

Isolation and Characterization of Revertant Cell Lines

III. ISOLATION OF DENSITY-REVERTANTS OF SV40-TRANSFORMED 3T3 CELLS USING COLCHICINE

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ABSTRACT Treatment of the SV40 transformed 3T3 cell line SV101 with colchicine permits the isolation of polyploid revertant sublines which have lower saturation densities than SV101. These low saturation density lines have also reverted to a high serum requirement for growth, and are unable to form colonies in methocel. Normal SV40 has been recovered from these revertants.

3T3 cells are more resistant to colchicine than SV3T3 cells at all cell densities. Colchicine revertants do not display a 3T3-like resistance to colchicine at low density, but do survive colchicine at confluent cell densities, presumably due to their increased contact inhibition.

Normal 3T3 cells grow to low saturation densities (Todaro and Green, '63), are unable to form colonies in methocel, and have a high serum requirement for growth (Jainchill et al., '70; Dulbecco, '70; Holley et al., '68). After SV40 infection, transformed 3T3 sublines altered in some or all of these parameters of growth control can be isolated. Density transformants continue to grow after extensive cell to cell contact (Todaro et al., '64), serum transformants continue to divide in medium with 1% calf serum or in serum depleted by preincubation with normal cells (Smith et al., '71), and anchorage transformants form colonies in methocel or agar (Stoker, '68). Clones recovered as transformed from any one of these three selective assays often exhibit the other transformed growth properties as well. For example, transformants selected as dense colonies overgrowing a 3T3 monolayer often grow in low serum and form colonies in methocel, and anchorage transformants grow to high saturation densities and have a transformed serum requirement.

On the other hand, the different criteria of transformation are not always equally expressed; SV3T3 colonies able to grow in 1% or depleted serum but not able to grow to higher densities in 10% calf serum have been described (Scher et al., '71).

Just as transformation is measured as a

multiparameter event, so also is reversion from transformation. Using negative selection, sublines can be isolated from SV40-transformed mouse lines which have altered properties, more like those of 3T3 than those of the transformed parent line (Pollack et al., '68; Ozanne et al., '70; Culp et al., '71, '72).

To date, the agents used in negative selection (e.g., FUdR, BUdR-light) have killed growing cells by interfering with DNA synthesis (Rueckert and Mueller, '60). Here we report that treatment of SV3T3 with colchicine, which kills cells in mitosis, but not cells synthesizing DNA, also permits the isolation of density revertants.

Unlike the previously described density-revertants selected with FUdR, colchicine-selected density-revertant sublines are also serum-revertant. Density revertants selected with FUdR or colchicine are both anchorage revertant.

MATERIALS AND METHODS

Stock cultures

Cell cultures were routinely grown in Dulbecco's modification of Eagle's medium, supplemented with 10% calf serum (Colorado Serum Co.) and 50 μ g/ml gentamycin (Schering). Stock cultures were grown in 28 cm² plastic Petri dishes (Falcon) and the medium changed twice a week. Colchicine (Sigma) was dissolved in

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distilled water at a concentration of 10 $\mu\text{g/ml}$ sterilized by filtration, and stored frozen.

Cell growth determinations

Cells were seeded at $0.1\text{--}0.2 \times 10^4$ cells/ cm^2 , and the number of cells per plate was determined daily thereafter in a Coulter counter after trypsinization with trypsin-EDTA. The medium was changed every three days in all growth determinations.

Colony formation assay

Cells were trypsinized, counted and diluted into dishes in order to obtain isolated colonies. The medium was changed twice weekly and large colonies appeared after 10–14 days. The cells were fixed in 10% formalin in PBS and stained with hematoxylin.

Tumor antigen assay

Cells were seeded onto cover slips and fixed in methanol-acetone (1:2). SV40 specific tumor antigen was assayed as immunofluorescence with hamster antiserum to SV40 T-antigen (Flow Laboratories) and fluorescein-conjugated rabbit anti-hamster globulin (Antibodies Incorporated).

Chromosomes

Chromosomes were prepared as described (Pollack et al., '70) and thirty well spread metaphases were counted for each determination.

Growth in methocel

Cells were grown as described by Stoker (Stoker, '68). Cells were plated at densities of 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 cells per plate. Plates were fed once a week with 4 ml of fresh methocel medium. Colonies were scored after three weeks. Only colonies visible to the naked eye (larger than 0.3 mm) were scored.

Assay of DNA content per cell

Cells were prepared for the Ios Alamos flow microfluorometry machine as previously described (Trujillo and Van Dilla, '72; Van Dilla et al., '69). 2×10^6 cells in a single cell suspension were fixed in 10% formalin overnight at 4°C, hydrolyzed with 4 N HCl, and stained with acriflavine. The stained cells were then ex-

posed to 488 m μ light from an argon laser, and the amount of fluorescence emitted by the cells was measured and analyzed in a pulse height analyzer. Approximately 10^5 cells were analyzed and a histogram of DNA content per cell was plotted. Peaks corresponding to cells in the G₁, S or G₂ phase of the cell cycle could easily be discerned. Cells in the G₂ peak contained twice the amount of DNA than cells in the G₁ peak. All the G₁ peaks from established tissue culture lines were normalized to the G₁ peak of diploid mouse embryo fibroblasts to determine the actual amount of DNA per cell. The results are expressed as C values, with 1C value of DNA being equal to a haploid amount of DNA.

Colchicine treatment

Cells were seeded at varying densities in 10% calf serum and 0.5 $\mu\text{g/ml}$ of colchicine was added 24 hours later. The cells were incubated in colchicine for two days. The medium was then removed and fresh medium was added for two hours. The attached cells were then trypsinized and plated out to determine the number of survivors able to form a colony. The results are expressed as the ratio of colony forming survivors to colony forming cells from untreated control plates.

RESULTS

Toxic effects of colchicine on normal, transformed and density revertant cells

The action of colchicine is limited to inhibition of mitosis and does not affect any other phase of the cell cycle (Puck, '64). Normal 3T3 cells, transformed SV3T3 cells and the density revertant F1SV101 cells were treated with colchicine (see METHODS) at different cell densities, ranging from sparse to super-confluence (fig. 1). The response to colchicine of the three lines was complex. As expected, at high density, non-dividing 3T3 cultures survived colchicine treatment (fig. 1). However, even sparse 3T3 cultures, in which cells were not in contact with each other, survived colchicine treatment (fig. 1). Colchicine was toxic to SV101 cells at all cell densities, presumably because SV101 cells continue to divide at all cell densities.

Only the FUDR-selected density-revertant subline F1SV101 showed a density-

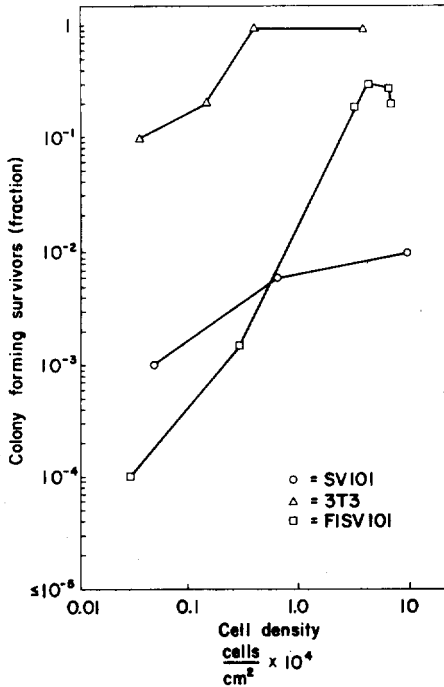


Fig. 1 Colony-forming survivors following treatment with colchicine at different cell densities. Δ , 3T3; \circ , SV101; \square , F1SV101.

dependent response to colchicine poisoning. In sparse culture, F1SV101 was killed by colchicine but high cell density protected these cells from colchicine (fig. 1). It is likely that these revertants were resistant to the toxic effects of colchicine because they ceased cell division at confluence (Pollack and Vogel, '73).

The resistance of sparse 3T3 to colchicine was unexpected, since FUDR, an agent toxic only to cells that synthesize DNA, killed sparse 3T3 as efficiently as it killed sparse SV101 or sparse F1SV101 (fig. 2). Presumably, 3T3 cells had the ability to cease traversing the cell cycle once they were exposed to colchicine, before irreversibly faulty mitoses could occur. Recently the drug Cytochalasin B has been shown to have a similar differential toxicity for normal and transformed cells (Kelly and Sambrook, '73; Defendi and Stoker, '73).

Whatever the mechanism of the differential toxicity of colchicine to 3T3 and SV3T3, it is clear in the case of F1SV101, that an increase in cell density can lead

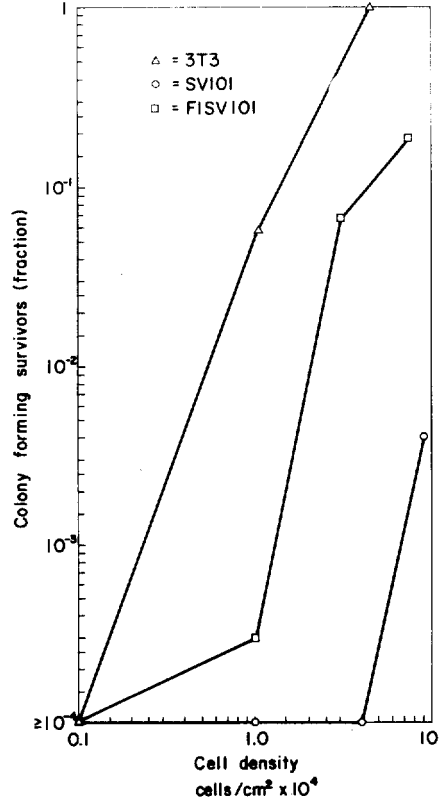


Fig. 2 Colony-forming survivors following FUDR treatment at different cell densities. Cells were treated with 25 $\mu\text{g/ml}$ FUDR and 250 $\mu\text{g/ml}$ uridine for 2 days, and then plated in the absence of FUDR to determine the fraction of cells capable of forming a colony. Δ , 3T3; \circ , SV101; \square , F1SV101.

to an increase in survival after colchicine treatment (fig. 1). Here it is likely that density-dependent inhibition of cell division (Pollack and Vogel, '73) is responsible for the enhanced survival at high density of F1SV101 treated either with colchicine (fig. 1) or with FUDR (fig. 2). These results suggest that treatment of dense cultures of SV101 with colchicine might permit the isolation of variants with increased density-dependent inhibition of cell division among the survivors.

Selection of revertant cells with colchicine

SV101 cells were seeded at a density of 4×10^4 cells/cm² and treated with colchicine as described in METHODS. Surviving colonies were trypsinized, pooled, replated

TABLE 1
Reversion of transformed state

| Line | Anchorage Growth in methocel ¹ | Density S. D. in 10% calf serum Cells/cm ² × 10 ⁴ | Serum | |
|---------|---|---|-------------------------------------|-----|
| | | | Doubling time in calf serum (hr) | |
| | | | 1% | 10% |
| 3T3 | 0.001 | 5 | 90 | 21 |
| SV101 | 20 | >45 | 30 | 16 |
| F1SV101 | 0.01 | 9 | 36 | 22 |
| Col SV1 | nt ² | 15 | 85 | 22 |
| Col SV2 | 0.10 | 11 | >100 | 21 |
| Col SV3 | nt ² | 15 | nt | 22 |
| Col SV4 | 0.07 | 14 | >100 | 24 |

¹ Visible spherical colonies per 100 cells cultured in methocel for 21 days.

² nt, not tested.

and allowed to grow to a density of 4×10^4 cells/cm², and the cells were then put through a second cycle of colchicine treatment. Most colonies surviving the second selection had a "flat" morphology (Pollack, '68). Four colonies, (ColSV1, ColSV2, ColSV3 and ColSV4) were cloned, grown in the absence of colchicine and further characterized.

Growth control of the revertants

Colchicine selected revertant lines were found to be reverted in their density and anchorage growth properties (table 1). With regard to density, all the colchicine revertants grew to lower saturation densities in 10% calf serum than did SV101.

With regard to anchorage, neither the colchicine revertants nor F1SV101 formed colonies in methocel (table 1). SV101 cells cannot form colonies unless they are provided with a solid substrate. Thus these density revertants have also reverted to an increased anchorage requirement for growth.

Density-dependence of colchicine resistance

Two lines surviving colchicine (ColSV2 and ColSV4) were tested at varying cell densities for their ability to survive a third cycle of colchicine treatment (fig. 3). The two colchicine-selected variants were killed by colchicine at low density, but were resistant at high density, a response similar to that of F1SV101 (fig. 1). The colchicine-selected variants did not revert to 3T3-like intrinsic resistance to colchicine, but were resistant to colchicine only

at high cell density (fig. 3). Presumably, the survival of ColSV2 and ColSV4, as well as their decreased saturation density, were consequences of their increased density-dependent growth control.

Negative selection with colchicine and negative selection with FUDR yield variant cell lines with lower saturation densities. This suggests that killing of cells in mitosis, as well as killing of those in S-period, can be used to recover density-revertants.

Serum requirement for growth of the revertants

The ability to grow in low concentrations of serum (Dulbecco, '70), in serum depleted of gamma globulin (Jainchill et al., '70), or in serum depleted by preincubation with normal 3T3 cells (Smith et al., '71; Holley et al., '68) is a characteristic of SV40-transformed 3T3 cells.

Whereas in 10% calf serum, both 3T3 and SV3T3 had approximately the same doubling time (table 1), in 1% calf serum only SV101 grew well (table 1). 3T3 cells in 1% serum grew very poorly, with a doubling time of 85–100 hours (table 1, fig. 4). Density revertants selected with FUDR (Pollack et al., '68) or BUdR (Vogel, unpublished) grew as well in 1% calf serum as SV101, albeit to a lower saturation density (fig. 4).

Surprisingly, the colchicine-selected revertants displayed a reduced ability to grow in 1% calf serum similar to that of 3T3 (table 1, fig. 4). Thus, although colchicine-selection was carried out in such a way as to select directly only for cells

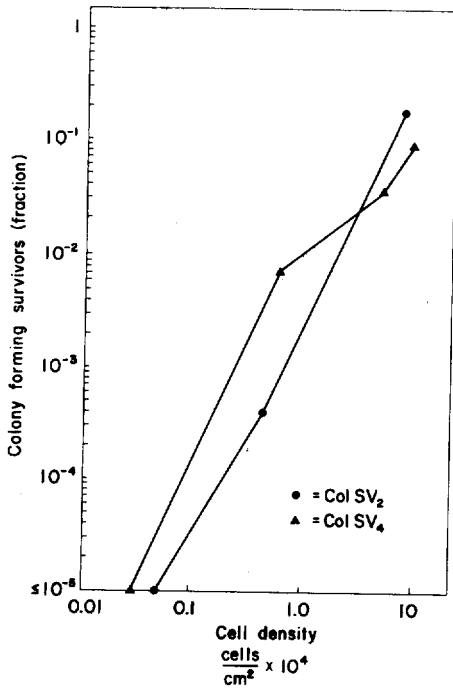


Fig. 3 Colony-forming survivors among colchicine-revertants after third cycle of colchicine treatment at different cell densities. (●), ColSV₂; (▲), ColSV₄.

unable to divide at high cell densities, the cell lines recovered had also acquired a higher serum requirement for growth even in sparse culture.

With colchicine, but not with FUdR or BUdR, it has been possible to select variants of SV3T3 which have reverted in three aspects of transformation. ColSV₂ and ColSV₄ have a low saturation density, a high anchorage dependence, and the high serum requirement of 3T3.

Viral properties of the colchicine-selected revertants

The isolation of variants from SV101 which have regained the serum-, anchorage-, and density-properties of an untransformed cell allows one to ask if any of these parameters of transformation are the consequence of the continued expression of any viral gene. Colchicine revertants contain SV40-specific T-antigen and SV40-specific RNA (table 2), and yield infectious virus upon fusion with permissive monkey cells (table 2).

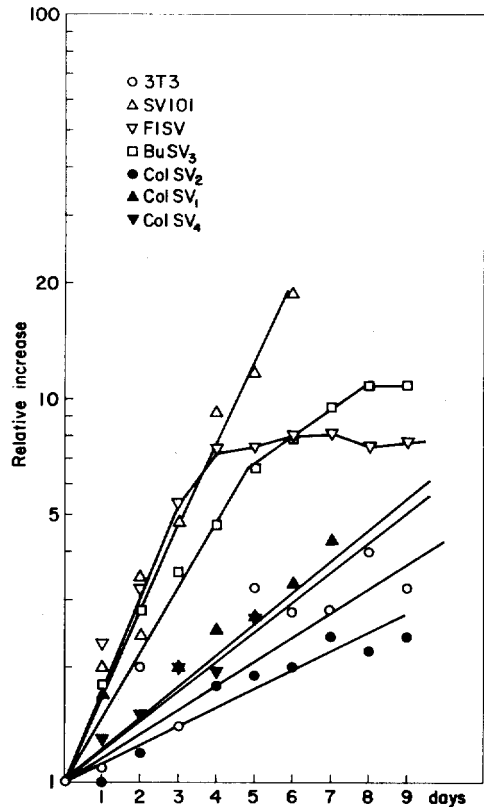


Fig. 4 Growth in 1% calf serum. Cells were seeded at $0.1-0.2 \text{ cells/cm}^2 \times 10^4$, and samples were counted daily. ○, 3T3; △, SV101; ▽, FISV101; □, BuSV3; ●, ColSV₂; ▲, ColSV₁; ▼, ColSV₄. BuSV3 is a density-revertant of SV101 selected with BUdR.

Revertants and SV101 parent cells differ only slightly in the transcription patterns of SV40-specific RNA. Revertants selected with colchicine contain the same percentage of RNA from the SV40 DNA strand involved in early functions in the lytic cycle as do SV101 cells, but more RNA complementary to the late strand (table 2).

SV40 virus rescued from ColSV₂, ColSV₃ and ColSV₄ is identical to wild type SV40 in its ability to transform 3T3 (table 2). Neither the FUdR nor the colchicine revertants are re-transformable by SV40, but both can be morphologically transformed by murine sarcoma virus (table 2), confirming and extending the results of Renger (Renger, '72).

SV101 and all its revertant sublines have enough SV40-specific DNA to ac-

TABLE 2

Retention of virus in mouse cell lines

| Line | SV40 T-antigen | SV40 virus rescued | | Transcription of SV40 RNA in mouse cells ³ | | Retransformable by: | |
|---------|-------------------|---|----------------------|---|------------------|------------------------|-------------------------------|
| | | PFU/10 ⁶ mouse cells ¹ | PFU/FFU ² | % Early strand | % Late strand | SV40 | MSV (Kirsten) ⁴ |
| 3T3 | - | 0 | 0 | 0 | 0 | + | + |
| SV101 | + | 2000 | 1600 | 81 | 4 | - | + |
| F1SV101 | + | 0 | — | 40 ⁵ | 5 ⁵ | - | + |
| Col SV1 | + | nt | — | 61 | 25 | nt ⁶ | nt ⁶ |
| Col SV2 | + | 700 | 1000 | nt | nt | - | nt ⁶ |
| Col SV3 | + | 100 | 1100 | 80 | 30 | nt ⁶ | nt ⁶ |
| Col SV4 | + | 20,000 | 760 | 69 | 22 | nt ⁶ | + |

¹ Virus recovered 4–6 days after sendai-mediated fusion to permissive BSC-1 monkey cells (Pollack and Vogel, in preparation).

² Ratio of plaques formed on BSC-1 cells to density-transformed foci formed on 3T3 cells.

³ Data taken from Ozanne et al. ('73).

⁴ Gift of B. Ozanne.

⁵ Non-saturating value. It has not been possible in these cases to saturate SV40 DNA with cell RNA.

⁶ nt, not tested.

count for more than one full SV40 genome per cell (B. Ozanne, personal communication). Therefore, the recovery of normal SV40 from a number of revertants does not prove the simple hypothesis that reversion in each case occurred solely by cellular mutation. The alternative, that each revertant carried a non-recoverable, mutated SV40 as well as a recoverable, nonmutant SV40, cannot yet be excluded. However, the data as far render unlikely the maximum hypothetical role of SV40 in the maintenance of the transformed state, that is, that all copies of one or more viral genes must be inactive in order for the cell to be reverted.

Chromosomes and DNA

All colchicine-selected revertant lines contained more chromosomes than SV101. Modal values varied from 80 chromosomes per cell for ColSV3 to 90 for ColSV1. In collaboration with S. Cram and H. Crissman at the Health Research Laboratories of the Los Alamos Scientific Laboratories, we have found that the DNA content per cell of the revertants is also increased (fig. 5).

Agents such as colchicine which disrupt spindle fibers can cause cells to become tetraploid or octaploid (Harris, '71), and many other revertants contain more chromosomes than their transformed parent (Pollack et al., '70; Hitotsumachi

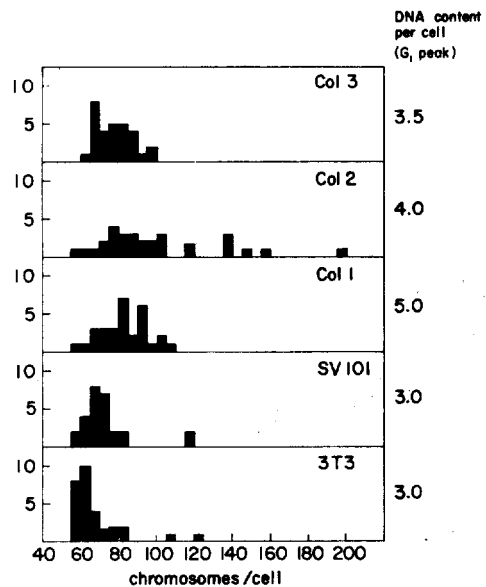


Fig. 5 Chromosome distribution and DNA content per cell of different cell lines.

et al., '71; Wyke, '71; Culp, '71, '72; Ozanne, '73). The mechanism and reason for this increase in DNA in revertants remain obscure. In colchicine selection of revertants, polyploidation may have been induced (Harris, '71). If this were so, a great deal of chromosome loss must have taken place during the growth of these surviving colonies, since the revertants

contain much less than twice the DNA and chromosomes per cell of the SV101 parent.

DISCUSSION

FUDR and BUdR are toxic to cells synthesizing DNA (Rueckert and Mueller, '60), while colchicine affects cells in mitosis (Puck, '64). Drugs toxic to cells in different parts of the growth cycle can, therefore, be used to isolate revertants with decreased saturation densities from transformed populations. At confluence, the FUDR-selected revertant behaves like 3T3 in that it ceases to make DNA (Pollack and Vogel, '73). The nature of the mechanism by which the colchicine-selected revertants maintain a low saturation density is not yet known. They may be unable to make DNA at confluence. Alternatively, because the selection was directed against cells entering mitosis, these revertants may be blocked just prior to mitosis, in G₂. A third possibility is that at confluence these variants may be shed into the medium (Scher and Nelson-Rees, '71).

Transformed cells can be distinguished from normal cells by differences in saturation density, serum requirement for growth, and anchorage requirement for growth. The data reported above suggest that selection for reversion in one property is accompanied by at least partial reversion in the other transformed properties. For example, density revertants isolated with FUDR, BUdR or colchicine do not grow as well as SV101 in methocel. Colchicine-selected revertants, but not FUDR- or BUdR-selected revertants, are also unable to grow as well as SV101 in 1% calf serum and thus have reverted in their serum requirement as well.

It is not clear whether the colchicine acted as a selecting or as an inducing agent. Density-revertants with more chromosomes exist in a transformed population before FUDR selection (Pollack, '70). Colchicine, by killing cells capable of growth in dense culture, may also have acted simply as a selective agent to allow the recovery of a pre-existing revertant. However, drugs that block microtubule polymerization can induce tetraploidy by interfering with cytokinesis (Harris, '71), and colchicine may have simply induced

hyperploidy. If so, and if hyperplod cells have increased density-dependent inhibition of growth, then such cells would have been recovered in this selection.

The increased chromosome number of the colchicine-selected revertants cannot explain their high serum requirement, because the FUDR and BUdR-selected density revertants have more chromosomes and DNA than SV101, yet maintain a transformed cell's low serum requirement.

The specific resistance of 3T3 to colchicine is quite surprising. After two days in the drug, the 3T3 cells are highly extended, and their morphology resembles that of an epithelial cell. Many cells contain two nuclei. Similar findings have been observed in BHK hamster fibroblasts (Goldman, '71). Although the 3T3 cells are morphologically affected by the colchicine, they are able to grow into colonies upon removal of the drug. 3T3 cells also survive treatment with cytochalasin B (Kelly, '73). Colchicine treatment leads to the depolymerization of microfilaments (Goldman, '73). Apparently, 3T3 cells are uniquely capable of surviving in the presence of drugs which reversibly effect structures responsible for the maintenance of cell morphology.

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