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Selection of Revertants of Kirsten Sarcoma Virus Transformed Nonproducer BALB/3T3 Cells

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Revertants of Kirsten sarcoma virus transformed nonproducer BALB/3T3 cells (KA31 cells) were isolated after exposing the transformed cells to 5-fluorodeoxyuridine at high cell density, or when suspended in methylcellulose. Revertants were also isolated by treating KA31 cells with the lectin, concanavalin A, which is manyfold more toxic to transformed cells than for normal cells. The revertants resemble BALB/3T3 cells in their morphology and growth characteristics in that they have a low saturation density, fail to grow in 1% calf serum or when suspended in methylcellulose, and cease to synthesize DNA after reaching their saturation density. Infection by murine leukemia virus rescues Kirsten sarcoma virus from only the concanavalin-A-selected variants, though all the revertants are susceptible to infection by leukemia virus. The concanavalin A revertants also become transformed after infection with murine leukemia virus. All the revertants can be transformed by Kirsten sarcoma virus but not by simian virus 40.

Like most (4) isolates of murine sarcoma virus (MSV), the Kirsten strain (Ki-SV) (30) is defective in that it requires the aid of murine leukemia virus (MLV) for growth but not for transformation (2). When cells of the BALB/C mouse embryo-derived cell line, 3T3 (3), are infected solely by a Kirsten sarcoma virus, they become transformed but yield no virus particles (2) nor express MLV antigens (33). Such transformed cells are called "nonproducers" (2). The sarcoma genome becomes stably associated with the cell (34) and can be rescued from transformed cells by either infection with MLV (2) or induction of the endogenous leukemia virus (1).

BALB/3T3 cells transformed by Ki-SV undergo a marked alteration in their phenotype (37). They become round and spindly, grow to high saturation densities, grow in low concentrations of serum, form colonies when suspended in medium containing methylcellulose, and become agglutinable by low concentrations of the lectin, concanavalin A (27). The means by which the virus effects these changes remains obscure, though continued expression of some viral gene(s) is apparently necessary for the maintenance of the transformed phenotype, since temperature-sensitive mutants of Ki-SV exist which render cells temperature sensitive for the expression of transformation (31).

One approach to sorting out the virus-cell interactions involved in transformation has been to select variant sublines of transformed cells which have regained some of the traits of normal cells (28). Variants have been isolated by a number of selection procedures, most of which have been devised for simian virus 40 (SV40)- or polyoma virus (PY)-transformed cells. The selection schemes depend upon specific differences in the behavior of normal and transformed cells, such as the inability of normal cells to synthesize DNA in dense culture (22) or when suspended in medium containing methylcellulose (41), or the resistance of normal cells to the toxic effects of the lectin, concanavalin A (con A) (17, 20). Revertants of SV40-transformed 3T3 cells isolated by these procedures behave like 3T3 cells in that they cease growing at low saturation densities, do not form colonies when suspended in methylcellulose-containing media, and are not agglutinated by con A. None of the variants selected by these procedures have reverted to a 3T3-like serum requirement for growth (17, 20, 38) nor do they display density dependent inhibition of DNA synthesis to the same degree as BALB/3T3 cells (17, 23, 29). As yet, the revertant phenotype of the variants cannot be ascribed to any altered viral function (13, 19, 21, 38) since the virus in the variants appears to be

functioning as in the transformed cells (19, 20, 38) and wild-type virus can be rescued from them (17, 20, 22, 38, 40).

To date, revertants of MSV-transformed cells have been isolated by screening under a microscope for spontaneously occurring "flat" clones (9, 16, 34, 35). Sometimes the frequency of revertants has been increased by pretreating the cells with sublethal doses of BUdR (10, 11), FUdR (15), or colcemid (15) before scanning for the morphological variants. Too few variants have been studied to make general statements as to their mode of occurrence, and, though the state of the transforming virus in the variants so far is unknown, in some cases the sarcoma genome can be rescued from the revertants (7, 10, 15, 16) after infection with MLV.

In this paper we describe the isolation of revertants of KA31 cells, a Ki-SV-transformed nonproducer BALB/3T3 cell line, by three different techniques and report some of their characteristics.

MATERIALS AND METHODS

Cells and viruses. All cell lines were grown on plastic tissue culture dishes, in Dulbecco's modification of Eagle medium supplemented with 10% calf serum at 37 C in a water saturated 5% CO_2 atmosphere. BALB/3T3 cells and KA31 (2) cells (a Ki-SV-transformed nonproducer BALB/3T3-derived cell line) were obtained from S. Aaronson (National Institutes of Health) and were recloned in our lab. Swiss 3T3 cells and BSC-1 cells were supplied by R. Pollack (Cold Spring Harbor Laboratory). XC cells were given to us by N. Hopkins (Massachusetts Institute of Technology).

Kirsten sarcoma virus in a Rauscher murine leukemia virus pseudotype (Ki-SV[R-MLV]) (S. Aaronson, N.I.H.), Rauscher murine leukemia virus (R-MLV) (S. Aaronson, N.I.H.), and Moloney murine leukemia virus (M-MLV) (N. Hopkins, M.I.T.) stocks were grown on BALB/3T3 cells. SV40 strain 777 stocks were grown on BSC-1 cells.

Determination of growth parameters. The saturation densities and the generation times of the various cell lines were determined as described elsewhere (38). Briefly, cells were plated on plastic dishes at a density of about 0.2×10^4 cells/cm^2 in media supplemented with either 10 or 1% calf serum and were incubated at 37 C. Every 24 h, cells were removed from a plate with trypsin and counted with a Coulter counter. Every third day the media was changed.

The colony forming ability of the cell lines in media containing methylcellulose was determined as described by Vogel et al. (40). Briefly, serial dilutions were made of cell suspensions starting with a cell suspension of 10^6 cells per ml, down to 10^2 cells per ml, and 1 ml of the cell suspension was plated into methylcellulose medium and incubated for 3 weeks at 37 C. A 4-ml amount of fresh methylcellulose medium was added to the culture once a week. Visible colonies (larger than 0.3 mm in diameter) were scored after 3 weeks.

Chromosomes. Cells blocked in mitosis for 1 h with 0.01 μg of Velban per ml were prepared for chromosome analysis as described by Pollack et al. (24). The chromosomes of 30 well-spread metaphases of each cell line were counted.

DNA synthesis. The fractions of cells synthesizing DNA were determined by autoradiography as described by Pollack and Vogel (23). The cells synthesizing DNA were labeled with [^{3}H]thymidine (10 μCi/ml, >15 Ci/mm) in the presence of 10^{-5} M unlabeled thymidine for 4 h at 37 C. Uptake of label was linear throughout the labeling period and the grains were observed to be localized over the nucleus with very few grains being seen above the cytoplasm.

Transformation by SV40 and SV40 tumor antigen assay. SV40 transformation assay was performed according to Aaronson and Todaro (3). The infected cells were incubated for 17 to 28 days with medium changes twice a week. The cells were fixed with 10% formalin in phosphate-buffered saline (PBS) and stained with hemotoxylin, and the number of transformed colonies was determined.

SV40-specific tumor antigen was assayed by immunofluorescence by using fluorescent antisera purchased from Flow Laboratories, Rockville, Md.

MSV and MLV assay. Ki-SV (M-MLV) was assayed according to Jainchill, Aaronson, and Todaro (13) on Swiss 3T3 or BALB/3T3 cells with several minor alterations. The cells were treated for 1 h with 25 μg of DEAE dextran per ml (8) and washed twice with serum-free medium before the addition of the virus. Medium was changed on days 3 and 6 and foci were counted on day 10.

MLV was assayed by the XC plaque assay (26).

Rescue of Ki-SV by infection with MLV. Ki-SV was rescued from KA31 cells and variants were derived from KA31 cells, as described by Aaronson and Rowe (2). Briefly, cells were infected with either R-MLV or M-MLV and at the time of medium changes, 3 and 6 days after infection, the medium was removed from the infected cells, centrifuged at 10,000 rpm for 10 min at 4 C, passed through a 0.22-μm membrane filter (Millipore Corp.), and assayed for Ki-SV and MLV on BALB/3T3 cells.

Selection of revertants from KA31 cells. (i) Methylcellulose revertants (41). To select cells unable to form colonies when suspended in methylcellulose-containing medium, 4×10^5 KA31 cells were plated in 5 ml of methylcellulose-containing medium (see above). Two days later, 25 μg of FUdR per ml and 250 μg of uridine per ml was added and the plates were incubated for an additional 2 days. The methylcellulose medium was harvested and the cells were centrifuged out of the methylcellulose and plated to determine the number of survivors. Approximately one in 5×10^3 cells survived the FUdR treatment. One of the surviving colonies had a "flat" morphology and was cloned and grown up. The progeny of this clone were subjected to two more selections in methylcellulose and FUdR. Forty colonies were picked after the third selection and tested for their ability to form

colonies in methocel. One of these colonies, M22, was characterized further.

(ii) FUdR revertants (22). KA31 cells (2×10^5) were plated onto a 90-mm plastic tissue culture plate and allowed to grow to 2×10^6 cells per plate. Growth medium containing 50 mg of FUdR and 250 mg of uridine per ml was then added. The plates were incubated for 3 days after which the FUdR-containing medium was removed and cells were washed three times with PBS. Fresh medium was added, and the cells were incubated for an additional day. Then the cells were removed from the plate with trypsin, serially diluted, and replated. Two weeks later the number of surviving cells was determined and "flat-looking" clones were isolated. One cell in 10^5 formed a colony after FUdR treatment, and approximately one in 10 of these appeared flat. Twenty-two flat clones were isolated, grown up, and retreated with FUdR. Nineteen out of twenty-two of these looked like the parent KA31 cells after 2 weeks in culture, and were killed at high cell density after retreatment with FUdR. The three flat clones behaved similarly with one in 10^3 cells surviving the second treatment of FUdR. Six subclones of these three original clones were isolated. Four were not stable and returned to a KA31-like morphology after 1 month in culture, whereas two remained flat. One of these, F-1, was characterized further.

(iii) Con A revertants (17, 20). KA31 cells (2×10^6) were plated onto a 90-mm plastic tissue culture plate. Twenty-four hours later con A (Miles Yeda) was added to the medium to give a final concentration of 300 μg of con A per ml, and the plates were incubated for another 24 h. The medium was removed and the plates were washed three times with PBS, and fresh medium was added. Two weeks later, several flat-looking clones were isolated, grown up, and recloned in the absence of con A. Out of 17 clones picked in the original selection, two of these remained flat and resistant to a second con A selection. These clones appeared to be stable in their "flat" morphology after a month of continuous culture. One of these, C-1, was characterized further.

RESULTS

Saturation density. BALB/3T3 cells in tissue culture have a flat polygonal shape in sparse culture and a cobblestone appearance after forming a monolayer of cells (3). Upon reaching a single confluent layer of cells, BALB/3T3 cells cease growing and have a characteristic low saturation density (1). By contrast, KA31 cells are round and spindly, form multilayers of cells, and attain a saturation density many times greater than do BALB/3T3 cells (2). The revertant clones isolated from KA31 cells resemble BALB/3T3 cells in their morphology, being flat and polygonal (Fig. 1), and in their low saturation densities (Fig. 2a, Table 1).

Serum requirement. KA31 cells also differ from BALB/3T3 cells in the concentration of serum required to sustain growth (35). The transformed cells are capable of growing in medium supplemented with 1% serum with only a slightly increased generation time. In 1% calf serum, BALB/3T3 cells and the revertants either fail to grow at all or do so with a greatly increased generation time (Fig. 2b, Table 1). Though the revertants were selected for reversion of different traits, all have reverted in their serum requirement. This serum reversion, though common for spontaneously occurring revertants of RNA tumor virus-transformed BALB/3T3 cells (35), does not occur in revertants of SV40-transformed 3T3 cells (17, 20, 38), unless directly selected for (39).

DNA synthesis. Under conditions where the growth of BALB/3T3 cells is greatly inhibited, either by the cells being in a confluent monolayer of cells, or in sparse culture with an insufficient amount of serum to support their growth (14), the cells' synthesis of DNA is also markedly reduced. Under similar conditions, KA31 cells continue to synthesize DNA at rates equivalent to the rate exhibited by log phase cultures. The revertants of KA31 cells behave like BALB/3T3 cells in that they also shut off DNA synthesis after forming a monolayer of cells, or after being placed in medium supplemented with 1% calf serum (Table 2). The decrease of DNA synthesis in the BALB/3T3 and revertant cells in low concentrations of calf serum probably reflects the cells' greatly increased generation time, though the decrease in DNA synthesis in a monolayer of cells may be due to a density dependent shut-off.

Growth in methylcellulose. Like BALB/3T3 cells, the three revertants need to be firmly anchored to the tissue culture dish in order to grow, as shown by their inability to form colonies when suspended in medium containing methylcellulose (Table 1). The parent KA31 cells, however, form colonies at a high efficiency when plated into methylcellulose-containing medium (Table 1). By this criterion, the revertants phenotype appears to be as stable as that of BALB/3T3 cells, since the revertants and BALB/3T3 cells form colonies in methylcellulose at frequencies of less than one cell in 10^6.

Number of chromosomes. The reversion of the transformed phenotype of virally transformed cells is often accompanied by an increase in the number of chromosomes per cell (10, 12, 15, 17, 19, 20, 24, 29, 38-40). Though an increase in the number of chromosomes per cell was observed for the F-1 and C-1 cells, no increase in the number of chromosomes per cell was found for M-22 cells (Fig. 3). Others have

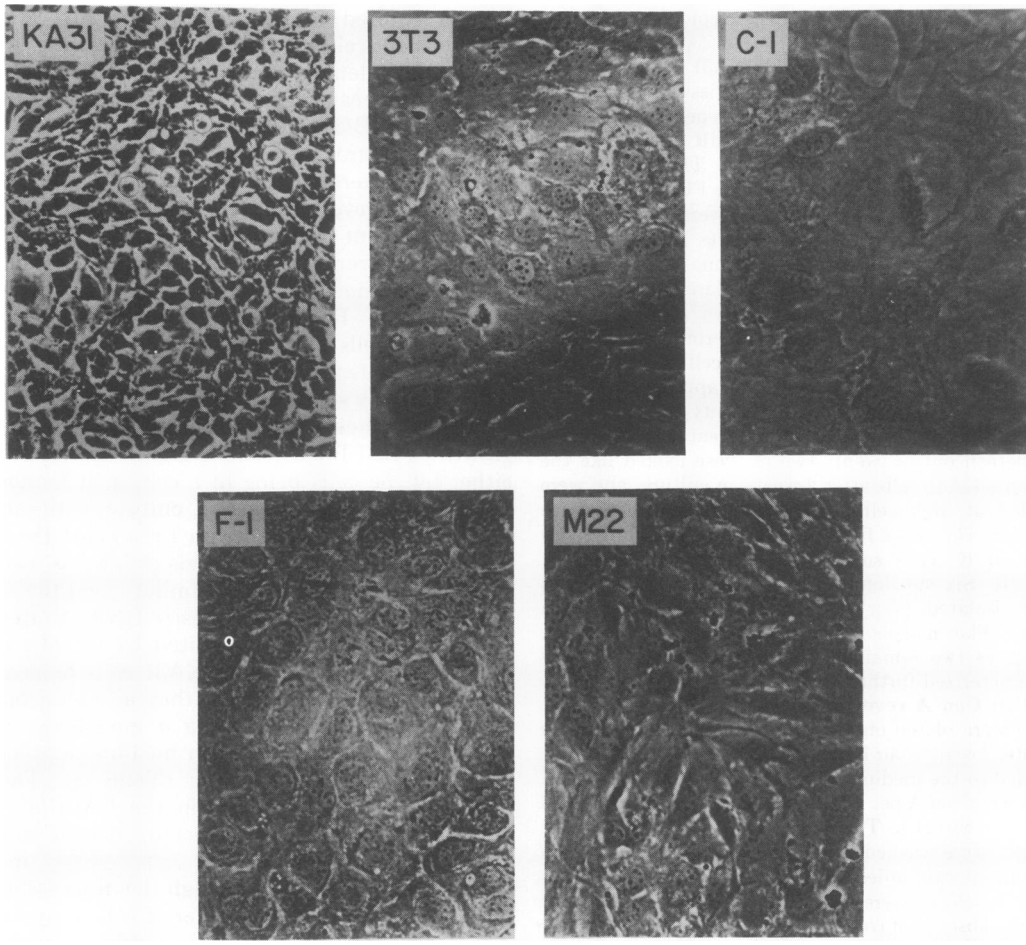

FIG. 1. *Photographs of the cell lines BALB/3T3, C-1, F-1, and M22 were taken 2 days after the cells had reached their saturation density; magnification ×200. The density of the KA31 cells was 20 × 10⁴ cells/cm².*

also found that the number of chromosomes per cell of revertants of MSV-transformed 3T3 cells has not always been increased when compared to the parent cells (10, 15). Thus, an increase in chromosome number per cell is not an absolute requirement for reversion of MSV-transformed cells.

Infection with MLV. Infection of KA31 cells with MLV results in the rescue of the sarcoma virus, with large quantities of both MLV and Ki-SV being produced (2). To determine if the revertants still harbor a Ki-SV genome in a rescuable form, the revertant cells were infected with MLV. Six days after infection, the medium from the infected cells was assayed for the presence of both MLV and Ki-SV (Table 3, columns 3 and 4). Only medium from MLV-infected C-1 cells contained focus-forming activity when tested on BALB/3T3 cells. The other revertants, M-22 and F-1, failed to yield any focus-forming activity (Table 3, column 4). However, all the revertant cell lines produced MLV in titers equal to those produced by similarly infected BALB/3T3 cells (Table 3, column 3). Furthermore, when a stock of MLV is assayed on either BALB/3T3 cells or the revertant cells, the same titer of MLV is observed, indicating that the revertants are as susceptible to infection by MLV as BALB/3T3 cells (Table 3, column 1). These results imply that the failure of F-1 and M-22 cells to yield any sarcoma virus after infection with MLV must be because the sarcoma genome in those cells, if present, is not in a rescuable form.

The C-1 cells behave uniquely after infection with MLV, not only because they yield Ki-SV, but because they also become transformed, forming typical MSV foci (Table 3, column 2).

No other cell line tested is transformable by MLV (Table 3, column 2). When C-1 cells are used as a focus-forming assay for MLV, the titer of MLV is 10 to 20 times lower than when the virus is assayed by the XC plaque assay, either on BALB/3T3 or C-1 cells (Table 3, columns 1 and 2). It appears that not all the C-1 cells become transformed after infection with MLV. This is unlike a class of S^+L^- cells (5) which also complete transformation after MLV infection, but give equal titers of MLV when the virus is assayed by either focus formation or by XC plaque assay (7). The C-1 cells vary from other revertants of KA31 cells which produce Ki-SV after MLV infection, but do not form foci as a result of the MLV infection (11).

Infection with Ki-SV (MLV). To determine whether the reversion in growth properties is the result of a cellular alteration which prevents the expression of the MSV-induced transformed state, the revertants were infected with Ki-SV (MLV). As seen in Table 4, the focus-forming ability of the Ki-SV (MLV) stock was the same when assayed on the revertants or BALB/3T3

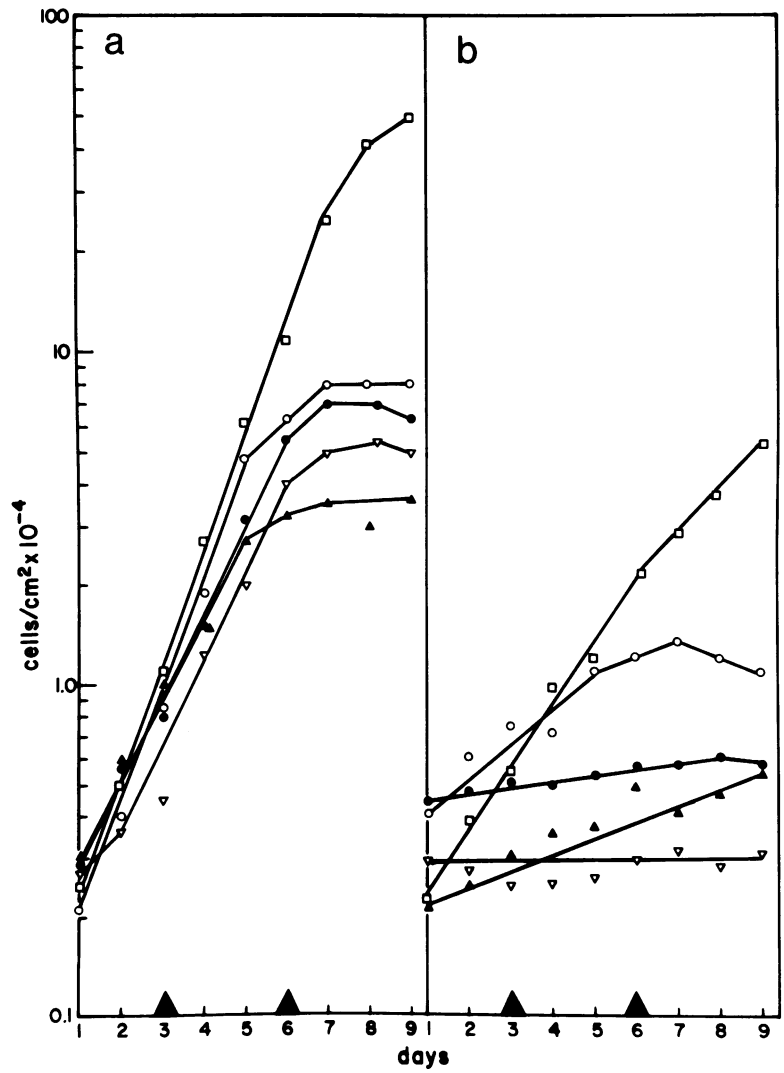

FIG. 2. *The growth curves in 10% calf serum were performed as described in the text. Cells were seeded at a cell density of 2 × 10³ to 4 × 10³ cells/cm². Media was changed on day 3 and day 6 (▲). (b) The growth curves in 1% calf serum were performed as described in the text. Cells were seeded at a cell density of 2 × 10³ to 4 × 10³ cells/cm². Media was changed on day 3 and day 6. Symbols: ○, 3T3; □, KA31; ▲, M22; ▽, C-1, ●, F-1.*

TABLE 1. *Growth properties of mouse cell lines*

Line	10% Calf serum			1% Calf serum
	Doubling time (h)[a]	Saturation density cells ($cm^2 \times 10^4$)[b]	% Colony formation in methyl-cellulose[c]	Doubling time (h)[d]
3T3	22	8	<0.0001	77
KA31	19	>50	20	38.4
M22	29	5	<0.001	98
F-1	25	7	<0.0001	>100
C-1	31	5	<0.0001	>100

[a] The doubling time of the cells in 10% serum was determined from growth curves while the cells were in log phase growth. The growth curves were performed as mentioned in the text.

[b] The saturation densities of the cell line were derived from growth curves and are the number of cells per unit area after the number of cells have ceased increasing for two consecutive days. The growth curves were done as described in the text.

[c] The colony forming ability of the different cell lines in methylcellulose containing medium was measured as described in the text. After 3 weeks in culture the viable colonies were counted and the percent colony formation was determined.

[d] The doubling time in 1% serum was determined from growth curves which were performed as described in the text.

TABLE 2. *Fraction of cells synthesizing DNA*

Line	Fractions of cells labeled		
	Sparse cultures[a]		Confluent cultures[b]
	1% Calf serum	10% Calf serum	10% Calf serum
3T3	0.07	0.51	0.03
KA31	0.38	0.41	0.20
M22	0.10	0.33	0.06
F-1	0.07	0.36	0.008
C-1	0.02	0.31	0.01

[a] Sparse cultures: Cells were seeded at 2.5×10^3 cells/cm^2 and 2 days later they were pulse labeled with [^{3}H]thymidine. The fraction of cells synthesizing DNA was determined by autoradiography as mentioned in the text.

[b] Confluent cultures: Cells were seeded at 2.5×10^4 cells/cm^2 and allowed to grow until they had reached their saturation density; the medium was then changed and 2 days later the cells were pulse labeled with [^{3}H]thymidine and prepared for autoradiography as described in the text.

(Table 4). Furthermore, the yield of Ki-SV and MLV from the revertant cells was equal to that from similarly infected BALB/3T3 cells (Table 4). These results rule out the possibility that the revertant phenotype resulted from a cellular alteration which permanently rendered these cells incapable of expressing the genetic information of the Ki-SV or the transformed state.

SV40 transformation. After infection of BALB/3T3 cells with a multiplicity of infection of 10 to 100 PFU of SV40, about 1 to 10% of the cells become stably transformed (3). When SV40 at a multiplicity of infection of 20 PFU was added to the revertants and BALB/3T3 cells, none of the revertants became stably transformed, whereas 2% of the BALB/3T3 cells did (Table 5). This lack of transformation of the revertant cells did not result from failure of the virus to enter the cells and to be expressed, since 48 h after infection both BALB/3T3 cells and the revertant cells expressed SV40-specific tumor antigen (Table 5). The failure of the revertant clones to be transformed by SV40 was a surprising result, because other revertants of RNA tumor virus-transformed BALB/3T3 cells do not display this resistance (35).

DISCUSSION

The work presented here reports the isolation and characterization of revertants of KA31 cells. They were selected for three different properties: (i) the inability of BALB/3T3 cells

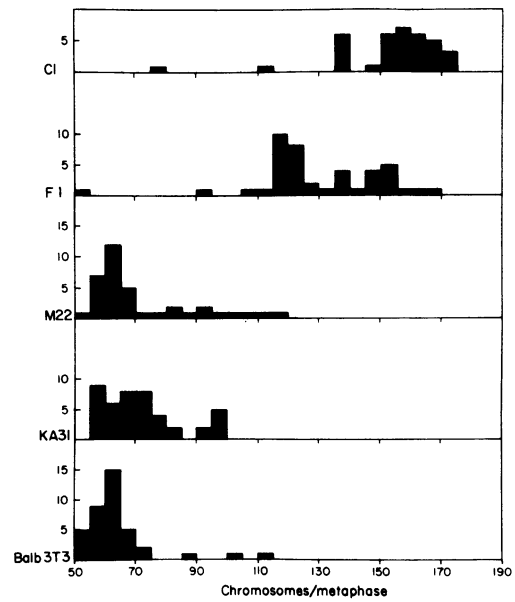

FIG. 3. *The number of chromosomes per cell for each cell line was determined as described in the text. The chromosomes in at least 30 well-spread metaphases of each cell line were counted.*

TABLE 3. *MLV infection*

Cell line	Input virus		Virus recovered from MLV infected cells[a]	
	MLV added[b]		MLV produced[c]	Ki-SV rescued by MLV[d]
	XC PFU/ml[e]	FFU/ml[f]	XC PFU/ml on BALB/3T3 cells	FFU/ml on BALB/3T3 cells
3T3	5.9×10^5	0	6×10^5	0
KA31	NT[g]	NT	2×10^5	2×10^4
M22	2.0×10^5	0	2.3×10^4	0
F-1	3.7×10^5	0	8×10^4	0
C-1	7.4×10^5	2.3×10^4	3.2×10^5	1.8×10^4

[a] Virus recovered: The supernatants from the MLV infected cells were assayed for the presence of MLV and Ki-SV.

[b] MLV added: M-MLV stocks were assayed for their ability to form foci on BALB/3T3 cells and XC plaques on the various cell lines.

[c] MLV produced: Medium was collected on day 6 from 10^{-1} dilution plates of M-MLV infected cells and assayed on BALB/3T3 cells by the XC plaque assay.

[d] Ki-SV rescued by M-MLV: Medium was collected on day 6 from 10^{-1} dilution plates of M-MLV infected cells and assayed for Ki-SV by the focus formation assay as described in the text.

[e] XC PFU/ml: The titer of M-MLV stocks was determined by the XC plaque assay on the various cell lines.

[f] FFU/ml: The focus-forming ability per milliliter of stocks of M-MLV was determined on the various cell lines.

[g] NT, Not tested.

TABLE 4. *Ki-SV (MLV) infection*

Cell line	Ki-SV (MLV) added[a]	Ki-SV (MLV) produced[b]	
	FFU/ml	FFU/ml on BALB/3T3 cells	XC PFU/ml on BALB/3T3 cells
3T3	2.5×10^5	5.1×10^5	8.5×10^5
M22	1.8×10^4[c]	2.0×10^4	NT[d]
F-1	2.0×10^5	4.3×10^4	4.1×10^5
C-1	3.1×10^5	6.1×10^4	7.9×10^5

[a] Ki-SV (MLV) added: Ki-SV (MLV) stocks grown on BALB/3T3 cells were assayed for focus-forming ability as described in the text.

[b] Ki-SV (MLV) produced: Ki-SV (MLV) stocks were grown on the various cell lines. Virus stocks were collected on day 6 from plates infected with a 10-fold dilution of the original virus stock and assayed for Ki-SV and MLV on BALB/3T3 cells as described in the text.

[c] Infected with 2×10^4 FFU/ml of Ki-SV (M-MLV) as assayed on BALB/3T3 cells.

[d] NT, Not tested.

to synthesize DNA at high cell density; (ii) the inability of BALB/3T3 cells to synthesize DNA when suspended in methylcellulose containing medium; and (iii) the resistance of BALB/3T3 cells to the toxic effects of con A. Although each of these procedures has been employed in the isolation of revertants of DNA virus-transformed cells (17, 20, 22, 41), they have not previously been applied to the selection of revertants of MSV-transformed 3T3 cells. Spontaneously occurring revertants of KA31 arise at a frequency of less than one in 10^4 cells (34). Variants isolated by these selection procedures appear to occur at a frequency of about one cell in 10^6. However, it may be that the selection techniques themselves induced the reversion, since it has been shown that sublethal doses of FUdR enhance the reversion frequency of S^+L^- cells (15). Although it cannot be excluded, it seems unlikely that the revertants reported here were induced by the isolation procedures, since all the selection techniques employed give revertants at about the same frequency and con A is not known to enhance revertant formation.

Others have demonstrated that transformation of BALB/3T3 cells by MSV renders the cells agglutinable by con A (27). This paper shows that MSV transformation also renders BALB/3T3 cells susceptible to the toxic effects of con A, and that the acquisition of resistance to con A by MSV-transformed BALB/3T3 cells is accompanied by a change in morphology and a return to 3T3-like growth characteristics. Increased growth control and resistance to con A, though commonly associated, are not inextricably entwined since con A-resistant SV40-transformed 3T3 cells exist which retain the growth characteristics of transformed cells (8). No analogous con A-resistant variants of MSV-transformed cells have yet been isolated, although Nomura et al. (15) reported that revertants of S^+L^- cells remained as agglutinable as the fully transformed parent cells. It is unknown whether their revertants also remain susceptible to the toxic effects of con A.

From the growth properties of each of the

TABLE 5. *SV40 transformation*

Cell line	Plating efficiency[a]		% Transformants[b]		% T-antigen[c]
	Uninfected	Infected	Uninfected	Infected	Positive cells
3T3	40	50	<0.0003	2	61
M22	10	15	<0.0005	<0.0005	29
F-1	50	50	<0.0002	<0.0002	39
C-1	25	25	<0.0002	<0.0002	10

[a] The plating efficiency of cells infected with 20 PFU/cell of SV40 and mock-infected cells was determined by removing the cells from the plates 24 h after infection, counting them in a Coulter counter, serially diluting them, and allowing them to grow into colonies which were fixed and then stained and counted. The plating efficiency is the number of cells forming colonies per 100 cells plated.

[b] Percentage of transformants was determined as mentioned in the text.

[c] Percentage of T-antigen-positive cells was determined by immunofluorescence as mentioned in the text.

revertants, it appears that regardless of the trait being selected for, the variants of KA31 cells return completely to a 3T3-like state of growth control. The three revertants described here grow to BALB/3T3-like saturation densities. After reaching their saturation densities, the revertants display density dependent inhibition of DNA synthesis. Furthermore, the revertants grow poorly in medium supplemented with 1% calf serum, as do BALB/3T3 cells. In their cessation of DNA synthesis after forming a monolayer of cells and in their inability to grow in 1% serum, the KA31 revertants differ from revertants of SV40-transformed 3T3 cells isolated by similar procedures. The revertants of SV3T3 cells grow as well as the transformed parent in 1% calf serum and do not shut off DNA synthesis to the extent that BALB/3T3 cells do (17, 20, 23, 39).

Another property by which the revertants' behavior mimics that of BALB/3T3 cells is in their inability to form colonies when suspended in medium containing methylcellulose (15). This inability to grow in methylcellulose containing medium can be used as a measure of the stability of the revertant phenotype. By this criterion, the revertants retain the untransformed phenotype as stably as do BALB/3T3 cells, returning to the transformed state at a frequency of less than one cell in 10^6. Furthermore, after 9 months of continuous passage, no spontaneous transformants have been observed in either the M22 or the F-1 revertant cell lines. C-1 seems to be less stable since several spontaneous transformants have been observed.

When the selection procedures used in this paper have been applied to SV40-transformed cells, essentially all of the revertants isolated have increased numbers of chromosomes (12, 17, 20, 24). What control, if any, this increase in chromosome number per cell has upon revertant phenotypes is unclear and in some cases, appears unnecessary, since M22 cells and other MSV revertant cells failed to show an increase in chromosome number.

The mechanism(s) by which the variants reported here became revertants is unclear, but three broad possibilities exist. (i) The cells may have lost the sarcoma virus genome; (ii) the sarcoma virus genome may be present but contain a mutation in a gene required for transformation; or (iii) a cellular alteration may have occurred which prevents the expression of the virus or the transformed phenotype. For the con A-resistant variants, the result that Ki-SV can be rescued after infection with MLV rules out the first possibility that these cells have lost the sarcoma virus genome. The other two explanations, however, remain feasible. Of the two remaining possibilities, the first, that the variants arose via a mutation in the sarcoma genome, seems more likely for two reasons. First, the purported mutation which leads to the revertant phenotype seems to be complemented by MLV because the cells release Ki-SV after MLV infection and become transformed. That MLV could complement the Ki-SV genome seems reasonable since the Ki-SV RNA is partially homologous to the MLV genome (30, 32), and a Ki-SV mutant exists which renders nonproducer cells temperature sensitive for the expression of transformation, but the transformed phenotype of nonproducer cells becomes temperature independent after infection with MLV (31). Secondly, the fact that the C-1 cells become transformed after infection with MLV or with a new sarcoma virus demonstrates that the C-1 cells have not permanently lost the ability to express the transformed phenotype. This result suggests that the mechanism by which the C-1 cells became resistant to con A differs from that mechanism responsible for the occurrence of con A-resistant SV3T3 cells which are unable to express the transformed state

after infection by Ki-SV (MLV) even though they can produce new virus (17, 18).

For the other two revertants, M22 and F-1, it is difficult to exclude any of the three possibilities as the mechanism by which they arose. The inability to rescue Ki-SV from either M22 or F-1 cells does not distinguish between the Ki-SV in those cells being a mutant virus which cannot be complemented by MLV, or the loss of the virus genetic material from the cells. However, since both sorts of cells become transformed after infection with Ki-SV, they appear not to have resulted from a cellular alteration which rendered them unable to express the transformed phenotype or the Ki-SV genome. The explanations for the occurrence of revertant behavior of these cells could perhaps be distinguished by a combination of nucleic acid hybridization studies, which could demonstrate the presence of the sarcoma virus genome in the cells and determine whether or not it was being transcribed, and genetic studies with temperature-sensitive Ki-SV mutants.

A result which is difficult to understand and which could be taken as support for the possibility that the revertants arose via a cellular alteration preventing the expression of the transformed phenotype is the failure of the revertants, M22, C-1 and F-1, to become transformed by SV40. However, because all these revertants are capable of expressing transformation in response to Ki-SV genomes, such an explanation seems unlikely. Whatever the explanation is though, it does not seem to be that the cells fail to become infected with the virus for they express SV40-specific T-antigen; nor does it seem that 3T3 cells are incapable of harboring both viruses, for revertants of SV40-transformed 3T3 cells which still contain active SV40 genomes can be infected and transformed with MSV (17, 18, 25, 38, 39).

As yet the origins of these revertants have not been identified as being viral or cellular. However, since the revertants isolated by various techniques differ from each other, it is plausible that they do not have common origins and haply, then, may be used as prisms with which to separate the viral and cellular components which make up the transformed state.

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ADDENDUM

After we had submitted this paper, the following articles reporting the isolation of revertants from murine sarcoma virus transformed 3T3 cells were reported. J. S. Greenberger and S. A. Aaronson, 1974. Morphologic revertants of murine sarcoma virus transformed nonproducer Balb/3T3: Selective techniques for isolation and biological properties in vitro and in vivo, Virology **57**:339–347. They selected revertants by using a methocel selection and obtained revertants with rescuable Ki-SV which were susceptible to retransformation by Ki-SV but not MLV. A. F. Gazdar, H. B. Stull, H. C. Choppra, and Y. Ikawa, 1974. Properties of flat variants of murine sarcoma virus transformed non-producer cells isolated after high temperature passage, Int. J. Cancer **13**:219–226. They isolated variants which were transformed after infection with MLV and rescued Ki-SV. These appear to be similar to our C-1 variants.

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