

## A Nonselective Analysis of SV40 Transformation of Mouse 3T3 Cells

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*Accepted February 5, 1974*

Mouse cells transformed by simian virus 40 show many alterations in their growth properties *in vitro*. In order to investigate the coordinate nature of these changes, we have analyzed the growth properties of 40 randomly selected colonies arising after SV40 infection of 3T3 cells. Clones of cells, established from these colonies, were characterized as to saturation density and doubling time in 10% and 1% calf serum, growth in methyl cellulose suspension, colony formation on monolayers of normal cells, and presence of viral antigens. This analysis revealed that only 5 of the clones were indistinguishable from 3T3 cells; the remaining 35 clones differed from 3T3 cells in that they grew as rapidly in 1% calf serum as standard SV40 transformed cells. Of these 35 clones, ten corresponded to standard transformants previously described. Another ten showed other growth properties intermediate between 3T3 cells and standard transformants. These intermediate clones had lower levels of viral T-antigen than standard transformants and showed considerable heterogeneity in staining from cell to cell. The remaining 15 clones were T-antigen negative and had saturation densities slightly higher than that of 3T3 cells. These changes in cellular behavior are stable on recloning.

### INTRODUCTION

Simian virus 40 can radically alter the *in vitro* behavior of fibroblastic cells. This transformation of cellular growth properties into patterns similar to those seen in tumor cells is detected in a minority of the infected cells by selective assays. Various approaches have been used to analyze the role of the virus in this process; in particular, viral mutants temperature-sensitive for lytic growth have been isolated and some have been found to affect transformation (Tegtmeyer, 1972; Kimura and Dulbecco, 1973; Robb *et al.*, 1972). Also cells reverted to a more normal cellular behavior have been selected from transformed populations, and SV40 recovered from them has been analyzed (Pollack *et al.*, 1968; Renger and Basilico, 1972; Ozanne, 1973; Vogel and Pollack, 1973; Vogel *et al.*, 1973; Culp and Black, 1972). Though many of these experiments indicate that viral functions are required for the transformation process, the relationship of a

viral function(s) to the observed alterations in cellular behavior is not at all clear. In this study we have approached the problem of virally induced growth alterations somewhat differently. Rather than subject infected cells directly to a selective transformation assay, we have obtained random clones of cells arising after SV40 infection and then tested them in a number of transformation assays. Such an analysis should detect changes in one or a few growth properties if such changes occur and thus define more completely the physiological effect of SV40 on most of the cells it infects.

The numerous assays which have been used to monitor *in vitro* transformation include growth to high density (Todaro *et al.*, 1964), growth in agar or methyl cellulose suspension (Macpherson and Montagnier, 1964; Stoker *et al.*, 1968), focus formation on monolayers of normal cells (Temin and Rubin, 1958), growth in depleted or low concentrations of serum (Smith *et al.*, 1971;

Holley and Kiernan, 1968) and morphological changes (Stoker and Abel, 1962). Additionally, a virus-specific nuclear antigen (T-antigen) has been found in cells acutely infected with or transformed by SV40 (Black *et al.*, 1963; Pope and Rowe, 1964).

Several observations suggest that each assay of transformation reflects a different change in cellular physiology. In particular, the frequency of cellular transformation induced by SV40 depends upon the particular assay used (Black, 1966). Also, it is possible to obtain cells which, though transformed in their ability to grow in depleted serum, are normal in their saturation density (Smith *et al.*, 1971; Scher and Nelson-Rees, 1971). The results presented here extend these observations, and demonstrate that after SV40 infection most clones show a reduced growth requirement for serum components. Furthermore, a high proportion of SV40 transformants show only intermediate changes in other cellular properties associated with oncogenic transformation. It is concluded from the data presented here that SV40 infection of 3T3 cells can induce several stages of transformed behavior. A preliminary report of these results has appeared elsewhere (Risser and Pollack, 1974).

#### MATERIALS AND METHODS

*Cell cultures.* Cell lines and clones were maintained in Dulbecco's modified Eagle's medium (DME) (Gibco-H21) containing 10% calf serum (Colorado Serum Company) with 50  $\mu\text{g}/\text{ml}$  Gentamicin. All cells were transferred weekly at an inoculation density of about  $5 \times 10^2$  cells/cm<sup>2</sup> on Falcon plastic dishes. A recently recloned line of mouse 3T3 cells (Todaro and Green, 1963) and a SV40 transformed 3T3 cell line, SV101 (Todaro *et al.*, 1964), were used routinely in each series of assays.

*Virus.* SV40 (strain 776, originally from NIH) was serially plaque purified three times on BSC-1 cells, a line of African green monkey kidney cells. The lysate obtained from a single plaque was passaged twice at low multiplicities of infection (<0.1 PFU cell), and virus was then purified according to the method of Black *et al.* (1964) and twice banded to equilibrium in CsCl. All plaque assays were done on BSC-1 cells in

medium containing 2% FCS, 98% DME mycostatin (50 units/ml) and 0.9% agar (Difco-Bacto). Plaques were detected at 10 days and counted at 14 days.

*Random cloning of SV40 cells.* A 60 mm plate containing  $5 \times 10^5$  3T3 cells was infected with 0.2 ml DME containing  $10^9$  PFU of purified SV40 or mock infected with 0.2 ml DME for 2 hr at 37°. Medium was then replaced, and the next day cells were trypsinized, counted, and replated at densities of  $5 \times 10^4$ ,  $5 \times 10^3$ ,  $5 \times 10^2$ ,  $5 \times 10^1$ , and 5 cells per plate. Plates were cultured for 2 weeks, and then most plates were stained with Harris hemotoxylin. The plating efficiency (EOP) was scored as the number of colonies formed per cells plated and the transformation efficiency as the number of dense colonies per total colonies. From unfixed duplicate plates containing 10–20 colonies, all well isolated colonies were circled, classified as morphologically normal or transformed, and cloned using a steel cloning cylinder. Colonies were scored as morphologically transformed if they showed several layers of cell growth and the tightly packed random orientation seen in isolated colonies of SV101 cells.

To clone, colonies were picked without preference for a particular morphology; 50–75% of the colonies on a given plate were cloned. Each clone was passaged for 1 month, and then tested in each assay or frozen for future testing.

*Viral antigens.* Assays for the presence of viral antigens were done by indirect immunofluorescence. Cells were plated on coverslips at a density of  $2 \times 10^4$  cells/cm<sup>2</sup>. Four to twelve hours before staining, medium was changed to 1% calf serum-DME. Coverslips were rinsed in phosphate-buffered saline medium (PBS) (at 4°), fixed 5–10 min in acetone (at –20°), and stained for 1 hr at 37° with direct antibody. Coverslips were rinsed and stained with rhodamine-conjugated bovine serum albumin and fluorescein-conjugated counterstain for 1 hr at 37°. After rinsing in PBS, coverslips were examined at 400 $\times$  magnification under dark-field ultraviolet illumination (Zeiss). Hamster anti-T antibody (Flow Laboratories), fluorescein-conjugated rabbit anti-hamster  $\gamma$ -globulins (Antibodies, Inc.) and rhoda-

mine-conjugated bovine serum albumin (Huntingdon Research Center) were used in PBS at dilutions of  $\frac{1}{5}$ ,  $\frac{1}{10}$  and  $\frac{1}{30}$ , respectively. Complement fixation assays were performed according to the method of Osborn and Weber (manuscript in preparation).

*Growth in medium containing 10% or 1% calf serum.*  $2 \times 10^4$  cells in 2 ml of medium containing 10% or 1% calf serum were seeded onto 35 mm Falcon dishes. Medium was changed every third day and cell counts taken daily using a Coulter counter. Saturation densities are the equilibrium densities which the cells maintain for the last 3 to 4 days of the growth experiment. Doubling times were calculated from the slope of the initial logarithmic growth curve before density-dependent inhibition of cell increase was seen.

*Growth in medium containing methyl cellulose.* Cells were plated in 4 ml of medium containing 10% calf serum, 1.2% Methocel (Dow Chemical Company, 4000 cps), 90% DME at densities of  $10^5$ ,  $10^4$ ,  $10^3$ , or  $10^2$  cells/60 mm dish over a layer of 0.9% agar (Difco-Bacto), 10% calf serum, 90% DME. Cultures were fed with an additional 4 ml of Methocel medium at 1 and 2 weeks and scored and measured at 3 weeks. Colonies  $\geq 100$  cells were scored as positive colonies in plating efficiency experiments. Measurements of colony size were done on cells and colonies by use of an eyepiece reticle. The reticle was calibrated using corn, pecan, ragweed, and mulberry pollen grains of diameters 87.5, 47.5, 19.5 and 13.5  $\mu\text{m}$ , respectively. From the diameter of the colony the volume was calculated. Volume increments were assumed to be proportional to increases in cell number, and the cell

number was inferred from the increase in volume.

*Growth on normal monolayers.* 100 or 1000 cells were plated on confluent 3T3 monolayers and on plastic dishes. Medium was changed every third day and dishes fixed with formalin-PBS and stained with Harris-hematoxylin at day 10. Colonies sufficiently dense to be seen without the aid of a microscope were scored. A single medium preparation was used throughout a plating experiment. The variation observed among the absolute plating efficiencies of different clones (Tables 1 and 2) is largely due to differences in serum preparations; a 2-fold difference in plating efficiencies is not considered significant.

RESULTS

*Random Selection of Clones of SV40 Cells*

Subconfluent 3T3 cells were infected with SV40 and plated at various dilutions as in Materials and Methods. After 2 weeks the transformation efficiency was determined and 40 randomly selected colonies were cloned from plates which received infected cells. The transformation efficiency of 10.5% in this experiment (Table 1) is comparable to that found by others for similar doses of virus. It is apparent from the low number of morphological transformants picked that no preference was shown in picking clones (Table 1). To serve as control normal lines, four clones were picked from plates which received mock infected cells. To serve as control transformed clones, four dense foci were picked from plates which received  $10^3$  infected cells. SV101 cells and the parental 3T3 cells served as additional controls. All

TABLE 1  
RANDOM SELECTION OF SV40 CLONES

Protocol	Virus Multiplicity (PFU/cell)	Transformation <sup>a</sup> efficiency ( $\frac{\text{transformed colonies} \times 100}{\text{total colonies}}$ )	Efficiency of plating ( $\frac{\text{colonies} \times 100}{\text{cells plated}}$ )	Clones picked	Clones morphologically transformed (%)
Virus Infected	$2 \times 10^3$	10.5	83	40	15
Mock Infected	0.0	$\leq 0.001$	91	4	0

<sup>a</sup> As in Materials and Methods.

**TABLE 2**  
**PROPERTIES OF SV40 CLONES**

SVR clone number	T-anti-gen	10% Calf serum		1% Calf serum		EOP <sup>b</sup> in methyl cellulose	EOP <sup>b</sup> on plastic	EOP <sup>b</sup> on 3T3 monolayer	EOP on monolayer/EOP on plastic
		Saturation density <sup>a</sup>	Doubling time (hr)	Saturation density <sup>a</sup>	Doubling time (hr)				
<b>Experimental clones</b>									
11	I	19.5	19	3.8	34	14.6	41.5	≤0.05	≤0.001
12	I	27.0	18	3.6	28	3.7	42.0	0.5	0.012
13	I	18.0	17	3.3	33	0.2	47.3	0.2	0.004
14	I	22.0	17	4.0	26	6.9	36.0	≤0.05	≤0.001
15	+	40.0	24	4.0	45	11.8	7.0	5.5	0.79
16	I	22.0	22	3.2	29	0.5	70.0	≤0.05	≤0.001
17	I	29.0	18	3.9	32	2.2	59.7	0.1	0.002
18	I	31.0	19	5.7	32	1.3	34.5	0.15	0.004
21	+	46.0	16	8.8	45	30.1	35.7	30.3	0.85
22	I	45.0	22	3.6	41	3.5	52.0	13.0	0.25
23	+	60.0	18	7.0	44	36.7	47.3	28.3	0.60
25	+	60.0	16	10.0	31	29.0	58.0	31.7	0.55
26	+	46.0	17	3.0	34	53.0	48.5	4.7	0.10
32	I	18.5	24	2.5	38	0.7	25.5	1.4	≤.055
33	-	8.5	26	0.9	84	≤0.001	22.0	≤0.03	≤0.001
34	-	8.5	28	1.0	88	≤0.001	27.3	≤0.03	≤0.001
35	-	7.6	26	0.9	100	≤0.001	25.0	≤0.03	≤0.001
36	-	8.0	24	0.6	63	≤0.001	7.8	≤0.03	≤0.001
41	-	9.5	19	2.0	48	≤0.001	38.5	≤0.03	≤0.001
42	-	12.5	15	2.4	37	≤0.001	40.5	≤0.03	≤0.001
43	-	8.0	20	2.5	32	≤0.001	44.7	≤0.03	≤0.001
44	-	11.0	24	1.8	43	≤0.001	36.0	≤0.03	≤0.001
45	-	12.5	22	1.8	38	≤0.001	17.3	≤0.03	≤0.001
47	-	11.5	24	1.8	26	≤0.001	32.0	≤0.03	≤0.001
51	-	16.5	17	1.8	36	≤0.001	27.0	≤0.03	≤0.001
54	-	15.0	17	1.9	29	≤0.001	30.7	≤0.03	≤0.001
55	-	13.0	22	2.3	46	≤0.001	42.0	≤0.03	≤0.001
56	-	16.0	19	3.0	49	≤0.001	51.8	≤0.03	≤0.001
57	-	16.0	17	3.0	31	≤0.001	58.0	≤0.03	≤0.001
58	-	11.5	22	0.7	55	≤0.001	31.0	≤0.03	≤0.001
63	I	15.0	15	2.5	29	≤0.001	43.0	≤0.03	≤0.001
81	+	50.0	17	10.5	24	10.7	53.0	52.0	1.0
82	-	12.0	20	3.0	29	≤0.001	37.3	≤0.03	≤0.001
84	+	45.0	16	11.0	27	10.6	15.3	40.0	2.6
85	+	43.0	14	7.6	22	44.0	29.0	23.5	0.81
86	+	46.0	17	8.0	34	58.0	36.5	39.5	1.1
87	+	35.0	19	9.3	34	38.6	27.7	28.3	1.0
94	-	11.0	22	3.4	26	≤0.001	51.0	≤0.03	≤0.001
95	-	13.0	24	2.9	31	≤0.001	37.3	≤0.03	≤0.001
97	-	10.7	19	3.2	48	≤0.001	31.7	≤0.03	≤0.001
<b>Control mock clones</b>									
101	-	9.0	20	1.0	67	≤0.001	36.0	≤0.03	≤0.001
103	-	7.0	22	1.3	66	≤0.001	32.7	≤0.03	≤0.001
104	-	7.0	26	0.8	70	≤0.001	35.5	≤0.03	≤0.001
106	-	8.5	19	1.4	86	≤0.001	26.5	≤0.03	≤0.001
3T3	-	7.6	23	0.9	66	≤0.001	39.0	≤0.03	≤0.001
<b>Control transformed clones</b>									
111	+	60	18	16.0	36	16.1	33.0	24.3	0.74
114	+	45	17	11.5	19	26.0	37.0	19.5	0.53
122	+	50	24	10.0	43	28.5	39.3	10.0	0.25
123	+	45	21	9.0	41	45.0	25.5	17.0	0.67
SV101	+	57	16	11.0	26	27.0	38.0	41.3	1.1

<sup>a</sup> Units are cells/cm<sup>2</sup> × 10<sup>4</sup>.

<sup>b</sup> Units are colonies/100 cells plated.

clones were passaged a minimum of 1 month before they were tested for transformed properties.

*Viral Antigens*

Though not a selective assay, the persistence of SV40 specific antigens has served as an indication of the presence of a functioning viral genome. For that reason all clones were stained for viral T-antigen by indirect immunofluorescence. Three patterns of staining were seen: positive, negative, and intermediate; examples are shown in the photographs in Fig. 6. Intermediate clones showed 10-20% bright positive nuclei, 50-80% weakly fluorescent nuclei and 10-30% dark nonfluorescent nuclei. Control transformants stained uniformly positive while negative clones did not show nuclear fluorescence. These staining patterns can be used to classify clones, and thus clones will be referred to as T-antigen negative, positive or intermediate (Table 2).

The staining method was checked by staining mixtures of SV101 and 3T3 cells at various ratios. When SV101 cells were mixed with 3T3 cells in ratios of 1/2 or 1/100, 215/1000 or 1 1/2/1000 cells, respectively, showed bright nuclear fluorescence. When a mixture of SV101 and 3T3 cells (ratio 1:4) were plated very sparsely and allowed to form colonies on coverslips, 3/25 colonies were uniformly T-antigen positive. No intermediate or mixed colonies were seen. These results suggest that T-antigen intermediate clones seen among the experimental clones are not due to artifacts introduced by the staining or cloning procedures used.

Levels of complement-fixing T-antigen were determined on equal numbers of cells from various clones. As can be seen in Table 3, indirect immunofluorescence and complement fixation show close agreement. Those clones designated intermediate by immunofluorescence have approximately a 10-fold reduction in complement-fixing titers. Clones designated negative by immunofluorescence show background complement fixing titers, comparable to that of 3T3.

V-antigen production was also determined by indirect immunofluorescence. Of the 25 clones tested, none were V-antigen positive, whereas BSC-1 cells infected 48 hr earlier with SV40 fluoresced brightly in the nucleus.

TABLE 3  
T-ANTIGEN DETERMINATION ON SV40 CLONES

SVR clone number	Immuno-fluorescence	Complement fixation
13	I	10.0
16	I	3.0
18	I	10.0
22	I	3.0
35	-	0.2
43	-	0.8
63	I	10.0
85	+	100.0
86	+	25.0
3T3	-	0.1
SV101	+	100.0

\* Complement fixation assays were performed on frozen-thawed lysates of approximately  $5 \times 10^6$  cells resuspended in about 100  $\mu$ l of PBS according to the procedure of Osborn and Weber (manuscript in preparation). Values are reported as the percentage of the SV101 complement-fixing titer.

*Growth in Medium Containing 10% and 1% Calf Serum*

Growth experiments were carried out on all clones in 10% and 1% calf serum (Table 2). As can be seen in Fig. 1, such experiments differentiated 3T3 cells and SV101 cells on the basis of saturation density in either serum concentration. As is graphically shown in the histograms in Fig. 2, a wide range of saturation densities was found among experimental clones in either 10% calf serum (Fig. 2A) or 1% calf serum (Fig. 2B). In contrast, control transformants or control mock-infected clones showed a relatively narrow distribution of saturation densities. The level of T-antigen in a clone is rather well correlated with its saturation density (Fig. 2).

The doubling times of all clones in 10% calf serum ranged from 16 to 24 hr, providing little distinction between normal and transformed cells. The doubling time in 1% calf serum, however, differed considerably between normal and transformed lines; 3T3 cells doubled in about 80 hr in 1% calf serum, whereas SV101 cells doubled in only 30 hr (Table 2, Fig. 1). Thirty-five of the experimental clones showed doubling times comparable to those of transformed cells in 1% calf serum (Fig. 3). As can be seen many of these clones lacked viral T-antigen.

*Growth in Medium Containing Methyl Cellulose*

Each clone was plated in suspension culture containing 1.2% methyl cellulose. Again, experimental clones showed a spectrum of plating efficiencies (Fig. 4). In this assay, however, less than one in  $10^5$  cells from any T-antigen negative clone plated, a result which did not distinguish them from mock-infected clones. T-antigen intermediate clones ranged from about 0.1% to 10% in plating efficiencies, whereas T-antigen positive clones plated efficiencies of 10–50%.

To determine whether cells from T-antigen negative clones were dividing in methyl cellulose, yet not reaching the size necessary for visual scores, measurements were made on individual cells or colonies. As can be seen in Fig. 5a, 3T3 cells underwent few if any divisions during the 3-week course of the assay, whereas SV101 cells underwent many divisions to finally give a mean colony size of 250 cells (Fig. 5b). This represents a significant lengthening of the doubling time of SV101 cells as compared to growth in medium lacking methyl cellulose (62 vs 16 hr), suggesting this is a more restrictive growth assay. T-antigen negative clones SVR82,

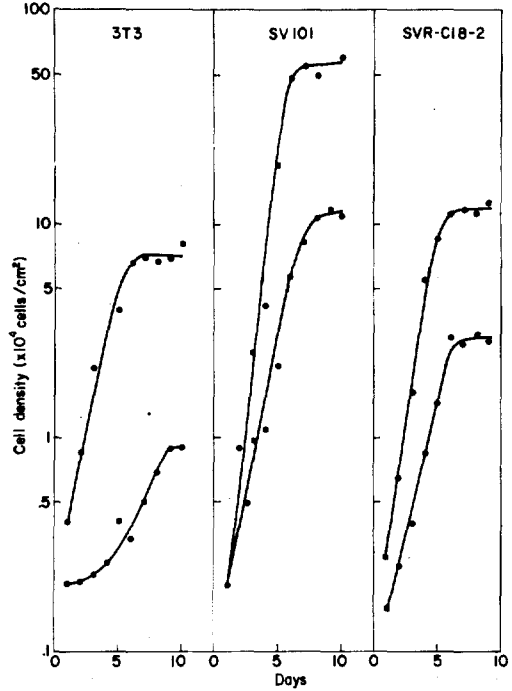


FIG. 1. Typical growth experiments of 3T3, SV101, SVR82. Cells were seeded as in Materials and Methods in 10% calf serum (upper curves) or 1% calf serum (lower curves), and cell counts taken daily. Medium was changed every third day.

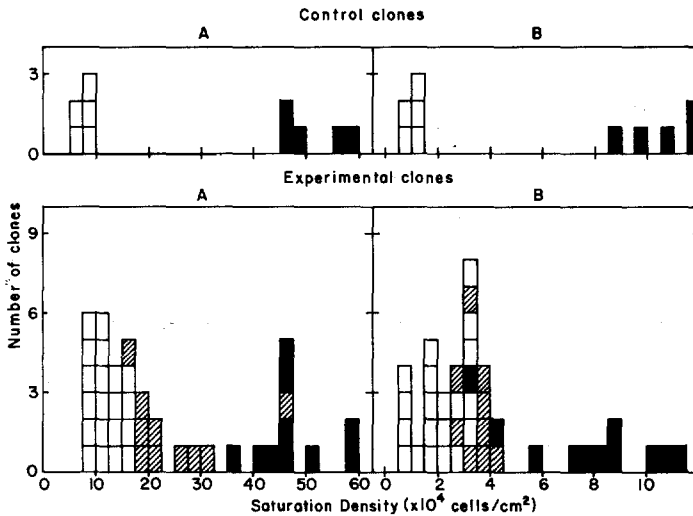


FIG. 2. Histograms of saturation densities of SV40 clones. Growth experiments were carried out in (A) 10% calf serum and (B) 1% calf serum as in Materials and Methods. □, T-antigen negative clones; ▨, T-antigen intermediate clones; ■, T-antigen positive clones

SVR34, and SVR42 and T-antigen intermediate clone SVR63 showed few divisions during the 3-week assay (Figs. 5k, 5h, 5i, 5j, respectively). The behavior of SVR63 is

unique, since the other five T-antigen intermediate clones measured showed some limited growth in methyl cellulose suspension. Cells from T-antigen intermediate clones SVR13 and SVR32 continuously divided during the three week assay (Figs. 5c and d; and Fig. 5f and g, respectively). In their continuous though slow division in methyl cellulose they differ from the abortive transformants described by Stoker (1968) which underwent 3 to 4 divisions and then ceased growing in suspension culture. The great majority of cells in these intermediate clones do not divide as rapidly as SV101 cells or other T-antigen-positive transformed cells. Most colonies from these clones are much smaller than SV101 colonies, and thus are not detected when plating efficiency is scored without a microscope.

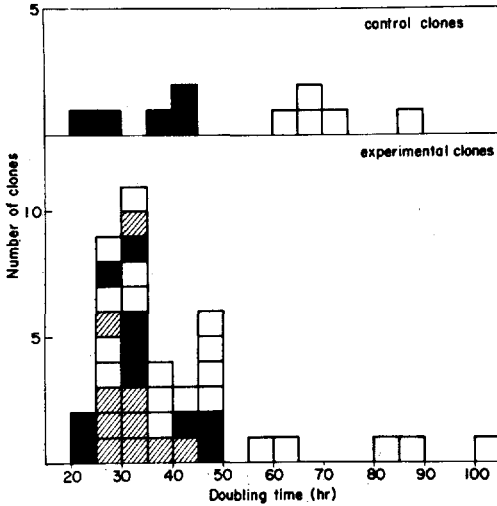


FIG. 3. Histogram of doubling times of SV40 clones in 1% calf serum. Doubling times were determined during the exponential growth phase before density-dependent inhibition of cell increase was seen. □, T-antigen negative clones; ▨, T-antigen intermediate clones; ■, T-antigen positive clones.

*Growth on Normal Monolayers*

When clones were plated on confluent 3T3 monolayers, a range of plating efficiencies was seen (Fig. 6). T-antigen negative clones form dense colonies on normal monolayers with efficiencies of  $\leq 10^{-3}$  as compared to plastic dishes, whereas T-antigen positive clones and control transformants plate as efficiently on normal monolayers as they do

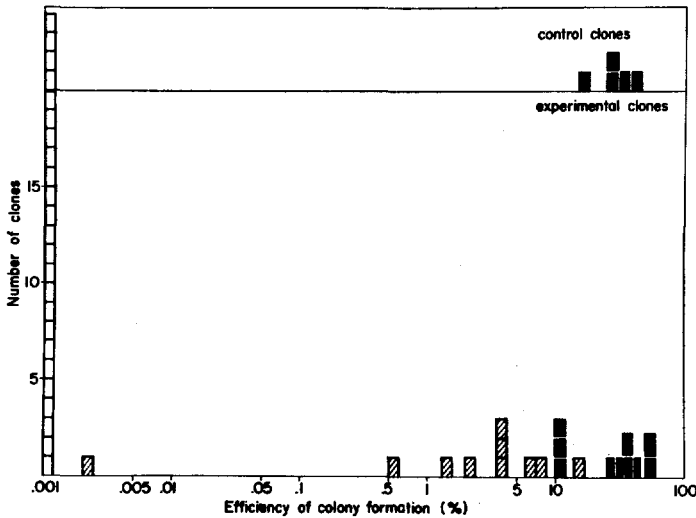


FIG. 4. Histogram of plating efficiencies of SV40 clones in methyl cellulose suspension. EOP was determined as in Materials and Methods. □, T-antigen negative clones; ▨, T-antigen intermediate clones; ■, T-antigen positive clones.

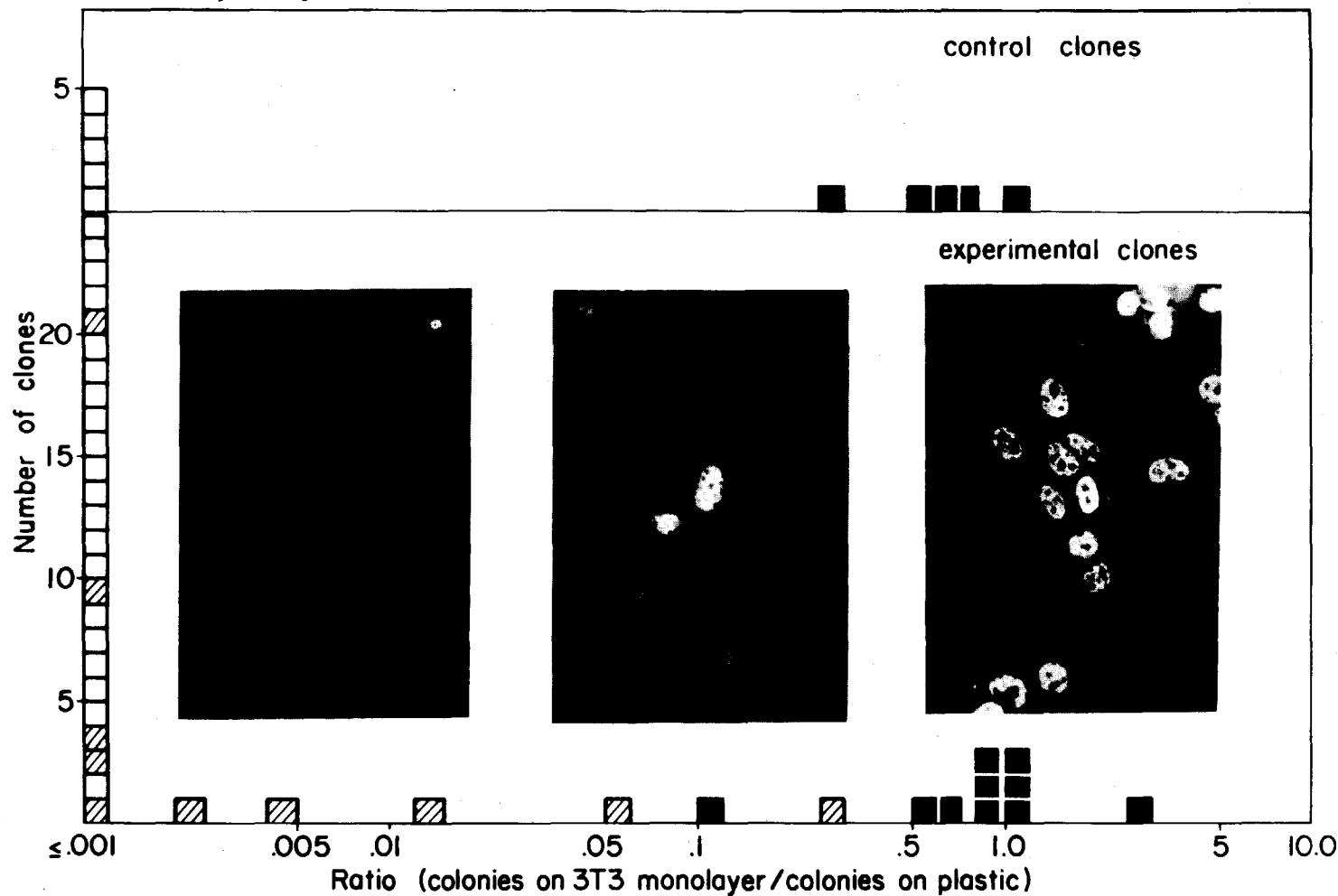


Fig. 6. Histogram of plating efficiency of SV40 clones on 3T3 monolayers as compared to plating efficiency on plastic dishes. EOP of SV40 clones was determined on plastic and on 3T3 monolayers as in Materials and Methods and the monolayer EOP divided by the EOP on plastic to give the values reported here. □, T-antigen negative clones; ▨, T-antigen intermediate clones; ■, T-antigen positive clones. The inserted photographs are examples of T-antigen staining patterns and are from left to right T-antigen negative 3T3 cells, T-antigen intermediate SVR16 cells, and T-antigen positive SV101 cells.



TABLE 4  
STABILITY OF GROWTH CHARACTERISTICS

SVR clone No.	T-antigen			Saturation density in 10% CS			Doubling time in 1% CS			EOP in methyl cellulose			EOP on monolayers EOP on plastic		
	1 mo.	5 mo.	5 mo. Reclone	1 mo.	5 mo.	5 mo. Reclone	1 mo.	5 mo.	5 mo. Reclone	1 mo.	5 mo.	5 mo. Reclone	1 mo.	5 mo.	5 mo. Reclone
42	-	-	-	8	12.5	10.0	24	37	47	0.001	NT <sup>b</sup>	0.001	0.01	NT	0.01
57	-	-	-	15	16	15	40	31	31	0.001	NT	0.001	0.01	NT	0.01
63	I*	I*	I*	9	15	15	33	29	29	0.01	0.001	0.001	0.01	NT	0.01
81	+	+	+	60	50	70	20	24	22	11	NT	20	1.0	NT	0.7
82	-	-	-	10.5	12	13.5	40	29	29	0.001	NT	0.001	0.01	NT	0.01
84	+	+	+	50	45	60	32	27	30	11	NT	28.5	2.6	NT	2.0

\* I\* Intermediate.

<sup>b</sup> NT, not tested.

TABLE 5  
PROPERTIES OF SUBCLONED LINES FROM  
INTERMEDIATE CLONES

Clone	T-antigen	EOP in methyl cellulose (%)	EOP on monolayer EOP on plastic
Parental clones			
13	I	0.2	0.004
32	I	2.2	0.055
Random subclones			
13-1	I	0.01	NT
13-2	I	0.11	NT
13-3	I	0.58	NT
Methyl cellulose subclones			
13MC1	+	65.0	1.2
13MC2	+	66.5	0.3
13MC3	I	102	0.5
13MC4	+	22.0	0.9
32MC1	+	16.0	1.5
32MC2	I	26.0	NT
32MC3	+	24.0	1.1
32MC4	NT	29.0	1.0

\* NT, not tested.

sion with low frequencies, comparable to those of the parental clone. When methyl cellulose selected subclones were picked and tested, however, they plated with high efficiencies comparable to those of standard transformants. In general, these MC subclones also plated better on normal monolayers. The T-antigen staining pattern of such clones also differs from that of parental lines in that methyl cellulose subclones show

more intense nuclear fluorescence. In some cases, e.g., 13MC 2, 13MC 4, heterogeneity is still seen from cell to cell. It is apparent, then, that cells which show fully transformed properties can be isolated from intermediate clones. Once such cells are obtained, i.e., by cloning in methyl cellulose, they continue to behave like standard transformants.

#### DISCUSSION

The transformation of mouse 3T3 cells by SV40 is a low-frequency event requiring input multiplicities of  $10^4$  PFU/cell to achieve 50% transformation in a standard assay (Black, 1966; Todaro and Green, 1966). In the present study we observed 10% transformation at a multiplicity of  $2 \times 10^8$  PFU/cell. When random clones of cells arising out of this infection were tested in a number of transformation assays, 87.5% were detectably different from 3T3 cells. Thus while the application of a selective assay detected only a small fraction of the cells as being affected by virus, most cells were in fact stably altered by SV40.

The cell types obtained from this experiment may be classed into four general categories (Table 6). The first two are those expected from the assay, namely clones indistinguishable from 3T3 cells and clones which behave like standard transformants. The percentage of clones which are classed as standard transformants is higher than that seen when clones were first morphologically scored (10 of 40 clones vs 6 of 40 clones).

TABLE 6  
CLASSES OF SV40 CLONES<sup>a</sup>

Class	Percent of clones	T-anti-gen	Saturation density in 10% CS ( $\times 10^4$ cells/cm <sup>2</sup> )	Doubling time in 1% CS (hr)	EOP in methyl cellulose (%)	Colony formation on normal cells <sup>b</sup>
Normal	12.5	-	8.5 (7.6-9.5)	78 (55-100)	$\leq 0.001$	$\leq 0.001$
Transformed	25	+	47 (35-60)	34 (24-45)	32 (11-58)	0.94 (0.1-2.6)
Minimal-transformed	37.5	-	15 (9.5-16.5)	34 (26-49)	$\leq 0.001$	$\leq 0.001$
Intermediate-transformed	25	$\pm$	25 (15-45)	32 (29-41)	4 (0.5-14.6)	0.03 (0.001-0.20)
<i>Control</i>						
Normal		-	7.8 (7.9)	70 (60-86)	$\leq 0.001$	$\leq .05$
Transformed		+	53 (47-60)	37 (26-43)	25 (16-30)	0.7 (0.25-1.1)

<sup>a</sup> The mean numerical value for each parameter of transformation is reported for each class of transformant, and in parentheses the range of values seen among different clones in each class is given.

<sup>b</sup> Expressed as the ratio of EOP on 3T3 monolayers to EOP on plastic dishes.

Delayed transformations have previously been observed (Stoker, 1963; Todaro and Green, 1966), and could well account for the increase in transformed clones. This is quite reasonable since only the center of the colony was observed when colonies were morphologically scored and thus a small transformed sector might have been missed.

The third class of cells obtained differ from 3T3 cells in their lower doubling time in 1% calf serum and their slightly higher saturation densities. They are, however, T-antigen negative. Two facts strongly suggest that these cells are significantly different from 3T3 cells. The serum dependence of such cells is stable on recloning, and no minimal transformed cells were detected among mock controls. Also, 3T3 cells continuously passaged during the course of this experiment retained their high serum requirement. In a preliminary report (Risser and Pollack, 1974) this class of cells was referred to as serum transformants; the term "minimal transformants" better describes their behavior and will be used in this and all subsequent reports.

The last category of cell types is actually a collection of cell lines showing growth properties intermediate between those of normal cells, e.g., 3T3, and transformed cells, e.g., SV101. All intermediate lines grow well in 1% calf serum and have a lower titer of complement fixing T-antigen as well as considerable intercellular variation in antibody

staining. The plating efficiency on 3T3 monolayers of T-antigen intermediate lines is approximately 30-fold reduced from that observed among standard transformants. The saturation density of T-antigen intermediate lines is about 50% of that of standard transformants, however. In their relative inability to plate on normal cells, SV40 intermediate lines resemble polyoma-transformed BHK cells (Stoker, 1964). When intermediate clones are plated in methyl cellulose suspension a small minority of the cells form large colonies (>200 cells) by the end of 3 weeks. Such segregated colonies behave in all assays like standard transformants and have increased levels of viral T-antigen.

Some lines found in this study are in some ways similar to those seen previously. Smith *et al.* (1971) and Scher and Nelson-Rees (1971) have described cell lines which grow to low or intermediate densities, grow in medium containing serum depleted of  $\gamma$ -globulin fractions, and contain viral T-antigen. Some of their lines may correspond to intermediate clones described here, though closer comparison is necessary to establish this. In addition we have detected a class of minimal transformants which lack viral T-antigen. It must be pointed out that the assay used by Smith *et al.* (1971) and Scher and Nelson-Rees (1971) differs from the assay used here in that in their assay cells are plated sparsely in depleted medium and

the plating efficiencies measured. In our assay cells are plated at  $\frac{1}{100}$  confluence in 1% calf serum and the growth rates measured. The former assay is probably more complicated in that cells are required to overcome the effects of depletion of medium on Balb/3T3 monolayers as well as removal of serum  $\gamma$ -globulin fractions.

It could be argued that the wide variation in transformed properties observed in this study resulted from the heteroploid condition of the 3T3 parental cell. This is probably not the reason for such a spectrum of transformed lines since SV40 infection of secondary embryonic rat cells gives rise to clones of cells showing a similar diversity of transformed expression (Pollack, Risser, Arelt, and Rifkin, unpublished observations). After SV40 infection we have obtained clones of established rat cells which grow to the same density as secondary rat embryo fibroblasts and do not plate in methyl cellulose suspension, clones which plate quite well in suspension and grow to high saturation densities, and clones intermediate in both these properties. All of these clones grow well in 1% serum.

The nonselective nature of this experiment should allow one to correlate given changes in growth behavior induced by SV40 without the preselection of a particular cell type. In general, this study shows quite good correlation of transformed properties; cells that grow to high saturation densities plate well on normal monolayers and in methyl cellulose suspension, and have high levels of SV40 specific T-antigen. Cell lines intermediate in densities plate with intermediate efficiencies on monolayers and in methyl cellulose and show intermediate levels of T-antigen. Furthermore, when transformed segregants are selected from such intermediate clones, they show coordinate increases in plating in suspension culture, plating on monolayers and levels of T-antigen.

There are, however, some significant exceptions to the coordinate nature of these changes. The great majority of the infected clones grow rapidly in 1% calf serum, regardless of the level of viral T-antigen expression. In 40% of the experimental lines this rapid growth in low serum concentrations is the

major difference from 3T3 cells. A somewhat analogous situation has been seen in various revertant lines obtained by negative selection of variants from transformed cell populations (Pollack *et al.*, 1968; Vogel and Pollack, 1973; Vogel *et al.*, 1973; Ozanne, 1973). When revertant cells are selected for the loss of transformed properties other than growth in low serum concentration, they generally retain the serum dependence of transformed cells. When they are selected for the inability to grow in 1% serum, they also revert in many other transformed properties (Vogel and Pollack, 1973). Taken together, these results suggest the primary physiological effect of SV40 infection on mouse 3T3 cells is a decrease in the cellular requirement for serum components. This change does not require continued viral T-antigen expression, and in revertant cells is retained even though other growth properties have returned to a more normal state.

This study was undertaken to better define what is meant by SV40 "transformation" of cellular growth properties. It is apparent from our data that several patterns of transformed behavior can be induced by SV40. These may correspond to different stages of transformed development as has been previously described for polyoma infected hamster cells *in vitro* (Vogt and Dulbecco, 1963). We do not, however, have any data that relates the stages described here sequentially one to another. It is entirely possible these different types of cellular behavior are the result of different cellular responses to viral infection. Either explanation of this diversity of transformed behavior suggests the process of cellular transformation by SV40 is the result of several complicated interactions of cellular and viral genes and not simply the result of a single viral gene acting to directly transform a cell. Previous *in vitro* transformation studies have emphasized the most "transformed" cells, e.g., cells resembling SV101, in attempting to correlate virus expression with transformed cellular behavior. It appears to us, however, that less "transformed" stages, e.g., minimal transformants or intermediate transformants, may more directly relate virus expression to cellular growth alterations.

ACKNOWLEDGMENTS

We thank Mary Osborn for performing complement fixation assays, Nancy Hopkins and Art Vogel for their helpful discussions, and Carole Thomason and Sue Arelt for their excellent technical assistance. This work was supported by a Public Health Service predoctoral fellowship to R.R. (4-F01-GM-49503) and a grant from the National Cancer Institute (CA13106).

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