JOURNAL OF VIROLOGY, Feb. 1986, p. 539–546
0022-538X/86/020539-08$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 57, No. 2

Properties of the Simian Virus 40 (SV40) Large T Antigens Encoded by SV40 Mutants with Deletions in Gene *A*

CHARLES N. COLE,[1]* JOANNE TORNOW,[2]† ROBIN CLARK,[3]‡ AND ROBERT TJIAN[3]

Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756[1]; Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510[2]; and Department of Biochemistry, University of California, Berkeley, California 94720[3]

Received 15 July 1985/Accepted 22 October 1985

The biochemical properties of the large T antigens encoded by simian virus 40 (SV40) mutants with deletions at *DdeI* sites in the SV40 *A* gene were determined. Mutant large T antigens containing only the first 138 to 140 amino acids were unable to bind to the SV40 origin of DNA replication as were large T antigens containing at their COOH termini 96 or 97 amino acids encoded by the long open reading frame located between 0.22 and 0.165 map units (m.u.). All other mutant large T antigens were able to bind to the SV40 origin of replication. Mutants with in-phase deletions at 0.288 and 0.243 m.u. lacked ATPase activity, but ATPase activity was normal in mutants lacking origin-binding activity. The 627-amino acid large T antigen encoded by *dlA*2465, with a deletion at 0.219 m.u., was the smallest large T antigen displaying ATPase activity. Mutant large T antigens with the alternate 96- or 97-amino acid COOH terminus also lacked ATPase activity. All mutant large T antigens were found in the nuclei of infected cells; a small amount of large T with the alternate COOH terminus was also located in the cytoplasm. Mutant *dlA*2465 belonged to the same class of mutants as *dlA*2459. It was unable to form plaques on CV-1p cells at 37 or 32°C but could form plaques on BSC-1 monolayers at 37°C but not at 32°C. It was positive for viral DNA replication and showed intracistronic complementation with any group A mutant whose large T antigen contained a normal carboxyl terminus. These findings and those of others suggest that both DNA binding and ATPase activity are required for the viral DNA replication function of large T antigen, that these two activities must be located on the same T antigen monomer, and that these two activities are performed by distinct domains of the polypeptide. These domains are distinct and separable from the domain affected by the mutation of *dlA*2465 and indicate that SV40 large T antigen is made up of at least three separate functional domains.

The simian virus 40 (SV40) *A* gene encodes the SV40 large tumor (T) antigen. This multifunctional phosphoprotein contains 708 amino acids, is encoded between 0.65 and 0.17 map units (m.u.) (Fig. 1), and plays a pivotal role during both lytic and transforming infections by SV40 (see reference 41 for a review). Genetic and biochemical analyses of large T antigen reveal that large T possesses both DNA-binding (12, 29, 38, 39) and ATPase (4, 10) activities. Monoclonal antibodies directed against sequences encoded between 0.33 and 0.28 m.u. specifically inhibit the ATPase activity, demonstrating a role in ATPase activity for amino acids encoded by these sequences (4). Studies with deletion mutants indicate that sequences encoded between 0.43 and 0.54 m.u. are required for the binding of large T to the SV40 DNA replication origin (5, 26, 34). Within this region, sequences between amino acids 126 and 132 are required for transport of large T antigen to the nucleus (13). Most of large T is located within the nucleus, but a small percentage can be found at the plasma membrane. The carboxyl-terminal portion of large T comprises a separate and physically separable functional domain (43), required for productive infection of most monkey kidney cell lines by human adenoviruses (9, 26) and also required for efficient production of SV40 progeny in many monkey kidney cell lines (20, 43).

These properties of large T antigen permit it to play several roles: (i) initiation of viral DNA synthesis in permissive cells (3, 36); (ii) autoregulation of the level of its own mRNA (1, 31, 38); (iii) initiation and maintenance of transformation in nonpermissive cells (14, 15, 37); (iv) facilitation of the growth of human adenoviruses in monkey kidney cells (7, 9, 11, 30); (v) efficient synthesis of late capsid proteins (43).

We described previously the construction and preliminary characterization of a series of mutants of SV40 with deletions at *DdeI* sites in the *A* gene (41–43). On the basis of studies with these mutants, we described experiments which demonstrated that SV40 large T antigen performs a function required after the onset of viral DNA replication and involved in the efficient production of late capsid proteins (41). In this paper, we report on additional properties of these and one additional deletion mutant. Representative mutants were analyzed for specific binding to the SV40 origin of DNA replication, ATPase activity, and intracellular localization.

These studies lead to the following conclusions. (i) Most large T antigens which contain the DNA-binding domain were able to bind to the SV40 origin of DNA replication. Small fragments of large T containing only the first 138 to 140 amino acids were unable to bind, as were large T antigens containing the alternate open reading frame at their carboxyl termini. (ii) Mutants with in-phase deletions at 0.288 and 0.243 m.u. lacked ATPase activity. ATPase activity was normal in a mutant with an in-phase deletion in the DNA-binding domain. ATPase activity was also normal with mutant *dlA*2465, whose deletion at 0.219 m.u. causes chain termination of large T antigen, producing a 627-amino acid

* Corresponding author.
† Present address: Thimann Laboratories, University of California, Santa Cruz, CA 95064.
‡ Present address: Department of Human Genetics, Cetus Corporation, Emeryville, CA 94608.

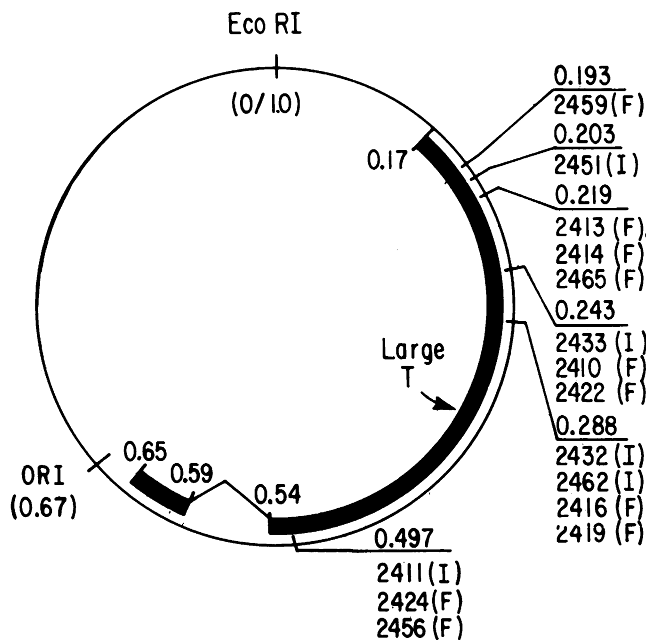

FIG. 1. Map positions of SV40 deletion mutants. The map shows the location of the SV40 origin of DNA replication and the coding regions for large T antigen. The map positions of the deletion mutants described in these studies are also shown. The designation I means that the mutant had a deletion which is a multiple of three bp, leaving the large-T-antigen translational reading frame in phase, while the designation F means that the deletion was not a multiple of three bp and that the deletion causes a frameshift out of the large-T-antigen reading frame. ORI, Origin.

fragment of large T. This large T antigen is the smallest large T displaying ATPase activity. The presence of the alternate carboxyl terminus also destroyed ATPase activity. (iii) All of the large T antigens produced by the mutants were located predominantly within the nucleus. The large T antigens of *dlA*2413, *dlA*2414, and *dlA*2415, containing the alternate carboxyl terminus, were also detected in the cytoplasm. (iv) Mutant *dlA*2465 belonged to the same class of mutants as *dlA*2459 (39, 40). It was positive for viral DNA replication and showed intracistronic complementation with any mutant whose T antigen contained a normal carboxyl terminus. In addition, it displayed the host range (42) and cold sensitivity (24) associated with mutants of this group.

These results and those reported in previous studies (5, 20, 25, 41–43) allow us to present a detailed model about the functional organization of SV40 large T antigen. Both DNA-binding and ATPase activity appear to be required for the viral DNA replication function of large T, and all data to date suggest that these functions must be found on the same large T antigen monomer, even though these two activities appear to be performed by distinct functional domains such that either function can be inactivated by mutation without affecting the other activity. This suggests the existence of at least three separate domains in SV40 large T antigen.

MATERIALS AND METHODS

Cells, cell culture, viruses, and plasmids. The growth of CV-1, CV-1p, BSC-1, Vero, Cos-7, and Cos-1 cells in Dulbecco modified Eagle medium (DMEM) containing 5% (all lines except Cos-1 and Cos-7) or 10% (Cos-1 and Cos-7) fetal bovine serum has been described previously (5, 8, 41). Our wild-type strain of SV40 (WT830) was the small-plaque strain originally characterized by Takemoto et al. (35). Para-cT is an SV40 mutant with a mutation in the *A* gene which prevents transport of the large T antigen to the nucleus (16). A plasmid carrying this SV40 mutant genome was a gift from Robert Lanford (Southwest Texas Medical Center).

Previously described methods were used for bacterial transformation (19), the preparation of minilysates (2), and the preparation of purified plasmid DNA (6) by CsCl-ethidium bromide buoyant density centrifugation. Wild-type SV40 and all mutants were maintained as recombinant DNA plasmids (inserted at the SV40 *Eco*RI site into the pBR322 *Eco*RI site). All of the mutants examined in these studies have been described previously (41–43) except *dlA*2465. All, except para-cT, have deletions at *Dde*I sites in the early region of SV40.

Infections and transfections. Plasmid DNA was first digested with *Eco*RI to separate viral and bacterial sequences and was then ligated at low concentration (5 μg/ml) with T4 DNA ligase to recircularize viral DNA. Subconfluent monolayers of cells were transfected, as previously described (43), by using a 30-min exposure of the cells to viral DNA (1 μg/100-mm plate) 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 500 μg of DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.) per ml. After exposure to DNA, cell monolayers were washed twice with Tris-buffered saline and exposed to DMEM containing 2% fetal bovine serum and 0.1 mM chloroquine diphosphate (Sigma) for either 4 h (Cos-1 cells) or 6 h (all other cell lines). At the appropriate time, this medium was replaced with DMEM containing 2% fetal bovine serum. The chloroquine diphosphate treatment increased the percentage of cells productively transfected from less than 2% to between 10% and 60% (18). This procedure was used for all transfections except those used in analyzing the ATPase activity of large T.

Stocks of SV40 mutants were prepared in Cos-7 cells as described previously (5). Infections were initiated with virus or with viral DNA, excised from recombinant plasmids, and recircularized as described above.

Analysis of DNA-binding activity of SV40 large T antigen. Unlabeled protein extracts were prepared from 100-mm plates of transfected CV-1 cells as previously described (43). A 0.1-ml sample of each extract was incubated with 25 ng of ^{32}P-labeled *Bst*NI fragments of SV40 DNA that were labeled at the 5′ ends. DNA bound to large T was immunoprecipitated and analyzed on 2% agarose gels as described by Scheller et al. (33).

DNA sequence analysis. DNA sequence analysis of mutant *dlA*2465 was performed by the partial chemical degradation method of Maxam and Gilbert (21). The $KMnO_4$ reaction was used to determine the positions of T residues (32). We first isolated from a polyacrylamide gel the *Dde*I fragment containing the deletion, 3′ end labeled this fragment by using the large fragment of DNA polymerase I and [α-^{32}P]dTTP, digested the fragment with *Bst*NI, and isolated the deletion-containing subfragment from a second polyacrylamide gel. This fragment was purified by chromatography on an Elutip-d column (Schleicher & Schuell, Keene, N.H.).

ATPase assays. Large T antigen was isolated and ATPase assays were performed as described previously (5). Briefly, T antigen in cell extracts was bound to 10-μm polyacrylamide beads by using monoclonal antibody Pab419 and tested for its ability to release $^{32}PO_4$ from [γ-^{32}P]dATP. Most mutants were analyzed by infection of CV-1 cells with stocks

of SV40 mutants prepared either in CV-1 cells (wild-type SV40 and viable deletion mutants) or Cos-7 cells (most nonviable deletion mutants). In some cases, Cos-7 stocks contained a high proportion of revertant viruses; in these cases, ATPase activity was measured after direct DNA transfection. CV-1 cells on 10-cm plates (approximately 50% confluent) were transfected with 0.75 μg of SV40 or mutant DNA in 0.5 ml of Tris-buffered saline containing 250 μg of DEAE-dextran per ml (22) for 1 h at 32°C followed by incubation in DMEM plus 70 μM chloroquine diphosphate for 6 h at 37°C. The cells were then incubated at 37°C in DMEM plus 2% fetal bovine serum until harvested at 28 to 32 h after transfection. The amount of large T antigen on polyacrylamide beads used in ATPase assays was quantitated by densitometric analyses of sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie blue (viral infections and most DNA transfections) or silver stained (some DNA transfections).

Immunofluorescence. CV-1 cells on 6-cm plates (approximately 70% confluent) were transfected with SV40 or mutant DNA as described above. At 30 h after transfection, cells were fixed with absolute methanol and air dried. The fixed cells were treated with Pab419 monoclonal antibody ascites fluid (a gift of L. Gooding, Emory University) and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (27).

Materials, enzymes, and immunological reagents. Restriction endonucleases were purchased from New England BioLabs (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or P-L Biochemicals (Milwaukee, Wis.). The large fragment of DNA polymerase was purchased from New England BioLabs. DNA ligase was purchased from Collaborative Research, Inc. (Waltham, Mass.). All enzymes were used according to the recommendations of the suppliers. Formalin-fixed, protein A-bearing *Staphylococcus aureus* was purchased from The Enzyme Center (Boston, Mass.). All radiochemicals were purchased from the Radiochemical Centre (Amersham, England). Cell line Pab419 was a gift of Ed Harlow (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.); Pab419 ascites fluid was a gift of Linda Gooding. Purified monoclonal antibody Pab204 was donated by David Lane (Imperial College; London, England). Goat IgG directed against mouse IgG light chain, a gift of John Kimura and Leon Wofsy (both at University of California, Berkeley, Calif.), was affinity purified by chromatography on Pab419-Sepharose (CNBr coupled).

RESULTS

In these studies, we used a large set of deletion mutants of SV40 (41 to 43). These are listed in Table 1. All of these mutants were prepared by digesting SV40 DNA, cloned into the *Eco*RI site of pBR322, with *Dde*I in the presence of ethidium bromide, yielding predominantly unit-length linear molecules. Limited digestion of these with nuclease S1 produced small deletions at the site of linearization. After ligation at low concentration, *Escherichia coli* HB101 was transfected, and the colonies obtained were screened for the presence of deletion-mutant genomes.

The set contains two main classes of mutants. One set has deletions which are multiples of three base pairs (bp). These deletions result in the production of mutant large T antigens lacking one to four amino acids at the site of the deletion. Occasionally, codon fusion results in the insertion at the deletion site of an amino acid not normally found at this position in large T. In this communication, this type of

TABLE 1. Deletion mutants used

Mutant no.	Frameshift or in-phase deletion[a]	Position of mutation (m.u.)	Size of deletion (bp)[b]	Amino acids in mutant T antigens[c]
2411	I	0.497	12 (4391–4380)	−(143–146)
2420	F	0.497	20 (4402–4383)	1–138 + 6
2456	F	0.497	17 (4397–4381)	1–140 + 5
2432	I	0.288	12 (3298–3287)	−(507–510) + 1
2462	I	0.288	3 (3293–3291)	−509
2416	F	0.288	10 (3295–3286)	1–508 + 2
2419	F	0.288	10 (3297–3288)	1–507 + 3
2433	I	0.243	9 (3059–3051)	−(587–589)
2422	F	0.243	10 (3060–3051)	1–585 + 26
2410	F	0.243	10 (3062–3053)	1–584 + 27
2413	F	0.219	22 (2946–2925)	1–624 + 96
2414	F	0.219	10 (2936–2927)	1–627 + 97
2465	F	0.219	14 (2937–2924)	1–626 + 1
2451	F	0.203	6 or 9 at 2850[d]	−2 or 3[d]
2459	F	0.193	14 (2798–2785)	1–673 + 3

[a] Deletions which were multiples of three bp are designated in-phase deletions (I); all others are designated frameshift deletions (F).

[b] Deletion size was determined by direct DNA sequence analysis except in the case of *dl*A2451, a viable mutant known from analysis of its properties to have an in-phase deletion. Numbers in parentheses show nucleotides deleted.

[c] The amino acid residues present in or missing from the mutant large T antigens were determined from the DNA sequence of the mutant.

[d] This mutant was not subjected to DNA sequence analysis. It is known to have an in-phase deletion based on the size of its large T antigen and its genetic complementation properties. It is a viable mutant, similar to the large number of other mutants with deletions in this region (8, 26). Based on restriction endonuclease digestion patterns (data not shown), it is believed to have a deletion of either six or nine bp, resulting in the loss of either two or three amino acid residues in the vicinity of amino acid 656.

mutant is called an in-phase deletion mutant (I). The second set of mutants has deletions which are not multiples of three bp. These deletions result in a translational reading-frame shift and are called frameshift deletion mutants (F). The large T antigens produced by these mutants are generally much shorter than wild-type large T and contain a stretch of amino acids at their carboxyl termini not normally found in large T. In the case of *dl*A2413, *dl*A2414, and *dl*A2415 (Fig. 1), the reading frame is shifted to the long open reading frame located at the 3′ end of the early region, resulting in the production of a large T, larger than wild-type large T, and with 96 to 97 new amino acids replacing the carboxyl-terminal 81 to 84 amino acids.

At many sites (0.497, 0.288, and 0.243 m.u.), both in-phase-and frameshift-producing mutants were obtained, and those studied in detail are listed in Table 1. At other sites, only in-phase (0.203 m.u.)- or only frameshift (0.193 m.u.)-producing mutants were obtained.

Initial characterization of *dl*A2465. Most of the mutants listed in Table 1 were characterized and reported previously (26, 41–43). We report here the characterization of an additional mutant from this set. *dl*A2465 lacks 14 bp at 0.219 m.u. (Fig. 1) and produces a large T antigen containing the first 626 residues of large T and a glutamic acid residue not normally found in large T at position 627. The mutant is unable to form plaques in CV-1p cells, but its defect can be complemented intracistronically by any SV40 *A* gene mutant which produces a large T antigen containing an intact carboxyl-terminal region. The mutant shows the same genetic complementation properties as *dl*A2459 (41–43) and

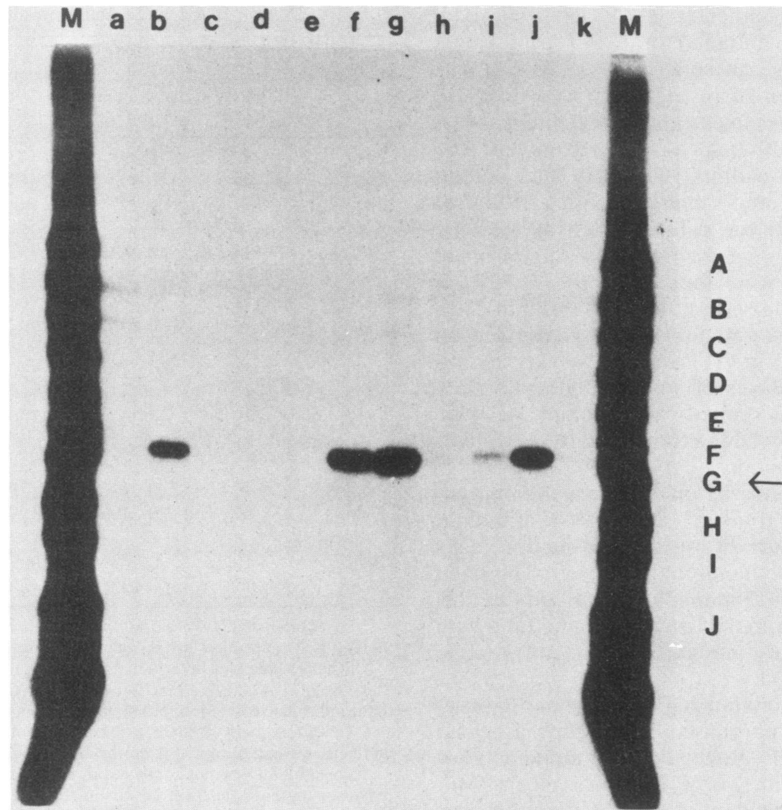

FIG. 2. Analysis of the ability of SV40 deletion mutant large T antigens to bind to the SV40 origin of DNA replication. Lanes: M, marker *Bst*NI fragments of SV40 DNA; a, mock infected; b, wild-type SV40; c, *dl*A2411; d, *dl*A2456; e, *dl*A2416; f, *dl*A2432; g, *dl*A2462; h, *dl*A2410; i, *dl*A2433; j, *dl*A2459; k, *dl*A2414. The position of each *Bst*NI fragment is shown on the right side of the figure.

belongs to that family of T-antigen mutants which are positive for viral DNA replication (42) and which show host-range plaque formation properties (43). AT 37°C, these mutants cannot form plaques in CV-1p cells but do form plaques in both BSC-1 and Vero cells; plaques are not formed in any of these lines at 32°C. The host-range property of SV40 *A* gene mutants will be described in detail elsewhere (C. N. Cole, manuscript in preparation).

Ability of mutant large T antigens to bind the SV40 origin of replication. Extracts of wild type-, mutant-, or mock-infected CV-1 cells were mixed with radiolabeled fragments of SV40 DNA produced by *Bst*NI digestion. After incubation, the reaction mixture was immunoprecipitated, and the immunoprecipitates were analyzed by electrophoresis in a polyacrylamide gel, followed by autoradiography. It can be seen (Fig. 2) that the *Bst*NI fragment spanning the SV40 origin of replication (fragment G, 311 bp; nucleotides 5027 to 95) was bound by the large T antigen from cells infected with wild-type SV40 (lane b), *dl*A2462 (lane g), *dl*A2432 (lane f), *dl*A2433 (lane i), *dl*A2410 (lane h), and *dl*A2459 (lane j). Thus, the large T antigens of all of the mutants with in-phase deletions except *dl*A2411 (lane c) bound to the SV40 origin of DNA replication. The origin-binding ability of truncated mutant T antigens was also tested. Binding was observed with *dl*A2459 (0.193 m.u.) and *dl*A2410 (0.243 m.u.) but not with *dl*A2416 (0.288 m.u., lane e) or *dl*A2456 (0.497 m.u., lane d). In a separate experiment, the large T antigen of *dl*A2465 was also able to bind to the origin (data not shown). The amount of SV40 origin fragment immunoprecipitated varied considerably and correlated well with the amount of large T antigen which could be immunoprecipitated from wild type- or mutant-infected cells (data not shown). This data is discussed further below.

Subcellular location of mutant large T antigens. The subcellular location of mutant large T antigens was examined by immunofluorescence. CV-1 cells were transfected with either mutant or wild-type DNA or mock transfected and analyzed for the location of large T antigen 30 h later. The data are presented in Table 2. All of the large T antigens were detected only in the nucleus, with the exception of the large T antigens from *dl*A2413 and *dl*A2414. These two mutants both have deletions at 0.219 m.u. and produce large T antigens which are fusions of the first 626 to 627 amino acids of large T and 96 to 97 amino acids derived from the alternate open reading frame at the 3′ end of the early region. With both of these mutants, weak cytoplasmic immunofluorescence could also be observed in most of the cells which showed nuclear fluorescence, suggesting that the unusual carboxyl-terminal domain had a minor effect on transport of the mutant large T antigens to the nucleus. However, even with these mutants, nuclear staining was predominant. The intensity of staining was strongest with those mutants which were viable or whose T antigens had been detected most easily by immunoprecipitation.

ATPase activity. The relative amount of T-antigen-related ATPase activity was determined by assaying extracts of infected or transfected cells. We initially attempted to analyze ATPase activity by infecting CV-1 cells with virus stocks. Stocks of nonviable mutants were prepared in Cos-7 cells as described previously (5). While it proved possible to

prepare stocks of many mutants in Cos-7 cells, there were some important exceptions. Revertants or pseudorevertants appeared with very high frequency (up to 10% of the progeny) in stocks of some mutants. These included those which contained frameshift-producing deletions distal to sequences required for replication of SV40 (deletions at 0.219 or 0.193 m.u.) and included some which were positive for DNA replication (e.g., *dl*A2459) and others which contained the long open reading frame at their carboxyl termini (e.g., *dl*A2413, *dl*A2414). In these cases, deletion or insertion of a single additional base pair would act to restore the proper translational reading frame distal to the second mutation, producing a large T antigen capable of expression of all the functions of large T required for complete productive infection. It was not possible to prepare stocks of *dl*A2432 or *dl*A2462. We do not know the reason for this, but it is possible that the defective mutant T antigens sequester the wild-type T antigen endogenous to Cos-7 cells in a mixed T-antigen oligomer, reducing the level of functional T antigen to a level insufficient for the production of progeny. Therefore, it was necessary to analyze the ATPase activity of some mutants by direct DNA transfection of CV-1 cells.

Table 3 presents the results of ATPase assays performed on extracts of CV-1 cells infected with virus stocks of wild type or deletion mutants or directly transfected with mutant or wild-type DNA. The amount of large T polypeptide was quantitated by densitometry of stained SDS-polyacrylamide gels (Fig. 3). Coomassie blue staining was used to quantitate the amount of T antigen in extracts prepared after infection of CV-1 cells, but the amount of T antigen obtained after direct DNA transfection of *dl*A2413 and *dl*A2414 was too low to permit Coomassie blue staining, and silver staining was employed in these cases.

All viable (*dl*A2451) and all replication-competent (*dl*A2459, *dl*A2465) large T antigens were positive for ATPase activity. The large T of *dl*A2465 was the shortest large T displaying ATPase activity; no ATPase activity was detected in cells infected with mutants containing frameshift deletions at 0.487, 0.288, or 0.243 m.u. Substantial ATPase activity was detected with *dl*A2411, which lacks origin-binding activity. Little or no ATPase activity was observed in cells infected with mutant *dl*A2432, *dl*A2462, or *dl*A2433. These mutants produce large T antigens lacking one to four amino acids at map positions 0.287 or 0.243. These results indicate that ATPase activity of large T antigen requires residues at these sites and extends the ATPase domain toward the carboxyl terminus of large T.

TABLE 2. Location of mutant large T antigens[a]

Mutant no.	Location of deletion (m.u.)	Location of T antigen[b]	Strength of immuno-fluores-cence[c]
2411	0.497	N	+ + +
2420	0.497	N	+
2432	0.288	N	+ +
2462	0.288	N	+ +
2416	0.288	N	+
2433	0.243	N	+ +
2410	0.243	N	+
2413	0.219	N/C	+ + +
2414	0.219	N/C	+ + +
2465	0.219	N	+ + + +
2459	0.193	N	+ + + +
SVS(wt)[d]		N	+ + + +
Para-cT[e]		C	+ +
Mock infected			0

[a] The location of mutant T antigens was determined by immunofluorescence with a hamster anti-T antigen serum and fluorescein-conjugated goat anti-hamster IgG.

[b] N, Nucleus; C, cytoplasm.

[c] The intensity of immunofluorescence was estimated by examination of more than 50 immunofluorescent cells in two separate experiments.

[d] SVS(wt) is the wild-type small-plaque strain of SV40 (35).

[e] Para-cT is an SV40 virus containing a mutation in T antigen which confines the mutant T antigen to the cytoplasm (16).

TABLE 3. ATPase activity of SV40 deletion mutants[a]

Mutant no.	Location of mutation (m.u.)	Frameshift (F) or in-phase (I) mutation	ATPase (relative sp act)[b]
WT830			1.00
2411	0.497	I	0.80[c]
2456	0.497	F	0[d]
2432	0.288	I	0.05[c]
2462	0.288	I	0.08[c]
2416	0.288	F	0[d]
2419	0.288	F	0[d]
2433	0.243	I	<0.08
2422	0.243	F	<0.1
2413	0.219	F	<0.3[c,e]
2414	0.219	F	<0.3[c,e]
2465	0.219	F	1.1
2451	0.203	I	0.8
2459	0.193	F	0.7

[a] ATPase activities were determined and are expressed relative to the level obtained in cells infected with wild-type SV40 (WT830). Where < appears, significant inhibition (>50%) of ATPase activity by Pab204 monoclonal antibody or rabbit anti-D2T IgG was not observed. Typically, the inhibition was >90% for wild-type SV40-infected extracts and mutant extracts which were ATPase positive. It is likely that there is no T-antigen ATPase activity in these cases.

[b] Initial rate of dATP hydrolysis per milligram of T antigen.

[c] T-antigen-containing extracts prepared by plasmid DNA transfection.

[d] No activity detected; insufficient protein for quantitation.

[e] Quantitated from amount of protein on silver-stained gel.

DISCUSSION

Studies of mutants affecting the SV40 large T antigen have permitted localization of some of the functions of large T to specific regions of the molecule. The studies described here permit further refinement of this mapping, and this is summarized in Fig. 4.

DNA binding. The inability of *dl*A2411 large T antigen to bind to the viral origin of replication is in agreement with previous studies in several laboratories (5, 28, 34) which indicate that the DNA-binding domain of large T is located between map positions 0.43 and 0.54, encoding amino acids 83 to 265. All mutant large T antigens which contained the DNA-binding domain were able to bind to the viral origin of DNA replication, except for the large T antigens of *dl*A2413, *dl*A2414, and *dl*A2415. These three mutants produced large T antigens in which residues encoded in the long open reading frame at the 3′ end of the early region replaced the normal carboxyl-terminal 81 to 84 amino acids. This alternative carboxyl terminus is considerably more hydrophobic than the normal carboxyl terminus and probably alters the prop-

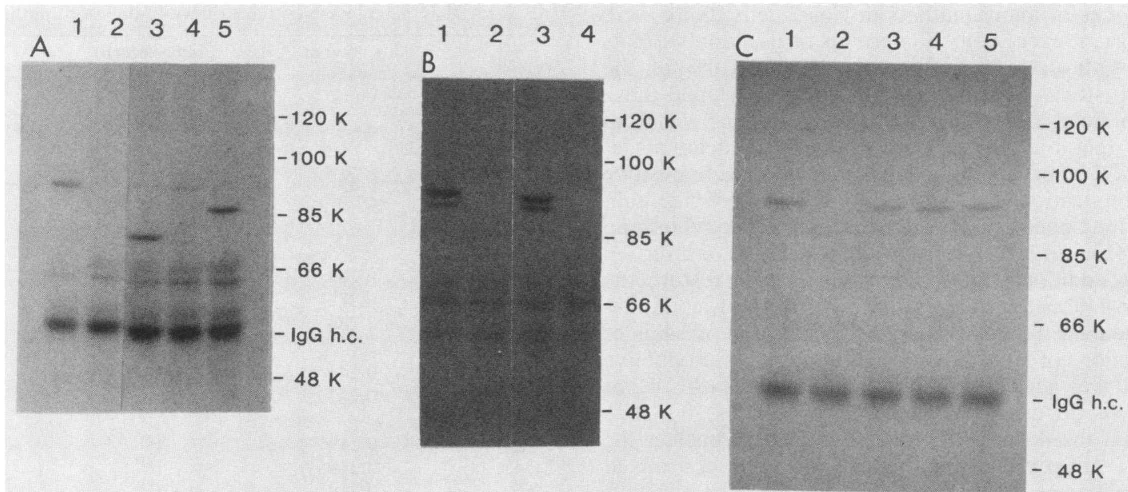

FIG. 3. SDS-polyacrylamide gel electrophoresis of mutant T antigens present in extracts used in ATPase assays. Proteins bound to polyacrylamide beads (1 μl settled volume) were eluted from the beads with SDS sample buffer, resolved on 7 to 15% gradient SDS-polyacrylamide gels, and stained with Coomassie blue. (A) Proteins from CV-1 cells infected with virus stocks: lane 1, wild-type SV40; lane 2, mock infected; lane 3, *dlA*2465; lane 4, *dlA*2433; lane 5, *dlA*2459. (B) Proteins from CV-1 cells infected with virus stocks: lane 1, wild-type SV40; lane 2, mock infected; lane 3, *dlA*2451; lane 4, *dlA*2422. (C) Proteins from CV-1 cells transfected with DNA: lane 1, wild-type SV40; lane 2, mock transfected; lane 3, *dlA*2411; lane 4, *dlA*2432; lane 5, *dlA*2462. Position and size (in kilodaltons [K]) of Ad2 + D2 marker proteins and Pab419 heavy chain (h.c., absent from panel B due to partial reduction of proteins) are shown at right.

erties of the protein so that it is unable to bind to SV40 DNA, even though the binding domain is retained.

With some of the mutants, the amount of labeled SV40 origin-containing fragment bound was quite low, but in these cases, the actual amount of mutant large T antigen present in infected cell extracts was also quite low, reflecting reduced stability of some mutant T antigens.

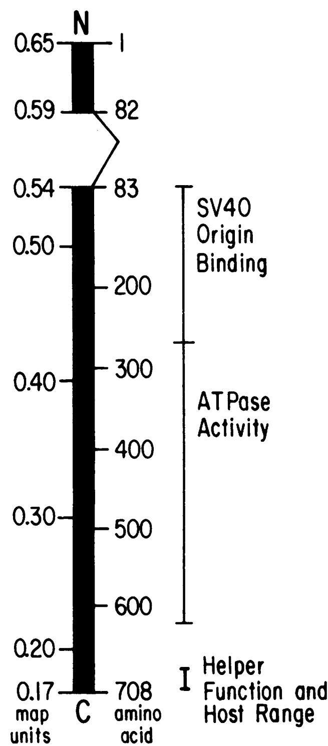

FIG. 4. Functional domains of SV40 large T antigen.

In addition, it is worth noting that the binding assay used in these experiments does not reveal whether the pattern and strength of binding of the mutant T antigens is identical to that of the wild-type polypeptide. It is possible that some mutant T antigens bind to the origin with reduced affinity or bind at slightly different sites or that the binding affinity is reduced over that observed with wild-type large T antigen. More-detailed studies will be required to examine in detail the binding of mutant large T antigens to the SV40 origin.

It is also interesting to note that the short fragments of large T encoded by *dlA*2420 and *dlA*2456 did not bind to the SV40 origin. These mutants produce large T antigens containing the amino-terminal 138 (*dlA*2420) or 140 (*dlA*2456) amino acids with an additional 6 or 5 amino acids, respectively, encoded in a different reading frame. Morrison et al. (23) have reported that a proteolytic fragment containing approximately 130 amino acids binds to the SV40 origin. The mutant fragments studied in our experiments may display different binding properties owing either to the additional amino acids encoded in a different reading frame or to the different number of normal amino acids in the different amino-terminal fragments studied here and in other experiments.

ATPase activity. Mutants lacking only one to four amino acids at map positions 0.288 and 0.243 showed little or no ATPase activity. Previously, ATPase activity was mapped to the region surrounding map position 0.30 by using monoclonal antibodies (4). Studies with deletion mutants (5) confirmed this result and suggested that additional regions (0.42 to 0.34 and 0.23 to 0.20) were involved in the ATPase activity. However, the mutants used in that study produced large T antigens lacking substantial carboxyl-terminal information or having large internal deletions. Our studies with mutants lacking only a few amino acids allow mapping with greater precision. The studies described here indicate that ATPase activity also requires residues at 0.287 and 0.243

m.u., suggesting that the ATPase domain is relatively large and continuous from the central portion toward the C terminus of the molecule. This domain does, however, show some degree of independence from the N-terminal DNA-binding domain since *dl*A2411 T antigen retains ATPase activity but lacks DNA-binding activity.

The large T antigen of *dl*A2465, which contains the amino-terminal 626 amino acids plus an additional glutamic acid residue at position 627, has normal levels of ATPase. The large T of *dl*A2465 is the smallest large T antigen fragment to display ATPase activity and demonstrates that sequences encoded distal to 0.219 m.u. (residues 627 to 708) are not required for ATPase activity.

It is therefore interesting to note that no ATPase activity was detected with mutant *dl*A2413 or *dl*A2414, even though both produce large T antigens containing the amino-terminal 624 to 627 residues. Just as we observed for origin-binding activity, it appears that the alternative carboxyl-terminal portion of these mutant polypeptides inactivates the ATPase activity of the polypeptide. It is interesting to note that *dl*A1135 T antigen shows a somewhat similar behavior (5, 25). That mutant, with an in-phase deletion of 33 bp, removing amino acids 17 to 27, was nonviable and defective for viral DNA replication, even though the residues deleted were also missing from the D2 adenovirus-SV40 hybrid protein, which is competent for ATPase. Thus, alterations in the structure of large T at either the amino terminus or the carboxyl terminus affected properties of the protein performed by residues located in more central portions of the polypeptide.

A mutant similar to *dl*A2413 and *dl*A2414 has been described previously (17) and is defective for viral DNA replication and transformation of NIH 3T3 cells; the DNA binding and ATPase activities of the mutant T antigen were not examined, but it is likely that its properties are the same as those of *dl*A2413 and *dl*A2414 large T antigens.

Requirements for viral DNA replication. A critical role for origin binding in DNA replication has long been postulated, but the need for ATPase activity in viral DNA replication has been less certain. The results we obtained when added to results obtained in previous studies (5, 25, 41–43) strongly suggest that ATPase activity is required for viral DNA replication. Replication has never been observed for an ATPase-defective mutant. The large T antigen of *dl*A2462 displays origin-binding activity, adenovirus helper function, complementation of *dl*A2459, and is relatively stable, yet shows no ATPase activity and is defective for viral DNA replication.

We reported previously that DNA replication did not occur in cells transfected with mutants *dl*A2411, *dl*A2462, *dl*A2432, and *dl*A2433, while DNA replication did occur in cells transfected with *dl*A2459 (42). None of these mutants were able to form plaques at 37°C in CV-1p cells, but plaques could be formed by complementation between *dl*A2459 and each of the other four mutants (41). Complementation to form plaques or to restore the replication function of large T was not observed when cells were cotransfected with any pair of the mutants *dl*A2432, *dl*A2433, *dl*A2462, and *dl*A2411. The current studies indicate that complementation to restore replication or to permit plaque formation does not occur between ATPase-defective, origin-binding-positive mutants and ATPase-positive, origin-binding-negative mutants. While it is possible that complementation might be observed if we prepared and studied additional binding-defective and ATPase-defective mutants, our results suggest that T antigen functionality requires the presence of both origin-binding and ATPase activities in a single T-antigen monomer. Even though these two activities of T are performed by different portions of the polypeptide, DNA replication initiated by T antigen appears to require the presence of both activities in a single monomer subunit. We cannot tell from our studies whether any of these mutants is defective in oligomerization or whether oligomerization is required for T-antigen functionality.

These studies indicate that the DNA-binding and ATPase activities of large T are performed by distinct portions of the polypeptide and that a mutant T antigen can display either or both of these activities. The carboxyl terminus of large T constitutes an additional separate and separable functional domain, required for efficient formation of capsid proteins and also involved in provision of adenovirus helper function. Thus, large T contains at least three distinct domains. Does large T antigen perform still further functions? This seems probable. Many of the mutants studied here have been analyzed for their ability to transform primary mouse embryo fibroblasts (M. J. Tevethia and C. N. Cole, unpublished data). We found that transformation occurred at normal frequencies with mutants defective in DNA binding (*dl*A2411), ATPase activity (*dl*A2432 and *dl*A2462), and in helper function-host-range activity (*dl*A2459). Although these mutant T antigens could retain very low levels of these functions, transformation does not require appreciable levels of the three activities of T antigen known to be required during the lytic cycle.

It also seems probable that the transformation functions of large T are also required for the normal lytic cycle of SV40. Presumably, transformation represents the consequence of a viral infection in which DNA replication and subsequent cell death are prevented. Major portions of the SV40 *A* gene have not been subjected to detailed genetic analysis, using the type of mutants used in the studies described here. Studies with additional mutants should reveal the location of the transformation functions of SV40 and permit analysis of the roles played in the normal SV40 lytic cycle by these oncogenic functions.

ACKNOWLEDGMENTS

We thank Mita Vogel and Deborah Klaus for excellent technical assistance, Maryellen Polvino-Bodnar and George Santangelo for helpful discussions, and Elmer Pfefferkorn and Steve Smale for critical reading of the manuscript.

This work was supported by grant PCM83-21805 from the National Science Foundation (to C.N.C.) and Public Health Service grants and CA-25417 (to R.T.) and CA-39259 (to C.N.C.) from the National Institutes of Health.

LITERATURE CITED

1. **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.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1413–1423.
3. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101–1109.
4. **Clark, R., D. P. Lane, and R. Tjian.** 1981. Use of monoclonal antibodies as probes of simian virus 40 T antigen ATPase activity. J. Biol. Chem. **256**:11854–11858.
5. **Clark, R., K. Peden, J. M. Pipas, D. Nathans, and R. Tjian.** 1983. Biochemical activities of T antigen proteins encoded by simian virus 40 *A* gene deletion mutants. Mol. Cell. Biol. **3**:220–228.
6. **Clewell, D. B., and D. R. Helinski.** 1970. A membrane filter technique for the detection of complementary DNA. Biochem-

istry 9:4428–4440.

7. **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.
8. **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.
9. **Fey, G., J. B. Lewis, T. Grodzicker, and A. Bothwell.** 1979. Characterization of a fused protein specified by the adenovirus type 2-simian virus 40 hybrid AD2+ND1 dp2. J. Virol. **40**:201–217.
10. **Giacherio, D., and L. P. Hager.** 1979. A poly dT stimulated ATPase activity associated with SV40 large T antigen. J. Biol. Chem. **254**:8113–8116.
11. **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.
12. **Jessel, D., J. Hudson, T. Landau, D. Tenen, and D. M. Livingston.** 1975. Interaction of partially purified simian virus 40 T antigen with circular DNA molecules. Proc. Natl. Acad. Sci. USA **72**:1960–1964.
13. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
14. **Kimura, G., and R. Dulbecco.** 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529–534.
15. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. Proc. Natl. Acad. Sci. USA **72**:673–677.
16. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**:801–813.
17. **Lewis, E. D., S. Chen, A. Kumar, G. Blanck, R. E. Pollack, and J. L. Manley.** 1983. A frameshift mutation affecting the carboxyl terminus of the simian virus 40 large tumor antigen results in a replication- and transformation-defective virus. Proc. Natl. Acad. Sci. USA **80**:7065–7069.
18. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
19. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA interaction. J. Mol. Biol. **53**:159–162.
20. **Manos, M. M., and Y. Gluzman.** 1985. Genetic and biochemical analysis of transformation-competent, replication-defective simian virus 40 large T antigen mutants. J. Virol. **53**:120–127.
21. **Maxam, A., and W. S. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
22. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
23. **Morrison, B., M. Kress, G. Khoury, and G. Jay.** 1983. Simian virus 40 tumor antigen: isolation of the origin-specific DNA-binding domain. J. Virol. **47**:106–114.
24. **Pipas, J. M.** 1985. Mutations near the carboxyl terminus of the simian virus 40 large tumor antigen alter viral host range. J. Virol. **54**:569–575.
25. **Pipas, J. M., K. W. C. Peden, and D. Nathans.** 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T antigen gene. Mol. Cell. Biol. **3**:203–213.
26. **Polvino-Bodnar, M., and C. N. Cole.** 1982. Construction and characterization of viable deletion mutants of simian virus 40 lacking sequences near the 3′ end of the early region. J. Virol. **43**:489–502.
27. **Pope, J. H., and W. P. Rowe.** 1964. Detection of specific antigen in SV40-transformed cells by immunofluorescence. J. Exp. Med. **120**:121–128.
28. **Prives, C., B. Barnet, A. Scheller, G. Khoury, and G. Jay.** 1982. Discrete regions of simian virus 40 large T antigen are required for non-specific and origin-specific DNA binding. J. Virol. **43**:73–82.
29. **Prives, C., Y. Beck, D. Gidoni, M. Oren, and H. Shure.** 1980. DNA binding and sedimentation properties of SV40 T antigens synthesized *in vivo* and *in vitro*. Cold Spring Harbor Symp. Quant. Biol. **44**:123–130.
30. **Rabson, A. S., G. T. O'Conor, 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.
31. **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. USA **73**:3083–3087.
32. **Rubin, C., and C. Schmid.** 1980. Pyrimidine-specific chemical reactions useful for DNA sequencing. Nucleic Acids Res. **8**:4613–4619.
33. **Scheller, A., L. Covey, B. Barnet, and C. Prives.** 1982. A small subclass of SV40 T antigen binds to the viral origin of replication. Cell **29**:375–383.
34. **Shortle, D., R. F. Margolskee, and D. Nathans.** 1979. Mutational analysis of the simian virus 40 replicon: pseudorevertants of mutants with a defective replication origin. Proc. Natl. Acad. Sci. USA **76**:6128–6131.
35. **Takemoto, K. K., R. L. Kirschstein, and K. Habel.** 1966. Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. J. Bacteriol. **92**:990–994.
36. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
37. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613–618.
38. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 *A* gene. J. Virol. **16**:168–178.
39. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen related protein. Cell **13**:165–179.
40. **Tooze, J.** 1981. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Tornow, J., and C. N. Cole.** 1983. Intracistronic complementation in the simian virus 40 A gene. Proc. Natl. Acad. Sci. USA **80**:6312–6316.
42. **Tornow, J., and C. N. Cole.** 1983. Nonviable mutants of simian virus 40 with deletions near the 3′ end of gene *A* define a function for large T antigen required after onset of viral DNA replication. J. Virol. **47**:487–494.
43. **Tornow, J., M. Polvino-Bodnar, G. Santangelo, and C. N. Cole.** 1985. 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. J. Virol. **53**:415–424.