Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 1931–1935, June 1976
Biochemistry

Regulation of viral transcription and tumor antigen expression in cells transformed by simian virus 40

(ts SV3T3 cells/G1 arrest/DNA·RNA hybridization)

CLAUDIO BASILICO AND DIMITRIS ZOUZIAS

Department of Pathology, New York University School of Medicine, New York, N.Y. 10016

Communicated by Severo Ochoa, March 30, 1976

ABSTRACT We have studied the expression of simian virus 40 (SV40) specific tumor antigen (T-antigen) and viral RNA in SV40-transformed mouse 3T3 cells that are temperature-sensitive for the expression of the transformed phenotype (ts SV3T3). Although transformed by wild-type SV40, ts SV3T3 cells at 32° behave like standard transformants, while at 39° they became arrested in G1 after reaching saturation density or under conditions of serum starvation. ts SV3T3 cells at 32° or exponentially growing at 39° are uniformly T-antigen positive. However, after G1 arrest at 39° the majority of the cells becomes T-antigen negative. Induction of proliferation in the resting cultures results in the reappearance of T-antigen in most of the cells, concomitant with the induction of DNA synthesis. The reason for the disappearance of T-antigen from ts SV3T3 cells arrested in G1 seems to reside in a transcriptional control operating on the integrated viral DNA, since these cells contain no appreciable amounts of SV40 specific RNA. Viral RNA can be easily detected in cells *growing* at 32° or at 39°. The results suggest that transcription of the viral genome in SV40-transformed cells is cell-cycle-dependent.

Cells transformed by the oncogenic DNA virus, simian virus 40 (SV40), exhibit several phenotypic changes with respect to their untransformed counterparts. Most or all of such changes appear to be directed by the presence of integrated viral DNA, which is transcribed and translated into viral gene-products. Only one viral protein has been identified in transformed cells. This is the virus specific tumor antigen (T-antigen), and recent evidence supports the notion that it is the product of the SV40 *A* gene (1–3). The SV40 *A* gene-product is required for the establishment (4, 5) and probably also the maintenance of cell transformation (4–7), and represents the only *early* viral product identified so far (8, 9).

It seems therefore important to define the relationship between T-antigen and cell growth, and to look for regulatory mechanisms that might possibly control T-antigen synthesis in transformed cells. We have thus studied T-antigen and viral RNA in SV40-transformed cells that were either exponentially growing or arrested in the G1 phase of the cell cycle. Since SV40-transformed cells do not generally respond to conditions that cause a viable G1 arrest in normal cells (10–14), we took advantage of the behavior of two lines of SV40-transformed cells (ts SV3T3), which, although transformed by wild-type (wt) SV40 are temperature sensitive (ts) for the expression of transformation, presumably because of a host cell mutation (15). ts SV3T3 cells express the transformed phenotype at 32°, but not at 39°, and as a result they became arrested in G1 after they reach saturation density at 39° (15–17). The results presented in this paper show that when these cells are arrested in G1, SV40 T-antigen can no longer be detected in their nuclei. The reason for this behavior seems to reside in a transcriptional control operating on the integrated viral DNA, since G1 arrested (G0) ts SV3T3 cells do not contain appreciable amounts of SV40 specific RNA.

MATERIALS AND METHODS

Cells. The properties of the two lines of ts SV40-transformed cells (ts SV3T3) used in this work (ts H6-15 and ts H1), have been described (15). Cell stocks were generally maintained at 32° in Dulbecco's medium containing 10% calf serum. For the experiments involving resting cells at 39°, cultures were generally grown in 5–10% calf serum. At the time they reached confluence, the medium was changed with medium containing 1% calf serum, unless otherwise stated.

T-Antigen Assay. Cells that had been grown on coverslips were fixed with acetone-ethanol (2:1) for 20 min at 4°. For T-antigen determination, the cells were incubated with hamster antiserum against SV40 T-antigen (Flow) for 45 min at 37°, and then with fluorescein-labeled antibody against hamster globulin (Caprine). Cells were observed under dark phase in a Zeiss UV microscope equipped with an automatic camera.

Autoradiography. Cells that had been grown on coverslips were incubated in the presence of [^{3}H]thymidine. At the end of the labeling period, they were fixed with 95% ethanol–acetic acid (9:1). Coverslips were washed extensively in 70% ethanol, dried, and mounted on slides. Slides were dipped in nuclear track emulsion (Kodak, NTB-2); after about 7 days of exposure they were developed and stained with Giemsa.

Preparation of Cellular RNA. RNA from transformed cells was prepared by the hot phenol method. For cytoplasmic RNA, cells were first fractionated into cytoplasm and nuclei as described (18). The RNA was treated with DNase I, extracted with phenol and $CHCl_3$, concentrated by precipitation with ethanol, and passed through a column of Sephadex G-75 (19). RNA eluting in the void volume was concentrated and used in hybridization experiments.

Preparation of ^{32}P-Labeled SV40 DNA. BSC-1 monkey cells were infected with plaque purified SV40 virus at a multiplicity of infection of 40 plaque-forming units/cell in phosphate-free medium containing 2% fetal calf serum. Carrier-free [^{32}P]-orthophosphate (100 μCi/ml) was added 16–24 hr after infection. SV40 DNA form I was extracted 2 days later and purified as described (20). Labeled SV40 DNA (1.5 to 2.4 $\times$ 10^6 cpm/μg) was sheared either by sonication or by boiling in 0.3 M NaOH for 10 min.

Separation and Purification of SV40 DNA Strands. Strands were separated essentially as described by Sambrook *et al.* (21), using complementary RNA (cRNA) synthesized *in vitro*. The synthesis of SV40 specific cRNA was catalyzed *in vitro* by *Escherichia coli* DNA-dependent RNA polymerase containing sigma factor (gift of Dr. S. Leffler). *E. coli* RNA polymerase transcribes supercoiled SV40 DNA asymmetrically (21).

Fragmented SV40 [^{32}P]DNA ($\simeq$0.04 μg) was denatured by

Abbreviations: ts, temperature-sensitive; wt, wild type; cRNA, complementary RNA; SV40, simian virus 40; SV3T3 cells, SV40-transformed mouse 3T3 cells; T-antigen, tumor antigen.

Table 1. DNA synthesis in ts H6-15 cells at 39° and 32°

Days after confluence	% Cells synthesizing DNA*		Cell no.†	
	39°	32°	39°	32°
−2‡	38	61	4	3
−1	29	45	8	5
0	10	ND	12	10
1‡	2.5	45.7	11	18
2	ND	49.7	13	40
3	4.2	38.5	13	82
4	3	42.2	12	85

* Cells were labeled for 1 hr with [^{3}H]thymidine (3 μCi, 0.11 μg/ml), then fixed and processed for autoradiography.
† Number of cells per 50-mm petri dish × 10^{-5}.
‡ Medium, containing 5% calf serum, was changed.

Table 2. T-antigen in ts SV3T3 cells at 39° and 32°*

Days after confluence	% T-antigen positive cells					
	ts H6-15		ts H1		ts 23A	
	39°	32°	39°	32°	39°	32°
−2	—	—	100	—	100	—
−1	100	100	—	—	—	—
0†	80	100	20	100	>90	—
1	40	—	5	—	>90	—
2	5.8	100	—	>90	—	—
3	2	100	5	—	> 90	—
4	3	—	3	—	—	—

* Cells were plated at 39° and 32° at day −3 in medium containing 5% serum. At the times indicated they were fixed and stained for T-antigen as described in *Materials and Methods.*
† Medium was changed with medium containing 1% serum at 39°, 5% serum at 32°.

boiling and incubated with 30 μg of SV40 cRNA at 68° for 30 min. The reaction mixture was then applied to a hydroxylapatite column at 60°. The late (L) strands of SV40 DNA were eluted in a small volume of 0.14 M phosphate buffer, 0.4% sodium dodecyl sulfate, and the early (E) strands were recovered as DNA·RNA hybrid with 0.4 M phosphate buffer. SV40 cRNA was destroyed by alkali treatment (0.6 M NaOH) at 37° for 6–8 hr; after neutralization with 6 M HCl, both eluates were left to "self-reanneal" at a sodium ion concentration of 1.0 M for 30–40 hr at 68°. After dilution, a second hydroxylapatite chromatography analysis was performed and purified, separated single-stranded DNA was recovered.

DNA·RNA Hybridization. The fraction of each SV40 DNA strand hybridizing to cellular RNA was determined by reacting increasing amounts of cellular RNA with small amounts of fragmented SV40 [^{32}P]DNA strands (19). Hybridization was carried out at 68° in 1 M NaCl, 0.1 M phosphate buffer, pH 6.8 and 0.4% sodium dodecyl sulfate for 36 hr. Single-stranded DNA was separated from DNA·RNA hybrids by hydroxylapatite chromatography, as described. The two fractions were mixed with Aquasol (New England Nuclear); radioactivity was determined directly in a liquid scintillation counter.

RESULTS

DNA synthesis and T-antigen in the ts SV3T3 cells at 39° and 32°

As already described (15, 16), the ts SV3T3 lines used in these experiments (ts H6–15 and ts H1) behave *in vitro* essentially like SV40-transformed 3T3 cells when grown at 32°, but at 39° their properties are similar to those of normal 3T3. This behavior is reflected in their response to density or to serum starvation at the two temperatures. At 32°, the DNA synthetic activity of these cells is high even in dense cultures or in low concentrations of serum. At 39°, on the other hand, DNA synthesis greatly decreases at confluence, especially if cells are incubated at low (1%) serum concentrations (15–17) (Table 1).

We determined the presence of T-antigen in the nuclei of the ts SV3T3 cells in various stages of growth at 39° and 32°. Cells were fixed at the desired times, and the presence of T-antigen was determined by immunofluorescence. The results are shown in Table 2. At 32°, the cells were uniformly T-antigen positive, at all cell densities. At 39°, cells of both the ts H1 and the ts H6-15 lines were uniformly positive as long as they were in exponential growth. When the cultures reached confluence, the intensity of the stain decreased in most cells. Some T-antigen negative cells could also be observed. By 1–2 days after confluence the majority of the cells had become T-antigen negative (Fig. 1). When the resting cells were trypsinized and replated at lower density at 39°, to allow them to resume growth, the cells again became T-antigen positive within 24 hr. If the confluent cultures were shifted from 39° to 32° without replating, most of the cells became T-antigen positive by about 48 hr after shift-down, a time similar to that required to again start DNA synthesis (15).

It would seem therefore that the expression of T-antigen in these cells is correlated with progression through the mitotic cycle. At 39°, approximately 24 hr after reaching a G1 arrest, most of the cells become T-antigen negative. At 32°, where even at high cell densities and in low serum DNA synthesis continues, the cells remained positive all the time. To ascertain whether the loss of T-antigen was related specifically to G1 arrest, or simply to cessation of growth, we examined cells of the ts 23A line. These SV40-transformed cell variants exhibit temperature-dependent serum requirements; their serum requirements for growth are much greater at 39° than at 32° (12). At 39° in standard serum concentrations (5–10%) these cells attain low saturation densities, and in 1% serum they do not grow. However, they are not arrested in G1, but continue to synthesize DNA and shed off the plate (12). When ts 23A cells were examined for T-antigen at 39°, they remained always more than 90% positive, even 2–3 days after they had stopped increasing in number (Table 2).

As shown in Table 2, a few positive cells were always present in the ts SV3T3 cultures even days after reaching confluence. Cultures of ts H6-15 cells that had been confluent at 39° for 2 days were labeled with [^{3}H]thymidine for 6 hr. At the end of the pulse, the coverslips were fixed, and duplicate coverslips were stained for T-antigen or processed for autoradiography. The frequency of T-antigen positive cells and of cells that had synthesized DNA was then determined. The cells (5.7%) were T-antigen positive, whereas 5.6% of the cells had incorporated radioactive thymidine in their nuclei. It would seem therefore that the few positive cells observed in resting cultures of ts SV3T3 reflected the fact that a certain proportion of the cells was still dividing. Most of these probably represented revertants, which have regained the ability to express the transformed phenotype and therefore grow beyond monolayer density at 39°. Such cells were generally observed in clusters, and with prolonged incubation the clusters of T-antigen positive cells became larger.

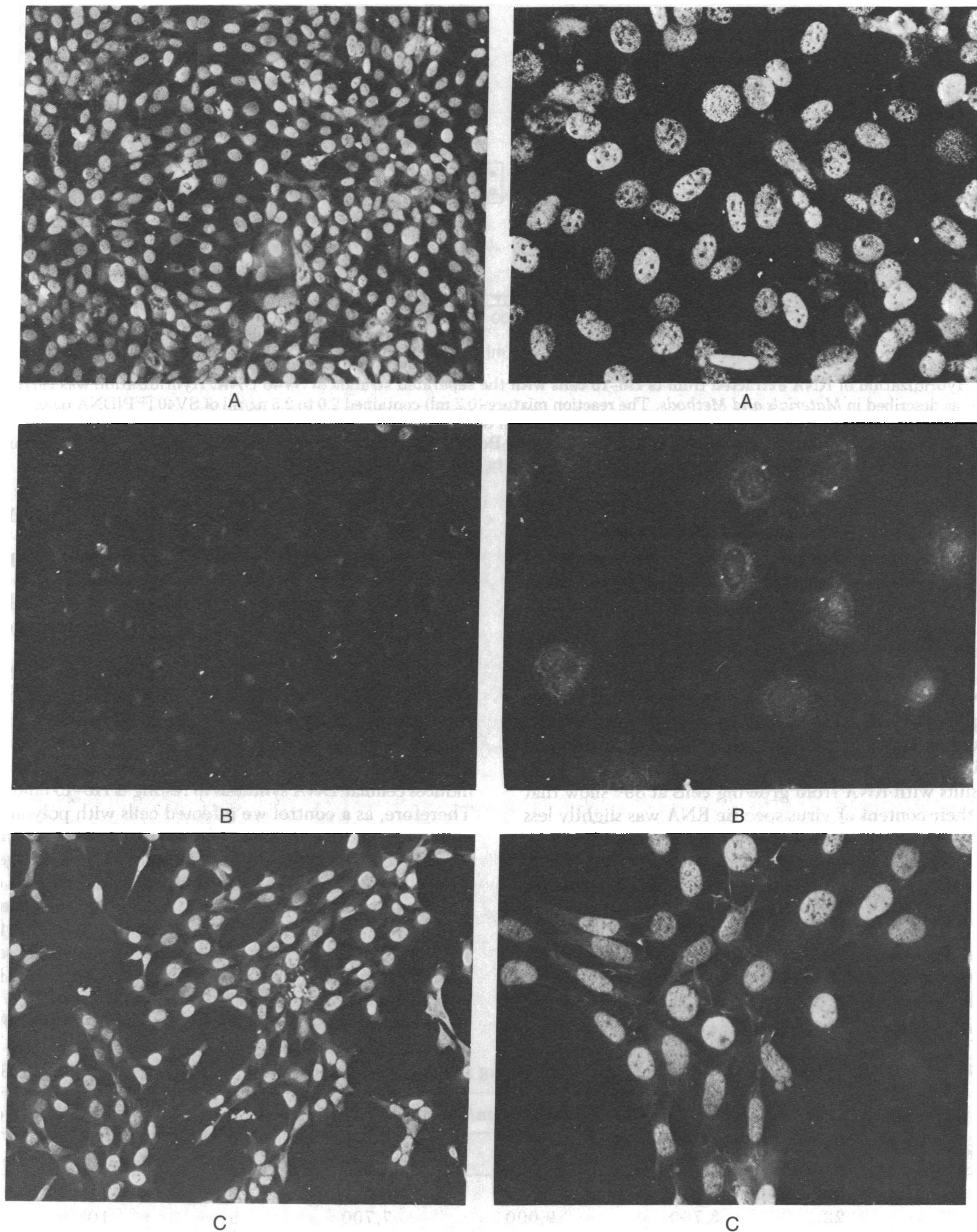

FIG. 1. Micrographs of ts H6–15 cells after immunofluorescent staining for SV40 T-antigen. Left, low power; right, high power. (A) Cells at 32°. (B) Cells at 39°, 2 days after reaching confluence. (C) Cells in exponential growth at 39°.

Virus specific RNA in resting or growing ts SV3T3 cells

The failure to detect T-antigen in resting ts SV3T3 cells might be due to some mechanism interfering with the synthesis or the assembly of T-antigen itself (increased lability, failure to bind to the cell chromatin, etc.) or could be due to a more general control mechanism, regulating transcription of the integrated viral DNA. ts H6–15 cells contain approximately 2.2 SV40 DNA equivalents per diploid cell genome, and the number of viral DNA equivalents does not change appreciably whether measured in DNA preparations isolated from ts H6–15 cells grown at 32° or from resting cultures at 39°. We determined the extent of viral transcription in ts H6–15 cells under different growth conditions by measuring the ability of RNA preparations extracted from these cells to form hybrid molecules with ^{32}P-labeled single-stranded SV40 DNA. Most SV40 specific stable RNA in transformed cells is complementary to the early strand

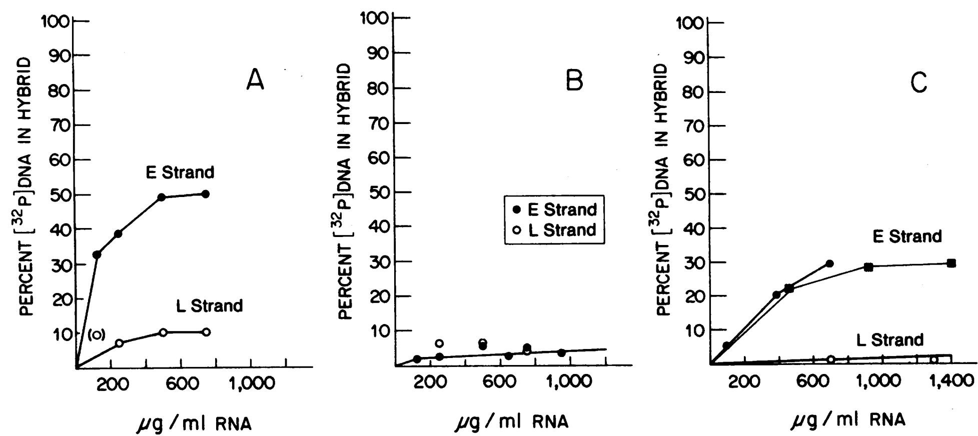

FIG. 2. Hybridization of RNA extracted from ts H6-15 cells with the separated strands of SV40 DNA. Hybridization was carried out at 68° for 36 hr, as described in *Materials and Methods*. The reaction mixture (0.2 ml) contained 2.0 to 2.5 ng/ml of SV40 [^{32}P]DNA (specific activity 5×10^5 to 1×10^6 cpm/μg). (A) Cytoplasmic RNA from ts H6-15 cells at 32°. (B) Cytoplasmic RNA from resting ts H6-15 cells at 39°. (C) Cytoplasmic (●, ○), or total (■) RNA from ts H6-15 cells growing at 39°. Both E and L SV40 DNA preparations gave at most 2-3% reannealing either in the presence or the absence of mouse cell RNA, or RNA from ts H6-15 cells after alkaline hydrolysis.

(19, 22), and therefore hybridization was performed with the separated early (E) and late (L) strands of SV40 DNA. The results of these experiments are seen in Fig. 2. RNA extracted from ts H6-15 cells at 32° hybridized with about 50% of the E strand of SV40 DNA. Some RNA also hybridized to about 10% of the L strand. In contrast, RNA extracted from resting ts H6-15 cells at 39° contained practically no RNA complementary to either strand of SV40 DNA. These results were obtained with several different RNA preparations, and did not change appreciably if total cellular, instead of cytoplasmic, RNA was used. It can be estimated that these cells contain at least 25 times less virus specific RNA than the cells at 32°.

The results with RNA from growing cells at 39° show that although their content of virus specific RNA was slightly less than at 32°, a saturating level of the E SV40 strand could be easily reached. Such a level was however somewhat lower than that obtained with RNA from cells growing at 32°. The possible significance of this difference will have to be investigated further.

In conclusion, the results presented suggest that the reason for the disappearance of T-antigen from G1 arrested ts H6-15 cells resides in the lack of transcription of the viral DNA into functional mRNA.

Superinfection of resting ts H6-15 cells with polyoma and SV40

From the data presented above, it appears that the expression of SV40 T-antigen in ts SV3T3 cells is dependent on the cell growth cycle, or at least that in G1 arrested (G0) cells some cellular control inhibits the expression of the T-antigen gene present in the integrated viral genome.

To determine whether SV40 DNA was subjected to this type of control also during acute infection, we superinfected resting cultures of ts H6-15 cells at 39° with SV40 and determined the frequency of T-antigen positive cells at various times after infection. However, infection with SV40 at high multiplicity induces cellular DNA synthesis in resting ts H6-15 cultures (17). Therefore, as a control we infected cells with polyoma virus, which also induces cellular DNA synthesis (17). In the polyoma-infected cultures, the frequency of SV40 T-antigen positive cells rose considerably, but only after induction of cellular DNA synthesis had taken place (Table 3). On the other hand, in the SV40 infected cultures, by 10 hr after infection the majority of the cells had become T-antigen positive. These results confirm the conclusion that T-antigen reappearance in these cells follows induction of proliferation. In addition, they suggest that nonintegrated SV40 DNA is not subjected to the G0 control,

Table 3. Induction of T-antigen and DNA synthesis in resting ts H6-15 cells after superinfection with polyoma or SV40*

Experiment	Hr after infection	DNA synthesis† (cpm/culture)			SV40 T-antigen‡ (% positive cells)		
		Control	Py	SV40	Control	Py	SV40
I	16	ND§	ND	ND	5	5	>90
	22	3,700	9,000	7,700	5	10	>90
	43	2,700	23,300	21,000	6	55	>90
II	11	5,400	4,800	4,000	7.4	5.4	47.8
	16	5,300	5,400	8,000	ND	8.0	44.0
	22	2,900	5,600	12,000	8.0	11.9	56.9
	36	3,600	17,000	15,200	6.4	34.8	61.6
	45	2,600	10,200	13,000	5.4	33.2	64.7

* Cells were plated at 39° in medium containing 10% calf serum. One day after reaching confluence they were infected with polyoma (Py) or SV40 at a multiplicity of infection of 200 plaque-forming units/cell in Exp. I and 100 plaque-forming units/cell in Exp. II. After 2 hr of virus adsorption at 39°, they received the old medium, diluted with fresh serum-free medium to bring the serum concentration to 2%.

† Cells were labeled for 1 hr with [^{3}H]thymidine (3 μCi, 0.11 μg/ml) at the times indicated. They were then lysed in 0.6% sodium dodecyl sulfate, and the trichloroacetic acid insoluble radioactivity was determined.

‡ Cells were fixed at the times indicated and the frequency of SV40 T-antigen positive cells was determined by immunofluorescence.

§ ND, not done.

since superinfecting viral genomes are capable of inducing T-antigen synthesis in resting ts H6–15 cells long before induction of cellular DNA synthesis (Table 3).

DISCUSSION

The results presented here show that after ts SV3T3 cells enter a resting state, T-antigen becomes undetectable in their nuclei. Concomitantly, the presence in these cells of SV40 specific RNA becomes undetectable. It appears that after the T-antigen produced before G1 arrest has decayed, no new T-antigen is formed. It has been shown (2, 3, 23, 24) that SV40 and polyoma T-antigen are not very stable *in vitro*, and our observation that T-antigen disappears from the cells about 24 hr after growth arrest is consistent with these findings.

It can be asked whether the lack of transcription of the viral DNA in ts SV3T3 is restricted to a state of G1 arrest (G0), and whether it is a unique property of these cell mutants or a general property of SV40-transformed cells. ts SV3T3 cells, although transformed by wt SV40, carry a host cell mutation which at 39° renders them similar to normal 3T3 cells (15, 16), and their behavior at 39° may be the result of such a mutation. Although more work will have to be done to answer these questions conclusively, we believe at this moment that the lack of or reduced transcription of SV40 DNA occurs throughout G1 and that it it likely to be a general property of SV40-transformed cells.

Swetly and Watanabe (25) have reported data suggesting that in synchronized SV40-transformed cells, SV40 transcription proceeds mainly during the S phase of the cell cycle. Preliminary experiments with our ts SV3T3 cells suggest that once resting cells enter a proliferative state, appreciable SV40 DNA transcription does not restart until the S phase has begun. Our results show that inhibition of SV40 DNA transcription and T-antigen production are correlated with G1 arrest and not with temperature. Thus, our cells are uniformly T-antigen positive when growing at 39° and also contain viral RNA. T-antigen negative cells at 39° can be induced to produce T-antigen by inducing them to proliferate, either by replating at lower density, or by superinfecting with polyoma virus. Furthermore, two reports suggesting cell cycle dependent transcription of integrated viral genomes have appeared, one related to SV40-transformed cells (25) and the other to cells transformed by adenovirus (26). Thus, we feel that it is likely that lack or impairment of transcription in G1 is a general phenomenon of SV40-transformed cells.

The lack of transcription of integrated SV40 DNA in G1 arrested (G0) cells might be due to several reasons: (*a*) a specific regulation of the transcription of the SV40 genome, similar to that occurring for histone mRNA (27), which is only synthesized during the S phase; (*b*) transcription of integrated SV40 DNA may occur only from newly replicated DNA, and therefore be dependent on cellular DNA synthesis; (*c*) transcription of SV40 DNA may be dependent on the integration site(s) on the host chromosomes. If SV40 DNA is transcribed together with the host cell DNA, integration at a cellular site which is normally not transcribed in G1 or G0 may produce the observed results.

In this respect it is important to note that, as shown here, nonintegrated SV40 DNA does not appear to respond to this type of control. Moreover, the overall transcription of putative cell mRNA does not appear to be much reduced in resting cells as compared with growing cells (28, 29). This finding strongly supports the hypothesis that the lack of SV40 transcription in resting ts H6–15 cells does not result from an overall depression of transcription in resting cells.

Finally, our work may provide a plausible explanation for some as yet unexplained findings in studies with SV40-transformed cells. Clones of SV40-transformed cells having an intraclonal variability in the expression of T-antigen have been described (30). This behavior could be explained by postulating that these cultures always contain some cells that are in a resting state, and are therefore unable to express T-antigen.

We thank Dr. Brad Ozanne for suggesting to us some of the initial experiments that led to this work, and E. Deustch and P. Santanello for their technical assistance. This investigation was supported by Grants CA 11893 and CA 16239 from the National Cancer Institute. C. B. is a scholar of the Leukemia Society.

1. Tegtmeyer, P., Schwartz, M., Collins, J. K. & Rundell, K. (1975) *J. Virol.* **16**, 168–178.
2. Tenen, D. G., Baygell, P. & Livingston, D. M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4351–4355.
3. Alwine, J. C., Reed, S. J., Ferguson, J. & Stark, G. R. (1975) *Cell* **6**, 529–533.
4. Tegtmeyer, P. (1975) *J. Virol.* **15**, 613–618.
5. Martin, R. G. & Chou, J. Y. (1975) *J. Virol.* **15**, 599–612.
6. Brugge, J. S. & Butel, J. S. (1975) *J. Virol.* **15**, 619–635.
7. Osborn, M. & Weber, K. (1975) *J. Virol.* **15**, 636–644.
8. Tegtmeyer, P. & Ozer, H. L. (1971) *J. Virol.* **8**, 516–524.
9. Chou, J. Y. & Martin, R. G. (1974) *J. Virol.* **13**, 1101–1109.
10. Pardee, A. B. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1286–1290.
11. Ley, K. D. & Tobey, R. A. (1970) *J. Cell Biol.* **4**, 255–262.
12. Toniolo, D. & Basilico, C. (1975) *Cell* **4**, 255–262.
13. Vogel, A. & Pollack, R. (1975) *J. Cell. Physiol.* **85**, 151–162.
14. Burstin, S. J. & Basilico, C. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2540–2544.
15. Renger, H. C. & Basilico, C. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 109–114.
16. Renger, H. C. & Basilico, C. (1973) *J. Virol.* **11**, 702–708.
17. Basilico, C., Renger, H. C., Burstin, S. J. & Toniolo, D. (1974) in *Control of Proliferation in Animal Cells* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), pp. 167–176.
18. Toniolo, D., Meiss, H. K. & Basilico, C. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1273–1277.
19. Ozanne, B., Sharp, P. A. & Sambrook, J. (1973) *J. Virol.* **12**, 90–98.
20. Prasad, I., Zouzias, D. & Basilico, C. (1975) *J. Virol.* **16**, 897–904.
21. Sambrook, J., Sharp, P. A. & Keller, W. (1972) *J. Mol. Biol.* **70**, 57–71.
22. Khoury, G., Byrne, J. C., Takemoto, K. K. & Martin, M. A. (1973) *J. Virol.* **11**, 54–60.
23. Paulin, D. & Cuzin, F. (1975) *J. Virol.* **15**, 393–397.
24. Gilden, R. V., Carp, R. I., Taguchi, F. & Defendi, V. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 684–692.
25. Swetly, P. & Watanabe, Y. (1974) *Biochemistry* **13**, 4122–4126.
26. Hoffmann, P. R. & Darnell, J. E. (1975) *J. Virol.* **15**, 806–811.
27. Borun, T. W., Scharff, M. D. & Robbins, E. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 1977–1981.
28. Rudland, P. S. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 750–754.
29. Williams, J. G. & Penman, S. (1975) *Cell* **6**, 197–206.
30. Risser, R. & Pollack, R. (1974) *Virology* **59**, 477–489.