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Methylation of Ribonucleic Acid in a Cell-Free System from Mouse Myeloma Cells†

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ABSTRACT: Isolated nuclei incorporate few methyl groups into RNA when they are incubated with *S*-adenosyl[*methyl*-$^3H_3$]methionine and four ribotriphosphates. When the nuclei were supplemented with a soluble total cell protein extract, the incorporation of methyl groups into RNA was stimulated 3–6-fold. All classes of RNA were methylated. Methylation of the 2′-OH of ribose and the bases of ribosomal RNA occurred predominantly on endogenous ribosomal RNA precursors, with a minority (20%) occurring on the newly synthesized rRNA precursor. Methylation of the tRNA precursor occurred on both endogenous (40%) and newly synthesized (60%) molecules. The methylation of adenosine in hnRNA occurred predominantly on molecules transcribed in vitro and was sensitive to 1 μg/mL α-amanitin. A final site of methylation was the 7 position of guanosine of the cap structure. About 10% of the RNA polymerase II transcripts were capped in vitro. Capping was blocked 90% by 1 μg/mL α-amanitin and was independent of the presence of the cell protein extract.

Following transcription, most RNAs undergo a series of processing reactions that result in a functional RNA molecule (Perry, 1976). Preribosomal RNAs are methylated on both specific bases and ribose moieties (Salim & Maden, 1973) and specifically cleaved to yield mature ribosomal RNAs. hnRNAs are extensively processed to yield mRNA: the 5′ end of the hnRNA is capped (Shatkin, 1976), the 3′ end of most molecules is polyadenylated (Brawerman, 1974), specific bases are methylated (Perry & Kelley, 1976), and the precursor is specifically cleaved and spliced (Tilghman et al., 1978; Roop et al., 1978; Schibler et al., 1978). The majority of these reactions, particularly on rRNA and mRNA precursors, occur in the cell nucleus, and the maturation of tRNA may also take place, at least partially, in the nucleus (Lönn, 1977; Melton et al., 1980). All classes of RNA are methylated: rRNA predominantly on the 2′-hydroxyl of specific riboses (Maden & Salim, 1974) and hnRNA and mRNA on the "cap" and on the 6-amino group of specific adenosines (Perry & Kelley, 1976; Salditt-Georgieff et al., 1976). Transfer RNA shows the largest variety and greatest number of methyl groups, with a variety of methylated bases as well as *O*-methylribose (Gauss et al., 1979).

Previously we (Marzluff et al., 1973; Cooper & Marzluff, 1978) have described a preparation of cell nuclei from mouse myeloma cells that is active in RNA transcription but that is deficient in some RNA processing reactions. Polyadenylation of RNA (Cooper & Marzluff, 1978) occurs only in the presence of a soluble protein extract derived from crude nuclei. Here we characterize the methylation of RNA. We show enzymes that methylate RNA are easily lost during nuclear preparation. The ability to methylate RNA is restored by the addition of a cell protein extract. A large proportion of the methylation of the tRNA precursor and of hnRNA on internal adenosines occurred on RNA transcribed in vitro. There was capping of the hnRNA in vitro, which was also dependent on transcription. The capping activity was tightly associated with the nuclei.

Materials and Methods

Materials. *S*-Adenosyl[*methyl*-$^3H_3$]methionine and [α-^{32}P]GTP were from New England Nuclear. α-Amanitin was from Calbiochem. Actinomycin D was a gift from Merck Sharp & Dohme. Nuclease P1 was from Yamasa Shoyu Co., and other enzymes were obtained from Sigma. Standard methylated nucleosides were obtained from P-L Biochemicals. Triethylamine and phenol were redistilled before use.

Growth of Cells and Preparation of Nuclei. Mouse myeloma cells (clone 66-2) were grown in Dulbecco's modified Eagle's media plus 10% horse serum. Cells were harvested at a concentration of (4–6) × 10^5 mL and nuclei prepared exactly as previously described (Marzluff, 1978).

Preparation of Cell Extracts. Cell extracts were prepared by a modification of the method of Cooper & Marzluff (1978). Cells (5 × 10^8/mL) were homogenized in 0.025 M KCl, 0.002 M $MgCl_2$, 0.01 M Tris,[1] pH 7.5, and 0.001 M DTT. The

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; 6MeAde, 6-methyladenine; 6MeAdo, 6-methyladenosine; DTT, dithiothreitol; hnRNA, heterogeneous nuclear RNA; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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homogenate was adjusted to 0.35 M KCl with 2 M KCl and homogenized an additional 10 strokes, and nuclei and membranes were removed by centrifugation at 8000*g* for 10 min. The supernatant was removed and centrifuged at 100000*g* for 1 h in an AH 650 rotor in a Sorvall OTD-2 centrifuge.

The 100000*g* supernatant was passed through a 4-mL DEAE-cellulose column equilibrated with 10% glycerol, 0.25 M KCl, 0.005 M $Mg(OAc)_2$, 0.01 M Tris, pH 7.5, and 0.001 M DTT, and the flow through was then passed through a Sephadex G-25 column equilibrated with 10% glycerol, 0.12 M KCl, 0.005 M $Mg(OAc)_2$, 0.01 M Tris, pH 7.5, and 0.001 M DTT. The protein peak (void volume) was pooled and frozen in small aliquots in liquid N_2.

Transcription and Methylation of RNA. Transcription was carried out essentially as described by Cooper & Marzluff (1978). Freshly prepared nuclei were suspended in protein extract or in the G-25 column buffer. In some experiments nuclei frozen in 25% glycerol, 0.005 M $Mg(OAc)_2$, 0.05 M Tris, pH 7.5, and 0.005 M DTT and stored in liquid N_2 were thawed and diluted with an equal volume of cell extract. The final DNA concentration was 300–600 μg/mL. Conditions of RNA synthesis were 0.12 M KCl, 7% (v/v) glycerol, 0.005 M $Mg(OAc)_2$, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 0.02 mM AdoMet, and 0.001 M DTT. When [^{3}H]GTP was used, the final GTP concentration was reduced to 0.05 mM GTP. When [*methyl*-$^3H_3$]AdoMet was used, the concentration of AdoMet was 0.01 mM (100 μCi/mL). Actinomycin D and α-amanitin were added from concentrated stock solutions (2 mg/mL) when they were used. Reactions were incubated in 0.5-mL aliquots in 10 × 75 mm test tubes at 25 °C. The total volume of the methylation reactions was 2–4 mL (containing 1–2 mg of DNA). [α-^{32}P]GTP (20–50 Ci/mmol) prepared as described by Reeve & Huang (1979) was used in some experiments.

Determination of Amount of Methyl Groups in Macromolecules. Nuclei were incubated with [*methyl*-$^3H_3$]AdoMet at 25 °C for 30 min. Duplicate aliquots were removed for determination of trichloroacetic acid precipitable radioactivity. The nuclei were then incubated with 100 μg/mL pancreatic RNase at 37 °C for 30 min and aliquots again removed for determination of trichloroacetic acid precipitable radioactivity. Another aliquot was then adjusted to 0.5% $NaDodSO_4$ and proteinase K added to a concentration of 50 μg/mL. The reaction was incubated for 30 min at 37 °C and then aliquots were again removed for determination of trichloroacetic acid precipitable radioactivity. The amount of methylated RNA was taken as the RNase-sensitive material and the amount of methylated protein as the protease-sensitive material. DNA methylation was taken as material resistant to RNase and protease after sequential digestion of a third aliquot with RNase and proteinase K. Control experiments showed that under these RNase conditions more than 95% of the ribosomal RNA and tRNA was rendered acid soluble. All of the methylated protein in the same extract was degraded by the protease under these conditions. In addition the amount of RNA methylation measured by this technique agreed with the amount of RNA isolated after phenol extraction. It is possible that some of the radioactivity resistant to both RNase and protease is not methylated DNA but tightly complexed RNA and proteins.

Preparation and Analysis of RNA. RNA was prepared by the hot phenol–$NaDodSO_4$ extraction at pH 5 as previously described (Marzluff et al., 1973). In some experiments the reaction was separated into nuclear and extranuclear fractions by centrifugation through an equal volume of 25% glycerol, 0.005 M $Mg(OAc)_2$, 0.05 M Tris, pH 8, and 0.001 M DTT at 1000*g* for 2 min. RNA was prepared from both the nuclear (pellet) and extranuclear (supernatant) fractions. RNA was analyzed by sucrose gradients as previously described (Marzluff, 1978) and by polyacrylamide gel electrophoresis under aqueous and denaturing conditions (Brown & Marzluff, 1978).

RNA samples were chromatographed on a Sephadex G-75 column equilibrated in 0.1 M NaCl, 0.01 M NaOAc, pH 5, 0.001 M EDTA, and 0.1% $NaDodSO_4$ to separate the tRNA precursor from the RNA larger than 5S rRNA (which eluted in the void volume of the column).

For analysis of the methylated nucleotides, the RNA was digested with T2 ribonuclease (1 unit/40 μg of RNA) and pancreatic ribonuclease (10 μg/mL) for 4 h at 37 °C in 0.01 M NaOAc, pH 5. The digests were diluted with 10 volumes of 7 M urea, chromatographed on a DEAE-cellulose column (4 × 15 cm) in 7 M urea–0.01 M Tris, pH 7.5, and eluted with a linear gradient (100 mL) of 0–0.35 M NaCl in 7 M urea (Tomlinson & Tener, 1963) at 8 mL/h. Radioactivity was detected by Cerenkov radiation, and fractions containing the desired nucleotides were diluted with 4 volumes of H_2O and bound to a 0.5-mL DEAE-cellulose column in a Pasteur pipet. The column was washed with 2 mL of water to remove all the urea and the RNA eluted with 1 mL of 2 M triethylammonium bicarbonate, pH 8. The nucleotides were recovered by repeated lyophilization.

The oligonucleotides were digested with nuclease P1, alkaline phosphatase, and nucleotide pyrophosphatase as previously described (Brown & Marzluff, 1978). After digestion the products were resolved by electrophoresis on cellulose thin-layer sheets at pH 3.5 (5% acetic acid, 0.5% pyridine, 2 mM EDTA) or by chromatography on cellulose thin-layer sheets. The methylated mononucleosides (prepared by alkaline phosphatase digestion) were identified by thin-layer chromatography in 1-butanol–NH_4OH–H_2O (86:5:14). After hydrolysis of the methylated adenosine derivatives by boiling in 0.1 N HCl for 30 min, the 6MeAde was identified by chromatography in 2-propanol–HCl–H_2O (68:14.4:11) (Furuichi et al., 1975). 7-Methylguanosine was identified by chromatography in acetonitrile–ethyl acetate–1-butanol–propanol–6 N NH_3 (7:2:1:1:2.7) (Fernandez-Munoz et al., 1977). "Caps" labeled with [α-^{32}P]GTP were digested with tobacco acid pyrophosphatase (BRL) and the released methylated 5′-phosphates analyzed by two-dimensional thin-layer chromatography (Silberklang et al., 1979). The first dimension was isobutyric acid–NH_3–H_2O (66:1:33), and the second dimension was 0.1 M sodium phosphate, pH 6.8–$(NH_4)_2SO_4$–1-propanol (100:60:2 v/w/w).

Results

Methylation of Macromolecules. When isolated nuclei were incubated with *S*-adenosyl[*methyl*-$^3H_3$]methionine, label was incorporated into macromolecules at a linear rate for at least 30 min (Figure 1). The great majority of the methyl groups were found in DNA and protein with only a small amount incorporated into RNA (Table I). However, in the presence of total protein extract there was no change in the methylation of DNA, but there was a stimulation of RNA methylation. This increased methylation of RNA was due to methylation of nuclear RNA (both endogenous and newly synthesized) by enzymes in the extract. If the endogenous RNA was removed from the protein extract by chromatography on DEAE-cellulose, there were no CH_3 groups incorporated into RNA in the extract incubated without nuclei (Table I, line 3). The total incorporation of CH_3 groups was not significantly affected

Table I: Methylation of Macromolecules[a]

	total (cpm/10 μg of DNA)	DNA		RNA		protein	
		% C^3H_3	cpm/10 μg of DNA	% C^3H_3	cpm/10 μg of DNA	% C^3H_3	cpm/10 μg of DNA
cell protein extract plus nuclei	5050	30	1500	30	1500	40	2000
nuclei in glycerol buffer	2100	80	1700	10	210	5	200
cell protein extract without nuclei	900	0	0	0	0	100	900

[a] Nuclei were incubated for 60 min with [*methyl*-$^3H_3$]AdoMet in cell protein extract, in glycerol buffer, and an equal amount of extract was incubated without nuclei. The incubations were treated sequentially with 100 μg/mL pancreatic RNase and proteinase K (50 μg/mL) in 0.5% $NaDodSO_4$ as described under Materials and Methods, and the trichloroacetic acid precipitable material was determined after each step. Results are expressed as percent of total counts in each fraction and per 10 μg of DNA (when nuclei were present and per equivalent volume of cell extract when nuclei were absent). The value for DNA is taken as that resistant to the two digestions. The nuclei incubated in extract showed a 3–6-fold stimulation of RNA methylation relative to nuclei in glycerol buffer in a number of experiments, due to differences in amount of methylation of the RNA in glycerol buffer, presumably due to different degree of retention of the RNA methylases in the nuclei.

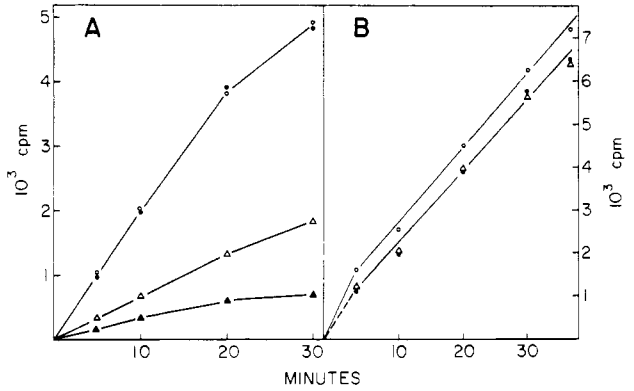

FIGURE 1: Methylation of macromolecules in vitro. Equal amounts of nuclei were incubated in glycerol buffer or in cell protein extract with [*methyl*-$^3H_3$]AdoMet and four ribotriphosphates. Duplicate aliquots (10 μL) were removed at the indicated times and the trichloroacetic acid precipitable counts determined: (A) (○) nuclei in cell protein extract, (●) nuclei in cell protein extract plus 1 μg/mL α-amanitin, (△) nuclei in glycerol buffer, and (▲) cell protein extract without nuclei; (B) (○) nuclei in cell protein extract, (△) nuclei in cell protein extract (plus 100 μg/mL α-amanitin), and (●) nuclei in cell protein extract (plus 80 μg/mL actinomycin D). Similar results with α-amanitin and actinomycin D were found in four separate experiments.

by low concentrations (1 μg/mL) of α-amanitin, but it was inhibited about 10% if 100 μg/mL α-amanitin or 80 μg/mL actinomycin D was added or if ribotriphosphates were omitted from the reaction, suggesting that some of the RNA transcribed in vitro was methylated (Figure 1B). Transcription was blocked at least 98% if triphosphates (other than [^3H]-GTP) were omitted or if 80 μg/mL actinomycin D was added. A total of 0.2–0.4 pmol of methyl groups were incorporated into RNA/μg of DNA in a 60-min incubation. Of the total methyl groups incorporated into macromolecules 10–30% were found in RNA.

Characterization of RNA Product. The methylated RNA was analyzed by sucrose gradient centrifugation (Figure 2A). About 30–40% of the RNA was low molecular weight and was found in the extranuclear fraction (Figure 2B). The remaining RNA was high molecular weight, ranging in size to larger than 28 S, and was retained in the nucleus.

The major methylated species released from the nuclei coelectrophoresed with the 4.5S tRNA precursors (Figure 3A). Two minor methylated species, which coelectrophoresed with the low molecular weight nuclear RNAs, N6 and N7 [nomenclature of Brown & Marzluff (1978)] known to be methylated, were also present. There was little methylated low molecular weight RNA retained in the nuclei. The presence of N6- and N7-methylated RNA in the extranuclear fraction was not surprising since newly made small nuclear

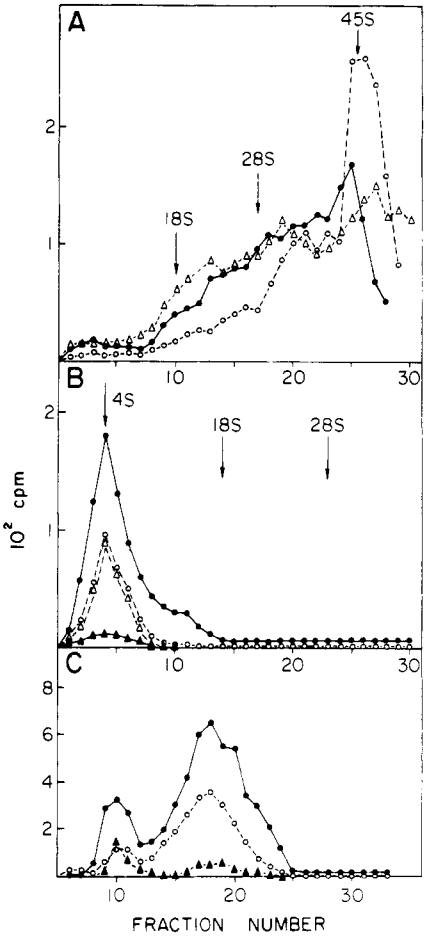

FIGURE 2: Size of methylated RNA. Nuclei were incubated with [*methyl*-$^3H_3$]AdoMet in the presence of cell protein extract, the nuclei were separated from the reaction, and the RNA was prepared from the nuclear pellet and the extranuclear supernatant fractions. The RNA was analyzed on 10–40% sucrose gradients for 16 h at 26 000 rpm in the AH627 rotor at 20 °C. The trichloroacetic acid precipitable counts in each fraction were determined. Equal amounts of nuclei were used in each experiment. (A) RNA retained in nuclei: (●) nuclei in cell protein extract, (○) nuclei in cell protein extract (plus 80 μg/mL actinomycin D), and (△) nuclei in cell protein extract (plus 100 μg/mL α-amanitin). There was not sufficient methylated RNA in nuclei incubated in glycerol buffer to analyze. (B) RNA released from nuclei: (●) nuclei in cell protein extract, (○) nuclei in cell extract (plus 80 μg/mL actinomycin D), (△) nuclei in cell extract (plus 100 μg/mL α-amanitin), and (▲) nuclei in glycerol buffer. (C) The small RNA [fractions 2–7 in (B)] was recovered and fractionated on Sephadex G-75. The void volume (fractions 9–11) contained the RNA larger than 5 S, while fractions 15–25 contained the tRNA precursor and tRNA (see Figure 3): (●) nuclei incubated in cell protein extract, (○) nuclei incubated in cell protein extract (plus 80 μg/mL actinomycin D), and (▲) nuclei incubated in glycerol buffer. Essentially the same result was obtained with nuclei incubated in the presence of 80 μg/mL actinomycin D and 100 μg/mL α-amanitin.

Table II: Effect of Inhibitors on Transcription and RNA Methylation[a]

	total extract	−XTP	% inhibition	+actinomycin D	% inhibition	+α-amanitin (1 μg/mL)	% inhibition	+α-amanitin (100 μg/mL)	% inhibition
RNA methylation									
extranuclear	450	225	50	225	50	450	0	200	58
nuclear	1500	1200	20	1200	20	1500	0	1500	0
RNA transcription									
extranuclear	8000	400	95	400	95	4000	50	400	95
nuclear	40000	800	98	800	98	20000	50	20000	50

[a] Nuclei were incubated in total protein extract with [*methyl*-$^3H_3$]AdoMet or [^{3}H]GTP in the presence of various inhibitors of RNA transcription. The reactions were separated into nuclear and extranuclear fractions, and RNA was prepared from each fraction. The results are expressed as cpm/μg of DNA. Specific activity of each isotope was 10 Ci/mmol (5000 cpm/pmol). This represented 0.3 pmol of CH_3 groups/μg of DNA in methylation experiments and 8 pmol of GMP/μg of DNA in the transcription experiments. The results are representative of three experiments with independent nuclei and cell extract preparations.

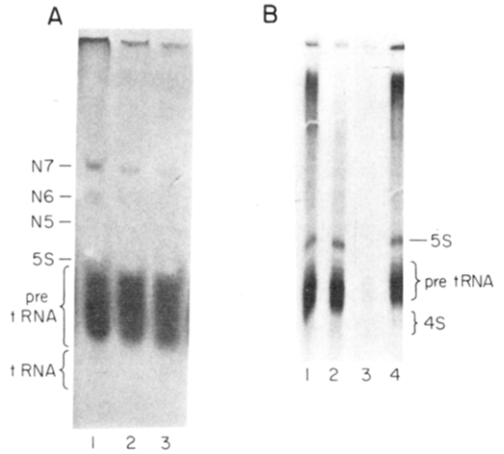

FIGURE 3: Small RNAs methylated in isolated nuclei. (A) Nuclei were incubated in the cell protein extract with [*methyl*-$^3H_3$]AdoMet. 4–8S RNA was prepared by sucrose gradient centrifugation and analyzed by gel electrophoresis: (lane 1) extranuclear RNA, (lane 2) extranuclear RNA methylated in the presence of 80 μg/mL actinomycin D, and (lane 3) extranuclear RNA methylated in the presence of 100 μg/mL α-amanitin. (B) Nuclei were incubated in the presence (lanes 1–3) or absence (lane 4) of cell protein extract with [α-^{32}P]GTP and analyzed as in (A): (lane 1) RNA synthesized in the presence of cell protein extract, (lane 2) RNA synthesized in the presence of 1 μg/mL α-amanitin, (lane 3) RNA synthesized in the presence of 100 μg/mL α-amanitin, and (lane 4) RNA synthesized in nuclei in the absence of cell protein extract.

RNAs are found in this fraction when these cells (Brown & Marzluff, 1978) and others (Zieve & Penman, 1976; Elicieri, 1976) are fractionated under aqueous conditions.

Most of the small RNA synthesized in isolated nuclei is synthesized by RNA polymerase III and migrates between 5S rRNA and tRNA (Figure 3B). We (Marzluff et al., 1974) have previously shown that most of this RNA is the tRNA precursors. Similarly, most of the methylated RNA released from nuclei probably represents methylated tRNA precursors. Analysis of the methylated nucleotides in this RNA fraction showed that 70% of the methyl groups were on the bases and 30% on the 2′-OH of ribose, consistent with their identification as tRNA precursors (data not shown).

The methylation of the extranuclear RNA was reduced by inhibitors of transcription. Low concentrations of α-amanitin had no effect on methylation of the extranuclear RNA (Table II). However, α-amanitin at 100 μg/mL, which blocked RNA polymerase III by 95% (as judged by labeling of 4.5S tRNA precursor and 5S rRNA with [α-^{32}P]GTP (Figure 3B), reduced methylation of extranuclear RNA 60% (Table II). A similar effect on methylation of extranuclear RNA was seen if transcription was blocked by actinomycin D or prevented by omitting ribotriphosphates (except GTP) (Table II) from the incubation. Either treatment reduced total transcription at least 98% and methylation of RNA found in the extranuclear fraction about 50%. Thus half of the methyl groups incorporated into small RNAs were added to RNAs synthesized in the isolated nuclei.

The remaining methylation of the 4.5S tRNA precursor was due to methylation of endogenous 4.5S tRNA precursor in the isolated nuclei by enzymes in the extract. There was no methylation of 4.5S RNA in the extract incubated without nuclei or in nuclei incubated without extract.

However, if RNA was not removed from the extract by DEAE-cellulose chromatography, the extract methylated endogenous RNAs in the extract. These RNAs were apparently unprocessed tRNA precursors as they migrated with the tRNA precursor on gel electrophoresis and not with mature tRNA (data not shown).

Characterization of Methylated Nucleotides in RNA Retained in the Nuclear Fraction. The high molecular weight RNA methylated in the presence of protein extract was digested with T2 and pancreatic ribonucleases and fractionated by DEAE-cellulose chromatography. Nuclei incubated in the cell extract showed prominent methylation of the dinucleotide fraction (ribose methylation) with a lesser methylation of mononucleotides (base methylation) and with a small amount of methyl groups incorporated into nucleotides with a charge of −5 to −6.5 (data not shown).

The methylated mononucleotides were analyzed by electrophoresis at pH 3.5. The major radioactive component migrated with AMP, while the minor components migrated with CMP and UMP (Figure 4A). After treatment with alkaline phosphatase, the nucleosides were separated by thin-layer chromatography. The major component migrated identically with 6-methyladenosine and the minor ones with 5-methylcytidine and ribothymidine (Figure 4D,E). After hydrolysis of the N-glycosidic bond of the material migrating with 6MeAdo with a 1 N HCl, the radioactivity migrated identically with authentic 6-methyladenine (Figure 4F). No 6,6-dimethyladenosine was found, although this is present in mature ribosomal RNA.

The methylation pattern seen was similar to that found on mature RNAs in vivo. The majority of the methylation of 18S and 28S rRNAs was found in dinucleotides, and a small amount (15% of the total) of the methyl groups was present in monocucleotides, in agreement with results of other workers (Salim & Maden, 1973) (data not shown). Mononucleotide methylation was primarily on 6,6-dimethyladenosine in 18S rRNA with lesser amounts on 5-methylcytosine and ribothymidine and on 5-methylcytosine and 6-methyladenosine in 28S rRNA (Salim & Maden, 1973; Desrosiers et al., 1974). In contrast, hnRNA contains many fewer methyl groups, which are found predominantly, if not exclusively, on the bases (primarily 6MeAdo) except for the methylation on the cap

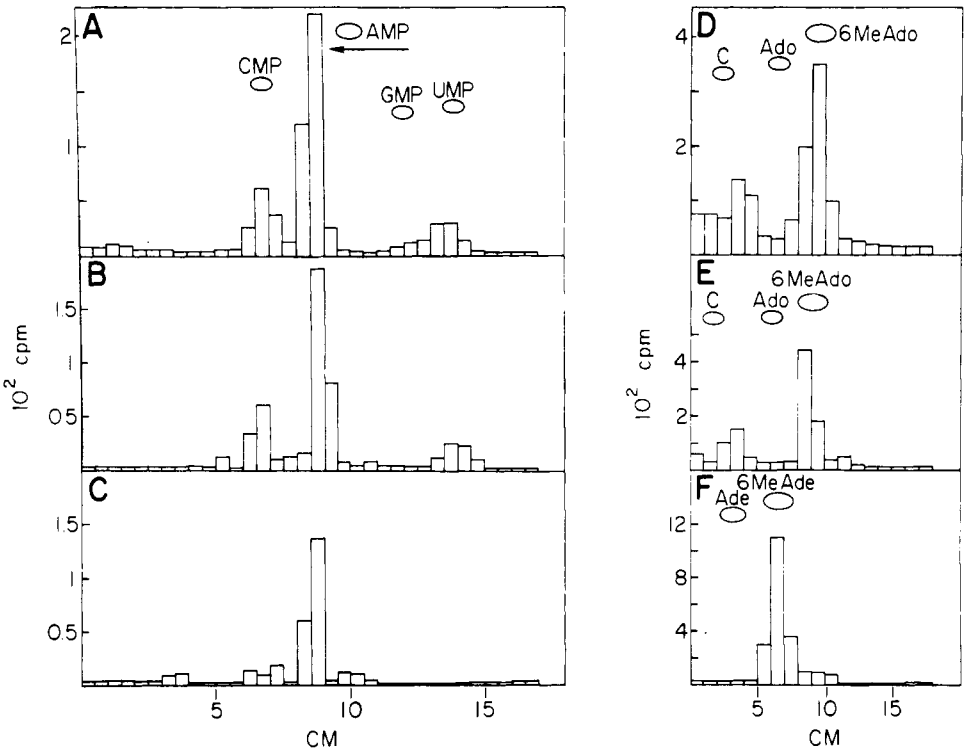

FIGURE 4: Identification of methylated mononucleotides. The mononucleotide fraction (charge −2) was recovered and analyzed by electrophoresis at pH 3.5 (A–C) or then treated with alkaline phosphatase and analyzed by thin-layer chromatography (D–F). The counts in 0.5-cm fractions were determined. RNA isolated from the same number of nuclei was analyzed in each experiment, except in (C) where twice as many nuclei were used. Positions of added marker nucleotides, nucleosides, or bases are indicated. (A) Nuclei were incubated in cell protein extract. (B) Nuclei were incubated in cell protein extract (plus 1 μg/mL α-amanitin). (C) Nuclei from cells preincubated in 0.04 μg/mL actinomycin D were incubated in cell protein extract (RNA from twice as many nuclei were used here). This treatment reduced rRNA synthesis 85–90% in this experiment. (D) The mononucleotides methylated in nuclei incubated in glycerol buffer were treated with alkaline phosphatase and chromatographed in 1-butanol–NH_3–H_2O (86:5:15). (E) The mononucleotides methylated in nuclei incubated in cell extract were analyzed as in (D). (F) The material in fractions 9 and 10 from (D) was recovered and hydrolyzed in 0.1 N HCl at 100 °C for 15 min and chromatographed in 2-propanol–12 N HCl–H_2O (68:14.4:11). 6,6-Dimethyladenine present in 18S rRNA was found in fractions 8–10 in a parallel chromatogram.

(Salditt-Georgieff et al., 1976).

The effect of inhibitors of RNA synthesis on the methylation of the mono- and dinucleotide fraction was determined (Table III). α-Amanitin (1 μg/mL) specifically blocked some of the methylation of the mononucleotide fractions, reducing it about 25% without affecting methylation of dinucleotides. Actinomycin D, at concentrations that blocked transcription at least 98%, inhibited methylation only about 20% but predominantly affected the mononucleotide fraction. Pretreatment of cells with 0.04 μg/mL actinomycin D to block rRNA synthesis (Perry, 1962) inhibited methylation of both fractions significantly but the dinucleotide fraction at least 80%. These data are consistent with a large portion of the dinucleotide and mononucleotide methylation occurring on preformed ribosomal RNA precursors, while a component (about 30%) of the methylation of the mononucleotides is on newly transcribed hnRNA (sensitive to α-amanitin). Some of the methylation may also occur on newly made ribosomal RNA precursors since two treatments that block transcription completely, actinomycin D and omission of triphosphates, reduce the methylation of the RNA retained in the nuclei by 20% (Tables II and III).

Capping of RNA with [α-^{32}P]*GTP.* A small proportion of C^3H_3 groups were found in oligonucleotides of charge −4.5 to −6.5 expected for cap structures (not shown). For confirmation of the presence of caps, the nuclear RNA larger than 12 S labeled with [α-^{32}P]GTP was digested with T2 and pancreatic ribonucleases and chromatographed on DEAE-cellulose. Material derived from RNA larger than 12 S eluting with charges of −4, −5, and −6 and −7 was pooled separately and analyzed by thin-layer electrophoresis after digestion with

Table III: Effect of Inhibitors of RNA Synthesis on Methylation of Oligonucleotides[a]

	mononucleotides		dinucleotides	
	cpm	% of control	cpm	% of control
control	2300		11500	
+1 μg/mL α-amanitin	1650	72	11850	100
+80 μg/mL actinomycin D	1225	53	9800	85
cells preincubated with 0.04 μg/mL actinomycin D	610	26	2100	18

[a] Equal amounts of nuclei were incubated in protein extract in the presence of 1 μg/mL α-amanitin or 80 μg/mL actinomycin D. In a separate experiment cells were preincubated with 0.04 μg/mL actinomycin D and nuclei prepared and incubated in the same protein extract at the same concentration of nuclei. RNA was prepared from the nuclear fraction, digested with RNases A and T2, and analyzed by DEAE-cellulose chromatography.

nuclease P1. Core cap structures were released in all samples, which were presumably derived from cap 0, cap I, and II structures, respectively (Figure 5A). In three experiments with independently prepared nuclei and cell extracts, the proportions of each structure varied from less than 10% cap II structures (>80% cap 0) to 50% cap II structures (<20% cap 0). These structures were confirmed as cap structures by digestion with nucleotide pyrophosphatase (Figure 5B). The capping base was identified as predominantly 7-methylguanosine monophosphate by two-dimensional thin-layer chromatography. A small amount of a second component (X) was also present. This material has a mobility consistent with

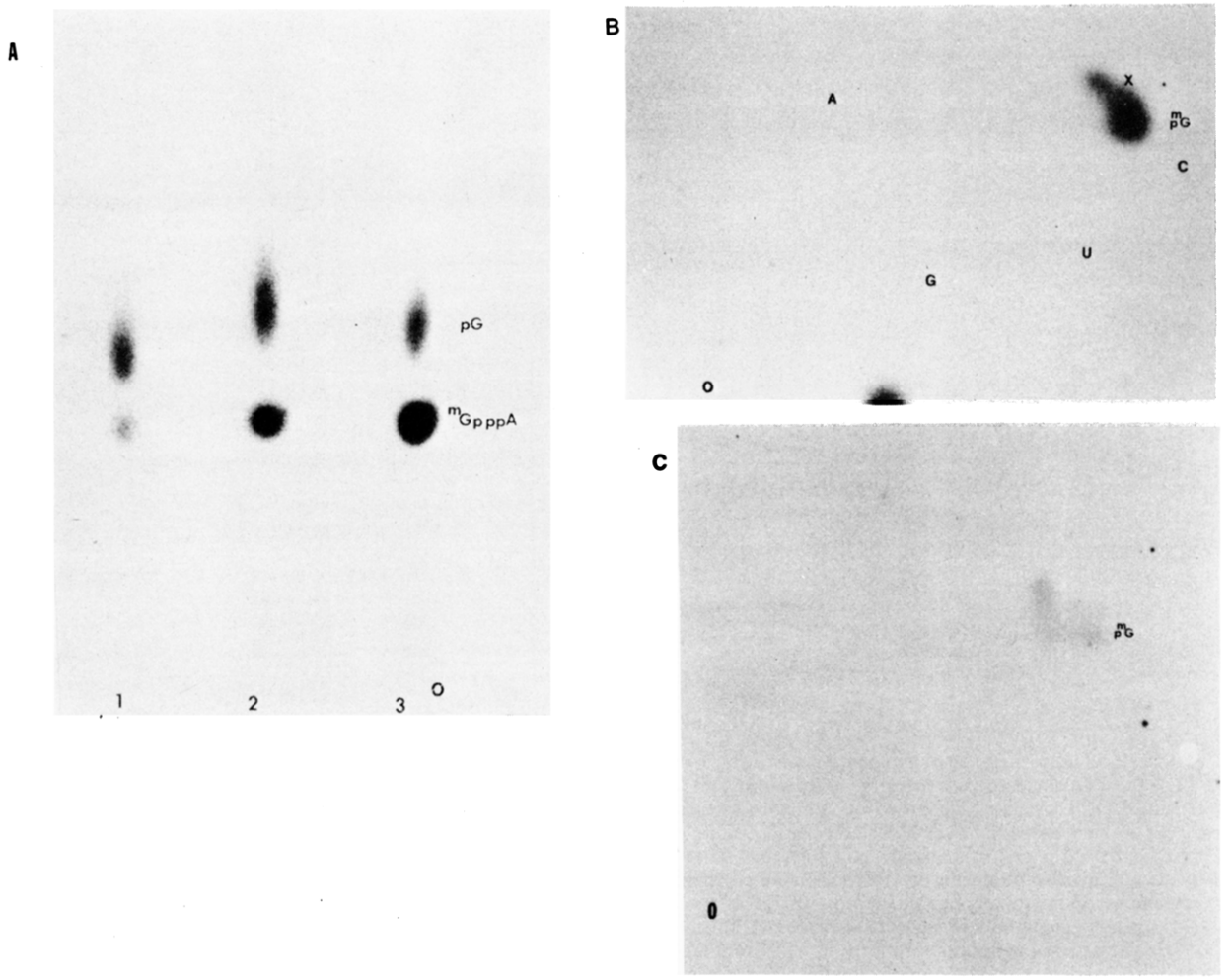

FIGURE 5: Isolation of caps from the nuclear fraction. (A) Nuclei were incubated with [α-^{32}P]GTP, and the RNA was isolated from the nuclear fraction. The RNA sedimenting faster than 12 S was pooled, digested, and analyzed by DEAE-cellulose chromatography as described in the legend to Figure 4. Material eluting with charges of −4, −5, and −6 and −7 was pooled separately and analyzed by thin-layer electrophoresis after treatment with nuclease P1: (lane 1) nucleotides with charge of −4 (cap 0), (lane 2) nucleotides with charge of −5 (cap I), and (lane 3) nucleotides with charge of −6 (cap II). (B) The core cap structures from lanes 1–3 were recovered, digested with tobacco acid pyrophosphatase, and analyzed by two-dimensional thin-layer chromatography. Most of the radioactivity migrated with 5′-end 7-methylguanosine phosphates, and a small amount of an unknown nucleotide (X). [The position of the four mononucleotides and the origin (O) are shown.] (C) The core cap structures isolated from RNA synthesis in the presence of 1 μg/mL α-amanitin were analyzed as in (B).

its being a modified guanosine phosphate and migrated similar to 2,2,7-trimethylguanosine phosphate prepared from ^{32}P-labeled small nuclear RNAs (unpublished results). Capping of the high molecular weight RNA fraction was inhibited 75–80% by 1 μg/mL α-amanitin, which completely blocked RNA polymerase II (Table IV). The resistant "caps" had predominantly the X base as the capping base so that labeling of 7-methylguanosine phosphate was blocked greater than 90% (Figure 5C).

About 2 molecules of GMP were incorporated into caps per 10000 molecules of GMP incorporated into RNA larger than 18 S. Similar results were found whether RNA was prepared from nuclei incubated in buffer or in extract (Table IV). This corresponds to capping of about 10% of the transcripts (see Discussion).

Discussion

These studies characterize an important reaction in RNA processing, methylation, as it takes place in a cell-free system prepared from mouse myeloma cells. Previously we have shown that another RNA processing reaction, polyadenylation of RNA polymerase II transcripts, occurs only when a protein fraction derived from crude nuclei is added to purified nuclei (Cooper & Marzluff, 1978). We show here that methylation of RNA is likewise stimulated by the addition of a concentrated protein extract to isolated nuclei. Much of the ability to methylate rRNA, hnRNA, and tRNA precursors is lost on preparation of the nuclei and is restored by the protein extract.

The methylated nucleotides incorporated into the rRNA precursor in vitro were similar to those found in vivo in many respects. About 10% of the methyl groups in HeLa cell rRNA are found on the bases (Salim & Maden, 1973; Desroisiers et al., 1974). These include 5-methylcytidine, ribothymidine, and methylated adenosines. A major site of base methylation is 6,6-dimethyladenosine in 18S rRNA (Maden & Salim, 1974). Some of these base methylations are events occurring relatively late after transcription, while others occur on the 45S rRNA precursor. We did not observe significant amounts of dimethyladenosine in the products of the cell-free methylation although it was a major component of 18S rRNA methylation in these cells (our unpublished results). Thus this methylation event did not occur in significant amounts. We have not obtained any direct evidence that the same sequences are methylated in vitro as are methylated in vivo.

The majority of the methylation of large RNA is on preexisting RNA (primarily pre-rRNA) and thus is not sensitive to treatments that block transcription. However, a fraction of the methylation of the bases is blocked by low

Table IV: Capping of RNA in Isolated Nuclei[a]

RNA sample	% GTP in caps	% polymerase II transcripts capped
nuclei buffer		
expt 1	0.010	8
expt 2	0.015	12
nuclei + cell extract		
expt 1	0.012	10
expt 2	0.025	20
expt 3	0.015	12
nuclei + cell extract +1 μg/mL α-amanitin		
expt 2	0.002	
expt 3	0.003	

[a] Nuclei were incubated with [α-^{32}P]GTP and cell extract. The RNA was isolated and the RNA larger than 12 S prepared by sucrose gradient centrifugation. The RNA was digested with pancreatic and T2 ribonucleases and chromatographed on DEAE-cellulose. The nucleotides eluting with charges −4 and −7 were recovered and digested with P1 and alkaline phosphatases. The amount of caps was determined after thin-layer electrophoresis (as shown in Figure 6). (5–10) × 10^6 cpm were analyzed in each experiment. When the same experiment was done in the presence of 1 μg/mL α-amanitin, total transcription was inhibited 40–50% (the amount of RNA polymerase II activity). The percent of RNA polymerase II transcripts capped was calculated from an average length of the in vitro transcript of 1600 bases and a composition of 25% guanosine.

concentrations of α-amanitin, showing that some of this methylation occurred on heterogeneous nuclear RNA transcribed in vitro. There is apparently little endogenous hnRNA in the nuclei that has not been methylated. Similar conclusions can be drawn from the earlier study of Winicov & Perry (1976), who reported substantial inhibition of mononucleotide methylation of hnRNA by α-amanitin. This is in direct contrast to the situation with ribosomal RNA. It is not known whether the same activity methylated the adenosine of both these classes of RNAs. However, as was previously found for polyadenylation (Cooper & Marzluff, 1978) and has been observed recently for several specific mRNAs (Schibler et al., 1978), processing of hnRNA is extremely rapid.

The methylation of the tRNA precursor was also dependent on the protein extract although it is possible that this is not normally a nuclear activity. The methylated RNA is larger than tRNA, and little RNA with the same electrophoretic mobility of tRNA was formed in these reactions. Endogenous tRNA is not methylated by the protein extract. However, there is endogenous RNA in the extract that is methylated, which is apparently recently synthesized tRNA precursor. This RNA can be removed by chromatography on DEAE-cellulose in 0.3 M KCl without affecting the methylation of nuclear RNA. At least 50% of the methylation of small RNA is blocked by inhibitors of transcription, including inhibition of RNA polymerase III by a high concentration of α-amanitin. Thus the RNA synthesized in vitro is the predominant substrate for these methylations.

The synthesis of the tRNA precursor, identified by hybridization to partially purified DNA coding for the tRNA genes, is about 2–4% of the total RNA synthesis in these nuclei (Marzluff et al., 1974). This represents about 0.2–0.4 pmol of GMP incorporated into tRNA precursor/μg of DNA in a 1-h incubation, in which 10 pmol of GMP was incorporated into RNA/μg of DNA. A total of 0.2–0.4 pmol of CH_3 groups was incorporated into RNA in the same incubation, about 15% of which were in the newly transcribed tRNA precursor. Thus about 0.1–0.3 pmol of CH_3 groups/pmol of GMP was incorporated into the 4.5S tRNA precursors. This represents 2–6 methyl groups per newly transcribed tRNA precursor (assuming 25 G's per tRNA precursor). Therefore, most of the tRNA precursor molecules synthesized in vitro probably serve as substrates for the methylases.

With [α-^{32}P]GTP as a precursor caps were found in high molecular weight RNA synthesized in isolated nuclei. These represented about 0.02% of the [α-^{32}P]GTP incorporated into RNA. The capping base was identified as 7-methylguanosine monophosphate. Synthesis of these caps was blocked 70–80% by 1 μg/mL α-amanitin. However the incorporation of [α-^{32}P]GTP into 7-methylguanosine monophosphate was blocked by more than 90% by α-amanitin. The α-amanitin-resistant caps had a different capping nucleotide. Other workers (Winicov & Perry 1976) also reported a proportion of the caps were resistant to 1 μg/mL α-amanitin.

The finding of labeled caps whose formation is blocked by α-amanitin suggests initiation of transcription may be occurring in vitro as has been found for the late adenovirus genes (Manley et al., 1979). We find about 1 cap per 5000 GMP residues incorporated into RNA. Of this RNA about 50% is RNA polymerase II transcripts. By assuming a guanosine content of 25%, this corresponds to 1 cap per 10000 nucleotides incorporated into RNA. In this system the number-average length of the portion of poly(A) RNA transcribed in vitro is 1000–1500 bases (Cooper & Marzluff, 1978). This implies that 10–15% of the polymerase II products are capped in vitro. Whether these actually represent chain initiation is not clear, but this is similar to the amount of initiated polymerase II transcripts found by Smith et al. (1978), using nucleotide γ-thiotriphosphates as a substrate.

These results, coupled with those previously reported (Cooper & Marzluff, 1978; Blanchard et al., 1978), point out that factors involved in RNA processing are relatively easily removed from nuclei during purification. They may be restored by addition of a soluble extract derived in part from crude nuclei. Systems such as this may provide a starting point for resolution and purification of factors involved in RNA processing.

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Specificity of Deoxyribonucleic Acid Cleavage by Bleomycin, Phleomycin, and Tallysomycin†

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ABSTRACT: The sites of cleavage of DNA by bleomycin A_2, bleomycin B_2, phleomycin, tallysomycin A, and Blenoxane (Bristol-Meyers) in reactions containing equimolar Fe^{2+} and atmospheric oxygen were analyzed by gel electrophoresis of ^{32}P end labeled DNA fragments. Bleomycin A_2 and bleomycin B_2 reactions cleaved DNA at all sites with a frequency equal to that of Blenoxane. At high concentrations of bleomycin the site specificity of cleavage was unchanged. Bleomycin cleavage sites and phleomycin cleavage sites are a subset of sites cleaved in reactions containing tallysomycin A. The nature of 5′ and 3′ termini induced by bleomycin cleavage was investigated. Electrophoresis of bleomycin-induced fragments after alkaline phosphatase or polynucleotide kinase treatment indicated that 5′ termini are phosphoryl groups but 3′ termini are not simple phosphoryl groups. Analysis of bleomycin cleavage of single-stranded DNA substrate showed that cleavage occurs only in regions of potentially double-stranded looped-back sequences. Possible mechanisms for determination of bleomycin cleavage sequence specificity are discussed.

Bleomycin is a glycoprotein antibiotic that is used clinically in the treatment of certain tumors (Blum et al., 1973; Hecht, 1979; Crooke & Bradner, 1976; Suzuki et al., 1969; Umezawa, 1978). The antibiotic is produced by *Streptomyces verticillus* (Umezawa et al., 1966). The clinical preparation in common use, Blenoxane (Bristol-Meyers), is a complex mixture of several different bleomycin species that differ from one another in the structure of the terminal amine group. Blenoxane is comprised predominantly of bleomycin A_2 (approximately 70%) and bleomycin B_2 (approximately 30%). The structure of these compounds is indicated in Figure 1.

Several other antibiotics structurally related to bleomycin have also been tested for antitumor effectiveness. These include the phleomycins. The phleomycin antibiotics differ from bleomycin in the bithiazole ring structure. One of the bithiazole rings is reduced in phleomycin. Recently, a new bleomycin-like compound, tallysomycin, has been introduced into clinical trials (Bradner, 1978; Kawaguchi et al., 1977). Tallysomycins differ from the bleomycins structurally in a few ways but principally by an additional sugar attached to the (aminoethyl)bithiazole moiety (Figure 1).

The bleomycin-like antibiotics bind to and break DNA (Suzuki et al., 1969; Haidle, 1971; Muller et al., 1972; Povirk et al., 1977; Strong & Crooke, 1978; Mirabelli et al., 1979, 1980). Strand scission by these compounds requires both ferrous ion and molecular oxygen (Sausville et al., 1976, 1978a; Lown & Sim, 1977; Gupta et al., 1979; Dabrowiak et al., 1979; Burger et al., 1979).

The available evidence supports a mechanism whereby a complex of DNA–bleomycin–chelated ferrous ion and molecular oxygen is formed. Oxidation of the bound ferrous ion occurs in this complex with concomitant reduction of oxygen to produce a reactive species (Burger et al., 1979; Povirk et al., 1979; Huang et al., 1980; Suguira, 1980). However, the precise details of the strand scission reaction have yet to be elucidated.

Strand scission by bleomycin is known to occur preferentially at specific sequences. D'Andrea & Haseltine (1978) and Takeshita et al. (1978) have demonstrated that under conditions of limited cleavage, DNA breaks occur preferentially at guanosine–cytidine (GC) and guanosine–thymidine (GT)

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