JOURNAL OF VIROLOGY, May 1979, p. 590-599
0022-538X/79/05-0590/10$02.00/0

Vol. 30, No. 2

Simian Virus 40 Gene *A* Regulation of Cellular DNA Synthesis

I. In Permissive Cells

J. B. HISCOTT AND V. DEFENDI*

Department of Pathology, New York University School of Medicine, New York, New York 10016

Received for publication 18 July 1978

The kinetics of host cellular DNA stimulation by simian virus 40 (SV40) *tsA58* infection was studied by flow microfluorometry and autoradiography in two types of productively infected monkey kidney cells (AGMK, secondary passage, and the TC-7 cell line). Prior to infection, the cell populations were maintained predominantly in G_0-G_1 phase of the cell cycle by low (0.25%) serum concentration. Infection of TC-7 or AGMK cells by wild-type SV40, viable deletion mutant *dl890*, or by SV40 *tsA58* at 33°C induced cells through S phase after which they were blocked with a 4N DNA content in the G2 phase. The infection of TC-7 cells by *tsA58* at 41°C, which was a nonpermissive temperature for viral DNA replication, induced a round of cell DNA synthesis in approximately 30% of the cell population. These cells proceeded through S phase but then re-entered the G1 resting state. In contrast, infection of AGMK cells by *tsA58* at 41°C induced DNA synthesis in approximately 50% of the cells, but this population remained blocked in the G2 phase. These results indicate that the mitogenic effect of the *A* gene product upon cellular DNA is more heat resistant than its regulating activity on viral DNA synthesis and that the extent of induction of cell DNA synthesis by the *A* gene product may be influenced by the host cell.

The isolation and characterization of temperature-sensitive mutants of simian virus 40 (SV40) have greatly fostered the investigation of the mechanisms by which papovaviruses induce cellular transformation. SV40 mutations imparting temperature sensitivity for viral DNA replication and cellular transformation have been localized to the distal half of the early region of the SV40 genome (*A* gene), particularly to the segment between 0.32 and 0.42 map units (16). Genetic mutations in this portion of the early region cause synthesis of a thermolabile polypeptide of 94,000 daltons (T antigen) without affecting a smaller protein of 17,000 to 20,000 daltons (t antigen), encoded for by the proximal portion of the early region of the genome (2, 8, 15, 30).

The *A* gene product has been shown to be involved in the initiation of new rounds of viral DNA replication (6, 27), autoregulation of early mRNA synthesis, and promotion of late virus-specific RNA synthesis (1, 12), probably mediated through binding to the origin of SV40 DNA replication (23, 31). In addition to these virus-related functions, the SV40 *A* gene product controls the establishment and maintenance of cellular transformation. Infection of the cells at the nonpermissive temperature with *tsA* mutants fails to induce transformation unless preceded by an incubation at the permissive temperature (4, 13, 14, 28). There is also good evidence that the *A* gene product may control DNA synthesis in transformed cells (3, 5). More specifically, Mueller et al. (20) recently demonstrated by microinjection of SV40 DNA fragments that, whereas viral DNA replication requires a completely intact early region, stimulation of cell DNA synthesis depends upon a subfragment of the early region encompassing 0.67 to 0.28 map units relative to the *Eco*RI site.

While the capacity of T antigen to induce cellular DNA synthesis has been demonstrated by direct intracellular microinjection of a highly purified preparation (32), previous studies (7) with *tsA* mutants have indicated that stimulation of host DNA synthesis still occurs during primary infection at the nonpermissive temperature, unless extreme and probably lethal temperatures (42.5°C) are used to inhibit functional *A* gene expression.

In this communication we have examined the kinetics of induction of cellular DNA synthesis in serum-arrested permissive cells and the proportion of the population mobilized into the cell cycle by infection with wild-type SV40, SV40 *dl890*, and SV40 *tsA58*.

MATERIALS AND METHODS

Virus and cells. SV40 *tsA58* virus was kindly provided by P. Tegtmeyer (29). The wild-type SV40 (wt SV40) used was strain RH-911. Viable deletion mutant *dl890* was obtained from T. Shenk (25). The monkey kidney TC-7 cell line was originally derived by J. Robb from CV-1 cells. Primary African green monkey cells (AGMK) were obtained from Flow Laboratories and grown to confluence in Dulbecco modified medium supplemented with 10% fetal calf serum (FCS; Grand Island Biological Co.).

Preparation of virus. Confluent TC-7 cells in 75-cm^2 flasks (Corning) were infected with SV40 at a multiplicity of infection of 0.1 to 0.5 PFU/cell. When cytopathic effect was advanced, cells and cellular debris were pelleted by low-speed centrifugation, resuspended in a small volume, and sonically disrupted (Branson Instruments Co.). The sonically treated cell extracts were treated with 0.1% (wt/wt) sodium deoxycholate for 60 min at 37°C and then centrifuged at 12,000 rpm for 60 min in a Beckman 21B centrifuge. The supernatant was layered over a 10-ml cesium chloride cushion (1.34 g/cm^2) in a 40-ml cellulose nitrate tube and centrifuged in an SW27 rotor at 25,000 rpm for 3 h. The virus band was collected, mixed with fresh cesium chloride (1.34 g/cm^2), and recentrifuged in an SW50.1 rotor at 37,000 rpm for 17 h. After collection the virus band was dialyzed against phosphate-buffered saline to remove CsCl.

Cultivation of cells and infection with virus. Monkey kidney (TC-7) cells and secondary AGMK cells were plated at a density of 8×10^4 cells per 35-mm dish in Dulbecco modified medium supplemented with 0.25 or 0.5% FCS. At 48 h after plating, the medium was changed and cells were incubated a further 48 h at 37°C. Prior to infection, the conditioned medium was removed, virions were diluted in conditioned medium, and the cells were either mock infected or infected at 5 to 10 PFU/cell with SV40 virions. In all experiments the minimum dilution of virions sufficient to produce 80 to 100% T antigen-positive cells at 48 h postinfection (p.i.) was used. After a 2-h incubation period, conditioned medium was returned to the cultures and the cells were incubated at 33 or 41°C.

Immunofluorescence for T and V antigen. Indirect immunofluorescence assay for SV40 T and V antigen was carried out with hamster anti-T sera and horse anti-SV40 capsid sera as previously described (17).

Measurement of DNA synthesis. The synthesis of DNA in uninfected and SV40-infected cells was measured in terms of the incorporation of tritiated thymidine ([^{3}H]dThd; New England Nuclear Corp.; 15 to 20 Ci/mmol). [^{3}H]dThd (10 μCi/ml) was added to the dishes at various times after infection. Cover slips (11 by 22 mm) with 3×10^4 to 4×10^4 cells were removed from the dishes, rinsed in four changes of phosphate-buffered saline, and allowed to dry in a vertical position. The cover slips were then fixed in Carnoy solution for 20 min, thoroughly dried, and introduced (cells facing up) into vials containing 5 ml of scintillation fluid (Liquifluor, New England Nuclear Corp.). After the radioactivity was assayed in a Beckman LS-350 scintillation counter, the cover slips were removed, rinsed in xylene, allowed to dry, and glued (cells facing up) on gelatinized microscope slides. The slides were filmed with Kodak AR-10 fine-grain autoradiographic stripping film. After 4 to 5 days in a tightly closed box containing desiccant, the slides were developed with Kodak D-19 developer. The slides were then stained with Giemsa, and the percentage of labeled cells was determined microscopically. On the average, 500 cells were counted per sample (19).

Preparation of cells for flow microfluorometry. Infected and control cultures (4×10^5 to 5×10^5 cells per 100-mm dish) were trypsinized at various times after infection and centrifuged at $150 \times g$ for 5 min. On ice, the cells were gently resuspended and fixed for 5 min with 10% (vol/vol) Formalin in water. After fixation the cells were centrifuged, suspended in 5 ml of distilled water, and centrifuged again. The cell suspension was treated with RNase (200 μg/ml) for 30 min at 37°C. The tubes were filled with distilled water and centrifuged at $150 \times g$ for 5 min. After resuspending, the cells were stained with 5 ml of propidium diiodide at 4°C for 15 to 20 min. The cells were then rinsed twice with distilled water and analyzed for DNA-specific fluorescence with a Biophysics Inc. Cytofluorgraf model 4800A (9).

Neutral sucrose gradient centrifugation. Cell suspensions in 0.9 ml of 0.15 M NaCl plus 0.015 M sodium citrate were gently lysed by the addition of 0.1 ml of 10% sodium dodecyl sulfate, and the DNA extracts were layered onto 32-ml sucrose density gradients (15 to 30% [wt/wt]) in 0.1 M NaCl-0.001 M EDTA-0.01 M Tris-hydrochloride (pH 7.4)-0.5% sodium dodecyl sulfate, formed over a 5-ml cushion of 70% sucrose. The gradients were centrifuged in an SW27 rotor (24,000 rpm for 12 h at 20°C) in a Beckman L5-75 preparative ultracentrifuge. After centrifugation, gradients were fractionated using an Isco model 185 density gradient fractionator. Sucrose gradient fractions (1.2 ml) were precipitated with an equal volume of 10% trichloroacetic acid, and the labeled acid-insoluble fraction was trapped on Whatman GF/C glass fiber filters. The filters were dried and assayed for radioactivity in toluene-based scintillation fluid (Liquifluor, New England Nuclear Corp.).

RESULTS

Viral DNA replication. The temperature sensitivity of SV40 *tsA58* DNA synthesis and the induction of virion capsid antigen (V antigen) were examined in productively infected AGMK and TC-7 cells. At a multiplicity of 1 to 5 PFU/cell, viral DNA synthesis commenced at approximately 48 h p.i. at 33°C and was maximal by 72 h in both wild type- and *tsA* mutant-infected cells. When cultures were infected and maintained at 41°C, viral DNA synthesis was detectable at 24 h p.i. in wt SV40-infected cells, but no viral DNA replication could be demonstrated in SV40 *tsA58*- infected cultures (Fig. 1, inset A). Likewise, no V antigen synthesis could be detected by immunofluorescence at 72 h in *tsA* mutant-infected cells maintained at 41°C. The kinetics of T antigen induction, however, at

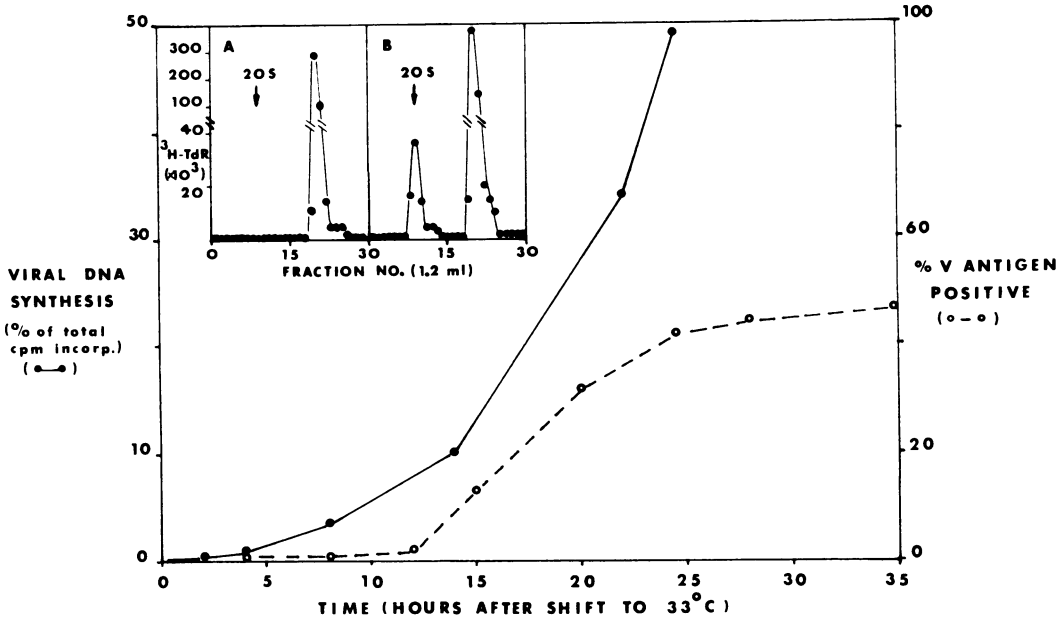

FIG. 1. *Induction of viral DNA synthesis and virion antigen in tsA58-infected TC-7 cells. Confluent TC-7 cells were infected with 1 to 5 PFU of SV40 tsA58 per cell and incubated at 41°C for 48 h. Cultures were then shifted to 33°C and incubated with [^{3}H]dThd (10 μCi/ml) for 2-h intervals. After labeling, cells were trypsinized, pelleted, resuspended in 0.15 M NaCl plus 0.015 M sodium citrate and lysed by addition of one-tenth volume of 10% sodium dodecyl sulfate. The lysates were poured onto a 15 to 30% (wt/wt) neutral sucrose gradient formed over a 5-ml cushion of 70% (wt/wt) sucrose. The gradients were spun in a Beckman L5-75 ultracentrifuge under the following conditions: SW27 rotor, 24,000 rpm, 12 h, 20°C. Fractions (1.2 ml) were analyzed for trichloroacetic acid-insoluble radioactivity. The amount of viral DNA synthesized was plotted as percent of the total [^{3}H]dThd incorporated during the labeling period (——). At various times after shift-down, parallel cultures were fixed and stained for induction of V antigen synthesis (---). (Inset A) Neutral sucrose velocity sedimentation profile of DNA synthesized in tsA58-infected cells at 0 to 2 h after shift-down. The arrow indicates the position of SV40 DNA form 1. (Inset B) Infected cells labeled at 12 to 14 h after shift-down and analyzed as in (A). In both insets, direction of sedimentation is left to right.*

the nonpermissive temperature was similar to that of the wild-type infection, with 70 to 100% of the cells becoming T antigen positive by 48 h p.i.

When *tsA58*-infected TC-7 cultures maintained at 33°C were shifted to 41°C at 72 h p.i., there was complete cessation of viral DNA synthesis within 2 h (not shown). When infected cultures were shifted from 41 to 33°C at 48 h p.i., viral DNA replication commenced within 2 to 4 h and virion capsid antigen became detectable at 12 to 15 h after shift (Fig. 1); for example, at 15 h after shift-down, 13% of the cell population was positive for V antigen, and between 12 and 14 h post-shift, 10% of the DNA synthesized during a 2-h pulse was viral (Fig. 1, inset B). Very similar results were obtained with AGMK cells (see later). These results essentially confirm the observations reported by Tegtmeyer (27).

Induction of host DNA synthesis. Having established that SV40 *tsA58* is temperature sensitive with respect to viral DNA replication and V antigen synthesis, the effect of the *tsA* gene product on cellular DNA synthesis was examined. In all the experiments described, cultures were maintained in low serum (0.25%) for 4 to 5 days to reach a quiescent state which reduced the labeling index to 7 to 10% (continuous labeling over a 12-h period). After infection at 33°C with purified virions or mock infection, conditioned medium was returned to the cultures to maintain a low background of DNA synthesis; cultures were returned to the appropriate temperature and at various times thereafter were analyzed for induction into S phase by [^{3}H]dThd incorporation and autoradiography and for cell cycle distribution by flow microfluorometry.

Autoradiography of wt SV40- and SV40 tsA58-infected cells. Infection with wt SV40 or *tsA* mutant at 33°C induced DNA synthesis in TC-7 and AGMK cells at approximately 30 h p.i. These data are not shown since the autoradiography technique prima facie does not distinguish between viral and cellular DNA synthesis.

The results obtained after infection at 41°C are illustrated in Fig. 2.

Several points concerning the induction of cell DNA synthesis can be made from these data (Fig. 2): (i) mock-infected cells maintained at 41°C remained approximately 10% positive for DNA synthesis between 14 and 60 h; (ii) infection of both TC-7 cells (Fig. 2A) and AGMK cells (Fig. 2B) by wt SV40 or *tsA58* at 41°C produced qualitatively similar time course profiles; (iii) a burst of DNA synthesis involving 30% of the *tsA* mutant-infected TC-7 cells occurred at 18 to 24 h p.i. after which the number of cells in S phase returned to near control levels; (iv) *tsA* mutant-infected AGMK cells produced a similar burst of DNA synthesis but the number of cells in S phase decreased more gradually with time; (v) autoradiography results of cells infected with wt SV40 or an early viable deletion mutant of SV40, *dl890* (data not shown), showed high numbers of cells synthesizing DNA throughout the time course although it should be reiterated that this reflects viral as well as cellular DNA.

Separation of newly synthesized viral and cellular DNA. From the autoradiographic results of Fig. 2, it appeared that a burst of DNA synthesis occurred at 20 to 24 h p.i. in SV40 *tsA58*-infected cells maintained at 41°C. To examine whether an abortive or partial viral DNA replication contributed to the observed stimulation of DNA synthesis, wt SV40- and *tsA58*-infected AGMK cells were labeled with [^{3}H]-dThd for 2 h at 20 to 22 h (Fig. 3a) and at 36 to 38 h (Fig. 3b) p.i. and cell lysates were analyzed by velocity sedimentation in 15 to 30% neutral sucrose. In wt SV40- or SV40 *dl890*-infected cells at 20 to 22 h p.i., a small peak of DNA corresponding to 20S viral DNA was detectable (pool 1), indicating that SV40 DNA replication was initiated at this time. At 36 to 38 h p.i. (Fig. 3b), the peak corresponding to viral DNA (20S) was greatly increased and the peak of high-molecular-weight cell DNA (70S, pool 2) showed a 2.5-fold increase in [^{3}H]dThd incorporation relative to the control. In *tsA58*-infected cultures maintained at 41°C, however, at both time points, there was no incorporation of [^{3}H]dThd in the 20S region, although there was a threefold increased incorporation into the newly synthesized cellular DNA (pool 2) when compared to the mock-infected control cells. These results indicate that the burst of DNA synthesis observed by autoradiography (Fig. 2) in a discrete cell population must reflect stimulation of cellular DNA.

Cellular DNA content of wt SV40- and deletion mutant-infected AGMK cells. The induction of serum-arrested secondary cultures of AGMK cells into the cell cycle following wt

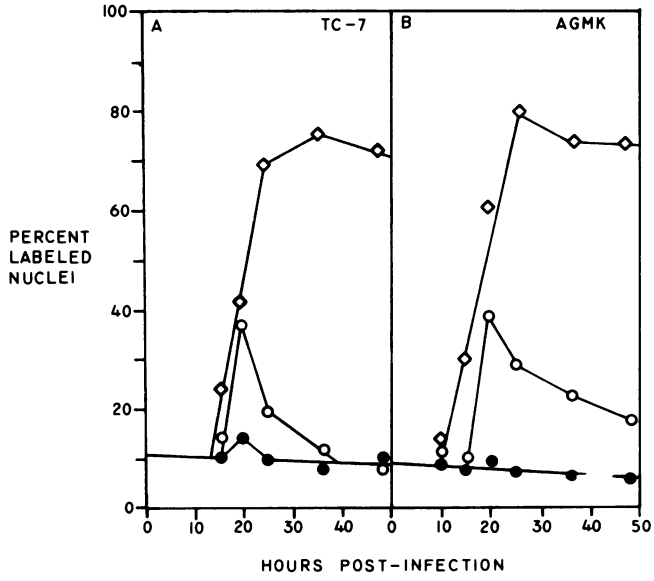

FIG. 2. *Autoradiography of wt SV40- and SV40 tsA58-infected TC-7 (A) and AGMK (B) cells. TC-7 and AGMK cells were plated and maintained in medium containing 0.25% FCS for 72 h. Cultures maintained at 41°C were infected with 5 to 10 PFU of purified virions per cell diluted in conditioned medium and, at the end of the adsorption period, conditioned medium was returned to the cells. At various times after infection, cultures were pulse labeled for a 1-h period with 10 μCi of [^{3}H]dThd per ml and prepared for autoradiography as outlined in the text. Symbols: ●, control; ○, tsA58; ◇, wt SV40.*

SV40 or deletion mutant *dl890* infection is illustrated in Fig. 4 and 5. After growth in low serum, the majority (70 to 90%) of the cells used in these experiments were in G1 phase of the cell cycle and possessed a 2N DNA content. Approximately 10 to 15% of the cells, with a 4N DNA content, were at some point in the G2 phase. Since few cells were binucleate prior to fixation in 10% Formalin, it appears that a minor proportion of the quiescent population was blocked at some point after DNA replication and prior to karyokinesis; this fraction may also include a small percentage of tetraploid cells. Those cells with an intermediate DNA content were in mid-S phase at the time of fixation.

Figure 4 and Table 1 illustrate the effects of wt SV40 infection upon G1-arrested AGMK cells. By 24 h after infection with wt SV40 at 37°C, the proportion of cells in G1 phase had decreased to 50% of the population, and the number of cells in S phase and G2 + M had increased proportionately from 10 to 20% and 15 to 30%, respectively. When compared to the control population, the DNA content profile at 24 h p.i. suggests the re-entry into the cell cycle of a population of relatively quiescent cells. At 36 h p.i. with wt SV40, most of the cell population possessed a 4N DNA content (Table 1) and presumably the cells were located at some G2 point in the cell cycle subsequent to S phase but prior to mitosis. However, from the 48-h DNA content profile, it appeared that the cells did not progress through mitosis but remained in G2 + M; it was also apparent that the bulk of the cell population had a greater than 4N DNA content. The shift in DNA content at 48 h probably reflects the accumulation of newly synthesized viral DNA between 36 and 48 h p.i.

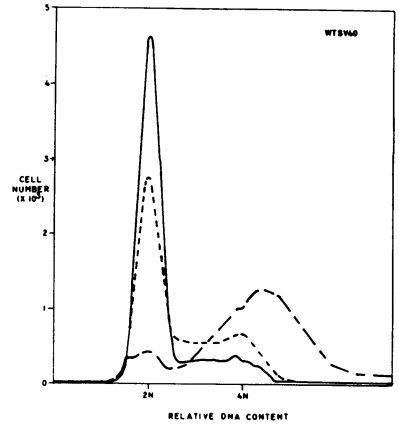

FIG. 4. *Cell DNA content of wt SV40-infected AGMK cells. AGMK cells were plated at 5 × 10⁵ cells per 100-mm Falcon dish in medium containing 0.25% FCS. These cultures were infected 96 h later, returned to conditioned medium, and placed at 37°C. At various times after infection, cultures were fixed in 10% Formalin, treated with 1 mg of RNase per ml for 30 min at 37°C, and stained with 0.05 mg of propidium diiodide per ml in 1.12% sodium citrate. The fixed-cell preparations were analyzed for DNA-specific dye fluorescence with a Biophysics Inc. Cytofluorograf model 4800A. Symbols: ——, control; -----, 24 h p.i.; — - —, 48 h p.i.*

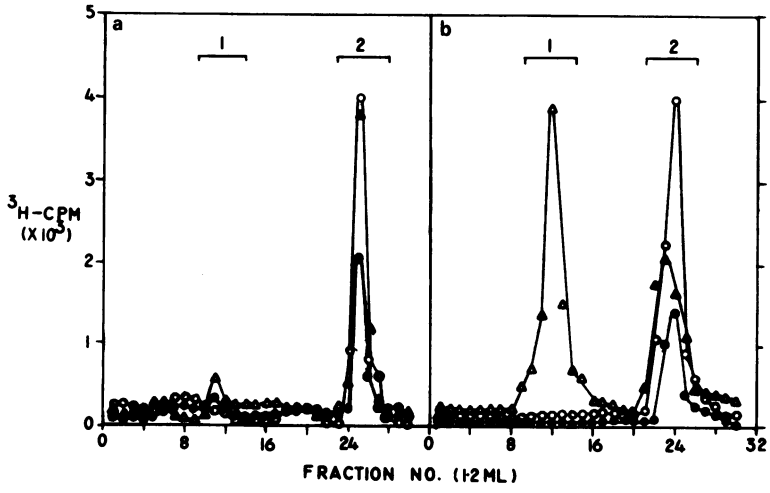

FIG. 3. *Neutral sucrose velocity sedimentation analysis of DNA synthesized in SV40-infected AGMK cells. Cultures of AGMK cells were infected by wt SV40 or SV40 tsA58 as described in the text. After 20 h (a) and 36 h (b) at 41°C, control and infected cells were labeled with [³H]dThd (10 μCi/ml) for 2 h. At the end of the labeling period, cells were harvested and analyzed as described in the legend of Fig. 1. Symbols: ●, control; ○, tsA58; △, wtSV40.*

TABLE 1. *Cell cycle distribution of wt SV40-infected AGMK cells*[a]

Hours p.i.	% of cell population		
	G1	S	G2 + M
Control (24 h)	76	11	13
24	49	21	30
36	9	16	75
48	8	12	80

[a] The percentage of cells in G1 and G2 + M was determined from the cell DNA content profiles by integration of the areas under the 2N and 4N peaks. These percentages were used to calculate an approximate value for the proportion of cells in S phase.

Since the rapid synthesis and accumulation of viral DNA in AGMK cells (as shown in Fig. 3, right) could affect the interpretation of flow microfluorometric results, the absolute quantities of viral and cellular DNA were measured. After infection by wt SV40 at 41°C for 48 h, cell lysates were centrifuged in neutral sucrose, and peaks of viral and cellular DNA were isolated and centrifuged to equilibrium in ethidium bromide-cesium chloride dye buoyant density gradients. After dialysis to remove cesium chloride, the quantities of viral and cellular DNA were measured by spectrophotometry. In these experiments, SV40 DNA accounted for 15 to 20% of the total DNA content of the cell. Thus, the maximal contribution of viral DNA to the total DNA content of the cell produced a 5- to 6-unit shift in the peak of DNA content (25 relative fluorescence units separate the 2N and 4N peaks of DNA in flow microfluorometry). Therefore, it appears that SV40 infection induced the quiescent cell population into the cell cycle by 24 h after infection and, by 36 h, the majority of the cells possessed a 4N DNA content. Between 36 and 48 h after infection the infected cells did not undergo mitosis but accumulated viral DNA and cellular DNA. Presumably the cytotoxic effect of virus replication inhibited mitosis and the cells eventually died.

The data of Fig. 5 and Table 2 demonstrated that quantitatively similar kinetics occurred when AGMK cells were infected with a viable deletion mutant, *dl890*, of SV40. In *dl890*-infected cells at 48 h p.i., the cell population had not shifted to a greater than 4N peak and suggested that large amounts of viral DNA had not yet accumulated. This result is consistent with the finding that viral DNA replication occurs more slowly in viable deletion mutant infected cells (25).

The results presented in Fig. 4 and 5, obtained by wt SV40 and *dl890* infection of secondary passage AGMK cells, were essentially identical to the results obtained with the TC-7 cell line.

Cell DNA content of *tsA* mutant-infected AGMK cells. The influence of the SV40 *A* gene upon cell cycle traverse and DNA content was examined in SV40 *tsA58*-infected AGMK cells maintained at the permissive (33°C) or nonpermissive (41°C) temperatures. At the permissive temperature for functional SV40 *A* gene product expression, the changes in DNA content were similar to the results produced by wt SV40 infection. At 26 h p.i. with *tsA58* at 33°C, the number of cells in S phase and G2 increased concurrent with the decrease in the G1 population (Fig. 6 and Table 3). Between 32 and 44 h p.i. the infected cell population progressed to a 4N DNA content with only 5% of the cells remaining in G1 (Fig. 6).

When secondary AGMK cells were infected with SV40 *tsA58* and maintained at 41°C, the cells were induced into DNA synthesis at approximately 20 h p.i. (Table 3). By 32 h p.i., about 50% of the cells contained a 4N cell DNA content. During the next 12 h, this profile changed only slightly with a small decrease in the 2N population and an increase in the number

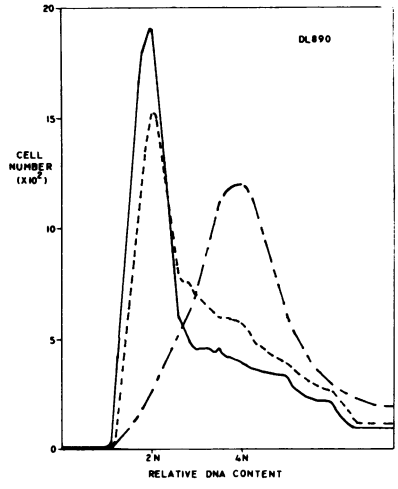

FIG. 5. *Cell DNA content of dl890-infected AGMK cells. AGMK cells were cultured, infected, and analyzed as described in Fig. 4. Symbols: ——, control; -----, 24 h p.i.; — - —, 48 h p.i.*

TABLE 2. *Cell cycle distribution of SV40 dl890-infected AGMK cells*[a]

Hours p.i.	% of cell population		
	G1	S	G2 + M
Control (24 h)	63	10	27
20	47	20	33
24	42	19	39
48	5	17	78

[a] The proportion of cells in G1 and G2 + M was determined as described in the legend of Table 1.

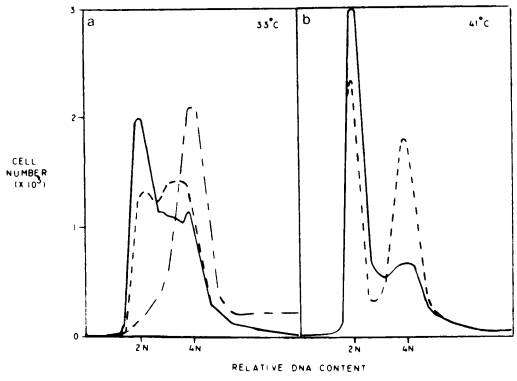

FIG. 6. *Cell DNA of SV40 tsA58-infected AGMK cells. AGMK cultures were plated and infected as described in the legend to Fig. 4. At the end of the adsorption period, conditioned medium was returned to the cells, and infected and mock-infected cultures were placed at 33 or 41°C. At various times after infection, cultures were fixed and prepared for flow microfluorometry as described in Fig. 4. Symbols: (a) SV40 tsA58-infected AGMK cells at 33°C: ——, 26 h p.i.; -----, 32 h p.i.; — - —, 44 h p.i. (b) SV40 tsA58-infected AGMK cells at 41°C: ——, mock-infected; -----, 44 h p.i.*

TABLE 3. *Cell cycle distribution of SV40 tsA58-infected AGMK cells*[a]

Temp (°C)	Hours p.i.	% of cell population		
		G1	S	G2 + M
33	Control (26 h)	67	11	22
	26	50	23	27
	32	29	36	35
	44	4	16	80
41	Control (26 h)	82	6	14
	21	42	28	30
	26	32	26	44
	32	36	11	53
	44	32	12	56

[a] The proportion of cells in G1 and G2 + M was determined as described in the legend to Table 1.

of 4N cells. Thus, infection of AGMK cells with *tsA58* at 41°C induced approximately half the cell population through S phase into G2 phase, with a 4N DNA content where the cells remained arrested. The appearance of the 4N population occurred under conditions which completely inhibited viral DNA replication (Fig. 3).

Cell DNA content of *tsA* mutant-infected TC-7 cell line. The infection of the TC-7 cell line with SV40 *tsA58* produced a difference in host DNA induction at the nonpermissive temperature when compared with the *tsA* mutant-infected AGMK cells. Infection with SV40 *tsA58* at 33°C induced the resting cell population from G1 into S phase commencing at 30 h p.i.; by 48 h p.i., analysis of DNA content indicated that over 85% of the cells were in S phase or G2 + M (Fig. 7a and Table 4). Integration of the areas under the G1 and G2 + M peaks of the flow microfluorometry profiles indicated that, while only 5% of a control population was in S phase, 70% of the infected population was mobilized by *tsA58* virus infection into the cell cycle (Table 4).

In contrast to the cell cycle pattern obtained at the permissive temperature, maintenance of SV40 *tsA58*-infected cells at 41°C for 48 h resulted in a cell population with a DNA content profile essentially indistinguishable from the control profile. This result was also in contrast to the results at 41°C with *tsA58*-infected AGMK cells which accumulated in G2 + M. Although both cell types appeared to be transiently induced into DNA synthesis as evidenced by the autoradiography results of Fig. 2 and by flow microfluorometry profiles analyzed at approximately 24 h p.i., TC-7 cells apparently returned to their low serum-induced resting state while induced AGMK cells became a population of cells with a 4N DNA content.

DISCUSSION

In the present study, the time course of induction of resting cells into DNA synthesis and the changes in cell cycle distribution were examined in monkey kidney cells (secondary AGMK and TC-7 cell line) productively infected with wt SV40, a viable deletion mutant of SV40 (*dl890*), and a temperature-sensitive gene *A* mutant (*tsA58*). Induction of host DNA synthesis and replication of viral DNA occurred in resting cultures after infection by wt SV40 or SV40 *dl890* at 33, 37, or 41°C, or by SV40 *tsA58* at

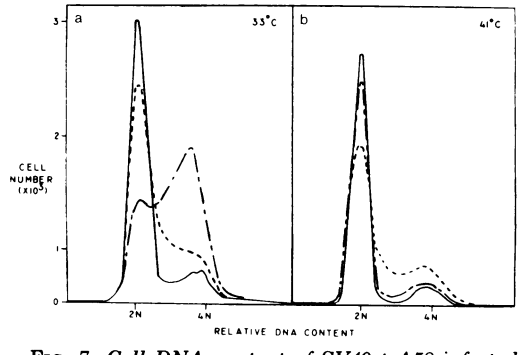

FIG. 7. *Cell DNA content of SV40 tsA58-infected TC-7 cells. TC-7 cells were treated as described in the legend to Fig. 4. Symbols: (a) SV40 tsA58-infected TC-7 cells at 33°C: ——, mock-infected; -----, 30 h p.i.; — - —, 48 h p.i. (b) SV40 tsA58-infected TC-7 cells at 41°C: ——, mock-infected; -----, 24 h p.i.; — - —, 48 h p.i.*

TABLE 4. *Cell cycle distribution of SV40 tsA58-infected TC-7 cells*[a]

Temp (°C)	Hours p.i.	% of cell population		
		G1	S	G2 + M
33	Control (48 h)	80	5	15
	32	61	22	17
	48	12	20	68
41	Control (48 h)	90	0	10
	24	49	20	21
	48	80	6	14

[a] The proportion of cells in G1 and G2 + M was determined as described in the legend to Table 1.

33°C. AGMK or TC-7 cells induced into DNA synthesis by SV40 infection did not continue cell cycle traverse but arrested in G2 phase with a 4N DNA content. The infected cell population did not progress through mitosis but at times later than 36 h p.i. accumulated significant quantities of newly synthesized viral DNA. By 48 h p.i., 15 to 20% of the total DNA in the cell was viral DNA which produced a shift of five to six channels in the flow microfluorometer. The DNA content profile indicated a cell population with a greater than 4N DNA content.

Infection of TC-7 cells at high temperature (41°C) with SV40 *tsA58* produced a transient induction of DNA synthesis involving a round of DNA synthesis in approximately 30% of the cell population at 20 to 24 h p.i. The stimulation of cell DNA synthesis in SV40 *tsA58*-infected TC-7 cells maintained at 41°C was not permanent, however, and at later times these cells returned to their low serum-induced G1 resting state although they remained T antigen positive by immunofluorescence. TC-7 cells infected by SV40 *tsA58* can be passaged at the nonpermissive temperature for several generations without evidence of any growth advantage conferred by T antigen (J.B.H., unpublished data).

In contrast to the results obtained with the permanent TC-7 cell line, secondary cultures of AGMK cells were more susceptible to induction by wt SV40 and SV40 *tsA58* as previously described (R. Weil, H. Turler, N. Leonard, and C. Ahman-Zadeh, INSERM, in press). AGMK cells responded to wt SV40 infection at 37 or 41°C or SV40 *tsA58* infection at 33°C similarly to TC-7 cells; the cells were induced from the low serum-induced G1 block through S phase to a G2 state with a 4N DNA content. However, *tsA* mutant infection of AGMK cells at 41°C induced approximately 50% of the cell population through S phase to G2 + M by 36 h p.i. The stimulation of resting AGMK cells by *tsA58* at 41°C was also abortive, since by autoradiography and flow microfluorometry the number of cells in S phase decreased after the initial burst of DNA synthesis at 20 to 24 h. Unlike the situation in TC-7 cells, the 4N DNA population of AGMK cells did not return to a G1 state. At later times after infection, the DNA content profile of SV40 *tsA58*-infected AGMK cells did not change significantly, suggesting that the cells did not continue to traverse the cell cycle but remained as either G1 or G2 populations. The induction in AGMK cells was apparently not multiplicity dependent since *tsA* mutant infection at 41°C with about 100 PFU/cell produced cellular DNA profiles identical to those obtained when cultures were exposed to 5 PFU/cell (data not shown).

Differences between AGMK and TC-7 cells in their inducibility into DNA synthesis suggest that susceptibility to induction of DNA synthesis by SV40 *tsA58* at high temperatures may have a host cellular component. This may be related to the inherent metabolic differences between diploid secondary cell cultures and heteroploid cell lines that have been in culture for many generations. In this context the BSC-1 monkey line has been reported not to be inducible into cell DNA synthesis by wt SV40 (24).

After the transient induction into the cell cycle of a fraction of the cell population by SV40 *tsA58* infection at 41°C, infected AGMK cells block at some point in G2 phase whereas TC-7 cells return to G1 phase. Although the data presented here do not explain this difference in arrest points, experiments are planned to follow the fate and test the viability of the two AGMK populations. No attempts were made in these experiments to follow the fate of productively infected cells blocked at 4N DNA content since, with wt SV40 and SV40 *tsA58* at the permissive temperature, cytopathic effects ensued. However, the fate of *tsA58*-infected 4N AGMK cells maintained at 41°C may be followed since no viral replication occurs at high temperature. It has been reported previously in nonpermissive cell populations (i.e., Chinese hamster embryo cells and mouse peritoneal macrophages) that a viable tetraploid population is eventually produced after SV40 infection (10, 18).

Previously, Chou and Martin (7) demonstrated that, in permissive CV-1 cells, cellular DNA synthesis was stimulated by *tsA* mutant infection at 40°C when measured at 24 h p.i., although this temperature inhibited viral DNA replication. However, when a temperature of 42.5°C was used, no stimulation of cellular DNA synthesis occurred. In our experiments, it was found that mouse 3T3 cells, Chinese hamster kidney cells, AGMK cells, and TC-7 cells were all nonviable (inability to replicate) at 42.5°C (unpublished observation), although at 41°C all

lines with the exception of 3T3 grew to saturation densities equivalent to those obtained at 37°C. It was not possible to find a temperature which completely abrogated the transient stimulation of cell DNA synthesis by *tsA* mutant infection while maintaining cell viability.

Since the viable deletion mutant, *dl890*, which affects a 17K species of T antigen (8) induced DNA synthesis in both permissive AGMK cells and nonpermissive Chinese hamster kidney cells (data not shown), this early SV40 gene product is not involved in stimulation of host DNA synthesis. The ability of the other early SV40 gene product, the *A* gene protein, to abortively stimulate resting cells to enter the cell cycle at 41°C suggests that the capacity of the *A* gene product to induce DNA synthesis in permissive cells is more heat resistant than the *A* gene activity regulating viral DNA replication. This may reflect two distinct functional activities associated with the *A* gene product or one activity with different specificities for viral and cellular DNA.

Tjian (31) has recently shown that a hybrid Ad_2D_2 protein closely related to the *A* gene product binds to and protects from DNase digestion a 30 to 120 nucleotide DNA fragment located near the origins of SV40 DNA replication and transcription. It was suggested that the protein may bind in vivo as an aggregate to regulate viral replication and late transcription. In this context, previous experiments of Kuchino and Yamaguchi (15) indicated that in *tsA* mutant-infected cells the *A* gene product failed to aggregate, and thus was unable to initiate viral replication.

If the binding of an aggregated *A* gene product regulates viral DNA replication, a possible explanation for the different sensitivities of viral and cellular DNA replication to *tsA* mutant infection at the nonpermissive temperature is that the residual mitogenic activity may be associated with a monomeric form of the *A* protein. In this configuration the *A* protein would then still be capable of transiently stimulating cell DNA synthesis but would fail to control viral DNA replication. Alternatively the *A* gene product may act together with another as-yet-unidentified viral or cellular protein with regulatory function to continuously stimulate cellular DNA synthesis.

In our experiments the evidence for the complete progression through S phase is consistent with the existence of a rate-limiting event (or transition) in G1 as proposed by Smith and Martin (26). The expression of the SV40 *A* gene product in infected cells is capable of forcing quiescent G1 cells past the critical transition to trigger a cascade of events in G1 phase which culminate in chromosomal DNA replication. Normally, eucaryotic DNA synthesis is preceded by enhanced membrane transport, increased RNA synthesis, and de novo protein synthesis in G1 phase (22). Circumstantial evidence of both a chronological (11, 32) and biochemical (21) nature supports the notion that the mitogenic activity of the *A* gene product acts at the G_0-G_1 level to stimulate cells from a serum-induced resting state.

ACKNOWLEDGMENTS

The technical assistance of Kate Smith and Helene Weiss is gratefully acknowledged.

This work was supported by Public Health Service grant CA-16239 and ACS VC 73.

LITERATURE CITED

1. **Alwine, J. C., S. I. Reed, and G. R. Stark.** 1977. Characterization of the autoregulation of simian virus 40 gene A. J. Virol. **24**:22-27.
2. **Anderson, J. L., C. Chang, P. T. Mora, and R. G. Martin.** 1977. Expression and thermal stability of simian virus 40 tumor-specific transplantation antigen and tumor-antigen in wild type- and tsA mutant-transformed cells. J. Virol. **21**:459-467.
3. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860-870.
4. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene A function in maintenance of transformation. J. Virol. **15**:619-635.
5. **Butel, J. S., and H. R. Soule.** 1978. Role of the simian virus 40 gene A product in regulation of DNA synthesis in transformed cells. J. Virol. **26**:584-594.
6. **Chou, J. Y., J. Avila, and R. G. Martin.** 1974. Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116-124.
7. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. **15**:145-150.
8. **Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeyer, K. Rundell, and P. Berg.** 1978. Organization and expression of early genes of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**:117-121.
9. **Crissman, H. A., P. F. Mullaney, and J. A. Steinkamp.** 1975. Methods and applications of flow systems for analysis and sorting of mammalian cells. Methods Cell Biol. **9**:179-205.
10. **Hirai, K., G. Campbell, and V. Defendi.** 1974. Changes of regulation of host DNA synthesis and viral DNA integration in SV40-infected cells, p. 151-166. *In* B. Clarkson and R. Baserga (ed.), Control of proliferation in animal cells. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Ide, T., S. Whelley, and R. Baserga.** 1977. Stimulation of RNA synthesis in isolated nuclei by partially purified preparations of simian virus 40 T antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:3189-3192.
12. **Khoury, G., and E. May.** 1977. Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T antigen. J. Virol. **23**:167-176.
13. **Kimura, G., and R. Dulbecco.** 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529-534.
14. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral

genetic property. Proc. Natl. Acad. Sci. U.S.A. **72**:673-677.

15. **Kuchino, T., and N. Yamaguchi.** 1975. Characterization of T antigen in cells infected with a temperature-sensitive mutant of simian virus 40. J. Virol. **15**:1302-1307.
16. **Lai, C.-J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70-81.
17. **Lehman, J., and V. Defendi.** 1970. Changes in DNA synthesis regulation in Chinese hamster cells infected with simian virus 40. J. Virol. **6**:738-749.
18. **Lehman, J., J. Mauel, and V. Defendi.** 1971. Regulation of DNA synthesis in macrophages infected with simian virus 40. Exp. Cell Res. **67**:230-233.
19. **Mauel, J., and V. Defendi.** 1971. Infection and transformation of mouse peritoneal macrophages by simian virus 40. J. Exp. Med. **135**:335-350.
20. **Mueller, C., A. Graessmann, and M. Graessmann.** 1978. Mapping of early SV40 specific functions by microinjection of early viral DNA fragments. Cell **15**:579-585.
21. **Postel, E. H., and A. J. Levine.** 1976. The requirement of SV40 gene A product for the stimulation of thymidine kinase activity after virus infection. Virology **73**:206-215.
22. **Prescott, D.** 1976. The cell cycle and the control of cellular proliferation. Adv. Genet. **18**:99-177.
23. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T antigen binds to simian virus 40 DNA at the origin of replication. Proc. Natl. Acad. Sci. U.S.A. **74**: 457-461.
24. **Ritzi, E., and A. J. Levine.** 1969. DNA replication in simian virus 40-infected cells. J. Virol. **5**:686-692.
25. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664-671.
26. **Smith, J. I., and L. Martin.** 1973. Do cells cycle? Proc. Natl. Acad. Sci. U.S.A. **70**:1263-1267.
27. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **18**:664-671.
28. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene A in transforming infection. J. Virol. **15**:613-618.
29. **Tegtmeyer, P., and H. L. Ozer.** 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516-524.
30. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and R. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. J. Virol. **16**:168-178.
31. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen related protein. Cell **13**:165-179.
32. **Tjian, R., G. Fey, and A. Graessman.** 1978. Biological activity of purified simian virus 40 T antigen proteins. Proc. Natl. Acad. Sci. U.S.A. **75**:1279-1283.