Clonal Selection versus Genetic Instability as the Driving Force in Neoplastic Transformation

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ABSTRACT

Recent clonal studies of spontaneous neoplastic transformation in cell culture indicate that it develops at confluence in a small minority of individual clonal populations before it does in the uncloned parental culture. Either preferential selection of spontaneous variants or genetic destabilization in clones can be inferred to explain the result. In the present experiments, using a subline of NIH 3T3 cells that is relatively refractory to transformation, we demonstrate unequivocally that transformed foci appear under selective conditions in some clones long before there is any sign of neoplastic change in the polyclonal culture from which they were derived. Because the transformed cells that appear in the susceptible clones are not inhibited in the size or number of foci formed on a confluent background of the uncloned parental population, the genetic events underlying transformation must occur much less frequently in the latter. This disparity can be accounted for by the much larger number of selectable cells in the susceptible clones at confluence than in the parental culture, where such cells are a minority. The preferential transformation exhibited by experimental isolation and expansion of susceptible clones accords with evidence from various sources that neoplastic transformation in culture is a multistep process dependent primarily on selection of spontaneously occurring genetic variants. There is no necessity to posit a significant role for genetic destabilization in neoplastic transformation. These considerations bolster computer models of human cancer that implicate selective expansion of rogue clones rather than genetic instability as the driving force in the origin of most tumors. Both the genetics of the selected clone and the epigenetics of the selective environment would then contribute to tumor development.

INTRODUCTION

It is well established that most human cancers are of monoclonal origin (1–4). Given the nature of neoplastic growth, selection is an intrinsic factor in the expansive growth of the progeny of a single cell that produces a tumor. In recent years there has been an emphasis on genetic instability as the driving force in the development of cancer (5). Much of this emphasis can be attributed to the multistep progression of cancer and the conventional estimates of normal somatic mutation rates that are far too low to account for its incidence (6). It was suggested that a mutator phenotype arises in which the rate of mutation is greatly increased. Mutations in mismatch repair genes do in fact raise the rate of mutation, particularly in simple sequence repeats, and underlie hereditary nonpolyposis colorectal cancer (7). Defects in mismatch repair do not, however, precede the APC4 mutations which provide the selective growth advantage that initiates most cases of the much more prevalent sporadic type of colorectal cancer (8, 9). Computer modeling indicates that selection is the driving force in the initiation of most human cancers and genetic instability is a late event in their development (10, 11). On this basis, mutation that confers selective growth advantage would be considered the critical event in the origin of cancer.

Although the studies of the genetics of human cancer have highlighted the role of selection, they provide little information on the dynamics of the process. However, studies of spontaneous neoplastic transformation in cell culture provide considerable insight on the subject. The first indication that selection plays a role in spontaneous transformation came from studies of the adaptation of mouse embryo fibroblasts to cell culture, which revealed that there were large increases in saturation density after multiple passages of cells at high density (12). Because the multiplication of normal cells is inhibited by contact at high population density, it appeared that there was selection of variants that overcome that inhibition, a characteristic of neoplastic cells. The role of selection was supported by the finding that mouse embryo fibroblasts passed at high density gained the capacity to produce sarcomas in syngeneic mice within 3 months, whereas those passaged at low density did not become tumorigenic through the entire 9 months of the study (13). Additional support for selection as the driving force in spontaneous transformation came from findings that it was produced in established lines of mouse fibroblasts by procedures that selectively inhibit the multiplication of normal cells, such as suspension in soft agar (14), long-term contact inhibition in confluent cultures (15), or reduced growth rate in either low serum concentration (16) or in fetal serum with suboptimal growth-supporting activity (15, 17).

All of the foregoing studies used fibroblasts, but most human cancers are of epithelial origin (18). It is therefore of particular significance for human cancer that selective conditions were found to promote transformation in epithelial cells. Diploid rat liver epithelial cells kept in a stationary state at confluence for 3 weeks between every monthly passage gained the capacity in only 20 cell divisions to produce liver carcinomas in syngeneic rats, whereas those passaged weekly before they became confluent did not produce tumors until they had undergone about 6 times as many divisions (19). A significant sidelight occurred when the liver epithelial cells were treated with a mutagenic carcinogen before being cultured under selective and nonselective conditions. The treated cells became tumorigenic under both regimes at about the same disparate times as they had in spontaneous transformation, indicating that selection was the dominant force in transforming carcinogen-treated cells as well. Indeed, the carcinogen seemed to add little to the spontaneous variation that provided the altered cells for selection. There was no correlation between aneuploidy and tumorigenicity in the spontaneously transformed populations: populations of aneuploid cells often were nontumorigenic. However, there was a correlation in the carcinogen-treated cells, indicating that the carcinogen treatment produced a specific type of aneuploidy that was associated with carcinogenesis.

The question of selection versus genetic instability arose again in spontaneous transformation of the NIH 3T3 line of mouse fibroblasts. Cells from the original transformation-sensitive stock of NIH 3T3 cells gave rise to clones among which there was a small minority that started to produce a few dense transformed foci at confluence sooner than the polyclonal population from which the clones were derived.
(20). When six of the clones were cocultured before the first round of
confluence, they produced many fewer dense foci in subsequent
rounds than expected from averaging the number that occurred in
parallel cultures of the individual clones (21). It was proposed that the
individual clones were genetically unstable, and intimate contact with
other clones stabilized them. We pursued this proposal further in the
present study using clones of a subline of NIH 3T3 cells that is
relatively refractory to spontaneous transformation. We report that a
minority of the clones produced well-defined transformed foci well
before there was any sign of transformation in the uncloned parental
culture. A quantitative analysis of the results shows that the disparity
can be fully accounted for by the much smaller number of transform-
able cells from the susceptible clones in the polyclonal parental
population at confluence than there are in each susceptible clone at
confluence. Simple numerical accounting thus eliminates the need for
the arbitrary assumption of genetic destabilization by clonal isolation
to explain preferential transformation in the minority of clones that
were susceptible.

This conclusion, of course, concurs with William of Ockham's
maxim, “Multiplicity ought not to be posited without necessity,”
otherwise known as Occam’s Razor.

MATERIALS AND METHODS

The A’ cells used here are a transformation-resistant subline (20) of the
original NIH 3T3 line of mouse embryo cells (22). They were derived by 146
LDPs from the primal stock of NIH 3T3 cells. The original transformation-
sensitive stock of NIH 3T3 cells (which we designated SA’) as well as its
clones readily underwent progressive neoplastic transformation in serial
rounds of incubation at confluence in a standard assay for transformed foci (15,
20). By contrast, the A’ subline was difficult to transform under these condi-
tions (23) and in the present experiments failed to exhibit unequivocal focus
formation through many serial rounds of the standard assay. Cells were
maintained by LDPs of 5 × 10^3 cells in 21-cm^2 plastic culture dishes. The
growth medium was MCDB 402 (24), containing 10% CS vol/vol. A primary
(1°) assay for transformed focus production was done by seeding 10^5 cells in
MCDB 402 with 2% CS, and incubating the cultures for 14 days with medium
changes every 3–4 days. The cells grew to confluence in 4–5 days, and
remained at confluence with no further increase in cell number between 7 days
and the end of the assay at 14 days. Under this selective condition, progres-
sively transforming and fully transformed cells continued to multiply to form
foi of overlapping cells interspersed among the surrounding monolayered
population. Secondary (2°) assays were duplicates of the 1° assay using sister
cultures of the latter, and further serial assays up to the sixth (6°) assay were
done in the same way using cells from the termination of the previous assay.
In some of the later serial assays, when heavy transformation was expected,
10^6 or 10^5 cells were also diluted with 10^5 nontransformed uncloned A’ cells
from the LDPs that had never been at confluence. Some assays were deliber-
ately prolonged by extending the incubation period to 28 days. The cell count
at the end of each serial assay represented the saturation density of the culture.

The cells were cloned by seeding an average of one cell/well in 96-well
plates (Falcon 3072) with growth medium MCDB 402 plus 10% CS vol/vol.
The approximate number of cells/well was monitored under the microscope
and recorded at 2, 4, 5, and 6 days. Wells containing more than one colony
were excluded. Clones were designated by the coordinate position of the well
they occupied. Twenty-seven clones were harvested by trypsinization at 5, 6,
and 7 days and divided into five groups, as shown in Figs. 1-3, depending on
their initial growth rates as estimated from the cell number in the wells. The
clones were expanded in 21-cm^2 dishes and subcultured five times in LDPs of
5 × 10^3 cells/dish every 4 days. The uncloned cells were maintained in parallel
with the clones by the same LDPs. The growth rate of each clone and of the
uncloned culture was estimated by the cell yield at each passage. At the fifth
passage, an aliquot of the cells was used to initiate the first set of six serial
assays at confluence for transformation, and the remaining cells were used to
continue the LDPs. At the 13th LDP, a second set of four serial transformation
assays was initiated.

The growth rate of every clone was determined after the first and second serial
assay of the first set and after each of three additional assays for groups 2 and 5.
Growth rates were initiated with the cells from the confluence assays culture by
passaging them once at low density (5 × 10^5 cells/dish) for 2 days to recover from
contact inhibition, and passaging them again at low density into 4 culture dishes.
Two of the cultures were counted at 1 day and another two at 4 days. These
measurements of growth rate were done in parallel with similar ones of cells from
the regular LDPs that had never experienced crowding. Growth rates were calcu-
lated as PD/D. The relative growth rate of cells recovered from the confluence
assay cultures was determined by dividing its PD/D by that of the control LDP cells.
All measurements of growth rate of the clones were accompanied by the same
measurements of the uncloned culture.

The best description of their morphology comes from viewing photographs of
selected clones and the uncloned culture. Those confluent assay cultures that
were not tympsinized for passage or growth rate determinations were fixed with
Bouin’s reagent and stained with 4% Giemsa buffered at pH 7.0 to determine
the extent of transformed focus formation. As is documented with photographs
(e.g., Fig. 4), the uncloned culture produced no discernible foci in the first set of
serial assays, but many of the clones did. However, the foci produced by the
A’ clones did not reach the large size and high cell density of those produced
by the transformation-sensitive SA’ cells in our previous studies (20, 23, 25).
The scale used to characterize the degree of morphological transformation is
shown in Fig. 6. Where the transformation was particularly strong, it resulted
in a marked increase in saturation density of the assay culture.

RESULTS

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The growth rate of every clone was determined after the first and second serial
Saturation Densities of Clones in Serial Assays at Confluence.

Fig. 3 shows the saturation densities after each of the six assays of the first set. The uncloned culture (dotted line in group 2) had a higher saturation density \(6 \times 10^5\) than any clone in the 1° assay, but it dropped to half that value in the 2° assay and remained at that low level for all of the subsequent assays. It produced no foci up to the 6° assay, when some very faint foci appeared (Fig. 4). Clone 1a of group 1 showed a steady increase in saturation density between assays 3° and 6° with a final value above \(10^6\) cells/culture in the final assay. This was consistent with the appearance of many small foci in clone 1a beginning in the 3° assay. Clone 10c of group 2 exhibited a marked increase in saturation density between assays 5° and 6° with a final value above \(10^6\) cells/culture in the final assay. This was consistent with the appearance of many small foci in clone 10c beginning in the 5° assay. Clone 9g of group 2 also increased significantly in saturation density in the 6° assay as well as in the number of moderately dense foci. Clone 6h displayed a more modest increase in saturation density in the 6° assay and had a large number of foci, but they were lighter than those of 10c and 9g. In group 3, clone 2c increased steadily in saturation density between assays 3° and 6° just as clone 1a of group 1 had. This was in keeping with a large increase in the number of moderately dense foci. The only clone in groups 4 and 5 to show a significant increase in saturation density was clone 11a. It had already produced a few light foci in the 1° assay. These became denser and larger in the 3° assay and increased in number in the 4°, 5°, and 6° assays, which had increases in saturation density. Fig. 3, bottom right, also shows the average and SE of saturation densities for all of the clones that began to exceed that of the uncloned culture in the 4° assay. Whereas the saturation density measurements of the late assays showed prominent increases for five of the clones with no increase for the uncloned culture, it was evident by inspection that many clones were producing visible foci that did not result in saturation density increases. An elementary way to document this, which captures the essence of the phenomenon that more sophisticated measurements cannot, was through photographs of the culture dishes. These included cultures from the first set of six serial assays and cultures from a second set of four serial assays as described below.
Table 1  Reduction in growth rates (PD/D) of clones and their uncloned parental culture after recovery from confluent assays

<table>
<thead>
<tr>
<th>Clonal group</th>
<th>Cells from LDPs (PD/D)</th>
<th>Cells from confluent culture (PD/D)</th>
<th>Assay no. (PD/D)</th>
<th>Column III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.77 ± 0.19</td>
<td>(1°) 1.33 ± 0.18</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.60 ± 0.10</td>
<td>(2°) 1.41 ± 0.17</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.72 ± 0.10</td>
<td>(1°) 1.36 ± 0.13</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.61 ± 0.12</td>
<td>(2°) 1.37 ± 0.10</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.54 ± 0.17</td>
<td>(1°) 1.17 ± 0.32</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.42 ± 0.21</td>
<td>(2°) 1.20 ± 0.26</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>A’</td>
<td>1.62 ± 0.11</td>
<td>(1°) 1.35 ± 0.12</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.52 ± 0.11</td>
<td>(2°) 1.40 ± 0.13</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.44 ± 0.17</td>
<td>(1°) 1.13 ± 0.31</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

Visual Demonstration of Preferential Progressive Transformation in Clones. Fig. 4A illustrates progressive transformation in 2 clones (1a and 2e) and its absence in the uncloned A’ parental cells spanning six serial assays of the first series. Clone 1a was negative in the 1° assay, and began showing light foci in the 3° assay (not shown); the light foci became confluent with one another in the 4° assay, and progressed to small, dense foci on a confluent background of light foci in the 6° assay. Clone 2e was also negative in the 1° assay but began to produce light foci in the 2° assay (not shown) that progressed to discrete, moderately dense foci in the 4° assay and to dense foci on a confluent background of lighter foci in the 6° assay. The background of light foci was not as thick as that of clone 1a in the 6° assay, which was reflected in the higher saturation density of the latter (Fig. 3). The uncloned A’ cells remained completely negative through the 5° assay but exhibited scattered, small, light foci in the 6° assay. The results indicate that the diverse cells of the uncloned parental culture, which must have contained many cells similar to those of clones 1a and 2e, underwent much less transformation than those clones.

We entertained the possibility that some transformation was occurring in the uncloned culture but was not expressed because of growth-suppressing interactions among the diverse cells of this polyclonal mixture that did not occur in the more homogeneous clones. To investigate this possibility, the individual clones and the uncloned parental cells were diluted for the 6° assay, and 10^5 cells were seeded with a 10-fold excess of 10^5 nontransformed cells from the LDPs of the uncloned A’ clone or delayed onset of transformation. The uncloned A’ cells failed to display any foci at either 14 days or 28 days. The clone 2e cultures at the bottom of Fig. 5 were from a later assay of the second set and showed development from light to dense foci between 14 and 28 days. The results show the diversity of dynamics of focus formation among the clones and reinforce the evidence of greatly reduced transformation in the uncloned population relative to some of its clones.

Overall Trends in Transformation among All of the Clones in the First Two Sets of Serial Assays. The degree of transformation of every clone and of the uncloned population was classified in each assay of the first two series according to the scale in Fig. 6. The scale of transformation is based on the seeding of 10^5 cells in each assay but was elaborated in the later assays by seeding 10^6 cells with an excess (10^5) of nontransformed cells from the LDPs of the uncloned A’ cells.

to determine whether foci would develop in cultures that were negative in the standard 14-day assay period and also to reveal expansion or progression of foci that were already apparent at 14 days. The extended assay was done with cells from the second set of serial assays and representative results can be seen in Fig. 5. The foci present in clone 2e at 14 days increased in size at 28 days, indicating the selective growth advantage of cells in the foci. Clone 4b exhibited no foci at 14 days, but a few moderately dense foci and many small, light ones could be seen at 28 days, indicating either slow growth of the transformed cells in this clone or delayed onset of transformation. The uncloned A’ cells failed to display any foci at either 14 days or 28 days. The clone 2e cultures at the bottom of Fig. 5 were from a later assay of the second set and showed development from light to dense foci between 14 and 28 days. The results show the diversity of dynamics of focus formation among the clones and reinforce the evidence of greatly reduced transformation in the uncloned population relative to some of its clones.
In those cases which included assays for $10^4$ cells, the classification in the relatively strong (+) and moderate (+) categories for $10^5$ cells was reinforced by the appearance of discrete foci in the assay of $10^4$ cells, which were not seen in the weak (−) category. The graphs in Fig. 7 show an increase in the number of transformed clones in each category with successive serial assays. About half of the clones had moderate-to-relatively strong transformation in the last two assays of the first series, whereas the uncloned A' cells were negative through assay 5° and only weakly transformed in assay 6°.

The proportion of clones in all categories of transformation was higher in all of the second set of assays than in comparable assays of the first set, indicating some progression during the eight additional LDPs between the beginning of the first and second sets. The uncloned culture was negative in assays 1° and 2° of the second series, but exhibited variable low-level transformation in assays 3° and 4°. Hence, some transformation appeared in the polyclonal parental culture of the second set of assays at an earlier point than it did in the first set and was associated with earlier transformation among the clones.

However, the parental transformation was at a much lower level than might be expected from the fact that one-third of the clones already exhibited relatively strong transformation in the 3° assay, and there would have been many thousands of such clones in the assay of $10^5$ cells of the parental culture. The apparent paucity of well-defined foci in the parental culture will be analyzed below in terms of the minority fraction of productive cells as compared with the number in some isolated clones. In this regard, it is notable that most of the clones that displayed unequivocal transformation in the first set of assays did so in the second set, indicating that transformability is a heritable property of the clones, although subject to population drift in multiple LDPs (25).

**DISCUSSION**

Our previous studies, which showed a higher rate of confluence-mediated transformation in recently cloned cell populations than in their polyclonal parental population (20, 21), left unresolved the
possibility that the mixed population simply suppressed the proliferation of transformed cells rather than somehow avoiding the events that underlie transformation. Here we showed that the transformed cells from about one-fifth of the clones produced characteristic multilayered foci against a confluent background of the nontransformed polyclonal parental population. (See Figs. 4B and 6.) Hence, in the regular seeding of 10^5 cells of the uncloned parental population in the assay for focus formation, there would be at least 2 \times 10^4 cells capable of undergoing and clearly exhibiting transformation if they were cultured as isolated clones, yet no clearly identified transformed foci were detected in six serial assays at confluence. The absence of such foci in the polyclonal parental population indicates that some feature of the clonal mixture reduced the number of genetic changes/culture that underlie the transformation seen in cultures of the isolated clones. This conclusion is augmented by the finding of large increases in saturation density of a number of clones with successive rounds of confluence when there was no such increase in the polyclonal population. Each of these clones was derived from a single uncloned parental cell. Hence, the transformed cell foci arising from the uncloned parental population exhibited essentially uniform transformation, with no significant clonal variation in their transformation with time in culture. This is consistent with the clonal analysis of the data obtained from the induction of transformation of the two individual parental strains. See Figs. 5 and 6.

Fig. 4. Preferential progressive transformation in clones and their capacity to produce transformed foci on a confluent background of uncloned A' cells. A. 10^5 cells of all of the clones and of the uncloned A' culture were seeded in six serial assays at confluence of the first set. Assays 1°, 4°, and 6° of clones 1a, 2e, and the uncloned cells are shown. B. 10^4 cells from the 6° assay of all of the clones and of the uncloned A' culture were mixed with 10^5 cells of the standard LDP of the A' cells and seeded for assays at confluence. The assay of clones 2e and 10c and the uncloned A' cells with a background of LDP A' cells are shown, as is the assay of 10^5 LDP cells by themselves at the lower right.

Fig. 5. Effect on focus formation of extending the assay at confluence to 28 days. The top three pairs are from the 2° assay of the second set; the bottom pair is from the 4° assay of the second set. Some cultures were fixed and stained at the standard assay time of 14 days and others at 28 days, as indicated.

Fig. 6. Scale of transformation of clones and the uncloned A' culture from the 6° assay of the first set. The assays were initiated with 10^5 cells in the left column, and 10^4 cells mixed with 10^5 cells of LDP uncloned A' cells in the right column. This was the first assay in which the uncloned A' cells exhibited any sign of transformation, although it was in the (-) category. Only the + and ++ categories produced distinct foci in the mixture of 10^4 cells with 10^5 LDP uncloned A' cells.
loration (Fig. 3). The failure of focus formation in the polyclonal population persisted even when the incubation period of the assay was increased from 14 to 28 days, whereas the foci initiated in the clonal cultures were increasing in size and number.

In isolating clones and expanding them to large populations in the selective assay at confluence for transformed foci, we increase the number of cells originating from a single cell in comparison with the number of clonally related cells in a polyclonal population. That is, the ratio of cell numbers in any particular clone at saturation density to the number of phenotypically similar cells in the polyclonal population is inversely proportional to the fraction of those clones in the polyclonal population. If we take as a class of clones, for example, those which eventually produce well-defined ++ transformed foci (Fig. 6), an isolated clone of that class will reach confluence with a much larger number of potential focus formers than the total number of similar cells in the polyclonal population, in inverse proportion to the fraction of ++ focus-forming clones among all clones in the latter population. The smaller the fraction, the larger is the differential.

There were 5 of 27 clones that consistently produced ++ foci in the last three assays of the first set, or roughly one-fifth the total number of clones. Therefore, the total number of ++ focus producers in the polyclonal population at confluence will be about one-fifth of the number in each of the isolated ++ clones at confluence. The saturation density of each of the cloned and uncloned cultures remains roughly constant between 2 and 4 × 10^5 cells during the first few assays (Fig. 3). Because the production of foci is dependent on the number of focus-forming cells at confluence, the isolated clones with the potential for producing ++ foci would be expected to display such foci in a much earlier assay than the polyclonal population. Well-defined focus production first appears in two clones in the 3° assay of the first set, and reaches a plateau of five consistent focus producers in the 4° assay. The failure of such foci to appear in the polyclonal population in any of the six assays of the first set (nor in any assay of the second set) follows from the 5-fold reduction in number of potential ++ cells in that population compared with the number in each isolated clone. According to this crude estimate, it would take at least 15 serial assays of the polyclonal population to produce foci of the ++ type. A similar calculation can be made for the combined number of + and ++ focus-producing clones, which constitute about half of the total number of clones, e.g., if there had been a large number of polyclonal cultures, and the number of serial assays extended beyond six, a fraction of them might be expected to exhibit foci by the 7° assay. If we include the somewhat marginal and sometimes transient ± focus-forming clones in the total, which reaches about half its maximum at about the 3° assay, it is not surprising that some weak foci appear in the polyclonal population by the 6° assay. Similar analysis can be applied to the second set of assays in Fig. 7. Therefore, the relative ease with which the clonal populations are transformed compared with the polyclonal parental population can be explained simply in terms of clonal diversity in the latter.

In earlier papers using the original transformation-sensitive line that produced large, dense foci (20, 21), and particularly where six isolated clones were compared with a mixture of the six clones, we found that some of the isolated clones progressed to dense focus formation before any were seen in the mixture (21). This was interpreted by assuming that isolated clones were genetically unstable at confluence, and that they were stabilized by metabolic cooperation with one another in the mixture, thereby preventing early steps in progression. The simpler explanation is that only three of the six clones progressed to dense focus formation in the 2° assay and did so at different rates. According to the inverse proportionality between a cloned and uncloned population in the number of potential focus-formers established above, there would be a 2-fold reduction of potential focus-formers in the mixture with a proportionate delay in focus formation to assays beyond those done in the experiment.

Because the argument of clonal diversity fully accounts for the paucity of foci in the polyclonal parental culture, the previous hypothesis of clonal destabilization is unnecessary. In fact, there is no independent verification that clonal isolation results in an increase in the rate of transformation-related variation. This possibility had been considered (20, 21) because transformation was frequently associated with a heritable reduction in the growth rate of cells at low population density (26–28), which could be ascribed to genetic damage with accompanying mutations. However, selection of progressively transformed cells by LDP in low CS concentration (16) or fetal bovine serum (15, 17) does not result in a reduction in growth rate. A complementary negative finding is that the heritable reduction in growth rate induced by methotrexate treatment of NIH 3T3 cells does not produce transformation (29). Perhaps more cogently, treatment of diploid liver epithelial cells with a mutagenic carcinogen only marginally increased their spontaneous rate of transformation under selective conditions and had no significant effect on the rate of transformation under nonselective conditions (19). Whereas we cannot exclude a role for genetic destabilization in transformation for all conditions, there is no reason to postulate such a role in the present case because the preferential transformation of minority clones can be fully accounted for by the selection and expansion of susceptible cells. Additional evidence against the destabilizing effect of clonal isolation is the existence of some clones even in the transformation-sensitive subline of NIH 3T3 cells that fail to transform in many repeated rounds of selection (25) as well as the many that show no transformation or only marginal effects in the present experiments (Fig. 7).

The conditions in culture for selective growth of cells in various
stages of transformation are those which preferentially inhibit multiplication of nontransformed cells. We achieved artificial selection in the present paper by isolating and expanding clones, a few of which were particularly susceptible to confluence-mediated transformation. The contact inhibition of confluent cultures was also used to drive selection of spontaneous transformants in a line of diploid liver epithelial cells (19). Because all epithelial cells in the organism are in direct contact with their homotypic neighbors, confluence per se does not act as a selective force for carcinoma development in vivo. In the case of colorectal cancer in humans, biallelic mutations at the APC locus apparently confer selective advantage on stem cells in the colonic crypt, which allows them to continue to multiply above the bottom two-thirds of the crypt and thereby to form dysplastic adenomas (7, 30). The particular mutations that underlie selection in culture are unknown. The differences in morphology of transformed foci (20, 31) and the number of steps in transformation (32) indicate that many genetic loci are involved in the process. The effect of these mutations in culture is formally the same as that of APC in the colon in that both endow selective advantage to the cells under conditions that regulate the multiplication of normal cells. Rapid exponential multiplication of cultured cells minimizes or even selects against transformation (17), perhaps because transformed cells tend to multiply, when unconstrained, at a lower rate than nontransformed cells (26–28). The only parallel in vivo might be the relatively unconstrained cell growth of early embryogenesis during which tumor development is minimized.

Another form of selection for transformation in culture is to lower the concentration of growth factors while maintaining exponential growth at low population density (16, 17). The corollary in vivo would be a long-term reduction in the concentration of a hormone that stimulates cell multiplication. Accordingly it has been suggested that the diminution of testosterone production that occurs in aging men selects for testosterone-independent cells and contributes to the steep increase in prostate cancer beyond the age of 50 (33). It has also been pointed out that deprivation of estrogenic hormones, which produces regression of spontaneous mammary tumors in mice, eventually results in progression of the residual neoplasia to a hormone-independent state (34).

Selection also plays a role in chemical carcinogenesis of experimental animals. Farber has proposed the resistant hepatocyte model of liver carcinogenesis based on the findings that the cells in early stages of tumor development multiply in the presence of a carcinogen which is inhibitory to the growth of the normal hepatocytes (35). There are a number of cases in rodents in which carcinogens apparently select for the growth of cells with preexisting mutations in ras genes (36, 37). Long-term repeated applications of nonmutagenic promoters alone give rise to a few liver tumors (38, 39) which apparently result from selection of preexisting enzyme-altered cells that have a defect in growth control (39, 40). The compensatory hyperplasia that is an early step in experimental carcinogenesis of the skin (41) and colon (42) may also favor clonal expansion of cells that are defective in growth control and thereby promote the accumulation of mutations associated with progression.

If selection is to play the dominant role in carcinogenesis, there must, of course, be sufficient rogue clones from which to select. It was recently pointed out that conventional estimates of mutation frequencies in normal tissues may be far too low (43). For example, 1 of 4,000 normal kidney cells in aging men was found to have a mutation at the X-linked HPRT locus (44), which encodes a much smaller gene than many tumor suppressor genes (43). Thus biallelic mutations of tumor suppressor genes may not be uncommon in normal tissues; mutations in micro- and minisatellites, some of which occur within functional genes, occur at much higher rates. There are enough cell divisions of normal intestinal stem cells and growing tumors to account for the 11,000 mutations/cell reported in colorectal polyps and carcinomas (45) without inferring an increased mutation rate. Simpson (43) suggests that the aging human body accumulates enough mutations to account for multistep carcinogenesis by selection of preexisting mutations. This would be especially true if selective conditions for clonal expansion were heightened with age. One indication that this is the case is that rat hepatocarcinoma cells are much more tumorigenic when inoculated into the liver of old rats than into that of young rats (46). Another indication of tissue-wide changes with age is the slowdown in multiplication rates of intestinal mucosa accompanied by a large increase in heterogeneity among the crypt cells in the onset of DNA synthesis (47, 48).

In fact, the combination of accumulated mutations and disturbances of the regulatory environment associated with aging raises the question of why cancer is not more common than it is. It may be that the normal architecture of a tissue maintains a regulatory environment that keeps rogue clones behaving normally or disables them by apoptosis. This would be in keeping with the first principle of Elsasser’s theory of organisms, which postulates that “there can be regularity in the large where there is heterogeneity in the small: ‘order above heterogeneity’” (49). This elementary principle has been expressed as macrodeterminism in developmental biology (50) and as holistic control of neuronal excitation in the brain (51). A classical example is the teratocarcinoma of mice that is produced when young mouse embryos are grafted into the testes of adult mice (52): inoculation of core cells of the teratocarcinomas into blastocysts results in their integration as normal elements in chimeric tissues of many types, including reproductively functional sperm (53). The primary principle of ordered heterogeneity subserves this behavior and several other genetic and epigenetic aspects of the cancer problem (54). It therefore provides a theoretical foundation to account for the long-term stability of multicellular structures despite the accumulation of many somatic mutations (55–57).

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Clonal Selection *versus* Genetic Instability as the Driving Force in Neoplastic Transformation

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