

Isolation and Characterization of Revertant Cell Lines

VI. Susceptibility of Revertants to Retransformation by Simian Virus 40 and Murine Sarcoma Virus

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The susceptibility of two classes of revertants of Simian virus 40 (SV40)-transformed 3T3 cells to retransformation by SV40 or murine sarcoma virus (MSV) was studied. Both serum-sensitive and density-sensitive revertants are not retransformable by SV40. MSV can transform both types of revertants. The MSV-transformed revertants grow to high cell densities and form colonies when suspended in semi-solid methylcellulose medium, but are unable to grow in 1% calf serum. The MSV-transformed revertants produce infectious MSV and murine leukemia virus and possess the same number of chromosomes as the untransformed revertants.

The proliferation of 3T3 cells in culture is controlled, at least in part, by serum concentration, cell density, and anchorage to the substratum. 3T3 cells cannot grow in low concentrations of serum (6-8, 25), grow to low saturation densities in serum concentrations which support cell growth (2, 23), and cannot form colonies when suspended in semi-solid methylcellulose medium (2; R. Risser, D. Rifkin, and R. Pollack, Cold Spring Harbor Symp. Quant. Biol., in press). Transformation by oncogenic viruses such as Simian virus 40 (SV40) or murine sarcoma virus (MSV) alters 3T3 cells in such a way as to render them less susceptible to growth regulation by these environmental factors. Thus, transformed cells are able to grow in conditions of low serum concentration, high cell density, or suspension in methylcellulose medium (1, 2, 6-8, 21, 22, 24, 25; R. Risser, D. Rifkin, and R. Pollack, Cold Spring Harbor Symp. Quant. Biol., in press).

This virus-induced alteration is phenotypically reversible since variant sublines have been isolated from SV40-transformed 3T3 cells which have regained 3T3-like growth properties. These revertant cell lines were selected to be susceptible to growth regulation by either serum concentration or cell density (Table 1) (3, 4, 10-12, 15, 16, 26, 27). Thus, serum-sensitive revertants cannot grow in 1% calf serum or 10% agamma-depleted calf serum (10, 26). Density-sensitive revertants grow to low cell densities in 10% calf serum (10, 15, 17, 27).

In addition to the specific growth property selected for, both serum and density revertants

display other 3T3-like growth properties (Table 1). The serum-sensitive revertant LsSV grows to low saturation density in 10% calf serum and cannot form colonies when suspended in methylcellulose medium. The other serum-sensitive revertant, A γ SV, also grows to low saturation density in 10% serum, but can form colonies in methylcellulose medium. The density-sensitive revertant selected with 5-fluorodeoxyuridine (FUdR) (F1SV) does not form colonies in methylcellulose, but retains the ability to grow in 1% calf serum. The density-sensitive revertant isolated with colchicine (ColSV) does not grow in 1% calf serum and cannot form colonies in methylcellulose medium.

Serum and density-sensitive revertants have not lost the viral genome, since they contain SV40-specific T-antigen, RNA, and DNA, and yield infectious SV40 after fusion to permissive monkey cells (11, 13-15, 26, 27). Thus, revertants represent a new stable equilibrium between viral and cellular genomes, which permits the cell to re-express all or part of the growth properties it displayed prior to SV40 infection. To probe the nature of this equilibrium, we have examined the effects on it of two specific perturbations: reinfection of the revertants by SV40 and infection of them by an RNA-transforming virus (Kirsten-SV [R-MLV]).

MATERIALS AND METHODS

Cells and media. Cells were grown as previously described (26). The serum-sensitive revertants, LsSV and A γ SV, were isolated by plating SV101 cells in sparse culture in 1% calf serum or 10% agamma-depleted calf serum (22) and adding bromodeoxyuri-

TABLE 1. Growth properties of mouse cells in 10% calf serum

| Cells | Line | Anchorage growth in Methocel ^a | Saturation density in 10% calf serum (cells/cm ² × 10 ⁴) | Doubling time in calf serum (h) | |
|------------------------------|----------------|---|---|---------------------------------|-----|
| | | | | 1% | 10% |
| Parent lines | | | | | |
| Normal | 3T3 | 0.001 | 5 | 90 | 21 |
| Transformed | SV101 | 20 | >45 (peels) | 30 | 16 |
| Serum-sensitive revertants | A γ SV4 | 2 | 15 | >120 | 22 |
| | A γ SV5 | 11 | 10 | >120 | 21 |
| | LsSV1 | 0.001 | 9 | >120 | 22 |
| | LsSV2 | 0.04 | 12 | >120 | 22 |
| Density-sensitive revertants | F1SV101 | 0.01 | 9 | 36 | 22 |
| | BuSV2 | 0.02 | 13 | 50 | 25 |
| | BuSV3 | 0.05 | 15 | 45 | 24 |
| | ColSV1 | NT | 15 | 85 | 22 |
| | ColSV2 | 0.10 | 11 | >100 | 21 |
| | ColSV3 | NT | 15 | NT | 22 |
| | ColSV4 | 0.07 | 14 | >100 | 24 |

^a Visible spherical colonies per 100 cells cultured in Methocel for 21 days. NT, Not tested.

dine (BUdR) to kill dividing cells (26). The density-sensitive revertant F1SV101 was isolated by plating SV101 cells in 10% calf serum at high cell density and adding FUdR (15). Colchicine revertants were isolated in a fashion similar to F1SV101, but with colchicine as the toxic agent (27).

Transformation by SV40. Cells were infected with 0.2 ml of an SV40 lysate for 2 h at 37 C. The infected cells were then trypsinized, diluted serially, and plated in medium containing 1 or 10% calf serum. Serum transformants were scored as isolated colonies in 1% calf serum. Density transformants were scored in 10% calf serum as dense colonies on a background monolayer of cells. Serum-transformation assays were fixed in formalin-PBS after 17 days, and density-transformation assays were fixed after 12 days. Fixed plates were stained with Harris hematoxylin and scored with a dissecting microscope.

Transformation by MSV. Kirsten sarcoma virus in a Rauscher murine leukemia virus pseudotype (Ki-SV [R-MLV]) was the gift of B. Ozanne, Cold Spring Harbor Laboratory.

Cells (10⁶) were plated in 60-mm dishes, and 25 μ g of DEAE-dextran (molecular weight 5 × 10⁵) (ref. 5) was added per ml 24 h later. After 1 h, the cells were washed twice in serum-free medium and infected with 0.2 ml of (Ki-SV [R-MLV]). The virus titer was approximately 5 × 10⁴ FFU per ml, as assayed on BALB/3T3 cells. After 1 h, fresh medium was added. Since (Ki-SV [R-MLV]) infection of revertants led to extensive cell death, particularly at confluence, it was not possible to score directly the fraction of cells forming dense foci on the monolayer. Instead, 3 days after (Ki-SV [R-MLV]) infection, cells were trypsinized, serially diluted, and plated in 10% calf serum. Transformed colonies, in which the cells appeared round and spindly, were picked and characterized further.

MSV and MLV assays. (Ki-SV [R-MLV]) was

assayed on Swiss or BALB/3T3 cells. Transformed foci of rounded cells were scored 10 days after infection. MLV was assayed by the XC plaque assay (20).

Chromosomes. The number of chromosomes per cell was determined as previously described (16).

RESULTS

SV40 infection. Both types of revertants were infected with SV40 and plated to determine the fraction of colonies which were density transformed (Table 2) or serum transformed (Table 3). The SV40 stock used had a low plaque-forming titer and a high transforming titer (Tables 2 and 3). None of the revertants were retransformable by SV40 in either assay.

As expected (R. Risser, D. Rifkin, and R. Pollack, Cold Spring Harbor Symp. Quant. Biol., in press) serum transformation of 3T3 by SV40 occurred more frequently than density transformation. SV40-infected 3T3 cells showed a density-transformation frequency of approximately 2% (Table 2) and a serum-transformation frequency of 90% (Table 3).

MSV infection. Although all the revertants were refractory to SV40, MSV was able to transform them. Both 3T3 and the various revertants were infected with 10⁴ FFU of (Ki-SV [R-MLV]). Two days postinfection, the frequency of round, spindly cells was approximately the same on both the infected 3T3 and infected revertant plates. Subcloning of the infected plates resulted in the appearance of typical MSV-transformed colonies on only the infected revertant plates. The round, spindly cells present on the MSV-infected 3T3 plates

TABLE 2. Density transformation of mouse cell lines by SV40

| Cell line | | MOI ^a | PE (%) ^b | Density-transformation frequency ^c |
|--------------------|----------|------------------|---------------------|---|
| 3T3 | Control | 0 | 38 | <0.0005 |
| | Infected | 0.75 | 25 | 2.5 |
| LsSV1 | Control | 0 | 44 | <0.0005 |
| | Infected | 0.17 | 50 | <0.0005 |
| A ₇ SV5 | Control | 0 | 19 | <0.0005 |
| | Infected | 0.17 | 22 | <0.0005 |
| ColSV4 | Control | 0 | 21 | <0.0005 |
| | Infected | 0.30 | 25 | <0.0005 |
| F1SV ^d | Control | 0 | NT | <0.01 |
| | Infected | 40 | NT | <0.01 |

^a Multiplicity of infection; plaque-forming units per cell.

^b Plating efficiency; total colonies per 100 cells plated.

^c Dense colonies \times 100/total colonies.

^d A different stock of SV40 was used which gave a transformation frequency of 10% on 3T3.

TABLE 3. Serum transformation of mouse cell lines by SV40

| Cell line | | MOI ^a | PE (%) ^b | Serum-transformation frequency ^c |
|--------------------|----------|------------------|---------------------|---|
| 3T3 | Control | 0 | 3.8 | None |
| | Infected | 0.75 | 38 | 90 |
| LsSV1 | Control | 0 | 0 ^d | None |
| | Infected | 0.17 | 0 ^d | None |
| A ₇ SV5 | Control | 0 | 0 ^d | None |
| | Infected | 0.17 | 0 ^d | None |
| ColSV4 | Control | 0 | 0 ^d | None |
| | Infected | 0.30 | 0 ^d | None |

^a See Table 1. F1SV was not tested since it grows in 1% calf serum.

^b See Table 1.

^c $[\text{PE (infected)} - \text{PE (control)}] \times 100/\text{PE (infected)}$.

^d Only microcolonies (<20 cells per colony) appeared.

never grew into large, dense foci and consequently we were unable to isolate clones of MSV-transformed 3T3 cells. The MSV-infected revertant colonies were picked and their properties were investigated.

All morphologically transformed clones isolated from revertants after MSV infection were able to grow to high cell density in 10% calf serum (Fig. 1A-D, closed circles) and were capable of forming colonies when suspended in medium containing methylcellulose (Table 4). To our surprise, however, transformation by

MSV did not restore the ability of the serum-sensitive revertants or the colchicine-isolated density-sensitive revertant to grow in low serum (Fig. 1A,B,C, open circles). In particular, while the SV40-transformed line SV101 and its density revertant F1SV grew in 1% serum (Table 1; Figure 1D and E, open triangles), clones of MSV-infected SV101 cells and MSV-transformed F1SV cells did not grow in 1% serum (Fig. 1D and E, open circles). This phenomenon may result from the production of a toxic substance by MSV-transformed mouse cells (9). In 1% serum the MSV-transformed 3T3 cells do not remain well attached to the plates and many floating cells are seen in the medium. A similar observation has been made in MSV-infected mouse embryo fibroblasts plated in low serum concentration (9).

All the (Ki-SV [R-MLV]) infected revertants produce infectious MSV and MLV (Table 5). Media overlaying stock cultures of the MSV-transformed revertants were assayed for MSV and MLV 2 to 3 months after the initial MSV-transformed colonies were isolated. None of the uninfected cells produce any leukemia or sarcoma virus.

The reversion in growth properties of SV40-transformed cells is often accompanied by an increase in chromosome number per cell (4, 13, 16, 26, 27; A. Vogel, B. Ozanne, and R. Pollack, manuscript in preparation). All of the revertants described here have more chromosomes than the transformed parent SV101. None of the MSV-transformed revertant clones decreased in chromosome number (Fig. 2). Thus, retransformation was accomplished without any detectable major reduction in chromosome number per cell.

DISCUSSION

With regard to superinfection, both types of revertants derived from SV40-transformed 3T3 are MSV sensitive and SV40 resistant. This implies, first, that the stable equilibrium between the SV40 and cell genomes in a revertant is not established at the expense of the cell's ability to express the transformed phenotype.

Second, SV40 and (Ki-SV [R-MLV]) cannot have exactly the same effect on the same cell, even though the end result of successful infection by either virus is detected in the same selective assay as overgrowth.

The nature of the resistance of these revertants to SV40 is not known. The persistence in them of SV40 viral genes and gene products such as T-antigen makes it difficult to rule out

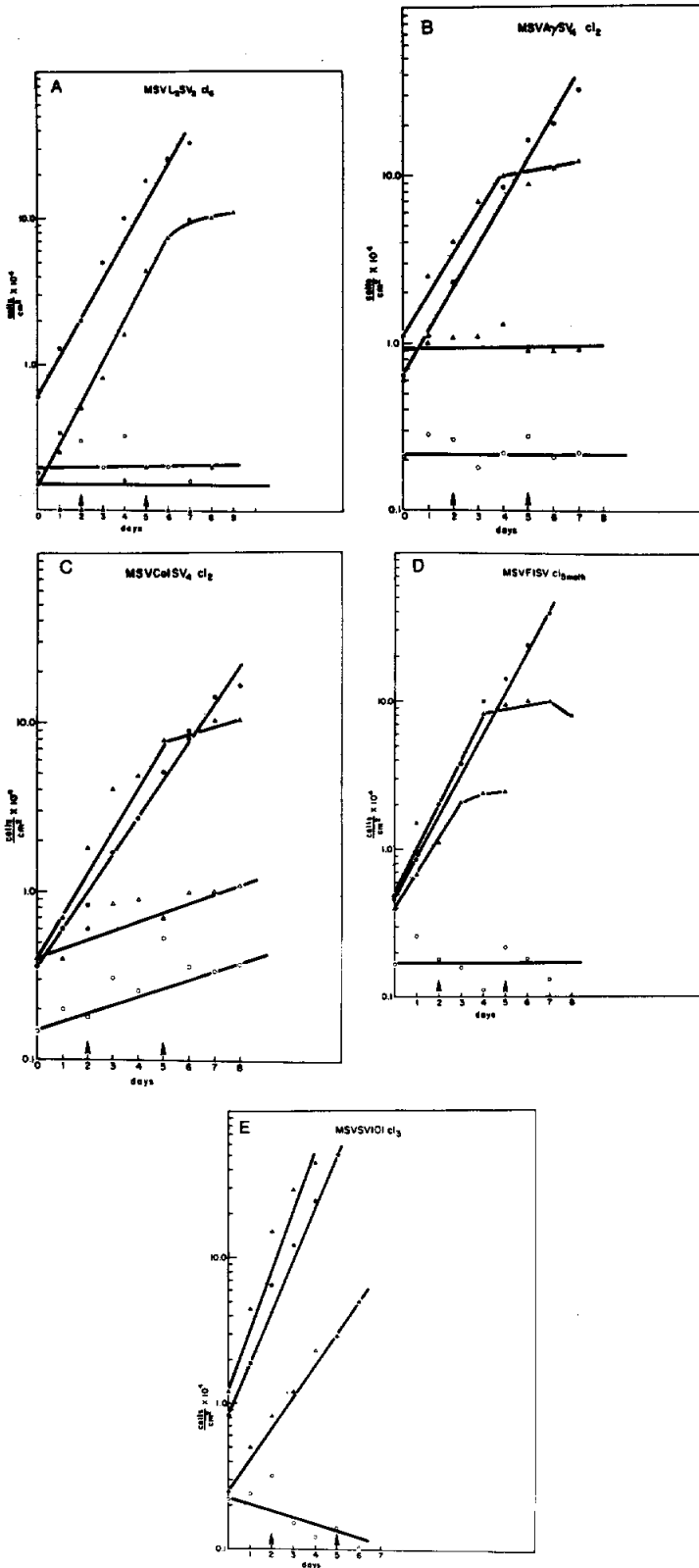


FIG. 1. Growth of revertants and MSV-transformed revertants in 1 and 10% calf serum. Uninfected revertant lines are represented by triangles. MSV-infected lines are represented by circles. Open circles or triangles represent growth in 1% serum; closed circles or triangles represent growth in 10% serum. (A) MSVL γ SV2; (B) MSVA γ SV4; (C) MSVColSV4; (D) MSVF1SV101; (E) MSVSV101.

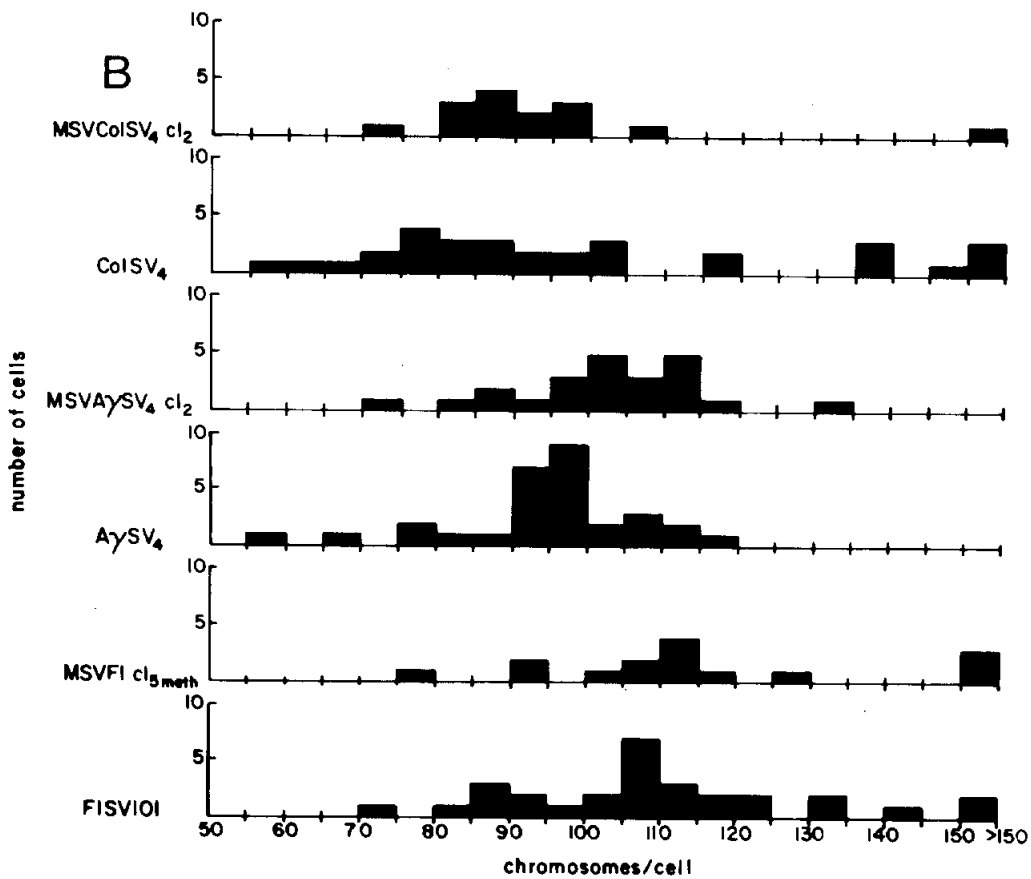
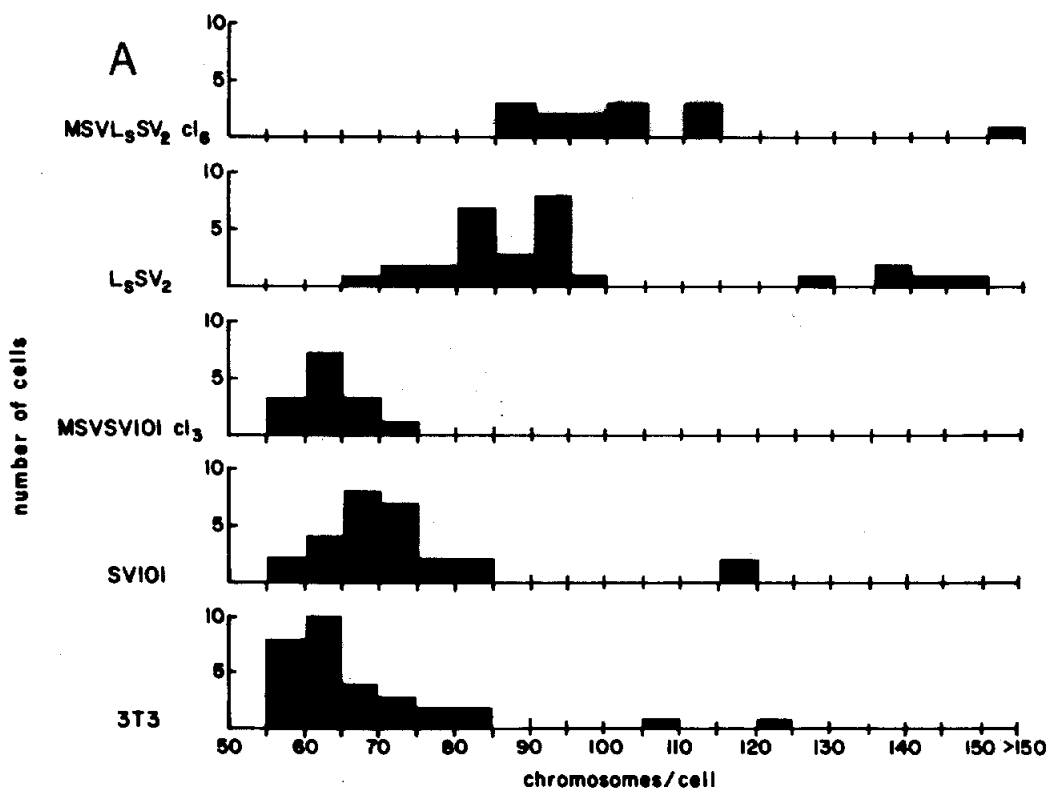


Fig. 2. (A and B) Chromosomes per cell in revertants and MSV-transformed revertants.

TABLE 4. Growth in Methocel of mouse cell lines

| Cell line | PE in Methocel ^a |
|------------------------|-----------------------------|
| F1SV101 | 0.01 |
| MSVF1SV cl 5 meth | 1.5 |
| MSVF1SV cl 8 | 1.0 |
| MSVF1SV cl 11 | 0.2 |
| LSV2 | 0.04 |
| MSVLSV2 cl 6 | 5 |
| A γ SV4 | 2 |
| MSVA γ SV4 cl 2 | 8 |
| ColSV4 | 0.07 |
| MSVcolSV4 cl 2 | 2.5 |
| SV101 | 20 |
| KA31 ^b | 20 |
| MSVSV101 cl 3 | 3 |

^a Colonies per 100 cells after 3 weeks. Only colonies visible to the naked eye (larger than 0.3 mm in diameter) were scored.

^b KA31 is an MSV-transformed BALB/3T3 nonproducer line kindly given to us by Stuart Aaronson, National Institutes of Health.

TABLE 5. MSV and MLV production by MSV infected revertant and transformed cell lines^a

| Cell line | MSV (FFU/ml) | MLV XC (plaques/ml) |
|------------------------|-------------------|---------------------|
| SV101 | 0 | 0 |
| MSV-3T3 ^b | 3×10^4 | 2×10^5 |
| MSVSV101 cl 1 | 7.5×10^3 | 1.3×10^5 |
| MSVSV101 cl 3 | 1.4×10^4 | ND |
| F1SV101 | 0 | 0 |
| MSVF1SV cl 5 meth | 1×10^4 | 5×10^5 |
| MSVF1SV cl 8 | 1×10^2 | 2.5×10^3 |
| MSVF1SV cl 11 | 2.5×10^4 | ND |
| LSV2 | 0 | 0 |
| MSVLSV2 cl 6 | 2.5×10^3 | 1.3×10^5 |
| A γ SV4 | 0 | 0 |
| MSVA γ SV4 cl 2 | 2.5×10^3 | 1.8×10^5 |
| ColSV4 | 0 | 0 |
| MSVcolSV4 cl 2 | 1.5×10^3 | 2×10^5 |

^a Cloned lines of MSV infected cells were tested 2 to 3 months after infection with MSV. Two-day supernatants were assayed. Focus formation and XC assays were done on BALB/3T3 cells. ND, Not done.

^b Two-day supernatants from (Ki-MSV [R-MLV]) infected Swiss/3T3 cells.

the possibility that superinfecting SV40 simply does not penetrate them.

Different types of revertants of MSV-transformed BALB/3T3 cells were also found to be transformable by MSV and not transformable by SV40 (B. Ozanne and A. Vogel, J. Virol., in

press). In these cells, it was possible to show that the SV40 had indeed entered the cells since SV40 T-antigen was detected in the SV40-infected MSV revertants. These experiments demonstrate that it is possible for SV40 to enter a cell, express T-antigen, and still not transform the cell.

These sets of data on the susceptibility of various types of revertants to retransformation by SV40 or MSV suggest that reversion per se may establish a cellular phenotype that permits transformation by MSV, but not by SV40. In earlier work, concanavalin A-resistant revertants of SV3T3 cells (11) and the temperature-sensitive SV40-transformed 3T3 cells infected with SV40 at the nonpermissive temperature (18, 19) were also unaffected by SV40.

MSV can render BALB/3T3 cells less susceptible to growth regulation by serum, since KA31, a Kirsten MSV-transformed BALB/3T3 nonproducer cell, grows well in 1% serum (B. Ozanne and A. Vogel, J. Virol., in press). Nevertheless, we found that after MSV infection the Swiss/3T3-derived lines F1SV and SV101 lost the ability to grow in 1% serum, and serum-sensitive revertants did not acquire this ability. This inability to grow in 1% serum may result from the production of a toxic substance by the MSV-transformed cells which inhibits cell growth in low concentrations of serum (9). Excess amounts of serum must be able to neutralize the effects of this toxin since the MSV-transformed revertants grow in 10% calf serum. The fact that MSV-transformed BALB-3T3 cells grow in 1% serum implies that these cells do not produce this toxin.

Although reversion in growth properties is accompanied by increases in chromosome number per cell (16, 26, 27), density transformation of the revertants by MSV is accomplished without affecting chromosome number. Thus, the initial increase in chromosome number by itself was not sufficient to prevent the expression of transformed growth properties by cells.

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