

Growth Factor Control of Skeletal Muscle Differentiation: Commitment to Terminal Differentiation Occurs in G₁ Phase and Is Repressed by Fibroblast Growth Factor

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Abstract. Analysis of MM14 mouse myoblasts demonstrates that terminal differentiation is repressed by pure preparations of both acidic and basic fibroblast growth factor (FGF). Basic FGF is ~30-fold more potent than acidic FGF and it exhibits half maximal activity in clonal assays at 0.03 ng/ml (2 pM). FGF repression occurs only during the G₁ phase of the cell cycle by a mechanism that appears to be independent of ongoing cell proliferation. When exponentially growing myoblasts are deprived of FGF, cells become postmitotic within 2–3 h, express muscle-specific proteins within 6–7 h, and commence fusion within 12–14 h. Although

expression of these three terminal differentiation phenotypes occurs at different times, all are initiated by a single regulatory “commitment” event in G₁. The entire population commits to terminal differentiation within 12.5 h of FGF removal as all cells complete the cell cycle and move into G₁. Differentiation does not require a new round of DNA synthesis. Comparison of MM14 behavior with other myoblast types suggests a general model for skeletal muscle development in which specific growth factors serve the dual role of stimulating myoblast proliferation and directly repressing terminal differentiation.

SKELETAL muscle development involves an initial period of myoblast replication, followed by a phase in which some myoblasts continue to proliferate while others undergo terminal differentiation. The latter process involves the permanent cessation of DNA synthesis, activation of muscle-specific gene expression, and the fusion of single cells into multinucleated muscle fibers. In vitro studies indicate that the onset of terminal differentiation is delayed by media that are rich in serum and/or embryo extract components (3, 15, 21, 22, 27, 28, 33, 37, 40, 42); and there is a general consensus that this is due to the influence of growth factors. Studies from our laboratory with a permanent skeletal muscle line derived from a mouse muscle satellite cell (MM14), indicated that the critical component in embryo extract was probably fibroblast growth factor (FGF)¹ (27, 28). However, since the FGF preparations available at that time were <1% pure, and since several forms of FGF were thought to exist, the role of FGF remained ambiguous. Subsequent studies of BC3H1 muscle cells have corroborated the role of FGF in repressing muscle differentiation (23, 24). However,

since BC3H1 cells may be of smooth muscle origin (i.e., the line was derived from a chemically induced mouse brain tumor and does not exhibit either the fusion or postmitotic skeletal muscle phenotypes), it is not certain that BC3H1 behavior can be extrapolated directly to the behavior of skeletal muscle cells.

While it is generally agreed that the absence of mitogenic components triggers the onset of skeletal muscle differentiation, the subsequent developmental events are controversial. Some studies indicate that mitogen-deprived myoblasts irreversibly lose their proliferative capacity (become postmitotic) and activate muscle gene expression before fusion (3, 27, 33, 40, 42), thus suggesting that mitogens play a direct role as repressors of an initial step in terminal differentiation. Other studies involving fusion-blocked cells concur that mitogen deprivation induces (derepresses) muscle gene expression, but suggest that a second phenotype, the postmitotic state, is not acquired without cell fusion (8, 12, 32). In the quail system, mitogen depletion is thought to regulate differentiation only indirectly by allowing cells to accumulate in an extended G₁ or G₀ phase, presumably the only cell cycle period in which muscle-specific genes can be activated and in which myocytes can fuse. An event after fusion is then presumed to block further mitogenic responsiveness.

To gain further insight as to how mitogens regulate skeletal muscle differentiation we have continued an analysis of the MM14 mouse myoblast line. In the following experiments we demonstrate that picomolar levels of both acidic and basic

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; F10C, Ham's F10 nutrient medium plus 0.8 mM additional CaCl₂; FGF, fibroblast growth factor; a-FGF, b-FGF, acidic and basic forms of FGF; [³H]Tdr, tritium-labeled thymidine, HS, horse serum; MHC, myosin heavy chain.

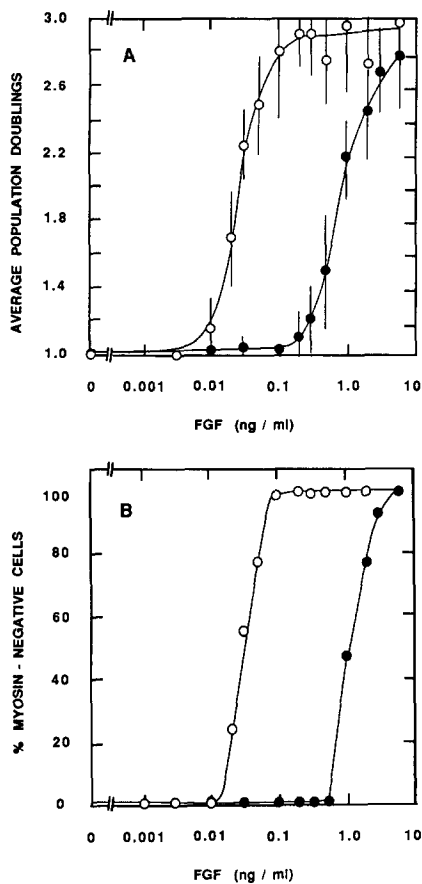


Figure 1. Dose response of MM14 myoblasts to a- and b-FGF. Clonal cultures were established in 60-mm dishes and grown for 37 h in 4 ml F10C + 15% HS containing various concentrations of pure a- and b-bovine FGF. Cultures were then fixed, immunostained for MHC, and 40–90 colonies per FGF concentration were counted and assessed for MHC-negative cells. (A) Growth response to acidic and basic FGF: (●) a-FGF; (○) b-FGF. Ordinate represents the mean number of population doublings starting from one-cell clones. In the absence of FGF virtually all cells form two-cell colonies and thus complete one population doubling. Error bars represent one standard deviation. (B) Differentiation-repression response to a- and b-FGF. Ordinate represents the total MHC-negative cells (cells that fail to differentiate)/(total cells in all clones) \times 100%. Each data point represents several hundred total cells counted.

FGF directly regulate muscle differentiation by repressing a G_1 -specific “commitment” event that directs expression of the muscle phenotype.

Materials and Methods

Cell Cultures and Culture Media

The MM14 line was derived by serial subcloning of myoblasts isolated from leg muscle of a 60-d-old male BALB/c mouse (16, 28). Cells used in these experiments were a near tetraploid subline designated MM14DZ. Unless stated otherwise, clonal cultures were established by plating 100–125 cells per 60-mm plate, feeding them once at 48 h, and then varying the media at 60 h. At the 60-h switch this protocol yields plates with \sim 50 clones containing 16–32 cells. The experiments were performed at low clonal density and with small colonies to avoid rapid depletion of added growth factors (28). With this protocol, all cells were growing exponentially with a 12.5-h cell cycle at the time of the media switches (see below). Mitotic myoblasts

were collected by rocking proliferating cultures several times and withdrawing the medium. The medium was centrifuged, and the cells were resuspended and plated in cloning cylinders (5 mm) placed in the center of the dish. 1 h later, the cylinders were removed and the dishes were rocked to displace unattached cells from the original area of plating. Media switches were performed by rinsing plates twice with 2 ml Ham's F10C (Ham's F10 supplemented with 0.8 mM $CaCl_2$) and then feeding cultures 3 ml of experimental medium that was preequilibrated to the proper pH and temperature. The standard growth medium (mitogen-rich) consisted of Ham's F10C plus 1% antibiotics (10,000 U/ml penicillin G, 0.5 mg/ml streptomycin sulfate), 15% horse serum (15% HS), and 3% embryo extract. An alternative growth medium contained FGF instead of embryo extract. FGF was initially purchased from Collaborative Research, Inc. (Waltham, MA) and handled as previously described (28). More recently the acidic (a-) and basic (b-) forms of FGF obtained from bovine brain have been purified to homogeneity by heparin affinity chromatography (4, 29, 36); these were active at <1 ng/ml. Low mitogen (differentiation) media were made without embryo extract or FGF and consisted of F10C, antibiotics, 1 μ M insulin (which prevents rapid myotube degeneration in the absence of serum), and 0–15% HS.

Assays

S-Phase Cells. Cultures were pulse-labeled 30 min with 3–5 μ Ci/ml [3 H]thymidine (3 H]Tdr), rinsed with saline, fixed in 70% ethanol, and air dried. Plates were coated with Kodak NT-B2 emulsion (diluted 3:2 with water), exposed 1–2 wk at 4°C, and developed with Microdol-X.

Postmitotic Cells. Postmitotic cells were detected by their inability to incorporate [3 H]Tdr in the presence of mitogen-rich medium. This assay consisted of refeeding cultures that had been deprived of FGF for various intervals with mitogen-rich medium and then, 12 h later, adding [3 H]Tdr (0.3 μ Ci/ml), and incubating an additional 18–30 h. [3 H]Tdr was added 12 h after mitogen restoration to prevent the labeling of cells that had not completed S phase during mitogen deprivation (see Fig. 5). To calculate the percentage of postmitotic cells at the time that colonies were reexposed to mitogen rich medium, the average number of unlabeled cells per clone in cultures refed at time t (and fixed about 30 h later) was divided by the average cell number per colony in parallel cultures fixed at time t . Loss of colony-forming ability was measured by dissociating cultures at various times after mitogen withdrawal, replating known cell numbers at clonal densities in mitogen-rich medium, and then counting the number of macroscopic colonies 4 d later. The percentage of loss in colony formation was then calculated by comparison with the percentage of colony formation in cultures assayed just before mitogen deprivation.

Myosin Heavy Chain (MHC). Cultures were rinsed with PBS, fixed with 70% ethanol/formalin/glacial acetic acid (20:2:1) at 4°C for 1 min, and stored at 4°C in PBS. MHC was detected with the MF20 monoclonal antibody (2), kindly provided by Drs. D. Bader and D. Fischman (Cornell University Medical College). MF20 culture medium was diluted 1:40 with 1% HS in 0.05 M Tris-saline pH 7.4. The diluted antibody was added to culture plates (0.5 ml) for 1 h. Plates were rinsed, then stained using a biotinylated rabbit anti-mouse IgG, avidin, and biotinylated horseradish peroxidase procedure (Vector Laboratories, Inc., Burlingame, CA).

Acetylcholine Receptor (AChR). AChR-positive cells were detected autoradiographically via [125 I]-labeled α -bungarotoxin binding as described previously (26).

Results

FGF-specific Regulation of Mouse Myogenesis

Fig. 1 compares the ability of pure acidic (a-) and basic (b-) forms of FGF to stimulate myoblast proliferation and to repress terminal differentiation. Myoblasts that had been growing exponentially in medium containing 6 ng/ml FGF (approximately a 1:1 ratio of a- and b-FGF) were replated in F10C + 15% HS plus increasing concentrations of either type of FGF. 37 h later (an interval during which log-phase myoblasts could undergo a maximum of three doublings, see below), the colonies were counted to determine the average population doublings per clone (Fig. 1A) and the percentage of MHC-negative (“undifferentiated”) cells in the entire population (Fig. 1B). The results support the following conclu-

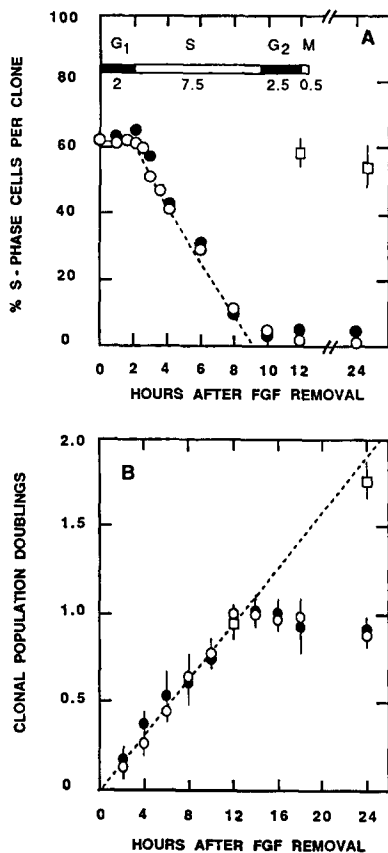


Figure 2. FGF regulation of MM14 cell replication. Clonal cultures of MM14 cells were grown exponentially for 60 h in FM (F10C + 15% HS + FGF), then rinsed twice with F10C, and refed with FM (\square), or switched to FGF-minus media; (\circ) F10C + 5% HS, or (\bullet) F10C + 15% HS. At the indicated times, cultures were labeled for 30 min with [3 H]Tdr and then fixed. After autoradiography, clones were scored for the total cell number and for the percentage of [3 H]Tdr-positive cells (cells in S phase). (A) Effect of FGF removal on the percentage of S phase cells per clone. The dashed line represents the predicted decline in S phase cells if after 2 h of FGF depletion all post-G₁ cells complete the cycle they are in at the normal rate but cells in G₁ fail to initiate DNA synthesis (see text). (Inset) Cell cycle parameters determined for exponentially growing MM14 cells (27). (B) Effect of FGF removal on the clonal growth rate. The dashed line represents the theoretical 12.5-h growth rate previously determined (27). Each data point represents an average from three experiments (30 clones/point/experiment; total, 90 clones, and >3,000 cells per point). FGF levels in different experiments were 10–25 ng/ml (Collaborative Research, Inc.).

sions: (a) FGF is, indeed, a critical factor for stimulating myoblast growth and for repressing differentiation, i.e., in media with 15% serum alone, no new cell cycles were initiated and all cells became myosin positive; (b) b-FGF is 30-fold more potent than a-FGF with respect to maintaining the myoblast, proliferative state; and (c) the dose response to each FGF type is virtually identical for stimulating cell proliferation and repressing terminal differentiation. Thus, the two processes are probably controlled by the same initial FGF-receptor interaction.

The FGF dose-response data shown in Fig. 1 were obtained in media containing 15% HS. In the absence of serum, FGF fails to stimulate proliferation, but it does repress

differentiation (see below). Although maximal clonal growth rates are obtained in FGF-supplemented media containing 15% HS, this serum concentration is not required. In the presence of high FGF the serum level can be reduced to 1–2% without the cessation of growth; but our attempts and those of others (17) to replace serum with epidermal growth factor, nerve growth factor, platelet-derived growth factor, insulin, insulin-like growth factor 1, transferrin, and fibronectin alone or in combination have been unsuccessful. Serum is not, however, required for MM14 differentiation. Cultures switched to F10C alone do not exhibit “normal” morphology, but they do exhibit “normal” differentiation as judged by the appearance of MHC-positive cells. The subsequent studies of clonal growth were thus all performed with medium containing 15% HS, while the studies of differentiation kinetics were performed in FGF-free media containing 5 or 15% HS. 5% HS was used because it supported rapid differentiation kinetics which were more easily correlated with the cell cycle kinetics of MM14 cells. Data from differentiating cultures switched to F10C + 15% HS are included because they represent the single-variable control for FGF deprivation.

Myoblasts Exposed to an FGF-Deficient Environment Stop Cycling after Completing Mitosis

To determine how FGF deprivation affects the myoblast cell cycle, DNA synthesis and clonal growth rate were monitored in cultures that had been switched to FGF-free media. For the first 2–3 h the percentage of cells in S phase remains constant (Fig. 2 A) and growth remains exponential (Fig. 2 B). After this lag period, however, the S-phase population declines and approaches zero over the next 8 h. The overall clonal growth rate during this interval remains exponential. This suggests that soon after FGF removal myoblasts stop entering S phase, while cells in S complete DNA synthesis and traverse G₂ and mitosis at the normal rate; the decline in S-phase cells approximates the 7.5-h length of S (Fig. 2 A, inset). The conclusion that the FGF-deprived population withdraws into a G₁ (or G₀) compartment is based on the observation that clones undergo an exact doubling at the maximum rate before DNA synthesis and growth stop (Fig. 2 B).

These results demonstrate that FGF is specifically required for the initiation of new MM14 cell cycles. In a statistical sense, the last G₁ cell to enter S phase 2–3 h after FGF removal is the last cell to divide at 13–14 h when the clone finally stops growing. We have compared this model of myoblast behavior to a mathematical cell cycle model that specifies the distribution of cells through the cycle (37, 41). The theoretical decline in S-phase cells after FGF removal closely resembles the experimental results (Fig. 2 A, dashed line).

FGF-deprived Myoblasts Express Muscle-specific Proteins before Fusing

After FGF removal, MHC-positive cells are detected in clones at 7–8 h (\sim 5 h after cells begin to withdraw from the cell cycle) and fusion is first observed at 12–16 h (Fig. 3). After 18 h in 5% HS, 96% of the cells contained MHC protein, while only 30% of the nuclei appeared in myotubes. Thus, nearly all cells had withdrawn from the cell cycle (Fig. 2) and had become MHC positive at a time when relatively few cells had fused. Additional experiments have shown that the mus-

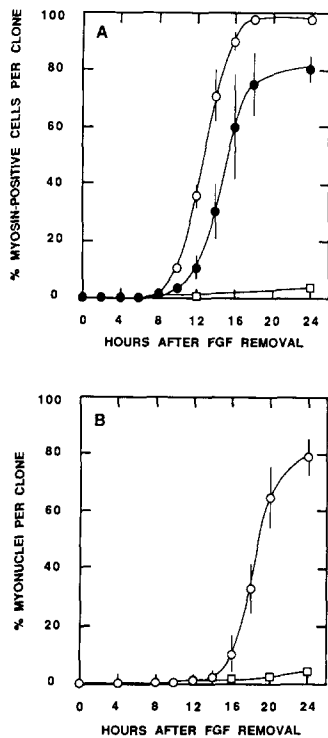


Figure 3. Kinetics of muscle-specific protein accumulation and fusion after FGF deprivation. Clonal MM14 cultures were grown exponentially for 3 d, then rinsed twice with F10C, and refed with F10C + 15% HS + FGF (□), or switched to FGF-minus media (○) F10C + 5% HS; (●) F10C + 15% HS. At the indicated times cultures were fixed for MHC and fusion detection. (A) Percentage of MHC-positive cells per clone. (B) Percent myotube nuclei per clone. For a colony to be scored as fused, it had to contain at least two multinucleated cells with a minimum of three nuclei/cell. Each data point represents an average from three experiments (90 clones, >3,000 total cells per point). FGF levels in different experiments were 10–25 ng/ml (Collaborative Research, Inc.).

cle forms of creatine kinase (6) and actin (5) also appear before MM14 fusion with the same kinetics as MHC. The onset of MHC accumulation, and the final percentage of MHC-positive cells, differed in clones as a function of serum concentration. Cultures switched to F10C + 15% HS behaved similarly except that fewer cells became MHC positive by 24 h and the percentage of nuclei in myotubes was somewhat lower.

Myoblasts Commit to Terminal Differentiation within 2 h of FGF Deprivation

FGF deprivation induces MM14 myoblasts to begin withdrawing from the cell cycle within 2–3 h, to begin accumulating detectable levels of MHC by 7–8 h, and to begin fusing by 12–14 h. When do these phenotypes become irreversibly activated (i.e., when do cells become “committed” to terminal differentiation)? Although the mechanism of commitment is not understood, the process can be delineated by determining the duration of FGF deprivation required before its restoration no longer represses the onset of differentiation. Log-phase cultures were exposed to FGF-free media for various times from 30 min to 8 h, then refed growth medium, incubated another 20–30 h to permit cell fusion, and scored for clones containing myotubes. According to this assay every myotube-positive colony must have contained several committed cells that could not be prevented from fusing after FGF readdition. The results (Fig. 4) indicate a significant increase in myotube-positive colonies in cultures deprived of FGF for as little as 2 h, and with a 5-h deprivation all colonies exhibit fusion. The failure of FGF restoration to prevent the initiation of fusion 10–14 h later (Fig. 3) indicates that FGF deprivation does, indeed, induce an irreversible commitment event. The reason that all colonies were not myotube positive unless subjected to ~5 h of FGF deprivation

is presumably due to cell cycle synchrony within individual small clones (i.e., some colonies had no G₁ phase cells during the first 3–4 h of FGF deprivation, see below).

FGF-deprived Myoblasts Commit to the Postmitotic State before Fusion

The inability of FGF to prevent cells that are deprived of FGF for as little as 2–3 h from differentiating suggests that without this mitogen cells lose the ability to initiate DNA synthesis. To test for postmitotic cells and to determine the rate at which this phenotype is established, log-phase myoblasts were switched to FGF-free medium for various intervals, and then returned to mitogen-rich medium. After a 12-h period to permit completion of DNA synthesis by any S-phase cells, [³H]Tdr was added and the cultures were incubated an additional 20 h. After autoradiography the postmitotic (unlabeled cells) could then be distinguished from the proliferative (labeled) cells. The kinetics of commitment to a postmitotic phenotype are shown in Fig. 5. Virtually all myoblasts remained proliferative in the presence of FGF. Postmitotic cells, however, appeared within 2 h after exposure to 5% HS, and the entire population was postmitotic by 14 h. The rate at which cells became postmitotic when switched to F10C + 15% HS was slightly slower and the final percentage again was lower than in 5% serum. However, in either serum concentration, FGF-deprived myoblasts committed to a postmitotic phenotype at essentially the same time that they started to withdraw from the cell cycle (Fig.

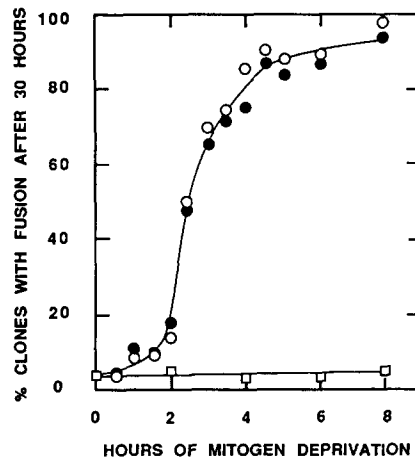


Figure 4. Commitment to fusion after FGF deprivation. Clonal MM14 cultures were grown exponentially for 96 h, then rinsed twice with F10C, and refed with F10C + 15% HS + FGF (□), or switched to FGF-minus media (○) F10C + 5% HS; (●) F10C + 15% HS. At the indicated times, cultures were refed with F10C + 15% HS + FGF; 22 h after the last refeeding point, cultures were fixed and analyzed for the percentage of clones with myotubes. To prevent depletion of FGF after its restoration, cultures were refed with FGF at 12 and 24 h. For a colony to be scored as fused, it had to contain at least two multinucleated cells with a minimum of three nuclei/cell. Each data point represents an average from two experiments (two duplicate plates in each experiment with >100 clones per point). Standard deviations for each point were <10%. FGF levels in different experiments were 10 and 25 ng/ml (Collaborative Research, Inc.).

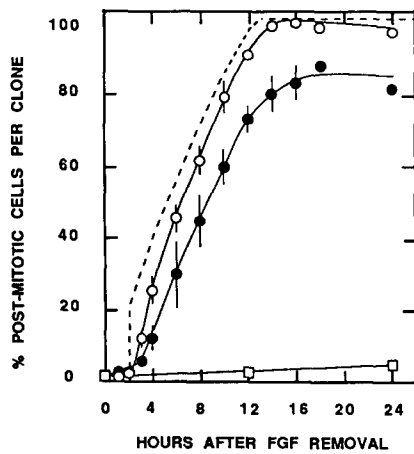


Figure 5. Commitment to the postmitotic state after FGF deprivation. Clonal MM14 cultures were grown exponentially for 60 h, then rinsed twice with F10C, and refed with F10C + 15% HS + FGF (□), or switched to FGF-minus media (○) F10C + 5% HS; (●) F10C + 15% HS. At the indicated times, cultures were refed with F10C + 15% + FGF. The assay for postmitotic cells was performed as described in Materials and Methods. Each data point is an average from three experiments (90 clones total) and unless indicated by error bars the standard deviation was <5%. The dashed line represents the theoretical percentage of postmitotic cells, assuming all G₁ cells become committed. It is based on the following assumptions: (a) exponential growth with 12.5-h population doubling; (b) G₁ phase lasts 2 h and thus contains 21% (see below) of the total cell population; (c) cells commit to the postmitotic state only in G₁ phase; (d) after FGF removal, a 2-h lag phase occurs before any cells commit; (e) after the lag, all cells entering G₁ commit. At time 0 the fraction of cells in G₁ is calculated by integrating over the 0–2-h interval of a 12.5-h exponential growth curve. At any time after the 2-h lag the fraction of cells in the G₁ compartment is calculated by integrating the area represented by the expanding G₁ compartment (e.g., 0–3 h, 0–4 h, etc.) beneath a 12.5-h exponential growth curve (37, 41).

2) and started committing to fusion (Fig. 4). Further proof that the postmitotic state, as assayed above, represents a permanent withdrawal from the cell cycle, and not simply a long lag period for reinitiating DNA synthesis, was obtained with a colony-formation assay that examined the proliferative capacity of cells for 4 d (see Fig. 6).

Commitment to the Postmitotic State, to Expression of Muscle-specific Genes, and to Fusion Occurs with Identical Kinetics

The concomitant commitment of MM14 myoblasts to both the postmitotic state and fusion suggests that the major phenotypes of skeletal muscle are initiated by a single FGF-regulated event. If this were so, FGF-deprived cells should commit to the expression of muscle-specific genes with the same kinetics. As shown in Fig. 6, MM14 myoblasts commit to an AChR-positive phenotype after the same lag period observed for the other muscle parameters (Figs. 4 and 5); and once initiated, the population kinetics of commitment to the expression of AChR is virtually identical to the kinetics for commitment to the postmitotic state. This observation is consistent with the suggestion that FGF represses a single regu-

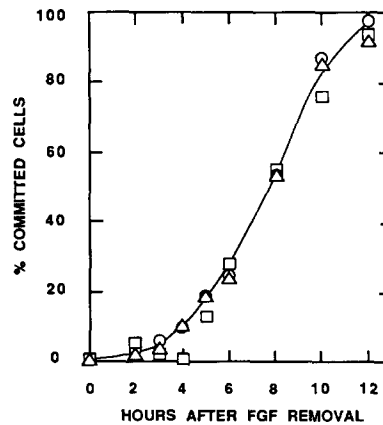


Figure 6. Comparison of the kinetics of commitment to the expression of a muscle-specific gene with the kinetics of commitment to the postmitotic state. Clonal MM14 cultures were grown exponentially for 60 h, then rinsed twice with F10C, and switched to FGF-depleted medium. At the indicated times, cultures were refed with growth medium and then subsequently assayed as described in Materials and Methods. (○) Percentage of postmitotic cells as determined by failure to incorporate [³H]Tdr; (□) percentage of postmitotic cells as determined by the loss of colony-forming ability; (Δ) percentage of cells that committed to AChR expression ([¹²⁵I] α-bungarotoxin binding at 24 h). Data points represent >200 cells counted for the labeled cell assays and ~250 clones for the colony-formation assay.

latory event which, after FGF removal, activates the terminal differentiation phenotype.

Mitogen Regulation of DNA Synthesis and Terminal Differentiation Occurs Exclusively during G₁

Our results suggest that FGF regulation of both DNA synthesis and terminal differentiation occurs during the G₁ phase of the cell cycle. To demonstrate this unambiguously we exposed mitotically synchronized cultures to either mitogen-rich or mitogen-depleted medium plus [³H]Tdr. After a 24-h incubation period, cultures were processed for detection of DNA synthesis and MHC protein (Table I). The results indicate that as myoblasts enter G₁ in the presence of FGF they replicate DNA and remain MHC negative, whereas in the absence of FGF they differentiate before initiating a new cell cycle. Clearly, DNA replication and the onset of terminal muscle differentiation are both regulated by mitogenic signals initiated in G₁. Even when the cell has traversed the previous cell cycle and entered mitosis in the presence of FGF, the absence of FGF in G₁ leads to rapid differentiation.

While this experiment demonstrates a G₁ phase regulation of terminal differentiation, it does not indicate whether S or G₂ phase cells would also be induced to commit when exposed to FGF-free medium. If commitment occurs exclusively in G₁, then the percentage of postmitotic cells per clone should be equal to or less than the number of cells that accumulate in G₁ during the final population doubling. Alternatively, if FGF-deprived S and G₂ phase cells also commit to differentiation, the total number of postmitotic cells would be augmented by the daughter cells of parental myo-

Table I. FGF Regulates Cell Replication and Terminal Differentiation during the G₁ Phase of the Myoblast Cell Cycle

Medium composition*	Cells Replicating DNA [‡]	Differentiated cells [§]
	%	%
15% HS + EE	98	1
15% HS + FGF [†]	96	3
15% HS	20	80
5% HS	1	98

The data are averages of two experiments in which a minimum of 500 cells was scored in each medium tested.

* Mitotic cells were plated into the indicated media containing [³H]Tdr (1.0 μCi/ml) and incubated for 24 h. Plates were then fixed, immunostained for myosin detection, and processed for autoradiographic detection of ³H-labeled nuclei.

[‡] The percentage of cells replicating DNA was determined by autoradiography. Since MM14 cells have a 12.5-h cell cycle and since the cells used in the experiment were mitotically synchronized (i.e., entered G₁ shortly after plating), it was assumed that each labeled cell detected 24 h after plating had completed one additional cell cycle, whereas unlabeled cells had remained in a G₁ or G₀ phase throughout the 24-h period. For 500 total cells of which 490 were [³H]Tdr positive, the percentage of the original G₁ cells replicating DNA was: (490/2)/[(490/2) + 10] × 100% = 96%.

[§] Differentiated cells were scored on the basis of being postmitotic (failure to incorporate [³H]Tdr) and positive staining for MHC. In accordance with the explanation given above, the percent of the original G₁ cells that differentiated was calculated as follows. For 500 total cells of which 330 were both unlabeled and MHC-positive: percentage of differentiated cells was: 330/[330 + (170/2)] × 100% = 80%.

^{||} EE = 3% chick embryo extract.

[†] 10 ng/ml FGF (Collaborative Research, Inc.).

blasts that had committed in S or G₂. The theoretical accumulation of cells in G₁ was calculated (37, 41) and compared with the actual increase in postmitotic cells after FGF removal (Fig. 5). As indicated, the percentage of postmitotic cells never exceeds the calculated percentage of G₁ cells (Fig. 5, dashed line) that accumulate during cell cycle withdrawal. After 3 h in medium containing 5% serum, about half of the G₁ population can reenter the cell cycle after FGF readdition. Thereafter, the increase in postmitotic cells matches the accumulation of cells in G₁. Although cells fed 15% serum withdraw from the cell cycle at the same rate as cultures fed 5% serum (Fig. 2), fewer cells become postmitotic per unit time. These data suggest that commitment to terminal differentiation in response to FGF removal occurs only after cells have completed the cell cycle and entered G₁.

FGF Represses Commitment Independently of Ongoing Cell Replication

FGF mediates two events in G₁ myoblasts: it stimulates cell replication and represses terminal differentiation. Is the FGF-mediated repression of terminal differentiation simply an "indirect" consequence of mitogenesis, or is there a "direct" repression mechanism that is independent of cell replication? These alternatives could be distinguished by taking advantage of the fact that MM14 cell proliferation requires both serum and FGF. Cultures could thus be exposed to FGF in the absence of serum (and proliferation) and be assayed for repression of terminal differentiation (Table II). In this experiment control cultures fed FGF and serum continued to replicate; clones doubled nearly twice with a high percentage of cells still replicating DNA after 24 h, and MHC-positive cells were absent. Not surprisingly, cultures de-

prived of FGF and serum completed one cell cycle and then differentiated. Interestingly, while cells exposed to FGF but no serum failed to replicate beyond a single doubling (only 6% of the cells were synthesizing DNA after 24 h), <10% of the cells became MHC positive and postmitotic. This response demonstrates that myoblasts can "stop" cycling in G₁ without differentiating or committing to the postmitotic phenotype. Thus, the repression of muscle differentiation by FGF occurs independently of cell proliferation.

Discussion

The principles of MM14 behavior after FGF removal typify those of most myoblast types adapted to tissue culture. It has been recognized for some time that differentiation is inhibited by factors present in serum and embryo extract, and that crude FGF preparations repress the differentiation of myoblasts isolated from a variety of sources (1, 10, 14, 18, 19, 23, 27). The observation that G₁-phase MM14 myoblasts are not obligated to replicate DNA before differentiation is unusual compared with most mitogen-deprived myoblasts that undergo additional rounds of DNA synthesis. This behavior is, however, consistent with previous studies using DNA synthesis inhibitors which indicated that additional DNA synthesis was not required after mitogen deprivation (9, 33). It is also consistent with some previous experiments with quail and rat myoblasts that had been switched to totally defined mitogen-free medium (11, 38).

FGF-deprived MM14 cells exhibit a "simultaneous" activation of terminal differentiation pathways leading to the postmitotic state, expression of muscle-specific genes, and fusion. The observation that these phenotypes occur in an ordered sequence does not imply a causal dependence among them. In fact, ~1% of mitotically synchronized FGF-deprived MM14 cells accumulate detectable levels of muscle gene products without being postmitotic (data not shown). This observation is consistent with the behavior of other skeletal muscle systems (8, 12, 23, 24, 32, 34); but in these cases a much larger percentage of the mitogen-deprived population can express muscle genes without becoming postmitotic. Muscle gene expression is thus not dependent upon a cell's prior acquisition of a postmitotic state.

The dependence of the postmitotic state on fusion remains controversial. In FGF-deprived MM14 cultures 75% of the cells are postmitotic before fusion begins. Similar behavior has been reported for other myoblasts (3, 33, 40, 42). In contrast, mitogen-deprived quail myoblasts activate myosin expression, and enter a prolonged G₁ or G₀ phase from which they can resume proliferation when mitogens are restored (8). This behavior may, however, be due to secondary effects of using EGTA to block cell fusion. (For example, see references 12, 20, 33 for conflicting data as to Ca⁺⁺ effects on rat L6 myoblast differentiation.) It may also be relevant to note that external Ca⁺⁺ is required for FGF-mediated mitogenesis (30).

It would be misleading to suggest that the differentiation of all myoblast types is regulated identically to MM14. For instance, the mouse line C2C12 which has FGF receptors does not require FGF for growth; and some sublines of the rat L6 have few, if any, FGF receptors and are unresponsive to FGF (Olwin, B. B., and S. D. Hauschka, unpublished data). The terminal differentiation of L6 myoblasts is, how-

Table II. FGF Prevents Differentiation in Noncycling G₁ Cells

Medium composition		Parameter assayed			
FGF [‡]	Serum [§]	Clonal doublings	S-phase cells	MHC-positive cells	Postmitotic cells
		<i>n</i>	%	%	%
+	+	1.7	53	0	0
-	-	0.8	0.5	93	99
+	-	0.9	6	8	8

Clones that were established in media containing 10 ng/ml FGF plus 15% HS were rinsed twice with F10C and switched to the three test media. 24 h later, the percentage of cells expressing each phenotype was determined. At least 30 clones and >500 cells were counted for each determination.

* All media contained F10C and were supplemented with 1 μM insulin to prevent the toxic effect of protein-free medium.

‡ Plus or minus 10 ng/ml FGF (Collaborative Research, Inc.).

§ Plus or minus 15% HS.

ever, regulated by insulin-like growth factors (13). It is also possible that the inhibition of differentiation in some muscle lines that is caused by serum may be due to the presence of transforming growth factor-β, which is known to inhibit terminal muscle differentiation (31, 35). In addition, it is possible, as proposed by stem-cell lineage models, that certain myoblasts that differentiate in the presence of mitogens are regulated by an intrinsic "probabilistic clock" (39). Alternatively, these myoblasts may undergo several additional cell cycles after being triggered to differentiate by mitogen deprivation. While it is clear that FGF is not the only mitogen to which myoblasts respond, it is important to reiterate that FGF is essential for many myoblast types and that FGF is present in both adult (18, 19) and embryonic chick muscle and early limb bud tissue (Seed, J., and S. D. Hauschka, unpublished data).

The molecular events involved in a cell becoming postmitotic are not understood, but two studies have provided instructive clues. First, heterokaryon analyses involving MM14 argue that myocytes contain a diffusible factor that inhibits intracellular mitogenic signals (7). Second, studies of mitogen receptor behavior indicate that commitment to terminal differentiation is accompanied by the permanent loss of both epidermal and fibroblast growth factor receptors (25; Olwin, B. B., and S. D. Hauschka, unpublished data). The postmitotic state might thus be initiated by the production of a diffusible inhibitor of mitogenesis, and then made permanent by the irreversible loss of mitogen receptors.

The model supplemented by our studies suggests that myoblasts proliferate indefinitely until they encounter insufficient levels of one or more specific growth factors. They then commit irreversibly to terminal differentiation, become postmitotic, express muscle-specific genes, and fuse. The control of commitment is deceptively simple in vitro where a homogeneous muscle population is instantaneously deprived of a single required growth factor. In vivo, where muscle differentiation lasts over periods of days to years, the regulation must be more complex. Within a single developing muscle, there is almost certainly a diversity of myoblast types with different mitogen sensitivities. The mechanisms responsible for regulating the relative populations of cells and mitogen levels within microenvironments of the developing tissue remain to be determined.

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References

- Allen, R. E., M. V. Dodson, and L. S. Luiten. 1984. Regulation of skeletal muscle satellite cell proliferation by bovine pituitary fibroblast growth factor. *Exp. Cell Res.* 152:154-160.
- Bader, D., T. Masaki, and D. A. Fischman. 1982. Biochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763-770.
- Bayne, E. K., and S. B. Simpson. 1980. Influence of environmental factors on the accumulation and differentiation of prefusion lizard myoblasts in vitro. *Exp. Cell Res.* 126:15-30.
- 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.
- Bulinski, J. C., S. Kumar, K. Titani, and S. D. Hauschka. 1983. Peptide antibody specific for the amino terminus of skeletal muscle α-actin. *Proc. Natl. Acad. Sci. USA.* 80:1506-1510.
- Chamberlain, J. S., J. B. Jaynes, and S. D. Hauschka. 1985. Regulation of creatine kinase induction in differentiating mouse myoblasts. *Mol. Cell Biol.* 5:484-492.
- Clegg, C. H., and S. D. Hauschka. 1987. Heterokaryon analysis of muscle differentiation: regulation of the postmitotic state. *J. Cell Biol.* 105:937-947.
- Devlin, B. H., and I. R. Konigsberg. 1983. Reentry into the cell cycle of differentiated skeletal myocytes. *Dev. Biol.* 95:176-192.
- Doering, J. L., and D. A. Fischman. 1974. The in vivo fusion of embryonic chick muscle without DNA synthesis. *Dev. Biol.* 36:225-235.
- Dollenmeier, P., D. C. Turner, and H. M. Eppenberger. 1981. Proliferation and differentiation of chick skeletal muscle cells cultured in a chemically defined medium. *Exp. Cell Res.* 135:47-61.
- Emerson, C. P. 1977. Control of myosin synthesis during myoblast differentiation. In *Pathogenesis of Human Muscular Dystrophies*. L. P. Rowland, editor. Excerpta Medica, Amsterdam. 799-809.
- Endo, T., and B. Nadal-Ginard. 1986. Transcription and post-transcriptional control of c-myc during myogenesis: its mRNA remains inducible in differentiated cells and does not suppress the differentiated phenotype. *Mol. Cell Biol.* 6:1412-1421.
- Florini, J. R., D. Z. Ewton, S. L. Falen, and J. J. Van Wyk. 1986. Biphasic concentration dependency of stimulation of myoblast differentiation by somatomedins. *Am. J. Physiol.* 250:C77-C78.
- Gospodarowicz, D., J. Weseman, J. S. Moran, and J. Lindstrom. 1976. Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. *J. Cell Biol.* 70:395-405.
- Hauschka, S. D. 1974. Clonal analysis of vertebrate myogenesis. II. Environmental influences upon human muscle differentiation. *Dev. Biol.* 37:345-368.
- Hauschka, S. D., T. A. Linkhart, C. H. Clegg, and G. F. Merrill. 1979. Clonal studies of human and mouse muscle. In *Muscle Regeneration*. A. Mauno, editor. Raven Press, New York. 311-321.
- Hayashi, I., and J. Kobylecki. 1982. Growth of myoblasts in hormone-supplemented serum-free medium. In *Growth of Cells in Horizontally Defined Media*. Vol. 9. B. G. Sato, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 857-865.
- Kardami, E., D. Spector, and R. C. Strohman. 1985. Myogenic growth factor present in skeletal muscle is purified by heparin-affinity chromatography. *Proc. Natl. Acad. Sci. USA.* 82:8044-8047.
- Kardami, E., D. Spector, and R. C. Strohman. 1985. Selected muscle and nerve extracts contain an activity which stimulates myoblast proliferation

- and which is distinct from transferrin. *Dev. Biol.* 112:353-358.
20. Konieczny, S. F., J. McKay, and J. R. Coleman. 1982. Isolation and characterization of terminally differentiated chicken and rat skeletal muscle myoblasts. *Dev. Biol.* 91:11-26.
 21. Konigsberg, I. R. 1971. Diffusion-mediated control of myoblast fusion. *Dev. Biol.* 26:133-152.
 22. Konigsberg, I. R. 1977. The role of the environment in the control of myogenesis in vitro. In *Pathogenesis of Human Muscular Dystrophies*. L. P. Rowland, editor. Excerpta Medica, Amsterdam. 779-798.
 23. Lathrop, B., 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.
 24. Lathrop, B., K. Thomas, and L. Glaser. 1985. Control of myogenic differentiation by fibroblast growth factor is mediated by position in the G₁ phase of the cell cycle. *J. Cell Biol.* 101:2194-2198.
 25. Lim, R. W., and S. D. Hauschka. 1984. A rapid decrease in epidermal growth factor-binding capacity accompanies the terminal differentiation of mouse myoblasts in vitro. *J. Cell Biol.* 98:739-747.
 26. Linkhart, T. A., and S. D. Hauschka. 1979. Clonal analysis of vertebrate myogenesis. VI. Acetylcholinesterase and acetylcholine receptor in myogenic and nonmyogenic clones from chick embryo leg cells. *Dev. Biol.* 69:529-548.
 27. Linkhart, T. A., C. H. Clegg, and S. D. Hauschka. 1980. Control of mouse myoblast commitment to terminal differentiation by mitogens. *J. Supramol. Struct.* 14:483-498.
 28. Linkhart, T. A., C. H. Clegg, and S. D. Hauschka. 1981. Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromolecular growth factors in the culture medium. *Dev. Biol.* 86:19-30.
 29. Lobb, R. R., and J. W. Fett. 1984. Purification of two growth factors from bovine neural tissue by heparin affinity chromatography. *Biochemistry.* 23:6295-6299.
 30. Magnaldo, I., G. L'Allemain, J. C. Chambard, M. Moenner, D. Barritault, and J. Pouyssegur. 1986. The mitogenic signalling pathway of fibroblast growth factor is not mediated through polyphosphoinositide hydrolysis and protein kinase C activation in hamster fibroblasts. *J. Biol. Chem.* 261:16916-16922.
 31. Massague, J., S. Cheifetz, T. Endo, and B. Nadal-Ginard. 1986. Type β -transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. USA.* 83:8206-8210.
 32. Merrifield, P. A., R. S. Compton, and I. R. Konigsberg. 1984. Cell cycle dependence of differentiation in synchronous skeletal muscle myocytes. In *Cellular Aspects of Myogenesis*. A. Wolsky, editor. S. Karger, Basel/New York. 1-9.
 33. Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell.* 15:846-855.
 34. 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.
 35. Olson, E. N., E. Sternberg, J. Hu, G. Spizz, and C. Wilcox. 1986. Regulation of myogenic differentiation by type β transforming growth factor. *J. Cell Biol.* 103:1799-1805.
 36. Olwin, B. B., and S. D. Hauschka. 1986. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. *Biochemistry.* 25:3487-3492.
 37. O'Neill, M. C. 1976. Population modeling in muscle cell culture: comparisons with experiments. *Dev. Biol.* 53:190-205.
 38. Pinset, C., and R. G. Whalen. 1985. Induction of myogenic differentiation in serum-free medium does not require DNA synthesis. *Dev. Biol.* 108:284-289.
 39. Quinn, L. S., H. Holtzer, and M. Nameroff. 1985. Generation of chick skeletal muscle cells in groups of 16 from stem cells. *Nature (Lond.).* 313:692-694.
 40. Slater, C. R. 1976. Control of myogenesis in vitro by chick embryo extract. *Dev. Biol.* 50:264-284.
 41. Stanners, C. P., and H. E. Till. 1960. DNA synthesis in individual L-strain mouse cells. *Biochim. Biophys. Acta.* 37:406-419.
 42. Yaffe, D. 1971. Developmental changes preceding cell fusion during muscle differentiation in vitro. *Exp. Cell Res.* 66:33-48.