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In Vivo and In Vitro Arginine Methylation of RNA-Binding Proteins

QING LIU AND GIDEON DREYFUSS*

Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148

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Heterogenous nuclear ribonucleoproteins (hnRNPs) bind pre-mRNAs and facilitate their processing into mRNAs. Many of the hnRNPs undergo extensive posttranslational modifications including methylation on arginine residues. hnRNPs contain about 65% of the total N^G,N^G-dimethylarginine found in the cell nucleus. The role of this modification is not known. Here we identify the hnRNPs that are methylated in HeLa cells and demonstrate that most of the pre-mRNA-binding proteins receive this modification. Using recombinant human hnRNP A1 as a substrate, we have partially purified and characterized a protein-arginine *N*-methyltransferase specific for hnRNPs from HeLa cells. This methyltransferase can methylate the same subset of hnRNPs in vitro as are methylated in vivo. Furthermore, it can also methylate other RNA-binding proteins that contain the RGG motif RNA-binding domain. This activity is evolutionarily conserved from lower eukaryotes to mammals, suggesting that methylation has a significant role in the function of RNA-binding proteins.

Pre-mRNAs undergo extensive processing reactions to form mRNAs in the eukaryotic cell nucleus. Immediately after transcription, pre-mRNA associates with at least 20 nuclear proteins, collectively called heterogeneous nuclear ribonucleoproteins (hnRNPs), and these remain bound throughout the time pre-mRNA remains in the nucleus. Many of the hnRNPs are as abundant as histones in the nucleus. However, the detailed mechanism of action and the entire range of functions of hnRNPs are not yet known. They are likely to influence RNA-RNA interactions and affect processing of pre-mRNA, and they may also play a role in the transport of mRNAs from the nucleus to the cytoplasm (17, 53). Many of the interactions between hnRNPs and pre-mRNA and mRNA are dynamic, suggesting that their binding to and dissociation from RNAs and other proteins are regulated. Such interactions may be controlled by posttranslational modifications, and most hnRNPs have indeed been found to be modified posttranslationally. These modifications include methylation (3, 39, 70), phosphorylation (3, 13, 23, 25, 50, 54, 70), and glycosylation (64). Early studies of hnRNPs have shown that several of them contain an unusual modified amino acid residue, N^G,N^G-dimethylarginine. Arginine is the only known methylated amino acid residue in hnRNPs (3, 70). Further studies have shown that N^G,N^G-dimethylarginine is the major methylated derivative, while N^G-monomethylarginine is present only in trace amounts (5, 25). Interestingly, hnRNPs contain about 65% of the total N^G,N^G-dimethylarginine found in the cell nucleus, and approximately 12% of the arginine residues in total hnRNPs appear to be methylated (5). Besides hnRNPs, several other proteins also contain methylated arginine residues. These include the U3 small nuclear RNA-binding protein fibrillarin (37), the pre-rRNA-binding protein nucleolin (38), p62, the GTPase-activating protein-associated protein which has RNA binding activity (72), myelin basic proteins (MBPs) (2), myosin (60), and heat shock proteins (67, 68).

Two protein arginine methyltransferases have been previously identified and partially purified. They both use *S*-adenosyl-L-methionine as the methyl donor and are reported to be specific for MBP and histones, respectively (20). The major methylation derivative of MBP is N^G,N'^G-dimethylarginine, in which the two methyl groups are distributed symmetrically (20). This is different from the methylation product of the hnRNPs, in which the methyl groups are asymmetrically arranged, suggesting that a unique enzymatic activity exists that is specific for these RNA-binding proteins (58). Wilk et al. (70) have shown that several hnRNPs in isolated hnRNP core complex contain this modification. hnRNP A1 contains N^G,N^G-dimethylarginine at residue 194 (30, 71), and there are at least two more arginine residues in this protein that can be methylated in vivo (3, 70). With improved techniques available for isolating hnRNP complexes, we were interested in identifying which hnRNPs other than the core proteins also contain this modification. Given the prevalence of this unusual modification in hnRNPs, it is likely that it plays an important role in regulating the functions of these proteins. Therefore, it is of great interest to understand the effect of methylation on the activity of hnRNPs.

In this investigation, we studied the in vivo methylation of hnRNPs, and we describe the partial purification of a protein arginine methyltransferase from HeLa cells that carries out this modification. This activity does not methylate MBP and methylates histone H4 to a much lesser extent than hnRNPs. The protein arginine methyltransferase activity that we partially purified acts on the same subset of hnRNPs in vitro as those that are methylated in vivo. Further characterization of this enzyme indicates that its substrates are not limited to pre-mRNA-binding proteins (hnRNPs). Rather, it has activity toward a wide range of RNA-binding proteins, all of which appear to contain the RGG box RNA-binding motif (27). Indeed, in vitro experiments have shown that a 23-amino-acid peptide derived from the RGG domain of hnRNP U can serve as an excellent substrate. Finally, we show that a similar activity is found in several evolutionarily divergent eukaryotes, which strongly suggests that this modification plays an important role in regulating RNA-binding protein functions.

* Corresponding author. Phone: (215) 898-0172. Fax: (215) 573-2000.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (SAM; specific activity, 84 mCi/mmol) was obtained from Amersham Corp. L-[*methyl*-^{3}H]methionine (1 mCi/ml, 83.1 Ci/mmol) and L-[^{35}S]methionine (10 μCi/μl, 1,000 Ci/mmol) were from DuPont NEN Research Products. Histone type II-S (H-6005) and MBP (M-1891) were from Sigma. Recombinant hnRNP A1 was produced in *Escherichia coli* and purified to homogeneity by D. Portman in our laboratory (56). The A1 mutant in which all six arginine residues were changed to lysine in the RGG domain from amino acids 194 to 234 (^{194}RGRSGSGNFGGGRGGGFGGND NFGRGGNFSGRGGFGGSRGG^{234}) was made by Mieyoung Choi in our laboratory (9a). The RGG and KGG peptides were made according to the sequence in the RGG domain of hnRNP U from amino acids 695 to 716. They were made by the Protein Biochemistry Laboratory at the University of Pennsylvania and purified by Megerditch Kiledjian in our laboratory (27a). SP-Sepharose Fast Flow and DEAE-Sephacel were purchased from Pharmacia. Phenyl-Sepharose was from Sigma. The Q-Sepharose Hiload (16/10) column was obtained from Pharmacia. The DEAE-TSK (8/8) glass column was from TosoHaas. All chromatography procedures were carried out with a Waters 650 gradient controller system.

Cell culture and labeling. HeLa JW36 cells were cultured in monolayer to subconfluent densities in Dulbecco's modified Eagle's medium, supplemented with penicillin and streptomycin and containing 10% fetal calf serum (medium A) at 37°C. For large-scale preparation, HeLa S3 cells were purchased from Cellex Bioscience, Minneapolis, Minn.

For in vivo methylation of hnRNPs, the following procedure, modified from the method described by Desrosiers and Tanguay (15), was used. Five plates (100-mm-diameter) of actively growing HeLa JW36 cells were incubated with cycloheximide (100 μg/ml) and chloramphenicol (40 μg/ml) in medium A for 30 min. The medium was then replaced with Dulbecco's modified Eagle's medium without methionine, supplemented with penicillin, streptomycin, and 10% fetal calf serum (medium B) and containing 10 μCi of L-[*methyl*-^{3}H]methionine per ml, and the methyl donor and cells were incubated for an additional 3 h in the presence of the same protein synthesis inhibitors. The cells were lysed in 10 mM Tris-HCl–100 mM NaCl–2.5 mM $MgCl_2$ (pH 7.4) (RSB-100) containing 0.5% Triton X-100, 1% aprotinin, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml, and 0.1 mM dithiothreitol. The nucleoplasmic fraction was prepared, and hnRNP complexes were immunopurified with monoclonal antibody 4F4 to the hnRNP C proteins (10, 16) as previously detailed (51). The proteins were then resuspended in 15 μl of sample buffer and resolved by two-dimensional gel electrophoresis (48). The protein gel was then fluorographed as described by Laskey and Mills (34), dried, and exposed to Kodak film (Kodak 165-1454) at −80°C for 2 weeks. The efficiency of the labeling was calculated by counting 1 μl of the sample in a Beckman LS6000IC scintillation counter, using 3 ml of CytoScint (ICN) scintillant.

For testing the effect of protein synthesis inhibitors, one plate (100-mm-diameter) of HeLa cells was incubated with cycloheximide (100 μg/ml) and chloramphenicol (40 μg/ml) in medium A for 30 min. This and another plate with an equal density of cells were then labeled with 10 μCi/ml L-[^{35}S]methionine for 3 h in medium B in the presence or absence of the same protein synthesis inhibitors. The cells were lysed in 500 μl of 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. The cell lysate was sonicated with a W-380 (Heat System Ultrasonic, Inc.) sonicator at setting 2.5 to 3 three times for 5 s each and boiled for 5 min. For Fig. 1C, 25 μl of each sample was loaded onto the SDS-polyacrylamide gel. The efficiency of labeling was measured by precipitating 2 μl of the lysate with 25% trichloroacetic acid and Casamino Acids mixture and counted in 3 ml of CytoScint (ICN) scintillant.

Fractionation of HeLa cells. For enzyme purification, 12 g of HeLa frozen cell pellet (equivalent to 12 liters of HeLa S3 cells grown to mid-log phase) was thawed on ice. After resuspension in 100 ml (8× volume of cell pellet) of RSB-100 buffer containing 0.5% Triton X-100, 1% aprotinin, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml, and 0.1 mM dithiothreitol at pH 7.4, the cell suspension was homogenized with at least eight strokes in a 40-ml dounce tissue grinder (Wheaton, Millville, N.J.). The cytoplasmic fraction was collected after pelleting of the nuclei at 3,000 × *g* for 10 min. The nucleoplasmic fraction was prepared as described previously (51).

Purification of hnRNP A1-specific protein arginine methyltransferase. All steps were carried out at 0 to 4°C unless otherwise indicated. HeLa cell cytoplasmic fraction was adjusted to 40% saturation by gradually adding solid $(NH_4)_2SO_4$ with constant mixing. After the mixture was stirred for an additional 20 min, the supernatant was recovered by centrifugation at 10,000 × *g* for 10 min and was then adjusted to 60% saturation. The resulting pellet from the 60% $(NH_4)_2SO_4$ precipitation was resuspended in 50 ml of 20 mM Tris-HCl (pH 7.4)–5% glycerol–0.1 mM EDTA–0.1 mM dithiothreitol (buffer A) and dialyzed against 4 liters of buffer A overnight. The dialysate was applied to an SP-Sepharose Fast Flow column (2.5 by 10 cm) equilibrated in buffer A. The flowthrough was collected and applied to a DEAE-Sephacel column (2.5 by 10 cm) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with buffer A containing 0.1 M NaCl until the A_{280} was less than 0.05. The enzyme activity was eluted with 150 ml of buffer A containing 0.3 M NaCl. The eluate was then diluted threefold with buffer A and loaded onto a Q-Sepharose Hiload (16/10) column preequilibrated with buffer A containing 0.1 M NaCl. The column was washed with buffer A containing 0.1 M NaCl until the A_{280} was less than 0.05 and eluted with a 150-ml linear NaCl gradient from 0.1 to 0.5 M. The fractions containing the enzyme activity eluting from 0.3 to 0.4 M were collected, adjusted to 0.2 M $(NH_4)_2SO_4$ with buffer A containing 1 M $(NH_4)_2SO_4$, and loaded onto a phenyl-Sepharose column (1.0 by 10 cm) preequilibrated with buffer A containing 0.2 M $(NH_4)_2SO_4$ and 10% glycerol. The column was eluted with a 30-ml linear decreasing gradient from 0.2 to 0 M $(NH_4)_2SO_4$ in buffer A containing 10% glycerol. The active fractions from 0.1 to 0.0 M were collected, loaded onto a DEAE-TSK column (7.5 cm by 8 mm), and eluted with a 30-ml NaCl concentration gradient from 0.1 to 0.36 M. Fractions containing the enzyme activity eluting from 0.2 to 0.3 M were pooled and stored at −20°C.

Assay for protein arginine methyltransferase. Thirteen microliters of each fraction was added to 4 μCi of lyophilized SAM and 2 μl (0.4 μg) of recombinant hnRNP A1. The mixture was incubated at 37°C for 25 min, and the reaction was terminated by adding 2× SDS sample buffer and boiling for 5 min. The extent of methylation was determined by a filter binding assay or SDS-PAGE analysis. For the filter binding assay, after the completion of the reaction, a portion of each reaction mixture (2 μl) was added to 1 ml of phosphate-buffered saline (PBS). This mixture was filtered through a 0.45-μm-pore-size nitrocellulose filter (HAWP 02500; Millipore Corp.) prewetted with 2 ml of PBS, using a 12-well filtration manifold (Millipore xx2702550) connected to a vacuum pump. Each sample well was then washed with 10 ml of PBS at 4°C. The filters were air dried, and the radioactivity present in each well was measured with a scintillation counter as described above. Background radioactivity obtained in the absence of A1 (usually <1% of input radioactivity) was subtracted from each datum point. Protein concentration was determined by using Bio-Rad protein assay dye reagent concentrate.

Gel electrophoresis. SDS-PAGE was performed as described previously (16), using 12.5% polyacrylamide in the separating gel. Separation of RGG and KGG peptides with tricine SDS-PAGE was carried out as described by Schagger and Jagow (61). L-[^{35}S]methionine and *methyl*-^{3}H-labeled proteins were detected by fluorography (34). The composition of different fractions was visualized by silver staining as described previously (44). Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) was done as described by O'Farrell et al. (48).

In vitro methylation of hnRNPs. hnRNP complexes were immunopurified from five culture plates (100-mm-diameter) of actively growing HeLa cells with monoclonal antibody 4F4 as described above. The complexes immobilized on the protein A-Sepharose beads were then incubated with 25 μl (0.5 μg of total protein) of the active enzyme fractions and 10 μCi of SAM under the standard assay conditions described above. The complexes were then isolated by centrifugation at 4,000 × *g* for 2 min and dissolved in 15 μl of electrophoresis sample buffer. Total radioactivity was calculated by counting 1 μl of the sample. Total sample was resolved by two-dimensional gel electrophoresis. The gel was then fluorographed, dried, and exposed to Kodak film at −80°C for 2 weeks.

Amino acid analysis of methylated hnRNP A1. Recombinant hnRNP A1 was methylated with SAM in the assay described above. Methylated product was separated from the enzyme by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell NC 21640). The membrane was then stained with 1% Coomassie blue in water, and the portion of the membrane containing hnRNP A1 was excised. Amino acid analysis of the membrane-bound A1 was performed by the Molecular Cell Biology Core Facility at the Children's Hospital of Pennsylvania, using an automated Applied Biosystems model 420A/H amino acid analyzer equipped with a C_{18} column (5 μm, 220 by 2.1 mm) for amino acid analysis. Samples were eluted from the column at a flow rate of 300 μl/min, and 75-μl fractions were collected. The radioactivity in 2 μl of each fraction was counted. Standard amino acids including N^G-monomethylarginine, N^G,N^G-dimethylarginine, and N^G,N'^G-dimethylarginine were also applied to the same column under exactly the same conditions. The elution profile of these standards was compared with the elution profile of radiolabeled sample. There was a lag time of 0.5 min between the time the fractions were collected by the fraction collector at the end of the analyzer and the time shown on the chromatograph.

RESULTS

Methylation of hnRNPs in vivo. Early studies on partially purified hnRNP particles had shown that hnRNP complexes contain a high level of the unusual modified amino acid N^G,N^G-dimethylarginine (3). Only a few individual modified hnRNPs, however, were identified (3, 71). With the ability to rapidly immunopurify hnRNP complexes, we sought to identify the individual hnRNPs that can be methylated in vivo. To do so, we modified the in vivo methylation assay developed by Desrosiers and Tanguay (15) in their study of histone methylation. The assay is based on the fact that the methyl group on the methyl donor *S*-adenosylmethionine is derived from free methionine in the cell. HeLa cells were labeled with L-[*methyl*-^{3}H]methionine in the presence of protein synthesis inhibitors. hnRNP complexes were then immunopurified from nucleo-

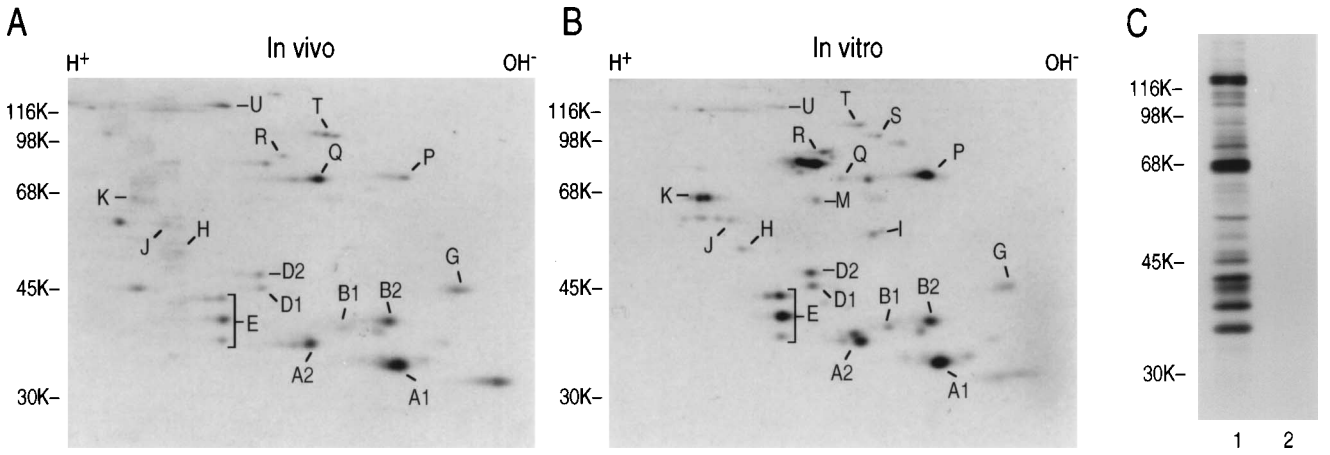

FIG. 1. In vivo and in vitro methylation of the hnRNP complex. (A) In vivo methylation of hnRNPs with L-[*methyl*-^{3}H]methionine in the presence of protein synthesis inhibitors cycloheximide and chloramphenicol. A total of 5,000 cpm was loaded onto the gel; the exposure time was 2 weeks. (B) In vitro methylation of hnRNPs, using the purest enzyme fractions. A total of 15,000 cpm was loaded onto the gel, and the exposure time was 2 days. (C) In vivo control for protein synthesis inhibition. Lane 1, hnRNP complex labeled with [^{35}S]methionine without the proteins synthesis inhibitors cycloheximide and chloramphenicol (loaded with 1.0×10^6 precipitable cpm); lane 2, the same complex incubated with [^{35}S]methionine and the two protein synthesis inhibitors. The same amount of protein was loaded in lane 1 and lane 2. This gel was exposed overnight.

plasm by using monoclonal antibody 4F4 against the hnRNP C proteins (16, 51), and the complexes were resolved by two-dimensional gel electrophoresis. Methylated proteins were then detected by fluorography. Interestingly, we found that most hnRNPs are methylated in vivo under these conditions (Fig. 1A). In addition to the previously described methylation of hnRNP A and B (3, 71), other hnRNP proteins, including D, E, G, H, J, K, P, Q, R, and U, are also methylated. The labeling is due to posttranslational methylation, not a low level of translational incorporation of L-[*methyl*-^{3}H]methionine, for the following reasons. First, as shown in Fig. 1C, coincubation of HeLa cells with protein synthesis inhibitors and L-[^{35}S]methionine followed by immunopurification of hnRNP complexes revealed that virtually no label is incorporated under these conditions. This finding indicates that the protein synthesis inhibitors are functioning properly. Second, even after very long exposure (1 month) of the gel shown in Fig. 1A, we could not detect any labeling of the highly abundant hnRNP C proteins. Lastly, and importantly, when we included a methyltransferase inhibitor, adenosine 2′,3′-dialdehyde or 5′-deoxy-5′-methylthioadenosine, in the in vivo methylation reaction, we did not see any labeling of the hnRNP complexes at all (data not shown). This evidence strongly argues against the possibility of a low-level translational incorporation. Interestingly, this experiment also shows that methylation does not have to be cotranslational. The prevalence of this modification further stimulated our interest in studying the hnRNP-specific methyltransferase and the effect of methylation on the function of hnRNPs.

The enzyme activity that methylates hnRNP A1 is enriched in the cytoplasmic fraction. All protein arginine methyltransferases studied to date are thought to be cytoplasmic enzymes (28). By contrast, hnRNPs reside predominantly in the nucleus (17). To provide some indication of the subcellular localization of the hnRNP methyltransferase in order to understand its potential functions and to determine a suitable source for purification, cytoplasmic and nucleoplasmic fractions were prepared from HeLa cells and assayed for hnRNP A1 methyltransferase activity. Recombinant hnRNP A1 was incubated with SAM and an equal amount of HeLa cytoplasmic or nucleoplasmic fraction. The labeling of hnRNP A1 was detected by SDS-PAGE and fluorography. The majority of the activity was found in the cytoplasmic fraction (Fig. 2, lane 1), suggesting that the enzyme is predominantly cytoplasmic. We therefore used HeLa cytoplasmic extract to further purify the activity.

Partial purification of hnRNP A1 arginine methyltransferase. The hnRNP A1 arginine methyltransferase was partially purified from HeLa cell cytoplasmic extract by six steps comprising ammonium sulfate precipitation, batchwise chromatography on SP-Sepharose and DEAE-Sephadex, and gra-

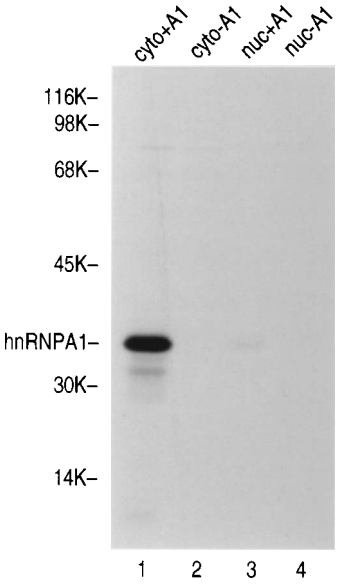

FIG. 2. Localization of the protein arginine methyltransferase in HeLa cells. HeLa cells were fractionated as described in Materials and Methods. Lane 1, 50 μg of protein from the cytoplasmic fraction was incubated with SAM and 0.4 μg of hnRNP A1 under standard assay conditions; lane 2, the same amount of cytoplasmic fraction was incubated with SAM but without A1; lane 3, the same amount of protein from the HeLa nuclear fraction was incubated with SAM and hnRNP A1; lane 4, as lane 3 but without hnRNP A1.

TABLE 1. Purification of arginine methyltransferase specific for RNA-binding proteins

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)[a]	Yield (%)	Purification (fold)
Cytoplasmic extract	797	36,100	45.1	100	1.0
$(NH_4)_2SO_4$ precipitation	385	32,500	84.4	90	1.9
SP-Sepharose Fast Flow	112	25,900	231.2	72	5.1
DEAE-Sephacel	30	19,500	650.0	54	14.4
Q-Sepharose Hiload	8.6	16,700	1,940	46	43.1
Phenyl-Sepharose	1.68	4,000	2,380	11	52.8
DEAE-TSK	0.11	2,700	24,500	7.5	550

[a] One unit is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of methyl group into hnRNP A1 per unit of time (25 min).

dient chromatography on Q-Sepharose, phenyl-Sepharose, and DEAE-TSK columns (Table 1). The overall purification was 600-fold, assuming 90% recovery of activity at the initial step. After this step, numerous attempts including gel filtration, affinity chromatography, SAM labeling, and other strategies have failed to purify further or identify the specific bands that represent the enzyme activity. The purest fraction that we obtained from these purifications contains about eight protein bands as determined by SDS-PAGE and silver staining, and the two most prominent bands have molecular masses of 100 and 45 kDa (data not shown). However, from these experiments, we learned that the native molecular mass of the activity determined by Superose-6 chromatography (Pharmacia) is 450 kDa (data not shown), indicating that it is probably a multimer of several subunits. It is not an abundant enzyme in HeLa cells, and its activity is relatively unstable when partially purified. Figure 3 shows the protein composition by SDS-PAGE after the various purification steps and the corresponding activity. We pooled the fractions containing the highest activity, fractions 29 and 30 from the DEAE-TSK column, and used them for further analysis of the biochemical properties of the enzyme.

Properties of the hnRNP arginine methyltransferase. The partially purified hnRNP arginine methyltransferase has apparent K_m values of about 2.5×10^{-6} M for hnRNP A1 and 5.8×10^{-6} M for SAM. The enzyme in the crude fraction was found to be quite stable up to 55°C under the experimental conditions used and has an optimum pH range of 7 to 7.4. It was inhibited by Zn^{2+} and Cu^{2+}, while Mg^{2+}, Ca^{2+}, and Mn^{2+} did not affect its activity. The activity was inhibited by sinefungin, adenosine 2′,3′-dialdehyde, and 5′-deoxy-5′-methylthioadenosine, known inhibitors of protein methyltransferases that use SAM as the methyl donor (reference 35 and data not shown).

Substrate specificity. To determine whether the enzyme has activity toward hnRNPs other than A1 in vitro, hnRNP complexes were purified by immunoprecipitation with monoclonal antibody 4F4 from HeLa cells as described in Materials and Methods. The complexes bound to protein-A Sepharose beads were then incubated with the purest enzyme fraction together with SAM under the standard assay conditions. The proteins were then resolved by two-dimensional gel electrophoresis, and the labeling was detected by fluorography. Under these conditions, most of the hnRNPs were methylated by the enzyme activity that we have partially purified (Fig. 1B). The most prominent ones are the A, B, D, and E proteins. Others, including G, H, I, J, K, M, P, Q, R, S, T, and U proteins, are

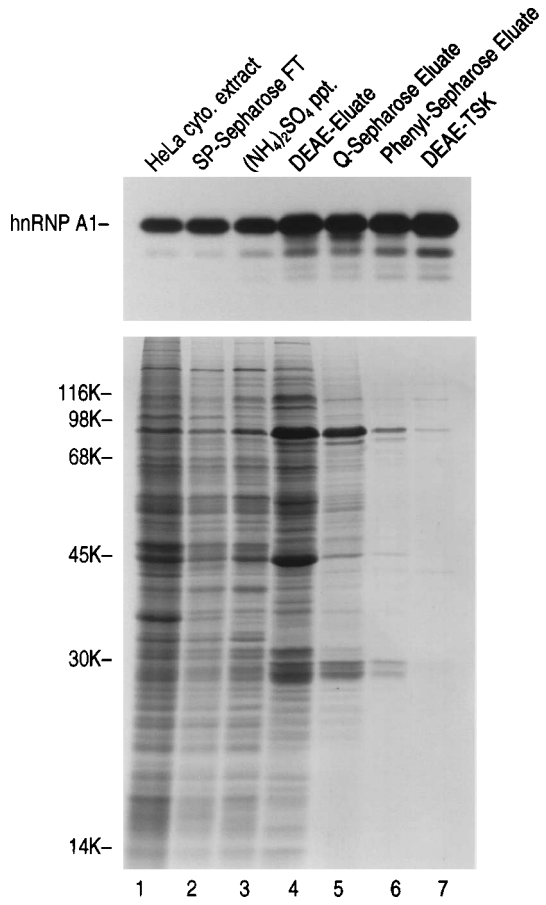

FIG. 3. Purification of the protein arginine methyltransferase from HeLa cytoplasmic extract. The top panel shows the activity in each step; the bottom panel shows the protein composition in each step by Coomassie blue staining. cyto., cytoplasmic; FT, flowthrough; ppt., precipitate.

methylated to a lesser extent. When the hnRNP complex was incubated with SAM alone, no labeling was detected (data not shown), indicating that no endogenous methyltransferase activity copurifies with the complex itself. Comparison of Fig. 1A and B indicates that the methylation patterns in vivo and in vitro are very similar, suggesting that the enzyme activity that we have partially purified is responsible for the in vivo methylation of hnRNPs.

To test the activity of the enzyme toward substrates other than hnRNPs, we incubated a variety of proteins known to be methylated in vivo with the partially purified enzyme. As shown in Fig. 4, recombinant human hnRNP A1, recombinant *Xenopus laevis* hnRNP K (42, 62), and recombinant bovine single-stranded DNA-binding protein UP2 (43) were significantly methylated. Histone H4 was only slightly methylated (Fig. 4; see Discussion). However, recombinant human hnRNP C and MBP were not methylated at all (Fig. 4). We believe that the MBP that we used can be methylated in vitro, since it was purified from the same source (bovine brain) as used by Ghosh et al. (20). This indicates that the activity that we have partially purified is an enzyme different from the previously characterized protein arginine methyltransferase specific for MBP (20).

When the sequences of various substrates were compared, we found that they all contain an RGG box RNA-binding motif, which is found in many RNA-binding proteins (8, 27). To examine if this domain alone can serve as a substrate for the

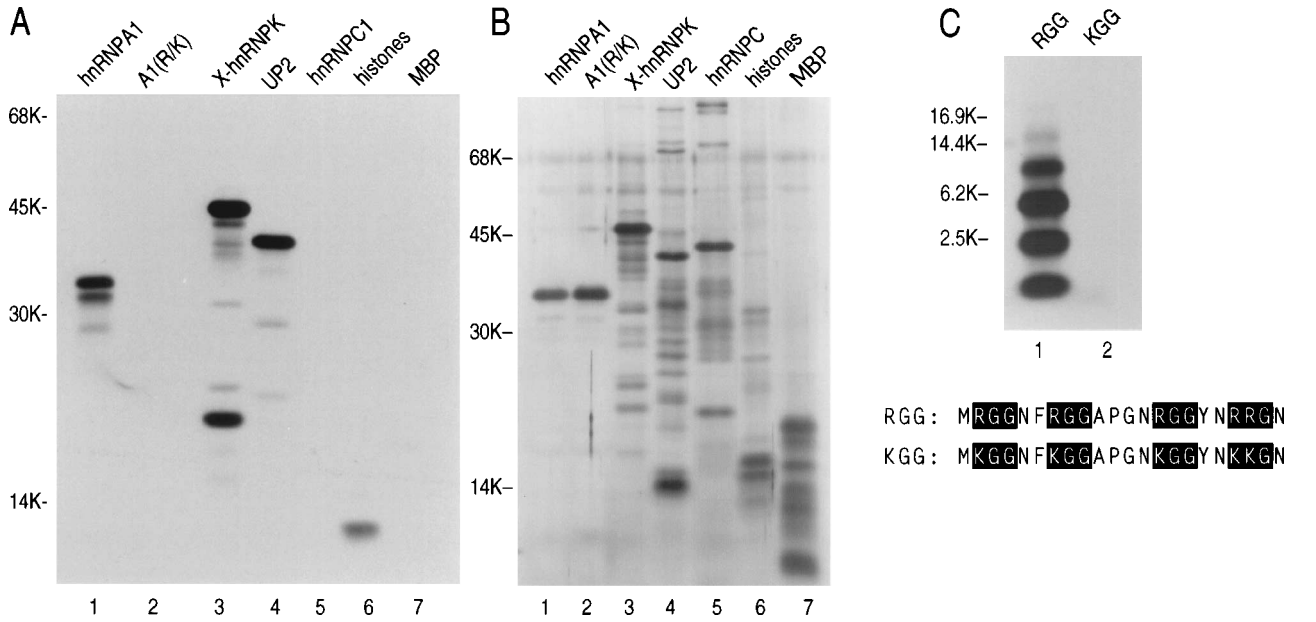

FIG. 4. Substrate specificity of the enzyme. (A) Methylation of different proteins, using the most purified enzyme fractions. Lane 1, recombinant hnRNP A1 (0.4 μg) methylation; lane 2, mutant A1 (0.4 μg) in which all arginine residues were changed to lysines in the RGG box region (see Materials and Methods); lane 3, recombinant *Xenopus* hnRNP K (0.4 μg); lane 4, recombinant maltose-UP2 fusion protein (0.4 μg); lane 5, recombinant human hnRNP C (0.4 μg); lane 6, histones (0.4 μg) from calf thymus (Sigma); lane 7, MBP (0.4 μg) from bovine brain (Sigma). The multiple bands are caused by protein degradation. (B) Silver staining of the different substrate proteins used in the assay. The proteins in the lanes are the same as in panel A. (C) Lane 1, methylation of the RGG peptide (MRGGNFRG GAPGNRGGYNRRGN) derived from human hnRNP U; lane 2, methylation of the KGG peptide. Both peptides were purified by high-pressure liquid chromatography and eluted from the C_{18} column as a single peak. However, both peptides appeared as four bands and ran aberrantly on tricine SDS-PAGE. The reason is currently unknown.

enzyme, we synthesized a small peptide whose sequence was derived from the RGG box of hnRNP U (RGG peptide, from amino acids 695 to 716; Fig. 4C). This peptide comprises the primary RNA-binding domain of hnRNP U (27). By incubation with the enzyme, we found that this peptide was an excellent substrate. To confirm that the enzyme is specific for arginine residues, we synthesized a mutant KGG peptide and also an hnRNP A1 mutant produced in *E. coli* in which all six arginines in the RGG domain were changed to lysines (see Materials and Methods). Neither of these peptides was methylated by the enzyme (Fig. 4A and C, lanes 2), thus confirming that the enzyme is specific for arginine residues.

To confirm that the in vitro enzyme products are the same as that in vivo, we carried out amino acid analysis on hnRNP A1 methylated in vitro, using SAM as a methyl donor. The results showed that the *methyl*-^{3}H-labeled amino acids elute from the amino acid analyzer in two peaks (Fig. 5). The first one elutes at 4.5 min, and the second elutes at 5.0 min. When standard methylated arginine derivatives N^G-monomethylarginine, N^G,N^G-dimethylarginine, and N^G,N'^G-dimethylarginine were used, they were found to elute at 4.5, 5.1, and 5.3 min, respectively (adjusted with the lag time between the fraction collector and the chromatograph). We therefore conclude that the first peak corresponds to N^G-monomethylarginine and the second peak corresponds to N^G,N^G-dimethylarginine (Fig. 5). Boffa et al. (5) reported that in vivo, hnRNPs contain N^G,N^G-dimethylarginine and a trace amount of N^G-monomethylarginine. By contrast, our result shows that there is slightly more N^G-monomethylarginine than N^G,N^G-dimethylarginine in the in vitro-methylated A1. This is likely to be due to a lower efficiency of the enzyme in vitro such that most of the methylation product is the intermediate N^G-monomethylarginine (28).

In conclusion, the activity described here appears to be responsible for methylating hnRNPs in vivo.

The RGG domain-specific arginine methyltransferase is present in divergent organisms. To determine if an A1 arginine methyltransferase is found in organisms other than humans, we tested rabbit reticulocyte lysate, *Drosophila melanogaster* Schneider cell extract, *Saccharomyces cerevisiae* extract, and *E. coli* extract for the ability to methylate the human hnRNP A1. Rabbit and *D. melanogaster* extracts contain such an activity, whereas *S. cerevisiae* and *E. coli* extracts do not (Fig. 6). Among *D. melanogaster* hnRNPs, hrp40, a major hnRNP in this organism, contains an RGG box (40, 41). This protein serves as an excellent substrate for the enzyme activity both in *D. melanogaster* extract and in the purified HeLa fractions (data not shown). It is somewhat surprising that no methyltransferase activity could be detected in the *S. cerevisiae* extract under the assay conditions, since there are several *S. cerevisiae* RNA-binding proteins that contain the RGG motif (27). Interestingly, *Schizosaccharomyces pombe* does have an enzyme activity that can methylate the hnRNP U RGG peptide but not hnRNP A1 (data not shown). The evolutionary conservation of the enzyme activity and the difference between the two yeast species *S. cerevisiae* and *S. pombe* are not understood (see Discussion).

DISCUSSION

hnRNPs are among the most abundant cellular targets for N^G,N^G-dimethylarginine modification, and it is likely that this modification has an important functional role in RNA metabolism. To better understand the function of hnRNPs and the role of dimethylarginine, we examined which hnRNPs can be posttranslationally methylated in vivo in HeLa cells, and we

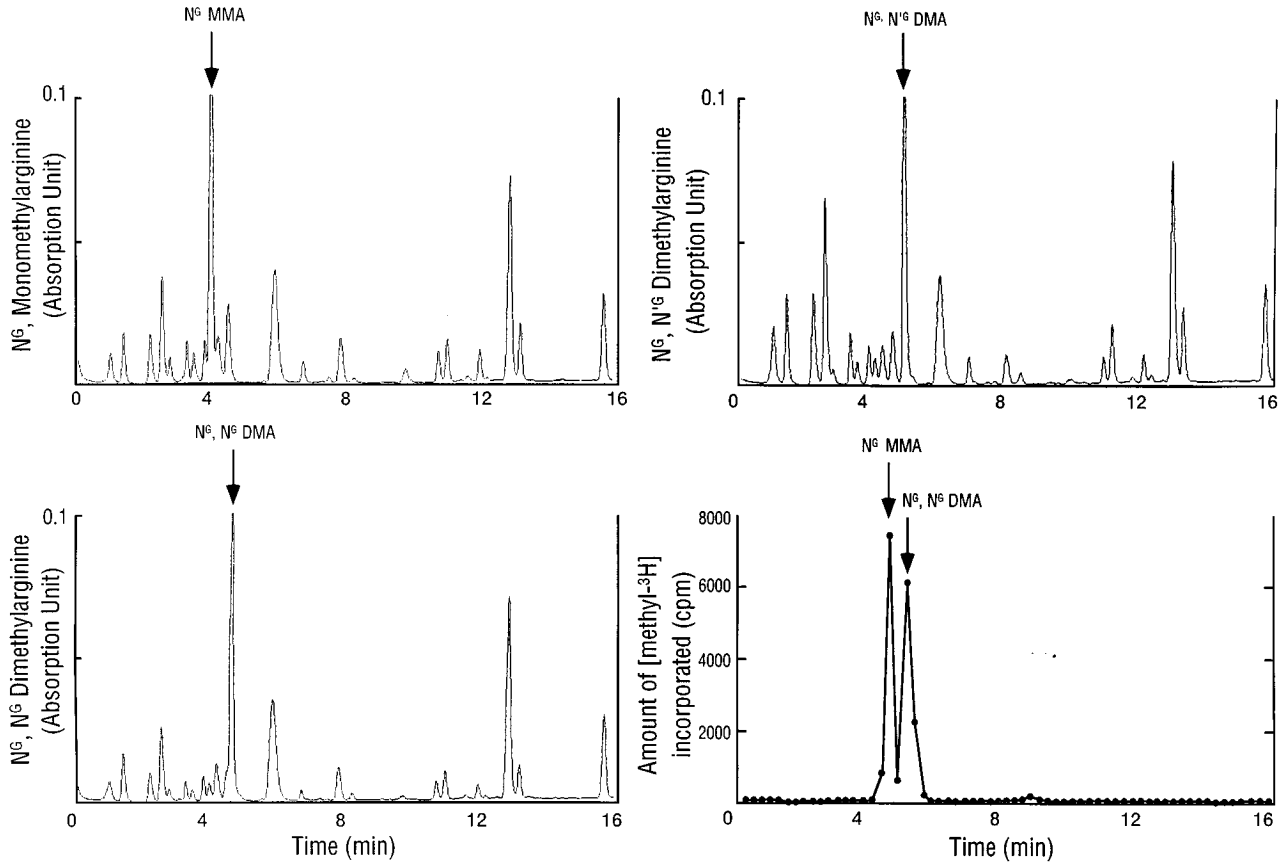

FIG. 5. Amino acid analysis of in vitro-methylated A1. The elution profiles of standard arginine methylation derivatives N^G-monomethylarginine (N^G MMA), N^G,N^G-dimethylarginine (N^G,N^G DMA), and N^G,N'^G-dimethylarginine (N^G,N'^G DMA) are shown. N^G-monomethylarginine peaks at 4.0 min, N^G,N^G-dimethylarginine peaks at 4.6, and N^G,N'^G-dimethylarginine peaks at 4.8. When the lag time between the fraction collector and the chromatograph is included, these times are 4.5, 5.1, and 5.3, respectively. The elution profile of radiolabeled hnRNP A1 is also shown. The two peaks of activity correspond to 4.5 and 5.0 min.

then undertook the purification and characterization of an enzymatic activity which can carry out this modification. As the enzyme that we partially purified methylates the same subset of hnRNPs in vitro as is methylated in vivo, it seems likely that this activity is responsible for the methylation of hnRNPs in vivo.

Using cellular fractionation, we have shown that this protein arginine methyltransferase is enriched in the cytoplasm, like other protein arginine methyltransferases studied to date (28). The in vivo methylation experiments showed that the majority of the hnRNPs are methylated. Importantly, the same proteins are also methylated in vitro by the partially purified methyltransferase. The hnRNP A, B, D, E group proteins are methylated more extensively than the others both in vivo and in vitro. This could simply be a reflection of the relative abundance of modification sites or of differences in the efficiency of methylation. However, it is difficult to ascertain that the in vivo data reflect the accurate stoichiometry of methylation on different proteins, since this assay was done in the presence of protein synthesis inhibitors. This may account for the discrepancy between our data and those of Wilk et al. (70). For example, they showed that the ratio of dimethylarginine in A1 to that of A2 is about 10, while our results showed that this ratio is at the most 5. This discrepancy and the varying degree of methylation on different proteins suggests that the extent of methylation could be regulated in the cell and that this regulation may be one way of controlling protein function. The in vivo methylation experiments also indicate that methylation can be posttranslational and that there are both methylated and unmethylated proteins at any one time in the cell. If the enzyme is indeed cytosolic, this would mean that these proteins are methylated completely either before they are imported into the nucleus or when they reenter the cytoplasm after nuclear export. It is interesting that hnRNP A1 has been found to shuttle between the nucleus and the cytoplasm (52), a property which may be shared by many other proteins. That the shuttling hnRNPs may be selectively methylated while the proteins are in the cytoplasm may have important implications for a role of methylation in the regulation of subcellular localization of pre-mRNA-binding proteins. As an interesting example along this line, Park et al. showed that methylation of cytochrome *c* promotes the import of this protein into mitochondria (49).

Our in vitro data show that the substrates of the enzyme comprise not only pre-mRNA-binding proteins (hnRNPs) but possibly other RNA-binding proteins which contain an RGG box. This domain is made up of repeated Gly-Gly dipeptides interspersed with arginines and aromatic residues. The RGG domain is present in proteins involved in diverse aspects of RNA metabolism (4, 8, 21, 27). It has been suggested to be involved in both protein-RNA and protein-protein interaction (12, 14, 27, 46). In hnRNP U, the RGG box is the primary RNA-binding domain, and this domain alone as part of a fusion protein is sufficient for RNA binding (27). In the case of hnRNP A1, its glycine-rich domain which contains the RGG

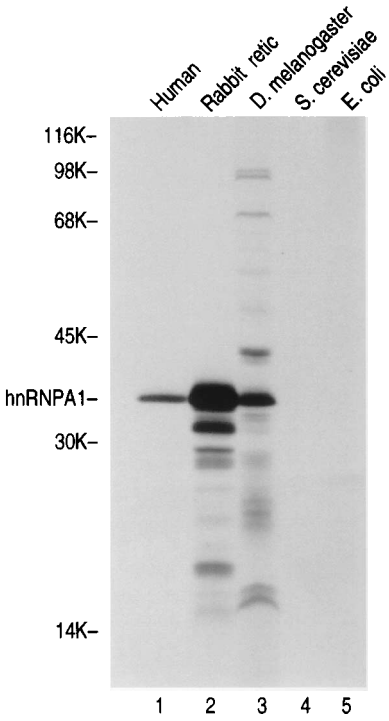

FIG. 6. The protein arginine methyltransferase is present in several organisms. HeLa total extract (lane 1), rabbit reticulocyte lysate (lane 2), Schneider cell extract (lane 3), *S. cerevisiae* extract (lane 4), and *E. coli* extract (lane 5) were assayed for activity toward 0.4 μg of human hnRNP A1 under standard conditions.

motif is capable of promoting RNA annealing (29, 45, 55). It is also responsible for RNA-binding cooperativity, which seems to involve both protein-protein and protein-RNA interactions (29). Here we have shown that both hnRNP U and A1 are methylated in vivo and in vitro in their RGG box regions. In hnRNP A1, this region contains the known methylated residue 194 (30, 71). Since on average there are three dimethylarginines per A1 molecule (3), our result with the R/K mutant hnRNP A1 shows that the two other dimethylarginines are located in the region from amino acids 195 to 234.

Other proteins that are known to contain N^G,N^G-dimethylarginine at the RGG box region are nucleolin (7, 37), fibrillarin (38), and ribosomal protein S2 (65). The RGG box region in nucleolin contains up to eight dimethylarginine residues (32, 33, 37). We predict that the enzyme that we partially purified can also methylate nucleolin in vitro because in their recent study on protein methylation, Najbauer et al. (47) used two small peptides containing two to three RGGF repeats derived from the methylated regions of fibrillarin and nucleolin and showed that these peptides can inhibit methylation of other endogenous proteins in PC12 cells. Most of these endogenous proteins are hnRNPs (47). This observation, along with our study, strongly suggests that the enzyme that methylates fibrillarin and nucleolin is the same as that which methylates hnRNPs. Interestingly, FMR1, the protein implicated in fragile X syndrome, also contains an RGG box (63). The recombinant form of this protein can also be methylated by our partially purified enzyme (data not shown). In conclusion, the enzyme activity that we have partially purified probably methylates not only hnRNPs but also other RNA-binding proteins that have an RGG box. This implies that all hnRNPs that can be methylated by this enzyme probably contain an RGG domain.

Another interesting finding is that the enzymatic activity that we characterized does not methylate MBP but does methylate histone H4 to a small degree. This histone methylation activity copurifies with the hnRNP A1 methylation activity during every step of purification. However, since previous studies of histone methylation have been unable to detect any methylarginine in histones from a variety of plant or animal sources (18), we suspect that the methylation of histone H4 under our assay conditions may be an in vitro artifact. Indeed, when the amino acid sequence of histones was examined, we found out that in histone H4 there is a region (^{3}GRGKGGKG^{10}) which bears some resemblance to an RGG domain. While this report was in preparation, two reports on this subject appeared (57, 59). Rajpurohit et al. (57) recently reported that the histone arginine methyltransferase that was partially purified by Ghosh et al. (20) from calf brain is more active toward hnRNP A1 than histones in vitro and that hnRNP A1 is more likely to be an in vivo substrate for the enzyme than histones. This nuclear protein/histone methyltransferase has a molecular mass of 275 kDa, with two nonidentical subunits of 110 and 75 kDa, and has a K_m for hnRNP A1 of 0.19 μM. Rawal et al. (59) showed that they have purified the same enzyme from rat liver and that it has a molecular mass of 450 kDa, possibly consisting of four identical-size subunits of 110 kDa. This enzyme has a K_m for hnRNP A1 of 0.54 μM. However, the latter enzyme can also methylate MBP and histones to a small extent. Both enzyme activities are inhibited by glycerol. The enzyme that we have partially purified has a molecular mass of 450 kDa, and the K_m for hnRNP A1 is 2.5 μM. It methylates histones to a small degree but is inactive toward MBP. It does not seem to be inhibited by glycerol. From these data, we conclude that all three enzymes are very similar and could be species- or tissue-specific isozymes. Further studies will be required to clarify these points.

The function of the enzyme is currently under study. Its importance is suggested by the prevalence of the modification and that the activity exists in organisms from lower eukaryotes such as *Physarum polycephalum* (11, 12) to mammals. Our study shows that there is an activity in *S. pombe* that can methylate the RGG peptide, but no such activity is detectable in *S. cerevisiae*. Several RNA-binding proteins in *S. cerevisiae* such as SSB-1 (24), NAB2 (1), GAR1 (21), and NOP1 (22), have been shown to contain RGG box motifs. We do not know whether the assay conditions or the substrates that we used are suitable for *S. cerevisiae* extract. However, so far, there is no evidence in the literature that any *S. cerevisiae* proteins contain dimethylarginine residues in vivo. Interestingly, it is known that RNA processing is more complex and extensive in *S. pombe* than in *S. cerevisiae*. For instance, *S. pombe* cells can correctly splice a simian virus 40 T antigen pre-mRNA, whereas *S. cerevisiae* cells cannot (26). Therefore, it could be that methylation of these proteins is a reflection of more complex RNA processing events.

The effect of methylation on protein function remains to be determined. As the substrate domain is involved in a diverse aspects of protein-RNA metabolism (4), one would expect its modification to affect its function in various different ways. Structure studies suggest that this domain in nucleolin forms a helical β spiral (19). Methylation of the arginine residues would not alter the positive charge of the side chain, but it would increase the steric constrains between the side chain and the RNA phosphate backbone and bases. This would potentially influence its binding to RNA. As an example of this, in the Tat protein of human immunodeficiency virus, one arginine residue within the basic region of the protein mediates specific binding of TAR RNA (66). On the other hand, meth-

ylation could affect protein-protein interactions, as in the case of MBPs, in which case it has been speculated that methylation helps to stabilize the insertion of MBPs into the myelin sheath (6, 9). Recently, there is increasing evidence that hnRNPs can interact with a variety of cellular factors besides binding to pre-mRNA (reference 69 and data not shown). Both hnRNP K and p62 bind to the Src SH3 domain through their proline-rich peptide motifs (69). In hnRNP K, this motif region can be methylated in vitro by using the partially purified enzyme (data not shown). p62, a protein which can bind to $p21^{ras}$ GTPase-activity protein, is known to contain N^G,N^G-dimethylarginine (72). Taken together, these findings suggest that methylation could play a role in regulating these protein-protein interactions. Finally, since it was observed that the methylarginine-containing peptide bond is partially resistant to trypsin digestion (6), the methylation of these proteins could regulate their stability and half-lives.

In conclusion, we have shown that most hnRNPs are methylated on arginine residues in vivo, and we have partially purified the protein arginine methyltransferase that is responsible for this methylation. This methyltransferase seems to be specific for the RGG box RNA-binding domain, and it is conserved among evolutionarily divergent organisms. Further studies on the arginine methyltransferase should reveal the function of this modification and its role in the function of RNA-binding proteins.

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