

# RNase Treatment of Yeast and Mammalian Cell Extracts Affects *in Vitro* Substrate Methylation by Type I Protein Arginine *N*-Methyltransferases<sup>1</sup>

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**Type I protein arginine *N*-methyltransferases catalyze the formation of  $\omega$ -*N*<sup>G</sup>-monomethylarginine and asymmetric  $\omega$ -*N*<sup>G</sup>, *N*<sup>G</sup>-dimethylarginine residues using *S*-adenosyl-L-methionine as the methyl donor. *In vitro* these enzymes can modify a number of soluble methyl-accepting substrates in yeast and mammalian cell extracts including several species that interact with RNA. We treated normal and hypomethylated *Saccharomyces cerevisiae* and RAT1 cell extracts with RNase prior to *in vitro* methylation by recombinant protein *N*-arginine methyltransferases and found that the methylation of certain polypeptides is enhanced up to 12-fold whereas that of others is diminished. 2-D gel electrophoresis of RNase-treated yeast extracts allowed us to tentatively identify the glycine- and arginine-rich (GAR) domain-containing proteins Gar1, Nop1, Sbp1, and Npl3 as major methyl-acceptors based on their known isoelectric points and apparent molecular weights. These results suggest that the methylation and RNA-binding of GAR domain-containing proteins *in vivo* may regulate protein-nucleic acid or protein-protein interactions.** © 1999 Academic Press

Type I protein arginine *N*-methyltransferases (PRMTs) catalyze the *S*-adenosyl-L-methionine-dependent formation of  $\omega$ -*N*<sup>G</sup>-monomethylarginine and asymmetric  $\omega$ -*N*<sup>G</sup>, *N*<sup>G</sup>-dimethylarginine residues on a variety of methyl-accepting polypeptides. Genes encoding these methyltransferases have been identified in yeast (1,2), rats (3,4), and humans (5). The Rmt1 yeast (*S. cerevisiae*) and the PRMT1 rat enzymes share 45% sequence identity and have been shown to act as functionally homologous proteins (1,4-6). A second rat enzyme, PRMT3, shares 46% sequence identity over its

catalytic domain with rat PRMT1 and has an N-terminal domain containing a putative C2H2 zinc finger motif. PRMT3, however, differs in its subcellular localization, regulation, and substrate specificity to other Type I enzymes (4).

PRMT1 has been implicated in a variety of cell signaling events. It was initially identified in a yeast two-hybrid screen based on its interaction with both mitogenically stimulated proteins TIS21 (BTG2) and BTG1 (3). In an independent yeast two-hybrid screen human PRMT1 (IRIB4) was identified based on its association with the cytoplasmic domain of the IFNAR1 chain of the interferon- $\alpha,\beta$  receptor (7). An antisense oligonucleotide to PRMT1 reduced growth inhibition by interferon while concomitantly curtailing arginine methyltransferase activity in human myeloma U266S cells (7). Finally, the human PRMT1 gene was also cloned as a multicopy suppressor (HCP1) of the inositol auxotrophy exhibited in *S. cerevisiae* *ire15* mutants (6). Its expression resulted in an upregulation of inositol 1-phosphate synthase transcription (6). The variety of screens which resulted in the isolation of PRMT1 suggests that the modification of arginyl residues may be important in some signal transduction reactions.

RNA-binding proteins are major *in vivo* substrates for Type I protein arginine methylation (for review, see (8)). These include mammalian hnRNP A1 (9-11), human GAP-associated tyrosine phosphoprotein p62 (also known as SAM68 for Src-associated in mitosis) (12), human poly(A)-binding protein II (13), rat fibrillarin (14), rat nucleolin (15), and yeast Npl3 (2,16). In fact, yeast *RMT1* was also found in a screen that caused a synthetic lethality in a strain containing a temperature-sensitive mutation in the Npl3 protein, a methyl-acceptor that plays an important role in poly(A)<sup>+</sup>-RNA maturation and transport (2). More recently, RNA-binding proteins Hrp1 and Hrb1 in yeast have been shown to be methylated *in vitro* by Rmt1, and functional studies suggest that they are methyl-

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ated *in vivo* (17,18). The site of arginine methylation for these substrates typically occurs within glycine- and arginine-rich (GAR) domains from which several consensus sequences have been derived (3,4,8,19,20).

To explore the relationship between the methyl-accepting substrates for Type I protein arginine *N*-methyltransferases and RNA binding, we pre-treated hypo- and normally-methylated yeast and RAT1 cell extracts with RNase prior to *in vitro* methylation by recombinant protein arginine *N*-methyltransferases. An enhancement of methylation for certain substrates was found to be dependent upon RNase treatment in extracts tested. Methylation under these conditions allowed us to tentatively identify the GAR domain-containing proteins Gar1, Nop1, Sbp1, and Npl3 from a list of candidate substrates as major methyl-acceptors based on their known isoelectric points (pIs) and apparent molecular weights by 2-D gel electrophoresis. Gar1 methylation, in particular, was dependent upon the pre-treatment of the extracts with RNase, suggesting that the *in vitro* methylation of Gar1 may be blocked by its interaction with the small nucleolar RNAs that interact with it (21). The results in this study suggest that protein methylation *in vivo* may be regulated by RNA interactions with methyl-accepting proteins.

## EXPERIMENTAL PROCEDURES

**Preparation of GST fusions of type I protein arginine *N*-methyltransferases.** GST fusion proteins of recombinant yeast protein arginine *N*-methyltransferase RMT1 (1), rat PRMT1 (3), and rat PRMT3 (4) were purified as previously described from extracts of *Escherichia coli* strain DH5 $\alpha$  harboring vectors pGEX-RMT1, pGEX(SN)-PRMT1, and pGEX(SN)-PRMT3, respectively. These fusion proteins were purified from extracts by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions and eluting with 30 mM glutathione, 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 2% glycerol. Aliquots of these enzymes were subsequently stored at  $-80^{\circ}\text{C}$ .

**Preparation of yeast soluble extracts.** *Saccharomyces cerevisiae* yeast strains CH9100-2 (*MAT $\alpha$* , *prc1-407*, *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*, *ycl57 $\Delta$ ::URA3*) (22), and JDG9100-2 (*MAT $\alpha$* , *prc1-407*, *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*, *ycl57 $\Delta$ ::URA3*, *rmt1::LEU2*) (1), also referred to as *RMT1* and *rmt1*, respectively, were grown in 250 mL YPD media on a shaker at  $30^{\circ}\text{C}$  to an  $A_{600\text{ nm}} = 2.5$ . Cell suspensions were then centrifuged at  $4,400 \times g$  for 10 min at  $4^{\circ}\text{C}$ , washed several times in buffer A (250 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA), and finally resuspended in approximately 3 mL buffer A/g (wet weight) of cells. These cell suspensions were passed twice through a French pressure cell at 20,000 p. s. i., and the resultant lysates were centrifuged at  $23,000 \times g$  for 50 min at  $4^{\circ}\text{C}$ . The soluble extracts were then collected and stored at  $-20^{\circ}\text{C}$ .

**Protein concentration determination.** A modification of the Lowry procedure was used to determine protein concentrations of GST fusion proteins and yeast soluble extracts following precipitation with 1 mL of 10% (w/v) trichloroacetic acid (23). A stock solution of bovine serum albumin (10 mg/mL) was used as a protein concentration standard.

**Preparation of RNase-treated soluble extracts.** 50  $\mu\text{L}$  of yeast soluble extracts (protein concentrations are as follows: 5.1 mg/mL

*RMT1*; 5.0 mg/mL *rmt1*) described above were incubated with or without 12.5 ng bovine pancreatic RNase (2.5  $\mu\text{L}$  of a 5  $\mu\text{g}/\text{mL}$  stock solution) for 30 min at  $23^{\circ}\text{C}$ . The RNase treatment was also performed using 50  $\mu\text{L}$  extracts from RAT1 cells (1.95 mg/mL) and adenosine dialdehyde-treated (AdOx-treated) RAT1 cells (0.12 mg/mL) generously donated by Drs. Jie Tang and Harvey Herschman at UCLA, and prepared as previously described (3). After the RNase treatment, samples were then placed on ice prior to methylation reactions described below.

**Methylation reactions.** RNase-treated and untreated yeast soluble extracts were incubated with or without varying amounts of recombinant methyltransferases in 0.83  $\mu\text{M}$  *S*-adenosyl-[methyl- $^3\text{H}$ ]-L-methionine (Amersham Pharmacia Biotech; 80.0 Ci/mmol; 2  $\mu\text{L}$ ) and buffer A in a final volume of 30  $\mu\text{L}$  for 1 hr at  $30^{\circ}\text{C}$ , or  $37^{\circ}\text{C}$  for some experiments. The reactions were either stopped by the addition of an equal volume of  $2 \times$  SDS-PAGE sample buffer for 1-D analysis (24), or by the addition of 1  $\mu\text{L}$  sample solution A (10% SDS, 150 mM DTT) for 2-D analysis, as recommended by Bio-Rad for their PROTEAN II xi 2-D Cell system.

**Gel electrophoresis.** Methylation reaction samples were heated for 5 minutes at  $100^{\circ}\text{C}$  prior to loading the entire reaction onto gels. Samples for 1-D analysis were loaded onto slab gels (1.5 mm  $\times$  10.5 cm resolving gel) using the buffer system described by Laemmli (1970) and run in either 10% or 12.6% (w/v) acrylamide, 1.1% or 1.4% (w/v), respectively, *N,N*-methylenebisacrylamide matrix with a constant current of 35 mA (25). Samples for 2-D analysis were run on a PROTEAN II xi 2-D Cell (Bio-Rad) per the manufacturer's instructions. Following electrophoresis, gels were stained for 1 hour with 0.1% (w/v) Coomassie Brilliant Blue R250 (Sigma) in 50% (v/v) methanol, 10% (v/v) acetic acid, and water. Destaining was performed in 5% (v/v) methanol, 10% (v/v) acetic acid with several solution changes over the course of several hours until bands Coomassie-stained protein bands were visible. Low molecular weight standards were obtained from Bio-Rad (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; hen egg lysozyme, 14.4 kDa). The destained gels were soaked in En $^3$ Hance (DuPont) for 1 hour and then in water for 30 minutes as suggested by the manufacturer, dried *in vacuo* onto a filter paper support for 1.5 hours at  $70^{\circ}\text{C}$ , and finally fluorographed by exposing to film (Kodak X-Omat AR) at  $-80^{\circ}\text{C}$  for varying lengths of time.

Total radioactivity of the major methylated species on 1-D gels described above was determined by a gel-slice method. Briefly, each slice (corresponding approximately to the size of the band on the fluorograph) of the dried gel was incubated with 3 mL 30% (v/v)  $\text{H}_2\text{O}_2$  in a capped scintillation vial for 24 hrs at  $55^{\circ}\text{C}$  to completely dissolve the slice. After the incubation, 10 mL scintillation fluid (Safety-Solve, Research Products International) was mixed into the vial and then the sample was counted to assess the extent methylation.

## RESULTS

The yeast *Rmt1* protein arginine *N*-methyltransferase has been previously shown to methylate several proteins in wildtype yeast soluble extracts and extracts prepared from *Rmt1*-deficient cells (1). In this study, we used purified GST-protein arginine methyltransferase fusion proteins to methylate polypeptides in various preparations of yeast extracts (Fig. 1). When comparing wildtype (*RMT1*) and *rmt1* mutant extracts as methyl-acceptors (lanes 5 and 6), polypeptide substrates with approximate molecular weights of 29 kDa and 44 kDa are methylated in *Rmt1*-deficient extracts, but are not methyl-accepting substrates in wildtype



TABLE I

RNase-Dependent Changes in Methyl-Accepting Activities of Specific Polypeptides in Yeast Soluble Extracts as Determined by the Gel Slice Assay Described Under Experimental Procedures

| Methylated species | Fold change in methylation <sup>a</sup> |                     |
|--------------------|---|---------------------|
|                    | <i>rmt1</i> extract                     | <i>RMT1</i> extract |
| R1                 | 12.1 ± 3.8                              | N.D.                |
| R1'                | N.D. <sup>b</sup>                       | 7.3 ± 5.6           |
| R2                 | 1.0 ± 0.22                              | 1.7 ± 0.5           |
| R3                 | 2.5 ± 0.39                              | 2.7 ± 1.1           |
| R4                 | 0.73 ± 0.067                            | 0.90 ± 0.26         |
| R5                 | 0.93 ± 0.20                             | 0.96 ± 0.18         |

<sup>a</sup> Average of cpm in RNase-treated sample/cpm in control sample ± standard error of the mean for 4 experiments.

<sup>b</sup> N.D., Not determined.

cantly, and R4 and R5 decreased slightly. These results suggest that the presence of endogenous RNA can either promote or inhibit the *in vitro* methylation of specific yeast proteins.

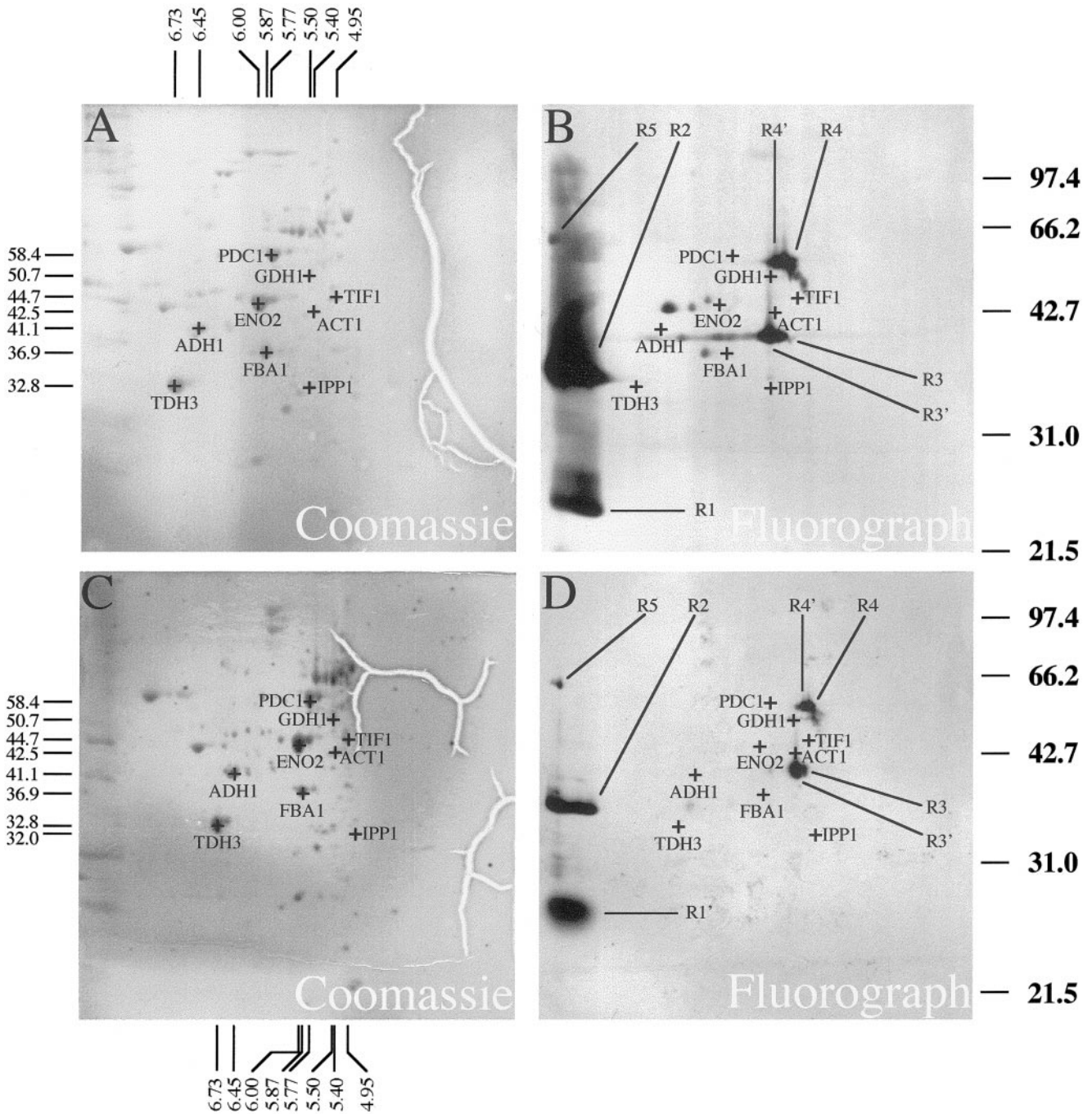
We used 2-D gel electrophoresis to characterize the major methylated species R1-R5 in RNase-treated extracts. Figure 2 shows the Coomassie gel and its corresponding fluorograph for reactions including either RNase-treated hypomethylated extracts (Fig. 2A and 2B, respectively) or RNase-treated wildtype extracts (Fig. 2C and 2D, respectively). R1, R1', R2 and R5 were found to be basic polypeptides that were not well-resolved in the isoelectric focusing direction. R3 and R4 resolved in a region of the gels in which previously identified proteins in 2-D gel analysis also migrate (Fig. 2A and 2C). Both methylated species appear to be heterogeneous in both their respective pIs, indicated by the additional labels R3' and R4' (Fig. 2B and 2D). Rajpurohit *et al.* (1994) have reported that recombinant rat hnRNP A1 differs in pI by 0.07 when comparing its unmethylated to methylated (less basic) form of the protein (26). This difference in pI may be reflected in the heterogeneous nature of the banding pattern observed for the R3/R3' pair and the R4/R4' pair of substrates, revealing various amounts of methylation.

The major methylated substrates R1-R5 were then matched with potential candidates for arginine methylation based on a comparison of apparent molecular weights on SDS gels, pIs, and the presence of arginine residues in a glycine-rich context. Many polypeptides containing GAR domains migrate anomalously slowly on SDS gels, and thus the observed apparent molecular weight values for these polypeptides in our comparisons were cited rather than their calculated weights when available (Table II). The methylated species R1 and R1' have been assigned as Gar1 and Rps2, respectively, although R1' might be an aberrantly migrating form of Gar1 whose molecular weight is different in the

*RMT1* background. Gar1 may be a more likely substrate because it contains a total of 18 potential sites for arginine methylation within its GAR domains, whereas Rps2 contains only 3 potential sites. R2, R3, and R4 have been assigned as Nop1, Sbp1, and Npl3, respectively. Each of these proteins contain several arginine-glycine repeats as potential methyl-accepting sites for Type I arginine *N*-methyltransferases. The R5 methylated species is poorly resolved in the basic region of the 2-D gels (Fig. 2B and 2D); however, 3 candidate substrates listed in Table II have similar basic pIs and molecular weights. Within their GAR domains, Ded1, Dbp2, and Psp2 proteins contain 2, 5, and 13 potential arginine methylation sites, respectively, but it is unclear as to which protein, if any, corresponds to R5 without additional analysis.

We then asked if mammalian Type I protein arginine *N*-methyltransferases could also demonstrate a change in the pattern of yeast extract methylation upon RNase treatment. We found that the GST fusion of the recombinant rat PRMT1 acts in a very similar manner as does GST-RMT1 (Fig. 3; lanes 1-8). These results are indicative of the fact that PRMT1 and Rmt1 are functionally homologous proteins (5). However, the results seen with the rat PRMT3 GST fusion protein show an entirely different substrate specificity (Fig. 3, lanes 9-12). This enzyme recognizes very few sites in the *rmt1* and *RMT1* extracts that have not been treated with RNase (Fig. 3, lanes 9 and 11). This result suggests that PRMT3 specifically recognizes substrates whose potential sites of methylation are otherwise blocked by RNA, or by proteins whose association with RNA prevent methylation. One of the most prominently methylated proteins observed for GST-PRMT3 in RNase-treated extracts is a 29 kDa protein previously reported to be its major substrate in *rmt1* yeast extracts (4). In all cases the 29 kDa band is substantially more methylated in wildtype extracts than in *rmt1* extracts, which may imply that prior methylation may confer some substrate availability, or that the amount of available substrate is less abundant in *rmt1* extracts. It is unclear at this point as to the identity of the 29 kDa substrate.

Finally, we asked whether RNase-treatment could also modify the methyl-accepting activity of mammalian extracts. By treating a RAT1 cell extract with RNase, the methylation of a 55 kDa species is enhanced, as well as several other proteins (Fig. 4; lanes 1-4); included is a 34 kDa methyl-acceptor which was previously shown to be hnRNP A1 (3). Lin *et al.* (1996) have reported that GST fusions of BTG1 and TIS21 augment the activity of an endogenous arginine methyltransferase in RAT1 cell extracts towards a 55 kDa protein (3). Mammalian extracts can be made hypomethylated by treating cells with AdOx, an effective *S*-adenosyl-L-homocysteine hydrolase inhibitor that hinders AdoMet-dependent methylation reactions as a



**FIG. 2.** 2-D analysis of *in vitro* methylation reactions of RNase-treated extracts. Yeast soluble extracts pre-incubated with RNase were subject to methylation by GST-RMT1 and separation by 2-D gel electrophoresis as described under "Experimental Procedures." The Coomassie gel of the methylation reaction with RNase-treated *rmt1* yeast soluble extract as the methyl-accepting substrate is shown in panel A, and its corresponding fluorograph (2 month exposure) is shown in panel B. The Coomassie gel of the methylation reaction with RNase-treated *RMT1* yeast soluble extract is shown in panel C, and its corresponding fluorograph (2 month exposure) is shown in panel D. Proteins previously identified in 2-D gel analysis (Swiss 2D-PAGE; <http://expasy.hcuge.ch/ch2d/ch2d-top.html>) are indicated as standards along with their reported isoelectric points (x-axis) and apparent molecular weights (y-axis): IPP1 (inorganic phosphatase; 5.41; 32.0 kDa), TDH3 (glyceraldehyde 3-phosphate dehydrogenase; 6.73; 32.8 kDa), FBA1 (fructose biphosphate aldolase II; 5.87; 36.9 kDa), ADH1 (alcohol dehydrogenase I; 6.45; 41.1 kDa), ACT1 (actin; 5.40; 42.5 kDa), ENO2 (enolase 2; 6.00; 44.7 kDa), TIF1 (translation initiation factor 4A; 4.95; 44.7 kDa), GDH1 (glutamate dehydrogenase; 5.50; 50.7 kDa), and PDC1 (pyruvate decarboxylase isozyme 1; 5.77; 58.4 kDa).

TABLE II

Candidate Type I Protein *N*-Arginine Methyltransferase Substrates in RNase-Treated Yeast Soluble Extracts

| Methylated species observed on 2-D gel <sup>a</sup> | Observed molecular weight on 2-D gel (Da) <sup>a</sup> | Observed isoelectric point on 2-D gel <sup>a</sup> | Candidate methyl-acceptors |                                |   |
|---|--|--|----------------------------|--------------------------------|---|
|   |  |  | Substrate                  | Apparent molecular weight (Da) | Calculated isoelectric point <sup>c</sup> |
| R1 (rmt1 only)                                      | 25,000   | >7.0   | GAR1 <sup>b</sup>          | 24,500 (29,30)                 | 11.48                                     |
| R1' (RMT1 only)                                     | 27,000   | >7.0   | RPS2/SUP44                 | 28,000 (34)                    | 10.59                                     |
| R2  | 37,000   | >7.0   | NOPI <sup>b</sup>          | 38,000 (36,37)                 | 10.37                                     |
| R3/R3'  | 40,000   | 5.4-5.5  | SBP1/SSBR1 <sup>b</sup>    | 45,000 (40)                    | 5.48                                      |
| R4/R4'  | 55,000   | 4.95-5.50  | NPL3/NOP3 <sup>b</sup>     | 55,000 (63)                    | 5.33                                      |
| R5  | 66,000   | >7.0   | PSP2/MSR15                 | 64,020 <sup>c</sup>            | 8.44                                      |
| R5  | 66,000   | >7.0   | DED1                       | 65,553 <sup>c</sup>            | 7.87                                      |
| R5  | 66,000   | >7.0   | DBP2 <sup>b</sup>          | 67,000 <sup>d</sup> (64)       | 8.97                                      |

<sup>a</sup> Identification of methylated species from the 2-D gel in Fig. 2.

<sup>b</sup> Putative Type I protein arginine methyltransferase substrates previously described in Gary and Clarke (1998).

<sup>c</sup> Values are provided by the Yeast Proteome Database (YPD) at <http://www.proteome.com/YPDhome.html>.

<sup>d</sup> This value is based on studies with the DBP2 human homolog, p68.

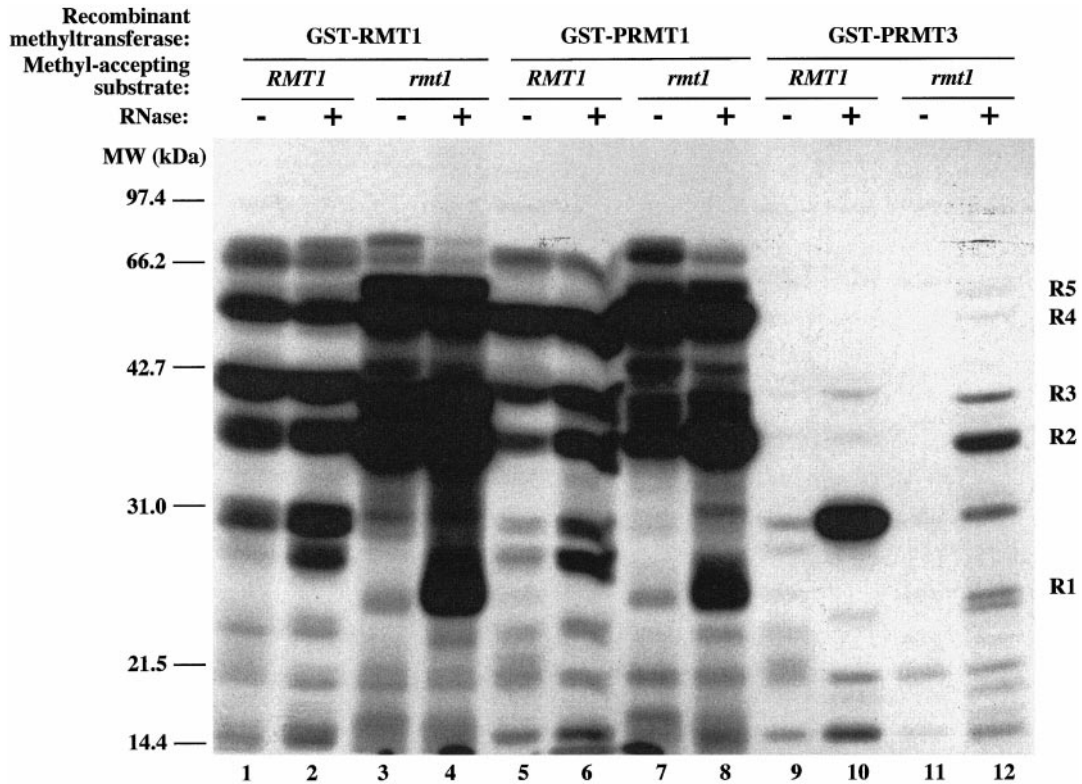
direct consequence of the substrate build-up (27). Previous studies have shown that AdOx treatment of PC12, RAT1, and human lymphoblastoid cells substantially increase the amount of available Type I methyl-acceptors (3,19,28). Although we do not observe any increase in the methylation of hnRNP A1 and several other substrates when AdOx-treated RAT1 cell extracts are pre-incubated with RNase, other polypeptides do appear to be methylated in an RNase-dependent manner (Fig. 4; lanes 5-8). Both BTG1 and TIS21 GST fusion proteins can enhance the GST-PRMT1-dependent methylation of hnRNP A1 in AdOx-treated RAT1 cell extracts (3). In light of these results it is tempting to speculate that one of the functions of both BTG1 and TIS21 may be to remove RNA from proteins whose sites for methylation can then be exposed to Type I methyltransferases.

## DISCUSSION

RNase-treatment of yeast and RAT1 soluble extracts can affect the availability of substrates for Type I protein arginine methylation. Previous studies have used hypomethylated extracts as the source of substrates to allow for maximal *in vitro* methylation (1,3,4,19,28). The combination of using hypomethylated extracts pre-treated with RNase can dramatically increase the number of methyl-accepting sites for certain proteins. The methylation enhancement of specific proteins upon RNase treatment of the extracts is methyltransferase-specific, where GST fusions of RMT1 and PRMT1 exhibit a distinct substrate specificity from that of PRMT3.

RNase treatment of yeast soluble extracts to enhance *in vitro*-methylation of specific proteins followed by 2-D gel electrophoretic analysis allowed us to tentatively identify some of the major substrates for GST-RMT1. The

first major methylated species, R1 and R1', whose methylation was enhanced dramatically upon RNase treatment of both wildtype and hypomethylated extracts, respectively, match two candidate substrates from the list in Table II, *GAR1* and *RPS2* (*SUP44*). *Gar1* was initially identified as containing GAR domains at both termini of the protein, and was subsequently shown to be an essential nucleolar protein that is associated with snoRNAs snR10 and snR30 and that is required for the pseudouridylation of rRNAs (29-31). Recently, evidence was presented for the contribution of the GAR domain of *Gar1* binding of snoRNAs *in vitro*, suggesting an accessory role for these domains in *Gar1* (21). Based on our results, we believe that the potential methylation sites of *Gar1* are completely blocked in the presence of RNA, and that the removal of RNA from our *in vitro* reaction allows for *Gar1* methylation. It is unclear under our assay conditions as to whether RNA is directly blocking methylation, or that another protein in complex with *Gar1* and RNA is preventing methylation. Both *Nhp2* and *Nop10* have been shown to associate with *Gar1* (32), and could possibly interfere with its methylation. Recently, the *Hrp1* protein, a requirement for mRNA 3'-polyadenylation formation in association with several other factors (33), was shown to be methylated by *Rmt1 in vitro*, and that this methylation was inhibited by the presence of *in vitro*-selected UAUUA-containing RNAs (18). Interestingly, sub-stoichiometric methylation (approximately 2 mol methyl group per mol of GST-*Hrp1*) of *Hrp1* did not affect its binding to RNA, but perhaps its methylation might mediate interactions with other proteins instead of RNA (18). *RPS2* encodes the yeast ribosomal protein S4 in which mutations have been shown to cause missense translation errors (34,35). Although the predicted 2-D migration of *Rps2* closely corresponds to R1', it is also possible that this species actually corresponds to an altered form of R1.



**FIG. 3.** RNase treatment of yeast soluble extracts highlight different substrate specificities between Type I arginine *N*-methyltransferases. Yeast soluble extracts indicated (50  $\mu$ g *rmt1* protein; 51  $\mu$ g *RMT1* protein) were pre-incubated with (+) or without (-) RNase and then incubated with 2.25  $\mu$ g GST-RMT1 (lanes 1-4), 2.85  $\mu$ g GST-PRMT1 (lanes 5-8), or 2.04  $\mu$ g GST-PRMT3 (lanes 9-12) in methylation reactions as described under "Experimental Procedures." The fluorograph represents a 10 day exposure.

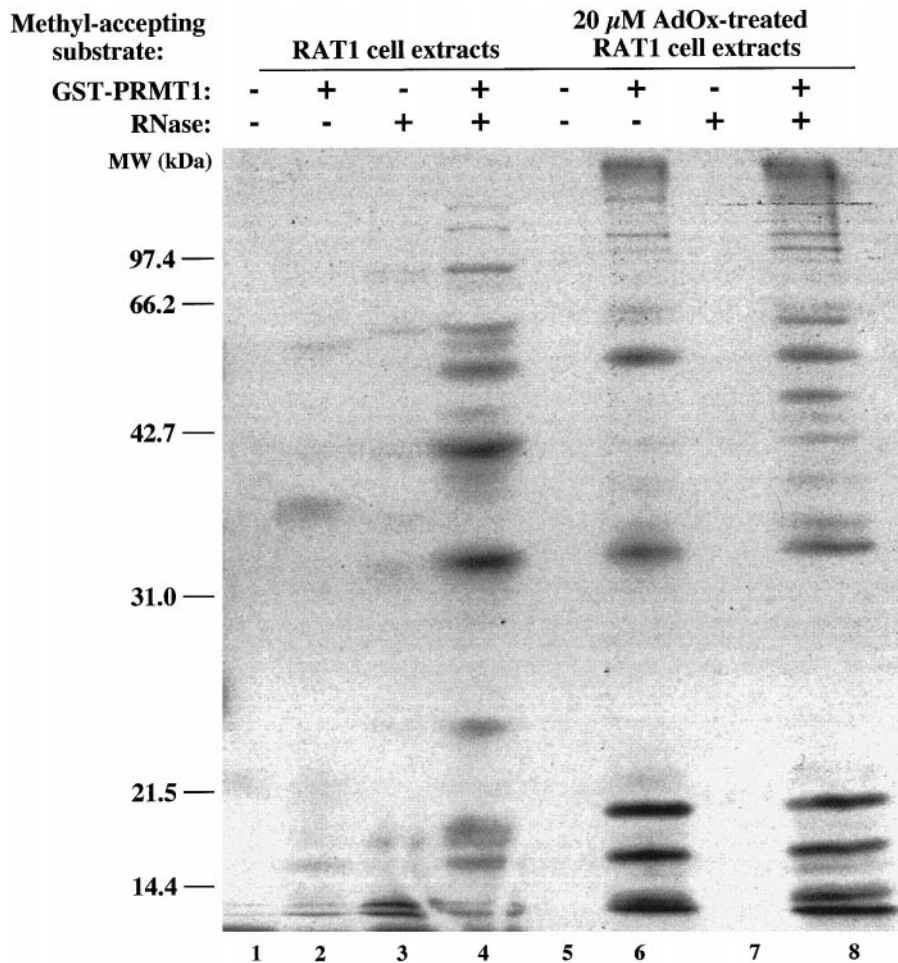
The R2 methylated species appears to correspond to Nop1, which is the essential nucleolar yeast homolog to mammalian fibrillarin associated with several snoRNAs (36,37). The N-terminal portion of fibrillarin has been shown to be asymmetrically dimethylated in its N-terminal GAR domain, yet the function of this modification remains unclear (14,38).

The R3/R3' methylated substrate matches Sbp1, which was originally purified by DNA affinity chromatography (39,40). Its homology to hnRNPs, particularly in the GAR domain in the middle of Sbp1, and its nucleolar localization led researchers to believe that the Sbp1 protein is involved in RNA metabolism (41). Immunoprecipitation of Sbp1 revealed that snRNAs snR10 and snR11 bound to it, implicating its role in pre-rRNA processing (42). Methylation of Sbp1 has not yet been studied, but it may help elucidate a role for arginine methylation.

The R4/R4' major methylated species matches to Npl3, whose C-terminal GAR domain has been shown to be mono- and asymmetrically dimethylated on its arginine residues, as well as being phosphorylated (2,16). Several studies have shown that Npl3 is involved in RNA export (43-46), and that its GAR domain is necessary for its nuclear localization (46,47), per-

haps through its association with Mtr10 at the Npl3 C-terminus (48).

The R5 major methylated species matches three candidate substrates from the list in Table II. The first candidate, *PSP2* was isolated as a nuclear multicopy suppressor (*MRS15*) that restores respiratory competence to the *mrs2-1* yeast mutant (49), and was also found to be a multicopy suppressor of temperature sensitive mutations in *POL1*, and a partial suppressor of mutations in *POL3* (50). The Psp2 protein contains a substantial C-terminal GAR domain believed to be involved in RNA binding (50). The other two candidates, Ded1 and Dbp2, are both "DEAD-box" proteins/putative RNA helicases thought to function in pre-mRNA splicing (51,52). Although overexpression of *DED1* can suppress a temperature sensitive mutation in *RPC31* (53), Chuang *et al.* (1997) have provided evidence supporting a cytoplasmic role for Ded1, showing that it is involved in translation initiation (54). Since the Psp2 protein contains significantly more potential sites for methylation than does Ded1 and Dbp2 combined, Psp2 appears to be the likely match for R5. Further work will be necessary to determine which, if any, of these polypeptides are in fact substrates of the Type I methyltransferases.



**FIG. 4.** RNase treatment of hypo- and normally-methylated RAT1 cell extracts affects *in vitro* methylation. Extracts from RAT1 and AdOx-treated RAT1 cells were pre-incubated with (+) or without (-) RNase as previously described. These extracts (19.5  $\mu$ g RAT1 protein; 1.2  $\mu$ g AdOx-treated RAT1 protein) were subject to methylation at 37°C for 1 hr with the addition of 2.85  $\mu$ g GST-PRMT1 when indicated (+), run on SDS-PAGE, and then fluorographed, all of which is described under "Experimental Procedures." The fluorograph represents a 2 week exposure.

GAR domain interactions may play a key role in understanding the function of Type I protein arginine methylation. The C-terminal GAR domains of hnRNP U and nucleolin have been shown to be essential for RNA binding (55,56). The GAR domain of hnRNP A1 was demonstrated to exhibit positive cooperativity with other RNA recognition motifs in binding to RNA, as well as promoting A1-A1 cooperativity (57). The methylation of hnRNP A1 did, however, decrease its affinity towards MS2-RNA (26). It has been considered that the methylation of arginine residues would effectively block H-bonding between the protein and the RNA phosphodiester backbone while keeping the same positive charge on the guanidino group of the arginine residue (58,59). In the case of Hrp1, methylation did not affect RNA binding (18). Therefore, it is still conceivable that the methylation of GAR domains might actually mediate both protein-protein and protein-RNA interactions.

Finally, RNase treatment can result in a similar activation of methyl-acceptors that was previously seen for treatment with the BTG1 and TIS21 proteins in RAT1 cell extracts (3). In that same study, BTG1 and TIS21 were both shown to interact with PRMT1 in a yeast two-hybrid screen, thus connecting the role of arginine methylation with cell growth (3). BTG1 and TIS21 have been implicated in cell cycle regulation demonstrating tumor suppressor and anti-apoptotic activities, respectively (60,61). In a separate yeast two-hybrid screen, BTG1 and TIS21 were shown to interact with mCAF, the murine homolog to the yeast CCR4 transcriptional regulatory complex (62). Interestingly, one of the genes obtained in our search of the *S. cerevisiae* genome for candidate methylation substrates is *RPC31*, which codes for a subunit of RNA polymerase III. Whether or not the Rpc31 protein is a methyl-acceptor remains to be seen, but it is tempting to speculate that

arginine methylation may even play a role in transcription in light of recent findings (62).

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