MITOGENIC EFFECT OF FIBROBLAST GROWTH FACTOR ON EARLY PASSAGE CULTURES OF HUMAN AND MURINE FIBROBLASTS

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Animal cells in tissue culture generally require serum for growth (10, 11). When serum is absent, or present at low concentrations (< 1%), growth is limited, i.e., there is no net increase in cell number over time under environmental conditions that, in the presence of serum, support growth. While the growth-promoting factors in serum remain mostly unidentified, there is evidence that steroids (1, 4, 7, 9, 11, 15) and polypeptides (1–11, 13, 14, 17, and footnote 1) may be required. One polypeptide, fibroblast growth factor (FGF),[2] purified from bovine brain[1] and pituitary (5) has been shown to stimulate the division of BALB/c (7, 9) and Swiss (11) 3T3 mouse fibroblasts and Y-1 mouse adrenal tumor cells (6) in the presence of limiting amounts of serum.

Since Todaro and Green (16) describe the establishment of the 3T3 cell line as a process of selection for mutant, usually heteroploid, cells adapted to in vitro culture, it cannot be assumed that growth control in these cells is the same as that in the population from which they originated. Therefore, in order to further ascertain the role of FGF in the control of cell division, we have examined the mitogenic effect of FGF on cells in early passage derived from normal (non-neoplastic) tissue from both human and murine sources.

[1] Gospodarowicz, D. 1975. Purification of fibroblast growth factor from bovine brain. Manuscript submitted for publication.

[2] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; Ha cells, mouse fibroblast cultures; HF cells, diploid human foreskin fibroblasts.

MATERIALS AND METHODS

Materials

FGF purified from bovine pituitary glands (5) was used throughout except in the experiment described in Fig. 3 wherein FGF from bovine pituitary was compared with FGF from bovine brain. Both FGF's were greater than 95% pure on acrylamide gel electrophoresis and had a mol wt of 13,400. Dexamethasone, insulin, and thymidine were obtained from Sigma Chemical Co., St. Louis, Mo. Crystalline bovine serum albumin was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y. Sera were obtained from Gibco (Grand Island Biological Co., Grand Island, N.Y.) and trypsin from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Tissue culture dishes were obtained from Falcon, Division of BioQuest, Oxnard, Calif. [methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from New England Nuclear, Boston, Mass.

Cell Lines

Diploid human foreskin fibroblasts (HF cells) were the gift of Dr. D. Kingsbury of Scripps Clinic and Research

Foundation, La Jolla, Calif. HF cells in passages 11–26 were used. Mouse fibroblast cultures (Ha cells) were prepared from fetuses of 14–18-day pregnant, random-bred, Ha/ICR Swiss mice from the Strong Foundation, San Diego, Calif. 8–12 fetuses were decapitated and eviscerated, then minced and dissociated by repeated pipetting in trypsin (0.25% in Ca^{++}-free buffered saline). The resulting suspension was filtered to remove clumps of tissue and centrifuged in 20% horse serum. The pellet was suspended in medium, and the suspended cells were plated in 6-cm dishes containing 5 ml of Dulbecco's modified Eagle's medium (DME) with 10% calf serum for primary cultures. Excess cells were plated in 15-cm dishes to create a stock which was later used (after one passage) to produce tertiary cultures. After 2 days in primary culture, and in all secondary and tertiary cultures, more than 90% of the cells were fibroblast-like in appearance.

Cell Culture

Cells were routinely cultured at 37°C in DME in 15-cm plastic dishes in a humidified, CO_2 incubator. Stocks of HF and Ha cells were grown in 5% and 10% calf serum, respectively. Subconfluent cultures were trypsinized (0.25% trypsin in Ca^{++}-free buffered saline). The trypsinization was stopped by adding horse serum to a final concentration of 20%, and the cell suspension was centrifuged. The pellet was suspended in medium, and the resulting suspension was distributed among 15-cm dishes (for continuance of the stocks) or 6-cm dishes (for experiments).

Sample Addition

FGF and insulin were added in 25–100 μl of a 0.5% solution of crystalline bovine serum albumin in DME. Dexamethasone was added in 25–50 μl of absolute ethanol.

Quantitative Determination of [^{3}H]Thymidine Incorporation

The incorporation of [^{3}H]thymidine into DNA was assayed as previously described (7).

Growth Rate Determinations

Cells were plated in 6-cm dishes containing 5 ml DME with 2.5% (HF cells) or 10% (Ha cells) calf serum. After 1 day (HF cells) or 3 days (Ha cells) the media were replaced with media containing 0.2% (HF cells) or 0.4% (Ha cells) calf serum. After 2 days (HF cells) or 1 day (Ha cells) in low serum, samples were added. FGF, insulin, and dexamethasone were added daily; serum was added only once.

Cells were counted in duplicate in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) after trypsinization.

RESULTS

Initiation of DNA Synthesis in Human Foreskin Fibroblasts

SPARSE CULTURES IN 0.05% SERUM: When 10% calf serum was added to sparese cultures of HF cells that had been made quiescent by maintaining them for 2 days in 0.05% serum, DNA synthesis was initiated (Table I). FGF (50 ng/ml) had only 5% of the effect of serum. The addition of either dexamethasone or insulin did not stimulate DNA synthesis or potentiate the response to FGF.

SUBCONFLUENT CULTURES IN 5% SERUM: In contrast to the results in 0.05% serum, quiescent cultures in 5% serum were very responsive to FGF. When calf serum or FGF was added, DNA synthesis was initiated (Fig. 1). The minimal effective dose of FGF was 0.5 ng/ml (4×10^{-11} M). At 10 ng/ml, FGF stimulated DNA synthesis as effectively as did the optimal amount of added serum. When 100 ng/ml of FGF was added, [^{3}H]thymidine incorporation was 11 times the control value and 47% higher than the maximal incorporation with serum.

In order to determine if the greater [^{3}H]thymidine incorporation in response to FGF as compared with serum was due to a larger proportion of the cells initiating DNA synthesis, autoradiography of cultures prepared in 5% calf serum as described above was performed. In controls less than 3% of the nuclei incorporated [^{3}H]thymidine in a 12-h labeling period. When 10% calf serum was added to the cultures (to result in a serum concentration of 15%), 17% of the nuclei were labeled. However, when 50 ng/ml FGF was added, 58% of the nuclei were labeled during the same period, and the addition of 10% serum plus 50 ng/ml FGF resulted in more than 85% labeling.

To see whether or not the initiation of DNA synthesis described in Fig. 1 was followed by cell division, the number of cells per dish, 63 h after sample additions, was determined (with a SE $<$ 5% of the mean). The cell densities were 4.5×10^4 cells/cm^2 when 10% serum had been added, 5.1×10^4 when 25 ng/ml FGF had been added, 6.8×10^4 when both 10% serum, and 25 ng/ml FGF had been added. The cell density in control dishes was 3.0×10^4 cells/cm^2.

TABLE I

Initiation of DNA Synthesis in Sparse, Quiescent Cells in Low Serum

Addition	[³H]thymidine	
	Human (HF) cells	Mouse (Ha) cells
	cpm/dish	
Calf serum (10%)	3,699	27,887
FGF (25 ng/ml)	338	6,669
FGF (50 ng/ml)	355	8,148
Dexamethasone (50 ng/ml)	196	730
Dexamethasone (500 ng/ml)	160	—
Insulin (500 ng/ml)	—	1,185
Insulin (1,000 ng/ml)	244	1,660
Dexamethasone (50 ng/ml) + FGF (25 ng/ml)	—	5,798
Dexamethasone (50 ng/ml) + FGF (50 ng/ml)	271	—
Dexamethasone (500 ng/ml) + FGF (25 ng/ml)	—	5,862
Dexamethasone (500 ng/ml) + FGF (50 ng/ml)	283	—
Insulin (500 ng/ml) + FGF (25 ng/ml)	—	5,372
Insulin (1,000 ng/ml) + FGF (50 ng/ml)	269	—
Control	180	1,635

Serum, FGF, and hormones were added to sparse (4.1×10^3 cells/cm² for HF cells and 7.1×10^3 cells/cm² for Ha cells) cultures that had been made quiescent by maintaining them for 2 days in low serum (0.05% for HF and 0.4% for Ha cells) with 5 ml of medium/6-cm dish. DNA was labeled with [³H]thymidine for a 24-h period beginning 12 h after sample addition. [³H]thymidine was recovered as cold trichloroacetic acid-precipitable material. Ha cells were in their third passage. Standard errors did not average more than 10% of the mean.

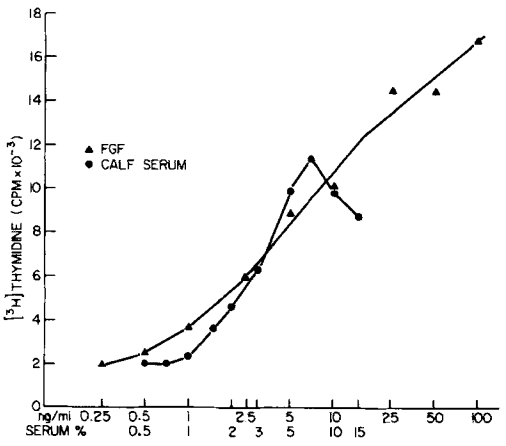

FIGURE 1 DNA synthesis in subconfluent human (HF) cells in response to various concentrations of serum or FGF. Serum and FGF were added to subconfluent (2.8×10^4 cells/cm²) cultures that had been made quiescent by maintaining them for 4 days in 5% calf serum (5 ml of medium/6-cm dish). DNA was labeled with [³H]thymidine for a 24-hr period beginning 12 h after sample addition. Abscissa, ³H cpm/dish in cold trichloroacetic acid-precipitable material. Ordinate, nanograms/milliliters FGF added (▲—▲) or percent serum added (●—●). Incorporation was 1,550 cpm/dish in controls. Standard errors did not average more than 10% of the mean.

Growth-Promoting Activity of FGF for Human Foreskin Fibroblasts

Since the initiation of DNA synthesis in response to FGF does not prove that FGF will induce mitosis in sparse cultures, the increases in cell number in cultures maintained in the presence of FGF, 10% serum, or 10% serum plus FGF were compared. When 10% calf serum was added to sparse, quiescent cultures maintained in 0.2% serum, the number of cells increased exponentially with an apparent doubling time of approximately 48 h, and the final density was 3×10^4 cells/cm² (Fig. 2). Culture growth followed a similar pattern in 20% fetal calf serum. When FGF (25 ng/ml added daily) was added to quiescent cultures in 0.2% serum, the cell number doubled. This result is in accord with the observation that FGF provoked only a twofold increase in [³H]thymidine incorporation in cultures maintained in low (0.05%) serum. When FGF was added along with serum, the apparent doubling time was half that observed with 10% calf serum or 20% fetal calf serum alone, and the final density was 10^5 cells/cm², 3.3 times as great as with serum alone.

The appearance of the cells in the presence of FGF was markedly different from that of cells in

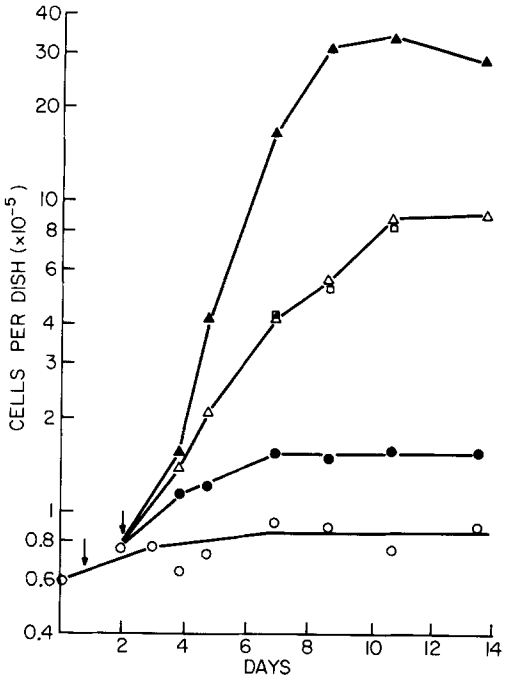

FIGURE 2 Growth curves of human (HF) cells. Cells were plated at 6 × 10^4 cells/6-cm dish in 5 ml of 2.5% calf serum on day 0. On day 1 (*arrow*), the medium was changed to medium containing 0.2% serum. On day 2 (*arrow*), either 10% calf serum (Δ—Δ), 20% fetal calf serum (□—□), 50 ng/ml of FGF (●—●), or 50 ng/ml FGF plus 10% calf serum (▲—▲) was added. Sera were added once; FGF was added daily. Controls (○—○). Standard errors did not average more than 10% of the mean.

either 0.2% or 10% serum alone. In the presence of FGF, the cells were elongated and no so flat as cells in serum alone. This morphological change took place within 2 days of the addition of FGF and was not dependent on cell density.

When cells that had been grown to a high density in the presence of FGF were replated, their growth characteristics and morphology in 0.2 and 10% serum were the same as those of cells that had never been exposed to FGF.

Initiation of DNA Synthesis in Mouse Fibroblasts

SPARSE CULTURES IN 0.4% SERUM: When calf serum or FGF was added to third passage cultures in 0.4% calf serum, the initiation of DNA synthesis was observed (Table I). FGF (50 ng/ml) had 25% of the effect of the optimal concentration (10%) of added serum. The addition of either dexamethasone or insulin did not stimulate DNA synthesis or potentiate the response to FGF.

CONFLUENT CULTURES IN 10% SERUM: When calf serum or FGF was added to tertiary cultures which had grown to confluence in 10% calf serum, DNA synthesis was initiated (Fig. 3). FGF was more effective than serum. The minimal effective dose of FGF was 1 ng/ml (8 × 10^{-11} M). At 10 ng/ml the incorporation of [^{3}H]thymidine was greater than that observed with the addition of the optimal amount of serum. At 100 ng/ml, a plateau value was obtained. At this concentration, FGF induced a 5.7-fold increase in [^{3}H]thymidine incorporation over controls. Since FGF purified from pituitary is more active at low concentrations than FGF from brain for BALB/c 3T3 (unpublished observation), brain FGF and pituitary FGF were compared. The minimal effective dose of brain FGF was 10 ng/ml, 10 times higher than for pituitary FGF, and the plateau was not reached at 500 ng/ml.

In order to confirm that FGF stimulated more cells to initiate DNA synthesis than did serum, confluent cultures prepared as described above were labeled with [^{3}H]thymidine for autoradiogra-

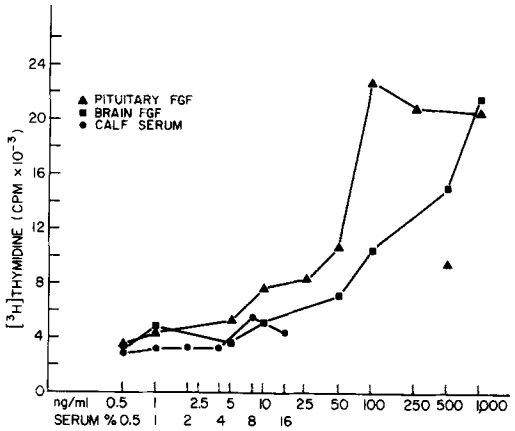

FIGURE 3 DNA synthesis in confluent mouse (Ha) cells in response to various concentrations of serum and FGF. Serum and FGF were added to confluent (7.6 × 10^4 cells/cm^2) third passage cultures that been made quiescent by maintaining them for 10 days in 10% calf serum (5 ml of medium/6-cm dish). DNA was labeled with [^{3}H]thymidine for a 24-h period beginning 12 h after sample addition. Abscissa, ^{3}H cpm/dish in cold trichloroacetic acid-precipitable material. Ordinate, nanograms/milliliter pituitary FGF (▲—▲) or brain FGF (■—■) added or percent calf serum (●—●) added. Incorporation was 4,000 cpm/dish in controls. Standard errors did not average more than 10% of the mean.

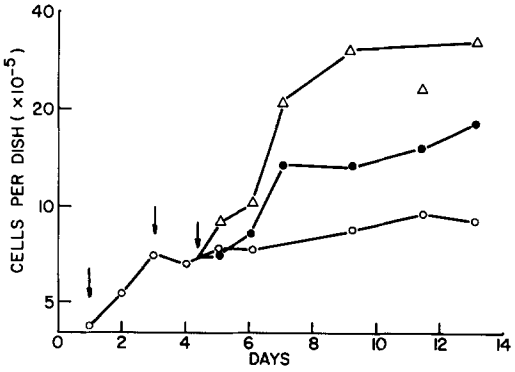

FIGURE 4 Growth curves of mouse (Ha) cells. Cells from minced, trypsinized, Swiss mice fetuses were plated in 6-cm dishes with 10% calf serum. On day 1 (*arrow*), the medium was renewed. On day 3 (*arrow*), the medium was changed to 0.4% calf serum. On day 4 (*arrow*), either 10% calf serum (Δ—Δ) or 50 ng/ml FGF (●—●) was added. Serum was added once; FGF was added daily. Controls (O—O). Standard errors did not average more than 10% of the mean.

phy. In a 12-h labeling period, 8% of nuclei were labeled in controls. When 10% calf serum was added, 26% of nuclei were labeled. The addition of 100 ng/ml FGF resulted in 67% labeling.

Growth-Promoting Activity of FGF for Mouse Fibroblasts

To see if mouse cells induced to synthesize DNA by FGF would continue through the division cycle through mitosis, the increases in cell number in subconfluent cultures maintained in 0.4% calf serum, 10% serum, or 0.4% serum plus FGF were compared. When primary cultures that had been grown for 3 days in 10% serum were fluid changed to 0.4% serum, culture growth slowed greatly; in the 10 days after the medium change, the cell density increased only about 75%, from 2.5×10^4 to 3.3×10^4 cells/cm^2 (Fig. 4). When, 2 days after the medium change, 10% serum was added, the cell number increased rapidly for 4 days and reached a final density of 1.1×10^5 cells/cm^2. When, instead of 10% serum, FGF (50 ng/ml) was added daily, there was a rapid increase in cell number for 3 days, and the final density was 6.4×10^4 cells/cm^2 (Fig. 4).

DISCUSSION

In this report we have shown that FGF is mitogenic not only for established heteroploid cell lines, but also for early passage cultures of diploid cells. Furthermore, we have shown that FGF lacks species specificity since it acts on human as well as mouse fibroblasts.

The responses of the human (HF) and murine (Ha) fibroblasts to FGF were essentially similar except that the addition of FGF to HF cultures growing in 10% serum resulted in a doubling of the growth rate, while the addition of FGF to Ha cells in 10% serum caused only a slight increase in the rate of growth of Ha cells. A large increase (60%) in growth rate in the presence of a pituitary growth factor, chondrocyte growth factor, has also been reported for lapin articular chondrocytes (13). The addition of FGF also increased the growth rate of skin fibroblasts from persons with cystic fibrosis. Such cultures grow much more slowly than do cultures from normal individuals (12). When FGF was added to cystic fibrosis-derived, cutaneous fibroblast cultures (CRL 1134 and CRL 1136 from the American Type Culture Collection, Rockville, Md.) maintained in 10% fetal calf serum, doubling times were decreased by 30–50% (unpublished observation).

The results with HF and Ha cells differ markedly from those obtained with the established murine lines of fibroblasts, BALB/c 3T3 (7, 9) and Swiss 3T3 (11), and from the results with Y-1 mouse adrenal cells (6). For 3T3 cells, the effect of FGF on DNA synthesis and cell division in low (less than 1%) serum is potentiated greatly by glucocorticoids and to a lesser extent by insulin; for Y-1 cells in 0.2% serum, FGF alone stimulates DNA synthesis and cell division as effectively as 10% serum. In contrast, FGF stimulation of the initiation of DNA synthesis was only 10% of that of serum in HF cells in 0.05% serum and only 20–80% of the maximal serum values for Ha cells maintained in 0.4% serum, and neither glucocorticoids nor insulin potentiated the response. Furthermore, while FGF alone induces one to two doublings in 3T3 cells in low serum, FGF plus dexamethasone induces multiple doublings (7). For both HF and Ha cells, neither dexamethasone nor insulin increased the final cell density of cells maintained in the presence of FGF in low serum.

The response to FGF of quiescent 3T3 cells has been shown to depend on the serum concentration in which the cells are maintained (9). Similarly, the response of chick embryo epidermis to epidermal growth factor requires the presence of serum (2). Likewise, the response of both HF and Ha cells to FGF was greatly enhanced by the presence of serum (5 or 10%), even though the serum had ceased supporting culture growth.

The clear morphological change in HF cells in the presence of FGF was reminiscent of changes induced by FGF and dexamethasone in 3T3 cells in 10% serum (8). A similar, but less marked change was noted in Ha cells. WI-38 fibroblasts respond to FGF with a morphological change very much like that observed in HF cells, although FGF is not mitogenic for WI-38 (unpublished observation). The significance of these changes remains unknown.

The results described in this paper that appear, at this time, to have special significance are the following: (*a*) the finding that the division of early passage mouse fibroblasts is not under the control of the same system of steroids and macromolecules as is the division of 3T3 cells (7, 9, 11) suggests that 3T3 cell division is not regulated by the same array of factors that regulate cell division in vivo, (*b*) the observation that the growth rate of cultures of human foreskin fibroblasts in high serum can be greatly increased by the addition of small amounts of a purified polypeptide indicates that for this cell type neither 10% calf serum nor even 20% fetal calf serum is sufficient to support a maximal growth rate. The identification of a factor that accelerates growth in the presence of serum opens a new area in the investigation of factors that control cell division.

SUMMARY

Fibroblast growth factor (FGF), a polypeptide that has been shown to stimulate division in 3T3 cells, was tested for mitogenic effects on diploid, early-passage cells from human and murine sources. The quantitative assay of [^{3}H]thymidine incorporation into acid-insoluble material showed that FGF at low concentrations (10^{-9} M) was more effective than additional serum for provoking the initiation of DNA synthesis in human foreskin fibroblasts or mouse fibroblasts maintained in 5 or 10% serum, respectively. The growth of the human fibroblasts was twice as fast in the presence of FGF plus 10% calf serum as it was in the presence of 10% calf serum or 20% fetal calf serum alone. The addition of FGF to primary cultures of mouse fibroblasts in 0.4% serum resulted in a twofold increase in cell number compared to controls. In contrast to results obtained with 3T3 cells, neither insulin nor a glucocorticoid potentiated the effects of FGF on either human or mouse cells.

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REFERENCES

1. ARMELIN, H. 1973. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proc. Natl. Acad. Sci. (U. S. A.).* **70**:2702–2706.
2. BERTSCH, S., and F. MARKS. 1974. Effect of foetal calf serum and epidermal growth factor on DNA synthesis in explants of chick embryo epidermis. *Nature (Lond.).* **251**:517–519.
3. COHEN, S., and J. M. TAYLOR. 1974. Epidermal growth factor: chemical and biological characterization. *Recent Progr. Horm. Res.* **30**:533–550.
4. GOSPODAROWICZ, D. 1974. Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature (Lond.).* **249**:123–127.
5. GOSPODAROWICZ, D. 1975. Purification of fibroblast growth factor from bovine pituitary. *J. Biol. Chem.* **250**:2515–2520.
6. GOSPODAROWICZ, D., and H. H. HANDLEY. 1975. Stimulation of division of Y-1 adrenal cells by a growth factor isolated from bovine pituitary glands. *Endocrinology.* In press.
7. GOSPODAROWICZ, D., and J. MORAN. 1974. Stimulation of division of sparse and confluent 3T3 cell populations by a fibroblast growth factor, dexamethasone and insulin. *Proc. Natl. Acad. Sci. (U. S. A.).* **71**:4584–4588.
8. GOSPODAROWICZ, D., and J. MORAN. 1974. Effect of a fibroblast growth factor, insulin, dexamethasone and serum on the morphology of BALB/c 3T3 cells. *Proc. Natl. Acad. Sci. (U. S. A.).* **71**:4648–4652.
9. GOSPODAROWICZ, D., and J. MORAN. 1975. Optimal conditions for the study of growth control in Balb/c 3T3 fibroblasts. *Exp. Cell Res.* **90**:279–284.
10. HOLLEY, R. W. 1974. Serum factors and growth control. Control of Proliferation in Animal Cells. Cold Spring Harbor Conf. Cell Proliferation. **1**:13–18.
11. HOLLEY, R. W., and J. A. KIERNAN. 1974. Control of the initiation of DNA synthesis of 3T3 cells: serum factors. *Proc. Natl. Acad. Sci. (U. S. A.).* **71**:2908–2911.
12. HOUCK, J. C., and F. L. CHENG. 1974. Defects in the cell cycle of fibroblasts obtained from patients with cystic fibrosis. *Proc. Soc. Exp. Biol. Med.* **147**:167–170.
13. MALEMUD, C. J., and L. SOKOLOFF. 1974. Some biological characteristics of a pituitary growth factor (CGF) for cultured lapine articular chondrocytes. *J. Cell. Physiol.* **84**:171–180.

14. TEMIN, H. M., G. L. SMITH, and N. C. DULAK. 1974. Control of multiplication of normal and RSV transformed chicken embryo fibroblasts by purified MSA with NS ILA and sulfation factor activities. Cold Spring Harbor Conf. Cell Proliferation. **1**:19–26.
15. THRASH, C. R., and D. D. CUNNINGHAM. 1973. Stimulation of division of density inhibited fibroblasts by glucocorticoids. *Nature* (*Lond.*). **242**:399–401.
16. TODARO, G. J., and H. GREEN. 1963. Mouse embryo cells in culture. *J. Cell Biol.* **17**:299–313.
17. VAN WYK, J. J., L. E. UNDERWOOD, R. L. HINTZ, D. R. CLEMMONS, S. J. VOINA, and R. P. WEAVER. 1974. The somatomedins: a family of insulin-like hormones under growth hormone control. *Recent Progr. Horm. Res.* **30**:259–294.