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Two Separable Functional Domains of Simian Virus 40 Large T Antigen: Carboxyl-Terminal Region of Simian Virus 40 Large T Antigen Is Required for Efficient Capsid Protein Synthesis

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The carboxyl-terminal portion of simian virus 40 large T antigen is essential for productive infection of CV-1 and CV-1p green monkey kidney cells. Mutant *dl*A2459, lacking 14 base pairs at 0.193 map units, was positive for viral DNA replication, but unable to form plaques in CV-1p cells (J. Tornow and C. N. Cole, J. Virol. 47:487–494, 1983). In this report, the defect of *dl*A2459 is further defined. Simian virus 40 late mRNAs were transcribed, polyadenylated, spliced, and transported in *dl*A2459-infected cells, but the level of capsid proteins produced in infected CV-1 green monkey kidney cells was extremely low. *dl*A2459 large T antigen lacks those residues known to be required for adenovirus helper function, and the block to productive infection by *dl*A2459 occurs at the same stage of infection as the block to productive adenovirus infection of CV-1 cells. These results suggest that the adenovirus helper function is required for productive infection by simian virus 40. Mutant *dl*A2459 was able to grow on the Vero and BSC-1 lines of African green monkey kidney cells. Additional mutants affecting the carboxyl-terminal portion of large T were prepared. Mutant *inv*2408 contains an inversion of the DNA between the *Bam*HI and *Bcl*I sites (0.144 to 0.189 map units). This inversion causes transposition of the carboxyl-terminal 26 amino acids of large T antigen and the carboxyl-terminal 18 amino acids of VP1. This mutant was viable, even though the essential information absent from *dl*A2459 large T antigen has been transferred to the carboxyl terminus of VP1 of *inv*2408. The VP1 polypeptide carrying this carboxyl-terminal portion of large T could overcome the defect of *dl*A2459. This indicates that the carboxyl terminus of large T antigen is a separate and separable functional domain.

The simian virus 40 (SV40) large T antigen performs many functions during lytic and transforming infections by SV40. It is involved in the initiation of viral DNA synthesis (10, 61), triggers host DNA replication (31), is involved in the autoregulation of the level of its own mRNA (2, 52, 63), and is required for both the initiation and maintenance of malignant transformation of nonpermissive cells (34, 35, 62). In addition, it provides a function that permits the growth of human adenoviruses in monkey cells (14, 30, 50). The polypeptide contains both DNA-binding (32, 64) and ATPase (11, 28) activities.

Analysis of the biochemical properties of mutants with deletions in the *A* gene, encoding large T antigen, indicates that sequences between 0.50 and 0.43 map units are required for specific binding to the SV40 origin of DNA replication (12, 48, 56), and sequences in the vicinity of 0.30 map units (m.u.) (11) are associated with ATPase activity. Thus, the polypeptide contains multiple functional domains. It is likely that both of these activities must exist within each active T antigen polypeptide, since complementation between binding-competent, ATPase-defective and ATPase-positive, binding-defective mutants has not been observed (65; J. Tornow, R. Clark, C. Cole, and R. Tjian, and C. N. Cole, manuscript in preparation), even though oligomeric forms of T antigen are found in virus-infected cells (8, 19, 20, 49).

There is considerable homology between the large T antigens of SV40 and polyomavirus, but each contains a region not found in the other large T antigen (Fig. 1) (24, 27, 51, 58). The carboxyl-terminal 113 amino acids of SV40 large T (residues 596 through 708, encoded by 0.25 through 0.174 m.u.) is the only portion of the polypeptide showing no homology to any portion of polyomavirus large T. This portion of large T is known to be involved in providing the adenovirus helper function (23, 47). In addition, infected cells contain a 64-base RNA that is complementary to early mRNA and that is encoded by sequences near map position 0.21 (1, 41). Many completely viable mutants of SV40 do not produce this small RNA (1, 47; M. Polvino-Bodnar, Ph.D. thesis, Yale University, New Haven, Conn., 1983), suggesting that it does not play an important role during the lytic infection cycle. Finally, this portion of the early region contains an alternate open reading frame of 95 condons. Almost all of this alternative reading frame is deleted in one or another viable mutant genome (15, 22, 25, 44, 45, 47, 66), suggesting that it also does not play an important role. There is no evidence that it is ever used. The role played by this portion of large T antigen during the normal SV40 infection cycle has remained a mystery.

We described previously a set of mutants with deletions at *Dde*I sites in the early region of SV40 (65, 66). One of these mutants, *dl*A2459, with a 14-base-pair (bp) deletion at 0.193 m.u., is nonviable, but differs from most nonviable T antigen mutants in its ability to catalyze the replication of SV40 DNA (66). This mutant defines a function that is provided by SV40 large T and that is required after the onset of viral DNA replication. The function defective in *dl*A2459 can be

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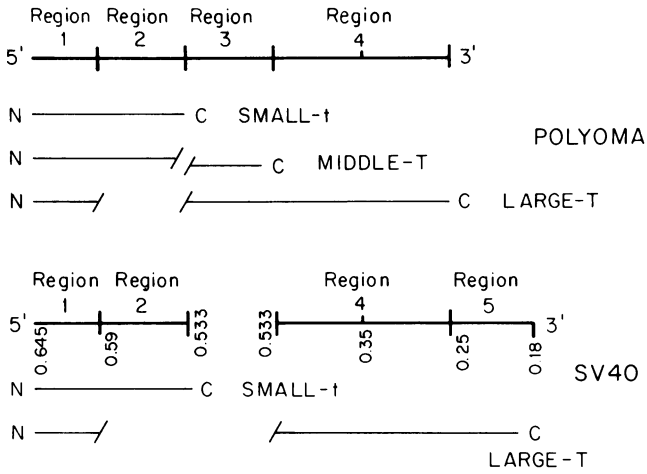

FIG. 1. Comparison of the early regions of SV40 and polyomavirus. The regions that show homology have the same numbers. Unique regions (polyomavirus region 3 and SV40 region 5) have different numbers. Homology was determined (27) from comparison of published sequence information for SV40 (24, 27, 51, 58). Region 1 corresponds to the NH_2-terminal regions shared by all papovavirus tumor antigens. Region 2 corresponds to the unique portion of small t antigen; the polyomavirus middle t antigen also contains this amino acid sequence. Region 3 encodes the unique portion of polyomavirus middle T antigen and, in a different reading frame, part of large T antigen. Region 4 encodes that part of large T antigen wherein map almost all of the *ts*A SV40 and *ts*a polyomavirus mutants. Region 5 encodes that portion of SV40 large T antigen which has no homology to polyomavirus large T antigen.

provided by group A mutants, which contain either point mutations or deletions that are multiples of 3 bp (65). Thus, mutants able to express the carboxyl-terminal domain of large T are able to complement *dl*A2459 intracistronically. This indicates that some critical function is provided by this portion of large T antigen and that this function can be performed by T-antigen molecules defective in other T-antigen properties.

In this report, the defect of *dl*A2459 is further defined. The mutant was positive for the synthesis and processing of late mRNA, but the level of capsid proteins found in infected CV-1 green monkey cells was drastically reduced. This defect resembles that of human adenoviruses in monkey cells (26, 37). Since *dl*A2459 large T antigen lacks those residues known to be required for helper function, these results suggest that adenovirus helper function is required for productive infection by SV40. Mutant *dl*A2459 is a host range mutant, since it is able to form plaques on BSC-1 and Vero green monkey kidney monolayers.

Additional mutations affecting the carboxyl-terminal portion of large T are also described. In one, *inv*2408, the carboxyl terminal 18 amino acids of VP1 and the carboxyl-terminal 26 amino acids of large T antigen were exchanged. This mutant was viable. The VP1-T antigen fusion protein of this mutant can overcome the defect of *dl*A2459. This indicates that the portion of large T that provides the late function defined by *dl*A2459 is a separate and separable functional domain.

MATERIALS AND METHODS

Construction of SV40 mutants. The construction of mutants *dl*A2459, *dl*A2411, and *dl*A2420 has been described previously (65, 66). Mutant *inv*2408 was constructed (Fig. 2) by digesting SV40 viral DNA to completion with *Bam*HI and *Bcl*I, yielding fragments of 237 and 5,006 bp. Although these two restriction endonucleases recognize different sequences, each produces 5'-GATC-3' termini at its cleavage site. These fragments were ligated with T4 DNA ligase at a DNA concentration of 20 μg/ml. Subsequently, the DNA was digested with *Eco*RI and ligated with *Eco*RI-digested pBR322. The ligation reaction contained 10 μg of each DNA per ml. The HB101 strain of *Escherichia coli* was transfected with this DNA, and colonies were selected on plates containing ampicillin (20μg/ml). Minilysate DNA preparations were screened by digestion with *Eco*RI, *Bam*HI, and *Sau*3AI. inv2408 contained two sites recognized by *Eco*RI and a single *Bam*HI site and produced the same *Sau*3AI digestion pattern as did wild-type SV40 cloned into the *Eco*RI site of pBR322.

dl(*inv*)2408E was constructed by combining the large *Apa*I-*Kpn*I fragment of *inv*2408 (containing the entire early region and including the inversion) with the small *Apa*I-*Kpn*I fragment of *dl*BC865 (containing most of the late region and covering the deletion at the *Eco*RI site) (Fig. 3). The ligation mixture was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and four times with ether, ethanol precipitated, and dissolved in 10 mM Tris-chloride (pH 7.5)–1 mM EDTA–10 mM NaCl. The DNA was digested with *Eco*RV and ligated with an equimolar concentration of *Eco*RV-digested pBR322 DNA, at a total DNA concentration of 20 μg/ml. Insertion of DNA into the *Eco*RV site of pBR322 inactivates the tetracycline resistance gene. After transfection, colonies were selected on plates containing ampicillin (20 μg/ml) and screened to identify those colonies sensitive to tetracycline (15 μg/ml).

dl(*inv*)2408L was constructed by combining the 946-bp *Apa*I-*Pst*I fragment of *inv*2408 (0.091 to 0.271 m.u. and including the inversion), the 2,004-bp *Pst*I-*Kpn*I fragment of *dl*A1209 (0.271 to 0.716 m.u. and covering the deletion between 0.587 and 0.650 m.u.) and the 6,326-bp *Kpn*I-*Apa*I fragment of pCC2, which contains the complete wild-type SV40 genome inserted into the *Eco*RI site of pBR322. For the ligation, an equimolar concentration of the three fragments and a total DNA concentration of 25 μg/ml were used.

Cells, viruses, and plasmids. The growth of CV-1 and CV-1p cells has been described previously (47). The same

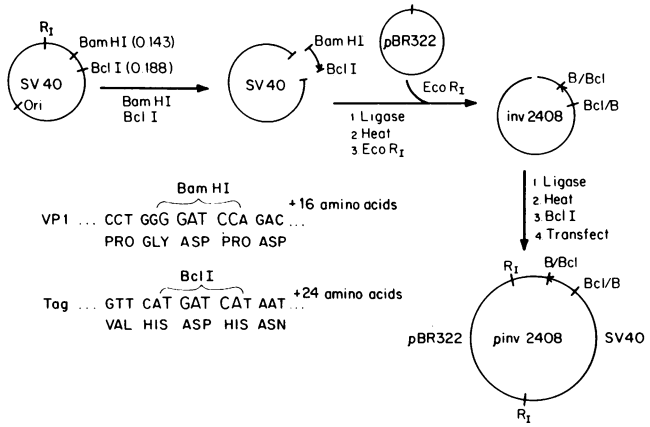

FIG. 2. Construction of SV40 mutant *inv*2408 and its insertion into pBR322. Also shown is the nucleotide sequence of the genes for VP1 and large T antigen at the *Bam*HI and *Bcl*I sites. Since GAT encodes aspartic acid in the reading frames of both polypeptides, this inversion results in transposition of the carboxyl termini of VP1 and large T antigen.

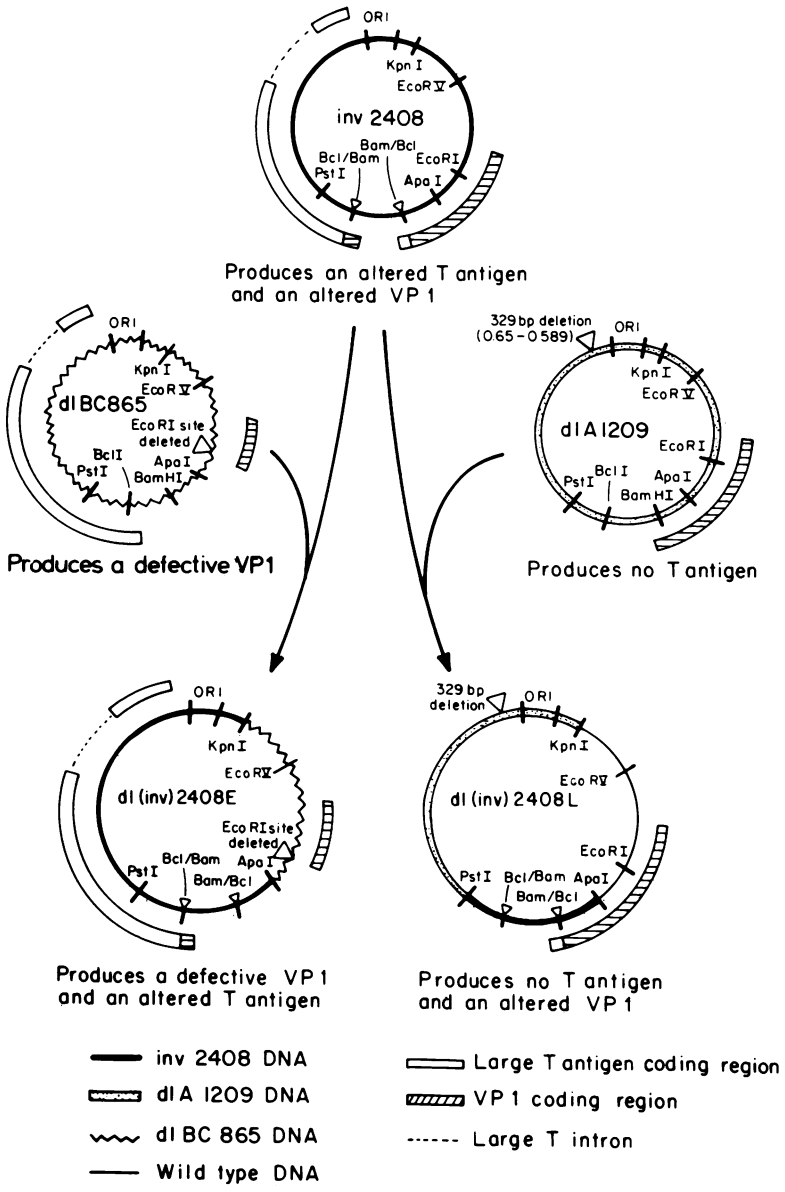

FIG. 3. Organization and origin of SV40 sequences in mutants *dl*(*inv*)2408E and *dl*(*inv*)2408L.

protocols were used for growth of BSC-1 and Vero cells, which are also African green monkey kidney lines. All were maintained in Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS). Our wild-type strain of SV40 (WT830) was the small-plaque strain originally characterized by Takemoto et al. (60). Wild-type SV40 DNA was digested with *Eco*RI and cloned into the *Eco*RI site of pBR322.

All mutants were maintained as recombinant plasmids (inserted into pBR322) in the HB101 strain of *E. coli*. Previously described methods were used for bacterial transformations (40) and for the preparation of minilysates (7) and purified plasmid DNA (13). All DNA preparations were purified by equilibrium buoyant density centrifugation in gradients containing CsCl and ethidium bromide (300 μg/ml). Mutants *dl*BC865 (9), *dl*A1209, and *dl*E1226 (15), and *ts*A1642 (16) were cloned in the *Bam*HI site of pBR322. All other mutants except *dl*(*inv*)2408E were cloned in the *Eco*RI site of pBR322; *dl*(*inv*)2408E was cloned in the *Eco*RV site of pBR322.

Transfection of eucaryotic cells. Plasmid DNA was first digested with either *Bam*HI, *Eco*RI, or *Eco*RV to separate bacterial and viral sequences. This DNA was then ligated at low concentration (5 μg/ml) with T4 DNA ligase to recircularize the viral DNA. Confluent monolayers of CV-1 cells were incubated in Tris-buffered saline (25 mM Tris-chloride [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.05 mM $MgCl_2$, 0.7 mM $CaCl_2$) containing 1 μg of DNA per ml and 500 μg of DEAE-dextran (5×10^5 daltons; Sigma Chemical Co., St. Louis, Mo.) per ml for 30 to 45 min at 37°C. Plates 100 mm in diameter received 0.6 to 1.0 mls of this mixture, 60-mm plates received 0.2 to 0.3 ml, and 35-mm plates received 0.1 to 0.2 ml. Cells were then washed twice with Tris-buffered saline and incubated with DMEM containing 2% FBS and 100 μM chloroquine diphosphate (Sigma) for 4 to 6 h at 37°C. The treatment with chloroquine

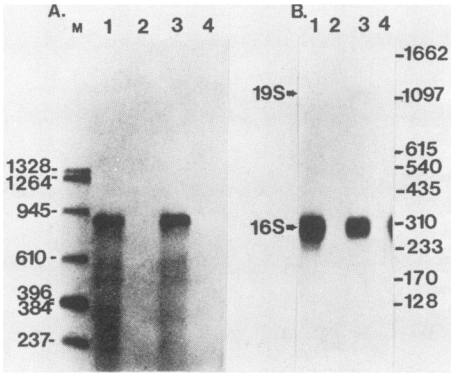

FIG. 4. S1 nuclease protection analysis of late mRNA produced in *dl*A2459-infected cells. Total cytoplasmic RNA was prepared 72 h after transfection. *Eco*RI-digested SV40 DNA was 3′ end labeled (A) or 5′ end labeled (B) as described in the text. The DNA probes were hybridized to 25 μg of RNA under conditions of DNA excess at 47°C for 15 h. After S1 nuclease treatment, samples were analyzed by electrophoresis in 1.4% alkaline agarose (30 mM NaOH, 2.5 mM EDTA) gels. After electrophoresis, gels were fixed in 7% trichloroacetic acid, dried, and exposed to Kodak XAR-5 film. Lanes (A and B): 1, wild-type SV40; 2, *dl*A2411; 3, *dl*A2459; 4, mock infected. In A, 5′ end labeled, *Mbo*I-digested SV40 DNA served for size markers; in B, 3′ end labeled, *Dde*I-digested plasmid containing SV40 inserted into the *Eco*RI site of pBR322 was used for size markers.

diphosphate increases the percentage of cells expressing transfected DNA from 1 to 2% to 40 to 60% (39). After the chloroquine diphosphate treatment, the chloroquine-containing medium was replaced with DMEM containing 2% FBS, and cells were incubated at 37°C.

Plaque assays and genetic complementation tests. Wild-type and mutant DNAs were separated from bacterial and plasmid sequences as described above. Confluent monolayers of CV-1p, BSC-1, or Vero cells were transfected with three different concentrations of each DNA (10, 1, and 0.1 ng per 60-mm plate) by using DEAE dextran as described by Mertz and Berg (42). The cells were incubated under an agar overlay containing DMEM, 1% Bacto-agar (Difco Laboratories, Detroit, Mich.), 4% FBS, and antibiotics for 10 to 12 days at 37°C (or other temperatures when temperature sensitivity was being determined). Plaques were visualized by staining with neutral red. Plaques were counted 24 h later. To determine rates of plaque enlargement, averages were determined for 50 plaques on a daily basis once plaques had appeared.

Genetic complementation analyses were done in the same way, except cells were cotransfected with dilutions of the DNA to be tested and 10 ng of helper DNAs.

RNA analysis. Cytoplasmic RNA was extracted from transfected CV-1 cells 48 to 72 h postinfection by the method of White et al. (68) and analyzed by the S1 nuclease method of Berk and Sharp (6) as described by Favaloro et al. (21). Probes were end labeled at the *Eco*RI site of SV40 by using either alpha[^{32}P]deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I (3′ end-labeled probes) or gamma[^{32}P]ATP and polynucleotide kinase (5′ end-labeled probes).

Analysis of viral DNA replication. DNA replication was analyzed after transfection of cultures of CV-1 cells in 60-mm dishes as described previously (47, 66).

Analysis of virus-specific proteins by immunoprecipitation. At appropriate times after infection (indicated in figure legends), cells were washed twice with Tris-buffered saline and incubated for 1 h in DMEM lacking methionine and containing 2% dialyzed FBS. This medium was removed and replaced with DMEM lacking methionine and containing 2% dialyzed FBS and 300 μCi of [^{35}S]methionine per ml (0.5 ml per 100-mm plate, 0.25 ml per 35-mm plate). The duration of the labeling period was as indicated in the figure legends. After the labeling period, cells were washed twice with Tris-buffered saline, and protein extracts were prepared. Ice-cold lysing buffer (0.15 M NaCl, 0.02 M Tris-chloride [pH 8.0], 1% Nonidet P-40) was added to the cells (0.5 ml per 100-mm plate, 0.25 ml per 35-mm plate). Cells were incubated in the lysing buffer for 30 min at 4°C. Cells and buffer were then scraped from the plates into Eppendorf tubes with a rubber policeman. Tubes were spun in a microcentrifuge to pellet the cellular debris, and the supernatant was removed to another tube and stored at −70°C.

A 0.1-ml sample of extract was immunoprecipitated as described previously (47). Either hamster antitumor serum (lot 8-00X from the National Institutes of Health), rabbit antiserum against sodium dodecyl sulfate (SDS)-disrupted virions, or monoclonal antibody was used to precipitate T antigens or capsid proteins. Monoclonal antibody recognizing determinants in the carboxyl-terminal region of T antigen was a gift from S. Tevethia. Samples were analyzed by electrophoresis on SDS-polyacrylamide gels. After electrophoresis, gels were fixed in 50% methanol–10% acetic acid–10% trichloroacetic acid, fluorographed with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and exposed to Kodak XAR-5 film for 1 to 5 days at −70°C.

Enzymes and Chemicals. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.), P-L Biochemicals (Milwaukee, Wis.), or Boehringer Mannheim (Indianapolis, Ind.) and used according to the instructions of the supplier. T4 DNA ligase was purchased from Collaborative Research (Waltham, Mass.) or New England Biolabs, T4 polynucleotide kinase was from New England Biolabs, calf intestinal alkaline phosphatase was from Boehringer Mannheim, S1 nuclease was from P-L Biochemicals or Boehringer Mannheim, and proteinase K was from Beckman

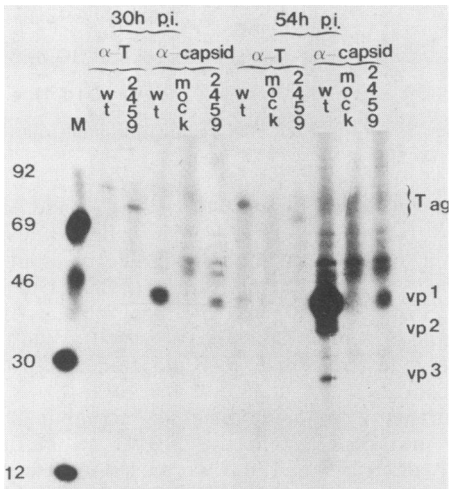

FIG. 5. Autoradiogram of [^{35}S]methionine-labeled viral proteins immunoprecipitated from wild-type-, *dl*A2459-, or mock-infected cell extracts. Extracts were prepared 30 or 54 h after transfection of CV-1 cells. Each extract was immunoprecipitated with either hamster antitumor serum or rabbit antiserum against SDS-disrupted SV40 virions. The numbers at the left represent the positions of size markers (sizes in kilodaltons).

TABLE 1. Complementation between *dl*A2459 and *ts*A1642[a]

Mutant no.	Location of mutation (m.u.)	Plaques formed (PFU/μg) by complementation with:		
		*dl*A2459	*ts*A1642	*dl*BC865
*dl*BC865	0 or 1.0	1×10^5	1.3×10^5	$<10^3$
*dl*A1209	0.650 to 0.587	$<10^3$	$<10^3$	9×10^4
*dl*A2411[b]	0.497	9×10^4	$<10^3$	2.0×10^5
*dl*A2420[b]	0.497	$<10^3$	$<10^3$	1.2×10^5
*dl*A2416[b]	0.288	$<10^3$	$<10^3$	1.4×10^5
*dl*A2432[b]	0.288	6×10^4	$<10^3$	9.5×10^4
*dl*A2410[b]	0.243	$<10^3$	$<10^3$	1.3×10^5
*dl*A2433[b]	0.243	7×10^4	$<10^3$	1.4×10^5
*dl*A2459[b]	0.193	$<10^3$	1.1×10^5	1.9×10^5
dl(*inv*)2408E[c]		$<10^3$	9×10^4	$<10^3$

[a] Monolayers of CV-1p cells were exposed to dilutions of mutant DNA and 10 ng of complementing mutant DNA. Plates were incubated at 37°C, and plaques were counted 12 days after infection.

[b] These mutants were constructed and characterized by Tornow and Cole (5). Mutants *dl*A2411, *dl*A2432, and *dl*A2433 produce nearly full-sized large T antigens, since a multiple of 3 bp is deleted. Mutants *dl*A2420, *dl*A2416, *dl*A2410, and *dl*A2459 produce truncated large T antigens, since the deletions are not multiples of 3 bp and therefore cause a shift in the translation reading frame.

[c] *dl*(*inv*)2408E contains an inversion of the *Bam*HI-to-*Bcl*I fragment of SV40 (0.144 to 0.189 m.u.) and the same deletion at the *Eco*RI site (0 or 1.0 m.u.) as mutant *dl*BC865.

Instruments Inc. (Fullerton, Calif.). All radiochemicals were purchased from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

The defect of *dl*A2459 affects a late stage of infection. SV40 mutant *dl*A2459 lacks 14 bp (nucleotides 2,785 through 2,798) at 0.193 m.u. (66). It directs the synthesis of a large T antigen that lacks the carboxyl-terminal 35 amino acids. Progeny viral DNA is synthesized in CV-1 cells transfected with *dl*A2459 DNA (66). To determine whether the block to productive infection by *dl*A2459 was at the level of late mRNA transcription, the synthesis of late mRNA was examined (Fig. 4).

CV-1 cells were transfected with *dl*A2459, *dl*A2411 (a replication-defective group A mutant), or wild-type SV40 DNA. Total cytoplasmic RNA was isolated 72 h later and analyzed by the S1 nuclease mapping procedure (6, 21). *Eco*RI-digested SV40 DNA was labeled at its 3′ end. This probe will be protected by all late mRNAs that extend beyond the *Eco*RI site to the polyadenylation site for the late mRNAs. Analysis of total cytoplasmic RNA prepared 72 h after transfection demonstrated that *dl*A2459 (Fig. 4A, lane 3) produced as much late mRNA as did wild-type SV40 (Fig. 4A, lane 1). No late mRNA was detected in cells transfected with *dl*A2411 DNA (Fig. 4A, lane 2) or mock-infected cells (Fig. 4A, lane 4). To determine whether the block to *dl*A2459 infection was at the level of mRNA processing, another S1 nuclease protection experiment was performed with a probe that was 5′ end labeled at the *Eco*RI site. This probe will be protected by that part of the late mRNAs that is between the acceptor splice junctions and the *Eco*RI site. Both 16S and 19S late mRNAs were produced in *dl*A2459-infected cells (Fig. 4B, lane 3) and the ratio of 16S/19S mRNAs was the same as in wild type-infected cells (Fig. 4B, lane 1). The probe was not protected by RNA prepared from either mock-infected (Fig. 4B, lane 4) or *dl*A2411-infected (Fig. 4B, lane 2) cells. Identical results were obtained when polyadenylated mRNA was used (data not shown). We conclude that late mRNAs are synthesized, polyadenylated, spliced, and transported in *dl*A2459-infected cells, and that the abundance of these species is comparable to that of wild-type SV40 late mRNA. We have not yet examined the structures of the leaders and the locations of splice donor sites of *dl*A2459 late mRNAs.

To determine whether the block to productive infection by *dl*A2459 was at the level of capsid protein synthesis, CV-1 cells were infected with *dl*A2459 or wild-type DNA or mock infected and labeled 30 or 54 h later with [^{35}S]methionine. Extracts were prepared, immunoprecipitated with rabbit anti-capsid protein antiserum or hamster anti-tumor antiserum, and analyzed by electrophoresis on SDS-polyacrylamide gels (Fig. 5). Although *dl*A2459 produces almost as much large T antigen as the wild type at both times (Fig. 5), the production of capsid protein VP1 is greatly reduced, especially at 54 h after infection. VP2 and VP3 could not be detected in protein extracts of *dl*A2459-infected cells. We conclude that *dl*A2459 does not form plaques in CV-1p cells due to an inability to produce sufficient levels of capsid proteins.

One other replication-competent group A mutant of SV40 has been described (16). Mutant *ts*A1642 produces viral DNA, late mRNA, and capsid proteins at the nonpermissive temperature, but does not form plaques. It is blocked at some stage after capsid protein synthesis, perhaps in virion morphogenesis. To determine whether *ts*A1642 and *dl*A2459 affected different functions of SV40 large T antigen, a genetic complementation experiment was performed (Table 1). *ts*A1642 complemented the defect of *dl*A2459, but not the defect of *dl*A2411. Therefore, *dl*A2459 and *ts*A1642 affect different functions of large T antigen.

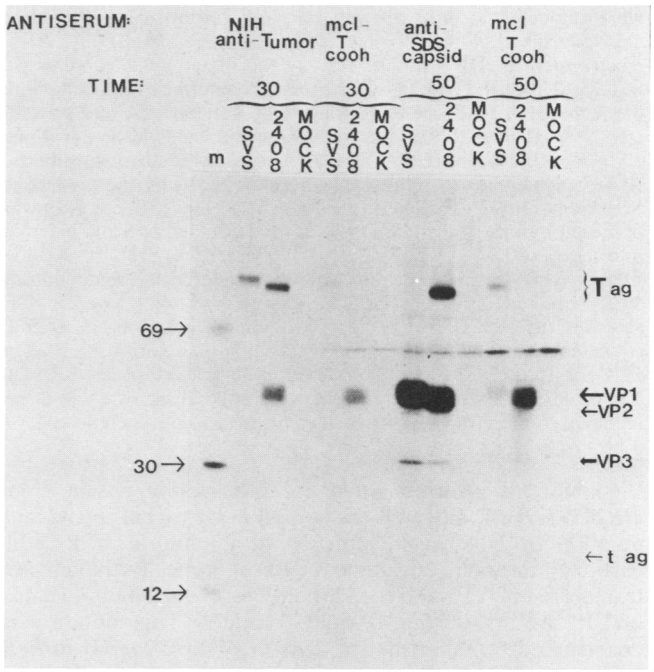

FIG. 6. Autoradiogram of [^{35}S]methionine-labeled viral proteins immunoprecipitated from wild type *inv*2408-, and mock-infected cell extracts. Extracts were prepared 30 or 50 h after transfection of CV-1 cells. Extracts were immunoprecipitated with either hamster antitumor serum, rabbit antiserum against SDS-disrupted SV40 virions, or tissue culture fluid containing a monoclonal antibody directed against the carboxyl terminus of large T antigen. The numbers at the left represent the positions of size markers (sizes in kilodaltons).

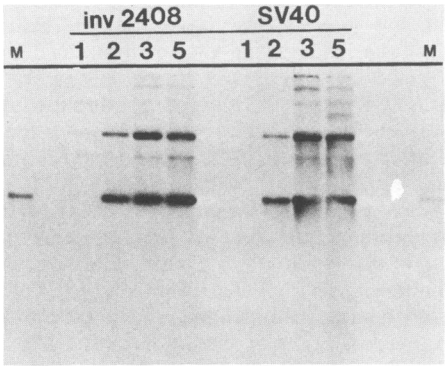

FIG. 7. Analysis of viral DNA replication in CV-1 cells infected by wild-type SV40 or *inv*2408. Confluent monolayers of CV-1 cells were transfected with wild-type SV40 or *inv*2408 DNA. Extracts were prepared at various times after infection (1, 2, 3, or 5 days). Cells were washed with Tris-buffered saline, and 0.3 ml of extraction buffer (0.01 M Tris-chloride, [pH 7.5], 0.6% SDS, 0.01 M EDTA); 0.1 ml of 5 M NaCl, and 0.025 ml of a solution (10 mg/ml) of proteinase K were added to each plate. Each plate also received 0.015 ml of ^{3}H-labeled M13 phage replicative-form DNA (approximately 30,000 cpm). The plates were incubated at 37°C overnight. The next day, lysates were scraped into Eppendorf tubes and stored at 4°C overnight. The next day, tubes were spun for 50 min at 18,000 rpm in a refrigerated Sorvall centrifuge. Supernatants were removed, phenol extracted once, and precipitated by the addition of 2 volumes of ethanol. After centrifugation, DNA pellets were dissolved in 0.1 ml of 10 mM Tris-chloride (pH 7.5)–1 mM EDTA–10 mM NaCl. A sample of each preparation was precipitated with trichloroacetic acid to determine the percent recovery of the ^{3}H-labeled phage DNA. Samples containing equal numbers of counts were subjected to electrophoresis in a 1% agarose gel in 89 mM Tris-chloride (pH 8.2–89 mM boric acid–2.5 mM EDTA. After electrophoresis, DNA in the gel was depurinated by soaking the gel in 0.25 M HCl for two 15-min periods, denatured by washing with 0.5 M NaOH and 1.5 M NaCl for two 30-min periods, and neutralized by washing with 0.5 M Tris-chloride (pH 7.5)–3 M NaCl for two 30-min periods. The DNA was then transferred to nitrocellulose (BA85; Schleicher & Schuell Co., Keene, N.H.) by the method of Southern (59). ^{32}P-labelled probe (specific activity, 10^8 cpm/μg) was prepared by nick translation of a plasmid containing SV40 inserted into the *Eco*RI site of pBR322 (54). The filter was prehybridized (8 h) and hybridized with probe (15 h) in standard buffers containing 50% formamide at 42°C. The filter was washed twice with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS at room temperature for 15 min and twice with 0.2× SSC–0.1% SDS at 55°C for 15 min. The filter was dried in air and exposed to Kodak XAR-5 film. The marker lanes (M) contained 20 ng of SV40 DNA included to monitor the evenness of transfer to nitrocellulose.

Viability is retained when the information absent from *dl*A2459 large T antigen is transposed to the carboxyl terminus of VP1. *inv*2408 is a viable SV40 mutant in which the carboxyl terminal 26 amino acids of large T antigen are transferred to VP1 and the carboxyl terminal 18 amino acids of VP1 are transferred to large T antigen. This mutant was constructed by inverting the small (237-bp) *Bam*HI-to-*Bcl*I fragment of SV40. That these domains have actually been exchanged was confirmed by immunoprecipitation of [^{35}S]methionine-labeled protein extracts from wild type- and *inv*2408-infected cells with appropriate antisera or monoclonal antibodies (Fig. 6). When antitumor serum was used, wild-type large T antigen was immunoprecipitated and both large T and VP1 were immunoprecipitated from *inv*2408-infected cells, indicating that *inv*2408 VP1 contains large T-antigen information. When a monoclonal antibody directed against the carboxyl-terminal portion of large T was used, large T was immunoprecipitated from wild type-infected cells, but not from *inv*2408-infected cells. Instead, *inv*2408 VP1 was immunoprecipitated, indicating that it carried information from the carboxyl terminus of large T antigen. When antiserum prepared against SDS-disrupted SV40 virions was used, the three capsid proteins, VP1, VP2, and VP3, were immunoprecipitated from wild type-infected cell extracts. The same three polypeptides and large T antigen were immunoprecipitated from *inv*2408-infected cells, indicating that the *inv*2408 large T antigen carried capsid protein antigenic information.

Plaques formed in CV-1p cells transfected with *inv*2408 DNA with the same efficiency (PFU per microgram) as with wild-type SV40. However, the plaques appeared later and enlarged more slowly than did wild-type SV40 plaques. The kinetics of progeny DNA synthesis in *inv*2408-infected cells was indistinguishable from that of wild-type SV40 (Fig. 7). This suggests that the slower rate of plaque enlargement reflects either a slower rate of assembly of *inv*2408 progeny, production of a smaller number of *inv*2408 virions, or reduced specific infectivity of *inv*2408 virions.

Construction and analysis of SV40 mutants expressing only one of the two fusion proteins expressed by *inv*2408. In *inv*2408, that portion of large T antigen missing from the large T of *dl*A2459 was fused to the carboxyl terminus of VP1. Since *inv*2408 was a viable mutant, this suggested that this information was able to function even when separated from the remainder of large T antigen. Two derivatives of *inv*2408 were constructed which expressed only one of two fusion polypeptides produced by *inv*2408 (large T with the carboxyl terminus of VP1 and VP1 with the carboxyl terminus of large T). These derivatives were constructed (Fig. 3) by replacing portions of the *inv*2408 genome with DNA fragments derived from other SV40 mutants.

*dl*BC865 is a nonviable SV40 mutant with a deletion at the *Eco*RI site (9), which is located near the beginning of the coding region for VP1. This mutant produces an 8-kilodalton

TABLE 2. Complementation properties of *dl*(*inv*)2408E and *dl*(*inv*)2408L[a]

Complementing mutant	Plaques formed (PFU/μg) by complementation with:	
	dl(*inv*)2408E	*dl*(*inv*)2408L
*dl*A1209	$<10^3$	$<10^3$
*dl*BC865	$<10^3$	3.5×10^4
*dl*E1226	2×10^5	2.5×10^5
*dl*A2459	$<10^3$	6.5×10^4
*dl*A2411[b]	2.5×10^4	$<10^3$
*dl*A2420[b]	$<10^3$	$<10^3$
dl(*inv*)2408E	$<10^3$	8.5×10^4
Controls		
SV40	3.0×10^5	
*inv*2408	1.7×10^5	
*dl*A2459	$<10^3$	
*dl*E1226	2.2×10^5	
dl(*inv*)2408E	$<10^3$	
dl(*inv*)2408L	$<10^3$	

[a] Monolayers of CV-1p cells were transfected with dilutions of *dl*(*inv*)2408E or *dl*(*inv*)2408L DNA and 10 ng of complementing mutant DNA per 60-mm plate.

[b] Mutant *dl*A2411 lacks 12 bp at 0.497 m.u.; mutant *dl*A2420 lacks 20 bp at 0.497 m.u. The large T antigen of *dl*A2411 contains the carboxy terminus of large T, whereas *dl*A2420 directs the synthesis of a 18-kilodalton fragment of large T (65).

fragment of VP1 (Cole and Goff, unpublished results). A portion of the *inv*2408 genome including the *Eco*RI site was replaced with an analogous fragment of *dl*BC865, as described above, to yield mutant *dl*(*inv*)2408E, which can express the early region fusion polypeptide (large T containing the carboxyl terminus of VP1) of *inv*2408, but not the late region fusion polypeptide. *dl*A1209 is another nonviable SV40 mutant with a 329-bp deletion between 0.650 and 0.587 m.u. (15; Polvino-Bodnar and Cole, unpublished results). This deletion removes the early mRNA cap site, the initiation site for large T antigen, and the donor splice junction for large T mRNA. No large T-related polypeptides have been detected in *dl*A1209-infected cells (Cole, unpublished results). A portion of the *inv*2408 genome, including the region between 0.650 and 0.587 m.u., was replaced by an analogous fragment of *dl*A1209 to yield mutant *dl*(*inv*)2408L, which can express the late region fusion polypeptide (VP1 containing the carboxyl terminus of large T antigen) of *inv*2408, but not the early region fusion polypeptide.

These two mutants were examined for their genetic complementation behavior (Table 2). Neither *dl*(*inv*)2408E nor *dl*(*inv*)2408L was able to form plaques in CV-1p cells alone. They were, however, able to complement one another to permit plaque formation. Furthermore, *dl*(*inv*)2408L was able to complement *dl*A2459, indicating that the information missing from *dl*A2459 large T antigen can be supplied in *trans* when this formation is attached to VP1. All of the control complementations agreed with predicted results: *dl*(*inv*)2408E and *dl*(*inv*)2408L were both able to complement *dl*E1226, which produces a defective VP2 (15), indicating that VP2 expression is normal in both mutants; *dl*(*inv*)2408L was unable to complement *dl*A1209 because it could not express large T antigen, but was able to complement *dl*BC865 since the VP1-T antigen fusion polypeptide could perform the functions of VP1; *dl*(*inv*)2408E was unable to complement *dl*BC865, since it could not express VP1, and was unable to complement *dl*A1209 or *dl*A2411, since it could not produce a large T antigen providing all of the functions of large T. We conclude that the carboxyl-terminal 26 amino acids, transferred to VP1 in *inv*2408 and *dl*(*inv*)2408L, constitute a separable functional domain of SV40 large T antigen.

Mutant *dl*A2459 can grow in some African green monkey kidney cell lines. Confluent monolayers of CV-1p, BSC-1, or Vero cells were transfected with *dl*A2459 or wild-type DNA. We found that *dl*A2459 was able to form plaques on BSC-1 and Vero cells; wild-type SV40 formed plaques on all three cell lines. The plaques formed in BSC-1 and Vero cells were approximately half the size of wild-type plaques formed in those cells. No plaques appeared in CV-1p cells transfected with *dl*A2459 DNA, even after 40 days.

DISCUSSION

The large T antigen of SV40 is a multifunctional protein and may therefore contain multiple functional domains. Studies with mutants and monoclonal antibodies demonstrate that some of the properties of large T antigen require different regions of the protein. The ATPase activity of T antigen can be inhibited specifically by monoclonal antibodies that recognize sequences encoded between 0.33 and 0.27 m.u. (11). The portion of T antigen encoded by sequences between 0.50 and 0.43 m.u. has been implicated in the binding of large T antigen to the SV40 origin of DNA replication (12, 48, 56). The carboxyl-terminal 35 amino acids of large T are sufficient for normal levels of adenovirus helper function (14, 23, 47). We and others have isolated and characterized many SV40 mutants with deletions in the *A* gene, encoding large T antigen (12, 14, 15, 46, 65, 66). These include mutants lacking ATPase activity, but retaining binding to the SV40 origin, mutants unable to bind to the SV40 origin, but retaining a wild-type level of ATPase activity, and mutants with drastically reduced levels of adenovirus helper function, but which are still completely viable. Therefore, at least three separate properties of T antigen have been localized to different regions of the protein.

We described previously (66) a mutant of SV40, *dl*A2459, lacking 14 bp at 0.193 m.u. The large T antigen encoded by *dl*A2459 lacks the carboxyl-terminal 35 amino acids and contains 3 new amino acids in their place. This mutant is capable of viral DNA replication, but is unable to form plaques on CV-1p monolayers. These results demonstrate that a late function, required after the onset of viral DNA replication, is performed by large T antigen.

In this report, we have further investigated the defect of *dl*A2459. Our results indicate that the block to productive infection by *dl*A2459 occurred after transcription and before translation of late mRNA. The defect of *dl*A2459 was very similar to the defect of human adenoviruses when they are grown in CV-1 cells. Adenovirus late mRNAs are produced, though at a level approximately 10% of that present in adenovirus-infected human cells (5); production of some capsid proteins (e.g., fiber) is reduced by as much as 100-fold. Since the deletion in *dl*A2459 affects that portion of large T antigen which provides adenovirus helper function (14, 23, 47) and the defect of *dl*A2459 resembles that of adenoviruses in monkey cells, we conclude that productive infection of CV-1 cells by SV40 requires the helper function.

This function can be provided by intracistronic complementation by any SV40 mutant whose large T antigen contains the information missing from *dl*A2459 large T antigen (65) and by mutant *dl*(*inv*)2408L, which contains this information transposed to the carboxyl terminus of VP1. This indicates that the helper function information of large T is a separable functional domain.

A large number of viable mutants with deletions near the 3′ end of the SV40 early region have been described (1, 15, 22, 25, 44, 45, 66). In all but one of these mutants, the deletion is located upstream from the deletion of *dl*A2459 and is a multiple of 3 bp. Thus, these mutant large T antigens contain the residues missing from *dl*A2459 large T antigen. Many of these mutants show normal levels of adenovirus helper function. The other mutant, *dl*1265, lacks the carboxyl-terminal 9 amino acids and contains 4 different residues in their place (67). This mutant shows a substantially reduced but measurable level (7%) of helper function (14). Therefore, productive infection of CV-1 cells by SV40 requires only a low level of adenovirus helper function. Of mutants with lesions in this region, only *dl*A2459 is completely defective for adenovirus helper function (66). In this paper, we have shown that a sufficient amount of the function was provided when only the 26 carboxyl-terminal residues were transposed to VP1. Since productive infection by SV40 requires only a low level of helper function, we cannot tell whether the 26 carboxyl-terminal residues provided wild-type levels of helper function. Mutant *inv*2408 provided little or no helper function to coinfecting adenovirus (Cole, unpublished results). However, in *inv*2408, helper function becomes a late SV40 function. Since, adenoviruses effectively inhibit both cellular and SV40 late protein synthesis (4, 29, 55), this probably accounts for our inability to measure helper function activity in *inv*2408-infected cells.

The genetic analysis of the altered genes in *inv*2408 supports the idea that some or all of the residues deleted in *dl*A2459 large T define a separate domain. Although *inv*2408 was viable, it grew more slowly than the wild type. To determine the genetic potentials of the two altered proteins, the altered genes were separated from one another. The results of complementation analyses indicated that the altered VP1 of *inv*2408 was a functional capsid protein, since *dl*(*inv*)2408L could complement *dl*BC865, a mutant which produces a defective VP1 (Table 2). However, the plaques enlarged at a significantly slower rate than those generated by control complementation [plaques formed by *dl*(*inv*)2408L and *dl*BC865 were not visible until 10 to 12 days after infection; plaques formed by coinfection with *dl*BC865 and *dl*A1209 (which encodes a wildtype VP1) were visible 7 days after infection]. This demonstrated that the altered VP1, although not completely defective, was impaired. Since *inv*2408 DNA replication proceeded with the same kinetics as wild-type SV40, this suggests that the slower growth rate of *inv*2408 was caused primarily by a partially impaired VP1.

It is not known whether oligomerization of T antigen is required for it to perform all of its functions. It is possible that oligomerization occurs as a consequence of the binding of T antigen monomers to DNA and that mutant monomers unable to remain oligomerized after dissociation from DNA might still possess full activity. The monomer fraction of T antigen displays both ATPase and specific DNA binding (8, 20). Our finding that the helper function of large T can be provided by a VP1 polypeptide carrying the carboxyl-terminal 26 residues of large T antigen suggests that oligomerization of large T is not required for this function. However, since VP1 does oligomerize in the formation of the viral capsid, the possibility that the helper activity of *inv*2408 requires the formation of VP1 oligomers cannot be eliminated.

SV40 large T antigen and the adenovirus 72-kilodalton DNA-binding protein show important similarities. Both are involved in DNA replication and in regulation of transcription (17, 33, 43). Although adenoviruses cannot grow productively in monkey cells, adenovirus mutants capable of productive infection of monkey cells can be isolated (3, 36, 38). The lesions in these host range mutants map to the 72-kilodalton DNA-binding protein and are located at a site in the polypeptide distant from the mutation sites of mutants affecting the replication and transcriptional regulation activities of the polypeptide (53). Thus, for both SV40 and the human adenoviruses, a separate domain of a major DNA-binding and regulatory polypeptide is involved in permitting the virus to grow productively in monkey cells. In both cases, sites of phosphorylation are located in the helper function region of the polypeptide. It will be interesting to determine whether expression of the adenovirus host range mutant 72-kilodalton DNA-binding protein will permit the growth of SV40 mutants such as *dl*A2459 in CV-1 or CV-1p cells in a manner analogous to the role played by the carboxyl terminus of large T antigen in permitting productive adenovirus infection of CV-1 cells.

The helper function activity of large T is provided by the only part of large T showing no homology with polyomavirus (Fig. 1). This suggests that this information entered the SV40 genome after the evolutionary separation of SV40 and polyomavirus. Although the large T antigen of the human papovavirus, BKV, shows homology to this portion of SV40 large T (57), the homology is much less than that between the remainder of SV40 and BK large T antigens.

The function defined by *dl*A2459 is essential only in some green monkey kidney cell lines. Although *dl*A2459 could not form plaques in CV-1p cells, plaques were formed at wild-type efficiency in both BSC-1 and Vero cells. Therefore, *dl*A2459 is a host range mutant. CV-1p, Vero, and BSC-1 cells are independent lines all derived from kidneys of *Cercopithecus aethiops*, but may have considerably different cellular biology. Human adenoviruses are able to grow in Vero cells without an SV40 helper (18). We found that adenovirus type 2 was also able to grow in BSC-1 cells (Cole, unpublished results). Eron et al. (18) reported that adenoviruses could not grow in BSC-1 cells. This discrepancy could reflect substantial differences between the BSC-1 line they used and the one we currently use. Another SV40 mutant, isolated by Pipas et al. (46), lacks 80 bp between 0.196 and 0.181 m.u.; it forms plaques in BSC-1 cells, but not in CV-1p cells (Pipas, personal communication). This suggests that the host range behavior of these mutants is a general property of mutants whose large T antigens lack carboxyl terminal information. At present, we do not understand the molecular basis for the host range behavior, but it may indicate that the carboxyl-terminal domain of large T antigen is involved in determining the tissue or species growth range (or both) of SV40.

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