S-Adenosylmethionine-dependent Methylation in Saccharomyces cerevisiae

IDENTIFICATION OF A NOVEL PROTEIN ARGinine METHYLTRANSFERASE*

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Agnieszka Niewmierzycka and Steven Clarke‡

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90095-1569

We used sequence motifs conserved in S-adenosylmethionine-dependent methyltransferases to identify 26 putative methyltransferases from the complete genome of the yeast Saccharomyces cerevisiae. Seven sequences with the best matches to the methyltransferase consensus motifs were selected for further study. We prepared yeast disruption mutants of each of the genes encoding these sequences, and we found that disruption of the YJL125c gene is lethal, whereas disruptions of YCR047c and YDR140w lead to slow growth phenotypes. Normal growth was observed when the YDL201w, YDR465c, YHR209w, and YOR240w genes were disrupted. Initial analysis of protein methylation patterns of all mutants by amino acid analysis revealed that the YDR465c mutant has a defect in the methylation of the δ-nitrogen atom of arginine residues. We propose that YDR465c codes for the methyltransferase responsible for this recently characterized type of protein methylation, and we designate the enzyme as Rmt2 (protein arginine methyltransferase). In addition, we show that the methylation of susceptible residues in Rmt2 substrates is likely to take place on nascent polypeptide chains and that these substrates exist in the cell as fully methylated species. Interestingly, Rmt2 has 27% sequence identity over 138 amino acids to the mammalian guanidinoacetate N-methyltransferase, an enzyme responsible for methylating the δ-nitrogen of the small molecule guanidinoacetate.

The recent explosion in the number of uncharacterized genes resulting from sequencing of entire genomes presents a new challenge in biology. Although several living organisms can now be completely defined in terms of their genetic codes, understanding the functions and interactions of macromolecules encoded by the multitude of genes is in its infancy. Methods are needed to assign function to new gene products in order to come closer to understanding what makes up a living system. With this goal in mind, we wanted to assign function to a fraction of the new sequences, specifically to yeast genes resulting from sequencing of entire genomes presents a new challenge in biology. Although several living organisms can now be completely defined in terms of their genetic codes, understanding the functions and interactions of macromolecules encoded by the multitude of genes is in its infancy. Methods are needed to assign function to new gene products in order to come closer to understanding what makes up a living system. With this goal in mind, we wanted to assign function to a fraction of the new sequences, specifically to S. cerevisiae which belong to the major supergroup of methyltransferases that share this common structural organization. In previous studies, we have successfully predicted several open reading frames to be yeast methyltransferases based on the

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‡ To whom correspondence should be addressed: UCLA Molecular Biology Institute, Box 951570, Los Angeles, CA 90095-1570. Tel.: 310-825-8754; Fax: 310-825-1968; E-mail: clarke@ewald.mbi.ucla.edu.

† The abbreviations used are: AdoMet, S-adenosyl-L-methionine; [3H]AdoMet, S-adenosyl-L-[methyl-3H]methionine; PCR, polymerase chain reaction; ORF, open reading frame.

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Fig. 1. Schematic representation of the conserved methyltransferase motifs in relation to S-adenosylmethionine. The conserved motifs I, post-I, II, and III are shown with the sequence of the human protein L-isoaspartate (D-aspartate) O-methyltransferase (12, 15; EC 2.1.1.77). The arrangement of motifs is predicted based on known three-dimensional structures (3–11).
conserved sequence motifs. For example, YBR236c (L12000 (GB)), which we predicted to be a methyltransferase (15) was later found to be an mRNA cap methyltransferase (18). Similarly, YML008c (YYAP_Yeast (SW)) was subsequently shown to be a sterol methyltransferase (15, 19). While we were compiling a list of putative methyltransferases for the present study, one of the sequences on our list, YML110c, was found to code for a methyltransferase involved in the biosynthesis of ubiquinone (20, 21), and another, YBR034c, was shown to code for a protein arginine methyltransferase (22).

There are now a limited number of methyltransferases in S. cerevisiae for which the corresponding genes have been identified (Table I). About half of these methyltransferases contain identifiable sequence motifs. The enzymes lacking motifs may have three-dimensional structures distinct from the family of methyltransferases for which the crystal structures are known or may have diverged sufficiently for the motifs to become unrecognizable. In addition to the enzymes for which the gene sequence has been described, several other methyltransferases are known based on characterization of the enzyme itself or on the presence of methyl groups on a substrate molecule (Table I). Putative methyltransferase genes identified by sequence motifs can be correlated with these activities by the analysis of their disruption mutants.

In this paper, we take advantage of the completion of the yeast genome sequence to analyze directly all of the open reading frames for potential methyltransferases. We present the conserved sequence motifs. For example, YBR236c (L12000 (GB)), which we predicted to be a methyltransferase (15) was later found to be an mRNA cap methyltransferase (18). Similarly, YML008c (YYAP_Yeast (SW)) was subsequently shown to be a sterol methyltransferase (15, 19). While we were compiling a list of putative methyltransferases for the present study, one of the sequences on our list, YML110c, was found to code for a methyltransferase involved in the biosynthesis of ubiquinone (20, 21), and another, YBR034c, was shown to code for a protein arginine methyltransferase (22).

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In this paper, we take advantage of the completion of the yeast genome sequence to analyze directly all of the open reading frames for potential methyltransferases. We present the identification of 33 sequences that contain conserved methyltransferase motifs. Seven of the sequences represent known methyltransferases and 26 are either proteins of unknown function or proteins not previously associated with methylation.

| Enzyme | EC number | Gene | Motifs | Ref. |
|--------|-----------|------|--------|------|
| mRNA cap MT | 2.1.1.56 | ABD1 | I, post-I, II, III | 18 |
| DHHB O-MT | 2.1.1.64 | COQ3 | I, post-I, II, III | 23 |
| Coq5 C-MT | 2.1.1.41 | DIM1 | I, post-I, II, III | 24 |
| 18S rRNA dimethyladenosine MT | 2.1.1.97 | ERG6 | I, post-I, II, III | 19 |
| Delta-sterol C-MT | 2.1.1.23 | MET1 | I, post-I, II, III | 25 |
| Protein arginine MT | 2.1.1.107 | STE14 | ND | 26 |
| tRNA guanine N7-MT | 2.1.1.32 | TRM1 | ND | 30 |
| tRNA dimethylguanine MT | 2.1.1.32 | TRM1 | ND | 30 |
| tRNA guanine N7-MT (2 activities) | 2.1.1.31 | I, post-I, II, III | 31 |
| Cytochrome c lysine MT | 2.1.1.59 | PEM1 | ND | 28 |
| tRNA ribose MT | 2.1.1.71 | PEM2 | ND | 28 |
| tRNA ribose MT | 2.1.1.71 | PET56 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |
| tRNA ribose MT | 2.1.1.71 | ND | 29 |

a ND, not detected. Motifs are based on consensus sequences derived by Kagan and Clarke (15).

b MT, methyltransferase.

c the methyl group is present on the δ-nitrogen atom of arginine residues (41). Previously, arginine methylation was only described on the ω-guanido nitrogens of arginine residues by enzymes such as the yeast Rmt1 methyltransferase (22, 42), although methylation of the δ-nitrogen atom on the small molecule guanidoacetate is known to occur (43). We provide evidence that the YDR465c gene encodes the methyltransferase activity responsible for this modification, and we designate the gene for this novel enzyme as RMT2 (protein arginine methyltransferase). Analyses of the other disruption strains have provided some clues to the possible function of their corresponding methyltransferases.

### EXPERIMENTAL PROCEDURES

**Cloning of Seven Putative Yeast Methyltransferases** — The genes designated as F1, F7, F8, F10, and F12 were subcloned into the pGEX-2T vector (Table II). Each gene was amplified by PCR from yeast genomic DNA using primers containing a BamHI site in the 5′ primer and EcoRI site in the 3′ primer (Table III). PCR products and the pGEX-2T vector (Amersham Pharmacia Biotech) were digested with BamHI and EcoRI, and gene inserts were ligated into the pGEX-2T vector in frame with the glutathione S-transferase coding region to allow for expression of each gene as a fusion protein. DH5α cells were transformed with each ligation mixture, and the plasmids containing gene inserts F1, F7, F8, F10, and F12 were designated pAN102, -100, -106, -107, and -108, respectively (Table IV).

The genes F3 and F4 were subcloned into the pCR2.1 vector (Table II) using the Original TA Cloning Kit (Invitrogen) according to manufacturer’s instructions (Invitrogen). Resulting plasmids containing the gene inserts F3 and F4 were designated pAN109 and -110, respectively (Table IV). Both genes were also cloned into the pGEX-3X vector by releasing the genes from pAN109 and pAN110 with BamHI and ligating the inserts into the BamHI site of pGEX-3X. The resulting plasmids were designated pAN130 and pAN130.

The yeast expression vector containing F3 was constructed by cutting the gene out from the vector pAN109 with BamHI and ligating it into the BamHI site of pCH1 (Table IV). The forward orientation of the insert was confirmed by restriction digests, and the resulting plasmid was designated pAN132. The yeast strain AN3-S was transformed with pAN132 or pCH1 as described below for yeast disruptions and selected on uracil-deficient SD plates to make the yeast strains AN-SR2 and...
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### TABLE II

| ORF | Code | Size | Cloning vector | Disruption sites | Auxotrophic marker | Resulting disruption plasmid | Enzymes used to cut out construct | Resulting yeast strains |
|-----|------|------|----------------|------------------|--------------------|-----------------------------|----------------------------------|------------------------|
| F1  | YDL510w | 858 | PGEX-2T | BglII | HIS3, TRP1 | pAN103, pAN115 | BamHI, EcoRI | AN1-H, AN1 |
| F3  | YDR465c | 1236 | pCR2.1 | ClaI | TRP1 | pAN126 | BamHI, Smal | AN3, AN3-S |
| F4  | YJL125c | 1149 | PGEX-3X | BglII (×2) | TRP1 | pAN122 | BamHI, Smal | AN4 |
| F7  | YCR047c | 825 | PspEI | HIS3, TRP1 | pAN101, pAN127 | BamHI, EcoRI | AN7-H, AN7 |
| F8  | YDR140w | 663 | PGEX-2T | NcoI | TRP1 | pAN123 | BamHI, EcoRI | AN8 |
| F10 | YHR209w | 873 | PGEX-2T | ClaI | TRP1 | pAN128 | BamHI, EcoRI | AN10, AN10-S |
| F12 | YOR240w | 1086 | PGEX-2T | NcoI | TRP1 | pAN114 | BamHI, EcoRI | AN12 |

* There are two BglII sites in F4; therefore, insertion of the TRP1 marker results in the deletion of a 218-base pair fragment. There are also two ClaI sites in F3, but the second site is blocked by dam methylation so it is not cut during linearization of the vector.

### TABLE III

| Primer | ORF No. | Sequence* | Restriction sites |
|--------|---------|-----------|------------------|
| D1075.1 N | (F1) | TGGAGTCATGGAAAGCCGCGCTA | BamHI |
| D1075.2 N | (F1) | CGAATTCGATTCAGAAGGCTCGG | EcoRI |
| GAM1.T. | (F3) | CGCGTACGATGCAATGTGCACTACATCG | BamHI |
| GCD.R | (F4) | AATTCGGGGCCATTGCGAATATTC | SmaI |
| YCT.1 | (F7) | CTGGAGTCATGGACAGCCTCGTGAAGA | BamHI |
| YCT.2 | (F7) | GTGAGTCATGGACAGCCTCGTGAAGA | BamHI, EcoRI |
| YD9302.F | (F8) | ACGAGTCGAAGGATGAATGCT | BamHI |
| YD9302.R | (F8) | TGGAGTCATGGACAGCCTCGTGAAGA | BamHI |
| YHO.9 | (F10) | TATGGGATCTGGGCTAATTGGAAGATC | BamHI |
| YHO.10 | (F10) | CGGAGTCATGGACAGCCTCGTGAAGA | EcoRI |
| YOR240.F | (F12) | GCGGAGTCATGGACAGCCTCGTGAAGA | BamHI |
| YOR240.R | (F12) | GCGGAGTCATGGACAGCCTCGTGAAGA | EcoRI |

* Underlined sequences show placement of restriction sites shown on the right.

### Yeast Chromosomal Disruptions of Seven Putative Methyltransferases—Yeast disruption was carried out using the one-step technique described previously (50). Disruption constructs were created by inserting auxotrophic markers into the middle of the coding region for each gene at a specific restriction site (Table II). The auxotrophically selectable markers for disruption were TRP1 or HIS3 and were obtained from YDp plasmids (44) either directly by digesting with BamHI or by PCR amplification using the YDp vectors as templates. The primers used for amplification of auxotrophic markers were specific for each gene at a restriction site (Table II). Each auxotrophic marker was cut out of its plasmid at the appropriate restriction sites as needed for disruption.

The disruption constructs were created by linearizing each of the vectors containing one of the seven genes at a restriction site in the middle of the coding region (Table II). The gene F4 contained two BglII sites, and therefore, digestion of the pAN120 vector resulted in the deletion of a 218-base pair fragment (Table II). An auxotrophic marker with matching restriction sites at each end was then obtained from the plasmids pAN116, pAN121, and pAN999 (Table IV) by digesting with the appropriate restriction enzyme and ligating the marker into one of the linearized vectors. In cases where BglII sites were used for disruption (F1 and F4; Table II), the auxotrophic marker was obtained from one of the YDp vectors by digesting with BamHI and ligating the marker directly into the BglII sites.

Disruption constructs were released from each vector using BamHI and either EcoRI or Smal (Table II). The disruption mutants of S. cerevisiae were created in the strain SEY6210 or in JDG9100-2, a strain defective in arginine methylation (Table IV). For diploid disruptions of F4 and F10, the strain GPY278 was used (Table IV). The transformation protocol involved a simplified lithium acetate procedure as described (51). Yeast transformants were selected on tryptophan or histidine-deficient SD plates using an amino acid dropout mix (Bio 101). The transformants were replated at least 3 times before they were confirmed by Southern blot or PCR. The yeast strains are listed in Table IV. The strain SEY6210-R was created in the strain SEY6210 as described previously (22) in order to disrupt the chromosomal RMT1 gene and was confirmed by PCR and by amino acid analysis of methylarginines (22).

### Tetrad Analysis—Sporulation and tetrad analyses were conducted as described by Sherman and Hicks (52). A haploid disruption mutant of F10 (AN10-S; Table IV) was obtained by sporulating the strain AN10 (Table IV) and isolating a spore with the genotype MATA, leu2-3, 112, ura3-52, his3-D200, trp1-D390, lys2-801, suc2-A9, ned, F10::TRP1. The isogenic strain SEY6210 was used as a control strain for characterization of AN10-S.

### Preparation of In Vivo Labeled Yeast Extracts—S. cerevisiae mutant and parent cells were labeled in vivo using [3H]AdoMet. Cells were grown to early log phase (A600 nm = 0.6–0.8) in YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) at 30 °C. Five A600 nm
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TABLE IV

Yeast strains

| Name  | Description | Ref.         |
|-------|-------------|--------------|
| GPY278| SEY6210/SEY6211 MATa/MATa, leu2–3, 112/leu2–3, 112, ura3–52/ura3–52, his3–Δ200/his3–Δ200, trp1–Δ901/trp1–Δ901, lys2–801/LYS2, ade2–101/ADE2, suc2–Δ9/suc2–Δ9, mel/mel | Greg Payne, UCLA |
| AN4  | GPY278, F4/F4::TRP1 | This work |
| AN10 | GPY278, F10/F10::TRP1 | This work |
| AN10-S| MATa, leu2–3, 112, ura3–52, his3–Δ200, trp1–Δ901, lys2–801, suc2–Δ9, mel, F10::TRP1 | This work |
| SEY6210| MATa, ura3–52, leu2–3, 112, his3–Δ200, trp1–Δ901, his2–801, suc2–Δ9, mel, F10::TRP1 | This work |
| SEY6210-R| SEY6210, rmt1::LEU2 | This work |
| CH9100–2| MATa, pre1–407, prb1–1122, pep4–3, leu2, trp1, ura3–52, ycl57wu::URA3 | 45 |
| JDG9100–2| CH9100–2, rmt1::LEU2 | 22 |
| AN1  | JDG9100–2, F1::TRP1 | This work |
| AN1-H| SEY6210, F1::HIS3 | This work |
| AN3  | JDG9100–2, F3::TRP1 | This work |
| AN3-S| SEY6210-R, F3::TRP1 | This work |
| AN3-SR2| AN3-S with pAN132 vector | This work |
| AN3-SV2| AN3-S with pCH1 vector | This work |
| AN7  | JDG9100–2, F7::TRP1 | This work |
| AN7-H| SEY6210, F7::HIS3 | This work |
| AN8  | JDG9100–2, F8::TRP1 | This work |
| AN12 | JDG9100–2, F12::TRP1 | This work |

Plasmids

| Name       | Description | Ref.         |
|------------|-------------|--------------|
| pAN099     | HIS3 flanked by BspEI in pBluescript | This work |
| pAN100     | YCR047c (F7) in PGEX-2T | This work |
| pAN101     | YCR047c (F7)::HIS3 in PGEX-2T | This work |
| pAN102     | YDL201w (F1) in PGEX-2T | This work |
| pAN103     | YDL201w (F1)::HIS3 in PGEX-2T | This work |
| pAN106     | YDR410w (F8) in PGEX-2T | This work |
| pAN107     | YHR209w (F10) in PGEX-2T | This work |
| pAN108     | YOR240w (F12) in PGEX-2T | This work |
| pAN109     | YDR465c (F9) in pCR2.1 | This work |
| pAN110     | YJL.125c (GCD14) in pCR2.1 | This work |
| pAN114     | YOR240w (F12)::TRP1 in PGEX-2T | This work |
| pAN115     | YDL201w (F1)::TRP1 in PGEX-2T | This work |
| pAN116     | TRP1 flanked by XbaI and NcoI in pCR2.1 | This work |
| pAN120     | YJL.125c (GCD14) in PGEX-3X | This work |
| pAN121     | TRP1 flanked by BspEI and ClaI in pCR2.1 | This work |
| pAN122     | YJL.125c (GCD14)::TRP1 in PGEX-2T | This work |
| pAN123     | YDR410w (F8)::TRP1 in PGEX-2T | This work |
| pAN126     | YDR465c (F9)::TRP1 in pCR2.1 | This work |
| pAN127     | YCR047c (F7)::TRP1 in PGEX-2T | This work |
| pAN128     | YHR209w (F10)::TRP1 in PGEX-2T | This work |
| pAN130     | YDR465c (F9) in PGEX-3X | This work |
| pAN132     | YDR465c (F9) in pCH1 | This work |
| YDp-W      | Plasmid carrying TRP1 | 44 |
| YDp-H      | Plasmid carrying HIS3 | 44 |
| pRS426     | Multigene shuttle vector with URA3 | 48 |
| pRS426-28  | pRS426 with CYC1 promoter inserted at EcoRI site | 49 |
| pGEX-2T    | GST+ expression plasmid | Amersham Pharmacia Biotech |
| pGEX-3X    | GST+ expression plasmid | Amersham Pharmacia Biotech |
| pBluescript | Plasmid | Stratagene |
| pCR2.1     | | Invitrogen |

* GST, glutathione S-transferase.

units of each culture were then harvested by centrifugation at 1,600 × g for 6 min at 25 °C. The pellets were resuspended in 820 μl of YPD, and 180 μl of [3H]AdoMet (NEIL Life Science Products, 77.9 Ci/ml, 550 μCi/ml in 10 mM H2SO4/ethanol (9:1, v/v)) was added. Cells were labeled for 30 min at 30 °C with shaking, pelleted at 16,000 × g for 5 s at 25 °C, and washed twice with water, and pellets were resuspended at 80 °C. The labeled cells were then harvested by centrifugation at 16,000 × g for 5 s. The cleared lysate was used for acid hydrolysis and amino acid analysis.

Determination of Protein Concentrations—Protein concentrations were determined using a Coomassie Plus Protein Assay Reagent (Pierce) against a standard of bovine serum albumin.

RESULTS

Identification of 26 Putative Methyltransferases in S. cerevisiae—We were interested in identifying new methyltransferases in the complete genome of the yeast S. cerevisiae based on conserved sequence motifs common to AdoMet-dependent methyltransferases. We used motifs I, post-I, II, and III, which we defined previously based on a large number of known methyltransferases. We used motifs I, post-I, II, and III, which we defined previously based on a large number of known methyltransferases (15), to search the yeast genome at the site Motifs in Protein Data Bases. The search resulted in 33 candidate genes with identifiable motifs (Table V). Seven of the

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Available on-line at the following address: http://alces.med.umn.edu/dbmotif.html.
genes turned out to be known methyltransferases responsible for modifying a variety of substrates including ubiquinone precursors, rRNA, the mRNA cap, an ergosterol precursor, uroporphyrin, and arginine residues on proteins (Tables I and V). We grouped the remaining 26 genes in two categories, “good matches” and “marginal matches” based on the number of
Fig. 2. Alignment of YDR465c (F3; Rmt2) and human guanidinoacetate N-methyltransferase (guanidinoacetate N-methyltransferase (GAMT); Ref. 43). The alignment was made using BLAST version 2.0. The conserved motifs I, post-I, II, and III are underlined (15). The deduced sequence of YDR465c is labeled RMT2. The guanidinoacetate N-methyltransferase sequence is GenBank accession number 2948404, amino acid residues that are similar between the two sequences are marked by a +.

![Alignment of YDR465c and human GAMT](image)

**Fig. 3.** Disruption of yeast YCR047c and YDR140w genes leads to slow growth of the resulting yeast strains AN7 and AN8. The parent strain JDG9100-2 and mutant strains AN7 and AN8 were grown in YPD-rich media at 30 °C, and cell numbers were estimated by the optical density of the culture at 600 nm.

![Disruption of yeast YCR047c and YDR140w](image)

There were several sequences, however, that displayed additional amino acid similarities outside of the conserved methyltransferase motifs. For example, the F7 putative methyltransferase could be aligned to five different classes of known methyltransferases, but even the best alignment resulted in the relatively high expect (E) value of 0.013 (Table VI). This observation suggested that although the F7 putative methyltransferase, the degree of sequence similarity to known methyltransferases was insufficient to determine its specific substrate. The low overall similarity scores most likely prevented the sequences identified in this study from being recognized as putative methyltransferases previously. In fact, only one (F7) of the 26 putative methyltransferases was identified as such in the GenBank data base.

Among the new sequences are four genes (GCD14, NOP1, RAD51, and COX1) which have previously been characterized but have not been associated with methyltransferase activity. Interestingly, Nop1, a fibrillarin homolog, is known to be important for proper methylation of pre-rRNA (54). It is possible that fibrillarin may itself be the rRNA ribose O-methyltransferase. However, its direct role as a methyltransferase has not been demonstrated. The remaining sequences were previously characterized open reading frames.

**Sequence Analysis of the Putative Methylation Transferases**—In order to identify likely substrates for each putative methyltransferase, we carried out BLAST 2.0 searches of the GenBank data base with the translated yeast ORF sequences (Table VI). Many of the putative methyltransferases displayed significant amino acid sequence similarities to known AdoMet-dependent methyltransferases in the data base. However, most of the observed similarities were found in the protein segments spanning the conserved methyltransferase motifs, and the overall similarity scores were low. For example, the F7 putative methyltransferase could be aligned to five different classes of known methyltransferases, but even the best alignment resulted in the relatively high expect (E) value of 0.013 (Table VI). This observation suggested that although the F7 putative methyltransferase likely encodes a methyltransferase, the degree of sequence similarity to known methyltransferases was insufficient to determine its specific substrate. The low overall similarity scores most likely prevented the sequences identified in this study from being recognized as putative methyltransferases previously. In fact, only one (F7) of the 26 putative methyltransferases was identified as such in the GenBank data base.

There were several sequences, however, that displayed additional amino acid similarities outside of the conserved methyltransferase motifs. For example, the F3 coding sequence was aligned with an E value of 2 × 10⁻⁶ to the human guanidinoacetate N-methyltransferase (GenBank accession number 2498404, Table VI) and showed sequence similarity in regions preceding motifs I and II (Fig. 2). These similarities suggested that the substrates of F3 and guanidinoacetate N-methyltransferase may share common features (see below). Other notable alignments included the alignment of Gcd14 to a putative β-aspartate methyltransferase from *Mycobacterium leprae* with an E value of 10⁻⁸ (GenBank accession number 2145817, Table VI), the alignment of F8 to several adenine methyltransferases, and the alignment of F2 to a ubiquinone/menaquinone biosynthesis methyltransferase from *Archaeoglobus fulgidus* with an E value of 3 × 10⁻⁷ (GenBank accession number 2650497, Table VI). The sequence similarity between F2 and the ubiquinone/menaquinone biosynthesis methyltransferase is consistent with the predicted location of F2 in the mitochondria and suggests that F2 may methylate a ubiquinone precursor or a similar molecule. Two of the gene products, F9 and F10, were aligned with an E value of 3 × 10⁻⁸ with each other, indicating that they may share similar functions. In addition, for a majority of the putative methyltransferases, a human sequence of unknown function with high similarity to each yeast ORF was found in the EST data base (Table VI).

**Yeast Disruption Mutants of 7 Putative Methyltransferase Genes Reveal One Lethal and Two Slow Growth Phenotypes**—As a first step to characterize the putative methyltransferases, we decided to make yeast disruption mutants of several genes which we considered good matches. Our goal was to...
find the methylation substrate for each new methyltransferase on our list, and the availability of yeast mutants facilitates identification of methylation defects. Disruptions were made in the genes F1, F3, F4, F7, F8, F10, and F12 by inserting either a TRP1 or a HIS3 cassette at a specific restriction site in each gene as described under “Experimental Procedures.”

We found that F4 (GCD14) is an essential gene. Dissection of six individual tetrads from the diploid disruption mutant AN4 resulted in 2:2 segregation of the lethal phenotype (data not shown). In addition, all of the viable spores of F4 were auxotrophic for tryptophan (data not shown), reflecting the absence of the TRP1 insert in the disrupted F4 gene. In contrast, disruption of the genes F1, F3, F7, F8, F10, and F12 resulted in viable colonies. In addition, we observed that the disruption of two of the genes, F7 and F8, leads to slow growth of the resulting yeast mutants. The doubling times for the strains AN7 and AN8 in rich media during exponential growth phase were 4.2 and 3.4 h respectively, compared with 2.1 h for the parent strain (Fig. 3). Similarly, a disruption of F7 in a SEY6210 yeast background resulted in a doubling time of 3 h for the AN7-H mutant compared with 1.5 h for the parent strain during exponential growth phase (data not shown). The remaining yeast mutants exhibited doubling times comparable to the corresponding parent strains (data not shown). We did not observe any additional growth defects of the mutants on glycerol, on minimal media, at 37 or 14 °C (data not shown).

Disruption of F3 Leads to a Methylation Defect of a Novel Type of Arginine Derivative—As a first step to identify the substrate for each new methyltransferase on our list, we decided to examine the disruption mutants for defects in protein methylation. These mutants were made in a strain lacking the Rmt1 protein arginine methyltransferase to reduce the level of N-ω-methylated arginine species that could obscure other methylated derivatives (22). Several different protein methyltransferases, for which the corresponding genes have not yet been identified, are predicted to exist in yeast (Table I). In addition, the sequence similarity between one of the sequences in this study, F3, and guanidinoacetate N-methyltransferase (Fig. 2) led us to suspect that F3 may be a methyltransferase responsible for modifying a molecule similar to guanidinoac-
We examined in vivo labeled extracts from all mutants for defects in protein methylation using amino acid analysis. Yeast proteins, methylated in vivo using [3H]AdoMet, were collected by trichloroacetic acid precipitation and acid-hydrolyzed. The methylated derivatives were analyzed using cation exchange liquid chromatography. The resulting pattern of amino acid methylation for the parent strain, JDG9100-2, is shown in Fig. 5. Constitutive expression of the RMT2 gene product on a multicopy plasmid restores the defect in arginine methylation. A and B, AN3-SR2 strain carrying the RMT2 expression plasmid pAN132. C and D, AN3-SV2 strain carrying the control plasmid pCH1. B and D are enlargements of A and C, respectively, in the region where methylated arginine derivatives elute. The yeast strains AN3-SR2 and AN3-SV2 (Table IV) were labeled in uracil-deficient defined medium in vivo with [3H]AdoMet, and extracts were prepared as described under “Experimental Procedures.” The hydrolysates were prepared and analyzed as described in Fig. 4 except 75 µl of each yeast extract was used for acid hydrolysis and amino acid analysis. DMA, N⁵,N⁷-dimethylarginine (asymmetric) standard; MMA, N⁷-monomethylarginine standard.

Fig. 5. Constitutive expression of the RMT2 gene product on a multicopy plasmid restores the defect in arginine methylation. A and B, AN3-SR2 strain carrying the RMT2 expression plasmid pAN132. C and D, AN3-SV2 strain carrying the control plasmid pCH1. B and D are enlargements of A and C, respectively, in the region where methylated arginine derivatives elute. The yeast strains AN3-SR2 and AN3-SV2 (Table IV) were labeled in uracil-deficient defined medium in vivo with [3H]AdoMet, and extracts were prepared as described under “Experimental Procedures.” The hydrolysates were prepared and analyzed as described in Fig. 4 except 75 µl of each yeast extract was used for acid hydrolysis and amino acid analysis. DMA, N⁵,N⁷-dimethylarginine (asymmetric) standard; MMA, N⁷-monomethylarginine standard.
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4A. The major peak of methylation corresponds to mono-, di-, and trimethyllysine eluting at 47 min. Since this parent strain is defective in N⁰-mono- and N⁰,N⁰-asymmetric dimethylation of arginine residues (22), there are no large peaks of radioactivity which migrate with the methylarginine standards (Fig. 4A). We found, however, no large differences in the methylation pattern of AN3 (Fig. 4C) or of the AN1, AN7, AN8, AN10-S, and AN12 mutants compared with the parent strains (data not shown).

However, closer analysis of the methylarginine region from the AN3 mutant acid hydrolysate reveals a defect in methylation of an amino acid derivative eluting at about 84 min (Fig. 4D), which is clearly present in similarly prepared hydrolysates of the parent strain (Fig. 4B). This derivative elutes in a position distinct from that of the N⁰,N⁰-dimethylarginine or N⁰-ω-monomethylarginine standards. The sequence similarity between F3 and guanidinoacetate N-methyltransferase (Fig. 2), which methylates this small molecule substrate on the ω-nitrogen of its guanidino group, suggested that the substrate of F3 may also be methylated on a ω-nitrogen of a guanidino group but on an arginine residue. In fact, we have recently demonstrated that yeast proteins contain N-ω-methylarginine residues (41). Direct chemical analysis of the acid hydrolysate product eluting at 84 min revealed that this derivative corresponds to free N-ω-methylarginine (41). Three other amino acid derivatives which elute in the methylarginine region at 74, 93, and 103 min are unaffected by the F3 disruption. The identity of these derivatives is unknown, but the peak eluting at about 92 min elutes with the N⁰-ω-monomethylarginine standard and suggests the existence of an additional type of N⁰-arginine methyltransferase.

The methylation defect at 84 min in extracts from the AN3 strain is also observed in the AN3-S strain (Table IV) which has an F3 disruption in a different rmt1 background (data not shown). In order to confirm that the methylation defect in the AN3 mutants is due to the absence of F3 activity, we reintroduced the F3 gene into the AN3-S strain on a multicopy plasmid as described under “Experimental Procedures.” The F3 expression plasmid restores the methylation defect in the AN3-S mutant (Fig. 5B). In contrast, a vector-only control fails to restore methylation of the new derivative (Fig. 5D). In additional control experiments, we analyzed the pattern of methylated molecules from extracts of AN3 and JDG9100-2 strains which had not been precipitated with trichloroacetic acid prior to acid hydrolysis. The pattern of methylated derivatives obtained was very similar to the pattern seen in Fig. 4, showing that the methylated derivative is not due to a small molecule contaminant.

The absence of N-ω-methylarginine in acid hydrolysates of extracts of the F3 disruption mutant coupled with the sequence similarity between F3 and a known ω-nitrogen methyltransferase clearly indicates that F3 is responsible for the formation of this novel arginine derivative. In contrast to guanidinoacetate methyltransferase, the F3 enzyme modifies an amino acid residue of a protein. The 176 amino acid extension at the N-terminal of F3, which is absent in the small molecule methyltransferase (Fig. 2), may represent a substrate binding domain which allows F3 to accommodate a much larger protein substrate. We designate the new enzyme as Rmt2.

Protein Synthesis Is Required for in Vitro Methylation of the Rmt2 Substrate—We wanted to find out whether formation of the ω-methylarginine derivative can occur in vitro. However, incubation of yeast extracts with [3H]AdoMet resulted in the formation of only a minimal level of the N-ω-methylarginine derivative (data not shown). We reasoned that this type of methylation may only occur in a short window of time immediately following translation, before the substrate becomes folded. This is the case for methylation of lysine residues of cytochrome c in yeast (55). We therefore decided to test whether inhibition of protein synthesis during in vivo labeling will prevent the new derivative from being formed. We labeled the JDG9100-2 strain in the presence or absence of cycloheximide and compared the resulting patterns of methylated amino acid residues (Fig. 6). We find that the N-ω-methylarginine peak at 87 min is completely absent upon cycloheximide treatment (Fig. 6B). Interestingly, there is also a significant reduction in the levels of methyllysine at 48 min (Fig. 6A), N⁰-monomethylarginine at 95 min (Fig. 6B), and the unknown derivative at 104 min (Fig. 6B). In contrast, the levels of the other methylated derivatives are unaffected by cycloheximide treatment.

The absence of N-ω-methylarginine under conditions where protein synthesis is inhibited suggests that this novel type of modification may be cotranslational or may in some other way be dependent on protein synthesis. For example, the Rmt2 substrate could have a high turnover rate or it could exist only in its fully methylated form. In order to test these possibilities, we carried out in vitro methylation reactions in which extracts from AN3 (which would be expected to contain unmethylated substrates) and JDG9100-2 (which would be expected to contain the Rmt2 activity) were mixed. Although methylation of each extract individually did not result in formation of N-ω-methylarginine, combining the extracts led to the formation of the new derivative (Fig. 7). This result suggests both that the
substrate of Rmt2 is stable when it is unmethylated and that it normally exists in its fully methylated form.

**DISCUSSION**

Since the completion of the DNA sequence for the entire genome of the yeast *S. cerevisiae*, there has been a focused worldwide effort to uncover the function of each gene product in this organism (56). This effort has included systematic disruption of individual genes and genome-wide phenotypic assays of the resulting disruption mutants (Ref. 56 and references within; see also Ref. 57). It is expected that these genome-wide approaches to functional analysis will contribute tremendously to the understanding of how an organism works. However, direct biochemical analysis will remain a powerful approach to the study of many classes of proteins.

In order to identify and characterize new AdoMet-dependent methyltransferases, we combined a genome-wide approach with biochemical analysis. The presence of conserved motifs in this class of enzymes allowed us to identify 26 potential methylation in the yeast genome that were not detected based on traditional sequence comparisons. Among the putative methyltransferases, we have identified a previously unknown enzymatic activity which catalyzes the unusual based on sequence similarity of this Rmt2 methyltransferase to guanidinacetate N-methyltransferase, an enzyme which also methylates the δ-nitrogen atom of a guanidino derivative, provided a clue about the substrate, but only in the context of what was already known about protein methylation. The recent disruption of the major yeast protein arginine methyltransferase gene *RMT1* (22) revealed the presence of a new arginine derivative and thus pointed to a possible connection between this derivative and the Rmt2 gene product. It is important to note that our identification of the activity of the Rmt2 enzyme was dependent upon having cells disrupted in two genes, a situation which would not occur when single mutants are analyzed en masse. We have therefore shown the added power of combining a direct biochemical approach with genome-wide approaches to elucidate the functions of new gene products.

The function of the new type of protein methylation by Rmt2 is unknown. Since the absence of Rmt2 does not affect general yeast viability or growth, N-δ-arginine methylation may be important under certain specific conditions or it may fine-tune the function of its protein substrate. Interestingly, in a recent study of yeast gene expression using DNA microarray technology, Rmt2 expression was found to be down-regulated after liquid cultures reached an A500 nm of 0.8, decreasing 3.6-fold at an A500 nm of 7.3 (58). The repression of RMT2 implies that N-δ-arginine methylation may be important during logarithmic growth and may be dispensable or even deleterious during post-exponential growth. Homologs of Rmt2 are present in *Schizosaccharomyces pombe* (GenBank™ accession number 1729243; E = 6 × 10^-56) and *Arabidopsis thaliana* (GenBank™ accession number 2927702; E = 7 × 10^-45), but so far, it has not been found in prokaryotes or mammals. Finding the substrate for Rmt2 will be the next step in identifying the function of this new type of arginine methylation.

Amino acid sequence similarities, as in the case of Rmt2 and guanidinacetate N-methyltransferase, may provide valuable starting points for elucidating the function of uncharacterized gene products. For example, the gene product of F4/Gcd14, found in this study to be essential, has been recently identified as a translational repressor of GCN4 (59). GCN4 is a transcription factor that activates at least 40 different genes encoding amino acid biosynthetic enzymes in response to amino acid or purine starvation (60, 61). Multiple translational repressors of GCN4 (GCD proteins) are required for efficient repression of GCN4 mRNA translation under non-starvation conditions (62).

All of the GCD proteins characterized thus far have general roles in protein synthesis initiation, in addition to GCN4 repression and deletions of many of these genes are lethal (Ref. 59 and references within). Several GCD proteins function to repress GCN4 by reducing the activity of the translation initiation factor 2 (59). The mechanism of GCN4 repression by F4/Gcd14 is not known, but our results suggest that it may involve a methylation reaction. In fact, F4/Gcd14 has sequence similarity to a family of putative protein carboxyl methyltransferases from Eubacteria and Archaea (for example, *M. leprae*, GenBank™ accession number 2145817, aligns with an E value of 10^-8 with F4/Gcd14). Carboxyl methylation can be reversible and is known to play a regulatory role in several systems (63).

Slow growth, as seen in the mutants AN7 and AN8, indicates a general metabolic defect (64), and there are various ways in which methylation may be involved in this phenotype. For example, disruption of the Erg6 methyltransferase, which is involved in the biosynthesis of ergosterol, leads to slow growth of the resulting yeast mutants (65, 66). Although the reason for slow growth is not known, it can be hypothesized that the altered sterols that are formed in the absence of the Erg6 methyltransferase may be unable to fulfill the normal cellular functions of ergosterol, such as its role in membrane structure (67). Interestingly, the gene disrupted in the slow-growing AN8 mutant (F8) has the sequence NPPY immediately following motif II which matches the signature motif of adenine methyltransferases (16, 68). It is conceivable that a defect in RNA methylation in the AN8 mutant could affect protein synthesis and lead to slow growth.

This initial study of seven of the putative methyltransferases has revealed one essential gene, two genes whose disruption leads to slow growth, and a novel arginine methyltransferase. There are a number of methyltransferases in yeast predicted to exist based on the presence of methyl groups on various substrates or on specific enzymatic activities that have been demonstrated (Table I). We hope to be able to match the other putative methyltransferases in this study with these activities. Studies are currently under way in our laboratory to analyze the pattern of methylation of proteins, nucleic acids, lipids, and small molecules of the remaining mutants described in this work.

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