

## Anchorage Independence: Analysis of Factors Affecting the Growth and Colony Formation of Wild-Type and dl 54/59 Mutant SV40-Transformed Lines

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We have studied in detail the parameters of anchorage-independent growth of normal rat cells and two clones of simian virus 40 (SV40)-transformed rat fibroblasts. Normal secondary rat embryo fibroblasts (REF) suspended in soft agar neither form large colonies (greater than 0.2-mm in diameter) nor show any appreciable increase in total cell volume. A clone of wild-type SV40-transformed REF grows in agar with a colony-forming efficiency of 54% and an increase in total cell volume greater than  $10^3$ . A clone of REF transformed by the SV40 early deletion mutant 884 has a colony-forming efficiency in agar of only 0.02%, but the total increase in cell volume is greater than 100-fold. In defining anchorage transformants, a distinction must be made between the ability to form large colonies and the ability to undergo a significant number of doublings. Transformation by the viable deletion mutant 884 apparently is impaired far more in the former aspect than in the latter.

### INTRODUCTION

Simian virus 40 (SV40) is a small DNA virus capable of transforming nonpermissive cells *in vitro*. A number of changes occur in the phenotypic growth properties of these transformants. The ability to form large colonies without anchorage is the most complete expression of *in vitro* transformation (Risser and Pollack, 1974) and best correlates with tumorigenicity (Shin *et al.*, 1975; Kahn and Shin, 1979; Barrett *et al.*, 1979).

The early region of SV40 codes for the two known T-antigens (T-ag). The larger protein, with a molecular weight of 94,000 (Rundell *et al.*, 1977), appears to be necessary for the initiation and efficient maintenance of transformation (Tegtmeyer, 1975; Martin and Chou, 1975; Brugge and Butel, 1975). The smaller protein, with a molecular weight of 17,000 (Prives *et al.*, 1977), is coded for by the proximal end of the early region and shares N-terminal sequences with the larger T-ag. Deletion mutants in the region from 0.54 to 0.59 on

the SV40 map code for a normal-sized large T-ag, but small t-ag is either shorter or completely absent (Crawford *et al.*, 1978; Shenk *et al.*, 1976; Sleigh *et al.*, 1978). In the absence of a ts little t-ag, it is not possible to decide on genetic grounds whether little t-ag is necessary for the maintenance of transformation.

The deletion mutant 884 lacks approximately 184 base pairs from the region 0.54-0.57 (Shenk *et al.*, 1976), and has one of the largest deletions of this series. We have used this viral mutant and wild-type virus to transform normal cells in tissue culture. We then used two of the resultant clones to quantitate, in detail, the anchorage transformation assay.

### MATERIALS AND METHODS

*Cells and culture conditions.* Rat embryo fibroblasts were precrisis secondary cells prepared from an inbred Fisher strain (CDF albino) by surgically removing embryos from 13- to 15-day pregnant rats. The exact age of the embryos could not be determined, but they were definitely at a stage of development which was less than optimal for trans-

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formation (Risser and Pollack, 1979). They were washed in phosphate-buffered saline (PBS), the heads removed, and the bodies dispersed through a 20-ml syringe followed by treatment with 0.25% trypsin (Gibco). Cells were counted, spun down, resuspended in Dulbecco's modified Eagle's medium (DME) (Gibco H21) plus 10% fetal calf serum (FCS), and plated at approximately  $10^7$  cells per 100-mm dish. Cells were routinely cultured in a moist atmosphere of 10% CO<sub>2</sub>, 90% air at 37°.

**Virus.** A plaque-purified strain of SV40, 776, was used as wild-type virus. Strain 884 was a gift of P. Tegtmeyer. Infections were carried out by rinsing cultures containing  $10^6$  cells/100-mm dish with PBS and adding 1.0 ml of virus diluted with spent media to a final m.o.i. of 300. Cells were incubated for 2 hr, agitated gently every 20 min, rinsed with PBS, suspended in 0.05% trypsin plus  $5 \times 10^{-4}$  M EDTA (Gibco), and replated in DME plus 10% FCS.

**Transformation assay.** Focus formation assays were carried out by plating the infected cells in triplicate at concentrations of  $10^5$ ,  $10^4$ , and  $10^3$  cells per 60-mm dish. Media were changed twice weekly. After 3 weeks, cells were fixed with 10% formalin in PBS and stained with hematoxylin, and dense foci and total colonies were counted.

**Plasminogen activator.** Extracts were made from cells and incubated for 2 hr in <sup>125</sup>I-fibrin-coated plates as described (Rifkin and Pollack, 1977). Values expressed are those determined with 2 µg protein.

**Agar growth.** Growth in agar was determined by suspending cells in 0.05% trypsin plus  $5 \times 10^{-4}$  M EDTA and diluting and plating in media plus serum containing 0.37% agarose (Difco) over a base layer of medium plus serum containing 0.9% agar (Difco). Cells were plated in triplicate at densities of  $10^5$ ,  $10^4$ , and  $10^3$  per 60-mm dish. They were fed twice weekly by the addition of 2 ml medium plus 10% serum plus 0.37% agarose. After 3 weeks, colonies greater than 0.2 mm in diameter were scored.

Determination of total growth in agar was done by growing cells as above. An inverted microscope (Leitz) was used to count the number of colonies greater than two cells in size, which were present in at least 50

randomly selected fields from each dish. At the same time, the diameters of each colony were measured, using a calibrated reticle positioned in one eyepiece. Where possible, at least 100 colonies were measured. The total number of colonies per dish was calculated by multiplying the average number of colonies per field by the number of fields per dish. Average colony diameter was calculated from the individual measurements and used to estimate the total cell volume per dish. The volume of a single cell was estimated to be  $3 \times 10^{-5}$  mm<sup>3</sup>. Colony size and colony number distributions were done with the aid of a computer.

**Cloning transformants.** Dense foci were surrounded by sterile cloning rings, and the cells suspended by trypsinization. The cells were then subcloned in microtiter wells (Falcon). Two densely growing colonies which were T-antigen positive by immunofluorescence (Steinberg *et al.*, 1978) were used for further experiments.

## RESULTS

### *Transformation Frequency*

The ability of wild-type SV40 and 884 to transform secondary rat embryo fibroblasts is shown in Table 1. Density transformation was determined by counting dense foci on semiconfluent cultures, and dividing by the total number of colonies found on sparse plates after 21 days. There was no difference in the transformation frequency for the two viruses. This data agree with previous reports (Bouck *et al.*, 1978; Martin *et al.*, 1979). Moreover, there was no appreciable difference in the total cell number per plate (data not shown). By the criterion of dense foci, 884 can transform as well as wild-type virus.

The transformation frequencies in the density and anchorage assays (Table 1) are not directly comparable. At the time dense foci were counted, some of the experimental plates were trypsinized and transferred into soft agar. This allowed full expression of the virus without anchorage deprivation, and assured that we were measuring the anchorage requirement for maintenance and not the establishment of transformation.

An aliquot was plated on plastic to de-

TABLE 1  
TRANSFORMATION BY WILD-TYPE AND 884 SV40

Virus	Density transformation (dense colonies/100 colonies)	Plating efficiency, plastic <sup>a</sup> (colonies/cell)	Plating efficiency, agar <sup>a</sup> (agar colonies/cell)	Relative plating efficiency <sup>b</sup>
Passage (days after infection)	1	21	21	—
WT	0.02	0.1	>0.001	>0.01
884	0.02	0.08	0.00005	<0.0007
Mock	<0.001	0.06	<0.000001	<0.00001

<sup>a</sup> Cells were suspended in trypsin and transferred en masse from a plate containing dense foci to soft agar 3 weeks after viral infection. A sample was plated on plastic at that time to determine plating efficiency.

<sup>b</sup> Plating efficiency (agar)/plating efficiency (plastic).

termine the proportion of cells capable of forming colonies. Plating efficiencies on plastic were similar for both infected and uninfected cells because only a small percentage of the REF was transformed. Feunteun *et al.* (1978) and Bouck *et al.* (1978) measured agar growth immediately after infection with dl 0.54/0.59 virus and found it reduced. We found that the accumulation of cells capable of expressing the anchorage-transformed phenotype over 3 weeks was less by 20-fold for 884 compared to wild-type SV40.

#### Isolation of Clones

Two density-transformed clones were isolated from plastic dishes by recovery and recloning of dense foci. Line WTA was transformed by wild-type SV40. It grew to a high density in 10% fetal calf serum and grew well in low serum. Line 884B, transformed by the early mutant 884, also grew to a high density in 10% FCS and grew in low serum. Both clones were greater than 99% T-antigen positive as determined by indirect immunofluorescence (Steinberg *et al.*, 1978). WTA contained both 94,000 and 17,000 molecular weight immunoprecipitable T-antigens. 884B contained the MW 94,000 band, but lacked the MW 17,000 band (A. Lo, personal communication). These two clones were used to study the parameters of the agar assay.

#### Serum Factors

Several years ago, Pollack *et al.* (1975) reported that growth of transformed rat cells in methocel could be enhanced by the addition of dog serum. They showed that the production of plasminogen activator correlated well with anchorage-independent growth, and that dog serum permitted high levels of fibrinolysis. Plasminogen activator from extracts of WTA and 884B was capable of digesting 19 and 27% of iodinated fibrin in 2 hr respectively (D. Rifkin, personal communication). These values are consistent with other SV40-transformed rodent cell lines (Rifkin and Pollack, 1977). Moreover, the two transformants have comparable levels of plasminogen activator.

We tested the ability of our two cloned transformants and normal secondary cells to grow in soft agar in the presence or absence of dog serum (Table 2). Since 5% dog serum appeared to be somewhat toxic when the cells were growing on plastic, the plating efficiency in agar was normalized to the plating efficiency on plastic in 10% FCS. This permitted a reasonable estimate of the number of viable cells which were capable of forming colonies. No colonies were formed by REF when the three types of cells were plated in agar in 10% FCS. WTA grew well, with a relative plating efficiency of 54%. 884B grew better than REF, since the inoculation of  $10^5$  cells resulted in an average of 17 colonies greater than 0.2 mm

TABLE 2  
EFFECT OF DOG SERUM ON COLONY GROWTH IN AGAR

Cells	Serum	Plating efficiency, plastic (colonies/cell)	Plating efficiency, (agar colonies/ cell)	Relative plating efficiency <sup>b</sup>
REF	10% FCS	0.004	<0.00001	<0.001
WTA	10% FCS	1.0	0.54	0.54
884B	10% FCS	0.83	0.00017	0.0002
REF	5% FCS, 5% dog	—	<0.00001	<0.001
WTA	5% FCS, 5% dog	—	0.018	0.018
884B	5% FCS, 5% dog	—	0.007	0.009

<sup>a</sup> Colonies >0.2 mm in diameter.

<sup>b</sup> Plating efficiency in agar/plating efficiency in 10% FCS on plastic.

in diameter. This value was 3000-fold less than WTA, confirming that this 884-transformed clone retained most of its anchorage requirement under standard assay conditions and that these two clones were each similar to the average behavior of WT and dl 0.54/0.59 clones studied by Sleight *et al.* (1978).

When a mixture of dog and fetal calf serum (Martin *et al.*, 1979; Bouck *et al.*, 1978) was used, the normal REF still did not grow. Growth of WTA was reduced about 10-fold, although a significant number of colonies still formed in agar. 884B grew much better in the mixture of dog and fetal calf serum, increasing colony formation nearly 50-fold. Thus, for this 884-transformed line, the block to anchorage independence was overcome under certain culture conditions which did not override the anchorage requirement of untransformed cells.

#### Total Cellular Increase in Agar

It was apparent in the earlier experiments that cells of the 884B clone grew into large numbers of tiny colonies, below the 0.2-mm threshold we had used for scoring growth. To quantitate this growth, we determined the actual increase in cellular volume after suspension of normal and transformed cells in agar.

Three weeks after inoculation, a large

number of fields were scored for colonies using an inverted microscope. All colonies in each field were counted, and their diameters were measured (see Materials and Methods). These data were used to determine the total number of colonies per dish and the average colony volume. From these values, the colony volume increase (CVI) (colony number  $\times$  average colony volume/total volume of cells inoculated) was calculated.

Table 3 shows the colony volume increase (CVI) in a 60-mm dish after 3 weeks of incubation. The rat embryo fibroblasts showed no significant CVI under any of the conditions used. No colonies greater than two cells in size were detected when  $10^4$  cells were plated in a mixture of fetal calf serum and dog serum. By even the most stringent criterion of anchorage independence, involving only a few doublings, these cells would clearly have been considered normal.

WTA grew to approximately the same final cell volume in 10% fetal calf serum whether inoculated at  $10^5$  or  $10^4$  cells per dish. At the lower inoculation density the addition of dog serum appeared to reduce somewhat the CVI of WTA, although the effect was not as great as the reduction seen earlier in determining numbers of colonies greater than 0.2 mm (see Table 2). The reason for this is not known, but may be related to the poorer plating efficiency

TABLE 3  
INCREASE IN TOTAL CELL VOLUME AFTER GROWTH IN AGAR

Cells	Final volume/initial volume			
	10 <sup>5</sup> Cells plated <sup>a</sup>		10 <sup>4</sup> Cells plated <sup>a</sup>	
	10% FCS	5% FCS + 5% dog	10% FCS	5% FCS + 5% dog
REF	1.6	1.2	1.7	1.0
WTA	1.6 × 10 <sup>3</sup>	2.8 × 10 <sup>3</sup>	2.9 × 10 <sup>4</sup>	5.2 × 10 <sup>3</sup>
884B	1.1 × 10 <sup>2</sup>	3.5 × 10 <sup>2</sup>	4.9 × 10 <sup>2</sup>	5.4 × 10 <sup>2</sup>

<sup>a</sup> Number of cells inoculated into 60-mm dish.

we see on plastic when small numbers of cells are plated. At 10<sup>5</sup> cells/dish inoculation density this effect was not observed.

Most 884B growth in agar was limited to tiny colonies under all the conditions used for this experiment. The total CVI generated by these colonies was 10-fold less than that seen for WTA (Table 3) at 10<sup>5</sup> cells/dish. The difference between WTA and 884B was 60-fold with 10<sup>4</sup> cells inoculated into fetal calf serum. These conditions allowed for the greatest increase in cell mass for WTA. The effect of dog serum on the final mass of 884B was slight, with only a 3-fold maximum enhancement. This was in sharp contrast to the 45-fold increase in large colonies under these conditions (see Table 2), suggesting that the presence of dog serum might have been affecting the distribution of colonies by sizes, rather than the overall growth capability of this line.

Since both WTA and 884B showed an increase in CVI when 10<sup>4</sup> cells rather than 10<sup>5</sup> were inoculated, we considered the possibility that a nutrient needed for rapid growth in agar was limited. If this were the case, then reducing the cell inoculum to 10<sup>3</sup> cells/dish would result in larger colonies in agar. When this was done, the CVI of WTA in either serum was slightly larger than that seen for 10<sup>4</sup> cells, while the CVI and total number of colonies of 884B were reduced (data not shown). Thus, nutrient deprivation is unlikely to be a major factor in the behavior of 884B. At 10<sup>3</sup> cells there was an increase in the CVI difference between WTA and 884B. However, since the

very small number of colonies of 884B increased the statistical uncertainty of our measurements, we chose a 10<sup>4</sup> cells/dish inoculum for further analysis.

#### Colony Volumes

To test the hypothesis that the distribution of colony size might be significantly different for WTA and 884B, we determined the volume (mm<sup>3</sup>) for each colony greater than two cells in size. Single cells and doublets were not measured and were not included in the calculations. Colony volumes are used in Fig. 1 to express each class of colony size as a fraction of the total number of colonies. These values allow us to compare the contribution of each colony size to the total number of colonies, and also allow a comparison of actual differences in growth among the different cell lines using the two types of serum.

The REF formed only a small number of colonies greater than doublets in 10% FCS. Of these, 80% were of the smallest measurable volume, and none were 0.2 mm in diameter or greater (Fig. 1A). When grown in a mixture of FCS and dog serum, there were no measurable colonies (Fig. 1B). This explains the finding that there was no appreciable CVI when 10<sup>4</sup> embryo fibroblasts were cultured in 10% FCS, and no detectable increase in 5% FCS plus 5% dog serum (Table 3). This supports our initial qualitative observation that normal secondary cells are markedly restricted in their ability to grow without anchorage.

Nearly all the colonies of WTA were 0.2

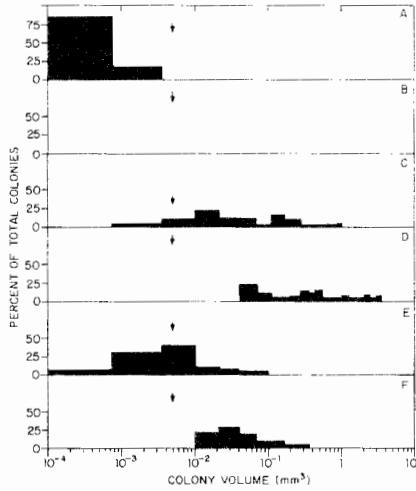


FIG. 1. Distribution of colony sizes in different sera. Plates (60 mm) were inoculated with  $10^4$  cells. Colony volumes were measured as described under Materials and Methods for each colony greater than two cells in size. The percentage of total colonies having a given volume is plotted against colony volume. The arrow represents 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) 884B growing in 10% FCS. (F) 884B growing in 5% FCS, 5% dog serum.

mm or greater in diameter when grown in 10% FCS (Fig. 1C). There was approximately a 100-fold range in the sizes of the colonies, suggesting that the WTA population may be somewhat heterogeneous with regard to its rate of growth without anchorage. This variability has been noted with other SV40-transformed cell lines as well (Risser *et al.*, 1974), even though all the lines had been previously cloned from single cells. Each category of colony volume made an appreciable contribution to the total number of colonies. Calculation of the total cell volume in each category of colony size showed that the greatest amount of cell volume was in the large colonies (data not shown).

When WTA was grown in a mixture of 5% FCS plus 5% dog serum, there was a similar distribution of colony sizes, with nearly a 100-fold variation (Fig. 1D). This distribution was shifted, so that the average colony volume was approximately 10-fold larger in

the presence of dog serum. However, the increase in the average colony size was more than offset by the reduction in the number of colonies of every size (data not shown), resulting in a decreased total cell volume (Table 3). In particular, the absolute number of large colonies decreased in fetal calf plus dog serum. Therefore the class of largest of colonies contained a total volume almost 6-fold less than in the presence of 10% FCS ( $1.5 \times 10^3$  as compared to  $9 \times 10^3$ ).

The ability to form very large colonies containing much of the total cellular volume seems to be the primary difference between WTA and 884B. The growth of 884B in 10% FCS in soft agar (Fig. 1E) was limited as compared to WTA. Most of the colonies were at or below the threshold generally used for scoring growth. Relative to the parental REF cells (Fig. 1A), however, the growth of 884B was very substantial. This cell line appears to be intermediate between the fully transformed WTA and the REF.

The presence of 5% FCS plus 5% dog serum did not appreciably alter the total cell increase of 884B (Table 3), but the distribution of colony sizes shifted, becoming 10-fold larger (Fig. 1F). Thus the enhancement of 884B growth in the presence of added dog serum seen in Table 2 was a consequence of a change in the colony size distribution. The increased size of the colonies was almost exactly balanced by a decrease in the total number of colonies. Even under these conditions, there was a significant difference between the growth of 884B and WTA. The largest colonies of 884B contained a volume only one-tenth that of the largest WTA colonies.

A summary of the data from Tables 2 and 3 is shown in Fig. 2, which compares the results of the two different assay methods. The REF cells grew only slightly, and formed almost no colonies. The WTA increased in cell number by four orders of magnitude, and most of the growth was in large colonies. The ratio of total cells to colony number was comparable for these cell lines. This was not the case for 884B, where the final number of cells (CVI) was much higher than would be predicted from the number of colonies. Thus the CVI increase and the standard

measure differ in the case of the 884-transformed clone but not in the REF or WTA.

#### DISCUSSION

This study is an attempt to understand the anchorage assay, using the apparent restriction on anchorage transformation by the SV40 viable deletion mutant 884 to generate clones with different responses to the anchorage assay. Previously, Bouck *et al.* (1978) and Feunteun *et al.* (1978) have reported that a large number of different early deletion mutants had a reduced but significant ability to establish anchorage-independent transformants using established cell lines. Their criterion for anchorage transformation was the ability to form large (i.e., 0.2 mm) colonies when suspended in soft agar. Sleight *et al.* (1978) reported a similar finding after isolating density transformants from several deletion mutants and testing their ability to form large colonies in methocel. The agreement of our data (Tables 1, 2) with these earlier studies made it possible to use the clones 884B and WTA as representative examples to study the anchorage assay in detail. In addition, we have recently found similar differences between CVI and formation of large colonies when comparing an SV40-transformed rat line and its T-negative revertant (Steinberg *et al.*, 1979). Several clones transformed by WT polyoma and early deletion mutants also show similar results when analyzed in the manner we describe in this paper (Griffin *et al.* 1980). In all cases the deletion mutants generated transformants whose CVI was less affected than their ability to form large colonies.

It would appear that the 884 deletion mutant is capable of transforming normal rat cells so that a resultant clone can undergo a limited number of doublings during the first 3 weeks in agar. The colonies of 884B appeared to slowly increase in size during the incubation period, suggesting that these cells have a lowered probability of transverting the G<sub>1</sub> phase of the cell cycle (O'Neill *et al.*, 1979). They did not double at the same frequency as WTA for a few days and then stop growing, as would be

expected if their growth were analogous to abortive transformation.

#### *Serum Factors*

O'Neill *et al.* (1979) have recently reported that the growth of NIL-8 hamster fibroblasts suspended in agarose was dependent on the concentration of serum. When serum levels were high enough, the transition probabilities through the cell cycle (and therefore the growth rates) of suspended cells equaled those of cells in attached cultures. This suggests that the levels of some serum factors are critical for rapid anchorage-independent growth. Our studies with a mixture of dog and fetal calf serum, compared to fetal calf serum alone, suggest that the factors may be cell line specific. Moreover, we found that the presence of dog serum in the agar affected the size of individual colonies of transformed cells rather than the overall increase in cell volume. Since Sleight *et al.* (1978) used only fetal calf serum and Martin *et al.* (1979) used only a mixture of dog and fetal calf serum, the differences we observed could explain some of the differences between their data.

Kahn and Shin (1979) have recently found that the use of McCoy's 5a medium, rather than DME, permits the anchorage-independent growth of a series of BHK21 hamster cells and several clones of virally transformed BHK21 cells. Since McCoy's 5a contains bactopectone, they suggest that the addition of purines may be necessary for some conditionally anchorage-independent lines. Therefore, the effects we have seen with different serum factors are not unique. There may well be many different factors which affect the ability of some, but not all, transformants to grow without anchorage.

#### *Colony Volumes*

The usual procedure for scoring anchorage-independent growth requires the presence of large colonies (Bouck *et al.*, 1978; Feunteun *et al.*, 1978; Shin *et al.*, 1975). Martin *et al.* (1979), scoring any-sized colonies as positive, have reported that they

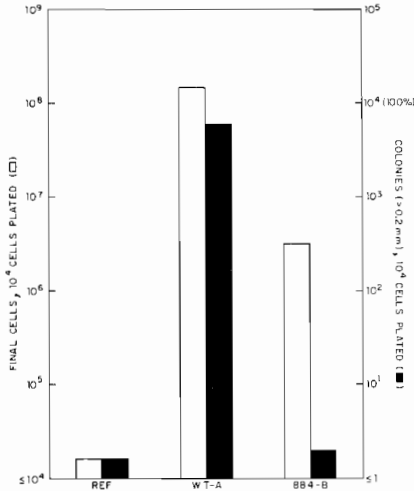


FIG. 2. Relative colony formation and increase in cell number for WTA, 884B, and REF. The final number of cells was calculated from the total increase in cell volume, assuming a constant unit cell volume regardless of colony size.  $10^4$  cells were inoculated.

found little or no difference in the agar growth of deletion mutant and wild-type SV40-transformed CHL cells. However, their category of largest-sized colonies begins just at the threshold used by others. An analysis of their data shows that only 3% of the deletion mutant-transformed lines isolated as dense foci form any large colonies, while 17% of comparable wild-type transformants form large colonies. It is now clear that with some cells these arbitrary methods can produce a set of results different from that obtained by scoring total colony volume increase (CVI) (Fig. 2). Conflicting data reported from different laboratories may be the consequence of variations in scoring criteria of the sort analyzed in this paper.

### Tumorigenicity

The ability of cells to form large colonies in agar with a high efficiency correlates very well with their tumorigenicity in nude mice (Shin *et al.*, 1975). It is possible to describe three classes of cells based on tumor formation in nude mice (Kahn and Shin, 1979). Class 1 includes fibroblast-like and epithelioid nontumorigenic cells. REF are

in this class. Class 2 cells are nontumorigenic under the standard assay of  $2 \times 10^6$  cells injected subcutaneously, but sometimes give tumors when  $10^7$  or more cells are injected. Rat-1, an established non-transformed line (Steinberg *et al.*, 1978), belongs in this class. It is perhaps significant that Rat-1 is capable of the same type of limited growth in agar as shown for 884B (Steinberg *et al.*, 1979). Class 3 cells are highly transformed, grow well in agar, and generate tumors with nearly 100% efficiency in the standard assay. WTA falls into this class.

### Intermediate Transformation

Risser and Pollack (1974) found that SV40 infection of 3T3 cells generated three types of transformants: minimal, intermediate, and full. The serum and density transformation phenotypes existed in the absence of anchorage transformation, but the converse was not true, and agar growth was the most fully transformed phenotype. Moreover, serum and density transformants had either no or low levels of T-antigen(s) as detected by immunofluorescence and complement fixation. Anchorage transformants had high levels of T-antigen(s). When the same analysis was applied to primary or secondary fibroblasts, a partial gradient of the transformation phenotype was again seen, but now all the cells contained large amounts of T-antigen (Risser *et al.*, 1974). This suggested that at least viral big T-antigen and possibly other tumor antigens (Pollack *et al.*, 1979; Lane and Crawford, 1979) were necessary to enable the primary cells to become a stable, established line capable of growth in sparse culture. The intermediate transformants they isolated from REF infected by wild-type SV40 had a phenotype similar to our 884B and the deletion transformants isolated by Sleight *et al.* (1978): growth in low serum, loss of density inhibition, T-antigen-positive nuclei, and an inability to form large colonies in agar. Taking these data together, we conclude that small t-antigen is not required for the initial conversion of a secondary fibroblast to an established line or to an

intermediate transformant but may be required for stable rapid growth without anchorage.

While it is not possible to generalize from these single clones of WT and 884 origin, recent *in vivo* studies are consistent with the observation that mutation of SV40 small t diminishes, but does not eliminate, the tumorigenic syndrome. Many 0.54/0.59 deletion mutant SV40 virus stocks have been injected into neonatal hamsters. In all cases the SV40 deletion mutant viruses have been tumorigenic. However, the tumors arising from dl 0.54/0.59 virus are always slow growing, and have a much longer latency than the tumors induced by injection of wild-type virus (Lewis and Martin, 1979; W. Topp, personal communication). Taken together, these data suggest that both SV40 viral antigens participate in tumorigenicity, and that the CVI assay is a closer measure of that aspect of anchorage independence which correlates with tumorigenicity than is the standard visible colony assay.

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