THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 2, Issue of January 14, pp. 1613–1624, 2005

*Printed in U.S.A.*

# 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*

Received for publication, August 13, 2004, and in revised form, October 26, 2004
Published, JBC Papers in Press, October 26, 2004, DOI 10.1074/jbc.M409306200

**Noriko Umeda‡§, Takeo Suzuki‡, Masashi Yukawa¶, Yoshikazu Ohya¶, Heisaburo Shindo§, Kimitsuna Watanabe¶‖, and Tsutomu Suzuki‡¶****

*From the ‡Department of Chemistry and Biotechnology, Graduate School of Engineering, the ¶Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 and the §School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachiouji, Tokyo 192-0392, Japan*

**Human mitochondrial (mt) tRNA$^{Lys}$ has a taurine-containing modified uridine, 5-taurinomethyl-2-thiouridine ($\tau m^5s^2U$), at its anticodon wobble position. We previously found that the mt tRNA$^{Lys}$, carrying the A8344G mutation from cells of patients with myoclonus epilepsy associated with ragged-red fibers (MERRF), lacks the $\tau m^5s^2U$ modification. Here we describe the identification and characterization of a tRNA-modifying enzyme *MTU1* (mitochondrial tRNA-specific 2-thiouridylase 1) that is responsible for the 2-thiolation of the wobble position in human and yeast mt tRNAs. Disruption of the yeast *MTU1* gene eliminated the 2-thio modification of mt tRNAs and impaired mitochondrial protein synthesis, which led to reduced respiratory activity. Furthermore, when *MTO1* or *MSS1*, which are responsible for the C5 substituent of the modified uridine, was disrupted along with *MTU1*, a much more severe reduction in mitochondrial activity was observed. Thus, the C5 and 2-thio modifications act synergistically in promoting efficient cognate codon decoding. Partial inactivation of *MTU1* in HeLa cells by small interference RNA also reduced their oxygen consumption and resulted in mitochondria with defective membrane potentials, which are similar phenotypic features observed in MERRF.**

The precise codon-anticodon pairing that occurs during translation requires a post-transcriptional modification at the anticodon first position (the wobble position) of the tRNA (1–4). In mammalian mitochondrial (mt)[1] tRNAs, we recently discovered novel taurine-bearing modified uridines at the wobble position, namely, 5-taurinomethyluridine ($\tau m^5U$) in mt tRNA$^{Leu(UUR)}$ and 5-taurinomethyl-2-thiouridine ($\tau m^5s^2U$) in mt tRNA$^{Lys}$ (5). We then showed that these wobble modifications are generated by the direct incorporation of taurine supplied by the medium. This is the first time it has been shown that taurine is a component of biological macromolecules.

We have also shown that the taurine-bearing modifications do not occur in mutant mt tRNAs that contain pathogenic point mutations that are associated with mitochondrial encephalomyopathies (6, 7). Point mutations in mt tRNA genes are known to be responsible for a wide spectrum of human diseases that are caused by mitochondrial dysfunction. We found that the mt tRNA$^{Leu(UUR)}$ molecules obtained from pathogenic cells of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) patients (8), who have either the A3243G or the T3271C mutation, lack the $\tau m^5U$ modification (6). To examine decoding disorder of the mutant tRNA due to the wobble modification deficiency independent of the pathogenic point mutation itself, we used a molecular surgery to construct a mt tRNA$^{Leu(UUR)}$ lacking the taurine modification but without the pathogenic mutation. This "operated" mt tRNA$^{Leu(UUR)}$ without the taurine modification showed severely reduced UUG translation but no decrease in UUA translation. We thus concluded that the UUG codon-specific translational defect of the mutant mt tRNA$^{Leu(UUR)}$ is the primary cause of MELAS at the molecular level (9). This result could explain the complex I deficiency observed clinically in MELAS. In addition, mt tRNA$^{Lys}$ molecules bearing the A8344G mutation that were obtained from cybrid cells of patients with MERRF (myoclonus epilepsy associated with ragged-red fibers) (10) were found to lack the $\tau m^5s^2U$ modification (7). Thus, the MELAS and MERRF mitochondrial diseases are both associated with a lack of taurine modification of their mutant tRNAs. We examined the translational ability of the mutant mt tRNA$^{Lys}$ molecules obtained from cybrid cells of MERRF patients who carry the A8344G mutation. This analysis revealed that the tRNA$^{Lys}$ that lacks the $\tau m^5s^2U$ modification was incapable of translating both of its cognate codons, AAA and AAG, due to complete loss of codon-anticodon pairing on the ribosome (11). This suggests that the taurine wobble modification-deficiency results in a translational defect and that this is likely to contribute significantly to the mitochondrial dysfunc-

* This work was supported by grants-in-aid for scientific research on priority areas from the Ministry of Education, Science, Sports, and Culture of Japan, and by a grant from the New Energy and Industrial Technology Development Organization (to T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB178028*

‖ Present address: Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-41-6 Aomi, Koto-ku, Tokyo 135-0064, Japan.

** To whom correspondence should be addressed: Tel.: 81-3-5841-8752; Fax: 81-3-3816-0106; E-mail: ts@chembio.t.u-tokyo.ac.jp.

[1] The abbreviations used are: mt, mitochondrial; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonus epilepsy associated with ragged-red fibers; ORF, open reading frame; RT, reverse transcription; LC/MS, liquid chromatography/mass spectrometry; APM, ((*N*-acryloylamino)phenyl)mercuric chloride; EGFP, enhanced green fluorescent protein; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $cmnm^5U$, 5-carboxymethylaminomethyluridine; $cmnm^5s^2U$, 5-carboxymethylaminomethyl-2-thiouridine; $\tau m^5s^2U$, 5-taurinomethyl-2-thiouridine; $mnm^5s^2U$, 5-methylaminomethyl-2-thiouridine; $s^2U$, 2-thiouridine.

tion associated with the pathogenic A8344G mt tRNA mutation. Notably, it has been suggested that it is the lack of the 2-thio modification of the MERRF mutant mt tRNA that is the main reason for the decoding disorder associated with this mutation, because it was observed that the 2-thio modification of $mnm^5s^2U$ (5-methylaminomethyl-2-thiouridine) in *Escherichia coli* $tRNA^{Lys}$ is responsible for stabilizing their codon-anticodon interactions (12). However, it has also been shown that the C5-taurinomethyl modification also apparently contributes to the efficient codon recognition of the $tRNA^{Lys}$ (5, 13).

We speculated that the pathogenic point mutation hinders the biosynthesis of the 2-thio as well as C5-taurinomethyl modifications of $\tau m^5s^2U$. It will be necessary to identify and characterize the enzyme genes that are responsible for introducing the 2-thio and C5-taurinomethyl groups onto the wobble position of $tRNA^{Lys}$ before we can elucidate the true molecular reason for the pathogenic nature of the mt mutations associated with MERRF, as well as with other mitochondrial diseases. This will hopefully lead to the development of therapeutic measures for these diseases. The biosynthesis of the $\tau m^5s^2U$ 2-thio and C5-taurinomethyl modifications of mt $tRNA^{Lys}$ probably involves complicated systems that require many genes, including several unidentified genes. In the case of the C5 modification of the wobble uridines, *mnmE* (*trmE*) and *gidA* are known to be required for the initial step in the modification of *E. coli* tRNAs, namely, $mnm^5U$ (5-methylaminomethyuridine) synthesis (14–16). *MSS1* and *MTO1* were found to be the respective homologs of the *mnmE* and *gidA* genes in both human and yeast (17, 18). The protein products of these genes were shown to localize in the mitochondria as a heterodimer complex, and their mutants are associated with respiratory defects (19). These observations suggest that the human *MSS1* and *MTO1* genes encode the enzymes that are responsible for the initial C5-taurinomethyl modification of mt tRNAs, although there is no direct evidence for this as yet. Concerning the 2-thio modification of the modified wobble uridines, it has been recently shown by Nakai *et al.* (20) that the yeast Nfs1p protein is involved in the 2-thio modification of both mitochondrial and cytoplasmic tRNAs. Nfs1p is a eukaryotic homolog of the bacterial IscS protein that serves to supply activated sulfur from cysteine to be utilized in iron-sulfur cluster biogenesis (21–23). IscS (cysteine desulfurase) was also found to be involved in the thio modifications of bacterial tRNAs (24, 25). It can be assumed that the sulfur of the cysteine is initially taken up by IscS and is then transferred onto unidentified sulfur mediators. An enzyme that is directly responsible for the 2-thio modification of the tRNAs finally then introduces the sulfur from the mediator onto its target tRNA molecules.

In this study, we report our identification of a mitochondria-specific tRNA-modifying enzyme that appears to be responsible for the 2-thio modification of $\tau m^5s^2U$ in $tRNA^{Lys}$. We have denoted this enzyme as MTU1 (mitochondrial tRNA-specific 2-thiouridylase 1). We also describe our observations that suggest that a deficiency in the 2-thio modification of mt tRNA is a major molecular cause of the pathogenesis of the MERRF-associated mutation in the mt $tRNA^{Lys}$ molecule.

## EXPERIMENTAL PROCEDURES

*Yeast Strains and Media*—Haploid *Saccharomyces cerevisiae* strains that lack single genes due to deletion by using the KanMX4 cassette (26) were purchased from the EUROSCARF collection (available at www.uni-frankfurt.de/fb15/mikro/euroscarf/). The BY4741 (*MATa*) strain was used as the wild type. $\rho^0$ cells of the BY4741 strain were constructed as described previously (27). Complete deletion of the mt DNA was checked by Southern blotting (data not shown). Homologous recombination in meiosis was employed to obtain haploid cells with double gene deletions. Thus, the Δ*MTU1* strain (EUROSCARF Y03729, *MATa*) was mated with the Δ*MTO1* or Δ*MSS1* strain (the EUROSCARF numbers are Y14603 and Y10598, respectively, *MATα*), and the diploid cells were selected on SD plates without methionine and lysine. These heterozygous strains were then sporulated and subjected to tetrad analysis. The tetrads displaying 2:2 segregation viability were selected on YPD (1% yeast extract (Difco), 2% bacto peptone (Difco), and 2% glucose) with G418, and the viable spores lacking the two genes were obtained as haploid cells with the *MATa* type. This was checked by mating tests. The growth properties and mitochondrial activities of these double deletion strains were compared with those of the single deletion strains with the *MATa* type, namely, BY4741 (*MATa*), Δ*MTU1* (EUROSCARF Y03729), Δ*MTO1* (EUROSCARF Y04603), and Δ*MSS1* (EUROSCARF Y00598). The media used to grow the yeast were the fermentable medium YPD, the non-fermentable medium YPG (1% yeast extract, 2% bacto peptone, and 3% glycerol), and the minimal medium SD (0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose supplied with the amino acids required). For tetrad analysis, presporulation medium (0.5% yeast extract, 1% peptone, and 0.17% yeast nitrogen base without ammonium sulfate and amino acids, 50 mM potassium phosphate buffer, pH 5.0, 1% potassium acetate, and 0.5% ammonium sulfate) and sporulation medium (1% potassium acetate with the appropriate nutrients as required) were used.

*Plasmid Construction and Site-directed Mutagenesis*—The yeast *MTU1* gene, including its promoter region (~1 kbp of 5′-untranslated region), was obtained from the *S. cerevisiae* genome by PCR using the following primers: yMTU1-5′XhoI (5′-gaccactcgagtggctaattctctggaaggc-3′) and yMTU1-3′BamHI (5′-gattcggatccttatcatcactatgcatggg-3′). The PCR product was cloned into pYO325LEU2 (derived from pRS315) (28) to yield pYMTU1. The ORF region of human MTU1 (GenBank™ accession number AB178028) was obtained by nested RT-PCR from the total RNA of HeLa cells. The first RT-PCR was performed with the primers hMTU1-F1 (5′-aaggcgcggaagcggcggtagctgc-3′) and hMTU1-R1 (5′-cccagcatgggtcctgctctccag-3′), which amplified the MTU1 ORF along with both untranslated regions. The second PCR was carried out with the primers hMTU1-F2 (5′-ggcaatcgattcaagtcagataacaatgcaggccttgcggcacgtcgtgtg-3′) and hMTU1-R2 (5′-aaggttcggatcctcagagcaagggactcaggcct-3′), which yielded cDNA of the human MTU1 ORF attached with sufficient edge remaining that could be used in PCR ligation that would attach the yeast promoter region to its 5′-end. To do this, the promoter region of the yeast *MTU1* gene was amplified with the primers yMTU1-5′XhoI and yMTU1-PR (5′-cacacgacgtgccgcaaggcctgcattgttatctgacttgaatcgattg-3′). The sequence of yMTU1-PR is complementary to the hMTU1-F2 sequence. The ORF region of human *MTU1* was then ligated to the promoter region of the yeast *MTU1* gene by using the primers yMTU1-5′XhoI and hMTU1-R2. The product was then cloned into pYO325LEU2 to yield pHMTU1. We employed a QuikChange site-directed mutagenesis according to the manufacturer's instruction to introduce the D38A mutation into the yeast MTU1 protein and the D18A mutation into the human MTU1 protein. The primers yMTU1D38A-F (5′-gatagtggcaatgtcatctggggtagcttcctctgtggcggcagcac-3′) and yMTU1D38A-R (5′-gtgctgccgccacagaggaagctacccagatgacattgccactatc-3′) were used to introduce the yeast MTU1-D38A mutation. The primers hMTU1D16A-F (5′-ctgtccggcggcgtggccagcgccgtggcc-3′) and hMTU1 D16A-R (5′-ggccacggcgctggccacgccgccggacag-3′) were used for human MTU1-D18A mutation.

*RNA Preparation and mt tRNA Isolation*—Total RNAs from yeast cells was extracted with phenol, precipitated with ethanol, and washed with 70% ethanol as described (29) and then fractionated with DEAE-Sepharose Fast Flow (2 × 30 cm) (Amersham Biosciences) with a linear gradient of NaCl (200–500 mM) and $MgCl_2$ (2–16 mM) in a buffer containing 20 mM HEPES-KOH (pH 7.5). The fractions enriched with tRNAs were pooled and precipitated with ethanol. The mt tRNAs was homogeneously isolated from the fraction by an improved solid-phase DNA probe method that we have named chaplet column chromatography (30). The 3′-biotin-labeled synthetic DNA probe for mt $tRNA^{Lys}$ employed in this study is as follows: 5-tggtgagaatagctggagttgaaccaa-3′. Eighty micrograms of DNA probe was immobilized on 200 μl of avidin-Sepharose (50% slurry, Amersham Biosciences) and packed in a small chaplet column. The tRNA fraction dissolved in binding buffer (1.2 M NaCl, 30 mM HEPES-KOH, pH 7.5, and 15 mM EDTA) was circulated through the column by a peristaltic pump at a temperature of 65 °C. After washing out nonspecifically bound tRNAs with wash buffer (0.6 M NaCl, 15 mM HEPES-KOH, pH 7.5, and 7.5 mM EDTA), each tRNA was eluted from the column with elution buffer (20 mM NaCl, 0.5 mM HEPES-KOH, pH 7.5, and 0.25 mM EDTA) at 65 °C. The eluted mt $tRNA^{Lys}$ was further purified by electrophoresis through a 12% polyacrylamide gel containing 7 M urea.

*Mass Spectrometry*—An LCQ ion-trap (IT) mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization source and an HP1100 liquid chromatography system (Agilent) was used to analyze

the total nucleosides and the RNase $T_1$-digested tRNA. Nucleoside analysis of total RNA was performed as described previously (30, 31). We identified each ribonucleoside by comparing its retention time in mass chromatogram, exact *m/z* values of proton adduct $[M+H]^+$ and base fragment ion $BH2^+$ with the data base for LC/MS analysis of ribonucleosides as described (53). As for $cmnm^5U$, we confirmed its retention time and mass spectrum by comparing with chemically synthesized $cmnm^5U$ (kindly provided by Dr. Murao). The exact mass of each RNA fragment produced by RNase T1 digest was determined by deconvoluting its multiply charged ions in the mass spectrum. Together with the information on a species of modified nucleosides in the tRNA and direct RNA sequencing by Donis-Keller's method (54), we could confirm the exact sequence of each RNA fragment.

Purified tRNA (~0.05 $A_{260}$/2.5 $\mu$g) was digested with RNase $T_1$ (2.5 units) in 20 mM $NH_4OAc$ (pH 5.3) at 37 °C for 30 min and then analyzed by mass spectrometry. The oligonucleotides produced by RNase $T_1$ digestion were analyzed as the negative ion form by LC/MS as described (32). Purified or unpurified tRNAs (0.02 $A_{260unit}$) were digested by nuclease $P_1$ for 3 h at 37 °C in 25 $\mu$l of reaction mixture containing 20 mM HEPES-KOH (pH 7.5) and 0.5 $\mu$l of 1 mg/ml nuclease $P_1$ and 0.5 $\mu$l of 0.05 unit/ml bacterial alkaline phosphatase.

*APM Gel Electrophoresis and Northern Blotting to Detect the Thiouridine Modification*—The presence of the thiouridine modification in the purified tRNAs was verified by the retardation of electrophoretic mobility in a polyacrylamide gel that contains 0.05 mg/ml ((*N*-acryloylamino)phenyl)mercuric chloride (APM) (30, 33), which is a method that was originally developed by Igloi (34). Thus, 0.05 $A_{260}$ unit of total RNA was separated by polyacrylamide gel electrophoresis and blotted onto Hybond $N^+$ membranes (Amersham Biosciences). Each tRNA fraction was detected with a 5′-$^{32}$P-labeled oligonucleotide probe whose sequence was based on a specific sequence in the corresponding tRNA. The following oligonucleotides were used: 5′-tggtgagaatagctggagttgaac-3′ for mt $tRNA^{Lys}$; 5′-tggttgaatcggtttgattcgaac-3′ for mt $tRNA^{Gln}$; 5′-tggtaaccttaatcggaatcgaac-3′ for mt $tRNA^{Glu}$; and 5′-tggctcctcataggggctcgaac-3′ for cytosolic $tRNA^{UUU}_{Lys2}$. After hybridization, the membranes were washed, dried, and then placed in contact with the imaging plate for 1 h to detect the cytosolic tRNAs and for more than 24 h to detect the mt tRNAs. The radioactivities were detected with a BAS5000 bioimaging analyzer (Fuji Photo Systems).

*Pulse-labeling of Mitochondrial Protein Synthesis*—The [$^{35}$S]methionine incorporation into mitochondrial proteins encoded by mt DNA was basically performed as described previously (35). Thus, yeast cells incubated with sulfur-lacking medium were harvested and cycloheximide (final concentration, 150 $\mu$g/ml) was added to inhibit cytoplasmic protein synthesis. The cells were then labeled with [$^{35}$S]methionine (54 mCi/mmol, Amersham Bioscience) at a concentration of $10^7$ cells/ml/80 $\mu$Ci for 10 min at 30 °C. The total proteins from the cells were separated by SDS-PAGE, and the gel was dried and exposed to an imaging plate. The radiolabeled mitochondrial proteins were detected with a BAS5000 bioimaging analyzer (Fuji Photo Systems).

*Subcellular Localization of MTU1 Protein*—The ORF of human *MTU1* was amplified from pHMTU1 by PCR using the primers hMTU1-5′XhoI (5′-ggccactcgagatgcaggccttgcggcacg-3′) and hMTU13′BamHI (5′-ggttcggatcccggagcaagggactcaggc-3′) and cloned between the XhoI and BamHI sites of pEGFP-N1 (Clontech) to yield pHMTU1/EGFP, which expresses a reporter MTU1 fused to the N terminus of EGFP. The resultant plasmid was transfected into $2 \times 10^5$ HeLa cells by using a LipofectAMINE reagent (Invitrogen) according to the procedure recommended by the manufacturer. After 24 h of transfection, the mitochondria were stained by MitoTracker Red (Molecular Probes), and the red fluorescence of MitoTracker Red and the green fluorescence of EGFP were simultaneously observed by confocal fluorescence microscopy (Leica DEMIRBE).

*siRNA Transfection*—Five siRNAs targeting *MTU1* were designed by an RNA interference activity-predicting algorithm that was developed by our group.[2] These siRNAs were synthesized by a method used to synthesize siRNAs derived from *in vitro* transcribed short hairpin RNA (36). The sequences of the sense strand of each siRNA are as follows: MTU1-1 (5′-aguuugcuguguucuacaa-3′), MTU1-2 (5′-acguaaaggaguauuggaa-3′), MTU1-3 (5′-gaaugaguaugaaaaagga-3′), MTU1-4 (5′-guuugaaguuagaaaugcg-3′), and MTU1-5 (5′-gucacuuuauuuccauaga-3′). The siRNA for luciferase (5′-cguacgcggaauacuucgaag-3′) was used as a negative control. The cells were cultured in Dulbecco's modified Eagle's medium with F-12 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin and $7.5 \times 10^4$ cells/well were then inoculated in 6-well plates and cultivated for 48 h. The medium was then changed to 1 ml of Opti-MEM I (Invitrogen) per well. The cells were transfected with 10 $\mu$mol of the siRNAs for *MTU1* or luciferase (final concentration, 50 nM) by using 10 $\mu$l of Oligofectamine (Invitrogen). They were then incubated for 4 h. Thereafter, 500 $\mu$l of Dulbecco's modified Eagle's medium with 30% fetal bovine serum was added. 96 h after the transfection, the total RNAs were extracted from the cells using TRI reagent (Sigma) according to the manufacturer's instruction. RT-PCR was carried out to measure the knocking down of the *MTU1* mRNA levels. Thus, 100 ng of the total RNAs from the cells that had been transfected with *MTU1*-siRNA or luciferase-siRNA were subjected to RT-PCR (Qiagen OneStep RT-PCR kit) as templates using the *MTU1* primers MTU1-F3 (5′-aggtgacaggggtgtttatg-3′) and MTU1-R3 primer (5′-cttgcatagtgacctgtggc-3′) and the glyceraldehyde-3-phosphate dehydrogenase primers GAPDH-F2 (5′-gtcgccagccgagccacat-3′) and GAPDH-R2 (5′-tcgttgtcataccaggaaatga-3′). The cycle number employed was 35.

*Visualizing Mitochondrial Membrane Potential*—72 h after the transfection of siRNA targeting MTU1, the cells were stained with both MitoTracker Red (final concentration, 50 nM) and MitoTracker Green (final concentration, 100 nM). As a control, we used cells that had been transfected with an siRNA-targeting luciferase. The cells were washed with phosphate-buffered saline, and 1 ml of medium was added. The cells were then visualized by confocal fluorescence microscopy (Leica DEMIRBE).

*Oxygen Consumption*—The rate of oxygen consumption by yeast cells ($A_{600}$ = 0.4) cultured in 4 ml of YPD was measured for 15 min at 30 °C or 37 °C by using a Clark type electrode (YSI 5300A Biological Oxygen Monitor System). The HeLa cells were trypsinized and adjusted to $5 \times 10^5$ cells/ml. The rate of oxygen consumption was monitored for 45 min at 37 °C.

## RESULTS

*Identification of Mitochondrial tRNA-specific 2-Thiouridylase (MTU1)*—It has been reported that the *Escherichia coli iscS* and *mnmA* (*trmU* and *asuE*) genes encode enzymes that are responsible for biosynthesizing the 2-thio group of $mnm^5s^2U$ (37, 38). Moreover, recombinant IscS has been shown to cooperate with MnmA to synthesize the 2-thio modification of tRNA *in vitro* (39). MnmA is believed to act in 2-thiouridylation by recognizing the substrate tRNA. Thus, we speculated that the mitochondrial *mnmA* homolog may be a candidate gene that introduces the 2-thio group of $\tau m^5s^2U$ in mt $tRNA^{Lys}$. We obtained the *mnmA* homologs in yeast, *Caenorhabditis elegans*, *Drosophila*, mice, and humans by BLASTP searches of protein databases. Sequence alignment revealed considerable conservation between these sequences (Fig. 1). The N-terminal regions contain a highly conserved SGGXDS sequence that is predicted to be a P-loop motif, which is a common ATP-binding motif found in the ATP pyrophosphate (pyrophosphate synthetase) family (40).

YDL033c is the *S. cerevisiae* yeast homolog of *mnmA*. It shows 29 and 24% homology to the sequences of the *E. coli mnmA* gene and its human homolog, respectively. It is a nonessential gene whose deletion strain is available (26). Thus, to examine the involvement of YDL033c in the 2-thio modification of the mt tRNAs for Lys, Glu, and Gln and the cytosolic $tRNA^{Lys}$, total RNAs obtained from the YDL033c-deletion strain were subjected to polyacrylamide gel electrophoresis containing (*N*-acryloylamino)phenylmercuric chloride (APM) combined with Northern blotting (33, 34) (see "Experimental Procedures") (Fig. 2). The four tRNAs from the wild type strain show specific retardation in the APM-containing polyacrylamide gel due to the strong affinity of the 2-thio group in these tRNAs with the mercuric compound in the gel (Fig. 2, *A–D*, *lane 1*). We have confirmed that no other thio modifications in these tRNAs are responsible for their gel-retardation in this experiment (data not shown). In the case of the tRNAs from the deletion strain, the retarded bands for the three mt tRNAs were not observed (Fig. 2, *A–C*, *lane 2*). However, the cytosolic $tRNA^{Lys}$ molecule was still specifically retarded. This suggests

[2] T. Katoh and T. Suzuki, manuscript submitted.

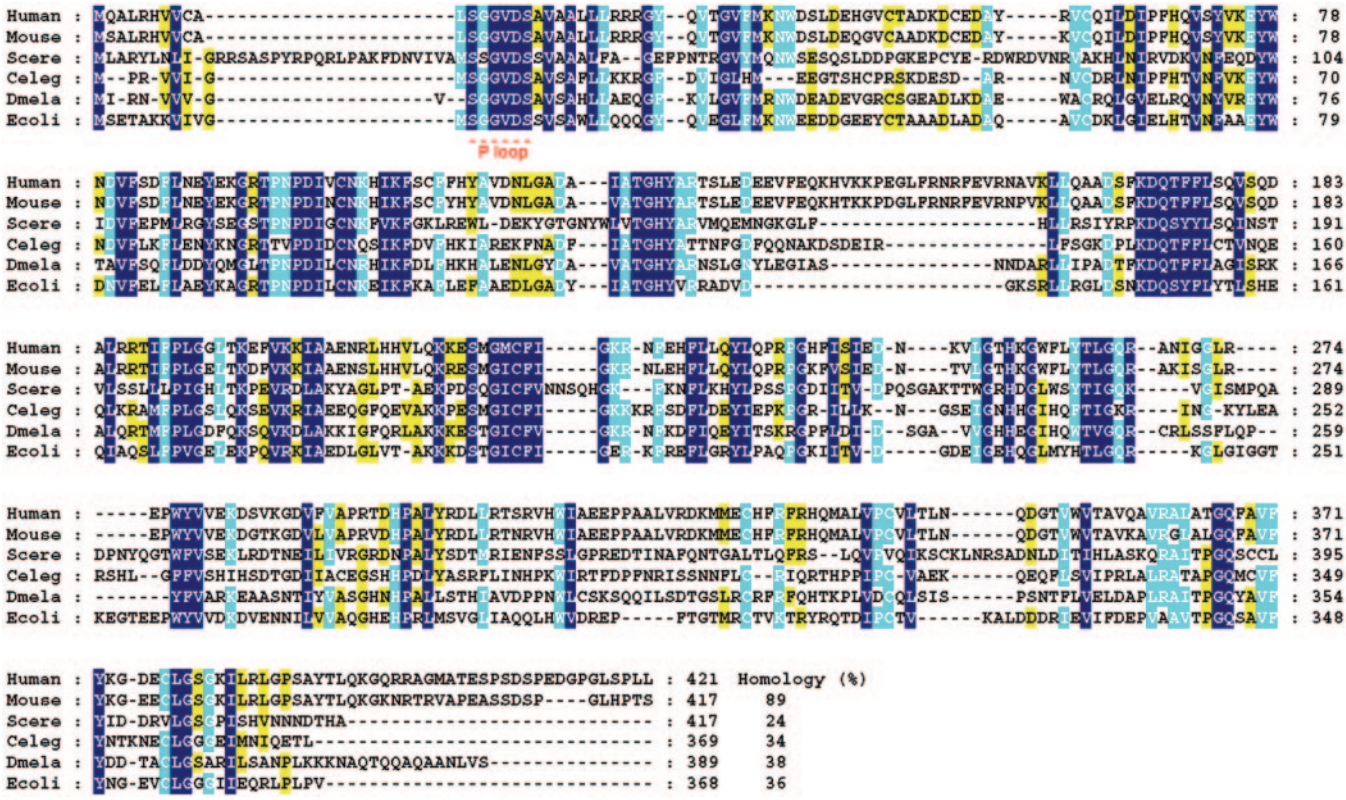


FIG. 1. **Sequence alignment of mt tRNA-specific 2-thiouridylases.** Human *MTU1* (AB178028) was aligned with its mouse (AAH61026), yeast (Scere, CAA98591), *C. elegans* (Celeg, Q17440), *D. melanogaster* (Dmela, AAE45578), and *E. coli* counterparts (P25745). Multiple alignment of each sequence was performed by using ClustalW (62) and displayed using the Genedoc multiple sequence alignment editor. Homology values (between the human sequence and the others), as calculated by Genedoc (63), are shown at the end of the alignment. The *colored boxes* indicate the degree of sequence similarity. The P loop motif (12–17) is indicated by a *dotted red line*.

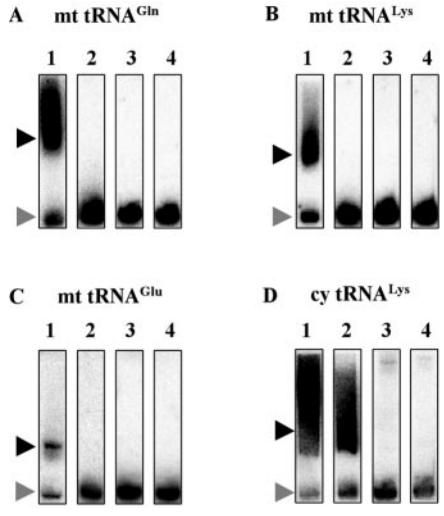


FIG. 2. **APM gel electrophoresis combined with Northern blotting of yeast mitochondrial tRNAs.** The presence of 2-thio modifications of the mt tRNA$^{Gln}$ (*A*), mt tRNA$^{Lys}$ (*B*), mt tRNA$^{Glu}$ (*C*), and cytoplasmic tRNA$^{Lys}$ (*D*) molecules in wild type yeast (*lanes 1* and *3*) or Δ*MTU1* yeast (*lanes 2* and *4*) were analyzed by APM gel electrophoresis and Northern blotting. The gels of *lanes 1* and *2* contain APM, whereas the gels of *lanes 3* and *4* lack APM. The retarded bands of the 2-thiolated tRNAs are marked by a *black arrowhead*, whereas the non-retarded bands of the unthiolated tRNAs are marked by a *gray arrowhead*.

that YDL033c is responsible for the 2-thio modification of mt tRNAs.

To confirm these observations, the mt tRNA$^{Lys}$ molecules (Fig. 3*A*) were isolated from the wild type strain and the Δ*MTU1* deletion strain and subjected to LC/MS analyses. Nucleoside analysis revealed that the mt tRNA from the wild type cells contains cmnm$^5$s$^2$U (5-carboxymethylaminomethyl-2-thiouridine) and its precursor cmnm$^5$U (5-carboxymethylaminomethyluridine) as the wobble modification (Fig. 3*B*, *left panel*). In the mt tRNA$^{Lys}$ from the deletion strain, however, there was a marked increase in the cmnm$^5$U peak, and the cmnm$^5$s$^2$U peak disappeared (Fig. 3*B*, *right panel*). The purified mt tRNAs$^{Lys}$ molecules from the wild type and mutant strains were also subjected to RNase $T_1$ digestion, which cleaves off the anticodon-containing fragment (Fig. 3*A*), and this fragment was then analyzed by LC/MS (Fig. 3*C*). In the wild-type mt tRNA$^{Lys}$, an anticodon-containing RNA fragment consisting of cmnm$^5$s$^2$U (5′-ΨCUcmnm$^5$s$^2$UUUt$^6$AAGp-3′ (3105.9 Da)) was prominently detected, whereas a fragment containing cmnm$^5$U instead of cmnm$^5$s$^2$U (5′-ΨCUcmnm$^5$UUUt$^6$AAGp-3′ (3089.9 Da)) was detected as a minor product. In contrast, in the tRNA molecules from the deletion strain, the cmnm$^5$U-containing fragment was detected as the major product and no cmnm$^5$s$^2$U-containing fragments could be seen. Thus, YDL033c encodes an enzyme that is responsible for synthesizing the 2-thio group on cmnm$^5$s$^2$U in the three mt tRNAs for Lys, Glu, and Gln. Thus, we have named this gene *MTU1* (mitochondrial tRNA-specific 2-thiouridylase 1).

*Direct Evidence of the Requirement of MTO1 and MSS1 in the C5 Modification of the Wobble Uridine in mt tRNAs*—In the modification of the *E. coli* tRNAs, the initial step of mnm$^5$U synthesis is known to require the *mnmE* (*trmE*) gene, because the disruption of *mnmE* resulted in defective mnm$^5$U synthesis (14, 41). The *gidA* gene also appears to be involved, as suggested by indirect genetic evidence (15, 16). Although *MSS1* and *MTO1* were found to be the respective homologs of the *mnmE* and *gidA* genes in both human and yeast (17, 18), it has

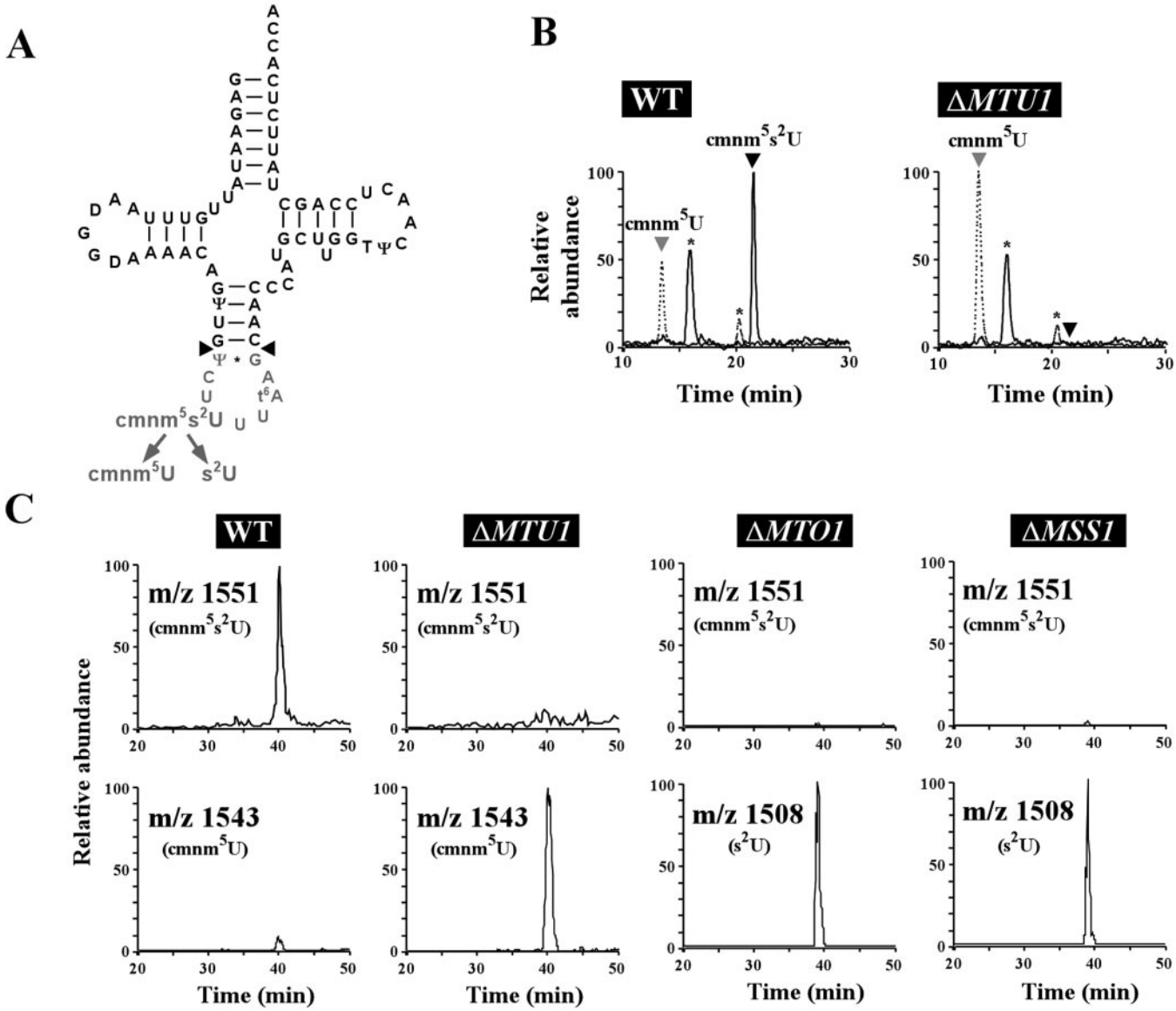


FIG. 3. **Mass spectrometric analysis of the tRNAs from the wild type and Δ*MTU1* yeast strains.** *A*, the secondary structure of yeast mt $tRNA^{Lys}$ bearing the following post-transcriptional modifications: dihydrouridine (*D*), pseudouridine (Ψ), ribothymidine (*T*), $N^6$-threonylcarbamoyladenosine ($t^6A$), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 5-carboxymethylaminomethyluridine ($cmnm^5U$), and 2-thiouridine ($s^2U$). The anticodon-containing fragment produced by digesting the tRNA with RNase $T_1$ is colored *gray*. The cleavage positions are shown by *arrowheads*. *B*, LC/MS analysis of the total nucleosides in the mt $tRNA^{Lys}$ molecules obtained from the wild type (*left panel*) and Δ*MTU1* (*right panel*) strains. Mass chromatograms of $cmnm^5U$ (*m/z* 332 at retention time 13.4, *dotted line*) and $cmnm^5s^2U$ (*m/z* 348 at RT 21.6, *solid line*) are shown by the proton adduct form of each nucleoside. Side peaks in RT 15.9 and RT 20.3 (*asterisks*) are assigned as artifacts with unknown chemical structures. *C*, LC/MS fragment analysis of RNase $T_1$-digested mt $tRNA^{Lys}$ molecules obtained from the wild type, Δ*MTU1*, Δ*MTO1*, and Δ*MSS1* strains. The mass chromatogram of doubly charged ions in the anticodon-containing fragment bearing $cmnm^5s^2U$ (*m/z* 1551) is shown in each of the *upper panels*. Mass chromatograms of the same fragments bearing $cmnm^5U$ (*m/z* 1543) are shown in the *lower left panels* for wild type and Δ*MTU1*, whereas the mass chromatograms of the same fragments carrying $s^2U$ (*m/z* 1508) are shown in the *lower right panels* for Δ*MTO1* and Δ*MSS1*.

never been demonstrated that these mitochondrial proteins are responsible for biosynthesizing the C5 modification of wobble uridines in mt tRNAs. To test this notion, yeast mt $tRNAs^{Lys}$ (Fig. 3*A*) molecules were isolated from *MTO1* and *MSS1* deletion strains and subjected to LC/MS analyses. First, total nucleoside analysis revealed that the mt $tRNAs^{Lys}$ from both deletion strains contains $s^2U$ (2-thiouridine) instead of the $cmnm^5s^2U$ found in the wild type $tRNA^{Lys}$ molecules (data not shown). RNA fragment analyses by LC/MS then also showed that the anticodon-containing RNA fragment of the mt $tRNAs^{Lys}$ molecules from the Δ*MTO1* and Δ*MSS1* strains bear $s^2U$ (5′-Ψ$CUs^2$UUU$t^6$AAGp-3′ (3018.9 Da)) but not $cmnm^5s^2U$ (Fig. 3*C*). These results directly show that *MSS1* and *MTO1* genes are both involved in the biosynthesis of the 5-carboxymethylaminomethyl group of $cmnm^5s^2U$ of mt $tRNA^{Lys}$.

*Synergism of MTU1 with MTO1 and MSS1 in Promoting Efficient Mitochondrial Decoding*—To determine the physiological effects of deleting yeast *MTU1*, the respiratory activity of the Δ*MTU1* strain was examined by analyzing its growth on non-fermentable (YPG) and fermentable (YPD) medium plates and by determining its oxygen consumption. As a control, $\rho^0$ cells grew normally on fermentable medium (YPD) but did not grow on the non-fermentable YPG plate (Fig. 4*A*). Growth defects of Δ*MTU1* on the YPD plate were not seen, but significant growth defect could be observed on the YPG plate. This can be explained by the fact that Δ*MTU1* showed strongly defective oxygen consumption (Fig. 4*B*). In addition, when we analyzed the mitochondrial protein synthesis in the wild type

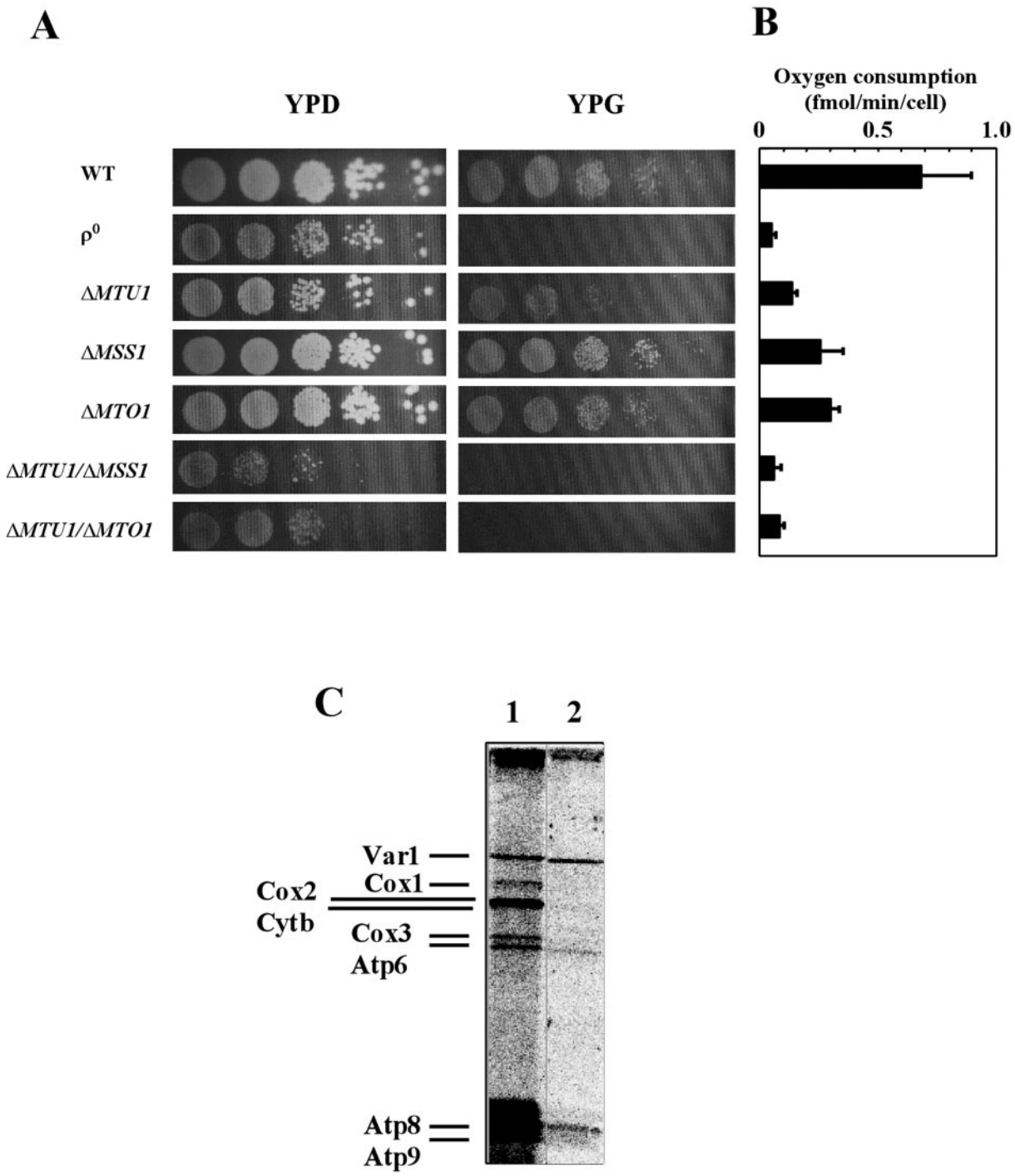


FIG. 4. **Growth properties and mitochondrial activity of strains lacking the enzymes that modify the wobble position of mt tRNAs.** *A*, growth of the wild type, three single deletion mutant (Δ*MTU1*, Δ*MTO1*, and Δ*MSS1*) and two double deletion mutant (Δ*MTU1*/Δ*MSS1* and Δ*MTU1*/Δ*MTO1*) strains. Each strain was serially diluted starting with $10^5$ cells and then spotted onto fermentable YPD and non-fermentable YPG plates and incubated at 30 °C for 72 h. *B*, oxygen consumption of each mutant strain. The rate of oxygen consumption is shown along with the S.D. values obtained from three independent experiments. *C*, mitochondrial protein synthesis in the wild type (*lane 1*) and Δ*MTU1* (*lane 2*) strains was determined by *in vivo* pulse-labeling of mt DNA products with [$^{35}$S]methionine. The mt DNA products are identified on the *left*.

and Δ*MTU1* strains by [$^{35}$S]Met pulse-labeling experiments, we found it was severely reduced in the Δ*MTU1* strain (Fig. 4*C*). These results suggest that the 2-thio modification of mt tRNAs is essential for mitochondrial protein synthesis, which directly reflects the respiratory activity of mitochondria.

Because *MSS1* and *MTO1* appear to be responsible for the biosynthesis of the C5 modification in cmnm$^5$s$^2$U, we assessed whether the 2-thio group and the C5 modification act synergistically in the decoding activity of mt tRNAs. The Δ*MSS1* and Δ*MTO1* strains show a growth phenotype that is similar to that of the wild-type cell on both YPD and YPG plates (Fig. 4*A*), as has been previously reported for cells with paromomycin-sensitive genetic backgrounds (19, 42). However, both Δ*MSS1* and Δ*MTO1* did show decreased rates of oxygen consumption (Fig. 4*B*), which indicates that defective C5 modification causes mitochondrial dysfunction to some extent, although the effect is weaker than that of Δ*MTU1*. We then constructed two double knock-out strains, namely, Δ*MTU1*/Δ*MSS1* and Δ*MTU1*/Δ*MTO1*, which should contain mt tRNAs with unmodified wobble uridines, because C5 and 2-thio modifications occur inde-

pendently. Both strains showed a severe growth defect on even the YPD plate, and no growth was observed on the YPG plate (Fig. 4*A*). Moreover, almost no respiratory activity could be seen in either strain (Fig. 4*B*). The severe phenotypes of these double knock-out strains demonstrate that both the 2-thio and C5 modifications are required for efficient decoding in mitochondrial translation.

*Identification and Characterization of the Human MTU1 Gene*—The human homolog of *MTU1* was introduced into the yeast Δ*MTU1* strain to see whether it can complement yeast *MTU1*. The Δ*MTU1* strain bearing the human *MTU1* gene (ΔMTU1+pHMTU1) was now able to grow on YPG plates (Fig. 5*A*), and its rate of oxygen consumption was increased slightly relative to the Δ*MTU1* strain (Fig. 5*B*). The 2-thio modification of the mt $tRNA^{Lys}$ in this strain was then examined by APM gel electrophoresis. This revealed that the introduction of human *MTU1* resulted in a slight but apparent recovery of the 2-thio modification in the Δ*MTU1* strain (Fig. 5*C*). The relatively weak complementation observed may be the result of the experimental system employed here, as we found that placing the yeast *MTU1* gene back into the Δ*MTU1* strain by transfecting the cells with pYMTU1 also did not completely restore the 2-thio modification or the oxygen consumption in the Δ*MTU1* strain (Fig. 5*C*). Thus, it seems that this experimental design does not lead to complete complementation. Nevertheless, these experiments still clearly show that the human *MTU1* gene works as a 2-thiouridylase in yeast cells.

It has been reported that certain genes responsible for tRNA modification work not only as tRNA-modifying enzymes but also as molecular chaperons for tRNAs, because the phenotype resulting from their gene deletion could be restored by introducing the enzyme genes that have been inactivated by mutating the active center of the enzymes (43). To determine whether the mitochondrial activity associated with *MTU1* depends on its 2-thio modification of the mt tRNAs or another function, we constructed inactive yeast and human *MTU1* mutants by, respectively, introducing the D38A and D16A point mutations into their P-loops. These mutant genes were then subjected to the complementation test, but neither promoted YPG plate growth or oxygen consumption of the Δ*MTU1* strain (Fig. 5, *A* and *B*). This indicates that the highly conserved Asp residue in the P-loop works as an essential residue for both yeast and human MTU1 and that the enzymatic function of MTU1 is required for its role in mitochondrial activity.

The subcellular localization of human MTU1 was then examined by transiently expressing EGFP-fused MTU1 (MTU1-EGFP) in HeLa cells. Judging from the N-terminal sequences of human and yeast MTU1, the probability that they localize in the mitochondria is 0.53% and 0.87%, respectively, according to PSORT II Prediction analysis (44) (available at psort.nibb.ac.jp/). As shown in Fig. 5*D*, MTU1-EGFP indeed localizes mainly in the mitochondria. Recently, it has been reported that mouse 5-methylaminomethyl-2-thiouridylate-methyltransferase (TRMU) homolog localizes in mitochondria, although there is no direct evidence of this gene to be responsible for mt tRNA modification (45). This is consistent with the observation that MTU1 is a mitochondrial tRNA-specific 2-thiouridylase.

*Knocking Down Human MTU1 Results in Mitochondrial Dysfunction*—We have shown that human *MTU1* complements yeast *MTU1* and localizes in mitochondria. To obtain direct evidence that human *MTU1* is responsible for the 2-thio modification of $\tau m^5 s^2 U$ in mt tRNAs, five siRNAs (MTU1-1 through -5, see "Experimental Procedures") that target human *MTU1* were designed by an algorithm that predicts efficacious siRNA sequences.[2] HeLa cells were transfected with each siRNA or with siRNA targeting luciferase as a control and harvested 72 h later. The total RNAs were then subjected to semi-quantitative reverse transcription (RT)-PCR to estimate the knockdown activity of each siRNA. Each of the five *MTU1* siRNAs caused a potent reduction in the *MTU1* mRNA levels but had no effect on the glyceraldehyde-3-phosphate dehydrogenase mRNA levels, which served as a control. We chose MTU1-1 siRNA for further experiment. Judging from the band intensity of the RT-PCR products, *MTU1* mRNA levels clearly decreased by MTU1-1 siRNA (Fig. 6*A*). APM gel-Northern analysis was then carried out to measure the degree of 2-thio modification of mt $tRNA^{Lys}$ in the *MTU1* siRNA-transfected cells. A large part of the 2-thiolated $tRNA^{Lys}$ molecules from control luciferase siRNA-transfected cells that were retarded in the APM gel disappeared when *MTU1*-targeting siRNAs were introduced (Fig. 6*B*). The rate of oxygen consumption of the *MTU1* siRNA-transfected cells was also clearly decreased as compared with the wild-type cells (Fig. 6*C*), although this is shown as raw data, because it is difficult to obtain large number of cells that are transfected by siRNA. We also examined the mitochondrial membrane potential of the knockdown cells by staining HeLa cells transfected by *MTU1*- or luciferase-targeting siRNAs with both MitoTracker Red and Green, which are fluorescent indicators for staining mitochondria (Fig. 6*D*). MitoTracker Red is an indicator of mitochondrial membrane potential ($\Delta\Psi$). The mitochondria of the control luciferase siRNA-transfected cells showed good staining with both dyes, and when the two images were superimposed they revealed a well developed meshwork. In contrast, the *MTU1*-knockdown cells had granular-shaped mitochondria that stained poorly with MitoTracker Red. Thus, knocking down human *MTU1* results in mitochondria with a defective membrane potential, which is consistent with a phenotypic feature of cells from MERRF patients who carry the A8344G mutation (46, 47).

## DISCUSSION

In this study, we describe our identification of *MTU1* as a mitochondrial-specific 2-thiouridylase that is responsible for the wobble modification of yeast and human mt tRNAs. The disruption of yeast *MTU1* results in a 2-thio modification defect in the three mt tRNAs (for Lys, Glu, and Gln) that were examined. Pulse-labeling experiments with the Δ*MTU1* strain indicated that the 2-thio modification of the mt tRNAs is required for efficient mitochondrial protein synthesis. The human homolog of *MTU1* complemented the Δ*MTU1* strain as its introduction caused the 2-thio modification of mt tRNAs and the oxygen consumption of the strain to recover slightly. Moreover, siRNA-induced knocking down of human *MTU1* in HeLa cells resulted in a 2-thio modification defect in their mt tRNAs. This is the first time a reverse genetic approach utilizing siRNA to study a mammalian RNA modification enzyme has been reported. This specific repression of the human *MTU1* gene resulted in reduced oxygen consumption and defective mitochondrial membrane potentials. This nicely explains our previous observation that the wobble modification deficiency of mt $tRNA^{Lys}$ bearing the 8344 mutation results in poor decoding of cognate codons (11). However, it should be noted that the effects of knocking down *MTU1* may not completely correspond to the consequences of the 8344 mt $tRNA^{Lys}$ mutation, because *MTU1* suppression may cause three mt tRNAs for Lys, Glu, and Gln to be inadequately modified. Consequently, it is likely that the knockdown of *MTU1* causes the wobble base of the mt tRNAs for Lys, Glu, and Gln to lack the 2-thio modification and therefore to only carry the $\tau m^5 U$ modification. The MERRF-associated 8344 mutation, on the other hand, results in the complete absence of any modifications of mt $tRNA^{Lys}$ only (*i.e.* both the C5 and 2-thio modifications are absent). Nevertheless, our observations indicate that the 2-thio modification defi-

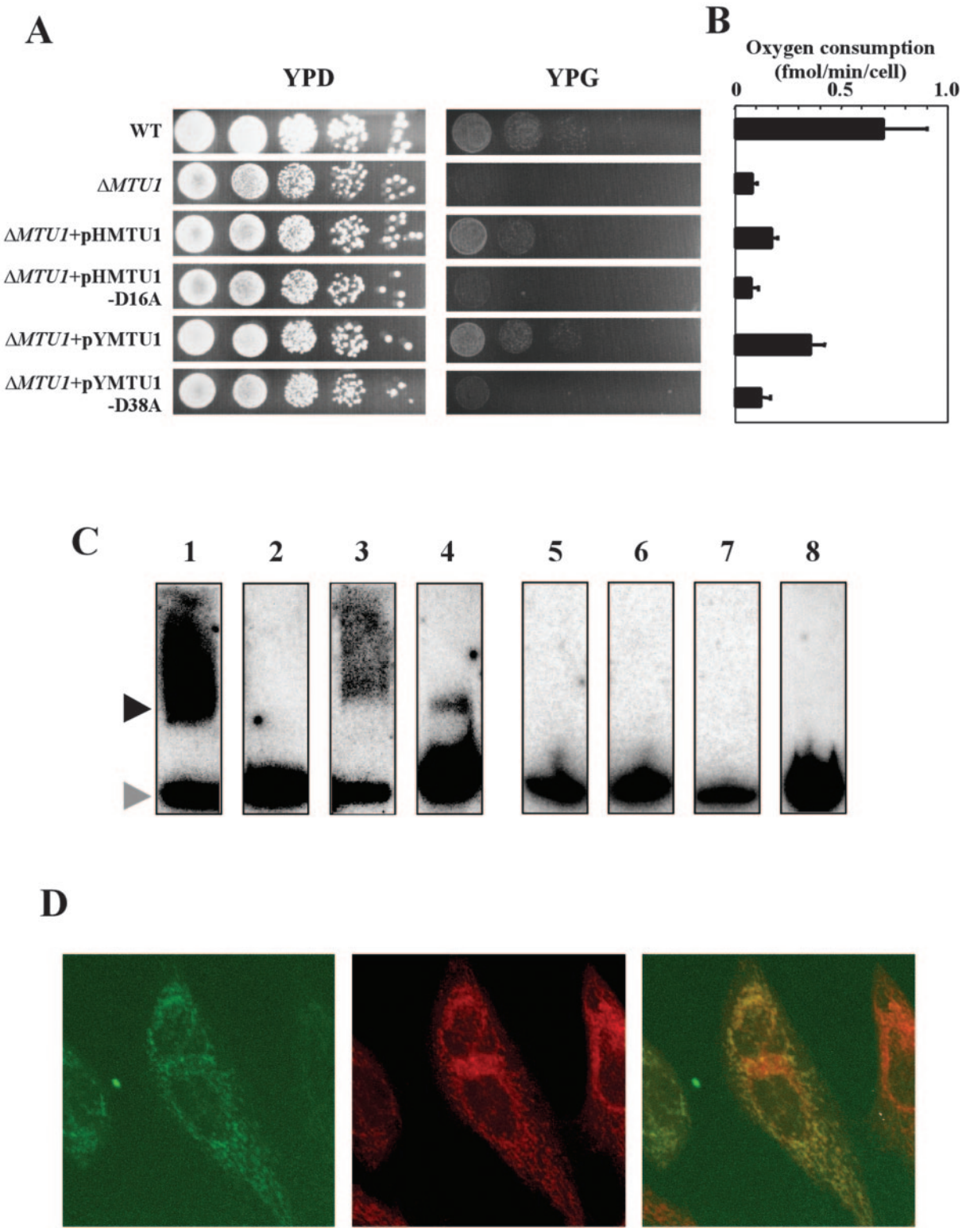


FIG. 5. **Suppression of the respiratory defect of Δ*MTU1* by introducing the human or yeast *MTU1* genes and mitochondrial localization of the MTU1 protein.** *A*, growth of the wild type, Δ*MTU1*, and the *MTU1* strain bearing a plasmid encoding the yeast *MTU1* (pYMTU1) or human *MTU1* (pHMTU1) gene. Also examined were *MTU1* strains bearing pYMTU1 or pHMTU1 containing an inactivating mutation (pYMTU1-D38A and pHMTU1-D16A, respectively). Starting with $10^5$ cells, serial dilutions were spotted onto YPD, and YPG plates and incubated at 30 °C for 72 h. *B*, rate of oxygen consumption for each strain. The rate of oxygen consumption is shown along with the S.D. values obtained from three independent experiments. *C*, APM-Northern analysis of the 2-thiolation in the mt $tRNA^{Lys}$ molecules from the wild type (*lanes 1* and *5*), Δ*MTU1* (*lanes 2* and *6*), Δ*MTU1* with pYMTU1 (*lanes 3* and *7*), and Δ*MTU1* with pHMTU1 (*lanes 4* and *8*) strains. The polyacrylamide gels of *lanes 1–4* contain APM, while the gels of *lanes 5–8* lack APM. The retarded bands of the 2-thiolated tRNAs are marked by a *black arrowhead*. The non-retarded bands of the non-thiolated tRNA are marked by a *gray arrowhead*. *D*, mitochondrial localization of the MTU1 protein. HeLa cells were transiently transfected with pHMTU1/EGFP, which expresses human MTU1 fused with EGFP. The intracellular localization of the MTU1-EGFP fusion protein was then visualized by the green fluorescence of EGFP by using a confocal fluorescence microscope (*left panel*). The mitochondria were stained by MitoTracker Red (Molecular Probes), and its red fluorescence was simultaneously observed (*middle panel*). Both panels were merged (*right panel*) to show the complete overlap of the two fluorescence patterns.

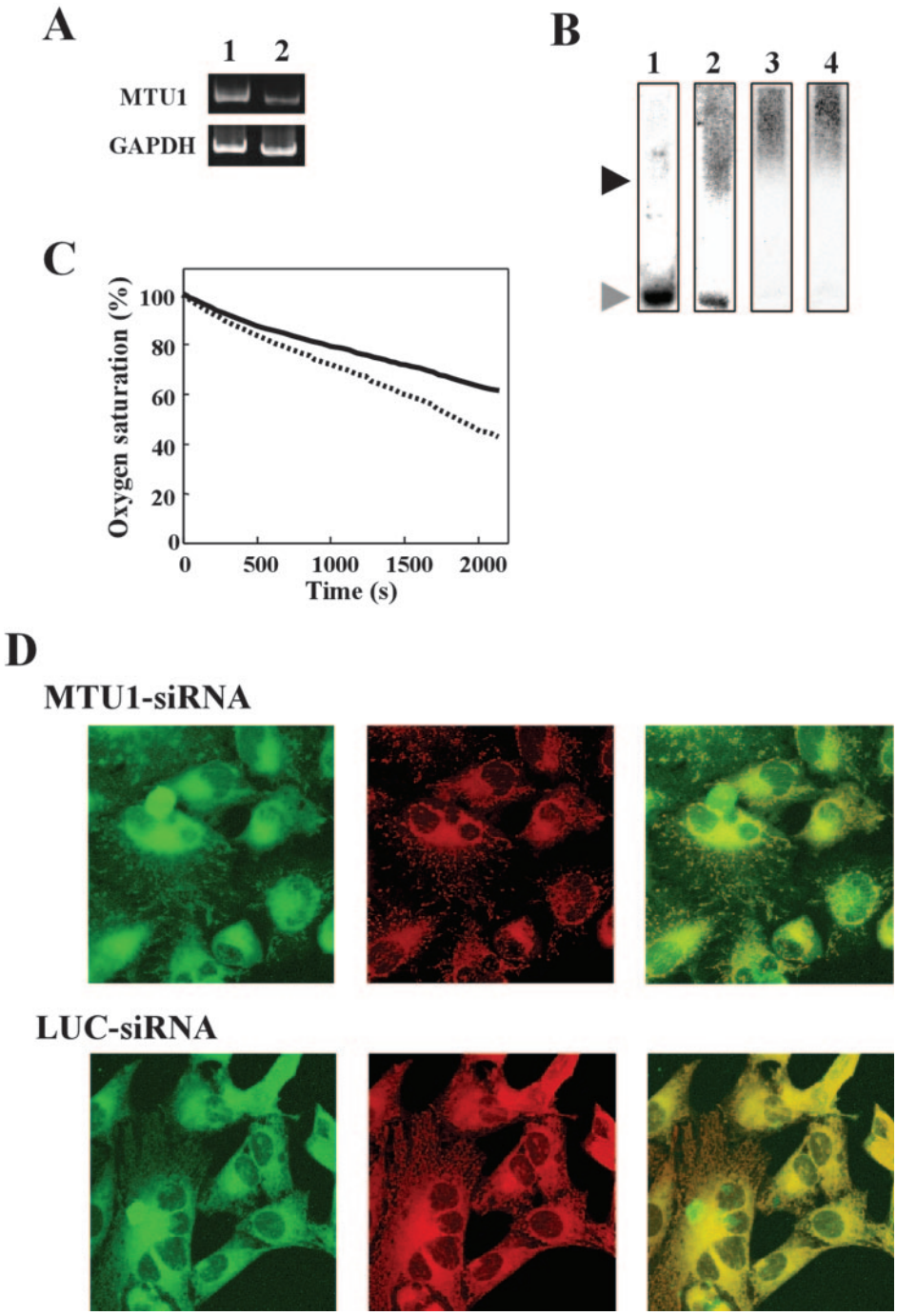


FIG. 6. **siRNA-induced knockdown of human *MTU1* results in mitochondrial dysfunction.** *A*, the *MTU1* mRNA levels of HeLa cells transfected with siRNAs that target *MTU1* (MTU1-1) (*lane 2*) or luciferase (*lane 1*) were quantified by RT-PCR (35 cycles). Glyceraldehyde-3-phosphate dehydrogenase mRNA was quantified as a control. *B*, APM-Northern analysis of the 2-thiolation of mitochondrial (*lanes 1* and *2*) or cytosolic (*lanes 3* and *4*) $tRNAs^{Lys}$ molecules from HeLa cells transfected with siRNAs that target *MTU1* (*lanes 1* and *3*) or luciferase (*lanes 2* and *4*). The retarded bands of the 2-thiolated tRNAs are marked by a *black arrowhead*. The non-retarded bands of non-thiolated tRNAs are marked by a *gray arrowhead*. *C*, the rates of oxygen consumption of HeLa cells transfected with siRNAs targeting *MTU1* (*dotted line*) or luciferase (*solid line*) were measured by a Clark type electrode. *D*, visualizing the mitochondrial membrane potential of *MTU1*-knocked down HeLa cells. The cells transfected with siRNAs targeting *MTU1* or luciferase are shown in the *upper* and *lower panels*, respectively. The mitochondria were visualized by staining the cells with MitoTracker Green (*left panels*), and its membrane potentials were simultaneously indicated by staining with MitoTracker Red (*middle panels*). Both panels were merged (*right panels*) to show the different intensities of the two fluorescence patterns.

ciency of mt tRNA induces strong mitochondrial dysfunction and that this may at least partly explain the mitochondrial disorders seen in MERRF patients.

It has been reported that tRNA-modifying enzymes have other function. An *E. coli* deletion strain of *truB* encoding pseudouridylase for position 55 shows growth defect in co-culturing with wild type strain. However, the phenotype of this strain is recovered by introducing a plasmid containing *truB* mutant whose product has no catalytic activity (43). In addition, *E. coli trmA* encoding $m^5U54$-methyltransferase is an essential gene, but its catalytic activity for methylation is not required for cell viability (48). These facts suggest that some RNA-modifying enzymes are multifunctional proteins. To test this possibility for yeast MTU1, point mutations D16A and D38A in the P-loop were respectively introduced into human and yeast MTU1 proteins. Because these mutants could not

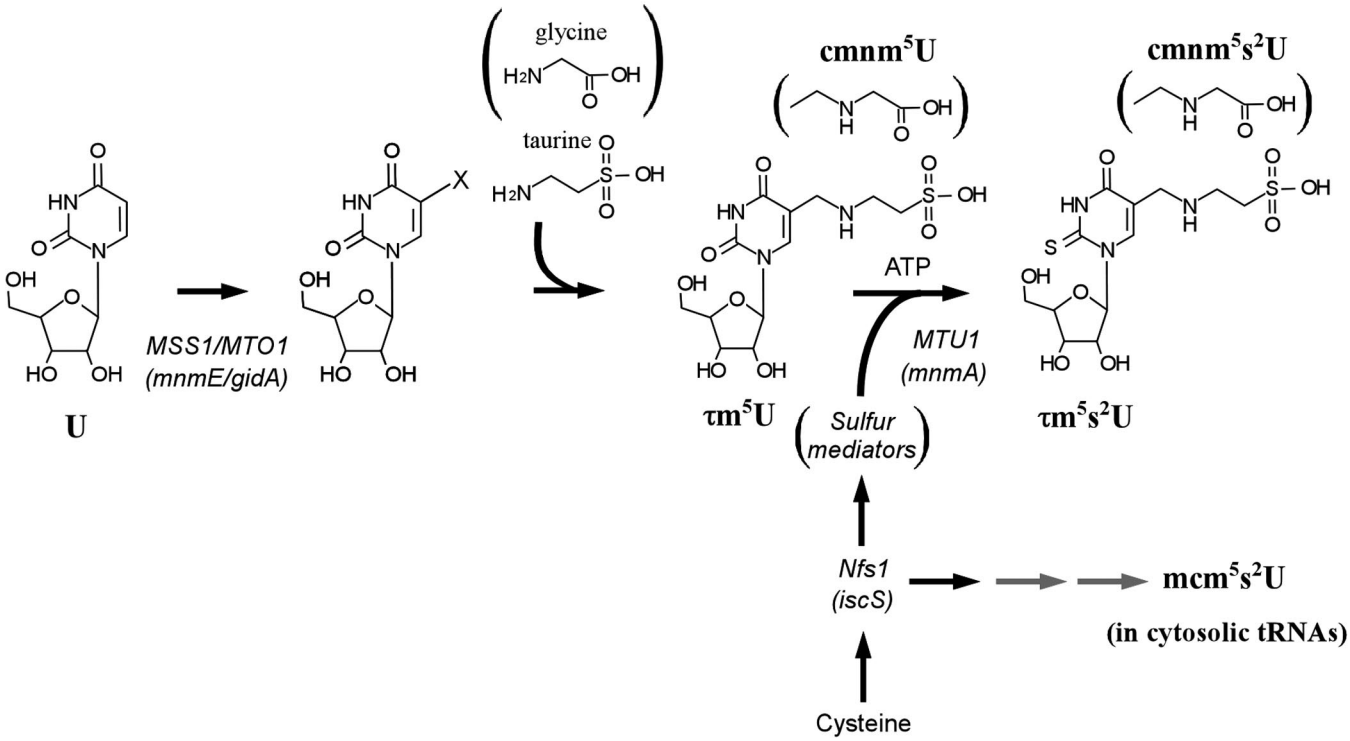


FIG. 7. **Biosynthetic pathway that introduces the $\tau m^5s^2U$ ($cmnm^5s^2U$) modification of mitochondrial tRNAs.** *MSS1* and *MTO1* are involved in the initial step of $\tau m^5s^2U$ ($cmnm^5s^2U$) synthesis on mt tRNAs. Mitochondrial taurine or glycine is subsequently incorporated into mt tRNAs by unidentified transferases to build $\tau m^5s^2U$ or $cmnm^5s^2U$, respectively. *Nfs1* is responsible for the initial step in the 2-thiolation of $\tau m^5s^2U$ ($cmnm^5s^2U$) in mt tRNAs and $mcm^5s^2U$ in cytoplasmic tRNAs (20). The sulfur from cysteine is transferred to unknown sulfur mediators by Nfs1p. MTU1 then acts as a mitochondria-specific 2-thiouridylase for $\tau m^5s^2U$ ($cmnm^5s^2U$) by using the activated sulfur from the mediators.

complement the Δ*MTU1* strain and restore the 2-thio modification, the conserved Asp residue in the P-loop is essential for enzymatic function of MTU1, which seems to be required for restoration of mitochondrial activity. However, introduction of a plasmid containing wild type MTU1 did not completely restore the 2-thio modification and oxygen consumption of Δ*MTU1* strain, indicating that there is an unknown cis element for MTU1 expression to be investigated.

*MTU1* bears the highly conserved P-loop motif (SGGXDS) in its N-terminal domain. This motif occurs widely in the pyrophosphate synthetase family (Fig. 1) (40). The P-loop is known to participate in the binding and hydrolysis of the α–β phosphate bond of ATP. Other tRNA-modifying enzymes with the P-loop motif also exist. One is *TilS*, an essential tRNA-modifying enzyme that was recently identified by our group to be responsible for synthesizing lysidine at position 34 of the bacterial $tRNA^{Ile}$ molecules that are specific for the AUA codon (31). Also bearing the P-loop motif is *ThiI*, a thiouridine synthetase that is responsible for the synthesis of 4-thiouridine at position 8 ($s^4U8$) of bacterial tRNAs (49, 50). Both enzymes require ATP as the energy source for the reaction, as does *E. coli mnmA*, the homolog of *MTU1* (39). More recently, *TtcA* was identified as a P-loop-containing modification enzyme for 2-thiocytidine at position 32 ($s^2C32$) (51). Thus, the P-loop motif is commonly found in enzymes responsible for RNA modifications that target the carbonyl carbon of bases in tRNAs, which suggests a similar reaction mechanism is involved in these modifications. According to the reaction mechanism employed by GMP synthetase, which belongs to the pyrophosphate synthetase family (52), it is thus likely that the first step of 2-thiouridine synthesis is the activation of the C-2 carbonyl group of the wobble uridine by the addition of AMP to form an adenylate intermediate. Nucleophilic attack on the adenylate by an activated sulfur carried on an unidentified protein probably completes the reaction. The details of the mechanism of 2-thiouridine formation will be clarified in the near future.

It has been shown previously that a mutation in *MTO1* caused yeast cells to exhibit a respiratory defect only when coupled with a paromomycin-resistant mutation in mitochondrial 15 S rRNA (19). This phenotype was similar to that induced by a mutation in *MSS1* (42). Indeed, Mto1p and Mss1p were shown to form a heterodimer complex (19). It has not been reported for *E. coli* that the products of the *gidA* gene (the bacterial *MTO1* homolog) and the *mnmE* (*MSS1* homolog) directly interact. However, genetic evidence has suggested that *gidA* is responsible for C5 modification along with *mnmE* (14–16). Nevertheless, direct evidence for this has not been reported. In our study reported here, we show by analyzing the tRNA molecules from the Δ*MTO1* yeast strains that *MTO1* (*gidA*) are indeed responsible for the initial step of C5 modification. Disruption of *MTO1* or *MSS1* resulted in a normal growth phenotype, even upon culture on YPG plates, which is consistent with previous reports (19, 42). However, both the Δ*MTO1* and Δ*MSS1* strains showed reduced oxygen consumption, which suggests that a decoding disorder arises from a deficiency in C5 modification of mt tRNAs. Notably, when *MTO1* or *MSS1* were disrupted along with Δ*MTU1*, a severe reduction in respiratory activity resulted. This indicates that the C5 and 2-thio modifications of the wobble uridine act synergistically in promoting cognate codon decoding efficiency. Because the human homologs of *MSS1* and *MTO1* have been shown to be localized in the mitochondria (17, 18), it appears that these proteins are responsible for the initial steps taken to synthesize the $\tau m^5U$ or $\tau m^5s^2U$ modifications of mt tRNAs. It is also likely that there are some unidentified genes other than *MSS1* and *MTO1* that participate in these modifications, including a putative taurine-transferase for the biosynthesis of $\tau m^5U$ (5). To understand how the C5 modification deficiency can contribute to the molecular pathogenesis of MELAS, it will be necessary to identify the enzyme genes that are responsible

for the biosynthesis of $\tau m^5U$ and to be able to reconstitute this whole biosynthesis pathway *in vitro*.

Fig. 7 shows a summary of the $\tau m^5s^2U$ biosynthetic pathway. Our analyses of the $tRNA^{Lys}$ modifications in the Δ*MTO1*, Δ*MSS1*, and Δ*MTU1* yeast (Fig. 3*C*) indicate that both the C5 and 2-thio modifications appear to proceed independently. Concerning the 2-thio modification, *Nfs1*, an eukaryotic homolog of bacterial *iscS*, has been found to be a master enzyme gene involved in the 2-thio modification of both mitochondrial and cytoplasmic tRNAs (20). Nfs1p works as a supplier of activated sulfur from cysteine to be utilized in iron-sulfur cluster biogenesis, and it is also known to be involved in thio modifications of tRNAs. Moreover, it has been reported that recombinant IscS and MnmA (the MTU1 homolog) cooperate to synthesize 2-thio modifications *in vitro* by using ATP and cysteine as substrates (39). However, we have found that additional genes are indispensable for the biosynthesis of 2-thio modifications in *E. coli* tRNAs.[3] Thus, there must be sulfur mediators that act between Nfs1p and MTU1. MTU1 may directly recognize mt tRNAs and synthesize the 2-thio group by using ATP and the sulfur served by the mediator. With regard to the C5 modification, we have shown that *MTO1* and *MSS1* are directly involved in the initial step of its biosynthesis. It is likely that subsequent steps that involve as yet unknown genes finally lead to the synthesis of $\tau m^5s^2U$ through the incorporation of taurine supplied from plasma (5).

We have shown that MTU1 is not involved in synthesizing 2-thio modification of $mcm^5s^2U$ in cytoplasmic tRNAs, showing the presence of an unknown enzyme in cytoplasm. It has been reported that yeast *Trm9* gene encodes methyltransferase which is responsible for C5 modification of $mcm^5U$ and $mcm^5s^2U$ (53). Although disruption of *Trm9* gene resulted in complete loss of $mcm^5s^2U$ in $tRNA^{Glu}$, it is not clear whether it is an unmodified uridine or $s^2U$ in this report. Recently, our group has demonstrated that $tRNA^{Glu}$ from the *Trm9* deletion strain has an unmodified wobble uridine, suggesting that 2-thio modification occurs in coupling with C5 modification in yeast cytoplasm.[4] It is a distinct biosynthesis with bacterial and mitochondrial 5-methyl-2-thiouridine type modifications ($xm^5s^2U$).

In bacterial and mitochondrial tRNAs for Lys, Gln, and Glu, the U at the wobble position is modified to $xm^5s^2U$ such as $mnm^5s^2U$, $cmnm^5s^2U$, $mcm^5s^2U$, and $\tau m^5s^2U$ (1, 3–5, 54, 55). Because these tRNAs are responsible for decoding two codon sets that end in purine (R) (*i.e.* NNR), the $xm^5s^2U$ modifications participate in preventing the misreading of the pyrimidine (Y)-ending near cognate codons (NNY) (56). $xm^5s^2U$ is largely fixed in the C3′-end form (56, 57). Due to this conformational rigidity, the $xm^5s^2U$ modification prefers to base pair with A and prevents misreading of NNY codons (56, 58, 59). In addition, it was reported that 2-thio modification of $mnm^5s^2U$ in *E. coli* $tRNA^{Lys}$ confers efficient ribosome binding (12, 55, 60). Furthermore, the C5-taurinomethyl group of $\tau m^5U$ was shown to be required for the efficient decoding of the UUG codon by stabilizing the U:G wobble-base pairing on the ribosomal A site (9, 61).

We have speculated that the MERRF A to G point mutation at the 8344 position of mt $tRNA^{Lys}$ may act as a negative determinant that inhibits the biosynthesis of both the C5 and 2-thio modifications involved in generating $\tau m^5s^2U$ (5, 7). Because we have found that human *MTU1* is a mitochondrial specific 2-thiouridylase that participates in generating $\tau m^5s^2U$ of mt tRNAs, it will be possible to determine whether and how MTU1 can recognize MERRF mt $tRNA^{Lys}$ molecules once the biosynthetic pathway leading to the 2-thio modification can be reconstituted *in vitro*. If we find that the 8344 mutation is in a critical position for MTU1 recognition, it may be that MTU1 recognizes the whole architecture of mt tRNAs, because position 8344 in mt $tRNA^{Lys}$ is far from the wobble position. Furthermore, if we could successfully design mutant MTU1 molecules that efficiently recognize MERRF mt $tRNA^{Lys}$ molecules, it may be possible to restore the 2-thio modification of MERRF mt $tRNA^{Lys}$ by introducing the mutant MTU1 from a plasmid vector. This could potentially lead to the development of a gene therapy for MERRF.

*Acknowledgments*—We are grateful to Dr. Takayuki Katoh (University of Tokyo) for the design of the siRNAs. We also thank Drs. Naoki Shigi (University of Tokyo), Yumi Nakai (Osaka Medical College), and Narumi Hino (University of Tokyo) for materials, fruitful discussions, and technical advice.

REFERENCES

1. Bjork, G. R. (1995) in *Biosynthesis and Function of Modified Nucleosides* (Soll, D., and Rajbhandary, U. L., ed) pp. 165–205, ASM Press, Washington, D. C.
2. Curran, J. F. (1998) in *Modified Nucleosides in Translation* (Grojean, H., and Benn, R., ed) pp. 493–516, ASM Press, Washington, D. C.
3. Yokoyama, S., and Nishimura, S. (1995) in *Modified Nucleosides and Codon Recognition* (Soll, D., and Rajbandary, U. L., eds) pp. 207–224, ASM Press, Washington, D. C.
4. Suzuki, T. (2005) in *Trends in Current Genetics* (Grosjean, H., ed) Chap. 1, Springer-Verlag, New York, in press
5. Suzuki, T., Suzuki, T., Wada, T., Saigo, K., and Watanabe, K. (2002) *EMBO J.* **21,** 6581–6589
6. Yasukawa, T., Suzuki, T., Suzuki, T., Ueda, T., Ohta, S., and Watanabe, K. (2000) *J. Biol. Chem.* **275,** 4251–4257
7. Yasukawa, T., Suzuki, T., Ishii, N., Ueda, T., Ohta, S., and Watanabe, K. (2000) *FEBS Lett.* **467,** 175–178
8. Goto, Y., Nonaka, I., and Horai, S. (1990) *Nature* **348,** 651–653
9. Kirino, Y., Yasukawa, T., Ohta, S., Akira, S., Ishihara, K., Watanabe, K., and Suzuki, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 15070–15075
10. Shoffner, J. M., Lott, M. T., Lezza, A. M., Seibel, P., Ballinger, S. W., and Wallace, D. C. (1990) *Cell* **61,** 931–937
11. Yasukawa, T., Suzuki, T., Ishii, N., Ohta, S., and Watanabe, K. (2001) *EMBO J.* **20,** 4794–4802
12. Ashraf, S. S., Sochacka, E., Cain, R., Guenther, R., Malkiewicz, A., and Agris, P. F. (1999) *RNA (N. Y.)* **5,** 188–194
13. Yarian, C., Townsend, H., Czestkowski, W., Sochacka, E., Malkiewicz, A. J., Guenther, R., Miskiewicz, A., and Agris, P. F. (2002) *J. Biol. Chem.* **277,** 16391–16395
14. Elseviers, D., Petrullo, L. A., and Gallagher, P. J. (1984) *Nucleic Acids Res.* **12,** 3521–3534
15. Nakayashiki, T., and Inokuchi, H. (1998) *J. Bacteriol.* **180,** 2931–2935
16. Bregeon, D., Colot, V., Radman, M., and Taddei, F. (2001) *Genes Dev.* **15,** 2295–2306
17. Li, R., Li, X., Yan, Q., Qin Mo, J., and Guan, M. X. (2003) *Biochim. Biophys. Acta* **1629,** 53–59
18. Li, X., and Guan, M. X. (2002) *Mol. Cell. Biol.* **22,** 7701–7711
19. Colby, G., Wu, M., and Tzagoloff, A. (1998) *J. Biol. Chem.* **273,** 27945–27952
20. Nakai, Y., Umeda, N., Suzuki, T., Nakai, M., Hayashi, H., Watanabe, K., and Kagamiyama, H. (2004) *J. Biol. Chem.* **279,** 12363–12368
21. Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 599–604
22. Urbina, H. D., Silberg, J. J., Hoff, K. G., and Vickery, L. E. (2001) *J. Biol. Chem.* **276,** 44521–44526
23. Muhlenhoff, U., and Lill, R. (2000) *Biochim. Biophys. Acta* **1459,** 370–382
24. Lauhon, C. T. (2002) *J. Bacteriol.* **184,** 6820–6829
25. Nilsson, K., Lundgren, H. K., Hagervall, T. G., and Bjork, G. R. (2002) *J. Bacteriol.* **184,** 6830–6835
26. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S. Storms, R. K., Véronneau, S., Voet, M., Volckhaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Phillippsen, P., Johnston, M., and Davis, R. W. (1999) *Science* **285,** 901–906
27. Guthrie, C., and Fink, G. R. (1991) in *Guide to Yeast Genetics and Molecular Biology* (Fox, T. D., Folley, L. S., Mulero, J. J., McMullin, T. W., Thorsness, P. E., Hedin, L. O., and Costanzo, M. C., eds) pp. 149–165, Academic Press, San Diego
28. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
29. Holley, R. W. (1963) *Biochem. Biophys. Res. Commun.* **10,** 186–188
30. Kaneko, T., Suzuki, T., Kapushoc, S. T., Rubio, M. A., Ghazvini, J., Watanabe, K., Simpson, L., and Suzuki, T. (2003) *EMBO J.* **22,** 657–667
31. Soma, A., Ikeuchi, Y., Kanemasa, S., Kobayashi, K., Ogasawara, N., Ote, T., Kato, J., Watanabe, K., Sekine, Y., and Suzuki, T. (2003) *Mol. Cell* **12,**

[3] N. Umeda, T. Suzuki, M. Yukawa, Y. Ohya, H. Shindo, K. Watanabe, and T. Suzuki, unpublished results.

[4] A. Noma *et al.*, manuscript in preparation.

689–698
32. Apffel, A., Chakel, J. A., Fischer, S., Lichtenwalter, K., and Hancock, W. S. (1997) *Anal. Chem.* **67,** 1320–1325
33. Shigi, N., Suzuki, T., Tamakoshi, M., Oshima, T., and Watanabe, K. (2002) *J. Biol. Chem.* **277,** 39128–39135
34. Igloi, G. L. (1988) *Biochemistry* **27,** 3842–3849
35. Barrientos, A., Korr, D., and Tzagoloff, A. (2002) *EMBO J.* **21,** 43–52
36. Katoh, T., Susa, M., Suzuki, T., Umeda, N., Watanabe, K., and Suzuki, T. (2003) *Nucleic Acids Res. Suppl.* **3,** 249–250
37. Mihara, H., Kato, S., Lacourciere, G. M., Stadtman, T. C., Kennedy, R. A., Kurihara, T., Tokumoto, U., Takahashi, Y., and Esaki, N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 6679–6683
38. Sullivan, M. A., Cannon, J. F., Webb, F. H., and Bock, R. M. (1985) *J. Bacteriol.* **161,** 368–376
39. Kambampati, R., and Lauhon, C. T. (2003) *Biochemistry* **42,** 1109–1117
40. Bork, P., and Koonin, E. V. (1994) *Proteins* **20,** 347–355
41. Hagervall, T. G., Pomerantz, S. C., and McCloskey, J. A. (1998) *J. Mol. Biol.* **284,** 33–42
42. Decoster, E., Vassal, A., and Faye, G. (1993) *J. Mol. Biol.* **232,** 79–88
43. Gutgsell, N., Englund, N., Niu, L., Kaya, Y., Lane, B. G., and Ofengand, J. (2000) *RNA* (*N. Y.*) **6,** 1870–1881
44. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911
45. Yan, Q., and Guan, M. X. (2004) *Biochim. Biophys. Acta* **1676,** 119–126
46. James, A. M., Wei, Y. H., Pang, C. Y., and Murphy, M. P. (1996) *Biochem. J.* **318,** 401–407
47. Antonicka, H., Floryk, D., Klement, P., Stratilova, L., Hermanska, J., Houstkova, H., Kalous, M., Drahota, Z., Zeman, J., and Houstek, J. (1999) *Biochem. J.* **342,** 537–544
48. Persson, B. C., Gustafsson, C., Berg, D. E., and Bjork, G. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3995–3998
49. Mueller, E. G., Buck, C. J., Palenchar, P. M., Barnhart, L. E., and Paulson, J. L. (1998) *Nucleic Acids Res.* **26,** 2606–2610
50. Kambampati, R., and Lauhon, C. T. (2000) *J. Biol. Chem.* **275,** 10727–10730
51. Jager, G., Leipuviene, R., Pollard, M. G., Qian, Q., and Bjork, G. R. (2004) *J. Bacteriol.* **186,** 750–757
52. Tesmer, J. J., Klem, T. J., Deras, M. L., Davisson, V. J., and Smith, J. L. (1996) *Nat. Struct. Biol.* **3,** 74–86
53. Kalhor, H. R., and Clarke, S. (2003) *Mol. Cell. Biol.* **23,** 9283–9292
54. Takai, K., and Yokoyama, S. (2003) *Nucleic Acids Res.* **31,** 6383–6391
55. Agris, P. F. (2004) *Nucleic Acids Res.* **32,** 223–238
56. Yokoyama, S., Watanabe, T., Murao, K., Ishikura, H., Yamaizumi, Z., Nishimura, S., and Miyazawa, T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4905–4909
57. Yokoyama, S., Yamaizumi, Z., Nishimura, S., and Miyazawa, T. (1979) *Nucleic Acids Res.* **6,** 2611–2626
58. Agris, P. F., Soll, D., and Seno, T. (1973) *Biochemistry* **12,** 4331–4337
59. Lustig, F., Boren, T., Claesson, C., Simonsson, C., Barciszewska, M., and Lagerkvist, U. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3343–3347
60. Yarian, C., Marszalek, M., Sochacka, E., Malkiewicz, A., Guenther, R., Miskiewicz, A., and Agris, P. F. (2000) *Biochemistry* **39,** 13390–13395
61. Kurata, S., Ohtsuki, T., Suzuki, T., and Watanabe, K. (2003) *Nucleic Acids Res.* **31,** e145
62. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
63. Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield II, D. W. (1997) *EMBNEW NEWS* **4,** 14

# 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

Noriko Umeda, Takeo Suzuki, Masashi Yukawa, Yoshikazu Ohya, Heisaburo Shindo, Kimitsuna Watanabe and Tsutomu Suzuki

*J. Biol. Chem. 2005, 280:1613-1624.*
*doi: 10.1074/jbc.M409306200 originally published online October 26, 2004*

---