Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1903–1907, March 1982
Cell Biology

Self-normalization of highly transformed 3T3 cells through maximized contact interaction

(magnesium/medium depletion/growth regulation)

HARRY RUBIN AND BERBIE M. CHU

Department of Molecular Biology, University of California, Berkeley, California 94720

Contributed by Harry Rubin, December 21, 1981

ABSTRACT **Nontransformed and moderately and highly transformed BALB/c 3T3 cells maintained on small coverslips in a large volume of medium multiplied to 2, 3, and 4 times higher population density, respectively, than they did in conventional cultures. Deprivation of Mg^{2+} caused highly transformed cells on coverslips to assume the appearance of nontransformed cells, decrease their rate of multiplication, and stop further growth at a much lower saturation density than the same cells in physiological Mg^{2+}. The latter cells reached a saturation density of $10^6/cm^2$ and their rate of DNA synthesis decreased progressively with increased crowding. At saturation density, cells in physiological Mg^{2+} took on an appearance and arrangement similar to normal fibroblasts. They developed a high requirement for serum to initiate DNA synthesis. When transferred at low density, they flattened out on a plastic surface and maintained the appearance of nontransformed cells for ≈1 day. Onset of DNA synthesis and multiplication in the transferred cells was delayed for periods characteristic of quiescent nontransformed cells stimulated by fresh medium or transfer. Cells from crowded coverslips were ≈1/10th as efficient at colony formation when suspended in agar as cells from uncrowded coverslips. They also had a significantly lower Mg^{2+} content. The crowded cells returned to their transformed morphological and growth behavior 2 to 3 days after transfer at low density. We conclude that a very high degree of crowding causes highly transformed cells to revert to the phenotype of nontransformed cells. Other treatments such as deprivation of Mg^{2+} or inorganic orthophosphate can achieve similar results. It appears that a balanced reduction in rates of metabolism and multiplication can restore the normal phenotype to transformed cells, implying that they differ only quantitatively from nontransformed cells. The putative role of Mg^{2+} in the regulation of multiplication and in transformation of animal cells is discussed.**

When the concentration of Mg^{2+} in the medium of transformed 3T3 cells is reduced below ≈40 μM (Mg^{2+} deprivation), their growth rate decreases and they assume the appearance and arrangement of nontransformed cells (1). They also acquire the serum dependency and Ca^{2+} content of nontransformed cells (2). In these particulars, Mg^{2+} deprivation "normalizes" the transformed cells but, in another particular, it does not appear to do so. Thus, nontransformed cells multiply to a maximal number ("saturation density") that is characteristic of the medium used; Mg^{2+} deprivation decreases that saturation density in proportion to its inhibition of the initial rate of multiplication (1). Transformed cells that have reached their saturation density under conventional culture conditions have not been shown to accumulate in the G_1/G_0 period to the same extent as nontransformed cells do (3–5). Although Mg^{2+} deprivation decreases the initial rate of multiplication of transformed and nontransformed cells to the same extent, the former usually continue to multiply until they approach the saturation density of cells in physiological Mg^{2+} concentrations (2).

The failure of Mg^{2+} deprivation to decrease the saturation density of transformed cells suggested that the limit on cell number was determined by depletion of metabolizable constituents of the medium rather than by growth inhibitory interactions between the cells. To minimize the problem of medium depletion and thereby evaluate the contribution, if any, of cellular contact interactions to the regulation of multiplication of transformed cells, cells were grown on coverslips of small surface area in a large volume of a medium that was regularly replaced. They were then found to reach a much higher saturation density than that found under conventional conditions and did so without depleting the medium. There was a progressive decrease in the rate of [^{3}H]thymidine incorporation as the population density increased and the cells flattened and took on a regular arrangement. This paper describes these and other changes indicating that population density has a profound effect on the transformed phenotype. We also show that Mg^{2+} deprivation decreases the saturation density of the transformed cells. The results are discussed in light of the view that transformed cells differ only quantitatively from nontransformed cells and that the intracellular Mg^{2+} state plays a role in expression of the transformed phenotype.

MATERIALS AND METHODS

Cells. The cells were derived from the A31 clone of BALB/c 3T3 cells. Clone 21 was a twice-recloned flat nontransformed line and clone 14 was a spontaneously transformed subclone of these cells. Clone 14c represented <30 passages of the transformed line and consisted mainly of slender and elongated cells with some spheroidal cells, randomly arranged, with an ≈10% colony-forming capacity when suspended in agar medium. Clone 14a represented a further transformation of clone 14 that appeared after ≈90 passages. In sparse populations, clone 14a consisted mainly of spheroidal cells with an ≈80% colony-forming efficiency in agar medium. The procedures for transferring and maintaining the cultures were as described (1, 2). The medium used was MCDB 402 (6)/10% calf serum and it was dialyzed against three changes of 0.15 M NaCl for use in the Mg^{2+} deprivation experiments.

Culture Methods. "Conventional culture" refers to the method of seeding cells at the appropriate densities over the full surface of 35-, 60-, or 100-mm plastic tissue culture Petri dishes with surface areas of 9.1, 21, and 56 cm^2 containing 2, 5, or 12.5 ml of medium, respectively. "Coverslip culture" refers to seeding cells on a coverslip 18 × 18 mm square (3.24 cm^2) in a small plastic Petri dish and transferring the coverslip with attached cells to a 100-mm dish with 12.5 ml of medium. On subsequent incubation, clone 21 cells remained attached to the coverslip and multiplied only there. A very small fraction of clone 14c cells and a somewhat larger fraction of clone 14a cells

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

detached from the coverslip during multiplication and started "outlying colonies" on the open plastic surface of the Petri dish. Since the cells of these outlying colonies were at a much lower density than the cells on the coverslips proper, their growth rate and appearance provided a control to distinguish between the effects of medium depletion and those of direct cell–cell interactions on the cells on the coverslips. Medium was changed every 2 or 3 days except as indicated and 17 hr before labeling with [^{3}H]thymidine to minimize the effects of medium depletion on incorporation of the labeled compound. Growth rates of cells on the plastic surface of the dishes and on the glass surface of the coverslips were indistinguishable.

Procedures for counting cells, labeling with [^{3}H]thymidine, determination of radioactivity by scintillation counting or autoradiography, and measuring protein content and Mg^{2+} content were as described (7).

To compare the appearance and growth potential of cells derived from sparse and crowded cultures under similar conditions, cells were trypsinized and seeded at $2 \times 10^3/cm^2$ in fresh medium. At intervals after the transfer, their appearance was noted and [^{3}H]thymidine incorporation and cell numbers were determined. The capacity of cells to multiply without attachment to a solid substratum was evaluated by seeding them in Bacto-Difco agar. A bottom layer was made in 60-mm dishes with 7.0 ml of 0.9% agar containing MCDB 402/10% calf serum/10% tryptose phosphate broth. The cells were seeded in a 1.5-ml top layer of 0.4% agar and the same nutrients as the bottom layer. Colonies [$\geq$150-μm diameter ($\approx$50 cells or more)] were counted in an inverted microscope after incubation for 3 weeks. To determine the degree of depletion of a used medium, clone 14a cells were seeded in the medium or in fresh medium at $2 \times 10^3/cm^2$, and the extent of multiplication of the cells was determined at 5 days.

RESULTS

Multiplication of Cells in Conventional and Coverslip Cultures. Cells of the three lines were seeded at the same density in conventional cultures and on coverslips that were transferred the next day to larger dishes with larger amounts of medium. The medium was changed and cells were counted at intervals of 2 to 3 days. The initial growth rate of clone 14a was higher than that of clone 14c and much higher than that of clone 21 (Fig. 1). There was little difference in the initial growth rate between conventional and coverslip cultures. The coverslip cultures of clones 14a, 14c, and 21 multiplied to population densities that were 4, 3, and 2 times higher, respectively, than the conventional cultures, indicating that the restriction on further increase in the conventional culture was the result of medium depletion rather than of direct cell–cell interaction.

When the cells of clone 14a were sparse, they were spheroidal or bipolar and plump (Fig. 2 *a*, *b*, *e*, and *f*). When very crowded, they tended to flatten and, when they reached their saturation density in the coverslip cultures, they aligned themselves in a side-by-side interdigitating fibrous pattern (Fig. 2*c*) reminiscent of crowded connective tissue cells.

Mg^{2+} Deprivation Reduces the Saturation Density of Clone 14a in Coverslip Cultures. When the Mg^{2+} concentration of the medium was reduced to 1/40th, cells of clone 14a on coverslips became well flattened and systematically arranged in patches by day 2, before they became confluent (see refs. 1 and 2). The rate of [^{3}H]thymidine incorporation in the Mg^{2+}-sufficient cultures was reduced progressively as the cultures became increasingly crowded, until, on day 10, it was 1/30th the initial rate (Fig. 3). The rate of [^{3}H]thymidine incorporation fell more rapidly in the Mg^{2+}-deficient cultures, reaching a minimum on day 8 that was 1/200th the initial rate. In the following 2 days,

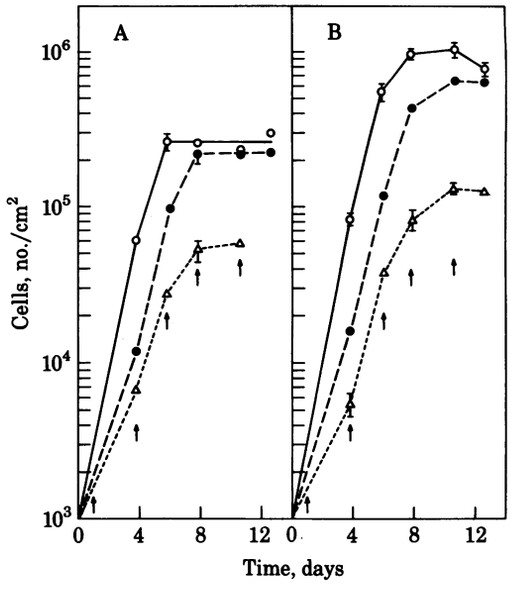

FIG. 1. Multiplication of transformed (*A*) and nontransformed (*B*) cells in conventional and coverslip cultures. Cells of clones 14a (○), 14c (●), and 21 (△) were seeded at $10^3/cm^2$ in 60-mm dishes with or without coverslips. Coverslips were placed in 100-mm dishes the next day and cells were trypsinized and counted as indicated. †, Medium change.

patches of cells appeared that had reverted to the original transformed phenotype, and the rate of [^{3}H]thymidine incorporation increased 10-fold.

The initial rate of multiplication of the cells was reduced to $\approx$1/2 by Mg^{2+} deprivation. The multiplication of both Mg^{2+}-sufficient and -deprived cells slowed down markedly after day 3, with the Mg^{2+}-deprived cultures showing no significant increase in number after day 6. Although Mg^{2+} deprivation decreased the saturation density by a factor of $\approx$7, the cells reached confluency before they stopped proliferating. The Mg^{2+}-sufficient cells were packed tightly together, in what appeared to be monolayers in some areas and multilayers in others (Fig. 2*c*). The Mg^{2+}-deprived cultures had a saturation density that was only slightly higher than that of the nontransformed clone 21 cells on coverslips in Fig. 1*B*.

Criteria for Normalization of the Transformed Phenotype at High Population Density. It seemed possible that the normalization of appearance of the transformed cells when they reached their saturation density was just a mechanical effect produced by extreme crowding. To evaluate this possibility, cells were transferred from coverslips before and after they had reached saturation density and observed for 3 days in low density subcultures. Cells were also transferred from the sparse outlying colonies of dishes containing coverslip cultures that had reached their saturation density. Cells transferred from actively multiplying coverslip cultures or from outlying colonies of saturation-density coverslip cultures underwent minimal spreading after attachment, and most had a spheroidal appearance (Fig. 2 *b* and *f*). Those transferred from the saturation-density coverslips spread and flattened out in a manner characteristic of nontransformed cells (1, 2) and maintained that appearance for about a day (Fig. 2*d*). At that time, some had begun to retract and a few had become spheroidal. In the next 2 days, most of the cells reverted to their original transformed appearance. Since the cells transferred from saturation-density coverslips retained their predominantly nontransformed appearance for a day after reseeding at low density, we conclude that their altered form in the crowded state is not a purely mechanical effect of crowding.

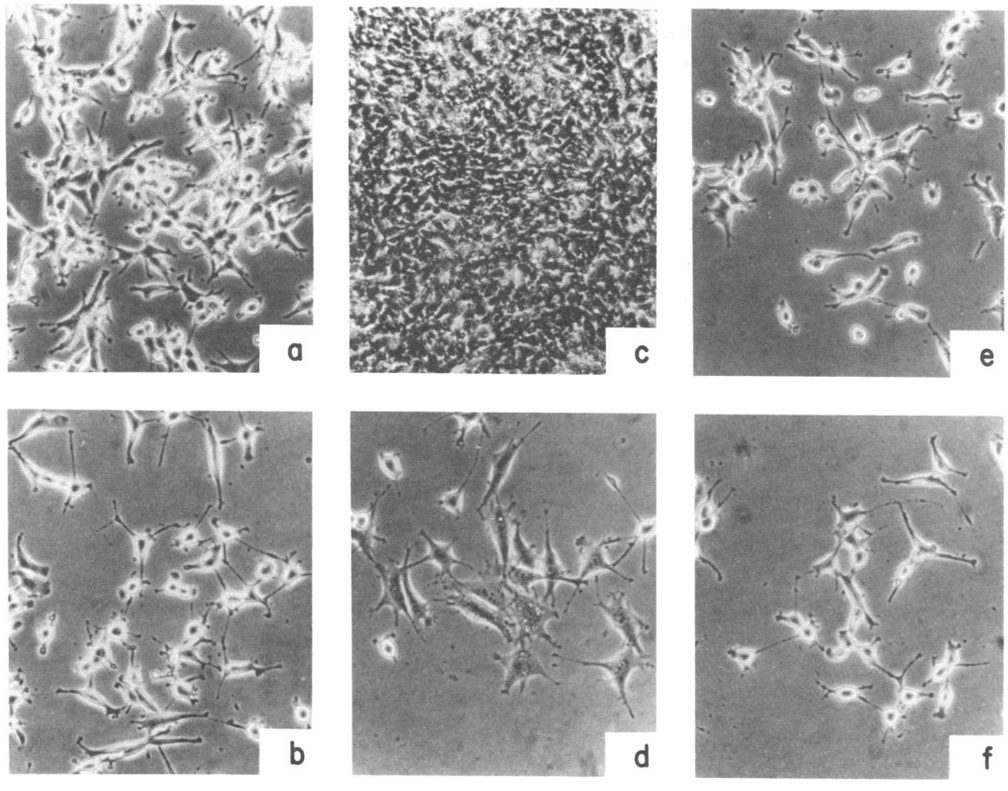

FIG. 2. Change in appearance of clone 14a cells with crowding and its persistence after transfer at low density. Clone 14a cells were seeded on coverslips at $10^4/cm^2$ and the medium was changed at 1, 4, and 6 days. Cells on coverslips were photographed on days 2 (*a*) and 7 (*c*), and cells in outlying colonies were photographed on day 7 (*e*). Cells in each category were then transferred to 35-mm dishes at $2 \times 10^3/cm^2$ and photographed 20 hr later. (*b*) Cells from *a*. (*d*) Cells from *c*. (*f*) Cells from *e*. (×90.)

The physiological state of the highly crowded cells was studied by measuring their growth rate after transfer and their capacity to grow into colonies when suspended in agar. Cells transferred after 4 days on coverslips—i.e., at a relatively low density and incorporating [^{3}H]thymidine at a high rate—increased 33-fold in number in the 3 days after transfer and had a high efficiency—82%—of colony formation in agar (Table 1). As they became more crowded on the coverslip and the rate of [^{3}H]thymidine incorporation decreased, their rate of multiplication on transfer and their efficiency of colony formation in agar decreased markedly, although there was no strict correlation between the two effects. The decreased growth rate on transfer of the very crowded cells was due to a lag of 10–13 hr before the increase in [^{3}H]thymidine incorporation occurred (not shown). If the cells from the day 10 coverslips were grown for 3 days after transfer and then seeded into agar, the efficiency of colony formation increased from 8.8% to 40%. The intracellular Mg^{2+} content of the coverslip cultures decreased by >30% as their population density increased to its maximum.

Another test of the physiological state of the coverslip cultures is their response to serum concentration. Transformed

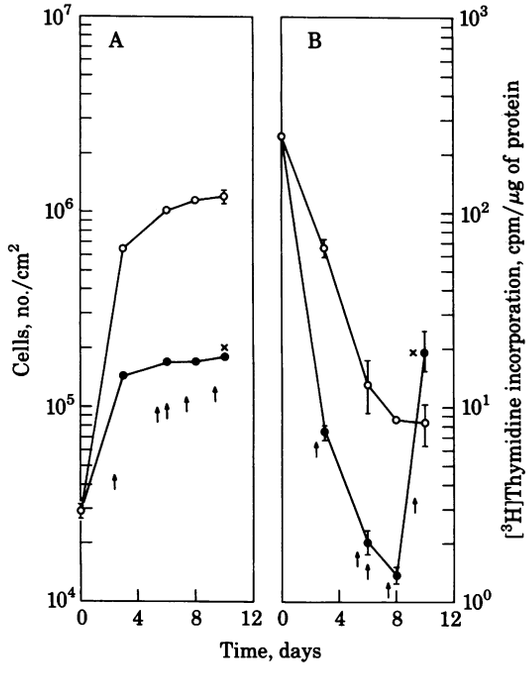

FIG. 3. Effects of Mg^{2+} deprivation on saturation density and rates of [^{3}H]thymidine incorporation in coverslip cultures of clone 14a cells. Cells of clone 14a were seeded on coverslips in 60-mm dishes at $10^4/cm^2$ and transferred to 100-mm dishes the next day in media with either 0.84 mM Mg^{2+} (○) or 0.02 mM Mg^{2+} (●). Cells from two coverslips were counted as indicated, and those on two coverslips were labeled with [^{3}H]thymidine and processed for scintillation counting and protein determination. Media were changed 17 hr before labeling cells (↑), and coverslips were moved to a new dish on day 6. Mg^{2+}-deprived cells were partially flattened on day 1 and fully flattened on day 2, but foci of rounded cells appeared on day 10 (×).

Table 1. Population density effects on growth potential and Mg^{2+} content of clone 14a cells

	Period of growth on coverslips		
	4 days	7 days	10 days
Population density, cells/cm²	2.4×10^5	11.0×10^5	12.5×10^5
Protein, μg/cm²	90.3	462.4	650.0
[³H]Thymidine incorporation, cpm/μg	192.9	20.4	8.0
Colony formation in agar, %	82	3.4	8.8
Factor by which cell number is increased 3 days after transfer	33.1	16.9	6.6
Intracellular Mg^{2+}, μmol/mg of protein	0.1020	0.07215	0.0673

Clone 14a cells were seeded on coverslips at $10^3/cm^2$, and the medium was changed on days 3, 5, 7, 8, and 9. Coverslips were placed in new culture dishes with a medium change on day 6.

cells in conventional culture, at densities that are low relative to those of coverslip cultures, require only low concentrations of serum for rapid multiplication (2). When various concentrations of serum were applied to low and high density coverslips, the rate of [^{3}H]thymidine incorporation in the former was independent of serum concentration from 1–30% (Table 2). In the high density cultures, however, the rate of [^{3}H]thymidine incorporation increased with serum over the entire concentration range. Even in 30% serum, however, the rate of [^{3}H]thymidine incorporation was <1/10th the rate in the low density cultures with 1% serum. Autoradiographs of the low and high density cultures in 10% serum showed that the differences in rates of [^{3}H]thymidine incorporation reflected differences in the proportion of cells in the S period (legend to Table 2) rather than in the rates of [^{3}H]thymidine uptake by the cells or in their rates of chain elongation. The high density cultures showed a 36-fold range of [^{3}H]thymidine incorporation from 0% to 30% serum, indicating that the cells were freely accessible to the macromolecular serum growth factors, and suggesting that availability of low molecular weight nutrients by diffusion was not a limiting factor in multiplication of these cells.

Possible Role of Medium Depletion in Altering Properties of Crowded Coverslip Cultures. Measurement of the rate of [^{3}H]thymidine incorporation in outlying colonies on the plastic of the dish containing the crowded coverslips showed that the cells in these colonies were multiplying at the same rate as those in low density cultures on coverslips (Table 3). Medium removed from the high density cultures after 17 hr of incubation supported a 32-fold increase in a sparse conventional culture of cells in 5 days. Although fresh medium supported more growth than used medium, the result showed that the limit on the number of cells on coverslips was not due to medium depletion.

DISCUSSION

Coverslip cultures were used to avoid depleting the medium and thereby permit cells to multiply to their full potential. In this way, the saturation density of nontransformed cells was doubled and that of moderately and highly transformed cells was increased 3- and 4-fold, respectively, when compared with conventional cultures. Since the increase in the ratio of medium volume to substratum area was more than 17-fold in the coverslip cultures—i.e., considerably greater than the increases in population density—it seemed *a priori* unlikely that limitation of cell multiplication was due to medium depletion. In the case of the highly transformed cells of clone 14a, the possibility of medium depletion as the limiting factor in the multiplication of coverslip cultures was ruled out by the findings that (*i*) a high proportion of cells in the sparse outlying colonies were incorporating [^{3}H]thymidine at the same time that the adjacent crowded coverslip cells were inhibited and (*ii*) the medium removed after 17 hr on stationary coverslip cultures with a low rate of [^{3}H]thymidine incorporation supported a >30-fold increase in cell number of an initially sparse population.

Table 2. Serum concentration effects on [^{3}H]thymidine incorporation in sparse and crowded clone 14a cells

	Period of growth on coverslips			
	2 days		7 days	
% serum	Protein, μg/cm^2	[^{3}H]Thymidine, cpm/μg of protein	Protein, μg/cm^2	[^{3}H]Thymidine, cpm/μg of protein
0	12.5	32.7	337.9	0.55
1	28.9	272.1	395.1	3.0
2	25.1	290.9	466.7	2.6
5	30.5	286.4	453.7	2.9
10	31.2	323.9*	500.2	7.4*
20	32.9	295.4	502.5	11.6
30	37.6	272.7	558.7	20.1

Coverslips seeded with clone 14a cells at 10^4/cm^2 in 10% calf serum were switched into medium with various concentrations of serum on days 1 and 6, and rate of [^{3}H]thymidine incorporation was determined immediately and 17 hr later (i.e., days 2 and 7). Protein contents of the cultures in 10% serum on days 1 and 6 were 7.6 and 418 μg/cm^2 and [^{3}H]thymidine incorporation rates were 543 and 7.8 cpm/μg of protein, respectively.

* Autoradiography of two samples labeled on days 2 and 7 showed that 65.3% and 1.2%, respectively, of the cells had labeled nuclei.

Table 3. Comparison of short-term (2 days) sparse cells with long-term (7 days) dense and sparse (outlying colonies) cells

	Coverslip cultures		Outlying colonies
	2 days	7 days	
Cells, no./cm^2	1.05×10^5	1.05×10^6	3.5×10^4
Protein, μg/cm^2	46.1	566.4	15.0
[^{3}H]Thymidine incorporation, cpm/μg of protein	215.2	13.1	230.7
Competence of medium,* (factor increase)	60.9	31.8	31.8†

Clone 14a cells were seeded on coverslips at 10^4/cm^2, and the medium was changed on days 1, 4, and 6.

* Used medium from cultures was tested for competence to support sustained multiplication of sparsely seeded clone 14a cells over a 5-day period. Results represent the increase in cell number in that period.

† Medium from outlying colonies was the same as that from day 7 coverslips.

The increase in saturation density of clone 14a cells made possible by coverslip culture showed that these highly transformed cells assume the appearance and behavior of nontransformed cells when allowed to multiply to a very high density. Most of them retain this appearance for at least 1 day after transfer at low population density, indicating that the structure of the cells has been modified by their intimate association. Actually, the change in appearance with increasing population density is a gradual one. The cells are mainly spheroidal when they occur as isolated individuals in a rapidly growing culture, although some are long, slender, and bipolar (Fig. 2 *b*, *e*, and *f*). As they multiply and form small groups, they start to flatten (Fig. 2*a*). When they first reach confluency, they are flattened but in an irregular relationship to one another. When they reach saturation density, they form a regular fibrous pattern (Fig. 2*c*). A progressive decrease in rate of incorporation of [^{3}H]thymidine occurs with increasing density despite systematic renewal of medium 17 hr before labeling to ensure maximum response to fresh medium. It appears that the changes in cell appearance and multiplication in response to local cell interactions go hand in hand.

A further test of the inhibited state of clone 14a cells at very high density is their growth behavior on transfer. The first increase in [^{3}H]thymidine incorporation occurs 10–13 hr after transfer, which is characteristic of our nontransformed BALB/c 3T3 cells stimulated by medium change (8). This indicates that the crowded transformed cells are held in the same part of the G_0/G_1 period as are quiescent nontransformed cells. Mitoses first appear in large numbers ≈24 hr after transfer of crowded cells and this delay could account for the small increase in cell number 3 days later relative to those of cells transferred from rapidly growing sparser cultures. When cells from highly crowded coverslip cultures are suspended in agar, they produce far fewer colonies of ≈50 or more cells than do cells from sparser

coverslip cultures. This indicates that suspension in agar inhibits the capacity of the "normalized" cells to revert to the transformed phenotype, which they do when seeded at low density on a solid substratum. Like nontransformed cells, therefore, normalized cells require attachment to a solid substratum for full expression of growth potential.

Still another indicator of the altered state of the transformed cells at very high density is their response to serum. Clone 14 cells grow to a high density with as little as 1% serum whereas nontransformed cells multiply to a much lower density in low serum concentrations (2). At low density, clone 14a cells reach their maximal rate of DNA synthesis in 1% serum whereas, at very high density, there is a large increase in [^{3}H]thymidine incorporation as the serum level is increased from 1% to 30%. In this respect also, they behave like crowded cultures of nontransformed cells, except that a 10-times-higher population density is required to bring out the regulatory response to cellular interaction and the dependence on serum concentration of transformed cells. The results indicate, however, that the difference between transformed and nontransformed cells is a quantitative rather than a qualitative one.

Effects similar to those of crowding can be produced among transformed cells at low cell densities by decreasing the external Mg^{2+} concentration (1, 2). Here we show that the saturation density of the cells is markedly reduced by Mg^{2+} deprivation. Previous attempts in conventional cultures to demonstrate a decrease in saturation density of transformed cells by Mg^{2+} deprivation failed because their multiplication was limited by medium depletion. Thus, cells in conventional cultures reached their limit at a much lower population density than those in coverslip cultures, even at normal Mg^{2+} concentration, and the slower growing Mg^{2+}-deprived cells kept on multiplying until they also depleted the medium. In coverslip culture, the problem of medium depletion is minimized, thereby allowing expression of a true density-dependent inhibition of transformed cells. The modulation of both the appearance and the saturation density of the transformed cells by Mg^{2+} raises the general question of its physiological role in regulating growth behavior of animal cells. In support of such a role is the observation that the Mg^{2+} content per milligram of protein of transformed cells decreases along with growth rate as the population density increases. Since similar reduction in Mg^{2+} content produced by depriving cells of extracellular Mg^{2+} results in a comparable decrease in growth rate, it becomes plausible that Mg^{2+} is involved in the response to crowding (9).

In addition to the effects of population density and Mg^{2+} deprivation, the appearance of clone 14a cells was normalized by depriving them of inorganic orthophosphate and by adding dimethyl sulfoxide (unpublished), as reported for simian virus 40-transformed 3T3 cells (10). High concentrations of K^+ also normalize transformed BALB/c 3T3 cells (11), and similar effects have been seen with cAMP (12) and retinoic acid (13), although the latter two are not effective with clone 14a cells. It will be of interest to determine whether these diverse treatments exert their normalizing effect through a common cellular pathway.

The only treatment among these that simulates a natural process is the increase in population density. This is, in effect, a change in intercellular relationships brought about by merely allowing cells to multiply to their limit in a physiological medium and maximize their contact interactions. It appears to be related to several observations on reversal of the malignant phenotype *in vivo*. The best known of these is the normalizing effect of implanting mouse teratocarcinoma cells into a normal blastula. A genetically mosaic mouse develops that has various normal tissues derived from the teratocarcinoma cells and no tumors (14). In adult newts, most tumors induced by chemicals in regions of high regeneration capacity differentiate to normal tissues whereas those produced in regions of low regeneration capacity become malignant (15).

There is, in addition, evidence that dense nondividing cultures of normal hamster or mouse fibroblasts inhibit multiplication of polyoma-transformed hamster cells with which they are in contact (16, 17). Our results show that transformed cells inhibit their own multiplication at sufficiently high density and reversibly take on aspects of the appearance and regulatory behavior of nontransformed cells. These results also provide an opportunity for quantitative analysis of the cellular interactions, nutritional conditions, and biochemical pathways that determine the transformed phenotype.

This research was carried out under Grants CA 15744 from the National Cancer Institute and DE-AT03-79EV10277 from the U.S. Department of Energy.

1. Rubin, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 328–332.
2. Rubin, H., Vidair, C. & Sanui, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2350–2354.
3. Bartholomew, J., Yokota, H. & Ross, P. (1976) *J. Cell. Physiol.* **88**, 277–286.
4. Lindegren, A. & Westermark, B. (1977) *Exp. Cell Res.* **104**, 293–299.
5. Moses, H., Proper, J., Volkenant, M., Wells, D. & Getz, M. (1978) *Cancer Res.* **38**, 3807–3812.
6. Shipley, G. & Ham, R. (1981) *In Vitro* **17**, 656–670.
7. Rubin, H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3551–3555.
8. Rubin, H., Terasaki, M. & Sanui, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4379–4383.
9. Sanui, H. & Rubin, H. (1977) *J. Cell. Physiol.* **92**, 23–32.
10. Matsuhisha, T., Mori, Y. & Tamura, H. (1981) *Cell Biol. Int. Rep.* **5**, 179–186.
11. Killion, J. & Davis, H. (1981) *J. Cell Biol.* **91**, 15 (abstr.).
12. Pastan, I. & Willingham, M. (1978) *Nature (London)* **274**, 645–650.
13. Jetten, A., Jetten, M., Shapiro, S. & Poon, J. (1979) *Exp. Cell Res.* **119**, 289–299.
14. Mintz, B. & Illmensee, K. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3585–3589.
15. Seilern-Aspang, F. & Kratochwil, K. (1965) in *Regeneration in Animals and Related Problems*, eds. Kiortsis, V. & Trampusch, H. (North Holland, Amsterdam), pp. 452–473.
16. Stoker, M. (1964) *Virology* **24**, 165–174.
17. Stoker, M., Shearer, M. & O'Neill, C. (1966) *J. Cell. Sci.* **1**, 297–310.