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The methylation state of poly A-containing-messenger RNA from cultured hamster cells

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ABSTRACT

The poly A-containing mRNA of cultured hamster (BHK-21) cells has been examined with regard to methylation status. Steady state-labeled mRNA was obtained by incubating cells for 20-22h in the presence of [methyl-^{3}H]-methionine and $^{32}P_i$. The degree of methylation of this RNA was 1.8 methyl groups per 1000 nucleotides, or 4-5 methyl groups on the average per molecule. The nature of the methylated residues was determined by paper chromatography and electrophoresis of acid and alkaline hydrolysates, by DEAE cellulose chromatography of alkaline hydrolysates and of T2 RNase digests, and by examining the effect of subjecting samples to "β-elimination." Approx. half of the methyl groups occurred in standard ("internal") linkage, 10% as m^5Cp and 40% as m^6Ap residues. The remainder occurred at least for the most part in "blocked" 5'-termini with the presumptive structure $m^7G(5')ppp(Nm)p..$, where Nm was Gm, m^6Am, Um, or Cm.

INTRODUCTION

Several workers have recently described messenger RNA (mRNA) fractions that are slightly but significantly methylated. Perry and coworkers have reported that mRNA from mouse (L) cells, isolated as the poly A-containing ["poly A(+)"] RNA of polyribosomes, contains 2.2 methyl groups per 1000 nucleotides, approx. half of which are on bases and half on ribose residues (ref. 1; also, as cited in ref. 2). A similar distribution between base and ribose methylation was reported for rat (Novikoff hepatoma) poly A(+) mRNA by Desrosiers et al[3], who characterized the most plentiful methylated nucleosides as 1-, and/or N^6-, methyladenosine. In addition, several animal viruses have been shown to catalyze the *in vitro* synthesis of mRNA containing "blocked" methylated 5'-termini, with the general structure $m^7G(5')pppNmp$ (refs. 4-6).

The present paper describes studies on the methylation status of the poly A(+) mRNA of cultured hamster (BHK-21) cells. This RNA was found to contain 1.8 methyl groups per 1,000 nucleotides. The major methylated bases were identified as 7-methylguanine and N^6-methyladenine. In addition, evidence was obtained for the presence of smaller amounts of m^5Cp and of

blocked methylated 5'-termini similar to those found in the viral mRNA's.

METHODS

Growth and labeling of cells were essentially as previously described.[7] Labeling periods of 20-22h were employed, and a low level (0.1μg/ml) of actinomycin D was added to suppress rRNA synthesis. Under these conditions incorporation into non-ribosomal RNA continued at a constant rate for 24-30h (see ref. 8).

RNA was prepared from mitochondrion-free cytoplasm, and fractionated by centrifugation in "standard salt" sucrose gradients.[7]

Column chromatography on oligo-T cellulose ("T3"; Collaborative Research, Inc.) was performed according to Hirsch and Penman.[9] The low-salt eluants from such columns will be referred to as poly A(+) RNA.

Chemical hydrolysis of RNA was accomplished by treatment with 1N HCl at 100° for 45 min. or 0.3 N NaOH at 37° for 18h. Digestion with RNase T2 (Sigma Chemical Co.) was performed as described by Furuichi et al (10), omitting prior denaturation of the RNA. Digestion with a mixture of venom phosphodiesterase and E. coli alkaline phosphatase (both from Worthington Biochemical Corp.) was carried out essentially according to Wei and Moss.[5]

Paper chromatographic systems were as follows: "System 1", 68% isopropanol, 17.6% 11.6N HCl, in H_2O; "System 4", nbutanol:H_2O, 86:14, in NH_3 atmosphere (both from ref. 11; run ascending, 26-28 cm, 16-18h). Thin layer chromatography was performed as described by Wei & Moss[5] (ethyl acetate:isopropanol:7.2 M NH_4OH:nbutanol, 3:2:2:1; run ascending, 18-20h, 20 cm). High voltage electrophoresis was performed in 0.05 N ammonium formate buffer, pH 3.5, using 110-cm Whatman 3MM sheets at 3000 V. Acid hydrolysates were spotted at the center of the paper and run for 2 to 3h, and alkaline hydrolysates were spotted near the cathode edge and run for 3h.

DEAE cellulose chromatography was performed essentially as described by Wei and Moss,[5] and nucleotides were recovered by barium chloride precipitation.[12]

"β-elimination" was performed as described by Fraenkel-Conrat and Steinschneider.[13]

Recoveries of methyl label after chemical or enzymatic hydrolysis of RNA were checked by summing the ^3H and ^{32}P after chromatography or electrophoresis, and comparing summed $^3H/^{32}P$ ratios with that of samples of intact RNA counted in parallel. These ratios agreed to ± 10%; indicating little or no preferential loss of methyl label (cf. ref. 3).

Counting of ^3H, ^{32}P-labeled samples was performed using two channels

of a Nuclear Chicago scintillation counter, adjusted so that spillover in either direction was less than 1%. Backgrounds were approx. 2.5 and 6 cpm for ^{3}H and ^{32}P respectively. In some analyses peaks of interest contained as few as 5-10 cpm of ^{3}H above background (e.g., Figs. 7,8); samples from such runs were counted for 3 to 7 10-min. intervals, together with appropriate backgrounds. To convert isotope ratios to molar ratios, a sample of tRNA from the same experiment was counted in parallel, and in exactly the same manner, as the mRNA samples.

Samples from sucrose gradients, and paper and "thin-layer" segments, were counted using "NCS" solubilizer-scintillator (Amersham-Searle). DEAE-cellulose column fractions were assayed for ^{3}H using "aquasol" (New England Nuclear Corp.). In some runs (e.g. Fig. 6) fractions were assayed for ^{32}P by Čerenkov radiation prior to counting aliquots in aquasol.

Methylated standards were obtained as follows: 1-, N^2-, N^2_2, and 7-methylguanine; 1-, 2-, N^6, and N^6_2-methyladenine; and m^6A and m^6_2A: from Cyclo Chemical Corp.; pm^7G, m^7G, and Am from P-L Biochemicals, Inc.; and Cm and Um from ICN Pharmaceuticals, Inc. ^{3}H-labeled BHK 28S rRNA (obtained by growing cells in the presence of [methyl-^{3}H]methionine) was also used as a source of methylated ribose standards. Approx. 85% of the methyl groups of this species is in 2'-O-methyl linkage (cf ref. 14), 94% of which appeared as a "-3" peak on DEAE cellulose chromatography after alkaline hydrolysis. Radioactivity recovered from this peak provided a standard for NmNp in acid hydrolysis (see Results) and also yielded a Gm standard on treatment with mixed alkaline phosphatase and phosphodiesterase.

RESULTS

Fig. 1 shows a representative density-gradient pattern for poly A(+) mRNA obtained from cells labeled for 22h with $^{32}P_i$ and [methyl-^{3}H]methionine. The RNA sedimented as a broad band centered at 18-20S. Aside from a small amount of residual low molecular weight material, the ^{3}H paralleled the ^{32}P, indicating a constant degree of methylation across the gradient. The average $^{3}H/^{32}P$ ratio of the mRNA was 2.1% that of a sample of gel-purified tRNA counted in parallel. Taking tRNA to contain 85 methyl groups per 1000 nucleotides (ref. 8) the value for mRNA was 1.8 per 1000. In a second experiment (cf Fig. 6) precisely the same value was obtained.

Fractions from the gradient of Fig. 1 corresponding to the 16S-30S region were pooled, and the RNA was concentrated and subjected to re-chromatography on oligo-T cellulose. Approx. 80% was again recovered in the low salt eluant, and the apparent degree of methylation was unchanged.

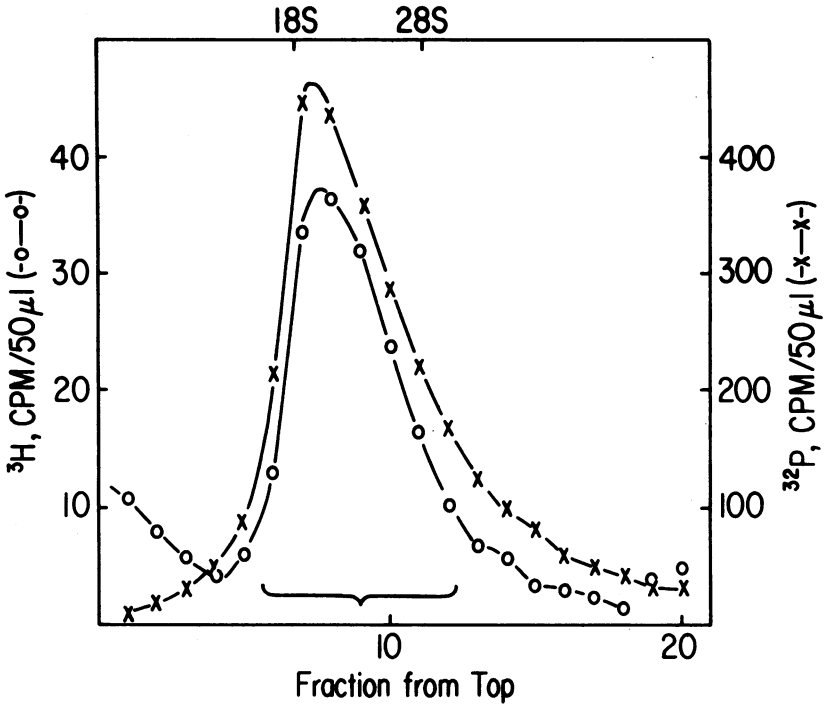

Fig. 1. Density Gradient Sedimentation of ^{3}H-Methyl-, ^{32}P-labeled Poly A(+) mRNA. RNA was prepared from cells labeled with [methyl-^{3}H]methionine (8mci, 3μg/ml) and $^{32}P_i$ (8mci, 0.65mM) for 22h as described in Methods, and 40% was subjected to density gradient sedimentation (40,000 rev/min, 3.5h in a Spinco SW 41 rotor). The positions of 18S and 28S rRNA were determined by absorbancy at 260mμ of added cytoplasmic RNA markers, and 20 0.6 ml samples were collected.

This rechromatographed RNA was used for more detailed analysis, as described below.

Since we expected to find 7-methylguanine plus 1- and/or N^6-methyladenine (cf ref. 2,3), we employed acid hydrolysis in an initial characterization of the methylated residues. Acid conditions preclude the degradation of 7-methylguanine and the isomerization of 1-methyl- to N^6-methyladenine that occur at alkaline or neutral pH's.[15] An especially useful system for separating the products of acid digests proved to be electrophoresis at pH 3.5, as illustrated in Fig. 2. The released purines move towards the cathode and pyrimidine nucleotides and ribose-phosphate derivatives towards the anode. The methylated guanines migrate largely as a group except for 7-methylguanine, which moves slightly faster than the

rest; the next fastest, N_2^2-dimethylguanine, is shown in the figure; N^2-, and 1-methylguanine run approx. with guanine. The methylated adenines also move largely as a group, beyond the guanines; again there is a prominent exception, 1-methyladenine, which moves considerably faster. These relationships are in general agreement with the pK values involved.[15] (It should be noted that the extra positive charge of 7-methylguanine-containing nucleosides or nucleotides depends on the presence of the

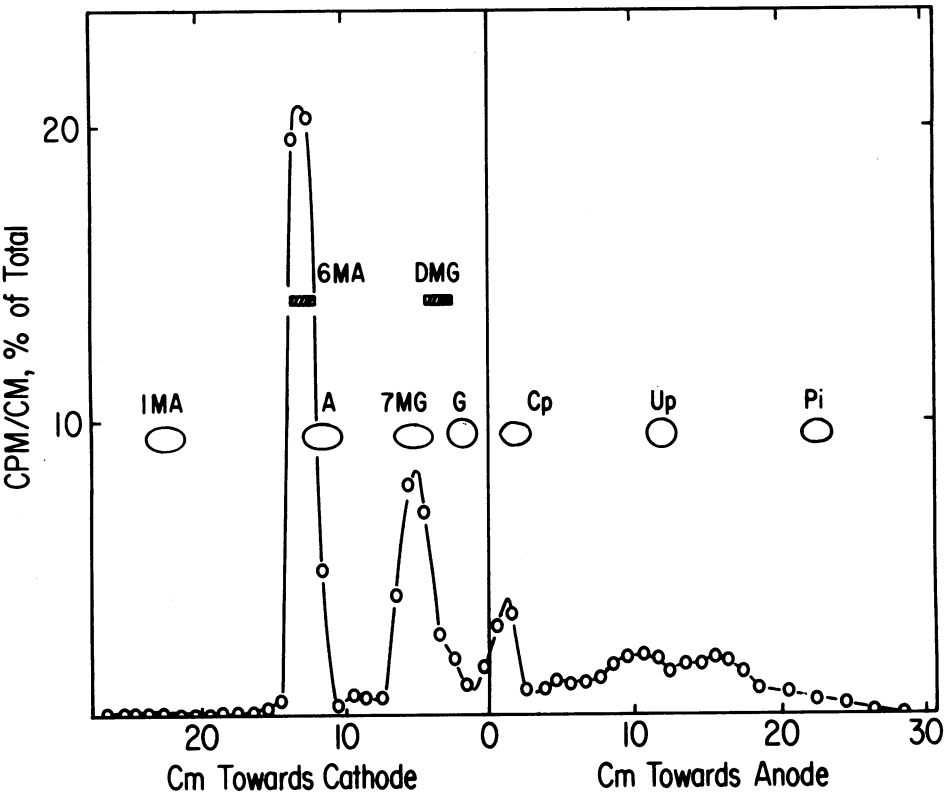

Fig. 2. Electrophoretic Analysis of an Acid Hydrolysate of Poly A(+) mRNA. The fractions indicated by the bracket in Fig. 1 were pooled and the RNA was precipitated with ethanol. After a second oligo-T passage (see Text) a portion (containing approx. 300 cpm in ^3H and 100µg of carrier RNA) was hydrolyzed with HCl (50µl containing 50mµmoles of m^7G) and subjected to electrophoresis as described in Methods. Markers derived from the hydrolysate are shown as ovals; parallel markers are shown as bars. Non-standard symbols: 1MA, 1-methyladenine; 6MA, N^6-methyladenine; A, adenine; 7MG, 7-methylguanine; DMG, N_2^2-dimethylguanine; G, guanine. In this and Figs. 3-5 only the methyl label has been plotted; to facilitate comparison of patterns, the results are expressed as % of total counts analyzed.

N-glycosidic bond.)

As shown in Fig. 2, there were two prominent methylated purine peaks in the mRNA patterns. One, containing 45-50% of the methyl label, ran with N^6-methyladenine; the second, containing 22-24% of the label, ran with 7-methylguanine.

The remaining 30% of the methyl label of acid hydrolysates ran towards the anode. A discrete peak containing 9-12% of the label moved slightly behind Cp, as expected for m^5Cp (ref. 16). Approx. 20% of the label migrated in heterodisperse fashion between the Cp and Pi markers. This resembles the behavior of the methyl label released by acid from the 2'-O-methyl residues of 28S RNA, presumably mainly as a mixture of ribose phosphate derivatives (cf ref. 17). Further evidence that the hetero-

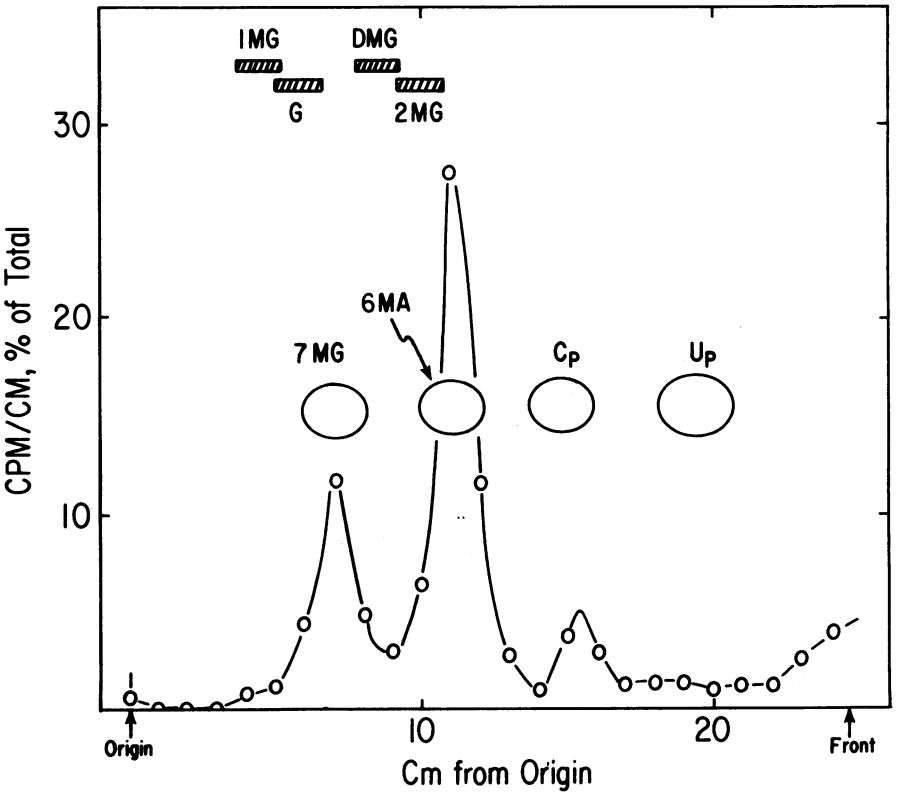

Fig. 3. Paper chromatography of an Acid Hydrolysate of Poly A(+) mRNA. A hydrolysate (containing approx. 200 cpm) was obtained as described for Fig. 2 and was subjected to paper chromatography in System 1, as described in Methods. Symbols are as for Fig. 2. Additional non-standard abbreviations: 1MG, 1-methylguanine; 2MG, N^2-methylguanine.

disperse mRNA methyl label of Fig. 2 arises from 2'-O-methyl residues is presented below.

A second generally useful system for separating the products of acid hydrolysis was paper chromatography in an acid system ("1"). As illustrated in Fig. 3, approx. 50% of the mRNA methyl label ran with N^6-methyladenine and 25% with 7-methylguanine. In addition, approx. 10% again ran as expected[16] for m^5Cp, as a peak just beyond Cp. In this system most of the label released by acid from 28S 2'-O-methyl residues ran as a shoulder at the solvent front, and smaller amounts trailed towards N^6-methyladenine. Approx. 15% of the mRNA methyl label ran heterogeneously between m^5Cp and the solvent front (Fig. 3), in general agreement with the inference regarding 2'-O-ribose methyl content drawn from electrophoretic analyses.

Further support for these identifications was provided by chromatography in an alkaline system ("4"). In this system (cf Fig. 7, below) negatively charged residues such as nucleotides and ribose phosphate derivatives remain at the origin. Approx. 30% of the mRNA methyl label did just this, equivalent to the sum of the label in m^5Cp plus presumed methylated ribose derivatives. System 4 cleanly separates N^6-methyladenine from the other methylated adenines that run near it on electrophoresis at pH 3.5, and again 45-50% of the mRNA methyl label ran with N^6-methyladenine.

To obtain information on the nature of the nucleotide linkages in which the various methylated residues were involved, samples of mRNA were subjected to alkaline hydrolysis. Fig. 4A shows a representative pattern obtained by electrophoresis of such a hydrolysate. The radioactivity appeared as 4 main fractions, numbered accordingly. Fractions 2 and 3 ran as discrete peaks in the positions expected for m^5Cp and m^6Ap (refs. 7, 16) and contained approx. 10% and 45% of the methyl label respectively. When the radioactivity in these peaks was eluted, concentrated, and subjected to acid hydrolysis followed by paper chromatography, compounds behaving like undegraded m^5Cp, and N^6-methyladenine, were recovered.

Electrophoretic fraction 1 moved as a neutral compound and fraction 4, often partially resolved into 2 moieties, ran in the Gp-Up region. These fractions contained approx. 8% and 35% of the total ^3H respectively. The mobilities of fractions 1 and 4 are compatible with their arising from "blocked" 5' end-groups containing pm^7G (cf ref. 4, and the pm^7G marker of Fig. 4). More definite evidence for blocked methylated 5'-termini was obtained by DEAE-cellulose chromatography. As illustrated in Fig.5, 35-40% of the ^3H of alkaline hydrolysates was eluted as a peak in the region of the tetranucleotide marker (the "-5" peak). The elution position of this

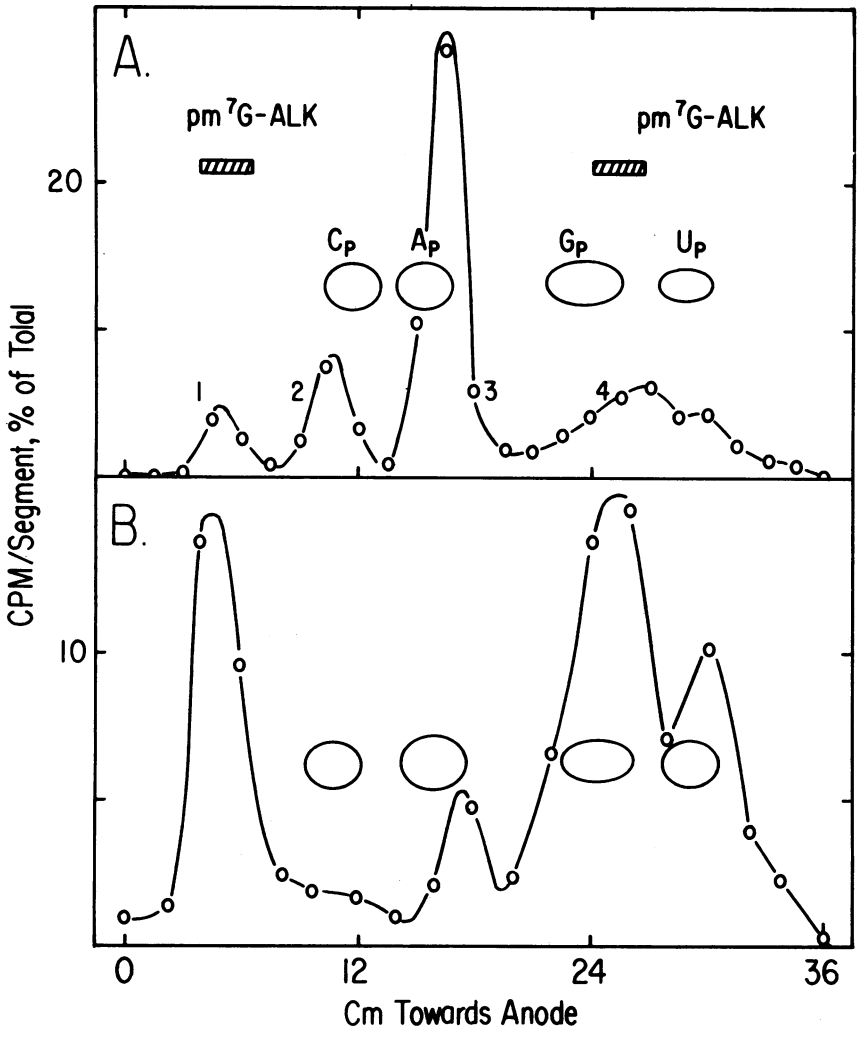

Fig. 4. Electrophoretic Analysis of Products of Alkaline Hydrolysis of Poly A(+) mRNA. The top panel (A) shows the pattern obtained from an alkaline hydrolysate of intact RNA, and the bottom panel (B) the pattern obtained from the end-group peak after DEAE-cellulose chromatography (bracketed fractions of Fig. 5, below). Portions of the RNA sample described for Fig. 2 were used, and hydrolysis and electrophoresis were as described in Methods. A sample of pm^7G was processed in parallel and yielded the 260 mμ-absorbing spots labeled "pm^7G-ALK". Approx. 200 cpm were run in A and 100 cpm in B.

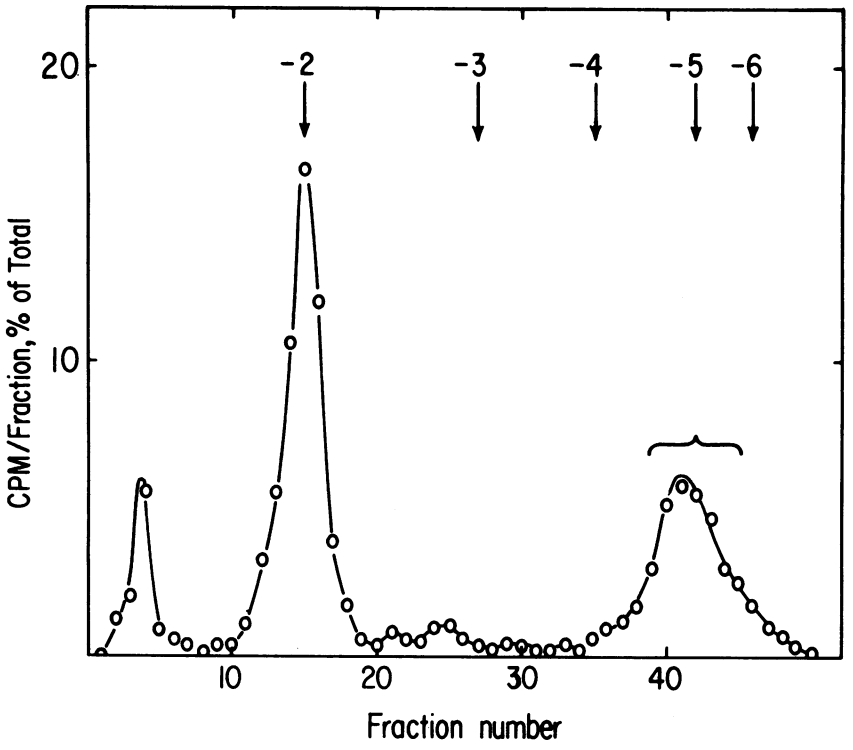

Fig. 5. DEAE-Cellulose Pattern of Methyl-labeled Poly A(+) mRNA. A portion (containing approx. 1,000 cpm) of the RNA sample described for Fig. 2 was subjected to alkaline hydrolysis followed by DEAE cellulose chromatography as described in Methods. The arrows indicate the mid-points of pancreatic ribonuclease digest markers as determined by monitoring 260 mμ-absorbancy. One-half (1 ml) of each fraction was counted.

peak corresponds well to that of the product generated by alkali from the Vaccinia mRNA end-group $m^7G(5')pppNmNp$ (ref. 5). Label recovered from this peak was markedly enriched for electrophoretic peaks 1 and 4, whereas little or no label corresponded to Cp or Ap (Fig. 4B). Another 8% of the ^3H appeared in the column wash. This material was found in subsequent studies to lack phosphate, and it presumably corresponds to the neutral compound ("1") of Fig. 4. It is likely that electrophoretic peak 1 is an alkaline degradation product of pm^7G-containing residues; apparently additional degradation (and/or release) occurs during recovery and processing of the -5 peak.

The remaining half of the methyl label eluted with the mononucleotides,

and, as expected, ran with m^5Cp and m^6Ap on electrophoresis at pH 3.5, and yielded m^5Cp and N^6-methyladenine on acid hydrolysis.

For more complete characterization of the presumed end-group peak, a second experiment was performed similar to the first except that more ^{32}P was added and analyses were accelerated, with the aim of measuring phosphate in isolated end-groups. In addition, to circumvent the degradation and partial release of 7-methylguanine from alkali-generated end-groups, we subjected the major portion of the mRNA to procedures designed to yield better defined structures. These were (1) digestion of intact RNA with RNase T2, which should yield end-groups with full complements of 7-methylguanine; and (2) alkaline hydrolysis of RNA that had been previously subjected to periodate oxidation followed by aniline treatment ("β-elimination"). This latter procedure releases m^7G from structures such as $m^7G(5')pppNmpN...$ (ref. 5, 10), theoretically yielding pppNmpNp after alkali.

Fig. 6 illustrates a DEAE-cellulose chromatography pattern of a T2

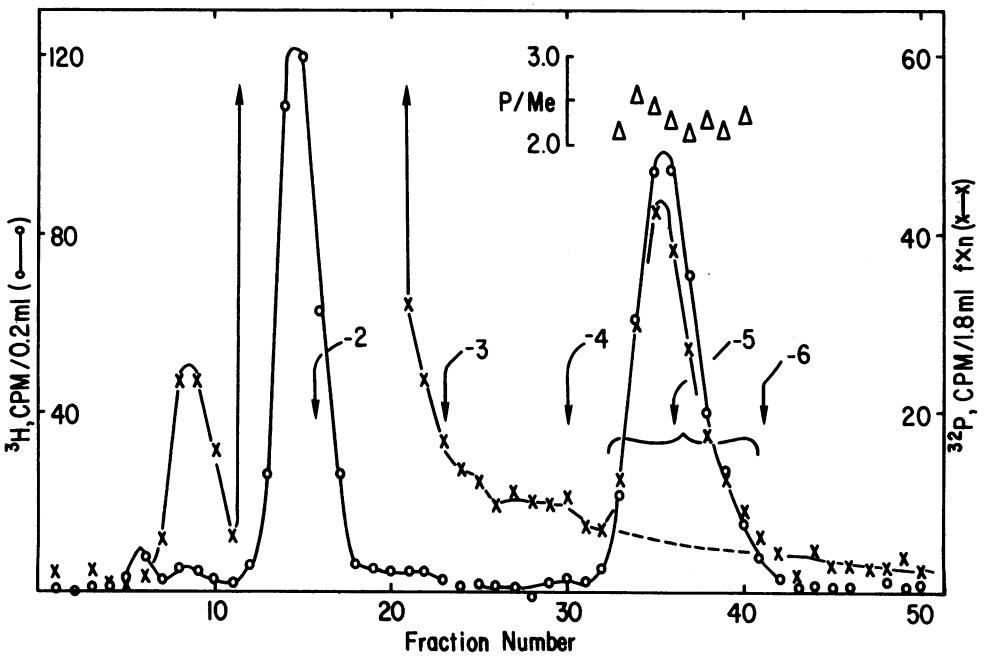

Fig. 6. DEAE-Cellulose Pattern of T2 RNase-Digested Poly A(+) mRNA. Cells were labeled essentially as for Fig.1, but with 16mci of ^{32}P and 10mci of ^3H, for a 20h period. 40% of the "16-30S" poly A(+) RNA (cf Fig. 1) was digested and chromatographed as described in the text. 1.8ml fractions were collected.

RNase digest of poly A(+) mRNA from this experiment. Less than 2% of the ^{3}H appeared in the wash, whereas 47% and 51% appeared in peaks corresponding to the "-2" and "-5" isostiches. There was also a well-defined ^{32}P peak corresponding to the -5 isostich. This peak contained 0.24% of the total phosphate, and 0.21% when a modest background (indicated by the dashed line in the Figure) was subtracted. Since the average size of the mRNA fraction examined was approx. 2600 nucleotides (based on sedimentation relative to 18S and 28S rRNA), this value is compatible with the "-5" peak's being an end-group oligonucleotide containing 5 or 6 phosphates [5/2600 = 0.19%; 6/2600 = 0.23%]. The phosphate/methyl ratios were fairly constant across the peak, and the overall ratio (Table 1) was in good agreement with that inferred from the more detailed compositional studies described below.

Fig. 7A shows the distribution of methylated residues from the -5 peak, as determined by acid hydrolysis followed by electrophoresis at pH 3.5. As expected, there was a prominent 7-methylguanine peak (40% of the total ^{3}H), as well as substantial amounts of heterodisperse label in the 2'-O-methyl derivative region (45% of the total ^{3}H). Unexpectedly, 13% of the ^{3}H ran as a peak in the adenine region. Parallel analyses were done on an RNA sample subjected to β-elimination followed by alkaline hydrolysis. DEAE chromatography of this sample yielded a pattern similar to that of Fig. 6, except for the expected lower methyl content of the -5 peak (Table 1). An acid hydrolysate of this "β-eliminated" -5 peak yielded the electrophoretic pattern shown in Fig. 7B. Most notable was the absence of the 7-methylguanine peak. In other experiments (not shown) comparable analysis of undigested RNA samples showed 90% loss of 7-methylguanine after periodate-aniline treatment, without altering the levels of the other methylated residues. These results, coupled with the presence of 3'-terminal poly A, indicate that the 7-methylguanine of mRNA is indeed at 5' termini in "inverted" 5'-phosphodiester linkage. Our best value for the 7-methylguanine complement of the mRNA methyl groups (from electrophoresis of acid hydrolysates as in Fig. 2) is 23%. Calculations similar to those presented above for the phosphate content of the -5 peak lead to a value of 1 7-methylguanine residue, on the average, per molecule (23% x 1.8/1,000 x 2,600 = 1.03), indicating that most or all of the molecules in the mRNA fraction studied do in fact end in $m^{7}G$.

The β-eliminated -5 peak retained the ^{3}H in methyladenine and in methyl ribose derivatives, in approx. the same relative amounts as in the intact peak (0.3 to 1), and as shown in Fig. 7C the methyladenine was all N^{6}-methyladenine.

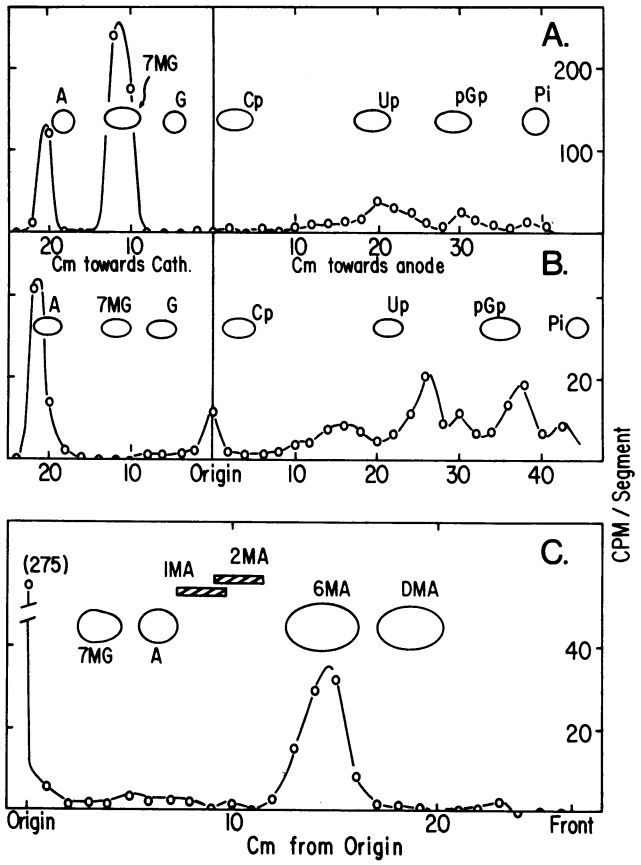

Fig. 7. Analysis of Acid hydrolysates of -5 peaks from T2 RNase-treated (Panel A) and "β-eliminated" (Panels B, C) Poly A(+) mRNA. Samples from the -5 peak of Fig. 6, or from an analogous peak from an RNA sample subjected to β-elimination followed by alkaline hydrolysis, were subjected to acid hydrolysis as described in Methods. Panels A and B: electrophoresis at pH 3.5; C, chromatography in System 4. Symbols are as for Fig. 2; in addition: 2MA, 2-methyladenine; DMA, N^6_2-dimethyladenine.

Table 1. Phosphate : Methyl Ratios of End-Groups from Poly A(+) mRNA

	Moles Phosphate per mole [Me]	
Treatment	Experimental	Theoretical
T2 RNAse Digestion	4.0	3.8
β-Elimination plus alkaline hydrolysis	2.3	2.2

The values represent weighted averages over -5 peaks such as that of Fig. 6, and are all from the experiment described in this figure. Conversion of isotope ratios to molar ratios and derivation of "theoretical" ratios were as described in the text.

Further information on the nature of the methylated ribosides of the end-groups was sought by subjecting portions of a "β-eliminated" -5 peak to combined alkaline phosphatase and venom phosphodiesterase treatment. When such digests were fractionated by thin layer chromatography (Fig. 8) two major peaks were observed. One, containing approx. 30% of the applied ^{3}H, corresponded to Gm; the other, containing approx. 36% of the ^{3}H, ran beyond all the standard Nm's, as well as $m^{6}A$. This latter peak was thought to be $m^{6}Am$, since its R_F relative to $m^{6}A$ resembled that of Am relative to A, and it contained about the right amount of radioactivity to account for the N^{6}-methyladenine in acid hydrolysates (v.i.); confirmation was provided by eluting the peak from a parallel run and demonstrating that acid treatment released approx. equal numbers of counts in N^{6}-methyladenine and in methyl ribose. Lower levels of ^{3}H (roughly 20% of the total in the β-eliminated -5 peak) ran with Cm plus Um (which were poorly resolved), while little (<10%) ran with Am or $m^{6}A$. 10% remained at the origin and was not examined

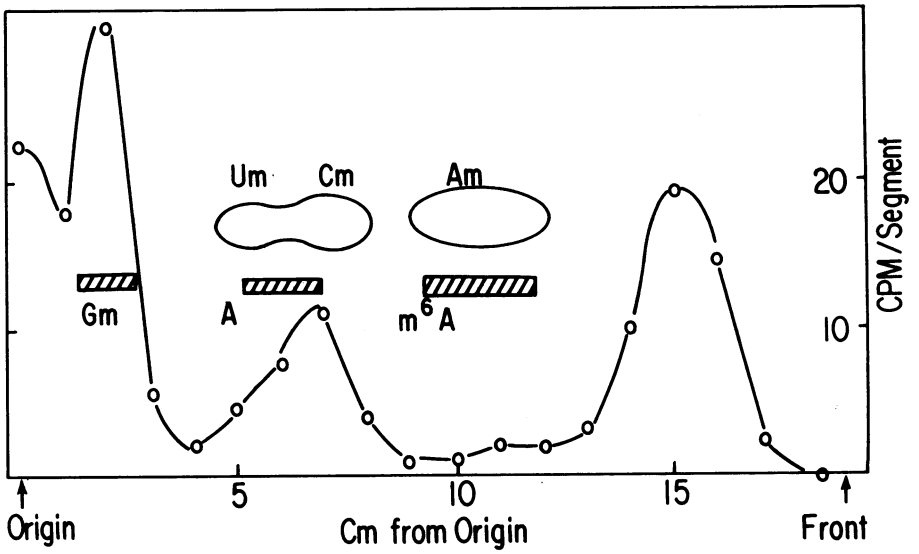

Fig. 8. Methylated Riboside Analysis of the -5 Peak of "β-eliminated" Poly A(+) mRNA. A portion of the -5 peak from the "β-eliminated" sample described in Fig. 7 was concentrated and subjected to digestion with alkaline phosphatase and phosphodiesterase (see Methods). The digest (in 30 µl) was lyophilized, re-dissolved in 10 µl of 0.5N NH_4OH, and subjected to thin layer chromatography as described in Methods. Symbols are as for previous Figs., except that standard abbreviations are used (i.e., all refer to ribosides). The major ribotides plus $pm^{7}G$ and $m^{7}G$ remained at the origin.

further. In summary, these results indicate that the predominant methylated nucleosides of the β-eliminated -5 peak are Gm, m^6Am, and Um plus Cm, in the approximate molar ratios 30:18:20. This distribution would yield a ratio of N^6-methyladenine/2'-O-methyl of 18/68 or 0.27, in general accord with the values obtained from acid hydrolysates (Fig. 7).

If we assume that a typical end group contains one m^7G, one Nm, and 0.3 N^6-methyladenine residues, then the average phosphate/methyl ratio of an intact end group would be 5/2.3, or 2.2; and that of a β-eliminated end group would be 5/1.3, or 3.8. This is the source of the "theoretical" values of Table 1, which are in reasonably good agreement with experimental.

The three types of end-groups examined (those from T2 RNase digestion and from alkaline hydrolysis with or without prior β elimination) were all eluted from DEAE-cellulose at approx. the same position. Apparently the presence, absence, or intactness of the 7-methylguanine residue has little influence on chromatographic behavior.

DISCUSSION

The present work indicates that hamster cell poly A(+) mRNA contains approx. 1.8 methyl groups per 1000 nucleotides. Half of these are in standard ("internal") nucleotide linkage, approx. 10% in m^5Cp and 40% in m^6Ap. The remaining methyl groups are clustered in "blocked" 5'-termini with the proposed structure $m^7G(5')ppp(Nm)_1pN_2p..$, where $(Nm)_1$ is largely Gm, m^6Am, or (Cm, Um) and N_2 is mainly unmodified. We cannot rule out minor amounts of other $(Nm)_1$'s or of modified N_2 residues (such as, e.g., m^6A or Nm). In fact, the slight excess of 2'-O-methyl derivatives over 7-methylguanine in acid hydrolysates of the T2 RNase-derived end group (Fig. 7A) suggests that a small proportion of end groups may indeed have a second Nm (v.i.). Our value for degree of methylation is in reasonably good agreement with the value found for mouse mRNA, 2.2 per 1000 (ref. 1). In addition, our findings with regard to characterization of methylated bases are largely in accord with, and extend, the earlier reports on both rat and mouse mRNA. These reports indicated the presence of 2 presumed base-methylated compounds: one whose properties were consistent with those of degraded 7-methylguanine residues (Perry and coworkers, cited in ref. 2; also, ref. 3) and a second identified as N^6mA under conditions allowing isomerization of lmA (ref. 3). The present work establishes the identifications of 7-methylguanine and N^6-methyladenine as the predominent methylated bases of mammalian cytoplasmic mRNA.

There are however some differences between our results and the earlier studies. The occurence of m^5Cp in mRNA has not been reported elsewhere.

In addition, the earlier studies indicated that 50% or slightly more of the mRNA methylation occurred on ribose residues, whereas our figure is approx. 20%. The source of these differences is not known, but an interesting possibility involves the labeling periods used. The 20-22 hr. incubations we employed probably yielded essentially uniformly labeled mRNA. The other investigators employed 3 or 4 hr. incubations, which would likely have resulted in preferential labeling of more rapidly-turning over mRNA moieties.[18,19] Perhaps different kinetic classes of mRNA vary in methylation status.

During preparation of this manuscript, detailed descriptions of mRNA methylation from two other mammalian systems appeared. Adams and Cory,[20] using mouse myeloma cells labeled with ^{32}P for 16.5h, reported findings strikingly similar to ours in most respects. In particular, their poly A(+) mRNA contained approx. 2 methyl groups per 1,000 nucleotides; internal m^6Ap residues accounted for 40% of these groups, and most of the rest occurred in 5' termini with the structure $m^7GpppNmpNp$.... In addition, "a fraction" of the end-group Am residues were thought to be base-modified. Furuichi et al[21] on the other hand presented somewhat differing results for HeLa cells. The mRNA from cells labeled for 3h with [methyl-^3H]methionine (but not from cells labeled under slightly different conditions with ^{32}P) contained equal amounts of end groups with one, and with two Nm residues. These workers also reported Am but no m^6Am in their end groups; however, it is unlikely that their analytical system (electrophoresis at pH 3.5) would have distinguished between these two nucleosides.

The presence of internal m^5Cp in mRNA is not unique. We have observed relatively large amounts (approx. 50% of the total methyl groups) of this compound in the "26S" mRNA synthesized in BHK cells infected with Sindbis virus (Dubin & Stollar, in preparation). Nevertheless, especially in view of its low levels in uninfected cell mRNA, it is legitimate to ask whether this component might arise from a contaminating non-messenger species. We cannot answer this question definitively. However, the relative amounts of m^5Cp were similar in samples treated with oligo-T once or twice, and (more importantly) in samples obtained from gradient "cuts" ranging from approx. 10S to 40S. It seems unlikely that a putative contaminant would mimic the centrifugal properties of the bulk of the poly A(+) fraction this closely.

The methylation pattern of the hamster mRNA is distinctive among cellular RNA's not only with regard to the unusual 5' terminal structures, but also in the relative abundance of internal N^6-methyladenine. The

present results, together with those on mouse,[1, 2, 20] rat,[3] and human[21] cells, indicate that these distinctive features are common and perhaps invariable in mRNA from mammalian systems. Coupling between transcription and methylation has been shown for reovirus mRNA,[22] and a specific requirement for terminal m^7G in translation has been demonstrated for cellular as well as viral mRNA's in an in vitro protein-synthesizing system.[23] The roles of the other terminal methyl groups, and of the internal methylated residues, remain to be specified, as does the significance of the apparent diversity of ribose methylated residues.

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