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Isolation and Characterization of Revertant Cell Lines

IV. DIRECT SELECTION OF SERUM-REVERTANT SUBLINES OF SV40-TRANSFORMED 3T3 MOUSE CELLS

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ABSTRACT SV101, the SV40-transformed subline of the mouse fibroblast line 3T3, is both serum- and density transformed, since it grows in both 1% and 10% calf serum, and grows beyond confluence in 10% calf serum. Negative selection at low cell density in 1% calf serum or in 10% agamma-depleted serum permits direct recovery of serum-revertant sublines of SV101. These sublines are unable to grow in 1% calf serum.

Although negative selection at high cell density in 10% calf serum is known to permit recovery of density-revertant sublines of SV101, most density-revertants are not serum-revertant. However, all serum-revertants isolated so far are density-revertant as well.

Normal 3T3 mouse cells have at least two modes of growth control: a high serum requirement for growth (Dulbecco, '70; Holley, '68) and the reversible cessation of growth in high serum upon the formation of a confluent monolayer (Todaro et al., '63). Transformation of 3T3 by SV40 leads to the loss of only the high serum-requirement (Scher, '71) or of both the density-inhibition and the serum-requirement (Dulbecco, '70; Jainchill et al., '70). A small minority of double-transformed SV3T3 cells spontaneously revert (Pollack et al., '68; Pollack, '70) to a 3T3-like regulation by density (Pollack and Vogel, '73). These density-revertants have been recovered by negative selection of SV3T3 in dense culture with FUdr (Pollack et al., '68), BUdr or colchicine (Vogel et al., '73). Density-revertants have also been recovered from transformed populations after selection for sublines which are resistant to concanavalin A (Ozanne, '70; Culp and Black, '72), and for sublines unable to grow in methocel (Wyke, '71).

In most cases, these density revertants have not reverted to a high serum requirement for growth, but rather have retained the ability to grow in low serum that is characteristic of transformed cells. Here we show that negative selection in sparse culture and low serum may be used to directly recover sublines of SV3T3 that have a serum requirement as great or greater than that of 3T3.

All the serum-revertants isolated are also reverted in their density property. That is, all sublines recovered that are unable to grow in low serum are also unable to grow dense in high serum.

MATERIALS AND METHODS

Cells and media

All cell lines were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Company) and 50 μg/ml Gentamycin (Schering). Stock cultures were carried in 28.3 cm^2 Falcon tissue culture plates, with medium changes twice a week. All revertant cell lines were derived from SV101, a clone of SV40-transformed Swiss 3T3 cells (Pollack et al., '68).

Preparation of agamma-depleted calf serum

Newborn agamma calf serum was purchased from North American Biological Incorporated and depleted by incubation with Balb-3T3 cells (Smith et al., '71). Balb 3T3 cells were grown in 100 mm dishes with 10% calf serum until they formed confluent monolayers. The medium was then removed and replaced with 10 ml of Dulbecco's modified Eagle's medium

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containing 20% agamma calf serum. After four days, the depleted medium was removed, centrifuged for 20 minutes at 5,000 rpm at 4°C, filtered through an 0.22 μ Millipore filter, diluted with an equal volume of serum free medium and frozen. Monolayers of 3T3-Swiss (Todaro, '63), the parent of SV101, would not deplete the agamma serum.

Cell growth

Cells were seeded at 0.1–0.2 $\times 10^4$ cells/cm^2 and the number of cells per dish was determined daily with a Coulter counter after trypsinization. The medium was changed every three days during the course of each experiment.

Efficiency of plating

Cells were trypsinized, diluted and plated to give isolated colonies. The medium was changed twice weekly. After 10–14 days the colonies were fixed in formalin-phosphate buffered saline (1:10), and stained with Harris hematoxylin.

Colony formation on 3T3 monolayers

Normal 3T3 cells were grown in medium containing 10% calf serum until they reached confluence. Medium with the appropriate serum concentration was then added and the plates incubated for two days. The medium was then removed and 200 test cells added in fresh medium with the appropriate amount of calf serum. The colonies were allowed to grow for 14 days, with medium changes every three days. Plates were then fixed and stained and the number of dense colonies counted.

Plating in methocel

One hundred to 10^5 cells were suspended per plate of methocel-medium containing 10% calf serum (Vogel et al., '73). Cells in methocel-medium were incubated in dishes over a thin layer of hard agar (Stoker, '68). The plates were fed once a week with 4 ml of fresh methocel-medium, and the colony-forming efficiency determined after three weeks. Only colonies large enough to see with the naked eye (0.3 mm in diameter) were counted.

Chromosomes

Chromosome preparations were made as described previously (Pollack et al., '70). At least 30 well spread metaphases were counted for each cell line.

Determination of DNA content per cell

DNA content was measured on the Los Alamos flow microfluorometry machine as previously described (Vogel et al., '73; Trujillo et al., '72; Van Dilla et al., '69). DNA content is expressed as C values, with 1 C value equal to a haploid amount of DNA.

RESULTS

Serum requirement of normal and SV40-transformed 3T3 cells

SV3T3 cells must grow well and 3T3 cells must grow poorly or not at all in sera that are to be useful in negative selection of serum-revertants. The growth properties of SV101, 3T3 and the density-revertant F1SV101 in 10% calf serum, 1% calf serum and 10% agamma-depleted calf serum are shown in table 1. SV101 grows well in all three sera, but 3T3 grows well only in 10% calf serum, doubling only every four to five days in either of the restrictive sera. The density revertant F1SV101, grows well in agamma-depleted medium (Jainchill et al., '70) and in 1% calf serum (table 1).

Both restrictive sera were able to support SV101 growth better than 3T3 growth, and so we had reason to expect that serum-requiring variants of SV101 could be isolated by negative selection.

Isolation of serum-requiring sublines of SV101

SV101 cells were seeded in medium with 10% calf serum at a density of 10^4 cells/cm^2 (fig. 1). This sparse initial density was chosen in order to avoid selecting cells which survived because of an increased susceptibility to contact inhibition of growth. One day after plating, the medium was changed to one containing a restrictive serum. After 24 hours in the restrictive medium alone, the cells were exposed to 200 μg/ml of BUdr for 96 hours. The restrictive medium was then removed and the cells exposed to a two second pulse of uv light from a General Electric 30 watt germicidal lamp at a distance of 18 inches. The uv light lowered the plating efficiency on non-BUdr treat-

TABLE 1

Mouse cell lines: Differential ability to grow in various sera

Line	Serum		Doubling time	Saturation density (cell/cm² × 10⁴)	Plating efficiency
	Type	Per cent			
			hr		%
3T3	calf	10	21	5	38
	calf	1	84	0.9	3.8
	depleted newborn Aγ calf	10	90	0.4	nt [1]
SV101	calf	10	16	>45	33
	calf	1	33	10	4.5
	depleted newborn Aγ calf	10	22	41	nt
FLSV101	calf	10	22	8	83
	calf	1	36	1.5	18
	depleted newborn Aγ calf	10	28	7	nt [1]

[1] nt, not tested.

ed cells by 50%, but lowered the plating efficiency of the BUdr treated cells by 10^{-4} (Kao et al., '68). Fresh non-restrictive medium with 10% calf serum, 10^{-5} M thymidine, and 8×10^{-5} M uridine (to prevent incorporation of any residual BUdr or Bu), was then added, and cells that had survived the uv pulse were permitted to grow out as colonies. The medium was changed daily for three days to remove dead cells (fig. 1).

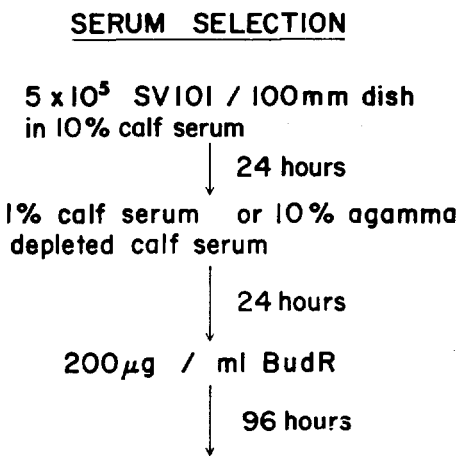

Fig. 1 Selection procedure for the isolation of serum revertants.

Isolated colonies appeared two to three weeks after removal of the BUdr and replacement with 10% calf serum. These colonies were picked and tested for their ability to grow in medium containing the restrictive serum (either 1% calf serum or 10% agamma-depleted calf serum). Clones demonstrating a decreased ability to grow in the restrictive serum as compared to SV101 were kept for further study. Clones isolated in agamma-depleted calf serum were designated AγSV, and clones isolated in 1% calf serum were designated LsSV. Approximately 1 SV101 cell in 10^5 gave rise to a serum-revertant colony.

Growth properties in 1% calf serum

3T3 cells grow slowly in 1% calf serum (table 1). Selection for serum-dependent SV101 sublines yielded revertants more serum-dependent than 3T3, since all serum revertants selected in agamma-depleted or 1% calf serum were absolutely unable to grow in 1% calf serum (fig. 2). In contrast selection for density-revertants usually yields cells able to grow in 1% calf serum (fig. 3). Because the preparations of agamma-depleted calf serum varied in their ability to support the growth of revertants, 1% calf serum was used to test all presumptive serum revertants.

To test if the serum revertants remained viable in 1% calf serum, they were plated in 10% and 1% calf serum, incubated for six days, and then trans-

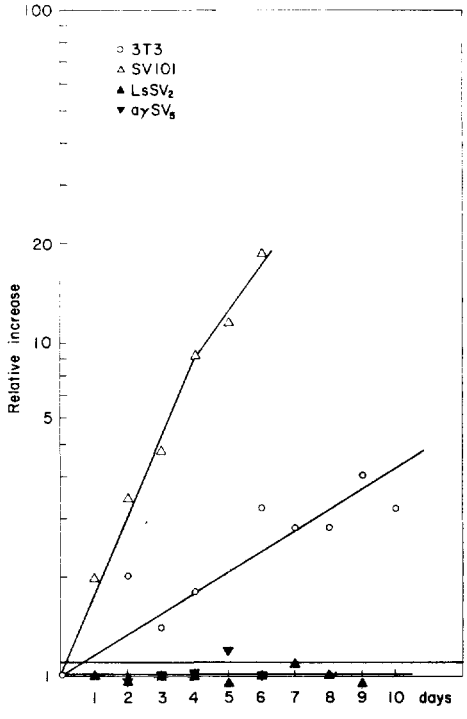

Fig. 2 Growth in 1% calf serum. Cells were seeded at 0.1–0.2 cells/cm² × 10⁴. (○), 3T3; (△), SV101; (▲), LsSV2; (▼), AγSV5.

ferred into 10% calf serum to test their ability to form a colony. No consistent decrease in plating efficiency was detected after six days in 1% calf serum (table 2). We therefore conclude that these revertants, although unable to divide (fig. 2), remain viable in the restrictive serum.

Density-dependent growth control of serum-revertants

Since serum-selection was carried out in sparse culture, no alteration in the degree of contact- or density-dependent growth control was expected in the surviving clones. Surprisingly, though, all the serum revertants ceased to grow at low cell densities in 10% calf serum. Their saturation densities in 10% calf serum were in the range typical for density-revertant clones (table 3).

In order to further characterize this unexpected difference between SV101 and its serum-revertants, we measured their saturation densities in different concentrations of serum (fig. 4).

The saturation density of every line tested was dependent upon serum concentration (fig. 4). Both density revertants grew to measurable plateaus in all sera tested, and the slopes of maximum-density versus serum concentration of 3T3, SV101 and the density-revertants were approximately the same (fig. 4a). Differences in density-dependent growth control among these lines are evident from figure 4a, since SV101 grew to a higher saturation density than 3T3 or the density-revertants, at all serum concentrations. The sharper rise in saturation density with increasing serum shown by the serum revertants distinguishes them from 3T3, SV101 and from the density revertants (fig. 4b). The mechanisms behind this difference in serum-response are unknown, but these data suggest that there are at least two different states of serum responsiveness which can result in a low saturation density in 10% serum. These data are consistent with the hypothesis (Holley et al., '68; Unkeless et al., '73) that alterations in such aspects of growth control as density-dependent cessation of growth are the consequence of alterations in cellular serum requirements.

Relative plating efficiency on 3T3 monolayers

Another assay for density dependent growth control is the formation of dense colonies on monolayers of 3T3. Most transformed cells can form dense colonies on monolayers (Pollack et al., '68). SV101 cells can form dense areas on monolayers at all serum concentrations tested (table 4). The serum revertants show a reduced ability to form dense colonies on monolayers in serum concentrations which support their growth on plastic. We therefore conclude that the ability of SV101 to form dense colonies on monolayers is not serum dependent since dense areas arise in serum concentrations which barely support growth on plastic (0.5%). The serum cells are reverted in this dense-colony forming ability. These data do not rule out the existence of cell-serum interactions (Lipton et al., '71; Unkeless et al., '73) which may be involved in growth on cell monolayers.

Anchorage requirement for growth

LsSV and AγSV clones are reverted in

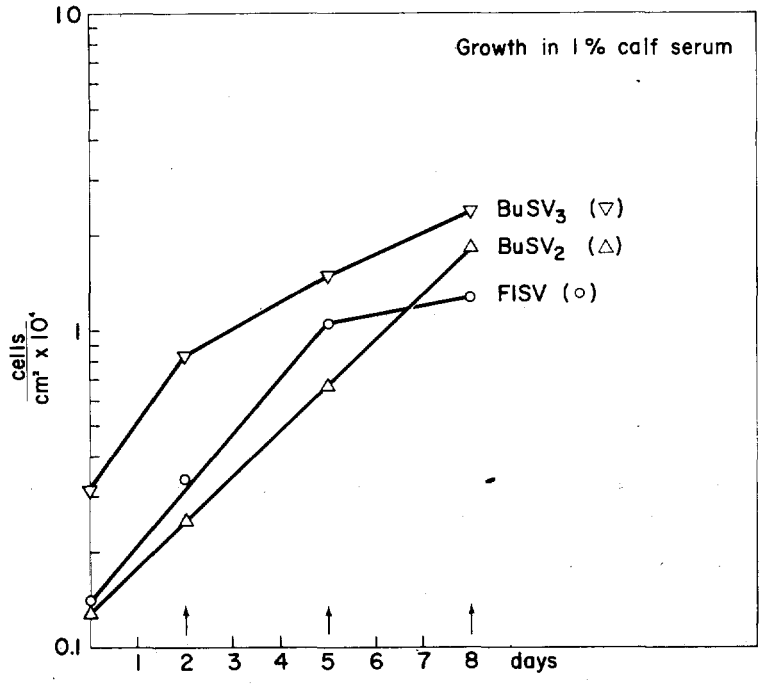

Fig. 3 Growth in 1% calf serum. (○), F1SV101; (△), BuSV2; (▽), BuSV3. BuSV2 and BuSV3 are density revertants isolated with BUdr as the selective agent.

both the serum and density parameters of transformation. A third independent parameter is the ability to grow in methocel, without attachment to a solid substrate (Stoker, '68). Normal 3T3 and the density-revertant F1SV101 will not form colonies in methocel, while SV101 grow well in methocel (table 3). The serum-revertants show different responses to methocel.

The LS revertants selected for their inability to grow in 1% calf serum cannot form colonies in methocel, while the Aγ revertants selected for their inability to grow in Aγ-depleted medium can form colonies in methocel (table 3).

TABLE 2

Line	E.O.P. [1] in 10% calf serum after six days in:	
	10% serum	1% serum
3T3	90	42
SV101	33	9
LsSV1	21	7
aγSV5	4	2
F1SV101	27	29

[1] Efficiency of plating: colonies/100 cells plated.

Transformed cells have less cyclic AMP than 3T3 or density revertants (Bürk, '68; Otten et al., '71; Sheppard, '72). When the concentration of cyclic AMP in all our revertants was examined (Oey, manuscript in preparation), growth in methocel correlated with a low intracellular cyclic AMP concentration in sparse culture in 10% calf serum. Thus, LsSV1 and LsSV2 have high cyclic AMP concentrations, comparable to the 3T3 value, while the AγSV clones have low cyclic AMP concentrations, similar to that of SV101.

Chromosome number and DNA content in the revertants

Many density revertants isolated in various laboratories have an increased number of chromosomes per cell, as compared with their transformed parent lines (Pollack et al., '70; Culp et al., '71; Vogel et al., '73; Wyke, '71). All serum revertants were found to have more chromosomes per cell than the SV101 parent (fig. 5). In collaboration with S. Cram and H. Crissman at

TABLE 3

Growth properties of mouse cells in 10% calf serum

	Line	Saturation density (Cells/cm² × 10⁴)	Growth in methocel (colonies/100 cells plated)
Parent line	3T3	5	0.001
	SV3T3(101)	>45(peels)	20
Serum revertants	aγSV4	15	2
	aγSV5	10	11
	LsSV1	9	0.001
	LsSV2	12	0.04
Density revertants	BuSV2	13	0.02
	BuSV3	15	0.05
	F1SV101	9	0.01

the Health Research Laboratory of the Los Alamos Scientific Labs, we have also found that these revertants contain more DNA per cell than either SV101 or 3T3 (fig. 5).

Incubation in restrictive serum with BUdr does not itself induce this increase, because clones that survived the BUdR negative selection procedure but which are still able to grow in 1% calf serum and grow to high cell densities in 10% calf serum (Aγ12d2, Aγ61, Aγ256; fig. 5) do not show this increase in chromosomes or DNA.

DISCUSSION

SV40 transformants selected for the ability to form dense colonies on a monolayer of 3T3 also have a reduced serum requirement for growth (Dulbecco, '70; Jainchill et al., '70). Depleted agamma newborn calf serum does not support the growth of 3T3 cells, but allows the growth of SV3T3 cells (Smith et al., '71). When this agamma-depleted serum was used as another selective condition for the isolation of SV40 transformants, the majority of SV40 infected-3T3 colonies able to grow in agamma-depleted calf serum also grew

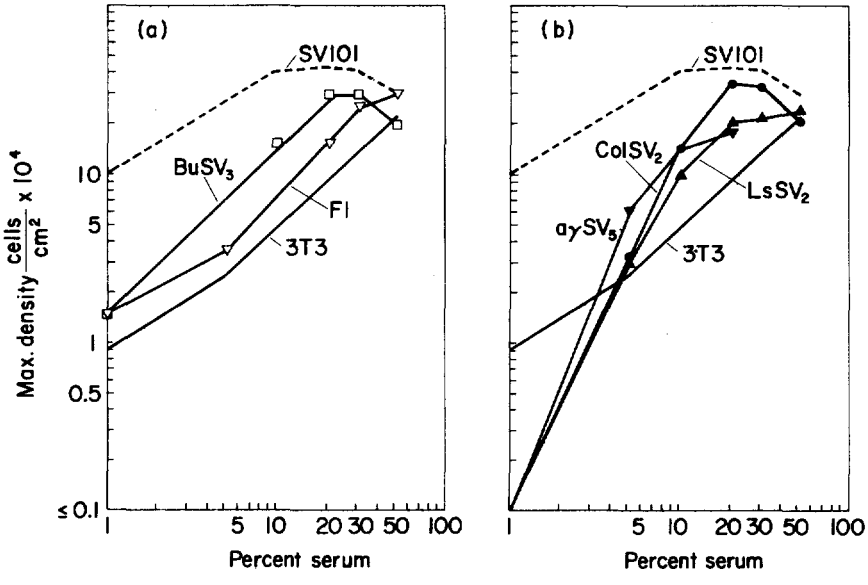

Fig. 4 Saturation density as a function of serum concentration. Cells were seeded in varying serum concentrations at a density of 0.1 × 10⁴ cells/cm² and samples were counted until growth stopped. Medium was changed every third day. ColSV_2 is density revertant isolated with colchicine (Vogel et al., '73).

TABLE 4

Ability of mouse cell lines to form colonies on 3T3 monolayers in different concentrations of serum

Line	RPE [1] in calf serum concentration				
	0.5%	1%	2%	5%	10%
3T3	nt [2]	<0.002	nt [2]	nt [2]	<0.0003
SV101	8.0	4.3	1.6	2,3	1.9
F1SV101	nt [2]	nt [2]	0.020	nt [2]	0.060
LsSV2	0 [3]	0	0	<0.003	0.10
AγSV5	0	0	0	<0.001	0.20

[1] RPE, relative plating efficiency = $\frac{\text{colonies on 3T3 monolayers}}{\text{colonies on bare Petri dish}}$.

[2] nt, not tested.

[3] 0, no colonies on bare dish or on monolayer.

in 10% calf serum to high saturation density (Scher and Nelson-Rees, '71). However, a significant fraction of these serum transformants continued to maintain a low saturation density in 10% calf serum. These flat-transformants were the first demonstration that serum-transformation could be uncoupled from density transformation (Scher and Nelson-Rees, '71).

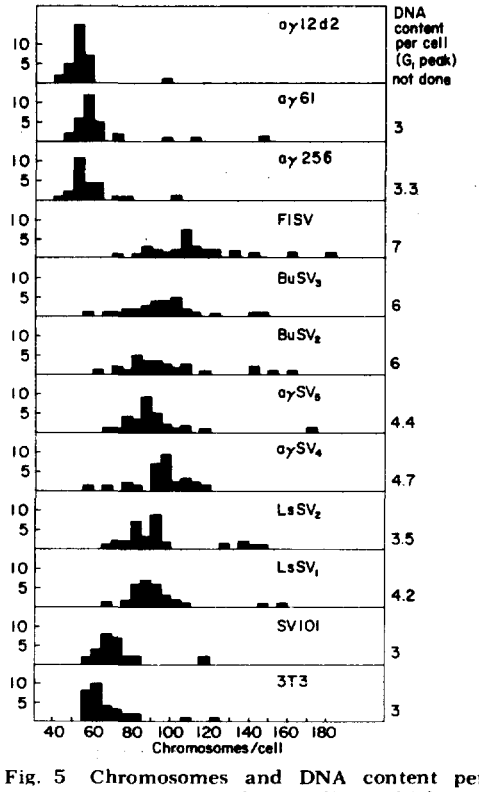

Fig. 5 Chromosomes and DNA content per cell. aγ12d2, aγ61 and aγ256 are lines which survived the serum selection but can still grow in 1% calf serum.

Density-revertants can be recovered from SV40-transformed 3T3 cells by using FUdR to kill cells capable of dividing at high cell density (Pollack et al., '68). Density-revertants still contain the SV40 genome (Ozanne et al., '73), virus specific T-antigen (Pollack et al., '68) and SV40-specific RNA (Ozanne et al., '73). However, these density revertants still maintain a serum requirement identical to that of the SV3T3 parent cell (Dulbecco, '70; Jainchill et al., '70). Therefore, direct selection for the reversion to low saturation density did not necessarily lead to the reversion of the serum property.

We report here the direct isolation of variants of SV3T3 cells which have reverted to a high serum requirement for growth. Such serum requiring lines also grow to low saturation density when cultured in serum that supports their growth.

Serum revertants contain SV40 T-antigen and the pattern of the SV40-specific RNA sequences transcribed in serum-revertants resembles the pattern found in the SV101 parent line (Ozanne et al., '73). Therefore, serum reversion did not result from the loss of the entire SV40 genome. However, fusion with permissive monkey cells does not result in the release of infectious virus from the LS revertants, but does result in the aγ revertants yielding a very small amount of virus (Vogel, unpublished results).

Reinfection of the serum revertants with SV40 does not result in a shift of the serum or density properties back to the transformed state, but the serum revertants

can be morphologically transformed by infection with murine sarcoma virus (Vogel and Pollack, unpublished data). These data suggest that the phenotypes of the serum-revertants are the result of a cellular, rather than viral alteration, and that these cellular events do not preclude all expression of transformation.

Transformation of normal cells can be assayed in at least three different ways: increase in saturation density, decrease in serum requirement for growth, or decrease in anchorage requirement for growth. One can ask if sublines selected to be revertant in any one of these parameters are also gratuitously reverted in any of the other properties.

Table 5 summarizes the properties of all revertants isolated in our laboratory with respect to serum, density and anchorage. Both the FUdr and BUdr revertants have a transformed serum-property while the colchicine revertants (Vogel et al., '73) behave like 3T3 with respect to growth in 1% serum. In general, reversion in one parameter is accompanied by reversion in one or both of the other two. Other laboratories have had similar results. For example, sublines of PyBHK selected for inability to grow in methocel grow to low saturation densities in 10% calf serum on plastic and cannot grow in 0.5% calf serum (Wyke, '71).

All serum revertants have reduced saturation densities in 10% calf serum. A class of revertants that has yet to be isolated would have a high serum requirement for growth but would be able to grow to high cell density in 10% calf serum. We screened 60 surviving colonies from the serum-selection, and found that the only stable serum revertants present were the three flat colonies described above. The other 57 survivors were dense, and all of these were able to grow in 1% calf serum. Perhaps the density-transformed serum-revertant class of cell cannot arise because the primary effect of SV40 on 3T3 is the alteration in serum requirement, with the loss of density-inhibition occurring only after the serum change has occurred (Scher and Nelson-Rees, '71).

As has been reported for density revertants selected with FUdr (Pollack et al., '70), BUdr and colchicine (Vogel et al., '73), serum revertants contain more DNA and more chromosomes per cell than their SV101 parent. This hyperploidy is not an artifact induced by selection techniques, because density-transformed clones recovered from the selections are not hyperploid. To date all cell lines derived from SV40 transformed 3T3 cells that are density-revertant are also hyperploid (Pollack et al., '70; Culp et al., '71, '72; Ozanne, '73).

3T3 and SV3T3 cells require different serum factors for growth, and sephadex

TABLE 5

Properties of revertants isolated from SV101

Line	Saturation density [1]	Serum requirement [2]	Anchorage requirement [3]
3T3	Normal	Normal	Normal
SV101	Transformed	Transformed	Transformed
Density revertants selected with			
FUdr	Normal	Transformed	Normal
BUdr	Normal	Transformed	Normal
Colchicine	Normal	Normal	Normal
Serum revertants selected in			
1% Calf serum	Normal	Normal	Normal
Aγ depleted calf serum	Normal	Normal	Transformed

[1] Normal cells have saturation densities less than 15×10^4 cells/cm^2 in 10% calf serum.
[2] Assayed by growth in 1% calf serum. Normal cells have doubling times greater than 90 hours; transformed cells double in 35 hours or less.
[3] Assayed by ability to form a colony in methocel. Normal cells do not form colonies in methocel; transformed cells do.

G-100 chromatography of rat serum at pH 2 resolves these factors from each other (Paul et al., '71). This result is in keeping with the earlier observations that serum can be depleted of 3T3 growth promoting activity while maintaining SV3T3 growth promoting activity (Holley et al., '68; Jainchill, '70). The density revertant F1SV101 requires the SV3T3 factors for growth, as expected from its SV3T3-like low serum requirement. Other serum factors have been described which are required for migration (Lipton et al., '71) and survival (Lipton et al., '72) of mouse cell lines. These are distinct from growth promoting factors and will not stimulate DNA synthesis in 3T3 cells. The factor allowing the survival of SV3T3 supports DNA synthesis, but not division of these cells (Lipton, '72).

Another class of factors is secreted by cells incubated in serum-free medium (Stoker, '72; Shodell, '72; Rubin, '66; Ossowski et al., '73; Unkeless et al., '73). For example, L cells adapted to grow in serum-free medium release factors which support the growth of BHK in serum free medium (Shodell, '72). The mitosis-stimulating factor produced by these cells can be separated from the factor maintaining DNA synthesis.

A highly specific fibrinolytic activity is found in medium after incubation with transformed cells and tumors, but not with normal cells. This fibrinolytic activity arises by interaction of a factor from the transformed cells with serum in the culture medium (Ossowski et al., '73; Unkeless et al., '73). Analysis of the factors produced and required by the serum revertants should prove useful in the further characterization of both the factors and the revertants.

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