JOURNAL OF VIROLOGY, June 1982, p. 854–864
0022-538X/82/060854-11$02.00/0

Vol. 42, No. 3

Mutant of Simian Virus 40 Large T-Antigen That Is Defective for Viral DNA Synthesis, but Competent for Transformation of Cultured Rat Cells

JAMES R. STRINGER

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received 26 October 1981/Accepted 8 February 1982

A mutant was isolated which demonstrates that the transforming activity of simian virus 40 large T-antigen is separable from its function in viral DNA replication. The mutant, SVR9D, is nonconditionally defective for viral DNA synthesis, but competent at wild-type level for morphological transformation of cultured rat cells. The lytic growth defect in SVR9D is complemented by the simian virus 40 *A* gene product present in the transformed CV1 cell line, COS1. The lesion in SVR9D DNA was mapped genetically by marker rescue of plaque formation and localized to a 214-base-pair segment of the viral genome bounded by nucleotide numbers 4100 and 4314. DNA sequence analysis showed the mutation to be an adenine-to-guanine transition at nucleotide number 4178. This change predicts a lysine-to-glutamic acid amino acid change at residue number 214 of the mutant large T-antigen polypeptide.

The large T-antigen of simian virus 40 (SV40) has long been known to possess multiple activities (for review see reference 31). During the viral replication cycle, large T-antigen initiates viral DNA replication and regulates viral gene transcription. In cells transformed by SV40, the large T-antigen induces a complex collection of morphological and physiological changes. Studies with temperature-sensitive conditional mutants have revealed much of what is known about the various activities of the large T-antigen. Most conditional mutants of large T-antigen, however, are equally temperature sensitive for lytic growth and transformation. Recently, two viruses were described in which growth and transformation are differentially sensitive to temperature (5, 20). The properties of these mutants support the idea that the lytic and transforming functions of large T-antigen are genetically separable (8). I report here the isolation and characterization of a new mutant of SV40, SVR9D, that clearly demonstrates that the transforming activity of SV40 large T-antigen is separable from its function in viral DNA replication. SVR9D T-antigen is nonconditionally incapable of supporting viral DNA replication, but the mutant protein retains wild-type potency for morphological transformation of cultured rat cells. Therefore, the viral DNA replication function of SV40 large T-antigen appears to be unnecessary to bring about transformation. The lesion is SVR9D falls in a region of viral A gene that has been previously implicated in the specific interaction between SV40 large T-antigen and the viral origin of DNA replication (24). SVR9D, and other mutants of its kind, may aid in the further localization of the viral DNA replication function within the large T-antigen polypeptide.

MATERIALS AND METHODS

Cells. SVRE9 cells were isolated and described by Pollack et al. (21). rat3 cells are a continuous line of Fisher rat cells isolated by Topp (30). Rat embryo fibroblasts (REF) are primary Fisher rat cells. CV1 cells are an established line of African green monkey kidney cells. COS1 cells are CV1 cells transformed by SV40 DNA that is deleted at the origin of DNA replication. COS1 cells have been described by Gluzman (7).

Transformation. Cells were transformed in 100-mm dishes overlaid with DME (Difco Laboratories, Detroit, Mich.) containing penicillin and streptomycin supplemented with 5% calf serum (Irvine Pharmaceuticals, Irvine, Calif.). The transformation assay was focus formation performed as follows. Monolayers of cells were transfected with viral DNA by the calcium precipitation method (32). Cells were incubated for 3 weeks at 37°C in DME with 5% calf serum and then scored for the presence of foci overgrowing the monolayer of normal cells. A single focus was picked from each plate either by teasing cells into the end of a pipette containing trypsin or by trypsinizing whole colonies within stainless-steel cloning cylinders. Cells derived from foci were subcloned by plating 100 to 200 cells in 100-mm plastic culture dishes. Individual colonies were ringed with cloning cylinders, and cells were removed by trypsin treatment. Growth in Methocel media was assayed as described by Frisque et al. (6) and in Table 3. SV40 T-antigen was visualized by

indirect immunofluorescence as described by Graessmann and Graessmann (10).

DNA transfection. Cells were transfected in 100-mm plastic culture dishes using either DEAE-dextran as described by Lai (15) or calcium precipitation as described by Van der Eb and Graham (32). Cells received 1 μg of sample DNA per 100-mm dish.

Marker rescue. Marker rescue assays were performed as described by Lai (15). Linearized mutant DNA was prepared from plasmid p9D by digestion with endo R·*Eco*RI. SV40 DNA fragments were purified either by cloning in pBR322 or by agarose gel electrophoresis. SV40 DNA fragments purified by cloning were as follows: *Hin*dIII fragments A, B, and D; *Mbo*I fragments D and F; both fragments produced by double digestion with endo R·*Taq*I and endo R·*Bam*HI; and the *Hpa*II-*Bam* fragment spanning 0.73 to 0.14 on the SV40 physical map. Two SV40 DNA fragments, *Hin*fI-C and *Alu*I-C, were isolated from gels. Mixtures were prepared which contained equimolar amounts of p9D DNA, the *Hpa*II-*Bam* late region fragment of SV40 DNA, and one other fragment of SV40 DNA. The *Hpa*II-*Bam* fragment was included to aid in circularization of heteroduplexes. Transfection of CV1 cells was mediated by DEAE-dextran. Each 60-mm culture dish received 1 μg of DNA.

Cell fusion. Cells were fused with polyethylene glycol as described by Botchan et al. (3). Low-molecular-weight DNA was extracted as described by Hirt (11).

Restriction endonucleases. Enzymes were purchased from either Bethesda Research Laboratories, Inc., Rockville, Md., or New England Biolabs, Inc., Beverly, Mass. DNA was digested under conditions recommended by the enzyme supplier.

Gel electrophoresis and blot hybridization. Agarose gels were formed with agarose (Sigma Chemical Co., St. Louis, Mo.) and run in 36 mM Tris–30 mM Na_2HPO_4—1 mM EDTA (pH 7.5). Gels were blotted by the method of Southern (25). Hybridization and radioautography were as described by Botchan et al. (2).

DNA sequencing. DNA was prepared and sequenced by the methods described by Maxam and Gilbert (17).

RESULTS

Origin of the SVR9D mutant. The SVR9D mutant was isolated from the viral DNA insertion present in the SV40-transformed rat cell line SVRE9. This cell line was originally described by Pollack et al. (21) who produced it by infection of REF cells with thrice-plaque-purified SV40. SVRE9 cells contain a single SV40 DNA insertion (2, 26). In previous work, the viral insertion was cloned from a library generated by ligation of endo R·*Sst*I cleaved SVRE9 cellular DNA into a modified λ WES:B phage (26).

Previous analysis of the cloned viral insertion by restriction endonuclease mapping had shown the SV40 DNA in SVRE9 cells to consist of 1.35 copies of the viral genome arranged in tandem (26; Fig. 1). By this structural criterion, then, the

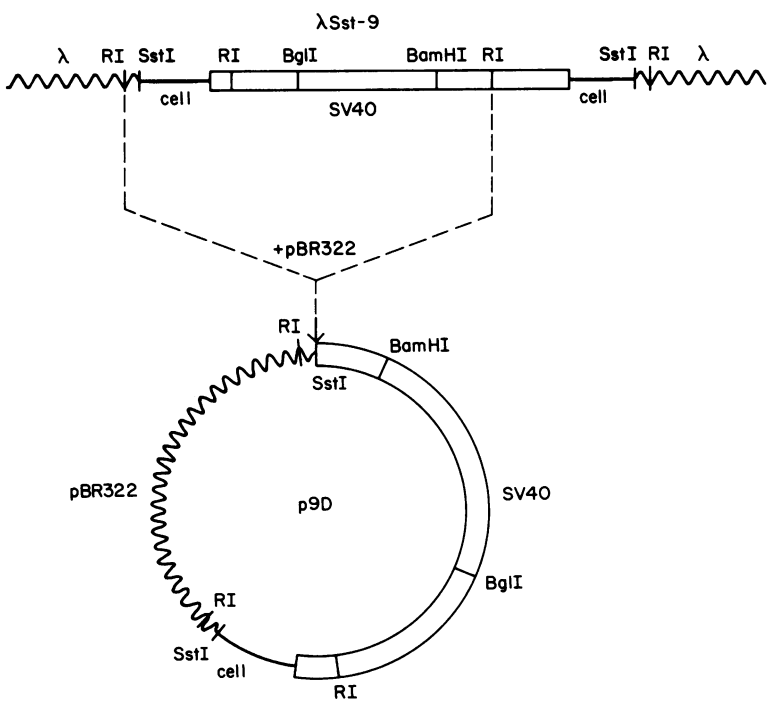

FIG. 1. SVR9D DNA clones. λ Sst9 is a chimeric λ phage, bearing a DNA fragment cloned from SVRE9 cells (25). Plasmid p9D was derived from λ Sst9 as indicated.

SV40 DNA insertion in SVRE9 cells appeared to comprise a full complement of viral genes. As a biological test for the presence of a lytically functional SV40 DNA insertion, I attempted to rescue virus by fusing transformed cells with CV1 cells. Surprisingly, fusion of SVRE9 cells with CV1 cells did not induce the production of infectious virus. Also, transfection of CV1 cells with the cloned SVRE9 viral DNA insertion failed to produce plaques.

SVR9D is defective for viral DNA replication. The failure to rescue virus either by cell fusion or by transfection of the cloned viral DNA insertion indicated that the SV40 genome integrated in SVRE9 cells was defective in some lytic function. Cell fusion and DNA transfection experiments were performed to determine the nature of block to lytic growth.

The first step in the rescue to SV40 by cell fusion is the production in the heterokaryon of freely replicating supercoiled forms of SV40 DNA (1, 3). SVRE9 cells were tested for the ability to produce free viral DNA as follows. SVRE9 cells (1×10^6) were fused to 2×10^6 CV1 cells by polyethylene glycol treatment as described in Materials and Methods. At 60 h after infection, cells were harvested and extracted by the method of Hirt (11) to obtain low-molecular-weight DNA. Samples were electrophoresed through an agarose gel, and the gel was blotted onto nitrocellulose. Hybridization to radiolabeled SV40 DNA followed by radioautography showed that SVRE9-CV1 heterokaryons produced no free SV40 DNA (Fig. 2, lane 3), whereas a control cell line (C1-4, which contained a wild-type, tandemly arranged SV40 DNA insertion) produced large amounts of form I SV40 DNA after fusion with CV1 cells (Fig. 2, lane 4). Thus, the lack of virus production by SVRE9 cells could be attributed to a failure to produce and replicate free circular SV40 DNA upon fusion to CV1 cells.

SVR9D large T-antigen is defective. Failure to produce free circular SV40 DNA could be due to either a defect in the viral origin of DNA replication or to a defect in the large T-antigen. A second cell fusion experiment, utilizing COS1 cells, showed that the failure of the SVRE9 viral DNA insertion to produce free SV40 DNA was due to a defect in the large T-antigen present in SVRE9 cells. COS1 cells are transformed CV1 cells that constitutively produce wild-type SV40 large T-antigen (7). Fusion with COS1 cells, therefore, endows heterokaryons with both functional SV40 large T-antigen and the SV40 permissiveness factors in monkey cells. SVRE9 cells (1×10^6) and 2×10^6 COS1 cells were fused by polyethylene glycol treatment and analyzed for the production of free SV40 DNA as described above. Figure 2 shows that fusion of

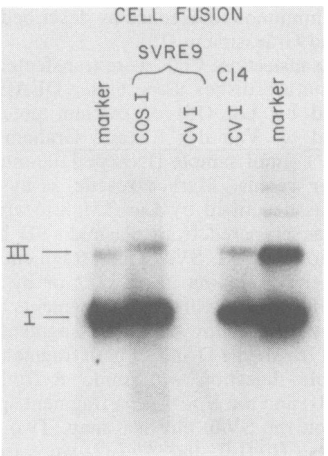

FIG. 2. Excision and replication of SVR9D DNA after fusion with permissive cells. Cells were fused as described in the text and harvested after 60 h. Low-molecular-weight DNA was extracted, and samples were electrophoresed through a 1% agarose gel. The gel was blotted onto nitrocellulose, and the blot was hybridized to radiolabeled DNA. Shown in the figure is a radioautograph of the hybridized blot. I and III mark the migration of supercoiled and linear SV40 DNA, respectively. Lanes labeled "marker" contained SV40 DNA. C14 is a rat cell line containing a wild-type SV40 DNA insertion.

SVRE9 and COS1 cells induced the production of form I SV40 DNA, which must have derived from the SVRE9 SV40 DNA insertion because the origin-defective SV40 DNA in COS1 cells cannot excise or replicate (7). Apparently, the endogenous large T-antigen in COS1 cells complemented the defective viral gene product in SVRE9 cells, allowing the production and replication of free, circular, mutant DNA.

Additional evidence in support of the idea that the SV40 genome in SVRE9 cells produces a defective large T-antigen came from DNA transfection experiments. Mutant DNA (SVR9D DNA) was prepared from λ Sst-9, a chimeric λ phage bearing a DNA fragment containing the SVRE9 viral insertion and flanking cellular sequences (Fig. 1). From this phage, a recombinant plasmid (p9D) was constructed that contained the *Eco*RI linear form of the SVR9D mutant genome, as shown in Fig. 1.

The SVR9D DNA, in plasmid p9D, was tested for the ability to replicate by an assay that measures the production of supercoiled viral DNA after transfection of cells with linear DNA molecules. Gluzman et al. (9) have shown that the transition of SV40 DNA from linear form to supercoiled form correlates with viral DNA replication. CV1 cells or COS1 cells were transfected with linear DNA produced by endo R·*Eco*RI digestion of either p9D DNA or SV40 DNA.

Transfection was mediated by DEAE-dextran as described in Materials and Methods. At various times after transfection, low-molecular-weight DNA was prepared by Hirt extraction, samples were fractionated by agarose gel electrophoresis, and SV40 DNA was detected by blotting, hybridization, and radioautography. Figure 3 shows that CV1 cells transfected with linear SVR9D DNA produced no detectable form I SV40 DNA even after 12 days (Fig. 3B), whereas form I DNA appeared 3 days after SVR9D DNA transfection of COS1 cells (Fig. 3A). Wild-type linear SV40 DNA circularized and replicated in both cell lines with the expected time course.

Production of SVR9D virus in COS1 cells. The experiments described above demonstrated that, when normal SV40 large T-antigen was

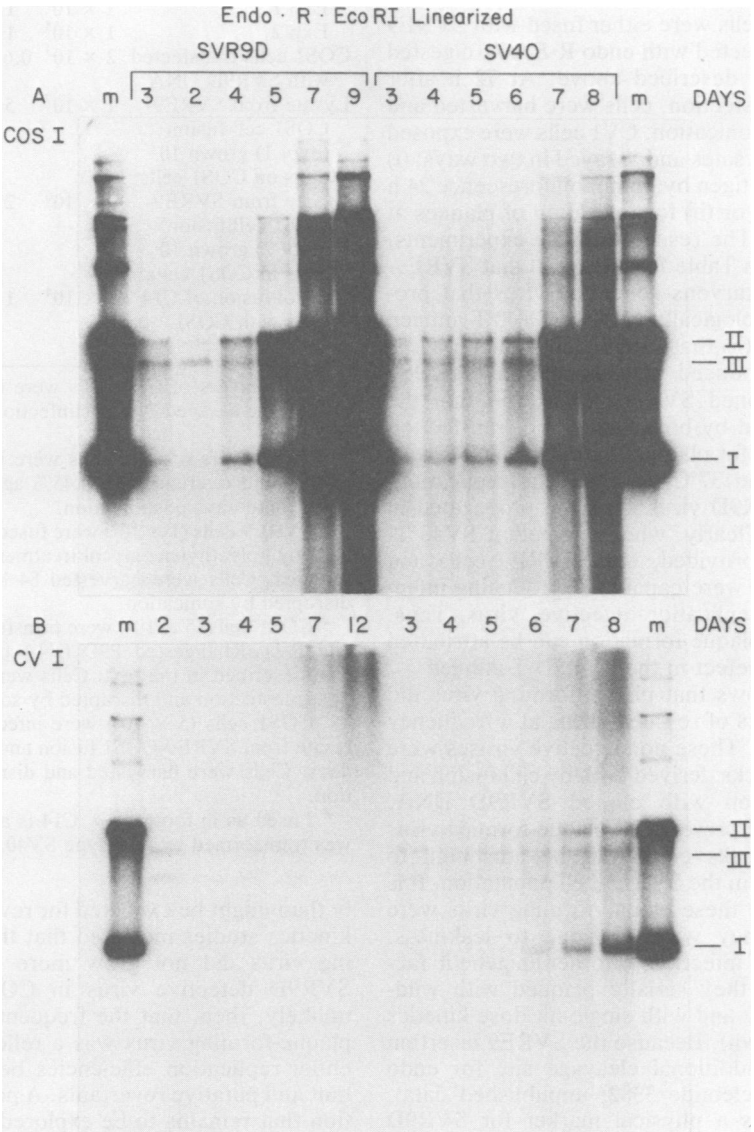

FIG. 3. Kinetics of SV40 and SVR9D DNA replication in CV1 and COS1 cells. Plates containing 5×10^6 cells were each transfected with 1 μg of either p9D DNA or SV40, both of which had been linearized by endo R·*Eco*RI. Cells were harvested after the number of days indicated, and low-molecular-weight DNA was extracted. DNA samples were electrophoresed through 1% agarose, and gels were blotted onto nitrocellulose. SV40 DNA was detected by hybridization to a plasmid clone of SV40 followed by radioautography. Roman numerals I, II, and III refer to the different forms of SV40 DNA. Lanes labeled M contained marker SV40 DNA. (A) Transfected COS1 cells; (B) transfected CV1 cells. The band running ahead of form III SV40 in lanes 2 through 5 of panel B is the input linear form of plasmid pBR322.

provided by COS1 cells, the SVR9D DNA insertion in SVRE9 cells was released from the integrated state and replicated. If SVR9D were defective in no other viral functions, fusion of COS1 cells with SVRE9 cells, or transfection of COS1 cells with SVR9D mutant DNA, should result in the rescue of SVR9D virus that would be replication defective in the absence of complementing T-antigen. Replication-defective SVR9D virus was rescued and assayed as follows. COS1 cells were either fused with SVRE9 cells or transfected with endo R·*Eco*RI-digested p9D DNA as described above. At 72 h after fusion or transfection, cells were harvested and disrupted by sonication. CV1 cells were exposed to sonicated lysates and assayed in two ways: (i) for SV40 T-antigen by immunofluorescence 24 h postinfection, or (ii) for formation of plaques at 32 and 37°C. The results of these experiments, summarized in Table 1, showed (i) that SVRE9-COS1 heterokaryons generated virus that produced immunologically reactive SV40 T-antigen in CV1 cells, (ii) that T-antigen-producing virus were also produced by transfection of COS1 cells with cloned SVR9D DNA, (iii) that the virus produced by both fusion and transfection was defective for plaque formation on CV1 cells at both 32 and 37°C, (iv) that the replication-defective SVR9D virus could be propagated in COS1 cells. Clearly, when competent SV40 T-antigen was provided, both SVRE9 cells and SVR9D DNA were capable of producing infectious, albeit replication-defective, virus. Thus, the block to plaque formation can be attributed entirely to a defect in the SVR9D T-antigen.

Table 1 shows that plaque-forming virus did occur in stocks of rescued virus, at a frequency of about 10^{-4}. These nondefective viruses were present in stocks derived both by cell fusion and by transfection with cloned SVR9D DNA. Therefore, the presence of plaque-forming virus in mutant stocks could not be attributed to heterogeneity in the SVRE9 cell population. It is not clear how these plaque-forming virus were generated. They were not due to leakiness, multiplicity of infection, or other epigenetic factors because they serially plaqued with wild-type efficiency and with single-hit dose kinetics (data not shown). Because the SVRE9 insertion contains an additional cleavage site for endo R·*Pst*I (at nucleotide 3382; unpublished data), that serves as a physical marker for SVR9D DNA, it could be conclusively shown that plaque-forming virus in SVR9D stocks were not contaminants but arose from the SVRE9 insertion. All plaque-forming variants were found to have the extra endo R·Pst cleavage site characteristic of SVR9D DNA (data not shown). Plaque-forming virus may have been revertants, but they occurred a hundredfold more frequently than might be expected for reversion. Growth kinetics studies indicated that the plaque-forming virus did not grow more efficiently than SVR9D defective virus in COS1 cells. It is unlikely, then, that the frequent occurrence of plaque-forming virus was a reflection of differential replication efficiencies between the mutant and putative revertants. A possible explanation that remains to be explored is that plaque-forming virus was frequently generated in COS1 cells by recombination between the mutant DNA and the integrated SV40 DNA in COS1 cells.

TABLE 1. Rescue and propagation of mutant SVR9D virus

Procedure	Virus titer of lysate		
	T-antigen-stained cells/ml[a]	Plaques/ml[b]	
		32°C	37°C
Fusion of SVRE9 cells with COS1 cells[c]			
Exp 1	1×10^5	1×10^1	1×10^1
Exp 2	1×10^5	1×10^1	1×10^1
COS1 cells transfected with SVR9D DNA[d]	2×10^3	0.6×10^1	0.6×10^1
Lysate from SVRE9-COS1 cell fusion (expt 1) grown 10 days on COS1 cells[e]	1×10^7	5×10^2	5×10^2
Lysate from SVRE9-COS1 cell fusion (expt 2) grown 10 days in COS1 cells[e]	2×10^6	2×10^2	2×10^2
Control fusion of C14 cells with COS1 cells[f]	3×10^4	1×10^4	1×10^4

[a] Monolayers of CV1 cells were infected with cell lysates and assayed 24 h postinfection by immunofluorescence.

[b] Monolayers of CV1 cells were infected with cell lysates and overlaid with 0.45% agar. Plaques were counted 10 days postinfection.

[c] SVRE9 cells (1×10^6) were fused to 2×10^6 COS1 cells by polyethylene glycol treatment as described in the text. Cells were harvested 84 h post-fusion and disrupted by sonication.

[d] COS1 cells (5×10^6) were transfected with 1 μg of endoR·*Eco*RI digested P9D DNA using DEAE-dextran described in the text. Cells were harvested 84 h posttransfection and disrupted by sonication.

[e] COS1 cells (5×10^6) were infected with 1 ml of lysate from SVRE9-COS1 fusion and incubated for 10 days. Cells were harvested and disrupted by sonication.

[f] Fused as in footnote *a*. C14 is a rat cell line that was transformed by wild-type SV40.

SVR9D DNA transforms rat cells with wild-type efficiency. Despite its defectiveness for lytic growth, SVR9D DNA retained full potency for transformation of cultured rat cells. The trans-

forming activity of SVR9D DNA was tested by transfection of endo R·*Eco*RI-digested p9D DNA into monolayers of three kinds of cells: (i) the rat3 line of Fisher rat fibroblasts (30), (ii) primary Fisher REF cells, and (iii) CV1 cells. DNA was introduced by the calcium precipitation method (32), and cells were incubated for 3 weeks and scored for the presence of foci overgrowing the monolayer of normal cells. The data (Table 2) showed that SVR9D DNA was as efficient as wild-type SV40 DNA in the production of foci in rat cells. All of the foci produced by SVR9D DNA expressed immunoreactive SV40 large T-antigen; a random sampling of 40 rat3 foci were all strongly immunofluorescent when stained for the viral protein. CV1 cells transformed by SVR9D DNA were T-antigen positive, as assayed by immunofluorescence, contained integrated viral DNA, as assayed by Southern blotting, and displayed the vacuolated morphology typical of transformed CV1 cells (data not shown). These cells have not been analyzed further.

Cells transformed by SVR9D DNA contain mutant T-antigen. SVR9D transformants should have the same phenotype for DNA rescue as SVRE9 cells; i.e., SVR9D-transformants should not produce free DNA when fused to CV1 cells, but should rescue SV40 DNA when fused to COS1 cells. Several clonal lines of SVR9D-transformed cells were tested for their ability to produce free viral DNA upon fusion with either CV1 cells or COS1 cells. Cells were fused with polyethylene glycol, and extracts of low-molecular-weight DNA were prepared as described above. Figure 4 shows that all of the cell lines tested produced low-molecular-weight SV40 DNA when fused to COS1 cells (Fig. 4A). Some of the cells (9.14 and SV40.1) produced discrete-size species of DNA homologous to the SV40 DNA radioactive probe. These discrete-size species of SV40-containing DNA molecules probably arise from recombination between tandemly repeated regions of SV40 DNA insertions. Cells may harbor more than one SV40 DNA insertion, and each insertion can be a complex arrangement of viral sequences in which part of the SV40 genome may be present in more than one copy, whereas other viral sequences are absent from the integrated SV40 DNA unit. The only constraint on SV40 DNA insertions seems to be the requirement that at least one intact copy of the viral A gene be preserved in each transformed cell (1–3, 7, 12, 26). Therefore, the appearance of multiple, variable-sized SV40-containing DNAs is not unexpected. Cells that did not produce discrete-size species of SV40-containing DNA upon fusion to COS1 cells did produce free DNA of heterogeneous size. Cells 17, 9-lb, 9-3h, 9-7b, 9-15b, D2F, and SVRIC all produced various amounts of heterogeneously sized DNA molecules containing sequences homologous with SV40 DNA. Such heterogeneous molecules are probably the products of imprecise excision of viral DNA insertions that are not arranged as partial tandem repeats. Indeed, Southern blot analysis of the SV40 DNA insertions in these cell lines showed them all to lack tandem-repeat arrangements (N. Sarvetnick, unpublished data). Imprecise excision of SV40 DNA insertions has been described in detail previously (1, 3).

TABLE 2. Transformation of cultured cells by SVR9D DNA

endo R·*Eco*RI-digested DNA[a]	Cell line transfected	Foci/μg of DNA[b]	% cells transformed to foci per μg DNA	(Foci expressing immunofluorescent T-antigen) foci tested
SVR9D	rat3	502 ± 52	0.02	40/40
	REF	50 ± 8	0.002	ND[c]
	CV1	2	0.00004	2/2
SV40 DNA	rat3	506 ± 39	0.02	20/20
	REF	47 ± 10	0.002	ND

[a] Subconfluent monolayers of 10^7 cells were transfected with 1 μg of endo R·*Eco*RI digests of either p9D SNA or SV40 DNA.

[b] Foci were counted at 3 weeks posttransfection. Values for rat3 and REF are averages of three plates ± standard deviation. CVI value is total number of foci from 0.5×10^7 cells.

[c] ND, Not done.

By contrast to the COS1 fusions, fusion to CV1 cells did not produce free SV40 DNA in any of the cells transformed with SVR9D DNA (Fig. 4B). Only SV40.1, a cell transformed by wild-type SV40 DNA, produced free SV40 DNA upon fusion to CV1 cells. Therefore, new SVR9D-transformants showed the same DNA rescue phenotype as SVRE9 cells, indicating that cells transformed by SVR9D DNA contain mutant T-antigen.

Properties of SVR9D-transformed rat cells. By most criteria of morphological transformation, SVR9D transformants did not appear different from wild-type SV40 transformants of the same cell lineage. Mutant and wild-type cell lines displayed similar morphologies and growth rates, plated with equal efficiency on plastic, and grew similarly in 1% fetal calf serum (data not shown).

Anchorage-independent growth is generally considered to be a more stringent criterion of transformation than those mentioned above. We tested the anchorage-independent growth of clonal rat cell lines transformed by either

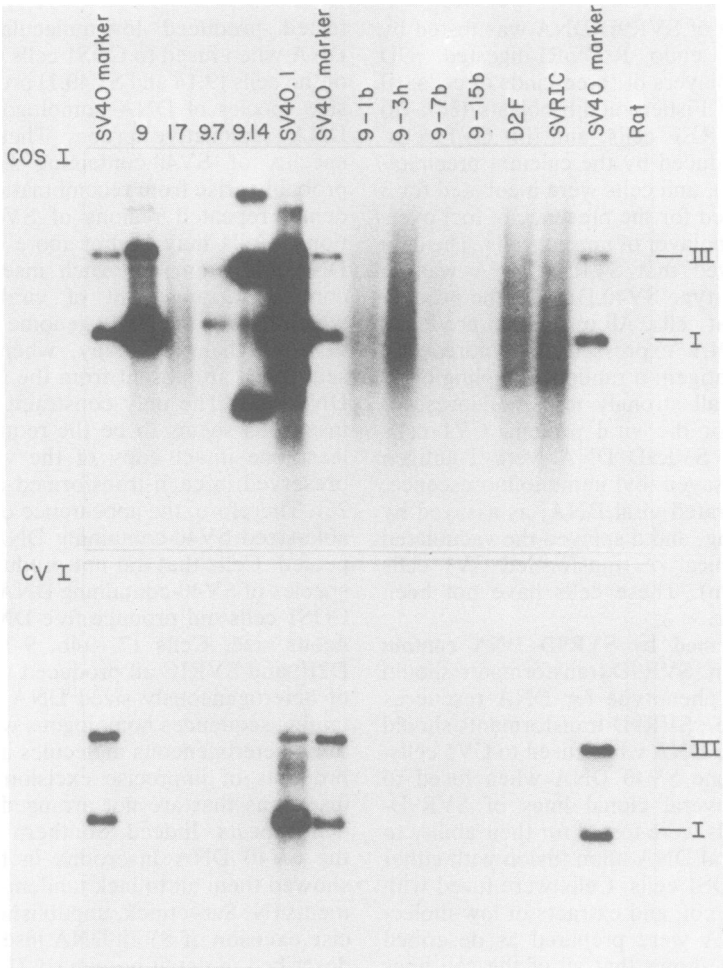

FIG. 4. Cell fusion phenotype of cells transformed by either SVR9D DNA or SV40 DNA. Clonal populations of cells transformed by DNA of either SV40 or SVR9D were fused with monkey cells as described in the text. Low-molecular-weight DNA was extracted from heterokaryons and electrophoresed through 1% agarose. The gels were blotted onto nitrocellulose, and blots were hybridized to radiolabeled SV40 DNA and radioautographed. Roman numerals I and III mark the positions of different forms of SV40 DNA. The key to the lane assignments is as follows: 9, SVRE9; 17, SVRE17, which is a sibling line of SVRE9 cells described by Pollack et al. (21); 9.7 and 9.14, REF cells transformed by SVR9D DNA; SV40.1, a REF transformed by SV40 DNA; 9-lb, 9-3h, 9-7b, 9-15b, D2F, and SVRIC, rat3 cells transformed by SVR9D DNA. Some cell lines (9, 9.7, 9.14, SV40.1) produced DNA of discrete size, whereas others (17, 9-16, 9-3h, 9-7b, 9-15b, D2F, and SVRIC) produced SV40 DNA molecules of heterogeneous size. Heterogeneous DNA molecules are probably the products of imprecise excision of viral DNA insertions. This phenomenon has been described in detail previously (1, 3).

SVR9D DNA or SV40 DNA, and the results of experiments using two different parental cell types are shown in Table 3. Table 3 shows that SVRE9 cells, the cell line from which SVR9D DNA was isolated, grew well in Methocel media. Recall that SVRE9 cells were derived from REF cells by Pollack et al. (21). We tested four new REF cell lines transformed by SVR9D DNA and found them all to form colonies in suspension at efficiencies comparable to SVRE9 cells. Interestingly, a REF cell line transformed by wild-type SV40 DNA, SV40.1, grew poorly compared to mutant transformants of REF cells. The significance of this difference cannot be assessed without comparison of more clonal cell lines of both mutant and wild-type origin. However, it is clear from these data that transformation by SVRE9D can induce REF cells to grow in suspension at efficiencies up to 10^3 times that of untransformed REF cells. Table 3 also shows the results of experiments done with rat3 cells that had been transformed by either SV40 DNA or SVR9D DNA. rat3 cells behaved quite differently from REF cells. Whereas all five mutant-

TABLE 3. Anchorage-independent growth of cells transformed by SVR9D DNA or SV40 DNA

Transformed cell line[a]	Parental cell line	Transforming DNA	Cloning efficiency in Methocel in 10% fetal calf serum (%)[b]
	rat3	None	0.001
SVRIG	rat3	SV40	0.5
9-D2F	rat3	SVR9D	0.04
9-1b	rat3	SVR9D	0.001
9-3h	rat3	SVR9D	0.001
9-7b	rat3	SVR9D	0.001
9-15b	rat3	SVR9D	0.001
	REF	None	0.001
SVRE9	REF	SVR9D	1
9.2	REF	SVR9D	1
9.7	REF	SVR9D	1
9.14	REF	SVR9D	0.3
9.20	REF	SVR9D	1
SV40.1	REF	SV40	0.1

[a] Cell lines were derived as follows: foci produced after transfection of subconfluent monolayers with endo R·*Eco*RI-linearized DNA (either SV40 or p9D), were picked, dispersed, and plated at low density on plastic. Individual colonies were picked, propagated, and tested for T-antigen by immunofluorescence. All cell lines tested were homogeneously expressing T-antigen.

[b] Cells were plated in 10 ml of 1.25% Methocel media at concentrations of 10^3 and 10^4 cells/ml. Colonies greater than 2 mm in diameter were scored after 4 weeks.

REF transformants grew well in Methocel media, only one of five SVR9D-transformed rat3 cell lines, 9-D2F, formed a measurable number of unanchored colonies. The wild-type rat3 transformant, SVRIG, grew well in Methocel media, but, again, more wild-type rat3 transformants must be tested before the significance of this difference between rat3 transformants of mutant and wild-type origin can be determined. Although it is possible that SVR9D is diminished in its capacity to induce anchorage-independent growth in rat3 cells, the growth properties of transformed REF cells clearly show that SVR9D transformants can grow well in suspension.

Genetic mapping of the SVR9D lesion by marker rescue. The location of the lytic growth lesion in SVR9D DNA was mapped by marker rescue of plaque formation on CV1 cells (15; and see Materials Methods). Monolayers of CV1 cells were transfected with heteroduplexes formed between endo R·*Eco*RI linearized SVR9D DNA (derived from plasmid p9D) and various fragments of wild-type SV40 DNA. Transfected cells were overlaid with 0.45% agar and assayed for plaques after 10 days at 37°C. The data (Fig. 5) showed the SVR9D mutation to lie between 0.443 and 0.484 map units on the SV40 physical map.

Determination of the SVR9D mutation by DNA sequence analysis. Marker rescue of plaque formation mapped the lytic growth lesion to DNA between the endo R·*Alu*I cleavage site at nucleotide 4314 and the endo R·*Mbo*I cleavage site at nucleotide 4100. p9D DNA was digested with either endo R·*Hin*fI or endo R·*Mbo*I and 3'-end labeled using the Klenow fragment of DNA polymerase I. endo R·*Hin*fI digests were then cut with endo R·*Mbo*I, and endo R·*Mbo*I digests were cut with endo R·*Hin*fI. End-labeled fragments corresponding to DNA between nucleotides 4100 and 4376 were isolated by electrophoresis on a 4.5% polyacrylamide gel and sequenced after Maxam and Gilbert (17). In the 214 base pairs determined by marker rescue to

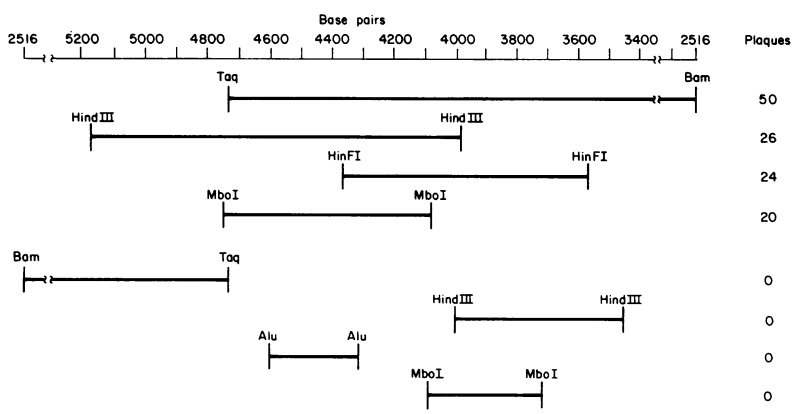

FIG. 5. Genetic mapping of the SVR9D mutation by marker rescue of plaque formation. The upper scale is the physical map of SV40 DNA numbered according to the BB convention (31). Lines below the scale show the map positions of the SV40 restriction endonuclease fragments used to form heteroduplexes with SVR9D DNA. Numbers to the right indicate the total number of plaques obtained in two 60-mm culture dishes of CV1 cells after transfection with 0.5 μg of DNA heteroduplexes per dish (see text for details).

contain the SVR9D lesion, a single base change was found. SVR9D DNA contained an adenine-to-guanine transition at nucleotide 4178 (Fig. 6). This mutation predicts a lysine-to-glutamic acid amino acid change at residue 214 of the large T-antigen.

DISCUSSION

SVR9D differentiates the lytic from the transforming activities of the SV40 large T-antigen and clearly demonstrates that part of this viral protein is dispensable for the initiation and maintenance of transformation. SVR9D T-antigen has lost the ability to support the measurable replication of viral DNA as assayed by either cell fusion or DNA transfection experiments (Fig. 2 and 3). This defect was not temperature sensitive in that SVR9D failed to produce plaques at either 32 or 37°C (Table 1). Despite its defectiveness for normal lytic function, SVR9D DNA produced dense foci on monolayers of cultured rat cells with efficiency equal to wild-type SV40 DNA (Table 2). Random screening by immunofluorescence indicated that all foci (40 out of 40) arising after SVR9D-DNA transfection expressed T-antigen (Table 2). The T-antigen in SVR9D transformants is a defective protein; cell fusion experiments showed that 7 out of 7 cloned cell lines derived from SVR9D-induced foci contained mutant T-antigen (Fig. 4).

REF cells transformed by SVR9D DNA are apparently fully transformed by the criteria of Pollack et al. (21). SVR9D and SV40 transformants of REF cells were indistinguishable by the criteria of clonability on plastic and growth in low serum. Furthermore, transformed cells derived from REF cells grew well in Methocel media regardless of the genotype of input viral DNA (Table 3). However, anchorage-independent growth proved to be a complex property strongly affected by the nature of the parental cell line. When SVR9D-transformed rat3 cells were picked as dense foci overgrowing a monolayer, and subcloned by cloned growth on plastic, the cells obtained did not grow in Methocel media at all, whereas a control rat3 cell line, transformed by SV40 DNA and subcloned on plastic, grew moderately well (Table 3). The significance of this apparent differential capacity of SVR9D and SV40 to induce anchorage-independent growth in rat3 cells is not yet clear, and comparison of more transformed cell lines of rat3 origin will be necessary to resolve this point.

The defect responsible for the block to lytic growth of SVR9D was complemented by wild-type T-antigen in COS1 cells (Fig. 2–4), indicating a lesion in the viral *A* gene of SVR9D. This lesion was mapped, by marker rescue of plaque formation, to a segment of the viral genome bounded by the endo R·*Alu*I cleavage site at nucleotide 4314, and the endo R·*Mbo*I cleavage site at 4100 nucleotides (Fig. 5). This segment of DNA lies just upstream of the region where nearly all TsA mutations have been mapped (see reference 4), and just downstream of the large T intron. Replication-defective SVR9D virus could be rescued from SVRE9 cells and propagated in COS1 cells (Table 1), indicating that the late genes of SVR9D are functional.

DNA sequence analysis showed the lesion in the SVR9D *A* gene to be a single adenine-to-guanine transition at nucleotide 4178 (Fig. 6). This change in the nucleotide sequence predicts the substitution of a glutamic acid amino acid residue for a lysine amino acid in the large T-

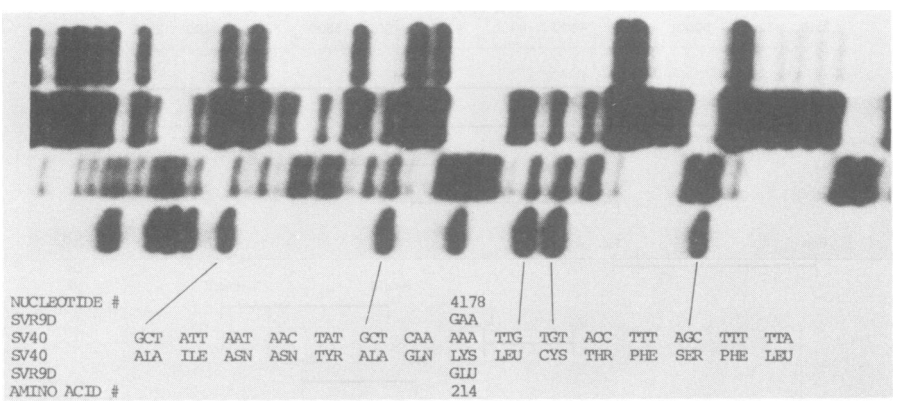

FIG. 6. DNA sequence at the site of the SVR9D mutation. The upper portion of the figure shows a segment of a sequencing-ladder radioautogram. Printed below the sequencing-ladder are the nucleotide and amino acid sequences of SV40 and SVR9D DNAs. The SVR9D lesion is an adenine-to-guanine change at nucleotide 4178 (BB numbering, reference 31). The change predicts a lysine-to-glutamic acid change at amino acid 214 of the viral large T-antigen.

antigen polypeptide. Such a change could, of course, result in a charge reversal at that position in the polypeptide.

Substitution of glutamic acid for lysine at amino acid 214 abolishes the ability of SV40 large T-antigen to support the efficient replication of viral DNA. The exact role of T-antigen in SV40 DNA synthesis is unknown, but one measurable activity of this protein that is almost certainly involved in viral DNA synthesis is the binding of the protein to the viral origin of DNA replication in vitro (14, 19, 22, 28). Preliminary in vitro binding experiments indicate that SVR9D T-antigen does not bind normally to the origin region of SV40 DNA. Other mutations in the same region of the T-antigen may also effect the specific DNA-binding activity of the protein. In their analysis of pseudorevertants of an SV40 mutant defective at the origin of DNA replication, Shortle et al. (24) mapped compensatory mutations to the region of the large T-antigen encoded between map units 0.43 and 0.50. In addition, there have recently been isolated two more mutants of SV40 C6 (Y. Gluzman, personal communication) and SV80 (M. Botchan, personal communication) that are defective for DNA synthesis due to lesions between 0.43 and 0.64 map units. It may be that SVR9D, C6, SV80, and pseudorevertants of SV40 origin mutants define a specific DNA-binding domain of SV40 large T-antigen.

Clearly, as proposed by Gluzman et al. (8), the function of SV40 large T-antigen that is responsible for the efficient replication of viral DNA is dispensable for the initiation and maintenance of morphological transformation. An attractive theory of SV40 transformation holds that the SV40 T-antigen induces unscheduled DNA synthesis and cell division through direct action on cellular origins of DNA replication (16, 27). Recently, DNA sequences that resemble those at the origin of SV40 DNA replication have been found in cellular DNA, and it has been suggested that these sequences may be cellular origins of DNA replication (13, 18). However, since SVR9D, which is defective in its normal association with the SV40 origin of DNA replication, can still transform cells with undiminished efficiency, it seems unlikely that transformation depends upon the ability of T-antigen to recognize sequences that resemble those at the SV40 DNA replication origin. In addition, Cosman and Tevethia (5) have described an SV40 mutant (TSA1642) that is the converse of SVR9D, in that it supports viral DNA replication but is temperature sensitive for transformation of rodent cells, as assayed by induction of anchorage-independent growth. The phenotype of TSA1642, therefore, suggests that something more than the viral DNA synthesis activity of SV40 large T-antigen is involved in the transformation of cells. SVR9D, C6, SV80, and TSA1642 do not rule out a transformation mechanism where the large T-antigen acts directly on cellular DNA, but they do suggest that the mode of such action may differ from that used to accomplish viral DNA synthesis.

Mutants like SVR9D will aid in the genetic resolution of the multiple activities ascribed to the SV40 large T-antigen. It will be particularly interesting to see if mutants that do not support viral DNA synthesis, and do not bind to the viral origin in vitro, are also relaxed in the autoregulation of early transcription. Mutant SVR9D arose spontaneously in SVRE9 cells in the absence of known selective pressure, and it is possible that other transformed cell lines harbor other mutant SV40 DNA insertions. Thus, transformed cells may act as reservoirs of SV40 lytic growth mutants. Many cell lines could be screened for large T-antigen mutants by the cell fusion assays applied in this study. Alternatively, the harvest of new mutants could be increased by using site-directed mutagenesis (23) to alter the viral genome in the vicinity of the SVR9D lesion. Mutants impaired in lytic growth, but still able to transform cells, could be selected by the transformation of permissive cells, as described by Gluzman et al. (8). Theoretically, it should be possible to produce mutants in every codon necessary for lytic growth but dispensable for transformation.

ACKNOWLEDGMENTS

I thank Laurel Garbarini for technical assistance, and Mike Ockler, Eric Lockhart, Patti Barkley, and Marilyn Goodwin for help in preparing the manuscript. Special thanks go to Yasha Gluzman for open communication of his results, for generously providing cloned viral DNA fragments, and for suggesting improvements in the manuscript.

This work was supported by Public Health Service grants CA-13106 and CA-06460 from the National Cancer Institute.

LITERATURE CITED

1. **Botchan, M., J. Stringer, T. Mitchison, and J. Sambrook.** 1980. Integration and excision of SV40 DNA from the chromosome of a transformed rat cell. Cell **20**:143–152.
2. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
3. **Botchan, M., W. Topp, and J. Sambrook.** 1979. Studies on simian virus 40 excision from cellular chromosomes. Cold Spring Harbor Symp. Quant. Biol. **43**:709–719.
4. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by TsA mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860–870.
5. **Cosman, D. J., and M. J. Tevethia.** 1981. Characterization of a temperature-sensitive, DNA-positive, nontransforming mutant of simian virus 40. Virology **112**:605–624.
6. **Frisque, R. J., D. B. Rifkin, and W. C. Topp.** 1980. Requirement for the large T and small T proteins of SV40 in maintenance of the transformed state. Cold Spring Harbor Symp. Quant. Biol. **44**:325–331.
7. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.

8. **Gluzman, Y., J. Davison, M. Oren, and E. Winocour.** 1977. Properties of permissive monkey cells transformed UV-irradiated simian virus 40. J. Virol. **22**:256–266.
9. **Gluzman, Y., R. J. Frisque, and J. Sambrook.** 1980. Origin-defective mutants of SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:293–300.
10. **Graessmann, M., and A. Graessmann.** 1976. Early simian virus 40 specific RNA contains information for tumor antigen formation and chromatin replication. Proc. Natl. Acad. Sci. U.S.A. **73**:366–370.
11. **Hirt, B.** 1967. Selective extraction of polyoma virus DNA. J. Mol. Biol. **26**:365–369.
12. **Hiscott, J. D., D. Murphy, and V. Defendi.** 1980. Amplification and rearrangement of integrated SV40 DNA sequences accompany the selection of anchorage-independent transformed mouse cells. Cell **22**:535–545.
13. **Jelinek, W. R., T. P. Toomey, L. Leinwand, C. H. Duncan, P. A. Biro, P. V. Choudary, S. M. Weissman, C. M. Rubin, C. M. Houck, P. K. Keininger, and C. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. U.S.A. **77**:1398–1402.
14. **Jessel, D., T. Landau, J. Hudson, T. Lalor, D. Tenen, and D. M. Livingston.** 1976. Identification of regions of the SV40 genome which contain preferred SV40 T-antigen-binding sites. Cell **8**:535–545.
15. **Lai, C.-J.** 1980. Mapping simian virus 40 mutants by marker rescue. Methods Enzymol. **65**:811–816.
16. **Martin, R. G.** 1981. The transformation of cell growth and transmorgification of DNA synthesis by simian virus 40. Adv. Cancer Res. **34**:1–68.
17. **Maxam, A. M., and A. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
18. **McCutchan, T., and R. Sager.** 1980. DNA sequences similar to those around the simian virus 40 origin of replication are present in the monkey genome. Proc. Natl. Acad. Sci. U.S.A. **78**:95–99.
19. **McKay, R. D. G.** 1981. An immunoassay for the interaction of the SV40 T-antigen related protein, D2T, and DNA. J. Mol. Biol. **145**:471–488.
20. **Pintel, D., Bouck, N., and DiMayorca, G.** 1981. Separation of lytic and transforming functions of the simian virus 40 A region: two mutants that are temperature-sensitive for lytic functions have opposite effects on transformation. J. Virol. **38**:518–528.
21. **Pollack, R., R. Risser, S. Colon, and R. Rifkin.** 1974. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **71**:4792–4796.
22. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T-antigen binds to simian virus 40 DNA at the origin of replication. Proc. Natl. Acad. Sci. U.S.A. **72**:1605–1609.
23. **Shortle, D., and D. Nathans.** 1978. Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. Proc. Natl. Acad. Sci. U.S.A. **75**:2170–2174.
24. **Shortle, D. R., R. F. Margolskee, and D. Nathans.** 1979. Mutational analysis of the simian virus 40 replicon: pseudo-revertants of mutants with a defective replication origin. Proc. Natl. Acad. Sci. U.S.A. **76**:6128–6131.
25. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
26. **Stringer, J. R.** 1981. Integrated simian virus 40 DNA: nucleotide sequences at cell-virus recombinant junctions. J. Virol. **38**:671–679.
27. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
28. **Tjian, R.** 1978. The binding site on SV40 DNA for a T-antigen related protein. Cell **13**:165–179.
29. **Tjian, R., G. Fey, and A. Graessmann.** 1978. Biological activity of purified SV40 T-antigen proteins. Proc. Natl. Acad. Sci. U.S.A. **75**:1279–1283.
30. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113**:408–411.
31. **Tooze, J.** 1980. The molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Van der Eb, A. J., and F. L. Graham.** 1980. Assay of transforming activity of tumor virus DNA. Methods Enzymol. **65**:826–839.