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Cellular epigenetics: Control of the size, shape, and spatial distribution of transformed foci by interactions between the transformed and nontransformed cells

(adaptive change/NIH 3T3 cells/neoplasia/morphogenesis)

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ABSTRACT **NIH 3T3 cells that are passaged frequently at low density in high (10%) calf serum lose their original capacity to produce transformed foci on a monolayer of nontransformed cells. They can then be used to form a monolayered background for the assay of the number of focus-forming cells from a transformed population. Continuation of the low-density passages for many weeks gives rise to a population that can suppress the full development of foci by a transformed line. The suppression appears to occur only after the background cells have become confluent and contact inhibited. It can also cause the disappearance of light foci that had developed before suppression began. Another subline of cells that were passaged at cloning density only once a week lose their focus-forming capacity more slowly than those passaged thrice weekly. When used as a background for the assay of a transformed line, they permit continuous expansion of the foci, with no sign of suppression. Not only the number and size of foci but also their detailed morphology is influenced by the background on which they are formed. A suppressive background can also determine the spatial distribution of foci, presumably as a result of gradients in local cell density of the background. The permissiveness of a nontransformed cell population for focus formation by transformed cells appears to be related to the capacity of the nontransformed population itself to undergo transformation when exposed to the constraints used to induce transformation. These findings indicate there are many degrees of capacity to suppress focus formation and to overcome suppression. They have significance for tumor development and for the epigenetic interactions of normal development.**

To determine the number of cells capable of forming transformed foci in a population containing many such cells, it is necessary to dilute the population and seed them together with a large number of cells that produce no foci. The reason is, of course, that the presence of many foci in a restricted space results in overlaps or confluent transformation, which defies precise quantitation of the number of transformed cells in the original population (1, 2). The addition of an excess of nontransformed cells is to provide a confluent background to exhibit the focus-forming capacity of the transformed cells by their capacity to continue multiplying when the background cells have stopped. This was the procedure followed in charting the course of changes in the competence of cells for transformation over many passages (3). It became apparent that the foci formed on the background in successive weekly assays of a transformed population were becoming fewer and smaller. This could have resulted from a reduction of the focus-forming capacity by the transformed cells or from an increased suppression of focus formation by the background cells that were passaged in parallel with the transformed cells. Using background cells that had undergone fewer passages than the standard cells then used for that purpose, or cells that had been passaged on a different regimen, it became evident that different non-focus-forming populations had different capacities to permit or to suppress focus formation by transformed cells. The presence of background cells also influenced the spatial distribution as well as the size of the foci produced by some cells. Both the morphology and spatial distribution of the foci varied with the passage history of the transformed cells. The significance of these effects for normal and neoplastic development is considered.*

MATERIALS AND METHODS

Cells and Culture Methods. Cells were all from the same stock of NIH 3T3 cells (4) obtained from S. A. Aaronson (National Cancer Institute). They were designated by the order in which they were thawed from the same cryopreserved stock—i.e., SA'17, -26, -27, -28, or -29. Numerical subscripts were used for the SA'17 subline to indicate the sequence in which it was refrozen; subscript letters indicate successive thaws of the refrozen cells—e.g., SA'17_3, 17_{3b}, 17_{3c}. The letter H, M, or L was appended to sublines SA'26, -27, -28, and -29 to denote the regimen used in the passage in 100-mm culture dishes (Falcon) of each subline (high, medium, or low density passage) (3). Briefly, H cells were passaged three times a week at 1–2 × 10^5 cells per dish; M cells were passaged three times a week at 2–4 × 10^4 cells per dish; L cells were passaged once a week at 400 cells per dish. Routine passages were made in molecular, cell, and developmental biology medium 402 (MCDB 402) (5) with 10% calf serum (CS), using the same batch of CS throughout. The SA' prefix is omitted in the text where it seemed dispensable.

The cells were assayed for focus formation in 60-mm dishes using MCDB 402 with 2% CS. In the present case, 10^5 cells were incubated for 2 weeks in a primary (1°) assay with two medium changes per week. They were then treated with trypsin, counted, and used in a secondary (2°) assay. The counts were recorded as the saturation density. In studying the effect of background cells on the development of foci, 10^2–10^4 cells from a 1° assay of a focus-producing subline were mixed in suspension with 10^5 non-focus formers and seeded in a 2° assay. The non-focus formers produced a flat monolayered background to contrast with the more crowded, frequently multilayered focus formers. Both 1° and 2° assay mixtures were incubated in MCDB 402 plus 2% CS for 2 wk, fixed with Bouin's reagent, and washed with pH 7.2 Tris/saline buffer. They were stained overnight with 4% Giemsa

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Abbreviations: CS, calf serum; 1° assay, primary assay; 2° assay, secondary assay; 3° assay, tertiary assay; H, M, and L, high, medium, and low density passages.

*This is paper no. 3 in a series on cellular epigenetics. Paper no. 1 is ref. 3. Paper no. 2 is ref. 21.

stain in pH 7.0 phosphate buffer, washed with tap water, and air dried; the foci were counted over a light box. In the experiment of Fig. 1, one of each of the 2° assay dishes on different backgrounds was treated with trypsin, counted, and used for a tertiary (3°) assay on a common background. Early passages of the M and L regimens were used for focus formation and late passages were used as backgrounds. Cells of the H regimen were used only for focus formation. The procedure for obtaining the saturation density of cells is in the legend for Table 3.

RESULTS

Effect of Background Cells on Expression of Focus-Forming Capacity of SA'28 H Cells in Successive 2° Assays. Cells of the SA'28 H passage regimen that had been used in 1° assays were at first tested in 2° assays only on a background of SA'17_{3c} cells (Table 1). Beginning at the third week, the 2° assays of the 28 H cells were done with a background of cells from several different passage regimens. The 17_{3c} background cells tended to reduce the size of the 28 H foci to such an extent that there was sometimes a marked reduction in the number of countable foci relative to the counts on other backgrounds. The 28 L cells were the most permissive backgrounds for multiplication of transformed 28 H cells as indicated by large and numerous 28 H foci, especially after the 8th wk of 28 H passage. By the same criteria, the 27 M and 28 M cells were more permissive than the 17_{3c} cells but less so than the 28 L cells.

Although the 28 H focal count and size in the 2° assays gave a rough idea of the permissiveness of the background cells for the multiplication and expression of transformed cells, a more precise accounting was sought. This was achieved by trypsin treating the cells from 2° assays on various backgrounds and doing a 3° assay of all the variations on a common background of 27 M cells. The results are shown in the photograph of Fig. 1 and in Table 2. The 17_{3c} cells almost completely suppressed visible focus formation in 2° assays of the 28 H cells except for a few barely detectable foci, while the 27 M and 28 L backgrounds were progressively more permissive (Fig. 1 *Left*). When a 3° assay was done from all three of the 2° assays on a common 27 M background, there were 10 times as many foci produced by cells derived from the 27 M and 28 L backgrounds as from the 17_{3c} background (Table 2). However, the size and general morphology of foci

Table 1. Focus formation in successive 2° assays of SA'28 H cells on backgrounds of different non-focus-forming sublines

Time of passage of SA'28 H, wk	Background population, % and size of SA'28 H foci			
	17_{3c} (92)	27 M (20)	28 M (2)	28 L (2)
0	4.0 S			
2	4.5 S			
3	2.2 S	4.5 S,M		
6	0.2 T	0.8 L		>0.85 VL
8	0.07 M	0.25 M	0.54 M	0.24 M
12	0.3 T	4.7 L		8.4 VL
14			1.1 S	4.2 VL
17*	2.5 S		2.9 M	2.6 L
20	1.2 T			4.1 L
21	0.7 S		0.6 M	1.0 L
22	1.5 T		1.8 T	
24			1.1 S,M	1.3 M,L

Numbers in parentheses in headings indicate weeks of passage of the background cells at the start of the experiment. Add weeks in left column to determine weeks of passage for background cells on that test. Foci: T, tiny (<1 mm); S, small (1–2 mm); M, moderate (2–3 mm); L, large (3–5 mm); VL, very large (>5 mm).
*Foci counted at 8 days instead of 14 days.

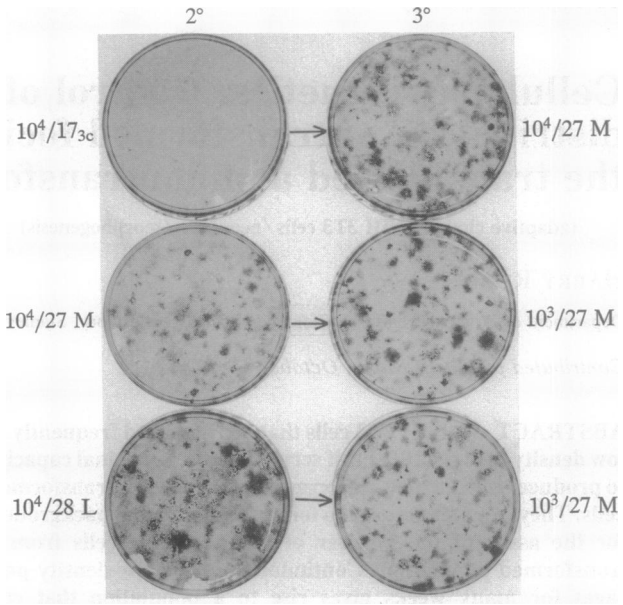

FIG. 1. Permissiveness of various background cells for expression of foci by SA'28 H cells and quantitation of the number of transformed cells in the foci. A 2° assay was made of 10^4 28 H cells (6 wk in passage) on backgrounds of SA'17_{3c}, -27 M, and -28 L cells. At 14 days, the dish on the left was stained and another dish was treated with trypsin; a 3° assay was made from all the 2° assays on a 27 M background and is displayed on the right. The number of cells from the focus-forming population is in the numerator, and the identity of the background is in the denominator. Note that 10^4 cells were used in the 3° assay of cells from the 17_{3c} background while only 10^3 cells were used in the 3° assay of cells from the 27 M and 28 L backgrounds. See Table 2 for counts of foci and weeks of passage of background cells.

on the 3° assays were similar to each other, indicating that no lasting change had been imposed on the transformed 28 H cells by the suppressive 17_{3c} background used in the 2° assay. The heterogeneity of focal morphology even within individual culture dishes is strikingly apparent in Fig. 1 and indicates the wide variety of degrees and kinds of transformation.

Saturation Densities of 2° Assays of 28 H Cells on Different Backgrounds. In 2° assays of 28 H cells on 28 L backgrounds as in Fig. 1, the cells in the large, densely growing 28 H foci are likely to greatly outnumber the number of nontransformed cells in the flat monolayers that form the background. While focus formation is a property of a fraction of the population, the permissiveness of the background cells is likely to be a property of a majority of those cells since they surround the focus formers. Cell counts have the advantage of greater objectivity (but lower sensitivity) than the counts of foci, particularly when there is great heterogeneity among the foci. When cells of the 28 M and 28 L regimens lost their

Table 2. Percentage of transformed cells detected in SA'28 H 2° assays on cellular backgrounds of different permissiveness by reassay on a common background

Background of 2° assay	SA'28 H foci, % and size of foci	
	2° assay (different backgrounds)	3° assay (27 M background)
SA'17_{3c} (98 wk)	0.2 T	1.02 L
SA'27 M (28 wk)	0.82 L	9.5 L
SA'28 L (8 wk)	>0.85 VL	11.8 L

The 2° assays were made of 10^4 28 H cells that had been passaged under the H regimen for 6 wk; 17_{3c}, 27 M, and 28 L cells were used as backgrounds. Then 3° assays were made of 10^3 and 10^4 cells from each 2° assay background category using 27 M cells as the sole background. Focal sizes T, L, and VL are as in Table 1.

Table 3. Saturation densities of 2° assays of SA'28 H cells on backgrounds of varying capacities for suppression

	SA'28 H cells × 10^{-6}			
	10 wk		17 wk	
Background cells	10^3	10^4	10^3	10^4
SA'17_{3c}	ND	ND	0.95	3.6
SA'28 M	0.22	4.7	2.1	2.4
SA'28 L	5.0	4.0	3.1	3.7

ND, not done. The 2° assays were made of 10^3 and 10^4 SA'28 H cells after 10 and 17 wk of passage with the indicated background cells. Cultures containing only the background cells were also made. Cells in all cultures were counted at 14 days, and the counts from the background-only cultures were subtracted from the mixed cultures to give the counts of SA'28 H cells shown. The times in passage for the background cells for the assays of the 10- and 17-wk SA'28 H cells were as follows: SA'17_{3c}, ND and 109 wk; SA'28 M, 12 and 19 wk; SA'28 L, 12 and 19 wk.

capacity for focus formation, their confluent monolayered sheets were not clearly distinguishable from one another. To determine whether they differed in permissiveness for focus formation by 28 H cells when they first lost their own capacity for focus formation, the early non-focus-forming passages of the 28 M and 28 L cells were used as background for 2° assays of different numbers of 10-wk 28 H cells. The results in Table 3 (10-wk cells) show that the "saturation densities" of 28 H cells from assays of 10^4 cells were equally high on both 28 M and 28 L backgrounds. By contrast, when only 10^3 28 H cells were assayed, the 28 M cells suppressed their growth, and the 28 L cells did not.

The same test was made 7 wk later, but this time the 17_{3c} cells were included as an additional background test. Table 3 (17-wk cells) shows that only the 17_{3c} cells were suppressive, and then only to the lower number of 28 H cells. Fig. 2 shows the appearance of the foci on the three backgrounds on the 8th day of the assay before most of the foci on the 28 L background had become confluent with one another, as they had by 14 days in Fig. 1. At this early point there is little to distinguish the foci on the 17_{3c} background from those on the 28 M background. Since the saturation densities (Table 3) and appearance of the foci (Fig. 1) are markedly different on these two backgrounds at 14 days, their similarity at 8 days (Fig. 2) suggests that the suppressive effect of the 17_{3c} cells is exerted late in the development of the foci, after the background cells have become confluent and suppressive to their own growth.

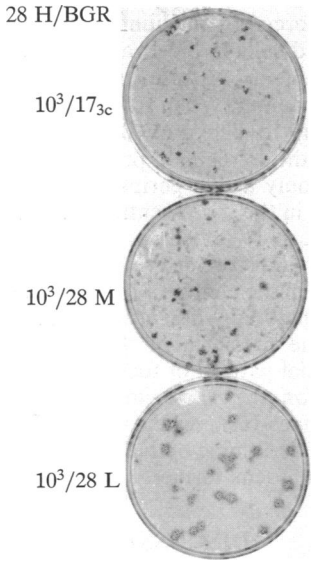

FIG. 2. An abbreviated 2° assay of 10^3 SA'28 H cells (17 wk in passage) on different backgrounds (BGR): relation of focus morphology to saturation density in Table 3. These cultures were stained at 8 days instead of the usual 14 days to minimize overlapping of foci on the 28 L background and show their developing features. Sister cultures were incubated further to 14 days and were counted to determine the saturation density of the 28 H cells on the different backgrounds as enumerated in Table 3.

The Fading Out of Foci Under Conditions of Suppression and Their Continuous Expansion Under Permissive Conditions. It was noted in the experiment of Fig. 2 that foci at 8 days on a 17_{3c} background were as numerous as on a 28 L background, but only a few of the former continued to increase in size (data not shown). Fig. 3 shows that light foci appearing early on a suppressive 17_{3c} background can even disappear with further incubation. Note, however, that two of the foci had become denser with longer incubation, indicating a selective effect of the 17_{3c} suppression. A markedly different fate of foci produced by the same 28 H cells is seen on backgrounds of 27 M and 28 L cells. Most of the foci present at 10 days on the 27 M and 28 L backgrounds continue to thicken and enlarge through 14 days. Although the foci on the 27 M and 28 L backgrounds share this capacity for continued expansion, the morphology of the foci differs; they are denser and more compact on the 27 M background, broader and more fragmented on the 28 L background.

How Soon After Starting Cells on Passage in Different Regimens Do They Diverge in Focal Morphology? Although it is known that cells passaged under the M and L regimens eventually lose their capacity for focus formation in the 1° assay, it is not known whether this loss is preceded by a change in focal morphology or, if so, how soon change occurs. To answer these questions, freshly thawed cells of

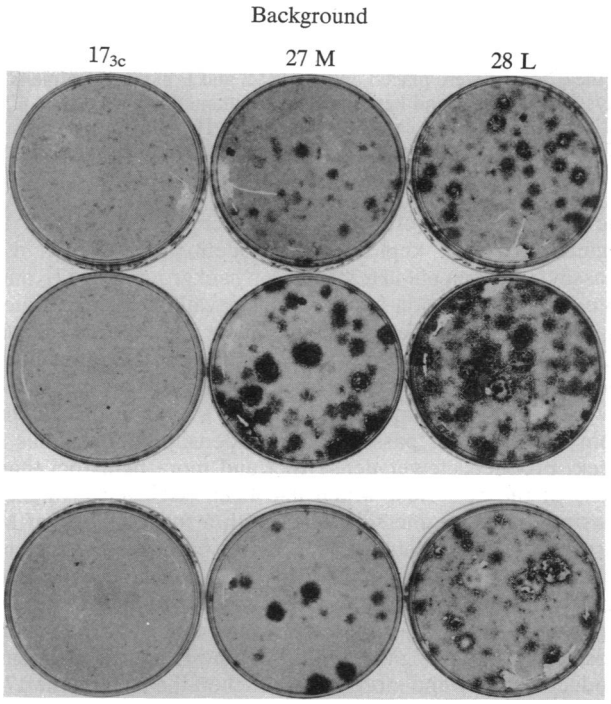

FIG. 3. Disappearance of early appearing light foci on a suppressive background contrasted with continuing expansion of foci on a permissive background. The 2° assays were made of SA'28 H cells (12 wk in passage) on SA'17_{3c}, -27 M, and -28 L backgrounds. The 10^3 cell seedings of the 28 H cells on backgrounds were stained at 10 and 14 days (top and middle rows), while the 10^2 cell seeding was stained only at 14 days (bottom row). Note the disappearance of many light foci between 10 and 14 days on the 17_{3c} background, in contrast to the continuing enlargement of most foci on the 27 M and 28 L backgrounds.

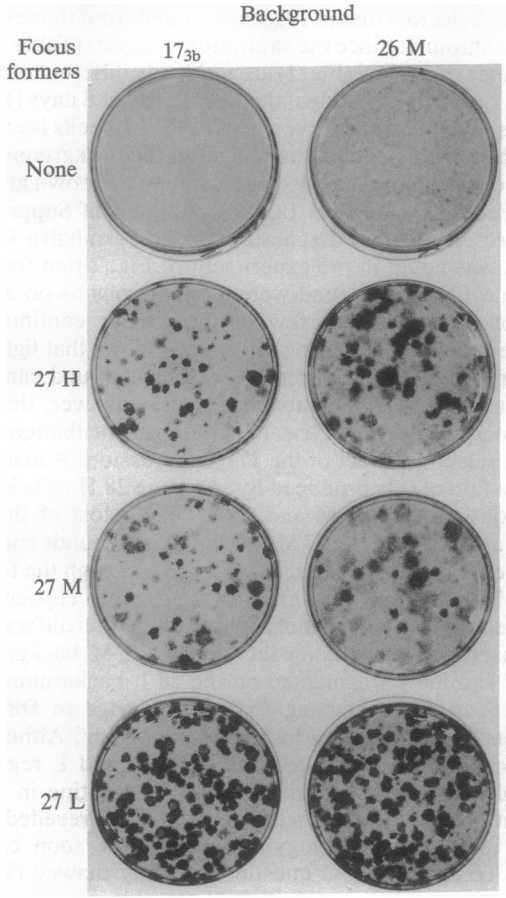

FIG. 4. Early divergence of focal morphologies in 2° assays of SA'27 cells after 1 week of passage under different regimens. Cells of the 27 series were placed on the H, M, and L passage regimens 2 days after thawing and kept on those regimens for 1 wk. After a 1° assay of 10^5 cells without background cells, 10^3 cells from the assay of each passage regimen were put on 2° assay with backgrounds of 17_{3b} (105 wk) or 26 M (8 wk) cells.

the SA'27 series were passaged under the H, M, and L regimens for 1 wk, kept 2 wk under identical conditions in a 1° assay, and then put in a 2° assay on backgrounds of 17_{3c} and 26 cells, the only non-focus formers available at that time. Fig. 4 shows that differences in numbers and types of foci on a 2° assay were already evident after 1 wk of passage under the three regimens, even though all of them had been kept for 2 wk under the common conditions of a 1° assay. The cells from the M series, passaged at low densities three times a week, produced fewer dense foci and more light foci than those of the H series, which had been passaged at higher densities on the same schedule. With a further 2 wk of M passage the cells lost the capacity to produce any foci in either 1° or 2° assays, regardless of background cells (see figure 2*a* in ref. 3). Cells of the L regimen in this series produced more of the dense foci and fewer light foci than those of the other regimens. Cells of the H and M regimens produce broader and more diffuse foci on the 26 than the 17_3 background, but foci of the L regimen were much the same on both backgrounds. The results illustrate the sensitivity of focal morphology produced by cells to only 1 wk of passaging the cells under different regimens of seeding density and frequency.

Effects of a Longer Term Change in Passage Regimen on Morphology and Spatial Distribution of Foci on Different Backgrounds. The 27 H cells of the previous section were continued on the H regimen for an additional 5 wk when some were switched to the M regimen for 3 wk and assayed. Those

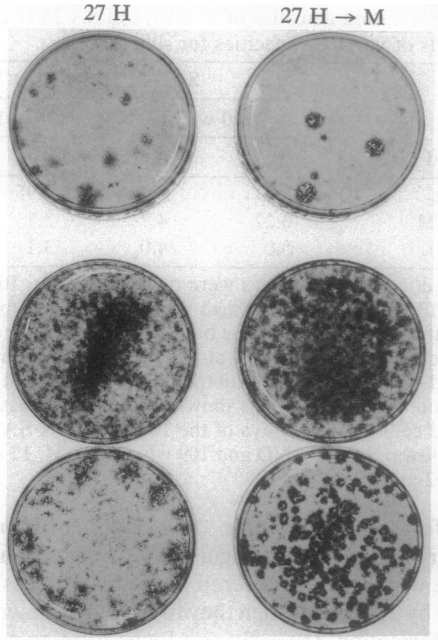

FIG. 5. Effects of longer term change in passage regimen on morphology and spatial distribution of foci on different backgrounds. Some cells of the SA'27 series that had been on the H regimen for 6 wk after thawing were switched to the M regimen for 3 wk. A 1° assay was then done with 10^5 cells (top row) of the cells kept on the H regimen and those switched to the M regimen. A 2° assay was done with 10^5 cells of both (middle row) and with 10^4 cells mixed with 10^5 SA'17_{3b} cells (bottom row) of 91 wk passage.

switched to the M regimen produced fewer but somewhat darker foci in the 1° assay than those maintained on the H regimen (Fig. 5). In a direct 2° assay of 10^5 cells, both sets tended to attach in the center of the dish, although the tendency was stronger in the cells maintained on the H regimen than those that had been switched to the M regimen. This is common in passage of cells that have been constrained by confluence and low serum concentration in the 1° assay and may be reinforced by medium depletion when there are many transformed cells in the 1° assay. These effects result in delayed attachment of trypsin-treated cells, which favors attachment in the center. When 10^4 of these cells were seeded in the 2° assay with 10^5 background cells, those that had been continuously on the H regimen produced large diffuse foci, which occurred only at the periphery of the cultures, with many small ones in the more central areas. By contrast, the cells that had been switched for 3 wk to the M regimen produced smaller and more discrete and compact foci, which tended to cluster in the center of the culture, although not to the degree that they did when seeded alone. It is apparent, however, that the presence of the 17_{3b} background cells changed the spatial pattern of focus formation by cells that had been kept on the H regimen. Thus, the particular combination of focus-forming and the presence or absence of background cells determine both the morphology and spatial distribution of the focus formers.

DISCUSSION

The SA'17 subline of cells, which had been in frequent low density passages for the longest time of those studied, was the most suppressive to the full development of foci initiated by cells of the SA'28 H subline. The capacity to suppress focus formation also increases with the frequency of passage (Fig. 2): cells passaged three times a week were more suppressive than those passaged once a week, although the latter were passaged at 1/100th the density of the former. There appears

to be a relationship between suppressiveness of focus formation of one subline on another and the capacity of the former to form foci after exposure to the constraints that ordinarily induce focus formation. Thus, the strongly suppressive SA'17 subline produced no foci even in 2° assay and the 28 M subline underwent a similar change with time. By contrast, the nonsuppressive 28 L subline, which was passaged only once a week and had lost the capacity for focus formation in a 1° assay, retained the capacity to do so in the more sensitive 2° assay. There is evidence that the capacity for suppression of transformed cell growth by nontransformed cells depends on contact and junctional communication among them (6). This would allow them to share small regulatory molecules, and if the nontransformed cells are in the majority, the transformed cells would be inhibited when the nontransformed cells are as, for example, in the stationary, postconfluent state (7, 8). However, there is not a simple relationship between self-inhibition and suppression of focus formation by another subline. The nontransformed 28 L subline, which is itself subject to contact inhibition at confluence (3), has little or no inhibitory effect on the growth of the transformed 28 H subline in 2° assay. The complexity of the relationship is further illustrated in Fig. 3 where cells of the SA'27 series that had been passaged under all three regimens for only 1 wk make many dense foci on a background of 17_{3b} cells, which had been in low density passage for some 2 years (Fig. 4). This indicates that strong focus formers can overcome the inhibitory effect of a suppressive background. Similar observations have been made with dense sheets of mouse cells that inhibit the growth of cells infected with polyomavirus but not those infected with Rous sarcoma virus (8). Dense sheets of chicken cells fail to inhibit the growth of either (8). The present results show that wide ranges of suppression and susceptibility to suppression are expressed within the same line of cells depending chiefly on their passage history. They also suggest that there is a relation between the resistance of a subline to "spontaneous" transformation and its capacity to suppress the growth of transformed cells from another subline. There may also be a relationship between these properties and the capacity of cells to suppress their own growth as suggested by the observation that the suppressive SA'17 line had a lower saturation density than the permissive SA'28 L line. As noted by others (6–8), the interactions between potential tumor cells and the cells that surround them may play an important role in the development of tumors *in vivo*.

The effect of background cells on the morphology of foci may be a subtle expression of the suppressive effect of the background cells. The morphological modification is reminiscent of those epigenetic interactions in normal development that determine the fate of tissues. Perhaps the best known of such interactions is the observation of Spemann and Mangold (9) in which the dorsal lip of the newt gastrula when transplanted into the ventral region of another gastrula induces the pigmented host cells to form a secondary axis (10). Within the secondary axis is the neural tube, which is induced in ectoderm by the underlying mesoderm, a process referred to as primary induction. It was later found that newt embryo ectoderm was so far predisposed that almost any substance could act as an inducer (11, 12). A more specific role of the inducing tissue in promoting differentiation in an adjoining tissue has been demonstrated in mouse and chicken embryos (13, 14). Explants of limb bud epidermis of the 5-day-old chicken embryo undergo different morphological changes depending on whether it is a combination with mesenchyme from gizzard, proventriculus, or heart of embryos of the same age (14). In the present case, different focal morphologies are presented by 28 H cells on background of cells with different passage histories (Figs. 2, 3, and 5). The background cells influence not only the morphology but the spatial distribution of foci as well (Fig. 5), possibly as a result of differentials in the local density of the background cells. These effects may be related to the spacing pattern of stomata in the leaves of plants (15). Sachs (16) attributes the spacing pattern to a process of "epigenetic selection" of the most appropriate developmental events from an excess of possibilities. Mature stomata were more evenly spaced than potential stomata. The concept of epigenetic selection is similar to that of *progressive state selection*, which was proposed to account for spontaneous transformation under conditions of growth constraint (17, 18). Both these forms of selection operate on fluctuations in the physiological state within all cells rather than the classical selection of rare genetic mutants among cells. They are related to the reaction patterns proposed by Dean and Hinshelwood (19) to account for bacterial adaptation and to the principle of creative selection enunciated by Elsasser (20) in his theory of organisms. This convergence of concepts from different perspectives suggests that the processes of adaptation, differentiation, and transformation can be structured under the single guiding principle of progressive selection among fluctuating metabolic states (17, 18).

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