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Simian Virus 40 Small-t Antigen Stimulates Viral DNA Replication in Permissive Monkey Cells

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The simian virus 40 (SV40) large-T antigen is essential for SV40 DNA replication and for late viral gene expression, but the role of the SV40 small-t antigen in these processes is still unclear. We have previously demonstrated that small t inhibits SV40 DNA replication in vitro. In this study, we investigated the effect of small t on SV40 replication in cultured cells. CV1 monkey cell infection experiments indicated that mutant viruses that lack small t replicate less efficiently than the wild-type virus. We next microinjected CV1 cells with SV40 DNA with and without purified small-t protein and analyzed viral DNA replication efficiency by Southern blotting. Replication of either wild-type SV40 or small-t deletion mutant DNA was increased three- to fivefold in cells coinjected with purified small t. Thus, in contrast to our in vitro observation, small t stimulated viral DNA replication in vivo. This result suggests that small t has cellular effects that are not detectable in a reconstituted in vitro replication system. We also found that small t stimulated progression of permissive monkey cells—but not of nonpermissive rodent cells—from G_0-G_1 to the S phase of the cell cycle, possibly leading to an optimal intracellular environment for viral replication.

In monkey cells infected by simian virus 40 (SV40), large-T antigen directs an ordered sequence of events leading from the early phase of infection, in which viral DNA replication starts, to the late phase, when viral particles are produced (2, 44, 46). The early phase is devoted to subversion of cellular control mechanisms to prepare the cell for the late phase. T antigen is an autoregulated phosphoprotein that accumulates in the nucleus during the early phase of infection; it alters cellular transcription patterns (6, 43) and stimulates cellular DNA synthesis in quiescent cells (11, 19, 20, 21, 30, 45). Upon transition to the late phase, T antigen sustains viral DNA replication and stimulates late gene expression and virion production.

The second early SV40 protein, the small-t antigen, is a 17-kDa polypeptide found predominantly in the cytoplasm of infected cells (13, 46). Small t shares 82 amino acids at its amino terminus with the large-T antigen; the remaining 92 amino acids are unique. Although small t can enhance transformation by the virus (3, 7, 8, 41), particularly when growth-arrested cells are used (28), little is known about its role in permissive infections of cycling cells. However, enhancement of permissive infections by small t is suggested by the reduced growth yields and small plaque sizes induced by viruses that carry mutations in the small-t antigen (40, 46, 47). Recently, it has been found that small-t antigen binds cellular protein phosphatase 2A (PP2A) (33, 50), inhibiting its activity toward several substrates, including large-T antigen itself (39, 51).

With a highly purified system for replication of SV40 DNA in vitro, PP2A was shown to dephosphorylate T antigen and stimulate replication (26, 49). It was shown that addition of small-t antigen to an in vitro cell-free replication system decreases SV40 DNA replication (5), a result consistent with the inhibition of PP2A by small t. To explore this apparent paradox between in vivo and in vitro experiments, we examined the effect of small t on replication of microinjected viral DNA and the effects of small-t antigen on the cell cycle in permissive (monkey) cells. Given its role in transformation, we also studied the effect of small t on cell DNA synthesis in nonpermissive (rodent) cells.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (CV1) cells were grown in Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum. Virus stocks were harvested from lysates initiated with a low multiplicity of virus, and titers were determined on CV1 cells. Lysates prepared by subjecting even uninfected cells to freeze-thaw cycles contain factors which induce cellular DNA synthesis. Consequently, serum-free mock and virus stocks were prepared. Cells were infected with 10 PFU per cell in medium containing serum. Medium was then replaced with serum-free DMEM until cytopathic effects were apparent by extensive vacuolization of the cells, but most cells remained attached to the dishes. At this time, medium containing virus was collected and the small number of detached cells was removed by centrifugation. Titers of these serum-free viruses were two- to threefold lower than titers obtained following freezing and thawing of infected cells. Mock lysates prepared in this way were unable to induce DNA synthesis in growth-arrested cells, in contrast to mock lysates prepared by freezing and thawing.

Rates of viral DNA synthesis. Confluent cultures of CV1 cells in 3.5-cm-diameter dishes were infected with 10 PFU of virus per cell or equivalent volumes of lysates of uninfected cells for 2 h and then kept in serum-free medium. At various times postinfection, 10 μCi of [^{3}H]thymidine was added for 1 h. Cells were washed with phosphate-buffered saline (PBS) and

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then extracted by the method of Hirt (23) to separate viral and cellular DNAs. Radiolabeled viral DNA from supernatant fractions was precipitated with 10% trichloroacetic acid, suspended in alkali, neutralized, and counted (see Table 1).

Flow cytometry. CV1 cells were grown to confluence in 10-cm-diameter dishes and then kept in serum-free medium for 96 h. Cells were then infected at 5 PFU per cell with serum-free virus prepared from cells infected in the absence of serum. Control and viral stocks were prepared without freezing and thawing of cells. The medium removed from the cells was added back at the end of the 2-h infection period. Theophylline was added in a final concentration of 1.8 mM to some cultures. At 36 h postinfection, cells were trypsinized to single cell suspensions, washed, and suspended in PBS containing 0.5% Nonidet P-40. Cells were over 90% viable as determined by trypan blue exclusion. Nuclei were collected by centrifugation, fixed, stained with propidium iodide, and analyzed by flow cytometry. Parallel control dishes were pulsed with 2 μCi of [^{3}H]thymidine from 36 to 48 h after infection. Cells were washed with PBS and then extracted by the method of Hirt (23) to separate viral and cellular DNAs. Radiolabeled viral DNA from supernatant fractions was precipitated with 10% trichloroacetic acid, suspended in alkali, neutralized, and counted (see Table 2).

Microinjection. CV1 cells, primary mouse kidney cells, and 3T3 cells, grown in DMEM supplemented with 10% fetal bovine serum and 1% gentamicin on glass slides (3 by 3 mm or 1 by 4 cm) with an imprinted grid, were used for microinjection experiments. Plasmid DNA (0.2 μg/μl) in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA was loaded into glass capillaries pulled from 1.2-mm-diameter glass with a capillary puller. Cells were microinjected as previously described (17). Small-t antigen used for microinjection was purified from bacterial sources as described previously (16), by using the dialyzable detergent *N*-octylglucopyranoside in the final stages of purification (48). Small-t preparations at 0.1 to 0.2 mg/ml were dialyzed extensively against PBS before injection.

Immunocytochemistry. After microinjection, cells were incubated for 2-h intervals in DMEM containing [^{3}H]thymidine (0.5 μCi/ml of medium; Dupont, NEN). Thereafter, the cells were washed in PBS solution, fixed in methanol at −20°C for 10 min, and air dried. The cells were then stained to detect T antigen with a mouse anti-T antibody (Ab-1; Oncogene Science) for 30 min at 37°C, washed with PBS, and further incubated with fluorescein-conjugated goat anti-mouse antibody (Cappel Laboratories) for 30 min at 37°C. Slides with the fixed cells were then dipped in emulsion (Ilford K.2 emulsion in gel form; Polyscience Inc.) and further processed for autoradiography (21).

DNA extraction and Southern blot analysis. Viral DNA was extracted from the microinjected cells as previously described (17). After agarose gel electrophoresis, DNA was transferred to a nylon membrane (GeneScreen Plus; NEN) and UV cross-linked with a UV-Stratalinker (Stratagene). The DNAs were hybridized with nick-translated, ^{32}P-labeled SV40 DNA by incubation with 2×10^8 cpm/μg as previously described (27). Filters were washed and exposed to X-ray film.

Densitometric analysis. To quantify the SV40 DNA synthesized in the microinjected cells, the Southern blot autoradiograms were subjected to densitometric analysis with the Image program, version 1.3 (available from the National Technical Information Service), on an Apple Macintosh IIci equipped with a Sierra Scientific MS-4030 high-resolution video camera and data Translation Quick Capture DT2255 Frame Grabber Board. Tryptic phosphopeptide maps of T antigen were analyzed in the same way.

Metabolic labeling. Confluent cultures of CV1 cells (10^6) were infected with wild-type (WT) SV40 or the 884 small-t deletion mutant (*dl*-884) at a final concentration of 25 PFU per cell. At different times postinfection (12 to 40 h), the medium was removed and cells were preincubated with P_i-free DMEM for 30 min. Cells were labeled with 3 mCi of $^{32}P_i$ (Amersham) per ml for 4 h in P_i-free DMEM supplemented with 5% fetal bovine serum. After labeling, cells were washed three to five times with ice-cold PBS, pelleted, and suspended at 10^6/ml of lysis buffer containing 20 mM $NaPO_4$ (pH 7.8), 250 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.01% sodium dodecyl sulfate supplemented with freshly prepared protease and phosphatase inhibitors (10 mM sodium fluoride, 0.1 mM sodium vanadate, 1 mM phenylmethylsufonyl fluoride, and leupeptin, pepstatin, and aprotinin at a 10-μg/ml final concentration). After 30 min of incubation on ice, cell extracts were clarified by centrifugation (Eppendorf 5415C centrifuge) at 12,000 rpm and then immunoprecipitated with anti-T-antigen antibody (Ab-1; Oncogene Science) preadsorbed to protein A-Sepharose beads (Pierce) for 2 h at 4°C. Immunoprecipitated proteins were washed six times in lysis buffer, resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis, and blotted onto nitrocellulose filters (27).

Phosphopeptide analysis of proteins. Phosphopeptide mapping of T antigen was carried out as described elsewhere (4, 9, 25, 36–39). Briefly, T antigen was localized on the blotting membranes by autoradiography and the corresponding bands were cut out. T antigen was digested with trypsin and pronase E. Digested phosphopeptides were oxidized with formic acid (90% formic acid and 10% hydrogen peroxide), lyophilized, and applied to thin-layer chromatography plates. Electrophoresis in the first dimension was carried out in 6% formic acid–1.25% acetic acid–0.25% (vol/vol) pyridine (pH 1.9) for 25 min at 13 kV. Ascending chromatography was performed in isobutyric acid-pyridine-acetic acid-butanol-H_2O (65:5:3:2:29). In comparative analyses of the phosphoproteins, approximately equal amounts of radioactive material were loaded onto the thin-layer plates. Alternatively, exposure times were varied.

RESULTS

Levels of viral DNA replication in mutant infections. The finding that small-t antigen could inhibit DNA replication in reconstituted in vitro replication systems was in apparent contradiction to initial reports (40, 46, 47) and observations of many laboratories, including ours, that small-t mutants grew more slowly and to lower yields than WT SV40. As shown in Fig. 1, levels of viral DNA which accumulate in mutant-infected cells were lower than levels found in WT infections. The data shown are for Hirt supernatant DNA obtained 72 h postinfection with mutant *dl*-888 and WT viruses. Similar patterns were obtained at 48 h postinfection. In addition, less mutant DNA was consistently obtained when extracted viral DNAs were purified on CsCl gradients (data not shown).

Rates of viral DNA synthesis were also analyzed at several times postinfection with WT and mutant viruses. As shown in Table 1, following infection of CV1 cells with 10 PFU of WT or mutant virus per cell, incorporation of [^{3}H]thymidine into Hirt supernatants following 1-h pulses was consistently reduced in *dl*-888-infected cells, suggesting that lower DNA levels did not simply reflect a delayed time course of the mutant infection. Maximum rates of viral DNA synthesis occurred between 35 and 39 h postinfection. The earlier peak of viral DNA synthesis by the mutant virus in data shown in Table 1 was not reproducible, and the exact time of peak synthesis varied from

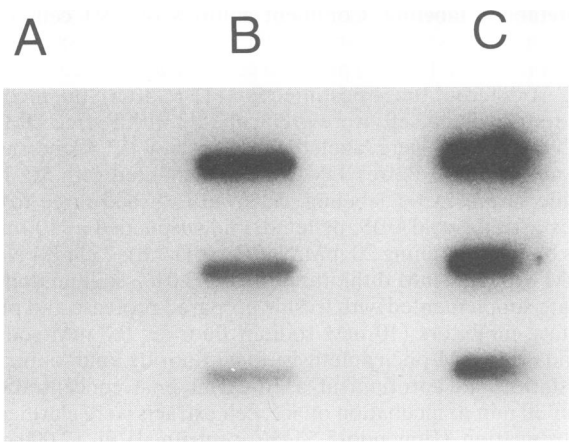

FIG. 1. Accumulation of viral DNA in WT and *dl*-888 infections. CV1 cells were mock infected (A) or infected at 10 PFU per cell with *dl*-888 (B) or WT SV40 (C). At 72 h postinfection, cells were extracted by the method of Hirt (23) and supernatant DNA was recovered following phenol extraction and ethanol precipitation. DNA was suspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, diluted 50-fold in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and then further diluted three- and ninefold. A 100-μl volume of each dilution was filtered onto nitrocellulose with a slot blot manifold. After baking of the filter and prehybridization, viral DNA was hybridized to a ^{32}P-labeled SV40 *Bam*HI-*Bst*XI fragment prepared by random oligonucleotide priming.

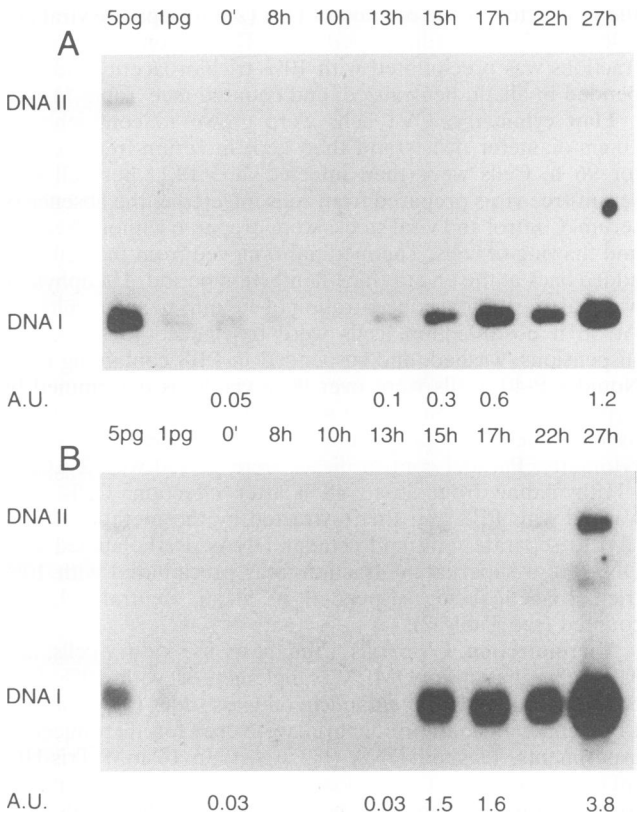

FIG. 2. CV1 cells grown on small glass slides (3 by 3 mm) were microinjected with WT SV40 DNA (A) or WT SV40 DNA mixed with purified small-t antigen (B). Each test point is based on 100 injected cells. At the time indicated (0 to 27 h after injection), the slides with the cells were removed from the culture medium and the DNA was extracted and separated on agarose gels as described in Materials and Methods. The blots were hybridized with ^{32}P-labeled SV40 DNA. The relative DNA concentrations are shown in arbitrary units (A.U.). The positions of covalently closed circular DNA (I) and relaxed circular DNA (II) are indicated.

experiment to experiment. Rates of synthesis of WT DNA exceeded those of the mutant even when the multiplicity of infection was reduced fourfold. Thirty to thirty-five percent of the acid-precipitable radioactivity was found to be form I DNA by agarose gel electrophoresis. The amount of labeled form I DNA always paralleled the total radioactivity in high-molecular-weight materials.

Effect of microinjected purified small t on viral DNA replication. To confirm that small t was responsible for the stimulation of DNA replication observed in infected cells, we used purified small-t antigen to microinject CV1 cells along with viral DNA. In the first set of experiments, WT SV40 DNA was microinjected into the nuclei of growing CV1 cells and viral DNA replication was assayed at various times after DNA transfer by Southern blot analysis. For each test point, 100 CV1 cells were microinjected. At the time points indicated in Fig. 2, glass slides with the injected cells were removed from the culture medium and washed with PBS and DNA was extracted by the modified Hirt extraction method (17). After agarose gel electrophoresis, Southern blotting, and autoradiography, the amount of viral DNA was estimated by densitometry. The data were converted to arbitrary units relative to the amount of microinjected DNA (DNA extracted from cells immediately after microinjection). In the experiment whose results are shown in Fig. 2A, SV40 DNA replication was first detectable 13 h after injection. At this time, the amount of viral DNA extracted from the cells was larger (0.1 arbitrary unit [AU]) than the amount of DNA injected into the cells (0.05 AU). Levels of viral DNA then increased over the course of the experiment (27 h) to 1.2 AUs.

The effect of purified small t on viral DNA synthesis was examined in parallel experiments. Small t was then coinjected with 0.1 μg of viral DNA per ml. As shown in Fig. 2B, the time of onset of viral DNA synthesis was similar but the extent of replication was far greater in the presence of small t, reaching a level of 3.8 AUs. It is unlikely that stimulation was due to a contaminating bacterial product in the small-t preparation; comparable preparations from bacteria carrying the expression plasmid but lacking the small-t insert did not stimulate viral replication following microinjection (data not shown).

Similar microinjection experiments were performed with small-t mutant *dl*-884 DNA (Fig. 3). As for WT DNA, purified

TABLE 1. Rates of viral DNA synthesis[a]

Time (h) postinfection	[^{3}H]thymidine incorporation/10^5 cells (cpm, 10^4)			
	dl-888	WT	WT (1:4)	Mock infection
22	1.23	1.19		
30	1.49	2.78		
35	1.90	3.62	2.81	0.37
39	1.49	3.86	2.85	0.27
43	1.43	2.92		
47	1.17	2.89		
55	0.86	1.74		

[a] CV1 cells were infected with WT or *dl*-888 at a multiplicity of 10 or with WT SV40 diluted 1:4 at a multiplicity of 2.5. Total precipitable counts are shown, with values for uninfected cells included for comparison.

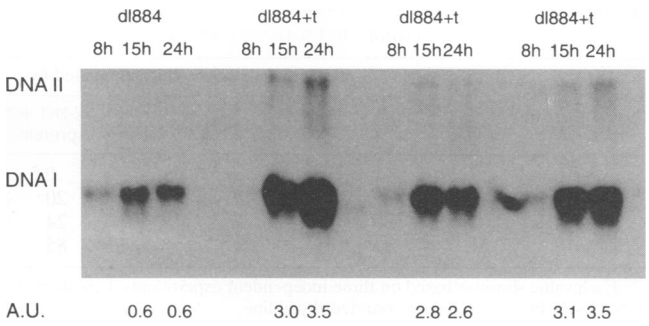

FIG. 3. Southern blot showing replication efficiency of *dl*-884 DNA and *dl*-884 DNA mixed with small-t antigen (results of three independent injection experiments are shown) after injection into CV1 cells. For details, see the legend to Fig. 2.

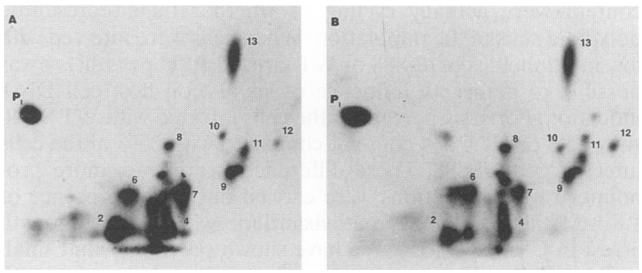

FIG. 4. Phosphopeptide analyses of large-T antigen obtained from CV1 cells infected with WT SV40 (A) and *dl*-884 (B). CV1 cells were labeled at 16 h postinfection for 4 h in the presence of 5 mCi of ^{32}P. Peptide analysis was carried out as described in Materials and Methods.

small-t antigen stimulated replication of *dl*-884 DNA by about fourfold. Initially, we might have predicted that the enhancement of replication would be greater when a mutant which could not synthesize its own small-t antigen was studied. However, in these experiments purified small-t antigen was coinjected with the template DNA, allowing high intracellular concentrations of small t long before similar levels could have been reached by de novo transcription and translation of the WT viral genome. Also, it is conceivable that the high intracellular levels of microinjected small t saturated the viral and cellular responses to small t.

Phosphorylation pattern of T antigen. In vitro experiments have shown that the small-t-antigen binds to the regulatory subunit of PP2A and inhibits the catalytic activity of the enzyme. Inhibition of dephosphorylation was demonstrated by using several substrates, including the SV40 T antigen (31, 39, 51). The PP2A-sensitive amino acids on the large-T antigen are Ser-120–Ser-123 and Ser-677–Ser-679 (36, 38, 39, 49). The phosphorylation status of these two clusters of serines together with Thr-124 seems to be critical for large-T-antigen-specific DNA replication activity (22, 36, 38, 39, 49).

To determine whether the small-t antigen mediates its stimulatory effect on viral DNA replication through alteration of the phosphorylation status of the large-T antigen, we could not use microinjection experiments; the large number of SV40-infected cells necessary to obtain enough T antigen for detection by tryptic map experiments precluded this approach. Consequently, we turned to studies of cells infected with either WT virus or small-t mutant virus *dl*-884. Cells were labeled for 4 h with $^{32}P_i$ at 16, 24, or 36 h after infection. At these times, cell proteins were extracted and T antigen was immunoprecipitated with a monoclonal antibody. Phosphorylated T antigens were isolated and digested with trypsin and pronase E, and peptides were separated as described in Materials and Methods. The peptide maps obtained from T antigen labeled between 16 and 20 h following infection with *dl*-884 showed somewhat reduced phosphorylation of one region of large T believed to play a role in DNA replication. For example, in the map shown in Fig. 4 (obtained 16 h after infection), T antigen from *dl*-884-infected cells (Fig. 4B) appeared to have less phosphorylation of peptide 7 and more of peptides 11 and 12 than the map of WT T antigen (Fig. 4A). Peptides 7, 11, and 12 are related to each other and differ mainly by charge (36, 38). Peptide 7 is labeled on Ser-120, Ser-123, and Thr-124; peptide 11 is labeled on Ser-120 or Ser-123 and on Thr-124; peptide 12 contains phosphate only on Thr-124 (36, 38). The differences in the WT and mutant tryptic maps were not striking and were limited mainly to peptide 7, which densitometric analyses indicated was twofold more phosphorylated in the WT tryptic map (Fig. 4A) than in the mutant tryptic map (Fig. 4B). These differences were observed only at this early time (16 h) after infection. No differences were found in maps of T antigens obtained at 24 and 36 h postinfection (data not shown).

Effect of small t on cell cycle progression. Since the differences in T-antigen phosphorylation in the presence or absence of small t were not impressive, we considered that small t might exert some other effects, not detectable in a cell-free replication system, responsible for the stimulation of viral DNA synthesis in vivo. In transformation assays, it has been suggested that small t is needed mainly when growth-arrested cells are assayed, suggesting that one function of small t might be stimulation of cell cycle progression. In permissive cells, such growth stimulation might account for the increased viral DNA synthesis observed in the presence of small t. To test this possibility, we analyzed cells infected with WT and mutant viruses by flow cytometry in parallel with thymidine incorporation.

It has been extremely difficult to arrest monkey kidney cells, and high levels of thymidine incorporation continue when these cells reach confluence. To reduce thymidine incorporation, it was necessary to maintain cells at confluence in serum-free medium for prolonged periods of time. Such cells undergo some morphological alteration and are easily damaged by microinjection; however, they maintain viability as measured by trypan blue exclusion.

As shown in Table 2, cells kept in serum-free medium and then infected with serum-free mock lysate showed largely G_1 DNA content (see Materials and Methods for technical details). It is not clear whether the cells with G_2 or S DNA

TABLE 2. Cell cycle analysis of uninfected and infected CV1 cells

Infection	Theophylline	% of cells in:			[^{3}H]thymidine incorporation (cpm, 10^4)	
		G_1	S	G_2-M	Cellular DNA	Viral DNA
Mock	−	86	9	4		
dl-888	−	74	15	11	4.3	1.2
WT	−	54	21	25	9.0	5.0
dl-888	+	85	10	6	1.0	0.2
WT	+	39	36	25	11.0	4.3

content were actually cycling or whether they represented polyploid cells in the population. When cells were infected with low multiplicities of *dl*-888 or WT virus (5 PFU per cell), it was possible to detect an influence of small t on host cell DNA induction. Forty-six percent of the cells infected with WT SV40 showed S or G_2 DNA content, compared with 26% of the cells infected with *dl*-888. These differences were even more pronounced when infections were carried out in the presence of 1.8 mM theophylline, a methylxanthine which causes growth arrest in CV1 cells (35). We have shown previously that small t allows CV1 cells to overcome a theophylline-induced growth arrest which may result from theophylline inhibition of the Na^+/H^+ antiporter (32). When infected cells were maintained in the presence of theophylline before flow cytometric analyses at 36 h, cells infected with small-t mutant viruses showed no evidence of progression through the cell cycle while most of the cells infected with WT SV40 had S or G_2 DNA content.

Some of the increase in DNA content may have resulted from accumulation of viral genomes. However, significant cellular DNA synthesis occurred in infected CV1 cells as well. Parallel dishes of infected cells were analyzed for [^{3}H]thymidine incorporation to determine the status of ongoing viral and cellular DNA synthesis. As shown in Table 2, about twice as much cellular DNA synthesis was detected in cells infected with the WT virus than in cells infected with the small-t mutant virus. Theophylline resulted in a 75% decrease in thymidine incorporation in mutant-infected cells, while theophylline had no effect on cells infected with WT SV40. Viral DNA synthesis showed the same general patterns, with little effect of theophylline on viral or cellular DNA synthesis in WT infections. Although not shown here, theophylline did not reduce levels of large-T antigen significantly in mutant-infected cells (34a).

Effect of microinjected small t on host DNA synthesis. We next attempted to repeat the results described in the previous paragraph (i.e., stimulation of cellular DNA replication by small t) by microinjection of purified small t into CV1 cells. However, we were unable to measure host cell DNA synthesis in the permissive CV1 cells successfully used to measure viral DNA synthesis. Growth of CV1 cells is extremely difficult to arrest. When CV1 cells were maintained in the absence of serum for several days, conditions required to reduce thymidine incorporation, they were easily damaged by microinjection. Consequently, we examined the effect of small t on the induction of host cell DNA synthesis by large T in nonpermissive cells. Because no viral DNA synthesis occurs in nonpermissive rodent cells, autoradiography following thymidine incorporation was used as a measure of ongoing cellular DNA synthesis.

Primary mouse kidney cells were chosen because these cells are terminally differentiated and do not have a proliferative capacity. Viral DNA (at a dilution of 0.01 μg/μl, corresponding to approximately 20 to 40 molecules) was injected into the nuclei of growth-arrested cells, which were then pulsed for 2-h intervals as indicated in Table 3. After these labeling periods, cells were fixed, stained for T antigen, and then processed for autoradiography (18). Data are presented as the percentage of T-antigen-positive cells showing ongoing incorporation of thymidine into DNA. In these cells, DNA from small-t mutant virus *dl*-884 induced cellular DNA synthesis as efficiently as did WT SV40 DNA and 85% of injected cells underwent DNA synthesis by 16 to 18 h following microinjection. Coinjection of small-t antigen had no effect on the time of onset or extent of cellular DNA synthesis. Similar results were obtained with a second nonpermissive cell type, mouse 3T3 cells (data not shown). The inability of purified small t to stimulate cellular DNA synthesis may be a consequence of the cell type we had to use in these microinjection experiments (nonpermissive rodent cells). It is possible that small t is able to stimulate cell DNA synthesis only in permissive cells.

TABLE 3. Stimulation of DNA synthesis in primary mouse kidney cells microinjected with viral DNA[a]

Labeling period (h postinjection)	% of T-antigen-positive cells stimulated by:		
	WT	*dl*-884	*dl*-884 + t protein
8–10	1	0.5	0.5
10–12	20	20	20
12–14	30	25	24
16–18	85	85	85

[a] Each value shown is based on three independent experiments. Less than 1% of the mock-injected cells incorporated thymidine.

DISCUSSION

SV40 T antigen is a multifunctional protein with several intrinsic biochemical activities required for virus production. The T antigen regulates the timing of the infection cycle in permissive cells, it represses its own transcription, and it initiates viral DNA replication. Initiation of viral DNA replication requires binding of the T antigen to the viral origin of replication (for a review, see reference 44). T antigen also induces cellular DNA synthesis in quiescent cells; it transforms tissue culture cells and induces tumor formation in animals (46). T antigen forms stable complexes with a number of cellular proteins that have been implicated in growth control (e.g., Rb1 and p53) and with the DNA polymerase-DNA primase complex (14).

Recently reported evidence indicates that the phosphorylation state of the T antigen is of importance for various T-antigen functions, such as viral DNA replication and cell transformation (9, 14, 31). The T antigen contains two clusters of phosphorylated serine and threonine residues. One is adjacent to its DNA-binding domain (Ser-106, Ser-111, Ser-112, Ser-120, Ser-123, and Thr-124), and the second is near the carboxy-terminal region of the protein (Ser-639, Ser-676, Ser-677, Ser-679, and Thr-701) (36–39). Some of these serine residues are selectively dephosphorylated by PP2A, and in vitro dephosphorylation of these amino acids increases the binding affinity of the T antigen for the viral DNA-binding site (II) and, hence, viral DNA replication (31, 34). Underphosphorylation of some of these amino acids is also associated with reduced transformation capacity of the large-T antigen (9). The SV40 small-t antigen binds cellular PP2A, reducing its activity against a variety of substrates which include the viral large-T antigen. Because PP2A has been shown to stimulate viral replication when added to highly purified cell-free DNA replication systems (26, 49), it was not surprising that small t reduced viral DNA synthesis in vitro by cellular extracts (5).

The paradox addressed by these experiments is that small-t antigen is known to increase virus yields and plaque size in infected monkey kidney cells, suggesting that it enhances rather than depresses viral replication. The data presented here suggest that reduced viral yields can be related directly to reduced viral DNA synthesis in the absence of functional small-t antigen. One possible explanation derives from observations of others that small t could induce continued cell cycling in nonpermissive cells (24) and that it played a role in transformation of nonpermissive cells primarily when growth-arrested cells were studied (28). These reports suggest that

small t functions to promote cell cycle progression in infected cells.

Support for this hypothesis comes from our flow cytometric analyses of infected CV1 cells, which showed that at low multiplicities, more cells infected with WT SV40 than those infected with the small-t mutant virus had DNA contents equivalent to the S or G_2 level. Because of the prolonged serum starvation needed with CV1 cells and the low virus multiplicities used, host cell induction was quite inefficient in these experiments and not all cells exited G_1. However, these conditions did allow an effect of small-t antigen to be observed. Additional evidence that small t can promote cell cycle progression was provided using theophylline to arrest cells. In theophylline-treated cells, small t is essential for release of cells from G_0. We have shown previously that theophylline does not appear to arrest cells through changes in cyclic AMP levels but does affect activation of the Na^+/H^+ antiporter (32), a key enzyme in growth induction. An effect of small t in stimulating progression of cells into the cell cycle might also explain our observation that coinjection of small t with either WT or mutant template DNA resulted in stimulation of replication to similar extents. Following microinjection, small t might trigger events which normally occur in G_0-G_1 progression leading to expression of enzymes which promote replication at G_1-S and the S phase. In this case, cells would be in an optimal environment for viral DNA replication once sufficient levels of large-T antigen were expressed from microinjected genomes. In addition to enzymes such as DNA polymerase, in vitro DNA replication studies (12) have also shown that the cyclin A-*cdc2* complex present in S-phase extracts can stimulate SV40 replication, possibly by promoting the required Thr-124 phosphorylation of large T.

Data shown in this report confirm those of several others (10, 15, 19, 21, 45) that large T is sufficient to induce cellular DNA synthesis in nonpermissive cells. Although we were unable to study the effect of microinjected small t on cellular DNA synthesis in permissive monkey cells because of the fragility of the cells under the long serum starvation conditions required for growth arrest, we did carry out such studies with nonpermissive cells. We were unable to demonstrate an effect of purified small-t protein on either the timing or the extent of induction of cellular DNA synthesis in microinjected nonpermissive cells. As in transformation (1), a role for small t in stimulating cell cycle progression might be apparent only when lower concentrations of T antigen are present. Alternatively, it has been shown in mouse embryo cells (24) that small t was not required to induce an initial round of cellular DNA synthesis but was required for subsequent rounds of division to occur. However, experiments with temperature-sensitive mutants indicated that in human cells permissive for SV40, induction of cellular DNA synthesis and mitosis depends only on the large-T antigen (46).

Our studies have demonstrated that under certain conditions, small t influences cell cycle progression. It should be noted, however, that even under conditions in which CV1 cells are not severely growth arrested, levels of viral DNA replication were consistently lower in the absence of small t. Thus, small t may promote viral DNA replication through some mechanism in addition to promotion of progression through the cell cycle. Alternatively, large T may induce G_1 progression by activating functions which normally occur in mid-G_1, bypassing some of the earlier steps of G_0-to-G_1 transitions or early G_1. This might create an intracellular environment which suffices for host DNA synthesis but is limited in key factors required for the additional demands placed on the cell by viral DNA synthesis.

The major finding of this study is that purified small t can stimulate DNA replication of microinjected WT or *dl*-884 genome. It does not seem likely that small t stimulates viral DNA synthesis in microinjected cells only by altering levels of large-T antigen. Similar levels of total large T appeared early after infection with either the WT or mutant virus, although T antigen from mutant infections appeared less phosphorylated at very early times postinfection, even in one-dimensional gels of immunoprecipitates. Furthermore, peptide maps suggested that early after infection, the large T isolated from mutant infections was less phosphorylated than that of the WT in peptide 7. Results from several laboratories suggest that dephosphorylation should correlate with increased, not decreased, viral DNA synthesis (22, 29, 34, 49). However, it should be pointed out that these results were obtained from in vitro experiments, which do not necessarily reflect in vivo conditions. Our flow cytometric studies of infected CV1 cells suggest that small t can promote cell cycle progression in permissive cells. This effect is probably mediated by the stimulation of the MAP kinase cascade, as reported very recently by Sontag et al. (42). The advantages of having cells in the late G_1 or S phase, if promoted by the small-t antigen, might then outweigh whatever influence small t might have on the phosphorylation state of proteins, such as the large-T antigen.

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