

# Control of Differentiation in BC<sub>3</sub>H1 Muscle Cells

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BC<sub>3</sub>H1 is a cell line that undergoes a musclelike pattern of differentiation under the appropriate conditions. We have examined the control of the synthesis of proteins characteristic of differentiated muscle in these cells as a function of their position in the cell cycle. We define two positions in the cell cycle where BC<sub>3</sub>H1 cells can remain stably quiescent. G<sub>1d</sub> is a restriction point early in the G<sub>1</sub> portion of the cell cycle that permits the synthesis of muscle-specific proteins and is probably identical to G<sub>0</sub>. The second restriction point, G<sub>1q</sub>, occurs approximately 4 hr later in the G<sub>1</sub> portion of the cell cycle and does not permit the synthesis of muscle-specific proteins. Movement of the cells from G<sub>1d</sub> to G<sub>1q</sub> occurs when fibroblast growth factor is added to the cells and is reversed when this growth factor is removed. Repression of the synthesis of muscle-specific proteins occurs when fibroblast growth factor is added to cells in G<sub>1d</sub>. In the case of the muscle form of creatine phosphokinase (M-CPK), the decline in the rate synthesis of this protein is a consequence of a decreased level of its mRNA. By contrast, the repression of  $\alpha$ -actin synthesis, a protein synthesized only in differentiated cells, appears to be controlled at the translational level. The effect of fibroblast growth factor and other mitogens in these cells require activation of tyrosine kinase(s), but the intracellular targets of these kinases are not known. Studies by others suggest that activation of the *ras* oncogene can mimic the action of mitogenic polypeptides on these and other muscle cells. However, these observations do not prove that activation of the *ras* protooncogene is directly responsible for the effect of mitogenic polypeptides on these cells.

## Introduction

The control of cell differentiation is one of the central problems of modern biology. Studies of muscle differentiation have been at the forefront of this field because of the availability of defined (often cloned) cells that can be induced to differentiate under controlled conditions in the laboratory.

It is frequently true that the study of slightly abnormal cells, in which the pathway of differentiation has been partially altered, provides unique insights into the normal sequence of events. The BC<sub>3</sub>H1 cell line (1) is a nonfusing muscle cell line that has been particularly useful in this regard. These cells do not fuse, but under appropriate conditions, differentiate. Differentiation is defined here as a large increase in the rate of synthesis of proteins characteristic of mature muscle such as M-CPK (1-4), vascular smooth muscle  $\alpha$ -actin ( $\alpha$ -actin) (5,6), and the nicotinic acetylcholine receptor (AChR) (1,7,8) (Fig. 1). The advan-

tage of the BC<sub>3</sub>H1 cell line is that the differentiation process can be reversed by the addition of serum or well-characterized mitogenic polypeptides such as fibroblast growth factor (FGF) (4,6,9-11) (Fig. 1). We summarize here our current knowledge derived from work in a number of laboratories in addition to our own of this process and speculate on the broader implications of this work for our understanding of muscle differentiation.

## Differentiation of BC<sub>3</sub>H1 Cells

Figure 2 provides a graphic description of our current knowledge of differentiation in BC<sub>3</sub>H1 cells. The experimental observations on which this is based are found in the original publications (4,6,9) and will only be mentioned briefly. In Figure 2 we show the cell cycle of eukaryotic cells, and we identify two restriction points at which cells can remain quiescent and viable, both in the G<sub>1</sub> portion of the cell cycle. G<sub>1d</sub> is equivalent to G<sub>0</sub> (12) and represents a restriction point permissive for cell differentiation, while G<sub>1q</sub>, 4 to 6 hr further in the G<sub>1</sub> phase of the cell cycle, is nonpermissive for cell differentiation (6,9).

When exponentially growing BC<sub>3</sub>H1 cells that are at a cell density high enough to allow extensive cell-cell contact are transferred from a medium containing high concentrations of serum to a medium containing low levels

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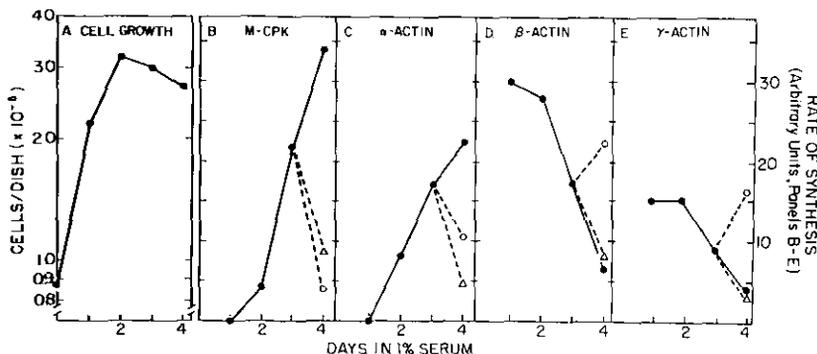


FIGURE 1. Relationship between cell growth and differentiation in BC<sub>3</sub>H1 cells. Logarithmically growing BC<sub>3</sub>H1 cells in 35-mm dishes were transferred from D-MEM containing 20% FCS to D-MEM containing 1% FCS on day 0. Cells were harvested at times indicated. (●) Cells in 1% FCS; (○) cells to which 20% FCS was added on day 3; (△) cells to which FGF was added on day 3. Cell number was determined using a Coulter counter. The rate of M-CPK synthesis (B) was measured essentially as described (4,9). Briefly, cells were labeled for 1.5 hr with <sup>35</sup>S-methionine and CPK was immunoprecipitated from cell homogenates. The muscle and brain isozymes were separated by SDS-gel electrophoresis. After fluorography of the gels, the amount of <sup>35</sup>S-CPK was quantitated by densitometry and normalized to the rate of total protein synthesis. The rate of isoactin synthesis (C-E) was determined as described (6). Briefly, cells were labeled for 1 hr with <sup>35</sup>S-methionine and the actins were purified from cell homogenates using DNase I affinity chromatography. The <sup>35</sup>S-actins were then subjected to isoelectric focusing to separate the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -isoforms or to SDS-gel electrophoresis to determine the amount of total <sup>35</sup>S-actin. The amount of <sup>35</sup>S-actin in the gels was quantitated as for M-CPK.

of serum, they arrest in G<sub>1d</sub> and initiate a differentiation program that results in the accumulation of high levels of M-CPK (1-4), AChR (1,7,9,13), and  $\alpha$ -actin (6), while the rate of synthesis of nonmuscle  $\beta$ - and  $\gamma$ -actin is decreased (6) (Fig. 1). In the case of M-CPK and  $\alpha$ -actin, it is known that their expression in differentiated cells requires the

synthesis of mRNAs absent in logarithmically growing cells (5,14); hence, induction of differentiation involves transcriptional control of these genes. The difference in the level of these proteins between growing and differentiating cells can be several hundredfold (Fig. 1).

When high concentrations of serum are added to differentiated BC<sub>3</sub>H1 cells, the cells reinitiate growth (3-11). Concomitantly, the synthesis of muscle-specific proteins ceases (3-11), and the cells initiate the synthesis of proteins characteristic of growing cells, for example  $\beta$  and  $\gamma$  actin (5,6) (Fig. 1). Serum is a complex mixture of molecules, and it is impossible to ascertain whether the effects of serum on the differentiated phenotype are due to a single effector molecule or whether multiple components are involved. As will be shown, the multiple components are probably involved.

Since the inhibition of the differentiation program occurs when cell growth resumes, it is tempting to examine known mitogenic polypeptides for their ability to influence growth and differentiation in BC<sub>3</sub>H1 cells. Lathrop et al. could show that either highly purified acidic FGF (aFGF) or relatively crude basic FGF (bFGF) when added to differentiated BC<sub>3</sub>H1 cells blocked expression of M-CPK (4). However, the addition of these polypeptides to cells initially in G<sub>1d</sub> was not sufficient to initiate cell growth (6,9). The  $\beta$ -transforming growth factor ( $\beta$ -TGF) affects differentiation of BC<sub>3</sub>H1 cells similarly to FGF (13). More recent observations suggest that in some subpopulations or clone(s) of BC<sub>3</sub>H1 cells, epidermal growth factor (EGF) has a similar effect on the differentiation program as FGF but EGF allows differentiated BC<sub>3</sub>H1 cells to reinitiate growth (15).

A variety of observations indicate that addition of FGF to BC<sub>3</sub>H1 cells in G<sub>1d</sub> results in movement of these cells from G<sub>1d</sub> to a new restriction point G<sub>1q</sub> about 4 hr closer to the G<sub>1</sub>-S boundary than G<sub>1d</sub>. These observations can be summarized as follows: a) when stimulated to divide by addition of serum, cells in G<sub>1q</sub> enter the S phase of the

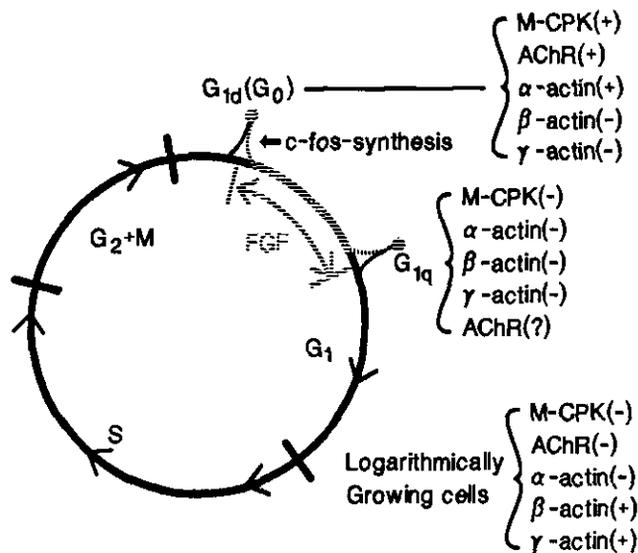


FIGURE 2. Differentiation and position in the cell cycle in BC<sub>3</sub>H1 cells. The figure illustrates in diagrammatic fashion the control of differentiation in BC<sub>3</sub>H1 cells and the induction of *c-fos*. Cells arrested early in the G<sub>1</sub> portion of the cell cycle (G<sub>1d</sub> or G<sub>0</sub>) differentiate while cells arrested later in G<sub>1</sub> at G<sub>1q</sub> are quiescent but cannot differentiate. Movement from G<sub>1d</sub> to G<sub>1q</sub> can be induced by addition of FGF and is reversed upon removal of FGF. Transit from G<sub>1d</sub> to G<sub>1q</sub> is accompanied by induction of a number of proteins characteristic of early portion of G<sub>1</sub>, the figure illustrates the induction of *c-fos*. Synthesis (+) or lack of synthesis (-) of the indicated protein. AChR (?) indicates that the effects of FGF on the synthesis of AChR has not been reported for BC<sub>3</sub>H1 cells, although  $\beta$ -TGF has been reported to inhibit the cell surface expression of AChR in these cells (13).

cell cycle 4 to 6 hr before cells in G<sub>1d</sub> (6,9); b) addition of FGF to cells in G<sub>1d</sub> results in a rapid and transient induction of the synthesis of the *c-fos* protooncogene characteristic of cells progressing from G<sub>1d</sub> (G<sub>0</sub>) to G<sub>1</sub> (6); c) addition of FGF to cells in G<sub>1d</sub> results in significant induction of the synthesis of ornithine decarboxylase (9), which is characteristic of cells transiting through early parts of G<sub>1</sub> (16); and d) the transit of cells from G<sub>1d</sub> to G<sub>1q</sub> requires 4 to 6 hr of exposure to FGF (9), although it seems likely that as soon as cells exit G<sub>1d</sub> they are no longer permissive for the synthesis of differentiated proteins.

The levels of control of the synthesis of various proteins associated with the differentiated phenotype appears to be different (Table 1). When cells exit from G<sub>1d</sub>, synthesis of M-CPK mRNA ceases (6,10) (Fig. 3), the message decays with a half-time of about 8 hr (independent of whether cells are in G<sub>1d</sub> or not, (10), and there is a corresponding decline in the rate of M-CPK synthesis (9). The rate of  $\alpha$ -actin synthesis also declines when cells exit G<sub>1d</sub> (5,6); however, this rate of synthesis is not a reflection of levels of  $\alpha$ -actin mRNA, which remain relatively high (6) (Fig. 3, Table 1). This is true whether the exit from G<sub>1d</sub> is induced by addition of FGF, which brings cells to G<sub>1q</sub> (6), or by addition of EGF or serum, which, in appropriate EGF responsive clones, allows cells to continue through the cell cycle (15). These observations suggest that translational control of  $\alpha$ -actin synthesis is possible under physiological conditions.

Translational control of the synthesis of muscle specific proteins has also been noted in other systems. In the absence of Ca<sup>2+</sup>, rat muscle cells fail to synthesize muscle-specific proteins in spite of the presence of appropriate mRNAs (17), but Ca<sup>2+</sup>-deprived avian or calf skeletal muscle myoblasts accumulate normal levels of muscle-specific proteins (17-21).

BC<sub>3</sub>H1 cells arrested in G<sub>1d</sub> show decreased rates of synthesis of  $\beta$  and  $\gamma$ -actin relative to growing cells (5,6), and the addition of FGF to such cells does not increase the rate of  $\beta$  and  $\gamma$  actin synthesis (6). In fact, the rate of  $\beta$  and  $\gamma$  actin synthesis in FGF-treated cells continues to decrease at the same rate as in differentiating cells which

remain in G<sub>1d</sub> (6) (Fig. 1). Addition of serum to cells in G<sub>1d</sub> does result, as expected, in a rapid increase in the rate of synthesis of  $\beta$  and  $\gamma$  actins. However, as discussed in Wice et al. (6), factors in serum affect the rate of synthesis of these proteins independent of position in the cell cycle.

Removal of FGF from the media of cells arrested in G<sub>1q</sub> allows the cells to return to G<sub>1d</sub> without going through the full cell cycle (9). Thus, transit from G<sub>1d</sub> to G<sub>1q</sub> is fully reversible, and, at least for these cells, induction of the differentiation program does not require a terminal cycle of DNA synthesis (22).

## Intracellular Signals that Control Differentiation of BC<sub>3</sub>H1 Cells

The receptor for a number of mitogenic polypeptides are tyrosine-specific protein kinases, and the FGF receptor appears to fall into this category as well (23). To assess whether the phosphorylation of proteins on tyrosine residues was important for the control of cell differentiation, we examined the effects of vanadate, an inhibitor of tyrosine-specific protein phosphatases (24-27) on differentiated BC<sub>3</sub>H1 cells. The effects of vanadate on cells are complex and results obtained with this compound can only be suggestive but not conclusive of an involvement of tyrosine phosphorylation (6). The experimental obser-

Table 1. Control of synthesis of muscle proteins by mitogens.<sup>a</sup>

Hour after addition of mitogen	Rate of $\alpha$ -actin synthesis, %	Relative level of $\alpha$ -actin mRNA, %	Relative level of M-CPK mRNA, %
0	100	100	100
4	86	—	80
8	71	127	55
12	29	—	40
24	29	105	—

<sup>a</sup>The table summarizes data on  $\alpha$ -actin synthesis from Wice, Milbrandt, and Glaser (6) and of M-CPK synthesis from Spizz et al. (10). Note that after addition of FGF to differentiated BC<sub>3</sub>H1 cells the rate of  $\alpha$ -actin synthesis, measured by incorporation of <sup>35</sup>S-methionine, decreases while no corresponding decrease occurs in the level of  $\alpha$ -actin mRNA measured by Northern analyses. Conversely, addition of serum to BC<sub>3</sub>H1 cells results in a steady decline of the mRNA for M-CPK. This decline in M-CPK mRNA fully accounts for the decrease in the rate of M-CPK synthesis in these cells [compare (4) and (10)].

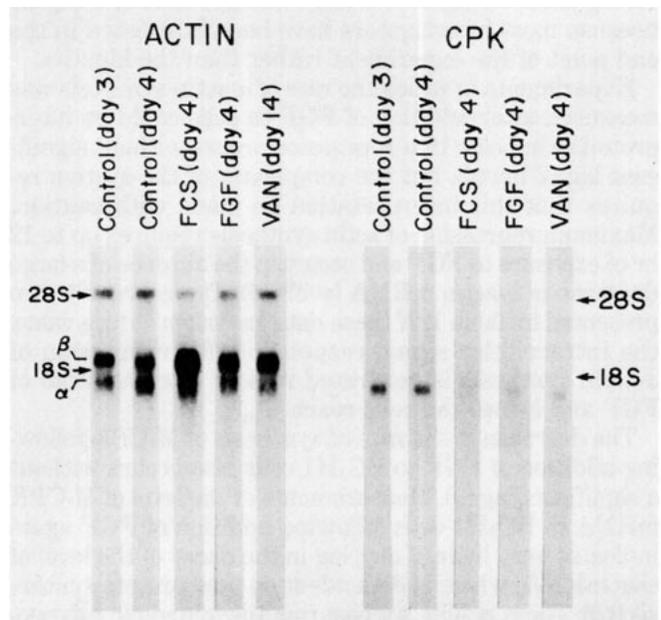


FIGURE 3. Northern analysis of M-CPK and actin mRNA levels. BC<sub>3</sub>H1 cells were plated in 150-mm tissue culture dishes (1.1 × 10<sup>6</sup> cells/dish) and were switched to DMEM containing 1% FCS 48 hr later (day 0). Total cellular RNA was prepared on day 3 or 4 as indicated. Some dishes received 20% FCS, FGF, or vanadate (VAN) for the final 24 hr. The  $\alpha$ - and  $\beta$ - plus  $\gamma$ -actin mRNAs are 1500 and 2100 nucleotides in length, respectively. CPK mRNA is ~1500 nucleotides in length. Each lane represent 6  $\mu$ g of total cell RNA. Reprinted in part with permission (6).

vations are that vanadate fully mimics the action of FGF on BC<sub>3</sub>H1 cells and blocks their differentiation program (6) (Figs. 1 and 3). By inference, we conclude that FGF reverses the differentiation program by activation of tyrosine-specific protein kinase of its receptor and that this activation is sufficient to result in movement of cells from G<sub>1d</sub> to G<sub>1q</sub>. It seems unlikely that  $\beta$ -TGF, which also inhibits BC<sub>3</sub>H1 cell differentiation (13), initially uses the same pathway as FGF, since activation of tyrosine kinases by  $\beta$ -TGF have not been reported. Multiple pathways may exist for the control of myogenic differentiation which ultimately must come together and affect a few or one central target molecule.

We do not know the targets of this tyrosine kinase and can only speculate on the nature of the molecules involved in the repression of the muscle phenotype in BC<sub>3</sub>H1 cells. Some of this speculation is by analogy to other muscle cells studied in a number of laboratories and implicitly assume that the fundamental processes that control muscle differentiation will be the same in all cells.

When FGF is added to BC<sub>3</sub>H1 cells, the cells move from G<sub>1d</sub> to G<sub>1q</sub>, and we estimate that this represents a movement of the cells about 4 to 6 hr closer to the G<sub>1</sub>-S boundary in the cell cycle. We can then ask at which time does repression or dedifferentiation take place. Does it follow immediately upon addition of mitogen, does it require 4 or 5 hr of exposure to the mitogen, or does it require even longer exposure to the mitogen? Important as these questions are, precise answers have not been forthcoming in part because of technical problems associated with measuring small changes over large backgrounds and in part because most investigators have been interested in the end point of the experiment rather than the kinetics.

Experiments in which the rate of  $\alpha$ -actin synthesis was measured after addition of FGF to cells could be interpreted to indicate that repression occurs without significant lag (2 hr) (6), but the complexity of the system requires that this interpretation be made with caution. Maximum repression of actin synthesis requires up to 12 hr of exposure to FGF and occurs in the absence of a large decrease in  $\alpha$ -actin mRNA levels (6). Typical results are presented in Table 1. If these data are taken at face value, the intracellular signal responsible for repression of  $\alpha$ -actin synthesis is generated rapidly after addition of FGF and before the cells reach G<sub>1q</sub>.

The decrease in the rate of synthesis of M-CPK following addition of FGF to BC<sub>3</sub>H1 cells also occurs without a significant lag (9). Measurements of the level of M-CPK mRNA in BC<sub>3</sub>H1 cells following addition of FGF again indicates very little, if any, lag in the decay of the level of this mRNA, which is dependent on new protein synthesis (10). These results suggest that the mitogenic polypeptide, FGF, induces the synthesis of one or more proteins that repress the muscle-specific differentiation program of BC<sub>3</sub>H1 cells.

FGF treatment of quiescent 3T3 fibroblasts results in their reentering and progressing through the cell cycle. Concomitantly, these cells transiently express several protooncogenes such as *c-fos* and *c-myc* in a sequential fashion (28,29), suggesting that these gene products are im-

portant for movement from G<sub>0</sub> into G<sub>1</sub>. It is therefore interesting to speculate that these gene products are involved in the inhibition of the muscle-specific phenotype since quiescent, differentiated BC<sub>3</sub>H1 cells express these proteins in a similar fashion following their treatment with FGF or serum (6). Two laboratories (30,31) have examined the effect of increasing levels of *c-myc* expression on myogenic differentiation. In one case (31), transfection of BC<sub>3</sub>H1 cells with the *c-myc* gene was only able to partially inhibit the expression of the muscle specific phenotype. In the second case (30), L<sub>6</sub>E<sub>9</sub>-B cells, which were biochemically differentiated but nonfused (fusion was blocked by chelating Ca<sup>2+</sup> with EGTA), responded to serum treatment by transiently expressing *c-myc* mRNA and reentering the cell cycle. However, the levels of muscle-specific mRNAs were not greatly affected by this treatment, even 96 hr after serum addition. These results suggest that *c-myc* expression can, at best, only partially block the differentiation of muscle cells. Conversely, the oncogenic forms of *N-ras* and *H-ras* have been shown to completely block myoblast differentiation (32). Transfection of the C-2 myoblast cell line with the *N-ras* oncogene under the control of a steroid-inducible promoter only blocked cellular differentiation when the cells are incubated with dexamethasone (33).

Can we conclude from these experiments that the expression of *c-ras* following addition of mitogen controls muscle differentiation? The answer is no. Induction of *c-ras* by mitogens (when observed) is a relatively late event after mitogen addition to cells (> 8 hr) (34). However, repression of the expression of muscle-specific proteins is initiated within 1 and maximally 2 hr after addition of mitogen to cells. Moreover, transfection of myoblasts with *c-ras* did not repress the muscle phenotype (32). Additionally, *c-ras* expression usually changes very little, if at all, as a function of position in the cell cycle (28).

In order to show that *c-ras* directly participates in the repression of muscle-specific differentiation, it would be necessary to demonstrate that the inhibition of *c-ras* expression blocks mitogen-induced repression of the muscle-specific phenotype. This could be accomplished by microinjecting antibodies directed against *c-ras* into differentiated BC<sub>3</sub>H1 cells. Such experiments have been successfully used to inhibit the NGF-induced differentiation of PC12 cells (35) and the serum-induced growth of quiescent 3T3 cells (36). Alternatively, antisense oligonucleoside methylphosphonates (oligo MP) (37) could potentially block the expression of endogenous *c-ras* by preventing the synthesis of new *c-ras* protein. Ideally, an antisense oligo (MP) directed against the 5' cap region of the mRNA would have the greatest chance of completely blocking the translation of *c-ras* mRNA (38). However, even if these experiments confirm that by blocking *c-ras* activity you were able to prevent FGF-induced repression of the muscle phenotype, it would not be possible to determine if this correlation was casual or causal. For example, blocking *ras* expression might prevent movement from G<sub>1d</sub> to G<sub>1q</sub> and might not be directly involved as a repressor of the muscle phenotype. Clearly, additional experiments using

different approaches to the problem will be required.

One powerful technique for potentially identifying a muscle-specific repressor is reverse genetics. However, this technique has met with mixed success. For example, growth-related genes are identified by screening for cognate RNAs whose levels are increased following serum or growth factor stimulation of quiescent cells. However, this approach has identified a human ADP/ATP translocase (39) and actin (40) as a growth-regulated gene. Other experiments have demonstrated that actin synthesis increases following serum stimulation of quiescent cells (40). Although these RNAs are certainly expressed at different levels in quiescent and mitogen-stimulated cells, their role as regulatory proteins would seem to be doubtful at best. Therefore, in order to identify FGF-induced muscle specific repressor proteins, a more restrictive screening protocol will have to be devised. For example, this repressor(s) should be expressed in BC<sub>3</sub>H1 cells that are in G<sub>1d</sub> and also in quiescent C2 cells that are expressing H-ras. Since quiescent, differentiated BC<sub>3</sub>H1 or C2 cells should not be expressing this repressor(s), a plus/minus DNA screening with RNA derived from these different cells and cell conditions might be useful in identifying this repressor(s).

The regulation of the expression of muscle-specific proteins is complex, at least four different control mechanisms have been identified (17): transcriptional control, differential splicing of certain gene products associated with differentiation process, translational control, which in rat cells can be controlled by Ca<sup>2+</sup> levels (low Ca<sup>2+</sup> prevents translation of muscle-specific mRNA), but which does not apply to avian cells where muscle-specific proteins are synthesized in low Ca<sup>2+</sup> media in the absence of fusion and finally mRNA stability. Mitogens have been shown to influence at least two of these regulator steps of transcriptional and translational control and may affect all of them (4,6,9,10,13). It is not clear, however, whether these four points of regulation can be controlled independently by mitogens or whether control is coordinate under all circumstances.

Myoblasts, unlike BC<sub>3</sub>H1 cells, fuse and irreversibly withdraw from the cell cycle and simultaneously become refractory to the repression by mitogens, but we do not understand the molecular basis of this phenomenon. Several laboratories have suggested that mitogens cease to be effectors because the cells lose mitogen receptors. This is an attractive idea but one for which the evidence is conflicting. For example, in L<sub>6</sub>E<sub>9</sub> cells induction of *c-myc* by mitogens can occur in differentiated cells, suggesting the presence of mitogenic receptors (30). Similarly, the ability of  $\beta$ -transforming growth factor ( $\beta$ -TGF) to block myoblast differentiation is not due to the disappearance of  $\beta$ -TGF receptors from these cells (41). By contrast, experiments with C-2 muscle cells suggest that fusion but not withdrawal from the cell cycle and differentiation results in downregulation of  $\beta$ -TGF and EGF receptors (41). Clearly the cause of the unresponsiveness of myotubes to mitogenic signals remains to be established.

While the intracellular signals that control differentia-

tion either in BC<sub>3</sub>H1 or other muscle cells remain to be established, the importance for stem cells to have a choice of quiescent states that are either permissive or nonpermissive for differentiation is the most important conclusion to be drawn from experiments with BC<sub>3</sub>H1 cells. In the context of a complex multicellular organism, muscle or other cells must be able to become quiescent with and without becoming differentiated. For several years many investigators have noted that some cells respond to specific mitogenic polypeptides by partial transit through G<sub>1</sub> portion of the cell cycle and have designated such mitogens as competence factors versus progression factors that allow cells to continue through the cell cycle (43-45).

We hypothesize that competence factors in general serve the function ascribed to FGF for BC<sub>3</sub>H1 cells, they control the movement of cells between G<sub>1d</sub> and G<sub>1a</sub> and therefore allow differentiation and quiescence to be uncoupled.

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