JOURNAL OF VIROLOGY, Oct. 1976, p. 123-132
Vol. 20, No. 1

Printed in U.S.A.

Methylation of High-Molecular-Weight Subunit RNA of Feline Leukemia Virus[1]

ARLEN R. THOMASON, DAVID A. BRIAN, LELAND F. VELICER, AND FRITZ M. ROTTMAN*

Department of Biochemistry and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824*

Received for publication 6 April 1976

The high-molecular-weight subunit RNA of feline leukemia virus (Rickard strain) (FeLV-R) was analyzed for the presence of methyl groups. After purification of native 50-60S FeLV-R RNA on nondenaturing aqueous sucrose density gradients, FeLV-R 28S subunit RNA, doubly labeled with [^{14}C]uridine and [*methyl*-^{3}H]methionine, was isolated by centrifugation through denaturing sucrose density gradients in dimethyl sulfoxide. As calculated from their respective ^{3}H/^{14}C ratios, FeLV-R 28S RNA was methylated to the same degree as host cell poly(A)$^{+}$ mRNA. When the 28S FeLV-R RNA was hydrolyzed to completion with RNase T2 or alkali, all of the *methyl*-^{3}H chromatographed with mononucleotides on Pellionex-WAX, a weak anion exchanger. The methyl-labeled material co-chromatographed with 6-methyladenosine if the mononucleotide fraction obtained by Pellionex-WAX chromotography was hydrolyzed to nucleosides by bacterial alkaline phosphatase or with 6-methyladenine if purine bases were released from the mononucleotides by acid hydrolysis. In another experiment in which FeLV-R 28S RNA uniformly labeled with ^{32}P was hydrolyzed and then analyzed by Pellionex-WAX chromatography, all of the ^{32}P label again co-chromatographed with mononucleotides. Thus FeLV-R 28S RNA does not appear to contain a 5′ structure, either methylated or nonmethylated, similar to those recently reported for cellular and some animal virus mRNA's.

To gain a better understanding of how RNA tumor viruses function, much study has been directed in recent years toward elucidating the nature of the virion RNA. That work, most of which was done with avian viruses, has shown that oncornavirus RNA consists of a high-molecular-weight species and several smaller ones (14). The high-molecular-weight RNA (usually designated 60-70S RNA) can be dissociated into high-molecular-weight subunit RNA (usually designated 30-40S RNA) plus smaller RNAs by heat or other hydrogen bond-disrupting agents. A previous report describes the size and kinetics of appearance of the RNA in a mammalian oncornavirus, the Rickard strain of feline leukemia virus (FeLV-R) (5). This communication describes the methylation of the high-molecular-weight subunits of FeLV-R RNA.

The high-molecular-weight subunit RNA of oncornaviruses resembles cellular RNA's in several ways. Both are single-stranded RNA molecules that can be translated in a cell-free protein synthesis system (28). Cellular mRNA's contain a post-transcriptionally added stretch of adenosine residues at their 3′ ends, and oncornavirus 30-40S RNA has been found to contain the same modification (13).

Recently it has been found that cellular mRNA (6, 23) and some animal virus RNAs (21, 27, 30) contain methylated nucleotides as an additional modification. It was originally postulated that structures containing 5′-terminal 7-methylguanosine and a 5′,5′-pyrophosphate linkage might be a general feature of all mRNA molecules (26). Such "cap" structures containing three adjacent phosphates have been found at the 5′ ends of mRNA's from several types of cells (2, 7, 24, 29) and from reovirus (11), vesicular stomatitis virus (1), vaccinia virus (31), cytoplasmic polyhedrosis virus (10), simian virus 40 (17), and two strains of an avian sarcoma virus (12, 15). In addition, internally located residues of 6-methyladenosine (m^{6}A) have been reported to be in cellular mRNA's (6) and in simian virus 40-specific mRNA (17), but appear to be absent in viral mRNA's synthesized in vitro (1, 10, 11, 30) or in vesicular stomatitis virus mRNA synthesized in vivo (22). The physiological functions of either the 5′ caps or the m^{6}A residues are unclear, but recent evidence suggests that the caps may be necessary for efficient translation of some viral mRNA's in vitro (4).

[1] Michigan Agricultural Experiment Station journal article no. 7645.

Erikson (9) first studied the methylation of oncornavirus RNA by growing avian myeloblastosis virus-producing cells in the presence of [*methyl*-^{3}H]methionine. It was found that avian myeloblastosis virus 4S RNA was highly methylated, and although the 60-70S RNA contained a low level of methylation, it was not determined whether this was due to methyl groups in the 30-40S RNA or in the 60-70S-associated 4S RNA. Others have looked for methyl groups in the high-molecular-weight RNA of Rous sarcoma virus (RSV) (3) and of murine sarcoma virus/murine leukemia virus (19) and have not found them. While the present study was in progress, two strains of RSV, RSV-Prague (15) and ASV-B77 (12), were reported to contain methylated nucleotides, including a 5′ cap structure, in their 30-40S RNA. We report here that the high-molecular-weight subunit RNA of FeLV-R, a mammalian leukemia virus, is methylated, and we describe its unique pattern of methylation.

MATERIALS AND METHODS

Virus. FeLV-R was purified from the culture fluid of feline thymus tumor cell line F-422 cells that were propagated as previously reported (5).

The Prague strain of RSV, type C, clone S312, was propagated in chicken embryo fibroblasts (CEF) obtained from SPAFAS, Inc., embryos. The cells were grown in roller bottles in the standard medium 199 and nutrient mixture F-10 combination essentially as previously reported for duck embryo fibroblasts (18), except that 5% calf serum was used. Cells were infected in the presence of 1 μg of DEAE-dextran per ml at a multiplicity of 0.01 focus-forming unit per cell at the time of cell seeding or within 24 h of seeding. The cells were allowed to grow to confluency and were then maintained in the same medium with 1% calf serum for 3 to 4 days until extensive transformation occurred, at which time the cells were used for radioactive labeling of RNA. The original virus stock and all primary CEF cells were kindly supplied by Eugene J. Smith at the U. S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich.

Isotopic labeling of RNA. For labeling FeLV-R RNA simultaneously with L-[*methyl*-^{3}H]methionine and [^{14}C]uridine, F-422 cells in mid-logarithmic growth phase were pelleted and resuspended to 2.5 × 10^6/ml in 400 ml of methionine-free medium, which, in addition to 15% fetal calf serum, contained 20 mM sodium formate, 20 μM each adenosine and guanosine, 25 μCi of L-[*methyl*-^{3}H]methionine per ml (5 to 15 Ci/mmol; New England Nuclear Corp.), and 0.03 μCi of [^{14}C]uridine per ml (50 mCi/mmol; New England Nuclear Corp.). Cells were incubated for 4.5 h, at which time they were pelleted and resuspended in 400 ml of normal medium and incubated for a second 4.5-h period. Virus was immediately purified from the tissue culture fluids, and RNA was extracted from pooled virus as soon as purification of virus from the second 4.5-h labeling interval was completed.

To label FeLV-R RNA with ^{32}P, F-422 cells at 2.5 × 10^6/ml were incubated in 200 ml of phosphate-free medium containing 10% fetal calf serum and 10 mCi of ^{32}P as orthophosphate (26 Ci/mg of P; Amersham/Searle) for 4.5 h, at which time the cells were pelleted and resuspended in 200 ml of normal medium and incubated for a second 4.5-h period. Virus was purified immediately from the tissue culture fluids and pooled, and the RNA was extracted immediately.

When labeling FeLV-R RNA with [^{14}C]uridine alone for use in the RSV mixing experiment, F-422 cells were handled essentially as above except that the cells were resuspended to 2.5 × 10^6/ml in 200 ml of normal medium containing 0.15 μCi of [^{14}C]uridine per ml (50 mCi/mmol; New England Nuclear Corp.).

For labeling RSV RNA, a large roller bottle of confluent, transformed CEF cells was incubated with 10 ml of medium containing 200 μCi of [^{3}H]uridine per ml (40 to 50 Ci/mmol; New England Nuclear Corp.). Cells were incubated for 12 h, at which time the labeling medium was removed and the cells were incubated for a second 12-h period in normal medium. Virus was purified from each supernatant fluid immediately after harvest, and the RNA was immediately extracted as described for FeLV-R.

Virus purification. FeLV-R was purified from F-422 cell tissue culture fluids clarified as previously described (5). Virus was sedimented onto a 4-ml cushion of 45% sucrose (wt/wt) made up in TNE (0.02 M Tris-hydrochloride, pH 7.5; 0.1 M NaCl; 0.001 M EDTA) at 25,000 rpm, 4°C, for 2 h in a Beckman SW27 rotor. The viral band, carefully collected from above to avoid any pellet, was diluted with TNE, and the virus was sedimented to an interface of 45% sucrose (wt/wt) (4 ml) and 20% sucrose (wt/wt) (20 ml). The viral band was collected from above, diluted with TNE, and pelleted through a barrier of 20% sucrose (wt/wt).

RSV was purified in a similar manner from the culture fluid of transformed CEF cells.

Purification of RNA. The viral pellet was resuspended in 0.5 ml of TNE, and the suspension was made 1.0% sodium dodecyl sulfate (SDS) by adding 10% SDS in TNE. Approximately 0.2 mg of proteinase K (EM Laboratories) was added, and the solution was incubated for 5 min at 37°C before being extracted three times with an equal volume of TNE-saturated phenol. The RNA was precipitated by addition of carrier RNA (5 A_{260} units/ml; Torula grade B RNA, Calbiochem) and 2 volumes of ethanol.

The RNA precipitate was dissolved in 0.1 to 0.2 ml of TNE-0.1% SDS, layered onto a 4.8-ml 5 to 20% sucrose gradient in TNE-0.1% SDS, and centrifuged in a Beckman SW50.1 rotor for 40 min at 45,000 rpm and 23°C. Fractions of approximately 0.2 ml were collected, and aliquots were counted in a scintillation counter. The fractions containing 50-60S RNA were pooled, and the RNA was precipitated by addition of carrier RNA and 2 volumes of ethanol.

To obtain FeLV-R subunit RNA, the 50-60S RNA

was dissolved in 0.01 ml of TNE-0.1% SDS and 0.2 ml of 99% dimethyl sulfoxide (Me_2SO)-1 mM EDTA-10 mM LiCl. The solution was heated to 60°C for 2 min, quickly cooled to room temperature, and layered onto a 4.8-ml 5 to 20% sucrose gradient in 99% Me_2SO-1 mM EDTA-10 mM LiCl. Centrifugation was in a Beckman SW50.1 rotor for 14 h at 45,000 rpm and 25°C. The gradients were fractionated, aliquots were counted, the fractions containing FeLV-R 28S RNA were pooled, and the RNA was precipitated by the addition of carrier RNA (5 A_{260} units/ml), 0.1 volume of 1 M sodium acetate, pH 5.1, and 2 volumes of ethanol.

For isolation of cellular RNA, F-422 cells were pelleted and washed once with a balanced salt solution. After swelling on ice for 8 min in hypotonic buffer (10 mM Tris-hydrochloride, pH 7.4, 10 mM NaCl, 1.5 mM Mg Cl_2), the cells were broken by Dounce homogenization. Nuclei were pelleted by centrifugation at 800 × *g* for 2 min, and mitochondria were removed by centrifugation at 10,000 × *g* for 7 min. The supernatant was made 0.1 M NaCl, 0.01 M EDTA, and 0.5% SDS and incubated with proteinase K, after which the RNA was isolated by phenol-chloroform extraction as described (6). The poly(A)-containing cytoplasmic RNA was isolated also as described (7).

Hydrolysis of RNA. RNA was alkaline hydrolyzed in a total volume of 0.5 ml of 0.4 M KOH for 14 to 18 h at 37°C. The solution was neutralized with perchloric acid on ice, and the $KClO_4$ was removed by centrifugation. The supernatant was lyophilized before being dissolved in 0.005 M sodium phosphate (pH 7.8)-7 M urea for analysis by high-speed liquid chromatography on Pellionex-WAX (see below).

RNase T2 digestion of RNA was performed by incubating 2 U of enzyme per A_{260} unit of RNA in the presence of 0.15 M sodium acetate (pH 4.5), 0.9 M NaCl, and 0.01 M EDTA for 2 h at 37°C. The solution was neutralized with KOH before analysis by Pellionex-WAX chromatography.

Acid hydrolysis of mononucleotides was performed as described (7).

Chromatography systems. The mono- and/or oligonucleotide products of alkaline or RNase T2 hydrolysis of RNA were analyzed by Pellionex-WAX (Reeve Angel) high-speed liquid chromatography as described (7), except that a gradient of 80 ml was used. When the mononucleotides from this column were to be recovered, the appropriate fractions were desalted by DEAE-cellulose (carbonate form) chromatography and subsequently lyophilized. The mononucleotides were then hydrolyzed to nucleosides by treatment with bacterial alkaline phosphatase (Worthington) and analyzed by high-speed liquid chromatography on Aminex A-5 (Bio-Rad) as previously reported (25). Purine bases (plus pyrimidine nucleotides) obtained by acid hydrolysis of the mononucleotide fraction from Pellionex-WAX were also analyzed on Aminex A-5 as described (7).

RESULTS

Size of methyl-labeled RNA. FeLV-R RNA that had been doubly labeled with [*methyl*-^{3}H]methionine and [^{14}C]uridine yielded two peaks after centrifugation through nondenaturing aqueous sucrose gradient, one at about 4*S* and the other at about 52*S* (Fig. 1a). These values are similar to those we have previously reported for [^{3}H]uridine-labeled FeLV-R RNA (5). About 17% of the ^{3}H and 48% of the ^{14}C were in the high-molecular-weight peak. The absence of significant amounts of material sedimenting between the two peaks suggested that very little nonspecific degradation of FeLV-R high-molecular-weight RNA had occurred.

When the FeLV-R 50-60*S* RNA thus purified was subjected to centrifugation under denaturing conditions in 99% Me_2SO, the majority of the ^{14}C sedimented at 28*S* (Fig. 2a), again similar to our results with [^{3}H]uridine-labeled FeLV-R RNA (5). Most of the *methyl*-^{3}H sedimented at 4*S*. This result would be expected if most of the ^{3}H were contained in 50-60*S*-associated 4*S* RNA, which has been shown to contain large amounts of methylated nucleosides (9). However, a significant amount (22 to 27%) of the ^{3}H sedimented at 28*S*, indicating that this RNA species also contains methylated nucleosides. The ^{3}H/^{14}C ratios of FeLV-R 28*S* RNA obtained in several experiments were the same as the respective ^{3}H/^{14}C ratios of the host cellular mRNA (Table 1). Thus FeLV-R 28*S* RNA and cellular poly(A)$^+$ mRNA are methylated to the same degree on the basis of methylated nucleosides/uridine.

In a control experiment, native high-molecular-weight RSV RNA that had been labeled with [^{3}H]uridine was mixed with [^{14}C]uridine-labeled FeLV-R before extraction and purification of RNA from the latter virus. Whereas the ^{14}C-labeled FeLV-R subunit RNA sedimented at 28*S* on Me_2SO gradients, the majority of the ^{3}H-labeled RSV subunit RNA was not reduced from its usual size of 33*S* (on Me_2SO gradients) (Fig. 3). Therefore, it seems unlikely that the 28*S* RNA we observe from FeLV-R is derived from a larger 30-40*S* molecule, similar to the high-molecular-weight subunit RNA of avian oncornaviruses, by degradation during RNA purification. Furthermore, it is unlikely that the FeLV-R 28*S* RNA is derived from a large precursor that is cleaved within the virus as a function of time, since virus labeled for a short time (60 min) with [^{3}H]uridine also yields RNA sedimenting at 28*S* (unpublished data).

Absence of methylated caps in 28*S* RNA. Within the last year, several animal virus RNAs (1, 10–12, 15, 17, 31) and eukaryotic cellular mRNA's (2, 7, 24, 29) have been reported to contain at their 5′ ends "caps" of the general type $m^7G^{5'}ppp^{5'}Nmp_{(1-2)}Np\cdots$. It was therefore of interest to determine whether FeLV-R

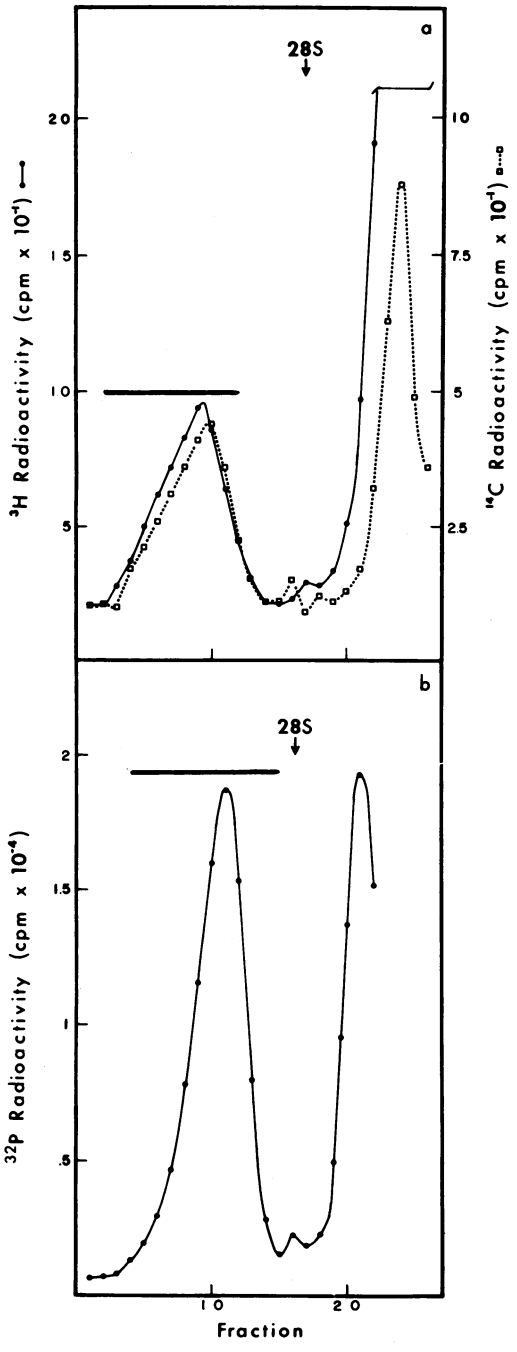

FIG. 1. *Isolation of FeLV-R native high-molecular-weight RNA by centrifugation through aqueous sucrose gradients. FeLV-R RNA was sedimented through a 5 to 20% aqueous sucrose gradient as described in Materials and Methods. F-422 cellular 28S rRNA was run as a marker on a parallel gradient. Fractions denoted by the bars were pooled, and the RNA was ethanol precipitated. (a) RNA labeled with [methyl-^{3}H]methionine and [^{14}C]uridine; (b) RNA labeled with $^{32}PO_4$.*

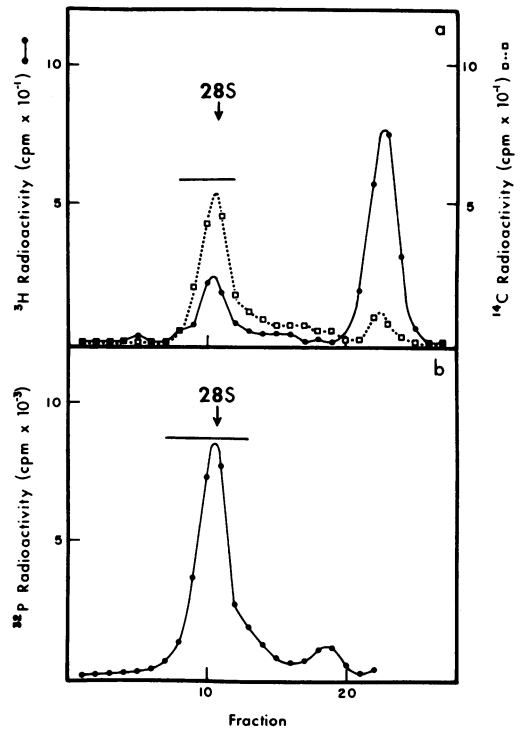

FIG. 2. *Isolation of FeLV-R high-molecular-weight subunit RNA by centrifugation through sucrose gradients in Me_2SO. FeLV-R high-molecular-weight RNA obtained as in Fig. 1 was sedimented through a 5 to 20% sucrose gradient in 99% Me_2SO as described in Materials and Methods. F-422 cellular 28S rRNA was run on a parallel gradient. Fractions denoted by the bars were pooled, and the RNA was ethanol precipitated and saved for further analysis. (a) RNA labeled with [methyl-^{3}H]methionine and [^{14}C]uridine; (b) RNA labeled with $^{32}PO_4$.*

TABLE 1. *Extent of methylation of FeLV-R 28S RNA relative to F-422 cellular poly(A)$^+$ mRNA*[a]

Expt	^{3}H cpm/^{14}C cpm of FeLV 28S RNA / ^{3}H cpm/^{14}C cpm of F-422 poly(A)$^+$ mRNA
1	0.91
2	1.04
3	1.00
4	1.04
Avg	1.00

[a] FeLV-R 28S RNA labeled with [*methyl*-^{3}H]methionine and [^{14}C]uridine was purified as described in Fig. 1a and 2a. The ^{3}H/^{14}C ratio was determined by liquid scintillation counting and was compared with the ^{3}H/^{14}C ratio of F-422 cellular mRNA obtained by oligo(dT)-cellulose chromatography in the same experiment.

28S RNA contains a similar structure. Methyl-labeled 50-60S FeLV-R RNA was first obtained by centrifugation through aqueous sucrose gradients as in Fig. 1a to reduce or eliminate possi-

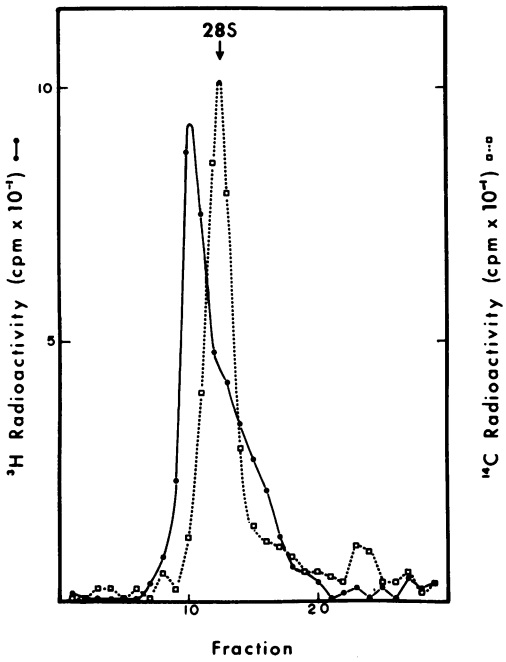

FIG. 3. *Me_2SO-sucrose gradient analysis of RSV RNA added to FeLV-R before RNA extraction. RSV native high-molecular-weight RNA labeled with [^{3}H]uridine was added to [^{14}C]uridine-labeled FeLV-R, and the RNA was immediately extracted as described in Materials and Methods. The native high-molecular-weight RNA was purified by aqueous sucrose gradient centrifugation and then centrifuged through a sucrose gradient in Me_2SO as described in Fig. 2. Ribosomal 28S RNA was run as a marker on a parallel gradient.*

ble contamination by host cell mRNA (see below). The 28S FeLV-R RNA was then obtained by centrifuging the 50-60S RNA through a sucrose gradient in Me_2SO as shown in Fig. 2a. The FeLV-R 28S RNA thus purified was hydrolyzed to completion with alkali or RNase T2. No resistant structures larger than mononucleotides were found in FeLV-R 28S RNA by Pellionex-WAX chromatography (Fig. 4). When analyzed by the same procedures, poly(A)$^+$ mRNA obtained from the cytoplasm of host cells in the same experiments contained oligonucleotides migrating between $(Up)_5$ and $(Up)_7$ markers (Fig. 4b), as expected for structures such as described above. Thus FeLV-R 28S RNA does not contain a detectable quantity of methylated caps at its 5′ ends, nor does it contain internal nucleosides that are methylated at the 2′ position, since such structures would be resistant to these hydrolysis procedures and would migrate with dinucleotide or larger markers. It was found that if the aqueous sucrose gradient centrifugation step for the isolation of native 50-60S FeLV-R RNA was omitted (i.e., if total viral RNA was applied directly to the Me_2SO sucrose gradient), then variable amounts of 2′-*O*-methylated nucleosides appeared in the final Aminex high-speed liquid chromatographic analysis of FeLV-R 28S RNA. These 2′-*O*-methylated nucleosides may have been derived from contaminating cellular mRNA, since chromatography of the RNA on oligo(dT)-cellulose did not result in their removal. However, when the aqueous sucrose gradient centrifugation step was included, no 2′-*O*-methylated nucleosides were found in FeLV-R 28S RNA.

m^6A is the only methylated nucleoside in 28S RNA. Since after alkaline or RNase T2 hydrolysis all of the methyl-labeled nucleotides co-chromatographed with mononucleotide standards, it was concluded that only base-methylated nucleotides are present in FeLV-R 28S RNA. The mononucleotides derived from FeLV-R 28S RNA and purified by high-speed liquid chromatography (Pellionex-WAX) were desalted by adsorption to DEAE-cellulose (carbonate form) and elution with 1 M ammonium carbonate. After removing the ammonium carbonate by lyophilization, the mononucleotides were dephosphorylated with bacterial alkaline phosphatase and chromatographed on Aminex A-5, a cation exchanger that separates nucleosides. Virtually all (90%) of the *methyl*-^{3}H co-chromatographed with 6-methyladenosine standard (Fig. 5a). In another experiment, purine bases were removed from the mononucleotides by acid hydrolysis and analyzed by high-speed liquid chromatography on Aminex A-5. More than 96% of the *methyl*-^{3}H co-chromatographed with authentic 6-methyladenine (Fig. 5b). The absence of any *methyl*-^{3}H radioactivity chromatographing in the position of adenine or guanine confirms that no ring labeling of purine bases occurred. Thus m^6A is the only methylated nucleoside in FeLV-R 28S RNA.

Absence of nonmethylated caps in 28S RNA. Although FeLV-R 28S RNA did not contain a methylated 5′ cap, we considered the possibility that the RNA of feline leukemia virus (grown in F-422 cells) might differ from other viral and cellular mRNA's by containing a nonmethylated cap of the type $N^{5'}ppp^{5'}Np \cdots$. We would not have detected such a structure in our experiments with methyl-labeled RNA. Therefore, we examined FeLV-R RNA that had been uniformly labeled with $^{32}PO_4$. RNA purified as in Fig. 1b and 2b was hydrolyzed with alkali and examined by Pellionex-WAX chromatography. No ^{32}P-labeled material eluted in the position of the $(Up)_4$ marker, as would be expected for a structure such as above (Fig. 6a). A structure with

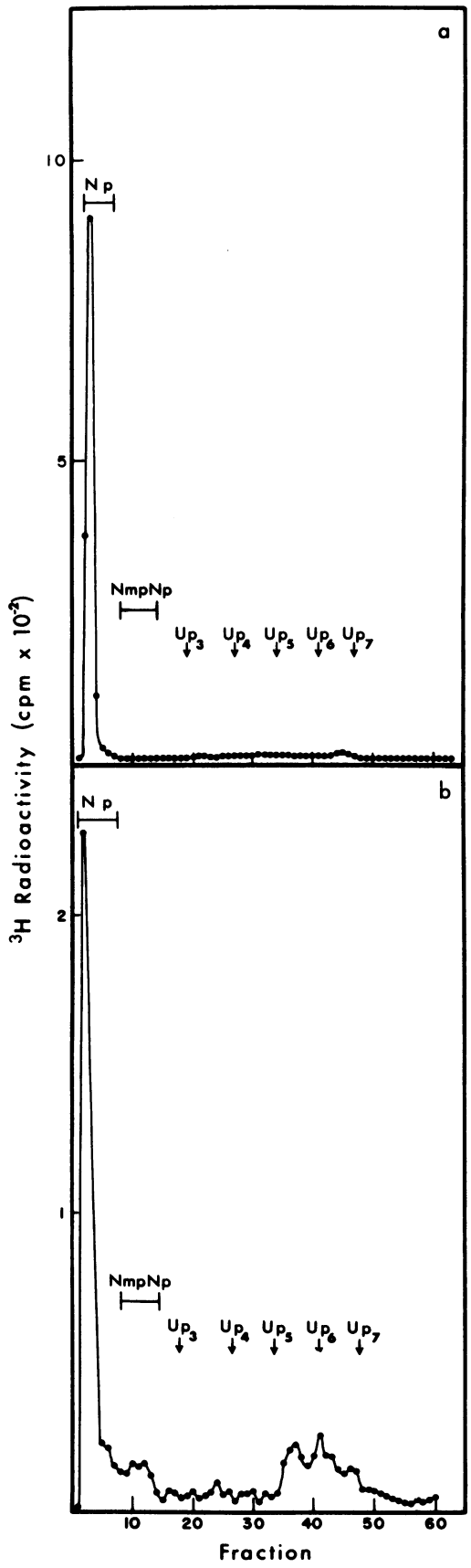

FIG. 4. *Analysis of the RNase T2 digestion products of methyl-labeled RNA. RNA labeled with [methyl-^{3}H]methionine and [^{14}C]uridine was hydrolyzed to completion with RNase T2 as described in Materials and Methods. Mixtures of oligomers of U, containing $(Up)_3$ through $(Up)_7$ [e.g., $(Up)_3$ is Up-UpUp], were mixed with the sample and quickly applied to a Pellionex-WAX high-speed liquid chromatography column equilibrated with 0.005 M sodium phosphate (pH 7.8)-7 M urea. The column was developed with a linear gradient of 0.0 M $(NH_4)_2SO_4$ (40 ml) to 0.2 M $(NH_4)_2SO_4$ (40 ml) in 0.005 M sodium phosphate (pH 7.8)-7 M urea. Markers were monitored by UV absorbance, and radioactivity in the collected fractions was determined by scintillation counting of aliquots. (a) FeLV-R 28S RNA; (b) F-422 cellular poly(A)$^+$ mRNA.*

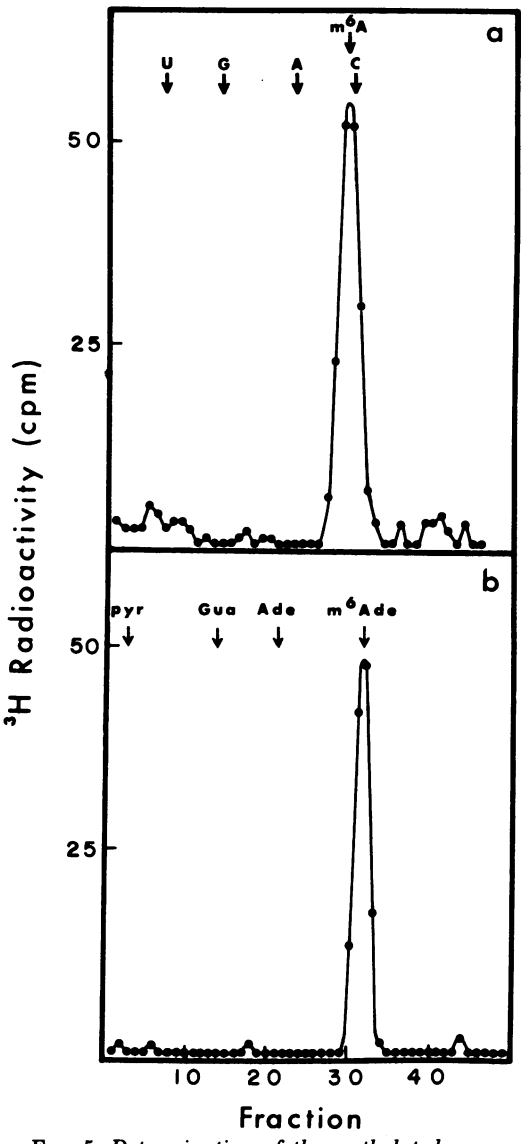

FIG. 5. *Determination of the methylated components of FeLV-R 28S RNA. (a) Mononucleotides obtained as in Fig. 4a were desalted and treated with alkaline phosphatase. The nucleosides thus generated were analyzed by Aminex high-speed liquid chromatography as described in Materials and Methods. Standards are uridine (U), guanosine (G), adenosine (A), and 6-methyladenosine (m⁶A). (b) Mononucleotides obtained as in Fig. 4a were desalted and purine bases were released by acid hydrolysis as described in Materials and Methods. The sample was analyzed by Aminex high-speed liquid chromatography. Standards are guanine (Gua), adenine (Ade), and 6-methyladenine (m⁶Ade).*

four phosphates should have contained 0.05 to 0.08% of the total cpm (600,000 cpm in the experiment shown in Fig. 6a), well within our limits of detection. The small peak eluting near the $(Up)_6$ marker represents only 40 cpm and was not reproducible. Again, in control experiments with host cell poly(A)$^+$ mRNA, alkali-resistant ^{32}P-labeled structures from 300,000 cpm of hydrolyzed RNA eluted between $(Up)_5$ and $(Up)_7$ markers on Pellionex-WAX (Fig. 6b). Therefore, FeLV-R 28S RNA lacks nonmethylated as well as methylated 5′ caps. We have not, however, been able to determine what is at the 5′ end of the FeLV-R 28S RNA, since no structures corresponding to pNp, ppNp, or pppNp were detected in $^{32}PO_4$-labeled RNA and our attempts to label the 5′ end of FeLV-R 28S RNA using polynucleotide kinase have been unsuccessful.

DISCUSSION

The results presented here show that FeLV-R 28S subunit RNA does contain methylated nucleosides. The possibility that these methylated components derive from contaminating 50-60S-associated 4S RNA seems very unlikely, since the FeLV-R 28S RNA was purified by centrifugation through a denaturing density gradient and since the methylation pattern of FeLV-R 28S RNA differs completely from that of 4S RNA (6). Furthermore, it is unlikely that the FeLV-R 28S RNA was contaminated with cellular 28S rRNA, since methylation of the latter molecule occurs principally on the 2′ position of ribose (6), whereas the only methylated nucleoside in FeLV-R 28S RNA is a derivative of adenosine methylated at the N-6 position. This was demonstrated both by analysis of the nucleosides produced by phosphomonoesterase digestion of the RNase T2-generated mononucleotides and by analysis of the bases released after acid hydrolysis of the methyl-labeled mononucleotides. Since FeLV-R 28S RNA is methylated to the same degree, on a per-uridine basis, as cellular poly(A)$^+$ mRNA (Table 1), the number of m⁶A residues per FeLV-R 28S RNA molecule can be calculated if the total number of nucleotides in the latter RNA is known. If the molecular weight of FeLV-R 28S RNA is the same as that of 28S rRNA, i.e., 1.8×10^6, then there are about 5,000 total nucleotides and about 10 m⁶A residues per FeLV-R 28S RNA molecule. However, if the molecular weight of FeLV-R 28S RNA is 2.8×10^6 to 3.2×10^6 (see below), then there are 8,700 to 10,000 total nucleotides and about 17 to 20 m⁶A residues per FeLV-R 28S RNA molecule. These calculations use the value of 0.2% methylation given by Perry and Kelley (23) for cellular mRNA. The error due to differences in the base compositions of FeLV-R 28S RNA and cellular poly(A)$^+$ mRNA is assumed to be small and is neglected.

Our results also indicate that FeLV-R 28S

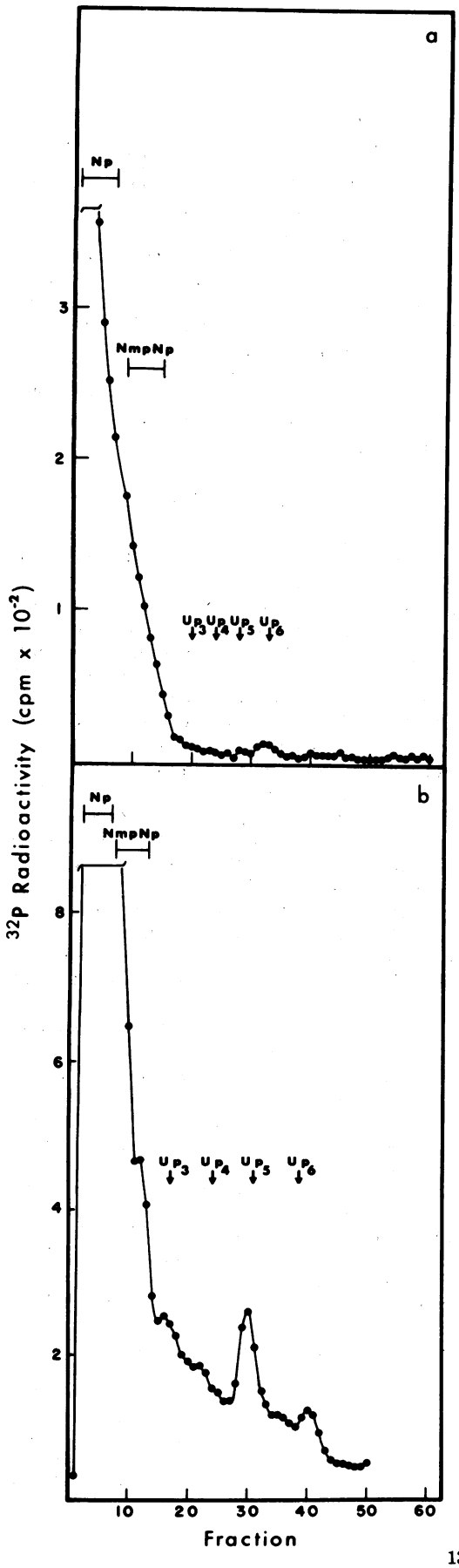

FIG. 6. *Analysis of the alkaline digestion products of RNA labeled with $^{32}PO_4$. RNA labeled with $^{32}PO_4$ was hydrolyzed to completion with alkali and analyzed by Pellionex-WAX high-speed liquid chromatography as in Fig. 4. (a) FeLV-R 28S RNA; (b) F-422 cellular poly(A)$^+$ mRNA.*

RNA contains no detectable "cap" (that is, a structure with an inverted 5',5'-pyrophosphate linkage [26]), either methylated or nonmethylated, at its 5' end. However, the amounts of radioactive FeLV-R 28S RNA used in these studies place limits on the conclusions that can be reached. Thus it can be concluded that less than one in ten FeLV-R 28S RNA molecules contains a cap. Cap structures have been found recently in cellular mRNA's (2, 7, 24, 29) and in animal virus mRNA's (1, 10, 11, 17, 31), including those from two strains of an RNA tumor virus (12, 15). The latter reports both dealt with an avian sarcoma virus, whereas this report concerns a mammalian leukemia virus. Whether or not this fact is important to the differences reported remains to be determined.

There are several possible explanations for the unexpected absence of a cap structure at the 5' end of FeLV-R 28S RNA. One possibility is that a 5' cap structure is present in an earlier transcript of FeLV-R high-molecular-weight subunit RNA, but that after packaging in the virus the 5' cap is removed either naturally or as an artifact by endonucleolytic hydrolysis of the RNA molecule. Since the FeLV-R subunit RNA isolated in our laboratories cosediments with 28S rRNA (both on aqueous and Me_2SO gradients), whereas the usually accepted value for RNA tumor virus subunit RNA is about 35S, it might be argued that degradation may have occurred during RNA isolation. Although this possibility cannot be rigorously excluded, the control experiment, in which RSV 30-40S RNA added to FeLV-R before RNA extraction was not reduced in size, argues against it. In addition, such degradation would have to result from a very specific nick since we always observe FeLV-R RNA subunit molecules that are 28S and since very little material sediments between the 28S and 4S peaks (Fig. 2). Furthermore, it does not appear that FeLV-R 28S RNA results from breakdown of RNA within the virion as a function of time, since FeLV-R subunit RNA labeled for as short a time as 1 h still sediments at 28S. That FeLV-R and other mammalian RNA tumor virus subunit RNAs can have a sedimentation coefficient of about 28S is supported by the results obtained by East et al. (8) for feline leukemia virus (Rickard), feline sarcoma virus (Rickard), RD-114, and Crandell virus, and by Manning et al. (20) for murine sarcoma virus (Moloney). Whether the 28S value represents a difference in chain length or conformation remains to be determined, but it is interesting to note that although FeLV-R subunit RNA cosediments with 28S rRNA, it nevertheless migrates more slowly than the same marker during polyacrylamide gel electrophoresis (with an estimated molecular weight of about 3.2×10^6) (5). In this regard RD-114 subunit RNA, which also cosediments with 28S rRNA (8), has a molecular weight of 2.8×10^6 as determined by electron microscopy (16). Therefore FeLV-R 28S RNA could have a molecular weight in the 2.8×10^6 to 3.2×10^6 range.

Another possible explanation for the absence of a 5' cap in FeLV-R 28S RNA is that the FeLV-R high-molecular-weight RNA destined for packaging into virions is not methylated by the normal post-transcriptional modification enzymes or that a methylated 5' end is removed from the RNA before packaging. This possibility cannot be eliminated, and in fact the finding of no caps in packaged viral RNA does not preclude their presence in similar polysomal viral RNA species. Studies to determine whether polysomal FeLV-R RNA does indeed contain a 5' cap structure are being actively pursued. Recent preliminary results in our laboratories indicate, however, that our stocks of FeLV-R, produced by F-422 feline thymus tumor cells, have only a low level of infectivity. Therefore the present results on FeLV-R RNA methylation may pertain mainly to defective particles. Experiments are in progress to determine whether this is indeed the case. If so, it will be interesting to examine the relationship between the absence of a cap structure in FeLV-R 28S RNA and the defective nature of the virus.

ACKNOWLEDGMENTS

We thank Alice Swanson, Catherine Sekerke, and Barbara Kennedy for technical assistance, and Allan Haberman for infectivity studies of FeLV-R and for helpful discussions.

This research was supported by Public Health Service grants CA-13175 (F.R.) and CA-12101 (L.V.) from the National Cancer Institute, and also by grant DRG-1230 (L.V.) from the Damon Runyon Memorial Fund for Cancer Research, Inc., and a grant (L.V.) from the Elsa U. Pardee Foundation. A.T. was partially supported by Public Health Service training grant GM-0191, and D.B. was partially supported by GM-01911, both from the National Institute for General Medical Sciences. L.V. is a recipient of Research Career Development Award CA-70808 from the National Cancer Institute.

LITERATURE CITED

1. **Abraham, G., D. Rhodes, and A. K. Banerjee.** 1975. The 5' terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis virus. Cell 5:51-58.
2. **Adams, J. M., and S. Cory.** 1975. Modified nucleosides and bizarre 5'-termini in mouse myeloma mRNA. Nature (London) 255:28-33.
3. **Bishop, J. M., W. E. Levinson, N. Quintrell, D. Sullivan, L. Fanshier, and J. Jackson.** 1970. The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virology 42:182-195.

4. **Both, G. W., A. K. Banerjee, and A. J. Shatkin.** 1975. Methylation-dependent translation of viral messenger RNAs *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 72:1189–1193.
5. **Brian, D. A., A. R. Thomason, F. M. Rottman, and L. F. Velicer.** 1975. Properties of feline leukemia virus. III. Analysis of the RNA. J. Virol. 16:535–545.
6. **Desrosiers, R., K. Friderici, and F. Rottman.** 1974. Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. Proc. Natl. Acad. Sci. U.S.A. 71:3971–3975.
7. **Desrosiers, R. C., K. H. Friderici, and F. M. Rottman.** 1975. Characterization of Novikoff hepatoma mRNA methylation and heterogeneity in the methylated 5′ terminus. Biochemistry 14:4367–4374.
8. **East, J. L., J. Knesek, P. Allen, and L. Dmochowski.** 1973. Structural characteristics and nucleotide sequence analysis of genomic RNA from RD-114 virus and feline RNA tumor viruses. J. Virol. 12:1085–1091.
9. **Erikson, R. L.** 1969. Studies on the RNA from avian myeloblastosis virus. Virology 37:124–131.
10. **Furuichi, Y., and K.-I. Miura.** 1975. A blocked structure at the 5′ terminus of mRNA from cytoplasmic polyhedrosis virus. Nature (London) 253:374–375.
11. **Furuichi, Y., M. Morgan, S. Muthukrishnan, and A. J. Shatkin.** 1975. Reovirus messenger RNA contains a methylated, blocked 5′-terminal structure: $M^7G(5')ppp(5')G^mpCp$. Proc. Natl. Acad. Sci. U.S.A. 72:362–366.
12. **Furuichi, Y., A. J. Shatkin, E. Stavnezer, and J. M. Bishop.** 1975. Blocked, methylated 5′-terminal sequence in avian sarcoma virus RNA. Nature (London) 257:618–620.
13. **Gillespie, D., S. Marshall, and R. C. Gallo.** 1972. RNA of RNA tumor viruses contains poly A. Nature (London) New Biol. 236:227–231.
14. **Green, M.** 1970. Oncogenic viruses. Annu. Rev. Biochem. 39:701–756.
15. **Keith, J., and H. Fraenkel-Conrat.** 1975. Identification of the 5′ end of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 72:3347–3350.
16. **Kung, H.-J., J. M. Bailey, N. Davidson, M. O. Nicolson, and R. M. McAllister.** 1975. Structure, subunit composition, and molecular weight of RD-114 RNA. J. Virol. 16:397–411.
17. **Lavi, S., and A. J. Shatkin.** 1975. Methylated simian virus 40-specific RNA from nuclei and cytoplasm of BSC-1 cells. Proc. Natl. Acad. Sci. U.S.A. 72:2012–2016.
18. **Long, P. A., P. Kaveh-Yamini, and L. F. Velicer.** 1975. Marek's disease herpesviruses. I. Production and preliminary characterization of Marek's disease herpesvirus A antigen. J. Virol. 15:1182–1191.
19. **McCain, B., N. Biswal, and M. Benyesh-Melnick.** 1973. The subunits of murine sarcoma-leukemia virus RNA. J. Gen. Virol. 18:69–74.
20. **Manning, J. S., F. Schaffer, and M. Soergel.** 1972. Correlation between murine sarcoma virus buoyant density, infectivity, and viral RNA electrophoretic mobility. Virology 49:804–807.
21. **Miura, K.-I., K. Watanabe, and M. Sugiura.** 1974. 5′-Terminal nucleotide sequences of the double-stranded RNA of silkworm cytoplasmic polyhedrosis virus. J. Mol. Biol. 86:31–48.
22. **Moyer, S., G. Abraham, R. Adler, and A. Banerjee.** 1975. Methylated and blocked 5′-termini in vesicular stomatitis virus *in vivo* mRNAs. Cell 5:59–67.
23. **Perry, R. P., and D. E. Kelley.** 1974. Existence of methylated messenger RNA in mouse L cells. Cell 1:27–42.
24. **Perry, R., D. Kelley, K. Friderici, and F. Rottman.** 1975. The methylated constituents of L cell messenger RNA: evidence for an unusual cluster at the 5′ terminus. Cell 4:387–394.
25. **Pike, L. M., and F. Rottman.** 1974. The determination of 2′-*O*-methylnucleosides in RNA. Anal. Biochem. 61:367–378.
26. **Rottman, F., A. J. Shatkin, and R. P. Perry.** 1974. Sequences containing methylated nucleotides at the 5′ termini of messenger RNAs: possible implications for processing. Cell 3:197–199.
27. **Shatkin, A.** 1974. Methylated messenger RNA synthesis *in vitro* by purified reovirus. Proc. Natl. Acad. Sci. U.S.A. 71:3204–3207.
28. **Van Der Helm, K., and P. H. Duesberg.** 1975. Translation of Rous sarcoma virus RNA in a cell-free system from ascites Krebs II cells. Proc. Natl. Acad. Sci. U.S.A. 72:614–618.
29. **Wei, C.-M., A. Gershowitz, and B. Moss.** 1975. Methylated nucleotides block 5′ terminus of Hela cell messenger RNA. Cell 4:379–386.
30. **Wei, C.-M., and B. Moss.** 1974. Methylation of newly synthesized viral messenger RNA by an enzyme in vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. 71:3014–3018.
31. **Wei, C.-M., and B. Moss.** 1975. Methylated nucleotides block 5′-terminus of vaccinia virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 72:318–322.