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Analysis of the Myogenin Promoter Reveals an Indirect Pathway for Positive Autoregulation Mediated by the Muscle-Specific Enhancer Factor MEF-2

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Transcriptional cascades that specify cell fate have been well described in invertebrates. In mammalian development, however, gene hierarchies involved in determination of cell lineage are not understood. With the recent cloning of the MyoD family of myogenic regulatory factors, a model system has become available with which to study the dynamics of muscle determination in mammalian development. Myogenin, along with other members of the MyoD gene family, possesses the apparent ability to redirect nonmuscle cells into the myogenic lineage. This ability appears to be due to the direct activation of an array of subordinate or downstream genes which are responsible for formation and function of the muscle contractile apparatus. Myogenin-directed transcription has been shown to occur through interaction with a DNA consensus sequence known as an E box (CANNTG) present in the control regions of numerous downstream genes. In addition to activating the transcription of subordinate genes, members of the MyoD family positively regulate their own expression and cross-activate one another's expression. These autoregulatory interactions have been suggested as a mechanism for induction and maintenance of the myogenic phenotype, but the molecular details of the autoregulatory circuits are undefined. Here we show that the myogenin promoter contains a binding site for the myocyte-specific enhancer-binding factor, MEF-2, which can function as an intermediary of myogenin autoactivation. Since MEF-2 can be induced by myogenin, these results suggest that myogenin and MEF-2 participate in a transcriptional cascade in which MEF-2, once induced by myogenin, acts to amplify and maintain the myogenic phenotype by acting as a positive regulator of myogenin expression.

The formation of skeletal muscle during vertebrate development involves the induction of mesoderm from primary ectoderm and the subsequent generation of proliferating myoblasts that ultimately terminally differentiate in response to environmental cues. The recent discovery of a family of related muscle-specific factors that can convert fibroblasts to myoblasts has contributed to rapid progress toward understanding the molecular events that underlie the establishment of the skeletal muscle phenotype (for reviews, see references 55 and 69). Members of this muscle regulatory gene family include MyoD (21), myogenin (25, 77), myf5 (9), and MRF4/herculin/myf6 (8, 48, 61), each of which can activate myogenesis when introduced into a wide range of nonmuscle cell types. Related myogenic factors have also been identified in a variety of vertebrate and invertebrate species (22, 30, 35, 39, 44, 47, 57, 65, 72). An additional gene, *myd*, that appears to be structurally unrelated to the MyoD family, has been identified as a genomic clone that can induce myogenesis in 10T1/2 cells, but it has not yet been characterized at the molecular level (59).

Members of the MyoD family share extensive homology within a basic region and adjacent helix-loop-helix (HLH) motif that mediate DNA binding and dimerization (11, 17, 20, 41, 52, 73). Basic-HLH (bHLH) motifs have also been identified in members of the *myc* family of oncogenes, several proteins that mediate lineage decisions during *Drosophila* embryogenesis (1, 16), and a growing number of transcription factors that are widely expressed (2, 29, 31, 36, 37, 51; see also reference 3). All bHLH proteins that bind DNA recognize the consensus sequence CANNTG, referred to as an E box. However, individual HLH proteins show distinct half-site preferences for binding that depend on the variable nucleotides within and surrounding the invariant dyad symmetry of the CANNTG consensus (5). Binding of myogenic HLH proteins to DNA is enhanced in the presence of the widely expressed E2A gene products (E12 and E47), with which they preferentially form hetero-oligomers (8, 14, 18, 20, 52, 53).

E boxes are present in the control regions of most muscle-specific genes and are important for muscle-specific transcription (14, 15, 26, 41, 45, 58, 63, 74, 75). However, there are examples of muscle-specific genes that do not contain E boxes within their regulatory regions (46, 71). Whether these genes are controlled by other muscle-specific regulatory factors that act in parallel with, or are induced by, members of the MyoD family remains to be determined. A candidate for such factor is the myocyte-specific enhancer-binding factor MEF-2, which binds an A+T-rich element within numerous muscle-specific enhancers and promoters (28). MEF-2 is upregulated during myogenesis and can be induced by myogenin and MyoD, suggesting it may function within a regulatory pathway controlled by myogenic HLH proteins (19, 42).

In addition to activating downstream genes involved in terminal differentiation, members of the MyoD family have the ability to positively activate their own expression and cross-activate one another's expression in cultured cells (7, 13, 48, 70). It is possible that autoregulation amplifies the expression of these factors above a threshold necessary to

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activate myogenesis and provides stability to the myogenic phenotype. Similar autoregulatory activity has been described for a number of genes that regulate cell fate in *Drosophila melanogaster* (e.g., fushi tarazu, ultrabithorax, and deformed [4, 33, 40]). Although it has generally been assumed that regulatory interactions among members of the MyoD family reflect direct binding of the corresponding proteins to their promoters, the regulatory regions that control transcription of these genes have not yet been characterized, leaving open the possibility that these interactions can be mediated indirectly through induction of intermediate regulatory factors.

Despite their apparent ability to activate one another's expression in transfected cells in culture, members of the MyoD family show distinct temporal and spatial patterns of expression during embryogenesis (6, 32, 56, 64) and are differentially regulated during myogenesis in tissue culture (7, 13, 50, 61), suggesting that additional cellular factors influence regulatory interactions among these genes. Myogenin, for example, is first detected in replicating myogenic precursors in the somite myotome at 8.5 days of mouse development, several days prior to the appearance of MyoD or MRF4 (61, 64, 77). Myogenin expression is undetectable during the migration of determined myogenic precursors into the limb, where it again becomes expressed at high levels (64). In tissue culture, MyoD and myf5 are generally expressed in proliferating myoblasts (7, 21), whereas myogenin does not appear until differentiation has been triggered by withdrawal of growth factors (25, 77). In contrast to MyoD, myf5, and MRF4, which are expressed only in subsets of skeletal muscle cells, myogenin appears to be expressed during differentiation of all skeletal muscle cell types, suggesting that the myogenin gene responds to a common myogenic regulatory pathway.

To begin to define the early events that activate the muscle differentiation program, we have analyzed the *cis*- and *trans*-regulatory system required to induce myogenin transcription and to establish the myogenin autoregulatory circuit during myogenesis. Here, we show that muscle specificity and positive autoregulation of myogenin transcription can be mediated by MEF-2, which binds a site in the myogenin promoter. The ability of MEF-2 to regulate expression of myogenin, which in turn can induce MEF-2 (19, 42), suggests that members of the MyoD family and MEF-2 function within a complex regulatory network of lineage-specific factors that cooperate to generate and maintain the skeletal muscle phenotype.

MATERIALS AND METHODS

Cell culture. C2 (78) and 10T1/2 cells were maintained as described previously (25). Differentiation of C2 cells was initiated by transferring cultures from growth medium (GM; Dulbecco's modified Eagle's medium with 20% fetal bovine serum) to differentiation medium (DM; Dulbecco's modified Eagle's medium with 2% horse serum). Primary cultures of chicken embryo muscle cells were prepared by modification of a previously described procedure (26). Briefly, muscle tissue from the breasts of 11- or 12-day-old chicken embryos was dissected and dissociated with 0.05% trypsin. Cells were preplated on 100-mm-diameter tissue culture dishes and were sequentially transferred to new dishes coated with gelatin after incubations of 45 min to remove adherent fibroblasts. Where indicated, myoblasts were incubated with 5-bromo-2′-deoxyuridine (BrdU; 5 μg/ml) for 4 days. Fibroblasts were obtained from skin tissue of the same embryos by 0.05% trypsin digestion and were plated into culture dishes.

Transfections and CAT assays. Transient transfections were performed by calcium phosphate precipitation as described previously (67). Forty-eight hours after transfection, cultures were transferred from GM to DM for an additional 48 h. Cell extracts were then prepared, and levels of chloramphenicol acetyltransferase (CAT) activity were determined by using aliquots of extract containing equivalent quantities of protein or aliquots normalized to β-galactosidase activity generated from cotransfected RSV-*lacZ*, which contains the Rous sarcoma virus (RSV) long terminal repeat linked to *lacZ* and is expressed constitutively. All transfections were performed with at least two separate preparations of each plasmid. Stable transfections were performed by using 10 μg of reporter plasmid and 500 ng of a neomycin resistance gene as described previously (25). Forty-eight hours after transfection, cells were transferred to GM with G418 (400 μg/ml) for 14 days.

Isolation of genomic clones. To isolate myogenin genomic clones, mouse genomic libraries, prepared by ligating an *Mbo*I partial digest of mouse genomic DNA into the *Bam*HI site of the lambda phage vector EMBL 3, were screened by hybridization to the full-length mouse myogenin cDNA (25). DNA was hybridized on duplicate filter lifts overnight at 42°C in 50% formamide–5× SSC (0.75 M NaCl plus 0.075 M sodium citrate [pH 7.0])–5× Denhardt's solution–0.05 M Na_2HPO_4 (pH 7.0)–5% dextran sulfate–100 μg of salmon sperm DNA per ml. The filters were then washed twice for 30 min in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature and once for 30 min in 0.1× SSC–0.1% SDS at 65°C. Positive plaques were carried through three successive rounds of screening. Four clones, designated MG1, MG2, MG4, and MG6, remained positive after plaque purification and were characterized further.

Subcloning and sequencing. Inserts from the phage clones for MG1, MG2, and MG6 were excised by digestion with *Sal*I or *Xho*I and were subcloned into pUC19 or pBluescript for restriction mapping and sequencing. Subcloned genomic fragments were sequenced by using the dideoxy-chain termination method in denatured double-stranded plasmid, using synthetic oligodeoxynucleotides for primers. Sequencing primers were synthesized by the Macromolecular Synthesis Facility, M. D. Anderson Cancer Center. Sequence analysis was performed using the Beckman Microgenie and University of Wisconsin Genetics Computer Group sequence analysis software package.

S1 protection analysis. Total cellular RNA was isolated from cell cultures according to the guanidinium isothiocyanate procedure as described previously (25). To determine the myogenin transcription start site, a fragment from MG2, which extended 1.8 kb upstream from within intron 1, was denatured by alkali treatment at 65°C, neutralized, ethanol precipitated, and annealed to an oligonucleotide corresponding to nucleotides 177 through 191 on the antisense strand of the myogenin cDNA. Klenow fragment of DNA polymerase I in the presence of unlabeled dATP, dGTP, and dTTP and ^{32}P-labeled dCTP was used to synthesize an antisense probe extending from nucleotide 191 several hundred bases into the 5′ flanking region of the gene. The labeled probe was separated on an alkaline denaturing gel and hybridized to 20 μg of total cellular RNA for 16 h at 55°C. RNA was then digested with 50 U of S1 nuclease for 1 h at 37°C, and digestion products were separated on a 5% polyacrylamide gel.

Primer extension. Synthetic oligonucleotides correspond-

ing to sequences within the myogenin cDNA or CAT were end labeled with T4 kinase. The myogenin oligomer annealed to sequence located between nucleotides 50 and 84 of the myogenin sequence, and the CAT oligomer was a 20-mer and annealed to a sequence 50 nucleotides downstream from the unique *Hin*dIII site in pSV0CAT. Primer extension was performed by adding 100,000 cpm of end-labeled product to 20 to 40 μg of total cellular RNA. The mixture (in 10 mM Tris [pH 7.5]–0.25 M KCl–1 mM EDTA, 20 μl in volume) was heated to 65°C for 60 min and then cooled slowly to room temperature. The reaction volume was adjusted to 67 μl and final concentrations of 10 mM Tris (pH 7.5), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate (dNTP), and 50 μg of actinomycin D per ml. Extension was performed at 37°C, using 400 U of Moloney murine leukemia virus reverse transcriptase for 90 min. Following ethanol precipitation, the products were electrophoresed on an 8% polyacrylamide gel. A dideoxy sequencing reaction was performed by using the same oligonucleotide primer, and samples were electrophoresed in adjacent lanes to determine more precisely the 3′ end of the extended species.

Mutagenesis. Mutagenesis was performed by using single-stranded template DNAs as described previously (11). All mutants were confirmed by sequencing, and mutagenic regions were subcloned into wild-type vectors to avoid extraneous mutations.

Myogenin reporter gene constructs. The myogenin-CAT reporter genes were constructed by cloning a *Hae*III-*Pst*I fragment that corresponded to nucleotides +18 to −184 relative to the myogenin transcription start site. This fragment was inserted into the pCATbasic vector (Promega) to yield the construct pMYO184CAT. pCATbasic was prepared for cloning by digestion with *Xba*I, filling in of the ends with dNTPs in the presence of the Klenow fragment, and digestion with *Pst*I. Myogenin-CAT reporters were created by cloning restriction fragments extending from the *Pst*I site at −184 to ~−3700 (*Pst*I site), −1565 (*Xba*I), −1102 (*Sst*I), or −335 (*Sma*I) into pMYO184CAT. These clones recreate the exact sequence of the myogenin genomic clones. pMYO1565lacZ was created in the same way, using the *lacZ* reporter plasmid pUCAUGbeta-gal (a gift from W. M. Perry, M. D. Anderson Cancer Center), which contains the *lacZ* coding sequences 3′ of a multicloning site. For deletion of E box E-1, a restriction fragment was made by digesting the appropriate DNA with *Ban*I, filling in the ends with Klenow polymerase, and then digesting the DNA with *Pst*I. The resulting fragment was cloned into pCATbasic as described above and yielded constructs whose 3′ ends correspond to the myogenin sequence at −18. Unidirectional 5′ deletions of the promoter were performed by using an exonuclease III-mung bean nuclease kit as recommended by the manufacturer (Stratagene).

In vitro transcription-translation and gel mobility shift assays. Myogenin and E12 were prepared for gel mobility shift assays by in vitro transcription-translation of the corresponding cDNAs as described previously (11). Nuclear extracts were prepared from tissue culture cells as described previously (28). Breast muscle from 13-day chicken embryos was dissected, and nuclei isolated were as described by Mar and Ordahl (46). Extracts were then prepared as for tissue culture cells. Five or ten micrograms of nuclear extract was used in each gel mobility shift assay. Oligonucleotide probes were synthesized by the Macromolecular Synthesis Facility at the M. D. Anderson Cancer Center and were end labeled with ^{32}P.

Nucleotide sequence accession number. The nucleotide sequence of the mouse myogenin gene has been entered into the GenBank data base under the accession number M95800.

RESULTS

Structure of the mouse myogenin gene. To begin to define the *cis*-acting elements that control myogenin transcription, we isolated myogenin genomic clones by screening a mouse genomic library with a full-length myogenin cDNA. Sequence analysis of overlapping recombinant phage revealed that the myogenin gene spans ~2.5 kb and contains two introns (Fig. 1). The sequence of human myogenin has also been reported recently by Salminen et al. (62). The first potential initiation codon for myogenin translation is located 50 nucleotides downstream of the transcription start site. The basic domain and HLH motif are contained within exon 1. Exon 2 encodes 27 amino acids that comprise an essential part of the transcriptional activation domain of the myogenin protein (66). Exon 3 is relatively rich in serine and threonine residues and contains a segment of 12 amino acids that is homologous to MyoD, myf5, and MRF4. The translation termination codon and the entire 3′ untranslated region are also contained in exon 3. At the 3′ end of the gene, there is a potential polyadenylation signal (AATAAA) 16 bases upstream of the poly(A) tail identified in the previously published analysis of the cDNA (25). Both introns are flanked by consensus sequences for splice donors and acceptors. The intron-exon organization of myogenin is the same as that of the mouse MyoD (38) and herculin (18a, 48) genes. In both of the latter genes, the bHLH region is encoded by exon 1; exon 2 encodes between 27 and 29 amino acids, and the conserved serine- and threonine-rich sequence is contained in exon 3. The *Drosophila* and *Caenorhabditis elegans* MyoD genes show a different organization (39, 47).

We analyzed the 5′ flanking region for sequence motifs known to participate in transcriptional regulation of muscle-specific genes as well as of genes more widely expressed. Two A+T-rich sequences are located in the proximal 5′ flanking region, −66 to −58 and −31 to −25; the latter sequence serves as the TATA box for transcription initiation (see below). The more distal sequence is similar to the binding site for MEF-2 (19, 28), which binds the muscle creatine kinase (MCK) and myosin light-chain one-third enhancers. E boxes (CANNTG) are present at −15 to −10 and −141 to −136. An element that resembles a CArG motif, $CC(A+T)_6GG$, which has been implicated in transcription of numerous muscle-specific genes, is present at −550 to −541 (49), and a sequence that corresponds to the binding site for nuclear factor 1 is located at −54 to −42 (23, 27). There is also an A+C repeat between −604 and −560 that could have the potential to form Z-DNA (54).

Determination of the transcription initiation site of myogenin. The transcription initiation site for myogenin was determined by S1 protection and primer extension. Using a labeled antisense probe that extended upstream from the first nucleotide of codon 48 in the open reading frame, we detected a major S1-resistant product of 191 nucleotides with RNA from C2 myotubes (Fig. 2A). The length of the protected species indicated that the major site for transcription initiation lies 25 bp downstream of the TTAAAT sequence in the 5′ proximal region (Fig. 1B). S1-resistant products were not generated by RNA from 3T3 fibroblasts (Fig. 2A), which do not express myogenin.

Primer extension confirmed the results of S1 analysis.

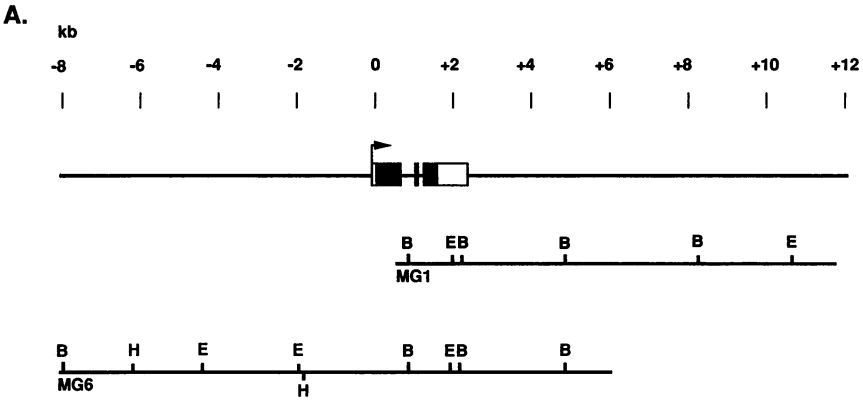

FIG. 1. Structure and nucleotide sequence of the mouse myogenin gene. (A) Gene structure. Genomic clones MG1 and MG6 were mapped by restriction endonucleases and Southern blot analysis with labeled fragments of the myogenin cDNA. Positions of exons, as determined by DNA sequencing, are indicated by boxes; black denotes coding sequence, and white denotes noncoding sequence. The arrow indicates the transcription initiation site. Positions of *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B) sites are indicated. (B) Nucleotide sequence of the transcription unit and 5′ flanking region of the gene. The intron sequence is shown in lower case letters. Nucleotides are numbered relative to the transcription initiation site (designated +1), which was determined by S1 protection and primer extension (see Fig. 2). The bHLH region in exon 1 and conserved serine- and threonine-rich region in exon 3 are underlined. The TATA box for transcription initiation is at −31 to −25. Additional sequence homologies within the 5′ flanking region include E boxes at −15 to −10 and −141 to −136, designated E-1 and E-2, respectively, a MEF-2 site at −66 to −58, a CArG box at −550 to −541, and a nuclear factor 1 (NF-1) site at −54 to −42. The asterisk above nucleotide 2503 denotes the last nucleotide before the poly(A) tail in the myogenin cDNA. We detected a nucleotide variation (C or T) at the −8 position during the sequencing of genomic clones derived from different libraries. It is unknown whether this represents normal allelic variation or a mutation of the nucleotide.

Using a synthetic oligomer that hybridized within the myogenin coding sequence, an extended product of 84 bp was detected in total RNA from BC3H1 myocytes (Fig. 2B) and C2 myotubes (data not shown). The size of the major extended product indicates that transcription initiates at the same nucleotide as determined by S1 protection. No extended product was detected in RNA from 10T1/2 cells, which do not express myogenin (Fig. 2B).

Sequences within 184 nucleotides of the myogenin transcription initiation site confer muscle specificity, growth factor responsiveness, and positive autoregulation. To begin to identify *cis*-acting sequences that regulate myogenin transcription, we used convenient restriction sites in the 5′ flanking region to generate a series of deletions extending upstream from nucleotide +18. Each DNA fragment was inserted immediately 5′ of a CAT reporter gene and tested for activity in a variety of cell types. As shown in Fig. 3A, reporter plasmids containing between 184 and 3,700 bp of myogenin 5′ flanking sequence were expressed at high levels in primary cultures of differentiated chick myotubes. In these cultures, we reproducibly observed higher expression with reporter plasmids containing 1,102 or 335 bp of 5′ flanking sequence than with those containing 3,700 or 1,565 bp, suggesting there may be a weak negative element between −1565 and −1102. We also observed a decrease in expression between −335 and −184. None of the reporter plasmids tested directed measurable expression in primary fibroblasts or in primary chick myoblasts treated with the thymidine analog BrdU, which extinguishes expression of myogenin and MyoD and inhibits myogenesis (68).

We also examined whether myogenin-CAT reporter genes were appropriately regulated in the C2 muscle cell line, which does not express myogenin until differentiation is initiated in response to mitogen withdrawal. As shown in Fig. 3B, pMYO1565CAT was not expressed at significant levels in stably transfected C2 myoblasts but was induced more than 12-fold in differentiated myotubes. A similar pattern of expression was observed in C2 cells stably transfected with other myogenin-CAT reporter plasmids shown in Fig. 3A (data not shown). In contrast, none of the myogenin-CAT reporter genes tested were expressed in either transiently or stably transfected 10T1/2, 3T3, or HeLa cells (24; data not shown). The activity of the myogenin promoter was also analyzed in C2 cells following stable transfection with a *lacZ* reporter gene linked to the 1,565-bp 5′ flanking region. In accordance with the pattern of expression of the endogenous myogenin gene, the myogenin-*lacZ* reporter was expressed at high levels in terminally differentiated myotubes but not in myoblasts prior to differentiation (Fig. 3C).

We next investigated whether the elements required for positive autoregulation of myogenin transcription were contained in the proximal upstream region by cotransfecting 10T1/2 fibroblasts with myogenin-CAT reporter plasmids and expression vectors encoding myogenin and MyoD. As shown in Fig. 3D, pMYO1565CAT and pMYO184CAT, which contain 1,565 and 184 bp of 5′ flanking sequence, respectively, were silent in 10T1/2 cells but could be *trans*-activated to high levels by myogenin or MyoD. The two reporter plasmids were induced to comparable levels by myogenin and MyoD, indicating that auto- and cross-activation of myogenin transcription can be maintained by sequences within the proximal promoter and do not depend on distal upstream elements. Together, these results demonstrate that DNA sequences that confer muscle specificity, positive autoregulation, and growth factor responsiveness to the myogenin gene are contained in the proximal 5′ flanking region. These results are consistent with those of Salminen et al. (62), who showed that the 211-bp sequence preceding the transcription initiation site of the human myogenin gene was sufficient for muscle-specific transcription.

Myogenin binds to a single site in its own promoter. The myogenin proximal upstream region contains E boxes at −15 to −10 and −141 to −136, designated E-1 and E-2, respectively, that could potentially serve as binding sites for

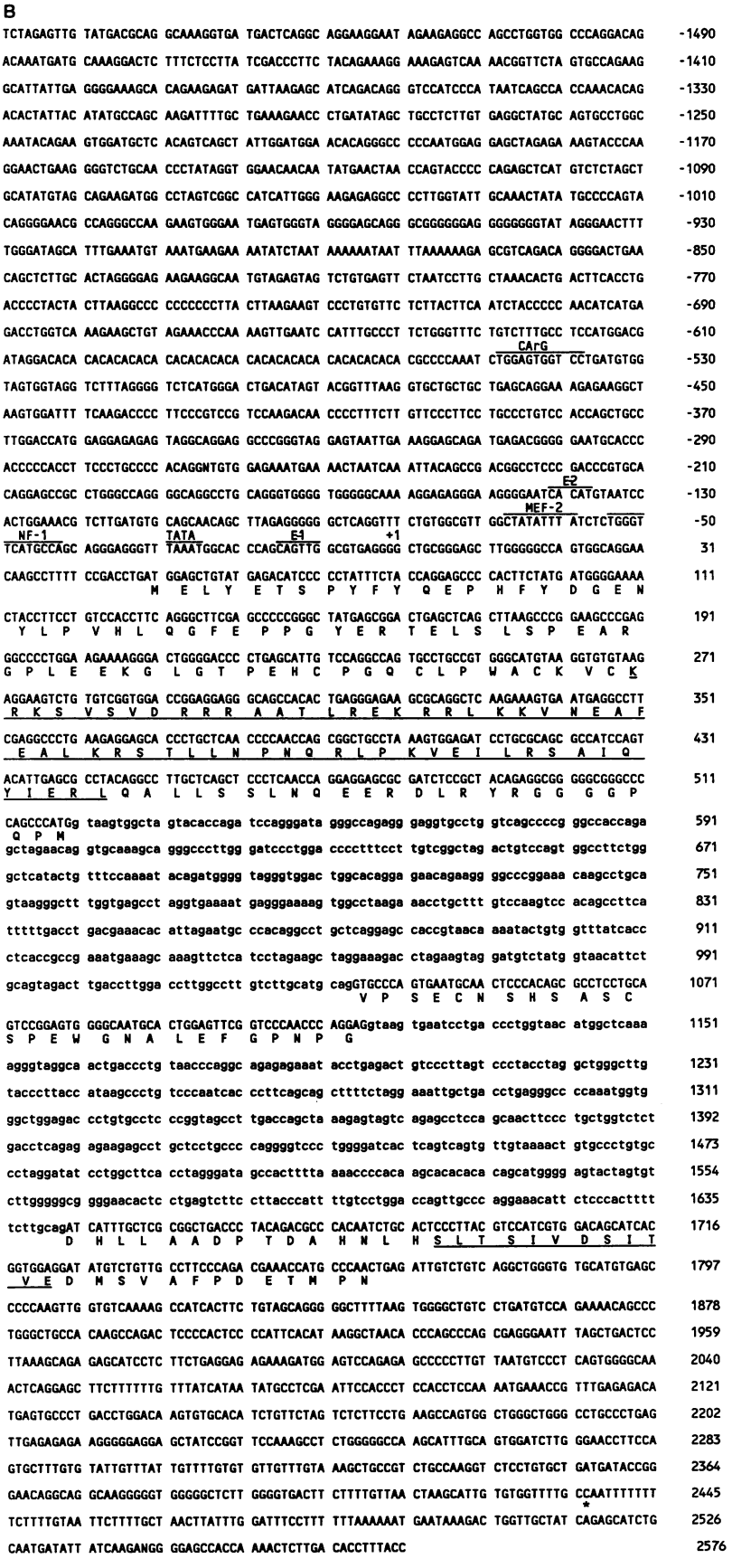

FIG. 1—*Continued.*

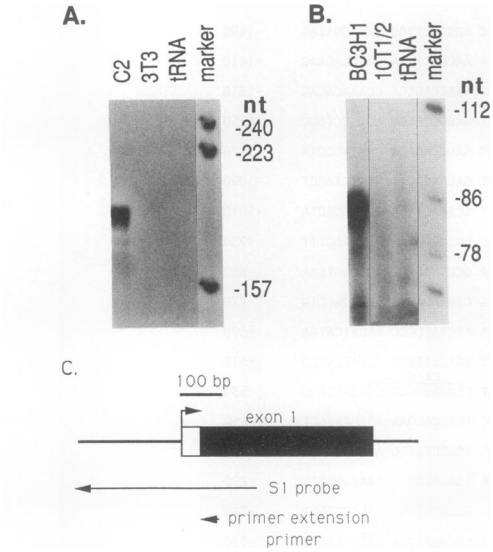

FIG. 2. Determination of the transcription initiation site for myogenin by S1 protection and primer extension. (A) S1 protection. The transcription initiation site for myogenin was determined by S1 protection as described in the text. The 3′ end of the labeled probe corresponds to the first nucleotide of codon 48 in the open reading frame. The labeled probe was incubated with RNA from C2 myotubes, 3T3 cells, or tRNA, as indicated. The protected species is 191 nucleotides (nt) in length and is exactly the predicted size of a transcript initiating 25 nucleotides downstream of the TATA-like element at −31 to −25. (B) Primer extension, performed as described in the text. The 3′ end of the primer used for extension corresponds to nucleotide 84 of the myogenin cDNA. The primer was annealed to RNA from BC3H1 myocytes, 10T1/2 cells, or tRNA, as indicated. The major extended product of 84 nucleotides indicates that the primary transcription start site begins 25 nucleotides 3′ of the TAAAT sequence, in exact agreement with S1 protection. A dideoxy sequencing reaction was performed by using the same primer used for the primer extension and was electrophoresed in adjacent lanes to determine the exact extent of protection (data not shown). (C) Positions of the S1 protection probe and primer extension primer within the first exon of the myogenic gene.

myogenin, MyoD, or other myogenic HLH proteins. To determine whether myogenin was able to bind either of these E boxes, we used oligonucleotide probes encompassing each E box in gel mobility shift assays with in vitro-translated myogenin and E12. As shown in Fig. 4, the more 3′ E box, E-1, strongly bound myogenin-E12 hetero-oligomers, whereas we did not detect significant binding to the more 5′ E box, E-2 (compare lanes 2 and 7). Binding of myogenin-E12 to E-1 was sequence specific, as shown by competition with unlabeled E-1 oligomer but not with E-2 or an unrelated sequence from another region of the promoter (Fig. 4, lanes 2 to 5). The E-1 oligomer was also able to compete for binding of myogenin-E12 to a labeled probe encompassing the high-affinity right E box from the MCK enhancer, which is the prototypical target sequence for myogenin and MyoD (Fig. 4, lane 13). The sequence of E-1 agrees with the consensus sequence for DNA binding of MyoD-E12 and myogenin-E12 hetero-oligomers, as determined by polymerase chain reaction-mediated binding site selection, whereas E-2 would not be predicted to bind these hetero-oligomers appreciably (5, 76).

It has been speculated that homo-oligomers of MyoD could play a role in positive autoregulation through interaction with the MyoD control region (3, 70). We therefore tested whether in vitro-translated myogenin or MyoD would bind E box E-1 or E-2 in the absence of E12. Under the conditions of these assays, we did not detect binding of either myogenic factor alone to E-1 or E-2 (data not shown). However, a bacterially expressed myogenin fusion protein, at concentrations significantly higher than can be achieved with in vitro translation products, will bind to E-1 (data not shown). We also examined whether there might be cryptic sites for binding of myogenin-E12 or MyoD-E12 within the 184-bp promoter by performing gel mobility shift assays with overlapping restriction fragments encompassing the entire region; the only measurable binding was observed with E box E-1 (data not shown).

The muscle-specific enhancer-binding factor MEF-2 binds the myogenin promoter. The myogenin promoter also contains an A+T-rich element at −66 to −58 that resembles a binding site for the muscle-specific factor MEF-2, which was originally identified as an MCK enhancer-binding factor that is induced during the myoblast-to-myotube transition (28). Although the A+T-rich element in the myogenin promoter deviates from the MCK MEF-2 site, it agrees with the preliminary consensus sequence recently deduced for MEF-2 binding (19; see also reference 60). Therefore, to determine whether MEF-2 could interact with this A+T-rich element, we used an oligomer encompassing this region as a probe in gel mobility shift assays with nuclear extracts from C2 myoblasts and myotubes. Figure 5A shows that this probe generated a complex specific to differentiated C2 myotubes (lane 4) and that the migration of this complex was indistinguishable from that of the complex generated with an oligomer corresponding to the MEF-2 site in the MCK enhancer (lane 1). Extracts from C2 myoblasts did not contain detectable MEF-2 activity but generated a heterogeneous series of more rapidly migrating complexes, one of which represents a previously characterized binding activity referred to as myoblast factor 1. Extracts from 10T1/2 cells did not show significant binding to the labeled probes under the conditions of these assays. All of the extracts showed equivalent binding activity with a labeled probe corresponding to the binding site for Oct-1, which is expressed ubiquitously (data not shown). Specificity of MEF-2 binding to the myogenin MEF-2 site was shown by competition by unlabeled MCK MEF-2 site and the lack of competition by an oligomer containing a single base substitution previously shown to abolish MEF-2 binding (19).

Nuclear extract from embryonic chicken breast muscle also contains the MEF-2 binding activity. Figure 5B shows that the MEF-2 complex produced with breast muscle extract (lane 4) is identical to the complex generated by C2 myotube extract (lane 1). This complex is competed for by excess homologous oligomer (lanes 2 and 5) but not by an oligomer containing a mutant MEF-2 site (lanes 3 and 6).

Muscle specificity of the myogenin promoter is E box independent. To further closely define the regulatory elements responsible for myogenin transcription, we created a series of exonuclease deletion mutants of the promoter, beginning at bp −184 (Fig. 6A). Deletion from −184 to −84 bp had only a modest effect on promoter activity in chick primary myotubes. However, deletion to −58 bp resulted in a greater than 20-fold decrease in activity (Fig. 6B), indicating that the region of the promoter containing the MEF-2 site is essential for transcriptional activity of the minimal promoter. The partial loss in activity when the region between −184 and −84 bp was deleted also suggests the presence of regulatory elements in this region that contribute to the

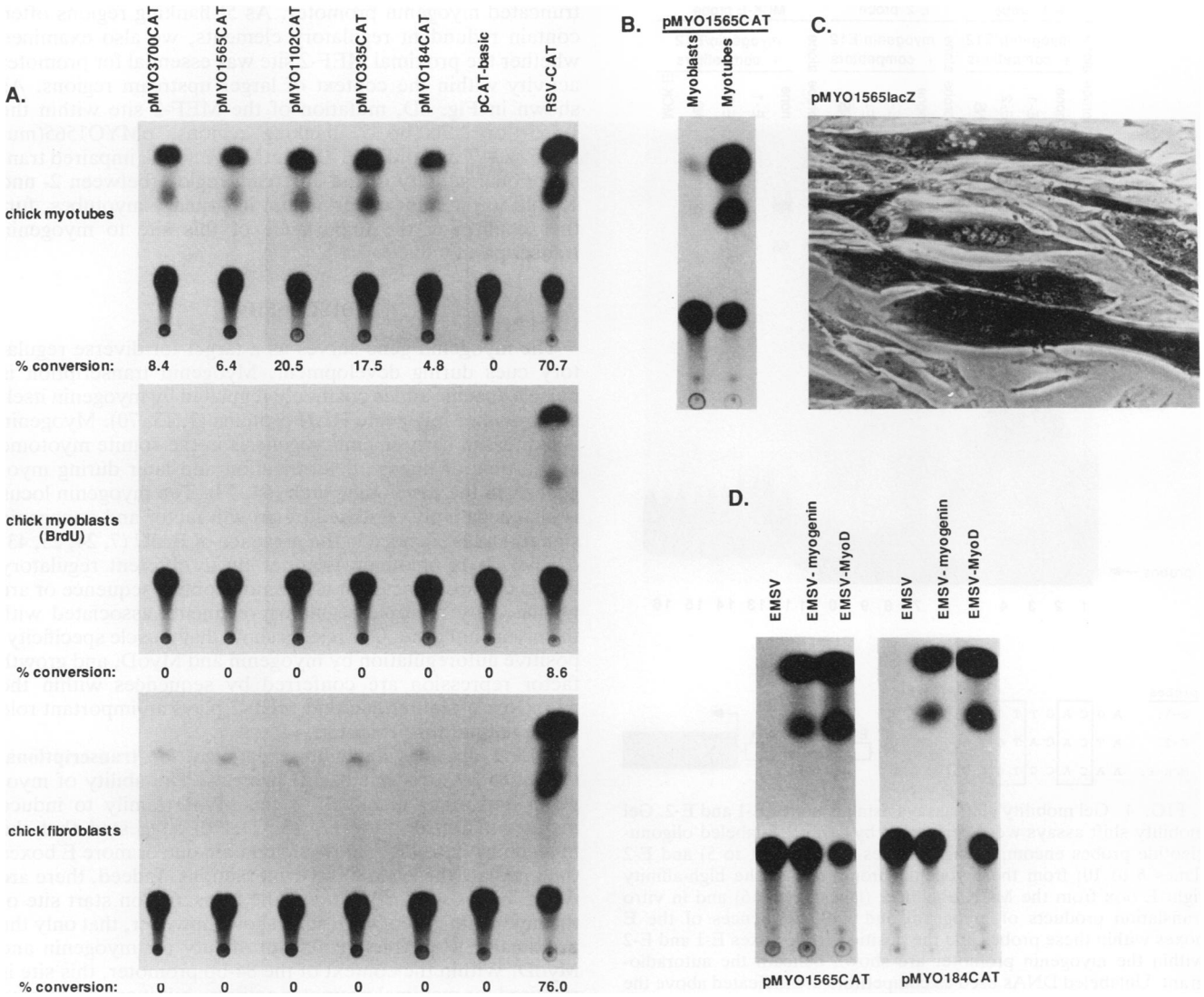

FIG. 3. Muscle-specific expression and *trans*-activation of myogenin-CAT reporter genes. (A) CAT activity in primary cultures of chick myotubes, myoblasts exposed to BrdU, or fibroblasts that were transiently transfected with the indicated reporter plasmids. CAT activity was assayed as described in the text and was normalized to β-galactosidase activity generated with cotransfected RSV-*lac*Z. CAT activity in BrdU-treated myoblasts and fibroblasts was not significantly different from the activity observed with the promoterless CAT reporter. (B) CAT activity in C2 myoblasts (in GM) and myotubes (in DM) stably transfected with pMYO1565CAT. (C) Assay of C2 cells stably transfected with pMYO1565lacZ and a neomycin resistance gene. After selection for G418 resistance, clones were stained for β-galactosidase activity. A representative clone, which contains intensely stained myotubes, is shown. (D) Assay of 10T1/2 cells transfected with 5 μg of EMSV (a mammalian expression vector containing the Maloney sarcoma virus LTR), EMSV-myogenin, or EMSV-MyoD and 5 μg of the indicated myogenin-CAT reporter plasmids. After transfer to DM for 48 h, CAT activity was determined in cell extracts. Thin-layer chromatograms from representative transfections are shown in panels A and B. Comparable results were obtained in at least five independent experiments.

overall level of transcription but are not essential for muscle-specific activity of the promoter.

To further assess the importance of the MEF-2 site and the E-1 E box to activity of the 84-bp promoter, we examined the effect of (i) a mutation in the MEF-2 site, (ii) deletion of the E-1 E box, and (iii) both (Fig. 6B). As shown in Fig. 6C, a single nucleotide insertion within the MEF-2 site, which prevents MEF-2 binding [pMYO84(mutMEF-2)CAT], resulted in an approximate fourfold loss in transcriptional activity in primary myotubes and abolished the ability of the promoter to be *trans*-activated by myogenin and MyoD in 10T1/2 cells. Deletion of E-1 [pMYO84(-E1)CAT] from the 84-bp promoter also diminished promoter activity in chick myotubes and reduced responsiveness to myogenin and MyoD, but the effect of the E-1 deletion was minimal relative to that of the MEF-2 site mutation. The activity of this reporter in muscle cells and in fibroblasts expressing exogenous myogenin and MyoD implies the existence of a mechanism for activation of muscle transcription independent of myogenic HLH proteins. Primer extension analysis of CAT RNA in transfected cells showed that deletion of E-1 did not affect the site of transcription initiation; transcription

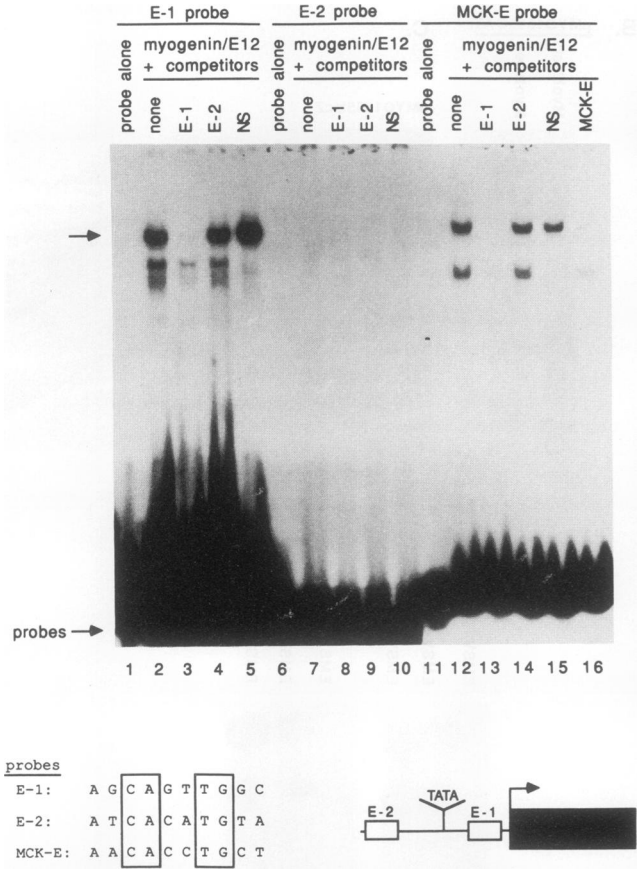

FIG. 4. Gel mobility shift assays using E boxes E-1 and E-2. Gel mobility shift assays were performed by using ^{32}P-labeled oligonucleotide probes encompassing E boxes E-1 (lanes 1 to 5) and E-2 (lanes 6 to 10) from the myogenin promoter and the high-affinity right E box from the MCK enhancer (lanes 11 to 16) and in vitro translation products of myogenin and E12. Sequences of the E boxes within these probes and the positions of E boxes E-1 and E-2 within the myogenin promoter are shown beneath the autoradiogram. Unlabeled DNAs used as competitors are indicated above the lanes. NS denotes a nonspecific DNA competitor corresponding to a fragment of the myogenin promoter that does not contain E-1 or E-2. Unlabeled competitors were added in a 20-fold molar excess over labeled probes. The top arrow at the left indicates the position of the major mobility-shifted complex.

initiated at exactly the same position relative to the TATA box in the endogenous gene and in reporter genes without E-1 (data not shown). Mutation of MEF-2 combined with E-1 deletion [pMYO84(mutMEF-2/-E1)CAT] eliminated the residual activity that remained with the single mutations and completely inactivated the promoter.

Because the MEF-2 site in the myogenin promoter is close to the transcription start site and resembles a TATA box, we also mutated the TATA box to a sequence that will not bind TFIID to determine whether it was essential for transcription and whether the MEF-2 site could direct transcription initiation. This reporter [pMYO(mutTATA)CAT] was devoid of transcriptional activity (Fig. 6C), indicating that the MEF-2 site in the myogenin promoter cannot mediate transcription initiation.

From the results for these mutants, it can be concluded that the MEF-2 site is essential for high-level activity of the truncated myogenin promoter. As 5′ flanking regions often contain redundant regulatory elements, we also examined whether the proximal MEF-2 site was essential for promoter activity within the context of larger upstream regions. As shown in Fig. 6D, mutation of the MEF-2 site within the 1,565- or 3,700-bp 5′ flanking regions, pMYO1565(mut MEF2)CAT and pMYO3700(mutMEF2)CAT, impaired transcriptional activity of the upstream region (between 2- and 10-fold in different experiments) in primary myotubes, further confirming the importance of this site to myogenin transcription.

DISCUSSION

The myogenin gene serves as a target for diverse regulatory cues during development. Myogenin transcription is muscle specific and is positively regulated by myogenin itself and by other myogenic HLH proteins (7, 13, 70). Myogenin is expressed early in embryogenesis in the somite myotome at the time of lineage determination and later during myogenesis in the developing limb (64, 77). The myogenin locus is also negatively regulated by growth factor and oncogenic signals and is silenced in the presence of BrdU (7, 24, 25, 43, 62, 68). It is unknown whether these different regulatory influences are directed at a common control sequence or are mediated by multiple regulatory elements associated with the myogenin gene. Our results show that muscle specificity, positive autoregulation by myogenin and MyoD, and growth factor repression are conferred by sequences within the myogenin promoter and that MEF-2 plays an important role in regulating this promoter.

MEF-2 provides an indirect pathway for transcriptional activation by myogenic bHLH proteins. The ability of myogenin and other members of the MyoD family to induce myogenin transcription (7, 13, 24, 70) suggested that the myogenin control region would contain one or more E boxes that mediate these regulatory interactions. Indeed, there are two E boxes within 150 bp of the transcription start site of the myogenin gene. Our results show, however, that only the proximal E-1 exhibits significant affinity for myogenin and MyoD. Within the context of the 84-bp promoter, this site is required for maximal promoter activity, but it can be deleted with only a partial loss in muscle-specific transcription or *trans*-activation by myogenin and MyoD. The ability of the proximal promoter, lacking an E box, to direct muscle-specific transcription and *trans*-activation by the myogenic regulators reveals the existence of a mechanism for activation of muscle transcription independent of direct binding of myogenic HLH proteins to the promoter (see below).

An unanticipated outcome of these studies was the finding that the myogenin promoter contains a MEF-2 site that is important for transcriptional activity. Myogenin and MyoD have been shown to activate expression of MEF-2 in transfected 10T1/2 and CV-1 cells (19, 42), suggesting that MEF-2 lies downstream of the myogenic HLH proteins in a dependent regulatory pathway. Thus, it seems paradoxical that MEF-2 participates in the regulation of myogenin transcription in differentiating muscle cells. How can these observations be reconciled? One possibility is that MyoD or myf5, both of which are expressed in myoblasts prior to myogenin and MEF-2, may activate the expression of MEF-2, which then collaborates with these myogenic regulators to induce myogenin transcription. Indeed, the rapid kinetics of MEF-2 induction upon withdrawal of growth factors from myoblasts are consistent with the involvement of MEF-2 in the regulation of myogenin expression (28). Induction of MEF-2

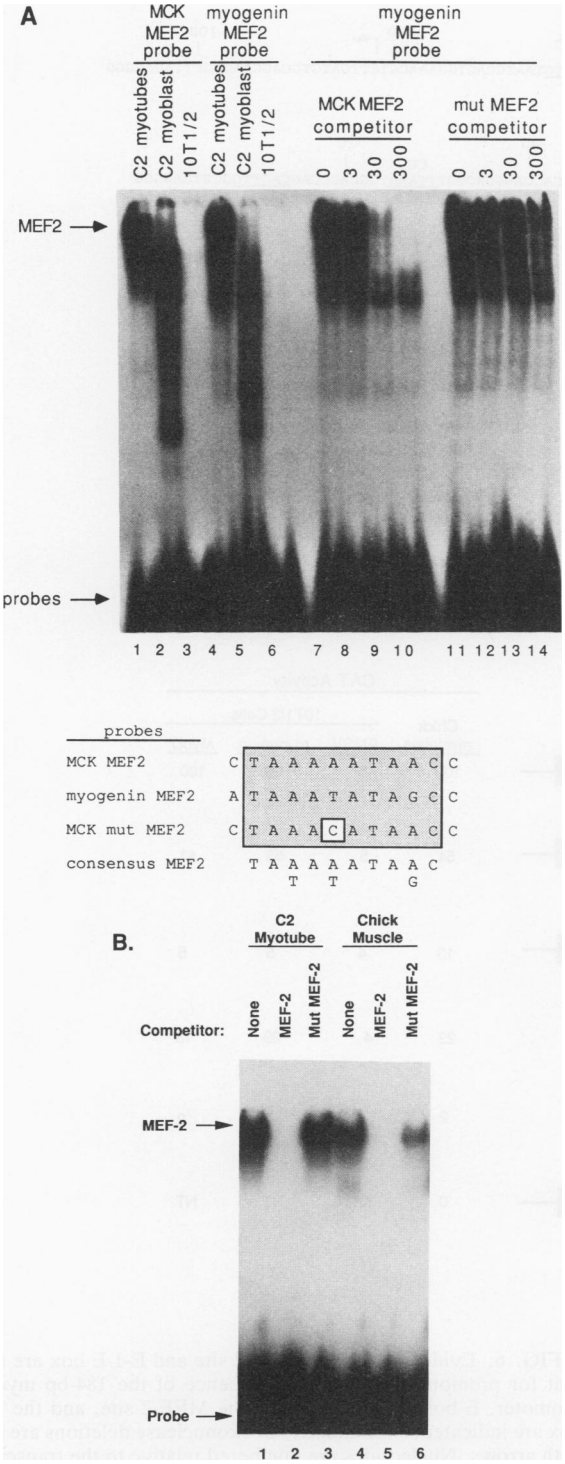

FIG. 5. Binding of MEF-2 to the myogenin promoter. (A) Nuclear extracts were prepared from C2 myoblasts, myotubes, and 10T1/2 cells, as indicated. Five micrograms of nuclear extract from each cell type was used in gel mobility shift assays with labeled oligonucleotide probes encompassing the MEF-2 site from the MCK enhancer, the putative MEF-2 site from the myogenin promoter, or a mutant MCK MEF-2 site. In competition experiments (lanes 7 through 14), unlabeled oligomers corresponding to the MCK MEF-2 site or mutant MCK MEF-2 site were used to compete for MEF-2 binding to the labeled myogenin MEF-2 probe. The molar excess of competitor oligonucleotides relative to the labeled myogenin MEF-2 probe is shown above lanes 7 to 14. Sequences of probes used in the gel mobility shift assays are shown beneath the autoradiogram. The core of the MEF-2 site is shaded, and a preliminary consensus for MEF-2 binding is shown. Longer exposure to film or use of greater amounts of nuclear extract in the assay reveals the presence of the ubiquitous MEF-2 binding site activity in 10T1/2 nuclear extracts, but its abundance is very low relative to that of MEF-2 in extracts from highly differentiated myotube cultures. (B) Ten micrograms of nuclear extract from C2 myotubes (lanes 1 to 3) or from chicken embryonic breast muscle (lanes 4 to 6) was incubated with a labeled oligonucleotide probe containing a MEF-2 site. For competitions, 100-fold molar excesses of unlabeled competing oligomers were added to the binding reaction mixture.

early in the differentiation program would lead to amplification of myogenin transcription, which would in turn further activate MEF-2 expression, leading to reinforcement of the decision to differentiate. Alternatively, MEF-2 may be regulated by factors in addition to myogenic HLH proteins. If myogenin and MEF-2 are initially upregulated in response to a differentiation inducing signal, their coexpression could serve to stabilize expression of both. The presence of functionally redundant sites that bind different factors that regulate one another provides stability to the transcription of both genes and could in this case contribute to commitment to the terminally differentiated state. A hypothetical model illustrating these types of regulatory interactions is presented in Fig. 7.

In addition to activating one another's expression, myogenin and MEF-2 can activate downstream muscle-specific genes separately or in combination. The ability of MEF-2 to activate transcription of target genes lacking E boxes can explain the observation that the mutant myogenin promoter lacking E-1 retained the ability to be *trans*-activated by myogenin and MyoD. Presumably, the induction of MEF-2 by these myogenic regulators indirectly activates myogenin transcription.

MEF-2 is one of several DNA-binding activities recognizing the MEF-2 site. In addition to the muscle-specific MEF-2 complex, several widely expressed DNA-binding activities have been identified that recognize the MEF-2 site (Fig. 5) (10, 19, 28, 34, 53a, 60). Insight into the nature of the factors involved in formation of these complexes has been provided by the recent isolation of cDNA clones encoding proteins that bind the MEF-2 consensus sequence (60). These proteins, termed RSRFs, for related to serum response factor, appear to represent a complex family of MEF-2 binding site factors, at least some of which are widely expressed. The RSRFs and serum response factor belong to the MADS box superfamily of regulatory factors, which regulate cell-type-specific gene expression in organisms ranging from plants and yeasts to mammals (see reference 60 and references therein). We have recently found that polyclonal antibodies directed against RSRFs quantitatively supershift the muscle-specific MEF-2 complex observed with the myogenin MEF-2 site (25a), indicating that MEF-2 is antigenically related to RSRFs. Whether MEF-2 represents the product of a muscle-specific RSRF gene or an alternate form of a widely expressed RSRF is unclear. It also remains to be determined why ubiquitous RSRFs expressed in myoblasts and nonmuscle cells are seemingly unable to induce myogenin transcription.

Paradoxically, MEF-2 is also expressed in cardiac muscle (53a), whereas myogenin is never expressed in the heart (25, 77). We are presently testing whether the apparent inability

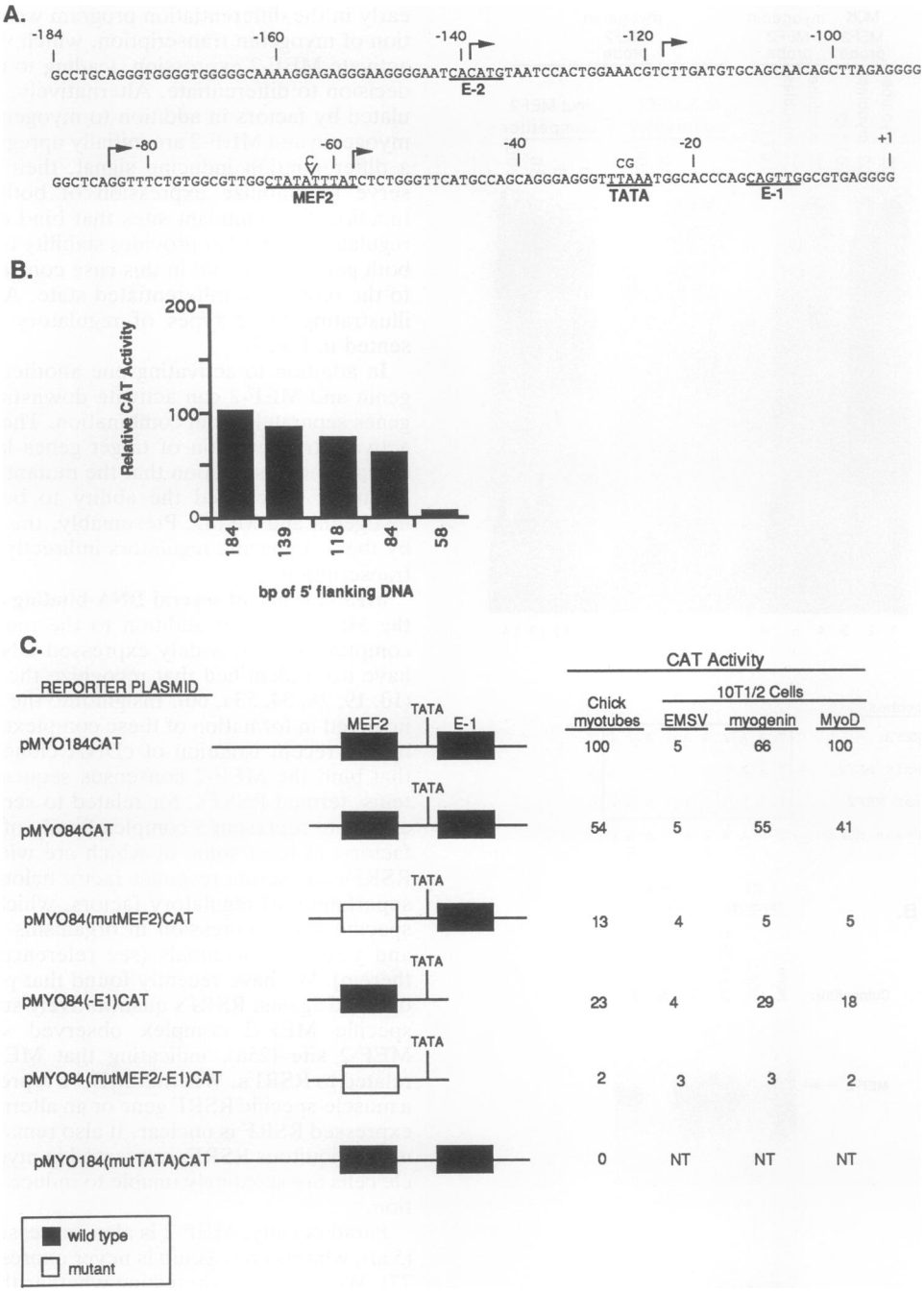

FIG. 6. Evidence that the MEF-2 site and E-1 E box are important for promoter activity. (A) Sequence of the 184-bp myogenin promoter. E boxes (E-1 and E-2), the MEF-2 site, and the TATA box are indicated; 5′ boundaries of exonuclease deletions are shown with arrows. Nucleotides are numbered relative to the transcription initiation site, designated +1. Nucleotides above the TATA box show mutations introduced at those positions. The MEF-2 site was mutated by insertion of a dC at the position indicated. (B and C) CAT assays. For panel B, myogenin-CAT reporter genes containing deletions of the promoter were assayed in primary cultures of chick myotubes. All myogenin 5′ flanking sequences contained the same 3′ end point, nucleotide +18 relative to transcription initiation. The 5′ deletion endpoint of each promoter mutant is indicated. For deletion of E box E-1, *Ban*I was used to remove sequences 3′ to −18. The normal TATA box continues to be used in these constructions (data not shown). For panel C, the MEF-2 site and E-1 E box

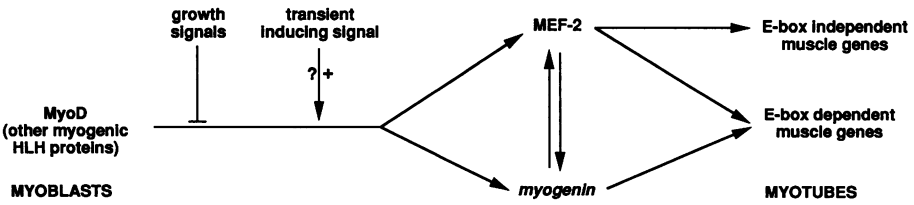

FIG. 7. Hypothetical model for the control of myogenin and MEF-2 expression during myogenesis. MyoD, or another myogenic HLH protein expressed in myoblasts, can induced myogenin and MEF-2 upon withdrawal of exogenous growth factors. Alternatively, a transient signal during development activates myogenin or myogenin and MEF-2. Myogenin and MEF-2 can activate one another's expression, thus stabilizing the myogenic phenotype, and in addition can activate downstream muscle-specific genes. Some muscle-specific genes may contain binding sites for myogenin and MEF-2, while others may be regulated by each factor independently.

of MEF-2 to activate myogenin transcription in cardiac myocytes reflects negative regulation in *cis* or in *trans* and whether regulatory elements in addition to the MEF-2 site influence the activity of the myogenin promoter in these two muscle cell types.

Growth factor signals repress myogenin transcription. Unlike MyoD and myf-5, which are expressed in proliferating myoblasts, myogenin is not upregulated until myoblasts are placed in an environment lacking mitogens or other serum factors that inhibit myogenesis (24, 25, 77). Our results show that serum represses myogenin expression by silencing the myogenin promoter. Such repression could, in principle, be mediated by negative elements in the promoter that bind inhibitory factors or by repression of positive-acting factors required for myogenin transcription. The finding that myogenic HLH proteins and MEF-2 contribute to promoter activity suggests that the latter type mechanism is responsible for repression of myogenin transcription in myoblasts because myogenic HLH proteins are present but functionally inactive (3, 12, 70) and MEF-2 is not expressed in proliferating myoblasts. There is therefore no need to invoke a mechanism for actively repressing the myogenin promoter in myoblasts.

Conservation of *cis*-acting elements and *trans*-acting factors that regulate members of the MyoD family. The shared intron-exon organization of the vertebrate myogenic regulatory factor genes suggests that these genes evolved from a common ancestral gene, with their unique patterns of expression arising as a result of divergence in their regulatory regions. Indeed, analysis of the mouse MRF4 5′ flanking region reveals a MEF-2 site and the E-1 E box in a spatial arrangement similar to that in the myogenin promoter (18a). It is therefore not unreasonable to anticipate that these elements may also be important in the regulation of MRF4 during development. MEF-2 and myogenic HLH proteins may also contribute to the regulation of other members of the MyoD family. The *Xenopus* MyoD gene *XLMF1* (65), for example, contains two E boxes and an MEF-2 site within its promoter (65a). Thus, the types of regulatory interactions described here for myogenin may represent a conserved mechanism for transcriptional activation of multiple members of the MyoD family.

The muscle specificity and autoregulatory activity of the myogenic regulatory factor genes imply that these genes are controlled by a common regulatory mechanism and suggest the existence of shared *cis*-acting control sequences. However, each member of this regulatory gene family also shows a unique pattern of expression during embryogenesis and during myogenesis of established muscle cell lines. Thus, these genes must also contain distinct regulatory elements that confer their individual patterns of expression. As the *cis*- and *trans*-regulatory systems governing the transcription of additional members of the MyoD family are defined, it should be possible to gain a better understanding of the regulatory relationship among these genes as well as the molecular details of this myogenic regulatory circuit.

reporters were assayed by transfection in chick primary myotube cultures or in 10T1/2 cells cotransfected with EMSV, EMSV-myogenin, or EMSV-MyoD. Filled boxes denote wild-type sequence, and open boxes denote mutant sequence. The TATA box was mutated within pMYO184CAT. Names assigned each plasmid are indicated at the left. NT, not tested. CAT activity in panels B and C was normalized to β-galactosidase activity generated by cotransfected RSV-*lacZ*. Levels of CAT activity directed by each plasmid are expressed relative to the level of expression of pMYO184CAT in each cell type. Values in panels B and C represent averages of at least three experiments and did not vary by more than 20% of the mean. (D) Transfection assay. Primary cultures of chick myotubes were transiently transfected with the indicated reporter plasmids, and CAT activity was determined. A representative thin-layer chromatogram showing the results of duplicate transfections is presented. The levels of expression of p1565mutMEF2 and p3700mutMEF2 are 10 and 15%, respectively, of the parental plasmid levels.

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The first two authors contributed equally to this work.

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