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Methylated, Blocked 5′ Termini in HeLa Cell mRNA

(7-methylguanosine/N⁶-methyladenosine)

Y. FURUICHI*, M. MORGAN, A. J. SHATKIN*, W. JELINEK†, M. SALDITT-GEORGIEFF†, AND J. E. DARNELL†

*Roche Institute of Molecular Biology, Nutley, New Jersey 07110; and †Rockefeller University, New York, N.Y. 10021

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ABSTRACT **Poly(A)-containing HeLa cell mRNA prepared from cells labeled with [*methyl*-³H]methionine or [³²P]phosphate was found to contain a variety of methylated, blocked 5′-terminal structures of two general types: m⁷GpppN^m-Np and m⁷GpppN^m-N^m-Np. In addition, about one-third of the [³H]methyl label was present in the N⁶-methyladenosine; this labeled nucleoside was not found in the 3′-terminal one-third of the mRNA chain and thus may also be in the 5′ portion of the mRNA.**

The 5′ end of a variety of viral mRNA molecules consists of an unusual type of methylated oligonucleotide with the general structure m⁷G(5′)ppp(5′)N^m-Np (1–6). Because the addition through pyrophosphate linkage of the terminal m⁷G renders the terminal dinucleotide resistant to digestion by the usual ribonucleases, the structure has been called a "cap" (7). Perry and Kelley (8) have shown that mammalian cell mRNA contains methyl groups, and, with the availability of methods used to characterize the cap structures in viral mRNAs, we have examined HeLa cell mRNA for the presence of caps. As pointed out by Rottman *et al.* (7), information about various steps of methylation of potential mRNA precursors by the cell should aid in understanding eukaryotic mRNA formation.

MATERIALS AND METHODS

Radioactive Labeling. Growth of suspension cultures of HeLa cells was in Eagle's medium. Cells were labeled with ³²P for 4 hr in phosphate-free medium as described (9). Labeling with [*methyl*-³H]methionine was carried out by resuspending cells, previously grown in Eagle's medium containing 30 μM adenine, at 2 × 10⁶ cells per ml in Eagle's medium lacking methionine and containing 30 μM adenine, 20 μM guanosine, 20 μM formate and 5% dialyzed serum plus 20 μCi/ml of [*methyl*-³H]methionine (specific activity 10 mCi/μmol). After 5 min, 20 μmol of unlabeled methionine per ml were added. Cells were harvested after 3 hr. Poly(A)-containing mRNA was prepared by poly(U)-Sepharose selection as described (9). The yield of mRNA labeled with [³H]methyl was about 20,000 cpm/mCi of input label.

Enzymatic Digestion. mRNA (about 20 μg plus 100 μg of yeast RNA) was digested with RNase T_2 (50 units/ml) in 0.05–0.2 ml of 0.01 M sodium acetate buffer at pH 4.5 for 2 hr at 37°. In some cases, RNA was first digested for 30 min at 37° with RNase A (100 μg/ml) in EDTA (1 μM), Tris·HCl buffer (1 μM) at pH 7, before addition of sodium acetate and T_2. Conditions for treatment with *Penicillium* nuclease (P_1), bacterial alkaline phosphate (BAP), or nucleotide pyrophosphatase were as described (1) except that nucleotide pyrophosphatase was used at a concentration of 0.05 units/ml (incorrectly stated as μg/ml in ref. 1).

Analytical Methods. Nucleotide and oligonucleotide analysis (1, 10) included column chromatography on DEAE-cellulose with 7 M urea, high voltage paper electrophoresis at pH 3.5 alone and coupled with DEAE paper electrophoresis in 7% formic acid, and descending paper chromatography. Samples eluted from DEAE columns were desalted by adsorption to DEAE-cellulose and elution with triethylammonium bicarbonate as detailed previously (10, 11).

Materials. Enzymes were purchased from Sankyo (RNase T_1 and T_2), Yamasa Ltd., Tokyo (P_1 nuclease), Sigma (nucleotide pyrophosphatase) and Worthington (RNase A and BAP). [*methyl*-³H]methionine (specific activity 10 Ci/mmol) and carrier-free [³²P]phosphoric acid were obtained from New England Nuclear.

RESULTS

Identification of ³H-Labeled Methylated Nucleotides in HeLa Cell mRNA. HeLa cells that had been exposed to [*methyl*-³H]methionine contained radioactivity in poly(A)-terminated molecules the size of mRNA. When this ³H-labeled RNA was digested to completion with RNase A and T_2 and subjected to DEAE-cellulose chromatography, peaks of radioactivity were observed that eluted in the positions of net charge −2 and approximately −4.5 to −5.5 (Fig. 1A). The material of −2 charge, which includes the mononucleotides, was desalted and re-analyzed by paper electrophoresis at pH 3.5. Most of the radioactivity migrated with adenylic acid (pA) (Fig. 1B). After digestion with BAP, the pA-like material was shown by paper chromatography to have the same R_F as N⁶-methyladenosine (Fig. 1C). This same compound was recently reported to be the predominant methylated mononucleotide in an alkaline hydrolysate of Novikoff hepatoma cell mRNA (12) and in poly(A)-containing cell and simian virus 40-specific RNAs from infected BSC-1 monkey cells (6).

The [³H]methyl-labeled oligonucleotides eluted from DEAE in two broad peaks with net charges from −4.5 to −5.5. The zones were pooled separately as indicated in Fig. 1A, desalted, and separately analyzed as "cap 1" and "cap 2". After *Penicillium* nuclease (P_1) digestion of cap 1, the majority of the radioactivity migrated during paper electrophoresis toward the anode in the position of pA (Fig. 1B), i.e., similar to the P_1-resistant portion of reovirus mRNA caps (1). The P_1-resistant part of the cellular cap 1 was eluted, digested with BAP, and compared by paper chromatography with marker 5′-terminal m⁷GpppG^m and m⁷GpppA^m derived

Abbreviations: pA, adenylic acid; pC, cytidylic acid; pG, guanylic acid; pU, uridylic acid; G^m, 2′-*O*-methylguanosine; A^m, 2′-*O*-methyladenosine; N⁶mA, N⁶-methyladenosine; m⁷G, 7-methylguanosine; BAP, bacterial alkaline phosphatase; P_1, *Penicillium* nuclease; VSV, vesicular stomatitis virus; N^m, any 2′-*O*-methylnucleoside; hnRNA, heterogeneous nuclear RNA.

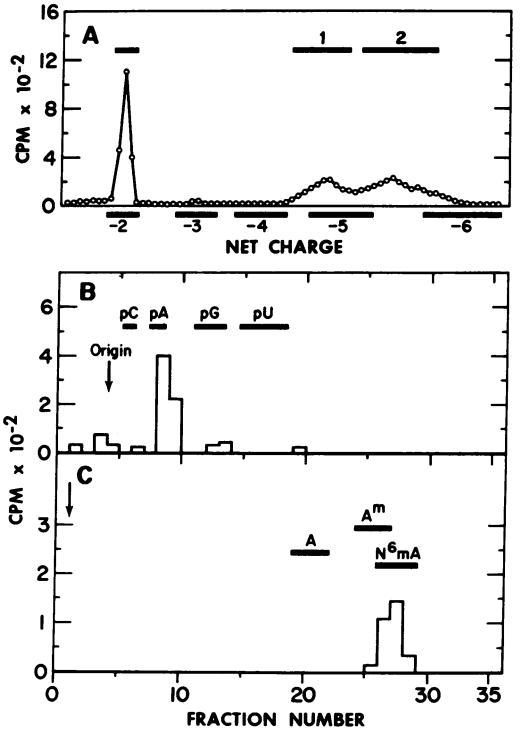

FIG. 1. Analysis of methylated nucleotides released from [*methyl*-^{3}H]mRNA by RNase T_2 digestion. (A) DEAE-cellulose chromatography: mRNA was digested with RNase T_2 as described in *Materials and Methods*, mixed with oligonucleotide markers, and applied to a 0.7 × 20 cm column in 0.05 M Tris·HCl buffer at pH 7.6, 0.05 NaCl, and 7 M urea. The column was eluted with a NaCl gradient (100 ml each of 0.05 M and 0.3 M in the same buffer containing 7 M urea) and a portion of each fraction counted. The positions of oligonucleotides of net charge −2 to −6 were determined by absorbancy at 260 nm. (B) Paper electrophoresis: The mononucleotides in A were pooled, desalted, and analyzed by high voltage paper electrophoresis in pyridine/acetate buffer at pH 3.5, as described previously (1, 10). (C) Paper chromatography: The material in the pA region of B was eluted, treated with BAP, and re-analyzed by descending paper chromatography in isobutyric acid:0.5 N NH_4OH (10:6 v/v).

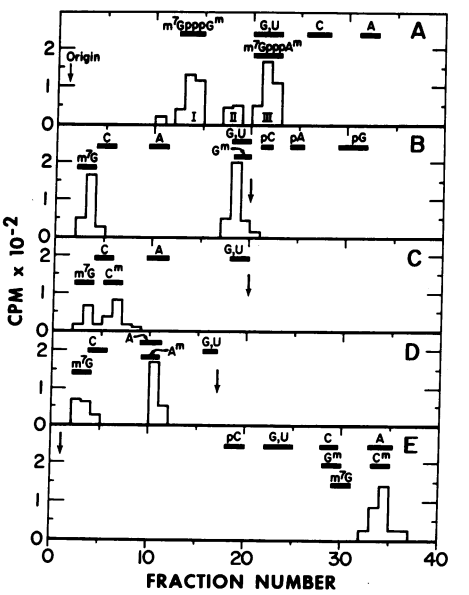

FIG. 2. Identification of methylated components in cap structures. (A) Separation of three 5' structures from cap 1 material: The pooled, desalted cap 1 material (Fig. 1A, pool 1) was digested with P_1-nuclease and BAP, and analyzed by descending paper chromatography in isobutyric acid:0.5 N NH_4OH (10:6 v/v). Papers were cut into 1 cm strips and counted in toluene-based scintillation fluid. (B, C, and D) Composition of Peaks I, II and III, respectively: Samples of P_1 and BAP-resistant material in A were eluted, digested with nucleotide pyrophosphatase followed by BAP, and re-analyzed by paper electrophoresis. Papers were dried, markers located under UV light, and 1 cm strips were counted in 1 ml of H_2O and 10 ml of Aquasol. (E) Release of 2'-*O*-methylcytidine from cap 2 by P_1 plus BAP digestion: Desalted cap 2 (Fig. 1A—pool 2) was digested with P_1 nuclease and BAP, and analyzed by paper electrophoresis with marker compounds. The region corresponding to cytidine was eluted and re-analyzed by descending paper chromatography in isobutyric acid:0.5 N NH_4OH.

by P_1 and BAP digestion of reovirus (1) and of cytoplasmic polyhedrosis virus (2) mRNA, respectively. Three peaks of phosphatase-resistant radioactivity were resolved: I, in the position of m^7GpppG^m; II, a minor component with an intermediate R_F; and III, migrating with m^7GpppA^m (Fig. 2A). Each of the three peaks was eluted, digested with nucleotide pyrophosphatase and BAP, and re-analyzed by paper electrophoresis. As shown in Figs. 2B–D, peaks I, II, and III each consisted of m^7G and a second methylated nucleoside which corresponded in R_F to 2'-*O*-methylguanosine and/or 2'-*O*-methyluridine (not resolved under these conditions but later data have shown both nucleosides), 2'-*O*-methylcytosine, and 2'-*O*-methyladenosine, respectively. The identity of m^7G and and the 2'-*O*-methylated nucleosides was confirmed by paper chromatography (Table 1).

Cap 2 material was similarly analyzed. After P_1 digestion and paper electrophoresis, radioactivity was present in material that migrated not only in the position of 5'-terminal structures as in cap 1, but also with pC, pG, and pU marker compounds. The mononucleotides were eluted, and after digestion with BAP, identified as the corresponding 2'-*O*-methylated nucleosides by paper chromatography as in Fig. 2E (Table 1). The results indicate that cap 2 contains two adjacent 2'-*O*-methylated nucleotides, one released as the mononucleotide by P_1 digestion and the other retained in the P_1-resistant portion of the cap. This P_1-resistant material, eluted from paper after electrophoresis, also was resolved by paper chromatography into the same three peaks as observed for P_1-treated cap 1 (see Fig. 2A). Nucleotide pyrophosphatase plus BAP treatment of each peak again released m^7G and G^m, U^m, C^m, and A^m (Table 1).

5'-Terminal Structures in ^{32}P-Labeled mRNA. Further confirmation of the nucleotides in cap structures came from an analysis of ^{32}P-labeled ribonuclease A- and T_2-resistant material obtained by enzymatic digestion of mRNA followed by separation of the digest in a "two-dimensional fingerprint," the technique originated by Sanger and colleagues for RNA sequence analysis (Fig. 3). Two major areas of radioactivity, (*a*) and (*b*), (each containing several compounds) and material (*c*) that failed to migrate toward the anode during the second dimension (electrophoresis in 7% formic acid on DEAE paper) were recovered for analysis. The areas marked (*a*) and (*b*) in Fig. 3 consisted predominantly (90 and 70%, respectively) of material which eluted from DEAE-cellulose at the same position (close to −5); small amounts of material of charge −6 to −7 were also present (data not shown). Material (*c*) was resolved by DEAE-cellulose chromatography into two peaks: most of the radioactivity eluting between −2 and −3 and the remainder in a broad peak between −5 and −6. The

TABLE 1. *Distribution of methylated constituents in cap 1 and cap 2 structures*

Treatment	7mGpppG^m, 7mGpppU^m†	7mGpppC^m†	7mGpppA^m†	pC^m†	pG^m†	pU^m†	pA^m
P_1 nuclease							
cap 1	40*	14	46	0	0	0	0
cap 2	12	12	26	13	20	16	0
Nucleotide pyrophosphatase plus BAP				C^m‡	G^m + U^m‡	A^m§	m^{7}G‡
P_1-resistant cap 1				8	22	29	41
P_1-resistant cap 2				12	14	36	37

* Percent of total [^{3}H]methyl cpm in cap 1 and cap 2 separated by DEAE-cellulose chromatography of RNase A plus T_2 digests of mRNA.

† Paper electrophoresis followed by BAP digestion and paper chromatography in isobutyric acid:0.5 N NH_4OH (10:6 v/v) or, for U^m, isopropanol:concentrated HCl:H_2O (68:17.6:14.4 v/v).

‡ Paper electrophoresis.

§ Paper electrophoresis followed by chromatography.

eluted peaks of radioactivity were desalted and re-analyzed by digestion with P_1 nuclease (and BAP) followed by paper electrophoresis and chromatography (Table 2). Only the predominant components in the DEAE profiles of (*a*) and (*b*) material contained caps, and almost all were of cap 1 type: m^{7}GpppN^m-Np, indicating that the caps were resolved into two spots in the "fingerprint" due to differences in base composition. Material from (*c*) also contained 6% of the radioactivity in cap 1 structures, but most of the radioactivity in (*c*) was released as P_i by digestion with P_1 nuclease plus BAP. The results indicate that the first nucleotide in the mRNA chain after the m^{7}G can be any of the four 2′-*O*-methylated nucleotides, but that under the conditions used for ^{32}P-labeling, there were few additional adjacent 2′-*O*-methylated nucleotides, i.e., cap 2 structures.

^{32}P-Labeled mRNA was also used to determine the number of phosphates in the P_1-resistant part of the caps. ^{32}P-Labeled oligonucleotides of net charge −4.5 to −5.5 were obtained by exhaustive digestion with RNase T_2 followed by DEAE-cellulose chromatography as in Fig. 1A. The desalted −4.5 to −5.5 material was treated with P_1 nuclease plus BAP and analyzed by paper electrophoresis. Two ^{32}P-labeled components were resolved: 43% of the radioactivity migrated in the position of inorganic phosphate and 57% migrated slightly faster than pA in the position of presumptive 5′ termini (Fig. 4A). The latter material was eluted and analyzed by DEAE-cellulose chromatography (Fig. 4B). The P_1- and BAP-resistant portion of the cap structures eluted with a net negative charge of −2.4 consistent with the presence of three phosphates, one of them partially neutralized by the positively charged m^{7}G, in the structure, m^{7}GpppN^m (1).

The foregoing results demonstrate clearly the presence of a variety of "capped" structures in HeLa cell mRNA, as well as the presence of N^{6}mA. The capped structure, like that in viral mRNA (1–6) is most likely at the 5′ end of the cell mRNA, although the present experiments do not rule out some unusual attachment of caps to an internal methylated site in mRNA.

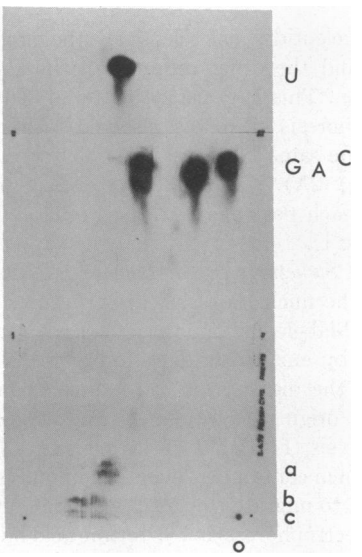

FIG. 3. Two-dimensional "fingerprint" of ^{32}P-labeled HeLa cell mRNA. After digestion with RNases A and T_2, a sample of HeLa cell mRNA RNA was subjected to electrophoresis at pH 3.5 on Whatman paper and labeled oligonucleotides "blotted through" with H_2O onto underlying DEAE (11). A second electrophoresis was then carried out in 7% formic acid. The marked areas (a, b, and c) were eluted and further analyzed. O denotes the origin and C, A, G, and U are the four mononucleotides.

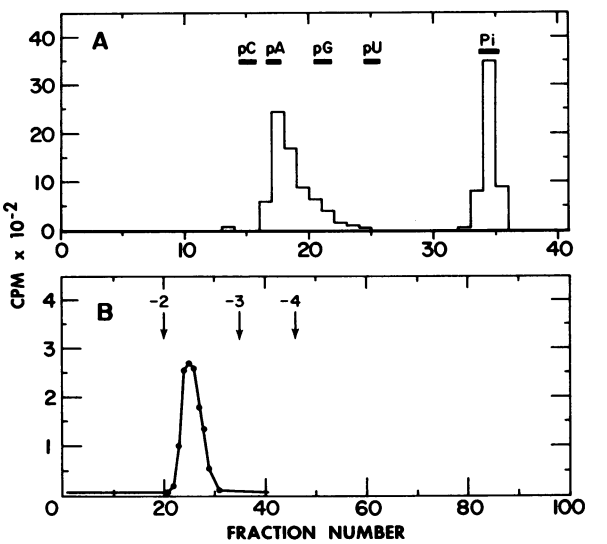

FIG. 4. Estimate of number of phosphates in caps. ^{32}P-Labeled mRNA was digested with RNase T_2 and analyzed by DEAE-cellulose chromatography. (A) Caps 1 and 2 were combined, desalted, digested with P_1 nuclease and BAP, and analyzed by paper electrophoresis. (B) The material in the pA region of the electropherogram was eluted and re-analyzed by DEAE-cellulose chromatography.

TABLE 2. *Composition of T_2 RNase-resistant ^{32}P-labeled oligonucleotides*

Region from autoradiograph	Enzyme treatment	m^7G-$pppN^m$	m^7G-$pppA^m$	m^7G-$pppC^m$	m^7G-$pppG^m$	m^7G-$pppU^m$	pA	pC	pG	pU	pU^m	P_i	Unidentified
(*a*)	P_1 nuclease	—	14	10	6.5	4.5	4	6.5	17	3	1	27.5	6
(*b*)	P_1 nuclease	—	4	10	8.5	5.5	<1	<1	13.5	22	2	23.5	9.5
(*c*)	P_1 nuclease + BAP	6*	—	—	—	—	0	0	0	0	0	94	—

Values in percent of total cpm as analyzed by paper electrophoresis at pH 3.5 followed by identification by descending paper chromatography in isobutyric acid:0.5 M NH_4OH, (10:6 v/v) with authentic marker compounds. Caps were also digested with pyrophosphatase after P_1 nuclease digestion and analyzed by paper electrophoresis and chromatography. From regions (*a*), (*b*), and (*c*) 34,800, 17,850, and 21,000 cpm, respectively, were analyzed.

* For (*c*), P_1- and BAP-resistant material was not further analyzed.

However, since the caps are (*a*) resistant to digestion by both *Penicillium* nuclease and alkaline phosphatase, (*b*) attacked by nucleotide pyrophosphatase, and (*c*) migrate in paper chromatography with authentic samples of terminal structures from viral mRNAs, we concluded that the 5′ end of many HeLa cell mRNA molecules consisted of a capped structure. In order to estimate the percentage of mRNA molecules that contained caps, we determined the ratio ^{32}P in caps to ^{32}P in poly(A) and to the radioactivity in the whole mRNA sample. This measurement indicated that there was 0.5–1 cap per mRNA molecule [5–6 cpm in cap/180 cpm in poly(A)/1500 cpm in mRNA]. This value is likely to be a minimum estimate since only the 3′ portion of degraded mRNA molecules would be selected by poly(U)-Sepharose binding.

Localization of the Methylated Nucleotides Within HeLa Cell mRNA. Further information on the distribution of ^3H-labeled methyl groups in the poly(A)-terminated mRNA molecules was sought using [*methyl*-^3H]methionine-labeled mRNA mixed with a small amount of ^{32}P-labeled mRNA. Samples were digested with a trace amount of RNase T_1 that was expected to make about 1 break per 500 nucleotides. The partially digested RNA was then exposed to poly(U)-Sepharose in order to separate the 3′ segment of the partially broken mRNA from regions closer to the 5′ end of mRNA (9). About 30% of the ^{32}P bound to poly(U)-Sepharose whereas greater than 90% of the [^3H]methyl label in the partially digested RNA passed through the poly(U)-Sepharose (Table 3). This result is consistent with an average of two enzymatic breaks per mRNA molecule. After the T_1 digestion, corresponding slower sedimentation of the mRNA through sucrose gradients was also observed (Fig. 5A–C). The ^3H-labeled RNA which failed to bind to poly(U)-Sepharose was collected, digested with RNase T_2, and analyzed by DEAE-cellulose chromatography. Caps 1 and 2 and the methylated mononucleotide peak were all present and in ratio similar to that observed for RNase T_2 digests of whole mRNA molecules (Fig. 5D).

TABLE 3. *Distribution of nucleotides labeled with [^3H]methyl in poly(U)-bound and unbound fragments of T_1 digested mRNA*

	^{32}P (cpm)		[^3H]Methyl (cpm)		Ratio mononucleotides/cap
	Bound	Unbound	Bound	Unbound	
Exp. 1	80,000 (29)	200,000 (71)	1000 (6)	16,000 (94)	1/2
Exp. 2	88,000 (32)	184,000 (68)	1250 (4)	35,000 (96)	1/2

Numbers in parentheses are percentages of total cpm. mRNA labeled with ^{32}P and [*methyl*-^3H]methionine (less than 50 μg total) was mixed with 200 μg of yeast RNA, precipitated with ethanol, and redissolved in 1.9 ml of sterile 1 μM Tris·HCl at pH 7, 1 μM EDTA, and 0.0075 ml of dilute T_1 RNase solution (enzyme was dissolved at 100 units/ml in 0.05 M acetate at pH 5 and heated at 80° for 10 min; this stock solution (diluted 100-fold for this digestion) was added for 5 min at 37°. The RNA was phenol extracted and excess phenol removed by several chloroform extractions. Poly(U)-Sepharose selection was carried out (9), and the unbound fraction was analyzed by DEAE-cellulose chromatography (see Fig. 5D).

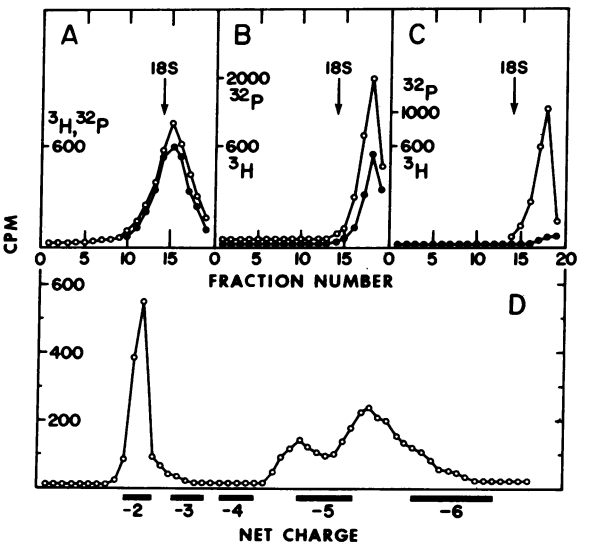

FIG. 5. Analyses of T_1 RNase partial digests of mRNA labeled with [^3H]methyl. As described in Table 2, mRNA labeled with ^{32}P and [^3H]methyl was partially digested with T_1 RNase and rechromatographed on poly(U)-Sepharose. A portion of undigested RNA (A), digested unbound (B), and bound (C) mRNA fractions were each analyzed on sucrose gradients (23,000 rpm, 25°, 16 hr, 40 rotor, Spinco; 15–30% sucrose in 0.1 M NaCl, 0.01 M Tris·HCl buffer at pH 7, and 0.01 M EDTA, 0.2% sodium dodecyl sulfate). (D) The unbound fraction, which contained almost all of the [^3H]methyl, was analyzed as in Fig. 1A by DEAE-cellulose chromatography.

DISCUSSION

HeLa cell mRNA appears to contain a number of forms of 5′-terminal, "capped" oligonucleotide structures. All are terminated with m^7G linked via three phosphates to either A^m, G^m, U^m, or C^m. In cells labeled with [*methyl*-^{3}H]methionine, about half the capped molecules contain a second 2′-*O*-methylated residue as the third nucleotide in the chain. The methylated cap structures, which can exist in many forms, can be grouped into two general types: m^7GpppN^m-Np and m^7GpppN^m-N^m-Np. The 2′-*O*-methylated nucleotide in pyrophosphate linkage to m^7G can vary considerably—A^m, G^m, U^m, and C^m were identified next to m^7G in both caps 1 and 2— and C^m, G^m, and U^m (but not A^m) were present next to the terminal nucleotides in cap 2. The low content of cap 2 structures in ^{32}P-labeled mRNA suggests that there may be important effects of cell growth conditions on the composition of caps in mRNA. Further work will be required to measure the exact percentages of each of the various capped oligonucleotides under different metabolic conditions.

In addition to the methylated caps, about $^1/_3$ of the ^{3}H from [^{3}H]methyl-labeled methionine was found in N^6-methyladenylic acid after RNase T_2 digestion, which indicates that this methylated nucleotide is not immediately adjacent to the capped 5′ terminus in mRNA. None of the methylated nucleotides appear to reside in the 3′-terminal one-third of the mRNA. Thus, there seems to be no methylation adjacent to the 3′-terminal polyadenylic acid.

Given the existence of caps on the 5′ ends of mammalian cell RNA, how can further study aid in understanding mRNA biogenesis? It has been proposed that a substantial fraction of 3′-poly(A)-terminated mRNA from HeLa cells arises by cleavage of a larger poly(A)-containing heterogeneous nuclear RNA(hnRNA) molecule (13). If this model is correct, then the methylated modifications at the 5′ end of the finished mRNA must be at sites internal to the originally transcribed 5′ end of the larger hnRNA that contains mRNA (7). It is of interest, though not of proven significance, that both cellular mRNA and mRNA of DNA viruses which replicate in the nucleus (simian virus 40 and Type 2-adenovirus) contain N^6mA, (ref. 6 and unpublished data) whereas the mRNAs of cytoplasmic viruses (vesicular stomatitis virus, reovirus, cytoplasmic polyhedrosis virus, and vaccina) all lack N^6mA (1–5). Clearly, the base- or 2′-*O*-methylations could occur at potential cleavage sites. However, the 5′-terminal m^7G, i.e., the cap, could only be added to a 3′-poly(A)-terminated hnRNA molecule after cleavage to final size or to a molecule not derived from a larger RNA.

The putative nuclease action which would create a 5′ terminus in the poly(A)-terminated hnRNA at the site of internal methylation(s) could leave at 5′ phosphate, e.g., RNase III, the double-stranded RNase from *Escherichia coli*, RNase P from *E. coli* (14), and endonuclease from liver cell nuclei (15) act in this way. The addition of ppm^{7}G would then be required to complete the structure. VSV mRNA synthesized *in vitro* in the presence of *S*-adenosylmethionine, contains the 5′ terminus m^7GpppA^m, apparently resulting from addition of ppm^{7}G to 5′-terminal pA, i.e., the middle phosphate of the cap is derived from the β-position of ppG (5). This mechanism of cap formation would be compatible with a cellular mechanism involving endonuclease cleavage of hnRNA and diphosphate transfer from pppG to cellular mRNA. The VSV capping mechanism is apparently different from that observed with reovirus (1) and cytoplasmic polyhedrosis virus (2), in which formation of 5′-terminal caps in both of these viral mRNAs occurs at the initiation of RNA synthesis (S. Muthukrishnan, Y. Furuichi, and A. J. Shatkin, unpublished results) (16). In these mRNAs, the middle phosphate of the m^7GpppN^m structure is derived from the β-phosphate of pppN [N = guanosine in reovirus mRNA (1) and adenosine in cytoplasmic polyhedrosis virus (2)], the first nucleotide in a new chain formed by a virion polymerase. Therefore, the cap is added to direct transcripts by a series of reactions involving transfer of guanosine, 1 phosphate, and 2 methyl groups to 5′-terminal ppN. If the cell possesses both capping mechanisms, the capped HeLa cell mRNA molecules may arise either by poly(A) addition followed by cleavage and capping or as direct RNA polymerase products which are capped and to which poly(A) is added to the 3′ terminus. Cap structures have in fact been observed in poly(A)-containing molecules isolated from HeLa cell nuclei but the ratio of cap structures to N^6mA is higher in the nuclear RNA than in the mRNA, perhaps indicating a step-wise methylation and cleavage process for at least some of the mRNA (unpublished results).

In addition to the possible biological function of caps in mRNA maturation discussed above, the *in vitro* translation of reovirus and VSV mRNA is methylation-dependent (17). m^7G in the cap structure appears to be essential for ribosome binding of mRNA (G. W. Both, Y. Furuichi, S. Muthukrishnan, and A. J. Shatkin, unpublished results). It should be noted that viruses which multiply in HeLa cells (vaccinia, VSV, and reovirus) have only $7mGpppA^m$ and $7mGpppG^m$ cap structures in contrast to all four kinds of $7mGpppN^m$ present in the cellular mRNA.

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