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Identification and mapping of N^6-methyladenosine containing sequences in Simian Virus 40 RNA

Dan Canaani, Chaim Kahana, Sara Lavi and Yoram Groner*

Department of Virology, The Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

Late SV40 16S and 19S mRNAs were found to contain an average of three m^6A residues per mRNA molecule. The methylated residues of both the viral and cellular mRNAs occur in two sequences; Gpm^6ApC and $(Ap)_nm^6ApC$, where n = 1-4. More than 60% of the m^6A residues in SV40 16S and 19S mRNAs occur in Gpm^6ApC even though there are twice as many $(A)_nAC$ than GAC sequences in these messengers. The m^6A containing oligonucleotides of late SV40 mRNAs were localized in the viral messengers. In the 16S mRNA two m^6A oligonucleotides were located at the 5' coding region between 0.95-0.0 map units. The third m^6A residue was mapped between 0.0-0.14 map units in the translated portion of this mRNA. The overall pattern of internal methylation in the 19S mRNA is similar. However, some differences between 16S and 19S mRNAs were observed in both the content and location of the longer $(Ap)_nm^6ApC$ nucleotides These results provide the first example of precise localization of internal methylation sequences in mRNA species with defined coding specificity. It implies that a) location of m^6A residues is not random but specific to a particular region of the RNA, b) apart from sequence specificity other structural features of the mRNA may influence internal methylation and c) m^6A residues are present in coding regions of SV40 mRNAs.

INTRODUCTION

Many viral and eukaryotic messenger RNAs are blocked at their 5' termini by methylated structure designated Cap (for review see 1). In addition, methylation of adenosine residues have been reported at internal positions in mRNA of animal cells and of DNA viruses that replicate in the nucleus (reviewed in 2). N^6-methyladenosines are not part of the poly(A) segment but their exact location or functional significance have not been established. In HeLa cells (3,4), L cells (5), and B77 Avian Sarcoma virus RNA (6), m^6A occurs mainly in two sequences; Gpm^6ApC and Apm^6ApC. This remarkable degree of sequence specificity and the conservation of these methylated residues, during processing of hnRNA (5,7), argues for an important biological function. However, various viral and cellular mRNAs lack m^6A (2). Therefore, it seems that this modified residue is not essential for mRNA translation. An interesting

possibility is that such groups constitute recognition sites for RNA processing or splicing enzymes.

During Simian Virus 40 lytic cycle the late region is transcribed into viral specific messengers which fall into the size classes of 16S and 19S (8,9). These SV40 mRNAs contain structural properties characteristic to many viral and cellular messengers; they are spliced (10-15), capped and contain internal m^6A (16-18). To learn more about the functional role of internal methylated residues we identified and compared the sequences containing m^6A in SV40 RNA (nuclear and mRNA) and in the host cellular mRNA. The average number of the m^6A oligonucleotides in SV40 16S and 19S mRNAs was determined and the methylated sequences were localized on the viral genome.

METHODS

Labeling and isolation of RNA

Labeling of SV40 infected BSC-1 cells with [methyl ^3H]-methionine or [^{32}P]-orthophosphate, extraction of poly(A)-containing mRNA and isolation of SV40 16S and 19S mRNAs were previously described (17), except that 5 mCi of [methyl ^3H]-methionine were used per 10^7 cells. For isolation of cellular mRNA free of contaminating rRNA (19), poly(A)-containing RNA which did not hybridize to SV40 DNA was heated at 60°C for 3 min in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.2% SDS, prior to a second chromatography on oligo(dT)-cellulose. SV40 specific nuclear RNA was isolated from nuclei washed with NP40 buffer (10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonident-P-40). After lysis in SDS buffer (10 mM Tris-HCl (pH 7.6), 0.6% SDS, 1 mM EDTA), RNA was extracted as described by Hirt (20). The supernatant was extracted twice with phenol-chlorophorm-isoamyl-alcohol and twice with chlorophorm-isoamyl-alcohol. RNA was precipitated by ethanol, resuspended in DNase buffer (20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$) and incubated with 20 µg/ml DNase for 60 min at 4°C. EDTA and SDS were added to a final concentration of 15 mM and 0.5%, respectively. RNA was re-extracted by phenol-chlorophorm and precipitated by ethanol as before. RNA was dissolved in hybridization buffer (0.1 M Hepes (pH 7.5), 0.75 M NaCl, 1 mM EDTA, 0.5% SDS, 50% formamide) and SV40 specific RNA isolated by hybridization to SV40 DNA immobilized on Sepharose (21).

Enzymatic digestion

Combined digestion with nuclease P_1 and alkaline phosphatase and digestion with RNase T_2 were carried out as described by Groner and Hurwitz (23). Digestion with RNase A (100 µg/ml) and RNase T_1 (50 µg/ml) in 10 mM Tris-HCl (pH 7.6),

was for 4 hrs at 37°C. Digestion with RNase A alone (100 μg/ml) or RNase T_1 alone (75 μg/ml) in 10 mM Tris-HCl (pH 7.6), was for 4 hrs at 37°C. Treatment of "blots" with RNase A (20 μg/ml) in 2xSSC was for 1 hr at 23°C. For partial digestion with nuclease P_1, oligonucleotides were incubated in 50 mM NaAC (pH 5.5) at 23°C with 0.75 μg/ml P_1 and 50 μg tRNA. Aliquots were removed at various times. EDTA was added to a final concentration of 10 mM and P_1 was inactivated by heating at 100°C for 5 min. Appropriate time points were combined, pH was raised to 8.0 and the sample was treated with 10 U/ml alkaline phosphatase for 60 min at 37°C prior to analysis by paper electrophoresis.

Chromatography and Electrophoresis

DEAE-cellulose chromatography in 7 M urea (pH 7.6) and paper electrophoresis in pyridinium acetate (pH 3.5) were carried out as before (23,24). Chromatography on acetylated dihydroxyboryl cellulose (25) was essentially as previously described (22) but was carried out at 4°C (26). Descending paper chromatography on Whatman 3MM was performed with isopropanol:NH_3:H_2O (70:1:30) for 48 hours. Electrophoresis on DEAE-paper (Whatman DE81) in pyridinium acetate (pH 3.5) was for 5 hrs at 30V/Cm.

Hybridization of SV40 RNA to Specific DNA Fragments and Analysis of Methylated Residues

Digestion of SV40 with restriction endonucleases and fractionation of the fragments were as previously described (14). In order to minimize non-specific adsorption of labeled RNA during annealing to the SV40 DNA fragments, SV40 RNA was purified by hybridization to SV40 DNA Sepharose prior to hybridization to "blots". Hybridized RNA was detected by cutting blots into 1.5 mm strips followed by 60 min incubation at 23°C in 2xSSC containing 20 μg/ml RNase A. Strips were washed twice with 0.5xSSC 0.5% SDS, dried and radioactivity determined in toluene-based scintillation fluid. For analysis of methylated residues RNase resistant RNA was eluted from the hybrids, digested with RNase A and T_1 and analyzed as outlined above. SV40 mRNA was also hybridized to restriction fragments of SV40 DNA bound to Sepharose. SV40 DNA fragments D (0.67-0.76) and E (0.76-0.83) obtained by digesting SV40 DNA with restriction endonucleases BglI, HaeII and HpaI were covalently-bound to Sepharose as previously described (21). Pre-selected 16S and 19S mRNAs were hybridized to each of the fragments for 12 hrs at 37°C in hybridization buffer (0.1 M Hepes KOH (pH 7.5), 0.75 M NaCl, 0.5% SDS, 1 mM EDTA, 50% formamide). Hybrids were washed 5 times with hybridization buffer, 5 times with RNase T_1 buffer (0.01 M Hepes KOH (pH 7.5), 0.3 M NaCl, 1 mM EDTA) and then digested with 23 μg/ml RNase T_1 at 25°C for 60 min. Digested hybrids were washed twice with RNase T_1 buffer and 5 times

with buffer I (0.01 M Hepes-KOH (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 0.5% SDS). Hybridized RNA which was protected against RNase T_1 was eluted at 60°C with elution buffer (10 mM Hepes-KOH (pH 7.5), 0.5% SDS, 98% formamide), and precipitated at -65°C by 3 volumes of ethanol in the presence of 100 µg tRNA and 0.3 M NaAc (pH 5.0). Eluted RNA was analyzed for caps and m^6A oligonucleotides as described above.

Average Size Determination

SV40 infected cells were labeled with [^{32}P]-orthophosphate for 8 hrs 44 hrs post-infection. Poly(A)-containing cytoplasmic RNA was isolated and a portion of the RNA analyzed by sedimentation through SDS sucrose gradients as described in Fig. 2. SV40 specific 16S and 19S mRNAs were localized and their proportion determined by hybridization of gradient fractions to SV40 DNA bound to nitrocellulose filters. It was found that under these labeling conditions the 19S mRNA species represent 15% of the cytoplasmic poly(A)-containing SV40 RNA. To isolate the SV40 poly(A) segment SV40 RNA was purified by preparative hybridization to SV40 DNA immobilized on Sepharose (21). A portion of the RNA was digested with RNases A and T_1 as described above and fractionated on oligo(dT)-cellulose. The bound poly(A) segment was eluted and the amount of radioactivity determined. It was found that the poly(A) segment represents 9.4% of the total radioactivity. Since late SV40 mRNA (16S plus 15% 19S) contain 1,735 nucleotides (27,28), the average length of the poly(A) segment is 163 residues. This number is in good agreement with the previous estimates (29).

MATERIALS

RNase T_2, penicillium nuclease (P_1) and restriction endonucleases were purchased from Calbiochem, Yamasa Shoyu Co., and New England Biolabs, respectively. Bacterial alkaline phosphatase, RNase T_1 and RNase A were from Worthington Biochemical Co. [Methyl ^3H]-methionine (80 Ci/mmole) and [^{32}P]-orthophosphate were obtained from New England Nuclear and Radiochemical Center (Amersham, England). Methylated nucleotides and nucleosides were from P.L. Biochemicals. Trinucleotides were a gift of Dr.Y. Lapidot from the Hebrew University, Jerusalem, Israel.

RESULTS

Quantitation of m^6A Residues in SV40 16S and 19S mRNAs

The average number of m^6A residues per mRNA molecule was determined by

two approaches. First, by measuring the amount of m^6Ap in uniformly ^{32}P-labeled SV40 mRNA, and second from the ratio m^6A/caps in [methyl ^3H]-labeled 16S and 19S mRNAs. For isolation of [^{32}P]-m^6A, labeled SV40 mRNA was purified, digested with RNase T_2 and passed through a column of DBAE-cellulose as described under Methods. RNase T_2 digestion of ^{32}P-labeled RNA generates 5' cap structures and 3' mononucleotides. Caps contain free 2'-3'-hydroxyl groups at the 5' terminal m^7G and are retained by the DBAE-cellulose column due to the affinity of the cis-diols for the dihydroxyboryl groups (22,25). Material unbound to the DBAE-cellulose contained a mixture of nucleosides monophosphate and was fractionated by paper electrophoresis at pH 3.5. Radioactivity comigrating with Ap was eluted and analyzed by paper chromatography under conditions that separates m^6Ap from Ap (Fig. 1). Most of the radioactivity chromatographed with pA. A portion of 0.4% chromatographed as m^6Ap slightly ahead of the pm^6A marker. Identification of this material as m^6Ap was further confirmed by two-dimensional chromatography as before (22).

An average number of 2.91 internal m^6A residues in late SV40 mRNAs was calculated from the following equation:

$$728 \times [0.4/100] = 2.91$$

where 728 represents the number of pA residues in late SV40 mRNA including the poly(A) segment and [0.4/100] is the percent of pm^6A.

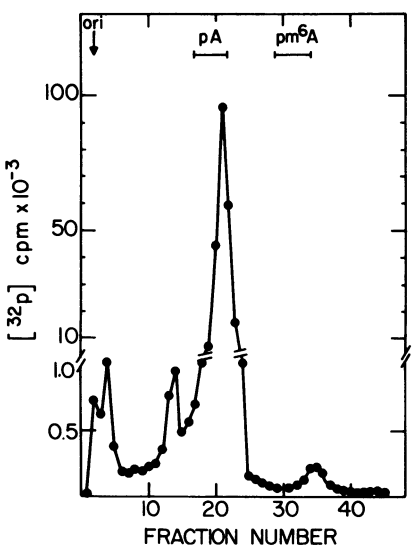

Fig. 1. Paper chromatography separation of Ap and m^6Ap derived from ^{32}P-labeled SV40 mRNA. ^{32}P-labeled material comigrating with the pA marker in paper electrophoresis at pH 3.5 was eluted and analyzed by descending paper chromatography in isopropanol:NH_3:H_2O (70:1:30) as described in Methods.

The number of pA residues in the poly(A) segment of late SV40 mRNA under the conditions of labeling with ^{32}P was determined as described under Methods. The total number of pA residues encoded in late SV40 mRNAs was obtained from the published DNA sequences (27,28) taking into account that under our ^{32}P-labeling protocol the 19S mRNA represented 15% of the late mRNAs (see Methods).

To estimate and compare the number of m^6A in each of SV40 16S and 19S mRNA species, [methyl ^3H]-labeled poly(A)-containing RNA was denatured by heating in 50% formamide and fractionated by velocity gradient centrifugation (Fig. 2). 16S and 19S mRNAs were localized by hybridization across the gradient to SV40 DNA filters. Fractions containing the 16S and 19S mRNAs were separately pooled and viral mRNA isolated by hybridization to and elution from SV40 DNA Sepharose. 16S and 19S mRNA species were digested with nuclease P_1 and alkaline phosphatase followed by separation of m^6A and caps by paper electrophoresis (Fig. 3).

Approximately 50% of the [methyl ^3H]-radioactivity derived from the viral mRNAs (Fig. 3A and 3B) migrated as m^6A and the remainder as cap cores and a small amount of Um which originated from Cap II (18). As previously reported (17,18) the majority of SV40 cap cores contain three methyl groups and have the structure $m^7GpppmAm$. From the ratio $m^6A/m^7GpppmAm \simeq 1$ we deduced that both the 19S and 16S mRNAs contain three m^6A per molecule. This number is in good agreement with the value of 2.91 derived by the direct measurement of m^6Ap in ^{32}P-labeled viral mRNA, described above.

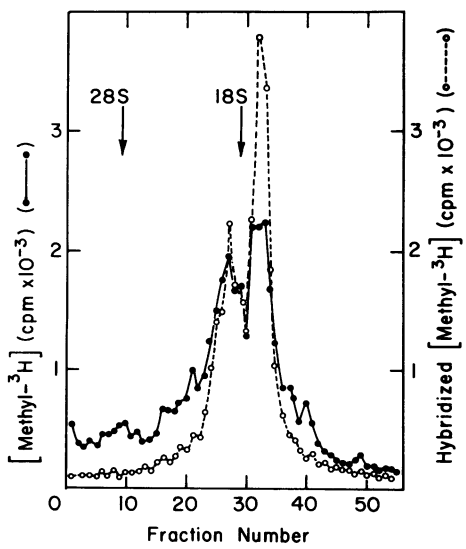

Fig. 2. Velocity gradient separation of SV40 16S and 19S mRNAs. [Methyl ^3H]-labeled poly(A)-containing RNA was layered on 15%-30% (W/V) sucrose gradient after heating 3 min to 60°C in 50% formamide. Gradient was centrifuged 24 hrs at 26 K rpm 20°C, in a Spinco SW 27.1 rotor. Radioactivity in 3 µl aliquots was determined and 15 µl were hybridized to SV40 DNA filters.

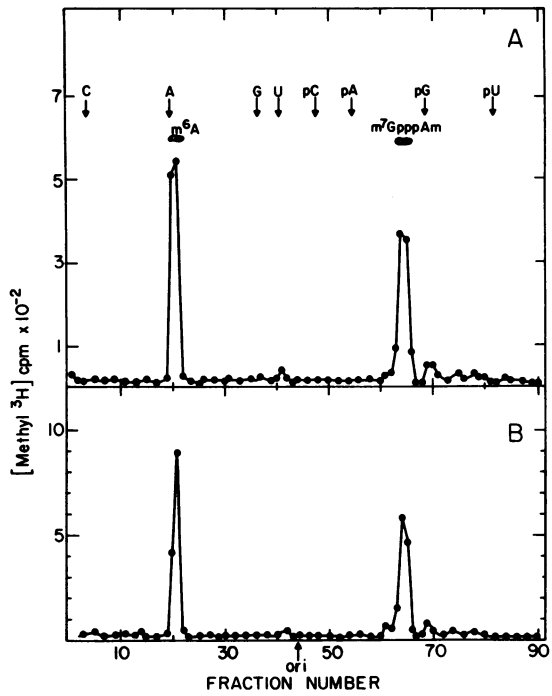

Fig. 3. Paper electrophoretic analysis of nuclease P_1 and alkaline phosphatase digestion of [methyl ^3H]-labeled SV40 16S and 19S mRNAs. Purified mRNA species were digested with nuclease P_1 followed by alkaline phosphatase and analyzed by paper electrophoresis as described in Methods.
A - SV40 19S mRNA; B - SV40 16S mRNA.

Sequences Surrounding Internal m^6A in Cellular BSC-1 and SV40 mRNA

To determine the sequences containing internal m^6A a similar procedure to that described by Wei et al. (4) and Schibler et al. (5) was applied. [Methyl ^3H labeled RNA was digested with a mixture of RNases A and T_1 (which cleave phosphodiester bonds adjacent to G, U and C but not A) and chromatographed on DBAE-cellulose column as described in Methods. The unbound material containing the internal m^6A oligonucleotides was desalted, treated with alkaline phosphatase and analyzed by paper electrophoresis. Figure 4 depicts the separation of m^6A containing oligonucleotides derived from SV40 19S mRNA. Five methylated oligonucleotides were obtained (numbered 1-5); two major peaks migrating slightl ahead of ApC and ApApC markers, plus three more minor peaks. Although methyl

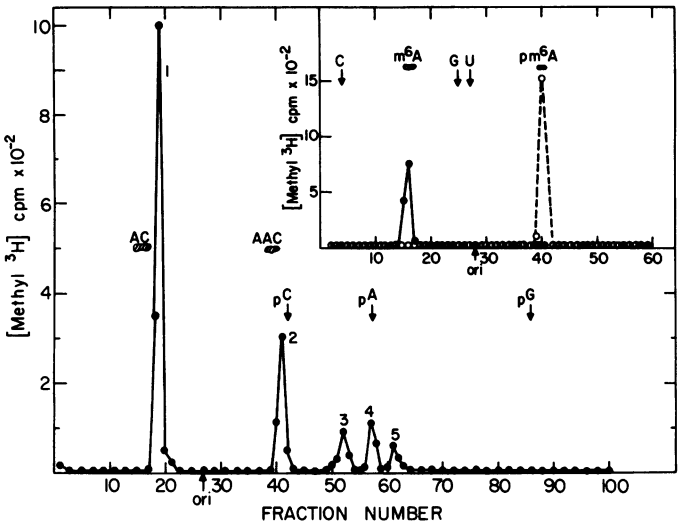

Fig. 4. Paper electrophoresis analysis of m^{6}A containing oligonucleotides derived from SV40 19S mRNA. m^{6}A oligonucleotides were isolated by passage through DBAE-cellulose as described in Methods, treated with alkaline phosphatase and subjected to paper electrophoresis in pyridimium acetate (pH 3.5). Insert - peak 1 (fractions 18-20) and peak 2 (fractions 40-43) in Fig. 4 were separately eluted, digested with nuclease P_1 and reanalyzed by paper electrophoresis in pH 3.5. ●——● peak 1; o----o peak 2. The arrows indicate the position of radioactive markers. Markers AC, AAC, m^{6}A and pm^{6}A were spots detected under ultra-violet light.

groups do not alter the charge of adenosine at pH 3.5, discrepancy between the unmethylated marker and methylated oligonucleotides was previously observed (5). To further characterize these oligonucleotides the appropriate fractions were eluted from the paper and analyzed both by chromatography on DEAE-cellulose columns in 7 M urea (pH 7.6) to determine their net negative charge (Fig. 5), and by electrophoresis on DEAE-cellulose paper (pH 3.5) to confirm their identification (Fig. 6). Material from peak 1 in Fig. 4 (#18-20) eluted with net negative charge of 1 (Fig. 5A), and comigrated with ApC in electrophoresis on DEAE-cellulose paper (Fig. 6B) and is, therefore, identified as m^{6}ApC. Peak 2 (#40-43) is a trinucleotide (charge of -2)(Fig. 5A) migrating with AAC marker in electrophoresis (Fig. 6A) and is interpreted as methylated AAC. To determine which A is methylated in AAC a portion of both peak 1 and peak 2 in Fig. 4 were digested with nuclease P_1 (which cleaves phosphodiester linkages to yield 5' mononucleotides) and analyzed by paper electrophoresis. Results are shown in

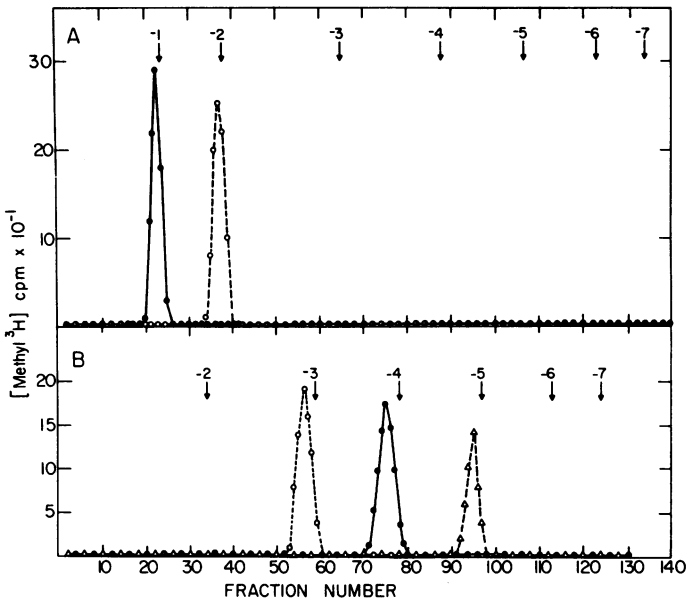

Fig. 5. DEAE-cellulose chromatography of m^6A oligonucleotides eluted from the electroperogram in Fig. 4. Material from each of the five radioactive peaks in Fig. 4 was separately eluted, mixed with tRNA hydrolysate and analyzed by chromatography on DEAE-cellulose in 7 M urea (pH 7.6) as described in Methods. Positions of markers with net negative charge 1 to 7 determined by absorbance at 260 nm are indicated by arrows.
A - ●——● peak 1 and o----o peak 2 from Fig. 4.
B - o----o peak 3; ●——● peak 4; Δ----Δ peak 5 from Fig. 4.

Fig. 4 insert. All the [methyl ^{3}H]-radioactivity from peak 1 moved with m^6A, whereas methyl label from peak 2 migrated as pm^6A. We concluded that the methyl group is located on the internal A and the sequence of the trinucleotide (peak 2) is Apm^6ApC.

From the known specificity of RNase A and T_1 the 5' neighbor of the dinucleotide m^6ApC released by the combined digestion of these enzymes must be either pyrimidine or guanosine. When SV40 19S mRNA was digested with RNase A alone and analyzed as in Figs. 4 and 5, virtually no radioactivity migrated as ApC indicating that the majority of m^6ApC are preceded by G.

Peaks 3, 4 and 5 from Fig. 4 have a negative charge of -3, -4, and -5, respectively, and are interpreted as methylated ApApAp(X), ApApApAp(X) and ApApApApAp(X) where (X) is either U, C or G. To identify the (X) and to determine

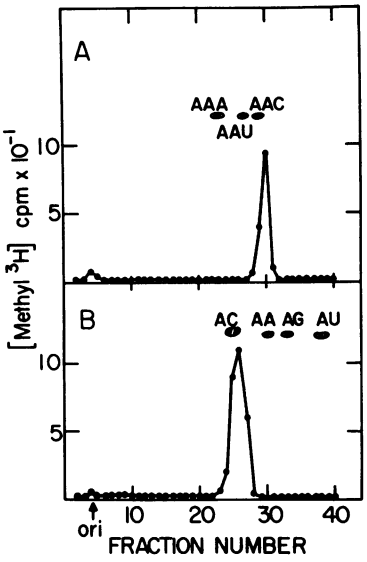

Fig. 6. Electrophoretic analysis on DEAE-cellulose paper of peaks 1 and 2 from Fig. 4. Peaks 1 and 2 in Fig. 4 were separately eluted and aliquots reanalyzed by electrophoresis on DEAE-cellulose paper in pyridimium acetate pH 3.5.
A - peak 2. Fig. 4.
B - peak 1. Fig. 4

which A is methylated, material from peaks 3-5 was subjected to partial digestion with nuclease P_1 followed by treatment with alkaline phosphatase (see Methods). Partial digestion of [methyl ^{3}H]-labeled oligonucleotides like ApApm^{6}ApX with P_1 should give rise to a series of radioactive products ranging in size from tetranucleotide down to methylated nucleoside m^{6}A. These methyl-labeled products can be separated by paper electrophoresis and their sequence can be established by a comparison to the appropriate markers. Figure 7 shows the separation of products generated by partial digestion of the tetranucleotide $(Ap)_3X$ (peak 3 in Fig. 4). In addition to m^{6}A, radioactivity migrated in spots corresponding to ApC and ApApC as well as some undigested material (fractions 67-70). From these results we deduced the sequence ApApm^{6}ApC for this m^{6}A containing oligonucleotide. Applying similar procedures, peaks 4 and 5 in Fig.4 were identified as ApApApm^{6}ApC and ApApApApm^{6}ApC, respectively.

The protocol outlined above was used to identify the internal m^{6}A containing oligonucleotides derived from SV40 16S mRNA, Fig. 8 as well as SV40 nuclear RNA and BSC-1 cellular mRNA (not shown). The relative proportions of the various methylated oligonucleotides in these RNAs are summarized in Table 1.

All RNAs have two major m^{6}A-containing oligonucleotides Gpm^{6}ApC and Apm^{6}ApC. However, some differences appeared between SV40 16S and 19S mRNA in the larger oligo(A)pc nucleotides. The 19S species, as well as SV40 nuclear RNA,

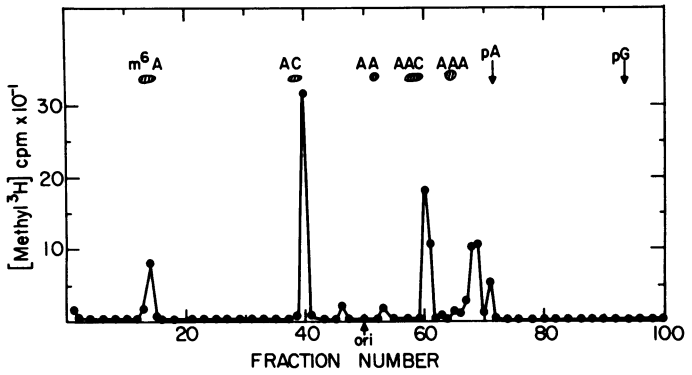

Fig. 7. Paper electrophoretic analysis of partial nuclease P_1 digest of peak 3 in Fig. 4. Fractions 50-54 in Fig. 4 were eluted and aliquots partially digested with nuclease P_1 as described in Methods. After treatment with alkaline phosphatase material was analyzed by paper electrophoresis at pH 3.5 m^6A, AC, AA, AAC and AAA are ultra-violet absorbing markers.

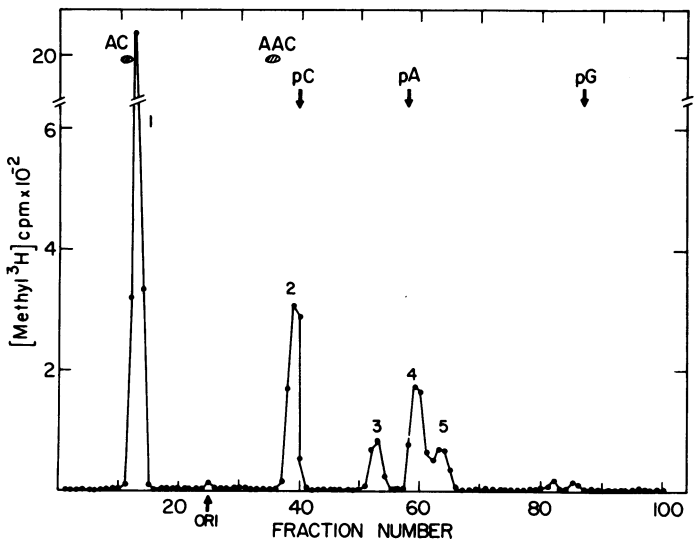

Fig. 8. Paper electrophoretic analysis of m^6A oligonucleotides derived from SV40 16S mRNA. Isolation of m^6A oligonucleotides and subsequent analysis was as described in Fig. 4.

TABLE 1: Relative Proportion of m^6A Oligonucleotides in Viral and Cellular RNA

m^6A oligonucleotides	RNA ANALYZED SV40 19S mRNA (%)	SV40 16S mRNA (%)	SV40 nuclear (%)	nuclear mRNA (%)
Gpm^6ApC	61.2	61.2	55.1	72.7
Apm^6ApC	21.6	23.2	20.3	16.4
$ApApm^6ApC$	6.0	2.1	9.3	2.9
$ApApApm^6ApC$	6.5	11.9	6.0	5.4
$ApApApApm^6ApC$	4.7	1.6	9.3	2.6

[Methyl ^3H]-labeled RNAs were digested with RNases A and T_1. m^6A containing oligonucleotides were isolated by passage through a DBAE-cellulose column, treated with alkaline phosphatase and analyzed by paper electrophoresis as in Fig. 4. Percents are the mean value of three different experiments.

contain considerably more methylated A_3C and A_5C than the 16S mRNA. On the other hand, the 16S species contain twice as much methylated A_4C compared to the 19S and nuclear RNA. The differences between the two viral mRNAs are even more emphasized when these m^6A oligonucleotides were mapped on the RNA (see below Table 3). The SV40 mRNA species contain an average of three m^6A per molecule. Therefore, from the data in Table 1 we deduce that, on the average, each mRNA molecule contains two Gpm^6ApC and one $(Ap)_nm^6ApC$ (where n = 1,2,3,or 4). This signifies that in addition to heterogeneity at the 5' end (18) the 16S and 19S mRNAs are also heterogeneous with respect to internal methylation.

The pattern of internal methylation in the viral 16S and 19S mRNAs as well as in the cellular mRNA was further evaluated by analysis of the m^6A oligonucleotides produced by digestion with RNase T_1 (Fig. 9). Virtually no radioactivity eluted at -1 as was expected by the absence of m^6ApG sequences. At (-2),position of the trinucleotide m^6ApCpG, a small peak appeared in the 19S (1.9%) indicating the low frequency of this methylated sequence in the 19S mRNA species (Fig. 9A). However, significantly larger amounts of the -2 material derived from the 16S (9.6%) and the cellular mRNA (7.6%)(Fig. 9B and C). Nevertheless most of the radioactivity eluted as larger oligonucleotides (Fig.9). The production of a variety of RNase T_1 oligonucleotides indicated that follow-

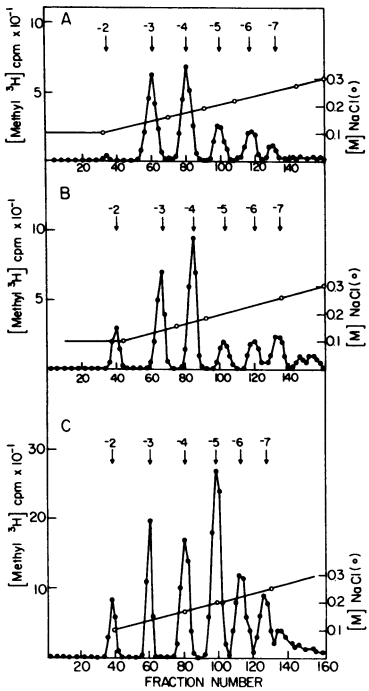

Fig. 9. DEAE-cellulose columns chromotography of m^{6}A-containing oligonucleotides produced by digestion of RNA with RNase T_1. SV40 16S and 19S mRNAs and cellular mRNA were digested with RNase T_1. m^{6}A oligonucleotides isolated by passage through DBAE-cellulose column, treated with alkaline phosphatase and analyzed by DEAE-cellulose chromatography in 7 M urea (pH 7.6).
●——● [methyl ^{3}H] cpm
o——o [M] NaCl
A - 19S mRNA
B - 16S mRNA
C - cellular mRNA

ing m^{6}ApC, G occurs randomly. The elution profiles of 16S, 19S and cellular mRNAs were similar except for a higher proportion of oligonucleotides eluting at -5 charge in BSC-1 mRNA (Fig. 9C). We concluded that in both the viral and cellular mRNAs the sequence $(\frac{G}{A})$pm^{6}ApC is specific but sequences surrounding it vary even in the same species of mRNA like the viral 16S and 19S mRNAs.

Mapping the m^{6}A Containing Sequences of Late SV40 16S and 19S mRNAs

The above experiments provide information regarding sequences surrounding the m^{6}A residues in the viral and cellular mRNAs. By comparing the number of various m^{6}A containing oligonucleotides found in SV40 16S and 19S mRNA species to the known nucleotide sequence of these mRNAs (27,28) it became evident that not all the potential GAC or AAC sequences are methylated. We, therefore, considered the possibility that the m^{6}A residues are located at specific points and we performed experiments to map the internal m^{6}A on the 16S and 19S mRNAs. Such experiments should also provide an answer to the open question as whether m^{6}A residues can occur in translated regions of the viral mRNA. To this end [methyl ^{3}H]-labeled SV40 specific 16S and 19S mRNAs were isolated by prepara-

tive hybridization to SV40 DNA Sepharose as described above and the purified RNAs were rehybridized to blots containing six DNA fragments obtained by digestion of SV40 DNA with EcoRI, BglI, BamI, HpaI and HaeII (Fig. 10). Blots were treated with RNase to digest the non-hybridized tails as described in Methods and the remaining radioactivity determined (Fig. 10). [Methyl ^{3}H]-radioactivity from both the 16S and 19S mRNAs was detected in fragments representing the entire late region of the genome. Nevertheless the distribution of methylated residues was not random as became evident from comparison with a parallel experiment performed with uniformly labeled ^{3}H-uridine RNA. The results of the two experiments are summarized in Table 2. Uridine labeled 16S and 19S mRNA hybridized most efficiently to DNA fragment D (0.0-0.14) which contain sequences represented in both late mRNAs (27,28,30). The 16S mRNA hybridized with lower efficiency to fragment C (0.83-0.0) as expected from the fact that only part of the sequences in this fragment are present in the 16S species (27,28,30). Radioactivity associated with fragment F

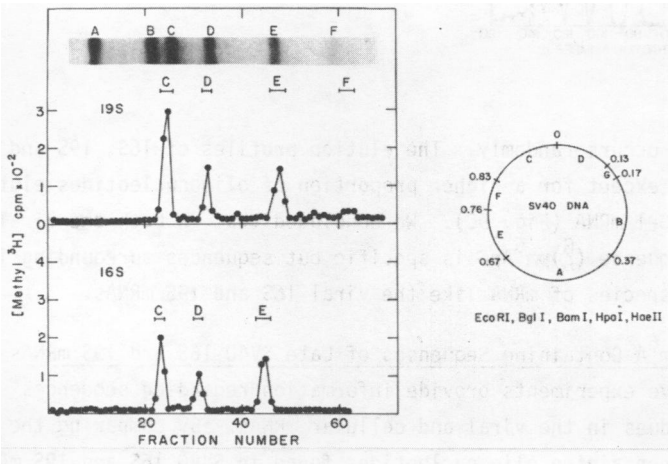

Fig. 10 Hybridization of [methyl ^{3}H]-labeled 16S and 19S mRNAs to "blots" containing DNA fragments obtained by digestion of SV40 DNA with EcoRI, BglI, BamI, HaeII and HpaI. SV40 16S and 19S were selected by hybridization to SV40 DNA bound to Sepharose. The purified mRNA species were then hybridized to filter blots containing the following SV40 DNA fragments: A - 0.37-0.67; B - 0.17-0.37; C - 0.83-0.0; D - 0.0-0.14; F - 0.67-0.76; F - 0.76-0.83. Blots were cut into 1.5 mm strips, treated with RNase A and radioactivity determined as described in Methods. Top - hybridization of 19S mRNA. Bottom - hybridization of 16S mRNA. Autoradiogram depicts hybridization of SV40 DNA labeled in vitro with ^{32}P to similar blots.

TABLE 2: Hybridization of [methyl ^{3}H] or [^{3}H]-uridine labeled SV40 16S and 19S mRNAs to DNA fragments produced by digestion with EcoRI, BglI, BamI, HaeII and HpaI

Frag.	Map Units	16S mRNA		19S mRNA	
		[^{3}H]-uridine	[methyl ^{3}H]	[^{3}H]-uridine	[methyl ^{3}H
		(cpm)		(cpm)	
C	0.83-0.0	200(22.1)	350(40.9)	470(41.7)	525(49.3)
D	0.0 -0.14	625(69.4)	195(22.8)	535(47.5)	210(19.7)
E	0.67-0.76	77(8.5)	310(36.3)	52(4.6)	285(26.7)
F	0.76-0.83	0(0)	0(0)	70(6.2)	45(4.3)

Preselected [^{3}H]-uridine labeled or [methyl ^{3}H]-labeled 16S and 19S mRNAs were hybridized to blots and analyzed as described in Methods and Fig. 10. Numbers in parenthesis represent the percent of total cpm hybridized.

(0.76-0.83) was detected only in the 19S mRNA since sequences in this fragment are absent from the 16S mRNA (28,28,30). A different distribution was found for the methylated nucleotides (Table 2). The relative [methyl ^{3}H]-radioactivity associated with fragments C (0.83-0.0) and E (0.67-0.76) was much higher than that found when the RNA was labeled with ^{3}H-uridine. Moreover, [methyl ^{3}H]-radioactivity in fragment E (0.67-0.76) is derived primarily from leader associated caps (11,14,18, and documented below), whereas, methyl groups in RNA sequences hybridized with fragment C are found only in $m^{6}A$ residues (see below). This clustering of $m^{6}A$ is more emphasized in the 16S mRNA which contain only part of the sequences present in fragment C, mainly those located between 0.95-0.0 map units (27,28).

To isolate and identify the $m^{6}A$ containing oligonucleotides that hybridized to the different fragments, radioactive RNA associated with fragments C and D in Fig. 10 was eluted, treated with RNases A plus T_1 followed by treatment with alkaline phosphatase and $m^{6}A$ oligonucleotides identified as detailed above. Results are summarized in Table 3. Two out of the three $m^{6}A$ residues of the 16S mRNA were localized in fragment C (0.83-0.0) and one in fragment D (0.0-0.14). Sequences from the region spanning 0.83-0.95 map units are not present in 16S mRNA (27,28) and our 16S mRNA preparation is practically free of 19S mRNA sequences (Tables 2 and 4). We, therefore, concluded that one $Gpm^{6}ApC$ and one of either $Apm^{6}ApC$ or $ApApApm^{6}ApC$ are present at the 5' region of the "body" of the 16S in the 390 nucleotide stretch, between 0.95-0.0 map units.

TABLE 3: m^6A oligonucleotides derived from regions in SV40 16S and 19S mRNAs that hybridized to DNA fragments C and D produced by EcoRI, BglI, BamI, HaeII, HpaI restriction endonucleases

mRNA species	Fragment Map Units	m^6A containing oligonucleotides %			
		Gpm^6ApC	Apm^6ApC	$ApApApm^6ApC$	$ApApApApm^6ApC$
16S	C (0.83-0.0)	39.5	34.0	26.5	N.D.
	D (0.0 -0.14)	100	N.D.	N.D.	N.D
19S	C (0.83-0.0)	52.0	34.6	8.5	4.9
	D (0.0 -0.14)	100	N.D.	N.D.	N.D.

Preselected [methyl ^3H]-labeled 16S and 19S mRNAs were hybridized to blots as described in Methods. Blots were treated with RNase A and radioactivity determined as described. Protected RNA was eluted and m^6A oligonucleotides identified as described in the text. N.D. means not detected.

The second Gpm^6ApC oligonucleotide was localized in fragment D (0.0-0.14) but none of the $(Ap)_nm^6A$ species was found in this fragment. The pattern of internal methylation in the 19S mRNA is very similar. Both Gpm^6ApC and methylated $(Ap)_npC$ (n = 2, 4 and 5) were detected in RNA hybridized to fragment C (0.83-0.0), whereas, only Gpm^6ApC was found in fragment D (0.0-0.14). Note, however,that there is three times more $ApApApm^6ApC$ in the 16S mRNA portion which hybridized to fragment C.

To examine whether the 19S mRNA contain m^6A in the region between 0.83-0.95 map units methyl-labeled 19S mRNA was hybridized to blots containing DNA fragments obtained by digestion with HindIII endonuclease. After treatment with RNase A as described in Methods mearly 5% of the total radioactivity were protected by HindIII fragment E (0.86-0.95). The rest of the RNA was distributed between fragments A (0.98-0.33) and C (0.65-0.86). From these results we concluded that in 19S mRNA similar to 16S the two methylated oligonucleotides are located between 0.95-0.0 map units. To identify the low amounts of radioactivity hybridized to fragment F (0.76-0.83) in Fig. 10 and to examine the possibility that m^6A residues are present in the leader RNA, methyl-labeled 16S and 19S viral mRNAs were hybridized to restriction endonucleases EcoRI, BglI, BamI, HaeII, HpaI DNA fragments E (0.67-0.76) and F (0.76-0.83) immobilized on Sepharose as described in Methods. The hybrids

were treated with RNase T_1 to remove non-complementary residues; the protected RNA was eluted, digested with P_1 and alkaline phosphatase prior to analysis by paper electrophoresis as in Fig. 2. m^6A and caps were identified and results are summarized in Table 4.

In both the 16S and 19S mRNAs most of the [methyl ^3H]-radioactivity protected by fragment E (0.67-0.76) were caps, in agreement with previous reports (11,14,18). Data in Table 2 shows that 36.3% of methylated residues in 16S mRNA and 26.7% of methyl groups in 19S mRNA were protected by DNA fragments spanning 0.67-0.76 map units. From the analysis described in Table 4 it is clear that only 13.5% and 10%, respectively, of these residues were m^6A and the rest caps. We, therefore, concluded that 4.7% of the m^6A derived from the 16S and 2.7% of those derived from the 19S mapped at this region. The proportion of methyl-labeled 19S mRNA hybridized to fragment F (0.76-0.83) in Table 2 was small (4.3%); from this 37.7% were caps and the rest m^6A oligonucleotides (Table 4). These m^6A oligonucleotides were analyzed by our standard procedure, and $ApApm^6ApC$ was the major component identified. We concluded from these experiments that a minor population in the 19S mRNA species contain methylated $(Ap)_3C$ in the region between 0.76-0.83 map units. The 16S mRNA does not contain sequences from this region and, therefore, did not hybridize to fragment F (0.76-0.83)(Table 4), confirming the data presented in Table 2 with both uridine-labeled and methyl-labeled 16S mRNA. These results strongly indicate that our 16S mRNA is not cross-contaminated by sequences derived from the 19S mRNA species.

TABLE 4: m^6A and caps in 16S and 19S SV40 mRNAs which are protected against RNase T_1 by hybridization to SV40 DNA fragments bound to Sepharose

		16S mRNA [methyl ^3H] cpm		19S mRNA [methyl ^3H] cpm	
Frag.	Map Units	m^6A	caps	m^6A	caps
(E)	0.67-0.76	184(13.5)	1184(86.5)	70(10.0)	618(90.0)
(F)	0.76-0.83	N.D.	N.D.	152(62.3)	92(37.7)

Preselected [methyl ^3H]-labeled 16S and 19S mRNAs were hybridized to DNA fragments immobilized on Sepharose. RNase T_1 treatment of hybrids and the subsequent analysis of protected RNA were as described in Methods. Numbers in parenthesis represent percent of total cpm hybridized.

DISCUSSION

The average number of m^6A residues in SV40 16S and 19S mRNA species was determined in two ways: a) by measuring the amount of m^6Ap in ^{32}P-labeled viral mRNAs and b) from the ratio m^6A/caps in [methyl ^3H]-labeled 16S and 19S mRNAs. The two values 2.91 and 3 m^6A residues per molecule agree very well with each other. SV40 16S mRNA (leader, body, poly(A)) contain 1574 nucleotides and the 19S species 2329 nucleotides (27,28). Thus, the 16S and 19S mRNAs contain one m^6A per 524 and 776 nucleotides, respectively. The number of m^6A residues in cellular and other viral mRNAs appears to be proportional to the size of the RNA. Poly(A)-containing cellular mRNAs have an average of one m^6A residue per 800-1000 nucleotides (7,31-33). Ten to twelve m^6A were found in the 10,000 nucleotide RNA genome of Rous Sarcoma virus (34,35), 15 m^6A per 9,000 nucleotides were reported for B77 Sarcoma virus RNA (6) and Adenovirus-2 mRNA which has an average size of 2,000 nucleotides contain four m^6A per molecule (36). It was, therefore, interesting to note that although SV40 19S mRNA is 755 nucleotides longer than the 16S it does not contain more m^6A residues suggesting that other factors besides size may play a role in internal methylation of mRNA.

The two major m^6A containing sequences Gpm^6ApC and Apm^6ApC that we have found in SV40 RNA and BSC-1 cellular mRNA are identical to those previously reported for mRNA from HeLa cells (3,4), L cells (5), and B77 Sarcoma virus RNA (6). This implies that in monkey cells too, the specificity of enzymes which modify the RNA has been conserved. In addition to the two major m^6A sequences we have identified a series of less abundant methylated $oligo(Ap)_nC$, which in the viral mRNA represent more than 15% of the m^6A oligonucleotides. These longer oligo(A)pC are methylated to a much lower extent in Hela and L cells mRNA (4,6), signifying that besides sequence specificity other structural features of the RNA may influence methylation.

The m^6A containing sequences present in SV40 16S and 19S mRNA species were mapped by hybridization of methyl-labeled RNA to specific DNA fragments. This provided the first example of precise localization of internal methylation sites in mRNA species with defined coding specificity. Two out of the three m^6A found in SV40 16S and 19S mRNAs are clustered in a rather small region of the RNA between 0.95-0.0 map units. The third m^6A residue was localized in the region spanning 0.0-0.14 map units and found in the sequence Gmp^6ApC. This portion of the 16S mRNA is part of the coding region of VP-1 (27,28). Moreover, assuming the differences in nucleotide sequence between SV40 strains 777 and 776 are not big, then from the published data (27,28) it is evident that

all the potential sites for internal methylation in the region 0.95-0.0 of the 16S mRNA are restricted to the translated region. Therefore, the 16S Gmp^{6}ApC and $(Ap)_n m^6$ApC protected by DNA fragment C (0.83-0.0) are also located in the coding portion of this message. As a matter of fact, in DNA fragment C (0.83-0.0) there is only one $(Ap)_4C$ (residues 1600-1604 Fiers et al. (27) or 1536-1541 Reddy et al. (28)) and one $(Ap)_5C$ (residues 1703-1707 (27) or 1640-1645 (28)). Hence, detection of ApApApm^{6}ApC in segments of 16S and 19S mRNAs that hybridized to fragment C (0.83-0.0) and ApApApApm^{6}ApC in 19S mRNA that hybridized to this DNA fragment allow us to pin-point these m^{6}A residues. In RSV RNA, m^{6}A residues were previously found in a region containing the src and part of env. genes, and it was suggested that these m^{6}A residues are present in translated regions of RSV mRNA (34).

The amounts of methylated $(A)_4C$ and $(A)_5C$ protected by DNA fragment C (0.83-0.0) represent the total amounts of those oligonucleotides in the 16S and 19S mRNAs (Table 1). We, therefore, deduce that these methylated sites are unique in the two SV40 mRNA species. It is interesting to note that in both the 16S and 19S mRNAs there are twice as many AAC than GAC sequences (27,28), nevertheless, two-thirds of the m^{6}A residues of SV40 late mRNA occur in Gpm^{6}ApC oligonucleotides. Fragment D (0.0-0.14), where only Gpm^{6}ApC was detected, contain 2.5 times more AAC than the region between 0.95-0.0 map units. These examples emphasize again the phenomenon of topographical differences in the pattern of internal methylations, namely the location of m^{6}A residues is not random but specific to a particular region of the RNA.

The functional role of the m^{6}A residues in mRNA is still unknown. It was proposed (37) that m^{6}A residues will be restricted to non-coding regions of mRNA since methylation of the N^6 position is known to destabilize Watson-Crick base-pairing (38,39). Nevertheless it is plausible that by virtue of destabilization of the hydrogen bonding, m^{6}A residues play a role in translation. For example, m^{6}A residues present at the 5' end of the 16S mRNA translated region, may have a role in opening-up secondary structures in that region, thus facilitating binding and movement of ribosomes. In this context it should be pointed out that the methylated pentanucleotide $(A)_4C$ in fragment C (0.83-0.0) is located right next to the VP-2 and VP-3 termination codon in the sequence UAAAm^{6}AC. The biological significance of this finding is not clear. Finally, an interesting possibility is that the m^{6}A residues which are located in the joining regions between the leader RNA and the body of the 16S mRNA constituted recognition sites for the splicing enzymes.

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* To whom correspondence should be addressed.

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