

From MECHANISMS OF VIRUS DISEASE,
Ed. by W. S. Robinson & C. F. Fox.
© 1974 by W. A. Benjamin, Inc.,
Menlo Park, Calif.

THE DIFFERENT STABLE PATTERNS OF GROWTH CONTROL
INDUCED BY SV40 INFECTION OF NORMAL CELLS

Robert Pollack and Rex Risser

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724, USA

ABSTRACT. When different assays of growth control are used, SV40 is found to induce more than one type of transformed cell.

INTRODUCTION

By definition, transformed cells must be able to grow where normal cells do not. Conditions which severely restrict the in vitro growth of normal cells while permitting the growth of transformed cells are presented in Table 1. A fully transformed cell line is one that can grow in all of these restrictive conditions.

Previous work on in vitro transformation has focused on cells which show unrestricted growth in all of these assays. We demonstrate here the existence of virus infected cells which show unrestricted growth in only one or a few of these assays and discuss their significance for the mechanism of virally induced transformation. In studying the process of in vitro transformation, we have used the small DNA-containing tumor virus SV40 (1,2,3) and two different types of "normal" cells.

The first cell type is the mouse fibroblast line 3T3, a post-crisis, established, heteroploid, clonable line derived from Swiss mouse embryo culture (4). The second cell type is a diploid culture of rat embryo cells called REF. REF cells are prepared directly from 14 day rat embryos by mincing and trypsinization (Risser and Pollack, unpublished observations).

Both REF and 3T3 cells are unable to grow in three of the four selective conditions (Table 1). However, they differ in their ability to grow when plated very sparsely,

a characteristic referred to as cellular autonomy. Like most cells taken directly from an animal, REF cells are not autonomous, i.e. they cannot form separate colonies when inoculated at less than 10 cell/cm². 3T3 cells, on the other hand, can form colonies at any initial density with an efficiency of about 50%.

We demonstrate here that SV40 induces a variety of new patterns in cellular behavior, not simply the fully transformed pattern. The most common alteration by SV40 of 3T3, which is already capable of forming colonies, is the acquisition of a reduced serum requirement for growth (Table 1). The most common alteration of REF by SV40 is simply the acquisition of the ability to form a colony. Furthermore, we have found that these two changes always occur in any REF or 3T3 cells which show the other changes more commonly thought of as transformation (Table 1).

SV40 TRANSFORMATION OF 3T3

Historically, transformed cell lines have been isolated from post-crisis "normal" cell lines by their ability to grow under conditions which prevent the growth of the normal cell lines. Since post-crisis lines are already able to form colonies with a high efficiency, such lines cannot be used to assay the acquisition of autonomy induced by SV40 infection. However, the three other selective assays (Table 1) do permit isolation of transformants. While cell lines recovered from any of these assays after SV40-infection of 3T3 are clearly different from uninfected 3T3 clones, no selective assay can provide information on the sum of possible physiological states that SV40 can induce in 3T3 cells. In particular, clones transformed by one selective assay do not always show transformed behavior in the other selective assays (5).

CONTINUUM OF STABLE ALTERATIONS IN 3T3 AFTER SV40-INFECTIOIN

To isolate all possible types of SV40 transformants, a sparse culture of 3T3 was infected with purified SV40 at a high multiplicity. After adsorption of virus, the culture was trypsinized and cells were plated at very high dilution, to give 10-20 colonies per dish. At the same time, mock-infected 3T3 cultures were plated at the same high dilution. After two weeks, 40 well-isolated colonies from dishes of

infected cells and four colonies each from dishes of mock-infected cells were picked by the cloning-ring technique.

The standard assay for density transformation (Table 1) was also carried out at the same time. Here, transformants are defined as densely growing foci. In the non-selective assay 15% of clones picked were judged to be density-transformed, indicating that our cloning procedure did not accidentally favor density-transformants. Four dense foci from lower dilutions were also picked as standard transformants.

All clones were grown up for about one month, then frozen. The frozen clones were thawed a few at a time and their phenotypes systematically characterized. At no time was any culture subjected to low serum or high cell density. Thus, this assay yielded clones representative of all cell types arising after SV40 infection (6).

PROPERTIES OF CLONES

The clones isolated here were divisible into four classes (Table 2). The first class consists of clones identical to 3T3 in all of the selective assays (Table 1). The second class consists of clones identical to standard transformants. The third and fourth classes are composed of clones that are not identical to 3T3, but that would probably be indistinguishable from 3T3 in the standard transformation assay. Class III clones are able to grow well in 1% calf serum and are about two-fold denser than 3T3. Otherwise, they are indistinguishable from 3T3. Class IV clones are intermediate transformants. They contain SV101-like and 3T3-like cells.

The minimal and most common alteration in 3T3 after SV40 infection is the acquisition of the ability to grow in reduced serum (Table 2). Fully 88 percent of the experimental clones were able to grow in 1% calf serum, while none of the four mock-infected clones could do so (Table 2). Furthermore, the 12 percent of experimental clones that grew poorly in 1% calf serum were also untransformed in any other assay (6).

PERSISTENCE OF VIRAL Tag IN 3T3-CLONES TRANSFORMED BY SV40

Mock-infected clones, of course, were free of T-antigen by both assays (Table 2). The presence and amount of T-

antigen correlated well with the degree of anchorage-transformation and with the degree of density transformation. The intermediate clones, in particular, had low levels of T-antigen and a mosaic distribution of positive and negative cells. Clones in the largest class of transformants, the minimal-transformants (Table 2), did not contain T-antigen. Thus, the most common new clone is one with the ability to grow in restrictive serum, while lacking T-antigen and lacking the transformed properties which correlate with T-antigen--high density and growth in methocel.

EFFECTS OF SV40 ON RAT EMBRYO FIBROBLASTS

To isolate all possible types of SV40 transformants that form colonies, the following selective protocol was used. A confluent culture of REF cells (Figure 1A) was infected with purified SV40 at an input multiplicity of about 100 PFU/cell. After adsorption of virus, the culture was trypsinized and the cells were also plated at very high dilution, to give 1000 cells per dish. At the same time, mock-infected REF cultures were plated at the same high dilutions. While more than 100 colonies were seen on infected plates, less than one was seen on mock-infected plates (Figure 1B,C). All colonies of infected REF cells must have been altered in some way by SV40 in order to grow; we define this alteration as the acquisition of autonomy by normal fibroblastic cells. The colonies induced by SV40 did not all resemble each other. In particular, most colonies resembled the rare colony arising on a dish of mock-infected cells (Figure 2A,B). Other colonies of infected cells were slightly denser than the majority (Figure 2C). A small minority were very dense (Figure 2D).

PERSISTENCE OF VIRAL Tag IN REF COLONIES

To determine whether infected colonies contained Tag, SV40-infected and mock-infected REF cells were plated on glass coverslips at 10^2 cell/cm² and examined by immunofluorescent stain. To our surprise, many of the colonies did not contain T-antigen. We conclude that the minimal effect SV40 can have on a normal cell is to confer autonomy on that cell in the absence of persistent T-antigen and in the absence of any apparent transformation to high density.

PROPERTIES OF REF COLONIES

We cloned 12 colonies from an infected plate resembling Figure 1C. Each of these clones was grown up, recloned, and assayed for the retention or loss of growth control (Table 1). Since even uninfected REF cells grow to high densities in 10% serum, we were not surprised to find that all 12 clones also grew to high densities. However, the colonies differed markedly from one another and from uninfected REF cells in their ability to grow in low serum or in suspension in methocel. All clones grew in 1% serum, while REF cells did not. Fewer than one REF cell in 10^5 can form a colony in methocel. Each REF clone had a stable plating efficiency in methocel, which ranged from one cell in 10 to one cell in 10^5 . Preliminary results suggest that, for these SV40-transformed REF clones, growth in methocel and production of a cellular activator of plasminogen (7; Rifkin, this volume) are well correlated (Pollack, Risser, Arelt, and Rifkin, unpublished observations).

IMPLICATION FOR VIRAL GENETICS

SV40 apparently has many different effects on a transformable cell, but these fall into two distinct classes. The first is detected by the assays of autonomy and serum-transformation. The second is detected by the assays of density and anchorage. The second class seems to be dependent on the first: All transformants of the second class so far examined are also transformed in autonomy or serum. However, transformants of the first class need not be transformed in density or anchorage.

The role of viral genes in either class is unproven, but the presence of the one known viral gene product, T-antigen, correlates with the second class, and not the first. Most, if not all, attempts to assay variant SV40 strains for effects on transformation have been assays on the final steps in transformation, and thus have actually been attempts to isolate viruses that are simultaneously defective in their ability to cause both classes of transformation. We believe it unlikely that such mutants will be picked up. Rather, we suggest that new virus selective systems be constructed, in which viruses can be detected even if they are only ts or defective in one of the two classes.

IMPLICATION FOR DIFFERENTIATION

When any embryo is minced and its cells placed in culture, only a small minority of the cells survive the initial few days and divide (8). Of these, the fibroblasts are the most common (9). Other differentiated cells normally do not divide for long, even in dense culture. No normal cell can be expected to grow into a clone in sparse culture. We have shown that SV40 can confer the ability to plate on rat embryo fibroblasts with high efficiency. Many of these colonies lack T-antigen and appear normal. Other normal embryonic tissues should be infected with SV40, to determine whether SV40 can be used as a reagent for production of clonable, normal differentiated cell lines.

ACKNOWLEDGMENTS

We thank Sue Arelt and Carole Thomason for excellent, reliable technical assistance. Robert Goldman suggested the possible use of SV40 to establish differentiated normal lines. This research was supported by funds from the National Cancer Institute, NIH. R.R. was supported by NIH predoctoral fellowship 4-501-GM-49503-04.

REFERENCES

1. Black, P. and Rowe, W., *Virology* 19, 107 (1963).
2. Shein, H. and Enders, J., *Proc. Nat. Acad. Sci.* 48, 1164 (1962).
3. Todaro, G. and Green, H., *Virology* 23, 117 (1964).
4. Todaro, G. and Green, H., *J. Cell. Biol.* 17, 299 (1963).
5. Smith, H., Scher, C., and Todaro, G., *Virology* 44, 359 (1971).
6. Risser, R. and Pollack, R., *Virology*, in press (1974).
7. Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rifkin, D.B., and Reich, E., *J. Experimental Medicine* 137, 85 (1973).
8. Sato, G., Zaroff, L., and Mills, S., *Proc. Nat. Acad. Sci.* 46, 963 (1960).
9. Dulbecco, R., *Proc. Nat. Acad. Sci.* 38, 747 (1952).

Table 1. Selective Assays Yielding Transformed Cell Lines

<u>System</u>		<u>Typical Value</u>	
<u>Virus</u>	<u>Cell</u>	<u>Normal</u>	<u>Transformed</u>
SV40	REF	<0.1 colony per 10 ² cells	>10 colonies per 10 ² cells
SV40	3T3,REF	Doubling time >75 hr	Doubling time <30 hr
SV40	3T3,REF	Density: Ability to form a dense colony	5x10 ⁴ cell/cm ² 10 ⁶ cell/cm ²
Py SV40	BHK 3T3,REF	Anchorage: Ability to form a colony suspended in gel	<0.001 colony per 10 ² cells >10 colonies per 10 ² cells

Table 2. Classes of 3T3 Clones Induced by SV40

<u>Class</u>	<u>Percent of Clones</u>	<u>T-antigen</u>	<u>Density(a)</u>	<u>Doubling Time in 1% GS(b)</u>	<u>Anchorage(c)</u>
I Normal	12	-	8.5 (7.6-9.5)	78 (55-100)	<.001
II Transformed	25	+	47 (35-60)	34 (24-45)	32 (11-58)
III Minimal-transformed	38	-	15 (9.5-16.5)	34 (26-49)	<.001
IV Intermediate-transformed	25	±	25 (15-45)	32 (29-41)	4 (.5-14.6)
<u>Control</u>					
Normal		-	7.8 (7-9)	70 (60-86)	<.001
Transformed		+	53 (47-60)	37 (26-43)	25 (16-30)

(a) cells/cm² x 10⁻⁴

(b) hrs

(c) percent cells forming visible colonies (>0.3mm diameter) in 21 days in methocel

MECHANISMS OF CELL TRANSFORMATION BY DNA TUMOR VIRUSES

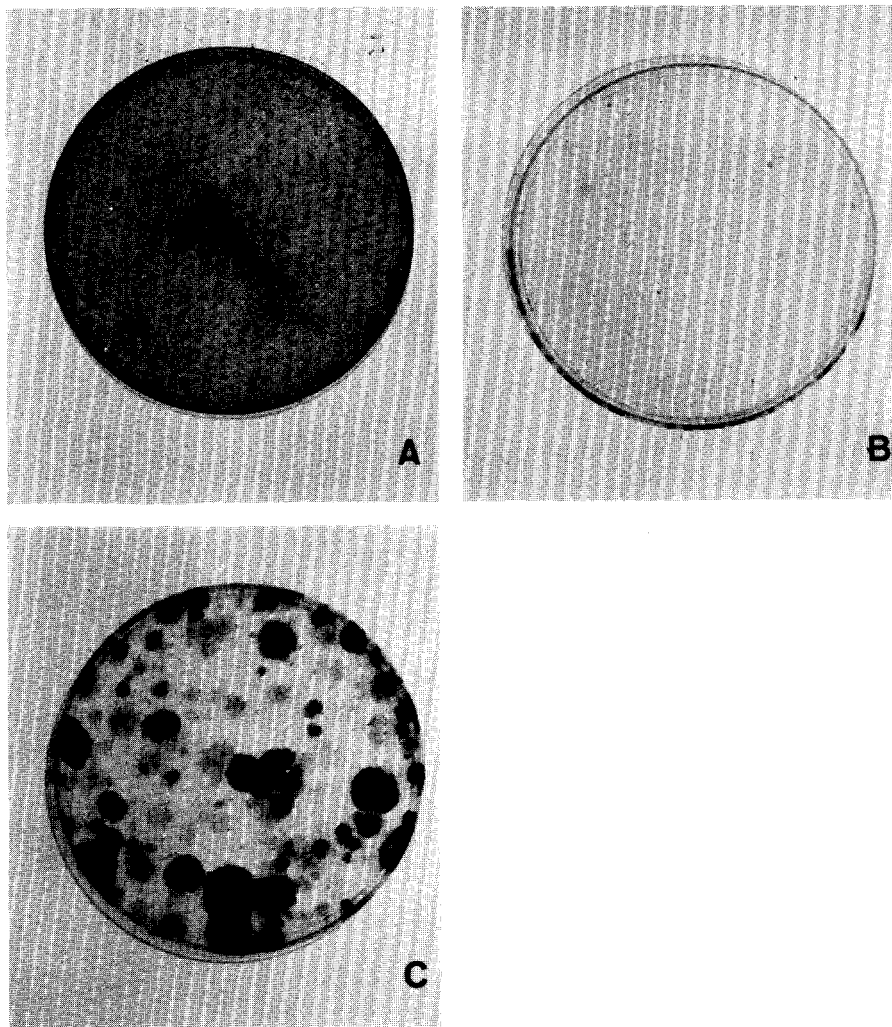


Fig. 1. Transformation of rat embryo fibroblasts by SV40. Fixed and stained plates: (A) confluent plate of REF cells at time of infection, (B) uninfected confluent plate was trypsinized and replated at 10^3 cells per plate (cells were cultured for 14 days), (C) infected confluent plate was trypsinized and replated at 10^3 cells per plate (cells were cultured for 14 days).

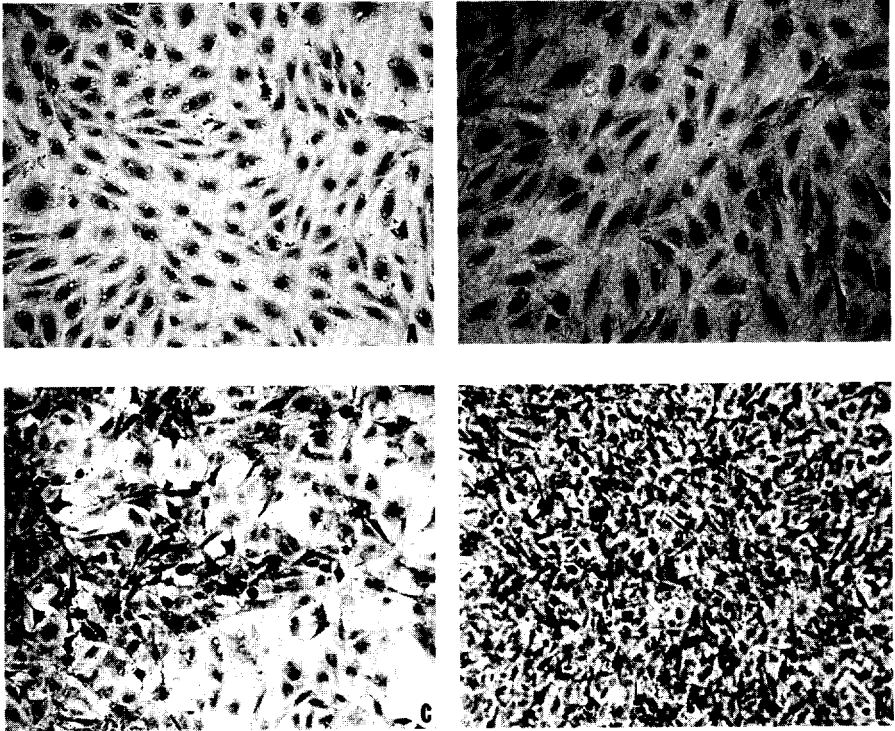


Fig. 2. SV40 transformed REF colonies. (A) Colony from mock-infected plate ($PE \sim 10^{-3}$), (B-D) colonies from infected plate ($PE \sim 10^{-1}$): (B) low density colony resembling mock-infected colony, (C) intermediate density colony, (D) high density colony. Relative frequency of different colony morphologies: $B > C > D$.