

Control of Myogenic Differentiation by Fibroblast Growth Factor Is Mediated by Position in the G₁ Phase of the Cell Cycle

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ABSTRACT We have used the expression of the muscle form of creatine phosphokinase (M-CPK) to assay myogenic differentiation in the cloned muscle cell line BC₃HI. BC₃HI cells express M-CPK when arrested in the G₀ portion of the cell cycle. Addition of the anionic form of brain fibroblast growth factor (B-FGF) rapidly represses synthesis of M-CPK with a half-time of 7 h. Even though B-FGF is not mitogenic for the cells, it causes quiescent BC₃HI cells to exit from the G₀ portion of the cell cycle, and to accumulate at a new restriction point ~4 to 6 h in the G₁ portion of the cell cycle. The repression of M-CPK synthesis by B-FGF is reversible upon removal of B-FGF, and cells which have re-initiated expression of M-CPK have also returned to the G₀ portion of the cell cycle. The primary control of M-CPK expression by B-FGF appears to be at the level of gene transcription. We conclude that arrest of cells at G₀ but not at other positions in the G₁ phase of the cell cycle provides permissive conditions for the expression of muscle-specific proteins, and that defined polypeptide growth factors, in this case B-FGF, are important in the control of the expression of muscle-specific proteins.

The process of muscle differentiation requires the coordinated expression (induction) of a group of proteins associated with the muscle phenotype (1–3). The induction of muscle-specific proteins occurs after myoblasts cease proliferation, and are arrested early in the G₁ portion of the cell cycle (4, 5).

A number of permanent cell lines can be induced to express proteins associated with the muscle phenotype (for review see reference 1). Some of these cell lines, which do not fuse to form multinucleated myotubes, also do not become irreversibly committed to the differentiated phenotype. Upon addition of mitogens, usually serum, these cells reinitiate growth and concomitantly the expression of muscle-specific proteins is repressed (5–7). We have used BC₃HI cells, a clonal muscle cell line, to examine the control of cell differentiation by mitogenic polypeptides using the muscle form of creatine phosphokinase (M-CPK)¹ as a probe to assay cell differentiation. We have reported recently that addition to differentiated BC₃HI cells of serum, commercial (impure) pituitary fibro-

blast growth factor, or pure anionic brain fibroblast growth factor (B-FGF) results in repression of M-CPK synthesis (7, 8). Under the conditions used, B-FGF is nonmitogenic for BC₃HI cells, suggesting that it is possible to dissociate the mitogenic response from the repression of muscle-specific protein synthesis. Observations have recently been reported in preliminary form showing that cell growth is not required for repression of muscle-specific functions (9).

In this communication we examine in detail the repression of M-CPK by addition of B-FGF to differentiated BC₃HI cells. We demonstrate that B-FGF causes quiescent BC₃HI cells to exit from G₀ into G₁ and become arrested at a new restriction point within G₁. The repression of M-CPK synthesis appears to be concomitant with, or follow shortly after, the exit of BC₃HI cells from G₀, and apparently reflects a rapid decline in the rate of transcription of the M-CPK gene.

MATERIALS AND METHODS

Cell Culture: BC₃HI cells (10) were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum in an atmosphere of 10% CO₂ as described (8). Cell growth was arrested at subconfluent densities

¹ *Abbreviations used in this paper:* B-CPK and M-CPK, the brain and muscle forms of creatine phosphokinase; B-FGF, the anionic form of brain fibroblast growth factor.

by re-feeding with DME supplemented with 1% serum. Unless otherwise indicated, B-FGF (5 ng/ml) was added to quiescent differentiated BC₃H1 cells in a simplified medium of DME and F12 at a 3:1 ratio supplemented with 250 µg/ml bovine serum albumin (BSA), 50 µg/ml transferrin, 0.25% serum, and antibiotics as described (8).

Determination of Rate of CPK Synthesis: The rate of CPK synthesis was determined by immunoprecipitation of Triton X-100-solubilized extracts that had been pulse labeled for 2 h with 100 µCi/ml [³⁵S]methionine as described (8). M- and B-isozymes of CPK were separated by SDS PAGE, and the radioactive bands were visualized by fluorography. The relative rate of synthesis of M-CPK was determined by densitometry of fluorographs (8).

Ornithine Decarboxylase Assay: Ornithine decarboxylase was assayed by the release of radioactive CO₂ from L-ornithine essentially by the method of Heller et al. (11). Cell monolayers were washed twice with cold phosphate-buffered saline and harvested by scraping in 10 mM Tris-HCl, pH 7.2, 50 mM KCl, 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, 5 mM dithiothreitol, and 0.025% Tween 20 at 4°C. Reactions were carried out in stoppered 12-ml glass conical centrifuge tubes at 37°C for 1 h. 150 µl of extract were added to 15 µl L-1-[¹⁴C]ornithine (Amersham Corp., Arlington Heights, IL) (8×10^{-8} M) at a final specific radioactivity of 7.5 Ci/mol, and 10 µl of 0.5 M Tris-HCl, pH 7.2. Reactions were quenched by injecting 0.2 ml of 10% trichloroacetic acid through the stopper, and liberated radioactive CO₂ was trapped with a wick soaked with 100 µl of 10% NaOH held in place by a center well (Kontes Co., Vineland, NJ). Wells with wicks were counted in 10 ml 3a70 scintillation fluid (Research Products International Corp., Mt. Prospect, IL). The assay was linear with respect to extract concentration and time.

Other Procedures: Fixing, mounting and developing cultures for autoradiography after labeling with [³H]thymidine was carried out essentially by the method of Cassel et al. (12). Cultures were grown on 35-mm dishes, and after labeling with [³H]thymidine, the cultures were washed once with 2 ml of phosphate-buffered saline, and fixed overnight with 2 ml of 4% paraformaldehyde in the same buffer. The rim of the dishes was removed and the bottom processed for autoradiography (12). 1 µCi/ml of [³H]methylthymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was used for 48-h labeling of BC₃H1 cultures. For 15-h cumulative labeling experiments, 3 µCi/ml were used.

The rate of thymidine incorporation was measured by trichloroacetic acid-precipitable radioactivity incorporated after a 1-h pulse with 3 µCi/ml of [³H]thymidine (6.7 Ci/mmol), as described by Whittenberger and Glaser (13). Uridine incorporation was measured by the same procedure after cultures had been pulsed with 2 µCi/ml of [³H]uridine (30 Ci/mmol; New England Nuclear) for 1 h. Labeling was linear with respect to time at these concentrations of isotopes. Epidermal growth factor was prepared as described (14).

RESULTS

When BC₃H1 cells are cultured in 1% serum, they cease to proliferate and synthesize muscle-specific proteins such as M-CPK. Fig. 1 shows that addition of B-FGF to differentiated cells, as described in Materials and Methods, results in a rapid decline of the rate of M-CPK synthesis as measured by immunoprecipitation from cells labeled with [³⁵S]methionine for 2 h. A half-life of 7 h for the decline in the rate of M-CPK synthesis can be estimated from the data in Fig. 1, and within the resolution of the method the decline is initiated without a significant time lag.

The decline in the rate of M-CPK synthesis observed in Fig. 1 could either reflect a decrease in the rate of transcription of the M-CPK gene or a specific destabilization of the M-CPK mRNA. A preliminary assessment of which of these alternatives applies can be obtained by the use of actinomycin D to block transcription in the absence of B-FGF. If the rate of M-CPK mRNA degradation is unaffected by the addition of B-FGF, then the decline in the rate of M-CPK should be the same whether B-FGF or actinomycin D is added to cells. The data in Fig. 2 document the decline in the rate of M-CPK synthesis after addition of actinomycin D, and these data have been incorporated in Fig. 1 B (triangles). The decline in the rate of M-CPK synthesis is the same whether B-FGF or actinomycin D are added to cells. The observations were similar using different levels of actinomycin D from 0.05 to

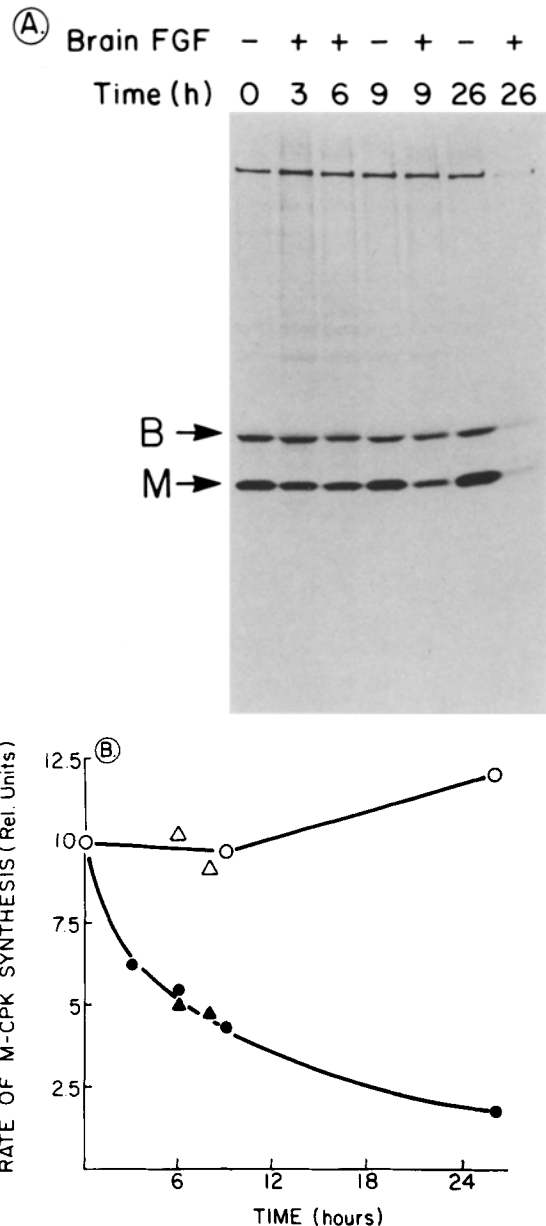


FIGURE 1 Repression of M-CPK synthesis by B-FGF. Differentiated BC₃H1 cells were re-fed with medium that contained B-FGF. At times indicated, the cultures were pulsed for 2 h with [³⁵S]methionine and CPK was immunoprecipitated and the B and M forms separated by PAGE, as described in Materials and Methods. A illustrates a fluorogram of a polyacrylamide gel, and B a densitometry tracing of such fluorogram. ○, controls not treated with B-FGF; ●, cultures treated with B-FGF; ▲, cultures treated with 0.05 µg/ml of actinomycin D; △, control cultures (see Fig. 2). B-FGF does not alter the rate of total protein synthesis in these cultures, and in some instances increases total rate of protein synthesis by 20% (not shown). The B and M isozymes of CPK in A are indicated by arrows.

0.2 µg/ml. The former level decreased the rate of uridine incorporation into RNA by 89% and the latter by 97%. Under the same conditions, the rate of synthesis of a number of major cellular polypeptides detected by one-dimensional gel electrophoresis was reduced by <25%, indicating that actinomycin-D does not significantly block protein synthesis nonspecifically under these conditions. While these observations will have to be confirmed by direct measurements of the cellular level of M-CPK mRNA, they strongly suggest that the major effect of B-FGF is at the level of gene transcription.

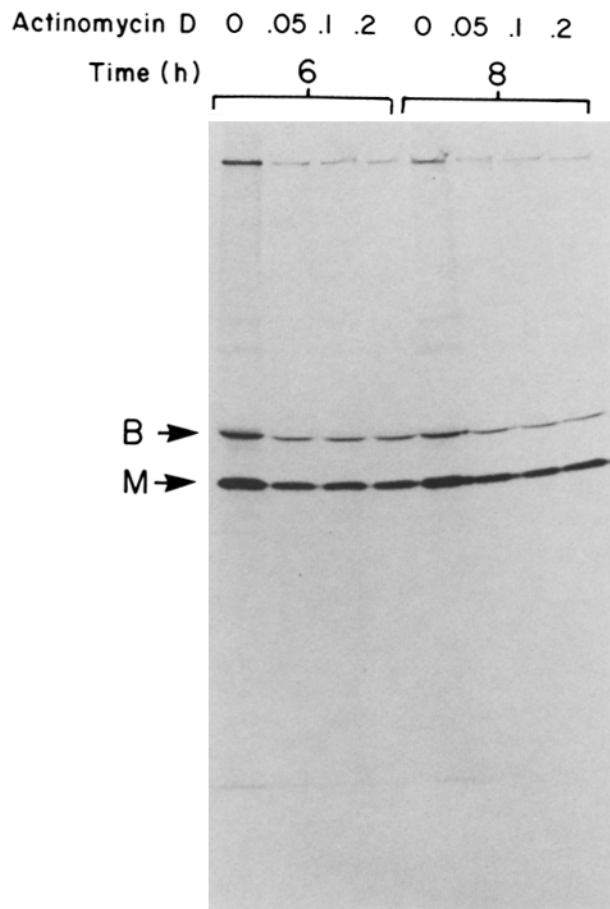


FIGURE 2 Effect of actinomycin D on the rate of M-CPK synthesis. Differentiated cultures were treated with actinomycin D for the duration and at the concentration indicated. Cultures were washed to remove actinomycin D, and the rate of M-CPK synthesis was determined, as described in Materials and Methods. A fluorograph of extracts which had been subjected to gel electrophoresis is shown. The M and B isozymes of CPK are indicated by arrows. The rate of M-CPK synthesis in the presence of 0.05 $\mu\text{g/ml}$ actinomycin D is shown in Fig. 1 by black triangles.

BC₃H1 Cells in the Presence of B-FGF Transit from G₀ to G₁

Previous observations (8) have indicated that, under our culture conditions, B-FGF was not mitogenic for BC₃H1 cells as determined by cell number 48 h after addition of B-FGF to cells. We have, in addition, used various combinations of B-FGF with insulin (1 $\mu\text{g/ml}$), epidermal growth factor (10 ng/ml), dexamethasone (0.5 $\mu\text{g/ml}$), and phorbolmyristic acid (100 ng/ml), and failed to observe an increase in cell number due to B-FGF after addition to quiescent BC₃H1 cells.

A more sensitive assay to determine whether B-FGF can cause even a small fraction of cells to traverse the cell cycle is to culture cells with [³H]thymidine in the presence of B-FGF and determine by autoradiography the fraction of cell nuclei labeled with [³H]thymidine as a measure of the fraction of cells that have entered S-phase in the cell cycle. 48 h after addition of B-FGF and [³H]thymidine to BC₃H1 cells, only $9.8 \pm .8\%$ of nuclei were labeled, compared to $13.4 \pm 1.6\%$ in the absence of B-FGF, confirming that B-FGF does not allow BC₃H1 cells to enter S-phase.

If B-FGF does not cause BC₃H1 cells to traverse the cell

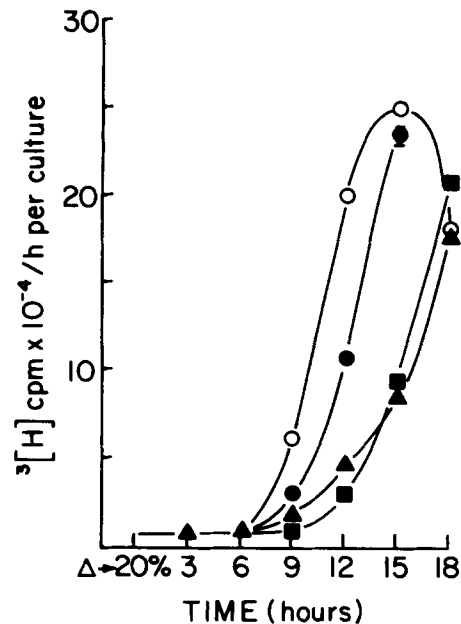


FIGURE 3 Effect of B-FGF on the duration of G₁. B-FGF was added directly to differentiated cultures at 5 ng/ml. Control cultures received no B-FGF. After incubation for the times indicated, cells were stimulated to enter S-phase by re-feeding with serum. The entry into S-phase was determined by the increased rate of incorporation of [³H]thymidine into a trichloroacetic acid-precipitable form. ▲, control cultures; ■, no pretreatment; ●, 4-h pretreatment; ○, 12-h pretreatment.

cycle, does it nevertheless move cells from G₀ to G₁? When quiescent BC₃H1 cells are stimulated with serum, they enter S-phase after ~12 h (Fig. 3). If B-FGF causes BC₃H1 cells to move into G₁, then when serum is added to BC₃H1 cells pretreated with B-FGF, the time required for these cells to enter S-phase should be shortened. Fig. 3 illustrates that this is in fact observed; pretreatment of BC₃H1 cells for either 4 or 12 h with B-FGF before addition of 20% serum decreases the time required to enter S-phase from 12 h to 7 or 8 h, consistent with B-FGF permitting the movement of BC₃H1 cells ~4 h into G₁. Since a 4-h pretreatment with B-FGF almost maximally reduces the duration of G₁ (Fig. 3), B-FGF must move cells rapidly out of G₀. The same observations have been obtained in three independent experiments. The time dependence of the movement of cells out of G₀ is consistent with time required to initiate repression of M-CPK synthesis.

There is a potential ambiguity in measuring the rate of entry of cells into S-phase by the use of acid-precipitable thymidine which may not correlate precisely with the number of cells entering S-phase. We have repeated the experiment in Fig. 3 by measuring the number of cells entering S-phase by autoradiography following labeling of cells with [³H]thymidine to determine directly the number of cells entering S-phase. The results in Fig. 4 confirm the data in Fig. 3 and clearly indicate that B-FGF addition to quiescent BC₃H1 cells brings these cells 3–4 h into G₁.

An independent measure of entry of cells into G₁ is the induction of ornithine decarboxylase (15). In Fig. 5 we show that addition of B-FGF to quiescent BC₃H1 cells causes a small but reproducible increase in ornithine decarboxylase, fully consistent with the entry of these cells into G₁, and arrest at a new restriction point ~4–6 h into G₁, at which point induction of ornithine decarboxylase is possible. The obser-

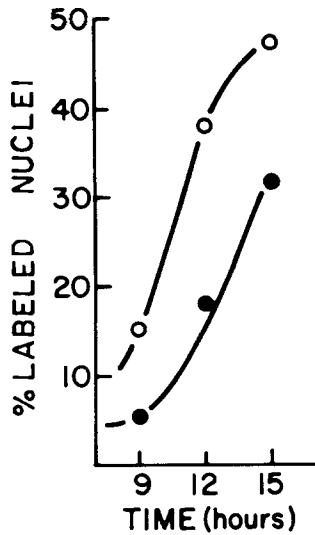


FIGURE 4 Autoradiography of B-FGF pretreated cultures labeled with $[^3\text{H}]$ thymidine. Cells were pretreated with 12 h with B-FGF as described in Fig. 3. Cells were then stimulated to enter S-phase by re-feeding with 20% serum in the presence of $1 \mu\text{Ci/ml}$ $[^3\text{H}]$ thymidine. Cultures were fixed for autoradiography at the times indicated, and the fraction of nuclei labeled with thymidine determined. ●, no treatment; ○, B-FGF pre-treatment.

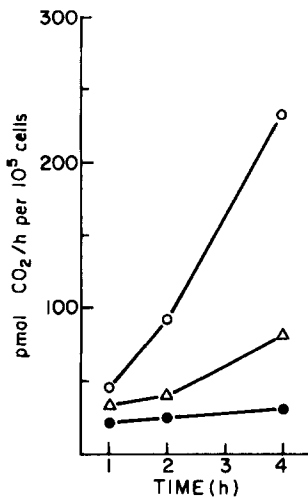


FIGURE 5 Stimulation of ornithine decarboxylase by B-FGF. B-FGF was added directly to differentiated cells at 5 ng/ml. Ornithine decarboxylase was measured at the times indicated. Cultures were re-fed when serum was added. This control presumably reflects the maximum possible stimulation of ornithine decarboxylase achievable under these conditions. ●, 1% serum; ○, 20% serum; △, B-FGF.

vation that ornithine decarboxylase is activated by B-FGF indicates that this growth factor activates at least some of the ordered series of events that occur when cells are stimulated to transit G_1 (16, 17). Because various components in serum may also influence the induction of ornithine decarboxylase, we do not believe that the absolute level of ornithine decarboxylase after the addition of B-FGF to quiescent cells can be compared in a meaningful way with the level observed after addition of serum.

Is the Repression of M-CPK Synthesis by B-FGF Reversible?

Since B-FGF causes BC_3HI cells to arrest within G_1 , and simultaneously represses M-CPK synthesis, is this repression reversible, and is it reversible in the absence of progression through the cell cycle? To answer this question, quiescent BC_3HI cells, which synthesize M-CPK, were treated with B-FGF for 48 h. Control cells received fresh B-FGF at 24 h. 24 h after addition of B-FGF, the rate of M-CPK synthesis is depressed (Fig. 6; compare lane 2 with lane 4). However, 48 h after addition of B-FGF (in the absence of daily addition of B-FGF), the rate of M-CPK synthesis has returned to the fully-induced level (compare lanes 3 and 5), indicating that the repression is reversible. The reinduction at 48 h most likely reflects the depletion of B-FGF from the medium, and

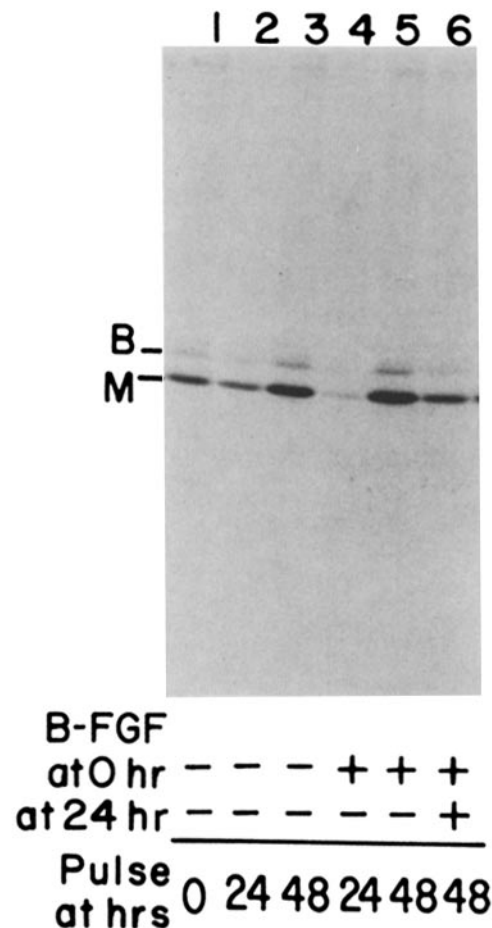


FIGURE 6 Reinduction of CPK expression after repression by B-FGF. B-FGF was added directly to differentiated cultures at 5 ng/ml at the times indicated. The rate of M-CPK synthesis was determined with $[^3\text{S}]$ methionine. Note that 48 h after addition of B-FGF the cells synthesized M-CPK maximally. If B-FGF is added each 24 h (lane 6), the cells remain repressed. B-FGF thus has a short functional half-life in these cultures, probably <24 h. The B and M isozymes of CPK are indicated at left.

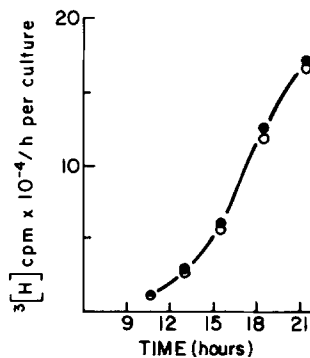


FIGURE 7 Re-induced cells reside in G_0 . Cultures were treated with B-FGF as described in Fig. 6. After 48 h, serum was added to these cells and control cultures, and the duration of G_1 was determined by $[^3\text{H}]$ thymidine incorporation as described in Fig. 3. Note that under these conditions (compare with Fig. 3), there is no difference in the duration of G_1 in the two cultures.

continued addition of B-FGF maintains the repressed state (compare lane 6 with lane 5).

We asked whether the cells under these conditions had returned to G_0 by measuring the time required for such cells to enter S-phase after stimulation by serum. The data in Fig. 7 are consistent with these cells having returned to G_0 . Cells which have re-entered the differentiation program after exposure to B-FGF are indistinguishable from cells never exposed to B-FGF when we measure the time required by these

cells to enter S-phase after addition of 20% serum (Fig. 7). Labeling of cells with [³H]thymidine during the 48-h reversal period, followed by autoradiography, indicates that only ~13% of the cells traversed the S-phase either in the presence or absence of B-FGF and, therefore, that the majority of the cells returned to G₀ without traversing the cell cycle. It is known from other cells that commitment to proceed through the cell cycle is a late event in G₁ (16). B-FGF-treated BC₃H1 cells are arrested early in G₁, presumably before this commitment point is reached.

DISCUSSION

The original definition of G₀ was that of a restriction point early in G₁, at which normal cells arrested in the absence of mitogens (16). Several observations suggest that G₀ represents a unique metabolic state of quiescent cells and that, during logarithmic growth, cells bypass G₀ and never enter this metabolic state (see, for example, references 16, 18, and 19). Our observations define the metabolic state of BC₃H1 cells and, by inference, of other muscle cells as the state which is permissive for the expression of muscle-specific proteins.

The addition of polypeptide growth factors to quiescent BC₃H1 cells initiates movement of the cells from G₀ to G₁. Exit of the cells from G₀ appears to be closely associated with repression of the synthesis of M-CPK. Though our data suggest that B-FGF allows BC₃H1 cells to progress rapidly into G₁ (see Fig. 3), the repression of M-CPK synthesis seems to start immediately after addition of B-FGF so that, 3 h after addition of this growth factor, the rate of M-CPK synthesis is already decreased by 40%.

Although all the experiments reported here were carried out with B-FGF, we have recently carried out similar experiments with identical results with highly purified pituitary FGF prepared as described by Bohlen et al. (20). Although these two polypeptide growth factors are distinct proteins, their metabolic effects on BC₃H1 are very similar.

Differentiation of a few other cell lines has been examined carefully regarding the role of different compartments in the cell cycle in the differentiation process. For example, the conversion of 3T3 preadipocytes to adipocytes occurs in G₀, but arrest of cells later in the cell cycle is nonpermissive for adipocyte differentiation (21, 22).

We would suggest that the control by polypeptide growth factors of cell differentiation is not only relevant to established cell lines in culture, but may also be of importance in controlling the expression of muscle cell proteins *in vivo*, first in preventing differentiation during periods of active cell proliferation, but also in mature cells by controlling the expression of gene products characteristic of differentiated cells in the absence of cell division. Note that in BC₃H1 cells it is possible to go from a differentiated phenotype (high rate of synthesis of M-CPK) to a relatively undifferentiated state (low rate of

synthesis of M-CPK) and back again to the differentiated state in the absence of cell division, and to do so without traversing the cell cycle by simply controlling the level of B-FGF in the culture medium. A critical test of the validity of this speculation will require an increased understanding of the role of B-FGF in the whole animal.

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REFERENCES

- Merlie, J. P., M. E. Buckingham, and R. G. Whalen. 1977. Molecular aspects of myogenesis. *Curr. Top. Dev. Biol.* 11:66-114.
- Buckingham, M. E. 1977. Muscle protein synthesis and its control during the differentiation of skeletal muscle cells *in vitro*. *Int. Rev. Biochem.* 15:269-332.
- Pearson, M. L. 1980. Muscle differentiation in cell culture: a problem in somatic cell and molecular genetics. In *Molecular Genetics of Development*. T. Leighton and M. F. Loomis, editors. Academic Press, Inc. New York. 361-413.
- Devlin, B. H., and I. R. Konigsberg. 1983. Reentry into the cell cycle of differentiated skeletal myocytes. *Dev. Biol.* 95:175-192.
- Devlin, B. H., P. A. Merrifield, and I. R. Konigsberg. 1982. The activation of myosin synthesis and its reversal in synchronous skeletal muscle myocytes in culture. In *Muscle Development: Molecular and Cellular Control*. M. L. Pearson and H. F. Epstein, editors. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. 355-366.
- Nguyen, H. T., R. M. Medford, and B. Nadal-Ginard. 1983. Reversibility of muscle differentiation in the absence of commitment: analysis of a myogenic cell line temperature-sensitive for commitment. *Cell* 34:281-293.
- Olson, E. N., K. L. Caldwell, J. I. Gordon, and L. Glaser. 1983. Regulation of creatine phosphokinase expression during differentiation of BC₃H1 cells. *J. Biol. Chem.* 258:2644-2652.
- Lathrop, B. H., E. Olson, and L. Glaser. 1985. Control by fibroblast growth factor of differentiation in the BC₃H1 muscle cell line. *J. Cell Biol.* 100:1540-1547.
- Compton, R. S., P. A. Merrifield, and I. R. Konigsberg. 1985. Nerve and growth factor regulation of muscle development. *J. Cell. Biochem. Suppl.* 9B:39. (Abstr.)
- Schubert, D., A. J. Harris, C. E. Devine, and S. Heineman. 1974. Characterization of a unique muscle cell line. *J. Cell Biol.* 61:389-413.
- Heller, J. S., K. Y. Chen, D. A. Kyriakidis, W. F. Fong, and E. S. Chanellakis. 1978. The modulation of the induction of ornithine decarboxylase by spermine, spermidine, and diamines. *J. Cell. Physiol.* 96:225-234.
- Cassel, D., P. M. Wood, R. P. Bunge, and L. Glaser. 1982. Mitogenicity of brain axolemma membranes and soluble factors for dorsal root ganglia Schwann cells. *J. Cell. Biochem.* 18:433-445.
- Whittenberger, B., and L. Glaser. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. *Proc. Natl. Acad. Sci. USA.* 74:2251-2255.
- Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative. *J. Biol. Chem.* 247:7609-7611.
- Tabor, C. W., and H. Tabor. 1976. 1,4-diaminobutane (putrescine), spermidine, and spermine. *Annu. Rev. Biochem.* 45:285-306.
- Pardee, A. B., R. Dubrow, J. L. Hamlin, and R. F. Kletzien. 1978. Animal cell cycle. *Annu. Rev. Biochem.* 47:715-750.
- Rozenfurt, E. 1980. Stimulation of DNA synthesis in quiescent cultured cells: exogenous agents, internal signals, and early events. *Curr. Top. Cell. Regul.* 17:59-88.
- Thompson, C. R., P. B. Challoner, P. E. Neiman, and M. Groudine. 1985. Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. *Nature (Lond.)* 314:363-366.
- Hahn, S. R., C. B. Thompson, and R. W. Eisenman. 1985. c-myc oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature (Lond.)* 314:366-369.
- Bohlen, P., A. Baird, F. Esch, N. Ling, and D. Gospodarowicz. 1984. Isolation and partial characterization of pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 81:5364-5368.
- Willie, J. J., Jr., and R. E. Scott. 1982. Topography of the predifferentiation G_D growth arrest state relative to other growth arrest states in the G₁ phase of the cell cycle. *J. Cell. Physiol.* 112:115-122.
- Scott, R. E., D. L. Florine, J. J. Willie, Jr., and K. Yun. 1982. Coupling of growth arrest and differentiation at a distinct state in the G₁ phase of the cell cycle: G_D. *Proc. Natl. Acad. Sci. USA.* 79:845-849.