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Isolation of Variant Cells from SV40-transformed Human Diploid Fibroblasts[1]

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SUMMARY

Variant cell lines that are sensitive to density-dependent inhibition of growth have been isolated from three of four SV40-transformed human fibroblast cell lines. Variants were isolated by plating the transformed cells at low density, by treating them with 5-fluorodeoxyuridine, or by growing them on glutaraldehyde-fixed monolayers of normal cells. The variant cell lines, isolated at a frequency of about 2% of all cells forming colonies after treatment, were initially recognized by colonial morphology, and the variant phenotypes were confirmed, after subculturing, by saturation-density determinations. The variant cell lines reach saturation densities that are 40% or less than those of the parent cell lines, and plate in soft agar medium at reduced efficiency, compared with the parent cells. They retain SV40 T antigen. The modal chromosome numbers of two of the variant cell lines were increased, compared with those of the parent cell lines; two other variants were indistinguishable in chromosome number from the parent cells. Stability of these properties over a 6-month period was demonstrated with two of the variants.

INTRODUCTION

Cell lines that resemble certain normal cells in their sensitivity to density-dependent inhibition of growth have been isolated from transformed rodent cell lines by various techniques (3, 13, 22). These cell lines have been termed variants or revertants. They probably preexist in the transformed population, and the different isolation procedures allow them to be recognized. Variants have been isolated by treatment of transformed cells with FUdR[3] (3, 13, 16), concanavalin A (2, 14), or colchicine (28) and by selection of anchorage-dependent cells by means of bromodeoxyuridine and blue-light irradiation (29, 30). Cultivation of transformed cells at low density (22, 23) or growth of transformed cells on glutaraldehyde-fixed monolayers of normal cells has also been used to select for variant cells (21).

Variant cells isolated from rodent cell lines show a reduced saturation density, compared with their parent cell lines. They usually lack various properties of the transformed lines, such as high plating efficiency in liquid medium, the ability to form colonies in soft agar (22, 23), and sensitivity to agglutination by plant lectins (9, 15), and tumor-specific transplantation antigens (13, 25). Most variant cell lines also have a reduced ability to form tumors after inoculation in animals (17, 21). Rodent cells in culture can be transformed by exposure to chemical carcinogens (7) and can spontaneously evolve into permanent cell lines (26, 27). However, human diploid fibroblasts do exhibit strict growth control *in vitro* and, except in modified culture medium, do not undergo more than 50 ± 10 cell divisions (5, 6). Infection with SV40 is required to convert these cells into an immortal population, free from density-dependent growth control (4, 19). We were interested in determining the frequency of variants in transformed cell lines derived from human diploid fibroblasts and in testing the possibility that cells which express a variant phenotype revert to the finite life-span characteristic of normal human fibroblasts.

MATERIALS AND METHODS

Cells. The origin and history of the human diploid fibroblasts, WI-38, and the SV40-transformed human fibroblasts, WI-38VA13A, WI-38VA13/2RA and WI-26VA4, have been described (4, 6, 10, 11). WI-26VA4-1 was isolated by single cell cloning of WI-26VA4, using the ring cloning technique (20). The procedures for isolating variants were applied to this cell line within 1 month of cloning. All cells were grown in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (Grand Island Biological Co.). Cells were subcultured weekly using a solution of 0.025% trypsin and 0.02% Versene in PBS for cell dispersion.

Isolation of Variants. Variants were isolated by 3 procedures: (*a*) SV40-transformed cells were plated at low density

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[3] The abbreviations used are: FUdR, 5-fluorodeoxyuridine; PBS, phosphate-buffered saline (100 mg $CaCl_2$ per liter, 200 mg KCl per liter, 200 mg KH_2PO_4 per liter, 100 mg $MgCl_2 \cdot 6H_2O$ per liter, 8000 mg NaCl per liter, and 1150 mg $Na_2HPO_4 \cdot 2H_2O$ per liter).

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(100 to 500 cells/60 mm Petri plate); (*b*) SV40-transformed cells were seeded on monolayers of glutaraldehyde-fixed WI-38 cells by the procedure of Rabinowitz and Sachs (25); (*c*) exponentially growing SV40-transformed cells were treated with FUdR using a modification of the method of Pollack *et al.* (16). For FUdR selection, transformed cells were seeded at a concentration of 1 or 2.5 $\times$ 10^5 cells/60-mm plate. After 24 hr, the monolayers were washed twice with PBS and exposed for 48 hr to 4 ml of fresh growth medium containing FUdR (20 μg/ml) and uridine (200 μg/ml). The monolayers were then washed with PBS and trypsinized; the cells were transferred at a ratio of 1:5 or 1:10 to fresh growth medium without FUdR.

To ensure that all variant cell lines arose from single-cell clones, all cultures were observed microscopically 24 hr after plating. Any areas in which 2 or more cells were close together were marked and eliminated from further consideration. Potential variant colonies *i.e.*, those in which the growing cells did not pile up, were subcultured using the ring technique (20).

Growth Properties. Sensitivity of the cell lines to density-dependent inhibition of growth was measured by saturation-density analysis. To determine saturation densities, 3 $\times$ 10^5 cells were inoculated into 60-mm plates. The medium was changed every 48 hr, and growth curves were derived by counting the number of cells in duplicate plates every 24 to 48 hr. Variant cell lines were those in which, under these conditions, the number of cells did not increase or decrease significantly after the 1st 6 or 7 days of incubation. Saturation density was defined as the number of cells in these cultures after 12 days of incubation.

To determine the plating efficiency in liquid medium, 25 to 200 cells were inoculated into 35-mm plates, and the number of colonies was determined after 14 days. To determine the plating efficiency in soft agar, 50 to 10,000 cells were inoculated into 35-mm plates, using the procedure of Macpherson and Montagnier (12). The number of colonies was determined after 3 weeks.

To determine the population-doubling capacity, 5 $\times$ 10^5 cells were inoculated into T-75 flasks. The number of cells was determined after 7 days of incubation.

Chromosome Analysis. For *in situ* chromosome analysis, a modification of the method of Bauscher and Schaeffer (1) was used. Test cells were inoculated onto sterile glass coverslips at a density of 8 $\times$ 10^4 to 1 $\times$ 10^5 cells/35-mm plate. The cells were fed fresh growth medium after 24 hr and, after 40 to 48 hr, were treated with Colcemid (0.1 to 0.15 μg/ml) for 1 to 3 hr. The cultures were washed with Hanks' balanced salt solution, treated with 0.075 M KCl for 30 min, and immediately fixed for 5 min in fresh methanol:acetic acid, 3:1 v/v. After drying, the cells were stained with Giemsa.

Test for SV40 T Antigen. To test for the presence of SV40 T antigen by immunofluorescence, the cells, grown on coverslips, were fixed with acetone, dried, and treated with SV40-specific anti-T hamster serum (obtained from F. Jensen and A. Girardi, Wistar Institute), and then with fluorescein isothiocyanate-conjugated goat anti-hamster globulin (Antibodies, Inc., Davis, Calif.).

RESULTS

Isolation of Variants. Variant cell lines which resemble the parental, untreated lines in their sensitivity to density-

Table 1

Isolation of variants

Variant cell lines were isolated by treating 1.0 to 2.5 $\times$ 10^5 transformed cells with 20 μg FUdR per ml for 48 hr, by plating transformed cells at low density (100 to 500 cells/plate), or by plating transformed cells on glutaraldehyde-fixed monolayers of normal cells. After treatment and plating, surviving colonies were screened for flat morphology; the sensitivity of flat colonies to density-dependent inhibition of growth was determined by saturation-density analysis.

Selection technique	Cell line	Clones screened[a]	Clones selected[b]	Variant clones[c]
FUdR	WI-26VA4	191	19	2
	WI-26VA4-1	59	11	1
	WI-38VA13/2RA	0[d]		
	WI-38VA13A	237	19	0
Low-density plating	WI-26VA4	115	6	0
	WI-26VA4-1	125	10	0
	WI-38VA13/2RA	60	9	1
	WI-38VA13A	NT[e]		
Glutaraldehyde	WI-26VA4	119	23	1
	WI-26VA4-1	135	28	1
	WI-38VA13/2RA	126	20	3
	WI-38VA13A	NT		

[a] Clones screened, clonal morphology observed by microscopic scanning.

[b] Clones selected, morphologically flat clones.

[c] Variant clones, clones confirmed by saturation density analysis as being sensitive to density-dependent growth inhibition.

[d] No cells formed colonies after FUdR treatment in 2 trials.

[e] NT, not tested.

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dependent inhibition of growth were isolated by the glutaraldehyde and FUdR techniques from 3 (WI-26VA4, WI-26VA4-1, and WI-38VA13/2RA) of the 4 cell lines tested. One variant cell line was isolated from WI-38VA13/2RA by low-density plating. No variants were isolated from WI-38VA13A (Table 1).

The initial screening criterion was colonial morphology, and it was found that 5 to 20% of the cells plated at low density or on glutaraldehyde-fixed monolayers gave rise to flat colonies in which the cells did not pile up. Screening of these flat colonies by saturation-density determination after first growing them to mass culture demonstrated that 10% of the flat colonies, or 1 to 2% of the total colonies arising after selection, were variant cell lines as defined by density-dependent growth control (Table 1).

Using FUdR concentrations and cell densities that reduced the plating efficiency of parent cell lines to approximately 2.5 to 5.0×10^{-5}, 8 to 20% of the surviving colonies were flat. Saturation-density determinations confirmed that 10% of the flat clones derived from WI-26VA4 and WI-26VA4-1 were variant cell lines.

All the cells in all variant cell lines isolated were positive for SV40 T antigen as determined by immunofluorescence.

Growth Properties. Under identical growth conditions for 12 days, the variant cell lines attained saturation densities that were one-half to one-third lower than those of their parent cell lines (Table 2).

Plating efficiencies in liquid medium of 3 of the 4 variant cell lines tested were 3 to 15 times lower than those of the parent cell lines; 1 variant had a plating efficiency approximately equal to that of its parent. Plating efficiencies in soft agar medium of the 3 variant cell lines tested were 2.5 to 100 times lower than those of the parent cell lines (Table 2).

Chromosome Analysis. The modal chromosome numbers of the parent line, WI-26VA4, and a clone of this line, WI-26VA4-1, were determined within 1 month of the isolation of variants. The chromosome numbers of 4 variant cell lines derived from these parents were also determined within 1 month of isolation. The parent cell lines and the 2 variants isolated by the FUdR technique (WI-26VA4-FU3 and WI-26VA4-1-FU5) have a range of 58 to 70 chromosomes per cell. (A minimum of 60 mitoses per cell line was analyzed.) Six nonvariant cell lines isolated after treatment with FUdR or by low-density plating also have modal chromosome numbers within the parent cell range (data not shown). Two variant cell lines isolated by glutaraldehyde plating have higher chromosome numbers than the parent cells: WI-26VA4-fb25 has 80 to 95 chromosomes per cell and WI-25VA4-1-fb40 has 100 to 120 chromosomes per cell.

Stability of Variant Cell Lines. The stability of the variant phenotype was studied in 2 variant cell lines over a 6-month period. Monitoring of saturation density during this period indicated that the variants retained sensitivity to density-dependent inhibition of growth (Table 3). The chromosome number, monitored every 2 or 3 passages, was also stable. The cells remained positive for SV40 T antigen. These properties were also stable in the parent cell line over the same test period. During this time, no increases in the numbers of piled-up colonies were observed when the variant cells were plated at low density. The number of population doublings at each passage of the 2 variant cell lines and their transformed parent was also monitored for 6 months and found to be stable (Table 4).

Table 2

Growth properties of cell lines

To determine the saturation density of cell lines, 3×10^5 cells were plated in 60-mm plates. The cultures were fed fresh growth medium every 48 hr, and the saturation density was determined after 12 days of incubation. To determine the plating efficiency in liquid medium, 25 to 200 cells/35-mm plate were inoculated; the number of colonies was scored after 14 days. To determine the plating efficiency in soft agar medium, 50 to 10,000 cells/35-mm plate were seeded; the number of colonies was counted after 21 days.

Cell line[a]	Saturation density (cells/sq cm $\times 10^4$)	% Plating efficiency[b]	
		Liquid medium	Soft agar medium
WI-26VA4	107.0	28, 33[c]	19, 15
WI-26VA4-FU11	22.0	NT[d]	NT
WI-26VA4-fb25	28.4	3, 6	1, 0.1
WI-26VA4-FU3	28.9	27, 35	7, 6
WI-26VA4-1	114.0	32	13
WI-26VA4-1-fb40	18.6	13	1
WI-26VA4-1-FU5	15.6	7	NT
WI-38VA13/2RA	26.0	NT	NT
WI-38VA13/2RA-nfnb10	10.3	NT	NT
WI-38VA13/2RA-fb5	14.9	NT	NT
WI-38VA13/2RA-fb10	14.5	NT	NT
WI-38VA13/2RA-fb13	13.1	NT	NT
WI-38 (Passage 16)	22.4	NT	NT

[a] Variants are designated by the name of the parent cell line, a code identifying their mode of isolation (fb for glutaraldehyde, FU for FUdR, and nfnb for low-density plating) and a clone number.

[b] Number of cells forming colonies/number of cells plated (× 100).

[c] The WI-26VA4 cell line and its 2 variants were tested twice, 5 passages apart.

[d] NT, not tested.

DISCUSSION

The isolation of variants from SV40-transformed human cells demonstrates that these populations contain cells that do not express all of the properties associated with the transformed phenotype of the mass culture. The frequency of variants in the cultures was low, about 2%, and probably constant, since variants were isolated with equal frequency from recently cloned and uncloned cell stocks.

The inability to isolate variants from the WI-38VA13A cell line probably reflects the homogeneous nature of the cell types within this population. Since large numbers of colonies were screened, the frequency of variants in the population must be low, if any do exist at all.

Many of the growth properties of the human cell variants investigated in this study resembled those of the rodent cell variants studied by other workers. The saturation densities of the human variants were 2 to 7 times lower than the

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Table 3
Stability of saturation density

Saturation density was monitored over a 6-month period. Cells were plated at a concentration of 3 × 10⁵ cells/60-mm plate. The cultures were fed fresh growth medium every 48 hr, and the number of cells and the saturation density were determined after 12 days.

Cell line	Passage	Saturation density (cells/sq cm × 10⁴)
WI-26VA4-FU3	2	40.5
	3	28.9
	5	25.2
	5	18.5
	9	36.1
	16	40.5
	20	40.6
	24	48.4
WI-26VA4-fb25	1	33.6
	3	28.4
	4	28.4
	6	19.0
	9	21.5
	15	25.7
	19	32.4
	23	28.3
WI-26VA4	182	90.0
	200	107.0
	207	83.0
	210	107.0
	215	76.0

Table 4
Stability of population-doubling capacity

To determine population doublings, 5 × 10⁵ cells were inoculated into 250-ml flasks. The number of cells was determined after 7 days. All cell lines were grown in parallel, using the same reagents throughout the experiment.

Cell line	Passage	Population doublings[a]
WI-26VA4	198-204	4.5 ± 0.8[b]
	204-209	4.4 ± 0.7
	209-218	4.8 ± 0.4
Average[c]	198-218	4.6 ± 0.8
WI-26VA4-FU3	7-12	3.6 ± 0.4
	12-18	4.1 ± 0.5
	18-25	4.0 ± 0.4
Average	7-25	3.9 ± 0.5
WI-26VA4-fb25	6-11	3.2 ± 0.2
	11-17	3.5 ± 0.6
	17-25	3.7 ± 0.4
Average	6-25	3.5 ± 0.5

[a] 3.3 log (number of cells at Day 7/number of cells at Day 0).
[b] Mean ± S.D.
[c] Average of all passages tested for each cell line.

saturation densities of their respective parents, and the 3 variants tested had a reduced ability to form colonies in soft agar.

As with variants of virus-transformed rodent cell lines isolated by similar techniques, the human variants retained at least a part of the virus genome present in the parental cells. This was indicated by the fact that they continued to synthesize SV40 T antigen.

Chromosome studies of human variant cell lines did not reveal any identifiable pattern consistent with the chromosomal models of carcinogenesis proposed by other workers (8, 18, 24). No consistent pattern of loss or gain of specific chromosomes or chromosome groups could be detected in any of the variant cell lines examined.

Long-term study of 2 variant cell lines (WI-26VA4-fb25 and WI-26VA4-FU3) and their parent cell line (WI-26VA4) demonstrated that these 3 lines were stable with respect to the properties studied. The lack of significant numbers of piled-up colonies after low-density plating of these variants indicated that the stability of the variant phenotype was reflected at the single-cell level by nearly all cells in the population. The stability of the doubling capacity of these 2 variant cell lines throughout the 6-month test period demonstrated that these lines did not undergo an aging phenomenon analogous to the *in vitro* senescence observed in normal human fibroblasts.

Experiments are in progress to characterize further the variant cells lines biologically and to determine the state of the SV40 genome within them.

REFERENCES

1. Bausher, J., and Schaeffer, W. I. A Diploid Rat Liver Cell Culture. I. Characterization and Sensitivity to Aflatoxin B_1. In Vitro, *9:* 286-293, 1974.
2. Culp, L. A., and Black, P. H. Contact-Inhibited Revertant Cell Lines Isolated from Simian Virus 40-Transformed Cells. III. Concanavalin A-Selected Revertant Cells. J. Virol., *9:* 611-620, 1972.
3. Culp, L. A., Grimes, W. J., and Black, P. H. Contact-Inhibited Revertant Cell Lines Isolated from SV40-Transformed Cells. I. Biologic, Virologic, and Chemical Properties. J. Cell Biol., *50:* 682-690, 1971.
4. Girardi, A. J., Jensen, F. C., and Koprowski, H. SV40-Induced Transformation of Human Diploid Cells: Crisis and Recovery. J. Cellular Comp. Physiol., *65:* 69-84, 1965.
5. Hayflick, L. The Limited *In Vitro* Lifetime of Human Diploid Cell Strains. Exptl. Cell Res., *37:* 614-636, 1965.
6. Hayflick, L., and Moorhead, P. S. The Serial Cultivation of Human Diploid Cell Strains. Exptl. Cell Res., *25:* 585-621, 1961.
7. Heidelberger, C. Chemical Oncogenesis in Culture. Advan. Cancer Res., *18:* 317-366, 1973.
8. Hitotsumachi, S., Rabinowitz, Z., and Sachs, L. Chromosomal Control of Chemical Carcinogenesis. Intern. J. Cancer, *9:* 305-315, 1972.
9. Inbar, M., Rabinowitz, Z., and Sachs, L. The Formation of Variants with a Reversion of Properties of Transformed Cells. III. Reversion of the Structure of the Cell Surface Membrane. Intern. J. Cancer, *4:* 690-696, 1969.
10. Jensen, F. C., Girardi, A. J., Gilden, R. V., and Koprowski, H. Infection of Human and Simian Tissue Cultures with Rous Sarcoma Virus. Proc. Natl. Acad. Sci. U. S., *52:* 53-59, 1964.
11. Jensen, F. C., Koprowski, H., and Pontén, J. A. Rapid Transformation of Human Fibroblast Cultures by Simian Virus 40. Proc. Natl. Acad. Sci. U. S., *50:* 343-348, 1963.
12. Macpherson, I., and Montagnier, L. Agar Suspension Culture for the Selective Assay of Cells Transformed by Polyoma Virus. Virology, *23:* 291-294, 1964.

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13. Mondal, S., Embleton, M. J., Marquardt, H., and Heidelberger, C. Production of Variants of Decreased Malignancy and Antigenicity from Clones Transformed *In Vitro* by Methylcholanthrene. Intern. J. Cancer, *8:* 410-420, 1971.
14. Ozanne, B., and Sambrook, J. Isolation of Cells Resistant to Agglutination by Concanavalin A from 3T3 Cells Transformed by SV40. *In:* L. Silvestri (ed.), The Biology of Oncogenic Viruses, pp. 248-357. Amsterdam: North Holland Publishers, 1971.
15. Pollack, R. E., and Burger, M. M. Surface-Specific Characteristics of a Contact-Inhibited Cell Line Containing the SV40 Viral Genome. Proc. Natl. Acad. Sci. U. S., *62:* 1074-1076, 1969.
16. Pollack, R. E., Green, H., and Todaro, G. J. Growth Control in Cultured Cells: Selection of Sublines with Increased Sensitivity to Contact Inhibition and Decreased Tumor-Producing Ability. Proc. Natl. Acad. Sci. U. S., *60:* 126-133, 1968.
17. Pollack, R. E., and Teebor, G. W. Relationship of Contact Inhibition to Tumor Transplantability, Morphology, and Growth Rate. Cancer Res., *29:* 1770-1772, 1969.
18. Pollack, R. E., Wolman, S., and Vogel, A. Reversion of Virus-Transformed Cell Lines: Hyperploidy Accompanies Retention of Viral Genes. Nature, *228:* 938, 967-970, 1970.
19. Pontén, J., Jensen, F., and Koprowski, H. Morphological and Virological Investigation of Human Tissue Cultures Transformed with SV40. J. Cellular Comp. Physiol., *61:* 145-163, 1963.
20. Puck, T. T., Marcus, P. I., and Cieciura, S. J. Clonal Growth of Mammalian Cells *In Vitro*. Clonal Characteristics of Colonies from Single HeLa Cells with and without "Feeder" Layer. J. Exptl. Med., *103:* 273 284, 1956.
21. Rabinowitz, Z., and Sachs, L. Reversion of Properties in Cells Transformed by Polyoma Virus. Nature, *220:* 1203-1206, 1968.
22. Rabinowitz, Z., and Sachs, L. The Formation of Variants with a Reversion of Properties of Transformed Cells. I. Variants from Polyoma-Transformed Cells Grown *In Vivo*. Virology, *38:* 336-343, 1969.
23. Rabinowitz, Z., and Sachs, L. The Formation of Variants with a Reversion of Properties of Transformed Cells. II. *In Vitro* Formation of Variants from Polyoma-Transformed Cells. Virology, *38:* 343-346, 1969.
24. Rabinowitz, Z., and Sachs, L. Control of the Reversion of Properties in Transformed Cells. Nature, *255:* 136-139, 1970.
25. Rabinowitz, Z., and Sachs, L. The Formation of Variants with a Reversion of Properties of Transformed Cells. IV. Loss of Detectable Polyoma Transplantation Antigen. Virology, *40:* 193-198, 1970.
26. Stoker, M., and Macpherson, I. Syrian Hamster Fibroblast Cell Line BHK21 and Its Derivatives. Nature, *203:* 1355-1357, 1964.
27. Todaro, G. J., and Green, H. Quantitative Studies of the Growth of Mouse Embryo Cells in Culture and their Development into Established Lines. J. Cell Biol., *17:* 299-313, 1963.
28. Vogel, A., Risser, R., and Pollack, R. Isolation and Characterization of Revertant Cell Lines. III. Isolation of Density-Revertants of SV40-Transformed 3T3 Cells Using Colchicine. J. Cellular Physiol., *82:* 181-188, 1973.
29. Wyke, J. A Method of Isolating Cells Incapable of Multiplication in Suspension Culture. Exptl. Cell Res., *66:* 203-208, 1971.
30. Wyke, J. Phenotypic Variation and Its Control in Polyoma-Transformed BHK21 Cells. Exptl. Cell Res., *66:* 209-223, 1971.

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