JOURNAL OF VIROLOGY, June 1978, p. 584-594
0022-538X/78/0026-0584$02.00/0

Vol. 26, No. 3
Printed in U.S.A.

# Role of the Simian Virus 40 Gene A Product in Regulation of DNA Synthesis in Transformed Cells

JANET S. BUTEL* AND HOWARD R. SOULE

*Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030*

Received for publication 27 September 1977

Cells transformed by *tsA* mutants of simian virus 40 (SV40) are temperature sensitive for the maintenance of the transformed phenotype. The kinetics of induction of DNA synthesis were determined for hamster cell transformants shifted to the permissive temperature after a 48-h serum arrest at the nonpermissive temperature. DNA synthesis was initiated in the *tsA* transformants by 8 h after shiftdown and was maximal by 12 h. The presence or absence of fetal bovine serum at the time of temperature shift had no effect on the kinetics of initiation of DNA synthesis. Analysis of TTP in *tsA* transformants revealed similar levels of incorporation of [$^3$H]thymidine into TTP at both permissive and nonpermissive temperatures. Autoradiography revealed that by 12 h after a shift to the permissive temperature, approximately 50% of the cells exhibited labeled nuclei after a 60-min pulse with [$^3$H]thymidine, indicating that a majority of the cells were actively synthesizing DNA. By 8 to 12 h after a shiftup of confluent *tsA* transformants to the nonpermissive temperature, the number of labeled nuclei was reduced to approximately 16%, regardless of serum concentration. These data indicate that the SV40 gene *A* product, either directly or indirectly, regulates cellular DNA synthesis in transformed cells.

The papovavirus simian virus 40 (SV40) has proven to be a suitable model for investigating the regulation of viral DNA synthesis because its genetic expression is under strict coordinate control. Approximately 50% of the SV40 genome is expressed early after infection of permissive primate cells, before the onset of viral DNA synthesis, whereas the remaining 50% of the genome functions only after the initiation of viral DNA replication (2, 14, 31, 37, 46). To date, a single gene, the *A* gene, has been ascribed to the early region of the SV40 genome (16, 27, 29, 31, 51, 54, 58), which codes for a single polypeptide product with an apparent molecular weight of about 100,000 (1, 15, 41, 44). Rundell et al. (44) were able to equate the gene *A* product with the SV40 tumor (T) antigen by the technique of immunoprecipitation, using serum from hamsters bearing SV40-induced tumors. The tumor serum precipitated a protein of smaller apparent molecular weight from extracts of cells infected with an SV40 mutant containing a deletion in the early region.

The essential role of the gene *A* product in viral DNA synthesis has been elucidated through the use of conditional-lethal, temperature-sensitive (ts) mutants of SV40. A rapid cessation of initiation of viral DNA replication was observed when cells productively infected by SV40 *tsA* mutants were shifted to the nonpermissive temperature (17, 51). The stimulation of host cell DNA synthesis, another early SV40 function (24, 43), was also diminished (17, 51). Therefore, it appears that in lytically infected permissive cells, the SV40 gene *A* product functions directly or indirectly at the level of viral DNA synthesis.

Nonpermissive or semipermissive cells transformed by SV40 are temperature sensitive for the maintenance of the transformed phenotype (8a, 9, 12, 28, 32, 39, 53) and revert to a degree of normality when propagated at the elevated temperature. This effect is attributed to the mutant gene *A* product, since it has been established that only early SV40 information is expressed in transformed cells (7, 13, 26, 40, 45); the possible role of another early gene function defined by deletion mutants mapping between 0.54 and 0.59 on the SV40 physical map (18, 48) remains to be determined. The biological parameters of transformation that can be monitored in culture (i.e., colony formation in soft agar and on plastic surfaces, saturation density levels, and cytoskeletal organization) do not define primary events mediated by the SV40 gene *A* protein. Rather, they represent secondary pleiotropic or polar effects of the virus-specified protein upon the host cell. The definitive mechanism by which the SV40 gene *A* protein initiates and maintains cellular transformation, either di-

rectly or indirectly, has not yet been elucidated, although several hypotheses have been presented (12, 30, 33, 34, 38, 57).

In recognition of the association of the SV40 gene *A* product with the control of DNA replication, studies were initiated to correlate host cell DNA synthesis with the expression of the transformed phenotype in *tsA* transformants. By using this approach, it was possible both to monitor the degree of reversion to normality by the mutant-transformed cells at the conditional temperature and to ascribe a possible control function to the gene *A* protein in SV40 transformants.

This report describes a series of temperature shift experiments that demonstrate the ability of the SV40 gene *A* product to control, either directly or indirectly, host cell DNA synthesis in SV40-transformed rodent cells. The results presented here demonstrate that host cell DNA synthesis can be utilized as a convenient marker to determine the magnitude and kinetics of reversion to the normal phenotype by *tsA* transformants under restrictive conditions, as well as the conversion to the transformed phenotype upon return to permissive conditions.

## MATERIALS AND METHODS

**Cells.** Normal hamster embryo fibroblast (HEF) cells were prepared by trypsinizing decapitated Syrian hamster embryos (Con-Olson Co., Inc., Madison, Wis.) followed by culturing in Eagle medium supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, Rockville, Md., or Grand Island Biological Co., Grand Island, N.Y.), 0.075% sodium bicarbonate, and 50 μg of gentamicin sulfate per ml (Schering Corp., Bloomfield, N.J.).

Two SV40 wild-type (wt)-transformed cell lines were included: a line of wt-transformed weanling Syrian hamster kidney cells, designated THK-1 (8), was obtained from A. M. Lewis, and a line of wt-transformed Chinese hamster lung cells, CHLWT15 (32), was obtained from R. G. Martin. Four SV40 *tsA*-transformed lines were used: a line of Chinese hamster lung cells transformed by SV40 *tsA*239, designated CHLA239, was provided by R. G. Martin (32); three Syrian hamster *tsA* transformants were established in this laboratory by transforming secondary HEF cells with SV40 *tsA*28 or *tsA*30 (51, 54), using methods previously described (9) and viruses generously provided originally by P. Tegtmeyer. The characteristics of the independently transformed lines, designated $Ha_2$/A28, $Ha_2$/A30, and $Ha_3$/A30, are summarized in Table 1. The methodologies for immunofluorescence tests (42), saturation density determinations (9), and colony formation assays on plastic (9) have been described.

All transformed cell lines were grown in plastic tissue culture vessels (Corning Glass Works, Corning, N.Y.) in Dulbecco-modified Eagle minimal essential medium (DMEM) (Grand Island Biological Co.) supplemented with 10% FBS, 50 μg of gentamicin sulfate per ml, and 0.3% sodium bicarbonate in a humidified atmosphere of 10% $CO_2$. Permissive and nonpermissive temperatures of 33 and 40.5°C, respectively, were used.

**Temperature shift protocols.** The protocol for the temperature shiftdown experiments is represented schematically in Fig. 1. Cell lines to be examined were first passaged two times at the nonpermissive (40.5°C) temperature and were then subcultured into 25-$cm^2$ plastic flasks (Falcon Plastics, Oxnard, Calif.) and incubated at the permissive temperature (33°C). At confluence, the cultures were washed three times with Tris-buffered saline (TBS: 2 mM Tris, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.4 mM $Na_2HPO_4$, 6 mM dextrose, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$), and then half of the cultures were refed with DMEM supplemented with 0.5% FBS, while the other half received DMEM supplemented with 10% FBS. One-half of the cultures with each serum concentration were then submerged in water baths (Blue M Electric Co., Blue Island, Ill.) calibrated to 33 and 40.5°C. After 48 h of incubation, the medium on a portion of the cultures at 40.5°C that had been serum depleted (0.5% FBS) was replaced with DMEM containing 10% or 0.5% FBS, and the cultures were shifted down to 33°C. The medium on the remaining cultures at both temperatures at the time of the temperature shift was replaced with fresh medium containing the same serum concentration used in the preceding 48-h incubation. At 0, 4, 8, 12, and 24 h after the temperature shift, cultures were pulse-labeled in duplicate for 60 min with 1 μCi of [$^3$H]thymidine per ml (TdR; 6.7 Ci/mmol; New Eng-

TABLE 1. *Characteristics of Syrian hamster embryo cells transformed by SV40 tsA mutants*

| Transformed cell line | Property | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of in vitro passages | Passage level when 100% T-antigen positive | Saturation density | | Colony formation on plastic | | Plating efficiency | |
| | | | No. of cells ($\times 10^{-5}$)/culture at 33°C | P/NP[a] | No. of colonies/$10^3$ cells plated at 33°C | P/NP | % of cells that plated at 33°C | P/NP |
| $Ha_2$/A28 | 180 | 23 | 54 | 10.6 | 193 | 27.6 | 19 | 2.6 |
| $Ha_2$/A30 | 150 | 25 | 40 | 7.1 | 172 | 37.4 | 23 | 7.7 |
| $Ha_3$/A30 | 150 | 35 | 16 | 4.7 | 610 | 33.9 | 74 | 1.9 |

[a] Ratio of value obtained at permissive temperature to that obtained at nonpermissive temperature.

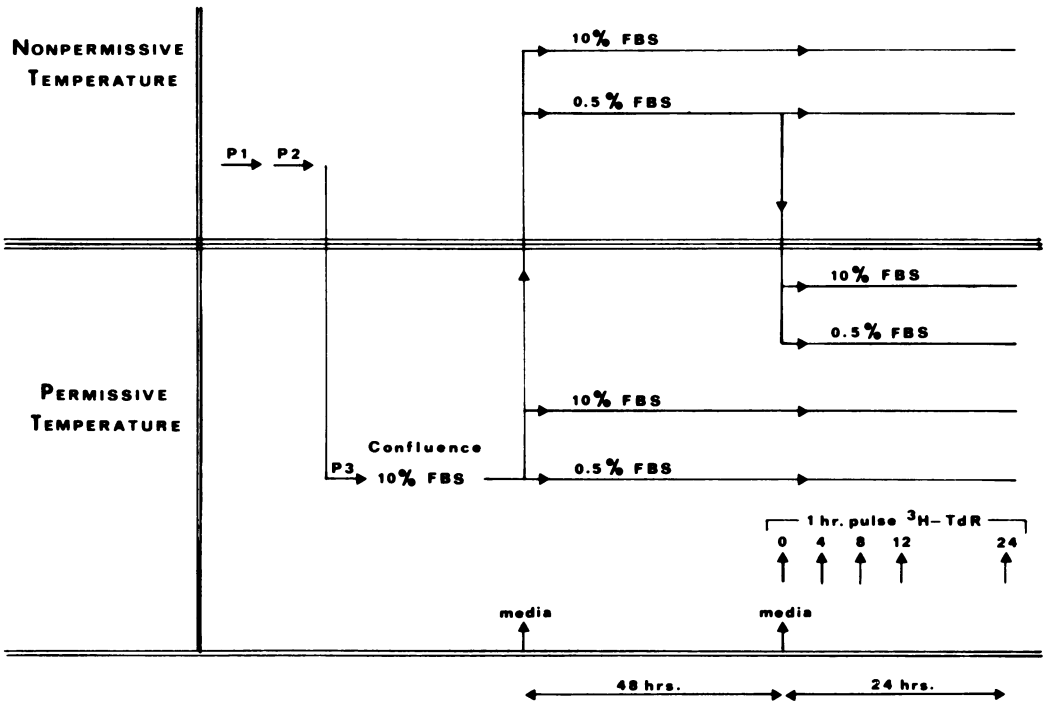


FIG. 1. *Experimental procedure for temperature shiftdown experiments. This design is described in detail in Materials and Methods.*

land Nuclear Corp., Boston, Mass.) in medium supplemented with the same serum concentration currently on the culture. Labeling was performed at the temperature of incubation before the pulse. The pulse was terminated by plunging the cultures into ice followed by three washes with cold TBS. The cells were dispersed with a 0.25% trypsin solution and precipitated with 1 volume of cold 10% trichloroacetic acid. Precipitates were collected on GF/C filters (Whatman, Clifton, N.J.), and radioactivity was determined by counting in a toluene-fluoralloy scintillation mixture (Beckman Instruments, Fullerton, Calif.) in a liquid scintillation spectrometer (model LS-250, Beckman Instruments).

Essentially the same protocol as that shown in Fig. 1 and described above for the temperature shiftdown experiments was followed for the temperature shiftup studies with the following modifications. The transformed cells were not passed twice at the nonpermissive temperature at the beginning of the experiment, and after 48 h of serum depletion (0.5% FBS) at 33°C, cultures were shifted up to 40.5°C in the presence of 10 or 0.5% FBS.

**Analysis of TTP.** TTP was extracted and chromatographed as described by Nazar et al. (36). Freshly passaged cells were seeded into 75-cm$^2$ plastic flasks and were propagated for 12 h at 33 or 40.5°C in medium consisting of DMEM supplemented with 10% dialyzed FBS and 4 μCi of $^{32}P_i$ per ml (carrier-free; Union Carbide, Tuxedo, N.Y.). This labeling period was found to be sufficient to uniformly label the TTP with $^{32}P_i$ as judged by similar specific activities of TTP for these samples and samples labeled for a longer period of time (96 h). Cells were then pulse-labeled for 1 h in fresh, prewarmed medium supplemented with 10% dialyzed FBS, 4 μCi of $^{32}P_i$ per ml, and 1 μCi of [$^3$H]TdR per ml at the temperature of previous incubation. After the pulse, monolayers were rapidly washed with TBS, and 5 ml of 1 N acetic acid was added to each flask. To extract the nucleotides, the cells were subjected to four cycles of freezing and thawing, and the lysate was lyophilized. The lyophilized sample was suspended in 1 ml of ice-cold water and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant fraction was removed, and 100 μl of this fraction plus 0.5 μmol of TTP (Sigma Chemical Co., St. Louis, Mo.) as UV-absorbing standard was applied to a polyethyleneimine-impregnated cellulose sheet (Brinkmann Instruments, Westbury, N.Y.). The sample was chromatographed in the first dimension for 15 cm with 1 N acetic acid–1 M $LiCl_2$ and in the second dimension for 15 cm with 3 M ammonium acetate–5% (wt/vol) boric acid, pH 7.0. The dried chromatogram was scanned with UV light to detect the standard TTP, the spot was excised, and the content of $^{32}P$ and $^3H$ was determined by counting in a toluene-fluoralloy mixture.

**Autoradiography.** Autoradiography was performed by the method of dip-coating described by Baserga and Malamur (5). Cultures of transformed cells were propagated and pulse-labeled with [$^3$H]TdR as described above. At the end of the pulse, the cells were dispersed with 0.25% trypsin and sedimented by centrifugation at 500 × *g* for 5 min at 4°C. The cell pellet was fixed in a 1:3 (vol/vol) solution of glacial acetic acid–80% ethanol for at least 15 min at room

temperature and were then washed three additional times in fixative. The fixed cells were aspirated onto a pre-wetted, acid-washed microscope slide and were air dried at 37°C. The slides were dipped in NTB-2 tracking emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted 1:3 in water, exposed in light-tight boxes at 4°C for 4 to 7 days, developed in D19 (Eastman Kodak Co.), and fixed in F5 (sodium thiosulfate, 240 g; sodium sulfite, 15 g; 28% acetic acid, 48 ml; boric acid, 7.5 g; water to 1,000 ml). The cells were stained through the emulsion with a 0.5% solution of toluidine blue (Sigma Chemical Co., St. Louis, Mo.) in acetate buffer, pH 4.5, for 60 min and were viewed with a light microscope (Bausch & Lomb, Rochester, N.Y.) at ×430. At least 500 cells were analyzed for each time point.

## RESULTS

### Effect of temperature shiftdown on DNA synthesis by SV40 *tsA* transformants.

The level of DNA synthesis in normal and transformed hamster cells under various conditions was monitored by the conversion of [$^3$H]TdR into trichloroacetic acid-precipitable radioactivity, as described in Materials and Methods. A representative experiment is illustrated with the Chinese hamster *tsA* transformant, CHLA239 (Fig. 2). Serum-arrested confluent cultures from 40.5°C initiated DNA synthesis between 4 and 8 h after a shiftdown to 33°C. Incorporation of [$^3$H]TdR was maximal by 12 h after the shift. The concentration of FBS at the time of the temperature shift had no effect on either the kinetics or the maximum level of DNA synthesis achieved. A minimal base line of thymidine incorporation was observed in *tsA*-transformed cultures maintained for the duration of the ex-

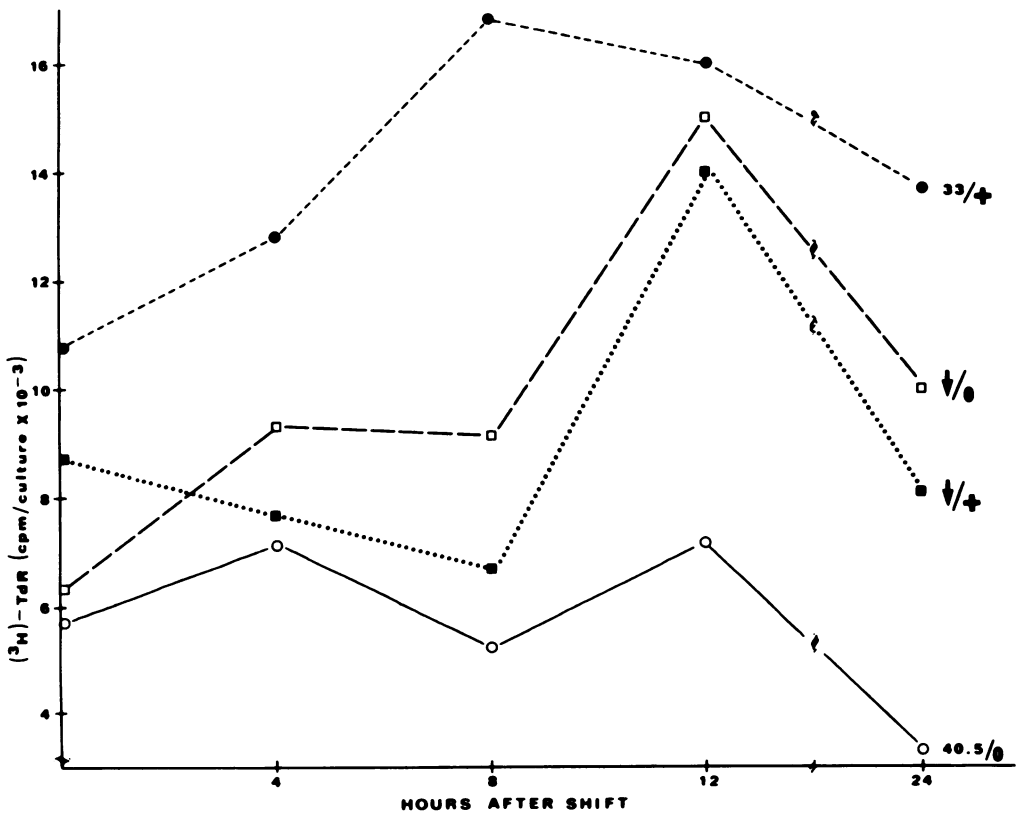


FIG. 2. *Effect of temperature shiftdown and serum concentration on DNA synthesis in the SV40 tsA-transformed Chinese hamster lung cell line, CHLA239. Before the temperature shift, cultures in 25-cm$^2$ flasks were incubated in DMEM supplemented with 0.5 or 10% FBS at the permissive (33°C) or nonpermissive (40.5°C) temperature as described in Materials and Methods and in Fig. 1. At the indicated times after the temperature shift, duplicate cultures were pulse-labeled for 60 min with [$^3$H]TdR (1 μCi/ml) in DMEM supplemented with the same serum concentration present at the time of the temperature shift. After the pulse, cells were dispersed and trichloroacetic acid-precipitable counts per minute (cpm) were determined. Symbols: (○) Cultures maintained at 40.5°C in medium supplemented with 0.5% FBS; (●) cultures maintained at 33°C in medium supplemented with 10% FBS; (□) serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 0.5% FBS; and (■) serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 10% FBS.*

periment at 40.5°C in the presence of either 0.5 or 10% FBS, indicating that DNA synthetic activity by the cells did not totally cease under nonpermissive conditions. In contrast, confluent *tsA*-transformed cultures incubated at 33°C exhibited relatively asynchronous DNA synthesis throughout the 24-h period of observation, regardless of the serum concentration. The results of several additional temperature shiftdown experiments are summarized in Table 2. The pattern of DNA synthesis observed in each experiment was similar to that described above. It should be emphasized that the resumption of DNA synthesis by the depleted *tsA*-transformed cells after shiftdown to permissive conditions was independent of serum concentration.

TABLE 2. *DNA synthesis in SV40 tsA-transformed hamster cells after temperature shift*

| Cell line | Expt no. | Serum added at shift | Hours after temperature shift | | cpm at maximum incorporation with shifted culture/cpm with arrested culture maintained at 40.5°C |
|---|---|---|---|---|---|
| | | | Initiation | Maximum | |
| $Ha_2$/A28 | 1 | + | 4-6 | 12 | 4.6 |
| | | 0 | 4-6 | 12 | 5.2 |
| | 2 | + | 8 | 12 | 1.7 |
| | | 0 | 8 | 12 | 1.4 |
| $Ha_2$/A30 | 1 | + | 4-6 | 9 | 5.0 |
| | | 0 | 4-6 | 9 | 6.3 |
| | 2 | + | 5-7 | 10 | 1.4 |
| | | 0 | 5-7 | 10 | 1.6 |
| CHLA239 | 1 | + | 4 | 12 | 4.6 |
| | | 0 | 4-7 | 12 | 3.9 |
| | 2 | + | 4-6 | 12 | 3.0 |
| | | 0 | 4-6 | 12 | 3.1 |

In marked contrast to the serum-depleted *tsA* transformants maintained at 40.5°C, no such arrest of DNA synthesis was observed with wt SV40 transformants, e.g., THK-1 cells (Fig. 3). Similar results were obtained with the CHLWT15 cells (data not shown). As could be predicted for cells able to express the transformed phenotype independent of the temperature of incubation (25, 49), wt virus-transformed cell lines synthesized DNA equally well at 33 and 40.5°C in either the presence or absence of serum.

Patterns of DNA synthesis by normal HEF cells demonstrated some different conditional requirements. In contrast to the SV40 *tsA* transformants that were temperature dependent for DNA synthesis and the wt SV40 transformants that were both temperature independent and serum independent for DNA synthesis, normal HEF cells (Fig. 4) exhibited a serum-dependent DNA synthetic profile predictable for a normal cell (20). Both HEF cultures maintained at 33°C in the absence of FBS and serum-depleted cultures shifted to 33°C in the absence of FBS failed to initiate DNA synthesis during the 24-h

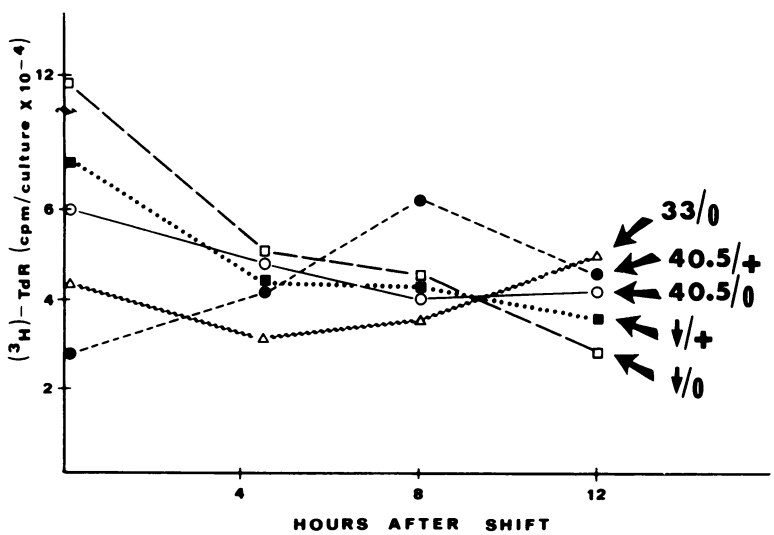


FIG. 3. *Effect of temperature shiftdown and serum concentration on DNA synthesis in the wt SV40-transformed Syrian hamster kidney cell line, THK-1. Experimental procedures were the same as those detailed in the legend to Fig. 2. Symbols: (○) Cultures maintained at 40.5°C in medium supplemented with 0.5% FBS; (●) cultures maintained at 40.5°C in medium supplemented with 10% FBS; (△) cultures maintained at 33°C in medium supplemented with 0.5% FBS; (□) serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 0.5% FBS; and (■) serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 10% FBS.*

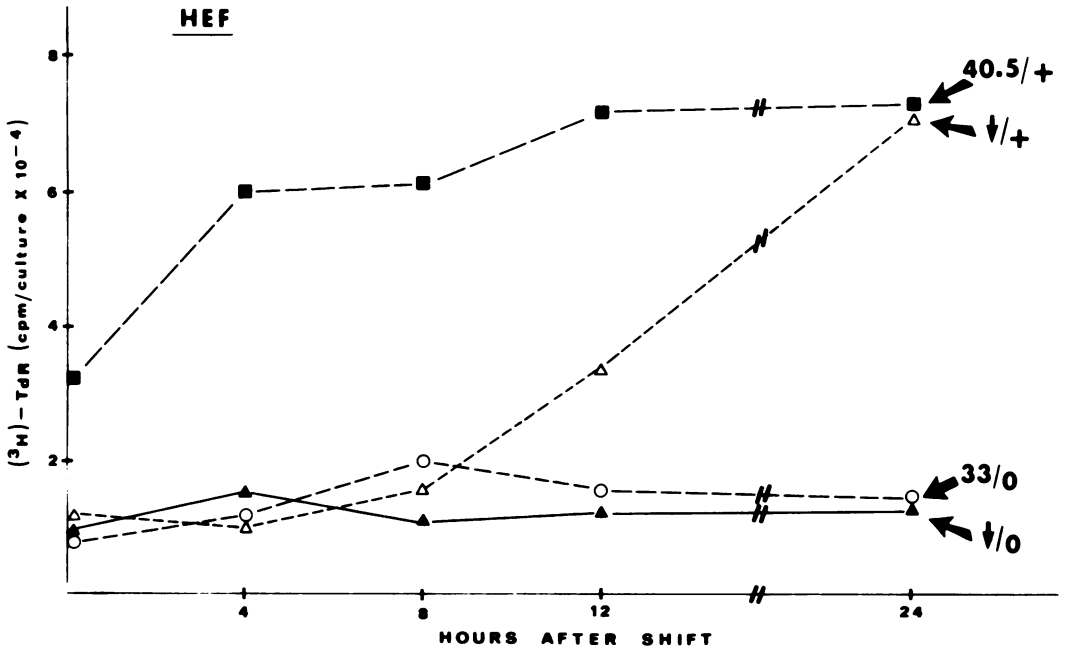


FIG. 4. *Effect of temperature shiftdown and serum concentration on DNA synthesis in normal Syrian hamster embryo fibroblasts. Experimental procedures were the same as those detailed in the legend to Fig. 2. Symbols:* (■) *Cultures maintained at 40.5°C in medium supplemented with 10% FBS;* (○) *cultures maintained at 33°C in medium supplemented with 0.5% FBS;* (△) *serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 10% FBS; and* (▲) *serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 0.5% FBS.*

period of observation. If 10% FBS was added to the serum-depleted cultures of HEF cells at the time of the shiftdown to 33°C, the incorporation of [$^3$H]TdR was initiated about 8 h later.

**Effect of temperature shiftup on DNA synthesis by SV40 *tsA* transformants.** After the determination of kinetics of induction of DNA synthesis by SV40 *tsA* transformants shifted to permissive conditions after a serum arrest at the nonpermissive temperature, the effect of a reverse temperature shift was examined. Asynchronously growing *tsA* transformants propagated at 33°C were shifted up to 40.5°C, with the serum concentration adjusted to either 0.5 or 10% at the time of transfer. Table 3 presents data obtained with the $Ha_2$/A28 cell line. There was a rapid cessation (~4 h) of the incorporation of [$^3$H]TdR after a shiftup to 40.5°C. The observed inhibition of DNA synthetic activity was independent of serum concentration. The CHLA239 and $Ha_3$/A30 cell lines responded similarly to the $Ha_2$/A28 cells in shiftup studies (data not shown). Incorporation of [$^3$H]TdR by the CHLA239 and $Ha_3$/A30 cell lines had dropped to base-line levels by 8 h after shiftup, regardless of serum concentration, and no change was observed in incorporation for the remainder of the 24-h observation period.

**Determination of relative labeling of TTP**

TABLE 3. *Effect of temperature shiftup on DNA synthesis in the SV40 tsA-transformed hamster cell line, $Ha_2$/A28*[a]

| Experimental condition | Hours after shift | [$^3$H]TdR cpm | cpm experimental[b]/ cpm 33°C, serum |
|---|---|---|---|
| 33°C, serum[c] | 0 | 3,751 | 1.00 |
| | 4 | 4,768 | 1.00 |
| | 8 | 8,213 | 1.00 |
| 40.5°C, no serum[d] | 0 | 1,659 | 0.44 |
| | 4 | 2,205 | 0.46 |
| | 8 | 1,309 | 0.16 |
| 33°C → 40.5°C, serum | 0 | 5,826 | 1.55 |
| | 4 | 594 | 0.12 |
| | 8 | 651 | 0.08 |
| 33°C → 40.5°C, no serum | 0 | 4,628 | 1.23 |
| | 4 | 681 | 0.14 |
| | 8 | 890 | 0.11 |

[a] The temperature shiftup protocol is described in Materials and Methods. Cultures growing at 33°C in the presence of 0.5% FBS were shifted to 40.5°C and at the indicated times were pulse-labeled with [$^3$H]-TdR (1.0 μCi/ml) for 1 h, followed by a determination of trichloroacetic acid-precipitable [$^3$H]TdR counts per minute (cpm).

[b] Ratios were calculated by dividing each experimental point with the value obtained for cultures maintained at 33°C in the presence of 10% serum during the same time period.

[c] Medium supplemented with 10% FBS.

[d] Medium supplemented with 0.5% FBS.

**in *tsA* transformants.** The relative incorporation of [$^3$H]TdR into TTP was determined in the *tsA*-transformed cell lines at the permissive and nonpermissive temperatures. These analyses were performed to rule out the possibility that apparent differences in DNA synthetic activity were actually reflections of differential incorporation of [$^3$H]TdR into TTP as a function of temperature. Nucleotides were extracted from cells uniformly labeled with $^{32}P_i$ at 33 and 40.5°C and pulsed for 60 min with [$^3$H]TdR.

Since the cells were uniformly labeled with $^{32}P_i$, the ratio of $^3$H to $^{32}$P in TTP after a pulse with [$^3$H]TdR is a measure of equilibration of [$^3$H]TdR into the total cellular TTP pool. Analyses of TTP for the SV40 *tsA* transformants CHLA239 and $Ha_2$/A28 are presented in Table 4. The ratio of $^3$H to $^{32}$P in TTP was nearly identical for each cell line at 33 and 40.5°C, indicating similar levels of equilibration of [$^3$H]TdR into TTP during a 1-h pulse at both the permissive and nonpermissive temperatures. Thus, the difference in incorporation of [$^3$H]TdR into DNA does not appear to be due to differential labeling of the precursor TTP in the indicated cell lines at the two temperatures.

**Autoradiographic analysis of the effect of temperature on DNA synthesis by SV40 *tsA* transformants.** When DNA synthesis is monitored by the conversion of [$^3$H]TdR into trichloroacetic acid-precipitable radioactivity, it is essential to determine whether that incorporation is representative of the complete cell population or only a smaller subpopulation. To eliminate the latter possibility, temperature shift experiments were executed as described above,

TABLE 4. *Determination of relative incorporation of [$^3$H]TdR into TTP in SV40 tsA-transformed cell lines CHLA239 and $Ha_2$/A28 at 33 and 40.5°C*[a]

| Cell line | Temp of incubation (°C) | TTP cpm | | $^3$H cpm/$^{32}$P cpm |
|---|---|---|---|---|
| | | $^3$H | $^{32}$P | |
| CHLA239 | 33 | 3,087 | 148 | 20.9 |
| | 40.5 | 4,074 | 155 | 26.3 |
| $Ha_2$/A28 | 33 | 9,113 | 447 | 20.4 |
| | 40.5 | 7,573 | 343 | 22.1 |

[a] Cell lines were propagated at 33 or 40.5°C in DMEM supplemented with 4 μCi of $^{32}P_i$ per ml for 72 h. Medium was removed and replaced with DMEM supplemented with 4 μCi of $^{32}P_i$ and 1 μCi of [$^3$H]TdR per ml for 1 h. Nucleotides were extracted and chromatographed on polyethyleneimine-impregnated cellulose thin-layer plates as described in Materials and Methods. Unlabeled TTP (0.5 μmol) was added to each sample before chromatography to allow visualization of TTP with UV light. The TTP spot was cut out and quantitated for relative amounts of $^{32}$P and $^3$H.

and the cultures were processed for autoradiography as detailed in Materials and Methods. Data obtained with the $Ha_2$/A28 cell line are shown in Fig. 5. When serum-arrested cultures propagated at 40.5°C were shifted down to 33°C, DNA synthesis was initiated between 8 and 12 h after the shift, regardless of serum concentration. These cultures were maximal for DNA synthesis by 12 h, at which time approximately 50% of the nuclei were labeled. Conversely, asynchronous cultures propagated at 33°C and then shifted to 40.5°C exhibited a rapid reduction in labeled nuclei commencing after only 4 h at the elevated temperature. A minimum base line was obtained between 8 and 12 h after the shiftup, with 10 to 15% of the cells continuing to demonstrate labeled nuclei. The kinetics of induction and time of maximum DNA synthesis after a temperature shiftdown were comparable when ascertained by the method of [$^3$H]TdR incorporation and by autoradiography. Similar kinetics were also observed by both techniques in shiftup studies.

An average background of about 14% labeled nuclei was observed in cultures maintained at 40.5°C. Whether this population represents leak of the mutant gene *A* protein, a reversion to the wild-type phenotype, or simply the imperfection of serum synchronization is not known.

## DISCUSSION

SV40 *tsA*-transformed cells are unable to maintain many of the phenotypic properties of transformation when grown under nonpermissive conditions (9, 28, 32, 35, 39, 53). As one possible explanation for these observations, it has been proposed that the SV40 gene *A* product may function in the transformed cells to supercede normal cell control mechanisms and regulate host cell DNA synthesis (12, 30, 33, 38, 52, 57). This premise was tested experimentally in this study by temperature shift experiments with SV40 *tsA*-transformed hamster cells. Confluent cultures of SV40 *tsA* transformants, serum starved at 40.5°C for 48 h, subsequently failed to initiate DNA synthesis during the following 24 h, whether or not FBS was replaced in the medium. Since minimal levels of DNA synthesis were observed with confluent cultures of normal HEF cells at either 33 or 40.5°C in the absence of serum, this observation indicates that *tsA* transformants propagated under conditions restrictive for the expression of gene *A* acquire the normal characteristic of density-dependent inhibition of growth. In contrast, *tsA* transformants maintained at 33°C in either the presence or absence of serum continued asynchronous DNA replication, comparable to the serum-in-

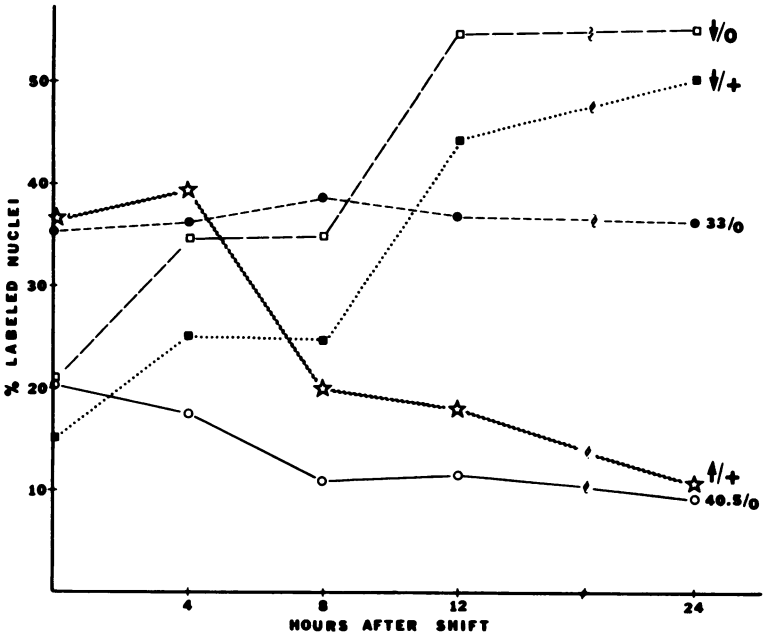


FIG. 5. *Autoradiographic analysis of the effect of serum concentration and temperature shiftup and shiftdown on DNA synthesis in the SV40 tsA-transformed Syrian hamster cell line, Ha$_2$/A28. Experimental procedures for temperature shiftdown were the same as those detailed in the legend to Fig. 2. Procedures for temperature shiftup and autoradiography are described in Materials and Methods. Symbols:* (○) *Cultures maintained at 40.5°C in medium supplemented with 0.5% FBS;* (●) *cultures maintained at 33°C in medium supplemented with 0.5% FBS;* (■) *serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 10% FBS;* (□) *serum-depleted cultures shifted from 40.5 to 33°C in medium supplemented with 0.5% FBS; and* (☆) *serum-depleted cultures shifted from 33 to 40.5°C in medium supplemented with 10% FBS.*

dependent patterns exhibited by wt SV40 transformants grown at either temperature. Hence, with respect to cellular DNA synthesis, a near-normal growth pattern was acquired by the SV40 *tsA* transformants at 40.5°C, and a transformed phenotype was maintained at 33°C. It should be mentioned that the cell lines used in this study had been transformed under liquid medium. Similar studies have not yet been performed on SV40 transformants selected on the basis of anchorage-independent growth in semisolid medium.

Analysis of the kinetics of effects on cellular DNA synthesis after a temperature shift with the *tsA* transformants provided further evidence of the requirement for a functional SV40 gene *A* product in the transformed cells. Approximately 8 h after a temperature shift to either the nonpermissive or permissive temperature, cessation or reinitiation of DNA synthesis, respectively, was observed. That these observations were not reflections of variable transport and equilibration of [$^3$H]TdR into intracellular TTP was demonstrated by direct measurements of relative incorporation of $^3$H into TTP in the *tsA* transformants at 33 and 40.5°C. The fact that a large proportion of the cell population was involved in the DNA synthetic events, rather than only a small subpopulation, was substantiated by autoradiography. The temperature effect on DNA synthesis by the *tsA* transformants was found to be serum independent. It is assumed that the cessation of DNA synthesis after a shiftup of the tsA transformants to the nonpermissive temperature is a reflection of the heat inactivation of the thermolabile mutant gene *A* product. The reinitiation of DNA synthesis after a return to the permissive condition could be due either to resumption of an active configuration by the mutant protein or to new synthesis of the gene *A* product. Therefore, it appears that the SV40 gene *A* product has the ability to regulate cellular DNA synthesis in these transformed hamster cells.

Martin and Stein (35) utilized the CHLA239 cell line in serum, glutamine, and isoleucine depletion studies to determine the point in the cell cycle at which the SV40 *tsA* transformants were arrested under nonpermissive conditions. They observed similar kinetics to those reported here for the onset of DNA synthesis when the *tsA*-transformed cells were shifted to the permissive temperature in the presence of 10% FBS. However, a 50-h lag was observed for [$^3$H]TdR in-

corporation when the depleted cultures were shifted to the permissive temperature in the absence of serum. The basis for the discrepancy in kinetics after a shiftdown is unclear, but could be the result of differences in the experimental approach. Although extensive cell cycle analyses have not been performed in this laboratory, cell doubling times with the *tsA* transformants used in this study are consistent with an arrest between mitosis and S phase, in agreement with the conclusions drawn by Martin and Stein (35).

Brockman (8a) investigated cell growth and DNA synthesis for a clone of *tsA*-transformed BALB/c3T3 cells grown in 1% serum. At the permissive temperature, the *tsA*-transformed cells were able to undergo cell division and synthesize DNA, but were arrested for both parameters at the nonpermissive temperature. After shiftdown to the permissive temperature, DNA synthesis resumed without the addition of higher concentrations of serum after a lag period of approximately 25 h, a value intermediate between the 4- to 8-h lag observed in our system and the 50-h lag reported by Martin and Stein (35).

Temperature shift experiments similar in design to those described here have been performed by Bell et al. (6) for chicken embryo fibroblasts infected with a mutant of Rous sarcoma virus (RSV) temperature sensitive for the maintenance of transformation. Mutant-infected chicken embryo fibroblasts became stationary upon serum depletion at the nonpermissive temperature and resumed incorporation of [$^3$H]TdR approximately 6 h after a shift to the permissive temperature. The resumption of the transformed phenotype after a shiftdown was serum independent. Autoradiography revealed that at least 25% of the cells incorporated [$^3$H]TdR (during a 1-h pulse) by 10 h after the shift to 35°C. The remarkable parallels in the two systems are intriguing because they suggest that a similar control over DNA synthesis may be exerted by an oncornavirus (RSV) and a papovavirus (SV40). Whether the primary modes of action of the transforming functions specified by RSV and SV40 are identical remains to be elucidated.

Toniolo and Basilico (56) have selected temperature-sensitive SV40-transformed mouse 3T3 cells (ts23A) that have lost the ability to grow in serum-depleted medium at the elevated temperature but continue to synthesize DNA, in contrast to the hamster *tsA* transformants described here, which arrest for DNA synthesis at 40.5°C. The ts23A cells express other transformed growth characteristics at the conditional temperature, leading the authors to conclude that the line possesses a cellular mutation in the ability to transport serum factors. Therefore, a distinction can be made between a cellular mutation affecting a single parameter of transformation and the *tsA* mutation, which induces a spectrum of alterations in the transformed phenotype.

The role of the SV40 gene *A* product in viral DNA replication is well documented (17, 31, 51). The data presented here support previous hypotheses (12, 30, 33, 38, 57) that there may be an association between gene *A* function and the regulation of DNA synthesis in cells transformed by SV40. An additional approach would be to microinject purified *tsA*-coded T antigen into normal cells. The mutant T antigen should manifest a temperature-sensitive effect on DNA synthesis if it possesses such a regulatory property. Alternatively, the induction of DNA synthesis in avian erythrocyte nuclei within heterokaryons of *tsA* transformants and avian erythrocytes could be assayed. Reactivation potential should be temperature sensitive if T antigen is an effector of cellular DNA synthesis. However, a sensitive cell-free system that measures the initiation of cellular DNA replication would be necessary to determine conclusively whether the antigen can exert a direct control over DNA synthesis.

The exact control mechanism underlying the observed phenomena is unknown. The gene *A* product may act as an aberrant initiator substance at preexisting host cell replicons, at integrated SV40 replicons, or a combination of both (12, 33). In fact, it has been reported that more replicons are active in SV40-transformed cells than in normal cells and that the *tsA*-transformed cells revert at the nonpermissive temperature and mimic normal cells (34).

Alternatively, the postulated level of control of DNA synthesis by gene *A* would not necessarily have to be restricted solely to the nucleus in the transformed cells. Theoretically, regulation could occur at other levels, such as the plasma membrane, either singly or in concert with nuclear activity. Evidence is available which relates SV40 nuclear T antigen to the viral-specific transplantation antigen (TSTA) (3, 4, 19). At the nonpermissive temperature, the lesion in the mutant gene *A* product might prevent proper cleavage or processing of the protein, resulting in faulty membrane insertion of TSTA. The subsequent absence of membrane perturbation could then result in the acquisition of density-dependent growth regulation (10, 11, 22, 47) by the *tsA* transformants at the elevated temperature.

The data in this report have been interpreted based on current genetic evidence that the early region of SV40 consists of a unique gene that

codes for a single gene product responsible for viral DNA synthesis in the productive infection, as well as the initiation and maintenance of transformation. Recent data have indicated a different genetic strategy for the expression of polyoma early genes. Genetic investigations have revealed two unique early determinants, one responsible for viral DNA synthesis and stable transformation defined by the *tsA* mutation and another necessary for maintenance of transformation defined by the hr-t mutation (21, 23, 50). Since viable deletion mutants of SV40 have been isolated which map in the portion of the early region of the genome in which no *tsA* mutants have been localized (29, 48), the possibility cannot be ruled out that the early region of SV40 may segregate into more than one genetic determinant. Sequence analysis of the SV40 genome supports this possibility in that one-third of the way into the early region, termination codons are found in all three phases (55). It has been suggested that mutants with deletions between 0.54 and 0.59 on the SV40 physical map fail to synthesize a small-molecular-weight (~17,000) protein and exhibit a decreased transforming frequency (cited in reference 18; P. Tegtmeyer, personal communication). The role of this putative early gene in regulation of DNA synthesis in SV40-transformed cells remains to be determined.

It is obvious that the precise mechanism by which the SV40 gene *A* product assumes control of DNA synthesis in transformed cells remains to be established. However, it may be concluded from the data presented in this report that the gene *A* product acts either directly or indirectly as a potent mitogen and releases stationary cultures from a metabolic arrest to initiate cellular DNA synthesis.

## ACKNOWLEDGMENTS

This study was supported by Public Health Service research grants CA-10893 and CA-22555 from the National Cancer Institute.

The excellent technical assistance provided by Elizabeth Torres is appreciated. The advice of Marion Steiner in the analysis of TTP is gratefully acknowledged.

## LITERATURE CITED

1. **Ahmed-Zadeh, C., B. Allet, J. Greenblatt, and R. Weil.** 1976. Two forms of simian virus 40 specific T-antigen in abortive and lytic infection. Proc. Natl. Acad. Sci. U.S.A. **73**:1097-1101.
2. **Aloni, Y., E. Winocour, and L. Sachs.** 1968. Characterization of the simian virus 40-specific RNA in virus-yielding and -transformed cells. J. Mol. Biol. **31**:415-429.
3. **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.
4. **Anderson, J. L., R. G. Martin, C. Chang, P. T. Mora, and D. M. Livingston.** 1977. Nuclear preparations of SV40 transformed cells contain tumor-specific transplantation activity. Virology **76**:420-425.
5. **Baserga, R., and D. Malamur.** 1969. Autoradiography; techniques and application. Harper and Rowe, New York.
6. **Bell, J. G., J. A. Wyke, and I. A. Macpherson.** 1975. Transformation by a temperature sensitive mutant of Rous sarcoma virus in the absence of serum. J. Gen. Virol. **27**:127-134.
7. **Benyesh-Melnick, M., and J. S. Butel.** 1974. Oncogenic viruses, p. 403-485. *In* H. Busch (ed.), The molecular biology of cancer. Academic Press Inc., New York.
8. **Black, P. H., and W. P. Rowe.** 1963. An analysis of SV40-induced transformation of hamster kidney tissue in vitro. I. General characteristics. Proc. Natl. Acad. Sci. U.S.A. **50**:606-613.

8a. **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.

9. **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.
10. **Burger, M.** 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature (London) **227**:170-171.
11. **Burger, M., and K. Noonan.** 1970. Restoration of normal growth by covering agglutinin sites on tumor cell surface. Nature (London) **228**:512.
12. **Butel, J. S., J. S. Brugge, and C. A. Noonan.** 1974. Transformation of primate and rodent cells by temperature-sensitive mutants of SV40. Cold Spring Harbor Symp. Quant. Biol. **39**:25-36.
13. **Butel, J. S., S. S. Tevethia, and J. L. Melnick.** 1972. Oncogenicity and cell transformation by papovavirus SV40: the role of the viral genome. Adv. Cancer Res. **15**:1-55.
14. **Carp, R. I., G. Sauer, and F. Sokol.** 1969. The effect of actinomycin D on the transcription and replication of simian virus 40 deoxyribonucleic acid. Virology **37**:214-226.
15. **Carroll, R. B., and A. E. Smith.** 1976. Monomer molecular weight of T antigen from simian virus 40-infected and transformed cells. Proc. Natl. Acad. Sci. U.S.A. **73**:2254-2258.
16. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101-1109.
17. **Chou, J., and R. G. Martin.** 1975. Products of complementation between temperature-sensitive mutants of simian virus 40. J. Virol. **15**:127-136.
18. **Cole, C. N., T. Landers, S. P. Goff, S. Manteuil-Brutlag, and P. Berg.** 1977. Physical and genetic characterization of deletion mutants of simian virus 40 constructed in vitro. J. Virol. **24**:277-294.
19. **Deppert, W., and G. Walter.** 1976. Simian virus 40 tumor-specific proteins in nucleus and plasma membrane of HeLa cells infected by adenovirus 2-SV40 hybrid virus Ad2$^+$ND2. Proc. Natl. Acad. Sci. U.S.A. **73**:2505-2509.
20. **Dulbecco, R.** 1970. Topoinhibition and serum requirement of transformed and untransformed cells. Nature (London) **227**:802-806.
21. **Eckhart, W.** 1977. Complementation between temperature-sensitive (ts) and host range nontransforming (hr-t) mutants of polyoma virus. Virology **77**:589-597.
22. **Eckhart, W., R. Dulbecco, and M. Burger.** 1971. Temperature-dependent surface changes in cells infected or transformed by a thermosensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **68**:283-286.
23. **Fluck, M. M., R. J. Staneloni, and T. L. Benjamin.** 1977. Hr-t and ts-a: two early functions of polyoma

virus. Virology **77**:610-624.
24. **Hatanaka, M., and R. Dulbecco.** 1966. Induction of DNA synthesis by SV40. Proc. Natl. Acad. Sci. U.S.A. **56**:736-740.
25. **Jainchill, J., and G. J. Todaro.** 1970. Stimulation of cell growth *in vitro* by serum with and without growth factor. Exp. Cell Res. **59**:137-146.
26. **Khoury, G., and M. A. Martin.** 1975. A transcriptional map of the SV40 genome in transformed cell lines. Virology **63**:263-272.
27. **Kimura, G., and R. Dulbecco.** 1973. A temperature sensitive mutant of simian virus 40 affecting transforming activity. Virology **52**:529-534.
28. **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.
29. **Lai, C. J., and D. Nathans.** 1974. Mapping temperature sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. Virology **60**:466-475.
30. **Levine, A. J.** 1973. A model for the maintenance of the transformed state by polyoma and SV40, p. 61-76. *In* M. Pollard (ed.), Perspectives in virology, vol. 8. Academic Press Inc., New York.
31. **Levine, A. J.** 1976. SV40 and adenovirus early functions involved in DNA replication and transformation. Biochim. Biophys. Acta **485**:213-241.
32. **Martin, R. G., and J. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599-612.
33. **Martin, R. G., J. Y. Chou, J. Avila, and R. Saral.** 1974. The semi-autonomous replicon: a molecular model for the oncogenicity of SV40. Cold Spring Harbor Symp. Quant. Biol. **39**:17-24.
34. **Martin, R. G., and A. Oppenheim.** 1977. Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. Cell **11**:859-869.
35. **Martin, R. G., and S. Stein.** 1976. Resting state in normal and simian virus 40 transformed Chinese hamster lung cells. Proc. Natl. Acad. Sci. U.S.A. **73**:1655-1659.
36. **Nazar, R. N., H. G. Lawford, and J. T. Wang.** 1970. An improved procedure for extraction and analysis of cellular nucleotides. Anal. Biochem. **35**:305-313.
37. **Oda, K., and R. Dulbecco.** 1968. Regulation of transcription of the SV40 DNA in productively infected and in transformed cells. Proc. Natl. Acad. Sci. U.S.A. **60**:525-532.
38. **Osborn, M., and K. Weber.** 1974. SV40: T antigen, the A function and transformation. Cold Spring Harbor Symp. Quant. Biol. **39**:267-276.
39. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. **15**:636-644.
40. **Ozanne, B., P. A. Sharp, and J. Sambrook.** 1973. Transcription of simian virus 40. J. Virol **12**:90-98.
41. **Prives, C., E. Gilboa, M. Revel, and E. Winocour.** 1977. Cell-free translation of simian virus 40 early messenger RNA coding for viral T-antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:457-461.
42. **Rapp, F., S. Pauluzzi, and J. S. Butel.** 1969. Variation in properties of plaque progeny of PARA (defective simian papovavirus 40)-adenovirus 7. J. Virol. **4**:626-631.
43. **Ritzi, E., and A. J. Levine.** 1970. Deoxyribonucleic acid replication in simian virus 40-infected cells. III. Comparison of simian virus 40 lytic infection in three different monkey kidney cell lines. J. Virol. **5**:686-692.
44. **Rundell, K., J. K. Collins, P. Tegtmeyer, H. L. Ozer, C. J. Lai, and D. Nathans.** 1977. Identification of simian virus 40 protein A. J. Virol. **21**:636-646.
45. **Sambrook, J.** 1972. Transformation by polyomavirus and simian virus 40. Adv. Cancer Res. **16**:141-180.
46. **Sauer, G., and J. R. Kidwai.** 1968. The transcription of the SV40 genome in productively infected and transformed cells. Proc. Natl. Acad. Sci. U.S.A. **61**:1256-1263.
47. **Sefton, B. M., and H. Rubin.** 1970. Stimulation of glucose transport in cultures of density-inhibited chick embryo cells. Proc. Natl. Acad. Sci. U.S.A. **68**:3154-3157.
48. **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.
49. **Smith, H. S., C. D. Scher, and G. J. Todaro.** 1971. Induction of cell division in medium lacking serum growth factor by SV40. Virology **44**:359-370.
50. **Staneloni, R. J., M. M. Fluck, and T. L. Benjamin.** 1977. Host range selection of transformation defective hr-t mutants of polyoma virus. Virology **77**:598-609.
51. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591-598.
52. **Tegtmeyer, P.** 1974. Altered patterns of DNA synthesis in infection by SV40 mutants. Cold Spring Harbor Symp. Quant. Biol. **39**:9-15.
53. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613-618.
54. **Tegtmeyer, P., and H. L. Ozer.** 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516-524.
55. **Thimmappaya, B., and S. M. Weissman.** 1977. The early region of SV40 may have more than one gene. Cell **11**:837-843.
56. **Toniolo, D., and C. Basilico.** 1975. SV40-transformed cells with temperature-dependent serum requirements. Cell **4**:255-262.
57. **Weil, R., C. Salomon, E. May, and P. May.** 1974. A simplifying concept in tumor virology: virus-specific "pleiotropic effectors." Cold Spring Harbor Symp. Quant. Biol. **39**:381-395.
58. **Yamaguchi, N., and T. Kuchino.** 1975. Temperature-sensitive mutants of simian virus 40 selected by transforming ability. J. Virol. **15**:1297-1301.