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Sensitivity of transformation to small differences in population density during serial passage of NIH 3T3 cells

(adaptation/epigenetics/tumorigenesis/metastasis)

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ABSTRACT **Early passages of the NIH 3T3 mouse cell line undergo spontaneous neoplastic transformation leading to the development of transformed foci if grown to confluence in 2% (vol/vol) calf serum (CS) and left there for more than a week. Transfer of the postconfluent cultures results in the appearance of large numbers of transformed foci; many of them are larger and denser than those in the original culture. If the cells are continually kept at low population densities by frequent passages in 10% CS, they lose the capacity to undergo spontaneous transformation. If however the low-density passages are made in 2% CS or in 10% (vol/vol) fetal bovine serum, both of which support lower growth rates and saturation densities than does 10% CS, they gain the capacities to grow to high saturation densities and produce more foci when grown to confluence in 2% CS. These increases are proportional to the population densities used in the frequent passages, although the densities are all kept well below confluence. We conclude that the combined constraints of submaximal serum plus those of the limited cell contacts of the low cell densities used here elicit an adaptive response that endows the entire population with increased growth capacity. The increased growth capacity of the heterogeneous population in turn increases the capacity of a fraction of the population to initiate distinctive transformed foci. Similar studies have indicated that the capacity of cells to produce tumors and metastases in mice and rats is enhanced by prior maintenance at high density in culture. We propose the concept of progressive state selection to account for the general increase in the growth capacity of cells that is elicited by moderate constraints on their growth and metabolism.**

The NIH 3T3 line of cells produces foci of neoplastically transformed cells when grown to confluence in low calf serum (CS) concentrations [2% (vol/vol)], but the number and morphology of the foci depend on the passage history of the cells (1, 2). If they are repeatedly passaged at low population density in a high CS concentration (10%), they gradually lose the capacity to produce transformed foci. If the low-density passages are done in low CS concentrations or in high fetal bovine serum (FBS) concentrations, or if the cells are grown to confluence in high CS concentrations and the cells are then transferred for use in the assay procedure, many foci are formed (1–4). These observations led to the proposal that transformation is an adaptive response to moderate constraints on cell growth and/or metabolism. The response to those constraints permits some cells to multiply in postconfluent cultures at a higher rate than the surrounding, contact-inhibited cells, which results in the formation of discrete foci of transformed cells. The epigenetic nature of the transformation is indicated by its occurrence only under certain conditions (i.e., physiological constraint). It receives support from the fact that most, if not all, of the cells of a responding population that are not overtly transformed to form dense foci show an increase in saturation density and an increase in sensitivity to transformation among cloned progeny populations (1). Furthermore, the foci from independent transforming events are expressed in different morphologies (3). Although the overtly transformed state is heritable if the conditions of moderate constraint are maintained, it is gradually diminished and finally reversed over a number of cell generations if the cells are grown under unconstrained conditions of low density in a high CS concentration (1, 5). Cells from the dense, but not the light, foci form sarcomas in nude mice within a few weeks, but progeny of cells from the dense foci lose that capacity when their focus-forming capacity is reversed (5). Cells obtained from the sarcomas and reinoculated into mice form tumors even more rapidly than any cells from culture, indicating that further adaptation to growth in the mouse occurs during development of the first sarcoma (5). The combination of all these features shows clearly that spontaneous transformation is not the result of mutation and is likely to involve a change in equilibria of many biochemical pathways in the responding cells.

The aim of the work presented here was to gauge just how sensitive the cells are to constraining conditions, in particular to population density. In examining this question experimentally, it was important to recognize that the sensitivity of the cells to focus formation changes as the cells are passaged in culture depending on the conditions used in passage and how many passages have occurred. Instead of using a single constraining condition in passage such as confluence, the cells were passaged at various subconfluent densities in the presence of reduced CS concentration or in FBS. The use of growth-moderating concentrations of serum revealed that sensitivity to transformation in the assay varies with the cell density used in passage even when the passages are maintained at subconfluent densities.

MATERIALS AND METHODS

Cells and Culture Methods. The cells used in all experiments were of the NIH 3T3 line derived from mouse embryos by five successive isolations of cells from flat, monolayered growth following low-density seedings (6). They were kindly provided by S. A. Aaronson of the National Cancer Institute. Cells were maintained in "60-mm" plastic culture dishes (21-cm^2 actual area) at subconfluent densities by a successive passage of 2, 2, and 3 days each week at densities of 5×10^4, 5×10^4, and 2×10^4 cells per dish, respectively. The medium was MCDB 402 (7), and CS was added to a final concentration of 10% for standard passages. Cells were detached by washing in Tris and treatment with 0.1% trypsin in 0.5 mM EDTA. The serum concentration or type was changed for the experiments as described in the text. Cells were counted in a

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Abbreviations: CS, calf serum; FBS, fetal bovine serum; MCDB 402, molecular cell and developmental biology medium 402.

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Coulter electronic counter. Since cell counts were made at every passage, an approximation of cellular growth rates could be made. The approximation does not take into account the attachment efficiency of the cells and is based on the formula: PD/day = 3.32 [(log C_t − log C_0)/days], where PD/day = population doublings per day, C_t = number of cells on the day of transfer, and C_0 = number of cells seeded.

Dishes of day 14 from each assay were fixed with Bouin's fixative and stained with 4% (vol/vol) Giemsa solution. Fifteen photographs at a magnification of ×125 were randomly taken from some of the stained dishes in the Zeiss microscope with Polaroid type 53 film. The number of cells per field was counted to determine the population density per field and the distribution of population densities among fields.

The assay for transformation was done by seeding 10^5 cells in 2% CS and changing the medium every 3 or 4 days. At day 14, the cells were fixed and stained as above, and the dark-staining, discrete focal areas of high cell density were counted over a light box, without magnification.

Cells were considered in contact if their borders touched or overlapped. To count the number of cells in contact with other cells, dishes from different passage densities were fixed and stained as above 1 and 2 days after the cells had been shifted down to 2% CS. Counting was done in an optical microscope with a magnification of ×125. Twenty fields were counted from each passage density each day.

RESULTS

Effects of the Subconfluent Population Density used in Passage in Low CS Concentrations on the Saturation Density of Late Passage Cells. Growth curves of NIH 3T3 cells in 10% CS show that multiplication at this serum concentration is exponential up to 10^6 cells per dish (5×10^4 per cm^2) (Fig. 1). This indicates that there is no constraint of growth up to this density, which is twice the density required to make a confluent sheet. Cells in 2% CS multiply only 0.8 times as fast as those in 10% CS during the first day of measured growth and only 0.6 times as fast during the second day. There was more than a 5-fold difference in saturation densities for cells grown in 2% and 10% CS. The reduction in growth in 2% CS between the first and second days of measurement indicates that growth inhibitory interactions of cells occur in low CS concentrations when there are $<2 \times 10^5$ cells per dish. We

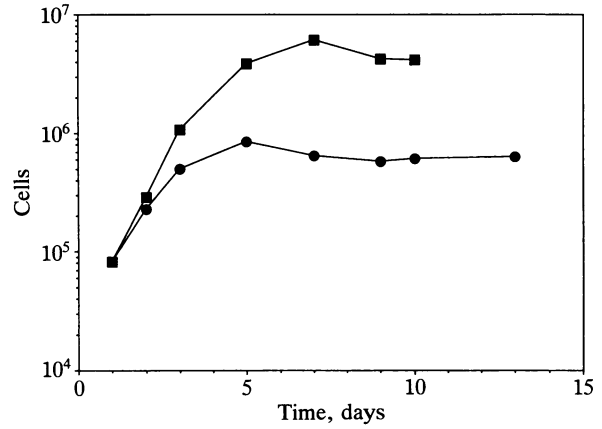

FIG. 1. Growth curves of nontransformed NIH 3T3 cells in low and high serum concentrations. Cells that had been in culture for only 4 days after thawing were transferred to a large number of dishes in 2% CS at 10^5 cells per dish. At day 1 the cells on two dishes were counted, and the medium on the remaining dishes was changed with 2% CS on half of them and 10% CS on the other half. The medium was changed at 2- to 3-day intervals up to day 8 and every day thereafter. Cell counts were made on two dishes of 2% and 10% CS at the indicated times. ■, 10% CS; ●, 2% CS.

therefore decided to examine whether the cells in 2% CS would respond adaptively to population density in cultures seeded at densities between 2×10^4 and 10^5 per dish and passaged every 2 or 3 days. The adaptive response measured was an increase in saturation density.

The first experiment combining multiplication in low CS concentrations with variations in subconfluent population density was done with cells that had been passaged for 5 months (late passage cells) in 10% CS. They no longer produced any distinct foci when grown to confluence in 2% CS, and no large or dense foci appeared on reassaying the cells after they reached confluence. They were divided into three groups for passage in 2% CS at high density (1×10^5, 1×10^5, and 5×10^4), medium density (5×10^4, 5×10^4, and 2.5×10^4), and low density (2×10^4, 2×10^4, and 1×10^4) at 2, 2, and 3 days, respectively, over a period of 3 consecutive weeks. Every third passage, three cultures from three parallel passage sublines of each density were used separately for a determination of saturation density by seeding them on six dishes at 5×10^4 in 2% CS and counting two of them at 7, 10, and 14 days. In addition, cultures were fixed and stained after 14 days, and photographs were taken to determine the distribution of cells. A parallel control determination of saturation density was included by using cells that had undergone the standard passage series in 10% CS.

The growth rates of cells at the different seeding densities measured over many passages as population doublings per day with standard errors were 0.605 ± 0.043, 0.742 ± 0.037, and 0.824 ± 0.034 for the high, medium, and low seeding densities, respectively. The significant differences in rates indicate a modulating effect of population density in 2% CS, even at these relatively low densities, and imply a great sensitivity to even limited contact in the presence of low serum concentration.

The percentage of cells at each seeding density that was in contact with at least one other cell is shown in Table 1. Although the lowest seeding density was <1/30th the saturation density in 2% CS (see Fig. 1), about 20% of the cells had at least one contact with other cells, and the percentage in contact rose on day 2 with the increase in cell number. The highest seeding of 1×10^5 was less than one-sixth the saturation density, but about 75% of the cells were in contact with one another. The high percentage of contacts among cells so far below saturation density arises from their great flattening out after attachment and their asymmetric shape. The difference in percentage of cells in contact as a function of seeding density accounts for the inverse relationship between seeding density and extent of multiplication in frequent passage. It is also likely to be the major factor in the longer term direct relationship between seeding density used in frequent passage and acquired increases in saturation density described below.

In all groups, cultures derived from replicate cultures passaged at the same density had the same saturation density, thereby establishing that there was no significant variation in parallel sublines independent of the conditions used (Fig. 2). Cells at all three passage densities in 2% CS increased their saturation densities in each successive week of passage (Fig.

Table 1. Percentage of cells in contact with at least one other cell

No. cells seeded per dish	Percentage of cells in contact	
	Day 1	Day 2
2×10^4	21.0 ± 26.4	30.7 ± 20.7
5×10^4	53.8 ± 23.9	70.1 ± 19.7
10^5	74.4 ± 18.6	82.5 ± 16.8

Twenty fields were counted for each determination, and the means ± SD are shown. Counts were made at a magnification of ×125.

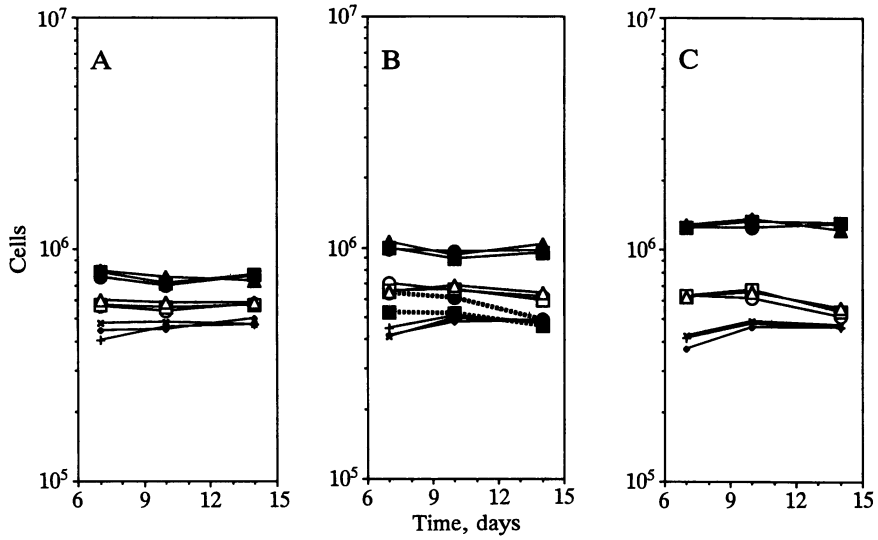

FIG. 2. Changes in saturation density of cells passaged in 2% CS at different densities over a 3-week period. Cells were seeded at 2×10^4 (*A*), 5×10^4 (*B*), and 10^5 (*C*) cells per dish in 2% CS and transferred at those densities twice each week at intervals of 2 days and once at half those densities for a 3-day interval. Three parallel lines of cells were passaged at each density. One control line of cells was passaged in 10% CS at 5×10^4 (and 2.5×10^4) cells. At every third passage in 2% CS, 10^5 cells from every group were seeded on six dishes, and counts were made at 7, 10, and 14 days to determine the saturation density. Saturation densities were determined for three parallel sets transferred in 2% CS at the three different densities for three passages (◆, ×, +), six passages (□, △, ○), and nine passages (■, ▲, ●). The broken lines in *B* show the saturation densities in 2% CS of the cells that had been transferred in 10% CS for 64 passages.

2). Those that had been passaged for 3 weeks at the highest density, however, reached the highest saturation density, followed by those that had been passaged at the middle and low densities, respectively. The control cultures, which had been passaged in 10% CS, remained at their initial saturation density throughout (Fig. 2*B*).

By the second week of passage, cells from the high- and middle-density passages generated many small, light foci when grown to confluence. Similar foci, but in smaller numbers, also appeared in the low-density cultures when assayed after 3 weeks of passage in 2% CS. No foci were visible in the control cultures passaged in 10% CS. Since the foci were too small and too numerous to count in the assay of the 3-week passage without magnification, the number and distribution of cells were determined by photographing and counting many microscopic fields. The mean ± SD number of cells per photographic field increased from 99 ± 15 in cells from the 10% CS controls to 162 ± 24 in cells from the low-density passages and 239 ± 102 in cells from the high-density passages (see Fig. 3). A much broader distribution of cell number from field to field was found in cells from the high-density passage than those from the low-density passage or from the controls. Most of the increased heterogeneity came from an increase rather than a decrease in the number of cells per field, which reflects the presence of small, lightly stained foci of crowded cells. The results show that small differences in passage density of cells in the presence of low serum concentrations lead to significant differences in the saturation density of those cells when they are allowed to grow to confluence.

Effects of Serum Type and Population Density Used in Passage on the Focus-Forming Capacity of Early Passage Cells. The demonstration of population density effects in the previous section depended on the use of a low (2%), growth-moderating concentration of CS during the passage series. It has been established that the use of 10% (vol/vol) FBS during early passage of cells leads to the formation of many more foci on assay than the use of 10% CS (2, 8). It was noted that the yield of cells at each passage of cells in 10% FBS was consistently lower than the yield of cells in 10% CS (data not shown), which suggests that FBS has less growth stimulatory activity for these cells than CS. To test this point, the growth rates of cells that had been frequently passaged over a 32-day period (nine passages) in either 10% FBS or 10% CS were determined in both 10% FBS and 10% CS. Cells that had been passaged in FBS attached less efficiently than those passaged in CS, as indicated by the cell yield 1 day after transfer, regardless of whether the cells were seeded in FBS or CS (Fig. 4). Cells from both FBS and CS passages multiplied faster in CS than they did in FBS and reached significantly higher saturation densities. The cells from passage in CS began to slow down in FBS at an earlier time and lower density than did those from FBS passage in the same medium, which resulted in a higher saturation density for cells from the FBS passage. The saturation density of the cells grown in 10% CS could not be reliably determined because the cell sheets started retracting on day 7. It is evident on the

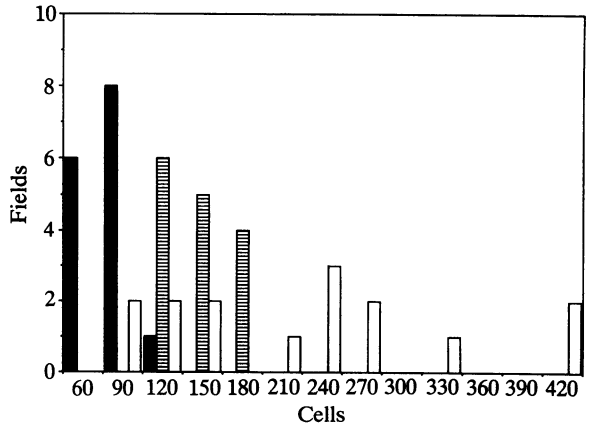

FIG. 3. Distribution of cell numbers at saturation density per microscopic field of cells that had been passaged nine times at different densities or in different serum concentrations. The procedure was the same as that of Fig. 2. Black bars, cells passaged nine times in 10% CS at 5×10^4 cells per dish (and 2.5×10^4); hatched bars, cells passaged nine times in 2% CS at 2×10^4 (and 1×10^4); open bars, cells passaged nine times in 2% CS at 10×10^4 (and 5×10^4). The cell counts were made 14 days after seeding 10^5 cells in 2% CS.

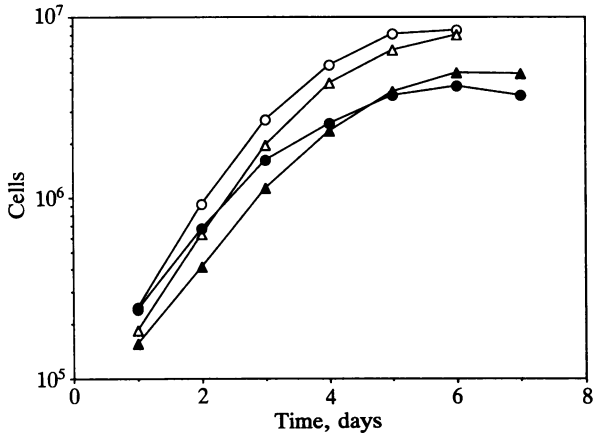

FIG. 4. Growth curves of cells in CS and FBS that had previously been passaged in 10% CS or 10% FBS. Cells were passaged for 33 days at 10^5, 10^5, and 5×10^4 cells per dish for 2-, 2-, and 3-day intervals, respectively, in either 10% CS or 10% FBS. Cells from both sera were then seeded at 10^5 cells per dish in 10% CS and in 10% FBS and counted at the indicated times. ○, Cells from 10% CS passage growing in 10% CS; ●, cells from 10% CS passage growing in 10% FBS; △, cells from 10% FBS passage growing in 10% CS; ▲, cells from 10% FBS passage growing in 10% FBS.

basis of reduced attachment of cells from FBS passage and the reduced growth rates and saturation densities of cells multiplying in FBS that FBS has less growth-stimulatory activity than does an equal concentration of CS.

We then explored the effect of using FBS in early cell passages on the relationship of the population density used in those early passages to the focus-forming capacity of the cells. The focus-forming capacity of the cells passaged in 10% FBS was assayed in three different concentrations of CS (2%, 5%, and 10%) and compared with that of cells that had been passaged in 10% CS. The results are shown in Fig. 5. Cells passaged at the highest density of 10×10^4 in FBS quickly increased their focus-forming capacity when assayed in 2% CS and retained a high focus-forming capacity for the rest of the experiment (Fig. 5A). Those passaged at lower densities

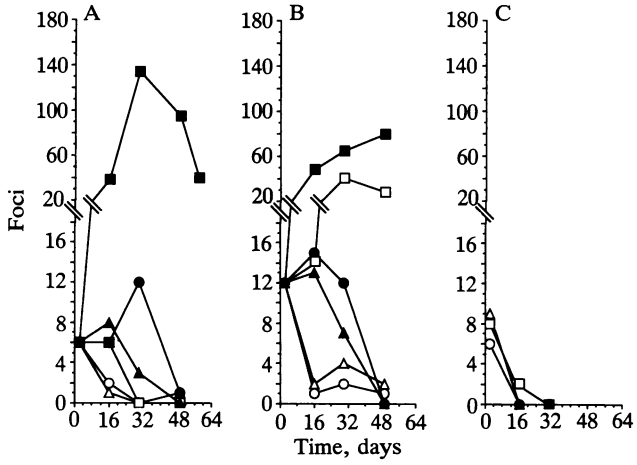

FIG. 5. Focus formation in various concentrations of CS of cells passaged at various densities in either 10% CS or 10% FBS. Cells that had been in culture 2 days were passaged in 10% CS (open symbols) or 10% FBS (closed symbols) at low (○ and ●; 2×10^4 cells per dish), medium (△ and ▲; 5×10^4 cells per dish), and high (□ and ■; 10^5 cells per dish) densities. After varying numbers of passages, the cells were used in the standard assay in 2% CS (A), 5% CS (B), or 10% CS (C) to determine their focus-forming capacities in those serum concentrations.

in FBS and those passaged in CS at all densities tended to lose their focus-forming capacities in 2% CS.

Cells passaged at the highest density in either FBS or CS maintained a high focus-forming capacity in 5% CS, though the cells passaged in FBS remained higher in all assays in 5% CS (Fig. 5B). Cells from the lower population densities passaged in either FBS or CS lost most or all of their focus-forming capacities in 5% CS during the course of the experiment. Even the highest density passages in both sera lost all their capacity for focus formation in 10% CS by the 32nd day of passage, regardless of the type of serum used in the passages.

The results, therefore, show that cells passaged in the slightly lower growth-supporting capacity of FBS as compared with CS increase and then maintain their focus-forming capacity in 2% and 5% CS if the population density at passage is 10×10^4 cells per dish ($\approx 5 \times 10^3$ cm^{-2}) but not if the density is lower. If passaged at the high density in CS, they only retain their focus-forming capacity in 5% CS. It is clear, therefore, that the focus-maintaining effects of the population density used during passage in CS depend on the conditions used in the assay, thereby illustrating the great sensitivity of transformation to the conditions used in both passage and assay of the cells.

DISCUSSION

The results described here show that heritable increases in transformation-related growth properties of NIH 3T3 cells are correlated with the population density used in passage of the cells, even when those densities are kept well below confluence. The growth properties assayed after these passage variations were saturation density, which is an expression of the capacity of the population to multiply when crowded, and focus formation, which reflects the ability of a minority of the cells to continue multiplying when the surrounding cells are contact inhibited. These enduring effects became manifest in the assay only when the preceding subconfluent density variations in passage were made in growth-modulating concentrations of CS or FBS. It seems likely that the constraints of suboptimal serum and slight increases in population density combine to elicit a stronger adaptive growth response than does either constraint alone. The direct growth-constraining effect of 2% CS versus 10% CS is manifest chiefly in a reduction of the saturation density of the cell population when the population is grown to confluence. However, even the highest density populations of the frequent passages used here exhibited a high multiplication rate during their 2- to 3-day passage intervals. Although maintained at relatively low average densities in these experiments, the cells are spread out in elongated shapes, and a high proportion are in contact with one another over part of their borders. Of course the percentage of surface in contact increases with increases in the number of cells seeded. Furthermore, the distribution of cells on the surface of the dish after seeding them is not completely uniform, with the result that many cells, especially in the highest passage densities, are in extensive contact with neighboring cells in the more crowded areas. This is most apparent after the 3-day passage interval, when the overall increase in cell number may be as much as 20-fold, thereby creating local high densities of cells and contact with the leading edge of neighboring microcolonies. Hence, there is ample opportunity for contact interaction among the cells, especially at the higher seeding densities, and this results in some decrease in the rate of multiplication. Such a decrease presumably follows a lowering of the metabolic rate among the cells, and it seems likely that the cells are adapting to that biochemical change rather than the decrease in multiplication *per se*. The fact that the population as a whole responds when passaged

and grown to confluency by an increase in saturation density offers further evidence that the response is an epigenetic one (1–5, 9), rather than one involving the selection of rare mutants. When we add to the present evidence that transformation is acutely dependent on culture conditions and that it involves a large fraction of the population the finding that the transformation is reversible (1, 4), there can be little doubt of the epigenetic nature of the changes in growth properties linked to transformation.

There have been other reports of persistent changes in cell growth behavior related to population density in other rodent cell lines. Cells from random-bred Swiss mouse embryos passaged in 10% CS every 3 days at densities of 6×10^5 or higher per 20-cm^2 dish developed a capacity to multiply to much higher saturation density than did cells passaged at 3×10^5 cells per dish (10). Cells from BALB/c mouse embryos passaged every 3 days in 20-cm^2 dishes at 12×10^5 cells per dish in 10% CS became tumorigenic within 3 months, whereas those passaged at 3×10^5 cells per dish remained nontumorigenic at 9 months (11). The high-density passages of the BALB/c cells grew to 3 times higher saturation density in culture than the low-density passages after 3 months of passage and 20 times higher after 9 months. A diploid line of adult rat liver epithelium began producing tumors in syngeneic hosts after about 20 cell divisions if kept at confluence for 3 weeks at every monthly passage but only became tumorigenic after about 100 divisions if transferred weekly just as the cells became confluent (12). The metastatic capacity of a line of mouse melanoma cells was increased more than 4-fold by a single round of growth to high density (13). In another report on the melanoma cells, it was claimed that their metastatic capacity upon inoculation into mice was increased by simply increasing the total number of cells in the culture population by growing them over a greater surface area without increasing their population density (14). Analysis of the data however shows that the population density was 2.4-fold higher in the larger than the smaller dishes. While such an increase may have been considered insignificant by the authors, our results indicate that it cannot be disregarded as the source of the increased metastatic capacity.

This survey shows that growth-constraining conditions elicit heritable, graded increases in the capacity of cells to multiply to high densities and produce transformed foci in culture as well as to initiate tumors and metastases in animals (1, 4, 5). We have proposed the concept of progressive state selection to account for the increase in growth capacity of the responding cells. It is based on the finding of a very high rate of phenotypic diversification with consequent heterogeneity of growth capacity among the cells (1, 5) and the progressive selection of those phenotypic states that are best suited to overcome the constraint. This type of selection is distinct from the selection of rare genetic mutations in that it modifies the growth behavior of the entire population of cells to varying extents in a heritable manner but also is gradually reversible if the growth constraints are lifted (1, 5).

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