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Cellular epigenetics: Topochronology of progressive "spontaneous" transformation of cells under growth constraint

(heterogeneity/adaptation/tumor progression)

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ABSTRACT **Early passages of NIH 3T3 cells yield about 10 transformed foci for every 10^5 cells seeded after the cells multiply to confluence in a standardized 2-week assay. The question arose whether more cells would give rise to foci if given more time for their development. This question could not be answered simply by extending the incubation period, since the original foci spread to cover much of the area of the culture dish. Transformed cells can also detach into the medium from the original foci to initiate new foci by reattaching at a distance. These problems were averted by growing cells in multiwell plates which in effect simulated partitioned culture dishes. All the wells in a given plate were assayed for focus formation at successive intervals up to 14 weeks. The results indicated the spatial pattern and sequence of transformation on different parts of the "partitioned" dish. The number of multiwells containing focus-forming cells increased steadily with time, indicating that all parts of a dish eventually undergo transformation. Also, most of the transformations were recorded long after confluence in the multiwells was reached. Hence such a transformation is much more likely to occur in the nondividing state rather than in the dividing state of the cells and is thus inconsistent with a mutational basis. The results suggest that "spontaneous" transformation is a population-wide, epigenetic phenomenon. This agrees with the results from clonal analysis and other studies and is well described by the concept of *progressive state selection*, in which "spontaneous" transformation represents a heterogeneous, adaptive response of competent cells to moderate constraints on cell growth.**

When cryopreserved NIH 3T3 cells are thawed and maintained in confluent cultures for 10 days or longer in a low concentration of calf serum (CS), a small number of transformed foci appear (1–5). The foci are heterogeneous in appearance. They consist of cells which are densely packed and often multilayered, in contrast to the surrounding flat monolayer of cells. If each of the foci represents the progeny of individual cells from the original seeding, they reflect the growth behavior of about 0.01% of the cell population, and their area covers only a fraction of the surface of the culture. Further incubation of the cultures results in an increase in area of the foci so that they cover much of the remaining monolayer. This prevents precise determination of the delayed production of new foci in the monolayer, although their number does not appear to be large. In any case, it would be difficult to determine whether the newly appearing foci arose from newly transformed cells or were secondary foci initiated by cells that detached from the early-arising foci. This problem complicates the interpretation of experiments (6) in which, for example, cells were isolated from nonfocal areas of mouse prostate fibroblast cultures which had formed foci after treatment with a chemical carcinogen. The fact that subcultures from the nonfocal areas eventually formed some foci could have been due to contamination by cells from the original foci.

A method for averting the contamination problem is to subdivide the cultures into many small areas separated by physical barriers. This can be achieved by initially seeding cells in large numbers of small wells and assaying the wells by transferring cells from a representative group of them to large dishes for focus formation at intervals. This procedure not only reveals whether the nonfocal areas had the capacity to ultimately undergo transformation but also provides insight into the kinetics of transformation under conditions of growth constraint. By assessing the changes in morphology of the foci produced by cells assayed from the wells at intervals after their initial seeding, information is gained about progression of the transformed states from light to dense focus formation.

The results of such experiments are presented here. They indicate that all cells—regardless of location—in a culture dish have the potential to undergo transformation if kept long enough under the contact-inhibiting state of confluence. In fact, most of the transformations occurred long after each of the multiwells became confluent. This implies that transformation is associated with crowding when the cells are not dividing and a mutation is least expected. The results also show that the number of positives and their degree of transformation increase with time in the presence of the constraints. All these data reinforce the hypothesis that there is an epigenetic basis for cellular transformation. In terms of the concept of *progressive state selection*, an immense number of states are available for each living cell. An application of moderate physiological constraints—confluence and low serum concentration are considered here—selects those cells in a heterogeneous population that are in states better suited for growth under the constraints. In time, the culture as a whole becomes progressively better adapted to the constraints. This describes well what we observed in the long-term multiwell cultures of NIH 3T3 cells. The term topochronology in the title signifies that the transformation process is examined both in space and in time†.

MATERIALS AND METHODS

NIH 3T3 mouse fibroblasts were used throughout the studies reported below. These cells were originally derived from mouse embryos by successive low-density seedings and isolations of cells from flat areas of monolayer in culture dishes (7). The cells were provided to us by S. A. Aaronson of the National Cancer Institute. Upon arrival, they were given one passage in a serum-enriched medium before transfer to glass vials for cryopreservation in liquid nitrogen. The serum-enriched medium contained, 10% (vol/vol) CS (Hy-

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Abbreviation: CS, calf serum.

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†This is paper 2 in a series on cellular epigenetics. Paper 1 is ref. 11.

Clone) in the synthetic medium MCDB 402 (8). The cells were frozen in serum-enriched medium containing 10% (vol/vol) dimethyl sulfoxide. Vials of cells were later thawed for experiments.

To determine long-term effects of confluence on cells in stationary culture while the total number of cells per sample was kept relatively low, multiwell plates with small flat-bottom wells (Falcon 3072) were used. Stationary culture here means maintaining the cells without transfer but with changes of medium twice weekly. The culture area of the wells used was ≈ 0.32 cm^2, which is about 1/70th that of a 60-mm Petri dish. A seeding of 1400 cells per well in 70 μl of MCDB 402 with 2% CS was made, which gave the same seeding density as 10^5 cells per 60-mm dish. The multiwell plates were then wrapped with Parafilm to prevent evaporation of medium and kept in a moisturized 37°C incubator flushed with 5% CO_2/95% air to keep the medium at pH 7.2–7.4. At various times in each series of experiment, cells from 48 such wells were assayed each time for focus formation.

To prepare for an assay of focus formation, disposable pipettes were used to carefully aspirate medium from the wells to be assayed. Each of the wells was then rinsed with Tris-buffered saline solution, trypsinized, and mixed with 10^5 non-focus-forming cells in the synthetic medium MCDB 402 with 2% CS in a 60-mm Petri dish. This so-called assay dish was then incubated as above and the culture medium was changed twice weekly for 2 weeks. At the end of this period, assay dishes were treated with Bouin's fixative for 1 hr, rinsed with Tris-buffered saline, stained with 4% Giemsa reagents for 1 day, and finally rinsed with distilled deionized water. Dishes were then scored for the number and the morphology of foci.

Four series of experiments were performed. A brief description of each series will be given after the following introduction of notations. Table 1 is a summary of the experimental conditions. SA′ 26 is a shorthand for the 26th thaw from the cryopreserved stock mentioned above. This labeling is necessary, since cells of different thaws were found to behave differently—say, in terms of focus formation. They are to be regarded as sublines of the original NIH 3T3 cells. Starting with the 27th thaw, different passaging regimes were used. SA′ 27M refers to a seeding density of 2–4 $\times 10^4$ per 100-mm Petri dish at these thrice-weekly passages. This passaging regime was used for SA′ 26 as well. For SA′ 27H, a seeding density of 1–2 $\times 10^5$ per 100-mm Petri dish was used. SA′ 17 is from the 17th thaw of the stock that had been maintained by low-density (2–4 $\times 10^4$ cells per 100-mm Petri dish) frequent (thrice weekly) passages at high (10% by volume) serum concentration. They gave no foci in assays for focus formation and were used as background cells which provide an overall seeding density sufficiently high (10^5 per 60-mm Petri dish in 2% CS) to reach confluence (4–5 $\times 10^5$ per 60-mm Petri dish) in 3–4 days.

In series 1, 1400 cells of SA′ 26 were seeded per well. They were assayed on the SA′ 17 background. Series 2 was similar except for the materials used. In series 3, 100 test cells (SA′ 27H) were mixed with 1300 SA′ 17 cells (which were thought to be nontransforming) in each of the wells; the point was to determine whether a large number of test cells was essential for the transformation. Series 4 was similar to series 3 except for the materials used.

Table 1. Characteristics of the four series of experiments

Series	Cells seeded per well	Assay background	Wells per assay
1	1400 SA′ 26	SA′ 17	48
2	1400 SA′ 27M	SA′ 26	48
3	100 SA′ 27H + 1300 SA′ 17	SA′ 17	40
3C	1400 SA′ 17	SA′ 17	8
4	100 SA′ 26 + 1300 SA′ 17	SA′ 17	40
4C	1400 SA′ 17	SA′ 17	8

RESULTS

Cells in 96-well plates that had been confluent in stationary culture for various lengths of time were assayed for focus formation. The stained assay dishes were evaluated on the basis of their appearances (or "phenotypes"). Basically, the number of foci and their intrafocal density—reflected in the darkness of staining—area, and shape determine the appearance of a stained assay dish. To demonstrate cellular progression of transformation, a semi quantitative scale of 5 is found sufficient. Fig. 1 illustrates the scale used. Fig. 2 shows the regular assays in 60-mm dishes of the same cells as those seeded in the multiwells at the start of their corresponding series. Figs. 3–5 represent the basic results of this study.

On each graph in Figs. 3–5, the number of transformed wells per assay is plotted versus the time they had been in stationary culture (confluence was reached at 3–4 days after the initial seeding). Each curve on any one graph records the number of stained assay dishes at or below a particular degree of transformation. For example, the lowest-lying curve of each graph records only the number of very strongly transformed, degree 4 wells. The next curve above records transformed wells of degrees 3 and 4. The top curve on each graph includes wells at all degrees, 1–4, of transformation. An unambiguous trend of more and stronger transformation

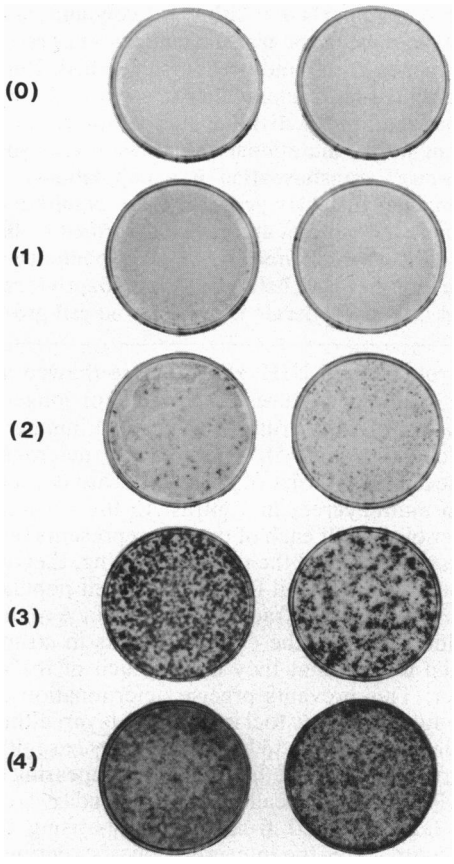

FIG. 1. Scale used in scoring the degree of transformation. Starting from the top of each of the two columns, nontransformed (degree 0), weakly transformed (degree 1), moderately transformed (degree 2), strongly transformed (degree 3), and very strongly transformed (degree 4) assay dishes are shown. Dishes in the left column were all chosen from the 11-week assay of series 3. Dishes in the right column are all chosen from the 10-week assay of series 4.

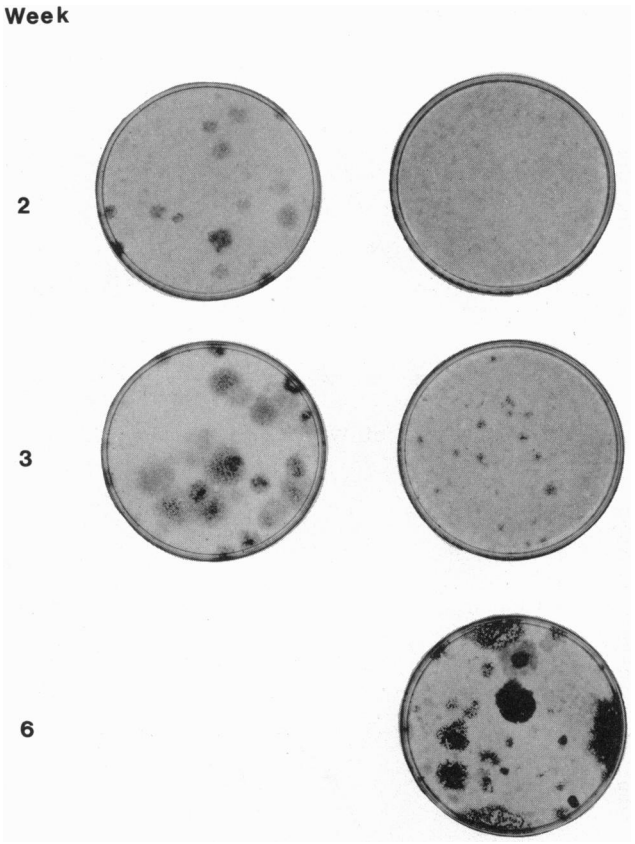

FIG. 2. Regular assays from the same group of cells seeded in the multiwell plates. The column on the left shows regular assays of the materials seeded in the multiwell plates at the start of series 2. The upper dish was stained at 2 weeks, the lower one at 3 weeks. The column on the right shows the assay of cells in the same proportion as used in series 4, but scaled up to the seeding density for the regular assay: 7000 SA′ 27H cells plus 93,000 SA′ 17 cells per 60-mm Petri dish. The corresponding seeding for the multiwells is 100 SA′ 27H cells plus 1300 SA′ 17 cells per well. The top dish was stained at 2 weeks, the middle one at 3 weeks, and the bottom one at 6 weeks.

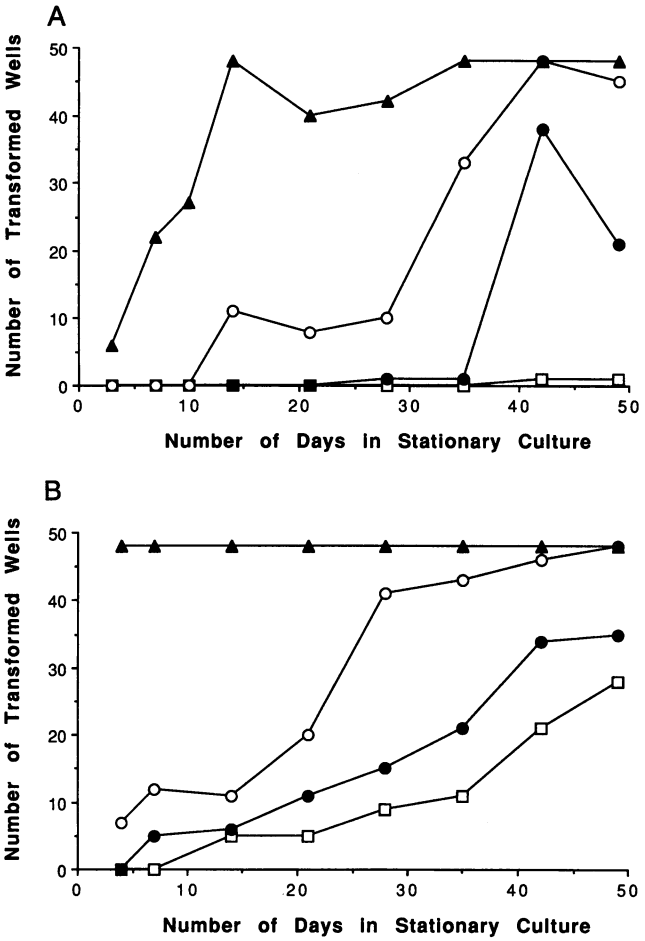

FIG. 3. Transformation in series 1 and series 2. Degree of transformation was as defined in Fig. 1:▲, degrees 1–4; ○, degrees 2–4; ●, degrees 3 and 4; □, degree 4. (*A*) Series 1, 1400 SA′ 26 cells seeded per well. (*B*) Series 2, 1400 SA′ 27M cells seeded per well. Other details are in Table 1.

over time can be seen in each graph. Problems concerning borderline cases in class assignment and the comparison of results across the four series of experiments will be discussed in the next section. Some remarks about the graphs should be made here first.

Fig. 3*A* summarizes experiment series 1. Despite the apparent anomalies at days 14 and the 49, where the trend of monotonic increase in the number of transformed wells starts to reverse, the general trend shown is still that of more and stronger transformation over time. Starting on day 14, most of the wells per assay show at least weak transformation (see the uppermost curve in Fig. 3*A*). A more selective curve, such as the second curve from the top in Fig. 3*A*—which includes wells that show at least moderate transformation, i.e., of degrees 2–4—affords a clearer picture of the progression. However, an even more selective curve in this experiment, such as the bottom one in Fig. 3*A*, which includes only very strongly transformed wells, is not informative about full progression. This category is better represented in the succeeding series maintained. For the cells (SA′ 26) used in this series and over that period of 7 weeks, there were simply not many "very strongly" transformed wells.

Fig. 3*B* summarizes experiment series 2. Note that the SA′ 26 cells were used as background after they had lost the capacity for strong focus formation in the standard 2-week assay. They were known (H. R., unpublished work) to be less suppressive than the SA′ 17 cells when used as a background for focus formation. However, the SA′ 26 cells formed weak foci by themselves, making it difficult to determine whether the weak foci seen came from the minority test cells—SA′ 27M, in this case—or from the majority background cells used in the assay. As a result, the top curve in Fig. 3*B* is not an indicator of progression, since all assay dishes showed at least weak transformation throughout the entire series 2. Progression of the SA′ 27M cells can be seen only by the other three curves in Fig. 3*B*: more and more transformed wells of higher degrees were seen following the course of series 2, but the background controls (the SA′ 26 cells by themselves) in each assay continued to show only weak transformation.

Series 1 and 2 demonstrate that the focus-forming transformation being reported here is a population-wide phenomenon. Nearly all wells per assay manifested moderate to strong transformation toward the end of the respective series. The progression was systematic—the increase in the number and degree of the transformed wells per assay was almost monotonic.

In series 3 and 4, only 100 cells of the types relatively susceptible to transformation were used per well. Thirteen hundred of the more stable (and ideally assumed nontransformable) line SA′ 17 were added to and mixed with the 100 cells to give an overall seeding density of 1400 cells per well. The point here was to lower the lower limit in the number of test cells necessary to reproduce the same phenomenon. Longer-term experiments were planned in anticipation of

using a smaller number of test cells. Figs. 4 and 5 summarize experiments series 3 and series 4, respectively.

The SA′ 27H cells had been routinely passaged prior to use at a cell density higher than that of the SA′ 27M cells. They were thawed at the same time and had undergone the same number of passages. Numerous other observations in this lab have indicated that, of all the cell lines considered in this report, the SA′ 27H cell line would be the most susceptible to transformation. Indeed, the first points at 3 weeks on all four curves in Fig. 4*A* reveal a number of transformed wells from weak (degree 1) to strong (degree 3), but none that was very strong (degree 4). It is likely that there were a number of transformed cells in the staring material. The top curve in Fig. 4*A* shows that most assayed wells were at least weakly transformed throughout series 3. However, progression can still be seen on more selective curves. The number of moderately to very strongly transformed wells per assay fluctuated about a trend of increase after the fourth week. In contrast, the SA′ 26 cells used in series 4 were less susceptible to transformation than the SA′ 27H cells. The SA′ 26 cells had been in culture for a larger number of passages than the SA′ 27 cells. In fact, the SA′ 26 cells had lost their ability for strong focus formation in our standard 2-week assays prior to the start of experiment series 4. It turns out, however, that progression in the SA′ 26 cells (in series 4) under the applied physiological constraints is more easily seen than that in other series reported here. This is evident in Fig. 5*A*.

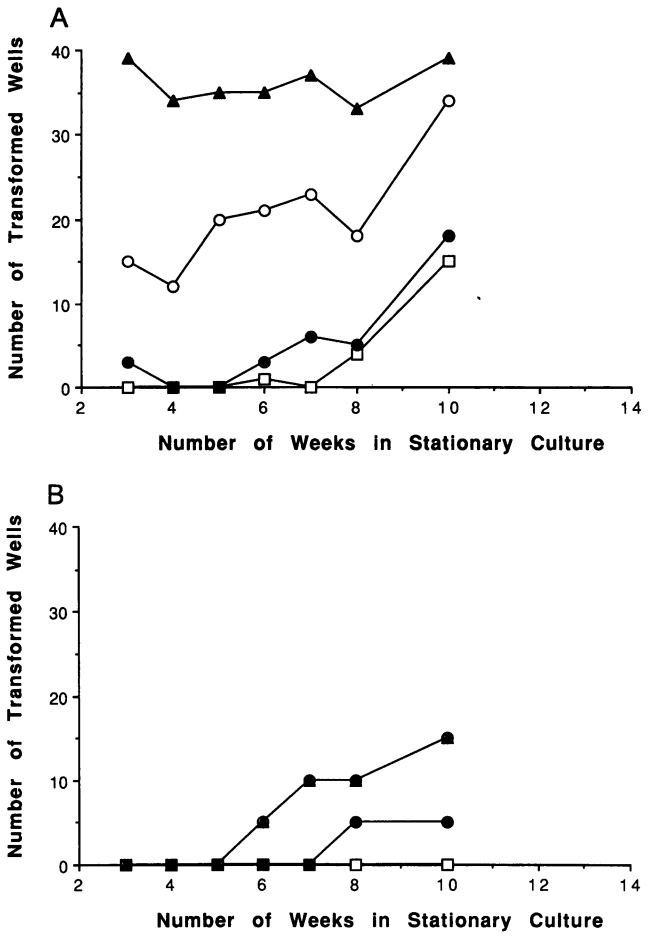

FIG. 4. Transformation in series 3. The notations of Fig. 3 are used here. (*A*) 100 SA′ 27H cells plus 1300 SA′ 17 cells (non-focus formers in the standard assay). (*B*) Control, 1400 SA′ 17 cells, 8 wells per assay scaled up to 40 for comparison with *A*. Cells from none of the control wells produced "very strongly" transformed assay dishes.

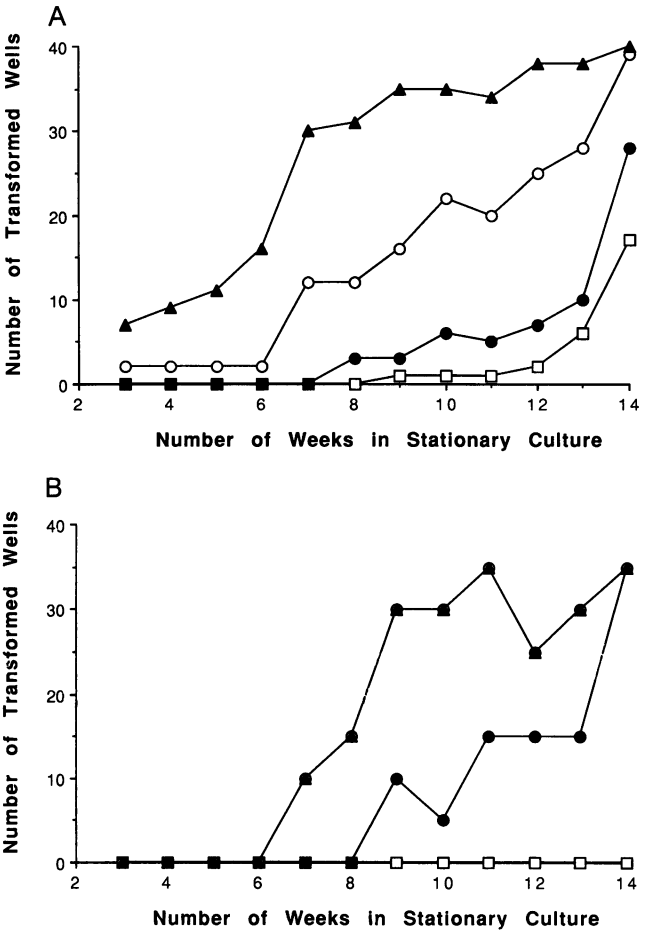

FIG. 5. Transformation in series 4. The notations of Fig. 3 are used here. (*A*) 100 SA′ 26 cells plus 1300 SA′ 17 cells. (*B*) Control, 1400 SA′ 17 cells. Cells from none of the control wells produced "very strongly" transformed assay dishes.

Series 3C and 4C are controls for series 3 and 4. Wells with 1400 SA′ 17 cells were housed in the same plates from which the weekly assays of series 3 and 4 were made. Fig. 4*B* and Fig. 5*B* (the number of wells shown has been scaled up to facilitate comparison with Figs. 4*A* and 5*A*) show that the SA′ 17 cells, which were thought to be least susceptible to transformation, also transformed. However, the degrees of transformation shown in Figs. 4*B* and 5*B* are clearly below that seen in Figs. 4*A* and 5*A*. In particular, a "very strongly" transformed assay dish was never observed throughout series 3C and 4C. This establishes that the major contribution to the transformation seen in Figs. 4*A* and 5*A* had come from the SA′ 27H cells and SA′ 26 cells, respectively, and not from the 1300 SA′ 17 cells added.

DISCUSSION

The classification based on the overall appearance of transformed dishes into five categories, 0–4, was somewhat arbitrary. Details such as the size, shape, density, and number of foci have been coarse-grained. Despite the apparent agreement in the gradation of transformed dishes chosen from two different series at two different assays in Fig. 1, comparison across different series of experiments should be made only with caution. Even within a series, there were borderline cases where a dish could be placed into the next higher category. Uncertainty of this sort has been estimated at about 10% for the boundary between degrees 0 and 1—that between the nontransformed and the weakly transformed

dishes—and ≤ 5% for the 2–3 and 3–4 boundaries. Error bars have been omitted from the graphs for clarity. Also, the inclusive curves – one such as ●—● in Fig. 3, which includes transformed dishes of degrees 3 and 4—did not incur cumulative errors because ambiguity applied, in this case, only at the 2–3 boundary. The aim of the graphs has been to track the global temporal trend of progression which has been unambiguously established in Figs. 3–5.

An interpretation of the above results is given here. In a typical assay for focus formation, a preconfluent 60-mm dish of cells from a standard frequent passage would be left in stationary culture for 2 weeks in 2% CS. The cells would multiply to confluence in 3–4 days, and perhaps 10–20 foci would form in 2 weeks. The results from the multiwells imply that, given enough time, cells of the regions without foci will also transform to produce foci of their own. In particular, the above results say that the smaller foci seen in assay dishes in the presence of larger foci can really be late primary foci of the original population rather than secondary foci derived from detachments of the larger foci. Indeed, the weekly assays of the "partitioned dishes" (of many wells each) over an extended period of time show that nearly all the partitions in late assays manifested transformation. The degree of transformation generally increased with time under the imposed constraints. These results strongly suggest a population-wide progression induced by the applied physiological constraints, the meaning of which is to be refined by the observations described below.

The number of foci seen within individual assay dishes, each derived from a multiwell, increased with time in all four experiments. After the first week, with the exception of some assay dishes that display only weak transformation where small numbers of foci might be hard to detect or to enumerate precisely, all other assay dishes that displayed moderate to very strong transformation contained a rather large number (≥ 50) of foci. In other words, assay dishes that exhibited a clearly defined transformation with a small number (≤ 5) of foci were rarely seen. In addition to this, the foci observed within an assay dish had similar phenotypes in terms of density, size, and shape, but differed visibly from foci observed in other assay dishes. This reveals that the cells of each well, identically prepared at the start, might have taken their own course of progression. The cells responded to the imposed constraints in a very heterogeneous way: some cells adapted sooner than others. It is likely that one or a few cells per well had undergone weak transformation at first. They then multiplied and progressed further. Those advanced (≥ moderate, in the sense of Fig. 1) cells were quickly selected for under the constraints. The large numbers and the similarity of foci seen within individual assay dishes that display transformation can be understood on the basis of proliferation of this sort. The observation here agrees with that of Grundel and Rubin (5), who used a different setup.

The distribution of positive wells per assay indicates that transformation may occur on any part of a culture dish and eventually in every cell in the culture. Furthermore, that distribution allows an estimate of the probability of transformation per cell (ρ) since the onset of confluence. If each positive well arose from one transformed cell, a slightly underestimated ρ is given by the number of positive wells per assay divided by the total number of cells assayed per assay. The latter number is equal to the total number of wells assayed per assay times the number of test cells at confluence inside a well, which is about 7000 for series 1 and 2. A simple calculation base on Figs. 3–5 (counting only moderate transformation and stronger as positive) then shows that ρ changed from 0 (no positive wells per assay) to 1.43×10^{-4} in experiment series 1 and 2. A similar estimate for the longer-term series 3 and 4 shows that ρ changed from 0 to 2.0×10^{-3}. The result here is consistent with the demonstration (2) that prolonged constraint changes the state of all cells so that their descendents have an increased probability for transformation. Similar results have been reported for cells exposed to chemical and physical carcinogens (6, 9). Since most of the transformations were detected here after the cells had reached saturation density, it can be assumed that the process is more likely to occur in nondividing cells than in dividing cells. A similar picture has been reported (10) for diploid liver epithelial cells, with or without carcinogens. Such results are contrary to expectation for a mutation which is most likely to occur as an error of DNA replication. It lends further support to the epigenetic origin of cellular transformation through the proposed process of *progressive state selection (2).*

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