

## Plasminogen Activator Production Accompanies Loss of Anchorage Regulation in Transformation of Primary Rat Embryo Cells by Simian Virus 40

(semi-solid medium/oncogenic viruses/fibrinolysis/serum)

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**ABSTRACT** We have isolated several lines of rat embryo cells transformed by simian virus 40. All these lines are fully transformed with regard to saturation density and serum sensitivity, but they differ greatly in their anchorage dependence, as assayed by efficiency of plating in methyl cellulose suspension. This set of lines reveals a consistent relation of plasminogen activator production to plating efficiency in methyl cellulose. T-antigen-positive transformed lines that synthesize activator grow in methyl cellulose suspension, while T-antigen-positive transformed lines that do not synthesize activator fail to form colonies in suspension. Normal rat embryo cells produce very little plasminogen activator and do not grow in methyl cellulose. Sera that permit high levels of plasmin formation and activity support growth in semi-solid medium better than sera whose plasminogen is activated poorly and/or sera that contain inhibitors to plasmin.

When a small DNA tumor virus, such as simian virus 40 (SV40) or polyoma, infects susceptible mammalian fibroblasts *in vitro*, a number of physiological and social characteristics of the cells are drastically and permanently altered. The sum of these behavioral changes, especially the loss of sensitivity to the growth-inhibiting effects of suspension in semi-solid media, extensive cell-cell contact, and serum-depletion, is termed transformation (1-8). The multiplicity of changes has been a major problem in understanding the relation of viral transformation to *in vivo* tumorigenicity (9-15). However, loss of sensitivity to these three different environmental stimuli is not coordinate, and transformants have been isolated that are still sensitive to one or even two of them (4, 7, 8).

The demonstration that analysis of nonselected SV40 transformation permits easy isolation of clonal transformed lines having widely differing *in vitro* properties (7) encouraged us to attempt to determine whether plasminogen activator production, a biochemical alteration which may be related to malignancy (16-27), was correlated with any of the above mentioned measures of transformation. In this paper we report the correlation of anchorage dependence with production of plasminogen activator in a set of SV40-transformed clones derived from infected rat embryo cells. A preliminary report of this work has appeared elsewhere (28).

### METHODS

**Culture Conditions.** All experiments were carried out at 36.5° in an atmosphere of 10% CO<sub>2</sub>, 90% air, with a relative humidity of 100%. Stock cultures were grown in Dulbecco's

modified Eagle's medium (Gibco, H21) with 10% fetal calf serum (Reheis Laboratories).

**Preparation of Rat Embryo (RE) Cells.** This procedure has been described (38). In brief, embryos were removed from 15-day pregnant rats of the Fisher inbred strain (CDF albino—Charles River), minced and disaggregated in 0.25% trypsin in phosphate-buffered saline (PBS)—Ca<sup>++</sup>, Mg<sup>++</sup>. Rat embryo (RE) cells were washed, centrifuged, and resuspended in medium containing 10% fetal calf serum.

**SV40 Infection of RE Cells.** RE primary cells were inoculated at  $2 \times 10^6$  cells per 60-mm dish immediately after dissociation of the embryos. Approximately 10% of the cells attached and spread, which resulted in cultures whose sparse density (approximately  $10^4/\text{cm}^2$ ) assured that contact inhibition would not interfere with any SV40-cell interaction. After 24-48 hr, the RE plates were washed with PBS and infected with SV40 at a multiplicity of infection of 400 for 2 hr (7). The cultures were then trypsinized and replated at appropriate dilutions to give  $10^8$  and  $10^2$  cells per 60-mm dish.

**Isolation of RE Clones Transformed by SV40 (SVRE Clones).** Colonies that appeared after 2 weeks of growth were ringed by steel cylinders, dispersed by trypsin, removed, and replated in 35-mm dishes. After 1-2 weeks, when the dishes became confluent, the cells were passaged at high dilution and a reclone was picked from the descendants of each original clone. Reclones were frozen in liquid nitrogen for later study.

**Virus Specific Antigens.** Subconfluent cultures of a clone (or of uninfected RE cells) were grown for 48 hr on glass coverslips, washed in PBS (Gibco), fixed in acetone (-20°) for 10 min, air dried, and stained for detection of viral antigens by indirect immunofluorescence. To detect SV40-specific T-antigen, fixed coverslips were incubated for 1 hr at 37° with hamster antibody to T-antigen (Flow, 1:10 in PBS), washed in PBS, and incubated for 1 hr at 37° with fluorescein-conjugated rabbit antibody to hamster IgG (Antibodies Incorporated, 1:10 in PBS). The coverslips were washed again in PBS, mounted in Elvanol, and examined under UV-epi illumination with a Zeiss Planapo 63× objective. T-antigen-positive cells contained bright green nuclei under these conditions, while nuclei of T-antigen-negative cells did not fluoresce.

**Chromosome Number.** Chromosomes were prepared as in ref. 29. Chromosome numbers were obtained from photographs of well-spread Giemsa-stained metaphase cells.

Abbreviations: SV40, simian virus 40; RE, rat embryo; PBS, phosphate-buffered saline; SVRE, rat embryo cells transformed by simian virus 40.

**Saturation Density.** To determine the maximum density a clone could attain in different serum concentrations, 10 sister cultures were inoculated at  $2 \times 10^3$  cells per  $\text{cm}^2$  in 35-mm dishes for each serum concentration, and these cultures were fed 1.5 ml of appropriate medium two times per week. Dishes were counted every second day. A clone was considered to have achieved saturation if three successive counts showed no more than a 10% increase in cell density. Some clones were unable to maintain a stable saturation density in 10% fetal calf serum, growing so dense as to peel off the dish instead.

**Serum Sensitivity.** From the growth curve obtained in the assay of saturation density of a clone, the slope of the log phase of growth was taken as the doubling time of the clone in a given serum concentration.

**Anchorage Requirement.** Methyl cellulose (Methocel) cultures (7, 35) were used to assay the ability of cells of a clone to form spherical colonies without attachment to a solid substrate. Sera [fetal calf, dog, or monkey (Gibco)] were added to a final concentration of 10%. Colonies  $>0.2$  mm were scored at 21 days, after three additions of 4 ml of Methocel medium (7).

**Plasminogen Activator.** Plasminogen activator present in live cultures, activator secreted by these cultures, and activator present in solubilized cells were all assayed by a method based on digestion of iodinated fibrin (19, 22–24). When serum plasminogen is cleaved by a cellular activator, it is activated to plasmin. The plasmin will degrade insoluble fibrin to soluble peptides. Activator is thus indirectly assayed by the solubilization of  $^{125}\text{I}$ -labeled peptides from iodinated fibrin.

To assay the ability of live cells to activate plasminogen, 2 to  $5 \times 10^5$  cells (a subconfluent density) were plated on  $^{125}\text{I}$ -labeled fibrin-coated dishes ( $10 \mu\text{g}/\text{cm}^2$ ;  $4 \times 10^4$  cpm; 60 mm diameter) in 2.5% fetal calf serum and allowed to attach for 5 hr. The cultures were then washed twice in serum-free medium, and then medium containing 2.5% dog serum was added. Since dog serum plasminogen is rapidly cleaved to plasmin by murine activator, the medium over cultures rich in activator becomes radioactive with time. To detect this, one-tenth of the medium was removed from each plate at 7–20 hr after switching to dog serum, mixed with 10 volumes of Aquasol, and its radioactivity was measured. Solubilization of radioactive material was usually linear during this period, suggesting that activator production was the rate-limiting step. At 20 hr the remaining medium was discarded, the subconfluent fibrin cultures were fixed and stained, and the cells per dish were calculated from counts of cells per field obtained using a Zeiss microscope (40 $\times$  objective). From the radioactivity released by the cells or by an excess of trypsin and from the cell density, cellular activator was calculated as a rate: average cpm  $^{125}\text{I}$ -labeled fibrin solubilized per hr/ $10^5$  cells per  $10^5$  trypsinizable cpm. This calculation of the rate of release of label permitted comparison of experiments by normalizing small differences from experiment to experiment in cell density and available fibrin.

To assay the activator released by cells, subconfluent cultures of a given clone were incubated overnight in petri dishes with serum-free medium. Under these conditions, plasminogen activator is liberated by the cells into the medium in a soluble form (19, 20). The medium was then assayed as above on  $^{125}\text{I}$ -labeled fibrin-coated dishes in the presence of 2.5% monkey serum, but in the absence of cells.

**Intracellular Plasminogen Activator.** Confluent and subconfluent cultures were grown on large (150-mm) dishes, washed in Tris buffer (24), suspended in 2 ml of Tris buffer (24), centrifuged at  $1000 \times g$  for 3 min, and solubilized in 0.25% Triton X-100, 0.1 M Tris·HCl at pH 8.1. Total protein was determined by the method of Lowry (30). Known aliquots of protein were then placed on  $^{125}\text{I}$ -labeled fibrin-coated plates with 2 ml of 0.1 M Tris·HCl at pH 8.1 and assayed using 2.5% monkey serum as the source of plasminogen. The total cell activator was monkey serum expressed as cpm released per hr/ $10^5$  available cpm per mg of protein.

## RESULTS

**Generation of SV40 Rat Clones.** When normal embryo cells are placed in culture at sparse densities, they rarely grow into separate colonies, and those colonies that do arise are not capable of continued growth *in vitro* (1, 3, 7, 15, 28). Infection with simian virus 40 increases the plating efficiency, and furthermore establishes the colonies into cell lines (28). Following SV40 infection at a multiplicity of infection of 400, the plating efficiency of rat embryo cells was 5–10%. Two morphologies of colonies could be distinguished among infected cells: dense and flat (28). Several colonies of both morphologies were cloned using steel cloning cylinders and propagated in 35-mm dishes. All clones that could grow into established cell lines expressed SV40 T-antigen uniformly in the nucleus of  $>95\%$  of their cells. No clone showed any viral capsid antigen by indirect immunofluorescence.

**Growth Properties.** The *in vitro* growth properties of 13 clones of SV40 rat embryo cells were analyzed in detail. Normal rat embryo cells were fully sensitive to the growth-inhibiting requirements for anchorage, serum, and density (Table 1).

Each of the thirteen SV40 rat clones was assayed for its ability to respond to these three restrictions that operate to limit the proliferation of normal rat embryo cells. With regard to serum sensitivity, no significant difference was found among the clones. All 12 clones had acquired the ability to grow in 1% fetal calf serum, and to grow more rapidly than rat embryo cells in 10% fetal calf serum (Table 1). As such, all clones were considered to be fully serum-transformed.

With regard to density sensitivity, all clones were also fully transformed. Only SVRE 12 was able to maintain a high stable saturation density in 10% fetal calf serum, while cultures of all other SVRE clones grew to higher densities and eventually peeled off the dish (Table 1). All SVRE clones, including SVRE 12, grew in 1% fetal calf serum to about the same saturation density (Table 1).

Surprisingly, the SVRE clones differed greatly from one another in their abilities to grow when suspended in semi-solid medium containing 1.2% methyl cellulose (Table 1) and 10% fetal calf serum. Clones SVRE 5 and SVRE 9 formed colonies more than 100 times more efficiently than SVRE 12, and more than 1000 times more efficiently than uninfected RE cells. With one exception (SVRE 5) the plating efficiency in methyl cellulose suspension of a given clone was stable over many months.

**Chromosomes.** The diploid chromosome number of the rat is 42. After two passages *in vitro* uninfected RE cells had a mean of 40.7 chromosomes per cell, but with some variation from cell to cell (SD = 2). The SVRE clones were examined after

TABLE 1. Expression of parameters of transformation by SV40-transformed rat embryo cell clones

	Serum sensitivity [doubling time (hr) in fetal calf serum]		Saturation density [(cell/cm <sup>2</sup> × 10 <sup>-4</sup> ) in fetal calf serum]		Anchorage dependence [(colonies/100 cells) innoculated in Methocel]	
	1%	10%	1%	10%		
	∞	35	—	25, 30		
Uninfected rat embryo cells	∞	35	—	25, 30	<0.001	
SV40-trans-formed clones						
SVRE 1	25	17	6	++	0.2	
SVRE 2	33	20	13	++	ND	
SVRE 3	33	13	12	++	0.2, 0.10	
SVRE 4	35	20	11	++	0.80	
SVRE 5	27	23	14	++	2.75, 2.40	
SVRE 6	50	20	9	++	++	0.01
SVRE 7	28	15	~10	++	ND	
SVRE 8	30	13	20	++	2.15	
SVRE 9	27	16	14	++	0.75, 1.77	
SVRE 10	30	18	14	++	0.30	
SVRE 11	38	23	≥14	++	++	0.002
SVRE 12	33	20	11	25	0.013, 0.09	
SVRE 13	40	20	9	++	++	0.70

— = No growth in 1% fetal calf serum; ++ = grows so dense that cell layer peels at >30 × 10<sup>4</sup> cells per cm<sup>2</sup>, never maintaining a stable saturation density; ND = not determined. When two figures are given, each represents a separate experiment.

three months in culture. SVRE 5 remained pseudodiploid, while all other SVRE clones became subtetraploid, and showed greater heterogeneity from cell to cell (SD > 5). The pseudodiploid chromosome number of SVRE 5 was unexpected (3), since that clone was fully transformed by all three criteria tested (Table 1).

**Activation of Plasminogen by Live Cells.** Normal rat embryo cells, like the cells of most normal tissues (19, 20, 22, 24), produce only small amounts of an activator of serum plasminogen. The SVRE clones produced different amounts of plasminogen activator, ranging from the low rate typical of normal rat embryo cells, to about a 12 times higher rate (Table 2). In four experiments carried out over 1 year, the SVRE clones held to their different rates of activation fairly well, excepting SVRE 5, in which the rate of activation decreased with time in culture (Table 2).

Cells that activate plasminogen to plasmin rapidly are caused to round up by the high level of plasmin (21, 25). A fibrin substrate enhances the morphological alteration (25). In our experiments, too, the extent of the morphological alteration of SVRE clones plated on fibrin correlated closely with the rate of activation of plasminogen (Table 2).

The different plasminogen activator rates seen in live cell assays may be the result of the clones' synthesizing different amounts of activator, or all clones may synthesize the same high amount, while some (such as SVRE 12) may be unable to secrete the activator so that it can come in contact with plasminogen. To decide between these possibilities, we assayed the secreted activator, and the total activator present in detergent-disrupted cells, in three SVRE clones and in unin-

TABLE 2. Activation of dog serum plasminogen by sparse cultures of rat cell lines

	Normalized rate of activation of plasminogen* (normal RE cells = 1.00†)				Morphological response‡
	Experiment No.				
	1	2	3	4	
Normal RE cells	1.0	1.0	1.0	1.0	—
SVRE 1	7.2	ND	ND	ND	++
SVRE 3	1.4	ND	1.5	1.7	—
SVRE 4	2.1	ND	ND	ND	—
SVRE 5	13.7	7.8	1.5	ND	++
SVRE 6	2.2	ND	ND	ND	+
SVRE 8	5.1	ND	ND	ND	++
SVRE 9	7.1	11.1	6.2	24.9	++
SVRE 10	2.2	ND	ND	ND	+
SVRE 11	0.6	ND	ND	ND	—
SVRE 12	2.0	1.6	1.0	1.1	—
SVRE 13	13.4	ND	ND	ND	+

\* Activation of dog serum plasminogen by live cells as assayed by rate of subsequent digestion of fibrin (see *Methods*). ND = not determined.

† Range: 36–198 cpm/hr solubilized per 10<sup>6</sup> cells per 10<sup>6</sup> available cpm of iodinated fibrin.

‡ Appearance of fixed cultures on fibrin substrates after 20 hr in 2.5% dog serum: ++ = all cells rounded up, + = curling of confluent areas, — = flat cells.

ected RE cells (Table 3). Clones SVRE 12 and SVRE 3, which in the live cell assay showed a very little activator, also showed relatively little activator in the assays of secreted material and disrupted cells. The assay of secreted material detected the activity accumulated by a population over many hours, resulting in higher absolute activity for all lines. Clone SVRE 9 had the most activator by all three assays. We conclude that the clones indeed differ in production of activator.

**Correlation of Plasminogen Activator Production with Loss of Control.** Uninfected RE cells and the SVRE clones show a striking inverse relation of plasminogen activator production to anchorage dependence (Fig. 1). All the SVRE clones contain viral T-antigen. Also, all are fully transformed with regard to serum sensitivity and saturation density (Table 1). Thus, the differences in production of plasminogen activator cannot be directly related to expression of the viral gene(s) for T-antigen, changes in serum requirement, or density regulation.

**Release of Anchorage Dependence by Dog and Monkey Sera.** Anchorage dependence normally was assayed using Methocel supplemented with 10% fetal calf serum, which was the serum used to isolate the RE cells and to clone the SVRE transformed lines. Fetal calf serum supports only low levels of fibrinolysis by SVRE lines. Dog and monkey sera, however, permit high levels of fibrinolysis (19, 20). If plasmin plays any role in growth in semi-solid medium, then the SVRE lines might be expected to grow more efficiently in methyl cellulose supplemented with dog or monkey serum.

To determine if this was so, we plated sister cultures of primary RE cells and three SVRE clones in methyl cellulose and in regular medium on plastic dishes in media containing

TABLE 3. Location of plasminogen activator

	Average plasminogen activator (normal RE cells = 1.00)		
	Live cell*	Cell extract†	Medium‡
Uninfected RE cells	1.00	1.00	1.00
SVRE 12	1.44	3.3	42.5
SVRE 3	1.53	4.0	85
SVRE 9	12.31	12.7	112, 570

\* Data in this column are averages from Table 2.  
 † Cells were disrupted by 2% Triton X-100. See *Methods*.  
 ‡ Activator secreted into serum-free medium. See *Methods*.

four different serum mixtures, each making up 10% of the medium (Table 4). Colony formation on plastic dishes, where anchorage was permitted, was insensitive to monkey serum for each of the three cell lines and for the primary RE cells, and was greater than 10% for all cultures in fetal calf serum or monkey serum (Table 4). Dog serum was slightly toxic, but this toxicity was not specific for any line (Table 4).

Growth in semi-solid medium was, on the other hand, strikingly dependent on the species of serum as well as on plasminogen activator production. Primary RE cells, which produced little activator, did not grow in any of the sera tested (Table 4). All three SVRE clones grew better in Methocel in media with either dog or monkey serum than in fetal calf serum alone (Table 4). SVRE 12 showed an especially marked increase in anchorage-independent growth, with a 1000 times increase in plating efficiency in methyl cellulose in dog serum as compared with fetal calf serum (Table 4).

DISCUSSION

We have shown a clear connection between growth of cells in a specific *in vitro* oncogenic transformation assay and their biosynthesis of a specific protease activator, the cell factor of the fibrinolysin system. While these data do not exclude a role for other biochemical alterations, they do show that the alteration leading to plasminogen activation is tightly correlated with growth in semi-solid medium.

It should be noted that the cell factor is itself a protease (22, 24), and direct proof has not yet been obtained that plas-

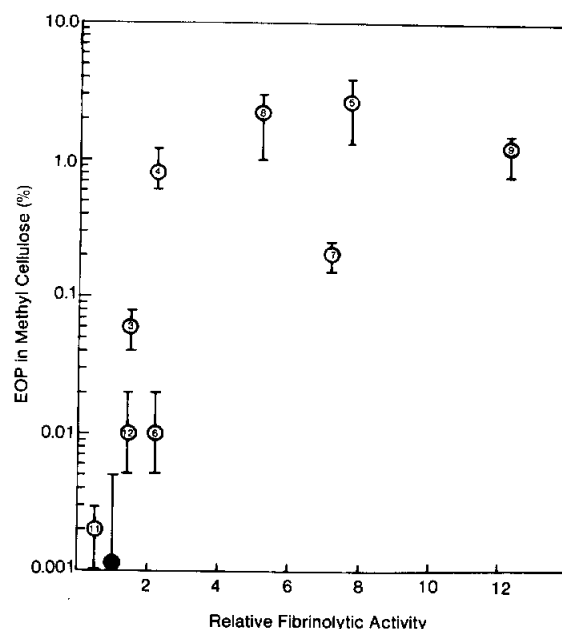


FIG. 1. Anchorage dependence vs fibrinolytic activity of normal and SV40 transformed clones of rat embryo cells. EOP = Efficiency of plating: colonies per 100 cells inoculated. Data on colony formation in methyl cellulose are taken from Table 1. Data on relative fibrinolytic activity are taken from Table 3. Fibrinolytic activity is that of plasminogen activated to plasmin by cellular factor. Each open circle is a separate SVRE clone; the filled circle is normal RE cells. The data for RE cells and clones 12, 3, 5, and 9 represent the averages of four separate assays of fibrinolytic activity and two assays of efficiency of plating in methyl cellulose. Other lines were assayed once.

min itself, rather than its activator, is required for growth in semi-solid medium. However, it has already been shown that hamster embryo cells transformed by SV40 form colonies less efficiently in the absence of plasminogen or in the presence of soybean trypsin inhibitor in semi-solid medium, even though plasminogen was not required for growth on a solid substrate (21). These findings, coupled with our data (Table 4), imply that plasmin generated by the activator that these cells produce, as well as the absolute level of activator synthesized, are both important for growth in semi-solid medium.

The evidence that tumors and cell lines derived from them synthesize large amounts of an activator of plasminogen, while

TABLE 4. Species-specific serum release of anchorage dependence

Line	Plasminogen* activator production	Anchorage permitted	Plating efficiency (colonies/100 cells inoculated)			
			Serum			
			Fetal calf-10%	Fetal calf-2.5% Dog-7.5%	Fetal calf-5% Dog-5%	Fetal calf-2.5% Monkey-7.5%
RE primary	1.0	Yes	30	1.5	8	20
		No	≤0.001	≤0.001	≤0.001	0.008
SVRE 12	1.4	Yes	34	30	34	53
		No	0.01	11	9.2	1.5
SVRE 5	7.6	Yes	75	37	75	77
		No	1.6	10.1	7.2	10.4
SVRE 9	12.3	Yes	60	21	45	88
		No	4.8	11.6	9.4	10.2

\* From Table 2, average of all experiments.

most normal tissues do not, has accumulated for many years and seems to be quite general (16–22, 24, 26, 31). However, *in vitro* studies have found that many “normal” cell lines, including for instance BHK21 and 3T3, do synthesize appreciable amounts of activator (ref. 24 and 26; D. Rifkin, R. Risser, and R. Pollack, in preparation); and some, such as BHK21, are in fact tumorigenic (9, 10, 12, 13). This raises a serious question about the usefulness of established “normal” cell lines for *in vitro* studies of oncogenic transformation. In the present study we bypassed this problem by transforming diploid primary cultures of rat embryo cells.

Finally, it should also be mentioned that the meaning of the loss of anchorage dependence is not yet understood (7, 32–37). If this property is a manifestation of the ability of cells to metastasize and grow while located in the three-dimensional structure of a tissue (38), the strong correlation of plasminogen activator level and growth in Methocel may well be related to the tumorigenic properties of metastasis and invasiveness. The SVRE clones isolated in this study have been employed in such an experiment by determining their tumorigenic potential by injection in *nude* mice. Preliminary results (39) are consistent with this hypothesis.

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