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CELLULAR AND VIRAL CONTRIBUTIONS TO MAINTENANCE OF THE SV40-TRANSFORMED STATE

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The transformation of a cell line by one of the small deoxyribonucleic acid (DNA) viruses is a complex interaction, divided into two sets of events: those which are time-dependent and relate to the immediate fate of the virus and cell, and those which relate to the new time-independent equilibrium that is eventually established between the cell and the virus.

In terms of the state of the virus (1–3) this stable equilibrium resembles lysogeny. For the transformed cell, the new equilibrium includes, in addition, a stable, heritable loss of growth control (4). The role that viral genes play in assisting the cell to maintain this new state is most likely different from the role that they play in the initial events of transformation (4–6).

The loss of growth control is reversible. Variant sublines with increased growth control have been recovered from virus-transformed cells by (a) negative selection with 5-fluoro-2-deoxyuridine (FUdR) (7), (b) passage on cell layers fixed with glutaraldehyde (8), and (c) loss of the viral genome from transformed intraspecific hybrid lines (9).

This paper discusses some aspects of the mechanism by which a cell normally maintains growth control, the stability of various states of growth control, and the role of the virus in the maintenance of these states.

Variant Cell Lines with Increased Growth Control

To provide experimental material, variant lines with increased growth control (7–9) were selected from the SV40-transformed murine fibroblast cell line, SV101 (Table 1). Selection of variants was carried out as follows (7):

1. Determination of the minimum lethal FUdR dose for exponentially growing cell lines. FUdR, an inhibitor of thymidylate synthetase (10, 11), left fewer than 10^{-5} viable survivors when given for 2 days to exponentially growing, sparse cultures.*

2. Treatment of cell lines with FUdR at high cell densities. FUdR given at cell densities below about 5×10^3 cells per cm^2 left fewer than 10^{-5} survivors. Above that density the fraction of cells surviving FUdR increased sharply with increasing density (7).

3. Cloning of variant cells from among the survivors. Surviving cells were transferred at appropriate dilution to give discrete colonies, which could be compared with colonies from cells that had not been exposed to FUdR. Control colonies from dense cell lines had mitoses throughout, and were densest at the center. Most surviving cells from FUdR-treated dense cell lines produced colonies of this morphology, but some colonies were flat or thin. While cells were dividing actively at the periphery of such colonies, very few mitoses were seen at their centers (12). These colonies were descended from cells with a higher degree of growth control. The flat colonies were cloned twice and then tested for reduced saturation density in mass culture (Table 1).

3T3 is a line of very low saturation density (13) (Fig. 1). SV101 is a dense transformed subline of 3T3 (14). Fl²SV101 is a flat variant isolated from SV101 by FUdR selection (7).

Fl²SV101 had the following properties in culture.

1. Fl²SV101 had a lower saturation density than SV101 (Table 1).

2. At cell densities high enough to permit survival to FUdR, more Fl²SV101 cells than SV101 cells survived the treatment (Fig. 2).

3. Enhanced survival to FUdR was in all cases density-dependent; at low enough density, cells of all lines were killed by FUdR

* In order to prevent any fluorouracil, produced by breakdown of the deoxyriboside, from interfering with RNA synthesis, a 10-fold greater quantity of uridine was added with the FUdR (10).

TABLE 1

PROPERTIES OF MURINE FIBROBLAST LINES*

Line	Saturation Density	T Antigen†	Experiment	Agglutinability‡: Wheat Germ Agglutinin				
				666	333	33	17	3
						μg/ml		
3T3	4.0	None	(1)	+	0	0		
Fl²SV101	7.0	SV40	(1)	0	0	0		
			(2)	++	+	0		
3T3E	18	None	(1)	++++	++	+	(+)	0
3T12	35	None	(1)	++++	++++	+		
			(2)		++++	(+)		(+)
			(3)		++++	+		+
SV101	46	SV40	(1)			++++	++(+)	+
			(2)				++(+)	(+)

* All cultures were maintained at 36.5°C in 20-cm² plastic Petri dishes in Dulbecco and Vogt's modification of Eagle's medium supplemented with 10% calf serum. The medium was changed twice weekly. The cells used in these experiments were established fibroblast lines originating in this laboratory. Under identical conditions of culture the lines differed greatly in their saturation densities.

† T antigen is a virus-specific protein found in transformed cells. Nuclei of T antigen-positive cells were stained with fluorescein-conjugated serum from a hamster that bore tumors of SV40-transformed cells.

‡ Agglutination: growing cells were suspended with 5.4×10^{-4} M ethylene diaminetetraacetate and agglutination was initiated at 2.5×10^6 cells per ml. Each agglutination was scored at two times by more than one person. For each cell type and each experiment a control suspension without agglutinin was observed to detect possible nonspecific clumping of the cells. No such aggregation occurred.

(Fig. 2). Although an FUdR-resistant mutant cell would in principle rapidly overgrow a population subjected to repeated FUdR treatments, no FUdR-resistant cells were observed.

4. The flat colony morphology that was the basis of selection of Fl²SV101 was stable in mass culture (Fig. 3).

5. Fl²SV101 grew poorly when plated on pre-existing monolayers of 3T3, while SV101 grew as well on 3T3 as on bare plates (7, 15, 16) (Fig. 3).

FLUCTUATION ANALYSIS

Because the possibility existed that lines such as Fl²SV101 were induced by FUdR treatment (17), it was necessary to determine whether flat variant lines arose spontaneously in cloned transformed cell lines. An experiment modeled on the fluctuation analysis of Luria and Delbruck (18) was carried out. The experiment consisted of two similar but not identical FUdR treatments of SV101.

For the first treatment an inoculum of 10^3 SV101 cells was grown up to 5×10^6 cells in one 20-cm² plate. The cells were then given FUdR for 2 days. As predicted for this cell density (Fig. 2), about 10^4 cells survived. These were plated out at 1:100 dilution in 30 dishes. The separate colonies were fixed and stained at 10 days, and the numbers of flat and parental colonies were determined. There were between zero and three flat colonies per plate, and the majority of the colonies were parentally dense. In this experiment the fraction of flat cells detected in the SV101 population was $1 \times 100/(5 \times 10^6)$ or 2×10^{-5}.

For the second treatment, 10^3 SV101 cells were inoculated into not one but 30 plates. The 30 plates were grown up as before and each was treated separately with FUdR. The survivors from each plate were transferred at 1:100 into one plate, to give 30 sets of survivors, each from a separate treated plate. These were grown up and examined for flat colonies. Again, most of the surviving colonies were parental. However, some survivor plates contained more than three flat colonies, and one plate had more than 100 flat colonies. The first treatment showed that this many flat variant cells could not have been put in one survivor plate by random assortment during 1:100 dilution of the survivors. Therefore, the survivors must have descended from a flat variant cell that had been present and dividing, before FUdR was added.

Together these experiments showed that

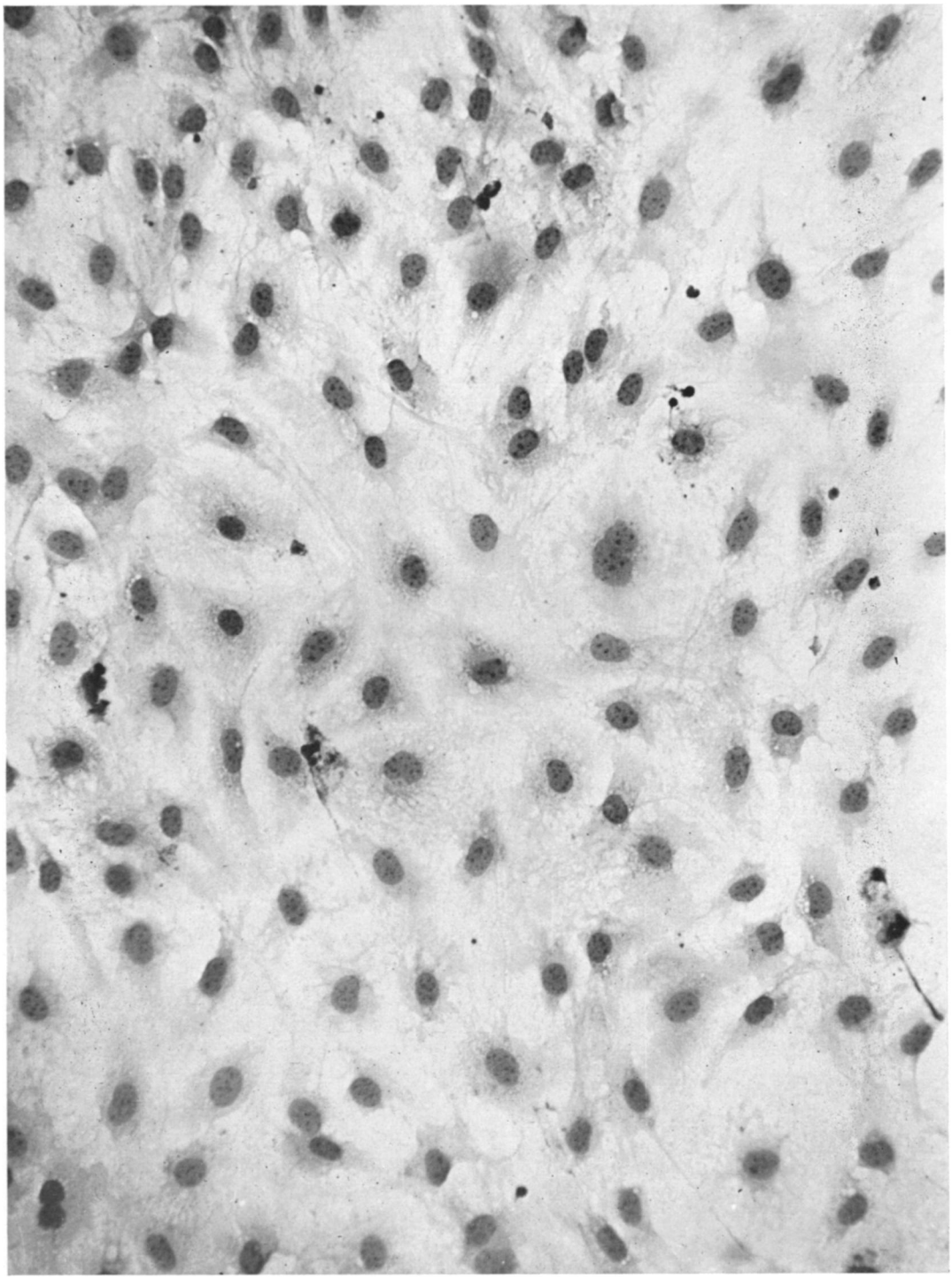

FIG. 1. Cells of the 3T3 murine fibroblast line at saturation density. The culture was fixed in formalin-phosphate-buffered saline (1:10) and stained with hematoxylin. At this density there were 4×10^4 cells per cm^2. Note the even distribution of nuclei, without overlap, and the absence of mitoses.

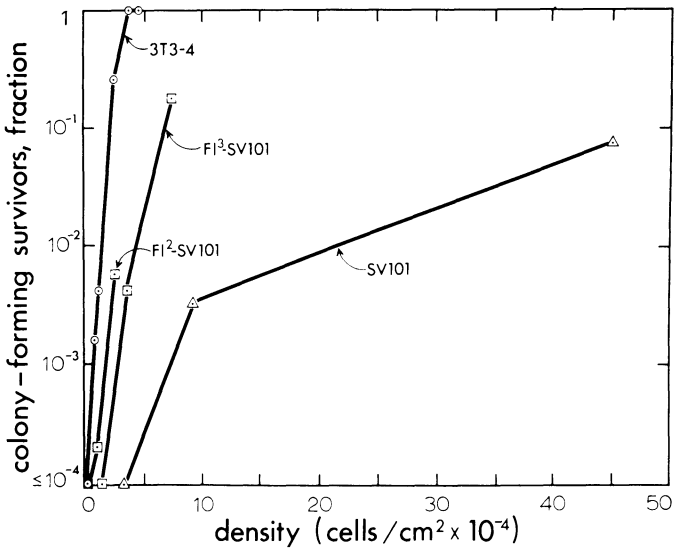

FIG. 2. Fraction of murine fibroblast cells that survived a pulse of FUdR versus cell density at the beginning of the pulse. 3T34 is a clone of 3T3, an established murine line of very low saturation density. SV101 is a cloned, dense SV40-transformed subline of 3T3. Fl^{2}SV101 is a flat variant subline selected from SV101 by two cycles of FUdR treatment. Note that, at all cell densities, the fraction of Fl^{2}SV101 surviving FUdR is about 10^2-fold higher than the fraction of surviving SV101 cells. The colony-forming survivor fraction was determined 10 to 14 days after transfer of FUdR-treated and control cells from sister plates.

about one cell in 10^5 of the SV101 population was of a spontaneously arising flat variant type. An in vivo implication is that, despite the common observation of inexorable tumor progression (19–22), the malignant state is probably genetically unstable.

CLUSTER-DEPENDENT GROWTH CONTROL

Twelve cell lines of murine and hamster origin were subjected to FUdR treatment. Although stable flat variant sublines could not always be recovered, these 12 different lines responded similarly to FUdR in one respect: the fraction of cells surviving FUdR always increased with increasing cell density for nine of these lines, the surviving fraction at the maximum cell density was 0.1 or higher. Saturation in these lines was the consequence of growth control, rather than the fortuitous equilibrium between increased toxicity and undiminished mitotic activity seen with such lines as Cl 1D.

Saturation densities of the nine lines ranged from 4 to 50 $\times$ 10^4 cells per cm^2 under identical culture conditions (7). By normalizing cell density to percentage of saturation density, the FUdR survivor fractions of the different lines could be compared. A log-log plot of normalized density (d) versus colony-forming survivor fraction (f_1) brought the survivor fractions of the nine lines together (Fig. 4). This suggested that they shared a common relation between growth control and density.

In particular, the points grouped about a line that rose from $f_1 = 10^{-4}$ at $d = 0.1$, to $f_1 = 0.1$ to 1 at $d = 1$ (Fig. 4). The line that fit these points has the formula $\log_{10} f_1 = m \log_{10} d$, where m was between 3 and 4.

If $\log_{10} f_1 = m \log_{10} d$, then $\log_{10} f_1 = \log_{10} d^m$, and $f_1 = d^m$. When $d = 1$, $f_1 = 1$ for any value of m, which defined saturation as the density at which all cells survive FUdR.

If we assume that cells are migrating freely at all densities up to saturation, and are therefore making contact with each other, the fraction (f_2) of cells contacting a given number (c) of other cells is also a simple function of cell density and cluster number. At any normalized density (d) the fraction of cells that are clustered in groups of C cells each will be related to density. Since each cell in the cluster has a probability of being present that is proportional to cell density, the fraction of cells in the clusters of C cells will be

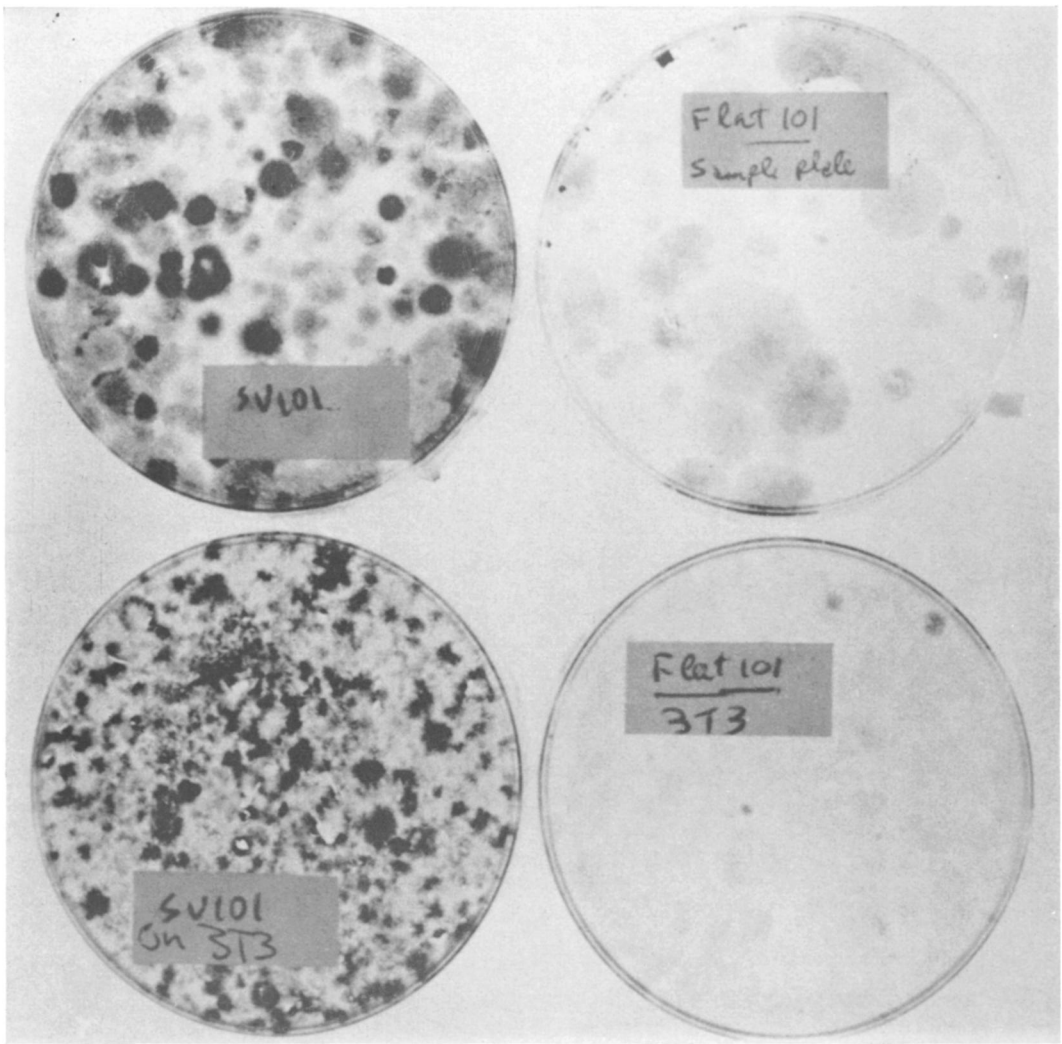

FIG. 3. Colony morphology of SV101 and Fl²SV101. Cells (100) of each line were distributed onto bare 20-cm² plates (*top*) and onto saturation density cultures of 3T3 (*bottom*). After 14 days, the four plates were fixed in formalin-phosphate-buffered saline and stained with hematoxylin. Growth of SV101 was slightly enhanced on the 3T3 monolayer, while growth of Fl²SV101 was markedly inhibited. On bare plates, colonies of Fl²SV101 were thin throughout, while colonies of SV101 were densest in the center.

$f_2 = Kd^c$, where K is a constant. At saturation, $d = 1$, so $K = f_2$ (saturation). For any density less than saturation, $f_2 = f_2$ (saturation) d^c.

Comparing the empirical formula that best approximated the data of Fig. 4 with this formula for the fraction of cells expected to be in clusters of different sizes,

$$f_1 = d^m$$

and

$$f_2 = f_2 \text{ (saturation) } d^c$$

If f_2 (saturation) $= 1$, then $f_2 = f_1$ when $m = c$; that is to say, c is the same as the slope of the line fitting the *points* in Fig. 4. Therefore, if saturation is the density at which all cells are in clusters of c, the fraction of cells surviving FUdR at any density is the same as the fraction of cells expected to be in clusters of three to five cells.

The FUdR survival data (Fig. 4) were

quantitatively not precise enough to decide among slopes, or cluster sizes, from three to five. Clearly, however, the slope was neither one nor two. This ruled out density (23) (a cluster of one) and cell-cell contact per se (12, 13, 24) (a cluster of two) as sufficient for growth control, although both are prerequisites to cluster formation.

Recently, time lapse cinematography provided a striking confirmation of the hypothesis that inhibition of cell division in the 3T3 line is cluster-dependent (25). Some 40 separate 3T3 cells and their descendents were followed, frame by frame, through log growth to confluence, over an 8-day period. Intermitotic times were measured directly from the film. Intermitotic times greater than 48 hr were detected only among cells that had been in contact with three or more cells. Extended intermitotic time did not correlate either with high local cell density or with contact between two cells (25).

Alterations of the Surface of the Cell

Since growth control was dependent upon a complex type of cell-cell contact, specific differences in the chemical composition of the surfaces of cell lines with different saturation densities were expected (26). A specific agglutinating compound, the phytoagglutinin WGA, isolated and purified (27, 28) from a commerical preparation of wheat germ lipase, was used to detect such a chemical difference (29).

WGA has been used to distinguish between cells from normal tissues and cells from tumors (27, 30) and between some cell lines with growth control and their viral and spontaneous transformants (31, 32). Suspensions of tumor cells and of transformed cells were agglutinated rapidly and completely by purified WGA at concentrations below 10 μg per ml, while untransformed cells and normal tissue cells remained in suspension. At WGA concentrations above 1,000 μg per ml, only slight agglutination of untransformed cells was observed.

In order to extend these studies, five fibroblast lines of murine origin, all related to 3T3, were assayed for WGA-dependent agglutinability.

Agglutinability of these five lines was in strict order of their saturation density (29) (Table 1). This proportionality was consistent with previous work (31). Significantly, the flat variant Fl^{c}SV101 was precisely as agglutinable as predicted from the relation between saturation density and agglutinability determined for the other four lines (Table 1).

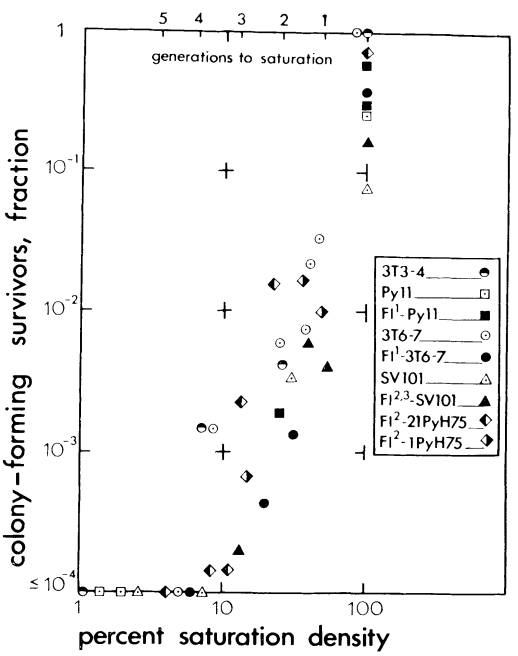

Fig. 4. The fraction of cells that survived a pulse of FUdR versus normalized cell density at the beginning of the pulse. The nine cell lines had stable saturation densities of from 4 to 50 $\times$ 10^{4} cells per cm^{2}. Density was normalized to percentage of saturation density for each line.

Gentle proteolytic digestion of untransformed, poorly agglutinating cells had been shown to make them as agglutinable as cells of a dense, transformed subline (32). Similar treatment of transformed cells did not increase their agglutinability. Evidently, both cell lines had about the same number of WGA receptor sites on their surface, but most sites were masked on the untransformed cell. Proteolysis was able to remove the group masking these WGA receptor sites (32).

A similar situation was found in the 3T3 series. When either 3T3 or Fl^{c}SV101 cells were exposed to trypsin, the cells became as agglutinable as SV101 (Table 2). Treatment of the highly agglutinable SV101 cells with trypsin

TABLE 2

AGGLUTINABILITY OF LINES FOLLOWING MILD TRYPSINIZATION*

Line Trypsinized	Agglutinability: Wheat Germ Agglutinin				
	666	333	33	17	3
	μg/ml				
3T3		++++	+++	+++	++
Fl²SV101			++++	+++	+
SV101		++++	++++	(+)	+

* 3T3 and SV101 were incubated with 0.01% trypsin for 10 min, pelleted, and resuspended for assay. Fl²SV101 was incubated with 0.1% trypsin for 10 min, pelleted, and resuspended for assay.

did not increase their agglutinability (Table 2).

FUdR permitted selection of pre-existing variant cells only because of their heritable ability to extend their intermitotic time beyond the period of FUdR treatment at cell densities below the saturation density of the general population. Unless this type of regulation of cell division required that WGA receptor sites be masked, there was no way for the FUdR system to have selected a cell line with masked sites, such as Fl²SV101. Therefore, the ability of a cell line to synthesize a trypsin-sensitive group that masks the WGA receptor site must be another prerequisite to growth control.

T ANTIGEN

The cells studied with WGA were also examined for evidence of persistent SV40 gene function. SV40 T antigen is a virus-specific protein that is found in nuclei of transformed cells (33). The antigen does not react with antibody to purified virus particles (34). SV40-specific T antigen was detected in all cells in the SV101 line. Under identical conditions, T-antigen was also detected in all Fl²SV101 cells. As expected, 3T3 and the untransformed dense lines 3T12 and 3T3E all were negative for SV40 T antigen (Table 1). SV40-specific T antigen was not sufficient to maintain the transformed state.

Recently, SV40 virus recovered from transformed murine fibroblasts was reported to have a higher transformation-plaque formation ratio (TFU:PFU) than wild type SV40 (35). However, when SV40 was isolated from SV101 and Fl²SV101 the two recovered virus strains and wild type SV40 were all found to have the same TFU:PFU ratio over a 100-fold range of input multiplicities (36). Evidently, cellular growth control was regained in Fl²SV101 cells despite the continued presence of the entire SV40 genome, as well as at least one viral gene function.

SUMMARY

1. A complex type of intercellular contact, requiring cluster formation among three cells or more, is a prerequisite to growth control for a large number of established fibroblast lines.

2. This form of contact occurs at lowest cell densities among cells that synthesize a specific protein that blocks intercellular WGA agglutination.

3. High saturation density cells, lacking this protein, are rapidly agglutinated by WGA.

4. Low saturation density cells, including a line containing SV40 T antigen and the SV40 genome, make this protein, and consequently are agglutinated poorly.

5. The presence of the SV40 genome and SV40 T antigen are not sufficient to guarantee that a cell line will have a high saturation density or that it will respond well to the WGA agglutinin.

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