Characterization and Partial Purification of mRNA N⁶-Adenosine Methyltransferase from HeLa Cell Nuclei

INTERNAL mRNA METHYLATION REQUIRES A MULTISUBUNIT COMPLEX*

(Received for publication, February 3, 1994, and in revised form, April 25, 1994)

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N⁶-Methyladenosine is found at internal positions of mRNA in higher eukaryotes. This post-transcriptional modification occurs at a frequency of one to three methylations/average mRNA molecule in mammalian cell lines and is sequence-specific. A highly conserved consensus recognition site for the methyltransferase has been determined from both viral and cellular messages, consisting of the sequence Pur(G/A)AC(U/A) (with A being methylated). Despite the ubiquity and the specificity of this modification, little is known about the mechanism of formation of N⁶-methyladenosine.

Utilizing an in vitro methylation system from HeLa cell nuclear extracts, and a substrate RNA derived from the mRNA coding for bovine prolactin, the mRNA N⁶-adenosine methyltransferase has been characterized and partially purified. Unique among other characterized nucleic acid methyltransferases, the enzyme is composed of three components which are separable under non-denaturing conditions. The molecular masses of the components are 30, 200, and 875 kDa as determined by gel filtration and glycerol gradient sedimentation. The 200-kDa component appears to contain the S-adenosylmethionine-binding site on a 70-kDa subunit. The 875-kDa component has affinity for single-stranded DNA-agarose, suggesting that it may contain the mRNA-binding site. N⁶-Adenosine methyltransferase is not sensitive to treatment with micrococcal nuclease, nor to immunodepletion using an anti-trimethylguanosine antibody, suggesting that it does not contain an essential RNA component.

* This work was supported by National Institutes of Health Grant CA31810 from the National Cancer Institute (to F. M. R.). 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.

‡ Supported by a Howard Hughes Medical Institute Physician Research Fellowship.

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‡ The abbreviations used are: mA, N⁶-methyladenosine; bPRL, bovine prolactin; SV40, simian virus 40; RSV, Rous sarcoma virus; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; snRNA, small nuclear RNA.

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it change the cytoplasmic half-life of cellular mRNA (31). Carrol et al. (24) showed that treatment of cells expressing a bPRL minigene construct with the methylation inhibitor neplanocin caused accumulation of bPRL pre-mRNA in the nucleus, although steady-state cytoplasmic levels of bPRL mRNA were unaffected. Similar results have been found with B77 avian sarcoma virus and SV40 RNA, suggesting that m5A may affect the rate of pre-mRNA splicing or transport of mRNA from the nucleus to cytoplasm (29, 32). The interpretation of these studies is limited, however, because such inhibitors affect multiple cellular processes requiring methylation and are not specific for m5A.

An alternative approach was undertaken by Kane et al. (33) and Cegpany et al. (34), by studying point mutations of several methylation sites in the RSV src mRNA. Mutation of four of the major m5A sites did not affect methylation at remaining sites and did not have any detectable effect on levels of mature src mRNA relative to pre-mRNA in total cellular RNA. No difference was detected in the rate of transport of mRNA to the cytoplasm. The lack of effect of these mutations may indicate that the remaining sites of methylation may be sufficient to preserve the function of m5A.

To address the mechanism of m5A formation and its potential role in mRNA processing, we set out to isolate and characterize the enzymatic machinery required for mRNA formation. We report that mRNA N6-adenosine methyltransferase (m6A-MT) from HeLa cell nuclei is different from previously described methyltransferases in that it is composed of three readily dissociable components (hereafter designated MT-A1, -A2, and -B) which are required for efficient methylation. The substrate RNA sequence specificity of the purified enzyme complex is similar to the sequence specificity for methylation observed in vivo. The methyltransferase does not appear to contain a nucleic acid component which is required for activity. The purification and characterization of this novel enzyme complex will facilitate future studies aimed at discovering the role of m5A in mRNA metabolism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Nuclear Extracts**—HeLa S3 cells were grown in suspension to a density of 5 x 10^6 cells/ml in Dulbecco's modified Eagle's medium (Whitaker Bioproducts Inc.) containing 10 nm penicillin-streptomycin, 10 nm 1-glutamine, 20 nm HEPES, pH 7.6, and 5% horse serum (Life Technologies, Inc.). Nuclear extract was prepared from 6-liter cultures (3 x 10^6 cells) by the method of Dignam et al. (35), with the following exception: after the nuclei were lysed in Dignam buffer C and centrifuged, the supernatant was dialyzed against buffer A containing 50 nm Tris-HCl, pH 8.0, 10% glycerol, 1.5 mm MgCl2, 0.5 mm EDTA (buffer A), with 100 nm (NH4)2SO4. The extract was stored at -80 °C. N5-Adenosine methyltransferase activity remained stable for at least 6 months.

**Plasmid Construction and RNA Synthesis**—Plasmid pBR600 was generated by subcloning a 62-nucleotide sequence of bPRL containing the major m5A methylation site into the vector pBSM13+ using the polymerase chain reaction (Perkin-Elmer Cetus) as previously described (26). Substrate RNA for the methylation assay (Fig. 1A) was generated using T7 polymerase (Stratagene) as recommended by the manufacturer and was labeled using [35S]S-RNA, and 0–15 of nuclear extract or partially purified fractions in a total volume of 40 ml. Salt concentration varied from 30 to 150 mm KCl or NaCl depending on the salt concentration of the fraction being assayed. Reaction mixtures were incubated at 30 °C for 30 min, then 55 °C for 5 min, 0.4 ml of 2 x loading buffer (20 mm Tris-HCl, pH 7.6, 1 M LiCl, 1 mm EDTA, 1% SDS, and 50 μl of glycerolate (10 mg/ml) in 1 x loading buffer were added, and the mixtures were incubated at room temperature with shaking for 30 min. Reaction mixtures were transferred to a 96-well filtration plate attached to a vacuum apparatus (Millipore). Each sample was washed sequentially with 2.4 ml aliquots of 1 x oligo(dT) loading buffer containing 0.5%, 0.1%, and no SDS. Finally, RNA was eluted with 300 ml of dH2O into a 96-well collection plate. Samples were transferred to scintillation vials, 10 ml of Ecoscint (DuPont-NEN) was added, and samples were counted in a Beckman LS1801 counter in window settings 0–400 (H channel) and 400–1000 (S channel). H radioactivity and S radioactivity were corrected for 35S spill into the H channel. One unit of activity is defined as the amount of enzyme necessary to transfer 1 fmol of methyl/pmol RNA under standard assay conditions.

**Column Chromatography**—All chromatographic steps were performed using an FPLC system (Pharmacia LKB Biotechnology Inc.) at 4 °C. All chromatography resins were purchased from Pharmacia unless otherwise noted. Nuclear extract was loaded onto a DEAE-Sepharose CL-6B column (1.6 x 10 cm, 10 mg protein/ml resin) which had been equilibrated in buffer A containing 150 mm NaCl and eluted with buffer A containing 150 mm NaCl. The concentrated fraction was washed with 2–3 column volumes of equilibration buffer at a rate of 1 ml/min. Eluant with an A260 of greater than 0.1 was collected (fraction DS-A, 30 ml, Fig. 1B). The column was then eluted with buffer A containing 500 mm NaCl. Eluant with an A260 of greater than 0.1 was collected, yielding a 20-ml fraction (designated DS-B).

**Fraction DS-A** was dialyzed for 4 h against 2 liters of buffer A containing 150 mm KCl, with one buffer change. The dialyzed fraction was centrifuged at 15,000 x g for 30 min. The supernatant was loaded onto a Q-Sepharose column (10 x 50 mm) which had been equilibrated with buffer A containing 150 mm KCl. The column was washed with 2–3 column volumes of loading buffer at a rate of 1 ml/min and the flow through fraction (fraction QS-A1, 35 ml) was collected. Fraction QS-B (8 ml) was eluted with buffer A containing 300 mm KCl.

MT-A1 was further purified as follows. Fraction QS-A1 was dialyzed against buffer B (20 mm HEPES, pH 7.6, 10% glycerol, 1.5 mm MgCl2, 0.5 mm EDTA) containing 250 mm NaCl. It was then loaded at a rate of 1 ml/min onto an SP-Sepharose column (1.6 x 5 cm) which had been equilibrated with buffer B containing 150 mm NaCl. The column was washed with 2–3 column volumes of loading buffer at a rate of 1 ml/min. The supernatant was then eluted at a flow rate of 0.15 ml/min, and fractions of 2 ml were collected. Methyltransferase assays were performed using 5-μl aliquots of each fraction supplemented with 5 μl of QS-A2 and 5 μl of QS-B (see below) and the conditions described above. Fractions determined to contain MT-A1 were pooled (fraction Sup6-A1).

MT-A2 was further purified as follows. Fraction QS-A2 was dialyzed against buffer B containing 100 mm NaCl and then loaded onto a Mono S HR 5/5 column (0.5 x 10 cm) containing 150 mm NaCl, 1 mm EDTA, 0.5 mm MgCl2, and 10% glycerol, equilibrated with buffer B containing 150 mm NaCl. The concentrated fraction was loaded onto a Superose 6 Prep Grade gel filtration column (1.6 x 60 cm) which had been equilibrated with buffer B containing 200 mm KCl. The column was eluted in the same buffer at a flow rate of 0.15 ml/min, and fractions of 2 ml were collected. Methyltransferase assays were performed using 5-μl aliquots of each fraction supplemented with 5 μl of QS-A2 and 5 μl of QS-B and assayed. The fractions determined to contain MT-A2 were pooled (fraction Sup6-A2).

MT-B was further purified as follows. Fraction DS-B was dialyzed against buffer A containing 200 mm KCl and then loaded onto a Q-Sepharose column (1 x 5 cm) which had been equilibrated in the same buffer. The column was then sequentially eluted with buffer A containing 350 mm KCl, 550 mm KCl, and 1 x KCl at 1 ml/min. Assays of each of the eluted fractions were performed by supplementing the reactions with 5 μl of QS-A2 and 2 μl of QS-B. MT-B eluted in the 550 mm step (fraction QS-B, 8 ml). Fraction QS-B was diluted 1:2 with buffer B and concentrated to 1 ml by binding to a Mono Q HR 5/5 column and eluting with buffer B containing 1 x NaCl. The concentrated fraction was loaded onto a Superose 6 Prep Grade gel filtration column (1.6 x 60 cm) which had been equilibrated with buffer B containing 200 mm KCl. The column was eluted in the same buffer at a flow rate of 0.15 ml/min, and fractions of 2 ml were collected. Methyltransferase assays were performed using 5-μl aliquots of each fraction supplemented with 5 μl of QS-A2 and 5 μl of QS-B and assayed. The fractions determined to contain MT-B were pooled (fraction Sup6-B).

**RNA Gradient Fractionation**—Linear gradients (11 ml) from 0 to 400 mm NaCl containing 0.5%, 0.1%, and no SDS were prepared in buffer A containing 200 mm KCl. Protein samples in a volume of 1 ml were layered on top. Gradients were centrifuged in a Beckman SW41 rotor at 4 °C for 24 h at 35,000
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The sequence AGACU (where the minor methylation site is shown). This coincides with the major site of methylation in bPRL mRNA. The sequence specificity of in vitro methylation of bPRL mRNA was determined using a series of DNA and RNA methyltransferase assays.

RESULTS

It has been previously shown that HeLa cell nuclear extract contains an enzymatic activity that is capable of catalyzing the formation of m\textsuperscript{6}A in vitro using a synthetic RNA substrate. The sequence specificity of this activity, when using a synthetic bPRL mRNA sequence as substrate, is similar to that observed in methylated bPRL mRNA isolated from bovine pituitary. We have developed a rapid and sensitive assay that measures the transfer of [\textsuperscript{3}H]methyl groups from [\textsuperscript{3}H]AdoMet and aliquots of Sup6-A, -A2, and -B using the standard assay conditions described above; 10 \mu g of RNA was added, and the sample was ethanol precipitated and resuspended in \textit{dH}_{2}O. An aliquot of the recovered RNA was removed for scintillation counting. The remainder was digested to completion with 10 \mu g of ribonuclease P1 (Calbiochem) in 5 mM sodium acetate, pH 6.0, in a final volume of 45 \mu l from 37 °C. The nucleotides were then treated with calf intestinal phosphatase (50 units, Boehringer Mannheim) overnight. The reaction was then dried under vacuum and resuspended in 20 \mu l of \textit{dH}_{2}O. HPLC was performed using a Brownlee anion-exchange guard column connected in series to a C18 reversed-phase column (JSM Instruments Inc.) as described previously (31). Fractions were collected directly into scintillation vials and counted. The [\textsuperscript{3}H]nucleosides were cocromatographed with unlabeled nucleoside and cap standards whose elution was monitored at 20°.

Ribo-nuclease T1 Mapping of Methylated Nucleosides—Unlabeled RNA substrate was methylated with [\textsuperscript{3}H]AdoMet and aliquots of Sup6-A, -A2, and -B using the standard assay conditions. The five-nucleotide consensus is shown in larger type. Double asterisk indicates the minor UC site.

To better understand the molecular mechanism of m\textsuperscript{6}A formation in mRNA, we have undertaken the purification of m\textsuperscript{6}A-MT. The purification scheme is summarized in Fig. 1B. HeLa cell nuclear extracts were fractionated by FPLC chromatography using non-denaturing conditions. Initial attempts to purify the enzyme using anion-exchange or gel filtration chromatography were unsuccessful due to dramatic losses of activity which occurred during a single fractionation step. The most striking property of m\textsuperscript{6}A-MT is the finding that enzymatic activity can be reconstituted by mixing together column fractions that had little activity when assayed alone. Crude nuclear extract was loaded onto a DEAE- Sepharose column equilibrated with 100 mM \(\text{NH}_4\text{SO}_4\) and the bound protein was eluted with 500 mM \(\text{NH}_4\text{SO}_4\). Neither the flow-through fraction (DS-A) nor the fraction that binds to the column (DS-B) contained substantial methyltransferase activity by itself; however, when aliquots of DS-A and DS-B were mixed, activity was quantitatively recovered (Fig. 1C). The methyltransferase component(s) that does not bind to the column is designated MT-A, and the bound component is designated MT-B.
In an effort to copurify these multiple components as a complex, crude nuclear extracts were dialyzed against buffer containing salt concentrations as low as 50 mM KCl and were then fractionated by Superose 12 chromatography or by glycerol gradient centrifugation. Even under these low salt, non-denaturing conditions the majority of the methyltransferase activity could not be maintained without reconstitution by mixing high and low molecular weight fractions (data not shown). The small amount of activity that could be detected without reconstitution most likely represented the overlap of the MT-A and MT-B fractions because this activity migrated at a position between the MT-A and MT-B activities, and not as a complex with a molecular weight greater than the largest of the individual factors. This result suggests that even in unfractonated extract m^A-MT exists as multiple, separate components. Therefore, the initial fractionation steps chosen represent conditions that most effectively separate each of the components. Assays of each fraction for activity throughout subsequent steps of purification required reconstitution by supplementing with the other components. Quantitation of activity of each of the components was estimated by assaying limiting amounts of individual fractions in the presence of excess amounts of partially purified fractions of the other required components. Because the stoichiometry of the complex is not known, the resulting quantitation can only be used as a crude estimate for the purpose of tracking activity. Therefore, the true recovery of enzyme, and the degree of purification reported represent estimates.

MT-B was further purified by Q-Sepharose (anion-exchange) chromatography, eluting in the 350-550 mM KCl gradient step (fraction QS-B). Assays for MT-B activity were performed by supplementing the reaction mixtures with equal amounts of fraction DS-A. Fraction DS-A was further fractionated by Q-Sepharose chromatography as described under "Experimental Procedures." The flow-through fraction (QS-A1) was not sufficient to complement fraction QS-B. The fraction that eluted in the 150-300 mM KCl gradient step (fraction QS-A2) contained an activity that was sufficient to complement QS-B (Fig. 1C), but quantitation of this activity revealed that the recovery of component A activity was very low (typically about 10-15%). When reactions containing aliquots of both QS-A1 and QS-A2 along with fraction QS-B were analyzed, recovery of methyltransferase activity dramatically improved (90-100%). These experiments suggest that at least three separate components (MT-A1, -A2, and -B) are all required for m^A-MT activity. This result alone does not prove that MT-A1, -A2, and -B differ from each other, but further purification of these fractions reveals strikingly different physical properties for each of the three components indicating that this is in fact the case (see below).

MT-A1, which is found in the flow-through fraction of both the DEAE-Sepharose and the Q-Sepharose ion-exchange steps, was further purified by fractionation on a cation exchange resin, SP-Sepharose, and by gel filtration on a Superose 6 Prep Grade column (Fig. 1B). The activity profile of the gel filtration fractions is shown in Fig. 2. Aliquots of each fraction were assayed in the presence of the same excess amount of QS-A2 and QS-B. MT-A1 elutes with an apparent molecular mass of 30 kDa. Purification of MT-A1 was estimated to be 16.7-fold based upon enrichment of specific activity, with a recovery of activity of 12% (Table I).

MT-A2 was further purified by Mono S chromatography and gel filtration (Fig. 1B). Assays of the Mono S fractions without supplemental QS-B did not have detectable activity (not shown). When the fractions were assayed with supplemental QS-B, but no QS-A1, a small peak of activity was reproducibly detected (Fig. 3A). When assays contained both supplemental QS-B and QS-A1, a substantially larger peak of activity was detected (Fig. 3A). Fractions containing MT-A2 activity were pooled and further fractionated on Superose 6 Prep Grade (Fig. 3B). The apparent molecular mass of MT-A2 as measured by gel filtration using Superose 6 Prep Grade resin is 200 kDa. The overall purification of MT-A2 is approximately 200-fold, and the recovery is 13% (Table I). QS-A2 was also fractionated on glycerol gradients (not shown); however, the activity migrated with the bulk of the protein and no significant purification was achieved with this step. The sedimentation coefficient estimated (9 S) is in approximate agreement with the molecular weight estimated by gel filtration alone.

MT-B was further purified using gel filtration on Superose 6 and affinity chromatography on single-stranded DNA agarose (Fig. 1B). All assays of MT-B activity were performed by supplementing the reactions with aliquots of QS-A1 and -A2 in quantities which were in excess. In the absence of MT-A1 and -A2, all fractions of MT-B had no detectable methyltransferase activity. These chromatographic steps resulted in approximately 1360-fold purification of MT-B, with 26% recovery (Table I). The apparent molecular weight of MT-B as estimated by gel filtration is approximately 1 x 10^6, and the Stokes radius was estimated.
collected. A represents activity measured when assays of fractions were gradient of 0.1
QS-M was loaded onto the Mono S and loaded onto the gel filtration column as described under “Experimental Procedures.” Buffer, elution, and molecular weight standards are as in Fig. 2. All fractions were assayed with supplemen-
tation of equal excess amounts of QS-B. B, chromatographic profile of MT-A2 on Superose 6 column. Fraction MS-A2 was concen-
trated to be 118 kDa is estimated to be 200 kDa.

Due to the complexity of m5A-MT, and to the large size of the components, micrococcal nuclease digestion of crude nuclear extract was performed to determine whether a nucleic acid component was necessary for activity. Extract was incubated with nuclease using conditions previously shown to inactivate snRNA-containing splicing factors (37). No loss of activity was observed (data not shown). This finding is in agreement with similar results reported by Harper et al. (28). We have also shown that no loss of activity occurred when nuclear extract was immunodepleted of snRNA using an anti-trimethylguanosine antibody under conditions in which >85% of snRNA was removed (data not shown). However, this does not rule out the possibility that a nucleic acid cofactor which is both protected from nuclease digestion and does not contain an exposed trimethyl-G cap is required for methyltransferase activity.

UV cross-linking of [methyl-3H]AdoMet to partially purified fractions was employed to determine which of the three components contains the active site for AdoMet binding. Aliquots of
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A. Units of Activity

B. "-" - "'

C.

Fractions at various stages of purification were incubated with [methyl-3H]AdoMet under buffer and salt conditions similar to those employed for the methylation assay. Reactions were incubated at room temperature under UV light as described, and the products were then analyzed by separation on a 10% SDS-polyacrylamide gel and fluorography. In the nuclear extract and in the early purification steps of all three fractions, multiple proteins were labeled (data not shown). An aliquot of MS-A2 and aliquots of all the Superose 6 A2 fractions were assayed by UV cross-linking in an effort to determine if the elution of MT-A2 activity coincided with the elution of any of the AdoMet-binding proteins. A major [methyl-3H]AdoMet-cross-linked band and several less intense bands were observed in MS-A2 (Fig. 5A). Analysis of the Superose 6 A2 fractions revealed that a protein with a molecular mass of 70 kDa that cross-linked to AdoMet was reproducibly present in fractions 34–36 (Fig. 5A), correlating with the elution of MT-A2 activity in the Superose fractions (compare with Fig. 3B). Other cross-linked proteins seen in MS-A2 were resolved from the MT-A2 activity by Superose 6 chromatography, and therefore are unlikely to be components of m6A-MT. Specifically, the most intense cross-linked band seen in MS-A2 increases in intensity in fractions 37 and 38, while MT-A2 activity is absent by fraction 38. The [methyl-3H]AdoMet labeling of the 70 kDa band is dependent on UV irradiation (data not shown). Competition experiments using a 10-fold molar excess of either D- or L-AdoHcy revealed a selectivity for the L-stereoisomer (Fig. 5B), indicating that the interaction is stereoselective, as would be expected for an enzyme-substrate interaction (38). Interestingly, both D- and L-AdoHcy appear to compete for binding somewhat more efficiently than would be expected by mass action kinetics alone. Because the detection of these weak signals required a 1-month exposure of the fluor-impregnated gel, the differences in band intensity may not represent linearly related differences in actual complex. Therefore, the qualitative differences imply that a stereospecific interaction exists, but cannot be accurately quantitated using this technique. Similar experiments performed with Sup6-A1 fractions and Sup6-B fractions did not identify cross-linked bands that copurified with enzymatic activity.

Aliquots of the most purified fractions of MT-A1, A2, and -B were assayed individually and in combination to determine which components are absolutely required for m6A formation. No significant activity was detected with any of the components individually or in pairs. However, substantial activity was seen when all three are present (Fig. 6A). This result suggests that all three components are absolutely required for efficient methylation.

Finally, it was important to verify that the RNA methyltransferase that has been purified is specific for catalyzing the formation of m6A and maintains the RNA sequence specificity observed in vivo and with unfractionated nuclear extract. Aliquots methylated with aliquots of fractions of Sup6-A1, -A2, and -B digested with ribonuclease P1, and then treated with calf intestinal phosphatase. The ribonucleosides were analyzed by HPLC as described under "Experimental Procedures." All detectable 3H comigrated with the m6A standard. C, cytosine; Cm-2'-O-methylcytosine; mC, N6-methylcytosine; U, uridine; Um, 2'-O-methyluridine; G, guanosine; Gm, 2'-O-methylguanosine; A, adenosine; Am, 2'-O-methyladenosine; mA, N6-methyladenosine; mAm, 2'-O-methyl-2'-O- methyladenosine; m2A, N5-dimethyladenosine; C, ribonuclease T1 analysis of pBRL60 mRNA: unmethylated [3P]GTP-labeled pBRL60 RNA (lane A) and [3H]methyl pBRL60 RNA (no 32P) (lane B) was digested with RNase T1, the oligonucleotides were separated by denaturing gel electrophoresis, transferred to Zetaprobe membrane, and fluorographed. The sizes of the oligonucleotides are shown on the left. The methylated 6-mer oligonucleotide migrates slightly slower than the unmethylated 6-mer oligonucleotide.
of the most purified fractions, Sup6-A1, Sup6-A2, and Sup6-B, were used to methylate the bPRL mRNA-derived substrate. For HPLC analysis, 3H-methylolated RNA was digested to completion with ribonuclease P1 and then treated with calf intestinal phosphatase. The ribonucleosides were then analyzed by HPLC on a C18 column as previously described (31). All detectable 3H radioactivity comigrated with the m6A standard (Fig. 6B), indicating that the three-component enzyme we have characterized fully maintains specificity for formation of m6A.

Unlabeled bPRL60 RNA substrate was also methylated with the purified components and [methyl-3H]AdoMet and was then digested with T1 nuclease. The digested RNA was analyzed by denaturing polyacrylamide gel electrophoresis, along with T1-digested (o-32P)RNA substrate run in a parallel lane to serve as size markers. 3H radioactivity was detected only in the unique six-nucleotide T1 fragment (Fig. 6C). The only adenosine residue in this oligonucleotide corresponds to the major methylation site observed in bPRL mRNA in vivo. No detectable methylation occurred at any of the other adenosine residues found within the bPRL sequence portion of the substrate, nor in the 50-adenosine residue poly(A) tail.

DISCUSSION

Although it has been more than 20 years since internal methylation of mRNA was first described (39), both the mechanism of formation and the biological function of m6A remain obscure. This is the first report that provides a physical description of mRNA-(N6-adenosine-methyltransferase), and the complexity of the enzyme is somewhat surprising. Using a synthetic RNA substrate which contains the sequence of the major methylation site described for bPRL mRNA (27), three independent enzyme components have been partially purified from HeLa cell nuclear extracts. We have yet to determine whether the presence of one or both substrates promotes complex assembly. The most purified fractions of these three components in concert specifically methylate only the N6-amino group of adenosine and also retain the sequence specificity observed for the formation of m6A in mRNA in vivo.

The largest methyltransferase component, MT-B, has an apparent molecular mass of 875 kDa. It does not copurify with an AdoMet binding activity but does have affinity for single-stranded DNA-agarose, a common property of RNA-binding proteins (40). To date, RNA cross-linking studies have been unsuccessful due to the inability to demonstrate sequence-specific UV-RNA cross-linking with proteins in the fractions containing MT-B. Due to the large size of MT-B, and the likelihood that at least one component of the methyltransferase recognizes specific RNA sequences, we considered the possibility that MT-B may be a small nuclear ribonucleoprotein. Extensive micrococcal nuclease digestion of HeLa nuclear extract and immunoprecipitation with anti-trimethylguanosine antibodies both failed to have any effect on methyltransferase activity. Although these experiments do not rule out the possibility that an RNA cofactor is present in MT-B, it is unlikely that an snRNA is required for methyltransferase activity.

MT-A2 has a molecular mass of 200 kDa and is also absolutely required for activity. The results of UV cross-linking experiments with [methyl-3H]AdoMet reveal that MT-A2 copurifies with a 70-kDa protein that binds AdoMet, and AdoMet binding can be inhibited by AdoHcy with stereospecificity. While this result does not prove that the 70-kDa protein in Superox fractions 54–56 is in fact the AdoMet-binding component of m6A-MT, the enzymatic activity and the binding activity are highly correlative. Coupled with the finding that the MT-A1 and -B activities do not copurify with a protein that cross-links to AdoMet under conditions suitable for m6A-MT activity, this finding strongly suggests that MT-A2 contains the enzyme active site for AdoMet binding and that MT-A2 is a multimeric protein containing a 70-kDa subunit. MT-A1 is the smallest of the three components with an approximate molecular mass of 30 kDa. Its role in the enzyme complex is unknown.

Our findings are very different from, but not entirely inconsistent with those recently reported by Tuck (41). He reported partial purification of two independent m6A-MT activities from HeLa cell nuclear extract using DEAE-cellulose. The major activity (fraction A) was dependent on the presence of a cap1 5' terminus. A minor peak of activity (fraction B) methylated uncapped- and cap1-dihydrofolate reductase substrates with similar efficiencies and also methylated cap1-bPRL substrate. Neither Fraction A nor B could be further purified without substantial loss of activity. Our finding that m6A-MT requires three dissociable components is consistent with the loss of activity observed by Tuck with multiple sequential chromatographic fractionations. However, in contrast to these findings, we do not detect differences in methylation efficiency between uncapped and cap1-containing bPRL mRNA sequences (data not shown). It remains to be determined whether there is any relationship between the two activities that Tuck has reported and the individual components which are described in this study.

In addition to mRNA, m6A is found in tRNA, rRNA, and snRNA (42–44). In contrast to mRNA, there does not appear to be any consensus sequence for methylation sites in these RNAs, although methylation remains highly specific for individual sites. The mechanism for this specificity remains unknown, as does the function of the base modifications in these RNA. There are no published reports of purification of m6A-MT specific for these other RNAs from eukaryotes. It will be interesting to determine whether the mRNA m6A-MT components which are characterized and partially purified are capable of recognizing other RNA substrates. One possibility is that the mRNA m6A-methyltransferase and other RNA m6A-methyltransferases share some, but not all, of their components. Conversely, the mRNA m6A-methyltransferase may have evolved in the presence of other transcriptional or mRNA processing components and maintains a dependence on these components.

The m6A-methyltransferase that methylates internal mRNA adenosine residues is very different from other RNA methyltransferases described previously. Nucleolar 2'-O-methyltransferase (45), N6-guanine methyltransferase (46), and N7-guanine tRNA methyltransferase (47) have been partially purified and characterized from eukaryotic cells. In each instance, the enzyme was purified as a single active component under native conditions, and their sizes ranged from 56 to 145 kDa.

We have partially purified and characterized the multicomponent enzyme system responsible for the formation of m6A residues at specific internal positions in eukaryotic mRNA. The overall chemical reaction catalyzed by this enzyme is basically quite simple. Characterization of the methyltransferase which catalyzes this reaction, however, raises several interesting questions. Why is this enzyme so complex, containing three separate components, two of which are very large? Could this activity in some way be coupled to other mRNA processing events which are equally complex and are influenced by additional interdependent components? It will be interesting to determine whether methylation is mechanistically linked with or utilizes factors required for other post-transcriptional events in mRNA metabolism such as splicing or transport. Furthermore, characterization and cloning of the genes that encode the individual subunits of this multicomponent enzyme will allow a better understanding of the underlying complexity of this enzymatic activity and the biological function of the post-transcriptional modification it produces.
REFERENCES