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Capping structures of simian virus 40 19S and 16S mRNAs

Yoram Groner *, Pnina Carmi* and Yosef Aloni**

Departments of Virology* and Genetics**, The Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

In vivo [methyl ^{3}H]-labeled SV40 19S and 16S mRNA species were purified and their internal methylation as well as their capping structures analyzed. SV40 viral mRNA sedimenting in the 19S region contains approximately equal proportions of $m^{7}GpppAm$ and $m^{7}Gpppm^{6}Am$, while the 16S mRNA contains mainly $m^{7}Gpppm^{6}Am$. N^{6} methyl adenosine is located internally within the RNA chains of both the 19S and 16S species.

INTRODUCTION

The 5' termini of many eukaryotic and viral mRNAs have been found to contain capping structures of the type $m^{7}G^{5}ppp^{5}'Xm$ (7-methylguanosine linked by 5'-5' pyrophosphate bridge to the adjacent nucleotide) (for review, see 1 and 2). Capping structures appear to be important for ribosome binding and mRNA translation (3,4,5,6) and cap analogs can cause translational inhibition of capped mRNA (7,8,9,10). It was also suggested (11,12,13) that during biogenesis of mammalian mRNAs and those of viruses replicating in the nucleus $N^{6}mA$ and 2'0-methyl substituents may mark the sites of cleavage of the nuclear precursors and thus have a role in processing and transport of mRNA to the cytoplasm.

Late in the lytic infection poly(A) containing SV40 viral RNA consists of two prominent cytoplasmic species sedimenting as 19S and 16S (14,15,16). It was previously reported that SV40 mRNA is methylated (17) and contain caps (18). However, it was not known whether both the 16 and 19S mRNAs are capped, nor was their capping structures identified. In this work we have analyzed the in vivo labeled capping structures and internal methylation of the two SV40 mRNA species.

RESULTS

Isolation of in vivo methylated SV40 mRNA.

Late SV40 mRNA species containing label derived from [methyl ^{3}H]-methionine were isolated from SV40 infected BSC-1 cells as outlined under Material and Methods. The RNA was chromatographed on oligo(dT)-cellulose and the poly(A)-containing RNA was fractionated by velocity gradient centrifugation, Fig. 1. The 19S and 16S species were localized by hybridization across the gradient to SV40 DNA filters. Fractions containing the 19S and 16S RNA species were separately pooled as indicated in Fig. 1. The viral RNA species were then isolated by hybridization to and elution from SV40 DNA filters as described under Material and Methods.

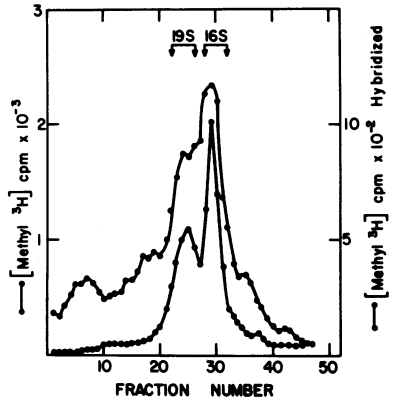

Fig. 1. Sedimentation analysis of Methylated RNA isolated from SV40-infected cells.
RNA containing label derived from [methyl ^{3}H]-methionine was isolated from infected cells as described in Experimental Procedures. The labeled RNA was layered on 15%-30% (W/V) linear sucrose gradients in SDS buffer and centrifuged for 22 hrs at 25,000 RPM at 20°C in a Spinco SW 27.1 rotor. Radioactivity in 3 µl aliquots of each fraction was determined and 20 µl from each fraction were hybridized to SV40 DNA filters as detailed under Experimental Procedures.

Analysis of the 5' termini of in vivo methylated SV40 19S and 16S RNA species.

Methylated SV40 cytoplasmic 19S and 16S RNA species, which were purified as described above, were digested with nuclease P_1 from penicillium citrinum (which cleaves phosphodiester linkages in polynucleotides, including those

containing 2'-0-methylated residues, to yield 5'-nucleotides) (19) followed by incubation with alkaline phosphatase and the products were analysed by paper electrophoresis. As shown in Fig. 2A, about 60% of the radioactivity in the 19S RNA digest migrated towards the anode as a single peak between the markers adenosine 5'-monophosphate and guanosine 5'-monophosphate, in a position identical with that of the cap marker $m^7GpppAm$. The remaining radioactivity migrated towards the cathode in a spot corresponding to adenosine. The 16S RNA digest was similarly analysed. Two peaks of radioactivity coinciding with the peaks from the 19S were resolved, Fig. 2B. Methyl-labeled material comigrating with the adenosine marker in both Fig. 2A and 2B was eluted from the paper and further identified as m^6A by paper chromatography in solvents 1 and 2 (not shown).

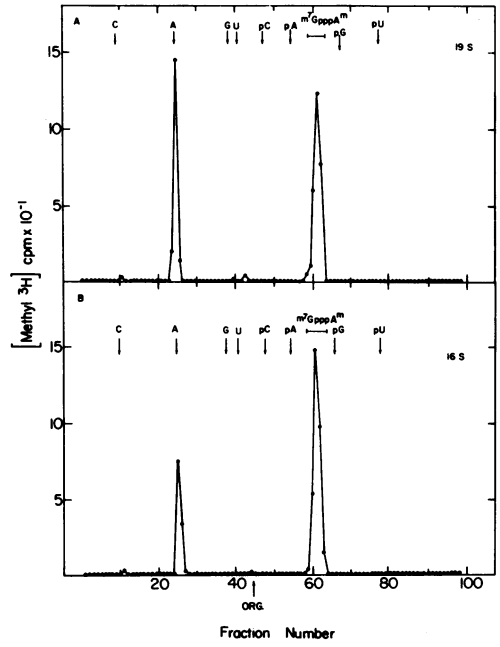

Fig. 2. Paper electrophoretic analysis of enzymatic digestion of [^{3}H]-methylated-SV40 RNA products.
SV40 19S and 16S RNAs isolated from infected cells labeled with [methyl ^{3}H]-methionine were digested with P_1 nuclease followed by alkaline phosphatase and analyzed by paper electrophoresis as described in Material and Methods. The arrows indicate the position of radioactive markers. Marker $m^7GpppAm$ is a spot detected under ultraviolet light A - SV40 19S mRNA, B - SV40 16S mRNA.

In order to identify the methylated nucleosides, material comigrating with the cap marker in Fig. 2A and 2B was eluted, digested with nucleotide pyrophosphatase followed by alkaline phosphatase and subjected to paper electrophoresis, (Fig. 3A and 3B). After the enzymatic digestion, radioactivity from both the 19S and 16S caps migrated as two spots corresponding to 7-methyl guanosine and adenosine. Approximately 33% of the labeled nucleosides derived from the 19S cap and 21% of those derived from the 16S cap coelectrophorased with m^7G. The rest, 67% in the case of 19S cap, and 79% in the case of 16S cap, did so with methylated adenosine. The relative high proportion of radioactivity associated with the methylated adenosine suggested that some of it may contain more than one methyl group.

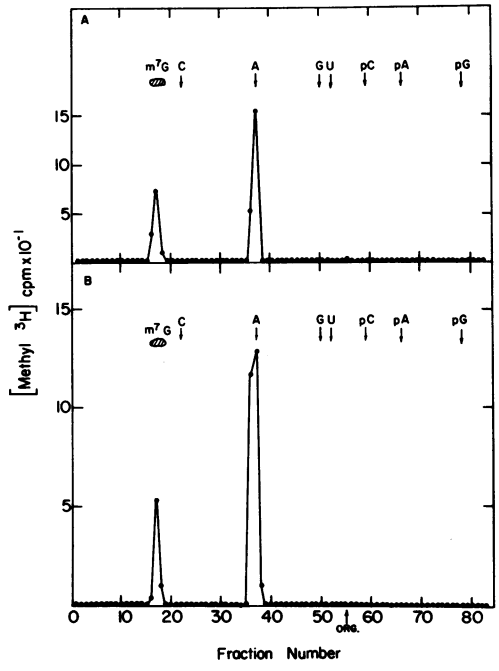

Fig. 3. Electrophoretic analysis of nucleotide pyrophosphatase digest of 19S and 16S mRNA caps. Fractions corresponding to 19S caps and 16S caps in Fig. 2 wer eluted from the paper treated with nucleotide pyrophosphatase and analyzed again by paper electrophoresis. A - Fraction 61-63 from Fig. 2A. B - Fraction 60-63 from Fig. 2B. The arrows indicate the position of radioactive markers. Marker m^7G is a spot detected under ultraviolet light.

In order to test this, material comigrating with adenosine in paper electrophoresis was eluted and analyzed by paper chromatography in solvent 1, Fig. 4A, and in solvent 2, Fig. 4B. As can be seen, the major component comigrated with the di-methylated marker N^6-methyl, 2'0-methyl adenosine

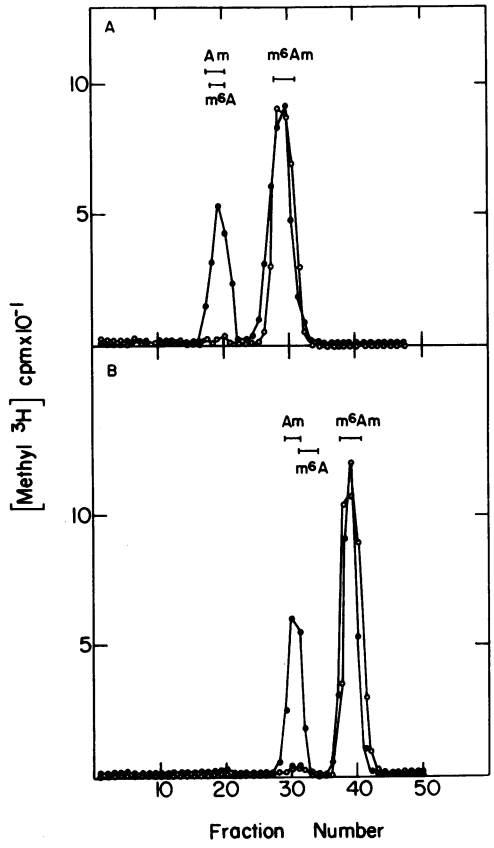

Fig. 4. Paper chromatography analysis of methylated adenosine derived from nucleotide pyrophosphatase treated caps. [Methyl ^3H]-labeled material comigrating with the adenosine marker in Fig. 3A and B was separately eluted from the paper and analyzed by descending paper chromatography. Authentic markers of 2'-0-methyl-adenosine (Am), N^6-methyl-adenosine (m^6A) and N^6-2'-0-dimethyl-adenosine (m^6Am) were included with the samples and located by ultravoilet light.
A - Chromatography in solvent 1; 1-butanol-NH_4OH-H_2O (86:5:14) ●——● methylated adenosine derived from 19S caps. o——o methylated adenosine derived from 16S caps.
B - Chromatography in solvent 2; isopropanol-H_2O-NH_4OH (7:2:1) ●——● methylated adenosine derived from 19 S caps. o——o methylated adenosine derived from 16S caps.

(m^6Am), and faster than monomethylated adenosine. 70% of the radioactivity derived from the adenosine in the 19S caps migrated as m^6Am.

Taking into account the presence of two labeled methyl groups in m^6Am and only one in Am, it can be deduced that in the 19S caps approximately 50% of the penultimate nucleotide was m^6Am, and the rest Am. The 16S caps, on the other hand, containing more than 90% di-methyl adenosine (m^6Am) as their penultimate nucleotide Fig. 4A and B. Thus, the 5' terminal sequences of the in vivo SV40 late mRNA are m^7Gpppm^6Am and $m^7GpppAm$. Same results were independently obtained by S. Lavi (Lavi, S., personal communication).

DISCUSSION

In vivo [methyl ^3H]-labeled SV40 specific 19S and 16S RNAs made at late time after infection were isolated and analysed. After P_1 nuclease and alkaline phosphatase treatment, two types of capping structures were isolated. SV40 mRNA sedimenting in the 19S region has 50% of 5' caps $m^7GpppAm$ and the rest m^7Gpppm^6Am, whereas the 16S RNA yields mostly m^7Gpppm^6Am. It has been previously shown (20) that in order to completely separate the 19S and 16S Polyoma Virus mRNAs it is essential to denature and sediment the RNA in 85% formamide. Therefore, we cannot rule out the possibility that some of the 16S mRNA species is sedimenting in the 19S region of the sucrose gradient (Fig. 1). Thus, it is possible that the 19S mRNA contain only $m^7GpppAm$ cap and the m^7Gpppm^6Am found in the 19S region of the gradient represents contamination of 16S RNA. In adenovirus mRNAs, another nuclear virus, similarly a mixture of A-caps have been found (80% m^7Gpppm^6Am, 20% $m^7GpppAm$) (21,22).

A more highly modified terminus containing an additional 2'O-methylated nucleotide Ym ($m^7GpppXmpYmpZp$) and which was designated cap II, has been found in mRNA of higher eukaryotes (11,12,23) and have also been reported to occur on vesicular stomatitis virus mRNAs (24,25,21) and in vitro synthesized caps (26,27). Under the labeling conditions applied in this report, cap II structures were not found. However, more recently when increased amounts of [methyl ^3H]-methionine

were used for labeling the RNA, SV40 cap II structures ($m^7Gpppm^6AmpUmp$) in which the sub-penultimate U is also methylated were observed (Canaani,D and Groner,Y. unpublished results).

Materials and Methods

Virus, Cells and Infection.

Plaque-purified stock (strain 777) was produced by infecting BSC-1 cells at a multiplicity of 25 PFU per 10^7 cells, with a single plaque isolate which had been subjected to two sequential plaque-purification procedures. BSC-1 cultures of 10^7 cells were infected with plaque-purified virus at a multiplicity of 25 PFU/cell in Eagle's medium with 2% calf serum.

Labeling and Isolation of SV40 RNA.

For *in vivo* labeling of viral mRNA with [methyl ^3H]-methionine, 36 hr post-infection, medium was changed to Eagle's medium containing a reduced concentration (1%) of methionine. At about 46 hrs post-infection, the low methionine medium was replaced by 10 ml (per 10^7 cells) of Eagle's medium lacking methionine containing 2% dialyzed serum. The minus methionine medium was also supplemented with 20 mM sodium formate, 20 µM guanosine and adenosine (to minimize incorporation of methyl groups into the purin biosynthetic pathway (Perry and Kelly, 1974). One millicurie of [methyl ^3H]-methionine was added per 10^7 cells and incubation was continued for 8-10 hrs. After labeling, cells were washed twice with cold phosphate-buffered saline at 4°C and monolayers treated with hypotonic buffer (10 mM Tris·HCl pH 8, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40. After cell lysis (5 min at 4°C), the nuclei were removed by centrifugation (7500xg 10 min) and the cytoplasmic extract made 0.5% with respect to sodium dodecyl sulfate (SDS) and 5 mM with respect to EDTA. The lysate was extracted 3 times with phenol-chlorophorm-isoamyl alcohol and twice with chlorophorm-isoamyl alcohol at room temperature. The RNA was precipitated from the aqueous phase with 2.5 volumes of ethanol at -20°C. The precipitate was collected by centrifugation, dissolved in oligo(dT)-cellulose binding buffer (10 mM Tris·HCl pH 7.5, 250 mM NaCl, 0.5 mM EDTA 0.1% SDS) and polyadenylated RNA was purified by binding to and elution from oligo(dT)-cellulose. The poly(A)-containing RNA was fraction-

ated by centrifugation in 15%-30% (W/V) sucrose gradients containing SDS buffer, for 22 hrs at 25,000 RPM in Spinco SW 27.1 rotor at 20°C. Fractions were collected and radioactivity in aliquot was determined in a dioxane-based scintillation fluid. The amount of [methyl ^{3}H]-RNA hybridizable to SV40 DNA was determined in aliquots of the gradient fractions. Each aliquot was adjusted to 0.5% SDS 4xSSC in a final volume of 0.5 ml and hybridization was performed with 1 µg SV40 DNA immobilized on 7 mm Millipore filters at 68°C for 20 hrs. Filters were washed extensively first with 2xSSC, and then with 0.5xSSC containing 0.5% SDS and finally with ethanol. The 19S and 16S RNAs were separately pooled and precipitated with ethanol at -20°C. For isolation of SV40 specific RNA by hybridization to and elution from SV40 DNA filters, RNA from the pooled fractions was dissolved in aliquots of 1.5 ml in 5 mM Tris·HCl pH 7.4, 70% (v/v) formamide, 0.5% SDS, 300 mM NaCl (modified from Weinberg et al. (14) by J.R. Hartman and E. Winocour) and incubated in vials with Millipore filters (45 µm pore size, 23 mm diam.) on which 20 µg SV40 DNA had been immobilized. After 48 hr at 37°C in a shaker, each filter was washed in the vial once with 10 ml of 0.5xSSC 0.5% SDS and then shaken for 30 min at 37°C with 2.5 ml of washing buffer (10 mM Tris·HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.1% SDS, 37.5% formamide). Washing buffer was removed, filters were washed each with 20 ml of 0.5xSSC 0.5% SDS and RNA was eluted by shaking for 60 min at 37°C with 1.5 ml of elution buffer (10 mM Tris·HCl pH 7.5, 1 mM EDTA, 0.1% SDS, 90% formamide). The eluted RNA was brought to 0.3 M NaCl and precipitated with ethanol at -20°C, prior to further characterization.

Chromatography and Electrophoresis.

Descending paper chromatography on Whatman 52 paper was performed with 1-butanol-NH_4OH-H_2O (86:5:14) solvent 1 and with isopropanol-H_2O-NH_4OH (7:2:1) solvent 2. Paper electrophoresis on Whatman 3MM was carried out at 1.85 V/cm^2 for 2-3 hrs in pyridine-acetate buffer (pH 3.5). The dried paper was cut into 1 cm strips and radioactivity was determined either in toluene-based scintillation fluid or after extracting the radioactivity with water in Triton X-100 based scintillation fluid.

Enzymes Treatment.

For combined digestion with Penicillium nuclease (P_1) and bacterial alkaline phosphatase, RNA was dissolved in 50-100 µl of 10 mM sodium acetate buffer (pH 6.0) and incubated with nuclease P_1 (1 mg/ml) for 60 min at 37°C. The pH of the mixtures was then brought to 8.0 with 1 M Tris base and incubation was continued for 60 min with bacterial alkaline phosphatase. Two units of enzyme were added four times at 15 min intervals. Digestion with nucleotide pyrophosphatase was carried out for 60 min at 37°C in 50 mM Tris·HCl buffer (pH 7.4) containing 2 mM $MgCl_2$ and 0.3 unit of enzyme/ml.

Enzymes and Chemicals.

Penicillium nuclease and nucleotide pyrophosphatase were purchased from Yamasa Shoyu Co. and Sigma Chemical Co., respectively. Bacterial alkaline phosphatase was from Worthington Biochemical Corp. [methyl ^3H]-methionine (10-80 Ci/mmole) was purchased from New England Nuclear. Nucleotides as well as cap markers were obtained from P.L.Biochem.Co.

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