

## Tumorigenicity of Revertants From an SV40-Transformed Line

Bettie M. Steinberg, Daniel Rifkin, Seung-il Shin, Charles Boone, and Robert Pollack

*Department of Biological Sciences, Columbia University, New York, New York 10027 (B.M.S. and R.P.), Department of Cell Biology, New York University Medical School, New York, New York 10016 (D.R.), Department of Genetics, Albert Einstein College of Medicine, Bronx, New York, 10461 (S.S.), and Cell Biology Section, Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20014 (C.B.)*

A syndrome of *in vitro* properties correlates with the tumorigenicity of SV40-transformed rodent cells. These properties are: plasminogen activator production, loss of large actin cables, and anchorage-independent growth. An established rat fibroblast line, its SV40 transformant, several T-antigen negative revertants, and a spontaneous retransformant isolated from one of the revertants were analyzed *in vivo* for their tumorigenicity and *in vitro* for the syndrome. The two transformed lines were highly tumorigenic, and had clearly abnormal *in vitro* properties. The parental rat line was weakly tumorigenic in nude mice and demonstrated a slightly transformed response in the *in vitro* assays. The revertants were completely nontumorigenic. Expression of the *in vitro* syndrome was not uniform for all revertants; however, most cell lines maintained the correlation of the syndrome and tumorigenicity.

**Key words:** SV40 transformation, tumorigenicity, anchorage independence

SV40-transformed murine cells demonstrate a number of cellular properties which differ from normal cells and can be studied *in vitro*. Selective characteristics include a reduced need for serum [1], density-independent growth [2], and the ability to grow without anchorage [3]. Among the nonselective changes which occur are decreased organization of the actin-containing cytoskeleton [4], increased production of proteolytic enzymes [5], and a reduction of cell surface fibronectin [6]. Anchorage independence is the change that best correlates with tumorigenicity [7], although there is also a fairly good correlation with plasminogen activator production and with disruption of intracellular actin cables [7–9]. This syndrome of changes would appear to be interrelated, since a change in any one characteristic usually results in changes in the others (Fig. 1).

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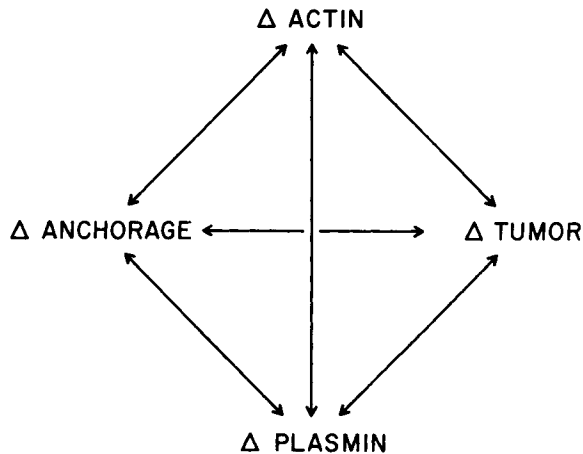


Fig. 1. Tumorigenicity syndrome. The relationship of changes in tumor forming ability to changes in anchorage independence, the structure of actin cables, and the production of plasminogen activator is shown. Double-headed arrows indicate that the changes are coordinate in both directions.

Revertants from transformed cells which have been previously isolated regained some of the normal *in vitro* growth properties and showed a reduced degree of tumorigenicity [10–12]. We have isolated a group of revertants from an SV40-transformed rat line which contained a single copy of the SV40 genome [13]. These revertants have lost detectable large T and small t antigens and regained the *in vitro* growth control of the non-transformed grandparental line. In this article we report on the tumorigenicity, and the *in vitro* characteristics which best correlate with tumor formation, of revertants and of a spontaneously occurring retransformed clone derived from one of the revertants.

## MATERIALS AND METHODS

### Cells and Culture Procedure.

The cell line 14B, the revertants F1<sup>1</sup> 1-4, 3-3, 3-5, 3-7 and 3-8, and parental line Rat-1 have been previously described [13]. 1-4 MCA was a spontaneous retransformant of F1<sup>1</sup> 1-4. It was isolated by picking a single large colony growing in methocel, and recloned by plating in microtiter wells (Falcon). 14B/nu 1 and T 14B were isolated from tumors excised from a nude mouse and a neonatal Fisher rat respectively. All cells were cultured in Dulbecco's modified Eagle's Medium (Gibco H21), supplemented with 10% fetal calf serum (Reheis) and 100 units/ml of penicillin and streptomycin (Gibco).

### Plasminogen Activator Levels.

Assay methods for plasminogen activator have been previously described [14].

### Actin Containing Cytoskeleton.

Presence of large actin cables was determined by indirect immunofluorescence. Cells seeded on glass coverslips at  $10^3/\text{mm}^2$  were grown for 2 days, fixed with 10% formalin for 15 minutes, treated with 1% NP40 in PBS for 20 minutes at room temperature, and stained with rabbit anti-actin serum (gift of K. Burridge, CSH) followed by goat anti-rabbit IgG (Cappel) [15].

**Anchorage Independence.**

Cells were plated in triplicate at  $10^5$ ,  $10^4$ , and  $10^3$  cells/60mm dish in 3 ml DME 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-DME-10% FCS and cultured for 3 weeks. Large colonies greater than 0.2mm in diameter were scored using a dissecting microscope. Total colony volume (CVI) increase in agar was determined as described [16].

**Tumorigenicity.**

Ability of cells to form tumors in nude mice and Fisher rats was previously described [17, 18]. Briefly, cells for injection into nude mice were trypsinized, resuspended in phosphate buffered saline (PBS) at the desired concentration and 0.2 ml injected subcutaneously at a single site.

Tumorigenicity in rats was determined by trypsinizing cells, suspending in DME at the desired concentration and injecting 0.2 ml subcutaneously. Newborn rats were inoculated within 48 hours of birth.

**Chromosome Number**

Determination of chromosome number was carried out by the method of Vogel et al [19].

**RESULTS****In Vitro Properties**

Table I shows 3 in vitro properties of the cells: plasminogen activator production, the presence of well defined actin cables, and the ability to grow in soft agar. Rat-1, an established rat fibroblast cell line, produced low levels of plasminogen activator, in any

**TABLE I. Syndrome of In Vitro Properties Which Correlate With Tumorigenicity**

Cells	Production of plasminogen activator (% counts released)			Cytoskeletal organization % Cells with actin cables	Anchorage independence	
	Fibrin plates	Cell extract	Harvest fluid		Relative EOP agarose/plastic	Colony volume increase in agarose <sup>a</sup>
Rat 1	8	4	0	59	$7.0 \times 10^{-5}$	$1.3 \times 10^1$
14B	43	27	0	17	$1.5 \times 10^{-1}$	$3.8 \times 10^2$
FI 1-4	74	51	44	64	$3.0 \times 10^{-5}$	$2.1 \times 10^1$
FI 3-3	29	21	0	78	$5.0 \times 10^{-5}$	ND
FI 3-5	ND	ND	0	72	$4.0 \times 10^{-5}$	ND
FI 3-7	13	10	0	81	$3.0 \times 10^{-5}$	ND
FI 3-8	9	6	0	90	$4.0 \times 10^{-5}$	1.0
1-4 MCA	72	ND	ND	2	$5.4 \times 10^{-2}$	$4.2 \times 10^3$

<sup>a</sup> $10^5$  cells plated.

ND, not determined.

of the 3 assays. The majority of Rat-1 cells contained large actin cables. This line did not grow well in soft agar when scored for the presence of large ( $> 0.2\text{mm}$ ) colonies ( $7 \times 10^{-3}\%$  relative plating efficiency). However, this arbitrary criterion of colony size does not detect a significant increase in cell volume if most or all of the colonies are smaller than the threshold at the time they are measured. Therefore we also determined the line's colony volume increase (CVI), defined as the (average colony volume  $\times$  total number of colonies)/(total initial volume of the cells plated in agar [16]). There was a 10-fold increase in colony volume when Rat-1 was grown in soft agar.

14B, an SV40 transformant of Rat-1, grew in low serum, grew to a high density on plastic and contained both large and small T-antigens [13]. It produced approximately 6 times more plasminogen activator than Rat-1, with all of the enzyme cell-associated. There was no detectable plasminogen activator released in the harvest fluid. This cell line showed a reduction in actin cables, compared to Rat-1, but a significant percentage of the cells still contained some cables. 14B formed colonies in soft agar with a 15% relative plating efficiency. The CVI of 14B was  $3.8 \times 10^2$ . For comparison, the CVI of a clone of rat embryo fibroblasts transformed by wild-type SV40 was  $1.6 \times 10^3$  when  $10^5$  cells were inoculated into soft agar [16]. 14B has a CVI capability appropriate for a fully transformed line.

The revertants FI<sup>1</sup> 1-4, 3-3, 3-5, 3-7 and 3-8 were all isolated from 14B [13]. They were all contact inhibited and did not contain either large or small T antigen. FI<sup>1</sup> 1-4 appeared to contain the intact SV40 genome, while 3-3 and 3-5 had undergone deletions within the SV40 early region, and 3-7 and 3-8 lacked detectable viral sequences [13]. Base sequence analysis will be necessary to determine whether FI<sup>1</sup> 1-4 contains a point mutation in the early region, or a deletion too small to be detected by the methods used.

The cured cells 3-7 and 3-8 were at least as growth controlled as Rat-1. They produced very little plasminogen activator, contained large numbers of well defined cables, and had a very low plating efficiency in soft agar. In fact, the CVI of 3-8 was 10-fold lower than Rat-1. No cell growth in agar by 3-8 was detectable.

FI<sup>1</sup> 3-3, containing a large deletion in the early region of SV40, produced a significant amount of plasminogen activator. The counts released by growing the cells on fibrin coated plates were less than for 14B, but extracts from the cells were comparable. FI<sup>1</sup> 3-3, and FI<sup>1</sup> 3-5 contained fewer actin cables than the cured revertants, but more than Rat-1. Neither line was able to form large colonies in agar. The CVI of these cells has not been determined.

FI<sup>1</sup> 1-4 contains a full sized SV40 genome, as measured by the Southern blotting technique [13]. This revertant produced twice the plasminogen activator of 14B, as assayed by growth on fibrin-coated plates or assay of cell extracts. It was the only cell line of this set to release significant amounts of enzyme into the surrounding media. As with many other PA secreting cell lines, FI<sup>1</sup> 1-4 growth in agar was enhanced by the presence of dog serum [16]. FI<sup>1</sup> 1-4 had an actin cable composition comparable to Rat-1. Like the other revertants, FI<sup>1</sup> 1-4 had a very low plating efficiency when counting large colonies in agar. Its CVI was  $2.1 \times 10^1$ , more like Rat-1 than like 3-8.

The last cell line described in Table 1 is 1-4 MCA. This line was isolated from a rare colony of FI<sup>1</sup> 1-4 growing in methocel. It had no fixed saturation density. When plated sparsely, it formed very dense colonies, but if plated at 1/10 confluence, the saturation density of this line in 10% FCS was only  $2.3 \times 10^5$  cells/cm<sup>2</sup>. Its doubling times in 1% and 10% FCS were 58 hours and 33 hours respectively, compared to 22 and 14 for 14B, and 56 and 25 for FI<sup>1</sup> 1-4 [14]. 1-4 MCA was negative for nuclear T-antigen as measured by indirect immunofluorescence.

TABLE II. Tumorigenicity of 14B, Revertants and a Spontaneous Retransformant in Nude Mice

Cells	Inoculum	No. of tumors / No. of animals	Days after inoculation
Rat 1	$2 \times 10^6$	0/4	> 150 <sup>a</sup>
Rat 1	$1 \times 10^7$	2/3	84
14B	$1 \times 10^7$	3/3	15
14B	$1 \times 10^6$	2/2	60
14B	$1 \times 10^5$	1/1	90
FI 1-4	$1 \times 10^7$	0/4	> 150 <sup>a</sup>
FI 3-3	$1 \times 10^7$	0/4	> 150 <sup>a</sup>
FI 3-5	$1 \times 10^7$	0/4	> 150 <sup>a</sup>
FI 3-7	$1 \times 10^7$	0/4	> 150 <sup>a</sup>
FI 3-8	$1 \times 10^7$	0/4	> 150 <sup>a</sup>
1-4 MCA	$5 \times 10^6$	3/3	28

<sup>a</sup>Discontinued at 150 days – still free of tumors.

When assayed for those in vitro properties which correlate with tumorigenicity, 1-4 MCA was found to more like 14B than like F<sup>1</sup> 1-4 (Table 1). The plasminogen activator activity of live 1-4 MCA cells on fibrin plates was 72%. This cell line contained almost no actin cables. It formed large colonies in soft agar with a relative efficiency of 5.4%, and a total CVI of  $4.2 \times 10^3$ . The CVI of 1-4 MCA was greater than the CVI of 14B. Also, although the total number of 1-4 MCA colonies larger than 0.2 mm was fewer than 14B, the 1-4 MCA colonies grew to a significantly larger size. Based on these results, 1-4 MCA had a highly transformed phenotype.

### Tumorigenicity

The ability of the various cell lines to form tumors in the nude mouse was determined (Table II). Rat-1 did not form tumors at an inoculation density of  $2 \times 10^6$  cells. It did slowly form tumors if  $10^7$  cells were injected. None of the revertants were able to form tumors, even at the higher inoculation density. 1-4 caused the formation of small nodules which eventually regressed. The SV40 transformed parent, 14B, was able to generate a tumor with an inoculation as small as  $10^5$  cells. The time of appearance of 14B tumors in nude mice was inversely proportional to cell number at inoculation. 1-4 MCA was highly tumorigenic, forming tumors with an efficiency comparable to 14B. This correlated well with its CVI, anchorage independence, disorganization of actin, and production of plasminogen activator (Table I).

We also measured the ability of 14B and the revertants to form tumors in inbred Fisher rats (Table III). 14B generated tumors in both newborn and weanling rats, but not in adults. Four months after inoculation, there were still no tumors in any of the newborn rats injected either with Rat-1 or the revertants. These experiments are being continued, but at the present time we conclude that the tumorigenic pattern of 14B and the revertants is the same in nude mice and in syngenic rats.

**TABLE III. Tumorigenicity of 14B and Revertants in Fisher Rats**

Cells	Inoculum	No. of Tumors/ No. of animals		
		Newborn	Weanling	Adult
Rat 1	$1 \times 10^7$	0/3	0/3	0/3
14B	$1 \times 10^7$	5/5	6/6	0/3
14B	$1 \times 10^6$	3/3	2/5	0/3
14B	$1 \times 10^5$	0/3	ND	ND
FI 1-4	$1 \times 10^7$	0/3	ND	ND
FI 3-3	$1 \times 10^7$	0/3	ND	ND
FI 3-8	$1 \times 10^7$	0/3	ND	ND

ND: not determined.

**TABLE IV. Properties of Cells Isolated From Tumors**

Cells	Tumor source	Doubling time (1%FCS/10%FCS)	Relative plating efficiency <sup>a</sup> (agarose/plastic)	Average No. of chromosomes
14B	—	0.44	0.14	41 (36–50)
TI4B	Fisher rat	0.32	0.27	33 (31–38)
14B/nu	Nude mouse	0.45	0.38	52 (48–60)

<sup>a</sup>agar colonies > 0.2mm

### Characteristics of Tumor Cells

Since 14B can spontaneously revert to a non-tumorigenic, stable “normal” cell [13], we considered the possibility that this cell line is markedly heterogenous, and that a small, highly transformed subset of the population actually gave rise to the tumors. We therefore isolated 14B tumor cells from both a Fisher rat and a nude mouse. A comparison of these tumor cells to the original 14B is shown in Table IV. The relative doubling time in 10% FCS and 1% FCS was comparable for the 3 lines, although not identical. Both tumor lines grew somewhat better than 14B in soft agar, using the standard assay of large colony formation. This selection is not surprising, since cells recovered from rare tumors which arose in nude mice after injection with other revertants had an increased capacity to grow in agar [12]. Here however, plating efficiency in soft agar of 14B/nu is increased only 2.7-fold over that of 14B because the plating efficiency in soft agar of 14B is already quite high.

Klinger et al [20] reported that both anchorage-independent growth and tumorigenicity in the nude mouse were accompanied by an increased ploidy in aneuploid Chinese hamster cells. We determined the chromosome numbers for 14B, T-14B, and 14B/nu (Table IV). As previously reported [13] 14B has a near diploid number of chromosomes. 14B/nu showed an increase in chromosomes, but not to a tetraploid number. T-14B had undergone a reduction in total chromosomes. A complete karyotype will have to be done to determine whether the ratios of specific chromosomal sections were consistently altered.

## DISCUSSION

We have shown that the series of T-antigen negative revertants isolated from 14B have not only regained normal growth properties *in vitro*, but have lost the ability to form tumors after injection into nude mice or Fisher rats.

As already reported by us and others [7, 8, 12] the best *in vitro* correlation of tumorigenicity is anchorage-independent growth (Table I). Determination of CVI provides more information than simply scoring visible agar colonies. The lines studied here show a consistent correlation of CVI with tumorigenicity. We have recently observed with other cell lines that there can be a very large discrepancy between CVI and the formation of colonies greater than 0.2mm [16].

Rat-1 was capable of a 10-fold increase in cell volume while suspended in agarose. Although its ability to form large colonies was very low, this cell line was weakly tumorigenic. F<sup>1</sup> 1-4 had a CVI comparable to Rat-1. While it did not form tumors, it did form long-lasting nodules at the site of the injection. FI 3-8 had a CVI as low as REF [16] and was not tumorigenic. 1-4 MCA and 14B were comparable and high in tumorigenicity.

Many factors can affect the ability of a given line to grow in agar. Folkman and Moscona [21] recently observed that a minimal degree of endothelial or fibroblast cell spreading *in vitro* was critical for DNA synthesis. O'Neill et al [22] reported that the growth of Nil 8 hamster fibroblasts in agarose was a linear function of serum concentration. Some hamster lines require additional purines for anchorage-independent growth [P. Kahn and S. Shin, personal communication]. Regardless of the underlying cause(s), it is clear that in the group of cells we studied, there is a gradation of anchorage-independent growth, and that this gradation tends to parallel the observed tumorigenicity.

The presence of actin cables also paralleled both the tumorigenicity of these cells and their ability to grow without anchorage. Plasminogen activator levels correlated well with tumorigenicity, with the exception of 1-4. This revertant line had very high levels of this enzyme by all 3 assays but contained a large number of actin cables, did not grow well in agar, and did not form tumors.

The re-transformant 1-4 MCA has regained all of the *in vitro* properties which correlate with tumorigenicity (Table I), and is highly tumorigenic (Table II). It contains neither of the early SV40 viral proteins [24]. It is clear that, while FI<sup>1</sup> 1-4 is non-tumorigenic, it can give rise to a tumorigenic variant. We have not yet determined if 1-4 MCA contains any SV40 sequences different from those in 1-4.

The revertants we have described have almost completely regained normal phenotype. This is in contrast to earlier SV40 revertants that continue to express SV40 T-antigens [9, 11, 23]. Most significantly, the revertants described here are not only less tumorigenic than their transformed parent, but are less tumorigenic than the original grandparental line.

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