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Construction and Characterization of Viable Deletion Mutants of Simian Virus 40 Lacking Sequences near the 3′ End of the Early Region

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Five viable deletion mutants of simian virus 40 (SV40) were prepared and characterized. These mutants lack 15 to 60 base pairs between map positions 0.198 and 0.218, near the 3′ end of the early region of SV40 and extend further into the body of the A gene, encoding the large T antigen, than previously described deletion mutants. These mutants were isolated after transfection of monkey kidney CV-1p cells with full-sized linear DNA prepared by partial digestion of form I SV40 DNA with restriction endonucleases *Hin*fI or *Mbo*II, followed by removal of approximately 25 base pairs of DNA from the 5′ termini using λ-5′-exonuclease and purification of the DNA in agarose gels. Based on comparisons of the DNA sequence of SV40 and polyoma virus, these mutations map in the 19% of the SV40 A gene that shares no homology with the A gene of polyoma virus. The mutations exist in two different genetic backgrounds: the original set of mutants (*dl*2401 through *dl*2405) was prepared, using as a parent SV40 mutant *dl*862, which has a deletion at the single *Hpa*II site (0.725 map unit). A second set (*dl*2491 through *dl*2495) contains the same deletions in a wild-type SV40 (strain SV-S) background. Relative to wild-type SV40, the original mutants showed reduced rates of growth, lower yields of progeny virus and viral DNA, and smaller plaque size; in these properties the mutants resembled parental *dl*862, although mutant progeny yields were usually lower than yields of *dl*862, suggesting a possible interaction between the two deletions. The second set of mutants had growth properties and progeny yields similar to those of wild-type SV40; however, Southern blotting experiments indicated that viral DNA replication proceeds at a slightly reduced rate. All of the mutants transformed mouse NIH/3T3 cells and mouse embryo fibroblasts at the same frequency as wild-type SV40. Mutants *dl*2402, *dl*2492, and *dl*2405 consistently produced denser and larger foci in both types of cells. All mutants directed the synthesis of shortened large T antigens. Adenovirus helper function was retained by all mutants.

The early region of the simian virus 40 (SV40) genome encodes two polypeptides, the large T and the small t antigens (15). The large T antigen has a molecular weight of approximately 81,600 (21, 59) and is encoded by two noncontiguous regions of the genome, 0.647 to 0.600 map units (mu) and 0.533 to 0.174 mu, which are brought together in the mature mRNA by splicing (6, 21, 59). The small t antigen, of molecular weight 20,500, is encoded by a continuous open reading frame between map coordinates 0.647 and 0.546. Thus, the two polypeptides share 82 codons at their N termini and possess unique C termini (17, 52, 53).

The large T antigen, the product of the A gene, is a multifunctional protein which plays a pivotal role in the life cycle of SV40. It is involved in the initiation of viral and host DNA replication (11, 73); regulation of the level of early mRNAs (3, 60, 75); initiation (14) and, in some cases, maintenance of late transcription (1); provision of adenovirus helper function (12, 29, 57); induction and maintenance of increased levels of a host 53,000-dalton (53 kd) to 54 kd nuclear protein (39, 42, 43); surface antigenicity (78); and tumor-specific transplantation antigenicity (4, 56, 76, 77). Located primarily in the nucleus, there may be low levels of it associated with the plasma membrane (68, 69), but it is not known if this species is identical to the nuclear T antigen. The large T antigen is a DNA-binding protein (33, 79) and a DNA-dependent ATPase (80). In the nonpermissive cell, the large T antigen is required for the initiation of transformation (35, 74). Whether large T antigen is required for the maintenance of the transformed state depends on parameters such as host cell species, cellular growth rate before and immedi-

ately after infection, multiplicity of infection (MOI), and serum factors. Under many conditions, maintenance requires the continued presence of the large T antigen (8, 9, 26, 35, 46, 51, 58, 63, 64, 74).

Most of our knowledge about the biological properties and functions of the T antigen derives from studies of *tsA* mutants (11, 73) which map in the central third of the A gene between 0.32 and 0.43 mu (38). Much of the rest of the A gene has not been subjected to detailed genetic analysis.

We described previously (13) two viable mutants of SV40 that contain small deletions in the C-terminal portion of the A gene. These were obtained by transfecting monkey kidney cells with full-sized linear DNA molecules obtained by treating form I SV40 DNA with S1 nuclease. Mutant *dl*1263 lacks 33 nucleotides at 0.195 mu, and *dl*1265 lacks 39 nucleotides at 0.175 mu (82). Both *dl*1263 and *dl*1265 direct the synthesis of altered large T antigens and have adenovirus helper activities which are 30 and 7%, respectively, of the activity of wild-type SV40 (12, 17). In all other respects, including their ability to transform cells for anchorage-independent growth, the biological properties of these mutants are normal (7, 13).

In this report we describe the construction and characterization of viable mutants of SV40 with small deletions (15 to 60 base pairs [bp]) in the C-terminal portion of the early region. The lesions in these mutants extend further into the body of the large T antigen than the lesions of *dl*1263 or *dl*1265 and demonstrate that substantial portions of the C-terminal 10% of the large T antigen may be deleted without affecting the viability of the virus. These mutants resemble wild-type SV40 in growth properties and in yields of viral DNA and progeny virions. However, analysis of DNA replication by Southern blotting demonstrated that DNA replication in mutant-infected cells proceeds more slowly than in wild-type-infected cells. All mutants direct the synthesis of shortened large T antigens, transform nonpermissive cells at frequencies indistinguishable from that of wild-type SV40, and provide normal levels of adenovirus helper function.

MATERIALS AND METHODS

Cells and cell culture. CV-1 and CV-1p cells, established sublines of AGMK cells (32), and HeLa cells (obtained from T. Shenk) were cultured in 100-mm plastic dishes in Dulbecco-modified Eagle medium (GIBCO Laboratories) (DMEM) supplemented with 5% fetal calf serum (FCS) or newborn calf serum. NIH/3T3 cells were obtained from R. Weinberg, maintained in DMEM containing 10% FCS, and subcultured by published protocols (5). Primary mouse embryo cells were derived from C57BL/6 mice (56) and maintained in DMEM containing 10% FCS. In all cases, DMEM contained 100 μg of streptomycin per ml and 500 U of penicillin per ml.

Virus and viral DNA. The wild-type strain for the mutants described in this report is the small-plaque strain (SV-S) originally characterized by Takemoto et al. (72). Monolayers of CV-1 cells in 100-mm dishes were infected with SV40 wild type or mutants at an MOI of 0.01 PFU/cell or transfected with wild-type or mutant DNA (0.01 μg/plate). Virus stocks were prepared as previously described (11). Adenovirus type 2 (Ad2) was grown in HeLa cells from a seed stock supplied by T. Grodzicker (Cold Spring Harbor Laboratory). SV40 wild-type and mutant DNAs were prepared as described by Carbon et al. (10).

Construction and isolation of deletion mutants. The original parent for these mutants was *dl*862, an *Hpa*II-resistant viable deletion mutant of SV40, constructed by Carbon et al. (10) and derived from the SV-S strain of SV40. This mutant lacks approximately 12 bp (Polvino-Bodnar and Cole, unpublished data) at the single *Hpa*II cleavage site (0.725 mu).

Form I DNA was converted to form III (linear) DNA by limited digestion with either *Mbo*II or *Hin*fI restriction endonuclease. After phenol extraction and ethanol precipitation, DNA was dissolved in exonuclease buffer (67 mM glycine [pH 9.6], 3 mM $MgCl_2$, 3 mM 2-mercaptoethanol) and incubated with λ-5′-exonuclease under conditions allowing removal of approximately 25 nucleotides from the 5′-phosphoryl termini (10). Linear molecules between 95 and 100% of full-size were isolated by two cycles of electrophoresis in 1 to 1.6% horizontal agarose slab gels in Tris-borate-EDTA buffer (TBE; 89 mM Tris-OH, 89 mM boric acid, 2.5 mM EDTA [pH 8.2]). Under the conditions of electrophoresis (10 V/cm) linear molecules migrated well ahead of uncleaved form I and nicked circular form II DNA. Subsequently, DNA was recovered from the gel by electrophoresis into a dialysis bag, using E buffer (40 mM Tris-OH, 12.5 mM NaOAc, 2.5 mM EDTA) at 100 V for 14 h followed by polarity reversal for 3 min. The DNA was extracted with phenol-chloroform-isoamyl alcohol (50:49:1) (four times), $CHCl_3$-isoamyl alcohol (49:1) (two times), and ether (four times) and ethanol-precipitated.

To isolate deletion mutants, confluent monolayers of CV-1p cells were transfected with the isolated linear DNAs as described by Mertz and Berg (48). Plaques were isolated, and mutants were cloned by two cycles of plaque purification, followed by preparation of stocks of viral DNA and virions.

Physical mapping and DNA sequence analysis of the mutants. The locations and approximate sizes of the deletions were determined by restriction endonuclease mapping. Each of the mutants was digested with a variety of restriction endonucleases, including *Bst*NI, *Hae*III, *Hin*dII + III, *Hin*fI, *Rsa*I, *Mbo*II, *Msp*I, and *Dde*I. Digests were analyzed by electrophoresis either in horizontal 1% agarose slab gels or in vertical polyacrylamide gradient slab gels, using TBE buffer. Gels were stained with ethidium bromide (1 μg/ml) for 30 min and visualized with a short-wavelength UV lamp.

DNA sequence analysis was carried out by the chemical degradation method of Maxam and Gilbert (47). Mutants *dl*2491, *dl*2493, and *dl*2494 were sequenced by isolating *Hin*fI fragment E (0.204 to 0.305 mu) from a 6% polyacrylamide gel (acrylamide/bis-

acrylamide ratio was 20:1), treating with calf intestinal alkaline phosphatase, labeling with polynucleotide kinase and [γ-^{32}P]ATP (Amersham Corp.; 3,000 Ci/mmol [1 Ci = 3×10^{10} becquerels]), digesting with *Pst*I (which cuts at 0.274 mu), and isolating the fragment which includes the deletion from a second 6% polyacrylamide gel. Mutant *dl*2492 was sequenced in a similar manner, but *Hin*fI fusion fragment EH (0.194 to 0.305 mu) was used. Mutant *dl*2495 was sequenced by labeling phosphatase-treated, sucrose gradient-purified, *Bcl*I-digested *dl*2495 DNA with polynucleotide kinase and [γ-^{32}P]ATP, digesting with *Bam*HI, and purifying the 5-kilobase (kb), deletion-containing fragment in a second sucrose gradient (5 to 20% sucrose in 10 mM Tris-chloride [pH 7.5], 100 mM NaCl, 10 mM EDTA; Beckman SW41 rotor, 18 h, 36,000 rpm, 4°C).

Plaque assays. Titers of stocks of wild-type SV40 and viable mutants were determined by plaque assay on monolayers of CV-1p cells as previously described (48); adenovirus titers were measured by plaque assay on HeLa cells (83).

Plaque morphology and rates of plaque development were studied by transfecting CV-1p monolayers with wild-type or various mutant DNAs. At various times after infection, cultures were stained with crystal violet (31) and photographed.

All of the mutants were tested for temperature sensitivity by titration of virus stocks at 32, 37, and 41°C.

Analysis of DNA replication. Cultures of subconfluent CV-1 cells in 60-mm plates were infected with wild-type SV40 or deletion mutants. At various times after infections, cultures were harvested. Monolayers were washed twice with Tris-buffered saline (TS). To each culture was added 0.2 ml of Hirt solution (0.01 M Tris-chloride [pH 7.5], 0.6% SDS, 0.01 M EDTA). After 10 min at room temperature, each culture received 60 μl of 5 M NaCl, 20 μl of proteinase K (10 mg/ml in water, obtained from Beckman), and 20 μl of M13 bacteriophage RF II [^{3}H]DNA (20,000 to 30,000 cpm, about 20,000 cpm/μg; a generous gift from A. Wu or R. Kahn). The phage [^{3}H]DNA was included to allow correction for losses of material during the harvesting procedure. After overnight incubation at 37°C, the contents of each plate were scraped into 1.5-ml Eppendorf tubes and held at 4°C for 12 to 24 h. The Hirt pellet was removed by centrifugation at 12,000 × *g* for 10 min in an Eppendorf centrifuge. The supernatant was extracted once with phenol-chloroform-isoamyl alcohol (50:49:1), once with chloroform-isoamyl alcohol (49:1), and three times with ether. DNA was precipitated with 2.5 volumes of ethanol, collected by centrifugation, dried briefly under vacuum, and dissolved in 100 μl of TEN (10 mM Tris-chloride [pH 7.5], 10 mM NaCl, 1 mM EDTA). A sample was precipitated with trichloroacetic acid to calculate the percent recovery of M13 [^{3}H]DNA. Samples with identical numbers of ^{3}H counts were mixed with sample buffer and loaded into wells of a 1.0% horizontal agarose slab gel (3 mm thick by 14.5 cm by 22.5 cm) in TBE buffer. Electrophoresis was carried out for 12 to 16 h at 20 to 25 V (approximately 1 V/cm). Gels were stained with ethidium bromide (0.5 μg/ml for 30 min) and photographed on a long-wavelength UV lamp. DNA in the gel was depurinated by treatment with 0.25 M HCl for two 15-min periods; denatured with 0.5 M NaOH–1.5 M NaCl for two 30-min periods; neutralized with 0.5 M Tris-chloride (pH 7.5)–3 M NaCl for two 30-min periods; transferred to nitrocellulose (S & S BA85), using the method of Southern (70; and probed with nick-translated (61) pMP1 [^{32}P]DNA (pMP1 is a plasmid containing the entire SV40 genome inserted into pBR322 at the *Bam*HI site). Nitrocellulose filters were hybridized under standard conditions in 50% formamide at 41°C with approximately 1×10^6 to 5×10^6 cpm of ^{32}P-labeled probe (specific activity, 1×10^8 to 4×10^8 cpm/μg) for 12 to 24 h. Filters were washed twice for 15 min at room temperature with 2× SSC–0.1% sodium dodecyl sulfate (SSC is 0.075 M NaCl, 0.0075 M sodium citrate) and twice for 15 min at 55°C with 0.1× SSC–0.1% sodium dodecyl sulfate. Filters were exposed to Kodak XAR-1 film for 12 to 48 h.

Analysis of T antigens. Confluent monolayers of CV-1 cells in 35-mm plastic dishes (approximately 10^6 cells) were infected with 10 PFU/cell of wild-type SV40 or various deletion mutants. After 48 or 72 h at 37°C, the medium was removed, the monolayer was washed once with TS, and the medium was replaced with medium lacking methionine. After 1 h, [^{35}S]methionine (50 μCi/plate in a volume of 0.2 ml; 1,000 Ci/mmol from Radiochemical Centre, Amersham, England) was added for 1 h. At the end of the labeling period, extracts were prepared as previously described (12).

Immunoprecipitation and alkylation of cytoplasmic extracts were done as described previously (12, 16), using either hamster anti-T serum (lot 78X-386 obtained from the National Cancer Institute, Biological Carcinogenesis Branch) or mouse anti-T serum (prepared in BALB/c mice and a generous gift from S. Tevethia). Normal mouse serum was obtained for Accurate Chemical and Scientific; normal hamster serum was a gift from L. Fink. Formalin-fixed, protein A-bearing *Staphylococcus aureus* were obtained from The Enzyme Center (Boston, Mass.).

Samples were subjected to electrophoresis on discontinuous polyacrylamide gradient gels (7 to 20% acrylamide) with an acrylamide-bisacrylamide ratio of 30:0.8 (13, 37).

Adenovirus helper function. Confluent monolayers of CV-1 cells in 60-mm dishes (approximately 3×10^6 cells) were mock-infected or infected with SV40 deletion mutants or wild-type virus at an MOI of 20. After 24 or 48 h, cultures were superinfected with Ad2 at an MOI of 3 PFU/cell. After adsorption for 2 h at 37°C, cultures were washed twice with TS and fed with 5 ml of DMEM containing 2% FCS. After an additional 70 to 72 h, cells were scraped off the plate and sonicated (three times for 45 s in a Heat Systems Ultrasonics sonifier). Because the level of enhancement of Ad2 growth depends on the SV40 MOI (12), the titers of all stocks were determined just before each experiment. The adenovirus progeny yield was determined by plaque titration on HeLa cells.

Transformation. (i) Mouse embryo fibroblasts. Viral transformation was assayed in mouse embryo fibroblasts provided by M. Tevethia or prepared by the procedures described by Pretell et al. (56) and Tevethia et al. (76). After 21 to 25 days, cultures were washed, fixed, and stained with methylene blue (0.5% in 70% isopropanol). Both the size of foci and the frequency of transformation were determined.

(ii) NIH/3T3 cells. Subconfluent cultures of NIH/3T3

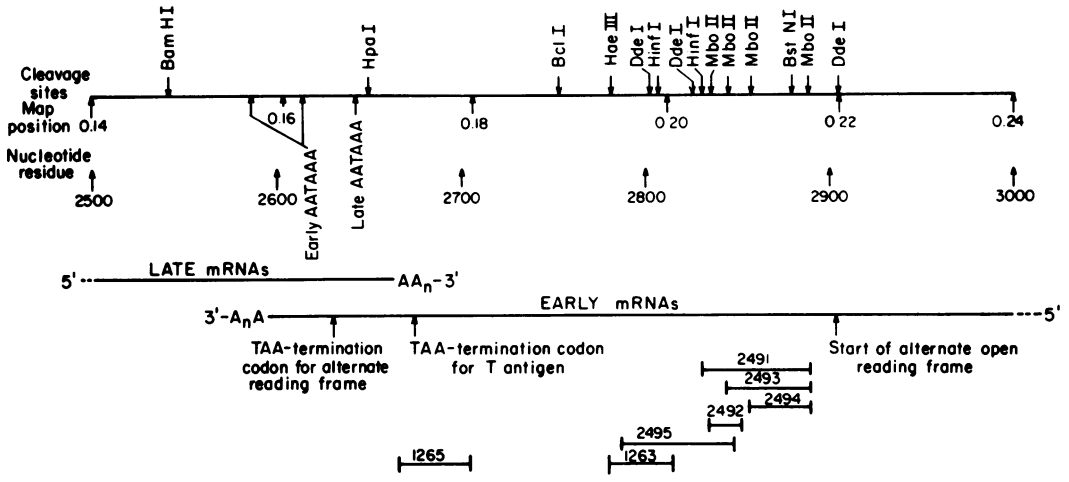

FIG. 1. Map of the SV40 genome near the 3'end of the early region. Map coordinates are expressed as the fractional length of wild-type SV40 where 0.00 is the map position of the single cleavage site for restriction endonuclease *Eco*RI. Nucleotide numbers refer to the sequence as reprinted in Tooze (81). The 3' ends of late and early mRNAs are shown in the lower part of the figure. Beneath this are shown the positions of the deletion mutants described in this report.

cells on 100-mm plates (approximately 1×10^6 to 1.5×10^6 cells) were infected with wild-type SV40 or deletion mutants at an MOI of 50 PFU/cell. After a 2-h adsorption period, cells were fed with 10 ml of DMEM + 10% FCS and incubated overnight at 37°C. After 24 h, cells were trypsinized and replated with 5×10^2, 5×10^3, and 5×10^4 cells per 100-mm plate. After 3 to 4 weeks, cultures were washed twice with TS and stained with methylene blue (0.5% in 70% isopropanol).

Enzymes. Most restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and used according to the instructions provided by the supplier. Restriction endonuclease *Msp*I, an isoschizomer of *Hpa*II, was prepared in this laboratory by chromatography on phosphocellulose. Bacteriophage λ 5'-exonuclease, prepared by the method of Little et al. (44), was used to digest the 5'-ends of linear SV40 DNAs as described by Carbon et al. (10). Proteinase K was from Beckman Instruments Inc., Fullerton, Calif. Calf intestinal alkaline phosphatase was from Boehringer-Mannheim Corp., New York, N.Y.

RESULTS

Isolation of the mutants. Previous work had established that mutants of SV40 with deletions near the 3' terminus of the early region (0.18 to 0.20 mu; Fig. 1) are viable, have normal growth rates, encode altered or shortened large T antigens, and have normal frequencies of transformation (7, 13). One of these, *dl*1265, encodes a large T antigen in which the nine amino acids at the C terminus are replaced by four not normally present; in *dl*1263 T antigen, amino acid residues 35 to 46, counting from the C terminus, are deleted, and one new amino acid exists in their place (17, 82). We were interested in determining whether mutants with deletions upstream from these would be viable and what effect deletions extending further into the early region would have on the biological properties of SV40.

Examination of the nucleotide sequence of SV40 (Fig. 1 and 2) upstream from the deletion of mutant *dl*1263 reveals the presence of one *Hin*fI and four *Mbo*II sites in the next 75 nucleotides. Using the procedures developed by Carbon et al. (10) and Cole et al. (13) for the construction of mutants with small deletions, form I DNA was digested briefly with either *Hin*fI or *Mbo*II restriction endonucleases, treated with λ-5'-exonuclease under conditions which remove approximately 25 nucleotides from the 5' terminus, and subjected to electrophoresis in 1% agarose gels. This allowed the recovery of nearly unit-length linear DNA, which was used to transfect CV-1p monkey kidney cells, both with and without helper *ts*B201 DNA. Approximately 1.6 times as many plaques were obtained with helper than without helper. We originally used, as a parent for these mutants, an *Hpa*II^r viable deletion mutant of SV40, *dl*862 (10). This parent was chosen to allow separation of mutant DNA from DNA of any helper which might have been required for the propagation of the mutants. Since only viable mutants were obtained, this differential restriction endonuclease sensitivity was unnecessary.

After two cycles of plaque purification in CV-1p cells, CV-1 cells were infected, viral DNA was isolated, and deletion mutants were identified on the basis of altered patterns of digestion with various restriction endonucleases. Four mutants were obtained using *Mbo*II (*dl*2401 through *dl*2404) and one using *Hin*fI (*dl*2405).

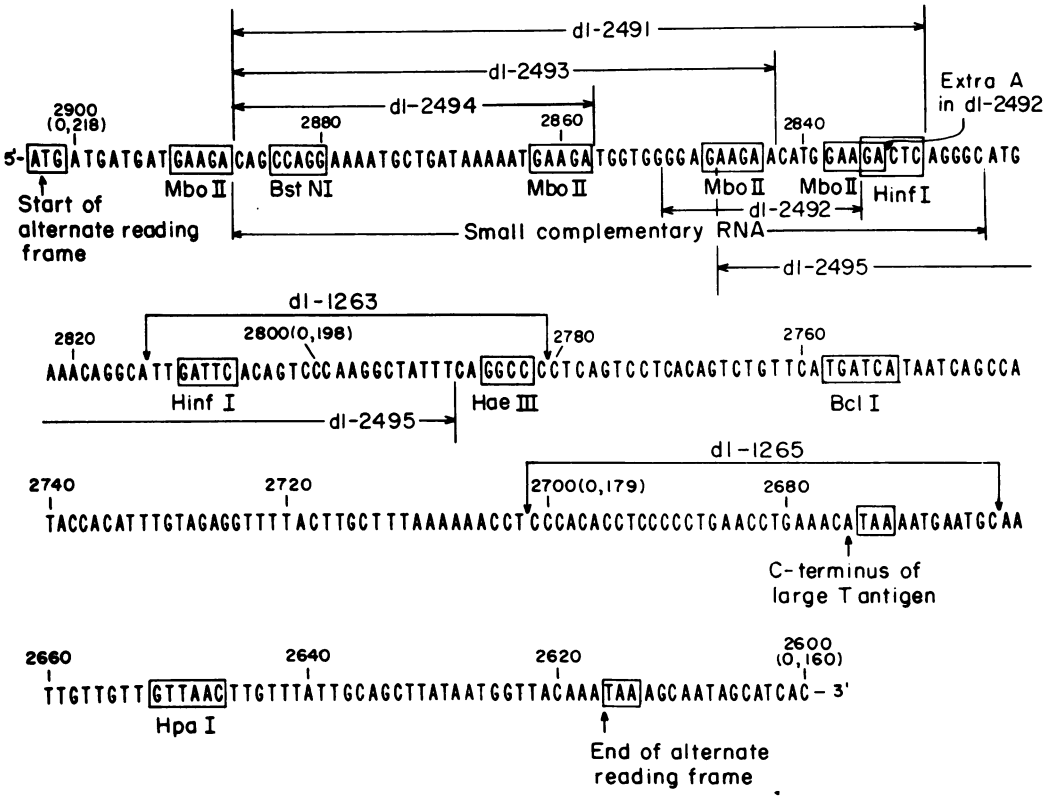

FIG. 2. Nucleotide sequences of mutants *dl*2491 through *dl*2495 (and *dl*2401 through *dl*2405). The sequence of the strand of SV40 DNA corresponding to early mRNA is shown. Numbers refer to residue numbers as designated by Tooze (81). Deleted nucleotides are bracketed, and where repeated nucleotides at both ends of a deletion make assignment of precise endpoints impossible, the deletion is placed such that endpoints are shared by different mutants. For comparison, the nucleotide sequences deleted in *dl*1263 and *dl*1265 (82) and the region complementary to the 62-nucleotide small SV40-associated RNA (1, 45) are also shown.

Characterization of these mutants revealed alterations in growth rates and plaque morphology, as well as reduced yields of viral DNA and progeny virions when compared with both wild-type SV40 and parental *dl*862. Since *dl*862 shows a reduced growth rate and altered plaque morphology (10) when compared with wild-type SV40, it was necessary to move the new deletions from the *dl*862 background into a wild-type SV40 background to study the effect of the new deletions on the biological properties of SV40. This was accomplished by isolating, from each mutant DNA, the small *Pst*I fragment (0.04 to 0.27 mu) and ligating it to the large *Pst*I fragment of wild-type SV40 DNA, obtained by *Pst*I digestion of a pMP1, a recombinant DNA clone of SV40 strain SV-S (72) cloned into the *Bam*HI site of pBR322. CV-1p cells were transfected with the ligated DNAs, and plaques were obtained and used for the preparation of viral DNA in CV-1 cells. These were screened for the presence of the deletion mutation, using restriction endonuclease digestion. These new isolates were designated *dl*2491 through *dl*2495.

Restriction endonuclease and DNA sequence analysis. Table 1 and Fig. 2 summarize data derived from restriction endonuclease digestion and direct DNA sequence analysis of the deletion mutant DNAs. The amount of DNA missing from the mutants ranges from 15 to 60 bp. For each mutant except *dl*2492 (and *dl*2402), the deletion removes a multiple of 3 bp, leaving the large T antigen reading frame unaffected. In the case of *dl*2492 (and *dl*2402), 16 bp are deleted, but a single A:T pair is inserted 2 bp from one end of the deletion, restoring the large T antigen reading frame. This insertion, indicated by the arrow in Fig. 3, destroyed the *Hin*fI and *Dde*I sites at 0.204 mu.

Biological properties. Each set of mutants resembles its parent in its biological properties. Thus, mutants *dl*2401 through *dl*2405 produce yields of progeny DNA and virions which are two- to fivefold lower than those produced by

TABLE 1. Position and size of deletions

Mutant no.	Restriction enzyme site[a]										Size of deletion (bp)[b]	Missing nucleotide residues[c]
	*Hpa*II (0.725)	*Hae*III (0.194)	*Hin*fI (0.199)	*Dde*I (0.204)	*Hin*fI (0.204)	*Mbo*II (0.204)	*Mbo*II (0.206)	*Mbo*II (0.209)	*Bst*NI (0.213)	*Mbo*II (0.215)		
Wild-type SV40	+	+	+	+	+	+	+	+	+	+		
*dl*862	−	+	+	+	+	+	+	+	+	+		
*dl*2401	−	+	+	−	−	−	−	−	−	+		
*dl*2491	+										57	2887–2831
*dl*2402	−	+	+	−[d]	−[d]	−	−	+	+	+		
*dl*2492	+										15[e]	2851–2836
*dl*2403	−	+	+	+	+	+	−	−	−	+		
*dl*2493	+										45	2886–2842
*dl*2404	−	+	+	+	+	+	+	+	−	−		
*dl*2494	+										30	2893–2864
*dl*2405	−	+	−	−	−	−	−	+	+	+		
*dl*2495	+										60	2847–2788

[a] Determined as described in the text. +, Presence of indicated restriction endonuclease cleavage site; −, absence of indicated site.

[b] Base pairs are calculated from nucleotide sequence analysis as described in the text.

[c] Determined as described in the text. The nucleotide residues are numbered according to the wild-type SV40 DNA sequence as reprinted by Tooze (81). In many cases, there is some ambiguity in the missing nucleotide residues due to the presence of short (1 to 7 bp) repeated sequences in SV40 DNA, one copy of which was deleted at the deletion endpoints. See the text.

[d] These sites are missing because an A residue was inserted into the mutant genome at this location; the deletion does not extend as far as these sites.

[e] The net deletion in this mutant is 15 bp due to deletion of 16 bp and insertion of 1 bp.

wild-type SV40 and similar to those produced by parental *dl*862. Mutants *dl*2491 through *dl*2495 produce yields of progeny DNA (Fig. 4) and virions (data not shown) which are approximately equal to those produced by wild-type SV40. The former set of mutants resembles parental *dl*862 in that plaques appear later and enlarge more slowly than those formed by wild-type SV40. Mutants *dl*2491 through *dl*2495 produce plaques which appear at the same time or 1 day later and enlarge at approximately the same rate as wild-type SV40 plaques. We conclude that the deletions do not have a dramatic effect on the growth properties and plaque morphology of SV40.

All of the mutants were tested for temperature sensitivity by incubating infected plaque assay cultures at 32, 37, and 41°C. In all cases, the mutants showed no sensitivity to temperature (data not shown).

Analysis of DNA replication in mutant-infected cells. Although dramatic differences between mutants and wild-type SV40 in growth properties and plaque morphology were not observed, subtle differences in viral DNA replication would not have been detected in the above experiments. Since the SV40 large T antigen plays an essential role in the initiation of viral DNA replication, experiments were conducted to see whether the lesions in these deletion mutants affect the DNA replication properties of large T antigen. Cultures of CV-1 cells were infected with wild-type SV40 or deletion mutants. At various times after infection, cultures were harvested, and viral DNA was isolated by a modified Hirt extraction procedure, as described above. The addition of ^{3}H-labeled M13 bacteriophage DNA to each culture at the time of harvesting allowed correction for any losses of viral DNA during the harvesting procedure. Since each culture contains approximately the same number of cells, each sample analyzed should contain viral DNA from an identical number of infected cells. Samples were analyzed by electrophoresis in 1% agarose gels, followed by transfer to nitrocellulose by the method of Southern (70) and hybridization with nick-translated SV40 [^{32}P]DNA. Figure 4 presents the results of an experiment in which replication of *dl*2491, *dl*2492, *dl*2493, and SV-S were compared in cells infected at an MOI of 5 to 10 PFU/cell. DNA replication follows similar kinetics and reaches similar maximum levels in each case.

The results obtained in experiments such as that in Fig. 4 are very sensitive to the MOI; a two- threefold decrease in MOI causes a significant alteration in the kinetics of DNA replication by delaying for 12 to 24 h the time at which the synthesis of progeny DNA is complete (Polvino-Bodnar and Cole, unpublished data). Therefore, DNA replication was compared in cells infected by wild-type SV40 or *dl*2492 at an MOI of 0.1 PFU/cell. In this experiment, errors in determining titers of virus stocks will affect the number of

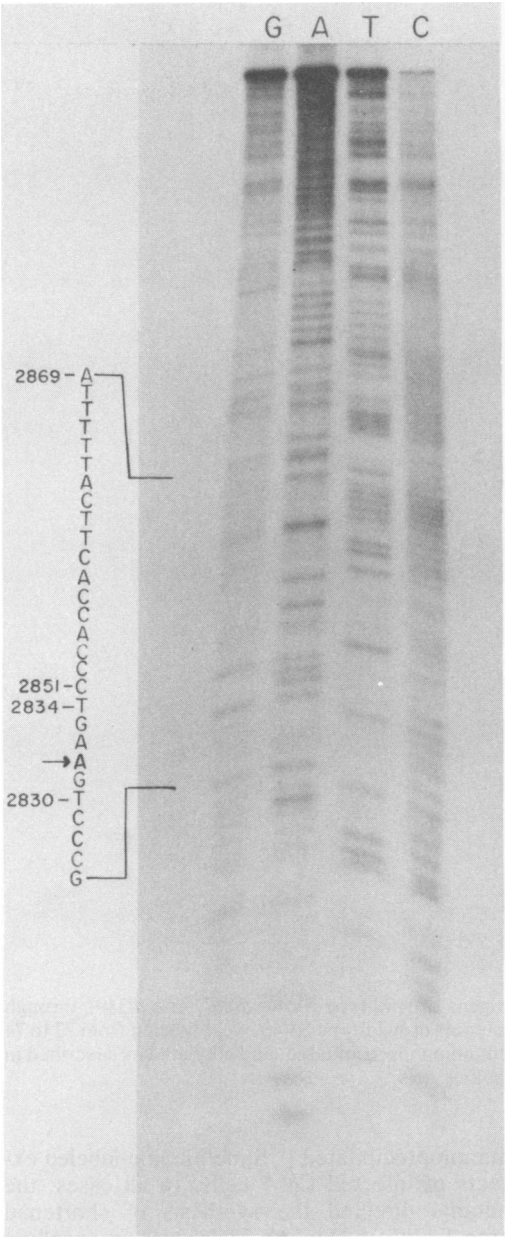

FIG. 3. Autoradiogram of the sequencing gel showing the deletion and nearby insertion in mutant *dl*2492. After the sequencing reactions, samples were denatured and loaded onto a 0.3-mm-thick 12% polyacrylamide–8 M urea slab gel. The gel was subjected to electrophoresis at 800 V until the bromophenol blue dye marker had reached two thirds of the way to the bottom. The sequence, read from the top to the bottom, corresponds to the late strand of SV40 DNA read in a counterclockwise direction. The site of the inserted A residue is indicated by an arrow. Comparison of this sequence with that of wild-type SV40 DNA (81) allowed determination of the location of the deletion.

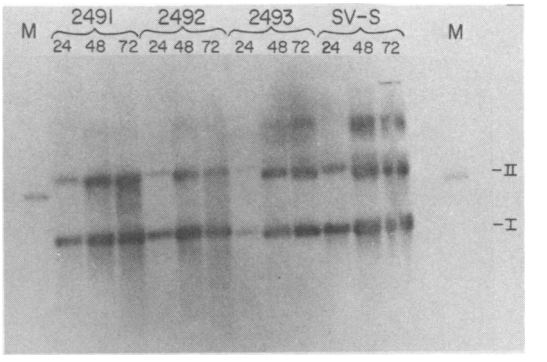

FIG. 4. Autoradiograph of Southern hybridization comparing DNA replication in cultures infected by *dl*2491, *dl*2492, *dl*2493, or wild-type SV40. Cultures of CV-1 cells were infected by wild-type SV40 or deletion mutants at an MOI of 5 to 10 PFU/cell. At the times indicated, cultures were harvested, treated, and analyzed as described in the text. The lanes marked M contain marker 4.3-kilobase linear pBR322 DNA. I and II indicate the positions of closed circular superhelical (form I) and nicked-circular (form II) SV40 DNA.

cells which become infected and, hence, the yield of viral DNA from each culture, but almost all of the cells which become infected will be infected at an MOI of 1 PFU/cell. This allows accurate comparison of the kinetics of viral DNA replication to be made.

The titers of virus stocks were determined immediately before each experiment, and the virus stocks were sonicated before titration and before infection to disrupt aggregates of virions. Virus titers were always identical within a factor of 2. From Fig. 5, it can be seen that DNA

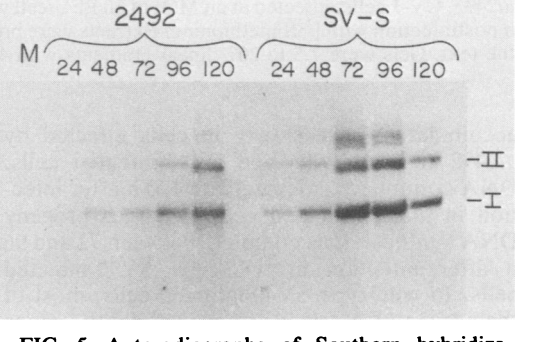

FIG. 5. Autoradiographs of Southern hybridizations comparing DNA replication in cultures infected by *dl*2492 or wild-type SV40 at an MOI of 0.1 PFU/cell. Cultures of CV-1 cells were infected by wild-type SV40 or *dl*2492. At the times indicated, cultures were harvested, treated, and analyzed as described in the text. The lanes marked M contain marker 4.3-kilobase linear pBR322 DNA. I and II indicate the positions of closed circular superhelical (form I) and nicked circular (form II) SV40 DNA.

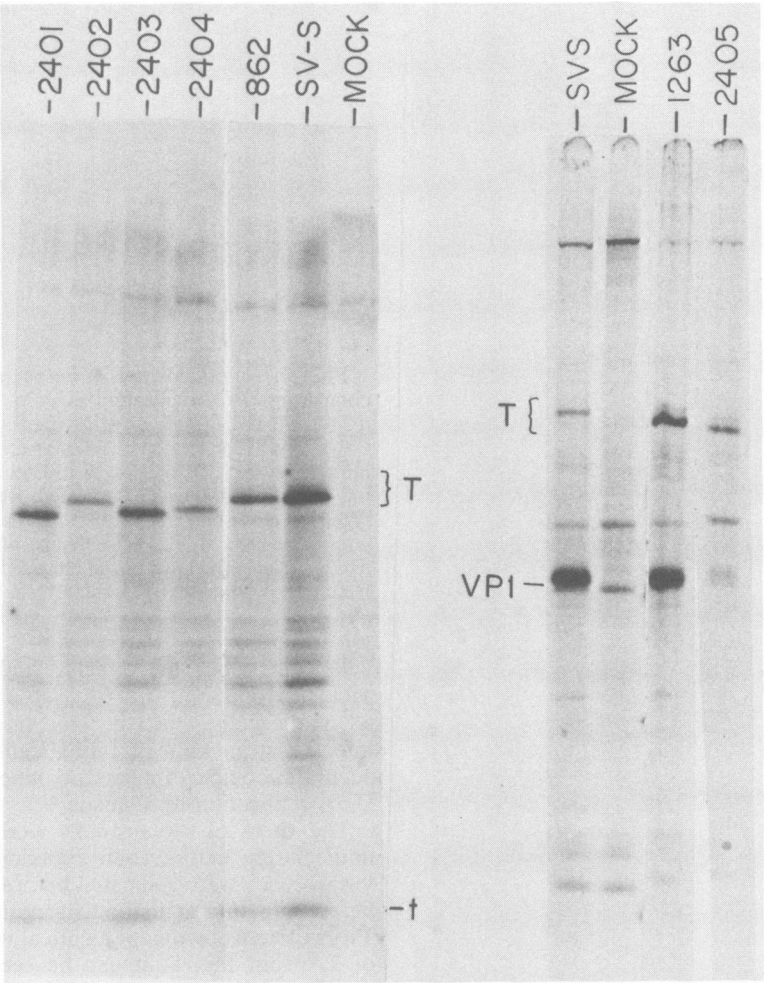

FIG. 6. Polyacrylamide gel electrophoresis of the T antigens of wild-type SV40, *dl*862, and *dl*2491 through *dl*2495. CV-1 cells, infected at an MOI of 10 PFU/cell with mutants or wild-type SV40, were labeled from 72 to 74 h postinfection with [^{35}S]methionine. Extracts were prepared, immunoprecipitated, and alkylated as described in the text. Gels were 7.5 to 20% linear gradients with 4% stacking gels.

accumulates more slowly in cells infected by *dl*2492 than in wild-type SV40-infected cells. DNA continued to accumulate 120 h after infection in *dl*2492-infected cells, whereas progeny DNA synthesis was complete between 72 and 96 h after infection in wild-type SV40-infected cells. In wild-type SV40-infected cells, most of the progeny DNA was encapsidated into virions which were released from infected cells by 120 h after infection. We found that this difference in the kinetics of accumulation of progeny DNA is reproducible and obtained identical results with mutants *dl*2491, *dl*2493, and *dl*2495 (data not shown).

Altered large T antigens encoded by deletion mutants. The T antigens encoded by the deletion mutants were examined by electrophoresis of immunoprecipitated [^{35}S]methionine-labeled extracts of infected CV-1 cells. In all cases, the mutants directed the synthesis of shortened large T antigen (Fig. 6). There was an excellent correlation between the size of the large T antigen produced and the size of the deletion. Mutant *dl*2402, which has the smallest deletion (15 bp), directed the synthesis of the large T antigen migrating closest to the position of wild-type large T antigen. Those mutants with the largest deletions (*dl*2401 and *dl*2405) directed the synthesis of large T antigens which showed the greatest size reduction. The deletions in this set of mutants should remove between 5 and 20 amino acids from large T antigen. All of the mutants encoded normal small t antigen. Small t antigen of *dl*2405 does not appear in Fig. 6

TABLE 2. Ability of SV40 mutants to provide adenovirus helper function

Exp no.	SV40 mutant no.	AD2 yield[a] (PFU)	Enhancement[b]	Relative enhancement[c]
1	— (Ad2 alone)	5.0×10^7	1	
	SVS	6.3×10^9	126	100
	2401	1.6×10^{10}	320	254
	2402	1.7×10^{10}	326	270
	2403	3.7×10^9	74	59
	2405	1.2×10^{10}	240	190
	1263	2.7×10^9	54	43
	1265	1.2×10^9	24	19
	862	2.1×10^9	114	91
2	— (Ad2 alone)	2.5×10^7	1	
	SVS	7.4×10^9	294	100
	2401	7.9×10^9	314	107
	2402	7.4×10^9	294	100
	2403	8.4×10^9	336	114
	2404	7.3×10^9	292	99
	2405	4.2×10^9	188	64
	1263	2.6×10^9	105	35
	1265	1.5×10^9	61	21
	862	6.3×10^9	252	86
3	— (Ad2 alone)	8.0×10^7	1	
	SVS	1.5×10^{10}	175	100
	2401	1.1×10^{10}	125	71
	2402	1.3×10^{10}	150	85
	2403	8.4×10^9	100	57
	2404	2.1×10^{10}	250	142
	2405	1.1×10^{10}	125	71
	1265	2.1×10^9	26	15
	862	1.5×10^{10}	175	100

[a] Determined in HeLa cells and expressed as plaque-forming units obtained for the entire harvested, sonicated culture.

[b] Ratio of Ad2 yield in the presence of helper (plaque-forming units) to Ad2 yield in the absence of helper (plaque-forming units).

[c] In each experiment the enhancement level due to wild-type SV40 is defined as 100%. Others are expressed relative to it.

because the antiserum used with this culture did not immunoprecipitate small t antigen; small t antigen was synthesized in *dl*2405- and *dl*2495-infected cells examined in other experiments (data not shown).

Adenovirus helper function. The ability of these deletion mutants to promote the growth of Ad2 in monkey cells was tested by infecting cultures of CV-1 cells with wild type or various mutants of SV40 and superinfecting, 24 or 48 h later, with Ad2. The yield of Ad2 from cultures infected with both Ad2 and SV40 (wild-type or mutants) was compared with the yield of Ad2 obtained from a culture of cells infected only by Ad2. The data from three separate experiments are shown in Table 2. All of the newly isolated deletion mutants tested were able to provide adenovirus helper function at levels comparable to that provided by wild-type SV40. In contrast, mutants *dl*1263 and *dl*1265 were found to have consistently lower levels of helper function, as previously reported (12).

Transformation of nonpermissive cells. The deletion mutants were tested for their ability to transform nonpermissive cells, using a dense-focus assay. Both the continuous NIH/3T3 line and mouse embryo fibroblasts derived from strain C57BL/6 were used. In both cells systems, all of the mutants transformed at frequencies equal to that of wild-type SV40. Data from typical experiments are summarized in Table 3. Since transformation frequencies are sensitive to MOI, and titers of virus stocks are not accurate to more than a factor of 2, a small difference in frequency of transformation may reflect differences in MOI. Mutants *dl*2402, *dl*2405, and *dl*2492 consistently produced foci that were denser and larger than those produced by wild-type SV40 (Fig. 7) or *dl*862 (data not shown).

TABLE 3. Transformation of nonpermissive cells by viable deletion mutants

Exp no.	Cell type	Mutant no.	No. of transformed colonies per 2×10^4 cells[a]
1	NIH/3T3[b]	862	21
		SV-S	28
		2401	22
		2402	27
		2403	25
		2404	19
		2405	35
2	C57BL/6[c]	862	19
		2401	21
		2402	54
		2403	10
		2404	12
		2405	69
3	C57BL/6[c]	SV-S	18
		2491	30
		2492	31
		2493	26
		2402	30
		1263	45
		1265	40

[a] Visible colonies were counted after 21 to 25 days after staining with methylene blue.

[b] Subconfluent NIH/3T3 cells were infected at an MOI of 25 PFU/cell. Twenty-four hours after infection, cells were trypsinized and replated at 2×10^4 cells per 100-mm culture dish. Cells were cultivated in DMEM + 2% FCS.

[c] Third-passage mouse embryo fibroblasts, derived from C57BL/6 mice, were seeded at a density of 2×10^4 cells per 25-cm^2 flask in DMEM + 10% FCS. After overnight incubation, cells were infected at an MOI of 25 PFU/cell. After adsorption, cells were cultivated in DMEM + 2% FCS.

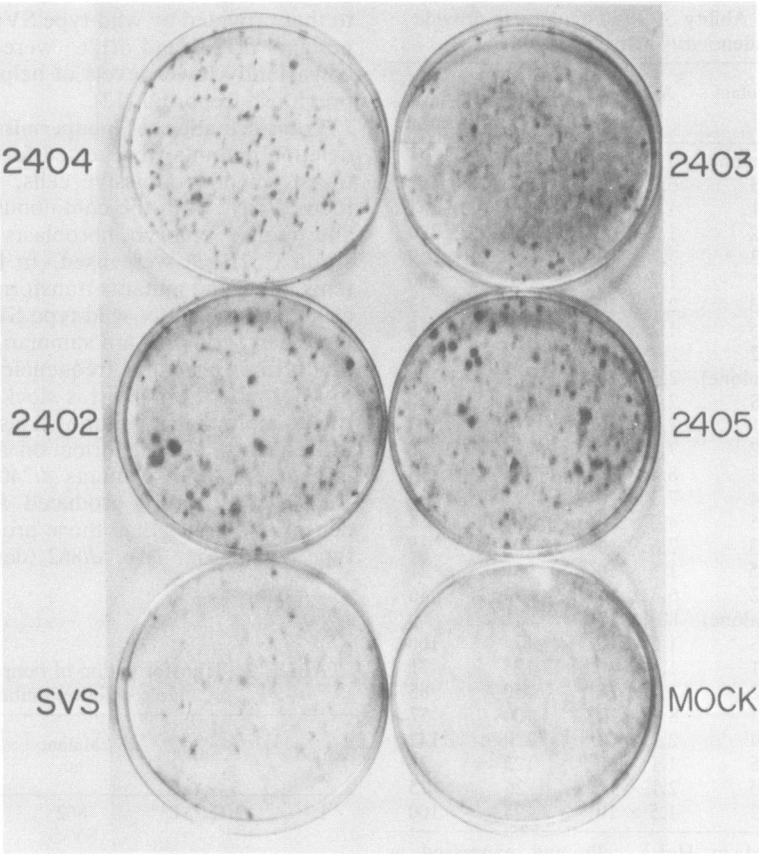

FIG. 7. Dense-focus transformation assay. Subconfluent cultures of NIH/3T3 cells were infected with viable deletion mutants or wild-type SV40 at an MOI of 50 PFU/cell. After 24 h, cells were trypsinized and seeded at 5×10^2, 5×10^3, and 5×10^4 cells per plate in DMEM containing 2% FCS. After 3 weeks, foci were visualized by staining with methylene blue. The plates shown above were seeded at 5×10^3 cells per plate.

From these data we conclude that these mutants are not impaired in their ability to transform both primary and established mouse cell lines.

DISCUSSION

Comparison of the DNA sequences of SV40 (21, 59) and polyoma (25, 67) reveals that these two papovaviruses share substantial sequence homology and that there are also regions unique to each virus. There exists a sequence of approximately 500 bp in the middle of the early region of polyoma DNA which has no counterpart in SV40. This portion of the genome encodes the portion of the polyoma virus middle T antigen not shared with other tumor antigens and, in another reading frame, a portion of the large T antigen. The unique portion of the SV40 early region spans approximately 400 bp between map positions 0.25 and 0.174 and encodes the C-terminal 19% of the large T antigen.

This portion of the SV40 early region is associated with (i) surface antigenicity and some determinants for tumor-specific transplantation antigenicity, based on studies with adenovirus-SV40 hybrid viruses (41, 50); (ii) adenovirus helper function, for which only the C-terminal 50 to 60 amino acids of the large T antigen appear to be necessary (20); (iii) a small SV40-associated RNA of 62 nucleotides, complementary to early mRNA at map position 0.21 (1, 45); (iv) an unassigned reading frame of 95 codons beginning at map position 0.22 (with a run of four consecutive methionine codons) and terminating at map position 0.169, beyond the normal termination site for large T antigen.

In this paper, we have described the construction, isolation, and preliminary characterization of two sets (*dl*2401 through *dl*2405 and *dl*2491 through *dl*2495) of five viable deletion mutants of SV40 which lack 15 to 60 bp in the middle of this portion of the early region, between map posi-

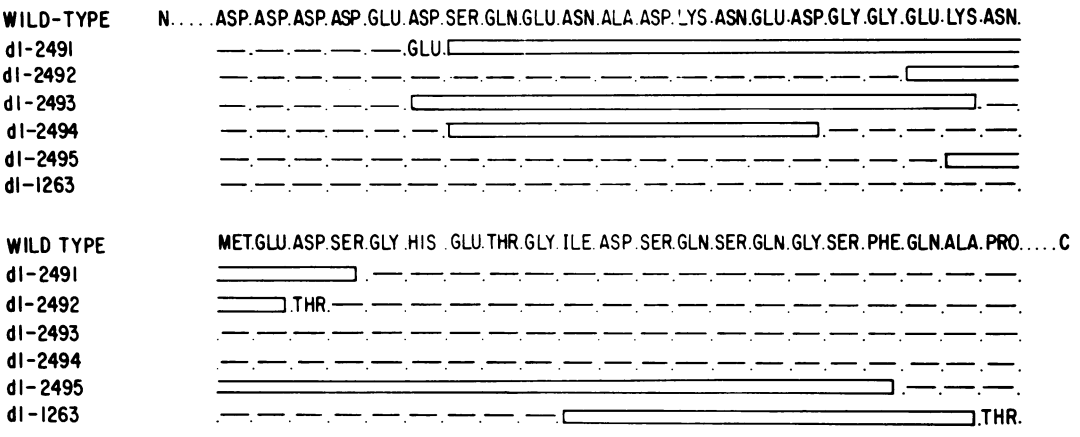

FIG. 8. Amino acids sequences of deletion mutant large T antigens. The amino acid sequences of the large T antigens of deletion mutants were determined from the nucleotide sequences (Fig. 2). Amino acids deleted are indicated by boxes. For the mutant T antigens, amino acids are indicated only where codon fusion or nucleotide insertion (in the case of *dl*2492) causes the insertion of a new amino acid into large T antigen. For comparison, the amino acid sequence of *dl*1263 large T antigen (17) is also indicated.

tions 0.218 and 0.198 mu. The first set contains, in addition, a deletion at the single *Hpa*II site (0.725 mu).

T antigens of the mutants. Correlation of the DNA sequence of the mutants with the amino acid sequence deduced for large T antigen allows determination of amino acid changes which occur in mutant large T antigens (Fig. 8). This region of the A gene encodes an unusual run of six consecutive acidic amino acids. Three of the mutants have one deletion endpoint adjacent to this portion of the A gene. *dl*2491 retains a run of six acidic residues, but the sixth residue becomes a glutamic acid instead of the aspartic acid found in wild-type T antigen. *dl*2493 lacks the final acid residue from this run, whereas the entire acidic sequence is retained in mutant *dl*2494. In the case of *dl*2494, there is ambiguity with respect to the actual location of the deletion because of the presence of short repeated sequences in the A gene in this region. Regardless of where the deletion is drawn, the six acidic residues are retained by the large T antigen of *dl*2494.

Mutants *dl*2492 and *dl*2495 have endpoints well downstream from this acidic region. The deletion in mutant *dl*2492 removes six amino acids from large T antigen and inserts a threonine as a result of codon fusion. T antigen is known to be phosphorylated on a threonine residue distal to this site near the C terminus of the protein. Perhaps this newly introduced threonine is also a site for phosphorylation. The deletion in *dl*2495 removes 20 amino acid residues.

Previous studies have shown that adenovirus helper function is provided by the C-terminal portion of large T antigen (12, 20). Any mutant with a deletion that causes a reading frame shift upstream from the helper function region should direct the synthesis of a T antigen lacking helper function. The existence of normal levels of helper function for all deletion mutants examined is consistent with our finding that the deletions in these mutants do not affect the large T antigen reading frame. Furthermore, the deletions must be sufficiently far upstream from the helper function region to leave it functionally intact, since none of the mutants examined showed reduced helper function. Mutant *dl*1263, lacking 33 bp at 0.195 mu (Fig. 2) retains only 30 to 40% helper function (Table 2 and reference 12). The deletion in mutant *dl*2405 overlaps that of *dl*1263, yet *dl*2405 shows normal helper function. Since helper function assays lack sufficient sensitivity to detect defects of less than 50% in helper function, it is possible that these mutants have a slightly lower level of helper function than wild-type SV40. These results suggest, however, that approximately full levels of helper function can be provided by a mutant T antigen (from *dl*2405) containing only 37 amino acids encoded distal to the site of the deletion.

Transformation. Polyoma and SV40 have different genetic requirements for transformation. Both viruses encode large and small T antigens, but polyoma encodes a middle T antigen as well. When a viral infection initiates the transforming event, polyoma large T antigen is required to establish the transformed state (18, 19, 23, 24, 62) but is not required for its maintenance (30, 40), and most transformed lines do not continue

to produce large T antigen. Thus, only middle or small t antigen or both are involved in the maintenance of transformation by polyoma.

Although a functional small t antigen enhances the frequency of transformation by SV40, only a functional large T antigen appears necessary for the initial stabilization of transformation (7, 64, 66, 71). In many cases, continued expression of large T is required to maintain the transformed state (8, 9, 46, 51, 58). Thus, SV40 large T antigen must be performing functions distinct from and in addition to those performed by polyoma large T antigen. It is possible that this function is contributed by the C-terminal 19% of the SV40 large T antigen, which is the only part lacking homology to polyoma large T antigen.

None of the mutants described in this report is defective in its ability to transform continuous and primary mouse cell lines, based on a focus assay. Furthermore, foci induced by *dl*2492, *dl*2402, and *dl*2405 are generally larger and denser than foci induced by wild-type SV40 or the other deletion mutants. It is interesting to note that Griffin and Maddock (27) have isolated a mutant (*dl*8) of polyoma virus with a deletion in the region encoding middle and large T antigens that also results in larger foci. Cells transformed by *dl*2491 and *dl*2493 have been tested for their ability to grow in soft agar; both have cloning efficiencies of approximately 10%, similar to the cloning efficiency in soft agar of wild-type SV40-transformed mouse cells (Polvino-Bodnar and Cole, unpublished data). More extensive experiments, including transformation assays conducted in soft agar, will be necessary for a thorough understanding of the transformation behavior of these mutants.

Limits of viability. Viable deletion mutants originally isolated by Shenk et al. (65) contained lesions which mapped in three different regions of the genome: 0.54 to 0.59, 0.68 to 0.75, and 0.165 to 0.175 mu. The first class of mutants affect only small t antigen, which is dispensible for lytic infection (15, 65). The other two sets of mutants map in intergenic regions. Subsequently, viable deletion mutants were isolated which have lesions in the coding regions for VP1, VP2, VP3, or large T antigen (13). From the studies described in this communication, it is now clear that substantial portions of T antigen distal to map position 0.218 are not required during either the lytic or transforming infection. None of the mutants described here showed any temperature sensitivity. This contrasts with the behavior of mutant *dl*(*ts*)1499, which has a deletion of 81 bp in this region and which is temperature sensitive for lytic growth (54, 55). Recently, a revertant of this mutant has been isolated (N. Bouck, A. Pater, C. Chang, and G. di Mayorca, personal communication). The revertant no longer shows temperature sensitivity, but appears to retain the original deletion, suggesting that the phenotype of *dl*(*ts*)1499 may be due to the presence of a previously undetected second mutation. Alternatively, a second mutation could have occurred to generate the revertant.

Mutants *dl*2491 through *dl*2495, *dl*1263, *dl*1265, and *dl*(*ts*)1499 all map in this area and together delete much of the sequence distal to 0.218 mu. However, all of these mutants retain the 77 nucleotides between the deletions of *dl*1263 and *dl*1265. Recently, defective (nonviable) mutants with deletions causing a reading frame shift at 0.22 mu have been isolated (J. Tornow and C. N. Cole, unpublished data), indicating that the C-terminal portion of large T antigen plays some essential role. Detailed characterization of additional mutants with lesions in this portion of the SV40 genome should allow determination of the functions performed by this portion of the A gene product in both lytic and transforming infections.

In addition, substantial portions of the alternate reading frame have been deleted in various mutants which have been described. These include the mutants reported here, as well as mutants with deletions between the end of the A gene (nucleotide 2677, Fig. 2) and the end of the alternate reading frame (nucleotide 2632) (22, 65). All mutants with deletions in the proximal part of this alternate coding frame do not cause frame shifts; only those mutants with deletions very near the end of this alternate reading frame are nontriplet deletions. Therefore, we cannot yet determine whether this alternate reading frame encodes an as yet undiscovered SV40 polypeptide.

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LITERATURE CITED

1. **Alwine, J. C., and G. Khoury.** 1980. Effect of a *tsA* mutation on simian virus 40 late gene expression: variations between host cell lines. J. Virol. 33:920–925.
2. **Alwine, J. C., and G. Khoury.** 1980. Simian virus-40 associated small RNA: mapping on the simian virus 40 genome and characterization of its synthesis. J. Virol. 36:701–708.

3. **Alwine, J. C., S. I. Reed, and G. R. Stark.** 1977. Characterization of the autoregulation of simian virus 40 gene A. J. Virol. **24**:22–27.
4. **Anderson, J. L., R. G. Martin, C. Chang, P. T. Mora, and D. M. Livingston.** 1977. Nuclear preparations of SV 40-transformed cells contain tumor-specific transplantation antigen activity. Virology **76**:420–425.
5. **Andersson, P., M. P. Goldfarb, and R. A. Weinberg.** 1979. A defined subgenomic fragment of *in vitro* synthesized Moloney sarcoma virus DNA can induce cell transformation upon transfection. Cell **16**:63–75.
6. **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.
7. **Bouck, N., N. Beales, T. Shenk, P. Berg, and G. di-Mayorca.** 1978. New region of the SV40 genome required for efficient viral transformation. Proc. Natl. Acad. Sci. U.S.A. **75**:2473–2477.
8. **Brockman, W. W.** 1978. Transformation of Balb/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of transformed phenotype and retransformation of wild-type virus. J. Virol. **25**:860–870.
9. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene A function in maintenance of transformation. J. Virol. **15**:619–635.
10. **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. U.S.A. **72**:1392–1396.
11. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101–1109.
12. **Cole, C. N., L. V. Crawford, and P. Berg.** 1979. Simian virus 40 mutants with deletions at the 3′ end of the early region are defective in adenovirus helper function. J. Virol. **30**:683–691.
13. **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.
14. **Cowan, K., P. Tegtmeyer, and P. D. Anthony.** 1973. Relationship of replication and transcription of simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **70**:1927–1930.
15. **Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeyer, K. Rundell, and P. Berg.** 1978. Organization and expression of early genes of SV40. Proc. Natl. Acad. Sci. U.S.A. **75**:117–121.
16. **Crawford, L. V., and P. Z. O'Farrell.** 1979. Effect of alkylation on the physical properties of simian virus 40 T-antigen species. J. Virol. **29**:587–596.
17. **Denhardt, D. T., and L. V. Crawford.** 1980. Simian virus 40 T-antigen: identification of tryptic peptides in the C-terminal region and definition of the reading frame. J. Virol. **34**:315–329.
18. **DiMayorca, G., J. Callender, G. Marin, and R. Giordano.** 1969. Temperature sensitive mutants of polyoma virus. Virology **38**:126–133.
19. **Eckhart, W.** 1969. Complementation and transformation by temperature sensitive mutants of polyoma virus. Virology **38**:120–125.
20. **Fey, G., J. B. Lewis, T. Grodzicker, and A. Bothwell.** 1979. Characterization of a fused protein specified by the adenovirus 2-simian virus 40 hybrid Ad2$^+$ ND1dp2. J. Virol. **30**:201–217.
21. **Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. van de Voorde, H. van Heuverswyn, J. van Herreweghe, G. Volckaert, and M. Ysebaert.** 1978. Complete nucleotide sequences of SV40 DNA. Nature (London) **273**:113–120.
22. **Fitzgerald, M., and T. Shenk.** 1981. The sequence 5′-AAUAAA-3′ forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell **24**:251–260.
23. **Fried, M.** 1965. Cell-transforming ability of a temperature-sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **53**:486–491.
24. **Fried, M.** 1970. Characterization of a temperature-sensitive mutant of polyoma virus. Virology **40**:605.
25. **Friedmann, T., A. Esty, P. LaPorte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV-40. Cell **17**:715–724.
26. **Gaudray, P., M. Rassoulzadegan, and F. Cuzin.** 1978. The expression of simian virus 40 early genes in transformed rat cells is correlated with the maintenance of the transformed phenotype. Proc. Natl. Acad. Sci. U.S.A. **75**:4987–4991.
27. **Griffin, B. E., and C. Maddock.** 1979. New classes of viable deletion mutants in the early region of polyoma virus. J. Virol. **31**:645–656.
28. **Griffin, J. D., S. Light, and D. M. Livingston.** 1978. Measurements of the molecular size of the simian virus 40 large-T antigen would require almost all the coding capacity of the early region. J. Virol. **27**:218–226.
29. **Grodzicker, T., J. B. Lewis, and C. W. Anderson.** 1976. Conditional lethal mutants of adenovirus type 2-simian virus 40 hybrids. II. Ad2$^+$ND1 host-range mutants that synthesize fragments of the Ad2$^+$ND1 30K protein. J. Virol. **19**:559–571.
30. **Hassell, J. A., W. C. Topp, D. B. Rifkin, and P. E. Moreau.** 1980. Transformation of rat embryo fibroblasts by cloned polyoma virus DNA fragments containing only part of the early region. Proc. Natl. Acad. Sci. U.S.A. **77**:3978–3982.
31. **Holland, J. J., and L. C. McLaren.** 1959. Improved method for staining cell monolayers for virus plaque counts. J. Bacteriol. **78**:596–597.
32. **Jensen, F., A. Girardi, R. Gilden, and H. Koprowski.** 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **52**:53–59.
33. **Jessel, D., J. Nudson, T. Landan, D. Jenen, and D. Livingston.** 1975. Interaction of partially purified SV40 T antigen with circular viral DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **72**:1960–1964.
34. **Kelly, S., M. A. R. Bender, and W. W. Brockman.** 1980. Transformation of BALB/c 3T3 cells by *tsA* mutants of simian virus 40: effect of transformation technique on the transformed phenotype. J. Virol. **33**:550–552.
35. **Kimura, G., and R. Dulbecco.** 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529–534.
36. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. Proc. Natl. Acad. Sci. U.S.A. **72**:673–677.
37. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
38. **Lai, C.-J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of SV40. Virology **66**:70–81.
39. **Lane, D. P., and L. V. Crawford.** 1979. T-antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
40. **Lania, L., D. Gandini-Attardi, M. Griffiths, B. Cooke, D. deCicco, and M. Fried.** 1980. The polyoma virus 100K large T-antigen is not required for the maintenance of transformation. Virology **101**:217–232.
41. **Lewis, A. M., Jr., A. S. Levine, C. S. Crumpacker, M. J. Levin, R. J. Samaha, and P. H. Henry.** 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol. **11**:655–664.
42. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17**:43–52.
43. **Linzer, D. I. H., W. Maltzman, and A. J. Levine.** 1979. The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen. Virology **98**:308–318.
44. **Little, J. W., I. R. Lehman, and A. D. Kaiser.** 1967. An

exonuclease induced by bacteriophage g. I. Preparation of the crystalline enzyme. J. Biol. Chem. **242**:672–678.
45. **Mark, D. R., and P. Berg.** 1979. A third splice site in SV40 early mRNA. Cold Spring Harbor Symp. Quant. Biol. **44**:55–62.
46. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599–612.
47. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
48. **Mertz, J. E., and P. Berg.** 1974. Defective SV40 genomes: isolation and growth of individual clones. Virology **62**:112–124.
49. **Moore, J. L., K. Chowdhury, M. A. Martin, and M. A. Israel.** 1980. Polyoma large tumor antigen is not required for the tumorigenesis mediated by viral DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:1336–1340.
50. **Morrow, J. F., P. Berg, T. J. Kelly, Jr., and A. M. Lewis, Jr.** 1973. Mapping of simian virus 40 early functions on the viral chromosome. J. Virol. **12**:653–658.
51. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene A function and maintenance of transformation. J. Virol. **15**:636–644.
52. **Paucha, A., A. Mellor, R. Harvey, A. E. Smith, R. W. Hewick, and M. D. Waterfield.** 1978. SV40 large and small T antigens have identical amino termini mapping at 0.65 map units. Proc. Natl. Acad. Sci. U.S.A. **75**:2165–2169.
53. **Paucha, E., and A. E. Smith.** 1978. The sequence between 0.59 and 0.54 map units on SV40 DNA code for the unique region of small-t antigen. Cell **15**:1011–1020.
54. **Pintel, D., N. Bouck, and G. di Mayorca.** 1981. Separation of lytic and transforming functions of the simian virus 40 A region: two mutants which are temperature sensitive for lytic functions have opposite effects on transformation. J. Virol. **38**:518–528.
55. **Pintel, D., N. Bouck, G. di Mayorca, B. Thimmapaya, B. Swerdlow, and T. Shenk.** 1979. SV40 mutant tsA1499 is heat-sensitive for lytic growth but generates cold-sensitive rat cell transformants. Cold Spring Harbor Symp. Quant. Biol. **44**:305–309.
56. **Pretell, J., R. S. Greenfield, and S. S. Tevethia.** 1979. Biology of simian virus 40 (SV40) transplantation rejection antigen (TrAg). V. In vitro demonstration of SV40 TrAg in SV40 infected nonpermissive mouse cells by the lymphocyte mediated cytotoxicity assay. Virology **97**:32–41.
57. **Rabson, A. S., G. T. O'Connor, I. K. Berezesky, and F. J. Paul.** 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exp. Biol. Med. **116**:187–190.
58. **Rassoulzadegan, M., B. Perbal, and F. Cuzin.** 1978. Growth control in simian virus 40-transformed rat cells: temperature-independent expression of the transformed phenotype in *tsA* transformants derived by agar selection. J. Virol. **28**:1–5.
59. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subrammanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–502.
60. **Reed, S. I., G. R. Stark, and J. C. Alwine.** 1976. Autoregulation of simian virus 40 gene A by T antigen. Proc. Natl. Acad. Sci. U.S.A. **73**:3083–3087.
61. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
62. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cell induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:722–728.
63. **Seif, R., and R. G. Martin.** 1979. Growth state of the cell early after infection with simian virus 40 determines whether maintenance of transformation will be A gene dependent or independent. J. Virol. **31**:350–359.
64. **Seif, R., and R. G. Martin.** 1979. Simian virus 40 small t antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. J. Virol. **32**:979–988.
65. **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.
66. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14**:79–88.
67. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
68. **Soule, H. R., and J. S. Butel.** 1979. Subcellular localization of simian virus 40 large tumor antigen. J. Virol. **30**:523–532.
69. **Soule, H. R., R. E. Lanford, and J. S. Butel.** 1980. Antigenic and immunogenic characteristics of nuclear and membrane-associated simian virus 40 tumor antigen. J. Virol. **33**:887–901.
70. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
71. **Steinberg, B. M., and R. Pollack.** 1979. Anchorage independence: analysis of factors affecting the growth and colony formation of wild-type and dl 54/59 mutant SV40-transformed lines. Virology **99**:302–311.
72. **Takemoto, K. K., R. L. Kirschtein, and K. Habel.** 1966. Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. J. Bacteriol. **92**:990–994.
73. **Tegtmeyer, P.** 1972. Simian virus 40 DNA synthesis: the viral replicon. J. Virol. **8**:516–524.
74. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene A in transforming infection. J. Virol. **15**:613–618.
75. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. J. Virol. **16**:168–178.
76. **Tevethia, S. S., D. C. Flyer, M. J. Tevethia, and W. C. Topp.** 1980. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). VII. Induction of SV40 TrAg in nonpermissive mouse cells by early viable SV40 deletion (0.54/0.59) mutants. Virology **107**:488–496.
77. **Tevethia, S. S., D. C. Flyer, and R. Tjian.** 1980. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). IV. Mechanism induction of SV40 transplantation immunity in mice by purified SV40 T antigen (D2 protein). Virology **107**:13–23.
78. **Tevethia, M. J., and S. S. Tevethia.** 1976. Biology of SV40 transplantation antigen (TrAg). I. Demonstration of SV40 TrAg on glutaraldehyde fixed SV40-infected African green monkey kidney cells. Virology **69**:474–489.
79. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
80. **Tjian, R., and A. Robbins.** 1979. Enzymatic activities associated with a purified simian virus 40 T antigen-related protein. Proc. Natl. Acad. Sci. U.S.A. **76**:610–614.
81. **Tooze, J.** 1980. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
82. **Van Heuverswyn, H., C. Cole, P. Berg, and W. Fiers.** 1979. Nucleotide sequence analysis of two simian virus 40 mutants with deletions in the region coding for the carboxyl terminus of the T antigen. J. Virol. **30**:936–941.
83. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. **9**:251–255.