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Control of Mouse Myoblast Commitment to Terminal Differentiation by Mitogens

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Regulation of the transition of mouse myoblasts from proliferation to terminal differentiation was studied with clonal density cultures of a permanent clonal myoblast cell line. In medium lacking mitogenic activity, mouse myoblasts withdraw from the cell cycle, elaborate muscle-specific gene products, and fuse to form multinucleated myotubes. Addition of a purified mitogen, fibroblast growth factor, to mitogen-depleted medium stimulates continued proliferation and prevents terminal differentiation. When mitogens are removed for increasing durations and then refed, mouse myoblasts irreversibly commit to terminal differentiation: after 2–4 h in the absence of mitogens, myoblasts withdraw from the cell cycle, elaborate muscle-specific gene products, and fuse in the presence of mitogens that have been fed back. Population kinetics of commitment determined with ^{3}H-thymidine labeling and autoradiography suggest the following cell-cycle model for mouse myoblast commitment: 1) if mitogens are present in the extracellular environment of myoblasts in G_1 of the cell cycle, the cells enter S and continue through another cell cycle; 2) if mitogens have been absent for 2 or more hours, cells in G_1 do not enter S; the cells commit to differentiate, permanently withdraw from the cell cycle (will not enter S if mitogens are refed), and they subsequently elaborate acetylcholine receptors and fuse (even if mitogens are refed); 3) cells in other phases of the cell cycle continue to transit the cell cycle in the absence of mitogens until reaching the next G_1. The commitment kinetics and experiments with mitotically synchronized cells suggest that the commitment "decision" is made during G_1. Present results do not, however, exclude commitment of some cells in other phases of the cell cycle.

Key words: myoblast differentiation, muscle cell culture, mitogens, growth factors, myoblast cell lines

Skeletal muscle differentiation is initiated as proliferating myoblasts withdraw from the cell cycle, elaborate muscle-specific gene products, and fuse to form multinucleated myotubes. Many studies of myogenesis in vitro suggest that regulation of this transition from proliferation to terminal differentiation is related to regulation of myoblast proliferation. Stimulation of myoblast proliferation by mitogenic components of the culture

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medium prevents (or delays) the onset of terminal differentiation [7, 20, 23, 30, 38, 47, 50]; myoblasts eliminate the mitogenic activity of the culture medium before differentiating [20, 23, 38]; and shifts in medium mitogen content from high to low bring about precocious terminal differentiation [7, 20, 23, 27, 38, 47, 50]. Although the relative stringency of control by mitogens may vary between species, it appears that a reduction of mitogenic activity in the culture medium environment stops myoblast proliferation and triggers initiation of the program of terminal differentiation.

Knowledge of how cell proliferation is controlled by mitogens has been greatly advanced by the purification of several different polypeptide mitogens. These molecules have different cell type and species specificities; they support cell proliferation in vitro in cooperation with other hormones and factors that usually are components of the tissue culture medium. We and others have found that bovine fibroblast growth factor (FGF) in the presence of serum stimulates proliferation and delays differentiation of myoblasts from cow [11], chick [44], and mouse [24].

We have been examining the transition from proliferation to terminal differentiation in permanent clonal mouse myoblast cell lines. These cell lines were derived in our laboratory from single primary mouse myoblast clones, are homogeneous with relatively stable diploid or near tetraploid chromosome numbers, and are similar in most respects to mouse myoblasts of primary cultures. We have found that mouse myoblast proliferation and differentiation are tightly controlled by specific mitogens in the culture medium. In this report we describe evidence that FGF stimulates mouse myoblast proliferation and prevents terminal differentiation, and we describe the irreversible response of mouse myoblasts to removal of mitogens from the culture medium.

MATERIALS AND METHODS

Cell Culture

Cell lines. Two tetraploid sublines (Y and Z) of mouse myoblast line MM14D were used in this study. The line was derived in our laboratory by serial subcloning of myogenic cells from the leg muscle tissue of a 60-day Balb/C male mouse [14, 15]. Homogeneity of the line was maintained by periodic subcloning from frozen stocks; subsequently 20–30 aliquots of cells were frozen (in 7.5% dimethylsulfoxide in complete medium) from each subclonal expansion to be used for experiments. For most experiments, frozen cells were thawed and plated into 100 mm culture plates for 1–2 days; then cells were collected counted in a hemacytometer, and inoculated into 60 mm culture plates at clonal densities (50–100 per plate). Clonal plating efficiencies were 50–70%.

Early passage cultures. Primary cultures were established from a limb muscle tissue cell suspension obtained from 3-day-old Swiss Ha/ICR mice as described for chick muscle cultures [16]. The primary cells were grown for several days and before any fusion occurred were collected and frozen in multiple aliquots. The secondary cultures established from frozen stocks were about 70% myogenic in clonal assays.

Medium. Fresh medium (FM) consisted of Ham's F10 with 15% horse serum, 3% chick embryo extract [19], 1% antibiotics solution (10,000 units/ml penicillin-G, 0.5 mg/ml streptomycin sulfate), and 0.8 mM extra $CaCl_2$ (final concentration of Ca^{++} = 1.1 mM). Conditioned medium (CM) was made by exposing 20 ml FM per 100 mm culture plate to mouse myoblast clones (5,000 per plate) from day 3 to day 6 after plating. The cells remove

mitogenic activity from the medium in this time and differentiate [23]. CM was Millipore filtered and frozen at −70°C. Mitogen-depleted medium (DM) was prepared from thawed CM by diluting 1:1 with Ham's F10 supplemented with 0.8 mM $CaCl_2$ and used within 24 h. All cultures were grown in Corning tissue culture plates coated with 0.67% gelatin [13]. Cells were dissociated for 3–5 min with 0.05% crude collagenase (Worthington, CLS) for passaging.

^{3}H-Thymidine Incorporation and Autoradiography

To determine the number of cells in S phase of the cell cycle, cultures were pulse labeled for 30 min with ^{3}H(methyl)-thymidine (TRK 418, Amersham), 3 μCi/ml medium, rinsed twice with saline and fixed. To determine the number of cycling cells, cultures were continuously labeled in 0.2 μCi/ml ^{3}H(methyl)-thymidine. Fixation was with 70% ethanol or 4% formaldehyde in saline. Plates were extracted several times in 70% ethanol, washed in distilled water, and air dried. Plates were coated with Kodak NT-B2 emulsion diluted 3:2 with water, exposed in the cold for 5–7 days, and developed with Microdol-X. Cells were stained with hematoxylin for scoring clonal assays; autoradiographs were also stained with hematoxylin.

Labeled metaphase preparation. To determine cell cycle parameters [35], proliferating cultures (5,000 clones per 100 mm plate) were fed daily and pulse labeled for 15 min with ^{3}H-thymidine (1 μCi/ml). The cultures were then rinsed twice with F10 supplemented with $CaCl_2$, incubated for 15 min with either FM or CM, then fed FM or CM again. After 15 min incubations with colcemid (0.5 μg/ml), attached cells were collected with collagenase and pooled with cells in the medium, then swollen in hypotonic KCl, fixed in methanol: acetic acid (3:1), and spread and air dried on glass slides. The slides were processed for autoradiography as described above and exposed in the cold for 10 days.

Acetylcholine Receptor Assay

Acetylcholine receptors on differentiating myoblasts were visualized by binding ^{125}I-αBungarotoxin to the cells and examining autoradiographs [25]. Decamethonium (2×10^{-4}M) was used to compete with labeled αBungarotoxin to determine the extent of nonspecific binding, which was 5% or less than total binding in differentiated cultures.

Fibroblast Growth Factor (FGF)

FGF from bovine pituitary [10] was purchased from Collaborative Research, Inc. (Waltham, MA). Lyophilized preparations (with bovine serum albumin as carrier) were reconstituted, diluted 5X in Ham's F10, and frozen at −70°C. Reconstituted FGF could be stored for at least 2 months without loss of mitogenic activity.

Mitotic Cells

Myoblasts in mitosis were collected by gently rocking 100 mm plates containing proliferating clones (5,000 per plate, 3 days after plating) and withdrawing the medium containing detached mitotic cells. This procedure is based on the observation of Terasima and Tolmach [41] that mitotic cells in culture are less firmly attached to the substratum. The cells were centrifuged, resuspended in DM, and plated into 60 mm plates preincubated with DM or FM.

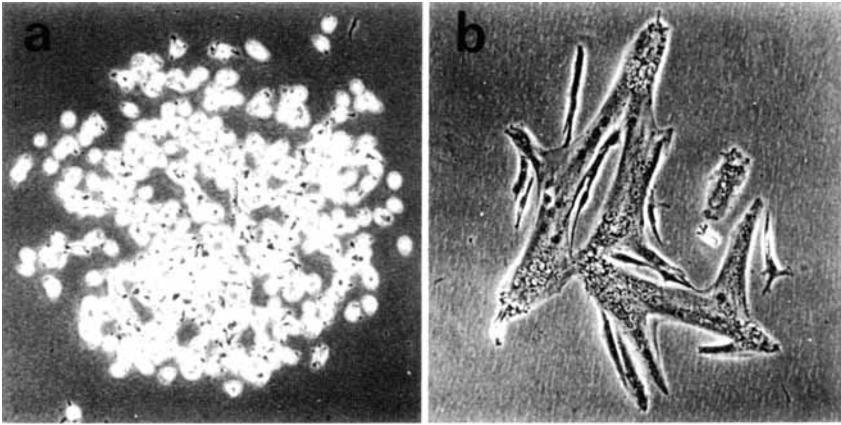

Fig. 1. Proliferation and differentiation in mouse myoblast clones fed FM and CM. Clonal density MM14DY myoblasts 3 days after plating were rinsed twice with F10 (supplemented with $CaCl_2$) and fed FM (A) or CM (B). Phase-contrast photomicrographs of two representative clones were taken 24 h after feeding. The clone fed FM continued to proliferate, while the clone fed CM underwent extensive myotube formation.

RESULTS

Control of Growth and Differentiation by Mitogens

In our initial characterization of mouse myoblast cell lines we observed an effect of cell and clonal density on the time of onset of myotube formation that suggested, as others had shown in avian myoblast cultures [5, 19, 30], that mouse myoblasts alter the culture medium before differentiating [14, 23]. We found that medium conditioned by exposure to high clonal density mouse myoblast cultures brought about precocious myotube formation when fed to low clonal density test cultures, while parallel cultures continued to proliferate when fed FM. Medium conditioning was shown to eliminate activity from FM which was inhibitory to mouse myoblast differentiation. The major contributor of inhibitory components to FM was the macromolecular fraction of embryo extract. In further studies we found that the addition of a purified mitogen, FGF, to conditioned medium delayed differentiation [24]. These findings suggested that mouse myoblasts are stimulated to proliferate and prevented from differentiating by growth factors in FM and that myoblasts eliminate the mitogenic activity before differentiating. Experimental results supporting these conclusions are illustrated in Figures 1 and 2 and Table I.

The typical response of mouse myoblast clones to refeeding on day 3 with FM or CM is shown in Figure 1. Twenty-four hours after refeeding, clones fed FM (containing mitogens) continued to proliferate and maintained the spherical, phase bright morphology characteristic of growing mouse myoblasts of both permanent cell lines and early passage cultures (Fig. 1a). In contrast, clones fed CM (medium depleted of mitogenic activity) underwent extensive myoblast fusion (Fig. 1b). The percentage of clones containing myotubes at different times following a switch to FM or CM is shown in Figure 2. Over half of the clones fed CM in this experiment contained myotubes within 24 h, while myotube formation was delayed for more than 48 h in clones fed FM. Myotube formation is generally delayed 2–3 days by feeding FM, but it is not permanently prevented; cells fed FM continue to proliferate, eventually "self-condition" the FM, and differentiate. Addition of

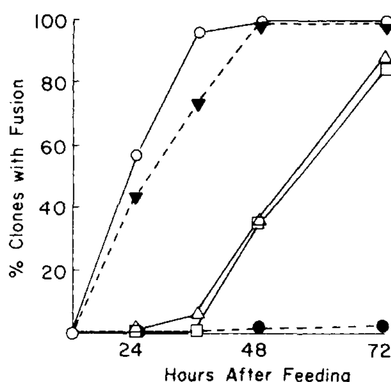

Fig. 2. Delay of myotube formation in mouse myoblast clones by FGF. Clonal density MM14DZ myoblast cultures 3 days after plating were rinsed and fed FM (•), CM (○), CM + 1 ng/ml FGF (▾), CM + 10 ng/ml FGF (△), or CM + 100 ng/ml FGF (□). The percent colonies containing myotubes was determined in cultures fixed at each time point (average of 2 plates).

TABLE I. Stimulation of Clonal Growth by FGF*

Medium	Average doubling time (hours)	
	No FGF	20 ng/ml FGF
FM	13.0	
CM	> 67	21.1
DM	–	21.2
F10 + 15% horse serum	> 67	12.7

*MM14DY myoblasts were plated (200 per 60 mm plate) into 5 plates each containing the media listed. The plating efficiency was similar in all media. Plates were fixed at approximately 12 h intervals from 18 to 67 h after plating, and stained with hematoxylin. The number of cells per clone in 40-100 clones per time point was counted by observation with a stereomicroscope. Average clone size at each time point was used in an exponential curve-fitting program to compute the doubling times. > 67 indicates no growth (1.5–1.7 cells per clone at all time points).

FGF to CM delays myotube formation (Fig. 2), although not as long as FM. Both 10 ng/ml and 100 ng/ml FGF added once at the time of the medium switch were effective. The shorter delay in myotube formation (compared to FM) may be due to differences in mitogenic activity between FM and CM + FGF (cf Table I) or to a more rapid loss of FGF mitogenic activity from the CM + FGF medium than the loss of mitogenic activity from FM.

To determine whether FGF promotes mouse myoblast proliferation, clonal growth rates were assayed (Table I). There was no clonal growth in CM, DM (50% CM, 50% F10), or F10 + horse serum; cells plated in these media completed one cell cycle and did not proliferate further. Embryo extract (in FM) and FGF added to 15% horse serum stimulated the maximum rate of mouse myoblast growth, with doubling times of about 12.8 h. FGF added to CM or DM also stimulated clonal growth; however, the rate of proliferation was slower, with doubling times of 21 h. Although horse serum alone does not support growth of these particular myoblast lines, the difference in FGF growth-promoting activity when added to CM or to medium containing 15% HS may arise from loss during medium conditioning of serum components that potentiate FGF activity. The observation that FGF

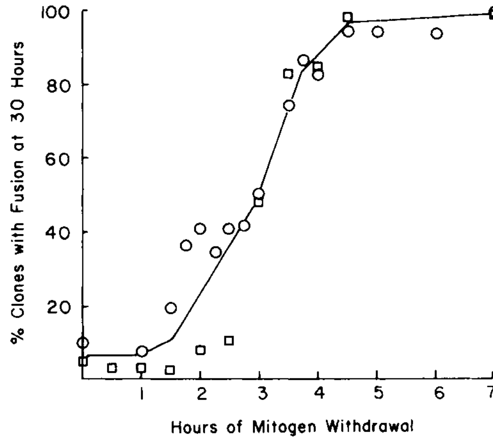

Fig. 3. MM14DZ myoblast commitment to myotube formation. Clonal density cultures of MM14DZ myoblasts plated in FM (100 per 60 mm plate) were rinsed twice with F10 + $CaCl_2$ and fed DM on day 3 after plating. At each time point the medium in duplicate plates was replaced with FM. All plates were fixed at 30 h after the initial switch to DM, and the percent clones with myotubes (containing 3 or more nuclei) was determined. The data points (average from two plates each) are from two separate experiments. In similar experiments it was found that commitment to myotube formation after mitogen refeeding was often delayed when different batches of undiluted CM were used. To reduce the variability apparently due to residue mitogenic activity in different CM batches, CM was diluted 1:1 with F10, and the resulting DM (which has no detectable activity in assays with mouse myoblasts) was used in most subsequent experiments. The persistence of some mitogenic activity in CM could account for the relatively low percent clones with fusion observed after 24 h in CM (Fig. 2) compared to the 100% clones with fusion observed here at 30 h in clones exposed to DM for only 5 h.

activity in similar assays was less at lower horse serum concentrations is consistent with this possibility. The delay of myoblast differentiation in clones fed CM + FGF (Fig. 2), together with the results of Table I, strongly suggests that growth-promoting activity in the embryo extract fraction of FM stimulates mouse myoblast proliferation and thereby prevents differentiation. When fed FM, myoblasts deplete the medium's mitogenic activity before initiating terminal differentiation.

Commitment to Differentiate Following Mitogen Depletion

The role of mitogens in controlling mouse myoblast proliferation and differentiation suggested questions about the response of myoblasts to lowered mitogen levels. Initially we asked how long mouse myoblasts needed to be without mitogens before the cells began initiating the process of terminal differentiation, and whether the response to mitogen removal could be reversed by subsequently refeeding mitogens. MM14DZ myoblast clonal cultures were rinsed and fed mitogen-depleted medium (DM) for increasing periods and then were refed with medium rich in mitogens (FM). The cultures were all fixed at 30 h after the initial switch to DM, and the percent clones containing myotubes was determined (Fig. 3). No myotube formation occurred in clones fed FM immediately after the switch to DM, but after 2 h in mitogen-depleted medium some clones contained sufficient numbers of cells that had irreversibly responded to mitogen withdrawal to permit myotube formation even after mitogens were fed back. However, most of the cells in clones refed FM after only 2 h in DM continued to proliferate. With increasing exposure to mitogen-depleted medium, more clones exhibited fusion after refeeding with FM, and by 5 h in DM all clones contained a sufficient number of myoblasts that were irreversibly "committed"

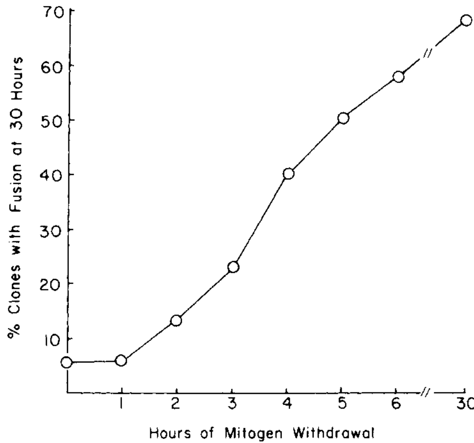

Fig. 4. Commitment of early passage mouse myoblasts. Secondary mouse myoblasts from frozen stocks were plated (1,000 per 60 mm plate) in FM. On day 4 after plating, the cultures were rinsed and fed DM (time 0). At each time point two plates were refed FM. All cultures were fixed at 30 h after the initial switch to DM, and were scored for the percent of total clones that contained myotubes (average of two plates). The 30 h point, from plates that were not fed back FM, indicates the percentage of colonies that are myogenic. The clonal plating efficiency in this experiment was 8%; each point represents a total of 160 colonies scored.

to differentiate so that fusion occurred in the subsequent presence of mitogens. The results in Figure 3 suggested that after a lag period of approximately 2 h in the absence of mitogens mouse myoblasts undergo a commitment to terminal differentiation that cannot be reversed by refeeding mitogens. In this context it is important to emphasize that while commitment occurred as rapidly as 2 h following mitogen withdrawal, fusion was not detected until 12 h after mitogen removal.

Because MM14DZ is a permanent cell line, we were concerned whether the commitment response to mitogen depletion was a property that had evolved during its selection or whether the results shown in Figure 3 were representative of mouse myoblasts in general. The same experiment described in Figure 3 was therefore performed with early passage mouse myoblast cultures, which contained approximately 70% myogenic clones (Fig. 4). Following increasing times of mitogen withdrawal and refeeding with FM, the cultures were fixed and the percent clones with fusion was determined. The results were essentially the same as for myoblasts of the MM14DZ cell line. After a lag period in the absence of mitogens, sufficient numbers of cells in some clones committed so that myotubes were subsequently formed even after mitogens were fed back to the cultures. Since, however, these cultures contained non-myogenic colonies as well as muscle clones, the percent clones with fusion did not attain the 100% level observed with the permanent clonal myogenic cell lines. As in the previous experiment, most of the myogenic clones contained committed cells by 5 h of mitogen withdrawal. These results suggest that the response to low mitogen levels seen in the MM14DZ cell line is common to all cultured mouse myoblasts. Commitment and irreversible loss of sensitivity to mitogens are the first detectable steps in mouse myoblast terminal differentiation.

Withdrawal From the Cell Cycle

The results shown in Figures 3 and 4 demonstrate that after a few hours in the absence of mitogens, some mouse myoblasts commit to differentiate and will fuse to form

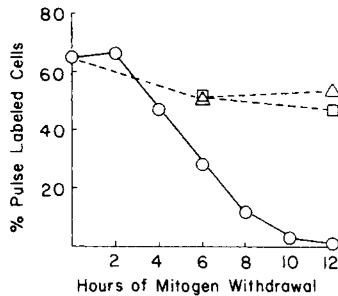

Fig. 5. Withdrawal of mouse myoblasts from the S phase of the cell cycle. Clonal density MM14DZ cultures 3 days after plating were rinsed and fed DM (○), FM (△), or DM + 20 ng/ml FGF (□). At 30 min before each time point, ^{3}H-thymidine was added in a small volume to one culture plate. After a 30 min incubation, the plate was rinsed twice in saline and fixed. Autoradiographs were scored for the percent cells that had incorporated ^{3}H-thymidine. Twenty clones were examined for each time point (500–1,000 cells).

myotubes even if mitogens are fed back. Although the relative numbers of cells that fused was observed to increase with the time the cells had been in the absence of mitogens, the commitment assay described in Figures 3 and 4 does not provide precise kinetic data at the level of individual cells. Since myoblasts stop proliferating as they terminally differentiate, we examined the ^{3}H-thymidine pulse-labeling index of mouse myoblasts following removal of mitogens from the culture medium to determine how rapidly the population responded to the absence of mitogens (Fig. 5). Clonal density MM14DZ myoblasts were rinsed and fed DM 3 days after plating. The percent cells labeling during 30 min pulses with ^{3}H-thymidine was essentially constant for 2 h, then decreased steadily until virtually no cells were synthesizing DNA by 10 h. This result suggests that all of the myoblasts stopped proliferating within one doubling time (12.5 h) of removal of mitogens from the culture medium. In similar experiments with early passage myoblasts, all the cells in myogenic clones stopped DNA synthesis within 12 h of removing mitogens from the medium.

In the same experiment, FGF (20 ng/ml) was added to the DM at the time of the initial medium switch. The pulse labeling index of cells exposed to FGF was the same at 6 and 12 h as that of cells fed FM. This result shows that a purified growth factor, FGF, prevents the withdrawal of mouse myoblasts from the cell cycle and provides additional evidence that mitogenic activity in FM inhibits myoblast differentiation.

Cell Cycle and Commitment Kinetics

The results in Figure 5 suggest that mouse myoblasts withdraw from the cell cycle in response to mitogen depletion, but they do not indicate whether the cells withdraw at a specific point or stop cycling in all phases of the cell cycle. To begin answering this question we determined the cell cycle parameters of proliferating mouse myoblasts and myoblasts that were responding to mitogen depletion. High clonal density cultures were pulsed for 15 min with ^{3}H-thymidine and allowed to cycle in FM or CM; the accumulation of radioactively labeled cells in mitosis was used to measure the durations of the cell cycle phases, as described initially by Quastler and Sherman [35]. The results and calculated cell cycle parameters are shown in Fig. 6. The doubling time (t) of 12.5 h was independently deter-

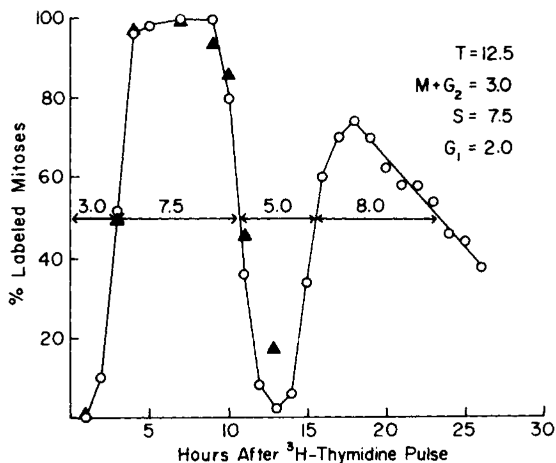

Fig. 6. Cell cycle parameters of mouse myoblasts in FM and CM. High clonal density plates were pulse labeled with ^{3}H-thymidine and fed either FM (○) or CM (▲). At hourly intervals cells were collected from one plate each (colcemid added 15 min before each time point) and processed for metaphase spreads. The percent labeled metaphase figures is plotted as a function of time after the ^{3}H-thymidine pulse (50 metaphase figures counted per time point). The cell cycle durations were estimated from the points of 50% labeled mitoses [40]; the generation time (12.5 h) was determined independently from clonal growth rates. No mitotic figures were observed after 13 h in cultures fed CM. In a parallel experiment performed at the same time, the ^{3}H-thymidine pulse labeling index decreased to essentially zero by 10 h, as in Figure 5.

mined in clonal growth rate experiments. The relatively long S (7.5 h) and short G_1 (2 h) fractions of the total generation time are similar to the cell cycle parameters observed by others in proliferating avian myoblast cultures [2, 20, 21].

In cultures switched to CM, the kinetics of accumulation of labeled mitotic figures during the first 12 h were identical to the kinetics for cells which continued to proliferate in FM. However, after 13 h, mitotic figures were no longer observed in cultures fed CM. These results indicate that in the absence of mitogens cells initially pulse-labeled in S continued with normal kinetics through the cell cycle to mitosis. Thus the S and G_2 + M periods of the mouse myoblast cell cycle do not seem to be altered by mitogen removal.

The evidence in Figures 5 and 6 suggests that when mitogens are removed for 2 or more hours, mouse myoblasts stop entering S and accumulate in G_1. The decrease in the percent cells in S between 2 and 10 h (Fig. 5) takes approximately the same time as the length of S determined in Figure 6. This evidence and the finding that mouse myoblasts cycle normally from S to mitosis in the absence of mitogens suggest that after 2 h in mitogen-depleted medium the myoblasts in G_1 stop entering S while those in S continue to leave at a normal rate (leaving fewer cells in S) until the S phase compartment is totally depleted by about 10 h (Fig. 5). By 13 h the last cohort of cells (from the beginning of S period) to continue cycling reaches mitosis, and subsequently mitotic figures are no longer observed (Fig. 6). All of the mouse myoblasts accumulate in the G_1 period within one cell cycle time of mitogen withdrawal.

The evidence in Figures 3 and 4, that mouse myoblasts irreversibly commit to differentiate, and the evidence in Figures 5 and 6, that they withdraw from the cell cycle in G_1, suggests that mouse myoblasts commit *and* withdraw irreversibly from the cell cycle

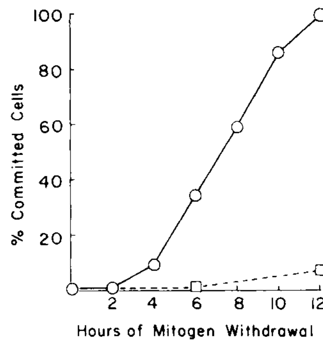

Fig. 7. Irreversible withdrawal of committed mouse myoblasts from the cell cycle. Clonal density MM14DZ cultures 3 days after plating were rinsed and fed DM (○) or DM + 20 ng/ml FGF (□). At each time point the medium in one plate was replaced with FM + ^{3}H-thymidine (0.2 μCi/ml). The cultures were rinsed with saline and fixed at 30 h after the initial switch to DM. The number of unlabeled nuclei per clone (N_C) was determined in autoradiographs (average of 20 clones per time point). The average clone size (N_T) at the time of mitogen refeeding was determined in a parallel series of cultures that were fixed at each time point (average of 20 clones per time point). The percent cells committed at increasing times of exposure to DM was calculated as % cells committed = $N_C/N_T \times 100\%$. This experiment was performed with the same cells and at the same time as the experiment in Figure 5.

as they cycle into G_1 in the absence of mitogens. We tested this by withdrawing mitogens from clonal cultures for increasing times (as in Figs. 3 and 4), then refeeding mitogens (FM) plus ^{3}H-thymidine for another 18–30 h. For this experiment it was assumed that cells that did not incorporate ^{3}H-thymidine following mitogen refeeding represented cells that had committed and irreversibly withdrawn from the cell cycle, whereas uncommitted cells would continue to proliferate in FM and would incorporate ^{3}H-thymidine. To normalize the number of committed cells to the entire population at each interval of exposure to mitogen-depleted conditions, the number of cells per clone at the time of the mitogen refeeding was determined in parallel plates which were fixed at each time point. The percent committed cells at increasing times of mitogen depletion was calculated by dividing the average number of committed cells per clone at the time of mitogen refeeding (ie, those cells that did not incorporate ^{3}H-thymidine during the subsequent 18–30 h) by the average clonal size of parallel clones fixed at the beginning of each refeeding with mitogens and ^{3}H-thymidine (Fig. 7).

The data in Figure 7 are from the same experiment in which the pulse labeling index was followed (Fig. 5). During the 2 h lag period, in which the percent cells in S did not change (Fig. 5), no cells committed (ie, all cells had incorporated ^{3}H-thymidine during the ensuing 28–30 h following mitogen refeeding). However, after 2–3 h in the absence of mitogens, cells appeared that did not incorporate ^{3}H-thymidine when mitogens were fed back. The percentage of such committed cells increased until all of the myoblasts had committed by 12 h. These results show that the mitogen-regulated withdrawal of mouse myoblasts from the cell cycle is not reversible by readdition of mitogens. Myoblasts commit to terminal differentiation and withdraw from the cell cycle several hours before cell fusion begins (at 12 h after mitogen removal).

Assuming that cells in S continue to cycle to mitosis (Fig. 6) and that the pulse labeling index decreases (after the lag period) due to cells leaving S without cells entering S (Fig. 5), the data in Figure 7 suggest that mouse myoblasts commit as they cycle into G_1 in the absence of mitogens. The time from the end of the lag period until the last cells

commit (10 h) is roughly equivalent to the time required for cells at the beginning of the S period to cycle through S (7.5 h) and G_2 + M (3 h). If commitment occurs in G_1, cells that do not cycle into S after the 2 h lag period (as suggested in the pulse labeling experiment) would commit and would not enter S after mitogen refeeding. The last cells to reach G_1 (of the following cell cycle) and to commit would be the cells that had just entered S at 2 h (the end of the 2 h lag period). The increase in committed cells from 2 to 12 h, then represents the cycling of cells into G_1.

Addition of FGF (20 ng/ml) to the DM in the commitment experiment described in Figure 7 prevented the accumulation of unlabeled cells. This provides further evidence that mouse myoblasts are prevented from differentiating by mitogens in the culture medium.

Differentiation of Committed Cells

One critical assumption of the analysis of commitment described in Figure 7 is that cells that do not incorporate ^{3}H-thymidine after mitogens are refed are committed to differentiate and are not, in the worst case, simply dead. We tested this assumption by comparing the kinetics of appearance of cells that fail to incorporate ^{3}H-thymidine after mitogen refeeding (as in Fig. 7) to the kinetics of appearance of cells that elaborate a muscle-specific gene product in the presence of mitogens that have been refed. We examined myoblasts that had been without mitogens for increasing times, then refed mitogens for 18–30 h, for the appearance of acetylcholine receptors (AChR). AChR is elaborated during myoblast differentiation [8, 34, 46] and is easily detected by autoradiographic analysis of ^{125}I-αBungarotoxin-treated cultures [25]. To facilitate examination of autoradiographs and quantitation of nuclei, we used low-density mass cultures immediately after plating rather than clonal cultures, as in the experiment described in Figure 7.

Proliferating MM14DZ myoblasts were collected and plated (20,000 per 60 mm plate) in DM. After increasing times without mitogens, the cultures were fed FM and incubated until 30 h after plating. At each time point one set of cultures was fed FM + ^{3}H-thymidine and was then fixed at 30 h, and autoradiographs of the plates were analyzed for the percent cells (at 30 h) that had not incorporated ^{3}H-thymidine. The other set was fed FM without radioactive thymidine, and at 30 h was incubated with ^{125}I-αBungarotoxin and fixed; autoradiographs of the plates were analyzed for the percent cells positive for AChR. The results (Fig. 8) show that following mitogen withdrawal and a 2 h lag period, the percent cells not incorporating ^{3}H-thymidine as well as the percent that elaborated AChR in the presence of mitogens increased, and the kinetics of commitment determined by the two criteria are the same. This suggests that all of the cells that failed to incorporate ^{3}H-thymidine after mitogen refeeding (as in Fig. 7) were committed to differentiate and elaborated muscle-specific gene products. The appearance of some AChR-positive cells at the early time points when no ^{3}H-thymidine-negative cells were observed is not yet understood, but it may arise from uncommitted cells that incorporated ^{3}H-thymidine in the first cell cycle after refeeding FM but that committed (due to some self-conditioning of the medium) during the next cell cycle (ie, the 2 h time point represents cells that were in FM for 28 h after refeeding).

Commitment of Mitotically Synchronized Mouse Myoblasts

The kinetic data in Figures 5–7 are most easily interpreted by a model in which mouse myoblasts commit and withdraw from the cell cycle as they cycle into G_1 in the absence of mitogens. Another approach to the evaluation of this model is to synchronize proliferating mouse myoblasts and withdraw mitogens during different phases of the cell cycle. As a first attempt, mitotic cells were collected from proliferating cultures by a shake-

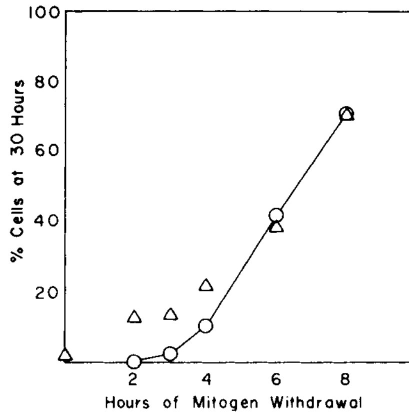

Fig. 8. Committed myoblasts elaborate acetylcholine receptors. Proliferating MM14DZ cells were plated (20,000 per 60 mm plate) into DM at 0 h and refed FM at each time point. One group was fed FM containing ^{3}H-thymidine, all plates were fixed at 30 h, and the percent cells not incorporating label (○) was determined in autoradiographs. The other group was fed FM, at 30 h, all plates were incubated with 1×10^{-8}M ^{125}I-αBungarotoxin, and the percent cells positive for acetylcholine receptor (with greater than background binding) was determined in autoradiographs (△). Control plates for nonspecific binding (co-incubation of ^{125}I-αBungarotoxin with 2×10^{-4}M decamethonium) had no positive cells.

TABLE II. Commitment of Mitotically Synchronized Cells in G_1*

	+ Mitogens	– Mitogens
% Cells not incorporating ^{3}H-thymidine	0.7	96.8
% Cells elaborating AChR	1.2	100

*Mitotic cells collected from proliferating MM14DZ cultures were plated (1,200 per 60 mm plate) into FM (+ Mitogens) or DM (– Mitogens). At 1 h the media were replaced to remove unattached cells. For determination of the percent cells which initiated DNA synthesis ^{3}H-thymidine (0.2 μCi/ml) was present from the time of plating until the plates were fixed at 30 h. No ^{3}H-thymidine was added to the plates that were incubated with ^{125}I-αBungarotoxin at 30 h for determination of the percent cells that elaborated AChR. Each value is based on analysis of 140–220 cells. The cell preparation that was plated consisted primarily of cells that appeared to be in mitosis (condensed chromosomes, large cell sizes, dumbell-shaped doublets) and was free of contamination by S phase cells. Cells plated into FM + ^{3}H-thymidine did not incorporate label until 4 h. The apparent G_1 period was longer than that determined in Figure 6, with 50% of the cells labeling by 7 h and 100% labeling at 10 h.

off technique and plated into media with and without mitogens (FM and DM). ^{3}H-thymidine was fed to one subset of each at the time of plating; these plates were fixed at 30 h, and the percent cells not incorporating label was determined from autoradiographs. Another subset of cultures that had been plated in DM or FM was incubated with ^{125}I-αBungarotoxin at 30 h, fixed, and the percent AChR positive cells was determined from autoradiographs. The results (Table II) show that mitotically synchronized mouse myoblasts entering G_1 in the absence of mitogens all committed: less than 5% incorporated ^{3}H-thymidine, and all were AChR positive at 30 h. This evidence supports the idea suggested by kinetic data that mouse myoblasts commit to terminal differentiation when they cycle into G_1 in the absence of mitogens.

DISCUSSION

Control of Myoblast Proliferation and Differentiation by Mitogens

We have found that mitogenic activity in the culture medium stimulates mouse myoblasts to proliferate and prevents terminal differentiation. Previous work has shown that the mitogenic activity is eliminated by mouse myoblasts as they "condition" the culture medium before differentiating [23]. A purified polypeptide mitogen, FGF, added once in nanomolar concentrations to conditioned or mitogen-depleted medium stimulated logarithmic clonal growth for more than 60 h after plating (Table I); prevented cells in proliferating clones from committing and from withdrawing from the cell cycle for 12 h (Figs. 5 and 7); and delayed myoblast fusion in clones for at least 36 h (Fig. 2). In preliminary experiments, epidermal growth factor [4] was inactive in all of these assays, and multiplication stimulating activity [6] and platelet-derived growth factor [37] were inactive in preventing mouse myoblast fusion. Since serum alone (without FGF or embryo extract) did not support myoblast proliferation, it is also unlikely that mouse myoblasts of the clonal line that was studied would be stimulated to proliferate by other known growth factors that have been derived from serum, such as the somatomedins [45] or non-suppressible insulin-like activities [36]. The mitogenic activity in chick embryo extract to which mouse myoblasts are sensitive is apparently from one or more tissue-derived molecules that are similar in specificity to FGF. FGF has been reported in the presence of serum to stimulate proliferation of chick myoblasts [44] and bovine myoblasts [11]. Other laboratories have reported the use of fetal calf serum and horse serum in mouse myoblast cultures [13, 28, 32, 48]. It is possible that during its derivation the MM14D line lost sensitivity to mitogens other than FGF-like molecules. Further work with FGF and other purified mitogens will be done to learn the details of mouse myoblast mitogen specificity and the mechanisms by which mitogens prevent terminal differentiation.

The idea that myoblast proliferation is stimulated and differentiation delayed by growth-promoting activity in the culture medium has been suggested by in vitro studies of avian myogenesis [19, 20, 30, 38]. The differences in growth-promoting activity between media with different embryo extract and serum concentrations and between fresh and conditioned media are often exploited to manipulate the transition of myoblast cultures from proliferation to differentiation. We have found that the control of mouse myoblast proliferation and differentiation by mitogens in clonal density cultures is striking compared to other myoblast systems, and that experimental manipulation of the timing of differentiation is very precise. The advantages of the mouse system arise partly from the characteristics of the cells and the homogeneity of the permanent cell lines, and partly from the clonal density culture system, in which alteration of the culture medium by the cells during an experiment (self-conditioning) is minimized.

Commitment for Terminal Differentiation

The first detectable event in mouse myoblast terminal differentiation following the removal of mitogens from the culture medium is the failure of cells to enter the S period and loss of the capacity to initiate DNA synthesis even if mitogens are fed back. At present we do not know whether the loss of mitogen response involves the loss of mitogen receptors or the inhibition of intracellular processes that mediate the proliferative response. The lag or latent period following mitogen removal from the medium (before any withdrawn or committed cells are detected) is 2–4 h (in different experiments) and may represent the dissociation of bound mitogens from cell surface receptors, the turnover of mitogen-

receptor complexes on the cell surface, the decay of an intracellular "second messenger" that mediates the stimulation of proliferation, or the build-up of an intracellular commitment signal that inhibits the signal to initiate DNA synthesis and proliferate. The relatively short duration of the lag period compared to the cell cycle time suggests that the rate-limiting cellular component for the initiation of DNA synthesis (in the presence of mitogens) turns over rapidly or that an inhibitory component is generated rapidly in the absence of mitogens.

The results from the kinetic experiments and the experiment with mitotically synchronized cells suggest the following model for mouse myoblast commitment in response to lowered mitogen levels: 1) After the lag period cells in G_1 stop entering S and cannot be stimulated to enter S by subsequent exposure to mitogens; these cells have permanently withdrawn from the cell cycle and are committed to terminally differentiate. 2) Cells in other phases of the cell cycle continue through the cell cycle in the absence of mitogens and withdraw in the next G_1 if mitogens are still absent. This model is consistent with the evidence that myoblast differentiation in other species occurs in G_1 [22, 27, 30, 31, 39, 40].

The results are compatible with the commitment "decision" being made at or near the time that mouse myoblasts withdraw from the cell cycle in G_1. The experiments do not, however, rigorously exclude the possibility that *some* cells commit before G_1 and withdraw from the cell cycle when they reach G_1. Evidence that *most* of the cells commit in G_1 at the time they withdraw from the cell cycle comes from comparing the times required for all cells to commit and withdraw (which fit the cell cycle phase durations as already discussed) and from comparison of the percent cells that have withdrawn to the percent that have committed at intermediate times of mitogen removal. The percent withdrawn cells that failed to enter S is estimated from Figure 5 (subtracting the pulse labeling index from the steady state value of 65%) to be 37% and 53% for 6 and 8 h of mitogen withdrawal, the percent committed cells at 6 and 8 h was 34% and 59% respectively (Fig. 7). Since these values are derived from three independent measurements (labeling index, clone size, and number of cells per clone), the close correlation of committed to withdrawn cell numbers of cells per clone), the close correlation of committed to withdrawn cell numbers suggests that most, if not all, mouse myoblasts withdraw and commit in G_1 when deprived of mitogens. This does not, however, imply that commitment is obligatorily associated with withdrawal. The causal relationship between these two phenomena is being examined.

Avian myoblasts do not withdraw from the cell cycle within one generation time of the depletion of growth-promoting activity from the culture medium [7, 20], nor does withdrawal of avian myoblasts appear to precede the onset of myoblast fusion by very long [7, 20, 21, 29, 30]. Unlike mouse myoblasts, which accumulate indefinitely in G_1 (or G_0) and commit to differentiate, avian myoblasts in proliferating clones appear to increase the length of the G_1 period, when mitogens are depleted, without permanently withdrawing from the cell cycle until they fuse [2, 20, 30]. Contrary to the behavior of mouse myoblasts, the avian studies suggest that commitment does not precede myoblast fusion, although fusion per se is not required for terminal differentiation [7, 16, 26, 42]. Results from several mass culture studies, however, have provided evidence that some avian myoblasts are committed at the time of plating and subsequently differentiate several hours later [5, 31, 38, 43]. Recent work [17, 18] suggests that the number of doublings preceding terminal differentiation of some avian myoblasts may be determined in the embryo.

Initial work by Yaffe [47] provided evidence that rat myoblasts in primary cultures commit to differentiate in response to lowered mitogenic activity and subsequently cannot

be prevented from fusing by refeeding mitogen-rich media. Unlike mouse myoblasts, however, rat primary myoblasts appear to require exposure to lowered mitogen concentrations (10% horse serum) for periods longer than one cell cycle before they commit [22]. Similarly, rat myoblasts of permanent cell lines at subconfluent densities continue to proliferate for more than two doublings before fusing in medium containing 10% horse serum [27, 49]. This "differentiation medium" may not be sufficiently low in mitogenic activity to allow rapid commitment of rat myoblasts. Rat (cell line) myoblasts at higher densities do withdraw from the cell cycle and accumulate in G_1 within one cell cycle; however, their subsequent commitment occurs slowly over the equivalent of several generation times [27]. Unlike mouse myoblasts, which commit at least 10 h before fusion is observed, commitment of rat myoblasts (failure of mitogen-rich media to prevent fusion or loss of proliferative capacity) appears to be coincident with fusion in primary cultures [22] and a permanent cell line [in reference 27, compare Fig. 4c to Fig. 5). In yet another system, lizard myoblasts of a permanent cell line withdraw from the cell cycle in G_1 without fusing (in low medium calcium ion concentrations) and commit to differentiate coincident with the elaboration of myosin [1].

The kinetics of differentiation of avian and rat myoblasts have suggested stochastic mechanisms for commitment and terminal differentiation [21, 27, 29, 30]. A stochastic mechanism has also been proposed for the commitment of murine erythroleukemia (MEL) cells to terminally differentiate following induction by dimethylsulfoxide [12]. Like mouse myoblast commitment, the commitment of avian and rat myoblasts and MEL cells occurs in G_1 [9, 21, 22, 30, 33]. Unlike mouse myoblasts, populations of rat myoblasts and MEL cells commit gradually over the equivalent of several generation times, so commitment in these systems is thought to involve probabilistic mechanisms. Mouse myoblasts all commit as they cycle into G_1 during the first cell cycle after mitogen removal, suggesting that commitment occurs by a deterministic rather than a stochastic mechanism (or at least that the probability of commitment during the first cell cycle is very high).

The permanent clonal mouse myoblast cell lines are a useful model system for the study of a developmental commitment process because the timing of commitment can be precisely controlled with mitogens and the cells commit synchronously in G_1. Studies of the commitment of mouse myoblasts synchronized in G_1 will allow careful kinetic studies from which the molecular details of the transition from proliferation to terminal differentiation may be learned.

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