METHYLATED SV40 mRNAs *

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1. Introduction

Recent experiments indicated that messenger RNA from animal cells [1,2] and viruses [3–5] are methylated. However, it is not yet known whether methylation is a general property of mRNA in eukaryotes and what the biological significance of this post-transcriptional event may be. It has been suggested that methylation plays a role in RNA translation [6] and processing [7].

Simian virus 40 (SV40) demonstrates a productive lytic infection in monkey cells. During the late portion of the lytic cycle, thwo prominent cytoplasmic viral RNAs 19s and 16s species are observed [8,9]. A precursor-product relationship between these components is indicated by their labeling kinetics [9,10], by pulse-chase experiments in enucleate SV40 infected cells [10], and by finding that the 19s species shares nucleotide sequences with and is less stable than the 16S RNA [9–11].

The experiments presented here show that the 19S and 16S viral RNA species are methylated. Each one of them contains about 1.8 methyl groups per 1000 nucleotides or about 6 and 4 methyl groups in the 19S and 16S RNA species, respectively.

2. Materials and methods

Growth of plaque-purified SV40 (strain 777) on monkey cells of the BSC-1 line as well as concentration and purification of the virus from the tissue culture lysates and preparation of SV40 DNA component I were described [12]. In all experiments, cultures (4×10^6 cells) were infected with 1.0 ml of the same stock of SV40 [2×10^8 plaque-forming units (PFU/ml]. For labeling the cells with [^{3}H]methyl methionine the cells were maintained after infection for 24 hr in growth medium containing 1 μM methionine (1% of the normal methionine concentration). Then 20 mM sodium formate and 20 μM each of adenosine and guanosine were added for another 24 hr. The inclusion of these chemicals in the labeling medium has been shown to inhibit effectively the incorporation of methionine-derived methyl groups into purine rings via the H_4-folate pathway [13]. At 48 hr post-infection, the cells were labeled with 0.1 mCi/ml [^{3}H]methyl methionine (The Radiochemical Centre, 7.4 Ci/mmol) and 0.05 μCi/ml [U-^{14}C]-uridine (The Radiochemical Centre, 514 mCi/mmol). The cells were harvested after 5 hr incubation at 37°C. Cytoplasmic fractions were prepared with NP_{40} detergent [14] and RNA extracted from them [15]. The RNA samples were digested with 50 μg/ml DNase (Worthington, RNase-free electrophoretically purified). The digest was extracted with SDS-phenol and fractionated on sucrose gradients [16]. Polyadenylated RNA was obtained by fractionation on oligo-dT cellulose column [17].

RNA-DNA hybridization with DNA immobilized on Millipore filters (25 mm diameter, 0.45 μm pore size), were performed as described [16]. For selecting the viral RNA by preparative hybridization, the labeled RNA was hybridized with 40 μg of plaque-purified SV40 DNA component I immobilized on two Millipore filters, as described [12]. For recovery of the hybridized RNA, each filter was incubated for 1 hr at 37° in 1 ml elution buffer [8]

* This is article IV in the series 'Transcription of SV40' Paper III is ref. 10.

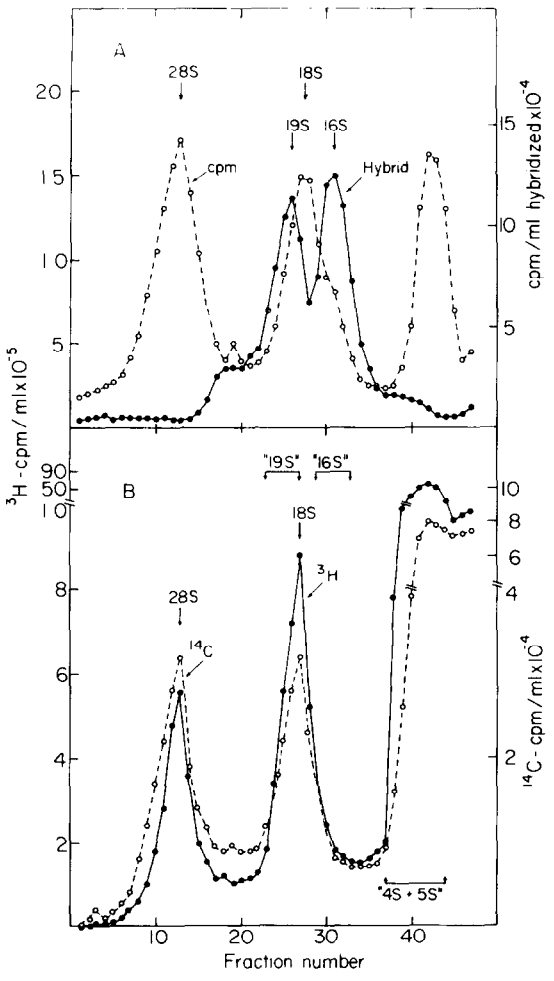

Fig.1. Purification of viral RNAs by sedimentation through sucrose gradients. In A, BSC-1 cells were infected with SV40 and labeled for 5 hr with [5,6-^{3}H]uridine (0.1 mCi/ml, 48 Ci/mmol) at 48 hr post-infection. [^{3}H]RNA was extracted from the cytoplasmic fraction [14] and centrifuged through 15–30% (w/w) sucrose in SDS buffer [16]. Centrifugation was for 22 hr at 25 000 rev/min at 20°C in a Spinco SW27 rotor. 10 μl aliquots of each fraction were counted directly [16]. The cpm hybridized represent the radioactivity in each fraction that was bound to 7 mm filters containing 0.5 μg SV40 DNA [16]. In B, the cells were labeled with [^{3}H]methyl methionine and [U-^{14}C]uridine and RNA extracted as described in Materials and methods. Centrifugation was performed as in A. The fractions containing the 16S and 19S RNA components were determined from fig.1A.

(90 volumes of 100% formamide, 9 volumes of distilled water and 1 volume of SDS buffer; final pH adjusted to 8.4). About 5% of the input ^{3}H-radioactivity was bound and eluted from SV40 DNA filters and less than 0.1% was bound and eluted from filters containing no DNA. The eluted RNA was precipitated from elution buffer by ethanol. Low molecular weight RNAs (5S and 4S) were further fractionated by electrophoresis of the corresponding fractions from the sucrose gradients (see fig.1B), using 10% polyacrylamide gels [18]. Radioactive samples were counted in a Tricarb liquid scintillation counter with channel and gain settings adjusted for the minimum ^{14}C crossover (10%).

3. Results

Fig.1A shows that late lytic viral RNA consists of two prominent cytoplasmic species sedimenting at 19S and 16S [8–10]. Virtually all RNA molecules of these two types are known to contain poly A tails [11]. In order to determine whether the two species of viral RNA are also methylated, RNA was extracted from the cytoplasm of SV40 infected cells labeled with [^{3}H]methyl methionine and [^{14}C]uridine and fractionated on a sucrose gradient (fig.1B). The fractions containing the 19S and 16S RNA species were separately collected from the gradient and the poly A containing RNA in each fraction was selected by chromatography on oligo dT cellulose column [17]. The viral RNA species was then purified from each poly A-RNA fraction by hybridizing to and eluting from SV40 DNA filters [8]. Fig.2 shows the sedimentation patterns of the '19S' and '16S' viral RNA species. There is a good correspondence between the ^{3}H and ^{14}C radioactivity patterns indicating that the [^{3}H]methyl groups were incorporated into the viral RNAs. A plot of ^{3}H/^{14}C ratio along the portion of the gradient containing the viral RNAs (fig.2A and 2B) indicate a similar ^{3}H/^{14}C ratio in the 19S and 16S RNA components. The presence of a pronounced 16S component in the '19S' profile (fig.2A) may arise from a mild degradation of some of the 19S molecules during the hybridization/elution step so that the broken 19S molecules sediment in the 16S peak and/or it may result from competition between their common sequences [11] and the 16S sequences which are in excess [10]. Evidence indicating

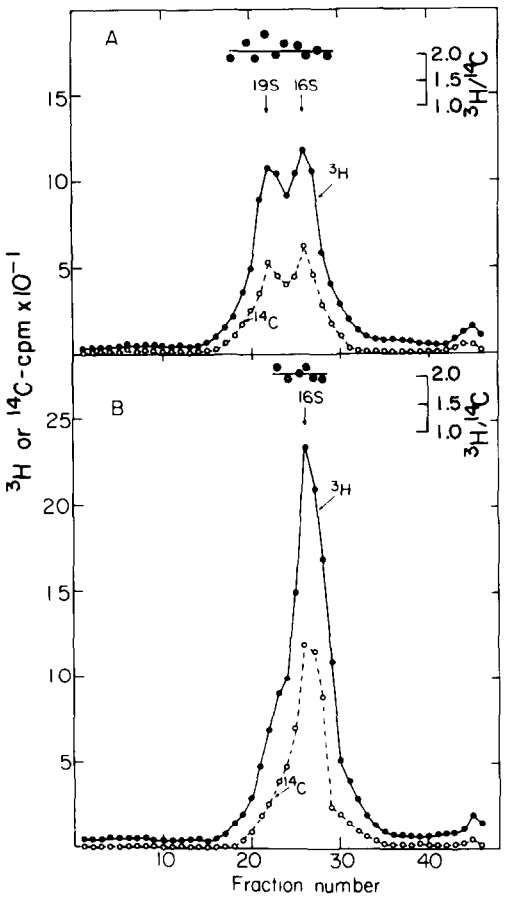

Fig.2. Sedimentation of the $^3H/^{14}C$ labeled viral RNAs in sucrose gradients. The fractions containing the 19S and 16S viral RNA species (fig.1B) were separately collected, the viral RNAs were selected as detailed in the text and they were then run through two sucrose gradients as in fig.1. A) '19S' B) '16S'.

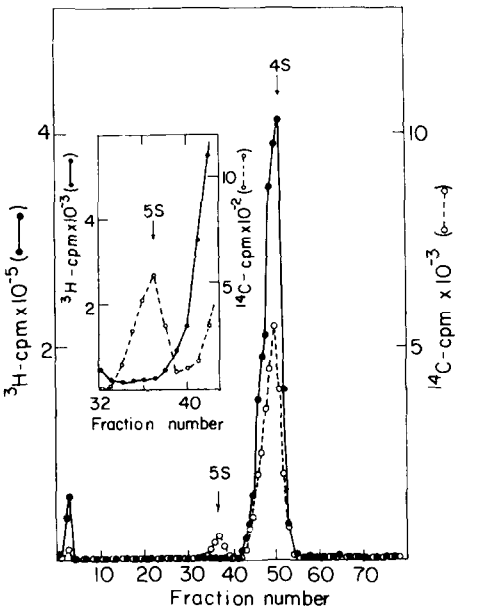

Fig.3. Analysis by polyacrylamide-gel electrophoresis of $^3H/^{14}C$ labeled 4S and 5S RNAs. The components sedimenting in the 2 to 7S region of the gradient in the pattern shown in fig.1B were collected by ethanol precipitation and centrifugation and run through a polyacrylamide gel (10%acrylamide) at 5 mA for 3.5 hr, as described before [18]. In the insert the gel pattern of the 5S region is given on a larger scale.

that the ^3H activity was not due to incorporation of methionine methyl group into the purine rings comes from gel electrophoresis analysis of the low molecular weight RNA components. A substantial methylation of t-RNA, but negligible methylation of 5S RNA, was observed (fig.3). Since the 5S RNA component is known not to contain any methyl groups, this negative finding provides good evidence that under our labeling conditions there was no incorporation of the methionine methyl group into the purine rings [1]. An additional evidence comes from the absence of ^3H activity in the guanylic and adenylic acid regions after chromatography of polynucleotide phosphorylase digested RNA (Aloni, Sorek and Littauer, unpublished results).

The results of the measurements of the $^3H/^{14}C$ ratio on the various types of RNA purified in this study are summarized in Table 1. From such ratios one can make an estimate of the absolute levels of methylation using the known degree of methylation of the rRNA components. There is excellent agreement with previously published findings [1] in regard to the degree and the ratio of methylation of the rRNA components and t-RNA, indicating the validity of such analysis. Furthermore, the $^3H/^{14}C$ ratio of the poly A-RNA indicates that on the average there are about 2.2 methyl groups per 1000 nucleotides, as was previously found for mRNA in L cells [1]. The $^3H/^{14}C$ ratio for the viral RNA species is somewhat lower than that of the total cellular poly A-RNA. There was no difference in the $^3H/^{14}C$ ratio between 19S and 16S species (fig.2). Since the uridine content

Table 1
Estimated number of methyl groups in various RNA species
The $^{3}H/^{14}C$ ratio was determined after purification of the various RNA components on sucrose gradients

RNA species	Ratio of $^{3}H/^{14}C$	Number of methyl groups per 1000 nucleotides		
		Estimated	Previously reported	
28 S ribosomal RNA	16	(14)	(14)	[21,22]
18 S ribosomal RNA	24	21	21	[21,22]
5 S ribosomal RNA	0.2	negligible	negligible	[21,22]
4 S RNA	78	68	70	[21,22]
Poly A-containing cytoplasmic RNA	2.5	2.2	2.2	[1]
19 S SV40 RNA	2.1	1.8	–	
16 S SV40 RNA	2.1	1.8	–	

The percentage of methylated nucleotides was calculated from the ^{3}H to ^{14}C ratios and from the known content of methyl groups of cytoplasmic 28S RNA [21].

of the two viral RNA species is almost identical [19], I conclude that they are methylated to the same degree, having 1.8 methyl groups per 1000 nucleotides or about 6 and 4 methyl groups in the 19S and 16S RNA species respectively.

4. Discussion

The results presented here establish that SV40 mRNA in productively infected cells are methylated. The two prominent viral RNAs, the 19S and 16S species have about 1.8 methyl groups per 1000 nucleotides or 6 and 4 methyl groups on the 19S and 16S RNAs, respectively. The viral RNA species were found previously to be also adenylated [11,23]. Since methylation, like polyadenylation may constitute a posttranscriptional modification of messenger RNA in eukaryotic cells, it appears that maturation of the viral RNAs controlled by the same mechanism which operates on the general cell message. Enzymes that carry out the methylation of the viral mRNA are presumably of cell origin. Although in the cases of polyhedrosis virus [4] reovirus [3] and Vaccenia virus [5], mRNA methylation activity is associated with the virion RNA polymerases, it has not been shown whether the methylase is of cell or virus origin. The pattern of methylation in all mRNAs studied including total cytoplasmic SV40 RNA [20] includes 5′ terminal structures of the type $m^{7}G(5')ppp(5')N^{m}$ [3-5,7,20] where the ^{7}mGp is apparently derived from GTP by a post-transcriptional 'capping process' [7].

Previous studies have shown that the 19S and 16S viral RNA components share common nucleotide sequences [11] and that they exhibit a precursor-product relationship [10]. The mechanism required for the processing of the 19S to the 16S RNA is unknown. However, the pattern of methylation in the two viral RNAs may help to elucidate this mechanism and the quantitation of the methyl groups in the 19S and 16S RNAs is a step in this direction. A model that fits the estimated number of methyl groups in the two prominent viral RNA species could be suggested (fig.4). According to this model, the six methyl groups in the 19S molecule are distributed as follows: two methyl groups

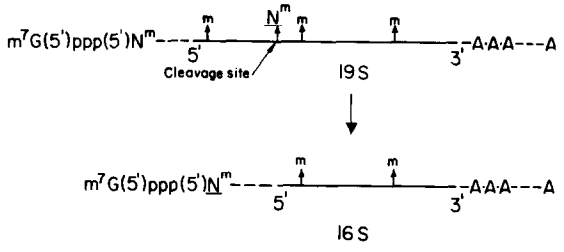

Fig.4. A proposed model for the distribution of methyl groups in the 19S and 16S viral RNAs. $m^{7}G$-7 methylguanosine. N^{m}- any 2′-0-methylnucleoside. m-methyl.

are located at the 5′ termini and pertain to the 'capping structure' [7]; of the other four, one is located in the region which is lost during the conversion of the 19S to the 16S species, while the other three are located in that portion which is shared by the two molecules. Three of the four methyl groups in the 16S molecule are those which are found in the 19S molecule and the remaining one pertains to the new 'capping structure' and is added with the m^7G, following the cleavage, at the N^m site. Other models that will fit the observed number of methyl groups are also possible.

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