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Linker Insertion Mutants of Simian Virus 40 Large T Antigen That Show *trans*-Dominant Interference with Wild-Type Large T Antigen Map to Multiple Sites within the T-Antigen Gene

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Linker insertion mutants affecting the simian virus 40 (SV40) large tumor (T) antigen were constructed by inserting a 12-base-pair oligonucleotide linker into restriction endonuclease cleavage sites located within the early region of SV40. One mutant, with the insertion at amino acid 5, was viable in CV-1p and BSC-1 cells, indicating that sequences very close to the amino terminus of large T could be altered without affecting the lytic infection cycle of SV40. All other mutants affecting large T were not viable. In complementation assays between the linker insertion mutants and either a late-gene mutant, *dl*BC865, or a host range/helper function (*hr*/*hf*) mutant, *dl*A2475, delayed complementation was seen with the 6 of the 10 nonviable mutants. Of these 10 mutants, 5 formed plaques 3 to 4 days later than in control complementations, while complementation by one of the mutants, *in*A2827, with an insertion at amino acid 520, was delayed more than 1 week. Most mutants which showed delayed complementation replicated less well in Cos-1 cells than did a control mutant, *dl*A1209, which produced no T antigen. The replication of *in*A2827(aa520) was reduced by more than 90%. Similar interference with viral DNA replication was seen when CV-1, HeLa, or 293 cells were cotransfected with an origin-defective plasmid encoding wild-type large T antigen and with *in*A2827(aa520). Only one of the mutant T antigens, *in*A2807(aa303), was unstable. These results indicate that some of the mutant T antigens interfered with functions of wild-type T required for viral DNA replication. However, not all of the mutants which showed delayed complementation also showed interference with viral DNA replication. This indicates that mutant large T antigens may interfere *trans* dominantly with multiple activities of wild-type large T antigen.

Simian virus 40 (SV40) large T antigen is a multifunctional, 708-amino-acid phosphoprotein. T antigen is located primarily in the nucleus, but a small amount is found in the cytoplasm and at the cell surface. T antigen plays essential roles during both lytic and transforming infections by SV40. It binds specifically to sequences at the SV40 origin of replication and nonspecifically to cellular DNA. Large T antigen also possesses ATPase and helicase activities (52, 64).

Sequences within T antigen sufficient for binding to the SV40 origin of DNA replication have been localized to the region between amino acids 131 and 259 (3, 54); however, mutations affecting the putative metal-binding finger region (amino acids 302 to 320) show altered DNA binding, suggesting that this region might interact with and regulate the DNA binding domain (3). Genetic and immunological studies indicate that the ATPase and helicase activities of large T are contained within the region between amino acids 350 and 600 (10, 14). The C terminus of T antigen constitutes a separate functional domain involved in providing adenovirus helper function and a host range function (hr/hf function) (12). *hr*/*hf* mutants replicate to the same level as wild-type SV40 in all monkey kidney cell lines examined but produce progeny virus inefficiently in CV-1 monkey kidney cells (40, 66, 67).

SV40 T antigen is phosphorylated (59), glycosylated (23), ADP ribosylated (22), acylated (27), and adenylylated (7). Phosphorylation sites are clustered in two regions. Several are located between Ser-106 and Thr-124, while others are located near the C terminus, from Ser-639 to Thr-701 (42, 68). T antigen exists as monomer and oligomerizes into dimeric, tetrameric, and higher-order forms (35). A cell cycle-related protein, p53, is physically associated with and metabolically stabilized by T antigen in both permissive and nonpermissive cells (28). T antigen is also associated with transcription factor AP-2 (33) and DNA polymerase α (47). The binding of DNA polymerase α to T antigen can be blocked in vitro by p53. This suggests that DNA polymerase α and p53 bind to the same or physically adjacent sites on large T antigen (18). Recently, T antigen has been found to form a complex with the retinoblastoma susceptibility gene product Rb (15). T-antigen sequences located between amino acids 105 and 114 are important for complex formation between T and Rb (15). Whether other T-antigen sequences are also important for Rb binding has not been determined.

Large T antigen performs multiple functions during lytic and transforming infections by SV40. The DNA binding and ATPase-helicase activities of T are required for viral DNA replication (47, 64, 69). For T antigen to be active in DNA replication, these activities must be located within a single T-antigen monomer (14). T antigen autoregulates synthesis of its own mRNA (2, 60). At low levels, T stimulates transcription of SV40 early mRNA (25), while at higher concentrations, T acts to repress early mRNA synthesis (2). T antigen can transactivate transcription from a number of viral and cellular promoters, including the SV40 late (8, 24), adenovirus E2 (29), Rous sarcoma virus long terminal repeat (1), and rRNA promoters (48). T antigen induces host cell DNA synthesis (17, 49). It also plays central roles in immortalization of primary cells (61, 62) and in the malignant

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transformation of established cell lines (26, 58). Mutants with defects in DNA binding, ATPase-helicase, or hr/hf function retain the ability to immortalize cells at the same frequency as wild-type SV40, indicating that none of these activities of T antigen are required for immortalization (14, 62).

Mutational analysis has played a central role in assigning functional activities to specific domains of T antigen. To study further the organization, interaction, and activities of the various domains of T antigen, we inserted a 12-base-pair (bp) synthetic oligonucleotide linker into multiple sites within the SV40 early region. Here we present the initial characterization of these mutants. Ten were nonviable, belonged to the A complementation group, and were defective in viral DNA replication. Several of these mutants interfered with the functions of wild-type T antigen. Plaque formation was delayed in cells coinfected by a capsid protein mutant and some linker insertion mutants. In addition, replication of viral DNA was reduced up to 20-fold in Cos-1 cells transfected by some mutants; these cells produce wild-type large T antigen constitutively (19). Not all mutants that showed delayed complementation showed reduced replication in Cos-1 cells. Mutations which resulted in *trans*-dominant interference were located at many sites.

MATERIALS AND METHODS

Cells, tissue culture, and plasmid DNAs. CV-1, CV-1p, and BSC-1 cells are permanent lines of African green monkey kidney cells. Cos-1 cells are a derivative of CV-1 cells transformed with an origin-defective SV-40 and produce SV40 large T antigen constitutively. Cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), unless specified otherwise. Our wild-type strain of SV40 was the small-plaque strain originally characterized by Takemoto et al. (56). Standard techniques were used for bacterial growth and transfection (31), minilysate DNA preparation and analysis (5), and large-scale plasmid purification by CsCl-ethidium bromide buoyant density centrifugation.

Construction of mutants. A plasmid containing the complete SV40 genome inserted into the *Eco*RI site of pUC18 (pCC2Pa) was used for mutant constructions. The early region of SV40 from the *Kpn*I site (nucleotide 294) (65) to the *Bam*HI site (nucleotide 2533) was isolated from pCC2Pa and subcloned between the *Bam*HI and *Kpn*I sites in the polylinker of pUC18. This plasmid DNA was digested with either *Alu*I, *Dra*I, or *Ssp*I, in the presence of ethidium bromide (33 μg/ml) for 1 to 2 h, to limit cleavage to once per molecule (45). After digestion, the DNA was fractionated by electrophoresis in a 0.7% agarose gel in TBE buffer (89 mM Tris hydroxide, 89 mM boric acid, 2.5 mM EDTA [pH 8.3]). Unit length, linear DNA was isolated from the gel and purified by extraction with phenol and chloroform, followed by ethanol precipitation. These linear molecules were then ligated, by using T4 DNA ligase, with a large molar excess of a 12-bp non-phosphorylated synthetic oligonucleotide linker, d(TCG CGATCGCGA), purchased from New England BioLabs. This linker contains two *Nru*I sites and a single *Pvu*I site. After ligation, the DNA was heated at 90°C for 30 s, cooled at room temperature for 30 min, and transformed into *Escherichia coli* HB101. The sites of linker insertion were determined by restriction endonuclease digestion of minilysate DNA preparations, which indicated which *Alu*I, *Dra*I, or *Ssp*I site was missing and where *Nru*I and *Pvu*I sites had been inserted. The mutant SV40 early regions, as *Kpn*I to *Bam*HI fragments, were then isolated from 0.7% agarose gels and ligated with the *Bam*HI to *Kpn*I fragment of pCC2Pa, which contains the remainder of the SV40 genome and pUC18. These mutants were assigned SV40 mutant numbers in the 2800 series.

Plaque and complementation assays. Viral DNAs were separated from pUC18 sequences by digestion with *Eco*RI and circularized by ligation at low concentration (5 mg/ml) with T4 DNA ligase. Agarose gel electrophoresis was used to monitor digestion and ligation. CV-1 cells in 60-mm plates were incubated for 45 min with 0.3 ml of DNA solution containing 500 mg of DEAE-dextran per ml in Tris-buffered saline (TS; 25 mM Tris chloride [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.05 mM $MgCl_2$, 0.7 mM $CaCl_2$) and various dilutions of viral DNAs. Cells were then washed twice with TS and were overlaid with 3 ml of DMEM containing 4% FCS and 1% Bacto-Agar (Difco Laboratories). Plates were overlaid 3 to 4 days later and stained 6 to 7 days after transfection with 0.01% neutral red. The plaques were counted every day after staining for about 2 weeks.

Complementation assays were performed in the same manner, except cells were transfected with a mixture of two mutant DNAs, as described previously (66). Each 60-mm plate received 0.1 mg of helper DNA and 0.1, 0.01, or 0.001 mg of mutant DNA.

Viral DNA replication. Mutant SV40 DNAs were separated from pUC18 sequences, circularized as described above, and introduced into CV-1p or COS-1 cells in 60-mm plates by using DEAE-dextran, as described above. After 45 min at 37°C, cells were washed twice with TS and then fed with 5 ml of DMEM containing 2% FCS–100 mM chloroquine diphosphate (Sigma Chemical Co., St. Louis, Mo.). After 6 h, chloroquine-containing medium was replaced with DMEM containing 2% FCS. Viral DNAs were harvested 0, 1, 3, and 5 days after transfection as described previously (40, 66, 67). The DNAs were digested with *Mbo*I and fractionated on a 1.5% agarose gel. Input DNAs had been isolated from *dam*$^+$ bacteria and were therefore methylated (GA^mTC) and resistant to *Mbo*I digestion, while DNA which had replicated in mammalian cells was not methylated at GATC and was therefore sensitive to *Mbo*I digestion. After electrophoresis, DNAs were transferred to Nytran nylon (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized under standard conditions with ^{32}P-labeled, nick-translated pCC2Pa DNA. After hybridization and washing, the nylon blot was exposed to Kodak XAR-5 film for autoradiography.

Plasmid p6-1 (a gift from J. Alwine) contains the complete genome of an SV40 mutant lacking 6 bp at the *Bgl*I site in the origin of replication (21, 24). In some experiments, CV-1, HeLa, or 293 cells were cotransfected with insertion mutant and p6-1 DNAs by the calcium phosphate method (70). Cells were incubated in the presence of the calcium phosphate precipitate for 18 h. Cells received 0.5 mg of each recircularized viral DNA and 5 mg of salmon sperm DNA (Sigma) as a carrier. Cultures were harvested and low-molecular-weight DNAs were isolated 1, 2, 3, and 5 days after transfection, as described above. These DNAs were digested with *Mbo*I and *Eco*RI and analyzed as described above.

Pulse-chase labeling and immunoprecipitation of T antigens. BALB/c 3T3 cell lines expressing mutant or wild-type SV40 large T antigens were prepared by cotransfecting cells with mutant or wild-type SV40 DNAs and pSV2neo (51). Colonies were isolated after selection in DMEM containing 10% FCS and 500 mg of G418 per ml, screened for the production of SV40 large T antigen by immunofluorescence,

and maintained in DMEM containing 10% FCS and 250 mg of G418 per ml. The production of large T antigen in the cell lines was confirmed by Western blotting (immunoblotting) analysis.

To examine the stabilities of mutant T antigens, 2×10^5 cells were seeded onto 60-mm plates the day before labeling. The cells were washed twice with TS and incubated for 1 h at 37°C in 2 ml of DMEM lacking methionine and containing 2% dialyzed FCS. The medium was removed, and the cells were labeled for 2 h with 100 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.) in 0.7 ml of DMEM lacking methionine and containing 2% dialyzed FCS. Cells were washed twice with TS and incubated in DMEM containing 10% FCS. Cultures were harvested after chase periods of 0, 5, and 17 h and were lysed with 0.5 ml of EBC buffer (50 mM Tris chloride [pH 8.0], 100 mM NaCl, 0.5% Nonidet P-40, 0.1 U of aprotinin per ml [15]) for 20 min at 4°C. The cellular debris were removed by centrifugation for 15 min at 4°C in a microcentrifuge. Extracts from identical numbers of cells were mixed with 10 μl of a mixture of mouse ascites fluids containing monoclonal antibodies PAb901 and PAb902 (a gift from S. Tevethia and J. Tevethia). These antibodies recognize denaturation-resistant determinants located at the carboxy and amino termini of large T antigen, respectively (J. Tevethia and S. Tevethia, personal communication). Mixtures were rocked for 1 h at 4°C. Protein A-Sepharose beads (20 μl; Sigma) (1:1 mixture in Tris-buffered saline-bovine serum albumin [15]) were added to the mixture, and rocking was continued for 30 min at 4°C. The Sepharose beads were pelleted by centrifugation and washed three times with 1 ml EBC buffer at 4°C. Immunoprecipitated proteins were released by heating in sample buffer for 15 min at 70°C and analyzed by electrophoresis in sodium dodecyl sulfate–7.5% polyacrylamide gels, followed by fluorography. The relative intensities of T-antigen bands were determined by densitometric scanning. Serial dilutions of ^{35}S-labeled proteins were electrophoresed on a parallel gel and analyzed in the same way. This indicated that the densitometric readings reflected accurately the amount of ^{35}S-labeled large T antigen.

Mutant Numbers	Insertion Sites	Codon	Frames Inserted	Wildtype / Mutant
2801	5150	5	2/3	4 5 6 Val Leu Asn 4 6 Val Phe Arg Asp Arg Glu Asn
2803	5060	35	2/3	34 35 36 Tyr Leu Lys 34 36 Tyr Phe Arg Asp Arg Glu Lys
2815	4316	168	1/3	167 168 169 Lys Ala Ala 167 169 Lys Val Ala Ile Ala Thr Ala
2817	4162	219	2/3	218 219 220 Phe Ser Phe 218 220 Phe Ser Arg Asp Arg Asp Phe
2807	3910	303	2/3	302 303 304 Cys Leu Lys 302 304 Cys Phe Arg Asp Arg Asp Lys
2819	3782	346	1/3	345 346 347 Leu Ala Lys 345 347 Leu Val Ala Ile Ala Thr Lys
2809	3592	409	2/3	408 409 420 Phe Leu Lys 408 409 Phe Phe Arg Asp Arg Asp Lys
2811	3546	424	0/3	424 425 Phe Lys 424 425 Phe Ser Arg Ser Arg Lys
2821	3438	460	0/3	460 461 Glu Leu 460 461 Glu Ser Arg Ser Arg Leu
2823	3428	464	1/3	463 464 465 Val Ala Ile 463 465 Val Val Ala Ile Ala Thr Ile
2827	3259	520	2/3	519 520 521 Gln Ile Phe 519 521 Gln Ile Arg Asp Arg Glu Phe
2828	3259	520	2/3	519 520 521 Gln Ile Phe 519 521 Gln Ile Arg Glu Phe
2829	4643	173 (small t)	0/3	173 174 Lys Leu Stop 173 174 Lys Ser Arg Ser Arg Leu Stop

FIG. 1. Linker insertion mutants of SV40. Mutants were prepared as described in Materials and Methods. The T antigen codon interrupted by or immediately upstream of the inserted linker is listed. The frame of insertion refers to whether the linker was inserted between 2 codons (0/3), after the first nucleotide of the disrupted codon (1/3), or after the second nucleotide of the disrupted codon (2/3). The amino acid sequences of wild-type and insertion mutant T antigens, at the site of insertion, are shown. All insertions maintain the normal reading frames on both sides of the insertion.

RESULTS

Construction of linker insertion mutants. A set of mutants was prepared by insertion of a 12-bp oligonucleotide linker, d(TCGCGATCGCGA), into *Dra*I, *Ssp*I, and *Alu*I sites within the SV40 early region. All three enzymes cleave multiple times in the large T-antigen-coding region and leave blunt ends on cleaved DNA. Digestion of SV40 DNA with these enzymes was performed in the presence of ethidium bromide so that a large fraction of the DNA was full-length linear molecules. Full-length, linear DNA was isolated from an agarose gel and ligated with the linker. The linker contains two *Nru*I sites and a single *Pvu*I site. Mutants were analyzed by restriction endonuclease digestion. All mutants were assigned numbers in the 2800 series; for clarity in this paper, mutants are also referred to by the position within the T antigen where additional amino acids were inserted. For example, the mutant *in*A2803 contains an insertion of 4 amino acids within codon 35; we refer to this mutant as *in*A2803(aa35). These linker insertion mutants encode large

T antigens which differ from the wild type by the addition of four amino acids at the site of the linker insertion (Fig. 1). The sequence of amino acids inserted depends on the reading frame of the insertion. Mutant *in*A2828(aa520) was derived from *in*A2827(aa520) by digestion to completion with *Nru*I followed by ligation. This excised 6 bp from the DNA, resulting in a mutant which encodes a large T antigen containing 2 amino acids inserted at the position of amino acid 520.

Biological properties of the mutants. The effects of the mutations on the SV40 lytic infection cycle were examined by plaque assay. Circularized viral DNAs were transfected into CV-1p and BSC-1 cells. Mutants *in*A2801(aa5) and *in*A2829(small t) were viable and formed plaques of the same size and with the same efficiency as wild-type SV40 in CV-1p cells at 33, 37, and 41°C and in BSC-1 cells at 37°C. The mutation in *in*A2829(small t) is in the region encoding the carboxy terminus of small t antigen; its viability is consistent with the previous observation that mutants affecting only small t antigen have little effect on lytic infection by SV40. Mutant inA2801(aa5) has a linker inserted at amino acid codon 5, suggesting that a mutation near the amino terminus of T antigen does not have major effects on the functions of T antigen required during the lytic cycle. None of the other mutants formed plaques in CV-1p and BSC-1 cells.

The SV40 large T antigen plays an essential role in initiation of viral DNA replication (57). To see whether any of these new nonviable mutants were positive for viral DNA replication, mutant DNAs were transfected into CV-1 cells. Cells were harvested 0, 1, 2, and 3 days after transfection, and viral DNA was isolated. Sensitivity of DNA produced in eucaryotic cells to digestion by *Mbo*I was used to distinguish the progeny DNA from input DNA. After *Mbo*I digestion, the DNAs were analyzed by Southern blotting. No viral DNA replication was detected in the cells transfected by any of the nonviable mutants.

Genetic complementation analysis. The ability of the nonviable insertion mutants to complement early and late gene mutants was tested by cotransfecting CV-1p cells with insertion mutant DNA and DNA from early- and late-gene mutants. Mutant *dl*A1209 (12) lacks 329 bp (nucleotides 4862 to 5191), including the normal early mRNA cap site, the entire first exon of the large T-antigen-coding region, and the 5′ splice site of the large T-antigen intron. It encodes no T antigen but can provide capsid proteins. As expected, none of the nonviable insertion mutants was able to complement *dl*A1209 (Table 1). Mutant *dl*BC865 contains a small deletion at the SV40 *Eco*RI site (nucleotide 1782) and produces a short amino-terminal fragment of VP1 and a wild type T antigen (9). All early gene mutants were expected to complement this mutant. All the nonviable insertion mutants did indeed complement dlBC865. However, the time of appearance and the numbers of plaques formed varied from mutant to mutant. In control plates which received 50 ng of both *dl*A1209 and *dl*BC865 DNA, about 50 plaques appeared on each 60-mm plate 6 to 7 days after transfection, and the number reached a maximum of about 200 plaques on day 10 or 11. Mutants *in*A2815(aa168), *in*A2817(aa219), *in*A2807 (aa303), *in*A2809(aa409), *in*A2811(aa424), and *in*A2823 (aa464) complemented *dl*BC865 as well as *dl*A1209 (Table 1). Mutants *in*A2803(aa35), *in*A2819(aa346), *in*A2821(aa460), and *in*A2827(aa520) showed delayed complementation with *dl*BC865. In plates transfected with *in*A2803(aa35) and *dl*BC865, plaques appeared 3 to 4 days later and grew more slowly than the control (*dl*BC865 and *dl*A1209). However, the specific infectivities (in PFU per milligram) of these mutants were similar to that of *dl*A1209. Complementation between *dl*BC865 and either *in*A2819(aa346) or *in*A2821(aa460) was also delayed by 3 to 4 days. Fewer plaques (20 to 50% of the total in the control) were formed, but the plaques formed were normal in size and rate of enlargement. In the case of *in*A2827(aa520), the plaques appeared at least 7 days later than on control plates. The specific infectivity of *in*A2827(aa520) DNA was 10 to 20% of that of *dl*A1209. We do not know whether more plaques would have appeared after further incubation because these cells died after 20 days of incubation, even without SV40 infection. Complementation between *dl*BC865 and *in*A2817 (aa219) or *in*A2823(aa464) was delayed by 1 to 2 days in some experiments and was normal in other experiments.

TABLE 1. Mutant DNA replication in COS-1 cells and complementation analysis

Mutant	Codon of insertion	Complementation[a]			Viral DNA replication in COS-1 cells (% of *dl*A1209)[b]
		*dl*A1209	*dl*BC865	*dl*A2475	
2801[c]	5	NT	NT	NT	210
2803	35	−	Delayed	Delayed	78
2815	168	−	+	+	99
2817	219	−	+	Delayed	50
2807	303	−	+	+	119
2819	346	−	Delayed	−	20
2809	409	−	+	+	125
2811	424	−	+	+	107
2821	460	−	Delayed	−	20
2823	464	−	+	Delayed	37
2827	520	−	Delayed	−	9
2828	520	NT	NT	NT	10
WT[d]		NT	NT	NT	314
A1209		−	+	−	100
BC865		+		+	NT
A2475		−	+	−	NT

[a] Complementation assays are described in Materials and Methods. +, Plaques appeared at the same time as in the control (*dl*BC865 or *dl*A1209), that is, 6 to 7 days after transfection; Delayed, plaques appeared more than 10 days after transfection. NT, Not tested; −, no plaques appeared.

[b] The numbers represent the relative levels of DNA replication in Cos-1 cells (level in *dl*A1209 = 100) averaged from samples isolated 3 and 5 days after transfection. The values were derived from densitometric scanning of comparable bands on Southern blot autoradiographs from one experiment. The experiments were repeated, and similar levels of DNA replication were observed.

[c] *in*A2801(aa5) was viable and was not tested for complementation.

[d] WT, Wild type.

The C-terminal 80 amino acids of SV40 large T antigen provide the hr/hf function (13, 66, 67). Mutants whose T antigens lack this domain fail to help human adenoviruses to grow in monkey cells and are unable to form plaques on CV-1p cells. These mutants produce plaques on BSC-1 cells and produce normal progeny yields in Vero cells (13). A well-characterized members of this class, *dl*A2475, produces a T antigen lacking the C-terminal 26 amino acids (13). We reported previously that this mutant is able to complement intracistronically other group A mutants that produce T antigens containing an intact C terminus (66). Formation of plaques by complementation (Table 1) between *dl*A2475 and *in*A2803(aa35), *in*A2817(aa219), or *in*A2823(aa464) was delayed by 3 to 5 days. Mutants *in*A2819(aa346), *in*A2821 (aa460), and *in*A2827(aa520) did not complement *dl*A2475. These results indicate that some of the insertion mutant T antigens, even though they contain normal C termini, were unable to complement an *hr/hf* mutant. This could reflect

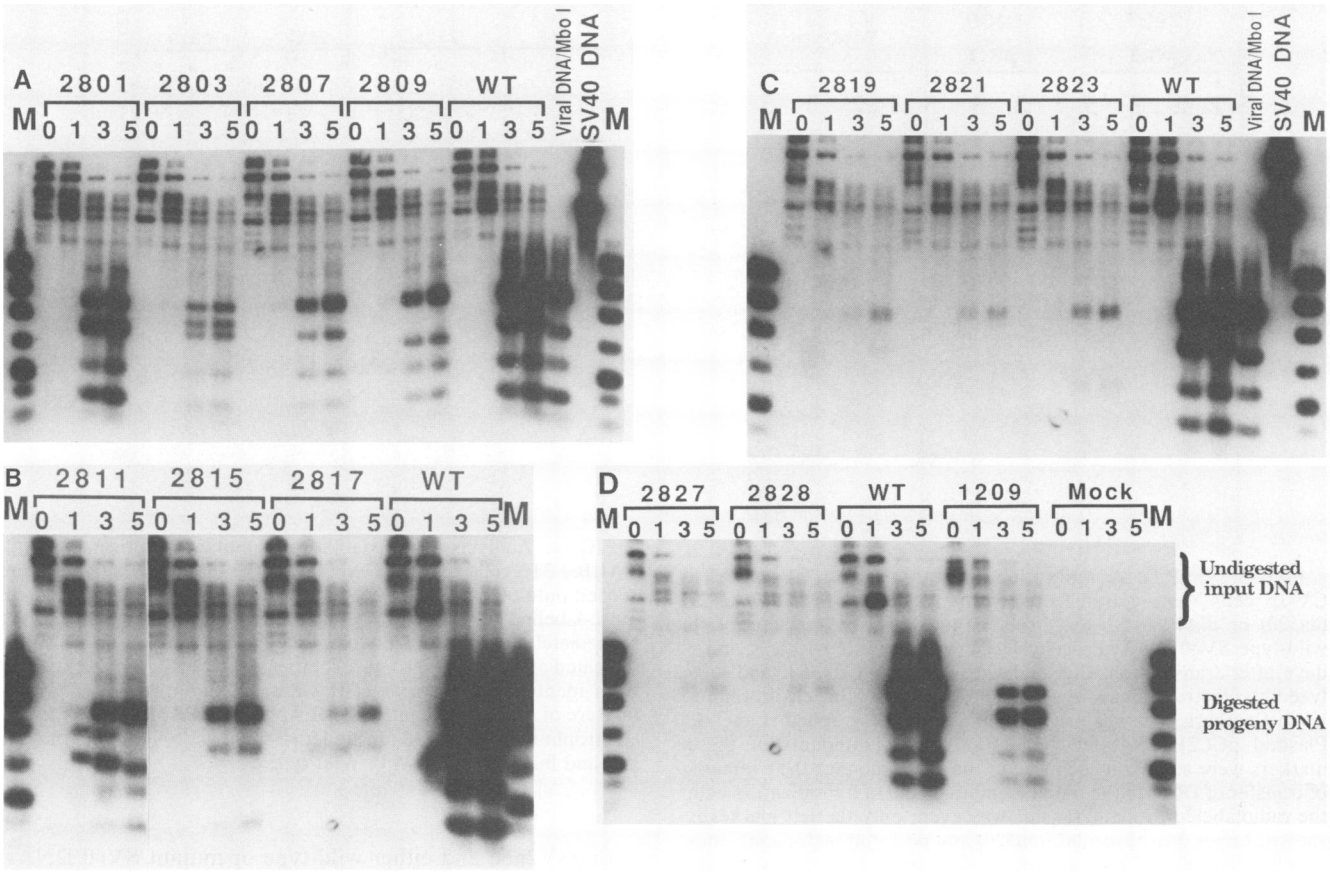

FIG. 2. Mutant SV40 DNA replication in Cos-1 cells. Cos-1 cells were transfected with mutant DNAs. Progeny DNA was harvested 0, 1, 3, and 5 days later, digested with *Mbo*I, and analyzed by electrophoresis in 1.5% agarose gels. After electrophoresis, a Southern blot was prepared (50) and autoradiographed. Lanes M, Plasmid pCC2Pa digested with *Hin*fI. Equal amounts of these markers were added on both sides of each gel to monitor the evenness of transfer of DNA to the nylon membrane and of hybridization with the radiolabeled probe. The lanes marked viral DNA/*Mbo*I contained *Mbo*I-digested SV40 DNA isolated from infected cells. The presence of an *Mbo*I site within the linker caused different mutants to have fragments of different sizes. WT, Wild type.

either their inability to provide the hr/hf function or, more likely, their interference with replication activities of *dl*A2475 T antigen.

Insertion mutant viral DNA replication in Cos-1 cells. The interference of mutant T antigens with wild-type (or *dl*A2475) T antigen could occur during viral DNA replication or at some other steps during the lytic infection cycle. To examine the interference further, viral DNA replication was analyzed in Cos-1 cells transfected by various insertion mutants. In Cos-1 cells, the large T encoded by the integrated SV40 genome permits replication of introduced DNA molecules containing a functional SV40 origin of DNA replication. Viral DNAs were isolated 0, 1, 3, and 5 days after transfection. *Mbo*I digestion was again used to distinguish the input DNA from progeny DNA in Cos-1 cells (Fig. 2). Mutant *dl*A1209 served as a positive control; because *dl*A1209 encodes no T antigen, the replication of *dl*A1209 DNA reflected the activity of the endogeneous large T antigen encoded by the integrated SV40 genome. Wild-type SV40 replicated to a higher level in Cos-1 cells than *dl*A1209; this probably reflects the contribution to replication of both the endogenous T antigen and the wild-type T encoded by transfected SV40 DNA (Fig. 2D). The replication of *in*A2817(aa219) (Fig. 2B), *in*A2819(aa346) (Fig. 2C), *in*A2821(aa460) (Fig. 2C), *in*A2823(aa464) (Fig. 2C), and *in*A2827(aa520) (Fig. 2D) DNAs was significantly reduced (50 to 95%) compared with that of *dl*A1209. Mutant *in*A2828(aa520) (Fig. 2D), which contains a 6-bp insertion at the same site as the 12-bp insertion of *in*A2827(aa520), replicated to the same reduced extent as *in*A2827(aa520), which replicated 5 to 10% as well as *dl*A1209. All these mutants showed reduced complementation with either a late-gene mutant (*dl*BC865), an *hr*/*hf* mutant (*dl*A2475) or both. The data in Fig. 2 demonstrate that some of the insertion mutant T antigens interfered with the replication functions of wild-type T antigen.

The replication of *in*A2803(aa35) (Fig. 2A) in Cos-1 cells was slightly reduced compared to that of *dl*A1209. Interestingly, this mutant also showed delayed complementation with *dl*BC865 and *dl*A2475. In contrast, mutants *in*A2817 (aa219) (Fig. 2B) and *in*A2823(aa464) (Fig. 2C) replicated much less well in Cos-1 cells than mutant *in*A2803(aa35), even though they complemented *dl*BC865 normally. This could indicate that *in*A2803(aa35) interfered with functions of wild-type T antigen distinct from viral DNA replication. Finally, some of the insertion mutants [*in*A2807(aa303) (Fig. 2A), *in*A2809(aa409) (Fig. 2A), *in*A2811(aa424) (Fig. 2B), and *in*A2815(aa168) (Fig. 2B)] replicated as well as *dl*A1209.

Interactions between wild-type and mutant T antigens in CV-1, HeLa, and 293 cells. The ability of *in*A2827(aa520) to

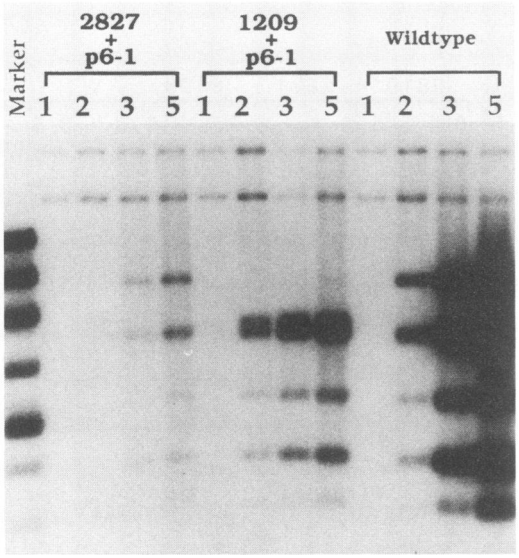

FIG. 3. Viral DNA replication in cotransfected CV-1p cells. CV-1p cells were cotransfected with plasmid p6-1 and *in*A2827 (aa520) or *dl*A1209 DNAs. Other cultures were transfected with wild-type SV40 DNA. Progeny DNA was harvested 1, 2, 3, and 5 days after transfection, digested with *Mbo*I and *Eco*RI, and analyzed by electrophoresis in a 1.5% agarose gel. After electrophoresis, a Southern blot was prepared and autoradiographed. Lane M, Plasmid pCC2Pa digested with *Hin*fI. Equal amounts of these markers were added on each side of the gel to monitor the evenness of transfer of DNA to the nylon membrane and of hybridization with the radiolabeled probe. Transfer was even; only the left marker is shown. Lanes 2 to 5, *in*A2827(aa520) and p6-1 cotransfection; lanes 6 to 9, *dl*A1209 and p6-1 cotransfection; lanes 10 to 13, wild-type SV40 transfection.

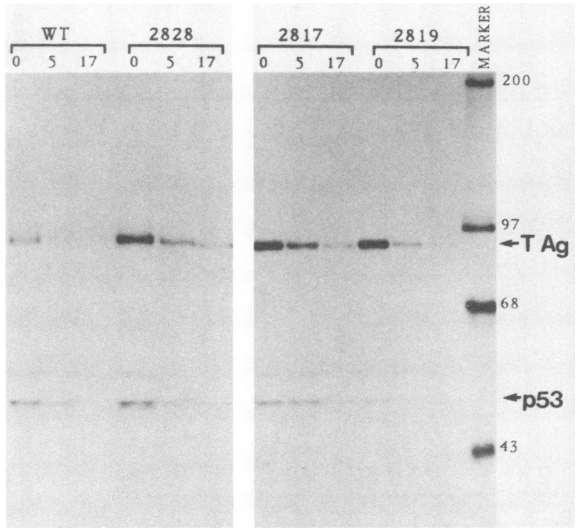

FIG. 4. Analysis of the stabilities of mutant T antigens (T Ag). BALB/c 3T3 cells (2×10^5) expressing the mutant T antigens were seeded onto 60-mm dishes the day before radiolabeling. Cells were pulse-labelled for 2 h with [^{35}S]methionine, and then either lysed immediately or chased in complete medium for 5 or 17 h (as indicated at the top of the figure) before lysis. Portions of cell lysates from identical numbers of cells were immunoprecipitated with a mixture of monoclonal antibodies PAb901 and PAb902, analyzed by electrophoresis on a sodium dodecyl sulfate–7.5% polyacrylamide gel, and fluorographed. WT, Wild type.

interfere with wild-type T antigen activities was also examined in cells cotransfected with *in*A2827(aa520) and a plasmid expressing wild-type T antigen. We used plasmid p6-1, an origin-defective plasmid expressing wild-type T antigen. The absence of a functional origin of replication prevented replication from occurring in cells which received only p6-1 (data not shown). Viral DNA replication could not occur in cells which received only mutant DNA, since all mutants examined in this experiment were replication defective. Only cotransfected cells contained both a functional origin of replication (provided on the plasmid expressing mutant T antigen) and a replication-competent T antigen (provided on plasmid p6-1).

Much less viral DNA replication occurred in CV-1 cells (Fig. 3) cotransfected by *in*A2827(aa520) and p6-1 (lanes 2 to 5) than in cells transfected by wild-type SV40 (lanes 10 to 13) or cotransfected by *dl*A1209 and p6-1 (lanes 6 to 9). We estimate that *in*A2827(aa520) replicated to only 5 to 10% of the level of the control plasmid, *dl*A1209. We observed a similar interference by *in*A2827(aa520) in cotransfected HeLa and 293 cells (data not shown).

Stabilities of mutant T antigens. The inability of a mutant T antigen to carry out some functions of T could be caused either by absence of the protein, due to instability, or to a functional defect in the mutant polypeptide. Analogously, interference with wild-type T may be a common effect of a stable mutant T antigen; the lack of interference by some of the insertion mutant T antigens could reflect their instability.

To examine this possibility, cell lines expressing mutant T antigens were prepared by cotransfecting BALB/c 3T3 cells with pSV2neo and either wild-type or mutant SV40 DNAs, followed by selection for resistance to G418. The stabilities of the mutant T antigens were measured by pulse-labeling cells for 2 h with [^{35}S]methionine, followed by incubation in complete medium for 0, 5, or 17 h. The T antigens were immunoprecipitated with anti-T antigen monoclonal antibodies PAb901 and PAb902, which recognize epitopes near the C terminus and N terminus of large T antigen, respectively (J. Tevethia and S. Tevethia, personal communication). Some examples of the fluorograms obtained are shown in Fig. 4. The fraction of labeled T antigen remaining after 5- and 17-h chase periods was determined by densitometric scanning of fluorograms; these data are presented in Table 2. All of the mutant T antigens except *in*A2807(aa303) were as stable as wild-type T. Mutant *in*A2807(aa303) produces a T antigen containing additional amino acids at amino acid 303. This T antigen was very unstable since labeled T antigen was not be detected after a 5-h chase. For the wild type and most of the other mutants, 30 to 50% of the labeled T antigen remained after the 5-h chase. The fraction of T antigen remaining after the 17-h chase ranged from 50% [*in*A2819 (aa346)] to 170% [*in*A2827(aa520)] of the amount of wild-type T antigen remaining after 17 h. These findings indicate that the behavior of most mutant T antigens in genetic complementation tests and the replication patterns of most mutants in Cos-1 cells reflect the properties of the mutant T antigens, rather than their instability.

We were unable to obtain cell lines which expressed *in*A2803(aa35) T antigen. The transfection protocol used a 10:1 molar ratio of insertion mutant DNA to pSV2neo. Under those conditions, most G418^r clones isolated were expected to contain the insertion mutant genome. Fewer G418^r clones were obtained with *in*A2803(aa35); of the 16 clones examined, all were negative for expression of

TABLE 2. Stabilities of large T antigens encoded by SV40 insertion mutants[a]

Mutant no.	Fraction of T antigen remaining after chase	
	5 h	17 h
2801	0.47	0.15
2807	<0.03	<0.03
2809	0.45	0.14
2811	0.44	<0.02
2815	0.48	0.13
2817	0.41	0.10
2819	0.24	0.06
2821	0.32	0.08
2823	0.15	0.11
2827	0.56	0.22
2828	0.35	0.09
WT-84 KD[b]	0.44	0.11
WT-super T[b]	0.47	0.10

[a] The data are from densitometric scannings of fluorograms like the one shown in Fig. 4. The numbers were normalized to the amount of T antigen present after the 2-h pulse-labeling.

[b] There were two T antigen bands immunoprecipitated from BALB/c 3T3 cells transformed by wild-type (WT) SV40 (Fig. 4). One migrates at the position expected for wildtype SV40 large T antigen. A larger species, super T, has been described previously (8a) and is often found in SV40-transformed cells.

*in*A2803 T antigen. This suggests that expression of *in*A2803(aa35) T antigen is deleterious or lethal.

DISCUSSION

In this paper, we have described the preparation and initial characterization of a set of mutants which contain a 12-bp oligonucleotide linker inserted into restriction endonuclease cleavage sites located at positions within the SV40 early region. The ability of these mutants to produce plaques was tested in CV-1p cells at three different temperatures and in BSC-1 cells at 37°C. The only mutant affecting large T antigen which was able to produce plaques under any of the conditions tested was *in*A2801(aa5), which has a linker inserted at amino acid codon 5 of both small and large T antigens. Since small t is not required for the SV40 lytic cycle in monkey kidney cell lines, our studies do not indicate whether this alteration affects any of the properties of small t. The finding that alterations can be made near the amino terminus of large T without affecting viability is interesting. No other SV40 mutants with lesions in this region have been described. Mutants with insertions or deletions located slightly further from the N terminus have been prepared, are nonviable, and belong to the A complementation group. These include *in*A2803(aa35), described in these studies, and *dl*A1135, lacking amino acids 17 to 27 (11, 39). Mutant *dl*A1135(aa35) is defective for binding to the SV40 origin of DNA replication and for ATPase activity. The adenovirus-SV40 hybrid T antigen, D2, is competent for SV40 DNA replication, even though it lacks the amino-terminal 114 amino acids of large T and has, in their place, 104 amino acids encoded by adenovirus DNA sequences (4, 63, 64). These data are consistent with the amino-terminal portion of large T playing a structural role in the overall conformation of the protein or with its being needed for other activities of T antigen or both. The changes in the protein caused by the insertion at codon 5 did not alter the ability of the protein to induce DNA replication. In preliminary experiments, we have found that the T antigen encoded by *in*A2801(aa5) was less active than wild-type T antigen in transactivation of the SV40 late promoter and in transformation of mouse 10T1/2 cells and rat REF52 cells (Zhu and Cole, manuscript in preparation).

Six of the mutants we described, with the linker inserted at codons 35, 219, 346, 460, 464, and 520, had reduced abilities to complement late-gene mutant *dl*BC865, *hr/hf* mutant *dl*A2475, or both. These defects in genetic complementation could reflect additional mutations in the mutant genomes, affecting the SV40 origin of replication, VP1 (the BC gene product), or the hr/hf region (the C terminus of T antigen). However, the inability of some insertion mutants to complement either a *BC* mutant or an *hr/hf* mutant would require multiple additional lesions, making this possibility unlikely. The T antigens encoded by *dl*BC865 and *dl*A2475 are both competent for viral DNA replication; the insertion mutants provide capsid protein and hr/hf activity. Our results suggest that the complementation patterns reflect the activities of the mutant large T antigens. We believe that these T antigens interfere directly with wild-type large T functions required during the lytic cycle.

To determine whether the mutant T antigens could inhibit viral DNA replication mediated by wild-type T, we analyzed viral DNA replication after transfection of mutant genomes into Cos-1 cells. All of the mutants which showed delayed complementation showed reduced levels of replication. This reduction could be caused by defective origins of replication. We have replaced the origin regions of two of these insertion mutants without affecting their ability to interfere. This indicates that *trans*-dominant interference is a property of the mutant T antigens.

The degree of interference with viral DNA replication in Cos-1 cells varied from mutant to mutant. *in*A2803(aa35) replicated slightly but reproducibly to a level about 25% below that of the control DNA, *dl*A1209, which produced no T antigen. DNA replication of mutant *in*A2817(aa219) was inhibited by more than 50% and *in*A2827(aa520) and *in*A2828(aa520) by more than 90% (Fig. 2, Table 1). Similar levels of interference with viral DNA replication were seen in CV-1, HeLa, or 293 cells cotransfected with *in*A2827 (aa520) and an origin-defective plasmid encoding wild-type T antigen (Fig. 3).

It is interesting that *in*A2803(aa35) replicated better than *in*A2817(aa219) or *in*A2823(aa464), even though *in*A2817 (aa219) and *in*A2823(aa464) complemented *dl*BC865 more efficiently than *in*A2803(aa35). This raises the possibility that there are multiple mechanisms by which mutant T antigens can interfere with wild-type T functions. We are currently conducting experiments to determine whether activities of T other than viral DNA replication are susceptible to *trans*-dominant interference by mutant T antigens.

We also noted that several of the mutant T antigens did not interfere with the replication activity of wild-type T antigen. The possibility that this reflects instability of these mutant T antigens was examined (Fig. 4, Table 2). Only one of the T antigens encoded by noninterfering mutants [*in*A2807 (aa303)] was unstable. Loeber et al. (30) described several point mutations affecting the region from amino acid 302 to 320 and suggested from their results that this region may be involved in the overall structural framework of the protein.

Mutant T antigens that are stable and do not interfere might not oligomerize, either with themselves or with wild-type T. Alternatively, these T antigens might be unable to form complexes with host DNA replication proteins. Interference might be possible only if T antigen contained specific

posttranslational modifications, and some mutant T antigens might show a pattern of modifications different from those of wild-type T.

Other T-antigen mutants which show *trans*-dominant interference have been described. Mutants 5061 and 5062 (16) produce T antigens containing amino acid insertions or substitutions in the vicinity of amino acid 430, in the domain involved in nucleotide binding and ATPase-helicase activity. Some of the mutants examined in our experiments affect sites relatively close to the position of the mutations of 5061 and 5062; others affect sites quite distant from them. Mutants of polyomavirus showing *trans*-dominant interference with polyomavirus DNA replication have also been reported (37) and have lesions in the ATPase region of polyomavirus large T antigen.

The ability of some purified SV40 mutant T antigens to interfere with viral DNA replication in vitro has also been reported. Mutants C6-2, T22, C8A, C2, and C11, with amino acid changes at positions 153, 203, 224, 516, and 523 or 549, respectively, were able to inhibit viral DNA replication in a concentration-dependent manner. Addition of a threefold molar excess of the mutant T antigen resulted in a 75 to 90% reduction in the amount of radiolabeled substrate incorporated into SV40 DNA. In contrast to our studies and those of Farber et al. (16), the mutant T antigens described by Stillman et al. (53) did not interfere with viral DNA replication when monkey cells producing the mutant T antigens were infected with wild-type SV40 (20).

There are multiple ways in which a mutant T antigen could interfere with wild-type DNA replication. It could bind to and block origins of DNA replication, preventing wild-type T antigen from using them. Alternatively, the mutant T antigen could oligomerize with wild-type T, forming mixed oligomers inactive in viral DNA replication. We do not know whether all of the monomers in an oligomer must be active for the oligomer to participate in viral DNA replication. The monomeric form and possibly the dimeric form (5S-7S) of T antigen are less phosphorylated (6, 36, 43, 55) and more active in DNA binding than higher-order oligomers (6, 41). Phosphorylation of T antigen down-regulates the DNA replication mediated by T antigen (34, 46). Finally, T could interfere by binding to and sequestering some host component essential for viral DNA replication. T is known to interact with host DNA polymerase α (18, 47) and might interact with other cellular DNA replication proteins.

trans-Dominant interference in procaryotes, where the formation of oligomers is essential for regulation to occur, has been described. The ability of mutant polypeptides to interfere with wild-type activity through formation of mixed oligomers has been described in the *lac* repressor system (32) and in the case of *recA* (38, 44, 71, 72). Additional experiments will be required to determine whether there are multiple mechanisms for *trans*-dominant interference by SV40 T antigen and how these mutant T antigens interfere.

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