

# A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program

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**MyoD1 is a nuclear phosphoprotein that is expressed in skeletal muscle in vivo and in certain muscle cell lines in vitro; it has been shown to convert fibroblasts to myoblasts through a mechanism requiring a domain with homology to the *myc* family of proteins. The BC<sub>3</sub>H1 muscle cell line expresses skeletal muscle-specific genes upon exposure to mitogen-deficient medium, but does not express MyoD1 at detectable levels. To determine whether BC<sub>3</sub>H1 cells may express regulatory genes functionally related to MyoD1, a cDNA library prepared from differentiated BC<sub>3</sub>H1 myocytes, was screened at reduced stringency with the region of the MyoD1 cDNA that shares homology with *c-myc*. From this screen, a cDNA was identified that encodes a major open reading frame with 72% homology to the *myc* domain and basic region of MyoD1. The mRNA encoded by this MyoD1-related gene is expressed in skeletal muscle in vivo and in differentiated skeletal myocytes in vitro and is undetectable in cardiac or smooth muscle, nonmuscle tissues, or nonmyogenic cell types. During myogenesis, the MyoD1-related mRNA accumulates several hours prior to other muscle-specific mRNAs and therefore represents an early molecular marker for entry of myoblasts into the differentiation pathway. Transient transfection of 10T½ or 3T3 cells with the MyoD1-related cDNA is sufficient to induce myosin heavy-chain expression and to activate a reporter gene under transcriptional control of the muscle creatine kinase 5' enhancer, which functions only in differentiated myocytes. Expression of this cDNA in stably transfected 10T½ cells also leads to fusion and muscle-specific gene expression upon exposure to mitogen-deficient medium. Thus, the product of this MyoD1-related gene is sufficient to activate the muscle differentiation program and may substitute for MyoD1 in certain developmental situations. Together, these results suggest the existence of a family of myogenic regulatory genes that share a conserved motif with *c-myc*.**

[Key Words: MyoD1; *myc* similarity region; myogenesis; muscle differentiation]

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Expression of a differentiated muscle phenotype involves determination of pluripotent stem cells to the myogenic lineage and subsequent differentiation to form terminally differentiated myotubes that express an array of muscle-specific genes. Conversion of stem cells to the myogenic lineage appears to be directed by a hierarchy of regulatory genes. Initial evidence for such regulatory genes was obtained by Jones and co-workers, who showed that brief exposure of the embryonic mouse fibroblast line C3H10T½ to 5-azacytidine was sufficient to generate stable cell lineages that differentiated into myocytes, adipocytes, and chondrocytes (Constantinides et al. 1977, 1978; Taylor and Jones 1979, 1982). Conversion of 10T½ cells into determined cell lineages is presumed to be due to incorporation of 5-azacytidine into DNA, resulting in hypomethylation and subsequent activation of specific regulatory loci (Jones and Taylor 1980). The high frequency of conversion to myo-

blasts (up to 50%) led Konieczny and Emerson (1984) to propose that one locus, or a few closely linked loci, was responsible for establishment of the myogenic lineage. This hypothesis was supported by transfection studies in which genomic DNA from 5-azacytidine-derived myoblasts, mouse C2C12 myoblasts, and quail embryonic myoblasts was shown to generate myogenic clones from 10T½ cells with frequencies consistent with a single myogenic regulatory locus (Konieczny et al. 1986; Lassar et al. 1986). The existence of a myogenic regulatory gene was demonstrated directly by Davis et al. (1987), who identified a cDNA, referred to as MyoD1, that converts fibroblasts to myoblasts when placed under transcriptional control of a strong viral long terminal repeat (LTR). Emerson and co-workers also obtained evidence for a myogenic regulatory gene, referred to as *myd*, which directs myogenic conversion when transfected into 10T½ cells as cloned unmethylated genomic DNA

(Pinney et al. 1988). MyoD1 expression is activated in *myd* transfectants, which led to the proposal that these genes may function sequentially in a dependent myogenic regulatory pathway.

While MyoD1 and *myd* clearly play important roles in establishing the myogenic lineage, there is considerable evidence for the involvement of additional regulatory genes in myogenic determination and differentiation. Exposure of 10T½ cells to 5-azacytidine, for example, gives rise to myoblasts at high frequency, whereas other fibroblast lines are converted at low frequency, suggesting that 10T½ cells may already express one or more genes that are involved in conversion to the myogenic lineage (Taylor and Jones 1979). Similarly, cells of mesodermal origin are more efficiently converted to myoblasts by MyoD1 than are nonmesodermally derived cells (Davis et al. 1987). These observations suggest that MyoD1 may normally cooperate with additional genes to confer the myogenic phenotype. Additional evidence for positive and negative myogenic regulatory factors has been provided by heterokaryon experiments (Blau et al. 1983, 1985; Wright and Aronoff 1983; Wright 1984). Finally, the mouse muscle cell line BC<sub>3</sub>H1 does not express MyoD1 at detectable levels (Davis et al. 1987), yet these cells express skeletal muscle-specific gene products upon exposure to mitogen-deficient medium (Munson et al. 1982; Olson et al. 1983, 1984, 1986; Caffrey et al. 1987; Hu and Olson 1988; Kelvin et al. 1989). Thus, it is reasonable to predict that expression of a differentiated muscle phenotype may involve interactions between multiple regulatory genes.

Following conversion of stem cells to the myogenic lineage, the muscle differentiation program becomes subject to negative control by serum mitogens and certain nonmitogenic peptide growth factors (Konigsberg 1971; Linkhart et al. 1981; Konieczny and Emerson 1985; Lathrop et al. 1985; Florini et al. 1986; Olson et al. 1986; Spizz et al. 1986; Clegg et al. 1987). Recent studies have implicated various oncogene products in the intracellular pathway whereby growth factors suppress myogenesis. In particular, *c-myc*, a putative intranuclear mediator of growth factor signals (Kelly et al. 1983; Armelin et al. 1984), has been shown to be downregulated prior to the onset of differentiation, and deregulated *c-myc* or *v-myc* alleles have been shown to delay or to prevent the induction of muscle-specific genes (Falcone et al. 1985; Denis et al. 1987; Endo and Nadal-Ginard 1987; Schneider et al. 1987; Spizz et al. 1987). Oncogenic *ras* proteins, which are thought to activate specific intracellular growth factor cascades (Barbacid 1987), have also been shown to prevent the normal decline of *c-myc* and to block the morphological and molecular events associated with myogenesis (Caffrey et al. 1987; Olson et al. 1987; Payne et al. 1987; Gossett et al. 1988; Kelvin et al. 1989).

The potential involvement of *c-myc* in suppression of myogenesis is intriguing, considering that MyoD1 converts fibroblasts to myoblasts through a mechanism requiring a segment similar to a conserved region of the *myc* family of proteins (Tapscott et al. 1988). In light of

the importance of the *myc* homology of MyoD1 for myogenic conversion and the antagonistic roles of *c-myc* and MyoD1 in the control of myogenesis, we explored the possibility that BC<sub>3</sub>H1 cells, which appear to be MyoD1-independent, might express MyoD1-related genes sharing the *myc* homology. Here, we describe a MyoD1-related cDNA that was identified by screening cDNA libraries from BC<sub>3</sub>H1 myocytes, as well as C2 myotubes, at reduced stringency with a segment of the MyoD1 cDNA corresponding to the *myc*-like domain. The mRNA encoded by this MyoD1-related gene is expressed in differentiated myocytes in vitro and in skeletal muscle in vivo, with no detectable expression in cardiac or smooth muscle, nonmuscle tissues, or nonmyogenic cell types. During myogenesis, this mRNA accumulates several hours prior to other muscle-specific mRNAs and thus represents an early marker for entry of myoblasts into the differentiation pathway. Sequence analysis of the MyoD1-related cDNA reveals 72% homology at the amino acid level to a region encompassing the basic and *myc*-similarity regions of MyoD1. Expression of the MyoD1-related cDNA in stably or transiently transfected 10T½ cells is sufficient to establish a regulatory program that leads to expression of muscle-specific genes upon exposure to mitogen-deficient medium. Together, these results provide evidence for a family of myogenic regulatory factors that share a conserved motif with *c-myc* and suggest that the product of this MyoD1-related gene functions, either directly or indirectly, as a transcriptional activator of multiple muscle-specific genes.

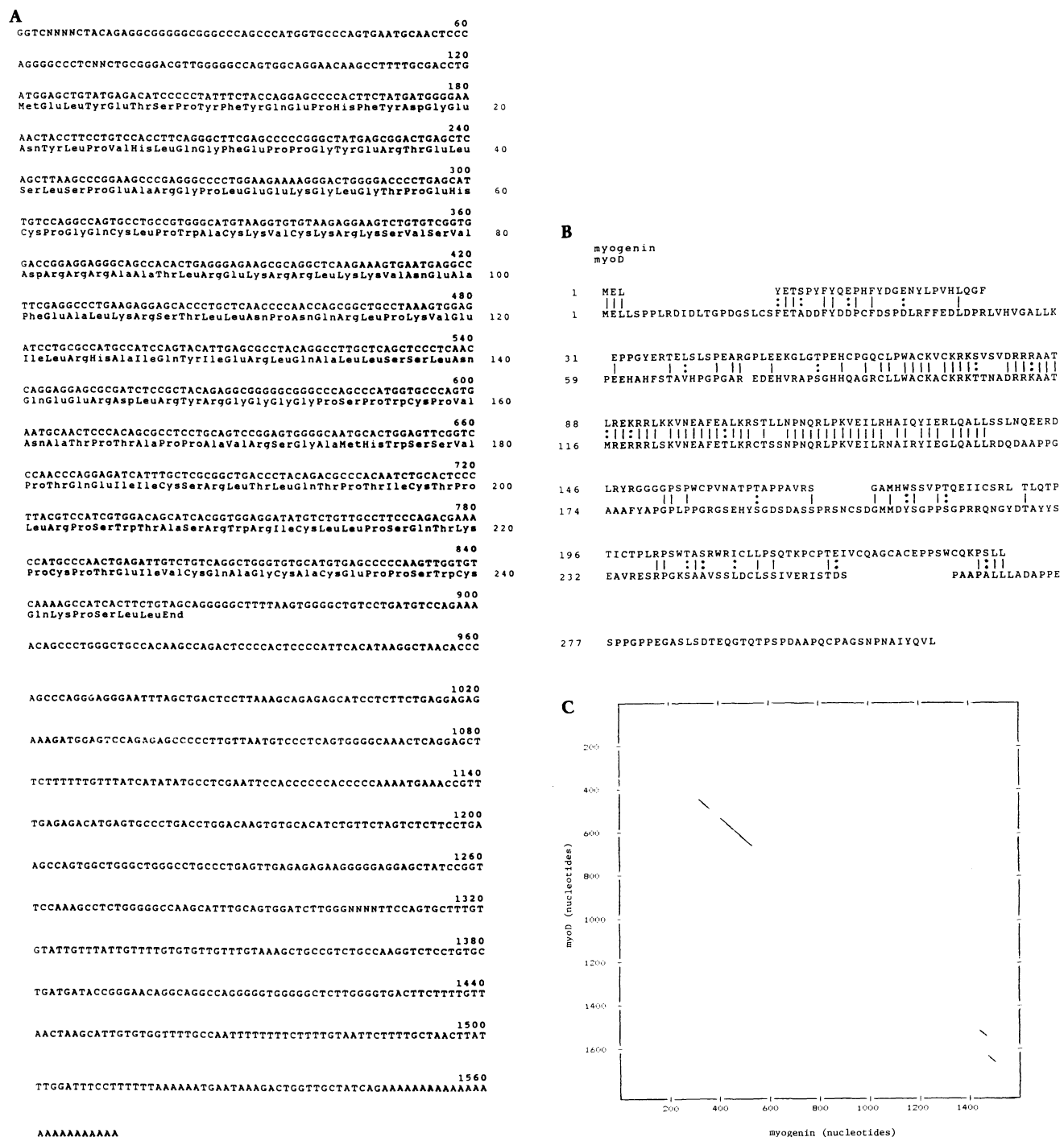
## Results

### *Screening of myocyte cDNA libraries with MyoD1 at low stringency and identification of a MyoD1-related cDNA*

Our initial interest was to investigate whether the BC<sub>3</sub>H1 muscle cell line, which does not express MyoD1 at detectable levels (Davis et al. 1987), might express a functionally related gene product. BC<sub>3</sub>H1 cells exhibit a fibroblast-like morphology when maintained at low density in mitogen-rich medium (Schubert et al. 1974). Upon exposure to mitogen-deficient medium, these cells cease dividing and express an array of muscle-specific genes. However, unlike other skeletal myoblasts, BC<sub>3</sub>H1 cells withdraw reversibly from the cell cycle and do not fuse or become committed to terminal differentiation (Munson et al. 1982; Olson et al. 1983, 1984, 1986; Strauch and Rubinstein 1984; Lathrop et al. 1985; Spizz et al. 1986; Strauch et al. 1986; Hu and Olson 1988).

To investigate whether BC<sub>3</sub>H1 cells might express a myogenic regulatory gene related to MyoD1, a cDNA library prepared from the poly(A)<sup>+</sup> mRNA of differentiated BC<sub>3</sub>H1 myocytes was screened at low stringency with a 221-bp fragment of the MyoD1 cDNA. The DNA fragment used for the screen encompassed the region of MyoD1 that shows similarity to a conserved sequence within chicken *c-myc* and *v-myc*, human and mouse *c-myc*, mouse *L-myc* and *N-myc*, and the predicted

## Edmondson and Olson



**Figure 1.** Nucleotide and predicted amino acid sequence of the MyoD1-related cDNA and homology to MyoD1. (A) The nucleotide sequence of the MyoD1-related cDNA is shown with the longest uninterrupted ORF. The cDNA shown was isolated from a C2 cDNA library. Sequence comparison with a rat cDNA clone for myogenin isolated by Woody Wright and co-workers (Wright et al. 1989) revealed 92% homology between the two sequences. (B) Homology between the predicted amino acid sequences of the MyoD1-related cDNA and MyoD1 are indicated. Bars indicate identity and dots indicate conservative amino acid substitutions. (C) Dot matrix analysis of MyoD1 and the MyoD1-related cDNA (myogenin). Diagonal lines indicate homology between the two cDNAs.

products of the *Drosophila achaete-scute* locus, which is involved in neurogenic determination. We selected this region from MyoD1 as a probe because of the demonstrated importance of the *myc* homology for myogenic conversion and because of the ability of *c-myc* to antagonize myogenesis. A cDNA library from C2 myotubes,

which express MyoD1, was screened in parallel with the same probe.

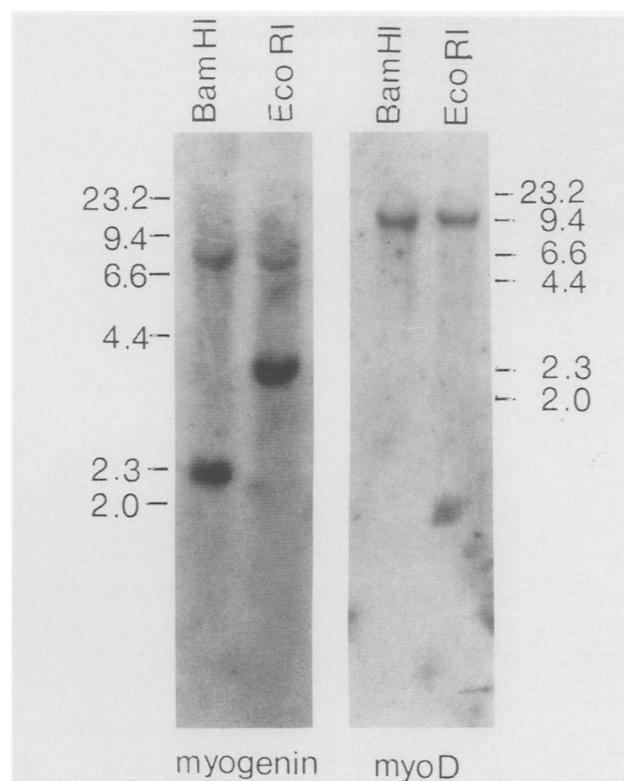
Several putative MyoD1-related cDNA clones were identified within the BC<sub>3</sub>H1 and C2 cDNA libraries from the low-stringency screens. The majority of positive clones from the C2 library encoded MyoD1; how-



ever, one of these positive clones was found to hybridize to the labeled probe at low stringency, but only weakly under high-stringency conditions. Sequence analysis of this clone and two clones from the BC<sub>3</sub>H1 library showed that they corresponded to the same gene product. The largest of the cDNAs was obtained from the C2 library and was 1571 bp in length. Within this cDNA was a single major uninterrupted open reading frame (ORF) preceded by a sequence (ACCTGATGG) that agrees reasonably well with the consensus for translation initiation (Fig. 1A) (Kozak 1984). This ORF would encode a polypeptide with a predicted  $M_r$  of 27.9 kD, which is approximately equivalent to the size of the primary translation product obtained following in vitro transcription and translation of the full-length MyoD1-related cDNA (T. Brennan, D. Edmondson, and E. Olson, unpubl.). We notice that the sequence between nucleotides 9 and 61 in the 5'-untranslated region was repeated between nucleotides 561 and 613 in the coding region. The significance of this, if any, is unclear. The 5' end of the largest of the BC<sub>3</sub>H1 cDNAs corresponded to nucleotide 231 of the longest C2 cDNA and extended to the poly(A) tail. The C2 and BC<sub>3</sub>H1 cDNAs were identical within this 1340-bp region. Although all of the clones obtained were identical, this does not rule out the possibility that additional genes, which share homology with MyoD1, might exist within BC<sub>3</sub>H1 or C2 cells.

While this work was in progress, we became aware of a cDNA isolated by Wright and co-workers that also showed homology with the basic and *myc* domains of MyoD1 (Wright et al. 1989). Their cDNA, designated myogenin, was isolated by subtraction-hybridization of cDNA from rat L6 myoblasts early in the differentiation program against cDNA from undifferentiated L6 myoblasts. Comparison of the sequence of the mouse MyoD1-related cDNA with the rat myogenin cDNA revealed 92% homology and indicated that they represented the same gene product. Therefore, we will refer to the MyoD1-related cDNA, hereafter, as myogenin. There are, however, some apparent differences between the mouse and rat sequences. In particular the mouse sequence encodes a protein of 246 amino acids, whereas the rat sequence encodes a protein of 287 amino acids. There are also nonconservative amino acid substitutions between the mouse and rat sequences at positions 124, 156, 158, 189, 234, 237, and 244.

Comparison of the sequence of the myogenin cDNA with the sequence of MyoD1 revealed 69% homology at the nucleotide level within a region of MyoD1 encompassing the basic and *myc* similarity domains, with limited homology outside of these domains. The basic domain also shares homology with *c-myc*. The homology between the MyoD1 probe used for the screen and the corresponding region of the myogenin cDNA was 58%; however, several stretches with greater than 90% homology were contained within the region encompassed by the probe. At the amino acid level, the homology between the two predicted sequences was 72% over the region of the *myc* and basic domain and, with conservative amino acid substitutions, the predicted polypeptides were 83% identical within this region (Fig. 1B). Dot

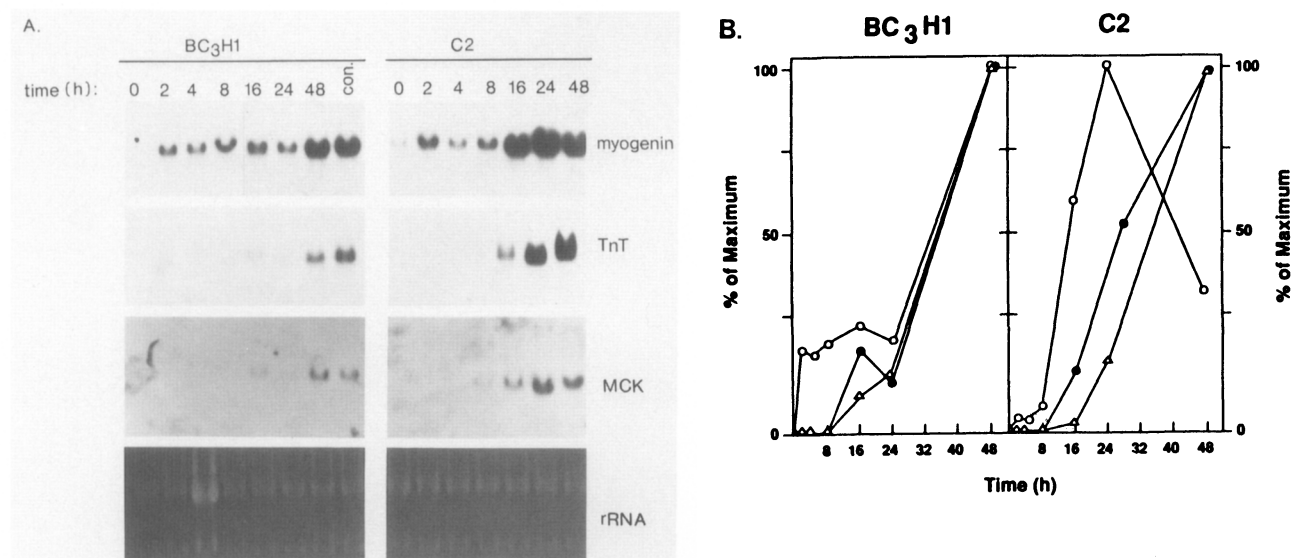


**Figure 2.** Southern analysis of mouse genomic DNA with myogenin and MyoD1 cDNA probes. Genomic DNA was isolated from C2 myoblasts, digested with *EcoRI* or *BamHI*, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, blots were hybridized with the full-length myogenin and MyoD1 cDNA probes. Sizes of DNA markers in kilobases are indicated.

matrix comparison of the myogenin cDNA and MyoD1 shows the relative position of the homologous segment and the extent of homology between the two cDNAs (Fig. 1C). Nucleotide mismatches dispersed throughout the *myc* and basic domains indicate that the myogenin mRNA does not arise by differential splicing from the MyoD1 gene. Southern blot analysis of mouse genomic DNA with the myogenin and MyoD1 cDNAs confirms that these represent distinct single-copy genes (Fig. 2).

The ORF of myogenin contains several interesting domains and is remarkably similar to MyoD1, even in regions that lack strong amino acid homology. An acidic domain is found between residues 1 and 59. Although myogenin and MyoD1 share only limited homology within this region, the first 60 residues of MyoD1 also have been reported to comprise an acidic domain (Tapscott et al. 1988). A domain rich in cysteine and histidine, which resembles the zinc finger motif found in several DNA-binding proteins (Berg et al. 1986), is found between residues 60 and 73. MyoD1 contains a similar Cys/His-rich region between residues 62 and 101. A basic domain is found between residues 74 and 96. This domain lies within the region of nucleotide homology with MyoD1. Like MyoD1, myogenin also shares homology with the *myc* family and the *Drosophila achaete-scute* complex (Villares and Cabrera 1987), as





**Figure 3.** Northern analysis of myogenin mRNA in cell lines. Total cellular RNA was isolated from the cell lines indicated and analyzed for expression of myogenin, MCK, or Tn-T transcripts, as specified. (A) BC<sub>3</sub>H1 or C2 myoblasts were transferred from growth medium to differentiation medium for the indicated times. A set of BC<sub>3</sub>H1 cultures was also allowed to differentiate at confluency in growth medium (con). Longer exposures failed to reveal Tn-T or MCK transcripts at times earlier than 8 hr after exposure to cells of differentiation medium (data not shown). The slight increase in myogenin RNA expression observed in C2 cells at 2 hr in differentiation medium (as compared to 4 and 8 hr) appears to represent a variation in this particular RNA preparation. (B) Relative levels of individual RNAs in A were quantitated by densitometry and are expressed relative to the maximum level of expression of each RNA which was assigned a value of 100%. (○) Myogenin; (△) MCK; (●) Tn-T. (C) 10T<sup>1/2</sup>, 3T3, or A7r5 cells were maintained as proliferating cultures (lane P) at subconfluent densities in growth medium or as quiescent cultures (lane Q) at confluency in differentiation medium for 5 days, as indicated. The lane designated C2 contains RNA from C2 cells exposed to differentiation medium for 48 hr; 10 µg of RNA was applied to each lane. Ethidium bromide staining of the gels is shown to confirm that equivalent quantities of RNA were electrophoresed on each lane.

well as the *Drosophila* gene *daughterless* (Caudy et al. 1988; Cronmiller et al. 1988), which is necessary for sex determination and formation of the peripheral nervous system. We also detected significant homology between the *myc* and basic regions of myogenin and the *Drosophila* gene *twist*, which is expressed in presumptive mesodermal cells and is required for germ layer formation (Thiesse et al. 1988). The amino acid sequence throughout this region appears to possess the potential to adopt a helix-loop-helix structure, as has been proposed for several other proteins that share the *myc* homology (Murre et al. 1989).

*Myogenin mRNA is induced during myogenesis and is restricted to skeletal muscle in vivo*

The pattern of expression of myogenin was examined during differentiation of BC<sub>3</sub>H1 and C2 cells. Myogenin

mRNA was undetectable in proliferating BC<sub>3</sub>H1 cells and was present at very low, but detectable, levels in C2 myoblasts at subconfluent density in mitogen-rich medium (Fig. 3A,B). Following transfer to mitogen-deficient medium, this mRNA was upregulated in both muscle cell lines within 2 hr. After 16–24 hr in mitogen-deficient medium, an additional increase in myogenin mRNA expression was observed. As reported previously, MyoD1 mRNA was undetectable in BC<sub>3</sub>H1 cells, regardless of their state of differentiation (Davis et al. 1987). MyoD1 mRNA was present in C2 myoblasts and increased in abundance during differentiation (data not shown).

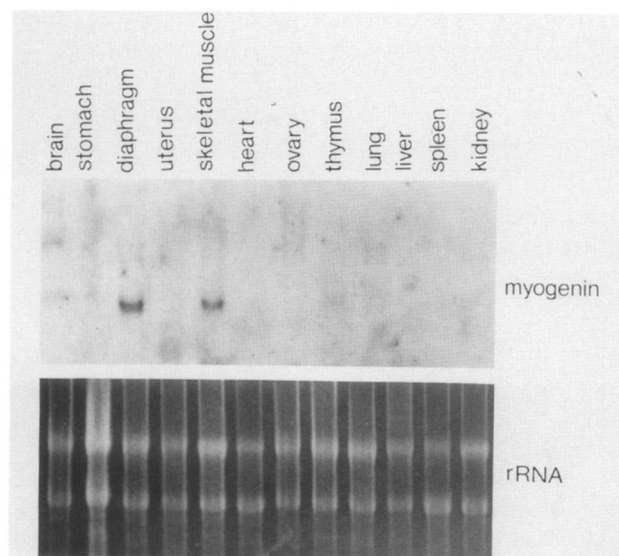
To establish whether myogenin mRNA was induced prior to or in parallel with other muscle-specific gene products, its kinetics of induction were compared with those of troponin-T (Tn-T) and muscle creatine kinase

(MCK). As shown in Figure 3, A and B, accumulation of the myogenin transcript preceded expression of these muscle-specific genes by several hours. Together, these results demonstrate that myogenin represents an early marker for myoblast differentiation and that its expression does not require MyoD1.

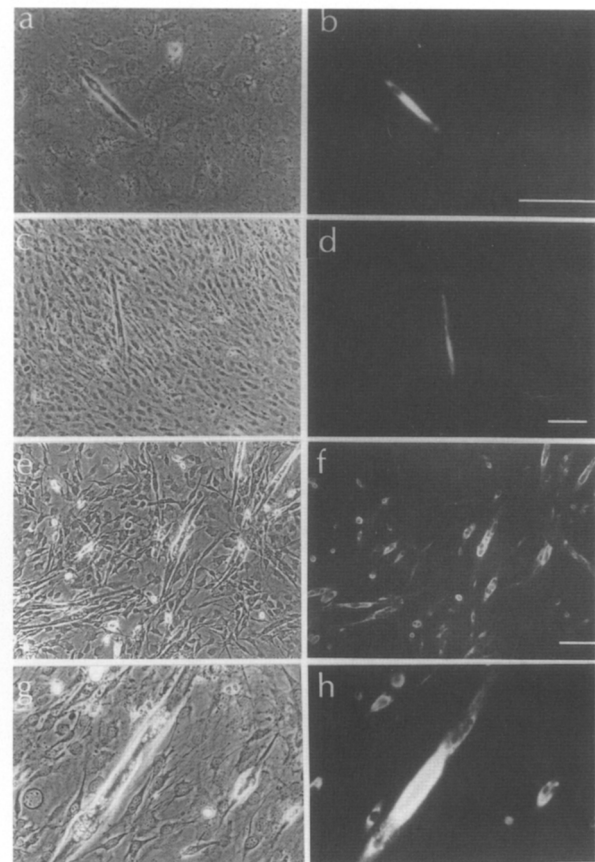
Myogenin mRNA was undetectable in proliferating or quiescent 3T3 cells or 10T½ cells (Fig. 3C). In light of the reported similarities between BC<sub>3</sub>H1 cells and smooth muscle, we also examined the A7r5 smooth muscle-like cell line for the myogenin transcript, but did not detect its presence in proliferating or quiescent cells (Fig. 3C). Analysis of adult mouse tissues for expression of myogenin mRNA showed that this mRNA was restricted to skeletal muscle, with no detectable expression in heart, smooth muscle, or nonmuscle tissues (Fig. 4).

#### *The myogenin cDNA converts 10T½ cells to myoblasts*

We investigated whether expression of myogenin was sufficient to mediate myogenic conversion by placing the cDNA under transcriptional control of a strong viral promoter and transfecting 10T½ and 3T3 fibroblasts. To allow direct comparison with MyoD1, we used the pEMSVscribeα2 expression vector used previously to characterize MyoD1 (Davis et al. 1987; Tapscott et al. 1988). The relative efficiencies of myogenin and MyoD1 in myogenic conversion were tested initially by immunostaining transiently transfected cul-



**Figure 4.** Northern analysis of myogenin mRNA in mouse tissues. RNA was isolated from the indicated tissues of adult mice and subjected to Northern analysis using a labeled myogenin probe; 10 µg of RNA was applied to each lane. Ethidium bromide staining of the gels is shown to confirm that equivalent quantities of RNA were electrophoresed on each lane. The faint bands which are detectable in RNA from brain appear to represent nonspecific hybridization to 18S and 28S ribosomal RNAs.



**Figure 5.** Myosin immunostaining of 3T3 and 10T½ cells following transfection with the myogenin cDNA expression vector. Cultures of 3T3 cells (a and b) or 10T½ cells (c–h) were transfected with the myogenin cDNA expression vector. (a–d) Results of transient transfections; (e–h) stably transfected clone. Following transfection, cultures were maintained in growth medium for 48 hr. For transient transfections, cultures were then transferred to differentiation medium for 3 days. Stable transfections were carried out with pSV2neo as a dominant selectable marker. G-418 was added to cultures 48 hr after transfection and cultures were maintained in selection medium for 14 days, at which time they were transferred to differentiation medium for 4 days. After exposure to differentiation medium, cultures were fixed and stained with anti-myosin heavy-chain antibody, MF-20, as described in Materials and methods. (a, c, e, and g) Phase-contrast photomicrographs; (b, d, f, and h) immunofluorescent photomicrographs. Bar, 100 µm. Frequencies for conversion for myosin-positive cells are shown in Table 1.

tures with a myosin heavy-chain antibody. As shown in Figure 5, a–d, exposure of transiently transfected 3T3 or 10T½ cells to mitogen-deficient medium for 3 days led to the appearance of myosin-positive cells, which were generally distinguishable morphologically by their elongated appearance compared to surrounding cells. The frequency of myosin-positive cells observed with this assay is summarized in Table 1. The results showed that the myogenin cDNA gave rise to myosin-positive cells with a frequency about two- to fourfold lower than MyoD1 (Table 1). The efficiency of myogenic conversion



**Table 1.** Frequency of myosin-positive cells following transient transfection with the myogenin expression vector

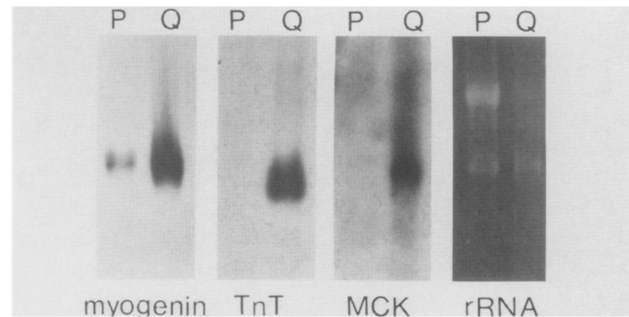
Expression vector	Frequency of myogenic conversion	
	10T1/2	3T3
Myogenin	$1.2 \times 10^{-4}$	$1.1 \times 10^{-5}$
MyoD1	$2.7 \times 10^{-4}$	$4.4 \times 10^{-5}$

Cultures of 10T $\frac{1}{2}$  and 3T3 cells were transfected transiently with 10  $\mu$ g of the indicated cDNA in the vector pEMSV-scribe $\alpha$ 2. Forty-eight hours later, cultures were transferred to differentiation medium. After 4 days, cultures were fixed and stained with anti-myosin antibody as described in Materials and methods. Cells transfected with antisense myogenin cDNA showed no myogenic conversion. Frequencies were determined by inspection of between 1 and  $2 \times 10^7$  cells.

was nearly an order of magnitude higher in 10T $\frac{1}{2}$  cells compared with 3T3 cells.

We also examined the ability of the myogenin cDNA to convert 10T $\frac{1}{2}$  cells to myoblasts following stable transfection. 10T $\frac{1}{2}$  cells stably transfected with the myogenin cDNA and pSV2neo as a dominant selectable marker proliferated and failed to express the skeletal muscle phenotype when maintained at subconfluent densities in mitogen-rich medium. After transfer to mitogen-deficient medium, stable clones that formed myotubes and stained with the anti-myosin antibody were observed. We observed no myogenic clones following transfection with the expression vector lacking an insert or with myogenin in the antisense orientation. A representative myogenic clone generated by transfection with the myogenin cDNA is shown in Figure 5, e–h. Multinucleate myotubes that are myosin-positive are readily apparent throughout this clone. Within individual myogenic clones, we observed highly differentiated cells, in addition to cells that neither fused nor stained for myosin heavy chain. This heterogeneity was maintained when these clones were expanded into cell lines. Similar observations were reported previously for MyoD1 and *myd* transfectants (Davis et al. 1987; Pinney et al. 1988). Since all cells within a given colony are clonal, this may suggest that there is a threshold of myogenin expression that is required for activation of the differentiation program and that cells within a clonal population exhibit a distribution in the level of myogenin expression. We plan to address this question using an antibody against myogenin to quantitate levels of myogenin expression at the single-cell level.

Representative myogenic clones obtained following transfection of 10T $\frac{1}{2}$  cells with the myogenin cDNA were passaged into stable cell lines and analyzed for expression of the myogenin transcript, as well as other muscle-specific mRNAs. The clone shown in Figure 6, designated 10TFL4, constitutively expressed myogenin at subconfluent density in mitogen-rich medium. The rate of cell division of mitogen-rich medium was similar in myogenin-transfectants and transfectants harboring only the neomycin-resistance gene. Following transfer to fusion-promoting medium, myogenin mRNA increased approximately 10-fold. Preliminary results indicate that this increase in myogenin expression upon re-



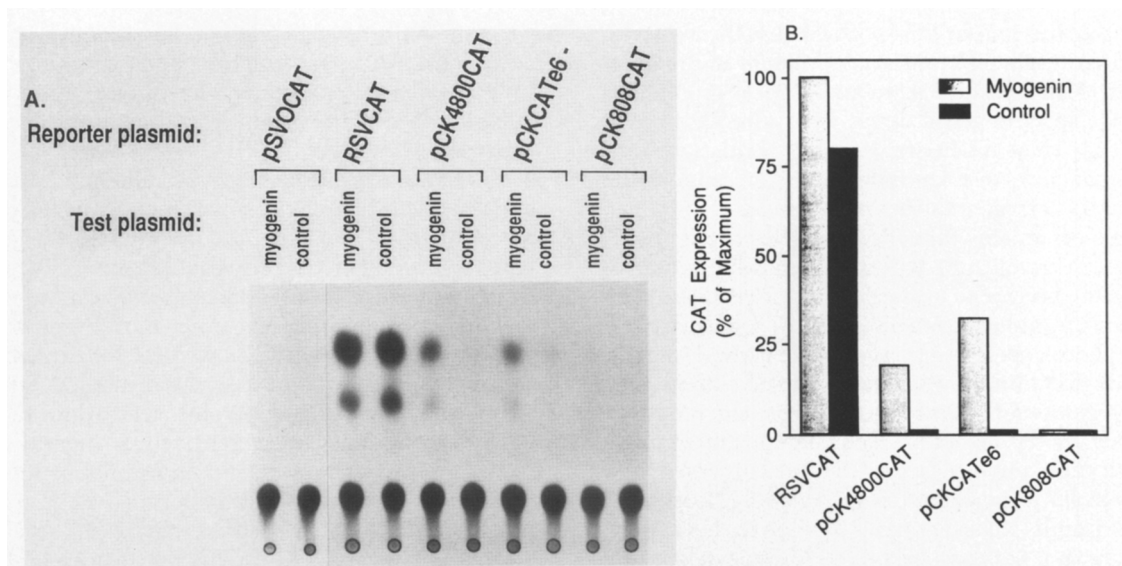
**Figure 6.** Expression of myogenin and muscle-specific mRNAs in 10T $\frac{1}{2}$  cells stably transfected with the myogenin cDNA expression vector. A representative clone of 10T $\frac{1}{2}$  cells, denoted 10TFL4, stably transfected with the myogenin cDNA and pSV2neo as a dominant selectable marker, was isolated and passaged into a stable cell line. Total cellular RNA, isolated from proliferating cultures (lane P) at subconfluent density in growth medium and from quiescent cultures (lane Q) at confluency in differentiation medium, was analyzed by Northern analysis for expression of myogenin, MCK, and Tn-T mRNAs, as specified. Ten micrograms of RNA was applied to each lane.

moval of mitogens is due to activation of the endogenous myogenin gene (D. Edmondson and E. Olson, in prep.). Tn-T and MCK mRNAs were undetectable in proliferating 10TFL4 cells, but were induced to high levels following transfer of this cell line to fusion-promoting medium. Similar results were obtained with multiple clones of 10T $\frac{1}{2}$  cells harboring the myogenin expression vector (data not shown). Together, these results show that upon removal of mitogens, the myogenin cDNA leads, either directly or indirectly, to fusion and activation of multiple muscle-specific genes.

#### *The myogenin cDNA activates a muscle-specific enhancer upstream from the mck gene following transient transfection into nonmyogenic cells*

Developmental regulation and muscle-specific expression of the *mck* gene has been shown to be dependent on an upstream muscle-specific enhancer located between –1204 and –1048 bp relative to the transcription initiation site (Jaynes et al. 1988; Sternberg et al. 1988, 1989). This enhancer is inactive in myoblasts and nonmyogenic cells and directs high levels of transcription from the *mck* promoter or from heterologous promoters in myotubes. To test whether the myogenin cDNA was able to activate the *mck* 5' enhancer, 10T $\frac{1}{2}$  cells were cotransfected with the myogenin cDNA expression vector and a reporter gene (*cat*) under transcriptional control of a series of *mck* 5' sequences or viral control elements. As shown in Figure 7, cotransfection of the myogenin cDNA and pCK4800CAT, which contains the 4.8 kb of DNA immediately preceding the *mck* gene, led to significant levels of CAT expression in 10T $\frac{1}{2}$  cells. In contrast, CAT expression was undetectable following cotransfection of pCK4800CAT with the myogenin cDNA in the antisense orientation. To begin to define the DNA sequences responsible for myogenin-dependent activation of the *mck*–*cat* gene, we examined the





**Figure 7.** CAT activity in 10T½ cells transiently transfected with the myogenin cDNA expression vector and *mck-cat* reporter genes. 10T½ cells were transfected transiently with 10 µg of each of the indicated reporter plasmids and 2 µg of test plasmids. At 48 hr following transfection, cultures were transferred to differentiation medium for 96 hr. Cells were then harvested and levels of CAT activity were determined as described in the text. (A) Thin-layer chromatographs of a representative CAT assay. (B) Results from several assays were quantitated and are expressed relative to the level of CAT activity in 10T½ cells transfected with RSV-CAT and the myogenin expression vector in the sense orientation, which was assigned a value of 100%. Control refers to transfections with the indicated CAT vectors and the expression vector with myogenin in the antisense orientation. Cotransfections using pUC19 as a control yielded comparable results.

ability of myogenin to activate expression of pCK808CAT, which contains only the *mck* promoter, and pCKCATE6-, which contains the promoter plus the 156-bp enhancer shown to be responsible for muscle-specific activity. The detailed structures of these plasmids are described in Materials and Methods. Myogenin showed little or no effect on pCK808CAT, whereas it directed expression of pCKCATE6- at a level approximately equivalent to pCK4800CAT (Fig. 7). These results suggest that the upstream enhancer mediates the actions of myogenin on the *mck* gene. The magnitude of induction of the *mck* enhancer by myogenin exceeds 20-fold, but is difficult to quantitate absolutely because there is no detectable expression in 10T½ cells in the absence of the myogenin expression vehicle. We reproducibly observed a weak positive effect of the myogenin cDNA on expression of RSV-CAT (Fig. 7) and a two- to fourfold stimulation of pSV2CAT (data not shown). Whether this reflects a generalized effect on several enhancers or a specific interaction with elements that may be shared between the SV40, RSV, and *mck* enhancers remains to be determined. Together, these results demonstrate that myogenin can function, either directly or indirectly, as a *trans*-activator of muscle-specific regulatory elements associated with the *mck* gene.

## Discussion

### *Identification of a MyoD1-related gene that is induced early in the myogenic differentiation program*

MyoD1 is expressed exclusively in skeletal muscle in vivo and in vitro and exhibits the ability to convert a

variety of cells of mesodermal origin to myoblasts. Deletion mutagenesis has revealed that a 68-amino-acid segment encompassing the basic and *myc*-similarity domains of MyoD1 is both necessary and sufficient for MyoD1-dependent conversion of fibroblasts to the myogenic lineage (Tapscott et al. 1988). The apparent importance of the *myc* domain as a positive effector for myogenic conversion led us to examine whether the BC<sub>3</sub>H1 muscle cell line, which does not express MyoD1 at detectable levels, might express a functionally equivalent regulatory gene product. Screening of a BC<sub>3</sub>H1 cDNA library at low stringency with a region of the MyoD1 cDNA corresponding to the *myc* domain resulted in the identification of a MyoD1-related cDNA. The deduced MyoD1-related polypeptide exhibits 72% homology to MyoD1 over the basic and *myc* regions and only limited homology outside of these regions. Within the 68-amino-acid segment of MyoD1 defined as the critical region for myogenic conversion, the two proteins are identical at 49 residues and with conservative substitutions are conserved over 54 residues. The organization of domains within the two predicted polypeptides is also remarkably similar, even over regions that lack strong amino acid homology. The rat homolog of this MyoD1-related cDNA was cloned independently by Wright and co-workers using subtraction hybridization, and has been designated myogenin (Wright et al. 1989).

### *Myogenin expression is sufficient to activate the muscle differentiation program*

In addition to the structural similarities between MyoD1 and myogenin, these gene products exhibit func-

tional similarities in that they both possess the ability to activate the muscle differentiation program in nonmyogenic cells. Although myogenin and MyoD1 might activate muscle-specific genes directly, an alternate possibility is that they modulate a set of regulatory gene products that may, in turn, modulate expression of the genes that are activated during myogenesis. To begin to define the mechanisms that allow muscle-specific genes to respond to myogenin, we examined whether myogenin would direct the expression of a reporter gene under transcriptional control of *mck* regulatory sequences. These experiments revealed that the 156-bp 5' enhancer region of *mck* was necessary and sufficient to confer myogenin-dependent regulation on the *mck* promoter. This 156-bp region has been demonstrated to interact with three nuclear factors that are expressed with kinetics similar to myogenin in C2 and BC<sub>3</sub>H1 cells following a stimulus to differentiate (D. Kelvin, L. Gossett, E. Sternberg, and E. Olson, in prep.). A myocyte-specific factor that interacts with this region has also been identified in nuclear extracts from mouse MM14 myotubes (J. Buskin and S. Hauschka, in press). Studies are in progress to determine whether any of these enhancer-binding factors may represent myogenin. In this regard, Murre et al. have recently identified two cDNAs whose products show homology to the basic and *myc* domain of myogenin. These factors bind specifically to the KE2 motif of the immunoglobulin  $\kappa$ -chain enhancer through a mechanism dependent on this domain (Murre et al. 1989). Because an element has been identified within the *mck* enhancer that shares 12 of 14 nucleotides with the KE2 motif, it is plausible that myogenin may interact directly with the *mck* enhancer at this site (Jaynes et al. 1988; Sternberg et al. 1988).

Although it is clear that constitutive expression of myogenin can direct the expression of muscle-specific genes in nonmyogenic cells, it is important to emphasize that the activity of myogenin is modulated in a negative manner by mitogens. This was demonstrated most clearly by stable transfection of 10T $\frac{1}{2}$  cells with the myogenin expression vector. In the presence of high levels of mitogens, these cells expressed the transfected myogenin gene, but did not express muscle-specific gene products. It appears, therefore, that myogenin, which is normally expressed at high levels in C2 and BC<sub>3</sub>H1 cells only after withdrawal of mitogens, may play an important role in a transcriptional regulatory program that is antagonized by mitogenic signals.

#### *Interactions between myogenic regulatory genes*

It is interesting to consider the potential relationship between myogenin, MyoD1, and *myd*. Southern blot analysis of *myd* transfectants with a myogenin cDNA indicates that *myd* and myogenin represent distinct genetic loci (C.P. Emerson, pers. comm.). Whereas *myd* is postulated to mediate the initial conversion of stem cells to the myogenic pathway and would therefore be expected to be expressed in myoblasts (Pinney et al. 1988), the temporal pattern of myogenin expression, combined with its ability to activate the differentiation program,

suggests that this gene product may function as a differentiation-specific transcription factor that controls multiple muscle-specific genes. Thus, myogenin may be required toward the end of a myogenic regulatory pathway.

The relative positions of MyoD1 and myogenin in the hypothetical hierarchy of myogenic regulatory genes are more equivocal and may vary in different myogenic cell types or developmental situations. Because BC<sub>3</sub>H1 cells do not express MyoD1 at detectable levels, we conclude that neither myogenin nor other muscle-specific genes are absolutely dependent on MyoD1 for expression. An attractive hypothesis is that the structural differences between myogenin and MyoD1 may allow these proteins to interact with different cellular factors or DNA sequences and thereby modulate parallel or interacting regulatory pathways. In this regard, the transfection assays that have been used to analyze the functions of these genes seem to require high levels of expression of the cDNAs to observe myogenic conversion and, as a result, might lead to a loss of subtle specificities that might be observed at more physiological levels of these gene products. It is important to emphasize that expression of these regulatory factors is not mutually exclusive, because differentiated C2 cells and, more importantly, muscle tissue express both myogenin and MyoD1. Thus, both of these regulatory factors may be required in vivo to express the full myogenic phenotype properly.

#### *BC<sub>3</sub>H1 cells may represent a fusion-defective skeletal muscle line*

The classification of BC<sub>3</sub>H1 cells as skeletal or smooth muscle has been controversial. This cell line, which was isolated from a nitrosoethylurea-induced brain tumor (Schubert et al. 1974), expresses a broad array of genes specific to skeletal muscle; however, they do not fuse or commit irreversibly to the postmitotic state in mitogen-deficient medium (Munson et al. 1982; Olson et al. 1983, 1984, 1986; Spizz et al. 1986; Hu and Olson 1988; Lathrop et al. 1985). These aspects of the BC<sub>3</sub>H1 differentiation program have contributed to speculation about the embryonic origin of these cells as well as their precise classification as smooth or skeletal muscle. The observation that myogenin is restricted to skeletal muscle in vivo and in vitro, and is undetectable in smooth muscle tissue or in the A7r5 smooth muscle-like cell line, suggests that BC<sub>3</sub>H1 cells represent a line of skeletal muscle origin. The reported expression of smooth muscle  $\alpha$ -actin by BC<sub>3</sub>H1 cells (Strauch and Rubinstein 1984; Strauch et al. 1986) is not inconsistent with a skeletal muscle phenotype, as other skeletal muscle cells appear to express this actin isoform (Buckingham et al. 1982; Pinset and Whalen 1984). Because myogenin expression in 10T $\frac{1}{2}$  cells can lead to fusion, the inability of BC<sub>3</sub>H1 cells to fuse or commit to terminal differentiation may be attributable to a defect in a regulatory pathway under the control of myogenin or to the lack of one or more factors that cooperate with myogenin to control these events. The fact that myogenin is ex-

pressed at high levels in BC<sub>3</sub>H1 myocytes, which retain the ability to reenter the cell cycle in response to mitogenic stimulation, also suggests that myogenin expression alone is insufficient to confer the postmitotic state.

#### *Potential interactions between c-myc and members of the MyoD1 family*

There is considerable evidence to suggest that *c-myc* plays a critical role in the control of differentiation of diverse cell types (Coppola and Cole 1986; Dmitrovsky et al. 1986; Prochownik and Kukowska 1986; Cole 1987). In myoblasts, for example, expression of *c-myc* declines dramatically prior to the onset of differentiation (Endo and Nadal-Ginard 1986; Spizz et al. 1987). Conversely, growth factors that suppress myogenic differentiation induce *c-myc* expression, and certain differentiation-defective muscle cell lines fail to downregulate *c-myc* following exposure to mitogen-deficient medium (Sejersen 1985; Olson et al. 1987; Payne et al. 1987; Spizz et al. 1987). The potential involvement of *c-myc* as a negative regulator of differentiation is suggested further by the ability of deregulated *c-myc* alleles or of *v-myc* to disrupt the differentiation program of a variety of cell types including myoblasts (Falcone et al. 1985; Coppola and Cole 1986; Dmitrovsky et al. 1986; Prochownik and Kukowska 1986; Denis et al. 1987; Schneider et al. 1987). Considering the homology between *c-myc* and myogenin/MyoD1, it is tempting to speculate that *myc* may compete with these myogenic regulatory factors for interaction with critical cellular targets. Within this context, the inability of *c-myc* to abolish the induction of muscle-specific genes in BC<sub>3</sub>H1 cells (Schneider et al. 1987) or to suppress muscle-specific genes in L6E9 myotubes (Endo and Nadal-Ginard 1986) may indicate that the level of *myc* expression cannot reach a sufficient level to counterbalance the positive influences of myogenin or MyoD1 on myogenesis.

The presence of the *myc* homology in multiple genes that function as positive effectors for cellular determination and differentiation supports the prediction of Davis et al. (1987) that the *myc*-like motif may represent a specialized protein domain common to multiple regulatory factors. In the future, it will be interesting to identify additional genes that share this conserved motif and ultimately to identify the cellular targets with which they interact.

## Materials and methods

### *Cell culture*

BC<sub>3</sub>H1 (Schubert et al. 1974) and C2 (Yaffe and Saxel 1977) cells were maintained at subconfluent densities in growth medium containing Dulbecco's modified eagle's medium (DMEM) with 20% fetal bovine serum (FBS) and were split every 48–72 hr to ensure that the cells did not make extensive cell–cell contact. To initiate differentiation, BC<sub>3</sub>H1 cells were transferred to DMEM with 0.5% FBS and C2 cells were exposed to DMEM with a 2% horse serum (differentiation media). C3H10T½,

NIH-3T3, and A7r5 cells (Kimes and Brandt 1976) were maintained in DMEM with 20% FBS unless otherwise specified.

### *Screening cDNA libraries*

Oligo(dT)-primed cDNA libraries were prepared from the poly(A)<sup>+</sup> mRNA of C2 myotubes or BC<sub>3</sub>H1 myocytes in the λ ZAP phage (Stratagene). Initial identification of MyoD1-related cDNAs were performed by hybridization of ~200,000 phage plaques from each library at a density of 10,000 plaques/15-cm dish with a *Dde*I fragment from mouse MyoD1 (Davis et al. 1987). This region of MyoD1 encompasses the basic and *myc* domains as defined by Davis et al. (1987). After hybridization at 42°C for 16 hr in 35% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 × Denhardt's solution, 50 mM sodium phosphate, and 10% dextran sulfate, filters were washed for 60 min at room temperature and for 30 min at 55°C in 2 × SSC, 0.1% SDS. Positive clones were plaque-purified, and clones that hybridized to the MyoD1 probe at reduced stringency, but not at high stringency, were analyzed by restriction mapping. Clones obtained from low-stringency screens were used to rescreen the BC<sub>3</sub>H1 and C2 libraries at high stringency to obtain additional cDNAs.

### *RNA isolation and Northern analysis*

Total cellular RNA was isolated from cells and tissues by the guanidinium method (Chomczynski and Sacchi 1987). RNA was electrophoresed on formaldehyde–agarose gels, transferred to nitrocellulose, and hybridized to labeled DNA probes as described (Spizz et al. 1986). Probes were labeled with <sup>32</sup>P to a specific activity of 1–5 × 10<sup>9</sup> cpm/μg as described by Feinberg and Vogelstein (1983). The following probes were used: *mck*, a 775-bp *Sma*I–*Pvu*I fragment of a canine *mck* cDNA (Roman et al. 1985); Tn-T, 800-bp *Pst*I fragment from a rat Tn-T clone (Garfinkel et al. 1982); myogenin, an *Eco*RI fragment from the coding region of the myogenin cDNA. Blots were exposed to Kodak XAR film at –70°C with intensifying screens. For quantitation of mRNAs, films were exposed for periods during which band intensity was linear with respect to time. Films were then scanned with a densitometer, and mRNA abundance was determined from the area under the peak corresponding to individual mRNAs. Hybridization of cDNA probes to total cellular RNA was linear with respect to RNA concentration.

### *Southern analysis*

Genomic DNA was extracted from C2 cells as described (Maniatis et al. 1982), digested with the indicated restriction enzymes, and electrophoresed on 0.8% agarose gels. Following transfer to nitrocellulose, DNA was hybridized to labeled probes as described above. Myogenin and MyoD1 sequences were detected using the full-length cDNAs. After hybridization, blots were washed at 68°C for 30 min in 0.1 × SSC, 0.1% SDS.

### *DNA sequence analysis*

Putative MyoD1-related phage clones were converted to the plasmid form by the plasmid rescue procedure (Stratagene Catalog) and were sequenced using the Sequenase Sequencing Kit (United States Biochemical Company). The universal and reverse sequencing primers were used to sequence the ends of the cDNAs, and oligonucleotides corresponding to determined sequences were synthesized and used to obtain the complete sequence of the MyoD1-related cDNAs. The sequence of the ORF



was determined for multiple cDNAs isolated from the C2 and BC<sub>3</sub>H1 libraries. Because of the discrepancy in the position of the stop codon in the mouse and rat sequences, two separate cDNAs from the BC<sub>3</sub>H1 library and one cDNA from the C2 library were sequenced on both strands in the region containing the stop codon. All sequences were in agreement. Dot matrix analysis was performed using the Microgenie System for sequence analysis with a window setting of 20 nucleotides at 75% homology.

#### Construction of expression vectors and stable transfections

To express the myogenin cDNA, the full-length cDNA was excised from the EcoRI site of the  $\lambda$  ZAP plasmid by partial digestion with EcoRI and was cloned into the EcoRI site of the vector pEMSVscribe, described by Davis et al. (1987). For comparison to MyoD1, the MyoD expression vector, EMC11s, was provided by A. Lassar. For stable transfections, 10  $\mu$ g of the appropriate expression vector was mixed with 1  $\mu$ g of pSV2neo as a dominant selectable marker. Transfections were performed as described above except that cultures were transferred to growth medium with G-418 (400  $\mu$ g/ml) 2 days after transfection. Fourteen days later, stable clones were exposed to differentiation medium. After an additional 4 days, clones were analyzed for the extent of fusion and for myosin immunostaining. At the same time, clones were picked and passaged into stable cell lines.

#### Immunostaining

Myosin immunostaining was performed by fixing cultures in 70% ethanol, 3.7% formaldehyde, 5% acetic acid for 5 min. Cultures were washed extensively with phosphate-buffered saline, and incubated overnight at 4°C with the anti-myosin antibody, MF-20 (Bader et al. 1982). After washing to remove unbound antibody, cultures were incubated sequentially with biotinylated goat anti-mouse IgG, and fluoresceine-labeled avidin.

#### CAT assays

The construction and properties of *mck-cat* vectors have been described previously. Briefly, pCK4800CAT and pCK808CAT contain the 4800 and 808 bp, respectively, of DNA that precedes the transcription start site of the *mck* gene linked immediately upstream of *cat* in the vector pSV0CAT (Sternberg et al. 1988). The vector pCKCATe6- was constructed by insertion of an *Ava*I–*Bam*HI fragment, extending from bp –1204 to –1048 of *mck*, into the unique *Bam*HI site of pCK246CAT, which contains the *mck* promoter region (*mck* –246 to +1) linked immediately upstream of *cat*. RSV–CAT contains the *cat* gene under transcriptional control of the RSV long terminal repeat (Gorman et al. 1982).

Subconfluent cultures of 10T $\frac{1}{2}$  or 3T3 cells were transfected by calcium phosphate precipitation as described (Graham and VanderEb 1973), with 10  $\mu$ g of *cat* reporter genes and 2  $\mu$ g of plasmids that were tested for their ability to *trans-activate mck* 5' sequences. At 4 hr after transfection, medium was removed and cultures were rinsed with DMEM and refed with growth medium. Two days later, cultures were exposed to differentiation medium for an additional 2 days. Cells were then harvested, and CAT activity was determined as described (Sternberg et al. 1988). All transfections were performed on at least three separate sets of cultures with at least two different DNA preparations of each plasmid. Levels of CAT activity were quantitated by excising the regions of the thin-layer chromatographs corresponding to chloramphenicol and its acetylated de-

rivatives followed by scintillation counting. CAT activity in cell extracts was linear with respect to the concentration of plasmid used in the transfection and with respect to the amount of cell extract used in the assay.

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## Corrigendum

Genes & Development 3: 628–640.

### A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program

Diane G. Edmondson and Eric N. Olson

The sequence for the open reading frame of the mouse myogenin cDNA contains two errors, which were detected while sequencing the corresponding genomic clone. Codon 124 encodes Ser instead of His, and an additional dC is present at codon 155. This results in a frameshift in the carboxy-terminal portion of the protein. These errors have been confirmed by resequencing the cDNAs on both strands. The corrected sequence is shown below and has been submitted to GenBank.

```

      10      20      30      40      50      60      70      80
GGCAGGAACAAGCCTTTCCGACCTGATGGAGCTGTATGAGACATCCCCCTATTTCTACCAGGAGCCCCACTTCTATGAT
      M E L Y E T S P Y F Y Q E P H F Y D

      90     100     110     120     130     140     150     160
GGGGAAGAACTACCTTCTGTCCACCTTCAGGGCTTCGAGCCCCCGGGCTATGAGCGGACTGAGCTCAGCTTAAGCCGGGA
      G E N Y L P V H L Q G F E P P G Y E R T E L S L S P E

      170     180     190     200     210     220     230     240
AGCCCCAGGGCCCCCTGGAAGAAAAGGGAAGTGGGAGCCCCCTGAGCATTTGCCAGGCCAGTGCCTGCCGTGGGCATGTAAGG
      A R G P L E E K G L G T P E H C P G Q C L P W A C K

      250     260     270     280     290     300     310     320
TGTGTAAGAGGAAGTCTGTGTCGGTGGACCGGAGGAGGGGAGGCCCACTGAGGGAGAAGCGCAGGCTCAAGAAAGTGAAT
      V C K R K S V S V D R R R A A T L R E K R R L K K V N

      330     340     350     360     370     380     390     400
GAGGCCTTCGAGGCCCTGAAGAGGAGCACCCTGCTCAACCCCAACGAGCGGCTGCCTAAAGTGGAGATCCTGCGCAGCGC
      E A F E A L K R S T L L N P N Q R L P K V E I L R S A

      410     420     430     440     450     460     470     480
CATCCAGTACATTGAGCGCTACAGGCCTTGTCTCAGCTCCCTCAACCCAGGAGGAGCGCATCTCCGTACAGAGCGGGG
      I Q Y I E R L Q A L L S S L N Q E E R D L R Y R G G

      490     500     510     520     530     540     550     560
GCGGGCCCCAGCCCATGGTGGCCAGTGAATGCAACTCCACAGCGCTCCTGCAGTCCGGAGTGGGGCAATGCACATGGAG
      G G P Q P M V P S E C N S H S A S C S P E W G N A L E

      570     580     590     600     610     620     630     640
TTCGGTCCCAACCCAGGAGATCATTTGCTCGGGGTGACCTACAGACGCCCACTTGCACCTCCCTTACGTCCATCGT
      F G P N P G D H L L A A D P T D A H N L H S L T S I V

      650     660     670     680     690     700     710     720
GGACAGATCACGGTGGAGGATATGTCTGTTCCTTCCAGACGAAACCATGCCCACTGAGATTGTCTGTCAAGCTGGG
      D S I T V E D M S V A F P D E T M P N U

      730     740     750     760     770     780     790     800
TGTGCATGTGAGCCCCAAGTTGGTGTCAAAAGCCATCACTTCTGTAGCAGGGGGCTTTAAGTGGGGCTGTCTGTATGT
      C C A G A A A C A G C C C T G G G C T G C C A A G C C A G A C T C C C C A C T C C C C A T T C A C A T A A G G C T A A C A C C C A G C C C A G C G A G G G

      810     820     830     840     850     860     870     880
AATTTAGCTGACTCCTTAAAGCAGAGAGCATCCTTCTGAGGAGAGAAAGATGGAGTCCAGAGAGCCCCCTTGTTAATG
      A A T T T A G C T G A C T C C T T A A A G C A G A G A G C A T C C T T C T G A G G A G A G A A A G A T G G A G T C C A G A G A G C C C C T T G T T A A T G

      890     900     910     920     930     940     950     960
TCCCTCAGTGGGGCAAACTCAGGAGCTTCTTTTGTATTATCATAATATGCTCGAATTCACCCCCACCCCAAAATG
      T C C C T C A G T G G G G C A A A C T C A G G A G C T T C T T T T G T T A T C A T A A T A T G C T C G A A T T C A C C C C C A C C C C A A A A T G

      970     980     990     1000     1010     1020     1030     1040
AAACCGTTTGAGAGACATGAGTGCCCTGACCTGGACAAGTGTGCACATCTGTTCTAGTCTCTTCTGAAGCCAGTGGCTG
      A A A C C G T T T G A G A G A C A T G A G T G C C C T G A C C T G G A C A A G T G T G C A C A T C T G T T C T A G T C T C T T C T G A A G C C A G T G G C T G

      1050     1060     1070     1080     1090     1100     1110     1120
GGCTGGGCCTGCCCTGAGTTGAGAGAGAAGGGGAGGAGCTATCCGTTCCAAAGCCTCTGGGGCCAAGCATTTTCAGT
      G G C T G G G C C T G C C C T G A G T T G A G A G A G A A G G G G A G G A G C T A T C C G T T C C A A A G C C T C T G G G G C C A A G C A T T T T C A G T

      1130     1140     1150     1160     1170     1180     1190     1200
GGATCTTGGGAACCTTCCAGTGCTTTGTGTATTGTTTATTGTTTGTGTGTTGTTGTAAGCTGCCGTCTGACCAAGGT
      G G A T C T T G G G A A C C T T C C A G T G C T T T G T G T A T T G T T A T T G T T T G T G T G T T G T A A G C T G C C G T C T G A C C A A G G T

      1210     1220     1230     1240     1250     1260     1270     1280
CTCCTGTGCTGATGATACCGGGAACAGGCAGGCCAAGGGGTGGGGGCTCTTGGGGTGACTTCTTTTGTAACTAAGCAT
      C T C C T G T G C T G A T G A T A C C G G G A A C A G G C A G G C C A A G G G G T G G G G G C T C T T G G G G T G A C T T C T T T T G T T A A C T A A G C A T

      1290     1300     1310     1320     1330     1340     1350     1360
TGTGTGTTTGGCAATTTTTTTCTTTTGTAAATCTTTTGCTAACTATTGTTGATTCTCTTTTAAATAATGAATAAA
      T G T G T G T T T G G C A A T T T T T T T C T T T T G T A A T C T T T T G C T A A C T A T T T G G A T T C T C T T T T T A A A A A T G A A T A A A

      1370     1380     1390     1400     1410     1420     1430     1440
GACTGGTTGCTATCAG
      G A C T G G T T G C T A T C A G

      1450

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