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Mechanisms of Interference with Simian Virus 40 (SV40) DNA Replication by *trans*-Dominant Mutants of SV40 Large T Antigen

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Mutations at multiple sites within the simian virus 40 (SV40) early region yield large T antigens which interfere *trans* dominantly with the replicative activities of wild-type T antigen. A series of experiments were conducted to study possible mechanisms of interference with SV40 DNA replication caused by these mutant T antigens. First, the levels of wild-type T antigen expression in cells cotransfected with wild-type and mutant SV40 DNAs were examined; approximately equal levels of wild-type T antigen were seen, regardless of whether the cotransfected mutant was *trans* dominant or not. Second, double mutants that contained the mutation of *in*A2827, a strong *trans*-dominant mutation with a 12-bp linker inserted at the position encoding amino acid 520, and various mutations in other parts of the large-T-antigen coding region were constructed. The *trans*-dominant interference of *in*A2827 was not affected by second mutations within the p105^{Rb} binding site or the amino or carboxy terminus of large T antigen. Mutation of the nuclear localization signal partially reduced the *trans* dominance of *in*A2827. The large T antigen of mutant *in*A2815 contains an insertion of 4 amino acids at position 168 of large T; this T antigen fails to bind SV40 DNA but is not *trans* dominant for DNA replication. The double mutant containing the mutations of both *in*A2815 and *in*A2827 was not *trans* dominant. The large T antigen of *dl*A2433 lacks amino acids 587 to 589, was unstable, and failed to bind p53. Combining the *dl*A2433 mutation with the *in*A2827 mutation also reversed the *trans* dominance completely, but the effect of the *dl*A2433 mutation on *trans* dominance can be explained by the instability of this double mutant protein. In addition, we examined several mutants with conservative point mutations in the DNA binding domain and found that most of them were not *trans* dominant. The implications of the results of these experiments on possible mechanisms of *trans* dominance are discussed.

Simian virus 40 (SV40) large T antigen is a multifunctional nuclear phosphoprotein. Within infected cells, it is found in both monomeric and oligomeric forms and with a diverse range of posttranslational modifications, including phosphorylation (52), glycosylation (30), and ADP ribosylation (25). It plays essential roles in lytic infection and in immortalization and malignant transformation of nonpermissive cells by SV40. Large T antigen binds DNA both specifically and nonspecifically (8, 68) and has helicase and ATPase activities (29, 62). ATP binding and hydrolysis change the conformation and oligomerization states of large T antigen (4, 6, 41).

Since not all T-antigen molecules contain the same modifications, there are multiple species of T antigen within infected cells, and different species of T antigen may have different biological and biochemical activities (5). For example, phosphorylation of certain sites on T antigen regulates its activity in DNA replication (42, 53), but current understanding of the role of these various modifications is incomplete. Large T antigen associates with a number of cellular proteins: p53 (34), the retinoblastoma susceptibility gene product, p105^{Rb} (15), a cellular protein related to p105^{Rb} with a molecular weight of 107,000 to 120,000 (17, 18), DNA polymerase α (16, 21, 57), transcription factor AP-2 (44), cellular heat shock protein hsp73 (51), and probably others.

T antigen causes immortalization of primary rodent cell cultures (66, 67) and malignant transformation of both primary cultures and established cell lines (33, 65). Interaction of T antigen with host proteins, including p53 and p105^{Rb}, the products of two tumor suppressor genes, probably plays essential roles in immortalization and transformation. T antigen also transactivates a number of viral and cellular genes (7, 32, 58). In addition, large T antigen can induce host cell DNA synthesis in quiescent cells (10, 59).

During the lytic infection by SV40, large T antigen initiates viral DNA replication by binding to a palindromic sequence within the origin of DNA replication (36, 63, 64) and unwinding the double-stranded DNA bidirectionally from the origin (for a review, see reference 3). Large T antigen is the only viral protein required for viral DNA replication (64). DNA binding and ATPase and helicase activities of large T antigen are essential for viral DNA replication (1, 14) and must be contained within a single T-antigen monomer. The interactions between T antigen and cellular proteins involved in viral DNA replication are crucial for initiation and elongation of SV40 DNA replication (3, 47).

A number of mutant large T antigens were found previously to interfere with SV40 DNA replication catalyzed by wild-type T antigen in vivo (19, 75) and in vitro (63). These have been called *trans*-dominant mutants. Mutations causing this *trans*-dominant effect on DNA replication were located at various sites throughout much of the T-antigen coding region and included mutations within the DNA binding and ATP binding and ATPase domains. The degree of inhibition of DNA replication varied among the mutants and ranged from 50% to greater than 90%.

In this article, we present experiments designed to investigate further the dominant negative behavior of mutant large T antigens. The drastically reduced replication of some mutant DNAs in Cos-1 cells could be explained if the ratio

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between wild-type and mutant T antigens in cotransfected cells were substantially lower with *trans*-dominant mutants than with other mutants which were not *trans* dominant. We therefore compared these ratios in CV-1 cells cotransfected by a plasmid encoding wild-type T antigen and different *trans*-dominant or non-*trans*-dominant mutants. No differences were seen. We also prepared double mutants containing the relevant mutation of a *trans*-dominant mutant and other insertion, deletion, or missense mutations, to determine whether specific properties of T antigen were required for *trans*-dominant activity. In some cases, the double mutant retained the *trans*-dominant behavior of its *trans*-dominant parent, while in other cases, this behavior was lost. Taken together, the data suggest that there are multiple mechanisms of *trans* dominance and that no single property of large T antigen is required for *trans* dominance.

Additional mutants, including several with point mutations in the DNA binding domain of T antigen (40, 55, 56), were also screened for *trans* dominance. Some of these mutants displayed *trans* dominance, while others did not. Possible mechanisms of *trans* dominance are discussed in terms of these results.

MATERIALS AND METHODS

Plasmid DNAs, cells, and tissue culture. CV-1 cells are a continuous line of African green monkey kidney cells (31). Cos-1 cells are an SV40-transformed cell line derived from CV-1 cells by transformation with an origin-defective mutant of SV40 (22). Cos-1 cells express SV40 large and small T antigens constitutively. Murine cell lines expressing various mutant or wild-type large T antigens were made by cotransfection of BALB/c 3T3 clone A31 cells (69) with mutant or wild-type SV40 DNAs and pSV2neo, a plasmid containing the bacterial neomycin phosphotransferase gene under control of the SV40 early promoter and enhancer (61). After selection for growth in the presence of 500 μg of G418 per ml, single colonies were subcloned and checked immunohistochemically for expression of T antigen. In some cases, surviving cells (100 to 300 colonies per plate) were expanded into pools and used directly; these pools contained more than 95% T-antigen-positive cells (data not shown). All cells were cultured in Dulbecco's modified Eagle medium containing 10% newborn calf serum (monkey cells) or 10% fetal calf serum (FCS) (mouse cells).

pCC2Pa contains the complete SV40 genome cloned into the *Eco*RI site of pUC18, in the orientation which places the *Bam*HI site of pUC18 close to the *Bam*HI site of SV40. p6-1 is a nonreplicative version of pCC2Pa which contains a 6-bp deletion at the *Bgl*I site within the origin of replication (24). *dl*A1209 is a deletion mutant lacking 329 bp (nucleotides 4862 to 5191), including the early mRNA start site, the entire first exon of the large-T-antigen coding region, and the splice donor site for the large-T mRNA intron (12). pSV2aPAP contains a human placental alkaline phosphatase gene under the control of SV40 early promoter (28). Mutants *in*A2801, *in*A2803, *in*A2815, *in*A2817, *in*A2807, *in*A2809, *in*A2811, and *in*A2827 were described previously (75) and contain 12-nt linkers inserted at the positions encoding amino acids (aa) 5, 35, 168, 219, 303, 409, 424, and 520, respectively. Mutant *in*A2831 contains an in-frame deletion extending from codon 5 to 35 and a linker d(TCGCGATCGCGA) at the deletion site. Mutants *dl*2441, *dl*A2411, *dl*A2432, *dl*A2433, and *dl*A2459 contain small deletions at *Dde*I sites and produce T antigens which lack amino acids 105 and 106, 143 to 146, 507 to 510, 587 to 589, and 674 to 708, respectively (70). Fusion of codons as a result of the deletions resulted in the insertion of an additional amino acid at the site of the deletion of mutant *dl*A2432; the T antigen of *dl*A2459 contains 3 additional residues at its C terminus because of a reading frameshift at the site of the deletion. All mutant genomes were maintained as recombinant clones in pUC18 with the same orientation as that of pCC2Pa. The nomenclature used for deletion and insertion mutants is to list the amino acids deleted or the site of the insertion in parentheses following the mutant number. For point mutants, the mutant number is followed by a designation such as S147T which indicates that residue 147 of large T is normally serine but was changed to threonine in that particular mutant.

Plasmid pBS-SV40 and mutants S147T, A149G, S185T, H187R, and R204K (55, 56) were obtained from Dan Simmons (University of Delaware, Wilmington). The first letter is the single amino acid code for the amino acid present at the numbered position in wild-type T antigen; the second letter is the amino acid which replaces the wild-type residue in the mutant protein. The parental plasmid for these point mutants is pBS-SV40, which contains the entire SV40 genome inserted into the *Bam*HI site of Bluescript (Stratagene). Mutant *dl*A2840 was prepared from pBS-SV40 by deleting the DNA between the two *Nde*I sites in the SV40 early region. This deletion removed 1,018 bp, including sequences extending from the middle of the intron for large T antigen to a position in the middle of the large-T-antigen coding region. Since the deletion removes the 3′ splice site for large-T mRNA, the mutant was unable to produce any large T antigen. Point mutants C-2(K516R) and C-6-2 (N153T) were described previously (22, 23, 40) and were obtained from Carol Prives (Columbia University, New York, N.Y.).

Standard techniques for bacterial growth and transfection (38) and DNA preparation (39) were used.

Construction of double mutants. Double mutants were constructed by combining appropriate restriction endonuclease fragments of various mutant DNAs, all cloned into the *Eco*RI site of pUC18.

Double mutants were constructed from mutants *in*A2827 (aa520) and *in*2801(aa5) or *dl*A2831(aa5-35) by utilizing the *Bst*XI site in the large-T-antigen intron and the *Xba*I site in the polylinker of pUC18. The *Xba*I-*Bst*XI fragment contains sequences from pUC18, part of the late region of SV40, the origin region, the first exon, and part of the intron. The *Bst*XI-*Xba*I fragment of *in*A2827(aa520) contained the remainder of the intron, the entire second exon of large T antigen (including the *in*A2827 mutation), the remainder of the late region of SV40, and the remainder of pUC18.

Double mutants combining the mutation of *in*A2827 (aa520) with *dl*2441(aa105-106), SVcT(K128T), or *dl*A2411 (aa143-146) were made by three-fragment ligations using *Bam*HI, *Bst*XI, and *Nde*I sites. The 1,275-bp *Nde*I-*Bam*HI fragment of *in*A2827 extends from the middle of the T-antigen coding region through the site of the insertion of *in*A2827 to the *Bam*HI site near the end of the VP1 gene. The 951-bp *Bst*XI-*Nde*I fragment of *dl*2441, SVcT, *in*A2815, and *dl*A2411 extends from the *Bst*XI site in the middle of the large-T intron through these mutations located in the proximal part of the second exon to the *Nde*I site in the middle of the large-T-antigen coding region. The 5.7-kb *Bam*HI-*Bst*XI fragment contains the remaining portions of SV40 and the complete pUC18 genome. Double mutants containing the mutations of *in*A2827(aa520) and *in*A2815(aa168) were made by a similar three-fragment ligation strategy using *Bst*HI, *Nde*I, and *Apa*I.

Double mutants of *dl*A2459(aa674-708) with *dl*A2411 (aa143-146), *in*A2815(aa168), *in*A2809(aa409), *in*A2811(aa424), *dl*A2432(aa509), or *in*A2827(aa520) as well as double mutant *in*A2827(aa520)/*dl*A2433(aa587-589) were prepared by three-fragment ligation using *Apa*I, *Bst*XI, and *Pst*I. *Apa*I and *Bst*XI digest each DNA once; there are three *Pst*I sites in all plasmids used, one in the polylinker region of pUC18, and two within SV40 sequences. The 946-bp *Pst*I-*Apa*I fragment of *dl*A2459 or *dl*A2433 extends from the *Pst*I site at nucleotide 3204 of SV40, located upstream of the *dl*A2433 and *dl*A2459 deletions, through the deletions, to the *Apa*I site located within the VP1 gene. The 1,555-bp *Bst*XI-*Pst*I fragment extends from the *Bst*XI site within the large-T intron, through the mutations of *dl*A2411, *in*A2815, *in*A2809, *in*A2811, *dl*A2432, and *in*A2827, to the *Pst*I site at nucleotide 3204. The 5.43-kb *Apa*I-*Bst*XI fragment contains the remaining portions of SV40 and the complete pUC18 genome.

To isolate fragments, DNAs were digested with restriction enzymes and subjected to electrophoresis on gels of low-melting-point agarose. The gel slices containing the desired fragments were excised, melted, and used for ligation either directly or after purification with GeneClean (Bio 101, La Jolla, Calif.).

Cell transfection and double staining. CV-1 cells (4×10^5) in 60-mm-diameter plates were cotransfected with equal amounts of plasmids of p6-1 and pSV2aPAP (1.0, 2.5, or 7.5 μg of each plasmid) and carrier salmon sperm DNA (to give a total DNA concentration of 15 μg per plate) for 16 h using the calcium phosphate method (73). At 48 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) (1.9 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 150 mM NaCl; pH 7.4) and fixed with 95% methanol–5% PBS for 10 min at 4°C. The cells were stained for alkaline phosphatase with 240 μg of fast violet B salt (Sigma) per ml in 0.01% naphthol AS-MX phosphate alkaline solution (Sigma) for 15 min at room temperature (28). After the cells were gently washed with PBS, they were blocked with PBS–4% bovine serum albumin–10% FCS for 1 h at room temperature and incubated with monoclonal antibody PAb419 supernatant (1:5 dilution) for 25 min at room temperature. The cells were washed three times with PBS–1% FCS and incubated with a 1/200 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.) for 25 min. The cells were again washed three times with PBS–1% FCS, incubated in enzyme reaction mixture (50 mM Tris HCl [pH 7.6], 0.6 mg of 3,3′-diaminobenzidine tetrahydrochloride [Sigma] per ml, 0.03% H_2O_2) for 10 min (27), and examined under the microscope for cytoplasmic alkaline phosphatase staining (pink) and nuclear peroxidase staining (brown).

Pulse-labelling and immunoprecipitation. CV-1 cells (4×10^5 cells per 60-mm-diameter plate) were cotransfected with p6-1 and an SV40 double mutant DNA (2 μg of each) and 11 μg of salmon sperm carrier DNA by the calcium phosphate method. After 1, 2, or 3 days, the cells were washed twice with Tris-buffered saline (25 mM Tris-chloride [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.05 mM $MgCl_2$, 0.7 mM $CaCl_2$) and incubated for 1 h at 37°C in 2 ml of Dulbecco's modified Eagle medium lacking methionine and containing 2% dialyzed FCS. The medium was removed and the cells were labelled for 1 h with 100 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.) in 0.7 ml of Dulbecco's modified Eagle medium lacking methionine and containing 2% dialyzed FCS. Nuclear extracts were prepared and immunoprecipitated with PAb419 monoclonal antibody as described previously (75). Antibody PAb419 was present in excess of large T antigen (data not shown).

Analysis of viral DNA replication. Cos-1 cells were transfected with wild-type or mutant DNAs by the DEAE-dextran method (72) and harvested by the Hirt method as described previously (75). The low-molecular-weight DNAs were digested with *Mbo*I and fractionated on a 1.5% agarose gel. The input plasmids, isolated from bacterial cultures, are methylated on 5′-G^{m}ATC-3′ sequences and therefore were resistant to *Mbo*I digestion. Unmethylated progeny viral DNAs are sensitive to digestion by *Mbo*I and could therefore be separated from the input DNAs by electrophoresis. The DNAs were then transferred to Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, N.H.), probed with ^{32}P-labelled pCC2Pa DNA (labelled by nick translation), and visualized by autoradiography. To monitor the efficiency and uniformity of transfer of DNA to nylon, *Hin*fI-digested pCC2Pa DNA was included in flanking lanes.

RESULTS

In our original studies of *trans*-dominant mutants which affected viral DNA replication, we discussed possible mechanisms that could permit mutant T antigens to interfere with the replicative activities of wild-type T antigen. *trans*-dominant mutant T antigens could bind to the SV40 origin of replication and block or hinder binding by wild-type T antigen. Alternatively, *trans*-dominant mutant T antigens, but not other T antigens, might be able to sequester some limiting host cellular factors required for viral DNA replication. Finally, *trans*-dominant mutant T antigens, but not other T antigens, might be able to sequester wild-type T antigen in inactive mixed heterooligomers.

In these models, the actual amounts of mutant and wild-type T antigen and their ratios could be important factors in determining how much viral DNA replication occurred. Several properties of large T antigen could result in different ratios of mutant T antigen to wild-type T antigen within cotransfected cells. First, large T antigen, through binding to site I within the SV40 origin region, represses early transcription (50). T antigen also activates transcription from many viral and cellular promoters. The ability of different mutant T antigens to autoregulate viral early transcription, to transactivate early transcription, or some combination of these could be better or worse than that of wild-type T antigen. Most of the mutant T antigens that we have examined have a stability similar to that of wild-type T in stably transformed rodent cells (75). However, differences in stability would clearly affect the amounts and ratios of mutant T antigen to wild-type T antigen in transiently transfected Cos-1 and CV-1 cells. We decided to examine these ratios under conditions similar to those in which interference with replication occurred.

Coexpression of mutant and wild-type T antigens in CV-1 cells. In our previous report, SV40 DNAs encoding *trans*-dominant mutant T antigens replicated poorly when transfected into Cos-1 cells because of interference by mutant T antigens. By cotransfecting CV-1 cells with an origin-defective plasmid encoding wild-type T antigen and a replication-competent plasmid encoding mutant T antigen, we saw that the level of interference with viral DNA replication by *trans*-dominant mutants was equivalent to that seen previously with Cos-1 cells (75). Cos-1 cells were not suitable for analyzing the ratios of mutant T antigen to wild-type T antigen, since only a modest fraction of cells can be transfected with viral DNA. The expression of wild-type T

TABLE 1. Efficiency of cotransfection in CV1 cells

Amt (μg) of DNA transfected[a]			No. (%) of cells with staining pattern[b]				
SV40	pSV2aPAP	Salmon sperm	T^+ pap^-	T^+ pap^+	T^- pap^+	T^+ pap?	T? pap^-
7.5	7.5	0	28 (12)	192 (79)	12 (5)	8 (3)	2 (1)
2.5	2.5	10	61 (22)	190 (69)	4 (1)	20 (7)	0 (0)
1.0	1.0	13	37 (14)	211 (80)	2 (1)	13 (5)	0 (0)

[a] CV-1 cells (4×10^5 cells per 60-mm-diameter plate) were transfected with the indicated DNA by the calcium phosphate method.

[b] The cells were stained for alkaline phosphatase, incubated with PAb419, and stained for horseradish peroxidase. Seven randomly chosen fields from each stained plate were scored under the microscope. T^+ pap^+ cells were positive for both brown nuclear horseradish peroxidase (HRP) staining and pink cytoplasmic placental alkaline phosphatase (PAP) staining. T^+ pap^- and T^- pap^+ cells were positive for either PAP or HRP staining, respectively. Some cells (T^+ pap? and T? pap^+) lost either cytoplasm or nuclei during staining. In all the plates examined, about 50% of the cells showed staining for PAP, HRP, or both.

antigen alone in all nontransfected Cos-1 cells created too high a background to permit us to determine accurately whether *trans*-dominant mutants and non-*trans*-dominant mutants affected the amounts of mutant and wild-type T antigen and their ratios differently.

To examine the levels of wild-type and mutant T antigens in cells expressing both proteins, we used CV-1 cells. We wished to know what fraction of cells that received one type of plasmid would also take up and express a second plasmid. CV-1 cells were cotransfected with p6-1 (defective origin, encoding wild-type T antigen) and pSV2aPAP, encoding human placental alkaline phosphatase. Two days after transfection, the cells were stained for alkaline phosphatase and cells expressing T antigen were identified immunohistochemically. The results (Table 1) indicated that 75 to 90% of the cells positive for T antigen also stained positive for alkaline phosphatase and that more than 90% of the cells positive for alkaline phosphatase also expressed T antigen. Cotransfected CV-1 cells were therefore used to study the effect of mutant large T antigens on the expression of wild-type T antigen in the same cells.

Several mutants were chosen for this study on the basis of the location of their mutations within the gene for T antigen and the stabilities of the mutant T antigens. *dl*A2411(aa143-146) and *dl*A2432(aa509) have small in-frame deletions. Mutants *in*A2815(aa168), *in*A2809(aa409), *in*A2811(aa424), and *in*A2827(aa520) are linker insertion mutants with a 12-bp oligonucleotide inserted at the positions indicated. Mutants *dl*A2411(aa143-146) and *in*A2815(aa168) are defective in both site I and site II binding, while the other mutants showed normal binding to viral DNA (76). *dl*A2411(aa143-146), *dl*A2432(aa509), and *in*A2827(aa520) are *trans*-dominant mutants, whereas *in*A2815(aa168), *in*A2809(aa409), and *in*A2811(aa424) are not (75). In order to distinguish these mutant T antigens from wild-type T antigen, double mutants were made by combining each of these mutants with the mutation of *dl*A2459(aa674-708). *dl*A2459 encodes a truncated T antigen lacking residues 674 to 708, and its smaller T antigen can be separated electrophoretically from wild-type T antigen. The C terminus of T antigen provides host range and adenovirus helper function, and *dl*A2459 mutant T antigen is fully functional in viral DNA replication (11, 13). These double mutant T antigens interfered with viral DNA replication in a manner similar to that of the full-length mutant T antigens (data not shown).

CV-1 cells were cotransfected with origin-defective p6-1 DNA and origin-containing DNA of various double mutant DNAs. Since none of the mutant T antigens are replication competent, there should be no viral DNA replication in cells which received only p6-1 (defective origin, encoding wild-type T antigen) or a double mutant plasmid (functional origin, replication-defective mutant T antigen) alone. This cotransfection procedure ensured that viral DNA replication would occur only in cells transfected by both plasmids. Mutant *dl*A1209 was used as a control because it contains a near-full-length SV40 DNA with a functional origin and does not express T antigen (12). Cells were pulse-labelled for 1 h with [^{35}S]methionine on days 1, 2, and 3 following transfection. After the cells were labelled, extracts were prepared and immunoprecipitated with anti-T antigen monoclonal antibody PAb419, and the proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels (Fig. 1). In all cases, the upper band represents full-length wild-type T antigen and the lower bands represent mutant T antigens lacking the normal carboxy terminus. In the absence of any mutant T antigen (p6-1 plus *dl*A1209), the synthesis of wild-type T antigen was the greatest on day 1 and was reduced to a very low level by day 3. Previous experiments (75) suggested that little input plasmid remains within cells by day 3.

In cells cotransfected by p6-1 and double mutants, the pattern of wild-type expression was similar to that seen with p6-1 and *dl*A1209. The pattern of mutant T-antigen expression was similar or identical with all mutants examined. There was slightly less mutant T antigen than wild-type T antigen produced on the first day. By the second day, the amount of mutant T produced equalled or slightly exceeded the amount of wild-type T, reflecting reduced copy number of wild-type DNA and increased copy number of each mutant genome. By the third day, mutant T production exceeded wild-type T-antigen production in all cases. None of the mutant T antigens had any significant effects on the expression of wild-type T antigen, regardless of whether or not they were *trans* dominant. We conclude that the *trans*-dominant behavior cannot be explained by differential effects on the levels of wild-type T or on the ratio of mutant T antigen to wild-type T antigen.

Replication of double mutants in Cos-1 cells. One possible mechanism of *trans* dominance involves occupancy of the SV40 origin region by *trans*-dominant T antigens, preventing wild-type T from gaining access to and catalyzing the replication of the viral DNA. This possibility was investigated by determining whether a double mutant containing the *in*A2827(aa520) mutation and a mutation in the DNA binding domain would retain *trans* dominance. We also wondered whether *trans* dominance would be affected by combining the *in*A2827 *trans*-dominant mutation with mutations affecting other properties of T antigen.

We therefore prepared double mutants with the strongest *trans*-dominant mutant, *in*A2827, and mutants defective for other properties of T antigen. The other mutants used are shown in Fig. 2. The double mutant SV40 DNAs were

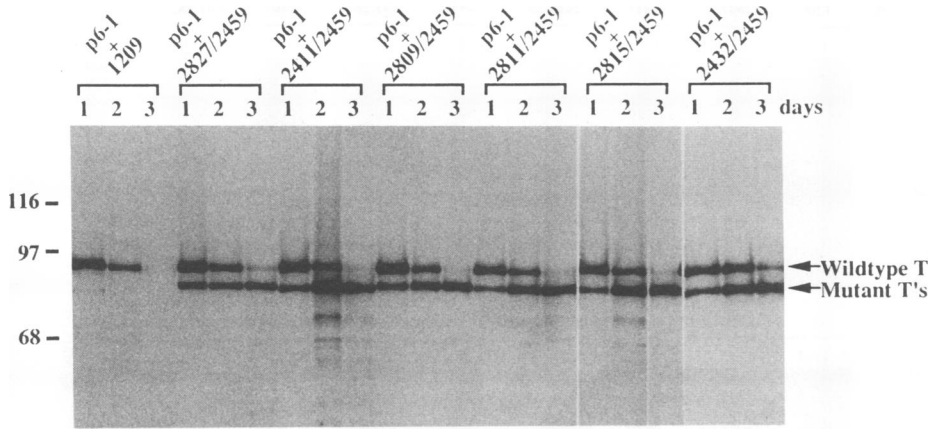

FIG. 1. Coexpression of mutant and wild-type T antigens in CV-1 cells. CV-1 cells were transfected with origin-defective plasmid encoding wild-type T antigen and origin-plus DNAs encoding mutant T antigen as described in Materials and Methods. The numbers of days after transfection are indicated over the lanes. The cells were pulse-labelled with [^{35}S]methionine for 1 h. Nuclear extracts were prepared, immunoprecipitated with monoclonal antibody PAb419, and analyzed by electrophoresis on sodium dodecyl sulfate–7.5% polyacrylamide gels. The positions of molecular weight markers are indicated at the left.

transfected into Cos-1 cells, and viral DNAs were isolated 0, 1, 3, and 5 days after transfection. These were digested with *Mbo*I restriction endonuclease to distinguish input DNA (resistant to *Mbo*I due to methylation in *Escherichia coli*) from progeny DNA (sensitive to *Mbo*I digestion), fractionated electrophoretically, and analyzed by Southern blotting (60), as shown in Fig. 3. The analyses of many of these mutants were repeated in additional experiments and identical results were obtained.

The 329-bp deletion of *dl*A1209 removes the early mRNA cap site, the first exon of large T antigen, and the large-T-antigen splice donor site. Several monoclonal antibodies to T antigen failed to detect cross-reacting material by either immunoprecipitation or immunofluorescence in cells transfected with *dl*A1209. Therefore, replication of this plasmid in Cos-1 cells should reflect only the activity of the endogenous T antigen, which is constitutively expressed in Cos-1 cells. Mutant viral DNA replication in *in*A2827(aa520)-transfected

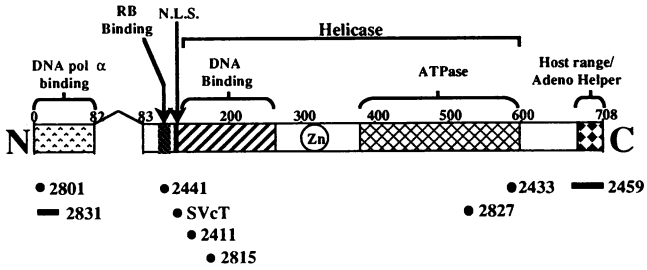

FIG. 2. Map of mutations used to construct double mutants of *in*A2827 and other T-antigen mutants. The two exons of the SV40 large-T-antigen coding region are shown as two rectangular boxes with amino acid numbers listed above. The DNA polymerase (pol) α binding domain, retinoblastoma protein (RB) binding site, nuclear localization signal (N.L.S.), DNA binding domain, ATP binding and ATPase domain, the sequences required for helicase activity, and the region encoding host range and adenovirus (Adeno) helper functions are indicated at the top of the figure. The circled Zn represents the zinc finger motif. N and C refer to the amino and carboxyl termini, respectively. Second mutations are indicated at the bottom with point, linker insertion, and small deletion mutations shown as dots and larger truncations shown as horizontal bars.

cells was about 10% of the level seen in cells transfected with *dl*A1209.

*in*A2815(aa168) encodes a mutant T antigen which does not bind to the SV40 origin. *in*A2815 DNA replicated as well as *dl*A1209 (Fig. 3B), indicating that *in*A2815 T antigen did not interfere with DNA replication. The double mutant *in*A2815/*in*A2827 T antigen also did not interfere with DNA replication, suggesting that DNA binding may be required for the strong *trans* dominance of *in*A2827(aa520), although other explanations are also possible (discussed below). Both *in*A2815 and *in*A2827 encode stable T antigens, and the double mutant T antigen had similar stability (data not shown); furthermore, this double mutant protein appeared to have a relatively normal conformation because it, like both parents, showed equivalent and strong reactivity with almost all members of a large panel of anti-T-antigen monoclonal antibodies tested (2).

The mutant T antigen encoded by *dl*A2411(aa143-146) does not bind to the SV40 origin. DNA replication in cells transfected with *dl*A2411 was considerably less than in cells transfected with *dl*A1209 but greater than in *in*A2827-transfected cells (Fig. 3A). *dl*A2411 therefore has a moderate *trans*-dominant effect on viral DNA replication. A double mutant combining *dl*A2411 and the strongly *trans*-dominant *in*A2827(aa520) was examined. Viral DNA replication in cells transfected with *dl*A2411/*in*A2827 was intermediate between that seen in cells transfected with each of the two parental plasmids. In other experiments (data not shown), replication in cells transfected with *dl*A2411/*in*A2827 more closely approximates the level of replication in *dl*A2411-transfected cells.

*dl*2441(aa105-106) contains a small in-frame deletion within the binding site for the retinoblastoma susceptibility protein, p105^{Rb}. This mutant T antigen is less able than wild-type T to form complexes with p105^{Rb} but was able to form a reduced level of T-p105^{Rb} complexes (77). This mutant is able to immortalize primary mouse embryo fibroblast cells but does not cause full transformation of those cells (67). Viral DNA replication in *dl*2441/*in*A2827-containing cells was similar to that in *in*A2827-transfected cells (Fig. 3A), suggesting that wild-type levels of p105^{Rb} binding were not important for the *trans* dominance of *in*A2827 T antigen.

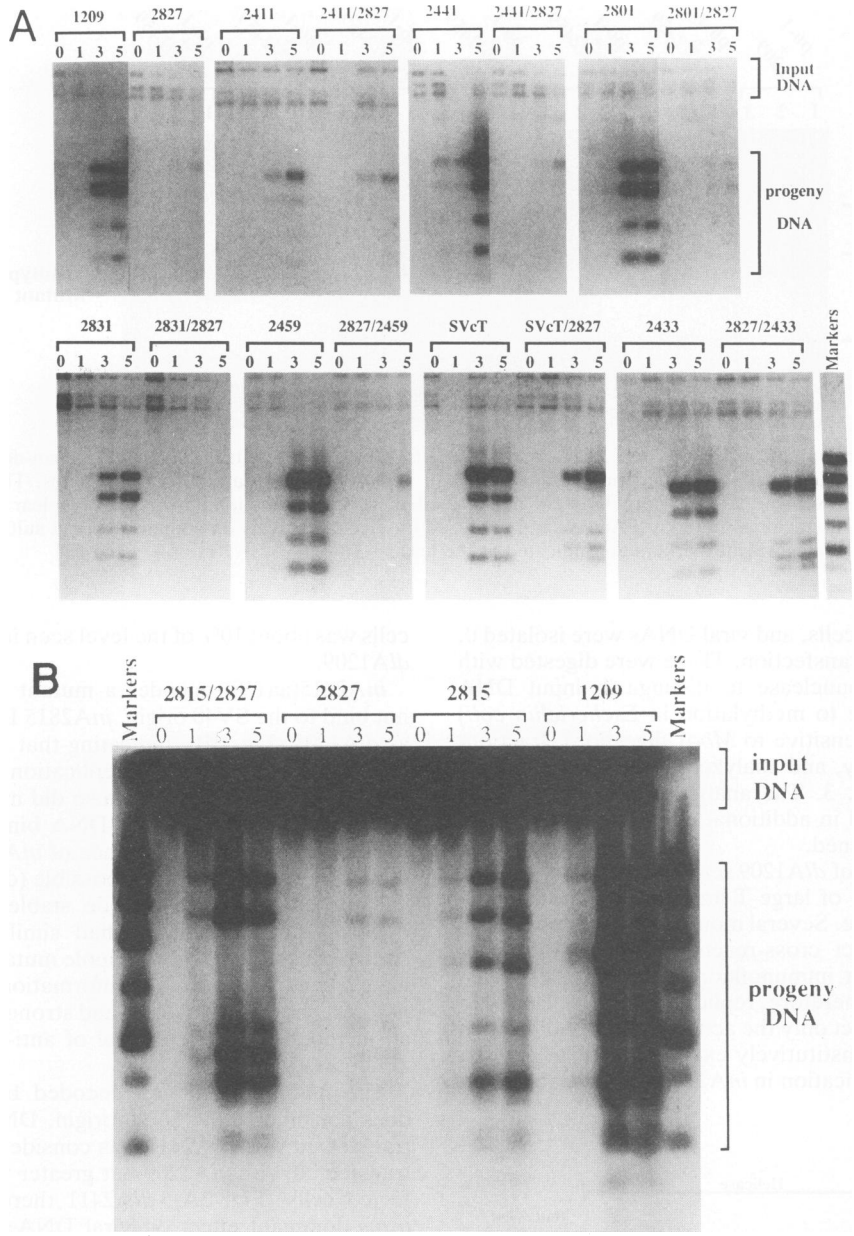

FIG. 3. Replication of double mutants in Cos-1 cells. Cos-1 cells were transfected with DNAs of the double mutants, and low-molecular-weight DNA was harvested 0, 1, 3, and 5 days after transfection. After digestion with *Mbo*I, the DNAs were fractionated by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and hybridized to a ^{32}P-labelled probe specific for SV40 sequences. To monitor the efficiency and uniformity of transfer of DNA to nylon, *Hin*fI-digested pCC2Pa DNA was included in flanking lanes. The data presented in panel A come from two experiments which contained different mutants and the same controls (*dl*A1209 for endogenous wild-type T activity and *in*A2827 for strong *trans* dominance). The relative amounts of replicated viral DNA in the controls were the same in each experiment, allowing direct comparison of the data from each experiment. The mutants in panel B were analyzed separately. The presence of extra *Mbo*I sites located at different positions in mutant genomes caused some mutants to have fragments of different sizes.

Introducing the mutations of *in*2801(aa5), *dl*A2831(aa5-35), or *dl*A2459(aa674-708) into *in*A2827(aa520) did not eliminate or significantly reduce the *trans*-dominant behavior of *in*A2827 T antigen. Therefore, these amino acids near the amino and carboxy termini of the *in*A2827 T antigen appeared to play no role in the *trans*-dominant interference.

SVcT(K128T) T antigen contains a point mutation (35) within the nuclear localization signal, and the mutant protein is not transported efficiently to the nucleus. There was a similar amount of viral DNA replication in SVcT-transfected cells and *dl*A1209-transfected cells. Viral DNA replication in cells transfected by the SVcT/*in*A2827 double mutant was slightly less than in cells transfected with the SVcT single mutant but considerably more than viral DNA replication in cells transfected with *in*A2827(aa520) (Fig. 3A). This double mutant T antigen resembled SVcT T antigen in being localized to the cytoplasm, as determined by immunofluorescence staining (data not shown). This suggests that nuclear

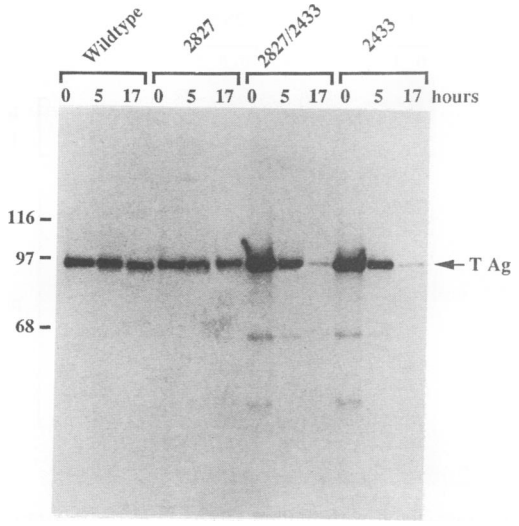

FIG. 4. Stabilities of mutant T antigens in CV-1 cells. CV-1 cells were transfected with plasmids encoding wild-type or mutant T antigens. Two days later, cells were pulse-labelled for 1 h with [^{35}S]methionine and lysed either immediately (0 h) or after culturing in complete medium for 5 or 17 h. Nuclear extracts were immunoprecipitated with PAb419 and analyzed by electrophoresis on a sodium dodecyl sulfate–7.5% polyacrylamide gel. The positions of molecular weight markers are shown at the left. T Ag, T antigen.

localization of a mutant T antigen may be necessary for maximum *trans* dominance. The fact that DNA replication caused by SVcT/*in*A2827 T antigen was less than that caused by SVcT T antigen probably reflects the presence within the nucleus of a small amount of this double mutant T antigen, leading to some interference with replication catalyzed by wild-type T antigen. Retention of some wild-type T antigen in the cytoplasm through oligomerization with the double mutant T antigen is also possible.

Another second mutation which completely reversed the *trans*-dominant behavior of *in*A2827(aa520) T antigen was *dl*A2433(aa587-589) (Fig. 3A). *dl*A2433(aa587-589) contains a small in-frame deletion and the mutant protein does not bind p53 (67, 71, 74). There are several possible explanations for this finding. Since *in*A2827(aa520) T antigen binds p53 while *dl*A2433(aa587-589) does not, p53 binding might be essential for a T antigen to show *trans*-dominant interference. A related possibility is that *trans* dominance reflects interactions between mutant T antigen and either wild-type T antigen or some host factor other than p53 but that the loss of residues 587 to 589 alters the conformation of the double mutant protein, affecting other protein-protein interactions. Instability of the double mutant T antigen could also prevent its interfering with viral DNA replication.

To examine the last possibility, the stabilities of mutant T antigens were examined by labelling cells for 1 h with [^{35}S]methionine followed by chase periods of 5 and 17 h. The data show (Fig. 4) that *in*A2827(aa520) T antigen was as stable as wild-type T antigen while *dl*A2433(aa587-589) T antigen was much less stable, as reported previously (75). The *in*A2827/*dl*A2433 T antigen was as unstable as *dl*A2433 T antigen. Therefore, the failure of this double mutant T antigen to interfere with wild-type T antigen in viral DNA replication can be explained by its instability.

***trans* dominance of additional SV40 mutants.** We examined a number of additional mutants for their ability to interfere *trans* dominantly with SV40 DNA replication. These mutants included several mutants with conservative amino acid substitutions in the DNA binding domain (S147T, A149G, S185T, H187R, and R204K) (55, 56) and one with an insertion [*in*A2817(aa215)] in this region. All five point mutants were replication defective and differed widely in their ability to bind DNA and in helicase activity (55, 56). Mutant *in*A2817(aa215) is replication defective but retains wild-type DNA binding activity. We also examined two mutants cloned from CV-1 cells transformed by replication-defective SV40 genomes (22, 23). These mutants had been shown previously to be *trans* dominant in vitro (63) but had not been studied in vivo. Mutant C-6-2(N153T) is defective for DNA binding, while mutant C-2(K516R) retains normal DNA binding but is defective for ATPase activity (40).

Mutants S147T, A149G, S185T, H187R, and R204K were not *trans* dominant (Fig. 5). Because the genomes of mutants S147T, A149G, S185T, H187R, and R204K were prepared in a plasmid background different from those of our other mutants, we used as a control for these studies a new plasmid, *dl*A2840, in which the 1,018-bp small *Nde*I fragment (nucleotides 3808 to 4826) was deleted from a wild-type SV40 genome inserted into the same plasmid background as these DNA binding domain point mutants. This plasmid cannot encode a large T antigen. In Cos-1 cells, its replication level was similar to that of *dl*A1209, the control plasmid for study of deletion and linker insertion mutants cloned into pUC18. Mutant *in*A2817(aa215) was strongly *trans* dominant. Mutant C-6-2 was moderately *trans* dominant, while mutant C-2 was strongly *trans* dominant (Fig. 5).

DISCUSSION

trans-dominant or dominant-negative mutants have been described in many systems. In prokaryotes, dominant negative *lac* repressor mutants form inactive heterotetramers with wild-type protein (43). In eukaryotes, several dominant negative mutants of transcription factors or transactivating proteins were found to inhibit transcriptional activation by wild-type protein. Mutant c-Jun proteins with point and deletion mutations in the basic domain were able to form heterodimers with Fos protein and suppress the wild-type c-Jun/Fos DNA binding activity and transcriptional activation function (48). Heterodimers were formed between wild-type and dominant-negative mutants of herpes simplex virus type 1 transcriptional regulatory protein ICP4. These heterodimers retained DNA binding activity but failed to activate transcription (54). Deletion of the acidic transcriptional activation domain of another herpes simplex virus type 1 transcriptional regulatory protein, VP16, and the activating region II of human immunodeficiency virus type 1 *trans*-activator protein, Tat, also created dominant negative mutants. Presumably the mutant proteins competed with wild-type proteins for binding to a cellular DNA-binding protein but failed to activate because they lack the activating domain (20, 26). Point and deletion mutations in one of the functional domains of human immunodeficiency virus type 1 Rev protein and the analog protein, Rex, of human T-cell leukemia virus type I resulted in *trans*-dominant suppression of sequence-specific nuclear export of incompletely spliced viral mRNAs (37, 49). Finally, adeno-associated virus contains a multifunctional regulatory gene, *rep*, required for viral DNA replication and regulation of viral gene expression. A point mutation in the nucleotide binding site in the Rep protein resulted in overproduction of the protein which was able to inhibit in *trans* wild-type adeno-associated virus

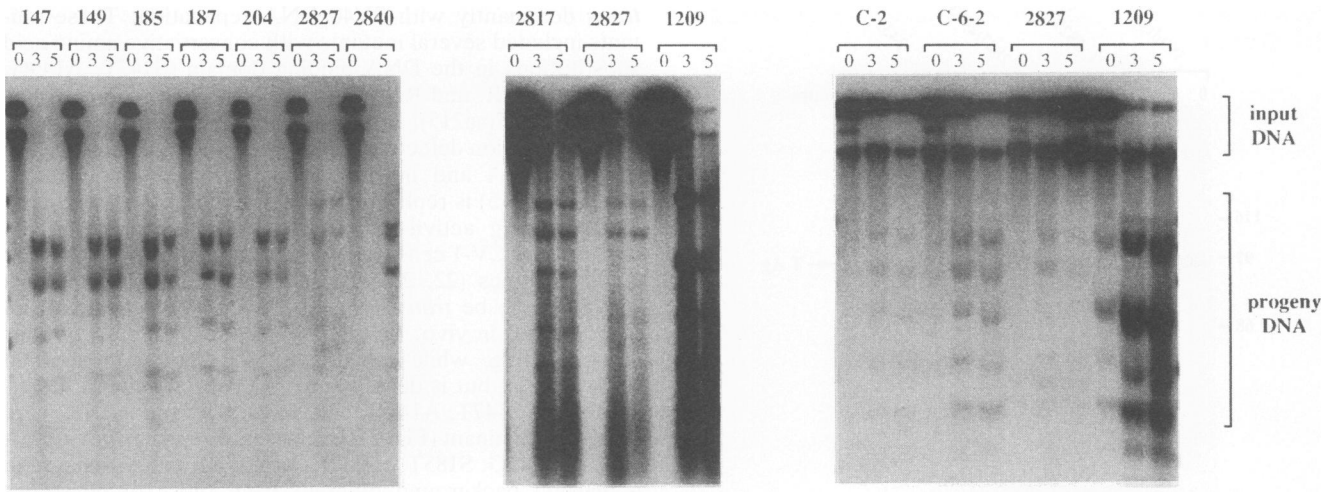

FIG. 5. Analysis of additional mutants for *trans* dominance. Cos-1 cells were transfected with mutant DNAs, and the low-molecular-weight DNA was harvested 0, 3, and 5 days after transfection. After digestion with *Mbo*I, the DNAs were fractionated by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and hybridized to a ^{32}P-labelled probe specific for SV40 sequences. *dl*A1209 and *dl*A2840 were controls for the level of replication mediated by endogenous T, and *in*A2827 was included for comparison. Because of differences among the mutant genomes and plasmid backgrounds, the *Mbo*I digestion patterns were not identical.

DNA replication (9). This last situation closely resembles the *trans* dominance we have been studying and could occur by similar mechanisms.

SV40 large T antigen is the only viral protein required for viral DNA replication. It binds specifically to the SV40 origin of replication, nonspecifically to double- and single-stranded DNA; it interacts specifically with p53 and cellular DNA polymerase α and perhaps also with other cellular replication factors, and it forms oligomers. An understanding of the mechanisms of interference with viral DNA replication by *trans*-dominant mutant T antigens could provide important insights into these processes. *trans*-dominant interference with viral DNA replication has been reported for SV40 (19) and polyomavirus (45). In both systems, the *trans*-dominant interfering T antigens possessed mutations which affected their ATPase domains. We showed that mutants in other regions of SV40 large T antigen could also inhibit viral DNA replication (75). Stillman et al. (63), showed that some mutant T antigen could inhibit SV40 viral DNA replication in vitro, and the data presented above indicate that two of these mutants, C-6-2(N153T) and C-2(K516R), were also *trans* dominant in vivo.

In this report, we have extended our previous study of *trans*-dominant mutants (75) in an attempt to learn more about mechanisms of the interference. We first eliminated the possibility that *trans*-dominant mutant T antigens acted by suppressing the expression of the wild-type T antigen. Large T antigen activates viral and cellular genes and autoregulates its own expression. However, the presence of various mutant T antigens in the same cells as wild-type T had no significant effects on wild-type T antigen synthesis, regardless of whether the mutants were *trans* dominant or not. The conditions used to examine T-antigen expression were exactly the same as those used to demonstrate the ability of *trans*-dominant mutants to interfere with viral DNA replication in CV-1 cells. Since 75 to 90% of transfected cells were cotransfected (Table 1), it should have been relatively easy to detect any significant changes in wild-type T antigen expression caused only by *trans*-dominant mutants. Although the amount of replication of the various mutant DNAs varied considerably from one mutant to another (75), the synthesis rates of different mutant T antigens showed little variation, even after 3 days. This may reflect autoregulation of the early promoter by T antigen.

One concern about these experiments was that homologous recombination would occur between the replication-defective plasmid encoding wild-type T antigen and the replication-competent plasmid encoding mutant T antigen, making interpretation of the results difficult. The replication-defective plasmid p6-1 has a single lesion within the origin of replication. The plasmids encoding mutant T antigens in this experiment were double mutants. Each contained two lesions, the deletion of *dl*A2459(aa674-708) and a second mutation located at various sites within the T-antigen gene. Recombination would generate a variety of recombinants, including replication-competent plasmids encoding wild-type T, and plasmids encoding full-length forms of mutant T antigens and truncated forms of wild-type T antigen. Such replication-competent plasmids would then have replicated in the presence of wild-type T antigen, and this would be expected to lead to an increase in the amount of full-length T antigen at later times after infection. The fact that nearly identical patterns of wild-type and mutant T-antigen synthesis were seen with *trans*-dominant and non-*trans*-dominant mutants and with mutants with lesions both nearest to [*dl*A2411(aa143-146)] and furthest from [*in*A2827(aa520)] the origin region mutation of p6-1 argues against recombination being a significant factor in this experiment.

We next analyzed a series of double mutants in order to find out whether there might be some specific functions of T antigen required for *trans* dominance to occur. We chose to prepare a set of double mutants which contained the *in*A2827(aa520) mutation because it is the strongest *trans*-dominant mutant; its mutation is located in the ATPase domain, where a majority of previously described *trans*-dominant mutations map. The *trans* dominance of *in*A2827(aa520) was reversed by the DNA-binding-defective mutation of *in*A2815(aa168) and by the mutation of *dl*A2433(aa587-589). However, the loss of *trans* dominance in *dl*A2433/*in*A2827 probably can be fully explained by the

fact that this double mutant protein was extremely unstable. In contrast, the T antigens of *in*A2815(aa168), *in*A2827 (aa520), and *in*A2815/*in*A2827 were all as stable as wild-type T antigen. One possible explanation for loss of *trans* dominance in the *in*A2815/*in*A2827 double mutant is that the *trans*-dominant behavior of *in*A2827 T antigen requires that it bind to DNA. Alternatively, DNA binding per se may not be required, but an intact DNA binding domain may be needed for the *in*A2827 T antigen to interact with wild-type T antigen or with some host cell factor.

Clearly, DNA binding and a wild-type DNA binding domain are not general requirements for *trans*-dominant T antigens, since DNA binding domain mutants *dl*A2411 (aa143-146) and C-6-2(N153T) both displayed moderate *trans* dominance. Both of these mutant genomes replicated to a greater extent in Cos-1 cells than did *in*A2827(aa520) DNA. We constructed a double mutant from the DNA binding mutant *dl*A2411(aa143-146) and *in*A2827(aa520). The double mutant was also *trans* dominant, to about the same extent as *dl*A2411(aa143-146) and to a lesser extent than *in*A2827 (aa520). If *in*A2827(aa520) interferes with wild-type T antigen's replicative ability by binding to DNA, *dl*A2411(aa143-146) would have to use a different mechanism of *trans* dominance, and the double mutant, *dl*A2411/*in*A2827, would have to be using *dl*A2411's mechanism. Alternatively, if protein-protein interactions underlie *trans*-dominant interference, the *dl*A2411/*in*A2827 and *in*A2815/*in*A2827 T antigens could differ quantitatively in the strength of their interactions with other proteins.

No effect on *trans* dominance was seen when mutations within the retinoblastoma-binding domain, the N-terminal 35 amino acids, or the C-terminal 35 amino acids were combined with the mutation of *in*A2827. Interestingly, a mutation in the nuclear localization signal, SVcT(K128T), combined with *in*A2827 substantially reduced the degree of interference. SVcT T antigen localizes to the cytoplasm, as does the double mutant *in*A2827/SVcT (unpublished results). The fact that *in*A2827/SVcT allowed more DNA replication than *in*A2827 alone indicates that the strong dominant negative effect of *in*A2827(aa520) T antigen on wild-type replicative function probably requires nuclear location of the interfering T antigen. Were binding to viral DNA important for its *trans* dominance, this would be the case. This finding is also consistent with *in*A2827 interfering by sequestering a cellular nuclear factor in an inactive complex. It has been reported that SVcT T antigen inhibits the nuclear localization of wild-type T antigen in Cos-1 cells (35), presumably by forming heterooligomers with wild-type T. The data presented here show that Cos-1 cells transfected by either SVcT or *in*A2827/SVcT contain sufficient wild-type T in the nucleus to permit a substantial amount of viral DNA replication. Furthermore, the result that *in*A2827/SVcT showed less replication than SVcT did indicates that either a small percentage of *in*A2827/SVcT is able to reach the nucleus to cause *trans* dominance or that *in*A2827 can carry out its dominant negative effect on DNA replication to a lesser degree while it is localized in the cytoplasm.

We also screened a number of additional mutants for *trans* dominance. These included five mutants with conservative amino acid substitutions in the DNA binding domain. Interestingly, none of them showed any interference. These DNA binding domain mutants vary considerably in helicase activity and in both specific and nonspecific DNA binding (55, 56). Among other mutants examined with lesions in the DNA binding domain, mutants C-6-2(N153T) and *dl*A2411(aa143-146) were moderately *trans* dominant, *dl*A2815(aa168) was not *trans* dominant and mutant *in*A2817(aa217) was strongly transdominant. We see no pattern relating these activities of T antigen to *trans* dominance. All of these mutant T antigens have approximately the same stability as wild-type T antigen. Thus, there is no simple model that explains the patterns of *trans* dominance we have seen. Rather, our data suggest that multiple mechanisms are involved.

Oligomerization may be central to the dominant-negative behavior of some mutant T antigens. Perhaps only *trans*-dominant mutant T antigens are able to form heterooligomers with wild-type T. Alternatively, both *trans*-dominant and non-*trans*-dominant mutants might be able to form heterooligomers with wild-type T antigen, but the properties of heterooligomers between wild-type and *trans*-dominant mutant T antigens might be distinctly different from the properties of heterooligomers between wild-type and non-*trans*-dominant mutant T antigens. In the presence of ATP, T antigen assembles in hexamers (41, 46). DNA is not required but stimulates hexamer formation cooperatively (46). This means that hexamer formation does not require an intact DNA binding domain. Some *trans*-dominant mutants may sequester wild-type T antigen in mixed hexamers, thus reducing the amount of wild-type T antigen available for replication. Other mutant T antigens might assemble on SV40 DNA into hexamers containing wild-type and mutant subunits; it is not known whether there are any conditions under which such mixed hexamers, formed from wild-type and mutant subunits, would be active in DNA replication. We reported previously that a DNA-binding-defective T antigen could not complement an ATPase-defective T antigen to restore viral DNA replication (70). However, other combinations of various mutants might restore this activity, and hexamers containing wild-type T antigen and T antigens encoded by some mutants might retain partial or full activity for DNA replication.

Perhaps mutants with conservative amino acid substitutions in the DNA binding domain retain full ability to oligomerize at the origin of replication, and oligomers containing wild-type and mutant subunits might retain normal activity for viral DNA replication. Mixed oligomers between T antigens which showed moderate interference with viral DNA replication [*dl*A2411(aa143-146) and C-6-2(N153T)] might retain some replicative activity but less than that of the wild type. Finally, mutants which are strongly *trans* dominant might form mixed oligomers totally defective for viral DNA replication. We have recently begun to examine the ability of mutant T antigens to oligomerize and to form heterooligomers with wild-type T antigen.

ATP-binding and ATPase activity might also play roles in some mechanisms of *trans* dominance. In an oligomer, binding of ATP to one T antigen molecule appears to induce allosteric changes in other T-antigen molecules (4, 46). Although we have not analyzed ATP binding by all mutant T antigens, many have mutations in the ATP binding or ATPase domain. The T antigens of mutants *dl*A2433(aa587-589), *dl*A2432(aa509), and *dl*A2462(aa507-510) are known to be defective for ATPase activity, while *trans*-dominant mutant *dl*A2411(aa143-146) T antigen showed wild-type ATPase activity (14). Altered ability to interact with ATP could result in the formation of hexamers unable to undergo important ATP-mediated, allosteric changes. Some mutant T antigens are likely to bind ATP normally but be defective for ATPase activity, since the latter activity requires a larger portion of T antigen than the former. Such ATPase-defective mutants might form mixed hexamers bound to SV40 DNA, and ATP binding might bring about the normal allosteric

changes; however, inability to hydrolyze ATP could result in such mixed hexamers remaining bound to SV40 DNA but unable to catalyze critical steps requiring ATP hydrolysis.

Each step performed by T antigen in DNA replication is a potential step at which *trans*-dominant interference could occur. The use of in vitro SV40 DNA replication systems has added substantially to our understanding of viral DNA replication in recent years. These systems offer the best prospect for further elucidating the mechanisms by which mutant T antigens interfere with the replication activities of wild-type T antigen.

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