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The Predominant Protein-arginine Methyltransferase from *Saccharomyces cerevisiae**

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Jonathan D. Gary, Wey-Jinq Lin‡, Melody C. Yang§, Harvey R. Herschman‡, and Steven Clarke§¶

From the Molecular Biology Institute, the §Department of Chemistry and Biochemistry, and the ‡Department of Biological Chemistry, UCLA, Los Angeles, California 90095-1569

We have identified the major enzymatic activity responsible for the *S*-adenosyl-L-methionine-dependent methylation of arginine residues (EC 2.1.1.23) in proteins of the yeast *Saccharomyces cerevisiae*. The *RMT1* (protein-arginine methyltransferase), formerly *ODP1*, gene product encodes a 348-residue polypeptide of 39.8 kDa that catalyzes both the N^G-mono- and N^G,N^G-asymmetric dimethylation of arginine residues in a variety of endogenous yeast polypeptides. A yeast strain in which the chromosomal *RMT1* gene was disrupted is viable, but the level of N^G,N^G-[^{3}H]dimethylarginine residues detected in intact cells incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine is reduced to less than 15% of the levels found in the parent strain, while the N^G-[^{3}H]monomethylarginine content is reduced to less than 30%. We show that soluble extract from parent cells, but not from mutant *rmt1* cells, catalyzes the *in vitro* methylation of endogenous polypeptides of 55, 41, 38, 34, and 30 kDa. The hypomethylated form of these five polypeptides, as well as that of several others, can be mono- and asymmetrically dimethylated by incubating the mutant *rmt1* extract with a purified, bacterially produced, glutathione *S*-transferase-RMT1 fusion protein and *S*-adenosyl-L-[*methyl*-^{3}H]methionine. This glutathione *S*-transferase-RMT1 fusion protein is also able to methylate a number of mammalian polypeptides including histones, recombinant heterogeneous ribonucleoprotein A1, cytochrome *c*, and myoglobin, but cannot methylate myelin basic protein. RMT1 appears to be a yeast homolog of a recently characterized mammalian protein-arginine methyltransferase whose activity may be modulated by mitotic stimulation of cells.

Evidence for the posttranslational methylation of arginine residues in proteins was first provided by the presence of radioactive species chromatographing at positions near that of arginine in acid hydrolysates of isolated calf thymus nuclei incubated with *S*-adenosyl-L-[*methyl*-^{14}C]methionine (Paik and Kim, 1967). The methylated species were later determined to be arginine derivatives that had been mono- and dimethylated on their guanidino group (Paik and Kim, 1968; Nakajima *et al.*, 1971). *S*-Adenosyl-L-methionine-dependent methyltransferase activities that catalyze these reactions have now been characterized in a number of eukaryotic tissues and organisms.

When the partially purified protein-arginine methyltransferase activity from calf brain was incubated with histones as a methyl-accepting substrate, the products of the reaction, after acid hydrolysis, were determined to be N^G-monomethylarginine, N^G,N^G-dimethylarginine (asymmetric), and N^G,N'^G-dimethylarginine (symmetric) (Lee *et al.*, 1977) (see Fig. 1). However, the substrate specificity of the enzyme at each stage of the purification suggested that two distinct methyltransferases are responsible for the creation of the three types of methylated species (Miyake and Kakimoto, 1973; Lee *et al.*, 1977). In support of this conclusion, two distinct protein methylases have been partially purified from calf brain. One enzyme activity specifically mono- and symmetrically dimethylates myelin basic protein (Ghosh *et al.*, 1988) (Fig. 1) on arginine residue 107 (Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971). The second activity was initially described as a histone-specific methyltransferase (Ghosh *et al.*, 1988), although it is now known to be much more efficient for substrates such as the heterogeneous ribonucleoprotein A1 (hnRNP A1)[1] and catalyzes the mono- and asymmetric dimethylation of arginine residues (Fig. 1). The site of arginine methylation in hnRNP A1 is within a domain that has been designated GAR for glycine and arginine-rich and contains multiple repeats of a consensus arginine methylation site RGG (Rajpurohit *et al.*, 1994a). Several other potential substrates for this enzyme also contain a similar GAR domain (Najbauer *et al.*, 1993). Members of this family of methyl-accepting substrates include nucleolin and fibrillarin proteins that contain 10–12 residues of asymmetric dimethylarginine (Lischwe *et al.*, 1985a; Lischwe *et al.*, 1985b). These proteins, like hnRNP A1, are involved in the processing of pre-RNAs.

A number of purifications of a histone/hnRNP A1-specific mammalian arginine methyltransferase activity have been reported (Ghosh *et al.*, 1988; Rawal *et al.*, 1994; Liu and Dreyfuss, 1995). However, the polypeptide composition of this enzyme is still not established. Similarly, no genes encoding for a protein-arginine methyltransferase have been identified to date. Two purifications of the enzyme activity resulted in preparations that demonstrated multiple polypeptide species present after SDS-gel electrophoresis. Ghosh *et al.* (1988) identified two polypeptides (110 and 75 kDa) associated with a histone-specific arginine methyltransferase from calf brain. The purest fraction obtained from HeLa cells by Liu and Dreyfuss (1995) still had eight polypeptide bands present, with two prominent species of 100 and 45 kDa. A single 110-kDa polypeptide by SDS-gel electrophoresis was identified by Rawal *et al.* (1994)

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¶ To whom correspondence should be addressed. Tel.: 310-825-8754; Fax: 310-825-1968; E-mail: clarke@ewald.mbi.ucla.edu.

[1] The abbreviations used are: hnRNP, heterogeneous ribonucleoprotein; AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; GST, glutathione *S*-transferase; GAR, glycine and arginine-rich region; kbp, kilobase pair(s); bp, base pair(s); LB, Luria Bertani; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SCD, synthetic complete medium with dextrose; YPD, yeast peptone and dextrose medium.

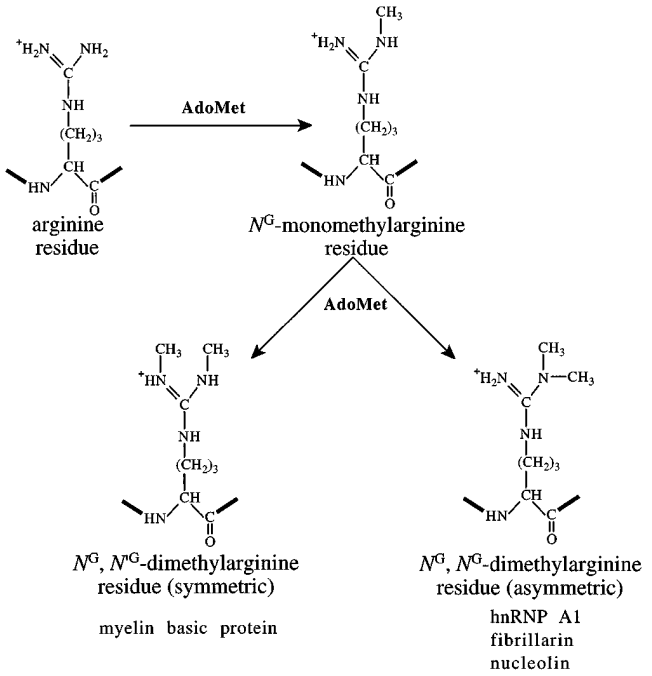

FIG. 1. **Structures of *N*-methylated arginine residues found in proteins.** Protein arginine methyltransferases catalyze the transfer of methyl groups from AdoMet to the terminal guanidino nitrogen atoms of arginine residues.

after a four-step purification from rat liver. However, only a small amount of material was analyzed by electrophoresis so that additional polypeptides of lower molecular mass may have escaped detection.

We have recently described the cDNA cloning and analysis of a mammalian gene product designated PRMT1 for protein-arginine methyltransferase (Lin *et al.*, 1996). The 40.5-kDa protein encoded by this rat cDNA was found to interact, in a yeast two-hybrid screen (Fields and Song, 1989), with the murine primary response/immediate early gene product TIS21 (Fletcher *et al.*, 1991; Varnum *et al.*, 1994) and its family member the mouse antiproliferative gene product BTG1 (Rouault *et al.*, 1992). A search of the sequence data base revealed that the mammalian *PRMT1* gene product was similar in sequence to the yeast *Saccharomyces cerevisiae ODP1* gene product. *ODP1* has been identified as a partial open reading frame downstream of the *PDX3* gene (Loubbardi *et al.*, 1995), and its complete sequence was determined with the yeast chromosome II genomic sequencing effort (Feldmann *et al.*, 1994). No function for ODP1 has been suggested. Using the combined biochemical and genetic approaches that can be taken in yeast, we were interested in exploring whether this gene might encode a protein-arginine methyltransferase with a function related to the mammalian enzyme. However, recent studies attempting to find an hnRNP A1 arginine methylating activity in yeast have been unsuccessful (Liu and Dreyfuss, 1995). Nevertheless, a search of the GenBank data base with the consensus arginine methylation sequence FGGRGGF has revealed numerous potential substrates for an enzyme of similar activity in yeast (Najbauer *et al.*, 1993), including the nucleolin homolog NSR1, the fibrillarin homolog NOP1, as well as the SSB1, GAR1, and NPL3 proteins.

In this paper we provide evidence that the *ODP1* gene product is, in fact, a protein-arginine methyltransferase that we now designate *RMT1* (protein arginine methyltransferase). This enzyme can catalyze both the mono- and asymmetric dimethylation of the guanidino nitrogens of arginine residues present in a number of yeast polypeptides. We have constructed a yeast strain in which the *RMT1* gene has been disrupted. The mutant cells are viable, but analysis of *in vivo* and *in vitro* methylated proteins from this strain demonstrated a dramatic decrease in the levels of mono- and asymmetrically dimethylated arginine residues present. The *RMT1* gene product is therefore required for the majority of the mono- and asymmetric dimethylation of arginine residues in yeast. We also created and purified an N-terminal fusion of RMT1 with glutathione *S*-transferase. Incubation of the fusion protein with the methyl donor *S*-adenosyl-L-methionine results in the mono- and asymmetric dimethylation of the hypomethylated substrate proteins from the *rmt1* soluble extract. These results demonstrate that the *RMT1* gene product is a protein-arginine methyltransferase that plays a major role in modifying proteins containing a GAR domain, many of which interact with RNA. The methylation of arginine residues within these proteins may modulate this interaction, and may regulate other activities as well.

EXPERIMENTAL PROCEDURES

Cloning and Chromosomal Disruption of the Yeast Protein-arginine Methyltransferase Gene RMT1—The *RMT1* gene (formerly *ODP1*) was PCR-amplified (Scharf, 1990) from genomic DNA isolated (see below) from *S. cerevisiae* strain JM43 (*MATα, leu2–3, leu2–112, ura3–52, trp1–289, his4–580*) (McEwen *et al.*, 1986) using the single-stranded DNA primers RMT1-N1 (5′-AAAGAAA**CATATG**AGCAAGACAGCC) and RMT1-C1 (5′-CTCCAGCAAACAA**AAGCTT**TAATGC). These primers overlap the ATG initiation codon and the TAA termination codon, respectively (underlined). The resulting 1.1-kbp PCR product was purified from a 1% agarose gel using Geneclean II (Biolabs 101) and digested with *Nde*I and *Hin*dIII, sites that were engineered into primers RMT1-N1 and RMT1-C1, respectively (bold). This fragment was then ligated into a similarly digested and purified pT7-7 vector (Tabor and Richardson, 1985) to create pJG-RMT1, whose insert contains the entire coding region of the gene (Fig. 2).

Plasmid pJG-RMT1 was digested at the unique *Bst*XI site in the middle of the coding region (Fig. 2), and the 3′ overhang was removed by subsequent treatment with T4 DNA polymerase. A *LEU2* disruption cassette was obtained from plasmid YDp-LEU2 (Berben *et al.*, 1991) by digestion with *Bam*HI, and the resulting 5′ overhang of the cassette was filled in by treatment with T4 DNA polymerase. The blunt-ended *LEU2* cassette was then purified from a 1% agarose gel as described above and ligated into the *Bst*XI-linearized pJG-RMT1 to create the disruption vector pJG-RMT1::LEU2. The orientation of the insertion was confirmed to be in the direction indicated in Fig. 2 by restriction digests with *Nde*I/*Hin*dIII, *Nde*I/*Eco*RV, and *Ssp*I.

Replacement of the wild-type *RMT1* chromosomal locus with the disruption construct was accomplished in the strain CH9100-2 (*MATa, prc1–407, prb1–1122, pep4–3, leu2, trp1, ura3–52, ycl57wΔ::URA3*) (Hrycyna and Clarke, 1993) using the one-step technique described by Rothstein (1983). Briefly, the disruption plasmid pJG-RMT1::LEU2 (10 μg) was digested with *Nde*I and *Hin*dIII, and the entire mixture was used to transform CH9100-2 cells by the lithium acetate method (Rose *et al.*, 1990). The transformed cells were then selected by plating onto leucine-deficient SCD plates (Rose *et al.*, 1990). Positives were re-screened on selective plates twice. Genomic DNA was isolated from cells remaining after the three screens. The replacement of the wild-type *RMT1* locus by the *LEU2* disrupted version was confirmed by PCR analysis using primers RMT1-N2 (5′-TTCGTACCTTATCTTACA-GAAACGC) and RMT1-C2 (5′-CAGTGAGTGTATGGAGCATGAGGAC) (Fig. 2). The wild-type locus produces a PCR product of 700 bp, but the disrupted locus gives a 2.4-kbp product. All putative positives tested from the auxotrophic screen produced only the 2.4-kbp product upon PCR analysis. The new *rmt1* strain, in which the genomic copy of *RMT1* has been disrupted by a *LEU2* cassette transcribed in the same direction, is designated JDG9100-2 (*MATa, prc1–407, prb1–1122, pep4–3, leu2, trp1, ura3–52, ycl57wΔ::URA3, rmt1::LEU2*).

Isolation of Genomic DNA from S. cerevisiae—We used a modified method of the procedure described by Hoffman and Winston (1987). Yeast were grown in YPD (Rose *et al.*, 1990) at 30 °C to an $A_{600\ nm}$ of 3–4. 10–20 $A_{600\ nm}$ units were then pelleted into a 1.5-ml microcentrifuge tube by centrifugation at 13,600 × *g* for 5 min at 25 °C. The pelleted cells were washed with water and resuspended in 500 μl of a 50 mM Tris-HCl solution containing 20 mM sodium EDTA and 1% (w/v) SDS at pH 7.5. Baked glass beads (425–600 μm, Sigma) (0.6 g) were

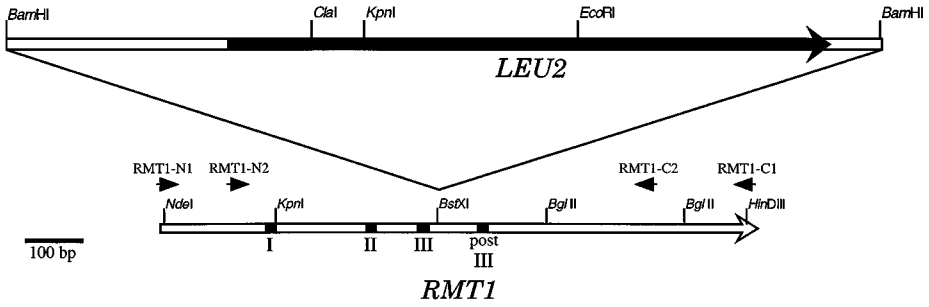

FIG. 2. **A schematic diagram of the wild-type *RMT1*, formerly *ODP1*, gene in *S. cerevisiae*, and of the constructed chromosomal insertion mutant.** The *lower portion* represents a PCR product containing the complete coding region of *RMT1*. The initiator codon ATG is within the *Nde*I site and the *Hin*dIII site is 1 bp downstream of the termination codon TAA. Relevant restriction enzyme sites and the locations of conserved methyltransferase regions I, II, III, and post III (Kagan and Clarke, 1994; Kagan and Clarke, 1995) are included in the figure. The *Nde*I and *Hin*dIII sites were engineered into the 1.1-kbp fragment using primers RMT1-N1 and RMT1-C1, respectively, as described under "Experimental Procedures." *Arrowheads* indicate the location of the 25-bp single-stranded DNA primers that were used for PCR analysis. The *upper portion* of the figure displays the insertional cassette from the plasmid YDp-LEU2 (Berben *et al.*, 1991); the *LEU2* coding region is represented by the *filled arrow*. The insertion of the *LEU2* cassette into the unique *Bst*XI site of *RMT1* is described under "Experimental Procedures."

then added and the suspension was vortexed for 1 min and then cooled at 0 °C for 1 min; this process of vortexing and cooling was repeated five times. The lysed cells were then incubated at 70 °C for 10 min before the addition of 200 μl of 5 M potassium acetate and 150 μl of 5 M NaCl. The mixture was vortexed and kept at 0 °C for 20 min. Cellular debris and denatured proteins were pelleted by centrifugation at 13,600 × *g* for 20 min at 25 °C. The supernatant was removed to a fresh tube, and two volumes of ethanol were added. The DNA was pelleted by centrifugation as just described and then washed once with 70% ethanol. The final DNA pellet was resuspended in 50 μl of a 10 mM Tris-HCl solution with 1 mM sodium EDTA at pH 7.0 containing 5 μg of DNase-free RNase.

Isolation of Soluble Proteins from S. cerevisiae—Cultures (500 ml) of both the parent (CH9100-2) and the mutant (JDG9100-2) strains were grown to an $A_{600\ nm}$ of 2.5 in YPD media at 30 °C. Cells were harvested by centrifugation at 4,400 × *g* for 10 min at 4 °C. The pelleted cells were then washed with buffer (25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA at pH 7.5) and centrifuged as described above. The resulting pellets (2.6–3.0 g, wet weight) were resuspended in 2 ml of buffer/g (wet weight) of cells, and 40 μl of a protease inhibitor mixture was added to give final concentrations of 1 mM benzamidine, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin. The cell suspensions were passed twice through a French pressure cell at 20,000 p. s. i., and the homogenates were centrifuged at 23,000 × *g* for 40 min at 4 °C. The supernatants were aliquoted and stored at −20 °C for use as soluble extracts.

Protein Concentration Determination—A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein after precipitation with 1 ml of 10% (w/v) trichloroacetic acid. Bovine serum albumin was used as a standard.

Fractionation of Polypeptides by SDS-Gel Electrophoresis—Samples were analyzed by mixing them 1:1 with SDS-containing sample buffer (Hrycyna *et al.*, 1994), heating to 100 °C for 5 min, and then loading onto slab gels (1.5 mm × 10.5-cm resolving gel) using the buffer system described by Laemmli (1970). A 10% or 12.6% (w/v) acrylamide, 0.28% (w/v) *N*,*N*-methylenebisacrylamide matrix was utilized with a constant current of 35 mA. Gels were then stained for 1 h with 0.1% (w/v) Coomassie Brilliant Blue in 50% (v/v) methanol and 10% (v/v) acetic acid in water. For fluorography, gels were destained overnight in destain, a 5% (v/v) methanol and 10% (v/v) acetic acid solution in water, at 25 °C, then the gels soaked in EN^{3}HANCE (DuPont) for 1 h, and finally in water for 30 min as suggested by the manufacturer. The gels were then vacuum dried at 65 °C for 1.3 h onto a filter paper support. The dried gels were exposed to film (Kodak, X-Omat AR) at −80 °C. For other applications, the destained gels were simply vacuum dried at 65 °C for 1 h onto a filter paper support.

Acid Hydrolysis of Homogenized S. cerevisiae Cells Labeled with [^{3}H]AdoMet—Parent (*RMT1*, CH9100-2) and mutant (*rmt1*, JDG9100-2) cells were grown to early log phase ($A_{600\ nm}$ = 0.6–0.8) in YPD media at 30 °C. Five $A_{600\ nm}$ units of each culture were then harvested by centrifugation at 1,600 × *g* for 6 min at 25 °C. The pelleted cells were washed with sterile water and centrifuged as just described. The washed pellets were then each resuspended in 820 μl of YPD and transferred to a 1.5-ml microcentrifuge tube. [^{3}H]AdoMet (DuPont NEN, 73 Ci/mmol, 550 μCi/ml in dilute H_2SO_4 (pH 2.0):ethanol (9:1, v/v)) was added to give a final [^{3}H]AdoMet concentration of 1.4 μM (180 μl, 99 μCi), and the cells were labeled for 30 min at 30 °C. The cells were then pelleted at 13,600 × *g* for 1 min at 25 °C, washed once with sterile water, and resuspended in 50 μl of lysis buffer, a 1% SDS solution containing 0.67 mM PMSF. To each of the mixtures, 0.2 g of baked zirconium beads were added and the samples were vortexed in 1-min bursts and then cooled at 0 °C for 1 min; this process of vortexing and cooling was repeated seven times. An aliquot containing 100 μg of protein from each extract was then mixed with an equal volume of 25% (w/v) trichloroacetic acid in a 6 × 50-mm glass vial and incubated at 25 °C for 10 min before pelleting the precipitated material at 4,000 × *g* for 20 min at 25 °C. The pellets were then washed once with acetone at −20 °C, dried, and then acid-hydrolyzed with 200 μl of 12 M HCl at 110 °C for 20 h in a Waters Pico-Tag vapor-phase apparatus. The hydrolyzed samples were then resuspended in 50 μl of water, and 25 μl of each sample was analyzed by cation exchange chromotography.

Construction, Expression, and Purification of a GST-RMT1 Fusion Protein—The *RMT1* coding region was amplified by PCR using the plasmid pJG-RMT1 as a template and the primers RMT1-N3 5′-CAA**GGATCC**AGCAAGACAGCCGTGAAA and RMT1-C3 5′-CAGAT**GAATTC**CTCTTAATGCATTAAATAAG. Primer RMT1-N3 has the ATG initiation codon deleted and a *Bam*HI site (bold) inserted for in-frame ligation into the GST expression vector pGEX-2T (Pharmacia Biotech Inc.). RMT1-C3 incorporates an engineered *Eco*RI site (bold) just downstream from the *RMT1* termination codon (underlined) for cloning purposes. The 1.1-kbp PCR product was digested with *Bam*HI and *Eco*RI, purified from a 1% agarose gel using Geneclean II, and ligated into the pGEX-2T vector, which had been similarly digested and isolated to generate the fusion construct pGEX-RMT1, that contains the *Schistosoma japonicum* glutathione *S*-transferase gene in frame with the entire coding region of the *RMT1*. This plasmid was propagated in the *Escherichia coli* host strain DH5α (Life Technologies Inc). To express the fusion protein, bacteria were grown at 37 °C in Luria Bertani medium (Sambrook *et al.*, 1989) with 100 μg/ml ampicillin until the $A_{595\ nm}$ reached 0.5–0.6. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 1 mM, and the culture was incubated for an additional 5 h at 37 °C to induce the expression of the fusion protein. The bacteria were harvested by centrifugation (5,000 × *g* for 10 min at 4 °C), washed twice with phosphate-buffered saline (10 mM dibasic sodium phosphate, 1.8 mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl at pH 7.4), resuspended in extraction buffer (phosphate-buffered saline, 5% glycerol, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, 1 mM PMSF, and 0.5% (v/v) Triton X-100), and disrupted by sonication with a microtip (Heat Systems-Ultrasonics, Inc.) using a 15-s continuous pulse and then cooling to 0 °C for 1 min; this process of sonication and cooling was repeated four times. Cell debris was removed by centrifugation (16,000 × *g* for 20 min at 4 °C), and the supernatant was collected. GST fusion proteins were purified by affinity chromotography with glutathione-Sepharose 4B (Pharmacia) beads according to the manufacturer's instructions. An identical procedure was used to create the mammalian PRMT1 fusion protein, except primers corresponding to the *PRMT1* gene were used.[2]

Growth of RAT1 Cells and Preparation of Soluble Extracts—RAT1 fibroblast cells were grown to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemeni Bioproducts, Inc.). Prior to harvesting, cells were washed once with phosphate-buffered saline (10 mM dibasic sodium phosphate, 1.8

Methyltransferase Region	Sequence	
I	VLDVGCGTG	ODP1/RMT1 (Yeast)
	VLDVGSGTG	PRMT1 (Rat)
	VIDFGCGSG	L11 Methyltransferase (*E. coli*)
post I	VIGVD	ODP1/RMT1 (Yeast)
	VIGIE	PRMT1 (Rat)
	AIGID	L11 Methyltransferase (*E. coli*)
II	PKVDIIIS	ODP1/RMT1 (Yeast)
	EKVDIIIS	PRMT1 (Rat)
	MKADVVVA	L11 Methyltransferase (*E. coli*)
III	YLVEGGLIFP	ODP1/RMT1 (Yeast)
	WLAPDGLIFP	PRMT1 (Rat)
	VLPVSGGLLG	L11 Methyltransferase (*E. coli*)

FIG. 3. **An alignment of the conserved methyltransferase regions from the *S. cerevisiae* RMT1, the rat protein-arginine methyltransferase PRMT1, and the *E. coli* L11 methyltransferase.** The yeast RMT1 (PIR S45890) shares an overall 45% identity with its mammalian counterpart PRMT1 (Lin *et al.*, 1996). The *E. coli* L11 protein methyltransferase (GenBank U18997, *cf.* GenBank Z26847) is the highest scoring protein found using a BLAST search with the RMT1 sequence and shares an 11% identity with RMT1. However, when methyltransferase regions I, post I, II, and III (Kagan and Clarke, 1994) for the three proteins are aligned, the sequences are highly conserved as is the inter-region spacing (not shown).

mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl at pH 7.4) and then removed from culture plates (20 × 100 mm) by treatment with trypsin (0.5 μg/plate) at 37 °C for 1 min. The trypsinized cells were washed with cold phosphate-buffered saline, pelleted by centrifugation, and resuspended in extraction buffer (25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, 1 mM PMSF at pH 7.4). Cell disruption was performed at 0 °C by Dounce homogenization with a glass tissue grinder. The crude homogenate was cleared of insoluble debris by centrifugation at 18,000 × *g* for 50 min at 4 °C, and the supernatants were either used immediately as cellular extract or stored at −80 °C for later use.

RESULTS

The S. cerevisiae ODP1 Gene Encodes a Protein with Sequence Similarities to the Mammalian Protein-arginine Methyltransferase PRMT1—We have recently identified a rat cDNA that encodes the catalytic subunit of a mammalian protein arginine methyltransferase (Lin *et al.*, 1996). A search of the non-redundant protein data base (PDB+SwissProt+PIR+SPUpdate+GenPept+GPUpdate) using the BLAST method (NCBI) (Altschul *et al.*, 1990) revealed that the *S. cerevisiae* gene previously designated *ODP1* (GB Z35903) encodes a protein whose sequence shares a 45% identity with the rat protein-arginine methyltransferase. The *ODP1* gene was originally described as a partial open-reading frame adjacent to the *PDX3* gene (Loubbardi *et al.*, 1995) with no assigned function. Because of these sequence identities to the mammalian enzyme, we were interested in determining whether the *ODP1* gene product is also a protein-arginine methyltransferase. The yeast gene encodes a 39.8-kDa polypeptide of 348 residues with a calculated pI of 5.3. Several of the regions of sequence similarity between the yeast ODP1 and the rat PRMT1 proteins have been identified previously as conserved regions among a large number of methyltransferases (Kagan and Clarke, 1994, 1995) (Fig. 3). Regions I, II, III, and post region I have been shown to be involved in recognizing the methyl-donating cofactor *S*-adenosyl-L-methionine (Schluckebier *et al.*, 1995).

The ODP1/RMT1 Gene is Required for Protein Methylation—To determine whether the *ODP1/RMT1* gene is in fact the gene for a protein methyltransferase, we created a *S. cerevisiae* strain in which this gene is disrupted by the insertion of a *LEU2* cassette into its unique *Bst*XI site, as described under "Experimental Procedures" (Fig. 2). The *LEU2* cassette inserts an additional 1.6 kbp into the *RMT1* gene, and the disrupted gene is predicted to create a non-functional protein because the insertion would cause an early translational termination 5 amino acid residues downstream from the insertion site. Analysis of the mutant cells demonstrated no obvious growth defects when compared to the parent cells. A light microscopic analysis of the two strains revealed no gross morphological abnormalities.

We then examined the endogenous protein methylation patterns in the crude soluble fraction from both strains by incubating these extracts with [^{3}H]AdoMet and then separating the polypeptides by SDS gel electrophoresis (Fig. 4*A*). At least five polypeptides (55, 41, 38, 34, and 30 kDa) that are methylated in the lane containing parent *RMT1* soluble extract show little or no methylation in the mutant *rmt1* soluble extract lane. Therefore ODP1/RMT1 is required for a protein methyltransferase activity in yeast.

To characterize the stability of the [^{3}H]methylated residues, we compared the total radioactivity present with the base-dependent volatile radioactivity in parallel gel slices. The pattern of total radioactive methyl group incorporation present in each slice (Fig. 4*B*) closely matches the data obtained from the fluorograph, as expected (Fig. 4*A*). The major methylated polypeptides at 55, 41, and 36 kDa from the endogenously methylated parent soluble extract are again absent when extract obtained from mutant *rmt1* cells was used (Fig. 4*B*). The peak of radioactivity at 97 kDa and the broad signal from 20–29 kDa are found in both the parent and the mutant extracts and presumably represent methylation reactions not dependent upon the *ODP1/RMT1* gene product. Gel slices from lanes parallel to those shown in Fig. 4*B* were then analyzed after base treatment using a vapor phase diffusion assay (Fig. 4*C*) that detects [^{3}H]methyl groups in either ester linkages or linkages to the guanidino groups of arginine residues (Paik and Kim, 1980; Najbauer *et al.*, 1991; Hrycyna *et al.*, 1994). The results obtained from the analysis of the parent cell extract indicate that the major 55-, 41-, and 36-kDa substrates for the RMT1-dependent methyltransferase also represent the majority of the base-labile, methylatable species present. To distinguish volatile [^{3}H]methylamine (derived from [^{3}H]methylarginine residues) from [^{3}H]methanol (derived from [^{3}H]methyl ester residues), we neutralized the base-treated gel slices with HCl prior to the determination of volatility. We found that the base-labile radioactivity derived from the 55-, 41-, and 36-kDa polypeptides in the parent extract are not observed under these conditions (data not shown), suggesting that the product of the base treatment was, in fact, [^{3}H]methylamine as the methylammonium cation would not be expected to be volatile.

The results shown in Fig. 4 suggest that the yeast *ODP1/RMT1* gene product is required for a large fraction of the total protein methylation as well as the predominant fraction of potential protein-arginine methylation reactions *in vitro*, since most of the base-labile methyl linkages observed are dependent upon the presence of RMT1. The remaining base-labile, volatile radioactivity seen at 42 kDa in the mutant extract in Fig. 4*C* may represent the C-terminal leucine methyl ester in the catalytic subunit of protein phosphatase 2A (Xie and Clarke, 1993, 1994). Similarly, the radioactivity peak in the 22-kDa region may represent the C-terminal isoprenylcysteine methyl esters in small G-proteins or the formation of methyl esters on tRNA molecules (Hrycyna *et al.*, 1994).

The ODP1/RMT1 Gene Product is Necessary for the in Vivo and in Vitro Mono- and Asymmetric Dimethylation of Arginyl Residues in S. cerevisiae—To directly demonstrate the chemi-

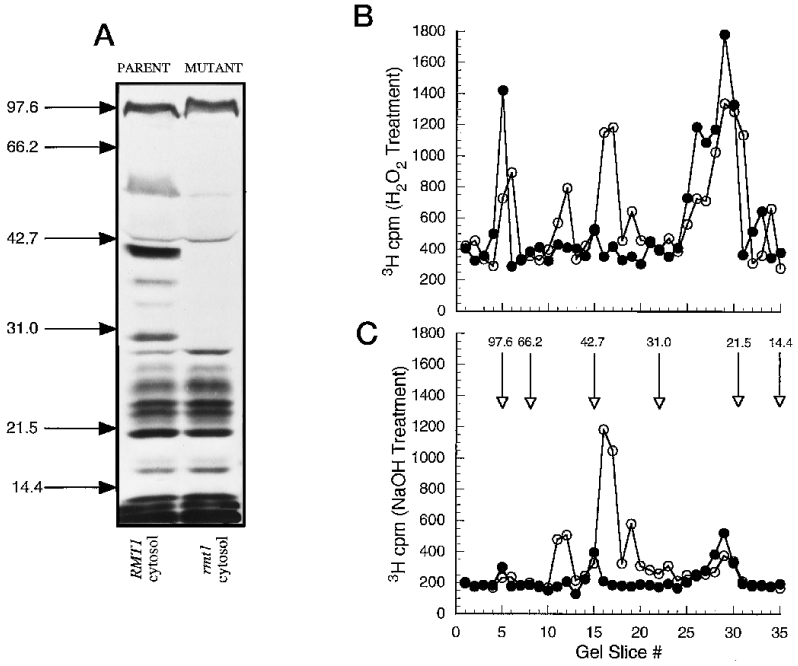

FIG. 4. **Loss of *in vitro* protein methylation in soluble extract from the yeast rmt1 mutant strain.** In *panel A*, cytosolic fractions containing parent extract (269 μg of protein) or mutant extract (338 μg of protein) (see "Experimental Procedures") were incubated in 0.8 μM [^{3}H]AdoMet (2.2 μCi) and a buffer of 25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5 in a final volume of 34 μl. After 30 min at 30 °C, the reactions were stopped by adding an equal volume of 2 × SDS-gel electrophoresis sample buffer (Hrycyna *et al.*, 1994). The samples were then separated by SDS-gel electrophoresis (10% acrylamide) and the gel fluorographed as described under "Experimental Procedures." This panel shows the result of a 2-month exposure at −80 °C. The *arrows* indicate the position of molecular mass standards (Bio-Rad) for each gel (rabbit phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg lysozyme, 14.4 kDa). In *panels B* and *C*, endogenously methylated polypeptides from either the parent (CH9100-2) or *rmt1* mutant (JDG9100-2) strain were analyzed by gel-slice methods. Reactions, prepared as described above, contained either CH9100-2 cytosol (336 μg of protein) or JDG9100-2 cytosol (423 μg of protein), 0.57 μM [^{3}H]AdoMet (2.2 μCi), and buffer in a final volume of 49 μl. After incubation and SDS-gel electrophoresis as described above, gel slices were analyzed as described below. After gel staining and destaining, each lane in the dried resolving gel was cut into 35 separate 3-mm slices. In *panel B*, total radioactivity was determined after each gel slice (with the filter paper backing removed with forceps by wetting the paper with water) was incubated with 1 ml of 30% (v/v) H_2O_2 in a capped scintillation vial at 70 °C for 24 h. After the gel dissolved, 5 ml of scintillation fluid (Safety-Solve, Research Products International) was added to the vial and the sample was counted to determine the radioactivity present as methyl groups. In *panel C*, identical slices from duplicate lanes were incubated with 100 μl of 2 N NaOH for 24 h at 70 °C. Using the vapor phase diffusion assay described by Xie and Clarke (1993), the quantity of base-labile, volatile methyl groups was determined. In both *panels B* and *C*, data from the parent strain is displayed with the *open circles*, and data from the *rmt1* mutant strain with *closed circles*. The *arrows* indicate the positions of molecular mass standards described in *panel A*.

cal nature of the RMT1-dependent methylated products, acid hydrolysates were prepared from *in vivo* labeled parent and mutant cells, as well as from endogenously methylated crude extracts from both strains as described under "Experimental Procedures." These hydrolysates were then analyzed by amino acid analysis using high resolution cation exchange chromotography (Lee *et al.*, 1977) under conditions where N^G-monomethylarginine, N^G,N^G-dimethylarginine (asymmetric), and N^G,N'^G-dimethylarginine (symmetric) are resolved from each other. The results from experiments where intact yeast cells were incubated with [^{3}H]AdoMet (Fig. 5, *B* and *D*) show that a disruption of the *RMT1* gene reduces the peak of asymmetrically [^{3}H]dimethylated arginine by greater than 85% compared to the level in the parent cells, with a smaller reduction in the amount of [^{3}H]monomethylarginine formed. We observed little or no change in the earlier eluting [^{3}H]methylated hydrolytic products between the parent and mutant strains (Fig. 5, *A* and *C*). Similar results were obtained when we analyzed the acid-hydrolyzed products of *in vitro*, endogenously methylated parent and mutant soluble extracts (Fig. 6). Incubation of the parent extract with [^{3}H]AdoMet produced equal levels of both mono- and asymmetrically dimethylated arginine residues (Fig. 6*B*), whereas only monomethylarginine was produced in the mutant extract (Fig. 6*D*). No changes were observed in the amounts of the previously eluted methylated species in the parent and mutant extracts (Fig. 6, *A* and *C*).

The results of Figs. 5 and 6 provide conclusive evidence for a protein-arginine methyltransferase activity that is dependent on the *RMT1* gene product. Furthermore, this activity represents the major arginine methyltransferase activity present in intact yeast cells. The analysis of *in vivo* methylated cells indicates that the *RMT1* gene product is required for about 89% of the asymmetrically dimethylated arginine residues and about 66% of monomethylated arginine residues (Fig. 5, *B* and *D*). *In vitro*, the *RMT1* gene product is responsible for 83% of the asymmetrically dimethylated arginine residues, but does not seem to be required for the production of monomethylarginine (Fig. 6, *B* and *D*). These results indicate the presence of at least one RMT1-independent protein-arginine methyltransferase in yeast.

Methylation of Hypomethylated Yeast Substrates Using a GST-RMT1 Fusion Protein—The absence of protein-arginine methyltransferase activity in the yeast *rmt1* mutant allowed us to use the soluble fraction from these cells as a source of hypomethylated protein substrates for this enzyme. We prepared an N-terminal GST fusion construct in which the *RMT1* gene is translated in frame with the 26-kDa GST polypeptide and expressed the fusion protein in bacteria. When the soluble fraction from the *rmt1* mutant strain was incubated with the purified GST-RMT1 fusion protein and [^{3}H]AdoMet, a large number of methylated products were present that were not observed in the control reactions lacking the GST-RMT1 fusion protein (Fig. 7) or containing *rmt1* soluble extract and purified GST itself (data not shown). The major methylated species

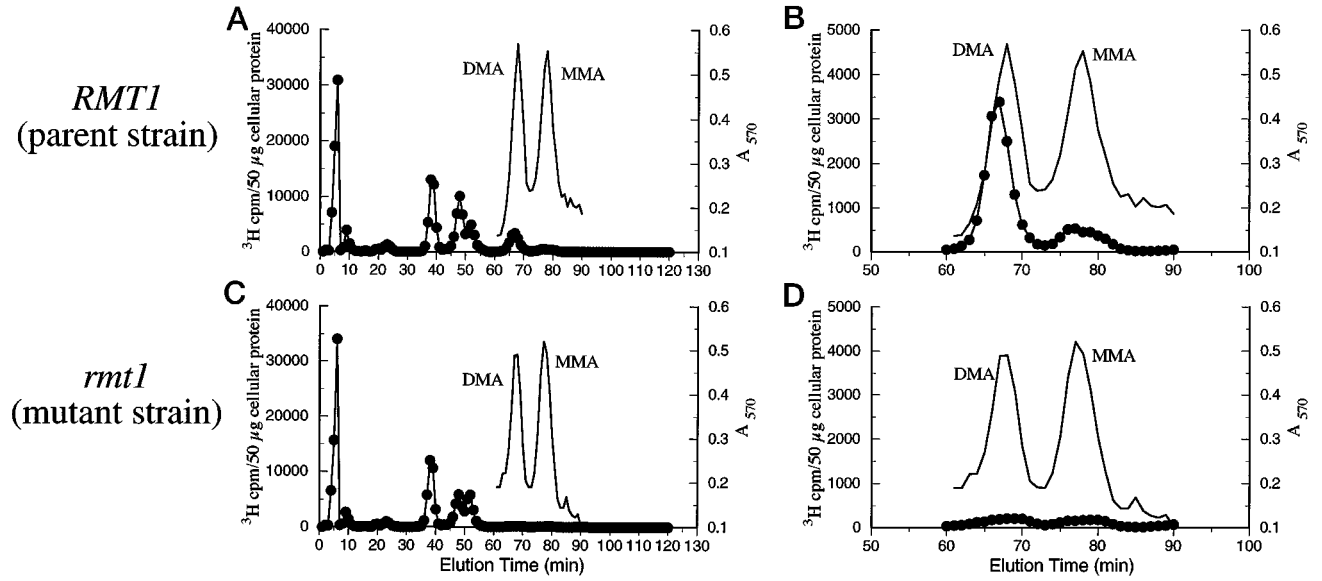

FIG. 5. **The *in vivo* mono- and asymmetric dimethylation of arginine residues in yeast is dependent upon the *RMT1* gene product.** *Panels A* and *C* show the fraction of N^G,N^G-dimethylated (asymmetric) arginine (*DMA*) and N^G-monomethylated arginine (*MMA*) present in acid hydrolysates of lysed parent (*RMT1*) or mutant (*rmt1*) yeast cells after a 30-min *in vivo* labeling with [^{3}H]AdoMet, as described under "Experimental Procedures." The samples were mixed with 1 μmol of each of the non-isotopically labeled standards, N^G,N^G-dimethylarginine (asymmetric) and N^G-monomethylarginine (both obtained from Sigma). An equal volume of citrate sample dilution buffer (0.2 M in Na^+ containing 2% thiodiglycol and 0.1% phenol at pH 2.2) was then added, and the resulting mixture was loaded onto a high resolution amino acid analysis cation exchange column. The column (Beckman AA-15 sulfonated polystyrene, 0.9 cm diameter × 11 cm height) was equilibrated with sodium citrate buffer (0.35 M in Na^+, pH 5.27) at 55 °C and eluted at approximately 1 ml/min. After each run the column was washed with 0.2 N NaOH for 20 min prior to the next run. One minute fractions were collected, and [^{3}H] radioactivity was determined by counting a 700-μl aliquot of each fraction (*filled circles*) by liquid scintillation in 5 ml of fluor. An additional 100 μl of each fraction was analyzed for the non-isotopically methylated amino acid standards using the ninhydrin method (Gary and Clarke, 1995) (*solid line*). Briefly, the 100 μl column sample was diluted with 600 μl of water and mixed with 300 μl of ninhydrin reagent (2% (w/v) ninhydrin and 3 mg/ml hydrindantin in a solvent of 75% (v/v) dimethyl sulfoxide and 25% (v/v) 4 M lithium acetate at pH 4.2). The mixture was heated for 15 min at 100 °C, and the absorbance at 570 nm was measured. It should be noted that the standard N^G,N'^G-dimethylarginine (symmetric) always elutes between the positions of N^G,N^G-dimethylated (asymmetric) arginine and N^G-monomethylated arginine (see text and Fig. 7*B*). The slightly earlier elution of the [^{3}H]methylarginine derivatives compared to their cold standards is due to the change in molecular weight and pI of the [^{3}H]species *versus* the hydrogenated form (Gottschling and Freese, 1962; Xie and Clarke, 1993). *Panels B* and *D* are enlargements of *panels A* and *C*, respectively, in the region where methylated arginines elute.

include polypeptides of 57, 55, 42, 38, 35, 33, 30, 29, 27, 26, 20, and 13 kDa and a minor methylated species at 66 kDa. These results directly show that the *RMT1* gene product is necessary for a yeast protein-arginine methyltransferase activity.

The yeast polypeptides methylated when incubated with the purified GST-RMT1 fusion protein (Fig. 7, *lane 1*) are mono- and asymmetrically dimethylated (Fig. 8*A*). The yeast GST-RMT1 fusion protein was incubated with [^{3}H]AdoMet in the presence or absence of *rmt1* mutant extract. The proteins were then precipitated and acid-hydrolyzed to quantitate the amount of [^{3}H]mono- and asymmetrically dimethylated arginine residues formed (Fig. 8, *A* and *C*). In the absence of *rmt1* extract, only a small background of methylated arginine residues was detected (Fig. 8, compare *A* with *C*).

We also compared the ability of the yeast GST-RMT1 and the mammalian GST-PRMT1 fusion proteins to cause the methylation of the hypomethylated substrates present in the yeast *rmt1* soluble extracts. The purified GST fusion protein containing the rat PRMT1 sequence was able to efficiently methylate only a 55-kDa species, while the corresponding yeast GST fusion protein promoted the methylation of 10 or more polypeptide species (Fig. 7, compare *lanes 1* and *3*). Thus the rat GST fusion enzyme appears to have a quite restricted substrate specificity for yeast hypomethylated proteins when compared to the yeast GST fusion protein. This narrow specificity demonstrated by the rat fusion protein also reflects the activity of the native rat enzyme, since the 55-kDa species is also the major methylated polypeptide when a soluble extract of RAT1 cells is used to methylate *rmt1* mutant cytosol. We do find, however, two additional minor methylated polypeptides at 34 and 24 kDa when *rmt1* extract is incubated with RAT1 extract and [^{3}H]AdoMet (Fig. 7). We determined that the purified GST-PRMT1 fusion protein specifically mono- and asymmetrically dimethylates the 55-kDa yeast substrate (Fig. 8, *B* and *D*).

The Yeast RMT1 Fusion Protein is a Protein-arginine Methyltransferase and Has a Broad Substrate Specificity for Purified Exogenous Proteins—The studies described above demonstrate that RMT1 is required for a protein-arginine methyltransferase activity in *S. cerevisiae*, but do not directly demonstrate that the product of the *RMT1* gene encodes the catalytic activity. We thus tested the ability of the GST-RMT1 fusion protein to methylate the peptide R1 (GGFGGRGGFG-amide) (a gift from D. Aswad, University of California, Irvine), which contains a consensus arginine methylation site (Najbauer *et al.*, 1993). This fibrillarin/nucleolin-related peptide inhibits the methylation of endogenous protein-arginine methyltransferase substrates in PC12 cells and can be methylated by a partially purified bovine brain protein-arginine methyltransferase (Najbauer *et al.*, 1993). This peptide is also an efficient substrate for the purified GST-RMT1 fusion protein; this result shows that the *RMT1* gene product is itself an arginine methyltransferase, rather than an activator of a separate yeast catalytic activity. GST-RMT1 only monomethylates the arginine residue in the R1 peptide, with an initial velocity of 105 pmol/min/mg of fusion protein (data not shown). We propose that the lack of a proper tertiary structure in the 10-residue peptide is the cause for its specific monomethylation. Because this peptide contains the RGG-consensus sequence found in the mono- and asymmetric arginine methylation sites in fibrillarin (Lischwe *et al.*, 1985b), nucleolin (Lischwe *et al.*, 1985a), and hnRNP A1 (Rajpurohit *et al.*, 1994a), we chose to test whether specific mammalian proteins

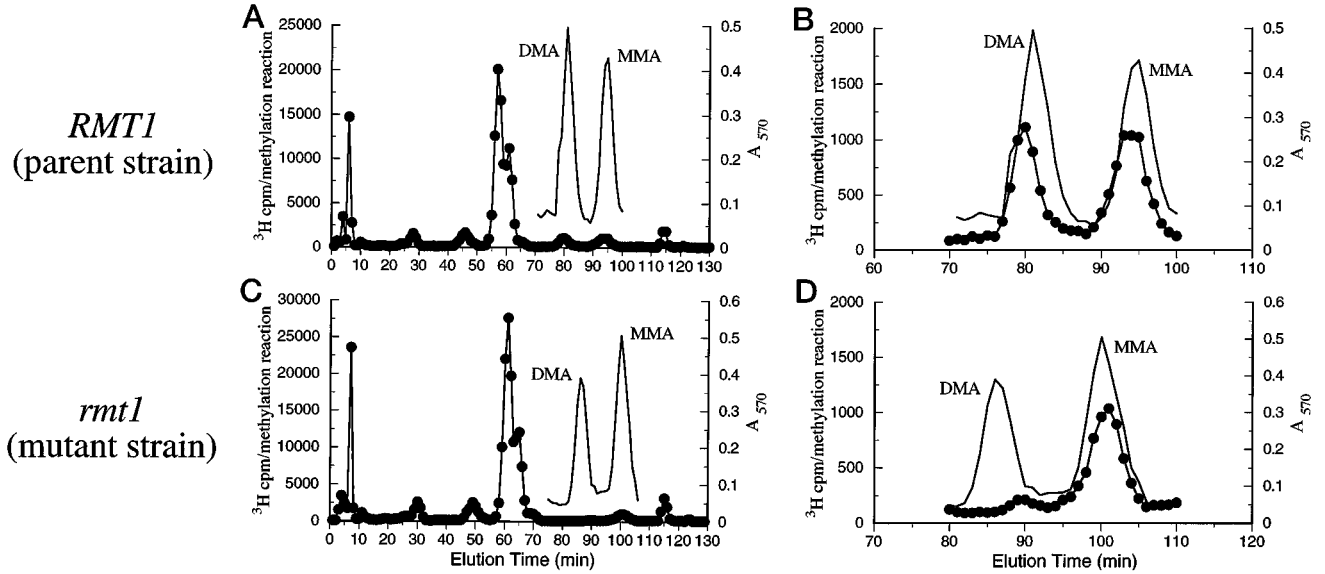

FIG. 6. **RMT1 is required for the asymmetric dimethylation of arginine residues in cellular extracts.** *Panels A* and *C* show the fraction of asymmetrically dimethylated arginine (*DMA*) and monomethylated arginine (*MMA*) formed during *in vitro* methylation reactions containing parent (*RMT1*) or mutant (*rmt1*) yeast extract with a 1-h incubation at 30 °C with [^{3}H]AdoMet. The reactions contained either 269 μg of CH9100-2 extract or 338 μg of JDG9100-2 extract, buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5), and 0.82 μM [^{3}H]AdoMet (2.2 μCi) in a final volume of 34 μl. After 30 min, 15 μl of each reaction was removed and placed in a glass vial (6 × 50 mm) and the protein was precipitated by the addition of an equal volume of 25% (w/v) trichloroacetic acid. The mixture was incubated at 25 °C for 10 min before pelleting the precipitate at 4,000 × *g* for 20 min at 25 °C. The protein pellet was washed with −20 °C acetone and dried before being acid hydrolyzed as described under "Experimental Procedures." The hydrolyzed pellet was resuspended in 50 μl of water, and 10 μl was loaded onto the sulfonated polystyrene column as described in Fig. 5. *Panels B* and *D* are enlargements of *panels A* and *C*, respectively, in the region where methylated arginines elute.

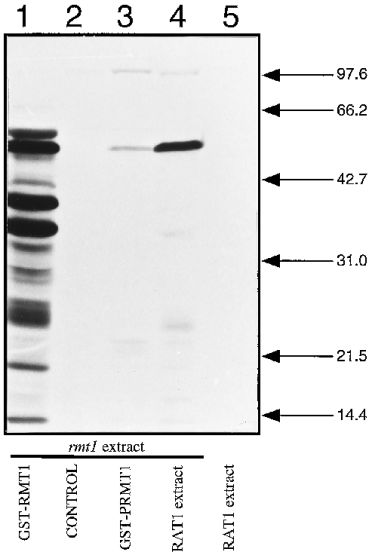

FIG. 7. **The addition of the yeast GST-RMT1 or the mammalian GST-PRMT1 fusion protein complements the methylation deficiency in *rmt1* extract.** Reactions (50 μl) in *lanes 1–3*, contained 169 μg of *rmt1* soluble extract protein, 0.7 μM [^{3}H]AdoMet (2.75 μCi), buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5), and either 1.95 μg of GST-RMT1 protein, 2.0 μg of GST-PRMT1 protein (see "Experimental Procedures"), or no fusion protein, as the control. The reactions were incubated and analyzed as described in Fig. 4*A*. In *lanes 4* and *5*, reactions (50 μl) contained 59 μg of soluble extract protein from RAT1 cells (see "Experimental Procedures"), buffer, 0.7 μM [^{3}H]AdoMet (2.75 μCi), with or without the addition of 169 μg of extract protein from the *rmt1* mutant cells. The reactions were incubated and analyzed as described above. This panel shows the result of a 5-day exposure at −80 °C. Molecular mass markers are indicated by the *arrows*.

could serve as substrates for the yeast protein-arginine methyltransferase fusion protein. Several substrates have been used for both purification and differentiation of the two types of arginine methyltransferases (Lee *et al.*, 1977; Farooqui *et al.*, 1985; Ghosh *et al.*, 1988; Rawal *et al.*, 1994).

We found that the purified GST-RMT1 fusion protein is able to methylate crude histones, recombinant hnRNP A1 (a gift from A. Krainer and A. Mayeda, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and to a lesser extent cytochrome *c* and myoglobin (Fig. 9*A*). On the other hand, GST-RMT1 did not appear to methylate myelin basic protein (Fig. 9*A*). We found that hnRNP A1 is mono- and asymmetrically dimethylated on arginine residues by the GST-RMT1 fusion protein (Fig. 9*B*). We also determined that the chemical identity of the methylated residues in crude histones is mono- and asymmetric dimethylarginine (data not shown).

Sensitivity of the Yeast and Mammalian Protein-arginine Methyltransferases to Inhibitors—We compared the yeast and mammalian protein-arginine methyltransferase fusion proteins for their ability to be inhibited by AdoMet analogs that have been used to study cellular methylation reactions (Kujubu *et al.*, 1993; Law *et al.*, 1992). We incubated each of the fusion proteins with 5′-methylthioadenosine and *S*-adenosyl-L-homocysteine using *rmt1* soluble extract as a source of methyl-accepting polypeptides. We found that neither enzyme was inhibited by 220 μM MTA (data not shown). However, *S*-adenosyl-L-homocysteine was found to be an inhibitor of both enzymes (Fig. 10, *A* and *B*). Using this assay, it appears that the yeast fusion protein is at least 20-fold less sensitive to *S*-adenosyl-L-homocysteine than the mammalian fusion protein. We estimate that the concentration needed to inhibit half of the activity of the yeast fusion enzyme is 250 μM, while that of the mammalian fusion enzyme is at least an order of magnitude lower.

DISCUSSION

Although a number of proteins have been identified that contain methylated arginine residues and protein-arginine methyltransferase activities have been characterized in a variety of eukaryotic cells, the functional significance of this type of protein modification is not well understood (Kim *et al.*, 1990; Lischwe, 1990; Clarke, 1993). For myelin basic protein, the

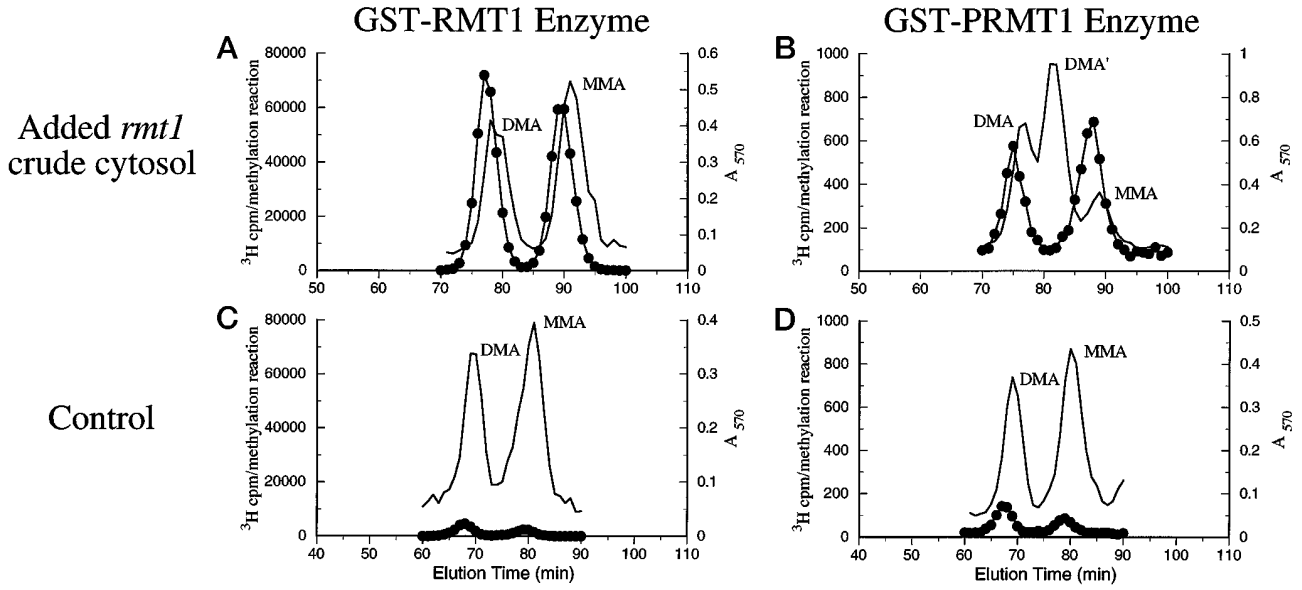

FIG. 8. **Polypeptides from the yeast *rmt1* mutant extract are mono- and asymmetrically dimethylated on arginine residues when incubated with the GST-RMT1 and GST-PRMT1 fusion proteins.** In *panels A* and *B*, the methylated polypeptides seen in *lanes 1* and *3* of Fig. 7 were analyzed for mono- and asymmetrically dimethylated arginine content. For *panel A*, 3.9 μg of GST-RMT1 protein was incubated at 30 °C for 30 min with 253 μg of *rmt1* extract protein, 0.82 μM [^{3}H]AdoMet (2.2 μCi), and buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5) in a final volume of 34 μl. For *panel B*, 1.55 μg of GST-PRMT1 was incubated at 30 °C for 30 min with 338 μg of *rmt1* extract protein, 0.97 μM [^{3}H]AdoMet (2.2 μCi), and buffer in a final volume of 29 μl. Both reactions were stopped by the addition of an equal volume of 25% (w/v) trichloroacetic acid. The samples were then acid-hydrolyzed and analyzed as in Fig. 5. Corresponding control reactions (*panels C* and *D*) were also prepared with only the fusion proteins, [^{3}H]AdoMet, and buffer. For the control reactions, 20 μg of bovine serum albumin was added to the reaction just prior to trichloroacetic acid precipitation to obtain a higher recovery of protein. All samples were co-chromatographed with 1 μmol of N^G,N^G-dimethylarginine (asymmetric) (*DMA*) and 1 μmol of N^G-monomethylarginine (*MMA*), shown as *solid lines* in the elution profile. In *panel B*, 1 μmol of N^G,N'^G-dimethylarginine (symmetric) (*DMA'*) was also included.

only known protein that contains a symmetrically dimethylated arginine residue (Kim *et al.*, 1990), methylation has been suggested to enhance the compaction of the opposing plasma membranes in myelin (Amur *et al.*, 1986; Young *et al.*, 1987; Rawal *et al.*, 1992). For the group of proteins containing asymmetrically dimethylated arginine residues, a common thread has emerged; many of these species interact with RNA (Lischwe, 1990; Najbauer *et al.*, 1993; Liu and Dreyfuss, 1995). Asymmetric dimethylation of arginine residues in these proteins could cause RNA binding to shift from a specific to a nonspecific mode due to the loss of specific hydrogen bonds (Calnan *et al.*, 1991). Consistent with this model, there is reduced nucleic acid binding of methylated hnRNP A1 compared to the unmethylated form (Rajpurohit *et al.*, 1994b). It is thus possible that arginine methylation modulates the activity of hnRNP A1 in pre-mRNA splicing or the activities of nucleolin and fibrillarin in processing preribosomal RNA (Lischwe *et al.*, 1985; Aris and Blobel, 1991). Asymmetric dimethylation of arginine residues has also been proposed as a means of modulating nuclear localization of these and other proteins. For example, only the high molecular weight forms of basic fibroblast growth factor that contain methylated arginine residues are specifically found in the nucleus (Burgess *et al.*, 1991). Additional roles of arginine methylation in the heat shock response (Desrosiers and Tanguay, 1988; Wang *et al.*, 1992) and in virus-induced cell transformation (Enouf *et al.*, 1979; Wang *et al.*, 1992) have been suggested as well.

No genes encoding a protein-arginine methyltransferase activity have been previously identified. In this work, we show that the yeast *ODP1* gene product, now designated *RMT1*, is a protein-arginine methyltransferase that is similar to a rat gene product we have recently characterized (Lin *et al.*, 1996). The RMT1 protein catalyzes the formation of N^G-monomethylarginine and N^G,N^G-dimethyl- (asymmetric) arginine residues on a number of endogenous yeast substrates and on exogenous mammalian proteins, including those containing a glycine and arginine-rich GAR domain.

In vivo analysis of methylated proteins in a *rmt1* mutant strain indicates that the major activity responsible for asymmetrically dimethylating as well as monomethylating arginyl residues in *S. cerevisiae* is dependent upon a functional *RMT1* gene product. Using soluble extracts from the parent and the *rmt1* mutant yeast cells in *in vitro* assays, we confirmed that the predominant asymmetric dimethylarginine methyltransferase activity is dependent upon RMT1. However, the homogenization of the yeast cells appears to release an RMT1-independent monomethylarginine methyltransferase from a cellular compartment where the activity could not be detected *in vivo*. Direct evidence that RMT1 is a mono- and asymmetric dimethylarginine methyltransferase comes from the acid hydrolysis and amino acid analysis of *in vitro* reactions containing the GST-RMT1 fusion protein and recombinant hnRNPA1 methyl-accepting protein.

It should be noted that our results are in contrast to those of Liu and Dreyfuss (1995), where arginine methylation was not observed in yeast extracts when recombinant hnRNP A1 was used as a substrate. One possible explanation for this difference is that the yeast strains used here are protease-deficient strains and that the methyltransferase activity may be highly susceptible to proteolysis when cells are disrupted for extract preparation.

This RMT1-dependent methylation is clearly not essential for viability since the *rmt1* cells grow similarly to the parent cells in YPD. Assays with purified substrates, using the GST-RMT1 fusion protein, suggest that this enzyme has a broad specificity for methyl-accepting substrates. The yeast fusion protein is not only able to methylate mammalian histones and recombinant hnRNP A1, but also cytochrome *c* and myoglobin. The latter two proteins have been characterized as substrates for a *Euglena gracilis* protein-arginine methyltransferase

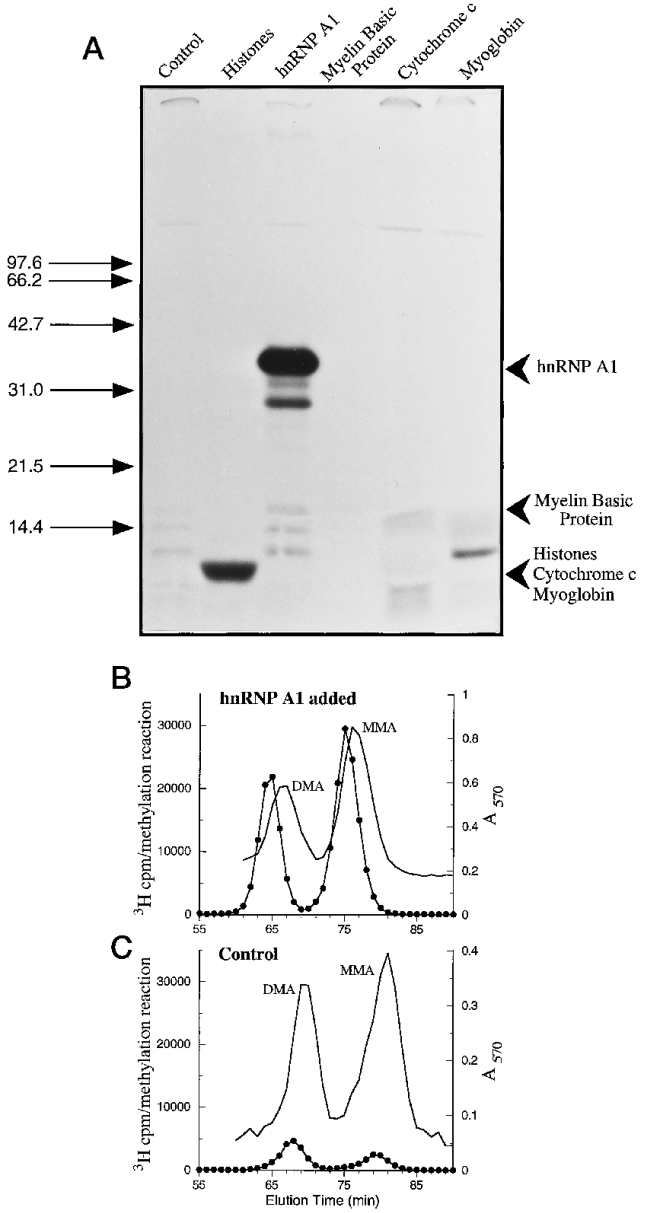

FIG. 9. **The GST-RMT1 fusion protein methylates a wide variety of substrates *in vitro*.** In *panel A*, purified GST-RMT1 protein (1.95 μg) was incubated with each of the following: 100 μg of histones (calf thymus, Sigma; type IIAS), 490 ng of recombinant human hnRNP A1 protein, 100 μg of myelin basic protein (bovine brain, Sigma), 100 μg of cytochrome *c* (horse heart, Sigma), and 100 μg of myoglobin (sperm whale skeletal muscle, Sigma). The reaction also contained 0.93 μM [^{3}H]AdoMet (2.2 μCi) and buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5) in a final volume of 30 μl. The reactions were incubated at 30 °C for 30 min and then stopped by the addition of an equal volume of 2 × SDS-gel electrophoresis sample buffer. The samples were then loaded onto a 12.6% SDS-acrylamide gel and fluorographed. This panel represents a 7-day exposure at −80 °C. *Arrowheads* indicate the position of the substrate proteins as determined by Coomassie stain. The *arrows* indicate the position of molecular mass standards described in Fig. 5. In order to determine the identity of the methylated residue, reactions identical to those described in *panel A* (*lanes 1* and *2*) were incubated at 30 °C for 30 min. Bovine serum albumin (20 μg) was then added to each reaction, and the entire mixture was transferred to a 6 × 50-mm glass vial. An equal volume of 25% (w/v) trichloroacetic acid was added, and the mixture was incubated at 25 °C for 10 min. Precipitated protein pellets were acid-hydrolyzed and analyzed by cation exchange chromotography as in Fig. 4. In *panels B* and *C*, the elution of [^{3}H] radioactivity is shown to coincide with the peaks of N^G,N^G-dimethylarginine (asymmetric, *DMA*) and N^G-monomethylarginine (*MMA*).

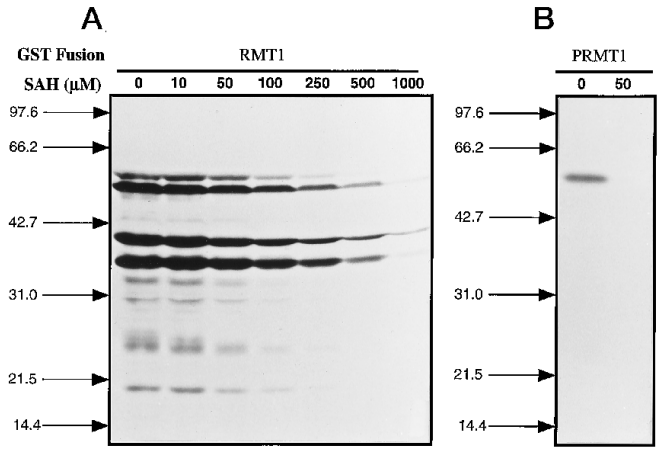

FIG. 10. **The yeast and mammalian arginine methyltransferase-GST fusions are differentially inhibited by *S*-adenosyl-L-homocysteine.** rmt1 soluble extract (169 μg of protein) was incubated with 1.95 μg of GST-RMT1 protein or 0.78 μg of GST-PRMT1 protein, 0.93 μM or 1.16 μM [^{3}H]AdoMet (2.2 μCi) respectively, the indicated concentrations of *S*-adenosyl-L-homocysteine (*SAH*) and buffer (25 mM Tris-HCl, pH 7.5, 1 mM sodium EDTA, and 1 mM sodium EGTA) in a final volume of 30 μl (*panel A*) and 24 μl (*panel B*). The reactions were performed at 30 °C for 30 min and were stopped by the addition of an equal volume of 2 × SDS-gel electrophoresis sample buffer. The samples were then loaded onto a 10% SDS-acrylamide gel and fluorographed. Both panels represent 4-day exposures at −80 °C. The *arrows* indicate the positions of molecular mass standards described in Fig. 4.

(Farooqui *et al.*, 1985), and the former two proteins as substrates for the mammalian enzyme. The broad specificity of the yeast RMT1, indicated both by the ability of the fusion protein to methylate purified substrates and by the many substrate proteins present in hypomethylated *rmt1* mutant extracts, suggests the wide-spread use of this post-translational modification in yeast. The comparatively restricted substrate specificity of the rat PRMT1 enzyme suggests the functions of a single yeast protein-arginine methyltransferase may be distributed among a family of related enzymes in higher eukaryotes, as is the case for other enzymes responsible for the posttranslational protein modifications (*e.g.* cyclin-dependent protein kinases; Grana and Reddy (1995)). In fact, analysis of the expressed sequence tag data base indicates that there appear to be at least two related human cDNA sequences that are highly similar to the rat PRMT/yeast RMT1 sequences.

Ghosh *et al.* (1988) partially purified two distinct protein-arginine methyltransferase activities from calf brain. One enzyme specifically methylated myelin basic protein (Ghosh *et al.*, 1990), and the other was a histone arginine methyltransferase that was later shown to be more efficient toward hnRNP A1 (Rajpurohit *et al.*, 1994a). Our substrate analysis of the yeast protein-arginine methyltransferase supports this idea of at least two distinct classes of arginine methyltransferases. The myelin basic protein-arginine methyltransferase specifically mono- and symmetrically dimethylates arginine residue 107 (Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971), while the histone/hnRNP A1-specific enzyme mono- and asymmetrically dimethylates multiple arginine residues present in a GAR domain (Rajpurohit *et al.*, 1992). The GST-RMT1 fusion protein methylated histones, hnRNP A1, cytochrome *c*, and myoglobin: substrates that can be modified to contain mono- or asymmetrically dimethylated arginine residues (Paik and Kim, 1969; Beyer *et al.*, 1977; Karn *et al.*, 1977; Farooqui *et al.*, 1985). However, GST-RMT1 was not able to methylate myelin basic protein.

The potential *in vivo* yeast arginine methyltransferase substrates identified by Najbauer *et al.* (1993) have molecular sizes similar to those of the methylated polypeptides observed when

GST-RMT1 was incubated with hypomethylated *rmt1* cytosol and [^{3}H]AdoMet. For example, the NSR1 polypeptide has a predicted size of 45 kDa, but migrates as a 67-kDa polypeptide by SDS-gel electrophoresis. *NSR1* is an essential gene in yeast, encoding a protein that not only binds nuclear localization sequences (Lee *et al.*, 1991) but also is involved in the processing of pre-rRNA and is the homolog to the mammalian nucleolin (Kondo and Inouye, 1992; Kondo *et al.*, 1992). Another potential RMT1 substrate is NPL3, whose gene was isolated from a mutant defective in localization of nuclear proteins and whose protein product migrates as a 55-kDa species (Bossie *et al.*, 1992). The RNA and single-stranded DNA-binding protein SSB1 also contains a GAR domain and migrates as a 45-kDa protein (Jong *et al.*, 1987), while NOP1, the essential yeast fibrillarin homolog, migrates as a 38-kDa protein (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990). The *GAR1* gene was identified by Southern analysis with a cDNA probe corresponding to the GAR domain of *Xenopus* fibrillarin. The encoded protein migrates at 24.5 kDa by SDS-gel electrophoresis (Girard *et al.*, 1992). We plan to determine if these GAR domain-containing proteins are in fact the major substrates *in vivo* and whether the lack of methylation affects their biological activities. The hnRNP A1 methylation reaction is of interest because of the availability of purified, recombinant protein as a substrate and the recent partial purifications of a methyltransferase activity capable of specifically methylating this substrate (Rawal *et al.*, 1994; Liu and Dreyfuss, 1995). Furthermore, the ability of hnRNP A1 to preferentially promote splicing to more distal 5′-splice sites has been well characterized (Mayeda and Krainer, 1992; Mayeda *et al.*, 1993) and provides an assay for determining the potential role of methylation in this context. The investigation into the identity of other GST-RMT1 substrates will also be of great importance in determining functions for arginine methylation.

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