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Cell Biology

The cellular ecology of progressive neoplastic transformation: A clonal analysis

(clonal selection/carcinogenesis/aging and cancer)

MING CHOW AND HARRY RUBIN*

Department of Molecular and Cell Biology and Virus Laboratory, 229 Stanley Hall, University of California, Berkeley, CA 94720-3206

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ABSTRACT **A comparison was made of the competence for neoplastic transformation in three different sublines of NIH 3T3 cells and multiple clonal derivatives of each. Over 90% of the neoplastic foci produced by an uncloned transformed (t-SA′) subline on a confluent background of nontransformed cells were of the dense, multilayered type, but about half of the t-SA′ clones produced only light foci in assays without background. This asymmetry apparently arose from the failure of the light focus formers to register on a background of nontransformed cells. Comparison was made of the capacity for confluence-mediated transformation between uncloned parental cultures and their clonal derivatives by using two nontransformed sublines, one of which was highly sensitive and the other relatively refractory to confluence-mediated transformation. Transformation was more frequent in the clones than in the uncloned parental cultures for both sublines. This was dramatically so in the refractory subline, where the uncloned culture showed no overt sign of transformation in serially repeated assays but increasing numbers of its clones exhibited progressive transformation. The reason for the greater susceptibility of the pure clones is apparently the suppression of transformation among the diverse membership that makes up the uncloned parental culture. Progressive selection toward increasing degrees of transformation in confluent cultures plays a major role in the development of dense focus formers, but direct induction by the constraint of confluence may contribute by heritably damaging cells. In view of our finding of increased susceptibility to transformation in clonal versus uncloned populations, expansion of some clones at the expense of others during the aging process would contribute to the marked increase of cancer with age.**

The common view of chemical carcinogenesis is that the agent in question induces genetic alterations in the treated population that lead to tumor formation. For example, the frequent occurrence of a specific mutation in the H-ras 1 gene in rat mammary tumors initiated by *N*-nitroso-*N*-methylurea (NMU) was at first assumed to result from the mutagenic action of NMU (1). However, it was subsequently discovered that patches of normal mammary epithelium with the H-ras 1 mutation had already occurred spontaneously in the young rats at the time the NMU was applied and that the NMU selected for the growth of the mutated cells that eventually developed into mammary cancer (2). The specific H-ras 1 mutation associated with NMU initiated mammary tumors was absent in mammary tumors that were initiated by dimethylbenzanthracene (1) or that arose spontaneously (2). The results showed that at least some tumors originate through the imposition of conditions that select for preexisting cell variants in normal tissue rather than the induction of oncogenic mutations.

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The original NIH 3T3 line of mouse fibroblasts undergoes spontaneous neoplastic transformation when maintained under the constraint of confluence for extended periods of time (3). The transformation is manifest in the appearance of dense, multilayered foci on a monolayered background of nontransformed cells or an increase in saturation density upon passage of the cultures and proliferation to confluence. The dense foci do not appear in standardized primary (1°) assays at confluence in 2% calf serum, and repeated passage of the original line at low density resulted in development of a subline that required many rounds of prolonged confluence before even light foci appeared. Prolonged confluence, which is necessary for display of the foci, also seemed to be required to induce the transformation because no foci appeared in 1° assays. However, it was possible that transformed cells or their precursors were selected against during low-density passages of the cells and even when present their expression in focus formation was suppressed in the first round of confluence. It was indeed found that some sublines of NIH 3T3 cells did suppress focus formation when they were used to provide a confluent background for transformed cells (4), which also tended to multiply at a lower rate than nontransformed cells in the low-density passages used to minimize transformation in routine maintenance of the cultures (5). These findings raised the possibility that confluence selected for cells in early stages of spontaneously occurring transformation and allowed their progression to more advanced states during expansion of the transforming population. We therefore undertook an extensive clonal analysis of transformation in the original NIH 3T3 cells that were highly sensitive to confluence-mediated transformation, and a relatively refractory subline that resulted from many low-density passages of the original cells (6, 7). Clones were obtained from uncloned cultures of both groups and from a heavily transformed culture of the susceptible line. The susceptibility of the clones to transformation was compared with that of the respective uncloned parental cultures in each of the two nontransformed groups. The proportion of dense and light focus formers in assaying cells from the uncloned heavily transformed t-SA′ culture was compared with the proportion of clones producing either type of foci. A variety of observations were made that indicate that selection plays a major role in confluence-mediated transformation, that there is suppression of focus formation in the uncloned cultures, and that transformation, especially in the refractory group, is far more common in clones than in the uncloned culture from which they came. We discuss the potential relevance of these findings for the age-related incidence of human cancer.

MATERIALS AND METHODS

Cells and Culture Methods. The original line of NIH 3T3 mouse cells (8) was obtained in 1988 from S. A. Aaronson, (Mt.

Abbreviations: NMU, *N*-nitroso-*N*-methylurea; 1°, 2°, . . . , serial transformation assays at confluence for focus formation; MCDB 402, molecular, cellular, and developmental biology medium 402; CS, calf serum; PD/D, population doublings per day.

*To whom reprint requests should be addressed. e-mail: hrubin@uclink4.berkeley.edu.

Sinai School of Medicine, New York). They were passaged once, collected in vials in growth medium plus 10% dimethyl sulfoxide and frozen in liquid nitrogen. More than a decade later, the cells, designated SA′, were thawed and used for the study of neoplastic transformation in the present experiments. The SA′ cells produced no dense foci in a 1° assay at confluence, a few dense foci in a 2° assay, and a very large number in a 3° assay (9). In addition, a subline of the same cells, designated A′, that had been serially passaged 108 times at low density and become relatively refractory to confluence-mediated transformation was used to provide a monolayered background for the display of foci by the transformation-competent SA′ cells. The standard low-density passage of both sublines consisted of seeding 2×10^4 cells per 56 cm^2 plastic Petri dish (Falcon 3003) in molecular, cellular, and developmental biology medium 402 (MCDB 402) (10) containing 10% vol/vol calf serum (CS). After a 3 day incubation period at 37°C, the cells were suspended by treatment with 0.01% trypsin in 0.5 mM EDTA/Tris saline buffer. Cells were counted electronically and reseeded as before.

The cells were assayed (1° assay) for the production of transformed foci or determination of saturation density by seeding 10^5 cells in 21-cm^2 plastic culture dishes (Falcon 3002) with MCDB 402 plus 2% vol/vol CS. The cells reached confluence in 3–5 days and were incubated a total of 14–15 days with medium changes every 3 days. Prolonged confluence promotes transformation in the SA′ cells, which was detected by assaying them in a second round of confluence (2° assay). Progression to more advanced and numerous transformed cells was monitored by further rounds of confluence (3°, 4°, etc., assays). When it seemed likely that the number of transformed foci would be so large that they would overlap, the cells were diluted in 10-fold steps and mixed with 10^5 cells of the transformation-refractory A′ line to obtain discrete, countable foci. At 2 weeks, cultures were fixed with Bouin's reagent and stained with 4% Giemsa buffered at pH 7.0 to highlight the transformed foci, which were counted and characterized over a light box. To determine the saturation density of the transformed populations, cultures originally seeded with 10^5 cells of the undiluted populations were trypsinized and counted electronically. The counted cells were also used in the successive rounds of assay.

When the growth rate of cells from the confluent cultures was to be determined, they were trypsinized, seeded at low density as in the standard passage with MCDB 402 plus 10% CS, incubated for 2 days to recover from contact inhibition, and reseeded at 5×10^3 cells/dish on eight 21 cm^2 Petri dishes with MCDB 402 plus 10% CS. The cells were counted daily for 4 days or at 1 and 4 days to determine growth rate that was expressed as population doublings per day (PD/D). Individual isolated colonies from single cells were formed by seeding 50 cells per dish on the Petri dishes in MCDB 402 plus 10% CS for 6–8 days when they were fixed and stained.

Clonal analysis was initiated by seeding cells in each well of 96-well microtiter plates. Two such multiwell plates were used for the nontransformed SA′ cells, designated here as SA′ cells, that had been continuously maintained by low-density serial passages, and an average of one cell was seeded per well in MCDB 402 plus 10% CS. In anticipation of low plating efficiencies, three multiwell plates were used for cells from a culture that was highly transformed as a result of a 1° assay for 22 days of freshly thawed SA′ cells followed by a 2° assay for 14 days and designated t-SA′. Cells from the t-SA′ culture were seeded at two cells per well directly from the 2° assay (no recovery period) to avoid selection of faster growing nontransformed cells that might be in the culture. Each well was observed twice during the development of the clones between 4 and 7 days to estimate the extent of cell proliferation and the presence of abnormal cells. All were observed again at 8 days; 31 single clones from the t-SA′ culture, and 29 from the nontransformed control SA′ culture were then trypsinized from the wells and picked for expansion in 21 cm^2 dishes. Each clone was serially passaged 5 times at low cell density, 5×10^3 cells per 21 cm^2 dishes, to determine growth rate, and then seeded in a 1° assay for focus formation and saturation density. A 2° assay and a 3° assay were done with all the control SA′ clones and many of the clones from the transformed t-SA′ cultures. Growth rates of all the clones were determined by cell counts of sister cultures of each clone at 1 and 4 days after seeding. Cells that had been through a focus assay received a 2 day passage at low density to recover from the inhibiting effects of confluence before their growth rates were measured. Another cloning experiment under the same conditions was carried out with 27 clones of the transformation-refractory A′ cells that had no previous incubation at confluence. The procedure was the same as that used for the nontransformed SA′ cells.

Aliquots of cells from 5 low-density passages of the t-SA′ and SA′ cells that preceded the first series of assays at confluence were similarly passaged another 6 times (11 passages total) to initiate a second series of assays at confluence with all the clones, and growth rates were determined with small aliquots of cells after each assay. Each series of assays is designated by number as a prefix to the assay number of the series, e.g., 1–2°, 1–3°, etc.; 2–1°, 2–2°, etc. Similar series were done with the A′ cells. The idea of the repeated series of assays and growth rate measurements was to determine how reproducible the results were with each clone, and whether transformation occurred during the low-density passages (i.e., clonal expansions).

RESULTS

Characterization of Cells from the Uncloned Transformed t-SA′ and Nontransformed SA′ Cultures. The focus forming and colony forming capacities of the SA′ cells from the transformed t-SA′ and nontransformed SA′ cultures were determined on Petri dishes at the same time the cells were seeded for clonal isolation in multiwell plates. The focus forming efficiency of the t-SA′ cells averaged ≈11% when seeded with an excess of transformation-refractory A′ cells to form a monolayered background. More than 90% of the t-SA′ foci were of the dense, multilayered type (Fig. 1) whereas no foci of any type were seen in the assay of the A′ cells by themselves. The 1° assay of 10^5 SA′ cells produced no discrete, countable foci but there were some very faint local densities distributed through the culture (Fig. 1) indicative of heterogeneity in growth properties of the cells.

The SA′ cells seeded for colony formation had proliferated to form large distinct colonies during the 8-day incubation period with a relatively high cloning efficiency of 39%. The colonies from the t-SA′ culture were much smaller and lighter than those from the SA′ cultures. Counting all the colonies that were visible to the unaided eye gave an efficiency of 27%, half of which were fragmented as well as small, indicative of damage and slow growth. Cloning efficiencies were also determined from the multiwell plates by the proportion of colonies with 100 cells or more after the 8 days of incubation and gave values of 53% from the SA′ culture and 16% from the t-SA′ culture. Less than 1% of these colonies from the SA′ cultures had two or more abnormally enlarged cells, whereas 63% of the colonies from the t-SA′ culture had such cells. These results showed that most of the cells from the t-SA′ culture were heritably damaged and a high proportion produced dense foci on a background of A′ cells. Cells from such foci give rise to rapidly growing sarcomas in immunologically deficient mice (47).

Characterization of Clones from the Transformed t-SA′ and Nontransformed SA′ Cultures. The growth rate averages for the first five passages of each colony isolated from the multi-

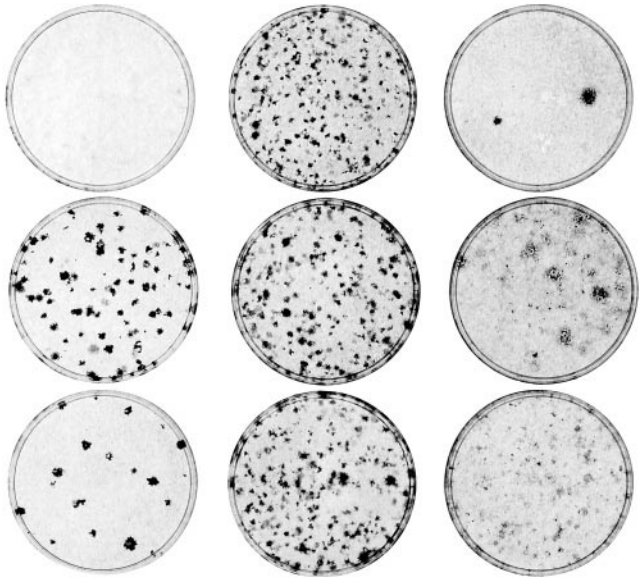

FIG. 1. Morphology of foci produced from the uncloned SA′ and t-SA′ cultures and from clones of the latter. *Left* column: *(Top)* 10^5 cells in a 1° assay of SA′ cells; (*Middle* and *Bottom*) 10^3 and 10^2 t-SA′ cells of the 3° assay. *Center* column: 3 t-SA′ clones that produced dense foci such as those produced by cells from the uncloned t-SA′ culture in the middle and bottom of the left column. *Right* column: 3 t-SA′ clones that produced foci different from those of the uncloned culture, and from each other. (*Top*) 10^3 cells; *Middle*, 10^5 cells; *Bottom*, 10^4 cells. Any assay with $<10^5$ cells was combined with 10^5 nontransformed A′ cells.

wells are shown in Fig. 2. It is immediately evident that most of the clones from the t-SA′ culture multiply at a markedly lower rate than those from the SA′ culture—an overall average of 1.01 ± 0.16 PD/D vs. 1.41 ± 0.18 PD/D, respectively. However, three of the SA′ clones multiplied at a distinctly lower rate than their sister clones, suggesting that, even in the absence of confluence, heritable damage was occurring continually among a small fraction of cells in low-density passages. The saturation densities of most of the clones from the t-SA′ culture were distinctly higher than those from the SA′ culture in the 1° assay after the first five low-density passages (Fig. 3)

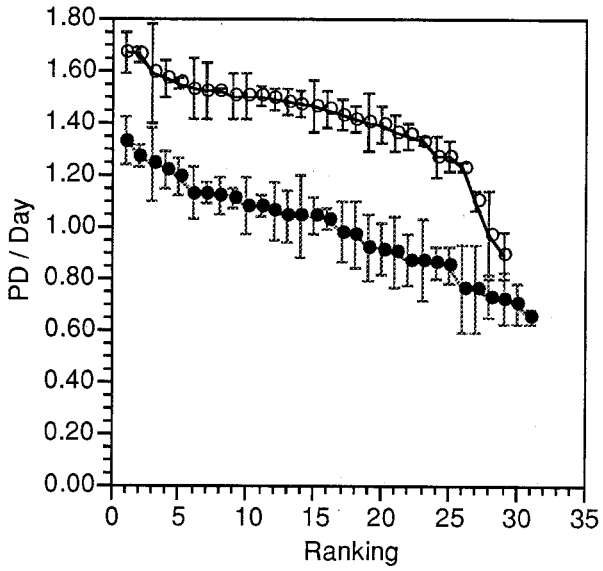

FIG. 2. Growth rates of clones from SA′ and t-SA′ cultures. Growth rates are averaged for the first four low-density passages, and are arranged in rank order of PD/D. ○, SA′ clones; ●, t-SA′ clones.

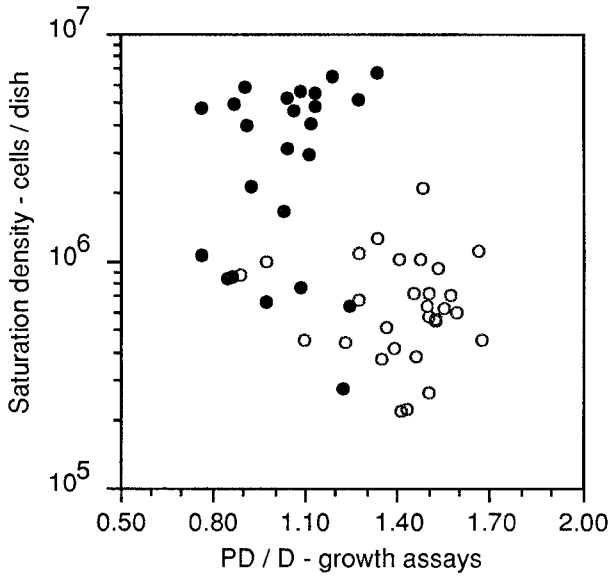

FIG. 3. Relation of saturation density of SA′ and t-SA′ clones in 1° assay to their growth rates averaged for the first four passages. ○, SA′ clones; J, ●, SA′ clones.

with overall averages of 2.95 ± 2.13 × 10^6 vs. 6.95 ± 3.79 × 10^5 cells per culture, respectively. The clones from the transformed t-SA′ culture with higher saturation densities are chiefly distributed among the slow growers but there are a few slow growers with low saturation density (Fig. 3). High saturation density is, of course, one of the defining characteristics of transformed cells, and its correlation with slow growth at low density is reflected in the small size of the colonies from the transformed culture mentioned earlier.

As might be expected from the high saturation densities, the direct assay for focus formation of many of the clones from the transformed culture yielded thick multiple layers of cells in which it was impossible to count individual foci. To characterize the morphology of the foci of each clone, another 1° assay was done with smaller numbers of cells after a total of 11 low-density passages of the cells. Fifteen of the 31 t-SA′ clones produced dense, multilayered foci such as the great majority of foci seen in the assay of the uncloned culture (Fig. 1). However, 14 of the t-SA′ clones produced light foci that were almost absent in the assay of the uncloned t-SA′ cells on a background of A′ cells (Fig. 1). Twelve of the light focus formers were carried through serial 2° and 3° assays with 10 producing thick sheets of heavily transformed cells by the 3° assay; one clone had scattered dense foci in the 3° assay and one had large numbers of broad light foci with a few pinpoint, fragmented dense foci. The two clones that had no foci in the 1° assay had many broad, light foci by the 3° assay and several small, moderately dense foci. In summary, about half of the transformed clones produced dense, multilayered foci such as those produced in assay of the uncloned culture and were probably derived from one or a few heavily transformed clones that selectively overgrew the other cells during the two rounds of prolonged confluence before cloning. Most of the other transformed clones in the 1° assay produced light foci that differed in morphology from clone to clone and probably originated from independent transforming events.

The SA′ clones presented a markedly different appearance than the t-SA′ clones during their assay at confluence. The different degrees of transformation produced by the SA′ clones are shown and classified in Fig. 4, and the number of clones in each category in two sets of serial assays are in Fig. 5. Twenty-seven of the 29 nontransformed clones in a 1° assay of the first series (1–1°) after five low-density passages formed

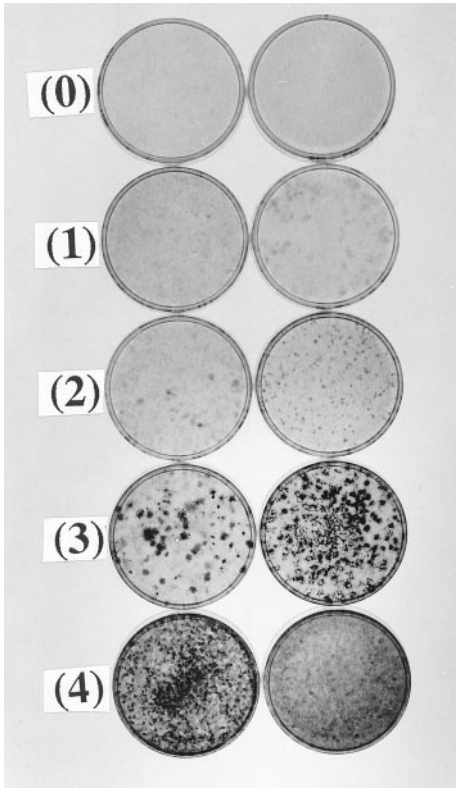

FIG. 4. Scale used in classifying the degrees of transformation. Starting from the top of each of the two columns: Nontransformed, degree 0; very weakly transformed, degree 1; weakly transformed, degree 2; moderately transformed, degree 3; strongly transformed, degree 4. In degrees 1–4, the two cultures indicate the lower (*Left*) and upper bounds (*Right*) of transformation of the category. The two negatives of category 0 were from the first 1° assay of the SA′ clones and the others from the 3° assay of the second series.

extremely clean monolayers with not even a trace of foci or even local densities (Fig. 5). The slowest growing SA′ clone produced a large number of light foci, and one of the faster growing clones produced 4 dense pinpoint foci (Fig. 6). In the 2° assay of the first series (1–2°), all but one of the clones produced foci, most of them of lower grades of categories 1 and 2 (Fig. 5). A second series of assays was initiated after the clones had been passaged at low density a total of 11 times. Sixteen of the clones that had produced no foci in the 1° assay of the first series produced light foci in the 1° assay of the second series (Fig. 5). This was the first evidence that transforming events occur during low-density passages of the cells, but they are not expressed in assays of the uncloned parental culture that was passaged at the same low density in parallel with the clones. By the 3° assay of the second series, all of the SA′ clones produced foci, the majority of them the dense foci of categories 3 and 4 (Fig. 5). This indicates that all the SA′ cells were sensitive to progressive transformation promoted by the selective constraint of confluence.

The SA′ clone, designated 1A, that produced the dense, pinpoint foci in the 1° assay was of particular interest to understanding the origin of large, dense foci in serial assays of the uncloned SA′ cells because it produced extremely large, dense foci in the 2° assay (Fig. 6). However, these were reduced in size, although greatly increased in number, when diluted and grown with an excess of A′ cells in a 3° assay (Fig. 6). The pinpoint size of the dense foci in the 1° assay of clone 1A indicated that this advanced form of transformation occurred after the cells became confluent and only a few days before the cells were fixed and stained. The great increase in size of foci in the 2° assay when there were no added A′ cells, and their

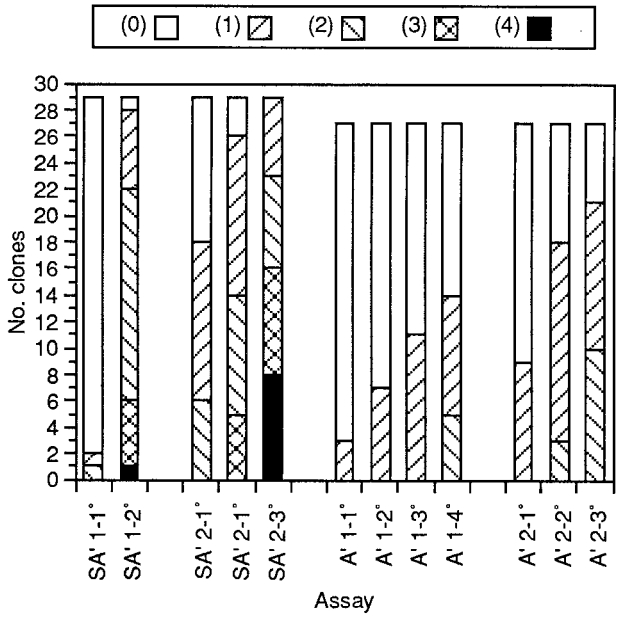

FIG. 5. Degrees of transformation in two series of assays of SA′ and A′ clones. The subline, series numbers and assay numbers are displayed under the appropriate bar. The scale of transformation is shown in Fig. 4.

subsequent decrease in size in the 3° assay with an A′ nontransformed background suggests that the growth of these fully transformed cells was more inhibited by a background of uncloned A′ cells in the 3° assay than when they were surrounded by nontransformed sister cells of the same clone in the 2° assay. To determine whether the occurrence of pinpoint foci in the 1° assay was merely a chance occurrence, the 1A clone was reassayed after it had been passaged at low density a total of 11 and 21 times. Each time the clone produced the same pinpoint foci in the 1° assay (data not shown), indicating that this clone had cells with a high probability of conversion to dense focus formation when they accumulated at confluence in a 1° assay. Because dense pinpoint foci were not expressed in the 1° assay of the uncloned SA′ cells, the expression of focus formation must have been suppressed in its early stages when the clone was surrounded by cells of other clones, although foci were produced in the 2° assay of the uncloned SA′ cells.

Characterization of Clones from the Transformation-Refractory A′ Culture. A preliminary series of eight serial assays at confluence of the uncloned A′ cells produced no dense foci, although a small fraction of the cells in the later assays of the series produced light foci (data not shown). Clonal analysis was initiated with a culture of freshly thawed cells and each of the serial assays of the clones at confluence was accompanied by a parallel serial assay of the uncloned parental culture. Cloning efficiency in the wells was 55%.

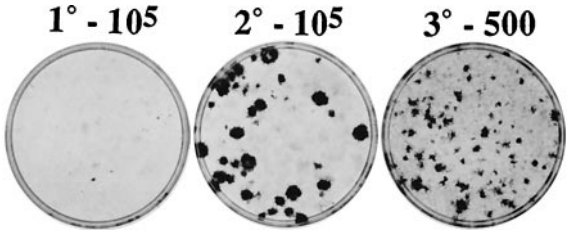

FIG. 6. Three serial assays for focus formation of the 1A clone. *Left*, 1° assay of 10^5 cells; *Center*, 2° assay of 10^5 cells; *Right*, 3° assay of 500 cells with 10^5 A′ cells to form a confluent background. Note the tiny dense foci in the 1° assay, the very large dense foci in the 2° assay, and the much smaller ones in the 3° assay.

Twenty-two of the 27 clones gave 1° assays completely free of irregularity, and two were slightly mottled (Fig. 5). Three of the clones had one or two focal areas that were slightly denser than the rest of the culture, but only one of these produced foci on serial assays, so the other two are considered negative. Seven of the 27 produced a small number of light foci and/or mottling in the 2° assay. The number of positives increased to 11 and 14 in the 3° and 4° assays, respectively. Although well defined foci were apparent in many of the clones in the 4° assay, none produced the dense foci seen in more than half the SA′ clones by the 3° assay. The uncloned parental A′ cells remained completely clear of any sign of transformation through the 4° assay despite the fact the culture must have contained hundreds, if not thousands, of clones such as those that produced foci in pure clonal populations. A second series of assays at confluence of the clones was begun when they had undergone eight additional low-density passages beyond the five that preceded the first series. The three clones that had exhibited continuing evidence of transformation from the 1° through the 4° assay of the first series (1–1° through 1–4°) registered clearer evidence of transformation in the 1° assay of the second series (2–1°). There were six additional clones that registered positive in the 1° assay of the second series. Eighteen and 22 of the clones had morphological evidence of transformation in the 2° and 3° assays (2–2° and 2–3°) of the second series but none produced dense foci. Again, there was no sign of transformation in any of the parallel assays of the uncloned A′ culture. Hence, the transformation-refractory A′ subline illustrated, in a more dramatic manner than the SA′ subline, that clonal populations are more likely to exhibit transformation than their uncloned progenitor population.

DISCUSSION

About 90% of the foci produced by the uncloned t-SA′ cells were uniformly dense when assayed on a background of A′ cells, suggesting that they arose from one or a few transforming events (11). However, about half of the clones obtained from the t-SA′ culture produced light foci in a 1° assay that varied in morphology from clone to clone. Twenty-two of the 31 t-SA′ clones were carried through three serial assays at confluence and all but one of them were initially dense focus formers in the 1° assay or progressed to dense focus formation. The exceptional clone produced only light foci in the 3° assay. Therefore, the impression from the focus morphology in the assay of the uncloned t-SA′ culture that only a few cells had undergone transformation is incorrect. The dense focus formers had undergone selective growth during two serial assays at confluence and had become a dominant element in the population before the clonal study. The impression of this dominance was heightened by the failure of the varied light focus formers to register as such when diluted and mixed with a large number of nontransformed A′ cells that served as a clean background for displaying foci.

Cloning and serial assay of the nontransformed SA′ cells also showed that all the SA′ clones would exhibit transformation within three serial assays although, as expected, a smaller percentage of them than of the t-SA′ clones had progressed to dense focus formation by the 3° assay because they had not undergone the two preliminary rounds of confluence that had resulted in the t-SA′ culture that preceded cloning. The uncloned SA′ culture did not display any dense foci in its 1° assay at confluence although one of its 29 clones, designated 1A, did produce a number of dense foci in a 1° assay. The 1A clone produced the same type and number of small, dense foci in two more 1° assays repeated after additional passages at low density, showing that this was an inherent property of the clone and not just a random event that could have occurred in any clone. One would expect there to be many other clones in the original SA′ population of 10^5 cells that had the capacity to produce small, dense foci in a 1° assay at confluence. The absence of even a single dense focus in the 1° assay of the uncloned SA′ culture could be attributed either to suppression of focus formation by surrounding clones or to the failure of that level of transformation to occur in the mixed population. There was evidence of suppression in the fact that a 2° assay, consisting only of cells of the 1A clone, produced extremely large foci, but when the cells from that assay were diluted on a background of the transformation-refractory A′ cells in a 3° assay, the foci were greatly reduced in size (Fig. 6). Suppression of focus formation by certain sublines of NIH 3T3 cells at confluence has been described (4). Similar suppression of transformed cell growth by surrounding normal cells has been described in several cell culture systems, including chicken embryo cells infected with Rous sarcoma virus (12), hamster cells infected with polyoma virus (13), and mouse embryo cells infected with polyoma virus (14). Cell to cell contact was required for inhibition of the tumor cells in these systems, and junctional communication has to be established (15).

Epigenetic regulation of tumor cells to normal behavior has been described in experimental animals. Epithelial tumor cells induced in regenerative regions of the newt differentiate to normal tissues, whereas those induced in nonregenerative parts are lethal (16). Mouse teratocarcinoma cells inoculated into the blastocyst of the developing mouse embryo are incorporated as a normal part of many tissues, including reproductively functional sperm (17). Rat hepatocarcinoma cells assume the appearance of normal hepatocytes when inoculated into, or are transported into, the liver of young rats (18). These and other examples (see ref. 19) provide ample evidence of the capacity of the cellular microenvironment to regulate the neoplastic expression of transformed cells.

The absence of foci in the first few assays of a series with various sublines of the NIH 3T3 cells such as the A′ cells and the appearance of the foci in later assays has contributed to the impression that the constraint of confluence plays an important role not only in the expression of transformation in foci but in the induction of the transformed state (20). Of course, other forms of growth inhibition, such as limiting concentrations of serum (21) and suspension in semisolid medium (22), promote the appearance of transformed cells. But the present findings that a few low-density passages lead to transformation in some clones and that further low-density passages result in transformation of more clones show that the various forms of growth inhibition are not required to induce transformation. Rather, it appears that their major function is to select for the preneoplastic and neoplastic cells that have a growth advantage under those limiting conditions and foster progressive development of the neoplastic state. The role of selection had been obscured by the heritable damage incurred in the process of transformation that frequently results in a slowdown of growth and negative selection at low population densities but confers a selective advantage under growth limiting conditions (5). Some contribution of growth limiting conditions to the induction of transformation cannot be ruled out, however, because high cell density markedly increases mutation frequency in human cells deficient in mismatch repair proteins (23) and serum limitation induces a hypermutable state in hamster cells (24). In view of the importance of chromosome rearrangements (25, 26) and deletions (27) in human cancer, it is noteworthy that the stationary state in bacteria leads to chromosome rearrangements (28) and deletions in simple repeats of nucleotides in DNA (29). While the case for induction of transformation by growth inhibition remains largely inferential, the evidence for a major role of progressive selection of spontaneously occurring transformed variants is straightforward.

A central role for progressive selection of clones in tumor development has been frequently suggested (30–33). The particular nature of the cellular microenvironment determines

the type of cell to be selected. This is illustrated by the finding that a specific carcinogen, NMU, produces mammary cancer in Fisher 344 rats by selecting for the growth of cells with preexisting H-ras 1 gene mutations in the mammary epithelium of young females (2). Development of the tumor is accompanied by an irreversible alteration in the conformation of the H-ras 1 promoter, which normally occurs in reversible fashion under hormonal control (34). Even in untreated rats there is selection for patches of H-ras 1 mutated mammary epithelial cells because they constitute a 5-fold higher proportion of the gland in old than in young rats (35). It is, in addition, established that most human cancers are monoclonal in origin (36–38). Topographically distinct head and neck tumors arise from the same clone (38). Adjacent areas of tissue varying from benign squamous hyperplasia to invasive head and neck carcinoma share common genetic changes, although the more advanced areas have additional genetic alterations (39). Large areas of metaplasia in Barrett's esophagus with a high risk of progression to dysplasia and adenocarcinoma share a common aneuploidy (40). Clonal expansion of p53 mutations is an early marker of neoplastic progression in ulcerative colitis (48). Clonal expansion to form hepatocyte nodules is an early step in experimental carcinogenesis of the liver in rats (49). This is an adaptive response, because over 95% of the nodules redifferentiate into normal liver, but progression to hepatocarcinoma occurs in the rest. These results indicate that large expanded clonal populations are favored sites for tumor development. It seems likely that clonal diversity is reduced in stem cell populations during the aging process due to the death of some of the cells from the accumulation of genetic damage (41). This would allow expansion of the remaining stem cell populations with increased potential for genetic change and tumor development. The frequent association of atrophy followed by hyperplasia with the onset of cancer of the stomach (42) and prostate (43) is compatible with a role of clonal elimination and expansion of survivors in the origin of cancer. An apparently related sequence occurs in cirrhosis of the liver, which is a precursor to hepatocarcinogenesis (44). This concept of clonal elimination and expansion resonates with our finding of a much greater incidence of transformation in clones than in their progenitor uncloned populations. This suggests that a decrease in clonal diversity of stem cell populations and an increase in clone size with aging contribute to the exponential increase with age in the incidence of the major human cancers (45, 46).

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