

58
Lestary

Correlation of *in Vitro* Growth Properties and Tumorigenicity of Syrian Hamster Cell Lines¹

J. Carl Barrett,² Brian D. Crawford,³ Laura O. Mixter, Leonard M. Schechtman,⁴ Paul O. P. Ts'o,⁵ and Robert Pollack

Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205 [J. C. B., B. D. C., L. O. M., L. M. S., P. O. P. T.], and Department of Biological Sciences, Sherman Fairchild Center for the Life Sciences, Columbia University, New York, New York 10027 [R. P.]

ABSTRACT

Several *in vitro* phenotypic characteristics frequently associated with neoplastic cells were examined in a series of spontaneous and benzo(a)pyrene-induced Syrian hamster clonal cell lines which differed in their degree of tumorigenicity. Nonparametric statistical analysis demonstrated cloning efficiency in semisolid agar, enhanced fibrinolytic activity, decreased serum requirement for growth, decreased organization of intracellular actin, and increased cloning efficiency in liquid medium to be correlated with tumorigenicity. These correlations were not only qualitative but also quantitative. This suggests that the factors determining the degree of tumorigenicity of a cell can be cellular growth properties.

INTRODUCTION

One of the difficulties in studying neoplastic transformation is the lack of one or several definitive phenotypic characteristics which distinguish tumorigenic and normal cells. The identification of *in vitro* cellular properties highly correlated with cancer *in vivo* would be of value in the development of assay systems for the detection of induced neoplastic transformation. Accordingly, extensive effort has been expended by cancer biologists to understand the relationship between the properties of both normal and transformed cells in culture and tumorigenicity *in vivo*. Several *in vitro* phenotypic transformations, including alterations in cell morphology (22, 35), increased saturation density (1, 14, 44), reduced serum requirement for growth (14, 18, 39), anchorage independence (13, 15, 21, 25, 38), enhanced proteolytic activity (20, 21, 23, 33), and alterations in membrane structure (17, 19, 20, 27, 30), have been associated with tumorigenicity. Recent observations suggest that *in vitro* transformations may reflect different cellular properties, controlled noncoordinately, which distinguish normal cells from their neoplastic counterparts (5, 7, 34). It is not surprising, therefore, that exceptions exist for the correlation of each of these phenotypic transformations with tumorigenicity (22, 23, 26, 29, 37, 42). These exceptions may prove significant in the understanding of the relationship between specific phenotypic alterations and neoplastic transformation. Although

many studies have demonstrated positive or negative correlations between *in vitro* phenotypes and tumorigenicity, the criteria for positive correlation frequently have been qualitative, based upon both the presence of a specific phenotypic characteristic and the production of tumors in a suitable host following inoculation with a large number (e.g., 10⁶) of cells. It remains possible that certain *in vitro* phenotypic characteristics can be correlated quantitatively to tumorigenicity (i.e., correlated with the inoculum size and time required to produce a tumor).

As an experimental parameter, tumorigenicity is often difficult to delineate since it is influenced, among other factors, by the immunological response of the host. During our studies of neoplastic transformation of GSHE⁶ cells *in vitro*, we have observed that several tumorigenic cell lines differed in their degree of tumorigenicity, (i.e., the number of cells required to produce a tumor). Syrian hamsters are unique among rodents in that they are rare and have a restricted geographical distribution. All domestic stocks of these animals are known to be derived from the progeny of 3 littermates found in a burrow near Aleppo, Syria in 1930 (4). Although they have the capacity to reject homografts with foreign transplantation antigens as well as do other mammals (10), Syrian hamsters appear unique in their ability to accept homografts of malignant tissue and even some heterografts of both normal and malignant tissues (9). Spontaneous and carcinogen-induced primary tumors of the Syrian hamster can be transplanted with a high frequency of success to other hamsters, even when they arise from different stocks (12). An unusual lymphatic drainage from the skin of the hamster has been postulated (8) based upon the observation that these animals have very loose skin, such that folds 2 inches deep can be pulled out from any part of the trunk. Skin grafts exchanged between hamsters of the same closed but randomly bred stocks are usually accepted for long periods of time, often indefinitely (2, 3). Even skin homografts transplanted to members of certain different stocks are accepted (8, 10). These and other experiments have indicated that Syrian hamsters are unusual in having a very small number of histocompatibility genes segregating in the various hamster strains available (10-12). Therefore, the majority of skin homografts exchanged between members of noninbred strains behave as if they are isografts.

Since homografts of malignant tissues are readily accepted in animals of this species, it was possible that the quantitative differences we observed in the tumorigenicities of Syrian ham-

¹ This research is supported in part by Contract N01-CP 55713 and Grant P01-CA16043-04 from the National Cancer Institute and on Contract EY-76-S-02-3280 from the Department of Energy.

² Present address: National Institute of Environmental Health Sciences, NIH, P. O. Box 12233, Research Triangle Park, N. C. 27709.

³ Predoctoral Fellow (Training Grant T32 CA04110-04).

⁴ Present address: Department of Biomedical Oncology, Metropolitan Associates, Bethesda, Md. 20816.

⁵ To whom requests for reprints should be addressed.

Received July 24, 1978; accepted January 30, 1979.

⁶ The abbreviations used are: GSHE, golden Syrian hamster (*Mesocricetus auratus*) embryo; TD₅₀, number of cells that produced tumors in 50% of inoculated animals; PBS, phosphate-buffered saline [0.14 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄ (pH 7.4)].

ster cell lines were attributable to differences in the growth properties of the cells rather than to antigenic differences. In this report, we have examined various phenotypic characteristics frequently associated with neoplastic cells and demonstrate that there exist significant correlations between tumorigenicity and several *in vitro* growth properties of spontaneous and benzo(a)pyrene-induced Syrian hamster transformed clonal cell lines.

MATERIALS AND METHODS

Cells and Growth Medium. GSHE cell cultures were established from 13-day-gestation fetuses collected aseptically by cesarian section from inbred Syrian hamsters, strain LSH/ssLAK (Lakeview Hamster Colony, Newfield, N. J.). Pools of primary cultures from littermates were stored in liquid nitrogen. Secondary cultures were initiated from frozen stocks, and all experiments were performed with tertiary or later cultures. BHK 21 clone 13 (hereafter designated BHK 21 cl.13) cells were obtained from American Type Culture Collection (Rockville, Md.). BP6, BP6T, BP6T-LC, BP12, BP12A, BP14, and BP17 cell lines (see Table 1) were established in this laboratory by treatment of GSHE cells with benzo(a)pyrene. LMS is a mass culture of GSHE cells that was grown continuously for 45 *in vitro* passages. Fol 1 to 4 are clonal cell lines which were isolated from cultures of GSHE cells which previously had escaped senescence (7) at passage 18. These clonal strains were grown for an additional 6 to 15 passages after isolation prior to characterization. The growth properties and tumorigenicity of each cell line were measured at the same *in vitro* passage level.

The cell culture medium used was IBR modified Dulbecco's Eagle's reinforced medium (Biologicals, Northbrook, Ill.) supplemented with NaHCO₃ (0.22 g/100 ml) and 1 or 10% Reheatin fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.) without antimicrobial agents. Cells were transferred by gentle trypsinization with 0.1% trypsin solution (1:250, Grand Island Biological Co., Grand Island, N. Y.) for 5 min at 37°C. All cells were tested by Microbiological Associates (Bethesda, Md.) and found free of *Mycoplasma* contamination.

Measurement of Growth Rates and Saturation Densities. Cells were plated at a density of 10³ cells/60-mm Petri plate in medium containing either 1 or 10% serum. The number of attached cells was determined 15 hr after plating. Cell counts were determined with a Coulter counter (Coulter Electronics, Hialeah, Fla.) following trypsinization of cell monolayers with 2 ml of 0.25% trypsin (1:250, Grand Island Biological Co.) containing 0.1% EDTA. Cell counts were performed daily for 10 days, during which time the medium was changed every 2 days. Logarithmic growth was observed for at least 4 days; the population doubling times were calculated from this portion of the growth curve. Saturation densities were measured 7 to 10 days after plating. (Accurate measurements of this parameter were often difficult since some cell lines did not cease growing, but rather, floated into the medium when high cell densities were attained.)

Cloning Efficiencies in Liquid and Semisolid Media. To determine the efficiency of colony formation in liquid medium, 10³ to 10⁴ cells were plated in triplicate 100-mm tissue culture dishes containing 8.0 ml of medium supplemented with 10% serum. After 8 days incubation at 37°C, the number of colonies

Cell line designation	Origin
GSHE	GSHE cells derived from 13-day-gestation fetuses
LMS	Spontaneously derived, established cell line of GSHE cells
Fol	Four spontaneously derived cell lines of GSHE cells, cloned at passage 26 from fibrin:agarose overlay plates (7)
BHK 21 cl.13	Baby hamster kidney cell line isolated by Stoker and MacPherson (43), obtained from American Type Culture Collection at passage 51
BHK-A	Subclone of BHK 21/cl.13 isolated as a colony growing in semisolid agar
BP-14	Cell line derived from a morphologically transformed clone of benzo(a)pyrene-treated GSHE cells
BP6	Subclone of 18 Cl-10, a benzo(a)pyrene-transformed cell line derived from GSHE cells (36) following isolation of a morphologically transformed colony
BP12	Subclone of 18 Cl-10, a benzo(a)pyrene-transformed cell line derived from GSHE cells (36) following isolation of a morphologically transformed colony
BP12-A	Subclone of BP12, isolated as a colony growing in semisolid agar
BP17	Cell line derived from morphologically transformed clone of benzo(a)pyrene-treated GSHE cells
BP6T	Tumor cell line derived from a fibrosarcoma, induced by injection of 10 ⁶ BP6 cells in a newborn hamster
BP6T-LC	Subclone of BP6T cells isolated as a small colony in semisolid agar; colony reached a size of less than 25 cells and ceased growing

containing greater than 50 cells was determined following fixation with 100% methanol and staining with 10% aqueous Giemsa stain.

The efficiency of colony formation in semisolid medium was measured by the procedure described by MacPherson and Montagnier (25), as modified by Kakunaga and Kamahora (22). Cells suspended in 4.0 ml 0.3% Difco agar supplemented with complete medium and 0.1% Bacto-Peptone were plated in 60-mm dishes over a layer of 0.6% agar containing complete medium. The Bacto-Peptone supplement was required for efficient colony formation by hamster cells when suspended in semisolid agar (24). Plates were incubated at 37°C in a 5% CO₂:95% humidified air atmosphere for 14 to 28 days. Colony formation efficiency in semisolid agar was expressed as the percentage of total cells which formed colonies containing at least 50 cells.

Quantitation of Extracellular Fibrinolytic Activity. To measure the release of extracellular plasminogen activator, 5 × 10⁵ cells were plated in 60-mm tissue culture dishes. After attachment, the cultures were washed twice with PBS to remove serum inhibitors of fibrinolysis. Two ml of Eagle's minimal essential medium supplemented with 5% fetal calf serum treated previously with acid (6) were then added, and the cultures were incubated at 37°C in a 5% CO₂:95% air atmosphere for 18 hr. The resulting cell-free media obtained from the cultures were assayed for fibrinolytic activity by the release of soluble [³H]fibrinopeptides from [³H]fibrin-coated plates (6). Released radioactivity was expressed as a percentage of the activity released by urokinase (Calbiochem, San Diego, Calif.);

Table 2
Growth properties and tumorigenicity of Syrian hamster cell lines

Cloning efficiencies in liquid and semisolid media were calculated as the percentages of inoculated cells which formed colonies containing greater than 50 cells. Population doubling times in medium containing 10 or 1% serum were calculated from growth curves during periods of exponential growth. Saturation densities were calculated from 7- to 10-day cultures grown in medium containing 10% serum, as described in the "Materials and Methods." Cells from some cell lines detached at high cell densities, for these lines, approximate values are provided. Extracellular fibrinolytic activities were measured by the degradation of [³H]fibrin to soluble [³H]fibrinopeptides in a cell-free assay (6). The pattern of distribution of intracellular actin was examined by specific immunofluorescence, as described previously (30, 31). Tumorigenicity studies were performed in nonimmunosuppressed neonatal Syrian hamsters.

	Cloning efficiency (%) ^a		Population doubling time (hr)		Saturation density (cells cm ⁻² × 10 ³)	Fibrinolysis (extracellular) ^b	Actin cables (% of cells positive) ^c	TD ₅₀
	Liquid medium	Semisolid agar	10% serum	1% serum				
BP6T	85	85-95	12.3	16.1	5.5	42 ± 1 ^d	0	≤10 ¹
BP6	75	42	13.1	30.2	≥5.0	47 ± 9	13	5 × 10 ¹
BP12-A	74	22	12.3	40.0	4.6	22 ± 2	23	5 × 10 ¹
BP6T-LC	23	20	12.9	29.4	3.5	35 ± 1	22	3 × 10 ²
BP17	33	2.5	12.9	37.6	≥5.0	4.8 ± 0.7	20	1 × 10 ³
BP12	60	3.9	12.3	67.7	≥5.0	5.3 ± 0.9	7	1 × 10 ⁴
BP14	57	2.2	16.9	46.1	~4.0	3.5 ± 0.2	41	1 × 10 ⁴
BHK	30	1.7	13.9	60.9	~7.5	4 ± 1	56	3 × 10 ⁵
21/cl.13								
BHK-A	26	0.8	11.2	81.0	≥3.5	4 ± 1	53	≥10 ⁵
GSHE	2-4	No growth	14.3	No growth	1.5	3.1 ± 0.2	57-90	>10 ⁷
LMS	2-5	No growth	19.8	No growth	2.0	4.2 ± 0.2	57	>2 × 10 ⁶
Fol 1	15	<0.001	17.8	No growth	0.8	30 ± 5	35	>2 × 10 ⁶
Fol 2	19	<0.001	16.0	154	0.7	38 ± 4	68	>2 × 10 ⁶
Fol 3	17	<0.001	20.9	128	0.7	N.D. ^e	55	>2 × 10 ⁶
Fol 4	14	<0.001	21.7	Not determined	0.7	N.D.	55	>2 × 10 ⁶

^a Means from a minimum of 3 determinations

^b Total units of plasminogen activator activity present in 2 ml of medium incubated with cells for 18 hr.

^c Percentage of cells demonstrating organized actin cables spanning the length of the cell at the plane of focus of the cell edge

^d Mean ± S.D.

^e N.D., not done

1 unit of fibrinolytic activity was defined as that which released 10% of the radioactivity liberated by 100 units of urokinase in 2 hr at 37°. Each unit of fibrinolytic activity represents approximately 0.2 to 0.3% of the available counts present on the [³H]fibrin-coated plates.

Visualization of Actin Organization by Indirect Immunofluorescence. The pattern of distribution of intracellular actin was examined by specific immunofluorescence (30, 31). Coverslips with cells grown for 24 hr in medium containing 0.5% serum were drained of excess medium and immersed in PBS containing 4.0% formaldehyde. After treatment with absolute acetone for 5 min at -10°, coverslips were incubated for 1 hr at 37° with 10 µl of rabbit anti-actin antibody (gift of Dr. K. Burridge) diluted 1:80 in PBS. They were then washed with PBS and incubated for 1 hr at 37° with 10 µl fluorescein-labeled goat anti-rabbit IgG diluted 1:10 in PBS (Cappel Laboratories, Lancaster, Pa.) prior to viewing with a Zeiss microscope equipped with a ×63 epifluorescence Planapochromat lens. Cells were scored for the extent of organization of intracellular actin at the adherent plane of the cell, as determined by focusing on the cell edge (30, 31). At least 100 cells were scored on each coverslip. Coverslips were blind scored by 2 persons, scores always agreed to within 10%.

Tumorigenicity Studies. Cells were trypsinized and suspended in complete medium at varying concentrations (10⁷ to 10¹¹/ml in logarithmic increments), and 0.1 ml was injected s.c. into nonimmunosuppressed neonatal littermates (1 to 3 days

old) of outbred Syrian hamsters (Lakeview). A minimum of 4 to 6 animals was used for each determination.

All animals were checked weekly for the appearance of palpable tumors over a period of 4 to 6 months, and TD₅₀ was calculated. Pathological and histological examination demonstrated that tumorigenic cells formed invasive anaplastic fibrosarcomas. Inoculated animals were observed for one year.

Calculation of Coefficients of Rank Correlation (Nonparametric Analysis). Determination of the correlation between tumorigenicity and the various cellular growth properties was achieved by nonparametric statistical analysis. Computation of the Kendall coefficient of rank correlation, τ , between the TD₅₀ of only the tumorigenic cell lines and their respective growth properties (see Table 2) was performed by using the formula

$$\tau = \frac{N}{\sqrt{\{n(n-1) - \sum T_1\} \{n(n-1) - \sum T_2\}}}$$

Where n = the number of ranks of the variables and $\sum T_1$ and $\sum T_2$ are the sums of correction terms for ties in the ranks. N measures how well the ranking of the second variable corresponds to the order of the first and is defined by the formula

$$N = 4 \sum C - n(n-1)$$

where C is the sum of counts following ranking. For further details, see Sokal and Rohlf (40).

The significance of the computed correlation coefficients was examined by using a normal approximation to test the null hypothesis that the true value of τ was equal to 0 (no correlation). For sample sizes ≤ 10 , the 5 and 1% (2-tailed) critical values for N (the numerator for τ) were obtained from Table 12.3 in Sokal and Rohlf (40).

RESULTS

Tumorigenicity of Syrian Hamster Cell Lines. All benzo(a)pyrene-induced cell lines examined in this study were tumorigenic in nonimmunosuppressed newborn hamsters. The degree of tumorigenicity varied, however, between cell lines (Table 2). For example, the BP6T cell line produced tumors in 100% of animals given injections of 10 cells *s.c.*, whereas other cell lines required as many as 10^4 cells to produce tumors in 50% of inoculated animals. Even related subclones (e.g., BP12 and BP6) exhibited disparate values for TD_{50} (Table 2). In summary, the degree of tumorigenicity among the chemically induced cell lines examined varied by nearly 4 orders of magnitude.

In contrast, normal hamster embryo cells (not treated with carcinogen) were generally nontumorigenic even after many *in vitro* passages (7). In our laboratory, GSHE cultures can be grown for 10 to 20 passages prior to senescence. Nine of 10 cultures senesced by the 20th passage and were always nontumorigenic. Over 110 animals were administered inocula of 10^6 to 10^7 cells from 20 different cultures from passages 6 to 20, and no tumors were observed one year after injection (Table 3). Isolated colonies from GSHE cultures senesced *in vitro* within a similar number of passages as did mass cultures (60–80 total population doublings) (7). These clonal strains resembled the mass cultures with regard to their lack of the transformed and tumorigenic phenotypes examined in this study.

Approximately 1 of 10 embryo cultures continually passaged in our laboratory has escaped senescence and become an established cell line. These cell lines have been generally nontumorigenic, even after as many as 45 passages *in vitro* (Table 3). Of 5 spontaneously established cell lines studied, only one became tumorigenic. After 34 *in vitro* passages, this cell line was capable of producing tumors following inoculation of newborn hamsters with 2×10^7 cells. These data indicate that, while rare, spontaneous neoplastic transformation of GSHE cells can occur in cultures which escape senescence. The spontaneously established Syrian hamster cell line BHK 21/cl.13 (43) was also tumorigenic when tested at its 55th *in vitro* passage (Table 2).

Correlation of Tumorigenicity and *in Vitro* Growth Properties. The wide variation in the tumorigenic potential of the cell lines permitted an investigation of whether specific growth properties could be correlated with tumorigenicity. The benzo(a)pyrene-induced cell lines as well as the spontaneously transformed BHK cell lines and normal GSHE cells were examined for various growth properties frequently associated with neoplastic cells *in vitro* (Table 2). To determine whether any of these *in vitro* properties correlated with the quantitative aspects of tumorigenicity, the data for tumorigenic cell lines were subjected to nonparametric statistical analysis (Table 4). The Kendall coefficient of rank correlation, τ , provided a measure of association between 2 variables; positive deviation (maximum value = 1) from $\tau = 0$ was taken as an indication of

Table 3
Tumorigenicity of untreated hamster embryo cells

Passage	No. of cells injected	No. of tumors/no. of animals injected
6-7	1×10^7	0/6
	2×10^6	0/17
	1×10^6	0/4
10-14	1×10^7	0/6
	2×10^6	0/19
	1×10^6	0/17
15-18	1×10^7	0/15
	2×10^6	0/6
	1×10^6	0/21
22	1×10^6	0/2
30-33	1×10^6	0/6
	1×10^6	0/7
44-45	2×10^6	0/11

Table 4
Correlation among *in vitro* growth properties and tumorigenicity of Syrian hamster cell lines

Computation of the Kendall coefficient of rank correlation, τ , between the TD_{50} of the cell lines and their respective growth properties was performed as described in the Materials and Methods.

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

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

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

positive correlation. The significance of this correlation was tested by the use of a normal approximation to test the null hypothesis that $\tau = 0$ (no correlation). The following sections summarize the results concerning the correlation of specific *in vitro* growth properties with tumorigenic potential.

Generation Time. The population doubling time of early passage GSHE cells in medium containing 10% serum was 14.3 to 19.8 hr. For clonal isolates of GSHE cells also demonstrated a similar range in population doubling times (16.0 to 21.7 hr). The tumorigenic cell lines had generation times ranging from 12.3 to 16.9 hr. The generation time in medium containing 10% serum was not correlated with tumorigenicity ($\tau = 0.062$).

In medium supplemented with 1% serum, early passage GSHE cells had no measurable growth after 10 days, and late-passage clonal isolates of GSHE cells exhibited either no growth or extremely long population doubling times (>120 hr). In contrast, all tumorigenic lines did grow progressively in medium containing 1% serum; the population doubling times varied from 16.1 to 81 hr (Table 2). The generation times in 1% serum were found to be significantly correlated with tumorigenicity ($\tau = 0.754$).

Saturation Density. Normal GSHE cells grew to high cell densities and occasionally formed multiple layers, yet distinct plateaus in the saturation densities for these normal cells could

be measured accurately (Table 2). The saturation densities for GSHE cells and the clonal strains of GSHE cells ranged from 0.7 to 2.0×10^6 cells cm^{-2} . All tumorigenic cell lines had saturation densities greater than had normal GSHE cells. However, in contrast to the normal GSHE cells, the exact value of the saturation density was often not measurable since at high densities cells of these lines would detach from the plates and grow in suspension. Saturation density was not correlated with tumorigenicity ($r = 0.246$); however, this may be attributable to the difficulties in quantitating this parameter. Notably, all tumorigenic cell lines exhibited increased saturation densities (≥ 3.5 to 7.5×10^6 cells cm^{-2}) in comparison to normal Syrian hamster cells.

Cloning Efficiency in Liquid Medium. Normal Syrian hamster cells had low cloning efficiencies (2 to 5%) on a solid substrate (polystyrene tissue culture dishes) when inoculated in liquid medium at densities of 0.5 to 2×10^4 cells/100-mm dish (Table 2). Established clonal lines of nontumorigenic GSHE cells demonstrated enhanced cloning efficiencies (14 to 19%) in comparison to early passage GSHE cells, even at lower inocula (0.5 to 1×10^4 cells/100-mm dish). Tumorigenic cell lines had increased cloning efficiencies (25 to 85%) at lower cell inocula (100 to 200 cells/100-mm dish). The cloning efficiencies of the tumorigenic cell lines in liquid medium varied slightly; this variation appeared correlated with tumorigenicity ($r = 0.638$).

Colony Formation in Semisolid Agar. Normal Syrian hamster cells did not grow when suspended in semisolid agar. Over 7×10^7 cells from passages 3 to 20 were tested and no colonies were observed. Established clonal lines of GSHE cells which escaped senescence grew in agar at late passages but at a very low efficiency. All tumorigenic lines exhibited colony formation in agar, with the efficiency of colony formation ranging from 0.8 to 95%. Analysis of the data from tumorigenic cell lines demonstrated a significant correlation ($r = 0.986$) between tumorigenicity and the efficiency of growth in agar.

It was possible that cell lines exhibiting a low efficiency of anchorage-independent colony formation were mixed populations of cells, only some of which could grow in agar (all tumorigenic lines were cloned, however, prior to characterization). For this reason, colonies of cells growing in agar were isolated from BP12 cells and BHK cells and designated BP12-A and BHK-A, respectively. Two results were obtained when these isolated strains were retested for their efficiency of anchorage-independent colony formation. The BP12-A strain displayed increased growth efficiency, the BHK-A strain remained unchanged. The tumorigenicities of these 2 lines were determined and found to be increased for BP12-A but not for BHK-A, which supported the correlation between tumorigenicity and anchorage-independent growth.

Additionally, we noted that some colonies which formed in semisolid agar were smaller than 50 cells. For example, BP6T cells which grew with high efficiency in agar (95% efficiency, colonies greater than 300 cells after 14 days) also demonstrated a low frequency (approximately 1%) of colonies comprised of 10 to 20 cells. One such colony (BP6T-LC) was isolated from agar and retested for anchorage-independent cloning efficiency. Its efficiency of anchorage-independent growth (20%) was reduced in comparison to BP6T cells, but most (>90%) of the colonies which formed were greater than

50 cells. The tumorigenicity of this cell line was also reduced (Table 2).

Togelther, these data indicate that the efficiency of anchorage-independent colony formation may be a cellular characteristic which is significantly correlated with tumorigenicity *in vivo*, as has been reported previously (15, 21, 38).

Fibrinolytic Activity. The normal GSHE cells did not have measurable extracellular fibrinolytic activity elevated above background [3.1 ± 0.2 units (S.D.)]. For clonal isolates of late-passage GSHE cells had significantly elevated activity, consistent with their origin as colonies which gave detectable lysis in the fibrin-agarose overlay (7). All transformed lines had elevated fibrinolytic activity, although this activity was often less than twice the background level of activity (Table 2). The more tumorigenic cell lines (TD_{50} in the range of 10^1 to 10^2) had demonstrably much higher fibrinolytic activity than had the less tumorigenic lines. Analysis of the data demonstrated a significant correlation ($r = 0.754$) between tumorigenicity and fibrinolysis.

Organization of Intracellular Actin Cables. Normal GSHE cells demonstrated a high percentage (7 to 90%) of cells with organized actin cables spanning the length of the cell. Clonal lines of late-passage GSHE also demonstrated organization of actin, although to a somewhat reduced degree (22 to 68%). This change may be associated with the origin of the F01 lines as cells with enhanced fibrinolytic activity (7, 31). Additionally, less tumorigenic cell lines (e.g., BP14 and BHK) had demonstrable organization of actin in a large proportion of cells. In contrast, several of the highly tumorigenic cell lines (e.g., BP6T and BP6) exhibited a dramatic decrease in the percentage of cells with organized actin. The loss of this organization was correlated with tumorigenicity ($r = 0.570$).

DISCUSSION

The Syrian hamster cell system offers features useful for studies of *in vitro* cellular properties associated with tumorigenicity. First, the normal cells exhibit a low frequency of spontaneous transformation and are nontumorigenic even as large inocula ($>10^7$ cells/newborn hamster). Second, clonally derived neoplastic cell lines can be generated following treatment with carcinogens; such cell lines may vary greatly in their degree of tumorigenicity. Some cell lines are highly tumorigenic, requiring only 10 cells or less to produce tumors, whereas other lines may require 10^4 to 10^5 cells. This study describes our preliminary attempts to correlate these quantitative differences in tumorigenicity with *in vitro* growth properties.

As summarized in Table 4, anchorage-independent cloning efficiency, elevated fibrinolytic activity, reduced serum requirement for growth, increased cloning efficiency, and loss of intracellular actin organization appear to be *in vitro* phenotypes associated with tumorigenicity in the cells examined in this study. These correlations were not only qualitative but also quantitative. This suggests that the factors determining the degree of tumorigenicity of a cell can be cellular growth properties.

As discussed in the "Introduction," the immunological response of the host may also exert an influence on the tumorigenicity of a cell line. The results from these experiments,

however, suggest that in assaying the tumorigenicity of transformed GSHE cells with the newborn Syrian hamster, the immunological response of the host may not be an overriding factor. The highly significant degree of correlation between certain growth properties and tumorigenicity (e.g., for growth in soft agar, $r = 0.986$ and $p \leq 0.01$) suggests that, in this test system, the factors controlling tumorigenicity are the growth properties of the cells rather than their antigenicity in the host.

In agreement with previously published work (15, 21, 38), anchorage independence appeared to be the *in vitro* growth property most highly correlated with tumorigenicity *in vivo* ($r = 0.986$ and $p \leq 0.01$). Interestingly, 2 other positive correlations, enhanced fibrinolytic activity and altered actin organization, are phenotypes which previously have been correlated with anchorage-independent and/or tumorigenic cell lines (21, 23, 30-32). The results described in the present article with Syrian hamster cell lines thus agree with other reports of a relationship among cell shape or anchorage dependence and cellular growth control (16, 30, 41). It has been suggested previously that correlations among the different *in vitro* phenotypes may be indicative of their relationship (i.e., coordinate control) (28, 31, 32). However, nonselective analyses of virally induced transformants (34) and the independent temporal acquisition *in vitro* of transformation-associated traits (5, 7), as well as the known exceptions to the phenotypic correlations, have indicated a noncoordinate control of different transformed cellular properties. The results of this study, which indicate that a spectrum of phenotypic changes may correlate with the quantitative aspects of tumorigenicity, perhaps can provide additional insight concerning this problem.

For a cell line to produce a tumor upon injection of only a small number of cells e.g., 10^1 - 10^2 , the cells must be able to survive and grow from a low cell density under adverse conditions. It is possible that the efficiency of tumor formation *in vivo* is governed by several cellular functions rather than the presence or absence of specific phenotypic characteristics. Such a control mechanism would not require that every tumor cell exhibit all phenotypic characteristics associated with cancer; thus, exceptions such as those detected by qualitative correlation studies would be expected. If, however, certain phenotypic characteristics confer advantages upon the tumor cell with regard to its growth as a neoplasm *in vivo*, it would be expected that, upon examination of several cell lines, such characteristics would tend to demonstrate positive quantitative correlations with tumorigenicity. Finally, some cellular characteristics might be strictly required for tumorigenicity; these characteristics would exhibit excellent correlations with tumorigenicity. In accord with the last prediction, current results in the Syrian hamster system suggest that anchorage independence may be required for tumorigenicity of these cells (5, 7, 13). Perhaps the additional *in vitro* correlates described in these and other studies, such as decreased serum dependence, elevated fibrinolytic activity, and altered membrane structure (all of which vary widely among transformed lines and have exceptions) represent cellular functions which contribute to efficient growth of the cell as required for a neoplasm.

The Syrian hamster system thus offers the possibility of studying cellular growth and survival mechanisms involved in tumor formation. In addition to being tumorigenic, most of the transformed cell lines described in this study are invasive and

form metastases. Other tumorigenic cell lines derived from Syrian hamster embryo cells form noninvasive, benign tumors.⁷ Therefore, these cell lines offer a useful system for the comparison of benign and malignant neoplasms. Extension of these preliminary studies to include other *in vitro* phenotypic characteristics associated with the neoplastic state, as well as an examination of GSHE cells transformed by other chemical, physical, and viral agents, should permit a critical assessment of the *in vitro* cellular properties correlated with the *in vivo* neoplastic and malignant states.

ACKNOWLEDGMENTS

We thank Sue Conlon for technical assistance.

REFERENCES

1. Aaronson, S. A., and Todaro, G. Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. *Science*, **162**: 1024-1026, 1968.
2. Adams, R. A. Recent experiments with skin grafting in Syrian hamsters. *Transplant. Bull.*, **5**: 24-26, 1958.
3. Adams, R. A., Patt, D. I., and Lutz, B. R. Long term persistence of skin homografts in untreated hamsters. *Transplant. Bull.*, **3**: 41-42, 1956.
4. Adler, S. Origin of the Golden Hamster *Cricetus auratus* as a Laboratory Animal. *Nature (Lond.)*, **162**: 256, 1948.
5. Barrett, J. C., Crawford, B. D., Graly, D. L., Hester, L. D., Jones, P. A., Benedict, W. F., and Ts'o, P. O. P. Temporal acquisition of enhanced fibrinolytic activity by Syrian hamster embryo cells following treatment with benzo(a)pyrene. *Cancer Res.*, **37**: 3815-3823, 1977.
6. Barrett, J. C., Crawford, B. D., and Ts'o, P. O. P. Quantitation of fibrinolytic activity of Syrian hamster fibroblasts using ³H-labeled fibrinogen prepared by reductive alkylation. *Cancer Res.*, **37**: 1182-1185, 1977.
7. Barrett, J. C., and Ts'o, P. O. P. Evidence for the progressive nature of neoplastic transformation *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.*, **75**: 3761-3765, 1978.
8. Billingham, R. E., and Hildeman, W. H. Studies on the immunological responses of hamsters to skin homografts. *Proc. R. Soc. Lond. B Biol. Sci.*, **149**: 216-233, 1958.
9. Billingham, R. E., and Hildeman, W. H. Studies of transplantation immunity in hamsters. *Ann. N. Y. Acad. Sci.*, **73**: 676-686, 1958.
10. Billingham, R. E., Sawchuck, G. H., and Silvers, W. K. Studies on the histocompatibility genes of the Syrian hamster. *Proc. Natl. Acad. Sci. U. S. A.*, **46**: 1079-1090, 1960.
11. Billingham, R. E., Sawchuck, G. H., and Silvers, W. H. The induction of tolerance of skin homografts in Syrian hamsters. *Transplant. Bull.*, **26**: 446-449, 1960.
12. Billingham, R. E., and Silvers, W. K. Syrian hamsters and transplantation immunity. *Plast. Reconstr. Surg.*, **34**: 323-353, 1964.
13. Dimayorca, G., Greenblatt, M., Trauthen, T., Soller, A., and Giordano, R. Malignant transformation of BHK₂₁ clone 13 cells *in vitro* by nitrosamines: a conditional state. *Proc. Natl. Acad. Sci. U. S. A.*, **70**: 46-49, 1973.
14. Dulbecco, R. Topoinhibition and serum requirement of transformed and untransformed cells. *Nature (Lond.)*, **227**: 802-806, 1970.
15. Freedman, V. H., and Shiu, S. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell*, **3**: 355-359, 1974.
16. Folkman, J., and Moscona, A. Role of cell shape in growth control. *Nature (London)*, **273**: 345-349, 1978.
17. Gallimore, P. H., McDougall, J. K., and Chen, L. B. *In vitro* traits of adenovirus-transformed cell lines and their relevance to tumorigenicity in nude mice. *Cell*, **10**: 669-678, 1977.
18. Holley, R. W., and Kiernan, J. W. "Contact inhibition" of cell division in 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.*, **60**: 300-304, 1968.
19. Hynes, R. O. Cell surface proteins and malignant transformation. *Biochim. Biophys. Acta*, **458**: 73-107, 1976.
20. Hynes, R. O. Role of surface alterations in cell transformation: the importance of proteases and surface proteins. *Cell*, **7**: 147-156, 1974.
21. Jones, P. A., Laug, W. E., Gardner, A., Nye, C. A., Fink, L. M., and Benedict, W. F. *In vitro* correlates of transformation in C3H/10T^{1/2} clone 8 mouse cells. *Cancer Res.*, **36**: 2863-2867, 1976.
22. Kakunaga, T., and Kamahora, J. Properties of hamster embryonic cells transformed by 4-nitroquinoline-1-oxide *in vitro* and their correlation with the malignant properties of the cells. *Biken J.*, **11**: 313-332, 1968.

J. C. Barrett, manuscript in preparation.

- 23 Laug, W. E., Jones, P. A., and Benedict, W. F. Relationship between fibrinolysis of cultured cells and malignancy. *J. Natl. Cancer Inst.*, **54**: 173-179, 1975.
- 24 Leavitt, J. C., Crawford, B. D., Barrett, J. C., and Tso, P. O. P. Regulation of requirements for anchorage independent growth of Syrian hamster fibroblasts by somatic mutation. *Nature (Lond.)*, **269**: 63-65, 1977.
- 25 MacPherson, I., and Montagnier, L. Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology*, **23**: 291-294, 1964.
- 26 Mott, D. M., Fabisch, P. H., Sani, B. P., and Sorof, S. Lack of correlation between fibrinolysis and the transformed state of mammalian cells. *Biochem. Biophys. Res. Commun.*, **61**: 621-627, 1974.
- 27 Nicolson, G. L. Trans-membrane control of the receptors on normal and tumor cells. II. Surface changes associated with transformation and malignancy. *Biochim. Biophys. Acta*, **458**: 1-72, 1976.
- 28 Ossowski, L., Quigley, J. P., Kellerman, G. M., and Reich, E. Fibrinolysis associated with oncogenic transformation—requirement of plasminogen for correlated changes in cellular morphology, colony formation in agar, and cell migration. *J. Exp. Med.*, **138**: 1056-1064, 1973.
- 29 Pearlstein, E., Hynes, R. O., Franks, L. M., and Hemmings, V. J. Surface proteins and fibrinolytic activity of cultured mammalian cells. *Cancer Res.*, **36**: 1475-1480, 1976.
- 30 Pollack, R., Osborn, M., and Weber, K. Patterns of organization of actin and myosin in normal and transformed cultured cells. *Proc. Natl. Acad. Sci. U. S. A.*, **72**: 994-998, 1975.
- 31 Pollack, R., and Rifkin, D. Actin-containing cables within anchorage-dependent rat embryo cells are dissociated by plasmin and trypsin. *Cell*, **6**: 495-506, 1975.
- 32 Pollack, R., Risser, R., London, S., and Rifkin, D. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by SV-40. *Proc. Natl. Acad. Sci. U. S. A.*, **71**: 4792-4796, 1974.
- 33 Reich, E. Tumor associated fibrinolysis. *Fed. Proc.*, **32**: 2174-2175, 1973.
- 34 Risser, R., and Pollack, R. A non-selective analysis of SV40 transformation of mouse 3T3 cells. *Virology*, **59**: 477-489, 1974.
- 35 Sanford, K., Barker, B. E., Woods, M. W., Farshad, R., and Law, L. W. Search for indicators of neoplastic conversion *in vitro*. *J. Natl. Cancer Inst.*, **39**: 705-733, 1967.
- 36 Schechtman, L. M. A mammalian system for the mechanistic study of I. *in vitro* neoplastic transformation by chemical carcinogens and II. The uptake and fate of exogenous nucleic acids. Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1975.
- 37 Shields, R. Transformation and tumorigenicity. *Nature (Lond.)*, **262**: 348, 1976.
- 38 Shin, S., Freedman, V. H., Risser, R., and Pollack, R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.*, **11**: 4435-4439, 1975.
- 39 Smith, H. S., Scher, C. D., and Todaro, G. J. Induction of cell division in medium lacking serum growth factor by SV40. *Virology*, **44**: 359-370, 1971.
- 40 Sokal, R. R., and Rohlf, F. J. Correlation. *In: Introduction to Biostatistics*, pp. 279-283. San Francisco, W. H. Freeman and Co., 1973.
- 41 Steinberg, B., Pollack, R., Topp, W., and Botchan, M. Isolation and characterization of T antigen-negative revertants from a line of transformed rat cells containing one copy of the SV40 genome. *Cell*, **13**: 19-32, 1978.
- 42 Stiles, C. D., Desmond, W., Chuman, L. M., Sato, G., and Saier, M. H., Jr. Relationship of cell growth behavior *in vitro* to tumorigenicity in athymic nude mice. *Cancer Res.*, **36**: 3300-3305, 1976.
- 43 Stoker, M., and MacPherson, I. Syrian hamster fibroblast cell line BHK21 and its derivatives. *Nature (Lond.)*, **203**: 1355-1357, 1964.
- 44 Todaro, G., Green, H., and Goldberg, B. Transformation of properties of an established cell line by SV40 and polyoma virus. *Proc. Natl. Acad. Sci. U. S. A.*, **51**: 66-73, 1964.