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Evidence for Methylation of B77 Avian Sarcoma Virus Genome RNA Subunits

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B77 avian sarcoma virus RNA was labeled with [*methyl*-^{3}H]methionine under conditions that prevent non-methyl incorporation of ^{3}H radioactivity into purine rings. From the determined values for the extent of methylation of 4*S* RNA isolated from infected chicken embryo cells, it was estimated that 30 to 40*S* RNA subunits that result from heat denaturation of the 60 to 70*S* RNA contain approximately 21 methyl groups, of which 14 to 16 are present at internal positions as N^6-methyladenosine residues. In addition, each of the virion RNA subunits appears to contain about two methyl groups in the "capped" 5'-terminal structure m^{7}G(5')ppp(5')G^m. These properties are consistent with the hypothesis that the 30 to 40*S* genome RNA of oncornaviruses also serves an mRNA function in infected cells.

Recent evidence indicates that many viral (1, 11, 12, 33) and cellular (7, 13, 20, 23) mRNA molecules are methylated. The methylated nucleosides in a number of viral mRNA's are contained in a unique blocked 5'-terminal structure: m^{7}G(5')ppp(5')N^m (m^{7}G: 7-methylguanosine; N^m: 2'-*O*-methylated nucleoside) where the two terminal nucleosides are linked by 5'-5' pyrophosphate bonds (12). In addition, certain cellular mRNA's contain not only these "capped" termini but also internal methylated bases, predominantly N^6-methyladenosine (m^{6}A) residues (7, 13, 20). [The complete structures of m^{7}G(5')ppp(5')G^m and m^{6}A are given in Fig. 1.] RNA molecules from oncornaviruses have many of the properties expected of an mRNA; they contain poly(A) sequences (14, 18) and can be translated in cell-free protein-synthesizing systems (26, 29, 31). We describe here evidence for another set of properties that the avian oncornavirus B77 sarcoma virus RNA appears to have in common with cellular mRNA's: "capped" and methylated 5'-termini and internal methylated bases, predominantly N^6-methyladenosine.

MATERIALS AND METHODS

Propagation and purification of virus. B77 avian sarcoma virus was propagated and purified as described in detail elsewhere (28). In summary, the B77 avian sarcoma virus (obtained from P. Vogt) was propagated in leukosis-free chicken embryo fibroblasts (SPAFAS, Inc., Norwich, Conn.) and purified by: (i) centrifugation of the culture fluid at 7,000 rpm in the Beckman J21B centrifuge for 15 min to remove cell debris; (ii) pelleting of the virus from the culture fluid through 20% (wt/vol) sucrose onto a shelf of 70% (wt/vol) sucrose; and (iii) isopycnic banding of the virus in a linear 20 to 70% sucrose gradient.

Radioactive labeling of B77 virus. To each of 10 confluent plates of B77-infected cells was added 5 ml of 200 μCi of L-[*methyl*-^{3}H]methionine per ml (specific activity, 2 to 5 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) and 60 μCi of carrier-free [^{32}P]phosphate per ml (ICN Pharmaceuticals, Irvine, Calif.) in methionine-free, phosphate-free Eagle minimal essential medium containing 20 mM sodium formate, 0.02 mM guanosine, 0.02 mM adenosine, 2% dialyzed calf serum, and 0.3% sodium bicarbonate. After an initial 12-h labeling period, the medium was removed and fresh medium was added containing the same components except that nonradioactive phosphate was added to a final concentration of 0.01 mM. The virus was collected again after a 12-h period. Virus labeled with [^{14}C]uridine and [*methyl*-^{3}H]methionine was made in a similar manner except that the Eagle medium contained phosphate. [^{14}C]uridine was used at a concentration of 0.2 μCi/ml.

Isolation of RNA. Viral RNA was isolated by diluting 1:2 (vol/vol) the virus-containing band from an isopycnic sucrose gradient with a buffer containing 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride, pH 7.5, and extracting with an equal volume of phenol-chloroform (1:1, vol/vol) at room temperature in the presence of 0.5% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) mercaptoethanol, and 100 μg of yeast tRNA (Miles Laboratories, Elkhart, Ind.) as a carrier. The extraction was repeated once, the aqueous phases were pooled, and the RNA was precipitated with 2 volumes of 95% ethanol at −20 C.

Enzymatic digestion. T2 RNase (Sigma, St. Louis, Mo.) was used at a concentration of 45 U/ml in 0.01 M sodium acetate, pH 4.5, and 0.001 M EDTA. *Penicillium* nuclease (P1 nuclease) was a

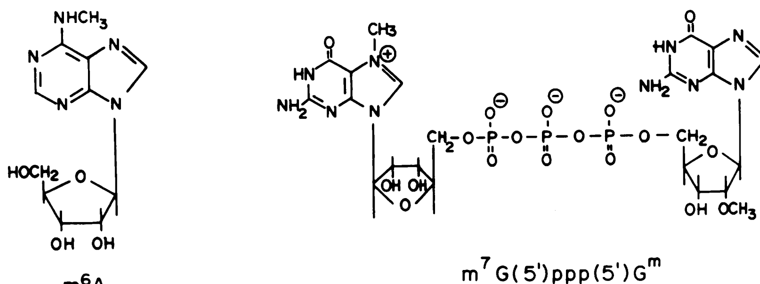

FIG. 1. *Chemical structures of* m^6A *and* m^7G *(5')ppp(5')*G^m.

generous gift from Amiya K. Banerjee, Roche Institute of Molecular Biology, Nutley, N.J. It was used at a concentration of 500 μg/ml in 5 mM sodium acetate, pH 6.0. Bacterial alkaline phosphatase (Worthington, Freehold, N.J.) was used at a concentration of 10 to 20 U/ml in 10 mM Tris-hydrochloride, pH 8.0. RNase A (Worthington) was used at a concentration of 100 μg/ml. Nucleotide pyrophosphatase (Sigma, St. Louis, Mo.) was used at a concentration of 0.06 U/ml in 10 mM Tris-hydrochloride, pH 7.5, and 1 mM $MgCl_2$. All digestions were carried out for 30 min at 37 C except for RNase T2, where a 3-h digestion was used.

Chromatography and electrophoresis of nucleotides and nucleosides. High-voltage electrophoresis was carried out on Whatman 3MM paper at 2,000 V for 2 h at pH 3.5 in pyridine-acetic acid-water (1:10:89, vol/vol). DEAE-cellulose chromatography in the presence of 7 M urea was carried out as previously described (27) except that a linear gradient was generated between 0.05 M NaCl and 0.4 M NaCl. Chromatography on Whatman no. 1 paper was done in three solvent systems: (i) isobutyric acid-5 M ammonia (10:6); (ii) isopropyl alcohol-water-ammonia (7:2:1); and (iii) isopropanol-concentrated HCl-water (680:170:144).

Oligo(dT)-cellulose chromatography of RNA. Oligothymidylate-cellulose [oligo(dT)-cellulose] chromatography was used to separate 30 to 40*S* viral RNA containing poly(A) sequences from that which did not contain poly(A). The 30 to 40*S* RNA sample was dissolved in 0.2 ml of a solution containing 0.5 M KCl, 0.001 M EDTA, and 0.01 M Tris-hydrochloride, pH 7.5, and heated at 60 C for 30 s. Adsorption and elution from the oligo(dT)-cellulose column were then carried out according to previously published procedures (2). Fractions of 0.5 ml were collected. Aliquots were removed from these fractions and counted for 5% trichloroacetic acid-precipitable radioactivity to determine the location of the poly$(A)^+$ and poly$(A)^-$ components of the RNA samples. These fractions were then precipitated with 2 volumes of 95% ethanol after addition to the poly$(A)^+$ fractions of 10 μg of carrier yeast tRNA and NaCl to a final concentration of 0.1 M.

Sedimentation of RNA on gradients containing formamide. Linear 2 to 10% (wt/vol) sucrose gradients (11 ml) containing 85% (vol/vol) formamide, 0.001 M EDTA, 0.01 M Tris-hydrochloride, pH 7.5, were prepared in polyallomer centrifuge tubes. Precipitated RNA samples were dissolved in 30 μl of 0.001 M EDTA, 0.01 M Tris-hydrochloride, pH 7.5, to which was added 170 μl of formamide (Matheson, Coleman and Bell, Norwood, Ohio). The sample was incubated 3 min at 60 C, layered onto the top of the gradient, and centrifuged at 25 C for 20 h at 35,000 rpm in the Spinco SW41 rotor.

Fractions (0.5 ml) were collected from the bottom of the tube, and aliquots were removed and assayed for radioactivity. The peak fractions of 30 to 40*S* RNA, which migrated about halfway through the gradient under these conditions, were combined, adjusted to a final concentration of 0.1 M NaCl, and precipitated with 2 volumes of 95% ethanol after the addition of 50 μg of yeast tRNA as a carrier.

Materials. Methylated nucleosides were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Oligo(dT)-cellulose was a generous gift from S. Kerwar, Roche Institute of Molecular Biology, Nutley, N.J.

RESULTS

Estimation of the number of methyl groups in B77 sarcoma virus RNA. To estimate the extent of possible methylation of the B77 sarcoma virus RNA subunits, virus was labeled with [*methyl*-^{3}H]methionine under conditions that prevent non-methyl incorporation into purine rings via the tetrahydrofolate pathway (23) and with [^{32}P]phosphate. The 60 to 70*S* RNA and its constituent 30 to 40*S* subunits were isolated from glycerol gradients shown in Fig. 2). The ^{3}H/^{32}P ratio was constant across the pooled fractions of both the 60 to 70*S* and 30 to 40*S* RNA peaks. Greater than 95% of the ^{3}H and ^{32}P label in the purified RNA was rendered trichloroacetic acid soluble after treatment with 40 μg of RNase A per ml for 30 min at 37 C, indicating that the material was not contaminated to a significant extent with labeled protein or DNA.

To estimate the fraction of the nucleosides methylated in the viral RNA, it was necessary to determine the extent of methylation of suitable control RNA species isolated from infected cells. The 18*S* and 28*S* rRNA's and 4*S* RNA were isolated from B77 sarcoma virus-infected cells labeled with [^{32}P]phosphate and [*methyl*-

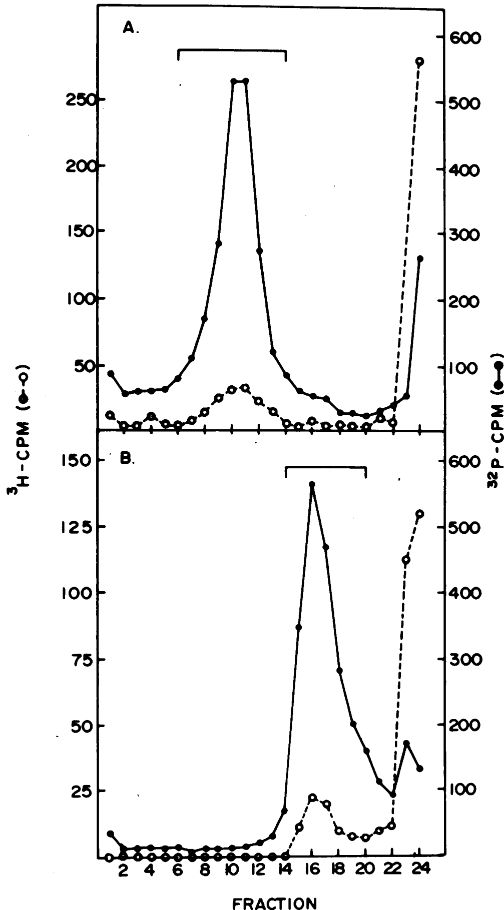

FIG. 2. *Profiles of methyl-³H- and ³²P-labeled RNA from B77 sarcoma virus. (A) B77 sarcoma virus was labeled with [methyl-³H]methionine and [³²P]phosphate, and the RNA was isolated as described in the text. The precipitated RNA was redissolved in 0.2 ml of a buffer containing 0.01 M Tris-hydrochloride, pH 8.0, 0.001 M EDTA, and 0.2% SDS, heated briefly to 37 C, and layered on a 5 to 30% glycerol gradient containing 0.02 M Tris-hydrochloride, pH 7.5, 0.1 M NaCl, and 0.001 M EDTA. Centrifugation was carried out at 35,000 rpm for 3.5 h in the Spinco SW41 rotor. The gradient was collected, 10-µl aliquots from the 0.5-ml fractions were analyzed for radioactivity, and the peak fractions corresponding to the 60 to 70S RNA (shown in figure) and the 4 to 7S RNAs were precipitated with 2 volumes of ethanol at −20 C after addition of 50 µg of yeast tRNA as a carrier. The top of the gradient is on the right. (B) The precipitated 60 to 70S RNA from the first glycerol gradient was redissolved in a buffer containing 0.01 M Tris-hydrochloride, pH 8.0, 0.001 M EDTA, and 0.2% SDS, heated at 80 C for 3 min, quenched in ice, centrifuged, and assayed for radioactivity as described in (A). Peak fractions of 30 to 40S RNA were combined as shown in the figure and precipitated with ethanol after addition of 50 µg of yeast tRNA as a carrier.*

³H]methionine for 24 h under conditions identical to those described above for radiolabeled virus. The 28S rRNA was then digested to completion with RNase A and T2 RNase. Under these conditions, any 3′,5′-phosphodiester bonds containing a methyl group at the C2 position of the ribose are resistant to cleavage (6). Consequently a dinucleotide is produced in the limit digest whenever a 2′-*O*-methylated nucleoside appears in a sequence of RNA. Since most of the methylations of rRNA from eukaryotic cells occur at the 2′-hydroxyl position of the ribose (5, 16, 19, 30, 32), it was expected that after T2 RNase digestion the majority of the *methyl*-³H radioactivity would appear in dinucleotides (with the general structure N^mpNp) and that most of the ³²P label would appear in mononucleotides. Dinucleotides and mononucleotides can be separated by chromatography on DEAE-cellulose columns (described below). By measuring the $^3H/^{32}P$ ratio of the entire 28S RNA molecule before cleavage with T2 RNase and the same ratio in the dinucleotide fraction after cleavage, it was possible to determine the absolute value for the extent of methylation of the 28S rRNA species. Then, since all stable RNA species were labeled to constant specific activity with [³²P]phosphate, it was possible to also determine the percentage of the nucleosides methylated in the 18S rRNA and 4S RNA species. These data are given in Table 1. The values given in Table 1 correspond well with measurements for the extent of methylation determined for rRNA's and tRNA's of a number of other eukaryotic cells (5, 16, 19, 30, 32).

Since 7.8% of the bases are methylated in the host chick cell 4S RNA (Table 1) and it is known that the extent of methylation of virion-associated 4S RNA is similar to that of the 4S RNA of the host chicken embryo cell (3), it was estimated that the virion RNA subunits are methylated to the extent of 0.23% (Table 2). Assuming a genome subunit size of 9,000 bases (17), this corresponds to a value of about 21 methylated nucleosides per RNA subunit. The value for the percentage of methylation of 4S RNA is reasonable since it can be determined from the difference in the $^3H/^{32}P$ ratios of the 60 to 70S and 30 to 40S RNAs that the 60 to 70S RNA contains about 5% 4S RNA, in good agreement with a previous determination (9) and our unpublished observations.

Evidence for base methylation of B77 RNA subunits. T2 RNase digests phosphodiester bonds in RNA to yield 3′-mononucleotides, but will cleave neither pyrophosphate bonds nor phosphodiester bonds when 2′-*O*-methylated nucleosides are present (6). B77 RNA subunits labeled with [*methyl*-³H]methionine and

TABLE 1. *Determination of the extent of methylation of RNA species isolated from B77 sarcoma virus-infected cells*[a]

Material	^{32}P counts/min	^{3}H counts/min	^{3}H/^{32}P	Calculated % methylation
28S dinucleotide[b]	2,821	29,344	10.40	–[c]
28S mononucleotide	99,782	1,756	0.017	0.08
28S RNA[d]	17,506	4,607	0.263	1.26
18S RNA	8,542	3,295	0.385	1.85
4S RNA	1,921	3,095	1.61	7.75

[a] Chicken embryo fibroblasts infected with B77 sarcoma virus were labeled for 24 h with [*methyl*-^{3}H]methionine and [^{32}P]phoshate under conditions described in the text for labeling viral RNA. The cells were harvested and the RNA was extracted by the hot phenol-sodium dodecyl sulfate technique (22). RNA was separated from 4S RNA and residual DNA by precipitation with 1.5 M LiCl. The 28S and 18S RNA species were then separated by glycerol gradient centrifugation. The 4S RNA was further purified by preparative electrophoresis on 10% polyacrylamide gels. By measurement of the absorbance at 260 nm of a sample with a known number of counts per minute, it was determined that these RNA species were labeled with [^{32}P]phosphate to the same specific radioactivity.

[b] rRNA (28S) was digested with RNase A and T2 RNase according to procedures given in the text. The hydrolysate was diluted to 1 ml with 0.05 M Tris-hydrochloride, pH 8.0, 0.05 M NaCl, and 7 M urea and applied to a DEAE-cellulose column. The column was eluted and counted for radioactivity as described in the legend to Fig. 3 except that 0.2 M NaCl was substituted for 0.4 M NaCl in the gradient. The ^{3}H and ^{32}P radioactivities in the mononucleotide and dinucleotide peaks were determined at appropriate channel settings in the Packard 3375 liquid scintillation counter.

[c] The dinucleotide peak has the general structure N^{m}pNp (one methyl group per two phosphates). Percentage of methylation = (^{3}H/^{32}P) × (50/10.4).

[d] Appropriate aliquots of intact 28S, 18S, and 4S RNAs were counted for radioactivity under the same conditions used to count the fractions from the DEAE-cellulose column.

TABLE 2. *Estimation of extent of methylation of B77 sarcoma virus RNA*

RNA	^{3}H/^{32}P[a]	Calculated % methylation	Calculated no. of methylated residues per 35S subunit[b]
Virion-associated 4S	1.77 ± 0.02	7.8[c]	
60 to 70S	0.144 ± 0.007	0.63	56.7
30 to 40S	0.054 ± 0.003	0.23	20.7

[a] ^{3}H/^{32}P ratios were determined by measuring radioactivity in 10 ml of scintillation fluid of a 20-μl aliquot of the designated RNA solution at appropriate channel settings in the Packard 3375 liquid scintillation counter. The limits express the standard errors due to counting statistics. The ^{3}H/^{32}P ratio of the virion-associated 4S RNA was calculated from the observed ratio of the total virion-associated low-molecular-weight RNA (1.56) and the following distribution of RNA species determined by direct analysis of ^{32}P- and *methyl*-^{3}H-labeled material on 10% polyacrylamide gels: 4S (88%); 5S (2%); 7S (10%). The peak of ^{32}P-labeled 5S RNA did not contain detectable *methyl*-^{3}H radioactivity, and the 7S peak was methylated to the extent of 8% that of the 4S RNA as determined by ^{3}H/^{32}P ratios.

[b] Number of methyl groups per subunit was determined by assuming a value of 9,000 nucleotide bases per 35S subunit (17).

[c] The percentage of methylation of 4S RNA was obtained from Table 1, row 5.

[^{14}C]uridine were digested with T2 RNase, and the digest was applied to a DEAE-cellulose column equilibrated with 7 M urea at pH 8 and eluted with a linear salt gradient as shown in Fig. 3. Such a column separates oligonucleotides on the basis of their net negative charge. As expected for a completely digested sample, the only detectable ^{14}C radioactivity emerged at −2 net negative charge, the position of mononucleotides. However, multiple peaks of *methyl*-^{3}H radioactivity were observed in this experiment: one major peak eluting at a net negative charge of −2 corresponding to mononucleotides and a minor peak eluting at about −5 net negative charge. In addition, minor amounts of *methyl*-^{3}H radioactivity emerged at a position greater than −5. The distribution of ^{3}H radioactivity is given in Table 3. The −2 peak was desalted, treated with bacterial alkaline phosphatase (BAP) in order to convert the mononucleotides to their corresponding nucleosides, and subjected to paper chromatography (Fig. 4). In both chromatography systems used, most of the *methyl*-^{3}H-labeled material chromatographed in the same position as the m^{6}A marker. Since 1-methyladenosine (m^{1}A) is converted to N^{6}-methyladenosine at neutral and weakly alkaline pH (21), the peaks of m^{6}A seen here contain an unknown contribution from m^{1}A and should be more precisely designated (m^{6}A + m^{1}A). Note that *methyl*-^{3}H radioactivity was not present in the position of guanosine (G) or adenosine (A), confirming the efficiency of the labeling technique in preventing non-methyl purine ring incorporation of [*methyl*-^{3}H]methionine. Further evidence for the absence of non-methyl purine ring incorporation was obtained by polyacrylamide gel analysis of ^{32}P and *methyl*-^{3}H-labeled, virion-associated low-molecular-weight RNA; no *methyl*-^{3}H radioactivity was detectable in the position of the ^{32}P-labeled 5S RNA (data not shown). This is expected since cellular 5S RNA molecules are not methylated (10) and the viron-associated 5S RNA is identical to that of host cell (9).

Characterization of the presumptive 5′-terminal structure of the B77 RNA subunits. The T2 RNase-resistant material that elutes from the DEAE-cellulose column at a net negative charge of about −5 is consistent with a structure m^{7}G(5′)ppp(5′)N^{m}pNp, but could also

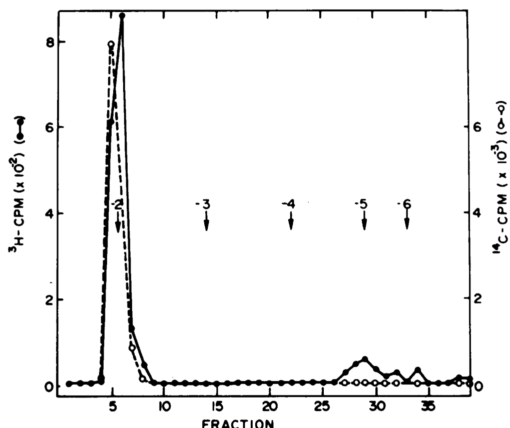

FIG. 3. *DEAE-cellulose chromatography of an RNase T2 digest of methyl-^{3}H- and [^{14}C]uridine-labeled B77 30 to 40S RNA. The 30 to 40S RNA was digested with 100 μg of RNase A per ml for 30 min and then with RNase T2 according to conditions given in the text. The digest was adjusted to a final concentration of 7 M urea, 0.05 M NaCl, 0.05 M Tris-hydrochloride, pH 8.0, mixed with an RNase A digest of 200 μg of yeast tRNA, and applied to a column (0.7 by 15 cm) of DEAE-cellulose. The chromatogram was developed with a linear salt gradient between 0.05 M NaCl, 0.05 M Tris, pH 8.0, 7 M urea, and 0.40 M NaCl, 0.05 M Tris, pH 8.0, 7 M urea (100 ml each). Fractions of 2 ml were collected, and 0.5-ml fractions were analyzed for radioactivity in 15 ml of Aquasol (New England Nuclear Corp., Boston, Mass.). The elution positions of marker oligonucleotides were monitored by measurement of absorbance at 260 nm and are shown in the figure by arrows corresponding to their net negative charges.*

result from the presence of multiple, adjacent 2'-O-methylated nucleosides in internal positions of the RNA. To characterize the sites of methylation further, direct digestion of the *methyl*-^{3}H-labeled 30 to 40S RNA with *Penicillium* nuclease (P1) and BAP was carried out according to the procedures of Lavi and Shatkin (20). P1 nuclease cleaves all phosphodiester bonds including those containing 2'-O-methylated residues. This enzyme, however, does not digest the pyrophosphate linkages in "capped" 5' termini (12). B77 sarcoma virus 30 to 40S RNA was digested with P1 and BAP and directly analyzed by paper electrophoresis (Fig. 5). The *methyl*-^{3}H radioactivity was distributed in three major peaks. One peak moved toward the cathode at a position corresponding to that of the m^{6}A marker, confirming the results given in the previous section. One peak remained at or close to the origin at the position of G^m and U^m. This peak as well as a minor peak that moves toward the cathode at a position beyond m^{6}A probably are digestion products from a trace of 4S RNA that is not removed by heat denaturation or reanneals with the 30 to 40S RNA after its isolation (see next section). In addition, one peak migrated toward the anode between the 2',3'-AMP (Ap) and 2',3'-GMP (Gp) markers, a position characteristic of the "capped" structure m^{7}G(5')ppp(5')N^m

TABLE 3. *Distribution of methyl-^{3}H radioactivity in fractions from DEAE-cellulose columns*

Charge of peak	% of total counts/min
−2	87
−5 to −6	13

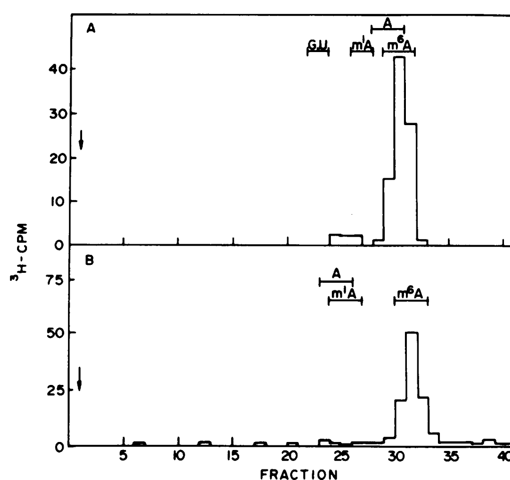

FIG. 4. *Paper chromatography of the mononucleotide peak from a DEAE-cellulose column. (A) The -2 peak from the DEAE-cellulose column shown in Fig. 3 was desalted by a procedure involving a fivefold dilution with 0.01 M triethylammoniun bicarbonate, adsorption to a small DEAE-cellulose column, and elution with 1 M triethylammonium bicarbonate. The material was lyophilized to dryness, dissolved in 10 mM Tris hydrochloride, pH 8, and digested with BAP as described in the text. The digest was then subjected to paper chromatography in system (i) (see text). After development of the chromatogram, the radioactivity was located by cutting the paper into 1-cm strips and counting for ^{3}H radioactivity. The locations of authentic nonradioactive markers in this and subsequent figures were determined by shining a UV lamp on the developed chromatogram. Their positions are indicated in the figure (adenosine, A; uridine, U; guanosine, G). In this chromatography system cytidine and m^{7}G co-migrate with m^{1}A. The efficiency of counting on papers was approximately 15% of that when an equivalent volume was counted in solution in scintillation solvent. (B) The fractions corresponding to the peak of ^{3}H radioactivity in Fig. 4A were eluted and subjected to paper chromatography in system (ii) (see text). In this system, m^{7}G migrates at a position with an R_f of about one-half that of adenosine.*

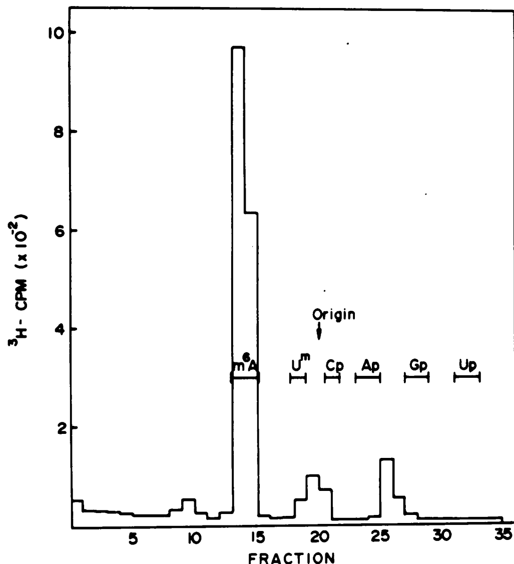

FIG. 5. *High-voltage paper electrophoresis of the P1 nuclease and BAP digest of heat-denatured B77 30 to 40S RNA. B77 sarcoma virus 30 to 40S RNA labeled with [methyl-^{3}H]methionine was digested with P1 nuclease and BAP as described in the text. The digest was spotted on Whatman 3MM paper and subjected to high-voltage paper electrophoresis at pH 3.5 in the presence of markers, as shown in the figure. The anode is on the right. After electrophoresis, the paper was dried, cut into 1-cm strips, and counted for radioactivity.*

(12). The distribution of *methyl*-^{3}H radioactivity in the three peaks is given in Table 4. The percentage of ^{3}H radioactivity in the presumptive "capped" termini (11%) is approximately the same as that (13%) found in the −5 to −6 region of the DEAE-cellulose column (Table 3).

The material in the presumptive "capped" 5'-termini peak was eluted, treated with nucleotide pyrophosphatase and BAP, and re-analyzed by paper electrophoresis (Fig. 6). Approximately equal amounts of *methyl*-^{3}H radioactivity were found in a peak migrating at the position of m^{7}G (52%; peak 1) and in a peak near the origin at the position of G^m and U^m (48%; peak 2). These results are consistent with a structure m^{7}G(5')ppp(5')N^m at the 5' ends of each of the 30 to 40S subunits of viral RNA where N^m is either U^m or G^m. These peaks were further characterized by paper chromatography using system (iii) (see Materials and Methods). The radioactive material in peak 1 co-migrated with m^{7}G (Fig. 7A), whereas the material in peak 2 migrated at the postion of G^m (Fig. 7B). No radioactivity was present at the position of U^m. In a separate experiment where the RNA was labeled with both [*methyl*-^{3}H]methionine and [^{32}P]phosphate, most of the ^{32}P label migrated as inorganic phosphate after digestion with P1 nuclease and BAP, as expected, but approximately 0.04% of the total ^{32}P label was associated with the presumptive 5'-terminal structure (20 counts/min in "capped" termini and 50,000 counts/min as ^{32}P$_i$). Assuming that the RNA subunits contain 9,000 bases (17), this is consistent with the presence of 3.6 phosphates in "capped" termini per RNA subunit and thus the structure m^{7}G(5')ppp(5')G^m. More accurate determination of this number will require analysis of larger amounts of ^{32}P-labeled RNA. It should be restated at this point, however, that the net negative charge of the major peak of T2 RNase-resistant material is −5 (Fig. 3), which is also consistent with the structure m^{7}G(5')ppp(5')G^{m}pNp at the 5' terminus of the avian sarcoma virus RNA.

TABLE 4. *Distribution of methyl-^{3}H radioactivity in peaks from paper electropherogram of P1 and BAP digest of heat-denatured B77 30 to 40S RNA*

Peak	% of total counts/min[a]		
	Expt 1	Expt 2	Avg
m^{6}A[b]	70	76	73
Caps	12	10	11
Origin	18	10	14

[a] Analysis was carried out on two separate preparations of *methyl*-^{3}H-labeled 30 to 40S RNA.

[b] This peak has an unknown contribution from m^{1}A due to its conversion to m^{6}A (see text).

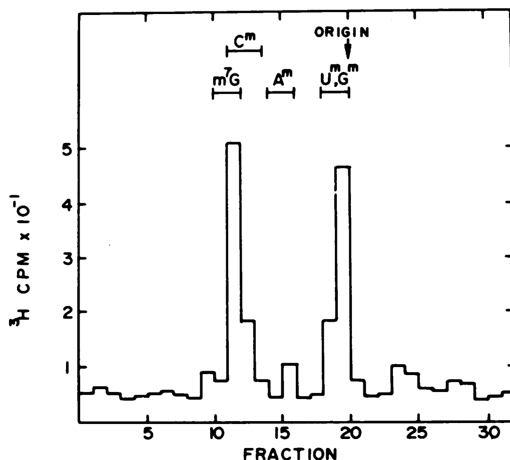

FIG. 6. *High-voltage electrophoresis of the nucleotide pyrophosphatase and BAP digest of presumptive 5'-terminal structures. The peak of radioactivity migrating toward the anode in Fig. 5 was eluted and digested with nucleotide pyrophosphatase and BAP as described in the text. Paper electrophoresis was carried out as described in the legend to Fig. 5.*

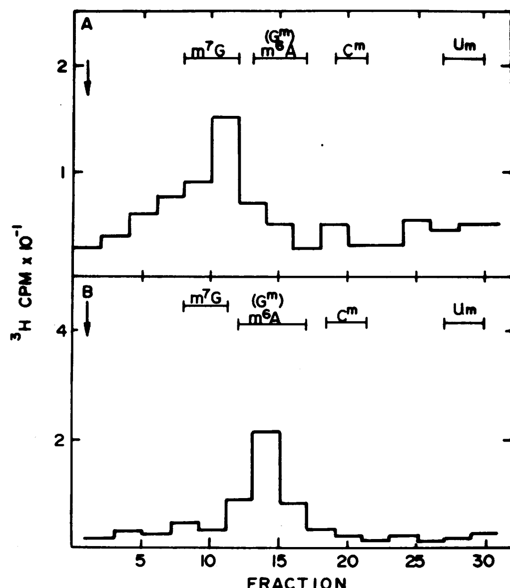

FIG. 7. *Paper chromatography of nucleosides obtained from the nucleoside pyrophosphatase and BAP digest. The peaks at the positions of m^7G (A) and the origin (B) from Fig. 6 were eluted and subjected to paper chromatography in system (iii) (see text). Marker compounds m^7G, m^6A, C^m, and U^m were also run. No marker G^m was available, but the R_f of G^m in this system is virtually the same as m^6A (15). In this experiment, 2-cm rather than 1-cm strips were analyzed for radioactivity.*

Removal of the origin peak from P1 nuclease and BAP digests of 30 to 40S RNA by further purification of the RNA. Significantly different amounts of *methyl*-^{3}H radioactivity were recovered at the origin of the electropherogram when two independent preparations of 30 to 40S RNA were analyzed after BAP and P1 nuclease digestion (Table 4). Furthermore, we found that, although a P1 and BAP digest of *methyl*-^{3}H-labeled 60 to 70S-associated 4S RNA contained no radioactivity at the position of "capped" termini, 74% of the radioactivity remained at the origin of the electropherogram, the position of methylated derivatives of guanosine and uridine. These two observations suggested the possibility that the material at the origin of the electropherogram might arise as a result of contamination of the 30 to 40S RNA with trace amounts of the highly methylated 4S RNA that either remain bound after heat denaturation or reanneal with the RNA before sedimentation on density gradients. To test this possibility, heat-denatured [*methyl*-^{3}H]methionine- and ^{32}P-labeled 30 to 40S RNA was further purified by binding to and eluting from oligo(dT)-cellulose columns followed by density gradient centrifugation in sucrose gradients containing 85% formamide (Fig. 8). The peak of radioactivity was isolated and digested with P1 nuclease and BAP (Fig. 9). A similar profile was obtained from the poly(A)$^-$ fraction (data not shown). It can be seen that purification on formamide gradients resulted in the removal of the peak of radioactivity at the origin from the digest. The minor peak seen in Fig. 5 that moved toward the cathode beyond m^6A was also absent. It appears, therefore, that the small amounts of material found at the origin of the P1 nuclease and BAP digests are products arising from traces of 4S RNA that are bound to the 30 to 40S RNA under normal conditions of heat denaturation, precipitation, and gradient centrifugation under nondenaturing conditions. The proportion of total ^{3}H radioactivity in the "capped" termini (12%) and in the m^6A peak (88%) corresponds to the data given in Table 4, from which it can be calculated that the distribution of radioactivity is 13% in caps and 87% in m^6A if the material at the origin is ignored. Therefore the m^6A and the "capped"

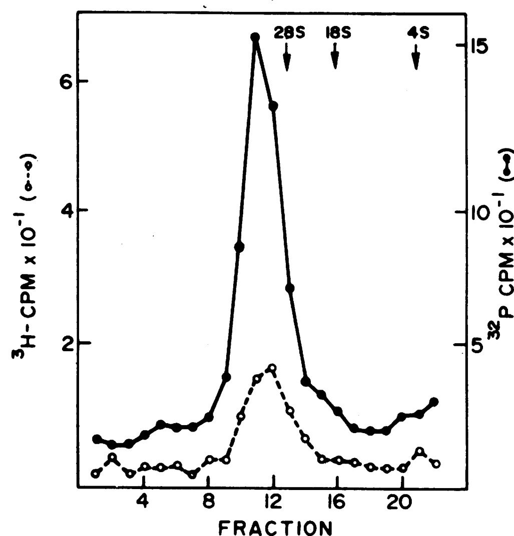

FIG. 8. *Formamide gradient of the poly(A)$^+$ fraction from an oligo(dT)-cellulose column. Oligo(dT)-cellulose column chromatography was carried out on heat-denatured 30 to 40S RNA labeled with [methyl-^{3}H]methionine and [^{32}P]phosphate as described in the text. The poly(A)$^+$ fraction, which represented 76% of the total ^{32}P radioactivity, was centrifuged on gradients containing 85% formamide as described in the text. Aliquots were removed to determine the positions of the radioactivity. ^{32}P-labeled chicken embryo fibroblast 4S RNA, 18S rRNA, and 28S rRNA were run on a separate gradient, and the positions are shown in the figure. Fractions 10 through 13 were combined, precipitated with 95% ethanol, and digested with P1 nuclease and BAP.*

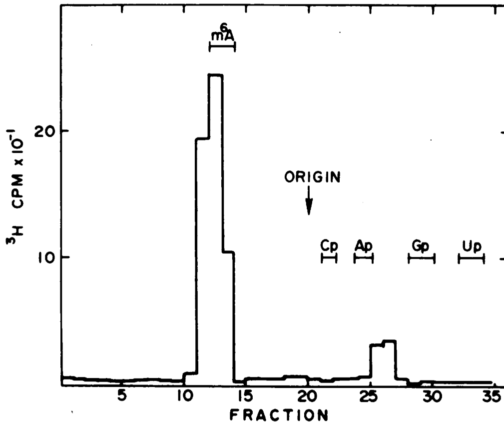

FIG. 9. *High-voltage electrophoresis of the P1 nuclease and BAP digest of B77 30 to 40S RNA that was further purified by oligo(dT)-cellulose chromatography and on sucrose gradients containing formamide. High-voltage paper electrophoresis was carried out as described in the legend to Fig. 5.*

termini appear to be integral constituents of the B77 RNA subunits.

DISCUSSION

The results given above are consistent with the hypothesis that each of the RNA subunits of B77 sarcoma virus contains the methylated 5'-terminal structure $m^7G(5')ppp(5')G^m$, a structure of the type that has been found at the 5' termini of a number of other cellular and viral mRNA's (1, 11, 12, 13, 20, 24, 33). From the data obtained in Tables 1 and 2 we estimated that about 21 bases per subunit, on the average, are methylated when the virus is labeled with [*methyl*-^{3}H]methionine under conditions that prevent non-methyl incorporation into purine rings. This is an upper limit since the results given in Fig. 9 indicate that the *methyl*-^{3}H-labeled 30 to 40S RNA isolated under these conditions is probably contaminated with trace amounts of annealed 4S RNA (on the order of 10 to 20% of the total ^{3}H radioactivity or, since the 4S RNA is methylated 30 times more heavily than the 30 to 40S RNA [Table 2], approximately 0.3 to 0.6% of the total mass). Thus, a value of 17 to 19 methylated bases is probably a better estimate for the number of methyl groups per 30 to 40S subunit. It should be stressed that we consider this number to be a reasonable estimate based on our present evidence. More accurate determinations will require additional types of analysis. From this number it is then possible to calculate the number of methylated bases present in the presumptive 5'-terminal structure. We found that 10 to 12% of the total *methyl*-^{3}H radioactivity was contained in the presumptive 5' terminal "cap" structure after P1 nuclease and BAP digestion (Tables 3 and 4). Thus we calculate that each subunit on the average contains approximately two methyl groups in the "capped" terminus. It was then found that treatment of this material with nucleotide pyrophosphatase and BAP liberated equal amounts of radioactivity in two nucleosides with the electrophoretic and chromatographic properties of m^7G and G^m. Thus it appears that the two methylated nucleosides in the presumptive 5'-terminal structure are m^7G and G^m and that they are linked by pyrophosphate bonds.

The results of the T2 RNase and RNase A digestion given in Fig. 3 indicate that the resistant material bears a net negative charge of about -5. Since the m^7G carries a positive charge, this is consistent with a structure $m^7G(5')ppp(5')G^mpNp$ with a net negative charge of about -5. In addition, it was noted that minor amounts of *methyl*-^{3}H radioactivity emerged at a position indicating a negative charge greater than -5. One possibility to explain the presence of this material is that there are a small number of "capped" termini containing a second 2'-*O*-methylated base, with the structure $m^7G(5')ppp(5')G^mpN^mpNp$. Such termini have been found in HeLa cell mRNA (13) and in mRNA isolated from vesicular stomatitis virus-infected cells (24). Another possibility is that the terminal m^7G base is partially converted to the ring-opened derivative 2,6-diamino-4-hydroxy-5-methylformamido-pyrimidine. If this occurred, the structure $m^7G(5')ppp(5')G^mpNp$ would lose a positive charge and thus assume a net negative charge of about -6. Further work is required to examine these possibilities.

In a similar manner to that described above for the "capped" termini, it can be calculated that there are about 15 to 17 (m^6A + m^1A) residues on the average per genome subunit. Since these modified bases are present even after extensive purification of the RNA, it suggests that they as well as the "capped" termini are integral components of B77 RNA subunits rather than contaminants derived from the heavily methylated 4S RNA. These bases are present in internal positions since they emerged with mononucleotides after T2 RNase digestion. Thus, the B77 sarcoma virus RNA subunits resemble cellular mRNA's (7, 13, 20) and simian virus 40-specific mRNA's (20) in that these mRNA's contain internal methylated bases recovered predominantly as m^6A.

The possible biological significance of the blocked, methylated termini and internal methylations of oncornavirus RNA is not yet clear. It has been shown that blocked, methyl-

ated termini are required for reovirus and vesicular stomatitis virus mRNA's to serve as messengers in cell-free protein-synthesizing systems (4). This function would also be expected in oncornavirus RNAs if the virion RNA serves a dual role: both mRNA and genome RNA. There is evidence suggesting that the 30 to 40S RNA indeed serves as a messenger in oncornavirus-infected cells (8, 25). If the virion RNA is formed in the nucleus from an integrated proviral intermediate, as present evidence suggests, internal methylations may have a role in affecting processing or transport of viral RNA after its synthesis. It will be of interest to determine whether these base methylations occur in specific positions on the RNA genome and whether they occur in regions of the RNA that are involved in RNA subunit linkage or in binding RNA primers for RNA-dependent DNA synthesis.

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ADDENDUM

During preparation of this manuscript, we became aware that essentially similar results to those given here for B77 sarcoma virus have been obtained by Y. Furuichi, E. Stavenezer, J. M. Bishop, and A. J. Shatkin (Nature [London], in press).

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