Reversion of Virus-transformed Cell Lines: Hyperploidy accompanies Retention of Viral Genes

by

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An increase in chromosome number accompanies reversion of subtetraploid mouse cell lines from malignant transformed phenotype to growth-controlled normal phenotype. When growth control is once again lost, the chromosome number drops back to the subtetraploid mode.

Growth control of a cell line is measured in vitro as a reversible cessation of cell division. Cell lines are maintained in culture by a periodic replacement of medium and serum. Populations with a degree of growth control will divide while sparse, but will cease to increase in number when they reach some cell density beyond confluence. Those cultures with maximum growth control cease dividing at the lowest cell density, that is, at confluence. Populations with less growth control reach higher densities before stopping, and those with no growth control continue to divide until their medium is exhausted. Growth control can therefore be measured by assay of saturation density.

Permanent fibroblast cell lines derive from primary tissue cell populations by passage through three distinct phases of growth: an initial period of generally rapid growth; a dormant period called crisis during which few cells are able to divide; and an extended period of rapid growth, as an established cell line. Established cell lines are the descendants of the minority of cells able to divide through crisis. This defines the crisis as a period of selection, and in fact cell lines can be selected to have either high or low degrees of growth control. Cell to cell contact seems to be the operative variable in this selection, for if cells are permitted to make contact during crisis, then a cell line will arise that has the ability to divide despite this contact, and such a line will retain little or no growth control. Keeping cells from contact during crisis will yield a line with a very high degree of growth control, like the 3T3 mouse line.

In constant culture conditions, constant frequency of medium changes, and constant concentration of serum in the medium, saturation density is the parameter of a cell line that correlates best with its ability to grow into a tumour in vivo. This point is shown clearly with the small polyoma and SV40 DNA viruses. These viruses induce a rapid loss of growth control in primary cells and in established lines of high growth control. Such cell lines, called transformed lines, are almost always more tumorigenic than their uninfected parent lines.

Reversion

The loss of growth control is reversible. By a choice of selective conditions, cell lines with high growth control (revertants) can be obtained from cloned populations with low growth control, including virus transformed cell lines. Revertants have been isolated by: (a) negative selection of transformed cells at high cell density with FUDR, a drug toxic to dividing cells; (b) passage of transformed cells on cell layers fixed with glutaraldehyde, which presumably selects for cells with an altered surface that permits them to grow on this substratum; and (c) selection for hybrid hamster transformed lines with reduced chromosome complements that have lost the chromosome(s) bearing the viral genome. Such cells are oriented and do not grow in agar. Like the untransformed hamster line BHK21, however, they grow to a high saturation density.

The viral genome, or part of it, is present in polyoma and SV40 transformed cells. Viral genes and their products must be responsible for the initial malignant transformation and viral genes are probably responsible for the maintenance of the transformed state in descendants of the original transformants, which cells are isolated by (c), which lack the viral antigens and are retransformable, arise as the viral genome was lost with some chromosomes of the polyplid hybrid line. Presumably, the genome was integrated into one of those chromosomes. Similarly, hybrids between SV40-transformed human cells and untransformed mouse cells originally retained the SV40 T-antigen, but lost it as human chromosomes were lost from the hybrid cells. By contrast, revertants of virus transformed cells isolated by methods (a) and (b) must retain at least part of the genome of the transforming virus, because they have viral T-antigen.

Revertant cell lines regain other phenotypic properties of untransformed cells in addition to increased growth control. Revertants isolated by methods (a) and (b) regain a completed cell surface glycoprotein, rendering them unagglutinable by a series of plant agglutinins that specifically agglutinate transformed cells and tumour cells. Flat revertants of spontaneous and polyoma transformed hamster cell lines isolated by (a) and (c) are less tumorigenic. Revertants of polyoma transformed hamster cells isolated by method (b) are in some cases more tumorigenic than their parental transformed lines.

Stability of Reverted Cell Lines

Back reversion, or the reappearance of low growth control cells within a reventant population, occurs at a low rate without experimental intervention in any population kept at maximum cell density for even a few days. Only variant cells with diminished growth control can divide at saturation, and in time their descendants overgrow the population. Measured against this background of spontaneous "transformation", stability of the reverted phenotype varies with the selection system. Selections (a), (b) and (c) yield revertants as stable as untransformed cells. That is, it is possible to select dense variants from the revertant populations isolated by system (a) and these arise with about the same frequency as such dense variants arise in untransformed populations.

Chromosomes of Transformed and Reverted Cell Lines

Immediately on transfer from the animal to tissue culture, somatic mouse cells have forty acrocentric and telocentric and no meta centric chromosomes. They grade smoothly in size. As the mouse cells in culture pass...
through crisis and cell lines are established, the population ceases to have a single chromosome number, and the median number of chromosomes/cell increases to slightly less than twice the diploid number, that is, subtetraploid. In addition to the loss of diploidy, cell populations cease to reject modified chromosomes on transition to cell lines, and chromosomes appear that are not found in preparations made from primary cells. Metacentric chromosomes, for example, are a common morphological indication of chromosomal abnormality in mouse cells, which normally have only acro and telocentrics.

An increase in chromosome number accompanies establishment and transformation of diploid primary cells; the subtetraploid mode is characteristic of most transformed cell lines. For example, when normal cells go through crisis to become established lines, they do so with an increase in chromosome number. Also, although transformation of already subtetraploid lines is not accompanied by any change in chromosome number, chromosome number increases when subtetraploid transformed lines are recovered from primary diploid cells after viral infection. Thus, we would have predicted that revertants should have either fewer chromosomes or the same number of chromosomes as transforms. But neither result was found. We report now that a consistent, anomalous increase in chromosome number above subtetraploidy accompanied reversion of spontaneous and virus-transformed cell lines from the malignant transformed phenotype to the growth-controlled normal phenotype, and that subtetraploidy returned with back reversion.

The histogram (Fig. 1) shows the heteroploid spread of 100 selected cells from each cell line, while the median range data (Table 2) give the actual frequency of cells in the median range for each cell line.

Of the four established cell lines, 3T3 had growth control and the other three lines did not. The chromosome distributions of 3T3, 3T6, SV3T3 and Py3T3 were very similar. All showed a major subtetraploid peak with a minor hyperploid component (Fig. 1 a, b, d, g). SV3T3 had the most homogeneous chromosome distribution. 97 per cent of the nuclei scanned at low power fell in the range of the major peak (Table 2). 3T3, 3T6 and Py3T3 had about the same hyperploid component. The subtetraploid peak was still the major chromosome pattern, even in Py3T3, the line with the most hyperploid cells (Table 2). In both transformation of 3T3 and establishment of the low growth control line 3T6, sub-

![Fig. 1. Histograms of chromosomes/cell against number of cells. For each mouse cell line 100 metaphase preparations were counted at 1,500 x magnification. The diploid number (2n) of the mouse is 40. The median number of chromosomes for each line is indicated (M).](image)
tetraploid remained the dominant mode as the population lost growth control.

Polysomy and SV40 transformation of primary cells is accompanied by heteroploidy, with the appearance of many abnormal chromosomes. Such tetraploid cells were, however, already heteroploid and about 11 per cent of 3T3 cells contained metacentrics (Table 2). There were 13 metacentrics per hundred cells for 3T6 cells, and 10 for Py 3T3 (Table 2). Thus, no consistent increase in metacentrics accompanied transformation in these cell lines.

The median chromosome number of 3T6 went from 76 to 195 on selection of variant F13T6 cells. 10 per cent of the F13T6 cells had more than 240 (= 18 n) chromosomes. One cell counted had 340 chromosomes (= 17 n). The overlap of distributions between 3T6 and F13T6 was slight: 10 per cent of 3T6 cells had more than 100 chromosomes, and 8 per cent of F13T6 cells had fewer than 100 chromosomes (Fig. 16, c).

As expected, 3T6 cells were bigger than 3T6 cells.

The frequency of cells lacking metacentrics declined slightly, from 84 per cent to 63 per cent, but the majority of 3T6 cells still lacked metacentrics (Table 2), and the total number increased slightly, from 19/ to 48/100 cells.

The subtotraploid population, once committed to the state of low growth control as a cell line, reverted to a state of high growth control (F13T6 with massive additional hyperploidy. This was unexpected, because 3T3, a subtotraploid cell line with growth control, was obtained with no more hyperploidy than the dense cell subcultures of 3T6 (Fig. 16, b) by selection from the diploid population before establishment.

Revertants selected from the two virus transformed 3T3 lines also had a marked increase in chromosome number and in addition an increased number of metacentrics per cell. The median chromosome number of F1ISV3T3 and 3T3 was increased from 75 to 115 with revertant populations. The F1ISV3T3 population had a much broader histogram than 3T3 (Fig. 1d, e), but overlap between F1ISV3T3 and SV40 chromosomes was limited: 6 per cent of SV40 cells had more than 90 chromosomes, and 10 per cent of F1ISV3T3 had fewer than 90 chromosomes. Five per cent of SV40 cells counted had more than 160 (= 8 n) chromosomes. In contrast to 3T6 and F13T6, F1ISV3T3 had many more metacentrics/100 cells than SV40 (196 vs 32, Table 2). Only 9 per cent of F1ISV3T3 cells were free of metacentrics, as compared with 74 per cent of SV40 cells (Table 2).

F1Py3T3, selected from Py3T3, did not shift entirely to another chromosome number. F1Py3T3 showed a bismodal distribution of counted chromosomes/cell (Fig. 1g, h). Low power scans showed that F1Py3T3 cells were evenly distributed between the two ranges (median = 60,120). No overlap occurred between the two peaks (Fig. 1h). The higher peak is very near to the peak of F1ISV3T3. The lower peak clearly is not identical to the major peak of Py3T3; its median is half that of the higher peak.

Metacentric abnormal chromosomes increased most strikingly in the selection of F1Py3T3 from Py3T3. The percentage of cells with metacentrics increased from 4 per cent to 91 per cent. The number of metacentrics/100 cells went up almost forty-fold, from 10 to 353 (Table 2).

The absolute number of metacentrics/100 cells increased in all three flat revertants. In the two virus-transformed revertants, the ratio of metacentrics to total chromosomes increased in addition (Table 5), whereas in F13T6 the increase in metacentrics just paralleled the increase in total chromosomes per 100 cells.

Both F1ISV3T3 and F1Py3T3 grew as rapidly in sparse culture as their parent lines, and both flat lines contained viral T-antigen (Table 2). Thus, the increase in chromosome accompanying flat revertant did not eliminate the entire viral genome, and did not produce slow-growing lines.

Table 2. CHROMOSOME CONSTITUTION OF MOUSE CELL CULTURES

<table>
<thead>
<tr>
<th>Chromosome number (abnormal)</th>
<th>Metacentrics (abnormal)</th>
<th>Median</th>
<th>Number of cells with Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>Median/ range, fraction</td>
<td>metaphase chromosomes metaphase chromosomes/metaphase/ counted</td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>MEF</td>
<td>46 (= 2 n)</td>
<td>1-9</td>
<td>100</td>
</tr>
<tr>
<td>3T6</td>
<td>73</td>
<td>0.65</td>
<td>89</td>
</tr>
<tr>
<td>3T3</td>
<td>76</td>
<td>0.72</td>
<td>84</td>
</tr>
<tr>
<td>F13T6</td>
<td>105</td>
<td>0.68</td>
<td>63</td>
</tr>
<tr>
<td>SV40</td>
<td>75</td>
<td>0.97</td>
<td>74</td>
</tr>
<tr>
<td>F1SY3T3</td>
<td>118</td>
<td>0.95</td>
<td>91</td>
</tr>
<tr>
<td>ReSV3T3</td>
<td>70</td>
<td>0.67</td>
<td>91</td>
</tr>
<tr>
<td>F1Py3T3</td>
<td>60</td>
<td>0.74</td>
<td>91</td>
</tr>
<tr>
<td>RePy3T3</td>
<td>65</td>
<td>0.79</td>
<td>91</td>
</tr>
</tbody>
</table>

For preparation of chromosome spreads, a cell line was incubated at about one-tenth density, to give a defined population. Medium was changed one day after transfer. On the second day, cells were exposed to 0-1 μg/ml. Vinblast (Vincristine, Osolob) for 4 h suspended in 0.25 per cent trypsin for 10 min, centrifuged at 800 r.p.m. for 10 min, resuspended in 20 per cent foetal calf serum in distilled H2O for 30 min, resuspended in 200 per cent foetal calf serum for 10 min, fixed for 30 min at 4°C in cold Carnoy’s fixative, air-dried on slides, and stained with 1 per cent acetic acid-gold chloride. Each cell line was obtained from extremely well-spread metaphases. In addition about 1,000 cells were examined under low power (1,000) and only the clearly anaphase figures were counted. More than 10 per cent of cells were counted. In the F1ISV3T3 cells, this was done to eliminate the bias against counting cells with higher chromosome numbers, where inadequate spreading and an overlap made accurate counts difficult. The metaphase preparations that were counted exactly for total chromosome number were those that showed no overlap of chromosomes. Because few unstable configurations of the sort that might interfere with mitosis (dissections, rings) were present, it is safe to assume most of the cells examined were capable of further division and representative of the population.

* Cells in the "median range" had chromosome numbers corresponding roughly to the major peak of cells contiguous with the median number on the bar graph.

Back revertants of T-antigen positive flat lines

A few foci inevitably arose in cultures of F1Py3T3 and F1ISV3T3 kept at saturation for more than a week. These foci were cloned to provide stable populations with saturation densities of more than 5x10^6 cells/cm^2. The back revertant lines derived from flat revertants were called ReSV3T3 and RePy3T3. The back revertant of F1ISV3T3, like F1ISV3T3, remained positive for SV40 T-antigen. Neither back revertant line was selected by re-infection with a transforming virus; both arose spontaneously.

Chromosomes in RePy3T3 and ReSV3T3 returned in both cases to distributions close to those of the respective dense transformed parent lines SV3T3 and Py3T3 (Fig. 1f, i). The SV40 transformed series show this most clearly (Fig. 1d, e, f). Only about 2 per cent of the F1ISV3T3 cells are in the lower range of SV3T3, and probably a cell of this minority was the ancestor of the ReSV3T3 population.

In the polyoma transformed series, RePy3T3 lost the hyperploid peak but did not return to the distribution of
Py3T3. Apparently, RePy3T3 arose from one of the low
mode cells of F1Py3T3 (Fig. 1g, 2, 4).
The back revertant populations lost metacentrics also,
to return to the range of their transformed parents. A
few hyperploid cells contained most of the metacentrics
in RePy3T3 (Table 2).
These data show that an increase in chromosome number
accompanied reversion, and a decrease in chromosome
number accompanies back reversion. The cell lines
studied here were selected as discrete variants in at most
two selective steps. Yet in each case the revertant popula-
tion had a hyperploid chromosome distribution that
overlapped only slightly with the parental distribution.
Selection in each case was exerted on growth control,
yet in each case the chromosome constitution was mar-
kedly changed. We conclude, therefore, that increase in
chromosome number is a necessary part of the mechanism
for maintenance of the reverted state of these cell lines.
Is hyperploidy sufficient, as well as necessary, to main-
tain the revertant state in flat cells? Hyperploid cells
are larger than their transformed parents; they therefore
have a lower surface/volume ratio. This renders them
less susceptible to the stressful loss of synthesized metabo-
lites to the medium. This may be the factor behind the
inhibition of hyperploid revertants by culture at low cell
density. Such revertants are very unstable, and polyploidy
generally results in reversion. Once any revertants arise after only 10 cell divisions, an instability
much greater than the frequency of re-reversion of the
flat revertants selected for by passage in FUDR.
An origin consistent with the transient nature of those
revertants would be that the cells first became hyper-
ploid in response to the stressful conditions of high cell
dilution, and that polyploidy resulted in reversion. Once
the cell number had increased enough to permit the cells
to condition the medium, hyperploidy would no longer
be selective, and any cell with the standard subtetraploid
complement of a back revertant would be able rapidly to
overgrow the culture.
If one can select for reversion by requiring polyploidy
or select for polyploidy by requiring reversion, then any
mechanism for maintenance of the reverted state should
require hyperploidy as a necessary step. At present the
mechanism of maintaining reversion is largely unknown,
as is the mechanism for maintaining transformation. Recent findings seem to indicate that the maintenance of
the transformed state in viral and spontaneous trans-
formation is mediated through alterations in the glyco-
proteins of the cell surface. Correspondingly, flat cells
complete the normal surface, and are unagglutinable.
These alterations, assayed with plant agglutinins, are
depicted as an increase in agglutinability of cells accom-
panying a loss of growth control. Increases in
agglutinability occur as well in cells cytologically infected with
SV40 as polyoma.
Two mutations of polyoma virus have recently been de-
scribed, of genes which effect the maintenance of the trans-
formed state through interference with cell surface. One is a
host range mutant that cannot grow on 3T3, but that
can grow on polyoma-transformed 3T3. This mutant
cannot transform either rat or hamster cells, and is unable
to induce an increase of agglutinability in lytic infection
of mouse cells. The other transformation mutant is
temperature-sensitive. When BHK hamster cells are
transformed with this mutant and kept at low tempera-
ture, they resemble other transformants and are agglutin-
able. When transformed cells are shifted from low to
high temperature, they regain orientation and lose agglutinability.
The agglutinins apparently detect a cell surface struc-
ture responsible for maintenance of growth control, and
a concomitant structure. The surface structure detected with agglutinins on transformed cells,
however, cannot itself be a viral gene product, because
(a) spontaneous, chemical, and X-ray induced trans-
formants from 3T3 and BHK, as well as tumour cells of
spontaneous origin, agglutinate as well as viral trans-
formants; (b) brief treatment with very low con-
centrations of proteolytic enzyme can bring about a
change in the cell surface similar to that occurring in the
transformation of normal to malignant cells; (c) the
SV40-induced surface antigen (S-antigen) is apparently
a cell constituent, since transformed cells, once con
formed spontaneously or by polyoma virus and non-
transformed lines can be made to display SV40 S-antigen
by mild proteolytic digestion of their cell surfaces;
and (d) a transient loss of growth control resembling transforma-
cion can be induced directly in a 3T3 population
by the addition of small amounts of trypsin to the
culture.
Because our flat revertants are unagglutinable, hyper-
ploidy must in some way act to permit the revertant cell
to mask agglutinin receptor sites, thereby reconstructing
a normal cell surface. Increase in cell size alone thus is not the cause of reversion.
What role does the increase in metacentrics have in
reversion from viral transformation that is not required
for reversion from non-viral transformation? A virus-
transformed cell may revert with chromosome loss,
elminating all viral genes, or with chromosome gain,
de spite the continued presence of the viral genome.
Transformation, with chromosome gain, may be a way for
virus-transformed cells to revert by inactivating viral
products responsible for maintenance of trans-
formation, rather than by losing the entire viral
genome. Whatever the mechanism, inactivation of some
but not all viral gene products must occur in revertants,
since they contain T-antigen, but are growth controlled.
Only the gene products necessary for the cell surface
alteration and the maintenance of the transformed state
would need to be inactivated, so the virus-specific
T-antigen should have no part in maintenance of the
transformed state (Table 1).
Does the relation of hyperplopy to reversion predict
the behaviour of hybrids between malignant and non-
malignant cell lines? All viable mouse x mouse hybrids
are, in a chromosomal sense, revertants: both types of
cell lines contain more mouse chromosomes than any
typical established mouse cell line. Recent reports show
that hyperploidy of mouse chromosomes accompanies
growth control in hybrids. When mouse x mouse hybrid
infantile pedigrees between 3T3 (M = 70) and a
spontaneous mouse tumour line with little growth control
(M = 53), the hybrids had between 100 and 120 chromo-
somes and were as growth controlled as 3T3. With time in
culture, chromosomes were lost and growth control of
the hybrid population dropped, variants with higher
saturation density appearing.
Two hybrid lines were isolated from mixtures of
SV40-transformed human cells (M = 71), and 3T3 mouse
cells (M = 70) containing the SV40 T-antigen (and
presumably the SV40 genome), but both hybrids were as
growth controlled as the 3T3 parent. Both hybrids had
15 human and 125 mouse chromosomes. This increase
in mouse chromosomes (70 to 135/cell) is very similar to
the increase seen in the F1SV3T3, which are also growth
controlled despite the presence of the SV40-T-antigen.
If the generalization is accepted that malignancy in vivo
and lack of growth control in vitro occur by similar
mechanisms, then in vivo results are consistent with
the hypothesis that hyperplopicy is related to rever-
sion. Malignancy was suppressed when malignant
and non-malignant cells were fused to give rise to viable
hybrids with between 85 and 128 mouse chromosomes/cell.
These hybrid lines gave rise to spontaneous malignant
re-revertants with fewer chromosomes.

Is Reversion of Clinical Interest?
The most significant test of this hypothesis would be
by inversion, determining whether agents causing hyper-
ploidy (for example, low concentrations of Velban or DNA base analogues) revert transformed and malignant populations. Recent observations on the suppression of malignancy in melanotic melanoma cells with subtoxic levels of BUdR may be relevant here. BUdR damages chromosomes and induces hyperploidy. BUdR-treated melanoma cells revert, lose pigment and become growth controlled. Revertant cell nuclei are at least twice the diameter of nuclei of the melanoma cells (ref. 49, Fig. 5). It would be interesting to see whether these "suppressed" cells are also hyperploid.

Selective killing of cancer cells by agents that interfere with cell division has been the rationale of most chemotherapy of tumours. X-irradiation, uracil, cytoxan, vincristine, vinblastine, amethopterin, daunomycin and nitrogen mustards are all effective against leukaemias and solid tumours because they are preferentially toxic to dividing cells. Chemotherapy is, however, rarely a complete success. Remission is often followed by relapse, with the reappearance of tumour cells, especially in the leukaemias. Non-proliferative leukaemia cells have been implicated as the source of regrowth in relapse.

If it is true in vivo as well as in vitro that hyperploid variants can be more growth controlled than their parents then these drugs and others used for cancer chemotherapy must be assayed for the possible induction of hyperploidy in the tumour cells that do not actually kill. Should revertant hyperploid cells be induced, they would then (as non-dividing cells) be resistant to further doses of the drug, and back revertants would be able to grow out from them once the drug was removed.

BUdR, FUDR, daunomycin, X-rays, and colchicine have already been shown to induce ploidy in cells surviving low doses. Therefore these agents must be suspected of inducing revertant hyperploid cells in vitro. Such revertants would be non-proliferative cells from which relapsing tumours might grow out, by back reversion.

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