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Physiological induction and reversal of focus formation and tumorigenicity in NIH 3T3 cells

(neoplastic transformation/adaptation/progressive state selection)

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ABSTRACT **NIH 3T3 cells undergo morphological transformation in response to conditions of constrained growth, such as occur in low serum concentrations or at confluence. Transformation is expressed in a small fraction of the cells by the appearance of discrete foci of multiplying cells on a confluent monolayer of quiescent cells. We isolated and expanded cell populations from three dense and three light foci. Cells from each of these populations efficiently reproduced foci of the same morphotype when grown on a background of nontransformed NIH 3T3 cells. Using cultures derived from one of the dense foci (subline D/2), we found that the number of focus-forming units was stable and the cells remained tumorigenic when they were subjected to repeated thrice-weekly passage in 2% calf serum. However, equivalent passage in 10% calf serum eventually rendered the cells incapable of both focus production and tumor formation. The results show that the capacity to produce tumors as well as morphological transformation are produced as a response to physiological constraints of growth and/or metabolism in the absence of carcinogens and that both properties can be reversed by lifting the constraints. This behavior is typical of an adaptational response and, taken together with other supporting evidence, shows that tumorigenesis does not require conventional genetic alteration.**

Precise description of the physiological conditions that favor progression or regression of the tumor phenotype is a major goal of cancer research. The strong association between age and the likelihood of developing the most common solid cancers (1) implies that physiological changes occurring during aging play an important role in the development of cancer. Cell culture offers the opportunity to study the effect of physiological conditions on neoplastic transformation because those conditions are easily controlled. We have shown that the NIH 3T3 cell line, established originally from mouse embryo cultures (2) and widely used as a target for oncogene-mediated transformation, undergoes spontaneous morphological transformation at a high rate under conditions in which certain physiological constraints on growth are introduced (3, 4). These constraints are achieved by lowering the serum concentration or by exposing the cells to confluent densities but not by lowering the glutamine concentration, which inhibits transformation (5). When the inducing conditions are removed, morphological reversion of the transformed phenotype occurs (4). In the present experiments we use highly transformed populations of NIH 3T3 cells isolated directly from spontaneously transformed foci to demonstrate the high correlation between the ability to initiate a focus in low serum concentrations and tumorigenicity in nude mice. This correlation extends to cell populations in which reversal of the focus-forming phenotype has occurred as a result of repeated subculture at low densities in medium supplemented with high serum concentrations. We also show that, while the process of transformation can yield cells that produce at least two distinct morphological types of foci, the ability to produce a focus of a particular morphotype is stably transmitted, even when the number of foci in a population is declining. These experiments provide fresh support for the view that the heritable changes underlying tumor development result from physiological modifications made by cells responding to their environment (6, 7).

MATERIALS AND METHODS

Cell Culture. NIH 3T3 cells, derived by repeated cloning of flat variants from partly inbred mouse embryos (2), were supplied from the original, cryopreserved stock by S. A. Aaronson (National Cancer Institute, Bethesda, MD). After one passage in molecular, cellular, and developmental biology (MCDB) 402 medium (8) containing 10% calf serum (CS) and subsequent refreezing in liquid nitrogen, the cells were thawed and subjected to a standard regimen of frequent passages on a weekly sequence of 2, 2, and 3 days, with seedings of 2×10^4, 2×10^4, and 1×10^4 cells per 60-mm dish, respectively, in 10% CS. Transformed foci, visible in dishes that had been fixed in methanol and stained in a 4% aqueous solution of Giemsa, were distinct from the nontransformed surrounding areas by their higher cell density. Focal populations were isolated from the nontransformed surrounding cells by application of cloning cylinders. The assay for the number of focus-forming units in cells that had already undergone transformation was done by plating 10^2 or 10^3 cells in the presence of 10^5 nontransformed background cells in 2% CS and incubating them for 10 or 14 days with twice-weekly medium changes. Cells were also assayed for their ability to form colonies >0.14 mm at the interface between 0.4% and 0.6% agar as described (9).

Tumorigenicity. The tumorigenic potential of cells undergoing experimental treatments was determined by subcutaneous injection of 5×10^5 cells suspended in 2% CS into athymic mice [Strain N:NIH(S)II-*nu*/*nu* (10)]. The mice were checked weekly for tumor formation at the site of injection.

RESULTS

Induction of Transformation. NIH 3T3 cells subjected to extended periods at confluence undergo morphological transformation. Transformed foci may not easily be distinguished from background irregularities when only the original dishes are examined, even after 21 days in 2% or 10% CS (Fig. 1 *A* and *B*, left columns). However, when cultures grown in 2% CS for 14 days were subsequently trypsinized, diluted, and grown for 2 additional weeks in 2% CS, transformed foci

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Abbreviations: CS, calf serum; CFE_{ag}, colony-forming efficiency in agar.

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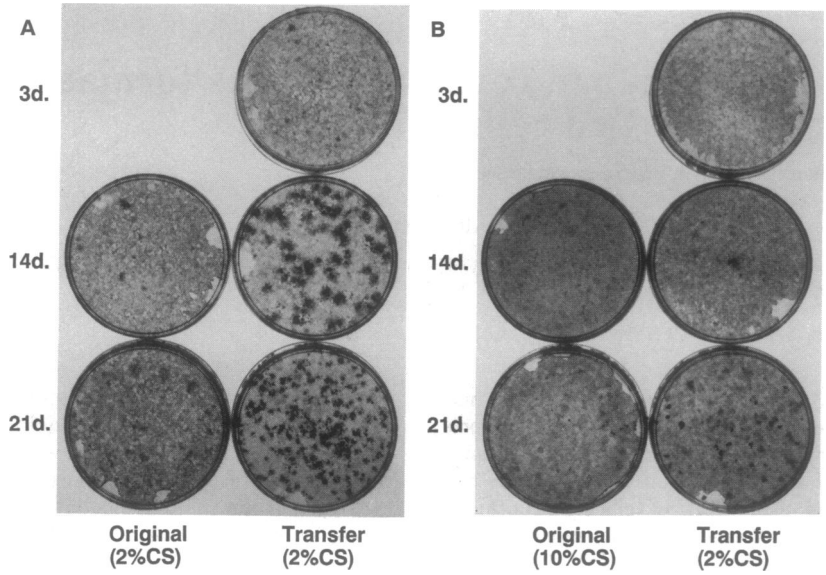

FIG. 1. Effect of CS concentration on spontaneous transformation of NIH 3T3 cells. Cells were seeded at 10^5 cells per 60-mm dish in MCDB 402 medium containing 2% CS (*A*) or 10% CS (*B*). After 14 and 21 days (d.) one of the original dishes was fixed and stained in 4% Giemsa (left columns), and a sister dish was trypsinized, diluted, and replated at 5×10^4 cells per 60-mm dish in 2% CS-containing medium. These cultures were then stained after 14 more days (right columns). The top right-hand dish in *A* and *B* represents a culture plated at 5×10^4 in 2% CS after having been plated at 10^4 cells and grown for 3 days in 2% CS or 10% CS. This procedure was done to show that short incubations at subconfluent densities were insufficient to render the cells transformed regardless of serum concentration.

became readily visible (Fig. 1*A*, right column). By 21 days the number of focus-forming units had further increased in the 2% CS population, while their morphology became denser and more discrete. That the confluent state played an important role in the transformation is indicated by the absence of foci in cultures transferred after only 3 days in 2% CS. Unlike the transfers done at 14 and 21 days that used cells that had been plated at 10^5 cells per 60-mm dish, this transfer used cells that had been plated at 10^4 cells per dish and, thus, had no chance in 3 days to achieve confluence. Transfer dishes from parallel cultures kept in 10% CS showed much less evidence of transformation than those that had been incubated in 2% CS; only a small number of foci appeared even after transfer of the 21-day cultures (Fig. 1*B*, right column). We conclude from this experiment that the development of newly transformed cells had been induced by specific environmental conditions that were more effective in 2% CS than in 10% CS medium. Because the saturation density and, therefore, the number of cell divisions per culture was more than 2-fold greater in 10% CS than in 2% CS, and mutations, i.e., copying errors, depend on division, the disproportionately high frequency of transformation in 2% CS argues against a mutational origin of transformation.

Dependence of the Transformed State in Focal Sublines on Culture Conditions. When a small number of the 2% CS/21-day cells were transferred in the presence of excess nontransformed NIH 3T3 cells, at least two focal morphologies were apparent (Fig. 2, far left). One focal type was composed of densely packed cells that underwent fragmentation into subfoci after establishment; we refer to these as "dense" foci. The other was much less dense, though more cohesive in long-term incubations; we refer to these as "light" foci. To determine the stability of these focal types we selectively isolated three representative populations of each focal type in 2% CS using cloning cylinders. After allowing 3 days for expansion on separate dishes, all six populations were trypsinized, and 1000 cells of each were replated and grown for 10 days in 2% CS in the presence of 10^5 nontransformed NIH 3T3 cells. In every case the morphology of the original focus, whether dense or light, was reproduced when a small number of the cells was seeded with a much larger number of nontransformed cells. Furthermore, during repeated transfer in 2% CS of cells from one dense (D/2) and one light (L/1) focus, focal morphology remained distinct (data not shown). This result suggested that these two focal morphotypes represented transformations that had occurred down separate pathways, possibly due to heterogeneity of the original population (4) at the time it was originally subjected to the transforming (confluent) conditions. In contrast to morphology, the number of focus-derived cells capable of reinitiating foci *was* reduced by thrice-weekly transfer at very low densities in 10% CS (Fig. 3*A*). Thus, after passaging subline

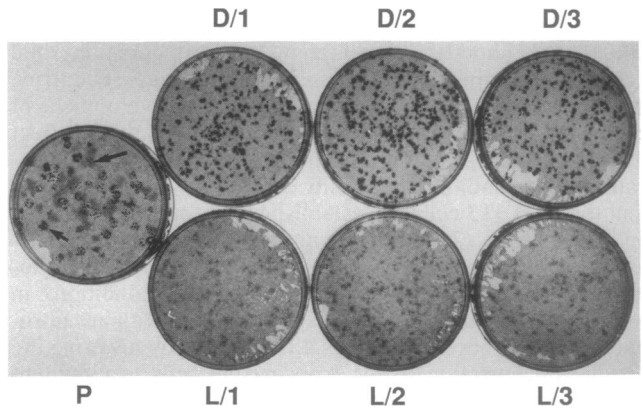

FIG. 2. Stability of the dense (D) and light (L) focal morphotypes after isolation and expansion of individual foci. Cultures from the experiment of Fig. 1 were trypsinized after 21 days in 2% CS and diluted, and 10^4 cells were plated in the presence of 10^5 nontransformed NIH 3T3 cells, generating after 2 weeks a culture containing two distinct focal morphotypes. The light morphotype is indicated in this parental culture (P) by a long arrow. The dense, fragmenting morphotype is indicated by a short arrow. At this time three representative foci from each morphotype were isolated using cloning cylinders and replated in 2% CS on separate dishes. After 3-day growth each culture was trypsinized, and 1000 cells were replated in 2% CS with 10^5 nontransformed cells. Staining was done at 10 days to avoid merging of foci. D/1, D/2, and D/3 represent the three dense focal populations. L/1, L/2, and L/3 represent the three light populations.

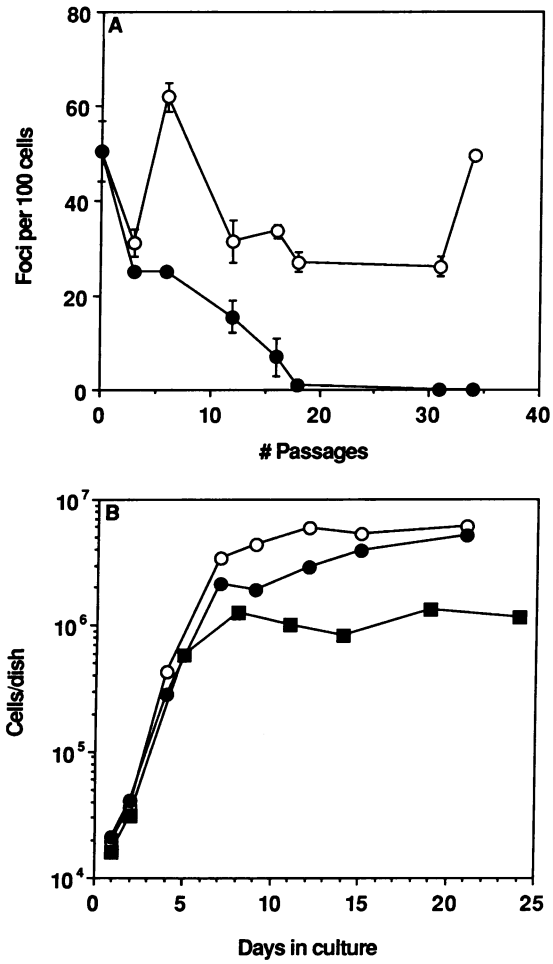

FIG. 3. (*A*) Effect on number of focus-forming units of frequent, low-density passage in 2% CS of cells from population D/2. Cells were passaged three times per week at 2×10^4, 2×10^4, and 10^4 cells per 60-mm dish. At the indicated passages 100 cells were plated in 2% CS in the presence of 10^5 nontransformed NIH 3T3 cells. Number of foci were counted after staining at 10 or 14 days. Error bars represent the range of duplicate determinations. ○, Cells previously passaged in 2% CS; ●, cells previously passaged in 10% CS. (*B*) Effect of frequent, low-density passages in 2% CS and 10% CS on saturation density achieved by D/2 cells and control cells in 2% CS. Cells were passaged as in *A*. After 16 thrice-weekly passages they were plated at 2×10^4 cells per 60-mm dish and counted electronically over the ensuing days. Points represent the average of duplicate determinations. ○, D/2 cells previously passaged in 2% CS; ●, D/2 cells previously passaged in 10% CS; and ■, control cells previously passaged in 10% CS.

D/2 three times in 10% CS the number of focus-forming units had dropped by ≈50% and continued to decrease with further passage, so that by 31 passages no foci could be discerned even if 10^4 cells were plated. Despite some fluctuation, the number of focus-forming units in the parallel cultures passaged in 2% CS remained >26 per 100 cells plated. The drop in focus-forming capacity of cells passaged in 10% CS was also reflected in a difference in the saturation density measured in 2% CS. Thus 16 low-density passages in 10% CS generated a population subject to density-dependent growth inhibition at $\approx 2 \times 10^6$ cells per 60-mm dish in 2% CS compared with almost 4×10^6 cells per dish for the population maintained in 2% CS (Fig. 3*B*). The control parental population saturated at slightly above 10^6 cells per dish, one indication that the experimental 10% CS population had not undergone complete reversal. This conclusion is supported by the observation that this population undergoes a secondary rise after 9 days, so that its final density rivals that of the population passaged in 2% CS. This result is most likely due to the presence of a minority of highly transformed cells among cells of declining levels of transformation. In addition to the change in the number of focus-forming units and saturation densities in 2% CS, it was also evident that both size of the remaining foci and their tendency to become fragmented were diminished after passage in 10% CS (Fig. 4). This result indicates that exposure to 10% CS generated adaptive changes in the focus-producing cells that resulted in the loss of focus-forming capacity in some cells and a decreased focal growth rate in others. It is also possible that selective forces operative in 10% CS favored the few nontransformed cells that may have contaminated the original focal isolation. A similar trend in focus formation was apparent for the populations deriving from the light focus, although this focal type became difficult to distinguish from the background after the sixth passage.

Correlation of Tumorigenicity with Focus-Forming Capacity but not with Colony Formation in Agar. We then tested the capacity of cultures derived from the foci and of control cultures to form tumors in nude mice. Table 1 shows that 100% of animals injected with 5×10^5 cells derived from the dense focus developed tumors within 25 days. While six passages in 10% CS decreased the number of foci formed >2-fold (Fig. 3*A* and Table 1), neither the number of animals developing tumors nor their latency was affected. However, after 34 passages in 10% CS, when no foci were formed by 100 cells, tumor formation was so markedly suppressed that no distinction could be made between this population and the control nontransformed population. In both cases a single animal developed a tumor by 97 days, due most likely to transformation that had occurred sometime *after* the injection. By contrast, cells passaged 34 times in 2% CS retained their focus-forming capacity and were tumorigenic in 100% of the injected mice within 33 days. When the maintenance regimen was altered so that transfers were done only at 2-week intervals, thus exposing the cells to confluent conditions for over a week in 2% CS during each passage cycle, the latent period for tumor formation decreased somewhat while the cells retained their extremely high focus-forming capacity and dense morphology. Cells derived from an explant of a 1-mo-old tumor initiated from this group had the shortest latencies of all, showing evidence of tumor formation within 5 days of injection. This finding suggests that there are conditions in the organism that more effectively foster the further development of the tumorigenic phenotype than exist in culture. In contrast, the populations derived from the light focus (L/1), which had lost their capacity for focus formation in the course of frequent passage in either 2% or 10% CS, failed to produce tumors. Focus-

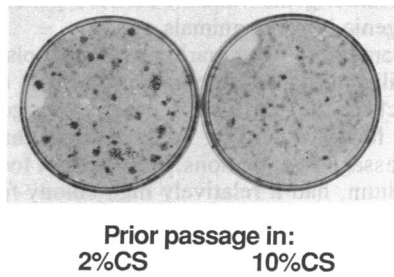

FIG. 4. Evidence that deadaptation to growth in 2% CS is involved in the reversal of transformation in D/2 cells frequently transferred in 10% CS. After 16 thrice-weekly transfers in 2% CS (*Left*) or 10% CS (*Right*), 100 cells were plated in the presence of 10^5 nontransformed cells in 2% CS. After 14 days the cultures were fixed and stained. Note that not only do the number of foci differ but the size of foci from the 10% CS population is smaller than many of those from the 2% CS population.

Table 1. Effect of culture conditions on focus formation and tumorigenicity by cells from a dense (D/2) and a light (L/1) focus

	Regimen	Passages, no. 2%	Passages, no. 10%	Foci/100 cells*	Tumor Incidence	Tumor Latency, days
Population D/2						
A	3×/week	6	0	65, 59	4/4	25
A	3×/week	34	0	48, 50	4/4	33
B	3×/week	0	6	24, 26	4/4	25
B	3×/week	0	34	0, 0	1/4	97†
C	1×/2 week	1	0	39, 34	4/4	18
D	Explant‡	1	0	55, 53	4/4	5
Population L/1						
A_2§	3×/week	6	0	2, 1	0/3¶	
B_2§	3×/week	0	6	1	0/4	
C	1×/2 week	1	0	6, 5	4/4	39
C	1×/week	4	0	32, 28	4/4	40
Control NIH 3T3	3×/week	0	86	0, 0	1/4	97‖

Cells were subjected either to thrice-weekly passage at 2×10^4, 2×10^4, and 10^4 cells per 60-mm dish or to passage once every 2 weeks at 10^5 cells per 60-mm dish and analyzed simultaneously for focus-forming units and for tumorigenicity. Tumorigenicity was determined by subcutaneous injection of 5×10^5 cells suspended in 0.5 ml of 2% CS into athymic mice. Mice were checked weekly for tumor formation at the injection site. Control NIH 3T3 cells were passaged thrice-weekly in 10% CS at 2×10^4, 2×10^4, and 10^4 cells per 60-mm dish or, later, at 5×10^5, 5×10^4, and 2×10^4 cells per 60-mm dish.

*Cells were plated in the presence of 10^5 nontransformed background cells.

†Only a single animal in this group developed a tumor after 97 days. Significantly, this is the same time when an animal from the control group also developed a tumor. Therefore, this transformation probably occurred subsequent to injection. One of the four animals died tumor-free after 47 days. Of the remaining two animals, one died tumor-free at 172 days, and the other died tumor-free at 187 days.

‡Cells from a 1-mo-old tumor deriving from population D/2C were explanted in 2% CS after dissociation with collagenase (type 1A, 1 mg/ml), transferred the next day at 10^5 cells per 60-mm dish, and assayed for transformation and tumorigenicity 13 days later.

§These cells had lost their focus-forming capacity with frequent passage in 2% or 10% CS.

¶Two of three animals died at 39 days and 56 days, respectively. The third was killed at 83 days. In each case the mice were dissected and found tumor-free at death.

‖Only a single animal in this group developed a tumor after 97 days. This transformation probably occurred subsequent to injection. One of the four animals died tumor-free after 52 days. Of the remaining two animals, one died tumor-free at 125 days, and the other was killed and found to be tumor-free at 188 days.

forming capacity was regained and the density of the light foci was increased when the cells were left without passage in 2% CS for 2 weeks, which kept them in the confluent state for >1 week. These cells also gained the capacity to form tumors, but the latent period was longer than that of tumors produced by the dense foci. Even after four such 2-week cycles of confluency when the number of focus-forming units had become comparable to the parallel dishes from the dense foci (Table 1), the latent period for tumor production was unchanged. The origin of these cells from a light focus was thus strongly reflected in their capacity for tumor formation, even though the efficiency of light focus formation was as high as that of the dense foci. This result is reminiscent of an earlier observation by Reznikoff *et al.* (11) that carcinogen-induced light (type I) foci were not tumorigenic while the denser types II and III foci were tumorigenic in some animals.

Another commonly used marker of the neoplastic phenotype, the ability to make large colonies (>0.14 mm) in soft agar (9), lacked the correlation with tumorigenicity that existed with focus formation. Table 2 shows that all of the frequently passaged populations, regardless of focal origin or passage medium, had a relatively high colony-forming efficiency in agar (CFE_{ag}) despite large differences in tumorigenicity and focus formation (Table 1). Thus, population D/2A which was both highly tumorigenic and capable of making foci, also showed high CFE_{ag}; populations D/2B, L/1A, and L/1B also had high CFE_{ag}, but these were not tumorigenic and lacked focus-forming capacity. Furthermore, the cells with the shortest latent periods for tumor production—i.e., those of D/2C derived from a dense focus and subjected to repeated 2-week passage cycles or of D/2D, their derivative cells after a 1-mo period of tumor growth in the animal—had little or no capacity for agar growth. Such discordance between agar growth and tumorigenicity has been observed in human epidermoid carcinoma cells which gain in ability to produce colonies in agar during the process of adaptation to growth on tissue culture plastic but lose their ability to grow as a tumor (12).

DISCUSSION

The present results demonstrate the effects of certain environmental alterations on the behavior of highly transformed NIH 3T3 cells. These cells were isolated from spontaneously transformed foci arising under conditions of extended exposure to high population densities and low CS concentrations

Table 2. Effect of culture conditions on colony-forming efficiencies in agar by cells from a dense (D/2) and a light (L/1) focus

	Regimen	Passages, no. 2%	Passages, no. 10%	CFE_{ag}, %
Population D/2				
A	3×/week	22	0	7.4*
B	3×/week	0	22	12.7
C	1×/2 week	4	0	0.45
D	Explant	1	0	0.00
Population L/1				
A	3×/week	22	0	15.6
B	3×/week	0	22	15.1
C	1×/2 week	4	0	12.0
Control NIH 3T3	3×/week	0	74	0.30

Cells subjected to the indicated passage routines (details are in legend to Fig. 1) were assayed for their ability to form a colony with a diameter >0.14 mm at the interface between 0.4% and 0.6% agar.

*Average of duplicate determinations.

(2%). Transformation fails to occur at all over many months of culture when the cells are maintained at low density in a high serum concentration (10% CS) before assay. This failure plus the dependence of transformation on high density and low serum concentration form a potent argument against its mutational origin. The additional evidence provided by these experiments that the transformation is reversible, even in populations in which a very high proportion of cells can initiate a focus, is fully consistent with an adaptational origin of the transformed phenotype. Maintenance of the transformed state, at least with respect to the percentage of focus-forming cells and tumorigenicity, depends, like the initial transformation itself, on the conditions under which the cells are maintained. This conclusion is based on three observations. (*i*) When the CS concentration is kept at 2%, the cells retain the capacity to produce foci at high levels. (*ii*) When the cells are exposed to confluent conditions during each passage cycle, they undergo progression both in the sense that foci in the light focal populations become gradually darker and more numerous and that all the cells become more tumorigenic. Similar observations on the enhancing effect of confluence on neoplastic behavior have been made beginning with diploid mouse fibroblasts (13) and with normal rat liver epithelial cells (14). (*iii*) When the serum concentration is raised to 10% while the cells are kept in a constant state of exponential growth, the percentage of transformed cells in the population declines quickly; this is reflected in measurements of saturation density, focus production, and tumorigenicity. Phenotypic reversal by low-density cultivation has previously been reported with hamster embryo cells (15) and with irradiated mouse C3H/10T½ cells (16). Although it is possible that selection of a small minority of nontransformed cells occurs under these conditions (R. Grundel and H.R., unpublished data), we present two types of evidence that loss of focus-forming capacity by once-transformed cells is also involved. (*i*) The saturation density declines to an intermediate level after 16 passages in 10% CS, reflecting the intermediate level of transformation in these cultures. (*ii*) The size of the foci (as well as their numbers) is reduced compared with those produced by the parallel cultures maintained and passaged in 2% CS. This evidence mirrors our previous demonstration that induction of spontaneous transformation under serum-restricted conditions is itself an adaptive change (4). Also, we should emphasize that the ability to produce tumors is correlated with the ability to produce dense foci in culture amid nontransformed cells in the presence of a low serum concentration. Such conditions mimic the *in vivo* environment, in which tumor cells are surrounded by normal cells and the concentration of growth factors in extracellular fluid, which is a filtrate of plasma, is low. These conditions would appear to better approximate the *in vivo* conditions than the agar suspension assay, which correlates poorly in NIH 3T3 cells with their tumor-forming capacity.

Certain similarities exist between the process of tumor progression *in vivo* and the types of enduring adaptive changes noted in these cell populations. As pointed out by Foulds (17), progression is independent of growth and may occur most reliably when growth is repressed. This was well-established by Foulds and by Noble (18) for hormone-responsive tumors in rodents but also probably applies to human tumors regressing after treatment with drugs, surgery, or endocrine therapy (17). Haddow (19) recognized the critical role played by cellular inhibition in the carcinogenic effect of many varied physical and chemical agents, noting also that the association of cancer with aging depends on the relative inhibition of growth in aging tissues. Franks (20) has also observed an association between sclerotic atrophy and carcinoma of the prostate.

We have proposed that adaptive change in cell populations occurs through a process called *progressive state selection* (4). Progressive state selection is based on the demonstrated occurrence of phenotypic variation in cell populations (4, 21). Because each cell is unique, it has available to it a slightly different set of potential states from which to select when confronted with environmental change. The whole population becomes heritably different because the physiological adjustments made by individual cells result in the progressive alteration of future states available to them. Such a process presumably underlies both progression and regression of tumors, depending on the physiological environment to which they are exposed. Of course, progressive state selection may proceed to the point that tumor growth becomes independent of environmental conditions, and the tumor is said to be autonomous.

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1. Jones, H. B. (1956) *Trans. N.Y. Acad. Sci.* **18,** 298–333.
2. Jainchill, J. L., Aaronson, S. A. & Todaro, G. J. (1969) *J. Virol.* **4,** 549–553.
3. Rubin, H. & Xu, K. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1860–1864.
4. Rubin, A. L., Yao, A. & Rubin, H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 482–486.
5. Rubin, A. L. (1990) *Cancer Res.* **50,** 2832–2839.
6. Farber, E. (1984) *Cancer Res.* **44,** 5463–5474.
7. Rubin, H. (1990) *Am. J. Physiol.* **258,** L19–L24.
8. Shipley, G. D. & Ham, R. G. (1981) *In Vitro* **17,** 656–670.
9. Rubin, H., Chu, B. & Arnstein, P. (1983) *J. Natl. Cancer Inst.* **71,** 365–375.
10. Azar, H. A., Hansen, C. T. & Costa, J. (1980) *J. Natl. Cancer Inst.* **65,** 421–430.
11. Reznikoff, C. A., Bertram, J. S., Brankow, D. W. & Heidelberger, C. (1973) *Cancer Res.* **33,** 3239–3249.
12. Ossowski, L. & Reich, E. (1980) *Cancer Res.* **40,** 2310–2315.
13. Aaronson, S. A. & Todaro, G. J. (1968) *Science* **162,** 1024–1026.
14. Lee, L. W., Tsao, M.-S., Grisham, J. W. & Smith, G. J. (1989) *Am. J. Pathol.* **135,** 63–71.
15. Rabinowitz, Z. & Sachs, L. (1968) *Nature (London)* **220,** 1203–1206.
16. Brouty-Boyé, D., Gresser, I. & Baldwin, C. (1979) *Int. J. Cancer* **24,** 253–260.
17. Foulds, L. (1969) *Neoplastic Development* (Academic, New York), Vol. 1, p. 73.
18. Noble, R. L. (1977) *Cancer Res.* **37,** 82–94.
19. Haddow, A. (1938) *Unio Int. Contra Cancrum* **4,** 342–352.
20. Franks, L. M. (1954) *J. Pathol. Bacteriol.* **68,** 617–621.
21. Rubin, H. (1990) *Cancer Met. Rev.* **9,** 1–20.