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# 5′-Terminal sequences and coding region of late simian virus 40 mRNAs are derived from noncontiguous segments of the viral genome

**(19S and 16S simian virus 40 mRNAs/leader sequences/5′ capping structures/$N^6$mA residues)**

SARA LAVI AND YORAM GRONER

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

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**ABSTRACT** The region of the simian virus 40 genome complementary to the 5′ end of the most abundant poly(A)-containing 19S and 16S mRNAs was mapped by hybridization of double-labeled RNA ([$^3$H]methyl group and [$^{14}$C]uridine) to specific DNA fragments. Chemical identification of methylated residues indicated that a common "leader" sequence adjacent to the 5′ terminus of both 19S and 16S mRNA is transcribed from DNA sequences located between 0.67 and 0.76 map units. The estimated size of this "leader" RNA, which does not code for any known viral protein, is 170–200 nucleotides. Our results indicate that sequences complementary to the "leader" region and coding portion of 16S mRNA are located in separate parts of the simian virus 40 genome.

Various eukaryotic mRNAs contain nontranslated 5′-terminal segments (1). Two characteristics of these 5′-terminal segments have been described. First, they contain structures of the type $m^7G^{5'}ppp^{5'}Nm$, referred to as cap structures (for review see ref. 2). Second, the 5′-terminal sequences of the mRNA for the hexon protein and of several other adenovirus-2 (Ad-2) messengers consist of three segments of RNA that are complementary to three regions of the Ad-2 genome. Furthermore, these three segments are spliced onto the coding portion of the mRNA (3, 4). Two of the most abundant Ad-2 mRNAs (100,000 molecular weight and fiber) isolated late during infection have a common capped T1 RNase-resistant oligonucleotide at the 5′ end that is encoded between positions 14.7 and 17.0 on the physical map of Ad-2 and not by DNA sequences adjacent to their respective structural genes (5).

Late in the lytic cycle of simian virus 40 (SV40), two distinct viral cytoplasmic mRNA species sedimenting in sucrose gradients as 19S and 16S species occur (6). Although hybridization to restriction fragments (7) and electron microscopy (8) permitted mapping of the bulk of the late 19S and 16S mRNA species on the SV40 genome, these approaches proved unsatisfactory for the assignment of the 5′ end of these mRNAs.

It has been reported that late SV40 mRNA is capped and methylated (9, 10), and the mRNA cap structure has been identified as $m^7Gppm^6Am$ (11) (S. Lavi and A. Shatkin, unpublished results;). It has also been reported that cell-free translation of SV40 late mRNA is totally inhibited by cap analogs like $m^7GTP$ and $m^7GpppGm$ (12). Since the cap structure is unique to the 5′ terminus of mRNA, it can serve as a convenient marker for mapping the 5′ region of messenger RNA. Earlier unpublished experiments (S. Lavi and A. Shatkin) indicated that the RNA sequences associated with the cap structure of 16S SV40 mRNA hybridized to the region between 0.73 and 0.84 on the SV40 genome contrary to the expected (7) result. The recent findings with Ad-2 mRNA (3–5) suggested an explanation for the earlier findings with SV40.

In this report, the region of the SV40 genome complementary to the 5′ end of the late mRNAs has been mapped by hybridization of [$^3$H]methyl-labeled RNA (labeled in its cap structure) to specific restriction endonuclease fragments of SV40 DNA. Our results indicate that the 5′-terminal sequences and coding regions of late SV40 mRNA are derived from noncontiguous regions of the SV40 genome.

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## MATERIALS AND METHODS

**Labeling and Isolation of Poly(A)-Containing mRNA from SV40-Infected Cells.** BSC-1 cells were infected with plaque-purified SV40 (multiplicity of infection of 50). At 36 hr after infection the medium was changed to Eagle's medium containing 1% methionine. At 46 hr after infection the medium was replaced by Eagle's medium lacking methionine supplemented with 20 mM sodium formate, 20 $\mu$M guanosine, and 20 $\mu$M adenosine. One millicuric of [*methyl*-$^3$H]methionine (80 Ci/mmol, New England Nuclear) and 10 $\mu$Ci of [$^{14}$C]uridine (462 mCi/mmol, Radiochemical Center) were added per $10^7$ cells and incubation was continued for 7 hr. Poly(A)-containing RNA was isolated from the cytoplasm (11).

**Hybridization to Specific DNA Fragments.** SV40 DNA was digested with restriction enzymes and the fragments were fractionated by electrophoresis through 1.4% agarose. *In situ* denatured DNA was transferred onto nitrocellulose filters by Southern's procedure, as modified by Botchan *et al.* (13). Hybridization with labeled RNA was carried out in 70% formamide at 37° (11). In some experiments RNA was fragmented (4–7S) by incubating 19S and 16S RNAs in 0.1 M NaOH at 38° for 6 min. The mixture was cooled and neutralized to pH 7.5 before hybridization to DNA fragments. The hybridized RNA was detected by cutting the "blots" into 1.5-mm strips. Radioactivity associated with each strip was determined in toluene-based scintillation fluid.

**Analysis of Methylated Residues.** RNA was eluted from the hybrids, digested with Penicillium nuclease and alkaline phosphatase, and analyzed by paper electrophoresis at pH 3.5 (11).

## RESULTS

**Selection of 19 and 16S SV40 Specific mRNAs.** BSC-1 cells infected with plaque-purified SV40 were labeled with [$^{14}$C]-uridine and [*methyl*-$^3$H]methionine. Consequently, the RNA was uniformly labeled in uridine as well as in its methylated nucleotides (the 5′-terminal cap, $m^7GpppNm$, and internal $N^6$mA). Cytoplasmic poly(A)-containing RNA was isolated and

Abbreviations: SV40, simian virus 40; Ad-2, adenovirus-2.

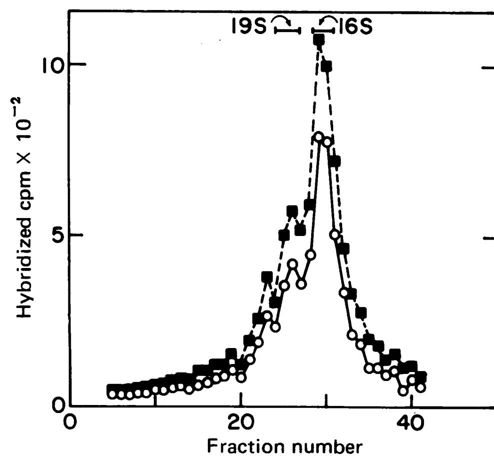


FIG. 1. Purification of 19S and 16S SV40 specific mRNA by sedimentation through sucrose gradient. Poly(A)-containing RNA labeled with [$^{14}$C]uridine and [*methyl*-$^{3}$H]methionine was isolated from SV40-infected BSC-1 cells. Labeled RNA was layered on 15–30% (wt/vol) linear sucrose gradients in sodium dodecyl sulfate buffer and centrifuged for 22 hr at 25,000 rpm at 20° in a Spinco SW 27.1 rotor. Aliquots of 15 $\mu$l from each fraction were hybridized to 3 $\mu$g of total SV40 DNA immobilized on filters. O, [$^{3}$H]methyl cpm; ■, [$^{14}$C]uridine cpm.

sedimented through a 15–30% sucrose/sodium dodecyl sulfate gradient. The 19S and 16S SV40 specific mRNA species were localized by hybridization of gradient fractions to SV40 DNA bound to nitrocellulose filters. As shown in Fig. 1, both the 19 and 16S RNA species were methylated. Fractions corresponding to 19 and 16S RNA species were pooled separately, as indicated in Fig. 1, and were used in all subsequent experiments. Recentrifugation of the pooled mRNAs indicated that the 19S mRNA was contaminated less than 10% by 16S mRNA.

**SV40 DNA Sequences Coding for 19 and 16S SV40 Late mRNAs.** To determine the region of the SV40 genome complementary to the sequences in the 19 and 16S RNA species, we digested RNA by mild alkali to produce 4–7S fragments and the fragments were hybridized to blots containing five DNA segments obtained by digestion of SV40 DNA with *Eco*RI, *Bgl* I, and *Hpa* I (Fig. 2). Both RNA species hybridized specifically to fragments representing the entire late region of the genome (see Fig. 4). The 19 and 16S RNA hybridized most efficiently to fragment D; this was expected since it is known (7) that this DNA segment (0.0–0.17) is represented in both late mRNA species. The 16S RNA hybridized with lower efficiency to fragment B (0.76–1.0), as expected from the fact that only part of the sequences in this fragment are present in the 16S species (7). Surprisingly, 16S RNA also hybridized to fragment E (0.67–0.76), although this region is remote from the known coding region of 16S mRNA. Moreover, the ratio of [$^{3}$H]methyl group to [$^{14}$C]uridine that hybridized to fragment E was relatively high for both 19 and 16S RNAs, suggesting that the RNA sequences hybridized with fragment E are enriched in the methylated residues.

**DNA Sequences Coding for RNA That Is Linked to Cap Structures of 19 and 16S RNA Species.** To isolate and identify the methylated nucleotides that hybridized to the different fragments derived from the late region of SV40 DNA, we eluted the radioactive RNA associated with the three late DNA fragments, B, D, and E, and pooled them as indicated in Fig. 2. The RNA was then digested with $P_1$ nuclease followed by incubation with alkaline phosphatase, and the product was analyzed by paper electrophoresis (as shown in Fig. 3 and summarized in Table 1). The majority (84%) of the 16S cap structures (Fig. 3*C*)

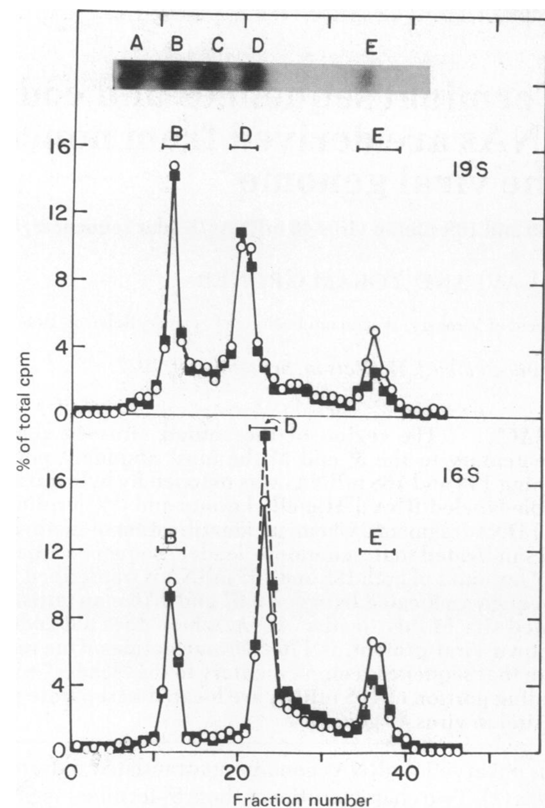


FIG. 2. Hybridization of fragmented 19S and 16S SV40 RNA to DNA fragments obtained by digestion of SV40 DNA with *Bgl* I, *Hpa* I, and *Eco*RI. RNA species corresponding to 19S and 16S RNA were pooled as indicated in Fig. 1, digested by mild alkali to fragments of 4–7S, and annealed to filter blots containing DNA fragments *Bgl* I, *Hpa* I, and *Eco*RI. The fragments are derived from the following portions of the SV40 map: A, 0.37–0.67; B, 0.76–0.0; C, 0.17–0.37; D, 0.0–0.17; and E, 0.67–0.76. The blots were cut into 1.5-mm strips and the radioactivity associated with the strips was determined in toluene-based scintillation fluid. (*Top*) Hybridization of 19S RNA. (*Bottom*) Hybridization of 16S RNA. O, [$^{3}$H]methyl cpm; ■, [$^{14}$C]-uridine cpm. Autoradiogram depicts hybridization of SV40 DNA labeled *in vitro* with $^{32}$P to a similar blot.

were associated with RNA that hybridized to the DNA fragment spanning from 0.67 to 0.76 map units. This suggested that the 5′ terminus of the 16S RNA molecules is coded by DNA sequences within this region. Only 16% of the 16S caps (Fig. 3*A*) were associated with RNA that hybridized to fragment B (0.76–1.00), and could represent 5′ caps on nonhybridized tails. No radioactivity associated with caps was present in fragment D (0.0–0.17) (Fig. 3*B*). In contrast to the caps, $N^6$mA residues were evenly distributed in fragments B and D (40 and 46%, respectively), while a lower proportion of $N^6$mA (14%) was found in fragment E. The exact location of the $N^6$mA residues is under further investigation. All the $^{14}$C-radioactivity migrated with the uridine marker (Fig. 3), indicating that digestion by $P_1$ nuclease and alkaline phosphatase was complete.

As a result of alkali treatment, ring opening of the imidazole portion of $m^7$G occurred. Since the opening of the $m^7$G ring results in loss of a positive charge, the cap structures with an open ring, *[$m^7$G]pppm$^6$Am, migrated between pG and pU (shown in Fig. 3). Material migrating in this position was eluted and identified further by paper electrophoresis after digestion with nucleotide pyrophosphatase and alkaline phosphatase. Radioactivity migrated in two spots; about 34% of the resulting $^{3}$H-labeled nucleosides migrated in the position of the opened

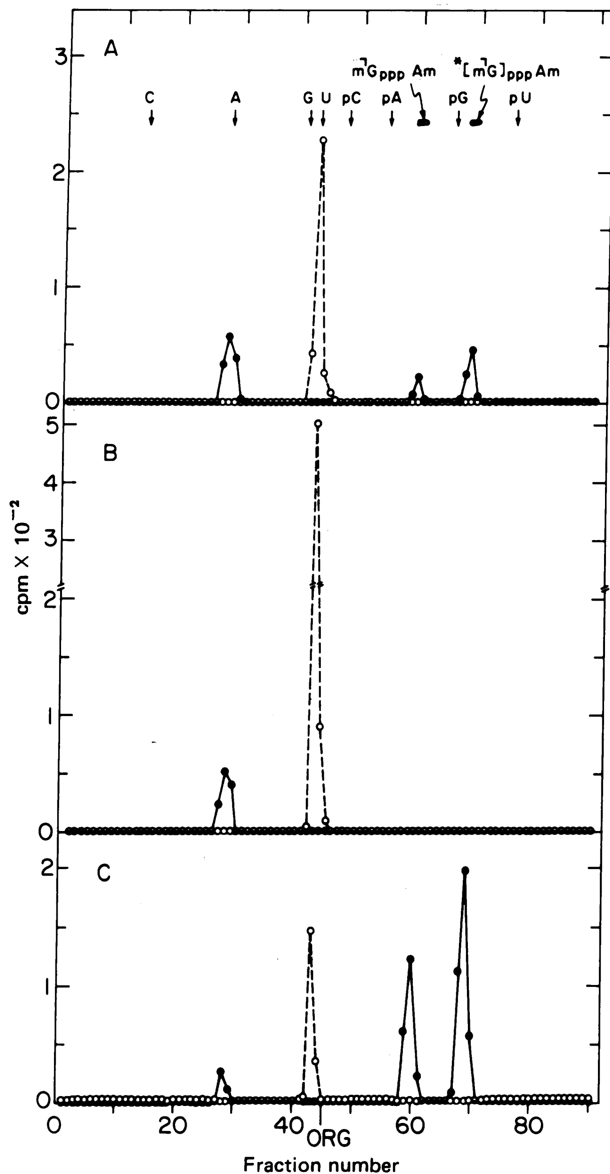


FIG. 3. Paper electrophoretic analysis of enzymatic digestion of 16S RNA fragments eluted from the blot in Fig. 2. [$^3$H]Methyl- and [$^{14}$C]uridine-labeled SV40 16S RNA fragments that hybridized to DNA fragments B (*A*), D (*B*), and E (*C*) were pooled as indicated in Fig. 2. RNA fragments were eluted from the hybrids, digested with $P_1$ nuclease followed by alkaline phosphatase, and analyzed by paper electrophoresis. The arrows indicate the position of radioactive markers. The markers $m^7$ GpppAm and *[$m^7$G]pppAm were detected under ultraviolet light. *[$m^7$G]pppAm, which contains an open ring structure, was prepared from $m^7$GpppAm by incubation with 10 M $NH_4OH$ for 60 min at 40°. ●, [$^3$H]methyl cpm; ○, [$^{14}$C]uridine cpm. ORG, origin.

ring structure *[$m^7$G], while 66% migrated with adenosine, as expected (not shown).

19S RNA eluted from the "blot" (Fig. 2) was similarly analyzed by enzymatic digestion and paper electrophoresis (summary in Table 1). The majority (68%) of the 19S cap structures were associated with RNA that hybridized to DNA fragment 0.67–0.76. This suggested that RNA sequences adjacent to the cap are similar in 19 and 16S RNAs. Thirty-two percent of the 19S caps were associated with sequences present in fragment B and could represent 5′ caps of nonhybridized tails. No caps were found in RNA that hybridized to fragment D (Table 1). Similar amounts of $N^6$mA were recovered from

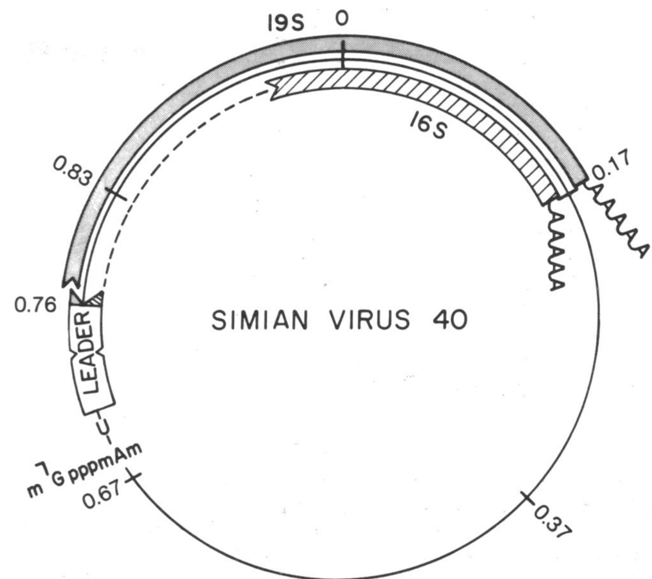


FIG. 4. Schematic representation of the relationship between the "leader" region and the coding portion of late SV40 mRNAs. The leader RNA maps within the region 0.67–0.76; its exact location has not yet been determined.

fragments B and D (42 and 36%, respectively), while fragment E contained somewhat fewer $N^6$mA residues. These results indicate that the capped sequences at the 5′ end of the 19 and 16S mRNA are transcribed from sequences within the region extending from 0.67 to 0.76 map units. These results further suggest that SV40-late mRNAs contain a 5′-terminal "leader" sequence analogous to that described for Ad-2 (3–5). Taking into account the amount of [$^{14}$C]uridine liberated from each of the fragments (Table 1), it is possible to estimate that the size of the E fragment covered by the 16S "leader" RNA is about 170–200 nucleotides.

**"Leader" RNA Is Covalently Bound to Coding Region of 19 and 16S mRNAs.** It was shown above that the 5′ caps of both 19 and 16S mRNAs are associated with sequences that hybridize to the DNA region between 0.67 and 0.76 map units. However, we did not know whether the capped RNA that hybridized to this region was covalently linked to the main portion of 16S mRNA, which codes for VP-1 (0.95–0.17 map units). To examine this question, we further purified intact [$^3$H]-methyl and [$^{14}$C]uridine-labeled 19 and 16S mRNAs by hybridization to and elution from DNA fragment E, 0.67–0.76 ("leader" region). The RNA selected in this way was rehybridized to DNA fragment 0.0–0.17 map units (complementary to the main portion of 16S mRNA). As shown in Table 2, RNA molecules from both the 19 and 16S species, preselected by hybridization to the "leader" region, rehybridized efficiently (>30%) to the DNA fragment 0.0–0.17. Thus, in both 19 and 16S mRNA species, sequences that are homologous to the DNA region 0.0–0.17 are covalently linked to sequences containing the "leader" region.

From these results it seems that sequences at the 5′ and 3′ ends of both 19 and 16S mRNA species are coded by the same region of the SV40 genome. Since the 16S RNA is shorter (by about 4 × $10^5$ daltons) than the 19S RNA, it was important to show that the 19 and 16S RNA species differ in some of their sequences. For this purpose, intact 19 and 16S RNA molecules were hybridized with specific DNA fragments derived by digestion of SV40 DNA with *Hap* I, *Bgl* I and *Hae* II. As shown in Table 3, the 19S mRNA molecules hybridized very similarly to the three fragments from the late region representing map

Table 1. Methylated nucleotides and capping structure in 19S and 16S SV40 RNA hybridized to the SV40 DNA fragments obtained by *Eco* RI, *Hpa* I, and *Bgl* I

| SV40 DNA | | | 19S RNA, cpm | | | 16S RNA, cpm | | |
|---|---|---|---|---|---|---|---|---|
| Fragment | Map units | No. of base pairs | [$^{14}$C]-Uridine | [$^{3}$H]Cap | [$^{3}$H] N$^{6}$mA | [$^{14}$C]-Uridine | [$^{3}$H]Cap | [$^{3}$H]N$^{6}$mA |
| B | 0.76–1.00 | 1300 | 320(44) | 97(32) | 111(42) | 270(26) | 112(16) | 138(46) |
| D | 0.00–0.17 | 920 | 230(32) | 0 | 94(36) | 590(56) | 0 | 117(40) |
| E | 0.67–0.76 | 490 | 179(24) | 206(68) | 57(22) | 190(18) | 584(84) | 42(14) |

The proportion of uridine residues, cap structures, and N$^{6}$mA in the RNA hybridized to fragments B, D, and E was calculated for 16S RNA from the data shown in Fig. 3. The values represent radioactivity found on the paper after electrophoresis. The values for 19S RNA were calculated from similar electrophoretic analysis (not shown). The numbers in parentheses represent the percent of total cpm hybridized.

units 0.83–0.17, 0.67–0.76, and 0.76–0.83, respectively. The 16S mRNA molecules hybridized 57 and 36% to fragments representing map units 0.83–0.17 and 0.67–0.76, respectively, while only 7% of the molecules hybridized to the fragment 0.76–0.83. These data indicate that in contrast to the 19S RNA, the majority of the 16S mRNA molecules do not share sequences with the DNA fragment spanning from 0.76 to 0.83. Thus, the 16S mRNA "leader" portion and the 16S mRNA coding portion are not transcribed from contiguous regions of the DNA. A summary of the results described above is presented schematically in Fig. 4.

## DISCUSSION

Hybridization experiments, followed by chemical analysis of methylated residues, showed that "leader" RNA sequences associated with the 5′ termini of the late poly(A)-containing 19S and 16S RNAs hybridize to DNA sequences located between 0.67 and 0.76 map units. The approximate size of the 16S "leader" region is 170–200 nucleotides. We reached this conclusion by comparing the ratio of [$^{14}$C]uridine radioactivity released from the RNA hybridized to fragment 0.67–0.76 to [$^{14}$C]uridine released from RNA hybridized to fragment 0.0–0.17 with the ratio of the size of the two fragments (Table 1). However, it is not clear whether the "leader" sequences are coded by a continuous DNA segment located within 0.67–0.76. Since the "leader" portions of both 19 and 16S mRNAs hybridized to the same DNA fragments, it is possible that the "leader" sequences are identical for both RNA species. This would be consistent with our observation that the subpenultimate nucleotide in capping structures of the 19 and 16S RNA species is uridine, (D. Canaani, S. Lavi, and Y. Groner, unpublished results). The DNA region 0.67–0.76 does not code for any known protein. Nevertheless, the capping structure, which is important for translation (2, 12), is attached to the 5′ end of RNA transcribed from this region. It is therefore tempting to speculate that the "leader" sequence may play a role in expression of viral genetic information; for example, it may contain part of the ribosome binding site. Such an arrangement might be very economical, since the SV40 genome is small (5400 base pairs) and contains only a limited amount of information.

Hybridization of intact RNA molecules with specific DNA fragments showed that sequences in the region 0.76–0.83 are not represented in the 16S species while they do appear in the 19S RNA [this sequence codes for the $NH_2$-terminal portion of VP-2 (14)]. Both 19 and 16S RNA species share sequences hybridizing to DNA from 0.83–0.17. Although DNA sequences homologous to the "leader" region and those homologous to the coding portion of 16S RNA are located in separate parts of the SV40 genome, they are covalently linked in the 16S RNA molecule. Thus, 16S RNA molecules that were preselected by hybridization to DNA fragment 0.67–0.76 rehybridized to DNA fragment 0.0–0.17 as efficiently as did the 19S molecules. An identical conclusion was reached independently by Aloni *et al.* (14) and Ming-Ta Hsu (unpublished).

Various models may be suggested to explain the biogenesis of such RNA molecules. Most of them fall into two major classes; the first proposes that the 19 and 16S RNA molecules are produced by processing of a larger precursor RNA molecule. This hypothesis requires specific mechanism(s) for cleavage and splicing to join the "leader" region with the "body" of the RNA. The second class proposes that the 16S mRNA molecules are transcribed on a DNA template folded in such a way that the RNA polymerase will be able to transcribe nonadjacent sequences continuously. Such folding of the DNA might be facilitated by duplex formation between two inverted repeats (16), one of which is adjacent to the sequence coding for the "leader" and the other of which is adjacent to the region coding for VP-1.

Table 2. 19S and 16S mRNAs preselected by hybridization to DNA fragment 0.67–0.76 rehybridized to DNA fragment 0.00–0.17

| Step | mRNA species | DNA fragment | cpm hybridized $^{3}$H | cpm hybridized $^{14}$C |
|---|---|---|---|---|
| 1 | 19S | 0.67–0.76 | 802 | 1177 |
| | 16S | 0.67–0.76 | 2299 | 3013 |
| 2 | 19S | 0.00–0.17 | 340 | 551 |
| | 16S | 0.00–0.17 | 781 | 1004 |

Intact 19S and 16S RNA were hybridized with fragment E (0.67–0.76) obtained by digestion of SV40 DNA with *Hpa* I, *Bgl* I, and *Eco*RI and immobilized on nitrocellulose filters. Radioactivity associated with the filters was determined (step 1) and then eluted. The selected RNA was rehybridized to DNA fragments of the same digest ranging between 0.00 and 0.17, and radioactivity was determined (step 2).

Table 3. Hybridization of intact 19S and 16S RNA species with DNA fragments obtained by digestion with *Hae* II, *Hpa* I, and *Bgl* I

| Fragment | Map unit | 19S RNA, $^{14}$C cpm | 16S RNA, $^{14}$C cpm | DNA, $^{32}$P cpm |
|---|---|---|---|---|
| A | 0.83–0.17 | 1957 (38) | 4570 (57) | 3638 (34) |
| B | 0.37–0.67 | — | — | 2782 (26) |
| C | 0.17–0.37 | — | — | 2100 (20) |
| D | 0.67–0.76 | 1575 (31) | 2659 (33) | 1177 (11) |
| E | 0.76–0.83 | 1602 (31) | 783 (10) | 963 (9) |

19S and 16S RNA molecules were hybridized to blots containing DNA fragments derived by digestion with *Bgl* I, *Hpa* I, *Hae* II. Blots were cut into 1.5-mm strips and the $^{14}$C radioactivity associated with each strip was determined. Control SV40 DNA labeled with $^{32}$P *in vitro* hybridized to similar blots. The numbers in parentheses represent the percent of total cpm hybridized.

The mechanism for biogenesis of mRNA in which the 5′-terminal sequences are transcribed from nonadjacent DNA segments may not be restricted to adenovirus (3–5) or SV40 mRNAs. For example, the variable and constant regions of immunoglobin mRNA are derived from different portions of the genome (17) and may be spliced together by a similar process.

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