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Simian Virus 40 Large T-Antigen Function Is Required for Induction of Tetraploid DNA Content during Lytic Infection

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Infection of quiescent CV-1 cells with simian virus 40 mutant *ts*A30 at 37°C resulted in the induction of two rounds of cellular DNA synthesis in T-antigen-positive cells, as previously described for wild-type simian virus 40. Following infection with *ts*A30 at 40.5°C, T-antigen-positive cells were induced into S phase and reached a diploid G_2 DNA content; however, a second S phase was not initiated. The failure of *ts*A30-infected CV-1 cells to enter tetraploid S phase at 40.5°C identifies a T-antigen function, distinct from T-antigen functions responsible for stimulation of cell DNA synthesis, which is required for initiation of a second round of DNA synthesis without mitosis.

Simian virus 40 (SV40) stimulates both permissive and nonpermissive cells into DNA synthesis (4, 6). A number of experimental approaches have demonstrated that SV40 T antigen, in the absence of other viral components, is capable of inducing quiescent cells to enter S phase (1, 2, 5, 24, 26, 27, 31). Flow cytometric (FCM) analysis has confirmed that quiescent monkey kidney cells are induced into S phase following SV40 infection and has revealed that T-antigen-positive cells do not enter mitosis but continue to synthesize DNA, reaching DNA contents greater than that of a tetraploid G_2 cell prior to cell lysis (17, 19). The induction of two rounds of cellular DNA synthesis without mitosis implies that SV40 overrides the normal cell cycle controls which prevent initiation of a second S phase within a cell cycle.

To understand the mechanisms involved in DNA rereplication during SV40 infection, it is important to determine whether T-antigen function is required for entry into tetraploid S phase. Previous reports indicated that several SV40 mutants encoding temperature-sensitive T antigen were capable of inducing quiescent cells into DNA synthesis, but entry into tetraploid S phase was not examined (7, 25).

Confluent CV-1 cells (ATCC CCL70) were inoculated at a multiplicity of infection of 80 to 100 PFU per cell with wild-type SV40 strain RH911 or *ts*A30 (20, 29). Cells and virus stocks were mycoplasma free. Following adsorption for 1 h at 37°C, modified Eagle's medium with 2% fetal bovine serum was added and cultures were placed at either 34, 37, or 40.5°C. At each time point, a minimum of two cultures were harvested and processed independently. Following fixation in 90% methanol–phosphate-buffered saline (PBS), cells were stained with propidium diiodide (PI) for quantification of DNA and treated with monoclonal antibody against T antigen (PAB101, ATCC TIB 117) that was quantified via indirect immunofluorescent staining with fluorescein isothiocyanate (FITC). Data acquisition was performed with a Cytofluorograph IIs model 50 H-H and a 2151 data analysis system using an Omnichrome air-cooled argon laser (model 532) at a wavelength of 488 nm and 20 mW. Details on all procedures are available in references 9, 13, and 19.

***ts*A30-infected CV-1 cells enter tetraploid S phase at 34 and 37°C.** To determine whether a temperature-sensitive mutant of T antigen was capable of inducing tetraploidy, confluent CV-1 cells were infected with *ts*A30 and analyzed by quantitative two-color FCM for DNA content and levels of large T antigen. Uninfected confluent CV-1 cells were T-antigen negative and found only in the G_1, S, and G_2 phases of the cell cycle (Fig. 1A, left panel). When confluent cultures were infected with *ts*A30 at the permissive temperature of 34°C and analyzed at 72 h postinfection (p.i.), the majority of T-antigen-positive cells had a DNA content greater than that of cells in G_2 phase ($>G_2$), demonstrating that the infected cells were entering a second round of DNA synthesis (Fig. 1A, right panel). CV-1 cells infected with *ts*A30 at 37°C also accumulated in $>G_2$ phase, but movement of T-antigen-positive cells through the cell cycle proceeded more quickly than in cultures infected at 34°C (Fig. 1B and C). The levels of T antigen in each cell cycle phase were similar at 34 and 37°C (Fig. 1D). These results demonstrated that *ts*A30-infected CV-1 cells enter $>G_2$ phase when cultured either at 34 or 37°C. To minimize the difference between the permissive temperature and the nonpermissive temperature of 40.5°C, 37°C was used as the permissive temperature for the experiments that follow.

***ts*A30-infected cells are induced to enter S phase at 40.5°C.** Having established that *ts*A30-infected cells at 37°C enter a tetraploid S phase, it was of interest to determine whether maintenance at the nonpermissive temperature of 40.5°C would also result in two successive rounds of cellular DNA synthesis. The induction of quiescent cells into diploid S phase was examined first. At early times after infection, T-antigen-positive cells exited G_1 phase at a slightly faster rate in 40.5°C cultures than in 37°C cultures (Fig. 2A). This progression into S phase at 40.5°C indicated that the T-antigen function required for the induction of cellular DNA synthesis was retained in *ts*A30-infected cells at the nonpermissive temperature. The ability of *ts*A30-infected cells to move through the cell cycle at the nonpermissive temperature was further substantiated by a slightly faster rate of movement of T-antigen-positive cells into G_2 phase in 40.5°C cultures than in 37°C cultures (Fig. 2A). Maintenance at 40.5°C did not have any effect on the amount of T antigen expressed in G_2 phase, but lower quantities of T antigen were present in G_1 phase at 40.5°C (Fig. 2B). Therefore, lower levels of T antigen may be sufficient to induce the G_1-to-S transition at 40.5°C than are needed at 37°C. Mock-

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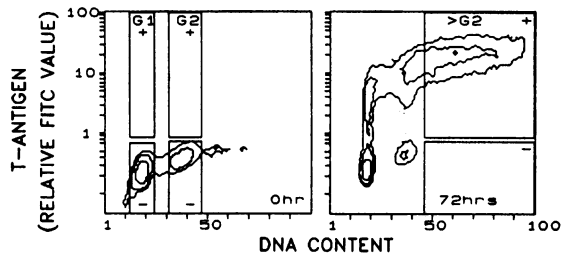
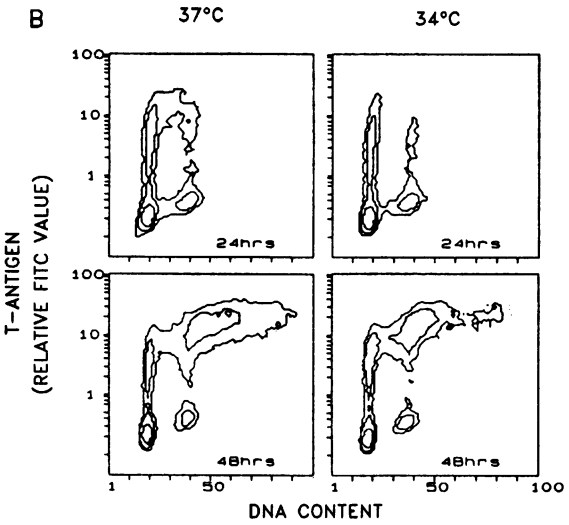
infected cells were not induced into DNA synthesis at either temperature (data not shown).

***ts*A30-infected CV-1 cells do not enter tetraploid S phase at 40.5°C.** As described above, CV-1 cells infected with wild-type SV40 or with *ts*A30 at 34 or 37°C do not enter mitosis but rather initiate a second round of cellular DNA synthesis. When *ts*A30-infected cultures at 40.5°C were analyzed at 48 and 52 h p.i., movement of T-antigen-positive cells into $>G_2$ phase was not observed (Fig. 3). In contrast, entry of T-antigen-positive cells into $>G_2$ phase was observed in parallel *ts*A30-infected cultures at 37°C. Cells infected with wild-type SV40 moved into $>G_2$ phase at 37 and 40.5°C (Fig. 3), demonstrating that the failure to enter $>G_2$ phase at the nonpermissive temperature was specific for *ts*A30. Contour plots of *ts*A30-infected cells at 52 h p.i. (Fig. 4A) clearly show that cells at 37°C have entered $>G_2$ phase and that the leading edge of the population has reached a tetraploid G_2 DNA content. However, *ts*A30-infected cultures at 40.5°C have not entered $>G_2$ phase. Absence of $>G_2$ cells at 40.5°C did not appear to result from cell death. Cell counts at 48 h p.i. were 20 to 30% higher in 40.5°C cultures than in 37°C cultures (data not shown), suggesting that a portion of the population was passing through mitosis. T-antigen-negative cells did not enter $>G_2$ phase at either temperature (Fig. 4A).

A



B



C CELL CYCLE DISTRIBUTION OF THE POSITIVE POPULATION

HRS•POST INF./ C	G1	G2	>G2
24/34	68	20	-
24/37	57	24	-
48/34	25	39	27
48/37	20	27	48

D MEAN Tag VALUE

HRS•POST INF./ C	G1	G2	>G2
24/34	4.3	5.3	-
24/37	5.1	8.7	-
48/34	4.7	11.9	19.6
48/37	3.9	9.3	17.3

To address the possibility that the failure of *ts*A30-infected cells to enter $>G_2$ phase at 40.5°C was due to insufficient quantities of T antigen, the levels of T antigen in each phase at 52 h p.i. were examined. The amount of T antigen in *ts*A30-infected G_2 phase cells at 40.5°C was slightly higher than at 37°C (Fig. 4B), but G_2 cells at 40.5°C do not enter $>G_2$ phase (Fig. 4A). Therefore, the failure of *ts*A30-infected cells to enter $>G_2$ phase at 40.5°C did not result from insufficient T antigen but appeared to be due to a loss of T-antigen function.

The results presented here demonstrate that entry into a second round of DNA synthesis is dependent on T antigen and that the T-antigen function responsible for reinitiation of DNA synthesis is lost in *ts*A30-infected cells at 40.5°C. The finding that *ts*A30 induces one round of cellular DNA synthesis at 40.5°C demonstrates that the temperature-sensitive T-antigen function required for entry into tetraploid S phase is distinct from the functions required for the induction of cellular DNA synthesis in quiescent cells.

The involvement of T antigen in the stimulation of the G_0/G_1-to-S transition is well established, but there is also evidence that T antigen can regulate events in G_2 phase. Rat embryo fibroblasts transformed with *ts*A58 (10) or *ts*A640 (22) arrest in G_1 and G_2 phases at the nonpermissive temperature. Superinfection of blocked cells with wild-type T antigen is followed by reentry of G_1 and G_2 cells into the cell cycle. T antigen has also been shown to overcome a G_2 block in temperature-sensitive rat 3Y1 cells (32). These results suggest that in some transformed cells, T antigen acts as a positive factor in G_2 progression, in addition to its role in G_1 phase.

Early FCM studies suggested that SV40-infected CV-1 cells were induced into one round of cellular DNA synthesis followed by a block in G_2 phase (3, 7). In one report,

FIG. 1. Cell cycle distribution and T-antigen expression in *ts*A30-infected CV-1 cells at permissive temperatures. Infected cultures incubated at 34 and 37°C were fixed in 90% methanol at 0, 24, 48 and 72 h p.i. Then 10,000 cells per sample were examined by FCM. Data in panels A and B are presented as contour plots with gates identifying the T-antigen- negative (−) and -positive (+) cells in the G_1, G_2, and $>G_2$ populations at 0 and 72 h p.i. and 34°C (A) and at 24 and 48 h p.i. and 37 and 34°C (B). (C and D) Summary of the average values of gated populations from duplicate samples for each time point illustrated in panel B. Listed are the percentages of the positive populations in the G_1, G_2, and $>G_2$ phases (C) and the FITC values (mean T-antigen values) of the positive population (D). T-antigen-positive cells are not found in $>G_2$ phase at 24 h p.i. at either temperature. The percentage of T-antigen-positive cells at 24 h p.i. was 32% at 34°C and 43% at 37°C and after 48 h was 68% at 34°C and 66% at 37°C.

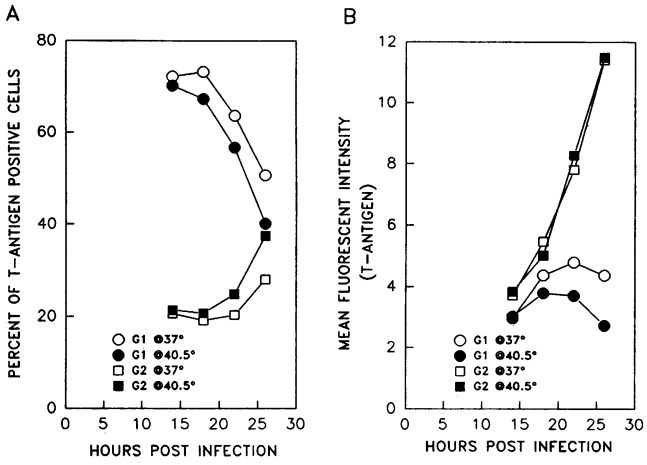


FIG. 2. (A) Effects of temperature on entrance into first S phase induced by *ts*A30. Confluent CV-1 cultures were infected with *ts*A30 and incubated at 37 and 40.5°C. At 14, 18, 22, and 26 h p.i., cells were fixed and processed for analysis of DNA and T-antigen content by FCM. Gates were placed on the T-antigen-positive G_1 and G_2 cells. Each time point represents the average of T-antigen-positive G_1 and G_2 cells from two cultures. (B) Quantitation of T antigen in G_1 and G_2 phases of *ts*A30-infected CV-1 cultures at 37 and 40.5°C. FCM data from time courses presented in Fig. 2A were examined for levels of T-antigen expression. The mean FITC value (T antigen) for gated T-antigen-positive G_1 and G_2 populations was the average from two cultures, and the results are displayed for each time point.

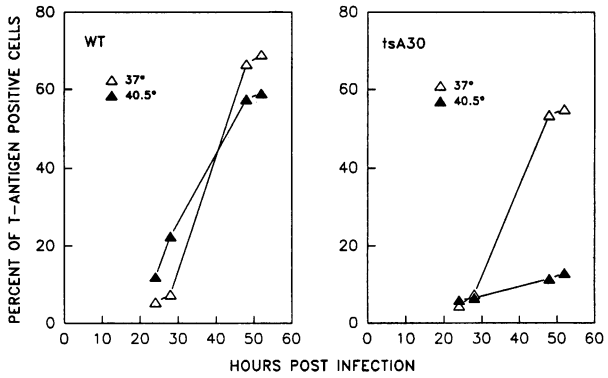
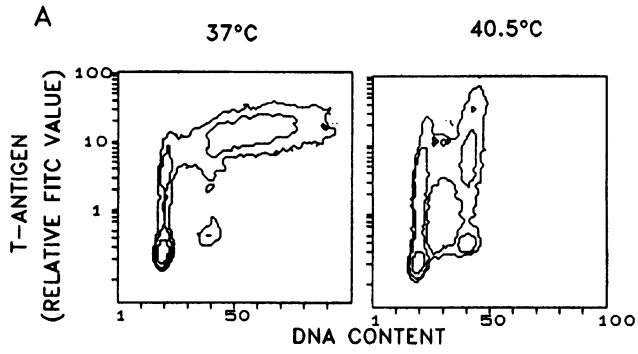


FIG. 3. Effects of temperature on second S phase induced by SV40. Wild-type and *ts*A30 SV40-infected confluent CV-1 cultures were maintained at 37 and 40.5°C for 24, 28, 48 and 52 h. The percentage of the T-antigen-positive population which entered a second S phase was determined by placing a gate on cells with a $>G_2$ DNA content. The averages from two cultures at each time point for both wild type and *ts*A30 are presented.

accumulation of infected cells with a DNA content 20% greater than that of 4C cells appeared to result from G_2-blocked cells containing replicated viral DNA (7). The detection of T-antigen-positive cells with DNA contents equivalent to those of tetraploid G_2 cells, as described here, is most likely the result of improved reagents, techniques, and FCM instrumentation. SV40-induced tetraploidy does not appear to be restricted to CV-1 cells, because a similar entry into $>G_2$ phase is observed following SV40 infection of Vero, BSC-1, and AGMK cells (16).

Temperature-sensitive T antigens are overproduced at the nonpermissive temperature, as determined by immunoprecipitation, but are also degraded more rapidly, leading to a reduced level of T antigen (30). Quantitative analysis of T antigen in G_2 phase *ts*A30-infected CV-1 cells demonstrated slightly higher levels of T antigen at 40.5°C than at 37°C, suggesting that a loss of function rather than a loss of quantity was responsible for failure to enter $>G_2$ phase. This finding is not inconsistent with previous reports. The values presented here are T antigen quantities in G_2 phase T antigen-positive cells, whereas immunoprecipitation measures the amount of T antigen in the total population. FCM analysis of SV40 lytic infection has established that the total amount of T antigen in a population is dependent on the cell cycle distribution of the population and that the highest levels of T antigen are expressed in $>G_2$ phase (16). The amount of T antigen in the total population at the permissive temperature increases relative to the total amount of T antigen at the nonpermissive temperature, because cells accumulate in $>G_2$ phase only at the permissive temperature.

Introduction of wild-type adenovirus E1A protein into quiescent BRK cells results in the stimulation of DNA synthesis followed by mitosis (11, 28). However, BRK cells infected with an E1A mutant containing a deletion of amino acids 121 to 150 are stimulated into S phase and then enter $>G_2$ phase rather than undergoing mitosis (21). Further investigation revealed that infection with E1A mutants containing smaller deletions in the region between amino acids 111 and 138 results in an increased accumulation of cells in $>G_2$ phase (8). The most efficient inducer of $>G_2$ cells, *dl*1108/520, contains a deletion between amino acids 124 and 127. This mutant exhibited loss of pRB and cyclin A binding and decreased p107 binding in HeLa cells, suggesting that the failure to form specific protein complexes may be in-



B

T-ANTIGEN EXPRESSION (MEAN FITC VALUE)

TEMP	G1	G2
37° C	4.02	10.27
40.5° C	4.37	12.26

FIG. 4. Cell cycle and T-antigen distributions late in the lytic cycle at 37 and 40.5°C. The 52-h *ts*A30-infected cultures at 37 and 40.5°C, described in Fig. 3, are displayed as contour plots illustrating the DNA content (A) and mean FITC (T-antigen) values (B). T-antigen values for gated T-antigen-positive G_1 and G_2 populations are averages from two cultures.

volved in the entry into $>G_2$ phase. The similar influences of wild-type T antigen and E1A *dl*1108/520 on the cell cycle suggest that interactions between T antigen, pRB, and p107 may be involved in the entry into $>G_2$ phase. In addition, formation of these T antigen complexes may be associated with changes in the activity of $p34^{cdc2}$ or other protein kinases involved in cell cycle regulation.

The presence of tetraploid-polyploid cells following the SV40 infection of nonpermissive cells in vivo and in vitro has also been reported (12, 14, 15, 18, 23). These cells are recognized as the potential precursors for emerging neoplastic cells. Therefore, the mechanisms responsible for the induction of multiple rounds of DNA synthesis in permissive cells may define a similar mechanism in the transformation process.

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