A Rapid Decrease in Epidermal Growth Factor-binding Capacity Accompanies the Terminal Differentiation of Mouse Myoblasts In Vitro

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ABSTRACT Specific mitogens stimulate the proliferation and repress the differentiation of mouse myoblasts (MM14). When mitogens are depleted, MM14 cells cease proliferation, commit to terminal differentiation, and become refractory to growth stimulation. The behavior of mitogen receptors during the transition from a proliferative to a permanently postmitotic state was examined using the epidermal growth factor receptor (EGFR) as a model system. Whereas proliferating myoblasts bound substantial amounts of EGF, their binding capacity declined rapidly upon exposure to low-mitogen medium. The decline became irreversible when a cell differentiated. Within 24 h, <5% of the original EGF binding capacity remained. Since the ability to internalize and degrade bound EGF was unaffected, the change presumably reflected a decrease in EGFR availability.

Several observations indicated that loss of EGFR following mitogen removal is related to differentiation rather than the result of starvation or cell-cycle arrest. First, the decline is correlated with the absence of a single mitogen (fibroblast growth factor) and is independent of serum concentrations. Second, myoblasts that are either cycling through G_1 or arrested at G_0, but prevented from differentiating, all bind large amounts of EGF. These findings suggest that specific reduction in mitogen receptors could be part of a mechanism whereby terminally differentiating cells become refractory to mitogenic stimulation.

Terminal differentiation of skeletal muscle myoblasts is known to be influenced by mitogen levels in the culture environment (1–3). When mitogens are reduced below some threshold value, myoblasts withdraw from the proliferative cycle and initiate a differentiation program (4–7). Although identification of the mitogens that regulate myoblast proliferation and differentiation is just beginning, it appears that myoblasts derived from different species, or even within the same species, may differ with respect to their mitogen responsiveness. For example, the established L6 line of rat myoblasts responds to the somatomedin multiplication stimulating activity (8), whereas the permanent mouse myoblast line (MM14) is not responsive to multiplication stimulating activity or to unfractionated serum, but is responsive to fibroblast growth factor (FGF)[1] when serum is provided (6, 7, 9). Interestingly, MM14 myoblasts, as well as primary cultures of mouse and human myoblasts, also exhibit receptors for epidermal growth factor (EGF), yet even in the presence of serum, myoblasts do not proliferate in response to EGF (10). In contrast, differentiation-defective myoblasts (DD-1 cells), a variant cell type that arises at high frequency from MM14 myoblasts, appear to acquire mitogenic responsiveness to EGF simultaneously with their loss of differentiation ability (10).

Studies with MM14 myoblasts demonstrate that within 2–3 h of exposure to medium lacking FGF, G_1-phase cells permanently withdraw from the cell cycle, and within one generation time (12.5 h) the entire population becomes postmitotic and exhibits muscle-specific properties (6, 7, 11). This irreversible withdrawal from proliferation that occurs 8–12 h prior to the initiation of myogenic fusion has been termed "commitment to terminal differentiation." Similar irreversible behavior has been observed in rat muscle cultures (5). Quail myoblasts also withdraw from the cell cycle and initiate differentiation in response to mitogen depletion, but in this

[1] *Abbreviations used in this paper:* CHS, conditioned 15% horse serum; EE, 3% embryo extract; EGFR, epidermal growth factor receptors; FGF, fibroblast growth factor; FM, standard fresh medium; LM, low mitogen medium; TdR, thymidine.

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system some myosin-positive myocytes (single differentiated muscle cells) retain the ability to reinitiate DNA synthesis when mitogens are restored (12).

Since myogenesis involves the transition from a proliferative state to a state that is refractory to mitogenic stimuli, it is of interest to determine whether the transition includes modifications in the capacity of muscle cells to interact with external mitogenic signals. Ideally, studies of mouse myoblasts would involve FGF and FGF receptors, but a lack of pure FGF and difficulties associated with demonstrating specific FGF binding have temporarily precluded such analyses. Attention has therefore been focused on the behavior of EGF receptors (EGFR).

In a preliminary study, a large decrease in EGFR has been shown to accompany MM14 cell differentiation (10). While a direct correlation between EGFR behavior and the mitogenic responsiveness of differentiating myoblasts is not immediately obvious, the fact that EGFR declines during muscle differentiation suggests that its regulation is important. In this report, the alteration in EGF binding capacity is characterized further and shown to be a differentiation-related phenomenon—the decline in EGF binding in differentiating myoblasts is rapid and irreversible, and is unrelated to cell density, nutrient depletion or withdrawal into a quiescent state. It thus appears to be a novel form of EGFR regulation. Functional implications of the rapid decline in EGFR with respect to the control of proliferation and differentiation are discussed.

MATERIALS AND METHODS

Cell Line: The derivation and characterization of MM14 mouse myoblasts and a differentiation-defective variant, DD-1, have been described previously (11, 13). Both cell lines are near-tetraploid in karyotype (C. Maxwell and S.D. Hauschka, unpublished observation).

Growth Factors: Bovine pituitary FGF and receptor grade mouse EGF were purchased from Collaborative Research (Lexington, MA) in lyophilized form and reconstituted according to the instructions provided; aliquots were frozen at −70°C until used.

Culture Conditions: Cells were passaged by dissociation with 0.05% crude collagenase (Worthington, Freehold, NJ) in Ca^{++}, Mg^{++}-free Puck's Saline G. Unless otherwise stated, all cultures were grown in gelatin-coated tissue culture dishes (Corning Medical and Scientific, Medfield MA) with a standard fresh medium (FM) consisting of Ham's F10 plus 0.8 mM extra $CaCl_2$ (F10C), 15% horse serum, 3% chick embryo extract (EE), and 1% antibiotic solution (10,000 U/ml penicillin-G, 0.5 mg/ml streptomycin sulfate). Rapid myogenic differentiation was initiated by rinsing plates twice with F10C and exposing them to low mitogen medium (LM) which contains horse serum but lacks EE.

Conditioned 15% horse serum (CHS) was prepared by exposing 15% horse serum in F10C (10 ml/100-mm plate) to high-clonal density MM14 cultures ($0.5–1 \times 10^4$ colonies/plate) for 24 h beginning 2 d after plating.

Mitotically synchronized cells were collected by shake-off from proliferating cultures and were rinsed and resuspended in CHS. Aliquots were plated into glass cloning cylinders (Bellco Glass, Vineland, NJ), set without grease in the center of a 60-mm plate containing 3 ml of the desired medium. Cells were allowed to attach for 1 h and cylinders were then removed.

Analysis of Withdrawal and Commitment: Withdrawal of cells from the proliferative cycle was determined by pulse-labeling cells with 2 μCi/ml [^{3}H]thymidine (TdR) for 30 min. Cultures were then fixed with 70% ethanol and processed for autoradiography (6). The decline in labeling index served as an indication of cell cycle arrest. Commitment to terminal differentiation was defined as the inability of cells to resume DNA synthesis after complete growth medium had been restored (6). Cells were labeled continuously with [^{3}H]TdR (0.5 μCi/ml) for at least 24 h starting immediately after refeeding with FM, and were then fixed and processed for autoradiography. For clonal density cultures, the average number of unlabeled cells present per colony was determined. The percent commitment was then calculated as the ratio between this number and the average colony size determined from a parallel plate which had been fixed at the time of mitogen restoration. For high density cultures, the percentage of unlabeled cells present at the end of the labeling period was used directly as an indication of commitment. Because of the likelihood that [^{3}H]TdR-positive cells have undergone cytokinesis during the 24 h period following mitogen restoration, this index of commitment underestimates the actual percentage of committed cells. Accordingly, data from mitotically-synchronized high density cultures were adjusted assuming that 100% of the proliferating cells had undergone one doubling since the mitogen feedback (9). In the text, single committed cells are referred to as myocytes.

Preparation of ^{125}I-EGF: ^{125}I-labeled EGF was prepared by iodinating receptor-grade EGF with chloramine T (14). Specific activity of various batches of ^{125}I-labeled EGF was $1–2 \times 10^5$ cpm/ng. Aliquots of the labeled growth factor were stored at −70°C in the presence of 0.1% BSA.

EGF Receptor Assays: Apparent EGF binding capacity was measured after cultures were rinsed twice with F10C containing 0.5% horse serum. Unless otherwise stated, the binding medium consisted of F10C plus 0.1% BSA and $0.5–1.0 \times 10^6$ cpm/ml of ^{125}I-EGF. Incubation was for 1 h in a 37° CO_2 incubator. Binding was terminated by aspirating the radioactive binding medium and rapidly rinsing the cultures eight times with cold phosphate-buffered saline containing 0.1% BSA (2 ml/plate). The amount of ^{125}I-labeled EGF remaining associated with the cell layer was then quantitated either by solubilizing the cells in 0.5 M NaOH and determining the radioactivity in the extract with a gamma counter or by fixing the cells in phosphate-buffered saline plus 2% glutaraldehyde, followed by autoradiography and microscopic examination. (A camera-lucida attachment was used so that separate grains could be drawn over the outline of individual cells.) When the gamma counting method was used, specific binding was determined by subtracting from total binding the nonspecific background observed in the presence of 0.5 μg/ml unlabeled EGF. Binding values were obtained from at least two replicate plates. Whenever applicable, binding data were normalized to cell number determined with cell suspensions obtained from parallel plates. Since binding assays were performed at 37°C to mimic physiological conditions, the observed binding capacity was actually a function of the entire process of EGF binding, internalization, and degradation. Results from binding studies at 4°C were similar to those obtained during the first hour after ^{125}I-EGF addition at 37°C. This suggests that the apparent EGF binding capacity observed at 37°C is representative of the actual EGF binding capacity (see Discussion). Detailed binding studies at 4°C have not been conducted, because MM14 myoblasts detach from the culture dishes before binding equilibrium is achieved.

[^{125}I]EGF Internalization: The extent of EGF internalization was determined as described by Haigler et al. (15). Cultures were incubated at 37°C with ^{125}I-EGF ($2–3 \times 10^5$ cpm/ml) for short periods of time. After removing the unbound EGF by rapid rinsing with cold buffer, cultures were extracted at 4°C for 4 min with 0.2 M acetic acid, 0.5 M NaCl (pH 2.5). Each plate was rinsed once with the same buffer. The cell layer was then solubilized by incubation in 0.5 M NaOH. Radioactivity in the acid and NaOH extract was quantified separately and represents respectively the amount of externally bound and internalized ^{125}I-EGF.

RESULTS

EGF-binding Capacity in Proliferating and Differentiated Muscle Cultures

The capacity of differentiated MM14 cells to interact with EGF was examined and compared with that of proliferating myoblasts. Results from a typical experiment are shown in Fig. 1. EGF binding capacity of differentiated cultures was determined 19 h after switching proliferating myoblasts to LM medium. By this time, >95% of the cells had committed to terminal differentiation (6, 9, 11). Whereas proliferating myoblasts maintained in growth medium exhibited a substantial amount of specific ^{125}I-EGF binding, the EGF binding capacity of differentiated cells (myocytes) was much reduced. Nonspecific binding in the presence of excess unlabeled EGF constituted <15% of the total binding observed in proliferating cultures, and was subtracted prior to data analysis. In contrast, the extent of total EGF binding was not affected by the presence of excess FGF in the binding medium.

Since decreased binding relative to proliferating cells was observed at all concentrations of ^{125}I-EGF examined, these data suggest that reduction of EGF-binding capacity in committed myocytes was due primarily to a change in the number of receptor sites. However, a definitive conclusion cannot be

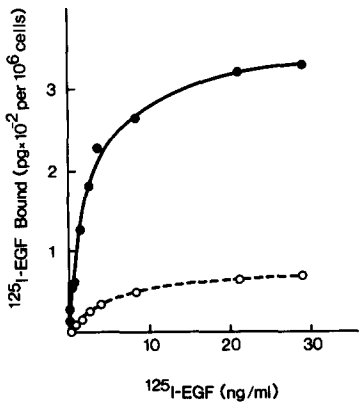

FIGURE 1 ^{125}I-EGF binding capacity of proliferating and differentiated muscle cultures. High density log-phase cultures of MM14 myoblasts were rinsed twice with F10C and either exposed to low mitogen medium (F10C plus 5% horse serum) to induce commitment (○) or refed with FM to maintain proliferation (●). 19 h later, the apparent EGF-binding capacities of the differentiated cultures and proliferating myoblasts were analyzed by incubating at 37°C for 1 h in a serum-free binding medium containing the indicated concentrations of ^{125}I-labeled EGF. (Myoblasts continue traversing the cell cycle during a 1-h exposure to serum-free medium [6, 9, 11].) Specific binding was determined as described in the text. Data shown represent the average of two determinations, with variations between samples generally <15%.

reached since the continual internalization of mitogen-receptor complexes prevents attainment of equilibrium conditions (16). Nonetheless, based on the plateau level of ^{125}I-EGF-binding proliferating myoblasts appeared to contain ~3×10^4 EGF binding sites per cell, whereas cells that had been exposed to LM for 19 h contained on average 7,000 EGF binding sites per cell. Estimations of the apparent binding constants (K_A) from the concentration of ^{125}I-EGF needed to achieve half-maximal binding disclosed roughly similar values (3.3×10^9 M^{-1} for proliferating myoblasts and 1.3×10^9 M^{-1} for committed cells). Subsequent studies revealed that there is a considerable distribution of EGF-binding capacity among individual cells in the population (cf. Fig. 3) and that longer exposure to LM leads to the virtual absence of specific EGF binding (cf. Fig. 2).

Time Course of Reduction in EGF-binding Capacity

Since the foregoing experiment indicated that EGF binding sites decrease in differentiated cells, it was interesting to determine when this decrease began and how rapidly it occurred relative to the kinetics of commitment. Cultures of proliferating myoblasts were shifted to LM and after increasing times, one set of cultures was rinsed and incubated with ^{125}I-EGF, while parallel cultures were either pulsed with [^{3}H]TdR or fed FM containing [^{3}H]TdR to measure cell-cycle withdrawal and commitment to differentiation. As shown in Fig. 2, the decline in EGF binding occurs at a very rapid rate: a 30% decrease is detectable as early as 3 h after the medium switch; within 12 h, only ~30% of the initial ^{125}I-EGF binding capacity remains and binding is reduced to an almost negligible level (<5%) after 24 h. By comparison, and as expected from previous studies (6, 9, 11), withdrawal of proliferating myoblasts from the cell cycle does not begin until 2–3 h after the medium switch and is completed within one generation time (12.5 h). Withdrawal is accompanied by a reduction in the percentage of uncommitted cells, such that after 12 h of exposure to LM, <10% of the nuclei become labeled during a 24-h period following mitogen restoration. Since a small number (3–5%) of uncommitted cells remain after 24 h of LM exposure, these cells could be responsible for the low residual level of EGF binding. If so, the differentiated myocytes (95–97% of cells in a 24-h population) would then be completely devoid of specific EGF binding capacity.

To determine whether a decline in EGF binding actually precedes the commitment decision, the early time courses of the two processes were analyzed in greater detail (Fig. 2 *inset*). Whereas no significant commitment was observed during the first 3 h, a decline in EGF binding was detected almost immediately after the medium switch. It thus appears that while there is a general correlation between the kinetics of withdrawal, commitment, and the decline in apparent EGF binding, the decline in binding is initiated prior to the onset of commitment and continues after all the myoblasts have committed to terminal differentiation. That the reduction in EGF binding is not simply a spurious result of switching cells to LM medium is shown below.

Analysis of Reduction in EGF-binding Capacity in Single Cells

The time course of EGF-receptor decrease was also analyzed at the level of individual cells. Cultures that had been exposed to LM medium for different lengths of time were incubated with ^{125}I-EGF, rinsed, and processed for autoradiography. The amount of ^{125}I-EGF bound by individual cells or myotubes was then determined by counting the number of grains located over each cell using a camera-lucida equipped microscope. Histograms depicting the relative frequency of cells containing different grain numbers were generated and the mean and standard deviation of the grain density distribution were determined (Fig. 3).

Although there is no a priori basis for associating a particular grain density with committed vs. uncommitted cells, the initial cells that exhibited a significant decrease in EGF binding are presumably those that were the first to commit to

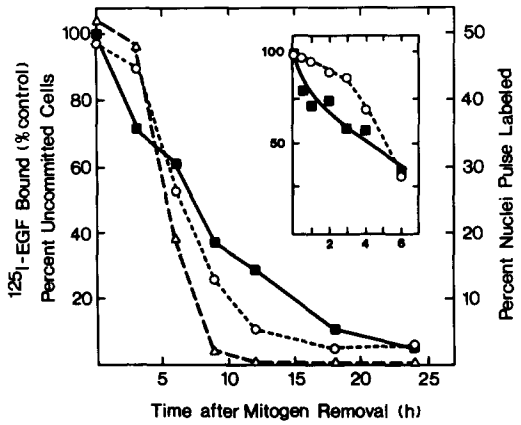

FIGURE 2 Temporal relationship between decline in EGF binding capacity, withdrawal from DNA synthesis, and commitment to terminal differentiation. Proliferating, high density MM14 cultures were rinsed 2 times with F10C and refed with medium containing 5% horse serum. At the times indicated, the apparent EGF binding capacity (■), pulse labeling index (△), and percentage of uncommitted cells (○) were determined as described in the text. The EGF binding capacity at each time point was normalized to cell number and expressed as a percentage of the 0-h value (cf. Fig. 1). Data represent an average of three experiments, with <12% variations from the mean. Myogenic fusion was not observed until 12–15 h after the medium switch. (*inset*) Early time course of the modulation in EGF binding capacity (■), and fraction of uncommitted cells (○) from an independent experiment.

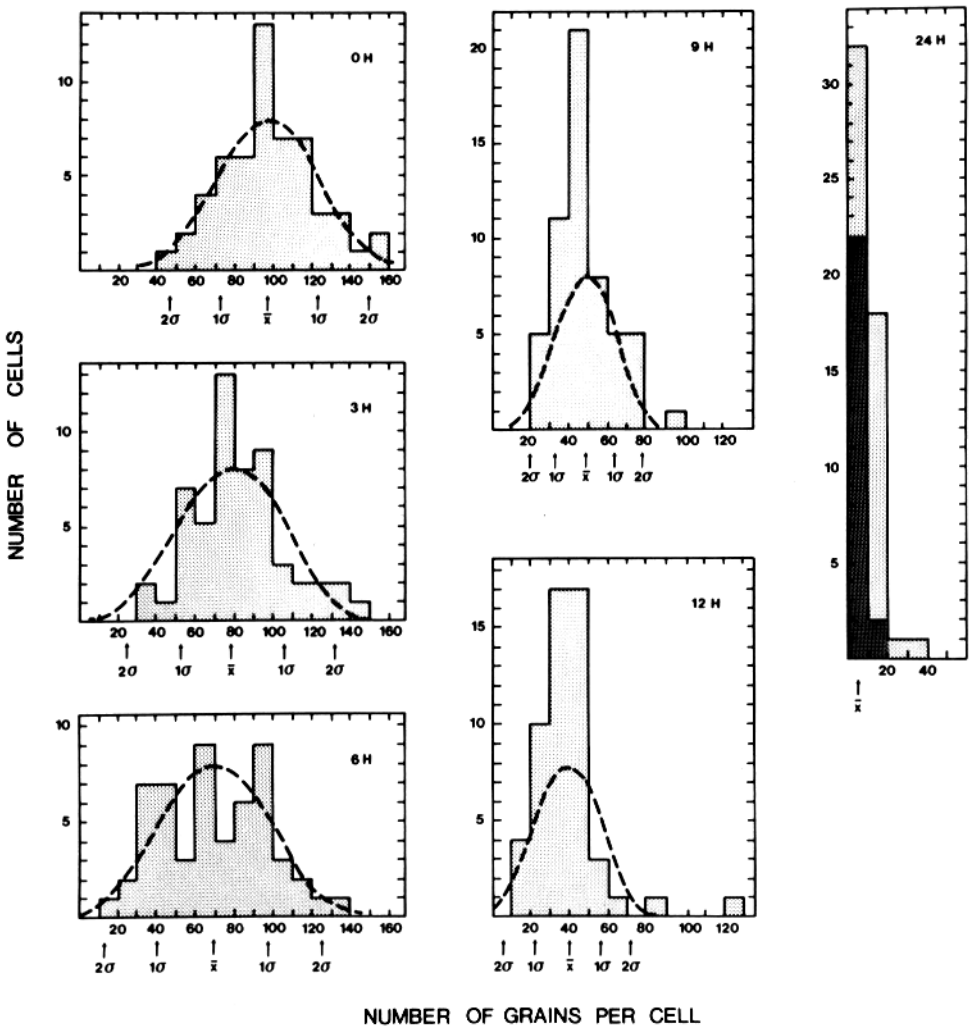

FIGURE 3 Autoradiographic analysis of the distribution in EGF-binding capacity among myoblast populations after increasing length of exposure to a low-mitogen medium. MM14 cultures were "induced" to initiate differentiation as described under Fig. 2. After the indicated length of exposure to LM medium (F10C plus 5% horse serum), cultures were incubated in ^{125}I-EGF, rinsed extensively, fixed and processed for autoradiography. EGF binding by individual cells was quantitated by counting the number of silver grains associated with each cell using a camera-lucida equipped microscope. For the 24-h time point, grains located above multinucleated syncitia were treated as though each nucleus contributed equally to the grain density. 55 cells were scored for each time point. The average grain number per cell ($\bar{x}$) was determined along with the standard deviation (σ) of the sample. These values were then used to derive a theoretical normal distribution curve (dotted line). Nonspecific binding, determined in the presence of 0.5 μg/ml unlabeled EGF accounted for an average of <10 grains per cell (see 24-h figure, darkly stippled data). Similar background binding was observed at the other time points. Because each data entry comes from a single cell, average nonspecific binding was not subtracted from the value entered.

terminal differentiation. If the minimum grain density exhibited by proliferating myoblasts at time zero (50 grains/cell, a value that is two standard deviations from the mean) is used as a cut-off value, it is evident that the proportion of cells with grain densities below this value increases at about the same rate as that of cells committing to terminal differentiation (Fig. 4).

Effect of Medium Composition on Myoblast Commitment and EGF Binding

Results from additional experiments indicated that the reduced EGF-binding capacity does not result from a nonspecific effect of lowering the serum level. Varying the serum concentrations from 0–15% in the absence of EE did not significantly affect either the extent of commitment to terminal differentiation or the magnitude of the decline in EGF binding. Furthermore, the addition of nanogram amounts of fibroblast growth factor inhibited commitment and substituted for EE in preventing the decline in EGF binding (see below). The effect of EE depletion on EGF binding thus appears to be related to the absence of specific factors that repressed myogenic differentiation.

This conclusion is strengthened by the observation that the decline in EGF binding occurred in parallel with myogenic differentiation even when cultures were permitted to differentiate on their own without a medium switch. For example, while 2-d-old proliferating myoblasts could bind substantial amounts of ^{125}I-EGF (Fig. 5*a*), the binding capacity became much reduced in a 4-d-old differentiated culture: the extent of binding by multinucleated myotubes was virtually indistinguishable from background (Fig. 5*b*).

Irreversibility of the Decline in ^{125}I-EGF Binding

Another approach to establishing a correlation between differentiation and reduced EGF binding is to examine the reversibility of the change in EGF binding. Whereas a truly differentiation-related event is likely to be permanent, changes that are dependent merely on environmental conditions should be reversed by restoring the initial environment (17–20). When differentiated MM14 cultures were refed with mitogen rich medium, their EGF binding capacity was not restored. Even 24 h after refeeding, EGF binding remained indistinguishable from the low level exhibited by cultures maintained throughout the experiment in LM medium.

Commitment to Terminal Differentiation Is Coincident with Commitment to an Irreversible Decrease in EGF-binding Capacity

To determine whether the decline in EGF binding becomes irreversible with the same time course as myogenic commitment, clonal density cultures of proliferating myoblasts were

exposed to LM medium for various time intervals (3–12 h) before refeeding with FM. 24 h after the initial switch to LM, the EGF binding capacity of individual cells in these cultures was determined autoradiographically. Since each culture had at least 12 h to recover from exposure to LM before EGF binding was assayed, cells that had simply modulated their binding should have had ample time to recover their binding capacity, while cells that had been "triggered" to reduce their EGF binding capacity permanently should bind very low levels of ^{125}I-EGF. The rate of appearance of such EGFR-negative cells can then be compared with the time course of cell-cycle withdrawal and commitment to differentiation (Fig. 6). Within 6 h of exposure to LM, 40–60% of the population had committed to terminal differentiation. With the same length of LM exposure, 30–50% of the myoblast population were "committed" to a permanent reduction of their EGF binding capacity and bound little or no EGF when assayed 18 h afterwards. The time course of "commitment" to a permanently reduced EGF binding capacity therefore appears to correlate closely with the time course of commitment to differentiation.

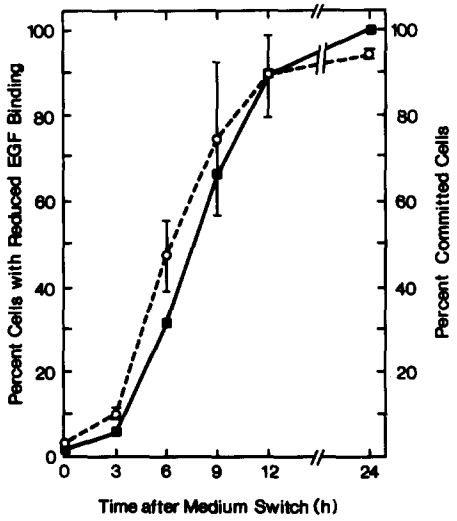

FIGURE 4 Comparison between the kinetics of commitment to terminal differentiation and appearance of cells with reduced EGF binding capacity. Using data from Fig. 3, the fraction of cells with EGF binding capacity falling below two standard deviations (i.e. <50 grains/cell) of the mean binding capacity of proliferating myoblasts (97.6 grains/cell) was determined and expressed as a function of time in LM medium (5% horse serum). Data for the commitment kinetics were obtained from Fig. 2 and represent the average of three experiments with standard errors as indicated. (■) EGF binding, (○) percentage of cells committed.

EGF Binding by Mitotically Synchronized Myoblasts

The data presented above do not indicate whether the permanent loss of EGF binding capacity is related to differentiation per se or simply to the cessation of proliferation and the resulting accumulation of cells in G_1. Evidence suggesting that the irreversible decline in EGF binding is linked to the differentiation process has been obtained from several approaches.

One approach was to examine the EGF binding capacity of G_1-synchronized undifferentiated myoblasts. Mitotic MM14 cells collected by a shake-off technique were replated into either FM or mitogen-depleted CHS medium and EGF binding was assayed 2.5 h later using the quantitative autoradiographic technique described above. Regardless of whether the mitotic cells were seeded into FM or CHS, a high level of EGF binding was observed (Fig. 7*a*). Since <10% of the cells

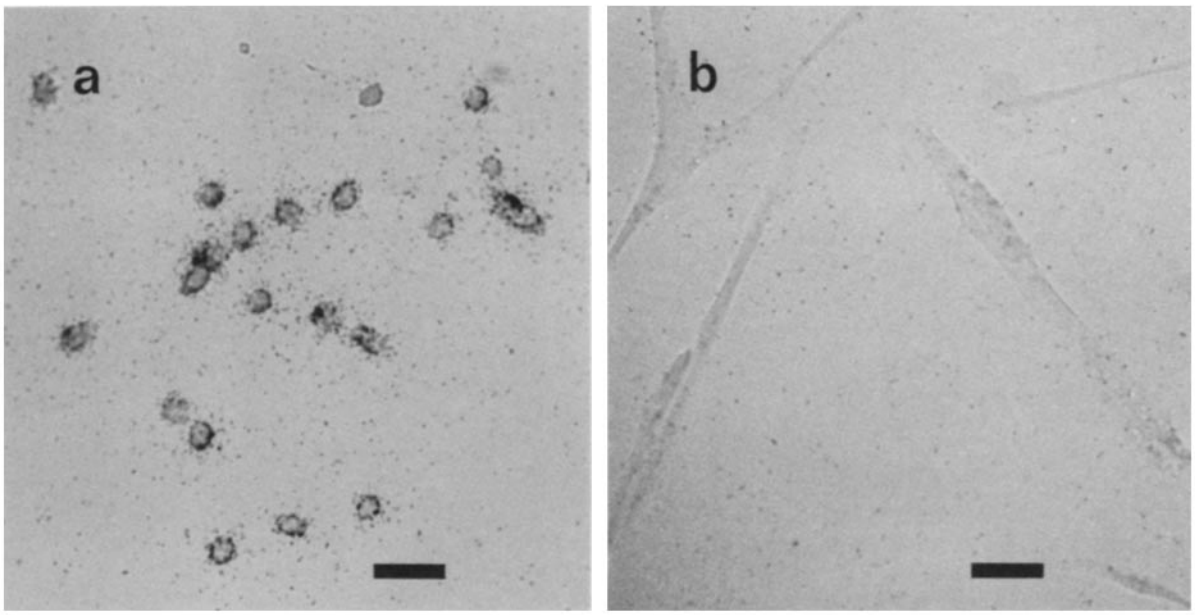

FIGURE 5 Comparison of ^{125}I-EGF binding to muscle cells before and after differentiation. 1 × 10^4 MM14 myoblasts were seeded into 60-mm dishes containing 3 ml FM. Cultures were permitted to differentiate without any medium changes. 2 and 4 d after seeding, cultures were rinsed and incubated with ^{125}I-EGF at 37°C for 1 h. After removing unbound ^{125}I-EGF, cultures were fixed and processed for autoradiography. To emphasize the contrast between EGF binding in proliferating vs. differentiated cells, autoradiographs were deliberately over-exposed, thus raising the frequency of background grains. Shorter exposure times were used when quantitative grain counts were desired. (*a*) 2-d-old culture consisting mainly of proliferating, mononucleated cells. (*b*) 4-d-old culture consisting mainly of multinucleated syncitia and committed single cells. Bar, 40 μM. × 250.

added to parallel plates containing [^{3}H]TdR became labeled during this period (Fig. 7*b*), the majority of the population was presumably still in G_1. In addition, since >90% of the

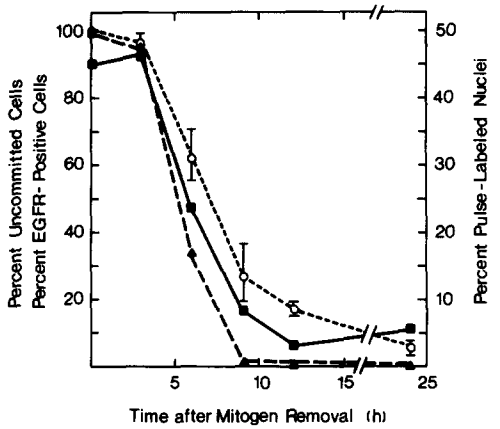

FIGURE 6 Commitment to an irreversible reduction in EGF-binding capacity. Clonal density cultures of MM14 myoblasts (100 colonies per 60-mm dish) were rinsed and exposed to LM medium (5% horse serum) for various time intervals. At the times indicated, one set of cultures was refed with complete growth medium and the ability to bind ^{125}I-EGF was assayed 24 h after the initial switch to LM. After removal of the unbound ^{125}I-EGF, the plates were processed for autoradiography and the percentage of cells retaining EGF-binding capacity was determined. Parallel cultures were either pulse-labeled with [^{3}H]TdR at the time of the feedback to measure the extent of cell cycle withdrawal or labeled continuously with [^{3}H]TdR after the feedback to detect cells that had not yet committed to terminal differentiation. (○) EGFR positive cells; (▲) pulse labeling index; (■) percentage uncommitted cells. Data represent results from two separate experiments. The range of variation for percent EGFR positive cells is indicated. Less than 10% variation was observed for the pulse-labeling index and for the percent uncommitted cells from experiment to experiment.

mitotically synchronized myoblasts incorporated [^{3}H]TdR after refeeding with FM (Fig. 7*c*), most of them were also not committed to terminal differentiation during the 2.5-h period. By comparison, when the assay was performed 20 h after seeding, myoblasts exposed to CHS exhibited a much reduced EGF-binding capacity, while that of myoblasts seeded into FM remained high (Fig. 7*a*). Furthermore, whereas ~100% of the mitotic cells seeded into FM re-entered S and resumed active proliferation, most cells seeded into CHS remained unlabeled (Fig. 7*b*), and were apparently committed to terminal differentiation (Fig. 7*c*). Thus a decline in EGF binding was observed only when myoblasts became committed to differentiation but not during the normal G_1 phase of a proliferative cycle. This finding was consistent with earlier data indicating that the entire population of log phase myoblasts could bind ^{125}I-EGF (cf. Fig. 3).

Effect of FGF on the Reduction of EGF-binding Capacity

Even though G_1 myoblasts bound EGF, the decline in binding capacity following mitogen depletion might still be an intrinsic property of myoblasts arrested in a quiescent (G_0) state, rather than the result of a differentiation process. This possibility was investigated by examining the EGF-binding capacity of MM14 myoblasts under conditions that allowed them to become quiescent while remaining undifferentiated. Previous studies have shown that when myoblasts are exposed to medium containing low levels of serum and optimal levels of FGF, the cells fail to proliferate, but the presence of FGF prevents commitment to differentiation (9). Proliferating MM14 cultures were thus exposed to medium containing either 15 or 2% horse serum with or without FGF and their ability to bind EGF was compared. Since high density cultures rapidly deplete FGF from the growth medium (7), the assays

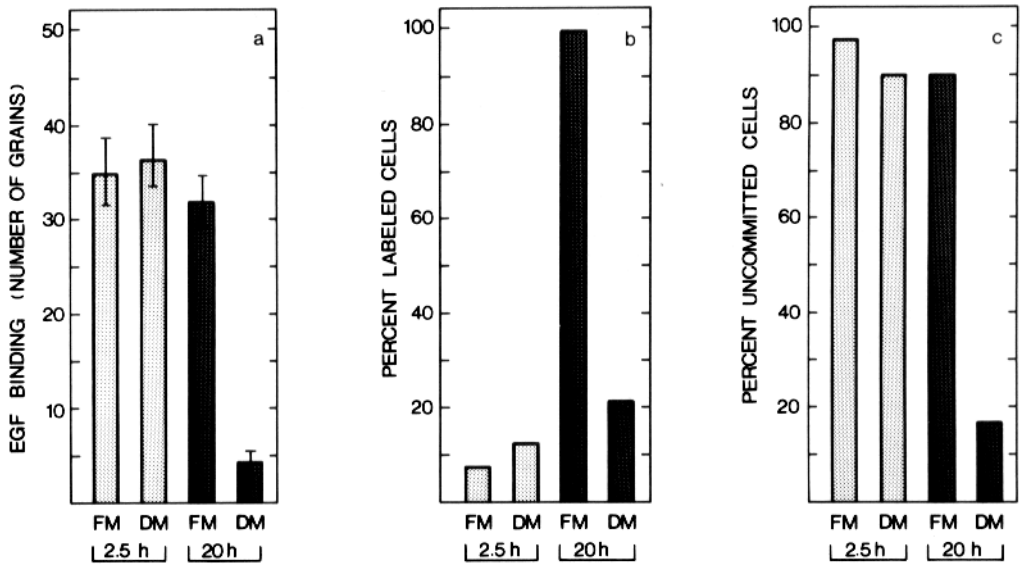

FIGURE 7 Comparison of the EGF binding capacity of MM14 myoblasts during a proliferative G_1 state vs. the committed G_0 state. Mitotically synchronized populations of MM14 myoblasts obtained from proliferating cultures using a shake-off technique were plated onto a small region of a gelatin-coated plate containing either complete growth medium (*FM*), or depleted medium (*DM*) (7.5% CHS). (*a*) Apparent EGF binding capacity was determined either 2.5 or 20 h after the cells were seeded and quantitated using autoradiographic techniques as described under Fig. 3. An average nonspecific background of ~10 grains per cell has been subtracted. (*b*) Entry into S was determined by seeding cells into [^{3}H]TdR-containing medium and fixing plates at the indicated times. (*c*) Cells that had not yet committed to terminal differentiation at the time of the EGF assay were detected by feeding back with growth medium containing [^{3}H]TdR.

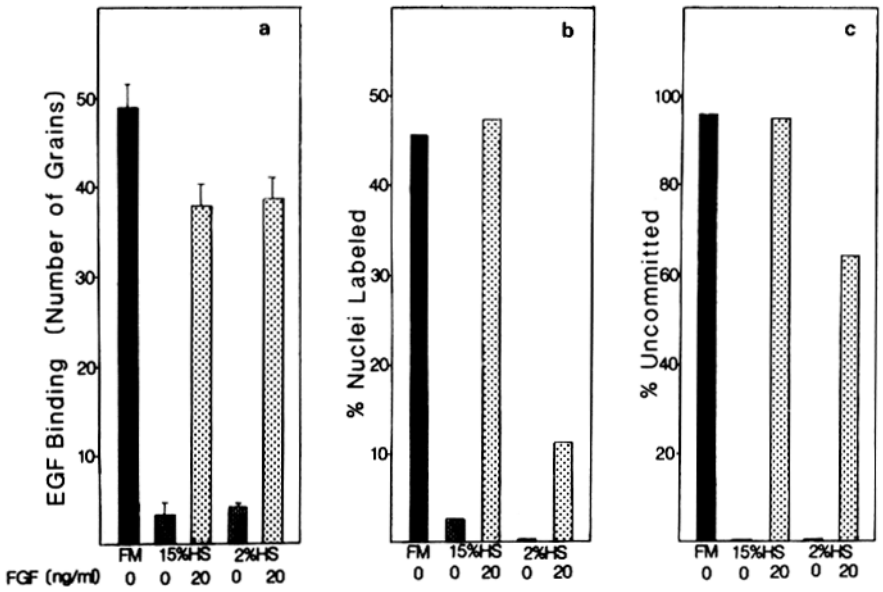

FIGURE 8 FGF effect on commitment and the decline in EGF binding. 2-d-old clonal density cultures (~16 cells per colony) of MM14 myoblasts were rinsed and switched to medium containing either 2 or 15% horse serum (*HS*) with or without FGF supplement (20 ng/ml). (*a*) EGF binding capacity; (*b*) pulse labeling index; and (*c*) fraction of uncommitted cells were determined 28 h after the medium switch. EGF binding was analyzed autoradiographically as described under Fig. 3. An average nonspecific background of about five grains per cell has been subtracted.

were performed using 2-d-old clonal density cultures and binding was quantitated autoradiographically. Parallel cultures were examined for cell-cycle withdrawal and commitment to differentiation.

As expected, 28 h after exposure to medium containing either 15 or 2% horse serum without FGF, EGF-binding capacity was greatly reduced (Fig. 8*a*). Almost all myoblasts withdrew from the cell cycle (Fig. 8*b*) and committed to terminal differentiation (Fig. 8*c*). Also as expected, supplementing 15% horse serum with 20 ng/ml of FGF allowed myoblasts to continue cycling (Fig. 8*b*), inhibited their commitment to terminal differentiation (Fig. 8*c*), and maintained EGF binding at a high level (Fig. 8*a*). In contrast, when FGF was added to medium containing only 2% horse serum, the pulse-labeling index declined from ~50 to 10% indicating that most myoblasts were accumulating in a G_1 or G_0 state (Fig. 8*b*). However, the majority of the myoblasts were still prevented from committing to differentiation (Fig. 8*c*) and the EGF-binding capacity of this mostly quiescent and uncommitted population remained at a fairly high level (Fig. 8*a*). In fact, even though ~35% of the cells had committed to differentiation (presumably because FGF was depleted too rapidly for its repression of commitment to persist over the 28-h exposure), the average EGF-binding capacity of the entire population remained at ~80% of the level observed in actively proliferating myoblasts maintained in FM. The high level of EGF binding observed in this quiescent but predominantly uncommitted myoblast culture suggests that the large decline in EGF binding activity observed following specific mitogen removal is indeed associated with commitment rather than with a reversible cell-cycle arrest.

Possible Mechanisms Involved in the Decrease in Apparent EGF-binding Capacity during Muscle Differentiation

Results from the foregoing experiments indicated that proliferating myoblasts bind high levels of EGF whereas fully differentiated myocytes and myotubes bind essentially no EGF. Since the EGF binding capacity (observed at 37°C) reflects the binding to cell surface receptors as well as EGF internalization and degradation (16–18), several experiments were carried out to determine whether changes in one or more of these processes could be correlated with the rapid decline in apparent EGF binding associated with muscle differentiation. In all of the experiments reported below, the behavior of proliferating myoblasts was compared with differentiating cultures that had been exposed to LM medium for 16 h. This exposure period was selected so as to provide cultures in which "all" of the cells had committed to terminal differentiation while most of the myocytes would still retain sufficient EGF binding capacity to permit EGFR analysis.

Initial experiments involving comparisons of EGF-binding kinetics disclosed no differences between proliferating myoblasts and differentiating cells. Cell-associated radioactivity reached maximal levels 40–60 min after ^{125}I-EGF addition and was followed by a decline that continued for the next 2 h. Similar decreases in cell-associated ^{125}I-EGF had been observed in many nonmuscle cell types and are believed to be caused by the degradation and release of internalized EGF-EGFR complexes (17, 18, 21).

Internalization of ^{125}I-EGF can be demonstrated directly by measuring the amount of radioactivity resistant to acid extraction in cultures incubated for brief periods with ^{125}I-EGF (Fig. 9). Regardless of whether MM14 cells were proliferating or committed, 60–70% of the total cell-associated radioactivity became resistant to acid extraction within the first 3 min after ^{125}I-EGF addition. Since extraction with acidic buffer removes surface-bound EGF without affecting internalized ligands (15) the size of the acid-resistant component reflects the extent of ^{125}I-EGF internalization. The data indicate that both proliferating and committed MM14 cells could internalize bound-EGF at very rapid rates.

To determine whether the decrease in apparent EGF-binding capacity of committed myocytes is due to an alteration in the degradative process, the time course of EGF binding by proliferating and committed cells was reexamined in the presence of 10 mM ammonium chloride, an inhibitor of lysosomal degradation of internalized ^{125}I-EGF (17, 21). In contrast to what was observed in the absence of NH_4Cl, the amount of cell-associated radioactivity continued to increase with increased length of EGF incubation (Fig. 10). Since the amount of radioactivity accumulating within the committed myocytes remained lower than that in proliferating myoblasts,

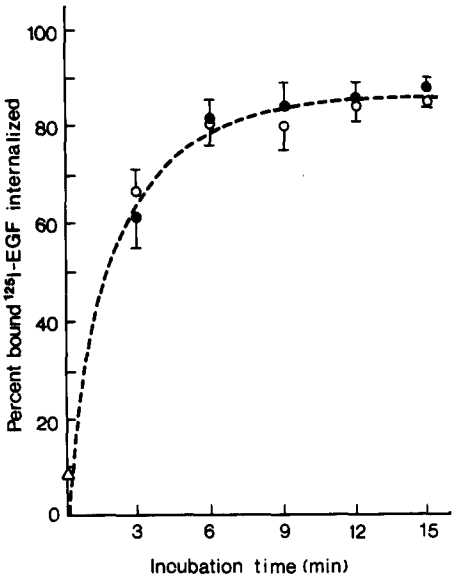

FIGURE 9 Internalization of bound ^{125}I-EGF by proliferating and differentiating MM14 cells. Proliferating and differentiating MM14 cultures were incubated at 37°C with ^{125}I-EGF for the indicated times. After removal of unbound EGF, the cultures were extracted and quickly rinsed once with a pH 2.5 saline at 4°C. Radioactivity in the acid-extract and that remaining associated with the cell layer were quantified separately and the percentage of acid-resistent (internalized) radioactivity was determined. Data represents an average of three independent experiments in which the cell density and amount of ^{125}I-EGF added were slightly varied. In a typical experiment, the total specific binding at 15 min was 3,000 and 600 cpm for proliferating myoblasts and committed myocytes respectively. ●, Proliferating myoblasts; ○, committed myocytes. The time zero estimate (△) represents the fraction of acid-resistant radioactivity associating with cells after a 2-h incubation with EGF at 4°C.

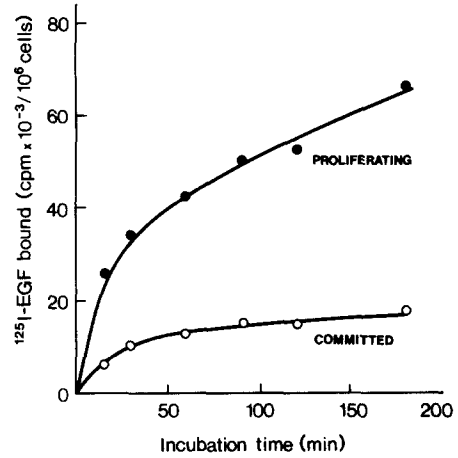

FIGURE 10 Effect of NH_4Cl on the time course of EGF binding by proliferating and differentiating MM14 cells. Proliferating and differentiating MM14 cultures were incubated with 10 mM NH_4Cl for 20 min at 37°C. ^{125}I-EGF was then added and the cells were incubated in the presence of NH_4Cl for the indicated times. The amount of EGF specifically bound and accumulated by each culture was determined as described in the text and normalized to the cell number determined on parallel plates. ●, myoblasts; ○, myocytes. Data were obtained from duplicate determinations with variations generally <15% between samples.

the decrease in apparent EGF binding following exposure to LM medium was not due to a change in the rate at which internalized EGF is degraded.

An alternative mechanism by which cells could reduce their apparent EGF-binding capacity is the production and secretion of molecules that compete with EGF binding to EGFR (22). If committed MM14 cells produce such molecules, then co-culturing committed with uncommitted cells might inhibit the latter from binding EGF. However, as indicated in Fig. 6, EGFR-positive and -negative cells can co-exist in the same colony, and analysis of the percentage of each cell class indicated that they are present at the same ratio as that of uncommitted vs. committed cells. Thus committed cells do not affect the EGF-binding capacity of adjacent noncommitted myoblasts.

DISCUSSION

Data presented in this study demonstrate that MM14 myoblasts regulate their EGF-binding capacity in a novel manner. When the principal mitogenic components (EE or FGF) are removed, the ability of myoblasts to bind EGF declines rapidly to <5% of the original level (Figs. 2, 3, and 8). This decline becomes irreversible as soon as myoblasts commit to terminal differentiation (Fig. 6). A similar decline in FGFR could be a causal element in the process of commitment. The data presented above also suggest that the decline in apparent EGF binding capacity was not due to changes in EGF binding affinity (Fig. 1) or to differences in internalization and degradation of bound EGF (Figs. 9, 10). In addition, since EGFR-positive and -negative cells can co-exist in the same colony (Fig. 6), involvement of a diffusible inhibitor of EGF binding seems unlikely. Instead, the decrease in EGF binding appears to involve a reduction in the number of available cell surface receptors. Such a decline in EGFR availability could result from altered rates of receptor synthesis, degradation, or transport to the cell surface.

The loss of EGF-binding capacity in terminally differentiating MM14 cells is not a cell cycle phenomenon (Fig. 7) or a general feature of the quiescent state (Fig. 8). Furthermore, since EGF-binding capacity is reduced at the same time that differentiating myocytes are rapidly accumulating muscle-specific gene products (including such cell surface molecules as acetylcholine receptors), the decrease in EGFR could not be due to a generalized effect on protein synthesis or on cell surface assembly. Finally, since myogenic fusion is not detected until 12–14 h following mitogen-depletion (6, 7, 11), when EGF binding is already substantially reduced, the decline in binding is unlikely to be a secondary effect of fusion-related cell surface changes (23–26).

Instead, the cumulative data support a correlation between the reduction in EGF binding and commitment to terminal differentiation, (Figs. 4–8), and suggest that both are triggered by the depletion of specific mitogens (Figs. 2 and 8). the commitment decision is correlated temporally to an EGF-binding capacity that is >2 standard deviations below the average binding capacity of proliferating cultures (Fig. 4). Although it is not clear whether this observation has any causative implications, data from nearly 20 separate experiments indicate that this empirical rule is always obeyed. Since myoblasts "commit" to a permanent reduction of EGF binding with the same kinetics as observed for their commitment to terminal differentiation (Fig. 6), the irreversible loss of EGF binding capacity appears to be an early step in the terminal differentiation process.

Such differentiation-related changes in EGF-binding capac-

ity are not unique to MM14 cells: a similar decrease occurs in differentiating primary cultures of mouse and human myoblasts (unpublished data). Other differentiating cell types also exhibit changes in EGFR. For example, exposure of teratocarcinoma cells to retinoic acid results in the formation of cells with endodermal properties and the simultaneous acquisition of EGF-binding activity and EGF-responsiveness (27). In contrast, addition of nerve growth factor to PC12 pheochromocytoma cells causes neurite formation and a decline in both EGF receptors and EGF responsiveness (28).

Since MM14 myoblasts do not proliferate in response to EGF, the functional implication of a reduced EGF binding capacity in differentiated muscle cells is not clear. However, EGF has been shown to regulate general metabolic (29) and developmental states (30, 31) of many cell types without stimulating a proliferative response. The EGFR of MM14 myoblasts may be involved in mediating similar hormonal functions. Thus, the reduced EGF binding may represent part of a coordinated change in the capacity of differentiating muscle cells to interact with their hormonal environment. The increase in β-adrenergic and insulin receptors during muscle differentiation is consistent with such a possibility (32, 33).

Another possibility is that the decline in EGF binding reflects a general reduction in the availability of mitogen receptors. Evidence from other studies with a differentiation-defective variant of the MM14 line (Lim, R. W., and S. D. Hauschka, submitted manuscript) suggests that a functional EGF response pathway could be present in MM14 myoblasts, and that its detection is masked by the rapid commitment to terminal differentiation and concomitant loss of EGF receptors (cf. Fig. 2).

If receptors for FGF and other mitogens are indeed regulated in a manner analogous to that of the EGF receptors, a large decline in binding capacity during muscle differentiation would presumably restrict the ability of differentiating muscle cells to respond to these mitogens. It is tempting to speculate that a diminished mitogen responsiveness due to reduction in mitogen binding capacity may be a means of insuring that terminally differentiated cells are prevented from resuming proliferation. During in vivo muscle development, temporal and spatial variations in mitogen levels as well as the distribution of myoblast types (35, 36) differing in mitogen-receptor regulation could be important factors in determining when and where muscle differentiation takes place.

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