Effects of Large and Small T Antigens on DNA Synthesis and Cell Division in Simian Virus 40-Transformed BALB/c 3T3 Cells

JOAN B. CHRISTENSEN AND WILLIAM W. BROCKMAN*

Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109

Received 27 April 1982/Accepted 28 July 1982

The roles of the large T and small t antigens of simian virus 40 in cellular DNA synthesis and cell division were analyzed in BALB/c 3T3 mouse cells transformed by wild-type, temperature-sensitive A (*tsA*), or *tsA*-deletion (*tsA/dl*) double mutants. Assessment of DNA replication and cell cycle distribution by radioautography of [³H]thymidine-labeled nuclei and by flow microfluorimetry indicate that *tsA* transformants do not synthesize DNA or divide at the restrictive temperature to the same extent as they do at the permissive temperature or as wild-type transformants do at the restrictive temperature. This confirms earlier studies suggesting that large T induces DNA synthesis and mitosis in transformed cells. Inhibition of replication in *tsA* transformants at the restrictive temperature, however, is not complete. Some residual cell division does occur but is in large part offset by cell detachment and death. This failure to revert completely to the parental 3T3 phenotype, as indicated by residual cell cycling at the restrictive temperature, was also observed in cells transformed by *tsA/dl* double mutants which, in addition to producing a *ts* large T, make no small t protein. Small t, therefore, does not appear to be responsible for the residual cell cycling and plays no demonstrable role in the induction of DNA synthesis or cell division in stably transformed BALB/c 3T3 cells. Comparison of cell cycling in *tsA* and *tsA/dl* transformants, normal 3T3 cells, and a transformation revertant suggests that the failure of *tsA* transformants to revert completely may be due to leakiness of the *tsA* mutation as well as to a permanent cellular alteration induced during viral transformation. Finally, analysis of cells transformed by *tsA/dl* double mutants indicates that small t is not required for full expression of growth properties characteristic of transformed cells.

The early transcription region of simian virus 40 (SV40) codes for two proteins (large T and small t antigens), both of which have been implicated in the neoplastic transformation of mammalian cells. The establishment of cellular transformation requires the presence of a functional large T antigen (15, 35). The requirement for small t antigen in the establishment of transformation, however, is not absolute; in the absence of small t antigen transformation occurs with a low efficiency in resting cells, but with the same efficiency as wild-type (*wt*) transformation in growing cells (2, 23, 31, 34). In addition, continued expression of large T antigen is required for maintenance of at least some parameters of the transformed phenotype (3, 4, 16, 20, 25, 36). The latter conclusion is based on the observation that many cells transformed at the permissive temperature by SV40 *tsA* mutants which produce a temperature-sensitive (*ts*) large T antigen revert to a more normal phenotype at

the restrictive temperature. A role for small t antigen in the maintenance of transformation has not been established.

One characteristic of SV40-transformed cells which we and others (3, 5, 24) have observed to revert in *tsA* transformants at the restrictive temperature is the ability to synthesize DNA and divide under conditions which do not allow growth of normal cells. Robinson et al. (26, 27), however, have examined DNA synthesis in *tsA*-transformed hamster cells by flow microfluorimetry (FMF) and found that whereas these cells, when shifted to the restrictive temperature, reverted to a normal phenotype in terms of morphology and saturation density, they did continue to synthesize DNA; low saturation density apparently resulted from a balance between cell replication and cell death. Our earlier analyses of DNA synthesis in *tsA*-transformed BALB/c 3T3 mouse cells (3) could not distinguish between cells which are quiescent and cells in

which a balance is maintained between cell division and cell detachment. To distinguish between these two possibilities, we have assessed DNA replication and cell division in *tsA*-transformed BALB/c 3T3 cells by radioautography of [³H]thymidine-labeled nuclei and by FMF analysis of cell cycle distribution. In this communication, we report the results of these analyses which indicate that *tsA* transformants do not synthesize DNA or divide at the restrictive temperature to the same extent as they do at the permissive temperature or as *wt* transformants do at the restrictive temperature; they do so, however, to a greater degree than do untransformed cells. We also report that this failure of *tsA* transformants to revert completely to the BALB/c 3T3 phenotype, as indicated by residual cell cycling, occurs in cells transformed by *tsA*-deletion (*tsA/dl*) mutants. These mutants contain a deletion in the region coding solely for small t antigen as well as a *ts* defect in large T antigen (32). Residual cell cycling cannot be attributed, therefore, to activity of small t antigen. In fact, we are unable to detect any contribution of small t antigen to the growth properties of transformed cells. Comparison of cell cycling in various *tsA* transformants, normal 3T3 cells, and in a transformation revertant, however, suggests that the failure of *tsA* transformants to revert completely may be due in part to leakiness of the *tsA* mutation as well as to a permanent cellular alteration induced during viral transformation.

MATERIALS AND METHODS

Virus and cells. The propagation of BALB/c 3T3 mouse cells and the derivation of the various lines of *wt*- and *tsA*-transformed 3T3 cells have been described previously (1, 3, 13). *tsA/dl* mutants (A209Δ287, A209Δ288, A209Δ289, and A209Δ290) and the parental *tsA209* were generously supplied by Robert Martin (32). 3T3 cells were infected by *tsA209* or one of the *tsA/dl* mutants at multiplicities of infection between 5 and 50 PFU per cell. Transformants were selected as foci overgrowing monolayers at 33°C (3) and recloned by end-point dilution (1). All cell lines were grown in Eagle minimal essential medium supplemented with either 10% fetal calf serum (FCS) (complete medium) or 1% FCS (serum-deficient medium).

Authoradiographic analysis of [³H]thymidine incorporation. Subconfluent monolayers growing in 2-cm² wells were labeled for 1 h with 1 μCi of [³H]thymidine, fixed with ethanol-acetic acid (3:1) at room temperature followed by 5% trichloroacetic acid at 4°C, and finally washed at 4°C with 70% ethanol followed by 100% ethanol. Cells fixed to the 2-cm² wells were coated with NTB-2 emulsion (Eastman Kodak) diluted 1:2 with water and exposed for 4 days at 4°C. After developing, the cells were stained through the emulsion with Giemsa and examined at ×100 to 400. At least 300 cells were counted for each determination.

Cellular DNA content analysis. Medium was removed from the cell culture, and the monolayer was

rinsed with phosphate-buffered saline. Nuclei were stained by treating with a hypotonic solution of propidium diiodide (0.05 mg/ml in 0.1% sodium citrate, 0.1% Triton X-100) for 15 min at 4°C. Stained nuclei were suspended by pipetting, and their DNA content was measured on a TPS-1 cell sorter (Coulter Electronics, Inc.), using an argon ion laser. A total of 128 channels were used for collection of data from approximately 50,000 cells per sample. For comparison of cell cycle distributions in different cell lines, histograms were plotted with the 2N DNA peaks of the various cell lines normalized to the same height.

Transformation assays. The ability to form colonies on monolayers of normal cells was assessed as previously described (3). Colony formation in soft agar was assessed as described (3) except that assays were performed in 6-cm dishes, the volumes of basal and suspension layers were increased accordingly, the concentration of sodium bicarbonate in minimal essential medium was reduced to 550 mg/liter, and colonies were counted at 3 to 4 weeks. To measure saturation density in serum-deficient medium, approximately 2 × 10⁴ cells were seeded into 2-cm² wells and allowed to attach at 33°C in minimal essential medium containing 10% FCS. The following day, medium was replaced with minimal essential medium containing 1% FCS, and replicate cultures were incubated at 33 or 39.5°C. Cell counts and medium changes were then performed at daily intervals.

Detection of SV40 T antigens. Cells were radiolabeled for 2 h in minimal essential medium lacking methionine and supplemented with 10% FCS and [³⁵S]methionine (200 μCi/ml; 1,440 Ci/mmol). Cell extracts were prepared and immunoprecipitated with hamster anti-SV40 tumor serum (Office of Program Resources and Logistics, National Cancer Institute) as described by Hutchinson et al. (12). Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels containing 12% acrylamide. After being stained with Coomassie blue, the gels were prepared for fluorography with a 1-h wash in 4 volumes of En³Hance (New England Nuclear Corp.).

RESULTS

Authoradiographic analysis of [³H]thymidine incorporation. Our earlier studies (3, 13) demonstrated that when SV40 *tsA*-transformed BALB/c 3T3 cells are shifted to the restrictive temperature, they revert to a normal phenotype as assessed by saturation density, requirement of serum for growth, and ability to overgrow monolayers of normal cells. In addition, analysis of DNA synthesis and cell division suggested that, under conditions of serum deprivation (3), *tsA* transformants, unlike *wt* transformants, are reversibly blocked in their passage through the cell cycle upon shift to the restrictive temperature. In these studies, however, DNA synthesis was assessed by incorporation, during continuous labeling, of [³H]thymidine in cells attached to culture dishes. It therefore was not possible to distinguish between cells which are quiescent and cells which are synthesizing DNA and dividing but maintaining a low cell density and con-

stant levels of [^3H]thymidine incorporation by a balance between cell replication and cell detachment.

As a first approach to differentiating between these two possibilities, we have assessed DNA synthesis in *wt*- and *tsA*-transformed as well as untransformed 3T3 cells by autoradiography of [^3H]thymidine-labeled nuclei. These and all other *tsA* transformants we analyzed have been shown to be temperature sensitive for the ability to grow in serum-deficient medium, to grow to high saturation density, and to grow on normal monolayers (3, 13). Table 1 shows the autoradiographic results of cells labeled for 1 h 4 days after plating in serum-deficient medium (1% FCS) at the permissive and restrictive temperatures. Cell monolayers were subconfluent at the time of labeling. Under these conditions approximately 10% of normal 3T3 cells were incorporating thymidine at either temperature. In contrast, one-half of the *wt* transformants incorporated thymidine regardless of incubation temperature. The three *tsA* transformants behaved in a fashion similar to *wt* transformants at the permissive temperature but showed a reduction in percentage of labeled nuclei at the restrictive temperature. The level of thymidine incorporation in the *tsA* transformants at 39.5°C, however, was still greater than that in the normal 3T3 cells.

FMF analysis of DNA content. The results of the autoradiographic analysis indicate that *tsA* transformants do not fully revert to the normal 3T3 phenotype at the restrictive temperature. To investigate further the nature of the residual thymidine incorporation, the distribution of cells at various stages of the cell cycle was analyzed by FMF determination of cellular DNA content. The DNA content distributions for 3T3 cells and several *wt* and *tsA* transformants, as well as A209B4a cells which were retransformed at the restrictive temperature by superinfection with *wt* SV40 (3), grown in serum-deficient medium (1% FCS) at 33 and 39.5°C are shown in Fig. 1. The *wt* transformants and retransformed *tsA* cells appear to actively synthesize DNA at both temperatures. At 33°C approximately 60% of the *wt*-transformed cells have the 2N DNA content of G1 cells, whereas the remainder appear to be in the process of, or have recently completed, a round of DNA replication before cell division. At 39.5°C the percentage of *wt* cells with DNA content greater than 2N is slightly greater than that observed at 33°C. In contrast, little DNA synthesis occurs in 3T3 cells in serum-deficient medium, approximately 90% of cells at either temperature having the 2N DNA content of G1-arrested cells. The *tsA* transformants behave as *wt* transformants at 33°C; at 39.5°C they show varying degrees of reduction in cell cycling but

TABLE 1. Autoradiography of [^3H]thymidine-labeled normal and transformed 3T3 cells

Cell line	% Labeled nuclei	
	33°C	39.5°C
3T3	10	8
<i>wt</i> B1a	48	52
<i>wt</i> F10a	49	50
A7B4b	48	18
A255ΔB1b	48	34
A58ΔF2b	40	22

are not blocked in G1 as completely as are normal 3T3 cells.

tsA transformants, then, fail to revert fully to the 3T3 phenotype at the restrictive temperature when DNA content distribution is analyzed in subconfluent cells exposed to serum-deficient medium, conditions under which 3T3 cells do not synthesize DNA or undergo cell division. Cessation of 3T3 cell growth also occurs in complete medium (10% FCS) when cells reach confluence or are plated onto confluent cell monolayers. Transformed cells, in contrast, have the ability to overgrow one another and can grow to high density and form dense colonies when seeded onto a normal monolayer. To determine whether the FMF distribution of *tsA* transformants at the restrictive temperature might more closely approach that of 3T3 cells under conditions of high cell density, analysis of DNA content distribution was performed on cells which had reached confluence or were plated on monolayers of normal 3T3 cells in complete medium. The results of these analyses (data for cells plated on normal monolayers not included), however, were similar to those obtained with subconfluent cells in serum-deficient medium. *wt* cells continue to traverse the cell cycle at both the permissive and restrictive temperatures, whereas normal 3T3 cells are blocked in G1 at both temperatures. The *tsA*-transformed cells behave as *wt* transformants at the permissive temperature. At the restrictive temperature cell cycling is reduced, but not as completely as in 3T3 cells.

Cell replication. FMF analysis demonstrates that when *tsA*-transformed cells are shifted to 39.5°C, under conditions in which 3T3 cells cease to grow, a significant number of the *tsA* transformants contain more DNA than the 2N DNA content of G1-arrested 3T3 cells. This suggests that some *tsA* transformants are continuing to synthesize DNA and traverse the cell cycle, an interpretation which must be reconciled with the earlier observation that growth of *tsA*-transformed cell cultures appeared to cease (3) under these same conditions. This discrepan-

FIG. 1. Cell DNA content in BALB/c 3T3 cells and in 3T3 cells which have been transformed by *wt* or *tsA* SV40. Cells were grown in medium containing 1% FCS at 33°C (solid lines) and 39.5°C (dotted lines) and analyzed by FMF as described in the text.

cy would be resolved if *tsA* transformants continued to replicate at 39.5°C but maintained a constant population density by a balance between cell division and release of cells from the surface of the culture vessel. An alternative explanation of the FMF profile of the *tsA*-transformed cells, although one which is not consistent with the autoradiographic data, is that these cells are in fact not traversing the cell cycle at 39.5°C but have been blocked in all stages of the cell cycle. To distinguish between these two possibilities, we have measured cell replication by quantitating cells released into culture medium as well as those remaining attached to the culture dish. In our earlier study (3), cell division was assessed by counting only

those cells attached to the vessel surface. Although in that study few detached cells were observed by microscopic examination, medium changes were performed at daily intervals. It is, therefore, possible that significant numbers of detached cells might accumulate over a several-day period. In the present study, at the time medium was replaced it was examined for the presence of detached cells by analysis in an electronic particle counter.

When 3T3 cells are plated at low density in serum-deficient medium at either 33 or 39.5°C, essentially all cells remain attached to the culture dish and little growth is observed (Fig. 2). *wt* transformants grow at both temperatures, the majority of cells remaining attached. *tsA*-trans-

FIG. 2. Growth of normal and transformed BALB/c 3T3 cells in medium containing 1% FCS. Symbols: ○, number of cells attached to dish; ×, total number of cells including those released into medium and those attached to dish. Each point represents the average value of duplicate samples.

formed cells behave as *wt* transformants at the permissive temperature. At the restrictive temperature the number of *tsA*-transformed cells attached to the culture dish remains relatively constant as reported previously (3). However, when floating particles as well as attached cells are counted, it appears that cell growth, although reduced from that observed at 33°C, is not inhibited as completely as that of 3T3 cells. The majority of particles counted in the supernatant medium appear to be whole cells and not fragments, as determined by size distribution in the electronic particle counter. Nevertheless, when these cells are replated in complete medium they do not grow.

tsA-transformed 3T3 cells, then, upon shift to the restrictive temperature in serum-deficient medium maintain a low population density by two mechanisms. First, the frequency of cell division is reduced. Second, although some residual cell division does occur, this is in large

part balanced by cell detachment and death. A similar but more striking phenomenon occurs when *tsA* transformants are plated on a monolayer of normal 3T3 cells (Fig. 3). Whereas some *wt* transformants at both temperatures and some *tsA* transformants at the permissive temperature are shed from the monolayer, the number of attached cells increases in all cases. In contrast, *tsA* transformants at the restrictive temperature do not remain attached to the monolayer, the majority of cells being shed within 2 to 3 days after temperature shift up. When, however, both attached and floating cells are counted, the *tsA* transformants are seen to continue replication, although at a reduced rate at the restrictive temperature. By the criterion of ability to overgrow a monolayer, then, *tsA* transformants are fully temperature sensitive for maintenance of the transformed phenotype but thermal inhibition of DNA synthesis and cell division under these conditions is not complete.

FIG. 3. Growth of *wt*- and *tsA*-transformed cells when seeded on monolayers of normal BALB/c 3T3 cells in medium containing 1% FCS. Symbols: O, number of transformed cells attached to monolayer; x, total number of transformed cells including those released into medium and those attached to monolayer. Each point represents the average value of duplicate samples.

Transformation by *tsA*/dl mutants. The preceding analyses demonstrate that thermal inactivation of large T antigen in SV40-transformed cells, although resulting in reduction in DNA synthesis and cell division under conditions in which 3T3 cell growth is arrested, does not cause complete reversion to the resting state of 3T3 cells. FMF analyses of cellular DNA content after infection of mouse cells by SV40 (11) and rat cells by polyoma virus (29) have indicated that small t antigen of papovaviruses may have mitogenic activity. This suggests that fail-

ure of *tsA* transformants to revert completely to the 3T3 phenotype at the restrictive temperature might be due to activity of small t antigen. To assess this possibility, we have analyzed 3T3 cells transformed by mutants containing lesions affecting both the large T and small t antigens. A209/dl mutants have a deletion within the region between 0.54 and 0.59 map units, which is specific to small t antigen, as well as a *tsA* mutation mapping between 0.32 and 0.37 map units, which results in production of a *ts* large T antigen (17, 32). The four A209/dl mutants used

FIG. 4. SV40 T antigens in transformed BALB/c 3T3 cells; autoradiogram of ³⁵S-labeled immunoprecipitates separated by gel electrophoresis. The positions of large T, small t, and NVT antigens in the *wt* transformant are indicated. (A) T antigens in cells transformed by *tsA/dl* double mutants. (Lanes a to g) Immunoprecipitates made with anti-SV40 tumor serum; (lane h) immunoprecipitate made with normal hamster serum: lane a, normal 3T3 cells; lanes b and h, *wt* B1a; lane c, A209b1; lane d, A209Δ287d1; lane e, A209Δ288a1; lane f, A209Δ289c1; lane g, A209Δ290b1. (B) T antigens in a transformation revertant. (Lane a) Immunoprecipitate made with normal hamster serum; (lanes b to d) immunoprecipitates made with anti-SV40 tumor serum: lanes a and b, line B1-0, the parental transformant; lane c, line B1-3, the transformation revertant; lane d, normal 3T3 cells.

in this study contain deletions ranging from approximately 23 to 180 base pairs in length and have been shown to produce no detectable small t antigen in lytic infections of monkey cells (32) or in transformed hamster cells (22). It is possible that some of these mutants make fragments of small t which turn over too rapidly to be detected. The minimum extent of the deletions in A209Δ288 and A209Δ289, however, includes the *DdeI* site at 0.545 map unit (unpublished data). This lies within 1 base pair of the position corresponding to the donor splice site in the small t message. Deletions which remove the

proximal small t splice junction have been shown to produce no stable cytoplasmic small t mRNA (14).

BALB/c 3T3 cells infected by *tsA209* or one of the four A209/dl mutants were selected for their ability to form dense foci in liquid medium. Cloned lines were then tested for the presence of immunoprecipitable T antigens and the ability to express several parameters of the transformed phenotype. Lines transformed by *wt*, A209, or A209/dl virions contain the 94K large T antigen (Fig. 4). The *wt* and *tsA209* transformants contain the 21K small t antigen as well. Small t,

TABLE 2. Growth properties of *tsA209/dl*-transformed cells

Transforming virus	Cell line	Saturation density (cells $\times 10^{-4}/\text{cm}^2$) in serum-deficient medium		EOP on monolayer ^a			EOP in agar ^b
		33°C	39.5°C	33°C	39.5°C	33/39.5°C	
<i>wt</i>	<i>wt</i> B1a	16.0	14.0	28	9	3.1	17.3
				44	18	2.4	28.2
				63	25	2.5	18.8
				90	43	2.1	
<i>tsA209</i>	A209b1	≥ 10.0	3.4	50	0.1	500	18.2
	A209d1			48	<0.01	>4,800	
	A209f1			60	0.4	150	
A209Δ287	A209Δ287b1	8.1	3.0	63	0.5	126	32.8
	A209Δ287c1			16	<0.01	>1,600	2.8
	A209Δ287d1			25	0.05	500	4.0
	A209Δ287d2			43	0.3	143	9.3
A209Δ288	A209Δ288a1	20.0	4.6	56	0.3	183	13.3
	A209Δ288b1			27	<0.01	>2,700	
	A209Δ288c1			22	<0.01	>2,200	
A209Δ289	A209Δ289a1	≥ 12.0	4.0	58	0.3	193	2.9
	A209Δ289b1			25	0.1	250	
	A209Δ289c1			65	13	5	
A209Δ290	A209Δ290a1	≥ 10.0	2.6	43	<0.01	<4,300	4.0
	A209Δ290b1			38	<0.01	>3,800	
	3T3	2.4	2.2	<0.01	<0.01		<0.002

^a Efficiency of plating; expressed as number of colonies per 100 cells plated. Results of four separate experiments with *wt* B1a are recorded.

^b Tested at 33°C.

however, is not detectable in the A209/dl transformants. In spite of the absence of small t in the A209/dl transformants, these lines appear to be fully transformed (3, 13) as assessed by the criteria of ability to grow to high density in serum-deficient medium, to form colonies on monolayers of normal cells, and to grow suspended in semisolid medium (Table 2). The A209/dl lines behave similarly to *tsA* transformants when their abilities to grow in serum-deficient medium and on monolayers are compared at the permissive and restrictive temperatures (Table 2), all those tested being temperature sensitive for the former property and all but 1 of the 12 showing a marked reduction in the ability to express the latter property at the restrictive temperature.

FMF analysis of *tsA/dl* transformants. To determine whether small t is responsible for the residual DNA synthesis and cell cycling observed in *tsA* transformants at the restrictive temperature, A209/dl transformants were subjected to FMF analysis. Figure 5 shows the DNA content distributions for several of the A209/dl transformants, as well as for two A209

transformants, at the permissive and restrictive temperatures. This analysis was performed on cells which had reached confluence in complete medium; similar results were obtained when DNA content distributions were assessed in subconfluent cells in serum-deficient medium or in cells plated on monolayers of normal 3T3 cells in complete medium. The majority of A209/dl-transformed cell lines behave similarly to *tsA* transformants. They traverse the cell cycle at the permissive temperature, whereas normal 3T3 cells do not. At the restrictive temperature, cell cycling is reduced by various amounts in all but one line, but in none as completely as in 3T3 cells. The one A209/dl transformant (A209Δ289c1) which shows no difference in DNA content distribution at the permissive and restrictive temperatures is relatively temperature resistant for the ability to form colonies on monolayers (Table 2). It thus resembles the rare *tsA*-transformed 3T3 cells which are temperature resistant for expression of the transformed phenotype (3, 13). The A209/dl transformants then fail to revert completely to the 3T3 phenotype at the restrictive temperature. Since these

FIG. 5. Cell DNA content in normal 3T3 cells, *tsA209* transformants, cells transformed by various *tsA/dl* double mutants, and a transformation revertant. FMF was performed on cells which had reached confluence in complete medium at 33°C (solid lines) or 39.5°C (dotted lines).

cells contain no detectable small t antigen, residual cell cycling of *tsA* transformants at the restrictive temperature cannot be attributed to small t-antigen function.

FMF analysis of a transformation revertant. The failure of BALB/c 3T3 cells transformed by *tsA* or A209/dl mutants to revert completely to the 3T3 phenotype could be due to leakiness of the *tsA* mutation. To address this possibility, we have examined DNA content distribution in line B1-3, a revertant of an SV40-transformed BALB/c 3T3 line. This revertant arose as a result of rearrangement of integrated SV40 sequences, and although it retains some SV40 DNA, it no longer expresses either the large T or the small t antigens (1). The T antigens are not produced because the single insert of SV40 DNA contains only between 2 and 7% of the region coding for large T antigen and lacks the major portion of the region coding for small t antigen as well as the small t messenger splice sites (unpublished data). As a result, it is no longer able to grow to high density or to grow in the absence of a solid substrate. It also does not express the 54K nonviral T antigen (NVT) at a level above that of untransformed BALB/c 3T3 cells (Fig. 4B). If residual cell cycling in *tsA* transformants were due solely to failure to thermally inactivate large T antigen, then the revertant line should not traverse the cell cycle under conditions in which normal 3T3 cells do not synthesize DNA or divide. However, the revertant B1-3 cells, when confluent, are not blocked in G1 as completely as are normal 3T3 cells which have been propagated under similar conditions (Fig. 5). Similar results are obtained when B1-3 cells are plated at low density in serum-deficient medium (data not shown). In this revertant line, it appears that some factor other than residual activity of either large T or NVT antigen is responsible for the failure to fully revert to the 3T3 phenotype.

DISCUSSION

The 94K large T antigen of SV40 influences replication of both viral and cellular DNAs. This protein binds to the SV40 origin of replication (38) and is involved in the initiation of viral DNA replication (35). In addition, infection of both rodent and primate cells by SV40 results in stimulation of host DNA synthesis. Large T antigen appears to be required for such stimulation although the results of studies with *tsA* mutants are equivocal. These mutants, although somewhat temperature sensitive for this function, are less so than for the initiation of viral DNA replication (6, 10, 11, 32). Deletion mutants which lack major portions of the large T coding region, however, fail completely to stimulate cellular DNA synthesis (30). Moreover,

microinjection of purified proteins related to large T antigen induces synthesis of cellular DNA (39). These observations are consistent with the notion that T antigen may initiate replication of host as well as viral replicons.

In the present study we have examined the effect of large T antigen on DNA synthesis in cells neoplastically transformed by SV40. In previous studies, we and others (3, 5, 24) have found that DNA synthesis, assessed by incorporation of [³H]thymidine into acid-precipitable material, is arrested when *tsA*-transformed rodent cells are incubated at the restrictive temperature under conditions in which *wt* transformants do but untransformed cells do not replicate. This suggested that *tsA*-transformed cells enter a normal resting state at the restrictive temperature and that T antigen may maintain transformation by directly initiating cell DNA synthesis (4, 18, 21). Robinson et al. (26, 27), however, have analyzed DNA content distribution by FMF in hamster cells transformed by *tsA* mutants and found that DNA synthesis in these cells is not arrested at the restrictive temperature. Although these cells revert at high temperature to a normal phenotype as assessed by saturation density and colony-forming ability, replication continues. Monolayers appear to be maintained at low density by balanced cell replication and cell death. To determine whether such a dissociation between cell replication and transformation exists in SV40-transformed BALB/c 3T3 cells, we have analyzed DNA replication and cell division by radioautography and FMF. The results of these studies confirm our previous finding that thermal inhibition of DNA synthesis occurs in *tsA*-transformed mouse cells which are also temperature sensitive for other transformation parameters. We are thus unable to separate DNA synthesis and cell replication from other phenotypic characteristics of transformation. Large T antigen may therefore initiate DNA synthesis, either directly or indirectly, in transformed cells and thus free cell replication from normal control mechanisms.

Inhibition of replication in *tsA*-transformed BALB/c 3T3 cells at the restrictive temperature, however, is only partial. Whereas at any given time many cells cease synthesis of DNA and division, a portion continue to replicate. This residual cell cycling might result from an activity of small t antigen, residual large T antigen activity, or a permanent cellular alteration induced during transformation. Although small t antigen is not required for viral DNA replication (33), studies from several laboratories suggest that this protein may play at least an indirect role in the stimulation of host DNA synthesis in cells newly infected by SV40 (11, 28, 32). The *tsA*/dl-

transformed 3T3 cells which contain no detectable small t antigen, however, behave similarly to *tsA* transformants; whereas DNA synthesis is reduced at the restrictive temperature, it is not inhibited completely. Small t antigen, therefore, does not appear to be responsible for the residual cell cycling observed in *tsA* transformants. On the other hand, we and others have previously noted that the relative temperature sensitivity of *tsA* transformants could be correlated with the intracellular level of large T antigen (3, 37). It is possible, then, that residual cycling at the restrictive temperature is due to residual large T antigen activity and that cells such as A7B4b and A209Δ290b1, which are nearly completely arrested (Fig. 1 and 5), have relatively low intracellular concentrations of large T. Residual large T antigen activity, however, may not be the sole explanation since some residual cycling is observed in the revertant B1-3, which produces neither large T nor small t antigen.

Residual cell cycling in the revertant suggests that a permanent cellular alteration may occur during transformation which is independent of continued expression of the viral T antigens. Such an alteration could be directly mitogenic. A potential candidate for such an effector is the 54K NVT which is induced by SV40 infection of 3T3 cells (19) and displays a cell cycle dependence in normal 3T3 cells (7). The revertant, however, does not express the 54K NVT at levels above that of untransformed 3T3 cells (Fig. 4; unpublished data), indicating that continued expression of T antigen is required for stimulation of the 54K NVT, a conclusion also derived from analysis of *tsA*-transformed cells by Linzer et al. (19). Alternatively, in addition to stimulating DNA synthesis, T antigen could, as proposed by Robinson et al. (27), be important for viability or attachment (see Fig. 3) of cells once they have been transformed. Replication of some cells then would occur when topoinhibition (8) was relieved as other cells died or were lost from the monolayer.

Not only are we unable to detect a role for small t antigen in the stimulation of host DNA synthesis in transformed cells, but we find little evidence that this protein is required for full expression of the transformed phenotype. Transformants derived from *tsA*/dl mutants were as fully transformed as those derived from *wt* or *tsA* virions when saturation density, monolayer overgrowth, and growth in agar were assessed at the permissive temperature. This contrasts with the results of Sleight et al. (34) and Frisque et al. (9), who reported that some rat cells transformed by mutants lacking small t antigen were less transformed than *wt* transformants as assessed by ability to reach high saturation density and to grow in semisolid

medium. Our results, however, are in agreement with those of Martin et al. (22, 23) and Frisque et al. (9), who found little difference between the growth properties of hamster cells transformed by mutants with deletions within 0.54 to 0.59 map units and those transformed by *wt* virus.

ACKNOWLEDGMENTS

We thank Carol Taylor for assistance in performing some of the transformation assays and Steven Kelley for assistance in computer analysis of FMF data.

This research was supported by Public Health Service grant CA-19816 from the National Institutes of Health.

LITERATURE CITED

1. Bender, M. A., and W. W. Brockman. 1981. Rearrangement of integrated viral DNA sequences in mouse cells transformed by simian virus 40. *J. Virol.* 38:872-879.
2. Bouck, N., N. Beales, T. Shenk, P. Berg, and G. di Mayorca. 1978. New region of simian virus 40 genome required for efficient viral transformation. *Proc. Natl. Acad. Sci. U.S.A.* 75:2473-2478.
3. Brockman, W. W. 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. *J. Virol.* 25:860-870.
4. Butel, J. S., J. S. Brugge, and C. A. Noonan. 1975. Transformation of primate and rodent cells by temperature-sensitive mutants of SV40. *Cold Spring Harbor Symp. Quant. Biol.* 39:25-36.
5. Butel, J. S., and H. R. Soule. 1978. Role of the simian virus 40 gene A product in the regulation of DNA synthesis in transformed cells. *J. Virol.* 26:584-594.
6. Chou, J. Y., and R. G. Martin. 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. *J. Virol.* 15:145-150.
7. DeLeo, A., G. Jay, E. Appella, G. Dubois, L. Law, and L. J. Old. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 76:2420-2424.
8. Dulbecco, R. 1970. Topoinhibition and serum requirement of transformed and untransformed cells. *Nature (London)* 227:802-806.
9. Frisque, R. J., D. Rifkin, and W. Topp. 1980. Requirement for the large T and small t proteins of SV40 in the maintenance of the transformed state. *Cold Spring Harbor Symp. Quant. Biol.* 44:325-332.
10. Hiscott, J. B., and V. Defendi. 1979. Simian virus 40 gene A regulation of cellular DNA synthesis. I. In permissive cells. *J. Virol.* 30:590-599.
11. Hiscott, J. B., and V. Defendi. 1981. Simian virus 40 gene A regulation of cellular DNA synthesis. II. In nonpermissive cells. *J. Virol.* 37:802-812.
12. Hutchinson, M. A., T. Hunter, and W. Eckhart. 1978. Characterization of T antigens in polyoma infected and transformed cells. *Cell* 15:65-77.
13. Kelly, S., M. A. Bender, and W. W. Brockman. 1980. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: effect of transformation technique on the transformed phenotype. *J. Virol.* 33:550-552.
14. Khoury, G., P. Gruss, R. Dhar, and C.-J. Lai. 1979. Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. *Cell* 18:85-92.
15. Kimura, G., and R. Dulbecco. 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. *Virology* 52:529-534.
16. 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.

17. Lai, C.-J., and D. Nathans. 1974. Mapping temperature-sensitive mutants of simian virus 30: rescue of mutants by fragments of viral DNA. *Virology* 60:466-475.
18. Levine, A. J., and M. M. Burger. 1972. A working hypothesis explaining the maintenance of the transformed state by SV40 and polyoma. *J. Theor. Biol.* 37:435-446.
19. Linzer, D., W. Maltzman, and A. J. Levine. 1979. The SV40 gene A product is required for the production of a 54,000 MW cellular tumor antigen. *Virology* 98:308-318.
20. Martin, R. G., and J. Y. Chou. 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. *J. Virol.* 15:599-612.
21. Martin, R. G., and A. Oppenheim. 1977. Initiation points for DNA replication in non-transformed and simian virus 40-transformed Chinese hamster lung cells. *Cell* 11:859-869.
22. Martin, R. G., V. P. Setlow, and C. A. Edwards. 1979. The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells: studies with simian virus 40 double mutants. *J. Virol.* 31:596-607.
23. Martin, R. G., V. Setlow, C. A. F. Edwards, and D. Vembu. 1979. The roles of simian virus 40 tumor antigens in transformation of Chinese hamster lung cells. *Cell* 17:635-643.
24. 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.
25. Osborn, M., and K. Weber. 1975. Simian virus 40 gene A function and maintenance of transformation. *J. Virol.* 15:636-644.
26. Robinson, C. C., and J. M. Lehman. 1978. Simian virus 40 A gene function: DNA content analysis of Chinese hamster cells transformed by an early temperature-sensitive virus mutant. *Proc. Natl. Acad. Sci. U.S.A.* 75:4389-4398.
27. Robinson, C. C., D. E. Swartzendruber, and J. M. Lehman. 1980. Replication of Chinese hamster embryo cells transformed by temperature-sensitive T-antigen mutants of simian virus 40. *J. Virol.* 35:246-248.
28. Rundell, K., and J. Cox. 1979. Simian virus 40 t antigen affects the sensitivity of cellular DNA synthesis to theophylline. *J. Virol.* 30:394-396.
29. Schlegel, R., and T. L. Benjamin. 1978. Cellular alterations dependent upon the polyoma virus hr-t function: separation of mitogenic from transforming capabilities. *Cell* 14:587-599.
30. Scott, W. A., W. W. Brockman, and D. Nathans. 1976. Biological activities of deletion mutants of simian virus 40. *Virology* 75:319-334.
31. Self, R., and R. G. Martin. 1979. SV40 small t-antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. *J. Virol.* 32:979-988.
32. Setlow, V. P., M. Persico-Dilauro, C. A. F. Edwards, and R. G. Martin. 1980. The isolation of SV40 tsA/deletion, double mutants, and the induction of host DNA synthesis. *Virology* 101:250-260.
33. 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.
34. Sleight, M., W. C. Topp, R. Hanich, and J. E. Sambrook. 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. *Cell* 14:79-88.
35. Tegtmeyer, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. *J. Virol.* 10:591-598.
36. Tegtmeyer, P. 1975. Function of simian virus 40 gene A in transforming infection. *J. Virol.* 15:613-618.
37. Tenen, D. G., R. G. Martin, J. Anderson, and D. M. Livingston. 1977. Biological and biochemical studies of cells transformed by simian virus 40 temperature-sensitive gene A mutants and A mutant revertants. *J. Virol.* 22:210-218.
38. Tjian, R. 1978. The binding site on SV40 DNA for a T-antigen related protein. *Cell* 13:165-179.
39. Tjian, R., G. Fey, and A. Graessmann. 1978. Biological activity of purified simian virus 40 T antigen proteins. *Proc. Natl. Acad. Sci. U.S.A.* 75:1279-1283.