Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization

Thomas J. Brennan and Eric N. Olson

Department of Biochemistry and Molecular Biology, The University of Texas, MD Anderson Cancer Center, Houston, Texas 77030 USA

Myogenin is a member of a family of muscle-specific factors that can activate the muscle differentiation program in nonmyogenic cells. Using antibodies directed against unique domains of myogenin, we show in the present study that myogenin resides in the nucleus of differentiated muscle cells. Myogenin translated in vitro does not exhibit detectable DNA binding activity; however, when dimerized with the ubiquitous enhancer-binding factor E12, it acquires high affinity for an element in the core of the muscle creatine kinase (MCK) enhancer that is conserved among many muscle-specific genes. Antibody disruption experiments show that myogenin, synthesized during differentiation of the BC3H1 and C2 muscle cell lines, is part of a complex that binds to the same site in the MCK enhancer as myogenin–E12 translated in vitro. Mutagenesis of the myogenin–E12-binding site in the MCK enhancer abolishes binding of the heterodimer and prevents trans-activation of the enhancer by myogenin. The properties of myogenin suggest that it functions as a sequence-specific DNA-binding factor that interacts directly with muscle-specific genes during myogenesis. The dependence of myogenin on E12 for high-affinity DNA binding activity also suggests that the susceptibility of various cell types to the actions of myogenin may be influenced by the cellular factors with which it may interact.

[Key Words: Myogenin; enhancer-binding factor E12; DNA-binding factors; MCK enhancer; muscle-specific genes]

Received December 28, 1989; revised version accepted February 7, 1990.

Establishment of a fully differentiated skeletal muscle phenotype involves the expression of a battery of muscle-specific genes that encode proteins required for the specialized functions of the mature myofiber. The cell-type-specificity and developmental regulation of this broad array of unlinked genes suggest the existence of a regulatory program that coordinately controls gene expression in developing muscle cells. Numerous studies have described cis-regulatory sequences that confer appropriate regulation on muscle-specific genes. In many cases, these elements have been found to be conserved among multiple muscle-specific genes and to serve as binding sites for putative nuclear regulatory factors (for review, see Rosenthal 1989). The precise specificity of expression of muscle-specific genes can be envisioned to be mediated by one or more muscle-specific transcription factors, by interactions between muscle-specific regulatory factors and general transcription factors, or by combinations of ubiquitous factors. Alternatively, negative regulatory factors could influence the expression of muscle-specific genes by ensuring that they are not expressed at inappropriate times during development or in inappropriate cell types. Indeed, evidence has been obtained for each kind of mechanism in the control of gene expression during myogenesis.

Recent studies have demonstrated the existence of a family of regulatory factors—myod (Pinney et al. 1988), MyoD1 (Davis et al. 1987), myogenin (Edmondson and Olson 1989; Wright et al. 1989), Myf-5 (Braun et al. 1989a), and MRF-4 (Rhodes and Konieczny 1989)—that are able to convert the multipotential mesodermal stem cell line 10T½ to the myogenic lineage. MyoD1, myogenin, Myf-5, and MRF-4 have been isolated as cDNAs and have been shown to be expressed exclusively in skeletal muscle (Davis et al. 1987; Braun et al. 1989a; Edmondson and Olson 1989; Rhodes and Konieczny 1989; Wright et al. 1989). In addition to activating the expression of genes associated with myogenesis, these regulatory factors positively autoregulate their own expression and activate one another’s expression (Braun et al. 1989b; Thayer et al. 1989; Brennan et al. 1990). It has, therefore, not yet been established unequivocally whether each factor can regulate muscle-specific genes independently or whether one factor mediates the actions of the others. The individual roles of myogenin, MyoD1, and Myf-5 in the control of myogenesis are complicated further by the fact that many muscle cell
lines express only a subset of these factors [Davis et al. 1987; Braun et al. 1989b; Edmondson and Olson 1989].

Myogenin, MyoD1, Myf-5, and MRF-4 show extensive homology within a basic region and a conserved helix–loop–helix motif [Murre et al. 1989a,b]. A similar domain is found in two immunoglobulin enhancer-binding gene products, E12 and E47, which appear to be expressed ubiquitously [Murre et al. 1989a], in several regulatory factors from Drosophila that control cell determination, includingachaete scute [Villares and Cabrera 1987; Alonso and Cabrera 1988], daughtercell [Caudy et al. 1988; Cronmiller et al. 1988], twist [Thissle et al. 1988], enhancer of split [Klaembt et al. 1989], and hairy [Rushlow et al. 1989], in the lyt-1 gene product [Mel-lentin et al. 1989], and in members of the myc family of oncogenes [Battey et al. 1983; DePinho et al. 1986]. The helix–loop–helix domain has been shown recently to mediate heterodimerization between either the daughtercell gene product, E12, or E47 and the achaete scute gene product or MyoD1 [Murre et al. 1989b]. The helix–loop–helix domain and adjacent basic region of MyoD1 is necessary and sufficient for myogenic conversion [Tapscott et al. 1988] and for binding of MyoD1 to the MCK enhancer [Lassar et al. 1989], which confers skeletal muscle-specific expression [Jaynes et al. 1988; Sternberg et al. 1988, 1989].

Despite the structural and functional similarities between myogenin, MyoD1, and Myf-5, there is increasing evidence that these factors do not function identically. The genes are expressed, for example, at different times during embryonic development in vivo [Sassoon et al. 1989], and during myogenesis of established muscle cell lines in vitro. In particular, MyoD1 and Myf-5 are expressed in several muscle cell lines prior to differentiation [Braun et al. 1989a; Davis et al. 1987], whereas myogenin is expressed only after withdrawal of mitogens from myoblasts [Edmondson and Olson 1989; Wright et al. 1989]. Paradoxically, myogenin is also expressed in embryonic somites prior to the expression of MyoD1 or other muscle-specific genes [Sassoon et al. 1989]. On the basis of these unique patterns of expression, it is reasonable to expect that the functions of myogenin and MyoD1 are not entirely redundant and that they may modulate the expression of different genes through interaction with different cellular factors and regulatory elements. In this regard, muscle-specific expression of the gene encoding the $\delta$ subunit of the acetylcholine receptor has been shown recently to be mediated by a DNA element that does not contain a binding site for MyoD1 [Baldwin and Burden 1989], indicating that other factors are necessary for manifestation of the complete myogenic phenotype.

To define further the functional relationships between the different myogenic regulatory factors, we prepared antisera against synthetic peptides corresponding to segments of myogenin that lie outside of the region conserved between myogenin, MyoD1, and Myf-5. By use of these antibodies in this study, we show that myogenin exists in the nucleus and acquires high affinity for a single site in the MCK enhancer on dimerization with the helix–loop–helix protein E12. The myogenin–E12-binding site is essential for high-level, muscle-specific activity and for myogen-independent regulation of the MCK enhancer. Antibody disruption experiments show that myogenin, synthesized during differentiation of the BC1H1 and C2 muscle cell lines, is part of a complex that binds to the same site in the MCK enhancer as myogenin–E12 synthesized in vitro. These results suggest that myogenin regulates myogenesis through its interactions with regulatory elements associated with muscle-specific genes. The dependence of myogenin on E12 for sequence-specific DNA binding also indicates that myogenin acts in concert with other cellular factors to regulate muscle-specific gene expression and suggests that the susceptibility of various cell types to its actions are dictated by the repertoire of factors with which myogenin can interact.

Results

Myogenin is localized to the nucleus

The mouse myogenin cDNA contains a major uninterrupted open reading frame with the potential to encode a polypeptide with a predicted molecular mass of 25.2 kD [Edmondson and Olson 1989]. The region between amino acids 63 and 136 of the predicted myogenin polypeptide shows 80% homology to the basic myc domain of MyoD1 that has been shown to be necessary and sufficient for myogenic conversion [Tapscott et al. 1988] and 75% homology to the corresponding region of human Myf-5 [Braun et al. 1989a]. To obtain antisera specific for myogenin that would not cross-react with MyoD1 or Myf-5, we immunized rabbits with two 15-amino-acid synthetic peptides corresponding to regions of myogenin outside of the MyoD1/Myf5 homology domain (see Materials and methods).

Antipeptide antisera were tested initially by Western blot analysis and reacted specifically with myogenin produced in bacteria [data not shown]. The antipeptide antisera also specifically immunoprecipitated in vitro-translated myogenin, which was effectively decreased by competition with an excess of the immunogenic peptides [data not shown].

Our initial interest was to determine the subcellular location of myogenin by indirect immunofluorescence. Figure 1 shows that myogenin was localized to the nuclei of C2 myotubes. Weak staining was also detectable in the cytoplasm of myotubes, which may represent newly synthesized protein en route to the nucleus. Staining of 10T½ cells and undifferentiated myoblasts with the antitymyogenin antisera did not result in detectable immunofluorescence [data not shown].

Myogenin gene expression was also examined by immunoprecipitation from extracts of metabolically labeled C2 cells. The antitymyogenin antisera specifically recognized a protein with an apparent molecular mass of 32 kD in nuclear extracts from [35S]methionine-labeled C2 myotubes [Fig. 2]. Prolonged exposure to film also revealed a species with a molecular mass of 34 kD that...
was specifically immunoprecipitated by the antisera. The identity of these proteins as myogenin was confirmed by the ability of the unlabeled myogenin peptides to compete for immunoprecipitation by the antisera. Extracts from 10T½ cells (Fig. 2) and C2 myoblasts (data not shown) did not contain proteins that were recognized specifically by the antmyogenin antisera. We do not yet know why myogenin migrates as a doublet, but it may reflect differences in the extent of phosphorylation, as the larger species is phosphorylated to a higher stoichiometry than the lower one [T.J. Brennan and E.N. Olson, unpubl.].

Myogenin binds to the MCK enhancer as a heterooligomer with the ubiquitous helix–loop–helix protein E12

We reported previously that myogenin activates the MCK enhancer in transfected 10T½ cells [Edmondson and Olson 1989]. It was unclear, however, whether the effects of myogenin on the enhancer were direct or were mediated by other myogenic regulatory factors such as MyoD1, which is expressed in myogenin-transfected 10T½ cells [Thayer et al. 1989, Brennan et al. 1990]. To determine whether myogenin interacts directly with the MCK enhancer, we initially examined whether a β-galactosidase–myogenin or TrpE–myogenin fusion protein from bacterial lysates would bind to the MCK enhancer in gel retardation assays. Although weak binding was detectable, the affinity of the fusion proteins was relatively low [T. J. Brennan and E. N. Olson, unpubl.].

During the course of these studies, Murre et al. (1989a,b) reported that MyoD1 forms a heterodimer with the immunoglobulin enhancer-binding factor E12 and that this heterodimer shows high affinity for the κE2 motif [or E-box], which is contained within the immunoglobulin κ and heavy-chain enhancers [Church et al.]

Figure 2. Immunoprecipitation of myogenin from nuclear extracts from C2 and 10T½ cells. C2 myotubes and 10T½ cells were labeled with [³⁵S]methionine for 5 hr as described in the text. Nuclear extracts were then prepared and equivalent amounts of radioactivity from each extract were immunoprecipitated with the antmyogenin antisera in the presence (+) or absence (−) of unlabeled myogenin peptides. Proteins migrating above 42 kD are nonspecific. Exposure time was 5 days. Large and small arrowheads denote the major and minor forms, respectively, of myogenin. They are present only in C2 myotubes and are competed by unlabeled myogenin peptides.
1985; Ephrussi et al. 1985; Sen and Baltimore 1986; Lenardo et al. 1987) and which resembles two elements within the core of the MCK enhancer (Jaynes et al. 1988; Sternberg et al. 1988). To ascertain whether myogenin shared this property with MyoD1, myogenin and E12 were prepared by in vitro translation of the corresponding RNAs in a rabbit reticulocyte lysate. The in vitro translation products were then incubated with a 32P-labeled DNA fragment that encompassed 68 bp (between −1204 and −1137, see below, Fig. 7) of the MCK enhancer core. As shown in Figure 3 (lane 2), myogenin showed no detectable binding to the MCK enhancer in the gel mobility-shift assay. E12 alone also did not show significant binding to the enhancer [lane 3], although more recently we have detected a low level of binding of E12 under slightly different assay conditions [data not shown]. When mRNAs encoding myogenin and E12 were translated together, however, they gave rise to a specific mobility-shifted complex with the enhancer (Fig. 3A, lane 4). Competition experiments with a variety of homologous and heterologous DNAs showed that binding of myogenin–E12 to the enhancer core was sequence specific (see below). The presence of myogenin in this DNA–protein complex was confirmed by the ability of antmyogenin antibody to abolish its formation [Fig. 3A, lane 6]. In contrast, preimmune rabbit serum had no effect on generation of the myogenin–E12-dependent complex (Fig. 3A, lane 7). Together, these results show that the affinity of myogenin alone for the MCK enhancer is low, but that, in the presence of E12, myogenin acquires the ability to bind with high affinity to the MCK enhancer core. On the basis of the intensity of the myogenin–E12-dependent complex and the relative inefficiency of heterodimerization (see below), we estimate that the affinity of myogenin–E12 for the MCK enhancer core is at least 100-fold higher than the affinity of myogenin alone.

To confirm that myogenin and E12 formed a heterodimer and to determine whether dimerization between these proteins required DNA, the two mRNAs were co-

Figure 3. Gel mobility-shift assays using fragments of the MCK enhancer and in vitro-translation products of myogenin, MyoD1, and E12. mRNAs for the indicated proteins were translated in vitro and translation products were incubated with the indicated 32P-labeled DNA fragments from the MCK enhancer. DNA–protein complexes were resolved on native acrylamide gels as described in the text. (A) In vitro-translation products of myogenin, E12, and MyoD1, as indicated, were incubated with a 32P-labeled DNA probe corresponding to the MCK enhancer core (−1204/−1137). The middle panel of A shows a gel retardation assay with myogenin plus E12 in the absence [control, lane 5] and presence of antmyogenin antibodies [antiMYO, lane 6] or preimmune rabbit serum [preimmune, lane 7]. The specific DNA–protein complex generated with myogenin–E12 is denoted with an arrow. In the right panel of A, myogenin (lane 8), myogenin plus E12 (lane 9), and myogenin plus MyoD1 (lane 10) were translated in vitro and the translation products were incubated with the enhancer core. (B) In vitro-translation products for myogenin and E12 were incubated with a 32P-labeled probe corresponding to a peripheral region of the MCK enhancer (−1094/−1048), which contains the binding site for MEF-2. Nuclear extract refers to a gel retardation assay performed with nuclear extract from differentiated BC3H1 cells. The position of the MEF-2 complex is indicated. “None” in A and B refers to reticulocyte lysate alone without translation of exogenous mRNAs.
translated in vitro and immunoprecipitated with the antimyogenin antisera. As shown in Figure 4 [lane 6], E12 precipitated with myogenin when the two mRNAs were translated together, but not when it was translated alone [lane 5]. These results demonstrate that myogenin and E12 form a stable complex in vitro and that association of these factors occurs in the absence of DNA. Heterodimerization also occurs when myogenin and E12 are translated separately and combined subsequently [data not shown]. Although E12 and myogenin were present in approximately equal amounts in the immunoprecipitation reactions [Fig. 4, lanes 1–3], only about 10% of the E12 translation product was precipitated by the antimyogenin antibody [lane 6], suggesting that heterodimerization in vitro is relatively inefficient. On the basis of the behavior of E12 and E47 [Murre et al. 1989a,b], we interpret these results to indicate that E12 and myogenin heterodimerize. It is possible, however, that higher order oligomers could be formed, as well.

We also examined whether myogenin would acquire the ability to bind to the MCK enhancer in the presence of MyoD1, but observed no DNA binding when myogenin and MyoD1 were translated together [Fig. 3A, lane 10]. Moreover, in contrast to the ability of bacterially expressed MyoD1 to bind to the MCK enhancer [Lassar et al. 1989], in vitro-translated MyoD1 did not show significant binding to the enhancer under the conditions of these assays [Fig. 3A, lane 10]. Cotranslation of myogenin and MyoD1, followed by immunoprecipitation, also failed to show heterodimerization between myogenin with MyoD1 [Fig. 4, right panel, lanes 7–9]. We estimate that the sensitivity of these assays is such that we would be able to detect heterodimerization between myogenin and MyoD1 if it occurred with an efficiency of about 10% the efficiency of heterodimerization between myogenin and E12. We also searched for potential interactions between myogenin and USF, a helix–loop–helix protein that is expressed in a wide range of cell types; no interactions between these proteins were detected [T.J. Brennan, M. Sawadogo and E.N. Olson, unpubl.].

Myogenin is distinct from MEF-2

We recently identified a muscle-specific enhancer-binding factor, MEF-2, that is expressed during differentiation of the C2 and BC3H1 muscle cell lines with kinetics similar to the kinetics for appearance of myogenin [Gossett et al. 1989]. The binding site for MEF-2 is adjacent to the MCK enhancer core, lying between -1077 and -1062 relative to the transcription initiation site of MCK, and is also found within regulatory regions of several other muscle-specific genes. The possibility that myogenin may share identity with MEF-2 was investigated by gel retardation assays using a labeled fragment of the MCK enhancer encompassing the MEF-2 binding site; however, no interaction between myogenin–E12 and this region of the enhancer was detectable [Fig. 3B]. The myogenin–E12 heterodimer thus shows sequence specificity for binding to the core of the MCK enhancer.

To determine whether MEF-2 is composed of a complex between myogenin and one or more other proteins that have a different recognition sequence from myogenin–E12, we examined whether the antimyogenin antibodies would recognize MEF-2 from nuclear extracts of differentiated BC3H1 myocytes. Addition of antimyogenin antibodies to nuclear extracts had no apparent effect on generation of the MEF-2 complex with the MCK enhancer (data not shown). Together, these results indicate that MEF-2 is distinct from myogenin.

Identification of the binding site for the myogenin–E12 heterodimer

The recognition sequence within the MCK enhancer for the myogenin–E12 heterodimer was determined by methylation interference. As shown in Figure 5, methylation of two guanines on the coding strand and four guanines on the noncoding strand strongly interfered with binding of the heterodimer to the MCK enhancer.
The contact points for the myogenin–E12 heterodimer on the MCK enhancer correspond to the downstream κE2-like motif (or E-box), which has been referred to as the right MyoD1-binding site by Lassar et al. [1989]. The most upstream guanine on the coding strand of this E-box (nucleotide −1146) corresponds to the nucleotide that was shown to interact with bacterially expressed MyoD1 [Lassar et al. 1989]. Methylation of the guanine at −1143 on the coding strand did not affect binding of MyoD1, but partially interfered with binding of myogenin–E12. On the noncoding strand, methylation of guanines at −1151, −1149, −1148, and −1145 interfered with binding of myogenin–E12. Of these, only guanines −1151 and −1145 interfered with binding of bacterial MyoD1 [Lassar et al. 1989]. Thus, the myogenin–E12 heterodimer binds to the same region of the MCK enhancer as MyoD1, but shows a unique set of guanine contact points. The contact points for MyoD1–E12 or MyoD1–E47 heterodimers have not yet been reported.

It should be pointed out that an additional E-box with similarity to the κE2 motif has been identified in the MCK enhancer 20 nucleotides upstream of the myogenin–E12-binding site [Jaynes et al. 1988; Sternberg et al. 1988]. A stretch of 10 d(C-G) residues that does not appear to be required for binding lies between the two sites (see below, Fig. 7A). MyoD1 has been shown to exhibit low affinity for this upstream E-box, which has been referred to as the left MyoD1-binding site [Lassar et al. 1989]. We did not detect any interaction, however, between the myogenin–E12 heterodimer and this region of the enhancer, referred to as E-boxL (Fig. 5 and see below).

To investigate whether the κE2-like motif in the MCK enhancer was sufficient for binding of myogenin–E12, we examined binding of the heterodimer to an oligomer corresponding to this site. As shown in Figure 6A, myogenin–E12 gave rise to a specific DNA–protein complex with the MCK oligomer, indicating that sequences surrounding this motif in the complete enhancer are not required for binding. Binding of myogenin–E12 to the labeled oligomer was inhibited by the corresponding unlabeled oligomer, but was not by an oligomer encompassing the upstream κE2 site [Fig 6B]. These results confirm the methylation interference data and indicate that myogenin–E12 is specific for the downstream κE2 site. Gel retardation assays with a labeled oligomer corresponding to the upstream κE2 motif also failed to reveal binding of myogenin–E12 [data not shown].

Mutagenesis of the myogenin–E12 binding site abolishes binding of myogenin–E12 and prevents activation of the MCK enhancer by myogenin

To define further the binding site for myogenin–E12 within the MCK enhancer, we created a site-specific mutation at the site that was shown by methylation interference to bind the heterodimer [Fig. 7A]. By use of a 179-bp fragment of the mutant enhancer encompassing this region in the gel retardation assay, no binding of the myogenin–E12 heterodimer was detectable [Fig. 7B], whereas the same DNA fragment from the wild-type enhancer gave rise to a shifted complex in the presence of myogenin–E12. The DNA fragment used in these assays also contained the upstream κE2 motif that binds MyoD1 [Lassar et al. 1989]. These results confirm the
were inserted 3' of a CAT gene, which itself was fused to 246 bp of the MCK promoter. CAT reporter genes containing the wild-type and mutant enhancers were then transfected transiently into 10T½ cells with an expression vector encoding myogenin under transcriptional control of the Moloney sarcoma virus long terminal repeat. Whereas myogenin was able to trans-activate the wild-type MCK enhancer in 10T½ cells, the enhancer that was mutated at the myogenin-E12-binding site was impaired in its ability to confer myogenin-dependent regulation on CAT [Fig. 7C]. When transfected at a ratio of 5:1 (CAT : myogenin), CAT expression from the mutant was not significantly different from background levels of expression from the MCK promoter alone. Thus, the myogenin-E12-binding region is important for trans-activation of the enhancer by myogenin in 10T½ cells.

Myogenin interacts with the MCK enhancer in nuclear extracts from differentiated BC3H1 and C2 cells

To establish whether myogenin interacts in vivo with the same site as myogenin-E12 produced in vitro, we examined whether nuclear extracts from BC3H1 and C2 cells contained factors that interacted with the oligomer corresponding to the myogenin-E12-binding site. We were particularly interested in comparing the spectrum of binding activities for the myogenin-E12 site in extracts from these cell lines because C2 cells express myogenin and MyoD1 whereas BC3H1 cells express only myogenin [Davis et al. 1987; Edmondson and Olson 1989]. We also examined extracts from 10T½ cells for factors that may bind to the myogenin-E12 site.

The pattern of DNA–protein complexes generated with each of the extracts and the labeled oligomer is shown in Figure 8A. All of the extracts gave rise to a common DNA–protein complex, denoted complex-1. In addition, extracts from differentiated BC3H1 and C2 cells gave rise to a myocyte-specific DNA–protein complex, denoted complex-2. Interestingly, the pattern of DNA–protein complexes generated by BC3H1 and C2 extracts was similar to the labeled oligomer as probe. Complex-1 and -2 result from the binding of sequence-specific factors because they were eliminated in the presence of homologous, but not heterologous, DNA competitors. As shown in Figure 8B, competition with the oligomer corresponding to the myogenin-E12-binding site (MCK-OLs) abolished formation of the myocyte-specific complex-2 from BC3H1 extracts, whereas oligomers corresponding to the upstream E-box (MCK-OLu) or the MEF-2-binding site had no effect on the formation of complex-2. Identical results were obtained with extracts from C2 myotubes [data not shown]. Formation of complex-1 with extracts from 10T½ cells (Fig. 8B) or myoblasts [data not shown], was also inhibited specifically by MCK-OLs, but not by MCK-OLu. Together, these results suggest that the myogenin-E12-binding site in the MCK enhancer interacts with a variety of sequence-specific factors and that this
Sequence-specific DNA binding of myogenin

A. MCK-OL$_L$ MCK-OL$_R$

Wild type

GGGTAGATCTGGTCTAGAC Mutant

Figure 7. Mutagenesis of the myogenin–E12 binding site prevents trans-activation of the MCK enhancer by myogenin. (A) The sequence of the MCK enhancer core (−1204 to −1134) is shown. The region of the enhancer core encompassing the myogenin–E12 binding site was mutated. Sequences of the wild-type and mutant enhancer within this region are shown. The E2-like sites are underlined and the sequences contained in the oligomers MCK-OL$_L$ and MCK-OL$_R$ are indicated. (B) A DNA fragment encompassing the region between −1269 and −1090 of the wild-type and mutant MCK enhancers was end-labeled and used in gel retardation assays with myogenin and E12 translated in vitro. The specific DNA–protein complex generated by myogenin–E12 is indicated with an arrow. (C) A DNA fragment encompassing the region between −1350 and −1048 of the wild-type and mutant MCK enhancers was inserted 3′ of CAT, in the vector pCK246CAT, which contains the 246-bp MCK promoter. Wild-type and mutant CAT constructions, designated pCKCAT$_{wt}$ and pCKCAT$_{mut}$, respectively, in addition to pSV2CAT and pCK246CAT were transfected transiently into 10T1A cells in the presence of an expression vector encoding myogenin. Twenty-four hours after transfection, cultures were transferred to Dulbecco's modified Eagle medium (DMEM) containing 2% horse serum for 48 hr. Levels of CAT expression are expressed as the percentage of $[^{14}C]$chloramphenicol converted to acetylated products.

site may normally be occupied by factors in cells in which the enhancer is inactive.

To determine whether any of the mobility-shifted complexes observed with myocyte nuclear extracts were attributable to the binding of myogenin, antimyogenin antibody or preimmune serum was added to the gel retardation assay. In the presence of antimyogenin antibody, we observed a diminution in intensity of the myocyte-specific complex-2 generated by extracts from BC$_3$H1 and C2 myocytes [Fig. 8C, cf. lane 3 with 4 and lane 7 with 8]. In contrast, preimmune serum had no measureable effect on the pattern of DNA–protein complexes formed with the labeled oligomer [Fig. 8C, lanes 5 and 9]. The identity of a component of the myocyte-specific complex-2 as myogenin was indicated further by the ability of excess myogenin peptides to prevent disruption of the complex by the antimyogenin antibody [Fig. 8C, lanes 6 and 10]. The antimyogenin antibody had no effect on formation of complexes with extracts from 10T½ cells [Fig. 8C, lanes 1 and 2] or myoblasts [data not shown]. These results indicate that myogenin synthesized in vivo binds to the same site as in vitro-translated myogenin–E12.

It is apparent from the assays in Figure 8C that the antimyogenin antibody did not completely abolish formation of the myocyte-specific complex. We attempted to obtain more complete disruption of this complex by adding greater amounts of antimyogenin antibody to the assay, but did not observe a further decrease in the intensity of this complex [see Discussion]. These results suggest that myocyte-specific factors in addition to myogenin may interact with the same region of the MCK enhancer as the myogenin–E12 heterodimer synthesized in vitro. Alternatively, a population of myogenin may be unable to react with the antimyogenin antibodies because peptide epitopes are masked, perhaps by heterodimerization with a different helix-loop-helix protein.

Discussion

Myogenin binds as a heterodimer to a conserved motif in the core of the MCK enhancer

We showed previously that the MCK enhancer is active only in differentiated skeletal muscle cells [Sternberg et al. 1988, 1989; Gossett et al. 1989] and that myogenin can trans-activate this enhancer in 10T½ cells, where it is normally inactive [Edmondson and Olson 1989]. In the present study, we extend these observations to show that myogenin resides in the nucleus of differentiated muscle cells and interacts as a heterodimer with a single site in the core of the MCK enhancer.

By use of myogenin translated alone in vitro, we were unable to detect significant binding to the MCK enhancer. Similarly, MyoD1 translated in vitro failed to
Figure 8. Nuclear extracts from myoblasts, myocytes, and 10T½ cells contain factors that bind to the myogenin–E12-binding site in the MCK enhancer. (A) The oligomer corresponding to the myogenin–E12-binding site in the MCK enhancer, denoted MCK-OLR (see Fig. 7A), was end-labeled and used as a probe in gel retardation assays with 5 μg of nuclear extract from 10T½ fibroblasts, undifferentiated and differentiated C2 cells, and differentiated BC3H1 cells. Complexes containing a ubiquitous factor (complex-1) and myogenin (complex-2) are indicated. Complexes migrating near the center of the gels reflect the binding of nonspecific factors. (B) Specificity of factor binding to MCK-OLR was determined by competition with homologous and heterologous DNA. Gel retardation assays were performed with 5 μg of nuclear extract from differentiated BC3H1 cells or 10T½ fibroblasts in the absence of competitor DNA (0) or in the presence of the indicated molar excess of MCK-OLR, MCK-OLL (see Fig. 7A), or an oligomer corresponding to the MEF-2-binding site (Gossett et al. 1989). A 50-fold molar excess was used in the competition experiment with 10T½ extract in the right panel of B. (C) Gel retardation assays were performed with 5 μg of nuclear extract from the indicated cell types. Antimyogenin antibody (M) or preimmune serum (P) in the presence (+) or absence (−) of myogenin peptides was added to the nuclear extract prior to incubation with the probe. Densitometry of this and other autoradiographs showed that myocyte-specific complex-2 decreased threefold in the presence of antimyogenin antibody. To resolve complex -1 and -2 in A and C, a 19-cm 4% polacrylamide gel was used. Assays in B employed a 5% gel, which does not resolve these complexes.
bind to the MCK enhancer alone or in combination with myogenin. In contrast, in the presence of the ubiquitous factor El2, myogenin formed a heterodimer and bound with high affinity and specificity to the enhancer. There was considerable selectivity in the factors with which myogenin would interact since it did not dimerize to a measurable extent with MyoD1 or with the helix–loop–helix protein USF, which is expressed constitutively in many cell types [M. Sawadogo, pers. comm.]. The ability of myogenin to heterodimerize specifically with El2 is consistent with the recent proposal of Murre et al. [1989b], that helix–loop–helix proteins can be divided into different classes on the basis of their cell-type distributions and abilities to form heterodimers. Ubiquitous proteins such as the *daughterless* gene product, E12, and E47 seem to dimerize preferentially with cell-type-specific proteins such as the *achaete scute* gene product, MyoD1, and myogenin.

The lack of binding of in vitro-translated MyoD1 to the MCK enhancer agrees with the results of Murre et al. [1989b], but contrasts with recent results of Lassar and co-workers, who showed that bacterially expressed MyoD1 interacts with the MCK enhancer at two sites [Lassar et al. 1989]. Bacterially expressed myogenin also has been found to bind to the MCK enhancer [W. Wright and A. Lassar, pers. comm.]. A likely explanation for this disparity may be the high concentrations of bacterially expressed MyoD1 or myogenin necessary to detect sequence-specific binding. In their assays, Lassar et al. [1989] used up to 0.5 μg of purified MyoD1, which we estimate to be at least three orders of magnitude higher than the amount present as a heterodimer with E12 in our in vitro-translation reactions. In vitro-translated myogenin also would presumably bind to the MCK enhancer when present at high concentrations. Considering the large quantities of bacterially expressed MyoD1 that are required to detect binding to the MCK enhancer, it is questionable whether MyoD1 normally binds alone to the enhancer in vivo, particularly because E12 has been reported to be ubiquitous (Murre et al. 1989a) and would presumably be available for heterodimerization with MyoD1 or myogenin in myogenic cells. Assuming that oligomerization is required for binding, it seems likely that at high concentrations of MyoD1 or myogenin in vitro, these proteins may form homodimers that can bind to the MCK enhancer. The ability of the helix–loop–helix protein E47 to form a homodimer at high concentrations has, in fact, been demonstrated [Murre et al. 1989a]. That heterodimerization with E12 (or homodimerization at high concentrations) is required for interaction of myogenin with the MCK enhancer suggests that the basic domain of each member of the oligomer is important for mediating sequence-specific DNA binding. In this regard, mutations within the basic domain of myogenin do not affect heterodimerization with E12, but abolish the ability of the heterodimer to bind the enhancer [T. J. Brennan and E. N. Olson, in prep.].

The sequence in the MCK enhancer to which myogenin–E12 binds corresponds to the site for binding of MyoD1 [Lassar et al. 1989]. The 10-bp motif, CACCTGCTGC, which constitutes this site, is conserved perfectly in the rat MCK enhancer (Horlick and Benfield 1989) and is also found in the rat myosin light-chain 1/3 enhancer [Donoghue et al. 1988], where it has been shown to bind MyoD1 [Lassar et al. 1989]. Divergent versions of this motif are also found in regulatory regions of the chicken acetylcholine receptor α-subunit [Wang et al. 1988], mouse acetylcholine receptor δ-subunit [Baldwin and Burden 1988], quail troponin I [Yutzey et al. 1989], hamster desmin [Pieper et al. 1987], rat tropomyosin [Helfman et al. 1986], chicken α-cardiac actin [Quitschke et al. 1989], and mouse myogenin and MRF-4 [D. E. Edmondson, T. C. Cheng, and E. N. Olson, unpubl.] genes. A similar sequence, referred to as a E2 motif or E-box, has also been identified within the enhancers of several genes that are not specific to skeletal muscle, such as the immunoglobulin heavy- and light-chain enhancers [Church et al. 1985; Ephrussi et al. 1985; Sen and Baltimore 1986; Lenardo et al. 1987] and the insulin enhancer [Moss et al. 1988]. Although these various E-boxes share the motif NCANNTGNN, there are clearly additional sequence requirements for binding of different helix–loop–helix proteins, as demonstrated by the inability of the myogenin–E12 heterodimer to bind with high affinity to the upstream E-box in the MCK enhancer. Moreover, even between different heterodimers that recognize the same site, methylation interference reveals subtle differences in the contact points [Murre et al. 1989b]. In this regard, bacterially expressed MyoD1 shows a different pattern of methylation interference from that of myogenin–E12 over the downstream E-box in the MCK enhancer.

Site-directed mutagenesis showed that the downstream E-box in the MCK enhancer is important for activation of the enhancer by myogenin in 10T1/2 cells. This element is also required for muscle-specific activity of this enhancer in differentiated C2 myotubes [Buskin and Haushka 1989; Lassar et al. 1989; E. Sternberg and E. N. Olson, in prep.]. It should be pointed out, however, that although the myogenin–E12-binding site is essential for high-level activity of the enhancer in muscle cells, it is not the only site within this enhancer that interacts with sequence-specific DNA-binding factors [Gossett et al. 1989; Buskin and Haushka 1989; Horlick and Benfield 1989], nor is this site alone sufficient to generate high-level muscle-specific enhancer activity. Moreover, mutagenesis of adjacent regions of the enhancer core that do not affect binding of myogenin or MyoD1 result in a dramatic loss of enhancer activity (E. Sternberg and E. N. Olson, unpubl.). The observation that the myogenin–E12-binding site is necessary, but not sufficient, for enhancer activity indicates that interactions between factors at the enhancer is highly cooperative.

It is not clear why myogenin and MyoD1, which are coexpressed in many muscle-cell types, should both bind to the same site in the MCK enhancer. Several possibilities can be envisioned. Myogenin and MyoD1 could differ with respect to the proteins with which they in-
teract and could, in principle, exhibit different specifici-
ties as activators of muscle genes. Alternatively, the dis-
tinct temporal and cell-type-specific patterns of expres-
sion of myogenin and MyoD1 could determine the pattern of gene expression in a particular muscle-cell type. Finally, because MyoD1 appears to possess the po-
tential to interact with the E-box located upstream from
the myogenin–E12-binding site, it is also conceivable
that myogenin and MyoD1 could bind simultaneously
to the MCK enhancer in muscle cells that express both
factors. This possibility is attractive, in light of the fact
that putative MyoD1 and myogenin binding sites occur
in pairs within regulatory regions of numerous muscle-
specific genes. It should also be emphasized, however,
that muscle-specific genes are expressed normally in
several muscle-cell lines that do not express MyoD1
[Braun et al. 1989b; Edmondson and Olson 1989; Wright
et al. 1989], whereas there have not been any reported
examples of muscle-cell types that do not express myo-
genin.

The binding site for myogenin serves as a site for
binding of numerous factors

The site in the MCK enhancer that binds myogenin has
also been reported by Hauschka and co-workers to in-
teract with a myocyte-specific factor, referred to as
MEF-1, which is expressed during differentiation of the
MM14 and C2C12 muscle-cell lines [Buskin and
Hauschka 1989]. Using antibodies against MyoD1,
Lassar et al. (1989) reported that MyoD1 shares antigenic
similarity with MEF-1.

By use of nuclear extracts from BC3H1 and C2 cells in
gel retardation assays, we observed a myocyte-specific
complex with the myogenin–E12-binding site as a la-
beled probe. Since this complex was recognized specifi-
cally by antibodies directed against segments of myo-
genin that share no homology with the other known
myogenic regulatory factors, we conclude that myo-
genin synthesized in vivo binds (probably as a compo-
nent of a complex) to the same site in the MCK en-
hancer as myogenin–E12 produced in vitro. The fact
that myogenin binds to the MCK enhancer in extracts
from C2 cells, which coexpress myogenin and MyoD1,
also indicates that myogenin can bind in the presence of
MyoD1.

It is intriguing that nuclear extracts from C2 and
BC3H1 myoblasts and 10T½ cells, none of which ex-
presses myogenin, also contain factors that interact spec-
ifically with the myogenin-binding site. The identities
of these factors will be particularly interesting because
their binding is apparently insufficient for transcrip-
tional activation. Perhaps these factors antagonize the
actions of myogenin.

The actions of myogenin may be dictated by factors
with which it can interact

Although myogenin clearly shows high affinity for E12
in vitro, the present results do not allow us to conclude
whether myogenin normally interacts with E12 in vivo.
E12 has been reported to be expressed ubiquitously
[Murre et al. 1989a], so it or a similar factor is likely to
be available for heterodimerization with myogenin in
muscle cells. In this context, we have isolated cDNAs
from a BC3H1 myocyte cDNA library encoding at least
three distinct types of helix–loop–helix proteins with
homology to segments of E12 and E47 [L. Li and E.N.
Olson, unpubl.]. The apparent dependence of myogenin
on E12, and perhaps other helix–loop–helix proteins,
for activity indicates that the susceptibility of various
cell types to the actions of myogenin may be dictated by
the nature of the cellular factors with which myogenin
may interact. This may begin to explain the resistance of
certain cell types to myogenic conversion by myogenin
[D. Edmondson and E.N. Olson, unpubl.] and MyoD1
(Weintraub et al. 1989). If, in certain cell types, for ex-
ample, E12 were absent or other helix–loop–helix pro-
teins were present, the activity of myogenin or MyoD1
could be modulated.

In addition to being expressed during myoblast differ-
entiation, myogenin has been shown to be expressed at
high levels in the myotome at 8.5 days of development,
prior to the appearance of differentiated muscle [Sassoon
et al. 1989; Wright et al. 1989]. The expression of myo-
genin at this early stage of development suggests that it
may function in establishing the myogenic lineage. It is
interesting to speculate that the apparent dual role of
myogenin as a regulator of myogenic determination and
differentiation may be dependent on its interactions
with unique sets of helix-loop–helix proteins. Future
studies will address the mechanisms that modulate
myogenin's activities in different cell types during deter-
mination and differentiation of the myogenic lineage.

Materials and methods

Cell culture and metabolic labeling

The C2 (Yaffe and Saxel 1977), BC3H1 [Schubert et al. 1974],
and 10T½ [Reznikoff et al. 1973] cell lines were cultured as
described previously [Edmondson and Olson 1989]. Labeling
with [35S]methionine was performed as described [Olson et al.
1983], except that cultures were incubated with isotope for 5 hr.
At the end of the labeling period, nuclear extracts were prepared
as described below.

Peptide synthesis and immunization

Peptides [MP1: PGYERTELSPEAR; MP2: SSSLNQEERDLRYRGG] were synthesized by the Macromolecular Synthesis
Facility at the MD Anderson Cancer Center [Houston, Texas].
To cross-link peptides to Keyhole Limpet hemocyanin [Sigma],
peptide [1 mg/ml in phosphate-buffered saline [PBS] and carrier
protein [1 mg/ml in PBS] were combined in a 1 : 1 ratio, to
which 16 μl of 2.5% glutaraldehyde [final concentration = 4
mm] was added per milliliter, and the total volume mixed on a
rotator for 30 min at room temperature. The peptide–protein
conjugates were then dialyzed for 5 hr in PBS. An equivalent of
500 μg of peptide was injected in Freund's complete adjuvant
into New Zealand White rabbits which were boosted every 3
weeks with 250 μg in Freund's incomplete adjuvant. Injections
were performed by Bethyl Laboratories [Montgomery, Texas].
Affinity purification

MP1/MP2-conjugated epoxy-activated Sepharose 6B was prepared according to manufacturer’s recommendations (Pharmacia). Briefly, 2 grams of epoxy-activated Sepharose 6B resin was swelled in water for 15 min and washed in a scinttered glass funnel with 200 ml of water. Two milliliters of 30 μM MP1 or MP2 (pH 11.0 in water) was mixed with the resin on a shaker for 16 hr at 37°C. The resin was then washed in a scinttered glass funnel with 100 ml of each of the following: water, 0.1 M NaHCO₃ (pH 8.0), water, 0.1 M sodium acetate (pH 4.0), and water. The excess reactive groups were then blocked with 1 μ ethanolamine at 42°C for 12 hr. The coupled resin was then washed in a scinttered glass funnel with 100 ml of 0.1 M sodium acetate (pH 4.0)/0.5 M NaCl followed by 0.1 M borate (pH 8.0)/0.5 M NaCl, and 10 mM Tris (pH 7.5) before 1 ml of the coupled resin was loaded onto a 10-ml Dispo column (Bio-Rad) and washed with 20 bed volumes of each of the following: 10 mM Tris (pH 7.5), 100 mM glycine (pH 2.5), 10 mM Tris (pH 8.8), and 10 mM Tris (pH 7.5). For affinity purification, 10 ml of a 1 : 4 dilution of either anti-MP1 or anti-MP2 serum in 10 mM Tris (pH 7.5) was loaded onto the 1-ml MP1 or MP2 affinity column, respectively. The serum was passed through the column 4 times by gravity, and the column was then washed with 20 bed volumes of each of the following: 10 mM Tris (pH 7.5), 500 mM NaCl/10 mM Tris (pH 7.5), and 10 mM Tris (pH 7.5). The bound antibodies were eluted with 100 mM glycine (pH 2.5), and 1-ml fractions were collected and immediately neutralized with 200 μl of 2 M Tris (pH 8.0). The peak fractions were combined and concentrated using micro-concentrators (Amicon). One milliliter of immune serum would typically yield 150–200 μg of affinity-purified antibody. Purified serum against both peptides was combined for all subsequent experiments.

Immunofluorescence

Cells were grown on plastic petri dishes, washed three times in PBS, fixed for 10 min in freshly prepared 2% paraformaldehyde/PBS, washed three times in PBS, incubated for 10 min in 0.15% Triton X-100/PBS, and washed 3 times in PBS. The cells were then blocked for 30–60 min in PBS containing 2% bovine serum albumin, incubated with a 1 : 20 dilution of purified anti-MP1/MP2 (200 μg/ml) in blocking buffer for from 4 hr to overnight, and washed 3 times for 10 min in blocking buffer. The dishes were incubated for 1–2 hr in fluorescent-conjugated goat anti-rabbit IgG (1 : 1000, Cappel) in blocking buffer, and washed 4 times, for 15 min each wash, in blocking buffer before being visualized at 495-nm wavelength.

Preparation of nuclear extracts

Nuclear extracts were prepared according to a slightly modified method of Dignam et al. (1983), as described by Gossett et al. (1989), with the exception that the dialysis buffer consisted of 100 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 20% (vol/vol) glycerol. Metabolically labeled nuclear extracts were not dialyzed.

Immunoprecipitation

For in vitro translations, 20 μl of affinity-purified anti-MP1/MP2 (200 μg/ml), 300 μl RIPA buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS], and 25 μl of the in vitro translation (see below) were incubated on a rotator at 4°C for 2 hr before addition of 100 μl of a 10% suspension of formalin-fixed Staphylococcus aureus. For nuclear extracts, 750 μl of RIPA buffer, 20 μl preimmune sera, and 250 μl of nuclear extract was incubated for 1 hr, to which 100 μl of S. aureus was added for 1 hr. The S. aureus was precipitated, and 40 μl of anti-MP1/MP2 (200 μg/ml) was added to the supernatant and rotated overnight. S. aureus cells [100 μl] were then added and rotated for 1 hr at 4°C. Immunoprecipitates from in vitro translations and nuclear extracts were washed three times by resuspension in 1% Triton X-100 and 1.0 M NaCl in PBS, and layering over a solution of 1% Triton X-100, 1 M sucrose in PBS, and reprecipitated. The pellets were then washed in PBS before being resuspended in sample buffer [0.25 M Tris (pH 6.8), 2% SDS, 4% β-mercaptoethanol, 10% glycerol] and resolved on 0.1% SDS/10% polyacrylamide gels. For peptide competition, 50 μl of a 15 μM mixture of MP1 and MP2 in water was preincubated with the anti-MP1/MP2 antiserum for 1 hr before addition to the binding reaction. For nondenaturing conditions, immunoprecipitates were prepared according to the method of Murre et al. (1989b).

In vitro transcription and translation

The myogenin vector used for in vitro transcription contained a full-length mouse myogenin cDNA cloned into the EcoRI site of pBluescript (Stratagene). Plasmid E12 (Murre et al. 1989a) was a gift from C. Murre and D. Baltimore. Plasmid PVZC11B, containing mouse MyoD1, was a gift from A. Lassar. RNA synthesis was performed using 1 μg of linearized plasmid and 10 units of T3 polymerase in a 25-μl reaction volume for 1 hr at 37°C as suggested by the manufacturer (Stratagene, mRNA Capping Kit). After extraction with phenol/chloroform, the RNA was precipitated and resuspended in 25 μl of TE (10 mM Tris [pH 7.6], 1 mM EDTA). Two μl of the RNA was used for in vitro translation with rabbit reticulocyte lysate in a 50-μl reaction volume as recommended by the manufacturer (Promega). The reactions were allowed to proceed for 1 hr at 30°C.

Gel retardation assays and methylation interference

The indicated in vitro-translation products [3 μl] or 5 μg of the indicated nuclear extracts was incubated for 5 min at room temperature with 1 μg poly[dI-C]2, 2 μl of 10× binding buffer [final concentrations, 40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, and 5% glycerol], and the indicated concentrations of DNA competitors in a 20-μl reaction volume. DNA competitors (90 ng) were used unless indicated otherwise. End-labeled enhancer fragments were then added and allowed to incubate for an additional 20 min before being loaded onto either 4% or 5% polyacrylamide gels and electrophoresed at 175 volts. Synthetic oligomers encompassing either of the two E-boxes in the MCK enhancer core were cloned into the BamHI site of pUC19 using 10-bp BamHI linkers and were excised from the vector by digestion with BamHI. The enhancer core (−1204 to −1134) and the peripheral region containing the MEF-2 site (−1094 to −1048) were cloned into the BamHI site of pUC19 and were excised with BamHI (Gossett et al. 1989). For assays involving antibody disruption, 8 μl of affinity-purified anti-MP1/MP2 (150 μg/ml, dialyzed in 1× binding buffer) or preimmune serum (dialyzed in 1× binding buffer) was preincubated with the extracts in the binding mixture for 10 min before addition of the probe. For peptide competition analysis, 2 μl of a 15 μM solution of MP1/MP2 in water was preincubated with the antibody in the binding reaction mixture for 10 min before the addition of nuclear extract. Methylation interference...
Brennan and Olson

and A + G reactions were performed as described previously [Baldwin and Sharp 1988].

Preparation of reporter genes and transient transfections

The MCK enhancer was assayed by inserting a 302-bp fragment encompassing the region between −1350 and −1048 into the unique BamHI site of the vector pCK246CAT [Stemberg et al. 1988], which contains the MCK basal promoter fused immediately upstream of CAT. This vector was referred to previously as pCKCATe4 [Stemberg et al. 1988]. Mutagenesis was performed by cloning the 302-bp enhancer fragment into the BamHI site of M13. A mutant oligomer was used to create the site-specific mutation at the myogenin−E12 binding site. The mutant 302-bp fragment was then recloned into pCK246CAT, and its identity was verified by sequencing. Mutant and wild-type enhancers in the + and − orientation were tested in transfection assays with the same results.

10T1/2 cells were transfected transiently as described previously [Stemberg et al. 1988] with 10 µg of CAT reporter plasmids and 2 µg of an expression vector encoding full-length mouse myogenin under transcriptional control of the Moloney sarcoma virus long terminal repeat (Edmondson and Olson 1989). Twenty-four hours after transfection, cultures were transferred to DMEM with 2% horse serum for 48 hr. Cells were then harvested and CAT activity was assayed as described [Stemberg et al. 1988]. Transfections were performed three times with two separate plasmid preparations with similar results.

Acknowledgments

We thank E. Stemberg for the mutated MCK enhancer, D.G. Edmondson for myogenin expression vectors and for helpful discussions, W. Michael Perry for critical comments on the manuscript, and Mamta Kalides for help with initial antibody characterization. We are grateful to C. Murre and D. Baltimore for the gift of El2 and to A. Lassar for MyoD1. We also acknowledge S. Jasser for tissue culture, M.E. Perry for technical assistance, and E. Madson for help in preparing the manuscript. This work has been supported by grants from the National Institutes of Health [NIH] and the American Cancer Society [ACS] to E.N.O., who is an Established Investigator of The American Heart Association. T.J.B. has been supported in part by an NIH training grant.

References

Alonso, M.C. and C.V. Cabrera. 1988. Theachaeta-scute complex of Drosophila melanogaster comprises four homologous genes. EMBO J. 7:2585−2591.

Baldwin, A. and P. Sharp. 1988. Two transcription factors, H2af1 and NFκB, interact with a single regulatory sequence in the class 1 MHC promoter. Proc. Natl. Acad. Sci. 85:723−727.

Baldwin, T.J. and S.J. Burden. 1988. Isolation and characterization of the mouse acetylcholine receptor β-subunit gene: Identification of a 148-bp cis-acting region that confers myotube-specific expression. J. Cell Biol. 107:2271−2279.

———. 1989. Muscle-specific gene expression controlled by a regulatory element lacking a MyoD1-binding site. Nature 341:716−720.

Battey, J., C. Moulding, R. Taub, W. Murphy, T. Stewart, H. Potter, G. Lenoir, and P. Leder. 1983. The human c-myc oncogene: Structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 34:779−787.

Braun, T., E. Bober, G. Buschhausen-Denker, S. Kotz, K. Grzeschik, and H.H. Arnold. 1989a. Differential expression of myogenic determination genes in muscle cells: Possible autoregulation by the Myf gene products. EMBO J. 8:3617−3625.

Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H.H. Arnold. 1989b. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. 8:701−709.

Brennan, T.J., D.E. Edmondson, and E.N. Olson. 1990. Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of the Bcjh1 muscle cell line. J. Cell Biol. [in press].

Buskin, J.N. and S.D. Hauschka. 1989. Identification of a myocyte-specific nuclear factor which binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell. Biol. 9:2627−2640.

Caudy, M., H. Vässin, M. Brand, R. Tuma, L. Yeh Jan, and Y.N. Jan. 1988. daughterless a Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to myc and the achaete-scute complex. Cell 55:1061−1067.

Church, G.M., A. Ephrussi, W. Gilbert, and S. Tonegawa. 1985. Cell type specific contacts to immunoglobulin enhancers in nuclei. Nature 313:798−801.

Crommiller, C., P. Schell, and R.W. Cline. 1988. Molecular characterization of daughterless, a Drosophila sex determination gene with multiple roles in development. Genes Dev. 2:1666−1676.

Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987−1000.

DePinho, R.A., E. Legoyou, L.B. Feldman, N.E. Kohl, G.D. Yancopolous, and F.W. Alt. 1986. Structure and expression of the murine N-myc gene. Proc. Natl. Acad. Sci. 83:1827−1831.

Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475−1489.

Donoghue, M., H. Ernst, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal. 1988. A muscle-specific enhancer is located at the 3′ end of the myosin light chain 1/3 gene locus. Genes Dev. 2:1779−1790.

Edmondson, D.G. and E.N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. 3:628−640.

Ephrussi, A., G.M. Church, S. Tonegawa, and W. Gilbert. 1985. B-lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science 227:134−140.

Gossott, L.A., D. Kelvin, E. Sternberg, and E.N. Olson. 1989. A new myocyte-specific enhancer binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. 9:5022−5033.

Helfman, D.M., S. Cheley, E. Kuismanen, L.A. Finn, and Y. Yamawaki-Kataoka. 1986. Nonmuscle and muscle tropomyosin isoforms are expressed from a single gene by alternative RNA splicing and polyadenylation. Mol. Cell. Biol. 6:3582−3595.

Horlick, R.A. and P.A. Benfield. 1989. The upstream muscle-specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol. Cell. Biol. 9:2396−2413.
Sequence-specific DNA binding of myogenin

Jaynes, J.B., J.E. Johnson, J.N. Buskin, C.L. Gartside, and S.D. Hauschka. 1988. The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. Mol. Cell. Biol. 8: 62–70.

Klaembt, C., E. Knust, K. Tietze, and J.A. Campos-Ortega. 1989. Closely related transcripts encoded by the neurogenic gene complex Enhancer of split of Drosophila melanogaster. EMBO J. 8: 203–210.

Lassar, A.B., J.N. Buskin, D. Lockshon, R.L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58: 823–831.

Lenardo, M., J.W. Pierce, and D. Baltimore. 1987. Protein-binding sites in Ig enhancers determine transcriptional activity and inducibility. Science 236: 1573–1577.

Maxam, A.M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65: 449–560.

Mellentin, J.D., S.D. Smith, and M.L. Cleary. 1989. Merrer, J.L., J.B. Moss, and W.J. Rutter. 1988. Systematic binding analysis of the insulin gene transcription control region: Insulin and immunoglobulin enhancers utilize similar transactivators. Mol. Cell. Biol. 8: 2620–2627.

Murre, C., P.S. McCaw, and D. Baltimore. 1989a. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56: 777–783.

Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, A.B. Lassar, H. Weintraub, and D. Baltimore. 1989b. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58: 537–544.

Olson, E.N., K.C. Caldwell, J.I. Gordon, and L. Glaser. 1983. Regulation of creatine phosphokinase expression during differentiation of BC2H1 muscle cell line. J. Biol. Chem. 258: 2644–2652.

Pieper, F.R., R.L. Slobbe, F.C.S. Ramaekers, H.T. Cuypers, and H. Bloemendal. 1987. Upstream regions of the hamster desmin and vimentin genes regulate expression during in vitro myogenesis. EMBO J. 6: 3611–3618.

Pimney, D.F., S.H. Pearson-White, S.F. Konieczny, K.E. Latham, and C.P. Emerson, Jr. 1988. Myogenenic lineage determination and differentiation: Evidence for a regulatory gene pathway. Cell 53: 781–793.

Quitschke, W.W., L. DePonti-Zilll, Z.E.-Y. Lin, and B.M. Patterson. 1989. Identification of two nuclear factor-binding domains on the chicken cardiac actin promoter: Implications for regulation of the gene. Mol. Cell. Biol. 9: 3218–3230.

Reznikoff, C.A., D.W. Brankow, and C. Heidelberger. 1973. Establishment and characterization of a cloned line of C3H mouse embry cy cells sensitive to postconfluence inhibition of division. Cancer Res. 33: 3231–3238.

Rhodes, S.J. and S.F. Konieczny. 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. 3: 2050–2061.

Rosenthal, N. 1989. Muscle cell differentiation. review. Curr. Opin. Cell Biol. (in press).

Russhlow, C.A., A. Hogan, S.M. Pinchin, K.M. Howe, M. Lardelli, and D. Ish-Horowicz. 1989. The Drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc. EMBO J. 8: 3095–3103.

Sassoon, D., G. Lyons, W.E. Wright, V. Lin, A. Lassar, H. Weintraub, and M. Buckingham. 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature 341: 303–307.

Schubert, D., J. Harris, C.E. Devine, and S. Heinemann. 1974. Characterization of a unique muscle cell line. J. Cell Biol. 61: 398–413.

Sen, R. and D. Baltimore. 1986. Multiple factors interact with the immunoglobulin enhancer sequences. Cell 46: 705–716.

Sternberg, E.A., G. Spizz, W.M. Perry, D. Vizard, T. Weil, and E.N. Olson. 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. 8: 2896–2909.

Sternberg, E., G. Spizz, M.E. Perry, and E.N. Olson. 1989. A ras-dependent pathway abolishes activity of a muscle-specific enhancer upstream from the muscle-creatine kinase gene. Mol. Cell. Biol. 9: 594–601.

Tapscott, S.J., R.L. Davis, M.J. Thayer, P.-F. Cheng, H. Weintraub, and A.B. Lassar. 1988. MyoD1: A nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. Science 242: 405–411.

Thayer, M.J., S.J. Tapscott, R.L. Davis, W.E. Wright, A.B. Lassar, and H. Weintraub. 1989. Positive autoregulation of the myogenenic determination gene MyoD1. Cell 58: 241–248.

Thissle, B., C. Stoetzel, C. Gorostiza-Thisse, and F. Perrin-Schmitt. 1988. Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. EMBO J. 7: 2175–2183.

Villares, R. and C.V. Cabrera. 1987. The acheate-scute gene complex of D. melanogaster: Conserved domains in a subset of genes required for neurogenesis and their homology to myc. Cell 50: 415–424.

Wang, Y., H.-P. Xu, X.-M. Wang, M. Ballivet, and J. Schmidt. 1988. A cell type-specific enhancer drives expression of the chick muscle acetylcholine receptor α-subunit gene. Neuron 1: 527–534.

Weintraub, H., S.J. Tapscott, R.L. Davis, M.J. Thayer, H.A. Adam, A.B. Lassar, and A.D. Miller. 1989. Activation of muscle specific genes in pigment, nerve, fat, liver and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. 86: 5434–5438.

Wright, W.E., D.A. Sassoon, and V.K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. Cell 56: 607–617.

Yaffe, D. and O. Saxel. 1977. Serial passaging and differentiation of mouse embryonic carcinoma cells. Cold Spring Harbor Symp. Quant. Biol. 42: 855–862.
Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization.

T J Brennan and E N Olson

Genes Dev. 1990, 4:
Access the most recent version at doi:10.1101/gad.4.4.582

References
This article cites 50 articles, 24 of which can be accessed free at: http://genesdev.cshlp.org/content/4/4/582.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.