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

II. In Nonpermissive Cells

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The stimulation of host macromolecular synthesis and induction into the cell cycle of serum-deprived G_0-G_1-arrested mouse embryo fibroblasts were examined after infection of resting cells with wild-type simian virus 40 or with viral mutants affecting large T antigen (*tsA*58) or small t antigen (*dl*884). At various times after virus infection, cell cultures were analyzed for DNA synthesis by autoradiography and flow microfluorimetry. Whereas mock-infected cultures remained quiescent and displayed either a 2N DNA content (80%) or a 4N DNA content (15%), mouse cells infected with wild-type simian virus 40, *tsA*58 at 33°C, or *dl*884 were induced into active cell cycling at approximately 18 h postinfection. Although *dl*884-infected mouse cells were induced to cycle initially at the same rate as wild type-infected cells, they became arrested earlier after infection and also failed to reach the saturation densities of wild-type simian virus 40-infected cells. Infection with *dl*884 also failed to induce loss of cytoplasmic actin cables in the majority of the infected cell population. Mouse cells infected with *tsA*58 and maintained at 39.5°C showed a transient burst of DNA synthesis as reflected by changes in cell DNA content and an increase in the number of labeled nuclei during the first 24 h postinfection; however, after the abortive stimulation of DNA synthesis at 39.5°C shift experiments demonstrated that host DNA replication was regulated by a functional *A* gene product. It is concluded that both products of the early region of simian virus 40 DNA play a complementary role in recruiting and maintaining simian virus 40-infected cells in the cell cycle.

A variety of approaches have demonstrated that the products of the *A* region of the simian virus 40 (SV40) DNA are responsible for the establishment and, under selective conditions, maintenance of cellular transformation (4, 5, 9, 10a, 17, 18, 23, 25, 28). The *A* locus codes for two specific polypeptides: a 94,000-dalton large T antigen, coded by the region extending from coordinates 0.66 to 0.17 on the conventional map with sequences between 0.54 and 0.59 map units spliced out from the large T mRNA, and a 17,000-dalton small t antigen coded for by sequences from 0.66 to 0.54 (3, 7, 29, 30). Temperature-sensitive mutants of SV40 (*tsA* mutants) which alter the structure and function of the large T antigen (1, 2, 35, 37, 38) generally map by marker rescue (19) to the segment between 0.32 and 0.43 map units, and thus do not affect the small t antigen. Because of the splice between 0.54 and 0.59 map units in the large T mRNA, viable deletion mutants (34) which lack sequences within this DNA segment affect only small t antigen.

Since the SV40 *A* gene product is involved in the initiation of viral DNA replication and the stimulation of host macromolecular synthesis (6, 15, 35, 40), models have been proposed which predict a direct role of the *A* gene product as an initiator of host DNA replicons (5, 24). The demonstration that microinjection of early SV40 DNA fragments (27), and more specifically the microinjection of purified T-related protein (39), stimulates cellular DNA synthesis supports the notion of a critical role of the *A* gene product in the induction of cellular DNA replication. In addition, mutants lacking a segment of the early region encoding large T antigen fail to stimulate cell DNA synthesis (32).

Previous studies with *tsA* mutants, however, have yielded equivocal results with contradictory conclusions about the capacity of a thermolabile T antigen to recruit cells into DNA synthesis and mitosis. Thus, induction of host cell DNA synthesis by *tsA* and *tsA*-deletion mutants has been reported in permissive CV-1 cells at the nonpermissive temperature of 40.5°C (6, 12, 34). Using the ability of *tsA* and viable deletion mutants to induce abortive or stable transformation, measured as the transient loss of anchorage independence, Fluck and Benjamin concluded that *A* gene mutants retain the capacity at high temperature to induce growth in

agar of NIH-3T3 cells, but that the emergence of stable transformants requires small t function (9). No role has been assigned to the small t antigen in the induction of host DNA synthesis, although it has been reported that theophylline, at concentrations which were insufficient to inhibit wild-type SV40 (wt SV40)-induced host DNA synthesis, completely inhibited induction by viable deletion mutants (31).

In previous work, we have studied the kinetics of DNA synthesis induction in resting permissive monkey cells (AGMK and TC-7) after infection by SV40 *tsA*58 and by the viable deletion mutant *dl*890 (12). It was shown that: (i) at the nonpermissive temperature of 41°C, *tsA*58 induced a transient stimulation of cellular DNA synthesis only in a fraction of the infected cell population (30% in TC-7 and 50% in AGMK); however, TC-7 cells after completion of the S period reentered a G1 resting state with no further cycling, whereas AGMK cells remained arrested in the G2 phase; (ii) the *dl*890 mutant induced host DNA synthesis with the same kinetics as wt SV40 at all the temperatures tested.

In an effort to further clarify the requirement of the two early viral gene products in the continuous metabolic stimulation of infected cells, we have studied the induction of serum-deprived G_0-G_1-arrested mouse embryo fibroblasts into the cell cycle by autoradiography and flow microfluorimetry after infection with wt SV40, SV40 *tsA*58, and SV40 *dl*884.

MATERIALS AND METHODS

Cells. Primary mouse embryo fibroblasts were derived from 15-day embryos of Swiss white mice (Blue Spruce Farms). The embryos were trypsinized extensively, plated in 100-mm Falcon petri dishes, and grown to confluence in Dulbecco-modified Eagle medium (DMEM) containing 10% fetal calf serum (GIBCO Laboratories). The mouse embryo cells were subcultured and at confluence were trypsinized and placed in Nunc cryovials in DMEM containing 10% fetal calf serum and 10% dimethyl sulfoxide and frozen in liquid nitrogen. To thaw these cells, or other cell lines, the 1-ml cell suspension (approximately 10^7 cells) was mixed with 25 ml of fresh DMEM containing 10% fetal calf serum and plated into a Corning 75-cm^2 flask. After the cells settled, fresh medium was added to the flask.

Virus. SV40 strain RH-911 was grown in confluent monolayers of TC-7 cells at an input multiplicity of 0.001 PFU/cell. The temperature-sensitive *A* gene mutant of SV40, *tsA*58, was kindly provided by P. Tegtmeyer (36); the *tsA* mutant was grown in TC-7 cells at an input multiplicity of 0.1 PFU/cell. The viable deletion mutant of SV40, *dl*884, was originally isolated by Shenk et al. (34) and was kindly provided to us by J. Melero.

Cultivation of cells and infection with virus. To study the effects of virus infection on resting cells, secondary mouse embryo fibroblasts were grown in low concentrations of fetal calf serum. In general, mouse cells were plated at a density of 10^5 cells per 35-mm dish or 2×10^5 cells per 60-mm dish in DMEM supplemented with 10% fetal calf serum. After the cells had settled, the medium was changed to DMEM plus 2% fetal calf serum. Cultures were maintained at 37°C in this medium for 72 to 96 h before virus infection.

Before infection, the conditioned medium was removed, and purified virions or viral lysates were diluted in conditioned medium. Cells were either mock infected with conditioned medium alone or were virus infected at 50 to 100 PFU per mouse cell. In all experiments the minimum dilution of virus sufficient to produce 80 to 100% T antigen-positive cells at 48 h postinfection (p.i.) was used. After a 2-h incubation period with gentle shaking at 20-min intervals, conditioned medium was returned to the cultures, and the cells were incubated at the appropriate experimental temperature.

Measurement of DNA synthesis and autoradiography. The synthesis of DNA in uninfected and SV40-infected cells was measured by the incorporation of tritiated thymidine ([^{3}H]dThd) (New England Nuclear Corp.; 15 to 20 Ci/mmol) as previously described (12).

Immunofluorescence assay for T and V antigen. The indirect immunofluorescence assay for SV40 T antigen was carried out with hamster anti-T serum as previously described (12).

Detection of actin cables. Actin cable structure was visualized by an indirect immunofluorescence technique after cell fixation in 3.5% formaldehyde for 10 min. After washing in phosphate-buffered saline the cells were further incubated in acetone-methanol (7:3) for 20 min at −20°C. The rabbit anti-actin serum was kindly supplied by Keith Burridge, and the goat anti-rabbit immunoglobulin G was fluorescein conjugated (Microbiological Associates, Inc.).

Preparation of cells for flow microfluorimetry (FMF). At various time intervals p.i., medium was aspirated from mock-infected and virus-infected cultures, and the monolayer was rinsed with phosphate-buffered saline. A preparation of nuclei was made directly from the monolayer by treating with STP staining solution (0.1% sodium citrate, 0.1% Triton X-100, 0.05 mg of propidium diiodide per ml). The dishes were incubated at 4°C for 1 h, and then the cell monolayers were scraped from the dish and placed in test tubes on ice. After blending in a Vortex mixer, the nuclear preparations were analyzed for DNA-specific fluorescence.

The average DNA content per cell was measured on a Cytofluorograf model 4800A (Biophysics, Inc.). Laser power was supplied by an argon ion laser. One hundred channels were used for the collection of data from 50,000 cells per sample. Data were analyzed by the least squares method of Fried et al. (10).

RESULTS

The stimulation of host macromolecular synthesis and induction into the cell cycle of serum-deprived G1-arrested mouse embryo fibroblasts were examined after infection of resting cells with wild-type virus or viral mutants affecting

large T antigen (*tsA*58) or small t antigen (*dl*884). The scheme of these experiments was similar to that outlined previously (12). In general, mouse embryo cells were arrested by growth in 2% serum for 72 to 96 h before infection; after infection with purified virions or viral lysates, conditioned medium was returned to the cultures, and at various times after infection the cultures were analyzed for DNA synthesis by autoradiography and flow microfluorimetry. Under these conditions mock-infected cells were 3 to 4% positive for DNA synthesis as determined by a 1-h pulse with [^{3}H]dThd. Infected cells at 33°C became essentially 100% T antigen positive as determined by immunofluorescence by 30 h p.i., whereas cells infected at 40°C were 50 to 70% T positive and usually fluoresced rather faintly.

Induction of host DNA synthesis by wt SV40. Initially we examined the kinetics of stimulation of DNA replication by wt SV40 to compare these results with the mutant-induced stimulation. After serum deprivation and mock infection, control mouse cell cultures were predominantly arrested in the G_0-G_1 phase of the cell cycle (80%), although a fraction of the cells (15%) had a 4N DNA content (Fig. 1A); this subpopulation represented a mixture of cells in the G_2 + M phase and tetraploid G_1-arrested binucleate cells. Infection of the mouse embryo population with wt SV40 induced the quiescent cells into S phase commencing at approximately 18 h as measured by changes in cellular DNA content and [^{3}H]dThd incorporation (Fig. 1B; see Fig. 3). By 27 h after infection (Fig. 1C) the number of cells with 2N DNA content had decreased from 80% in mock-infected cultures to 35% in wt SV40-infected cells; approximately 30% of the cells were actively synthesizing DNA, whereas the remainder possessed a 4N or greater DNA content. Analysis of the wt SV40-infected cultures by FMF at 47 and 68 h p.i. (Fig. 1D and E) demonstrated an actively cycling cell population with an increasing proportion of cells with 4N DNA content and a population of cells with 2N DNA content reduced to only 20% of the total (Fig. 1E). At 4 to 5 days after infection (Fig. 1F) the FMF profile of the wt SV40-infected mouse cells resembled qualitatively the mock-infected FMF profile (Fig. 1A), suggesting that the high cell density and depletion of serum growth factors in the conditioned medium was rate limiting to further cell growth. The mobilization of the resting cell population by wt SV40 infection was accompanied by a fivefold increase in cell number, with most of the cells having undergone two to three divisions within the 108-h time course.

Infection with *dl*884. The viable deletion mutant of SV40, *dl*884, contains a 217-nucleotide deletion which spans one of the splice junctions of small t RNA; consequently, no stable cytoplasmic mRNA for small t antigen is produced (16). Therefore, infection of resting cells with the *dl*884 mutant permits the analysis of host DNA stimulation by large T antigen in the absence of functional small t. The analysis of *dl*884-infected cells by FMF (Fig. 2) and autoradiography (Fig. 3) indicated that the infected population was induced into the cell cycle at the same time and rate as wt SV40-infected cells. The cell DNA content profile was characteristic of a cycling population between 20 and 48 h (Fig. 2B, C, and D); however, by 60 to 72 h the number of cells with a DNA content characteristic of S-phase cells decreased to approximately 10% of the total population.The majority of the cells appeared to be arrested either in the G_1 phase (50 to 60%) or in the G_2 + M phase (30%) as illustrated in Fig. 2F. From these results it was concluded that although *dl*884-infected mouse cells were induced to cycle initially at the same rate as wild type-infected cells, they became arrested earlier after infection than did wild type-infected cells and also failed to reach the saturation densities of wild type-infected cells (see Fig. 8). Clearly, the small t gene was not required for the initial induction into the cell cycle of serum-deprived G_1-arrested cells, but may be required for successive cell cycles since *dl*884-infected cultures displayed a decreased number of S-phase cells during the latter part of the time course.

The indirect influence of small t protein on DNA synthesis may be related to cytoplasmic morphological changes which are dependent on functional small t protein (10a, 11). Cytoplasmic alterations as reflected by loss of actin cables did not occur in *dl*884-infected mouse cells (Fig. 4 and 5). These cells in culture remained well spread, resembled uninfected cells morphologically (compare Fig. 4A and B), and contained abundant well-defined actin cables as determined by indirect immunofluorescence. On the other hand, infection of mouse embryo cells with SV40 *tsA*58, which produces a temperature-sensitive large T protein and functional small t protein, disrupted actin cable organization irrespective of temperature (Fig. 4C and D). The loss of actin cables, as measured by immunofluorescence, was clearly not an all-or-none phenomenon as illustrated in Fig. 5. Control cultures were 80 to 90% actin cable positive during the experiment, whereas mouse cells infected with wt SV40 or the SV40 *tsA* mutant were 20 to 50% cable positive. SV40 *dl*884-infected cells remained 60 to 70% positive for cytoplasmic actin cables.

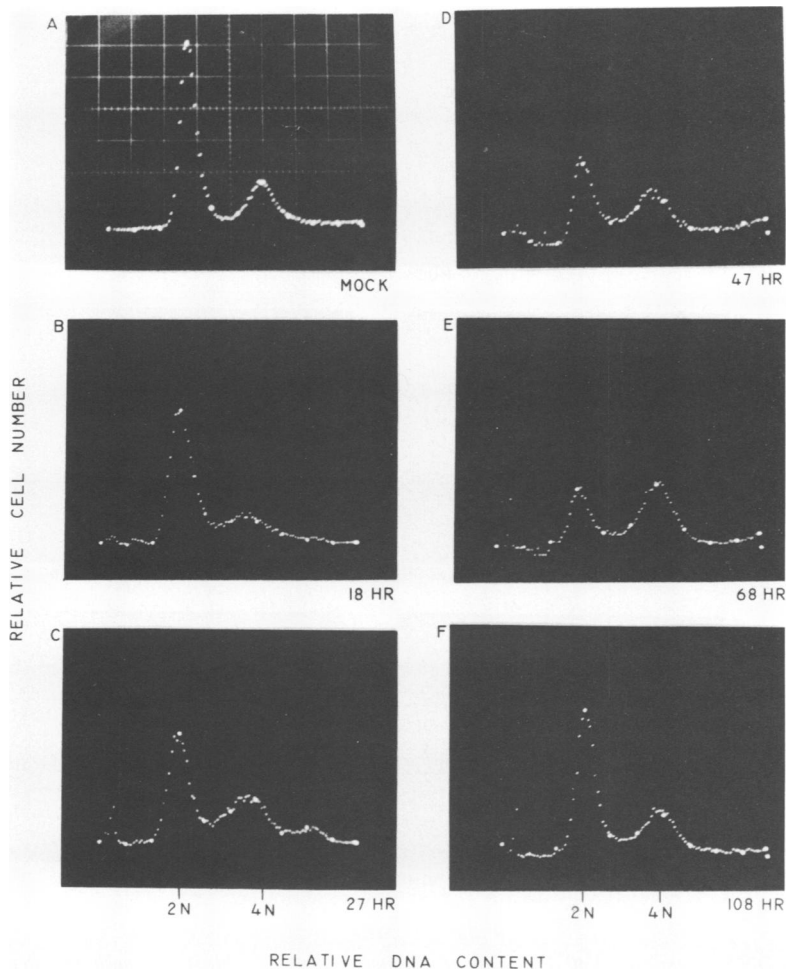

FIG. 1. *Cell DNA content of wt SV40-infected mouse embryo fibroblasts. In all experiments mouse embryo cells were plated and infected as described in the text. At various times after infection with wt SV40, 60-mm dishes were rinsed with phosphate-buffered saline and stained directly with staining solution (0.1% sodium citrate, 0.1% Triton X-100, 0.05 mg of propidium diiodide per ml) (10). The dishes were placed at 4°C for 1 h, and then the cell monolayers were scraped from the dish and placed in test tubes. After blending in a Vortex mixer, the nuclear preparations were analyzed for DNA specific fluorescence by using a Cytofluorograf model 4800A. One hundred channels were used for the collection of data from 50,000 cells per sample. The photographs represent the cumulative oscilloscope histograms.*

Infection with *tsA*58. The infection of quiescent mouse cells with SV40 *tsA*58 at 33°C induced active cell cycling at 33°C by 24 to 30 hours p.i.; the temporal delay in the initial stimulation of DNA synthesis relative to wt SV40 infection was produced by the lower incubation temperature. Although the FMF profile of the *tsA* mutant-infected population maintained at 33°C for 18 h (Fig. 6A) appeared similar to that of the mock-infected control (compare Fig. 6a and Fig. 1a), by 27 and 47 hours p.i. (Fig. 6B and C) the *tsA*58-infected cultures were actively cycling and behaved as wild type-infected cells. In contrast, when mouse cells were infected with *tsA*58 and maintained at 39.5°C, only a transient change in the DNA content of the cell population was observed, as illustrated in Fig. 6D, E, and F. At 27 h p.i., the proportion of cells with a 2N DNA content had decreased from 80% in mock-infected cultures to approximately 50% and was accompanied by an increase in the number of cells with a 4N or greater DNA content from 15 to 30% (Fig. 6E). However, by 48 h p.i., the DNA content profile of *tsA* mutant-infected cells maintained at 39.5°C was essentially indistinguishable from that of mock-in-

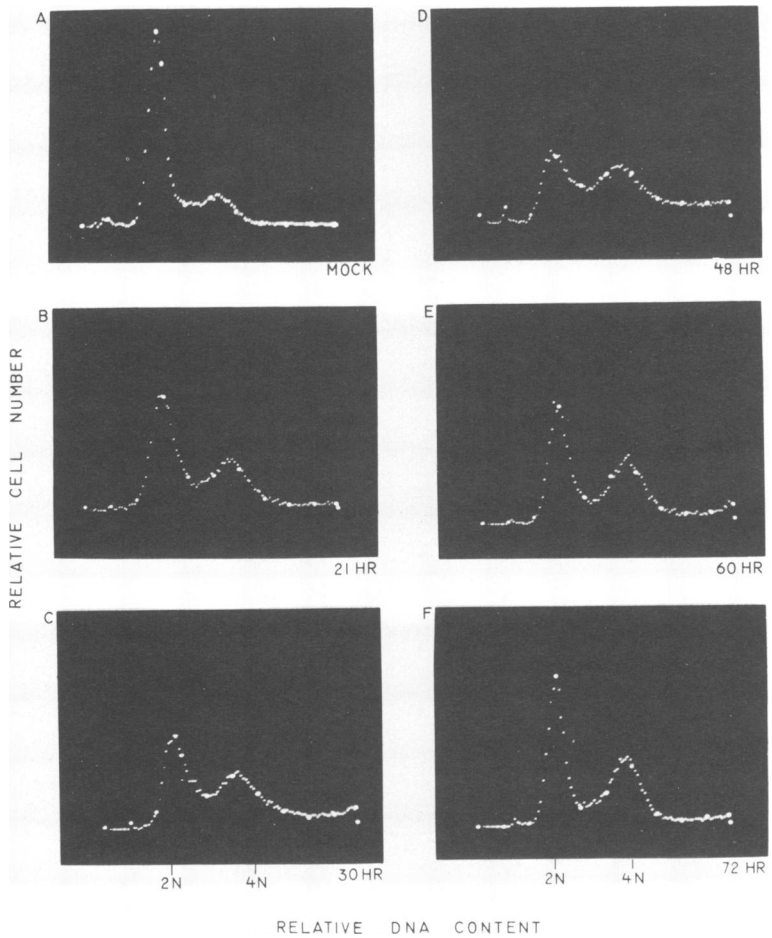

FIG. 2. *Cell DNA content of SV40 dl884 infected mouse embryo fibroblasts. Growth-arrested mouse embryo cells were infected with 20 to 50 PFU/cell of SV40 dl884 and at various times after infection were analyzed for cell DNA content as described in the legend to Fig. 1.*

fected cells (Fig. 6F).

The stimulation of DNA synthesis by *tsA* mutant infection was also examined by autoradiography after a 1-h pulse with [^{3}H]dThd at various intervals after infection. Infection with SV40 *tsA*58 at 33°C produced a 10- to 15-fold increase in the number of labeled nuclei between 30 and 48 h after infection (Fig. 7A). As suggested by the FMF data, infected cells maintained at 39.5°C showed a two- to fivefold increase in dThd incorporation and number of labeled nuclei at 18 to 24 h p.i. After the transient burst of DNA synthesis in a fraction of the cell population, the number of S-phase cells returned to near control levels by 48 h p.i. Cumulative 12-h labeling experiments with wt SV40- and *tsA*58-infected mouse embryo cells supported the pulse-labeling data of Fig. 7A. wt SV40-infected cells incubated at either 33 or 39.5°C or *tsA*58-infected cells incubated at 33°C maintained a labeling index of 60 to 75% at 24 to 72 h p.i., whereas *tsA*58-infected cells incubated at 39.5°C initially had a labeling index of 35% at 12 to 24 h p.i., but the number of labeled nuclei decreased to 10% between 36 and 72 h p.i. (data not shown).

To test the temperature sensitivity and *A* gene regulation of host DNA induction, cultures of *tsA* mutant-infected mouse cells were shifted from the nonpermissive to the permissive temperature or vice versa at 48 h p.i. (Fig. 7B). The incorporation of [^{3}H]dTHd during a 1-h pulse was monitored and expressed as a ratio of the incorporation in infected versus control cells. Infected cultures maintained at 39.5°C incorporated [^{3}H]dThd at approximately a twofold-higher rate than did control cultures throughout the experiment; a shift-down to 33°C at 48 h p.i. induced cells into DNA synthesis by 24 h after

shift. By 48 h after shift-down, an eightfold increase in [^{3}H]dThd incorporation was observed in *tsA*58-infected mouse cells relative to similarly shifted control cultures. Conversely, *tsA*-infected cycling cells maintained at 33°C for 48 h before shift-up to 39.5°C displayed a precipitous decrease in [^{3}H]dThd incorporation 12 to 24 h after shift. Whereas *tsA*58-infected mouse cells maintained at 33°C incorporated [^{3}H]dThd at 20 times the rate of control cultures, the rates of DNA synthesis in infected and control cells were identical by 24 h after shift-up. FMF results indicated that infected cells shifted to 39.5°C completed a round of DNA synthesis and then became quiescent, arrested primarily in the G_1 phase.

Growth of wild type- and mutant-infected mouse cells. The autoradiographic and FMF results suggested that only limited stimulation of cell growth occurred as a result of infection with viral mutants which inactivated either of the SV40 early gene functions. Analysis of cell growth in the wt SV40- and mutant-infected mouse cell populations confirmed this fact directly, as illustrated in Fig. 8. Mock-infected cultures maintained in conditioned medium throughout the experiment remained at a cell density of 3.5×10^5 to 3.8×10^5 cells per 60-mm dish. Infection of serum-deprived G_1-arrested mouse cells with wt SV40 or *tsA*58 at 33°C, also in the presence of conditioned medium, induced continuous cell growth commencing between 24 and 48 h p.i. Infection with either the *dl*884 or the *tsA*58 mutant at 40°C likewise produced a doubling in cell number between 24 and 48 h p.i.; however, during the next 72 h no further increase in cell number was observed.

These results would predict that coinfection of quiescent mouse embryo cells with *dl*884 and *tsA*58 at the nonpermissive temperature would complement to produce a stimulation of DNA synthesis and cellular growth equivalent to that induced by wt SV40 infection at the nonpermissive temperature. As illustrated in Fig. 8, coinfection with the two viral mutants does stimulate cell growth to levels similar to those obtained with wt SV40 at either 33 or 40°C or with SV40 *tsA*58 at 33°C.

DISCUSSION

The requirement for two SV40 early gene functions in the initial metabolic stimulation of nonpermissive mouse cells has been examined by measuring the induction into the cell cycle of serum-deprived G_1-arrested embryonic fibroblasts. An analysis of these results together with results previously obtained with permissive cells (12) allows us to reach several conclusions concerning SV40-induced host DNA induction. (i) The products encoded by the *A* locus are potent mitogens for resting cells, capable of inducing, in the absence of additional growth factors, multiple rounds of DNA synthesis and cell division in the wild type-infected population. (ii) A thermolabile large T antigen is not capable of inducing host DNA synthesis continuously; at a temperature of 40°C for mouse cells and 41°C for monkey cells, a small fraction of cells (depending upon the cell type) is recruited into DNA synthesis; this is a transitory effect which is not followed by continuous cycling. (iii) The small t antigen function is not required for induction of cells into DNA synthesis, although it appears necessary for subsequent cycles of DNA replication and cell division (Fig. 8); it is also not sufficient to sustain further cell cycling once large T antigen has become thermolabile (Fig. 7B). It appears, therefore, that both products of the *A* region play a complementary role in recruiting and maintaining in cycle SV40-infected cells as demonstrated by coinfection experiments with *dl*884 and *tsA*58 at 40°C (Fig. 8). When each of the proteins is functional by itself, a transitory DNA synthesis stimulation occurs;

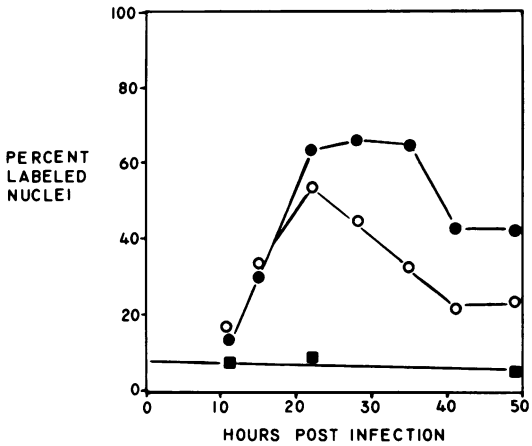

FIG. 3. *Autoradiography of wt SV40- and dl884-infected mouse embryo cells. Cultures were plated and infected with virus as detailed in the text. At various times after infection, cell cultures were pulse-labeled for a 1-h period with 10 μCi of [^{3}H]dThd per ml. The pulse was terminated by rinsing with phosphate-buffered saline, and the cells were fixed in Carnoy solution and prepared for autoradiography as previously described (12). On the average, 500 cells from various parts of the slide were counted to give percent labeled nuclei. Uninfected controls maintained under similar experimental conditions in 2% serum showed 3 to 4% positive nuclei throughout the experiment. Symbols: (●) wt SV40-infected cells; (○) dl884-infected cells; (■) mock-infected cells.*

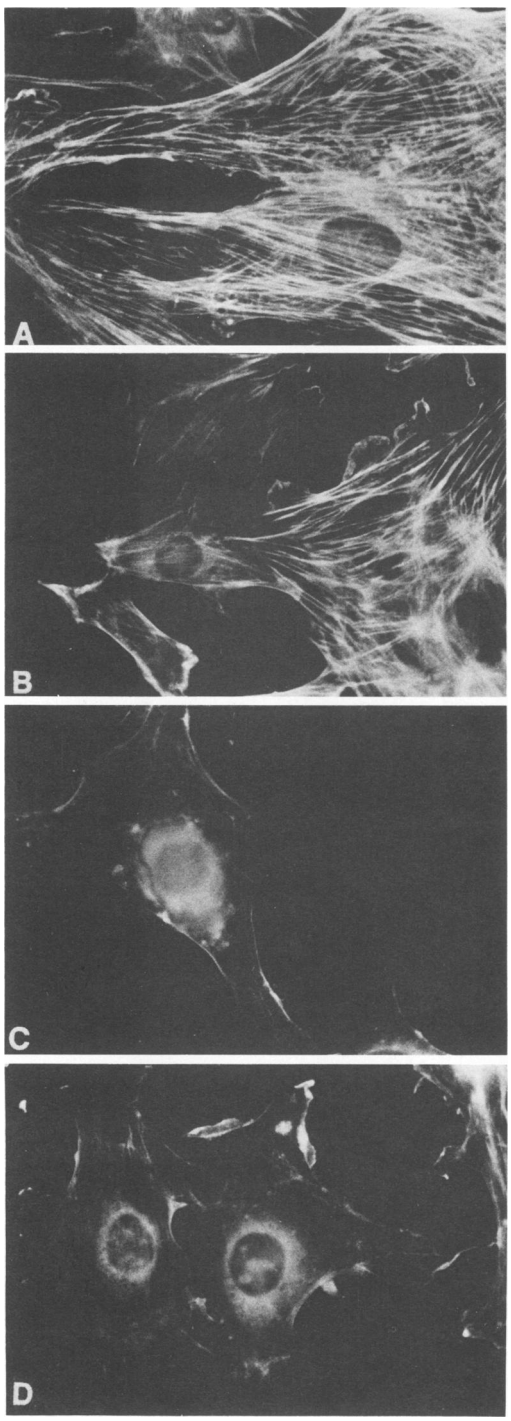

FIG. 4. *Presence of actin cables in SV40-infected mouse embryo cells. The fixation conditions used were similar to those employed for T antigen immunofluorescence. Fixed cover slips of tsA58- or dl884-infected mouse cells were stained with rabbit anti-actin antibody for 30 min, rinsed in phosphate-buffered saline, and stained with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum for 30 min at 37°C (×250). A, Mock-infected mouse embryo cells; B, dl884-infected mouse embryo cells at 37°C for 48 h; C, tsA58-infected mouse embryo cells at 33°C for 48 h; and D, tsA58-infected mouse embryo cells at 40°C for 48 h.*

however, the mechanism underlying the transitory stimulation by each product is quite different.

Thus, infection of mouse cells with viral mutants which fail to synthesize small t protein induces resting cells into the cell cycle initially with the same kinetics as wild type-infected cells, and the population undergoes a doubling in cell number during the first 48 h. However, the number of cells in S phase between 24 and 48 h after infection is approximately 50% lower than that in wild type-infected cells, and the final cell density is also threefold lower than that of the wild type-infected cells. The small t protein, which has been localized to the cytoplasm of infected and transformed cells, is also involved in the disruption of the actin-containing cytoskeletal elements (11). Whether mediated directly or indirectly by the small t protein, depolymerization of actin cables may be necessary for the infected cells to reach high saturation

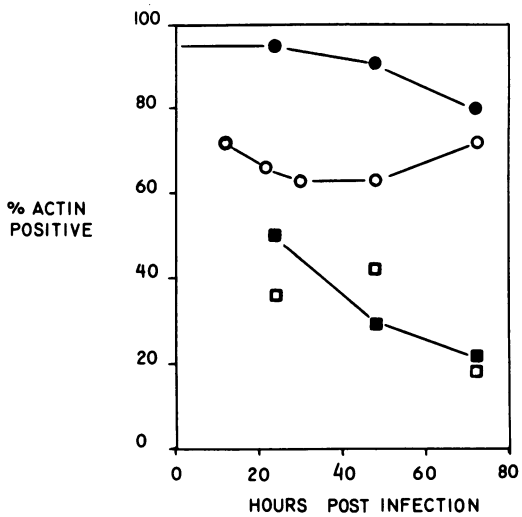

FIG. 5. *Quantitation of actin cable-positive cells in SV40-infected mouse embryo cells. The number of actin cable-positive cells was quantitated by two persons who did not know the identity of the slides. On the average 500 cells per slide were counted. Symbols: (●) mock-infected cells; (○) dl884-infected cells; (■) tsA58-infected cells at 33°C; and (□) tsA58-infected cells at 40°C.*

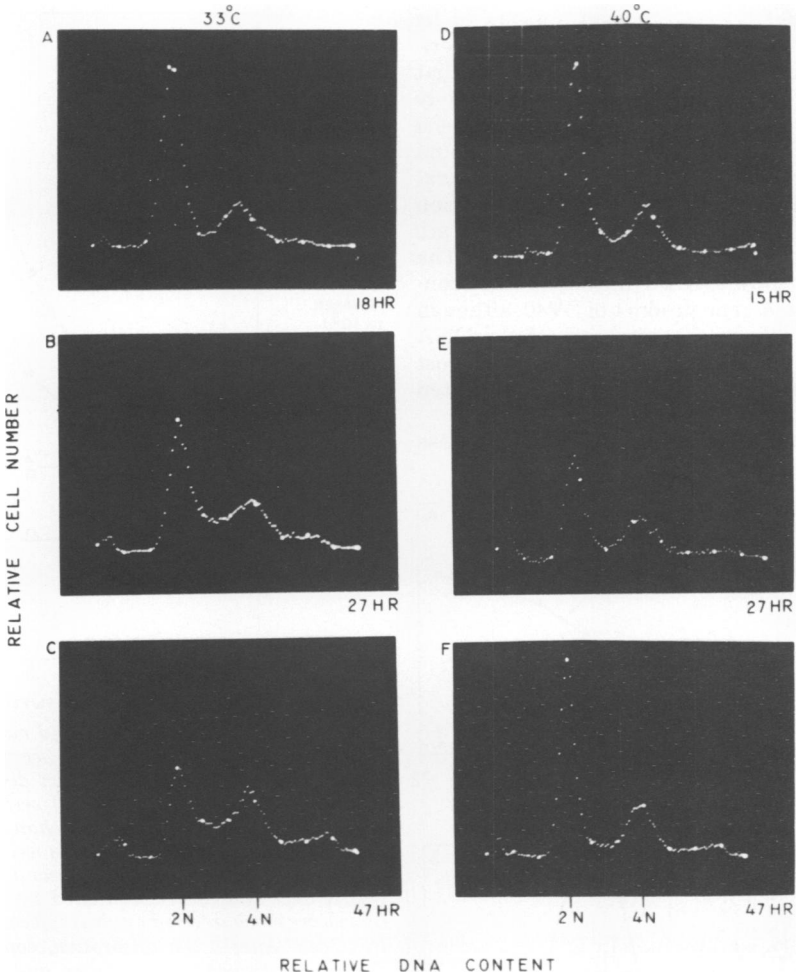

FIG. 6. *Cell DNA content of SV40 tsA58-infected mouse embryo fibroblasts. Mouse embryo cells were cultured and infected as described in the text. At various times after infection, the cells were fixed and analyzed for DNA specific fluorescence by FMF as described in the legend to Fig. 1.*

density or could act as a cytoplasmic trigger for the cascade of nuclear events culminating in DNA replication. The stimulation of DNA synthesis observed in *dl*884-infected cells could represent a round of DNA synthesis induced by the mitogenic effect of large T antigen on the subconfluent cells which divide until a normal saturation density for mouse cells is reached. Alternatively, several rounds of division could occur in cells that do have disrupted cytoskeletal elements; quantitation of the actin cable-positive cells (Fig. 5) indicated that 20 to 25% of the *dl*884-infected cells did have diffuse actin cable networks.

The abortive stimulation of DNA synthesis which occurs at 40°C after infection with *tsA* mutants is qualitatively similar to the transient stimulation of host DNA synthesis which occurs at 41°C in permissive monkey cells, even though detectable viral DNA replication is inhibited at this temperature (6, 12). Our previous results, which suggest a differential sensitivity of host and viral DNA to the *A* gene product, support the present results suggesting that the round of host DNA synthesis observed in *tsA* mutant-infected mouse cells at 40°C is induced by residual activity of the *A* gene product. In this respect, our data are qualitatively similar to the results of Martin and co-workers, who reported that *tsA* mutants, or *tsA*-deletion double mutants (33) induced some host DNA synthesis in permissive CV-1 cells at 40°C.

After the transient induction of DNA synthesis in 30 to 40% of the infected mouse cells, the

regulation of DNA metabolism appears to be tightly *A* gene dependent because shift experiments beginning at 48-h p.i. demonstrate that the number of cells in S phase is coordinately associated with expression of a functional *A* gene product. Taken together, these results would suggest that once the small t function is expressed, perhaps as it relates to the disruption of cytoplasmic actin cables, nuclear events are initiated which result in DNA synthesis. The regulation of host DNA synthesis is then controlled by the *A* gene product of SV40, although the different affinities for viral and cellular DNA (14) may produce a transient induction of host DNA replication when rodent cells are infected with *tsA* mutants at 40°C.

It is noteworthy that interspersed 300-base pair repeat sequences in human and rodent cells have been tentatively identified as origins for cellular DNA replication (13). These sequences, which are highly conserved throughout evolution, share sequence homology with the origin of SV40 DNA replication, including a portion of a perfect inverted repeat located at or near the origin of replication. The origin of SV40 DNA replication represents the major binding site for the *A* gene product, T antigen (38), and the homology between nucleotide sequences at the viral T binding site and the interspersed re-

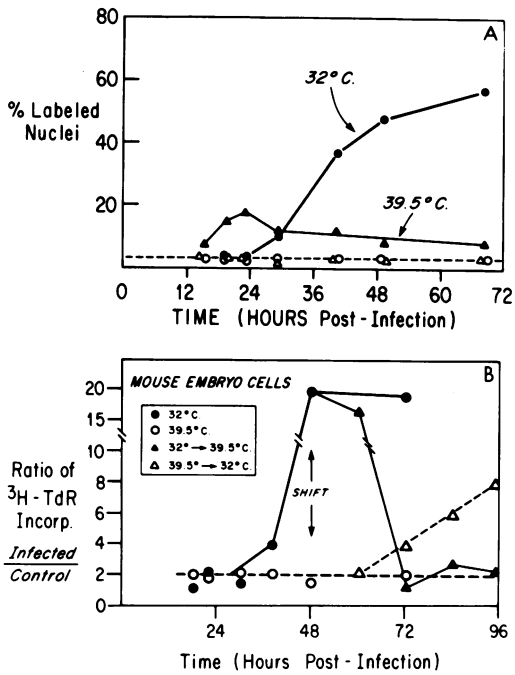

FIG. 7. *DNA synthesis in SV40 tsA58-infected mouse embryo fibroblasts. SV40 tsA mutant-infected mouse embryo cultures were pulse-labeled with [³H]dThd (10 μCi/ml) for 1 h at various times after infection and after temperature shift. Cover slips containing labeled cells were prepared for autoradiography or assayed for incorporation of [³H]dThd into acid-insoluble materials as previously described (12). Symbols in A: (●) tsA58-infected cells at 32°C; (○) mock-infected cells at 32°C; (▲) tsA58-infected cells at 39.5°C; and (△) mock-infected cells at 39.5°C. Symbols in B: (●) tsA58-infected cells at 32°C; (○) tsA58-infected cells at 39.5°C; (▲) tsA58-infected cells shifted to 39.5°C from 32°C at 48 h p.i.; and (△) tsA58-infected cells shifted to 32°C from 39.5°C at 48 h p.i.*

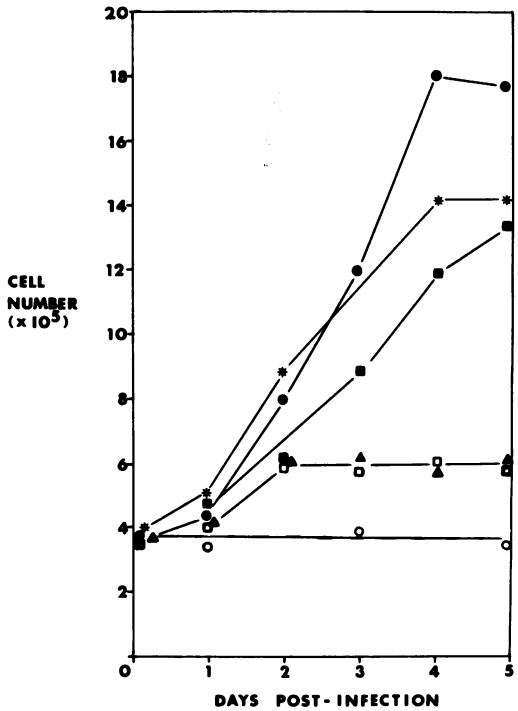

FIG. 8. *Growth of wild type- and mutant-infected mouse cells. Mouse embryo cells were plated at a density of 2 × 10⁵ cells per 60-mm dish in DMEM supplemented with 10% fetal calf serum. After the cells had settled, the medium was changed to DMEM plus 2% fetal calf serum, and cells were maintained in this medium for 72 h before virus infection. Viral lysates were diluted in conditioned medium, and cultures were mock infected or virus infected at 50 to 100 PFU/cell. After a 2-h adsorption, conditioned medium was returned to the cultures, and the cells were incubated at the appropriate experimental temperature. At 24-h intervals, duplicated cultures were trypsinized and counted in a Coulter counter; the points represent the average of duplicate counts. Symbols: (●) wt SV40-infected cells at 37°C; (○) mock-infected cells at 37°C; (■) tsA58-infected cells at 33°C; (□) tsA58-infected cells at 40°C; (▲) dl884-infected cells at 37°C; (*) dl884- and tsA58-infected cells at 40°C.*

peated cellular DNA sequences suggests that T antigen may interact with cellular DNA at these repeated sequences.

The *A* gene product has also been shown to complex in infected and transformed cells with a family of species-specific host nuclear antigens which are induced by SV40 infection (20, 21, 26); a functional *A* gene product is required for induction of a 54K cellular protein during primary infection of 3T3 cells (22). The host protein which has also been identified in chemically transformed, papovavirus-transformed, and retrovirus-transformed mouse cells, displays a cell cycle dependence in normal 3T3 cells; stimulation of arrested 3T3 cells by serum or tumor-promoting agents results in the detection of the host protein within 2 to 4 h (8). It would be attractive to postulate that separate viral and cellular functions are complexed in the physiological state to regulate in a coordinated fashion progression of infected cells into S phase. The multifunctional T antigen, acting at a different regulatory level, may likewise interact directly with host polynucleotide sequences which share homology with the viral replication origin. In this way, regulation of host DNA synthesis could be mediated both directly and indirectly by a pleiotropic *A* gene function (40) acting within the cell nucleus. Continuous metabolic stimulation in infected cells would also require cytoplasmic small t function to release the cell population in vitro from growth restraints imposed by contact inhibition.

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