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Characterization of Novikoff Hepatoma mRNA Methylation and Heterogeneity in the Methylated 5′ Terminus†

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ABSTRACT: KOH digestion of methyl-labeled poly(A)$^+$ mRNA purified by (dT)-cellulose chromatography produced mononucleotide and multiple peaks of a large oligonucleotide (−6 to −8 charge) when separated on the basis of charge by Pellionex-WAX high-speed liquid chromatography in 7 *M* urea. Heat denaturation of the RNA before application to (dT)-cellulose was required to release contaminants (mostly 18S rRNA) that persisted even after repeated binding to (dT)-cellulose at room temperature. Analysis of the purified poly(A)$^+$ mRNA by enzyme digestion, acid hydrolysis, and a variety of chromatographic techniques has shown that the mononucleotide (53%) is due entirely to N^6-methyladenosine. The large oligonucleotides (47%) were found to contain 7-methylguanosine and the 2′-*O*-methyl derivatives of all four nucleosides. No radioactivity was found associated with the poly(A) segment. Periodate oxidation of the mRNA followed by β elimination released only labeled 7-methylguanine consistent with a blocked 5′ terminus containing an unusual 5′–5′ bond. Alkaline phosphatase treatment of intact mRNA had no effect on the migration of the KOH produced oligonucleotides on Pellionex-WAX. When RNA from which 7-methylguanine was removed by β elimination was used for the phosphatase treatment, distinct dinucleotides (NmpNp) and trinucleotides (NmpNmpNp) occurred after KOH hydrolysis and Pellionex-WAX chromatography. Thus Novikoff hepatoma poly(A)$^+$ mRNA molecules can contain either one or two 2′-*O*-methylnucleotides linked by a 5′–5′ bond to a terminal 7-methylguanosine and the 2′-*O*-methylation can occur with any of the four nucleotides. The 5′ terminus may be represented by $m^7G^{5'}ppp^{5'}$ $(Nmp)_{1or2}Np$, a general structure proposed earlier as a possible 5′ terminus for all eucaryotic mRNA molecules (Rottman, F., Shatkin, A., and Perry, R. (1974), *Cell 3,* 197). The composition analyses indicate that there are 3.0 N^6-methyladenosine residues, 1.0 7-methylguanosine residue, and 1.7 2′-*O*-methylnucleoside residues per average mRNA molecule.

Only in recent years has considerable understanding of the structure and composition of eucaryotic mRNA been obtained. The existence of a poly(A)[1] tract not only accounts for a portion of the large untranslated region but also has greatly facilitated the isolation of pure mRNA from cells. More recently, poly(A)$^+$ mRNA has been found

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[1] Abbreviations used are: poly(A), poly(adenylic acid); Am, 2′-*O*-methyladenosine; Gm, 2′-*O*-methylguanosine; m^7G, 7-methylguanosine; m^6A, 6-methyladenosine; poly(A)$^+$ mRNA, mRNA containing poly(A); poly(A)$^-$ RNA, RNA lacking poly(A).

to contain a low level of methylated nucleotides (Perry and Kelley, 1974) and the distribution of methylation is distinct from rRNA and tRNA (Desrosiers et al., 1974). The finding of a simple distribution of base methylnucleosides in cellular mRNA is interesting since many different species of messenger coding for many proteins were actually present in the analyzed material (Desrosiers et al., 1974).

The ability to synthesize high specific activity methylated viral mRNA in vitro using virion associated RNA polymerase has facilitated the characterization of the viral mRNA methylated nucleotides. In these in vitro systems, the methylation occurs exclusively as a large alkali-stable oligonucleotide with a charge estimated to be −5 to −6 by DEAE chromatography in 7 *M* urea (Urushibara et al., 1975; Wei and Moss, 1975; Furuichi et al., 1975; Rhodes et al., 1974; Furuichi, 1974). Furuichi et al. (1975) have analyzed this large oligonucleotide of reovirus mRNA synthesized in vitro and found that it contains 7-methylguanosine and 2′-*O*-methylguanosine in approximately equal proportions. After labeling with [β,γ-^{32}P]GTP, the large oligonucleotide was found to be blocked with respect to removal of ^{32}P by alkaline phosphatase. Periodate oxidation followed by reduction with [^{3}H]borohydride labeled the 7-methylguanosine of the large oligonucleotide, consistent with the structure originally postulated by Rottman et al. (1974) to be present on the 5′ end of eukaryotic mRNA. Furuichi et al. (1975) proposed the structure m^{7}G$^{5'}$ppp$^{5'}$GmpNp for the 5′ terminus of reovirus mRNA. Wei and Moss (1975) have analyzed the vaccinia virus mRNA made in vitro and have concluded that the 5′ terminus consists of a similar structure, only the 2′-*O*-methylnucleoside is a mixture of Gm and Am. An analogous structure m$_3^{2,2,7}$G$^{5'}$pp$^{5'}$AmpUmp has been reported for the 5′ terminus of certain low molecular weight nuclear RNAs (Ro-Choi et al., 1974, 1975).

Quantitative analysis of the methylated components of cellular mRNA has progressed less rapidly. This is probably due at least in part to the greater complexity of methylation. About 50% has been found in N^6-methyladenosine that runs as a mononucleotide after KOH hydrolysis (Desrosiers et al., 1974; Perry et al., 1975). This mononucleotide has not yet been observed with viral mRNAs synthesized in vitro. Its presence has recently been reported in SV-40 specific mRNA of infected cells (Lavi and Shatkin, 1975) but Abraham et al. (1975) have reported its total absence in VSV specific mRNA of infected cells. A dinucleotide peak has also been observed in the poly(A)$^+$ mRNA fractions of L cells (Perry et al., 1975) and HeLa cells (Wei et al., 1975) but Perry et al. (1975) suspected this may have been due to rRNA contamination, since the dinucleotide peak was not observed in cells that were labeled with a dose of actinomycin D that suppresses rRNA synthesis. As with viral mRNA made in vitro, a large oligonucleotide was also observed in these cellular mRNAs. Perry et al. (1975) have presented evidence that the oligonucleotide is derived from a blocked 5′ terminus, since it was resistant to phosphorylation by polynucleotide kinase even after phosphatase treatment and phosphatase treatment only reduced its charge by two units. Wei et al. (1975) have found that periodate oxidation and β elimination remove the 7-methylguanosine from the large oligonucleotide and have concluded that a 5′–5′ bond similar to the viral 5′ terminus is also present.

In the characterization of methyl-labeled Novikoff hepatoma poly(A)$^+$ mRNA, we have found radioactive contamination with other species that cannot be eliminated by standard room temperature (dT)-cellulose procedures. We present evidence that the only methylated constituents of the poly(A)$^+$ mRNA are the base-modified mononucleotide and the heterogeneous large oligonucleotide produced by alkaline hydrolysis. Extensive compositional and structural analyses are shown including depurination by acid hydrolysis under conditions that preserve 7-methylguanine structure and do not allow conversion of 1-methyladenine to N^6-methyladenine, and periodate oxidation followed by β elimination of 7-methylguanosine from the blocked 5′ terminus.

Materials and Methods

(*a*) *Materials.* Cell culture materials were purchased from Grand Island Biologicals Co. L-[*methyl*-^{3}H]Methionine (5–8 Ci/mmol) was obtained from Amersham Searle. Chromatographically pure proteinase K was purchased from EM laboratories (Elmsford, N.Y.). Methylated bases and nucleosides used as standards in chromatography were obtained from Sigma Chemical Co. and Calbiochem. Oligo(dT)-cellulose was prepared as described by Gilham (1964). Sigma urea was passed over a mixed bed ion exchange resin before use. AL-Pellionex-WAX was purchased from Reeve Angel. [^{14}C]Poly(A) was prepared using polynucleotide phosphorylase as described (Rottman and Heinlein, 1968).

(*b*) *Cell Culture and Preparation of Methyl-Labeled RNA.* Novikoff hepatoma cells (N1S1 strain) were maintained in Swimm's S-77 medium with 10% calf serum essentially as described (Desrosiers et al., 1974). Cells in late-log phase were harvested and resuspended at approximately 10^6 cells/ml in fresh warm medium containing 10% calf serum and the normal methionine concentration (0.1 m*M*). Labeling (generally 5 mCi/200 ml of cells) was performed with L-[*methyl*-^{3}H]methionine for 13 hr in the presence of 20 m*M* sodium formate and 40 μM of adenosine and guanosine to suppress purine ring labeling. No radioactivity was detected in adenosine or guanosine.

At the completion of labeling, cells were pelleted and washed once with a balanced salt solution. After swelling for 8 min in hypotonic buffer (10 m*M* Tris-HCl (pH 7.4)–10 m*M* NaCl–1.5 m*M* $MgCl_2$), the cells were disrupted by dounce homogenization. Nuclei were removed by centrifugation at 800*g* for 2 min and mitochondria at 10,000*g* for 7 min. The supernatant was made 0.1 *M* NaCl, 0.01 *M* EDTA, and 0.5% dodecyl sulfate and the RNA was isolated with a proteinase K digestion and phenol–chloroform extraction as previously described (Desrosiers et al., 1974; Singer and Penman, 1973).

(*c*) *Oligo(dT)-Cellulose Chromatography.* RNA containing a poly(A) segment was isolated by two passes through (dT)-cellulose essentially as described (Desrosiers et al., 1974). RNA was applied to the column in 0.12 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.4), 1 m*M* EDTA, and 0.2% dodecyl sulfate at room temperature and the RNA lacking a poly(A) segment was eluted by continued washing with this buffer. The material retained by the column was then collected by elution with the same buffer lacking NaCl. The material eluted by buffer without NaCl in the first pass was chromatographed a second time on (dT)-cellulose. Generally, 80% or more of the radioactivity eluted as poly(A)$^+$ RNA when passed a second time through the column. Approximately 0.5% of the original radioactivity present in total cytoplasmic RNA was recovered in the final poly(A)$^+$ mRNA.

For studies on the effect of heat denaturation on reten-

tion of RNA on (dT)-cellulose, the poly(A)$^+$ RNA isolated by two passes through (dT)-cellulose was dissolved in 0.2% dodecyl sulfate, heated to 60° for 2 min, and chilled rapidly in an ice bath. An equal volume of 0.24 *M* NaCl, 0.02 *M* Tris-HCl (pH 7.4), and 2 m*M* EDTA was added and the RNA was rechromatographed on (dT)-cellulose at room temperature as above.

(*d*) *KOH Hydrolysis and Pellionex-WAX Chromatography.* Alkaline hydrolysis was performed in a volume of 0.5 ml of 0.4 *N* KOH overnight at 37°. The solution was neutralized with perchloric acid in the cold and the insoluble $KClO_4$ removed by centrifugation. The supernatant was lyophilized prior to chromatographic analyses.

The nucleotides produced by alkaline hydrolysis were separated on the basis of net negative charge by high speed liquid chromatography in 7 *M* urea on Pellionex-WAX, a weak anion exchanger. This system was found to separate principally on the basis of charge since purine and pyrimidine oligonucleotide markers eluted identically and the 16 dinucleotides produced by KOH digestion of methyl-labeled rRNA eluted in a narrow band with −3 charge. The alkaline hydrolysate from above was dissolved in 0.005 *M* sodium phosphate–7 *M* urea at pH 7.8 along with oligonucleotide markers and injected onto the Pellionex-WAX column. A 100-ml linear gradient of 0–0.2 *M* $(NH_4)_2SO_4$ in 0.005 *M* sodium phosphate–7 *M* urea at pH 7.8 was used to develop the column. The eluate was passed through a Gilford uv monitor for location of the markers and collected either in test tubes or directly in scintillation vials. The mononucleotide (−2) and dinucleotide (−3) were located from the uv absorbance of the hydrolyzed RNA while oligouridylate markers were used for the location of more highly charged species.

When particular fractions were to be recovered from Pellionex-WAX and further analyzed, the nucleotide was bound to charcoal to remove salt and urea and the nucleotide eluted with 10% pyridine in water (Dlugajczyk and Eiler, 1966). The mononucleotide was dephosphorylated by treatment with alkaline phosphatase for 16 hr at 37°. The dinucleotide and large oligonucleotide were degraded to nucleosides by treatment with pancreatic ribonuclease, snake venom phosphodiesterase, and alkaline phosphatase (Pike and Rottman, 1974).

(*e*) *Enzyme and Acid Hydrolysis and Analysis of the Products.* Techniques for the enzymatic degradation of RNA to nucleosides as well as separation into 2′-*O*-methylnucleoside and base-methylnucleoside fractions by DEAE-borate chromatography and analysis by Aminex high-speed liquid chromatography have been completely described (Pike and Rottman, 1974; Desrosiers et al., 1974). The inclusion of pancreatic ribonuclease in the digestion mixture has been found to be unnecessary and was frequently left out in these studies.

Acid hydrolysis was performed with 88% formic acid at 100° for 2 hr in sealed Pyrex tubes as described (Munns et al., 1974). These conditions were chosen since complete depurination without ring opening of 7-methylguanine or conversion of 1-methyladenine to N^6-methyladenine has been reported to occur (Munns et al., 1974). The hydrolysates were dried under a stream of N_2 and used for analysis by Aminex A-5 high speed liquid chromatography. The column was developed with 0.4 *M* ammonium formate (pH 5.6). Unhydrolyzed material (from 2′-*O*-methylnucleotides) eluted in the solvent front and excellent resolution was obtained among the purine bases studied. The radioactivity present as 2′-*O*-methylnucleotides was determined by adding the amount present in the solvent front in Aminex chromatography to the radioactivity lost by conversion of the 2′-*O*-methyl to methanol (Baskin and Dekker, 1967).

(*f*) *Isolation of the Poly(A) Segment.* [^{3}H]RNA was mixed with a known amount of [^{14}C]poly(A) and digested with 5 μg/ml of pancreatic ribonuclease and 6 units/ml of T1 ribonuclease in 0.3 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.4), and 0.01 *M* EDTA for 60 min at 37°. Upon completion, dodecyl sulfate was added to 0.5% and a brief proteinase K digestion was performed to remove nuclease activity. The poly(A) segment was isolated by phenol–chloroform extraction, ethanol precipitation, and chromatography on (dT)-cellulose. The yield of nuclease-resistant ^{3}H radioactivity was corrected to obtain the amount in poly(A) based on the recovery of [^{14}C]poly(A). The recovery of [^{14}C]poly(A) was usually 50–60%. Poly(A) isolated in this manner from 12-hr adenosine-labeled mRNA has a size of 140–200 nucleotides estimated by 10% polyacrylamide gel electrophoresis.

(*g*) *Periodate Oxidation and β Elimination.* Periodate oxidation and β elimination were performed as described by Fraenkel-Conrat and Steinschneider (1967). After cleavage of terminal residues that contained free 2′ and 3′ hydroxyls, the RNA was precipitated with ethanol. The alcohol supernatant was dried and the residue acid hydrolyzed as described above to identify the species released by β elimination. Half of the RNA precipitate was used for direct KOH hydrolysis and the other half was treated with alkaline phosphatase (0.15 mg/ml) for 2 hr at 45° in 0.05 *M* ammonium formate–0.002 *M* $MgCl_2$ (pH 9) prior to KOH hydrolysis and Pellionex-WAX chromatography.

Results

The methyl-labeled Novikoff mRNA purified by repeated binding to (dT)-cellulose appeared pure by aqueous sucrose gradient centrifugation and further (dT)-cellulose chromatography. Other findings suggested to us that in fact it was contaminated with other RNA species. Aqueous sucrose gradient centrifugation in 10 m*M* Tris-HCl (pH 7.4) and 5 m*M* EDTA revealed no hint of rRNA or tRNA contamination in the broad mRNA profile, but sedimentation in Me_2SO–sucrose under denaturing conditions produced a rather sharp peak at 18 S superimposed on a heterogeneous profile (Desrosiers et al., 1974). Furthermore, we discovered that when poly(A)$^+$ mRNA was recovered from Me_2SO–sucrose gradients, the expected 100% binding to (dT)-cellulose was no longer achieved. Figure 1A shows that approximately 95% of the labeled mRNA elutes as poly(A)$^+$ RNA when passed a third time through (dT)-cellulose at room temperature. When the mRNA isolated by two passes was first heat denatured as described in Materials and Methods before room temperature application to (dT)-cellulose, only about 70% was retained as poly(A)$^+$ material (Figure 1B).

To characterize the RNA released by heat denaturation as non-messenger contaminants, the poly(A)$^+$ and the poly(A)$^-$ RNA isolated by heat denaturation and (dT)-cellulose chromatography (heat-denatured poly(A)$^+$ and heat-denatured poly(A)$^-$) were analyzed by Me_2SO–sucrose gradient centrifugation. The heat-denatured poly(A)$^-$ RNA produced three peaks that correspond to the cytoplasmic 4S, 18S, and 28S RNAs (Figure 2B). The 18S peak always predominated and in some preparations made up over 80% of the heat-released radioactivity. We assume

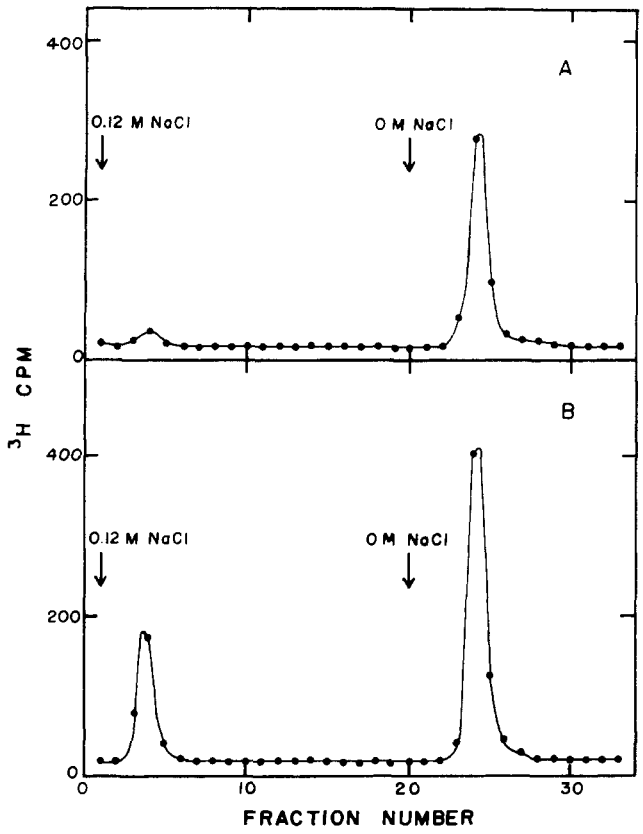

FIGURE 1: The effect of heat denaturation on the elution of poly(A)$^+$ RNA from (dT)-cellulose. Poly(A)$^+$ RNA that had been isolated by two passes through (dT)-cellulose as described in Materials and Methods was rechromatographed on (dT)-cellulose after: (A) no treatment; (B) heating to 60° for 2 min in 0.2% dodecyl sulfate.

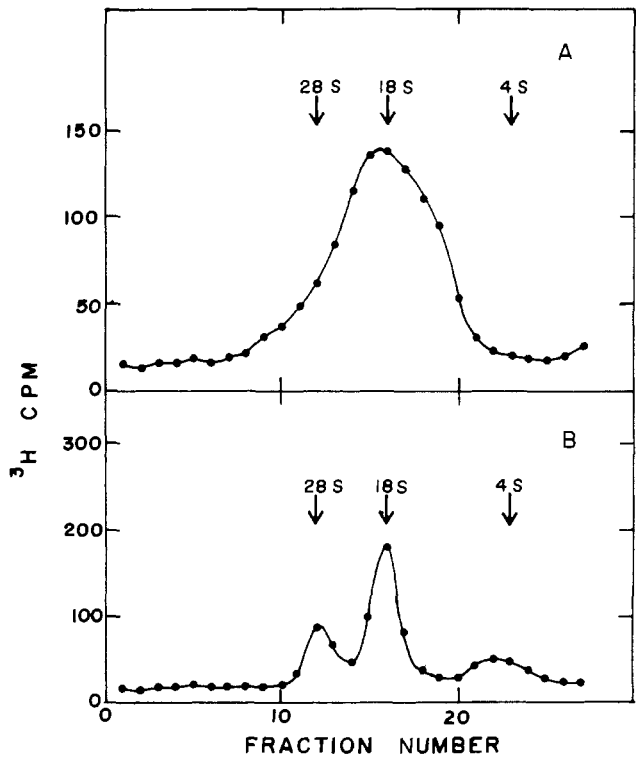

FIGURE 2: Me_2SO-sucrose gradient centrifugation of poly(A)$^+$ and poly(A)$^-$ RNA obtained from heat denaturation and (dT)-cellulose of poly(A)$^+$ RNA. The poly(A)$^-$ and poly(A)$^+$ fractions from Figure 1B were pooled and precipitated in ethanol. An aliquot of each RNA fraction was made 90–95% Me_2SO in 10 m*M* LiCl–1 m*M* EDTA and heated to 60° for 2 min. All of this material was layered over 4.8 ml of a linear 5–20% sucrose gradient in 99% Me_2SO (10 m*M* LiCl–1 m*M* EDTA). Centrifugation was for 14 hr at 26° and 45,000 rpm in a SW 50.1 rotor. Marker RNA was run in parallel tubes. (A) Heat-denatured poly(A)$^+$; (B) heat-denatured poly(A)$^-$

this peak is due to 18S rRNA contamination. The heat-denatured poly(A)$^+$ RNA sedimentation profile in Me_2SO–sucrose (Figure 2A) reveals no sharp peak at 18 S and retains the size distribution expected of mRNA.

Alkaline hydrolysis of RNA produces mainly mononucleotide, but a ribose 2′-*O*-methyl group confers alkali stability on the adjacent phosphodiester bond. A dinucleotide (−3 charge) is thus produced by alkaline hydrolysis for each 2′-*O*-methylnucleotide while a mononucleotide (−2 charge) is produced for each base methylnucleotide. Alkaline hydrolysis followed by identification of the charged species produced is an efficient method of characterization of methyl-labeled RNA since most known RNAs have distinctive patterns of methylation. While rRNA methylation is approximately 90% 2′-*O*-methylation, tRNA is approximately 80% base methylation (Desrosiers et al., 1974, Munns et al., 1974). Alkaline hydrolysis of 18S + 28S rRNA from the poly(A)$^-$ RNA of the first (dT)-cellulose pass produced 82.6% CH_3-labeled dinucleotide and 9.9% mononucleotide (Figure 3A). Mammalian rRNA is thought to contain two sequences of two adjacent 2′-*O*-methylnucleotides and one sequence of three adjacent 2′-*O*-methylnucleotides (Choi and Busch, 1974; Slack and Loening, 1974; Maden and Salim, 1974). The resolving power of the Pellionex-WAX column can be seen by the elution of radioactivity in the trinucleotide and tetranucleotide region of Figure 3A.

Figure 3B shows that KOH hydrolysis of poly(A)$^+$ mRNA isolated without heat denaturation produced mononucleotide, dinucleotide, and large oligonucleotides similar to what has been observed in L cells (Perry et al., 1975) and HeLa cells (Wei et al., 1975). The amount observed as dinucleotide in Novikoff hepatoma cells has varied with the preparation (18%, cf. Figure 3B) but appears slightly higher than that reported for the other two cell lines mentioned above. Note that the large oligonucleotides elute as a series of three peaks of charge −6, −7, and −8, with the predominant species being the −7 charged oligonucleotide. This is, on the average, at least one charge unit larger than has been observed by others for the large oligonucleotides of methylated viral mRNA (Urushibara et al., 1975; Wei and Moss, 1975; Furuichi et al., 1975; Rhodes et al., 1974) and cellular mRNA (Perry et al., 1975; Wei et al., 1975) using DEAE-Sephadex chromatography in 7 *M* urea. The reason for the observed differences between Pellionex-WAX and DEAE-Sephadex is presently uncertain. The Pellionex-WAX profile obtained after KOH hydrolysis of heat-denatured poly(A)$^+$ mRNA (Figure 3D) reveals that almost all the dinucleotide peak is removed by the heat-denaturation procedure. The ratio of counts in mononucleotide to large oligonucleotides is 53:47. In the profile obtained from heat-denatured poly(A)$^-$ RNA (Figure 3C), approximately 66% of the radioactivity elutes as dinucleotide and 34% as mononucleotide. 18S rRNA is known to contain a higher percentage of base methylnucleotides than 28S rRNA (Maden and Salim, 1974) and any contribution from tRNA would be mostly as mononucleotides. The elution profile of the heat-denatured poly(A)$^-$ RNA is thus consistent with it being rRNA and tRNA contamination.

Previously our analyses of 3-hr labeled mRNA using enzyme digestion yielded 50% base and 50% ribose methyl-

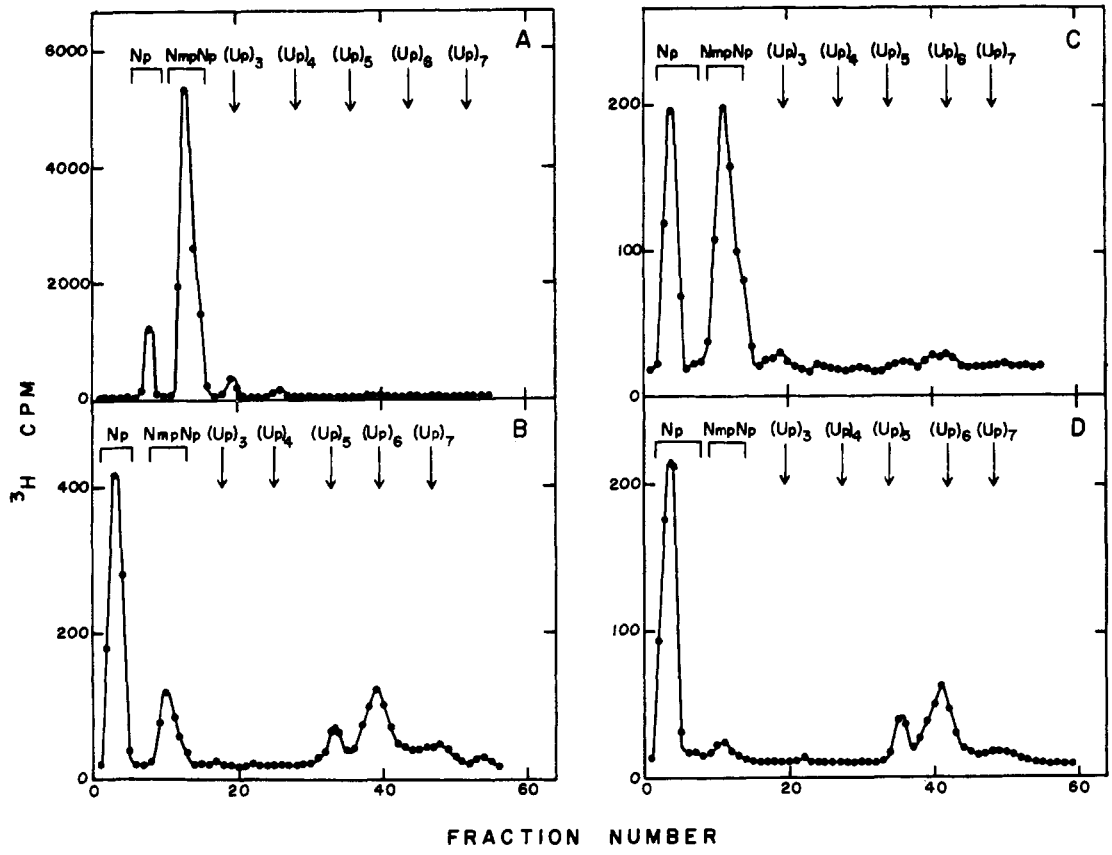

FIGURE 3: Pellionex-WAX high speed liquid chromatography of KOH hydrolysates. The KOH hydrolysates of each RNA fraction were dissolved in 0.005 *M* sodium phosphate–7 *M* urea at pH 7.8 and injected onto a 53-cm Pellionex-WAX column made from ⅛-in. stainless steel tubing. The column was developed at room temperature under 600 lb maximum pressure with a 100-ml 0–0.2 *M* $(NH_4)_2SO_4$ gradient in 0.005 *M* sodium phosphate–7 *M* urea at pH 7.8; 20 drop fractions (~1.0 ml) were collected. (A) 28S + 18S rRNA: isolated from a sucrose gradient of the poly(A)$^-$ material of the first (dT)-cellulose pass. (B) Poly(A)$^+$ RNA: isolated by two passes through (dT)-cellulose with no heat denaturation of the RNA. (C) Heat-denatured poly(A)$^-$ RNA: RNA that elutes as poly(A)$^-$ when poly(A)$^+$ RNA isolated by two (dT)-cellulose passes is heat denatured before a third cycle through (dT)-cellulose. (D) Heat-denatured poly(A)$^+$ RNA: RNA that elutes as poly(A)$^+$ when poly(A)$^+$ RNA isolated by two (dT)-cellulose passes is heat denatured before a third cycle through (dT)-cellulose.

ation. The base methylnucleosides identified were N^6-methyladenosine (40%), N^6,N^6-dimethyladenosine (~2.5%), and an unidentified component (7.5%). The unidentified component was probably 7-methylguanosine since the digestion conditions cause ring opening of 7-methylguanosine and the unidentified component migrated with ring-opened 7-methylguanosine. The 2′-*O*-methylnucleosides were distributed among all four species but heat denaturation was not used in the isolation procedure. The methylnucleoside composition of the 13-hr labeled poly(A)$^+$ mRNA obtained by heat denaturation was determined using enzyme digestion and acid hydrolysis techniques. The results are summarized in Table I. N^6,N^6-Dimethyladenosine was no longer detected in the mRNA, consistent with the removal of 18S rRNA contamination. 18S rRNA is known to contain two such moieties (Maden and Salim, 1974). The methylated species present in 13-hr labeled mRNA are the same as found in 3-hr labeled mRNA. The lower value for percent *O*-methylation reported here is due to the removal of residual contamination by the heat treatment–(dT)-cellulose procedure.

Acid hydrolysis proved useful in further analyses since the conditions did not ring open 7-methylguanosine and did not convert 1-methyladenosine to the N^6 derivative. Control digestions with methyl-labeled tRNA revealed the expected percentage of 1-methyladenine and almost no N^6-methyladenine as has been previously reported (Munns et al., 1974).

Table I: Summary of Methylnucleoside Composition Analysis.

Species	Method of Hydrolysis	% of Total m^7G	% of Total m^6A	% of Total Nm
Poly(A)$^+$ mRNA[a]	Enzymes	9.5	48	42
Poly(A)$^+$ mRNA[a]	Acid	19	51	30
Poly(A)$^+$ mRNA[a]	β elimination	17		
Mononucleotide[b]	Enzymes		53	
Oligonucleotides[b]	Enzymes	14.5		32.5

[a] Analyses were performed with poly(A)$^+$ mRNA obtained with heat denaturation and (dT)-cellulose. The RNA contained approximately 4% dinucleotide contamination. [b] Analyses were performed with fractions isolated from Pellionex chromatography.

Figure 4 shows the separation of the bases released upon acid hydrolysis of the heat-denatured poly(A)$^+$ mRNA by Aminex A-5 high-speed liquid chromatography. The identity of N^6-methyladenine and 7-methylguanine were further confirmed by descending paper chromatography in 2-propanol, concentrated HCl, and H_2O (680:170:144). These results identify 7-methylguanine and N^6-methyladenine as the base-methylated species of poly(A)$^+$ mRNA. Determinations of the percent 7-methylguanosine by enzyme hydrolysis consistently yielded lower values than when measured by acid hydrolysis or by β elimination which will be

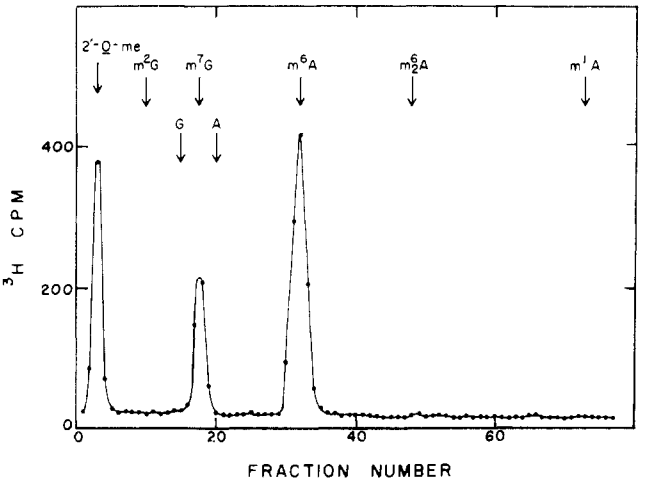

FIGURE 4:. Aminex A-5 high-speed liquid chromatography of poly(A)$^+$ mRNA acid hydrolysate. The acid hydrolysate of poly(A)$^+$ mRNA obtained by heat denaturation and (dT)-cellulose was dried under a stream of N_2, dissolved in column buffer, and applied to a 90-cm stainless steel Aminex A-5 column. The column was developed at 42°, 4200 lb maximum pressure with 0.4 *M* ammonium formate (pH 5.6); 20-drop fractions (~0.8 ml) were collected. The position of the arrows indicates the location of purine base markers.

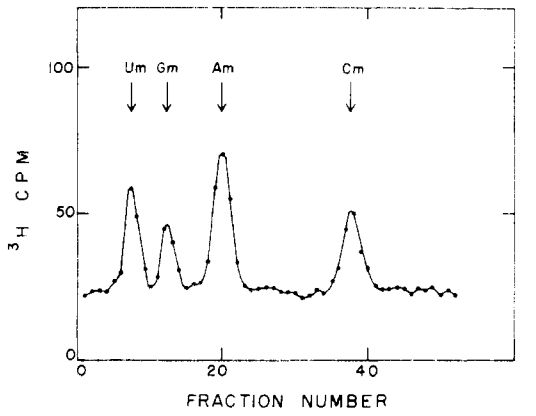

FIGURE 5: Aminex A-5 high-speed liquid chromatography of the 2'-*O*-methylnucleoside fraction of the large oligonucleotides. The large oligonucleotides of poly(A)$^+$ RNA were recovered from Pellionex chromatography as in Figure 3B and enzymatically digested to the nucleside level. The nucleosides were separated into a 2'-*O*-methylnucleoside fraction and a base methylnucleoside fraction by DEAE-borate chromatography. The 2'-*O*-methylnucleoside fraction in column buffer was applied to a 90-cm Aminex A-5 column. The column was developed with 0.4 *M* ammonium formate and 40% ethylene glycol (pH 4.25) at 32° and 3200 lb maximum pressure; 13-drop fractions (~0.5 ml) were collected.

described below. A decreased recovery of the ring-opened form of 7-methylguanosine has been previously reported (Hall, 1971) and a similar phenomenon is probably responsible for the low values obtained here using the enzyme digestion procedure. Thus the value of 17–19% 7-methylguanosine determined by acid hydrolysis and by β elimination is most probably correct. Analysis of the 2'-*O*-methylnucleosides of heat-denatured poly(A)$^+$ mRNA produced by enzyme digestion revealed the presence of all four ribose-methylated species (Um, Gm, Am, Cm) (data not shown).

The mononucleotide and all large oligonucleotides were recovered from Pellionex-WAX chromatography (Figure 3) and enzymatically digested to nucleosides. The dephosphorylated mononucleotide was composed entirely of N^6-methyladenosine and this accounted for all of the N^6-methyladenosine present in the poly(A)$^+$ mRNA. This agrees well with the findings of Perry et al. (1975) for the mononucleotide of L cell mRNA. Analysis of the large oligonucleotides after digestion revealed that they were approximately one-third 7-methylguanosine and two-thirds 2'-*O*-methylnucleosides. The distribution of 2'-*O*-methylnucleosides obtained from the large oligonucleotide (Figure 5) agrees well with the 2'-*O*-methylnucleoside distribution obtained when entire heat-denatured poly(A)$^+$ mRNA was analyzed. This heterogeneity in 2'-*O*-methylnucleosides contrasts with the distinct species observed with viral mRNAs (Furuichi et al., 1975; Wei and Moss, 1975; Urushibara et al., 1975) and probably reflects the heterogeneous population of messengers being analyzed.

The high percentage of N^6-methyladenosine in mRNA suggested to us that at least part of these moieties could be present in the poly(A) segment (Desrosiers et al., 1974). A nuclease digestion was thus performed on methyl-labeled mRNA using conditions that are known to leave the poly(A) segment intact. Less than 2% of the total methyl radioactivity was found associated with the poly(A) segment and we conclude that the poly(A) is devoid of any methylated species (data not shown). This conclusion has also been reached by Perry et al. (1975) for L cell mRNA.

Periodate oxidation of RNA and β elimination of the oxidized nucleoside require that both 2' and 3' hydroxyls be free. Since the poly(A) tract occupies the 3' end of each of these RNA molecules, β elimination of radioactive nucleoside from methyl-labeled poly(A)$^+$ mRNA would occur if a methylated 5'–5' structure were present at the 5' terminus. Furthermore, β elimination of such a blocking group should free the phosphate in the original 5'–5' bond for removal by alkaline phosphatase. When periodate oxidation and β elimination were performed on heat-denatured poly(A)$^+$ mRNA, 17% of the radioactivity was released from the polynucleotide chain and greater than 95% of this material was recovered as 7-methylguanine upon acid hydrolysis (Figure 6).

Incubation of the methyl-labeled heat-denatured poly(A)$^+$ mRNA (no β elimination) with alkaline phosphatase prior to KOH hydrolysis resulted in the same alkali-stable oligonucleotides as previously shown when the RNA received no phosphatase treatment (Figure 7A). Thus phosphatase treatment of mRNA had no effect on the migration of the KOH-resistant large oligonucleotides. KOH hydrolysis of the RNA after β elimination (7-methylguanosine removed) should have produced an increased negative charge of the large oligonucleotides of one unit. The migration of the large oligonucleotides actually changed by more than this amount (Figure 7B), consistent with the somewhat anomalous migration of NTPs observed on Pellionex-WAX chromatography (R. Derosiers, unpublished observation). For example, GTP was found to elute three negative charges greater than authentic G$^{5'}$ppp$^{5'}$G rather than the expected one charge difference (data not shown). The small amount of radioactivity remaining in the $(Up)_6$ region in Figure 7B is probably due to incomplete β elimination, but at least 90% of the oligonucleotides are of the blocked type. When RNA obtained from β elimination (7-methylguanosine removed) was treated with alkaline phosphatase prior to KOH hydrolysis, radioactivity then eluted in the distinct dinucleotide (NmpNp) and trinucleotide (NmpNmpNp) regions (Figure 7C). These di- and trinucleotides accounted for 95% of the oligonucleotides of increased charge after β elimination seen in Figure 7B. No te-

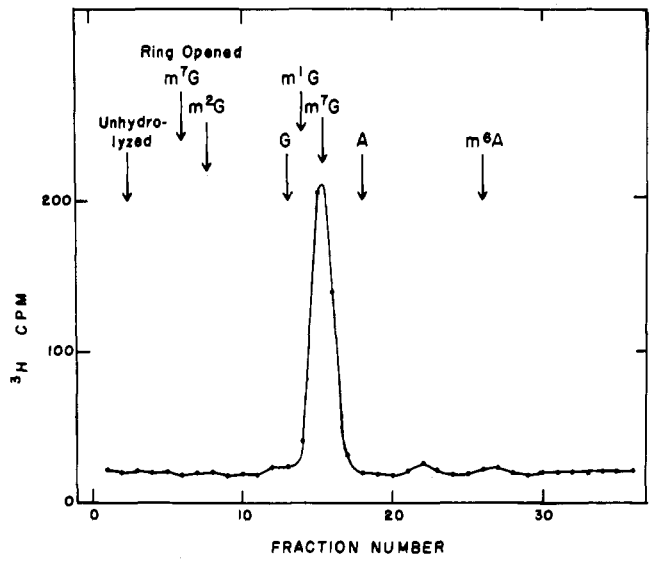

FIGURE 6: Aminex A-5 high-speed liquid chromatography of the material removed by β elimination. Heat-denatured poly(A)$^+$ mRNA was used for periodate oxidation and β elimination. The remaining polynucleotide was precipitated with ethanol and the supernatant dried and analyzed by acid hydrolysis and Aminex A-5 chromatography as described in Materials and Methods and the legend to Figure 4. The arrows indicate the position of purine base markers.

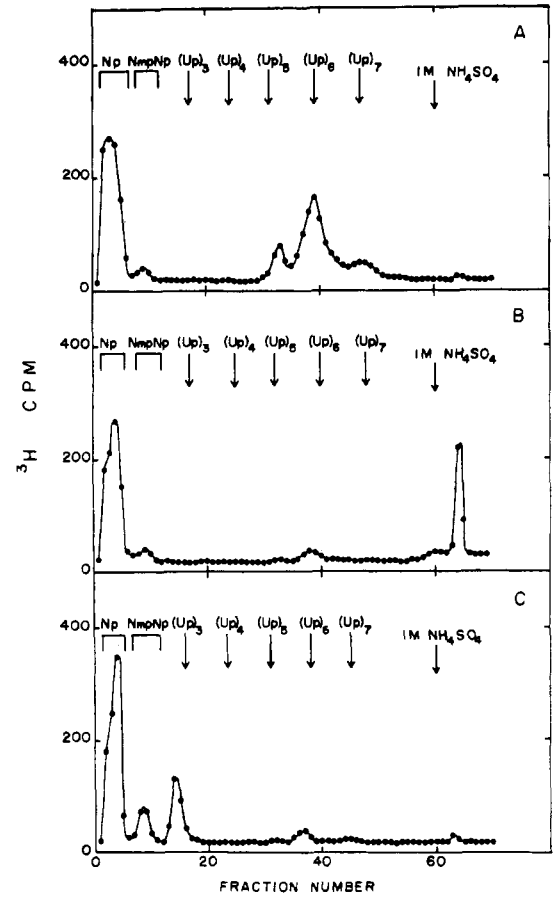

FIGURE 7: Pellionex-WAX chromatography of KOH hydrolysates of poly(A)$^+$ mRNA following β elimination and/or alkaline phosphatase treatment. The conditions of chromatography were the same as described in the legend to Figure 3. (A) Heat-denatured poly(A)$^+$ mRNA was treated with alkaline phosphatase (0.15 mg/ml for 2 hr at 45° in 0.05 *M* ammonium formate–0.002 *M* $MgCl_2$ (pH 9)) prior to KOH hydrolysis. (B) The RNA recovered from β elimination by ethanol precipitation was hydrolyzed with KOH. (C) The RNA recovered from β elimination was treated with alkaline phosphate (0.15 mg/ml for 2 hr at 45° in 0.05 *M* ammonium formate–0.002 *M* $MgCl_2$ (pH 9)) prior to KOH hydrolysis.

tranucleotide was observed; 2.6 times more radioactivity was shifted to the trinucleotide region than to the dinucleotide region, indicating there are 1.3 times more structures containing two 2′-*O*-methylnucleotides than there are with one 2′-*O*-methylnucleotide.

Discussion

Several lines of evidence may be used to show that the material released by heat denaturation is primarily 18S rRNA. These include Me_2SO–sucrose gradient centrifugation, the high level of N^6,N^6-dimethyladenosine, and the oligonucleotide patterns after KOH hydrolysis. The interaction of the RNA contaminants with the poly(A)$^+$ mRNA must be fairly strong since the contaminants are not evident in nondenaturing aqueous sucrose gradient centrifugation or (dT)-cellulose chromatography run without prior heat denaturation of the RNA. A contamination of less than 5% could result in a 25% radioactive contamination when a methyl labeling is used since the level of methylation in the mRNA is quite low (Perry et al., 1975). It should be emphasized that repeated passage of poly(A)$^+$ mRNA through (dT)-cellulose without prior denaturation of the RNA does not remove the RNA contamination.

Using values we have obtained here for the methylnucleoside composition of poly(A)$^+$ mRNA and a value for the number of methyl groups in an average mRNA molecule derived from Perry et al. (1975), it is possible to calculate the frequency of occurrence of each methylnucleoside. We have done this in Table II using a value of 5.7 methyl groups per average messenger (Perry et al., 1975). A considerable number of assumptions are used in making such a calculation but the result does allow for several interesting observations. There is sufficient 7-methylguanosine for each mRNA molecule to have one such species and for each 7-methylguanosine there are three N^6-methyladenosine residues. The amount of 2′-*O*-methylnucleosides does not seem to be an integral number when compared to the other two methylated species but actually occurs in an amount expected if there are 1.3 times more 5′ structures with two 2′-*O*-methylnucleosides than with one 2′-*O*-methylnucleoside. The results obtained from Figure 7C indicate that indeed there are 1.3 times more structures with two 2′-*O*-methylnucleotides. Although the results do not conclusively show that all poly(A)$^+$ mRNA molecules have such a methylnucleoside composition, the numbers we have obtained are consistent with it. Perry et al. (1975) have observed a great-

Table II: Distribution of Methylated Species in an Average Poly(A)$^+$ mRNA Molecule.

Species	Fraction of Total[a]	×	Total number of Methyls per Average Messenger Molecule[b]	=	Number of Each Species Per Average Messenger Molecule
m^{7}G	0.18		5.7		1.0
m^{6}A	0.52		5.7		3.0
2′Nm	0.30		5.7		1.7

[a] The numbers used in this column are composite values obtained from Pellionex chromatography and Table I. [b] The value of 5.7 was derived from the work of Perry and Kelley (1974) and Perry et al. (1975).

er frequency of methylation per 1000 nucleotides in the smaller mRNA molecules, as would be the case if all mRNA molecules had either five or six methyl groups.

Two factors are involved in the heterogeneity we have observed in the 5′-terminal structure: the 2′-*O*-methylation occurs with the four common nucleosides (Um, Gm, Am, Cm) and the 5′ structure may contain either one or two 2′-*O*-methylnucleotides. The exact nature of each of the large oligonucleotide peaks obtained from direct KOH hydrolysis of the poly(A)$^+$ mRNA and Pellionex-WAX chromatography is not entirely certain. Three peaks were consistently observed and there is no simple way to correlate their percentages with the percentages observed as dinucleotide (NmpNp) and trinucleotide (NmpNmpNp) after β elimination, phosphatase treatment, and KOH hydrolysis. It seems possible that the unusual structure, the variable base composition, and the variable size (one vs. two 2′-*O*-methynucleotides) can completely account for the heterogeneous oligonucleotide pattern. If the heterogeneity is indeed due to the fact that a mixed population of messengers is being examined, methyl labeling and isolation of a specific mRNA should simplify the analysis.

Both et al. (1975) have recently found that the initiation and subsequent in vitro translation of viral mRNA are dependent on the presence of 7-methylguanosine in a blocked 5′ structure. It is thus possible that the methylation serves some important function in vivo in the translation of viral and cellular mRNA or its control. In addition, 2′-*O*-methylation may serve some role in the stabilization of certain RNA sequences to nuclease degradation. Although the maturation process of rRNA conserves only about 50% of the original molecule, the conserved regions retain all of the methylated sequences (Wagner et al., 1967). The presence of 2′-*O*-methylnucleotides stabilized synthetic RNA molecules against hydrolysis by a processing 3′-OH exoribonuclease (Stuart and Rottman, 1973) and Wei et al. (1975) have observed that the blocked 5′ terminus is resistant to 5′-nuclease action. Any increased stabilization caused by methylation may be important not only for nuclear processing but also for cytoplasmic turnover of the mRNA. In this latter regard, Singer and Penman (1973) have observed two populations of poly(A)$^+$ mRNA that were distinguished on the basis of half-life (7 hr and 24 hr) and it is interesting to speculate whether these populations are differentiated by the number of 2′-*O*-methylnucleotides at the 5′ terminus. Kinetic studies on the rate of appearance of the structures with one vs. two 2′-*O*-methylnucleotides should reveal whether termini containing one 2′-*O*-methylnucleotide are derived from mRNA that turns over more rapidly.

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