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Novel mechanism for RNA maturation: The leader sequences of simian virus 40 mRNA are not transcribed adjacent to the coding sequences

[spliced RNA/mRNA·DNA hybrids/RNA processing/cap structures/poly(A)]

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ABSTRACT **The 5′-terminal 100–200 ribonucleotides of late simian virus 40 (SV40) mRNAs are not transcribed immediately adjacent to their coding sequences. This conclusion is based on the following observations. The major late SV40 cytoplasmic RNA species, 16S and 19S, were purified from poly(A)-containing cytoplasmic RNA by hybridization to and elution from an SV40 DNA fragment that maps between 0.67 and 0.76. This fragment is remote from the DNA fragments that include the coding sequences. The RNA transcripts from the fragment located between 0.67 and 0.76 were found in abundance. Even though selected on oligo(dT)-cellulose columns, the 5′-terminal sequences did not contain poly(A) tails directly adjacent to their 3′ termini. The 5′ terminus of the 16S mRNA, as monitored by hybridization of the sequences adjacent to the "cap" structure, was found adjacent to the coding sequences when intact [^{3}H]methyl-labeled RNA was hybridized with restriction fragments. However, after fragmentation, the methyl label of this same RNA hybridized with a fragment that is remote from the coding sequences and maps between 0.67 and 0.73. These results imply a novel mechanism for biosynthesis of SV40 mRNA.**

The use of animal viruses as model systems for probing the complexities of molecular control mechanisms has been particularly fruitful. It is generally thought that an understanding of genetic regulation in viruses will provide an insight into similar regulatory processes in eukaryotic cells. The molecular biology of simian virus 40 (SV40) has been under intensive investigation for a number of years. These studies have provided considerable information regarding the regulation of gene expression and, in particular, transcription and the post-transcriptional processing of mRNA (1, 2). In this paper we report the finding of a novel mechanism of RNA processing. The leader sequences and the adjacent coding sequences of mRNA are not transcribed from contiguous segments of viral DNA. This implies either a "copy choice" at the transcriptional level or a fusion of RNA at the post-transcriptional level.

MATERIALS AND METHODS

Cells and Viruses. Growth of plaque-purified SV40 on monkey kidney cell cultures, the concentration and purification of the virus from the tissue culture lysates, and the preparation of SV40 DNA component I have been described (3). In these experiments, secondary African green monkey kidney cells were infected with 20 to 60 plaque-forming units of stock 776 SV40 per cell.

Labeling and Extraction of RNA. For labeling with [*methyl*-^{3}H]methionine, the infected cells were pretreated (4) and 1 mCi of [*methyl*-^{3}H]methionine (80 Ci/mmol, New England Nuclear, Boston, MA) was added in a volume of 0.6 ml per 1×10^6 cells. For labeling with $^{32}PO_4$, the cells were pretreated (3) and 2 mCi/10^7 cells (Amersham/Searle, Arlington Heights, IL) was used. Poly(A)-containing cytoplasmic RNA was prepared as described (4).

Filter Hybridization. SV40 DNA was cleaved with the desired enzyme, and the fragments were separated by 1.4% agarose gel electrophoresis (5). The DNA was transferred from the gel into nitrocellulose paper (B-6, Schleicher and Schuell, Keene, NH) with 6× standard saline–citrate (SSC) by using the technique of Southern (6). Each transfer filter (10 cm wide) contained 25 μg of SV40 DNA. The hybridizations in 4× SSC at 68° and in formamide at 37° were as described (7). Bands were traced with a Gilford spectrophotometer, model 2400.

Analysis of [^{3}H]Methyl-Labeled RNA. The radioactivity associated with the bands was eluted by boiling in 0.5 ml of H_2O in the presence of 20 μg of tRNA, followed by rapid cooling and precipitation with ethanol. The RNA pellet was dissolved in 10 μl of 0.05 M ammonium acetate buffer, pH 5.2, containing T_2 (1 unit), T_1 (5 units), and pancreatic (2 μg) RNases and incubated at 37° for 3 hr. The RNA digest was analyzed by electrophoresis on DEAE-paper at 1500 V for 3 hr. Sequential 0.5-cm strips were cut out and the radioactivity was eluted into 1 ml of 2 M LiCl and assayed. "Caps" were identified as reported previously migrating between the origin and an orange G marker (8). The 6-methyladenosine was identified with an unlabeled marker detected under UV light.

RESULTS

RNA Complementary to a DNA Fragment Not Known to Code for Any Protein. The SV40 genome is comprised of early and late genes that are localized to symmetrical halves of the viral DNA (9). The segment between 0.67 and 0.17 map unit is transcribed in a counterclockwise direction prior to the onset of viral DNA replication and codes for the early viral proteins. The second segment is transcribed in abundance, after initiation of viral DNA replication, from 0.67 to 0.17 map unit in a clockwise direction. It encodes the information for the late proteins: VP_1, VP_2, and VP_3. These capsid proteins have been approximately mapped at 0.95–0.17, 0.76–0.95, and 0.83–0.95 map unit, respectively (see refs. 1, 2, and 10 and Fig. 5). The viral DNA segment between 0.67 and 0.76 map unit is transcribed at late times during infection, but the genetic infor-

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Abbreviations: SV40, simian virus 40; SSC, standard saline–citrate (0.15 M NaCl/0.015 M Na citrate, pH 7); 6× SSC, SSC at 6 times the standard concentration.

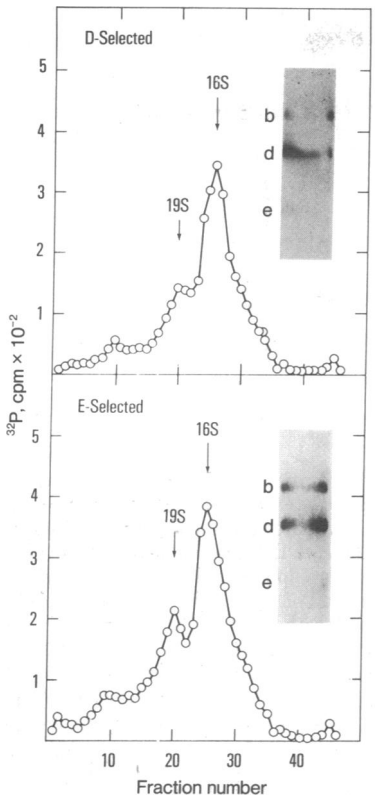

FIG. 1. Analysis of ^{32}P-labeled poly(A)-containing RNA that was annealed to and eluted from fragments *d* and *e* produced by cleavage of SV40 DNA with *Eco*RI, *Hpa* I, and *Bgl* I restriction endonucleases. ^{32}P-Labeled cytoplasmic poly(A)-containing RNA was prepared from 2×10^7 infected cells. It was divided into two halves, of which the first was hybridized in formamide at 37° with 20 μg of fragment *e* DNA and the second, with 20 μg of fragment *d* DNA. The fragments were produced by cleavage of SV40 DNA with *Eco*RI, *Hpa* I, and *Bgl* I restriction endonucleases and transferred to nitrocellulose paper as described in *Materials and Methods*. The ^{32}P-labeled RNAs were eluted from the nitrocellulose paper; one portion of each eluate was rehybridized to *Eco*RI, *Hpa* I, and *Bgl* I restriction enzyme fragments (see *insets*), and the second portion was analyzed by sedimentation through neutral sucrose gradients. Rehybridization experiments were performed in 5× SSC/0.2% sodium dodecyl sulfate at 68° for 24 hr. The nitrocellulose papers were then washed extensively with warm 2× SSC, dried, and exposed to x-ray films. The ^{32}P-labeled RNAs were sedimented in 15–30% sucrose gradients in sodium dodecyl sulfate buffer (12) in a Spinco SW 27 rotor at 25,000 rpm for 18 hr at 20°. Fractions were collected and radioactivity was assayed in Triton-based scintillation fluid. ^{3}H-Labeled 28S and 18S rRNAs were included for sedimentation markers (not shown).

mation encoded in this region is unknown. To analyze the RNA transcribed from this region, ^{32}P-labeled poly(A)-containing cytoplasmic RNA was extracted from cells 48 hr after infection and annealed to a fragment of SV40 DNA spanning the region 0.67–0.76 immobilized on nitrocellulose paper. The hybridization was performed in formamide at 37° in order to minimize thermal degradation. After annealing, the virus-specific RNA was eluted from the paper and subjected to two tests. In the first, ^{32}P-labeled viral RNA was reannealed to viral DNA fragments produced by cleavage with restriction endonucleases *Eco*RI, *Hpa* I, and *Bgl* I. In a second analysis, the ^{32}P-labeled RNA was sedimented through a neutral sucrose gradient. In a similar set of experiments, ^{32}P-labeled poly(A)-containing RNA was hybridized to and eluted from a fragment between 0.0 and 0.17 map unit, a region of the genome known to encode the 3′ portions of the 16S and 19S cytoplasmic RNAs (9, 11).

Fig. 1 shows that both of the eluted ^{32}P-labeled RNAs rehybridized in a similar pattern to the fragments produced by restriction endonucleases *Eco*RI, *Hpa* I, and *Bgl* I. Hybridization occurred with the *b* (0.76–0.0), *d* (0.0–0.17), and *e* (0.67–0.76) fragments. Analysis of the eluted ^{32}P-labeled RNAs on sucrose gradients showed that both of them contained the 16S and 19S RNA species. Because the 16S and 19S RNAs were mapped previously between 0.95 and 0.17 map unit and between 0.77 and 0.17 map unit, respectively, their hybridization with fragment *e* (0.67–0.76) was unexpected and prompted further investigations.

Template Location of 16S Poly(A)-Containing RNA. Fig. 2*A* shows the profile of the 16S species obtained after two sequential centrifugations in sucrose gradients and hybridization of the corresponding fractions with filters containing excess amounts of SV40 DNA. A sharp and homogeneous 16S component is apparent with almost no recognizable virus-specific radioactivity in the 19S region of the gradient. The peak fraction of purified ^{3}H-labeled 16S RNA (fraction 17) was annealed with a filter containing the five *Eco*RI, *Hpa* I, and *Bgl* I restriction endonuclease fragments. Fig. 2*B* shows that the 16S RNA hybridized predominantly with fragment *d* (0.0–0.17); lower levels of hybridization were obtained with fragment *b* (0.76–0.0) and fragment *e* (0.67–0.76). A densitometer tracing of this film showed that the radioactivity in fragments *b* and *e* corresponded to about 30% and 20% of that of fragment *d*, respectively. Because fragment *d* represents 17.5% of the physical size of the viral genome (or about 900 nucleotides), the sequence content of the 16S RNA represented in fragments *b* and *e* would correspond to 270 and 180 nucleotides, respectively. The hybridization results with the purified ^{3}H-labeled 16S RNA with fragment *e* were in agreement with the results described above.

Mapping the 3′ Ends of Cytoplasmic Viral RNAs. Dhar *et al.* (13) have recently shown that the transcripts of the late strand of SV40 DNA from 0.70 to 0.76 map unit are abundantly represented in the cytoplasmic RNA fraction from SV40-infected cells, which is also retained on oligo(dT)–cellulose. To determine whether this RNA is in fact polyadenylylated, the following experiment was performed. Unlabeled poly(A)-containing cytoplasmic RNA was obtained by extraction of cytoplasmic RNA from infected cells at 48 hr after infection and fractionation on an oligo(dT)–cellulose column. The retained RNA [poly(A)-containing] was annealed with restriction fragments produced by a combination of *Eco*RI (0.0), *Taq* I (0.55), and *Hae* II (0.83) restriction endonucleases. The hybridization was carried out in 70% formamide at 37° in order to minimize thermal degradation. The RNA·DNA hybrids were then annealed with [^{3}H]poly(U) in order to detect the free poly(A) tails.

Fig. 3 shows that the [^{3}H]poly(U) hybridized mainly to fragment *a* (0.0–0.55) which includes the 3′ ends of the 16S and 19S species (0.17 map unit) (9, 11). The low levels of radioactivity found in fragments *c* (0.83–0.0) and *b* (0.55–0.83) seems to be proportional to the fraction of 16S and 19S RNAs represented in these DNA regions. When the unlabeled RNA was fragmented to about 10S size prior to the annealing, the radioactivity of [^{3}H]poly(U) in the second step of hybridization was detected only with fragment *a*. Thus, we conclude that the abundant sequences transcribed from 0.70 to 0.76 map unit lack poly(A) directly adjacent to their 3′ end.

Mapping the 5′ End of 16S Viral RNA. The experiments described above have shown that the sequences transcribed from 0.67 to 0.76 map unit are found in high concentration, that they sediment in sucrose gradients together with the 16S and 19S SV40 RNA species, and that they are retained on oli-

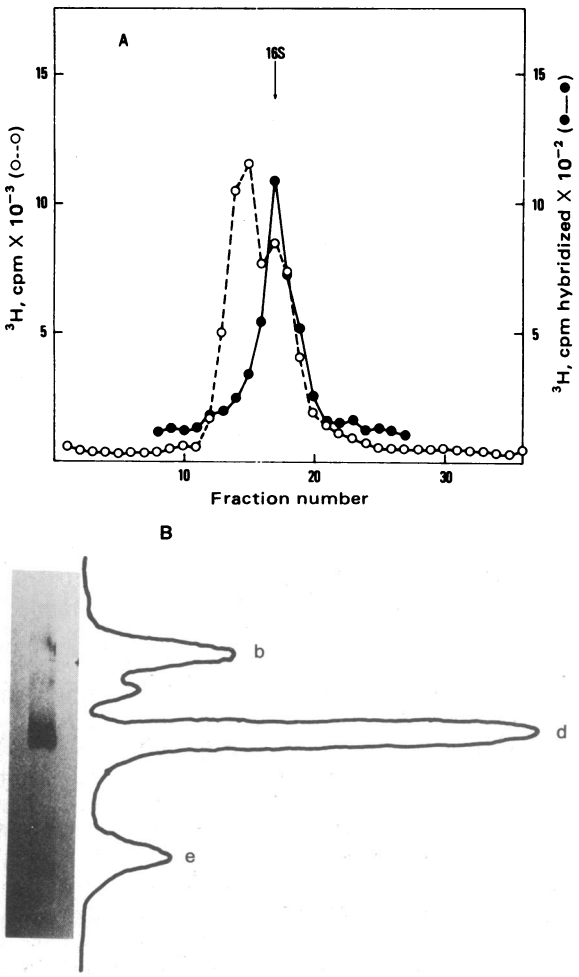

FIG. 2. Purification of 16S viral RNA by sedimentation through sucrose gradients and hybridization to restriction fragments. (*A*) African green monkey kidney cells were infected with SV40 and labeled for 5 hr with [5-6-^{3}H]uridine (0.5 mCi/ml; 41 Ci/mmol) at 48 hr after infection. [^{3}H]RNA was prepared from the cytoplasmic fraction and chromatographed on an oligo(dT)–cellulose column. The poly(A)-containing RNA was collected with ethanol and centrifuged through 15–30% (wt/wt) sucrose in sodium dodecyl sulfate buffer (12) in an SW 41 rotor for 18 hr at 28,000 rpm at 20°. Radioactivity in aliquots (5 μl) of each fraction was assayed directly (data not shown). The 10S–23S region of the gradient was collected and concentrated by ethanol precipitation, resuspended in sodium dodecyl sulfate buffer and run through a second sucrose gradient as above. Radioactivity in aliquots (5 μl) was assayed directly and another 5 μl of the corresponding fractions was hybridized with filters containing 0.2 μg of SV40 DNA (12). (*B*) The 16S RNA peak fraction was annealed to a filter blot containing *Eco*RI, *Hpa* I, and *Bgl* I restriction enzyme fragments. The map positions of these fragments are: fragment *a*, 0.37–0.67; *b*, 0.76–0.0; *c*, 0.17–0.37; *d*, 0.0–0.17; *e*, 0.67–0.76. The conditions for DNA transfer and DNA-RNA hybridization with the peak fraction (fraction 17) are described in *Materials and Methods*. The densitometer tracing of the bands is shown adjacent to their positions.

go(dT)–cellulose even though they lack adjacent 3′ poly(A) tails.

A model that will accommodate all these results suggests that these abundant sequences located between 0.67 and 0.76 map unit are covalently linked to the 5′ ends of the coding sequences of 16S and 19S viral RNAs.

To test this model we performed experiments to map the 5′ end of the 16S RNA. Infected cells were incubated with [*methyl*-^{3}H]methionine to label the methylated "cap" structure at the 5′ end and the internal 6-methyladenosine residues. The

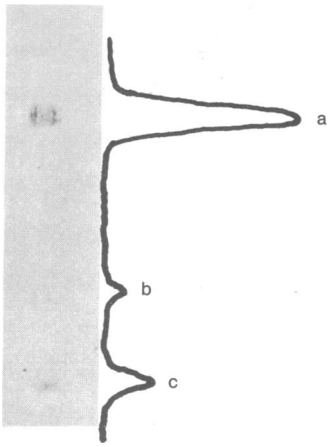

FIG. 3. Hybridization of [^{3}H]poly(U) to unlabeled poly(A)-containing cytoplasmic RNA-SV40 DNA hybrids. Cytoplasmic poly(A)-containing RNA prepared as described in Fig. 2 was hybridized with *Eco*RI, *Taq* I, and *Hae* II restriction fragments in 70% formamide/0.3 M NaCl/0.5% sodium dodecyl sulfate/0.01 M Tris·HCl, pH 7.4 for 36 hr at 37°. At the end of the incubation, the nitrocellulose sheet was washed extensively with 2× SSC and then annealed with 100,000 cpm of [^{3}H]poly(U) (New England Nuclear, 0.5 Ci/mmol) in 2× SSC for 3 hr at 30°, after which the nitrocellulose sheet was washed again in 2× SSC, dried, dipped in 2,5 diphenyloxazole/toluene (23 g/100 ml), and exposed to x-ray film. The fragments generated by cleavage with the restriction endonucleases *Eco*RI, *Taq* I, and *Hae* II are: *a*, 0.0–0.55; *b*, 0.55–0.83; *c*, 0.83–0.0. The densitometer tracing of the bands is shown adjacent to their positions.

labeled RNA was then hybridized with the five *Eco*RI, *Hpa* I, and *Bgl* I restriction endonuclease fragments under two sets of conditions. In the first experiment, the ^{3}H-labeled poly(A)-containing RNA was hybridized in formamide at 37° to minimize thermal degradation. Under the second protocol, the ^{3}H-labeled RNA was first fragmented to pieces 100–200 nucleotides long under limited alkaline conditions and the hybridization was carried out at 68°. The filters were processed as described in *Materials and Methods* and the remaining radioactivity bound to the nitrocellulose paper was detected with x-ray film. The position corresponding to each of the fragments was cut from the nitrocellulose paper and eluted for further analysis.

A typical x-ray film from a filter hybridization experiment is shown in Fig. 4*A*. Radioactivity was detected in fragments *b* (0.76–0.0), *d* (0.0–0.17), and *e* (0.67–0.76). The relative radioactivity found with fragment *e* was higher than that found with this fragment when the RNA was labeled with [^{3}H]uridine (see Fig. 2). Table 1 summarizes the radioactivity bound to these fragments when intact or fragmented RNA was used for hybridization. The percentage radioactivity associated with fragment *e* increased about 2-fold and that associated with fragment *b* increased about 1.5-fold when the RNA was fragmented prior to hybridization. This indicates that the radioactivity associated with fragment *d* was in part due to nonhybridized RNA sequences covalently attached to hybridized sequences. This analysis, of course, does not discriminate between "caps" and internal methyl label.

The radioactivity associated with each of the fragments was then eluted from the nitrocellulose paper and digested with T_2, T_1, and pancreatic ribonucleases; the products were analyzed by electrophoresis on DEAE-paper at pH 3.5. Radioactivity was found only in the "cap" structures and 6-methyladenosine residues as was found previously (ref. 14; Y. Groner, P. Carmi, and Y. Aloni, unpublished data). Furthermore, Table 2 shows that, when intact RNA was analyzed, labeled "caps" were found

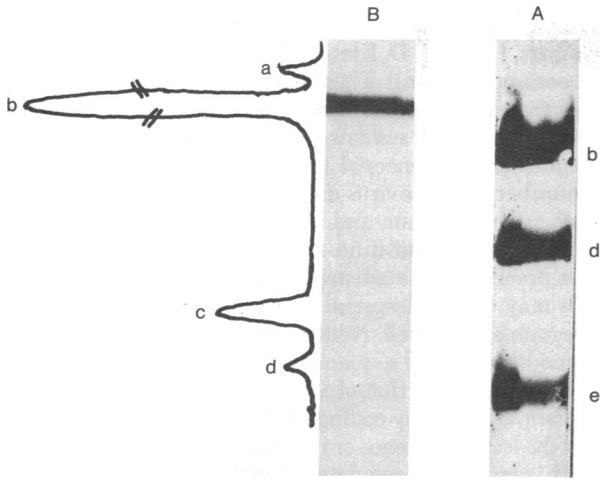

FIG. 4. Hybridization of [^{3}H]methyl-labeled RNA to restriction fragments. (*A*) Poly(A)-containing cytoplasmic RNA was prepared as described in Fig. 2, except that the cells were labeled with [*methyl*-^{3}H]methionine (see *Materials and Methods*). The RNA was nicked with 0.025 M KOH for 3 hr at 4° to produce 4S–7S fragments, neutralized with 1% perchloric acid, and hybridized with *Eco*RI, *Hpa* I, and *Bgl* I fragments on nitrocellulose sheets. The conditions for DNA transfer and RNA·DNA hybridization are described in *Materials and Methods*. (*B*) The hybridization of [^{3}H]methyl-labeled RNA was as described in *A*, but with fragments generated by cleavage with restriction endonucleases *Hae* II, *Bam* I, *Taq* I, and *Hpa* II. The positions of the fragments are shown in Table 3. The densitometer tracing of the bands is shown adjacent to their positions. The tracing of band *b* is reduced 4-fold.

associated with fragment *b* (43.7%), fragment *d* (46.7%), and fragment *e* (9.6%). However, analysis of fragmented RNA showed that more than 80% of the labeled "caps" were associated with fragment *e* and none with fragment *d*.

The most plausible interpretation of these results is that the 5′ end of the coding region of the 16S poly(A)-containing RNA previously found to map from 0.95 to 0.17 is covalently linked to sequences transcribed from fragment *e* (0.67–0.76).

In order to locate more precisely within the *Eco*RI, *Hpa* I, and *Bgl* I fragment *e* the sequences that are adjacent to the "cap," similar hybridization experiments with fragmented [^{3}H]-methyl-labeled poly(A)-containing RNA were performed. The ^{3}H-labeled RNA was annealed with the four fragments produced by a combination of *Hae* II, *Bam* I, *Taq* I, and *Hpa* II restriction endonucleases. Fig. 4*B* shows that a portion of the radioactivity was associated with each of the fragments, predominantly with fragment *b* (0.83–0.14). The labeled RNA was eluted from each band, digested with T_2, T_1, and pancreatic ribonucleases, and analyzed for both "cap" structures and internal 6-methyladenosine as described above.

Table 1. Hybridization of [^{3}H]methyl-labeled poly(A)-containing RNA with fragments produced by *Eco*RI, *Hpa* I, and *Bgl* I restriction endonucleases

Cytoplasmic RNA	cpm bound to fragments		
	b (0.76–0.0)	*d* (0.0–0.17)	*e* (0.67–0.76)
Intact	343 (37.3%)	478 (52.2%)	97 (10.5%)
Fragmented	1063 (53.8%)	513 (25.9%)	401 (20.3%)

[^{3}H]Methyl-labeled poly(A)-containing RNA was prepared as in Fig. 4. Hybridization with intact RNA in formamide and with ^{3}H-labeled RNA fragmented in 0.025 M KOH in 4× SSC was carried out as described in *Materials and Methods*. The corresponding fragments were cut from the nitrocellulose sheet and assayed for radioactivity in Triton-based scintillation fluid.

Table 2. Location of "caps" as determined from hybridization of intact and fragmented RNA with *Eco*RI, *Hpa* I, and *Bgl* I restriction fragments

Cytoplasmic RNA	"Cap" cpm bound to fragments, %		
	b (0.76–0.0)	*d* (0.0–0.17)	*e* (0.67–0.76)
Intact	43.7	46.7	9.6
Fragmented	18.0	0	82.0

The regions corresponding to each of the fragments were cut and placed in 0.5 ml of H_2O containing 20 μg of tRNA. The labeled RNA was eluted by heating to 100° followed by rapid cooling. The RNA was collected by ethanol precipitation and centrifugation. Digestion with T_2, T_1, and pancreatic ribonucleases, electrophoresis on DEAE-paper at pH 3.5, and identification of the radioactivity associated with "caps" and 6-methyladenosine were carried out as described in *Materials and Methods*. About 50% of the radioactivity found on the nitrocellulose sheet was recovered from the DEAE-paper (the radioactivity pertaining to 6-methyladenosine is not shown).

Table 3 shows that the most of the radioactivity associated with fragment *b* was accounted for by 6-methyladenosine. About 70% of the radioactivity associated with the "cap" structures was found in fragment *c* (0.55–0.73). These results indicate that the sequences adjacent to the "cap" structure of the 16S RNA are transcribed from a region between 0.67 and 0.73 map unit.

The results described in this paper are summarized in Fig. 5. The coding region for VP_1 is transcribed into RNA which is complementary to sequences from 0.95 to 0.17 map unit. One hundred fifty to 200 ribonucleotides transcribed from a segment of DNA that maps between 0.67 and 0.76 are spliced to the 5′ end of the coding sequences. These leader sequences contain, at their 5′ end, the "cap" structure. The sequences closest to the "cap" are transcribed from a DNA segment that maps between 0.67 and 0.73. A similar mechanism is also suggested for primary processing of the 19S RNA component.

DISCUSSION

The region of the SV40 genome between 0.67 and 0.76 map unit is transcribed at late times after infection (13), but the genetic information encoded by these sequences is unknown. The results presented in this paper show that a portion of these sequences is represented at the 5′ termini of the late 16S and 19S mRNAs. Because the sequences located between 0.67 and 0.76 map unit are not reiterated in the SV40 genome and because the coding sequences found in the bodies of the 19S and 16S late SV40 mRNAs are clearly separated from this region of the genome, we conclude that the leader sequences and coding sequences are not transcribed across a contiguous region. Similar observations by S. Lavi and Y. Groner (personal communication) and electron microscopic studies by M. T. Hsu

Table 3. Percentage of "cap" and 6-methyladenosine (6mA) cpm bound to restriction fragments produced by *Bam* I, *Taq* I, *Hpa* II, and *Hae* II restriction endonucleases

Fragment	Map unit	"cap" cpm, %	6mA cpm, %
a	0.14–0.55	0	6.5
b	0.83–0.14	10.4	84.7
c	0.55–0.73	71.5	4.9
d	0.73–0.83	18.1	3.9

Fragment cytoplasmic RNA was used. Analysis of the radioactivity associated with the fragments shown in Fig. 4*B* was carried out as described in Table 2.

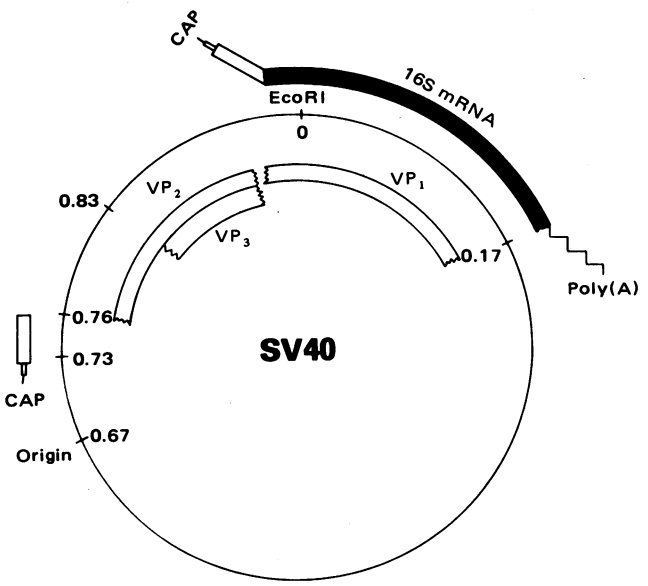

FIG. 5. Scheme for splicing of the 5′ end of 16A viral RNA. It should be noted that the number of components in the leader sequence as well as the position of the sequences adjacent to the cap within the region 0.67–0.73 map unit has not been determined. For details see *text*.

(personal communication) are in agreement with this conclusion.

If these leader sequences are indeed needed for the expression of late viral mRNA, then we predict that deletions in the 0.67–0.76 region might result in a polar effect on the synthesis of the late SV40 proteins.

The late SV40 19S RNA species shares nucleotide sequences with, and is less stable than, the late 16S RNA (12). Experiments with enucleated cells have demonstrated a precursor–product relationship between these two viral RNAs (12) and, furthermore, have suggested that the "cap" of late 16S RNA is methylated in the cytoplasm (Y. Groner, P. Carmi, and Y. Aloni, unpublished data). Because the mature 19S and 16S SV40 cytoplasmic RNAs appear to contain 5′ leader sequences, this suggests a rather complex mechanism for bioconversion of the precursor to product in the cytoplasm. Although a number of models could fit these data, we hesitate to speculate on such a mechanism without more information. While this work was in progress we learned of similar conclusions made by S. Berget, C. Moore, and P. Sharp, and by T. Broker, L. Chow, A. Dunn, R. Gelinas, J. Hassel, D. Klessig, J. Lewis, and R. Roberts (personal communications). These investigators used several techniques to examine mRNA extracted from cells infected with adenovirus 2. In their systems, three short sequences were joined to form leader sequences of 100–200 nucleotides at the 5′ end of a number of adenovirus mRNAs.

Both the mechanism and the reason for RNA splicing are unclear at the present time. Nevertheless, the observation of such a process in both adenovirus and SV40 mRNAs suggests that it may be a widespread phenomenon and may occur in eukaryotic cells as well. Neither the leader sequences nor the intermediate deleted sequences code for known functions. In a genome as small as that of SV40, however, it would seem extravagant to waste any coding capacity. Thus, it is possible that either the leader sequence or its adjacent deleted RNA segment, or both, may serve a regulatory function either at the level of RNA or by a heretofore undetected protein.

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