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Adaptive evolution of degrees and kinds of neoplastic transformation in cell culture

(tumor progression/epigenetics/state selection)

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ABSTRACT **Human cancers undergo protracted complex development from benign to malignant states, as most thoroughly documented in the mole-to-melanoma sequence. The early stages of the sequence tend to redifferentiate into normal tissues; the later stages progress to ever increasing multiplication and malignancy. When placed under the growth constraint of either crowding or low serum concentrations, the NIH 3T3 line of mouse cells readily undergoes transformation, expressed in the development of foci of cells that continue to multiply at confluence when the rest of the population has stopped. If the nontransformed cells are maintained for 3 months by frequent low-density passages in high concentrations of calf serum, they gradually lose the capacity to undergo transformation when the constraints are applied. The same conditions of passage have been used to reverse the transformation, both processes resembling in principle the reversal of the early stages of the mole-to-melanoma sequence. When the frequent low-density passages are made in high concentrations of fetal bovine serum, which supports a slightly lower growth rate than an equal concentration of calf serum, the degree of transformation is gradually increased, so that the foci become more numerous, broader, and thicker, reaching a maximum in successive assays at about 3 months of passaging. A diversity of focal morphologies is sporadically generated in the calf serum passage by exposing the cells to various concentrations of calf serum for 14 days of growth and confluency before assaying them. The dependence of the number, density, and morphology of foci on the environment in which the cells had been grown before assay reinforces the evidence that the transformation is an epigenetic process. The fact that these effects develop in culture gradually over an extended period of time suggests parallels to the characteristically long-term early regression and later progression, as well as the diversity of the mole-to-melanoma sequence, and may also be representative of other cancers.**

The development of the most common types of solid cancer in humans is a long-term process, often spanning one-third to two-thirds of the lifetime of the individual (1). The lesions produced by carcinogens are, at the outset, focal proliferations that are orderly in form and temporally restricted in their growth (2). The prolonged sequence of events is encompassed by the term "tumor progression." In a number of cases of both experimental and clinical cancers, the initial benign lesions are either reversible or may persist for extended periods without progression. In many neoplastic systems, initial lesions are numerous, and progression to the next lesional class is rare (2). Squamous papillomas, common acquired melanocytic nevi (ordinary moles), adenomatous colonic polyps (tubular adenomas), and hepatocyte nodules are examples of initial lesions. Because such growths may serve a physiological need or a protective function, they have been referred to by Farber (3) as examples of clonal adaptation.

A form of progression has been studied during sequential passage of cultured cells (4) or after treatment with carcinogens (5). The markers used to assess progression include morphological transformation, increased plating efficiency, increased saturation density, multiplication in low serum concentrations, increased fibrinolytic activity, colony formation in agar, and tumor formation in animals (4, 5). It is not clear whether these parameters reflect different underlying alterations in cellular functions or the accumulation of similar functional changes with different thresholds for detection by each technique. In the present report, a single property is used to assess the degree of transformation, namely, the ability to multiply at high densities, which inhibit the multiplication of nontransformed cells. This property is expressed for an entire population of cells as an increase in the saturation density of a culture and for individual cells as the formation of foci by cells that continue to multiply at confluence when the surrounding cells have virtually stopped multiplying. Emphasis is placed on focus formation and, in particular, on the size, density, and morphology of the foci. The changes in this property have a formal resemblance to those of the mole-to-melanoma sequence. The opportunity for such study initially arose from the observation that the NIH 3T3 line undergoes "spontaneous" transformation when exposed to moderate constraints on growth and metabolism, such as occur when the population becomes confluent or when the cells are grown in low concentrations of serum (6, 7).

This report provides evidence from long-term analysis that either progression or regression of transformation-related behavior may occur in culture depending on the type of serum used in maintaining the cells during their passage. It also shows that the morphology of foci can be affected in a less dramatic way by simply varying the concentration of a single type of serum for a relatively short period of time before assaying the cells for focus formation under constant conditions.

MATERIALS AND METHODS

Culture Methods and Cell Passage Regime. The NIH 3T3 cells (8) used in these studies were obtained from S. A. Aaronson (National Cancer Institute, Bethesda, MD) and were stored in liquid nitrogen. The synthetic medium used was MCDB 402 (9), supplemented with either fetal bovine serum (FBS) or calf serum (CS) (HyClone). The concentration of serum used in regular passage of the cells was 10% (vol/vol). The cells were cultured in plastic Petri dishes (Falcon) with a surface area of 21 cm^2 in a humidified (37°C) incubator with CO_2 input to maintain pH 7.2–7.4. The cells were passaged (transferred) on a weekly schedule of 2, 2, and

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Abbreviations: CS, calf serum; FBS, fetal bovine serum.

3 days, and the number of cells seeded at each passage was 1×10^5, 1×10^5, and 5×10^4, respectively. The cells were suspended for passage by treatment with 0.01% trypsin/a 0.5 mM solution of EDTA/0.02 M Tris, pH 7.27/0.14 M NaCl. Cells were counted in a Coulter electronic counter.

Primary Assay for Focus Formation and Saturation Density and Secondary Assay for Focus Formation. The primary assay for focus formation was done by seeding 10^5 cells from the early passages in 2% and 5% CS; in later passages, a primary assay in 10% CS was added (see Fig. 1). The medium was changed every 3 or 4 days, and the incubation was carried on for 14 days. When it was expected that there would be too many foci to count, the cells were diluted, and from 10^2 to 10^4 cells were mixed with 10^5 cells of a subline that had been passaged for more than a year in 10% CS and produced no foci in a primary assay at any CS concentration. Some of the cultures were fixed in Bouin's solution at 14 days, washed three times with 80% ethanol, and stained with 4% (vol/vol) Giemsa in water overnight. The unfixed cultures were trypsinized at 14 days and counted to determine their saturation density. They were also used for a secondary or transfer assay by transferring 10^5 cells, or dilutions of the cells mixed with 10^5 cells of the nontransformed culture (Fig. 1). All secondary assays were done in 2% CS and the cells were fixed and stained at 14 days as above. As the foci were of various sizes and densities down to a barely perceptible level, only dense foci were counted. Two sets of the NIH 3T3 cells, thawed 57 days apart, were used to study the evolution of the growth and focus-forming capacity of the cells. The same batch of CS was used in passaging and assaying both sets of cultures, but different batches of FBS designated FBS-A and FBS-B were used in passaging the first and second set, respectively. The doubling time of the cells was 14.4 hr in 10% CS and was 18.2 hr in 10% FBS, or about 0.8 the rate in CS. The saturation density of the cells in 10% FBS was 0.58 of that in 10% CS.

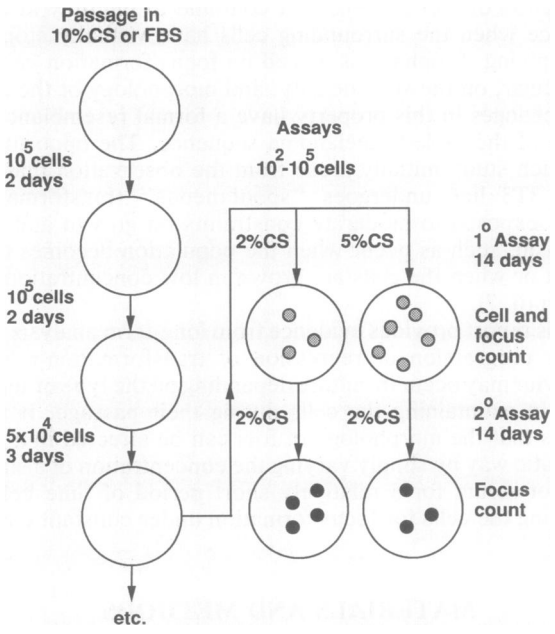

FIG. 1. Procedure for passaging cells and testing them for focus formation in primary and secondary assays. The large circles represent 21-cm² plastic Petri dishes. The left column represents the standard 2- to 3-day passages in either 10% CS or 10% FBS. At intervals, a primary assay (1°) was done in 2% or 5% CS by seeding 10^5 of the passaged cells; or 10^2–10^4 of these cells together with 10^5 cells of a line that no longer produced foci in either primary or secondary assays. The latter then formed a confluent lawn to display the focus-forming capacity of the minority. The cells were incubated 14 days with medium changes every 3–4 days. Some cultures were fixed and stained for focus counts. Others were trypsinized, counted for saturation density, and reseeded in a secondary assay (2°) at various cell densities, as in the primary assay. All dishes in secondary assays were in 2% CS.

RESULTS

Effect on Saturation Density of Serum Type Used in Low-Density Passage. As described above, frequent low-density passage was initiated at various times in two sets of cultures, each set containing one series in 10% CS and the other in 10% FBS. Similar results were obtained in measuring saturation density in the two sets of cultures. Since measurements were available from an earlier stage of passaging for the second set, only those will be described in detail, as represented in Fig. 2. Cells sampled from the early passages in 10% FBS had a slightly higher saturation density in 2% CS than those sampled from the early passages in 10% CS. The saturation density in 2% CS of the cells sampled from the FBS passage began a steady increase after 28 days of frequent passage, from 1×10^6 cells per dish to 7×10^6 at 96 days of passage and beyond (see also Table 1). By contrast, the cells sampled from the CS passage remained at relatively constant saturation densities in both 2% and 5% CS throughout the experiment, except for unaccountably high points at 61 days and a decrease at 136 days. The saturation density in 5% CS of the FBS-passaged cells showed an increase when tested at 96 days or later. The eventual result was that the cells passaged in FBS grew to a much higher density when tested in 2% and 5% CS than did the cells that had been passaged in CS (Fig. 2 and Table 1). The saturation density in 10% CS was only about 50% higher for the cells passaged in FBS than in CS, because the value in CS was already high (Table 1). A separate group of NIH 3T3 cells from the same stock, which had undergone passage in CS for 502 days, reached much lower saturation densities in all concentrations of CS than any cells passaged for shorter periods of time in CS (Table 1 and the legend of Fig. 2).

As noted above, FBS has lower growth stimulatory power than equal concentrations of CS, so its effect in gradually raising the saturation density of cells is consistent with an adaptive response to mildly constraining growth conditions.

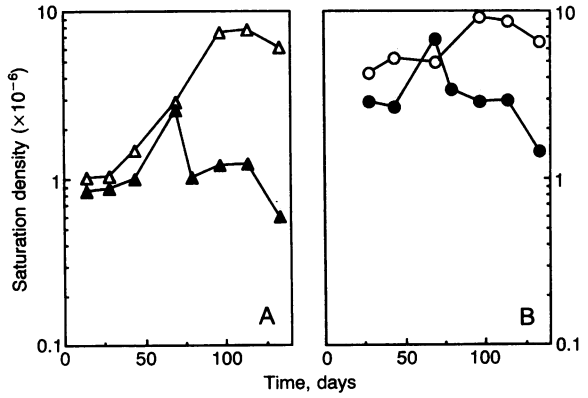

FIG. 2. Saturation densities of cells at various times after successive passages were started in 10% CS or 10% FBS-B. At intervals of ≈2 weeks, 10^5 cells passaged in 10% CS or 10% FBS were seeded in 2% or 5% CS for a primary assay. They were trypsinized and counted at 14 days to obtain the saturation density. (*A*) Cells assayed in 2% CS from passage in 10% CS (▲) or 10% FBS (△). (*B*) Cells assayed in 5% CS from passage in 10% CS (●) or 10% FBS (○). Abscissa shows the number of days the cells had been in passage at the time of the seeding for the primary assay. The figure does not show the cell line that had been in passage for a year in 10% CS at the start of the experiment, which had a saturation density in 2% CS of 5.2 (± 1.6) × 10^5 cells and in 5% CS of 10.0 (± 1.7) × 10^5 cells.

Table 1. Saturation densities

Cells	Days in culture	Serum for passage	Saturation density, cells per culture ($\times 10^{-6}$) 2% CS	5% CS	10% CS
Set 1	153	CS	1.16	2.46	6.87
		FBS-A	6.58	8.47	9.78
Set 2	96	CS	1.22	2.88	6.65
		FBS-B	7.49	9.04	9.25
Long term	502	CS	0.49	1.08	2.94

Saturation densities of cells that had been passaged serially in 10% CS or 10% FBS for the indicated number of days. At that point, 1×10^5 cells were seeded in 2, 5, or 10% CS and counted at day 14.

Focus-Forming Capacity of Serum Type Used in Low-Density Passage. Transformed foci vary greatly in area, cell population density, and detailed morphology (e.g., see Fig. 4). Only densely populated dark-staining foci can be counted reliably, and such foci occur more frequently in a secondary assay than in a primary assay. Even in a secondary assay there is variation in size and staining intensity of the foci, so a full appreciation of the extent of transformation can only be achieved through photographs, which will be considered below. The trends of dense focus formation in secondary assays were similar in the two sets of cultures initiated at different times, but since results were available from earlier passages of the second set, they are used to illustrate the main features of the response to passage in the two different sera. The number of dense foci in secondary assays of cell passages in CS after primary assay in 2% CS decreased to zero during 114 days of passage (Fig. 3). The same passage series also tended to decrease in dense focus formation in the secondary assay of cells that had been in 5% CS in the primary assay, but to a much lesser extent than cells that had been in 2% CS. The cells that had been passaged in FBS and had been through a primary assay in 2% CS produced increasing numbers of dense foci in the secondary assay to 44 days of passage, with the proportion fluctuating around 10% in that and later assays. The FBS cells from a primary assay in 5% CS tended on the average to produce fewer dense foci in the secondary assay than those from primary assay in 2% CS, but the difference was not great. However, the foci of the former tended to be lighter than those of the latter. The divergence in focus-forming capacity depending on whether passage was in CS or FBS will become more evident in the photographs that follow.

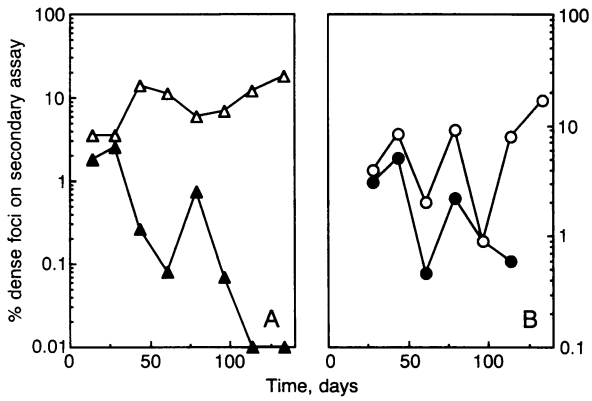

FIG. 3. Focus formation in secondary assays of cells at various times after successive passages in 10% CS or 10% FBS-B. The procedure for cell passage and primary assay was the same as that of Fig. 2. After the cells were counted to obtain saturation densities, they were reseeded for the secondary assay in 2% CS using 10^5 cells from the primary assay or 1:10 dilutions of those cells plus 10^5 cells from a line that no longer produced foci. Only dense foci are included in the figure. (*A*) Primary assay in 2% CS from passage in 10% CS (▲) or 10% FBS (△). (*B*) Primary assay in 5% CS from passage in 10% CS (●) or 10% FBS (○).

No distinct dense foci were made at any time during the course of the experiment in either primary or secondary assays by the group of cells that had been through frequent low-density passages in 10% CS for >1 year before the experiment was initiated. However, there were many small, pale foci in secondary assays of these cells that had been in 10% CS in the primary assay, but not in secondary assays of those that had been in 2% CS or 5% CS. By contrast, the optimal CS concentration in the primary assay of the CS cells of both experimental sets for producing foci in the secondary assays was 5% CS; and the optimum for the FBS cells in the primary assay for maximal focus production in secondary assays was 2% CS. This suggests that the more transformed the cells are the lower the serum concentration required in the primary assays to elicit or advance focus formation in the secondary assays.

Effects of Long-Term Passage in CS or FBS on Focal Morphology in Secondary Assays. Fig. 4 shows the morphology in secondary assays of cells after various lengths of time in frequent passage in 10% CS or FBS. The secondary assays in 2% CS used cells that had spent 14 days in 2% CS in primary assays, so the photographs represent cells that had all been in the same medium of 2% CS for a total of 28 days. The cells passaged in 10% CS for 27 days produced light foci when 10^5 cells were assayed. After 52 days of passage in 10% CS, there were large dark foci and small light ones. After 101 days, only small foci were produced, and at later times, no foci were produced.

When 10^5 cells were passaged in 10% FBS for 27 days, many more foci and denser foci were produced than when 10^5 cells from parallel cultures were passaged in 10% CS. There were, in addition, many light foci in the FBS cultures. Subsequent assays of FBS-passaged cultures had so many transformed cells that they had to be diluted to 10^3 cells and seeded with 10^5 nontransformed cells to obtain discrete foci. When this was done, after 52 days of passage, there was a mixture of small dark and small light foci. After 101 days of passage, some large dark foci appeared in the secondary assay, as well as the small dark and small light foci previously noted. In later assays there was a predominance of large dark foci, although light foci of various sizes were discerned between the dark ones.

In subsequent assays out to 213 days, the cells passaged in 10% CS produced few or no foci in secondary assays after primary incubation in 2% CS whereas the cells passaged in 10% FBS continued to produce large dark foci. The results illustrate the radical difference in cellular response to confluence in 2% CS depending both on whether the cells had been passaged in CS or FBS and on how long the passages had been carried on.

Differences in Foci of Primary and Secondary Assays of Cells Passaged Separately in Two Lots of FBS. Cultures passaged in two lots of FBS continued to make foci in both primary and secondary assays (Fig. 5). However, there were both dense and light foci in the primary assay of cells passaged in FBS-A, but there were only light foci in the primary assay of cells passaged in FBS-B. Most of the foci in the secondary assays of FBS-A cells were larger and denser than any foci in the primary assay. Most of the foci produced in the secondary assay of FBS-B cells were small and dense. There remained some light foci in the secondary assays of both sets of cultures. Once this pattern was established, it remained constant in subsequent assays, indicating that an equilibrium had been established between the physiological state of the cells and the treatment to which they had been repeatedly exposed. The results illustrate the diversity and short-term progression of focus-producing cells in two batches of sera

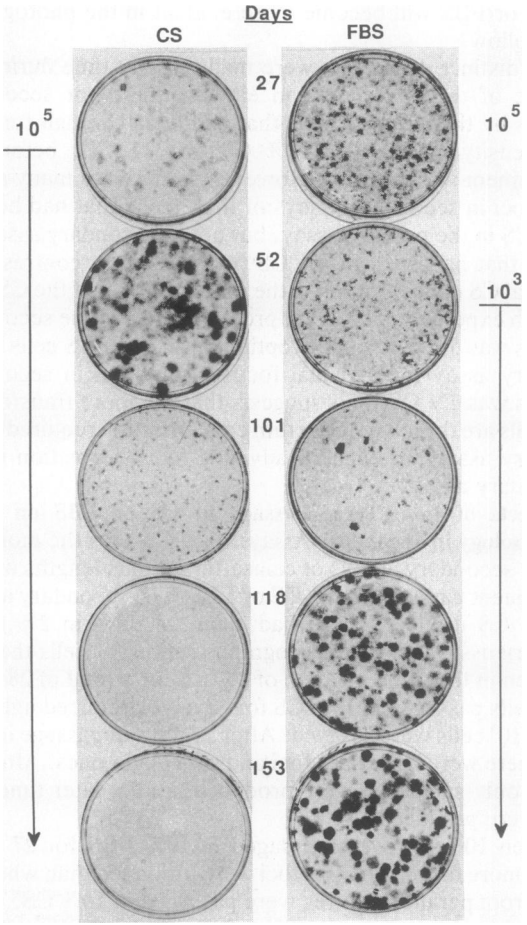

FIG. 4. Effects of long-term passage in CS or FBS-A on the morphology of foci in secondary assays. This series shows the secondary assay in 2% CS of cells that had been in 2% CS in the primary assay. The number of days of passage preceding the primary assay is shown between the two sets of dishes. Cells had been passaged in 10% CS (left column) and in 10% FBS-A (right column). Note that all the secondary assays of the CS passages were of 10^5 cells, whereas only the earliest secondary assay of the FBS cells used 10^5 cells, the later assays using only 10^3 cells plus 10^5 nontransforming cells to form a confluent lawn. Note also that, regardless of the serum used in passages, the cells of all the cultures shown had been in medium of the same composition of 2% CS for two assays, or a total of 28 days, before fixation and staining. Thus, the differences in focus formation represented a stable difference produced by their prior passage history in 10% CS or 10% FBS-A.

originally assumed to be equivalent in their growth-promoting activity.

Occasional Foci of Diverse Morphology in Cells Passaged in CS. After long-term passage in CS, the cells no longer made foci in primary assays in 2% CS, but some scattered small dense foci were formed in 5% and 10% CS (Fig. 6). Usually the cells that had been kept in 2% and 10% CS in the primary assay produced no foci in secondary assays. Occasionally, foci did appear in secondary assay of cells that had been in 5% CS in the primary assay. In the assay shown in Fig. 6, however, foci appeared in the secondary assays of cells from all three CS concentrations in the primary assay. The striking feature of the foci is that they differed in appearance depending on the concentration of CS in the primary incubation. Those that had been in 2% CS in the primary assay without producing foci produced large fuzzy foci in the secondary assay. Those that had been in 5% CS in the primary assay gave rise to many large fragmented foci in the secondary assay. And those that had been in 10% CS in the primary assay produced an extremely large dark focus in the secondary assay. The foci of the secondary assays from all three serum concentrations used in the primary incubation differed markedly from the compact dense foci produced by cells that had been passaged in FBS. The results show that different focal morphologies were produced by cells that differed only by a single passage in different concentrations of the same CS.

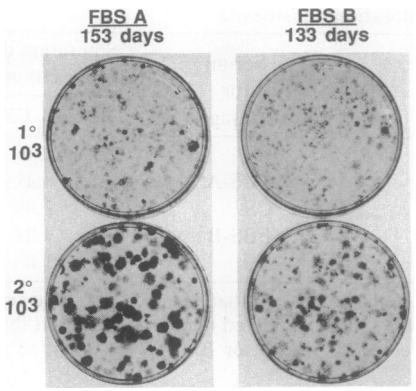

FIG. 5. Differences in foci of primary and secondary assays of cells passaged in different lots of FBS. Cells were passaged in FBS-A or FBS-B for 153 or 114 days, respectively. Primary (1°) and secondary (2°) assays were carried out in 2% CS with 10^3 cells plus 10^5 cells from a line no longer producing foci in either assay. Note the increase in size and/or density of foci in passing from primary to secondary assay in each case and the difference in foci between cells that had been passaged in FBS-A and FBS-B.

DISCUSSION

The results show that cells can follow either of two divergent pathways over a period of 3–4 months with regard to transformation. If they are kept at a maximum rate of multiplication by frequent low-density passage in a relatively high concentration of CS (10%), they gradually lose their capacity to produce transformed foci, even after enhancing that capacity by a preliminary incubation under the selective constraint of confluency. Focus formation is not an all or none phenomenon. The foci become smaller and smaller in successive tests after increasing number of passages, finally grading into undetectability.

If they are passaged on the same schedule and at the same density but are maintained in 10% FBS, which decreased

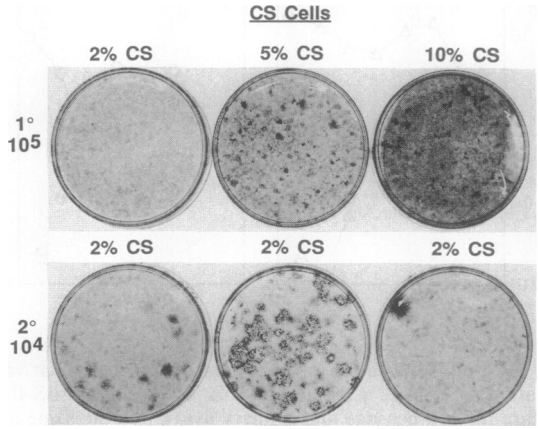

FIG. 6. Occasional foci of diverse morphology in cells passaged in CS. Cells were passaged for 96 days in 10% CS and a primary (1°) assay was done in 2, 5, or 10% CS with 10^5 cells. At 14 days one culture was fixed and stained (upper row) and 10^4 cells from a sister culture were transferred along with 10^5 nonfocus producers into 2% CS for the secondary (2°) assay (lower row).

their growth rate by ≈20% relative to that in CS, the capacity for focus formation gradually increases in successive tests as measured by both the proportion of cells producing foci and the size and density of the foci. The capacity for focus formation reaches a maximum in about the same time of 3–4 months that parallel passages in CS completely lose that capacity. The size and density of foci can be considered a measure of the degree of transformation since they represent the degree to which the cells continue to multiply when surrounded by contact-inhibited nontransformed cells. As the number and size of large dense foci increase, the proportion of light foci decreases, but some heterogeneity remains. The continued appearance of some light foci may result from partial reversion of some highly transformed cells during passage or progression of some less-transformed cells in the postconfluent state. The heritability of the differences in transformability and transformation depending on the serum type used in passage is emphasized by the fact that the focal assays are all carried out in equivalent concentrations of the same serum, namely, CS. Indeed, the secondary assays are made after 14 days in primary assay in equivalent CS concentrations and are counted after an additional 14 days in 2% CS. Furthermore, the large dense foci contain $>10^4$ cells, so the initiating cell must undergo >13 divisions during development of a focus. The conventional explanation for spontaneous transformation associated with growth at high density is selection (10), presumably meaning selection of spontaneously occurring genetic variants, since no other type of selection had been suggested at that time. Our analysis, however, indicates that selection occurs but that it is not primarily of spontaneously occurring mutants. One reason is that no cells capable of producing the large dense foci appear in early passages in either serum or in any passage in CS, so there are no spontaneous variants of this type from which to select. In fact, after prolonged passage in CS, cells lose the capacity to produce any type of focus in either primary or secondary assay, indicating that focus formation is dependent on the physiological state of cells when they are subjected to certain constraints. We have also shown that the capacity for focus formation is, in principle, reversible in populations (7) and in cells isolated from foci (11) of transformed cells. The latter demonstration is particularly significant, since the foci originate from single cells and, therefore, represent clones. Furthermore, the reversal is gradual, as is the degree of transformation, so the foci get smaller and smaller when cells are assayed after successive unconstrained passages in CS. The phrase "in principle" should be emphasized, since we cannot rule out the possibility that mutations and chromosomal changes occur after many passages of cells under mild constraint and that these will stabilize the transformed state. Certainly, such changes eventually occur in genetic assimilation of phenotypically induced changes of *Drosophila* that mimic specific mutations (12). However, the basic process of transformation is more like the Dauermodifikationen [enduring modifications] induced in *Paramecium* (13), which are heritable for many generations but reversible upon mating or autogamy (14).

This laboratory has proposed that transformation occurs by a process designated progressive state selection (7). The term "state" signifies the integrated metabolic pattern of the entire cell, which neither excludes nor requires genetic change for transformation to occur. The proposal is based on the observation of an extremely high rate of phenotypic variation in the capacity for focus formation, so that every clone may be unique (7). Other related properties, such as morphology and capacity to multiply in agar (15), also vary at a high rate. I propose that physiological constraints on growth select for those states that are more efficient for functioning under the constraint and that such selection may involve most if not all cells in a population. An immense number of states is presumed to become available at each stage of progression. The successive traverse of many such states confers a degree of heritable stability to the altered phenotype. The process resembles the principle of creative selection originally proposed by Elsasser (16), with the qualification that the observable diversification of transformation is recorded at the level of the cellular phenotype rather than that of the molecular composition of the cell and organism, and the effects are cumulative.

Progression to the malignant state in the intact organism (2) no doubt involves different conditions than those of cell culture, but the basic principle of state selection may be the same. Cells are maintained in an orderly state in the organism by a hierarchy of organizational controls (17). If the hierarchical controls are persistently disrupted as they are by carcinogens or by alterations in physiological state such as aging, conditions are created for state selection. If the disruption is severe enough, or greatly prolonged, progressive state selection can lead to malignancy.

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