JOURNAL OF VIROLOGY, Apr. 1986, p. 165–172
0022-538X/86/040165-08$02.00/0

Vol. 58, No. 1

# Spontaneous Deletion Mutants Resulting from a Frameshift Insertion in the Simian Virus 40 Agnogene

SHIGEKO NOMURA, GILBERT JAY, AND GEORGE KHOURY*

*Laboratory of Molecular Virology, National Cancer Institute, Bethesda, Maryland 20892*

Received 20 August 1985/Accepted 13 December 1985

**The 61-amino-acid agnoprotein is a nonessential polypeptide encoded by the late leader region of simian virus 40 which appears to play a role in viral assembly. A 2-base-pair (bp) insertion mutant (in2379) was created by altering the coding region of this protein. This mutation prevents the synthesis of the agnoprotein and, in contrast to the more extensive deletion mutations previously described in this region, might be expected to have a lesser effect on the template for late viral transcription. In fact, the 2-bp insertion mutant grew significantly less well than most mutants containing larger deletions in the agnoprotein region and frequently gave rise to stock containing second-site alterations in the same region. These observations suggested that the defect in mutant in2379 extends beyond the loss of the agnoprotein. Characterization of a number of second-site mutants indicated that all of them grew more efficiently than the original 2-bp insertion mutant. Based on the nucleotide sequence of these mutants, we suggest possibilities for the deleterious effect induced by the insertion in mutant in2379.**

In addition to the three viral structural proteins, VP-1, VP-2, and VP-3, the late region of simian virus 40 (SV40) encodes in its leader region a 61-amino-acid, highly basic polypeptide which has been termed the agnoprotein (7, 16, 17, 26, 28). Several viable deletion mutants with mutations in the agnogene coding sequence have been characterized, and the results are consistent with this protein being nonessential for viral growth in cell cultures (4, 5, 12, 13, 24, 25, 33, 35, 37). Nevertheless, most of these mutants grow with decreased efficiency and produce plaques of altered morphology. In addition, there is a curious lack of correlation between the size and position of the late leader deletion and the relative growth rate of the mutant (2, 33).

There is now evidence from both genetic (21) and biochemical (27) studies to suggest that the agnoprotein plays a role late in the lytic cycle, perhaps in the assembly of virions. In addition, based on its DNA binding properties (17) as well as some of the characteristics of the deletion mutants, there is speculation that the agnoprotein may also directly affect the synthesis or processing, or both, of late mRNA (1, 14).

Due to the complexity and overlapping structure of the SV40 control sequences and the ill-defined nature of the late transcriptional signals, it has been difficult to ascribe the defects which result from the various mutations in the agnogene either to the loss of the agnoprotein or to alterations in either the template or control signals for late mRNA synthesis. In the present study, we attempted to construct a simple 2-bp insertion mutation which would prevent production of the normal agnoprotein and yet, presumably, represent a minimal alteration in the late transcriptional template. The generation and analysis of the properties of the 2-bp insertion mutant are described below.

## MATERIALS AND METHODS

**Cells and viruses.** Wild-type SV40 strain 776 and derived SV40 mutants were grown in either primary AGMK cells (M.A. Bioproducts; Flow Laboratories, Inc.) or the established AGMK cell line, CV-1. Cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS) and maintained in Eagle minimal essential medium with 2% FCS after infection. Transfection and plaquing experiments were carried out in CV-1P cells (24) which were grown and maintained in Dulbecco modified Eagle medium supplemented with 5% FCS. Media and sera were purchased from Meloy Laboratories, Inc. and GIBCO Laboratories, respectively.

**DNA transfection.** Twenty-four hours before transfection, CV-1P cells were seeded at $3 \times 10^4$ cells per microwell (Falcon MicroTest II Tissue Culture Plates). Cultures were refed 4 h before transfection. Transfections were performed by the DEAE-dextran method (DEAE-dextran molecular weight $2 \times 10^6$; Pharmacia) (23) using a modification of the method of Brockman and Nathans (4). DNA concentrations were determined by optical density readings and by photographic comparisons with the known concentrations of SV40 DNA in agarose gel electrophoresis after ethidium bromide staining. Cultures were washed once with prewarmed Dulbecco phosphate-buffered saline and then incubated with transfection mix, consisting of 3.2 to 32 ng of DNA, 30 μl of DEAE-dextran (2 mg/ml in minimal essential medium), 15 μl of 1 M Tris hydrochloride (pH 7.5), and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in a final volume of 0.3 ml. After 60 min at 37°C, the inoculum was removed and cells were trypsinized and transferred to 60-mm plates containing 2 ml of prewarmed Dulbecco modified Eagle medium with 5% FCS. Plates were incubated at 37°C for 2 to 3 h to permit attachment, and then $10^6$ CV-1P cells in 3 ml of medium were added to each plate. After 24 h of incubation at 37°C, medium was replaced with 5 ml of agar overlay containing 0.9% agar (Agar-Noble; Difco Laboratories) and 5% FCS in minimal essential medium. Thereafter, 2 ml of agar overlay was added to each plate every 3 days. Cultures were stained with neutral red at 10 to 13 days posttransfection to identify plaques.

**Analysis of DNA from plaques.** Well-separated plaques were picked and suspended with a Pasteur pipette in 1 ml of minimal essential medium. The suspension was heated at

* Corresponding author.

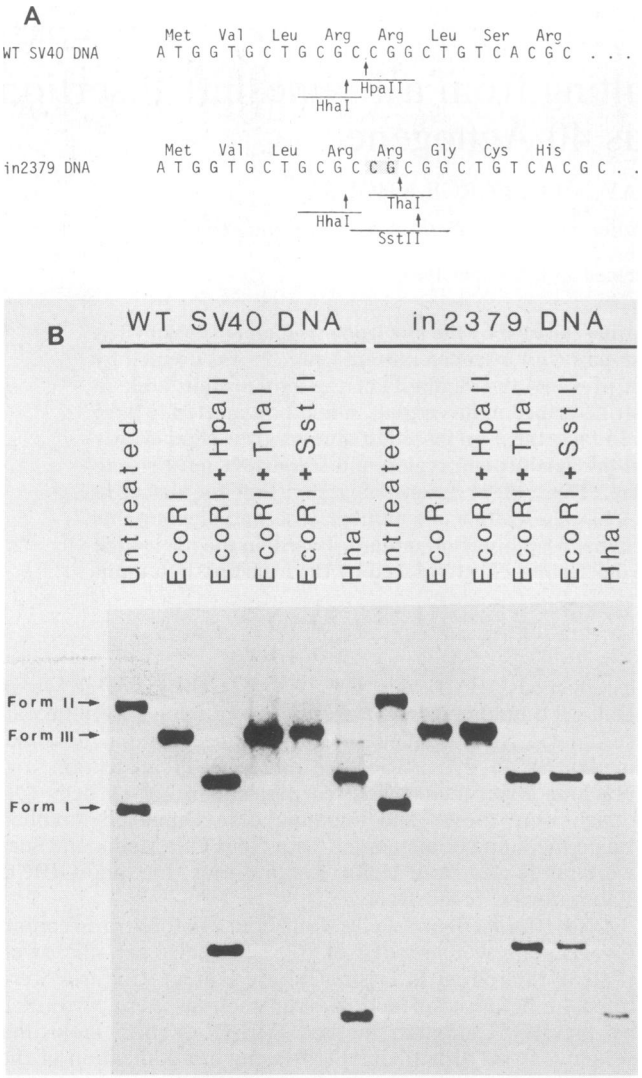


FIG. 1. Construction and analysis of SV40 mutant in2379. (A) Diagram of changes in the translational reading frame and restriction endonuclease recognition sites resulting from a 2-bp (CG) insertion at the *Hpa*II site (nt 346) in mutant in2379. (B) Restriction endonuclease analysis of wild-type and mutant in2379 DNA. Wild-type and mutant in2379 SV40 DNAs were digested by *Eco*RI alone, by *Eco*RI plus *Hpa*II, *Sst*II, or *Tha*I, or by *Hha*I alone. DNA digests were electrophoresed in a 1.4% agarose gel. (Forms I, II, and III represent the positions of migration of supercoiled, nicked circular, and linear SV40 DNAs, respectively.)

68°C for 10 min with 1% sodium dodecyl sulfate and 1 mM EDTA, extracted with phenol and chloroform, and ethanol precipitated. Pellets were suspended in 25 μl of 10 mM Tris hydrochloride (pH 7.5) plus 1 mM EDTA, and 5-μl samples were subjected to digestion with restriction endonucleases *Bam*HI and *Sst*II. To confirm the completion of digestion, pBR322 DNA and φX174 RF I DNA (New England Biolabs), which were cleaved once with *Bam*HI and *Sst*II, respectively, were included in each reaction. DNA digests were electrophoresed in a 1.4% agarose gel, checked under a UV light to confirm that the control pBR322 and φX174 DNA had been cleaved, transferred to a nitrocellulose membrane (34), and hybridized with a $^{32}$P-labeled probe prepared by nick-translation of SV40 DNA (31).

**Mutants.** To introduce a frameshift mutation, wild-type SV40 DNA (form I) was cleaved at the unique *Hpa*II site (nucleotide [nt] 346). Nucleotide numbering follows the BBB system (36). Repair of the 2-base sticky ends with DNA polymerase I was followed by blunt-end ligation with T4 DNA ligase (20). A mutant virus stock, in2379, was prepared in AGMK cells from one of the plaques produced by transfection of AGMK cells with the altered DNA.

**Recombinant DNAs.** The plasmid construct in2379p was obtained by inserting the DNA of in2379 into pBR322 at the *Bam*HI site; this recombinant plasmid was introduced into *Escherichia coli* HB101 by the $CaCl_2$ method (18). Clones containing mutant DNA were analyzed by restriction endonucleases (*Sst*II from Bethesda Research Laboratories, Inc. and the others from New England Biolabs) and by DNA sequencing. For large-scale preparation, the recombinant DNA was amplified in *E. coli* with chloramphenicol and was purified from bacterial cell lysates by two consecutive equilibrium centrifugations in cesium chloride-ethidium bromide (20).

For reconstruction of in2379r, the DNAs of wild-type

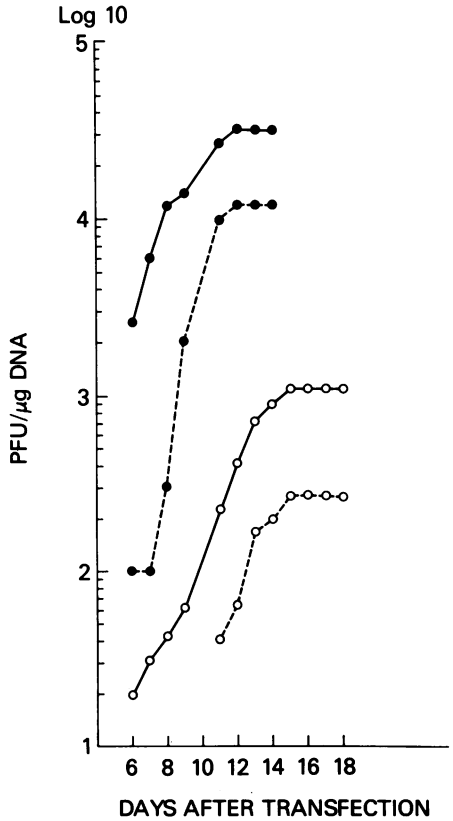


FIG. 2. Viral growth curves after DNA transfection. Plasmid pBR322 sequences were excised from the recombinant in2379p DNA by *Bam*HI digestion, and the SV40 segment was recovered from a 1.4% agarose gel in linear form. Wild-type 776 form I DNA was similarly treated to isolate it as a linear form. A portion of both DNAs was subjected to recircularization by T4 DNA ligase with efficiencies greater than 50%. CV-1P cells were transfected with wild-type or in2379 DNA, as either circular or linear molecules, using the DEAE-dextran technique as described in Materials and Methods. Cultures were stained on day 10, and plaques were scored from day 6 to day 17. Symbols: ●——●, wild-type SV40, circularized DNA; ●- - -●, wild-type SV40, linear DNA; ○——○, in2379, circularized DNA; ○- -○, in2379, linear DNA.

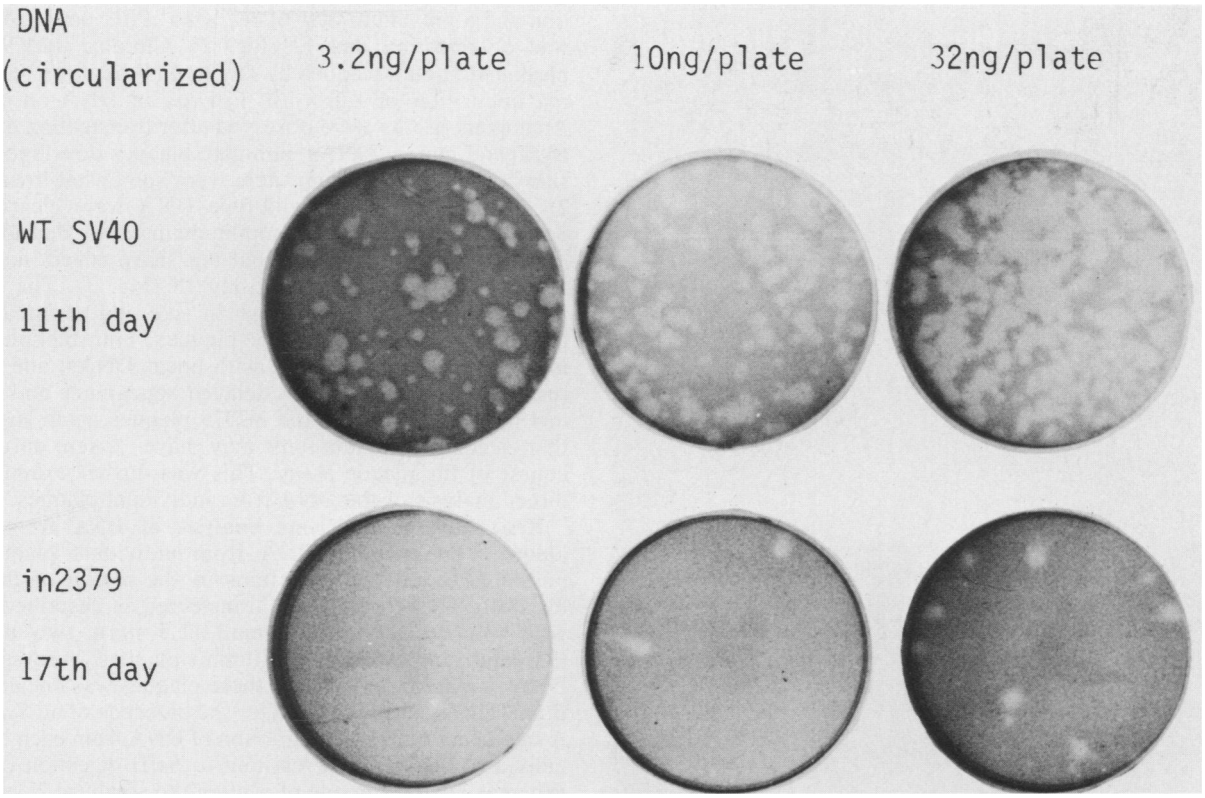


FIG. 3. Morphology of plaques formed after transfection. CV-1P cells were transfected with varying concentrations of either circularized wild-type or mutant in2379 DNA. Transfections were performed as described in Materials and Methods.

SV40 and in2379p were digested with restriction endonucleases *Kpn*I and *Eco*RV at SV40 nt 294 and 768, respectively. The small fragment (475 bp) from in2379p and the large fragment (4,768 bp) from wild-type SV40 were recovered by polyacrylamide and agarose gel electrophoresis, respectively. The large wild-type SV40 fragment was dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals) and joined to the smaller in2379p fragment by overnight incubation at 15°C in a 10-μl reaction mixture containing 2 U of T4 DNA ligase (Collaborative Research, Inc.). The resulting reconstructed in2379r DNA was inserted into pBR322 at the *Bam*HI site and used to transform *E. coli* LE392.

In this report, the terms in2379 and in2379r DNA indicate the complete SV40 genomic DNA excised from vector pBR322. Recombinants in2379p or in2379rp are plasmid DNA with the SV40 insert.

**DNA sequence analysis.** DNA sequencing was performed by the method of Maxam and Gilbert (22). Cloned DNAs were treated with *Dde*I endonuclease. A 435-bp fragment (nt 287 to 722) was isolated by agarose gel electrophoresis followed by 3′-end labeling with 10 U of DNA polymerase I (Klenow enzyme; Boehringer Mannheim Biochemicals) and [α-$^{32}$P]dTTP (New England Nuclear Corp.). The labeled ends were separated by a second cleavage with *Hpa*I endonuclease at SV40 nt 501. The 214-bp fragment (nt 287 to 501) was isolated by polyacrylamide gel electrophoresis and elution (22). Single end-labeled fragments were sequenced on 0.4-mm gels (32) of 8% polyacrylamide with 7 M urea or of 20% polyacrylamide with 8 M urea and on gradient gels as described by Biggin et al. (3). Gels were subjected to autoradiography at −70°C.

## RESULTS

**Construction and analysis of an agnogene mutant.** To produce a minimal mutation in the agnogene coding sequence, two nucleotide pairs (CG) were inserted at the unique *Hpa*II site in SV40 DNA as described in Materials and Methods (Fig. 1A). This insertion mutant (in2379) potentially encodes a 33-amino acid polypeptide of which only the first 5 amino acids are common to the wild-type agnogene product. The insertion both destroys the *Hpa*II site and creates overlapping cleavage sites for restriction enzymes *Tha*I and *Sst*II, which allows one to easily distinguish the mutant in2379 DNA from wild-type DNA (Fig. 1A). Digestion of either wild-type SV40 DNA or the insertion mutant DNA with *Eco*RI plus either *Hpa*II, *Tha*I, or *Sst*II, or digestion with *Hha*I alone, results in the expected cleavage patterns (Fig. 1B). This confirms the structure of the 2-bp insertion mutant.

**Growth of in2379 in monkey kidney cells.** In preliminary experiments, CV-1 cells were transfected with circularized in2379 DNA (from which the plasmid sequences had been excised), and the resulting plaques were isolated. Viruses from several plaques were individually propagated in CV-1 cells, and the growth characteristics of one such mutant virus stock were studied. Cultures of CV-1 cells were infected with either wild-type SV40 or the mutant virus in2379 stock at a multiplicity of 20 PFU per cell. Whereas the cytopathic effect appeared at 48 h in wild-type SV40-infected cells, it was not observed until 72 h postinfection with the mutant virus. The SV40 agnoprotein was detected by 48 h, using polyacrylamide gel electrophoresis, only in those cultures infected with the wild-type virus. The putative

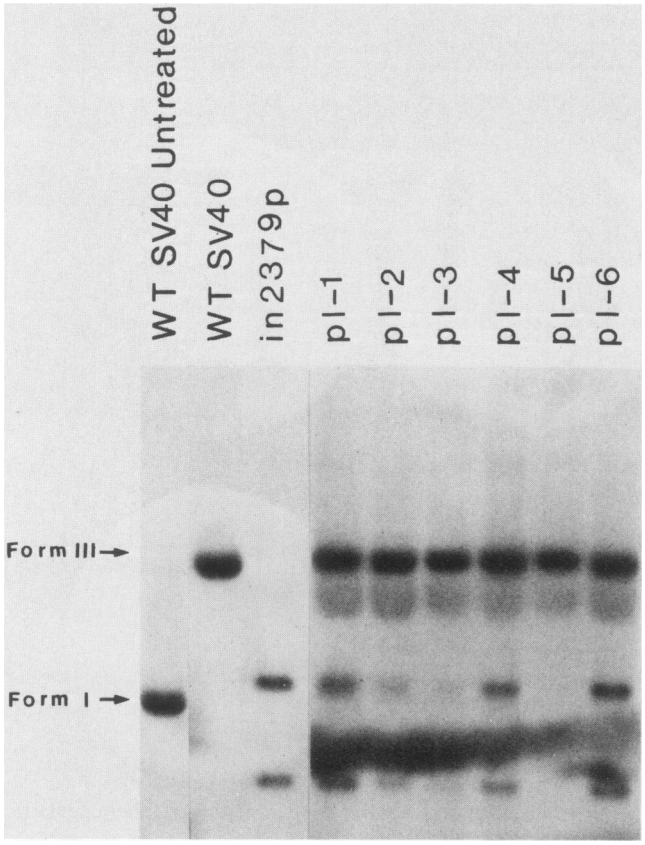


FIG. 4. Restriction endonuclease analysis of in2379 DNA plaques. DNAs were prepared from six individual plaques resulting from transfection of CV-1P cells with circular in2379 DNA; one-fifth of the total DNA from each plaque was employed for digestion with *Bam*HI and *Sst*II (lanes 4 through 9). To assure completeness of digestion, each digest included unlabeled φX174 RF I DNA and pBR322 DNA, which were cleaved once by *Sst*II and *Bam*HI, respectively. Also shown are uncut (form I) wild-type SV40 DNA (lane 1), linear (form III) wild-type SV40 DNA (lane 2), and recombinant in2379p DNA cleaved with *Sst*II and *Bam*HI (lane 3).

33-amino acid polypeptide encoded by the agnogene region of in2379 was not observed under similar conditions (data not presented); we assume that this polypeptide either is produced at subdetectable levels or is unstable. After the mutant virus stock was passaged twice in AGMK cells, a significant portion of the viral DNA was found to be resistant to digestion by both restriction enzymes *Hpa*II and *Sst*II. This suggested that second-site mutations were occurring at reasonably high frequency in the region of the original 2-bp insertion. To further investigate this observation and to exclude the possibility of artifacts which might arise from the initial passage of the virus stock, we repeated these experiments starting with a plasmid clone with SV40 in2379 DNA inserted at the *Bam*HI site of pBR322 (in2379p).

**Plaque formation by wild-type and mutant SV40 DNAs.** CV-1P cells were transfected in the presence of DEAE-dextran with three different concentrations of wild-type SV40 or in2379p DNA. The viral DNA molecules were cleaved from pBR322 by *Bam*HI restriction enzyme and either transfected as linear molecules or circularized before transfection (see Fig. 2, legend). With circular wild-type SV40 DNA, plaques were observed by day 5 posttransfection and a maximum titer of $3.2 \times 10^4$ PFU per μg of DNA was obtained by day 12 (Fig. 2). Circular in2379 DNA produced minute plaques by day 6, and the virus reached a maximum titer of $1.1 \times 10^3$ PFU/μg of DNA on day 15. Analogous results were observed after transfection of linear SV40 and mutant DNAs, although plaques developed more slowly and the maximum titers were somewhat lower (Fig. 2). Plaques formed by wild-type DNA were clear, sharp edged, and greater than 12 mm in diameter by day 18. Most mutant plaques were clear, but not sharp edged, and were about 5 mm in diameter by day 18 (Fig. 3). The mutant plaques continued to increase in size and eventually approached the size of wild-type plaques. Transfection experiments were repeated twice with linear DNAs, and similar results were obtained. This delayed appearance and continued increase in size of the in2379 plaques again suggested that second-site mutations may have arisen during the course of the plaque assay. This was further examined by direct analysis of the DNA from individual plaques.

**Restriction endonuclease analysis of DNA from single plaques.** To examine DNA from individual plaques for possible second-site alterations in the region of the 2-bp insertion, CV-1P cells were transfected as described above with in2379 DNA; two small (1.5-mm), two medium (3.0-mm), and two large (5.0-mm) plaques were selected. DNA prepared from each of these plaques was linearized by *Bam*HI digestion and tested for the presence of an *Sst*II site. A significant but variable fraction of DNA from each of these individual plaques was resistant to *Sst*II digestion (Fig. 4). Whereas DNA from one of each of the small (pl-2), medium (pl-3), and large (pl-5) plaques was largely resistant to *Sst*II cleavage, about 50% of the DNA from the other plaques (pl-1, pl-4, and pl-6) remained sensitive to *Sst*II. The rapid development of second-site alterations in this region, as indicated by *Sst*II resistance, suggested a selection against the original mutant in2379.

**Nucleotide sequence of in2379 variants.** To determine the nature of the second-site alterations, virus from one of the plaques, pl-1, was amplified in primary AGMK cells. Form I viral DNA was prepared by the Hirt procedure (15) and was cloned into pBR322 at the *Bam*HI site for sequence analysis. Of 30 clones with SV40 inserts, 7 were found to have DNA resistant to *Sst*II cleavage. Three *Sst*II-resistant clones (cl 1-5, cl 1-22, and cl 1-24) and two *Sst*II-sensitive clones (cl 1-14, cl 1-21) were examined further. All of these clones contained SV40 DNA which was resistant to *Hpa*II cleavage and thus did not represent wild-type DNA contamination or reversion to the wild-type SV40 sequence. Comparison of the restriction enzyme cleavage pattern of parental in2379 DNA with DNAs from the five clones suggested that a deletion had occurred in the region of the original 2-bp insertion as a second-site alteration in each of the clones. To determine the specific change, the nucleotide sequence of each clone in the region of the original *Hpa*II site was determined by the method of Maxam and Gilbert. DNA from in2379p and from each of the five clones was digested with *Dde*I, and the 3′ ends of a fragment from SV40 nt 287 to 722 were $^{32}$P labeled as described in Materials and Methods. After *Hpa*I cleavage, the SV40 fragments from nt 287 to 501 were purified and sequenced (Fig. 5).

The positions of the deletions in cl 1-5 and cl 1-22 DNAs are identical, from nt 318 to 368 (51 bp); in cl 1-24 the position of the deletion is from nt 297 to 365 (69 bp). The sequences deleted in these clones include the SV40 major late RNA cap site (9, 10, 12, 29) and the translation initiation codon for the agnoprotein. Two deletions were present in cl

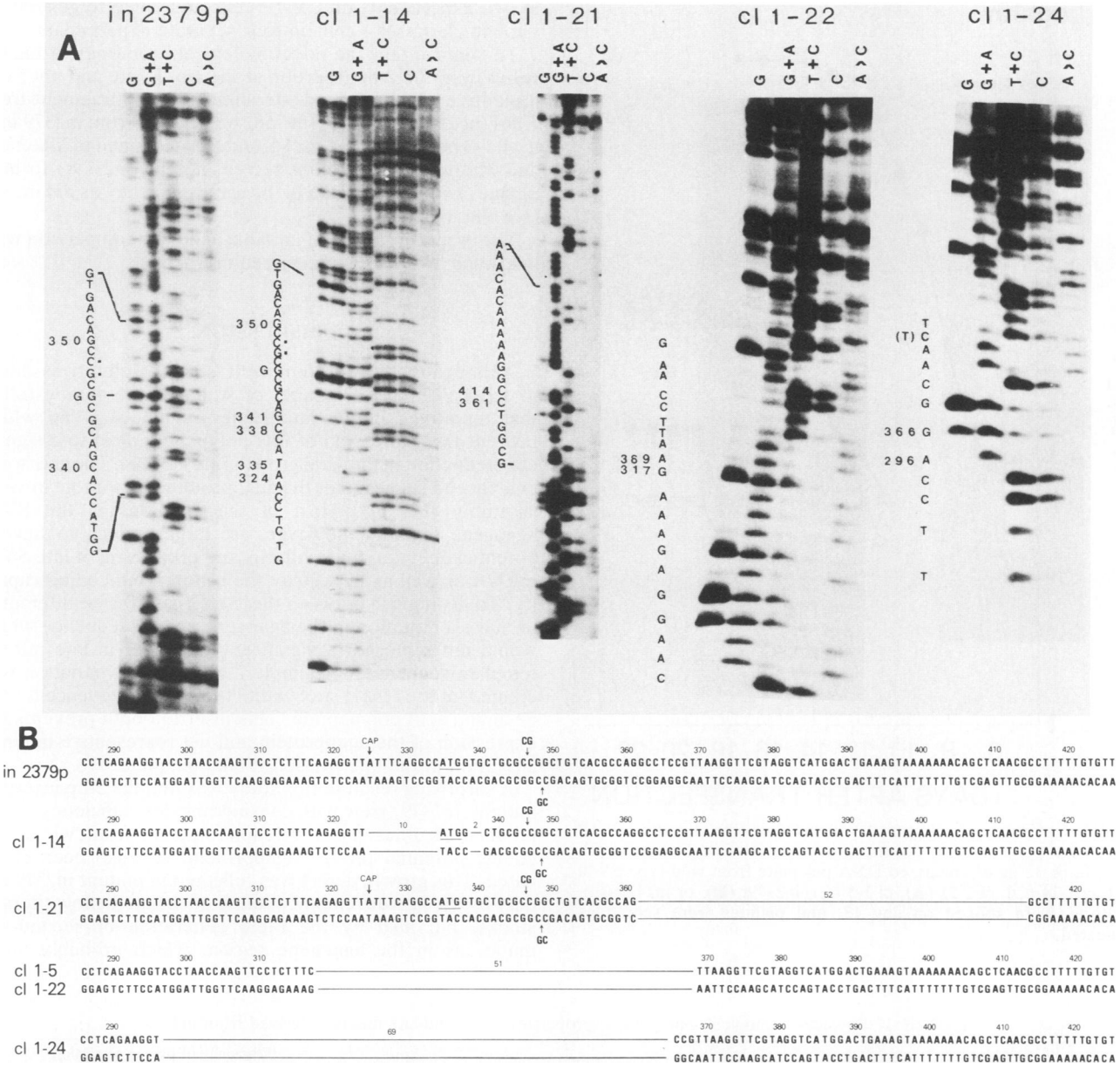


FIG. 5. Location of the deletions in second-site mutant DNAs. (A) Autoradiograms of the sequencing gels from which the positions of the insertions in mutant in2379p and cl 1-14 and the second-site alterations in clones cl 1-14, cl 1-21, cl 1-22, and cl 1-24 were determined. The sequence of cl 1-5 was identical to that of cl 1-22. cl 1-21 was analyzed in a gradient gel, whereas the others were analyzed in 20% polyacrylamide–8 M urea gels (see Materials and Methods). (B) Nucleotide sequences of mutations in the late leader region of SV40. Deleted sequences are represented by solid lines. The position of the major late SV40 cap site (↓) and the initiation codon for the agnoprotein (ATG) are indicated. Nucleotide numbers follow the BBB system (36).

1-14 DNA (which remained *Sst*II sensitive); a 10-bp segment (nt 325 to 334) as well as two additional nucleotide pairs (nt 339 and 340) were removed. Although this mutant lacks the major late SV40 cap site, it could potentially encode a 62-amino acid polypeptide initiating at the agnogene AUG which would differ from the agnoprotein in its first 4 amino acids. The size of the deletion in cl 1-21 DNA is 52 bp (nt 362 to 413). This clone retains the major late SV40 cap site and the initiation codon for agnoprotein. A putative truncated polypeptide would be only 23 amino acids in length.

**Biological properties of second-site deletion mutants.** The plaquing efficiency in CV-1P cells of DNA from these deletion mutants was compared with that of wild-type SV40 and in2379 DNA. Experiments were performed by transfecting CV-1P cells with linearized viral DNA as previously described.

Plaques appeared 15 days after transfection of in2379, and a maximum titer of $1.0 \times 10^2$ PFU per μg of DNA was reached on day 19 (Fig. 6). The deletion mutants cl 1-5, cl 1-14, and cl 1-21 grew seven- to ninefold better than the parental in2379. Although these mutants differ in the size and location of deleted sequences, in two separate studies they all grew with rather similar kinetics. cl 1-24 DNA did not form any plaques after transfection with even 100 ng of DNA

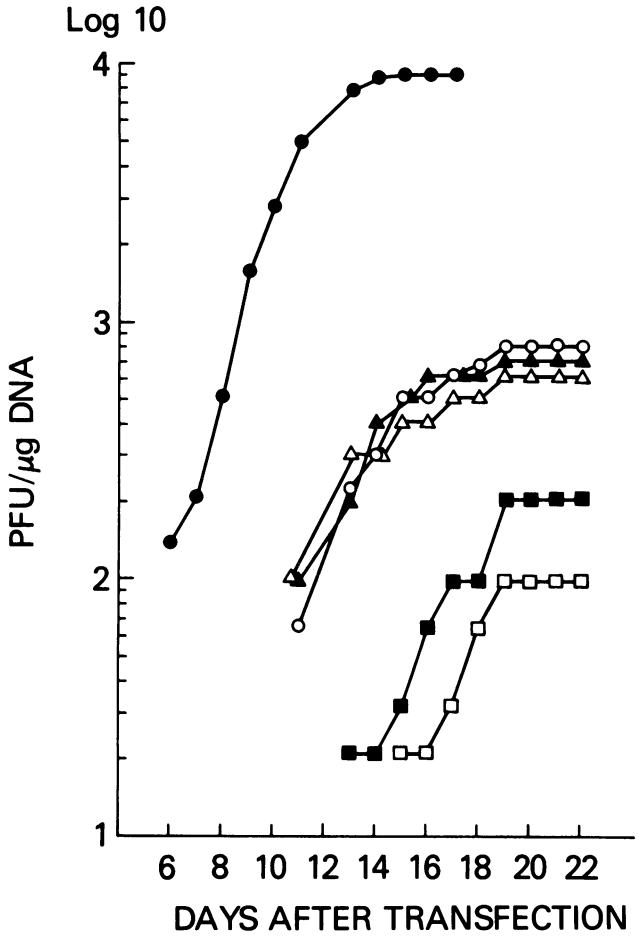


FIG. 6. Growth of wild-type SV40 and agnogene mutants. CV-1P cells were transfected as described in Materials and Methods with 3.2, 10, or 32 ng of linearized DNA per plate from wild-type SV40 (●), cl 1-14 (○), cl 1-21 (▲), cl 1-5 (△), in2379r (■), or in2379 (□). Cultures were stained on day 13, and plaques were scored as indicated.

in two experiments on CV-1P cells and failed to generate a viral stock in CV-1 cells in four separate experiments.

To confirm that the novel biological properties of in2379 result from the 2-bp insertion at the *Hpa*II site and not from a putative distant second-site alteration, the fragment from *Kpn*I (nt 294) to *Eco*RV (nt 768) was cloned from in2379 into a wild-type SV40 DNA background as described in Materials and Methods. This reconstructed mutant (in2379r) formed plaques with approximately the same efficiency as did in2379 (see Fig. 6).

The properties of these mutants and their comparison with wild-type SV40 are summarized in Table 1.

## DISCUSSION

Although the agnoprotein itself is not absolutely essential to virus viability, a number of studies have demonstrated that mutations in the late leader region of SV40 which prevent the expression of this polypeptide lead to a significant reduction in the viral growth rate (13, 25). There is some evidence to suggest that the agnoprotein plays a role in virus assembly (21, 27). Most of the deletions in the SV40 agnogene mutants, however, are large enough to have a potential effect on the synthesis and processing of late SV40 mRNA as well as to destroy the agnoprotein coding capacity. To distinguish between these alternatives, we attempted to make a mutation in the agnogene coding sequence which would not represent a significant alteration in late mRNA template sequences. A simple 2-bp insertion mutation was created (at the *Hpa*II site) within the coding sequence for the 62-amino acid polypeptide. This insertion both prevents the expression of the agnoprotein and yet represents a minimal alteration in the late transcriptional template.

A surprising result of this study was that the 2-bp insertion mutant, in2379, grew with considerably less efficiency than a number of other SV40 deletion mutants from which the coding potential for the agnoprotein had also been eliminated. This growth defect was reflected in mutant in2379 not only by the late development of plaques and the low titers of stocks, but also by the rapid generation of second-site mutations in the agnogene region, which probably arose

TABLE 1. Physical, biological, and genetic properties of second-site mutants derived from in2379

| Viral DNA | Deletion | | Major cap site | Agnoprotein (AUG) | Efficiency of plaque formation[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Titer | | Plaque size (mm) |
| | Size (bp) | Location (nucleotide residues)[a] | | | PFU per μg of DNA | % of wild type | |
| Wild-type strain 776 | | | + | + | $9.2 \times 10^3$ | 100.0 | 10.0–12.0 |
| in2379[c] | | | + | +[d] | $1.0 \times 10^2$ | 1.1 | 0.5–3.0 |
| in2379r[e] | | | + | +[d] | $2.1 \times 10^2$ | 2.2 | 1.0–3.0 |
| cl 1-5 | 51 | 318–368 | − | − | $6.3 \times 10^2$ | 6.8 | 1.0–4.0 |
| cl 1-14 | 10 + 2 | 325–334<br>339–340 | − | +[f] | $8.3 \times 10^2$ | 9.0 | 0.5–5.0 |
| cl 1-21 | 52 | 362–413 | + | +[g] | $7.3 \times 10^2$ | 7.9 | 0.5–5.0 |
| cl 1-22 | 51 | 318–368 | − | − | NT[h] | | |
| cl 1-24 | 69 | 297–365 | − | − | $<1.0 \times 10$ | <0.1 | |

[a] Nucleotide residues are numbered according to the BBB system (see reference 36).
[b] Eighteen days posttransfection with linear DNA.
[c] A 2-base insertion at the *Hpa*II site, nt 346.
[d] A putative 33-amino acid polypeptide with only the first five residues homologous to the wild-type protein.
[e] Reconstructed in2379.
[f] A putative 62-amino acid polypeptide whose sequence is identical to that of the wild-type protein except for the first four residues.
[g] A putative 23-amino acid polypeptide with the first five residues homologous to the wild-type protein.
[h] NT, Not tested (the deletion is the same as that of cl 1-5).

during growth in monkey kidney cells but could have been generated at low levels in plasmid stocks.

Although each of the second-site mutants contained a more extensive deletion in the leader region, except for cl 1-24 they grew more efficiently than did the original mutant in2379. There was no obvious common pattern to the second-site mutations such as regeneration of the agnoprotein reading frame, deletion of the agnoprotein translation initiator AUG, or elimination of the major late cap site. In fact, each of these changes was found in at least one of the second-site mutants. Only one of the second-site mutants (cl 1-14) appeared able to regenerate a reading frame similar to that of the wild-type SV40 agnoprotein; thus it is clear that, while beneficial, the agnoprotein is not essential to the life cycle of SV40. This may be either because the virus does not absolutely need the protein for growth in cell culture or because a cellular protein can substitute (although perhaps inefficiently) for the wild-type agnoprotein. In cl 1-24, as in cl 1-5 and cl 1-22, both the major cap site and the AUG for agnoprotein translation were removed. In cl 1-24, an additional 21 bp upstream (nt 297–317) were deleted. This clone failed to replicate in AGMK cells in four separate attempts.

Since the agnoprotein is nonessential, the pronounced inefficiency of growth by the 2-bp insertion mutant and the rapid alterations that it appears to undergo in monkey kidney cells remain puzzling. It is possible that this 2-bp insertion has a more profound effect either on the SV40 late transcriptional template or on the secondary structure of the SV40 late mRNA precursor molecule (2, 8, 9, 29) than do the larger deletions in the late leader region. Another alternative is that the 2-bp insertion in some way interferes with translation of late mRNA molecules (6, 8, 11, 19, 30). Depending on the initiator codon, the 2-bp insertion can produce a translational frameshift that would read through the AUG for VP-2 at nt 562 and thus, in some forms of the spliced 19S mRNA (splice from nt 373 to 558), interfere with initiation of VP-2 (J. Resnick and T. Shenk, personal communication). It is also conceivable that the truncated form of the agnoprotein generated by in2379 is in some way deleterious to the life cycle of SV40. Since we were unable to definitively demonstrate the presence of such a truncated protein, we suspect that if it is synthesized, it may be highly unstable.

The nucleotide sequence of the second-site mutant clones indicates that they could circumvent either of the potential problems discussed above. Clones cl 1-22 and cl 1-24 have the agnogene transcriptional initiation codon deleted. The donor splice site at nt 373, as well as most of the agnoprotein coding sequence, is absent from cl 1-21. In cl 1-14, the deletion of 2 bp just beyond the agnogene AUG (nt 339–340) establishes a new reading frame with a termination codon at nt 521, just before the 19S mRNA splice site. Thus, reinitiation could presumably take place at the VP-2 AUG in the spliced late 19S mRNA of this mutant.

Since the second-site mutants grow better than in2379, it is clear that this mutant suffers from a significant defect beyond the loss of agnoprotein. One approach to studying the role of the late agnoprotein apart from the potentially deleterious effects of mutations in the late mRNA template involves establishing cell lines which constitutively express the agnoprotein. Such studies are in progress in our laboratory as well as in others (J. Alwine and J. Mertz, personal communications).

## ACKNOWLEDGMENTS

We thank R. Dhar and R. Koller for helpful advice and M. Priest for preparation of the manuscript.

## LITERATURE CITED

1. **Alwine, J.** 1982. Evidence for simian virus 40 late transcriptional control: mixed infections of wild-type simian virus 40 and a late leader deletion mutant exhibit *trans* effects on late viral RNA synthesis. J. Virol. **42**:798–803.
2. **Barkan, A., and J. E. Mertz.** 1981. DNA sequence analysis of simian virus 40 mutants with deletions mapping in the leader region of the late viral mRNA's: mutants with deletions similar in size and position exhibit varied phenotypes. J. Virol. **37**:730–737.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and $^{35}S$ label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
4. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. USA **71**:942–946.
5. **Carbon, J., T. E. Shenk, and P. Berg.** 1975. Biochemical procedure for production of small deletions in simian virus 40 DNA. Proc. Natl. Acad. Sci. USA **72**:1392–1396.
6. **Celma, M. L., R. Dhar, J. Pan, and S. M. Weissman.** 1977. Comparison of the nucleotide sequence of the messenger RNA for the major structural protein of SV40 with the DNA sequence encoding the amino acids of the protein. Nucleic Acids Res. **4**:2549–2559.
7. **Dhar, R., K. N. Subramanian, J. Pan, and S. M. Weissman.** 1977. Structure of a large segment of the genome of simian virus 40 that does not encode known protein. Proc. Natl. Acad. Sci. USA **74**:827–831.
8. **Ghosh, P. K., M. Platak, J. E. Mertz, S. M. Weissman, and P. Lebowitz.** 1982. Altered utilization of splice sites and 5′ termini in late RNAs produced by leader region mutants of simian virus 40. J. Virol. **44**:610–624.
9. **Ghosh, P. K., V. B. Reddy, J. Swinscoe, P. V. 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.
10. **Ghosh, P. K., V. B. Reddy, J. Swinscoe, P. Lebowitz, and S. M. Weissman.** 1978. Heterogeneity and 5′-terminal structures of the late RNAs of simian virus 40. J. Mol. Biol. **126**:813–846.
11. **Ghosh, P. K., P. Roy, A. Barkan, J. E. Mertz, S. M. Weissman, and P. Lebowitz.** 1981. Unspliced functional late 19S mRNAs containing intervening sequences are produced by a late leader mutant of simian virus 40. Proc. Natl. Acad. Sci. USA **78**:1386–1390.
12. **Haegeman, G., D. Iserentant, D. Gheysen, and W. Fiers.** 1979. Characterization of the major altered leader sequence of late mRNA induced by SV40 deletion mutant dl-1811. Nucleic Acids Res. **7**:1799–1814.
13. **Haegeman, G., H. Van Heuverswyn, D. Gheysen, and W. Fiers.** 1979. Heterogeneity of the 5′ terminus of late mRNA induced by a viable simian virus 40 deletion mutant. J. Virol. **31**:484–493.
14. **Hay, N., H. Skolnik-David, and Y. Aloni.** 1982. Attenuation in the control of SV40 gene expression. Cell **29**:183–193.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
16. **Jackson, V., and R. Chalkley.** 1981. Use of whole-cell fixation to visualize replicating and maturing simian virus 40: identification of new viral gene product. Proc. Natl. Acad. Sci. USA **78**:6081–6085.
17. **Jay, G., S. Nomura, C. W. Anderson, and G. Khoury.** 1981. Identification of the SV40 agnogene product: a DNA binding protein. Nature (London) **291**:346–349.
18. **Kretshmer, P. J., A. C. Y. Chang, and S. N. Cohen.** 1975. Indirect selection of bacterial plasmids lacking identifiable phenotypic properties. J. Bacteriol. **124**:225–231.
19. **Lai, C.-J., and G. Khoury.** 1979. Deletion mutants of simian virus 40 defective in biosynthesis of late viral mRNA. Proc. Natl. Acad. Sci. USA **76**:71–75.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Margolskee, R. F., and D. Nathans.** 1983. Suppression of a VP1

mutant of simian virus 40 by missense mutations in serine codons of the viral agnogene. J. Virol. **48**:405–409.
22. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–560.
23. **McCutchan, J., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyldextran. J. Natl. Cancer Inst. **41**:351–357.
24. **Mertz, J. E., and P. Berg.** 1974. Defective simian virus 40 genomes: isolation and growth of individual clones. Virology **62**:112–124.
25. **Mertz, J. E., and P. Berg.** 1974. Viable detection mutants of simian virus 40: selective isolation by means of a restriction endonuclease from *Haemophilus parainfluenzae*. Proc. Natl. Acad. Sci. USA **71**:4879–4883.
26. **Mertz, J. E., A. Murphy, and A. Barkan.** 1983. Mutants deleted in the agnogene of simian virus 40 define a new complementation group. J. Virol. **45**:36–46.
27. **Ng, S.-C., J. E. Mertz, S. Sanden-Will, and M. Bina.** 1985. SV40 maturation in cells harboring mutants deleted in the agnogene. J. Biol. Chem. **260**:1127–1132.
28. **Nomura, S., G. Khoury, and G. Jay.** 1983. Subcellular localization of the simian virus 40 agnoprotein. J. Virol. **45**:428–433.
29. **Piatak, M., P. K. Ghosh, L. C. Norkin, and S. M. Weissman.** 1983. Sequences locating the 5′ ends of the major simian virus 40 late mRNA forms. J. Virol. **48**:503–520.
30. **Piatak, M., K. N. Subramanian, P. Roy, and S. M. Weissman.** 1981. Late messenger RNA production by viable simian virus 40 mutants with deletions in the leader region. J. Mol. Biol. **153**:589–618.
31. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
32. **Sanger, F., and A. R. Coulson.** 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Lett. **87**:107–110.
33. **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.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
35. **Subramanian, K. N.** 1979. Segments of simian virus 40 DNA spanning most of the leader sequence of the major late viral messenger RNA are dispensable. Proc. Natl. Acad. Sci. USA **76**:2556–2560.
36. **Tooze, J. (ed.).** 1981. Molecular biology of tumor viruses, part 2, 2nd ed., revised. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Villarreal, L. P., R. T. White, and P. Berg.** 1979. Mutational alterations within the simian virus 40 leader segment generate altered 16S and 19S mRNAs. J. Virol. **29**:209–219.