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Density-Dependent Inhibition of Both Growth and T-Antigen Expression in Revertants Isolated from Simian Virus 40-Transformed Mouse SVT2 Cells

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Phenotypic revertants were isolated from simian virus 40-transformed cells in order to examine the relationship between simian virus 40 T-antigen expression and G1 arrest of growth. Revertant clones with increased adherence were selected from cultures of SVT2, a simian virus 40-transformed BALB/c mouse cell line, and screened to find arrestable revertant clones which inhibited DNA synthesis when crowded. The clones selected from untreated SVT2 were unstable and showed little or no inhibition of DNA synthesis when crowded. Stable revertants were found after treatment of SVT2 with Colcemid to increase ploidy. The stable revertants all lost most transformed growth properties tested, including tumorigenicity, but only a few showed the same degree of inhibition of DNA synthesis at high cell density as BALB/3T3. All revertant clones expressed T antigen at low cell density. Three revertants showed coordinate inhibition of DNA synthesis and apparent loss of T antigen at high cell density. We suggest that changes in gene dosage rather than mutations caused the altered properties of the new revertants and that continued DNA synthesis in confluent cultures may be the transformed phenotype that requires the least simian virus 40 T antigen.

The transformation of rodent cells by simian virus 40 (SV40) appears to require the synthesis and function of T antigen, a nuclear protein specified by the early region of the SV40 genome (20, 40). Some evidence for the function of T antigen in both initiation and maintenance of transformation has come from the properties of the *tsA* mutants of SV40. At restrictive temperatures, the *tsA* mutants overproduced an unstable form of T antigen in an infection of monkey cells (2, 54) and had a greatly reduced ability to transform rodent cells (28, 53). Transformants obtained at permissive temperatures under certain conditions showed temperature-dependent expression of transformation (19). At permissive temperatures, these *tsA* transformants displayed typical transformed characteristics, including continued cellular DNA synthesis in dense cultures. However, when shifted to restrictive temperature, the same *tsA*-transformed cells lost most, but not always all, transformed characteristics and often, but not always, lost T-antigen expression as well (6, 7, 9, 19, 25, 26, 28, 30, 33, 42, 47). The relationship between T-antigen expression and cellular transformation established by the viral mutants, therefore, suggests that T-antigen expression leads to transformation, but the evidence to date has some unexplained exceptions.

The relationship between the function of SV40 early genes and cellular transformation has also been studied in phenotypically "normal" clones selected from populations of SV40-transformed rodent cells. The mouse cell revertants isolated by Pollack and his co-workers (34, 38, 56, 58), who used several different selection procedures, lacked most, but not all, characteristics of transformed cells and continued to express detectable T antigen. The one transformed characteristic not lost by any of these mouse revertants was continued cellular DNA synthesis in dense cultures (57), which was, therefore, the transformed phenotype best correlated with retention of T antigen. Steinberg et al. (52) isolated revertants from SV40-transformed rat cells and found both clones which expressed T antigen and clones which did not have T antigen, but the effect of cell density on DNA synthesis in the revertants which lacked T antigen has not been reported. Renger and Basilico (43) isolated mouse cells (ts SV3T3) which carried a wild-type SV40 genome but were temperature sensitive for transformation. The ts SV3T3 cells were transformed in all respects at 32°C but lost all transformed properties at 39°C in medium containing low serum concentrations. Under the latter restrictive conditions, confluent ts SV3T3 cells entered a reversible G1 arrest of growth

and lost both T antigen and mRNA representing the SV40 early region (3, 43). T antigen reappeared before DNA synthesis when G1-arrested ts SV3T3 cells were stimulated to resume growth, although other experiments indicated that the synthesis of T antigen was not sufficient to stimulate DNA synthesis (60). Study of the variant cells isolated to date, therefore, reinforces the tentative correlation between T antigen and transformation suggested by the results obtained with the *tsA* viral mutants and furthermore suggests that continued DNA synthesis in dense cultures is the transformed phenotype best associated with the presence of T antigen.

We report here the isolation of new revertants of SV40-transformed mouse cells with properties which lend further support to the correlation between T antigen and cellular transformation. The new revertants were isolated from SVT2, an SV40-transformed mouse cell line obtained after SV40 infection of an early passage of BALB/c mouse embryo fibroblasts during derivation of BALB/3T3 (S. A. Aaronson, personal communication). SVT2 cells are pseudodiploid (24) and highly tumorigenic (1) and contain one complete copy of the SV40 genome plus about five additional copies of the early region (4). Revertant clones were isolated from cultures of SVT2 by selection for cells which adhered more tightly to the growth surface. One population of SVT2 cells was pretreated with Colcemid to induce tetraploidy (22) because many revertants previously isolated showed about a twofold increase in ploidy compared with their transformed progenitors (37, 41). Tightly adhering revertants were screened to find those which inhibited DNA synthesis at high cell densities. Some of the new revertants had no transformed characteristics and showed coordinate depression of T-antigen expression and DNA synthesis in crowded cultures.

MATERIALS AND METHODS

Cell culture. SVT2 and BALB/3T3 clone A31 mouse cells were cloned and used within 10 passages after cloning. Cells were grown in the Dulbecco and Vogt modification of Eagle medium (DME) containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, 30 to 48 mM $NaHCO_3$, and 2 to 20% calf serum as monolayers on plastic petri dishes at 37°C in a humid incubator equilibrated with 7% CO_2 in air. Cells were harvested with 0.25% crude trypsin (1:250; Difco)–3 mM EDTA in phosphate-buffered saline (PBS) (17).

Saturation density was calculated from 7- to 9-day growth curves by using initial seedings of 10^5 cells per 60-mm dish in DME containing 5% calf serum and changing the medium every third day. Cloning efficiency in soft agar was determined by counting clones with more than 10 cells after 23 days of incubation of 10^3 or 10^6 cells in DME containing 10% calf serum and 0.35% agar above a base layer containing 0.5% agar in the same medium.

Adherence assay. Cells were seeded at 2×10^5 cells per 60-mm dish (Falcon) in DME containing 5% calf serum and incubated for 4 or 5 days. Each dish was rinsed twice with PBS, filled with 1 ml of PBS alone or with 1 ml of PBS plus 10 to 200 μg of crystalline trypsin, and allowed to stand for 2 to 3 min at 20 to 25°C. Then the PBS or PBS plus trypsin was pipetted vigorously across the surface of the dish in a regular pattern including one 90° rotation and removed from the dish. The dish was next rinsed gently with 1 ml of PBS. The gentle rinse was combined with the vigorous rinse. The cells remaining attached to the dish after vigorous and gentle rinsing were next harvested as described above. Detached cells and attached cells were counted.

Colcemid treatment. A culture of SVT2 cells at 3×10^6 cells on a 100-mm dish was incubated for 28 h with 0.1 μg of Colcemid per ml in DME containing 2% calf serum.

Selection of adherent cells. SVT2 cells with or without prior Colcemid treatment were seeded at 2×10^3, 2×10^4, or 2×10^5 cells per 100-mm dish (Falcon). Cells were incubated for 7 days, rinsed with PBS, and covered with 2 ml of PBS. After 2 to 3 min at room temperature, the buffer was pipetted vigorously as in the adherence assay described above and removed from the dish. The dish was rinsed three times with PBS, filled with 15 ml of DME containing 5% calf serum, and incubated for several days. Each culture was subjected to three cycles of pipetting and incubation to detach loosely attached cells and permit adherent cells to multiply. After the third selection treatment, clones of adherent cells were removed from the selection dishes with stainless steel cloning rings and transferred to dishes containing 3-mm square glass cover slip pieces for recloning by a procedure similar to that of Martin and Tuan (27). Twice-cloned lines were stored in liquid nitrogen at an early passage after slow freezing in DME containing 20% calf serum and 12% dimethyl sulfoxide.

Spot culture assay of growth control. The fractions of cells synthesizing DNA on the edge and in the center of a dense group of cells were compared to determine the effect of cell density on growth. A method originally developed for chick cultures (21) was modified for use with mouse cell lines as follows. A spot culture of about 0.5 cm^2 was made by planting 10^4 to 10^5 cells in 0.03 ml of culture medium in the center of a 35-mm (8-cm^2) dish, incubating for 30 min at 37°C, and then adding 2 ml of DME containing 5% calf serum. After 3 days, [^{3}H]thymidine (17 Ci/mmol) was added to the medium already in the dish to a final concentration of 0.5 μCi/ml. After 18 h of incubation, the cells were fixed with Bouin fluid (35), rinsed with 70% ethanol, allowed to dry, and coated with photographic emulsion (Ilford K-5). The autoradiograms were developed with Kodak D-19 developer after 10 to 14 days of exposure at 2°C and observed at ×500 magnification with phase-contrast optics. The fraction of labeled cells at both the center and the periphery of each spot culture was determined. The same autoradiograms were examined for cytoplasmic labeling as

evidence for contamination by mycoplasma. No contamination was found with any of the cells discussed here.

Flow microfluorometric analysis. Cells were harvested with crystalline trypsin and EDTA and fixed with formaldehyde by using the methods of Deaven and Petersen (15). James Bartholomew (University of California, Berkeley) kindly stained the cells with acriflavine and determined the distribution of DNA contents by using the methods of Deaven and Petersen (15) and the flow microfluorometer at the Lawrence Livermore Laboratory of the University of California.

Determination of chromosome number. Rapidly growing cells were exposed to 0.06 μg of Colcemid per ml in culture medium at 37°C for 2 to 3 h, harvested, swelled at 37°C for 10 min in hypotonic buffer (0.25 × PBS), fixed with acetic acid-methanol (1:3), and spread on slides (31). The cells were stained for 10 min at room temperature with 5% Giemsa blood stain (MCG) diluted immediately before use in 0.01 M sodium phosphate buffer, pH 6.8. Chromosomes were counted in 50 isolated metaphase spreads for each cell line.

Tumorigenicity. Rapidly growing cells were harvested, resuspended in cold PBS, and counted with a hemacytometer. Samples of these cells were sedimented and resuspended at several concentrations up to 10^8 cells per ml in culture medium lacking serum. The cell suspensions were kept on ice for 0 to 2 h and injected (0.10 to 0.15 ml/mouse) subcutaneously into the right flank of 6- to 7-week-old male BALB/c mice. Some mice were exposed to 300 rads of ^{60}Co 24 h before injection. The mice were observed at weekly intervals for the development of tumors. Only tumors larger than 5 mm in diameter were scored.

SV40-specific T antigen. T antigen was detected by the indirect immunofluorescence assay, using the methods of Pope and Rowe (39) and Robb (45). Cells were grown on glass cover slips (11 by 22 mm; no. 2), fixed with methanol as described by Robb (45), air dried, and stored in polyethylene vials at −70°C. The antisera used were hamster anti-T (1:20 dilution in PBS; Flow Laboratories) and fluoresceinated rabbit anti-hamster-immunoglobulin G (Sylvana). The latter reagent was fractionated on DEAE-cellulose as described by Kawamura (23), and the fractions with low ratios of fluorescein to protein and minimal cytoplasmic staining were used for the assay. The fixed cells were rinsed with water and exposed to each antiserum for 30 min at room temperature with three rinses with PBS between antisera. The cover slips were mounted on slides with buffered glycerol (glycerol-PBS, 9:1), sealed with clear nail polish, and stored at −70°C. The cells were observed and photographed with a Leitz microscope (incident illumination, xenon light source; BG38 and K510 filters) and Kodak Tri-X Pan film developed with Acufine developer (ASA 1000).

RESULTS

Adherence of SVT2 and BALB/3T3 to plastic culture dishes. SVT2 cells were attached much less tightly to the growth surface than were BALB/3T3 cells. When rinsed with PBS alone, only 8% of the SVT2 cells remained attached, whereas 83% of the BALB/3T3 cells adhered to the dish under the same conditions (Fig. 1).

Spot culture assay of growth control. The different growth properties of BALB/3T3 and SVT2 cells gave distinctly different patterns of labeling after exposure of spot cultures to [^{3}H]-thymidine. Autoradiograms of spot cultures prepared with BALB/3T3 and SVT2 cells are shown in Fig. 2A and B, respectively. Most (94%) of the BALB/3T3 cells on the perimeter of the spot culture (Fig. 2A) synthesized DNA during the 18-h labeling period, but few (1.3%) of the crowded cells in the center became labeled. Fisher and Yeh (18) observed a similar pattern of thymidine labeling with clones of 3T3 cells after the 60-cell stage. In addition to the cells on the free edge, most of the BALB/3T3 cells for several rows inward from the edge also synthesized DNA; therefore, relatively sparse cells synthesizing DNA formed a ring around more crowded cells which rarely synthesized DNA. In other experiments, we observed that the thickness of the ring of labeled BALB/3T3 cells was directly related to the concentration of serum in the culture medium. In sharp contrast to the ring pattern of the labeled cells in the BALB/3T3 spot, the SVT2 spot culture (Fig. 2B) was a dense mass of uniformly labeled cells. The fraction of SVT2 cells labeled in the center (98%) was not significantly different from the fraction labeled on the edge (100%), indicating that SVT2 cells synthesized DNA regardless of their position in a group of cells. In work described below,

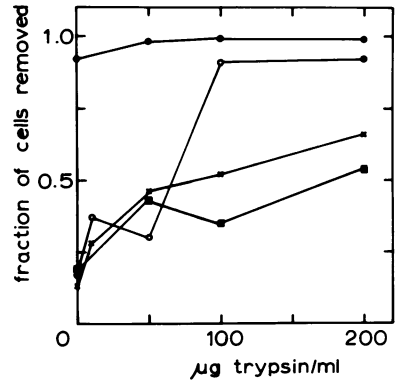

FIG. 1. *Adherence of SVT2, BALB/3T3, and revertants C16-9 and D3. Cells were seeded at 2 × 10^5 cells per 60-mm dish. After 5 days of growth, cells were tested for adherence by pipetting without and with trypsin as described in the text. Attached cells and detached cells were counted to determine the fraction of cells removed without trypsin and at each trypsin concentration. Symbols: ●, SVT2; ○, BALB/3T3; ×, C16-9; ■, D3.*

we used the difference in frequency of cells labeled between the inside and outside of spot cultures as our primary criterion for screening revertant clones.

Isolation of revertants. The loose attachment of SVT2 cells to plastic culture dishes was exploited to select rare adherent cells, which were then screened to identify clones which exhibited growth control similar to that of BALB/3T3. Tightly adhering cells were selected from two SVT2 populations by three cycles of rinsing off all loosely attached cells with PBS, with intervening periods of incubation to allow growth. At the end of this procedure, clones of flat, nonoverlapping cells were visible on the rinsed plates. Such clones occurred at a frequency of greater than 10^{-5}. Well-isolated adherent clones were recloned on small pieces of glass and then tested for growth control by using the spot culture assay. A total of 11 revertant clones selected from SVT2 without Colcemid treatment and 11 revertant clones selected from SVT2 after Colcemid treatment designed to induce tetraploidy were analyzed for growth control (Fig. 3). Most of the revertants showed only a slight reduction (less than threefold) in the fraction of cells which became labeled with [^{3}H]thymidine when crowded. Two clones derived after Colcemid treatment, C16-9 and C17-2, showed a reduction of more than fivefold. Clone C17-2 showed an 11-fold reduction of labeling frequency in the spot culture assay, but the cells had heterogeneous morphologies one passage after being recloned and were, therefore, cloned a third time.

A total of 16 subclones of C17-2 were obtained. These subclones probably represented different isolates of a few revertants and were classified into three groups according to labeling frequencies in the spot culture assay (Table 1). The first group of six, including D5, had a 2- to 6-fold reduction in labeling frequency, and the second group of five subclones had about a 10-fold reduction. The five subclones of the third group (A18, D3, D8, D9, and E2) consistently showed the same labeling pattern as BALB/3T3 (Fig. 2C), with at least a 20-fold reduction in [^{3}H]-thymidine labeling frequency of crowded cells and are hereafter called arrestable revertants.

G1 inhibition of growth. The cell cycle distribution of cells from growing and quiescent cultures was determined by flow microfluorometry of acriflavine-stained cells to determine the point of growth arrest in arrestable revertants. Cells from rapidly growing cultures of revertants A18, D3, and D8 yielded patterns similar in shape to that of SVT2 (Fig. 4A), with an appreciable fraction of the cells in the S and G2 phases of the cell cycle. In confluent cultures of BALB/3T3 and the revertant D3 (Fig. 4B and C), the vast majority of the cells contained the G1 amount of DNA. Revertants A18 and D8 showed

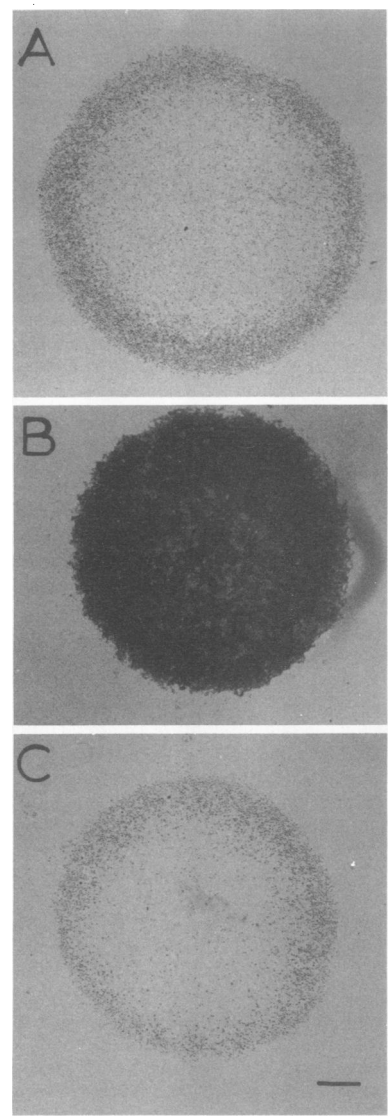

FIG. 2. *Visual comparison of transformed cells and cells with growth control in autoradiograms of spot cultures. Cells were seeded in spot cultures and labeled for 18 h with [^{3}H]thymidine after 3 days of growth as described in the text. At the end of the labeling period, the cells were fixed and autoradiographed. The autoradiograms were photographed at low power through a dissecting microscope. The same magnification was used for all three micrographs. Bar = 1 mm. (A) BALB/3T3 cells. (B) SVT2 cells. (C) Revertant D3 cells. Note the reduced frequency of labeling in the center of the spot culture of revertant cells (1% in this culture) and the similarity to the labeling pattern of BALB/3T3 cells.*

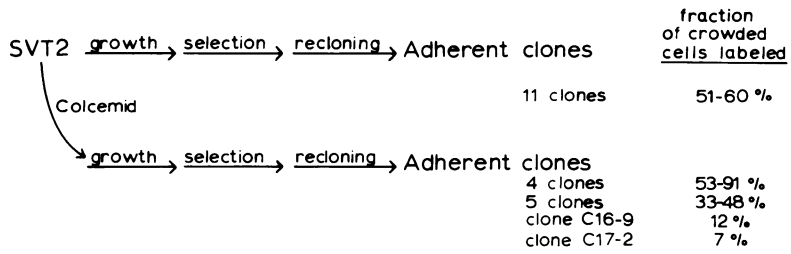

FIG. 3. *Selection of adherent cells from SVT2 and screening for revertant cells with growth control. Untreated SVT2 cells or SVT2 cells which had been exposed to Colcemid were incubated for 1 week before loosely attached cells (initially >90% of the total) were removed by pipetting PBS across the surface of the dishes and rinsing with additional PBS. Fresh medium was added, and the cultures were given two additional treatments with several days of incubation between the two to allow growth. After the third selection, adherent clones were harvested with cloning rings and recloned immediately. Spot cultures were used to determine the fraction of crowded cells labeled with [^{3}H]thymidine in 18 h as an assay of growth control. More than 80% of the cells on the perimeter of the spot cultures were labeled except for C16-9, where 68% of the cells were labeled in this experiment.*

TABLE 1. *Growth properties of C17-2 subclones*

Cell line	% of labeled cells[a]		Relative adherence[b]	Cellular morphology	Saturation density ($\times 10^{-4}$ cells/cm^2)	Cloning efficiency in soft agar	Growth in 1% calf serum
	Edge	Center					
SVT2	97 (134)	83 (116)	0.08	Round or spindly	>40	0.12	+
BALB/3T3	95 (170)	0.7 (817)	0.83	Flat	4–7	$<10^{-6}$	–
D6	96 (263)	47 (369)	ND[c]	Flat	9	ND	ND
D5	89 (260)	30 (390)	0.80	Flat	7	$<10^{-6}$	–
D13	92 (210)	29 (404)	ND	Flat	5	ND	ND
A7	89 (143)	28 (304)	0.96	Flat	8	$<10^{-6}$	–
A20	95 (400)	23 (505)	ND	Flat	13	ND	ND
A26	98 (361)	17 (349)	ND	Flat	9	ND	ND
D2	94 (327)	13 (407)	ND	Flat	6	ND	ND
D10	96 (244)	11 (380)	ND	Flat	4	ND	ND
D1	95 (220)	10 (255)	0.88	Flat	8	ND	ND
A21	97 (356)	9.3 (447)	ND	Flat	11	ND	ND
E1	99 (331)	9.0 (366)	ND	Flat	4	ND	ND
E2	97 (338)	2.5 (482)	0.50	Flat	4	$<10^{-6}$	–
D9	97 (283)	2.1 (424)	0.98	Flat	4	$<10^{-6}$	–
A18	94 (207)	1.1 (452)	ND	Flat	6	$<10^{-6}$	–
D3	98 (259)	0.7 (305)	0.81	Flat	5	$<10^{-6}$	–
D8	98 (289)	0.4 (558)	ND	Flat	4	$<2 \times 10^{-6}$	–

[a] Numbers of cells counted are in parentheses.

[b] Relative adherence is the fraction of cells remaining on a plate after PBS alone (no trypsin) was pipetted over the culture as described for the adherence assay.

[c] ND, Not determined.

the same pattern. An increase in the concentration of serum in the medium (from 5 to 20%) stimulated many confluent D3 cells to reenter the cell cycle within 24 h (Fig. 4D). Therefore, the revertant cells showed the same reversible G1 inhibition of growth shown by 3T3 and BALB/3T3 (16, 32, 55).

Other cellular properties. All adherent cells selected from SVT2 had a flattened appearance quite different from the spindle and round shapes typical of SVT2. Unlike 3T3 and BALB/3T3, which were fibroblastic at low densities, the revertants tended to have the same epithelioid morphology at all cell densities. The revertants attached to the growth surface more firmly than did SVT2 (Fig. 1 and Table 1) and grew as a monolayer without the overlapping piles formed by SVT2. Cultures of the revertants selected from SVT2 without exposure to Colcemid rapidly accumulated cells with the mor-

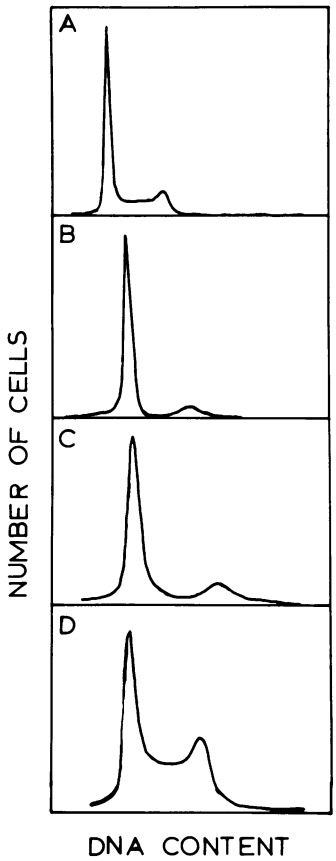

FIG. 4. *Comparison of the relative amounts of DNA per cell in SVT2, BALB/3T3, and the revertant D3. Cells grown as indicated below were harvested, fixed with formaldehyde, stained with acriflavine, and analyzed by flow microfluorometry. The peak on the left in each flow microfluorometer tracing was considered to be the position of G1 cells, and the peak on the right was assumed to represent G2 + M cells. Values between the two peaks and above the base line were assumed to represent S-phase cells. The cell number scale (ordinate) was approximately linear. (A) SVT2 cells were seeded at 10^5 cells per 60-mm dish in DME containing 5% calf serum and harvested after 3 days. (B) BALB/3T3 cells were seeded at 8×10^5 cells per 60-mm dish in DME containing 5% calf serum. The cultures were confluent after 2 days and had very few mitotic cells when harvested after 3 days. (C) Revertant D3 cells were seeded at 8×10^5 cells per 60-mm dish in DME containing 5% calf serum. The culture became confluent within a day and had very few mitotic cells when harvested after 3 days. (D) Revertant D3 cells were seeded as in (C). After 3 days, the medium was replaced with DME containing 20% calf serum. The cells were harvested 24 h after the medium change.*

phology and adherence characteristic of SVT2. The growth properties of these unstable revertants were not examined further.

The revertants selected from SVT2 after Colcemid treatment, in contrast, were quite stable since the cells remained similar to BALB/3T3 in cellular and colonial morphology and in adherence through at least 20 passages in culture. Furthermore, no cells with the cellular morphology or the overlapping growth pattern of SVT2 appeared in confluent cultures of C16-9, A7, D3, D5, D9, or E2 which were maintained for 3 weeks. Like BALB/3T3 but unlike SVT2, seven subclones of C17-2 did not form clones in soft agar and grew poorly in medium containing 1% calf serum (Table 1). All of the subclones of C17-2 had low saturation densities regardless of the degree of growth control measured in spot cultures (Table 1). The stable revertants, therefore, shared most growth properties of BALB/3T3, although only the five arrestable revertants showed the same degree of inhibition of DNA synthesis at high density as BALB/3T3.

Ploidy. Since the stable revertants were derived from SVT2 after Colcemid treatment intended to induce tetraploidy, mitotic figures of cells arrested in metaphase were examined. All of the chromosomes in SVT2, BALB/3T3, and eight revertants were telocentric or acrocentric, as is characteristic of mouse chromosomes. The nomograph in Fig. 5 shows the tight distribution of chromosomes obtained for SVT2, with 40 chromosomes (the mouse diploid number) in 72% of the spreads counted. In contrast, BALB/3T3 and five revertants had subtetraploid numbers of chromosomes, with modal numbers of 66 to 71 chromosomes per cell and a broader distribution of chromosome numbers than found with SVT2 (Fig. 5). Similar data were obtained for revertants A18 and D8. Revertant C16-9 had 56 chromosomes per cell, fewer than the seven C17-2 subclones which were examined. The distribution of DNA contents revealed by flow microfluorometric analysis of SVT2, BALB/3T3, and revertant D3 (Fig. 4) indicated that G1 phase BALB/3T3 and D3 cells contained an amount of DNA consistent with their subtetraploid number of chromosomes.

Tests for tumorigenicity. BALB/3T3, SVT2, and revertants derived from SVT2 were injected subcutaneously into male BALB/c mice, the inbred strain from which the cells were originally obtained, to test the capacity of the cells to induce tumors (Table 2); 10^3 SVT2 cells were sometimes able to induce a tumor in mice irradiated with ^{60}Co to reduce their immune response. About one-half of the animals inoculated with 10^4 SVT2 cells and 94% of the animals inoculated with 10^5 SVT2 cells developed tumors whether or not the animals were irradiated before inoculation. All of the tumors which appeared in animals inoculated with SVT2 cells

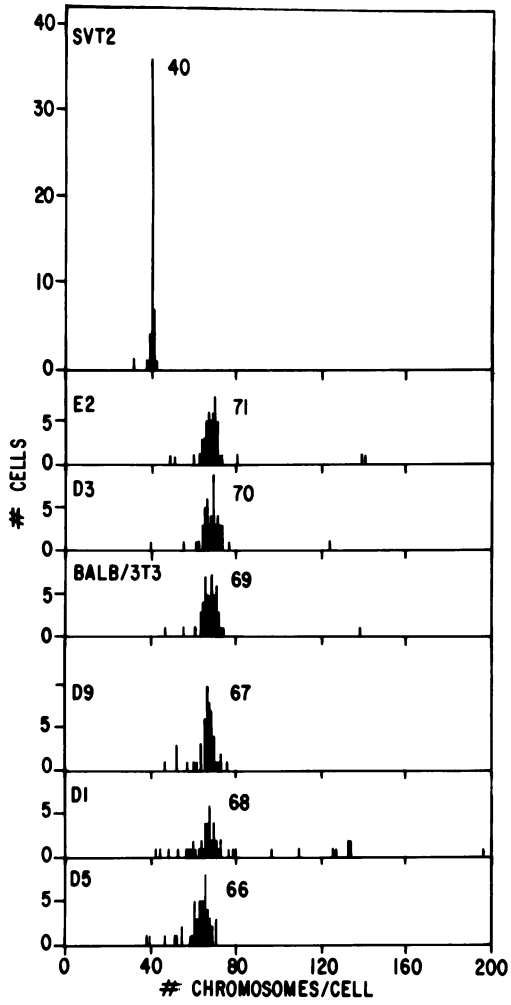

FIG. 5. *Chromosome distributions of BALB/3T3, SVT2, and revertants D1, D3, D5, D9, and E2. Metaphase spreads were prepared for each cell line as described in the text. The modal number of chromosomes is indicated beside the nomograph for each cell line.*

developed at the site of injection, and all were detectable within 5 weeks after inoculation. Most tumors were incapacitatingly large by 7 weeks. None of the tumors regressed, and the mice with tumors all died within 3 months after inoculation. Similar tumors with similarly rapid rates of growth developed in all of the irradiated mice which were inoculated with 5×10^6 or 10^7 cells of R1-15, an unstable revertant isolated without Colcemid treatment. R1-15 cells were not tested in smaller doses or in nonirradiated mice. The SVT2-like cells which arose frequently in R1-15 cultures may explain the tumorigenicity observed with R1-15. No tumors developed when 10^7 cells of BALB/3T3, D3, E2, or D5 were injected subcutaneously into irradiated or nonirradiated mice. All of the animals were alive and healthy 3 months after inoculation. Therefore, three revertants obtained after Colcemid treatment of SVT2 were not tumorigenic in syngeneic mice when tested at 1,000 times the dose at which SVT2 cells caused one-half of the injected animals to develop tumors.

T-antigen expression. SV40-specific T antigen, detected by indirect immunofluorescence using hamster tumor serum, was found in the nuclei of SVT2 and of rapidly growing cells of all of the adherent revertants of SVT2. In cultures of SVT2 and most of the revertants, virtually all of the cells showed bright nuclear fluorescence regardless of cell density. In rapidly growing cultures of revertant D8, however, only about one-half of the cells seemed to contain T antigen, and few cells stained brightly. Cells with similar variable T-antigen expression have been described previously (42, 44, 46). Two other arrestable revertants, D3 and E2, did not seem to have T antigen when the cells were confluent, although rapidly growing cells were brightly fluorescent. Spot cultures of D3 or E2 cells prepared as for the assay of growth control showed the same ring pattern of T-antigen-containing cells as described above for thymidine-labeled cells; cells on the free edge of a spot usually had T antigen, but few cells (about 1%) in the center of a spot culture had detectable T antigen (Fig. 6). Revertant D5, which showed an intermediate degree of growth control by thymidine labeling (25 to 30% of crowded cells labeled in spot cul-

TABLE 2. *Tumorigenicity*

Cell line	Dose (no. of cells injected)	Fraction of irradiated mice with tumors	Fraction of nonirradiated mice with tumors
BALB/3T3	10^7	0/13	0/5[a]
SVT2	10^3	2/9 (22)[b]	0/10
	10^4	6/11 (56)	6/10 (60)
	10^5	17/18 (94)	15/16 (94)
	10^6	9/10 (90)	9/9 (100)
	10^7	3/3 (100)	NT[c]
R1-15[d]	5×10^6	2/2	NT
	10^7	3/3	NT
D3	3×10^6	0/3	NT
	10^7	0/13	0/5[a]
E2	10^7	0/3	0/5[a]
D5	10^7	0/1	0/5[a] + 0/20

[a] One-quarter of the cell dose was injected each week for 4 weeks.

[b] Numbers in parentheses are percentages.

[c] NT, Not tested.

[d] Unstable revertant isolated from SVT2 without exposure to Colcemid.

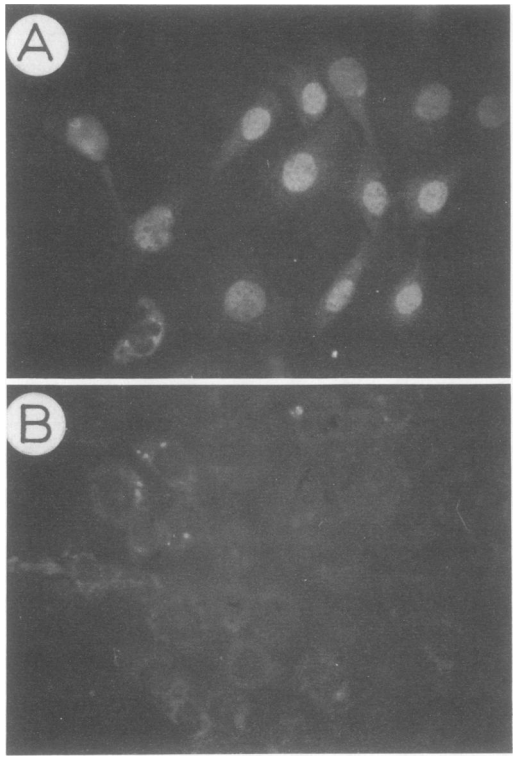

FIG. 6. *Photomicrographs of SV40-specific T antigen in revertant D3. T antigen was visualized in methanol-fixed D3 cells by indirect immunofluorescence and photographed as described in the text. (A) Cells in sparse culture which had bright nuclear fluorescence. (B) Cells in crowded center of spot culture. No nuclear fluorescence was observed.*

ture), also gave an intermediate degree of inhibition of T antigen. About one-quarter of the cells in the center of a spot culture had detectable T antigen, whereas almost all of the free cells on the edge of the spot culture had T antigen. Therefore, three revertants showed coordinate inhibition of DNA synthesis and apparent loss of T antigen when the cells were crowded.

Failure to rescue virus. We have rescued infectious SV40 from SVT2 by Sendai virus-induced fusion of SVT2 cells with BSC-1 monkey cells, but no virus has yet been obtained from revertants D3, E2, and D5 despite serial passage on BSC-1 cells. Further experiments will be necessary to determine whether the viral DNA present in the revertant cells includes an entire viral genome capable of infecting and transforming cells.

DISCUSSION

The phenotypic revertants which we isolated from SVT2 included clones that had regained all of the tested growth characteristics of BALB/3T3, including reversible G1 arrest in confluent culture. These five arrestable revertants were not isolated independently and may represent different isolates of the same cell. Our other revertants showed loss of most transformed characteristics but continued to synthesize DNA in confluent cultures at levels above the level of BALB/3T3 and, therefore, resembled revertants isolated by Pollack and co-workers (49, 57). The arrestable revertants resembled the ts SV3T3 clones isolated by Basilico and collaborators (3, 43, 60) in some, but not all, respects. The arrestable revertants coordinately inhibited both DNA synthesis and T-antigen expression at confluence, as did ts SV3T3 at confluence under restrictive conditions. Unlike ts SV3T3, however, arrestable revertants did not require high temperature (39°C) or low serum (1%) to inhibit T-antigen expression or DNA synthesis. The serum requirement for DNA synthesis in arrestable revertants was the same as the relatively high serum requirement of BALB/3T3. As with ts SV3T3, however, the simultaneous reduction in T antigen and DNA synthesis supports the proposal that T antigen plays a role in maintenance of the cell cycle in transformed cells by affecting initiation of cellular DNA synthesis (8, 29).

The selection method we used to obtain revertants from SVT2 was a rinsing procedure which allowed separation of a culture into more adherent and less adherent groups of cells. Adherence is probably determined by cell surface properties which also affect cellular morphology, with flat cells being more tightly attached than rounded cells. We assumed a correlation between loose adherence and transformation in SVT2 cells because tumor cells and other transformed cells have been shown to be less adherent than untransformed cells (48, 51, 59). Furthermore, revertants selected from transformed cells have all been described as flat in morphology (5, 12, 13, 34, 38, 52, 56, 58). However, not all cells with the morphology and adherence of 3T3 show the G1 inhibition of growth of dense 3T3. For example, the flat revertants isolated from SV3T3 by Pollack and co-workers and the flat transformants isolated from SV40-infected BALB/3T3 by Scher and Nelson-Rees continued to synthesize DNA after attaining confluence (49, 57). For this reason, we screened all of the revertants selected by adherence to identify arrestable revertants which inhibited DNA synthesis to the same extent as did BALB/3T3.

Two general categories of hypothetical mechanisms have been proposed to explain the loss of transformed characteristics in revertant

clones. In gene dosage models (37, 41), revertants were proposed to arise because of changes in chromosome complements of hyperploid cells. The dosage of certain cellular genes was proposed to increase to offset expression of viral genes or other genes conferring the transformed phenotype. Evidence for the model came from chromosome counts of transformed cells and revertant clones derived from them. In alternate mutation models, reversion was explained by mutation of an integrated viral gene or mutation of a cellular gene which affected viral gene expression. For example, the ts SV3T3 cells of Renger and Basilico (43) have been presumed to be mutant in a cellular gene because wild-type SV40 was rescued from ts SV3T3 despite the temperature sensitivity of transformation. Some revertants of 14B rat cells have been shown to be mutant in integrated viral sequences (52). The mutation model would be favored over the gene dosage model in reversion of diploid cells where changes in chromosome complements were not observed (52).

We cannot make a clear choice between gene dosage and mutation for the origin of our revertant clones, but three considerations make us favor some form of a gene dosage model at present. (i) Reversion of transformation was found with a frequency $>10^{-5}$, similar to the frequency observed by Pollack (36). This frequency is greater than that usually observed for structural gene mutations in unmutagenized cultures (10, 14, 50). (ii) Reversion was found in hyperploid cells after Colcemid treatment of diploid SVT2. It seems reasonable to expect variations in chromosome complements among cells undergoing chromosome losses after Colcemid treatment. Furthermore, Colcemid has been observed to yield subtetraploid cells which never duplicated all of their chromosomes as well as tetraploid cells (11). (iii) The C17-2 subclones all showed some degree of density-dependent control of DNA synthesis but differed among each other in the degree of control. Three levels of growth control were distinguishable. If mutation were the basis of this aspect of reversion in the C17-2 subclones, then it would be necessary to assume at least two mutations. On the other hand, it seems more reasonable to assume continuing slow chromosome loss in clones after Colcemid treatment as the basis of continuing divergence among C17-2 subclones. In a gene dosage theory, we might expect that there would be different ratios of SV40 gene products to other cellular gene products in the different subclones, which would be specified by the chromosome complements of the subclones. The theory in its simplest form might predict a dilution of T antigen, but it cannot explain the disappearance of T antigen at G1 arrest in some of the revertants. As a minimal addition to the gene dosage theory, we suggest an additional provision, which is that during G1 or G0 arrest occurring under the control of excess cellular gene products, T antigen is eliminated by turnover and lack of synthesis. The disappearance of T antigen and its lack of synthesis during G1 arrest was previously shown in ts SV3T3 (3). Characterization of immunoprecipitable T antigens, virus-specific transcripts, and viral DNA in our arrestable revertants may clarify their origin.

In our work as in previous work, lines showing 3T3-like control of DNA synthesis have been relatively rare. Lines showing loss of other transformed characteristics have been easier to find. Perhaps low levels of T antigen can prevent G1 arrest in crowded cells, but higher levels of T antigen are required for other aspects of the transformed phenotype.

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