can be calculated. These calculated values are shown in Table 1 along with the experimental data of Rubin *et al.* (3). As in the present paper, not all the data presented in the earlier work (3) can be so easily accounted for by clonal selection and expansion of rare transformed cells. The point is that in many cases, results interpreted to support an epigenetic mechanism can as easily be applied to selection processes associated with rare genetic events.

Table 1 *Increase of focus-forming cells as a function of time in 2% serum as calculated assuming clonal expansion*

Wk	From Ref. 2	Calculated[a]
0	0.001	0.001
2	0.075	0.076
3	0.27	0.40
5	0.65	0.55
6	0.59	0.55
7	0.35	0.55
8	0.56	0.55

[a] Calculated assuming that normal cells exhibit a growth rate of 0.75/day and a saturation density of 6.5×10^5; transformed cells grow at a rate of 0.85/day and have a saturation density of 1.5×10^6. These values were extracted from Fig. 1 of Ref. 2. Data shown was calculated using a competitive Gompertz model of the form

$$N_{i,t} = N_{i,0} \exp\left(\ln \frac{i}{M_{i,j}}\right) \cdot (1 - {}^{M}e^{-kt})$$

where the number of type i cells at time t, $N_{i,t}$ is a function of the initial number of cells ($N_{i,0}$), the saturation density M_i, and the growth rate k. The total number of cells in the culture is represented by $N_{i,j}$.

Dr. Rubin's contributions have clearly demonstrated the inherent complexity of cellular processes involved in neoplastic transformation. However, current research into molecular mechanisms associated with initiation, as well as progression of the transformed phenotype, demonstrates that mutational and related genetic mechanisms may be at least as complex as putative epigenetic phenomena (4, 5). It is obvious that such phenomena must play some role in carcinogenesis, but genetic changes are difficult to rule out in any system and, based on the wealth of data from many models, are likely to be critical events during multistage malignant transformation.

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Reply

In the preceding Letter to the Editor (1), Garte questions our proposal that nonrandom, adaptive changes underlie the neoplastic transformation and progression of NIH-3T3 cells (2, 3). He prominently states that the appearance of foci in our experimental cultures can be explained by rare mutational events followed by a selective growth advantage of the mutant cell. He only passingly acknowledges that much of our data cannot be explained by such clonal expansion of rare mutant cells.

The main part of our work is, in fact, inconsistent with this mutation-driven mechanism. For instance, we find that the probability of transformation of NIH-3T3 cells is strongly influenced by the nutrient and growth factor environment of the cells (4). Also, the transformed state of NIH-3T3 cells exhibits characteristics of diversity and reversibility which are unlikely to be mutationally based.

The dependence of transformation on the cellular environment is well illustrated in an experiment mentioned by Grundel and Rubin (3). In that experiment, a clonal population of NIH-3T3 cells was continually maintained in log phase growth for 21 weeks and was assayed repeatedly during that period for the ability to form transformed foci. Not a single focus formed in any of 50 assays performed during this period. Near the end of this experiment an aliquot of these non-focus-forming cells was transferred to a medium containing a substantially lower concentration of serum. After only 2 days of growth in this low serum environment these NIH-3T3 cells were assayed for focus formation. Numerous foci formed. The cells maintained in low serum and the cells maintained in higher serum were assayed for focus formation using an identical assay procedure. Therefore, the only reason for differences in focus-forming ability between the two groups of cells was the environment to which the cells had been exposed prior to assay. Cells made foci after a short period of growth in low serum; cells maintained in high serum consistently did not make foci. Obviously the ability to express the focus-forming, or transformed, phenotype was dependent on the environmental history of the cells. This is not the hallmark of a process in which mutation is the necessary and sufficient explanation of causation.

Garte does admit that feasible mutation and cell proliferation rates cannot explain certain changes we have observed, such as an NIH-3T3 population whose ability to form foci increased over 1000-fold in just 3 days (3). Nonetheless, he has produced a graph, his Fig. 1, which purports to show that other results we present can be simply explained by mutation and cell proliferation. In that figure, Garte predicts that the number of foci per culture should reach an uncountable level by 28 days after the appearance of the first focus-making cell in the population. This matches what we observed. However, his selected curve ignores a >50% decline in number of foci that occurs after the 28-day cutoff on his graph (see Ref. 3, Fig. 9). Thus his prediction does not describe what we observed. This decline can hardly be explained on his assumption of selective growth of mutated cells. This decline is consistent, however, with the suppression or reversal of the ability to form foci which we have already documented to occur in the NIH-3T3 system (2, 4, 5).

Garte takes data on rates of focus formation from two of our papers and states that these data could have been generated from populations of cells which mutated at rates of 2×10^{-7}

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and 10^{-5}/cell/generation. The 2×10^{-7} rate of mutation he proposes is incorrectly calculated and also needs to be near 10^{-5} for two reasons: (*a*) saturation densities are between 3 and 7×10^5 cells, not 3×10^6 as he supposed; (*b*) Garte assumes that half of the cultures transformed when originally plated in 2% calf serum at 10^5 cells when, in fact, all of the cultures transformed (see Ref. 3, Fig. 3). The predicted mutation rates, 10^{-5}, are nearly 100-fold greater than Garte's stated reasonable mutation rate for mammalian genes, $1.8–2.2 \times 10^{-7}$. We realize that this 100-fold difference between predicted and observed mutation rates can be explained by the fact that mutation rates might vary over a wide range. Nevertheless, if a mutation-driven mechanism is solely responsible for transformation then the probability of transformation of a cell should at least be positively related to the number of divisions the cell undergoes. Garte fails to note that NIH-3T3 populations that underwent more cell divisions in our study were less likely to make dense foci than were populations which underwent fewer cell divisions (see Ref. 3, Figs. 3–7). In other words, transformation frequency can be inversely related to number of cell divisions, an outcome not expected based on a strictly mutational mechanism of transformation.

The ease with which the transformed phenotype can be modified or reversed is a key to understanding the nature of the process which drives transformation. Here too we find that prior cellular history has an important effect. Populations grown from cells taken from a single focus illustrate this point. Because such a population is derived from a single dense focus most, if not all, of the cells in the population are capable of making foci (see Ref. 5, Fig. 3). When one such population is split into two and one of the resulting subpopulations is maintained in high serum and the other subpopulation in low serum, differences in the morphology of foci arise between the subpopulations. Because the cells maintained in low serum and in high serum are assayed for focus formation with identical procedures, these resulting differences in focus morphology can be mainly ascribed to the nutrient history of the subpopulations between the time the original population was split and the time the resulting subpopulations were assayed for focus formation. The transformed subpopulation maintained in low serum makes larger foci than the subpopulation maintained in high serum (see Ref. 5, Fig. 4). This is consistent with our repeated finding that maintenance of NIH-3T3 cells in low serum media tends to enhance many aspects of transformation including increasing the frequency of focus formation and increasing the size and density of foci (2). The influence of serum concentration on focus morphology implies that there is gradation in the transformed phenotype, in this case gradation in focus size and density, and that cellular history can heritably modify that phenotype even after the transformed state has been achieved. As we point out, reversal of the transformed phenotype is not confined to "spontaneously" transformed NIH-3T3 cells but is found in clones of X-ray-transformed C3H10T½ cells where the transformed phenotype can revert to a nontransformed phenotype when the transformed cells are maintained at low densities (6, 7). Reversal of transformation occurs in more than 90% of carcinogen-induced early lesions of the liver of rats (8) and in naturally occurring dysplastic pigmented nevi in humans, only a small fraction of which ever lead to melanoma (9).

While we disagree with Garte's specific explanation of our results, we nonetheless do not deny the importance of spontaneous, random, heritable change and selection in cellular transformation. Indeed these processes form the basis of a model of transformation and progression we have proposed, termed "progressive state selection" (10). We suggest, however, that the heritable changes leading to transformation need not affect only a miniscule portion of cells, as would be true for mutation. Instead, heritable, transformation-related changes can happen to most cells in a population in response to environmental conditions such as low serum media (2, 11). While it is true that only a small fraction of the cells eventually become so altered as to produce dense foci when placed in low serum, the bulk of the cells undergo transformation-related changes in low serum. These transformation-related changes in which most cells participate include increased saturation density of the population as a whole (2, 11). The progressive state selection model recognizes that cells can express a multitude of metabolic states. The environment serves as an agent which selects from among these metabolic states both by inducing metabolic variation and by allowing cells spontaneously expressing certain metabolic variations to proliferate. In a low serum environment this selection produces an adaptation of cells to the initially growth-constraining milieu. An increased saturation density emerges from this adaptation as cells initially poor at proliferating in low serum become metabolically more competent to do so. We have documented that within a clonal population the metabolic states achieved by this process can be rather stable and passed down to the following generations of descendant cells (12). In a similar manner, Farber (10) has shown that rat hepatocyte metabolic adaptation to toxic environments is a reversible, metabolically mediated process and that cells having undergone this adaptation are capable of dividing to form nodules of hepatocytes which can survive in this hostile environment. These nodules, in turn, are the precursors to malignant liver tumors. The implication in both Farber's *in vivo* model and our *in vitro* model is that heritable, reversible, metabolic changes, changes selected by environmental conditions, are usually a fundamental part of the carcinogenic process.

Understanding the relationship of phenotypic heterogeneity to transformation is a major aspect of our work ignored by Garte, although heterogeneity comprises part of the title of both papers he cites. The heterogeneity we describe includes differences in the capacity of every clone of cells to undergo transformation and differences in the morphology of foci resulting from each independent transformation (2, 3, 13). It is certainly not characteristic of mutation that each resulting phenotype is so unique. Garte acknowledges that mutation cannot explain all the phenomena we describe. However, adaptive, epigenetic change can. A definition of epigenesis is the origin of entirely new structure during development (14). It results from interactions between cells and their environment which includes other cells. It does not exclude genetic changes but these genetic changes are seen as mediating cellular responses to the environment instead of being seen only as random events.

The totality of our laboratory's work is an attempt to develop a network of observations which together can convince researchers that a good part of cellular transformation is not a random event divorced from the environmental conditions in which the cells are maintained or from the inherent heterogeneity of cellular growth characteristics which is expressed when cells are liberated from the hierarchical constraints of the intact tissue and organism (15). Researchers are often critical of the approach we take to studying the nature of malignant transformation because we are not proposing a discrete molecular

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mechanism, such as mutational activation or inactivation of one or more genes, to describe the cellular changes taking place. While mutations may play a role in transformation, they are not seen as the primary source of the cellular metabolic heterogeneity which the environment selects from in the pathway leading to transformation. Although environmental effects can certainly increase the rate of mutation, the level of responsiveness to environmental conditions which we observe in the transformation of NIH-3T3 cells effectively precludes mutation as being the driving force of the process. We view the cell as an active agent responding to its environment and are concerned with those responses which underlie transformation. While Garte, and we too, often couch description of this response in terms of genetics *versus* epigenetics, it is relevant here to cast our questions instead in terms of the roles random *versus* adaptive changes play in transformation. Adaptation is a central process in biology yet is generally forgotten by cancer biologists despite the presence of a large body of information demonstrating the critical role cellular adaptation can play in transformation (10). Unfortunately, few investigators have utilized the inherent advantages of cell culture to examine this phenomenon, as we have endeavored to do. Nearly 5 decades ago, the question of whether induced change or spontaneous change was the agent of cell population response to challenge and perturbation was investigated in the classic experiments of Luria and Delbrück (16). Their conclusions on the spontaneous nature of change have become pervasively established as the foundation for understanding the nature of cellular response to the environment. We question whether this is the best foundation to build upon in studying phenomena, such as cellular transformation, which are not as discrete as the ability of a cell to synthesize a single molecule under conditions where the cell quickly dies if it does not produce that molecule in appropriate quantities. The cell-environment interactions we have discussed here indicate that an alternative conceptual framework, such as the progressive state selection model, will more effectively account for the diversity of cell behavior associated with transformation.

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