JOURNAL OF VIROLOGY, Jan. 1979, p. 209-219
0022-538X/79/01-0209/11$02.00/0

Vol. 29, No. 1

Mutational Alterations Within the Simian Virus 40 Leader Segment Generate Altered 16S and 19S mRNA's

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Received for publication 17 August 1978

We have analyzed the structure of the late cytoplasmic RNAs made after infection with wild-type simian virus 40 and a set of viable mutants, four of which have deletions and one an insertion within the nucleotide sequence specifying the leader segment of the 16S and 19S mRNA's. The principal findings are: (i) simian virus 40 16S and 19S mRNA's made during infections with wild-type virus are heterogeneous with respect to the map location of their 5′ ends and possibly in the nucleotide sequence comprising the "leader" segments. (ii) "Spliced" 16S and 19S mRNA's are made during infections with each of the mutants although, in some cases, the ratio of 19S to 16S mRNA species is reduced. (iii) The deletion or insertion of nucleotides within the DNA segment defined by map position 0.70 to 0.75 causes striking alterations in the types of leader structures in the late mRNA's. (iv) Many of the late RNA leader segments produced after infection with the mutants appear to be multiply spliced, i.e., instead of the major 200- to 205-nucleotide-long leader segment present in wild-type 16S mRNA, the RNAs produced by several of the deletion mutants have leaders with short discontiguous segments.

Recently, it was discovered that simian virus 40 (SV40) late mRNA's, which are transcribed clockwise from the DNA segment between map coordinates 0.67 and 0.17 and code for the three virion polypeptides (VP1, VP2, and VP3), have unusual structures (2, 5, 8, 11, 13, 15, 16). Instead of being homologous to a contiguous length of DNA, these mRNA's are composite structures, containing nucleotide sequences transcribed from nonadjacent DNA segments. Each mRNA contains a 5′-terminal segment (the "leader") transcribed from the L-strand between map coordinates 0.70 and 0.76. Joined to the leader sequence, the 19S class of mRNA's has a segment of RNA homologous to the L-strand between map coordinates 0.77 and 0.17 (the 19S "body"); the body of the 16S late mRNA is transcribed from the L-strand of the DNA between map coordinates 0.94 and 0.17. The physiological function or formation of such "spliced" mRNA's is not yet clear.

Some time ago we obtained a set of viable mutants, four of which have deletions and one an insertion within the DNA segment coding for the leader sequence (6, 21, 23). In this study we examine the effect of these deletions on the formation and structure of SV40 late mRNA's. Our experiments demonstrate that spliced 19S and 16S mRNA's are formed after infection with each of the mutants, although the structures of their leader sequences are decidedly altered.

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MATERIALS AND METHODS

Cell culture and viruses. The history, growth, and protocols for virus infection of the CV-1 monkey kidney cell line have already been described by Mertz and Berg (20), as has the isolation of the mutants *dl* 804, *dl* 810, *dl* 861, *dl* 894, and *in* 872 (6, 21, 23).

Enzymes. Restruction endonucleases *Bgl*I, *Bam*HI, *Eco*RI, *Hpa*I, *Hin*cII, and *Hin*dIII were purchased from New England Biolabs. *Taq* endonuclease was obtained from S. Goff. S1 nuclease was purchased from Sigma Chemicals, and exonuclease VII was a gift from S. Goff (12).

Preparation of SV40 DNA. Semiconfluent monolayers of CV-1 cells were infected with wild-type (SVS) and mutant virus at a multiplicity of infection of 50. To obtain ^{32}P-labeled DNA, [^{32}P]orthophosphate (carrier free, 1 mCi per plate) was added 30 h postinfection to the cells in phosphate-free medium containing 2% dialyzed fetal calf serum. Viral DNA was extracted 18 h later by Hirt's method (14) and purified as described previously (24); the specific activity of the viral DNA was generally 1×10^6 to 2×10^6 cpm/μg.

Polyacrylamide gel analysis of the viral DNAs. Each purified viral DNA (1 μg) was cut with *Hin*cII and *Hin*dIII endonucleases, after which sodium dodecyl sulfate (to 1%) and EDTA (to 20 mM) were added, and the mixture was heated to 65°C for 5 min. Glycerol (5%) and bromophenol blue indicator dye (1%) were added, and the samples were layered on a 3 to 7% gradient polyacrylamide gel and subjected to electrophoresis as previously described (10). The lo-

cation of the DNA bands was determined with a short-wavelength UV light transilluminator after staining with 1 μg of ethidium bromide per ml.

Preparation of hybridization probes. Viral DNA fragments, specific for the leader and body of the late mRNA's, were prepared by digesting SVS DNA with *Hpa*I and *Bgl*I endonucleases (see Fig. 2B). The A fragment of this digest (the segment bounded by map coordinates 0.765 and 0.175) contains the nucleotide sequence coding for the bodies of the late mRNA's. The D fragment (defined by map coordinates 0.670 and 0.760) spans the nucleotide sequence coding for the leader of the late mRNA's. The fragments were separated by electrophoresis in horizontal 1.5% agarose slabs (10), eluted electrophoretically into dialysis tubing, and labeled with ^{32}P by nick-translation (22). To assay the purity of these fragments prior to their use as hybridization probes, the *Hpa*I-*Bgl*I endonuclease digest of 5 μg of SVS DNA was transferred from an agarose gel to a sheet of nitrocellulose according to Southern (25). The nitrocellulose strips were then annealed with 2×10^5 cpm of each of the ^{32}P-labeled probes, and then autoradiograms were prepared. The probe for the body of the mRNA's hybridizes predominately to the A fragment, with only a trace of hybridization to the B, C, and D fragments (see Fig. 2B). Similarly, the probe for the leader segment hybridizes principally to the D fragment, but here too some hybridization occurs with the other fragments.

Isolation of SV40 RNA. RNA was extracted from semiconfluent cell monolayers 48 h after infection (multiplicity of infection, 50) with SVS, *dl* 804, *dl* 810, *dl* 861, *in* 872, and *dl* 894. The cells were washed twice with cold Tris-buffered saline and once, briefly, with a cold solution of 10 mM Tris (pH 7.5) containing 10 mM NaCl and 5 mM $MgCl_2$, followed with 1 ml per plate of the same buffer plus 0.5% Nonidet P-40 and 0.05% deoxycholate. The cell monolayer was scraped off with a rubber policeman and mixed with an equal volume of 100 mM Tris (pH 8.5) containing 300 mM NaCl and 15 mM EDTA, and the cell aggregates were dispersed by forcing the mixture in and out, several times, through a Pasteur pipette. The suspension was extracted twice with an equal volume of water-saturated phenol-chloroform (1:1), and the RNA was precipitated with 2 volumes of ethanol. This procedure extracts mainly cytoplasmic RNA, as the nuclei do not lyse and remain in the first phenol-chloroform aqueous interface. The precipitates were washed with 70% ethanol, drained, and dissolved in a small volume of 20 mM Tris (pH 7.5) containing 10 mM NaCl and 10 mM $MgCl_2$. DNase I, freed of RNase by iodoacetate treatment (26), was added (20 μg of DNase per mg of nucleic acid), and the mixture was incubated at room temperature for 45 min. Then, 0.5 volume of a solution of 80 mM Tris (pH 8.5) containing 50 mM EDTA, 300 mM NaCl, 0.5% sodium dodecyl sulfate, and proteinase K (100 μg/ml) was added. After 10 min at room temperature, the mixture was extracted with an equal volume of water-saturated phenol-chloroform (1:1). The RNA was either precipitated with ethanol or passed over polyuridylic acid-Sepharose (Pharmacia) (17) to select polyadenylic acid-terminated RNAs.

Analysis of SV40 RNAs. Polyadenylic acid-containing RNAs were denatured with glyoxal and subjected to electrophoresis in a horizontal 1.5% agarose slab gel (10 μg per gel track) in 10 mM sodium phosphate buffer (pH 7.0) (19). After electrophoresis, the gel was immersed in 50 mM NaOH containing ethidium bromide (1 μg/ml) for 1 h to dissociate the RNA-glyoxal compound and to stain the RNA bands. About 30 min after the gel was immersed in alkali, the diazobenzyloxymethyl-treated cellulose sheet was "diazotized" (3). After 60 min of alkali treatment, the gel was neutralized by washing twice in 100 mM phosphate buffer (pH 6.8) for 15 min, followed by three washes of 20 mM phosphate buffer for 10 min each. During neutralization the diazotized cellulose was washed four times with cold water for 10 min each and then twice with cold 20 mM phosphate buffer for 10 min each. The RNA was transferred by blotting to the diazotized cellulose (3) and then hybridized for 24 h at 42°C with approximately 2×10^6 cpm of the appropriate ^{32}P-labeled DNA probe. The cellulose sheets were then washed for 2 h at 42°C with six changes of 100 ml each of 50% formamide and fivefold-concentrated standard citrate-saline, followed by one wash at room temperature with Tris-buffered saline. The sheets were air dried and autoradiographed using Kodak XR-5 film exposed to −70°C for 4 h with a Dupont Cronex lightning-plus intensifying screen.

Analysis of spliced structure of the RNAs. The contiguity of the nucleotide sequences in the mRNA's was determined by the method of Berk and Sharp (4). About 50 μg of the cytoplasmic RNA was hybridized with 1×10^4 to 3×10^4 cpm of intact ^{32}P-labeled viral DNA fragments for 40 h at 47°C in 50 μl of 80% formamide as described by Casey and Davidson (7). A 25-μl aliquot was diluted 10-fold into cold 30 mM sodium acetate (pH 4.5) containing 280 mM NaCl and 4.5 mM $ZnCl_2$ and digested with 8 U of S1 nuclease at 45°C for 45 min as described by Shenk et al. (24). The remaining 25 μl of the hybridized sample was diluted with 10 volumes of 10 mM Tris (pH 7.5) containing enough exonuclease VII to digest 1 μg of denatured SV40 DNA in 45 min at 45°C. After the digestion, samples were precipitated with ethanol, and the resistant DNA was subjected to electrophoresis in horizontal 1.5% agarose slab gels containing 30 mM NaOH (18). Dried gels were autoradiographed for 12 h as described above.

RESULTS

Verification of the DNA structure of SV40 deletion and insertion mutants. We have previously observed high levels of transcriptionally active defective genomes accumulating during a single growth cycle after high-multiplicity infection, particularly by mutant virus stocks (unpublished data). Since the mRNA's being analyzed were to be isolated from cells infected with high multiplicities of the wild-type and mutant viruses, it was advisable to verify that the intranuclear viral genomes were predominately those of the infecting species. Accordingly, viral DNA, extracted by Hirt's procedure (14) 48 h after infection, was digested

with *Hinc*II and *Hin*dIII endonucleases, and the fragments were subjected to electrophoresis in polyacrylamide gels (Fig. 1). Strain *Rh* 911 is the parent virus to mutants *dl* 804 and *dl* 810 (21), whereas strain SVS is the progenitor of *dl* 861, *dl* 894, and *in* 872 (6, 23). *dl* 804 and *dl* 810 DNA lack the *Rh* 911 C fragment but contain a new fragment that comigrates either with the F fragment (*dl* 804) or just behind the G fragment (*dl* 810); this corresponds to deletions of 170 ± 10 and 190 ± 10 nucleotides, respectively. Compared to their parent virus, SVS, mutants *dl* 861 and *dl* 894 also lack the C fragment, but yield new fragments comigrating with the D or E fragments, respectively, indicating deletions of 20 to 30 and 150 ± 10 nucleotides, respectively. Mutant *in* 872 was reported to contain a deoxyribosyladenine:deoxyribosylthymine insertion at map position 0.73 (6), a contention supported by our finding that the C fragment is about 50 nucleotides larger than the corresponding fragment from wild-type DNA. Because the digestion patterns are uncomplicated, i.e., free of fragments, we conclude that there are not substantial amounts of defective or rearranged genomes in these stocks or in the vegetatively multiplying viral DNA pool.

Judging from the size of the deletions and insertion and the fact that each of these mutants lacks the *Hpa*II endonuclease cleavage site (map position 0.73) (mutant *dl* 810 also lacks the *Kpn* endonuclease cleavage site at map position 0.72), the location of the deletions and insertion can be fixed within the segment bounded by map coordinates 0.70 and 0.76, the putative leader sequence of SV40 late mRNA's (11, 13).

Spliced 19S and 16S cytoplasmic, polyadenylated viral mRNA's are produced after infection with SV40 mutants. Cytoplasmic, polyadenylated RNA, synthesized after infection of CV-1 cells with SVS or each of the mutants, was isolated, denatured, subjected to electrophoresis on agarose gel, and then transferred to sheets of diazotized cellulose as described in Materials and Methods. One set of RNA samples was hybridized with ^{32}P-labeled DNA derived from the region defined by map position 0.67 to 0.76 (the late mRNA leader probe), and an identical set of electrophoresed RNA samples was hybridized with ^{32}P-labeled DNA containing the nucleotide sequences from map position 0.76–0.175 (the late mRNA body probe). The results (Fig. 2) demonstrate that the mature 19S and 16S late mRNA classes are produced during infections with the mutants; mutants *in* 872 and *dl* 894 produce considerably less of the 19S size class, but on longer exposures the 19S RNA band is clearly visible in the autoradiograms. Since both mRNA species hybridize with the leader and body probes, we infer that the cytoplasmic mRNA's produced by the mutants are probably similar in structure to the wild-type mRNA's.

Evidence that the mutant mRNA's are indeed discontiguous (spliced) structures was obtained using Berk and Sharp's technique (4) for detecting such spliced RNAs. SV40 cytoplasmic RNA, isolated 48 h after virus infection, was hybridized to a 2.5-kilobase (kb) ^{32}P-labeled DNA fragment (the segment between map coordinates 0.67 and

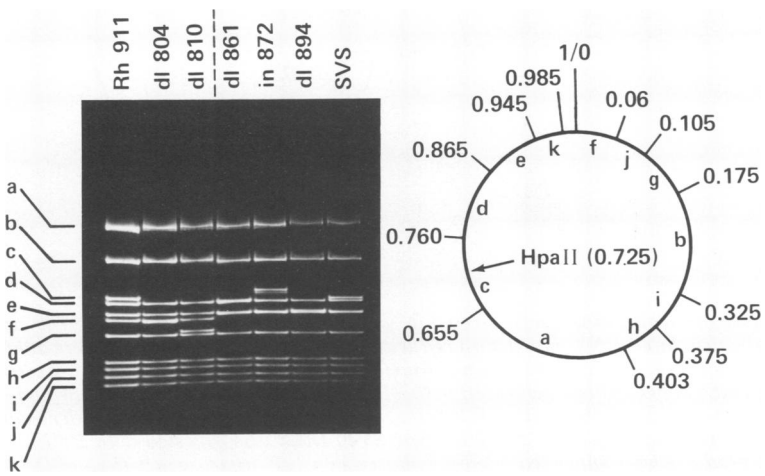

FIG. 1. *Analysis of wild-type and mutant viral DNAs by HincII and HindIII endonuclease digestion and electrophoresis on polyacrylamide gel. The DNAs were isolated by Hirt extraction (14) 48 h after high-multiplicity infections, digested with HincII and HindIII endonucleases, subjected to electrophoresis on a 3 to 7% gradient polyacrylamide slab gel, stained with ethidium bromide, and photographed on UV light transilluminator as described in the text.*

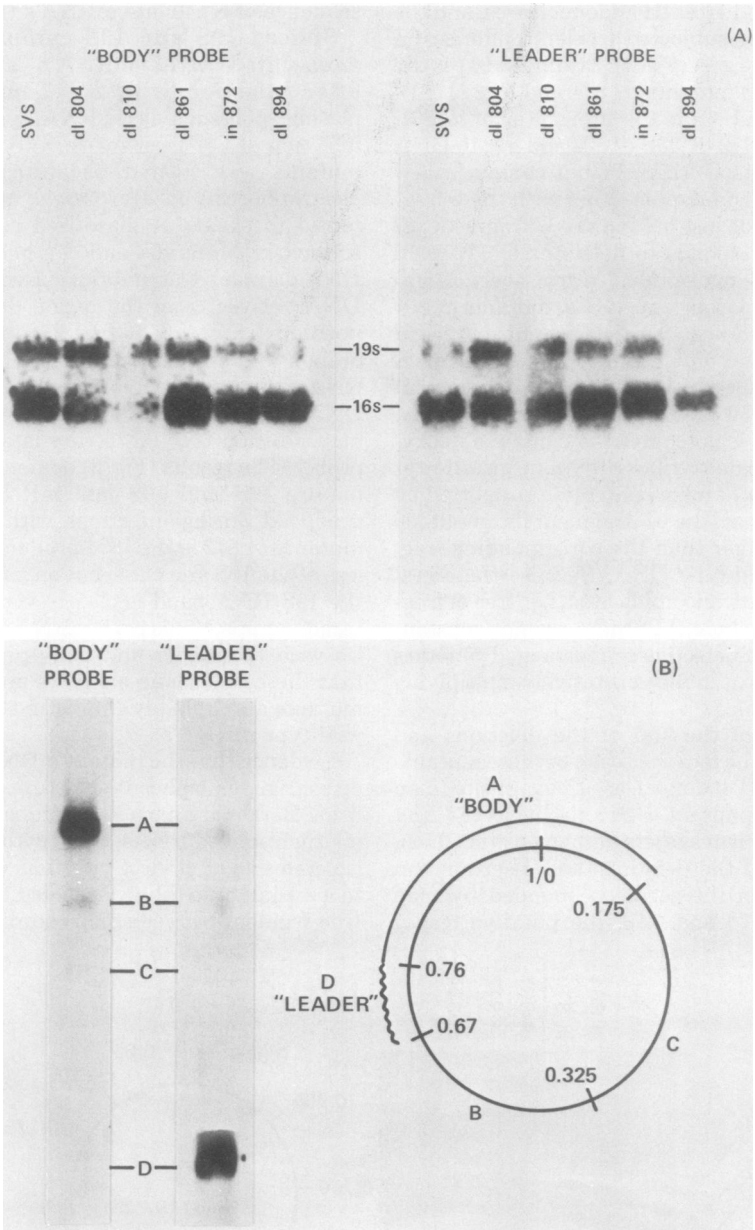

FIG. 2. *Analysis of viral RNAs synthesized late after infection with wild-type SV40 and mutants. (A) Cytoplasmic, polyadenylated RNA was denatured with glyoxal, duplicate samples were subjected to electrophoresis on 1.5% agarose gels, and each was transferred by blotting to sheets of diazotized cellulose as described in the text. One sheet was incubated with ^{32}P-labeled DNA homologous to the body of the mRNA's, and the other sheet was incubated with ^{32}P-labeled DNA corresponding to the leader sequence (see text). Autoradiograms were exposed for 4 h. (B) Wild-type SV40 DNA was digested with BglI and HpaI endonucleases, and the products were subjected to electrophoresis on 1.5% agarose gels (5 μg of DNA per track). The DNA was transferred by blotting to nitrocellulose as described in the text, hybridized to the ^{32}P-labeled body or leader DNA probes, and autoradiographed for 2 h.*

0.15), the hybrids were digested with S1 nuclease to degrade the unhybridized single-strand DNA, and the resistant DNA was subjected to electrophoresis in alkaline agarose gels. Figure 3 shows that hybridization with RNA isolated after infections with either wild-type or each of the mutant viruses generates two S1 nuclease-resistant segments, approximately 2 and 1.1 kb in length; there are also smaller bands, in the range of 0.2 kb and smaller, but these are not easily resolvable or visualized with this electrophoresis system and are dealt with subsequently. The preservation of the 2- and 1.1-kb DNA fragments with each of the RNA preparations is indicative of the presence of two RNAs, each homologous to only part of the labeled DNA probe. The 1.1-kb DNA fragment results from hybridization with 16S RNAs having a discontiguity between the RNA and DNA nucleotide sequence at about 0.94, thereby yielding a DNA fragment of about 1.1 kb (map position 0.94 to 0.15). Similarly, hybridization with the 19S RNA species reveals a discontiguity in the two nucleotide sequences at about map position 0.76, thereby yielding an S1 nuclease-resistant fragment of 2 kb (map position of 0.76 to 0.15). If a ^{32}P-labeled DNA fragment extending from map position 0.67 to 1.0 is used as the probe, the S1 nuclease-resistant fragments are only 1.25 and 0.3 kb in length (data not shown), establishing that the contiguous part of the RNAs maps to the distal end of the indicator fragment. Note that the mutant and wild-type RNAs are equally efficient in protecting the smaller fragment, i.e., they contain about equal quantities of the 16S RNA species. Similarly, the RNAs obtained after infection with mutants *dl* 804, *dl* 810, and *dl* 861 generate about the same amount of the longer fragment as does the wild-type RNA. However, there is considerably less of the longer fragment after hybridization with the RNA obtained from the infection with mutants *in* 872 and *dl* 894, although the larger fragment is visible on a longer exposure of these autoradiograms. This is consistent with the earlier conclusion that the RNA produced after infection with mutants *in* 872 and *dl* 894 has less of the 19S RNA, relative to the 16S RNA, than is found in wild-type infections.

Altered late RNAs are produced after infection with mutants having deletions and insertions in the leader segment. The data presented in the previous section show that the spliced late RNAs are produced after infection with mutants having deletions and insertions in the leader segment. However, the late RNAs generated by each of the mutants are distinguishable from those formed in wild-type infections, particularly in the nucleotide sequences included in the leader segment. This is demonstrated by a more detailed analysis of the structure of the late RNAs using Berk and Sharp's technique (4) for mapping the termini of RNAs and sizing the individual segments of spliced RNAs.

Cytoplasmic RNAs obtained 48 h after infection with wild-type or mutant viruses were hybridized to a ^{32}P-labeled DNA fragment (the segment between map coordinate 0.57 to 1.0), and the hybrids were digested with exonuclease VII, an enzyme which degrades the unhybridized DNA from the 5′ and 3′ ends exclusively (9). The products of the digestion (i.e., the protected ^{32}P-labeled DNA segments) were subjected to electrophoresis on an alkaline agarose gel and autoradiographed. Figure 4 shows that RNA obtained from infections with wild-type virus yields two classes of protected fragments, 1.62 and 1.45 kb in length. This is consistent with the existence of two major classes of late RNA, one whose 5′ ends extend to about map position 0.72, yielding the 1.45-kb protected DNA segments, and the other having 5′ ends at or about map position 0.69, to generate the 1.62-kb protected DNA fragment. Since the RNA from infections with mutant *dl* 894 is deficient in the 19S RNA species (see Fig. 2A and 3) and appears to produce little or none of the protected 1.62-kb DNA fragments, we tentatively identify the larger fragment as the one protected by the 19S mRNA class and the 1.45-kb fragment as the one that hybridized with the 16S mRNA class.

Comparing the results obtained with the wild-type and mutant RNAs reveals that mutants *dl* 804, *dl* 810, *dl* 861, and *in* 872 produce, predominately, RNAs with 5′ ends at or near map position 0.69. Mutant *dl* 810 also appears to make a small amount of another RNA, one indicated by the occurrence of a 1.28-kb protected DNA fragment; this indicates the existence of a minor RNA species whose 5′ terminus is at about map position 0.75. Since 16S mRNA's are made after infection with each of these mutants and wild-type 16S mRNA's have their 5′ ends at about map position 0.72 (11, 13), we conclude that the mutants produce 16S RNAs with altered 5′ ends. From this experiment we cannot determine whethere the 5′ ends of the 19S mRNA's made by the mutants are or are not altered by the mutations. The RNA obtained from infections with mutant *dl* 894 protects mainly the 1.45-kb DNA fragment, indicating that the 5′ end of most of the RNAs produced by this mutant occurs close to map position 0.72.

A more precise estimate of the map location of the 5′ termini of wild-type and mutant 19S

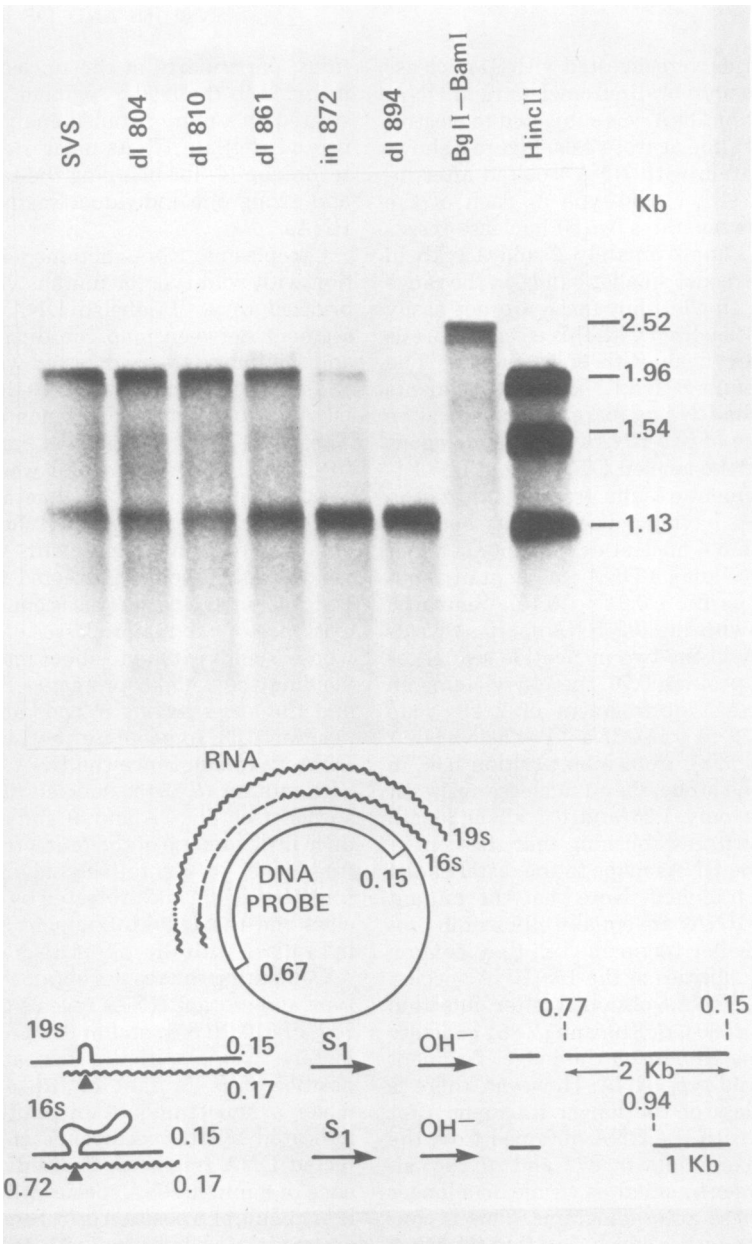

FIG. 3. *^{32}P-labeled SV40 DNA segment made resistant to S1 nuclease digestion after hybridization with cytoplasmic RNA made after infection with wild-type and mutant SV40. The ^{32}P-labeled DNA fragment, defined by map coordinates 0.67 and 0.15 (made by digestions with BglI and BamHI endonucleases), was annealed with cytoplasmic RNAs from cells infected with wild-type and mutant viruses, digested with S1 nuclease, incubated with alkali, and subjected to electrophoresis on a 1.5% alkaline agarose gel (see the text); autoradiograms were exposed for 12 h. The molecular length markers were obtained by digesting SV40 DNA with HincII endonuclease as well as the starting ^{32}P-labeled DNA fragment. The diagram below outlines features of the experimental protocol: the ^{32}P-labeled probe is shown as a single solid line, the putative cytoplasmic RNAs are indicated by wavy lines, the small triangle represents the position of the missing nucleotide sequence in the splice junction of RNAs, and the loops of solid line are the DNA sequences absent (spliced out) from the RNA. The products of the exonuclease VII and alkaline digestions are the DNA segments that define the 5′ and 3′ termini of the RNAs. In this experiment the protected DNA fragments represent the segments that comprise the leader and the body. Since the portions of the spliced RNA fragments corresponding to the leader segment are not well resolved or visualized under these electrophoresis conditions, that portion of the autoradiograms has not been reproduced here.*

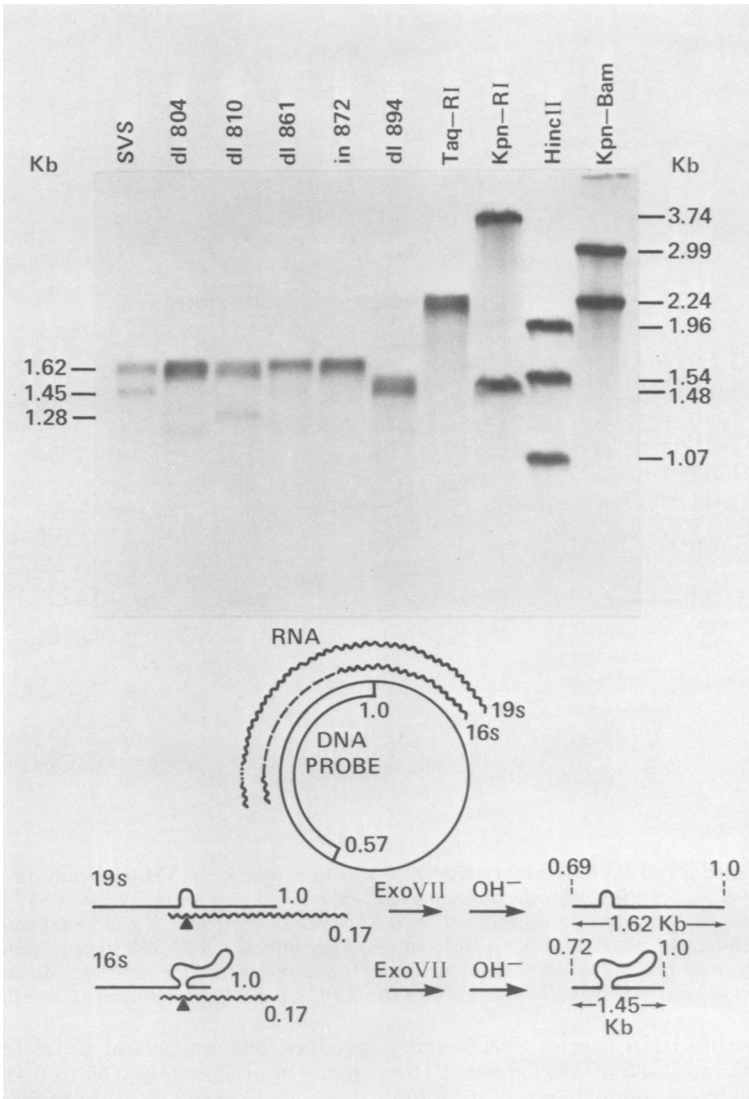

FIG. 4. *^{32}P-labeled SV40 DNA segment made resistant to exonuclease VII digestion after hybridization with cytoplasmic RNAs made after infection with wild-type and mutant SV40. A ^{32}P-labeled SV40 DNA fragment, defined by map coordinates 0.57 to 1.0 (made by TaqI and EcoRI endonuclease cleavages), was annealed with excess cytoplasmic RNA isolated 48 h after infection with wild-type and mutant virus. The annealed mixture was incubated with exonuclease VII, incubated with alkali (0.03 M), and subjected to electrophoresis on a 1.5% alkaline agarose gel as described in the text. The gels were neutralized, dried, and autoradiographed (12 h). The molecular length markers are generated from SV40 DNA by digestion with HincII, KpnI, and EcoRI, or TaqI and EcoRI endonucleases. The diagram below outlines features of the experimental protocol; see legend to Fig. 3 for explanation.*

mRNA's was obtained by repeating this analysis with a ^{32}P-labeled fragment extending from map coordinate 0.67 to 0.82; this DNA segment hybridizes preferentially to 19S RNAs, since it does not contain nucleotide sequences present in the 16S RNA body segment. Consequently, the experiment permits a measure of the length of that portion of the 19S RNA that maps from map coordinate 0.83 to the position of their 5′ ends.

Figure 5 shows the autoradiogram of the result with wild-type and mutant late cytoplasmic RNAs. The uppermost band of each track is the full-length segment (0.82 kb) used as the probe. With RNA from wild-type virus infections there are a closely spaced pair of fragments of 0.70 and 0.645 kb and another pair of 0.575 and 0.515 kb.

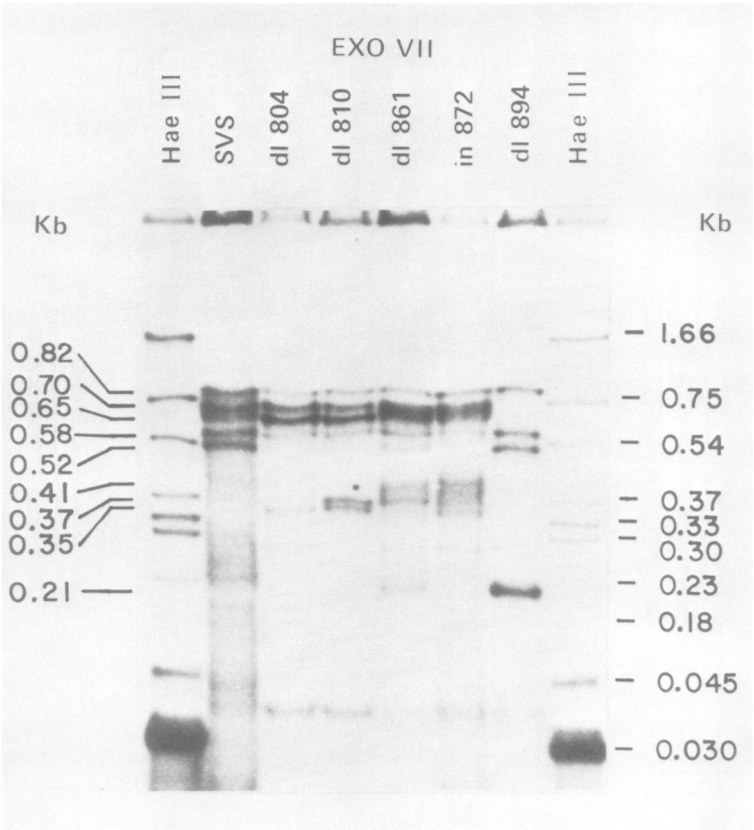

FIG. 5. *^{32}P-labeled SV40 DNA segment made resistant to exonuclease VII digestion after hybridization with cytoplasmic RNAs made after infection with wild-type SV40 and mutant viruses. A ^{32}P-labeled SV40 DNA fragment, defined by map coordinates 0.67 to 0.82 (made by BglI and HaeII endonuclease cleavages), was annealed with excess cytoplasmic RNA isolated 48 h after infection with wild-type and mutant virus. The annealed mixture was incubated with exonuclease VII, denatured with glyoxal (1 M), and subjected to electrophoresis on a 3 to 7% polyacrylamide gel (see the text); autoradiograms were exposed for 40 h.*

These represent 19S RNA species with 5′ ends that map to positions 0.685 to 0.695 and 0.710 to 0.720, respectively. A faint band of 0.20-kb length is also observed during a longer exposure of the autoradiogram. This band probably corresponds to the DNA fragment protected by hybridization to the 16S RNA leader. Its relatively low amount is due to the conditions of hybridization which favor the more stable hybridization of the longer 19S RNA with the DNA probe. The RNA obtained from infections with *dl* 804, *dl* 810, and *in* 872 contains predominately the pair of RNAs extending to 0.686–0.695 and some to 0.720. Mutant *dl* 861 RNA contains the 5′ termini characteristic of wild-type 19S RNA, whereas *dl* 894 RNA contains only 19S RNA species with 5′ ends extending to 0.710–0.720. There are also other notable features of the experimental result: (i) RNA from infections with mutants *dl* 804, *dl* 810, *dl* 861, and *in* 872 produce one or several protected DNA fragments approximately 0.35 to 0.41 kb in length; these could result from hybridization of a 16S mRNA leader segment to the portion of the labeled DNA mapping between 0.76 and 0.70 to 0.68. (ii) Mutant *dl* 894 RNA protects principally a DNA fragment of about 0.22 kb, probably as a result of hybridization between the leader segment of its 16S RNA and the probe; this seems reasonable in light of the paucity of 19S RNAs produced after infection with this mutant. (iii) There are a large number of discrete smaller DNA bands that are protected by the hybridizations with the mutant and not with wild-type RNAs, but their origin is difficult to decipher.

SV40 mutants produce late mRNA's with altered leader segments. The experiments described thus far show that the length and structure of the 19S and 16S mRNA body segments produced in the infections with wild-type and

mutant viruses are indistinguishable; all of the changes occur in the leader segment.

To compare the wild-type and mutant leader segments more closely, the late cytoplasmic RNA was hybridized to a 1.67-kb ^{32}P-labeled DNA fragment (defined by map position 0.67 to 1.0), and, after digestion of the hybrids with S1 nuclease, the products were denatured with glyoxal and subjected to electrophoresis on a gradient polyacrylamide gel; these conditions resolve the small fragments derived from the leader segments (Fig. 6). The wild-type and mutant RNA samples each protect DNA fragments of 1.25 and 0.30 kb; these correspond to the body portion of the 19S and 16S mRNA species, respectively. The wild-type RNA produces, in addition to the 1.25- and 0.3-kb fragments, a fragment of 0.20 kb and three minor fragments ranging in size from about 0.04 to 0.15 kb. The 0.20-kb fragment is probably the leader segment of the major 16S mRNA (11, 13), and the others are the leader segments of minor 16S mRNA's and the 19S mRNA species. Except for mutant *dl* 894, none of the RNAs contains leader segments corresponding to the major 16S RNA species. Instead, each generates several small fragments resembling, but distinct from, the putative 19S mRNA leader segments. The RNA obtained from infections with mutant *dl* 861 contains a predominant leader segment of about 0.17 kb and two smaller ones about 0.035 to 0.045 kb in length. The pattern produced with mutant *dl* 894 RNA is consistent with the existence of a 16S mRNA-like leader and no fragments diagnostic of 19S mRNA's.

Although considerably more work is needed to assign the different leader segments to specific classes of wild-type and mutant late mRNA's, our experiments demonstrate clearly that deletions and insertions within the leader region do not block the production of spliced 16S and 19S mRNA's, but they do alter the location of the splices and the lengths of the leader segments.

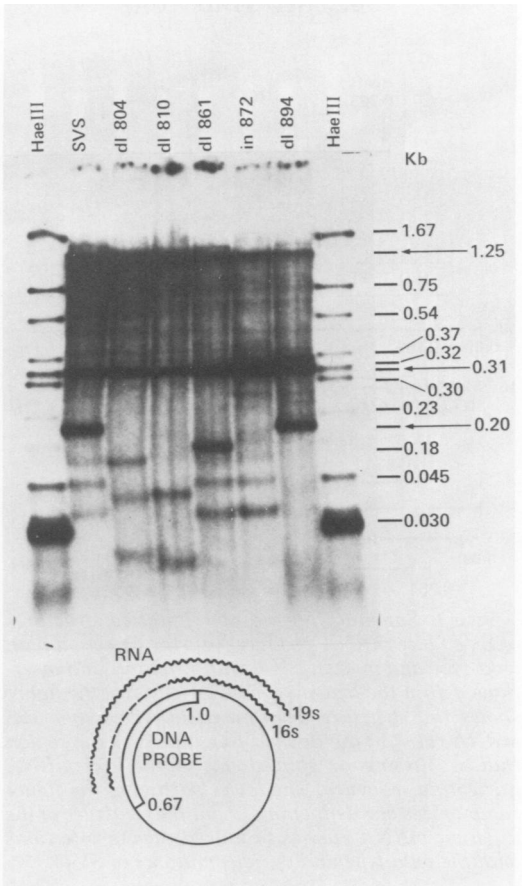

FIG. 6. *Analysis of the size of the leader segments of cytoplasmic RNAs produced after infection with wild-type and mutant SV40. The ^{32}P-labeled DNA fragment, defined by map coordinates 0.67 to 1.0 (made by digestion with BglI and EcoRI endonuclease digestions), was annealed with excess cytoplasmic RNAs as described in the legend to Fig. 3. The annealed mixtures were digested with S1 nuclease, denatured with glyoxal, and subjected to electrophoresis on a 3 to 7% gradient polyacrylamide gel (see the text); autoradiograms were exposed for 94 h. The molecular length markers are the digestion products of SV40 DNA with HaeIII endonuclease.*

DISCUSSION

Recently, it was shown that the late mRNA's of SV40 are composite molecules containing nucleotides transcribed from at least two noncontiguous DNA segments (2, 5, 8, 11, 13, 15, 16). This raises several interesting questions about the formation and function of what has been termed spliced mRNA's (13). Is it the splicing event or the spliced RNA per se that is essential for late gene expression? Which nucleotides in the sequence are essential for generating the properly spliced mRNA's? What function does the leader have, and how critical are the nucleotides comprising the 5′ and 3′ termini and the internal portions of the 19S and 16S mRNA leader segments for that function?

One approach to answering these and other questions makes use of virus mutants, particularly those whose nucleotide sequence within and adjacent to the leader and body segments of the mRNA's are altered. For example, the 5′ end of the major late 16S mRNA (presumably bearing the "cap" structure [1]) has been located at map coordinate 0.72, between the *Kpn*I and *Hpa*II endonuclease cleavage sites, and the 3′ end of the leader segment has been located at

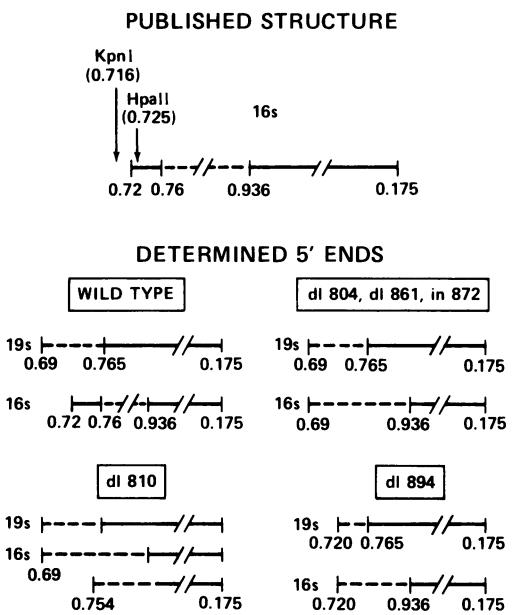

FIG. 7. *Summary of findings. Putative structures of SV40 late mRNA produced during infection with wild-type and mutant SV40 with deletions and insertions within the leader segment. The top of the figure shows the structure determined for the major 16S mRNA (11, 13); the dashed line indicates the region that is missing or spliced out of the 16S mRNA nucleotide sequence. The lower portion of the figure summarizes our deductions about the structure of the different mRNA classes produced during infections with the wild type and various mutants of SV40.*

map coordinate 0.76 (11, 13) (see top of Fig. 7). Yet a mutant exists which lacks the nucleotides specifying the 5′ end of the major late 16S mRNA. Mutant *dl* 810 lacks both the *Kpn*I and *Hpa*II endonuclease cleavage sites, but still synthesizes VP1 and propagates without a helper virus (21). Similarly, there are several mutants with deletions that eliminate the 3′ end of the late mRNA leader segment (map coordinate 0.76) (M. Dieckmann, R. Mulligan, S. P. Goff, and P. Berg, unpublished data); these, however, fail to make any of the late viral proteins after infection (10; Dieckmann et al., unpublished data) because of an inability to generate stable cytoplasmic 16S and 19S mRNA's from the late nuclear transcripts (R. T. White and L. P. Villarreal, unpublished data).

In the present work we have compared the structure of the late cytoplasmic RNAs made after infection with wild-type SV40 and a set of viable SV40 mutants, four of which have deletions and one an insertion within the nucleotide sequence specifying the leader segment of the 19S and 16S mRNA's. Several particularly striking results were noted. (i) The leader segments of wild-type SV40 16S and 19S mRNA's are heterogeneous with respect to the map location of their 5′ ends and possibly in the nucleotide sequence comprising the leader segments. (ii) Spliced 16S and 19S mRNA's are made after infection with each of the mutants, but in two cases (i.e., *in* 872 and *dl* 894) the ratio of 19S to 16S mRNA is reduced. (iii) The elimination or introduction of nucleotides within map positions 0.70 and 0.75 strikingly alters the types of structures found in the leader segments of the late mRNA's (see Fig. 7 for summary). For example, the late RNAs made after infection with mutants *dl* 804, *dl* 861, *dl* 810, and *in* 872 have their 5′ termini at or about map position 0.69 and lack the major species with a 5′ end at map coordinate 0.72; conversely, after infection with *dl* 872, very few of the late RNAs have 5′ ends mapping to 0.69, the preferred terminus being map position 0.72. (iv) Many of the late RNA leader segments produced after infections with the mutants appear to be multiply spliced (except for *dl* 894), since the major 200- to 205-nucleotide-long leader segment characteristic of the wild-type 16S mRNA's is replaced by leaders with several short discontinuous segments.

The failure of relatively large deletions or an insertion within the leader nucleotide sequence of the 16S and 19S mRNA's to render the mutants nonviable suggests that a precise structure for the mRNA leader sequences is not critical for its function. Alternatively, there may be a multiplicity of spliced structures, each capable of fulfilling the others' functions, and mutational alterations merely change the relative proportions of the different spliced molecular species.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants GM-13235, GM-13212, and GM-13213 from the National Institute of General Medical Sciences and by grant VC 23-D from the American Cancer Society. L.P.V. was supported by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

LITERATURE CITED

1. **Aloni, Y.** 1975. Methylated SV40 mRNAs. FEBS Lett. **54**:363–367.
2. **Aloni, Y., R. Dhar, O. Laub, M. Horowitz, and G. Khoury.** 1977. Novel mechanism for RNA maturation: the leader sequences of simian virus 40 mRNA are not transcribed adjacent to the coding sequences. Proc. Natl. Acad. Sci. U.S.A. **74**:3686–3690.
3. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
4. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**: 1274–1278.
5. **Bratosin, S., M. Horowitz, O. Laub, and Y. Aloni.** 1978. Electron microscopic evidence for splicing of SV40

late mRNAs. Cell **13**:783-790.
6. **Carbon, J., T. E. Shenk, and P. Berg.** 1975. Construction *in vitro* of mutants of simian virus 40: insertion of a poly (dA·dT) segment at the *Hemophilis parainfluenza* II restriction endonuclease cleavage site. J. Mol. Biol. **98**:1-15.
7. **Casey, J. W., and N. Davidson.** 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. Nucleic Acids Res. **4**:1539-1552.
8. **Celma, M. L., R. Dhar, and S. M. Weissman.** 1977. Comparison of the nucleotide sequence of the messenger RNA for the major structural proteins of SV40 with the DNA sequence encoding the amino acids of the protein. Nucleic Acids Res. **4**:2549-2559.
9. **Chase, J. W., and C. C. Richardson.** 1974. Exonuclease VII of *Escherichia coli*. J. Biol. Chem. **249**:4553-4561.
10. **Cole, C. N., T. Landers, S. P. Goff, S. Manteuil-Brutlag, and P. Berg.** 1977. Physical and genetic characterization of deletion mutants of simian virus 40 constructed in vitro. J. Virol. **24**:277-294.
11. **Ghosh, P. K., V. B. Reddy, J. Swinscoe, P. Choudary, P. Lebowitz, and S. M. Weissman.** 1978. The 5'-terminal leader sequence of late 16S mRNA from cells infected with simian virus 40. J. Biol. Chem. **253**: 3643-3647.
12. **Goff, S. P., and P. Berg.** 1978. The excision of DNA segments introduced into cloning vectors by the poly(dA:dT) joining method. Proc. Natl. Acad. Sci. U.S.A. **75**:1763-1767.
13. **Haegerman, G., and W. Fiers.** 1978. Evidence for 'splicing' of SV40 16S mRNA. Nature (London) **273**:70-73.
14. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365-369.
15. **Hsu, M.-T., and J. Ford.** 1977. Sequence arrangement of the 5' ends of simian virus 40 16S and 19S mRNAs. Proc. Natl. Acad. Sci. U.S.A. **74**:3686-3690.
16. **Lavi, S., and Y. Groner.** 1977. 5'-Terminal sequences and coding region of late simian virus 40 mRNAs are derived from non-contiguous segments of the viral genome. Proc. Natl. Acad. Sci. U.S.A. **74**:5323-5327.
17. **Lindberg, U., and T. Perrsson.** 1974. Messenger RNA. Isolation with poly(U) agarose. Methods Enzymol. **34**: 496-499.
18. **McDonell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119-146.
19. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835-4838.
20. **Mertz, J., and P. Berg.** 1974. Defective SV40 genomes: isolation and growth of individual clones. Virology **62**: 112-124.
21. **Mertz, J., and P. Berg.** 1974. Viable deletion mutants of SV40: selective isolation using *Hpa* II restriction endonuclease. Proc. Natl. Acad. Sci. U.S.A. **71**:4879-4883.
22. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237-251.
23. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664-671.
24. **Shenk, T. E., C. Rhodes, P. Rigby, and P. Berg.** 1975. Biochemical method for mapping mutational alterations in DNA with S1 nuclease: the location of deletions and temperature-sensitive mutations in SV40. Proc. Natl. Acad. Sci. U.S.A. **72**:989-993.
25. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.
26. **Zimmerman, S. B., and G. Sandeen.** 1966. The ribonuclease activity of crystallized pancreatic deoxyribonuclease. Anal. Biochem. **14**:269-277.