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E-Box- and MEF-2-Independent Muscle-Specific Expression, Positive Autoregulation, and Cross-Activation of the Chicken *MyoD* (*CMD1*) Promoter Reveal an Indirect Regulatory Pathway

CLAUDE A. DECHESNE,† QIN WEI, JUANITA ELDRIDGE, LEILA GANNOUN-ZAKI,† PHILIPPE MILLASSEAU,‡ LYDIE BOUGUELERET,§ DOMINIQUE CATERINA,§ AND BRUCE M. PATERSON*

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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Members of the MyoD family of gene-regulatory proteins (MyoD, myogenin, myf5, and MRF4) have all been shown not only to regulate the transcription of numerous muscle-specific genes but also to positively autoregulate and cross activate each other's transcription. In the case of muscle-specific genes, this transcriptional regulation can often be correlated with the presence of a DNA consensus in the regulatory region CANNTG, known as an E box. Little is known about the regulatory interactions of the myogenic factors themselves; however, these interactions are thought to be important for the activation and maintenance of the muscle phenotype. We have identified the minimal region in the chicken *MyoD* (*CMD1*) promoter necessary for muscle-specific transcription in primary cultures of embryonic chicken skeletal muscle. The *CMD1* promoter is silent in primary chick fibroblast cultures and in muscle cell cultures treated with the thymidine analog bromodeoxyuridine. However, CMD1 and chicken myogenin, as well as, to a lesser degree, chicken Myf5 and MRF4, expressed in *trans* can activate transcription from the minimal *CMD1* promoter in these primary fibroblast cultures. Here we show that the *CMD1* promoter contains numerous E-box binding sites for CMD1 and the other myogenic factors, as well as a MEF-2 binding site. Surprisingly, neither muscle-specific expression, autoregulation, or cross activation depends upon the presence of these E-box or MEF-2 binding sites in the *CMD1* promoter. These results demonstrate that the autoregulation and cross activation of the chicken *MyoD* promoter through the putative direct binding of the myogenic basic helix-loop-helix regulatory factors is mediated through an indirect pathway that involves unidentified regulatory elements and/or ancillary factors.

The transcriptional cascade that establishes the muscle cell lineage in the somites of the newly forming vertebrate embryo and ultimately leads to terminal myogenesis and the formation of muscle has been only partially defined by the isolation and characterization of the MyoD family of muscle-specific gene-regulatory proteins: MyoD, myogenin, Myf5, and MRF4 (8, 16, 51, 74). Members of this family of gene regulators have the unusual property that when any one factor is introduced into a variety of nonmuscle cells of different germ layers or tissue origins, myogenesis is activated (7, 13, 16, 71). Closely related proteins have also been isolated from *Drosophila melanogaster* (35, 44), *Caenorhabditis elegans* (25), and sea urchins (65), and all have the ability to convert 10T1/2 mouse fibroblasts into muscle. This conversion involves not only the activation of the downstream muscle-specific genes but also the positive activation of one or more of the endogenous *MyoD*-related genes, through poorly understood mechanisms. It is thought that this regulatory circuitry between the myogenic genes plays an important role in the activation and maintenance of the muscle cell phenotype (for reviews, see references 10, 40, 56, and 67).

* Corresponding author. Phone: (301) 496-1746. Fax: (301) 402-3095.

† Present address: INSERM U 300, Faculté de Pharmacie, 34090 Montpellier Cedex 01, France.

‡ Present address: Genethon, 91002 Evry Cedex, France.

§ Present address: Centre d'Étude du Polymorphisme Humain, 75010 Paris, France.

It has also been assumed that these regulatory interactions involve the direct binding of the myogenic factor proteins to the promoters of the responding genes; however, little is known about the promoter regulatory regions that control the transcription of these myogenic regulatory proteins. This leaves open the formal possibility that these interactions are indirect and may involve the induction or action of additional regulatory factors. The differences in the in vivo and in vitro expression patterns for the myogenic factors during myogenesis and in different muscle cell lines suggest that additional cellular factors are involved in the myogenic regulatory pathway (10, 23, 56, 58).

To begin to understand the regulatory circuitry involved in the regulation of these myogenic factors, we have analyzed the tissue-specific expression, autoregulation and cross activation of the chicken *MyoD* (*CMD1*) promoter in various cell backgrounds. Here we demonstrate that the muscle specificity, positive autoregulation, and cross activation of *CMD1* transcription do not involve either E-box or MEF2 sites in the *CMD1* promoter and, therefore, that the putative autoregulatory/cross-activating circuits utilize either additional regulatory regions in the gene and/or ancillary cellular factors yet to be identified.

MATERIALS AND METHODS

Cell cultures. All of the mammalian cell lines and the primary chick fibroblasts (SL-29) were from the American Type Culture Collection (Rockville, Md.) and were grown and

maintained as previously described (30, 43). The chick fibroblast cell strain SL-29 was developed by standard trypsinization of a decapitated 11-day SPAFAS Leghorn embryo. The fibroblast-like population can be propagated through a total of about 35 doublings, according to the American Type Culture Collection. Primary cultures of chicken embryonic breast muscle were also prepared and maintained by previously described methods (43). Under standard conditions, 50 to 70% of the cells in the muscle cultures would fuse. When required, chicken myoblast cultures were treated with 5-bromo-2′-deoxyuridine (BrdU) at a concentration of 5 μg/ml for 3 to 5 days (3, 41).

Cell transfections, CAT, and histochemical β-galactosidase assays. Transient transfections of mammalian and chick cells were carried out by the calcium phosphate precipitation procedure as described before (30, 48). Forty-eight hours after transfection, the mammalian cultures were switched from 10% fetal calf serum to either 2 or 10% horse serum, to trigger differentiation. After a further 48 to 72 h, cell extracts were prepared from 60- or 100-mm-diameter plates for the analysis of chloramphenicol acetyltransferase (CAT) activity, either on thin-layer plates (30, 48) or by using the nonchromatographic procedure (Promega). Assays were normalized to equivalent amounts of protein. Transfection efficiencies were determined by cotransfection with either a β-galactosidase or a luciferase expression plasmid (Promega). Conversion values represent four to seven independent assays. The 1.2-kb *Nco*I fragment (+174 to −955) and the 322-bp *Pst*I-*Sma*I fragment (−327 to −5) were subcloned into the β-galactosidase expression vector pNASS-β (Clontech) for transfection studies, and the histochemical β-galactosidase staining of the cultures was carried out as described previously (4).

Isolation and mapping of genomic clones. A chicken genomic library in lambda EMBL3 (Clontech) was screened with the full-length *CMD1* cDNA clone (30), in duplicate, by using standard procedures (32). Filter hybridization was carried out in 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 100 μg of polyriboadenylate per ml, and 100 μg of denatured calf thymus DNA per ml for 36 h at 42°C by using 10^6 cpm of probe per 150-mm-diameter-pore-size nitrocellulose filter. Filters were washed twice in 0.1× SSPE–0.1% SDS at 50°C for 1 h. Positive plaques were carried through three successive rounds of purification. Seven clones were isolated, of which four gave identically strong signals with the 5′ and 3′ probes. These were further characterized and mapped by the partial digestion procedure previously described (50). A single 17-kb *Sal*I fragment contained the entire cDNA, with roughly 8 kb of 5′ sequence and 6.5 kb of 3′ sequence. A further subclone, a 9-kb *Hin*dIII fragment with 5 kb of promoter sequence, was capable of converting 10T1/2 cells to muscle, so this fragment was selected for sequence analysis (data not shown).

Subcloning and sequence analysis. The 9-kb *Hin*dIII fragment was sonicated, and the 300- to 500-bp fragments were cloned into the *Sma*I site of M13. The sequence was determined by the shotgun procedure (59), using the Applied Biosystems model 370A/373A sequencer. Appropriate deletions, based either on convenient restriction sites in the promoter region or on exonuclease III deletions (32), were grown in pKS+ (Stratagene), excised, blunt ended, and then subcloned into blunt-ended *Hin*dIII-*Sal*I-cut p8CAT for transient CAT expression (48). The 3′ end of all the deletions utilizes the *Nco*I site at the translational initiator ATG. All clones were verified by sequence analysis.

Primer extension, S1, and RNase protection analysis. Total RNA was prepared from embryonic chicken muscle and liver as described previously (17). tRNA or embryonic chicken liver RNA was used as a negative control. Primer extension was carried out under previously described conditions (42), with an end-labeled double-stranded 89-bp CMD1 cDNA restriction fragment (*Apa*I-*Nco*I) labeled at the *Nco*I site. A total of 25,000 cpm of ^{32}P-labeled probe was hybridized with 20 μg of total RNA at 50°C and was extended with avian myeloblastosis virus reverse transcriptase. For S1 nuclease protection studies (32), 20 μg of total RNA was hybridized at 60°C with 20,000 cpm of ^{32}P-labeled probe, a 1-kb *Xho*I-*Nco*I genomic fragment labeled at the *Nco*I site. For RNase protection, the 1.2-kb *Nco*I fragment (+174 to −955) was subcloned into pKS+II (Stratagene), and the antisense probe was transcribed from the T7 promoter. The hybridization and digestion were carried out according to the protocol in the RPAII RNase protection kit (Ambion). Digestion, extension, and protection products were analyzed on a 6% sequencing gel along with the G reaction for the S1 probe (32).

Gel mobility shift assays. All of the chicken myogenic factors for MyoD (CMD1), myogenin, Myf5, MRF4, and E12 were prepared in *Escherichia coli*, purified by histidine tag affinity chromatography (Qiagen), and used in band shift assays as previously described (57). MEF-2 protein was prepared by in vitro transcription-translation of the MEF-2 cDNA clone, obtained from Eric Olson (34). Nuclear extracts were prepared from embryonic chicken muscle or cultured cells by previously well-established methods (21, 33). Five to ten micrograms of nuclear extract protein was used in each band shift reaction. Oligonucleotide probes were synthesized on a Milligen 8750 by Juanita Eldridge in our department and were end labeled by fill-in reaction with ^{32}P[dCTP] and Klenow polymerase (32). The complementary oligonucleotide strand is offset enough to allow the fill-in of 1 to 3 added G nucleotides. The sequences for the oligonucleotides used in the assays are as follows: E-1, TCCCTTGCACAGCTGCAGTTAAAT; E-5, GCTGTGAAGGCACGTGTGCCTGTGA; E-7, AGAATTC ACCAGCTGGTCATGGCT; E-9, GCCAATGCACAGCTG TTATACCAT; E-10, GCCTCAGAACAGCTGGGAGATA CA; E-13, GAAATCACACAGCTGAAGTGCTGC; MEF2, CACTGCAGTAAATATAGCACTCAAACTC; SMEF-2, GT ATTCTCCAGTAAAAATACTGCATTGTGC; TATA box at −30, GGGGCTCCGGCATAAATACGGCCC; and NS, TC GGGTCCTGAAGGGGGGCTATAAAAGGGGGTGGGG GCGCGTTCGTCC. The adenovirus MLP TATA box was used as nonspecific competitor oligonucleotide.

Nucleotide sequence accession number. The nucleotide sequence (7,389 bp) of the chicken (*Gallus domesticus*) *CMD1* (*MyoD*) gene has been entered into the GenBank database under accession number L34006.

RESULTS

The chicken *CMD1* gene. In order to begin to define the *cis*-acting promoter elements that regulate the transcription of the chicken *MyoD* gene *CMD1*, we isolated seven genomic clones from an EMBL3 chicken genomic library (Clontech) by screening with the full-length *CMD1* cDNA clone (30). Restriction mapping and sequence analysis revealed that a single 17-kb *Sal*I insert contained the entire *CMD1* gene in approximately 3 kb of DNA that was flanked 5′ and 3′ by 8 and 6.5 kb of sequence, respectively. The transcribed portion of the gene contains two introns (Fig. 1B) and is organized like the mammalian *MyoD* genes (61, 78): exon 1 includes the 5′ untranslated region (UTR) and encodes the basic helix-loop-helix (bHLH) domain (underlined in Fig. 1B), exon 2 encodes

A


TTTTAAATAT ACCACATTTT TATGAAATTG GGGTTGGCTC CTGGAAAGAT TCTTACTTTT ATGTGAAAAC AATGTGGCTT -4112
AATGTGGAAT AATCCAATTC CATATTAAAA AATGGTACTG GCATTTACAC TGTATTCCTT ATTTTACATT ATGAGCATGA -4032
CAGTACTTCC CAAGAGATTT TGCGTACAAT TTGAAATGAC ACCAATAGTT AACTGTGATT GAGAAAGTTG CATGGATGAT -3952
TTTAAAGATG AAAGCAACAG ACCACAGAAT GAAAATAAAT GCTAGAAGAC TGTATTAAGG TGACAAAAAA AAGTACAATG -3872
AAACACTTTC AGTAAATGCT TTGACTCCCT TGCACAGCTG CAGTTAAATA CCCATTTCTG TAAAGCTGTG TAGACCACTT -3792
E-1
CATTTCTGTT AGTGTTGCAA CACAAGACAA ATGATTACAG CACTGATGTT TCCCCATCTT TCTAGTACAG TGACTCAACC -3712
E-2 AP1
TTTTTTTTTC TTTTAGTTGG GCAAGTTACT TTAAAACATC TCCTGTATTC TCCAGTAAAA ATACTGCATT GTGCCACAAG -3632
sMEF2
AACAGTTTGA AATGCTTTTA GCAACATGCT GGATTTGTCT GTTTGGGATG GCCGCTCTCA AATACAGCAG TAGTTTAACA -3552
GATATTTCCA TCTGGGCTGG ATTCCAACAC CCACCCTCAC ACTGAGCATA CCTTATTCAA CAATTAGTTC CACTGACTGC -3472
E-3
AACTCAATAT GAATAAGAGC AATGGAATCC ATTCTTCTCA GTATTAATAC ATAGCTCACT AAGTATGAAT GGAGCAAAAC -3392
TGCTAGTTGA CTTATAAACT AGTGCCATAT AGCTAGTACA GGCAACATTT GGTATACAGC AGCCTCCTTA AGAGTTGTTA -3312
E-4
ACCAACAATA CAGGCATTGG TTTCAAGCAC TGAAGACCTT AAATGAATTC CAACAGAACA AGGAGCCCAA CATCTAAACT -3232
TTTCAATGTG AGACTTACTT GCTGCTGCTT TTTCCGTGAA ATGTTGCTCT ATGTGCTTTT CAGAGACTCT TCTCATCTTT -3152
GTAGCCAAAC ATGCCAGTAC TTTTTCAGAT TTTAGGCATT TCTCTCTCTT TCTTTCTTCC ACAATACCTT CTCGGACTCT -3072
GCCTGAAGAG ACAGTTCTAT TAATTCTAGG TAATCTCACA GTCACCGTTT TCCCAGTCTA TCCTCTGAAA AAGAAAAAAA -2992
TCAGTAACTG TAGATAACCA AACCTTTTCT TGTCTATGTG ATGCTTGTGC TGTGAGGCAC GTGTGCCTGT GATCTCTTTG -2912
E-5
TCAGCAGTAC CCGTCTGTTG TGTACAGGAT GATCCTCTCC ATCTTCGCAG CGCAGGAAGT ATAACCTGCC ACTCTCTTCT -2832
CAGCTTCATT TCAACCACAC CCTGAGGAAA TGAGATGAAG AATCAGTGTG AAATAGGTAT AAAGTCAACA GCATTTAAAG -2752
CAACTGTACA GATACTCTTC CTCTGCTGAA TGCTCGGTCG TACATGCACT CTGTATCTGT TCTTGCAAAG CAAAGCCCAA -2672
E-6
GTAATCCAAA ATAGCCACAG AAACATCACT GCAGTAAATA TAGCACTCAA ACTGGAAGCT CTGAAGAGAC CTATTCTTTA -2592
MEF-2
CTTAAAATTA TGAGTTACAA CAACAGAGGC TTCCAAACAG GAATTAAAGA TGGTGTATTT CTAAACGCTG CCATAAGAAA -2512
TTCCTGTGGT TGTAGGGAAA GGGAGCTTGT CTGCAATCAG AGTGAGGAAT TCACTTCAAG TCTCTTGGTG GAGATCATGC -2432
CATATCCTTC TTCTCCTCCA CTCTGGCCGC ACAACATTGA GAAGAGCATC TCAGAATACT GTTCTTTGCA AAGACTTGTT -2352
TCTGGCCCAC AACATCTTTT TGGACACAAC TATCTTCGGG CAAAATACTA GAAAGATAAT TTGGGTTTTG GTGCCAGCAA -2272
CAGGGAACAT ATCCTTGCAG AATCAGAGGC CAGAAGGGAC CTCTGGAGAT GATCTAGTCT AACTCCCCGC TAAAGCAGTT -2192
TGAAGCTGCT CAAGGGCTGC TTCACTGCAA GACAATTCTG AGCAGAATTC ACCAGCTGGT CATGGCTTTC TCCTGACTTT -2112
E-7
TCCACTTAGG TGAGCTCATG CACTTACGAG CATTCATAGG CAATTACTGG CAGTCTCTCA GTATTGCCTG TCCAAGTCTC -2032
ACCATCTGTT ACCGCTCTGC CCAAATTAAC CACTGCGCAT GCCAGTCCTC AAATGCCTTT TCCTGGGGTC AGACACAACC -1952
E-8
TAGATGCACA CTCACAGATT ATGCTGTAGC TTTAGGTAAA TACCAGCAAA ATTAGCGTTT CCACAAATTA AACCAATGAC -1872
CTGAGGAAAG TTTCTGTGTT TTGAGTGGGT CAGCAAGGCC AGCACCAATG CCTGACACCC AATGCACAGC TGTTATACCA -1792
E-9
TACAATTTTT TCTCACTTAT ACTTGTTTTG TCTTACTGTA TACAAAACAT GGTTTTGAAT ACTAATTATT ATTACTTTAG -1712
CTGTTCCGTA AAGGTTAAAA CAAACATTCT GTTCTGTCCT TCCGGATCAA AGAGCCTCTA CAGTAACAGT GGAGTGTTTC -1632
CATGAGCTTG CTGCTCTGCC TCAGAACAGC TGGGAGATAC ACTTCTGCAT GTGTAGGGCT AATCAATAGC TGCTCTCCTT -1552
E-10
GGGGTGGATC GTTTTTCAGC ACTGGGTACA GTGAAAGAAC AAACTTGTAC AGAGTCCAAA ACATCTACTT AAAAAAAAAA -1472
ATAAATTGAT CTTCGGGGTA ATGACAGAAT GTGACTAACA ATTCTTATCT TTGGACTTAA AAACACTTAA GAAAGTAGAA -1392
AP1
AACGAGGGCT GCAGACTGGG AATTTTTTGC AAAAATGGAA TGATAGAACA ACAGAAGACC CCACCATGGT GACAGCCCAT -1312
E-
CTGAGCTGCC ACAGGCGTGG GGACCGTGGA CCCGACCGCC AACTGACGTT CTGTCACCTT CCGAGATGCT CTCAGCTTTG -1232
11 E-12
CTGCAGGGGG AGCTTTGCCT TTCATTTTAA AGGAGTCAGA ATGCCACCAG CACGTATAAC TGCAGAACGG AGGTTAATTT -1152
TAGCCCACGG TAAATTAAAA GTATCCATAA CTACCTGAAT CATAAAGTTA ATGAAATCAC ACAGCTGAAG TGCTGCAGTA -1072
E-13
CTTCTGTATC AGAGAAAGAA CCATTCTTCC CAGCTTAAGT GATAGTCCAA ACCCAGACTC CGGCAGCCTT GAGAGACATT - 992
NcoI
ACCAAAGCCA TCCTGCTCTT CCACATGCCC AACTCTGCCA TGGCTATCTG CCCTAAGTAA CACACTCTTA TTGCACAAGC -912
E-14 E
AATTGGACTG CAGTGCGGTT ACGTGTGCCG CACAATGCTA CTCATGAGGC AGCGTCGTCT CTTCAAAGGC TTTAGAATTA - 832
-15
AGGTAAACTG AGGCTCGAGT TCGAAGAAGA ACCTGAAACG CAAATGTATA CATCTGAGCC GACGTGCAAA GAGTCACTTA - 752
E-16 E-17
CTGTAACTCA GGTATGCCAC TGCAGAGCCA CGGCTGCCCG CAGAGCTCCC GCCGGCTACT CGGCCAGGCC TCGCCCTCCC - 672
GCTCATCCCC GTGCTTCAGG CCAAGCTCCG GCAGCCGTGC CCGAGCCCCG CCCGACACCT CGCACTCCGC GGCACGACTC - 592
AP2 SmaI
GCCGATGCGG GCACCGGCCC CAGCCGACAG CCCGTCCTGC GGGCCTCCGC CGGGGTTCTA CAGAACAGCT CCCGGGCGGC - 512
GC-box
GTTACGCGCC CGTCTGCCGG CAGAAGGCAC GCGGCGGCAG TCCCGGCACG TCGCTGCGGG GCGAAGGACG CAGTCCGCGA - 432
GCCGCCGACG GGCCCGGAGC CTTTTCTCGC AGCTCCGACG GGGCGGTTGC TCTCGTTCCC TCCGCTTCCC CGAGCTCCGC - 352
PstI GC-box ExoIII
CGAGCCGCGG GCCGCTACCC CCCCCTGCAG CGGCCGCGCC TCCGCAGCCT CGACCCCATC GCTGCCCTCG CCCCGCCGCC -272
AP2 ExoIII
CCGGCTCGTC GCGCTGCCCC CCGGCTCCCG GCTCCGTCGC GGACGCCCCC GCGGTAACCG TGCGGCCGCG GCCGTCCCGC -192
KpnI
CGGGCGAGGC GCGGAGCTCC GGTACCCGCG GCCGCTCGGC CCTTCCCCCG CCGCCGCGCG CAGCTCCCCA TCCCCCCCGC -112
SacII
AGGGGGCGAG GCTCCGGGGG CTCCGTCAGC CGCGGCCCCC TCCCCGCCCC GCCGCGCCCC GCCGGCCCGC CGGGGGCTCC - 32
SmaI AP2 AP2
GGCATAAATA CGGCCCCGAG CGGCGCTCCC G -1
TATA-box

B

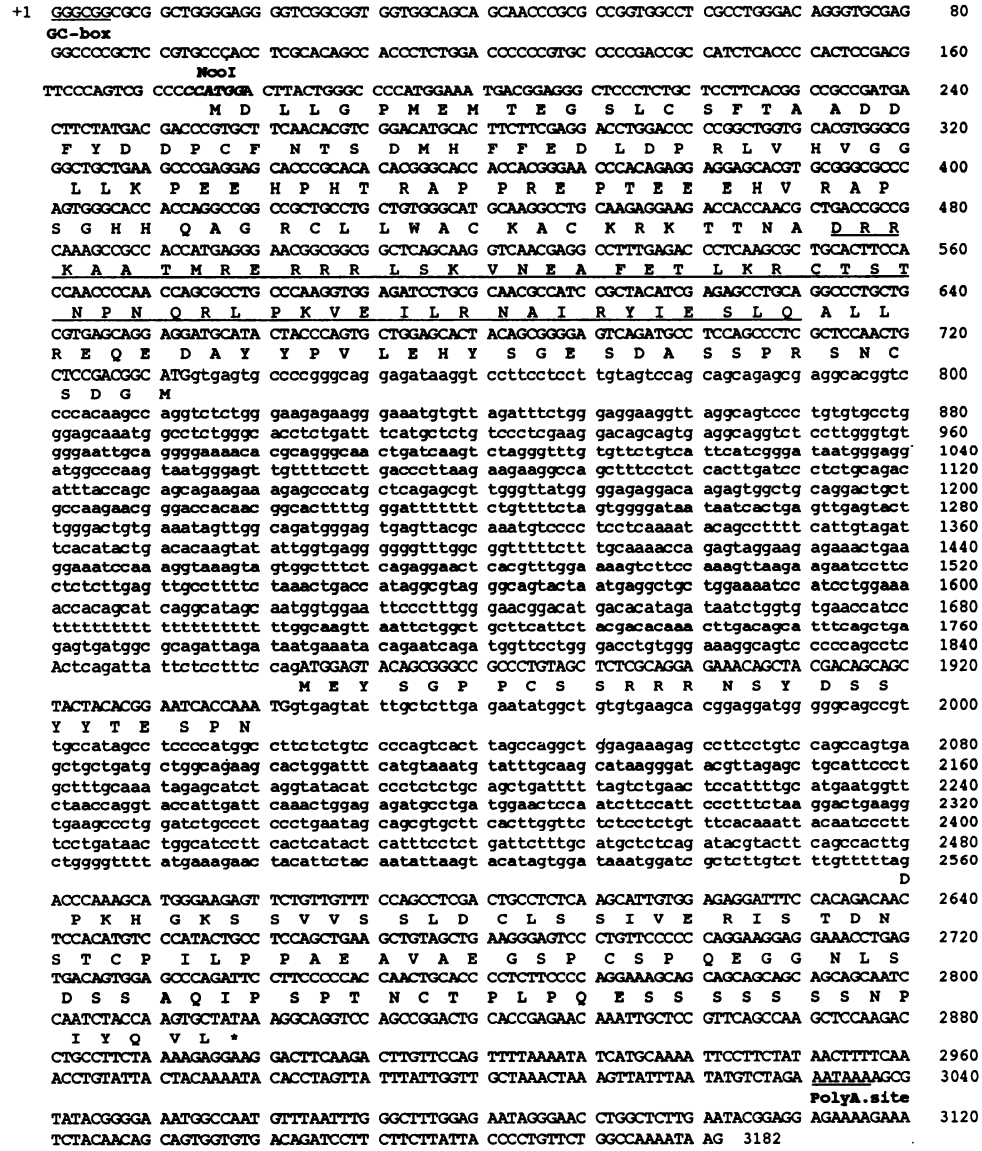

FIG. 1. Structure and nucleotide sequence of the chicken *CMD1* (*MyoD*) gene. (A) The nucleotide sequence of the promoter region of the gene: E boxes are labeled E-1 to E-17; MEF-2 sites are labeled sMEF-2 (a degenerate site with no function) and MEF-2; restriction sites or "Exo III" mark positions of the promoter deletions; AP1, AP2, and GC boxes are according to standard nomenclature (78); and ATAAATA is assumed to be the TATA-like element. The +1 position marks the transcriptional start as determined by the primer extension and S1 protection experiments described in the text. (B) Nucleotide sequence of the *CMD1* transcription unit. The introns are shown in lowercase letters and all have the consensus GT/AG ends. The bHLH domain is underlined, and a possible poly(A) addition site is indicated. The cDNA clone includes this poly(A) site, suggesting that additional sites are present in the gene.

a short stretch of 26 amino acids, and exon 3 contains the termination codon, the 3′ UTR, and a polyadenylation signal. The previously published *CMD1* cDNA sequence (30) includes this putative polyadenylation signal 258 bp from the 3′ end of the clone, suggesting that there are additional adenylation sites in the gene beyond the one shown for the genomic sequence in Fig. 1. We previously reported a similar result for the chicken vimentin gene, in which four different adenylation sites are used randomly during myogenesis (77).

Approximately 4 kb of sequence 5′ to the transcriptional start site (see below) is shown in Fig. 1A. Numerous sequence elements known to be important for muscle-specific gene expression are noted, including 17 E boxes (CANNTG) (19, 26, 45, 55, 72), two GC boxes (55), a MEF-2 consensus, and a MEF-2 related site (22). In addition to an A+T-rich TATA-like motif at −30, AP1 and AP2 sequence elements are also present in the promoter region. Elements resembling a CArG motif, $CC(A+T)_6GG$, known to play an important role in

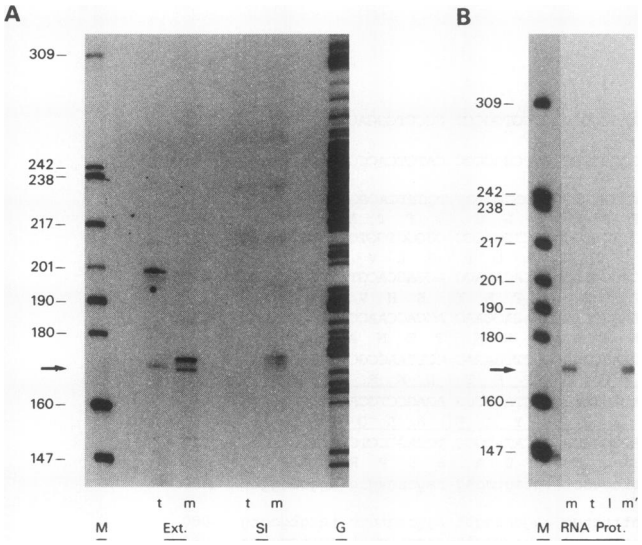

FIG. 2. Mapping the transcription initiation site in the *CMD1* gene. (A) Embryonic chicken muscle total RNA was analyzed by primer extension (Ext) and S1 nuclease protection (S1). tRNA (t) was used as a negative control. The sizes of the products were determined by using a labeled *Msp*I digest of pBR322 and the G-lane sequence reaction for the S1 probe, sequenced by the Maxam-Gilbert procedure (32). The arrow shows that the position of the extended product and the S1-resistant fragments which map to 177 bp 5′ of the initiator ATG. A minor cap site was repeatedly detected by primer extension 4 bp 3′ to the major start site. (B) Nuclease protection assay. m, 30 μg of total embryonic chicken muscle RNA; t, 30 μg of tRNA control; l, 30 μg of total embryonic chicken liver RNA; m, 2 μg of poly(A) RNA from embryonic chicken muscle. Note the protected or elongated band in the same position in all three assays, marking the transcription initiation site approximately 177 bp 5′ of the ATG.

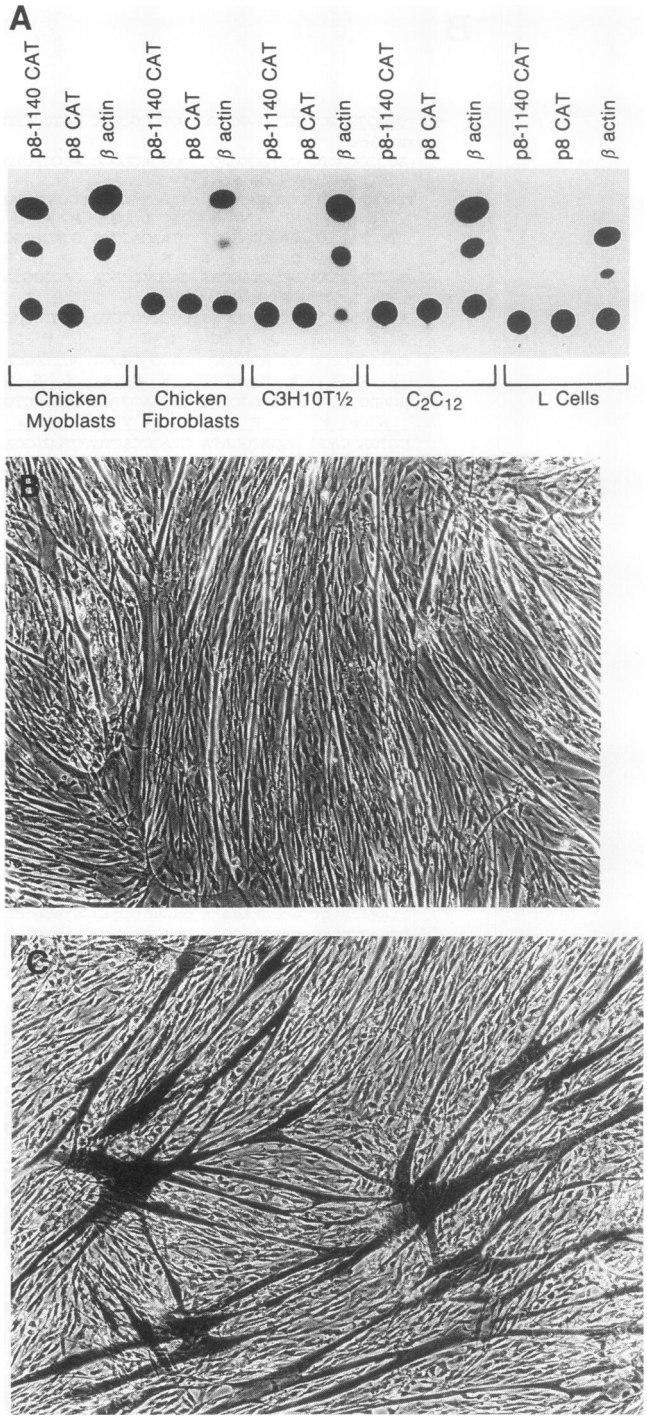

FIG. 3. Specificity of the *CMD1* promoter in various cell backgrounds. (A) An *Nco*I promoter fragment starting at −963 bp was tested in transient CAT expression assays in cultures of primary chick myoblasts and fibroblasts and in 10T1/2, C2C12, and L cell lines. In each transfection, promoter activity was compared with those of the empty p8CAT vector and of the chicken β-actin promoter p8CAT plasmid (49), which is constitutively expressed in different cell backgrounds. CAT activity in cell extracts from 60-mm-diameter plates 48 h after transfection was determined by using aliquots of cell extract containing 20 μg of protein. Similar results were obtained in four independent transfections. (B and C) The same 1.2-kb *Nco*I promoter fragment confers muscle-specific expression of β-galactosidase activity. (B) Vector alone. (C) 1.2-kb *Nco*I promoter fragment.

muscle-specific actin gene expression (36), or M-CAT elements, important for cardiac troponin-T expression (33), were not found.

The transcription initiation site for the *CMD1* gene was identified by S1 nuclease protection, primer extension, and RNase protection. A convenient *Nco*I site at the initiator ATG provided a common end for all the probes so that the sizes of the primer extended product and the S1- and RNase-protected fragments would be the same. As shown in Fig. 2A, a comparison of the sizes of the primer-extended products and the S1 nuclease-protected fragments, using the size markers and the sequence ladder, indicates that the *CMD1* transcripts initiate predominantly from a position 177 nucleotides 5′ to the initiator ATG. A minor band in the primer extension reaction also suggests a secondary start site 4 nucleotides 3′ to the main site. This result was independently confirmed by an RNase protection assay with embryonic chicken muscle RNA using liver RNA as a negative control for tissue specificity (Fig. 2B). The position of the major start site places initiation approximately 30 bp 3′ to the putative TATA element ATAAATA, at −30 in the promoter region (Fig. 1A).

The *CMD1* promoter confers muscle-specific expression in primary chick muscle cultures. In order to begin to define important *cis*- regulatory elements within the *CMD1* promoter, two promoter fragments, one extending to −8 kb and the other to −1140 bp, were tested for muscle-specific activity in primary chick muscle cultures, fibroblasts, and a variety of muscle and nonmuscle cell lines. As shown in Fig. 3A, when the 1,140-bp *Nco*I fragment, from −963 to the initiator ATG at +177, was

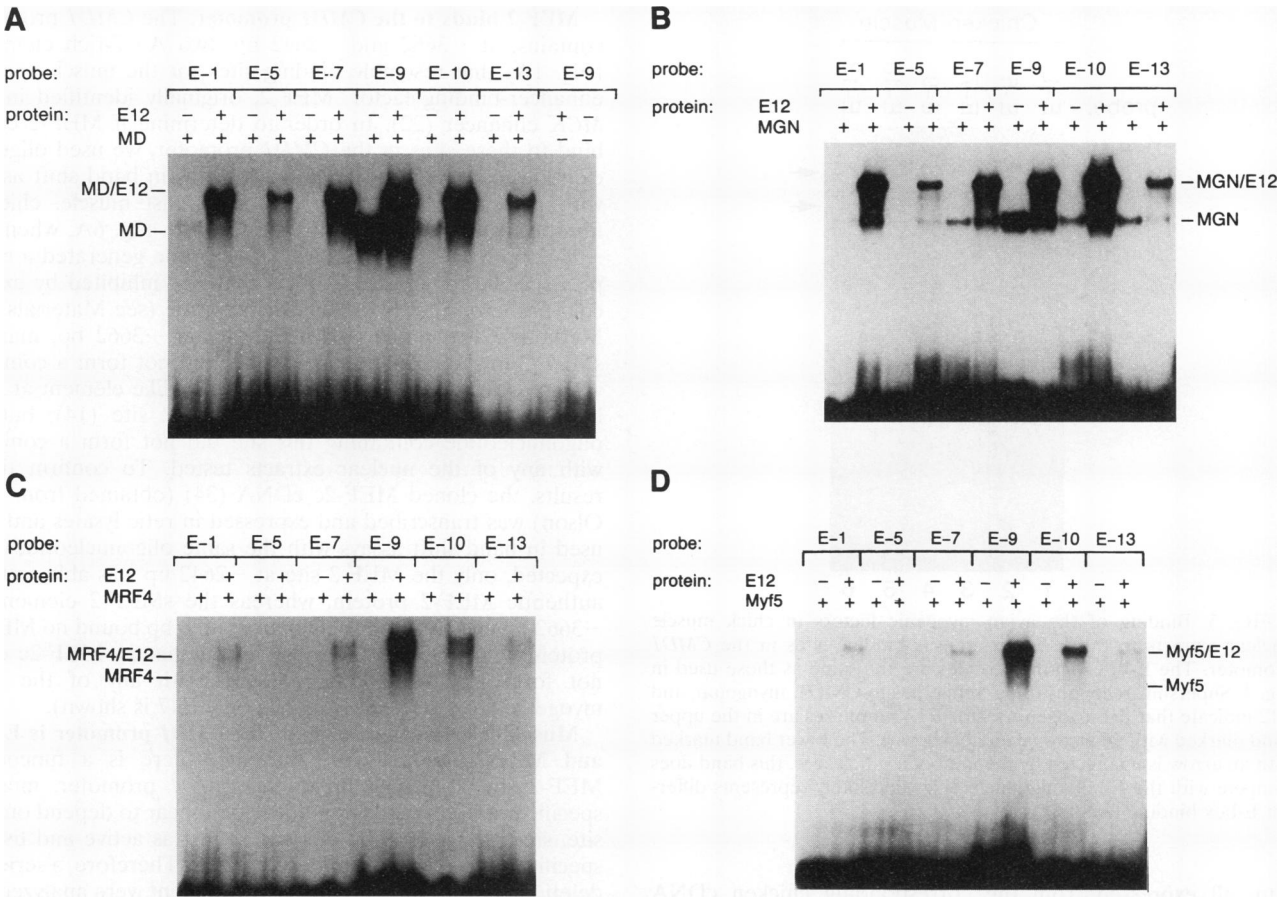

FIG. 4. Gel mobility shift assays using the myogenic factor E-box consensus binding sites in the *CMD1* promoter. Twenty-four-base-pair probes encompassing E boxes E-1, E-5, E-7, E-9, E-10, and E-13 were used in gel shift assays with proteins produced in *E. coli* for all the avian myogenic factors and avian E12. One hundred nanograms of each bacterially expressed protein was used per assay. The position of the myogenic factor homodimer is marked by MD (MyoD), MGN (myogenin), MRF4, and Myf5. The corresponding heterodimer, the upper band, is indicated by (factor) E12. The binding affinities for the indicated E boxes are shown for CMD1 (A), myogenin (B), MRF4 (C), and Myf5 (D). A 50-fold molar excess of unlabeled oligonucleotide completely inhibited binding, whereas oligonucleotides without an E-box motif did not compete in the binding assay (data not shown). The sequences for each oligonucleotide, derived from the sequence in Fig. 1, are described in Materials and Methods.

used, high levels of CAT activity comparable to the β-actin promoter CAT control plasmid were seen only in primary chick myoblast cultures. Similar results were obtained for the 8-kb promoter fragment (data not shown). No activity was seen in chick fibroblasts or in 10T1/2 cells, C2C12 cells, or L cells grown in 10% fetal calf serum and then switched to either 2 or 10% horse serum. The β-actin promoter, however, showed comparable levels of activity in all of these cell backgrounds (Fig. 3A). Surprisingly, a 7-kb fragment from the mouse *MyoD* promoter region was also active only in chick primary muscle cultures and failed to demonstrate reliable activity above background in numerous commonly used muscle cell lines, including mouse C2C12, F3, and BC3H1 cells, rat L6 cells, and human RD rhabdomyosarcoma cells (61). Our results may also be due to the fact that we are using primary cell cultures and not heterologous lines to analyze the regulation of the *CMD1* gene in its natural cell background. Muscle specificity for this 1,140-bp *CMD1* promoter fragment was also confirmed by using a *lacZ* reporter construct to demonstrate myofiber-specific β-galactosidase staining in the transfected myoblast cultures (Fig. 3B). Secondary cultures prepared from these primaries also demonstrated β-galactosidase expression in numerous single cells, suggesting that this promoter fragment is active in myoblasts as well (data not shown). However, experiments to demonstrate myoblast-specific expression are complicated by the fact that expression can be studied only in transient transfections where early single-cell expression is difficult to detect because of the rapid differentiation kinetics of chick primary muscle cultures and the extended period in culture required to detect β-galactosidase activity (43). β-Galactosidase activity was never seen in fibroblast cultures.

CMD1 and the other HLH myogenic factors bind to multiple E-box sites in the *CMD1* promoter. The 4-kb *CMD1* promoter region shown in Fig. 1A contains 17 E boxes that could potentially serve as binding sites for the different myogenic bHLH proteins and play a role in the muscle-specific activation of the *CMD1* promoter. In order to test this possibility and to determine if a subset of these E boxes could bind the myogenic factors, we tested numerous 24-bp oligonucleotide E-box probes in gel mobility shift assays. E boxes were selected with reasonable consensus binding site preferences for MyoD and myogenin homodimers or MyoD/E12-E47 heterodimers (5, 73). Binding assays were performed by using *E. coli*-produced CMD1, myogenin, MRF4, Myf5, and E12 pro-

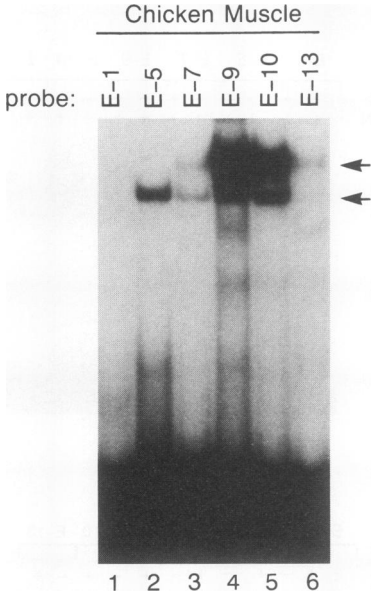

FIG. 5. Binding of the avian myogenic factors in chick muscle nuclear extracts to the E-box consensus binding sites in the *CMD1* promoter. The E-box oligonucleotides are the same as those used in Fig. 4. Supershift reactions using antibodies to CMD1, myogenin, and E12 indicate that the myogenic factor-E12 complexes are in the upper band marked with an arrow (data not shown). The lower band marked with an arrow is unaffected by the antibodies; however, this band does compete with the E-box oligonucleotides and likely represents different E-box binding factors.

tein, all expressed from the corresponding chicken cDNA clones, as previously described (57). As shown in Fig. 4A, the E boxes 1, 5, 7, 9, 10, and 13 (marked in Fig. 1A) all efficiently bind the CMD1/E12 heterodimer, especially boxes 7, 9, and 10, whereas box 9 binds the CMD1 homodimer very efficiently compared with the other E-box elements. Similar results are seen for the myogenin homodimer and the myogenin/E12 heterodimer (Fig. 4B). By contrast, MRF4 (Fig. 4C) and Myf5 (Fig. 4D) show a preference for heterodimer binding to E boxes 9 and 10, and there is essentially very little homodimer binding at these comparable protein concentrations and exposures. Weak homodimer binding and heterodimer binding to the other sites is seen with longer exposures (data not shown) or with a different E-box consensus (57). The sequence for E box 9 is a 9-of-10 match for the consensus sequence for DNA binding by the MyoD and myogenin homodimers, but none of the selected sites show a strong consensus for the E12-MyoD heterodimer, as determined by previous PCR-mediated binding site selection studies (5, 73).

Nuclear extracts from embryonic chicken breast muscle also contain E-box binding activity and, as shown in Fig. 5, E boxes 9 and 10 form a complex with these extracts. The complex is inhibited by excess homologous oligonucleotide but is unaffected by competition with nonspecific oligonucleotide (data not shown). Furthermore, the upper band, marked with an arrow, is partially depleted by antibodies to CMD1 (MyoD), and myogenin and is completely shifted by antibodies to chicken E12 (data not shown). The lower band appears to be nonspecific in the supershift assay in that it does not react with antibody; however, it competes with specific oligonucleotide. This complex likely represents an additional E-box-recognizing protein(s) in the muscle nuclear extracts unrelated to CMD1, myogenin, or E12.

MEF-2 binds to the *CMD1* promoter. The *CMD1* promoter contains, at −3662 and −2642 bp, two A+T-rich elements (Fig. 1A) that resemble binding sites for the muscle-specific enhancer-binding factor, MEF-2, originally identified in the *MCK* enhancer (22). In order to determine if MEF-2 could bind to these sites in the *CMD1* promoter, we used oligonucleotides containing these potential sites in band shift assays with nuclear extracts from chicken breast muscle, chicken fibroblasts, and 10T1/2 cells. As shown in Fig. 6A, when the −2642-bp MEF-2 site was used, this probe generated a complex specific for muscle extracts that was inhibited by excess cold probe but not by a nonspecific probe (see Materials and Methods). The potential MEF-2 site at −3662 bp, marked sMEF-2 in the sequence in Fig. 1A, did not form a complex with muscle nuclear extracts. The TATA-like element at −30 bp also resembles a degenerate MEF-2 site (14), but an oligonucleotide containing this site did not form a complex with any of the nuclear extracts tested. To confirm these results, the cloned MEF-2c cDNA (34) (obtained from Eric Olson) was transcribed and expressed in retic lysates and was used in band shift assays with the same oligonucleotides. As expected, only the MEF-2 site at −2642 bp was able to bind authentic MEF-2 protein, whereas the sMEF-2 element at −3662 bp and the TATA element at −30 bp bound no MEF-2 protein (Fig. 6B). Furthermore, in our hands, MEF-2c does not form a DNA-binding complex with any of the four myogenic factors tested (Fig. 6B; only E47 is shown).

Muscle-specific expression of the *CMD1* promoter is E box and MEF-2 independent. Although there is a functional MEF-2 site at −2642 bp in the *CMD1* promoter, muscle-specific promoter activation does not appear to depend on this site, since the −1140-bp deletion is just as active and tissue-specific as the −8-kb promoter fragment. Therefore, a series of deletions within the 1,140-bp *Nco*I fragment were analyzed for the ability to direct either muscle-specific CAT or β-galactosidase activity (Fig. 7A and B). Because of the strong CAT activity of these various deletions, only 1/20 of the normal volume of cell extract used in the assays shown in Fig. 3A was tested here, in order to keep the assay in the linear range. Under these conditions, moderate differences in the deletions were apparent. Deletions from −963 up to −322 bp increased eightfold in CAT activity and then progressively decreased in similar fashion as deletions continued, up to −165 bp (Fig. 7A). These variations in CAT activity suggest the removal of various positive and negative elements in the promoter. We also observed an increase in expression with deletions between −165 and −77 bp. However, unlike the longer deletions, this activity was also relatively high in fibroblast cultures (see below, Table 1). None of these constructs demonstrated measurable activity in myoblast cultures grown in the thymidine analog BrdU (Fig. 7B), an inhibitor of *CMD1* expression in myoblasts (30, 60), a reversible inhibitor of myogenesis in chick primary muscle cultures (3, 41), and an inhibitor of transcription from muscle-specific promoters (2). None of the deletions down to −165 bp were active in any muscle or nonmuscle cell lines tested; however, the −77-bp deletion was active in 10T1/2 cells (data not shown). Taken together, these results indicate that neither the MEF-2 site nor the E-box elements in the proximal promoter are essential for muscle-specific transcriptional activity, since the −322-bp deletion is fully active in primary myoblast cultures, yet has none of these binding sites. Histochemical staining of cultures transfected with a *lacZ* expression plasmid under the control of the −322 *CMD1* promoter fragment also confirmed the muscle-restricted nature of this promoter element (Fig. 7C).

CMD1 autoactivates its own promoter in the absence of

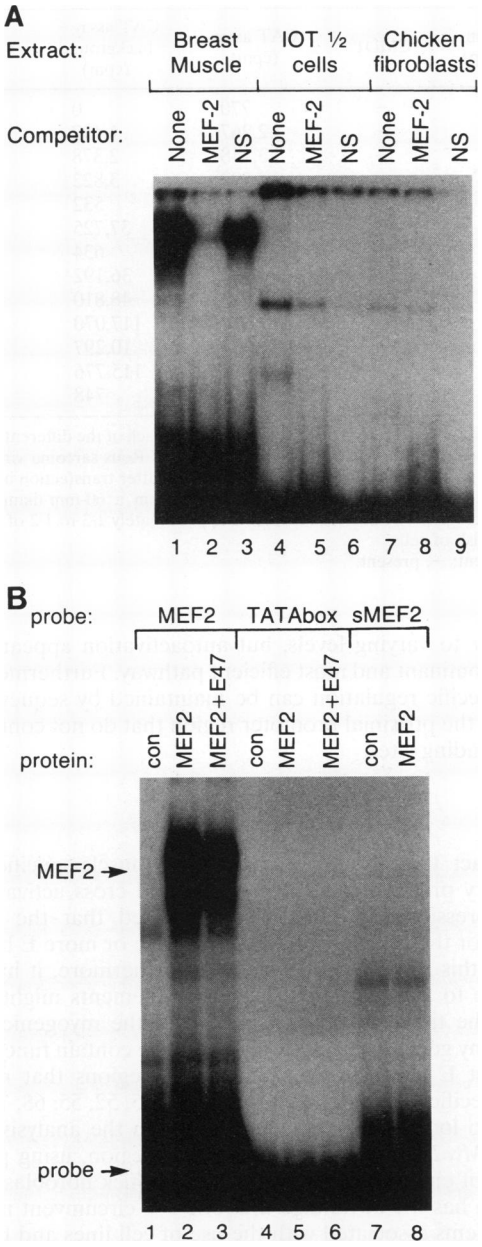

FIG. 6. Binding of MEF-2 to the *CMD1* promoter. (A) Binding to the CMD1 MEF-2 site is tissue specific. When nuclear extracts from chick breast muscle were used, chick fibroblasts, and 10T1/2 cells were used, only muscle extracts formed a complex with the CMD1 MEF-2 site at −2642 bp; this was inhibited with cold MEF-2 oligonucleotide but not with nonspecific oligonucleotide. (B) In vitro-transcribed-translated MEF-2c protein was used in gel shift assays with the three A+T-rich elements in the *CMD1* promoter at −3662 bp (sMEF-2), −2642 bp (MEF-2), and −30 bp (TATA-like). Only the MEF-2 site at −2642 bp has binding activity for authentic MEF-2c protein, and this binding activity is not influenced by the E protein E47 or any of the other myogenic factors (data not shown) (100 ng of *E. coli*-produced avian E47). The TATA-like element at −30 bp and the sMEF-2 site at −3662 bp do not bind MEF-2 protein in this assay.

E-box elements. From the transfection experiments it was clear that the *CMD1* promoter was very active in primary chicken myoblast cultures but was inactive in primary chicken fibroblasts (Fig. 3A). To begin to determine if CMD1 protein expressed in *trans* could autoactivate the *CMD1* promoter, we cotransfected primary chick fibroblast cultures with a Rous sarcoma virus CMD1 expression plasmid and the various promoter deletions in the CAT expression vector tested earlier, extending from −8 kb down to +177 bp, to see which fragments, if any, could respond to *CMD1* in *trans*. As shown in Table 1, when the promoter deletion fragments down to −165 bp were used, CAT activity was activated approximately 20- to 30-fold by CMD1 expression in *trans*. Similar activation experiments carried out with 10T1/2 cells gave no detectable activity, even though CMD1 expression can convert 10T1/2 cells to muscle (30). The −77-bp deletion showed relatively high activity levels in fibroblasts in the absence of exogenous CMD1 protein and was only slightly activated by CMD1 in *trans*, suggesting the removal of a tissue-specific negative element with this deletion. However, there are no obvious muscle-specific factor binding motifs in this G+C-rich sequence element between −165 and −77 bp (Fig. 1A). From the results with these deletions it is concluded that regulatory sequences in the proximal *CMD1* promoter from −322 bp through an unknown point between −165 and −77 bp confer high levels of muscle-specific expression which can be positively autoregulated in fibroblasts by CMD1 protein in *trans* in the absence of any E boxes or MEF-2 sites.

Cross activation of the *CMD1* promoter by myogenin, Myf5, and MRF4. We next determined if the *CMD1* promoter could be cross activated by the other members of the MyoD family of gene-regulatory proteins. As mentioned earlier, we have isolated the chicken homologs for these other factors and all, including CMD1, were initially cloned into the murine sarcoma virus (EMSV) expression vector to test 10T1/2 cell conversion, as previously described (16). However, the EMSV long terminal repeat is not as strong a promoter as the Rous sarcoma virus long terminal repeat used in the initial experiments to express CMD1 (Table 1), so expression levels for these various factors are reduced somewhat in the experiments to be discussed concerning cross activation. All these expression constructs were able to convert 10T1/2 cells to muscle, albeit at different efficiencies, in agreement with previous results obtained by using the mammalian factors (76): in our studies, on a scale of 1 to 10, MRF4 was the most efficient (10), CMD1 and myogenin were very similar (5), and Myf5 was the least efficient (1) in converting 10T1/2 cells to muscle (data not shown). Using the −8-kb promoter CAT construct p8-8000CAT, in cotransfections with primary chick fibroblasts and EMSV expression plasmids for each of the myogenic factors, CMD1 was the most effective activator (Table 2) (greater than 10-fold), whereas myogenin and MRF4 showed only a four- and a twofold activation, respectively, and Myf5 was able to transactivate reproducibly the *CMD1* promoter slightly above background levels (50%). Similar cross-activation results were obtained with the −322-bp promoter deletion by using CMD1 and myogenin; however, Myf5 (again) and MRF4 were able to activate transcription reproducibly only just above background levels (50%) (data not shown). It should be noted that this activation is not a secondary effect due simply to myogenic conversion, since MRF4, which is the most efficient avian myogenic conversion factor, is a weak cross activator of the *CMD1* promoter (Table 2), compared with the autoactivation by CMD1. From these experiments it can be concluded that both auto- and cross-regulatory pathways involving the MyoD family of bHLH proteins are capable of activating the *CMD1*

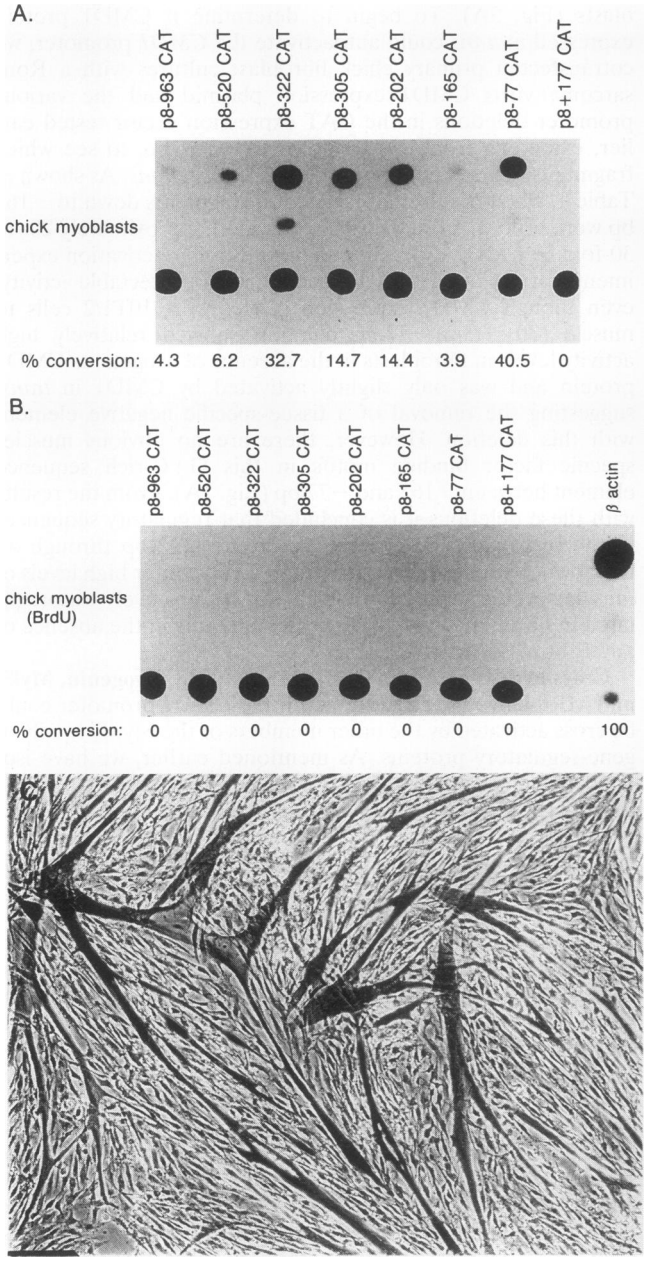

FIG. 7. Muscle-specific expression of various *CMD1* promoter deletions. Promoter deletions, named according to the 5′ end-point of the deletion, were transfected into primary chick myoblast cultures (A) and into myoblast cultures grown in the presence of 5 μg of BrdU per ml (B). Cellular extracts were diluted 20-fold, by comparison with those extracts shown in Fig. 3, in order to keep the assay within the linear range. The β-actin promoter CAT plasmid was used as a control in the BrdU-treated cultures, since promoter activity is unaffected by the analog (2, 49). The percentage conversion is given for each deletion. Comparable results were obtained in four independent experiments. Primary cultures of differentiated chick myoblasts transfected with the −322 *lacZ* reporter construct were stained for β-galactosidase activity to show the well-stained myotubes (C). Fibroblasts in these cultures were never observed as β-galactosidase positive.

TABLE 1. Cotransfection of chick fibroblasts with the different *CMD1* promoter deletions and a CMD1 expression plasmid autoactivates the *CMD1* promoter[a]

Deletion in p8CAT (bp)	CMD1[b]	CAT assay (cpm)	CAT assay − background (cpm)	Fold activation
None	−	770	0	0
−8000	−	2,267	1,497	1
−963	−	3,148	2,378	1
−322	−	4,592	3,822	1
−165	−	1,302	532	1
−77	−	38,495	37,725	1
+177	−	1,404	634	1
−8000	+	36,962	36,192	24.2
−963	+	49,580	48,810	20.1
−322	+	117,840	117,070	30.6
−165	+	11,067	10,297	19.4
−77	+	116,546	115,776	4.4
+177	+	1,518	748	1.2

[a] Chick fibroblasts were transfected with 2 μg of each of the different promoter fragment p8CAT plasmids with or without 2 μg of Rous sarcoma virus CMD1 expression plasmid. CAT activity was assayed 48 h after transfection by using 20 μg of protein from the cell extract prepared from a 60-mm-diameter dish. Twenty micrograms of protein represents approximately 1/3 to 1/2 of a 60-mm-diameter dish of cells.

[b] −, absent; +, present.

promoter to varying levels, but autoactivation appears to be the predominant and most efficient pathway. Furthermore, this tissue-specific regulation can be maintained by sequence elements in the proximal promoter region that do not contain any E-box binding sites.

DISCUSSION

The fact that the MyoD family of muscle-specific gene-regulatory proteins can autoregulate and cross activate their own expression has tentatively suggested that the control regions for these genes would contain one or more E boxes to mediate this regulatory interaction. Furthermore, it has been attractive to assume that these E-box elements might play a role in the tissue-specific expression of the myogenic genes, since many genes expressed only in muscle contain functionally important E boxes in their regulatory regions that mediate tissue-specific expression (11, 19, 26, 29, 45, 52, 55, 68, 72). We set out to look at these assumptions with the analysis of the chicken *MyoD* (*CMD1*) gene promoter region, using primary cultures of embryonic chick muscle and chick fibroblasts. This approach has the advantage that one can circumvent many of the problems associated with the use of cell lines and heterologous cell backgrounds (61), so that promoter regulation can be studied under conditions that more closely resemble the in vivo situation.

The minimal *CMD1* muscle-specific promoter has no E boxes. In primary chick cell cultures, when the *CMD1* promoter fragments from −8 kb to −165 bp were used, tissue-specific expression was maintained in transient assays. Activity was never seen in fibroblasts or in various muscle and non-muscle cell lines including C2C12, L6, L8, BC3H1, 10T1/2, and 3T3, and L cells (Fig. 3A). This is in contrast to the human *MyoD* enhancer/promoter that was very active in several nonmyogenic heterologous cell lines (20). The most active *CMD1* promoter fragment extends to −322 bp and contains no E-box elements or MEF-2 sites. Although the −77-bp deletion is inhibited by BrdU, it is active in fibroblasts (Table 1) so the −165- to −77-bp deletion appears to have removed a tissue-

TABLE 2. Transactivation of p8-8000CAT, the −8-kb *CMD1* promoter fragment, in chick fibroblasts, by the various myogenic factors[a]

Transactivator	CAT assay (cpm)	CAT assay − background (cpm)	Fold activation
None (EMSV)	1,539	761	1
CMD	11,088	10,310	13.6
CMGN	3,990	3,212	4.2
MRF4	2,736	1,958	2.6
Myf5	1,893	1,115	1.5

[a] Chick fibroblasts were cotransfected with 2 μg of p8-8000CAT, the −8-kb promoter fragment from the *CMD1* gene, and 2 μg of one of the MSV expression plasmids for the chicken myogenic factors EMSV-CMD1, EMSV-myogenin, EMSV-MRF4, or EMSV-Myf5. CAT activity was assayed 48 h after transfection by using 20 μg of protein from the cell extract prepared from a 60-mm-diameter dish.

restricted negative element of some sort. Unexpectedly, the *CMD1* promoter was not active in any mammalian muscle cell lines tested, and these included C2C12, L6, L8, and BC3H1 cells. Similar results were reported for promoter constructs from the mouse *MyoD* gene extending up to −7 kb, in which transient assays in various mouse muscle cell lines gave no reliable activity above background (61). However, a −160-bp fragment of the mouse *MyoD* promoter did show activity in primary chick cell cultures and, like the chicken *CMD1* gene, this activity was E box independent. Activity of the proximal mouse *MyoD* promoter was dependent upon a G+C-rich region with an SP-1 consensus site and a region containing a CCAAT sequence (64). We have not identified any consensus binding sites in the −322-bp region of the minimal *CMD1* gene promoter fragment that could account for muscle-specific activity. Although the mouse *MyoD* promoter was silent in all the muscle cell lines tested, a 720-bp distal promoter element from the −5.39- to −4.67-kb region could restore transcriptional activity to an integrated proximal promoter fragment in C2C12 cells but did not enhance promoter activity in chick muscle (61). This distal element contains three E-box consensus elements and an A+T-rich region of unknown function. No experiments were presented, however, to determine if the three E boxes in the distal regulatory region bound any myogenic factors or had an essential role in the restoration of promoter activity. Although promoter fragments extending to −8 kb in the *CMD1* promoter showed no transient activity in 10T1/2 cells, stable integration of the entire gene as a 9-kb *Hin*dIII fragment, with the complete coding region and 5 kb of promoter sequence, resulted in the myogenic conversion of 10T1/2 cells (data not shown), similar to the results reported for the *Myd* cosmid clone described earlier (46). Whether the conversion we observed was due to some sort of position effect or to the activation of the promoter through a similar integration-dependent distal regulatory region is not clear.

Transient expression experiments using primary chick muscle and fibroblast cultures with the *Xenopus MyoDa* (*XMyoDa*) promoter have also shown that a −55-bp promoter fragment containing no E-box motifs was sufficient for muscle-specific expression (27). However, in this instance the promoter fragment contains overlapping binding sites for TFIID (TBP) and the muscle-specific enhancer factor MEF-2, suggesting that MEF-2 stabilizes *XMyoDa* transcription in the muscle cell lineage. Transcription from the minimal *XMyoDa* promoter in nonmuscle cells is MEF-2 dependent (27), indicating a major role for MEF-2 in the stable activation of XMyoDa in muscle.

The minimal muscle-specific mouse myogenin promoter (from −88 to +1 bp) also contains a functional MEF-2 site and a single functional E box; however, the MEF-2 site is required for high levels of muscle-specific transcription in 10T1/2 cells, whereas the E box can be deleted with only a twofold effect (18). Transgenic studies with the minimal mouse myogenin promoter by Olson and coworkers reveal a similar trend in that mutation of the E box in the *lacZ* transgene had little or no effect on expression of the transgene in somites but greatly diminished expression in the limb buds and visceral arches. However, expression of the transgene occurred in the limb buds about 1 day later, suggesting that the pattern of myogenin activators changes during development (12). This result disagrees with that of Yee and Rigby, who reported that the E box in the minimal myogenin promoter was essential for transgene function (75). This difference has not been clarified. However, both groups agree that the MEF-2 site was required for appropriate temporal regulation of the transgene in the limb buds and in a subset of somites. Recent studies on cardiac muscle gene expression have likewise implicated MEF-2 in an E-box-independent pathway for muscle-specific expression in ventricular cardiac muscle cells (39, 63). Although there is a functional MEF-2 binding site in the *CMD1* promoter, it is not present in the minimal muscle-specific promoter. Furthermore, the *CMD1* TATA-like element is also not a degenerate MEF-2 binding site, as shown here. In no instance do E-box elements appear to be essential for the function of the minimal muscle-specific promoters for MyoD or myogenin, suggesting that direct promoter binding of the bHLH proteins does not play a role in tissue-specific expression of these muscle-regulatory genes. Moreover, there does not appear to be a conservation of *cis*-acting elements involved in the muscle-specific expression of the MyoD family of gene regulators that has been identified to date.

Auto activation of the *CMD1* promoter is E box and MEF-2 independent. It is well established that MyoD expression in a variety of cell types activates the endogenous *MyoD* gene (for reviews, see references 40, 56, 67, and 69) and, as mentioned above, this result has led to the general assumption that auto activation involves the direct binding of these bHLH proteins to the promoter regions of the activated genes. Promoter interaction is thought to occur through the well-characterized consensus binding site for these proteins, the E box, CANNTG (5, 26, 73). This autoregulatory/cross-regulatory loop would then maintain the expression of the myogenic factors in mesoderm and muscle precursor cells that form the muscle cell lineage, until the appropriate developmental cues trigger the terminal stages of myogenesis. It was therefore not unreasonable to anticipate that the multiple E-box and MEF-2 sites in the *CMD1* promoter might play a role in the regulation of CMD1. Although muscle-specific expression from the *CMD1* promoter was not dependent upon the direct binding of CMD1 to E-box elements or the binding of MEF-2 to a functional MEF-2 site, the current paradigm of autoactivation/cross regulation favored this type of regulatory mechanism in the establishment and maintenance of the muscle cell lineage. Unexpectedly, our results show that autoactivation of the *CMD1* promoter in primary chick fibroblasts is both E box and MEF-2 site independent and, therefore, is likely to involve an indirect pathway yet to be defined. Autoactivation of the myogenin promoter in 10T1/2 cells is also E box independent but, unlike with the *CMD1* promoter, there is an absolute requirement for an MEF-2 site in the minimal promoter in order for autoactivation to occur: myogenin activates MEF-2 expression, and once it is induced, MEF-2 is thought to amplify and maintain the myogenic phenotype in a positive autoregulatory loop without the direct binding of myogenin to its own

promoter, thus defining an indirect pathway for auto regulation (18). Likewise, the overlap of an MEF-2 site and the TATA element in the *XMyoDa* promoter, taken together with the dual role of these promoter elements in the muscle-specific expression of the gene, suggests that autoactivation in this instance involves the indirect action of MEF-2, as with the myogenin promoter.

Since the *CMD1* promoter autoactivates in transient expression experiments in the absence of E boxes and MEF-2 sites, it must be assumed that distinct regulatory elements confer this activity through an indirect pathway. This conclusion would be consistent with the different developmental expression patterns for the myogenic factors in vertebrate embryos (10, 15, 23, 56, 58). The one similar sequence element found in all the minimal muscle-specific promoters for the *MyoD* and myogenin genes is the TATA motif: ATAAATA for the different *MyoD* genes and TAAAT for myogenin. Previously described results (66), obtained by using muscle-specific enhancers from the *MCK* gene and the myoglobin gene in combination with the myoglobin and simian virus 40 TATA elements, suggest that there is a functional heterogeneity in the TATA element, since only certain combinations of TATA box-binding factors can interact productively with muscle-specific enhancers to form active transcription complexes. Such a mechanism may be utilized in the autoactivation of the *CMD1* promoter through an indirect pathway involving the TATA element, the CMD1 protein, and specific TATA-associated factors. In this scenario, CMD1 protein, either as a heterodimer with one of the E proteins (57) or as a phosphorylated monomer (37), could interact with ancillary TATA-associated factors to mediate muscle-specific expression from the *CMD1* promoter. This is being explored further.

Cross activation of the *CMD1* promoter appears to involve distinct regulatory elements and an indirect pathway. Our results demonstrate that there is a hierarchy in the cross-activating efficiency of the various myogenic factors on the *CMD1* promoter: CMD1 > myogenin > MRF4 > Myf5 (Table 2). Furthermore, this difference is not dependent upon any known muscle-specific transcriptional regulatory elements, since it is seen with the −322-bp minimal promoter fragment which has no E boxes or MEF-2 sites. Cell culture studies have also shown that MyoD will activate itself and myogenin or vice versa, suggesting an autoregulatory/cross-regulatory loop (62, 67, 70). Our results support this observation and further demonstrate that the loop is indirect. Transgenic studies also indicate that the mouse myogenin promoter without functional E boxes is still capable of activating muscle-specific *lacZ* expression, about 1 day later than normal. This delay in expression also implies that the spectrum of E-box independent myogenin activators changes during development (12). In order to explain this E-box independent regulation, one might speculate that each myogenic factor differentially activates or is capable of indirect interaction with a broad range of additional transcription factors that can modulate promoter activity. Alternatively, myogenic factor–E-protein complexes may preferentially recognize unknown common factor(s), and differences in the transcriptional activation by these complexes are due to protein-protein interactions and the specificity of the activator domain(s) for each myogenic factor. Data consistent with the first notion come from direct biochemical evidence showing that the amino terminus of Jun can mediate repression of the transcriptional activation by myogenin and MyoD through the bHLH domain (28). In addition, the above-mentioned transgenic experiments with the myogenin promoter, the different developmental expression patterns for the myogenic factors in embryos (10, 56), and the observation that even established muscle cell lines show different expression patterns for the myogenic factors (58), all suggest that there are distinct regulatory elements and factors responsible for the variation in myogenic bHLH protein expression during development. Results consistent with the second possibility come from the observations that the differences in the transcriptional activation of the troponin I and myosin light-chain 1 reporter genes by myogenin and MyoD, respectively, map to activation domains outside the basic helix-loop-helix region (1, 31).

Gene knockouts for the various myogenic factors in mice have also given a complicated picture with regard to the regulatory circuitry between the myogenic bHLH genes (9, 24, 38, 53, 54). Although MyoD$^-$ or Myf5$^-$ mice produce normal muscle, the double-knockout mouse expresses no detectable muscle-specific transcripts. The myogenin$^-$ mouse, on the other hand, produces normal numbers of myoblasts that do not differentiate efficiently in vivo but appear normal in vitro. The regulatory pathway then places *MyoD* and *Myf5* as redundant essential genes upstream to myogenin, the latter of which is required for efficient further differentiation. The developmental role of MRF4 is unknown at present, but its expression pattern suggests that MRF4 acts very late in the developmental program (6) and may, speculatively, be involved in the regulation of isoform switching. The myogenin$^-$ mouse expresses normal levels of MyoD but fourfold lower levels of MRF4 RNA, indicating that myogenin is not required for MyoD expression. This is consistent with the observation that myogenin is a weaker activator of the *CMD1* promoter than is CMD1. The MyoD$^-$ mice are completely normal and upregulate Myf5 in response to the mutation, whereas the Myf5$^-$ mice have no ribs and die at birth because of respiratory failure. Among the various combinations of the MyoD/Myf5 knockouts, it is interesting that mice with only one copy of the *MyoD* gene express almost normal amounts of MyoD and myogenin RNA, whereas mice with one copy of the *Myf5* gene produce half the normal levels of Myf5 and myogenin RNA (54). Our results, demonstrating that CMD1 is an efficient autoactivator (Table 1), are consistent with the observations that MyoD appears to autoactivate to normal levels in mice with a single copy of the *MyoD* gene. Myf5 does not appear to autoactivate effectively in vivo nor was it a good activator of the *CMD1* promoter in our studies, suggesting that Myf5 could not upregulate MyoD directly.

Regardless of the mechanisms that mediate this regulation among the myogenic factor genes, the assumptions concerning the pathways involved in the autoactivation and cross regulation will have to be reevaluated in terms of mechanisms that do not involve the direct binding of these factors to E-box elements, at least in the case of the *CMD1* promoter. Our results are consistent with the notion that MyoD (CMD1) is autoregulated through an indirect pathway that may involve the indirect action of myogenin, since the *CMD1* promoter is efficiently autoactivated in fibroblasts and responds moderately to myogenin but practically not at all to either Myf5 or MRF4. Unlike Myf5 in mammals (10), CMD1 (qmf1) is the first myogenic factor expressed in the developing avian somite (47). The phylogenetic reversal in the developmental appearance for MyoD and Myf5 in mammals and birds is not understood but it is consistent with the redundant nature of these two factors in mice with regard to myogenesis. Unfortunately there is no avian system amenable to gene targeting as yet, so this cannot be demonstrated directly.

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