trans-Dominant Defective Mutants of Simian Virus 40 T Antigen

JOSHUA M. FARBER,* KEITH W. C. PEDEN, AND DANIEL NATHANS

Howard Hughes Medical Institute Laboratory and the Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 14 July 1986/Accepted 23 October 1986

We constructed a collection of linker insertion mutants in the simian virus 40 (SV40) genome and studied several of these with changes limited to a part of the large T antigen gene corresponding to an amino acid sequence shared with other ATPases. Two of these mutants were found to have a novel phenotype in that they could not be complemented for plaque formation by a late-region deletion mutant. These two mutants, in contrast to other mutants in this region, were able to transform rat cells in culture at a frequency close to that of the wild-type gene. The noncomplementing mutants were found to be potent inhibitors of SV40 DNA replication despite the presence of wild-type T antigen in the transfected cells. This inhibition was shown to be the result of the introduced mutations in the large T antigen gene. We conclude that the large T antigens of the noncomplementing mutants can act as inhibitors of SV40 DNA replication.

The large tumor antigen (T antigen) of simian virus 40 (SV40), the major regulatory protein product of the early region of the viral genome, functions in the viral life cycle to initiate DNA replication (83), enhance late region transcription (1, 17, 35, 36, 58, 65), and down-regulate early region transcription (1, 36, 64, 85). In addition to its role in regulating the program for propagation of the virus, T antigen exerts profound effects on cellular metabolism, including stimulation of cellular DNA synthesis (11, 20, 31, 32, 69, 77, 86), transcription of specific cellular genes (76-78), and in some cell lines morphological transformation (5, 6, 24, 37, 38, 50, 57, 73, 79, 82, 84).

Mutational analysis has indicated that some of the activities of T antigen are localized to functional domains on the protein (13, 20, 23, 34, 47-49, 60, 62, 77, 81). Among the biochemical activities of T antigen is an ATPase (21, 28, 87) whose biological role remains obscure, although it has been implicated in DNA replication (15, 48, 80). Attempts have been made to localize the ATPase and nucleotide binding domains of T antigen based on analysis of deletion (13) and point (47, 48) mutants, monoclonal antibodies to T antigen that eliminate this activity (12), and affinity labeling (14, 15). Recently, Walker et al. (91) pointed out related sequences among a number of adenine nucleotide-requiring enzymes, including the α and β subunits of ATP synthase, myosin, adenylate kinase, and *RecA* protein. All proteins have an invariant sequence of Gly (X₄) Gly Lys. Furthermore, Seif (71) had noted significant sequence homologies between *RecA* protein and T antigen, and Clertant and Seif (16) noted homologies between T antigen and papillomavirus E1 proteins, all sharing the invariant sequence noted by Walker et al. among enzymes requiring adenine nucleotide. On the basis of these experimental studies and considerations of sequence homologies, we decided to construct mutants with changes in the T antigen gene in the vicinity of the invariant amino acid sequence and to determine the functional consequences of such mutations. In the course of this study, we identified two mutants with changes limited to the T antigen gene that were found to have a novel phenotype, namely, they could not be complemented for plaque formation by a late region deletion mutant due to *trans*-dominant inhibition

of SV40 DNA replication. In this report, we present the initial characterization of these mutants.

MATERIALS AND METHODS

Cells, DNAs, and bacterial strains. BSC40 and REF52 cells have been described previously (45, 60, 90). Cells were grown in MEM10 (Earle minimum essential medium supplemented with 10% fetal bovine serum; Hyclone Laboratories, Inc., Logan, Utah). The plasmid pSVB3, which contains wild-type SV40 strain 776 cloned into the *Bam*HI site of pBR322, has been described previously (61). pKP54 is a deletion mutant of pBR322 from nucleotides 29 (*Hind*III site) to 2457, with the sequences replaced by a polylinker containing sequences for the restriction enzymes *Hind*III, *Sal*I, *Bam*HI, *Sma*I, *Kpn*I, and *Xba*I (K. W. C. Peden, unpublished data). pSVKB1 contains the *Kpn*I-to-*Bam*HI early region of SV40 (nucleotides 2533 to 296) cloned into pKP54. pSVT+T' plasmids were derived from pSVKB1 by insertion of the 3.26-kilobase *Hpa*II fragments from recombinant plasmids containing the SV40 mutants 5060, 5061, 5071, 5062, 5072, and 1042 into the *Cla*I site. pMtSV.neo contains the SV40 T antigen gene (nucleotides 2533 to 5171) under the control of the mouse metallothionein-I promoter and carries resistance to the aminoglycoside antibiotic G418. pRSVCAT (25) was obtained from American Type Culture Collection, Rockville, Md. *Escherichia coli* MM294 (52) and its *recA* mutant (29), DH1, have been described previously.

Preparation of plasmid DNA. Both small-scale (1- to 4-ml cultures) and large-scale preparations (300-ml cultures) of plasmid DNA were done according to the sodium dodecyl sulfate-alkaline procedure of Birnboim and Doly (3) as modified by Ish-Horowicz and Burke (33). For large-scale preparations, the isopropanol precipitates were dissolved in TE (10 mM Tris hydrochloride, pH 8; 1 mM EDTA) and banded twice in CsCl-ethidium bromide gradients. After removal of the ethidium bromide with isobutanol, the DNA was precipitated with ethanol, dissolved in TE and purified by proteinase K digestion, phenol extraction and isopropanol precipitation.

Construction and screening of mutants. To construct mutants with an *Xba*I linker inserted at random, form I pSVB3 DNA was first incubated at 26°C with DNase I (6 ng/ml) in ethidium bromide (100 μ g/ml) for 50 min as described by Shortle and Botstein (74) to convert approximately 90% of

* Corresponding author.

the molecules to form II. After phenol extraction and ethanol precipitation, 14 μ g of this DNA was treated with *Micrococcus luteus* DNA polymerase I to convert nicks into short, single-stranded gaps (75). This DNA was phenol extracted and twice ethanol precipitated before digestion with S1 nuclease to convert form II to form III, as described previously (72), except that the reaction was done at pH 5 (92). Gapped DNA (7 μ g) was incubated at 10°C for 120 min with S1 nuclease (2,310 units; P-L Biochemicals, Inc., Milwaukee, Wis.) in 4.5 mM ZnCl₂–250 mM NaCl–30 mM sodium acetate, pH 5.0, in a final volume of 1.75 ml. The reaction was terminated with 0.2 volume of 1 M Tris hydrochloride, pH 7.5, and 0.2 volume of 200 mM EDTA, pH 7.5, followed by phenol extraction and ethanol precipitation. These linear molecules were fractionated by electrophoresis on a 1% low-melting-temperature agarose gel and isolated by chromatography on NACS columns (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and ethanol precipitation. The ends of these linear molecules were made flush by incubation with T4 DNA polymerase as previously described (8, 46), before the addition of *Xba*I linkers (Collaborative Research, Inc., Lexington, Mass.) phosphorylated with T4 polynucleotide kinase and ATP (46). Full-length linear molecules were digested with *Xba*I, gel purified as described above, diluted to ca. 0.1 μ g/ml, and ligated to circles with T4 DNA ligase. Transformation (29) of *E. coli* DH1 yielded approximately 4×10^4 ampicillin-resistant transformants. These colonies were harvested, glycerol was added to 50%, and the colonies were stored at –70°C. This produced a library of 4×10^4 independently isolated colonies, each of which should contain a plasmid with an *Xba*I linker inserted at random in pSVB3.

To isolate plasmids with inserts in the presumed ATPase region, colonies were plated, grown, and immobilized on nitrocellulose filters by the method of Hanahan and Meselson (30). Duplicate filters were hybridized (44, 59) with two different oligonucleotides that had been labeled by use of T4 polynucleotide kinase and [γ -³²P]ATP (46). The sequence of one oligonucleotide (probe 1) was derived from the region of the T antigen gene encompassing the region of interest—nucleotides 3516 to 3530—whereas that of the other oligonucleotide (probe 2) was from another region of the gene to serve as control—nucleotides 3090–3104. Colonies that did not hybridize with probe 1 but did with probe 2 were picked and subjected to a second round of screening. From colonies showing persistent differential hybridization, plasmid DNA was prepared (33) and analyzed.

DNA sequence analysis. Mutants were subjected to sequence analysis by the methods of Maxam and Gilbert (51) and Chen and Seeburg (10). In the first method, mutant DNA was digested with *Sau*96I and *Eco*RV and labeled at the *Sau*96I site at nucleotide 3538 by use of the Klenow fragment of DNA polymerase I, [α -³²P]dTTP, and cold dGTP and dCTP. The labeled fragments were purified after electrophoresis on 5% polyacrylamide gels and subjected to the chemical cleavage reactions (51). The products were resolved by electrophoresis on 8% polyacrylamide gels (66). In the second method, dideoxy sequencing reactions were done by use of alkali-denatured form I DNA as templates. Oligonucleotides that were used as primers for the sequencing of both strands corresponded to SV40 nucleotides 3636 to 3620 (5' → 3') and 3409 to 3425 (5' → 3'). Denaturation, neutralization, and annealing to the primer were done as previously described (10), except that base treatment was for 15 min at 37°C. Sequencing reactions were done as previously described (67) by use of [α -³⁵S]dATP, and the reaction prod-

ucts were analyzed by electrophoresis on 8% acrylamide gels with a buffer gradient (2).

Marker rescue. Marker rescue was performed by use of modifications of published procedures (23, 40, 60). SV40 fragments used for marker rescue were either cloned into pBR322 or twice purified from low-melting-temperature agarose gels with NACS columns. The wild-type viral *Hind*III D fragment was inserted into the *Hind*III site of pBR322, and the *Rsa*I F fragment of SV40 was inserted into the *Sca*I site of pBR322. The *Hpa*I A fragment of wild-type SV40 was gel purified. Two methods were used to prepare heteroduplexes for transfection. For the marker rescue of mutants 5060 and 5063, 45 ng of *Bam*HI-digested mutant DNA was incubated with 67 ng of the gel-purified *Hpa*I A fragment alone or in combination with 300 ng of the appropriately digested plasmids containing either the *Hind*III D or *Rsa*I F wild-type fragments. The DNA mixtures were denatured with 0.1 N NaOH at 37°C for 20 min in a total volume of 232 μ l, neutralized by the addition of 38 μ l of 0.5 M HCl–0.5 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, and the fragments were annealed by incubation at 68°C for 45 min. Transfection with this material by the DEAE-dextran method was done as previously described (60). The procedure was identical for mutants 5061 and 5062 except that attempts were made to minimize potential interference caused by mutant DNA not hybridized to a wild-type rescue fragment. The presence of this mutant DNA might be expected to prevent plaque formation even if marker rescue were to occur (see Results and Discussion). After the denaturation, neutralization, and annealing steps, heteroduplexes with DNA from 5061 and 5062 were phenol extracted and ethanol precipitated. In an attempt to make the molecules completely double stranded, the heteroduplexes (0.4 μ g) were incubated with DNA polymerase I, Klenow fragment (0.6 U; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 5 μ l of a solution containing 10 mM Tris hydrochloride, pH 7.9, 6.6 mM MgCl₂, 60 mM NaCl, 6 mM dithiothreitol, and dATP, dTTP, dGTP, and dCTP (each at 0.4 mM) for 20 min at 30°C. After this time, 1 μ l of a solution containing 0.6 U of the Klenow fragment and dATP, dTTP, dGTP, and dCTP (each at 2.3 mM) was added, and the mixture was incubated for a further 20 min. The DNA was phenol extracted and ethanol precipitated, digested with *Xba*I, again phenol extracted and ethanol precipitated, and used for transfection of BSC40 cells. The amounts of DNA of each of the four mutants used as starting material for heteroduplex formation were identical.

Chloramphenicol acetyltransferase assay. Chloramphenicol acetyltransferase activity in monkey cells was assayed as previously described (25, 26), after transfection with pRSVCAT.

Viability, complementation tests, and indirect immunofluorescence. The assays for viability, complementation, and indirect immunofluorescence have been described previously (60). Viability and complementation tests used 30 ng of excised and cyclized DNA with or without 30 ng of excised and cyclized *d*/1007 (39), an SV40 late deletion mutant.

DNA replication in monkey cells. Replication of mutant DNA and plasmid DNA was determined as described previously (60–62). Replication was quantified when appropriate by cutting out and counting bands from nitrocellulose filters.

Transformation of rat cells. The dense-focus assay of Todaro and Green (88) was done as described previously (60).

Construction of monkey cell lines expressing wild-type or

mutant T antigens. BSC40 cells (approximately 2×10^5) grown in MEM10 (in 5-cm-diameter dishes) were transfected by the calcium phosphate-glycerol method (19, 27) with derivatives of pMtSV.neo in which the *Nde*I-*Bam*HI fragment (SV40 nucleotides 2533 to 3808) from the wild-type gene was replaced by the corresponding fragment from mutants 5060, 5061, 5062, and 5063. The day after transfection, the cells from each dish were trypsinized and plated in four dishes, and the next day the medium was changed in MEM10 + G418 (300 μ g/ml; GIBCO Laboratories, Grand Island, N.Y.). The cells were fed every 3 days, and at about 2 weeks G418-resistant colonies were visible. Several colonies from each transfection were picked, expanded to cell lines, and assayed for the expression of T antigen by indirect immunofluorescence in the presence or absence of 100 μ M ZnCl₂. Only the cell lines with the most intensely staining cells were used. MKSV6 is an African green monkey cell line expressing the wild-type SV40 T antigen gene under control of the mouse metallothionein-I promoter (Peden, unpublished data).

RESULTS

Mutant construction. Our initial goal was to isolate in-frame, deletion-insertion mutants with alterations in the T antigen gene localized to the region presumed to be involved in the ATPase activity of the protein. A collection of *Xba*I linker insertion mutants was constructed as described in Materials and Methods. Each of these mutants has a single, randomly positioned linker insertion, creating a unique restriction site in the plasmid molecule. Approximately 9×10^3 transformants were grown on duplicate nitrocellulose filters. The colonies were lysed, DNA was bound to the filters in situ, and one of each of the duplicates was screened by hybridization with one or the other of two ³²P-labeled oligonucleotides. The oligonucleotide sequences were derived from positions 3516 to 3530 (probe 1) and 3090 to 3104 (probe 2) on the SV40 genome (89). Probe 1 sequence corresponds to the region in the T antigen amino acid sequence with similarity to that of other adenine nucleotide-requiring enzymes (91) (Fig. 1), and probe 2 corresponds to a site outside the region of interest. DNA was prepared from 12 colonies giving positive signals for hybridization with probe 2 but not probe 1, indicating the presence of a mutation in the region 3516 to 3530. Three of those DNAs that yielded no detectable changes in mobility of restriction fragments were chosen for further study.

Sequence determination of mutants. The nucleotide sequences with the predicted amino acid sequences of four linker insertion in-frame mutants are shown in Fig. 1. Three mutants were isolated from the original screening, and the fourth, 5063, was constructed by recombining two of the original mutants at their *Xba*I sites, after first removing the *Xba*I unpaired nucleotides with S1 nuclease. Three of the four mutants were predicted to alter one or more of the residues of the invariant sequence among adenine nucleotide-binding proteins described by Walker et al. (91). These three mutants were likewise predicted to yield products with a change in the number of amino acid residues.

Included in Fig. 1 are the sequences of two frameshift mutants constructed by repairing the *Xba*I unpaired nucleotides of mutants 5061 and 5062. The sequences of these two mutants, 5071 and 5072, predict the production of protein products terminating after the 13th residue after interruption of the wild-type sequence. These out-of-frame derivatives were used as controls in some of the experiments described below.

FIG. 1. Sequences of deletion-insertion mutants. The nucleotide sequence of SV40 DNA with the corresponding amino acid sequence of T antigen from amino acid residues 424 to 435 is shown across the top. Amino acids all in capitals are the invariant residues in the sequence described by Walker et al. (91) for adenine nucleotide-binding proteins. Mutant designations are in the left column. For each mutant, inserted nucleotides are underlined, as are novel amino acid residues.

Biological activities of the mutants. The biological activities assayed were viability, the ability to transform nonpermissive rodent cells, and the cellular localization of the T antigens. All of the mutants were tested for viability by their ability to form plaques on monolayer cultures of BSC40 cells at 32, 37, and 40°C. None was viable at the three temperatures tested. The ability of the mutant T antigens to transform nonpermissive rodent cells was measured by the dense-focus assay of Todaro and Green (88). REF52 cells were transfected with plasmid DNA from the mutants and the SV40 wild-type plasmid, pSVB3, and foci were scored at 30 days (Table 1). At 30 days, mutants 5060 and 5063 showed few foci, whereas 5061 and 5062 yielded transformed foci at an efficiency (when compared with pSVB3) of approximately 65 and 40%, respectively. Localization of the mutant T antigens was determined by indirect immunofluorescence at 24 and 48 h after transfection of BSC40 cells. The mutant T antigens in cells transfected with 5060, 5061, 5062, and 5063, as well as in the T antigen-producing cell lines described below, were localized to the cell nuclei.

Ability of mutants to complement viral DNA with a late deletion. The ability of the mutants to complement *d*11007 DNA (containing a late deletion [39]) for plaque formation was assayed on BSC40 cell monolayers at 37°C. Whereas

TABLE 1. Transformation and complementation of mutants

Mutant	Complementation ^a of T mutant		Transformation of REF52 cells ^c
	Alone	+ <i>dl1007</i> ^b	
pSVB3	>100,>100	ND ^d	22,27,32,34,61,85
5060	0,0	>100,>100	0,0,0,0,0,2
5061	0,0	1,3	14,24,26,30,32,36
5071	0,0	>100,>100	0,0,0,0,0,0
5062	0,0	3,4	14,16,18,19,20,26
5072	0,0	>100,>100	0,0,0,0,0,0
5063	0,0	>100,>100	0,0,1,1,1,3

^a PFU per dish at 37°C.^b *dl1007* alone produced no plaques.^c Foci per dish at 30 days after transfection with a total of 2 µg of DNA.^d ND, Not done.

mutants 5060 and 5063 complemented *dl1007* with frequencies comparable to that of early deletion mutants (62) and point mutants (60), mutants 5061 and 5062 unexpectedly failed to do so (Table 1). (The few plaques that are found are presumably caused by recombination.) One possibility for this lack of complementation was that mutants 5061 and 5062 carried additional mutations outside the early region. To eliminate this possibility, genomic reconstructions were done to replace much of the mutant viral genomes with wild-type sequences. The *BclI*-to-*NdeI* small fragment (nucleotides 2770 to 3808) from the mutant genomes was used to replace the corresponding fragment in wild-type DNA; these reconstructed molecules still failed to complement *dl1007*, demonstrating that the mutation causing this phenotype resides within the 1038-nucleotide-long segment of the SV40 genome between nucleotides 2770 and 3808.

To show that the lack of complementation was a consequence of the production of a mutant T antigen, out-of-frame mutants producing a truncated protein were derived from the noncomplementing mutants 5061 and 5062. Digestion of the DNAs from mutants 5061 and 5062 with *XbaI*, repair of the unpaired nucleotides, and ligation to circles produced mutants 5071 and 5072, respectively, each of which has a 4-base-pair insertion causing a frame shift (Fig. 1). These out-of-frame mutants were now able to complement *dl1007* for plaque formation (Table 1), and therefore the noncomplementing phenotype must be due to a mutation in the gene for large T antigen.

Marker rescue of the mutants. Marker rescue experiments were done to confirm that the early region changes responsible for the mutant phenotypes were due to the mutations introduced. The wild-type fragments used to rescue the mutants were the *HindIII* D fragment (nucleotides 3476 to 4002) and the *RsaI* F fragment (nucleotides 3225 to 3576). Both fragments were able to rescue plaque formation (Table 2), indicating that the mutation causing the defective phenotype must reside in the 100-base-pair region between nucleotides 3476 and 3576. This region was sequenced for each of the mutants; except for the changes presented in Fig. 1, all

sequences were the same as wild type. Together with the data on complementation with *dl1007*, these results indicate that the sequence changes shown in Fig. 1 are responsible for the noncomplementing, defective phenotype of mutants 5061 and 5062.

DNA replication in BSC40 cells. The ability of each of the mutant T antigens to support viral DNA replication in BSC40 cells was tested by the *DpnI* assay described previously (61). The ability of the mutant viral DNAs to replicate was evaluated after transfection with or without the addition of helper *dl1007* DNA. The results shown in Fig. 2 indicate, first, that mutants 5061 and 5062 were unable to replicate their DNA at 37°C and, second, that even after cotransfection with *dl1007* these mutant DNAs failed to replicate. However, with a long exposure, a very faint band of replicated 5061 and 5062 DNA was seen after cotransfection with *dl1007*. In contrast, other defective T antigen mutants with lesions in the same region (5060 and 5063), as well as the out-of-frame derivatives of 5061 and 5062 (5071 and 5072), were complemented by *dl1007*.

Replication of molecules containing both mutant and wild-type T antigen genes. The results described above suggested that mutants 5061 and 5062 were producing T antigens able to act in a dominant fashion in the presence of a wild-type T antigen to inhibit viral DNA replication. To demonstrate this more directly, plasmids were constructed that contained a copy of the wild-type T antigen gene as well as a copy of a mutant T antigen gene. Cells transfected with this DNA would presumably make both wild-type and the mutant T antigens. Replication of the plasmids should not occur if the mutant T antigen is 5061 or 5062 but should occur if the plasmid contains any of the other mutants. The plasmids, described in Materials and Methods and depicted in Fig. 3A, contain the *KpnI*-to-*BamHI* early region fragment of wild-type SV40 in tandem with the *HpaII*-to-*BamHI* early region fragment of the mutants cloned into pKP54, such that both genes are transcribed in the same direction. The T antigen genes of mutants *dl1042*, 5060, 5061, and 5062 and the out-of-frame derivatives of 5061 and 5062 (5071 and 5072) were tested in replication experiments with these plasmids. *dl1042* is an N-terminal deletion mutant predicted to produce a truncated T antigen of 43 amino acids (62). The results are shown in Fig. 3B and indicate as predicted that the T antigens from mutants 5061 and 5062, but not from the others, prevent replication of the transfecting plasmid. Faint bands of replicated DNA can be seen with mutant 5062 at 48 and 72 h and with both 5061 and 5062 at 48 h with *dl1007*. This suggests that 5061 is a more potent inhibitor of DNA replication than is 5062 and that the inhibition of DNA replication by both mutants can be partially overcome with an excess of wild-type T antigen (see below). Restriction analysis demonstrated that the replicated DNA did not show rearrangements or recombination between the tandemly positioned T antigen genes.

Replication in BSC40 cell lines producing mutant T antigens. As a further demonstration of the difference between complementing and noncomplementing T antigen mutants, we constructed BSC40 cell lines that produce mutant T antigens and used these lines to determine whether wild-type T antigen has replication activity in the presence of preexisting mutant T antigen. The plasmid pSVKB1, which contains the SV40 origin and T antigen gene from the *KpnI* site to the *BamHI* site cloned into pKP54, was introduced into cell lines producing T antigens 5060, 5061, 5062, and 5063. At 24, 48, and 72 h posttransfection, low-molecular-weight DNA was isolated and assayed for DNA replication. To

TABLE 2. Marker rescue results

Mutant	Plaques per dish produced by restriction fragments		
	<i>HpaI</i> -A	<i>HpaI</i> -A + <i>HindIII</i> -D	<i>HpaI</i> -A + <i>RsaI</i> -F
5060	0,0	13,16	15
5061	1,0	8,13	4,6
5062	0,0	19,20	2,3
5063	0,0	31,35	19,30

FIG. 2. (A) Replication of mutant DNA in BSC40 monkey cells. Transfections were done as described previously (59), with 100 ng of excised and cyclized mutant DNA alone or with 50 ng of helper *dl1007* DNA. Hirt DNA was prepared (59, 61) at different times after transfection, purified, digested with *DpnI* and *Bam*HI, fractionated by electrophoresis on 1% agarose gels, transferred to nitrocellulose, and hybridized with 32 P-labeled SV40 DNA. The mutant DNAs transfected are indicated above the lane designations. DNA was prepared at the following times (in hours) posttransfection (lanes): 24 (a), 48 (b), 72 (c), and 48 with *dl1007* (d). Lane e contains 0.25 ng of wild-type SV40 DNA cleaved at the *Bam*HI site and run as a marker. (B) Replication of mutant DNA plus helper *dl1007* in BSC40 monkey cells. The procedure was followed as for panel A except that dishes were transfected with 300 ng of mutant plasmid DNA and 30 ng of *dl1007* plasmid DNA. DNA was harvested at 48 h after transfection. The DNA used was as follows (lane): 5060 (a), 5061 (b), 5071 (c), 5062 (d), 5072 (e). Lane f contains 0.1 ng of SV40 DNA cut at the *Bam*HI site and run as a marker.

eliminate the possibility that differences in the replication between the cell lines could be accounted for on the basis of variations in either transfection efficiencies or uptake and expression of the added DNA species, the plasmid pRSVCAT (25) was included in each transfection. Assays for chloramphenicol acetyltransferase expression indicated insignificant differences from one experiment to another. The different lines affected the replication of pSVKB1 to various degrees (Fig. 4). There was almost no replication of the plasmid in cell lines producing T antigens from mutants 5061 and 5062 (<1% of 5060 rate), although if the cells were passaged without ZnCl_2 , there was slightly more replication than if the cells were grown in its presence. The reduced replication of pSVKB1 in the presence of ZnCl_2 was presumably caused by the higher levels of the mutant T antigens in cells exposed to zinc. In the case of mutant T antigens that complement the late deletion mutant *dl1007*, such as 5060 and 5063, little reduction in replication was seen (Fig. 4). We conclude that the presence of the noncomplementing mutant T antigen in the cell does not permit significant replication activity of an introduced wild-type T antigen.

Replication of mutant virus in a cell line producing wild-type T antigen. The above experiment demonstrated that the replication activity of wild-type T antigen was inhibited in cells producing the mutant T antigens 5061 and 5062. The results shown in Fig. 3B suggested that the inhibition of DNA replication by 5061 and 5062 could be partially overcome by providing additional wild-type T antigen by cotransfection with *dl1007*. This would be expected if the inhibition by 5061 and 5062 were due to interference by the mutant T antigens with the function of the wild-type protein. Replication of the mutant DNAs might be further enhanced if the mutant molecules were transfected into cells producing wild-type T antigen. Accumulation of the mutant DNAs might then occur before mutant T antigens reached a level sufficient to prevent further replication. To test this possibility, mutant viral DNA was transfected into MKSV6 cells, an African green monkey cell line that produces wild-type T antigen under mouse metallothionein promoter control, and replication was assayed at 24, 36, and 48 h after transfection. Although there was slight replication of the mutant DNAs, the *trans*-dominant effect of mutants 5061 and 5062 is clearly evident even when wild-type T antigen preexists in the transfected cells (Fig. 5). This result suggests that the mutant T antigen is inhibitory at a very low ratio of mutant to wild-type T antigen.

DISCUSSION

In our continuing effort to define functional domains of the SV40 T antigen by directed mutagenesis, several in-frame *Xba*I linker insertion mutants were constructed in a part of the T antigen gene corresponding to the protein sequence shared with other ATPases. All mutants were defective for plaque formation and for DNA replication. Two of the mutants (5060 and 5063) induced a low level of transformation of REF52 cells, whereas two others (5061 and 5062) induced transformed foci at 40 to 65% of the wild-type level. All mutant T antigens were localized to the nucleus by indirect immunofluorescence.

Mutants 5061 and 5062 had a distinctive phenotype: they were unable to complement a late deletion mutant of SV40. The possibility that this phenotype was due to the presence of an additional defective mutation either in the late region or in the origin of replication was eliminated both by recon-

FIG. 3. Replication of pSVT+T' DNA. (A) The structure of pSVT+T'. (B) Replication in BSC40 monkey cells. The replication assay used was that described in the legend to Fig. 2 with the following exceptions. Transfection was done with 250 ng of mutant DNA per dish with or without 30 ng of *dl1007* plasmid DNA. Mutant DNA was used directly without the excision and cyclization steps. Digestion of Hirt DNA was done with *EcoRV* and *DpnI*. DNA was prepared at the following times (in hours) posttransfection (lanes): 24 (a), 48 (b), 72 (c), 48 with *dl1007* (d), 48 mock with *dl1007* (e). (*dl1007* DNA was included in these experiments to demonstrate that differences in replication among the various constructs were not artifactual.)

struction with wild-type genomic segments and by marker rescue experiments. In addition, out-of-frame mutants derived from the noncomplementing mutants by repairing the 5' unpaired nucleotides at the *XbaI* site could complement the late deletion mutant. Thus, the dominant, noncomplementing phenotype must be a property of the mutant T antigens themselves.

To determine whether the noncomplementable T antigens of 5061 and 5062 inhibited viral DNA replication in the presence of wild-type T antigen, three experiments were done. First, the mutant and wild-type T antigens were introduced into the same cell by transfecting with a plasmid containing both the mutant and wild-type T antigen genes arranged in tandem. Inhibition of plasmid replication was

FIG. 4. Replication of pSVKB1 DNA in BSC40 monkey cell lines producing mutant T antigens. Cell lines producing the different mutant T antigens were grown in the absence or presence of ZnCl_2 , and dishes of each line were transfected with 100 ng of pSVKB1 and 1 μg of pRSVCAT DNA. Replication of pSVKB1 was determined by the procedure described in the legend to Fig. 2, except that Hirt DNA was digested with *DpnI* and *XbaI*. DNA was prepared at the following times (in hours) posttransfection (lanes): 24 (a and d), 48 (b and e), 72 (c and f). Lanes a, b, and c contain samples from cells grown without ZnCl_2 ; lanes d, e, and f are from cells grown in the presence of 100 μM ZnCl_2 . Lane g is from a mock transfection, and lane h contains 0.5 ng of pSVKB1 DNA digested at the *XbaI* site and included as a marker.

FIG. 5. Replication of SV40 mutant DNA in MKSV6 cells. Dishes of MKSV6 cells were transfected with 50 ng of excised and cyclized mutant plasmid DNA, and replication of the mutant DNA was analyzed by the procedure described in the legend to Fig. 2. DNA was prepared at the following times (in hours) posttransfection (lanes): 24 (a), 48 (b), 72 (c). As a negative control, lane d contains 0.5 ng of pSVB3 DNA which had been added to an extract of mock-transfected cells and digested with *DpnI* and *BamHI*. Lane e contains 0.5 ng of SV40 DNA cleaved at the *BamHI* site and included as a marker.

found only when the 5061 or 5062 T antigen gene was present; other mutant genes did not inhibit. Second, monkey cell lines were constructed that produced the mutant T antigen under the control of the mouse metallothionein promoter. The effect of the mutant proteins on replication in the presence of the wild-type T antigen was assessed after transfection of SV40 DNA into these cells. Little replication of SV40 DNA was observed in the cell lines producing the 5061 and 5062 T antigens compared with replication in lines producing other defective T antigens. Third, transfection of mutant DNA into cells already producing wild-type T antigen showed that, although some DNA replication occurred, the *trans*-dominant effect of mutants 5061 and 5062 was still evident. The results of these experiments demonstrate that 5061 and 5062 T antigens are potent inhibitors of viral DNA replication, even at a low ratio of mutant to wild-type T antigen.

There are examples of other mutant T antigens of SV40 that inhibit viral DNA replication to various degrees. These T antigens have amino acid substitutions at positions remote from the site of mutation seen in the 5061 and 5062 proteins (60, 80). In addition, the effect of ZnCl_2 induction on replication of pSVKB1 in the cell line producing mutant T antigen 5063 (Fig. 4) suggests that this mutant protein, when present in large initial excess, is also capable of interfering with viral DNA replication. However, the unique property of 5061 and 5062, even among mutants that demonstrate some inhibition of viral DNA replication, is that they fail to complement the SV40 late deletion mutant *dl1007*, presumably because of their potency as inhibitors of viral DNA replication. A *trans*-dominant mutant of SV40 T antigen has been described (41–43) that exhibits cytoplasmic localization

and also inhibits the nuclear accumulation of wild-type T antigen. Both the cellular localization of this mutant T antigen and its site of mutation (Lys-128) differ from those of 5061 and 5062. In addition, this mutant did not inhibit wild-type SV40 production in cells infected with both viruses, indicating that the mutant was not a potent inhibitor of viral DNA replication (41).

Although the mechanism by which 5061 and 5062 T antigens inhibit viral DNA replication is not understood, we speculate that inhibition could be the result of a nonproductive, stable interaction of the mutant protein with a host cell component required for viral DNA replication, or with the viral origin of replication, or with wild-type T antigen. Of these possible mechanisms, there is clear precedent in *E. coli* systems for the interaction of mutant and wild-type proteins to produce nonfunctional oligomers, for example in the case of the *recA* protein (55, 70, 95, 96), the *lac* repressor (53), the *mutD* (*dnaQ*) protein (18), and the *lon* (*capR*) protein (9). Where the negative complementation in *E. coli* perhaps differs from that seen with SV40 mutants 5061 and 5062 is in the ratio of mutant protein to wild-type protein required to cause significant inhibition of activity. In the case of the *recA* protein, the *lac* repressor, and the *lon* (*capR*) proteins, the mutant proteins must be present in excess of the wild-type protein before significant loss of wild-type function is observed, whereas with 5061 and 5062 under conditions in which we presume there are equal amounts of mutant and wild-type proteins, we see at least a 90% loss of replication activity. In their effectiveness as inhibitors of viral DNA replication, 5061 and 5062 also are likely to differ from the five mutants studied by Stillman et al. (80). These five mutants produced between 40 and 70% inhibition of DNA replication when evaluated in an in vitro assay with equal amounts of mutant and wild-type T antigen (80). Nonetheless, wild-type virus is able to replicate in cell lines producing these mutant T antigens (24, 73).

trans-acting defective mutants of the large T antigen of polyomavirus have been described by Nilsson and Magnusson (54) that inhibit the replication of wild-type viral DNA. These mutants have changes carboxy-terminal to the sequence, shared among ATPases, that is altered in 5061 and 5062. The mechanism of the *trans*-dominant inhibition shown by the polyomavirus mutants is unknown.

The inhibitory effect of 5061 and 5062 is profound, seen even in T antigen-producing cells that are transfected with a plasmid carrying the mutant gene. (However, there is evidence [22, 56, 63, 68] that newly synthesized T antigen in productively infected cells is most active in binding to the replication origin and is required for viral DNA replication [93, 94], and so the active molecules present in a T antigen-producing line may constitute only a small proportion of the accumulated protein.) Thus, one possible reason for the inhibitory potency of 5061 and 5062 T antigens is that multimers of T antigen are required for viral DNA replication and that these multimers are inactive if they contain even a minority of mutant subunits. Since most of the evidence implicates the monomer or dimer species of T antigen (or both) in origin binding (4, 7, 22, 63, 68), the putative formation of higher-order oligomers might occur on the DNA. Alternatively, 5061 and 5062 subunits may have a high propensity to associate, both with themselves and with the wild-type protein, thereby inactivating wild-type T antigen. A test of these and other hypotheses to explain the unusual properties of the 5061 and 5062 T antigens will require a comparison of the biochemical properties of purified mutant and wild-type proteins.

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