THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 25, Issue of June 21, pp. 15034–15044, 1996

Printed in U.S.A.

The Mammalian Immediate-early TIS21 Protein and the Leukemia-associated BTG1 Protein Interact with a Protein-arginine *N*-Methyltransferase*

(Received for publication, February 29, 1996, and in revised form, April 11, 1996)

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

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

The TIS21 immediate-early gene and leukemia-associated BTG1 gene encode proteins with similar sequences. Two-hybrid analysis identified a protein that interacts with TIS21 and BTG1. Sequence motifs associated with *S*-adenosyl-L-methionine binding suggested this protein might have methyltransferase activity. A glutathione *S*-transferase (GST) fusion of the putative methyltransferase modifies arginine residues, in appropriate protein substrates, to form N^G-monomethyl and N^G,N^G-dimethylarginine (asymmetric). We term the protein-arginine *N*-methyltransferase (EC 2.1.1.23) gene "PRMT1," for protein-arginine methyltransferase 1. GST-TIS21 and GST-BTG1 fusion proteins qualitatively and quantitatively modulate endogenous PRMT1 activity, using control and hypomethylated RAT1 cell extracts as methyl-accepting substrates. PRMT1 message appears ubiquitous, and is constitutive in mitogen-stimulated cells. Modulation of PRMT1 activity by transiently expressed regulatory subunits may be an additional mode of signal transduction following ligand stimulation.

The protein products of the immediate-early/primary response genes are thought to act as "third messengers," mediating phenotypic alterations in cells in response to ligands such as growth factors, hormones, neurotransmitters, cytokines, and neurotrophins. Many immediate-early genes encode transcription factors (*e.g.* Fos, Jun, Egr-1) that initiate transcriptional cascades required for proliferation or differentiation (Herschman, 1991). Other ligand-induced immediate-early genes encode paracrine mediators of cellular communication whose products (*e.g.* prostaglandin synthase-2, inducible nitric-oxide synthase, and cytokines such as MCP-1) modulate the behavior of neighboring cells (Smith and Herschman, 1995).

Because immediate-early/primary response genes have been cloned on the basis of their induction characteristics, rather than the functions of their protein products, a number of these genes encode proteins whose biological roles have not yet been determined. One such immediate-early gene is TIS21. The TIS21 cDNA was cloned by differential screening, both from a cDNA library prepared from mitogen-treated, quiescent murine Swiss 3T3 cells (Fletcher *et al.*, 1991) and from a cDNA library prepared from nerve growth factor-treated rat PC12 pheochromocytoma cells (Bradbury *et al.*, 1991). The predicted rat and mouse TIS21 proteins differ at only four out of 158 amino acid residues. We demonstrated, by metabolic labeling followed by immunoprecipitation, that maximal TIS21 protein synthesis occurs within the first hour after exposure to ligand, both in mitogen-stimulated Swiss 3T3 cells and in nerve growth factor-stimulated PC12 cells (Varnum *et al.*, 1994). Moreover, the half-life of both mitogen- and nerve growth factor-induced TIS21 protein is less than 15 min (Varnum *et al.*, 1994). Despite substantial investigation into both the structure of the TIS21 gene and the induced expression of the TIS21 message and protein, no function has been identified for this protein.

The human BTG1 gene was cloned and characterized (Rimokh *et al.*, 1991) because of its proximity to a chromosome translocation breakpoint (8;12) associated with a chronic lymphocytic leukemia. BTG1 overexpression in murine 3T3 cells decreases growth rate and reduces colony formation, leading to the suggestion that BTG1 is an "antiproliferative gene" whose loss of function contributes to uncontrolled growth (Rouault *et al.*, 1992). The human BTG1 and rodent TIS21 open reading frames share 59% identity and 75% conserved sequence similarity at the amino acid level. Moreover, the exon/intron structures of the TIS21 and BTG1 genes are remarkably similar (Varnum *et al.*, 1991; Rimokh *et al.*, 1991). However, BTG1 is not the human TIS21 orthologue; the murine BTG1 cDNA has been cloned and identified as a distinct entity with an amino acid sequence identical to human BTG1 (Rouault *et al.*, 1993). TIS21 and BTG1 are, therefore, members of a gene family.

The stringent regulation of TIS21 gene expression, the short time window for the presence of TIS21 protein following ligand stimulation, and the similarities between the TIS21 and BTG1 proteins suggest that these proteins play important roles in mediating ligand-induced biological responses. We previously speculated that, during its brief availability in mitogen-stimulated cells, TIS21 might reverse a BTG1 antiproliferative barrier by formation of a TIS21-BTG1 heterodimer (Varnum *et al.*, 1994). Alternatively, TIS21 and BTG1 might serve as regulatory partners that modulate the function of a common catalytic subunit. To test these hypotheses, we used the yeast two-hybrid interaction system (Fields and Song, 1989; Fields and Sternglanz, 1994) to determine (i) whether TIS21 and BTG1 proteins can form heterodimers and/or homodimers, and (ii) whether there exist proteins that interact with both TIS21 and BTG1.

EXPERIMENTAL PROCEDURES

Two-hybrid Analysis of Heterodimer and Homodimer Formation of BTG1 and TIS21 Proteins—The open reading frame from TIS21 cDNA

* This work was supported by National Institutes of Health GM26020 (to S. C.) and GM24797 (to H. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: 341 Molecular Biology Institute, 611 Circle Drive East, Los Angeles CA 90095-1570. Tel.: 310-825-8735; Fax: 310-825-1447; E-mail: Harvey@LBES.medsch.ucla.edu.

(Fletcher *et al.*, 1991) was subcloned by PCR[1] using AmpliTaq (Perkin Elmer) and the primers 5′-CATGTCGACGGGTTCTGGTTCTGGGAGCCACGGGAAGAGAACC-3′ and 5′-CTAGCGGCCGCCCAGGGTCGGGTGGCTCC-3′ to create a 5′ *Sal*I site, a 3′ *Not*I site, and a 5 amino acid linker (Gly-Ser-Gly-Ser-Gly) preceeding the TIS21 coding sequence, for flexibility between the GAL4 domains and TIS21 protein. The resulting cDNA was ligated to GAL4 activation domain fusion plasmid pGAD425 (Han and Colicelli, 1995) and GAL4 DNA-binding domain fusion plasmid pGBT10 (Han and Colicelli, 1995).

Murine BTG1 cDNA was prepared by reverse transcription and PCR. Total RNA was extracted from NIH3T3 cells grown to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gemini Bioproducts). Poly(A)$^+$ RNA was isolated using an Oligotex mRNA kit (Qiagen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). A PCR reaction was performed using primers 5′-CATGTCGACGGGTTCTGGTTCTGGGCATCCCTTCTACACCCGG-3′ and 5′-CTAGCGGCCGCATCCATCCAATAGACTATATC-3′ to create a 5′ *Sal*I site, a 3′ *Not*I site, and the 5 amino acid linker, as described for TIS21. BTG1 cDNA was then ligated to pGAD425 and pGBT10. The plasmids were sequenced using Sequenase 2.0, according to the manufacturer's instructions (U. S. Biochemical Corp.), to confirm the reading frame.

Yeast strain PCY2 (MATα Δgal4 Δgal80 URA3::GAL1-lacZ lys2–801^{amber} his3-Δ200 tryp1 Δ63 leu2 ade2–101^{ochre}) (Wang *et al.*, 1995) was transformed simultaneously with both a pGAD and a pGBT construct, according to the method of Schiestl and Gietz (1989), to analyze homodimerization and heterodimerization of TIS21 and BTG1. Briefly, PCY2 cells were grown in YPD (Rose *et al.*, 1990) medium until cell density reached 9–10 $\times$ 10^6 cells/ml. After harvest, the cells were resuspended in transformation buffer (1 M sorbitol, 0.1 M lithium acetate, pH 7.5, 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA) and incubated at 30 °C for 1 h. The cells were then mixed with plasmid DNA, carrier DNA, and PEG3350 (polyethylene glycol, molecular weight 3350), incubated at 30 °C for 30 min and heat-shocked at 42 °C for 5 min. For each transformation, 1 μg of each plasmid DNA was used with 66 μg of denatured salmon sperm DNA as carrier. Transformants containing both plasmids were selected on agar plates containing synthetic complete medium without leucine and tryptophan (SC−leu−trp) and analyzed for β-galactosidase activity by the yeast colony filter assay (Breeden and Naysmyth, 1985), using 5-bromo-4-chloro-3-indoyl β-D-galactoside as substrate.

Two-hybrid Analysis to Identify Proteins That Interact with TIS21 and BTG1—The GAL4 activation domain vector pGAD424 (Bartel *et al.*, 1993), which contains a leucine selective marker, was digested with *Sph*I. The *Sph*I cassette containing the GAL4 activation domain was removed. The *Sph*I cassette of pBTM116, which encodes the LexA DNA binding domain (Bartel *et al.*, 1993), was isolated by *Sph*I digestion and gel purification, using GeneClean II (BIO-LABS 101) and ligated into the pGAD425 whose original *Sph*I cassette had been removed. The resulting vector, containing the region encoding the LexA DNA-binding domain and the leucine selective marker, was named pLexA(L) and was used for two-hybrid interaction cloning.

TIS21 cDNA was subcloned by PCR, using pGAD425TIS21 as template and the primers 5′-GCAGAATTCGGTTCTGGTTCTGGGAG-3′ and 5′-CGAGTCGACGGGTCGGGTGGCTCCTA-3′, into pLexA(L) at an *Eco*RI/*Sal*I site, to create plasmid pLexA(L)TIS21. Murine BTG1 cDNA was amplified using pGAD425BTG1 as template and the primers 5′-GCAGAATTCGGTTCTGGTTCTGGGCA-3′ and 5′-CGTGTCGACATCCATCCAATAGACTA-3′. The BTG1 cDNA was then ligated to pLexA(L) at the *Eco*RI/*Sal*I site, to create plasmid pLexA(L)BTG1. The plasmids were sequenced using Sequenase 2.0 according to the manufacturer's instructions (U. S. Biochemical Corp.), or by using the DyeDeoxy Terminator Cycle sequencing kit and a 373 DNA sequencer (Applied Biosystems) to confirm the sequence and reading frame. The DNA encoding the LexA-Rin1-CT fusion was excised from pBTM116 (a gift from Limin Han and John Colicelli, UCLA) with *Sph*I and ligated to pGAD424 whose *Sph*I cassette had been removed, to create pLexA(L)Rin1-CT.

The pPC86 cDNA fusion library contains cDNAs fused to the GAL4 activation domain. cDNA was prepared from FAO cell poly(A)$^+$ mRNA, using the SuperScript plasmid system (Life Technologies, Inc.) according to the manufacturer's instructions, and ligated to the pPC86 vector (Chevray and Nathans, 1992) at the *Sal*I/*Not*I site. Plasmid pPC86 contains a tryptophan selective marker.

Escherichia coli DH5α (Life Technologies, Inc.) was the transformation recipient for all plasmid constructions, and was also used to recover expression plasmids from yeast. Yeast strain L40 (MATa his3D200 trp1–901 leu2–3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ gal4?? gal80??) (Hollenberg *et al.*, 1995) was used for two-hybrid library screening. To screen the cDNA library for clones encoding proteins that interact with TIS21 protein, the yeast L40 strain was transformed to leucine prototrophy with pLexA(L)TIS21. The transformant was grown in SC-leu-ura-lys (synthetic complete without leucine, uracil, and lysine) medium overnight and used to inoculate 450 ml of YPD medium. The culture was grown until the cell density reached 9–10 $\times$ 10^6 cells/ml, then harvested for transformation with 300 μg of the plasmid library. Cells were transfected as described in the previous section. After heat shock, the cells were incubated in YPD for 1 h at 30 °C, then washed thoroughly with TE buffer (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA) and resuspended in SC medium lacking tryptophan, leucine, and uracil. A sample was plated on SC medium lacking uracil, leucine, and tryptophan, to calculate transformation efficiency. After overnight incubation in SC-ura-leu-trp, the transformed cells were washed twice with TE buffer and plated on SC medium lacking tryptophan, leucine, histidine, uracil, and lysine, to select for interacting clones. Three days later, viable colonies were assayed for β-galactosidase activity using the yeast colony filter assay (Breeden and Naysmith, 1985). The filters were incubated at 37 °C for 2 h. Colonies positive for β-galactosidase activity were grown to saturation in SC-ura-trp liquid medium, a nonselective condition for pLexA(L), then replated on YPD plates. Replica plating of these plates to SC-ura-trp and SC-ura-leu identified colonies that had lost pLexA(L)TIS21 but still retained cDNA plasmids. The colonies that continued to give a positive β-galactosidase assay after loss of pLexA-(L)TIS21 were eliminated.

Thirty-three yeast colonies which demonstrated pLexA(L)TIS21-dependent histidine prototrophy and β-galactosidase activity were grown and treated with lyticase (3 mg/ml) and 0.1 M β-mercaptoethanol in SCE (1 M sorbitol, 0.1 M sodium acetate, 60 mM EDTA, pH 7.0) at 37 °C for 1 h to release spheroplasts. The pPC86 plasmids containing cDNA inserts encoding proteins that interact with TIS21 were isolated using a Qiagen plasmid miniprep kit and subjected to PCR using a GAL4 activation domain primer (5′-GGAATCACTACAGGGATG-3′) and a vector primer (5′-TTGATTGGAGACTTGACC-3′) to amplify cDNA inserts. Cross-hybridization was performed, using randomly selected cDNA inserts from these 33 clones as probes, to identify clones with common sequences.

Clones 3G, 4A, and 5A were sequenced using the DyeDeoxy Terminator Cycle Sequencing kit and a 373 DNA sequencer (Applied Biosystems). Sequence homology searches were performed using the BLAST program, through the National Center for Biotechnology Information (Altschul *et al.*, 1990).

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

Preparation of GST Fusion Proteins—To construct GST plasmids expressing TIS21, BTG1, and 3G, the cDNA insert fragments were excised from plasmids pSP64XβSN-TIS21, pSP64XβSN-BTG1, and pPC86–3G using *Sal*I and *Not*I, and ligated to plasmid pGEX-2T (Pharmacia) which had been modified to accept a *Sal*I/*Not*I insert (Han and Colicelli, 1995). Expression of GST fusion proteins was induced in transformed *E. coli* DH5α by isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 5 h. Bacteria were harvested by centrifugation and resuspended in extraction buffer (phosphate-buffered saline, pH 7.4, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). The cells were disrupted by sonication. Cell debris was removed by centrifugation (16,000 $\times$ *g*, 20 min, 4 °C), and the supernatants were collected. GST fusion proteins were isolated with glutathione-Sepharose 4B beads (Pharmacia), as described by the manufacturer.

Identification of ^{3}H-methylated Arginine Residues—Samples were lyophilized and hydrolyzed with 12 N HCl *in vacuo* (110 °C, 20 h), in a vapor-phase Waters Pico-Tag apparatus. The hydrolyzed material was resuspended in 50 μl of water and then 25 μl were analyzed on a sulfonated polystyrene column of Beckman AA-15 resin. Samples were mixed with 1 μmol each of N^G,N^G-dimethylarginine (asymmetric) and N^G-monomethylarginine (both obtained from Sigma). An equal volume

[1] The abbreviations used are: PCR, polymerase chain reaction; AdoMet, *S*-adenosyl-L-methionine; BLAST, basic local alignment sequence tool; GST, glutathione *S*-transferase; GAR, glycine and arginine-rich region; PRMT, protein-arginine transferase; hnRNP, heterogeneous ribonucleoprotein; AdOx, adenosine dialdehyde; PAGE, polyacrylamide gel electrophoresis.

of citrate sample dilution buffer (0.2 M in Na^+, containing 2% thiodiglycol and 0.1% phenol at pH 2.2) was added, and the mixture was loaded onto the column (0.9 cm in diameter, 11 cm in length). The column was equilibrated with Na^+ citrate buffer (0.35 M in Na^+, pH 5.27) at 55 °C. Elution was at approximately 1 ml/min. The column was regenerated with 0.2 N NaOH for 20 min prior to the next run. One-minute fractions were collected and ^3H radioactivity was determined by counting samples in a scintillation counter. Additional 100-μl samples from each fraction was analyzed for the nonradioactive methylated arginine standards, using the ninhydrin method (Gary and Clarke, 1995).

Electrophoretic Analysis of Methylated Proteins—Methylation reactions were stopped by the addition of an equal volume of 2 × SDS-PAGE sample buffer (Hyrcyna *et al.*, 1994), and subjected to SDS-PAGE analysis. The samples were heated to 100 °C for 5 min and then loaded onto slab gels using the buffer system described by Laemmli (1970). The samples were subjected to electrophoresis through either a 10 or 12.6% acrylamide, 0.28% (w/v) *N*,*N*-methylenebisacrylamide matrix by the application of a 35-mA constant current. Gels were stained with Coomassie, destained, and then treated with the fluor EN^{3}HANCE (DuPont) as described by the manufacturer. The dried gels were subjected to fluorography at −80 °C for 7–14 days.

Preparation of RAT1 Cytosolic Extracts—RAT1 cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were removed from the plates with trypsin, washed with cold phosphate-buffered saline, and resuspended in extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM sodium EDTA, 1 mM sodium EGTA, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Cell disruption was performed on ice, by homogenization with a glass tissue grinder. The homogenate was centrifuged at 18,000 × *g* for 50 min at 4 °C. Supernatants were either used immediately, or stored at −80 °C. To obtain hypomethylated cytosol, RAT1 cells were cultured with 20 μM adenosine dialdehyde (Sigma) for 2 days. After removal from the plates with trypsin, cells were washed twice with phosphate-buffered saline containing 1 mM EDTA, and disrupted as described above.

Isolation of Endogenous PRMT1 Activity with Glutathione-Sepharose Immobilized GST-TIS21 Fusion Protein—RAT1 cytosolic extract (500 μl, 233 μg of protein) or chromatographic fractions (200 μl) were incubated with either GST-TIS21 or GST immobilized to glutathione-Sepharose beads at 4 °C. After centrifugation, the supernatant was collected. The beads were washed thoroughly with buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.05% Tween 20, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), then incubated with 40 μl of 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, to elute GST-TIS21 (or GST) and the associated proteins.

Preparation of odp1/rmt1 Yeast Extract as Substrate for Methylation Reactions—Cultures (500 ml) of the *odp1/rmt1* mutant strain (Gary *et al.*, 1996) were grown to an absorbance at $A_{600\ nm}$ of 2.5 in YPD medium at 30 °C. Cells were harvested by centrifugation at 4,400 × *g* for 10 min at 4 °C. The pelleted cells were washed with buffer A (25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA at pH 7.5) and again harvested by centrifugation. The pellet (2.6–3.0 g) was resuspended in 2 ml of buffer A/g of cells and 40 μl of a protease inhibitor mixture was added to give final concentrations of 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 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 psi. Homogenates were centrifuged at 23,000 × *g* for 40 min at 4 °C. The supernatants were stored at −20 °C.

Northern Analysis of RNAs from RAT1 Cells and Rat Organs—Total RNA was isolated from RAT1 cells, using lithium chloride (Cathada *et al.*, 1983). RNA from rat tissues was prepared using acid guanidinium thiocyanate-phenol-chloroform (Chomczymski and Sacchi, 1987). RNA was subjected to electrophoresis on formaldehyde-agarose gels and transferred to nylon membranes (Thomas, 1981). The blots were hybridized to random-primed cDNA probes (Sambrook *et al.*, 1989) for TIS21, BTG1, 3G, and S2.

RESULTS

TIS21 and BTG1 Proteins Do Not Form Heterodimers or Homodimers—We prepared (i) vectors expressing both murine TIS21 and BTG1 as fusion proteins with the DNA-binding domain of the GAL4 transcription factor and (ii) vectors expressing both TIS21 and BTG1 as fusion proteins with the transcriptional activation domain of the GAL4 transcription factor (see "Experimental Procedures"). Appropriate constructs were then transfected in pairs into the yeast PCY2 strain, to analyze by two-hybrid interaction analysis (Fields and Song, 1989; Bartel *et al.*, 1993) the ability of TIS21 and BTG1 proteins to associate (see "Experimental Procedures). No combination of TIS21 and BTG1 GAL4 fusion proteins could activate the expression of β-galactosidase driven from a *GAL4* promoter, suggesting that BTG1 and TIS21 are unable to form either heterodimers or homodimers.

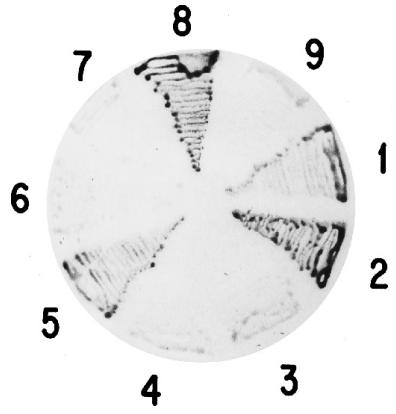

FIG. 1. **Two-hybrid interaction analysis identifies proteins that interact with TIS21 and/or BTG1 proteins.** Yeast strains containing plasmids pLexA(L)TIS21, pLexA(L)BTG1, or pLexA(L)Rin1-CT, along with plasmids pPC86-5A, pPC86-4A, or pPC86-3G, were assayed for β-galactosidase activity, using a yeast colony filter assay (Breeden and Naysmith, 1985). Blue color develops when the fusion proteins expressed from the two plasmids physically interact and activate transcription of a *LacZ* fusion gene in which β-galactosidase expression is under the control of the *LexA* operator. Rin1-CT, whose sequence is unrelated to either TIS21 or BTG1, was used as a specificity control. Rin1-CT interacts with Ras protein in the two-hybrid analysis (Han and Colicelli, 1995). *1*, BTG1/3G; *2*, TIS21/3G; *3*, Rin1-CT/3G; *4*, BTG1/4A; *5*, TIS21/4A; *6*, Rin1-CT/4A; *7*, BTG1/5A; *8*, TIS21/5A; *9*, Rin1-CT/5A.

Identification of a Protein That Interacts with TIS21 and BTG1 Proteins—To identify proteins that interact with the TIS21 protein, a yeast strain expressing a LexA-TIS21 fusion protein as "bait" was transfected with a plasmid library in which the GAL4 activation domain was fused to cDNAs prepared from rat FAO cells. Yeast cells in which a complex containing the LexA DNA-binding domain and the GAL4 activation domain can form are able to survive in histidine-free medium because a *LexA*-responsive promoter drives expression of the HIS3 protein (Fields and Sternglanz, 1994). This complex will form because of a physical association between the LexA-TIS21 protein and a library fusion protein with the GAL4 activation domain. These yeast cells also contain a reporter gene in which a *LexA*-responsive promoter drives expression of β-galactosidase. Colonies able to survive on histidine-deficient medium were subsequently tested for β-galactosidase activity. Colonies dependent on the presence of the LexA-TIS21 fusion protein for these two properties were then identified. Cross-hybridization studies indicate that, among 33 such histidine-independent clones that also express β-galactosidase, three distinct cDNAs express fusion proteins (5A, 3G, and 4A) that interact with TIS21. When tested in two-hybrid interaction analysis, clones 5A and 4A do not interact with BTG1. In contrast, clone 3G does interact with both TIS21 and BTG1 (Fig. 1). Sequence analysis of clone 5A identified the encoded protein as PICK-1, a protein recently cloned from a two-hybrid screen using the catalytic region of protein kinase C as bait (Staudinger *et al.*, 1995). Partial sequence analysis of clone 4A has not identified this open reading frame as similar to any known protein or expressed sequence tag. The sequence of the proposed open reading frame of the 3G protein is shown in Fig.

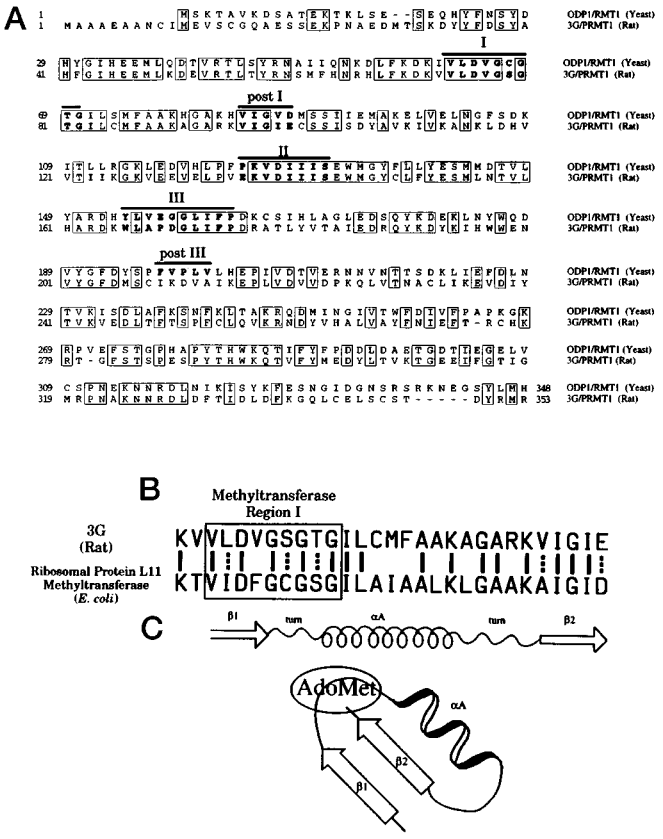

FIG. 2. **Sequence of the translated 3G cDNA identified by interaction with TIS21 and BTG1.** *A*, alignment of the amino acid sequences of the protein predicted by the 3G open reading frame and the protein predicted by the yeast ODP1 open reading frame. Identical amino acids are *boxed*. The predicted methyltransferase region I, post-region I, region II, region III, and post-III motifs are indicated. *B*, the highest scoring protein with a known function from a BLAST search, using the 3G protein as the query, is the *E. coli* L11 methyltransferase (SP P28637). In the 29 residue alignment shown, amino acid identities between 3G and the L11 methyltransferase are indicated by *solid lines*, similarities are indicated by *broken lines*. The conserved methyltransferase region I is *boxed*. *C*, the proposed secondary and tertiary structures for the L11 methyltransferase and 3G sequences shown in *panel B*, based on the structural determinations of two bacterial DNA methyltransferases and the rat catechol-*O*-methyltransferase (Schluckebier *et al.*, 1995).

2*A*. The longest predicted open reading frame of the 3G protein encodes a 40.5-kDa polypeptide composed of 353 amino acid residues.

To confirm the interaction of both the TIS21 and BTG1 proteins with the 3G protein, radioactive [^{35}S]methionine-labeled *in vitro* translated TIS21 protein or BTG1 protein were incubated with purified glutathione *S*-transferase-3G (GST-3G) fusion protein or with control GST protein. GST-3G or GST protein was first immobilized on glutathione-Sepharose beads. The beads were then incubated with radioactive TIS21 or BTG1. After washing, the bound material was eluted and subjected to SDS-PAGE electrophoresis, followed by autoradiography. Both *in vitro* translated BTG1 and *in vitro* translated TIS21 are able to bind to GST-3G, but not to the GST control (Fig. 3). These *in vitro* binding studies confirm the two-hybrid interaction analysis, and further demonstrate the interaction of the 3G protein with both BTG1 and TIS21 proteins.

The 3G Protein Is a Protein-arginine N-Methyltransferase—A BLAST search (Altschul *et al.*, 1990) of the available sequence data bases for sequences similar to the 3G protein identified an open reading frame (ODP1) (accession number GB Z35903) from yeast chromosome II (Loubbardi *et al.*, 1995;

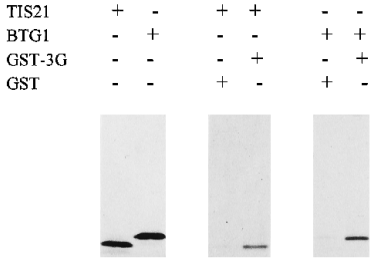

FIG. 3. ***In vitro* translated BTG1 and TIS21 proteins form stable complexes with recombinant GST-3G protein.** *In vitro* translated [^{35}S]TIS21 and [^{35}S]BTG1 were incubated with GST (*lanes 3* and *5*) or GST-3G (*lanes 4* and *6*) bound to glutathione-Sepharose beads. After washing, the immobilized proteins were eluted and subjected to electrophoresis followed by autoradiography. *Lanes 1* and *2* are products of the *in vitro* translation reactions of TIS21 and BTG1. For *in vitro* translation, the TIS21 cDNA was amplified by PCR from pLexA(L)-TIS21 using primers 5′-CTTGTCGACGAGCCACGGGAAGAG-AACCG-3′ and 5′-CTAGCGGCCGCCCAGGGTCGGGTGGCTCC-3′, and ligated to pSP64 XβSN (obtained from John Colicelli, UCLA) which had been linearized with *Sal*I and *Not*I. The BTG1 cDNA was amplified by PCR from pLexA(L)BTG1 using primers 5′-CTTGTCGACGCATC-CCTTCTACACCCGGG-3′ and 5′-CTAGCGGCCGCATCCATCCAATA-GACTATATC-3′ and similarly ligated to pSP64 XβSN. The plasmids were sequenced to confirm the reading frame. *In vitro* transcription and translation were performed using the TNT SP6 coupled reticulocyte lysate system (Promega), according to the manufacturer's instructions. [^{35}S]Methionine (1000 Ci/mmol; Amersham) was used to label the protein products. To assay for protein-protein interactions, 60 μl of bacterial extract containing either GST-3G fusion protein or GST protein was first incubated with 10 μl of glutathione-Sepharose beads at 4 °C for 1 h. The beads were washed twice with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.05% Tween 20, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The resulting GST-3G-Sepharose bead complex or GST-Sepharose bead complex was then resuspended in 200 μl of binding buffer containing 1% nonfat milk. Five μl of the *in vitro* translation reaction mixture containing [^{35}S]TIS21 or [^{35}S]BTG1 was added to the GST-3G-Sepharose bead complex or the GST-Sepharose bead complex. After incubation for 1 h at 4 °C, the Sepharose bead complexes were collected by centrifugation, washed five times with binding buffer, resuspended in 10 μl of SDS-PAGE sample buffer (Laemmli, 1970), and the entire mixture was subjected to electrophoresis in a 12% polyacrylamide-SDS gel. The gel was stained with Coomassie Blue, destained, dried, and subjected to autoradiography at −80 °C.

Feldman *et al.*, 1994; Fig. 2*A*) and expressed sequence tags from the genome sequencing projects for rice, *Arabidopsis*, *Caenorhabditis elegans*, and humans. However, these proteins have no identified functions. A short region of sequence similarity between the 3G protein and the enzyme that methylates *E. coli* ribosomal protein L11 (accession number SP P28637) was also identified (Fig. 2*B*). This region of sequence similarity between the 3G protein and the ribosomal protein L11 methyltransferase has previously been identified as "methyltransferase region I," a consensus sequence found in a wide variety of proteins that bind *S*-adenosyl-L-methionine (AdoMet) (Kagan and Clarke, 1994). Crystallographic studies have demonstrated the direct interaction of region I with AdoMet, in both a small molecule methyltransferase and two DNA methyltransferases (Schluckebier *et al.*, 1995; see also Fig. 2*C*). Further sequence comparisons of the 3G protein with the L11 methyltransferase, as well as other known methyltransferases, identified three additional motifs (post-I, -II, and -III) that are also highly conserved among methyltransferases and other AdoMet-binding proteins (Fig. 2*A*; Kagan and Clarke (1994)). The similarities in sequence for these regions of the 3G protein and members of the methyltransferase family suggested that the 3G protein and the protein encoded by the yeast *ODP1* gene might also have methyltransferase activity.

Methyltransferases can transfer methyl groups from AdoMet to oxygen, nitrogen, sulfur, and carbon moieties, and can use as substrates a wide variety of both small biological molecules and macromolecules that include DNA, RNA, lipids, and proteins

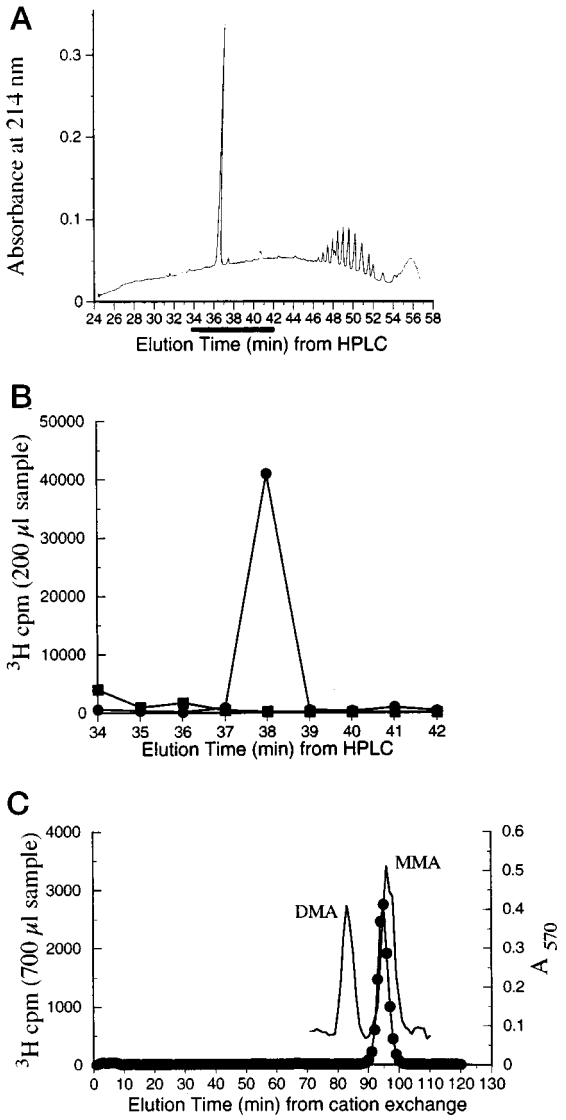

FIG. 4. **The 3G protein is an R1 peptide-arginine methyltransferase.** *A*, the elution profile, after separation by reverse phase high performance liquid chromatography, of the R1 peptide incubated with purified GST-3G fusion protein and [^{3}H]AdoMet. For methylation of the R1 peptide, the reaction mixtures contained 100 μM R1 peptide (GGFGG**R**GGFG-amide), 1.1 μM [^{3}H]AdoMet (DuPont NEN, 73 Ci/mmol) and either 0.8 μg of purified GST-3G protein or buffer (25 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA at pH 7.5) alone, as the control. The 25-μl reactions were incubated at 30 °C for 30 min. The mixture was then acidified by the addition of 10% trifluoroacetic acid (3 μl) to stop the reaction and prepare the sample for injection onto the high performance liquid chromatography. The entire reaction was injected onto a C18 reverse phase column (Alltech Econosphere; 5 micron spherical particles; column dimensions 4.6 × 250 mm) equilibrated at room temperature in buffer A (0.1% trifluoroacetic acid in water) at a flow rate of 1 ml/min for 10 min and was eluted with buffer B (0.1% trifluoroacetic acid, 0.9% water in acetonitrile) using the following gradient: 0–10 min, 0% B; 10–50 min, 0–100% B; 50–55 min, 100% B; and 55–58 min, 100–0% B. The column effluent was monitored at 214 nm and 1-min fractions were collected. The R1 peptide elutes as a single peak from the column at 36.5 min. The *bar* indicates the region that is expanded in *panel B*. *B*, the radioactivity profile following the R1 methylation reaction with GST-3G fusion protein. The *closed circles* represent data from the reaction where GST-3G is present. The 1.5-min delay in the elution of the radioactivity is due to the additional time required for the sample to get from the UV detector to the fraction collector. The *closed squares* represent data from the control reaction, with no enzyme present. *C*, cation exchange chromatography analysis of the methylated products of the hydrolyzed R1 peptide. Following the methylation reaction and separation by high performance liquid chromatography, the R1 peptide present in fraction 38 was acid hydrolyzed and subjected to cation exchange chromatography (see "Experimental Procedures"), along with standards for N^G,N^G-dimethylarginine (asymmetric, *DMA*) and N^G-monomethylarginine (*MMA*). The *closed circles* show the elution of radioactivity. The *solid line* indicates the elution of the co-injected standards, determined by analyzing each fraction with ninhydrin (Gary and Clarke, 1995). The slightly earlier elution of the radioactive material compared to the nonradioactive standard is due to the change in molecular weight and pI of the tritiated species *versus* the hydrogenated forms (Gottschling and Freese, 1962; Xie and Clarke, 1993).

(Clarke, 1993). Because of the similarities between the 3G protein and the L11 protein methyltransferase, an enzyme that appears to modify the N-terminal alanine residue and/or internal L11 lysine residues (Vanet *et al.*, 1993), we examined the possibility that the 3G protein is a protein methyltransferase. We first tested whether the 3G protein has enzymatic activity similar to that of the previously characterized types of soluble eukaryotic enzymes that catalyze methyl ester formation; *e.g.* protein L-isoaspartyl methyltransferase (Lowenson and Clarke, 1995) or protein phosphatase 2A C-terminal leucine methyltransferase (Xie and Clarke, 1994a). However, we found no evidence that the 3G protein has either type of activity. Recombinant GST-3G fusion protein does not methylate the isomerized aspartyl residues present in either the isoaspartyl-containing peptides KASA(isoD)LAKY or VYP(isoD)HA, nor is it able to methylate the catalytic subunit of protein phosphatase 2A from rat brain (a gift from Sandra Rossie, Purdue University).

Because the GST-3G fusion protein did not appear to be a protein carboxyl methyltransferase, we next investigated whether 3G might methylate nitrogen atoms in proteins. We began these studies by assaying the ability of GST-3G to methylate the guanidino nitrogens of arginine residues, because well defined substrates are available for these enzymes. Methylation of arginine residues is a common post-translational modification of proteins that mediate RNA processing (Rajpurohit *et al.*, 1994a; Lischwe *et al.*, 1985a, 1985b). Many of these proteins (*e.g.* fibrillarin, nucleolin, and hnRNP A1) contain N^G,N^G-dimethylarginine (asymmetric). The arginine residues subject to methylation in these RNA-binding proteins are present in a glycine and arginine rich "GAR domain" containing multiple repeats of the consensus arginine methylation site RGG (Najbauer *et al.*, 1993).

The R1 peptide, GGFGG**R**GGFG-amide, derived from the fibrillarin consensus methylation site, has been used to characterize protein-arginine *N*-methyltransferase activity present in cultured cells and in mammalian tissues (Najbauer *et al.*, 1993). When R1 peptide is incubated with GST-3G fusion protein and *S*-adenosyl-[*methyl*-^{3}H]L-methionine ([^{3}H]AdoMet), the peptide is readily methylated (Fig. 4, *A* and *B*). Acid hydrolysis of the methylated R1 peptide and subsequent amino acid analysis by cation exchange chromatography demonstrate that the radioactive methyl group has been incorporated to form an N^G-monomethylarginine residue (Fig. 4*C*). We conclude that the 3G fusion protein is a functional R1 peptide-arginine methyltransferase.

Histones, hnRNP A1, myelin basic protein, and cytochrome *c* have been used as substrates to identify, partially purify, and distinguish protein-arginine *N*-methyltransferases from several sources (Ghosh *et al.*, 1988; Farooqui *et al.*, 1985; Lee *et al.*, 1977). Previous studies with partially purified mammalian enzyme preparations have suggested that one enzyme is responsible for the mono- and asymmetric dimethylation of arginine residues in both histones and hnRNP A1 (Rajpurohit *et al.*, 1994a; Ghosh *et al.*, 1988), while a different enzyme mono- and symmetrically dimethylates arginine 107 in myelin basic protein (Ghosh *et al.*, 1988; Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971). The protist *Euglena gracilis* contains a third,

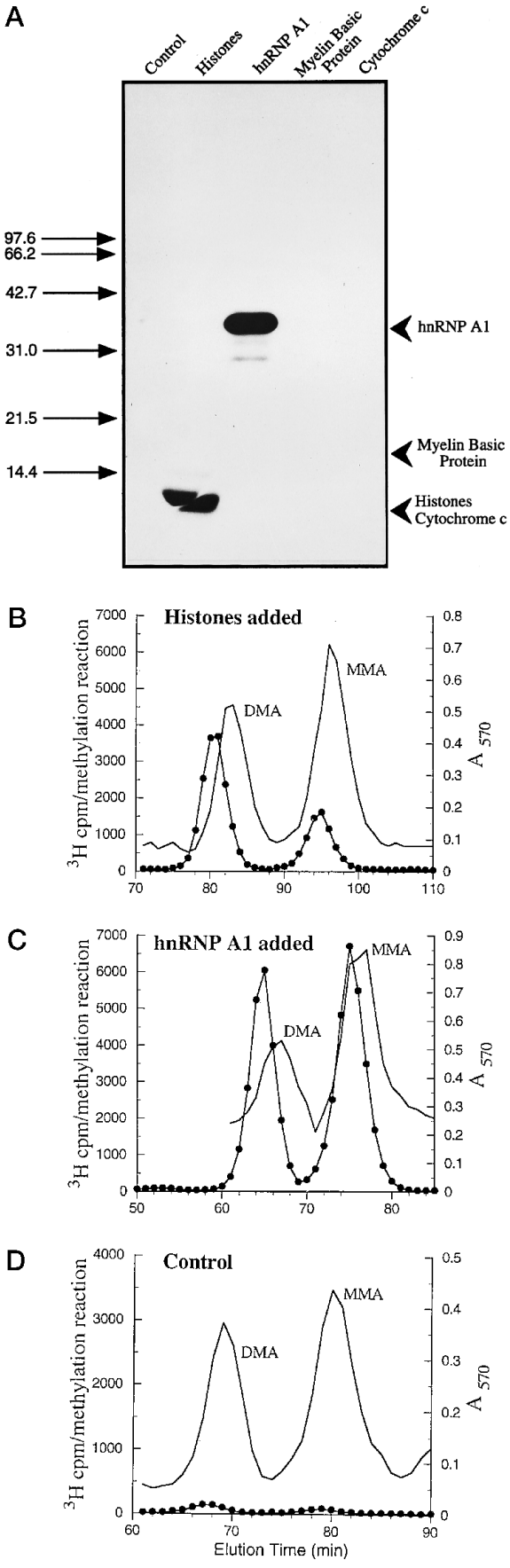

FIG. 5. **The 3G protein is a protein-arginine *N*-methyltransferase.** *A*, histones (100 μg; Sigma, type IIA from calf thymus), bacterially expressed recombinant human hnRNP A1 (490 ng, a gift from A. Krainer and A. Mayeda, Cold Spring Harbor), myelin basic protein (100 μg; Sigma, bovine brain), and cytochrome *c* (100 μg; Sigma, type VI horse heart) were used as potential substrates for the methyltransferase activity of the GST-3G fusion protein. Reactions contained one of the protein substrates (or buffer only as the control), 0.93 μM [^{3}H]AdoMet (2.2 μCi), 2.0 μg of 3G-GST, and buffer (25 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA at pH 7.5) to a final volume of 30 μl. After incubation with GST-3G fusion protein and [^{3}H]AdoMet, samples were subjected to SDS-PAGE. After staining with Coomassie Blue, the gel was dried and then subjected to fluorography. Migration of molecular mass standards, determined by position of stained markers, is indicated on the *left*. The positions of the substrate proteins, also determined by the Coomassie-stained bands, are indicated on the *right*. *B*, identity of the modified arginine residues present following methylation of histones. A reaction identical to that described above was incubated at 30 °C for 30 min, then stopped by addition of an equal volume of 25% trifluoroactic acid. The reaction products were placed in 6 × 40-mm glass hydrolysis tubes and kept at 25 °C for 10 min before pelleting the precipitated material by centrifugation (4,000 × *g*, 20 min). The protein pellets were washed with −20 °C acetone, dried, and acid hydrolyzed, as described for the R1 peptide. The hydrolyzed material was then resuspended in 50 μl of water. Half of the sample was analyzed by cation exchange chromatography, along with N^G-monomethylarginine (*MMA*) and N^G,N^G-dimethylarginine (*DMA*) standards (see "Experimental Procedures"). The *solid circles* indicate radioactivity (*C*). Identity of the modified arginine residues present following methylation of hnRNP A1. *D*, little or no methylated arginine products are present in the absence of appropriate substrate. GST-3G protein does not methylate itself.

distinct protein-arginine *N*-methyltransferase that is able to methylate arginine residues in mammalian cytochrome *c* (Farooqui *et al.*, 1985). The GST-3G fusion protein is able to methylate both histones and recombinant hnRNP A1 protein (Fig. 5*A*). Cation exchange chromatography of acid hydrolysates of these substrates demonstrates that mono- and asymmetric dimethylated arginine residues are the sole products of the enzymatic reactions (Figs. 5, *B* and *C*). In contrast, the GST-3G fusion protein cannot use either cytochrome *c* or myelin basic protein as substrate for methylation (Fig. 5*A*). We suggest that the 3G protein is the catalytic component of the previously described histone/hnRNP A1 arginine methyltransferase (Ghosh *et al.*, 1988; Rawal *et al.*, 1994; Liu and Dreyfuss, 1995), and have named this enzyme "protein-arginine transferase 1," or PRMT1.

GST-TIS21 Protein Can Interact with Native PRMT1 Present in a Macromolecular Complex from Cytosolic Extracts of RAT1 Cells—In parallel work, we have found (i) that the yeast *ODP1/RMT1* gene also encodes a protein-arginine *N*-methyltransferase (Gary *et al.*, 1996), and (ii) that soluble extract from an *odp1/rmt1*-deficient yeast strain contains a 55-kDa protein that is an excellent methyl-accepting substrate for the GST-PRMT1 fusion protein (Gary *et al.*, 1996). When *odp1/rmt1* mutant soluble extract is used as substrate with GST-PRMT1 and [^{3}H]AdoMet, this 55-kDa protein is, by far, the major methylated species (Gary *et al.*, 1996). Moreover, the 55-kDa protein present in *odp1/rmt1* methyltransferase-deficient yeast cytosol can also be methylated by an endogenous methyltransferase enzyme(s) present in soluble extracts of RAT1 cells (Gary *et al.*, 1996). We can, therefore, use the extract prepared from the *odp1/rmt1* methyltransferase-deficient yeast strain as substrate to assay for the presence of protein-arginine *N*-methyltransferase activity in mammalian cells.

We used GST-TIS21 fusion protein immobilized on glutathione-Sepharose beads to demonstrate that TIS21 protein can sequester protein methyltransferase activity present in RAT1 cell extracts. Using the 55-kDa protein present in *odp1/rmt1*-deficient yeast as a methyl-accepting substrate, we demonstrated protein methyltransferase activity was bound to the GST-TIS21 fusion protein (Fig. 6*A*). In contrast, GST protein immobilized on glutathione-Sepharose beads cannot sequester methyltransferase activity. When extracts of RAT1 cells are

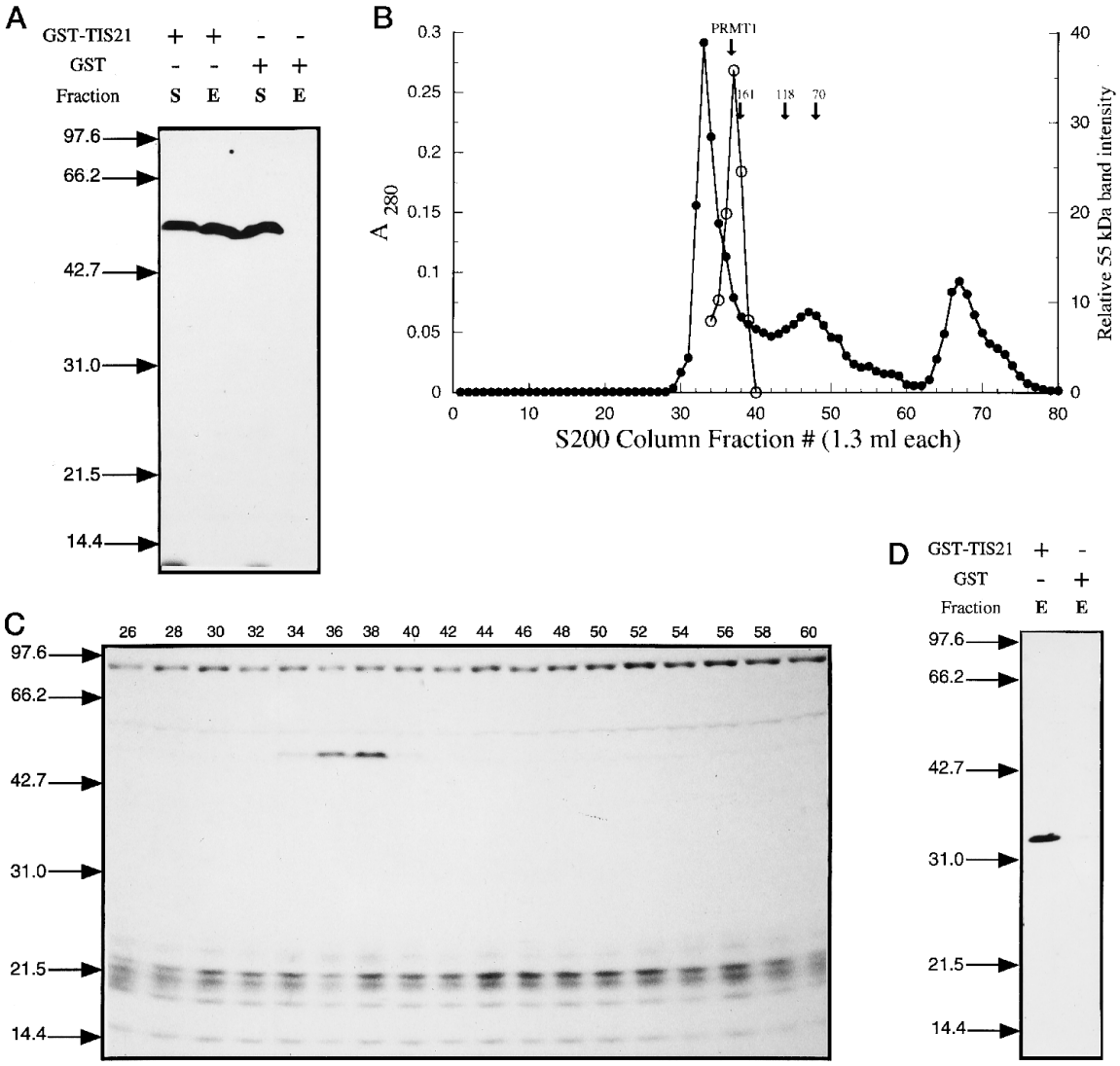

FIG. 6. **PRMT1 activity in soluble extracts of RAT1 cells.** *A*, RAT1 cells contain a protein methyltransferase that interacts with GST-TIS21 fusion protein. RAT1 extract was incubated with either Sepharose-immobilized GST-TIS21 fusion protein or Sepharose-immobilized GST protein. After centrifugation and washing, the beads were treated with glutathione to release bound proteins (see "Experimental Procedures"). The supernatants remaining after removal of the glutathione-Sepharose beads (*S*) and the glutathione eluates (*E*) from the beads were assayed for methyltransferase activity, using an extract from *odp1/rmt1* deficient yeast (10 μl, 169 μg) as substrate. Methylation reactions were incubated at 30 °C for 30 min, stopped by the addition of an equal volume of 2 × SDS-PAGE sample buffer, boiled for 5 min, separated on a 10% SDS-PAGE gel, and subjected to fluorography. *B* and *C*, RAT1 methyltransferase activity exists as a high molecular weight complex. A Superdex 200 Prep Grade (Pharmacia) gel filtration column (1.5 cm diameter, 58 cm in height; 102.5 ml) was equilibrated with buffer (25 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA at pH 7.5) at 4 °C. RAT1 cell crude cytosol (800 μl) was loaded onto the column, and eluted with buffer at a constant flow rate of 20.4 ml/h. Fractions (1.3 ml) were collected, and A_{280} was measured (*solid circles*). Samples were assayed for malate dehydrogenase (70 kDa), glucose-6-phosphate dehydrogenase (118 kDa), and aldolase (161 kDa) as described in the Worthington enzyme manual. Samples (20 μl) from every other fraction between fractions 26 and 60 were tested for PRMT1 activity (*panel C*). Crude cytosol (169 μg) from a yeast *odp1/rmt1* deficient strain was used as substrate. [^{3}H]AdoMet (0.82 μM, 2.2 μCi) was included in the reaction mixture (34 μl). Samples were incubated for 30 min at 30 °C. The reaction was stopped by addition of an equal volume of 2 × SDS-PAGE sample buffer, and analyzed on a 10% acrylamide-SDS gel. The methyltransferase reaction was repeated on fractions 35–40, and the intensity of the 55-kDa band in each lane was analyzed by quantitative densitometry. Relative densitometry units are shown by the *open circles* in *panel B*. *D*, the methyltransferase present in the high molecular weight Superdex column fractions can be isolated with immobilized GST-TIS21 protein, and can methylate hnRNP A1. Portions (200 μl) of fraction 37 from the Superdex column were incubated with either glutathione-Sepharose immobilized GST-TIS21 fusion protein or glutathione-Sepharose GST protein. After centrifugation and washing, the beads were treated with glutathione to release bound proteins. The eluates (*E*) from the beads were assayed for methyltransferase activity, using recombinant hnRNP A1 protein (490 ng) as substrate.

analyzed by gel filtration chromatography, the rat cell enzyme capable of methylating the 55-kDa yeast substrate present in *odp1/rmt1* mutant yeast extracts migrates as a single complex of approximately 180 kDa (Fig. 6, *B* and *C*). In contrast, the calculated polypeptide molecular mass of PRMT1 is approximately 40 kDa (Fig. 2). The protein methyltransferase activity present in the high molecular size fractions from the gel filtration column can be absorbed by immobilized GST-TIS21 and, when eluted, can methylate both the yeast 55-kDa substrate (not shown) and recombinant hnRNP A1 (Fig. 6*D*). We conclude (i) that native PRMT1 present in RAT1 cells, like the GST-PRMT1 fusion protein, is able to interact with TIS21 protein and (ii) that PRMT1 exists as a macromolecular complex in RAT1 cells.

Recombinant GST-PRMT1 Fusion Protein Can Methylate Endogenous Substrates Present in RAT1 Cytosolic Extracts—If [^{3}H]AdoMet is added to a soluble extract from RAT1 cells, methylated polypeptides are observed at molecular masses of 96, 60, 28, 22, and 20 kDa (Fig. 7*A*, *lane 1*), due to endogenous methyltransferase activity. If purified GST-PRMT1 fusion pro-

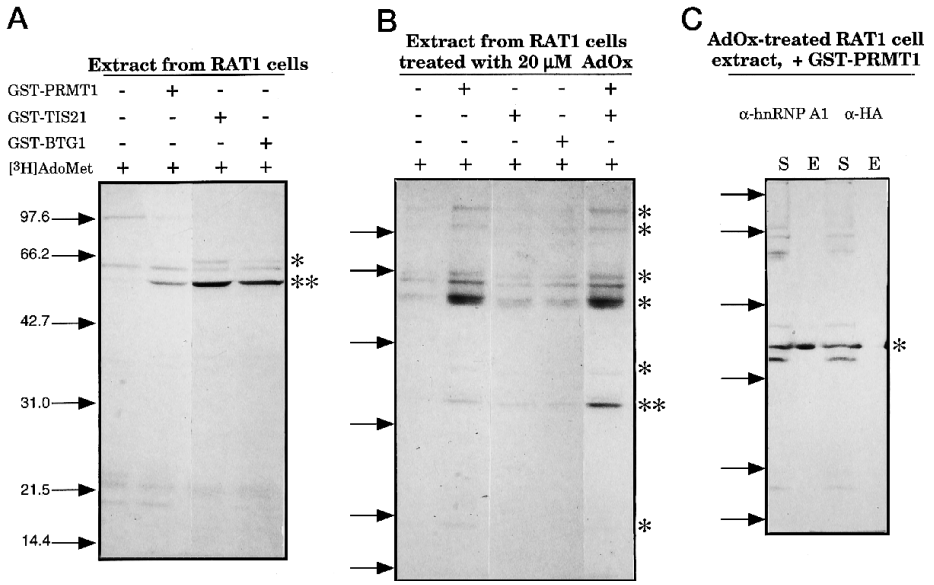

FIG. 7. **The TIS21 and BTG1 fusion proteins modulate endogenous methyltransferase enzymatic activity *in vitro*, using RAT1 cytosolic proteins as substrates.** *A*, GST-TIS21 and GST-BTG1 modulate endogeous methyltransferase activity, using cytosol prepared from untreated RAT1 cells as substrate. GST-TIS21 (2.2 μg), GST-BTG1 (2.55 μg), or GST-PRMT1 (2.0 μg) were added to RAT1 soluble extract (20 μl, 39 μg of protein), along with [^{3}H]AdoMet (0.88 μM, 2.8 μCi). Methylation reactions were carried out for 30 min at 30 °C. After the methylation reaction was stopped, the samples were subjected to polyacrylamide gel electrophoresis. Gels were stained, dried, and subjected to fluorography. The 65-kDa protein that becomes methylated only in the presence of BTG1 or TIS21 fusion proteins is indicated by a *single asterisk*. The 55-kDa protein methylated by GST-PRMT1 is indicated by a *double asterisk*. *B*, GST-TIS21 and GST-BTG1 fusion proteins do not modulate endogenous methyltransferase activity, using hypomethylated exacts prepared from RAT1 cells as substrate. Cytosolic extract was prepared from RAT1 cells grown for 2 days in the presence of adenosine dialdehyde (20 μM). Fusion proteins (as in *panel A*) and [^{3}H]AdoMet (0.7 μM, 2.8 μCi) were added to extract samples (30 μl, 3.6 μg of protein) as indicated in the figure. After the methylation reaction was stopped the samples were subjected to electrophoresis and fluorography. A *single asterisk* indicates the positions of proteins that are methylated by GST-PRMT1 fusion protein. The position of the 34-kDa substrate whose methylation is enhanced by the presence of GST-TIS21 are indicated by the *double asterisks*. *C*, hnRNP A1 present in hypomethylated extracts is methylated by GST-PRMT1 fusion protein. GST-PRMT1 (2.15 μg) was added to cytosolic extract (6 μg) prepared from cells grown for 2 days in the presence of adenosine dialdehyde (20 μM), along with [^{3}H]AdoMet. After the methylation reaction was stopped, samples (50 μl) were incubated with protein A-Sepharose beads bound either to a monoclonal antibody directed against hnRNP A1 (a gift from G. Dreyfuss, U. of Pennsylvania) or to a control monoclonal antibody directed to the hemagglutinin epitope (a gift from A. Berke, UCLA). Anti-hnRNPA1 monoclonal antibody or anti-HA hemagglutinin monoclonal antibody 12CA5 were incubated with 30 μl of protein A-agarose (Oncogene Science) at 4 °C for 1[1,2] h. The antibody-protein A complexes were collected by centrifugation, and washed three times with phosphate-buffered saline containing 0.05% Tween 20 prior to being used for immunoprecipitation. After methylation, portions of the reaction mixtures were incubated with the immobilized antibody complexes in an equal amount of buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, 40 μg/ml aprotinin, and 20 μg/ml pepstatin) for 2 h at 4 °C. After centrifugation, the supernatants were collected. The pellets were washed five times with the same buffer and resuspended in SDS sample buffer. The initial supernatant (*S*) was mixed with an equal volume of 2 × SDS buffer, and the initial supernatant and the eluate from the pellet (*E*) were analyzed by gel electrophoresis and fluorography. The single *asterisk* indicates the position of the 34-kDa hnRNP A1 protein.

tein is added to the RAT1 cytosolic extract along with the [^{3}H]AdoMet, methylation of an additional protein migrating at 55 kDa is observed (Fig. 7*A*, *lane 2*) and methylation of the 96- and 20-kDa proteins is reduced.

It is likely that many substrates for PRMT1 methyltransferase activity are already extensively methylated in cell extracts, and cannot be further methylated during *in vitro* reactions of the type shown in Fig. 7*A*. Najbauer *et al.* (1993) demonstrated that incubation of PC12 cells with adenosine dialdehyde (AdOx) results in the cytosolic accumulation of hypomethylated protein substrates that can subsequently be methylated *in vitro*. When purified GST-PRMT1 fusion protein and [^{3}H]AdoMet are added to extracts prepared from hypomethylated RAT1 cells, we again observe the extensive methylation of a 55-kDa substrate. In addition, methylated proteins of 130, 100, 38, 34, and 16 kDa are observed (Fig. 7*B*, compare *lanes 1* and *2*), suggesting that these proteins normally exist in RAT1 cytosol in an extensively methylated form.

GST-TIS21 and GST-BTG1 Fusion Proteins Can Modulate the Methyltransferase Activity of Endogenous PRMT1—Purified GST-BTG1 or GST-TIS21 recombinant protein, when added to RAT1 cytosolic extracts *without* addition of GST-PRMT1, activate methylation of the 55-kDa protein (Fig. 7*A*, *lanes 3* and *4*). In addition, a 65-kDa protein is methylated. Neither GST-TIS21 nor GST-BTG1 fusion protein alone have methyltransferase activity; they are unable to methylate histones (data not shown). Extracts from quiescent, non-dividing RAT1 cells apparently contain latent, endogenous PRMT1 methyltransferase activity, which can be activated by addition of either GST-BTG1 or GST-TIS21 fusion proteins. The data suggest that interaction of either TIS21 or BTG1 with PRMT1 can modulate endogenous PRMT1 methyltransferase activity present in RAT1 cytosolic extracts. It will, of course, be of great interest to identify the substrates methylated both by recombinant GST-PRMT1 and by the TIS21/PRMT1 and BTG1/PRMT1 complexes.

In contrast to the results observed with extracts from untreated RAT1 cells, there does not appear to be any latent PRMT1 activity present in extracts from RAT1 cells exposed to adenosine dialdehyde. Addition of either GST-TIS21 or GST-BTG1 fusion protein, in the absence of GST-PRMT1, does not enhance methylation of proteins in the hypomethylated extract (Fig. 7*B*, *lanes 3* and *4*). These data suggest that the expression and/or stability of PRMT1 in RAT1 cells may depend on continued cellular methylation activity.

GST-TIS21 Recombinant Protein Modulates the Activity of Recombinant GST-PRMT1 Methyltransferase—The apparent absence of endogenous PRMT1 in hypomethylated RAT1 cell extracts provided us with an opportunity to examine the effect of the GST-TIS21 fusion protein on the enzymatic activity of

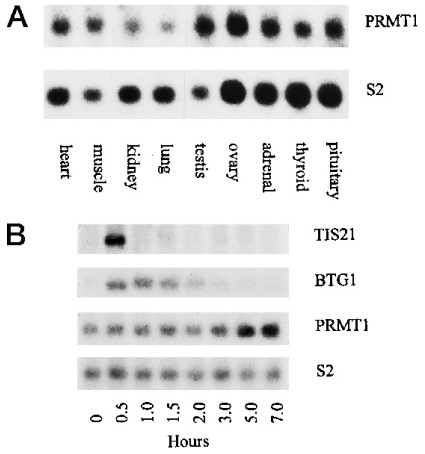

FIG. 8. **PRMT1 mRNA is present in all tissues tested and is constitutively expressed in RAT1 cells.** *A*, Northern blot analysis of PRMT1 expression in various rat tissues. Ten μg of RNA from each rat tissue indicated in the figure was subjected to electrophoresis, and analyzed for PRMT1 expression. *S2*, an mRNA encoding the ribosomal S2 protein, was used to normalize the RNA loading in each lane. *B*, Northern blot analysis of BTG1, TIS21, and PRMT1 mRNA levels in epidermal growth factor-stimulated RAT1 cells. Density-arrested RAT1 cells were stimulated with epidermal growth factor (20 ng/ml). At the times (in hours) following EGF stimulation indicated, cells were harvested. Total RNA was prepared and 5 μg from each sample was examined for BTG1, TIS21, PRMT1, and S2 message levels.

the GST-PRMT1 fusion protein, using endogenous rat cell substrates. When purified GST-TIS21 fusion protein is added to extracts of adenosine dialdehyde-treated RAT1 cells, along with GST-PRMT1 fusion protein, the methylation of the 34-kDa substrate is enhanced (Fig. 7*B*, *lane 5 versus lane 2*). Similar results were observed with the GST-BTG1 fusion protein (data not shown). The data from Fig. 7, *A* and *B*, suggest that the TIS21 and BTG1 proteins both qualitatively and quantitatively modulate PRMT1 activity.

Endogenous hnRNP A1 Present in Hypomethylated RAT1 Soluble Extracts Is a Substrate for GST-PRMT1—We speculated that hnRNP A1 might be the 34-kDa substrate present in hypomethylated RAT1 extracts that is methylated by the GST-PRMT1 fusion protein (Fig. 7*B*, *lanes 2* and *5*). Samples of hypomethylated RAT1 extract incubated with purified GST-PRMT1 fusion protein and [^{3}H]AdoMet were subjected to immunoprecipitation, either with an immobilized monoclonal antibody to hnRNP A1 or with an immobilized control monoclonal antibody to the HA hemagglutionation epitope. The proteins present in the supernatants and immunoadsorbed fractions were subjected to electrophoresis and fluorographic analysis. A ^{3}H-methylated 34-kDa protein could be recovered from the hypomethylated extract following methylation by the GST-PRMT1 fusion protein, using anti-hnRNP A1 antibody (Fig. 7*C*), confirming that the 34-kDa substrate is hnRNP A1. Control 12CA5 monoclonal anti-hemagglutinin antibody did not immunoprecipitate any methylated proteins. These experiments suggest (i) that the bulk of hnRNP A1 present in confluent, density-arrested RAT1 cells is normally methylated at PRMT1 substrate sites (Fig. 7*A*), and (ii) that BTG1 protein and TIS21 protein may modulate the ability of PRMT1 to methylate hnRNP A1 *in vitro* (Fig. 7*B*). Confirmation of this hypothesis will, of course, depend on further experiments with purified PRMT1, TIS21, and BTG1, expressed without the GST moiety.

The PRMT1 Gene Is Transcribed in all Rat Tissues Examined, and Is Constitutively Expressed in RAT1 Cells—The TIS21 gene is expressed in a wide variety of tissues (Fletcher *et al.*, 1991). BTG1 message is low in fully differentiated tissues such as brain or muscle, but is present in most other tissues (*e.g.* thymus, heart, lung, spleen, liver, and kidney) (Rimokh *et al.*, 1991). Northern analysis of rat tissue extracts demonstrates that the PRMT1 gene is expressed in all rat tissues tested (Fig. 8*A*).

The expression of the TIS21 gene is rapidly and transiently induced by a variety of ligands, in a number of different cell types (Herschman, 1991). BTG1 gene expression has been reported to be down-regulated when cells enter S phase (Rouault *et al.*, 1992). We compared the levels of expression of the BTG1, TIS21, and PRMT1 messages in mitogen-stimulated RAT1 cells. As expected (Fletcher *et al.*, 1991), TIS21 message is not present in quiescent, growth-arrested cells (Fig. 8*B*). Stimulation with epidermal growth factor induces a rapid appearance of TIS21 message, detectable only at 30 min. Accumulation of TIS21 message is transient; within 1 h TIS21 mRNA returns to baseline, undetectable values. In contrast to results previously reported for 3T3 cells (Rouault *et al.*, 1992), BTG1 message is also low in growth-arrested RAT1 cells (Fig. 8*B*). Following epidermal growth factor stimulation, BTG1 message is also elevated. The mitogen stimulation of BTG1 message accumulation peaks at a later time, 60 min, than that of TIS21 message. Even after 5–7 h, BTG1 message levels do not return to the baseline values observed in unstimulated, growth-arrested cells. Unlike the mitogen-induced elevations in BTG1 and TIS21 message levels, PRMT1 mRNA is present in growth-arrested RAT1 cells and does not change in response to epidermal growth factor stimulation. It seems likely that, if alterations in protein-arginine *N*-methyltransferase activity occur in response to ligand stimulation, such changes are likely to result from transient modulation of PRMT1 enzyme activity, rather than by alterations in PRMT1 gene expression.

DISCUSSION

Protein-arginine N-Methyltransferases—Paik and Kim (1967) provided the first evidence for post-translational methylation of protein arginine residues. They described two novel radioactive species present in acid hydrolysates of calf thymus nuclei that had been incubated with *S*-adenosyl-[*methyl*-^{14}C]-L-methionine. These methylated species were subsequently identified as arginine residues that had been mono- and dimethylated on their guanidino groups (Paik and Kim, 1968; Nakajima *et al.*, 1971). Partial purification of protein-arginine transferase activity from calf brain suggested that two enzymes are present (Ghosh *et al.*, 1988), one that methylates arginine residue 107 of myelin basic protein (Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971), and a second enzyme that was initially thought to be a histone-specific methyltransferase. Subsequent studies demonstrated that this second protein-arginine transferase utilizes other substrates, *e.g.* hnRNP A1, much more efficiently than it does histones (Rajpurohit *et al.*, 1994a; Liu and Dreyfuss, 1995). hnRNP A1 is methylated on an arginine residue present in the GAR domain (Rajpurohit *et al.*, 1994a).

Although a number of purifications of histone/hnRNP A1 arginine *N*-methyltransferase have been reported (Ghosh *et al.*, 1988; Rawal *et al.*, 1994; Liu and Dreyfuss, 1995), the polypeptide composition of the methyltransferase complex has not been conclusively established. Previous chromatographic studies on partially purified preparations suggested that the histone/hnRNP A1 methyltransferase is a high molecular mass complex, estimated to be between 275 and 450 kDa, and containing multiple polypeptide components. Like GST-PRMT1, these protein-arginine transferases (i) do not methylate myelin basic protein, and (ii) mono- and asymmetrically dimethylate hnRNP A1 and histones. Ghosh *et al.* (1988) suggested that histone arginine methyltransferase preparations from calf brain had

two major polypeptides, of molecular masses 110 and 75 kDa. In contrast, Rawal *et al.* (1994) report only a single 110-kDa polypeptide present in their rat liver preparation. SDS gel analysis of the preparation from HeLa cells obtained by Liu and Dreyfuss (1995) demonstrated eight distinct bands, the most prominent migrating at 110 and 45 kDa.

Our chromatographic data agree with the suggestion that native protein-arginine *N*-methyltransferase activity in mammalian cells and tissues exists as a macromolecular complex. Our present study describes the first cloning of a mammalian protein-arginine *N*-methyltransferase. The predicted molecular mass of the protein encoded by the PRMT1 cDNA is 40.5 kDa. Thus the 45-kDa band observed by Liu and Dreyfuss (1995) may represent the PRMT1 catalytic component. Coimmuoprecipitation experiments with antibody to the PRMT1 catalytic subunit should allow us to characterize the proteins present in the macromolecular complex and to investigate the effect of the TIS21 and BTG1 proteins on both the composition of the complex and the functions of its components.

Relationship between the Protein-arginine N-Methyltransferase Activity Present in Yeast and PRMT1—The PRMT1 open reading frame has extensive sequence similarity to the open reading frame of the yeast *ODP1* gene (Fig. 2). We have recently demonstrated, both by genetic and biochemical means, that the *ODP1* gene also encodes a protein-arginine *N*-methyltransferase (Gary *et al.*, 1996), and renamed this yeast gene *RMT1*, for arginine methyltransferase. Although the yeast RMT1 enzyme and the rat PRMT1 enzyme are both protein-arginine *N*-methyltransferases, the GST-RMT1 (yeast) fusion protein has substantially broader substrate specificity than does the GST-PRMT1 (rat) fusion protein (Gary *et al.*, 1996). Moreover, analysis of data from the expressed sequence tag data bases suggest that more than one gene with sequence similarities to the rat PRMT1 gene may exist in humans. These data are reminiscent of other regulatory enzymatic activities that modify protein function, such as cyclin-dependent kinases. In *Saccharomyces cerevisiae* a single gene, *CDC28*, encodes a single cyclin-dependent kinase with broad substrate specificity. In contrast, mammalian cells encode a family of cyclin-dependent kinases, each with a more restricted substrate specificities.

Protein Arginine Methylation as a Potential Mediator of Ligand-induced Signal Transduction—We initiated the studies described here with the goal of identifying the role of the TIS21 immediate-early gene product in ligand-induced signal transduction. Using two-hybrid interaction analysis, we identified PRMT1 as a protein that can associate with both TIS21 protein and with BTG1 protein, a member of TIS21 gene family. In addition to identifying PRMT1 as a protein-arginine *N*-methyltransferase, and demonstrating its ability to interact with TIS21 and BTG1 prepared by *in vitro* translation, we also demonstrated that the TIS21 and BTG1 fusion proteins can modulate the methyltransferase activity of endogenous PRMT1 (Figs. 7, *A* and *B*).

It is of particular interest that extracts of RAT1 cells contain latent PRMT1 activity that cannot methylate endogenous substrates present in these extracts, unless recombinant TIS21 or BTG1 fusion protein is added. Although GST-PRMT1 can methylate the 55-kDa substrate present in RAT1 extracts, endogenous PRMT1 can only methylate this substrate if recombinant TIS21 or BTG1 GST fusion proteins are added (Fig. 7*A*). A 65-kDa substrate, not methylated by recombinant GST-PRMT1, can also be methylated by the endogenous PRMT1 activity only if TIS21 and/or BTG1 fusion protein is added to the extract.

The use of hypomethylated cell extracts as substrates for methylation resulted in two provocative observations. First, unlike extracts from untreated cells, extracts from cells treated with adenosine dialdehyde do not appear to have latent protein-arginine *N*-methyltransferase activity present. These data suggest that the presence of the catalytic component of this enzymatic activity, PRMT1, may itself be regulated by methylation. However, GST-PRMT1 does not methylate itself (Fig. 5, *A* and *D*). Thus, if PRMT1 levels are regulated by methylation, it must be by a more indirect route. Second, when GST-PRMT1 and GST-TIS21 are used together to methylate endogenous substrates in extracts from hypomethylated RAT1 cells, the methylation of the 34-kDa protein identified as hnRNP A1 is greater than that observed with GST-PRMT1 alone (Fig. 7*B*).

The data obtained with RAT1 cell extracts suggest that the enzymatic activity of a constitutively expressed protein methyltransferase catalytic component, PRMT1, can be modified both with respect to substrate specificity and with respect to catalytic efficiency by the interaction of ligand-induced, transiently expressed TIS21 and BTG1 regulatory subunits. These results are similar to the qualitative and quantitative modulation of enzymatic activity of constitutively expressed catalytic protein kinase molecules by transiently expressed regulatory subunits such as the G1 and G2 cyclins. We suggest that PRMT1-dependent protein arginine methylation, mediated by the TIS21/BTG1 protein family, may be an additional pathway of ligand-induced signal transduction.

Protein methylation has previously been identified as a signal transduction mechanism in several biological systems. In bacteria, the γ-carboxyl methylation of glutamate residues in a class of chemoreceptor/transducer proteins by the CheR methyltransferase is necessary for proper chemotactic response (Shapiro *et al.*, 1995). In eukaryotic cells, carboxyl methylation of the COOH-terminal leucine residue in the protein phosphatase PP2A catalytic subunit has been shown to alter the subcellular localization of this molecule (Turowski *et al.*, 1995). Moreover this methylation is reversible by the action of a methyl esterase (Xie and Clarke, 1994b). Additionally, endotoxin treatment of B cells (Law *et al.*, 1992) and nerve growth factor treatment of PC12 pheochromocytoma cells (Kujubu *et al.*, 1993) cause an increase in membrane-associated protein methylation that is specifically inhibited by pretreatment with the protein methylation inhibitor 5′-methylthioadenosine.

Modulation of RNA Splicing as One Potential Target for PRMT1-mediated Signal Transduction—hnRNP A1 plays a role in determining alternative mRNA splicing. Modulation of the level of hnRNP A1, either *in vitro* (Mayeda and Krainer, 1992) or *in vivo* (Caceres *et al.*, 1994), can modulate 5′ splice site selection during the maturation of mRNA. Moreover, methylation can alter the nucleic acid binding properties of hnRNP A1 (Rajpurohit *et al.*, 1994b). Thus it seems likely that modulation of hnRNP A1 methylation may modify mRNA splicing *in vivo*. It is also possible that modulation of the methylation of hnRNP A1 may mediate other functions of this molecule, such as nuclear export (Michael *et al.*, 1995), nuclear localization (Siomi and Dreyfuss, 1995), or other aspects of RNA biogenesis (Dreyfuss *et al.*, 1993).

We suggest that transient expression of the TIS21 and BTG1 genes may mediate extracellular signals by modulation of splicing following ligand stimulation. Perhaps the best precedent for such a suggestion is the study of Shifrin and Neel (1993), who demonstrated that a novel form of PTP-1B, a nontransmembrane phosphotyrosine phosphatase, is expressed in human fibroblasts when they are stimulated with a variety of growth factors. The expression of the altered form of PTP-1B protein is the consequence of a growth factor-induced alteration in splicing of the PTP-1B message. Moreover, the growth factor-induced alteration in splicing of the PTP-1B message

requires protein synthesis, suggesting that the product of an immediate early gene (perhaps TIS21) is necessary. Similarly, Ogimoto *et al.* (1993) demonstrated that either anti-IgM antibody or the combination of phorbol ester and ionomycin induces alterations in splicing of the CD45 message in murine B cells, and Sarmay *et al.* (1995) demonstrated that interleukin 4, anti-IgM antibody, or phorbol ester treatment can induce alterations in splicing of the Fc γRII message in human B cells.

Many other protein components of the RNA processing machinery are also substrates for methylation. For example, glycine- and arginine-rich GAR domains containing multiple potential RGG substrate sites for PRMT1 methylation, are present in nucleolin and fibrillarin (Najbauer *et al.*, 1993). These proteins contain N^G,N^G-dimethylarginine (asymmetric) *in vivo* (Lischwe *et al.*, 1985a, 1985b). Nucleolin and fibrillarin participate in pre-rRNA processing, and are specifically localized to the nucleolus (Henriquez *et al.*, 1990). It seems likely that these proteins (i) will also be substrates for PRMT1 and (ii) their methylation by PRMT1 will be subject to modification by the interaction of the TIS21 and/or BTG1 proteins with the methyltransferase enzyme.

In addition to modulating mRNA splicing, protein arginine methylation may modulate growth factor localization and function. The high molecular weight form of fibroblast growth factor has a glycine- and arginine-rich domain at its NH_2 terminus (Prats *et al.*, 1989) that contains methylated arginine residues (Burgess *et al.*, 1991). Arginine methylation has been suggested as a mechanism for preferential targeting of the high molecular weight form of this growth factor to the nucleus (Burgess *et al.*, 1991).

Identification and cloning of the catalytic component of the protein-arginine transferase should now make possible previously inaccessible experiments. Elucidation of additional substrates for PRMT1, the functional consequences following methylation of PRMT1 substrates, the search for other protein-arginine transferases and their regulatory subunits, the nature of the interactions between protein-arginine transferases and their regulatory subunits, and the consequences of genetic alterations in these molecules all present important questions for future studies.

Acknowledgments—We thank Dana Aswad (University of California, Irvine) for R1 peptide, Arnold Berk (UCLA) for monoclonal antibody 12CA5, Gideon Dreyfuss (University of Pennsylvania) for antibody to hnRNP A1, Akila Mayeda and Adrian Krainer (Cold Spring Harbor Laboratory) for recombinant hnRNP A1, and Sandra Rossie (Purdue University) for catalytic subunit of protein phosphatase 2A. We also thank Douglas Black (UCLA) for his advice and helpful discussions at critical stages of this manuscript, and Limin Han and John Colicelli for plasmids and advice on yeast two-hybrid analysis. We also acknowledge the help the late Robert Andersen provided with the yeast two-hybrid analysis, and Dr. Andersen's gift of the pPC86 cDNA fusion library.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410

Bailey, J. L. (1967) in *Techniques in Protein Chemistry*, 2nd Ed., pp. 340–341, Elsevier Science Publishers B.V., Amsterdam

Baldwin, G. S., and Carnegie, P. R. (1971) *Science* **171,** 579–581

Bartel, P. L., Chien, C.-T., Sternglanz, R., and Fields, S. (1993) in *Cellular Interactions in Development, A Practical Approach* (Hartley, D. A., ed) pp. 153–179, Oxford University Press, Oxford

Bradbury, A., Possenti, R., Shooter, E. M., and Tirone, F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3353–3357

Breeden, L., and Naysmith, K. (1985) *Cold Spring Harbor. Symp. Quant. Biol.* **5**, 643–650

Brostoff, S., and Eylar, E. H. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 765–769

Burgess, W. H., Bizik, J., Mehlman, T., Quarto, N., and Rifkin, D. B. (1991) *Cell Regul.* **2,** 87–93

Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994) *Science* **265,** 1706–1709

Cathala, G., Savouret, J., Mendez, B., West, B. L., Karin, M., Martial, J. A., and Baxter J. D. (1983) *Lab. Methods* **2,** 329–335

Chomczymski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159

Clarke, S. (1993) *Curr. Opin. Cell Biol.* **5,** 977–983

Dreyfuss, G., Matunis, M. J., Pino-Roma, S., and Burd, C. G. (1993) *Annu. Rev. Biochem.* **62,** 289–321

Farooqui, J. Z., Tuck, M., and Paik, W. K. (1985) *J. Biol. Chem.* **260,** 537–545

Feldmann, H., Aigle, M., Aljinovic, G., André, B., Baclet, M. C., Barthe, C., Baur, A., Bécam, A. M., Biteau, N., Boles, E. et al. (1994) *EMBO J.* **13,** 5795–5809

Fields, S., and Song, O. (1989) *Nature* **240,** 245–246

Fields, S., and Sternglanz, R. (1994) *Trends Genet.* **10,** 286–292

Fletcher, B. S., Lim, R. W., Varnum, B. C., Kujubu, D. A., Koski, R. K., and Herschman, H. R. (1991) *J. Biol. Chem.* **266,** 14511–14518

Gary, J. D., and Clarke, S. (1995) *J. Biol. Chem.* **270,** 4076–4087

Gary, J. D., Lin, W.-J., Yang, M. C., Herschman, H., and Clarke, S. (1996) *J. Biol. Chem.* **271,** 12585–12594

Ghosh, S. K., Paik, W. K., and Kim, S. (1988) *J. Biol. Chem.* **263,** 19024–19033

Gottschling, H., and Freese, E. (1962) *Nature* **196,** 829–831

Han, L., and Colicelli, J. (1995) *Mol. Cell. Biol.* **15,** 1318–1323

Henriquez, R., Blobel, G., and Aris, J. P. (1990) *J. Biol. Chem.* **265,** 2209–2215

Herschman, H. R. (1991) *Annu. Rev. Biochem.* **60,** 281–319

Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. (1995) *Mol. Cell. Biol.* **15,** 3813–3822

Hrycyna, C. A., Yang, M. C., and Clarke, S. (1994) *Biochemistry* **33,** 9806–9812

Kagan, R. M., and Clarke, S. (1994) *Arch. Biochem. Biophys.* **310,** 417–427

Kujubu, D. A., Stimmel, J. B., Law, R. E., Herschman, H. R., and Clarke, S. (1993) *J. Neurosci. Res.* **36,** 58–65

Laemmli, U. K. (1970) *Nature* **227,** 680–685

Law, R. E., Stimmel, J. B., Damore, M. A., Carter, C., Clarke, S., and Wall, R. (1992) *Mol. Cell. Biol.* **12,** 103–111

Lee, H. W., Kim, S., and Paik, W. K. (1977) *Biochemistry* **16,** 78–85

Lischwe, M. A., Cook, R. G., Ahn, Y. S., Yeoman, L. C., and Busch, H. (1985a) *Biochem. J.* **24,** 6025–6028

Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., Reichlin, M., and Busch, H. (1985b) *J. Biol. Chem.* **260,** 14304–14310

Liu, Q., and Dreyfuss, G. (1995) *Mol. Cell. Biol.* **15,** 2800–2808

Loubbardi, A., Marcireau, C., Karst, F., and Guilloton, M. (1995) *J. Bacteriol.* **177,** 1817–1823

Lowenson, J. D., and Clarke, S. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., ed) pp. 47–63, CRC Press, Inc., Boca Raton, FL

Mayeda, A., and Krainer, A. R. (1992) *Cell* **68,** 365–375

Michael, W. M., Choi, M., and Dreyfuss, G. (1995) *Cell* **83,** 415–422

Najbauer, J., Johnson, B. A., Young, A. L., and Aswad, D. W. (1993) *J. Biol. Chem.* **268,** 10501–10509

Nakajima, T., Matsuoka, Y., and Kakimoto, Y. (1971) *Biochim. Biophys. Acta* **230,** 212–222

Ogimoto, M., Katagiri, T., Hasegawa, K., Mizuno, K., and Yakura, H. (1993) *Cell. Immunol.* **151,** 97–109

Paik, W. K., and Kim, S. (1967) *Biochem. Biophys. Res. Commun.* **29,** 14–20

Paik, W. K., and Kim, S. (1968) *J. Biol. Chem.* **243,** 2108–2114

Prats, H., Kaghad, M., Prats, A. C., Klagsbrun, M., Lélias, J. M., Liauzun, P., Chalon, P., Tauber, J. P., Amalric, F., Smith, J. A., and Caput, D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1836–1840

Rajpurohit, R., Lee, S. O., Park, J. O., Paik, W. K., and Kim, S. (1994a) *J. Biol. Chem.* **269,** 1075–1082

Rajpurohit, R., Paik, W. K., and Kim, S. (1994b) *Biochem. J.* **304,** 903–909

Rawal, N., Rajpurohit, R., Paik, W. K., and Kim, S. (1994) *Biochem. J.* **300,** 483–489

Rimokh, R., Rouault, J. P., Wahbi, K., Gadoux, M., Lafage, M., Archimbaud, E., Charrin, C., Gentilhomme, O., and Germain, D. (1991) *Genes Chromosomes Cancer* **3,** 24–36

Rose, M. D., Winston, F., and Heiter, P. (1990) in *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Rouault, J. P., Rimokh, R., Tessa, C., Paranhos, G., Ffrench, M., Duret, L., Garoccio, M., Germain, D., Samarut, J., and Magaud, J. P. (1992) *EMBO J.* **11,** 1663–1670

Rouault, J. P., Samarut, C., Duret, L., Tessa, C., Samarut, J., and Magaud, J. P. (1993) *Gene* (*Amst.*) **129,** 303–306

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 1060–1061, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sarmay, G., Rozsnyay, Z., Koncz, G., Danilkovich, A., and Gergely, J. (1995) *Eur. J. Immunol.* **25,** 262–268

Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346

Schluckebier, G., O'Gara, M., Saenger, W., and Cheng, X. (1995) *J. Mol. Biol.* **247,** 16–20

Shapiro, M. J., Chakrabarti, I., and Koshland, D. E., Jr. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1053–1056

Shifrin, V. I., and Neel, B. G. (1993) *J. Biol. Chem.* **268,** 25376–25384

Siomi, H., and Dreyfuss, G. (1995) *J. Cell Biol.* **129,** 441–560

Smith, J. B., and Herschman, H. R. (1995) *J. Biol. Chem.* **270,** 16756–16765

Staudinger, J., Zhou, J., Burgess, R., Elledge, S. J., and Olson, E. N. (1995) *J. Cell Biol.* **128,** 263–271

Thomas, P. S. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5205

Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) *J. Cell Biol.* **129,** 397–410

Vanet, A., Plumbridge, J. A., and Alix, J. H. (1993) *J. Bacteriol.* **175,** 7178–7188

Varnum, B. C., Reddy, S. T., Koski, R. A., and Herschman, H. R. (1994) *J. Cell. Physiol.* **158,** 205–213

Wang, H., Peters, A., Zeng, X., Tang, M., Wallace, I., and Khan, S. A. (1995) *J. Biol. Chem.* **270,** 23322–23339

Xie, H., and Clarke, S. (1993) *J. Biol. Chem.* **268,** 13364–13371

Xie, H., and Clarke, S. (1994a) *J. Biol. Chem.* **269,** 1981–1984

Xie, H., and Clarke, S. (1994b) *Biochem. Biophys. Res. Commun.* **203,** 1710–1715