Biochem. J. (1992) 288, 233–240 (Printed in Great Britain)

Partial purification of a 6-methyladenine mRNA methyltransferase which modifies internal adenine residues

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Two forms of a 6-methyladenine mRNA methyltransferase have been partially purified using a T7 transcript coding for mouse dihydrofolate reductase as an RNA substrate. Both enzyme forms modify internal adenine residues within the RNA substrate. The enzymes were purified 357- and 37-fold respectively from nuclear salt extracts prepared from HeLa cells using DEAE-cellulose and phosphocellulose chromatography. The activity of the first form of the enzyme eluted from DEAE-cellulose (major form) was at least 3-fold greater than that of the second (minor form). H.p.l.c. analysis of the hydrolysed, methylated mRNA substrates demonstrated that both forms of the enzyme produced only 6-methyladenine. The two forms of the enzyme differed in their RNA substrate specificity as well as in the dependence for a 5′ cap structure. The 6-methyladenine mRNA methyltransferase activity was found to be elevated in HeLa nuclei as compared with nuclear extracts from rat kidney and brain. Enzymic activity could not be detected in nuclei from either normal rat liver or regenerating rat liver. In the case of the HeLa cell, activity could only be detected in nuclear extracts, with a small amount in the ribosomal fraction. Other HeLa subcellular fractions were void of activity.

INTRODUCTION

The formation of 6-methyladenine (m6A) residues in mRNA is a post-transcriptional reaction in which *S*-adenosyl-L-methionine (AdoMet) serves as the methyl donor. This reaction has been observed in the mRNA of higher eukaryotic cells (Adams & Cory, 1975; Desrosiers *et al.*, 1975; Dublin & Taylor, 1975; Furuichi *et al.*, 1975; Lavi & Shatkin, 1975; Perry *et al.*, 1975) as well as plant (Kennedy & Lane, 1979; Nichols, 1979; Haugland & Cline, 1990) and viral systems (Beemon & Keith, 1977; Moss *et al.*, 1977; Chen-Kiang *et al.*, 1979). Numerous studies have also demonstrated that the m6A residues occur in two conserved sequences within the transcripts, Am6AC or Gm6AC (Dimock & Stoltzfus, 1977; Wei *et al.*, 1976; Canaani *et al.*, 1979). More recent reports have significantly expanded the knowledge of the sequence specificity by demonstrating the sequence RGACU, in which R is usually a guanine residue, to be a major target in order for methylation to occur (Csepany *et al.*, 1990; Harper *et al.*, 1990). The formation of m6A has been shown to occur in the nucleus of the cell in the same population of transcripts which are polyadenylated (Lavi *et al.*, 1977). In most cases the residues also appear to be conserved during the processing of the heterogeneous nuclear RNA (hnRNA) (Lavi *et al.*, 1977), thus suggesting m6A formation precedes RNA splicing. A few reports (Aloni *et al.*, 1979; Canaani *et al.*, 1979; Carroll *et al.*, 1990), however, have demonstrated that methylated adenine residues are located, in certain cases, in the intron regions of hnRNA. One of the most puzzling aspects of m6A formation is the fact that in certain systems, methylation occurs in mRNA in a non-stoichiometric manner. In the case of prolactin mRNA, Narayan & Rottman (1988) have demonstrated that only 20% of the transcripts were methylated *in vivo* and only 5% were methylated *in vitro*.

The biological function of internal m6A residues in mRNA has remained a mystery for a number of years. While most of the investigations regarding the function of m6A have produced inconclusive results, internal methylation has been suggested to play a significant role in the processing and/or transport of mRNA. Inhibition of m6A formation in both avian sarcoma viral RNA (Stoltzfus & Dane, 1982) and SV40 RNA (Finkel & Groner, 1983) using the inhibitor of methylation *in vivo* (cycloleucine) caused a decrease in the production of spliced mRNA in both systems. The methylation inhibitor *S*-tubercidinylhomocysteine has also been used to investigate the biological significance of cellular mRNA methylation (Camper *et al.*, 1984). These experiments have demonstrated that inhibition of m6A formation causes a delay in the cytoplasmic appearance of polyadenylated mRNA in HeLa cells without having a significant effect on the half-life of the transcripts. While these experiments have yielded significant observations regarding the biological function of m6A residues, the fact that in all cases general methylation inhibitors were used does not rule out the possibility that other methylation reactions may also be involved in mRNA processing.

The enzymology of m6A formation has significantly lagged behind investigations regarding the biochemistry or biological function of this process. The major reason for this delay has been the lack of an adequate assay for the enzyme for use *in vitro*. Narayan & Rottman (1988) have developed an assay *in vitro*, in which the enzymology of m6A formation could finally be investigated. Using a 7-methylguanine-capped T7 transcript for prolactin RNA, it was found that only one adenine residue in this transcript was modified *in vitro*, which was at a site identical to the methylation site observed *in vivo*. A modification of this assay has since been used to methylate a T7 transcript coding for mouse dihydrofolate reductase (DHFR) (Rana & Tuck, 1990). This transcript was shown to be a 3-fold better substrate than prolactin mRNA for the enzyme, partly because of the fact that DHFR contains multiple methylation sites.

In this investigation, two forms of an m6A mRNA methyltransferase, which modify internal adenine residues, have been partially purified from HeLa nuclei using a capped T7 transcript coding for mouse DHFR as an RNA substrate. The two enzyme forms have been characterized with respect to RNA-substrate specificity as well as their requirement for a 7-methylguanine cap. These studies have also demonstrated m6A mRNA methyl-

Abbreviations used: DHFR, dihydrofolate reductase; m6A, 6-methyladenine; AdoMet, *S*-adenosyl-L-methionine; hnRNA, heterogeneous nuclear RNA; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; DEPC, diethylpyrocarbonate.

transferase activity to be found exclusively in the nucleus, and to be enriched in HeLa cells as compared with nuclear extracts from other tissues investigated.

MATERIALS AND METHODS

Materials

S-[*methyl*-^{3}H]Adenosyl-L-methionine (78 Ci/mmol) and [α-^{32}P]GTP (3000 Ci/mmol) were purchased from Dupont/NEN (Boston, MA, U.S.A.). All restriction enzymes were obtained from New England BioLabs (Beverly, MA, U.S.A.). T7 RNA polymerase and RNAsin were purchased from Promega Biotec (Madison, WI, U.S.A.). RNAase-free DNAase, RNAase, nuclease P1 and mussel glycogen were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Standard nucleosides for h.p.l.c. analysis, ATP, GTP, CTP, and UTP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ribonuclease T2 and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). m7G(5′)ppp(5′)Gm (cap 1 structure) was obtained from Pharmacia LKB (Piscataway, NJ, U.S.A.). DEAE-cellulose and phosphocellulose were purchased from Whatman Inc. (Clifton, NJ, U.S.A.). All tissue culture supplies including calf serum were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). The prolactin cDNA clone, PRL 72 FL, was kindly provided as a gift from Dr. Fritz Rottman, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH, U.S.A.

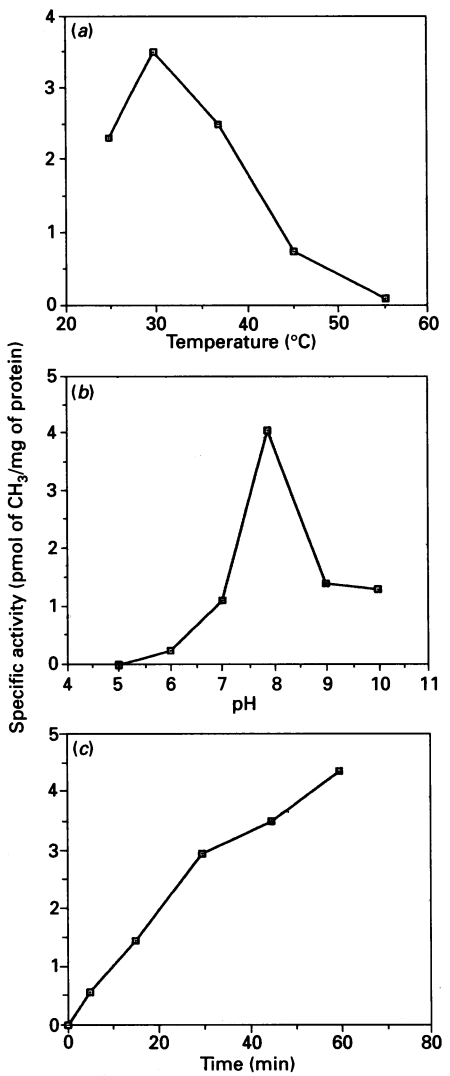

Fig. 1. Optimization of the m6A mRNA methyltransferase assay

An aliquot (35 μg) of fraction II was incubated in the methyltransferase assay described above in the presence of 2.5 μCi of AdoMet and 3.0 μg of a cap 1 DHFR T7 transcript. (*a*) Assay tubes were incubated for 30 min at the temperatures indicated. Specific activity values are expressed as pmol of CH_3 incorporated/30 min per mg of protein. (*b*) Assay tubes were incubated for 30 min at 30 °C in buffers of the different pH values reported. Specific activity values are expressed as described above. (*c*) Assay tubes were incubated at 30 °C for the different time periods described (assay buffer is as described in the Materials and methods section). Charted values are the average of triplicate assays.

Growth and maintenance of HeLa cells

HeLa cells were maintained in suspension using Joklik minimum essential medium containing 5% (v/v) calf serum and 10 mg of gentamicin/l. For enzyme purification, the cells were harvested at 2000 rev./min in a Sorvall HG4L rotor once the cells had reached a density of (4.0–6.0) $\times$ 10^5 cells/ml.

Subcellular fractionation of HeLa cell organelles

Nuclei from HeLa cells were isolated by the method described by Dignam *et al.* (1983). Crude ribosomes, mitochondria and a post-ribosomal supernatant were all isolated from the crude cytoplasmic supernatant of the lysed cells by a slight modification of the method described by Ensinger & Moss (1976). Briefly, the crude cytoplasmic supernatant from the lysed HeLa cells was further fractionated by centrifugation at 30000 ***g*** for 20 min to remove the mitochondria and lysosomes. This pellet was then resuspended in Buffer D [20 mM-Hepes, pH 7.9, 20% (v/v) glycerol, 0.2 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) and 0.5 mM-dithiothreitol (DTT)] and assayed for enzymic activity. A ribosomal fraction was then prepared by recentrifugation of the post-mitochondrial supernatant at 114000 ***g*** for 2 h. The supernatant of this step was labelled as the post-ribosomal supernatant and assayed. The pellet containing the ribosomes was resuspended and washed with KCl, again as described by Ensinger & Moss (1976). The final salt-washed ribosomal pellet was then resuspended in buffer D and assayed for enzymic activity.

Partial hepatectomies

Partial hepatectomies were performed on male Sprague–Dawley rats (weighing 200–250 g) following the procedure described by Higgins & Anderson (1931).

Transcription assays *in vitro*

Large-scale plasmid preparations were performed as described in the method of Krieg & Melton (1985). Linearized plasmid (10 μg) was incubated in a 100 μl transcription assay mixture *in vitro* as described (Rana & Tuck, 1990) except that the resulting RNA was precipitated with ethanol without the aid of a glycogen carrier. Uncapped transcripts were synthesized by omitting the cap structure and increasing the GTP concentration to 400 μM. T7 RNA transcripts were labelled with [^{32}P]GTP also as described (Rana & Tuck, 1990).

Enzymic assay

m6A mRNA methyltransferase activity was assayed as described (Narayan & Rottman, 1988; Rana & Tuck, 1990) with the modifications given below. Many of the additional modifications were made to make the assay less laborious for enzyme purification. Briefly, 3 μg of an RNA substrate was incubated

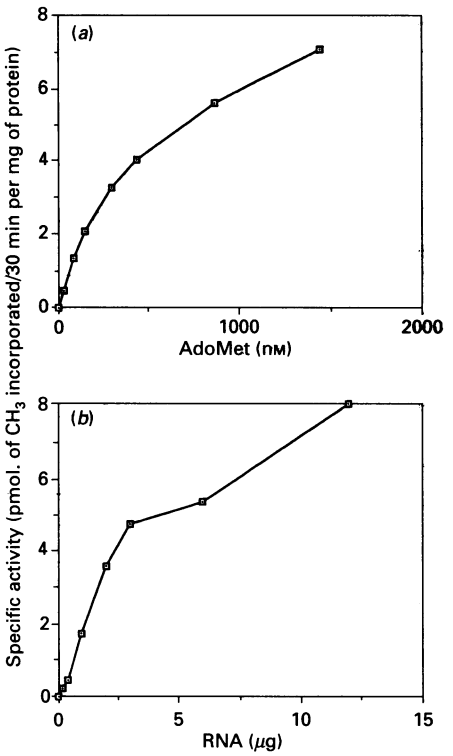

Fig. 2. Substrate versus velocity curves of the m6A mRNA methyltransferase assay

Fraction II (35 μg) was incubated in the methyltransferase assay. Optimal conditions of 30 °C and a pH of 7.9 were used in the assay. The assay tubes were incubated for 30 min. (*a*) Specific activity measurements are reported in the presence of increasing concentrations of radiolabelled AdoMet. The specific activity is again expressed as pmol of CH_3 incorporated/30 min per mg of protein. (*b*) Specific activity measurements are reported (as defined above) in the presence of increasing concentrations of a cap 1 DHFR T7 transcript. Charted values are the average of triplicate assays.

with 10–20 μl of enzyme preparation and 2.5 μCi of S-[*methyl*-^{3}H]adenosyl-L-methionine. The relationship between protein concentration and enzyme activity was determined at every step in the purification process (results not shown). Activity measurements reported all use enzyme concentrations which fall within the linear portion of this curve. Salt and buffer concentrations for the assay have been described (Rana & Tuck, 1990). The final volume of the assay was 50 μl and the mixtures were incubated for 30 min at 30 °C. As shown in Figs. 1(*a*) and 1(*b*), 30 °C and pH 7.9 were the optimum temperature and pH of the m6A mRNA methyltransferase. Assays were routinely performed for 30 min, which as shown in Fig. 1(*c*) was within the linear region of the time curve. The routine substrate concentrations of 660 nM for AdoMet and 3.0 μg/assay for RNA both fall within the mixed-order region of their respective velocity versus substrate concentration curves (Figs. 2*a* and 2*b* respectively). Ideally, the assays should be routinely performed in the zero-order range (saturating conditions) with respect to substrate concentration. However, to achieve these conditions the [^{3}H]AdoMet would have required significant dilution with non-radioactive AdoMet. Use of saturating AdoMet concentrations was initially attempted in these studies; however, the reduction in specific activity significantly reduced the limit of detection of the assay and hampered the purification process. The RNA concentration was limited because of the low levels of capped RNA that can be synthesized in the T7 transcription reaction *in vitro*.

The reactions were stopped by the addition of an equal volume of 100 mM-Tris (pH 7.6) containing 20 mM-EDTA, 20 mM-NaCl and 0.2% SDS. Diethylpyrocarbonate (DEPC)-treated water (75 μl) was also added to dilute further the polyvinylalcohol added to the reaction mixtures. The mixtures were then extracted once with an equal volume of phenol/chloroform (1:1, v/v). Aliquots (75 μl) of the aqueous layer were then spotted onto 2.4 cm diam. DE-81 filters. Each filter was then washed separately three times with 0.1 M-Na_2HPO_4 (pH 7.6), once with water, twice with ethanol/ether (1:1, v/v) and once with ether. Each wash used 10 ml of solution. The filters were then air-dried and the radioactivity counted in a scintillation counter in the presence of 6 ml of scintillation cocktail.

RNA digestion and h.p.l.c. analysis of methylated nucleosides

m6A mRNA methyltransferase assays were performed as described above except that the RNA contained in the aqueous layer of the phenol/chloroform extract was ethanol precipitated using 30 μg of glycogen as a carrier. The RNA pellets were then dissolved in 38 μl of 10 mM-sodium acetate and digested with ribonuclease T2, nuclease P1 and bacterial alkaline phosphatase by a procedure identical to that described by Alberts *et al.* (1981). The resulting mixtures of nucleosides were then analysed for m6A residues using h.p.l.c. by the method described by Backlund *et al.* (1986) employing an Altex ultrasphere octadecyl silica (ODS) column.

Enzyme purification

HeLa cells were grown in suspension and harvested as described above. The packed cells were then washed once with five × packed-cell vol. of phosphate-buffered saline. The cells were lysed, nuclei isolated and salt extracted by the procedure described by Dignam *et al.* (1983) with the following modification: the KCl was omitted in the final dialysis buffer (buffer D).

Nuclei from the different rat tissues analysed were isolated according to the method described by Duerre *et al.* (1977). Once isolated and washed, the nuclei were salt-extracted as described by Dignam *et al.* (1983) and the extracted proteins dialysed against buffer D as described above.

Nuclear extract was isolated from 4 l of HeLa cells at a time and frozen at −70 °C. Extracts from four preparations (16 l of cells) were then combined and used for the purification procedure described below. The dialysed extract was loaded on a DEAE-cellulose column (2.5 cm × 13 cm) which had been equilibrated with buffer D. After loading, the column was washed with buffer D and the bound proteins eluted with a 300 ml linear KCl gradient (0–0.5 M). Fractions (4.5 ml) were collected at a flow rate of 1.0 ml/min. Enzyme activity measurements were performed on 20 μl aliquots of the fractions using 3 μg of a cap 1 DHFR T7 transcript as an RNA substrate (prepared as described above). The two eluted enzyme peaks (fractions III-A and III-B, see Fig. 3) were pooled separately and assayed for enzyme activity and protein concentration to obtain specific activity measurements.

Fraction III-A was further purified by phosphocellulose chromatography. A column (2.5 cm × 10 cm) of phosphocellulose P11 was equilibrated with buffer D. Pooled fraction III-A, which had been dialysed for 4 h against buffer D, was loaded on to the column and the column washed with buffer D as described above for the DEAE-cellulose. The bound proteins were then eluted using a 240 ml linear gradient of $(NH_4)_2SO_4$ (0–0.6 M) in buffer D. The flow rate of the column was 1.0 ml/min. Fractions were collected and assayed as described above. The fractions containing the enzymic activity (see Fig. 4) were pooled and again assayed for specific activity.

Protein concentrations were determined using the Pierce BCA reagent with BSA as the standard. The enzyme activity is expressed as pmol of CH_3 incorporated/mg of protein.

RESULTS

Tissue specificity of the m6A mRNA methyltransferase

Nuclear salt extracts from five different cell types were assayed for m6A mRNA methyltransferase activity using a cap 1 DHFR T7 transcript (prepared as described above) as an RNA substrate (Table 1). It was found that only three of the cell types tested, cultured HeLa cells, rat brain and rat kidney, displayed detectable enzymic activity. Activity was not detectable in either rat liver or regenerating rat liver, suggesting that enzymic activity is not increased in rapidly dividing cells. It is interesting to note that HeLa cells appear to be greatly enriched in methyltransferase activity, as attested by the fact that these cells contained over 10-fold the enzymic activity of any other tissue analysed in these investigations.

Table 1. Tissue specificity of the m6A mRNA methyltransferase

One unit of methyltransferase activity = 1 pmol of CH_3 incorporated/30 min. Abbreviation: n.d., not detected (limit of detection was 0.02 units/mg of protein). Reported values represent the mean ± S.D. of triplicate assays from the same nuclear extract preparations. Endogenous enzymic activity for all tissue sources was determined by assaying the nuclear extract(s) in the absence of capped T7 DHFR transcripts. Reported values have been corrected for this activity.

Tissue	Specific activity (units/mg of protein)
HeLa cell	2.61 ± 0.07
Rat brain	0.07 ± 0.04
Rat kidney	0.20 ± 0.01
Rat liver	n.d.
Regenerating rat liver	n.d.

Table 2. Subcellular distribution of the m6A mRNA methyltransferase from HeLa cells

One unit of methyltransferase activity = 1 pmol of CH_3 incorporated/30 min. Abbreviation: n.d., not detected (limit of detection was 0.02 unit/mg of protein). Reported values represent the mean ± S.D. of triplicate assays from the same enzyme preparation. Values have also been corrected for endogenous enzymic activity by omitting the T7 DHFR transcript from the control assay reactions.

Fraction	Specific activity (units/mg of protein)
Nuclear	2.60 ± 0.07
Mitochondrial	0.12 ± 0.07
Ribosomal	0.32 ± 0.12
Post-ribosomal supernatant	n.d.

Subcellular distribution of the m6A mRNA methyltransferase activity from HeLa cells

Cultured HeLa cells were harvested by centrifugation. The plasma membranes were then disrupted and the cellular organelles isolated as described above. Enzymic activity associated with HeLa nuclear extract, mitochondria, ribosomes and the post-ribosomal supernatant was then quantified again using a cap 1 DHFR T7 transcript as an RNA substrate. Activity was found to be primarily associated with the nucleus of the cell, with a trace of activity localized to the mitochondria and ribosomes (Table 2). No activity was detectable in the post-ribosomal supernatant, suggesting that enzyme leakage from the nuclei during the isolation procedure occurred at a minimum.

Enzyme purification

Two forms of a m6A mRNA methyltransferase have been purified 357- and 37-fold respectively, with yields of 77% and 27% as shown in Table 3. The fact that the recovery of enzyme activity increased from 100 to 105% upon salt extraction of the nuclei indicates that the enzyme activity is present in the soluble portion of the nucleus or is weakly bound to chromatin. The presence of two enzyme forms was first detected using DEAE-cellulose chromatography in which the column was eluted with a linear KCl gradient (Fig. 3). Fig. 3 shows the chromatographic profile of protein eluted during the salt gradient, including the enzymic activity (d.p.m./assay) of these fractions. Protein fractions initially eluted with buffer D (the flow-through) were also assayed for m6A mRNA methyltransferase activity; however, these fractions were consistently devoid of enzymic activity (results not shown). As shown in the total activity column of Table 3, the major enzyme form, which eluted in low salt conditions, represented approx. 75% of the total m6A mRNA methyltransferase activity.

The fourth step utilized in the purification procedure employed a phosphocellulose column (Fig. 4). The two enzyme peaks from DEAE-cellulose were pooled separately, concentrated, dialysed and loaded on a phosphocellulose column. The column was then eluted with a linear $(NH_4)_2SO_4$ gradient as described above. This step increased the purification factor of the major enzyme form (fraction III-A, Table 3) by 3-fold (fraction IV-A, Table 3). Unfortunately, phosphocellulose chromatography of the fraction III-B peak resulted in significant losses of enzyme activity, with

Table 3. Purification of the m6A mRNA methyltransferase

One unit = 1 pmol of CH_3 incorporated/30 min.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (-fold)
I. Nuclei lysis	58	1028	925	0.9	100	1
II. Nuclear extract	32	190	969	5.1	105	5.7
III. DEAE-cellulose						
A	18.3	6.7	737	110	80	122
B	12.3	7.7	254	33	27.3	37
IV. Phosphocellulose						
A	19.5	2.2	713	324	76.9	357

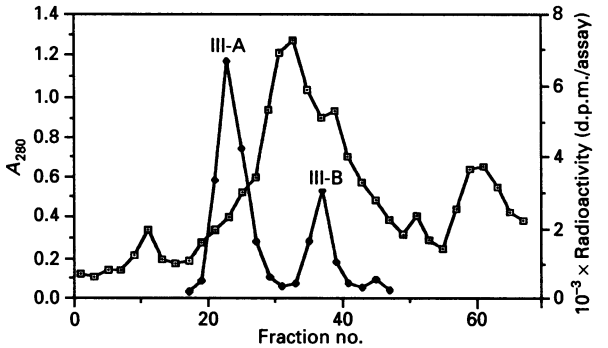

Fig. 3. DEAE-cellulose chromatography of fraction II

Fraction II (190 mg) was loaded on a DEAE-cellulose column (2.5 cm × 13 cm) and washed with buffer D. The column was then eluted using a linear KCl gradient as described under the Materials and methods section. Fractions (4.5 ml) (during gradient elution) were then collected at a flow rate of 60 ml/h. Aliquots (20 μl) from the fractions were then assayed as described above using 3 μg of a cap 1 T7 transcript coding for DHFR as an RNA substrate. Radioactivity (d.p.m.) incorporated in these assays (●) was charted along with the A_{280} profile (⊡). Details of the assay conditions have been described in the Materials and methods section.

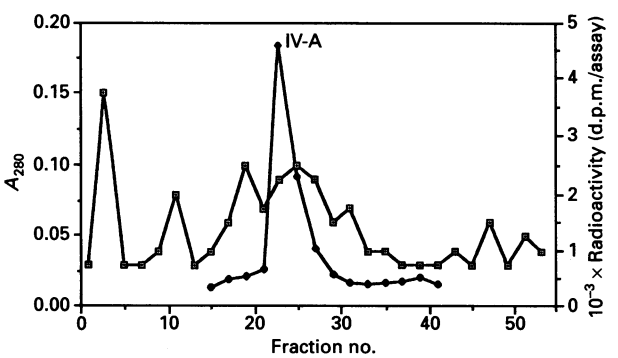

Fig. 4. Phosphocellulose chromatography of fraction III-A

Fraction III-A (2–3 mg) was loaded on a phosphocellulose column (2.5 cm × 10 cm) and again washed with buffer D. The column was then eluted with a linear ammonium sulphate gradient as described in the Materials and methods section. Fractions (4.5 ml) (during gradient elution) were collected at a flow rate of 60 ml/h. Aliquots (20 μl) were again assayed for enzymic activity under the conditions described in the Materials and methods section using 3 μg of a cap 1 DHFR T7 transcript as an RNA substrate. Radioactivity (d.p.m.) incorporated in these assays (●) was again charted along with the A_{280} profile (⊡).

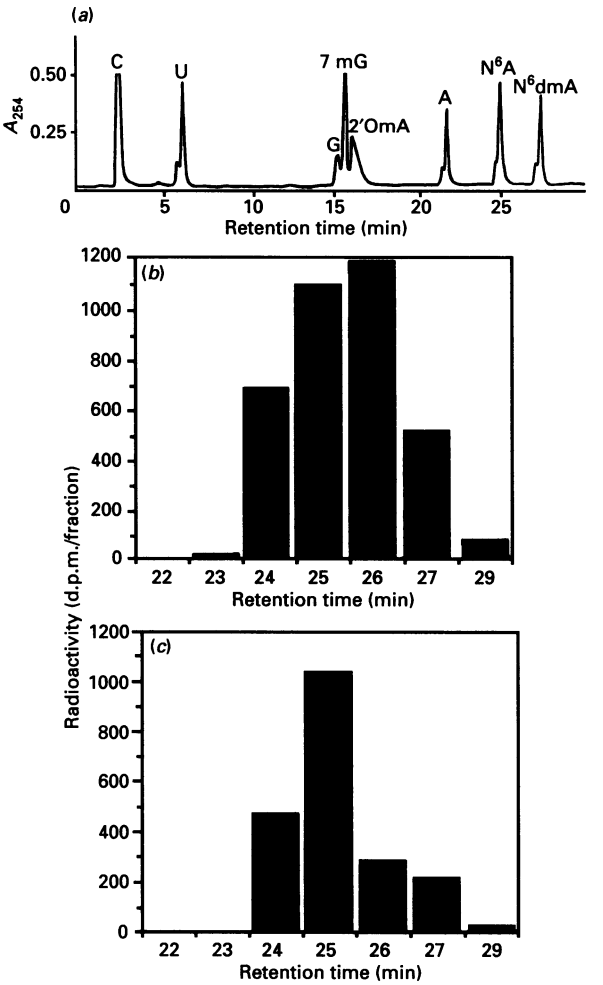

Fig. 5. H.p.l.c. analysis of the methylated nucleosides

DHFR T7 transcripts possessing 5′-cap 1 structures were methylated in separate reactions with enzyme fractions IV-A and III-B as described in the Materials and methods section. After enzymic hydrolysis, the ^{3}H-labelled nucleosides were chromatographed using h.p.l.c. Fractions (1 ml) were collected and counted for radioactivity using a liquid scintillation counter. (*a*) Absorbance profile of standard nucleosides from the system employed (C, cytosine; U, uridine; G, guanosine; 7mG, 7-methylguanosine; 2′OmA, 2′-*O*-methyladenosine; A, adenosine; N^6A, *N*-6-methyladenosine; N^6dmA, *N*-6-dimethyladenosine). (*b*) Radioactivity incorporated into the m6A peak from transcripts methylated with enzyme fraction IV-A. (*c*) Radioactivity incorporated into the m6A peak from transcripts methylated with enzyme fraction III-B. Results from (*b*) and (*c*) have been corrected for endogenous activity by assaying the enzyme fractions in the absence of added transcript.

no further increases in purity (results not shown). Further attempts at purifying both enzyme forms using gel filtration, affinity and hydroxyapatite chromatography have thus far met with limited success.

Product identification

Methylated nucleosides formed during the enzymic reaction were analysed using h.p.l.c. Cap 1 DHFR T7 transcripts methylated with ^{3}H-labelled AdoMet were enzymically hydrolysed using RNAase T2 and Nuclease P1. The resulting nucleosides were then chromatographed using the h.p.l.c. system described above. Fractions (1 ml) from the column were then counted for radioactivity in a scintillation counter. The chromatographic position of standard methylated nucleosides separated using this system is shown in Fig. 5(*a*). Radioactivity incorporated into the m6A peak using fractions IV-A and III-B as enzyme sources are also shown in Figs. 5(*b*) and 5(*c*), respectively. A control chromatograph was also performed in which the RNA substrate was omitted; the reported counts have therefore been corrected for this endogenous activity as well as AdoMet and possible AdoMet metabolites which may have carried through in the RNA isolation process. After this correction, the only peak of radioactivity observed using either enzyme fraction chromatographed in the region of m6A (Fig. 5). Figs. 5(*b*) and 5(*c*) therefore focus only on this region of the chromatograph. It should also be noted that m6A was the only radiolabelled

Table 4. RNA substrate specificity of the two m6A mRNA methyltransferase enzymes from HeLa cell nuclei

Reported values represent the mean ± S.D. of triplicate assays from the same HeLa nuclear extract preparation. Values have also been corrected for endogenous enzymic activity by omitting the T7 transcript from the control assay reactions.

	Specific activity (pmol of CH_3/mg of protein per pmol of RNA)	
Enzyme fraction... T7 transcript	IV-A	III-B
Cap 1 DHFR	4.06±0.27	0.41±0.01
Uncapped DHFR	0.09±0.05	0.35±0.002
Cap 1 prolactin	3.13±0.09	0.19±0.01
Cap 1 MS-7	0.52±0.05	0.05±0.001

Table 5. RNA recovery yields from the methyltransferase assays

Reported values represent the mean ± S.D. of triplicate assays using the same enzyme preparation (IV-A or III-B).

		Counts recovered (%)	
Enzyme fraction	DHFR transcript...	Capped	Uncapped
Control		100	100
IV-A		85±2.5	94±1.6
III-B		71±1.5	89±0.1

product detected when the cruder forms of the enzyme (fractions II and III) were used as an enzyme source (results not shown). In these experiments a cap 1 containing DHFR T7 transcript was also used as the RNA substrate.

RNA substrate specificity of the two m6A mRNA methyltransferase enzymes from HeLa cell nuclei

The RNA substrate specificities of enzyme fractions IV-A and III-B were also investigated (Table 4). Specific activity in these experiments is expressed as pmol of CH_3/mg of protein per pmol of RNA to correct for the differences in the molecular masses of each transcript tested. These results demonstrated significant differences in RNA specificity, as well as a relationship between enzymic activity and a 5′-cap structure. Activity of fraction IV-A was found to be highly dependent on the presence of a 5′-cap structure. When tested with DHFR T7 transcripts which lacked a cap structure, activity was found to be only slightly above the endogenous control values (Table 4). While fraction III-B was also found to prefer the capped transcript as a substrate, its dependence was much less than that of fraction IV-A. Initially the preference for capped RNA was thought to be at least partially caused by a difference in RNA stability. It is well known (Furuichi *et al.*, 1977; Lockard & Lane, 1978) that capped RNA is more resistant to cellular ribonucleases than uncapped transcripts. To test for ribonuclease activity in the enzyme fractions, 3 μg of ^{32}P-labelled T7 DHFR transcripts (capped and uncapped) were incubated separately with each enzyme fraction in the methyltransferase assay as described above. The radiolabelled AdoMet, however, was omitted from the reactions. Control assays omitting the enzyme fractions were also performed and RNA recoveries were then determined. As shown in Table 5 only slight differences (comparing capped and uncapped transcripts) in recovered RNA were observed for both enzyme fractions, IV-A and III-B, thus suggesting limited ribonuclease activity in either enzyme fraction or the presence of natural ribonuclease inhibitors. PAGE of the recovered substrate RNA confirmed limited degradation of the 1500 bp DHFR transcript. Little difference was observed in the intensity of either the capped or uncapped 1500 bp RNA band, regardless of the enzyme fraction used (results not shown).

Enzymic activity for both enzyme forms was also found to be dependent on the nature of the transcript. In this experiment, three different cap 1 T7 transcripts were tested as substrates: mouse DHFR, bovine prolactin and a synthetic transcript (MS-7) derived from the multiple cloning of methylation sites from Rous sarcoma viral DNA (Kane & Beemon, 1987). Transcripts from these sequences have been estimated to contain 20 possible methylation target sequences (Rana & Tuck, 1990).

Two cellular transcripts, bovine prolactin and mouse DHFR, were compared with regards to substrate activity. These two transcripts were selected for these investigations because of the fact that both had been shown previously to possess m6A residues *in vivo* (Horowitz *et al.*, 1984; Rottman *et al.*, 1986) as well as to act as excellent substrates *in vitro* for a crude form of the m6A mRNA methyltransferase (Narayan & Rottman, 1988; Rana & Tuck, 1990). In the case of enzyme fraction IV-A, very little difference was observed in the specific activity between the DHFR and prolactin transcripts (4.06 and 3.13 pmol of CH_3/mg of protein per pmol of RNA respectively). On the other hand, the specific activity of prolactin mRNA (0.19 pmol of CH_3/mg of protein per pmol of RNA) was half that of DHFR (0.41 pmol of CH_3/mg of protein per pmol of RNA) when fraction III-B was used as an enzyme source. Neither enzyme had a high affinity for the synthetic MS-7 transcript, despite the abundance of consensus methylation sequences.

DISCUSSION

The relationship between m6A formation and mRNA metabolism and function continues to remain a mystery. While a number of reports have suggested that this modification may be important for correct post-transcriptional processing of a number of viral (Stoltzfus & Dane, 1982; Finkel & Groner, 1983) and cellular transcripts (Camper *et al.*, 1984), the results of these experiments remain suspect because of the fact that general methylation inhibitors were employed. Using a totally different experimental design, recent research involving the construction of site-directed mutations at the methylation sites (to alter the consensus methylation sequences) of Rous sarcoma virus virion RNA (Csepany *et al.*, 1990) have unfortunately thus far produced mainly negative results with regards to determining biological significance.

A greater understanding of the enzymology of m6A formation should be extremely helpful in determining the functional significance of m6A formation. To date, information regarding the enzymology of this process has been difficult to obtain, mainly because of the lack of an adequate *in vitro* assay. The recent development of an accurate *in vitro* assay by Narayan & Rottman (1988) has already made its mark on the field with regards to mapping of methylation sites *in vitro* (Rana & Tuck, 1990) as well as recent investigations which have advanced the knowledge of the consensus methylation sequence (Harper *et al.*, 1990). In this report, a modification of this assay has been used to partially purify two forms of an m6A mRNA methyltransferase enzyme which modify internal adenine residues in mRNA. This is the first time this enzyme has been isolated from any tissue source.

Initial studies utilizing this assay produced very interesting

results regarding tissue specificity and the subcellular location of the m6A mRNA methyltransferase activity. Comparison of nuclear salt extracts from a number of different cell types demonstrated that the methyltransferase activity was highly enriched in cultured HeLa cells over any other tissue analysed (Table 1). Because the HeLa cells were harvested in mid-logarithmic phase, the enzyme activity was at first thought to be elevated due to the fact that the HeLa cells were actively undergoing cellular division. This hypothesis was tested by comparing the enzymic activity isolated from adult rat liver with that from regenerating rat liver (a tissue source in which rapid cell division was also occurring). These experiments demonstrated that neither tissue displayed detectable mRNA methyltransferase activity. Also, with regards to the relationship between m6A mRNA methyltransferase activity and cell division, it should be noted that nuclear extract isolated from HeLa cells during late-logarithmic phase (a cell density greater than 8×10^5 cells/ml) contained only slightly less activity than an extract from mid-logarithmic-phase cells (results not shown).

The preliminary observation that HeLa cells are enriched in m6A mRNA methyltransferase activity is an interesting one. It is therefore intriguing to speculate on the possible relevance of m6A formation in mRNA to the general carcinogenic process. While such speculation may be premature at this time, future research involving the investigation of this enzyme activity in other cancer cell lines, as well as tumour tissue, may lead to additional answers involving the relationship of mRNA processing to cancer development and metastasis.

Chromatography of the proteins from the salt-extracted nuclei of HeLa cells on DEAE-cellulose separated two forms of the enzyme (Fig. 3). While the possibility exists that one enzyme form is a degradation product of the other, the fact that the two forms separate so well on DEAE-cellulose coupled with the significant differences in RNA substrate specificity would seem to suggest otherwise. It should be noted that both forms of the enzyme at their respective purification stages are far from homogeneous. SDS/PAGE has shown the major form (fraction IV-A) to consist of six major bands, while the minor form consists of at least 15 major bands (after silver staining; results not shown). Attempts to purify further both enzymes utilizing other chromatography methods resulted in significant losses of activity. It is felt that part of this problem stems from the fact that even in HeLa cells m6A mRNA methyltransferase activity is present at extremely low levels for purification. This problem, coupled with the fact that HeLa cells are not an ideal cell source for enzyme purification, has severely hampered the further purification of the enzyme. It is the author's hope that in the future an additional tissue source can be found, which is more enriched in activity as well as a better-suited tissue source for enzyme purification than HeLa cells.

The two m6A mRNA methyltransferase enzymes were found to differ in RNA substrate specificity. The major form of the enzyme was also found to be highly dependent on the presence of a 5′-cap structure within the RNA substrate. The fact that uncapped transcripts have been found to be much less stable than capped transcripts may account for part of the difference; however, studies summarized in Table 5 indicate very little difference in RNA stability using either enzyme source. Therefore it is unlikely that such a dramatic drop in activity could be attributed to a difference in stability alone. It is again interesting to note that the synthetic transcript MS-7, which contains multiple consensus methylation sequences (Rana & Tuck, 1990), is such a poor substrate for both enzymes. This result seems to further support the hypothesis that the secondary and possibly tertiary structure of the RNA transcript seems to be critical for enzyme recognition. Further characterization of the enzyme activity, especially with regards to methylation site specificity and sequence specificity within the DHFR transcript, will be most interesting and may be extremely fruitful in determining the biological function of m6A formation.

In summary, this is the first report of the partial purification of an mRNA methyltransferase which modifies internal adenine residues. Enzymic activity was found to be enriched in HeLa cells as compared with other rat tissues analysed and was found exclusively in the soluble nuclear fraction. Partial purification of the enzymic activity resulted in the separation of two forms of the enzyme which differed in RNA substrate specificity as well as a 5′ 7-methylguanine cap requirement. While the isolation of the m6A mRNA activity is a significant finding in itself, it is hoped that a continuation of this work will produce additional information regarding the sequence specificity and genetic regulation of the enzyme. The major goal of this research is therefore to provide detailed information regarding the m6A mRNA methyltransferase enzyme which will in the future provide new avenues by which the biochemistry of m6A formation in mRNA can be studied, along with its physiological importance.

This research was supported in part by the Ohio University Baker Fund.

REFERENCES

Adams, J. M. & Cory, S. (1975) Nature (London) **255**, 28–33
Alberts, R. J., Coffin, B. & Rottman, F. M. (1981) Anal. Biochem. **113**, 118–123
Aloni, Y., Dhar, R. & Khoury, G. (1979) J. Virol. **32**, 52–60
Beemon, K. & Keith, J. (1977) J. Mol. Biol. **133**, 165–179
Backlund, P. S., Jr., Carotti, D. & Cantoni, G. L. (1986) Eur. J. Biochem. **160**, 245–251
Camper, S. A., Albers, R. J., Coward, J. K. & Rottman, F. M. (1984) Mol. Cell. Biol. **4**, 538–543
Canaani, D., Kahana, C., Lavi, S. & Groner, Y. (1979) Nucleic Acids Res. **6**, 2879–2899
Carroll, S. M., Narayan, P. & Rottman, F. M. (1990) Mol. Cell. Biol. **10**, 4456–4465
Chen-Kiang, S., Nevins, J. R. & Darnell, J. E., Jr. (1979) J. Mol. Biol. **135**, 733–752
Csepany, T., Lin, A., Baldick, C. J., Jr. & Beemon, K. (1990) J. Biol. Chem. **265**, 20117–20122
Desrosiers, R. C., Friderici, K. H. & Rottman, F. M. (1975) Biochemistry **14**, 4367–4374
Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
Dimock, K. & Stoltzfus, C. M. (1977) Biochemistry **16**, 471–478
Dublin, D. T. & Taylor, R. H. (1975) Nucleic Acids Res. **2**, 1653–1668
Duerre, J. A., Wallwork, J. C., Quick, D. P. & Ford, K. M. (1977) J. Biol. Chem. **252**, 5981–5985
Ensinger, M. J. & Moss, B. (1976) J. Biol. Chem. **251**, 5283–5291
Finkel, D. & Groner, Y. (1983) Virology **131**, 409–425
Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Salditt-Georgieff, M. & Darnell, J. E. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1904–1908
Furuichi, Y., LaFinandra, A. & Shatkin, A. J. (1977) Nature (London) **266**, 235–239
Harper, J. E., Micele, S. M., Roberts, R. R. & Manley, J. L. (1990) Nucleic Acids Res. **18**, 5735–5741
Haugland, R. A. & Cline, M. G. (1990) Eur. J. Biochem. **104**, 271–277
Higgins, G. M. & Anderson, R. M. (1931) Arch. Pathol. **12**, 186–202
Horowitz, S., Horowitz, A., Nilsen, T. W., Munns, T. W. & Rottman, F. M. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5667–5671
Kane, S. E. & Beemon, K. (1987) J. Biol. Chem. **262**, 3422–3427
Kennedy, T. D. & Lane, B. G. (1979) Can. J. Biochem. **57**, 927–931
Krieg, P. A. & Melton, D. A. (1985) Promega Biotec: Catalogue and Applications Guide, DB3
Lavi, S. & Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 2012–2016
Lavi, U., Fernandez-Monoy, R. & Darnell, J. E., Jr. (1977) Nucleic Acids Res. **4**, 63–69
Lockard, R. E. & Lane, C. (1978) Nucleic Acids Res. **5**, 3237–3247

Moss, B., Gershowitz, A., Stringer, J. R., Holland, L. E. & Wagner, E. K. (1977) J. Virol. **23**, 234–239
Narayan, P. & Rottman, F. M. (1988) Science **242**, 1159–1162
Nichols, J. L. (1979) Plant Sci. Lett. **15**, 357–367
Perry, R. P., Kelley, D. E., Friderici, K. & Rottman, F. M. (1975) Cell (Cambridge, Mass.) **4**, 387–394
Rana, A. P. & Tuck, M. T. (1990) Nucleic Acids Res. **18**, 4803–4808
Rottman, F., Narayan, P., Goodwin, R., Camper, S., Yao, Y., Horowitz, S., Albers, R., Ayers, D., Maroney, P. & Nilsen, T. (1986) in Biological Methylation and Drug Design: Experimental and Clinical Roles of *S*-Adenosylmethionine (Borchardt, R. T., Creveling, C. R. & Ueland, P. M. eds.), pp. 189–199, Humana Press, Clifton NJ, U.S.A.
Stoltzfus, C. M. & Dane, R. W. (1982) J. Virol. **42**, 918–931
Wei, C. M., Gershowitz, A. & Moss, B. (1976) Biochemistry **15**, 397–401

Received 6 January 1992/12 May 1992; accepted 18 May 1992