MyoD Functions as a Transcriptional Repressor in Proliferating Myoblasts*

(Received for publication, October 17, 1996, and in revised form, December 6, 1996)

Caryn Chu, John Cogswell, and D. Stave Kohtz§
From the Department of Pathology, Mount Sinai School of Medicine, New York, New York 10029 and the Department of Cellular Sciences, Glaxo Wellcome, Inc., Research Triangle Park, North Carolina 27709

The myogenic basic helix-loop-helix (myo-bHLH) proteins are a family of transcriptional regulators expressed in myoblasts and differentiated skeletal muscle. Ectopic expression of myo-bHLH regulators transdetermines some fibroblast cell lines into myoblasts, which exit the cell cycle and differentiate into skeletal muscle when cultured in low mitogen medium. While members of the myo-bHLH family have been shown to function as transcriptional activators in differentiating muscle, the molecular basis of their function in proliferating myoblasts has not been elucidated. In this report, we present evidence that MyoD functions as a transcriptional repressor in myoblasts. We show that transcription from a cyclin B1 promoter construct is repressed in proliferating myoblasts and that repression is mediated by a pair of MyoD binding sites. We also show that transcription from the cyclin B1 promoter is repressed in proliferating C3H10T1/2 cells by ectopic expression of MyoD. These results demonstrate that MyoD can repress transcription of specific genes in proliferating cells, a novel function that may be important to maintenance of the myogenic phenotype and to cell cycle regulation in myoblasts.

Cells committed to the myogenic lineage are referred to as myoblasts, and the stability of this phenotype is evident after several passages in culture (1). The development and differentiation of competent myoblasts is dependent on expression of the myogenic basic helix-loop-helix (myo-bHLH) regulators (2–7). Four myo-bHLH regulators have been identified (MyoD, myogenin, myf-5, MRF4/myf-6/hereculin; Refs. 8–13), and ectopic expression of any one will confer the myoblast phenotype to mitogenic cells 

1 The abbreviations used are: bHLH, basic helix-loop-helix; bFGF, basic fibroblast growth factor; USF, upstream stimulatory factor; CAT, chloramphenicol acetyltransferase.

2 C. Chu, J. Molkentin, E. N. Olson, and D. S. Kohtz, submitted for publication.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Repression of the Cyclin B1 Gene Minimal Promoter in C2C12 Myoblasts—The cyclin B1 minimal promoter has recently been identified, and analysis of its sequence has revealed consensus binding sites for several transcriptional regulators upstream of the transcription initiation site (34). Binding sites for AP-2 (−181 to −174; Refs. 43 and 44), SP1 (−263 to −258 and −144 to −139; Refs. 45–48), NF-Y (−49 to −42; Refs. 49 and 50), MyoD (−268 to −263 and −236 to −231; Refs. 36 and 51), USF (−190 to −185; Refs. 52 and 53), and E2A gene products (−224 to −218; Refs. 54–57) have been identified (Fig. 1A). A series of deletion mutants of the cyclin B1 promoter (−3800, −1050, −318, −224, −169, and −127; Fig. 1B) were assayed for activity as CAT reporter constructs in proliferating C2C12 myoblasts (the promoter displayed only low level activity in differentiated myotubes; data not shown). Of these constructs, pCycB1(−224)-CAT displayed the greatest transcriptional activity. The activity of pCycB1(−169)-CAT and pCycB1(−127)-CAT was significantly less than that of pCycB1(−224)-CAT, due to the loss of an essential USF binding site (Fig. 1B; Refs. 54 and 53). Interestingly, retention of the pair of MyoD binding sites in constructs pCycB1(−318)-CAT, pCycB1(−1050)-CAT, and pCycB1(−3800)-CAT resulted in repression of transcription in comparison to pCycB1(−224)-CAT. Since it is expressed at significant levels in C2C12 myoblasts (8), this result suggested to us that MyoD may mediate transcriptional repression of certain genes in proliferating myoblasts.

Paired MyoD Binding Sites Are Required for Repression of the Cyclin B1 Promoter—We next asked whether the MyoD binding sites specifically mediate repression of the pCycB1-CAT constructs, and whether both sites or a single site are required for this function. Single-base mutants eliminating the left (pCycB1(−290 5′ MyoDmut)-CAT), right (pCycB1(−290 3′ MyoDmut)-CAT), or both (pCycB1(−290 5′ + 3′ MYodmut)-CAT) MyoD binding sites were tested for expression in C2C12 myoblasts. Expression was compared to that of a wild-type construct (pCycB1(−290)-CAT) spanning the same promoter region (this construct contains the paired MyoD binding sites; Fig. 2A). Expression of all three mutant reporter constructs greatly exceeded that of the wild-type construct (Fig. 2B). Thus, mutation of either MyoD binding site abolishes repression of the pCycB1-CAT reporters in proliferating myoblasts. This result strongly suggests that binding to these sites is required for repression. Interestingly, disruption of either site blocked repression, indicating that repression requires binding to both sites.

Ectopic Expression of MyoD Mediates Repression of the Cyclin B1 Promoter in C3H10T1/2 Fibroblasts—We next asked whether repression of transcription from the pCycB1-CAT reporters is mediated by MyoD expression. The mouse fibroblast cell line C3H10T1/2 was transfected with pCycB1(−127)-CAT, pCycB1(−169)-CAT, pCycB1(−224)-CAT, and pCycB1(−318)-CAT either with or without a MyoD expression construct (pEMSV-MyoD). The transient expression assays were performed with C3H10T1/2 cells under mitogenic conditions (subconfluence in high serum medium). Under these conditions, activation of muscle gene transcription by ectopic MyoD is inhibited as it is in myoblasts cultured similarly. Expression of pCycB1(−127)-CAT, pCycB1(−169)-CAT, and pCycB1(−224)-CAT in proliferating C3H10T1/2 fibroblasts was either unaffected or stimulated by ectopic expression of MyoD (Fig. 3).
contrast, ectopic expression of MyoD inhibited expression of pCycB1(−318)-CAT, consistent with the presence of the paired MyoD binding sites on this construct. This result shows that MyoD can directly mediate repression of the minimal cyclin B1 reporter through interaction with the paired binding sites present in pCycB1(−318)-CAT. Interestingly, repression of pCycB1(−318)-CAT by ectopic expression of MyoD was not observed in CV-1 cells (data not shown), which are resistant to myogenic conversion (58).

The role of the paired MyoD binding site in mediating repression by MyoD was further investigated by transient expression assays of pCycB1(−290)-CAT site mutants in proliferating C3H10T1/2 cells. Ectopic expression of MyoD repressed pCycB1(−290)-CAT in proliferating C3H10T1/2 cells only when both binding sites were intact (Fig. 4). Indeed, disruption of the right (3') or both binding sites resulted in stimulation of pCycB1(−290)-CAT by ectopic expression of MyoD (Fig. 4). These results are consistent with those obtained using the same mutants in C2C12 myoblasts and emphasize the functional importance of both binding sites in mediating repression by MyoD. Disruption of other E-box sequences (including the USF site) in the pCycB1-CAT constructs does not affect repression (data not shown).

**DISCUSSION**

The possibility that E-box binding proteins may function as transcriptional repressors has been suggested by studies of the immunoglobulin heavy chain (IgH) enhancer. The activity of a bHLH protein (TFE3) that binds a E3 site in the IgH enhancer is repressed by binding of another factor to an adjacent E5 site (59). Displacement of the repressor factor by another E5-binding protein (ITF-1) has been shown to mediate activation of the IgH enhancer (59). The IgH enhancer contains E-box sequences that can bind MyoD; however, binding of MyoD to these sites does activate transcription (36, 60). Repression of transcriptional activation of the IgH enhancer by MyoD is also dependent on an adjacent E5 site; removal of this site allows MyoD to activate transcription from the mutant enhancer (61). The protein(s) binding E-box sequences have not been characterized as yet. Nonetheless, a functional parallel may be drawn to repression of the cyclin B1 promoter by MyoD, which may result from repression of transcriptional activation by USF. In support of this possibility, specific binding of the cyclin B1 promoter by MyoD and repression of transcriptional activation by adjacent E-box binding proteins have not been characterized as yet. Nonetheless, a functional parallel may be drawn to repression of the cyclin B1 promoter by MyoD, which may result from repression of transcriptional activation by USF. In support of this possibility, specific binding of the cyclin B1 promoter by MyoD and repression of transcriptional activation by adjacent E-box binding proteins have not been characterized as yet.

Although ectopic expression of MyoD in fibroblasts can induce autoactivation of the endogenous MyoD gene and activate expression of other myo-bHLH regulators, the molecular basis of these events is not clear. Electrophoretic gel mobility shift assays suggest that MyoD maintains significant sequence-specific DNA binding activity in myoblasts (36, 38, 62). On the other hand, activation of muscle gene transcription by all of the myo-bHLH regulators is restricted to mitogenically arrested cells. Repression of the transcriptional activity of the myo-bHLH regulators in proliferating cells has been well studied (63), and recent studies of autoactivation of the MyoD gene in myoblasts have supported an indirect mechanism (27). Binding of MyoD to its own promoter is not required for autoactivation in myoblasts, consistent with its lack of transcriptional activity.
in mitogenically active cells. As shown here, the repressor activity of MyoD is functional in proliferating myoblasts. From this observation, an indirect mechanism of autoactivation can be proposed in which the expression of a repressor of MyoD gene transcription is itself repressed by MyoD.

Understanding the role of MyoD in regulating expression of the cyclin B1 gene during the cell cycle will require further investigation. Similar to activation of the IgH enhancer by TFRE3 described above, activation of the cyclin B1 gene by USF and/or downstream elements may require displacement of TFE3 described above, activation of the cyclin B1 gene by USF

binding of inhibitory protein(s) to a region –90 to +1 of the cyclin B1 promoter requires translocation during late G1. In addition, their studies show that repression of the cyclin B1 gene during mitosis will require further investigation. As shown here, the repressor

activity of MyoD is functionally analogous to repressor proteins that are expressed during G1 and/or downstream elements may require displacement of TFE3 described above, activation of the cyclin B1 gene by USF.

In mitogenically active cells. As shown here, the repressor activity of MyoD is functional in proliferating myoblasts. From this observation, an indirect mechanism of autoactivation can be proposed in which the expression of a repressor of MyoD gene transcription is itself repressed by MyoD.

Understanding the role of MyoD in regulating expression of the cyclin B1 gene during the cell cycle will require further investigation. Similar to activation of the IgH enhancer by TFRE3 described above, activation of the cyclin B1 gene by USF and/or downstream elements may require displacement of TFE3 described above, activation of the cyclin B1 gene by USF.

binding of inhibitory protein(s) to a region –90 to +1 of the cyclin B1 promoter requires translocation during late G1. In addition, their studies show that repression of the cyclin B1 gene during mitosis will require further investigation. As shown here, the repressor