NOTE TO USERS

CELL CYCLE AND HEB-MEDIATED REGULATION OF GENE EXPRESSION DURING MYOGENIC DIFFERENTIATION

By

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Abstract

Embryonic studies clearly demonstrate that MyoD and Myf5 are required for specification of the myogenic lineage. Moreover, MyoD plays an essential role in initiating expression of myogenin and inducing differentiation. However, the factors and mechanisms which temporally regulate expression of myogenic genes are not fully understood. Furthermore, the controversy of whether cell cycle withdrawal precedes induction of MyoD transcriptional activity, or MyoD activity induces cell cycle withdrawal, still exist. In this thesis, I demonstrate that although MyoD transcriptional activity is regulated by cell cycle regulators, such as pRb, cdk4 and cyclin D1, these molecules are functioning in a cell cycle-independent manner. In addition, MyoD regulates cell cycle progression, and more specifically S-phase entry, by regulating the stability of cyclin E. Moreover, MyoD regulates expression of cyclin D1 indirectly by modulating activation of NF-κB. Importantly, I demonstrate how the E-protein, HEB, regulates myogenic gene expression in an isoform-specific, MRF-specific, and promoter-specific manner. In the absence of HEB, MyoD is unable to effectively induce expression of myogenin, and differentiation is inhibited. Therefore, ubiquitously expressed factors, such as pRb and HEB, are able to regulate the restricted pattern of gene expression in myogenic differentiation.

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Table of Contents

List of Figures

List of Abbreviations

bHLH – basic helix loop helix
cdk – cyclin dependent kinase
ChIP – chromatin immunoprecipitation
DM – differentiation medium
DMEM – Dulbecco's modified Eagle's medium
DML – dorsal medial lip
DNA – deoxyribonucleic acid
ERK – extracellularly regulated kinase
FBS – fetal bovine serum
G1 – gap phase 1
G2 – gap phase 2
GAPDH – glyceraldehyde phosphate dehydrogenase
GM – growth medium
HAT – histone acetyltransferase
HDAC – histone deacetylase
IGF – insulin-like growth factor
IGFBP – insulin-like growth factor binding protein
IP – immunoprecipitation
Luc - luciferase
M – mitosis
MAPK – mitogen activated protein kinase
MCK – muscle creatine kinase
MEK – MAP-ERK kinase
Mgn – myogenin
MLC – myosin light chain
MRF – myogenic regulatory factor
MyHC – myosin heavy chain
PCR – polymerase chain reaction
PMSF – phenylmethylsulfonyl fluoride
RNA – ribonucleic acid
RNase – ribonuclease
RT-PCR – reverse transcription-PCR
S – DNA synthesis phase
SDS – sodium dodecyl sulfate
siRNA – small interfering RNA
UTP – uridine triphosphate
VLL – ventral lateral lip
WT – wildtype

Chapter 1 - Introduction

Identification of the Myogenic Regulatory Factors

The study of myogenic lineage determination began with the observation that 5-azacytidine treatment of (C3H)10T½ fibroblasts produces myogenic, adipogenic and chondrogenic clones (Jones and Taylor, 1980; Taylor and Jones, 1979). These experiments were interpreted to suggest that the demethylation of only one or a few closely linked loci was necessary for myogenic conversion (Konieczny and Emerson, 1984). It was thus proposed that a set of regulatory determination loci exist that are essential for lineage specification.

This concept was further investigated, resulting in the identification of *MyoD* as a master regulatory gene necessary for myogenic determination (Davis et al., 1987). The cloning of *MyoD*, and the conversion of fibroblasts into myoblasts, was crucial in putting forth the notion that there are cell-type specific determination genes that act in addition to more ubiquitously expressed genes in lineage specification (Davis et al., 1987; Tapscott et al., 1988). On the basis of sequence homology, three additional family members were identified, namely *Myf5* (Braun et al., 1989), *myogenin* (Edmondson and Olson, 1989; Wright et al., 1989), and *MRF4* (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989). Together, these genes constitute the myogenic regulatory factors (MRFs), a family of basic helix-loop-helix (bHLH) transcription factors responsible for myogenic specification and differentiation (Choi et al., 1990; Weintraub et al., 1989).

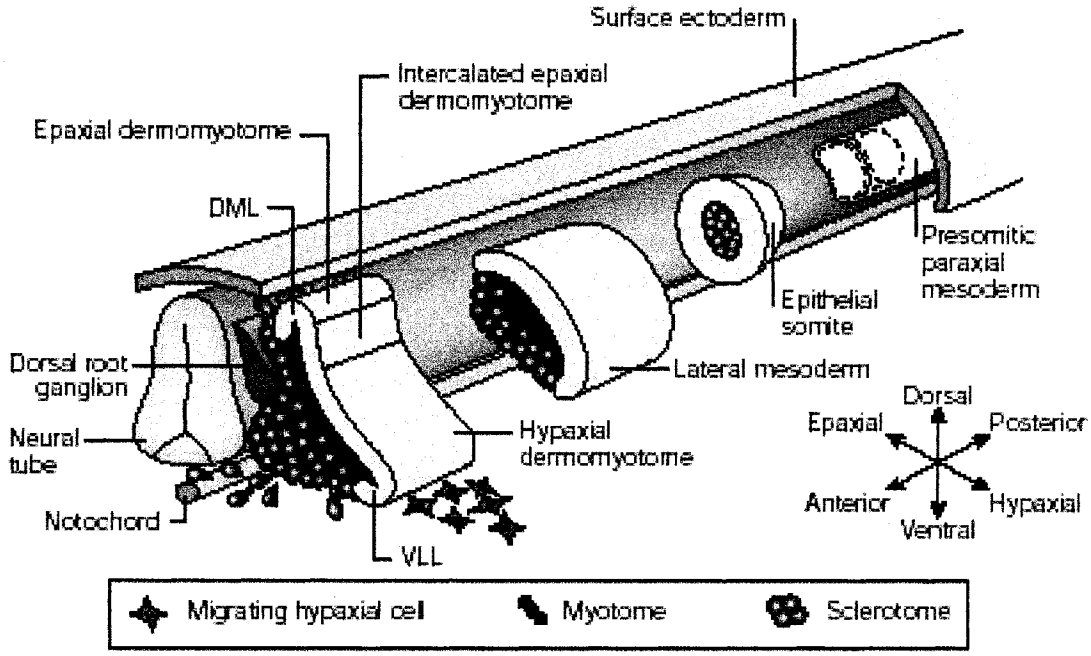
The ability of the MRFs to convert cells to the myogenic lineage *in vitro* is dependent on the type of cell, such that 10T½ fibroblasts are more amenable to conversion than Swiss 3T3 cells (Crescenzi et al., 1990; Davis et al., 1987; Hopwood and Gurdon, 1990; Schafer et al., 1990; Weintraub et al., 1991). This indicates that myogenic conversion requires a permissive environment, in which one or a number of factors must be present for efficient determination and differentiation. As such, progenitor cells within the embryo are thought to be pre-committed to the myogenic lineage prior to MRF expression, and express particular transcription factors, such as Pax3 (Williams and Ordahl, 2000).

Embryonic Myogenesis

The majority of embryonic skeletal myogenic progenitors, with the exception of some craniofacial and esophageal muscles, arise from a group of epithelial spheres known as somites. Somite pairs develop on either side of the neural tube as a result of condensation and segmentation of the paraxial mesoderm (reviewed in Pourquie, 2001; Pownall et al., 2002; Summerbell and Rigby, 2000). The somites differentiate along the dorsal-ventral axis to give rise to the dorsally located epithelial dermomyotome and the ventrally located mesenchymal sclerotome. The dermomyotome gives rise to the dermis and the skeletal muscle of the trunk and limbs, while the sclerotome develops into the cartilage and bone of the vertebrae and ribs. Moreover, a recent report describes a novel somitic compartment of tendon progenitors, which forms as a result of interactions between the myotome and sclerotome (Brent et al., 2003). Myogenic precursors of the

Figure 1. The embryonic origin of limb and trunk skeletal muscle.
During early embryonic development, the presomitic paraxial mesoderm, located on either side of the neural tube, condenses and segments, resulting in the formation of the epithelial somites. The somites differentiate along the dorsal-ventral axis to give rise to the dermomyotome and the sclerotome. The myotome is formed by cells from the dorsal medial lip (DML) extending beneath the dermomyotome and differentiating. A second wave of migration from the dorsal medial lip (DML) gives rise to the epaxial myotome, whereas migration from the ventral lateral lip (VLL) gives rise to the non-migratory hypaxial myotome. Furthermore, precursor cells from the VLL delaminate and migrate (migrating hyapxial cell) to the limb, ventral body wall, diaphragm, and tongue. Signals received from the lateral mesoderm, the neural tube, and the notochord, combine to regulate specification and differentiation.

Figure 1.


Surface ectoderm
Intercalated epaxial dermomyotome
Epaxial dermomyotome
DML
Presomitic paraxial mesoderm
Epithelial somite
Dorsal root ganglion
Lateral mesoderm
Neural tube
Hypaxial dermomyotome
Notochord
VLL
Dorsal
Epaxial
Posterior
Anterior
Hypaxial
Ventral
Migrating hypaxial cell
Myotome
Sclerotome

dermomyotome express the paired box transcription factors, *Pax3* and *Pax7*, and low levels of *Myf5* (Goulding et al., 1994; Hirsinger et al., 2001; Jostes et al., 1990; Kiefer and Hauschka, 2001).

The myotome is formed when the cells of the dorsal medial lip (DML) extend beneath the dermomyotome, exit the cell cycle, elongate, and terminally differentiate. The development of the myotome is augmented by a second wave of migration from the dorsal medial lip, resulting in the formation of the epaxial myotome, which gives rise to deep back muscles (Christ and Ordahl, 1995; Ordahl and Le Douarin, 1992). A similar pattern of events induces the ventral lateral lip (VLL) to establish the ventral non-migratory hypaxial myotome, which gives rise to lateral trunk muscle. Moreover, cells from the VLL delaminate from the dermomyotome and migrate ventrally to regions of presumptive muscle development in the limbs, ventral body wall, diaphragm and tongue.

As cells of the dorsal medial lip and ventral lateral lip migrate to form the epaxial and hypaxial myotome, respectively, expression of *MyoD* increases and expression of *Pax3* decreases. Premature activation of MyoD causes precocious differentiation in the dermomyotome and prevents migration of sufficient muscle precursors to the myotome and the limb buds. Thus, the dermomyotomal *Pax3*-expressing cells are maintained as a proliferative, undifferentiated population by signals secreted from the lateral plate mesoderm and surface ectoderm (Amthor et al., 1999).

Regulation of MRF Expression during Embryonic Development

Signals from the axial structures, namely the neural tube and the notochord, are important in determination of epaxial muscle precursors located in the dorsal-medial domain of the somite (Bober et al., 1994a; Buffinger and Stockdale, 1994; Gamel et al., 1995; Hirano et al., 1995; Pourquie et al., 1993; Pownall et al., 1996; Rong et al., 1992; Spence et al., 1996). In the absence of the notochord, epaxial musculature is absent and only the hypaxial muscle program is activated (Dietrich et al., 1999). The notochord and the floor plate produce Sonic hedgehog (Shh), a member of the Hedgehog family of signaling molecules, which activates myogenesis in somitic tissue and positively regulates the formation and survival of the dorsal myotome (Johnson et al., 1994; Munsterberg et al., 1995; Teillet et al., 1998).

Shh signaling regulates expression of *Myf5* directly through the Gli family of transcription factors, and specific Gli1 binding sites have been identified in the *Myf5* epaxial somite enhancer (Gustafsson et al., 2002). Importantly, Shh signaling through Gli does not initiate expression of *Myf5*, but rather ensures continued expression of *Myf5* (Teboul et al., 2003).

It was initially demonstrated that Shh null mice lacked epaxial musculature, and failed to activate *Myf5* expression specifically in the dorsal region of the somite (Borycki et al., 1999; Chiang et al., 1996). However, Kruger and colleagues demonstrate that *Myf5* expression is reduced, but not absent in the epaxial muscle of Shh null mice. This is more consistent with Shh playing a role in maintaining expression of *Myf5*, rather than initiating expression (Kruger et al., 2001; Teboul et al., 2003). Interestingly, these mice display a loss of hypaxial musculature, and a reduced number of cells populating the limb buds, suggesting that Shh may also be involved in expansion of the muscle precursor population prior to differentiation (Duprez et al., 1998).

Signals from the lateral plate mesoderm and the dorsal ectoderm combine to regulate hypaxial muscle formation (Cossu et al., 1996; Dietrich et al., 1997; Fan and Tessier-Lavigne, 1994; Kenny-Mobbs and Thorogood, 1987; Pourquie et al., 1995; Pourquie et al., 1996). The dorsal ectoderm provides signals that stimulate myogenesis and activate expression of *MyoD*, whereas the signals from the lateral plate mesoderm inhibit myogenesis (Cossu et al., 1996). Signals from the dorsal ectoderm can be mimicked by Wnt-4, Wnt-6, and Wnt-7a (Tajbakhsh et al., 1998). Wnt-7a is able to activate expression of *MyoD* in explants of paraxial mesoderm, which in turn leads to co-expression of both *MyoD* and *Myf5*. More recently, it was shown that Wnt5b is expressed in the presomitic mesoderm in chicks, and specifically activates *MyoD* expression (Linker et al., 2003). Thus, individual Wnts may exert unique effects, and a combination thereof may be required to fully activate the myogenic program.

Soluble Frizzled-related proteins (sFRPs), which specifically block Wnt signaling, inhibit myogenesis in explanted somitic mesoderm or in newly formed somites (Borello et al., 1999). Furthermore, injection of sFRP-transfected cells into the placenta of pregnant females abrogates myogenesis. This is accompanied by down-regulation of *noggin* and *Myf5* expression, and maintained expression of *Pax3*. Therefore, disruption of Wnt signaling by sFRPs does not alter early aspects of lineage pre-commitment, such as *Pax3* expression, but disrupts myogenesis by blocking the expression of *Myf5* or *MyoD*.

The lateral plate mesoderm produces signaling molecules that inhibit myogenic differentiation and promote proliferation of myogenic precursor cells. The numbers of cells that migrate to sites of hypaxial or limb myogenesis are small compared to the mass of muscle that differentiates during myogenesis, indicating that these muscle progenitors proliferate considerably prior to differentiation. Interestingly, these progenitors express *Myf5*, but not *MyoD*, and this expression is presumably regulated through signaling molecules and transcription factors, such as *Pax3* and *Pax7*. In birds, *Myf5* is expressed in proliferating progenitors, whereas *MyoD* is active in differentiated muscle, suggesting that MyoD acts downstream of Myf5 to induce differentiation (Delfini et al., 2000). Moreover, this indicates that Myf5 is required for proliferation and maintenance of myogenic lineage specification prior to differentiation.

In order to prevent precocious cell cycle withdrawal and induction of differentiation, *MyoD* expression is inhibited by bone morphogenic proteins (BMPs), Notch/Delta signaling, and transcription factors, such as Msx1 (Bendall et al., 1999; Delfini et al., 2000; Hirsinger et al., 2001; Houzelstein et al., 1999; Pourquie et al., 1996). Each of these factors downregulate expression of *MyoD*, yet maintain expression of *Pax3* and *Myf5*, and preserve myogenic lineage specification. Therefore, myogenic determination and differentiation requires the orchestrated effort of a number of signaling molecules and transcription factors which spatially and temporally regulate expression of *Myf5* and *MyoD*.

MRF Knockout and Knock-in Mice

Gene targeting in mice has proven to be invaluable in understanding lineage determination and maintenance in skeletal muscle. Cell culture work suggests that MyoD is essential for myogenic lineage determination and thus, it is surprising that *MyoD*

knock-out mice are viable and fertile and have grossly normal musculature (Rudnicki et al., 1992). It was suggested that this was the result of Myf5 compensation, as there is a 4-fold increase in expression of *Myf5* in the absence of *MyoD*. However, the viability of *MyoD*-null mice depends on the genetic background, such that in a C57/BL6 or Balb/C background, loss of *MyoD* is lethal, whereas *MyoD*-null mice in an SV129 background are viable (Rudnicki lab, unpublished observations). This indicates that there are unknown contributing factors that determine the MyoD-null phenotype.

Myf5 null mice also display apparently normal muscle formation, yet the original knockout mice die perinatally due to a severe rib defect (Braun et al., 1992). However, replacing the *Myf5* coding region with *FGF-6*, and removing the neomycin cassette, fully rescues the rib phenotype (Kaul et al., 2000). More importantly, simple deletion of exon 1 of *Myf5* using the Cre-recombinase system results in viable, fertile mice without any rib or skeletal defects. As expected, muscle development is delayed until the onset of *MyoD* expression, and at birth, the mice display grossly normal musculature.

MyoD$^{-/-}$:*Myf5*$^{-/-}$ mice completely lack myoblasts or muscle fibers, clearly demonstrating that either *Myf5* or *MyoD* is required for specification of the myogenic lineage (Kaul et al., 2000; Rudnicki et al., 1993). In addition, *MyoD*$^{-/-}$:*Myf5*$^{-/-}$:*MyoD-LacZ* mice demonstrate that although there are presumptive muscle precursor cells that are lacZ positive, these cells develop to assume non-muscle fates (Kablar et al., 1999). Thus, in the absence of *MyoD* or *Myf5*, the myogenic lineage cannot be specified.

Mice lacking *myogenin* specify the appropriate number of myogenic precursor cells, yet die perinatally due to a severe loss of differentiated muscle fibers, demonstrating that myogenin expression is essential for myogenic differentiation (Hasty et al., 1993; Nabeshima et al., 1993). Mice lacking *myogenin* and *MyoD* or *myogenin* and *Myf5*, are phenotypically identical to *myogenin*-null mice, suggesting that MyoD and/or Myf5 possibly function upstream of myogenin (Rawls et al., 1995). Moreover, mice lacking *MyoD* and *MRF4* have a phenotype similar to *myogenin*-null mice, clearly demonstrating that MyoD is required for activation of *myogenin* expression and initiation of differentiation (Rawls et al., 1998).

Myogenin expressed from the *Myf5* locus (*Myf5*$^{myg\text{-}ki}$) rescues the rib defect of the *Myf5*$^{-/-}$ mice, and the mice are viable (Wang et al., 1996). In a *MyoD*-null background, the mice die perinatally due to reduced muscle formation, demonstrating that *myogenin* cannot compensate for the loss of *MyoD* and *Myf5*, and that the MRFs are not functionally redundant (Wang and Jaenisch, 1997). In a *myogenin*-null background, the mice are born with a *myogenin*-null phenotype, indicating that *myogenin* expression from the *Myf5* promoter cannot substitute for expression from its own promoter in regulating differentiation (Wang and Jaenisch, 1997).

There are three different *MRF4* knockout mice with phenotypes ranging from perinatal lethality associated with loss of *Myf5* activation, to viable mice with normal *Myf5* expression and a 4-fold increase in *myogenin* expression (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). The range in the phenotypes observed in the *MRF4* knockout mice may be a function of the position and orientation of the neomycin cassette in the various *MRF4* alleles (Olson et al., 1996). In addition to the variations in alleles, the analysis of the *MRF4* knockout mice is complicated by the proximity of *MRF4* and *Myf5* on the mouse chromosome, suggesting that disruption of the *MRF4* gene may cause alterations in the expression of the *Myf5* gene (Yoon et al., 1997). Yet, the

expression pattern of *MRF4* in the developing mouse embryo suggests that this factor plays an important role in fiber formation, maturation, and maintenance.

Pax3 is expressed in the presomitic mesoderm and the early epithelial somites. However, upon differentiation of the somites, expression of *Pax3* is restricted to the dermomyotome, and eventually to the ventral-lateral domain of the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994). The naturally occurring *splotch* mouse, in which there is a loss-of-function mutation in the *Pax3* gene, displays diaphragm and limb muscle deficiencies (Bober et al., 1994b; Daston et al., 1996; Franz et al., 1993). Pax3 regulates expression of c-met, and both Pax3 and c-met/SF/HGF are implicated in controlling migration of muscle precursor cells to both the limbs and the myotome (Brand-Saberi et al., 1996; Epstein et al., 1996). Furthermore, the dermomyotome and the myotome fail to organize and elongate in *splotch* mice (Tremblay et al., 1998).

Pax3 plays an important role in myogenic lineage determination by acting upstream of MyoD. Wnts and Shh can induce expression of *Pax3* and *Pax7*, and overexpression of Pax3 in paraxial mesoderm induces expression of MyoD, Myf5 and myogenin (Maroto et al., 1997). In addition, *splotch/Myf5* double homozygous mutant mice lack all trunk and body muscles, indicating that *MyoD* expression is ablated in the absence of both *Myf5* and *Pax3* (Tajbakhsh et al., 1997). Therefore, although repression of MyoD expression involves a complex pattern of signaling and transcriptional events, activation of MyoD expression occurs either through Pax3 or Myf5.

Satellite Cells and Post-natal Muscle Growth

Satellite cells represent a unique lineage of myogenic precursors required for post-natal growth and repair of skeletal muscle (Bischoff, 1994; Hawke and Garry, 2001; Schultz, 1996; Seale and Rudnicki, 2002). These cells are quiescent mononuclear cells that reside between the basal lamina and sarcolemma of the muscle fiber, and first appear in the limb musculature at approximately day 17.5 of mouse embryonic development (E17.5).

The developmental origin of satellite cells has been subject to much speculation in recent years. Early experiments with quail/chick chimeras suggested that satellite cells were derived from the somite (Armand et al., 1983). However, more recent studies by DeAngelis *et al.* provide evidence that satellite cells may be derived from cells associated with embryonic vasculature, including the dorsal aorta (De Angelis et al., 1999). Interestingly, the dorsal aorta is colonized by migratory populations of somitic angioblasts derived from the paraxial mesoderm (Pardanaud and Dieterlen-Lievre, 2000). Thus, the presence of myogenic cells in dorsal aortic explants does not preclude the possibility of an indirect somitic origin for satellite cells. Moreover, recent studies describing heterogeneity in satellite cell populations may reflect multiple developmental origins for this lineage (Beauchamp et al., 2000).

Quiescent satellite cells do not express any of the MRFs, but do express c-met, M-cadherin, CD34, and Pax7 (Beauchamp et al., 2000; Cornelison et al., 2000; Seale et al., 2000). Activation of satellite cells through normal growth, weight bearing or injury, stimulates cell cycle re-entry and induces expression of *MyoD* or *Myf5*, followed by expression of both MRFs. Subsequently, *myogenin* is expressed; the cells exit from the cell cycle, differentiate, and fuse to the existing damaged myofiber. In addition, the

original pool of quiescent satellite cells is repopulated, suggesting that a subpopulation of satellite cells exists that do not differentiate and fuse to the fiber, but downregulate expression of the MRFs and become quiescent satellite cells again. This variation in function is likely to be represented, in part, by the presence or absence of particular transcription factors. For example, Msx1, which is expressed in satellite cells, may downregulate MRF expression in a subset of cells, directing them back to quiescence (Cornelison et al., 2000; Odelberg et al., 2000).

In the embryo, myogenic precursor cells express transcription factors, such as Pax3, prior to MRF-mediated specification. In adult skeletal muscle, Pax7 is expressed in both quiescent and activated satellite cells. Moreover, *Pax7*-null mice display a lack of satellite cells in post-natal skeletal muscle, and exhibit a significant reduction in muscle mass and fiber caliber, suggesting that Pax7 is important for satellite cell specification and post-natal growth of skeletal muscle (Seale et al., 2000).

Although Pax7, and other transcription factors, are important in preparing a cell for specification, the MRFs remain the master regulators of myogenic determination. The hind limb muscle of *MyoD*$^{-/-}$ mice displays a reduced capacity to differentiate, yet has an increase in the number of satellite cells associated with the myofibers (Megeney et al., 1996). Further analysis of satellite derived myoblasts *in vitro* revealed a significant delay in differentiation and a concomitant increase in proliferative potential (Sabourin et al., 1999).

Myf5 expression is increased 4-fold in *MyoD*$^{-/-}$ satellite cells, suggesting that Myf5 plays a distinct role from MyoD in satellite cell activation, commitment, and differentiation. The presence of lacZ-expressing satellite cells on freshly isolated muscle fibers from Myf5-nLacZ mice suggests that Myf5 may be expressed at low levels in quiescent satellite cells (Beauchamp et al., 2000). Although it is possible that these cells may be activated in response to the isolation procedure, it is intriguing to postulate that Myf5-positive cells, which do not up-regulate MyoD, define a subpopulation of cells required to replenish the satellite cell population (Sabourin et al., 1999).

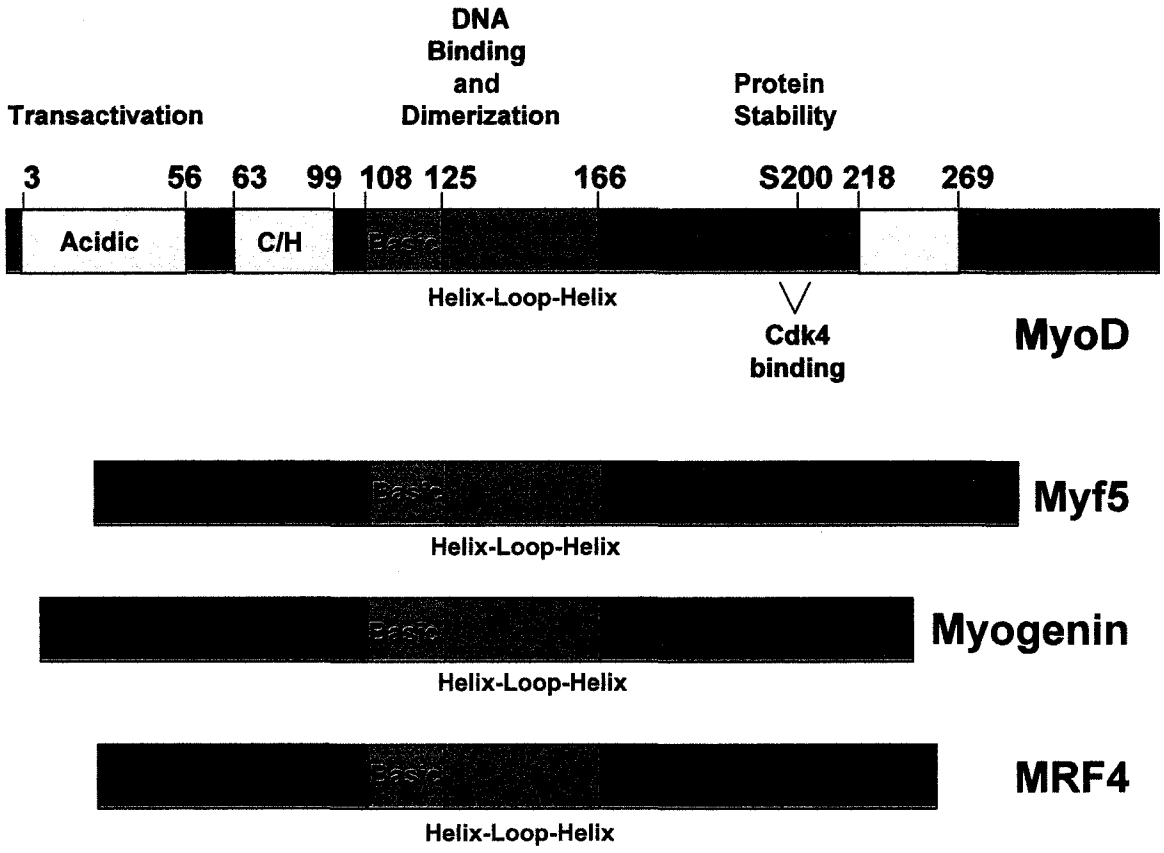
The MRFs as Transcription Factors

The MRFs control Myogenic specification and differentiation by regulating expression of genes necessary for the formation and function of skeletal muscle. Discrete functional domains within the MRFs play unique roles in the process of transcriptional activation. The basic domain of the MRFs mediates binding to the consensus E-box sequence, CANNTG, found in the promoters of muscle-specific genes. Specifically, two conserved amino acids in the basic domain, an alanine and a threonine, are important for binding of the MRFs to promoters and activating transcription in a myogenic-specific manner (Brennan et al., 1991; Weintraub et al., 1991). The helix-loop-helix (HLH) region, responsible for dimerization and other protein-protein interactions, is also essential for myogenic gene activation (Davis et al., 1989; Lassar et al., 1989; Tapscott et al., 1988).

The N- and C-terminal sequences are responsible for transactivation and protein-protein interactions involved in DNA binding and chromatin remodeling (Berkes et al., 2004; Gerber et al., 1997; Weintraub et al., 1991). The uniqueness of these regions suggests that although there may be a common mode of action, there is sufficient

Figure 2. Domain structure of the MRFs.
MyoD can be subdivided into a number of domains. The basic domain (amino acids 108-125) and the helix-loop-helix domain (amino acids 125-166) mediate DNA binding and dimerization, and the MRFs share a high degree of homology within these domains. Furthermore, the helix-loop-helix domain interacts with p300 acetyltransferase. The MyoD N-terminus contains a transcriptional activation domain (amino acids 3-56) and a putative chromatin remodeling domain (amino acids 63-99). Amino acids 96 and 98 specifically direct binding of MyoD to the E2 E-box of the myogenin promoter in association with Pbx1. The C-terminus of MyoD also contains a putative chromatin remodeling domain (amino acids 218-269), as well as a cdk4-binding region (amino acids 189-203). Moreover, phosphorylation of serine 200 by cdk2 or cdk1 targets MyoD for degradation during specific phases of the cell cycle.

Figure 2.


DNA
Binding
and
Dimerization
Transactivation
Protein
Stability
3
56
63
99
108
125
166
S200
218
269
Acidic
C/H
Basic
Helix-Loop-Helix
Cdk4
binding
MyoD
Basic
Helix-Loop-Helix
Myf5
Basic
Helix-Loop-Helix
Myogenin
Basic
Helix-Loop-Helix
MRF4

variation within the MRFs to ensure functional independence. In particular, a highly conserved C-terminal amphipathic α-helix has been shown to possess discrete functions in MyoD and myogenin (Bergstrom and Tapscott, 2001). In MyoD, this helix is important in initiating transcription from normally silent genes, thus functioning as a specification domain. In myogenin, on the other hand, this α-helix functions as a general transcriptional activation domain, likely amplifying expression from "open" genes.

The transcriptional activity of the MRFs is regulated in part by dimerization, as heterodimerization with E-proteins is required to activate transcription of muscle-specific genes (Lassar et al., 1991). The first proteins to be implicated as binding partners were the products of the E2A gene, E12 and E47. Originally isolated as immunoglobulin enhancer binding factors, E12 and E47 both contain a basic helix-loop-helix motif, important for dimerization and DNA binding (Henthorn et al., 1990; Murre et al., 1991). The role of E2A in myogenesis was first postulated upon finding that MyoD is able to heterodimerize with E12 and E47 *in vitro* (Murre et al., 1989). Co-transfection of MyoD and E47 is required for E-box transcriptional activity in COS cells (Lassar et al., 1991). Moreover, mobility shift assays using muscle cell nuclear extracts indicate the presence of a complex containing either MyoD or myogenin and E12/E47-like proteins. In contrast, an analysis of the cardiac actin promoter in P19 embryonic carcinoma (EC) cells demonstrates that E12 inhibits MyoD transcriptional activity 3-fold and E47 inhibits activity 5-fold (Petropoulos and Skerjanc, 2000).

In addition to independently cloning the E2A gene, designated ITF-1 in this case, an additional homologous E-protein was cloned, namely ITF-2 (Henthorn et al., 1990). Two alternatively spliced variants exist, ITF-2A and ITF-2B, which differ in their amino terminus. Each form appears to play a distinct role in myogenesis, as shown by the fact that ITF-2B inhibits MyoD activity on the cardiac actin promoter, while ITF-2A does not display any effect (Skerjanc et al., 1996).

Lastly, HEB was isolated based on homology to E2A and ITF-2 (Hu et al., 1992). HEB is able to heterodimerize with myogenin *in vitro*, as do E12 and ITF-2, suggesting that HEB plays a role in regulating MRF activity (Hu et al., 1992; Neville et al., 1998). Of particular interest is the fact that HEB is highly expressed in skeletal muscle, as well as the thymus and B-cells, and is thus postulated to play an important role in lineage-specific gene expression. Interestingly, HEB is alternatively spliced, and each variant displays a characteristic spatial and temporal distribution pattern (Klein et al., 1993). Thus, it is hypothesized that myogenic transcriptional activity is further regulated by variations in complex formation, which in turn may be defined by the sequences surrounding the E-boxes (Huang et al., 1996; Yutzey and Konieczny, 1992).

Myogenic determination and differentiation involves multiple subprograms of gene expression that are uniquely regulated (Bergstrom et al., 2002). To identify genes specifically induced by MyoD, Bergstrom and colleagues employed gene array technology to study the transcriptional profile of *MyoD*$^{-/-}$*:Myf5*$^{-/-}$ fibroblasts expressing an estradiol-inducible form of MyoD (MyoD-ER) (Bergstrom et al., 2002). The authors established that MyoD regulates the expression of a number of gene clusters, which in combination, orchestrate differentiation. Clustering MyoD-responsive genes into a temporal pattern of regulation revealed that nuclear regulatory factors, such as myogenin, Mef2a, and Hes6, and adhesion/matrix genes, such as cadherin-15, syndecan 2, and dystroglycan, were represented in early activation clusters. In contrast,

structural/cytoskeletal genes, such as myosin light chain (MLC), myosin heavy chain (MyHC), and troponin, comprise the later activation clusters. Moreover, cell cycle genes and growth factors were part of a cluster that decreased immediately, or after a transient increase in expression.

Regulation of MRF Activity through Signal Transduction

In the presence of mitogens, cultured myoblasts proliferate, and the majority of muscle-specific genes are not expressed. MyoD and Myf5 are expressed in order to maintain myoblast identity, yet transcriptional activity is tightly regulated to prevent premature differentiation. Upon removal of mitogens, MRF transcriptional activity increases, myogenin is expressed, and differentiation proceeds.

Many mitogenic growth factors, such as fibroblast growth factor (FGF), promote proliferation and inhibit differentiation (Allen et al., 1984; Allen et al., 1985; Clegg et al., 1987). Transforming growth factor-β (TGF-β), on the other hand, inhibits both proliferation and differentiation, while insulin-like growth factor (IGF-1) has a modest effect on proliferation and promotes differentiation (Allen and Boxhorn, 1989). A significant amount of effort has been directed towards determining the specific effect that each of these factors has on MRF activity, and characterizing of the pathways involved in the process.

Many growth factors stimulate phospholipase C-mediated hydrolysis of phosphoinositides, activating protein kinase C (PKC) in the presence of phospholipids and calcium. PKC phosphorylates a conserved threonine residue in the bHLH of myogenin, which in turn inhibits transcriptional activity and differentiation (Li et al., 1992b). This is specific to myogenin, since MRF4 and MyoD are not phosphorylated by PKC, suggesting that unique pathways exist for regulating each MRF (Hardy et al., 1993; Liu et al., 1998). Growth factors also stimulate cyclic AMP-dependent protein kinase, also known as protein kinase A. Activated protein kinase A (PKA) inhibits myogenic differentiation through the repression of the transcriptional activity of MyoD, Myf5, and myogenin (Li et al., 1992a; Winter et al., 1993). Surprisingly, this occurs independently of direct phosphorylation of the MRFs, or inhibition of DNA-binding.

The binding of a variety of growth factors to their cell-surface receptors initiates a cascade of events resulting in the activation of $p21^{ras}$. Activated $p21^{ras}$ binds Raf-1, which in turn activates the mitogen activated protein kinase pathway (MAPK) through MEK1/2. Activated MEK1/2 phosphorylates and activates ERK1/2, which in turn activates transcription of responsive genes, either through kinases such as $p90^{rsk}$ and MSK1, or through transcription factors such as Elk-1 or c-myc.

Coexpression of activated $p21^{ras}$ inhibits MRF-mediated conversion of 10T½ fibroblasts by a mechanism independent of MRF DNA binding and transcriptional activation (Kong et al., 1995). Further work attempting to delineate the pathway concluded that activated $p21^{ras}$ acts through a means other than the typical Raf/MEK or Rac/Rho pathways, and may at least partially involve NF-κB (Mitin et al., 2001; Ramocki et al., 1997). However, it is likely that high level expression of activated $p21^{ras}$ elicits a completely different response from that induced either by low level expression, or normal cellular activation.

Low level expression of an estradiol-inducible form of Raf in myoblasts enhances differentiation (Wang et al., 2004). However, high level expression inhibits muscle-

specific gene expression and myogenic differentiation, as a result of increased secretion of TGFβ into the medium. The TGF-β pathway activates Smad3, which interferes with MRF-E protein heterodimerization and transcriptional activity (Liu et al., 2001). Moreover, TGFβ activation of Smad3 specifically represses Mef2 activity (Liu et al., 2004).

It was initially suggested that FGF might not be linked to the MAPK pathway, but in fact, chemical inhibitors of the MAPK pathway demonstrate that MEK is important for FGF-induced proliferation and inhibition of differentiation (Milasincic et al., 1996; Weyman and Wolfman, 1998). Moreover, overexpression of MAP kinase phosphatase (MKP-1), which dephosphorylates ERK1/2, and thus inhibits MAP kinase activity, stimulates and accelerates the initiation of differentiation (Bennett and Tonks, 1997). Subsequent downregulation of MKP-1 activity is necessary for fusion and myotube formation, suggesting that the MAP kinase pathway plays a role in both proliferation and differentiation.

Lastly, coexpression of an activated form of MEK1, which specifically localizes to the nucleus, inhibits myogenic differentiation by abrogating MRF transcriptional activity (Perry et al., 2001). This occurs independently of direct phosphorylation, but instead involves an indirect interaction between MyoD and MEK1, in which the N-terminus plays an important role. This complex, which involves a number of other factors, may serve as a sensor in the switch from proliferation to differentiation, as well as providing a mechanism for maintaining myoblast identity prior to differentiation.

Unlike the growth factor stimulated pathways, the p38 signaling pathway is activated upon induction of differentiation (Wu et al., 2000). Moreover, abrogation of the pathway by chemical inhibitors, or stable expression of dominant negative MKK3, inhibits myogenic differentiation (Cabane et al., 2003). Importantly, p38-mediated signalling positively regulates a particular subset of myogenic genes, which mainly comprises the late activated structural genes (Bergstrom et al., 2002). Moreover, p38 regulates transcriptional activation, rather than DNA binding, suggesting that p38 promotes activation domain accessibility. This role of p38 may be MyoD-specific, but may also reflect p38-mediated stimulation of Mef2 activity (Han et al., 1997; Yang et al., 1999; Zetser et al., 1999; Zhao et al., 1999). The ability of signalling molecules and other regulatory factors to target specific promoters raises the question of what part of the promoter, or bound complexes, determines the specificity.

Chromatin Remodeling and the MRFs

The role of histone acetyltransferases (HATs) and histone deacetylases (HDACs) in myogenesis came to the fore with the demonstration that p300, as well as N-CoR, can interact with MyoD and regulate transcriptional activity (Bailey et al., 1999; Yuan et al., 1996). Normally, acetylation of histones is associated with an increase in transcriptional activity at a gene locus, and deacetylation is associated with gene silencing. Both p300 and pCAF can acetylate MyoD, and this acetylation significantly increases MyoD-dependent transcriptional activation, due to more efficient recruitment of these co-activators to muscle-specific promoters (Polesskaya et al., 2000; Polesskaya et al., 2001; Sartorelli et al., 1999). Activation of STAT3 disrupts the interaction of MyoD with p300 and pCAF, resulting in inhibition of transcriptional activity (Kataoka et al., 2003).

Examination of *p300*-null mice and embryonic stem (ES) cells has confirmed that p300 is required for myogenic differentiation (Roth et al., 2003). However, loss of *p300* severely reduced expression of MyoD and Myf5, without affecting expression of Pax3, suggesting that p300 functions upstream of the MRFs to regulate expression of MyoD and Myf5. This may reflect MyoD- or Myf5-mediated activation of *MyoD* and *Myf5*, or a role for p300 in Pax3 activation of MyoD.

MyoD also interacts with HDAC1, and this complex occupies the myogenin promoter prior to differentiation (Mal et al., 2001); (Mal and Harter, 2003). Furthermore, the transcriptional repressor prohibitin (PHB2) interacts with MyoD, and recruits HDAC1 to myogenic promoters (Sun et al., 2004). Upon initiation of differentiation hypophosphorylated Rb, or activated Akt, disrupts the MyoD-HDAC1 complex, and pCAF, a histone acetyltransferase, associates with the promoter (Mal and Harter, 2003; Puri et al., 2001; Sun et al., 2004). However, during adipogenic differentiation, a PPARγ-Rb-HDAC3 complex inhibits PPARγ activity and adipocyte differentiation (Fajas et al., 2002). Moreover, HDAC1/2 is postulated to be involved in non-targeted deacetylation, whereas HDAC3 is required for targeted gene silencing (Li et al., 2002). This indicates that regulation of myogenic differentiation at the level of chromatin may not simply be the result of a switch from HDAC1 to pCAF, but may also involve various HDACs and other regulatory factors with unique functions.

In addition to the action of HDACs and HATs, a family of ATP-dependent SWI/SNF complexes remodels chromatin to regulate gene expression and cell cycle withdrawal. Overexpression of a dominant negative form of the SWI/SNF protein brahma-related gene-1 (BRG1) or brahma (BRM) inhibits muscle-specific gene expression and differentiation, albeit in a non-specific manner (de la Serna et al., 2001a; Roy et al., 2002). Although BRM and BRG-1 have been implicated in Rb-mediated cell cycle arrest, the dominant negative enzymes did not alter induction of p21, cyclin D3, or Rb during myogenic differentiation (de la Serna et al., 2001b). Therefore, cell cycle exit may be separated from myogenic gene activation by the type of chromatin remodeling factors acting at a particular locus.

The MRFs and the Cell Cycle

The role of the retinoblastoma protein (pRb) in the control of cell cycle progression is well established. During myoblast differentiation, pRb expression is elevated and the protein is progressively dephosphorylated (Endo and Goto, 1992). These modifications in pRb have been demonstrated to be required for MyoD transcriptional activity. However, MyoD converts $Rb^{-/-}$ embryonic fibroblasts to the myogenic lineage, and early markers such as myogenin are expressed normally in these cells (Schneider et al., 1994; Skapek et al., 1996). Expression of myosin heavy chain (MyHC) is delayed, and although restimulation with serum induces DNA synthesis, these restimulated *Rb* null cells are blocked at mitosis.

The presence of a pocket domain mutant of pRb, pRb^{N757F}, in which the E2F binding site is not disrupted, permits DNA synthesis in myotubes upon restimulation with serum (Chen and Wang, 2000). Modifying the cdk phosphorylation sites of pRb^{N757F}, to create a hypophosphorylated mutant, inhibits this reinitiation of DNA synthesis. This indicates that the pocket domain of Rb binds to a factor, other than E2Fs, that prevents

Rb phosphorylation, and subsequently, entry into S phase, even in the presence of a mitogenic signal. Taken together, this underscores the importance of hypophosphorylated Rb in establishing the terminally differentiated state.

Differentiation requires both the inhibition of cdk activity and downregulation of cyclin expression in order to maintain Rb in a hypophosphorylated state. Cell cycle progression, on the other hand, requires that Rb is phosphorylated and this occurs in part through cyclin D1-cdk4 kinase activity. Surprisingly, overexpression of cyclin D1 inhibits MRF activity and myogenic differentiation through a mechanism independent of Rb or MyoD phosphorylation, such that cyclin D1 can inhibit muscle gene expression even in the presence of a hypophosphorylated mutant of Rb (Rao and Kohtz, 1995; Skapek et al., 1996; Skapek et al., 1995). Cyclin D1 expression and its related kinase activity is regulated through FGF, TGF-β, and IGF, suggesting a possible link between signaling molecules and the cell cycle in regulating MRF activity (Rao and Kohtz, 1995; Rosenthal and Cheng, 1995). Although the mechanism of this regulation is unclear, further work demonstrates that MyoD can interact with cdk4 (Zhang et al., 1999a; Zhang et al., 1999b). This interaction inhibits the binding of MyoD to DNA during proliferation, and the interaction in turn can prevent phosphorylation of Rb by cdk4 during differentiation.

Cdk-9, on the other hand, is able to stimulate MyoD transcriptional activity and enhance differentiation by forming a complex with and phosphorylating MyoD (Simone et al., 2002). This may be linked to cdk-9 mediated phosphorylation of RNA polymerase II during elongation by means of a transcriptional complex, once again illustrating how ubiquitous factors can regulate myogenic lineage determination and specification through modulation of MRF activity.

The cell cycle machinery also regulates the MRFs independent of transcriptional activity. Phosphorylation of MyoD by cdk2 or cdk1 targets it for ubiquitin-mediated degradation (Kitzmann et al., 1999; Song et al., 1998; Tintignac et al., 2000). Association of MyoD with $p57^{kip2}$, a cdk2 inhibitor, prevents this phosphorylation and stabilizes MyoD protein (Reynaud et al., 2000; Reynaud et al., 1999). Moreover, expression of MyoD harboring non-phosphorylatable mutations of the two cdk1 phosphorylation sites delays entry into mitosis (Tintignac et al., 2004). This corresponds with data that demonstrates that expression of MyoD and Myf5 is regulated in a cell-cycle dependent manner, such that MyoD expression is associated with G1 and G2-M, and Myf5 is predominantly present in G0, S, and G2 (Kitzmann et al., 1998). This cell cycle-mediated regulation of MRF expression may prove to be important to the cell in determining the timing of differentiation.

The MEF2 family of Transcription Factors

In addition to the MRFs, the myocyte enhancer factor-2 (Mef2) family of transcription factors is involved in activation of muscle-specific gene expression. The Mef2 family in vertebrates consists of four members, Mef2a, -b, -c, and –d, which are expressed in a distinct but overlapping manner (Edmondson et al., 1994; McDermott et al., 1993; Subramanian and Nadal-Ginard, 1996; Yu et al., 1992). The N-terminus contains a MADS domain (MCM1, Agamous, Deficiens, Serum response factor) that is

responsible for DNA-binding and dimerization. An adjacent Mef2-specific domain is also involved in DNA binding and various co-factor interactions. Lastly, the C-terminal domain of Mef2 is required for transcriptional activation.

Mef2 proteins are expressed in a variety of tissues, including skeletal, cardiac and smooth muscle, as well as the brain. In order to address where Mef2 is transcriptionally active, a transgenic Mef2 sensor mouse was designed in which Mef2 binding sites were placed upstream of the *lacZ* gene (Naya et al., 1999). This revealed that Mef2 transcriptional activity is restricted to developing cardiac, skeletal and smooth muscle. After birth, expression of the transgene is downregulated, but is upregulated in skeletal muscle during regeneration (Akkila et al., 1997; Naya et al., 1999).

The Mef2 family and the MRFs appear to act synergistically in activating myogenesis, as Mef2 was postulated to interact with MyoD-E protein dimers to activate muscle-specific gene expression (Black et al., 1998). Further, the Mef2 family and the MRFs form an autoregulatory loop, such that expression of myogenin and MRF4 is dependent on both sets of transcription factors (Naidu et al., 1995; Ridgeway et al., 2000; Wang et al., 2001). MyoD-converted $Rb^{-/-}$ fibroblasts, in which MyoD activity is compromised and late-stage differentiation is abrogated, Mef2 is functionally inactive (Novitch et al., 1999). This strongly suggests that the MRFs act in concert with the Mef2 family in orchestrating differentiation.

Mef2 is able to respond to specific growth factor signals by inducing expression of c-jun and thus playing a role in the regulation of cell cycle progression (Han and Prywes, 1995). It has been further demonstrated that this is mediated by ERK5/BMK1, which phosphorylates Mef2C in the C-terminal transactivation domain, thereby activating expression of c-jun (Kato et al., 1997; Kato et al., 2000; Yang et al., 1998). Yet, the main role of the Mef2 family in skeletal muscle is to promote differentiation through cooperative activation of gene expression. In order for Mef2 to function during differentiation, there must be regulation at the post-translational level to ensure proper temporal activity.

The p38 family ($\alpha,\beta,\gamma,\delta$) of MAP kinases has been shown to play an important role in stimulating differentiation (Lechner et al., 1996; Puri et al., 2000; Wu et al., 2000; Zetser et al., 1999). p38α and β preferentially phosphorylate Mef2A and Mef2C in the transactivation domain and enhance transcriptional activity (Han et al., 1997; Yang et al., 1999; Zetser et al., 1999; Zhao et al., 1999). In addition, activated MKK6, an upstream activator of p38, stimulates Mef2A and Mef2C transcriptional activity (Zhao et al., 1999). The PI-3′ kinase pathway, also shown to promote myogenic differentiation, activates Mef2 transcriptional activity and the expression of myogenin (Tamir and Bengal, 2000; Xu and Wu, 2000). This demonstrates that Mef2 acts to translate differentiation-stimulating signals into appropriate myogenic gene expression.

To ensure inactivation of muscle gene expression in proliferating myoblasts, the Mef2 family is transcriptionally inhibited by interactions with the class II histone deacetylases (HDACs), namely HDAC-4, -5, -7, and –9 (McKinsey et al., 2002). Calcium/calmodulin-dependent kinase (CaMK), which is activated in response to transient, high amplitude calcium spikes, phosphorylates two conserved serine residues in the N-terminus of HDAC-4, -5, -7 and –9, creating docking sites for 14-3-3 proteins (Grozinger and Schreiber, 2000; Wang et al., 2000). The association with 14-3-3 proteins masks the HDAC nuclear localization signal (NLS) and unmasks the nuclear

export signal (NES) (McKinsey et al., 2001; Wang and Yang, 2001). Therefore, the Mef2-HDAC interaction is disrupted, the HDACs are shuttled to the cytoplasm, and Mef2 is activated, presumably in part through association with the p300 histone acetyltransferase (Lu et al., 2000). Moreover, HDAC4 and 5 interact with heterochromatin protein 1 (HP1), which recognizes methylated lysine residues and recruits methyltransferase enzymes (Zhang et al., 2002). Thus, Mef2, when complexed with HDACs and HP1, is able to effectively repress myogenic transcriptional activation. Therefore, although Mef2 factors are not skeletal muscle-specific transcription factors, the muscle environment provides cues and coactivators that allow these proteins to acquire myogenic activity essential for differentiation.

Perspectives and Thesis Objectives

Although the importance of the MRFs in myogenic specification and differentiation has been clearly established, the precise mechanism by which the MRFs regulate myogenic gene expression is not well understood. Recently, the list of potential MyoD target genes has been expanded, and the possibility of gene-specific regulation has been introduced. Defining promoter-specific complexes and discovering the corresponding regulatory pathways will be essential for understanding muscle-specific gene expression and myogenic differentiation.

The objective of this thesis was to examine the role of auxiliary factors in regulating MRF transcriptional activity. Given that expression of E12 and E47 was undetectable in proliferating myoblasts, it was imperative to determine if another E-protein family member regulated MRF activity. As such, the first section of the thesis examined the role of HEBα and HEBβ in specifically regulating myogenic gene expression and differentiation.

Initiation of differentiation requires irreversible withdrawal from the cell cycle, yet the precise mechanism by which the MyoD and the MRFs regulate the cell cycle is not well defined. Therefore, this thesis sought to establish the role of MyoD in cell cycle regulation by assessing expression of cyclin genes in *MyoD*$^{-/-}$ myoblasts. Conversely, cell cycle regulators affect MyoD transcriptional activity. Thus, the last chapter of the thesis investigated the means by which pRb specifically regulates MyoD activity.

Preface to Chapter 2

The MRFs are hypothesized to activate muscle-specific gene expression as heterodimers with the E-protein family of transcription factors. Historically, E12 and E47 were postulated to be the preferential partners for the MRFs. However, in an attempt to discover the mechanism by which MEK1 inhibits MyoD, it was discovered that neither E12 nor E47 are expressed in C2C12 myoblasts or 10T½ fibroblasts (Perry et al., 2001). Therefore, this work sought to discover the role of HEB, another member of the E-protein family, which is highly expressed in skeletal muscle (Hu et al., 1992). This manuscript contributes to the field of skeletal muscle biology by demonstrating that HEB is the preferential partner for MyoD and myogenin, and that each isoform of HEB functions in a promoter-specific and MRF-specific manner to orchestrate myogenic differentiation. Moreover, this furthers the concept that each myogenic promoter must be considered individually, or as a member of a subset, when investigating regulatory mechanisms.

This manuscript will be submitted to Molecular Cell in the summer of 2004. Robert Perry was a Ph.D. student in the lab who did the initial expression studies demonstrating that E2A was not present, and analyzed synchronized cells for expression of HEB. Mélanie Fauteux is a technician in the lab who helped with transfections and luciferase assays. Charlotte Berkes is a Ph.D. student at the University of Washington who built the Mgn-Luc reporter vector, and the corresponding E-box mutants. I performed all of the experiments reported in the manuscript, constructed the figures and wrote the manuscript.

MyoD Synergizes with the E-Protein HEBβ to Induce Myogenic Differentiation

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Running Title: MyoD requires HEBβ for Differentiation

Key words: myoblasts, proliferation, differentiation, HEBβ, MyoD, Myogenin

Abstract

The MyoD family of bHLH transcription factors are postulated to function as heterodimers with members of the E-protein family to induce myogenic gene activation. The E-protein, HEB, is alternatively spliced to generate two isoforms, HEBα and HEBβ, however the molecular functions of the HEB proteins remain largely elusive. Our data demonstrated that both myoblasts and myotubes expressed HEBα, whereas expression of HEBβ was upregulated early in the differentiation program. In myoblasts, HEBα failed to synergize with MyoD to activate the myogenin promoter, and the E2 E-box specifically mediated the negative regulation. Upon induction of differentiation, a MyoD-HEBβ complex predominated and bound the E1 E-box of the myogenin promoter to stimulate transcription. Notably, forced expression of HEBβ with MyoD synergistically stimulated endogenous myogenin transcription whilst in growth conditions. However, after differentiation, HEBα synergized with myogenin, but not MyoD, to activate the MLC enhancer. Specific knock down of HEBβ by siRNA in myoblasts blocked differentiation and inhibited induction of myogenin transcription. Therefore, HEBα and HEBβ play a novel and central role in orchestrating the regulation of myogenic factor activity through myogenic differentiation.

Introduction

Basic helix-loop-helix (bHLH) containing transcription factors play an important role in the development of a variety of cellular lineages. Tissue-specific bHLH transcription factors homo- or heterodimerize to regulate the transcription of a restricted set of tissue-specific target genes (reviewed in Westerman et al., 2003). The myogenic regulatory factors (MRFs) are a group of basic helix-loop-helix (bHLH) transcription factors, consisting of MyoD, Myf5, myogenin, and MRF4, that control skeletal muscle development (Tapscott et al., 1988). Genetic and expression analysis has suggested that *Myf5* and *MyoD* act as determination factors that specify muscle progenitor cell identity. Although expression of MyoD or Myf5 commits a cell to the myogenic lineage, expression of MyoD is more effective at initiating differentiation. *Myogenin* and *MRF4* are induced upon differentiation and activate the differentiation program (reviewed in Parker et al., 2003)..

Transcription of muscle-specific genes is dependent upon dimer interactions between MRFs and the more ubiquitously expressed E-proteins, a process mediated by the helix-loop-helix motif. The E-protein family includes the E2A gene products (E12/E47), the E2-2 gene products (ITF-2A and 2B), and the HEB gene products (HEBα and HEBβ). Dimerization is dependent upon the relative abundance of each transcription factor, and/or the presence of other factors that may potentiate or inhibit dimerization. Therefore the ability of bHLH factors to homo- or heterodimerize in a variety of combinations raises the hypothesis that regulation of dimer formation is essential for control of tissue-specific gene expression.

The role of E2A in myogenesis was first postulated upon finding that MyoD was able to heterodimerize with E12 and E47 *in vitro* (Murre et al., 1989; Murre et al., 1991). Co-transfection of MyoD and E47 is required for E-box transcriptional activity in COS cells (Lassar et al., 1991). However, gene-targeting experiments have indicated that E12 and E47 are not essential for skeletal muscle formation or function (Zhuang et al., 1992).

HEB was isolated based on HLH region identity with E2A and ITF-2, and was shown to be highly expressed in skeletal muscle, as well as in the thymus and in B-cells (Hu et al., 1992). During embryonic development, HEB is widely expressed in the limb buds, somites, and proliferating neuroblasts. Expression is highest during the initial stages of differentiation and decreases once cellular differentiation has been completed (Neuman et al., 1993).

These results prompted us to investigate the importance of HEB in myogenesis. Our experiments establish an essential and novel role for HEBβ in regulating the switch from myogenin repression to myogenin activation as an immediate early step in the myogenic differentiation program.

Results

Induction of HEBβ during Myogenic Differentiation

The HEB gene is alternatively spliced to generate two isoforms, HEBα and HEBβ. The HEBβ isoform is distinguished by insertion of a 24-amino acid ankyrin-like motif, resulting from inclusion of a 72-base pair (bp) alternate exon (Klein et al., 1993). Prediction of the domain structure of HEB, based on studies of E12 and E47, reveals that this motif intersects the second putative activation domain (AD2), and may alter one face of the α-helix present in this domain (Goldfarb et al., 1998; Markus et al., 2002; Massari et al., 1999).

To investigate the expression pattern of the two isoforms of HEB in myogenic differentiation, RT-PCR was performed using RNA isolated from a differentiation time course of C2C12 myoblasts and 10T½ fibroblasts (Figure 1A). C2C12 myoblasts and 10T½ fibroblasts were grown to 85-90% confluency, and either harvested for RNA isolation (GM; DMEM + 10% FBS) or switched to differentiation medium (DM; DMEM + 2% horse serum). The cells were harvested and the RNA isolated daily over a four-day period after stimulation with DM.

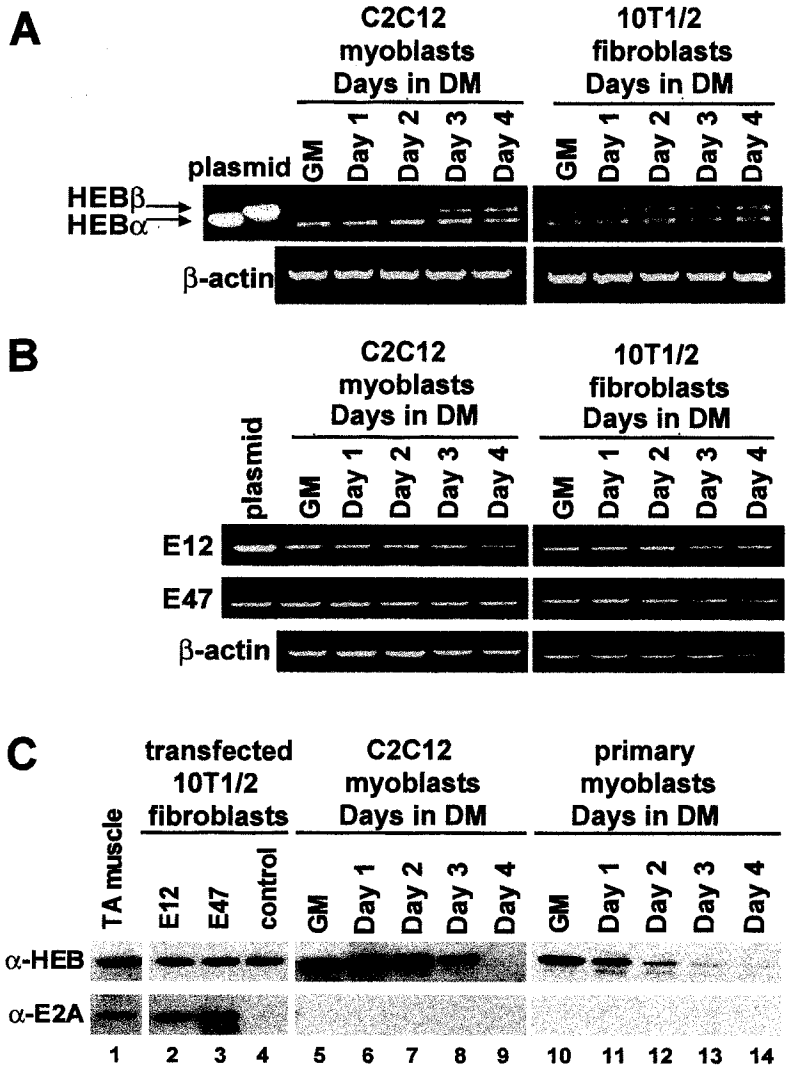
To distinguish HEBα from HEBβ by the size of the amplified product, PCR primers were designed that flanked the region surrounding the 72- bp ankyrin-like motif. RT-PCR analysis indicated that expression of HEBα mRNA remained constant throughout proliferation and differentiation in both C2C12 myoblasts (Figure 1A) and primary myoblasts (not shown). By contrast, expression of HEBβ was not detectable in proliferating myoblasts but was induced upon differentiation with increased expression evident as differentiation proceeded (Figure 1A). Notably, 10T½ fibroblasts displayed no modulation in HEB isoform expression in high versus low mitogen conditions (Figure 1A). Furthermore, levels of E12 and E47 mRNAs remained unaltered in myoblasts through differentiation or in 10T1/2 fibroblasts after serum withdrawal (Figure 1B).

Western blot analysis of protein extracts using an antibody that recognized both isoforms of HEB, demonstrated that HEB was abundantly expressed in proliferating primary myoblasts and C2C12 myoblasts (Figure 1C). However, expression of HEB protein was almost undetectable by 4 days after the onset of differentiation, suggesting that expression of HEB is further regulated at the post-transcriptional level. (Figure 1C). Moreover, this indicates that HEB plays an important role during the initial stages of differentiation.

Western analysis revealed readily detectable E12 and E47 protein in *tibialis anterior* (TA) muscle (Figure 1C, lane 1), transfected fibroblasts (Figure 1C, lanes 2 and 3), and adult mouse mammary tissue (not shown). Strikingly, expression of E12 and E47 was below the limit of detection in C2C12 myoblasts, primary myoblasts and 10T½ fibroblasts (Figure 1C, lanes 4-14). Therefore, the low levels of E12 and E47, and high expression of HEB proteins supported the notion that HEB was a significant transcriptional partner of the MyoD family of bHLH factors. Moreover, the absence of HEBβ in proliferating myoblasts and its upregulation during myogenic differentiation is consistent with a differentiation-specific role for HEBβ.

Figure 1. HEB expression is highly regulated in proliferating and differentiating myoblasts.
A. RNA was isolated from a differentiation time course of C2C12 myoblasts and 10T1/2 fibroblasts, and analyzed by RT-PCR. *HEBα* was expressed under both growth and differentiation conditions whereas *HEBβ* was induced upon differentiation of C2C12 cells.
B. RT-PCR detection of *E12* and *E47* mRNAs in C2C12 and 10T1/2 cells.
C. Western analysis of HEB and E2A expression reveals abundant HEB protein but no detectable E2A protein in myoblasts and fibroblasts .

Figure 1


A
C2C12 myoblasts Days in DM
10T1/2 fibroblasts Days in DM
plasmid
GM
Day 1
Day 2
Day 3
Day 4
HEBβ
HEBα
β-actin
B
C2C12 myoblasts Days in DM
10T1/2 fibroblasts Days in DM
plasmid
GM
Day 1
Day 2
Day 3
Day 4
E12
E47
β-actin
C
transfected 10T1/2 fibroblasts
C2C12 myoblasts Days in DM
primary myoblasts Days in DM
TA muscle
E12
E47
control
GM
Day 1
Day 2
Day 3
Day 4
α-HEB
α-E2A
1 2 3 4 5 6 7 8 9 10 11 12 13 14

HEB Regulates Binding of MyoD to Muscle-Specific Promoters

To determine if HEB is associated with promoters of myogenic-specific genes, chromatin immunoprecipitation (ChIP) assays were performed using either proliferating or differentiating C2C12 myoblasts (Figure 2A). Prior to the immunoprecipitation, a small volume of the extract from each sample was removed, the cross-links reversed, and the DNA purified. This "input" DNA was carefully quantitated, and the relative amount of immunoprecipitated (IP) DNA used in each PCR reaction was normalized for the amount of input DNA. PCR amplification of the IP DNA was undertaken with primers specific for myogenic promoter regions; primers specific for the amylase promoter served as a negative control.

In proliferating C2C12 myoblasts, binding of the MRFs and HEB to the myogenin promoter was minimal, even though MyoD, Myf5 and HEB were expressed (Figure 2A; data not shown). After 2 days in differentiation medium, MyoD, myogenin and HEB were all associated with the myogenin promoter. Therefore, MyoD and HEB associated with the myogenin promoter to a greater extent in differentiation. In a similar manner, binding of MyoD, myogenin and HEB to the MLC enhancer was also enriched after differentiation (Figure 2A). Interestingly, Myf5 was associated with the MLC enhancer in both proliferation and after 2 days in differentiation, but not with the myogenin promoter.

To determine if binding of MyoD and HEB to myogenic-specific promoters was mutually dependent, ChIP assays were repeated in transfected 10T½ fibroblasts. 10T½ fibroblasts are non-myogenic, but are amenable to myogenic conversion upon expression of MyoD. Co-expression of either HEBα or HEBβ enhanced binding of MyoD to the MLC enhancer (Figure 2B). Moreover, binding of endogenously expressed HEB to the MLC enhancer was dependant on expression of MyoD (Figure 2B). Taken together, these findings indicate that HEB binds to myogenic E-boxes in association with MyoD or myogenin.

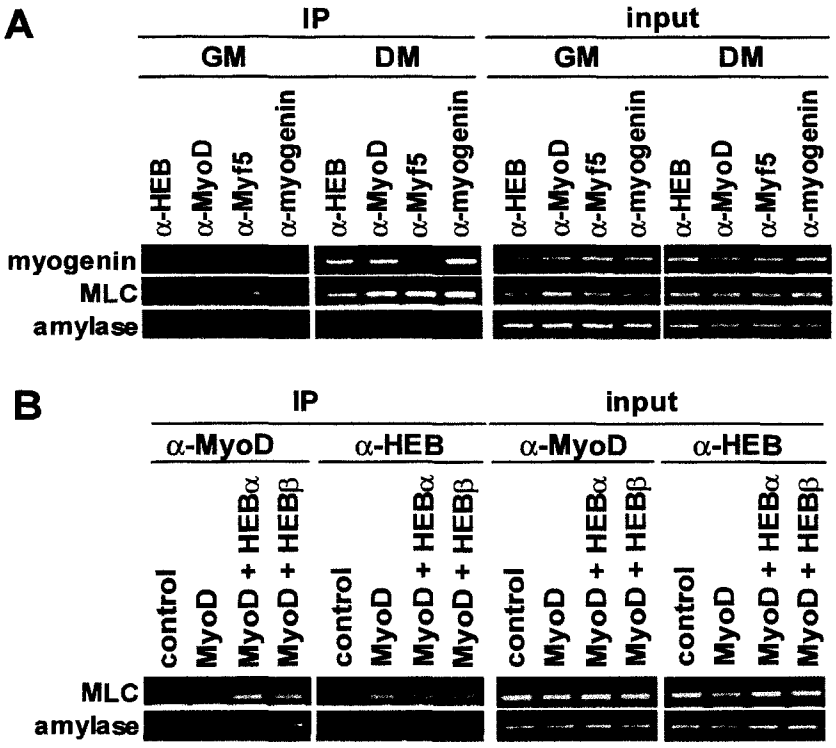
HEBα and HEBβ Display Promoter-Specific Activity

To study the role of HEB in transcriptional activation, the mouse cDNAs for both HEBα and HEBβ were cloned by RT-PCR, using mRNA isolated from differentiating wild type primary myoblasts. The full-length cDNAs were cloned into the EMSV vector, the same vector from which the MRFs were expressed. MRF transcriptional activity was assessed using reporter vectors containing either four repeats of the CACCTG E-box from the MCK promoter (*4R-Luc*), a portion of the murine myogenin promoter (*Mgn-Luc*), or rat myosin light chain enhancer (*MLC-Luc*).

Co-transfection of HEBα or HEBβ with *4R-Luc* into C2C12 cells augmented the activity of the reporter in both proliferation and differentiation (Figure 3A). Notably, the increase in *4R-Luc* activity was increased 1.8-fold with HEBα relative to HEBβ. The MLC enhancer (*MLC-Luc*) displayed increased activity in both proliferating and differentiating conditions only when HEBα was over-expressed. Neither E12 nor E47

Figure 2. HEB binds muscle-specific promoters.
A. Chromatin immunoprecipitation (ChIP) revealed that binding of HEB to the myogenin promoter and myosin light chain (MLC) enhancer increased markedly after differentiation. Amylase was amplified as a control for specificity, and input DNA was amplified with each primer set to ensure proper DNA quantitation.
B. ChIP analysis of transfected 10T1/2 fibroblasts revealed that HEB overexpression facilitated MyoD-binding to the endogenous MLC promoter. Moreover, HEB binding to the MLC enhancer requires MyoD expression. Amylase was amplified as a control for specificity, and input DNA was amplified to ensure proper quantitation.

Figure 2.


A
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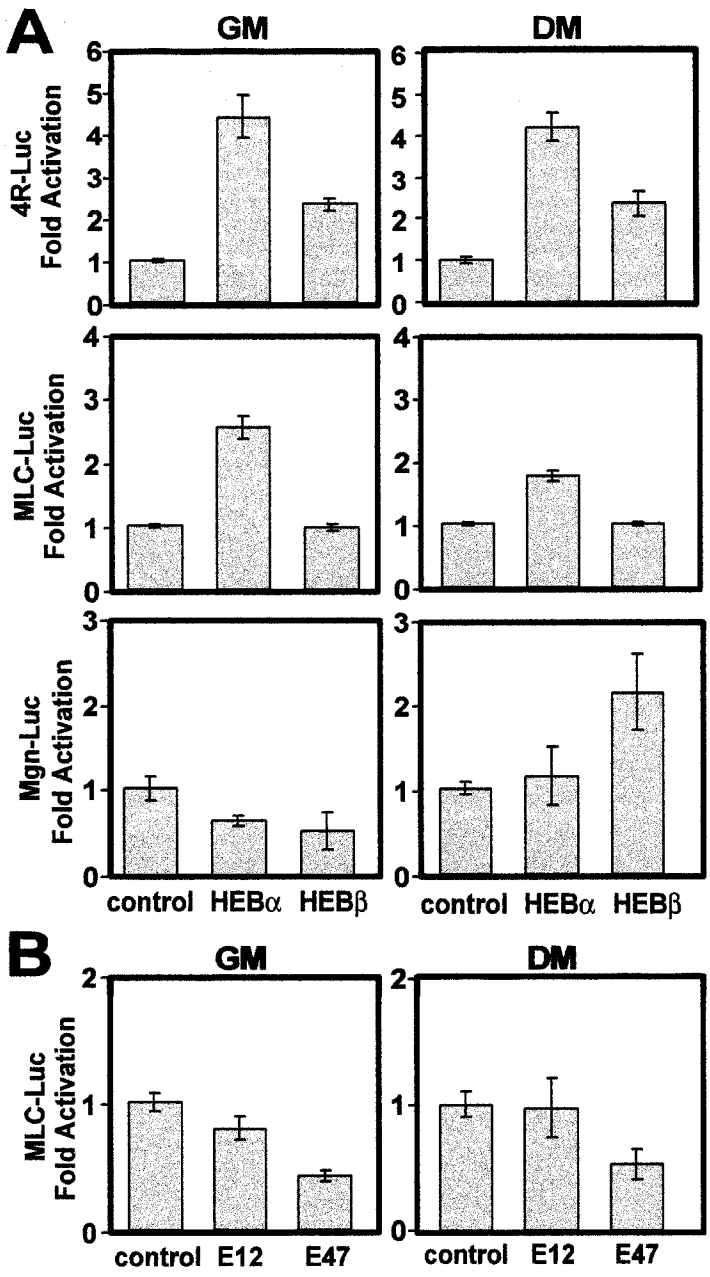
input
GM
DM
GM
DM
α-HEB
α-MyoD
α-Myf5
α-myogenin
myogenin
MLC
amylase
B
IP
input
α-MyoD
α-HEB
α-MyoD
α-HEB
control
MyoD
MyoD + HEBα
MyoD + HEBβ
MLC
amylase

Figure 3. Expression of HEB specifically enhances the activity of muscle-specific promoters in C2C12 myoblasts.
A. C2C12 myoblasts were transfected with the indicated reporter plasmids, in addition to either empty EMSV plasmid (control), or plasmid expressing HEBα or HEBβ. Luciferase activity was normalized to protein, and fold activation was determined by setting the activity of the control transfection as 1. The bars represent the mean fold activation, and the error bars represent the standard deviation (n=9).
B. C2C12 myoblasts were transfected with MLC-Luc as in A, but with either control plasmid or a plasmid expressing E12 or E47.

Figure 3.


A
GM
DM
4R-Luc
Fold Activation
MLC-Luc
Fold Activation
Mgn-Luc
Fold Activation
control
HEBα
HEBβ
B
GM
DM
MLC-Luc
Fold Activation
control
E12
E47

stimulated the activity of *MLC-Luc* in either proliferation or differentiation conditions (Figure 3B). Strikingly, the activity of the myogenin promoter (*Mgn-Luc*) was only enhanced in differentiation, and only in the presence of HEBβ (Figure 3A). Therefore, overexpression of HEB in myoblasts stimulated the activity of myogenic promoters in myocytes. Notably, HEBα and HEBβ isoforms stimulated transcription of different reporters suggesting that HEB isoforms either display binding preferences or have different activities on different promoters.

HEBα and HEBβ Differentially Synergize with MyoD and Myogenin in a Differentiation-Specific Manner

To determine the effect of HEB on each MRF, 10T1/2 fibroblasts were transfected with each reporter vector individually, and one of *EMSV-MyoD*, *-Myf5*, *-myogenin*, or *EMSV* control, in addition to *EMSV-HEBα*, *EMSV-HEBβ*, or *EMSV* alone. In proliferation conditions, both HEBα and HEBβ increased the activity of MyoD on *MLC-Luc*, but not Myf5 or myogenin activity (Figure 4A). In differentiation conditions, only the activity of myogenin was increased, and specifically through HEBα.

A potential complication was that two transcription factors, both capable of binding to the E-box sequence, were being assayed. Therefore, to determine if the increase in activity was additive (each transcription factor acting individually) or synergistic (both factors acting together), a value, designated as "synergy" was calculated (Markus et al., 2002). If the value for synergy was 1, or close to 1, then the effect of HEB and the MRF together on the activity of the enhancer was considered additive. If the value was significantly greater than 1, then the two transcription factors were considered to have functioned synergistically (see Materials and Methods).

HEBα and HEBβ both acted synergistically and specifically with MyoD in proliferation conditions to activate the MLC enhancer (Figure 4A). Although the cells were maintained in growth medium, they initiated differentiation, as indicated by the expression of endogenous *myogenin* gene (Figure 5A, lanes 4-6). This suggested that the increase in activity is representative of the initial stages of differentiation, rather than a true proliferative response. After 2 days in differentiation, HEBα synergized with myogenin to activate the MLC enhancer. This corresponded with the results observed in C2C12 myoblasts in high serum, in which only HEBα activated the MLC enhancer.
MyoD and HEBβ functioned synergistically to activate the myogenin promoter in growth conditions (Figure 4B). However, in differentiation conditions both HEBα and HEBβ synergized with myogenin to activate the myogenin promoter. Intriguingly, neither HEBα nor HEBβ functioned synergistically with the MRFs to activate *4R-Luc* (Figure 4C).

In order to further validate the data obtained using reporter vector assays, we exogenously expressed the MRFs in 10T½ cells, either alone or with HEBα or HEBβ. In agreement with the reporter vector assays, HEBβ was able to enhance expression of endogenous myogenin to a greater extent than HEBα (Figure 5A, lanes 4-6). This was not the result of increased MyoD or Myf5 expression as MyoD levels remained constant and Myf5 was undetectable in 10T1/2 cells transfected with MyoD. In addition,

Figure 4. HEB-dependent increase in MRF activity is synergistic and multifaceted.
A. 10T1/2 fibroblasts were transfected with the MLC-Luc reporter plasmid, and MyoD, Myf5 or myogenin expressing plasmids with either HEBα or HEBβ expression plasmids. The panels on the left represent fold activation. The activity of the MLC-Luc reporter plasmid alone was arbitrarily set at 1, and the activity in the presence of the effectors was determined relative to this value (Fold Activation). The bars represent the mean fold activation and the error bars represent the standard deviation (n=9). The panels on the right represent synergy (see Materials and Methods). The horizontal line represents the value at which the two effectors function independently, or additively. The bars represent the mean synergy value and the error bars represent the standard deviation (n=9).
B. The same transfection as A, but with the Mgn-Luc reporter plasmid.
C. The same transfection as A and B, but with the 4R-Luc reporter plasmid.

Figure 4.

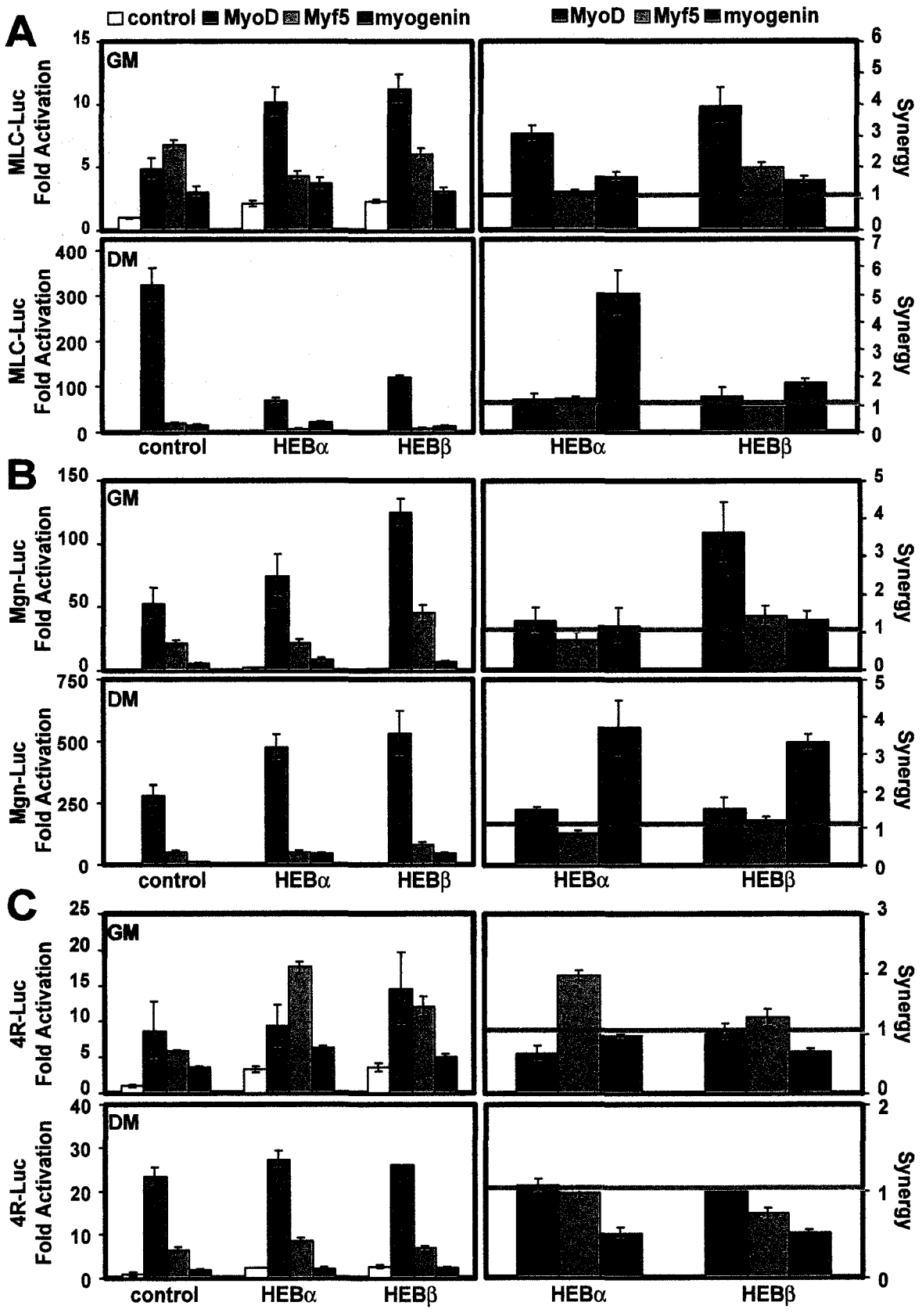
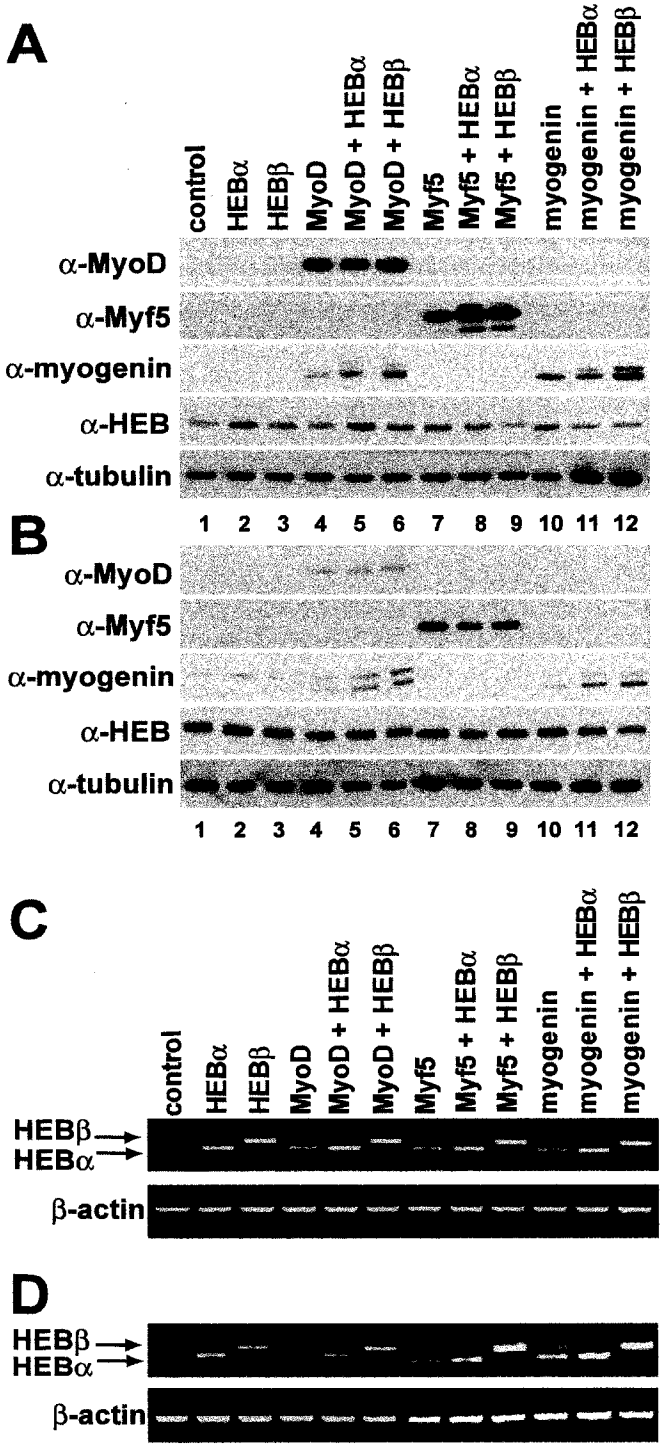

A
control MyoD Myf5 myogenin
MyoD Myf5 myogenin
GM
DM
MLC-Luc Fold Activation
Synergy
B
Mgn-Luc Fold Activation
C
4R-Luc Fold Activation
control
HEBα
HEBβ

Figure 5. HEBβ enhances MyoD-mediated expression of endogenous myogenin.
A. 10T1/2 fibroblasts were transfected as indicated, and the cells were maintained in growth medium (GM) or induced to differentiate for two days (DM). Protein was isolated and 25 μg was analyzed by western blot with the indicated antibodies. Expression of tubulin was monitored as a control for protein quantitation.
B. 10T1/2 fibroblasts were transfected as indicated, and the cells were maintained in growth medium (GM) or induced to differentiate for two days (DM). RNA was isolated and analyzed by RT-PCR. Forced expression of HEBα and HEBβ in 10T1/2 fibroblasts resulted in the exclusive expression of the overexpressed isoform.

Figure 5.


A
control
HEBα
HEBβ
MyoD
MyoD + HEBα
MyoD + HEBβ
Myf5
Myf5 + HEBα
Myf5 + HEBβ
myogenin
myogenin + HEBα
myogenin + HEBβ
α-MyoD
α-Myf5
α-myogenin
α-HEB
α-tubulin
1 2 3 4 5 6 7 8 9 10 11 12
B
α-MyoD
α-Myf5
α-myogenin
α-HEB
α-tubulin
1 2 3 4 5 6 7 8 9 10 11 12
C
control
HEBα
HEBβ
MyoD
MyoD + HEBα
MyoD + HEBβ
Myf5
Myf5 + HEBα
Myf5 + HEBβ
myogenin
myogenin + HEBα
myogenin + HEBβ
HEBβ
HEBα
β-actin
D
HEBβ
HEBα
β-actin

transfection with Myf5 did not induce expression of myogenin in GM, even when co-expressed with HEB isoforms (Figure 5A, lanes 7-9).

Interestingly, myogenin-mediated expression of myogenin in serum-starved 10T1/2 fibroblasts was enhanced in presence of exogenous HEBβ (Figure 5A, lane 12). This was not the result of myogenin-induced expression of endogenous MyoD, as MyoD was undetectable by western blot (Figure 5A, lanes 10-12). In addition, it was not the result of a HEB-mediated increase in the activity of the *MSV* LTR, since the expression of *EMSV-MyoD* and *EMSV-Myf5* were not altered in the presence of either HEBα or HEBβ. Taken together, these data reveal a hitherto unknown role for HEBβ in directing transcription during myogenic differentiation.

Binding to E1- and E2-E Boxes in the Myogenin Promoter by Specific MyoD-HEB Complexes Regulates Induction of Transcription during Differentiation

The observation that distinct combinations of MyoD and myogenin with HEBα or HEBβ functioned is a synergistic manner, either in growth or differentiation depending on the promoter, suggested the possibility that specific complexes were being recruited to individual E-boxes. Therefore, we investigated whether MyoD and HEBβ synergistic activation of the myogenin promoter was E-box specific.

The 224-bp minimal myogenin promoter required to recapitulate myogenin expression *in vivo* contains two consensus E-box sequences, E1 (CAGTTG) and E2 (CACATG) (Figure 6A). Mutation of the E1 E-box (*Mgn-LucE1^{mut}*) or deletion of the E2 box (*Mgn-LucΔE2*) decreased MyoD-mediated activation of the myogenin promoter during early differentiation (Figure 6B). In contrast, the low ability of HEBα and HEBβ to activate *Mgn-Luc* transcription was unaffected by mutation of E1, and was actually increased upon deletion of E2. Furthermore, HEBβ and more strikingly, HEBα, displayed significantly increased activity on the double E-box mutant reporter vector (*Mgn-LucE1^{mut}/ΔE2*).

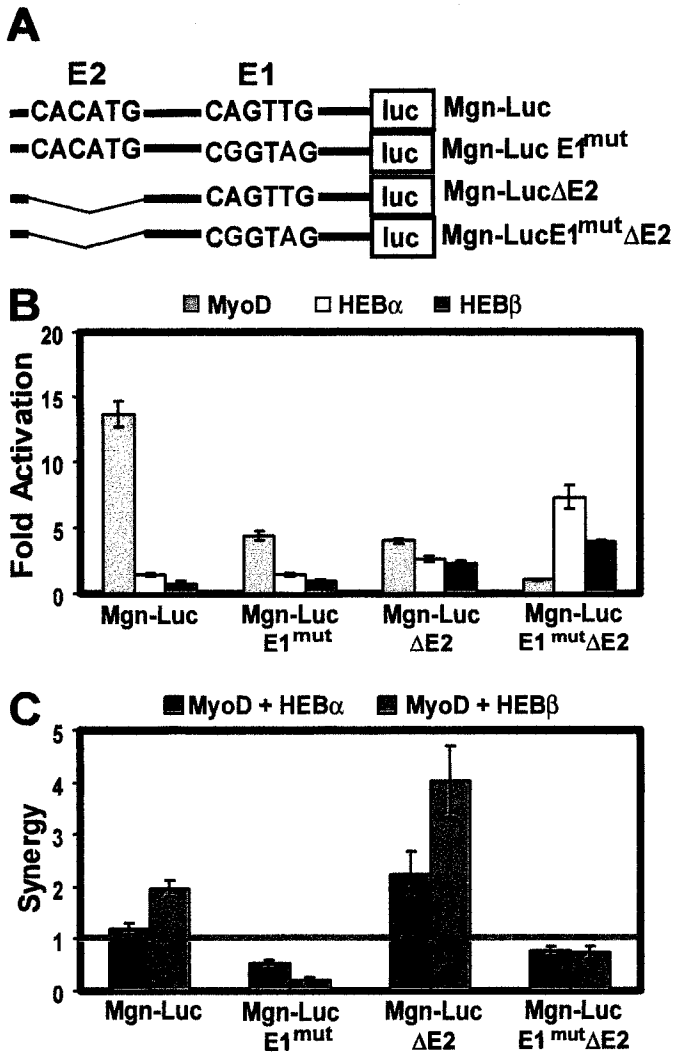
In absence of the E1 E-box, HEBα and HEBβ appeared to function as inhibitors of MyoD as evidenced by negative synergy (Figure 6C). Moreover, deletion of the E2 E-box resulted in a robust 2-fold increase in synergy between MyoD and HEBβ (Figure 6C). By contrast, both HEBα and HEBβ were unable to function synergistically with MyoD to activate *Mgn-LucE1^{mut}/ΔE2* (Figure 6C). We interpret these data to suggest that in growth conditions, a MyoD-HEBα complex binds the E1 E-box to inhibit myogenin transcription, and this inhibition requires the E2 E-box complex. During initiation of differentiation, a MyoD-HEBβ complex binds the E1 E-box to stimulate myogenin transcription. Together, these experiments underscore the dynamic role played by HEB isoforms in facilitating MyoD activity during myogenic differentiation.

Knock Down of HEBβ Inhibits Differentiation

Our experiments indicate that in growth conditions MyoD and HEBβ synergistically activate myogenin transcription. These data therefore suggest that

Figure 6. MyoD activity on the myogenin promoter is E1 E-box specific.
A. Schematic diagram of the E-boxes in the *Mgn-Luc* reporter vector and the mutant reporter vectors in which the E1 E-box has been mutated (*Mgn-LucE1*^{mut}), or the E2 E-box has been deleted (*Mgn-LucΔE2*), or both (*Mgn-LucE1*^{mut}*/ΔE2*).
B. Activity of *Mgn-Luc* in transfected 10T1/2 fibroblasts. Cells were transfected with either EMSV control, MyoD, HEBα, or HEBβ, in addition to each of the reporter plasmids indicated. The cells were maintained in growth medium, harvested and assayed for luciferase activity. The bars represent the mean fold activation, and the error bars represent standard deviation (n=3).
C. Synergy between MyoD and HEB isoforms in the activation of *Mgn-Luc* in transfected 10T1/2 fibroblasts. Cells were transfected with combinations of MyoD and HEBα or MyoD and HEBβ. The horizontal line represents the value at which the two effectors function in an additive manner. The bars represent the mean synergy value and the error bars represent the standard deviation (n=3).

Figure 6.


A
E2
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CACATG
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Mgn-Luc
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CGGTAG
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Mgn-Luc E1mut
CAGTTG
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CGGTAG
luc
Mgn-LucE1mutΔE2
B
MyoD
HEBα
HEBβ
Fold Activation
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Mgn-Luc
Mgn-Luc E1mut
Mgn-Luc ΔE2
Mgn-Luc E1 mutΔE2
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MyoD + HEBβ
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Mgn-Luc ΔE2
Mgn-Luc E1 mutΔE2

induction of HEBβ in myoblasts acts as a switch that permits progression through the immediate early differentiation program. To investigate the effect of loss of HEB expression on myogenic differentiation, C2C12 myoblasts were transfected with siRNAs specific for HEB and assessed for myogenin expression 48 hours after stimulation with differentiation medium (Figure 7). Typically, transient transfection resulted in about 10-12% of cells transfected as judged by Cy3-labeled siRNA uptake (Figure 7C).

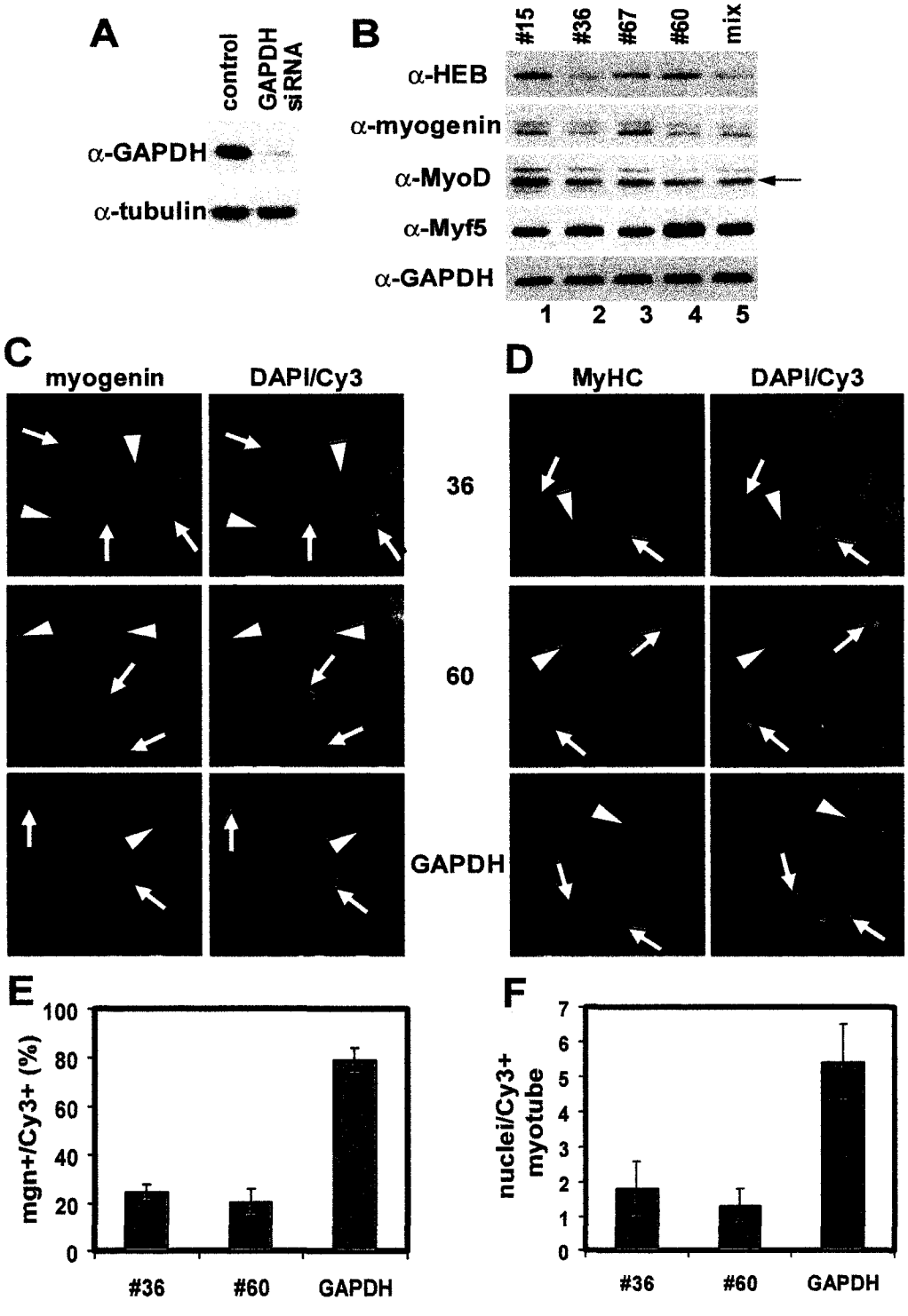
Transfection of GAPDH-specific siRNA effectively inhibited expression of GAPDH protein as demonstrated by western blot analysis (Figure 7A). Transfection of C2C12 myoblasts with HEB-specific siRNA#36 either alone, or in combination with the other HEB-specific siRNAs (mix), resulted in an apparent high level inhibition of HEB expression given the overall transfection efficiency (Figure 7B, lanes 2 and 5). By contrast, siRNA#15 had no effect, as thus served as a negative control (Figure 7B, lane 1). Moreover, siRNA#36 knock down of HEB expression resulted in a markedly decreased myogenin expression, despite a similar level of MyoD expression (Figure 7B, lane 2). Importantly, siRNA#60, designed to specifically bind HEBβ, and not HEBα, inhibited myogenin expression (Figure 7B, lane 4). Interestingly, siRNA#60 also induced an increase in Myf5 expression, raising the possibility that MyoD and HEBβ negatively regulates Myf5 transcription.

HEB-specific siRNAs were labelled with Cy3, and thus detectable by immunofluorescence following transfection. The transfection efficiency for each siRNA was approximately equal, with 9.8% of cells transfected with GAPDH siRNA, 12.8% with siRNA #36, and 12.4% with siRNA #60. As demonstrated in Figure 7C, C2C12 cells transfected with high levels of siRNA #36 and #60 exhibited an inability to upregulate endogenous myogenin (Figure 7C, arrows) relative to untransfected cells (Figure 7C, arrowheads). By contrast, cells transfected with GAPDH siRNA expressed normal levels of myogenin (Figure 7C, arrow in upper panel). In fact, myogenin is expressed in 78.7% cells transfected with GAPDH siRNA, but is expressed in only 24.6% of cells transfected with HEB siRNA #36, and 20.5% of cells transfected with HEBβ-specific siRNA #60 (Figure 7E).

After 4 days in DM, GAPDH and untransfected C2C12 cells displayed normal differentiation kinetics as evidenced by the presence of multiple nuclei and myosin heavy chain (MyHC) expression (Figure 7C, arrowheads in lower panels). By contrast, cells transfected with HEB-specific siRNAs displayed an overall delay in differentiation as judged by fusion index and reduced MyHC expression. For example, GAPDH siRNA transfected cells contained on average 5.4 nuclei per myotube (Figure 7C, arrow in lower panel, Figure 7F), whereas cells transfected with HEB siRNA#36 exhibited an average of 1.8 nuclei per myotube (Figure 7C, arrow in lower panel, Figure 7F). Moreover, those transfected with HEBβ-specific siRNA #60 displayed an average of 1.3 nuclei per myotube (Figure 7C, arrow in lower panel, Figure 7F). Together, these data therefore confirm that HEBβ is required for the switch from myoblast proliferation to myogenic differentiation.

Figure 7. Knock down of HEB expression inhibits induction of myogenin and differentiation.
A. Western analysis of C2C12 myoblasts transfected with GAPDH siRNA. Note the reduced levels of GAPDH protein only in cells transfected with siRNA specific for GAPDH. Expression of tubulin was monitored as a control for protein quantitation.
B. Western analysis of C2C12 cells transfected with HEB-specific siRNAs and then induced to differentiate for 48 hours. Note the reduced level of HEB and myogenin protein present in cells transfected with siRNAs specific for HEBα/β (#35, mix) and HEBβ (#60). HEB siRNA#15 did not reduce HEB expression.
C & D. Immunofluorescent detection of myogenin and myosin heavy chain (MyHC) in siRNA-transfected C2C12 cells. Cells were fixed either after 48 h of differentiation for detection of myogenin (C) or after 96 h for detection of MyHC (D). Nuclei were counterstained with DAPI. Note the inhibition in myogenin induction (C) and delayed formation of multinucleated myotubes (D) after HEBα/β (#36) and HEBβ (#60) induced knock down of HEB expression.

Figure 7.


A
control
GAPDH siRNA
α-GAPDH
α-tubulin
B
#15
#36
#67
#60
mix
α-HEB
α-myogenin
α-MyoD
α-Myf5
α-GAPDH
1 2 3 4 5
C
myogenin
DAPI/Cy3
36
60
GAPDH
D
MyHC
DAPI/Cy3
E
mgn+/Cy3+ (%)
100
80
60
40
20
0
#36
#60
GAPDH
F
nuclei/Cy3+ myotube
7
6
5
4
3
2
1
0
#36
#60
GAPDH

Discussion

Our experiments indicate that differentiation-dependent alternative splicing of *HEB* plays a hitherto unappreciated regulatory role during myogenic differentiation. HEBα is expressed in both proliferating and differentiating myoblasts. However, HEBβ is expressed exclusively during myogenic differentiation (Figure 1A, 1C). Expression of myogenin is a hallmark of early differentiation, and is highly regulated in order to prevent premature induction of differentiation. The MyoD and HEBβ-specific activation of the myogenin promoter is an important means of regulation since expression of HEBβ is not induced until differentiation has been initiated. In later differentiation, both HEBα and HEBβ activated the myogenin promoter synergistically and specifically with myogenin. This observation is consistent with the notion that MyoD plays a central role in regulating the switch from myoblast proliferation to initiation of differentiation. By contrast, myogenin functions to enforce terminal differentiation by enhancing transcriptional activity of differentiation-specific target genes (Bergstrom and Tapscott, 2001). Therefore, MyoD initiates expression of myogenic genes, but once myogenin is expressed, the two MRFs function cooperatively and synergistically with HEB, to fully activate transcription.

E12 and E47 have been proposed to function as the heterodimerization partners for the MRFs, thereby enhancing activation of muscle-specific genes (Lassar et al., 1991). Our experiments surprisingly suggested that while C2C12 myoblasts and primary myoblasts expressed E12/E47 mRNA, the level of E2A protein was below the limit of detection by Western and ChIP analysis using several commercial preparations of antibody. Moreover, in C2C12 myoblasts (Figure 3B), and 10T1/2 fibroblasts expressing MyoD or Myf5 (data not shown), neither E12 nor E47 were able to augment the activity of *MLC-Luc* or *4R-Luc*. Taken together, these data provide a compelling argument that HEB serves as an important E-protein partner of MyoD and myogenin, and functions in a promoter-specific manner to support and enhance myogenic differentiation.

One interesting possibility is that HEBα and HEBβ each participate in the formation of distinct transcription complexes. The activation domain of a sequence specific *trans*-acting factor typically associates with coactivators, such as elements of the basal transcriptional machinery or chromatin-modifying enzymes (Featherstone, 2002). Activators, in cooperation with coactivators, establish the pre-initiation complex (PIC), which includes the TATA-binding protein (TBP) and TBP-associated factors (TAFs). The PIC is not assembled unless a transcriptional activator is present, even if the DNA is accessible. Moreover, the PIC is not universal, and in fact, TBP can form a variety of complexes that may be promoter-specific, or unique to the state of the cell (Hochheimer and Tjian, 2003; Veenstra and Wolffe, 2001). Therefore, it is possible that HEBα and HEBβ facilitate the formation of unique PICs on different myogenic promoters.

A number of studies have demonstrated that HEB is able to function both as an activator and a repressor on a given promoter, depending on the presence of other factors (Mandolesi et al., 2002; Rose et al., 2001; Watanabe et al., 1993). In particular, HEB represses activation of the GAP-43 gene in PC12 cells, pending expression of

Nex1/MATH2, a member of the neuroD family of bHLH factors (Uittenbogaard et al., 2003). Nex1/MATH2 binds to the GAP-43 promoter, alleviates HEB-mediated repression and activates expression of the gene, resulting in neurite outgrowth and neuronal differentiation. Indeed, alleviating the repression may involve a switch from a HEBα-specific complex to a HEBβ-specific complex.

The ability of HEBβ to synergize with MyoD appeared to be promoter specific, given that MyoD and HEBβ were unable to synergistically activate the *4R-Luc* reporter plasmid. Although this may be the result of differences in core E-box sequence, it is more likely to be indicative of the importance of the context or the position of the E-box within a promoter. MyoD-mediated activation of the *Mgn-Luc* required at least one consensus E-box (Figure 6B); in the presence of both, activity is cooperative, resembling the *MCK* enhancer (Weintraub et al., 1990).

Although MyoD alone was able to activate a myogenin promoter containing only the E2 E-box (*Mgn-LucE1*^{mut}), overexpression of either HEBα or HEBβ repressed MyoD activity on this reporter construct. Moreover, the activity of the E1-complex was regulated through the E2-complex, given that MyoD-HEBβ synergy was enhanced approximately 2-fold in the absence of the E2 E-box, and surprisingly, MyoD and HEBα synergistically activated this same reporter construct (*Mgn-LucΔE2*). Importantly, binding of a MyoD-HEBβ complex to the E1 E-box was required to transcriptionally activate the myogenin promoter. Taken together, this indicates that an active MyoD-HEBβ complex replaces an inhibitory MyoD-HEBα complex on the E1 E-box of the myogenin promoter upon initiation of differentiation.

The E1 E-box is proximal to the TATA box and the initiator (Inr) element of the promoter. The ankyrin-like motif of HEBβ may play an essential role in recruiting the preinitiation complex to the E1 E-box. The role of the E2 E-box may be twofold, in that it regulates precocious activation by means of the E1 E-box, but may also bind myogenin to maintain expression of the gene. MyoD and myogenin bind to distinct portions of the MLC enhancer and function cooperatively in order to fully activate expression of MLC (Asakura et al., 1993). As such, HEBα or HEBβ may utilize this E-box in association with myogenin in later differentiation to sustain expression of myogenin.

Knock down experiments demonstrated that HEBβ is essential for myogenin expression and differentiation into multinucleate myotubes. Loss of HEB did not appear to inhibit expression of myosin heavy chain, but did alter differentiation, as the average number of nuclei per myotube is considerably reduced in cells positive for HEB siRNA. Moreover, siRNA transfected cells display a higher level of Myf5 expression (Figure 7, lane 4). This is reminiscent of primary myoblasts from *MyoD*$^{-/-}$ mice, which express more *Myf5*, and remain primarily mononuclear after mitogen withdrawal (Sabourin et al., 1999).

Therefore, HEB plays an important role in the formation of promoter-specific complexes on muscle-specific genes. HEBβ is expressed exclusively after initiation of differentiation in myoblasts and specifically forms a distinct activation complex in cooperation with MyoD to induce myogenin transcription. This study provides the first evidence that E-proteins, and specifically HEB, directly regulates the activity of the

MyoD-family of bHLH transcription factors in an isoform- and promoter-specific manner. Determining the factors that regulate differentiation-specific expression of HEBβ will be important for understanding the early aspects of myogenic differentiation. Indeed, splicing is regulated in other tissues, such as neurons, and the ratio of expression of HEBα to HEBβ is characteristic of each cell type. Altering the ratio in these tissues likely plays an essential role in the switch from proliferation to differentiation, as it does in skeletal muscle.

Materials and Methods

Cell Culture and Transfections

C3H10T½ fibroblasts (ATCC; CCL-226) and C2C12 myoblasts (ATCC; CRL-1772) were maintained in growth medium (GM), consisting of Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini Bioscience) and 0.1 units/ml of penicillin and 0.1 μg/ml of streptomycin (Invitrogen; 1X). Transfections were performed using the calcium phosphate method as previously described (Perry et al., 2001). Briefly, 1 day prior to transfections, cells were plated at 3.5 X 10^4 cells per 60-mm dish (Falcon) for 10T1/2 cells, or 2.5 X 10^4 cells per 60-mm dish for C2C12 cells. Cells were incubated with the calcium phosphate precipitate for 16 hours, washed twice with PBS and refed with GM. 24 hours after refeeding, cells were either harvested (GM) or washed once with PBS and refed with differentiation medium and cultured for an additional 48 hours (DM; DMEM + 2% horse serum (Invitrogen) and penicillin/streptomycin). Transfections were assayed for efficiency by inclusion of a plasmid expressing either β-gal or GFP.

Primary myoblasts were isolated as described previously (Sabourin et al., 1999) and maintained in Ham's F10 medium (Invitrogen) supplemented with 20% FBS, 2X penicillin/streptomycin, and 12.5 ng/ml basic FGF (Invitrogen). Primary myoblasts were induced to differentiate by washing the cells once with PBS and refeeding with DMEM supplemented with 5% horse serum and 2X penicillin/streptomycin.

Plasmids

Plasmids expressing MyoD, Myf5 or myogenin were constructed in pEMSV as previously described (Davis et al., 1987). Expression plasmids encoding HEBα and HEBβ were constructed by RT-PCR using RNA from differentiating primary myoblasts as template, and primers specific for the N- and C-terminus of murine HEB:

F1 (5') ATGAATCCCCAGCAGCAGCGCATG (3')

R1 (5') CCATGGTCAGATGACCCATAGGGTTGGT(3').

The PCR products were cloned into the EcoRI site of pEMSV. The plasmids encoding ITF-2A and ITF-2B were kind gifts of Dr. Ilona Skerjanc. The E12 expression plasmid was a kind gift of Dr. Lauren Snider, and the E47 expression plasmid was a kind gift of Cornelius Murre. The 4R-Luc plasmid was constructed by cloning the BamH1 fragment from 4RtkCAT (Weintraub et al., 1990) into the BglII site of the pGL3 Promoter reporter vector (Promega). The BamH1 fragment from MLC-CAT (Donoghue et al., 1988) was also cloned into the BglII site of this vector to obtain the MLC-Luc. Myogenin-luciferase (MG-Luc) and the E-box mutants were constructed as previously described (Berkes et al., 2004). Vectors lacking cDNA inserts or promoter elements were used as controls in the transfections.

RNA Isolation

C2C12 myoblasts and 10T1/2 fibroblasts were grown to approximately 85-90% confluency in 60-mm dishes and either harvested as a GM sample or washed once with PBS and refed with DM. The cells were then harvested at various time points after

refeeding with DM. RNA was isolated according to manufacturer's instructions using the RNeasy Mini Kit (Qiagen). The RNA was quantitated and 5μg was run on a formaldehyde gel to ensure integrity.

RT-PCR

RT-PCR was carried out using the RNA PCR Core Kit (Applied Biosystems). Briefly, 1 μg of RNA was reverse transcribed for 1 hour at 42°C, using random hexamers. One-eighth of the reaction was used in a PCR reaction (20 cycles) with primers specific for α-actin:

forward (5') TGAGACCTTCAACACCCCAG (3')
reverse (5') GAGCCAGAGCAGTAATCTCC (3')

in order to ensure proper quantitation of the RNA. One-fourth of the reaction was used in a PCR reaction (30 cycles) with primers specific for HEB:

forward (5') GATCTCCTTCACCTCTCACAG (3')
reverse (5') TAGTAGGCAGACTGGTAGAAG (3')

The primers for E2A were specific for the bHLH:

E12 forward (5') CCAGACGAGGACGAGGACGAC (3')
E47 forward (5') AGTACAGATGAGGTGCTGTCC (3')
E2A reverse (5') ACCACGCCAGACACCTTCTCC (3')

The PCR reactions were optimized to ensure that amplification was within the linear range.

Protein Extraction

Cells were washed twice with PBS and scraped with NP-40 lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 1.5 mM $MgCl_2$, 1 mM DTT, 10 mM NaF, 0.5 mM PMSF, 0.5 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and transferred to a 1.5 ml eppendorf tube. The cells were incubated on ice for 30-45 minutes with periodic vortexing, and the lysates cleared by centrifugation. Protein concentration was determined using the modified Bradford Assay (BioRad), using BSA as a standard.

Immunoblotting and Antibodies

Western blot analysis was performed as described previously (Perry et al., 2001). The antibodies used were: anti-HEB (A-20; Santa Cruz), anti-MyoD (C-20; Santa Cruz), anti-myogenin (F5D; Developmental Studies Hybridoma Bank), anti-Myf5 (C-20; Santa Cruz), anti-E2A (V-18; Santa Cruz), anti-tubulin (DM1A; Sigma), anti-GAPDH (Ambion). For immunoblotting, all antibodies were used according to manufacturer's instructions, and anti-myogenin was used at 1:10 (hybridoma supernatant). Anti-mouse and anti-rabbit secondary antibodies were used at 1:2000 (BioRad), and were detected using enhanced chemiluminescence (ECL; Amersham-Pharmacia)

Chromatin Immunoprecipitation Assays

For each immunoprecipitation, the protein and DNA in approximately 1 X 10^6 C2C12 myoblasts/myotubes or 1.5 X 10^6 transfected 10T1/2 fibroblasts were cross-

linked by the addition of formaldehyde directly to the culture medium to a final concentration of 1%, and incubated for 10 minutes at 37°C. The cells were washed twice with ice-cold PBS containing protease inhibitors (PBS+; 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The cells were lysed with 200 μl of SDS Lysis Buffer (Upstate) and the cross-linked protein DNA complexes were isolated according to the manufacturer's instructions (Chromatin Immunoprecipitation (ChIP) Assay Kit; Upstate). For input DNA, 50 μl of sample was removed prior to the immunoprecipitation (IP), the cross-links were reversed as instructed in the kit protocol, and the DNA was purified using the PCR Purification Kit (Qiagen). The amount of input DNA was quantitated using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).

The antibodies used to immunoprecipitate the complexes were all supplied by Santa Cruz: anti-MyoD (C-20), anti-HEB (A-20), anti-Myf5 (C-20), and anti-myogenin (M-225), and used at 1 μg per IP. After the IP, the immunoprecipitated complexes were washed according to the manufacturer's protocol, the cross-links were reversed and the DNA was purified.

8.2 ng of input DNA from 10T1/2 samples and 1.2 ng of input DNA from C2C12 samples were used in a 25-cycle PCR reaction. The amount of immunoprecipitated DNA used for each 35-cycle PCR reaction was determined based on the concentration of input DNA. PCR reactions were optimized to ensure that amplification was within the linear range and the primers used were previously described (Bergstrom et al., 2002).

Reporter Assays

Transfected cells were washed twice with PBS and scraped with 1X Reporter Lysis Buffer (Promega). The cells were incubated on ice for 30 minutes with periodic vortexing, followed by one round of freeze-thaw lysis. The extracts were centrifuged and the supernatant transferred to a new tube. Luciferase activity was assayed using 10-15 μl of extract and the Luciferase Assay System (Promega). Protein concentration was determined using the modified Bradford assay (BioRad), and the relative light units (RLUs) from the luciferase assay were normalized using protein concentration. Fold activity was determined by setting the relative light units (RLUs) for the reporter vector alone sample as 1. Synergy is defined as:

$$\text{SYNERGY} = \frac{\text{RLUs(MRF + HEB)}}{\text{RLUs(MRF)} + \text{RLUs(HEB)}}$$

where the RLUs represent luciferase activity normalized to protein concentration.

siRNA

HEB target sequences were chosen from a group of possible sequences from the siRNA Target Finder website (www.ambion.com/techlib/misc/siRNA_finder.html). DNA oligonucleotides were synthesized (Alpha DNA):

15 5'-AAT GAC AGT CGA TTA GGA ACC CCTGTCTC- 3' (anti-sense)
5'-AA GGT TCC TAA TCG ACT GTC A CCTGTCTC-3' (sense)

#36 5'- AAT CTC CTA GTT ACC CAT CTC CCTGTCTC -3' (anti-sense)
5'- AA GAG ATG GGT AAC TAG GAG A CCTGTCTC -3' (sense)

#67 5'- AAT GCA TCA ATT GGA AAC CTC CCTGTCTC -3' (anti-sense)
5'- AA GAG GTT TCC AAT TGA TGC A CCTGTCTC -3' (sense)

#60 5'- AAC TTC ACG AGC ATT TGC AAG CCTGTCTC -3' (anti-sense)
5'- AA CTT GCA AAT GCT CGT GAA G CCTGTCTC -3' (sense)

and siRNA molecules constructed using the *Silencer* siRNA Construction Kit (Ambion). GAPDH siRNA was synthesized using DNA oligonucleotides provided by Ambion, and served as a positive control. The siRNA was labeled with Cy3 using the *Silencer* siRNA Labeling Kit to permit visualization of transfected cells.

C2C12 myoblasts were plated at 1 X 10^4 cells per 35-mm plate. The following day, the cells were transfected with 18.75 pmol of siRNA using Oligofectamine and cultured for 2 days. The cells were washed once with PBS, refed with DM without penicillin/streptomycin, and cultured for 48 hours, or 96 hours.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA)/PBS for 5 minutes at room temperature, and the fixation was stopped with 100mM glycine/PBS for 5 minutes. The cells were washed three times with PBS, and permeabilized in 0.3% Triton X-100/PBS for 5 minutes, followed by another three washes in PBS. The cells were blocked in 10% horse serum/PBS for one hour, followed by incubation for one hour with primary antibody (F5D [anti-myogenin] or MF20 [anti-myosin heavy chain]; Developmental Studies Hybridoma Bank; 1:20 in 10% goat serum/PBS). The cells were then washed three times in PBS, and incubated with FITC-conjugated anti-mouse secondary (Chemicon; 1:200 in 10% goat serum/PBS) for one hour. The cells were washed three times in PBS and nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI; 0.25μg/ml in PBS) for 5 minutes. The cells were washed once in PBS and mounted with Fluorescence Mounting Medium (DAKO).

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Preface to Chapter 3

Myoblasts isolated from *MyoD*$^{-/-}$ mice proliferate more rapidly, display a delay in differentiation, and continue to incorporate BrdU after serum withdrawal (Sabourin et al., 1999). Therefore, the next logical course of action was to determine which cell cycle regulatory proteins were affected by the loss of MyoD, and the mechanism by which the regulation is achieved. This paper contributes to the field of skeletal muscle biology by demonstrating that MyoD induces cell cycle arrest by regulating expression of cyclin D1, D2, and E. Moreover, the loss of MyoD results in maintained activation of NF-κB under conditions which induce differentiation. NF-κB directly activates expression of cyclin D1 and components of the proteosome.

This manuscript will be submitted for publication in 2004. Robert Perry cultured the *MyoD*$^{-/-}$*:Myf5*$^{-/-}$ fibroblasts, and performed the transfections for Figure 6. Adele Girgis-Gabardo was a technician in the lab who isolated the initial *MyoD*$^{-/-}$ myoblasts and helped with cell culture. I performed the experiments shown in Figures 1 through 5 and 7, the RNase protection assay of Figure 6, constructed the figures, and wrote the manuscript.

Maintained Activation of NF-κB Inhibits Differentiation in MyoD-null Myoblasts

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Abstract

Myoblasts isolated from *MyoD*$^{-/-}$ myoblasts proliferate more rapidly that wildtype myoblasts, and continue to incorporate BrdU after serum withdrawal (Sabourin et al., 1999). The precise mechanism by which MyoD regulates cell cycle withdrawal is not well understood. We demonstrated that increased nuclear localization of cyclin E, and a concomitant increase in cdk2 activity, contributed to the increase in proliferation of the MyoD-null myoblasts and DNA synthesis in the absence of serum. Moreover, expression of cyclin D was maintained during differentiation, inhibiting expression of muscle-specific genes required for differentiation. Importantly, MyoD regulation of cell cycle was indirect and was the result of maintained activation of NF-κB. Therefore, MyoD regulates cell cycle withdrawal by indirectly regulating expression and stability of important cell cycle regulators.

Introduction

The myogenic regulatory factors (MRFs), namely MyoD, Myf5, myogenin, and MRF4/Myf6, play a well-defined role in orchestrating skeletal muscle development and differentiation (Parker et al., 2003). The MRFs share a highly homologous basic helix-loop-helix (bHLH) domain, which is required for DNA binding and dimerization with the E-protein family of transcription factors. MRF-E-protein heterodimers bind to the consensus E-box sequence, CANNTG, found in the promoters of muscle-specific genes. The DNA binding and transcriptional activity of these dimers is highly regulated by a variety of protein-protein interactions and extrinsic cues (Perry and Rudnicki, 2000; Puri and Sartorelli, 2000).

Analysis of regeneration in *MyoD*$^{-/-}$ muscle established an essential role for MyoD in the regulation of adult myogenesis. Specifically, increased numbers of satellite cells and a deficient regenerative processes in muscles of mice that lack *MyoD* (*MyoD*$^{-/-}$), or *MyoD* and *dystrophin* (*MyoD*$^{-/-}$*:mdx*), suggests that *MyoD*-deficient satellite cells have an increased propensity for self-renewal rather than differentiation (Megeney et al., 1996). Several labs have subsequently demonstrated impaired differentiation of cultured *MyoD*$^{-/-}$ satellite cell derived myoblasts, with a concomitant increase in *Myf5* expression (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999).

A further investigation of differentiation potential demonstrated a continued incorporation of bromodeoxyuridine (BrdU) in *MyoD*$^{-/-}$ myoblasts during differentiation, indicating continued DNA synthesis in the absence of mitogen stimulation (Sabourin et al., 1999). In addition, *MyoD*$^{-/-}$ cultures display a reduced number of differentiated cells, as assessed by myosin heavy chain (MyHC) expression, and notably, those cells that have differentiated are primarily mononuclear. These cells also demonstrate a delay in the expression of differentiation specific markers such as *myogenin*, *MRF4*, *α-actins* and the *acetylcholine receptor-δ*.

A number of previous studies have investigated the role of the cell cycle machinery in regulating MyoD activity, yet the only report to date examining the corollary describes how MyoD initiates the expression of p21, an inhibitor of cdk2 and cdk1 kinase activity (Halevy et al., 1995; Parker et al., 1995). Interestingly, *MyoD*$^{-/-}$ myoblasts have a similar level of *p21* mRNA as compared to *WT* myoblasts, and mice lacking *p21* display no apparent muscle abnormalities (Deng et al., 1995; Sabourin et al., 1999). In contrast, mice lacking *p21* and *p57*, another cdk2/cdk1 inhibitor, are phenotypically similar to *myogenin* knockout mice, in that they lack differentiated myofibers (Zhang et al., 1999). Although p21 alone does not regulate cell cycle exit in myoblasts, this suggests that inhibition of cdk2 activity is essential for myogenic differentiation.

Therefore, in this study we sought to examine the role MyoD plays in regulating cell cycle withdrawal and terminal differentiation. We demonstrated that continued S-phase entry was due to maintained expression of cyclin D and E, and increased cdk2 activity. Importantly, it is hypothesized that MyoD indirectly controls cell cycle withdrawal by regulating activation of the p50/NF-κB1 and p65/RelA transcription factors.

Results

MyoD$^{-/-}$ Myoblasts Maintained Expression of Cyclin D1 and D2 after Serum Withdrawal

In order to investigate the changes in cell cycle in the absence of MyoD, RNA was isolated from a differentiation time course of *MyoD*$^{-/-}$ and *WT* primary myoblasts, and the relative expression level of a variety of cyclins was determined using an RNase protection assay (Figures 3.1 and 3.2). The relative amount of mRNA present for each cyclin was quantitated using a phosphorimager, and normalized to a GAPDH control. Figure 1A and 2A depict the resulting autoradiographs of the gels and Figure 1B and 2B graphically depict the results. *MyoD*$^{-/-}$ myoblasts exhibited a higher level of *cyclin D1* and *D2* mRNA, and a lower level of *cyclin D3* and *cyclin G1* mRNA as compared to WT myoblasts (Figure 1). However, neither *cyclin E* nor *cyclin H* mRNA was affected by the absence of MyoD.

Loss of MyoD Stimulates S-phase Entry

To determine if the changes in mRNA are translated into changes in protein levels, nuclear protein extracts isolated from a similar differentiation time course were analyzed by Western blot analysis. In accord with RNase protection assay results, *MyoD*$^{-/-}$ myoblasts displayed a higher level of cyclin D1 and D2 protein in the nucleus and continued expression after mitogen withdrawal (Figure 3). The level of cdk4 in the nucleus was also elevated, and expression continued after mitogen withdrawal. Expression of cyclin D3 protein, on the other hand, was not altered in the absence of MyoD.

Although expression of *cyclin E* mRNA was not altered, *MyoD*$^{-/-}$ myoblasts displayed an increase in the level of cyclin E protein in the nucleus (Figure 4A). Moreover, the level of nuclear cdk2 protein was elevated, and more importantly, appeared to be the slower migrating, or activated, form. As expected, this correlated with an increase in cdk2 kinase activity (Figure 4B).

Although cdk activity is regulated by the expression of its corresponding cyclin partner, the cyclin-cdk complex is also activated by the cdk-activating kinase (CAK), which includes cyclin H and cdk7. Western blot analysis demonstrated that expression of cdk7 protein was increased in the nucleus of *MyoD*$^{-/-}$ cells, yet cyclin H expression was similar to that of *WT* myoblasts, as expected from the RNase protection assays (Figures 4C and 2B).

To determine if increased cdk2 kinase activity forced cells to enter S-phase more rapidly, proliferating *MyoD*$^{-/-}$ and *WT* myoblasts were subjected to fluorescent activated cell sorting (FACS) analysis. The greatest proportion of *WT* myoblasts were in the G1 phase of the cell cycle (84%) (Figure 5). Furthermore, 23% of *MyoD*$^{-/-}$ myoblasts were in S phase compared with 12% of *WT* cells. This increase in the proportion of *MyoD*$^{-/-}$ myoblasts in S-phase was accompanied by a decrease in the proportion of cells in G1

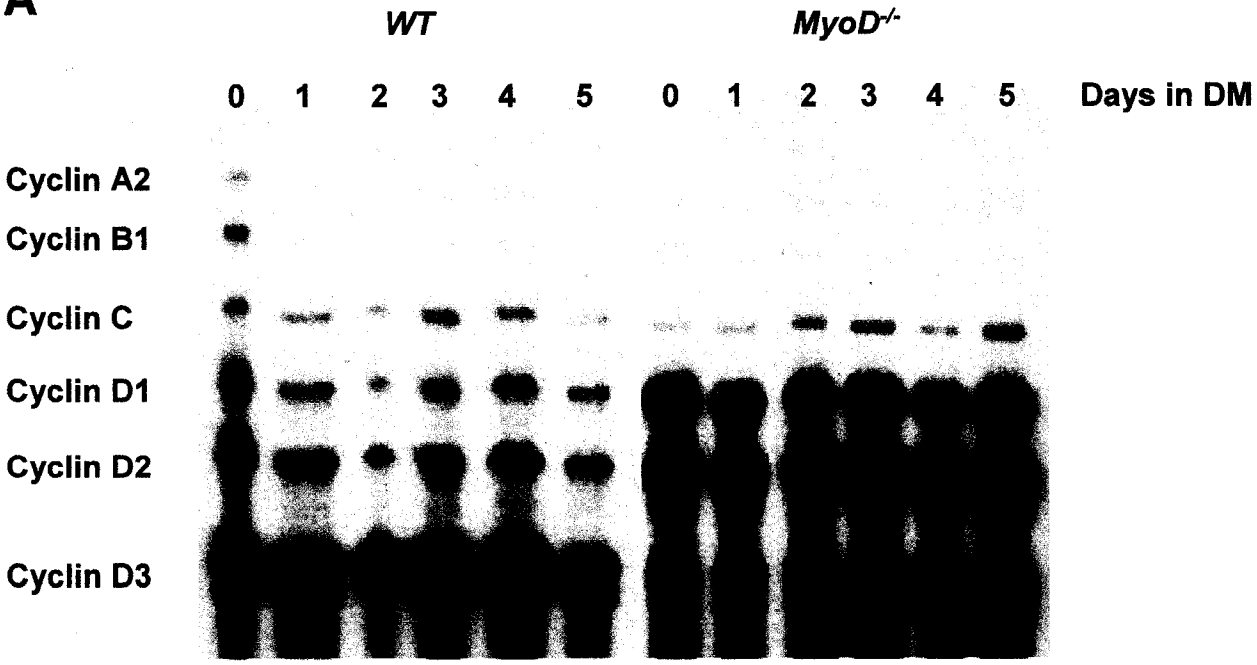
Figure 1. Maintained expression of cyclin D in *MyoD*$^{-/-}$ myoblasts after serum withdrawal.

A. RNA was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and an RNase protection assay was performed with the indicated probes. The protected ^{32}P-labeled fragments were separated on a polyacrylamide gel, the gel dried and exposed to X-ray film.

B. The amount of each protected fragment was quantitated using a phosphorimager. The values obtained were normalized relative to the GAPDH control and the L32 control, and graphed as a function of the number of days in differentiation medium (DM). Expression of *cyclin D1* and *D2* was increased in *MyoD*$^{-/-}$ myoblasts.

Figure 1.

A


WT
MyoD-/-
0 1 2 3 4 5 0 1 2 3 4 5 Days in DM
Cyclin A2
Cyclin B1
Cyclin C
Cyclin D1
Cyclin D2
Cyclin D3

B

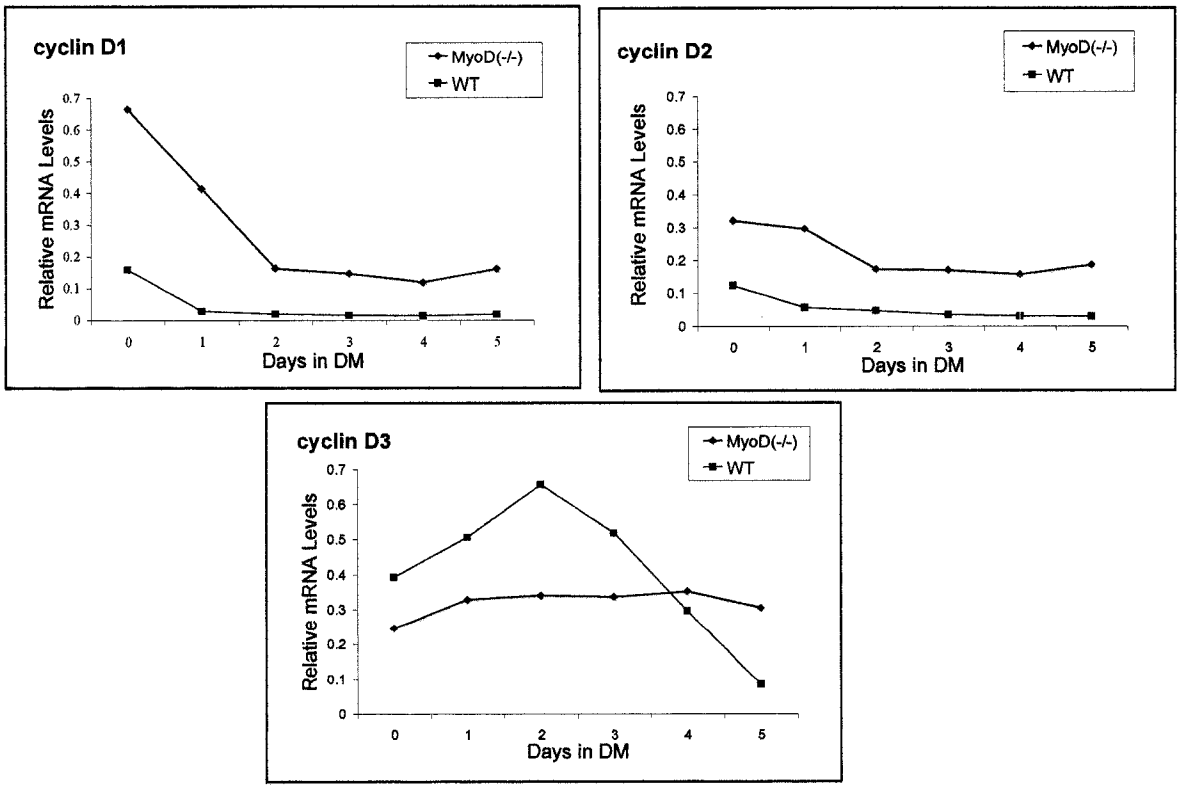

cyclin D1
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM
cyclin D2
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM
cyclin D3
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM

Figure 2. Decreased *cyclin G1* expression in *MyoD*$^{-/-}$ myoblasts.
A. RNA was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and an RNase protection assay was performed with the indicated probes. The protected ^{32}P-labeled fragments were separated on a polyacrylamide gel, the gel dried and exposed to X-ray film.
B. The amount of each protected fragment was quantitated using a phosphorimager. The values obtained were normalized relative to the GAPDH control and the L32 control and graphed as a function of the number of days in differentiation medium (DM). Expression of *cyclin G1* was decreased in *MyoD*$^{-/-}$ myoblasts, however expression of *cyclin E* was unchanged.

Figure 2.

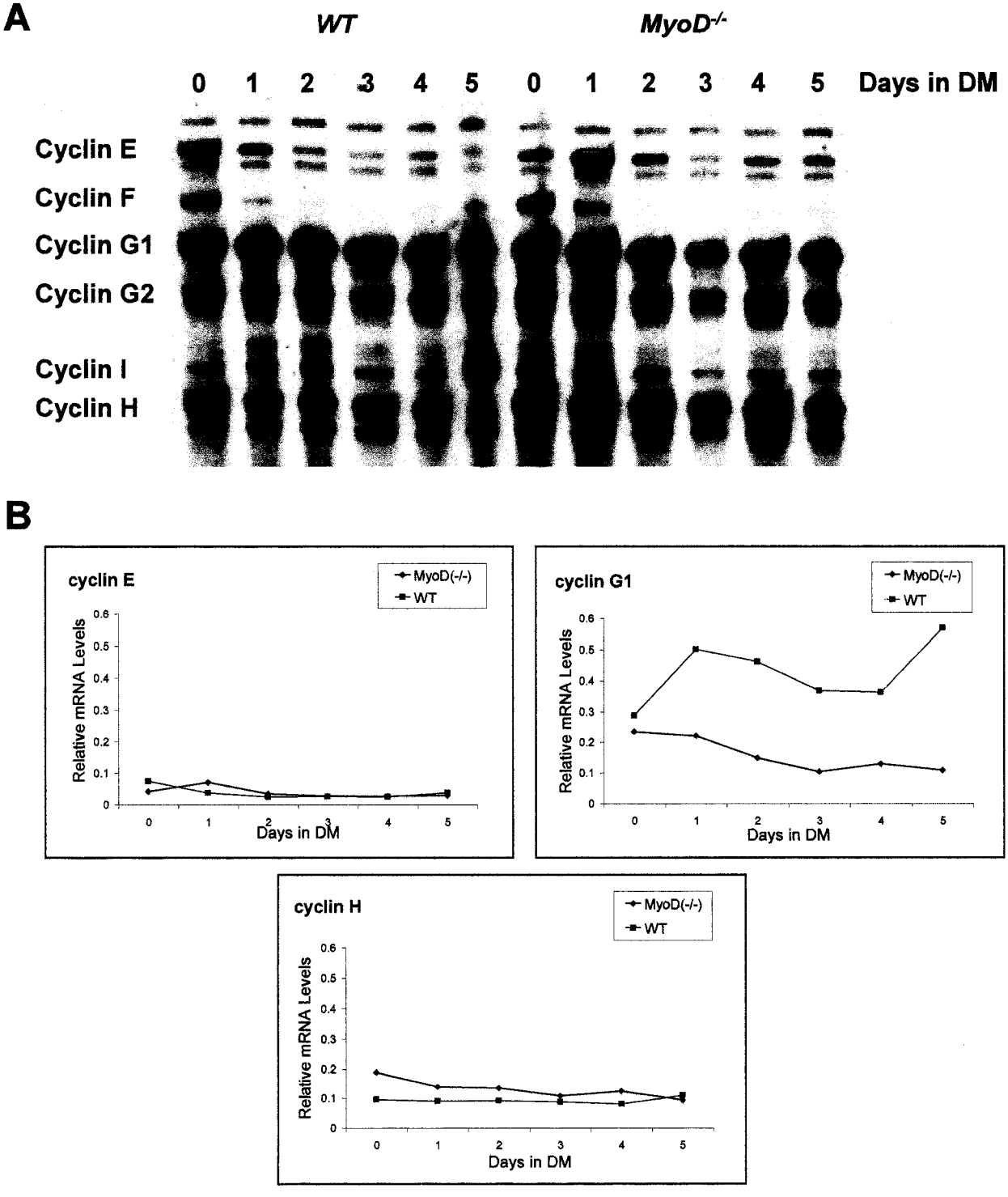

A
WT
MyoD-/-
0 1 2 3 4 5 0 1 2 3 4 5 Days in DM
Cyclin E
Cyclin F
Cyclin G1
Cyclin G2
Cyclin I
Cyclin H
B
cyclin E
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM
cyclin G1
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM
cyclin H
MyoD(-/-)
WT
Relative mRNA Levels
Days in DM

Figure 3. Cyclin D1 and D2 protein remained localized to the nucleus during serum withdrawal in *MyoD*$^{-/-}$ myoblasts.
Nuclear protein was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and a western blot was performed with the indicated antibodies. The level of cyclin D1 and D2 protein in the nucleus was elevated in *MyoD*$^{-/-}$ myoblasts, as was the level of its corresponding kinase, cdk4.

Figure 3.

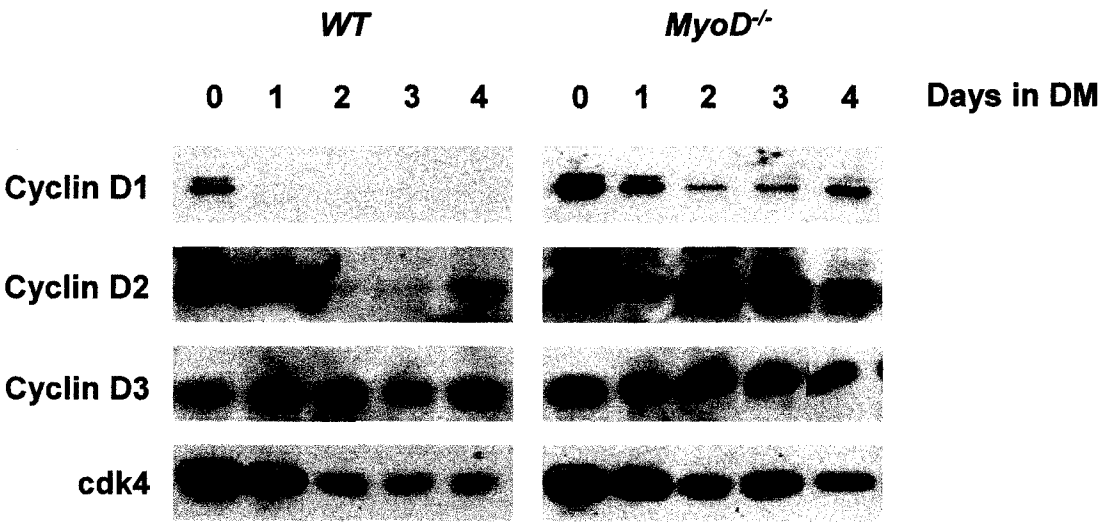

WT
MyoD-/-
0 1 2 3 4
0 1 2 3 4
Days in DM
Cyclin D1
Cyclin D2
Cyclin D3
cdk4

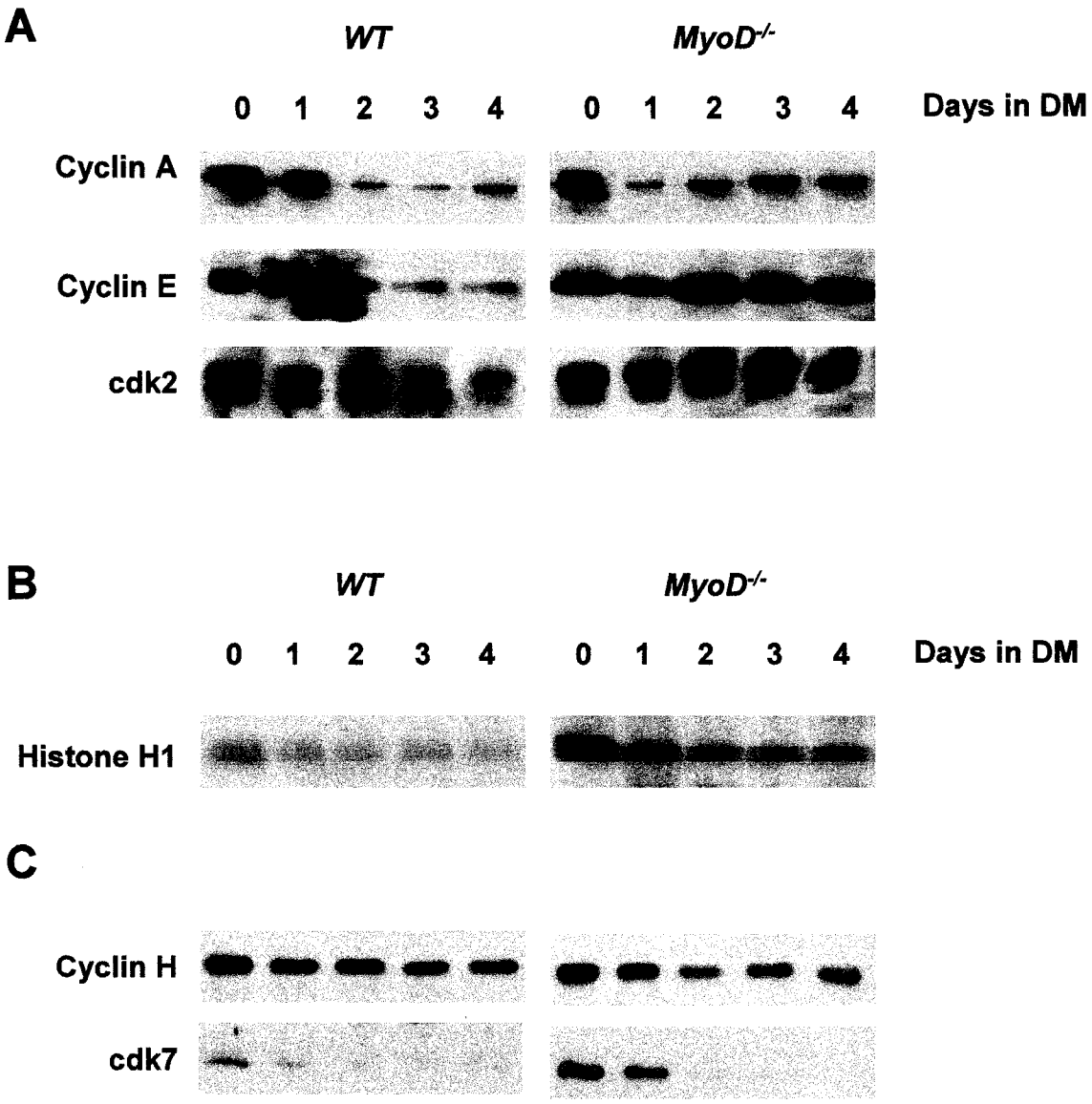
Figure 4. Maintained nuclear localization of cyclin E and increased cdk2 activity during serum withdrawal in *MyoD*$^{-/-}$ myoblasts.

A. Nuclear protein was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and a western blot was performed using the indicated antibodies. Cyclin E protein remained localized to the nucleus in *MyoD*$^{-/-}$ myoblasts, even after serum withdrawal

B. Cdk2 was immunoprecipitated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts, and assayed for kinase activity using histone H1 and γ-^{32}P-ATP as substrate. *MyoD*$^{-/-}$ myoblasts displayed a higher level of cdk2 kinase activity.

C. Nuclear protein was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and a western blot was performed using the indicated antibodies. *MyoD*$^{-/-}$ myoblasts exhibited an increased level of cdk7, a component of the cdk-activating kinase (CAK) complex.

Figure 4.


A
WT
MyoD-/-
0 1 2 3 4
0 1 2 3 4
Days in DM
Cyclin A
Cyclin E
cdk2
B
WT
MyoD-/-
0 1 2 3 4
0 1 2 3 4
Days in DM
Histone H1
C
Cyclin H
cdk7

(68% of *MyoD*$^{-/-}$ cells compared to 84% of *WT* cells). Moreover, 8.8% of WT cells were in mitosis compared to 4.1% of *MyoD*$^{-/-}$ myoblasts. Therefore, in the absence of MyoD, myoblasts enter into S-phase more readily.

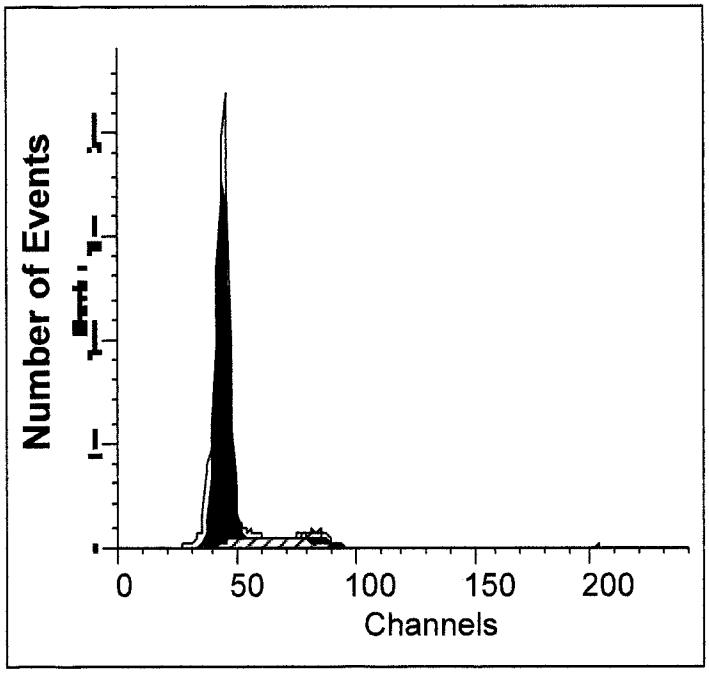
MyoD Regulation of Cyclin D Gene Expression is Indirect and Involves NF-κB

In order to address whether the changes in cyclin expression were a direct result of MyoD or Myf5 activity, embryonic fibroblasts lacking both *MyoD* and *Myf-5* (double knockout fibroblasts; DKO fibroblasts) were transfected with MyoD, Myf-5, or an estradiol-inducible form of MyoD (MyoD-ER). The MyoD-ER transfected cells were either treated with ethanol, thus keeping the MyoD-ER fusion protein in the cytoplasm, or exposed to estradiol, activating MyoD by inducing nuclear localization. RNA was isolated from proliferating cells and RNase protection assays were performed using the two cyclin probe sets shown earlier (Figure 6). Neither MyoD nor Myf5 expression altered the levels of cyclin mRNA as compared to control cells. Moreover, estradiol induction of MyoD-ER transfected cells had no effect on cyclin expression. This suggests that the cell cycle anomalies of the *MyoD*$^{-/-}$ myoblasts are not the direct result of MyoD or Myf-5 transcriptional activity.

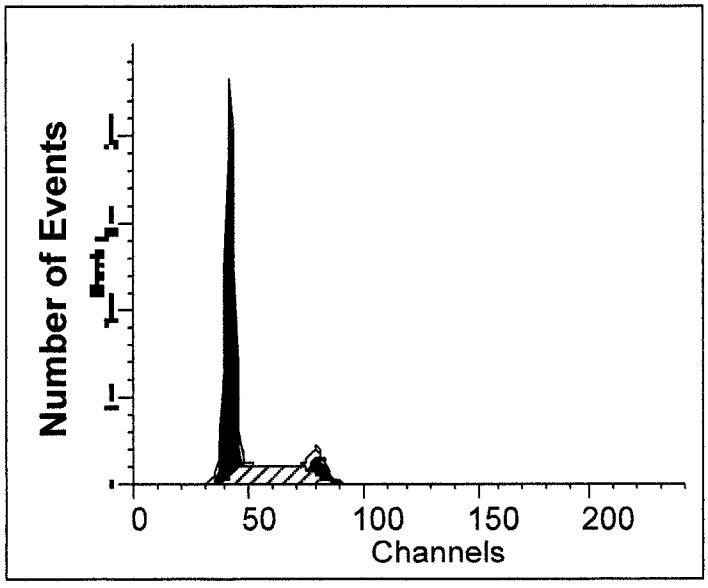
Indeed, NF-κB directly activates expression of cyclin D1, and blocks synthesis of the C3 subunit of the proteasome (Guttridge et al., 1999a). Strikingly, *MyoD*$^{-/-}$ myoblasts retained NF-κB in the nucleus, even after mitogen withdrawal, in contrast to *WT* myoblasts, which predominantly maintained cytoplasmic localization of NF-κB (Figure 7). This strongly suggests that differentiation-specific MyoD activity inhibits NF-κB activation, allowing cyclin D expression to be downregulated.

Figure 5. Proliferating *MyoD*$^{-/-}$ myoblasts displayed a greater percentage of cells in S-phase.
Proliferating *WT* and *MyoD*$^{-/-}$ myoblasts were fixed in ethanol and the DNA stained with propidium iodide (PI). The cells were then analyzed for DNA content by FACS analysis, and the percentage of cells in each phase of the cell cycle is indicated. *MyoD*$^{-/-}$ myoblasts displayed a greater propensity to enter S-phase, relative to *WT* myoblasts.

Figure 5.



WT Growth
G0-G1: 83.86 %
G2-M: 4.12 %
S: 12.01 %



MyoD(-/-) Growth #2
G0-G1: 67.94 %
G2-M: 8.75 %
S: 23.32 %

Figure 6. MyoD and Myf5 do not directly regulate expression of *cyclin D*.
Mouse embryonic fibroblasts isolated from *MyoD*$^{-/-}$*:Myf-5*$^{-/-}$ mice were transfected with MyoD, Myf-5, or an estradiol-inducible form of MyoD (MyoD-ER). The cells transfected with MyoD-ER were treated either with ethanol (MyoD-ER) or estradiol (MyoD-ER + estradiol). RNA was isolated from proliferating cells, and RNase protection assays were performed using the two cyclin probe sets described earlier. The first two lanes represent mouse control RNA, and the third lane, labeled "control", represents fibroblasts transfected with empty expression vector. The two sets of MyoD and Myf-5 lanes represent two separate experiments. The pattern of *cyclin* expression was not altered upon transfection of MyoD or Myf5, or estradiol induction of MyoD-ER.

Figure 6.

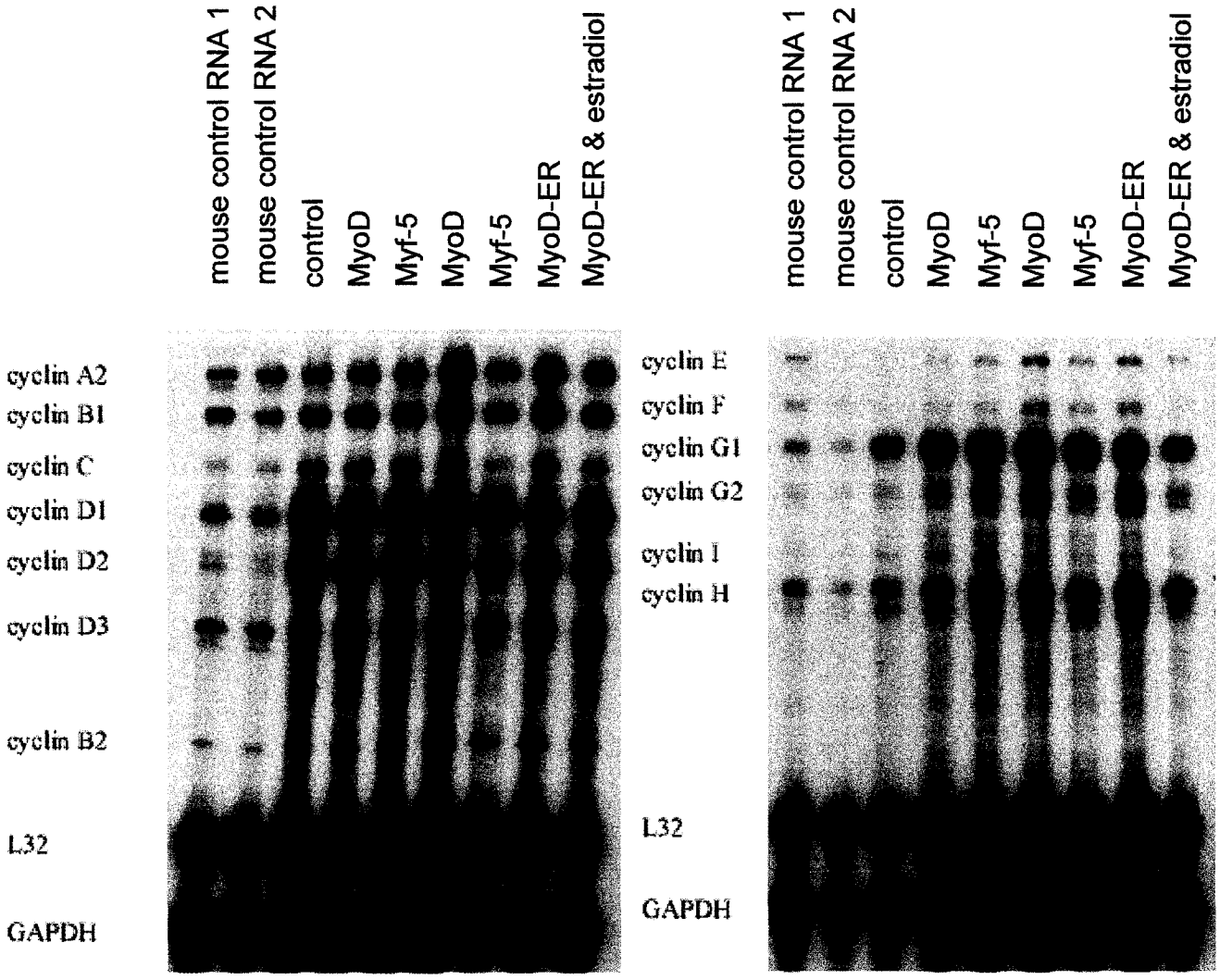
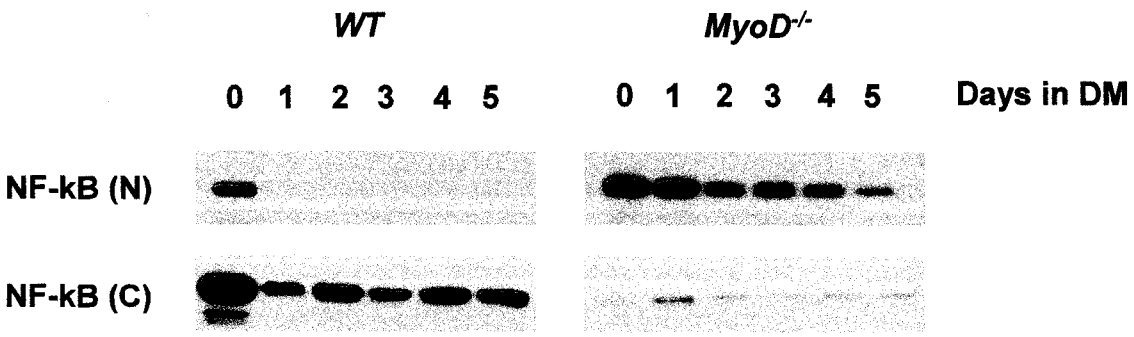

mouse control RNA 1
mouse control RNA 2
control
MyoD
Myf-5
MyoD
Myf-5
MyoD-ER
MyoD-ER & estradiol
cyclin A2
cyclin B1
cyclin C
cyclin D1
cyclin D2
cyclin D3
cyclin B2
L32
GAPDH
mouse control RNA 1
mouse control RNA 2
control
MyoD
Myf-5
MyoD
Myf-5
MyoD-ER
MyoD-ER & estradiol
cyclin E
cyclin F
cyclin G1
cyclin G2
cyclin I
cyclin H
L32
GAPDH

Figure 7. Sustained activation of NF-κB during serum withdrawal in *MyoD*$^{-/-}$ myoblasts.
Protein was isolated from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts and a western blot using the nuclear (N) and cytoplasmic (C) fractions was performed with an antibody specific for the p65 subunit of NF-κB. *MyoD*$^{-/-}$ myoblasts displayed a continued localization of NF-κB to the nucleus after serum withdrawal.

Figure 7.



Discussion

Expression of muscle-specific genes is regulated by the MRF family of bHLH transcription factors. Mice lacking both *MyoD* and *Myf5* display a complete absence of skeletal muscle, demonstrating that MyoD and Myf5 play an essential role in myogenic specification (Rudnicki et al., 1993). During embryonic development, progenitors present at sites of hypaxial and limb muscle formation express *Myf5*, but not *MyoD*, and proliferate extensively prior to differentiation (Amthor et al., 1999; Delfini et al., 2000). Moreover, the population of LacZ-expressing epaxial myogenic progenitors is reduced in the absence of *Myf5* in gene-targeted $Myf5^{nLacZ/nLacZ}$ embryos, and myoblasts from newborn $Myf5^{-/-}$ mice display reduced proliferative potential (Tajbakhsh et al., 1996)(Montarras et al., 2000). Taken together, these observations suggest a model in which Myf5 activity is required for proliferation and specification of the myogenic lineage, whereas MyoD functions predominantly to induce cell cycle arrest and differentiation. This is especially intriguing in light of the increase in *Myf5* expression in $MyoD^{-/-}$ myoblasts and their propensity to proliferate rather than differentiate.

Myogenic differentiation requires irreversible withdrawal from the cell cycle, and a number of studies have elucidated the importance of cell cycle proteins in regulating myogenic differentiation (Kitzmann and Fernandez, 2001; Wei and Paterson, 2001). Cell cycle progression, on the other hand, requires that pRb is phosphorylated, and this occurs, in part, through cyclin D1-cdk4 kinase activity. As such, the increase in expression of cyclin D1 and D2 in $MyoD^{-/-}$ myoblasts (Figure 1 and 3) may cause cell cycle progression in the absence of mitogens through increased phosphorylation of pRb. However, overexpression of cyclin D1 also inhibits MRF activity and myogenic differentiation through a mechanism independent of pRb or MyoD phosphorylation (Rao and Kohtz, 1995; Skapek et al., 1996; Skapek et al., 1995).

Cyclin D is able to bind transcription factors, such as the estrogen receptor, STAT3 and Beta2/NeuroD, or co-factors such as pCAF and HDACs, to inhibit transcriptional activity (Coqueret, 2002). Moreover, cyclin D blocks the interaction between Mef2 and its cofactor GRIP-1, thus inhibiting transcriptional activity of Mef2 (Chen et al., 2000; Lazaro et al., 2002). Given that Mef2 plays an important role in myogenic differentiation, this may represent an important mechanism by which continued expression of cyclin D1 blocks transcriptional activation of differentiation-specific myogenic genes.

Cyclin D1 is highly regulated at the level of transcriptional activation, protein expression, and subcellular localization. In *WT* myoblasts and C2C12 myoblasts, serum withdrawal results in export of cyclin D1, D2, and cdk4 protein out of the nucleus (Figure 3, data not shown), and downregulation of *cyclin D1* and *D2* expression (Figure 1). Normally, mitogens induce expression of *cyclin D1* through activation of the the Ras-MAP kinase pathway (Albanese et al., 1995). Given that the level of *cyclin D1* and *D2* mRNA is maintained in $MyoD^{-/-}$ myoblasts, even after induction of differentiation through mitogen withdrawal (Figure 1), one possibility is that differentiation-specific MyoD activity negatively regulates the MAP kinase pathway, thereby downregulating expression of *cyclin D1*.

Degradation of cyclins is another important mechanism by which cell cycle progression is regulated. Cyclin D1 associates with Cul-1, a component of the SCF ubiquitin E3 ligase complex, and is degraded through the 26S proteasome (Russell et al., 1999; Yu et al., 1998). Phosphorylation of cyclin D1 on Thr286 by GSK-3β forces cyclin D1 out of the nucleus and targets the protein for degradation (Alt et al., 2000; Diehl et al., 1998). In the absence of MyoD, the level of nuclear localized cyclin D1 protein is elevated (Figure 3) and although this may simply reflect an increase in the mRNA, it may also be due to a lack of cyclin D1 phosphorylation, or a failure to activate the components of the degradation pathway. The possibility of MyoD-dependent degradation of cyclin D1 is intriguing since the level of cyclin E protein is also maintained in the nucleus of *MyoD*$^{-/-}$ myoblasts in the absence of mitogens (Figure 4).

Although cyclin D1 may function outside of the cell cycle in order to inhibit myogenic differentiation, the role of cyclin E is likely to be more archetypal. pRb is phosphorylated by the cyclin E-cdk2 complex, and this phosphorylation is required for S-phase entry (Harbour et al., 1999). *Cyclin E* expression is controlled by the E2F family of transcription factors, whose activity is regulated by interactions with pRb, p107 and p130. Cyclin D-cdk4 phosphorylates pRb, disrupting the interaction with E2F, and allowing E2F to activate transcription of the *cyclin E* gene. In fact, the only cell cycle function of cyclin D-cdk4 is to induce expression of cyclin E. Moreover, forced expression of cyclin E bypasses the need for cyclin D/cdk4 activity in the progression from G1 to S phase (Geng et al., 1996; Geng et al., 1999).

In the absence of MyoD, myoblasts displayed an increase in the proportion of cells in S-phase relative to *WT* myoblasts (Figure 5). This corresponded with an increase in nuclear localized cyclin E and cdk2 protein, and an increase in cdk2 kinase activity (Figure 4). Expression of *cyclin E* mRNA was not changed in the absence of MyoD, indicating that the increase in cyclin D1 expression is not inducing cell cycle progression through stimulation of pRb phosphorylation and induction of E2F activity. However, cyclin E protein was stabilized and remained localized to the nucleus after mitogen withdrawal (Figure 4).

Targeted degradation of cyclin E depends on phosphorylation of four main serine and threonine residues - T62, S372, T380, and S384. The F-box protein, Fbw7, recognizes phosphorylated T380, which is is phosphorylated by GSK3β. The SCF^{Fbw7} ubiquitin ligase complex ubiquitinates cyclin E and targets the protein for degradation. Downregulation or inactivating mutations in the E3 ubiquitin ligase complexes have been implicated in a number of disease states, including cancer (Adams, 2003). Therefore, MyoD may inhibit S-phase entry by inducing the activity of SCF^{Fbw7}, in order to ensure that cyclin E protein is degraded, allowing cells to arrest in the G1 phase of the cell cycle and induce differentiation.

Neither MyoD nor Myf5 appeared to directly regulate expression of either *cyclin D1* or *D2* (Figure 6). As indicated above, MyoD may regulate expression of cyclin D1 by modulating the activity of Ras-MAP kinase pathway. Moreover, it is possible that MyoD regulates expression of another factor that is involved in transcriptional control of cell

cycle genes. During normal differentiation of *WT* myoblasts and C2C12 myoblasts, NF-κB DNA-binding activity decreases within 24 hours of serum withdrawal (Guttridge et al., 1999a). Therefore, it was intriguing to discover that *MyoD*$^{-/-}$ myoblasts displayed a continued nuclear localization of NF-kB, even after mitogen withdrawal (Figure 7). Furthermore, NF-κB regulates cyclin D1 expression at the mRNA level in myoblasts, and cyclin D2 expression in lymphoma cells (Guttridge et al., 1999b; Hinz et al., 2001).

Expression of NF-κB1 (p50) is downregulated in *MyoD*$^{-/-}$ myoblasts (P. Seale, J. Ishibashi, and M.A. Rudnicki, unpublished observations), however the antibody used for the western blot was specific for p65 (RelA). RelA/p65 is activated through the canonical pathway, and normally forms dimers with the NF-κB/p50 subunit. In fact, NF-κB1/p50 does not contain an activation domain, and homodimers are transcriptionally repressive. Moreover, the heterodimer or homodimer that is formed may function in cell-specific and a promoter-specific manner (Yamamoto and Gaynor, 2004).

Differentiation is accelerated in myoblasts expressing a non-phosphorylatable form of IκBα (IκBα-SR), which is unable to be degraded and inhibits NF-κB (p65) nuclear localization (Guttridge et al., 1999b). Furthermore, these IκBα-SR expressing myoblasts proliferate less rapidly and downregulate expression of *cyclin D1*. In myoblasts lacking MyoD, NF-κB was constitutively localized to the nucleus, the cells proliferated more rapidly, and maintained expression of *cyclin D1* after mitogen withdrawal (Figure 1 and 7)(Sabourin et al., 1999).

Normally, NF-κB is sequestered in the cytoplasm by inhibitory proteins, such as IκBα, IκBβ, and IκBε (Israel, 2000; Liou and Hsia, 2003; Yamamoto and Gaynor, 2004). In response to a stimulus, the IκB proteins are degraded and NF-κB localizes to the nucleus. Regulation of NF-κB subcellular localization is further complicated by the inherent differences in the IκB proteins. IκBα contains a nuclear export signal (NES), and is able to shuttle NF-κB out of the nucleus, as well as maintain cytoplasmic localization. The β and ε forms do not contain an NES, and thus function solely to sequester NF-κB in the cytoplasm.

IκBα expression is regulated by NF-κB, such that upon stimulated degradation and NF-κB activation, expression of IκBα is induced as a feedback mechanism. In embryonic fibroblasts (EFs) expressing either IκBβ or IκBε, in the absence of IκBα, the level of nuclear NF-κB steadily increases until a plateau is reached, whereas EFs expressing only IκBα have a steady oscillation of nuclear NF-κB (Beg et al., 1995). It has been suggested that an increase in IκBβ or IκBε relative to IκBα reduces the oscillation of NF-κB, and results in sustainable NF-κB activation. Moreover, *IκBα-/-* EFs infected with a MyoD-expressing retrovirus maintain NF-kB nuclear localization, resulting in the formation of fewer myotubes that are smaller (Ladner et al., 2003). Therefore, it is possible that the increase in NF-κB activation in *MyoD*$^{-/-}$ myoblasts is due to a loss of IκBα expression, or an increase in the expression of either IκBβ or IκBε relative to IκBα response to the stimulus.

Localization of NF-κB to the nucleus may also be regulated by the Ras-MAP kinase pathway. Expression of an activated form of Ras (H-RasG12V) induces NF-κB activity in MyoD-expressing 10T½ fibroblasts, resulting in an increase in cyclin D1 expression and inhibition of myogenesis (Mitin et al., 2001). Notably, the inhibition of

myogenesis by this form of activated Ras does not solely depend on the activity of NF-κB, although expression of NF-κB alone in 10T½ fibroblasts inhibits MyoD-mediated myogenesis (Guttridge et al., 1999b; Mitin et al., 2001).

Indeed, MyoD negatively regulates expression of H-Ras, suggesting that in the absence of MyoD, myoblasts maintain activity of the Ras-MAP kinase pathway (Bergstrom et al., 2002). Moreover, MyoD induces the expression of insulin-like growth factor binding protein 5 (IGFBP-5), which binds IGFs in order to modulate their activity (P. Seale, J. Ishibashi, and M.A. Rudnicki, unpublished observations)(Bergstrom et al., 2002; Rousse et al., 2001). In fact, in the presence of IGFBP-5, IGF-I inhibits myoblast proliferation and stimulates differentiation, presumably by exchanging the responding pathway from the Ras-MAP kinase pathway to the PI-3' kinase/Akt pathway (Coolican et al., 1997; Ewton et al., 1998; Tureckova et al., 2001). Therefore, in the absence of MyoD, myoblasts are unable to activate expression of IGFBP-5, and IGF-I continues to induce proliferation through the Ras-MAPK pathway, rather than stimulating differentiation through the PI-3' kinase/Akt pathway. Moreover, this inability to change signaling pathways may be due to the increase in NF-κB activity, given that TNFα, which activates NF-κB, blocks secretion of IGFBP-5 and inhibits IGF-I stimulated expression of myogenin (Broussard et al., 2003; Meadows et al., 2000).

Therefore, in the absence of MyoD, myoblasts are unable to exit the cell cycle in response to mitogen withdrawal. *MyoD*$^{-/-}$ myoblasts continue to express *cyclin D1* and *D2,* as well as maintain nuclear localization of cyclin D1, D2, and cyclin E. This may be due to the inability of *MyoD*$^{-/-}$ myoblasts to downregulate the activity of the Ras-MAP kinase pathway, or upregulate the activity of GSK3β and the SCF^{Fbw7} complex. The effects may also be a direct result of the loss of MyoD, the increase in Myf5 expression or the result of increased activation of NF-κB. Defining the precise gene targets of each of these transcription factors is essential for understanding the mechanism of Myf5-mediated proliferation of myogenic precursors and MyoD-mediated induction of differentiation.

Materials and Methods

Myoblast Isolation and Cell Culture

Myoblasts were isolated from 6 to 8 week old *wildtype (WT)* Balb/C mice and *MyoD*$^{-/-}$ mice as previously described (Sabourin et al., 1999). Briefly, the hindlimb muscle of the mouse was removed, washed in PBS, minced in 1.5 ml of collagenase/dispase (2.1 units/ml dispase (Roche), 1% collagenase, 2.5 mM $CaCl_2$), and incubated for 12 minutes at 37°C in a humidified incubator. The mixture was pipetted up and down ten to fifteen times to disperse the muscle, and returned to 37°C for a further 8 minutes. This was repeated until the mixture was smooth. The mixture was passed through a nylon filter (Nitex, Fisher Scientific) to remove undigested material. The flow-through was centrifuged for 5 minutes at 1000 rpm, and the pellet resuspended in 4 mls of isolation medium (Ham's F10 medium supplemented with 20% fetal bovine serum [FBS; Gemini Bioscience], 0.2 units/ml of penicillin and 0.2 μg/ml of streptomycin (Invitrogen; 2X), 10 ng/ml hepatocyte growth factor [HGF; Roche], and 5 ng/ml heparin [Sigma]). The cells were plated on a 6-cm plastic dish for 24 hours to allow the contaminating fibroblasts to adhere.

The following day, the medium and cells in suspension were centrifuged for 5 minutes at 1000 rpm, and the pellet resuspended in 4 mls of isolation medium and plated on a 6-cm plastic dish. After 20 minutes at 37°C, the medium and cells in suspension were transferred to a collagen-coated 6-cm dish. The following day, the isolation medium was removed and the cells were refed with myoblast growth medium (Ham's F10 medium supplemented with 20% FBS, 2X penicillin and streptomycin, and 12.5 ng/ml of basic fibroblast growth factor [bFGF; Invitrogen]). For the first 3 passages, the cells were pre-plated on plastic for 15-20 minutes prior to reseeding on collagen-coated dishes to remove fibroblasts and non-myogenic cells. To induce differentiation, the cells were washed once with PBS and transferred to differentiation medium (DMEM supplemented with 5% horse serum (Invitrogen), and 2X penicillin/streptomycin).

RNA Isolation

RNA was isolated using TriZol Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells from a 10-cm dish were lysed in 1ml of TriZol Reagent for 5 minutes at room temperature and the lysate transferred to a 1.5 ml eppendorf tube. The RNA was extracted from the mixture with 0.2 ml of chloroform, and precipitated with 0.5 ml isopropanol. The precipitate was washed with 1 ml of 75% ethanol, and the RNA dissolved in 250 μl of DEPC-treated water. In order to ensure purity, the RNA was re-precipitated with 500μl of ethanol and 10μl of 3M sodium acetate, and the pellet was washed once with 75% ethanol. The resulting RNA was resuspended in 30μl of DEPC-treated water, quantitated by spectrophotometry and analyzed on a formaldehyde-agarose gel.

RNase Protection Assay

The RNase protection assay was performed using the RiboQuant kit according to the manufacturer's instructions (BD Pharmingen). Briefly, probes corresponding to the indicated cyclins were transcribed from each multiprobe set using T7 RNA polymerase and deoxynucleotides, including $^{\alpha\text{-}32}$P-labeled UTP. The labeled RNA probes were hybridized to 1μg of RNA isolated from a differentiation time course of myoblasts. Single stranded RNA was digested with RNase, and the double stranded protected fragments of RNA were separated on a 4.75% acrylamide/8M urea gel, the gel dried and exposed to X-OMAT XAR-5 (Kodak) film overnight. A sample of mouse RNA provided in the kit was included as a positive control. A sample of labeled probe not hybridized to RNA was also separated on the gel as a standard. The relative amount of radioactivity present in each band was quantitated using a Phosphorimager (Molecular Dynamics), and the values obtained for each cyclin were normalized to the value for the GAPDH control.

Isolation of Nuclear and Cytoplasmic Protein

Each 10 cm plate of myoblasts from a differentiation time course was washed twice with PBS+ (PBS containing 1mM sodium vanadate, 0.1 M sodium fluoride). The cells were scraped from the plate using 1 ml of PBS+ and transferred to an eppendorf tube. The cells were centrifuged at 1000 rpm for 2.5 minutes at 4°C, and the supernatant was removed. The cell pellet was resuspended in 250 μl of Buffer A (10 mM Hepes [pH 7.8], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% NP-40, 1 μg/ml each of aprotinin, leupeptin, and pepstatin) and incubated on ice for 30 minutes with periodic mixing. The lysate was centrifuged at 10,000 X g for 5 minutes at 4°C and the supernatant transferred to a new tube (cytoplasmic fraction). The pellet was washed in 100 μl of Buffer A and re-centrifuged. This pellet was resuspended in 40 μl of Buffer C (20 mM Hepes [pH 7.8], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin), and incubated on ice for 20 minutes with periodic vortexing. The lysate was centrifuged at 10,000 X g for 5 minutes at 4°C and the supernatant transferred to a new tube (nuclear fraction) and diluted with 60μl of Buffer D (20 mM Hepes [pH 7.8], 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin). The protein concentration was determined with a Bradford assay (BioRad) using bovine serum albumin (BSA) as a standard.

Immunoblotting and Antibodies

Proteins were separated on 10 or 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore) according to established protocols. The membranes were blocked with 5% non-fat milk/PBS for 1 hour at room temperature, and probed with primary antibody for 1 hour at room temperature. The antibodies used were all from Santa Cruz Biotechnology: anti-cyclin D1 (C-20), anti-cyclin D2 (H-289), anti-cyclin D3 (C-16), anti-cdk4 (C-22), anti-cyclin A1 (C-19), anti-cyclin E (C-19), anti-cdk2 (H-298), anti-cyclin H (C-18), anti-cdk7 (C-19), anti-NF-kB p65 (C-20). For immunoblotting, all antibodies were used according to the manufacturer's instructions, normally at a dilution of 1:500 or 1:1000 in 5% non-fat

milk/PBS. After incubating with primary antibody, the blots were washed 3 times with PBS for 5 minutes each wash. The blots were then incubated with secondary antibody for 1 hour at room temperature. Goat anti-mouse and goat anti-rabbit secondary antibodies were used at 1:2000 (BioRad). The blots were washed 3 times with PBS for 5 minutes each wash, and the protein was detected using enhanced chemiluminescence (ECL; Amersham-Pharmacia) and X-OMAT 5 x-ray film.

Kinase Assay

Each 10-cm plate from a differentiation time course of *WT* and *MyoD*$^{-/-}$ primary myoblasts were washed twice with PBS+ and lysed with 300µl of NP-40 Lysis/IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 10% glycerol, 0.1 mM Na_2VO_3, 50 mM NaF, 20 mM β-glycerophosphate, 50 µg/ml PMSF, 2 µg/ml leupeptin, 1 µg/ml aprotinin, 10 u µg/ml pepstatin). The lysates were incubated on ice for 30 minutes with periodic vortexing. The lysates were cleared by centrifugation (10,000 X g, 5 minutes, 4°C) and the protein was quantitated using a Bradford assay (BioRad). For the kinase assay, cdk2 was immunoprecipitated from 25 ug of extract using 1 ug of anti-cdk2 antibody (..., Santa Cruz Biotechnology) for 1 hour at 4°C with agitation. The cdk2-antibody complex was recovered by adding 25 µl of Protein A-Sepharose (Amersham Pharmacia) and further incubating at 4°C for 1 hour with agitation. The beads were collected by centrifugation (1000 X g, 2 minutes, 4°C), and washed three times with NP-40 Lysis/IP buffer, followed by three washes with kinase buffer (50 mM Hepes [pH 7.5], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 100 uM sodium vanadate). Kinase activity was assessed by incubating the immunoprecipitated cdk2 with 1 µg of histone H1 (Invitrogen) and 5 µCi of γ-^{32}P-ATP (Amersham Pharmacia) in kinase buffer, and incubating at 30°C for 20 minutes. The proteins were separated on a 12% SDS-polyacrylamide gel, stained with Coomassie Blue, destained (90% methanol, 10% acetic acid), dried, and exposed to X-OMAT XAR-5 film (Kodak).

FACS Analysis

A 10-cm dish each of proliferating *WT* and *MyoD*$^{-/-}$ myoblasts was trypsinized, centrifuged at 1000 rpm for 5 minutes and the cell pellets washed twice with PBS. The cells were fixed in 70% ethanol/PBS for 10 minutes on ice, centrifuged at 1000 rpm for 5 minutes, and washed twice in PBS. DNA was stained with 5 µg/ml propidium iodide in PBS for 5 minutes. The stained cells were analyzed for DNA content using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson; McMaster University Clinical Laboratory, Health Sciences Centre).

MyoD/Myf5 Double Knockout Fibroblasts

MyoD$^{-/-}$*:Myf5*$^{-/-}$ embryonic fibroblasts were isolated as previously described (Perry, 2003). The cells were maintained in growth medium (DMEM supplemented with 10% FBS and penicillin/streptomycin), and transfected using the calcium phosphate precipitate method as previously described (Perry et al., 2001). Briefly, 1 day prior to transfections, cells were plated at 2 X 10^5 cells per 10-cm dish (Falcon). Cells were incubated with the

calcium phosphate precipitate for 16 hours, washed twice with PBS and refed with growth medium (GM; DMEM + 10%FBS). 24 hours after refeeding, the cells were harvested for RNA isolation.

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Preface to Chapter 4

pRb plays an important role in establishing terminal differentiation in skeletal muscle by preventing apoptosis and S-phase entry after initiation of differentiation. Moreover, pRb is essential for MyoD transcriptional activity and muscle-specific gene expression. *In vivo*, it has been technically difficult separating cell cycle and survival effects from muscle-specific gene expression. To specifically address myogenic gene activation, an activated hypophosphorylated mutant of pRb, pRbΔp34, in which 8 cdk phosphor-acceptor sites were mutated, was expressed in C12C12 myoblasts and 10T½ fibroblasts co-expressing MyoD. This mutant mimics the form of pRb present during differentiation. This work established that pRb activates MyoD by rescuing cdk4-mediated inhibition, and therefore pRb plays an important role during the switch from proliferation to differentiation. I performed all of the experiments, constructed the figures and wrote the manuscript. In addition, I participated in a collaboration with Michael Huh, addressing the role of pRb *in vivo*, by looking at a muscle-specific knock-out of Rb and the resulting myoblasts. This work has been submitted to the Journal of Cell Science and is in review (Huh et al., 2004).

Activated pRb increases MyoD activity by rescuing cdk4-mediated inhibition

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Abstract

In order to establish terminal myogenic differentiation, myoblasts must irreversibly withdraw from the cell cycle. Expression of the cell cycle regulator pRb increases upon initiation of differentiation, and is progressively dephosphorylated. However, the role of pRb in myogenic differentiation does not involve cell cycle regulation, but rather the activation of myogenic gene expression. pRb increased MLC enhancer activity, and activated a more rapid induction of myosin heavy chain (MyHC) expression. The increase in MyoD activity did not involve the N- or C-terminus in isolation, suggesting pRb targets the bHLH domain. However, an activated mutant of pRb was able to rescue cdk4-mediated inhibition of MyoD, which involves an 18-bp portion of the MyoD C-terminus. Therefore, pRb augments MyoD transcriptional activity by regulating the switch from a proliferation-specific to a differentiation-specific complex.

Introduction

The pRb-family, consisting of pRb, p107, and p130, is a group of nuclear phosphoproteins important in cell cycle regulation and cellular differentiation. The ability to control the cell cycle is mediated mainly by the interaction of hypophosphorylated pRb-family members with the E2F family of transcription factors. Cyclin D/cdk4, cyclin E/cdk2, and cyclin A/cdk2 complexes sequentially phosphorylate the pRb family during G1 and S-phase, disrupting the interaction with E2F factors, and stimulating activation of E2F-specific genes (Coqueret, 2002; Murray, 2004).

The mammalian genome encodes at least six E2F proteins that differ in transcriptional activity and pRb-family member preference. The binding of pRb-family members interferes with the ability of E2F factors to form complexes with members of the basal transcriptional machinery, such as TBP and TFIIB, thus inhibiting transcriptional activation (De Luca et al., 1998; Hagemeier et al., 1993; Kim et al., 1994; Pearson and Greenblatt, 1997). In addition, pRb has been shown to actively repress transcription through the recruitment of histone deacetylases (HDACs), Swi/Snf chromatin remodeling complexes, and histone methylases (Coqueret, 2002; Stevaux and Dyson, 2002).

pRb has been suggested to play its most significant role in ensuring that cells progress through the restriction point (R) in the first gap phase (G1) of the cell cycle and enter into the DNA-synthesis phase (S). Once past the restriction point, cells will continue through one more cell cycle, until the next G1 phase is reached. *In vitro*, myoblasts are induced to differentiate under low serum conditions, forcing the cells to exit the cell cycle from the G1 phase, prior to the restriction point. Once differentiation has been established, myoblasts are unable to re-enter the cell cycle, even upon serum restimulation. These myoblasts/myotubes are described as terminally differentiated. Since pRb plays an essential role in regulating passage through the restriction point, it has been postulated that the pRb family plays an important role in establishing the terminally differentiated state.

The level of *Rb* mRNA and protein increases during differentiation of C2C12 myoblasts (Corbeil et al., 1995; Endo and Goto, 1992). MyoD stimulates transcriptional activation of the *Rb* promoter, yet the mechanism by which this occurs is indirect (Bergstrom et al., 2002; Magenta et al., 2003; Martelli et al., 1994). Proliferating myoblasts predominantly maintain E2F in an uncomplexed form, or as a complex with p107, and to a lesser extent, complexed with p130. Interestingly, pRb-E2F complexes are conspicuously absent. By contrast, p130-E2F complexes predominate during muscle differentiation, while p107-E2F complexes disappear and pRb-E2F complexes remain absent. This indicates that during myogenic differentiation pRb plays a role distinct from the traditional repression of E2F transcriptional activity.

Studies using MyoD-converted *Rb*-null embryonic fibroblasts have shown that although p107 protein levels are increased in these cells, pRb is essential for both MyoD and MEF2 transcriptional activity, as well as maintaining the terminal differentiated state (Novitch et al., 1996; Novitch et al., 1999; Schneider et al., 1994). Although *Rb*-null fibroblasts expressing MyoD become myogenic and express early muscle markers, such

as myogenin, expression of late markers, such as myosin heavy chain, is reduced. In addition, serum restimulation of these differentiated *Rb*-null myoblasts results in bromodeoxyuridine (BrdU) incorporation and thus, S-phase entry and DNA synthesis.

In order to discriminate between the role of pRb in cell cycle and myogenic gene expression, a hypophosphorylated mutant of pRb was expressed and its effects on MyoD activity and myogenic differentiation were assessed. We demonstrate that pRb increased MyoD transcriptional activity by specifically rescuing cdk4-mediated inhibition.

Results

pRb regulated activity of the MLC enhancer during the initial stages of differentiation

To investigate the role of pRb in MRF activation and differentiation, C2C12 myoblasts were transfected with a myosin light chain enhancer reporter construct (MLCLuc), and either a plasmid expressing wildtype mouse pRb (pRb), a hypophosphorylated mutant of mouse pRb (pRbΔp34), or empty vector as control. A similar transfection was performed with a Gal4-MLCLuc reporter vector, in which five repeats of the Gal4 DNA binding site were inserted upstream of the MLC enhancer. For these transfections, Gal4-pRb or Gal4-pRbΔp34 vectors, in which the Gal4 DNA binding domain was fused to the N-terminus of both the mouse pRb and the pRbΔp34 sequence, were co-transfected with the Gal-MLCLuc.

In proliferating C2C12 myoblasts, pRb activated the MLC enhancer 5-fold above the control, and a hypophosphorylated mutant of pRb (pRbΔp34) increased the activity 12-fold. In addition to demonstrating the importance of pRb in muscle gene activation, this data indicates that the phosphorylation status of pRb is important for this phenomenon. Thus, the interaction of pRb with another protein may be involved in the mechanism. This is not likely to involve a direct pRb-MyoD interaction for two reasons. First, increasing the probability of this interaction by using the Gal4-MLCLuc and Gal4 fusions of pRb did not augment MyoD activity to any significant degree (Figure 1A). In fact, the ability of Gal4-pRb to enhance activation of the Gal4-MLCLuc reporter was reduced 2.5-fold, and reduced 4-fold in the presence of pRbΔp34. Second, Zhang and colleagues, investigating the effect of cdk4 on MyoD activity, demonstrate no direct interaction between MyoD and pRb by means of a mammalian 2-hybrid assay (Zhang et al., 1999a; Zhang et al., 1999b). Therefore, the ability of pRb to stimulate MyoD activity is not likely the result of a direct effect.

In differentiating C2C12 myoblasts (Fig. 1B), pRb and pRbΔp34 did not significantly affect activation of the MLC enhancer, indicating that pRb plays a role early in differentiation, likely during the coupling of cell cycle withdrawal and initiation of differentiation. To determine if pRb-enhanced MLC enhancer activity is directed to MyoD specifically, 10T½ fibroblasts were transfected with MyoD, MLCLuc, and either pRb, pRbΔp34, or empty vector. In proliferation medium (Fig 2A), pRb increased MyoD activity on the MLC enhancer approximately 2-fold, but a hypophosphorylated mutant of pRb (pRbΔp34) increased MyoD activity 5-fold. Therefore, the increase in activation of the MLC enhancer in C2C12 myoblasts is at least in part due to the stimulation of MyoD activity.

Moreover, in differentiation medium (Figure 2.2B), both pRb and pRbΔp34 inhibited MyoD activity on the MLC-Luc reporter approximately 4-fold. Under low mitogen conditions, the effect is not dependent upon pRb phosphorylation status since wild type pRb is no longer phosphorylated due to a normal loss of cdk activity. Transfection of either Gal4-pRb or Gal4-pRbΔp34 inhibited MyoD activation of a Gal4-

Figure 1. pRb induced MRF transcriptional activity in proliferating conditions. Proliferating C2C12 myoblasts were transfected with MLCLuc or Gal4-MLCLuc reporter plasmid, and either empty control vectors or plasmids encoding mouse pRb, a hypophosphorylated mutant of pRb (pRbΔp34), or fusions of pRb with the Gal4 DNA-binding domain. For proliferation (A), the cells were maintained in growth medium (GM; DMEM & 10% FCS) and harvested 36 hours after transfection. For differentiation (B), the cells were transferred to differentiation medium (DM; DMEM & 2% HS) 36 hours after transfection, and maintained for 48 hours prior to harvest. The luciferase assay values were normalized to protein concentration, and the activity of the MLCLuc and Gal4-MLCLuc reporter vector alone was set to 1. The bars represent the mean fold activation and the error bars represent the standard deviation (±SD; n=9).

Figure 1.

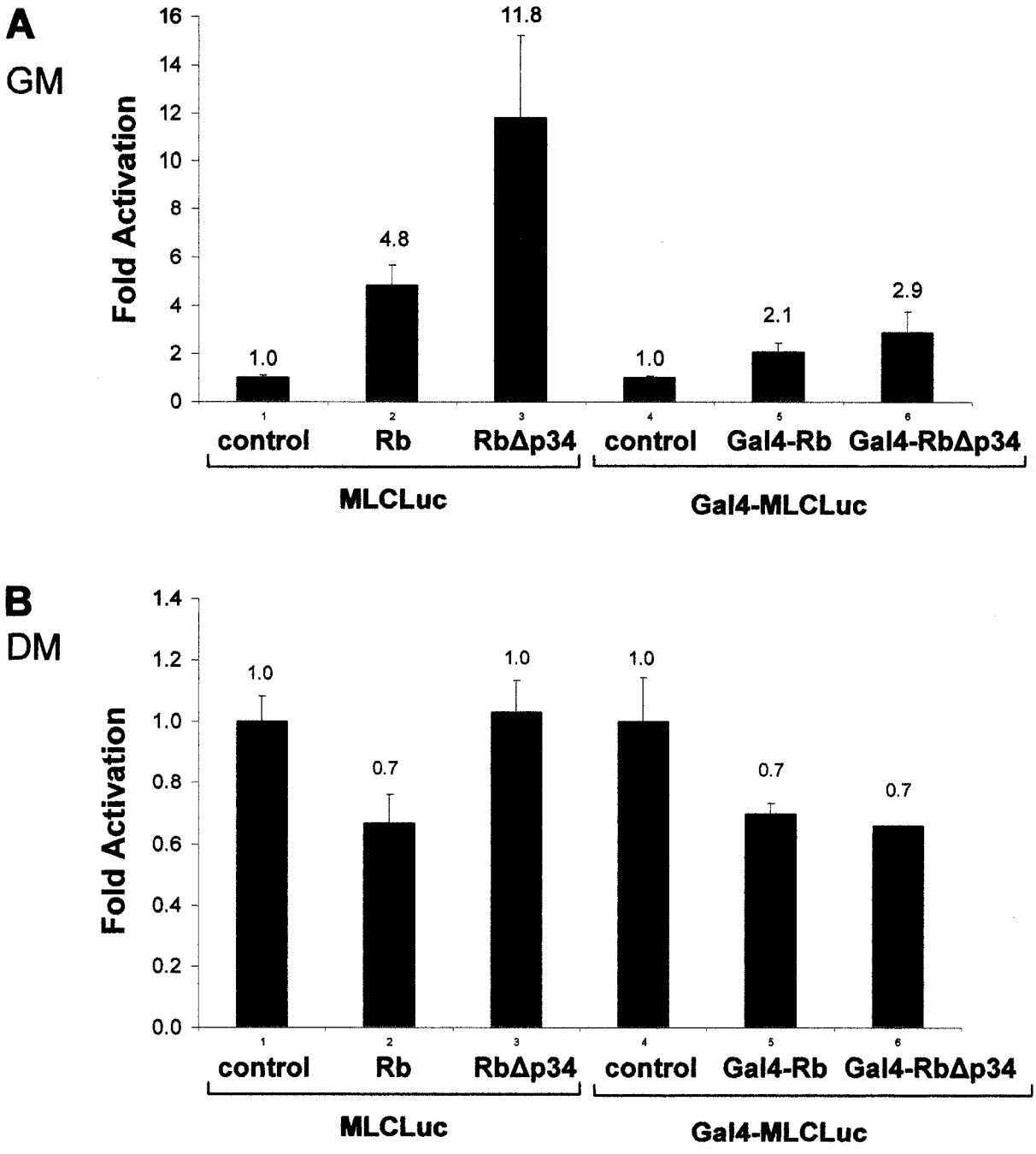
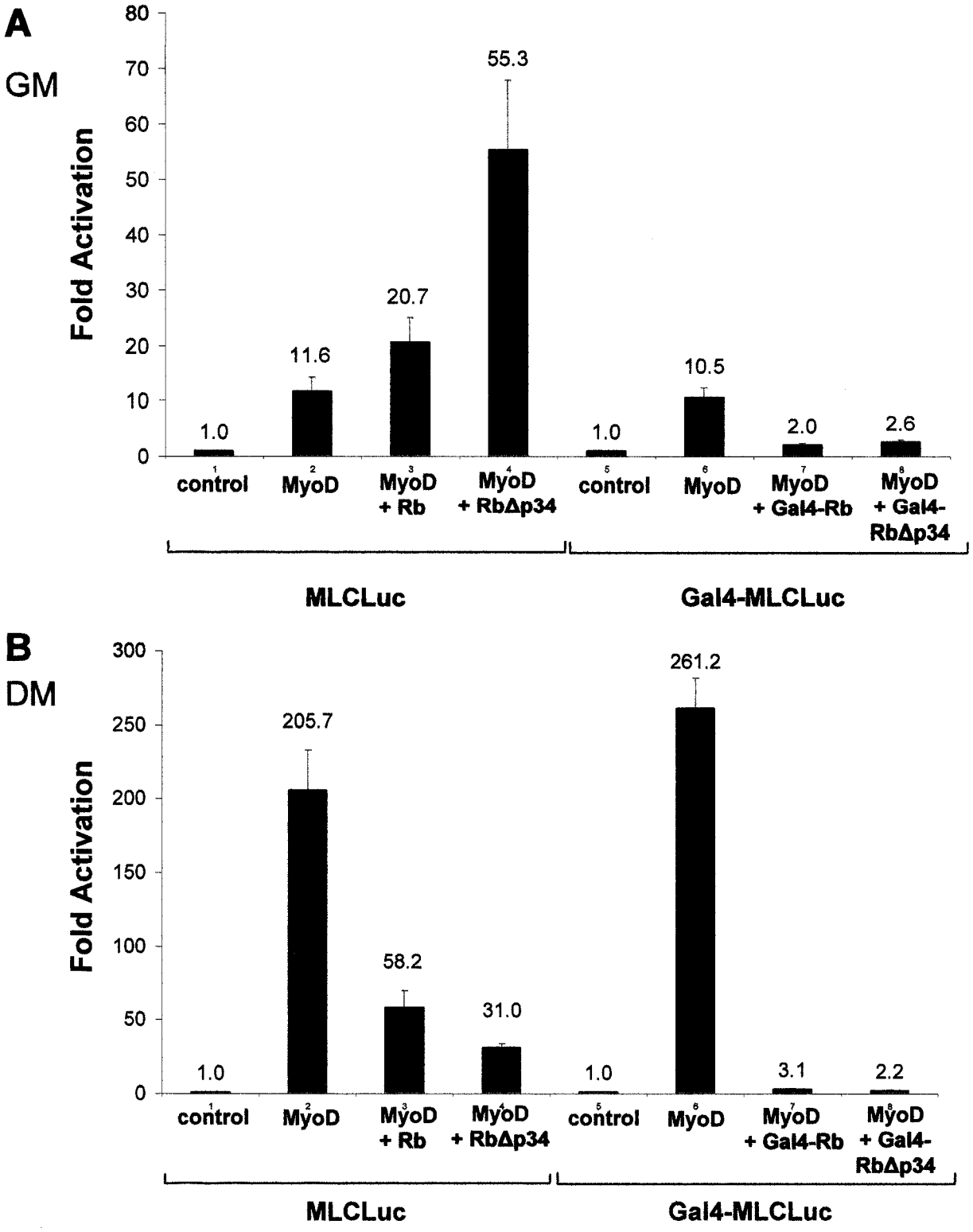

A
GM
Fold Activation
16
14
12
10
8
6
4
2
0
11.8
4.8
2.9
2.1
1.0
1.0
1
2
3
4
5
6
control
Rb
RbΔp34
control
Gal4-Rb
Gal4-RbΔp34
MLCLuc
Gal4-MLCLuc
B
DM
Fold Activation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.7
1.0
1.0
0.7
0.7
1
2
3
4
5
6
control
Rb
RbΔp34
control
Gal4-Rb
Gal4-RbΔp34
MLCLuc
Gal4-MLCLuc

Figure 2. pRb induced MyoD transcriptional activity in proliferating conditions. Proliferating 10T½ fibroblasts were transfected with MLCLuc or Gal4-MLCLuc reporter plasmid, MyoD, and either empty control vectors or plasmids encoding mouse pRb, a hypo-phosphorylated mutant of pRb (pRbΔp34), or fusions of pRb with the Gal4 DNA-binding domain. For proliferation (A), the cells were maintained in growth medium (GM; DMEM & 10% FCS) and harvested 36 hours after transfection. For differentiation (B), the cells were transferred to differentiation medium (DM; DMEM & 2% HS) 36 hours after transfection, and maintained for 48 hours prior to harvest. The luciferase assay values were normalized to protein concentration, and the activity of the MLCLuc and Gal4-MLCLuc reporter vector alone was set to 1. The bars represent the mean fold activation and the error bars represent the standard deviation (±SD; n=9).

Figure 2.


A
GM
Fold Activation
80
70
60
50
40
30
20
10
0
55.3
20.7
11.6
10.5
2.0
2.6
1.0
1.0
control
MyoD
MyoD + Rb
MyoD + RbΔp34
control
MyoD
MyoD + Gal4-Rb
MyoD + Gal4-RbΔp34
MLCLuc
Gal4-MLCLuc
B
DM
Fold Activation
300
250
200
150
100
50
0
261.2
205.7
58.2
31.0
3.1
2.2
1.0
1.0
control
MyoD
MyoD + Rb
MyoD + RbΔp34
control
MyoD
MyoD + Gal4-Rb
MyoD + Gal4-RbΔp34
MLCLuc
Gal4-MLCLuc

MLCLuc reporter construct, further supporting the hypothesis that pRb does not interact with MyoD in order to augment transcriptional activity.

pRb regulated MyoD transcriptional activity independently of the N- and C-terminal domains

To further investigate the role of pRb in differentiation, 10T1/2 cells were transfected with MyoD alone or MyoD and pRb, and the extent of differentiation was assessed by scoring the number of myosin heavy chain (MyHC) positive nuclei per 1000 nuclei. 10T½ fibroblasts transfected with MyoD and pRb exhibited a 2-fold increase in the number of MyHC positive nuclei, as compared to cells transfected with MyoD alone, consistent with the increase in MyoD activity on the MLC enhancer (Figure 3A).

C2C12 cells transfected with pRb displayed approximately 3-fold more MyHC-positive nuclei after 24 hours in differentiation medium, as compared to control cells (Figure 3B). The effect of pRb on MyHC expression was only apparent during the first 24 hours of differentiation, since at the 48-hour time point and beyond, there was no difference in the number of MyHC positive nuclei as compared with the control. Taken together, this indicates that pRb accelerates differentiation, likely through an indirect stimulation of MyoD activity.

In order to address which domain of MyoD is affected by the presence of pRb, Gal4 fusions of the N-terminus (Gal4-MyoD$^{1-99}$) and C-terminus (Gal4-MyoD$^{174-318}$) of MyoD were transfected into 10T½ fibroblasts with a Gal4-luciferase reporter (Gal4-Luc). The N-terminus of MyoD (amino acids 1-99) contains the transactivation domain and a protein-protein interaction domain implicated in chromatin-remodeling. The C-terminus (amino acids 174-318) also contains a chromatin-remodeling domain and has transcriptional activity. Neither pRb nor pRbΔp34 altered the activity of Gal4-MyoD$^{1-99}$ or Gal4-MyoD$^{174-318}$ on a Gal4-Luc reporter. This suggests that pRb affects the basic helix-loop-helix (bHLH) domain, responsible for DNA-binding, heterodimerization and other important protein-protein interactions.

Activated pRb rescued cdk4-mediated inhibition of MyoD

Cdk4 interacts with an 18-bp region of the C-terminus of MyoD (Zhang et al., 1999a; Zhang et al., 1999b). This interaction inhibits MyoD DNA binding and is independent of cdk activity. Indeed, both cdk4 and dominant negative cdk4 (DNcdk4), inhibited MyoD activity on the MLC enhancer in proliferating conditions. Co-expression of a hypophosphorylated mutant of pRb (mpRbΔp34) rescued this inhibition, yet wildtype pRb had little or no effect.

To determine if a kinase-activating cyclin of cdk4 is able to alter inhibition of MyoD by cdk4, cyclin D1 was cotransfected with cdk4 and MLCLuc into C2C12 myoblasts (Figure 6). Cyclin D1 enhanced cdk4 inhibition of MLC enhancer activity. Moreover, expression of cyclin D1, together with cdk4, inhibited the ability of mpRbΔp34 to rescue the cdk4-mediated inhibition (Figure 6).

Figure 3. pRb accelerated expression of MyHC in C2C12 myoblasts and MyoD-converted 10T1/2 fibroblasts.
(A) Proliferating 10T½ fibroblasts were transfected with MyoD alone, MyoD and pRb, or empty vector as a control. The cells were transferred to differentiation medium 36 hours after transfection, and cultured for the indicated times. Expression of myosin heavy chain (MyHC) was monitored, and the numbers represent the average number of MyHC positive nuclei per 1000 nuclei. (±SD; n=3). The control plates were negative for MyHC expression (data not shown).
(B) Proliferating C2C12 myoblasts were transfected with Rb, or empty vector as a control. The cells were transferred to differentiation medium 36 hours after transfection, and cultured for the indicated times. Expression of myosin heavy chain (MyHC) was monitored, and the numbers represent the average number of MyHC positive nuclei per 1000 nuclei. (±SD; n=3).

Figure 3.

A 10T½ cells

	MyoD control	MyoD & Rb
24 hours	12.0 ± 3.4	29.5 ± 6.5
48 hours	34.6 ± 10.0	94.9 ± 17.0
72 hours	63.6 ± 7.8	119.3 ± 15.1
96 hours	107.9 ± 8.5	220.1 ± 27.6

B C2C12 cells

	control	Rb
24 hours	10.0 ± 2.5	37.7 ± 6.1
48 hours	32.5 ± 6.1	45.8 ± 7.7
72 hours	176.0 ± 26.6	164.5 ± 21.8
96 hours	159.9 ± 22.1	151.1 ± 26.8

Figure 4. pRb did not affect the activity of the MyoD N- or C-terminus.
Proliferating 10T½ fibroblasts were transfected with the Gal4-Luc reporter and plasmid encoding either the N-terminus of MyoD (Gal4-MyoD$^{1-99}$) or the C-terminus of MyoD (Gal4-MyoD$^{174-318}$) fused to the Gal4 DNA-binding domain, in addition to plasmids encoding mouse pRb or a hypo-phosphorylated mutant of pRb (pRbΔp34). The cells were harvested 36 hours after transfection and luciferase activity was assayed. The luciferase assay values were normalized to protein concentration, and the activity of the Gal4-Luc reporter vector alone was set to 1. The bars represent the mean fold activation and the error bars represent the standard deviation (±SD; n=9).

Figure 4.

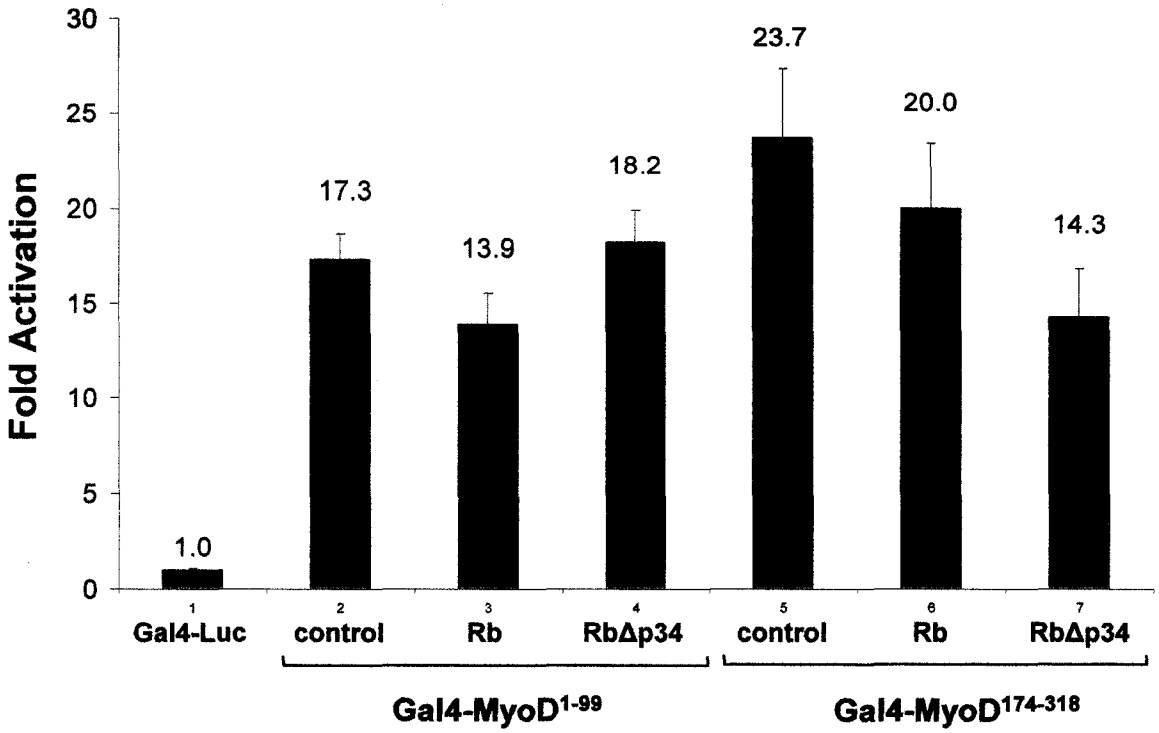

Fold Activation
30
25
20
15
10
5
0
1.0
17.3
13.9
18.2
23.7
20.0
14.3
1
Gal4-Luc
2
control
3
Rb
4
RbΔp34
5
control
6
Rb
7
RbΔp34
Gal4-MyoD1-99
Gal4-MyoD174-318

Figure 5. A hypophosphorylated pRb mutant rescued cdk4 inhibition of MRF activity.

Proliferating C2C12 myoblasts were transfected with MLCLuc and either empty control vector or plasmid encoding cyclin dependent kinase 4 (cdk4), or dominant-negative cdk4 (DNcdk4). These cells were also co-transfected with either control vector, or plasmid encoding pRb or pRbΔp34. The cells were harvested 36 hours after transfection, and luciferase activity was assayed. The luciferase assay values were normalized using the protein concentration, and the activity of the MLCLuc reporter vector alone was set to 1. The bars represent the mean fold activation and the error bars represent standard deviation (±SD; n=9).

Figure 5.

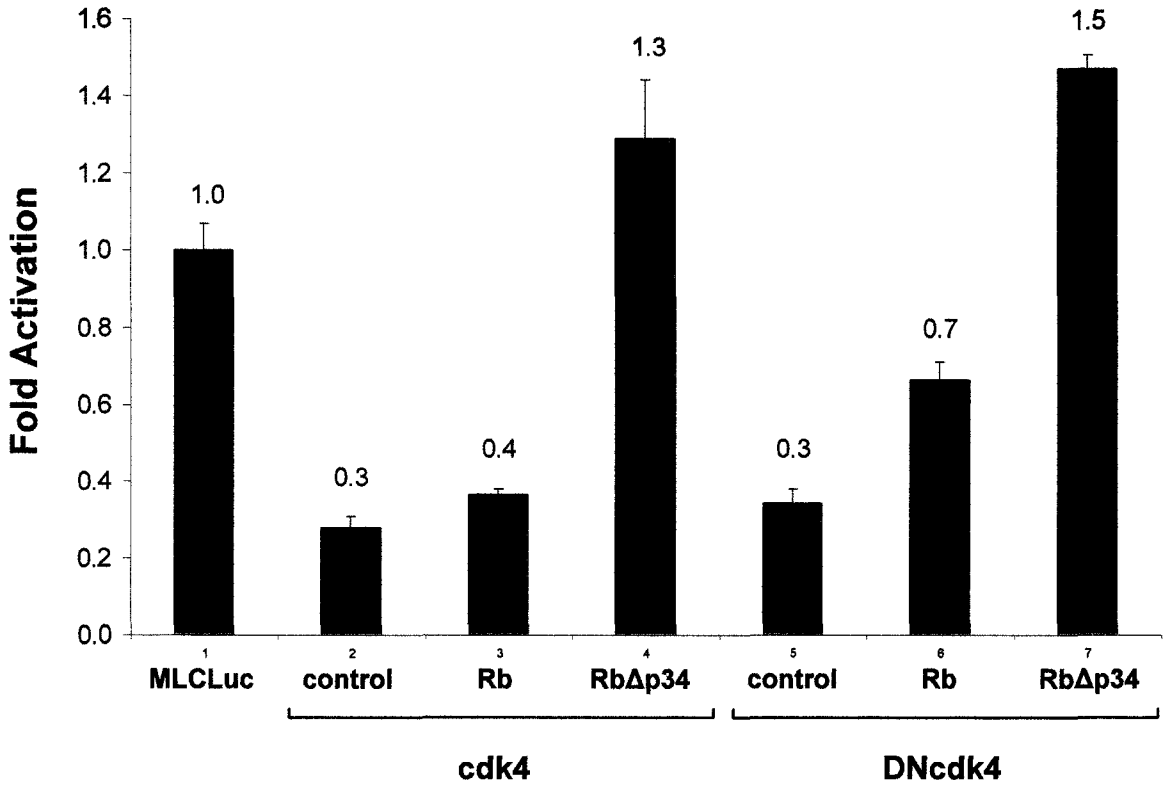
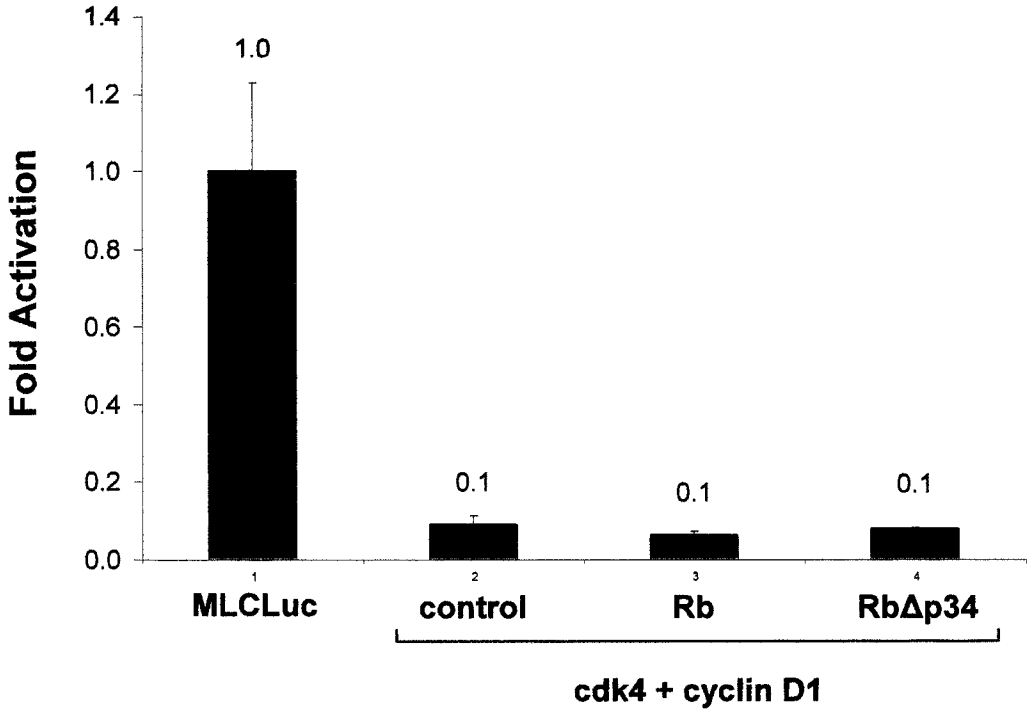

Fold Activation
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.3
0.4
1.3
0.3
0.7
1.5
1
MLCLuc
2
control
3
Rb
4
RbΔp34
5
control
6
Rb
7
RbΔp34
cdk4
DNcdk4

Figure 6. A hypophosphorylated pRb mutant was unable to rescue cdk4 inhibition of MRF activity when cyclin D1 is co-expressed.
Proliferating C2C12 myoblasts were transfected with MLCLuc, and plasmids encoding cyclin dependent kinase 4 (cdk4) and cyclin D1. These cells were also co-transfected with either control vector, or plasmid encoding pRb or pRbΔp34. The cells were harvested 36 hours after transfection, and luciferase activity was assayed. The luciferase assay values were normalized using the protein concentration, and the activity of the MLCLuc reporter vector alone was set to 1. The bars represent the mean fold activation and the error bars represent standard deviation (±SD; n=9).

Figure 6.


Fold Activation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.1
0.1
0.1
1
2
3
4
MLCLuc
control
Rb
RbΔp34
cdk4 + cyclin D1

Discussion

Mice lacking *Rb* die during embryonic development and display defects in erythroid differentiation, as well as massive cell death in the peripheral and central nervous system (PNS and CNS) (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Transgenic expression of low levels of *Rb* permits $Rb^{-/-}$ embryos to survive until birth (*mgRb:*$Rb^{-/-}$); however, the pups die soon after of skeletal muscle defects (Zacksenhaus et al., 1996). Increased cell death, in addition to smaller muscle fibers, reduced expression of muscle-specific genes, and ectopic S-phase entry characterize the abnormal muscle. Supplementing $Rb^{-/-}$ embryos with wildtype extraembryonic cells rescues the defect in erythroid differentiation, and enhances survival in the PNS and CNS, yet does not prevent endoreduplication (de Bruin et al., 2003; Wu et al., 2003). Importantly, skeletal muscle continues to exhibit severe defects, and the mice die shortly after birth due to diaphragm failure.

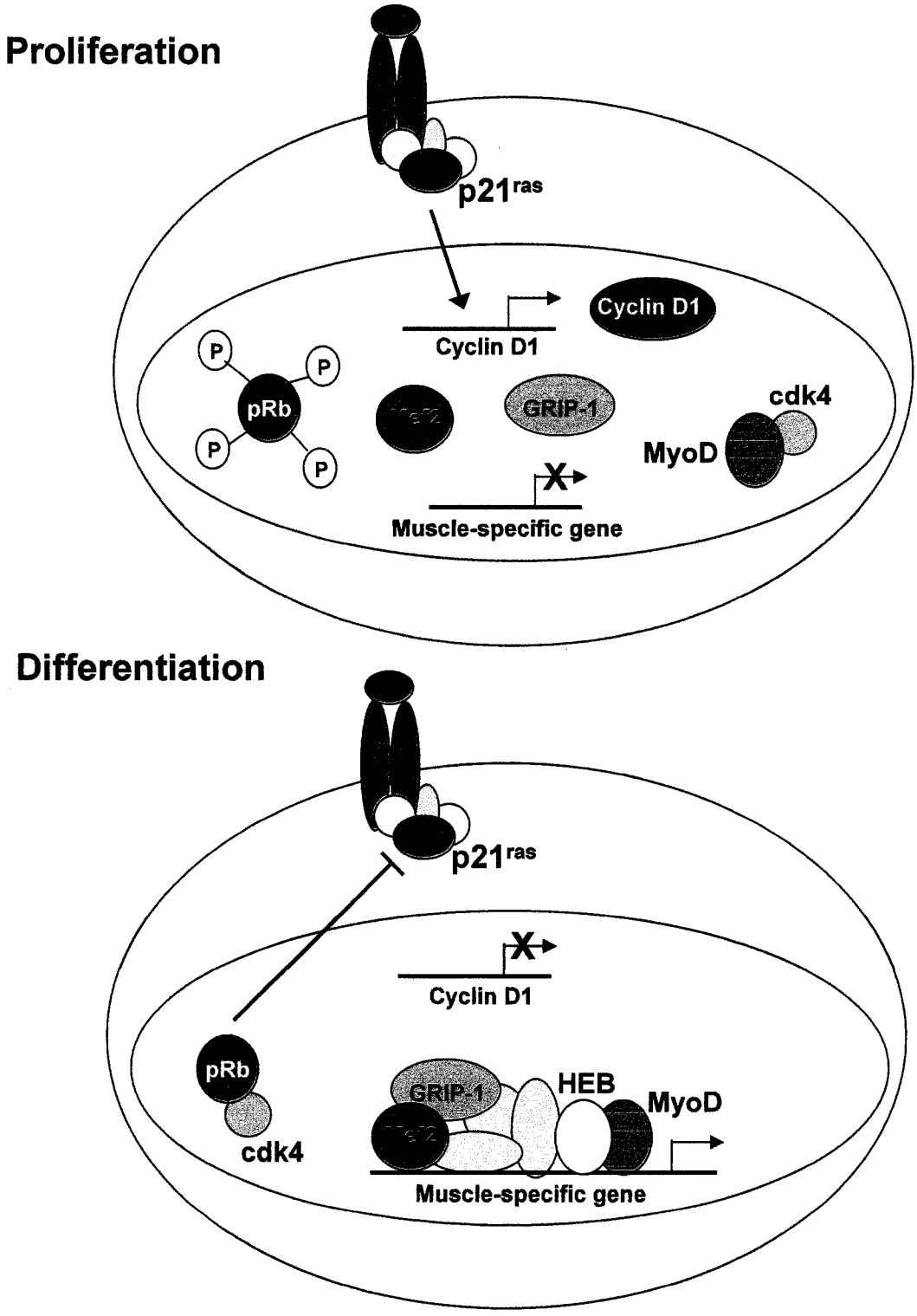
MyoD-expressing embryonic fibroblasts (EFs) lacking *Rb* are able to activate early differentiation markers, such as myogenin, but expression of late markers, such as MLC and MyHC, is abrogated (Novitch et al., 1996). In addition, the activity of muscle-specific reporter genes is severely reduced, illustrating the importance of pRb in myogenic gene expression. These cells fail to exit the cell cycle, are able to enter into S-phase even after induction of differentiation, but are unable to progress through mitosis. Thus, it was postulated that pRb was required for expression of late muscle markers and maintenance of the terminally differentiated state. Yet, the question remained: Is the lack of myogenic differentiation the result of the inability to exit the cell cycle?

Ectopic expression of pRb in C2C12 myoblasts accelerated expression of myosin heavy chain (MyHC) during initiation of differentiation, and doubled the number of MyHC-positive 10T½ fibroblasts co-expressing MyoD (Figure 3). This is most likely due to enhanced transcriptional activation of myogenic genes. Indeed, pRb enhanced activity of the myosin light chain (MLC) enhancer in C2C12 myoblasts and 10T½ fibroblasts expressing MyoD, without altering survival or transfection efficiency (Figure 1 and Figure 2, data not shown). Moreover, pRb was unable to enhance the activity of Gal4 DNA binding domain fusions with the N- or C-terminus of MyoD (Figure 4), suggesting that pRb targets the basic helix loop helix domain, and functions to enhance DNA binding or dimerization of MyoD with HEB.

A muscle-specific knockout of *Rb*, in which pRb expression is lost prior to differentiation (*Rbflox:Myf5-Cre*), recapitulates the muscle defects seen in other *Rb*-null mice (Huh et al., 2004). However, loss of pRb expression after onset of differentiation (*Rbflox:MCK-Cre*) results in mice with apparently normal muscle development and differentiation. Moreover, differentiated *Rbflox:MCK-Cre* satellite cell-derived myoblasts are unable to re-enter S-phase upon serum restimulation. Therefore, pRb is dispensible for maintenance of the terminally differentiated state once that state has been established.

Figure 7. Model of pRb activation of muscle-specific gene expression.
During proliferation, growth factors activate expression of cyclin D1, and maintain pRb in an inactive, phosphorylated state. Cyclin D1 prevents binding of Mef2 to its cofactor, GRIP-1, inhibiting Mef2 transcriptional activity. Moreover, MyoD interacts with cdk4, which precludes binding of MyoD to DNA. Together, this prevents activation of muscle-specific gene expression. Upon initiation of differentiation, pRb is progressively dephosphorylated, and hypophosphorylated pRb negatively regulates $p21^{ras}$ activation. In turn, cyclin D1 expression is inhibited, and Mef2 is able to interact with GRIP-1. Furthermore, hypophosphorylated pRb disrupts the MyoD-cdk4 interaction, allowing MyoD to bind DNA. Together, MyoD and Mef2 activate expression of muscle-specific genes.

Figure 7.


Proliferation
p21ras
Cyclin D1
Cyclin D1
P
P
pRb
P
P
GRIP-1
cdk4
MyoD
Muscle-specific gene
Differentiation
p21ras
Cyclin D1
pRb
cdk4
GRIP-1
HEB
MyoD
Muscle-specific gene

pRb regulates S-phase entry by inhibiting E2F-mediated gene expression, until phosphorylation of pRb releases E2F, allowing E2F to be transcriptionally active. C2C12 myoblasts ectopically expressing E2F1 are able to induce differentiation, but synthesize DNA and express cyclin A upon serum restimulation (Chen and Lee, 1999). Mice lacking both *Rb* and *E2F1* continue to display defects in myogenic differentiation, despite a reduction in apoptosis and S-phase entry in other tissues (Tsai et al., 1998). Taken together, this indicates that loss of pRb expression does not inhibit skeletal muscle differentiation by inducing cell cycle progression mediated by unregulated E2F1. However, other E2F family members may be compensating for the loss of E2F1, or may play a more important role in cell cycle progression in myoblasts.

Expression of an N-terminal deletion of pRb ($Rb^{\Delta 195\text{-}224}$) in *Rb*-null mice is unable to rescue the defect in skeletal muscle differentiation (Riley et al., 1997). This deletion encompasses a region outside of the pocket domain, and therefore this mutant is able to bind E2Fs and cyclins. Furthermore, mutations in the pocket domain, rendering pRb incapable of binding E2Fs, are able to cooperate with MyoD to activate the MCK enhancer and induce differentiation (Sellers et al., 1998). Therefore, pRb does not promote myogenic differentiation by inhibiting E2F activity and inducing cell cycle arrest.

pRb also interacts with Id2, a helix-loop-helix (HLH)-containing protein that lacks a basic domain, and inhibits transcriptional activity of the myogenic regulatory factors (MRFs) and E-proteins (Benezra et al., 1990; Langlands et al., 1997; Lasorella et al., 2000; Peverali et al., 1994). If the interaction of pRb and Id2 dissociates the Id-MRF complex, then loss of Id2 expression should rescue the defects in myogenic differentiation in $Rb^{-/-}$ mice. However, loss of *Id2* has no effect on myogenic differentiation, clearly indicating that the defects in $Rb^{-/-}$ skeletal muscle cannot be ameliorated by removing functional Id2.

$Rb^{-/-}$ embryonic fibroblasts (EFs) exhibit elevated levels of activated N- and K-Ras during the G1 phase of the cell cycle (Lee et al., 1999). This overstimulation of Ras can be rescued by ectopic expression of pRb, and recapitulated in WT cells expressing SV40 large T antigen (SVLT). Importantly, expression of dominant-negative Ras (Ras^{N17}) in $Rb^{-/-}$ EFs enhances MyoD activity on the muscle creatine kinase (MCKLuc) reporter and induces expression of myosin heavy chain (MyHC). This demonstrates that there is functional equivalency between introduction of Ras^{N17} and pRb with regards to muscle gene expression.

Mice lacking *Rb* and *N-ras* survive until between day 16.5 and 17.5 of embryonic development (E16.6 – E17.5), and the embryos appear normal, albeit smaller, until E15.5 (Takahashi et al., 2003). An examination of axial muscle at E14.5 reveals that concomitant loss of *N-ras* prevents the reduction in muscle fiber density and the abnormal myotube formation seen in the absence of *Rb*. Moreover, activity of the MCKLuc reporter and a MEF2-specific reporter is rescued. However, ectopic DNA synthesis and apoptosis persist. This clearly demonstrates that the role of Rb in myogenic differentiation is mediated by two pathways: (1) regulation of ras activation, which controls muscle

specific gene expression, and (2) regulation of S-phase entry, presumably through regulation of the E2F family members.

Activated Ras induces expression of cyclin D1 (Albanese et al., 1995; Filmus et al., 1994; Winston et al., 1996). In myoblasts, activation of the Ras-MAP kinase pathway by basic fibroblast growth factor (bFGF) specifically upregulates cyclin D1 and inhibits myogenic differentiation (Rao and Kohtz, 1995). Ectopic expression of cyclin D1 inhibits activation of an E-box reporter by MyoD and myogenin (Skapek et al., 1995). A mutant of cyclin D1, unable to bind pRb, is nonetheless able to inhibit MyoD transcriptional activity, indicating that cyclin D1-mediated inhibition of muscle specific gene expression is independent of pRb (Skapek et al., 1996).

Cyclin D1 interacts with p/CAF, and is able to regulate the activity of the androgen receptor (Reutens et al., 2001). It is possible that cyclin D1 prevents the interaction of p/CAF with MyoD, thereby inhibiting MyoD acetylation, which is necessary for MyoD activation (Sartorelli et al., 1999). Moreover, histone deacteylase 1 (HDAC1) interacts with MyoD in proliferating cells, resulting in both the deacetylation of MyoD and inhibition of MyoD transcriptional activity (Mal et al., 2001). A hypophosphorylated mutant of pRb (pRbΔp34), which occurs naturally in low serum conditions, interacts with HDAC1, disrupting the MyoD-HDAC1 complex, thereby activating MyoD (Puri et al., 2001).

However, Rb^{N757F}, which can bind E2F, but cannot interact with HDAC1, is able to cooperate with MyoD to stimulate muscle-specific gene activation and myogenic differentiation in $Rb^{-/-}$ EFs (Chen and Wang, 2000). Nevertheless, this mutant is unable to establish permanent growth arrest, given that serum restimulation induces phosphorylation of this pRb mutant and initiates S-phase entry in MyHC-positive cells. Therefore, this data questions the ability of pRb to enhance muscle-specific gene expression and myogenic differentiation by disrupting HDAC1-MyoD complexes. Moreover, HRDP an inhibitor of HDACs, which binds HDAC1 and HDAC3, is highly expressed in skeletal muscle, and expression increases as differentiation proceeds, providing an alternative mechanism for overcoming HDAC-mediated inhibition of MyoD activity (Zhou et al., 2000). Yet, the mechanism by which pRb is able to activate MyoD transcriptional activity is not apparent.

Cyclin D1 forms a complex with cdk4, the activity of which is required for activation of cyclin E expression. Cdk4 inhibits MyoD transcriptional activity by preventing binding to DNA, independently of kinase activity (Zhang et al., 1999a; Zhang et al., 1999b). Indeed, both cdk4 and a dominant negative cdk4 (DNcdk4), lacking kinase activity, inhibited MyoD activity on the MLC enhancer in proliferating conditions (Figure 5). Co-expression of a hypophosphorylated mutant of pRb (mpRbΔp34) rescued this inhibition, yet wildtype pRb had little or no effect, suggesting that a hypophosphorylated mutant of pRb binds cdk4, disrupting the MyoD-cdk4 interaction, allowing MyoD to activate transcription.

Expression of cyclin D1 together with cdk4 inhibited the ability of mpRbΔp34 to rescue the cdk4-mediated inhibition (Figure 6). Cyclin D1/cdk4 activity blocks the nuclear sub-localization of MEF2C and GRIP-1, a steroid receptor coactivator (SRC), necessary for muscle-specific gene expression (Lazaro et al., 2002). GRIP-1 interacts

with both MEF2C and myogenin and mediates synergistic transactivation of myogenic promoters (Chen et al., 2000). Specifically, MEF2 transcriptional activity is required for activation of the MLC enhancer (Esser et al., 1999; McGrew et al., 1996; Zetser et al., 1999). Moreover, in the absence of Rb, MyoD is able to induce nuclear localization of MEF2, which is able to bind DNA, but is functionally inactive (Novitch et al., 1999). Although the mechanism by which pRb activates MEF2 in unknown, the importance of pRb in muscle-specific gene expression is indisputable. Yet, cyclin D/cdk4 inhibition of MEF2 activity is unable to be overcome in the presence of an activated form of pRb (pRbΔp34), indicating a specific role for pRb in regulating MyoD activity.

Therefore, pRb plays an important role in myogenic differentiation and muscle-specific gene expression exclusive of cell cycle withdrawal. pRb negatively regulates Ras activation, resulting in downregulation of cyclin D1. Cyclin D1 inhibits muscle-specific gene expression, in part by inhibiting formation of an active MEF2 transcriptional complex. An activated form of pRb is unable to rescue cyclin D-mediated inhibition of myogenic transcriptional activation, suggesting the two molecules function independently. However, activated pRb is able to disrupt the cdk4-MyoD complex, allowing MyoD to bind DNA and activate transcription.

Materials and Methods

Cell Culture and Transfections

C3H10T½ fibroblasts (ATCC; CCL-226) and C2C12 myoblasts (ATCC; CRL-1772) were maintained in Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini Bioscience) and 0.1 units/ml of penicillin and 0.1 μg/ml of streptomycin (Invitrogen; 1X). Transfections were performed using the calcium phosphate method as previously described (Perry et al., 2001). Briefly, 1 day prior to transfections, cells were plated at 3.5 X 10^4 cells per 60-mm dish (Falcon) for 10T½ cells, or 2.5 X 10^4 cells per 60-mm dish for C2C12 cells. Cells were incubated with the calcium phosphate precipitate for 16 hours, washed twice with PBS and refed with growth medium (GM; DMEM + 10%FBS). 24 hours after refeeding, cells were either harvested (GM), or washed once with PBS and refed with differentiation medium and cultured for an additional 48 hours (DM; DMEM + 2% horse serum (Invitrogen) and penicillin/streptomycin). Transfections were assayed for efficiency by inclusion of a plasmid expressing either β-gal or GFP.

Plasmids

The plasmids expressing MyoD was constructed in pEMSV as previously described (Davis et al., 1987). Expression plasmids encoding Gal4-MyoD$^{1-99}$ and Gal4-MyoD$^{174-318}$ were constructed as previously described (Perry et al., 2001). Expression plasmids encoding murine pRb, Gal4-pRb, the hypophosphorylated mutant of pRb (pRbΔp34), and Gal4-pRbΔp34 were kind gifts of Paul Hamel (University of Toronto). Expression vectors encoding cdk4, dominant negative cdk4 (dNcdk4), and cyclin D1 were kind gifts of Peter Whyte (McMaster University, Hamilton). The BamH1 fragment from MLC-CAT (Donoghue et al., 1988), representing the rat myosin light chain 1/3 enhancer, was cloned into the BglII site of the pGL3 Promoter reporter vector (Promega) to give MLCLuc. Five repeats of the cognate Gal4 binding sequence were cloned into the XbaI site of pGL3 to give Gal4Luc, and also cloned into the MLC-Luc to give Gal4-MLCLuc. Vectors lacking cDNA inserts or promoter elements were used as controls in the transfections.

Reporter Assays

Transfected cells were washed twice with PBS and scraped with 1X Reporter Lysis Buffer (Promega). The cells were incubated on ice for 30 minutes with periodic vortexing, followed by one round of freeze-thaw lysis. The extracts were centrifuged and the supernatant transferred to a new tube. Luciferase activity was assayed using 10-15 μl of extract and the Luciferase Assay System (Promega). Protein concentration was determined using the modified Bradford assay (BioRad), and the relative light units (RLUs) from the luciferase assay were normalized using protein concentration. Fold activity was determined by setting the relative light units (RLUs) for the reporter vector alone sample as 1.

Immunocytochemistry

Cells were fixed with ice-cold 90% methanol for 10 minutes at -20°C. The cells were washed three times with PBS, and blocked in 5% non-fat milk/PBS for one hour, followed by incubation for one hour with primary antibody (MF20 [anti-myosin heavy chain]; Developmental Studies Hybridoma Bank; 1:10 in 5% non-fat milk/PBS). The cells were then washed three times in PBS, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (BioRad; 1:1000 in 5% non-fat milk/PBS) for one hour. The cells were washed three times in PBS and incubated with staining solution (0.06% diaminobenzamidine (DAB), 0.05M Tris [pH 7.6], 0.03% H_2O_2) until a brown colour appeared. The reaction was stopped by rinsing the cells with water. The nuclei were counterstained with hematoxylin.

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Chapter 5 - Discussion

Myogenic specification and differentiation is characterized by a spatially and temporally regulated pattern of gene expression. Although expression of any one of the myogenic regulatory factors (MRFs) defines a myogenic cell, the MRFs are not functionally redundant (Parker et al., 2003). Expression of Myf5 alone defines a population of proliferative cells that are committed to the myogenic lineage, and migrate to the limb buds or reside in the myotome prior to differentiation. However, in culture, proliferating myoblasts express both MyoD and Myf5.

Neither HEBα nor HEBβ synergistically activate Myf5, and Myf5 transcriptional activity was only marginally increased after induction of differentiation (Chapter 2, Figure 4). *Myf5* expression is increased 4-fold in *MyoD*$^{-/-}$ myoblasts, which proliferate more rapidly and exhibit a delay in differentiation (Sabourin et al., 1999). Moreover, myoblasts isolated from newborn *Myf5*$^{-/-}$ mice are unable to proliferate (Montarras et al., 2000). Taken together, this suggests that Myf5 regulates genes required for cell cycle progression rather than differentiation. However, expression of Myf5 in *MyoD*$^{-/-}$*:Myf5*$^{-/-}$ fibroblasts did not alter expression of cyclin D1 or D2 (Chapter 3 – Figure 6); yet this does not preclude a direct role for Myf5 in regulating cyclin expression in myoblasts. Furthermore, Myf5 was bound to the MLC enhancer in proliferating and differentiating C2C12 myoblasts, suggesting Myf5 may also mark genes to be activated by MyoD and myogenin later in differentiation (Chapter 2, Figure 2).

During myoblast proliferation, activated MEK1 forms a complex with MyoD, which inhibits MyoD activity and prevents myogenic differentiation (Perry et al., 2001). Moreover, a mutant of MyoD lacking amino acids 3 to 56 is unable to form a complex in association with activated MEK1. HEBα was expressed in both proliferating and differentiating myoblasts, however, HEBβ expression was restricted to differentiating myoblasts (Chapter 2, Figure 1). The hypothesis is that activated MEK1 forms a complex with MyoD and HEBα, inhibiting HEB-mediated activation of MyoD on the myogenin promoter. In the absence of the MyoD N-terminus, the inhibitory complex will not form, and HEBα should be able to synergize with MyoD to activate expression of myogenin.

HEBα was able to synergistically activate a mutant of MyoD, in which the N-terminus of MyoD is replaced with that of Myf5 (data not shown). Since HEBα is normally unable to activate MyoD on the myogenin promoter, this demonstrates that this domain is responsible for preventing HEBα-mediated activation of myogenin expression. This is an important form of regulation in view of the fact that HEBα is expressed in proliferating myoblasts, and precocious expression of myogenin induces cell cycle withdrawal and initiation of differentiation. Further dissection of the N-terminus indicates that amino acids 3 to 56, which represent a transactivation domain, are responsible for regulating the ability of MyoD to respond to HEBα.

Preliminary experiments addressing the effect of activated MEK1 on HEB-mediated activation of MyoD indicated that activated MEK1 inhibits synergy between MyoD and HEBα (data not shown). Interestingly, activated MEK1 had little or no effect on HEBα-mediated activation of MyoD$^{\Delta 3\text{-}56}$, suggesting that when MyoD is in complex with HEBα on the myogenin promoter, the N-terminus functions as a MEK1-responsive

inhibitory domain. On the other hand, activated MEK1 inhibited HEBβ-mediated synergistic activation of $MyoD^{\Delta 3-56}$ more dramatically than wildtype MyoD. Thus, in the context of a differentiation-specific MyoD-HEBβ complex, the N-terminus functions as an activation domain, and allows MyoD to be potentially unresponsive to MEK1-mediated inhibition. Determining the precise complexes formed in the presence of HEBα and HEBβ, with and without activated MEK1, will be important in determining the mechanism by which these factors regulate MyoD activity.

During differentiation, MyoD and myogenin bind to E-box sequences (CANNTG) in the promoters of muscle-specific genes and activate transcription. MyoD binds to a promoter just prior to transcriptional activation, which suggests that initiation of myogenic gene expression is regulated in part at the level of DNA binding (Bergstrom et al., 2002). During proliferation, cyclin-dependent kinase 4 (cdk4) interacts with MyoD and inhibits binding to DNA (Zhang et al., 1999a; Zhang et al., 1999b). Moreover, the cell cycle regulator, pRb, predominantly exists in its inactive, phosphorylated form, as a result of cdk activity. A hypophosphorylated mutant of pRb (pRbΔp34), which mimics the differentiation-specific activated form of pRb, augments MyoD activity by rescuing cdk4-mediated inhibition of MyoD (Chapter 4, Figure 5).

MyoD stimulates transcriptional activation of the *Rb* promoter indirectly, causing the level of pRb mRNA and protein to increase upon induction of differentiation (Bergstrom et al., 2002; Corbeil et al., 1995; Endo and Goto, 1992; Magenta et al., 2003; Martelli et al., 1994). MyoD also regulates cell cycle withdrawal by ensuring timely degradation of cyclin E protein and downregulation of cdk2 activity during initiation of differentiation (Chapter 3, Figure 4). Taken together, MyoD upregulates expression of pRb, and ensures the protein is in its active form by inhibiting cdk2 activity and preventing phosphorylation. Hypophosphorylated pRb disrupts the MyoD-cdk4 interaction, and enhances MyoD binding to promoter DNA.

Genes expressed in tissues other than skeletal muscle, are also regulated by MyoD and the MRFs. Indeed, MyoD regulates expression of cyclin D1 and D2, as well as IGFBP5 and NF-κB1/p50 (Chapter 3 – Figure 1, Discussion). Although MyoD may regulate expression of these genes by binding E-boxes in the promoter, expression may also be regulated by another transcription factor. As such, MyoD downregulated activation of NF-κB specifically during initiation of differentiation (Chapter 3, Figure 7). NF-κB binding sites in the cyclin D1 promoter specifically regulate muscle-specific expression, and thus, in the absence of MyoD, expression of cyclin D1 and D2 is maintained after serum withdrawal (Chapter 3 – Figure 1,3)(Guttridge et al., 1999). Cyclin D1 specifically inhibits myogenic gene expression independently of pRb phosphorylation (Skapek et al., 1996).

It is predicted that the E-box sequence can be found once every 256-bp in the genome. Indeed, E-boxes are located in the promoters of a number of genes, including neural-specific and B-cell specific genes. Moreover, the bHLH DNA binding motif, which recognizes the E-box sequence, is not restricted to the MRF family of transcription factors.

MyoD and myogenin bound to the E-boxes of the B-cell specific IgH enhancer (data not shown). E12 and E47, the E2A gene products, regulate expression of genes

involved in B-cell development and differentiation. Therefore, if E12 and E47 were expressed in myoblasts, and dimerized with MyoD and myogenin to activate gene expression, then IgH should be activated in muscle cells.

Interestingly, HEB did not bind to the IgH enhancer in C2C12 myoblasts, suggesting that binding of HEB to a promoter may be required for MRF-mediated transcriptional activation (data not shown). Moreover, binding of HEB to the MLC enhancer required expression of MyoD, indicating that HEB functions as a regulatory factor, rather than a directing factor (Chapter 2, Figure 2). However, HEB also plays a role in B-cell development and differentiation as a heterodimer partner of E12 and E47. If HEB binds to B-cell specific promoters in the presence of E12 or E47, this precludes co-expression of the two E-proteins in myogenic cells.

Transcription factors may direct binding of MyoD to specific E-boxes within a promoter that are flanked by the cognate DNA binding sequence for that particular transcription factor. Indeed, Pbx1 binding sites adjacent to the E2 E-box of the myogenin promoter, target MyoD complex formation on this E-box (Berkes et al., 2004). However, HEBβ-MyoD activation of myogenin expression specifically required the E1 E-box of the myogenin promoter (Chapter 1, Figure 6). Moreover, this E-box is proximal to the TATA box, and complexes bound to this E-box have more potential to be involved in associations with the basal transcriptional machinery. Importantly, it is hypothesized that the type of complex formed determines which activation and regulatory domains are accessible. Therefore, E-box specific complexes are likely to play distinct roles in gene expression and possess unique transcriptional activation potentials.

In the absence of HEBβ, expression of myogenin was inhibited, and myotubes remained mononuclear, indicating HEBβ is required for myogenic differentiation (Chapter 1, Figure 7). HEBβ differs from HEBα only by the presence of a 24-amino acid ankyrin-like motif, which intersects the putative second activation domain. This suggests that HEBα and HEBβ may form distinct transcriptional complexes with MyoD or myogenin. Moreover, the promoters of a particular cluster of myogenic genes, which exhibit a similar pattern of regulation, likely bind similar MRF-HEB complexes. This is important, since expression of these genes is also likely to be affected by the same signaling pathways and regulatory molecules. Ultimately, the ability to define the MRF complexes bound to a particular cluster of genes and identify a pattern of promoter-specific regulation is paramount to understanding myogenic specification and differentiation.

The formation of unique complexes suggests that particular domains of MyoD may play unique functions in each complex. Preliminary data indicates that in order for HEBβ to synergistically enhance MyoD activity on the myogenin promoter, the C-terminus of MyoD must be present (data not shown). Moreover, replacing the C-terminus of Myf5 with that of MyoD, converted Myf5 into a HEB-responsive transcription factor. Deletion of amino acids 170 to 209 or 218 to 269 of MyoD did not alter the ability of MyoD to respond to HEBβ, indicating that as yet unidentified domains are important in HEBβ-mediated transcriptional activation.

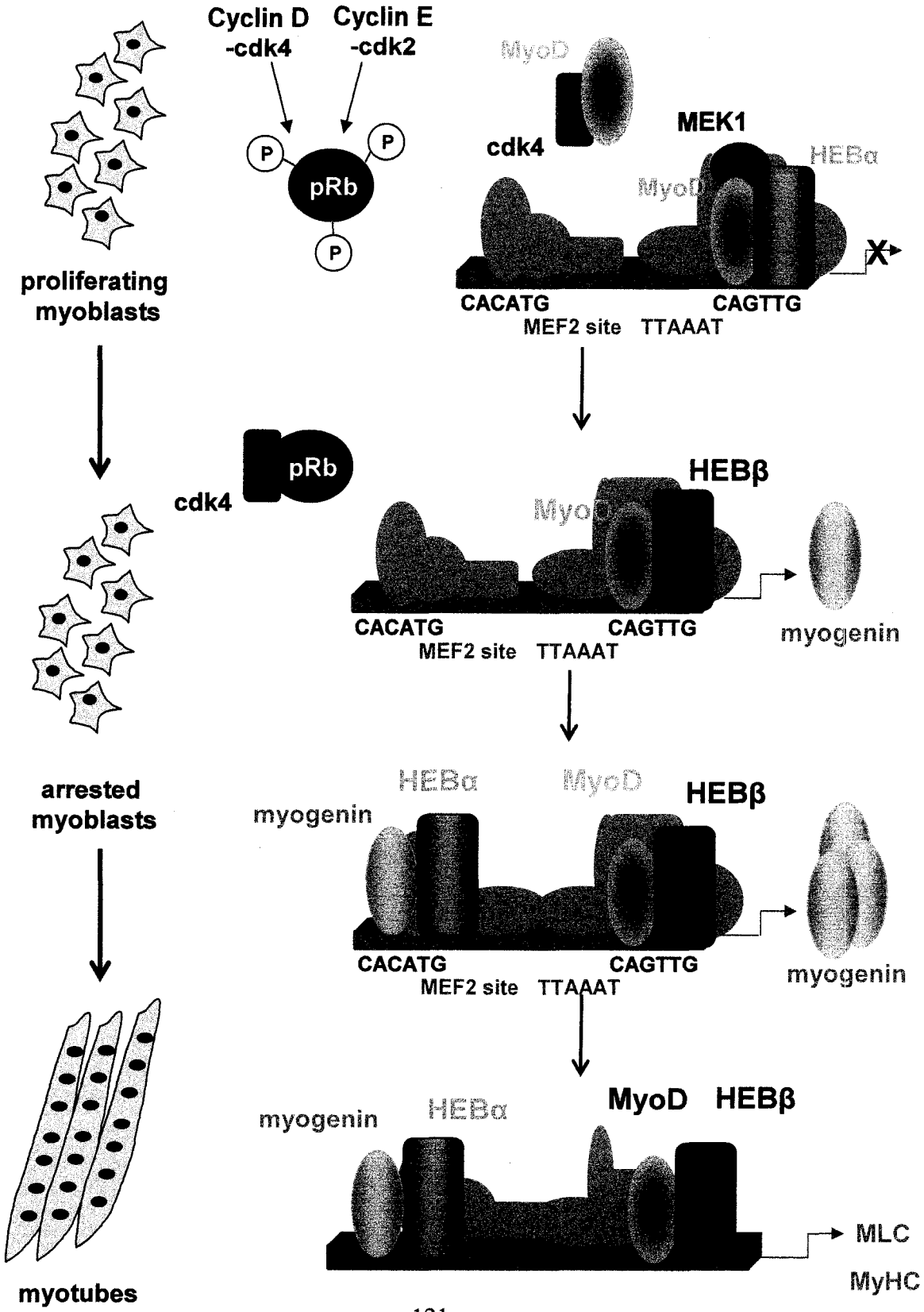
A mutant of MyoD lacking the N-terminal transactivation domain (amino acids 3 to 56) displayed an enhanced ability to respond to HEBβ (data not shown). Moreover,

HEBβ is unable to synergistically activate a mutant of MyoD lacking amino acids 63 to 99. Specifically, amino acids 96 (Trp) and 98 (Cys) are required for MyoD binding to the E2 E-box of the myogenin promoter in association with Pbx1 (Berkes et al., 2004). However, these amino acids do not appear to affect binding of MyoD to the E1 E-box, which is required for HEBβ-mediated activation of MyoD. Therefore, it would be interesting to test the ability of HEB to synergize with the MyoD$^{W96A/C98A}$ mutant, and determine if activity from the E1 E-box is affected.

The process of myogenic specification and differentiation involves a complex pattern of gene regulation, involving numerous transcription factors and regulatory mechanisms. Clusters of genes, in which the expression patterns is similar, are being defined, and guiding skeletal muscle biologists toward searching for promoter-specific complexes and patterns of regulation. Although we know that MEK1 affects early markers of differentiation, such as myogenin, and pRb and p38 regulate expression of late-induced differentiation genes, the question remains of how these regulatory molecules target particular promoters. Presumably, the formation of promoter-specific complexes accounts for the restricted pattern of regulation, but how are these complexes formed? Do the complexes change during the processes of specification and differentiation? What cellular stimuli and regulatory pathways are involved in complex construction and evolution? Thankfully, the questions, and thus the experimental possibilities, are endless.

Figure 1. Regulation of Myogenic Gene Expression in Proliferating and Differentiating Myoblasts.
MyoD binding to promoter DNA is inhibited in proliferating myoblasts by cdk4. MyoD also forms a repressive complex in cooperation with HEBα and MEK1. Moreover, during proliferation, cyclin D1-cdk4 and cyclin E-cdk2 maintain pRb in a phosphorylated state. Upon initiation of differentiation, MyoD downregulates expression of cyclin D1, and induces targeted degradation of cyclin E. pRb is progressively dephosphorylated, and disrupts the MyoD-cdk4 interaction. MyoD binds the E1 E-box of the myogenin promoter, as a complex with HEBβ, and activates expression. Myogenin cooperates with HEBα, and MyoD-HEBβ and myogenin-HEBα fully activate expression of myogenin. Moreover, MyoD and myogenin cooperate to induce expression of other muscle-specific genes, such as myosin light chain (MLC) and myosin heavy chain (MyHC).

Figure 1.


Cyclin D
-cdk4
Cyclin E
-cdk2
P
P
pRb
P
MyoD
cdk4
MEK1
HEBα
MyoD
X
CACATG
CAGTTG
MEF2 site
TTAAAT
proliferating
myoblasts
pRb
cdk4
HEBβ
MyoD
CACATG
CAGTTG
MEF2 site
TTAAAT
myogenin
arrested
myoblasts
HEBα
MyoD
HEBβ
myogenin
CACATG
CAGTTG
MEF2 site
TTAAAT
myogenin
myogenin
HEBα
MyoD
HEBβ
MLC
MyHC
myotubes

Chapter 6 -References

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