JOURNAL OF VIROLOGY, Jan. 1985, p. 120–127
0022-538X/85/010120-08$02.00/0

Vol. 53, No. 1

Genetic and Biochemical Analysis of Transformation-Competent, Replication-Defective Simian Virus 40 Large T Antigen Mutants

M. MICHELE MANOS†* AND YAKOV GLUZMAN
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received 11 July 1984/Accepted 28 September 1984

To study the role of the biochemical and physiological activities of simian virus 40 (SV40) large T antigen in the lytic and transformation processes, we have analyzed DNA replication-defective, transformation-competent T-antigen mutants. Here we describe two such mutants, C8/SV40 and T22/SV40, and also summarize the properties of all of the mutants in this collection. C8/SV40 and T22/SV40 were isolated from C8 and T22 cells (simian cell lines transformed with UV-irradiated SV40). Early regions encoding the defective T antigens were cloned into a plasmid vector to generate pC8 and pT22. The mutations responsible for the defects in viral DNA replication were localized by marker rescue, and subsequent DNA sequencing revealed missense and one nonsense mutation. The T22 mutation predicts a change of histidine to glutamine at residue 203. C8 has two mutations, one predicts lysine$_{224}$ to glutamamic acid and the other changes the codon for glutamic acid$_{660}$ to a stop codon; therefore, C8 T antigen lacks the 49 carboxy-terminal amino acids. pC8A and pC8B were constructed to contain the C8 mutations separately. Plasmids pT22, pC8, pC8A, and pC8B were able to transform primary rodent cell cultures. T22 T antigen is defective in binding to the SV40 origin. C8B (49-amino-acid truncation) is a host-range mutant defective in a late function in CV-1 but not BSC cells. Analysis of T antigens in mutant SV40-transformed mouse cells suggests that the replicative function of T antigen is important in generating SV40 DNA rearrangements that allow the expression of "100K" variant T antigens in the transformants.

Large T antigen, a product of the simian virus 40 (SV40) *A* gene, is necessary in both the lytic cycle of the virus and in the neoplastic transformation of cells by SV40 (for review, see reference 50). During the lytic cycle in permissive cells, the multifunctional T antigen is required for several processes, including the initiation of viral DNA synthesis and the regulation of both early and late viral gene expression. In the nonlytic pathway, such as the infection of nonpermissive cells, large T antigen is implicated in both the initiation and maintenance of the transformed phenotype. The 82,000-molecular-weight (82K) T-antigen phosphoprotein binds specifically to the SV40 origin of replication (40, 48), has an ATPase activity (8, 15), and forms a complex with a host cell phosphoprotein (p53) in both permissive and nonpermissive cells (21, 28). T antigen has numerous effects on host cell physiology (for review, see reference 50), including the stimulation of cell DNA and RNA synthesis. It also exhibits a helper function, which allows adenovirus to grow more efficiently in monkey cells.

Studies of deletion and point mutants suggest that T antigen contains distinct domains that are capable of functioning somewhat independently. For example, the adenovirus helper function of T antigen resides in the carboxy-terminal 38 amino acids (12, 38). Several T-antigen activities, such as origin binding, viral DNA replication, ATPase activity, stimulation of cell DNA synthesis, stimulation of rDNA transcription, transformation, and nuclear localization have been mapped to specific regions of the *A* gene (9, 24, 29, 37, 46). Single-amino acid alterations between residues 147 and 214 were found to affect the origin-binding activity of T antigen (17, 39, 47). Stimulation of cell DNA synthesis and stimulation of rDNA transcription are distinguishable activities; several deletion mutants stimulate DNA synthesis without activating rRNA genes (13, 46). The rDNA transcription activation requires a T antigen containing only amino acid residues 1 through 509, whereas cell DNA synthesis can be stimulated by a truncated T antigen comprised of only the amino-terminal 272 amino acids (46). Origin-binding activity is distinct from both of these functions; deletion mutants that are defective in origin binding but retain the ability to stimulate cell DNA synthesis have been described, as well as some that retain origin-binding activity but do not stimulate rDNA transcription (9).

The lytic and transforming functions of T antigen are genetically separable; studies of permissive cells transformed by UV-irradiated SV40 provided an example of viral DNA replication-defective, transformation-competent T antigens (18). Genetic and biochemical analyses of these and similar mutants (17, 30, 47) revealed that the replicative function of T antigen is not essential in the transformation process. Furthermore, neither the origin-binding activity (39, 47) nor wild-type levels of ATPase activity (30) are necessary for transforming activity.

Here, we describe the analysis of two viral DNA replication-defective, transformation-competent T-antigen point mutants, C8/SV40 and T22/SV40, and summarize the information we have obtained from our studies of a collection of mutants with similar properties (17, 30).

This work also addresses the role of the replicative function of T antigen in the duplication and rearrangement of SV40 DNA in transformed cells. Although mouse cells are considered completely nonpermissive for SV40 replication, cell lines transformed by replication-defective SV40 have somewhat different properties than wild-type transformants. Many SV40-transformed mouse cell lines contain duplications and rearrangements of the integrated viral DNA (2, 22, 23, 41), often accompanied by the expression of super T or truncated T antigens, in addition to normal T antigen (5, 26,

* Corresponding author.
† Present address: Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608.

31, 32). Numerous SV40-transformed mouse 3T3 cell lines express both normal size and larger, "100K" T antigens (7, 45). The viral origin of replication (6) and the replicative activity of T antigen (R. Pollack, personal communication) have been implicated in the generation of the rearrangements which promote "100K" T antigen synthesis. We have further investigated the role of the replicative function of T antigen in the generation of the "100K" variant by analyzing the SV40- and mutant SV40-transformed primary mouse embryo fibroblasts.

(This work constitutes part of the doctoral thesis of M. Manos, Department of Microbiology, State University of New York at Stony Brook.)

MATERIALS AND METHODS

Cells, cell fusion, and transformation. Fusion of transformed simian cell lines with COS-1 cells, and the transformation of primary mouse embryo fibroblasts (MEF) with cloned SV40 DNAs were described previously (30). CV-1 and BSC-1 cells (obtained from the American Type Culture Collection), C8, and T22 cells (44) were cultured in Dulbecco modified Eagle medium plus 5% fetal calf serum. C8 cells are a CV-1 cell line transformed with UV-irradiated SV40, and are a member of the C series which includes C2, C11, and C6 cells (18).

Bacterial strains and plasmids. Plasmids were propagated in *Escherichia coli* DH-1 (19). Plasmids pK1, pC6-1, and pC11B have been described previously (17, 30). Information regarding the plasmids containing wild-type SV40 early region fragments is available from the authors upon request.

Cloning SV40 sequences from C8 and T22 cells. The isolation and cloning of viral early regions from T22 and C8 cells was done as previously described for C2 and C11 cells (30).

Marker rescue. Marker rescue experiments were done with pT22, pC8, pC8A, and pC8B by methods described previously (30). Wild-type SV40 fragments were generated by digestion of recombinant plasmids. *Mbo*I D and C fragments contain nucleotides 4710 through 4100 and 3716 through 2771, respectively. The *Alu*I C fragment spans nucleotides 4643 through 4314. Nucleotide numbers are in the SV system (50).

Separation of C8 mutations. pC8 was digested with *Pst*I, and the two resultant fragments were purified by agarose gel electrophoresis. The larger (A) fragment was ligated with the wild-type *Pst*I B fragment (from plasmid pSVPstB) to produce pC8A. The smaller (B) fragment of pC8 was cloned into the *Pst*I site of pK1-P (30) to make pC8B.

DNA sequencing. The *Mbo*I D fragment (nucleotides 4710 through 4100) was isolated from pC8 and pT22, and the *Mbo*I C fragment (nucleotides 3716 through 2771) was isolated from pC8 by digestion with *Sau*3a and purification by acrylamide gel electrophoresis. The fragments were cloned into the *Bam*HI site of mp8 (34), and the recombinant phage DNAs were used in chain termination sequencing reactions (42, 43).

DNA replication analyses. Viral DNA replication in CV-1 cells was analyzed as described previously (30).

Origin binding. Nuclear extracts were prepared from transformed cells as previously described (30). SV40 DNA used in the assays was nick translated with ^{32}P-labeled deoxynucleotide triphosphates and then digested with *Bst*NI. Binding assays were performed by methods described previously (33). T antigen-DNA complexes were immunoprecipitated with monoclonal antibody PAb416 (10, 20) and protein A-Sepharose (Pharmacia Fine Chemicals, Inc.). The amount of T antigen present per volume of cell extract was determined by double-antibody radioimmunoassay (1) with monoclonal antibodies PAb416 and PAb419 (10, 20).

Immunoprecipitation of T antigens from transformed cells. Proteins were labeled by incubating cells with [^{35}S]methionine (300 μCi per 10-cm dish) for 1 h. Cell lysis and immunoprecipitations were performed by methods previously described (10), with some modifications. Preclearing with nonimmune serum was omitted, and protein A-Sepharose (Pharmacia) was used in place of *Staphylococcus aureus*. A mixture of monoclonal antibodies PAb419 and PAb431 (10, 20) was used. Proteins were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels.

RESULTS

Cloning C8 and T22 mutant early regions. C8 and T22 are SV40-transformed simian cell lines that synthesize T antigens defective in viral DNA replication. C8 cells were isolated after the infection of CV-1 cells with UV-irradiated SV40, and the C8 cell line is a member of the C series of transformed cell lines that includes C6, C2, and C11 (18). C8 cells are permissive for the replication of wild-type SV40 and have nuclear localization of T antigen as shown by immunofluorescence (unpublished data). The T22 cell line (44) was isolated after green monkey kidney cells were infected with UV-irradiated SV40 defective particles, and its properties are similar to those of C8.

SV40 early regions encoding the mutant T antigens were isolated as follows. T22 or C8 cells were fused with COS-1 cells (16) to rescue the viral DNA from the cellular genomes. Free viral DNA was isolated from the heterokaryons and cloned into the vector pK1 as described previously (30). Resultant plasmids pC8 and pT22 contain a complete viral genome: the mutant early regions (nucleotides 5243 through 2537, *Bgl*I-*Bam*HI site) from C8 and T22 cells, respectively, and the wild-type SV40 late region (nucleotides 2536 through 1, *Bgl*I-*Bam*HI site) from pK1.

Localization of mutations by marker rescue. To map the mutations responsible for the replication defects in pC8 and pT22 (M. M. Manos, Ph.D. thesis, State University of New York at Stony Brook, 1984), marker rescue studies were done as previously described (30). As expected, neither pC8 nor pT22 produced plaques when no wild-type SV40 early region fragments were included. pT22 was rescued by the SV40 *Hin*dIII B fragment (nucleotides 5171 through 4002) or the *Mbo*I D fragment (nucleotides 4710 through 4100) but was not rescued by the *Alu*I C fragment (nucleotides 4643 through 4314). Since nucleotides 4710 through 4644 of the *Mbo*I D fragment encode part of the large T-antigen mRNA intron, these results localized the T22 lesion to the region of the *Mbo*I D fragment between nucleotides 4314 and 4100. The results with pC8 were more complicated. Neither the *Hin*dIII A′ (nucleotides 3373 through 2848, *Hin*dIII-*Bam*HI), *Hin*dIII D (nucleotides 4002 through 3476), or *Hin*dIII B fragment could rescue pC8. But pC8 was rescued when both the *Hin*dIII A′ and B fragments were included. This suggested that there was at least one mutation within each of these fragments in pC8. The mutations were further localized with the *Mbo*I D, *Alu*I C, *Mbo*I C (nucleotides 3716 through 2771), and *Hin*fI E (nucleotides 3373 through 2848) fragments in various combinations. The mutations in pC8 were defined to two regions, one between nucleotides 4314 and 4100 of the *Mbo*I D fragment and another between nucleotides 2771 and 2848 of the *Mbo*I C fragment.

DNA sequence analyses. The regions to which the pC8 and pT22 mutations had been mapped by marker rescue were sequenced by the chain termination method (43). One nucleotide substitution was found in pT22, predicting a change of histidine to glutamine at residue 204 (Table 1). pC8 also has a mutation in this area, predicting a change at amino acid 224 of lysine to glutamic acid. The second lesion in pC8 is a nonsense mutation that changes codon 660 (glutamic acid) to an ochre (UAA) termination codon (Table 1). This predicts that C8 T antigen lacks the carboxy-terminal 49 amino acids found in wild-type T antigen. pC8 differs from the published SV40 sequence (50) at additional nucleotides between 2771 and 2848 in the *Mbo*I C fragment, nucleotides 2817 (cytosine to thymine) and 2781 (thymine to cytosine, early strand), and there is a tandem duplication of nucleotides 2795 to 2787, predicting an additional three amino acids. This tandem duplication was previously observed in the LP strain of SV40 (11). These additional differences are downstream from the pC8 nonsense mutation and, thus, do not influence the C8 T antigen structure. In the *Mbo*I D fragment, both pC8 and pT22 have an adenine rather than guanine at nucleotide 4110 (early strand). These differences are probably specific to SV40 strain 777, which was used to generating the C series transformed cell lines. The 2817 change has been noted in other SV40 strains (W. Gish and M. Botchan, personal communication). The 2817, 2781, and 4110 changes are all silent, third-base substitutions. Comparison of strains 777 and 776 by restriction mapping revealed several differences (25), some of which reflect the nine-nucleotide duplication.

Separation of the C8 mutations. To study their effects independently, the two C8 lesions were separated from each other into distinct viral genomes in pC8A and pC8B. pC8A contains the C8 mutant early region from the *Bgl*I site to the *Pst*I site (nucleotides 5243 through 3204), and the remaining early region is wild type. Therefore, pC8A encodes a T antigen of normal size, with the lysine-to-glutamic acid alteration at residue 224. pC8B contains the C8 mutant early region from the *Pst*I-*Bam*HI site (nucleotides 3204 through 2533), with the remaining wild-type viral sequences. pC8B encodes a T antigen that lacks the carboxy-terminal 49 amino acids.

The construction of pC8A and pC8B was confirmed by marker rescue (Manos, Ph.D. thesis). Neither plasmid produced plaques without the addition of wild-type early fragments. pC8A was rescued by the *Mbo*I D fragment, and pC8B was rescued by the *Mbo*I C fragment, as expected.

Replication of C8A and C8B mutants. To determine the independent effects of the two C8 mutations on viral DNA replication, we analyzed the replication of viral DNA from the plasmids in CV-1 cells. CV-1 cells were transfected with pK1 (a plasmid containing the wild-type SV40 genome), pC8, pC8A, pC8B, or pC11B (a pinpoint plaque mutant [30]),

TABLE 1. Mutations in pC8 and pT22, and predicted amino acid changes

Mutant	Nucleotide		Amino acid	
	No.[a]	Change	Number	Change
T22	4209	CAT → CAA	203	Histidine to glutamine
C8A	4148	AAA → GAA	224	Lysine to glutamic acid
C8B	2840	GAA → TAA	660	Glutamic acid to stop codon

[a] Nucleotide numbers are in the SV system (50).

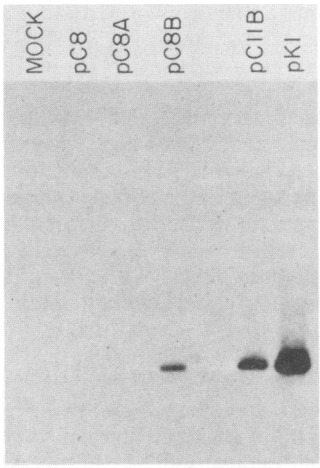

FIG. 1. Replication of mutant DNAs in CV-1 cells. CV-1 cells were transfected with the plasmids denoted as previously described (30). Low-molecular-weight DNA was extracted after 72 h, digested with *Dpn*I plus *Bam*HI, and analyzed by blot hybridization with [^{32}P]pK1 as a probe. pK1 contains the wild-type SV40 genome, and pC11B (30) is a pinpoint plaque mutant.

and replication products were isolated 72 h later and analyzed as previously described (30). pC8 and pC8A did not replicate, but pC8B replicated to a level similar to the pinpoint plaque mutant pC11B (Fig. 1). Therefore, C8B T antigen, which lacks the carboxy-terminal 49 amino acids, is able to replicate viral DNA to a level that is potentially sufficient for plaque formation (that is, a level comparable to pC11B). Since plaques were not observed on CV-1 cells transfected with pC8B, we conclude that C8B T antigen is defective in a function which is essential in the lytic pathway after the onset of DNA replication.

Growth of mutant C8B in CV-1 versus BSC cells. Several T-antigen mutants that are defective in a late function in CV-1 cells do not exhibit this defect in BSC cells (another African green monkey kidney cell line), and are able to form plaques on them (51; C. Cole, personal communication; J. Pipas, manuscript in preparation). Therefore, we tested the ability of pC8B to form plaques on BSC cells. Low-passage CV-1 or BSC-1 cells were transfected with viral DNA from pK1 (containing wild-type SV40 DNA), pC8B, or pC11B as previously described (30). pC8B was able to form plaques, although less efficiently than pK1, on BSC cells (Table 2). pC8B and pC11B (30) behaved similarly in BSC cells; both formed pinpoint plaques that appeared later than wild-type pK1 plaques. In contrast, in CV-1 cells the titer of pC8B is 0.1% or less than that of pC11B, and 0.017% or less than that of pK1. These results suggest that the C8B chain termination

TABLE 2. Growth of mutants C8B and C11B in CV-1 and BSC cells

DNA	Titer (per 100 ng)[a]	
	CV-1	BSC-1
pK1	6×10^3	5×10^3
pC8B[b]	0	8.6×10^2
pC11B[b]	1×10^3	1.2×10^3

[a] Plasmids were digested with *Eco*RI and ligated to promote the formation of circular viral genomes; the DNA was then used to transfect CV-1 or BSC cells by the DEAE-dextran technique with modifications (30). Samples of 100, 10, 1, or 0.1 ng of pK1, pC8B, or pC11B (30) were placed on 6-cm plates. Plaques were scored after 10 days for pK1 and after 18 days for pC8B and pC11B.

[b] Both pC8B and pC11B produced pinpoint plaques.

mutation has a host range effect. The absence of the 49 carboxy-terminal amino acids incapacitates T antigen in an activity that is essential for virus growth in CV-1 cells but is dispensable in BSC cells. It is predicted that C8B T antigen is also defective in adenovirus helper function, known to reside in the carboxy-terminal 38 amino acids (12, 38).

Analysis of T22 and C8 T antigens. SDS-polyacrylamide gel analysis of the various T antigens in T22, C8, MC8, MC8A, and MC8B cells is shown in Fig. 2. MC8, MC8A, and MC8B lines were isolated after the transformation of primary mouse embryo fibroblasts (MEF) with pC8, pC8A, and pC8B, respectively. T22 and MC8A T antigens migrate with the wild-type T antigen of COS-1 cells, whereas C8, MC8, and MC8B have a smaller T antigen as predicted by DNA sequencing (molecular weight: calculated, 76,000; apparent, 80,000). The p53 host tumor antigen is present in all lanes, indicating that the C8 and T22 T antigens are not defective in their association with this cellular protein in either mouse or simian cells.

Binding of T22 and C8 T antigens to the SV40 origin. The abilities of the variant T antigens to bind specifically to the SV40 origin of replication were analyzed (Fig. 3) by the immunoprecipitation method (33). T antigens from all cells except T22 were able to bind preferentially to the origin fragment (Fig. 3, G fragment, arrow). The relative amounts of T antigen present per volume of nuclear extract were determined by radioimmunoassay with the same monoclonal antibody used in the origin-binding experiments. The T22 and C8 extracts had amounts comparable with that of COS-1 cells, and MC8A and MC8B were comparable with MK1 values (Manos, Ph.D. thesis). These data indicate that T22 T antigen is defective in origin-specific binding, and that C8, C8A, and C8B T antigens are comparable with wild type in this activity. Although the C8 and C8A T antigens behave similarly to wild type in these assays, it is possible that they bind to the origin in a manner that is not suitable for the initiation of DNA replication.

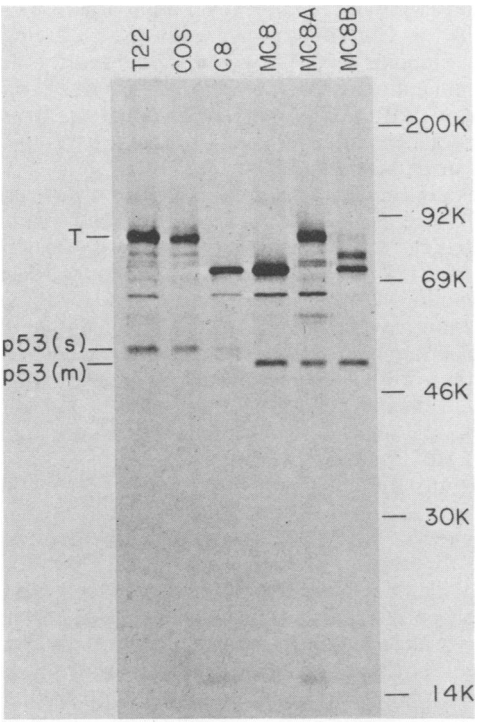

FIG. 2. Immunoprecipitation of [^{32}S]methionine-labeled nuclear extracts with anti-T antigen monoclonal antibodies. Extracts from the cells denoted were immunoprecipitated and analyzed on a SDS–10% polyacrylamide gel. Simian (s) and mouse (m) p53 proteins and normal-sized T-antigen (T) positions are noted.

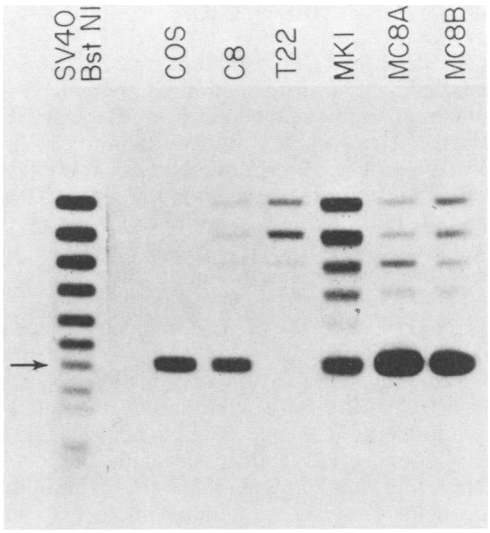

FIG. 3. Binding of mutant T antigens to the SV40 origin. Nuclear extracts of the transformed cells were incubated with ^{32}P-labeled *Bst*NI SV40 DNA fragments. The T antigen-DNA complexes were immunoprecipitated with PAb416, and the bound DNA fragments were separated on a 1.8% agarose gel. The arrow denotes the SV40 *Bst*NI G fragment which includes the viral origin of replication.

Comparison of T antigens in mouse cells transformed by wild-type or mutant SV40 DNAs. SV40-transformed mouse cells often synthesize ca. 100K forms of large T antigen in addition to the normal-sized 82K protein (7, 45). We analyzed the T antigens in primary MEF cultures transformed by plasmids encoding C series mutant T antigens or wild-type T antigen (pK1). Four separately isolated lines transformed by pK1 [MK1(1), MK1(2), MK1-2, and MK1-3] and seven MEF lines transformed by plasmids which encode replication-defective T antigens [MC2-1, MC8-1, MC8A-3, and MC11A-1 (Fig. 4) and MC8-3, MC2-2, and MC11-1 (not shown)] were analyzed. In addition, MC8B-4 cells were examined; these cells synthesize the C8B truncated T antigen, which supports a reduced level of viral DNA replication. Our data are consistent with the previously proposed model that the replicative function of T antigen is required for the DNA rearrangements that lead to the formation of genes encoding "100K" T antigens in mouse cells (R. Pollack, personal communication). None of the seven replication-defective mutant-transformed lines (MC2, MC11, MC11A, MC8, and MC8A isolates) had detectable amounts of a super T antigen. In contrast, all four of the independently isolated wild-type transformants (MK1 lines) synthesize the "100K" T antigen, and one of them (MK1-3) produces an additional ca. 200K variant. Comparison of MC8 and MC8B cell extracts (Fig. 4) also links the replicative function of T antigen to the generation of "100K" T antigens. MC8 cells synthesize a truncated T antigen which is replication defective; it contains the lysine-to-glutamic acid change at residue 224 as well as the 49-residue truncation. Only a ca. 80K T antigen is detected in MC8 cell extracts. In contrast, MC8B cells (which produce a replication-competent, truncated T antigen) synthesize a ca. 85K "mini super T antigen," in addition to the ca. 80K species.

DISCUSSION

We have described the isolation and analysis of two replication-deficient, transformation-competent SV40 T-antigen mutants. They were isolated from the genomes of C8 and T22 cells (African green monkey kidney cell lines that were transformed with UV-irradiated SV40 [18, 44]) where early viral DNA sequences encoding the mutant T antigens are integrated. Plasmids pC8 and pT22, containing the viral early regions from C8 or T22 cells, exhibited the predicted replication-defective phenotype in marker rescue experiments.

DNA sequence analyses of pT22 and pC8 revealed missense and nonsense mutations. pT22 has a nucleotide substitution predicting the replacement of histidine by glutamine at residue 203. pC8 has two mutations: one predicting a lysine-to-glutamic acid change at amino acid 224 and another replacing glutamic acid codon 660 with the ochre stop codon. Therefore, C8 T antigen lacks the 49 carboxy-terminal amino acids and has a predicted molecular weight of 76,000. The two pC8 mutations were separated from each other into two distinct viral genomes in pC8A (amino acid 224 substitution) and pC8B (termination at codon 660).

Mutant plasmids pC8, pC8A, pC8B, and pT22 are competent in the oncogenic transformation of cells in culture. Each was comparable with the wild-type plasmid pK1 in transforming primary MEF cultures (Manos, Ph.D. thesis) or primary baby rat kidney cells (data not shown).

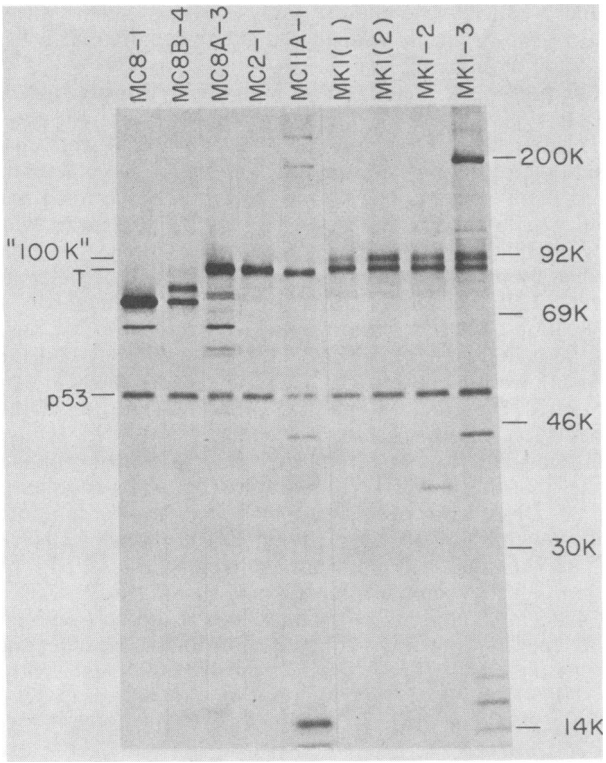

FIG. 4. [^{35}S]methionine-labeled extracts from transformed primary mouse embryo fibroblasts. Independent isolates of MEF cells transformed by pK1 (containing wild-type SV40 DNA) (lanes 6 through 9) or by pC8, pC8B, pC8A, pC2, or pC11A (29) (lanes 1 through 5) were analyzed. Extracts of the labeled cells were immunoprecipitated with monoclonal antibodies specific for T antigen, and were separated on an SDS–10% polyacrylamide gel. The positions of p53, normal-sized T antigen, and the "100K" super T antigen are noted.

The portion of T antigen including residues 83 through 215 has been implicated in origin-binding activity. Origin-binding activity is affected by missense mutations that cause alterations between amino acids 140 and 215 (39, 47), and the amino-terminal tryptic peptide extending to residue 130 was reported to bind efficiently to the origin of replication (35). We have shown that C8, C8A, and C8B T antigens are similar to wild-type T antigen in origin-binding activity. Therefore, the replicative functions that are defective in C8 are distinct from origin binding (Fig. 3). In contrast, the replication deficiency of T22 T antigen may be explained by a defect in origin-binding activity. The altered amino acid in T22 (residue 203) is within the presumptive origin-binding region discussed above, and T22 T antigen has no origin-binding activity in the assays described here.

The defective replicative functions of the mutant T antigens are probably distinct from p53 binding activity, since they are similar to wild type in this property in both monkey and mouse cells. The defective functions are also distinct from ATPase activity. C8 and T22 T antigens were found to have wild-type levels of ATPase activity (Manos, Ph.D. thesis).

A host range mutant phenotype results from the 49-carboxy-terminal-amino acid truncation of C8B T antigen. Others (J. Pipas, manuscript in preparation; C. Cole, personal communication) have found that mutations affecting the adenovirus helper function domain (the carboxy terminus of T antigen) sometimes confer a host range phenotype; that is, although they are viral DNA replication-competent in CV-1 and BSC cells, they produce virus only in BSC cells. We have shown that C8B behaves similarly. Mutant C8B supports viral DNA replication in CV-1 cells at a level comparable with that of C11B (30), ca. 10-fold less than wild-type pK1. Since C11B produces pinpoint plaques, we conclude that the decreased level of replication of C8B is sufficient for virus propagation. The lack of C8B virus growth suggests that the truncated T antigen is defective in a late activity that is essential in CV-1 cells but dispensable in BSC cells.

Studies concerning C8B gene expression in CV-1 versus BSC cells are necessary to define the late function that resides in the carboxy terminus of T antigen. This function is coincident with and may be identical to the adenovirus helper function. Further investigation of the SV40 host range phenomenon may reveal the role of this T-antigen function (which also manifests itself as adeno-helper activity) in the SV40 lytic cycle. The block in adenovirus growth in some simian cells, such as CV-1, is proposed to be at both the late transcriptional and late translational levels (for review, see reference 50). Presently, we are analyzing C8B late transcription and translation in a variety of African green monkey cell lines.

Polyomavirus host range transformation-defective (hr-t) mutants (for review, see reference 3), originally characterized by their poor growth on normal 3T3 cells and normal growth on polyomavirus-transformed 3T3 cells, differ from SV40 host range mutants, but they also have some similarities. Mutations that confer the polyomavirus hr-t phenotype map to the region encoding amino acids common between the small and middle tumor antigens and do not affect the polyoma large T antigen. The polyomavirus hr-t mutants are completely defective in transformation of cells in culture, in contrast to SV40 host range mutants like C8B which are transformation competent. However, polyomavirus hr-t mutants, like SV40 host range mutants, are defective in a late

TABLE 3. Summary of T-antigen mutants and their characteristics

Mutant[a]	Amino acid change (residue)	DNA replication[b]	Origin[c]	Virus growth[d]		ATPase	Transformation	
				CV-1	BSC		MEF[e]	Rat[f]
C11A[g]	Proline to serine (522)	−	+[h]	−	ND	−	+	+
C2[g]	Lysine to arginine (516)	−	+[h]	−	ND	+	+	+
C8A	Lysine to glutamic acid (224)	−	+	−	ND	+	+	+
C6-2[i]	Asparagine to threonine (153)	−	−[h]	−	ND	+	+	+
T22	Histidine to glutamine (203)	−	−	−	ND	+	+	+
C6-1[i]	Methionine to isoleucine (30) and lysine to asparagine (51)	−−/+	+[h]	−	ND	+	+	+
C8B	Glutamic acid to stop codon (660)	++/−	+	−	+PP	+	+	ND
C11B[g]	Proline to histidine (549)	++/−	+	+PP	+PP	+	+	+

[a] All of the mutants bind to the p53 host tumor antigen in both simian and mouse cells (21; this work).
[b] Viral DNA replication in CV-1 cells. Degrees of replication: + (wild-type level) > ++/− > −−/+ > − (negative).
[c] Preferential binding to the SV40 origin of replication.
[d] Plaque formation on CV-1 or BSC cells. Symbols: +, positive; −, negative. PP, Pinpoint plaques; ND, not done.
[e] Primary mouse embryo fibroblast cultures.
[f] Primary baby rat kidney cells or Rat2 cells.
[g] See reference (30).
[h] See reference (39).
[i] See reference (17).

function in their respective restrictive host cell types. In the case of polyomavirus hr-t mutants, the defective late function is thought to be involved in polyoma virion assembly (14). It is interesting that although polyoma and SV40 large T antigens are quite homologous, the SV40 host range mutant phenotype results from changes in SV40 T antigen in the region void of homology with polyoma large T antigen (that is, the carboxy-terminal region). Possibly the carboxy-terminal domain of SV40 large T antigen contains an activity that is analogous to the late lytic hr-t activity resident in polyoma small or middle tumor antigen.

SV40 strain 777 was used in the generation of C2, C8, C11, and T22 cell lines, whereas the published sequence (50) is that of strain 776. DNA sequence analyses of the complete region coding for T antigen in strain 777 (17, 30; and this work) have revealed numerous nucleotide differences between the two strains, some of which predict differences in T-antigen amino acids. The nine-nucleotide duplication, which was found in the LP strain (11), was also found in pC8 (see above), pC2, and pT22 (unpublished data) and is therefore a strain 777-specific property. This duplication predicts that the T antigen synthesized by strain 777 contains three additional amino acids (Pro-Ser-Gln) between residues 675 (glutamine) and 676 (serine) (strain 776 numbering). This small increase in T-antigen size is visible in polyacrylamide gel analyses (e.g., shorter exposures of gels in Fig. 3 and 4). T antigens with carboxy termini that are encoded by strain 777 sequences migrate slightly more slowly than those with strain 776-encoded carboxy termini. Additional strain 777-specific silent changes were found by DNA sequencing: early strand, nucleotide 4071 (adenine to thymine) and 3755 (thymine to cytosine); and two changes that have been noted in other strains (W. Gish and M. Botchan, personal communication), 2757 (cytosine to thymine) and 2741 (thymine to cytosine). Differences in the primary structure of T antigen between wild-type strains indicate alterations that do not appear to affect T-antigen function. The identification of these somewhat flexible residues may also be helpful in understanding the relationship between T-antigen structure and function.

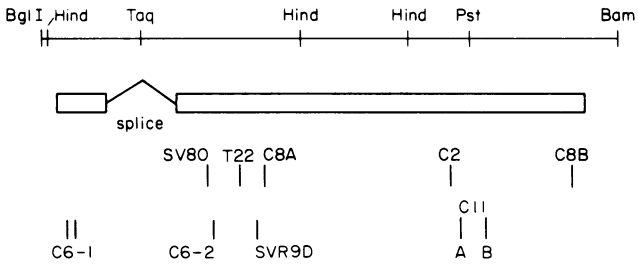

FIG. 5. Lesions in replication-defective, transformation-competent T-antigen point mutants. C6-1, C6-2 (17), C2, C11A, and C11B (30), SVR9D (47), and SV80 were described previously. All mutations are missense except C8B, which is a nonsense mutation.

Figure 5 diagrams the region coding for T-antigen and shows the location of missense and nonsense mutations that confer the replication-defective, transformation-competent phenotype. None of these mutations map within the middle of the gene, where most of the *tsA* mutations map (*Hin*dIII D fragment, nucleotides 4003 through 3372, [27]). The portion of T antigen encoded by this region may be important in maintaining a tertiary or quaternary structure that is essential for both replicative and transforming activities. It is also possible that it harbors the active site of a transforming function.

Table 3 summarizes the properties of the T-antigen mutants described by our laboratory here and previously (17, 30). All of them are defective in viral DNA replication, but all retain the ability to transform primary rodent cell cultures. These mutants are all able to form a complex with the p53 host tumor antigen in both simian and mouse cells. It is probable that p53 binding activity plays a role in the transformation process. Studies of mutant C11A indicated that a wild-type level of ATPase activity is not necessary for transformation. However, ATPase activity is probably essential for viral DNA replication, since no ATPase-defective, replication-competent mutants have been described to date (9, 30). C8B T antigen does have ATPase activity, indicating that the carboxy-terminal 49 residues are dispensible for this function. C8A T antigen has a drastic (glutamic acid for lysine) single-amino acid substitution close to the proposed origin-binding domain, yet it retains the ability to bind to the origin. In contrast, other mutations in this region render C6-2 and T22 T antigens unable to bind to the origin.

The extremely low level of viral DNA replication supported by C6-1 T antigen, without an obvious defect in origin-binding, is due to changes in the amino-terminal region of the protein. Alterations in this region have a variety of effects; small deletions can affect ATPase, origin-binding, and transformation (9, 37). Other data however indicate that the amino terminus of T antigen is not essential for origin-binding and ATPase activities; D2 T antigen lacks residues encoded by the first exon, but retains these activities (36, 49). These data suggest that this region may influence the tertiary or possibly quarternary structure of T antigen. Furthermore, point mutations in this region can render T antigen able to replicate DNA but defective in transforming cells (K. Peden and J. Pipas, personal communication). Changes in the carboxy-terminal third of T antigen can affect the level of DNA replication without affecting origin binding, as in C8B and C11B. C8B T antigen lacks a region that contains adenovirus helper function and it is deficient in a late function in CV-1 but not BSC cells. It is possible that some of the mutations described here influence the oligomerization of T-antigen molecules. For example, it has been suggested that oligomeric, but not monomeric, forms of T antigen possess ATPase activity (4). Perhaps the C11A mutation, which predicts the change of a proline residue to a serine, affects the oligomerization process.

ACKNOWLEDGMENTS

We thank P. Tegtmeyer, D. Hanahan, E. Harlow, K. VanDoren, C. Cole, J. Pipas, and C. Prives for helpful discussions. We thank F. McCormick and W. Herr for critically reading the manuscript. We also thank E. Jarvis and K. Levenson of Cetus Corporation for preparation of the manuscript.

This work was supported by Public Health Service grant CA 13106 from the National Cancer Institute.

LITERATURE CITED

1. **Benchimol, S., D. Pim, and L. V. Crawford.** 1982. Radioimmunoassay of the cellular protein p53 in mouse and human cell lines. EMBO J. **1**:1055–1062.
2. **Bender, M. A., and W. W. Brockman.** 1981. Rearrangement of integrated viral DNA sequences in mouse cells transformed by simian virus 40. J. Virol. **38**:872–879.
3. **Benjamin, T. L.** 1982. The hr-t gene of polyoma virus. Biochim. Biophys. Acta **695**:69–95.
4. **Bradley, M. K., J. D. Griffin, and D. M. Livingston.** 1982. Relationship of oligomerization to enzymatic and DNA-binding properties of the SV40 large T antigen. Cell **28**:125–134.
5. **Chang, C., D. T. Simmons, M. A. Martin, and P. T. Mora.** 1979. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. J. Virol. **31**:463–471.
6. **Chen, S., D. S. Grass, G. Blanck, N. Hoganson, J. L. Manley, and R. E. Pollack.** 1983. A functional simian virus 40 origin of replication is required for the generation of a super T antigen with a molecular weight of 100,000 in transformed mouse cells. J. Virol. **48**:492–502.
7. **Chen, S., M. Verderame, A. Lo, and R. Pollack.** 1981. Nonlytic simian virus 40-specific 100K phosphoprotein is associated with anchorage-independent growth in simian virus 40-transformed and revertant mouse cell lines. Mol. Cell. Biol. **1**:994–1006.
8. **Clark, R., D. 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.
9. **Clark, R., K. Peden, J. 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.
10. **Crawford, L., K. Leppard, D. Lane, and E. Harlow.** 1982. Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen. J. Virol. **42**:612–620.
11. **Feunteun, J., G. Carmichael, J. C. Nicolas, and M. Kress.** 1981. Mutant carrying deletions in the two simian virus 40 early genes. J. Virol. **40**:625–634.
12. **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. **30**: 201–217.
13. **Galanti, N., G. J. Jonak, K. J. Soprano, J. Floros, L. Kaczmarek, S. Weissman, V. B. Reddy, S. M. Tilghman, and R. Baserga.** 1981. Characterization and biological activity of cloned simian virus 40 DNA fragments. J. Biol. Chem. **256**:6469–6474.
14. **Garcia, R. L., and T. L. Benjamin.** 1983. Host range transforming gene of polyoma virus plays a role in virus assembly. Proc. Natl. Acad. Sci. U.S.A. **80**:3613–3617.
15. **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.
16. **Gluzman, Y.** 1981. SV40 transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
17. **Gluzman, Y., and B. Ahrens.** 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. Virology **123**:78–92.
18. **Gluzman, Y., J. Davison, M. Oren, and E. Winocour.** 1977. Properties of permissive monkey cells transformed by UV-irradiated simian virus 40. J. Virol. **22**:256–266.
19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
20. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for the simian virus 40 tumor antigens. J. Virol. **39**:861–869.
21. **Harlow, E., D. C. Pim, and L. V. Crawford.** 1981. Complex of simian virus 40 large-T antigen and host 53,000-molecular-weight protein in monkey cells. J. Virol. **37**:564–573.
22. **Hiscott, J., D. Murphy, and V. Defendi.** 1980. Amplification and rearrangement of integrated SV40 DNA sequences accompany the selection of anchorage-independent transformed mouse cells. Cell **22**:535–543.
23. **Hiscott, J. B., D. Murphy, and V. Defendi.** 1981. Instability of integrated viral DNA in mouse cells transformed by simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **78**:1736–1740.
24. **Kalderon, D., W. Richardson, A. Markham, and A. Smith.** 1984. Sequence requirements for nuclear localization of simian virus 40 large T antigen. Nature (London) **311**:33–38.
25. **Kay, A. C., G. R. Koteswara Rao, and M. F. Singer.** 1978. Comparison of two viable variants of simian virus 40. J. Virol. **25**:339–348.
26. **Kress, M., E. May, R. Cassingena, and P. May.** 1979. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J. Virol. **31**:472–483.
27. **Lai, C. J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70–81.
28. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
29. **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.
30. **Manos, M. M., and Y. Gluzman.** 1984. Simian virus 40 large T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. Mol. Cell. Biol. **4**:1125–1133.
31. **May, P., M. Kress, M. Lange, and E. May.** 1980. New genetic information expressed in SV40-transformed cells: characterization of the 55K proteins and evidence for unusual SV40 mRNAs. Cold Spring Harbor Symp. Quant. Biol. **44**:189–200.
32. **McCormick, F., F. Chaudry, R. Harvey, R. Smith, P. W. J. Rigby, E. Paucha, and A. E. Smith.** 1980. T antigens of SV40-transformed cells. Cold Spring Harbor Symp. Quant. Biol. **44**:171–178.
33. **McKay, R.** 1981. Binding of a simian virus 40 large T antigen related protein to DNA. J. Mol. Biol. **145**:471–488.
34. **Messing, J., and J. Vieria.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction frag-

ments. Gene **19**:269–276.
35. **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.
36. **Myers, R. M., M. Kligman, and R. Tjian.** 1981. Does simian virus 40 T antigen unwind DNA? J. Biol. Chem. **256**: 10156–10160.
37. **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.
38. **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.
39. **Prives, C., L. Covey, A. Scheller, and Y. Gluzman.** 1983. DNA-binding properties of simian virus 40 T-antigen mutants defective in viral DNA replication. Mol. Cell. Biol. **3**:1958–1966.
40. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T antigen binds to SV40 at the origin of DNA replication. Proc. Natl. Acad. Sci. U.S.A. **72**:1605–1609.
41. **Sager, R., A. Anisowicz, and N. Howell.** 1981. Genomic rearrangements in a mouse cell line containing integrated SV40 DNA. Cell **23**:41–50.
42. **Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463–5467.
44. **Shiroki, K., and H. Shimojo.** 1971. Transformation of green monkey kidney cells by SV40 genome: the establishment of transformed cell lines and the replication of human adenoviruses and SV40 in transformed cells. Virology **45**:163–171.
45. **Smith, A. E., R. Smith, and E. Paucha.** 1979. Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell **18**:335–346.
46. **Soprano, K. J., N. Galanti, G. J. Jonak, S. McKercher, J. M. Pipas, K. W. C. Peden, and R. Baserga.** 1983. Mutational analysis of simian virus 40 T antigen: stimulation of cellular DNA synthesis and activation of rRNA genes by mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3**:214–219.
47. **Stringer, J. R.** 1982. Mutant of simian virus 40 large T-antigen that is defective for viral DNA synthesis, but competent for transformation of cultured rat cells. J. Virol. **42**:854–864.
48. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
49. **Tijan, R., A. Robbins, and R. Clark.** 1979. Catalytic properties of the SV40 tumor antigen. Cold Spring Harbor Symp. Quant. Biol. **44**:103–111.
50. **Tooze, J. (ed.).** 1981. Molecular biology of tumor viruses, part 2, DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
51. **Tornow, J., and C. N. Cole.** 1983. Intracistronic complementation in the simian virus 40 *A* gene. Proc. Natl. Acad. Sci. U.S.A. **80**:6312–6316.