Aberrant Regulation of MyoD1 Contributes to the Partially Defective Myogenic Phenotype of BC_3H1 Cells

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Abstract. Two skeletal muscle-specific regulatory factors, myogenin and MyoD1, share extensive homology within a *myc* similarity region and have each been shown to activate the morphologic and molecular events associated with myogenesis after transfection into nonmyogenic cells. The BC_3H1 muscle cell line expresses myogenin and other muscle-specific genes, but does not express MyoD1 during differentiation. BC_3H1 cells also do not upregulate α-cardiac actin or fast myosin light chain, nor do they form multinucleate myotubes during differentiation. In this study, we examined the basis for the lack of MyoD1 expression in BC_3H1 cells and investigated whether their failure to express MyoD1 is responsible for their defects in differentiation. We report that expression of an exogenous MyoD1 cDNA in BC_3H1 cells was sufficient to elevate the expression of α-cardiac actin and fast myosin light chain, and to convert these cells to a phenotype that forms multinucleate myotubes during differentiation. Whereas myogenin and MyoD1 positively regulated their own expression in transfected 10T1/2 cells, they could not, either alone or in combination, activate MyoD1 expression in BC_3H1 cells. Exposure of BC_3H1 cells to 5-azacytidine also failed to activate MyoD1 expression or to rescue the cell's ability to fuse. These results suggest that BC_3H1 cells may possess a defect that prevents activation of the MyoD1 gene by MyoD1 or myogenin. That an exogenous MyoD1 gene could rescue those aspects of the differentiation program that are defective in BC_3H1 cells also suggests that the actions of MyoD1 and myogenin are not entirely redundant and that MyoD1 may be required for activation of the complete repertoire of events associated with myogenesis.

DIFFERENTIATION of skeletal myoblasts involves irreversible conversion to a postmitotic state, fusion to form multinucleate myotubes, and transcriptional activation of muscle-specific genes. Three muscle-specific regulatory factors, MyoD1 (3), myogenin (5, 41), and myf-5 (2), have recently been identified and shown to share a high degree of homology within a domain related to the myc protein family. When transfected into fibroblasts under the transcriptional control of a constitutive promoter, each of these genes can activate the morphologic and molecular events of myogenesis.

Initial evidence for a hierarchy of myogenic regulatory genes was obtained from studies that showed that exposure of the multipotential mesodermal stem cell line C3H10T1/2 to the demethylating agent 5-azacytidine resulted in the cells' conversion to myoblasts at high frequency (25–50%) (37). These observations led to the proposal that hypomethylation of a single gene or a few closely linked loci was responsible for conversion to the myogenic lineage (13). MyoD1 expression is activated in 5-azacytidine-derived myoblasts (3); however, it is not known whether the MyoD1 gene is the actual target for demethylation by 5-azacytidine. Nonetheless, the expression of MyoD1 as a result of 5-azacytidine treatment indicates that the MyoD1 gene itself or a gene that regulates MyoD1 is activated in 10T1/2 cells as a consequence of demethylation. In this regard, Emerson and co-workers have identified a gene referred to as *myd*, that efficiently converts 10T1/2 cells to myoblasts when it is transfected as demethylated cosmid DNA (27). MyoD1 and myogenin are expressed by *myd*-transfected 10T1/2 cells, which led to the proposal that these genes might be regulated by *myd* in a dependent myogenic regulatory cascade (27). Recent studies by Thayer et al. (38) also showed that MyoD1 positively autoregulates its own expression and that myogenin and MyoD1 regulate each others' expression. Similarly, MyoD1 is expressed by *myf*-5-transfected 10T1/2 cells (2).

The mouse muscle cell line BC_3H1 has been used extensively as a model for studying the mechanisms through which growth factors and oncogenes regulate myogenesis (10, 12, 17, 21, 22, 25, 30, 31, 32, 39). When maintained in the presence of high concentrations of serum, fibroblast growth factor, or type-β transforming growth factor, BC_3H1 cells exhibit a fibroblast-like morphology and do not express muscle-specific genes. Upon exposure to growth factor–deficient medium, these cells exit the cell cycle, and an array of skeletal muscle gene products is induced. However, unlike normal skeletal muscle cells, BC_3H1 cells do not fuse or commit to terminal differentiation, nor do these cells express

MyoD1 at detectable levels (3). α-Cardiac actin (33) and fast myosin light chain (MLC-1_f)[1] (36) also do not appear to be upregulated to normal levels in these cells. The lack of MyoD1 expression in BC_3H1 cells suggests that the myogenic program can be activated, at least in part, through a MyoD1-independent mechanism. That finding initially led us to search for regulatory genes related structurally or functionally to MyoD1. Using the *myc* similarity region of a MyoD1 cDNA as a probe to screen a BC_3H1 cDNA library under conditions of reduced stringency, a MyoD-related cDNA was identified (5). This cDNA was found to be homologous to a rat cDNA referred to as myogenin, which was isolated by subtraction–hybridization (41). Subsequent studies showed that myogenin possessed the potential to induce muscle-specific genes and fusion in transfected 10T1/2 cells (5).

That BC_3H1 cells express myogenin at high levels but do not form myotubes suggests that the actions of myogenin are more restricted in these cells than in 10T1/2 cells. To explore the mechanism that prevents myogenin from activating the complete myogenic program in BC_3H1 cells, we examined whether the MyoD1 gene can be expressed in BC_3H1 cells, and whether these cells possess the genetic potential to fully differentiate. We report here that expression of an exogenous MyoD1 cDNA in BC_3H1 cells is sufficient to restore their ability to form multinucleate myotubes and to elevate the expression of α-cardiac actin and MLC-1_f during differentiation. Whereas myogenin and MyoD1 positively regulate each others' expression in transfected 10T1/2 cells, this regulatory interaction is inactive in BC_3H1 cells, such that neither myogenin nor MyoD1, alone or in combination, can activate the endogenous MyoD1 gene. These results demonstrate that the failure of BC_3H1 cells to activate the complete repertoire of events associated with myogenesis is due to their inability to express MyoD1. The fact that neither myogenin nor MyoD1 are able to activate MyoD1 expression in BC_3H1 cells also suggests that the MyoD1 gene itself has undergone a stable alteration that prevents its activation, or that the regulatory pathway that activates the MyoD1 gene in 10T1/2 fibroblasts is nonfunctional in these cells.

Materials and Methods

Cell Culture

The BC_3H1 (31), C2 (42), and 10T1/2 cell lines were maintained in DME containing 20% FBS as described previously (21). Differentiation was initiated by replacing growth medium with differentiation medium containing 0.5% FBS or 2% horse serum, as indicated. BC_3H1 cells differentiate better in 0.5% FBS, whereas MyoD1-transfected BC_3H1 cells show higher levels of differentiation in 2% horse serum. For examination of the effects of 5-azacytidine, the cultures were treated with 10 μM 5-azacytidine for 24 h, after which they were plated at clonal density and allowed to form colonies for 14 d. The cultures were then transferred to differentiation medium and analyzed for myotube formation.

Transfections

BC_3H1 and 10T1/2 cells were transfected by calcium phosphate precipitation (8) as described previously (23) with 200 ng of pSV2neo and 4 μg of MyoD1 or myogenin expression vectors. The expression vector pEM275 (generously provided by C. P. Emerson, Jr. and S. Pearson-White, University of Virginia), which contains a mouse MyoD1 cDNA under transcriptional control of the β-actin promoter, was used to transfect BC_3H1 cells. The MyoD1 insert in that vector contains the complete coding region and lacks ~500 nucleotides from the 3′ untranslated region. 10T1/2 cells were transfected using a MyoD1 expression vector designated pEMC11s (generously provided by A. Lassar, Fred Hutchinson Cancer Research Center), which contains mouse MyoD1 under control of the Maloney sarcoma virus long terminal repeat (3). A mouse myogenin cDNA was also cloned into this vector and has been described previously (5). 48 h after transfection, plates were transferred to growth medium containing 20% FBS and 400 μg/ml G-418. After 14 d, individual clones were isolated and passaged into stable cell lines.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated using the guanidinium isothiocyanate procedure (5). 10 μg of RNA was electrophoresed on each lane of a denaturing formaldehyde agarose gel. After electrophoresis, RNA was transferred to nitrocellulose as described previously (32), and the blots were hybridized to ^{32}P-labeled probes for MyoD1 (3), troponin-T (Tn-T) (7), myogenin (5), muscle creatine kinase (28), and MLC-1_f, as indicated. The MLC-1_f probe (generously provided by Nadia Rosenthal, Boston University) was derived from the plasmid c91-JC and contains only light chain-1-specific sequences from the 5′ portion of the transcript. α-Cardiac actin was measured using a synthetic oligomer (generously provided by Michael Schneider, Baylor College of Medicine) corresponding to a unique sequence within the 3′ untranslated region (19). DNA probes were labeled by random priming (6), except that the α-cardiac actin oligomer was end labeled with T4 kinase.

DNA Isolation and Southern Analysis

Genomic DNA was extracted from BC_3H1 and C2 cells (18), digested with the indicated restriction enzymes, and electrophoresed on 0.8% agarose gels. After its transfer to nitrocellulose, the DNA was hybridized to labeled probes as described above.

Results

BC_3H1 Cells Possess a MyoD1 Gene

A straightforward explanation for the lack of expression of MyoD1 by BC_3H1 cells would be that the MyoD1 gene is deleted or rearranged such that it cannot be expressed. To examine these possibilities, we analyzed genomic DNA from BC_3H1 cells and C2 cells, which express MyoD1, for the MyoD1 gene by Southern analysis. As shown in Fig. 1, the DNA from both muscle cell lines showed an identical pattern of MyoD1 hybridization. The MyoD1 gene is, therefore, not deleted from the BC_3H1 genome and appears not to be grossly rearranged, at least within the limits of resolution of this analysis.

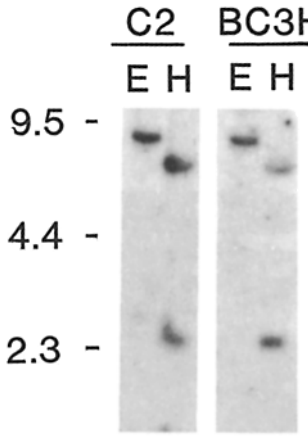

Figure 1. Southern analysis of the MyoD1 gene in C2 and BC_3H1 cells. Genomic DNA was isolated from BC_3H1 and C2 cells, digested with Eco RI (*E*) or Hind III (*H*) as indicated, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, blots were hybridized to a full-length mouse MyoD1 cDNA labeled with ^{32}P. Relative migrations of DNA standards are indicated.

1. *Abbreviations used in this paper*: MLC-1_f, fast myosin light chain; MSV-LTR, Maloney sarcoma virus long terminal repeat.

BC_3H1 Cells Can Be Converted to a Fusion-competent Phenotype by Transfection with MyoD1

If the inability of BC_3H1 cells to form myotubes is due solely to the lack of MyoD1 expression, BC_3H1 cells should form myotubes after transfection with an exogenous MyoD1 gene. If BC_3H1 cells instead possess multiple genetic alterations that prevent terminal differentiation and fusion, it is unlikely that these events could be rescued by transfection of a single gene. To determine whether the failure of BC_3H1 cells to fuse was due to the lack of MyoD1 expression, BC_3H1 cells were transfected with a mouse MyoD1 cDNA that was under transcriptional control of the β-actin promoter and with the neomycin resistance gene as a selectable marker.

We examined the morphology of MyoD1-transfected clones and found that ~25% of the colonies that had been transfected with MyoD1 formed extensive multinucleate myotubes after 4–5 d of exposure to mitogen-deficient medium. The morphologies of three representative clones that had acquired the ability to fuse as a result of transfection with the MyoD1 expression vector are shown in Fig. 2. No myotubes were observed in any of the MyoD1-transfected clones in growth medium. Examination of several hundred colonies transfected with the neomycin resistance gene alone also did not reveal any multinucleate myotubes.

To eliminate the possibility that the failure of the parental BC_3H1 cells to fuse was simply due to an insufficient level of myogenin expression, cells were also transfected with an

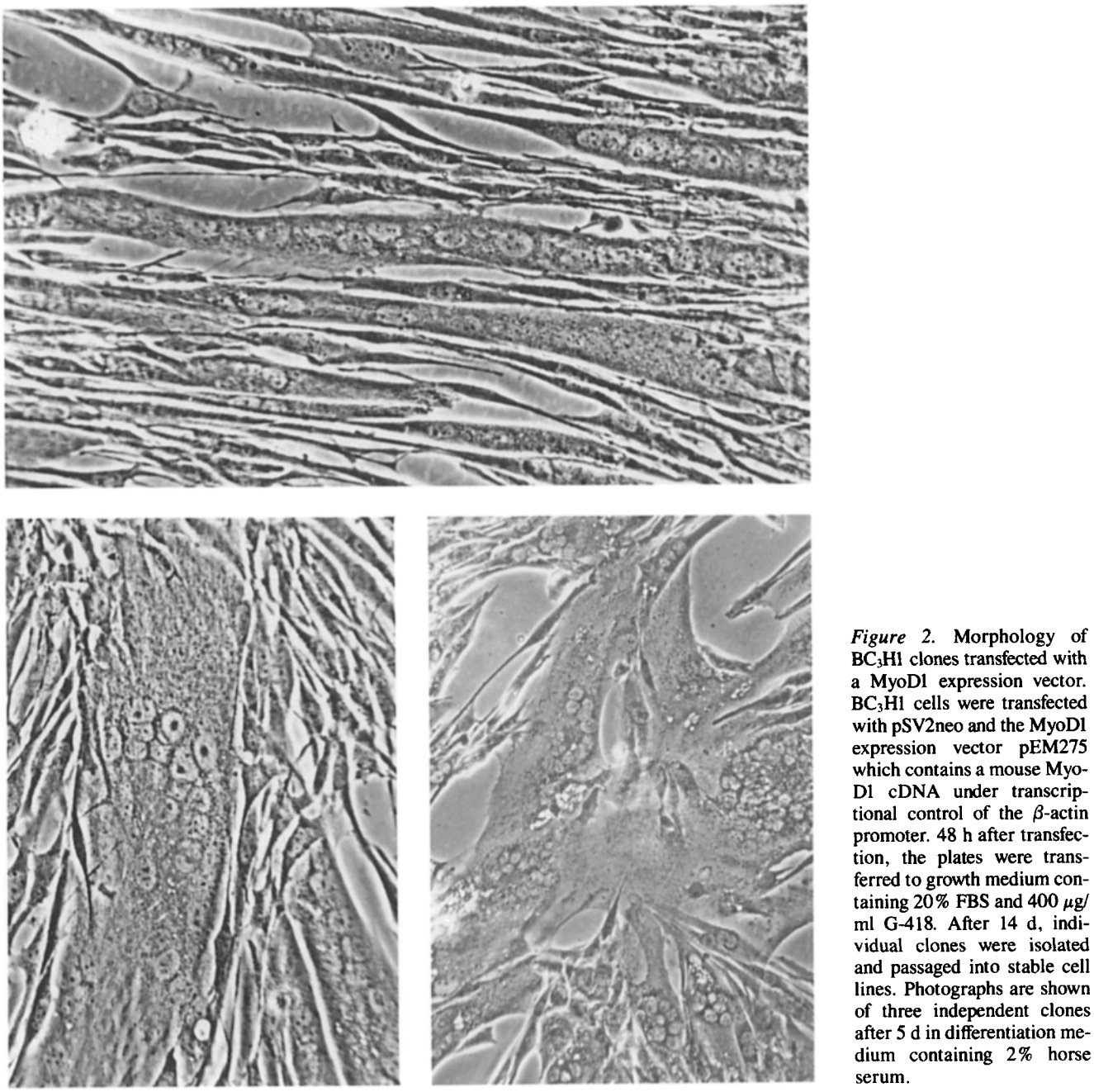

Figure 2. Morphology of BC_3H1 clones transfected with a MyoD1 expression vector. BC_3H1 cells were transfected with pSV2neo and the MyoD1 expression vector pEM275 which contains a mouse MyoD1 cDNA under transcriptional control of the β-actin promoter. 48 h after transfection, the plates were transferred to growth medium containing 20% FBS and 400 μg/ml G-418. After 14 d, individual clones were isolated and passaged into stable cell lines. Photographs are shown of three independent clones after 5 d in differentiation medium containing 2% horse serum.

expression vector encoding myogenin under transcriptional control of the Maloney sarcoma virus long terminal repeat (MSV-LTR) (5). None of the resulting clones formed myotubes after exposure to differentiation medium. We have not analyzed these clones in detail to confirm that they express myogenin at elevated levels. However, since myogenin-transfected 10T1/2 cells can form myotubes with levels of myogenin expression similar to those in differentiated BC_3H1 cells (5), it is unlikely that fusion can be restored in BC_3H1 cells simply by elevating the level of myogenin expression. These results support the conclusion that myogenin alone cannot induce fusion in BC_3H1 cells, and show that the lack of MyoD1 expression is responsible for the failure of BC_3H1 cells to form myotubes during differentiation.

The MyoD1 Gene Cannot Be Activated by MyoD1 or Myogenin in BC_3H1 Cells

To confirm that the exogenous MyoD1 gene was expressed in the stable transfectants that had acquired the ability to fuse, 14 independent clones were isolated before exposure to differentiation medium and passaged into stable cell lines. RNA was then isolated from cultures in growth and differentiation media and analyzed for MyoD1 expression. 7 of the 14 clones showed the ability to form myotubes to varying degrees after being expanded into cell lines. Results obtained with a representative fusing clone, designated BMD-1, are shown in Fig. 3. The transcript originating from the transfected MyoD1 gene is ~500 nucleotides shorter than the complete mouse MyoD1 mRNA due to the absence of sequences from the 3′ untranslated region. The transfected gene product is therefore distinguishable by Northern analysis. Although the exogenous MyoD1 gene was expressed at a high level in the BMD-1 cell line, there was no detectable expression of the endogenous transcript in either growth or differentiating conditions. Prolonged exposure of blots to film also failed to show endogenous MyoD1 expression. We also examined MyoD1 expression in two independent sets of pooled clones containing between 25 and 50 clones each. Only the exogenous MyoD transcript was detectable in these pools (data not shown). We also used a probe from the portion of the 3′ flanking region of MyoD1 that is absent from the transfected cDNA to confirm that the smaller transcript indeed arises from the endogenous gene.

It should be pointed out that all of the MyoD1 transfectants that we analyzed expressed the transfected gene at lower levels than the endogenous gene in differentiated C2 cells. Nonetheless, even when the exogenous MyoD1 transcript was present at levels 10-fold lower than the level of MyoD1 in C2 cells, extensive fusion was observed upon transfer to

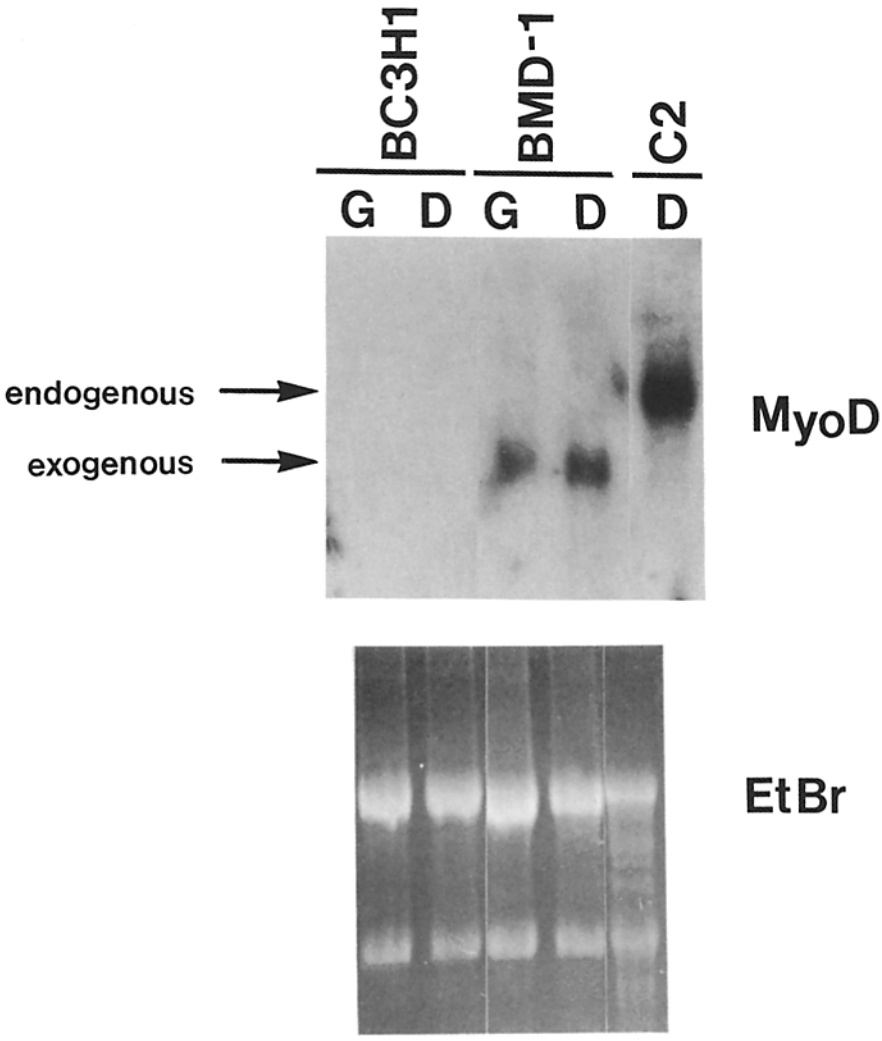

Figure 3. Expression of MyoD1 in MyoD1-transfected BC_3H1 cells. Total cellular RNA was isolated from BC_3H1, BMD-1, and C2 cells. Growth medium (*G*) contained 20% FBS; differentiation medium (*D*) contained 0.5% FBS for BC_3H1 cells, and 2% horse serum for BMD-1 and C2 cells. RNA blots were hybridized to ^{32}P-labeled probes for MyoD1. Ethidium bromide staining of the gel is shown to confirm that equivalent quantities of RNA were electrophoresed on each lane.

differentiation medium. These observations argue that fusion is not dependent simply on the level of expression of myogenin, but also requires additional factors.

Whereas MyoD1 was expressed in every clone that had the potential to form myotubes, analysis of seven clones that failed to form myotubes after transfection with MyoD1 showed no MyoD1 expression (data not shown). We also isolated three MyoD1-transfected cell lines that were able to form myotubes at early passages, but that subsequently lost that ability. Northern analysis of RNA from these cells showed that MyoD1 was no longer expressed after the cells became fusion defective (data not shown). We have continued to follow the properties of several clonal lines of MyoD1-transfected BC_3H1 cells over a period of several months and have found that the expression of MyoD1 and the ability to fuse are progressively lost with time. It is unclear whether BC_3H1 cells may have a mechanism for extinguishing MyoD1 expression.

Myogenin and MyoD1 Positively Regulate Their Own Expression in 10T1/2 Cells

The observation that MyoD1 was required for fusion of BC_3H1 cells prompted us to examine whether 10T1/2 cells that had acquired the ability to form myotubes after transfection with myogenin (5) might express MyoD1. Two stably transfected lines of 10T1/2 cells, designated 10TFL2-3 and 10TFL2-1, harboring a myogenin cDNA under control of the MSV-LTR were examined and were found to express myogenin at high levels under both growth and differentiating conditions (Fig. 4 *A*). Myogenin was expressed in these cell lines at approximately the level it was expressed in differentiated BC_3H1 myocytes (data not shown). MyoD1 mRNA was undetectable when the myogenin-transfected cell lines were maintained in mitogen-rich medium. However, upon transfer to mitogen-deficient medium, MyoD1 mRNA was detected. These results show that MyoD1 is regulated, either directly or indirectly, through a myogenin-dependent pathway and that activation of MyoD1 expression by myogenin is dependent upon the removal of mitogens from the medium.

Because myogenin is able to regulate the expression of MyoD1, we investigated whether MyoD1 could activate the expression of myogenin. Two clonal cell lines, designated M1 and M2, were isolated after stable transfection of 10T1/2 cells with MyoD1 under control of the MSV-LTR. Clone M1 showed low levels of myogenin in the presence of growth medium and high levels soon after transfer to mitogen-deficient medium (Fig. 4 *B*). In contrast, even when maintained at low density in mitogen-rich medium, clone M2 expressed MyoD1 at a fourfold higher level than clone M1 and had a tendency to differentiate precociously. The early differentiation of the M2 cell line is demonstrated by the expression of muscle creatine kinase in growth medium (Fig. 4 *B*). The behavior of this cell line was consistent with that observed by Davis et al. (3), who reported that high-level expression of MyoD1 causes cells to exit the cell cycle and differentiate in spite of high mitogen concentrations. Myogenin was expressed at high levels by M2 cells under both growth and differentiating conditions. The high level of expression in growth medium seems to reflect the fact that this cell line differentiates precociously. The behavior of stably transfected cell lines that we have analyzed thus far suggests that MyoD1 is more effective than myogenin in retarding the rate of cell growth in mitogen-rich medium, as several Myo-

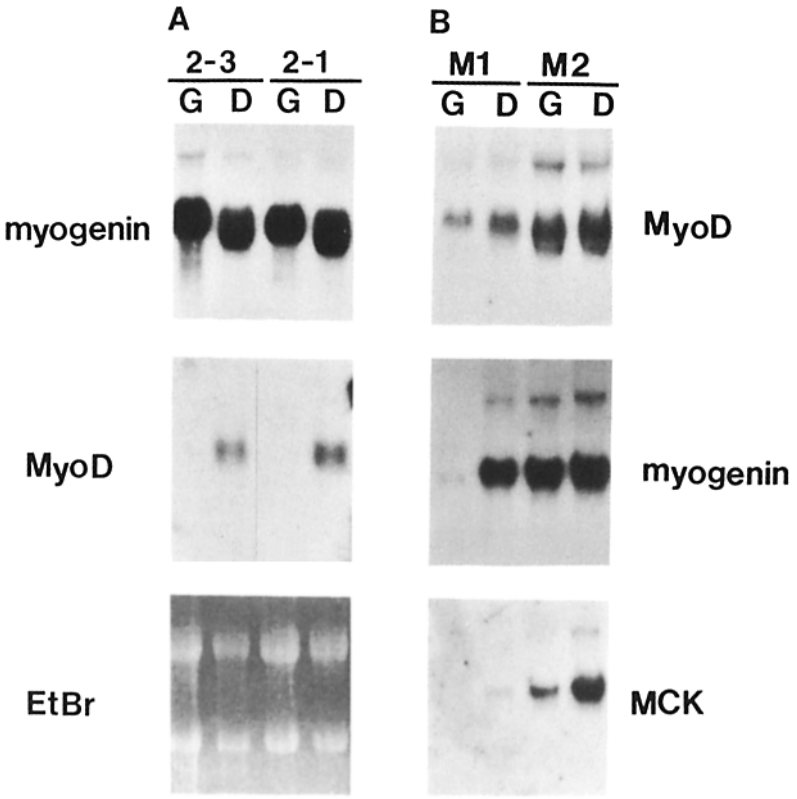

Figure 4. MyoD1 and myogenin regulate each others' expression in transfected 10T1/2 cells. Clonal cell lines derived from 10T1/2 cells, designated 10TFL2-3 and 10TFL2-1 (*A*), and M1 and M2 (*B*), were isolated after transfection with myogenin or MyoD1, respectively, under control of the MSV-LTR. Cells were maintained at subconfluent density in growth medium (*G*) or at confluent density in differentiation medium (*D*) for 4 d. Total cellular RNA was isolated from each cell line under the indicated conditions and was analyzed for expression of myogenin, muscle creatine kinase (*MCK*), and MyoD1 mRNAs. *A* shows ethidium bromide staining to confirm that equivalent quantities of RNA were electrophoresed on each lane. Ethidium bromide staining also confirmed that equivalent quantities of RNA were electrophoresed on each lane in *B* (data not shown).

D1-transfected clones differentiate precociously, whereas we have not observed this phenomenon with myogenin transfectants. Together, these results show that forced expression of MyoD1 or myogenin in 10T1/2 cells leads to activation of the other member of the pair. Similar results were reported recently by Thayer et al. (38).

α-Cardiac Actin and MLC-1$_f$ Are Expressed by MyoD1-transfected BC$_3$H1 Cells

α-Cardiac actin is the predominant α-actin isoform expressed during differentiation of C2 cells and primary myoblasts (1, 24). Strauch and Reeser (33) reported that α-cardiac actin mRNA was below the levels of detection in differentiated BC$_3$H1 myocytes. BC$_3$H1 cells also do not express skeletal muscle alkali MLC-1$_f$ during differentiation (36). MLC-1$_f$ and -3$_f$ are derived from a single gene and are generated by alternative splicing of a primary transcript that is transcribed from two promoters (26, 34). The MLC-1$_f$ and -3$_f$ promoters can be regulated independently (34), despite the fact that the MLC-1/3 locus is under the control of a muscle-specific enhancer that acts in a promoter-independent manner (4). BC$_3$H1 cells provide an example of independent regulation of these promoters, because these cells selectively express MLC-3$_f$ at high levels during differentiation (36). Since the expression of MyoD1 was sufficient to rescue the ability of BC$_3$H1 cells to fuse, we investigated whether α-cardiac actin and MLC-1$_f$ expression were also restored in MyoD1-transfectants.

In agreement with findings from previous studies (33, 36), there was no detectable expression of α-cardiac actin or MLC-1$_f$ mRNA in BC$_3$H1 cells (Fig. 5). However, these transcripts were induced in the BMD-1 cell line after transfer to differentiation medium. From densitometric analysis, the levels of expression of α-cardiac actin and MLC-1$_f$ mRNAs were ~10-fold lower in BMD-1 myotubes than in C2 myotubes, however, it is clear that they were significantly induced above the level in the parental BC$_3$H1 cell line. The lower level of expression of α-cardiac actin and MLC-1$_f$ in BMD-1 myotubes than in C2 cells cannot be attributed to an overall reduction in the extent of differentiation of these cells, because Troponin-T was expressed at similar levels in all of the cell lines under differentiating conditions. That the exogenous MyoD1 gene is expressed at a lower level in BMD1 cells compared to C2 cells may explain the corresponding reduction in MLC-1$_f$ and α-cardiac actin expression in these cells. Expression of α-cardiac actin mRNA was also observed in five additional MyoD1-transfected clones of BC$_3$H1 cells and in two independent pools of clones. MyoD1-transfected BC$_3$H1 cells that no longer expressed MyoD1 and had lost the potential to fuse also lost the ability to express α-cardiac actin and MLC-1$_f$ (data not shown). We conclude that the genes encoding α-cardiac actin and MLC-1$_f$ are potentially functional in BC$_3$H1 cells and that their expression can be activated by MyoD1.

We were able to detect faint signals in the region of the MLC-1$_f$ and α-cardiac actin transcripts after prolonged exposure to film of Northern blots of BC$_3$H1 RNA. If these signals indeed represented MLC-1$_f$ and α-cardiac actin mRNAs, their levels of expression were at least 100-fold lower than the levels in C2 myotubes (10-fold lower than the levels in BMD-1 cells). It should be mentioned that Taubman et al. (36) reported the expression of α-cardiac actin mRNA in BC$_3$H1 cells. This disparity could be explained by differential modulation of the actin gene in different subclones of BC$_3$H1 cells or under subtly different culture conditions.

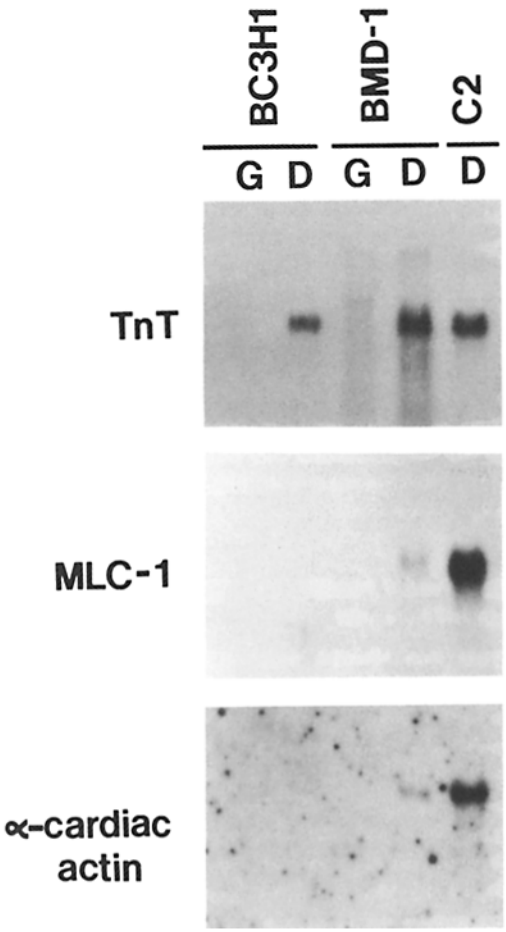

Figure 5. Expression of α-cardiac actin and MLC-1$_f$ in MyoD1-transfected BC$_3$H1 cells. Total cellular RNAs were isolated from BC$_3$H1, BMD-1, and C2 cells maintained in growth (*G*) or differentiation (*D*) medium, as indicated. RNA blots were hybridized to ^{32}P-labeled probes for α-cardiac actin, TnT, and MLC-1, as indicated. Ethidium bromide staining confirmed that equivalent quantities of RNA were electrophoresed on each lane (data not shown). Densitometry showed that α-cardiac actin and MLC-1 were expressed at a 10-fold higher level in C2 myotubes than in BMD-1 myotubes.

The Ability to Fuse Is Not Efficiently Rescued in BC$_3$H1 Cells by 5-Azacytidine

10T1/2 cells are efficiently converted to myoblasts that express MyoD1 and form multinucleate myotubes after brief treatment with 5-azacytidine (13, 14, 37). We therefore investigated whether treatment with 5-azacytidine could activate the MyoD1 gene and rescue the ability to fuse in BC$_3$H1 cells. To address this question, BC$_3$H1 cells were exposed to 10 μM 5-azacytidine for 24 h; cells were then plated at clonal density and allowed to form colonies. 14 d later, cultures were transferred to differentiation medium and their morphology was examined over the ensuing 10 d. In the more than 500 individual BC$_3$H1 colonies we examined, no

myotubes were observed. 10 individual clones of 5-azacytidine-treated BC_3H1 cells were isolated and analyzed for expression of MyoD1, but none was detected (data not shown). Consistent with previous studies (13, 14, 37), 23% (23 out of 100 colonies) of 10T1/2 colonies treated with 5-azacytidine generated extensive myotubes. Since BC_3H1 cells are already myogenic, we anticipated that they would be converted to a fusion-competent, MyoD1-positive phenotype by 5-azacytidine with an efficiency at least equivalent to that of 10T1/2 cells. However, our results show that if 5-azacytidine can convert BC_3H1 cells to a fusion-competent phenotype, the frequency for conversion must be at least two orders of magnitude lower than that for 10T1/2 cells.

Discussion

The results of this study show that the failure of BC_3H1 cells to activate the complete repertoire of events associated with myogenesis is caused by the lack of MyoD1 expression. Because an exogenous MyoD1 gene can rescue some aspects of the differentiation program that are defective in BC_3H1 cells, the genes required for normal differentiation are potentially functional in BC_3H1 cells and can be activated by MyoD1. The fact that myogenin is expressed at high levels in BC_3H1 cells but is unable to activate the complete differentiation program also suggests that the functions of myogenin and MyoD1, while overlapping, are distinct.

Although BC_3H1 cells possess a MyoD1 gene that appears at a gross level to be structurally normal, the gene is refractory to activation by MyoD1 or myogenin or by exposure to the demethylating agent 5-azacytidine, all of which are sufficient to activate MyoD1 expression in 10T1/2 cells. There are several possible explanations for the inability of myogenin or MyoD1 to activate expression of the MyoD1 gene in BC_3H1 cells. The MyoD1 gene may possess a structural alteration that is undetectable by Southern analysis but that prevents its activation. Alternatively, BC_3H1 cells may lack one or more cellular factors that cooperate with myogenin and MyoD1 to activate the MyoD1 gene. Finally, BC_3H1 cells may express a negative regulatory factor that prevents activation of the MyoD1 gene by myogenin or MyoD1. Heterokaryon experiments should distinguish among these possibilities.

Developmental studies have shown that myogenin and MyoD1 have distinct patterns of expression during development and are not always coexpressed (29, see also 3, 5, 41). BC_3H1 cells may represent a cell line derived from a specific stage of development in which MyoD1 is not normally expressed. Repression of MyoD1 expression in certain developmental stages could be due to positive or negative regulatory factors, or could be a consequence of chromosomal structure. If the inactivation of MyoD1 in BC_3H1 cells is not the consequence of a gene defect, these cells would present a particularly attractive system in which to study different myogenic developmental stages.

The frequency of conversion of 10T1/2 cells to myoblasts by 5-azacytidine is consistent with the involvement of one or a few closely linked loci (13, 14, 37). The low efficiency of conversion of BC_3H1 cells to a fusion-competent phenotype suggests either that multiple genes must be demethylated to rescue the ability to fuse, or that a single gene that is required for fusion cannot be activated by 5-azacytidine in these cells.

Our results show that myogenin and MyoD1 positively regulate each other's expression in 10T1/2 cells and that mitogens interfere with this transactivation. Thayer et al. (38) also reported recently that MyoD1 and myogenin activate expression of the endogenous MyoD1 allele in certain types of cells. Based on these observations, they proposed that this type of regulatory loop may provide a mechanism whereby these regulatory factors can amplify their own expression above a critical threshold required for activation of and commitment to myogenic differentiation. The fact that BC_3H1 cells are unable to terminally differentiate is consistent with their failure to regulate MyoD1. Considering the extensive homology between myogenin and MyoD1 within the *myc* similarity domain (3, 5, 35, 41), it is likely that the cross activation between these factors is mediated by their homologous domains.

That mitogens prevent myogenin-dependent activation of MyoD1 and other muscle-specific genes, suggests that myogenin acts on these genes through a common mechanism. In this regard, it has been shown that MyoD1 (15) and myogenin (T. J. Brennan and E. N. Olson, *Genes & Dev.*, in press) interact with the muscle creatine kinase enhancer core. In addition, myogenin binds to its own promoter (T. Chakraborty and E. Olson, our unpublished observations). The mechanism through which mitogenic signals block the actions of myogenin and MyoD1 also remain to be established. We have shown previously that growth factor–dependent repression of muscle-specific genes requires protein synthesis, suggesting that growth factor–inducible early gene products may act as negative regulators of myogenesis (32). Thus, growth factor–inducible nuclear factors might interfere either directly or indirectly with interactions between myogenin and MyoD1 and their appropriate cellular targets. Consistent with this hypothesis is the observation that fos (16, 40) and JunB (L. Li, T. Brennan, and E. Olson, unpublished results) prevent activation of the muscle creatine kinase enhancer by myogenin.

Thus far, the biologic actions of myogenin and MyoD1 have been found to be indistinguishable. However, since fusion and expression of the α-cardiac actin and MLC-1_f genes can be activated by MyoD1 in BC_3H1 cells, it appears that subtle differences exist between the activities of myogenin and MyoD1 as activators of muscle genes. The most straightforward interpretation of these observations is that the α-cardiac actin and MLC-1_f genes, in addition to one or more genes that are required for fusion and terminal differentiation, are directly dependent on MyoD1 or on factors that are regulated specifically by MyoD1. This model predicts that myogenin does not act alone to control all aspects of the differentiation program but instead cooperates with MyoD1 and perhaps other regulatory factors to regulate myogenesis. The susceptibility of various nonmyogenic cell lines to myogenic conversion by myogenin or MyoD1 (40) may, therefore, be dictated by whether either of the endogenous genes can be activated.

The expression of only certain aspects of the myogenic program is not unique to BC_3H1 cells. Rat L6 myoblasts, for example, also exhibit a defect in differentiation and do not express α-cardiac actin, MLCs 1 and 3 and one of two myosin heavy chain components when they fuse to form myotubes (9). The phenotype of BC_3H1 cells also closely resembles that of the L6E9-3b2 cell line, which was derived from L6 (20). These cells are temperature sensitive for fu-

sion and commitment to terminal differentiation; at nonpermissive temperatures they reversibly induce sarcomeric contractile protein genes, but do not fuse. Interestingly, L6 myoblasts also do not express MyoD1 at detectable levels (41). It remains to be determined whether an exogenous MyoD1 gene can restore all aspects of the differentiation program in this system. The fact that L6 myoblasts form myotubes but do not express MyoD1 may indicate that fusion can be triggered through a MyoD1-independent mechanism. Perhaps other regulatory factors, e.g., myf-5 (2), may be expressed in L6 myoblasts and compensate partially for the lack of MyoD1.

Elucidation of the mechanism that prevents activation of MyoD1 in BC_3H1 cells should shed light on the regulatory events that control MyoD1 expression during development. BC_3H1 cells should also provide an important system for identifying genes that depend specifically on MyoD1 for expression and should facilitate the analysis of the specific actions of myogenin in the absence of MyoD1.

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