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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[1,2].

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[3,4]. 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[3–5]. Keeping cells from contact during crisis will yield a line with a very high degree of growth control, like the 3T3 mouse line[4].

In constant culture conditions, constant frequency of medium changes, and constant concentration of serum in the medium[6,7], saturation density is the parameter of a cell line that correlates best with its ability to grow into a tumour *in vivo*[3,8,9]. 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[3,9–12].

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[13]. Such cells are bigger than their transformed parents and look *flat*; (*b*) passage of transformed cells on cell layers fixed with glutaraldehyde[14], 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[15] 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[16–18]. 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 transformant[19–21]. Thus the cells isolated by (*c*), which lack the viral antigens and are retransformable, arose as the viral genome was lost with some chromosomes of the polyploid hybrid line[22]. 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 most human chromosomes were lost from the hybrid cells[23]. 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[13,14].

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[24,25]. Flat revertants of spontaneous[8] and polyoma transformed[13,15] 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[26].

Stability of Reverted Cell Lines

Back reversion, or the reappearance of low growth control cells within a revertant 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[4]. 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 metacentric chromosomes. They grade smoothly in size[27]. As the mouse cells in culture pass

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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[11,28–30]. 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 transformants. 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-

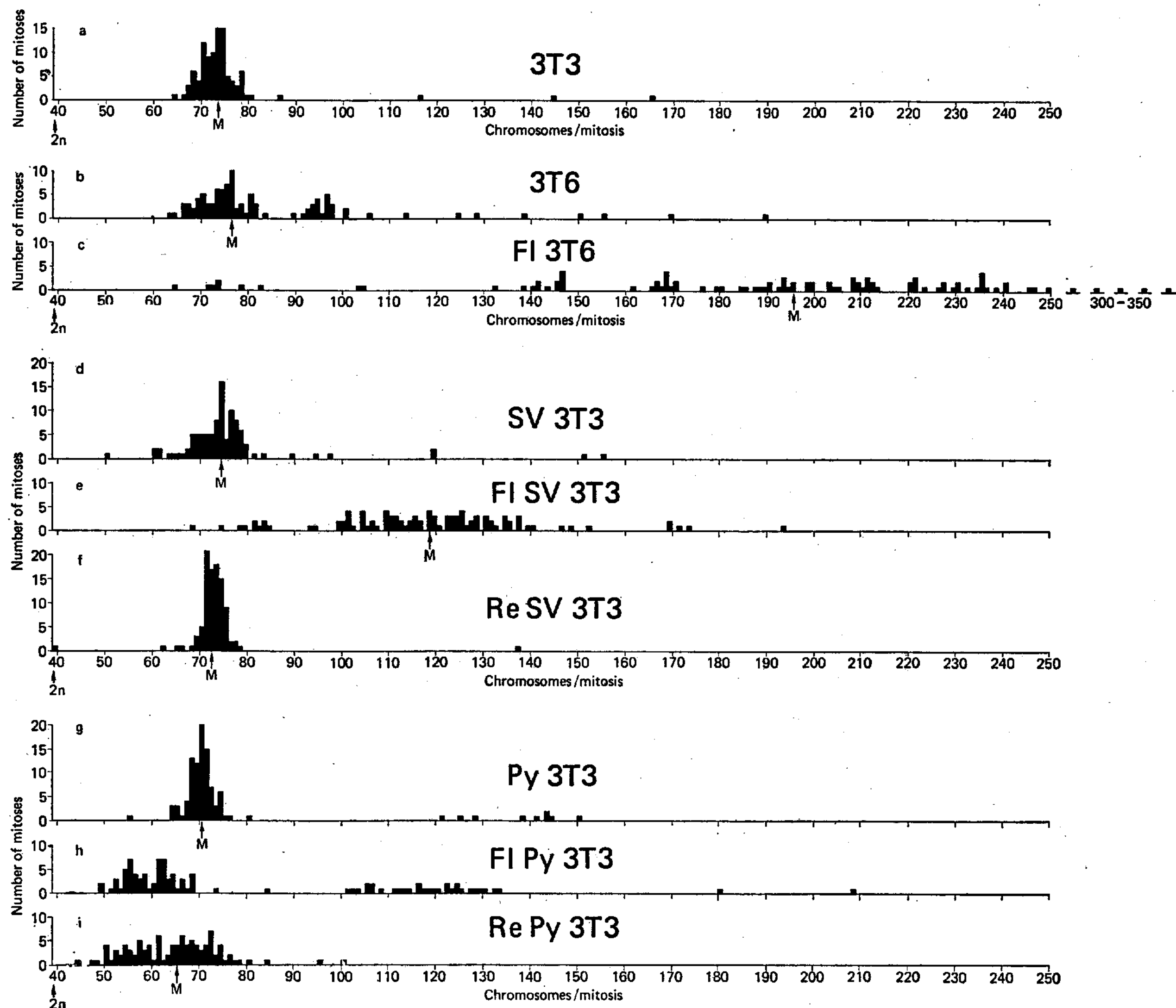

Fig. 1. Histograms of chromosomes/cell against number of cells. For each mouse cell line 100 metaphase preparations were counted at 1,500× magnification. The diploid number (2 n) of the mouse is 40. The median number of chromosomes for each line is indicated (M).

tetraploidy remained the dominant mode as the population lost growth control.

Polyoma and SV40 transformation of primary cells is accompanied by heteroploidy, with the appearance of many abnormal chromosomes[11,30]. 3T3 was, however, already heteroploid and about 11 per cent of 3T3 cells contained metacentrics (Table 2). There were 13 metacentrics per hundred cells for 3T3, 19 for 3T6, 32 for SV3T3, and 10 for Py3T3 (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 (=12 n) chromosomes. One cell of the 100 counted exactly 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. 1*b*, *c*). As expected, F13T6 cells were bigger than 3T6 cells.

The fraction of cells lacking metacentrics declined slightly, from 84 per cent to 63 per cent, but the majority of F13T6 cells still lacked metacentrics (Table 2), and the

Table 1. ORIGIN AND PROPERTIES OF MOUSE CELL CULTURES

Cell	Growth control	Established line	Transforming virus		Original selection	
			Name	T-ag	From	By
MEF	+	No	None	None	—	—
3T3	+	Yes	None	None	MEF	—
3T3	−	Yes	None	None	MEF	—
F13T6	+	Yes	—	None	3T6	FUdR
SV3T3	−	Yes	SV40	SV40	3T3	Focus
F1SV3T3	+	Yes	—	SV40	SV3T3	FUdR
ReSV3T3	−	Yes	—	SV40	F1SV3T3	Focus
Py3T3	−	Yes	Polyoma	Polyoma	3T3	Focus
F1Py3T3	+	Yes	—	Polyoma	Py3T3	FUdR
RePy3T3	−	Yes	—	Not done	F1Py3T3	Focus

Some years ago[4] two permanent cell lines were established from mouse embryo fibroblasts. One line, 3T3, retained maximal growth control, while the other, 3T6, did not. 3T3 was transformed by SV40 and by polyoma in 1963 (ref. 31). Transformed sublines were isolated and cloned twice. These lines were stored in liquid nitrogen. 3T3, 3T6, SV3T3 and Py3T3 were thawed and cloned twice in 1967 (ref. 13). They have been kept in culture since. Phenotypic revertants, designated as flat cell lines (F1) because of their low saturation density and their large, flat morphology in culture, were isolated by FUdR negative selection from 3T6, giving F13T6, and from the two virus transformed 3T3 lines, giving F1Py3T3 and F1SV3T3 (ref. 13). Recent spontaneously arising sublines with low growth control were isolated as dense foci from monolayers of F1Py3T3 and F1SV3T3. These were cloned twice and are designated in these experiments as RePy3T3 and ReSV3T3. All cell lines were maintained at 36·5° C in 20 cm² plastic Petri dishes (Falcon) in Dulbecco and Vogt's modification of Eagle's medium supplemented with 10 per cent calf serum. The medium was changed twice weekly. Growth control was assayed as saturation density. (+)=Saturation density less than 8×10^4 cell/cm² with 2 changes/week of DME+10 per cent CS. (−)=Saturation density more than 5×10^5 cell/cm² with 2 changes/week of DME+10 per cent CS.

Table 2. CHROMOSOME CONSTITUTION OF MOUSE CELL CULTURES

Cell	Chromosome number		Metacentrics (abnormal)					
	Median/cell	Median range, fraction	Number of cells with metacentric chromosomes 0	1	2	3	≥ 4	Counted metacentrics/100 cells
MEF	40 (=2 n)	1·0	100	0	0	0	0	0
3T3	73	0·85	89	9	2	0	0	13
3T6	76	0·72	84	13	3	0	0	19
F13T6	195	0·58	63	29	7	1	0	46
SV3T3	74	0·97	74	21	4	1	0	32
F1SV3T3	118	0·95	9	32	28	22	9	196
ReSV3T3	72	0·90	91	6	2	0	1	14
Py3T3	70	0·74	91	8	1	0	0	10
F1Py3T3	60,120	0·50, 0·50	4	18	16	17	45	353
RePy3T3	65	0·79	69	8	4	4	15	115

For preparation of chromosome spreads, a cell line was inoculated at about one-tenth of its saturation density, to give a dividing population. Medium was changed one day after transfer. On the second day, cells were exposed to 0·1 μg/ml. Velban (Vinca Leucoblastine, Lilly) for 4 h, suspended in 0·25 per cent trypsin for 10 min, centrifuged at 900 r.p.m. for 10 min, resuspended, swelled by exposure to 20 per cent foetal calf serum in distilled H_2O for 30 min, recentrifuged at 500 r.p.m. 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–orcein. A histogram for each cell line was obtained from exactly 100 well-spread metaphases. In addition about 1,000 cells were examined under low power (100×) and classified according to whether or not their number was in the median range* for that cell line. This was done to eliminate the bias against counting cells with higher chromosome numbers, where inadequate spreading and multiple overlaps made accurate counts difficult. The metaphase preparations that were counted exactly for total chromosome number were also counted for metacentrics. Because few unstable configurations of the sort that might interfere with mitosis (dicentrics, rings) were present, it is safe to assume that 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.

total number increased slightly, from 19/ to 46/100 cells.

The subtetraploid 3T6 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 subtetraploid cell line with growth control, was obtained with no more hyperploidy than the dense cell line 3T6 (Fig. 1*a*, *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 SV3T3 increased from 75 to 118 with reversion. The F1SV3T3 population had a much broader histogram than SV3T3 (Fig. 1*d*, *e*), but overlap between F1SV3T3 and SV3T3 chromosome number was limited: 6 per cent of SV3T3 cells had more than 90 chromosomes, and 10 per cent of F1SV3T3 had fewer than 90 chromosomes. Five per cent of F1SV3T3 cells counted had more than 160 (=8 n) chromosomes. In contrast to 3T6 and F13T6, F1SV3T3 had many more metacentrics/100 cells than SV3T3 (196 vs 32, Table 2). Only 9 per cent of F1SV3T3 cells were free of metacentrics, as compared with 74 per cent of SV3T3 cells (Table 2).

F1Py3T3, selected from Py3T3, did not shift entirely to higher chromosome number. F1Py3T3 showed a bimodal distribution of counted chromosomes/cell (Fig. 1*g*, *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. 1*g*, *h*). The higher peak is very near to the peak of F1SV3T3. 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 2), whereas in F13T6 the increase in metacentrics just paralleled the increase in total chromosomes per 100 cells.

Both F1SV3T3 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 number accompanying flat reversion did not eliminate the entire viral genome, and did not produce slow-growing lines.

Back Revertants of T-antigen Positive Flat Lines

A few dense foci inevitably arose in cultures of F1Py3T3 and F1SV3T3 kept at saturation for more than a week. These foci were cloned to provide stable populations with saturation densities of more than 5×10^5 cells/cm². The back revertant lines derived from the flat revertants were called ReSV3T3 and RePy3T3. The back revertant of F1SV3T3, like F1SV3T3, 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. 1*f*, *i*). The SV40 transformed series shows this most clearly (Fig. 1*d*, *e*, *f*). Only about 2 per cent of the F1SV3T3 cells are in the median range of SV3T3. Presumably 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. 1*g*, *h*, *i*).

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 accompanies 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 population 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 markedly 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 maintain 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 metabolites to the medium[1,28,32,33,35]. This may be the factor behind the induction of hyperploid revertants by culture at low cell density. Such revertants are very unstable, as they grow to form visible colonies[34]. Many back 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 hyperploid 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 transformation is mediated through alterations in the glycoproteins of the cell surface. Correspondingly, flat cells complete the normal surface, and are unagglutinable[23,24]. These alterations, assayed with plant agglutinins, are detected as an increase in agglutinability of cells accompanying a loss of growth control[36–41]. Increases in agglutinability occur as well in cells lytically infected with SV40 as polyoma[19,42].

Two mutations of polyoma virus have recently been described, of genes which effect the maintenance of the transformed 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 [20]. This mutant cannot transform either rat or hamster cells, and is unable to induce an increase of agglutinability in lytic infection of mouse cells[19]. The other transformation mutant is temperature-sensitive. When BHK hamster cells are transformed with this mutant and kept at low temperature, they resemble other transformants and are agglutinable. When transformed cells are shifted from low to high temperature, they regain orientation and lose agglutinability[21].

The agglutinins apparently detect a cell surface structure responsible for maintenance of growth control, and a polyoma gene can affect this 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 transformants from 3T3 and BHK, as well as tumour cells of spontaneous origin, agglutinate as well as viral transformants[36,37,39]; (*b*) brief treatment with very low concentrations of proteolytic enzyme can bring about a change in the cell surface similar to that occurring in the transformation of normal to malignant cells[38]; (*c*) the SV40-induced surface antigen (S-antigen) is apparently a component of the normal cells, since cell lines transformed 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[42]; and (*d*) a transient loss of growth control resembling transformation can be induced directly in a 3T3 population by the addition of small amounts of trypsin to the confluent culture[43].

Because our flat revertants are unagglutinable, hyperploidy 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, eliminating all viral genes[15,22], or with chromosome gain, despite the continued presence of the viral genome. Translocation, with chromosome gain, may be a way for virus-transformed cells to revert by inactivating[1,15,21,28,44] viral gene products responsible for maintenance of transformation, rather than by losing[15,21] 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 hyperploidy to reversion predict the behaviour of hybrids between malignant and non-malignant cell lines? All viable mouse × 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 × mouse hybrid lines were prepared[47] between 3T3 ($M=70$) and a spontaneous mouse tumour line with little growth control ($M=53$), the hybrids had between 100 and 120 chromosomes 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[47] 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 135 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[1,3,8–10,12], then *in vivo* results are consistent with the hypothesis that hyperploidy is related to reversion. Malignancy was suppressed when malignant and non-malignant cells were fused[48] 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[49]. BUdR damages chromosomes[50] and induces hyperploidy[52]. 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, imuran, cytoxan, vincaleukoblastine, amethopterin, dactinomycin and nitrogen mustard are all effective against leukaemias and solid tumours because they are preferentially toxic to dividing cells[52]. 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[53,54].

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 they 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[49], FUdR[13], dactinomycin[55], X-rays[56], and colcimide[1] have already been shown to induce polyploidy in cells surviving low doses. Therefore these agents must be suspected of inducing revertant hyperploid cells *in vivo*. Such revertants would be non-proliferative cells from which relapsing tumours might grow out, by back reversion.

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