

Biological Analysis of Clones of SV40-infected Mouse 3T3 Cells

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A variety of selective assays have been used to monitor the transformation of normal cells into tumor cells. These assays take advantage of the ability of transformed cells to grow to high saturation densities (Todaro, Green and Goldberg 1964), to grow in the absence of anchorage to glass or plastic (Macpherson and Montagnier 1964; Stoker et al. 1968), to form colonies or foci on monolayers of normal cells (Temin and Rubin 1958), to grow in reduced concentrations of serum components (Holley and Kiernan 1968; Jainchill and Todaro 1970; Smith, Scher and Todaro 1971), and to grow in disoriented patterns (Stoker and Abel 1962).

The transformation of mouse 3T3 cells by SV40 is usually monitored by the first assay and is a low-frequency event. At very high virus doses (10^8 infectious units/cell) only 50% of the cells are transformed in this assay (Todaro and Green 1966; Black 1966). When such transformants are tested in other assays, they are found to have reduced serum requirements, to form colonies on normal monolayers, and to form colonies in agar or methylcellulose suspension. Cells which show all of these transformed growth properties we term standard transformants.

Recently, however, Smith et. al (1971) and Scher and Nelson-Rees (1971) have isolated SV40-transformed cells which differ from standard transformants. These cells are primarily altered in their serum requirements, though they have saturation densities comparable to 3T3. The existence of such cells demonstrated that SV40 infection could yield cells with only a partially transformed character. This raised the possibility that selective transformation assays were not detecting the full range of cellular alterations that were induced by SV40.

To isolate all possible types of SV40 transformants, a nonselective scan of clones of 3T3 cells arising after SV40 infection was carried out. Forty clones of SV40-infected 3T3 cells were picked without regard to morphology and analyzed in each transformation assay.

A density transformation assay carried out simultaneously on these infected cells showed a standard transformation frequency of 10%. However the nonselective scan demonstrated that fully 90% of the clones differed from 3T3 cells in their ability to utilize low concentrations of serum.

In addition to this change 25% of the clones showed intermediate growth properties in other assays and another 25% had growth properties comparable to those of standard transformants. A more thorough description of all clones will appear elsewhere.

Scheme of the Experiment

A recently cloned 3T3 line was infected at subconfluence (2×10^4 cells/cm²) with SV40 or mock-infected with Dulbecco's modified Eagles medium (DME). The virus was prepared from a low-multiplicity passage on BSC-1 monkey cells of a triply plaque-purified clone of SV40 (Strain 776 originally NIH). The virus was purified by standard procedures and banded twice to equilibrium in CsCl (Black, Crawford and Crawford 1964). After collection it was dialyzed against serum-free medium (Dulbecco) and used immediately for infection. A separate aliquot of banded virus was used to determine the number of physical particles by optical density (Koch et al. 1967). The multiplicity of infection in this experiment was 2×10^8 pfu/cell, which corresponded to a physical multiplicity of 5×10^5 – 1×10^6 particles/cell.

The cells were allowed to recover from infection in medium with 10% calf serum. The next day they were trypsinized and replated at different dilutions in a standard density transformation assay (Sambrook and Pollack 1973). Medium (10% Colorado calf serum in DME) was changed every third day for two weeks. Some plates were then stained and others used for cloning. Dilution plates having between 10 and 20 colonies were scanned and well-isolated colonies were chosen without regard to colony morphology.

Each colony was photographed and then picked using trypsin within a steel cloning cyclinder. Clones were grown up and passaged weekly at a density of about 5×10^2 cells/cm² for one month. Forty isolated colonies were picked for experimental clones. Four isolated colonies from mock-infected plates and the 3T3 parental line served as control normal clones. Four dense foci picked from infected low-dilution plates and the standard transformant SV101 served as control standard transformants.

From stained plates the transformation frequency per colony was 11%. Of the clones picked 15% were judged to be transformed by the morphological criterion of high cell density. The plating efficiencies of virus-infected or mock-infected cells were 83% and 91%, respectively.

Properties of Clones of SV40-infected 3T3 Cells

Viral Antigens

Twenty-five of the 40 clones were tested for the presence of viral capsid antigen by indirect immunofluorescence. All clones were found to be negative, whereas BSC-1 cells infected three days earlier with SV40 fluoresced brightly in the nucleus.

SV40-specific T antigen was assayed by indirect immunofluorescence. Cells were plated onto 9-mm coverslips at a density of 2 – 4×10^4 cells/cm². Twelve hours before staining, coverslips were placed in DME containing only 1% calf serum. To stain, the coverslips were rinsed in phosphate-buffered saline (PBS), fixed in cold acetone and incubated one hour with hamster antibody to SV40 T antigen (Flow

Laboratories). Coverslips were then rinsed with PBS and incubated with goat antibody to hamster gammaglobulin, which had been conjugated to fluorescein (Antibodies, Inc.). After rinsing in PBS the coverslips were examined at $400\times$ magnification under dark-field ultraviolet illumination (Zeiss).

Three patterns of staining were seen: negative, positive and intermediate. Half of the 40 clones were negative by immunofluorescence, as were 3T3 cells and all mock-infected clones. In SV101 and standard transformants more than 95% of the cells stained uniformly positive; ten experimental clones stained identically to these transformants. In the remaining ten experimental clones, the staining pattern was heterogeneous. In such clones 10–30% of the cells stained as brightly as standard transformants, 50–80% of the cells stained weakly, and 10–20% of the cells did not show any nuclear fluorescence.

The validity of the staining pattern was tested by staining mixtures of SV101 cells and 3T3 cells. In such mixtures only positive or negative cells were seen; furthermore the percentage of positive cells depended on the proportion of SV101 cells in the original mixture. When SV101 and 3T3 cells were mixed in a ratio of 1 to 5, 215/1000 cells were positive; when SV101 and 3T3 cells were mixed in a ratio of 1 to 100, 11/1000 cells were positive. The cloning procedure was also checked by plating a mixture of SV101 and 3T3 (ratio 1 to 4) very sparsely and allowing colonies to form on coverslips. Seven of the 25 colonies scored were positive and 18 were negative. No intermediate or mixed colonies were seen.

T antigen levels have also been checked by complement fixation on equal numbers of cells of various clones. When two uniformly positive clones and SV101 were compared to eight T antigen intermediate clones, the complement-fixing titer of the three positive clones was tenfold greater than that of any of the intermediate clones. Two T antigen negative clones were also tested; both had background complement-fixing titers comparable to that of 3T3. Thus the indirect immunofluorescence and complement fixation assays both revealed similar levels of T antigen in the clones. In the intermediate clones a wide variation from cell to cell in intensity of staining was revealed by immunofluorescence.

Growth Properties in 10% and 1% Calf Serum

The saturation density and doubling times of each clone were obtained from growth curves derived from cultures in which cells were seeded sparsely on plastic dishes. Typical growth curves are given in Fig. 1. In 10% calf serum 3T3 cells grew to a density of 7.5×10^4 cells/cm² with a doubling time of 23 hours. SV101 cells grew to a density of 50×10^4 cells/cm² with a doubling time of 16 hours. Cells from one experimental clone, SVR-Cl 8–2, grew to a density of 15×10^4 cells/cm² with a doubling time of 20 hours.

In 1% calf serum these lines grew to densities of 0.9, 10 and 3×10^4 cells/cm² with doubling times of 76, 26 and 29 hours, respectively. Vogel and Pollack (1973) have found that the doubling times of cell lines in 1% calf serum can be used to distinguish a normal from transformed response to low serum. By this criterion SVR-Clone 8–2 is like the standard SV40-transformant SV101 in its utilization of serum to grow; it is, however, T antigen negative.

A compilation of data from each clone on saturation densities in 10% calf serum and doubling times in 1% calf serum is shown in Fig. 2 and 3. The saturation densities of control normal clones or standard transformed clones show little spread in

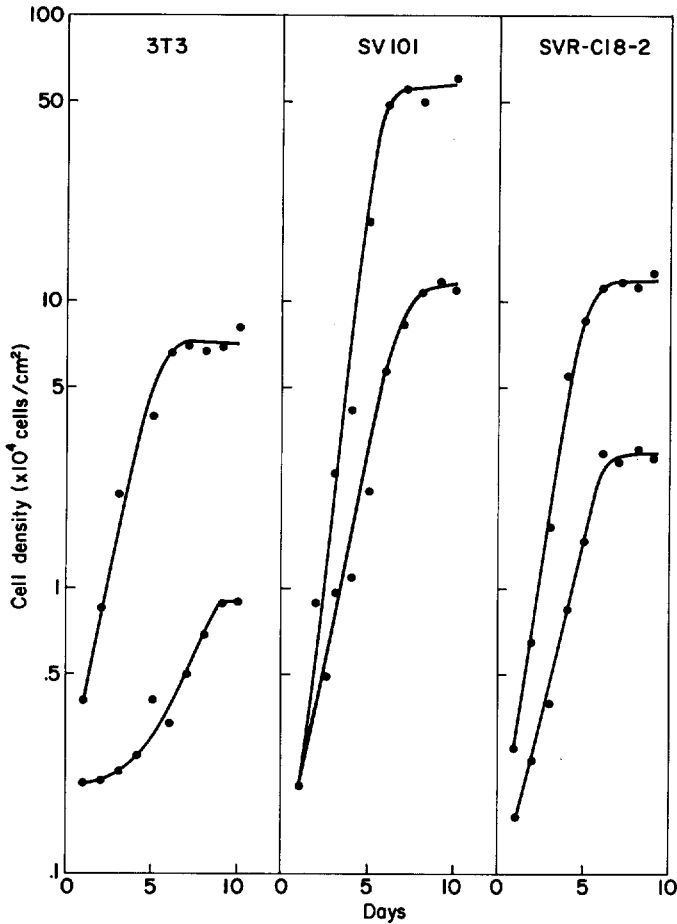


Figure 1

Growth curves of SV101, 3T3 and experimental clone SVR-CI 8-2. *Upper curve*, growth in 10% calf serum-DME; *lower curve*, growth in 1% calf serum-DME. Cells were seeded at a density of $1-2 \times 10^3$ cells/cm² in 2 ml of medium onto a 35-mm Falcon petri dish. Medium was changed every third day and cell counts were taken daily using a Coulter Counter.

values (Fig. 2); however, the 40 experimental clones show a spectrum of values ranging from that of 3T3 to that of standard transformants. In most cases the saturation density of a clone correlates with its T antigen level. Positive clones have high densities; intermediate clones have intermediate densities, and negative clones have densities closer to that of 3T3.

In contrast, the doubling time in 1% calf serum bears little or no correlation to the T antigen level of a given clone (Fig. 3). Only five of the experimental clones have doubling times comparable to 3T3 or mock controls; the remaining 35 double as efficiently in 1% calf serum as do transformants. The four experimental clones with doubling times of greater than 60 hours correspond to the four clones with saturation densities in 10% calf serum of $< 8 \times 10^4$ cells/cm².

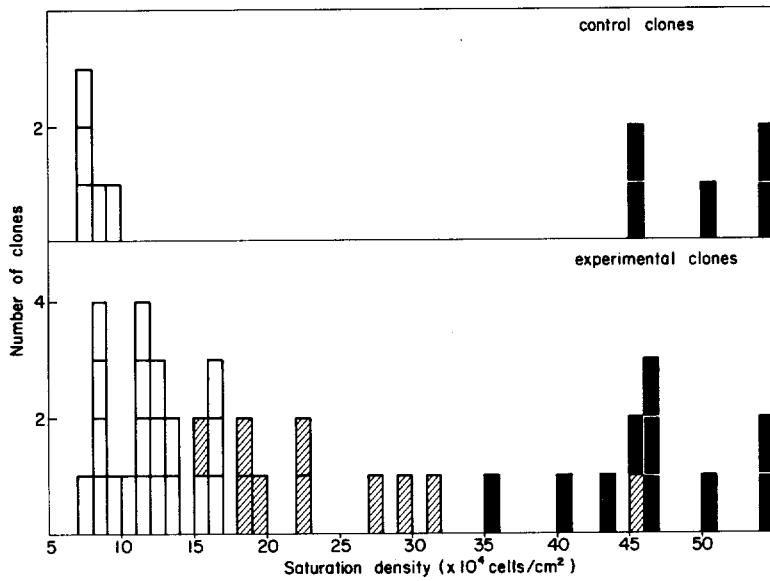


Figure 2
Histogram of saturation densities of control and experimental clones in 10% calf serum-DME. Black rectangles, T antigen positive clones; hatched rectangles, T antigen intermediate clones; white rectangles, T antigen negative clones.

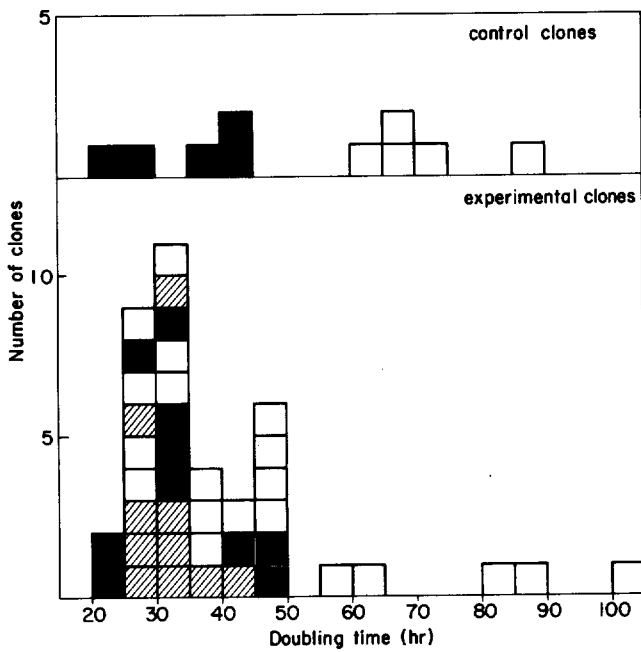


Figure 3
Histogram of doubling times of control and experimental clones during logarithmic growth in 1% calf serum-DME. Black rectangles, T antigen positive clones; white rectangles, T antigen intermediate clones; hatched rectangles, T antigen negative clones.

Anchorage Properties

Anchorage assays on each clone were carried out according to the method of Stoker et al. (1968) using methylcellulose. The colony-forming efficiencies of each clone in methylcellulose are presented in Fig. 4. As with saturation densities, a considerable range of colony-forming efficiencies can be seen. T antigen positive clones plated with an efficiency of 10–50%, whereas intermediate clones plated with an efficiency of 0.5–10%. One intermediate line (SVR-CI 6–3), however, formed colonies with a very low efficiency.

Additional information on anchorage dependence of various clones was obtained from measurements on individual cells or colonies. Figure 5 presents the results of such measurements as histograms of the number of colonies vs. colony size. From these data we conclude that most cells from clones with intermediate plating efficiencies (and intermediate T antigen) are capable of a number of divisions in methylcellulose, though they do not form a visible colony in the 3-week assay time. In methylcellulose the T antigen intermediate clone least capable of division, SVR-CI 6–3, corresponds to that with the lowest plating efficiency (0.002%). 3T3

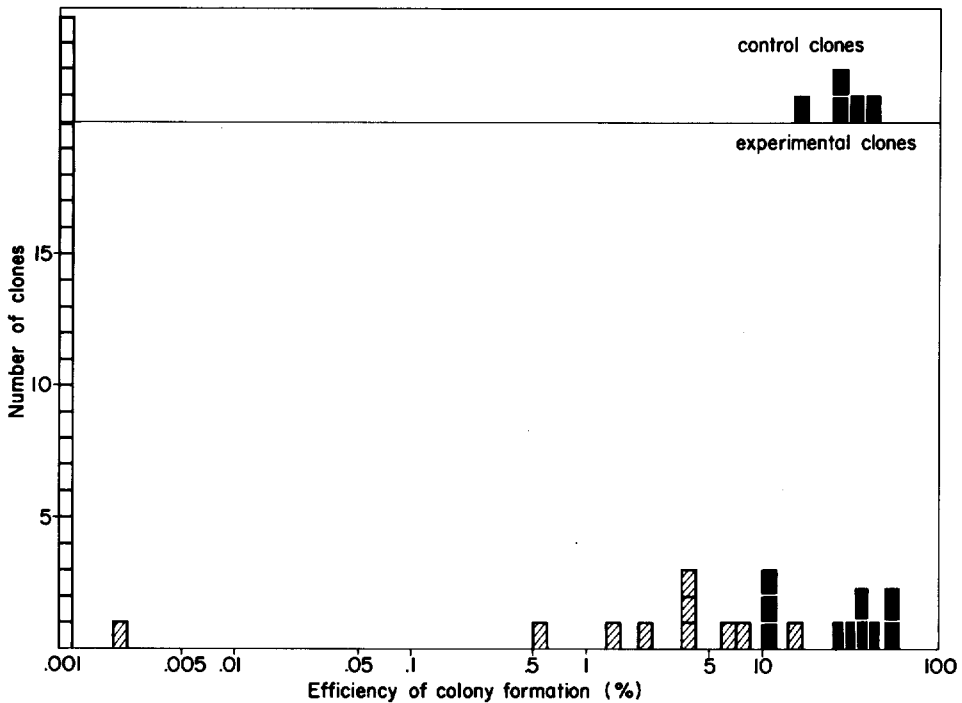


Figure 4

Histogram of efficiency of colony formation of control and experimental clones in 1.17% Methocel, 10% CS-DME. Medium and agar plates were prepared according to Stoker et al. (1968). Cells (10^5 , 10^4 , 10^3 or 10^2 in 4 ml Methocel medium) were plated in quadruplicate onto plates containing 3 ml solid agar. An additional 4 ml Methocel medium was added at 1 and 2 weeks. Colonies of 100–200 cells or greater were scored at three weeks with dissecting microscope. Plates having 30–200 colonies were used for scoring. Black rectangles, T antigen positive clones; hatched rectangles, T antigen intermediate clones; white rectangles, T antigen negative clones.

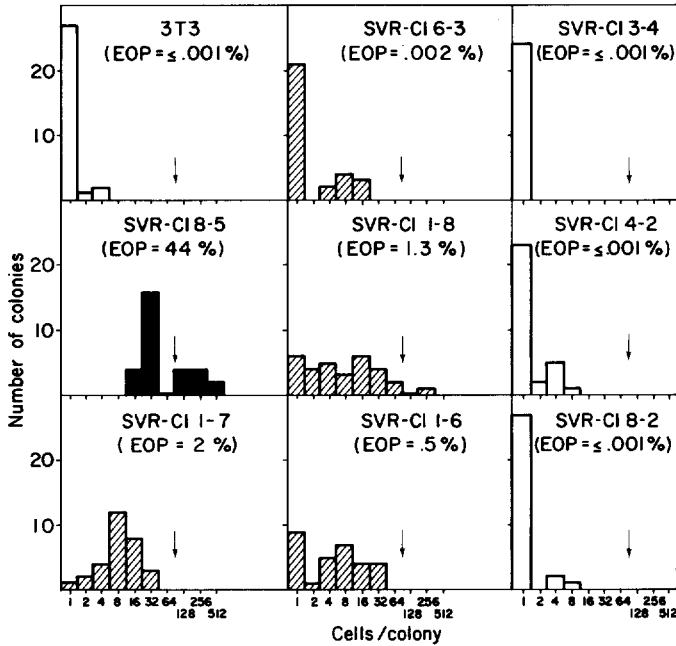


Figure 5

Histograms of colony sizes of various clones grown in Methocel. At 3 weeks 30 colonies were measured using a reticle eyepiece at random on plates inoculated with 10^4 cells. The eyepiece was calibrated using pollen grains of 13–90 μ in diameter. From measurements of 3T3 and SV101 the day after plating the mean cell diameter was 19.8 μ . The volume increase was calculated from the colony diameter and from this the colony size was inferred. Black rectangles, T antigen positive clones; hatched rectangles, T antigen intermediate clones; white rectangles, T antigen negative clones.

and other T antigen negative clones undergo few, if any, divisions in methylcellulose.

Colony Formation on Monolayers of Normal Cells

The ability of cells from each clone to form visible colonies on monolayers of 3T3 cells or on plastic dishes was tested. The plating efficiency of all clones on plastic dishes ranged from 10–60% with an average of about 40%. Standard transformants and most clones which are uniformly T antigen positive plated on plastic or on 3T3 monolayers with about equal efficiencies (Fig. 6). Intermediate clones, however, showed a wide range in their plating efficiency on 3T3 monolayers as compared to their plating efficiency on plastic. In general, the higher the saturation density of the intermediate clone, the higher its plating efficiency on 3T3 monolayers. A similar correlation of monolayer plating with saturation density has been seen by Pollack, Green and Todaro (1968).

T antigen negative clones did not form visible colonies on normal monolayers. It must be pointed out, however, that the density of these clones is only about twice that of 3T3. Colonies of that density might grow on a 3T3 monolayer but be indistinguishable from the monolayer itself.

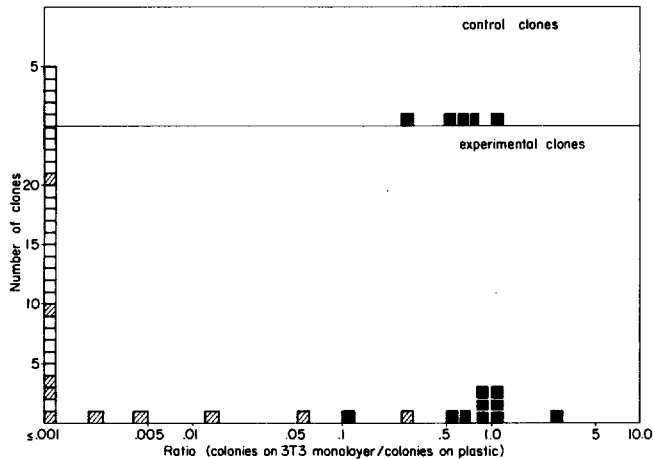


Figure 6

Histograms of efficiencies of colony formation on 3T3 monolayers for control and experimental clones. Cells 10^2 or 10^3 were plated onto confluent 3T3 monolayers or plastic dishes (60-mm) in 10% calf serum-DME in triplicate. Medium was changed every third day and plates fixed and stained with Harris-hemotoxylin on the tenth day. The plating efficiencies on plastic vary from 20–60%, averaging 40%. The data is normalized to the plating efficiency on plastic. Black rectangles, T antigen positive clones; hatched rectangles, T antigen intermediate clones; white rectangles, T antigen negative clones.

Stability of Growth Properties of SV40 Clones

One month after the initial cloning, six clones were tested in each assay. The clones were passaged for 4 months and then recloned. Both parental and recloned lines were retested in each assay one month after the recloning. The data in Table 1 demonstrate that the growth properties measures in this series of assays are quite stable.

Specifically the T antigen negative clones that initially grew well in 1% calf serum (e.g., SVR-Cl 4–2, 5–7 and 8–2) continued to do so even after recloning. The T antigen intermediate clone, SVR-Cl 6–3, was tested for saturation density in 10% calf serum, growth in 1% calf serum, plating in methylcellulose, and plating on 3T3 monolayers. By all of these tests both the reclone and the initial clone after 5 months of continuous passage were very similar to the initial clone after 1 month of continuous passage.

A more thorough investigation has been made of T antigen intermediate clones. Five clones showing heterogeneous T antigen by immunofluorescence and lower levels of T antigen by complement fixation were recloned. Eighteen subclones were picked and their T antigen staining characterized by indirect immunofluorescence. Approximately half of the clones showed a wide variation in staining pattern, much like the parental cells. The other subclones showed a uniform pattern of staining; the intensity of this staining was considerably less than that of standard transformants. Other growth properties of these subclones will be described elsewhere (Risser and Pollack in prep.). In no case was a T antigen negative subclone generated.

Table 1
Stability of Growth Characteristics of SV40-transformed 3T3 Clones

Line	T antigen		Saturation density (cells/cm ² × 10 ⁴)		Doubling time in 1% CS (hr)		EOP in Methocel (colonies/100 cells)		Ratio EOP on monolayers to EOP on plastic		
	1 mo.	5 mo.	5 mo. re- clone	1 mo.	5 mo.	5 mo. re- clone	1 mo.	5 mo.	1 mo.	5 mo.	
4-2	-	8	10.0	24	37	47	.001	NT†	.01	NT	.01
5-7	-	15	15	40	31	31	.001	NT	.01	NT	.01
6-3	I*	9	15	33	29	29	.01	.001	.01	NT	.01
8-1	+	60	70	20	24	22	11	NT	1.0	NT	.7
8-2	-	10.5	12	40	29	29	.001	NT	.01	NT	.01
8-4	+	50	60	32	27	30	11	NT	2.6	NT	2.0

* I= intermediate. † NT = not tested.

Table 2
Classes of SV40 Clones

Class	% of Clones	T antigen	Saturation density (cells/cm ² × 10 ⁴)	Doubling time in 1% CS (hr)	Anchorage (colonies in Methocel per 100 cells)	Ratio EOP on monolayers to EOP on plastic
I Normal	12	-	8.5 (7.6-9.5)	78 (55-100)	≤ .001	≤ .001
II Transformed	25	+	47 (35-60)	34 (24-45)	32 (11-58)	.94 (.1-2.6)
III Serum-transformed	38	-	15 (9.5-16.5)	34 (26-49)	≤ .001	≤ .001
IV Intermediate-transformed	25	±	25 (15-45)	32 (29-41)	4 (.5-14.6)	.03 (.001-.25)
Control						
Normal		-	7.8 (7-9)	70 (60-86)	≤ .001	≤ .001
Transformed		+	53 (47-60)	37 (26-43)	25 (16-30)	.7 (.25-1.1)

DISCUSSION

The nonselective nature of this assay has allowed us to isolate many transformants which have not been previously described. In fact we have found that most clones arising after SV40 infection are altered at least with respect to serum requirement for growth.

The variety of clones obtained in this manner is presented in Table 2. Clones of the first class, which are indistinguishable from 3T3 in these assays, may be unaffected by the virus or may be similar to the cryptic transformants of Smith et al. (1971) if they carry SV40-specific sequences in unexpressed form (Smith, Gelb and Martin 1972). They are presently being tested for SV40-specific DNA sequences.

Clones of the second class correspond to the standard transformants that have been described by many authors. The high proportion of these clones may be due in part to delayed transformation (Stoker 1963; Todaro and Green 1966). In fact four of the clones looked normal by morphological criteria when they were picked. Subsequent to their cloning they converted to transformants, perhaps because of the more rapid doubling time of transformed cells in 10% calf serum (16 hr vs. 23 hr). Reconstruction experiments on 3T3 and SV101 are currently in progress to test this hypothesis.

The third class of clones consists of cells which are altered in their ability to utilize low concentration of serum. The saturation density of these clones is about twice that of 3T3. The mechanism by which this saturation density is maintained has not yet been investigated. We do not yet know if class III clones contain viral-specific DNA sequences.

Serum transformants are expected from the work of others (Smith, Scher and Todaro 1971; Scher and Nelson-Rees 1971). What is surprising is the high proportion of clones which they represent and the lack of viral T antigen in these clones. Among a less extensive catalog of cells, Smith et al. (1971) reported one T antigen negative, serum-transformed clone and one T antigen positive, serum-transformed clone. It must be pointed out, however, that the assay used by Smith et al. (1971) differs from the assay used here in that it requires cells to form visible colonies in 10% agamma-depleted calf serum when plated sparsely.

The last class of clones does not represent a uniform category of cells, but rather cells with a spectrum of growth properties intermediate between 3T3 cells and SV101 cells. The pattern of staining and level of complement-fixing T antigen is significantly lowered from that of standard transformants. The saturation densities of such clones show a spectrum of values, much as seen by Scher and Nelson-Rees (1971). Intermediate saturation densities seen in this class of clones may well be the result of an equilibrium between cell growth and cell death and detachment as Scher and Nelson-Rees have demonstrated for their lines. The intermediate growth of these clones in methylcellulose medium demonstrates that such clones are not comprised of a mixture of normal and standard transformed cells, but rather are comprised of cells each with a limited anchorage independence, similar to the abortive transformants of Stoker (1968). Such cells differ from abortive transformants in that they continue to grow slowly throughout the three-week assay. It will be quite important to know if the few dense colonies which such lines form on 3T3 monolayers in fact represent segregation of standard transformants.

We have shown in this work that the effect of SV40 on 3T3 cells is more varied and of a greater extent than previously thought to be the case. Whether the variety

of cell growth patterns seen in this study is a result of an inherent instability of 3T3 cells due to their aneuploid chromosome complement is not yet clear. This range could result from a mechanism of SV40 transformation that effected each cell somewhat differently, leading to the simultaneous appearance of every different type of transformant. Alternatively, the range may be the result of secondary alterations that become possible only after a primary virus-mediated alteration, such as serum utilization, has occurred.

In any event the results reported here demonstrate that SV40 is capable of bringing about a variety of stable changes in growth properties in the majority of infected cells. Which of these changes, if any, is related to the uncontrolled proliferation of a cell in an animal host is not yet clear.

Acknowledgments

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