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# Methylation of Nuclear Simian Virus 40 RNAs

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Nuclear RNAs, pulse-labeled with [*methyl*-$^3$H]methionine and [$^3$H]uridine under optimal conditions, were prepared from cells infected with simian virus 40 at a late time after infection. The labeled RNAs were hybridized with restriction fragments of simian virus 40 DNA. Levels of *methyl*-$^3$H-labeled RNA annealing with the early (E) or late (L) strands of a particular restriction fragment were compared with [$^3$H]uridine-labeled RNA annealing with the same fragment. The *methyl*-$^3$H-labeled RNA hybridizing to the various restriction fragments was eluted and analyzed for caps and internal 6-methyladenosine residues. Caps of transcripts of both the L and E strands were primarily located in a fragment containing the origin for viral DNA replication. The highest level of the internal 6-methyladenosine residues in simian virus 40 nuclear RNA was located on the L strand within a fragment from 83 to 0.0 map units and in RNA transcripts from the E strand within a fragment from 37 to 67 map units. Portions of both these regions represent intervening sequences not represented in cyotplasmic mRNA's. Lower levels of internal methylation were found in other locations as well. The potential role of internal 6-methyladenosine residues in RNA processing and transport from the nucleus to the cytoplasm is discussed.

Many eucaryotic and viral mRNA's, as well as nuclear pre-mRNA's, contain methylated nucleotides. The methylated moieties are found in 5'-terminal cap structures consisting of an m$^7$G residue connected at its 5' position through a triphosphate bridge to the 5' position of the penultimate 2'-*O*-methylribonucleoside, which is often methylated in the N-6 position as well (for review see reference 30). Furthermore, a number of additional residues (N$^6$-methyladenosine or m$^6$A) have been found at internal positions in the RNA molecules (2, 3, 13, 15, 26, 29).

An understanding of the function of mRNA methylation and of the 5' capping structure is far from complete. Whereas there is some indication that the capping structure is involved in increasing mRNA affinity for ribosomes and preventing breakdown of mRNA, the functional significance of m$^6$A is still entirely unknown (for review see references 30 and 32).

The molecular biology of simian virus 40 (SV40) has been under intensive investigation for a number of years, and these studies have provided considerable information regarding the regulation of gene expression, in particular the transcriptional and posttranscriptional processing of mRNA (for review see references 1 and 19). At least two control mechanisms regulate SV40 gene expression in productively infected cells; one operates at the transcriptional level, and the second operates at the posttranscriptional level (9, 12, 20, 22, 23). Evidence for the first control mechanism is the observation that at late times after infection about 10% of the nuclear viral RNA is transcribed from the early (E) strand and 90% is transcribed from the late (L) strand (12, 22, 23). A posttranscriptional control mechanism is suggested by the different frequencies of E- and L-RNA transcripts found in the cytoplasm. There, only 1% of the viral RNA is complementary to the E strand (22). Additional evidence for posttranscriptional regulation is the splicing of SV40 RNAs, which has also been shown to occur at the posttranscriptional level (5, 6, 10, 16-18, 21, 25). Accordingly, RNA sequences located within the precursor molecule are degraded, and nonadjacent segments are subsequently ligated. As an approach to determining a potential role for the internal 6-methyladenosine (m$^6$A) in RNA processing, the question of the location of the m$^6$A residues within the E- and L-nuclear SV40 transcripts at late times after infection has been investigated.

## MATERIALS AND METHODS

**Viruses and cells.** Plaque-purified SV40 (776) was grown and titrated in primary African green monkey kidney (AGMK) cells (obtained from Flow Laboratories, Rockville, Md.).

**Infection of cells.** Confluent monolayers of pri-

mary or secondary AGMK cells grown in minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented with 2 mM glutamine, 10% fetal calf serum, and 25 μg of gentamicin (Schering Corp., Kenilworth, N.J.) per ml were infected with SV40 at a multiplicity of 20 to 50 PFU/cell. After a 1-h adsorption, cells were refed with minimal essential medium containing 2% fetal calf serum.

**Optimal conditions for labeling the viral RNAs with [*methyl*-$^3$H]methionine.** Hybridization of [*methyl*-$^3$H]methionine-labeled RNA to restriction fragments of SV40 DNA and analysis of the labeled nucleotides was chosen as a method to localize the internal m$^6$A residues on the physical map of SV40 DNA (6). Since this analysis should be carried out under DNA excess hybridization conditions (4), we first performed preliminary experiments to determine the best labeling conditions for obtaining *methyl*-$^3$H-labeled RNA of the highest specific activity.

Various numbers of infected cells were labeled with 25 mCi of [*methyl*-$^3$H]methionine (80 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 3 h. Nuclear and cytoplasmic fractions were prepared, labeled RNA was extracted from each fraction, and the acid-precipitable radioactivity was determined (see below for details). Figure 1 shows that for this amount of radiolabeled precursor the highest incorporation was obtained by labeling two 150-cm$^2$ bottles ($10^7$ cells per bottle); there was no increase in the incorporation of radioactivity with labeling larger numbers of cells. Consequently, in all the experiments described below we labeled two bottles with 25 mCi of [*methyl*-$^3$H]methionine (80 Ci/mmol; New England Nuclear Corp.).

**Preparation of nuclear Sarkosyl-high-salt fractions and RNA extraction.** At the end of the labeling period, the cultures were washed three times with 10 mM Tris-hydrochloride (pH 7.4)–0.15 M NaCl. Nuclei were isolated with Nonidet P-40 detergent (28).

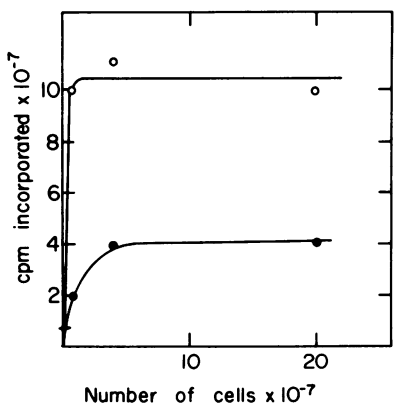


FIG. 1. *Labeling the infected cells with [methyl-$^3$H]methionine. Various numbers of infected cells grown in bottles ($10^7$ cells per bottle) were labeled with 25 mCi of [methyl-$^3$H]methionine. The acid-precipitable radioactivity incorporated into the nuclear and cytoplasmic RNAs was determined as described in the text. Nuclear RNA (○); cytoplasmic RNA (●).*

To prepare the Sarkosyl-high-salt fraction, the washed nuclei were resuspended in 1 ml of buffer containing 0.05 M Tris-hydrochloride (pH 7.9), 0.5 M NaCl, 0.02 M EDTA, and 0.2 M sucrose per $10^7$ nuclei. One-half volume of a solution containing 0.4 M NaCl, 0.75% Triton X-100, and 1.5% Sarkosyl (sodium lauryl sarcosinate) was added, and the mixture was centrifuged at 30,000 × *g* for 30 min at 2°C. The supernatant (Sarkosyl supernatant) was carefully removed from the chromatin pellet. RNA was extracted either from the Sarkosyl supernatant or from whole nuclei with sodium dodecyl sulfate (SDS)-phenol-chloroform-isoamyl alcohol (28) at room temperature and was collected by ethanol precipitation. The precipitate was suspended in TKM (50 mM Tris-hydrochloride, pH 6.7, 25 mM KCl, 2.5 mM $MgCl_2$) and digested with 25 μg of DNase (Worthington, RNase free, electrophoretically purified) per ml at 2°C for 60 min. The digest was extracted with SDS-phenol. Essentially all nuclear viral RNA was extracted in the Sarkosyl supernatant.

Sedimentation analysis of the labeled RNA after preliminary denaturation (0.5% SDS, 50°C, 3 min) was performed in 15 to 30% (wt/wt) sucrose gradients in SDS buffer (4). Centrifugation was carried out as indicated in the legend to Fig. 2. At the end of the run, fractions were collected, and the radioactivity in portions of each fraction was counted in a Triton-based scintillation fluid. Hybridization of the viral RNA to SV40 DNA immobilized on filters was performed as described previously (22).

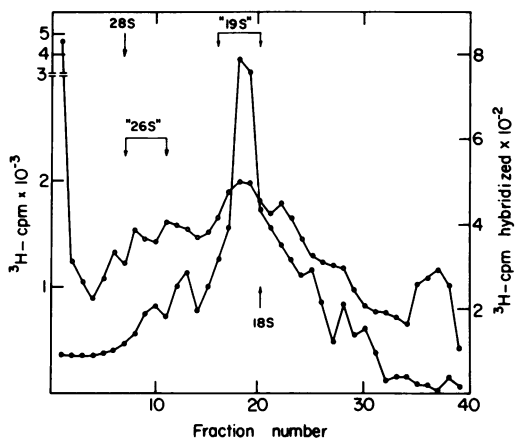


FIG. 2. *Sedimentation analysis of pulse-labeled viral RNA. Forty-eight hours after infection, $10^7$ cells were labeled with [$^3$H]uridine for 20 min. Nuclei were isolated, and Sarkosyl supernatant was prepared. The labeled RNA was placed on a linear 15 to 30% (wt/wt) sucrose gradient in SDS buffer and centrifuged for 16 h at 25,000 rpm at 20°C in a Spinco SW27 rotor. Arrows indicate the positions of $^{32}$P-labeled 28S and 18S rRNA markers. Aliquots (50 μl) from each fraction of the gradient were incubated in 0.4 ml of 4× SSC (SSC is 0.15 M NaCl–0.015 M trisodium citrate) with 7-mm nitrocellulose filters with 0.5 μg of bound SV40 DNA for 18 h at 68°C. The filters were then treated with 20 μg of RNase per ml. Blank filter contained less than 5 cpm. ○, Nuclear RNA; ●, viral RNA.*

**Transfer of SV40 DNA fragments to nitrocellulose filters and filter hybridization technique.** SV40 DNA was cleaved with the desired enzymes, and the fragments were separated by 1.4% agarose-gel electrophoresis (31). Separation of the strands of the two fragments generated by cleavage with the restriction endonucleases *Bam*HI and *Hpa*II was performed as described by Khoury and May (20), using a 1.4% agarose gel at 2 V/cm for 14 h. The DNA was transferred from the gel into a nitrocellulose paper (B-6; Schleicher and Schuell, Keene, N.H.) with 6× SSC (SSC is 0.15 M NaCl–0.015 M trisodium citrate) by using the technique of Southern (33). Each strip contained 1 to 2 μg of SV40 DNA. Nitrocellulose strips were incubated with a particular $^{32}$P-labeled RNA in 1.5 ml of 5× SSC–0.1% SDS for 24 h at 68°C. After hybridization the strips were treated with RNase (20 μg/ml in 2× SSC at 22°C for 45 min) and exposed to an X-ray film or cut into 1-mm fractions and counted. No cross-contamination of filter bands of the *Hpa*II/*Bam*HI-separated strands was found after hybridization to $^{32}$P-labeled separated strands of SV40 DNA or to SV40 complementary RNA (36).

## RESULTS

**Sedimentation analysis of nuclear SV40 RNA.** Figure 2 shows the sedimentation pattern of pulse-labeled nuclear RNA from the Sarkosyl–high-salt supernatant fraction. Previous experiments have shown that essentially all newly synthesized chromatin-coded RNA was pelleted during centrifugation, whereas viral RNA and DNA remained in the supernatant fraction (Aloni, unpublished data). Three distinct viral RNA peaks can be distinguished sedimenting at approximately 26S, 24S, and 19S. The heaviest of the viral RNA peaks (26S) is theoretically the size of a complete SV40 RNA transcript (about $1.8 \times 10^6$ daltons).

RNA species labeled with [$^3$H]uridine and sedimenting at about 26S and 19S were separately pooled as indicated in Fig. 2A and hybridized with restriction fragments produced by cleavage of form I SV40 DNA with restriction endonucleases *Eco*RI, *Hpa*I, and *Bgl*I (6). These enzymes cut the genome into five fragments, three of which (e, b, and d) are located within the "late" half of the genome and two of which (a and c) are contained within the "early" half of the genome (Fig. 3).

At the end of the incubation period, the nitrocellulose strips containing the five fragments were washed, dried, and exposed to an X-ray film. Figure 4A shows that the fastest-sedimenting viral RNAs (26S pool) hybridized to the five restriction fragments essentially in proportion to the length of the fragment. Since the hybridization was carried out under conditions of DNA excess, and because the early (E-strand) transcripts represent only a minor fraction of the

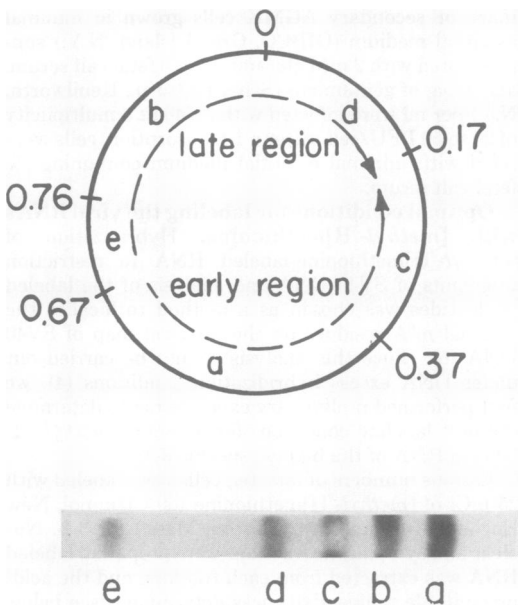


FIG. 3. *Map positions of restriction fragments produced by cleavage of form I SV40 DNA with EcoRI, HpaI, and BglI restriction endonucleases. The cleavage sites of the restriction enzymes are in map units (m.u.): EcoRI, 0.0; HpaI, 17, 37, 76; BglI, 67. The strip below the map shows the result of a fractionation of the restriction fragments on 1.4% agarose, blotted on a nitrocellulose membrane filter and hybridized with $^{32}$P-labeled nick-translated SV40 DNA. At the end of the hybridization the membrane filter was exposed to an X-ray film. Note that the intensity of the bands is proportional to the lengths of the fragments.*

RNA, these results suggest that transcripts which represent most or all of the L strand are present in this region of the gradient. The hybridization of the 19S RNA with the same blot (Fig. 4B) shows enrichment for the late fragments b, d, and e, indicating the relative absence of anti-early L-strand RNA sequences in this population compared to the 26S RNA. The low proportion of hybridization of 19S RNA to fragments a and c results at least in part from the presence of RNA transcribed from the E strand, as is evident from hybridization between this RNA and the separated strands of SV40 DNA (Fig. 5A). From Fig. 5 it is also estimated that the early transcripts represent about 10% of the total nuclear polyadenylated [poly(A)$^+$] SV40 RNA. In contrast, these transcripts represent only 1 or 2% of the cytoplasmic SV40 RNA (Fig. 5B and C), confirming previous observations (12, 22, 23). Since the proportion of E transcripts to L transcripts in the cytoplasm remains almost constant for various labeling periods (22), it seems likely that the E transcripts are less effi-

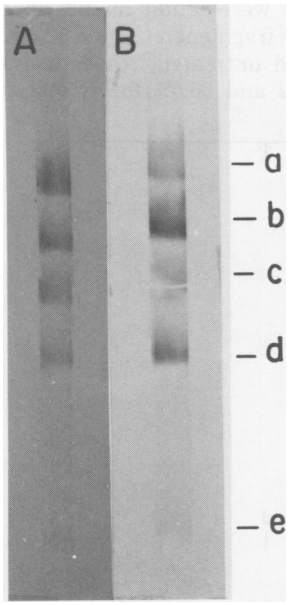


FIG. 4. *Hybridization between 26S and 19S RNAs and EcoRI, HpaI, and BglI restriction fragments. Hybridization was carried out between the pooled fractions 26S (A) and 19S (B) (as indicated in Fig. 2) and nitrocellulose filters containing the five SV40 DNA fragments as shown in Fig. 3.*

ciently exported from the nucleus to the cytoplasm than are the L transcripts.

To determine whether there is any correlation between the level of internal methylation and the efficiency of transport of the viral RNAs from the nucleus to the cytoplasm, we undertook the following experiments to determine the levels and general localization of internal $m^6A$ in the nuclear E and L transcripts.

**Localization of the internal $m^6A$ in nuclear viral RNA.** SV40-infected cells were incubated with [*methyl*-$^3$H]methionine at 48 h postinfection for 20 min to label methylated cap structures and internal $m^6A$ residues (6). The labeled RNA was fragmented to pieces about 500 nucleotides in length under limited alkaline digestion conditions (6); it was then annealed with the five *Eco*RI, *Hpa*I, and *Bgl*I restriction endonuclease fragments (see Fig. 3) and analyzed under two sets of conditions. In the first experiment, the nitrocellulose strips containing the five fragments were washed, dried, cut, and counted. In the second protocol, at the end of the hybridization period the nitrocellulose strips were treated with pancreatic RNase and then analyzed for radioactivity as described above. The results are shown in Fig. 6A and 6B, respectively. In both experiments radiolabeled material was detected in fragments *a* (37 to 67 map units [m.u.]), *b* (76 to 0.0 m.u.), *d* (0.0 to 17 m.u.), and *e* (67 to 76 m.u.). No radioactivity was detected in fragment *c* (17 to 37 m.u.). The relative radiolabel found in fragment *a* (37 to 67 m.u.) with nuclear RNA was much higher than that found with this fragment when cytoplasmic RNA was analyzed. Figure 6C shows a typical X-ray film from a similar hybridization experiment of [*methyl*-$^3$H]methionine-labeled cytoplasmic RNA and a blot containing the same five restriction fragments, in which RNA complementary to fragment *a* is undetectable. Similar results were obtained with [$^3$H]uridine-labeled nuclear and cytoplasmic RNA (see Fig. 5). It could also be seen in Fig. 6 that, after treatment of the nitrocellulose paper with RNase, the proportion of radioactivity associated with each of the fragments was slightly altered. This may indicate that some of the radioactivity associated with the fragments was in part due to nonhybridized RNA sequences covalently attached to hybridized sequences. The analysis of *methyl*-labeled SV40 RNA presented thus far, of course, does not discriminate between caps and internal *methyl* label.

The radiolabeled RNA associated with each of the fragments (Fig. 6) was next eluted from the nitrocellulose paper and digested with $T_2$, $T_1$, and pancreatic RNases. The products were analyzed by electrophoresis on DEAE paper at pH 3.5. As was the case when cytoplasmic RNA

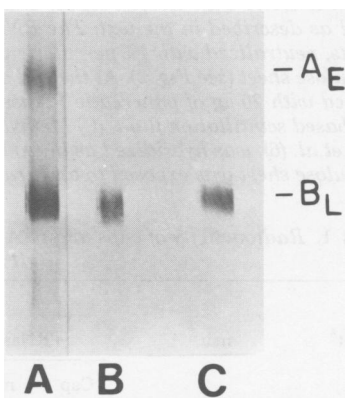


FIG. 5. *Hybridization of [$^3$H]uridine-labeled nuclear and cytoplasmic RNAs to separated strands of fragments on filters. The positions of fragments generated by cleavage with the restriction endonucleases BamHI and HpaII are detailed in the text and by Ferdinand et al. (12). The conditions for DNA transfer and DNA-RNA hybridization are described in the text. (A) Hybridization was carried out with 19S poly(A)$^+$ nuclear RNA; (B and C) hybridization was carried out with 19S and 16S poly(A)$^+$ cytoplasmic RNAs purified by sedimentation through sucrose gradients (6).*

was previously analyzed (6), radioactivity was found only in the cap structures and m$^6$A residues. Results in Table 1 show that in mRNA eluted from both RNase-treated and untreated blots, caps were found mainly in species annealed with fragment *a* (22 and 32.6% for RNase-treated and untreated, respectively) and fragment *e* (72 and 55.5% for RNase-treated and

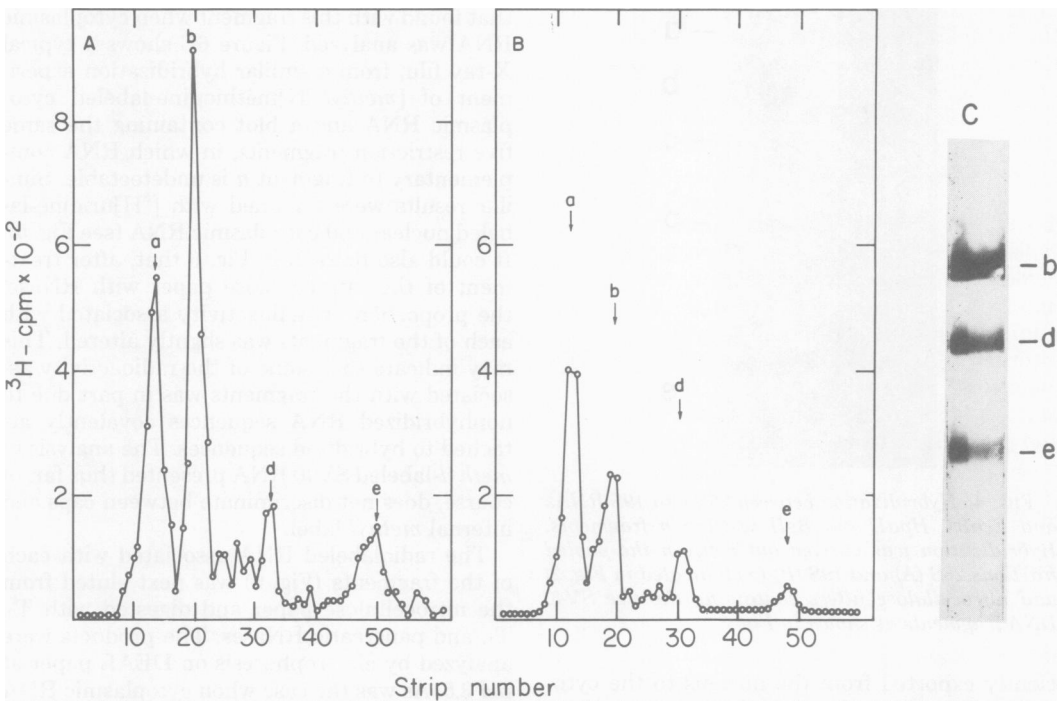


FIG. 6. *Hybridization of methyl-$^3$H-labeled nuclear RNA to restriction fragments produced by cleavage of form I SV40 DNA with EcoRI, HpaI, and BglI restriction enzymes. Methyl-$^3$H-labeled nuclear RNA was prepared as described in the text. The RNA was nicked with 0.025 M KOH for 3 h at 4°C to produce 4-7S fragments, neutralized with 1% perchloric acid, and hybridized with EcoRI, HpaI, and BglI fragments on a nitrocellulose sheet (see Fig. 3). At the end of the annealing period the nitrocellulose sheet was not (A) or was (B) treated with 20 μg of pancreatic RNase per ml (60 min at 22°C). Strips (1 mm) were cut and counted in toluene-based scintillation fluid. (C) Methyl-$^3$H-labeled cytoplasmic RNA, labeled and prepared as described in Aloni et al. (6), was hybridized as in (A) to the same restriction fragments. At the end of the incubation the nitrocellulose sheet was exposed to an X-ray film (see reference 6).*

TABLE 1. *Radioactivity of caps and m$^6$A bound to restriction fragments produced by EcoRI, HpaI, and BglI restriction endonucleases*[a]

| Fragment[b] | m.u. | cpm | | | | Percentage (cpm) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | +RNase | | −RNase | | +RNase | | −RNase | |
| | | Cap | m$^6$A | Cap | m$^6$A | Cap | m$^6$A | Cap | m$^6$A |
| d | 0.0–17.5 | 3 | 100 | 5 | 172 | 2.1 | 8.7 | 1.7 | 7.3 |
| c | 17.5–37.5 | NA | NA | NA | NA | NA | NA | NA | NA |
| a | 37.5–67.0 | 30 | 462 | 96 | 830 | 22.2 | 40.5 | 32.6 | 35.5 |
| e | 67.0–76.0 | 97 | 36 | 163 | 156 | 72.0 | 3.1 | 55.5 | 6.6 |
| b | 76.0– 0.0 | 5 | 542 | 30 | 1180 | 3.7 | 47.7 | 10.2 | 50.6 |

[a] The regions corresponding to each of the fragments as shown in Fig. 6A and B were cut and placed in 0.5 ml of water containing 20 mg of tRNA. The labeled RNA was eluted by heating to 100°C, followed by rapid cooling. The RNA was collected by ethanol precipitation and centrifugation. Digestion with $T_2$, $T_1$, and pancreatic RNases, electrophoresis on DEAE-paper at pH 3.5, and identification of the radioactivity (counts per minute [cpm]) associated with caps and m$^6$A were carried out as described by Aloni et al. (6). About 50% of the radioactivity found on the nitrocellulose sheet was recovered from DEAE-paper. NA, Not analyzed.

[b] See Fig. 3.

untreated, respectively). The localization of the labeled caps to fragment *e* is in agreement with previous results analyzing cytoplasmic SV40 RNA (6, 9a). It suggests that the majority of the 5′ termini found in SV40 nuclear RNA mapping at these positions are conserved and appear in the cytoplasmic viral RNA species. The annealing of the labeled caps with fragment *a* could be due in part to caps of L viral RNA which map in the portion of fragment *a* adjacent to fragment *e* (14, 16) and, in part, to caps of the E viral RNA. Experiments to distinguish between caps that pertain to the L and E viral RNAs are described below. It could also be seen that the radioactivity representing internal $m^6A$ annealed predominantly with fragment *a* (37 to 67 m.u.) and fragment *b* (76 to 0.0 m.u.). Only minimal amounts of radiolabel corresponding to internal $m^6A$ residues were detected with fragment *e* (67 to 76 m.u.) and fragment *d* (0.0 to 17 m.u.), which are the templates for the leaders and parts of the bodies of the late viral mRNA's, respectively. The sequences transcribed from fragment *b* contained the highest proportion of $m^6A$. It is noteworthy that the excision and ligation (splicing) of the late viral RNAs occur within the transcript of fragment *b*; but the role of $m^6A$ in this splicing event is presently unknown. The high level of $m^6A$ in RNA transcripts from the *a* fragment was unexpected. The proportion of these trancripts in the total nuclear RNA is about 10% (22; see also above); however, the abundance of $m^6A$'s in transcripts complementary to the *a* fragment (37 to 67 m.u.) was almost equal to that found in the transcripts complementary to the *b* fragment (76 to 0.0 m.u.).

Since $m^6A$ residues in transcripts complementary to the *a* fragment are virtually absent from cytoplasmic RNA, they appear to be lost during processing. In contrast, $m^6A$ residues appear to be conserved during the processing of rRNA (27, 30).

To locate more precisely the $m^6A$ residues within the late and early regions, a similar hybridization experiment was performed with fragmented *methyl*-$^3$H-labeled poly(A)$^+$ and poly(A)$^-$ nuclear RNA prepared from infected cells labeled for 20 min. In this experiment the *methyl*-$^3$H-labeled RNA (containing both cap and internal $m^6A$'s) was annealed with the four fragments produced by digestion of form I SV40 DNA with a combination of *Hae*II, *Bam*HI, *Taq*I, and *Hpa*II restriction endonucleases (6). This combination of enzymes was chosen since *Hae*II and *Taq*I cleave within *Eco*RI, *Hpa*I, and *Bgl*I fragments *b* and *a*, respectively. Figure 7 shows that for both poly(A)$^+$ and poly(A)$^-$ RNAs radioactivity was associated predominantly with fragments *b* (83 to 14 m.u.), *a* (14 to 56 m.u.), and *c* (56 to 72 m.u.). The labeled RNA from both poly(A)$^+$ and poly(A)$^-$ fractions eluted from each band was again digested with $T_2$, $T_1$, and pancreatic RNases and analyzed for both cap structures and internal $m^6A$ residues as described above.

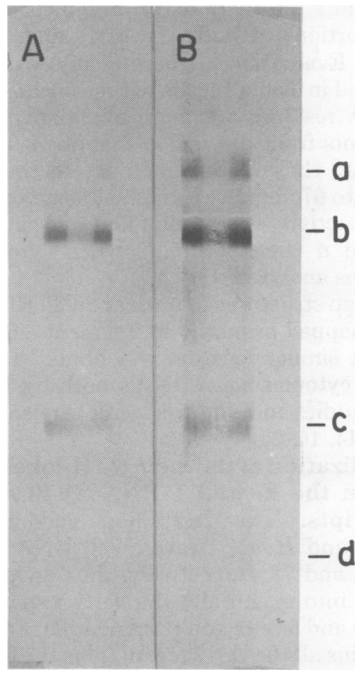


FIG. 7. *Hybridization of methyl-*$^3$*H-labeled nuclear RNA to restriction fragments produced by cleavage of form I SV40 DNA with HaeII, BamHI, TaqI, and HpaII restriction enzymes. Methyl-*$^3$*H-labeled nuclear RNA was prepared and fractionated on a oligodeoxythymidylic acid-cellulose column to molecules with (B) and without (A) poly(A) tails. Each preparation was hybridized with a nitrocellulose sheet containing the four fragments produced by cleavage of form I SV40 DNA with HaeII, BamHI, TaqI, and HpaII restriction enzymes. The map positions of the fragments are shown in Table 2. At the end of the hybridization the nitrocellulose sheets were exposed to X-ray films.*

Table 2 shows that most of the radioactivity associated with fragment *b* (83 to 14 m.u.) is accounted for by $m^6A$ residues. Comparison of these results with those shown in Table 1 leads to the conclusion that the transcripts of the late region from 83 to 0.0 m.u. contains the highest number of $m^6A$ residues. It is noteworthy that, with cytoplasmic viral RNA as well, transcripts representing this region were found to contain the highest number of $m^6A$ residues (6, 9a).

It could also be seen (Table 2) that $m^6A$ residues were present in RNA transcripts complementary to the early fragments *a* (14 to 56

m.u.) and *c* (56 to 72 m.u.). Fragment *c* represents portions of both the early and the late regions. Evaluation of these results with those presented in Table 1 leads to the conclusion that the $m^6A$ residues are more abundant in RNA transcripts from the region that spans 37 to 56 m.u. than those complementary to the region from 56 to 67 m.u. In contrast, almost no *methyl*-$^3H$ radioactivity was found to be associated with fragment *a* (14 to 56 m.u.) when cytoplasmic RNA was analyzed (6).

The cap structures of nuclear SV40 RNA (Table 2) mapped primarily in fragment *c* (56 to 72 m.u.). A similar location was obtained for the caps of cytoplasmic mRNA's both by hybridization studies and a more specific sequence analysis (6, 14, 16, 25).

**Localization of the *methyl*-$^3H$-labeled residues in the E- and L-RNA SV40 nuclear transcripts.** The restriction endonucleases *Bam*HI and *Hpa*II cleave SV40 DNA at map units 14 and 72, respectively, thus dividing the genome into essentially the early region (fragment A) and late region (fragment B). Fragment A contains all the early region (plus the terminal portion of the late region). Fragment B contains essentially all of the late region except for the terminal 3.5% (14 to 17.5 m.u.) and the initial 5% (67 to 72 m.u.).

The cleavage products of *Bam*HI and *Hpa*II were denatured in alkali and electrophoresed in 1.4% agarose gel as described (20; Materials and Methods), separating the early (E) and late (L) strands of the early (A) and late (B) DNA fragments. The four DNA bands were then transferred to nitrocellular paper (33) for subsequent hybridization studies. In a control experiment (not shown), $^{32}P$-labeled separated strands of SV40 DNA and $^{32}P$-labeled SV40 complementary RNA were used to locate the positions of the four bands (see 20).

*Methyl*-$^3H$-labeled poly(A)$^+$ and poly(A)$^-$ nuclear RNA labeled for 20 min at 48 h postinfection was prepared from SV40-infected cells. These RNAs were annealed to nitrocellulose blots containing the separated strands of SV40 DNA. Results (Fig. 8) show that radioactivity was associated with fragments $A_E$, $A_L$, and $B_L$. The labeled RNA was eluted from each band, digested with $T_2$, $T_1$, and pancreatic RNases, and analyzed as described above both for cap structures and internal $m^6A$ residues.

Data from the experiment (Table 3) indicate that about 20% of the methylated caps and $m^6A$'s are associated with the E transcripts, and 80% of the methylated caps and $m^6A$'s are associated with the L transcripts. Based on these results and those shown in Fig. 5, it appears that the relative level of internal methylation in early nuclear SV40 transcripts is at least as high as that in late nuclear SV40 transcripts. These results, together with those presented above, indicate (Fig. 6; Table 1) that the $m^6A$'s are present in nuclear RNA mapping between 37 and 67 m.u. on both the early and anti-early transcripts. It is noteworthy that, because of the

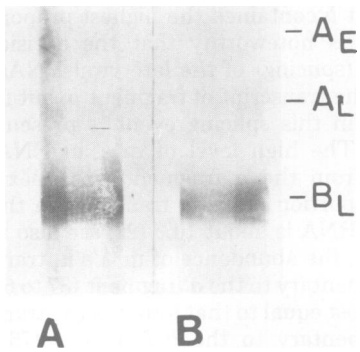


FIG. 8. *Hybridization of methyl-$^3H$-labeled nuclear RNA to the separated strands of HpaII-BamHI fragments. Methyl-$^3H$-labeled poly(A)$^+$ (A) and poly(A)$^-$ (B) nuclear RNA was prepared as described in Fig. 7 and hybridized with the separated strands of HpaII-BamHI fragments. At the end of the hybridization the nitrocellulose sheets were exposed to X-ray films.*

TABLE 2. *Radioactivity of caps and $m^6A$ bound to restriction fragments produced by BamHI, TaqI, HpaII, and HaeII restriction endonucleases*[a]

| Fragment | m.u. | cpm | | | | Percentage (cpm) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | poly(A)$^+$ | | poly(A)$^-$ | | poly(A)$^+$ | | poly(A)$^-$ | |
| | | Cap | $m^6A$ | Cap | $m^6A$ | Cap | $m^6A$ | Cap | $m^6A$ |
| *Bam*HI-*Taq*I (*a*) | 14–56 | 0 | 130 | 0 | 54 | 0 | 19.2 | 0 | 20.4 |
| *Taq*I-*Hpa*II (*c*) | 56–72 | 94 | 122 | 63 | 21 | 79 | 17.7 | 61.8 | 7.9 |
| *Hpa*II-*Hae*II (*d*) | 72–83 | 20 | 25 | 27 | 10 | 21 | 3.7 | 26.6 | 3.8 |
| *Hae*II-*Bam*HI (*b*) | 83–14 | 0 | 402 | 12 | 180 | 0 | 59.4 | 11.6 | 67.9 |

[a] The regions corresponding to each fragment as shown in Fig. 7 were cut. Elution of the labeled RNA and analysis of caps and internal $m^6A$'s were carried out as described in Table 1, footnote *a*.

TABLE 3. *Radioactivity of caps and m$^6$A bound to the separated strands of the restriction fragments produced by BamHI and HpaII restriction endonucleases*[a]

| Fragment | m.u. | cpm | | | | Percentage (cpm) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | poly(A)$^+$ | | poly(A)$^-$ | | poly(A)$^+$ | | poly(A)$^-$ | |
| | | Cap | m$^6$A | Cap | m$^6$A | Cap | m$^6$A | Cap | m$^6$A |
| $A_E$ (*BamHI-HpaII*) | 14-72 (E) | 30 | 125 | 13 | 55 | 21.5 | 18.2 | 6.6 | 15.7 |
| $A_L$ (*BamHI-HpaII*) | 14-72 (L) | 34 | 65 | 33 | 50 | 24.5 | 9.3 | 16.7 | 14.3 |
| $B_L$ (*HpaII-BamHI*) | 72-14 (L) | 75 | 503 | 151 | 245 | 54.0 | 72.5 | 76.7 | 70.0 |
| $B_E$ (*HpaII-BamHI*) | 72-14 (E) | NA | NA | NA | NA | NA | NA | NA | NA |

[a] The regions corresponding to each fragment as shown in Fig. 8 were cut. Elution of labeled RNA and analysis of caps and internal m$^6$A's were carried out as described in Table 1, footnote *a*. NA, Not analyzed.

very low level of the early cytoplasmic transcripts (Fig. 5B), internal m$^6$A residues are undetectable in that fraction (Fig. 6C).

## DISCUSSION

In the present study we have localized the internal m$^6$A's and cap structures of the late nuclear RNA transcripts on the E and L strands of SV40 DNA.

Several investigators have determined the sequence and locations of the internal m$^6$A residues in various mRNA's. In HeLa cell mRNA's, m$^6$A was found to occur predominantly in two sequences: G-m$^6$A-C and A-m$^6$A-C (34). The authors suggested that not all of the potential sites are methylated. If SV40 mRNA's are also "undermethylated," the relative abundance of internal m$^6$A's in the transcripts of the E and L strands complementary to the early fragment (37 to 67 m.u.; Table 1) may reflect a longer survival in the nucleus of these transcripts. This increased intranuclear lifetime would thus allow a larger number of residues to be methylated. A noteworthy observation in this study was the increased ratio of E- to L-strand transcripts in the nuclear RNA and the relatively high level of internal methylation (m$^6$A) in the E-strand-specific nuclear transcripts. Whether these observations are related in a functional way is not clear at present.

Efforts to localize the internal m$^6$A within various mRNA's have yielded a variety of results. Adams and Cory (2) reported that m$^6$A is present in both the 5′ and 3′ halves of mouse myeloma cell mRNA. Furuichi et al. (13) found that m$^6$A is not located close to the 3′ poly(A) segment of HeLa cell mRNA. Wei and Moss (35) have found that in HeLa mRNA's, although some m$^6$A residues are contained within 4-to-6S-size 5′ terminal fragments, the majority are located more internally. In Rous sarcoma virus, approximately 10 to 12 m$^6$A residues are located between 500 and 4,000 nucleotides from the 3′ poly(A) end (7). In the present study we have found that the highest levels of the internal m$^6$A's in SV40 nuclear RNA are (i) on the L strand, in the RNA transcripts of a fragment which spans 83 to 0.0 m.u. and (b) on the E strand, in the RNA transcripts of a fragment mapping between 37 and 67 m.u. Lower levels of internal methylation were found in other locations as well. The clustering of internal m$^6$A's on the L-RNA transcript at 83 to 0.0 m.u., a location which contains the 5′ end of the body of the 16S viral RNA, may suggest that internal methylation may play a role as a signal for the enzyme(s) that carries out the excision-ligation process. This assumption is consistent with the high level of internal m$^6$A's on the E-RNA transcripts in a fragment that spans 37 to 67 m.u. and contains the sequences within which the splicing of E transcripts occurs (8, 11). A more precise localization of the internal m$^6$A's in both nuclear and cytoplasmic SV40 RNA would clearly be helpful. Recent studies with late SV40 cytoplasmic RNAs have localized three m$^6$A residues, all within coding sequences (D. Canaani and Y. Groner, personal communication).

Splicing has been found to occur in several locations within the RNA transcripts of various genes. If internal methylation plays a role in the splicing process, then the irregularity of location of the internal m$^6$A's among various mRNA's could be explained.

We have localized the cap structures of the late nulcear RNA transcripts in two fragments which map from 67 to 76 m.u. and 37 to 67 m.u. Since the radioactivity for the cap was resistant to RNase treatment, the sequences immediately adjacent to the cap are not likely to be free tails covalently attached to true hybrids, but colinear transcripts of these two fragments. It appears, therefore, that there are several locations for the 5′ ends of the late nuclear SV40 RNAs. This conclusion is in agreement with previous results suggesting a heterogeneity in the 5′ ends and caps of the late SV40 transcripts (9a, 10, 14, 17, 21, 24).

The E-RNA transcripts were also found to contain cap structures located between 56 and 76 m.u., but the precise map location of the sequences immediately adjacent to these caps has not been determined.

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