The Yeast Translation Release Factors Mrf1p and Sup45p (eRF1) Are Methylated, Respectively, by the Methyltransferases Mtq1p and Mtq2p

Received for publication, July 14, 2005, and in revised form, November 21, 2005 Published, JBC Papers in Press, December 1, 2005, DOI 10.1074/jbc.M507651200

Bogdan Polevoda 1, Lisa Span 1, and Fred Sherman 3

From the Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14642

The translation release factors (RFs) RF1 and RF2 of Escherichia coli are methylated at the N6-glutamine of the GGQ motif by PrmC methyltransferase. This motif is conserved in organisms from bacteria to higher eukaryotes. The Saccharomyces cerevisiae RFs, mitochondrial Mrf1p and cytoplasmic Sup45p (eRF1), have sequence similarities to the bacterial RFs, including the potential site of glutamine methylation in the GGQ motif. A computational analysis revealed two yeast proteins, Mtq1p and Mtq2p, that have strong sequence similarity to PrmC. Mass spectrometric analysis demonstrated that Mtq1p and Mtq2p methylate Mrf1p and Sup45p, respectively, in vivo. A tryptic peptide of Mrf1p, GGGHVNTTD-SAVR, containing the GGQ motif was found to be ~50% methylated at the glutamine residue in the normal strain but completely unmodified in the peptide from mtq1Δ-Δ. Moreover, Mtq1p methyltransferase activity was observed in an in vitro assay. In similar experiments, it was determined that Mtq2p methylates Sup45p. The Sup45p methylation by Mtq2p was recently confirmed independently (Heurgue-Hamard, V., Champ, S., Mora, L., Merkulova-Rainon, T., Kisselv, L. L., and Buckingham, R. H. (2005) J. Biol. Chem. 280, 2439–2445). Analysis of the deletion mutants showed that although mtq1Δ had only moderate growth defects on nonfermentable carbon sources, the mtq2Δ had multiple phenotypes, including cold sensitivity and sensitivity to translation fidelity antibiotics paromomycin and geneticin, to high salt and calcium concentrations, to polymyxin B, and to caffeine. Also, the mitochondrial mit1 mutation, cox2-V25, containing a premature stop mutation, was suppressed by mtq1Δ. Most interestingly, the mtq2Δ was significantly more resistant to the anti-microtubule drugs thiabendazole and benomyl, suggesting that Mtq2p may also methylate certain microtubule-related proteins.

Post-translational modification of proteins extends molecular structures beyond the limits imposed by the 20 encoded amino acids and, if reversible, allows a means of control and signaling. A wide range of prokaryotic and eukaryotic proteins are methylated post-translationally, including, for example, cytochrome c, ribosomal proteins, translation factors, and histones (1). The modifications occur by either N-methylation or carboxymethylation reactions, with the former reactions usually involving N-methylation of lysine, arginine, histidine, alanine, proline, glutamine, phenylalanine, asparagine, and methionine, whereas the latter reactions usually involving O-methylation of glutamic and aspartic acids. The enzymes catalyzing these methylation reactions generally use S-adenosylmethionine (AdoMet) as the methyl donor to transfer the methyl group to the free amino group on the side chain of an amino acid residue (2). The extent of methylation can be complete or almost complete, as in the case of cytochrome c, or can be partial, as in case of ribosomal proteins. Once incorporated, the methyl groups do not appear to be removed from most proteins. However, reversible methylation of glutamic acid residues is involved in the chemotactic response of bacteria (3); also reversible methylation of the C subunit of the phosphoprotein phosphatase 2A (PP2A) at a conserved C-terminal leucine residue regulates PP2A activity (4). Furthermore, histones were recently shown to be demethylated at the N-terminal tails by the LSD1 enzyme, a process that impacts on chromatin structure and gene transcription (5).

Protein methylation affects various important cell processes, including protein-protein and protein-nucleic acid interactions, chromatin remodeling, transcriptional regulation, RNA processing, protein nuclear trafficking, protein metabolism, cellular signaling, and other basic cellular phenomena (1, 6, 7). Although methylation does not change the overall charge of amino acid residues, addition of the methyl groups increases steric hindrance and removes amino hydrogens that might be involved in the formation of bonds. Therefore, methylation could serve to modulate intra- or intermolecular interactions of the target proteins. Particularly, modification of the heterogeneous nuclear RNA proteins, ribosomal proteins, and translation factors may affect their affinity to RNA or play auxiliary role in RNA binding.

Methylation of the proteins involved in translation, including translation factors and ribosomal proteins, has been observed in diverse organisms, from Escherichia coli to higher eukaryotes. Furthermore, methylation of certain ribosomal protein orthologs, for example, E. coli L11, yeast Saccharomyces cerevisiae L12, and rat L12 and L3, as well as possibly methylation of translation release factors (RFs), is conserved throughout evolution (2, 8, 9). RFs recognize the stop codon in the A site of the ribosome and transfer this stop signal to the peptidyltransferase center (10). This induces the hydrolysis of peptidyl-tRNA, thus releasing the nascent polypeptides, but the mechanisms of this reaction are not clearly understood (11). There are two RFs classes, I, which actually recognizes a stop codon, and II, a recycle factor for RFI, which is a GTPase (10). In E. coli, two of the class I translation release factors, RF1...
and RF2, are required for recognition of the translation termination stop codons; RF1 recognizes UAG and UAA and RF2 recognizes UAA and UGA (12, 13). In eukaryotes a single protein, eRF1, recognizes all three translation stop codons (14); thus eukaryotic RFs are structurally and functionally distinct as compared with the prokaryotic counterparts. The corresponding genes of the class I release factors, RF1, RF2, and eRF1, are essential for viability in bacteria and yeast (11).

RF3 (class II RF) enhances the activity of RF1 and RF2 in a GTP-dependent manner and catalyzes the dissociation of RF1 from the ribosome following peptide release (15–17), although this has not been established in eukaryotes. RF3 has been shown not to be essential in E. coli (18); however the eukaryotic release factor, eRF3 (Sup35p in yeast), is an essential gene and is required for the activation of the release factor complex at all three stop codons (19–21). Also, eRF3 forms a ribosome-bound complex with eRF1 that in bacteria could be detected only in presence of ribosomes (16, 22).

Organellar release factors have been identified in the mitochondria of rat, yeast, and humans (23–25). The only release factor that acts at the translation termination stop codons in mitochondria is mRF1 (24, 26).

In the yeast S. cerevisiae, the MRF1 gene encodes a protein more similar to the prokaryotic RF1 than to RF2, and the MRF1 gene is required for proper translation in mitochondria (27). Mrf1p recognizes only the termination codons UAA and UAG on mitochondrial and bacterial ribosomes (25), the same codons identified by RF1. The UGA codon is not a stop signal in yeast mitochondria (28).

Bacterial RF1, mitochondrial RF1, and eRF1 are homologous proteins (supplemental Fig. 1), and although they are functionally similar, they belong to the different families and display differences in the protein domain structure (10). However, one tripeptide motif, the GGQ motif, is the most conserved feature of the release factors in prokaryotes, eukaryotes, and Archaea (14, 29). This motif was shown to be important for the hydrolysis of peptide- tRNA (22), and it has been proposed that the glutamine residue in the release factor GGQ motif is involved in the coordination of the water molecule necessary for the hydrolysis of the peptidyl-tRNA ester bond (29) and for normal functioning of the class I release factors. S. cerevisiae Mrf1p and Sup35p (eRF1) have extensive sequence similarities to the bacterial RF1, including the predicted site of methylation, GGQ.

The GGQ motif is essential for the function of both prokaryotic and eukaryotic RFs, and mutations of the glutamine residue in E. coli and S. cerevisiae result in lethality (29, 30). RF1 and RF2 proteins of E. coli are post-translationally methylated at the glutamine residue by PrmC (HemK) methyltransferase (31, 32). It has been confirmed in another study that the glutamine residues of the GGQ motif at position 235 in RF1 and at position 252 in RF2 are indeed N6-methylated (8). Moreover, methylation of glutamine at position 252 in the GGQ motif of E. coli RF2 correlates with increased efficiency of translation termination (8). The E. coli ribosomal protein L3 is the only other protein known to have an N6-methylglutamine modification (33).

In addition to high similarities of the protein sequences for translation release factors in bacteria and yeast, including the conserved GGQ motif, the proteins similar to E. coli PrmC methyltransferase could be identified in yeast as well. The protein methyltransferases responsible for these modifications generally have conserved functional domains, responsible for binding of the cofactor AdoMet. The orthologs of these protein methyltransferases share common functional domains, and consequently potential protein methyltransferases can be identified by sequence comparisons. To identify those methyltransferases that may modify the yeast translation release factors, we have searched the yeast S. cerevisiae proteome for potential candidates; this search revealed the two yeast proteins, Ynl063wp and Ydr140wp, that had strong sequence similarity to the E. coli PrmC (Fig. 1). The corresponding genes will be referred to as MTQ1 and MTQ2, respectively.

In this study we demonstrated that the yeast S. cerevisiae protein methyltransferases, Mtq1p and Mtq2p, methylate Mrf1p and Sup45p (eRF1), respectively, in vivo. We also tested the methyltransferase activity of Mtq1p and Mtq2p in vitro and the phenotypes of the corresponding deletion mutants. Although mtq1–Δ strains had only slightly diminished growth on YPG medium, the mtq2–Δ strains showed multiple phenotypes, including cold sensitivity and sensitivity to translation fidelity antibiotics paromomycin and gentamicin, to high salt and calcium concentrations, to polymyxin B, and to caffeine. Most interestingly, the mtq2–Δ mutant was significantly more resistant to the anti-mitotic drugs thiabendazole and benomyl as compared with the normal strain. While this work was in progress, Heurgue-Hamard et al. (35) independently found that YDR140wp was capable of methylating eRF1.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains—**The strains of S. cerevisiae used in this study are listed in Table 1. The analysis of N6-glutamine methylation was carried out with two isogenic series, which were derived from the following parental strains: B-8114 (MATa CYC1 cyq-767 lys5-10 ura5-52) and B-14276 (MATA his3Δ0 leu2Δ0 lys10Δ0 ura3Δ0). Several deletion mutant strains, which were used as a source to generate the PCR disruption products, and the MRF1::TAP strain were purchased from Invitrogen or Open Biosystems (Huntsville, AL).

**Media—**Standard media YPD, YPG, YPDG, and SD, containing appropriate supplements, and sporulation medium SP3 have been described (36). Other media contain 1% Bacto-yeast extract, 2% Bactopeptone, and either 2% ethanol (YPE), 2% glycerol and 1% ethanol (YPEG), or 2% raffinose. Unless stated otherwise, yeast strains were grown at 30 °C. Certain phenotypes of the mtq1–Δ and mtq2–Δ strains were determined with YPD medium containing the following amounts of different agents: 1 M NaCl, 1 M KCl; 0.3 M CaCl2; 0.15% caffeine; 200–1200 µg/ml paromomycin; 50–75 µg/ml genetin (G418); 100–200 µg/ml polymyxin B; 25–50 µg/ml thiabendazole; and 10–30 µg/ml benomyl.

**Construction of the Deletion Mutants—**Standard molecular biological procedures were used to generate deletions. The MTQ1, MTQ2, and MRF1 genes were disrupted by replacing portions of the genes with the URA3 gene of Kluveromyces lactis or the kanMX4 gene produced by PCR and then using the appropriate fragment for yeast transformation. For example, primers Oligo1 and Oligo2 (Table 2) and plasmid pAB2630 (pBS1539) were used to prepare the PCR fragment, which were used in the construction of the mtq1–Δ::URA3 disruption. After yeast transformation, the correct disruptions among the transformants were identified by PCR, using the set of primers Oligo3 and Oligo4. Similarly, the DNA fragment required for producing the mtq2–Δ::URA3 disruption was prepared with Oligo5 and Oligo6 and plasmid pAB2630. The transformants were screened by PCR with Oligo7 and Oligo8. To obtain mtq1, mtq2, and mrf1 deletion strains with the marker kanMX4 in the B-8114 background, yeast genomic DNAs, which were prepared from the corresponding ORF:kanMX4 deletion strain (Invitrogen), were used as template for PCR with primers Oligo3 and 4, Oligo7 and 8, and Oligo14 and 15, respectively.

**Construction of the SUP45::TAP Yeast Strain—**The SUP45::TAP strain was made by a PCR-based technique with Oligo9 and Oligo10 and with plasmid pAB2630 (pBS1539) as a template (Table 2). B-8114 was transformed with the resulting PCR product containing the SUP45::TAP::URA3 DNA fragment, and the transformants were
Mtq1p and Mtq2p Methylate Translation Release Factors

TABLE 1
Yeast strains

| Strain no. | Genotype | Ref./Source |
|------------|-----------|-------------|
| B-8114     | MATa CYC1 cycl7-67 lys5-10 ura3-52 | This study |
| B-8389     | MATa ade2-101 kar1-1 ura3-52 [cox2-25] | This study |
| B-15278    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq1-1::kanMX4 | This study |
| B-15313    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq2-2::kanMX4 | This study |
| B-15314    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq1-1::kanMX4 | This study |
| B-15354    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq2-2::kanMX4 | This study |
| B-15286    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq1-1::kanMX4 | This study |
| B-14276    | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | BY4742 |
| B-14483    | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mtq1-1::kanMX4 | Research Genetics |
| B-14071    | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mtq2-2::kanMX4 | Research Genetics |
| B-15403    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MRF1::TAP-HIS3 | Open Biosystems |
| B-15450    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MRF1::TAP-HIS3 mtq2-2::kanMX4 | This study |
| B-15492    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MRF1::TAP-HIS3 mtq1-1::kanMX4 | This study |
| B-15609    | MATa CYC1 cycl7-67 lys5-10 ura3-52 [p2u URA3 MRF1::TAP-HIS3] | This study |
| B-15610    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq1-1::kanMX4 | This study |
| B-15543    | MATa CYC1 cycl7-67 lys5-10 ura3-52 SUP45::TAP::URA3 | This study |
| B-15612    | MATa CYC1 cycl7-67 lys5-10 ura3-52 [p2u URA3 SUP45::TAP::URA3] | This study |
| B-15613    | MATa CYC1 cycl7-67 lys5-10 ura3-52 mtq2-2::kanMX4 [p2u URA3 SUP45::TAP::URA3] | This study |
| B-15593    | MATa kar1-1 leu1 [cox2-25] | M. Boguta |
| B-15594    | MATa kar1-1 leu1 [mtq1-1::mtq1-1::kanMX4 [cox2-25]] | M. Boguta |
| B-15598    | MATa kar1-1 leu1 [mtq1-1::kanMX4 [cox2-25]] | This study |
| B-15599    | MATa kar1-1 leu1 mtq1-1::kanMX4 [cox2-25] | This study |
| B-15891    | MATa kar1-1 leu1 mtq2-2::kanMX4 [cox2-25] | This study |
| B-15890    | MATa kar1-1 leu1 mtq2-2::kanMX4 [cox2-25] | This study |

TABLE 2
Oligonucleotides used in the construction and testing of the disrupted genes

The position of the first nucleotide is indicated in parentheses, where A of the open reading frame ATG initiation codon is assigned position 1. The underlined sequences represent standard overhangs used to ligate the PCR products.

| Open reading frame | Oligonucleotide | Sequence (5' → 3') |
|--------------------|----------------|-------------------|
| MTQ1               | Oligo1         | (-26) CTTCTCCCTAATCTCGTGAACAAGGCGATATGGAATGATGCGATTGGAATGTTGAACGG |
| MTQ1               | Oligo2         | (+1016) TCCACAGGTGTTCCGTTTAGTAACCCTCAAAAAACCTGCAATAATGTTGATACGG |
| MTQ1               | Oligo3         | (-51) GAGGACATGTTACGTAAATATGAG |
| MTQ1               | Oligo4         | (+993) AAATTCCACAAACAAAAAACCCTGAG |
| MTQ2               | Oligo5         | (+50) TCCTGAATATATAATATGATGAAAACATACACAGGTTAGAGATGATGTCGAGTCAACGG |
| MTQ2               | Oligo6         | (+707) GACGACATTATATATATAGGTATTGGAAGTTTGTCAAATGCATACGTACCAATG |
| MTQ2               | Oligo7         | (-100) CAGGAAACGGCGCCGATGATTC |
| MTQ2               | Oligo8         | (+719) CTGCCTGAGAATAAGACACAGGT |
| SUP45              | Oligo9         | (+1264) GAGGAGTAATATATTGAAAGATAGAGGATGCGGATGCGGATGCGGATGCGGATGCGG |
| SUP45              | Oligo10        | (+1390) AGCGAAATTTATTTGATCATGGTCATGGTCGATGGTCGATGGTCGATGGTCGATGGTCG |
| SUP45              | Oligo11        | (+151) CAGGATCAAATACGATGATCAGATAGAG |
| SUP45              | Oligo12        | (+1485) TCCCTATACCGTGATGTAC |
| SUP45              | Oligo13        | (+1111) GAATGCAGACGTAACTACAT |
| MRF1               | Oligo14        | (+44) TGGAAATTTCTGGAGCGACAGGAC |
| MRF1               | Oligo15        | (+1294) CAGAAATGTGATAGATGATAGATG |
| MRF1               | Oligo16        | (-7) AGAACATATGATGTGACGATCATCATACTACATT |
| MRF1               | Oligo17        | (+956) CATTCTATATCTGGATCCAAAATATGTTG |
| MTFQ1              | Oligo18        | (-6) TGAAGTGGGCTGAGACCCATTCATAG |
| MTFQ1              | Oligo19        | (+667) GTACCCTTGGACCGCGCATACCTGGAAG |
| MTFQ1              | Oligo20        | (+798) GCCATTCTGCGCTGCGCC |
| MTFQ1              | Oligo21        | (+171) ATTTTCAGAGGCGAGACAC |
| MTFQ1              | Oligo22        | (+720) ACTGGATTGTTCACTCCGAGG |
| ACT1               | Oligo1         | (+702) AGAGAGTGCGAGCGCTGTT |
| ACT1               | Oligo2         | (+73) GACGACCTCCTGCGTCTCTC |
| ACT1               | Oligo25        | (+646) GACGACCTCCTGCGTCTCTC |

screened by amplifying the SUP45 locus with Oligo11 and Oligo12. The SUP45::TAP from the positive clones was amplified by PCR, and the insert was sequenced to confirm the correct in-frame fusion. In addition, the cell extract of the positive strain, B-15543, was probed with peroxidase-anti-peroxidase complex antibody that recognizes the TAP tag within proteins. Because B-15543 grew similar to the normal strain, without a detectable phenotype, the SUP45::TAP fusion was inferred to be completely functional. It is important to note that SUP45, unlike MRF1, is an essential gene, and the sup45 deletion mutant is not viable. Also, MRF1::TAP was completely functional, although the mtq1-Δ strain does not grow on nonfermentable carbon sources.

Test for Suppression of the Readthrough Accuracy Using the cox2-25 Mutant—Testing gene deletions for readthrough accuracy in mitochondrial translation was performed by using strains B-15594, normal, and B-15593 that contain the cox2-25 allele, which has an early premature stop codon that interrupts the COX2 gene (37). These tester strains were obtained from Dr. M. Boguta (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). mtq1-Δ deletions were made in both strains B-15594 and B-15593 by replacing the MTFQ1 gene with the kanMX4 gene, as described above, to produce B-15598 and B-15599, respectively (Table 1). Similarly, mtq2-2 strains B-15891 and B-15890 were made
from B-15594 and B-15593, respectively. Subsequently, all strains were tested on YP0.1%DG medium.

The level of mitochondrial Cox2p expression was determined by Western blot analysis, using cell extracts of the yeast strains grown on YPDG and using mouse monoclonal anti-Cox2p antibody (a gift from Dr. T. Fox, Cornell University). We also used actin antibody as loading control (rabbit polyclonal antibody was a gift from Dr. A. Bretscher, Cornell University). The following isogenic sets of strains were used: normal B-15594 (mtz-); B-15593 (cox2-V25); and each of the strains containing mtq1-Δ or mtq2-Δ (Table 1). Yeast cell extract preparation, protein separation by SDS-PAGE, Western blotting, and probing with antibody were performed by standard techniques (38, 39).

Total yeast RNA was extracted by standard procedures with phenol: chloroform treatment of the glass-bead disrupted cells. The RNA samples were normalized after measuring their absorbance at 280/260 nm and were tested by electrophoresis on 1% agarose gel. COX2 and ACT1 cDNAs were obtained with ThermoScript RT-PCR System kit (Invitrogen) using gene-specific oligonucleotides 20 and 23, respectively (Table 2). Subsequently, specific DNA fragments were amplified by PCR with nested oligonucleotides 21 and 22 for COX2 and oligonucleotide 24 and 25 for ACT1. The expected PCR products for 550 bp for COX2 and 574 bp for ACT1 were observed in 1% agarose gel.

**MTQ1 and MTQ2 Overexpression in Bacterial System**—Cloning of the MTQ1 and MTQ2 genes for overexpression in the *E. coli* pet system was performed by using PCR with oligonucleotides Oligo16 and Oligo17 for MTQ1 and Oligo18 and Oligo19 for MTQ2 (Table 2). Genomic DNA prepared from the normal yeast strain served as template for PCR, and the primers were designed to introduce Xhol and BamH1 restriction sites to both ends of the resulting PCR products (Table 2). This allowed for the direct cloning of DNA fragments containing the modified MTQ1 and MTQ2 genes into the pET15b vector (Novagen, Madison, WI) that is used for making polyhistidine-tagged proteins. The positive transformants containing inserts were sequenced to confirm the correct in-frame fusion for MTQ1 and MTQ2 genes; subsequently the corresponding cell extracts were tested with anti-polyhistidine antibody (Sigma). The plasmids containing N-His-MTQ1 and -MTQ2 were transformed into the *E. coli* strain BL21 that is designed for production of proteins.

**Purification of N-His-Mtq1p and N-His-Mtq2p Proteins**—The bacterially overexpressed Mtq1p and Mtq2p His protein fusions were purified by immobilized metal affinity chromatography using TALON IMAC resin (Clontech). Bacterial cell extract preparation and affinity purification were carried out according to the manufacturer's protocol supplied with the TALON resin. Additional purification conditions were used to optimize protein purification of the Mtq1p and Mtq2p His fusion proteins. Four sets of Wash Buffers and corresponding Elution Buffers were made according to the manufacturer's protocol, using the following different reagents in the amounts indicated: 1% Nonidet P-40; 0.5% SDS and 0.1% deoxycholic acid; 4 m urea; and 0.8 m NaCl. The manufacturer's protocol was followed in all other aspects of affinity purification. The resultant eluted proteins were dialyzed against methanol containing the following: methyltransferase buffer (100 m

**In Situ Gel Protein Tryptic Digests and Peptide MS Analysis**—After staining, the protein bands were excised from the gel, washed, reduced, alkylated, and then incubated with trypsin essentially as described by Polevoda et al. (39). Briefly, the gel slices were washed in 500 μl of 100 mm NH4HCO3 for 1 h, then incubated in 150 μl of 100 mm NH4HCO3 with 10 μl of 45 µm DTT at 60 °C for 30 min, followed by the addition of 10 μl of 100 mm iodoacetamide, and treatment for 30 min at room temperature in the dark. The solvent was discarded, and the gel slices were washed in 50% acetonitrile, 100 mM NH4HCO3 for 1 h with shaking. The gel slices were cut into smaller pieces and dehydrated with 50 μl of acetonitrile followed by vacuum speed evaporation. Subsequently, the gel pieces were re-swelled with 10 μl of 25 mM NH4HCO3 containing sequencing grade trypsin (Promega, Madison, WI) in a substrate to enzyme ratio of 1:10. After 15 min, an additional portion of 25 mM NH4HCO3 was added to cover the gel pieces in order to keep them hydrated. The samples were incubated overnight at 37 °C, and the supernatants containing the peptide mixture were transferred to new tubes and, if necessary, concentrated by vacuum. The peptides were purified by Zip-tip (Millipore, Billerica, MA) before MALDI-TOF MS analysis in a Voyager-DE STR mass spectrometer (by Dr. T. McCarley at the Mass Spectrometry Facility, Chemistry Department, Louisiana State University (Baton Rouge, LA)). LC-MS/MS sequencing of the tryptic peptide digests was performed using QSTAR XL quadrupole time-of-flight MS with nano LC system (Applied Biosystems). Proteins or peptides were identified by searching a comprehensive nonredundant yeast protein database using the program MS-Fit/Prospector (University of California, San Francisco, prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

**Methyltransferase in Vivo Assay**—The standard methyltransferase assay was performed at 30 °C for 1.5 h in a 100-μl reaction mixture containing the following: methyltransferase buffer (100 mM HEPES, pH 7.9, 2 mM EDTA; 4 mM MgCl, 1 mM DTT); 2 μl of [3H]Ado-Met at 3.1 Tbq/mmol (PerkinElmer Life Sciences); TAP-purified Mrf1p or Sup45p; and bacterially overexpressed His-Mtq1p or His-Mtq2p. The reactions were stopped by placing the reaction mixture on ice, and the
products were precipitated by the addition of 125 μl of bovine serum albumin at 10 mg/ml and 950 μl of 20% trichloroacetic acid on dry ice. Subsequently the samples were thawed and spun for 20 min at 4 °C; the pellets were washed with 1 ml of 5% trichloroacetic acid and dissolved in 150 μl of 88% formic acid. A total of 3 ml of EcoscintH (National Diagnostics, Atlanta, GA) was added to the vials, and the samples were counted in a Beckman liquid scintillation counter. To visualize the products of the assays, reactions were precipitated with acetone and counted in a Beckman liquid scintillation counter. Also, both of the products were precipitated by the addition of 125 μl of bovine serum albumin at 10 mg/ml and 950 μl of 20% trichloroacetic acid on dry ice. Subsequently the samples were thawed and spun for 20 min at 4 °C; the pellets were washed with 1 ml of 5% trichloroacetic acid and dissolved in 150 μl of 88% formic acid. A total of 3 ml of EcoscintH (National Diagnostics, Atlanta, GA) was added to the vials, and the samples were counted in a Beckman liquid scintillation counter. To visualize the products of the assays, reactions were precipitated with acetone and counted in a Beckman liquid scintillation counter.

Identification of the Mrf1p and Sup45p as the Potential Substrates for the Methyltransferases Mtq1p and Mtq2p, Respectively—The protein sequence alignments of Mrf1p and Sup45p (supplemental Fig. 1) with multiple RF sequences from different species showed significant similarity, including the E. coli region of the conserved GGQ motif containing the methylated glutamine residue. Moreover, our search for PrmC orthologs in S. cerevisiae (NP_014336; and ScMtq2p, S. cerevisiae NP_010424) was used to align protein homologs. MegAlign sequence analysis software (DNAStar) was used to construct a phylogenic tree.

Results

Identification of the Mrf1p and Sup45p as the Potential Substrates for the Methyltransferases Mtq1p and Mtq2p, Respectively—The protein sequence alignments of Mrf1p and Sup45p with multiple RF sequences from different species showed significant similarity, including the E. coli region of the conserved GGQ motif containing the methylated glutamine residue. Moreover, our search for PrmC orthologs in S. cerevisiae (NP_014336; and ScMtq2p, S. cerevisiae NP_010424) was used to align protein homologs. MegAlign sequence analysis software (DNAStar) was used to construct a phylogenic tree.

FIGURE 1. Protein sequence alignment of E. coli PrmC and the S. cerevisiae homologs Mtq1p and Mtq2p showing conserved domains. Protein sequences were aligned using Multalin version 5.4.1. Highly conserved residues are in black boxes, and moderately conserved residues are in gray boxes. The predicted S-adenosylmethionine binding domain (motif f) and the NPPY motif are underlined. GenBankTM accession numbers for PrmC orthologs are as follows: EcPrmC, E. coli NP_415730; ScMtq1p, S. cerevisiae NP_014336; and ScMtq2p, S. cerevisiae NP_010424.
having glutamine at the end was higher (141.75 m/z) than if that glutamine were unmodified (on average 128 m/z). This indicates that the position of the glutamine residue is methylated in that peptide. However, it should be noted that the relative abundance of the unmodified peptide was higher as compared with the methylated one.

**Mtq1p Is Sufficient to Methylate Mrf1p in Vitro**—Mtq1p was shown to be sufficient for methylating Mrf1p in vitro. The substrate Mrf1p was purified by TAP protocol as described under "Experimental Procedures," and Mtq1p was overexpressed in the pET bacterial system as a His<sub>6</sub>-tagged protein and was purified by a common affinity chromatography procedure. An in vitro assay was performed with partially purified Mtq1p in the presence of [<sup>3</sup>H]AdoMet and with Mrf1p prepared from the normal strain or from the mtq1−/H9004 deletion strain (Fig. 3). The products of the methyltransferase assay were separated on an SDS-polyacrylamide gel, and the protein bands that incorporated [H] were visualized by autoradiography. [H]-Labeled Mrf1p was observed in the samples containing the protein purified from both the normal strain (A), and it is not modified in the mtq1− strain (B).

**TABLE 3**

Amino acid sequence of the GGQ-containing peptide of Mrf1p

The amino acid sequence was determined by LC-MS/MS mass spectrometry. Ion series for charge +1 is presented. The position of the methylated glutamine residue is marked with an arrow.

| Amino acid | B ion, m/z | Y ion, m/z |
|------------|-----------|-----------|
| Gly        | 57.05     | 1307.57   |
| → Gln      | 114.11    | 1307.57   |
| His        | 256.02    | 1250.55   |
| Val        | 393.15    | 1108.80   |
| Asn        | 492.22    | 972.46    |
| Thr        | 606.24    | 863.39    |
| Thr        | 707.34    | 749.35    |
| Asp        | 808.45    | 648.25    |
| Ser        | 923.38    | 547.13    |
| Ala        | 1010.52   | 432.09    |
| Val        | 1081.60   | 345.06    |
| Arg        | 1180.67   | 273.98    |

**FIGURE 2.** The yeast Mrf1p protein is not methylated in the mtq1− deletion strain. TAP fusion proteins from normal (A) and mtq1− strains (B) were purified by tandem affinity purification (TAP) protocol and separated on a 4–20% linear gradient SDS-polyacrylamide gel, and the corresponding protein bands were cut from the gel. The proteins were digested in the gel with trypsin, and the resultant peptide was analyzed by MALDI-TOF mass spectrometry. The complete trypsin digest of Mrf1p produces the peptide GGQHVNTDASVR, in which Q is the proposed methylation site. The peak at 1341 m/z atomic mass units corresponds to the unmethylated peptide (designated by N), whereas the peak at 1355 m/z atomic mass units corresponds to the methylated peptide (designated by Me). The 14-Da increase in the molecular mass of the second peptide corresponds to the molecular mass of the methyl group. Apparently, the peptide is only ~50% methylated in the normal strain (A), and it is not modified in the mtq1− strain (B).
Mtq1p and Mtq2p Methylate Translation Release Factors

Mtq2p Methylates Sup45p—Similar to Mrf1p/Mtq1p experiments, the Sup45p (eRF1) protein was found to be methylated by Mtq2p. TAP-purified proteins were prepared from the normal and mtq2-Δ strains and analyzed by MS, generating partially modified peptide GGQ/SLR with the molecular mass of 702 Da only in the sample from the normal strain as compared with unmethylated peptide of 688 Da in the mtq2-Δ strain (not shown). Moreover, it should be noted that methylation of Sup45p by Mtq2p both in vivo and in vitro was recently confirmed by Heurgue-Hamard et al. (35).

Phenotypic Analysis of mtq1-Δ and mtq2-Δ Yeast Strains—To determine whether the deletions of MTQ1 or MTQ2 would cause any defect in cell proliferation or abnormal sensitivities to various agents, we tested the growth of the corresponding mutants on a number of different media and at different temperatures (for a list of media, see “Experimental Procedures”). Phenotypic analysis using 1/10 serial dilutions of the deletion mutants mtq1-Δ (B-15545) and mtq2-Δ (B-15567) strains were tested at 16, 30, and 37 °C on the following media: YPD; YPG; YPD with 800 μg/ml paromomycin; YPD with 75 μg/ml genetin (G418); YPD with 0.3 M CaCl2; YPD with 1 M KCl; YPD with 30 μg/ml benomyl (Beno); YPD with 25 μg/ml thiabendazole (TBZ); and YPD with 0.15% caffeine (Caff). Unless indicated otherwise, the strains were tested at 30 °C. The mtq2-Δ strain grew slower on YPD, was cold-sensitive, and was also sensitive to paromomycin and geneticin, to high salt and calcium concentrations, and to caffeine and polymyxin B but was significantly more resistant to the microtubule-destabilizing drugs benomyl and thiabendazole.

FIGURE 3. Mtq1p methyltransferase modifies Mrf1p in vitro. Overexpressed Mtq1p was incubated in the presence of [3H]Ado-Met with the following: lane 1, Mrf1p purified from the normal strain; lane 2, Mrf1p from the mtq1-Δ strain; lane 3, no substrate added. The products of the reactions were separated on an SDS-polyacrylamide gel. The gel was stained with Coomassie Blue, treated with Amplify reagent, dried, and exposed to Hyperfilm. The position of Mrf1p-CBP is marked on the left, and the molecular markers are on the right.

FIGURE 4. The mtq2-Δ mutant strain showed multiple phenotypes. 1/10 serial dilutions of normal (B-8114), mtq1-Δ (B-15545), and mtq2-Δ (B-15567) strains were tested at 16, 30, and 37 °C on the following media: YPD; YPG; YPD with 800 μg/ml paromomycin; YPD with 75 μg/ml genetin (G418); YPD with 0.3 M CaCl2; YPD with 1 M KCl; YPD with 30 μg/ml benomyl (Beno); YPD with 25 μg/ml thiabendazole (TBZ); and YPD with 0.15% caffeine (Caff). Unless indicated otherwise, the strains were tested at 30 °C. The mtq2-Δ strain grew slower on YPD, was cold-sensitive, and was also sensitive to paromomycin and geneticin, to high salt and calcium concentrations, and to caffeine and polymyxin B but was significantly more resistant to the microtubule-destabilizing drugs benomyl and thiabendazole.

and was cold-sensitive as it grew substantially slower than the normal strain at 16 °C. The mtq2-Δ mutant was sensitive to the translation fidelity drug paromomycin at all temperatures and to another similar antibiotic of the aminoglycoside group, geneticin (G418). Increased sensitivity to paromomycin was observed at the higher temperature. The mutant strain mtq2-Δ, was also sensitive to high salt and calcium concentrations, such as 1 M KCl, 0.3 M CaCl2, as well as caffeine (0.15%). In addition, the mtq2-Δ strain was sensitive to polymyxin-B sulfate, which leads to an enhanced permeability of yeast (42), which is an inhibitor of protein kinase C of prokaryotic type membranes, and in mammals was shown to inhibit the activity of glycerol-3-phosphate acyltransferase that catalyzes the initial and rate-limiting step of triglycerolipid synthesis. Most interesting, the mtq2-Δ mutant was resistant to the anti-microtubule drugs thiabendazole (25 μg/ml) and benomyl (30 μg/ml), although the normal strain and the mtq1-Δ were highly sensitive to those agents. Some of the phenotypes mentioned above, like paromomycin and anti-microtubule drugs sensitivity, were reported previously for SLI45 mutations (43).

There were no significant phenotypes observed for the mtq1-Δ mutant on solid media. However, a quantitative growth analysis of the normal and mtq1-Δ mutant strains in liquid cultures with the nonfermentable carbon source, ethanol or glycerol, revealed that the mtq1-Δ deletion strain grew slower (Fig. 5A). By using a cell counting chamber, we determined that the doubling time during the exponential phase of growth in YPG medium of the mtq1-Δ strain was ~395 min as compared with 340 min for the normal strain. This suggests that mtq1-Δ affects mitochondrial function.

Testing the mtq1-Δ Deletion for Suppression of the Readthrough Accuracy in the cox2-V25 Mutant—The mitochondrial translation readthrough accuracy in the mtq1-Δ strain was investigated with the mitochondrial mt1-Δ mutation, cox2-V25, containing a premature stop mutation in the COX2 gene (37). The following four isogenic strains were tested on media containing nonfermentable carbon sources:
Mtq1p and Mtq2p Methylate Translation Release Factors

In this study we uncovered the two yeast protein methyltransferases, Mtq1p (YNL063w) and Mtq2p (YDR140w), that had extensive sequence similarity to PrmC of E. coli (Fig. 1). Although overall sequence homology is relatively low, these proteins are highly homologous in the regions responsible for binding AdoMet (40), particularly motif I (44) with consensus sequence hh(D/E)hGXXGXXG (where h is a hydrophobic residue) and the NPPY motif, which were both previously thought to be characteristic of DNA methyltransferases (45–47). Multiple sequence alignments and phylogenetic analysis demonstrated that E. coli PrmC and S. cerevisiae Mtq1p and Mtq2p, as well as their various orthologs, could be classified into two related subfamilies of protein methyltransferases (supplemental Fig. 2).

Mass spectrometry analysis of the peptides produced from the tryptic digests of the mitochondrial translation release factor Mrf1p of S. cerevisiae from normal and mtq1-Δ mutant strains revealed that Mrf1p was methylated only in the normal strain and not in the mtq1-Δ deletion strain (Fig. 2). Biochemical assays demonstrated that Mtq1p was sufficient to methylate Mrf1p (Fig. 3). Also, MS analysis of cytoplasmic translation release factor Sup45p proteins from the normal and mtq2-Δ strains revealed that Mtq2p methylates Sup45p. Apparently both Mtq1p and Sup45p are only partially (≈50%) methylated in vivo. Furthermore, both Mrf1p and Sup45p methylated peptides included the highly conserved GGQ motif with glutamine residue as a potential methylation site. In addition, using LC-MS/MS mass spectrometry, we demonstrated that the GGQ-containing peptide of Mrf1p is methylated at the conserved glutamine position (Table 3). The specific methyltransferase activities of Mtq1p and Mtq2p are similar to the previously observed glutamine methyltransferase activity of E. coli PrmC toward RF1 and RF2 (31, 32). Recently, it was independently shown that Mtq2p methylates the glutamine residue in the conserved GGQ motif of the eRF1 (Sup45p) of S. cerevisiae (35).

Although Mtq1p modifies the Mrf1p, the protein that is essential for mitochondrial function (27), the mtq1-Δ mutant had no significant phenotypes on solid media (Fig. 4). However, the quantitative growth experiment in nonfermentable liquid medium (YPE, ethanol) showed that the mtq1-Δ deletion strain grew slower than the normal strain (Fig. 6A). This is indicative of a biological role for Mtq1p related to the proper functioning of mitochondria and to mitochondrial translation that is required for normal growth on YPE. Moreover, mtq1-Δ deletion also affected the accuracy of readthrough in the cox2-2V5 strain. The double mutant cox2-2V5 mtq1-Δ was able to grow to a high culture density in a medium limited on fermentable carbon source as compared with the cox2-2V5 strain (Fig. 5B). Direct evidence for Mtq1p involvement in readthrough activity of the mitochondrial release factor was obtained when we examined the level of Cox2p in cox2-2V5 mtq1-Δ and related strains (Fig. 6). The mtq1-Δ deletion in the cox2-2V5 strain caused a minor but clearly detectable level of Cox2p as compared with the strain with the wild type Mtq1. In contrast, the mRNA levels were very similar in both the normal and cox2-2V5 strain and their corresponding mtq1-Δ mutants (Fig. 6B). Although very low, such an increase in Cox2p protein translation in the cox2-2V5 mtq1-Δ strain is comparable with the level of suppression of nonsense mutations of chromosomal genes by various SUP45 mutations that encode altered forms of the cytoplasmic translation release factor eRF1 (21).

In contrast, the mtq2-Δ deletion mutant showed multiple phenotypes (Fig. 4). First, the mtq2-Δ strain was sensitive to the translation fidelity drugs paromomycin and geneticin, indicating that the methylation of Sup45p (eRF1) is important for its function and affects the translation accuracy in cytoplasm. This is also consistent with the role of the universally conserved
GGQ motif in the translation peptide release. In addition, mtq2-Δ was cold-sensitive, grew slower at normal temperature, and was sensitive to high salt and calcium concentrations and to polymyxin and caffeine. Cold sensitivity in bacteria is often associated with defects in ribosomal assembly (2). Most interestingly, the lack of ribosomal protein L3 methylation caused a cold-sensitive phenotype (9, 31), and it was suggested that L3 methylation may serve as one of the “assembly factors” for E. coli ribosome (2). Although methylation of the yeast L3 protein was described, the position of the methylated residue is not known (48). Moreover, the yeast cytoplasmic L3 protein does not contain the QNQ motif that is methylated in E. coli L3. Both bacterial methyltransferases PrmC, which methylates RFs, and PrmB, which methylates L3, have extensive sequence homology to the yeast Mtq1p and Mtq2p; nevertheless, considering the other findings presented above, it is not clear whether yeast L3 protein is methylated by any of the Mtq1p or Mtq2p and whether a cold-sensitive phenotype of mtq2-Δ deletion is related to L3.

It is possible that the cold-sensitive phenotype is related to another mtq2-Δ defect, particularly the significantly increased resistance to anti-microtubule drugs benomyl and thiabendazole, which effect chromosome segregation at anaphase. Most interestingly, some mutants of SLIP45 and SLIP35 have been observed to have increased sensitivity to benomyl (43). This suggests that Mtq2p may be involved in the methylation of certain microtubule-related proteins and that Mtq2p may have an additional substrate or that the lack of Sup45p methylation affects its interaction with another protein that acts beyond the translation release. Recently, it has been suggested that Sup45p and Sup35p have functions outside of translation related to cytoskeleton organization and cell cycle regulation (49). Moreover, eRF1 was found to interact directly with myosin light chain protein Mlc1p to effect cytokinesis (50). The C-terminal domain of Mtq1p is homologous to the translation elongation factor EF-1α, which binds aminoacyl-tRNAs and is the most abundant actin-binding protein in eukaryotic cells (51). Repression of SLIP35 caused damaging effects, not related to translation, including the disappearance of actin cytoskeletal structures, defects in mitotic spindle formation, nuclei division, and segregation in mitosis (49). Accumulation of un acosedered nuclei with high DNA content was noticed when the SLIP45 gene was repressed (49). The observed benomyl and thiabendazole resistance in the mtq2-Δ mutant supports such function outside of translation for Sup45p or Sup35p. The benomyl and thiabendazole resistance of the mtq2-Δ mutant strain can be explained by the effect of glutamine methylation, or lack of thereof, on any of these functions and interactions of Sup45p and Sup35p. It is tempting to speculate that methylation of Sup45p or Sup35p is required for interaction with Mlc1p or another microtubule-related protein.

It is also possible that the lack of Sup45p methylation causes a secondary effect. For example, methylation could affect binding to Sup35p, which has a ribosome-dependent GTPase activity (16, 17). However, Sup35p could also function as a GTP exchange factor involved with microtubule proteins known to utilize GTP as an energy source. The GTPase activity of Sup35p may have an additional function related to cytoskeletal proteins.

The protein structure of Sup45p shows that the GGQ motif is located on the surface of the molecule and extended from a backbone, where it is easily accessible for modification. One approach would be to look for a similar fold within the pool of the known or predicted yeast protein structures containing the conserved GGQ motif that potentially could be methylated. It remains to be seen whether Mtq2p has any additional substrate besides Sup45p.
42. Boguslawski, G. (1985) Mol. Gen. Genet. 199, 401–405
43. Borchsenius, A. S., Tchourikova, A. A., and Inge-Vechtomov, S. G. (2000) Curr. Genet. 37, 285–291
44. Kagan, R. M., and Clarke, S. (1994) Arch. Biochem. Biophys. 310, 417–427
45. Malone, T., Blumenthal, R. M., and Cheng, X. (1995) J. Mol. Biol. 253, 618–632
46. Schubert, H. L., Phillips, J. D., and Hill, C. P. (2003) Biochemistry 42, 5592–5599
47. Yang, Z., Shipman, L., Zhang, M., Anton, B. P., Roberts, R. J., and Cheng, X. (2004) J. Mol. Biol. 340, 695–706
48. Lhoest, J., Lobet, Y., Costers, E., and Colson, E. (1984) Eur. J. Biochem. 141, 585–590
49. Valouev, I. A., Kushnirov, V. V., and Ter-Avanesyan, M. D. (2002) Cell Motil. Cytoskeleton 52, 161–273
50. Valouev, I. A., Urakov, V. N., Kochneva-Pervukhova, N. V., Smirnov, V. N., and Ter-Avanesyan, M. D. (2004) Mol. Microbiol. 53, 687–696
51. Condeelis, J. (1995) Trends Biochem. Sci. 20, 169–170