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Sequence Specificity of Internal Methylation in B77 Avian Sarcoma Virus RNA Subunits†

Kenneth Dimock and C. Martin Stoltzfus*,‡

ABSTRACT: Following ribonuclease digestion of methyl-^{3}H-labeled B77 avian sarcoma virus RNA subunits, methylated oligonucleotides were isolated by diethylaminoethylcellulose chromatography. Partial nucleotide sequences were deduced from the known enzymatic specificities of the ribonucleases. In addition to methylated nucleosides in the 5′-terminal cap structure, m^{7}G(5′)ppp(5′)G^{m}pCp, N^6-methyladenosine (m^{6}A) was found to be present in only two internal sequences of the RNA molecule, Gpm^{6}ApC and Apm^{6}ApC. The average numbers of methylated nucleosides per RNA subunit are about 12–13 in Gpm^{6}ApC, 1–2 in Apm^{6}ApC, and 2 in m^{7}GpppG^{m}pCp. The sequences containing m^{6}A in B77 sarcoma virus RNA are identical to m^{6}A-containing sequences previously reported for the bulk mRNA from HeLa cells (Wei, C. M., Gershowitz, A., and Moss, B. (1976), *Biochemistry 15,* 397–401). Analysis of the oligonucleotides produced by RNase A digestion indicated that the sequence of bases on the 5′ side of these trinucleotides is not specific. The oligonucleotide profile, however, was highly reproducible in different virus preparations. This suggests that the methylations occur at specific positions on the RNA molecule. Some of the methylated oligonucleotides produced by RNase A digestion appear to be present in less than molar amounts. Several hypotheses are proposed to explain this result.

The 35S RNA subunits, which comprise the 70S genome RNA of avian oncornaviruses, have many of the structural and functional properties of cellular mRNA. Each subunit contains 3′-terminal poly(A) sequences (Lai and Duesberg, 1972; Quade et al., 1974) and a capped and methylated 5′-terminal structure m^{7}G(5′)ppp(5′)Gm[1] (Furuichi et al., 1975c; Keith

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[1] Abbreviations used are: m^{6}A, N^6-methyladenosine; m^{7}G, 7-methylguanosine; N, any ribonucleoside; N^m, any 2′-*O*-methylribonucleoside; Pu, purine ribonucleoside; G^m, 2′-*O*-methylguanosine; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; P$_i$, inorganic phosphate.

and Fraenkel-Conrat, 1975; Stoltzfus and Dimock, 1976); both of these structural properties characterize many viral (Furuichi et al., 1975b; Furuichi and Miura, 1975; Lavi and Shatkin, 1975; Moyer et al., 1975; Sommer et al., 1976; Wei and Moss, 1975) and cellular mRNAs (Adams and Cory, 1975; Dottin et al., 1976; Furuichi et al., 1975a; Lavi and Shatkin, 1975; Perry et al., 1975; Sripati et al., 1976; Wei et al., 1975; Yang et al., 1976). In addition, viral-specific 35S RNA from oncornavirus-infected cells is found associated with polysomes (Fan and Baltimore, 1973) and the genome RNA has been shown to direct the synthesis of viral polypeptides in cell-free translation systems (Siegert et al., 1972; Twardzik et al., 1973; von der Helm and Duesberg, 1975). An additional structural property which avian sarcoma virus RNA has in common with cellular mRNA is the presence of a number of N^6-methyladenosine (m^6A) residues at internal positions (Furuichi et al., 1975c; Stoltzfus and Dimock, 1976). It is not yet clear what the functional role of these modifications might be.

As one approach to the problem and also to learn more about the structure of the viral RNA, we have asked the following questions. (1) Are the m^6A residues located in specific sequences or are they randomly distributed on the RNA? (2) Is each RNA subunit methylated to the same extent? (3) Do the methylated sequences resemble those of cellular mRNA and heterogeneous nuclear RNA (hn RNA)? In order to answer these questions we have carried out a partial sequence analysis of the methylated oligonucleotides of B77 sarcoma virus RNA and have determined the average number of these methylated oligonucleotides per 35S subunit.

Experimental Procedure

Propagation and Purification of Virus. Bratislava 77 (B77) avian sarcoma virus was propagated in leukosis-free chicken embryo fibroblasts (Spafas, Inc., Roanoke, Ill.) and purified from the cell culture medium by (1) removal of cell debris, (2) sedimentation onto a sucrose shelf, and (3) isopycnic centrifugation in linear sucrose gradients as previously detailed (Stoltzfus and Snyder, 1975). Cells from a single focus were used to obtain a stock of cloned sarcoma virus (Goldé, 1970).

Radioactive Labeling of Virus. Confluent plates of chicken embryo fibroblasts infected with B77 virus were labeled for 12 h with 5 ml of either (1) 100 μCi/ml of [*methyl*-^{3}H]methionine in methionine-free Eagle's minimal essential medium containing 20 mM sodium formate, 0.02 mM guanosine, and 0.02 mM adenosine in order to prevent nonmethyl incorporation of [*methyl*-^{3}H]methionine into purine rings or (2) 100 μCi/ml of carrier-free ^{32}P in phosphate-free Eagle's minimal essential medium. Both media contained 2% dialyzed calf serum and 1% $(CH_3)_2SO$. After the initial 12-h labeling period, the medium was replaced for two subsequent 12-h intervals with fresh medium containing either 0.005 mM nonradioactive methionine or 0.01 mM nonradioactive phosphate, respectively. The culture fluid from the three harvests was combined and used for virus purification.

Isolation and Purification of RNA. The isolation and purification of B77 sarcoma virus RNA subunits have been previously described (Stoltzfus and Snyder, 1975; Stoltzfus and Dimock, 1976). Viral RNA was isolated by chloroform–phenol extraction in the presence of 0.5% sodium dodecyl sulfate followed by ethanol precipitation. Native 60–70S viral RNA was isolated by centrifugation in linear 5–30% glycerol gradients. Centrifugation in linear 2–10% sucrose gradients containing 85% formamide was used to prepare 35S RNA subunits free of associated 4–7S RNA. Aliquots from gradients were assayed for radioactivity, peak fractions of radioactivity were pooled, and the RNA was precipitated with ethanol. RNA was then dissolved in the appropriate buffer for either the second centrifugation step or subsequent enzymatic digestion.

Enzymatic Digestion. RNase A was used at a concentration of 10 μg/ml in 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA (TE buffer). Complete RNase T_1 digestion was done at a concentration of 100 μg/ml in TE buffer plus 0.2 M NaCl. For combined RNase A plus T_1 digestion, RNase T_1 was used at a concentration of 57 μg/ml in TE buffer following RNase A digestion carried out as described above. For combined RNase A plus T_2 digestion, RNase A was used at a concentration of 100 μg/ml and T_2 RNase at 50 units/ml in 0.01 M sodium acetate, pH 4.5, 0.001 M EDTA. *Penicillium* nuclease (P_1) was used at a concentration of 500 μg/ml in 0.005 M sodium acetate, pH 6.0. Bacterial alkaline phosphatase (BAP) was used at a concentration of 12 units/ml in 0.02 M Tris-HCl, pH 8.0. Nucleotide pyrophosphatase was used at a concentration of 0.12 unit/ml in 0.01 M Tris-HCl, pH 7.5, 0.001 M $MgCl_2$. All digestions were carried out for 30 min at 37 °C in the presence of 500 μg/ml of yeast tRNA with three exceptions: RNase A digestions were for 2.5 h, complete RNase T_1 digestions were for 1.0 h, and RNase T_2 digestions were for 3.0 h.

Chromatography and Electrophoresis. Oligothymidylatecellulose chromatography, employed for the separation of 35S viral RNA containing polyadenylate regions from RNA lacking poly(A), has been detailed elsewhere (Aviv and Leder, 1972).

DEAE-cellulose chromatography on 0.7 × 40 cm columns in 7 M urea at pH 8.0 was carried out as previously described (Stoltzfus and Banerjee, 1972) using a linear gradient between 0.05 and 0.4 M NaCl for elution. Chromatography in 7 M urea at pH 3.5 was performed on 0.7 × 16 cm DEAE-cellulose columns eluted with a linear gradient between 0 and 0.2 M NaCl.

Aliquots from the fractions were assayed for radioactivity and peak fractions were pooled. The pooled fractions were then diluted 1:5 in 0.01 M triethylammonium bicarbonate, applied to 0.8 × 3.0 cm DEAE-cellulose columns equilibrated with 0.01 M $Et_3NH_2CO_3$, and eluted with 1.0 M $Et_3NH_2CO_3$. These desalted samples were evaporated to dryness and resuspended in the appropriate buffer for digestion. The recovery of radioactive oligonucleotides was complete.

Descending paper chromatography was carried out in 2-propanol–water–ammonia (7:2:1, v/v) for 48 h. High-voltage paper electrophoresis was performed in pyridine–acetic acid–water (1:10:89, v/v), pH 3.5, for varying lengths of time. Whatman 3MM paper was used in each case.

Materials. L-[*methyl*-^{3}H]Methionine (4–5 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y. $^{32}P_i$ was obtained from ICN Pharmaceuticals, Irvine, Calif. RNase A and bacterial alkaline phosphatase were purchased from Worthington, Freehold, N.J.; T_2 RNase and nucleotide pyrophosphatase from Sigma, St. Louis, Mo; RNase T_1 (Sankyo) from Calbiochem, LaJolla, Calif.; and *Penicillium* nuclease from Yomasa-Shoyu Co., Tokyo, Japan. Methylated nucleotides and nucleosides were obtained from P-L Biochemicals, Milwaukee, Wisc., and dinucleotides and ApApC from Miles Laboratories, Elkhart, Indiana.

Results

Quantitation and Distribution of m^6A in B77 RNA Subunits. We have previously estimated (Stoltzfus and Dimock,

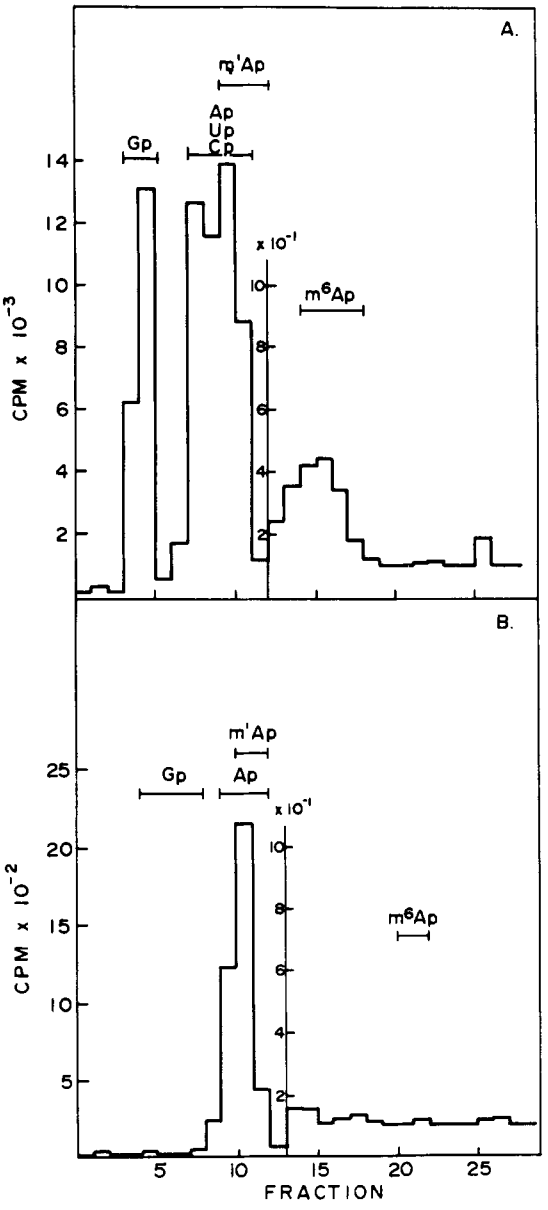

FIGURE 1: Paper chromatographic analysis of the distribution of m^6A in B77 35S RNA. (A) ^{32}P-labeled 35S RNA was digested with T_2 RNase and analyzed by descending paper chromatography (30 h). The chromatogram was dried and 1.0-cm strips were cut and counted for radioactivity in 5 ml of toluene-based scintillation fluid. (B) ^{32}P-labeled 35S RNA was digested to completion with RNase T_1. The digest was then made 0.5 M KCl and heated to 60 °C for 30 s. Adsorption to oligo(dT)-cellulose and elution were carried out according to previously published procedures (Aviv and Leder, 1972). Fractions of 0.5 ml were collected and aliquots were counted for radioactivity. Fractions containing poly(A) were precipitated with 2 volumes of 95% ethanol after addition of 25 μg of carrier yeast tRNA and NaCl to a final concentration of 0.1 M. The poly(A)-containing RNA was then digested with T_2 RNase and analyzed by paper chromatography (48 h).

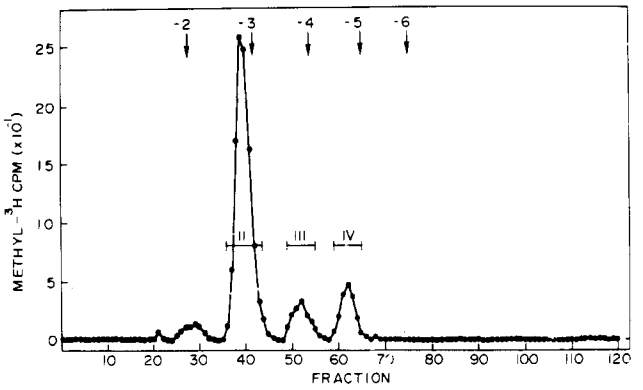

FIGURE 2: DEAE-cellulose chromatography of an RNase A and T_1 digest of methyl-^3H-labeled B77 35S RNA. The RNA was digested with RNase A and then with RNase T_1 as described in the text. The hydrolysate was then mixed with an RNase A digest of 200 μg of yeast tRNA, adjusted to 7 M urea, 0.05 M NaCl, 0.05 M Tris-HCl, pH 8.0, applied to the column, and eluted with a 200-ml 0.05 to 0.4 M linear NaCl gradient. Fractions of 1 ml were collected and 0.3-ml aliquots were analyzed for radioactivity in 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.). The positions of marker oligonucleotide peaks were monitored by measurement of absorbance at 260 nm and are shown in the figure by arrows corresponding to their net negative charges. Peak fractions migrating with each charge class were pooled and desalted as described in the text.

1976) that there are, on the average, 15 to 17 internal methylated bases in each B77 35S RNA subunit and that these methylated bases are predominantly m^6A. This number was based, however, on the assumption that virion-associated 4S RNA is methylated to the same extent as cellular 4S RNA. In order to quantitate the number of m^6A residues directly, without assumptions, and to determine the distribution of m^6A within the B77 genome, ^{32}P-labeled 35S RNA was digested to completion with RNase A and T_2. Together, these enzymes will hydrolyze all phosphodiester bonds in RNA, except at the 5′ terminus, to yield 3′-mononucleotides. Analysis of the hydrolysate by paper chromatography under conditions that separate m^6Ap from the four common mononucleotides resulted in the profile given in Figure 1A. Greater than 99% of the radioactivity chromatographed in the positions of the common 2′,3′-ribonucleotide markers. However, a small amount of radioactivity (0.17%) cochromatographed with the m^6Ap marker. If the 35S RNA is assumed to contain 9000 nucleotides (Jacobson and Bromley, 1975), this represents 15.3 residues of m^6A, on the average, in each subunit, in good agreement with our previous estimates (Stoltzfus and Dimock, 1976). Similar experiments were carried out with the poly(A) sequences isolated by RNase T_1 digestion of ^{32}P-labeled 35S RNA and oligo(dT)-cellulose chromatography. The poly(A) was digested with RNase T_2 and analyzed by paper chromatography to determine if m^6A was present. As shown in Figure 1B, there was no evidence for even a single m^6Ap residue in poly(A). All ^{32}P radioactivity cochromatographed with the Ap marker. These results indicate that all the m^6A is located within the heteropolymeric sequences of 35S RNA and that there is none in the poly(A).

Oligonucleotides Containing m^6A Released by RNase A Plus T_1 Digestion. Because m^6A was located internally within the B77 genome RNA, we asked whether the methylations occur in specific sequences or whether they are distributed at random positions on the molecule. To investigate these possibilities, methyl-^3H-labeled 35S RNA was digested to completion with RNase A and T_1. These conditions result in cleavage of the phosphodiester bonds adjacent to cytosine, uridine, and guanosine, but not adenosine. The released 3′-mononucleotides and oligonucleotides were then separated on the basis of their net negative charges by chromatography on DEAE-cellulose in 7 M urea. Four peaks were obtained, which eluted with net negative charges of −2 through −5 (Figure 2). Evidence from earlier studies suggested that peak IV represented the resistant 5′-terminal "cap" structure m^7GpppG^mpCp. In order to confirm its identity, the material in peak IV was digested with nucleotide pyrophosphatase and bacterial alkaline phosphatase. Electrophoresis of the hy-

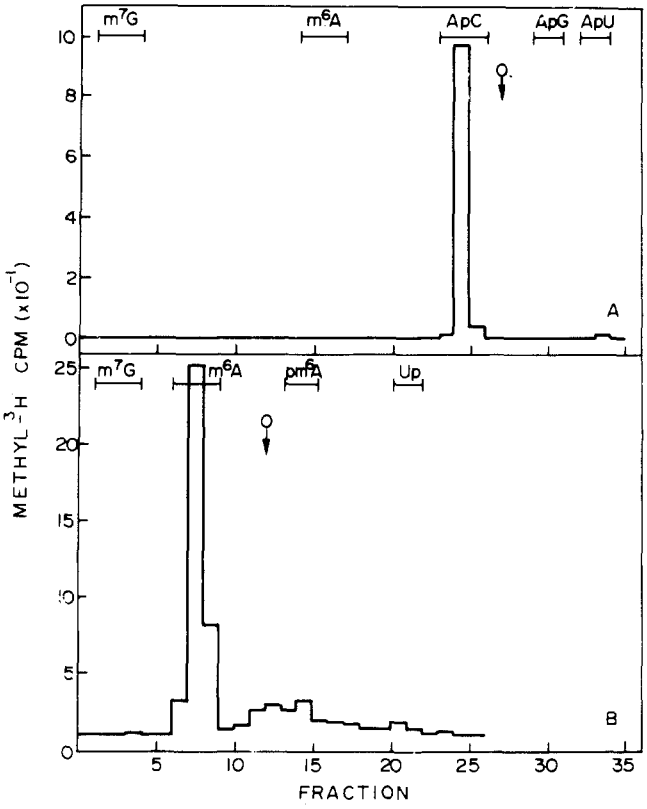

FIGURE 3: High-voltage paper electrophoresis of the dinucleotide peak from DEAE-cellulose column chromatography. (A) Peak II from the DEAE-cellulose column in Figure 2 was desalted and digested with bacterial alkaline phosphatase, spotted on Whatman 3MM paper with the markers indicated, and subjected to high-voltage paper electrophoresis at pH 3.5 and 2500 V for 3.5 h. After electrophoresis, the electropherogram was dried, cut into 1-cm strips and counted in 5 ml of toluene-based scintillation fluid. In this and in subsequent experiments given in Figure 4A, the counting efficiency of the methyl-^{3}H radioacitvity on paper was approximately 8% that of an equivalent amount of radioactive material counted in solution. Radioactivity coelectrophoresing with A_pC was eluted in 1 ml of water and the sample was evaporated to dryness. The peak was then digested with nuclease P_1, spotted, and electrophoresed 2 h at 2000 V under conditions as in A. The electropherogram was dried and 1-cm strips were cut and soaked overnight in 1 ml of water before being counted in 10 ml of Aquasol. O represents the origin.

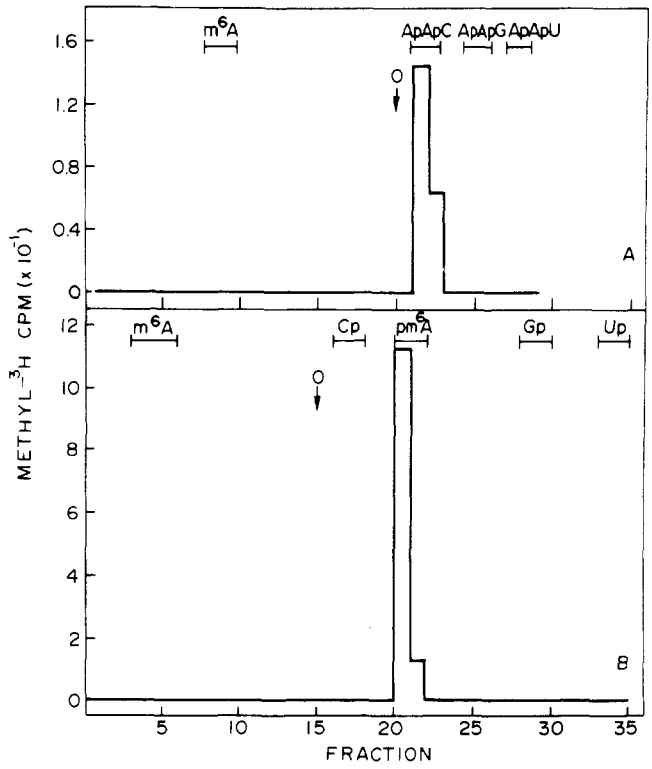

FIGURE 4: High-voltage paper electrophoresis of the trinucleotide peak from DEAE-cellulose chromatography. (A) Peak III from the DEAE-cellulose column in Figure 2 was desalted and digested with bacterial alkaline phosphatase and then analyzed by paper electrophoresis as in Figure 3A. ApApU and ApApG were isolated from an RNase A plus T_1 digest of yeast tRNA. (B) Radioactivity coelectrophoresing with ApApC was eluted, and the sample was treated as in Figure 3B, except that electrophoresis was for 2.5 h at 2500 V.

drolysate yielded the expected radioactive products m^7G and G^mpC in a 1:1 ratio (data not shown). Peak IV, therefore, consists of the "capped" 5′ termini of the 35S RNA. A small amount of radioactivity emerged at the position of mononucleotides (peak I) but was not further characterized due to insufficient radioactivity. Incorporation of labeled methyl groups into the guanine ring is a possible explanation for the presence of this material, although labeling conditions were employed that should block this metabolic step. (See Experimental Procedure.) It may also represent the digestion products arising from a low level of contamination with 4S RNA, although we have previously shown that purification on sucrose gradients containing formamide frees the 35S RNA subunits of most, if not all, of the associated 4S RNA. It is also possible that this material represents a minor amount of a modified nucleoside other than m^6A.

The radioactive material in peak II, the major digestion product, eluted at the position of dinucleotides and could possibly represent m^6ApCp, m^6ApUp, m^6ApGp, or a combination of these three oligonucleotides. In order to determine which of these products were present, the material in peak II was further digested with bacterial alkaline phosphatase and subjected to paper electrophoresis at pH 3.5 together with appropriate marker compounds ApC, ApU, and ApG. Since the N^6-methylated derivative of adenosine bears a net charge almost identical to adenosine at this pH, it is expected that the m^6A-containing dinucleotides should have virtually the same mobility as their unmethylated cognates. As shown in Figure 3A, all the methyl-^{3}H radioactivity migrates at the position of the ApC marker. The identity of this oligonucleotide was confirmed by DEAE-cellulose chromatography of peak II at pH 3.5 under conditions where ApCp is separated from ApUp and ApGp. All of the radioactivity eluted at the position of the ApCp marker (data not shown). To confirm that the label was indeed in m^6A, the m^6ApC peak was eluted from the electropherogram shown in Figure 3A and digested with *Penicillium* nuclease. Since this enzyme cleaves oligonucleotides to yield 5′-monophosphates (Fujimoto et al., 1969), the expected products would be m^6A and pC. The digest was again electrophoresed and, as shown in Figure 3B, almost all the radioactivity coelectrophoresed with m^6A. Thus, we conclude that the radioactive material in peak II is m^6ApCp.

The material in peak III should be composed of the trinucleotides ApApCp, ApApUp, ApApGp, and their methylated derivatives. The methyl group could possibly be found on either or both of the two adenosine residues. Following BAP digestion and paper electrophoresis, all the methyl-^{3}H radioactivity migrated with the ApApC marker (Figure 4A). After elution and nuclease P_1 digestion of the material in the ApApC spot, the digest was analyzed by paper electrophoresis. All the radioactive label coelectrophoresed with a pm^6A marker (Figure 4B). This implies, from the known specificity of nuclease P_1

TABLE I: Oligonucleotides from Combined RNase A plus T_1 Digestion of Methyl-^{3}H-Labeled B77 35 S RNA.

Peak	Net Negative Charge	% Total cpm[a]	Deduced Structures	No. of Methyl Groups per Subunit (1)[b]	(2)[c]
I	−2	6.0 ± 1.4	ND[d]	1.0	1.0
II	−3	71.9 ± 1.3	m^6ApCp	11.5	12.3
III	−4	9.6 ± 0.3	Apm^6ApCp	1.5	1.6
IV	−5	12.5 ± 1.5	m^7GpppG^mpCp	2.0	2.1

[a] Average error from three separate preparations of methyl-^{3}H-labeled 35S RNA. [b] Assuming 2.0 methyl groups per capped terminus. [c] Assuming 17 methyl groups per 35S subunit (see text). [d] Not determined.

TABLE II: Oligonucleotides from RNase A Digestion of Methyl-^{3}H-Labeled 35S RNA from Uncloned B77 Sarcoma Virus.

Peak	Net Negative Charge	% Total cpm	T_1 Products Deduced Structures	% Total cpm[a]	Deduced Structures	No. of Methyl Groups per 35S Subunit (1)[b]	(2)[c]
IP	−3	2.3	ND[d]	2.3	ND[d]	0.4	0.4
IIP	−4	15.3	m^6ApCp	12.9	Gpm^6ApCp	2.0	2.2
			Apm^6ApCp	2.4	Apm^6ApCp	0.4	0.4
IIIP	−5	46.6	m^6ApCp	30.2	$PupGpm^6ApCp$	4.8	5.1
			Apm^6ApCp	3.8	$GpApm^6ApCp$	0.6	0.6
			m^7GpppG^mpCp	12.6	m^7GpppG^mpCp	2.0	2.1
IVP	−6	5.2	ND[d]	5.2	$(Pup)_3m^6ApCp$	0.8	0.9
VP	−7	18.2	m^6ApCp	16.6	$(Pup)_3Gpm^6ApCp$	2.6	2.8
			Apm^6ApCp	1.6	$(Pup)_2GpApm^6ApCp$	0.3	0.3
VIP	−8	3.8	ND[d]	3.8	$(Pup)_5m^6ApCp$	0.6	0.6
VIIP	−9	8.6	m^6ApCp	8.6	$(Pup)_5Gpm^6ApCp$	1.4	1.5

[a] Peaks obtained from RNase A digestion of methyl-^{3}H-labeled 35S RNA from uncloned B77 sarcoma virus were desalted, further digested with RNase T_1, and rerun on DEAE-cellulose at pH 8.0. The fraction of the total radioactivity emerging in peaks II, III, and IV (see Figure 2) was multiplied by the value given in column 3. [b] Assuming 2.0 methyl groups per capped terminus. [c] Assuming 17 methyl groups per 35 S subunit (see text). [d] Not determined.

(see above), that all the m^6A is adjacent to the cytosine residue in the trinucleotide.

In summary, therefore, complete RNase A plus T_1 digestion of B77 35S RNA released only two m^6A-containing oligonucleotides: m^6ApCp and Apm^6ApCp. It must be emphasized that the profiles from DEAE-cellulose column chromatography were highly reproducible when RNA from different virus preparations were used. The values for the distribution of methyl-^{3}H radioactivity in each peak are shown in Table I and represent the average of three separate experiments.

Table I also lists the deduced structures of the methylated RNase A plus T_1 products and the average number of methyl groups per subunit calculated to be present in each of these products. The number of methyl groups in each oligonucleotide has been calculated in two ways: (1) by assuming that there are two methyl groups in each capped terminus and (2) assuming that there are a total of 17 methyl groups in each subunit (15 in m^6A plus 2 in each capped terminus). The calculated values agree very well with each other and the sum also corresponds well with the number determined above in ^{32}P-labeled RNA.

Oligonucleotides Containing m^6A Released Following RNase A Digestion. We then proceeded to determine whether the bases preceding m^6ApC and Apm^6ApC are also specific. B77 35S RNA was digested with RNase A alone and the hydrolysate was analyzed by DEAE-cellulose chromatography at pH 8.0 (Figure 5). A small amount of radioactivity (<3%) emerged at the position of dinucleotides (peak IP). However,

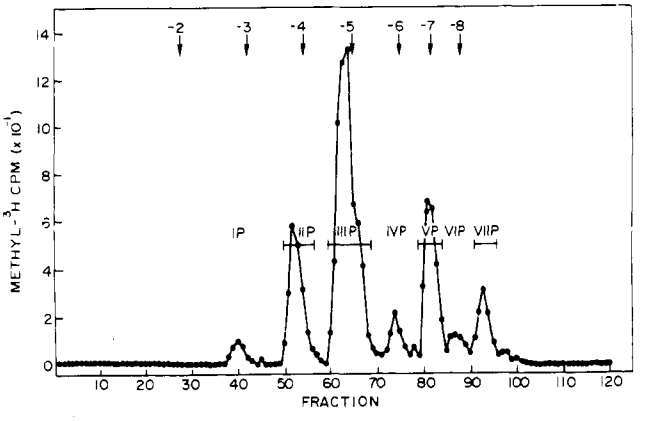

FIGURE 5: DEAE-cellulose chromatography of an RNase A digest of methyl-^{3}H-labeled B77 35S RNA. The RNA was digested with RNase A as described in the text and then treated as described in Figure 2.

the bulk of the radioactive material emerged at positions with net negative charges of −4 (trinucleotide) or greater. Since RNase A specifically cleaves at pyrimidines, this indicates that the m^6A is almost always preceded by a purine. The profile shown in Figure 5 was also highly reproducible in several independent preparations of methyl-^{3}H-labeled RNA.

In order to determine which of the RNase A digestion products are terminated with m^6ApCp and which are terminated with Apm^6ApCp, the material in peaks IIP, IIIP, VP,

TABLE III: Oligonucleotides from RNase A Digestion of Methyl-^{3}H-Labeled 35S RNA from Cloned B77 Sarcoma Virus.

Peak	Net Negative Charge	% Total cpm	T_1 Products Deduced Structures	% Total cpm[a]	Deduced Structures	No. of Methyl Groups per 35S Subunit (1)[b]	(2)[c]
IP	−3	3.3	ND[d]	3.3	ND[d]	0.6	0.6
IIP	−4	12.8	m^6ApCp	11.1	Gpm^6ApCp	2.1	1.9
			Apm^6ApCp	1.7	Apm^6ApCp	0.3	0.3
IIIP	−5	41.5	m^6ApCp	31.7	$PupGpm^6ApCp$	6.1	5.4
			Apm^6ApCp	3.0	$GpApm^6ApCp$	0.6	0.5
			m^7GpppG^mpCp	10.4	m^7GpppG^mpCp	2.0	1.8
IVP	−6	3.2	ND[d]	3.2	$(Pup)_3m^6ApCp$	0.6	0.5
VP	−7	21.4	m^6ApCp	19.7	$(Pup)_3Gpm^6ApCp$	3.8	3.3
			Apm^6ApCp	1.7	$(Pup)_2ApApm^6ApCp$	0.3	0.3
VIP	−8	4.0	ND[d]	4.0	$(Pup)_5m^6ApCp$	0.8	0.7
VIIP	−9	10.2	m^6ApCp	10.2	$(Pup)_5Gpm^6ApCp$	2.0	1.7

[a] Analysis as described for Table II, except that 35S RNA was prepared from cloned B77 sarcoma virus. [b] Assuming 2.0 methyl groups/capped terminus. [c] Assuming 17 methyl groups per 35S subunit (see text). [d] Not determined.

and VIIP was desalted, further digested with RNase T_1, which cleaves at guanosine residues, and rerun on DEAE-cellulose columns at pH 8.0 (there was insufficient radioactivity in peaks IVP and VIP for further analysis). The results obtained from this analysis are given in Table II. It can be observed that peaks IIP, IIIP, and VP contain both m^6ApCp and Apm^6ApCp. However, in each case, m^6ApCp is the predominant species. Peak VIIP appears to contain only m^6ApCp. Consequently, it can be deduced that the major methylated sequence is Gpm^6ApCp. Peak IIIP (−5 net negative charge) also contains the capped terminus, m^7GpppG^mpCp, which, as expected, is resistant to T_1 RNase treatment.

The distribution of methyl-^{3}H radioactivity in the RNase A digestion products both before and after treatment with RNase T_1 is given in Table II. The structures that can be deduced for each product considering the specificity of the two enzymes are also given, as well as the average number of methyl groups per RNA subunit calculated for each of the olignoucleotides. The values obtained for the number of methyl groups in each of the RNase T_1 products agree very well with those shown in Table I.

Note that, on the average, some of the methylated oligonucleotides are present in at least one copy per RNA subunit. However, other methylated oligonucleotides, in particular those containing Apm^6ApC, appear to be present in less than one copy per subunit. This suggests that the subunits are heterogeneous with respect to internal methylation.

It is possible that the apparent heterogeneity could arise from the presence of both "a" and "b" subunits in the virus preparations used in the analysis. The b subunits arise spontaneously during passage of avian sarcoma viruses and represent a deletion of part of the virus genome (Martin and Duesberg, 1972). To rule out this possibility, we carried out an identical analysis to that given in Table II on methyl-^{3}H-labeled 35S RNA from freshly cloned B77 sarcoma virus (Table III). Equivalent amounts of a and b subunits are present in uncloned virus 35S RNA, as determined by 0.5% agarose–2% polyacrylamide gel electrophoresis (Fisher and Dingman, 1971) of heat denatured 70S RNA. However, 95% of the 35S RNA in the cloned virus preparation is present in "a" subunits (data not shown). The deduced methylated sequences and stoichiometry in Table III are very similar to those given in Table II. The same methylated oligonucleotides which appear to be present in submolar amounts from the data in Table II are present in less than stoichiometric amounts in the cloned preparation. This suggests that the apparent heterogeneity in internal methylation exists in the "a" subunits and does not arise from the presence of a mixed population of a and b subunits.

Discussion

We have shown that the internal adenosine methylations in B77 sarcoma virus RNA are present in two sequences: Gpm^6ApC and Apm^6ApC, the former being the predominant sequence. This indicates that the sites of methylation are not distributed at random and suggests that the methylation enzymes recognize specific sequences on the RNA molecule, as has been found with certain other RNA methylases (Kuchino and Nishimura, 1974). From the results of the RNase A digestion of methyl-^{3}H-labeled RNA, we have found that this sequence specificity does not extend to the 5′ side of these three bases. If one assumes a random distribution of the four nucleotide bases and that the RNA subunits contain 15 m^6A residues per 9000 nucleotides, it can be calculated that it would require a sequence of four or five unique bases (i.e., $0.25^5 \times 9000 = 8.8$) to account for the number of m^6A residues if the only criterion for methylation of RNA is base sequence. Since we have not examined the sequence of bases following the m^6ApC on the 3′ side, it is impossible to determine from our present data whether this is true for the methylation of B77 RNA. However, since the profile obtained by RNase A digestion is highly reproducible in independent preparations of methyl-^{3}H-labeled B77 RNA and is also independent of the particular embryo cells or cell passage number, it suggests that the methylated bases are present at specific positions on the RNA molecule. Further work is required to confirm this possibility.

It is remarkable that the methylated sequences that we have determined for B77 sarcoma virus RNA are identical to those reported previously for the bulk cytoplasmic mRNA from human HeLa cells (Wei et al., 1976). While the latter material represents thousands of individual mRNA species, we have shown that this sequence specificity extends to one particular species of RNA. Also, since the viral RNA is transcribed from an integrated DNA provirus and, presumably, is modified by host methylases, this specificity has been conserved in such divergent species as the chicken and man.

In several aspects of its structure, however, the viral RNA

of B77 resembles the cellular heterogeneous nuclear RNA more closely than mRNA. First, its size is greater than normal cellular mRNAs. Second, the total number of m^6A residues per B77 RNA molecule is considerably higher than that reported for cytoplasmic mRNA from eukaryotic cells (1–3 m^6A residues per molecule; Wei et al., 1975, 1976: Salditt-Georgieff et al., 1976; Perry et al., 1975) and approaches the values reported for the heterogeneous nuclear RNA of HeLa cells (four to six times as much m^6A as HeLa cell mRNA; Salditt-Georgieff et al., 1976). Third, the B77 RNA also appears to contain mainly cap 1 termini of the type m^7GpppN^mpNp and little, if any, cap 2 structures of the type $m^7GpppN^mpN^mpNp$ (Keith and Fraenkel-Conrat, 1975; Furuichi et al., 1975c; Stoltzfus and Dimock, 1976). This is also true of nuclear RNA from Novikoff hepatoma cells (Rottman et al., 1976) and HeLa cells (Perry and Kelley, 1976), whereas the cytoplasmic mRNA contains both cap 1 and cap 2 structures. It will be of interest to examine viral-specific polysomal RNA to determine whether the methylation patterns, the ratio of cap 1 to cap 2, or the amount and location of the m^6A are similar to or different from the RNA assembled into virions.

Some of the methylated oligonucleotides appear to be present in less than one copy per RNA subunit (Table II). In view of the evidence to date that the two RNA molecules contained in each virion (King, 1976) are identical in sequence (Coffin and Billeter, 1976; Billeter et al., 1974; Beemon et al., 1974), these results are surprising. Since we have obtained very similar results using both cloned and uncloned B77 virus, it suggests that the apparent heterogeneity with respect to methylation is not due to the presence of a mixed population of a and b subunits (compare Tables II and III).

To account for the apparent heterogeneity, we propose two alternative explanations. One possibility is that the subunits are identical in base sequence but that each RNA molecule is not methylated uniformly. Since the internal base methylation of mRNA and hn RNA is thought to occur in the cell nucleus (Rottman et al., 1976; Perry and Kelley, 1976), the probability that a given RNA molecule is methylated at a particular site may depend on the relative abundance of methylating enzymes, on the affinities of nuclear methylating enzymes for particular nucleotide sequences, the rate of transport of RNA molecules to the cytoplasm, or other factors which might influence the rate and extent of the methylation process. Some ApApC and GpApC sequences might also be protected from methylation by secondary structure of the RNA or by proteins that bind to the RNA and cover these sites.

A second possibility is that different RNA molecules differ at least partially in base sequence and, thus, that the subunits are not completely identical. It should be mentioned here that the first indication that *Escherichia coli* ribosomal RNA contains some reiterated sequences was obtained by observing that the methylated oligonucleotides were present in twice their expected molar ratio (Fellner, 1969). We consider the possibility that subunits differ in base sequence to be unlikely in view of the evidence that the large T_1 RNase oligonucleotides from a number of oncornavirus RNAs invariably occur in equimolar ratios (Beemon et al., 1974; Coffin and Billeter, 1976). However, other evidence based on the rates of DNA–RNA hybridization has suggested the presence of nonidentical subunits in Rous sarcoma virus (Taylor et al., 1974) and Moloney murine leukemia virus (Fan and Paskind, 1974).

The remarkable coincidence in sequence specificity for internal methylation of mRNA in chicken and human cells suggests that there may be a selective advantage for preserving these particular RNA base modifications. However, the functional role of internal base methylation of mRNA remains unknown. It appears that the presence of internal m^6A is not required for translation, since a number of mRNAs from cytoplasmic viruses (Furuichi et al., 1975b; Furuichi and Miura, 1975; Moyer et al., 1975; Wei and Moss, 1975), as well as duck globin mRNA (Perry and Scherrer, 1975), lack such modified bases. However, it is possible that the presence of m^6A in mRNA may affect the affinity of mRNA for ribosomes or its base pairing with anticodons in tRNA. Methylation of adenosine at the N-6 position is known to destabilize Watson–Crick base-pair formation (Engel and von Hippel, 1974; Griffin et al., 1964). Thus, one factor in controlling the relative rates of translation of different mRNAs might be due to differences in internal base methylation. Further studies comparing the extents of methylation of purified mRNAs with their relative translational efficiencies may indicate whether this is a possible translational control mechanism.

The absence of m^6A in duck globin mRNA (Perry and Scherrer, 1975), as well as poly(A)-containing RNA from slime molds (Dottin et al., 1976) and yeast (Sripati et al., 1976) also suggests that the presence of m^6A is not required for posttranscriptional cleavage or transport of mRNA. However, since it appears that the poly(A)-containing nuclear RNA from slime molds is only slightly larger than the mRNA from this organism (Firtel and Lodish, 1973), whereas the hn RNA of higher organisms can be several times larger than mRNA (Holmes and Bonner, 1973; Derman and Darnell, 1974), the steps involved in the conversion on nuclear RNA to mRNA may be different in these two cases. It has been suggested, for instance, that base and sugar methylations may mark positions on large precursor hn RNAs where they are cleaved to form smaller mRNAs (Furuichi et al., 1975a). Further analysis to locate the sites of methylation on selected hn RNA precursors and their mRNA products may give us clues regarding the possible role of methylation in RNA processing.

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