

SV40 and Cellular Gene Expression in the Maintenance of the Tumorigenic Syndrome

R. POLLACK, A. LO,* B. STEINBERG, K. SMITH, H. SHURE,† G. BLANCK, AND M. VERDERAME

Department of Biological Sciences, Columbia University, New York, New York 10027;
**Department of Microbiology, State University of New York, Stony Brook, New York 11794*

The end point of the study of viral transformation is a greater understanding of the mechanism of host growth control. At present, this mechanism is totally unknown. Therefore, we are currently left with the task of tabulating the response of the cell to viral tumorigenic transformation. Such work provides a necessary context for any future hypotheses on the mechanism of cellular growth control, since such hypotheses will have to explain these responses. Fortunately, tumorigenic transformation correlates well with a relatively small number of phenotypic changes in the transformed cell. In rat fibroblast lines transformed by SV40, these changes tend to occur coordinately, forming a syndrome (Fig. 1) (Risser and Pollack 1974; Barrett and Ts'o 1978; Pollack and Kopelovich 1979; Steinberg et al. 1979).

This syndrome consists of (1) a loss of anchorage dependence for cell division (Stoker et al. 1968; Folkman and Moscona 1978; Steinberg and Pollack 1979), (2) a loss of cytoskeletal actin organization (Pollack et al. 1975; Tucker et al. 1978), (3) the production of a large amount of protease plasminogen activator (PA) (Rifkin and Pollack 1977), and (4) tumorigenicity of suspended cells in the nude mouse (Shin et al. 1975; Steinberg et al. 1979). We and others have found that each of the *in vitro* parameters of this syndrome can be separately manipulated by variation of any of the other three. Thus, for example, growth in

the absence of anchorage is enhanced under conditions where plasmin concentrations are high (Pollack et al. 1975), and plasmin can disrupt the cytoskeletal actin organization of normal rat fibroblasts (Pollack and Rifkin 1975).

Recently, we have been able to quantitate this correlation in a nonparametric analysis of a series of spontaneous and benz[α]pyrene-induced Syrian hamster clonal cell lines (Barrett et al. 1979). These 15 lines differed over a 10^6 -fold range in their degree of tumorigenicity, as measured by TD₅₀ in hamsters. Growth in the absence of anchorage, enhanced PA production and plasmin activity, decreased organization of intracellular actin, as well as decreased serum requirement for growth, and increased clonal efficiency in liquid medium were all quantitatively correlated with TD₅₀ (Table 1). As in many other sets of transformed fibroblasts (Kahn et al., this volume), the most closely correlated to tumorigenicity of all measured *in vitro* parameters of growth control is anchorage independence.

Viral Gene Contributions

The early region of the SV40 genome codes for at least two proteins, large T antigen (90K) and small T antigen (19K). Transformed cells usually express both of these proteins (Prives et al. 1977; Crawford et al. 1978; Sleight et al. 1978; Paucha et al. 1978). Both proteins have been shown to be virus-coded by direct *in vitro* translation of viral messages (Prives et al. 1977, 1978). Deletions in the region of 0.54–0.59 map units of the SV40 genome map affect small T antigen without affecting large T antigen (Crawford et al. 1978). With these deletion mutants of SV40, many laboratories have generated sets of transformed clones of various mammalian species in order to examine the contribution of large and small T antigens to maintenance of various parts of the syndrome, in particular anchorage-independent growth. Results of these examinations are in conflict. Some laboratories have found that mutants that carry a deletion at 0.54–0.59 map units are severely limited in their ability to generate transformed clones that grow in agar (Sleight et al. 1978; Bouck et al. 1978), whereas other laboratories report no significant difference in the ability of

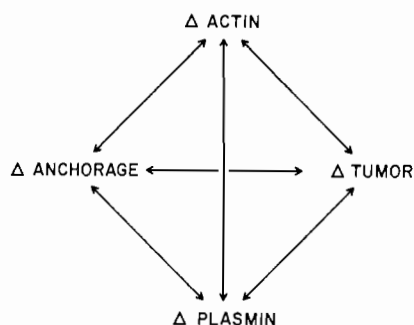


Figure 1. Tumorigenic syndrome in SV40-transformed rodent lines.

†Present address: Weizmann Institute, Rehovot, Israel.

Table 1. Correlation among In Vitro Growth Properties and Tumorigenicity of Syrian Hamster Cell Lines

Growth parameter	Coefficient of rank correlation, τ^a
Cloning efficiency, semisolid agar	0.986 ^b
Fibrinolytic activity	0.754 ^c
Generation time, 1% serum	0.754 ^c
Cloning efficiency, liquid medium	0.638 ^c
Organization of intracellular actin	0.570 ^c
Saturation density, 10% serum	0.246
Generation time, 10% serum	0.062

Adapted from Barrett et al. (1979). Fifteen hamster cell lines, some transformed by benz[α]pyrene, were tested for tumorigenicity in neonatal hamsters and for the in vitro parameters listed.

^aComputation of the Kendall coefficient of rank correlation, τ , between the TD₅₀ of the cell lines and their respective growth properties was performed as described in Barrett et al. (1979).

^bSignificantly correlated at the 99% confidence level ($p \leq 0.01$).

^cSignificantly correlated at the 95% confidence level ($p \leq 0.05$).

the deletion mutants versus the wild type to generate anchorage-independent transformed clones (Martin et al. 1979). These laboratories use different species of serum in their anchorage assay. We have used a new, refined measure of anchorage-independent growth in various serum species to examine a single wild-type clone transformed by SV40 and a single clone transformed by a deletion mutant (0.54–0.59 map units) of SV40, *dl884* (Shenk et al. 1976). This new analysis of total anchorage-independent cell growth resolves the conflict noted above.

Host Gene Contributions

In the past year, there has been a good deal of excitement about the presence in many SV40-transformed lines of a 54K nonviral T (NVT) antigen (Lane and Crawford 1979). This protein, which is entirely host-coded (Linzer and Levine 1979), is nevertheless immunoprecipitated by many antisera developed against SV40-transformed cells, in part because the 54K protein binds very well to large T antigens of SV40 (Lane and Crawford 1979; Linzer and Levine 1979). Apparently, the 54K protein is immunoprecipitated as a passenger molecule by antisera directed solely against the viral large-T-antigen molecule (Lane and Crawford 1979). However, the 54K NVT antigen is also a true T antigen, since antisera directed against tumors arising from SV40-transformed cells recognize the protein directly, even in the absence of large T antigen (Jay et al., this volume).

We have examined the presence of viral proteins and also of the 54K protein in our wild-type- and *dl884*-transformed clones. We have also assayed by immunoprecipitation for the presence of the two viral proteins and the 54K NVT protein in a family of four cloned rat cell lines derived from embryo fibroblasts

through a series of transformations, reversions, and retransformations. These results are presented below.

EXPERIMENTAL PROCEDURES

Cells and culture conditions. Hamster cell lines have been previously described in Barrett et al. (1979). All other cell lines are the descendants of second- to fourth-passage rat embryo fibroblast (REF) cells, prepared as described in Risser and Pollack (1979). The SV40 transformed REF lines *dl884B* and *wtA* have been previously described in Steinberg and Pollack (1979). The postcrisis rat line Rat 1, its transformant 14B, and the revertant 1-4 have been previously described in Steinberg et al. (1978). 1-4 MCA is a spontaneous retransformant of 1-4 (Steinberg et al. 1979). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; H21; GIBCO) supplemented with 10% fetal calf serum (FCS) (Reheis) and 100 units/ml of penicillin and streptomycin (GIBCO), unless otherwise indicated.

Anchorage independence. Cells were plated in triplicate at 10^5 , 10^4 , and 10^3 cells/60-mm dish in 3 ml DMEM plus 10% FCS containing 0.33% agarose (Difco) over a 2-ml layer of 0.9% agarose in the same medium. Cultures were fed twice weekly with an additional 2 ml of the soft agar and DMEM plus 10% FCS and cultured for 3 weeks. Large colonies more than 0.2 mm in diameter were scored using a microscope. Total colony volume increase (CVI) on agar was determined as described in Steinberg and Pollack (1979).

Tumorigenicity. Assay of ability of cells to form tumors in nude mice was previously described in Steinberg et al. (1979). Briefly, cells for injection into nude mice were trypsinized and resuspended in phosphate buffered saline (PBS) at the desired concentration; 0.2 ml was then injected subcutaneously at a single site. The first tumors were palpated after a latent period ranging up to many months. Tumor-free mice were maintained for at least 150 days. Tumor regression was only observed in animals injected with cells of 1-4 (Steinberg et al. 1979).

Immunoprecipitation. Cells were washed twice with sterile Tris-buffered saline (3.0 g Tris, 8.0 g NaCl, 0.38 g KCl, 0.1 g Na₂HPO₄/liter adjusted to pH 8) and labeled for 2 hours with [³⁵S]methionine (>1000 Ci/mole, 100 μ Ci/ml; Amersham) in methionine-free DMEM supplemented with 2% dialyzed FCS. After labeling, cells were washed three times with cold Tris-buffered saline (pH 8) and extracted for 30 minutes in 20 mM Tris (pH 8), 125 mM NaCl, 1 mM DTT, and 0.3 mg/ml PMSF. Soluble protein extracts were obtained by sequential centrifugation at 2000g for 10 minutes and 48,000g for 35 minutes. Normal or immune serum and extracts were incubated at room temperature for 90 minutes, 100 μ l of killed Staph A (10% suspension) (Kessler 1975) was added, and in-

incubation continued at room temperature for another 15 minutes. The bacteria-immunocomplexes were washed three times with 10 mM Tris, 10 mM EDTA, and 0.5 M NaCl (pH 8), incubated with electrophoresis sample buffer (0.0625 M Tris at pH 6, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) for 30 minutes at 50°C, and boiled for 5 minutes. Samples were electrophoresed according to the method of Laemmli (1970) in Tris-borate buffer in 10–15% gradient polyacrylamide gels. Autoradiographs were prepared from stained and dried gels. Normal sera did not immunoprecipitate large, small, or the host 54K T antigen from any cell line.

RESULTS

Slow Growth versus No Growth in the Absence of Anchorage

The assay of anchorage-independent growth used by most laboratories studying SV40 transformation of rodent fibroblasts measures the frequency with which cells of a given clone can form visible colonies after a period of 2–3 weeks in suspension on a bed of agar (Risser and Pollack 1974). Visible colonies contain between 10^3 and 10^4 cells. If a cell were able to grow in agar at a cumulative rate that generated fewer than 10–13 divisions over the duration of the experiment, the standard assay would fail to score that cell's progeny as a colony. Thus, the standard anchorage assay suppresses the first 10–13 divisions a cell may be capable of in agar and equates slow growth in agar with no growth (O'Neill et al. 1979; Steinberg and Pollack 1979). Cellular response in this assay can also vary with culture conditions. For example, it has been known for some time that dog serum presented to cells in agar can dramatically increase their efficiency of growth in the standard anchorage assay (Table 2).

Total CVI is a less-biased measure of anchorage-independent growth than visible colony formation (Steinberg and Pollack 1979). The CVI assay also quantitates differences in cell response to such in vitro variables as serum species (Steinberg and Pollack 1979). Because other laboratories have used different

sera in their studies of anchorage-transformation by deletion mutants (0.54–0.59 map units) of SV40 (Sleigh et al. 1978; Bouck et al. 1978; Martin et al. 1979), we measured visible colony formation and CVI for a wild-type-transformed clone (*wtA*) and a *dl884*-transformed clone (*dl884B*), as well as REF, in the presence of both FCS alone and a mixture of FCS and dog serum (Figs. 2 and 3).

The distribution of colony sizes as a percentage of total colonies for these three populations is shown in Figure 2. The arrows in Figure 2 indicate the threshold of visible colony size. A few REF cells divided more than once in FCS, whereas none divided in the serum mixture. Wild-type-transformed *wtA* colonies grew to visible size in either serum.

Colonies of *dl884B* showed a very strong differential response to serum species. Colonies of *dl884B* were mostly below visible size in the presence of FCS, but were all of visible size in the presence of the mixture of FCS and dog serum. Thus, by choice of serum in the standard assay, it is possible to generate data that show that a *dl884*-transformed rat clone either does (Martin et al. 1979) or does not (Sleigh et al. 1978; Bouck et al. 1978) grow to large colonies in the absence of anchorage.

Even in FCS, the small colonies of *dl884B* cells cumulatively represent a significant cell increase, as shown in Figure 3. Although the *dl884*-transformed clone does not grow in agar with sufficient rapidity to form a detectable number of visible colonies, the CVI assay reveals that this clone has a considerable capacity to grow on agar, albeit slowly. This capacity represents an average of seven to eight divisions over the 3 weeks in culture, compared to one division (at most) for untransformed REF cells (Fig. 3). In preliminary studies, injection of *wtA* and *dl884B* cells into nude mice yielded tumors at 2×10^6 cells per mouse at 6 weeks for *wtA*, but no tumors by 11 weeks for *dl884B* (P. Kahn and S. Shin, pers. comm.).

Although it is not possible to generalize from these single clones of wild-type and *dl884* origin, recent in vivo studies are consistent with the observation that mutation of SV40 small T antigen diminishes, but does not eliminate, the tumorigenic syndrome. Many

Table 2. Effect of Serum Species on Efficiency of Large-colony Formation in the Absence of Anchorage

Line	PA production ^a	Anchorage independence (%) ^b		
		10% fetal	2.5% fetal 7.5% dog	5% fetal 5% dog
REF	1.0	≤0.01	≤0.07	≤0.01
SVRE 12	1.4	0.03	36	26
SVRE 9	12.6	8	55	21

^aAdapted from Pollack et al. (1975).

^bIodinated fibrin (cpm) released by live cells/hr/ 10^5 trypsinizable counts, normalized to rate of release by REF cells.

^cTransformed clones isolated from REF after a nonselective infection with wild-type SV40. Both clones grow to high density on plastic. For this experiment, cells were inoculated on agar in DEM with the sera shown. Large colonies (>0.3 mm) were counted at 21 days, using a dissecting microscope and dark-field illumination.

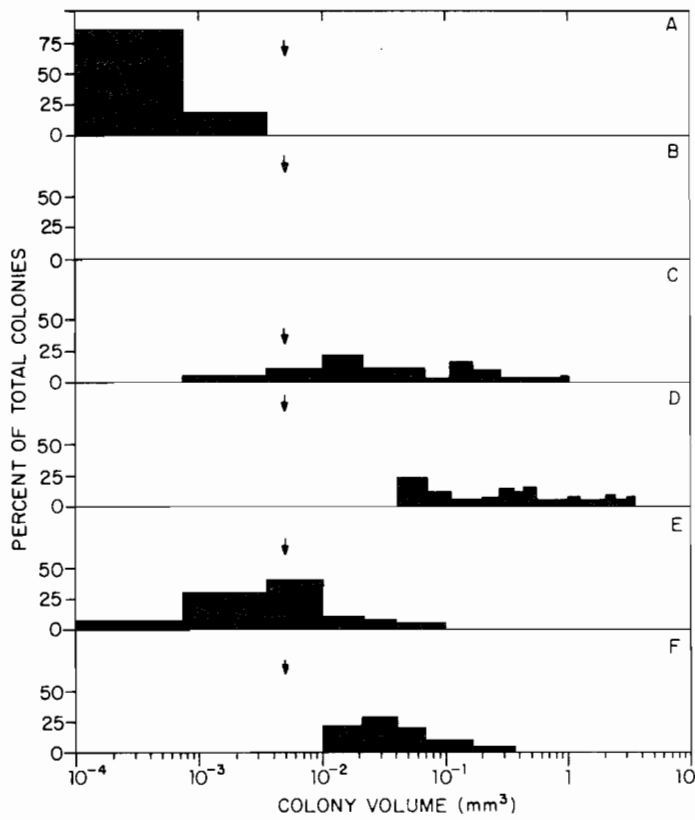


Figure 2. Contribution of various size colonies to total colony number in different sera. Plates (60 mm) were inoculated with 10⁴ cells. Colony volumes were determined as described in Steinberg and Pollack (1979) for each colony larger than two cells in size. The percentage of total colonies having a given volume is plotted against colony volume. Arrows indicate a colony diameter of 0.2 mm, at the threshold of visibility. (A) REF growing in 10% FCS; (B) REF growing in 5% FCS, 5% dog serum; (C) wtA growing in 10% FCS; (D) wtA growing in 5% FCS, 5% dog serum; (E) dl884B growing in 10% FCS; (F) dl884B growing in 5% FCS, 5% dog serum. (Adapted from Steinberg and Pollack 1979.)

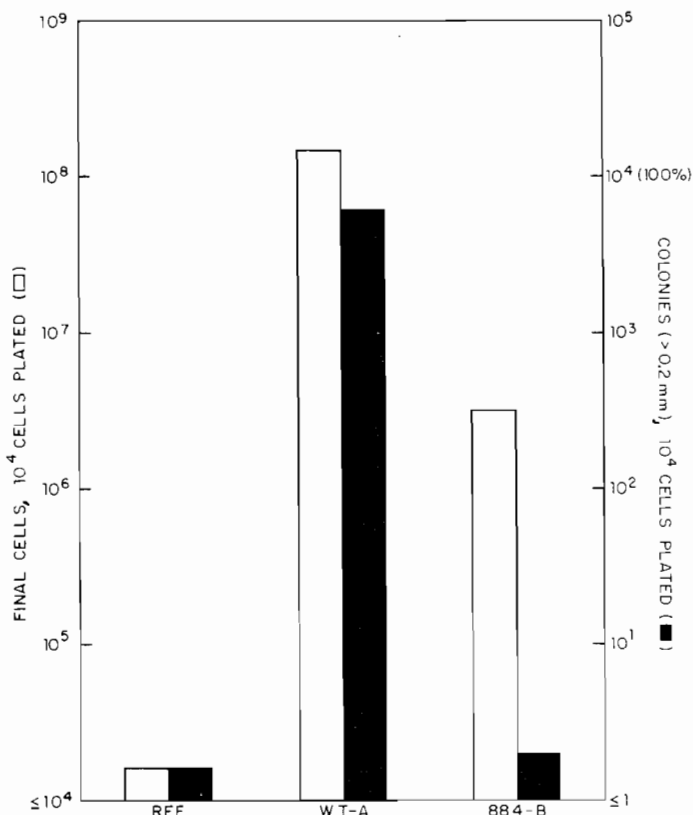


Figure 3. Cell growth in agar with 10% FCS. The standard anchorage assay and the total CVI are compared for REF, dl884A, and wtB. Inoculum was 10⁴ cells/60-mm dish in all cases, and a constant volume/cell was assumed regardless of colony size. Note the disparity between CVI and the standard assay, for dl884B. (Adapted from Steinberg and Pollack 1979.)

deletion mutant (0.54–0.59 map units) SV40 stocks have been injected into neonatal hamsters. In all cases the SV40 deletion mutant viruses have been tumorigenic; however, the tumors arising from these virus stocks are always slow-growing and have a much longer latency than the tumors induced by injection of wild-type virus (Lewis and Martin 1979; Frisque et al., this volume). Taken together, these data suggest that both SV40 antigens participate in tumorigenicity, and that the CVI assay is a closer measure of that aspect of anchorage independence that correlates with tumorigenicity than is the standard visible colony assay.

Host Gene Contributions to the Tumorigenic Syndrome

We have examined *dl884B* and *wtA* for T antigens by immunoprecipitation (Kessler 1975). The *dl884* transformant and the wild-type-transformed clone both contain the large T antigen, whereas only the wild-type clone contains the small T antigen. Both lines contain the 54K protein; REF lacks all three proteins (A. Lo, in prep.).

These two cell lines, and other sets of cells that contain viral proteins, cannot be used to determine the direct role of the 54K protein in the maintenance of the tumorigenic syndrome. The characteristic binding of the 54K protein to the viral large T antigen complicates immunoprecipitations by most antitumor sera. Even with more specific antisera, transformed lines carrying SV40 antigens as well as the 54K antigen do not easily yield data to support or eliminate the hypothesis that the 54K antigen is itself a correlate or a causal agent of the tumorigenic syndrome.

To answer this question, we turned to a set of cell lines generated by repeated selection for expression and nonexpression of the capacity for anchorage-independent growth. The tumorigenic syndrome is for the most part maintained for each line in this set. The efficiency of plating in agar and the CVI of this set are well correlated with each other (Table 3), as well as with tumorigenicity (Steinberg et al. 1979). With this set of lines, we have been able to show that the presence of the 54K protein does correlate quantitatively with the capacity of a cell line to grow in the

absence of anchorage, by both the standard and the CVI assays and in either the presence or absence of viral antigens.

This set of lines begins with the precrisis population of rat embryo fibroblasts, REF (Risser and Pollack 1979). From a population similar to REF, the clonable postcrisis contact-inhibited cell line Rat 1 was established as F2408 by Aaron Freedman (Mishna and Ryan 1973). Rat 1 has a high cloning efficiency and is able to grow in low serum; but it is not able to grow well in the absence of anchorage. Its CVI is approximately 13 (Table 3). Rat 1 was transformed by infection with wild-type SV40 DNA to generate the clone 14B. Southern blot analysis revealed that 14B contains one copy of SV40 DNA, presumably integrated in the genome (Botchan et al. 1976). 14B was subjected to negative selection by FdU killing of dividing cells at high cell density (Pollack et al. 1968). The revertant clone 1-4 was recovered from among the survivors of this treatment (Steinberg et al. 1978). Clone 1-4 contains SV40 DNA indistinguishable from 14B but does not express any detectable large or small T protein (Steinberg et al. 1978). About one cell in 10^5 of 1-4 is capable of growing into a large colony in agar. One such colony was picked and grown up as a clone, the anchorage-independent back transformant, 1-4 MCA (Steinberg et al. 1979).

We examined these five cell lines by immunoprecipitation for the presence of the two viral antigens and the host 54K antigen. We used two antisera. One, a gift of J. Gruber (National Institutes of Health), immunoprecipitates both large T antigen and the 54K protein directly. The other, prepared by D. Gidoni (Weizmann Institute), does not detect the 54K protein directly. It immunoprecipitates large T antigen, and only then is any 54K protein present and binding to large T antigen brought down. Neither serum recognizes any viral antigens or any 54K protein in the precrisis REF cells (A. Lo, in prep.). The immunoprecipitations are shown in Figures 4 and 5.

The NIH tumor antiserum immunoprecipitates 54K protein from all four postcrisis lines (Fig. 4). Rat 1 and 1-4, which are poorly tumorigenic, have a small amount of 54K protein (Fig. 4). Both 14B and 1-4 MCA, lines that are highly tumorigenic and grow with

Table 3. Coordinate Expression of Tumorigenic Syndrome in a Set of Rat Cell Lines

Cells	Production of PA (% counts released)			Cytoskeletal organization (% cells with actin cables)	Anchorage independence		Tumorigenicity in nude mice		
	fibrin	cell extract	harvest fluid		relative EOP agar/plastic	CVI in agarose ^a	inoculum	tumors/ animals	days to first tumor
REF	<5	<5	0	95	$<10^{-5}$	2.0	1×10^7	0/4	>150 ^b
Rat 1	8	4	0	59	7.0×10^{-5}	1.3×10^1	1×10^7	2/3	84
14B	43	27	0	37	1.5×10^{-1}	3.8×10^2	1×10^6	2/2	60
FL 1-4	74	51	44	64	3.0×10^{-5}	2.1×10^1	1×10^7	0/4	>150
1-4 MCA	72	n.d. ^c	n.d.	2	5.4×10^{-2}	4.2×10^3	5×10^6	3/3	28

Adapted from Steinberg et al. (1979).

^a 10^5 cells plated.

^bDiscontinued at 150 days, tumor-free.

^cn.d. indicates not determined.

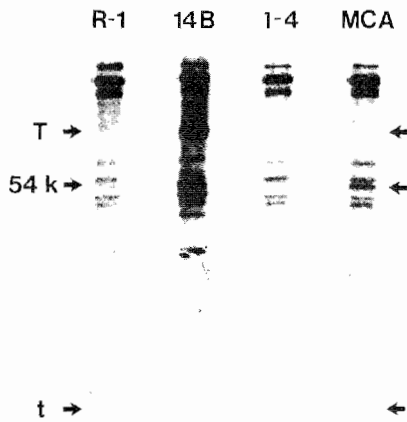


Figure 4. SV40 T antigens in a set of rat cell lines. Cells were labeled with [³⁵S]methionine (>1000 Ci/mmol, 100 μCi/ml; Amersham) in methionine-free DMEM supplemented with 2% dialyzed FCS for 2 hr. Extracts were immunoprecipitated, according to the method of Kessler (1975), with hamster antitumor serum, electrophoresed on a 10–15% gradient polyacrylamide gel according to the method of Laemmli (1970), stained, and autoradiographed. T indicates SV40 large T antigen (present only in 14B); 54K indicates host T antigen present in all four lines, but quantitatively increased in 14B and 1-4 MCA; t indicates SV40 small T antigen, present only in 14B.

high efficiency and rapidly in agar, have a large amount of 54K (Fig. 4). The viral large and small T antigens are present only in 14B. Significantly, they do not reappear in 1-4 MCA, which has reacquired tumorigenicity and the 54K protein.

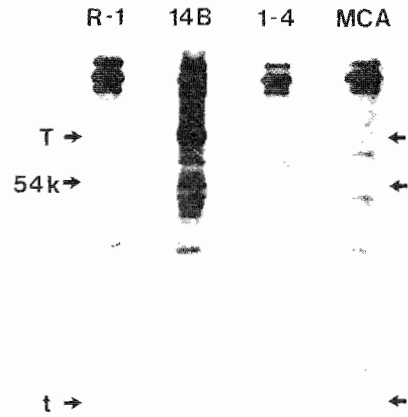


Figure 5. SV40 T antigens in a set of rat cell lines. Conditions were identical to those in Fig. 4, except that the tumor antiserum used here was obtained from D. Gidoni and C. Prives (Weizmann Institute). This antiserum is unable to detect the 54K T antigen directly. However, the 54K antigen is detected in the presence of SV40 large T antigen in extracts of 14B.

An antiserum that detects only viral antigens immunoprecipitates the 54K protein only through its binding to large T antigen (Lane and Crawford 1979). Using such a serum (Fig. 5), we see that only 14B, which contains large and small T antigens, has precipitable 54K protein. This antiserum fails to precipitate the 54K protein from 1-4 MCA (Figs. 4 and 5). We conclude that the line 1-4 MCA contains the 54K protein and not large or small T antigen.

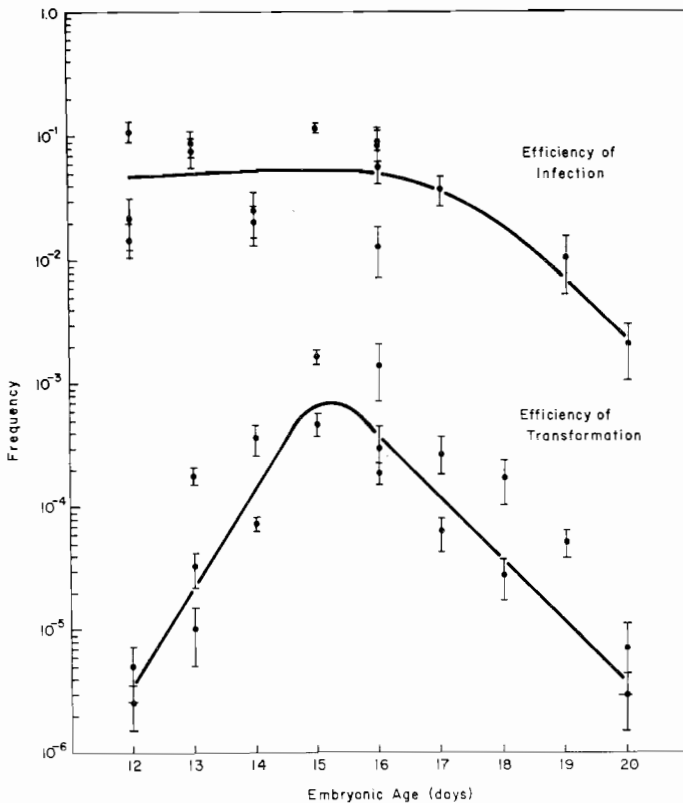


Figure 6. The effect of embryonic age on transformation induced by SV40. The upper curve is the frequency of T-antigen-containing cells 2 days postinfection. The lower curve is the frequency of dense focal colonies formed after 2 weeks of culture in 10% FCS. (Adapted from Risser and Pollack 1979.)

DISCUSSION

The simplest hypothesis to explain the data presented in this paper is that the ability to grow in agar and express the rest of the tumorigenic syndrome is the consequence of the presence in a cell of the 54K protein. The small and large T antigens would then have the hypothetical function of increasing the amount of this protein, and a line such as 1-4 MCA, which has the protein but neither large nor small T antigen would be the consequence of a host mutation leading to increased production of the 54K protein. From this hypothesis it would follow that other lines similar to 1-4 MCA might arise through host mutation in the total absence of SV40 gene products.

If their ability to increase 54K protein expression is the reason that small and large T antigens are transforming proteins, then any other gene (Wigler et al. 1979) or gene product that increases the expression of 54K-like proteins would also be expected to generate the tumorigenic syndrome. Indeed, if the 54K protein has any function in the maintenance of the anchorage-independent state in 1-4 MCA, this line must contain host proteins equivalent to SV40 large or small T antigen with which the 54K protein or its gene(s) can interact (Segawa et al. 1977).

The 54K protein is unlikely to be the only one of its kind. The polyoma middle T antigen (B. Griffin et al., this volume) and the endogenous *sarc* protein (Purchio et al. 1978; Erikson; Bishop; both this volume) come to mind as other examples, and it is possible that there are genes for many 54K-like proteins in the normal cell.

What is the function of the 54K protein, or sets of proteins? No answer springs easily to mind from the available data. However, it is intriguing to speculate that the host 54K is an example of a set of proteins used by cells at times of very rapid proliferation. Early embryogenesis is of course the paradigm of such moments in the life history of a cellular organism (Linzer and Levine 1979). Recently, we have found that fibroblasts obtained from rat embryos of different ages have markedly different efficiencies of transformation with wild-type SV40 (Fig. 6) (Risser and Pollack 1979). We have begun to ask whether the expression of 54K protein is enhanced in those periods of embryogenesis when cells are markedly more transformable by SV40.

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