Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 328–332, January 1981
Cell Biology

Growth regulation, reverse transformation, and adaptability of 3T3 cells in decreased Mg^{2+} concentration

(saturation density/normalization/phenotypic variation)

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Contributed by Harry Rubin, September 25, 1980

ABSTRACT **A nontransformed and a spontaneously transformed clone of BALB/c 3T3 cells were compared for their capacity to multiply in decreased concentrations of Mg^{2+}. Cells of the nontransformed clone were flat, formed regularly patterned, nonoverlapping arrays, required high serum concentration for multiplication, had a low saturation density, and did not make colonies in agar. Cells of the transformed clone were slender and spiky, formed random, overlapping arrays, multiplied in low serum concentrations, and had no fixed saturation density, and 20–30% of them formed colonies in agar. The saturation density of the nontransformed clone was decreased in a growth-limiting supply of Mg^{2+} in proportion to the reduction in initial rate of multiplication. At very low Mg^{2+} concentrations, saturation occurred when less than half of the surface of the dish was covered with cells. The transformed cells did not reach a stable saturation density in low Mg^{2+} concentrations, but their growth rate did slow down when they became crowded, and a transient saturation density was reached at the lowest Mg^{2+} concentrations that allowed multiplication. Limiting the supply of Mg^{2+} caused the transformed cells to flatten and to assume a regularly patterned, nonoverlapping relationship to one another, resembling that of the nontransformed cells. This also occurred in BALB/c 3T3 cells transformed by infection with Moloney mouse sarcoma virus. After 1 week in low concentrations of Mg^{2+}, the nontransformed cells began to multiply and to incorporate [^{3}H]thymidine at a rapid rate. The transformed cells did so also and, in addition, reverted to their transformed appearance. The intracellular content of Mg^{2+} was not significantly decreased when the extracellular concentration was decreased to 1/50th. The results suggest that: (*a*) limited contact among cells already multiplying at a reduced rate is sufficient to halt further multiplication; (*b*) a very small decrease in intracellular Mg^{2+} content or in membrane-associated Mg^{2+} causes transformed cells to assume aspects of the appearance and behavior of nontransformed cells (i.e., Mg^{2+}-regulated reactions may be involved in determining the transformed phenotype); and (*c*) cells multiplying at a slow rate in low concentrations of Mg^{2+} begin to multiply faster after about 1 week, due either to an adaptation of the cells or to a change in the cellular microenvironment.**

When animal cells in culture are treated with growth stimulants such as insulin or serum, various reactions in the cells are accelerated (1). This group of cellular reactions has been designated "the coordinate response" (2, 3). Many of the early reactions of the coordinate response are independent of one another and of macromolecular synthesis (1). The only intracellular substance that has the capacity, both theoretically and experimentally, to modulate the various reactions of the coordinate response is Mg^{2+}, and I have proposed that Mg^{2+} acts as a second messenger for these growth stimulants (2, 3). McKeehan and coworkers concurred in this conclusion and additionally proposed that cell transformation causes a selective loss of the regulatory role of Mg^{2+} (4, 5). Their conclusions were based on measurements of the size of colonies of normal and transformed cells formed in varying concentrations of Mg^{2+} and in other putative growth regulatory substances.

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I set out to extend their findings by comparing the effects of exposure to different concentrations of Mg^{2+} on growth rate and saturation density of a nontransformed clone of 3T3 cells and a spontaneously occurring transformed variant of that line. I found that the differential requirements of the two cell lines for Mg^{2+} depend on a complex set of variables which will be described elsewhere. The experiments did show a consistent relationship between the initial multiplication rate of nontransformed cells and their saturation density, which clarifies the relative contributions of growth stimulatory materials and population density in fixing limits to the size of a population. Deprivation of Mg^{2+} caused the transformed cells to resemble nontransformed cells in appearance and growth behavior. After about 1 week in Mg^{2+}-deficient medium, the rate of DNA synthesis began an increase which continued through the second week in both nontransformed and transformed cells, and the latter reverted to their original appearance.

MATERIALS AND METHODS

Cell Types, Transfers, and Labeling Techniques. The original cells used in these experiments were of the A31 clone of BALB/c 3T3 cells which had been recloned by J. Bartholomew to obtain a uniform culture of flat cells. This "parental clone" was obtained in January 1979 and maintained by weekly transfers at low population density in Dulbecco's modification of Eagle's medium containing 10% calf serum in the early passages and 10% fetal calf serum later on. As the work progressed, the cells were switched to an improved medium developed for 3T3 cells and designated MCDB 402 (6). From 2 to 5 × 10^4 cells were seeded per 100-mm plastic petri dish at each passage, and only those cultures that were subconfluent or had barely attained confluency were used for further passage. Small areas of refractile and somewhat rounded cells were observed after 4 months of transferring. The cells were seeded at very low density, and two different types of colonies were observed. Most of the clones were considered to be nontransformed and, like the original parental culture, consisted of cells that were flat and somewhat elongated before confluency but assumed a regular, cobblestone arrangement when confluent (Figs. 1*a* and 2*a*). About 2% of the clones appeared to be transformed and consisted of thin, randomly arranged cells in both sparse and crowded cultures (Figs. 1*b* and 2*b*), although some flattened cells were seen in the background in the crowded cultures.

One colony of each type was selected for further transfer; the nontransformed one was designated clone 2 and the transformed one, clone 14. The nontransformed clone required a minimum of 5% serum for sustained multiplication and failed to form colonies when suspended in an agar medium; the transformed clone multiplied rapidly even in 1% serum, and 20–30% of the cells formed colonies in agar.

For the experiments, cells were transferred by washing with Tris-buffered saline and suspended in 0.01% crystalline trypsin containing 0.5 mM EDTA in the buffered saline. The initial

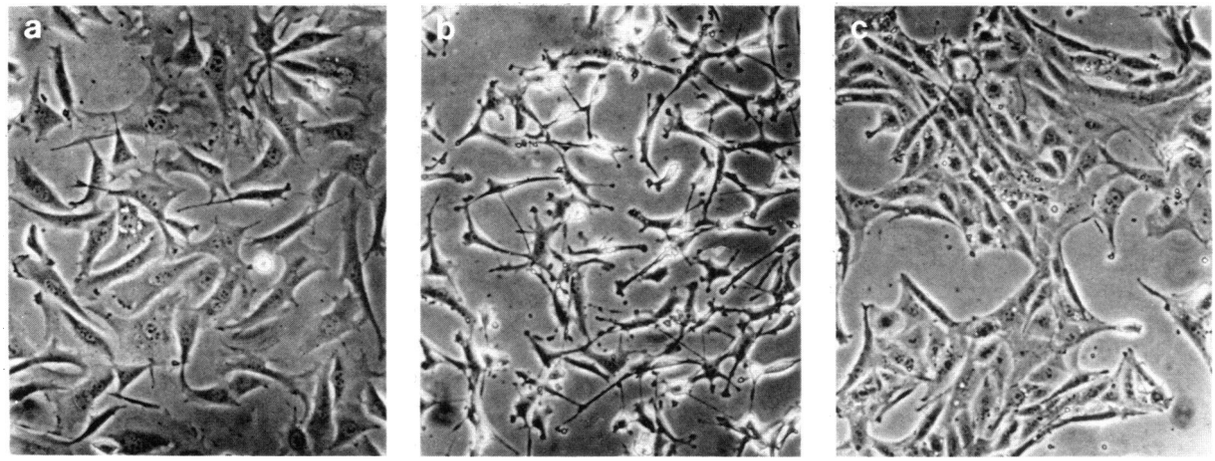

FIG. 1. Sparse cultures. (*a*) Clone 2 in 1.0 mM Mg^{2+} for 2 days. (*b*) Clone 14 in 1.0 mM Mg^{2+} for 2 days. (*c*) Clone 14 in 0.01 mM Mg^{2+} for 2 days. Initial seeding density was 4.0×10^4 cells per 60-mm dish. (×100.)

seeding densities are given in the figure legends. The cells were seeded in MCDB 402 medium with 5% fetal calf serum. The following day the cells were washed twice with Tris-buffered saline and MCDB 402 medium with the appropriate cation concentrations plus 10% dialyzed fetal calf serum. In all experiments involving variations in Mg^{2+} concentration, the Ca^{2+} concentration of the medium was constant at 1.0 mM instead of the usual 1.6 mM of this medium. The medium was changed every 2–3 days beginning on day 3. In the experiments in which the rate of [^{3}H]thymidine incorporation into DNA was studied, the medium was changed 18 hr before the labeling was done in order to allow a full response of the cells in the presence of fresh medium components.

The rate of DNA synthesis was assessed by labeling for 1 hr with [^{3}H]thymidine (20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at 1 μCi/ml in MCDB 402 containing the usual concentrations of all cations. The procedures for determining radioactivity by scintillation counting, labeled nuclei by autoradiography, and protein content by the Lowry procedure were as described (1). The Mg^{2+} content of the medium and the cells was determined in a Perkin–Elmer model 403 atomic absorption spectrophotometer as described (7).

RESULTS

Response of Clones 2 and 14 to Variations in Mg^{2+} Concentration. The growth rates of clones 2 and 14 in different concentrations of Mg^{2+} varied with the number of passages after thawing of the frozen cells. At 2 1/2 months after thawing there was little difference between clones 2 and 14 in the initial degree of growth inhibition produced by lowering the Mg^{2+} concentration (Fig. 3), but at 6 1/2 months, clone 2 was more severely inhibited, especially at the lowest concentration of Mg^{2+} (0.016 mM) used (Fig. 4). The differential response to Mg^{2+} of the two clones varied with cell population density, serum concentration, and length of treatment; this will be reported elsewhere. The saturation density of clone 2 varied from 2 to 4×10^4 cells per cm^2 and it decreased reproducibly with Mg^{2+} concentration in proportion to the decrease in the initial rate of multiplication. Saturation was reached at some low Mg^{2+} concentrations when only about one-half the surface area of the dish was covered, although most of the cells were in contact with other cells at this population density. At the lowest Mg^{2+} concentrations, there was loss of cells in clone 2 for a week, followed by a low rate of multiplication during the second week (Fig. 4).

The cells of clone 14 did not reach a fixed saturation density in a physiological concentration of Mg^{2+} nor in decreases to 0.0275 mM (Figs. 3 and 4). With 0.016 mM Mg^{2+}, however, the growth curve of clone 14 began to flatten out at a population density twice as high as the saturation density of clone 2 in physiological Mg^{2+} concentration and then seemed to reach saturation between 10 and 12 days but later underwent a further increase in cell number (Fig. 4).

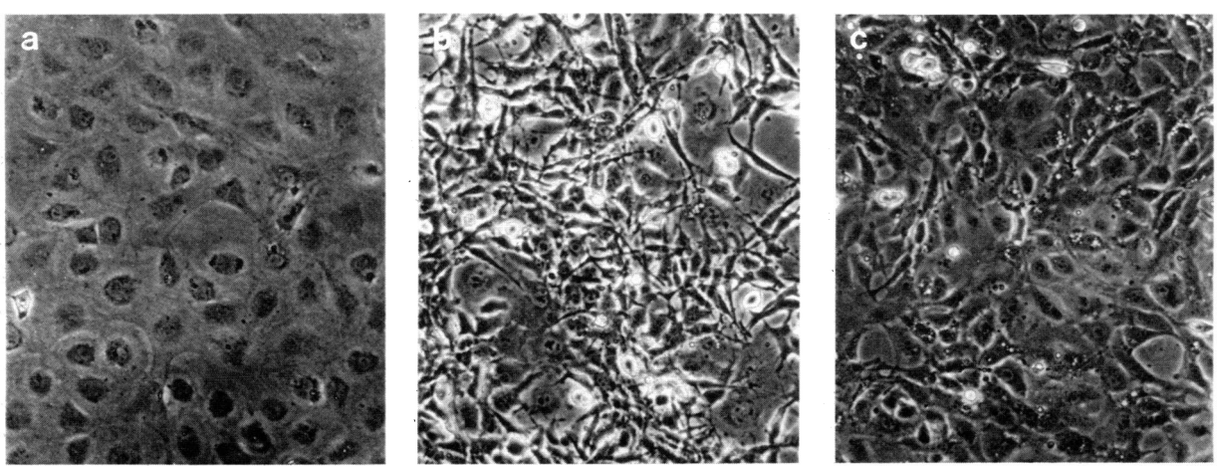

FIG. 2. Crowded cultures. (*a*) Clone 2 in 1.0 mM Mg^{2+} for 7 days. (*b*) Clone 14 in 1.0 mM Mg^{2+} for 7 days. (*c*) Clone 14 in 1.0 mM Mg^{2+} for 6 days and 0.01 mM Mg^{2+} for 1 day. Initial seeding density was 4.0×10^4 cells per 60-mm dish. (×100.)

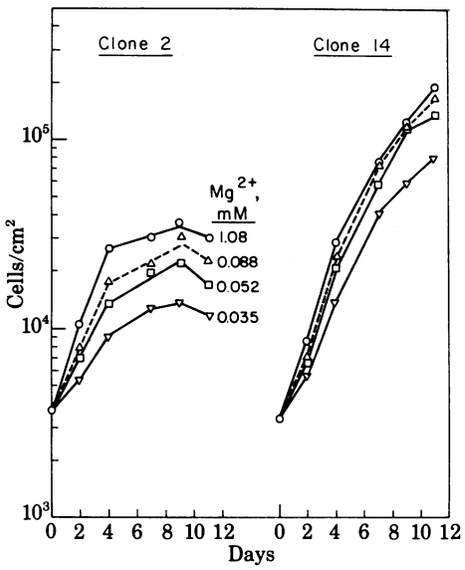

FIG. 3. Multiplication of clones 2 and 14 in various concentrations of Mg^{2+}. The cells had been passaged for 2 1/2 months after thawing. Initial seeding density was 1×10^5 per 60-mm dish.

When the concentration of Mg^{2+} was decreased to 0.0375 mM or less, not only was the multiplication rate of clone 14 detectably decreased but also there was a marked change in the appearance of the cells. Within a day after the Mg^{2+} concentration was decreased, the cells began to flatten out; within the following days, they assumed an appearance which resembled that of nontransformed cells in many respects except for the more common occurrence of vacuoles when the clone 14 cells in low Mg^{2+} were confluent (Figs. 1*c* and 2*c*). They also changed from a random, overlapping arrangement to a regularly patterned one with little overlap. The same change occurred in BALB/c 3T3 cells transformed by infection with Moloney sarcoma virus grown in low concentrations of Mg^{2+}. Graded reductions in Ca^{2+} or K^+ to growth inhibitory concentrations did not cause similar morphological alterations, nor did a graded substitution of either choline or sucrose for Na^+.

Beginning about 1 week after incubation in low Mg^{2+}, isolated groups of the flattened cells began to retract from the substratum and resumed the characteristic slender and spiky or rounded appearance and the random arrangement of transformed cells. The number of cells in these foci and the number of foci increased during the second week. Mitotic figures were commonly seen among the newly transformed cells. The predominance of such cells toward the end of the second week may have been responsible for the secondary spurt of growth in clone 14 at the lowest Mg^{2+} concentration of Fig. 4. A similar reversion to the original transformed phenotype could be brought about within 1 day in all the Mg^{2+}-deprived, flattened cells of clone 14 at any time by simply raising the concentration of Mg^{2+} in the medium to the physiological level.

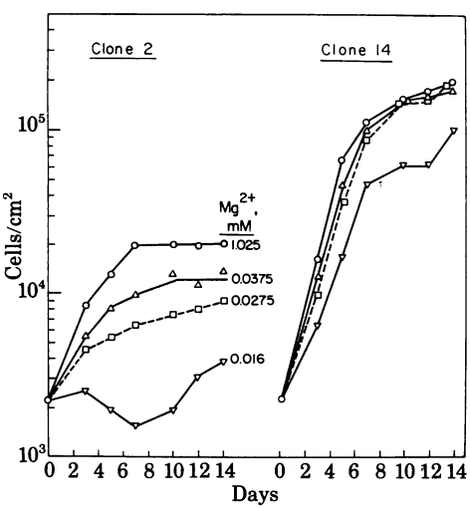

FIG. 4. Multiplication of clones 2 and 14 in various concentrations of Mg^{2+}. The cells had been passaged for 6 1/2 months after thawing. Initial seeding density was 2×10^4 per 35-mm dish.

Reduction in Saturation Density of Mg^{2+}-Deprived Cells of Clone 2 at Low Seeding Densities. The reduction in saturation density of clone 2 with reduction of Mg^{2+} concentration in Figs. 3 and 4 could have been due to a difference in the inhibitory effect of population density on cells growing at different rates, but it also could have resulted from some fixed limit in the number of divisions the cells could undergo in low Mg^{2+}. To distinguish between these possibilities, I compared the growth curves, in various Mg^{2+} concentrations, of clone 2 cells seeded at the same density as in the experiment of Fig. 3 and at 1/10th this density. At all but the lowest concentration of Mg^{2+} used, the cells seeded at the lower density continued to multiply until they became almost as numerous as those seeded at the higher density in equivalent concentrations of Mg^{2+} (Fig. 5). This indicated that population density was indeed the limiting factor in determining the maximum number of cells per culture at each Mg^{2+} concentration.

At the lowest concentration of Mg^{2+} used there was a delay of about 9 days before the sparsely seeded cells began to multiply, but then they did so at the maximal rate. This result plus the delayed multiplication seen in Fig. 4 at the lowest Mg^{2+} concentration with both nontransformed and transformed cells indicated that there was either an adaptation of the cells to low concentrations of Mg^{2+} or that the cellular microenvironment had changed to overcome the inhibition of Mg^{2+} deprivation.

Adaptation of Clones 2 and 14 to Low Concentrations of Mg^{2+}. In the previous experiments, hints of an adaptation to low Mg^{2+} arose from a delayed increase in the total number of cells

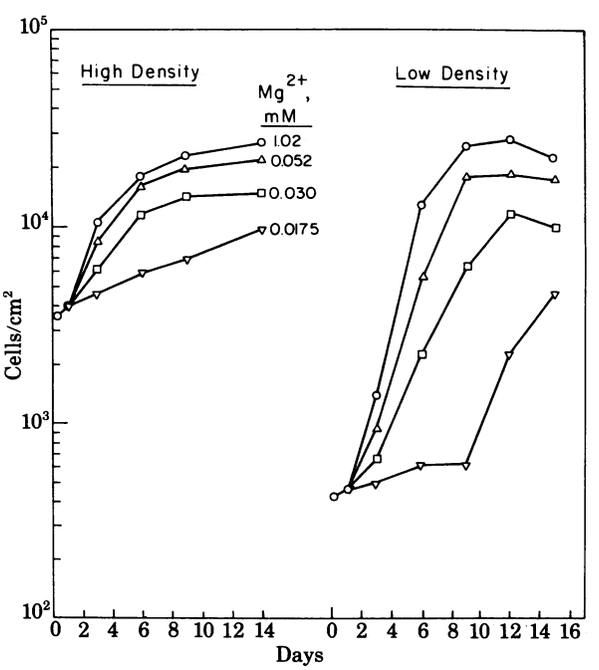

FIG. 5. Multiplication of relatively low and high densities of clone 2 in various concentrations of Mg^{2+}. The low seeding density was 1×10^4 and the high seeding density was 1×10^5 per 60-mm dish.

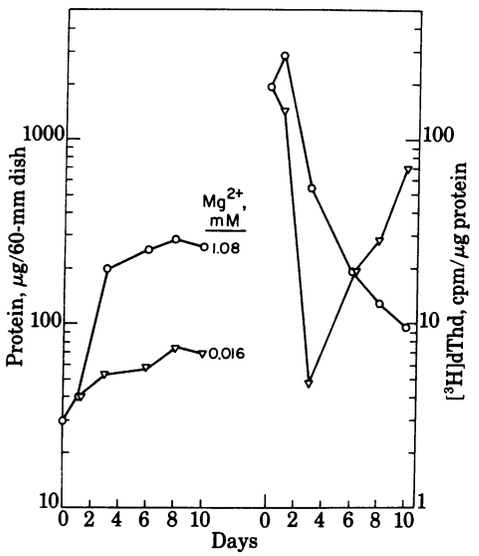

FIG. 6. Incorporation of [^{3}H]thymidine into DNA and multiplication of clone 2 in physiological and low concentrations of Mg^{2+}. The initial seeding density was 8×10^4 cells per 60-mm dish. Left side, protein content per dish; right side, [^{3}H]thymidine incorporation.

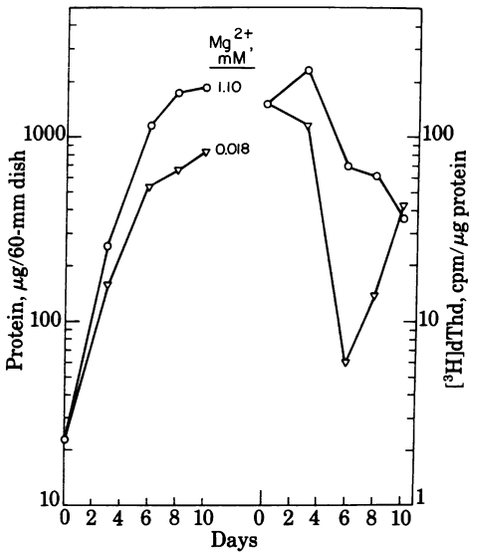

FIG. 7. Incorporation of [^{3}H]thymidine into DNA and multiplication of clone 14 in physiological and low concentrations of Mg^{2+}. Initial seeding density was 1×10^5 cells per 60-mm dish. Left side, protein content per dish; right side, [^{3}H]thymidine incorporation.

in the population. If the initial size of the cell population is large, detection of an increase would be a relatively insensitive indicator of adaptation, especially when it involves a minority of cells in the population. I therefore turned to a study of the incorporation of [^{3}H]thymidine into DNA as a potentially more sensitive indicator of adaptation. The rate of [^{3}H]thymidine incorporation was markedly decreased in the cells of clone 2 in 1.0 mM Mg^{2+} on day 3, when cell multiplication had greatly slowed down (Fig. 6). This trend to a decreased rate of [^{3}H]thymidine incorporation, which was associated with confluency of the cultures, continued on subsequent days. An even steeper decline in [^{3}H]thymidine incorporation was seen in low concentrations of Mg^{2+} on day 3, but subsequently there was a steady increase until day 10 to a rate almost 15-fold higher than the minimal value. At this time, paradoxically, the rate of [^{3}H]thymidine incorporation was about 7 times higher in the cultures with low Mg^{2+} than in those with a normal concentration.

The rate of [^{3}H]thymidine incorporation into the transformed cells of clone 14 in 1 mM Mg^{2+} showed no decline by day 3 and declined only slowly after that (Fig. 7). This difference from clone 2 was reflected in continued multiplication of these cells at a rapid rate until day 6 and even beyond. The clone 14 cells in low Mg^{2+} also continued to have a relatively high rate of [^{3}H]thymidine incorporation to day 3 but underwent a sharp decline by day 6, concomitant with their growth into a confluent sheet of relatively flat cells. Their rate of cell multiplication also declined markedly from this point on, although no fixed saturation density was reached. On day 7, some retracted and rounded cells were seen again in the low Mg^{2+} cultures, and their proportion increased markedly in the next 3 days. During the same period, there was a 7-fold increase in the rate of [^{3}H]thymidine incorporation, signifying adaptation of the culture to the low concentrations of Mg^{2+}. When the initial plating of clone 14 cells was low enough to produce isolated colonies in low Mg^{2+}, most of the colonies consisted of flat cells during the first week. Foci of newly transformed cells appeared among the flat cells in most of the colonies during the second week, indicating that the reversion to the transformed phenotype was not a rare mutation-like event.

Cellular Content of Mg^{2+} in Mg^{2+}-Deprived Cultures. The intracellular content of Mg^{2+} was determined in three sets of cultures of clones 2 and 14 at 3–4 days after the Mg^{2+} content of the medium was changed. In 1.0 mM Mg^{2+}, the average intracellular content of Mg^{2+} for both clones was 0.0883 μmol/mg of protein; and in 0.02 mM Mg^{2+}, it was 0.0825 μmol/mg of protein. This 7% decrease in the intracellular Mg^{2+} is within the SD of the measurement and is not considered significant.

DISCUSSION

My original aim in this study was to extend to 3T3 cells the finding of McKeehan and coworkers (4, 5) that transformed human diploid fibroblasts require much less Mg^{2+} for multiplication than do nontransformed ones and to use this hardy cell line to test their conclusion that transformation involves a selective loss of the regulatory role of Mg^{2+} in cellular multiplication.

To this end I compared two clones that differed markedly from each other in the *in vitro* criteria commonly used to distinguish nontransformed from transformed cells. I found that the requirement of the cells for Mg^{2+} varied with population density, serum concentration, and length of time in the varying concentrations of Mg^{2+}. These results, which will be reported elsewhere, indicated that no simple conclusion could be drawn about the differential requirements for Mg^{2+} in the growth of nontransformed and transformed cells. For the present, therefore, the findings of McKeehan *et al.* must be considered to apply only to the particular cell types and conditions used by them.

I did find that reductions in Mg^{2+} concentration which lowered the initial rate of multiplication of the nontransformed cells lowered their saturation density proportionately. This relationship between initial rate of multiplication and saturation density in nontransformed 3T3 cells was not evident from previous experiments in which various serum concentrations were used to regulate both features (8). Difficulty in interpreting those experiments is due in part to the variation, with serum concentration, in the number of cells which attached to the culture dish and in part to the complicating effects of serum depletion in determining saturation density. Both these complications were avoided in the present experiments because: (*a*) all cultures were seeded in the same medium before they were switched to experimental media, so that the starting density was the same for all groups; and (*b*) it was shown that the concentration of Mg^{2+} in the medium, which determined the initial growth rate,

did not decrease during the course of the experiment even when the initial concentration of Mg^{2+} was very low. The concentration of Mg^{2+} in the medium remained nearly constant because there was a large ratio of medium volume to cell volume.

A culture growing at a reduced rate in limiting Mg^{2+} may reach saturation density when less than half the dish is covered, showing that full confluency is not a necessary condition for restricting the growth of a cell population. This indicates that a limited interaction between cells is sufficient to stop further growth in a population already multiplying at a reduced rate. If, as seems likely, this interaction involves contact between cells, the contact need not involve the entire perimeter of the cells to produce its inhibitory effect. Indeed, it is well established that limited contact between two cells is sufficient to inhibit their locomotion (9), and it now appears that similarly limited contact can inhibit multiplication. The results suggest that the growth rate of a population is the summation of all the inhibitory and stimulatory influences acting on it, and there is no category of effect that can cancel or be ruled out by the other effects.

The inhibition of cell multiplication of the transformed population did not impose a stable saturation density on them but it did cause a distinct slowdown in multiplication at very low Mg^{2+} concentrations when the population became dense (Figs. 3, 4, and 7). At the same time, the rate of incorporation of [^{3}H]thymidine decreased sharply relative to that in the control (Fig. 7). Within a day after Mg^{2+} deprivation, the transformed cells underwent a striking change in appearance—from a slender or rounded to a flattened shape—and from a random, overlapping arrangement to a regular, nonoverlapping arrangement resembling that of normal cells. A similar "normalization" of growth behavior in low Mg^{2+} may account for their reduced rate of multiplication upon crowding—i.e., they had become subject to at least a partial density-dependent inhibition of multiplication. The apparent normalization of transformed cells in low Mg^{2+} could support the thesis (4, 5) that transformation involves a selective loss of the regulatory role of Mg^{2+} in multiplication.

Derivatives of cyclic AMP are known to alter the morphologic phenotype of transformed cells in a manner similar to that described here (10, 11), and these changes have been designated "reverse transformation" (10). The effects of cyclic AMP on the multiplication of transformed cells are different from those produced by limiting the concentration of Mg^{2+}. In one case it was reported that the addition of cyclic AMP limits the number of cell generations regardless of the initial density (12), and in another it was reported that growth on solid surfaces is unchanged by cyclic AMP (13). By contrast, limitation of Mg^{2+} clearly affects the growth behavior of the transformed cells on solid surfaces in a population-dependent manner which resembles, at least transiently, the growth behavior of nontransformed cells.

The inhibitory effect of Mg^{2+} deprivation on growth rate and cell behavior proved to be a transient one in both nontransformed and transformed cells alike. After 1 week the cultures resumed their maximal growth rate in the case of sparsely seeded nontransformed cells and reverted to a transformed shape in the case of the transformed cells. The evidence to date indicates that these late changes do not involve the selection of rare preexisting variants. Extrapolation to zero time of the curve for delayed multiplication of sparse cells in the lowest Mg^{2+} concentration of Fig. 4 indicates that any preexisting variants fully capable of growing in very low Mg^{2+} concentrations would have to constitute about 5% of the original population. This is a high figure for mutation, considering that no prior selection in low Mg^{2+} occurred, and the adaptive phenomenon has been observed in recently cloned populations. In the case of the transformed cells flattened in low Mg^{2+}, in which adaptation can be detected by the reappearance of cells with transformed shape, selection of mutants with low Mg^{2+} requirement is even less likely because reversion to the transformed phenotype occurs in most isolated colonies of clone 14 cells which have undergone normalization in low Mg^{2+}. Because the colonies consist of a few thousand cells at most, the putative mutation would have to occur with regularity at an extremely high frequency. The altered responsiveness of the cells could represent an adaptive change in cellular metabolism or a change in cellular microenvironment. An example of metabolic adaptation is found in the liver of rats where the level of citrate cleavage enzyme is 30- and 60-fold greater when the rats are on a diet high in glucose or glycerol, respectively, than when the diet is high in fat (14). Alternatively, the capacity of cells to multiply in low Mg^{2+} concentrations could be enhanced by microenvironmental changes such as increased population density or local accumulation of cellular secretions. At present we have not distinguished between the adaptive and microenvironmental hypotheses.

The measured decrease of intracellular Mg^{2+} content brought about by a large decrease in the Mg^{2+} concentration of the medium is not statistically significant. It is possible that very small changes in intracellular Mg^{2+} content are sufficient to bring about the observed reductions in both growth rate and saturation density as well as the "normalization" of the transformed cells, because protein synthesis and the onset of DNA synthesis appear to be very sensitive to changes in intracellular Mg^{2+} concentration (15). It is also possible that the large reductions in extracellular Mg^{2+} concentration which are necessary to inhibit growth and change cellular appearance act at the cell surface to inhibit the phosphorylation of proteins and lipids of the plasma membrane. Increased phosphorylation of membrane proteins is an early step in the stimulation of cell multiplication by epidermal growth factor (16) and it has been shown that membrane phosphorylations can be inhibited by limiting the supply of Mg^{2+} in the medium (17). Regardless of whether limitation of Mg^{2+} produces its biological effects within the cell or the membrane, the results illustrate the capacity of Mg^{2+} to act as a regulator in cell multiplication and transformation.

I thank Berbie Chu for her excellent technical assistance, H. Sanui for noting the transformed cells, and Charles Vidair for picking the clones used in this work. This research was supported by Research Grant CA 15744 from the National Cancer Institute and Contract DE-ATO3-79EV10277 from the Department of Energy.

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