JOURNAL OF VIROLOGY, Apr. 1972, p. 611-620
Vol. 9, No. 4

Contact-Inhibited Revertant Cell Lines Isolated from Simian Virus 40-Transformed Cells

III. Concanavalin A-Selected Revertant Cells

LLOYD A. CULP[1] AND PAUL H. BLACK

Department of Medicine, Harvard Medical School, and Infectious Disease Unit, Massachusetts General Hospital Boston, Massachusetts 02114

Received for publication 22 December 1971

Contact-inhibited variants have been isolated by treatment of simian virus 40 (SV40)-transformed Balb/c 3T3 cells (SVT2) with the plant lectin concanavalin A. These con A revertant cells exhibit the following properties: (i) they resemble 3T3 cells morphologically and grow to saturation densities which are similar to that of 3T3 cells; (ii) they synthesize the SV40-specific T antigen and yield infectious virus after fusion with permissive monkey cells; (iii) they contain a high sialic acid content similar to that of 3T3 cells and not to that of SVT2 cells; sialic acid composition was found to be independent of serum concentration; (iv) they contain more chromosomes with the average number in the tetraploid range than the SVT2 cells from which they were derived; and (v) SVT2 and revertant cells, confluent or subconfluent, produce more collagen than Balb/3T3 cells. The relationship of surface membrane properties to contact inhibition of growth and the mechanisms for generating revertant cells are discussed.

Much interest has been generated recently in the interaction of plant lectins with polysaccharides of defined isomeric composition (2, 13) on the surfaces of mammalian cells. This interaction results in the agglutination of transformed cells. Contact-inhibited cell lines in tissue culture, such as mouse 3T3 cells, are highly resistant to agglutination by concanavalin A (con A; an agglutinin obtained from the Jack bean) (13) or wheat-germ agglutinin (from wheat-germ lipase preparations) (2). These cells, after transformation with the oncogenic deoxyribonucleic acid (DNA) viruses, simian virus 40 (SV40), or polyoma, are readily agglutinated with either of these lectins. When normal 3T3 cells are subjected to mild proteolysis with trypsin or other proteolytic enzymes, the cells became agglutinable; it was suggested by Burger (3) that 3T3 cells contain surface coat material not found in transformed cells which may be important in determining the phenomenon of contact inhibition of growth.

The agglutinability of transformed cells can be described as one more of a number of pleiotropic properties resulting from oncogenic virus transformation of cells. However, it was soon discovered that contact-inhibited, revertant cells, obtained from transformed cell populations by the 5-fluorodeoxyuridine (FUdR) procedure (6, 18) or by growth on glutaraldehyde-fixed monolayer cultures (19), were resistant to agglutination by the various plant lectins, although they still contained viral genetic information (12, 17). These experiments lent strong credence to the hypothesis that the properties of the surfaces of cells which determine agglutinability potential are relevant to contact inhibition of growth.

We therefore wished to determine the correlation of contact-inhibitory phenomena to agglutination by studying the properties of variants of SV40-transformed Balb/c 3T3 cells which are resistant to agglutination by con A. These variants, termed con A revertant cells, are flat and contact-inhibited; their biologic, virologic, chemical, and cytogenetic properties are described below. These properties will be compared to those of revertants obtained by the FUdR procedure and described previously (6), with specific reference to the mechanism(s) by which revertant cells are generated in transformed cell cultures.

MATERIALS AND METHODS

Cells. Balb/c 3T3 cells, clone A31, and SV40-transformed A31 cells, clone SVT2, were obtained from Stuart Aaronson after 150 generations of growth. These cell lines were used only between their 5th and 15th passages in our laboratory. Similarly, revertant

[1] Present address: Department of Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106.

cells were used only between their 5th and 15th passages after cloning. The origin and history of Swiss 3T3, SV3T3, and the F1A and F1E revertant cells obtained by utilization of the FUdR technique have been described previously (6, 14). The details of cell culture and determination of saturation densities have also been described (6).

The Balb/c cell lines were routinely assayed and found to be free of *Mycoplasma* contamination as determined by the cultural assay of Madoff (15) and a biochemical assay utilizing radiolabeled thymidine kindly performed by R. Roblin. This latter assay was carried out as follows. Cells were plated thinly in 50-mm plastic petri dishes and allowed to grow for 24 or 72 hr after a change of medium. ^{3}H-thymidine (18 to 20 Ci/mmole) was added to the medium at a final concentration of 5 μCi/ml for 4 hr of incubation. The medium was then decanted, the cells were washed twice with phosphate-buffered saline, *p*H 7.4 (PBS), fixed with two washes of cold 5% trichloroacetic acid in water (w/v), and washed five times with cold 95% ethanol. Autoradiograms of the fixed cells were prepared by layering the dishes with Kodak AR10 film and setting at room temperature for 3 days before the film was developed and fixed. *Mycoplasma*-infected cells yielded patterns of silver grains over the cytoplasm of cells with a paucity of silver grains over the nucleus [probably due to the preferential use of thymidine by *Mycoplasma* in the cytoplasm where they grow (16)]. *Mycoplasma*-free cultures displayed heavy silver grain patterns only over the nuclei of cells. Balb/c cells were only utilized in the present studies when they were found to be free of contamination by *Mycoplasma* by both assays.

Isolation of revertants. Balb/c SVT2 cells were inoculated into 50-mm plastic petri dishes. After 3 days of growth, when the cells were just confluent (i.e., 95 to 100% of the surface of the dish was covered with cells), the medium was changed and con A was added to the medium at a final concentration of 200 μg/ml. (Con A was dissolved in saturated NaCl in PBS at a concentration of 25 mg/ml and sterilized by filtration. In some instances, when con A was added to medium containing serum, a flocculent solid formed and these dishes were not used. Control dishes were exposed to the same portions of saturated NaCl in PBS without con A, and no toxicity resulted from the small increase in NaCl concentration.) Within 24 hr in medium containing con A, most cells rounded up and came off the dish; 100% of these cells were stained with the vital dye trypan blue, indicating loss of viability. After 48 hr of exposure to con A, the few remaining cells on the surface of dishes were washed twice with PBS, dispersed with trypsin, and passed into new dishes with fresh medium (no con A). Within 1 week, 50 to 60 colonies appeared on the dishes (a survival rate of approximately 10^{-5}), and approximately half of these colonies had a flat morphology similar to the morphology of Balb/c 3T3 cells and not SVT2 cells. Four of these colonies were isolated as described previously (6), cloned from single cells, and designated revertant clones 80, 81, 83, and 84. The properties of the progeny of these clones will be described below.

SVT2 cells that were exposed to a concentration of con A of 400 μg/ml (twice the concentration used above) rarely yielded any survivors when the procedure outlined above was used. Cells exposed to 100 μg of con A per ml (one-half of the selection concentration) yielded several hundred colonies of which greater than 95% had the small, spindle-shaped morphology of parental SVT2 cells.

Virologic procedures. The presence of the SV40-specific T antigen was determined by the indirect immunofluorescence method as described previously (6).

Fusion of SVT2 or revertant cells with a continuous line of African green monkey kidney cells (MA-134) to rescue infectious virus from virogenic cells in the presence of Sendai fusion factor has been described (6). An alternate method of removing SVT2 or revertant cells from dishes with the chelating agent ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) was tested to determine the comparative effects of trypsinization and chelation on the fusability of cell lines since proteolysis presumably alters surface properties. The ability of EGTA, a specific Ca^{2+}-chelating agent, to remove cells from the surfaces with minimal removal of cell surface material will be described in a separate communication (*manuscript in preparation*). In the present studies, SVT2 cells or revertant cells, which were 70 to 80% confluent, were washed once with PBS, shaken gently at 37 C with 0.02% EGTA in PBS for 10 min, pelleted by centrifugation at 1,500 rev/min in an International PR-2 centrifuge, and resuspended in MEM $\times$4 supplemented with 10% fetal calf serum to give the desired concentration of cells for fusion with trypsinized MA-134 cells in the presence of Sendai fusion factor (6). The suspensions of mixed cells were then inoculated into roller tubes and incubated for 10 days. Triplicate tubes were then frozen-thawed three times between -70 and 36 C, treated sonically for 2 min in a Raytheon sonic oscillator, and assayed for plaque-forming ability on monkey kidney cells by using an agar overlay.

Sialic acid determination. The method for determining the sialic acid content of cells has been described (6), a micromodification of the method of Warren (23).

Collagen determination. Cells were inoculated into 29-oz (approximately 960-ml) Blake bottles. After 24 hr (the cells were only 5 to 10% confluent), 50 ml of fresh medium [MEM $\times$4 supplemented with 0.5 mg of L-proline, 100 μCi of proline-*2,3-*^{3}*H* (specific activity, 45.7 Ci/mmole), 50 μg of ascorbic acid per ml, and 1 μg of $FeSO_4 \cdot 7H_2O$ per ml] was added. Since proline is converted to hydroxyproline, which is a unique component of collagens, collagen production is determined by measuring the ratio of the two components (9). To measure the collagen-producing ability in growing cells, cultures were maintained in ^{3}H-proline-containing medium for 48 hr before harvesting; the cells were 50 to 60% confluent at the time of assay. To determine collagen production in confluent cultures, fresh ^{3}H-proline-containing medium was added to cultures which had just become confluent for an additional 3 days. Cells were then harvested by decanting

medium, washing twice with PBS containing 100 mg each of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ per liter, and scraping the cells into 5 ml of RSB buffer [0.01 M NaCl; 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 1.4; 0.0015 M $MgCl_2$). Cell extracts were then treated sonically for 5 min at 4 C in a Raytheon sonic oscillator, incubated at 4 C for 1 hr with 10 μg of deoxyribonuclease I per ml, dialyzed overnight at 4 C against three changes of distilled water, and lyophilized to dryness. Cell protein was hydrolized to amino acids with 6 N HCl as described previously (6) and analyzed by split-stream analysis with carrier proline and hydroxyproline on the UR-30 column of a Beckman amino acid analyzer. The radioactive content of each fraction was determined by counting fractions in Aquasol in a Packard (model 3320) scintillation counter.

Cytogenetic analsyis. Subconfluent cultures of growing cells were dispersed with trypsin, pelleted by centrifugation at 1,500 rev/min in an International PR-2 centrifuge, and suspended at a concentration of 10^6 cells/ml in PBS containing 1% fetal calf serum. Cell suspensions were then scanned for mitotic cells, whose chromosomes were examined after methanol–glacial acetic acid (3:1) fixation and staining with Giemsa. Thirty mitoses of each cell line were analyzed for chromosome numbers, and five or six typical chromosome preparations were photographed for evaluation of chromosome morphologies.

Materials. Con A (A grade) was purchased from Calbiochem, Los Angeles, Calif., and EGTA from Eastman Organic Chemicals, Rochester, N.Y. Other materials used in these studies were obtained from sources previously described (6).

RESULTS

Isolation of revertant cell lines. Many of the colonies that survived the con A treatment (*see above*) contained cells with a flat morphology very similar to that of Balb/c 3T3 cells and did not appear to grow over one another. These survivors contrasted with the small, spindle-shaped morphology of the parental SVT2 cells which formed multilayered colonies. Four single-cell clones of these flat cells were isolated (clones 80, 81, 83, and 84). These cells will be referred to as con A revertant cells, in that their morphologies and growth properties have "reverted" back to those characteristic of contact-inhibited Balb/c 3T3 cells.

In Fig. 1, the morphologies of revertant clones 81 and 84 are compared to the morphologies of Balb/c 3T3 cells and their SV40-transformed counterparts, SVT2 cells. Revertant cells maintained a flat morphology in either subconfluent or confluent cultures and formed a monolayer of cells rather than piling on top of neighboring cells.

The growth curves of these cell lines are depicted in Fig. 2. Balb/c 3T3 cells are highly contact-inhibited and achieve a saturation density of 1.7×10^6 cells per 50-mm dish, whereas SVT2 cells which are not contact-inhibited grow to densities greater than 25×10^6 cells per dish. Revertant clone 84 achieves a saturation density of 2.5×10^6 cells per dish; clone 81 of 3.7×10^6 cells per dish; clone 83 of 5.0×10^6 cells per dish; and clone 80 of 2.1×10^6 cells per dish. Thus, con A revertant clones grow only to densities slightly higher than Balb/c 3T3 cells and stop growing once this density has been achieved; these clones therefore exhibit the property of contact inhibition of growth characteristic of parental Balb/c 3T3 cells.

Virologic studies: presence of T antigen. The SV40-specific T antigen was detected in more than 90% of the nuclei in cell lines SVT2 and all four revertant clones by indirect immunofluorescence staining (Table 1). Thus, con A revertants have retained the ability to express at least one SV40-specific function.

Virus rescue. To determine whether revertant cells contain the complete viral genome and whether this could be rescued, fusion experiments were performed with a permissive monkey kidney cell line mediated by inactivated Sendai virus. The data in Table 1 indicate that fairly comparable yields of SV40 virus were rescued from all four revertant clones after the use of trypsin to suspend these cells, but that none could be rescued from parental SVT2 cells from which the revertant clones were derived. Three attempts to rescue virus from SVT2 cells after the use of trypsin were unsuccessful. It is possible that the surfaces of these cells are very labile and may be altered after trypsin treatment to the extent that Sendai virus may not adsorb; thus, heterokaryons would not be formed.

EGTA, which has been shown to be a milder agent for removing cells from surfaces while minimizing membrane damage (L. A. Culp and P. H. Black, *manuscript in preparation*), was used to remove revertant clone 81 and SVT2 cells from surfaces, followed by Sendai-mediated fusion with monkey kidney cells. The data in Table 1 indicate that comparable yields of virus were rescued from revertant clone 81 whether treated with EGTA or trypsin. Very low yields of virus were also rescued from SVT2 cells with EGTA, but four logs less virus than could be rescued from revertant clones.

Chemical properties: sialic acid content. The sialic acid compositions of Balb/c 3T3 and SV40-transformed 3T3 cells, SVT2, agree with the data obtained previously for these cells (10) and for Swiss 3T3 and SV3T3 cells (6). The Balb/c 3T3 cells have a high sialic acid content (Table 2) of

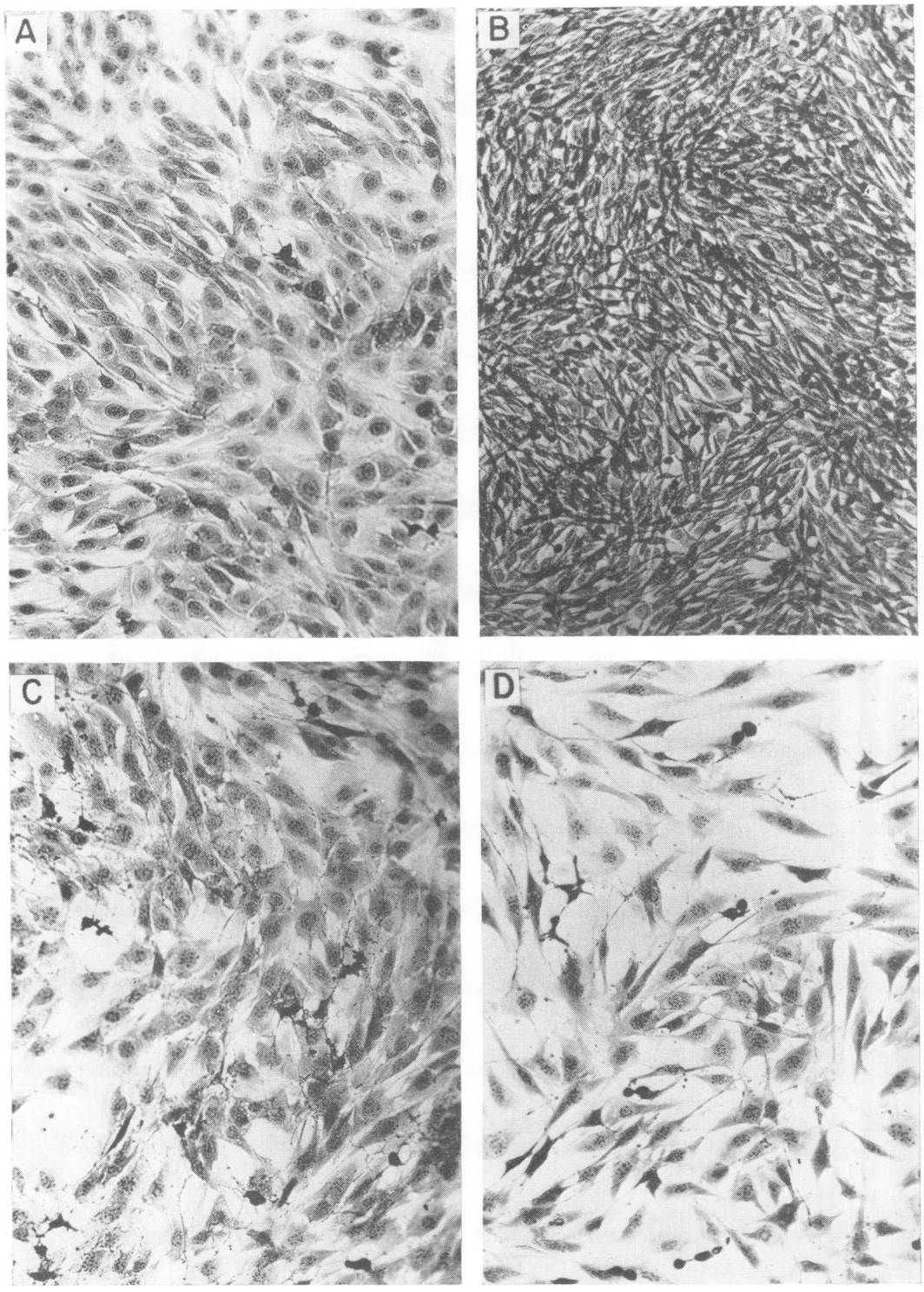

FIG. 1. *Morphology of Balb/c 3T3 cells, SV40-transformed 3T3 cells, and con A revertant cells. A, Confluent Balb/c 3T3 cells; B, confluent SVT2 cells; C, confluent con A revertant clone 84 cells; D, subconfluent con A revertant clone 81 cells. Cells were stained with Giemsa after methanol fixation.* ×120.

4.0 ± 0.3 μg of sialic acid per mg of protein, while SVT2 cells have a much lower level, 2.3 ± 0.2 μg. These values were found for both confluent and subconfluent growing cells (Table 2), indicating that sialic acid composition is not quantitatively influenced by growth control in 3T3 cells or by the establishment of cell-to-cell contacts in confluent cultures.

Revertant clones 80, 83, and 84 have a very high sialic acid composition (Table 2) with a combined average of 3.8 ± 0.3 μg of sialic acid per mg of protein, a value similar to that of Balb/c 3T3 cells and not to that of SVT2 cells. The consistently low sialic acid level found in clone 81 will be discussed later and probably reflects the very high propensity of this clone to "rerevert" to spindle-shaped cells which form multilayers.

Thus, con A revertant cells have high sialic acid composition which correlate with their contact inhibition of growth. These results are similar to those found with FUdR revertant cells (6) isolated by a different procedure.

To determine whether the level of fetal calf serum, which is rich in sialic acid-containing glycoproteins, influences the sialic acid composition of cells, sialic acid determinations of Balb/c 3T3 cells were carried out with medium containing varying serum concentrations. The results indicate that the sialic acid composition of Balb/c 3T3 cells does not fluctuate appreciably from the value normally found in these cells (4.0 ± 0.3 μg of sialic acid per mg of protein) over a range of serum concentrations from 3 to 25%. Therefore, if serum glycoproteins which are rich in sialic acid bind to cell surfaces, they must saturate cell sites at serum concentrations as low as 3%.

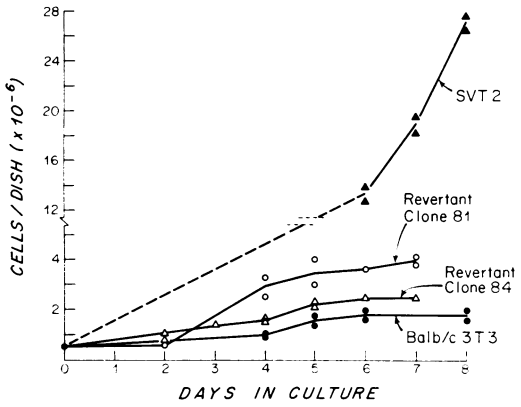

FIG. 2. *Growth curves and saturation densities of Balb/c 3T3, SV40-transformed 3T3 (SVT2), and revertant cell lines. Duplicate dishes (50-mm plastic petri dishes) were treated as described previously (6). 3T3 cells (●), SVT2 cells (▲), revertant clone 81 cells (○), and revertant clone 84 cells (△).*

Collagen content. The content of collagen in subconfluent and confluent cultures of mouse cell lines is indicated in Table 3. The ability of cells which were grown for long periods of time in the presence of radioactive proline to produce radioactive hydroxyproline was used as an indication of collagen-producing capacity (9). Both confluent SVT2 and con A revertant cells accumu-

TABLE 1. *Properties of revertant cells*

Cell line	Saturation density ($\times 10^{-6}$)[a]	T antigen	Rescue of SV40 virus[b]		Morphology[e]
			Trypsinized[c]	EGTA-treated[d]	
Balb/c 3T3	1.7	−	ND[f]	ND	Large, polygonal
SVT2	>25	+	0	14	Small, spindle-shaped
Revertant clone 84	2.5	+	8.9×10^4	ND	Large, polygonal
Revertant clone 81	3.7	+	6.5×10^4	16×10^4	Large, polygonal[g]
Revertant clone 83	5.0	+	14×10^4	ND	Large, polygonal
Revertant clone 80	2.1	+	37×10^4	ND	Large, polygonal

[a] Cells per 50-mm plastic petri dish.

[b] Plaques per milliliter of extract. Rescued from cells by fusion with indicator MA-134 cells in the presence of Sendai fusion factor. After fusion, the cells were maintained for 10 days in culture. Extracts were then prepared and assayed for virus content by plaque formation.

[c] SVT2 and revertant cells were removed from surfaces by trypsinization, pelleted by centrifugation at 600 × *g* for 10 min, and resuspended in medium before fusion with MA-134 cells as described in the text.

[d] SVT2 and revertant cells were removed from surfaces by treatment with 0.02% EGTA (a Ca^{2+}-chelating agent) in phosphate-buffered saline and then treated as described in 3.

[e] See Fig. 1.

[f] Not done.

[g] These cells demonstrated a pronounced tendency after several passages in culture to transform spontaneously into small, spindle-shaped cells in confluent cultures.

TABLE 2. *Sialic acid content of cell lines*

Cell line	Amt of sialic acid (μg/mg of protein)[a]			Saturation density (×10⁻⁶ cells per 50-mm dish)
	Expt 1	Expt 2	Expt 3	
Balb/c 3T3[b]	4.0 ± 0.3[c]			1.7
Subconfluent 3T3[d]	3.8 ± 0.0[c]			
Balb/c SVT2[b]	2.3 ± 0.2[c]			>25
Subconfluent SVT2[d]	2.3			
Revertant clone 84[b]	3.6[e]	4.2[e]	3.6[e]	2.5[f]
Revertant clone 83[b]	4.2[e]	4.0[e]		5.0[f]
Revertant clone 80[b]	3.6[e]	3.6[e]		2.1[f]
Revertant clone 81[b]	2.6[e]	2.7[e]	2.8[e]	3.7[f]

[a] Preparation of whole-cell extracts and the micromodification of the Warren method for determining sialic acid composition have been described previously (6). Cells were grown in four-times-concentrated minimal essential medium supplemented with 10% fetal calf serum.

[b] Cell cultures which had been confluent for at least 48 hr were used in these determinations.

[c] Sialic acid determinations were made on four to six different cell preparations.

[d] "Subconfluent" denotes that only 50% of the surface of the culture dish was covered with cells, which were growing exponentially.

[e] Sialic acid determinations were carried out on cells at the 7th to 10th passage levels after cloning.

[f] Saturation densities were carried out at the 4th passage level after cloning.

TABLE 3. *Collagen content*

Cell line[a]	Hydroxyproline (counts/min)/proline (counts/min)[b]
Balb/c 3T3 (confluent)	1.3×10^{-3}
Balb/c 3T3 (subconfluent)	1.7×10^{-3}
Balb/c SVT2 (confluent)	3.5×10^{-3}
Revertant clone 84 (confluent)	3.4×10^{-3}
Revertant clone 84 (subconfluent)	2.3×10^{-3}
Swiss 3T3 (confluent)	3.2×10^{-3}

[a] Cells were grown in the presence of proline-*2,3-*^3H as described by Green and Goldberg (9) and in the text. Radioactive proline was incorporated into cellular proteins and converted to radioactive hydroxyproline in collagens (9).

[b] Cell extracts were prepared and assayed for protein-incorporated proline and hydroxyproline as described in the text. The ratio of radioactive hydroxyproline eluted from the UR30 column over radioactive proline was calculated and is a relative measure of the content of collagen as compared to cellular proteins in extracts. Approximately 75 to 80% of the hydroxyproline was the 4 isomer, while the remainder was the 3 isomer.

lated larger quantities of collagen than BALB/c 3T3 cells; the 3T3 cells produced comparable amounts of collagen whether they were growing (subconfluent) or growth-inhibited (confluent). The collagen content of SVT2 and revertant cultures was comparable to that of Swiss 3T3 cells, which are known to be efficient producers of this material (9).

Cytogenetic analysis. The selection of revertant cells from SV40-transformed cells with con A presumably occurs by action of con A binding to the surface of cells with a resultant cytotoxicity. It was of interest to compare the chromosomal composition of con A revertants with that of the parental SVT2 cells and also with the composition of the FUdR revertants which were isolated by a completely different approach (6).

Balb/c SVT2 cells have an average (average number of chromosomes per cell determined by summing the total number of chromosomes in 30 cells and dividing by 30) chromosome number of 43 (Fig. 3A) with a range varying, in general, from 40 (the diploid number in mouse) to 45. The fact that this SV40-transformed cell line is just slightly hyperdiploid [confirmed by Stuart Aaronson (*personal communication*)] is quite unusual, because most SV40-transformed cell lines have much higher ploidies which generally are in the hypertriploid or hypotetraploid range.

Con A revertant clone 84 (Fig. 3B) has an average of 90 chromosomes per cell with great variability over a range from 73 to 108 chromosomes per cell. Con A revertant clone 81 (Fig. 3C) exhibited an average of 99 chromosomes per cell with a range from 82 to 118 chromosomes per cell. Thus, selection of revertants presumably by a surface selection procedure yields cell variants with chromosome complements which are approximately twice as numerous as parental SVT2 cells but which have similar qualitative cytogenetic characteristics.

Revertants which have been described previously (6), selected by the FUdR procedure, have increased ploidy as well. The Swiss F1A

revertant (Fig. 4B) has an average of 95 chromosomes per cell (range 70 to 107), which is considerably higher than the average of 63 chromosomes per cell (range 57 to 68) found in parental Swiss SV3T3 cells (Fig. 4A). Similarly, the F1E revertant (Fig. 4C) exhibits an average of 96 chromosomes per cell (range 80 to 109).

A unique feature of the FUdR revertant cells is the presence of four to six dot, or minute chromosomes in every cell. These are not found in parental Swiss SV3T3 cells nor in Balb/c SVT2 and con A revertant cells. Since FUdR affects DNA synthesis in the cell while con A presumably affects surface functions, one may hypothesize that these dot chromosomes may have arisen by extensive chromosomal damage caused by FUdR inhibition of DNA synthesis.

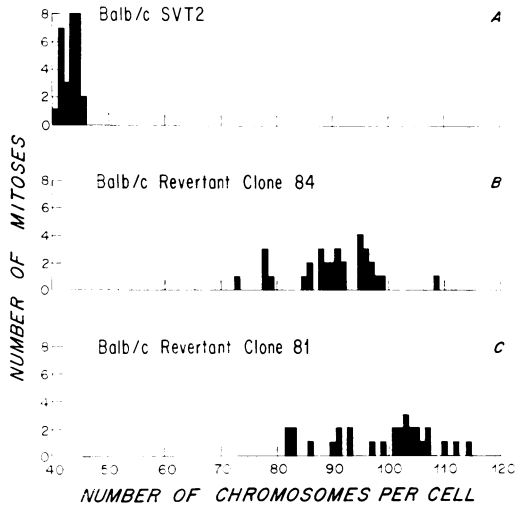

FIG. 3. *Cytogenetic analysis of Balb/c SVT2 and con A revertant cells. Cells were treated as described in the text, and chromosomes were counted in naturally occurring mitotic cells during exponential growth of the cultures.*

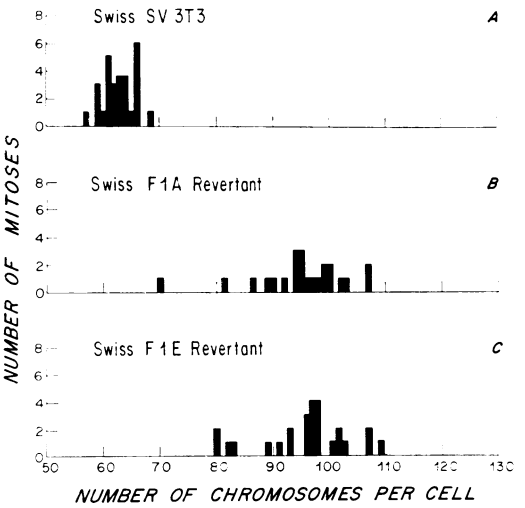

FIG. 4. *Cytogenetic analysis of Swiss SV3T3 and FUdR revertant cells. Analytical procedures are described in Fig. 3 and in the text.*

DISCUSSION

Contact-inhibited (so-called revertant) cells have been isolated from virus-transformed cell lines by: (i) selection of cells resistant to FUdR toxicity (6, 18) and (ii) selection of flat cells able to grow on a glutaraldehyde-fixed feeder layer (19). We now report a third method, based on the ability of con A to agglutinate and kill the least contact-inhibited SV40-transformed mouse 3T3 cells while sparing the more contact-inhibited cells. The mechanism whereby revertant cells are generated in a population of transformed cells is not known. It is possible that revertant cells arise by spontaneous mutation which are subsequently selected for by either FUdR or con A. It is not known whether FUdR is mutagenic for mammalian cells. However, there is no reason to believe that con A is a mutagen in mammalian cells, since its only biological functions to date have centered on its ability to bind with polysaccharides of defined specificity (8) at the surfaces of mammalian cells (4, 21).

Clones of the con A revertant cells have morphologies, growth curves, and saturation densities which are similar to those of Balb/c 3T3 cells; however, the stability of these revertants is somewhat variable (e.g., clone 81, *see below*). In contrast to the F1A revertants isolated by FUdR selection (6), con A revertants rarely form multinucleated or single-nucleated giant cells in either subconfluent or confluent cultures (see Table 4 for a summary and comparison of the properties of revertant cells isolated by the FUdR and con A procedures).

The con A revertant cells contain the SV40 genome(s) as proven by (i) their capacity to synthesize T antigen and (ii) the rescue of infectious SV40 virus by fusion with permissive monkey cells. It is unusual that SV40 virus could be rescued quite easily from the four con A revertant clones, but not from the parental SVT2 cells from which they were derived. A plausible explanation lies in the fact that Balb/c SVT2 cells are quite sensitive to trypsin treatment (L. A. Culp and P. H. Black, *manuscript in preparation*), which may have altered the ability of these cells to adsorb Sendai virus and to form effective heterokaryons with the monkey kidney cells. When these cells were removed from surfaces with EGTA, a Ca^{2+}-chelating agent which

TABLE 4. *Properties of revertant cells*

Property	Swiss FUdR Revertant[a]	Balb/c Con A Revertant[b]
Morphology	Large, flat	Large, flat
Giant cells in cultures[c]	+	−
Rescue of infectious SV40 virus[d]	+	+
Sialic acid composition	High, similar to Swiss 3T3 cells	High, similar to Balb/c 3T3 cells.
Collagen composition in confluent cultures	Low, similar to Swiss SV3T3 cells	Higher than Balb/c 3T3 cells.
Cytogenetic composition	Hypertetraploid (SV3T3-hypertriploid)	Hypertetraploid (SVT2-hyperdiploid
Unusual chromosomes	4–6 Dot chromosomes/cell	None

[a] The isolation and properties of a contact-inhibited variant of Swiss SV3T3 cells have been described previously (6). FUdR, 5-fluorodeoxyuridine.

[b] Isolation and properties of a contact-inhibited variant of Balb/c SVT2 cells are described in this report. Con A, concanavalin A.

[c] Propensity to form single-nucleated or multinucleated giant cells in confluent cultures.

[d] Virus was rescued subsequent to fusion with permissive monkey kidney cells by utilizing inactivated Sendai virus.

minimizes surface damage (Culp and Black, *manuscript in preparation*), some virus was rescued from SVT2 cells, although much less virus (four logs) than was rescued from revertant clone 81 cells after EGTA treatment.

The frequency with which these variants have been isolated, approximately 1 in 10^5 cells, is unusually high if these arose by mutation, since genetic variants should be generated with a frequency of 1 in 10^{12} mammalian cells. It is of interest that revertants obtained by the FUdR technique were isolated with a similar frequency (6, 18). It is probable, then, that genetic mutations do not account for these variants, but that they are evolved through a series of phenotypic changes in these cells which occur with a frequency of 1 in 10^5. These variants would never become prominent in a culture because they are contact-inhibited and would be rapidly overgrown by non-contact-inhibited parental cells.

We have confirmed the results of Pollack et al. (18) that FUdR-selected revertant cells (6) have an increased number of chromosomes in the tetraploid range. In addition, these revertant cells contain numerous dot chromosomes. It is of interest that both plant cells (1, 22) and Chinese hamster cells (11) contain fragmented dot chromosomes after treatment with FUdR. It is possible that chromosome pulverization occurs at the time of inhibition of DNA synthesis caused by FUdR. However, it has also been reported that fusion of cells into homokaryons in the presence of Sendai virus causes fragmentation of chromosomes (20). In addition to homokaryons, synkaryons are also present in the FUdR revertant cell population, and chromosomal loss and fragmentation may occur in the latter cells. Cytogenetic instability resulting from such events could conceivably affect the growth properties of a cell.

Such a mechanism may not explain the generation of con A revertants, since they lack fragmented chromosomes and since there was little tendency to form either single- or multinucleated giant cells. Cells of these revertant clones also had an increased number of chromosomes: 90 to 99 per cell. It is of interest that both FUdR revertants and con A revertants have similar average chromosome numbers, since they were derived from parental cells with widely differing ploidies. Balb/c SVT2 cells are slightly hyperdiploid, while Swiss SV3T3 cells are hypertriploid. The reason for the increased ploidy of the con A revertants is not known; con A presumably interacts with cells only at the surface and does not affect the genetic apparatus of the cell directly. It is possible that con A inhibits cytokinesis; however, multinucleated cells are rarely seen in these revertant cell populations, and we are not aware that con A produces this effect.

It has been shown previously (6) that FUdR revertants have a high sialic acid composition similar to that of Swiss 3T3 cells and not the SV3T3 cells from which they were derived. An inverse correlation (6) was found between the saturation density that cells display in culture and their content of sialic acid (on a per-milligram-of-protein basis). Con A revertant clones 80, 83, and 84 have high sialic acid compositions similar to Balb/c 3T3 cells and not the SVT2 cells from which they were derived. Thus, sialic acid composition is high in revertant cells isolated in different cell systems (Swiss and Balb/c mouse cells) by widely differing selective procedures (FUdR and con A treatment). There is evidence that as much as 80% of the cell's sialic acid is in surface membrane components (5, 7); these data therefore reflect quantitative differences in surface membrane components.

Revertant clone 81 consistently yielded sialic acid compositions which were lower than the

values observed for the other revertant clones. During the growth of these cells, there is a tendency for a portion of the cells to assume a small, spindle-shaped morphology characteristic of parental SVT2 cells and a marked tendency in confluent cultures of clone 81 cells to "rerevert" to the non-contact-inhibited growth properties of SVT2 cells. This tendency may explain the low sialic acid composition of this clone.

Studies are being initiated to investigate the qualitative distribution of surface glycoproteins in these different cell systems. It will be important to differentiate between several models: (i) similar distributions of glycoproteins with a few glycoproteins being quantitatively prominent in cells which exhibit growth control; (ii) widely different qualitative distributions of types of surface glycoproteins of which revertant cells have regained the ability to make those which are relevant to growth control; (iii) the same distribution of glycoproteins in normal, transformed, and revertant cells, but a greater concentration of these surface components per unit area of membrane in normal and revertant cells as compared to transformed cells; (iv) localized areas of surface membrane (perhaps only a very small fraction of the total surface membrane) where unique glycoproteins important in growth control are found while the vast majority of the membrane components from normal, transformed, and revertant cells are the same; and (v) the same protein "backbones" in surface glycoproteins but unique sequences or lengths of polysaccharide components bound to these proteins. A great deal of analytical work will be necessary to explain the chemical basis for contact inhibition of growth as it is mediated by cell surface components.

Previous evidence indicated that FUdR revertant cells (6) had a diminished ability to produce collagen and in this respect resembled Swiss SV3T3 cells from which they were derived. On the other hand, Swiss 3T3 cells produced large amounts of collagen when they became confluent (9). The data on collagen production by the BALB/c mouse cell lines indicated exactly the opposite. SVT2 cells, which were confluent and growing, and the con A revertant cells, which were confluent and nongrowing, accumulated more collagen than BALB/c 3T3 cells from which these lines were derived. Thus the SV40-induced transformation event which yielded the SVT2 clone either: (i) occurred in a variant 3T3 cell which had an increased capacity for collagen synthesis or (ii) "derepressed" collagen synthesis several-fold in a low-producing cell. Even subconfluent revertant cells produced more collagen than 3T3 cells. The 50% higher yields of collagen in confluent as compared to subconfluent revertant cells may not necessarily mean a stimulation of collagen production after growth inhibition (9), but perhaps may mean an inhibition of net protein synthesis because of the absence of further cell division and the continued synthesis of collagen at the normal rate. It has also been proposed that the increased production of collagen in confluent cultures is due to stimulation of the biosynthetic enzymes operative in the production of mature collagen. In any case, the inverse correlation of cell division with production of a differentiated cellular product is not apparent in this system (6, 9). It will now be of interest to determine the fluctuation of collagen-producing ability in different BALB/c 3T3 clones and whether this phenomenon occurs in different SV40 transformation events.

The ultrastructural properties of the surface membranes of BALB/c 3T3, SVT2, and con A revertant cells, as well as the relative distribution of the alpha microfilament system (14) in the cytoplasm of these cells, will be described in a separate communication (L. S. McNutt, L. A. Culp, and P. H. Black, *manuscript in preparation*).

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Sharon Wilbert, the hydroxyproline analyses of Andrew Kang, and the cytogenetic analysis of Leonard Atkins.

Lloyd A. Culp is a postdoctoral fellow of the National Institutes of Health. This work was supported by Public Health Service grant CA-10126-05 from the National Cancer Institute.

LITERATURE CITED

1. Ahnstrom, C., and A. T. Natarajan. 1966. Mechanism of chromosome breakage–a new theory. Hereditas 54:379–388.
2. Burger, M. M. 1969. A difference in the architecture of the surface membrane of normal and virally transformed cells. Proc. Nat. Acad. Sci. U.S.A. 62:994–1001.
3. Burger, M. M. 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature (London) 227:170–171.
4. Burger, M. M., and K. D. Noonan. 1970. Restoration of normal growth by covering of agglutinin sites on tumour cell surface. Nature (London) 228:512–515.
5. Codington, J. F., B. H. Sanford, and R. W. Jeanloz. 1970. Glycoprotein coat of the TA3 cell. I. Removal of carbohydrate and protein material from viable cells. J. Nat. Cancer Inst. 45:637–647.
6. Culp, L. A., W. J. Grimes, and P. H. Black. 1971. Contact-inhibited revertant cell lines isolated from SV40-transformed cells. I. Biologic, virologic and chemical properties. J. Cell Biol. 50:682–690.
7. Glick, M. C., C. A. Comstock, M. A. Cohen, and L. Warren. 1971. Membranes of animal cells. VIII. Distribution of sialic acid, hexosamines and sialidase in the L cell. Biochim. Biophys. Acta 233:247–257.
8. Goldstein, I. J., C. E. Hollerman, and E. E. Smith. 1965. Protein-carbohydrate interaction. II. Inhibition studies on the interaction of conconavalin A with polysaccharides. Biochemistry 4:876–883.
9. Green, H., and G. Goldberg. 1965. Synthesis of collagen by mammalian cell lines of fibroblastic and nonfibroblastic origin. Proc. Nat. Acad. Sci. U.S.A. 53:1360–1365.

10. Grimes, W. J. 1970. Sialic acid transferases and sialic acid levels in normal and transformed cells. Biochemistry 9: 5083–5092.
11. Hsu, T. C., R. M. Humphrey, and C. E. Somers. 1964. Responses of Chinese hamster and L cells to 2′-deoxy-5-fluorouridine and thymidine. J. Nat. Cancer Inst. 32:839–855.
12. Inbar, M., Z. Rabinowitz, and L. Sachs. 1969. The formation of variants with a reversion of properties of transformed cells. III. Reversion of the structure of the cell surface membrane. Int. J. Cancer 4:690–696.
13. Inbar, M., and L. Sachs. 1969. Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. Proc. Nat. Acad. Sci. U.S.A. 63:1418–1425.
14. McNutt, N. S., L. A. Culp, and P. H. Black. 1971. Contact-inhibited revertant cell lines isolated from SV40-transformed cells. II. Ultrastructural study. J. Cell Biol. 50:691–708.
15. Madoff, S. 1960. Isolation and identification of PPLO. Ann. N.Y. Acad. Sci. 79:383–392.
16. Nardone, R. J., J. Todd, P. Ganzales, and E. V. Gaffney. 1965. Nucleoside incorporation into strain L cells: inhibition by pleuropneumonia-like organisms. Science 149:1100–1102.
17. Pollack, R. E., and M. M. Burger. 1969. Surface-specific characteristics of a contact-inhibited cell line containing the SV40 viral genome. Proc. Nat. Acad. Sci. U.S.A. 62: 1074–1076.
18. Pollack, R., S. Wolman, and A. Vogel. 1970. Reversion of virus-transformed cell lines: hyperploidy accompanies retention of viral genes. Nature (London) 228:967–970.
19. Rabinowitz, Z., and L. Sachs. 1968. Reversion of properties in cells transformed by polyoma virus. Nature (London) 220:1203–1206.
20. Sanbe, K., T. Aya, T. Ikeuchi, and A. A. Sandberg. 1970. Electron microscopic study of fused cells, with special reference to chromosome pulverization. J. Nat. Cancer Inst. 44:1079–1089.
21. Shoham, J., M. Inbar, and L. Sachs. 1970. Differential toxicity on normal and transformed cells in vitro and inhibition of tumour development in vivo by concanavalin A. Nature (London) 227:1244–1246.
22. Taylor, J. H., W. F. Haut, and J. Tung. 1962. Effects of fluorodeoxyuridine on DNA replication, chromosome breakage, and reunion. Proc. Nat. Acad. Sci. U.S.A. 48: 190–198.
23. Warren, L. 1971. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:1959–1975.