MOLECULAR AND CELLULAR BIOLOGY, June 1984, p. 1125–1133 Vol. 4, No. 6
0270-7306/84/061125-09$02.00/0
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Simian Virus 40 Large T-Antigen Point Mutants That Are Defective in Viral DNA Replication but Competent in Oncogenic Transformation

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Received 6 February 1984/Accepted 21 March 1984

The large T antigen of simian virus 40 (SV40) is a multifunctional protein that is essential in both the virus lytic cycle and the oncogenic transformation of cells by SV40. To investigate the role of the numerous biochemical and physiological activities of T antigen in the lytic and transformation processes, we have studied DNA replication-deficient, transformation-competent large T-antigen mutants. Here we describe the genetic and biochemical analyses of two such mutants, C2/SV40 and C11/SV40. The mutants were isolated by rescuing the integrated SV40 DNA from C2 and C11 cells (CV-1 cell lines transformed with UV-irradiated SV40). The mutant viral early regions were cloned into the plasmid vector pK1 to generate pC2 and pC11. The mutations that are responsible for the deficiency in viral DNA replication were localized by marker rescue. Subsequent DNA sequencing revealed point mutations that predict amino acid substitutions in the carboxyl third of the protein in both mutants. The pC2 mutation predicts the change of Lys → Arg at amino acid 516. pC11 has two mutations, one predicting a change of Pro → Ser at residue 522, and another predicting a Pro → Arg change at amino acid 549. The two C11 mutations were separated from each other to form two distinct viral genomes in pC11A and pC11B. pC2, pC11, pC11A, and pC11B are able to transform both primary and established rodent cell cultures. The C11 and C11A T antigens are defective in ATPase activity, suggesting that wild-type levels of ATPase activity are not necessary for the oncogenic transformation of cells by T antigen.

The multifunctional large T antigen of simian virus 40 (SV40) is essential in both the lytic cycle of the virus and the oncogenic transformation of cells by SV40. T antigen, encoded by the SV40 *A* gene, is expressed early in the lytic pathway and functions in numerous processes throughout the infectious cycle. It is required for the initiation of viral DNA replication (59), autoregulates early viral RNA synthesis (1, 51, 52, 61), stimulates late viral transcription (30; J. Brady, J. Bolen, M. Radonovich, N. Salzman, and G. Khoury, Proc. Natl. Acad. Sci. U.S.A., in press), and induces host cell DNA and RNA syntheses (6, 15, 27, 28, 39, 42, 45, 55, 56, 64). T antigen also possesses a "helper function" that enables adenovirus to grow more efficiently in simian cells (13, 21, 31, 49). Recent evidence also suggests an essential role for T antigen after the onset of late gene transcription (67; C. Cole, personal communication). In nonpermissive cells, T antigen is implicated in both the initiation and maintenance of the transformed phenotype (5, 32, 33, 38, 43, 60). This 82-kilodalton protein specifically binds to the SV40 origin of replication (50, 63), has an ATPase activity (7, 17), and is complexed with a 53-kilodalton cellular phosphoprotein in vivo (24, 35).

Mutants that retain only a subset of the biochemical and physiological activities of T antigen have been valuable in localizing activities to specific regions of the protein. For example, the carboxy-terminal 38 amino acids of T antigen are sufficient for the adenovirus helper function (12, 46). Studies involving the binding of T-antigen tryptic peptides to the SV40 origin have implicated a fragment including amino acids 83 through 130 in the origin-binding activity (41). Single amino acid alterations between residues 147 and 214 affect origin binding (19, 47, 58; W. Gish and M. Botchan, personal communication). Studies of deletion mutants have facilitated the mapping of several T-antigen activites, such as viral DNA replication, ATPase, origin binding, stimulation of cell DNA synthesis, stimulation of host rDNA transcription, and transformation, to different portions of the *A* gene (8, 44, 56). Therefore, numerous data suggest that the T-antigen protein may contain distinct domains that are able to function somewhat independently.

The analysis of T-antigen functions in SV40-transformed permissive cells led to the speculation that the lytic and transformation activities of T antigen were genetically separable (20). Since then, numerous DNA replication-deficient, transformation-competent T-antigen mutants have been described (9, 19, 36, 58). Many of these mutant T antigens were found to be defective in origin binding (47, 58), providing the probable explanation for their replication deficiency. This class of mutants revealed that the origin-binding activity of T antigen is dispensable in its transformation function. Few mutants with the converse phenotype, that is, transformation deficient and replication competent, have been reported to date (10; J. Pipas, personal communication). Mutant *tsA*1642 has these properties, and its replicative and transforming functions are differentially sensitive to temperature (10). To further investigate the role of the numerous activities of T antigen in cellular transformation and the SV40 lytic cycle, we have extended our studies of early mutants that are deficient in viral DNA replication, but retain transforming activity. Here we describe the genetic and biochemical analysis of two such replication-deficient, transformation-competent T-antigen mutants, C2/SV40 and C11/SV40 (20). These variant T antigens were previously found to have retained the ability to bind to the origin (47). The mutations predict single amino acid changes in the carboxyl third of the protein, and one change specifies a defect in ATPase activi-

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ty. These and similar mutants may be useful in further defining the role of T antigen in the viral lytic cycle and transformation.

(This work constitutes part of the doctoral dissertation of Michele Manos through the Department of Microbiology, SUNY-Stony Brook, Stony Brook, N.Y.)

MATERIALS AND METHODS

Cells, cell fusion, and transformation. Cells were propagated as described previously (19). MEF cells (10) were obtained from M. Tevethia and cultured in Dulbecco modified Eagle medium plus 10% fetal calf serum. Cell fusions were induced with polyethylene glycol as described (4). The calcium phosphate technique (68) was used to deliver 250 ng of linearized plasmid per plate of cells. Samples of 10^6 and 10^5 Rat2 or mouse embryo fibroblast (MEF) cells per 10-cm dish, respectively, were treated. For baby rat kidney cell transformations, about 5×10^5 cells per 6-cm dish was used. Foci were counted after 3 weeks.

Bacterial strains and plasmids. Plasmids were grown in *Escherichia coli* DH-1 (22). Plasmids pK1 and pC6-1 have been described previously (19). Information regarding the plasmids containing wild-type SV40 early region fragments is available from the authors upon request.

Cloning SV40 early sequences from C2 and C11 cells. pK1 is a recombinant plasmid in which a complete wild-type SV40 genome has been inserted into the unique *Eco*RI site of pMK16#6 (19). Plasmid pMK16#6 is a derivative of pMK16 (29) in which about 800 base pairs of the tetracycline resistance gene, including the unique *Bgl*I and *Bam*HI restriction sites, have been deleted. Digestion of pK1 with *Bam*HI plus *Bgl*I produces two fragments: a large fragment, A, containing most of the SV40 late region and the pMK16#6 vector, and a small fragment, B, containing the SV40 early region. Both of these fragments were purified by agarose gel electrophoresis. The larger fragment was used as an acceptor for cloning the mutant early regions. The small fragment (B) was ^{32}P-labeled by nick-translation and used in screening as described below.

Low-molecular-weight DNA was prepared from fused C2/COS-1 and C11/COS-1 heterokaryons by Hirt extraction (26). The DNA was digested with *Bgl*I plus *Bam*HI and ligated to the *Bgl*I-*Bam* acceptor/vector fragment of pK1. The ligation mixtures were used to transform *E. coli* DH-1 (22). Kanamycin-resistant colonies were screened for the presence of SV40 early region DNA by hybridization to ^{32}P-labeled pK1 insert (B) fragment. Resultant plasmids pC2 and pC11 contain a complete SV40 genome, comprised of the mutated early region from cell lines C2 and C11, respectively, and the wild-type late region from the plasmid pK1.

Marker rescue. Marker rescue was done as described previously (19). All wild-type fragments were generated by digestion of recombinant plasmids containing the SV40 DNA fragment of interest. For example, *Hin*dIII fragments A, B, and D were released from their plasmid vectors by *Hin*dIII digestion. The *Hin*dIII A plasmid was further digested with *Bam*HI to release the *Hin*dIII-*Bam* fragment "*Hin*d A′" (nucleotides 3476 to 2533) to avoid overlapping the "patch" fragment (nucleotides 346 to 2533) in the heteroduplex. The *Pst*I B fragment was released from the vector with *Pst*I and further digested with *Bam*HI to give the "P-B" fragment spanning nucleotides 3204 to 2533.

Separation of C11 mutations. pK1 and pC11 were digested to completion with *Pst*I and then ligated to promote circularization. Resultant plasmids pK1-P and pC11-P contain only the large *Pst*I fragment of SV40 and were used as acceptors for *Pst*I B fragments to reconstruct complete viral genomes. The wild-type *Pst*I B fragment (from plasmid pSV*Pst*B) was inserted into the unique *Pst* site of pC11-P to generate pC11A. The C11 *Pst*I B fragment (from plasmid pC11) was cloned into the *Pst*I site of pK1-P to make pC11B.

ATPase assays. A modification of the monoclonal antibody-bound assay (7) was used. T antigen was extracted from the nuclei of transformed cells. Confluent 10-cm plates of cells were incubated with M.Y. buffer for 10 min at 4°C to swell the cells. M.Y. buffer is 10 mM morpholineethanesulfonic acid (pH 6.2)–10 mM NaCl–4 mM $MgCl_2$. The buffer was replaced by M.Y. buffer plus 0.1% Nonidet P-40 for 5 min at 4°C to lyse the cells, leaving the nuclei intact on the plate. The plates were washed carefully twice with M.Y. buffer and then incubated at 4°C with 3 ml of extraction buffer (10 mM Tris, pH 8–10 mM NaCl) for 45 min. The nuclei and buffer were collected by scraping and centrifuged (Eppendorf) for 15 min at 4°C. The supernatant was used in the assays.

For each assay, 0.4 ml of extract was incubated with 2 μg of monoclonal antibody PAb419 (11, 23) for 20 min on ice. A 100-μl volume of protein A-Sepharose (Pharmacia) slurry (30 mg/ml) was added and incubated at 4°C for 2 h with rocking. The beads were pelleted and washed once with 0.5 ml of 100 mM LiCl–10 mM Tris (pH 8)–100 mM NaCl–0.5% Nonidet P-40 and then with ATPase buffer [25 mM PIPES piperazine-*N,N′*-bis(2-ethanesulfonic acid) (pH 7)–5 mM $MgCl_2$–0.01% Nonidet P-40]. Then 100 μl of ATPase reaction mixture (ATPase buffer made 0.5 μM ATP plus 10 μCi of [γ-^{32}P]rATP) was added to the pellet and incubated at room temperature for 30 min with occassional mixing. The beads were pelleted, and 80 μl of the reaction mixture was added to 120 μl of Norit activated charcoal solution (7.5% in 50 mM HCl–5 mM H_3PO_4). The charcoal was pelleted by centrifugation, and the free radioactive phosphate in the supernatant was measured by Cerenkov counting.

The amount of T antigen per volume of cell extract was measured by the double-antibody radioimmunoassay technique (2), using PAb419 as the first antibody and [^{125}I]PAb602 as the second.

DNA sequencing. The *Hin*fI E fragment (nucleotides 3204 to 2533) was isolated from pC2 and pC11, and the protruding ends were "filled in" to produce blunt ends and cloned into the *Sma*I site of the replicative form of bacteriophage mp8 (40). The resultant hybrid phage DNAs were used to sequence the complete *Hin*fI E fragment by the "dideoxy" method (53, 54). [^{35}S]dATPαS label and gradient gels (3) 80 cm in length were used.

DNA replication analyses. Plasmids pK1, pC11, pC11A, pC11B, or pC6-1 (19) were prepared for use in transfections by digestion with *Eco*RI followed by ligation to generate bona fide circular viral genomes. Confluent monolayers of CV-1 cells (6-cm plates) were transfected with 1 μg of DNA by methods described (19), followed by a chloroquine "boost" (37). Low-molecular-weight DNA was prepared from the cells (26) 48 to 72 h after transfection and digested with *Bam*HI plus *Dpn*I. Digestion products were analyzed by blot hybridization (57) with nick-translated pK1 as probe.

RESULTS

Cloning SV40 early regions from C2 and C11 transformed cell lines. The transformed cell lines C2 anc C11 were isolated after infection of CV-1 cells with UV-irradiated SV40 and have been described previously (20). Both lines contain a single, incomplete copy of the SV40 genome integrated into the cellular DNA (34) and produce a nuclear

T antigen of normal size (48). Fusion of the C2 or C11 cell lines with COS-1 cells, providing wild-type T antigen in *trans*, results in rescue of the viral DNA sequences from the cellular genome (18). Free viral DNA sequences are replicated efficiently in the heterokaryons, thus facilitating the isolation of the SV40 early regions resident in C2 and C11 cells. Figure 1 shows the results of fusing C2 or C11 transformed simian cell lines with either CV-1 cells or COS-1 cells. No free viral DNA was detected after fusion with CV-1 cells, whereas fusion with COS-1 cells allowed efficient excision and replication of viral DNA sequences. Both cell lines liberated a *Bgl*I-*Bam* fragment that comigrated with the bona fide SV40 early *Bgl*I-*Bam* fragment (arrow). As predicted by previous studies (34), intact late region fragments were not found.

The *Bgl*I/*Bam*HI-digested DNA from the crude Hirt extracts was cloned into the vector pK1 as diagrammed in Fig. 2 and described in detail above. Resultant plasmids pC2 and pC11 contain the mutant early regions derived from C2 and C11 cells, respectively, and the wild-type SV40 late region of pK1, thus restoring a complete viral genome that can be excised by *Eco*RI digestion.

The use of a *Bgl*I site is important in reducing background in this cloning scheme. The *Bgl*I recognition sequence includes five variable nucleotide positions, and the enzyme cleaves symmetrically, producing 3′ protruding ends comprised of three of the variable nucleotides: GCCNNNN ↓ NGGC. This feature provides a ligation specificity to the pK1 vector molecule at its *Bgl*I end. Only the desired SV40 fragment's *Bgl*I terminus, or the small fraction (1/64) of other *Bgl*I termini with the same 3′ nucleotide protrusion, will ligate with the *Bgl*I end of the pK1 vector. Indeed, this scheme, combined with the amplification of viral DNA by COS cell fusion and the isolation of nonchromosomal DNA by Hirt extraction, provided efficient cloning of the C2 and C11 inserts; up to 20% of the kanamycin-resistant colonies contained the desired SV40 fragment.

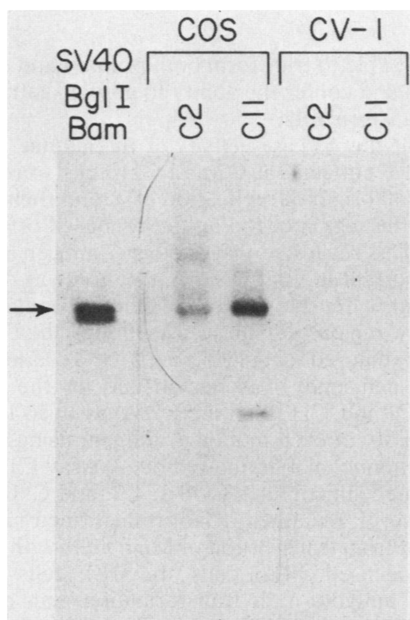


FIG. 1. SV40 DNA replication after fusion of transformed cells with COS-1 or CV-1 cells. Cells were fused with either COS-1 or CV-1 cells by using polyethylene glycol (4). Low-molecular-weight DNA was isolated 72 h later, digested with *Bgl*I and *Bam*HI, separated on a 1% agarose gel, and transferred to nitrocellulose. SV40 sequences were visualized by hybridization with SV40 [^{32}P]DNA. The first lane shows SV40 DNA digested with *Bgl*I and *Bam*HI, and the arrow denotes the resultant early region fragment (upper band of doublet).

Mapping of mutations by marker rescue. Plasmids pC2 and pC11 were analyzed in marker rescue experiments to localize the mutations responsible for the replication deficiency. Figure 3 illustrates the wild-type fragments that were used and shows the results. Neither pC2 or pC11 produced plaques when no wild-type early region fragments were included, as expected. Both pC2 and pC11 were rescued by the *Hin*dIII A′ fragment and by the smaller *Hin*fI E fragment (nucleotides 3373 to 2848). The pC2 mutation was further localized to the *Hin*dIII-*Pst*I (H-P) fragment spanning nucleotides 3476 to 3204, placing the mutation between nucleotides 3373 and 3204. pC11 was not rescued by the H-P fragment or the P-B fragment (spanning nucleotides 3204 to 2533), but fragments H-P and P-B together could rescue. These results suggested that there was at least one mutation on each side of the *Pst*I site in the *Hin*fI E fragment of pC11.

DNA sequence analyses. The complete *Hin*fI E fragment (nucleotides 3204 to 2533) from both pC2 and pC11 was sequenced by the chain termination method (54). One mutation was found to be specific to pC2, and two were found in pC11 (Fig. 4), one on either side of the *Pst*I site as predicted by marker rescue. The pC2 mutation predicts an amino acid change at residue 516 of large T antigen, replacing a lysine with arginine. One mutation in pC11 predicts the change of a proline to serine at residue 522, and the other specifies arginine in place of proline at residue 549. Both the pC2 and pC11 sequences differed from the published SV40 sequence (65) at two additional nucleotides (early strand, nucleotide 2951 A → G and 2907 A → T). These differences in common are not surprising, since strain 777 was used in generating C2 and C11, and the published sequence is for strain 776. Both of these nucleotide substitutions have been noted in other SV40 strains (W. Gish and M. Botchan, personal communication).

Separation of the mutations in C11. To analyze the effect of each pC11 mutation separately, two recombinant plasmids, pC11A and pC11B, were constructed, each containing one of the C11 mutations (Fig. 4). Marker rescue experiments confirmed the construction of these plasmids (Fig. 3). pC11A was rescued by the H-P fragment, as predicted. In contrast, pC11B did not retain the replication-deficient phenotype. When no wild-type early fragments were included, pC11B produced pinpoint plaques. Fragment P-B rescued the pinpoint plaque phenotype of pC11B, yielding wild-type plaques on a pinpoint plaque background.

DNA replication of mutant C11B. We investigated whether a defect in viral DNA replication influenced the pinpoint plaque phenotype of mutant C11B/SV40. CV-1 cells were transfected with pK1, pC11, pC11A, pC11B, or pC6-1 (19), and then relative levels of viral DNA replication were examined. Low-molecular-weight DNA was isolated 72 h after transfection (48 h for pK1) and digested with *Dpn*I and *Bam*HI. The products were analyzed by blot hybridization using pK1 as a probe. Figure 5 shows the resulting autoradiogram. pC11 and pC11A DNAs did not replicate, and pC6-1 DNA was severely impaired in replication, as previously described (19). pC11B DNA replicated more efficiently than that of pC6-1, but appeared less than 10 times as efficient as wild-type pK1. These data suggest that the decreased viability of mutant C11B is, at least in part, due to a decreased level of viral DNA replication.

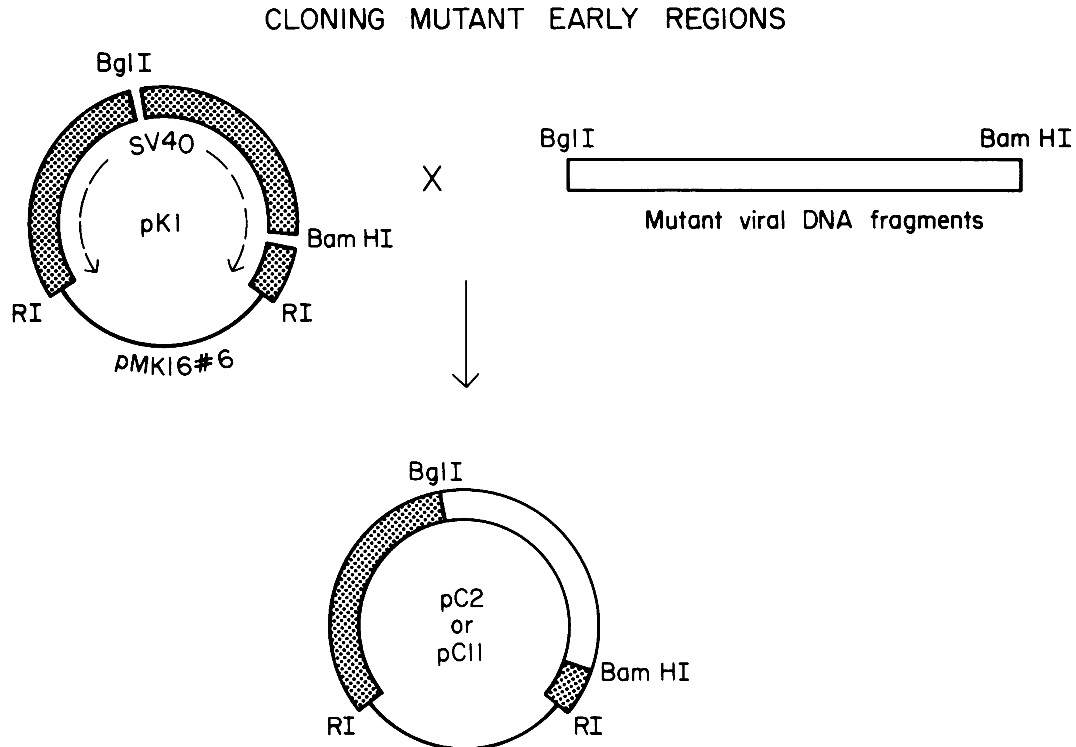


FIG. 2. Method of cloning mutant early regions from transformed cells. Low-molecular-weight DNA from C2 or C11/COS-1 heterokaryons was digested with *BglI* and *BamHI* and then ligated with the vector fragment of pK1 (containing the plasmid and SV40 late region sequences). Resultant plasmids pC2 and pC11 contain the early region from C2 and C11 cells, respectively, and the wild-type late region from pK1.

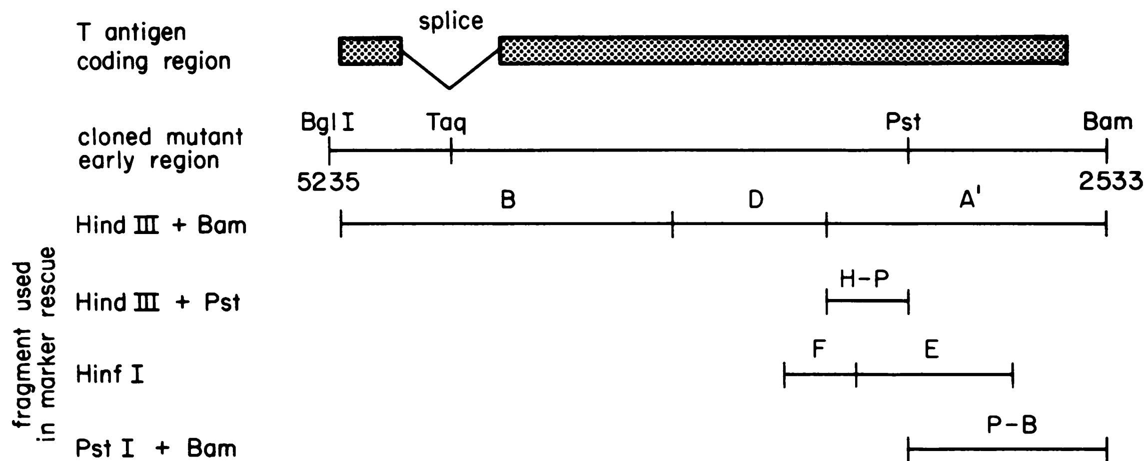
Transformation of rodent cells with the mutant DNAs. The cloned mutant DNAs were tested for their ability to transform both primary and established rodent cells. Using the calcium phosphate technique (68), pC2, pC11, pC11A, pC11B, or pK1 (the plasmid containing wild-type SV40 DNA) was used to transform the Rat2 cell line (66), primary baby rat kidney cells, or a primary MEF culture. The mutant plasmids transformed MEF cells with an efficiency comparable to pK1, although pC11A was consistently less efficient (Table 1). The difference in efficiency between pC11A and the other plasmids varied between experiments. The threefold decrease shown here represents the extreme of differences observed. In MEF cells, the resultant foci from transformation by pC11A were consistently smaller than those produced by wild-type (pK1) and the other mutant plasmids.

With the Rat2 cell line as a recipient, the plasmids gave efficiencies and focus sizes similar to those given by pK1, except that pC2 consistently gave about 10% the efficiency. Data from one experiment are shown in Table 1. In BRK cultures, although focus size was similar, the mutant plasmids tested were 30 to 50% as efficient as pK1.

Several foci were isolated from each MEF transformation assay and grown into mass culture. All lines had nuclear localization of T antigen as evidenced by indirect immunofluorescence. Also, transformants resulting from pK1 and all of the mutants were able to grow in soft agar. The plating efficiencies of pK1 and mutant transformants in soft agar varied greatly between focus isolates, ranging from 0.5 to 15%. Thus, all the mutant plasmids, pC2, pC11, pC11A, and pC11B, were able to transform both primary and established cell cultures and confer the ability to grow in soft agar to the resultant transformants.

Analysis of the ATPase activity of the mutant T antigens. The mutant T antigens of C2 and C11 cells were shown to bind the SV40 origin of replication (47), and their mutations lay outside the region encoding the proposed origin-binding "domain." This suggested that another T-antigen activity (activities) important in viral DNA replication was defective in these mutants. Recent studies of deletion mutants (8) revealed that a region of T antigen including the C2 and C11 mutations is required for ATPase activity. To assess whether an ATPase deficiency was responsible for the replication defects of C2 and C11 T antigens, we assayed the in vitro ATPase activity of each mutant T antigen, using a modification of the monoclonal antibody-bound assay (7).

Transformed simian lines COS-1, C2, and C11 were used as the source of T antigen. Also, transformed murine lines that resulted from transfection of MEF cells with the cloned mutants were used. MK1 cells are MEF cells expressing wild-type T antigen, after transformation with pK1. MC2, MC11, MC11A, and MC11B resulted from transformation by pC2, pC11, pC11A, and pC11B, respectively. Nuclear extracts were prepared from monolayer cultures of the cells and used in the assay as described above. Table 2 shows the results of the assay with and without the T antigen-specific monoclonal antibody PAb419. The amount of T antigen present per volume of cell extract was determined by radioimmunoassay, using the same monoclonal antibody that was used in the ATPase assays. For the last column of



	Early Region Fragments						
	None	Hind B	Hind A'	H-P	P-B	H-P P-B	Hinf E
pC2	0[a]	0	8,6	12,25	0	-	5-6
pC11	0	0	12,11	0	0	3,4	16,19
pC11-A	0	0	-	8,19	0	12,11	-
pC11-B	38,26[b]	0	-	40,36[b]	16,10[c]	7,6[c]	-

FIG. 3. Localization of mutations by marker rescue. Early region fragments used in marker rescue experiments are diagrammed. Fragments were heteroduplexed with pC2, pC11, pC11A, or pC11B and used to transfect monolayers of CV-1 cells. (a) Number of plaques per 6-cm dish; (b) pinpoint plaques; (c) normal-sized plaques on a background of pinpoint plaques.

Table 2 the activities were adjusted for the amount of T antigen and are expressed as a percentage of that found in COS-1 cells. C2, MK1, MC2, and MC11B cell T antigens had ATPase activities comparable to the wild-type activity of COS cells. C11, MC11A (Table 2), and MC11 (not shown) T antigens were defective in ATPase function, giving an activity similar to CV-1 cells (control) and untransformed MEF cells (not shown). These data suggest that the C11A mutation, predicting a pro → ser change at amino acid 522, confers a defect in the ATPase activity of T antigen.

DISCUSSION

We describe the genetic and biochemical analysis of two replication-deficient, transformation-competent SV40 T-antigen mutants. They were isolated from the genomes of C2 and C11 cells (CV-1 cell lines that were transformed with UV-irradiated SV40 [20]), where early viral DNA sequences encoding the mutant T antigens are present as single copies. Several enrichment steps were included in the procedures for the isolation and cloning of the viral sequences encoding the mutant T antigens. As explained above, fusion of C2 and C11 cells with COS cells to promote amplification of viral DNA, low-molecular-weight DNA purification, and the use of *Bgl*I site in the vector pK1 are important in the efficiency of the cloning procedure. The background could be reduced considerably by using a recently available enzyme, *Sfi*I (New England Biolabs), in place of *Bgl*I. *Sfi*I recognizes and cleaves the same site at the SV40 origin, but its recognition sequence is comprised of eight nucleotides plus five variable nucleotides: GGCC(N_5)GGCC. The number of *Sfi*I sites in the cellular DNA will be about 16-fold less than *Bgl*I sites, further reducing the number of undesired fragments able to ligate with the vector. This enzyme should be useful in any situation where cloning of sequences that are adjacent to the SV40 origin of replication is desired.

Plasmids pC2 and pC11, containing the early regions from C2 and C11 cells, revealed the predicted replication-defective phenotype in marker rescue experiments. Plaques were formed only when wild-type early region fragments were present to rescue the lesions in the mutants. Marker rescue and subsequent DNA sequence analyses revealed that the replication-deficient phenotypes of both pC2 and pC11 were due to point mutations in the region encoding the carboxy-terminal third of large T antigen. pC2 has a single nucleotide change predicting a substitution of an arginine for a lysine residue at amino acid 516. pC11 has one nucleotide change predicting a proline-to-serine substitution at residue 522 and two nucleotide changes resulting in the replacement of another proline, residue 549, with arginine. The two pC11 mutations were separated from each other in pC11A (amino acid 522 mutation) and pC11B (amino acid 549 mutation).

T antigen from C2 and C11 cells were previously shown to behave similarly to wild-type T antigen in binding to the SV40 origin of replication (47). The region of T antigen between amino acids 83 and about 215 has been implicated in origin-binding activity. An amino-terminal tryptic peptide extending to residue 130 was found to bind efficiently to the origin (41). Many point mutations affecting origin binding map to the region encoding amino acids 140 to 215, for example, mutants C6-1 (19), SVR9D (58), and SV80 (W. Gish and M. Botchan, personal communication). The muta-

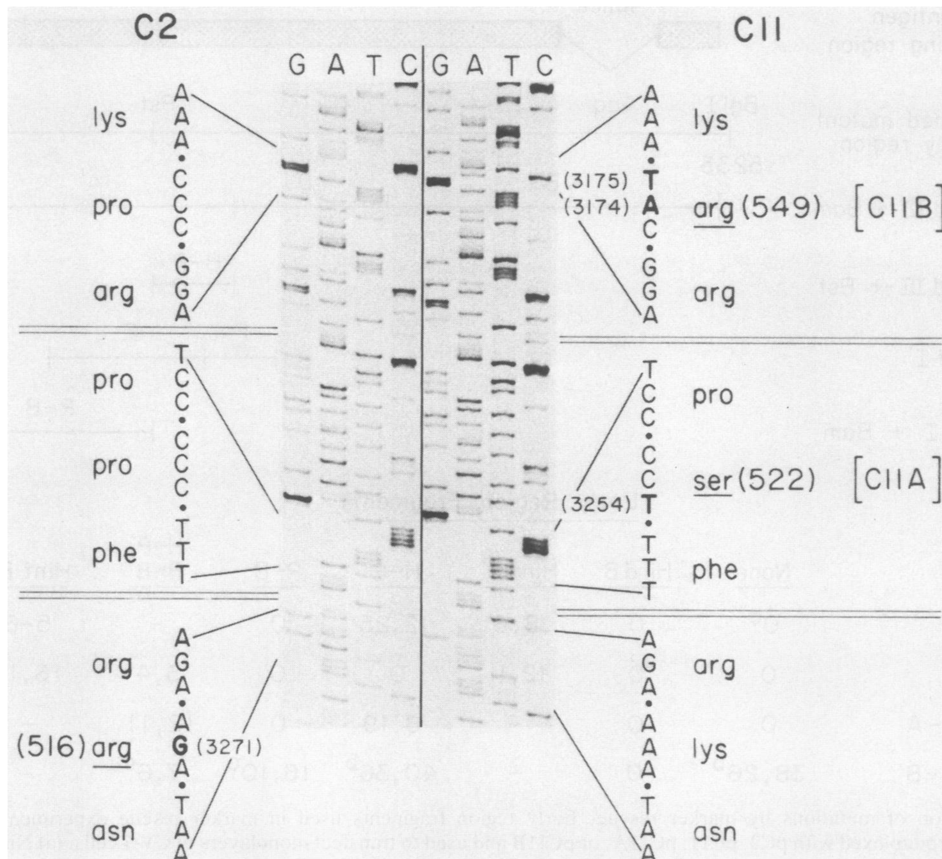


FIG. 4. Mutations in C2 and C11. The *Hin*fI E fragment (nucleotides 3204 to 2533) of pC2 and pC11 was sequenced using the chain termination method and [^{35}S]dATPαS. Nucleotides that differ from the wild-type sequence (65) are shown in boldface, and predicted amino acid substitutions are underlined. Nucleotide numbers are in the SV system (65). C11 mutations were separated into two distinct viral genomes in pC11A and pC11B, containing the mutations denoted. The early strand of SV40 is read from the gel, bottom to top.

tions responsible for the replication defect in pC2 and pC11 are far removed from this presumptive origin-binding region. The replicative functions that are defective in these mutants are distinct from origin binding and are dependent actively or structurally on the region between amino acids 500 and 550. The defective replicative functions are probably distinct from p53 binding activity as well, since C2 and C11 T antigens are similar to wild type in this activity in both monkey cells (24) and mouse cells (unpublished data).

Mutant plasmids pC2, pC11A and pC11B and double mutant pC11 were comparable to the wild-type plasmid pK1 in transforming MEF cultures. pC11A was usually less efficient, but never less than 35% of pK1. pC11, which contains both pC11A and pC11B mutations, does not exhibit the slightly lowered efficiency that is characteristic of pC11A. It is possible that a property of the C11B mutation compensates for the lower efficiency of pC11A in MEF cells.

Relative transformation efficiencies in the Rat2 cell line and primary baby rat kidney cells were quite different from those in MEF cells. In both rat cell types, pC2 has the lowest efficiency. We must address the possibility that additional mutations in the pC2 and pC11 early regions affect the transformation activity of the plasmids. Although the replication defects were localized to the *Hin*fI E fragment by marker rescue, there may be mutations elsewhere (e.g., in the region encoding small t antigen-specific residues) that could affect the transformation process. The relative transformation efficiencies of the wild-type and mutant plasmids vary between cell type and also between experiments with the same cell cultures, but all of the mutants are comparable to wild type in at least one type of cell tested. Resultant MEF transformants from all the mutant plasmids were able to form colonies in soft agar, a property that is considered a measure of complete transformation (for review see reference 67). When pC2 or pC11 was injected subcutaneously into newborn hamsters, both exhibited a tumorigenic potential comparable to that of pK1 (M. Manos, Y. Gluzman, and W. Topp, unpublished data).

Recent studies suggest that the carboxy-terminal half of T antigen, encoded between approximately nucleotides 2800 and 4000, is important for its ATPase activity and that the carboxy-terminal 49 amino acids are dispensable for this function (8; M. Manos and Y. Gluzman, manuscript in preparation). The amino acid alteration in C11A T antigen (amino acid 522, Pro → Ser) affects the ATPase activity, possibly providing an explanation for its replication deficiency. T antigens from C11 cells or mouse cells transformed with pC11A or double mutant pC11 (MC11A, MC11 cells) have no detectable ATPase activity in our monoclonal antibody-bound assays. We cannot conclude that no ATPase activity is present, since C11, MC11A, and MC11 cell extracts exhibit background (CV-1, MEF) levels, but we

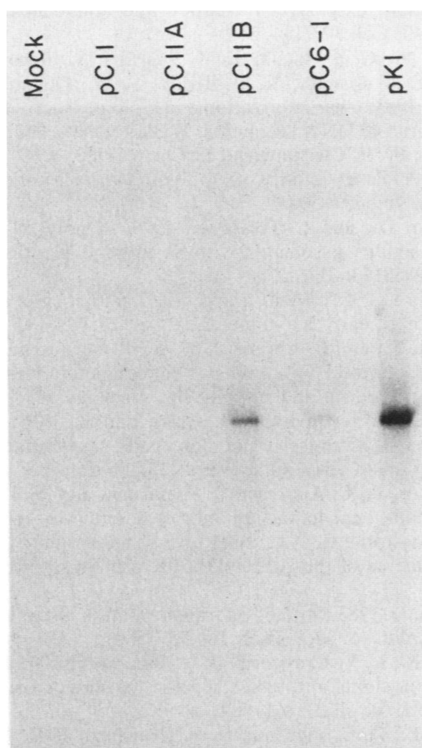


FIG. 5. Replication of mutant DNAs in CV-1 cells. CV-1 cells were transfected with the plasmids noted, using DEAE-dextran. Low-molecular-weight DNA was extracted after 72 h (48 h for pK1), digested with *Dpn*I plus *Bam*HI, separated on a 1% agarose gel, and transferred to nitrocellulose. ^{32}P-labeled pK1 was used as a hybridization probe.

have found that levels are decreased at least 10-fold. These results demonstrate that wild-type levels of ATPase activity are not necessary for T antigen to efficiently transform cells in culture or form tumors in hamsters. These findings suggest that ATPase activity is not necessary in the process of oncogenic transformation by T antigen. The analysis of the ATPase activity of purified C11A T antigen may further address this possibility, as well as the analysis of other transformation-competent ATPase mutants. On the other

TABLE 1. Transformation of cultured cells with the mutant DNAs[a]

DNA	Avg no. of foci per plate		
	MEF cells	Rat2 cells	Baby rat kidney cells
Carrier DNA alone	0,0	0,0	0,0
pK1	20,12	172,136	50,50
pC2	18,13	14,14	16,12
pC11	19,18	156,175	23,24
pC11A	4,8	126,146	20,28
pC11B	24,12	142,119	ND

[a] A 250-ng sample of linearized plasmid was delivered to each plate of cells, using the calcium phosphate technique (68), as described in the text. Foci were counted after 3 weeks. ND, Not done.

TABLE 2. ATPase activity of the mutant T antigens[a]

Cell extract	Counts on RIA[b]	ATPase ASSAY (avg cpm of free phosphate)[c]		% ATPase activity[d]
		Without PAb419	With PAb419	
CV-1	380	9,179	18,282	9
COS-1	82,849	208	207,406	100
C2	67,592	322	139,448	82
C11	7,200	0	1,793	10
MK1	46,169	10,260	100,551	88
MC2	45,930	5,414	63,928	56
MC11A	50,630	7,900	14,241	11
MC11B	53,408	1,552	105,246	80

[a] ATPase activity of T antigen in nuclear extracts was determined using the monoclonal antibody-bound technique (7), using monoclonal PAb419 (11, 23), with modifications described in the text.

[b] The relative quantities of T antigen per volume of cell extract were determined by radioimmunoassay (RIA) (2). The average of duplicate samples is shown.

[c] Average of duplicate samples. The value of free phospahte at time zero (reaction buffer plus Norit), 19,700 cpm, was subtracted from each.

[d] Values of activity were adjusted to reflect activity per unit of T antigen and then shown as a percentage of activity in COS cell extracts. For each extract (i) the number was calculated:

$$\frac{[\text{cpm with PAb419}]_i \ \ [\text{RIA counts}]_{\text{COS}}}{[\text{RIA counts}]_i \ \ [\text{cpm with PAb419}]_{\text{COS}}} \times 100$$

Because CV-1 cells do not have T antigen, ATPase activity was not adjusted for amount of T antigen.

hand, it is likely that the ATPase activity is crucial in the initiation of viral DNA synthesis, since replication and ATPase are strictly correlated in the analysis of various T-antigen mutants (8).

The ATPase defect may be responsible for the replication deficiency of C11A and C11. In contrast, C2 T antigen has an ATPase activity comparable to wild type, leaving the replicative function that is defective in C2 T antigen an unknown. In light of the very "conservative" amino acid change in C2 (amino acid 516, Arg → Lys), it is possible that this mutation resides in, or is close to, the active site of a yet undetermined replication activity of T antigen.

SV40 large T antigen shares considerable sequence homology with large T antigen of polyomavirus (Py T) (14), and the two proteins have a set of similar activities in the lytic cycles of their respective viruses. Py T binds to the origin of replication, initiates viral DNA synthesis, is important in regulating both early and late viral gene expression, and has an ATPase activity (16, 67). Both the C2 and C11A alterations occur in a stretch of SV40 large T where 33 of 39 residues are identical with Py T (14), i.e., between amino acids 493 and 531. We and others (19, 58; W. Gish and M. Botchan, personal communication) have isolated two types of transformation-competent, replication-deficient SV40 large T antigen point mutants: (i) those that are completely defective for viral DNA replication (undetectable within the limits of our experiments) and (ii) those that are partially defective in replication. Amino acid changes that confer these phenotypes occur in regions of homology with Py T. Moreover, the lesions conferring the replication-negative phenotype occur at positions where the amino acids are identical between the two proteins. In contrast, those mutations that affect only the level of replication, such as C6-1 (2)

and C11B, affect residues that are not strictly conserved. We have found that two other mutants, C8 and T22 (to be reported elsewhere), exhibit these characteristics as well. It would be interesting to determine whether altering the corresponding amino acids in Py T antigen would affect its replication or origin-binding activities. Several Py T mutants with alterations at amino acids that are conserved between polyomavirus and SV40 have been described (25, 62). Of particular interest is Py T mutant 7B-M (25), which has a single amino acid change in the "33 of 39" residue stretch of homology with SV40 large T, at a conserved residue (Py 642, SV40 495). The properties of polyomavirus 7B-M large T antigen are strikingly similar to those of C2 and C11 T antigens. All three are defective in viral DNA replication while retaining the ability to bind to their respective viral origins.

ACKNOWLEDGMENTS

We thank E. Harlow and T. Adams for monoclonal antibodies and many suggestions. We are grateful to R. Clark and C. Prives for helpful discussions, M. J. Tevethia for MEF cells, J. Moomaw for preparing BRK cells, and especially W. Herr for his expertise in dideoxy sequencing. We thank T. Grodzicker, E. Harlow, and J. Harper for critical reading of the manuscript. We also thank M. Goodwin and C. Timm for manuscript preparation.

This work was supported by Public Health Service grant CA13106 from the National Cancer Institute.

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