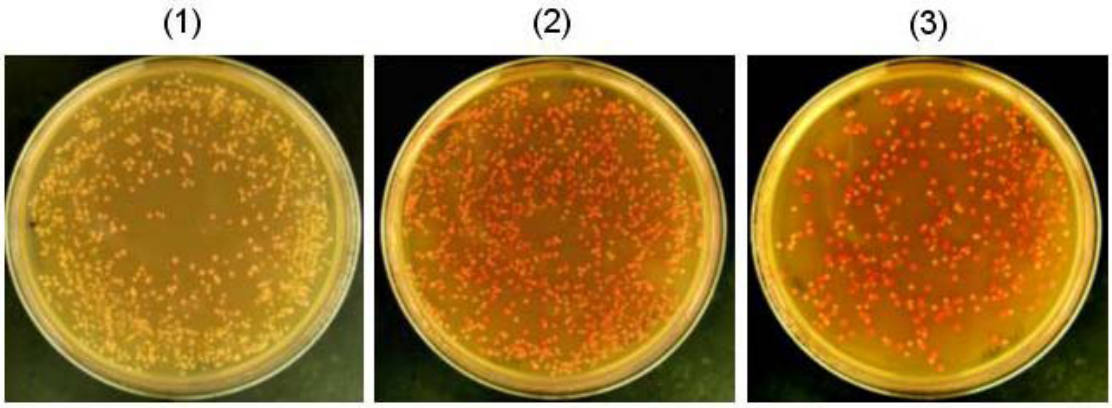

Figure 3.6: Tetrazolium chloride (TTC) assay. Panel (1) Cells containing pRS415 empty vector (Negative control). Panel (2) Cells with the pRS415-HIS3 construct. Panel (3) Cells with the pRS415-unORF construct. Cells were grown on YPD plates at 30^{o}C for 2 days, and then agar and TTC were overlaid. The plates were then incubated at room temperature for 3 hours.

3.1.3 Complementation groups analysis of the 8 synthetic petite mutant strains

Despite our failure to identify the nature of the synthetic petite mutation in the s.p.79-2 strain, the fact that the pRS415-unORF construct consistently complemented the petite phenotype of the s.p.79-2 strain allowed us to use this construct for complementation group analysis of the 8 synthetic petite mutant strains isolated. pRS415-unORF was transformed into the other 7 synthetic petite mutant strains. Table 3.2 summarizes the complementation group analysis. Transformants were spread onto a minimal medium plate containing histidine and methionine. After colony purification, they were restreaked onto 5-FOA plates to evict pRS416-FMT1. The resulting colonies were tested for the petite phenotype using two different methods: growth on YPEG and tetrazolium chloride (TTC) assay (Figure 3.7).

Only two mutant strains (s.p.175 and s.p.38) gave clear results in both assays, indicating that they represented different complementation groups from s.p.79-2.

Table 3.2: Summary of complementation analysis. Seven synthetic petite mutants were transformed with pRS415-unORF and tested for complementation of petite phenotype. Only s.p.175 and s.p.38 (highlighted by yellow) gave clear results in both assays, indicating different complementation groups from s.p.79-2.

S.P.strain / Methods	175	192	172-2	17-1	88	89	38
TTC	White	White	Red	Unclear	Red	Unclear	White
YPEG	No growth	Growth	Growth	Unclear	Growth	No growth	No growth

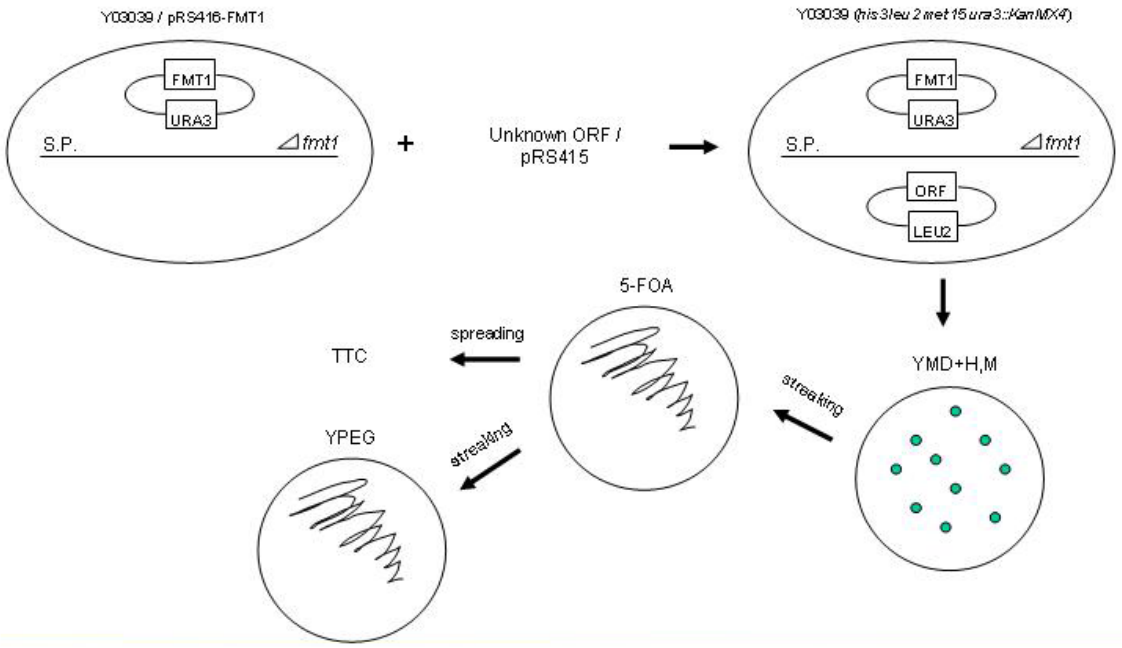

Figure 3.7: Isolation of complementation groups of synthetic petite mutants

3.2 Identification of a mutation in the *AEP3* gene

Based on complementation group analysis, the s.p.175 strain was used next to find a mutation in a gene that causes the synthetic petite phenotype in combination with deletion of the *fmt1* gene. Transformation with a yeast genomic DNA library was done as described above (Result 3.1). Transformants were spread onto 70 minimal media plates without uracil and leucine, and then replica-plated onto 70 5-FOA/YPEG plates. Four potential candidates were obtained out of 6,000 colonies, screened, and retested by YPEG streaking (Figure 3.8). After rescuing the library plasmids from these 4 candidates, sequencing and BLAST analysis identified overlapping regions on chromosome XVI (Figure 3.9). This region contained 3 verified genes, and we immediately focused on the *AEP3* gene, because the protein encoded by this gene is known to be a peripheral mitochondrial inner membrane protein (Ellis et al., 2004).

To test whether *AEP3* is responsible for complementation of the petite phenotype of the s.p.175 mutant strain, yeast genomic DNA was isolated from DAY4 cells and employed in PCR with specific primers to amplify the wild-type *AEP3* gene. Subsequently, a pRS415-AEP3 construct was generated (Experimental procedures 2.3.2). After transformation into the s.p.175 mutant strain, it was confirmed that the *AEP3* gene alone complemented the petite phenotype of the s.p.175 mutant strain (Figure 3.10).

A mutation on the *AEP3* gene created by UV radiation was subsequently identified by DNA sequencing. The *AEP3* locus in the s.p.175 mutant strain was amplified by PCR using several different primers either sitting upstream of the start codon or in the middle of the *AEP3* gene or downstream of the stop codon of the *AEP3* gene. The resulting PCR fragments were analyzed by DNA sequencing. A T at position 913 of the *AEP3* coding sequence was changed to A, as shown in the chromatogram of

the sequencing reaction (Figure 3.11). This mutation would cause a tyrosine to asparagine substitution at position 305 in the *AEP3* protein (Y305N).

We wanted to confirm the genetic interaction between *AEP3* and *FMT1*. Based on the design of the screen, the *aep3* mutation (Y305N) should only cause the petite phenotype in a Δ*fmt1* background. An *aep3* mutant construct (pRS415-AEP3-Y305N) was introduced into a yeast strain containing the wild-type *FMT1*, but the deletion of *AEP3* (Figure 3.12). Cells containing pRS415-AEP3-Y305N grew on non-fermentable carbon sources medium, confirming that *AEP3* genetically interacts with *FMT1*.

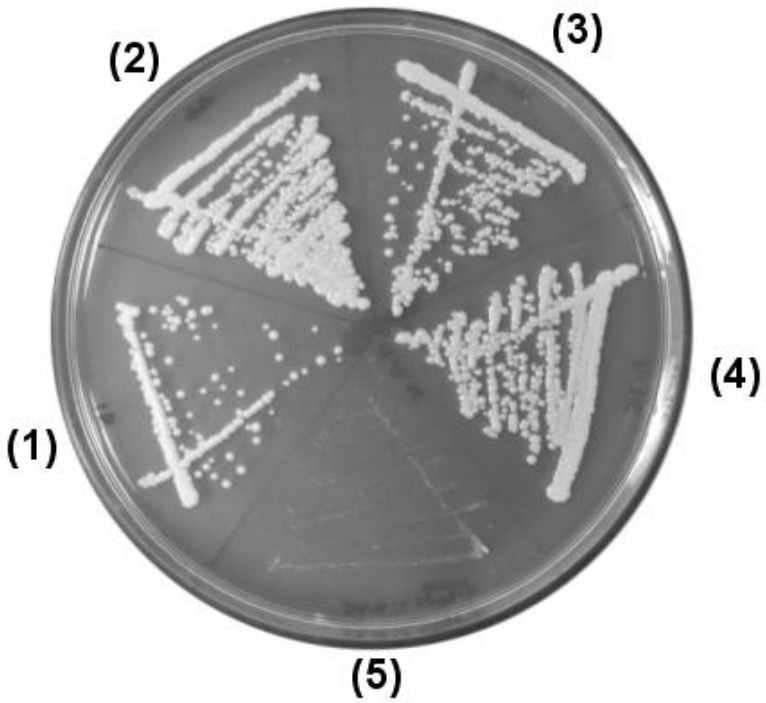

Figure 3.8: Confirmation analysis of 4 potential candidates from the s.p.175 mutant strain. Colonies identified on YPEG plates containing DNA library plasmids were spread onto 5-FOA/YPEG plate at 30^{o}C for 6 days. The four candidates, (1) – (4), are shown here. A DNA library plasmid rescued from (4) was transformed into s.p.79-2 (5). It did not complement the phenotype of s.p.79-2 mutant strain, indicating that the s.p.175 mutant strain is not in the same complementation group with the s.p.79-2 mutant strain.

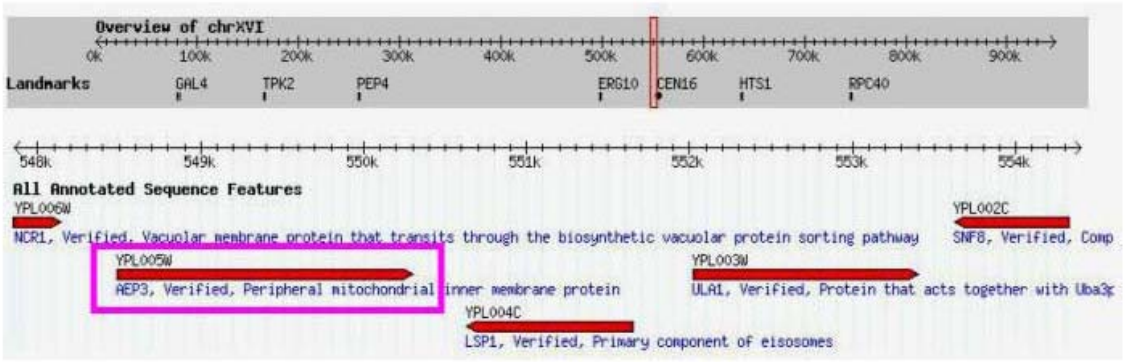

Figure 3.9: Partial map of Chromosome XVI containing overlapping DNA regions from 4 potential candidates to complement the phenotype of s.p.175 mutant strain. Red arrows are verified genes. The pink open box represents the *AEP3* gene.

Figure 3.10: Complementation of the s.p.175 mutant strain with the pRS415-AEP3 construct. Cells were grown at 30^{o}C for 5 days on 5-FOA/YPEG plate. The bottom represents a negative control with the vector transformed into s.p.175 mutant strain.

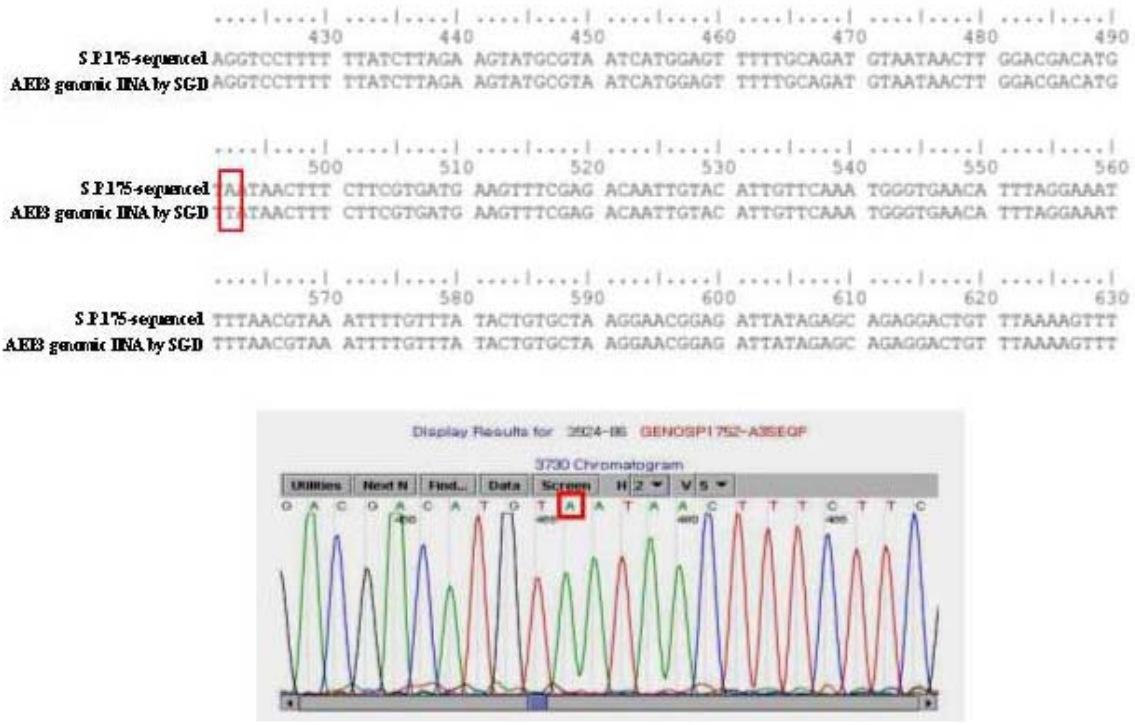

Figure 3.11: DNA sequencing results verifying a mutation on AEP3 gene. The red boxes indicate the site of mutation.

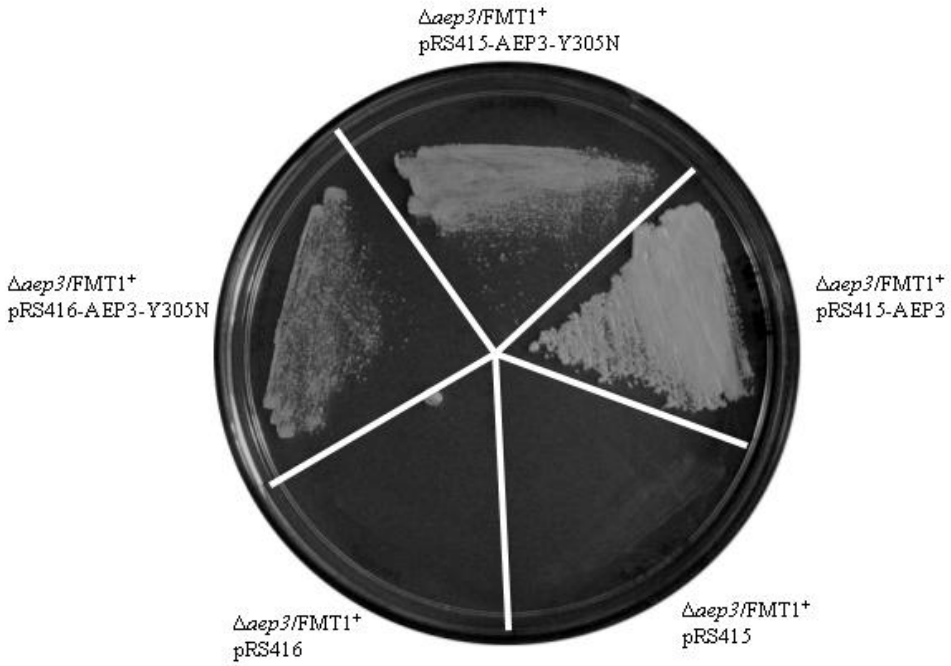

Figure 3.12: Confirmation test of genetic analysis. Each strain was incubated at 30^{o}C for 5 days on YPEG medium.

3.3 Overexpression and purification of Aep3p

The wild-type and Y305N *AEP3* genes were isolated from strain DAY4 or s.p.175, respectively. The resulting PCR fragment was digested with restriction enzymes (Experimental procedures 2.4), gel-purified, and inserted into pRSET A vector (Invitrogen, CA) in such a way that it contained a hexahistidine tag fused upstream of the start codon. Induction with IPTG (Experimental procedures 2.4) produced insoluble AEP3 protein (Aep3p) at all different conditions tested. Therefore, we decided to express Aep3p as a fusion protein with the maltose-binding protein (MBP) using a pMALc2H$_{10}$T vector (Experimental procedures 2.4). Immobilized Metal Affinity Chromatography (McCarthy and Brimacombe, 1994) with His·Bind matrix (Novagen) and Ni^{2+} as the ligand was used to purify the fusion protein (MBP-Aep3p). The purity of the recombinant fusion protein was as > 90% (Figure 3.13.A). The protein migrated at approximately 110kDa which is consistent with the combined size of Aep3p plus MBP (Figure 3.13.A). The purification of a mutant protein was performed by the same procedure as described for the wild-type protein (Figure 3.13.B).

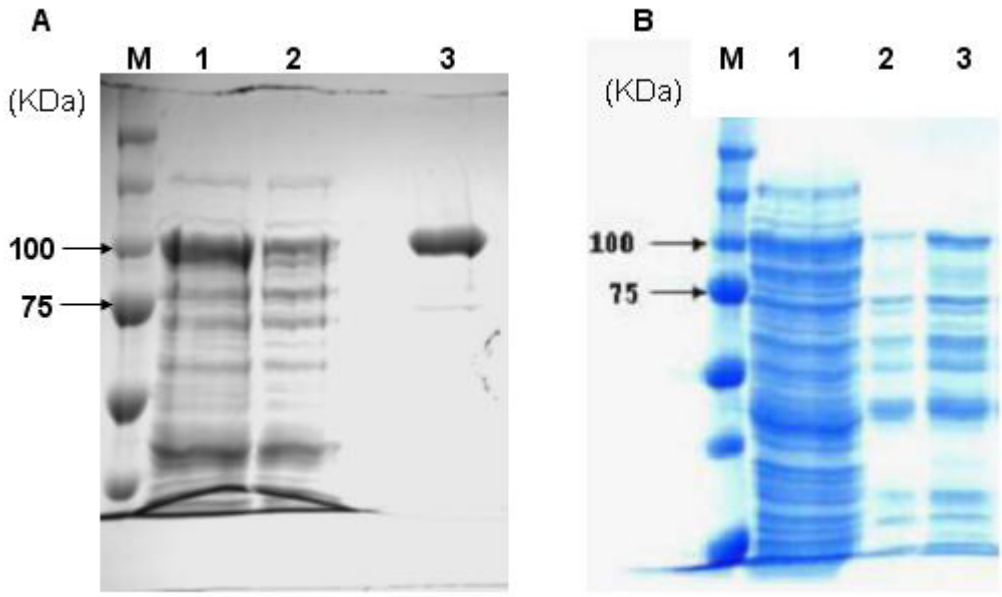

Figure 3.13: Analysis of purified MBP-Aep3p and MBP-Aep3p-Y305N by SDS-PAGE. Panel A. M: protein marker. Lane 1: soluble fraction after sonication. Lane 2: Flow-through fraction. Lane 3: purified, dialyzed MBP-Aep3p fraction (15 µl; 63 pmol). Panel B. Lane 1: soluble fraction after sonication. Lane 2: purified, dialyzed MBP-Aep3p-Y305N fraction (5 pmol). Lane 3: purified, dialyzed MBP-Aep3p-Y305N fraction (40 pmol). The 10% gel was stained with Coomassie blue.

3.4 Binding of MBP-Aep3p to ymIF2 or its C2 subdomain

The genetic interaction between *AEP3* and *FMT1* is observed under conditions where there is no formylation of mitochondrial initiator tRNA. We speculated that Aep3p might interact with factors that are involved in the initiation stage of protein synthesis. To date, the only initiation components that have been identified in yeast mitochondria are ymIF2 and the initiator tRNA. YmIF2 is responsible for binding and delivering the initiator tRNA to the 30S ribosome (Garofalo et al., 2003), and shows a 6 x preference for formylated versus unformylated Met-tRNA^{Met} (Garofalo et al., 2003). Aep3p might interact with ymIF2 to stabilize initiator tRNA binding. Alternatively, Aep3p might interact directly with the initiator tRNA.

To test whether Aep3p interacts with ymIF2, we used a MBP pull-down assay as described in Experimental procedures 2.6. Purified MBP-Aep3p and ymIF2 were incubated for 2 hours at room temperature. Complexes were isolated with amylose affinity beads and analyzed by SDS-PAGE and immunoblotting with antibodies against ymIF2. Figure 3.14 shows that ymIF2 is pulled down with MBP-Aep3p (lane 4), but not with MBP alone (lane 6).

We also tested whether Aep3p binds to the C2 subdomain of ymIF2. The C2 subdomain of ymIF2 is approximately 15kDa in molecular weight, and known as the binding site for initiator tRNA (Figure 3.15). Figure 3.15 shows that the C2 subdomain of ymIF2 binds to MBP-Aep3p (lane 7) about as well as the full-length ymIF2 (lane 4).

These results could be obtained if Aep3p is simply a “sticky” protein, and binds to everything. Therefore, the binding of Aep3p to another protein was tested. The purified yeast cytosolic C_1-THF synthase (Barlowe et al., 1989) was used as a negative control for the pull-down assay. The C_1-THF synthase is approximately 100kDa in molecular weight and contains three functional activities in yeast cytoplasm (Appling and Rabinowitz,

1985). Purified C_1-THF synthase was mixed with MBP-Aep3p, and the immunoblotting was performed using an antibody against C_1-THF synthase. Lane 4 in Figure 3.16 shows that there was no C_1-THF synthase in the pull-down fraction, indicating that Aep3p did not bind to cytosolic C_1-THF synthase.

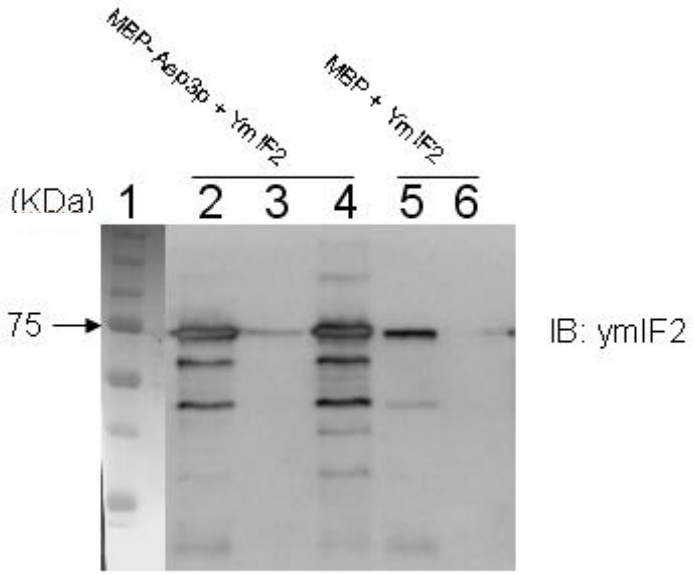

Figure 3.14: Aep3p binds to ymIF2 *in vitro*. Lane 1: protein marker. Lanes 2-4: binding test between Aep3p and YmIF2. Lane 2, unbound fraction. Lane 3, wash fraction. Lane 4: pull-down fraction. Lanes 5-6: binding test between MBP and ymIF2. Lane 5, unbound fraction. Lane 6, pull-down fraction. The immunoblotting was performed using an antibody against ymIF2. For lanes 2-4, 40 pmol of each protein was used, and lanes 5-6, 20 pmol of each used.

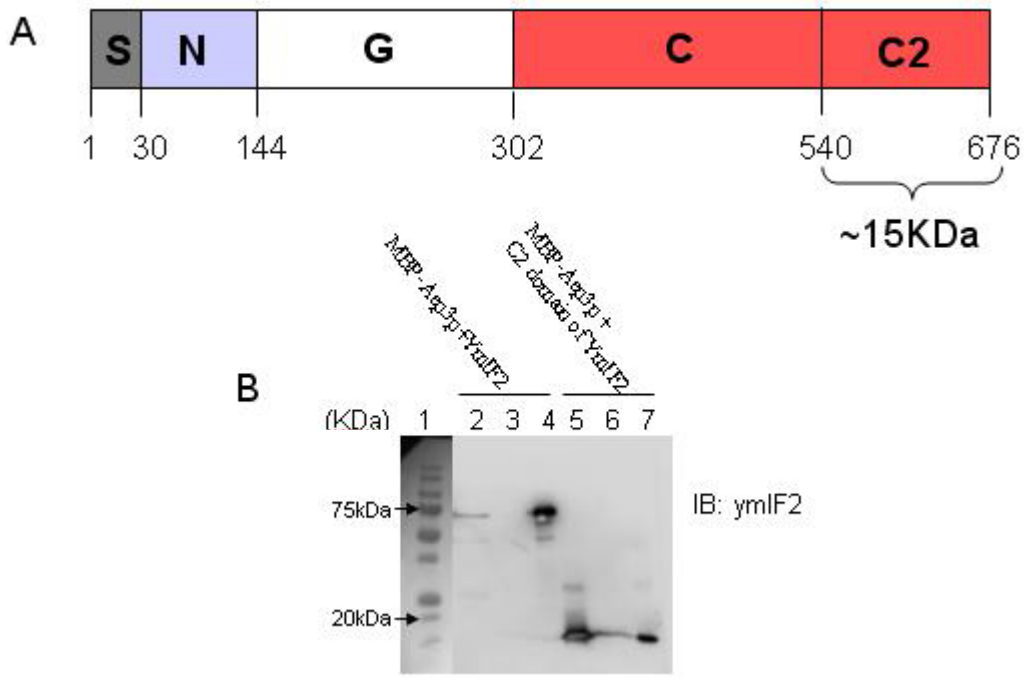

Figure 3.15: Aep3p binds to the C2 subdomain of ymIF2. A. the domain structure of ymIF2 is shown. B. Lane 1: protein marker. Lanes 2-4: binding test between MBP-Aep3p and YmIF2. Lane 2, unbound fraction. Lane 3, wash fraction. Lane 4, pull-down fraction. Lanes 5-7: binding test between C2 subdomain and MBP-Aep3p. Lane 5, unbound fraction. Lane 6, wash fraction. Lane 7, pull-down fraction. The immunoblotting was performed using an antibody against ymIF2. For lanes 2-4, 20 pmol of each protein was used, and lanes 5-7, 20 pmol of each used.

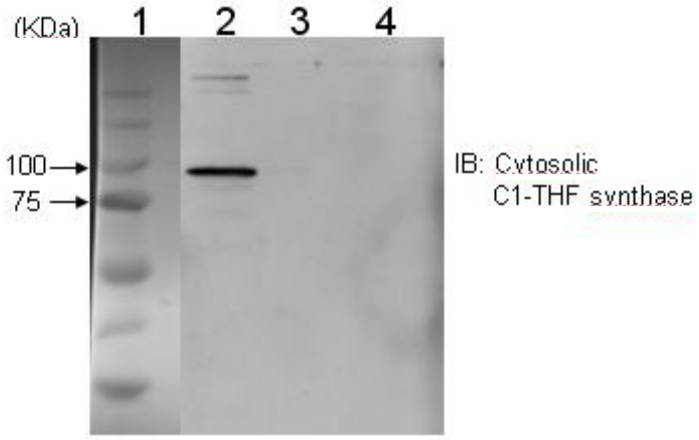

Figure 3.16: Aep3p does not bind to cytosolic C_1-THF synthase. Lane 1: protein marker. Lanes 2-4: binding test between Aep3p and C_1-THF synthase. Lane 2, unbound fraction. Lane 3, wash fraction. Lane 4, pull-down fraction. The immunoblotting was done using an antibody against cytosolic C_1-THF synthase. 20 pmol of each protein was used.

3.5 Binding of MBP-Aep3p to initiator tRNA

To test whether Aep3p binds directly to initiator tRNA, we used the filter-binding assay as described (Experimental procedures 2.5). Various purified proteins were incubated with unformylated [^{35}S]Met-tRNA^{Met} or [^{14}C]Lys-tRNA^{Lys} (negative control). Figure 3.17 shows that Aep3p did not bind to any tRNA tested. YmIF2 binding to Met-tRNA$^{Met}_{f}$ was used as a positive control (brown closed squares).

3.6 The effect of Aep3p on the binding of ymIF2 to Met-tRNA$^{Met}_{f}$

Although Aep3p did not bind directly to initiator tRNA (Figure 3.17), it did interact with ymIF2 *in vitro* (Figure 3.14). The filter-binding assay was used to test whether Aep3p affected the ymIF2-initiator tRNA binding. The gold diamonds show the binding of unformylated Met-tRNA^{Met} by ymIF2 alone. MBP-Aep3p, added in equimolar amount with ymIF2, significantly stimulated the binding of Met-tRNA^{Met} by ymIF2 (red circles) (Figure 3.18). MBP-Aep3p alone (green triangles) or MBP alone (blue inverted triangles) showed only background levels of tRNA binding. The point mutant of Aep3p (MBP-Aep3p-Y305N) was also tested in the filter binding assay. The mutant protein actually decreased the binding of Met-tRNA^{Met} by ymIF2 (data not shown). This needs to be repeated after we solve the proteolytic degradation problem (Figure 3.13).

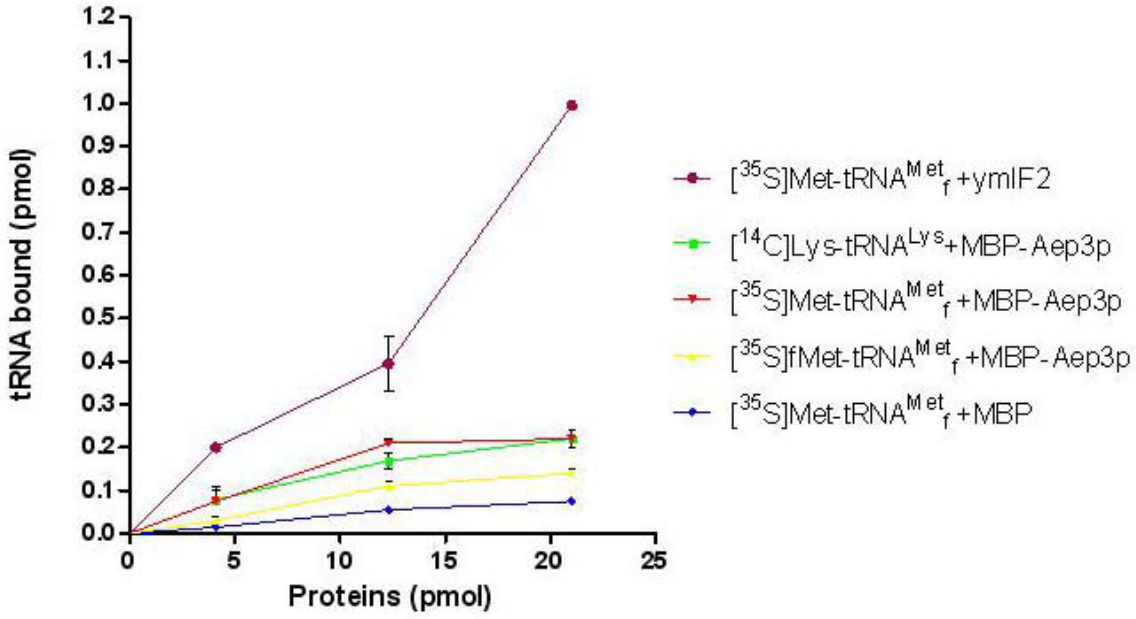

Figure 3.17: Aep3p does not bind to any tRNA. Each reaction is separately shown by color symbols and lines. Each assay was performed in duplicate. Nonspecific filter binding of each tRNA in the absence of proteins was subtracted. Data are expressed as means of two separate determinations ± S.E.

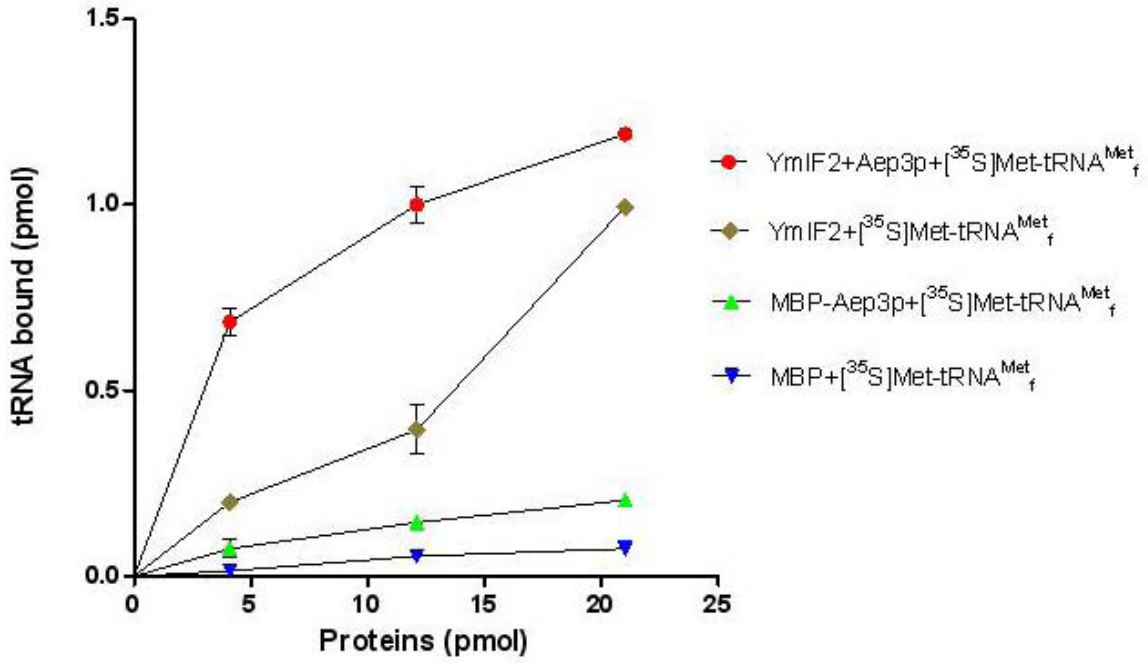

Figure 3.18: Aep3p promotes the binding of ymIF2 to Met-tRNA$^{Met}_{f}$. Each reaction is differently represented by colored symbols. Data are reported as means ± S.E., n = 2.

3.7 The binding assay of ymIF2 to Aep3p *in vivo* (Co-IP assay)

To test whether Aep3p binds ymIF2 *in vivo*, we prepared an HA epitope-tagged version of AEP3 construct in a pRS415 vector (pRS415-AEP3-HA). This construct was transformed into a heterozygous diploid *AEP3* strain (Y22823; Appendix C), sporulated, and dissected to obtain the strain Δ*aep3* containing pRS415-AEP3-HA. We checked that the HA tag did not interfere with *AEP3* function since cells containing pRS415-AEP3-HA were grown on non-fermentable carbon sources (data not shown). Cells with this construct were grown in 50 ml YPEG media at 30^{o}C for 2 days, followed by the Co-IP assay as described in Experimental procedures 2.8. As shown in Figure 3.19, we did not detect an interaction between Aep3p and ymIF2 *in vivo* (lane 4). For the control experiment, the pRS415-AEP3-HA construct was transformed into the strain Δ*ifm1* containing the pVT101U-bmIF2 construct (Appendix D). The Co-IP assay was performed as described above. We confirmed that several bands shown in lane 4 of Figure 3.19 were proteins unspecifically bound to antibodies against ymIF2 (data not shown).

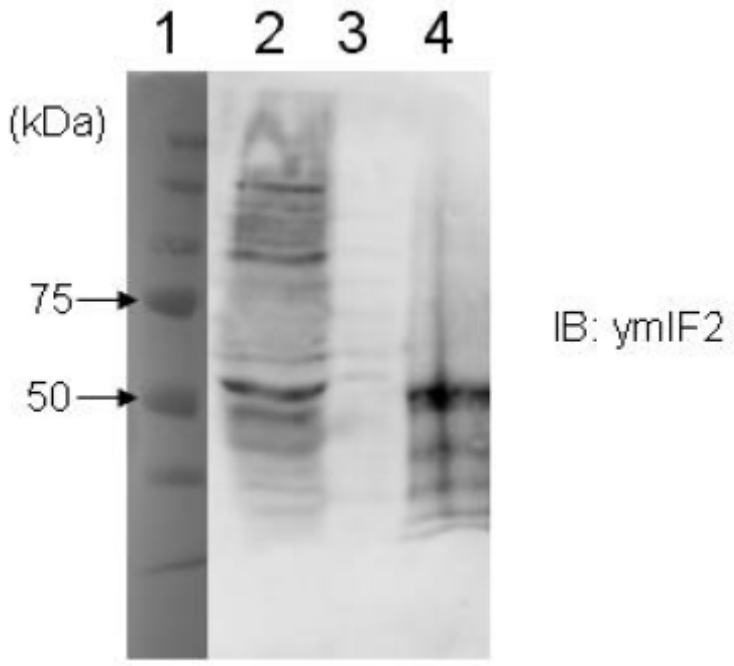

Figure 3.19: The Co-IP assay. Lane 1: protein marker. Lane 2: unbound fractions. Lane 3: wash fractions. Lane 4: co-ip fractions.

3.8 DISCUSSION

Formylation of the initiator Met-tRNA$^{Met}_{f}$ is required for initiation of protein synthesis in bacteria (Sundari et al., 1976). Also, organelles such as chloroplast and mitochondria use formylated initiator Met-tRNA$^{Met}_{f}$ for their protein synthesis (Bianchetti et al., 1971; Dickerman et al., 1967; Epler et al., 1970; Feldman and Mahler, 1974; Galper and Darnell, 1969, 1971; Halbreich and Rabinowitz, 1971; Marcker and Sanger, 1964; Schwartz et al., 1967; Smith and Marcker, 1968). However, previous work in the Appling lab had demonstrated that the formylation of initiator Met-tRNA$^{Met}_{f}$ is not necessary for mitochondrial respiration in *S. cerevisiae* (Li et al., 2000). Moreover, *in vivo* studies showed that protein synthesis in yeast mitochondria can be initiated under conditions where there is no formylation of initiator tRNA (Tibbetts et al., 2003). Therefore, we hypothesized that there might be additional factors that support the initiation process in yeast mitochondria. The fact that the *FMT1* gene is not essential for mitochondrial protein synthesis or respiratory growth led us to design a genetic screening system to search for genes that are synthetically respiratory-deficient (petite) with deletion of the *FMT1* gene. A mutation that causes synthetic petite phenotype in the absence of the *FMT1* gene was found in the gene *AEP3*.

Aep3p is a peripheral mitochondrial inner membrane protein. Aep3p is reported to stabilize the biscistronic transcript of the mitochondrially-encoded *ATP6* and *ATP8* genes. The *ATP6* and *ATP8* genes encode the subunits of the ATP synthase. Ellis et al. (2004) investigated the nature of the *aep3* mutant. Deletion of the *AEP3* gene causes respiratory incompetence (petite phenotype). They also found that the *aep3* mutant reduced levels of cytochrome b and cytochrome a+a3, and lacks oligomycin-sensitive ATPase activity. The decreased ATPase activity inspired them to determine whether it is the result of a loss of one or more of the mitochondrially-encoded subunits of ATP

synthase. *In vivo* labeling assay showed that subunit 9 (Atp9p) was not affected in the mutant strain, but both subunit 6 (Atp6p) and 8 (Atp8p) were almost undetectable. Therefore, they speculated that the lack of inhibition of the ATPase by oligomycin and the respiratory deficiency of the mutant strain resulted from the absence of both subunits. What these data suggested was that Aep3p may function to stabilize the transcript of *ATP6* and *ATP8*. Northern analysis showed that the transcript levels of ATP8/6, which its transcript isolated from the *aep3* mutant strain, were reduced by ~30% compared with that of the wild-type strain. Therefore, they concluded that the principal effect of the aep3 mutation is on the stability of the ATP8/6 mRNAs (Ellis et al., 2004).

However, one thing they ignored to point out was that the expression levels of other mitochondrially-encoded proteins in the *aep3* mutant strain were also reduced (Figure 3 of Ellis et al., 2004). This fact led us to hypothesize that Aep3p also may function in the mitochondrial protein synthesis. To date, no studies have been reported whether the *AEP3* is involved in initiation of protein synthesis in yeast mitochondria. Our genetic screening results suggest that the *AEP3* gene product in the absence of the *FMT1* gene product, the methionyl-tRNA formyltransferase, is involved in initiation process, raising the possibility of AEP3-interaction with components of the protein synthesis in yeast mitochondria.

Initiation factor 1, 2, and 3 (IF1, IF2, and IF3) take part in the assembly of initiation complexes including initiator fMet-tRNA$^{Met}_{f}$ in bacteria (Laursen et al., 2005). In mitochondria, the initiation of protein synthesis should be similar to the bacterial process as is believed so far. To investigate the initiation of protein synthesis in yeast mitochondria in regard to whether or not AEP3 is associated with the initiation process, we focused on yeast mitochondrial IF2 (ymIF2) that is the only factor which has been identified and characterized (Garofalo et al., 2003). Bacterial IF2 and *S. cerevisiae* ymIF2

are apparent homologs. However, there is no obvious homolog between bacterial IF1 or IF3 and yeast mitochondrial initiation factors.

The discovery that Aep3p binds to ymIF2, especially to its C2 subdomain raised the possibility for the association of Aep3p with initiator tRNA. However, Aep3p did not bind to any tRNA tested in this assay including Lys-tRNA^{Lys} as a negative control (Figure 3.17). Although Aep3p does not directly bind the initiator tRNA, the binding between Aep3p and ymIF2 left open the possibility that Aep3p could affect the interaction of ymIF2 with initiator tRNAs. Indeed, Aep3p promoted the binding of ymIF2 to unformylated initiator tRNA (Met-tRNA$^{Met}_{f}$) (Figure 3.18).

Although we have seen the binding of Aep3p to ymIF2 *in vitro*, we could not detect any interaction between them *in vivo* (Figure 3.19). The one possibility to explain why the *in vivo* assay did not work is that the expression level of ymIF2 is very low in yeast cells. The Appling lab unsuccessfully tried to detect ymIF2 in the yeast cells or in mitochondria using the same antibodies. However, when they cloned the wild-type *IFM1* gene into a pVT101U, a high copy yeast expression vector (Appendix D), and transformed it into the Δ*ifm1* strain, they found that the construct complemented the respiratory defect and ymIF2 expressed on the construct was detected by the same antibodies against ymIF2 (unpublished data).

Few other "factors" involved in initiation of protein synthesis in *S. cerevisiae* mitochondria have been characterized. A recent study showed that the *RSM28* gene encoding the mitochondrial small ribosomal protein Rsm28p genetically interacts with the *IFM1* gene encoding ymIF2 and the *FMT1* gene encoding the methionyl-tRNA formyltransferase in *S. cerevisiae* (Williams et al., 2007). Although Rsm28p does not appear to be a homolog of bacterial IF1 or IF3, its interaction with the *IFM1* and the *FMT1* suggests a role in the initiation process in yeast mitochondria. Our own genetic

and biochemical analyses suggest that Aep3p is also associated with the initiation process. AEP3 was discovered under conditions where there is no formylation of initiator tRNA. Therefore, we propose a model in which Aep3p binds to ymIF2 causing a structural change of ymIF2. This change facilitates the binding of unformylated initiator tRNA. *In vitro*, ymIF2 shows a binding preference for fMet-tRNA$^{Met}_{f}$ over Met-tRNA$^{Met}_{f}$ (Garofalo et al., 2005). Because Aep3p did not affect the binding of ymIF2 to fMet-tRNA$^{Met}_{f}$ (preliminary data), Aep3p function may become critical for unformylated Met-tRNA$^{Met}_{f}$ in the mitochondria, perhaps increasing the affinity between ymIF2 and Met-tRNA$^{Met}_{f}$.

Aep3p is not a homolog of bacterial IF1 or IF3 or mammalian IF3. However, the discovery of Aep3p gives more insights into the mechanism by which the initiation process of protein synthesis in yeast mitochondria occurs under conditions where there is no formylated initiator tRNA, and may open more possibilities for higher eukaryotes to follow the same mechanism when cells encounter in special situations.

PART II

Chapter 4: Introduction

Transfer RNA from all species contains various modifications on the four canonical nucleosides: adenosine (A), guanosine (G), uridine (U), and cytidine (C). There are more than 100 different post-transcriptional modifications characterized in tRNAs across archaea, bacteria, and eukaryotes (Rozenski et al., 1999). In particular, the position 37 (3' to the anticodon) contains one of the largest variety of modifications (Bjork et al., 2001), and that modification is often 1-methylguanosine (m^1G) (Figure 4.1). This m^1G37 modification is one of the most ancient tRNA modifications present in all organisms as well as mitochondria.

Many studies have reported on the effect of m^1G37 modification. This modification is important to prevent translational frameshifting (Bjork et al., 1989). The authors generated a temperature-sensitive mutant gene (*trmD3*) for the m^1G modification in the three proline tRNA species of *Salmonella typhimurium*. When grown at high temperatures, all proline tRNAs from the mutant strain were almost devoid of m^1G, and the cells exhibited high levels of frameshifting. Hagervall, et al. (1990) studied the functional aspect of the m^1G37 modification of tRNA in *Salmonella typhimurium*. They observed that the presence of m^1G improved the rate of cellular growth and the absence of m^1G reduced the rate of polypeptide elongation. They also found that m^1G prevented the tRNA from frameshifting (Hagervall et al., 1990).

Other reports also demonstrated that the m^1G37 modification improved the reading frame maintenance. Urbonavicius, et al. (2001 and 2003) tested whether m^1G37 modification affects the frameshifting. They used an assay system to measure +1 frameshifting. For this assay, the β-galactosidase activity was used to measure the

frequency of frameshifting. They found that m^1G37 modification improved the reading frame maintenance by preventing +1 frameshifting errors. In addition, these authors also tested whether m^1G37 influences reading frame maintenance by affecting -1 frameshifting. Interestingly, they found that m^1G37 modification did not influence or only slightly increased the frequency of -1 frameshifting. Therefore, they concluded that while m^1G at position 37 has a profound influence on preventing +1 frameshifting, it has little effect on -1 frameshifting errors (Urbonavicius et al., 2001; Urbonavicius et al., 2003).

Putz, et al. (1994) showed that the m^1G37 modification played an important role in the fidelity of tRNA aminoacylation. They found that m^1G37 prevented the mischarging of yeast $tRNA^{Asp}$ by arginyl-tRNA synthetase (ArgRS). The kinetic analysis indicated that the efficiency of arginylation on $tRNA^{Asp}$ with m^1G37 modification was reduced around 400-fold as compared with unmodified tRNA, indicating that the m^1G37 modification was responsible for the discrimination by ArgRS against $tRNA^{Asp}$, and was the strong anti-determinant preventing efficient $tRNA^{Asp}$ mischarging by yeast ArgRS (Putz et al., 1994).

Li, et al. (1997) also tested whether tRNAs (Leu-$tRNA^{Leu}$, Pro-$tRNA^{Pro}$, and Arg-$tRNA^{Arg}$) isolated from a mutant strain (*trmD3*) of *Salmonella typhimurium* show the deficiency of m^1G37 modification, and this deficiency affects the rate of aminoacyl-tRNA (aa-tRNA) selection. They used an assay called "speedometer" to determine the relative rate of the first step in the translation elongation cycle. If an aminoacyl-tRNA binds successfully to its test codon, no β-galactosidase is synthesized due to the absence of frameshifting. If it unsuccessfully binds to the test codon due to the frameshifting, it activates the β-galactosidase. Based on relative β-galactosidase activity, they determined the intrinsic rate (rate of the aa-tRNA/rate of the frameshift) in a *trmD3* mutant relative to

the wild-type. They found that m^1G37 strongly stimulated the selection rate of the three tRNA species specific for proline, and one tRNA species specific for arginine. Also, they showed that mutant tRNAs slowed aminoacyl-tRNA selection at the ribosome. Therefore, they concluded that the m^1G37 modification affects *in vivo* the aminoacyl-tRNA selection (Li et al., 1997).

4.1 The tRNA (m^1G37) methyltransferase

The enzyme, tRNA (guanine-N1-)-methyltransferase, is responsible for the methylation on position 37 of tRNA. In bacteria, *trmD* encodes this enzyme, and in archaea or eukaryotes, *TRM5* encodes the enzyme (Bjork et al., 2001; Christian et al., 2004; Sindhuphak et al., 1985). The bacterial *trmD* is composed of two domains. The N-terminal domain is known as the binding site for the AdoMet cofactor. The C-terminal domain binds the target tRNA. There is a flexible linker between two domains (Malone et al., 1995; Martin and McMillan, 2002; Schluckebier et al., 1995). When *TrmD* in *E.coli* or *TRM5* in yeast is disrupted, mutant cells showed a severe growth defect, indicating that m^1G modification of tRNA is important (Bjork et al., 2001).

The bacterial *TrmD*s are quite similar, sharing 33% identity and 75% similarity among themselves (Li and Bjork, 1999). Surprisingly, the *E.coli trmD* gene and yeast *S. cerevisiae TRM5* gene are not homologous to each other, sharing only 18% identity and 45% similarity (Bjork et al., 2001). Even the *TRM5* gene, which is responsible for the m^1G modification in archaeal tRNA, appears not to be a homolog of the *E.coli trmD* gene (Hou et al., 2006). Therefore, m^1G modifying enzymes may have evolved twice.

4.2 The purpose of present study

In *S. cerevisiae*, at least eight cytoplasmic tRNAs are methylated at guanosine 37 catalyzed by the TRM5 protein (Trm5p) (Bjork et al., 2001; Hiley et al., 2005). Trm5p catalyzes the transfer of the methyl group of AdoMet to the N1 of guanosine in position 37 in those tRNAs. In eukaryotic cells, the modification of nucleotides in tRNAs occurs in the nucleus, and continues further in the cytoplasm. If those tRNAs contain an intron, an additional maturation step must take place for the removal of the intron before tRNAs are transported to the cytoplasm. The enzymatic formation for the m^1G modification is hindered by an intron (Grosjean et al., 1997). Therefore, this modification takes place only after intron removal (Johnson and Abelson, 1983; Nishikura and De Robertis, 1981). For example, in yeast $tRNA^{Phe}$, the enzymatic formation of m^1G37 was completely hindered by a naturally occurring intron (Jiang et al., 1997). The m^1G37 modification is the first obligatory intermediate of yW37 (nucleoside of the Y-base) synthesis, and tRNA (guanine-N1-)-methyltransferase also participates in the synthesis of yW37. Therefore, all the enzymes required for yW37 synthesis, including the tRNA (guanine-N1-)-methyltransferase, are cytoplasmic (Droogmans and Grosjean, 1987).

In yeast mitochondria, at least eight tRNAs, including the initiator tRNA (Figure 4.2) and $tRNA^{Phe}$ are also methylated at guanosine position 37 (Canaday et al., 1980; Dunin-Horkawicz et al., 2006; Martin et al., 1978). However, the enzyme responsible for the methylation of mitochondrial tRNAs has not been identified (Figure 4.3). It has been proposed that nuclear-encoded Trm5p might be transported into mitochondria and be responsible for the methylation of mitochondrial tRNAs (Bjork et al., 2001; Brule et al., 2004). A potential mitochondrial targeting signal (MTS) exists at the N-terminal end of Trm5p, but only cytoplasmic and nuclear localization of Trm5p has been reported (Huh et al., 2003).

In the present study, genetic and biochemical analyses are used to answer these open questions. First, is Trm5p localized to both the cytoplasm and the mitochondrial matrix? Second, can Trm5p methylate guanosine at position 37 of mitochondrial tRNAs, especially initiator tRNA and $tRNA^{Phe}$? Third, is the product generated by Trm5p m^1G? Also, the mechanism by which the *TRM5* gene encodes a protein for dual localization and how the methylation of mitochondrial tRNAs affects on mitochondrial protein synthesis in *S. cerevisiae* are described.

Portions of this study have been published in the Journal of Biological Chemistry (Lee, C., Kramer, G., Graham, D.E., and Appling, D.R. 2007 *J. Biol. Chem.* **282**, 27744-27753). These sections are identifiable by quotation marks.

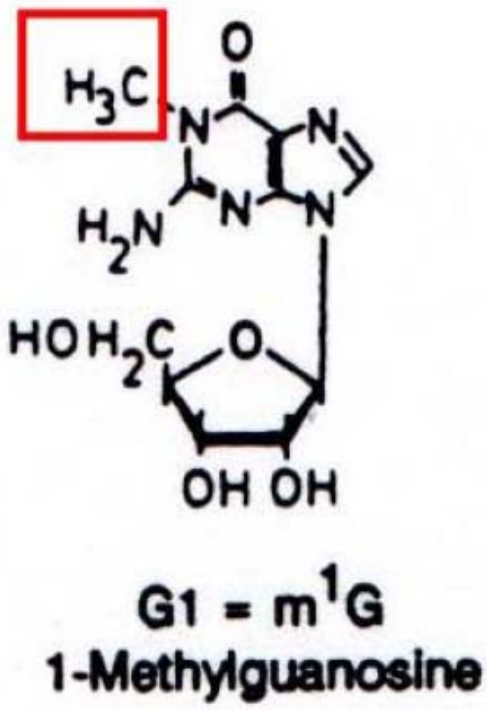

Figure 4.1: The structure of 1-Methylguanosine (m^1G). The methyl group is highlighted by a red open box

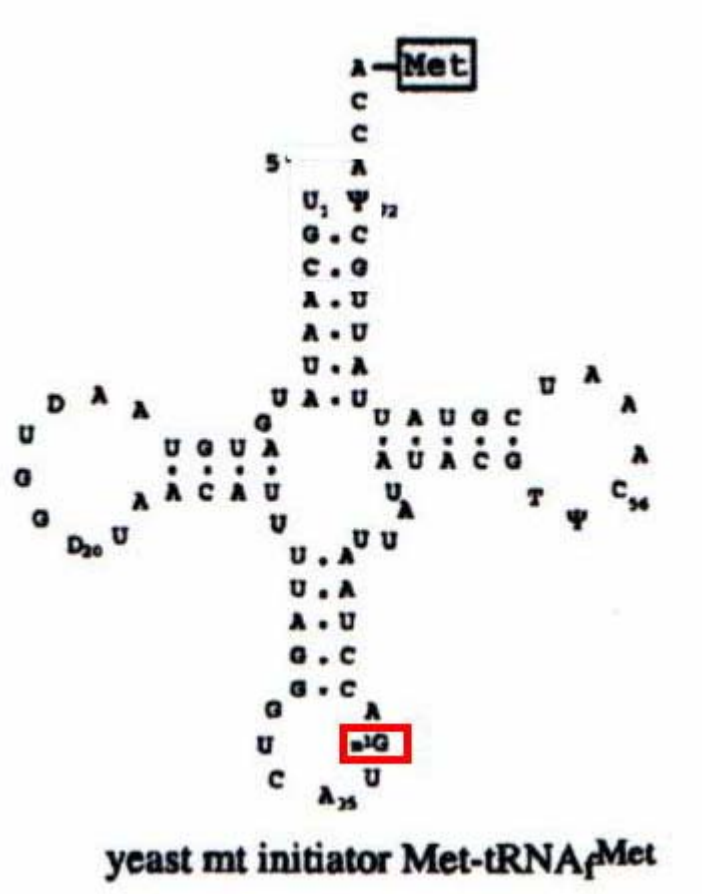

Figure 4.2: Secondary structure of mitochondrial initiator tRNA in *S. cerevisiae*. The m^1G37 modification is highlighted by a red box.

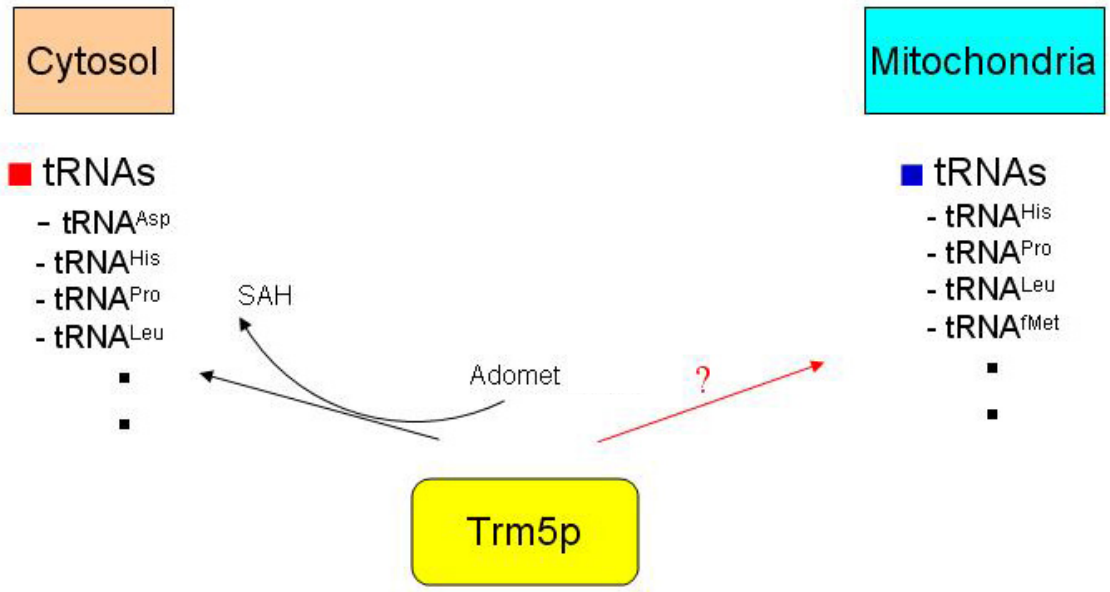

Figure 4.3: Overall view of m^1G modification on tRNAs by Trm5p

Chapter 5: Experimental procedures

The experimental methods used to study the *TRM5* gene in *Saccharomyces cerevisiae* are described in the following sections. Yeast strains and plasmids used for this work are summarized in Appendix C and D.

5.1 Chemicals, reagents, and strains

Chemicals and biochemicals, of the highest grade commercially available, were obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), or VWR (West Chester, PA) and used without further purification. Geneticin (G-418 sulfate) was purchased from American Bioanalytical (Natick, MA). From Ambion (Austin, TX), RPA III ribonuclease protection assay kit, RNA marker templates, and SUPERase-in were obtained. The commercially produced enzymes such as ribonuclease P1, phosphodiesterase I, bacterial alkaline phosphatase, T7 RNA polymerase Plus, Moloney murine leukemia virus reverse transcriptase, T4 DNA ligase, T4 polynucleotide kinase, KOD Hot Start DNA polymerase, and proteinase K were purchased from Sigma, Worthington, Fermentas, Ambion, Ambion, Epicenture, New England Biolabs, Novagen, and Fisher Biotech, respectively. [γ-^{32}P]ATP (PerkinElmer Life Sciences, Waltham, MA) and [methyl-^{3}H]S-adenosyl-L-methionine (PerkinElmer Life Sciences or MP Biomedicals) were purchased. From Sigma, *Saccharomyces cerevisiae* total tRNA (Type X) was obtained. Artificially synthesized oligonucleotide primers were purchased either from Bio-Synthesis (Lewisville, TX) or from Sigma-Genosys or Integrated DNA Technologies (Corallville, IA). Antibodies against GFP were obtained from AnaSpec (San Jose, CA). Dr. John Tesmer (University of Michigan) kindly gave us both the *Escherichia coli* expression vectors pMALc2H10T (Kristelly et al., 2004) and pRK793

(Kapust et al., 2001). DNA sequencing for recombinant plasmids with inserts was done at the DNA Core Facilities of the Institute for Cellular and Molecular Biology at The University of Texas at Austin.

E.coli Rossetta 2 (DE3) (Novagen) and E.coli JM109 (Promega) were grown aerobically in Luria-Bertani medium at 37^{o}C. The *S.cerevisiae* strains used in this work are summarized in Appendix C. Yeast media, YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose), YPEG (1% yeast extract, 2% Bacto-peptone, 2% ethanol, and 3% glycerol) , and YMD (0.7% yeast nitrogen base without amino acids and 2% glucose supplemented with the following nutrients as indicated (final concentration in mg/L): 20-histidine, 30-leucine, 30-lysine, 20-methionine, and 20-uracil) were used to grow yeast cells (Tibbetts et al., 2003).

To isolate yeast genomic DNA, DAY4 cells were used. Haploid Δtrm5 cells (DLY1) were obtained from Y21898 (Appendix C) by sporulation and tetrad dissection (Chan and Appling, 2003). The plasmid constructs used in this study are summarized in Appendix D.

5.2 Preparation of yeast mitochondria

Mitochondria were isolated by spheroplasting and differential centrifugation as described (Daum et al., 1982). Yeast cells were grown aerobically in 1L of semisynthetic galactose media containing 3 g of yeast extract, 10 g of galactose, 0.8 g of $(NH_4)_2SO_4$, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 1 g of KH_2PO_4, 0.4 g of $CaCl_2$, and 5 mg of $FeCl_3 \cdot 6H_2O$. Cells were harvested at mid-log phase and converted to spheroplasts using lyticase (Sigma, St.Louis, MO). Spheroplasts were resuspended in a buffer, and then they were homogenized using a tight-fitting dounce homogenizer with 15 strokes. Cell debris was pelleted by centrifugation at 4,000 x g. After one more centrifugation, the

supernatant was collected and centrifugated at 15,000 x g for 30 min. The mitochondria were resuspended and stored in aliquots at -70^{o}C. The final concentration of the isolated mitochondria was determined by the Bradford protein assay (Bradford, 1976).

5.3 Cloning of yeast *TRM5* for protein expression in *E.coli* and purification of MBP-Trm5p

The yeast genomic DNA was used to isolate the wild-type *TRM5* (YHR070w) ORF by PCR. The primers used: TRM5-F 5'-GGTCTAGA**ATG**AAAATCGCACTGCCAGT-3' (forward; restriction site XbaI is underlined; start codon is in bold) and TRM5-R 5'-GGAAGCT**TCA**TACGTTAGCTGGAAGTTGG-3' (reverse; restriction site HindIII is underlined; stop codon is in bold). The obtained PCR products were digested with the same restriction enzymes, ligated into the same sites of the digested vector pMALc2H$_{10}$T. The plasmid was transformed into *E.coli* JM109 cell. The recombinant construct was introduced by transformation into *E.coli* Rosetta2 (DE3) cells to express the TRM5 protein as a fusion protein (MBP-Trm5p) containing the maltose-binding protein (MBP) and decahistidine tag. Heterologous protein was expressed in these cells after induction with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 29^{o}C for three hours. The cells were harvested, washed and kept at -80^{o}C.

Cells from a 2-liter culture of *E.coli* expressing MBP-Trm5 were used and lysed by sonication in 15 ml of binding buffer containing benzamidine (1 mM) and phenylmethanesulfonyl chloride (PMSF, 1 mM) as protease inhibitors. After centrifugation at 16,000 rpm for 20 min, the supernatant was loaded onto a His-bind matrix which has a 5 ml-bed volume and was equilibrated in 1 X binding buffer containing 5 mM imidazole in 20 mM Tris-HCl buffer, pH 7.8. After washing, loosely

bound protein was eluted with wash buffer containing 60 mM imidazole and 500 mM NaCl in Tris buffer, and tightly bound protein was eluted with 250 mM imidazole in the same Tris/NaCl buffer. The protein fractions eluted by 250 mM imidazole were dialyzed in 500ml of 20mM Tris-HCl, pH 7.5 for four hours. The dialyzed proteins were stored in – 80^{o}C.

5.4 Subcellular fractionation of mitochondria and immunoblotting against GFP

Mitochondria were isolated as described in section 5.2. The commercially available strain EY0986/TRM5-GFP was used for isolation. After differential centrifugation, the post-mitochondrial supernatant was used as the cytoplasmic fraction. Each fraction containing 70 µg of mitochondria were loaded onto a 10% SDS-PAGE gel (Laemmli, 1970), and immunoblotted as described (Prasannan et al., 2003) using anti-GFP as primary antibodies in a 1:500 dilution or anti-Hsp60 antibodies in a 1:50,000 dilution. About 50 µg of mitochondria were resuspended in 100 µl of isotonic buffer containing 0.6 M sorbitol and 20 mM HEPES-KOH, pH 7.4. The 5 µl of proteinase K at 1 mg/ml was added to the mitochondria samples and incubated for 15 min on ice. After 10 min of proteinase K treatment, in some digestions, Triton X-100 having a final concentration of 0.5% was added to the mitochondrial samples to solubilize membranes. Digestions were stopped by addition of 1 µl of 100 mM phenylmethylsulfonyl fluoride in ethanol, and incubated for 10 minutes on ice. After centrifugation at 12,000 x g for 15 min, mitochondria were collected and resuspended in SDS sample buffer. Each sample was loaded onto a 7.5% SDS-PAGE gel, and protein was analyzed by immunoblotting as described above.

5.5 TRM5 Transcript mapping using 5'- RACE experiment

A 5'-RACE (RNA ligase-mediated rapid amplification of cDNA ends) experiment that is designed as a primer extension-based method was used to identify the transcriptional start sites for the *TRM5* gene using the FirstChoice RNA ligase-mediated RACE kit from Ambion. Total RNA was isolated from the yeast strain DAY4 (Appendix C) using the RNeasy Mini Kit (Qiagen). To eliminate DNA contamination of total RNA, a Turbo DNA-free kit from Ambion was used. The four primers were used for this experiment. Two of them were provided by the kit as two nested 5'-RACE primers. The other ones were designed to be specific to the *TRM5* transcript. The TRM5-specific inner primer 5'- ACTAAGCTTCACACTGATATTTTGGGTTCGG-3' (5R_AGA_IP; the restriction site BamHI is underlined) was complementary to nucleotides +183 to +161. The TRM5-specific outer primer 5'- GTACACTTGTCAGTTCGTCC -3' (5R_AGA_OP) was complementary to nucleotides +283 to +264. The 5'-RACE inner primer and the TRM5-specific inner primer had BamHI and HindIII restriction sites, respectively, at their 5' ends to facilitate cloning. PCR fragments created by "inner primers" were cloned into a pBluescript II KS(+/-) vector (Stratagene, La Jolla, CA) (Prasannan et al., 2003) and sequenced at the DNA Core Facilities at The University of Texas at Austin.

5.6 Construction of TRM5 Truncation mutants and their expression in yeast

To construct the wild-type *TRM5* gene in a plasmid, yeast genomic DNA obtained from DAY4 strain was amplified by PCR using KOD Hot Start DNA polymerase and two primers; 5'- AGTGAAGCTTCCCTGTGTTGTTATAG – 3' (TRM5_FOR; the BamHI site is underlined) and 5'- AGCTGGATCCGCCTTTTTGCCTTT - 3' (TRM_REV; the HindIII site is underlined). The resulting PCR fragment was gel-

purified, digested with same enzymes, and ligated into a pRS416 vector and a pRS415 vector to produce the pRS416-TRM5 and pRS415-TRM5 wild-type constructs, respectively. Both pRS416 and pRS415 vectors (Sikorski and Hieter, 1989) (Appendix D) are yeast low-copy vectors containing the *URA3* gene and the *LEU2* gene as markers respectively.

To generate the construct with codons 1-19 of the *TRM5* coding sequence deleted, sequence and ligation-independent cloning (SLIC) was used (Li and Elledge, 2007). The four different primers were used for this methods: T7 forward primer 5'-AATACGACTCACTATAGGG-3' versus T5_1/20_2 reverse primer 5'-CACTGGTGA**CAT**GCAATGAAAAATATCCTT-3' (stop codon is in bold) and T5_1/20_3 forward primer 5'-AGGATATTTTCATTGC**ATG**TCAGGAGTGTTCCCA-3' (start codon is in bold) versus T3 reverse primer 5'- AATTAACCCTCACTAAAGGG -3'. Two PCR fragments obtained from each PCR reaction, and the pRS415 vectors which were linerized with both BamHI and HindIII enzymes were gel-purified and treated with T4 DNA polymerase to generate 5' overhang at each end. The two PCR products and the linearized vector were annealed at a 2:2:1 molar ratio with 20 ng of RecA protein at room temperature for 30 min, and the reconstructed plasmid (pRS415-Δ1-19TRM5; Appendix D) was obtained from transformed *E.coli* XL1-Blue cells.

The Splice Overlap Extension (SOE) PCR method (Horton et al., 1993) was introduced for generating mutant constructs with codons 1-33 deleted (pRS415-Δ1-33TRM5; Appendix D) and 1-47 deleted (pRS415-Δ1-47TRM5; Appendix D). For the pRS415-Δ1-33TRM5 construct, the *TRM5* gene containing 300 bp of 5'-UTR in the pRS416-TRM5 plasmid was amplified with the T7 forward primer (upstream vector primer) and T5_1/34_2 5'-TTCTCG**CAT**GCAATGAAAAATATCCTT-3' (stop codon

is in bold). The DNA in the region between codon 34 and the stop codon of TRM5 was amplified by PCR using primers T5_1/34_3 5'-TTTCATTGC**ATG**CGAGAATTGGATAGG-3' (start codon is in bold) and T3 reverse primer 5'-AATTAACCCTCACTAAAGGG-3' (downstream vector primer). Primers T5_1/34_2 and T5_1/34_3 contain complementary nucleotides of 18 base pairs at their 5'ends for the second SOE PCR. Two separate reactions obtained from PCR were purified by a PCR purification kit (Qiagen, Germany) and mixed together for the second PCR using the T7 forward primer and the T3 reverse primer. The resulting PCR product was gel-purified, digested with HindIII and BamHI, and cloned into a pRS415 vector (Sikorski and Hieter, 1989). The pRS415-Δ1-47TRM5 construct was similarly generated using the T7 forward primer and T5_1/48_2 5'-TGCACA**CAT**GCAATGAAAAATATCCTT-3' (stop codon is in bold). T5_1/48_3 5'-TTTCATTGC**ATG**TGTGCAGTCAAGTTC-3' (start codon is in bold) and T3 reverse primer were used to generate a product for the first PCR. T5_1/48_2 and T5_1/48_3 contain 18 base pairs of complementarity at their 5' ends for the second SOE PCR. The procedure described above was used for the second PCR, and then cloned into the pRS415 vector. All truncation mutant constructs were DNA-sequenced at the DNA Core Facilities at The University of Texas at Austin, and they were transformed into the DLY1 strain containing pRS416-TRM5 for subsequent analysis.

5.7 Oxygen consumption assay

To measure the oxygen consumption rates of cells containing either pRS415-TRM5, pRS416-TRM5, pRS415-Δ1-19TRM5, or pRS415-Δ1-33TRM5, an oxygen consumption assay was used. A standard Clark-type electrode (model 5300A; YSI Inc.) was inserted into the airtight chambers containing the cells and oxygen consumption was measured at 30^{o}C for at least 10min. Rates (nmol oxygen min^{-1}) were normalized to the OD_{600} of each culture.

Chapter 6: Results and Discussion

6.1 Expression of Trm5p as a fusion protein

Several attempts to express *Saccharomyces cerevisiae* Trm5p in different *E.coli* expression systems fused to N- or C-terminal polyhistidine tags produced mostly an insoluble form of the protein. Therefore, the full-length *TRM5* ORF containing codons for 499 amino acids was cloned into the vector pMALc2H_{10}T to express the N-terminal maltose-binding protein, a decahistidine tag, and the tobacco etch virus (TEV) protease cleavage site just upstream of the coding sequence for Trm5p (MBP-Trm5; Figure 6.1). *E.coli* cells expressed this fusion protein at high levels in soluble form. Nickel affinity chromatography was used to purify this fusion protein, and almost pure fusion protein (>95% in purity) was obtained. The mass of this fusion protein when analyzed by SDS-PAGE was around 97kDa which is very similar to the calculated mass of 101.4kDa.

6.2 Methyltransferase activity of the MBP-Trm5 fusion protein and its cleavage product (performed by Dr. Gisela Kramer)

To separate Trm5p from MBP, the tobacco etch virus protease site was cleaved by its protease. The Trm5p fragment migrated on an SDS-PAGE gel with an apparent mass of 61 kDa, very close to its predicted mass of 57.2 kDa. The MBP fragment showed a band on the gel around 42 kDa which is similar to its predicted mass of 44.2 kDa. The fusion protein showed substantial methyltransferase activity in assay when Δ*trm5* tRNA was used as substrate (Table 6.1).

Figure 6.1: The Map of MBP-Trm5p.

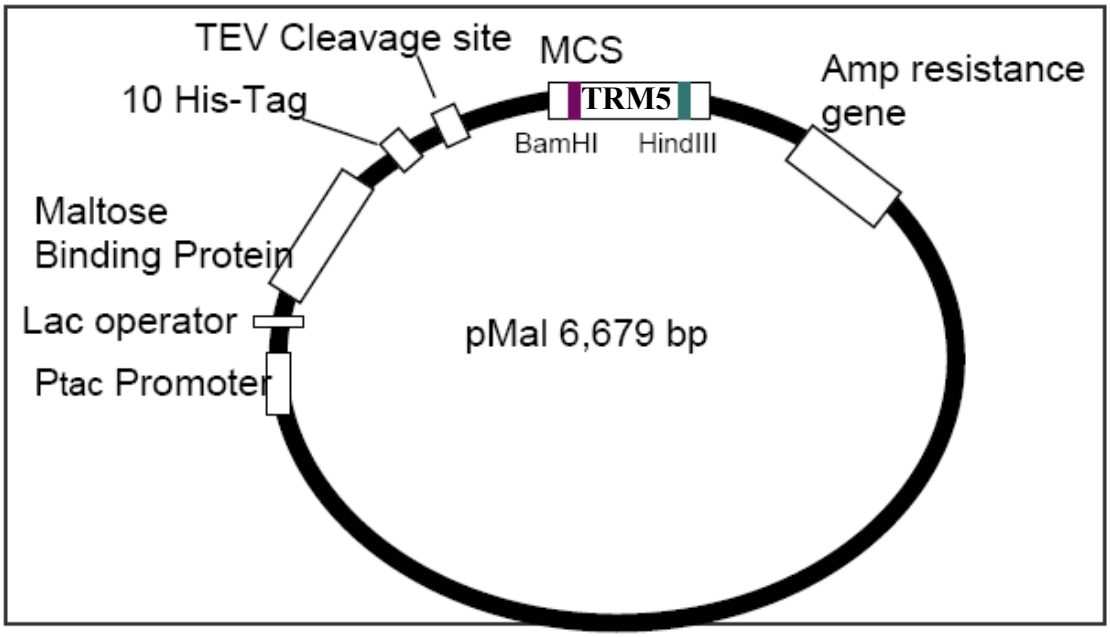

Table 6.1: Test of Enzymatic activity for MBP-Trm5

Loss of enzymatic activity upon incubation of MBP-Trm5[a]

Pre-incubation	MBP-Trm5	Methyl incorporated
	μg	*pmol*
No pre-incubation	0.3	195.3
No pre-incubation	0.5	235.7
No pre-incubation	1.0	237.5
Buffer	0.64	36.8
Buffer	1.28	65.8
Buffer + TEV protease	0.64	47.0
Buffer + TEV protease	1.28	73.5

[a] MBP-Trm5 was pre-incubated with TEV protease (0.5 μg) in 10 μl of 20 mM Tris-HCl, pH 7.8, or with buffer alone for 30 min at 30°C. Aliquots were then withdrawn to determine methyltransferase activity in the presence of [^{3}H]AdoMet and 628 pmol Δtrm5 tRNA. Methyl incorporation (*pmol*) was determined in a 30 min assay as described in "Experimental Procedures".

This table has been published as supplementary table 2 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

However, when MBP-Trm5p was preincubated with buffer alone or tobacco etch virus protease, methyltransferase activity was considerably reduced. This could be due to inherent instability of the enzyme. Therefore, all subsequent experiments described here were carried out with the uncleaved fusion protein (MBP-Trm5p). Maltose-binding protein (MBP) was purified from empty pMALc2H$_{10}$T vector in order to use it as a control for methyltransferase assay experiment. The MBP did not show any activity when incubated with Δ*trm5* tRNA and AdoMet (data not shown).

When a saturating amount of MBP-Trm5p and 50μM [^{3}H]AdoMet were used in the assay, a linear relationship was observed between the amount of Δtrm5 tRNA substrate and the incorporation of methyl groups (Figure 6.2A). A specific enzymatic activity of 30 ± 1.3 nmol of methyl incorporated per min per mg MBP-Trm5p was calculated when a small amount of enzyme and saturating amount of substrates were used at initial rate conditions. The fusion protein did not catalyze the methylation of wild-type yeast total tRNA probably due to full methylation of tRNA.

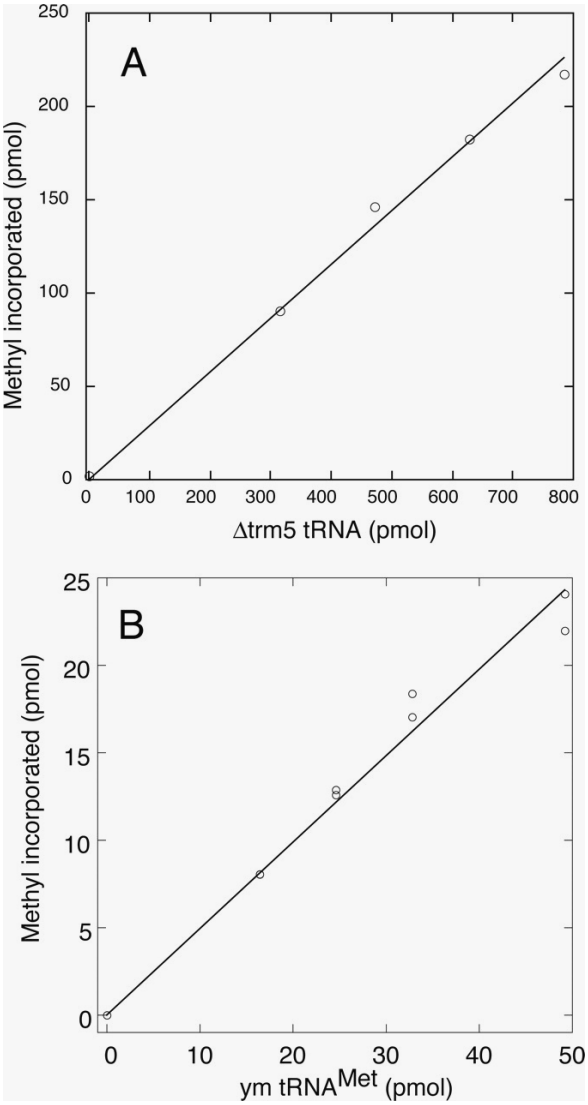

Figure 6.2: MBP-Trm5 methyltransferase activity. *A*, Δtrm5 tRNA as substrate. The methyltransferase assay was carried out as described under "Experimental Procedures" with 0.8 μg of MBP-Trm5 enzyme, 50 μM [methyl-3H]AdoMet, and Δtrm5 tRNA. *B*, synthetic $tRNA^{Met}_f$ as substrate. Synthetic $tRNA^{Met}_f$ was heated at 70 °C for 5 min, and then cooled to room temperature immediately before the reaction mixtures were assembled. The reactions contained 0.8 μg of MBP-Trm5 enzyme and the indicated amount of the $tRNA^{Met}_f$ in the standard assay. The results from duplicate reactions are shown fit to a linear model (R^2 = 0.98). This figure has been published as figure 1 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

6.3 Identification of the *TRM5* tRNA methylation site and product (performed by Dr. Gisela Kramer and Dr. David E. Graham)

To demonstrate that the MBP-Trm5 protein catalyzed the methylation of guanosine position 37 of tRNAs, on N1 position of the guanine base, primer extension assay was used (Jackman et al., 2003). The methylation of N1 position of guanine base interferes with Watson-Crick base pairing. Therefore, the reverse transcriptase cannot incorporate a complementary cytidylate, resulting in blocking further primer extension. Two different [^{32}P] labeled oligonucleotide primers that are specific for yeast mitochondrial tRNAs such as the initiator tRNA and mitochondrial Phe-tRNA^{Phe} were used. Phe-tRNA^{Phe} also in its native form has an m^{1}G37 modification following A36 (Martin et al., 1978). Total tRNAs obtained from cells expressing wild-type or Δ*trm5* were annealed to primers, and then the reverse transcriptase reaction was carried out. The primer extended samples were analyzed by denaturing gel electrophoresis (Figure 6.3). The reverse transcriptase extended the primers up to m^{1}G37 in the wild-type tRNAs, terminating the reaction at position 38 which is opposite the A (Figure 6.3; Lanes 1 and 4). In contrast, reactions containing undermodified Δ*trm5* tRNA showed that there is the presence of the full-length transcripts (Figure 6.3; Lanes 3 and 6). Lanes 2 and 5 showed that a significant amount of the transcripts were observed on the gel where the termination position is exactly same with one of the wild-type tRNA. Therefore, this experiment confirmed that the methylation occurred on position 37 of yeast mitochondrial tRNAs (Hou et al., 2006).

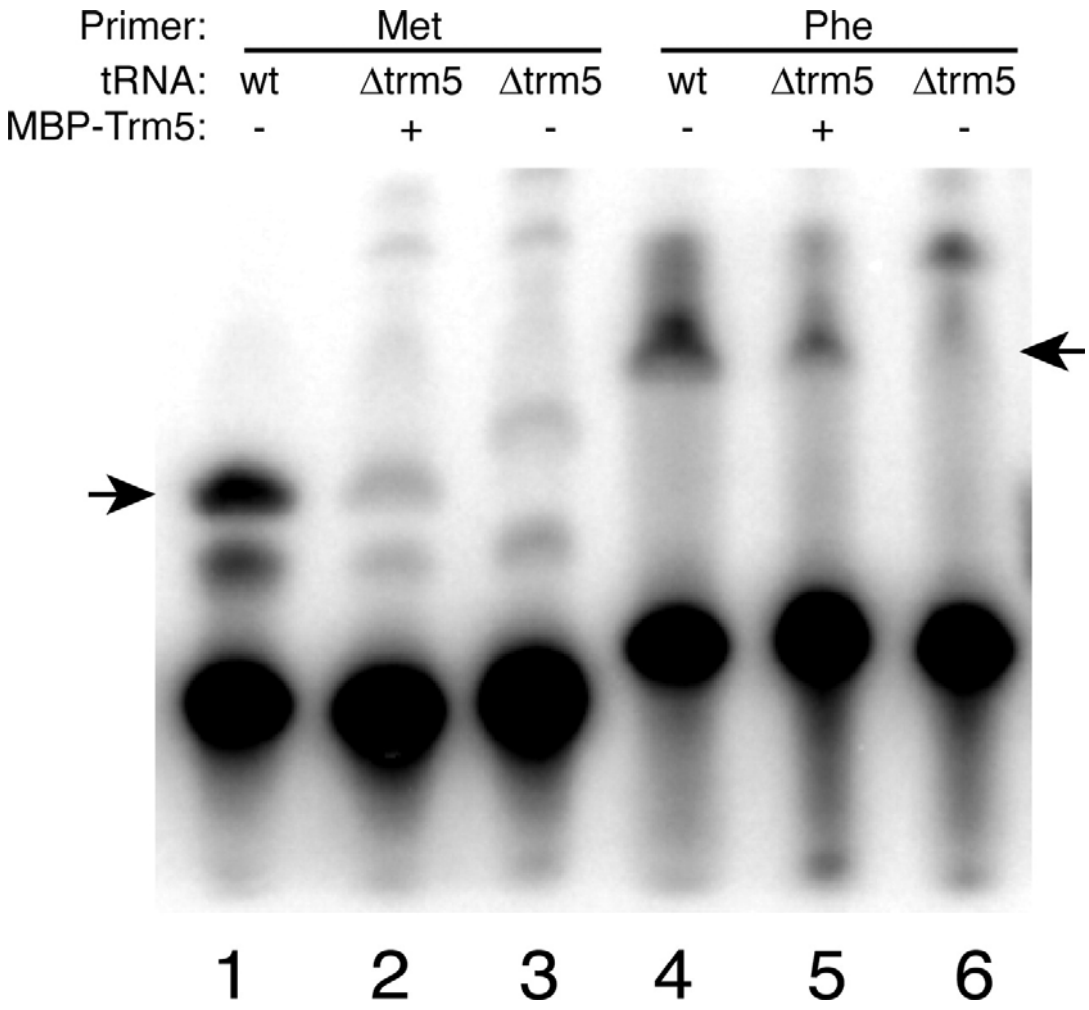

Figure 6.3: Primer extension demonstrates methylation of yeast mitochondrial tRNA. Total tRNA (cytoplasmic + mitochondrial) from wild-type (Newton et al.) or Δ*trm5* cells was incubated in the methyltransferase assay with or without MBP-Trm5 as indicated. ^{32}P-Labeled primers specific for yeast mitochondrial initiator $tRNA^{Met}_{f}$ (*Met, lanes 1–3*) or mitochondrial $tRNA^{Phe}$ (*Phe, lanes 4–6*) were then annealed and primer extension reactions performed. The *arrows* indicate the primer extension termination products caused by the presence of m^1G for $tRNA^{Met}$ (*left*) or $tRNA^{Phe}$ (*right*). This figure has been published as figure 2 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

To confirm whether the termination product obtained by the reverse transcriptase after methylation with MBP-Trm5 protein is A38 where it is just ahead of G37 site, the primer extension experiment was applied to perform the dideoxyribonucleotide triphosphate DNA sequencing. The mitochondrial initiator tRNA primer was annealed to Δ*trm5* tRNA, and then the reverse transcriptase reaction was carried out in the presence or absence of the four different ddNTPs. The termination product observed in wild-type yeast tRNA corresponds to dideoxy T incorporation which is complementary to A38 (Figure 6.4). Therefore, this experiment confirmed that the methylation of mitochondrial initiator tRNA occurred at guanosine position 37.

The product created by Trm5 is m^1G confirmed by chromatographic analysis of mitochondrial tRNAs. The synthetic $tRNA^{Met}_f$ which was made by in vitro transcription was incubated with MBP-Trm5 and [^{3}H]AdoMet. The radiolabeled product was hydrolyzed by nuclease P1, phosphodiesterase I, and alkaline phosphatase. The reverse phase HPLC was used to analyze products. A UV-absorbing compound in the hydrolysate co-elutes with a synthetic m^1G standard (Figure 6.5). Fractions of eluate were monitored by liquid scintillation counting, and m^1G peak in UV absorption profile corresponds to the maximum peak of radioactivity, indicating that the product of Trm5 methylation is m^1G.

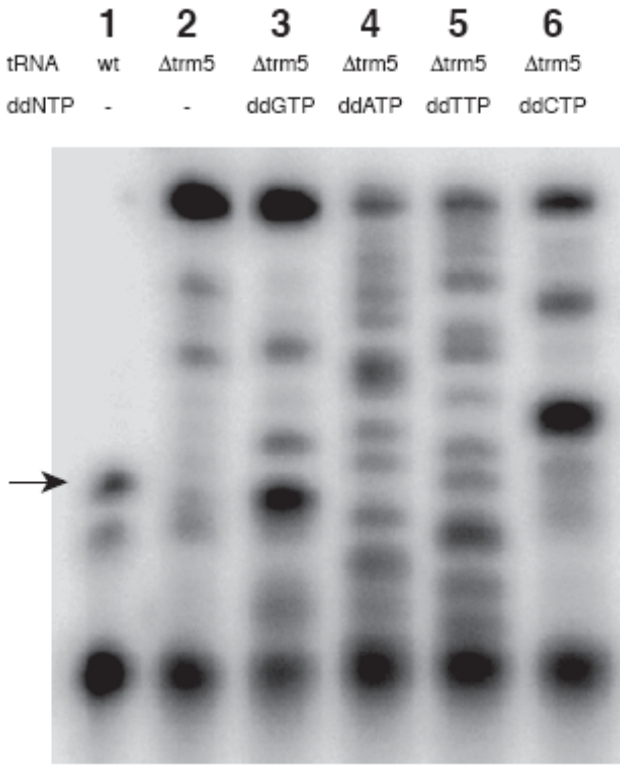

Figure 6.4: Mitochondrial $tRNA^{Met}_f$ is methylated at G37 in wild-type tRNA. The 32P-labeled primer specific for $tRNA^{Met}_f$ was annealed to wild-type yeast tRNA (Newton et al.) or to Δtrm5 tRNA as indicated. The latter sample was then divided into 5 aliquots, each containing dNTP and MMLV-RT. To samples 3-6, dideoxynucleoside triphosphates were added. After the reverse transcriptase reaction, the samples were electrophoresed on a 10% polyacrylamide-urea gel and analyzed by phosphorimaging. *Lane 1*, wild-type S. cerevisiae tRNA; *lanes 2-6*, Δtrm5 tRNA with ddNTPs as indicated. The band corresponding to ddT incorporation complementary to A38 is indicated as arrow. This figure has been published as supplementary figure 3 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

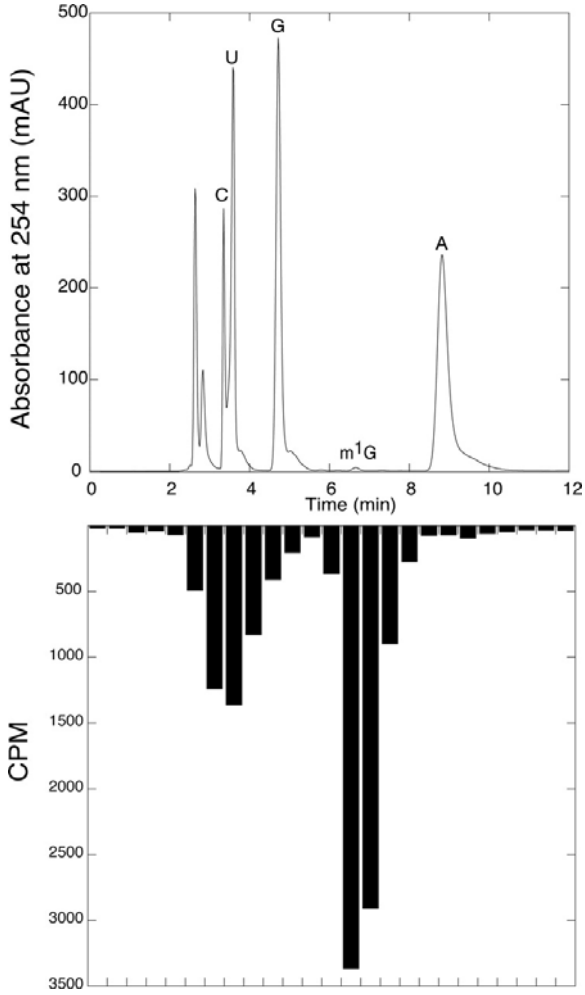

Figure 6.5: MBP-Trm5 catalyzes guanosine methylation in synthetic $tRNA^{Met}$ to produce 1-methylguanosine (m^1G). A synthetic $tRNA^{Met}$ transcript (720 pmol) was incubated with MBP-Trm5 and [^{3}H-methyl]AdoMet at 37 °C for 40 min. The ethanol-precipitated product was enzymatically hydrolyzed, and the nucleoside components were separated by reversed phase HPLC. The UV absorbance profile (*top panel*; in milliabsorbance units) shows an m^1G peak that corresponds to the maximum peak of radioactivity measured by liquid scintillation counting (*bottom panel*; in cpm). This figure has been published as figure 3 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

6.4 Mitochondrial localization of Trm5p

At least based on the *in vitro* results, it was determined that the Trm5p methyltransferase is able to methylate the N1 of guanosine at position 37 in mitochondrial tRNAs such as $tRNA^{Met}_{f}$ and $tRNA^{Phe}$. If Trm5p is responsible for the methylation of these mitochondrial tRNAs, the enzyme must also be present in mitochondria. To test this possibility, the commercially available *S.cerevisiae* strain EY0986/TRM5-GFP was used. This strain contains a GFP tag at the C-terminus of *TRM5* gene which is under the control of the endogenous *TRM5* promoter. The cells were grown in a semisynthetic galactose medium and fractionated by differential centrifugation into cytoplasmic and mitochondrial fractions. Both were analyzed by immunoblotting with antibodies against GFP (Figure 6.6). It showed that Trm5-GFP is localized to both the cytoplasm and mitochondria (Figure 6.6A). It also showed that the cytoplasmic protein migrated slightly faster than the mitochondrial protein. Antibodies against Hsp60, a mitochondrial matrix marker, were used as a control eliminating the possible contamination between the two fractions. Proteinase K treatment showed that the mitochondrial TRM5-GFP was protected from the enzymatic digestion as Hsp60 was. When Triton X-100 was introduced to the mitochondria to solubilize all membranes, TRM5-GFP showed similar localization with a matrix marker, Hsp60. These data indicate that the single *TRM5* gene encodes a protein which is localized to both the cytoplasm and the mitochondrial matrix.

6.5 Mapping of *TRM5* Transcript

To determine where the transcriptional start site of the *TRM5* gene is, RNA ligase mediated RACE experiment (Experiment produces 5.5) was used to explore the 5'end of the *TRM5* transcript. Ten μg of yeast total RNA was used, and the experiment was performed by reverse transcriptase. The first round of PCR by using "outer" primers showed no detectable specific product. Two μl of the outer PCR product were used for the second round of PCR with "inner" primers (nested primers), producing a specific product of around 400 bp. This performance was done several times to yield more PCR products. The final PCR products were gel-purified and subcloned into a pBluescript II KS(+/-) vector. Ten clones were screened by PCR and sequenced. Three of the ten clones exhibited the same 5'-end 24 bp upstream (-24) of the first AUG start codon (Figure 6.7). Two of them showed the same 5'-end 18 bp (-18) upstream of the start codon. Likewise, two of them exhibited at 12 bp (+12) downstream of the AUG codon, and three of them at 21 bp downstream of the AUG codon (+21). Four potential transcriptional start sites of the *TRM5* gene were revealed at -24, -18, +12, and +21 (Figure 6.7).

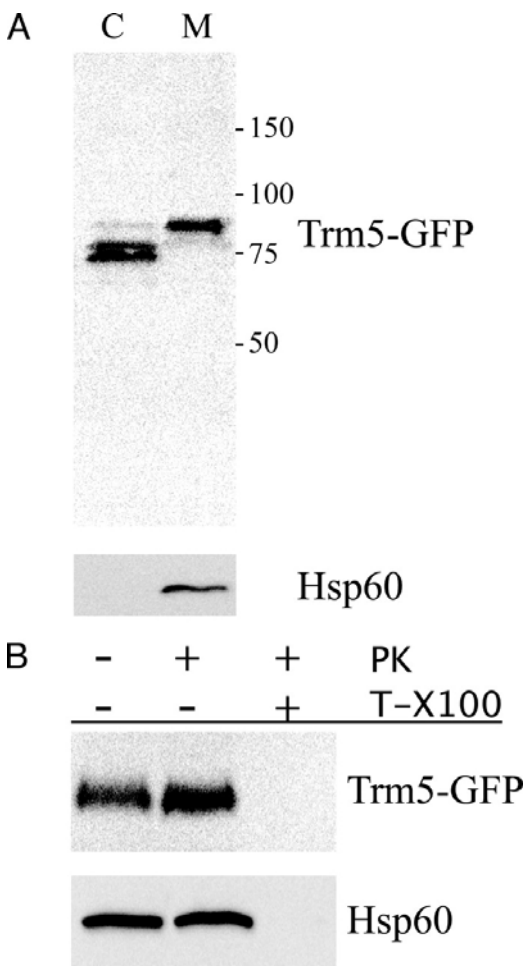

Figure 6.6: Trm5p shows dual localization to cytoplasm and mitochondria. *A*, cytoplasmic (*C*) and mitochondrial (*M*) fractions prepared from cells expressing Trm5-GFP were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted with antibodies against GFP. Each lane contains 75 μg of protein. Locations and size (kDa) of molecular mass markers are shown on the *right*. Full-length Trm5p (499 amino acids) fused to GFP (238 amino acids) would have a molecular mass of ~83.3 kDa. A duplicate gel was immunoblotted with antibodies against mitochondrial marker Hsp60 as a control. *B*, mitochondria (50 μg protein) were incubated with or without proteinase K (*PK*, 1 mg ml^{-1}) in the presence or absence of 0.5% Triton X-100 and immunoblotted with antibodies against GFP. A duplicate gel was immunoblotted with antibodies against mitochondrial matrix marker Hsp60 as a control. This figure has been published as figure 4 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

Figure 6.7: 5' -UTR and potential transcription and translation start sites of the yeast *TRM5* gene. Nucleotides are numbered with the A of the first ATG codon as 1. Codons are numbered with the first potential ATG start codon as 1. In-frame ATG codons (codons 1, 20, 34, and 48) are indicated in *bold type*. The *arrows* beginning at nucleotides –24, –18, +12, and +21 indicate transcriptional start sites based on 5'-RACE (this work). The *diamond* at –94 indicates the 5' end of the *TRM5* transcript mapped using a high density oligonucleotide array (David et al., 2006). This figure has been published as figure 5 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

6.6 Identification of translation start sites of *TRM5 in vivo*

"There are several mechanisms known that can generate dual localization of a protein encoded by a single gene (Karniely and Pines, 2005; Martin and Hopper, 1994). In most cases, alternative translational start sites result in alternative N-terminal sequences of the proteins, which in turn targets the proteins to different compartments (*e.g.* cytoplasm *versus* mitochondria). Inspection of the 5' end of the proposed *TRM5* ORF reveals four in-frame AUG codons (positions 1, 20, 34, and 48) within the first 48 codons (Figure 6.7). To try to identify the translational start site(s) used *in vivo*, we constructed three N-terminal truncation mutants of Trm5p, in which amino acids 1–19, 1–33, or 1–47 were deleted (Figure 6.8). The 5'-UTR up to position –1 adjacent to the first AUG codon remained intact in each construct. Each construct was expressed from the single-copy vector pRS415 (*LEU2* vector), driven by the *TRM5* promoter. These pRS415 constructs were transformed into the Δ*trm5* haploid mutant strain (DLY1) harboring a wild-type copy of *TRM5* on pRS416 (*URA3* vector). Leu+ Ura+ transformants were then streaked onto yeast minimal glucose (YMD) or ethanol+glycerol (YPEG) plates containing 5-fluoroorotic acid to evict the wild-type *TRM5* gene on the pRS416 plasmid. The Δ1–19 and Δ1–33 constructs support growth on glucose as well as the full-length *TRM5* construct, whereas cells expressing the Δ1–47 construct failed to grow (Figure 6.9). Likewise, all the constructs support growth on the nonfermentable ethanol + glycerol plate except the Δ1–47 construct (Figure 6.9). Thus, the first 33 amino acids of the proposed *TRM5* ORF are not necessary for the expression of a functional Trm5p, because *trm5* null mutants exhibit a severe growth defect on glucose (Bjork et al., 2001). The simplest interpretation of these data is that translation initiates at the third AUG codon (codon 34) to produce a Trm5p that functions in the cytoplasm to methylate cytoplasmic tRNAs".

"To determine the translation start site of the mitochondrial form of Trm5p, we examined the methylation status of mitochondrial tRNAs in DLY1 cells expressing the Δ1–19 and Δ1–33 truncation constructs. The primer extension product indicative of methylation at position 37 was undetectable in mitochondrial $tRNA^{Met}_f$ and $tRNA^{Phe}$ isolated from the two truncation mutants (Figure 6.10). Overexposure of the gel revealed a faint signal in the $tRNA^{Met}_f$ isolated from the Δ1–19 mutant (*lane 3*), but this was not reproducable in subsequent experiments. These data suggest that initiation at either the first or second AUG codon can produce a functional mitochondrial form of Trm5p, although use of the second AUG (codon 20) is much less efficient".

6.7 Oxygen consumption of Trm5p truncation mutants

"Although neither the Δ1–19 nor Δ1–33 truncation mutants showed a growth defect on YPEG plates, the lack of detectable methylation prompted us to take a closer look at mitochondrial function in these two mutants. Oxygen consumption rates were measured on DLY1 cells expressing either the full-length or the Δ1–19 or the Δ1–33 truncated TRM5p. As shown in Figure 6.11, cells expressing the Δ1–33 construct exhibited a significantly lower rate of oxygen consumption compared with cells expressing full-length *TRM5*. Cells expressing the Δ1–19 construct exhibited a smaller, nonsignificant decrease in oxygen consumption rate".

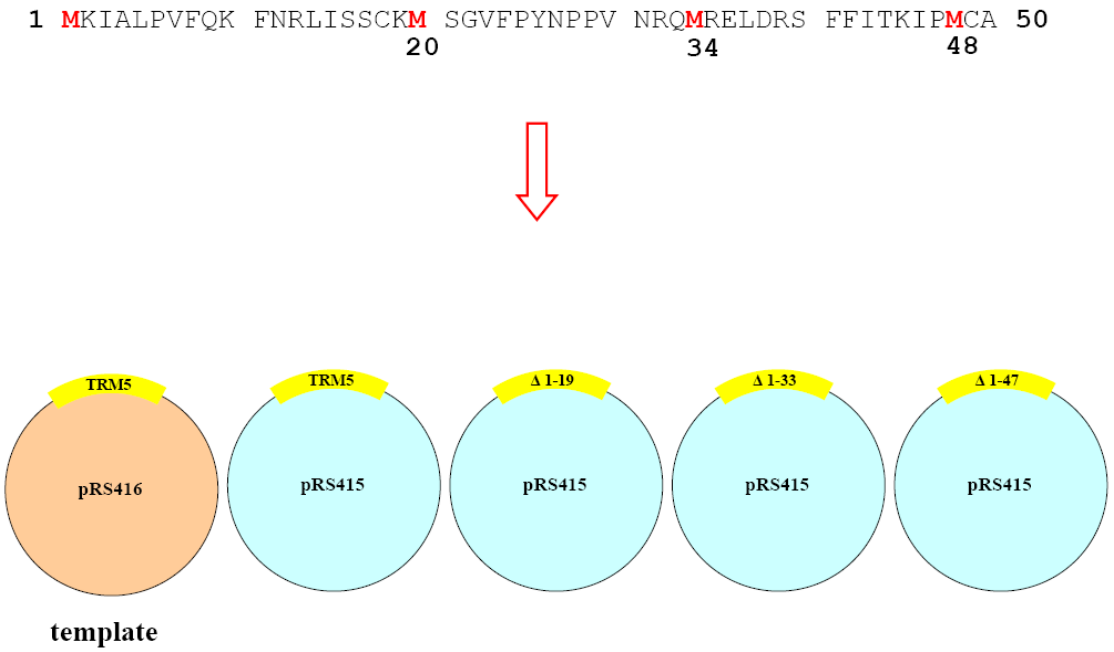

Figure 6.8: Three N-terminus truncation mutants of TRM5. The four potential translational start sites are colored as red.

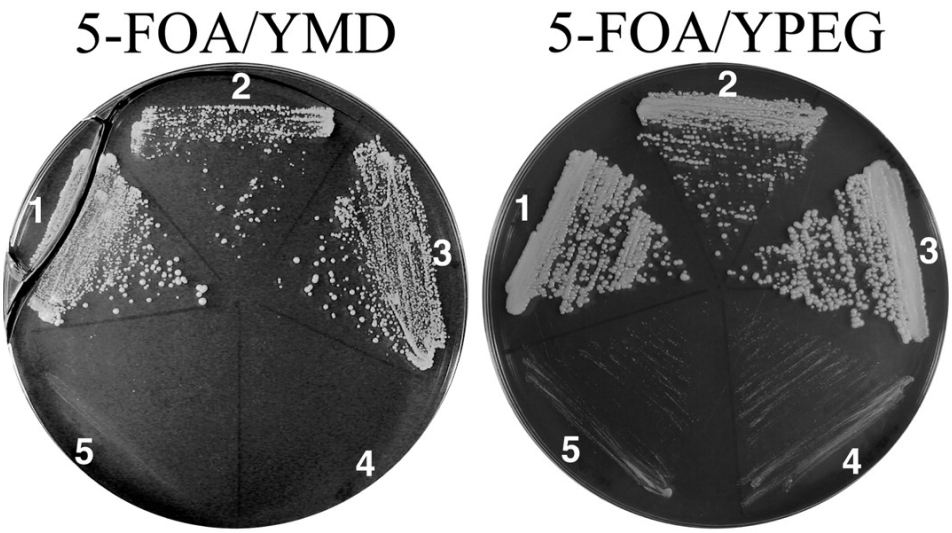

Figure 6.9: Growth phenotypes of Trm5p truncation mutants. A Δ*trm5* haploid strain (DLY1) harboring full-length *TRM5* on a *URA3* plasmid (pRS416-TRM5) was transformed with various *TRM5* constructs in *LEU2* plasmids (pRS415). Leu^+ transformants were then streaked onto 5-fluoroorotic acid (*5-FOA*)/YMD or 5-fluoroorotic acid/YPEG plates and incubated at 30 °C for 4 or 5 days, respectively. *Sector 1*, full-length *TRM5* (pRS415-TRM5); *sector 2*, pRS415-Δ1–33TRM5; *sector 3*, pRS415-Δ1–19TRM5; *sector 4*, pRS415-Δ1–47TRM5; *sector 5*, pRS415 empty vector (negative control). This figure has been published as figure 6A in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

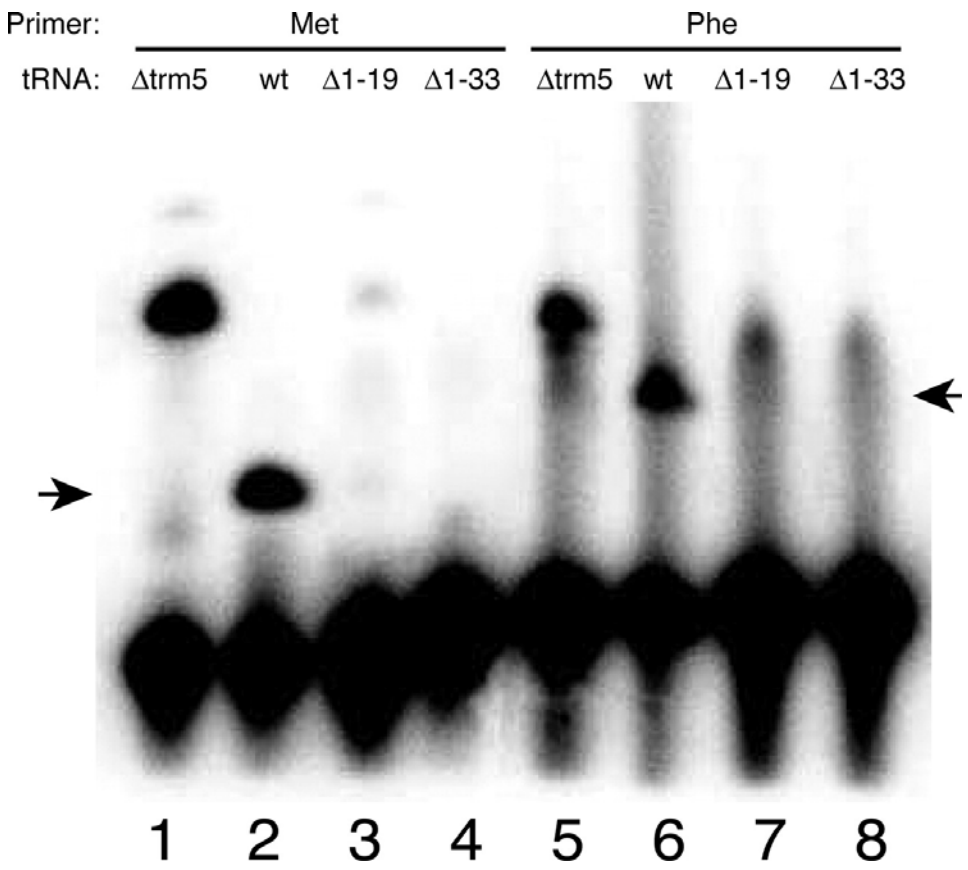

Figure 6.10: Primer extension analysis of the methylation status of mitochondrial $tRNA^{Met}_f$ and $tRNA^{Phe}$ in Trm5p truncation mutants**.** Total tRNA was obtained from wild-type (Newton et al.) cells, strain DLY1 (**Δ***trm5*), or DLY1 harboring plasmids pRS415-**Δ**1–19TRM5 (**Δ***1–19*) or pRS415-**Δ**1–**33**TRM5 (**Δ***1–33*). ^{32}P-Labeled primers specific for yeast mitochondrial initiator $tRNA^{Met}_f$ (*Met, lanes 1–4*) or mitochondrial $tRNA^{Phe}$ (*Phe, lanes 5–8*) were annealed, and primer extension reactions were performed. The *arrows* indicate the primer extension termination products caused by the presence of m^1G for $tRNA^{Met}$ (*left*) or $tRNA^{Phe}$ (*right*). This figure has been published as figure 7 in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

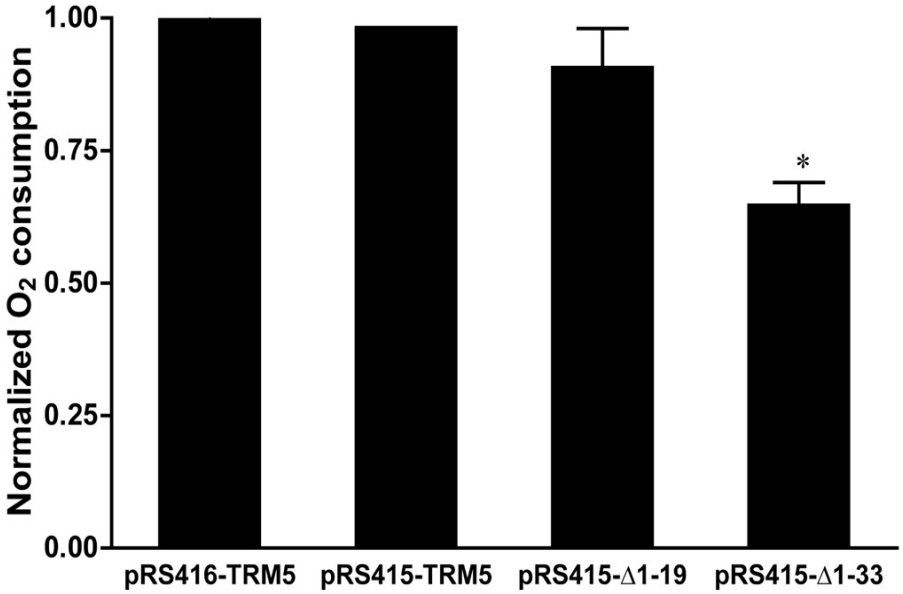

Figure 6.11: Oxygen consumption of strain DLY1 harboring various *TRM5* constructs grown in minimal medium with glucose as carbon source (YMD). All of the rates (nmol oxygen min^{-1}) were normalized to the A_{600} of the culture, and the mean oxygen consumption rate (nmol min^{-1} A_{600}^{-1}) of the pRS416-TRM5 (wild-type) culture was assigned a value of 1. Each column represents the mean ± the S.E. from two independent experiments ($n = 2$). *, $p < 0.02$ *versus* pRS416-TRM5, unpaired *t* test. This figure has been published as figure 6B in Lee, C. et al (2007) *J.Biol.Chem*, **282**, 27744-27753.

6.8 DISCUSSION

"At least eight mitochondrially encoded tRNAs are known to be methylated in the N1 position of G37 *in vivo* (Dunin-Horkawicz et al., 2006), yet the enzyme(s) responsible for modification of these mitochondrial tRNAs has not been previously identified. We have shown here that the tRNA (guanine-N1-)-methyltransferase encoded by the yeast *TRM5* gene is responsible for methylation of G37 in mitochondrial initiator tRNA ($tRNA^{Met}_f$). Its function in the yeast cytoplasm has been shown before (Bjork et al., 2001). Similar to the human Trm5p (Brule et al., 2004), the recombinant yeast enzyme uses *S*-adenosyl-L-methionine as a methyl donor and is specific for the N1 in the G residues at position 37 in natural tRNAs. In particular, yeast Trm5p efficiently methylates G37 in mitochondrial $tRNA^{Met}_f$, which contains U at position 36. Thus, like the human Trm5p and in contrast to prokaryotic TrmD (Brule et al., 2004; Takeda et al., 2006), the yeast enzyme appears to tolerate any nucleotide at position 36. The product of the reaction catalyzed by yeast Trm5p was shown to be m^1G by HPLC".

"Because Trm5p is also responsible for m^1G37 modification of cytoplasmic tRNAs (Bjork et al., 2001), the enzyme must have a dual localization to both the cytoplasm and the mitochondria. This was confirmed in yeast using a GFP-tagged version of Trm5p and antibodies against GFP. Proteinase K digestion showed that the mitochondrial form resides in the mitochondrial matrix, where it can access the mitochondrially encoded tRNAs. Early work in yeast indicated the existence of two tRNA (guanine-N1-)-methyltransferases in that organism, specific for tRNA sites 9 and 37 (Sindhuphak et al., 1985; Smolar et al., 1975). These two distinct enzymes are now known to be encoded by *TRM10* (Jackman et al., 2003) and *TRM5* (Bjork et al., 2001), respectively. Sindhuphak *et al.* even detected two peaks of m^1G37 methyltransferase

activity on a tRNA affinity column, foreshadowing the two forms of Trm5p (cytoplasmic and mitochondrial) reported here".

"The predicted amino acid sequence of Trm5p reveals four potential AUG start codons in the N terminus (codons 1, 20, 34, 48) (Illustration 6.2). Growth analysis (Figure. 6.8) revealed that a Trm5p active in the cytoplasm (as determined by normal growth on glucose) could be obtained with a construct lacking amino acids 1–33, whereas production of a Trm5p active in the mitochondria (as determined by the methylation status of mitochondrial tRNAs) required these first 33 amino acids. Oxygen consumption assays (Figure. 6.10) revealed decreased respiration efficiency for both the Δ1–19 and Δ 1–33 constructs, although only the Δ1–33 construct was significantly reduced. These data suggest a model in which the mitochondrial form initiates at the first AUG codon, producing a protein with an N-terminal mitochondrial targeting sequence. Helical wheel analysis indicates that residues 1–19 could fold into an amphipathic α-helix, with both a hydrophobic and a basic face (cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html). Our data cannot deduce whether or where the presequence is cleaved upon translocation into the mitochondrion. PSORT II analysis (psort.hgc.jp/form2.html) predicts mitochondrial localization only for the full-length ORF; initiation at any of the downstream AUG codons predicts a cytoplasmic localization. In our model, the shorter cytoplasmic form initiates at the third AUG codon, producing a protein lacking a mitochondrial targeting sequence. This model is consistent with the slightly larger size of the mitochondrial form compared with the cytoplasmic form on SDS gels (Figure 6.6). However, we cannot rule out the possibility that a non-AUG codon is used to initiate translation, as occurs in the dual localization of the *GRS1* and *ALA1* gene products (Chang et al., 2006; Chang and Wang, 2004). Attempts to address this question with N-terminal amino acid sequencing of Trm5p isolated from yeast were not successful".

"Mapping of the 5' ends of the *TRM5* transcript suggests how this differential translation initiation might occur. We observed two sets of transcriptional start sites: nucleotides –24 and –18 (upstream of the first AUG codon) and nucleotides +12 and +21 (between the first and second AUG codons). Transcriptional initiation at the upstream sites would allow translation to initiate from the first AUG, producing a protein with a mitochondrial presequence to direct translocation into the mitochondrion. Transcriptional initiation at the downstream sites would force translation initiation to the second or third AUG codon, bypassing the mitochondrial presequence, to yield a cytoplasmic protein".

"A transcription map of the yeast genome obtained using a high density oligonucleotide array (David et al., 2006) indicates the 5' end of the *TRM5* transcript at nucleotide –94, although this mapping method would not detect transcripts from the same gene with shorter 5' ends, such as those we observed with 5'-RACE mapping. Because we were unable to obtain reproducible results with ribonuclease protection mapping, the transcriptional start site(s) of the *TRM5* gene remain equivocal".

"It is also possible that the two Trm5p isoforms are produced from the same transcript, as a result of "leaky" ribosome scanning. Leaky scanning can occur when the first AUG resides in a suboptimal sequence context, leading to its inefficient utilization as initiator and shifting initiation to an AUG at a downstream location. This mechanism occurs in at least four yeast genes that produce two protein isoforms from a single transcript: *MOD5* (Slusher et al., 1991), *CCA1* (Wolfe et al., 1994), *GRS1* (Chang and Wang, 2004), and *ALA1* (Chang et al., 2006). Indeed, the sequence context of the first AUG (5'-UUGCAUGAAA-3') is a very poor match to the consensus for yeast genes (Cigan and Donahue, 1987); the sequences surrounding the second and third AUGs are much closer to the consensus".

"Finally, it is possible that mRNA localization plays a role as well. Total RNA isolated from a crude mitochondrial fraction was enriched for *TRM5* mRNA (data not shown). A number of nuclear-encoded mitochondrial proteins are known to be translated from mitochondria-associated mRNAs (Garcia et al., 2007), and the *TRM5* transcript has a fairly high mitochondrial localization of mRNA index level of 63 (Marc et al., 2002). Thus, it may be that the mitochondrial form of Trm5p is translated on cytoplasmic ribosomes bound to the surface of mitochondria".

"It is likely that the m^1G37 tRNA modification plays the same role in mitochondria as it does in cytoplasmic and bacterial translation systems, *i.e.* reading frame maintenance (Bjork et al., 1989). The results from the truncation mutants showed that m^1G37 methylation of mitochondrial tRNAs is not essential for mitochondrial protein synthesis, because the Δ1–33 mutant, which lacks this modification, can still respire (as evidenced by growth on ethanol + glycerol; Figure 6.8). However, loss of mitochondrial m^1G37 tRNA methylation did correlate with decreased oxygen consumption (Figure 6.10), indicating a defect in oxidative phosphorylation. This suggests that the efficiency or accuracy of mitochondrial protein synthesis is decreased in cells lacking m^1G37 methylation of mitochondrial tRNAs, probably because of accumulation of translational frameshift mutations".

"This same phenomenon is also seen in mutants of the *MSS1* and *MTO1* genes involved in 2-thiolation of the wobble position in yeast mitochondrial tRNAs (Umeda et al., 2005). Thus, whereas lack of these tRNA modifications may not cause observable growth defects in a laboratory setting, they can cause decreased mitochondrial translation efficiency. If these tRNA modifications provide even the slightest growth advantage to the organism, they would be retained, reflecting what has been called the ruthless delicacy of selection".

"The bacterial ancestor of mitochondria probably had a full complement of tRNA-modifying enzymes. During their organellar evolution, mitochondria have maintained distinct transcription, translation, and tRNA genes, even as their aminoacyl-tRNA synthetases and RNA-modifying enzymes were displaced by nuclear-encoded eukaryotic forms. Similar to the *TRM1* gene, whose product introduces the N2,N2-dimethylguanosine modification in cytoplasmic and mitochondrial tRNAs (Martin and Hopper, 1994), *TRM5* encodes multiple targeting sequences. This organization simplifies and consolidates the RNA-modifying systems but constrains the evolution of RNA processing and translation in both the nucleus and the mitochondria".

Appendix A

Abbreviations

AdoMet	S-adenosylmethionine
Amp	ampicillin
bp	base pair
BSA	bovine serum albumin
C_1	one-carbon
CH^+-THF	5,10-methenyltetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate
cpm	counts per minute
ddH_2O	distilled, deionized water
ddNTP	dideoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
fMet-tRNA	formylmethionyl-tRNA
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
His	histidine
HOAc	Acetic acid
HPLC	high performance liquid chromatography

HRP	horseradish peroxidase
IAA	Isoamyl alcohol
GFP	green fluorescent protein
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pair
kDa	kilo Dalton
KOAc	potassium acetate
LiOAc	lithium acetate
MBP	maltose binding protein
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
RACE	rapid amplification of cDNA ends
RbCl	rubidium chloride
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SOE	splice overlap extension
TBS	Tris-buffered saline
TEV	tobacco etch virus

THF	tetrahydrofolate
UTR	untranslated region
YT	Yeast extract and Tryptone

Appendix B

Materials

Material	Manufacturer
Agarose	Invitrogen Life Technologies™ (Carlsbad, CA)
Amino acids	Calbiochem Corp. (San Diego, CA)
Ampicillin	Sigma-Aldrich Co. (St.Louis, MO)
Amylose Resin	New England Biolabs (Ipswich, MA)
Anti-GFP antibodies	AnaSpec (San Jose, CA)
Anti-HA antibodies	Covance Inc. (Trenton, NJ)
Anti-Hsp60 antibodies	IMGENEX (San Diego, CA)
BSA	Sigma-Aldrich Co.
dNTPs	Invitrogen Life Technologies
ECL Western blotting reagents	GE Healthcare Bio-Sciences (Piscataway, NJ)
EDTA	Sigma-Aldrich Co.
DTT	Sigma-Aldrich Co.
Ethanol	AAPER Alcohol and Chemical Co.
Fast-Link™ DNA Ligation Kit	Epicentre® Technologies (Madison, WI)
FirstChoice™ RLM-RACE Kit	Ambion, Inc. (Austin, TX)
Formic acid	EM Science (Gibbstown, NJ)
Geneticin® (G418 Sulfate)	American Bioanalytical (Natick, MA)

His-Bind Metal Affinity Resin	Novagen (Gibbstown, NJ)
HRP-goat anti-mouse or rabbit IgG_γ	Zymed Laboratories, Inc. (San Francisco, CA)
Imidazole	Sigma-Aldrich Co.
IPTG	Research Products International Corp. (Mt. Prospect, IL)
KOD Hot Start DNA polymerase	Novagen (Gibbstown, NJ)
Lyticase	Sigma-Aldrich Co.
Yeast Media ingredients	Difco Laboratories (Sparks, MD)
2-mercaptoethanol	Sigma-Aldrich Co.
Methanol	Sigma-Aldrich Co.
Nitrocellulose membrane	Schleicher and Schuell Bioscience, Inc. (Keene, NH)
Oligonucleotides	Integrated DNA Technologies, Inc. (Coralville, VA)
PAGE supplies	Bio-Rad Laboratories, Inc. (Hercules, CA)
pBluescript KS (+/-)	Stratagene® (La Jolla, CA)
[α-^{32}P]dATP	PerkinElmer Life Sciences (Wellesley, MA)
Phenol:$CHCl_3$:IAA (25:24:1)	Ambion, Inc. (Austin, TX)
Phenylmethylsulfonyl fluoride	Sigma-Aldrich Co.
Protease inhibitor cocktail Tablet	Roche Applied Science (Indianapolis, IN)
Protein A Sepharose beads	Pierce (Rockford, IL)
Protein assay dye reagent	Bio-Rad laboratories, Inc.
DNA purification kits	QIAGEN Inc. (Valencia, CA)
Restriction endonucleases	New England Biolabs (Ipswich, MA)

Rosetta 2 (DE3)pLysS *E.coli* strain	Novagen
S-adenosylmethionine	Sigma-Aldrich Co.
Strip-EZ™ PCR Kit	Ambion, Inc.
Taq DNA polymerase	Invitrogen Life Technologies
Triton X-100	Beckman® Analytical Chemicals
XL1-Blue *E.coli* strain	Stratagene

Appendix C

Yeast Strains

Strain	Genotype	Source
DAY4	*MAT***a** *ser1 leu2 his4 trp1 ura3-52*	Tibbetts et al., 2000
Y03039	*MAT***a** *his3 leu2 met15 ura3 fmt1*::*KanMX4*	EUROSCARF
S.P.175[a]	*MAT***a** *his3 leu2 met15 ura3 fmt1*::*KanMX4/Y305N in AEP3*	This work
DLY2[b]	*MAT*α *his3 leu2 met15 ura3 aep3*::*KanMX4*	This work
LOY1	*MAT*α *his3 leu2 lys2 ura3 ifm1*::*KanMX4*	Tibbetts et al., 2003
Y21898	*MAT***a**/α *his3*/*his3 leu2*/*leu2 lys2*/*LYS2*	EUROSCARF
	MET15/*met15 ura3*/*ura3 trm5*::*KanMX4/TRM5*	
DLY1[c]	*MAT*α *his3 leu2 lys2 ura3 trm5*::*KanMX4*	This work
EY0986/TRM5-GFP	*MAT***a** *his3 leu2 met15 ura3 TRM5-GFP*	Invitrogen
Y22823	*MAT***a**/α α *his3*/*his3 leu2*/*leu2 lys2*/*LYS2*	EUROSCARF
	MET15/*met15 ura3*/*ura3 aep3*::*KanMX4/AEP3*	

[a] Transformed with a plasmid pRS416-FMT1

[b] Transformed with plasmid pRS416-AEP3 complementing the *aep3* deletion

c Maintained with plasmid pRS416-TRM5 complementing the *trm5* deletion

Appendix D

Plasmids

Plasmid	Description	Source
pVT101U	Yeast 2μm expression vector	Vernet et al., 1987
pVT103L	Yeast 2μm expression vector	Vernet et at., 1987
pRS415	Yeast centromeric low copy *LEU2* vector	Sikorski and Hieter, 1989
pRS416	Yeast centromeric low copy *URA3* vector	Sikorski and Hieter, 1989
pRS416-FMT1	A low copy *URA3* vector containing *FMT1*	This work
pRS415-PET56	A low copy *LEU2*vector containing *PET56*	This work
pRS415-HIS3	A low copy *LEU2* vector containing *HIS3* and unORF	This work
pRS415-unORF	A low copy *LEU2* vector containing unORF	This work
pRS415-PstI	A low copy *LEU2* vector containing disrupted unORF	This work
pRS416-AEP3	A low copy *URA3* vector containing *AEP3*	This work
pRS415-AEP3	A low copy *LEU2* vector containing *AEP3*	This work
pRS415-AEP3-Y305N	A low copy *LEU2* vector containing *AEP3* point mutation	This work
pMALc2H_{10}T	His-tagged MBP fusion *E.coli* expression vector	Kristelly et al., 2004
pVT101U-bmIF2	Wild-type bovine mitochondria initiation factor 2 in yeast 2μm expression vector	Tibbetts et al., 2003

pMAL-MBP-AEP3	MBP-AEP3 fusion construct in pMALc2H$_{10}$T	This work
pMAL-MBP-AEP3-Y305N	MBP-AEP3-Y305N fusion construct in pMALc2H$_{10}$T	This work
pRS415-AEP3-HA	HA-tagged *AEP3* in low copy *LEU2* vector	This work
pRS415-AEP3-c-Myc	c-Myc-tagged *AEP3* in low copy *LEU2* vector	This work
pRS415-AEP3-Flag	Flag-tagged *AEP3* in low copy *LEU2* vector	This work
pMAL-MBP-TRM5	MBP-TRM5 fusion construct in pMALc2H$_{10}$T	This work
pRS415-TRM5	Wild-type *TRM5* gene, including 300-bp 5'-UTR, in low copy LEU2 vector	This work
pRS416-TRM5	Wild-type *TRM5* gene, including 300-bp 5'-UTR, in low copy URA3 vector	This work
pRS415-Δ1-19TRM5	Truncated TRM5, with codons 1-19 deleted	This work
pRS415-Δ1-33TRM5	Truncated TRM5, with codons 1-33 deleted	This work
pRS415-Δ1-47TRM5	Truncated TRM5, with codons 1-47 deleted	This work

Appendix E

Protocols

Protocol 1: Standard Polymerase Chain Reaction (PCR)

The Vent DNA polymerase (New England Biolabs) was used for the majority of the PCR reactions. Cloning experiments were performed with KOD Hot Start DNA polymerase (Novagen) which has 3' to 5' exonuclease proofreading activity with high fidelity.

PCR on yeast genomic DNA:

A. Mixture: (50µl total reaction volume)

1. 5 µl of 10 x PCR amplification buffer (supplied with enzyme)
2. 5 µl of 2.5 mM dNTPs (supplied with enzyme)
3. 2 µl of MgSO4 (1mM final concentration, supplied with enzyme)
4. 1 to 100 ng template DNA
5. 1.5 µl of 10 µM 5' primer
6. 1.5 µl of 10 µM 3' primer
7. 1µl of 1U/µl KOD Hot Start DNA Polymerase
8. Sterile ddH_2O to a final volume of 50 µl

B. Amplification steps in a thermal cycler:

1. 94^{o}C, 2 min.; 1 cycle
2. 94^{o}C, 15 sec.; annealing at 55^{o}C, 30 sec.; elongation at 68^{o}C, 1 min per 1kb; 30 cycles
3. Extend 68^{o}C, 10 min; 1 cycle

PCR on DNA plasmid templates:

A. Mixture: (50µl total reaction volume)

1. 5 µl of 10 x PCR amplification buffer (supplied with enzyme)
2. 4 µl of 2.5 mM dNTPs
3. 10 to 100 ng template DNA
5. 1 µl of 10 µM 5' primer
6. 1 µl of 10 µM 3' primer
7. 1 µl of 1U/µl Vent DNA Polymerase
8. Sterile ddH_2O to a final volume of 50 µl

B. Amplification steps in a thermal cycler:

1. 94^{o}C, 3 min.; 1 cycle
2. 94^{o}C, 30 sec.; annealing at 55^{o}C, 30 sec.; elongation at 72^{o}C, 1 min per 1kb; 30 cycles
3. Extend 72^{o}C, 7 min.; 1 cycle

Protocol 2: Yeast Transformation

This method was originally derived from the Gietz Laboratory (University of Manitoba, Winnipeg, Manitoba, CANADA) (Gietz and Woods, 2002), and modified for our own purposes.

1. Start a 5 ml culture of yeast in minimal medium or rich medium at 30^{o}C overnight.
2. Transfer cells at OD_{600} of 1.0 to 25 ml culture of yeast and incubate at 30^{o}C overnight
3. Collect cells by centrifugation at 3,000 x g for 5 min., at room temperature
4. Resuspend pellets in 5 ml of pre-warmed rich media and take 0.5 ml of resuspended cells.
5. Transfer the cells into pre-warmed 50 ml of rich medium and incubate it at 30^{o}C until the cells reach 3 x 10^{9}. (Usually, it takes 3 hours)
6. Wash the cells in half volume of ddH_2O and resuspend pellets in 3 ml of 100 mM LiOAc.
7. Aliquot 100 µl of cell suspension into a 1.5 ml microcentrifuge tube for each transformation.
8. Incubate cell suspension at 30^{o}C for 15 min.
9. Microcentrifuge at 10,000 x g for 1 min. and take off the supernatant with a micropipette.

10. Add the transformation mix to each tube in the following order.
    a. 240 µl 50% (v/v) PEG, filter sterilized
    b. 36 µl 1 M LiOAc, filter sterilized
    c. 10 µl 10 mg/mL salmon sperm DNA (It should be boiled for 5 min before use)
    d. 100 ng to 5 µg of DNA plasmid
    e. 36 µl sterile ddH_2O

11. Vortex mixture vigorously for at least 1min. and incubate at 30^oC for 30 min.
12. Incubate mixture at 42^oC for 15 min (Invert tubes 5 times every 2.5 min)
13. Microcentrifuge at 10,000 x g for 1 min. and remove the supernatant with a micropipette.
14. Add 150 µl sterile ddH_2O into tubes and spread cells on the selective media plates
15. Incubate them at 30^oC for at least 3 days.

Protocol 3: Immunoblotting

(Tibbetts and Appling, 2000)

Solutions:

1. 20X Transfer Buffer (200ml)

0.2 M Tris base (4.84 g)

2M glycine (30.03 g)

200 ml of 20 X transfer buffer mixed with 400 ml of methanol and 3400 ml of ddH_2O to prepare 1 X transfer buffer

2. 20X TBS (100ml)

0.2 M Tris base (2.42 g)

3 M NaCl (17.53 g)

80 ml of ddH_2O, pH 8.0 with HCl

ddH_2O to final volume of 100 ml (if desired, autoclave)

dilute 20-fold to prepare 1X TBS (final concentration of 10 mM Tris-HCl, 150 mM, NaCl, pH 8.0)

3. 1X TBST

Add 0.05% of Tween-20 to 1 X TBS

Protocol:

1. Run protein samples as well as Bio-Rad protein markers on a SDS-PAGE gel.
2. Soak the gel in 1 X transfer buffer for 2 min.
3. Cut a piece of nitrocellulose membrane to fit the gel and two pieces of Whatman 3MM filter paper with a slightly larger size than the membrane
4. Wet the nitrocellulose membrane in 1X transfer buffer briefly and place it on top of the gel. Do not forget to get rid of any air bubbles in between.
5. Make a sandwich of the gel plus membrane between two pieces of filter paper. Make sure the gel is closer to the black (cathode) and membrane to the red (anode).
6. Assemble the electroblot apparatus (Bio-rad) in the cold room and run the transfer at 250mA (~200V) for 90 min while stirring.
7. Disassemble the apparatus and wash the membrane in ddH_2O.
8. Block the membrane with 2% dry milk powder (H.E.B) in TBS for 1 hour with shaking.
9. Remove the blocking solution and add primary antibody appropriately diluted in 1 X TBS with 1% dry milk powder [The antibodies and dilutions used are given under Experimental procedures]. Incubate with shaking for 1 hour.
10. Wash the membrane with TBST (three times, 5 min each)
11. Add secondary antibody in a 1:2000 dilution in TBST and incubate with shaking for 1 hour.
12. Wash the membrane twice in TBST for 5 min each.
13. Wash the membrane twice in TBS for 5 min each.

14. Rinse the membrane with ddH_2O immediately before using an enhanced chemiluminescence (ECL) detection system.
15. In a dark room, combine the ECL Plus Western Blotting reagents (GE Healthcare Bio-Sciences), Reagent 1 with Reagent 2 together in a 40:1 ratio and put it on the membrane. Incubate for 5 min.
16. Remove the reagents from the membrane and place it in a transparency sheet.
17. Visualize the signals in a Kodak Image Station 4000R.

Protocol 4: Isolation of mitochondria from *S. cerevisiae*

Semisynthetic galactose medium (per 1L)

3 g yeast extract

10 g galactose

0.8 g $(NH_4)_2SO_4$

0.7 g $MgSO_4 \cdot 7H_2O$

0.5 g NaCl

1 g KH_2PO_4

0.4 g $CaCl_2$

5 mg $FeCl_3 \cdot 6H_2O$

1. Start a 25 ml culture of yeast in semisynthetic galactose medium at 30^{o}C for growth overnight with shaking.
2. Transfer the culture into a flask containing 2 L galactose medium and incubate at 30^{o}C overnight with shaking.
3. Harvest cells at 5,000 rpm for 10 min and wash with ice-cold ddH_2O three times.
4. Resuspend cell pellets in 60 ml of 0.1 M Tris-HCl, pH 9.3, 10 mM DTT and incubate at 30^{o}C for 10 min with shaking.
5. Centrifuge at 3,500 rpm for 10 min.
6. Resuspend cell pellets in 100 ml 1.2 M sorbitol, 10 mM phosphate buffer, pH 7.4 and incubate at 3,500 rpm for 10 min.
7. Resuspend cell pellets in 40 ml 1.2 M sorbitol, 10 mM phosphate buffer, pH 7.4 and

add 40 mg of lyticase.

8. Incubate at 30^{o}C for 90 min. and then centrifuge at 4,200 rpm for 10 min.
9. Resuspend cell pellets in 7 ml of 0.6 M sorbitol, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA and homogenize cells with a douncer with 15 strokes.
10. Centrifuge at 4,200 rpm for 10 min. Keep the supernatant.
11. Resuspend cell pellets in 7 ml of same buffer above and homogenize cells again. Centrifuge at 4,200 rpm for 10 min and combine supernatants from both homogenization together.
12. Centrifuge at 10,000 rpm for 15 min.
13. Keep supernatants as a cytosolic fraction, and resuspend the mitochondrial pellets in 400 μl of 0.6 M sorbitol, 20 mM HEPES, pH 7.4.
14. Measure the protein concentration in the Bradford assay (Bradford, 1976).
15. Aliquot the resuspended mitochondria and store at -80^{o}C.

Protocol 5: Splice Overlap Extension (SOE) PCR

This method was originally devised by Dr. David E. Graham (University of Texas at Austin, TX), and modified for our own purposes.

1. Amplify separate DNA fragments using a standard PCR method.
2. Purify PCR products using a PCR purification Kit (Qiagen) and gel-purify each product.
3. Prepare SOE PCR

    5 µl of 10x PCR buffer for the DNA polymerase

    5 µl of dNTPs (final concentration of 0.2 mM)

    3 µl of MgSO4 (final concentration of 1.5 mM)

    50 to 100 ng each PCR product

    1 µl of KOD Hot Start DNA Polymerase (final concentration of 0.02 U/µl)

    sterile ddH_2O to a final volume of 50 µl

   - Note: Do not add any primers in this stage

4. Run a program in a thermocycler as follows.

    94^{o}C, 3 min; 1cycle

    94^{o}C, 30 sec; 55^{o}C, 30 sec for annealing; 72^{o}C, 2 min; 5 cycles

   - Note: two DNA fragments should be overlapping by at least 20 nucleotides.

     Annealing and elongation times are adjustable based on DNA fragments (1kb/1min).

5. Add 2 µl of each terminal primers (final concentration of 0.8 µM)
6. Continue the program for the rest of the PCR program as follows.

    94^{o}C, 30 sec; 55^{o}C, 30 sec; 72^{o}C, 2 min; 25 cycles

7. Analyze the PCR product on an agarose gel.

Protocol 6: Transformation of RbCl-Competent E.coli Cells

(Hanahan, 1985)

Solutions:

1. TFB1

30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$,

15% (w/v) glycerol, pH 5.8, sterilize by filtration (HOAc used to adjust the pH)

2. TFB2

10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% (w/v) glycerol, pH 6.5,

sterilize by filtration (adjust the pH with KOH)

Protocols:

1. Make competent cells

a. Inoculate a single colony of *E.coli* XL1-B into 5 ml of YT medium.
Incubate at 37^{o}C overnight.

b. Transfer the cell suspension into 250 ml culture of YT containing 20 mM $MgSO_4$.
Incubate at 37^{o}C until OD600 of 0.4 ~ 0.6 is reached.

c. Centrifuge cells at 4^{o}C for 5 min at 5,000 x g.

d. Resuspend pellets gently in 100 ml TFB1.

e. Incubate for 5 min on ice.

f. Centrifuge at 4^{o}C for 5 min at 5,000 x g.

g. Resuspend pellets gently in 10 ml TFB2

h. Incubate for 60 min on ice.

i. Aliquot 100 µl of cell suspension into 0.5ml Eppendorf tubes.

j. Freeze them right away in liquid N_2.

2. Transformation into RbCl-competent cells

a. Let cell thaw on ice for 5 min.

b. Add 10 ng – 500 ng of plasmid DNA

c. Incubate on ice for 60 min.

d. Heat shock at 37^{o}C for 45 sec.

e. Add 200 µl of YT and incubate at 37^{o}C for at least 30 min.

f. Spread cells onto selective medium plates.

Protocol 7: Plasmid Rescue from *S. cerevisiae*

(Robzyk and Kassir, 1992)

Solution:

STET: 8% sucrose, 50 mM Tris-HCl (pH 8.0), 50mM EDTA, 5% Triton X-100.

Protocol:

1. Start 3 ml of a yeast culture containing the plasmid in selective minimal medium and grow overnight.
2. Harvest cells at 5,000 rpm for 5 min.
3. Resuspend the cell pellets in 100 µl of STET
4. Add 0.2 g of washed glass beads (425-600µm; Sigma-Aldrich Co.) into the cell suspension and vortex for 5min.
5. Add another 100 µl of STET.
6. Vortex briefly and place in boiling water for 3 min and cool the tube on ice.
7. Centrifuge cells at 12,000 x g for 10 min in the cold room.
8. Take 100 µl of the supernatant and place into a 1.5ml Eppendorf tube containing 50 µl of 7.5 M ammonium acetate.
9. Incubate at -20^{o}C for 1 hour.
10. Centrifuge at 12,000 x g for 10 min in the cold room.
11. Take 100 µl of the supernatant into 200 µl of ice-cold ethanol to precipitate the DNA.
12. Incubate the tube on ice for 10 min.
13. Centrifuge at 12,000 x g for 10 min in the cold room.

14. Remove supernatant carefully and wash pellet in 70% ice-cold ethanol.
15. Centrifuge at 12,000 x g for 5 min and remove supernatant. Dry the resulting pellets.
16. Resuspend pellet in 20 µl of sterile ddH_2O.
17. Do the electroporation to transform *E.coli* JM109 cell with the resuspended pellet.

Protocol 8: UV mutagenesis of yeast cells

(Tibbetts, 2008)

1. Start a 4 ml culture of yeast in minimal medium (YMD) with the appropriate amino acids at 30^{o}C overnight.
2. Inoculate 25 ml YMD containing the amino acids with 2 ml of the overnight culture and grow again at 30^{o}C overnight until OD_{600} of 2.
3. Centrifuge cells at 1,750 x g for 5 min, and resuspend in 10 ml of sterile ddH_2O to a density of 1-5 x 10^7cells /ml.
4. Vortex for 30 sec and aliquot 1.5 ml of cells into 7 different screw-top microcentrifuge tubes.
5. Distribute each 1.5 ml of cells in pre-sterilized boats and expose to UV radiation at 6 sec intervals
   - Use Stratalinker autocrosslinker (Stratagen) to sterilize boats.
6. Try different time exposures such as 6, 12, and 18-sec. and save cells before UV radiation as control.
7. Put 750 µl each of UV-exposed cells into 2 sterile tubes and wrap with foil.
8. Dilute each cell at each time interval and plate onto YPD plates
9. Determine the killing efficiency relative to controls (No mutagenized cells).
   - Cutoff threshold: 10-40% cells survival

Bibliography

Altman, F.P. (1976). Tetrazolium salts and formazans. Prog Histochem Cytochem *9*, 1-56.

Appling, D.R. (1999). Genetic approaches to the study of protein-protein interactions. Methods *19*, 338-349.

Appling, D.R., and Rabinowitz, J.C. (1985). Regulation of expression of the ADE3 gene for yeast C1-tetrahydrofolate synthase, a trifunctional enzyme involved in one-carbon metabolism. J Biol Chem *260*, 1248-1256.

Attardi, G. (1985). Animal mitochondrial DNA: an extreme example of genetic economy. Int Rev Cytol *93*, 93-145.

Barlowe, C.K., Williams, M.E., Rabinowitz, J.C., and Appling, D.R. (1989). Site-directed mutagenesis of yeast C1-tetrahydrofolate synthase: analysis of an overlapping active site in a multifunctional enzyme. Biochemistry *28*, 2099-2106.

Beechey, R.B., Roberton, A.M., Holloway, C.T., and Knight, I.G. (1967). The properties of dicyclohexylcarbodiimide as an inhibitor of oxidative phosphorylation. Biochemistry *6*, 3867-3879.

Bender, A., and Pringle, J.R. (1991). Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involved in morphogenesis in Saccharomyces cerevisiae. Mol Cell Biol *11*, 1295-1305.

Bianchetti, R., Lucchini, G., Crosti, P., and Tortora, P. (1977). Dependence of mitochondrial protein synthesis initiation on formylation of the initiator methionyl-tRNAf. J Biol Chem *252*, 2519-2523.

Bianchetti, R., Lucchini, G., and Sartirana, M.L. (1971). Endogenous systhesis of formyl-methionine peptides in isolated mitochondria and chloroplasts. Biochem Biophys Res Commun *42*, 97-102.

Bjork, G.R., Jacobsson, K., Nilsson, K., Johansson, M.J., Bystrom, A.S., and Persson, O.P. (2001). A primordial tRNA modification required for the evolution of life? EMBO J *20*, 231-239.

Bjork, G.R., Wikstrom, P.M., and Bystrom, A.S. (1989). Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. Science *244*, 986-989.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132.

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

Brule, H., Elliott, M., Redlak, M., Zehner, Z.E., and Holmes, W.M. (2004). Isolation and characterization of the human tRNA-(N1G37) methyltransferase (TRM5) and comparison to the Escherichia coli TrmD protein. Biochemistry *43*, 9243-9255.

Canaday, J., Dirheimer, G., and Martin, R.P. (1980). Yeast mitochondrial methionine initiator tRNA: characterization and nucleotide sequence. Nucleic Acids Res *8*, 1445-1457.

Carter, A.P., Clemons, W.M., Jr., Brodersen, D.E., Morgan-Warren, R.J., Hartsch, T., Wimberly, B.T., and Ramakrishnan, V. (2001). Crystal structure of an initiation factor bound to the 30S ribosomal subunit. Science *291*, 498-501.

Chan, S.Y., and Appling, D.R. (2003). Regulation of S-adenosylmethionine levels in Saccharomyces cerevisiae. J Biol Chem *278*, 43051-43059.

Chang, K.J., Lin, G., Men, L.C., and Wang, C.C. (2006). Redundancy of non-AUG initiators. A clever mechanism to enhance the efficiency of translation in yeast. J Biol Chem *281*, 7775-7783.

Chang, K.J., and Wang, C.C. (2004). Translation initiation from a naturally occurring non-AUG codon in Saccharomyces cerevisiae. J Biol Chem *279*, 13778-13785.

Chomyn, A., Hunkapiller, M.W., and Attardi, G. (1981). Alignment of the amino terminal amino acid sequence of human cytochrome c oxidase subunits I and II with the sequence of their putative mRNAs. Nucleic Acids Res *9*, 867-877.

Christian, T., Evilia, C., Williams, S., and Hou, Y.M. (2004). Distinct origins of tRNA(m1G37) methyltransferase. J Mol Biol *339*, 707-719.

Chung, H.K., and Spremulli, L.L. (1990). Purification and characterization of elongation factor G from bovine liver mitochondria. J Biol Chem *265*, 21000-21004.

Cigan, A.M., and Donahue, T.F. (1987). Sequence and structural features associated with translational initiator regions in yeast--a review. Gene *59*, 1-18.

Dahlquist, K.D., and Puglisi, J.D. (2000). Interaction of translation initiation factor IF1 with the E. coli ribosomal A site. J Mol Biol *299*, 1-15.

Daum, G., Bohni, P.C., and Schatz, G. (1982). Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J Biol Chem *257*, 13028-13033.

David, L., Huber, W., Granovskaia, M., Toedling, J., Palm, C.J., Bofkin, L., Jones, T., Davis, R.W., and Steinmetz, L.M. (2006). A high-resolution map of transcription in the yeast genome. Proc Natl Acad Sci U S A *103*, 5320-5325.

Dickerman, H.W., Steers, E., Jr., Redfield, B.G., and Weissbach, H. (1967). Methionyl soluble ribonucleic acid transformylase. I. Purification and partial characterization. J Biol Chem *242*, 1522-1525.

Droogmans, L., and Grosjean, H. (1987). Enzymatic conversion of guanosine 3' adjacent to the anticodon of yeast tRNAPhe to N1-methylguanosine and the wye nucleoside: dependence on the anticodon sequence. EMBO J *6*, 477-483.

Dunin-Horkawicz, S., Czerwoniec, A., Gajda, M.J., Feder, M., Grosjean, H., and Bujnicki, J.M. (2006). MODOMICS: a database of RNA modification pathways. Nucleic Acids Res *34*, D145-149.

Ellis, T.P., Helfenbein, K.G., Tzagoloff, A., and Dieckmann, C.L. (2004). Aep3p stabilizes the mitochondrial bicistronic mRNA encoding subunits 6 and 8 of the H+-translocating ATP synthase of Saccharomyces cerevisiae. J Biol Chem *279*, 15728-15733.

Epler, J.L., Shugart, L.R., and Barnett, W.E. (1970). N-formylmethionyl transfer ribonucleic acid in mitochondria from Neurospora. Biochemistry *9*, 3575-3579.

Feldman, F., and Mahler, H.R. (1974). Mitochondrial biogenesis. Retention of terminal formylmethionine in membrane proteins and regulation of their synthesis. J Biol Chem *249*, 3702-3709.

Galper, J.B., and Darnell, J.E. (1969). The presence of N-formyl-methionyl-tRNA in HeLa cell mitochondria. Biochem Biophys Res Commun *34*, 205-214.

Galper, J.B., and Darnell, J.E. (1971). Mitochondrial protein synthesis in HeLa cells. J Mol Biol *57*, 363-367.

Garcia, M., Darzacq, X., Delaveau, T., Jourdren, L., Singer, R.H., and Jacq, C. (2007). Mitochondria-associated yeast mRNAs and the biogenesis of molecular complexes. Mol Biol Cell *18*, 362-368.

Garofalo, C., Kramer, G., and Appling, D.R. (2005). Characterization of the C2 subdomain of yeast mitochondrial initiation factor 2. Arch Biochem Biophys *439*, 113-120.

Garofalo, C., Trinko, R., Kramer, G., Appling, D.R., and Hardesty, B. (2003). Purification and characterization of yeast mitochondrial initiation factor 2. Arch Biochem Biophys *413*, 243-252.

Grosjean, H., Szweykowska-Kulinska, Z., Motorin, Y., Fasiolo, F., and Simos, G. (1997). Intron-dependent enzymatic formation of modified nucleosides in eukaryotic tRNAs: a review. Biochimie *79*, 293-302.

Gualerzi, C.O., and Pon, C.L. (1990). Initiation of mRNA translation in prokaryotes. Biochemistry *29*, 5881-5889.

Guillon, J.M., Mechulam, Y., Schmitter, J.M., Blanquet, S., and Fayat, G. (1992). Disruption of the gene for Met-tRNA(fMet) formyltransferase severely impairs growth of Escherichia coli. J Bacteriol *174*, 4294-4301.

Hagervall, T.G., Ericson, J.U., Esberg, K.B., Li, J.N., and Bjork, G.R. (1990). Role of tRNA modification in translational fidelity. Biochim Biophys Acta *1050*, 263-266.

Halbreich, A., and Rabinowitz, M. (1971). Isolation of Saccharomyces cerevisiae mitochondrial formyltetrahydrofolic acid:methionyl-tRNA transformylase and the hybridization of mitochondrial fMet-tRNA with mitochondrial DNA. Proc Natl Acad Sci U S A *68*, 294-298.

Hawley, E.S., and Greenawalt, J.W. (1970). An assessment of in vivo mitochondrial protein synthesis in Neurospora crassa. J Biol Chem *245*, 3574-3583.

Hiley, S.L., Jackman, J., Babak, T., Trochesset, M., Morris, Q.D., Phizicky, E., and Hughes, T.R. (2005). Detection and discovery of RNA modifications using microarrays. Nucleic Acids Res *33*, e2.

Horton, R.M., Ho, S.N., Pullen, J.K., Hunt, H.D., Cai, Z., and Pease, L.R. (1993). Gene splicing by overlap extension. Methods Enzymol *217*, 270-279.

Hou, Y.M., Li, Z., and Gamper, H. (2006). Isolation of a site-specifically modified RNA from an unmodified transcript. Nucleic Acids Res *34*, e21.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686-691.

Jackman, J.E., Montange, R.K., Malik, H.S., and Phizicky, E.M. (2003). Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. RNA *9*, 574-585.

Jiang, H.Q., Motorin, Y., Jin, Y.X., and Grosjean, H. (1997). Pleiotropic effects of intron removal on base modification pattern of yeast tRNAPhe: an in vitro study. Nucleic Acids Res *25*, 2694-2701.

Johnson, P.F., and Abelson, J. (1983). The yeast tRNATyr gene intron is essential for correct modification of its tRNA product. Nature *302*, 681-687.

Kapust, R.B., Tozser, J., Fox, J.D., Anderson, D.E., Cherry, S., Copeland, T.D., and Waugh, D.S. (2001). Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. Protein Eng *14*, 993-1000.

Karniely, S., and Pines, O. (2005). Single translation--dual destination: mechanisms of dual protein targeting in eukaryotes. EMBO Rep *6*, 420-425.

Koc, E.C., and Spremulli, L.L. (2002). Identification of mammalian mitochondrial translational initiation factor 3 and examination of its role in initiation complex formation with natural mRNAs. J Biol Chem *277*, 35541-35549.

Kozak, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol Rev *47*, 1-45.

Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. Gene *234*, 187-208.

Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. Gene *299*, 1-34.

Kristelly, R., Gao, G., and Tesmer, J.J. (2004). Structural determinants of RhoA binding and nucleotide exchange in leukemia-associated Rho guanine-nucleotide exchange factor. J Biol Chem *279*, 47352-47362.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Laursen, B.S., Sorensen, H.P., Mortensen, K.K., and Sperling-Petersen, H.U. (2005). Initiation of protein synthesis in bacteria. Microbiol Mol Biol Rev *69*, 101-123.

Lee, C.P., Seong, B.L., and RajBhandary, U.L. (1991). Structural and sequence elements important for recognition of Escherichia coli formylmethionine tRNA by methionyl-tRNA transformylase are clustered in the acceptor stem. J Biol Chem *266*, 18012-18017.

Li, J., Esberg, B., Curran, J.F., and Bjork, G.R. (1997). Three modified nucleosides present in the anticodon stem and loop influence the in vivo aa-tRNA selection in a tRNA-dependent manner. J Mol Biol *271*, 209-221.

Li, J.N., and Bjork, G.R. (1999). Structural alterations of the tRNA(m1G37)methyltransferase from Salmonella typhimurium affect tRNA substrate specificity. RNA *5*, 395-408.

Li, M.Z., and Elledge, S.J. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods *4*, 251-256.

Li, Y., Holmes, W.B., Appling, D.R., and RajBhandary, U.L. (2000). Initiation of protein synthesis in Saccharomyces cerevisiae mitochondria without formylation of the initiator tRNA. J Bacteriol *182*, 2886-2892.

Liao, H.X., and Spremulli, L.L. (1991). Initiation of protein synthesis in animal mitochondria. Purification and characterization of translational initiation factor 2. J Biol Chem *266*, 20714-20719.

Ma, J., and Spremulli, L.L. (1996). Expression, purification, and mechanistic studies of bovine mitochondrial translational initiation factor 2. J Biol Chem *271*, 5805-5811.

Mahler, H.R., Dawidowicz, K., and Feldman, F. (1972). Formate as a specific label for mitochondrial translational products. J Biol Chem *247*, 7439-7442.

Malone, T., Blumenthal, R.M., and Cheng, X. (1995). Structure-guided analysis reveals nine sequence motifs conserved among DNA amino-methyltransferases, and suggests a catalytic mechanism for these enzymes. J Mol Biol *253*, 618-632.

Marc, P., Margeot, A., Devaux, F., Blugeon, C., Corral-Debrinski, M., and Jacq, C. (2002). Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep *3*, 159-164.

Marcker, K., and Sanger, F. (1964). N-Formyl-Methionyl-S-Rna. J Mol Biol *8*, 835-840.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1997). Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell *89*, 951-961.

Martin, J.L., and McMillan, F.M. (2002). SAM (dependent) I AM: the S-adenosylmethionine-dependent methyltransferase fold. Curr Opin Struct Biol *12*, 783-793.

Martin, N.C., and Hopper, A.K. (1994). How single genes provide tRNA processing enzymes to mitochondria, nuclei and the cytosol. Biochimie *76*, 1161-1167.

Martin, R.P., Sibler, A.P., Schneller, J.M., Keith, G., Stahl, A.J., and Dirheimer, G. (1978). Primary structure of yeast mitochondrial DNA-coded phenylalanine-tRNA. Nucleic Acids Res *5*, 4579-4592.

Matsuo, H., Li, H., McGuire, A.M., Fletcher, C.M., Gingras, A.C., Sonenberg, N., and Wagner, G. (1997). Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. Nat Struct Biol *4*, 717-724.

McCarthy, J.E., and Brimacombe, R. (1994). Prokaryotic translation: the interactive pathway leading to initiation. Trends Genet *10*, 402-407.

Meinnel, T., Guillon, J.M., Mechulam, Y., and Blanquet, S. (1993). The Escherichia coli fmt gene, encoding methionyl-tRNA(fMet) formyltransferase, escapes metabolic control. J Bacteriol *175*, 993-1000.

Moazed, D., Samaha, R.R., Gualerzi, C., and Noller, H.F. (1995). Specific protection of 16 S rRNA by translational initiation factors. J Mol Biol *248*, 207-210.

Nathans, D. (1964). Puromycin Inhibition of Protein Synthesis: Incorporation of Puromycin into Peptide Chains. Proc Natl Acad Sci U S A *51*, 585-592.

Newton, D.T., Creuzenet, C., and Mangroo, D. (1999). Formylation is not essential for initiation of protein synthesis in all eubacteria. J Biol Chem *274*, 22143-22146.

Nishikura, K., and De Robertis, E.M. (1981). RNA processing in microinjected Xenopus oocytes. Sequential addition of base modifications in the spliced transfer RNA. J Mol Biol *145*, 405-420.

Ogur, M., St. John, R., and Nagai, S. (1957). Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science *125*, 928-929.

Prasannan, P., Pike, S., Peng, K., Shane, B., and Appling, D.R. (2003). Human mitochondrial C1-tetrahydrofolate synthase: gene structure, tissue distribution of the mRNA, and immunolocalization in Chinese hamster ovary calls. J Biol Chem *278*, 43178-43187.

Putz, J., Florentz, C., Benseler, F., and Giege, R. (1994). A single methyl group prevents the mischarging of a tRNA. Nat Struct Biol *1*, 580-582.

RajBhandary, U.L. (1994). Initiator transfer RNAs. J Bacteriol *176*, 547-552.

Roll-Mecak, A., Shin, B.S., Dever, T.E., and Burley, S.K. (2001). Engaging the ribosome: universal IFs of translation. Trends Biochem Sci *26*, 705-709.

Rozenski, J., Crain, P.F., and McCloskey, J.A. (1999). The RNA Modification Database: 1999 update. Nucleic Acids Res *27*, 196-197.

Sala, F., and Kuntzel, H. (1970). Peptide chain initiation in homologous and heterologous systems from mitochondria and bacteria. Eur J Biochem *15*, 280-286.

Schluckebier, G., O'Gara, M., Saenger, W., and Cheng, X. (1995). Universal catalytic domain structure of AdoMet-dependent methyltransferases. J Mol Biol *247*, 16-20.

Schmitt, E., Panvert, M., Blanquet, S., and Mechulam, Y. (1998). Crystal structure of methionyl-tRNAfMet transformylase complexed with the initiator formyl-methionyl-tRNAfMet. EMBO J *17*, 6819-6826.

Schwartz, J.H., Meyer, R., Eisenstadt, J.M., and Brawerman, G. (1967). Involvement of N-formylmethionine in initiation of protein synthesis in cell-free extracts of Euglena gracilis. J Mol Biol *25*, 571-574.

Schwartzbach, C.J., and Spremulli, L.L. (1989). Bovine mitochondrial protein synthesis elongation factors. Identification and initial characterization of an elongation factor Tu-elongation factor Ts complex. J Biol Chem *264*, 19125-19131.

Sebald, W., Wachter, E., and Tzagoloff, A. (1979). Identification of amino acid substitutions in the dicyclohexylcarbodiimide-binding subunit of the mitochondrial ATPase complex from oligomycin-resistant mutants of Saccharomyces cerevisiae. Eur J Biochem *100*, 599-607.

Shannon, K.W., and Rabinowitz, J.C. (1988). Isolation and characterization of the Saccharomyces cerevisiae MIS1 gene encoding mitochondrial C1-tetrahydrofolate synthase. J Biol Chem *263*, 7717-7725.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Sindhuphak, T., Hellman, U., and Svensson, I. (1985). Site specificities of three transfer RNA methyltransferases from yeast. Biochim Biophys Acta *824*, 66-73.

Skala, J., Van Dyck, L., Purnelle, B., and Goffeau, A. (1992). The sequence of an 8 kb segment on the left arm of chromosome II from Saccharomyces cerevisiae identifies five new open reading frames of unknown functions, two tRNA genes and two transposable elements. Yeast *8*, 777-785.

Slusher, L.B., Gillman, E.C., Martin, N.C., and Hopper, A.K. (1991). mRNA leader length and initiation codon context determine alternative AUG selection for the yeast gene MOD5. Proc Natl Acad Sci U S A *88*, 9789-9793.

Smith, A.E., and Marcker, K.A. (1968). N-formylmethionyl transfer RNA in mitochondria from yeast and rat liver. J Mol Biol *38*, 241-243.

Smolar, N., Hellman, U., and Svensson, I. (1975). Two transfer RNA (1-methylguanine) methylases from yeast. Nucleic Acids Res *2*, 993-1004.

Sorensen, H.P., Hedegaard, J., Sperling-Petersen, H.U., and Mortensen, K.K. (2001). Remarkable conservation of translation initiation factors: IF1/eIF1A and IF2/eIF5B are universally distributed phylogenetic markers. IUBMB Life *51*, 321-327.

Spencer, A.C., and Spremulli, L.L. (2004). Interaction of mitochondrial initiation factor 2 with mitochondrial fMet-tRNA. Nucleic Acids Res *32*, 5464-5470.

Spurio, R., Brandi, L., Caserta, E., Pon, C.L., Gualerzi, C.O., Misselwitz, R., Krafft, C., Welfle, K., and Welfle, H. (2000). The C-terminal subdomain (IF2 C-2) contains the entire fMet-tRNA binding site of initiation factor IF2. J Biol Chem *275*, 2447-2454.

Steffens, G.J., and Buse, G. (1979). Studies on cytochrome c oxidase, IV[1--3]. Primary structure and function of subunit II. Hoppe Seylers Z Physiol Chem *360*, 613-619.

Steffensen, S.A., Poulsen, A.B., Mortensen, K.K., and Sperling-Petersen, H.U. (1997). E. coli translation initiation factor IF2--an extremely conserved protein. Comparative sequence analysis of the infB gene in clinical isolates of E. coli. FEBS Lett *419*, 281-284.

Sundari, R.M., Stringer, E.A., Schulman, L.H., and Maitra, U. (1976). Interaction of bacterial initiation factor 2 with initiator tRNA. J Biol Chem *251*, 3338-3345.

Takeda, H., Toyooka, T., Ikeuchi, Y., Yokobori, S., Okadome, K., Takano, F., Oshima, T., Suzuki, T., Endo, Y., and Hori, H. (2006). The substrate specificity of tRNA (m1G37) methyltransferase (TrmD) from Aquifex aeolicus. Genes Cells *11*, 1353-1365.

Takeuchi, N., Kawakami, M., Omori, A., Ueda, T., Spremulli, L.L., and Watanabe, K. (1998). Mammalian mitochondrial methionyl-tRNA transformylase from bovine liver. Purification, characterization, and gene structure. J Biol Chem *273*, 15085-15090.

Tibbetts, A.S., Oesterlin, L., Chan, S.Y., Kramer, G., Hardesty, B., and Appling, D.R. (2003). Mammalian mitochondrial initiation factor 2 supports yeast mitochondrial translation without formylated initiator tRNA. J Biol Chem *278*, 31774-31780.

Tomoo, K., Shen, X., Okabe, K., Nozoe, Y., Fukuhara, S., Morino, S., Ishida, T., Taniguchi, T., Hasegawa, H., Terashima, A.*, et al.* (2002). Crystal structures of 7-methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5',5'-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. Biochem J *362*, 539-544.

Tzagoloff, A., and Dieckmann, C.L. (1990). PET genes of Saccharomyces cerevisiae. Microbiol Rev *54*, 211-225.

Tzagoloff, A., Macino, G., and Sebald, W. (1979). Mitochondrial genes and translation products. Annu Rev Biochem *48*, 419-441.

Umeda, N., Suzuki, T., Yukawa, M., Ohya, Y., Shindo, H., and Watanabe, K. (2005). Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. J Biol Chem *280*, 1613-1624.

Urbonavicius, J., Qian, Q., Durand, J.M., Hagervall, T.G., and Bjork, G.R. (2001). Improvement of reading frame maintenance is a common function for several tRNA modifications. EMBO J *20*, 4863-4873.

Urbonavicius, J., Stahl, G., Durand, J.M., Ben Salem, S.N., Qian, Q., Farabaugh, P.J., and Bjork, G.R. (2003). Transfer RNA modifications that alter +1 frameshifting in general fail to affect -1 frameshifting. RNA *9*, 760-768.

Varshney, U., and RajBhandary, U.L. (1992). Role of methionine and formylation of initiator tRNA in initiation of protein synthesis in Escherichia coli. J Bacteriol *174*, 7819-7826.

Walker, J.E., Lutter, R., Dupuis, A., and Runswick, M.J. (1991). Identification of the subunits of F1F0-ATPase from bovine heart mitochondria. Biochemistry *30*, 5369-5378.

Williams, E.H., Butler, C.A., Bonnefoy, N., and Fox, T.D. (2007). Translation initiation in Saccharomyces cerevisiae mitochondria: functional interactions among mitochondrial ribosomal protein Rsm28p, initiation factor 2, methionyl-tRNA-formyltransferase and novel protein Rmd9p. Genetics *175*, 1117-1126.

Wolfe, C.L., Lou, Y.C., Hopper, A.K., and Martin, N.C. (1994). Interplay of heterogeneous transcriptional start sites and translational selection of AUGs dictate the production of mitochondrial and cytosolic/nuclear tRNA nucleotidyltransferase from the same gene in yeast. J Biol Chem *269*, 13361-13366.

Wu, X.Q., and RajBhandary, U.L. (1997). Effect of the amino acid attached to Escherichia coli initiator tRNA on its affinity for the initiation factor IF2 and on the IF2 dependence of its binding to the ribosome. J Biol Chem *272*, 1891-1895.

Vita

Changkeun Lee was born in Taegu, Korea, on October 2, 1971, the son of Soyoung Lee and Sangtae Cho. In 1990, he graduated from Myoung-Deok High School in Seoul, Korea, and began attending The University of Suwon, Korea. The studies were interrupted by three years of mandatory military service in the Korean Army. He received the degree of Bachelor of Science in Chemistry with the 1st rank in February of 1998. He continued his education at Seoul National University, Korea, and received the degree of Master of Science in Biochemistry in February of 2000. In August of 2002, he entered the Cell and Molecular Biology Program in the Graduate School of The University of Texas at Austin.

Permanent address: 110-802 Jukong Apt.
Kuro-1dong, Kuro-gu,
Seoul, Korea 152-761

This dissertation was typed by the author.