

Chapter 5

Methods for Obtaining Revertants of Transformed Cells¹

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I. Introduction

Although *in vitro* cell culture techniques have facilitated the investigation of the mechanisms involved in viral carcinogenesis, we as yet know very little about the functions involved in maintaining the "normal" state of growth

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control, and consequently we know little about the mechanism(s) used by viruses to alter this regulation. There is no single path to understanding these functions.

Some investigators are searching for the existence of viral coded molecules (proteins and nucleic acids) in transformed cells with the hope of discovering a molecule required for either the induction or the maintenance of the transformed state.

Other investigators are looking at the properties of the transformed cells themselves, to understand how they differ from normal cells. Many differences have been established between normal and transformed cells, but no one yet knows if these differences are the primary result of the transformation process, or if they reflect changes secondary to the initial alternation. An excellent recent review by Sambrook summarizes much of this work (Sambrook, 1972).

Our way of investigating these alterations has been to isolate sublines of transformed cells which have reverted in one or many of the transformed properties.

Three systems for the isolation of transformed cells have been developed, based upon the ability of the transformed cells to grow in conditions where the normal cells cannot (Table I). These three assays measure (1) the maximum cell density attained by a line in excess serum, (2) the ability of a cell to establish an isolated colony suspended in agar or Methocel, and (3) the ability of a cell line to grow in limiting or depleted sera.

A. Density

Most normal cell lines grow in an oriented fashion and exhibit a density-dependent cessation of cell division. For example, untransformed 3T3 mouse cells stop growing at a saturation density of 5×10^4 cells/cm² whereas

TABLE I
SELECTIVE ASSAY FOR TRANSFORMATION

Assay	System		Initial reference
	Normal	Transforming agent	
Growth to high density	3T3 mouse Chick cells	SV40 RSV	Todaro and Green (1964) Temin and Rubin (1958)
Growth suspended in gel	BHK hamster	polyoma	Macpherson and Montagnier (1964)
Growth in reduced serum	3T3 mouse	SV40	Smith <i>et al.</i> (1971)

BHK cells cease division at 25×10^4 cells/cm². Infection of 3T3 cells by SV40 or murine sarcoma virus results in dense transformed foci appearing on the contact-inhibited monolayer. These dense clones, when isolated from the monolayer and grown on plastic dishes, grow to higher saturation densities ($> 20 \times 10^4$ cells/cm²) than the untransformed 3T3 cells. Transformants of BHK cannot be isolated in such a way because these normal cells grow to high densities.

B. Anchorage

Polyoma virus transformants of BHK can be isolated by an assay involving growth in semisolid medium. Normal cells (both BHK and 3T3) will grow only when attached to a plastic or glass surface and are therefore said to have a high anchorage requirement for growth. Infection by oncogenic viruses (containing either DNA or RNA) results in a decrease in this anchorage requirement. Transformed cells will grow into spherical colonies when suspended in a semisolid medium of agar or methocel. The transformed colonies picked in agar grow to high saturation density when cultured on plastic. When cultured on plastic tissue culture dishes, polyoma-transformed BHK cells grow in a randomly oriented manner and continue to divide until they eventually turn the medium acidic and die.

C. Serum

γ -Globulin-free calf serum incubated with confluent monolayers of Balb 3T3 loses the ability to support the growth of normal 3T3 cells. This depleted serum will allow SV40-transformed 3T3 cells to grow. Thus, serum transformants can be isolated by infecting 3T3 cells with SV40 and plating the infected cells in this agamma-depleted medium, because only transformed cells will grow (Smith *et al.*, 1971).

Transformed cells isolated in each of these systems display other properties which differentiate them from the parental normal cells (Table II). The most important difference is in tumorigenicity. In general, transformants form tumors when injected into an animal, while normal cells do not. Transformed cells exhibit new antigens. Some of these are presumably virus-coded (Black *et al.*, 1963), while other antigens are cell-coded, but induced by the virus (Hayri and Defendi, 1970). Alterations in the surfaces of cells occur upon transformation. The most completely studied such alteration is measured as an increased agglutinability by plant lectins such as concanavalin A and wheat germ agglutinin. Finally, transformed cells maintain a lower internal concentration of cyclic AMP than do normal cells.

TABLE II
NONSELECTIVE PROPERTIES OF TRANSFORMED LINES

Transformed property	System		Reference
	Normal	Trans- forming agent	
Increased tumorigenicity	3T3	SV40	Aaronson and Todaro (1968)
	Hamster	DMBA	Chen and Heidelberger (1969)
Increased agglutinability with lectins	3T3	SV40	Burger (1969)
	Mouse	MSV	Burger and Martin (1972)
	Hamster	Py	Inbar and Sachs (1969)
Decreased level of cAMP	3T3	SV40, Py	Otten <i>et al.</i> (1971); Sheppard (1972), Oey <i>et al.</i> (1973)
Increased sensitivity to drugs affecting cell morphology			
	Colchicine	SV40	Vogel <i>et al.</i> (1973)
Cytochalasin B	3T3	SV40	Kelly and Sambrook (1973)
Growth on monolayer of normal cells	3T3	SV40	Pollack <i>et al.</i> (1968)
New antigens	3T3	SV40	Black <i>et al.</i> (1963)

II. Isolation of Revertants from Transformed Cell Lines

A. Definition of a Revertant

A revertant cell line lacks at least one of the properties of a transformed cell line from which it is descended. A revertant may be *selected* by a modification of the protocol used to isolate the transformed parent. Three different assays for selecting transformants are in current use (Table I); therefore three different selective assays for reversion will be described. Once selected, a revertant may be *described* as either transformed or not with regard to the other two transformation properties in Table I, and also with regard to other nonselective properties of transformed cell lines (Table II).

One obvious pathway to reversion is to cure the transformant of its virus. This approach was used successfully by Marin and Littlefield (1968). Using somatic cell hybridization and the appropriate selection system, they were able to induce the loss of the viral genome from polyoma-transformed BHK cells. Having lost the viral genome, these cell lines now appear normal. They no longer contain the polyoma tumor antigen, are unable to grow in agar medium, and grow in a similar manner to the parental BHK cells.

In most cases, however, reversion is not the result of a lost viral genome, since most revertant cell lines still contain the viral coded antigens associated

TABLE III
REVERTANT CELL LINES ISOLATED THROUGH NEGATIVE SELECTION

Revertant selected for	Parent transformant	Selective agent	Transformed properties					
			Density	Serum	Anchorage	Agglutinability	Tumorigenicity	
Density	SV3T3	FUdR ^{a,b}	Normal ^a	Transformed	Normal	Normal ^a	NT	
Density	SV3T3	BUdR-light ^c	Normal	Transformed	NT ^a	NT	NT	
Density	SV3T3	Colchicine ^d	Normal	Normal	Normal	NT	NT	
Density	PyBHK	FUdR ^e	Normal	NT	NT	NT	Reduced ^{a,m}	
Density	Methylcholanthrene ^e transformed C3H mouse prostate cells ^f	FUdR	Normal	NT	NT	NT	Reduced ^g	
Serum-1% calf serum	SV3T3	BUdR-light ^c	Normal	Normal	Normal	Normal	NT	
Serum- γ depleted calf serum	SV3T3	BUdR-light ^c	Normal	Normal	Transformed	Transformed	NT	
Anchorage	PyBHK	BUdR-light ^c	Normal	Normal	Normal	NT	Transformed	
Concanavalin A resistance	SV3T3	Concanavalin A/ α	Normal	Transformed	Normal	Normal	NT	
Growth on glutaraldehyde monolayers	Py-hamster	Glutaraldehyde fixed cells ^h	Normal	NT	Normal	Normal	Reduced	
	Methylcholanthrene-transformed C ₃ H mouse ⁱ	Glutaraldehyde fixed cells ^h	Normal	NT	NT	NT	Reduced	
No selection	MSV-3T3	—	Normal	NT	Normal	NT	NT	
spontaneously arising rever-	MSV-NRK ^j	—	Normal	NT	NT	NT	NT	
tants	RSV-NRK ^k	—	Normal	NT	NT	NT	NT	
	RSV-BHK ^k	—	Normal	NT	Normal	NT	NT	

^aPollack *et al.* (1968).^bWyke (1971a,b).^cOzanne and Sambrook (1970).^dCulp *et al.* (1971).^eVogel and Pollack (1973).^fVogel *et al.* (1973).^gWolfe and Sachs (1968).^hFischer *et al.* (1972).ⁱOzanne and Sambrook (1970).^jCulp *et al.* (1971).^kMacpherson (1965).^lMacpherson (1965).^mPollack and Teebor (1969).ⁿPollack and Teebor (1969).^oNot tested.^pPollack and Vogel (1973).

with transformation (Culp *et al.*, 1971; Culp and Black, 1972; Ozanne with Sambrook, 1970; Pollack *et al.* (1968); Vogel *et al.*, 1973; Vogel and Pollack, 1973) as well as virus-coded RNA and DNA (Ozanne *et al.*, 1973; Shani *et al.*, 1972). The next most obvious way to isolate revertants is by observation. Cells with variant morphologies are present in cultures of transformed cells, and these variant cells have been isolated from the cultures and found to have lost transformed properties.

Finally, revertant cell lines have been isolated from both RNA and DNA virus transformed cell lines, and from populations of chemically transformed cells (Table III), through negative selection. These protocols will be described in detail below.

B. Negative Selection

This selection procedure involves placing a population of transformed cells in a situation which supports the growth of only transformed cells and then adding an agent which is toxic only to growing cells. The toxic drug and restrictive conditions are then removed, and conditions supporting the growth of cells with normal properties are restored to allow the recovery of surviving cells. Some of these cells escape the toxic effects of the drug because of their inability to grow and thereby have reverted to a more normal state.

For example, selection of revertants unable to grow beyond a monolayer in density involves plating transformed cells at high density and killing any cells capable of overgrowing the monolayer. Serum revertants are selected by placing the transformants in the restrictive serum and killing the cells capable of growth. Cells reverted in their ability to grow in semisolid medium are obtained by killing the cells in this medium, and recovering the survivors. Colonies grown up from cells surviving these selections can be tested directly for the reversion of the specific property or they can be recycled through the selection once or twice more to eliminate cells with transformed phenotype which have slipped through the killing process.

C. Agents Used in Negative Selection of Revertants

The choice of drug depends upon the type of selection desired. Selection for reversion in an ability to grow in certain conditions requires the use of a drug that kills growing cells. Selection for reversion in a surface property involves the use of compounds, such as concanavalin A, which are more toxic to transformed cells than to normal cells.

Among the substances specifically toxic growing cells are antimetabolites known to affect cells in different phases of the cell cycle. The most com-

monly used antimetabolites are the thymidine analogs bromodeoxyuridine (BUdR) and fluorodeoxyuridine (FUdR). Both these agents specifically affect cells synthesizing DNA. Although these drugs are both thymidine analogs, they kill by different mechanisms. BUdR is incorporated into newly synthesized DNA, and blue light will cause breaks in the DNA and lead to cell death (Kao and Puck, 1968). FUdR inhibits endogenous thymidine synthesis by inhibiting thymidylate synthetase, the enzyme that converts d-UMP to d-TMP (Rueckert and Mueller, 1960). The cells no longer synthesize thymidine and are killed. Thymidine can prevent the toxic effects of both FUdR and BUdR.

Dividing cells can also be killed by colchicine because of its ability to block cells in mitosis. This drug prevents the polymerization of microtubules (Borisey and Taylor, 1967) and thus prevents the completion of cytokinesis. Cells blocked in mitosis for long periods of time will die. In addition, colchicine is more toxic to growing SV40-transformed 3T3 cells than to growing 3T3 cells (Vogel *et al.*, 1973). This differential toxicity is not understood.

There are many other drugs that could probably be used as agents specifically toxic for growing cells. Cytosine arabinoside, 6-thioguanine, and aminopterin, are examples.

TOXICITY OF SELECTIVE AGENTS

Sparse and confluent cultures of the contact-inhibited mouse line 3T3 should be used to test a drug for specific toxicity to growing cells. At confluence, 3T3 cells are blocked in G_1 and do not synthesize DNA or enter mitosis (Nilhausen and Green, 1964). At sparse densities, 3T3 cells double in about a day. Treatment of confluent 3T3 cells with FUdR, BUdR, or colchicine does not affect the viability of the cells, but treatment of sparse 3T3 cultures with these agents will leave fewer than 1 cell in 10^4 alive. The following procedure can be used to evaluate the relative toxicity of drugs on growing versus nongrowing cells.

Seed 3T3 cells at varying cell densities in regular growth medium. Densities chosen should represent cell number which correspond to confluence, one-tenth confluence, and one-hundredth confluence (3T3 typically has a confluent saturation density of 5×10^4 cells/cm²). One day after plating, count a plate at each density, and add the drug to a test plate at each density. A range of the times of exposure to the drug and of concentrations of the drug should be tested to give each cell a chance to go through the cycle. After exposure for at least a day, remove the medium containing the drug and add fresh medium for 2–3 hours. This incubation with fresh medium serves to remove any residual drug. Then remove this medium and trypsinize the remaining cells. Count and plate them at suitable dilutions to determine the number of cells capable of forming colonies. Plot the ratio of colony-

forming survivors of the treated plates to colony formers on the control plates vs cell density at the time the drug was added. Drugs which are specific for growing cells kill the cells at non-confluent cell densities, but do not affect the confluent cells. Drugs toxic for nongrowing cells will show very few survivors at any cell density.

D. Selection of Density Revertants

The following selection procedures have been used to isolate variants of transformed cells which are reverted in the density, serum, or anchorage properties.

Transformed cells are seeded at a density of 5×10^4 cells/cm², and 24 hours later, the toxic agent is added. One can use per milliliter 25 μ g of FUdR, 100 μ g of BUdR, or 0.5 μ g of colchicine. Selections with FUdR or BUdR should be done in the presence of a 10-fold excess of uridine to prevent the incorporation of any fluorouracil or bromouracil into RNA. The optimum time of exposure of these drugs is 2 days. Stock solutions of FUdR (1 mg/ml), BUdR (10 mg/ml), uridine (10 mg/ml), and colchicine (10 μ g/ml) are dissolved in distilled water, sterilized by Millipore filtration, and stored frozen. After the 2 days in drug, the medium is removed, the cells are trypsinized and counted, and serial dilutions (with 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} of the original population) are plated to determine the number of surviving colonies. Survivors will arise at a frequency of approximately 1 per 10^4 cells initially plated.

It is generally a good idea to put the cells through a second cycle of selection before colonies are picked and tested. After 7–10 days of growth, the 10^{-1} or 10^{-2} plates from the colony surviving experiment are trypsinized, pooled and replated at 5×10^4 cells/cm². The next day, the selective agent should be added and the procedure repeated as described above. A third selection may be done, but in general, two selections with FUdR, BUdR, or colchicine are sufficient. Colonies with revertant properties are recognized by their variant, more normal, flat morphology. These generally arise at a frequency of 1 per 10^5 cells initially plated. Variant colonies should be recloned and their saturation densities be determined. Revertant lines generally have saturation densities of 7 to 15×10^4 cells/cm².

E. Selection of Serum Revertants

Either 1% calf serum or 10% agamma-depleted calf serum can be used to differentiate 3T3 from SV40-transformed 3T3, since neither support the growth of 3T3. The 1% calf serum requires no preparation and supports the

forming survivors of the treated plates to colony formers on the control plates vs cell density at the time the drug was added. Drugs which are specific for growing cells kill the cells at non-confluent cell densities, but do not affect the confluent cells. Drugs toxic for nongrowing cells will show very few survivors at any cell density.

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E. Selection of Serum Revertants

Either 1% calf serum or 10% agamma-depleted calf serum can be used to differentiate 3T3 from SV40-transformed 3T3, since neither support the growth of 3T3. The 1% calf serum requires no preparation and supports the

growth of transformed cells, but it reduces their doubling time and saturation density. The agamma-depleted serum has the advantage of not appreciably reducing the saturation density or doubling time of the transformants, but requires a good deal of preparation. Also, different batches of treated serum vary in their ability to differentiate between 3T3 and SV3T3. We have tried the heating method (Smith *et al.*, 1971) to deplete newborn agamma serum, but our preparations do not support the growth of SV3T3. The following depletion procedure developed by Smith and her colleagues, has worked for us (Smith *et al.*, 1971).

Allow Balb/3T3 to grow to a confluent monolayer in 100-mm Falcon tissue culture dishes in medium containing 10% calf serum. Balb/3T3 must be used because agamma serum depleted on Swiss/3T3 allows the growth of both Balb/ and Swiss/3T3. Remove the medium and add 10 ml of medium containing 20% newborn agamma calf serum (North American Biologicals Incorporated). Allow the plates to incubate for 4 days at 37°C, then remove the medium and centrifuge for 20 minutes at 5000 rpm at 4°C in a Sorvall GSA rotor to remove debris. Then filter through a Millipore apparatus and add an equal volume of serum-free medium to restore any small molecules that may have been depleted. This 10% agamma-depleted medium can be stored frozen. Balb/3T3 monolayers can be used 2 to 3 times to deplete serum. After removing one batch of depleted medium, add fresh 20% agamma serum and incubate for 4 days.

To select cells revertant in their ability to grow in restrictive sera, plate transformed cells at sparse cell density in either 10% agamma-depleted calf serum or 1% whole calf serum. These conditions will allow SV3T3, but not 3T3, to grow. Then add an agent to kill dividing cells, remove the drug and permit survivors to grow in 10% calf serum. One must be certain to start the selection at very sparse cell densities to avoid selecting cells which have ceased dividing because of a density-dependent shut-off of cell division. A specific protocol follows.

Seed the transformed cells at a density of 10^4 cells/cm² in 10% calf serum. The next day wash the cells in serum-free medium and add the restrictive serum. One day after adding the restrictive serum, add 200 μ g of BUdR per milliliter and incubate for 4 days. At the end of 4 days remove the medium containing BUdR and add fresh 10% calf serum medium for 2 hours. Remove the medium and expose the cells to blue light or a short pulse of UV light. The time of exposure necessary should be determined by the investigator. A two-second UV pulse from a 30-W GE germicidal lamp 18 inches from the cells decreases the plating efficiency of untreated cells by 50%, but reduces the plating efficiency of cells exposed to BUdR to 10^{-3} to 10^{-4} . The cells should then be trypsinized, counted and plated out for colony survivors in 10% calf serum containing 10^{-5} M thymidine and 8×10^{-5} M

uridine (to prevent the incorporation of any residual BUdR or bromouracil into nucleic acid).

It is advisable to put the cells through 2–3 cycles of selection before picking colonies. Trypsinize the treated plates, dilute them 15-fold, and replat them in 10% calf serum with uridine and thymidine. When the cells reach a density of 10^4 cells/cm², add the restrictive serum and, 1 day later, add the BUdR and repeat the procedure. After 2–3 cycles, surviving colonies should be picked, grown, and tested for their growth in the restrictive serum. Serum revertants generally show no growth in the restrictive serum, but sometimes lines can be isolated that can grow slightly in the restrictive serum, with a doubling time of 3–4 days.

F. Selection of Revertants in Agar

Either agar suspension or methocel suspension cultures can be used to select cells with a reduced anchorage dependence for growth. Methocel is the more advantageous medium because the cells are more easily recovered from it. Methocel is quite viscous and can be a problem to pipette, but wide-bore pipettes (Bellco) facilitate the dispensing of methocel.

1. METHOCEL MEDIUM

The method of preparation is that of methocel medium, Vogel *et al.* (1973), based on Stoker (1968).

Make up a 2.5% methocel suspension by adding 2.6 gm of methocel (Dow Chemical Co. standard grade 4000 centipoises) to 25 ml of 80–90°C distilled water. Shake vigorously to disperse all clumps. Make up the rest of the volume with 4°C distilled water, shake vigorously, and stir on a magnetic stirrer for 3 hours at 4°C. Autoclave the methocel and then store at 4°C. The suspension will clarify at 4°C. Allow the methocel to remain at 4°C for at least 1 day before use.

Methocel medium is prepared by adding to the methocel an equal volume of twice-concentrated growth medium containing 20% calf serum and twice the concentration of antibiotics (final concentration of methocel = 1.3%). The methocel medium should be stirred on a magnetic stirrer at room temperature for a few hours before use.

Plates are prepared by adding 3 ml of agar medium (equal volumes of sterilized 1.8% Bacto Difco agar in water, and twice-concentrated growth medium containing 20% serum) to 60-mm tissue culture plates, tilting the plates to coat the sides, and allowing the agar to harden. The agar prevents the cells from attaching to the bottom or sides of the dish. Different amounts of cells (10^1 to 10^5 per milliliter) are then suspended in the methocel medium, and 4 ml is added per agar plate. The plates are incubated for 3 weeks, 4 ml

of fresh methocel medium being added every week. Visible colonies (0.3 mm or larger) should appear within 2 weeks. SV101 cells typically give a plating efficiency of 10–50% in methocel, while 3T3 has a plating efficiency of 0.001%.

Isolated colonies can be picked from the methocel with a 100- μ l micropipette. The colony is placed in growth medium, vigorously suspended with a pipette, centrifuged, resuspended, and replated. Alternatively the picked colony can be dispersed in a small volume of trypsin-EDTA, centrifuged, and replated. It is also possible to harvest all the colonies on the plate by placing the plates at 4°C for 1 hour (methocel is less viscous at lower temperatures), removing the methocel medium with a pipette, washing the plates with 4 times the volume of 4°C serum free medium and centrifuging at 300 g at 4°C. The cells form a pellet at the bottom of the tube and the methocel can be poured off.

2. SELECTION OF REVERTANTS WITH AN INCREASED ANCHORAGE REQUIREMENT FOR GROWTH

The following procedure has been used by Wyke (1971a) to isolate variants of PyBHK no longer capable of growth in methocel.

Plate 10^5 transformed cells per milliliter of methocel medium. Incubate the cultures at 37°C for 45 hours to allow cells that can grow in methocel to begin to do so. Add 5×10^{-6} M BUdR in 0.5 ml of serum-free medium. Continue the incubation for an additional 45 hours, and then harvest all the cells in the methocel as described above. After centrifugation, seed the cells into tissue culture dishes and incubate for 4–6 hours to allow the cells to attach. Then expose the plates to blue light to kill the cells that have incorporated BUdR into DNA. The duration of exposure to the light should be determined by the investigator. Add fresh growth medium to allow the surviving colonies to grow. The cells should be cycled through the selection procedure three times before colonies are picked and tested for their ability to grow in methocel.

G. Selection of Revertants with Altered Surface Properties

Plant lectins such as concanavalin (Con A) and wheat germ agglutinin (WGA) are used to demonstrate differences in surface properties between transformed and normal cell. (Burger, 1969; Inbar and Sachs, 1969). Transformed cells that have been removed from plates with EDTA are agglutinable whereas normal cells treated in a similar fashion are not. Trypsinization renders all cells equally highly agglutinable.

Spontaneously transformed cells, transformants induced by various oncogenic viruses, and chemically transformed cells all manifest this agglutin-

ability. However, it appears that this difference in agglutination between normal and transformed cells does not arise because of the transformed cells binding more lectin than normal cells (Ozanne and Sambrook, 1971; Cline and Livingstone, 1971; Arndt-Jovin and Berg, 1971). A possible explanation for this difference in agglutinability may be that the lectin binding sites are clustered together on the surface of transformed cells (Nicholson, 1971). If confirmed by other groups, this observation may be very important.

In addition to agglutinating transformed cells, Con A will kill them. Con A does not affect normal 3T3 cells. Thus Con A can be used as a selective agent to isolate Con A-resistant sublines from transformed population. This procedure is not based upon the ability of a transformed cell to grow in conditions that will not allow normal cell growth, but uses a presumed difference in the surface structure as the selective condition. The surviving sublines resemble 3T3 in their resistance to subsequent Con A treatment and in their low agglutinability. They are revertant, because they grow to low saturation densities in 10% serum.

SELECTION OF CON A-RESISTANT CELLS

Plate SV40-transformed cells at a density of 4×10^5 cells per 100-mm dish in 10% calf serum. Twenty-four hours later, add 300 μ g of Con A per milliliter. Make stock solutions of Con A (25 mg/ml) in 5 M NaCl, and make a suitable dilution of the Con A in calcium- and magnesium-free phosphate-buffered saline (PBS) just prior to adding the Con A to the plates. Leave the cells in Con A for 24 hours, then remove the medium and wash the plates 3 times in PBS to remove dead cells. Add fresh growth medium, and allow the surviving colonies to grow. Survivors arise at a frequency of 1 in 10^4 treated SV3T3 cells. Cycle these survivors through Con A selection another 1-2 times. After the final selection, plate the cells at suitable dilutions to yield colonies. These surviving colonies can then be picked and further characterized. (Ozanne and Sambrook, 1970.)

H. Isolation of Revertants on Monolayers of Fixed Cells

Working with cultures of Syrian hamster embryo cells and polyoma virus, Sachs and co-workers have devised a technique to isolate revertants using growth on monolayers of glutaraldehyde-fixed normal cells as the selective system. This procedure presumably selects for cells with altered surface properties, but there is no direct evidence for this. As opposed to other selection procedures where a small minority of revertant cells arise, this procedure produces revertants at a very high frequency. However, these revertants are not very stable since they back-revert to a transformed phenotype within a few dozen generations in culture.

PREPARATION OF GLUTARALDEHYDE-FIXED MONOLAYERS

Mix a cold 25.3% glutaraldehyde solution containing barium carbonate (to absorb oxidized derivatives) with activated charcoal, and filter through Whatman filter paper. This stock solution should be stored at 4°C in the dark and not kept longer than 3 weeks.

Prepare the monolayers by seeding 3×10^6 syrian hamster secondary fibroblasts onto 50-mm petri dishes and fixing with glutaraldehyde 16 hours later. The fixing solution is 1% glutaraldehyde in PBS (pH 7.3) which has been sterilized by filtration through a 0.45- μ m filter. Wash the monolayers twice with PBS and add 5 ml of cold fixative. Store the plates at 4°C for 24 hours. Remove the glutaraldehyde and wash the cells with 10 ml of cold PBS. Follow with 12 washings with PBS at room temperature, two successive washings every hour.

To select revertants from polyoma-transformed Syrian hamster cells plate approximately 100 transformed cells on fixed monolayers and incubate for 10–14 days. To visualize the growing colonies, add a solution of 50 μ g/ml neutral red to the plates for 20 minutes. The viable cells will take up the stain and appear red, and they can be picked with a 2-ml pipette. The majority of the cells capable of growth on these fixed monolayers have an epithelioid morphology, when compared to the fibroblastic morphology of the transformed parent. These variant colonies also grow to low saturation densities, have a decreased ability to form colonies in agar, are less tumorigenic and less agglutinable than the transformed parents.

It is not clear why such a selection works, but 80–97% of the colonies that grow up on these fixed normal cells have a revertant phenotype.

I. Isolation of Revertants of Cells Transformed by RNA Tumor Viruses

Cells transformed by RNA tumor viruses grow to high saturation densities, form dense colonies on monolayers of normal cells, and can grow in suspension in agar. Presumably, then, any of the procedures mentioned previously can be used to isolate density or anchorage revertants from RNA transformed cells.

Spontaneous morphological revertants of MSV-transformed 3T3 and RSV-transformed hamster cells have been reported (Macpherson, 1965; Nomura *et al.*, 1972). These revertants were not selected, but appeared spontaneously in cultures of the transformed cells. Such cells have a "flat" morphology, grow to low saturation densities, and are unable to grow in agar suspension cultures.

Stephenson *et al.* have isolated revertants of MSV-transformed NRK rat

kidney cells and RSV-transformed NRK rat kidney cells after mutagenesis with BUdR. The cells were exposed to 200 $\mu\text{g}/\text{ml}$ of BUdR for 20 hours. Single cells were then plated out in separate microtest titer wells (Falcon) to score the number of morphological revertants. One revertant colony was recovered among the 3×10^3 colonies examined. This procedure did not use BUdR as a killing agent, but rather depended on the mutagenic affects of BUdR to yield mutant cells defective in some transformed function. Nevertheless these BUdR-induced revertants grew to low saturation densities.

III. Properties of Revertant Cells

A. Selection versus Induction

By the use of a fluctuation analysis (Luria and Delbruck, 1943), it has been shown that the density revertant of the type selected with FUdR does exist in untreated transformed populations (Pollack, 1970). This observation is consistent with the fact that some serum transformants, isolated for their ability to grow in a gamma-depleted serum, also grow to low saturation densities in 10% calf serum (Scher and Nelson-Rees, 1971). These cells directly isolated in a transformation assay are identical to the density revertants isolated by negative selection from fully transformed SV3T3 with FUdR. Revertants can be isolated without prior treatment of transformed cultures with mutagens. Some revertant cells therefore are preexistent in populations of transformed cells, and the above selection procedures allow the isolation and cloning of these preexisting cells as revertant cell lines. However, not all revertants need have arisen spontaneously. For example, any type of revertant isolated with BUdR as the killing agent could have undergone a mutagenic event permitting survival.

Similarly, colchicine may act simply as a selective agent by killing growing cells in mitosis. However, colchicine can cause the formation of polyploid cells by interfering with cytokinesis. Many revertant lines show an increased number of chromosomes when compared with the transformed parent cell (Pollack *et al.*, 1970; Culp *et al.*, 1971; Vogel *et al.*, 1973; Vogel and Pollack, 1973; Wyke, 1971b; Nomura *et al.*, 1972). If this polyploidiation is related to reversion, then it is possible that colchicine acts as an inducer of the revertant phenotype.

B. Virus versus Cell

One of the major hopes of workers who isolate revertants is to correlate the revertant phenotype with a defect in the viral genome integrated in the

transformed cell. Ideally one would like to isolate a revertant cell, rescue the virus by fusion with a permissive cell, and demonstrate that the rescued virus can no longer transform. Such a defective virus has not yet been found. The majority of revertants of SV3T3 cells isolated yield little or no virus upon fusion with permissive monkey cells. The virus that has been rescued can transform 3T3 cells as well as virus rescued from the transformed parent cells. Infectious virus cannot be rescued from polyoma transformed cells, so one does not know about the genotype of the virus. FUDR selection on 3T3 cells that were infected with mutagenized SV40 yielded temperature-sensitive transformants which grow to high saturation density at 32°C, but grow only to a confluent monolayer at 39°C (Renger and Basilico, 1972). The SV40 virus rescued from these cells is wild type at both temperatures with regard to growth and transforming ability. This result does not rule out the possibility of the existence in these cells of a defective virus causing the temperature-sensitive phenotype, but it does raise the possibility that these cells are temperature sensitive in a cell function involved in regulating saturation density.

Another way to investigate the question of defective viruses is to attempt to retransform the revertants with the same and other oncogenic viruses. None of the revertants of SV3T3 cells are retransformable by SV40 (Renger, 1972; Ozanne *et al.*, 1973; Vogel, 1973). That is, SV40 will not induce the growth of dense areas on density revertants and will not restore the ability to grow in 1% calf serum in the serum revertants. The temperature-sensitive SV3T3 cells, the FUDR-selected revertant, the colchicine-selected revertants, and the serum revertants all can be transformed by murine sarcoma virus (Renger, 1972; Vogel, 1973). However, the con A revertants are not retransformable by MSV (Ozanne, 1973).

The flat MSV revertants can be transformed by MSV. However, further characterization of the MSV in these revertants has not been possible because the cells do not yield virus after infection with murine leukemia virus (Nomura *et al.*, 1972). The revertants of RNA virus transformed cells isolated by Stephenson are not retransformable by the virus which originally infected them, but can be retransformed by other DNA or RNA oncogenic viruses (Stephenson *et al.*, 1973).

C. Gratuitous Reversion in Properties not Selected Against

Once cells reverted in any one of the transformed properties (density, serum, anchorage, lectin susceptibility) have been isolated, one may ask whether reversion in one property leads to the gratuitous reversion of the other transformed properties and if any other alterations in cellular physiology consistently accompany reversion (Table III). Density revertants of SV40-transformed 3T3 cells isolated with FUDR or BUDR are reverted in

their ability to grow in semisolid medium, but maintain a transformed serum requirement. Colchicine-selected density revertants also show a decreased ability to grow in methocel and have also reverted to a 3T3-like serum requirement. Thus, selection of density revertants with different drugs leads to the isolation of revertants with different properties.

The anchorage revertants of Wyke have reverted in other transformed properties in that they do not grow in 0.5% calf serum and grow to low saturation density when cultured on plastic culture dishes. Their susceptibility to lectins has not been determined.

Revertants selected with Con A grow to low saturation densities, but maintain a transformed serum requirement. They do not form colonies in methocel (Ozanne, personal communication).

Selection of variants with a higher serum requirement for growth surprisingly also leads to reversion in saturation density. Thus selection with either restrictive serum yielded serum-sensitive lines with reduced saturation density. However, selection in different restrictive sera lead to the isolation of revertants with different properties with regard to growth in methocel. Serum revertants isolated in 1% calf serum are unable to form colonies in methocel, while variants selected in agamma-depleted serum can grow in methocel. The reason for this difference is unknown, but the behavior in methocel is correlated with the cyclic AMP levels of these lines. The 1% calf serum, the serum revertant isolated in 1% calf serum has high cyclic AMP levels, when grown in sparse culture, while the agamma-depleted revertant culture has a low SV3T3-like cyclic AMP level (Oey *et al.*, 1973).

Cyclic AMP levels are generally higher in normal cells than in transformed cells (Otten *et al.*, 1971; Sheppard, 1972; Oey *et al.*, 1973). Intracellular concentrations of cAMP increase when cells are deprived of serum. Recent studies with the mouse fibroblast line 3T3, an SV40-transformed subline of 3T3, and six different revertant lines derived from this clone, show that a marked increase in cAMP occurs only when the serum concentration is reduced below the minimum necessary for growth of a given line. Conversely, density-dependent inhibition of growth is not accompanied by an increase in cyclic AMP in any line. The relation between reversion and cAMP levels might be better understood by developing a system which selects directly for an alteration in cAMP level (Oey *et al.*, 1973).

All revertants of SV40-transformed 3T3 cells show an increase in chromosome number and DNA content (Pollack *et al.*, 1970; Vogel *et al.*, 1973; Vogel and Pollack, 1973; Culp and Black, 1972; Ozanne *et al.*, 1973). The reason for this alteration in ploidy is not understood, but this relation raises the possibility of selecting directly for polyploid cells to test whether these large cells manifest any revertant properties.

IV. Conclusion

It is a bit surprising that revertants exist, since unarrested tumors almost always get progressively more abnormal with time, rarely if ever throwing off more normal variants. Nevertheless, revertants are found whenever they are selected for properly. As their properties are uncovered, we hope that they will continue to contribute to an understanding of the regulation of normal control of growth and its loss in oncogenesis.

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