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Cell Biology

Sensitivity of transformation to small differences in population density during serial passage of NIH 3T3 cells

(adaptation/epigenetics/tumorigenesis/metastasis)

ADAM YAO* AND HARRY RUBIN

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

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ABSTRACT **Early passages of the NIH 3T3 mouse cell line undergo spontaneous neoplastic transformation leading to the development of transformed foci if grown to confluence in 2% (vol/vol) calf serum (CS) and left there for more than a week. Transfer of the postconfluent cultures results in the appearance of large numbers of transformed foci; many of them are larger and denser than those in the original culture. If the cells are continually kept at low population densities by frequent passages in 10% CS, they lose the capacity to undergo spontaneous transformation. If however the low-density passages are made in 2% CS or in 10% (vol/vol) fetal bovine serum, both of which support lower growth rates and saturation densities than does 10% CS, they gain the capacities to grow to high saturation densities and produce more foci when grown to confluence in 2% CS. These increases are proportional to the population densities used in the frequent passages, although the densities are all kept well below confluence. We conclude that the combined constraints of submaximal serum plus those of the limited cell contacts of the low cell densities used here elicit an adaptive response that endows the entire population with increased growth capacity. The increased growth capacity of the heterogeneous population in turn increases the capacity of a fraction of the population to initiate distinctive transformed foci. Similar studies have indicated that the capacity of cells to produce tumors and metastases in mice and rats is enhanced by prior maintenance at high density in culture. We propose the concept of progressive state selection to account for the general increase in the growth capacity of cells that is elicited by moderate constraints on their growth and metabolism.**

The NIH 3T3 line of cells produces foci of neoplastically transformed cells when grown to confluence in low calf serum (CS) concentrations [2% (vol/vol)], but the number and morphology of the foci depend on the passage history of the cells (1, 2). If they are repeatedly passaged at low population density in a high CS concentration (10%), they gradually lose the capacity to produce transformed foci. If the low-density passages are done in low CS concentrations or in high fetal bovine serum (FBS) concentrations, or if the cells are grown to confluence in high CS concentrations and the cells are then transferred for use in the assay procedure, many foci are formed (1–4). These observations led to the proposal that transformation is an adaptive response to moderate constraints on cell growth and/or metabolism. The response to those constraints permits some cells to multiply in postconfluent cultures at a higher rate than the surrounding, contact-inhibited cells, which results in the formation of discrete foci of transformed cells. The epigenetic nature of the transformation is indicated by its occurrence only under certain conditions (i.e., physiological constraint). It receives support from the fact that most, if not all, of the cells of a responding population that are not overtly transformed to form dense foci show an increase in saturation density and an increase in sensitivity to transformation among cloned progeny populations (1). Furthermore, the foci from independent transforming events are expressed in different morphologies (3). Although the overtly transformed state is heritable if the conditions of moderate constraint are maintained, it is gradually diminished and finally reversed over a number of cell generations if the cells are grown under unconstrained conditions of low density in a high CS concentration (1, 5). Cells from the dense, but not the light, foci form sarcomas in nude mice within a few weeks, but progeny of cells from the dense foci lose that capacity when their focus-forming capacity is reversed (5). Cells obtained from the sarcomas and reinoculated into mice form tumors even more rapidly than any cells from culture, indicating that further adaptation to growth in the mouse occurs during development of the first sarcoma (5). The combination of all these features shows clearly that spontaneous transformation is not the result of mutation and is likely to involve a change in equilibria of many biochemical pathways in the responding cells.

The aim of the work presented here was to gauge just how sensitive the cells are to constraining conditions, in particular to population density. In examining this question experimentally, it was important to recognize that the sensitivity of the cells to focus formation changes as the cells are passaged in culture depending on the conditions used in passage and how many passages have occurred. Instead of using a single constraining condition in passage such as confluence, the cells were passaged at various subconfluent densities in the presence of reduced CS concentration or in FBS. The use of growth-moderating concentrations of serum revealed that sensitivity to transformation in the assay varies with the cell density used in passage even when the passages are maintained at subconfluent densities.

MATERIALS AND METHODS

Cells and Culture Methods. The cells used in all experiments were of the NIH 3T3 line derived from mouse embryos by five successive isolations of cells from flat, monolayered growth following low-density seedings (6). They were kindly provided by S. A. Aaronson of the National Cancer Institute. Cells were maintained in "60-mm" plastic culture dishes (21-cm^2 actual area) at subconfluent densities by a successive passage of 2, 2, and 3 days each week at densities of 5×10^4, 5×10^4, and 2×10^4 cells per dish, respectively. The medium was MCDB 402 (7), and CS was added to a final concentration of 10% for standard passages. Cells were detached by washing in Tris and treatment with 0.1% trypsin in 0.5 mM EDTA. The serum concentration or type was changed for the experiments as described in the text. Cells were counted in a

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Abbreviations: CS, calf serum; FBS, fetal bovine serum; MCDB 402, molecular cell and developmental biology medium 402.

*To whom reprint requests should be addressed.

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