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Control of the Initiation of DNA Synthesis in 3T3 Cells: Low-Molecular-Weight Nutrients

(amino acids/glucose/phosphate/cell cycle)

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ABSTRACT **Sparse 3T3 cells, in excess serum, become quiescent in the early G_1 (or G_0) phase of the cell cycle if the cells are cultured in low concentrations of amino acids, glucose, or phosphate ion. These quiescent cells then initiate DNA synthesis if the concentration of the limiting nutrient is increased. In normal medium, DNA synthesis in the same 3T3 cell line is controlled by serum factors. The results demonstrate the complexity of the control by external agents of DNA synthesis in these "normal," density-dependent cells.**

The growth of mouse 3T3 cells is normally controlled by serum factors (1–3). At least four serum factors appear to play a role in the initiation of DNA synthesis in quiescent cells (4). Three of the four serum factors can be replaced by known substances (4), the fibroblast growth factor of Gospodarowicz (5), insulin, and dexamethasone.

In the present paper it is shown that the initiation of DNA synthesis in 3T3 cells can also be controlled by concentrations of a number of different low-molecular-weight nutrients in the medium. The results indicate that DNA synthesis and growth in these "normal," density-dependent cells are subject to control by a variety of different external agents.

MATERIALS AND METHODS

Cell Cultures were maintained as described in the previous paper (4).

Flow Microfluorometric Analyses. The staining procedure was that of Tobey *et al.* (6). Trypsin-treated and washed cells were fixed in the cold with a 7.4% formaldehyde solution in saline for 2 hr to 6 days, and then the cells were stained with acriflavine (Aldrich Chemical). The stained cells were analyzed in a Los Alamos design microfluorometer (7) with an argon laser at 488 nm. The photograph obtained from the storage oscilloscope was analyzed graphically, as indicated in Fig. 1.

Arrest of 3T3 Cell Growth by an Individual Limiting Amino Acid. Cells were plated at 4×10^5 cells per 9-cm dish in medium with normal concentrations of all nutrients except with only 1% of the normal concentration of a single amino acid, plus 10% calf serum which had been filtered through Sephadex G-50 (8). After 3 days the cells were fixed for flow microfluorometry.

Arrest and Reinitiation of Growth by Lowering and Raising the Concentrations of All of the Amino Acids. Stock cells were plated at 10^5 cells per 5.5-cm dish in medium plus 10% calf serum. One day later the cells were rinsed and fluid changed into medium that contained the normal concentrations of salts and glucose but only 2% of the normal Dulbecco-Vogt medium concentrations of amino acids and vitamins, plus 10% Sephadex G-50-filtered calf serum. Subsequently, daily additions of 1 ml per dish were made of the same medium, containing 10% Sephadex G-50-filtered serum, but only 0.1% of the normal concentrations of amino acids and vitamins. On the third day after the fluid change, 0.5 ml of a 5-fold concentrate of all the amino acids was added to half of the dishes, to raise the concentrations of the amino acids in these dishes to approximately 30% of normal. Controls were fixed at the time of addition of the amino acids. Approximately 17 hr later, the cells to which amino acids were added were fixed and prepared for flow microfluorometric analyses (Fig. 3). Cell counts were made daily throughout the experiment (Fig. 4).

Arrest and Reinitiation of Growth by Lowering and Raising the Concentration of Phosphate. Growing stock cells were trypsin-treated and plated at 2×10^5 cells per 9-cm dish in medium plus 10% calf serum. One day later the cells were rinsed and fluid changed into medium that contained 1% of the normal Dulbecco-Vogt concentration of sodium phosphate, plus 10% Sephadex G-50-filtered calf serum. On the third day after the fluid change, 0.1 ml of a 0.1 M sodium phosphate solution (pH 7) was added (to give approximately 1 mM phosphate), or sodium phosphate plus 0.05 ml of a solution of 2 mg/ml of crystalline bovine pancreatic insulin (Sigma) in water at pH 2.5 (to give approximately 10 μg/ml of insulin), was added per dish and the treated and control cells were fixed 17 hr later.

Arrest and Reinitiation of Growth by Lowering and Raising the Concentration of Glucose. Stock cells were trypsin-treated and plated at 10^5 cells per 9-cm tissue culture dish in medium with 0.5% of the normal concentration of glucose plus 20% Sephadex G-50-filtered calf serum. On the third day after plating, 0.4 ml of an 0.6 M solution of glucose was added to half the dishes (to give approximately 0.024 M glucose) and these and control cells were fixed 16 hr later.

2-Deoxyglucose Uptake. The procedure was that of Hatanaka *et al.* (9). The cell cultures were the same as those used in assays for DNA initiation in confluent quiescent cells (4). At 6 days, the additions to be assayed were made, and the cultures were incubated for 30 min in a CO_2 incubator. The cultures were then rinsed twice with medium without glucose or serum and 5 ml of this medium containing 0.1 mM 2-deoxyglucose were added per dish. To each dish, 2 μCi of [^{3}H]2-deoxy-D-glucose (New England Nuclear Corp.) were added immediately and the cultures were returned to a CO_2 in-

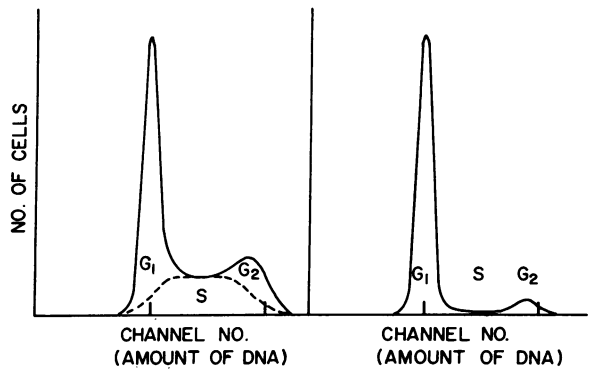

FIG. 1. Analyses, obtained with a Los Alamos design flow microfluorometer, showing DNA content per cell in: (*left*), a rapidly growing 3T3 cell population with approximately 40% of the cells in S phase; and (*right*), a quiescent 3T3 cell population in which almost all of the cells have the G_1 content of DNA. The percentages of cells in G_1, S, and G_2 + M were estimated graphically, based on the height of the curve in the middle of S, as is illustrated by the *broken line* in the analysis of the growing cell population.

cubator. At the end of 30 min, the medium was removed by aspiration, the cells were rinsed three times with cold Tris–saline (10), and 2 ml of 10% Triton X-100 (Bass Chemical) in water were added to each dish. After 10 min the cells were scraped or agitated off the dishes and 1 ml of the cell suspension was added to 10 ml of scintillation solution (2:1:0.084, toluene:Triton X-100:Liquifluor), and the mixture was shaken. The samples were counted the next day (to lower the background). If more than 20 dishes were used per experiment the dishes were divided into groups and these were treated at 20-min intervals.

Phosphate Uptake. The cell cultures were prepared in the same way as those used in assays for DNA initiation in sparse quiescent cells (4) except the medium contained only 1% of the normal concentration of phosphate. The additions were made (Table 1) approximately 24 hr after the cells were plated, and, after 30 min at 37° in the CO_2 incubator, 3 μCi of [^{32}P]phosphate (International Chemical and Nuclear) was added per plate. After 30 min of incorporation at 37°, the medium was removed by aspiration, the cells were rinsed three times with cold Tris–saline, and the acid-soluble material was extracted from the cells with 2 ml of 5% trichloroacetic acid solution for 30 min. The trichloroacetic acid solution was centrifuged and an 0.6-ml aliquot of the solution was mixed in a scintillation vial with 10 ml of the scintillation fluid described above, and 0.2 ml of 1 M sodium hydroxide, and the vials were counted immediately.

RESULTS

Flow Microfluorometric Analyses of Growing and Quiescent 3T3 Cells. Flow microfluorometric analyses of the DNA contents of cells in a growing and in a quiescent 3T3 cell population are shown in Fig. 1. Approximately 40% of the cells in the growing population were in the S phase of the cell cycle. In the quiescent population, with growth arrested by limitation of serum factors, almost all the cells had the G_1 content of DNA.

A quiescent 3T3 cell population, limited by serum, is obtained by allowing growing cells to deplete the medium of serum factors over a period of a few days. Alternatively, the serum concentration in the medium can be lowered abruptly, for example, from 10% to 0.4%. This latter treatment arrests the growth of the cells in the G_1 (or G_0) phase of the cell cycle within approximately 12 hr.

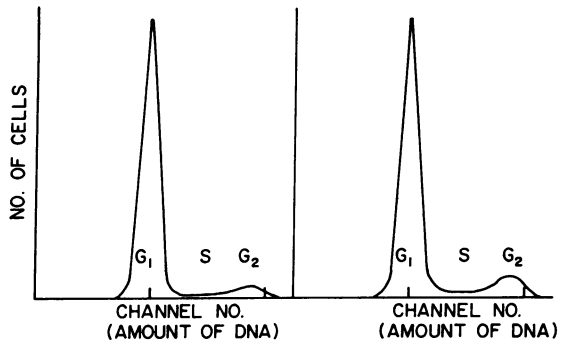

FIG. 2. Flow microfluorometric analyses of sparse 3T3 cells in 10% calf serum with growth arrested by limiting (*left*) phenylalanine and (*right*) lysine in the medium.

Arrest of 3T3 Cell Growth by Limitation of Individual Amino Acids. Limitation of individual amino acids in the medium of sparse 3T3 cells, growing in the presence of 10% serum, leads to preferential arrest of growth of the cells with the G_1 content of DNA in most cases. The percentage of cells in S phase, shown by flow microfluorometry, after 3 days of culture in medium starting with 1% of the normal Dulbecco-Vogt medium concentration of the individual amino acids, was: arginine, 20%; cystine, 10%; glutamine, 15%; histidine, 10%; isoleucine, 8%; leucine, 12%; lysine, 10%; methionine, 35%; phenylalanine, 6%; threonine, 8%; tyrosine, 15%; valine, 15%. (Since glycine and serine are not required for the initiation of DNA synthesis, and since initiation is substantial in the absence of added tryptophan, these three amino acids are omitted from the above list.) Flow microfluorometric analyses are given in Fig. 2 for cells grown with limiting lysine and with limiting phenylalanine.

Initiation of DNA synthesis and growth after the readdition of the single limiting amino acid is generally poor under these conditions, suggesting that the cells are in poor condition after arrest of growth by deprivation of a single amino acid.

Arrest and Reinitiation of Growth by Lowering and Raising the Concentrations of All of the Amino Acids. Fig. 3 shows a flow microfluorometric analysis of 3T3 cells after the arrest of

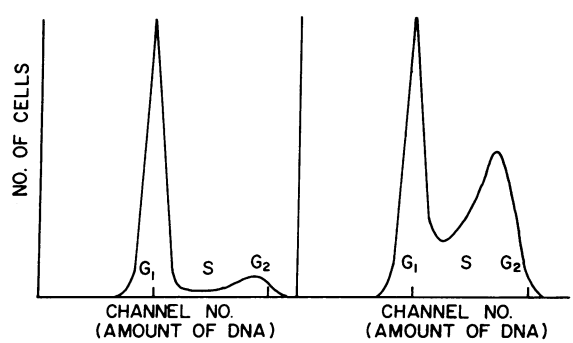

FIG. 3. Flow microfluorometric analyses of sparse 3T3 cells in 10% calf serum (*left*) with growth arrested by limiting amino acids, and (*right*) 17 hr after addition of amino acids to the arrested cells.

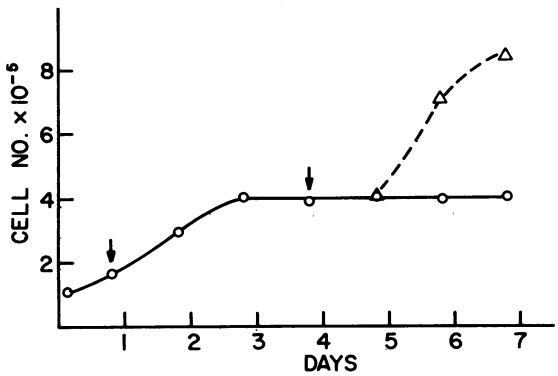

FIG. 4. Growth curves showing (*solid lines*) the arrest of growth of 3T3 cells by limitation of amino acids and (*broken line*) the reinitiation of growth after the addition of amino acids. At day 1, *first arrow*, the culture fluid was changed to medium that contained 2% of the normal concentrations of all of the amino acids. At day 4, *second arrow*, amino acids were added to half the dishes (*broken line*).

growth of sparse cells, in 10% Sephadex G-50-filtered serum, by limiting the concentrations of all of the amino acids in Dulbecco-Vogt modified Eagle's medium. The figure also gives the pattern obtained 17 hr after the reinitiation of growth by the addition of amino acids. The percentage of cells in the S phase increased from less than 10% to over 60%. Reinitiation of DNA synthesis takes place in this instance not only after the addition of all the amino acids but also after the addition of only cystine plus glutamine, suggesting that it is the concomittant limitation of these two amino acids that is responsible for the arrest of growth of the cells in G_1 (or G_0) phase under these conditions. The other amino acids must also be added to sustain DNA synthesis and growth.

Fig. 4 shows growth curves during this experiment. Studies by autoradiography indicated that the initiation of DNA synthesis requires 12–15 hr after the addition of amino acids.

Arrest and Reinitiation of Growth by Lowering and Raising the Phosphate Concentration in the Medium. Flow microfluorometric analyses (Fig. 5) showed that limitation of phosphate in the medium of sparse cells in 10% Sephadex G-50-filtered serum arrests the growth of 3T3 cells primarily in the G_1 phase. Reinitiation of growth results from the addition of phosphate, or, more synchronously, after the addition of phosphate plus 10 μg/ml of insulin [which stimulates the uptake of phosphate under the conditions of Table 1 in this paper as well as under the conditions used by Cunningham and Pardee (11) to show stimulation of uptake by serum (R. W. Holley and J. A. Kiernan, unpublished)]. Studies by autoradiography indicated that the initiation of DNA synthesis by phosphate or phosphate plus insulin requires 12–15 hr. In the autoradiographic experiments the percentage of cells in S phase rose from approximately 10% in low phosphate to 20% with added phosphate and to 40% with added phosphate plus insulin. Insulin alone had little effect.

TABLE 1. *Effects of the fibroblast growth factor and insulin on uptake of 2-deoxyglucose and phosphate ion by quiescent cells*

Additions	2-Deoxyglucose cpm ± SE	Phosphate cpm ± SE
None (depleted medium)	770 ± 37	590 ± 19
4% fresh serum	1530 ± 58	1100 ± 32
50 ng/ml of fibroblast growth factor	1600 ± 54	700 ± 20
50 ng/ml each of fibroblast growth factor + insulin	2330 ± 70	790 ± 20
50 ng/ml of insulin	1630 ± 70	700 ± 35
2 μg/ml of insulin	2580 ± 100	700 ± 18

For conditions see *Materials and Methods*. *SE* is standard error.

Arrest and Reinitiation of Growth by Lowering and Raising the Glucose Concentration in the Medium. Flow microfluorometric analyses (Fig. 6) showed that limitation of glucose in the medium of sparse cells in 20% Sephadex G-50-filtered serum arrests the growth of 3T3 cells primarily in the G_1 phase. Reinitiation of growth results from the addition of glucose. The percentage of cells in S phase increased from approximately 10% in low glucose to 30% after the adddition of glucose.

Stimulation of 2-Deoxyglucose and Phosphate Uptake. Table 1 summarizes the effects of serum, the fibroblast growth factor, and insulin on the uptake of 2-deoxyglucose and phosphate by quiescent 3T3 cells. Both pure factors stimulate uptake within 30 min.

DISCUSSION

It is clear from the results described above that the initiation of DNA synthesis in 3T3 cells can be regulated by the availability of a variety of low-molecular-weight nutrients: amino

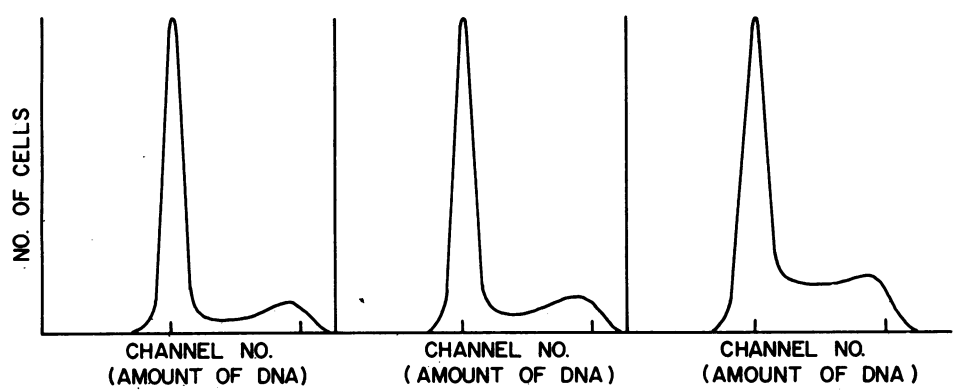

FIG. 5. Flow microfluorometric analyses of: (*left*) sparse 3T3 cells in 10% serum with growth arrested by a low phosphate concentration in the medium; (*center*) 17 hr after the addition of phosphate to the medium; and (*right*) 17 hr after the addition of phosphate plus insulin.

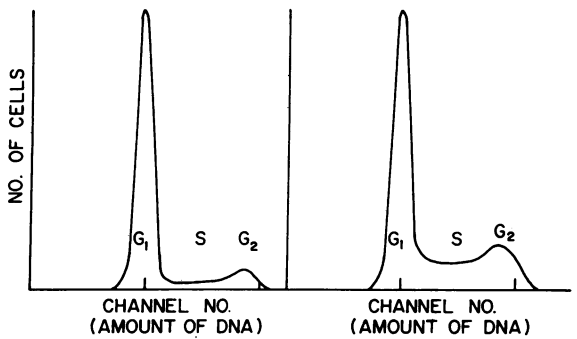

FIG. 6. Flow microfluorometric analyses of (*left*) sparse 3T3 cells in 20% serum with growth arrested by a low glucose concentration in the medium and (*right*) 16 hr after the addition of glucose.

acids, glucose, and phosphate ion. When one of the nutrients becomes limiting, growth of the cells is arrested in the early G_1 (or G_0) phase of the cell cycle. Raising the concentration of the limiting nutrient can lead to the initiation of DNA synthesis. DNA synthesis in these same cells is normally controlled by serum factors, or by insulin, the fibroblast growth factor of Gospodarowicz, and dexamethasone (4).

Prior to the work of Ley and Tobey (12) it was not anticipated that limitation of a nutrient would arrest growth in G_1. The present experiments extend their work and indicate that under the appropriate conditions DNA synthesis can be arrested or initiated by limiting or adding any one of a variety of different low-molecular-weight nutrients. Apparently, mechanisms exist within the cell that arrest the growth of the cell in the early G_1 (or G_0) phase if nutrients seem to be in insufficient supply to permit completion of a round of cell division. Such mechanisms would increase the chances of survival under conditions of starvation.

The many different factors that control the initiation of DNA synthesis in 3T3 cells fall into two groups. Certain factors, such as insulin and the fibroblast growth factor, presumably act at the cell surface, and others, low-molecular-weight compounds, presumably act inside the cell. An important action of factors that act at the cell surface may be to increase the uptake of low-molecular-weight nutrients (13). As is shown in the present paper, both insulin and the fibroblast growth factor stimulate uptake of nutrients by quiescent 3T3 cells, but it is not known whether this is a primary action of the factors.

The internal mechanisms by which the different types of factors control the initiation of DNA synthesis are not clear. With such varied factors, it seems likely that detailed mechanisms will differ, though the actions of the widely different factors may converge at some common internal mechanism. From some viewpoints, the internal control mechanisms are of the greatest interest. However, if one wishes to understand the external controls of growth, what is important is knowledge of the external agents that can control the initiation of DNA synthesis in a given cell. It is clear from the present work that the external agents can be numerous and varied. There is suggestive evidence that the same situation exists *in vivo* (14, 15).

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