MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3814–3824 Vol. 16, No. 7
0270-7306/96/$04.00+0

MEF2B Is a Potent Transactivator Expressed in Early Myogenic Lineages

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Received 24 January 1996/Returned for modification 20 March 1996/Accepted 3 April 1996

There are four members of the myocyte enhancer binding factor 2 (MEF2) family of transcription factors, MEF2A, -B, -C, and -D, that have homology within an amino-terminal MADS box and an adjacent MEF2 domain that together mediate dimerization and DNA binding. MEF2A, -C, and -D have previously been shown to bind an A/T-rich DNA sequence in the control regions of numerous muscle-specific genes, whereas MEF2B was reported to be unable to bind this sequence unless the carboxyl terminus was deleted. To further define the functions of MEF2B, we analyzed its DNA binding and transcriptional activities. In contrast to previous studies, our results show that MEF2B binds the same DNA sequence as other members of the MEF2 family and acts as a strong transactivator through that sequence. Transcriptional activation by MEF2B is dependent on the carboxyl terminus, which contains two conserved sequence motifs found in all vertebrate MEF2 factors. During mouse embryogenesis, MEF2B transcripts are expressed in the developing cardiac and skeletal muscle lineages in a temporospatial pattern distinct from but overlapping with those of the other *Mef2* genes. The mouse *Mef2b* gene maps to chromosome 8 and is unlinked to other *Mef2* genes; its intron-exon organization is similar to that of the other vertebrate *Mef2* genes and the single *Drosophila Mef2* gene, consistent with the notion that these different *Mef2* genes evolved from a common ancestral gene.

The myocyte enhancer binding factor 2 (MEF2) family comprises a group of transcription factors that have homology within a MADS (MCM1-agamous-deficients-serum response factor) box at their amino termini and an adjacent motif known as the MEF2 domain (reviewed in reference 39). There are four *Mef2* genes in vertebrates, *Mef2a-d*, whose products bind as homo- and heterodimers to the A/T-rich DNA sequence $CTA(A/T)_4TAG/A$ (2, 3, 5, 20, 29–31, 41, 50). MEF2 binding sites were first identified in the control regions of muscle-specific genes, where they are required for transcriptional activation (8). More recently, they have also been shown to be important for serum inducibility of the c-*jun* gene (10, 11). There is a single *Mef2* gene, *D-mef2*, in *Drosophila melanogaster*, which encodes a protein with the same DNA binding properties as the vertebrate MEF2 factors (21, 37). This gene has been shown to be required for differentiation of skeletal, cardiac, and visceral muscle cells in the *Drosophila* embryo (1, 22, 42).

The MADS and MEF2 domains mediate DNA binding and dimerization of MEF2 monomers (33, 41). MEF2 factors can also interact with heterodimers formed between myogenic basic helix-loop-helix (bHLH) factors, such as MyoD and myogenin, and ubiquitous bHLH proteins (32). Amino acids in the MADS and MEF2 domains appear to be the primary determinants of this type of protein-protein interaction (32). MEF2A, -C, and -D show similar DNA binding and transcriptional activation properties (2, 29–31, 41, 50). In contrast, it has been reported that human MEF2B is unable to bind the MEF2 consensus sequence unless the C terminus is deleted; this has led to the conclusion that MEF2B may have a function different from that of the other MEF2 factors and that its C terminus interferes with DNA binding of the N-terminal MADS and MEF2 domains (41, 50).

Expression of the *Mef2a*, *-c*, and *-d* genes marks early myogenic lineages during mouse and frog embryogenesis (3, 6, 49). *Mef2c* is the first member of the family to be expressed in the mouse, with transcripts appearing in the precardiac mesoderm at day 7.5 postcoitum. Soon thereafter, *Mef2a* and *-d* are expressed throughout the developing heart. In skeletal muscle cells, *Mef2c* is expressed within the somite myotome beginning at about 9.0 days postcoitus (d.p.c.) and *Mef2a* and *-d* are expressed immediately thereafter. The *Mef2* genes also show specific expression patterns in the developing brain (24). After birth, *Mef2a*, *Mef2b*, and *Mef2d* transcripts are expressed ubiquitously (2, 20, 29–31, 41, 50). *Mef2c* transcripts are restricted to skeletal muscle, brain, and spleen (20, 30, 31). Despite the widespread expression of *Mef2* mRNAs in adult tissues and established cell lines, MEF2 DNA binding activity is highly enriched in muscle cells and neurons. Alternative mRNA splicing of *Mef2a* and *Mef2d* transcripts gives rise to muscle-specific isoforms whose expression parallels MEF2 DNA binding activity (2, 29, 50).

To further define the functions and regulation of MEF2B, we characterized MEF2B cDNA and genomic clones. We show that MEF2B expression is restricted to myogenic lineages during early mouse embryogenesis and that, in contrast to previous reports, full-length MEF2B protein binds the MEF2 DNA consensus sequence with high affinity. The mouse *Mef2b* gene is located on chromosome 8 and has an intron-exon organiza-

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tion similar to that of *D-Mef2*, which suggests that they evolved from a common ancestral *Mef2* gene.

MATERIALS AND METHODS

Cloning and characterization of *Mef2b* cDNA and genomic clones. We began by screening a mouse SV129 genomic library in λ phage under conditions of reduced stringency with a region of the human MEF2A cDNA (50) encompassing the MADS and MEF2 domains. One of the genomic clones isolated did not correspond to *Mef2a*, *-c*, or *-d*, so it was chosen for further analysis. On the basis of DNA sequencing of the MADS and MEF2 domain coding regions, we concluded that this clone encoded mouse MEF2B. A 141-bp fragment of this genomic clone corresponding to the coding sequence immediately 3′ of the MEF2 domain was used as a probe to screen a 10.5-d.p.c. mouse heart cDNA library (uni-ZAP library; Stratagene, La Jolla, Calif.) to obtain the corresponding cDNA. Two positive clones were isolated and sequenced, and one was found to encode the full-length MEF2B protein. The full-length clone was rescued in pBluescript.

To define the intron-exon organization of *Mef2b*, we used primers based on the cDNA sequence to amplify regions of the gene by PCR. The same primers were also used for DNA sequencing of the genomic clone.

DNA binding assays. The MEF2B full-length cDNA was cloned into the *Nco*I and *Xba*I sites of the T7-directed expression vector pCITE 2a by PCR to allow efficient in vitro transcription and translation. pCITE-MEF2B plasmid was added with all the necessary components of a coupled in vitro transcription-translation reaction kit (TNT kit; Promega Corp., Madison, Wis.). Two microliters of this reaction mix was then incubated for 10 min at room temperature in an electrophoretic mobility shift assay with 40,000 cpm of a ^{32}P-labeled, double-stranded oligonucleotide corresponding to the consensus MEF2 binding site from the muscle creatine kinase (MCK) gene (8) in the presence of 1 μg of poly(dI-dC) · poly(dI-dC). The electrophoretic mobility shift assay buffer and electrophoresis conditions were as described previously (36).

Expression plasmids. The full-length MEF2B cDNA was cloned into the cytomegalovirus-directed mammalian expression plasmid pCI (Promega Corp.) by PCR with primers containing an *Nhe*I site at the 5′ end and an *Eag*I site at the 3′ end. Deletion mutants 1-272, 1-223, 1-170, 1-120, and 1-96 were also cloned into pCI by PCR with the same 5′ primer containing an *Nhe*I site and different 3′ primers containing an in-frame stop codon followed by an *Xba*I site. Expression of each clone was confirmed by T7-directed in vitro transcription-translation in the presence of [^{35}S]methionine followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The myogenin deletion mutant, Myo-bHLH, and the E12 mutant construct used in the MEF2 interaction assay are truncations that contain the bHLH regions but lack the transcriptional activation domains and are described elsewhere along with the 4RtkCAT reporter construct (32).

Site-directed mutagenesis. Mutations were introduced into the MEF2B expression vector by rolling-circle PCR (13) as described previously (34). Mutant proteins were tested for integrity by in vitro coupled transcription-translation (TNT kit) in the presence of [^{35}S]methionine followed by SDS-PAGE analysis.

Transient-transfection assays. The activities of the wild-type and mutant MEF2B proteins were assessed in transient-transfection assays with the MEF2-dependent reporter construct pE102MEF2x2CAT, which contains two MEF2 sites from the MCK enhancer upstream of the minimal promoter from the embryonic myosin heavy chain gene (50). Transfections were performed in 10T1/2 cells grown in Dulbecco's modified Eagle's medium with high glucose and L-glutamine and 10% fetal bovine serum. Ten micrograms of the pE102MEF2x2CAT reporter construct was used with 5 μg of the MEF2 test construct and 1 μg of pRSVβGAL to control for transfection efficiency. The cells were grown to 60% confluence in 6-cm-diameter plates and transfected by calcium phosphate precipitation for 16 h, washed, and harvested 48 h later. The cells were then lifted into 70 μl of 250 mM Tris (pH 7.5), sonicated, and centrifuged to pellet cellular debris. Five micrograms of this supernatant was then used to perform the chloramphenicol acetyltransferase (CAT) assays. CAT assays and subsequent thin-layer chromatography were performed as described elsewhere (47). The data shown in Fig. 6 and 7 represent the results of at least three separate transfections.

Interspecific backcross mapping. Interspecific backcross progeny were obtained by mating (C57BL/6J × *Mus spretus*)F_1 females and C547BL/6J males as described previously (4). A total of 205 F_2 mice were used to map the *Mef2B* locus (see Results for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described elsewhere (16). All blots were prepared with Hybond-N+ nylon membranes (Amersham, Arlington Heights, Ill.). The probe, an ~141-bp *Hae*III fragment of mouse genomic DNA, was labeled with [α-^{32}P]dCTP by using a random primed labeling kit (Stratagene). A fragment of 8.7 kb was detected in *Bam*HI-digested C57BL/6J DNA, and a fragment of 7.5-kb *Bam*HI *M. spretus*-specific fragment was monitored in backcross mice.

A description of the probes and restriction fragment length polymorphisms for the loci linked to *Mef2b*, including scavenger receptor (*Scvr*), lipoprotein lipase (*Lpl*), Jun protooncogene-related gene dl (*Jundl*), and mineralocorticoid receptor (*Mlr*), has been reported previously (46, 48). Recombination distances were calculated as described previously (9) with the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

In situ hybridization. The protocol which was used to fix and embed C57BL/6 × DBA/2 embryos is described in detail by Lyons et al. (26). Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight, dehydrated, and infiltrated with paraffin. Serial sections (5 to 7 μm) were mounted on gelatinized slides. Two sections were mounted per slide, deparaffinized in xylene, rehydrated, and postfixed. The sections were digested with proteinase K, postfixed, treated with triethanolamine-acetic anhydride, washed, and dehydrated.

In order to distinguish *Mef2b* transcripts from other members of the *Mef2* multigene family, we used a probe derived from the 3′ coding and noncoding region of the *Mef2b* mRNA that is not conserved between the different *Mef2* genes. The *Mef2b* probe was a 156-bp mouse cDNA (24) in pAMP1 (Gibco-BRL) that was linearized with *Not*I and transcribed with T7 polymerase for the antisense probe. The plasmid was linearized with *Eco*RI and transcribed with Sp6 for the sense control probe. The cRNA transcripts were synthesized according to the manufacturer's conditions (Stratagene) and labeled with [^{35}S]UTP (>1,000 Ci/mmol; Amersham).

Sections were hybridized overnight at 52°C in a mixture of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $NaPO_4$, 10% dextran sulfate, 1× Denhardt's solution, 50 μg of total yeast RNA per ml, and 50,000 cpm of ^{35}S-labeled cRNA probe per ml. The tissue was subjected to stringent washing at 65°C in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10 mM dithiothreitol and washed in PBS before treatment with 20 μg of RNase A per ml at 37°C for 30 min. Following washes in 2× SSC and 0.1× SSC for 10 min each at 37°C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 1 or 2 weeks in light-tight boxes with desiccant at 4°C. Photographic development was carried out in Kodak D-19 developer. Slides were counterstained lightly with toluidine blue and analyzed with both light- and dark-field optics of a Zeiss Axiophot microscope. Sense control cRNA probes (identical to the mRNAs) always gave background levels of hybridization signal. Embryonic structures were identified with the help of the atlases of Rugh (43) and Kaufman (17).

Western blots. Western blots (immunoblots) were performed as previously described (7). Briefly, equivalent quantities of whole-cell extracts from the various cell lines were separated on an SDS–10% polyacrylamide gel. Proteins were then transferred to nitrocellulose, incubated with one of the MEF2 polyclonal antibodies, and detected with an enhanced chemiluminescence kit (Amersham).

Antibodies. An affinity-purified rabbit antibody raised to a peptide corresponding to codons 487 to 507 of human *MEF2A* was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, Calif. Antibody to MEF2C was raised against an isoform-specific peptide representing codons 300 to 316 (7). The rabbit antisera to MEF2B and MEF2D were provided by Ron Prywes (Columbia University). They were prepared against polyhistidine fusion proteins corresponding to codons 234 to 365 of human MEF2B and codons 292 to 514 of mouse MEF2D, respectively, and have been described previously (10, 11).

RESULTS

MEF2B is the most divergent of the vertebrate MEF2 factors. To begin to characterize the structure and function of MEF2B, we isolated and sequenced a mouse MEF2B cDNA including the complete open reading frame (Fig. 1A). The longest MEF2B cDNA extended from 82 nucleotides 5′ of the initiating AUG to 196 nucleotides 3′ of the termination codon and encoded a MEF2B protein 349 amino acids in length with a predicted M_r of 37,347 and pI of 10.2. The first 257 amino acids of this open reading frame product were 75% identical to the corresponding region of human MEF2B reported previously (41, 50). However, beginning at amino acid 258, there was no further relatedness between the mouse and human proteins. The point of divergence between the mouse and human sequences corresponds to the center of a *Mef2b* exon, so this divergence seems unlikely to arise from alternative mRNA splicing. Analysis of the nucleotide sequences of the mouse and human cDNAs indicated that they continued to be highly related beyond the point of divergence of the conceptual open reading frames and suggested that there was a frameshift in the human sequence. We are certain that the mouse sequence is correct, because the introduction of an in-frame stop codon at codon 272 resulted in termination of MEF2B translation at that point, which would not have occurred if the

A.

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CCGAATTCGGCACGAGCAGCAGGTGTGGCCAGCAGGGACCAGACTCCCGGGACTCCTGAG     60
CCGCTCTGCACAGCTACCCACGATGGGGAGGAAAAAAATCCAGATCTCACGAATTCTAGA    120
                        M  G  R  K  K  I  Q  I  S  R  I  L  D     13
CCAAAGGAACAGGCAGGTGACTTTCACCAAGCGCAAGTTTGGACTGATGAAGAAGGCTTA    180
  Q  R  N  R  Q  V  T  F  T  K  R  K  F  G  L  M  K  K  A  Y     33
TGAGCTGAGCGTGCTTTGTGACTGCGACATTGCCCTGATCATCTTCAACAGTGCGCAACG    240
  E  L  S  V  L  C  D  C  D  I  A  L  I  I  F  N  S  A  Q  R     53
CCTCTTCCAGTATGCGAGCAGCGACATGGACCGGGTGCTGCTCAAATACACCGAGTACAG    300
  L  F  Q  Y  A  S  S  D  M  D  R  V  L  L  K  Y  T  E  Y  S     73
CGAGCCGCACGAGAGCCGCACCAACGCGGATATCCTTCAGACACTTAAGAGGAGGGGCGT    360
  E  P  H  E  S  R  T  N  A  D  I  L  Q  T  L  K  R  R  G  V     93
GGGCCTTGATGGGCCCGAGCTGGATATGGAGGAGGGGCCAGAAGGTCCTGGAGAGAAGCT    420
  G  L  D  G  P  E  L  D  M  E  E  G  P  E  G  P  G  E  K  L    113
GCTGAGGACACTGGGGGGTGACAGAGGCTCGGCCTCGCCCCCGCCTCGGATCTATCCCGT    480
  L  R  T  L  G  G  D  R  G  S  A  S  P  P  P  R  I  Y  P  V    133
GGCCCCTGCAATGTCGGTTTCAGAGCTGTCCTACCGGGTCCCACCAGCCACTCCAGGCTG    540
  A  P  A  M  S  V  S  E  L  S  Y  R  V  P  P  A  T  P  G  C    153
TGATCCTGGCGGGCTGGGGGAGGCCCCGTCTGTCCACAGCCGCCCGCCTCACTTTCGACC    610
  D  P  G  G  L  G  E  A  P  S  V  H  S  R  P  P  H  F  R  P    173
CCCGGGCCTGGGACACCCGATCTTCTCTCCAAGCCACCTTGCGAGCAAGACGCCCCCGCC    670
  P  G  L  G  H  P  I  F  S  P  S  H  L  A  S  K  T  P  P  P    193
TCTGTACCTGGCGACTGATGGGCGGAGGCCAGACCTGCCCCCTGGCCTGGTTGGTGCCCG    730
  L  Y  L  A  T  D  G  R  R  P  D  L  P  P  G  L  V  G  A  R    203
AGGGGGATTGGGCACCTCGAGAAGCCTGTACAGCGGCCTGCAGAGTCCAGGTGCCCCAGG    790
  G  G  L  G  T  S  R  S  L  Y  S  G  L  Q  S  P  G  A  P  G    223
CCCTGCTCTGGGTAGCTTTGCCTTCTTACCATCAGGCTCCACAGATTGCAGCCCGGGAGA    850
  P  A  L  G  S  F  A  F  L  P  S  G  S  T  D  C  S  P  G  D    243
CGCAGCTCAGGGACCCCTGCAACCCTCACCCTGGCCACCAACGAGGGACGCTGTAGACCC    910
  A  A  Q  G  P  L  Q  P  S  P  W  P  P  T  R  D  A  V  D  P    263
CGCCCGGCCAGTTGCCCGCAGTCTGTGCAAGGAGGGTCCCCCCAGCCGAGGGGCCTCCCC    970
  A  R  P  V  A  R  S  L  C  K  E  G  P  P  S  R  G  A  S  P    293
TCCGACACCTCCGGTCAGCATCAAGTCTGAGCGACTGTCTCCAGTCACTGGGACCTCTGG   1030
  P  T  P  P  V  S  I  K  S  E  R  L  S  P  V  T  G  T  S  G    313
CGACTTCCCCAGGTCCTTCCCCTACCCGCTGCTCCTTGCCAGGCCCCTTGCAGAGCCACT   1090
  D  F  P  R  S  F  P  Y  P  L  L  L  A  R  P  L  A  E  P  L    333
GCGGCCCTCGGCCTCCCTGCACCGCTTGACCCCTGACAGCTGGCCACGGTAGCGCTCCAA   1150
  R  P  S  A  S  L  H  R  L  T  P  D  S  W  P  R  *             349
GAGGTTCCTCCAGCCTCCACAGAGTTCCCAAAGTCTCATGTTCCCTGTACCACCAGGAGG   1210
ACGGACGAGGGCACCCACTGTGCATCACTAAGGCCCTGTCCCTTCCTGGGGGCCGCTGCA   1270
TCAATAAAGCCCAAGGACTCGGGAGGGCGCTGGGCAGCCCCTAGCTCAGAGCTCCCGAGC   1330
CCTCGTGC                                                       1338
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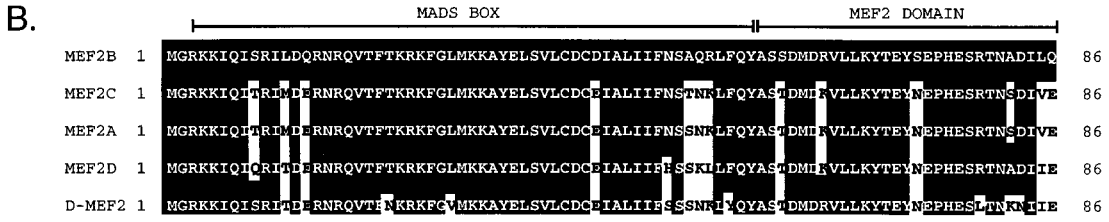
B.



C.

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MEF2B      187   ASKTPPPL    X6   RRPDLPPG    208
                 .:|:|||.         |:|||...
MEF2A      252   PTKSPPPP    X9   RKPDLRVV    276
                 ..|:|||.         |:|||...
MEF2C      237   QAKSPPPM    X6   RKPDLRVL    258
                 ..|:|||.         |:|||...
MEF2D      248   PAKSPPPP    X10  RKPDLRVI    273

Consensus        [K][S/T][P][P][P]  [X]  [R][R/K][P][D][L]


MEF2B      298   VSIKSERLSP          308
                 :|||||.:||
MEF2A      392   ISIKSEPISP          401
                 :|||||.:||
MEF2C      380   LSIKSEPVSP          389
                 :|||||.:||
MEF2D      429   ISIKSEPVSP          438

Consensus        [V/I/L][S][I][K][S][E][X][V/I/L][S][P]
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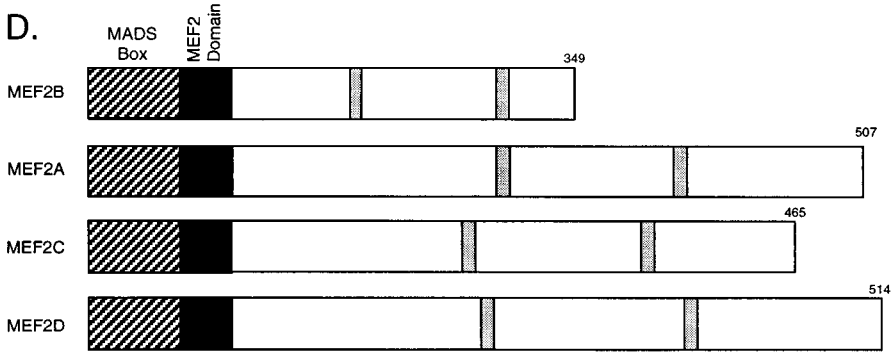
D.



mouse sequence had the same open reading frame as the human sequence (see below). The nucleotide sequence homology between the mouse and human MEF2B cDNAs continued to the termination codon of the mouse sequence but did not extend beyond that point into the 3′ untranslated region. The observation that the nucleotide homology extended only to the termination codon of the mouse sequence also suggests that the human MEF2B sequence contains a frameshift. We detected no significant nucleotide sequence homology between mouse and human MEF2 cDNAs in the 5′ untranslated regions. While this work was in preparation, the sequence of MEF2B was published by another group (14). The open reading frame of their sequence agrees with ours.

MEF2B is highly homologous to the other MEF2 factors within the MADS and MEF2 domains (Fig. 1B), whereas C terminal to the MEF2 domain, it showed relatively little homology to the other vertebrate MEF2 factors (Fig. 1B). However, by comparing the amino acid sequences of the four vertebrate *Mef2* gene products, we identified two segments of homology in the C-terminal regions of all the factors (Fig. 1C and D). Region I corresponded to the consensus sequence K-S/T-P-P-P-$(X)_{6-10}$-R-R/K-P-D-L and contained a potential phosphorylation site for proline-dependent protein kinase (S/T-P) (18). Region II corresponded to the consensus sequence V/I/L-S-I-K-S-E-X-V/I/L-S-P and contained potential phosphorylation sites for proline-dependent protein kinase and protein kinase C (S-X-K) (18). These conserved regions are also present in *Xenopus* (3, 49) and chick (45) MEF2 factors and would also be encoded by the human MEF2B cDNA in the reading frame corresponding to that of the mouse clone. Although exact sequence homology between MEF2B and other MEF2 factors in the C-terminal region was restricted to these two regions, like the other MEF2 factors, the C terminus of MEF2B was relatively rich in serine, threonine, and proline, which often characterize transcription activation domains.

Structure and chromosomal location of *Mef2b*. The structure of the mouse *Mef2b* gene was determined by sequencing and PCR of a genomic clone using primers derived from the cDNA sequence. As shown in Fig. 2A, the coding region of *Mef2b* contained seven exons and spanned about 3.5 kb. The positions of the introns in the region encoding the MADS box and MEF2 domain correspond exactly to those in the other vertebrate *Mef2* genes (28) and in the *Drosophila Mef2* gene, *Dmef2* (21, 37).

The mouse chromosomal location of *Mef2b* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. The interspecific backcross mapping panel has been typed for over 2,000 loci that are well distributed among all the autosomes as well as the X chromosomes (4). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms using a mouse genomic DNA probe. The 7.5-kb *Bam*HI *M. spretus* restriction fragment length polymorphism (see Materials and Methods) was used to monitor the segregation of the *Mef2b* locus in backcross mice. The mapping results indicated that *Mef2b* is located in the central region of mouse chromosome 8, linked to *Scvr*, *Lpl*, *Jundl*, and *Mlr*. Although 96 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 2B), up to 160 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies by using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere-*Scvr*-18/156-*Mef2b*-1/157-*Lpl*-0/150-*Jundl*-3/160-*Mlr*. The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are *Scvr*-11.5 ± 2.6 (*Mef2b*, *Lpl*, and *Jundl*)-1.9 ± 1.1 *Mlr*. No recombinants were detected between *Mef2b* and *Lpl* in 157 animals typed in common or between *Lpl* and *Jundl* in 150 animals typed in common, suggesting that the two loci within each pair are within 1.9 and 2.0 centimorgans of each other (upper 95% confidence limit), respectively.

The central region of mouse chromosome 8 has regions of homology with human chromosomes 8p, 19p, and 4q (summarized in Fig. 2B). Indeed, the human *Mef2b* gene was recently mapped to the syntenic region of chromosome 19p, and it was predicted that the mouse gene would map to mouse chromosome 8 (15).

Localization of *Mef2b* gene transcripts in muscle lineages. A series of sections of mouse embryos from developmental stages 7.5 to 16.5 d.p.c. were hybridized in situ with a ^{35}S-labeled cRNA probe for *Mef2b*. These gene transcripts were first detected in the precardiac mesoderm at 7.5 d.p.c. (Fig. 3A). At 8.0 d.p.c., the bilaterally symmetric cardiac anlage expresses a high level of *Mef2b* mRNA (Fig. 3B) prior to fusion to form a simple cardiac tube. After 9.5 d.p.c. (Fig. 3C), the level of *Mef2b* mRNA in cardiac muscle decreases gradually through 11.5 d.p.c. (Fig. 3D) and falls below the level of sensitivity of the in situ technique after 13.5 d.p.c. (not shown).

In skeletal muscle cells of myotomes, *Mef2b* mRNAs were first detected at 9.0 d.p.c. (Fig. 3C). *Mef2b* gene transcripts were detected in myogenic cells that migrate to populate the head, branchial arches, and limb buds at 11.5 d.p.c. (Fig. 3D). As the myotomes form the deep back muscles and intercostal muscles at 11.5 to 14.5 d.p.c., *Mef2b* mRNAs continue to be easily detected (Fig. 3D and E). These gene transcripts are also detected in body wall and hind-limb muscles at 16.5 d.p.c. (Fig. 3F). After 12.5 d.p.c., *Mef2b* mRNAs were expressed at the highest levels at the ends of developing myofibers (Fig. 3F, arrowheads).

Mef2b mRNA was not detected in vascular or visceral smooth muscle by in situ hybridization, but this does not rule out a low level of expression of this gene in the smooth muscle lineages. *Mef2b* gene transcripts were also detected in nonmuscle tissues such as neurons (Fig. 3D) (24), the neural crest and developing whisker follicles (data not shown), and calcifying cartilage (Fig. 3E).

Expression of MEF2B protein in established cell lines. There is considerable evidence indicating that MEF2 proteins are more restricted in their cell type distribution than MEF2 mRNAs, suggesting that posttranscriptional mechanisms regulate MEF2 expression (2, 6, 20, 29–31, 50). Using antibodies specific for each of the four MEF2 factors, we examined by Western blot analysis the expression of these proteins in sev-

FIG. 1. Nucleotide sequence of mouse MEF2B transcript and deduced open reading frame for the MEF2B protein. (A) The sequences of the mouse MEF2B cDNA and the deduced open reading frame product are shown. The MADS and MEF2 domains are in boldface. (B) Comparison of amino acid sequences of the MADS and MEF2 domains of mouse MEF2B, MEF2A (27a), MEF2C (30), and MEF2D (29). Amino acid sequence identities are shown in black. (C) Homology regions I and II are shown, and their positions in the different MEF2 factors are indicated. (D) Schematic representations of the four MEF2 factors. The number of amino acids in each protein is indicated at the end. Only one of the alternatively spliced forms for each factor is shown. The positions of homology regions I and II are indicated (grey bars).

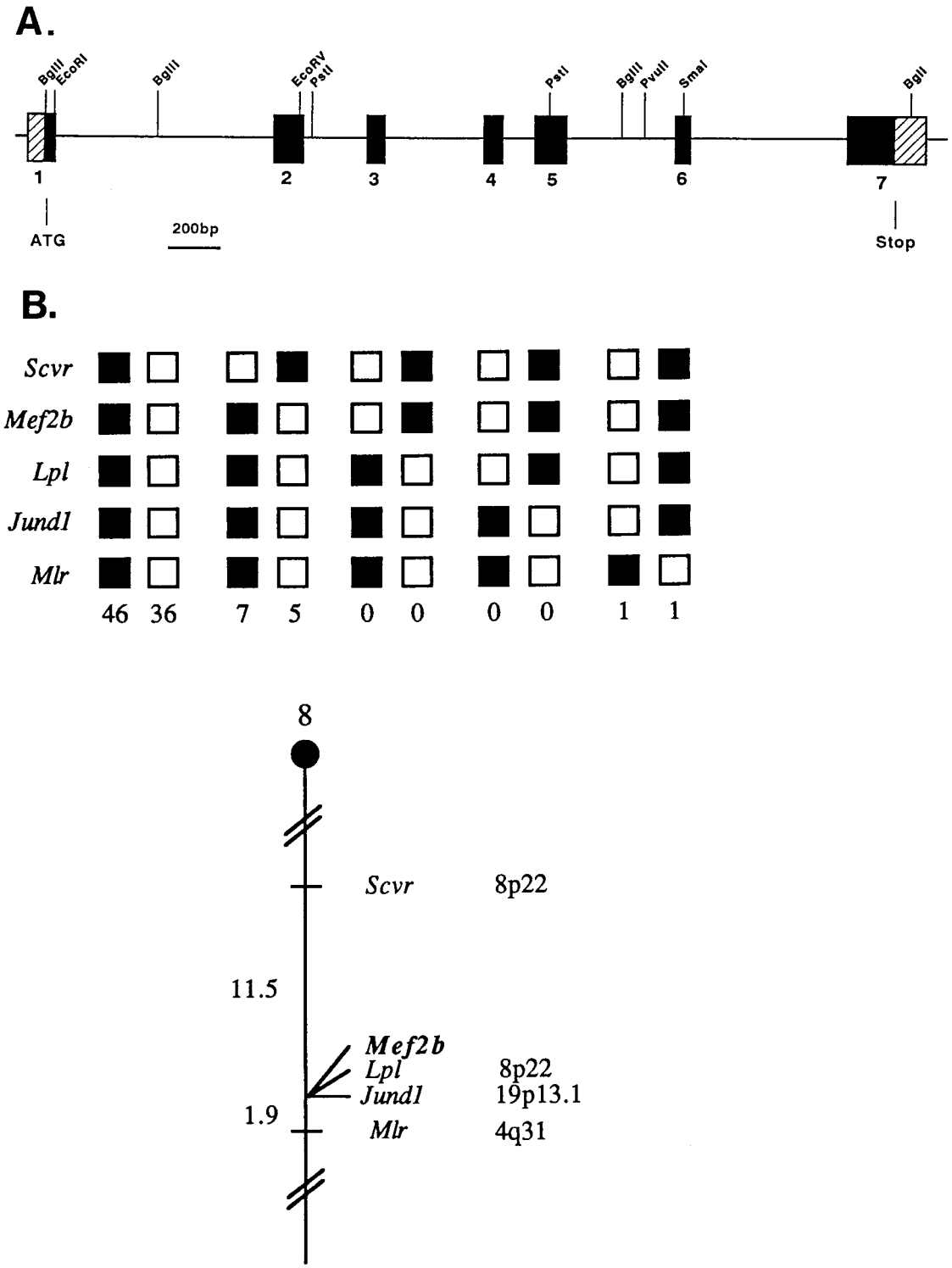


FIG. 2. Structure and chromosomal location of mouse *Mef2b*. (A) The intron-exon organization of the mouse *Mef2b* gene was determined by DNA sequence analysis of the *Mef2b* genomic clone and comparison with the cDNA sequence. PCR was also used to determine distances between exons. Coding and noncoding regions are indicated in black and striped areas, respectively. Amino acids of MEF2B encoded by each exon are as follows: exon 1, 1 to 17; exon 2, 18 to 86; exon 3, 87 to 131; exon 4, 132 to 174; exon 5, 175 to 247; exon 6, 248 to 277; exon 7, 278 to 349. (B) *Mef2b* was placed on mouse chromosome 8 by interspecific backcross analysis. The segregation patterns of *Mef2b* and flanking genes in 96 backcross animals that were typed for all loci are shown at the top of the panel. For individual pairs of loci, more than 96 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The black boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 8 linkage map showing the location of *Mef2b* in relation to linked genes is shown at the bottom. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the Genome Data Base, a computerized database of human linkage information maintained by the William H. Welch Medical Library of Johns Hopkins University (Baltimore, Md.).

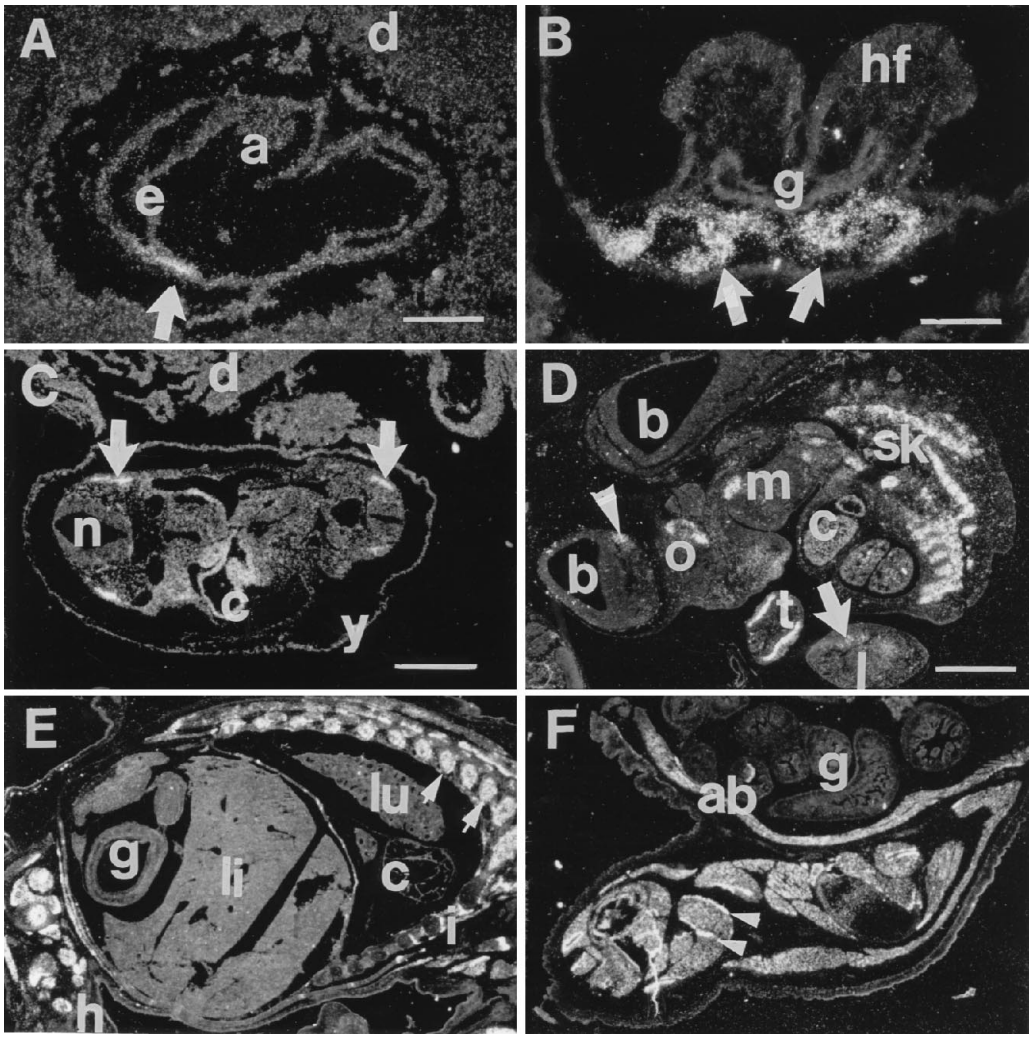


FIG. 3. *Mef2b* gene transcripts are detected at high levels in developing cardiac and skeletal muscle. (A) Dark-field micrograph of a parasagittal section of a 7.5-d.p.c. mouse embryo hybridized with the *Mef2b* antisense probe. The arrow points to precardiac mesoderm that is close to the rostral end of the embryo. a, allantois; d, decidua; e, ectoderm. (The ectoderm was separated from mesoderm and endoderm by a sectioning artifact.) (B) Frontal section through the head folds (hf), foregut (g), and cardiac anlage (arrows) of an 8.0-d.p.c. embryo. (C) A transverse section of a 9.5-d.p.c. embryo surrounded by yolk sac (y). Arrows point to myotomes in the rostral (left) and caudal (right) portions of the curved embryo. c, cardiac muscle; n, neural tube. (D) In a parasagittal section of an 11.5-d.p.c. embryo, *Mef2b* mRNAs are detected in cardiac muscle (c), extraocular muscles (o), trunk muscles (sk), premuscle muscle masses (arrow) in a limb bud (l), the mandibular arch (m), and myotomes of the tail (t). The arrowhead points to expression in the brain (b). (E) A parasagittal section of a 14.5-d.p.c. embryo shows that *Mef2b* mRNAs are detected in intercostal muscle (i), hind limb (h), and back muscles (top). These gene transcripts are also found in cartilage cells (small arrows). lu, lung; li, liver. (F) Parasagittal section of a 16.5-d.p.c. hind limb and a portion of the abdomen (ab). As muscles mature at 14.5 (E) and 16.5 (F) d.p.c., the highest levels of *Mef2b* mRNAs are detected at the ends of myofibers (arrowheads in panel F). Bars: A, 200 μm; B, 100 μm; C, 400 μm; D to F, 800 μm.

eral muscle and nonmuscle cell types. The specificities of the antibodies were established by Western blots of in vitro-translated MEF2 proteins (reference 7 and data not shown).

All of the MEF2 factors were expressed at the highest levels in differentiated C2 myotubes (Fig. 4). As reported previously (2), MEF2D was detected at low levels in C2 myoblasts, as was MEF2B. MEF2A and MEF2C were not detected in myoblasts, in the P19 embryonal carcinoma cell line, or in the NIH 3T3 and 10T1/2 fibroblast cell lines. MEF2B was barely detectable in P19 cells but was readily detectable in NIH 3T3 and 10T1/2 cells. Western blots with antitubulin antibodies confirmed that equal quantities of protein were in each lane (not shown). We conclude that MEF2B protein is enriched in but not specific to differentiated muscle cells.

MEF2B binds the MEF2 site. It was reported previously that human MEF2B failed to bind the MEF2 binding site unless the C terminus was truncated, which led to the suggestion that the conformation of the C terminus interferes with DNA binding (41, 50). However, when we tested the DNA binding activity of in vitro-translated mouse MEF2B, we found that it bound the MCK MEF2 site with an efficiency similar to that of MEF2C (Fig. 5). Moreover, deletion of the C terminus by introducing a termination codon at codon 170, 223, or 270 did not result in an increase in DNA binding activity (not shown).

Within its MADS box, MEF2B differs from the other MEF2 factors by the presence of a glutamine in place of an aspartic acid at position 14 (Fig. 1B). Mutational analysis of SRF has shown that aspartic acid at this position in the MADS box is critical for DNA binding (38, 44), which raised the possibility that this residue alters the DNA binding activity of MEF2B. To test this, we replaced Gln-14 with aspartic acid in the mutant MEF2B-Q14E. This mutant showed an approximately twofold

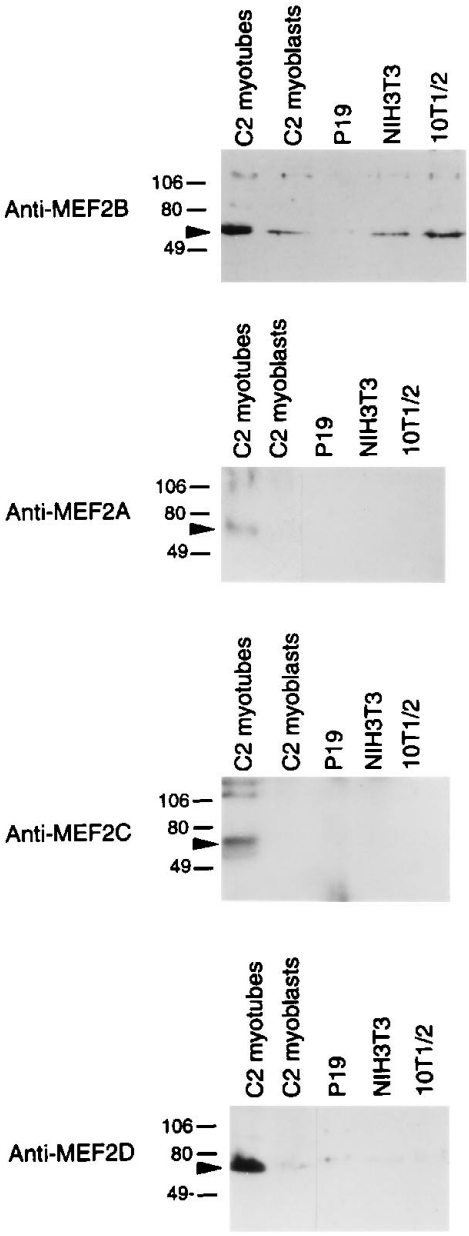


FIG. 4. Detection of MEF2 proteins in cell lines by Western blot analysis. Extracts were prepared from the indicated cell types as described in Materials and Methods, and proteins were resolved by SDS–10% PAGE followed by electrophoretic transfer to nitrocellulose. Blots were then probed with antibodies specific to each MEF2 protein. Molecular weight markers (in thousands) are shown to the left, and the arrowheads point to the position of each MEF2 protein.

increase in DNA binding activity relative to that of wild-type MEF2B (Fig. 5, lane 2). These results confirm that Gln-14 reduces DNA binding, but it clearly does not prevent binding of wild-type MEF2B to the consensus MEF2 binding site.

Transcriptional activity of MEF2B. To further investigate the properties of MEF2B, we tested its ability to transactivate the MEF2-dependent reporter pE102MEF2x2CAT, which contains two tandem copies of the MCK MEF2 site upstream of the embryonic myosin heavy chain promoter. As shown in Fig. 6, MEF2B efficiently transactivated the reporter in 10T1/2 cells. MEF2B mutant Q14E showed about twofold greater transcriptional activity than wild-type MEF2B, consistent with its increased DNA binding activity. Transactivation was dependent on the presence of the MEF2 sites and was not observed with a similar reporter plasmid in which they were mutated (not shown).

We also compared the transcriptional activities of a series of MEF2B C-terminal deletion mutants (Fig. 6). All mutant proteins were expressed at comparable levels in in vitro translation reactions (not shown). Deletion to residue 272 (mutant 1-272) had only a small effect on transcriptional activity, whereas deletion to residue 223 (mutant 1-223) resulted in an approximately fivefold reduction in activity. Further deletion to residue 170 (mutant 1-170) resulted in a complete loss of transcriptional activity. These results suggest that the C terminus of MEF2B, like those of other MEF2 factors, acts as a transcriptional activation domain.

We have previously demonstrated that MEF2A, -C, and -D proteins can synergize with myogenic bHLH proteins to activate skeletal muscle-specific gene expression (32). This interaction was shown to occur by direct protein-protein interaction between MEF2 and the heterodimer of a myogenic bHLH protein and E12 (32). It was of interest to determine whether MEF2B could also interact with members of the myogenic bHLH protein family. A trihybrid assay in which a myogenin deletion mutant containing just the bHLH region was expressed with a truncated E12 protein mutant and full-length MEF2B to drive expression of the E-box-dependent reporter plasmid 4R-tkCAT was employed. This E-box-dependent reporter contains four tandem copies of the right E box from the MCK enhancer upstream of the thymidine kinase promoter linked to CAT and is not activated by the truncation mutants of myogenin and E12, because each lacks a transactivation domain (32) (Fig. 7, lanes 1, 2, and 4). This reporter was also silent in the presence of MEF2B alone, MEF2B with Myo-bHLH, and MEF2B with E12 (lanes 3, 5, and 6). However, a

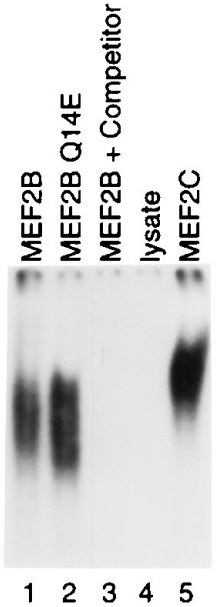


FIG. 5. DNA binding activity of MEF2B. MEF2 factors were translated in rabbit reticulocyte lysate and tested for binding to a labeled oligonucleotide probe corresponding to the MEF2 site from the MCK enhancer. Only the region of the gel containing the shifted probe is shown. Lanes: 1, MEF2B; 2, mutant MEF2B Q14E; 3, MEF2B plus a 100-fold excess of unlabeled MEF2 site as a competitor; 4, reticulocyte lysate alone; 5, MEF2C.

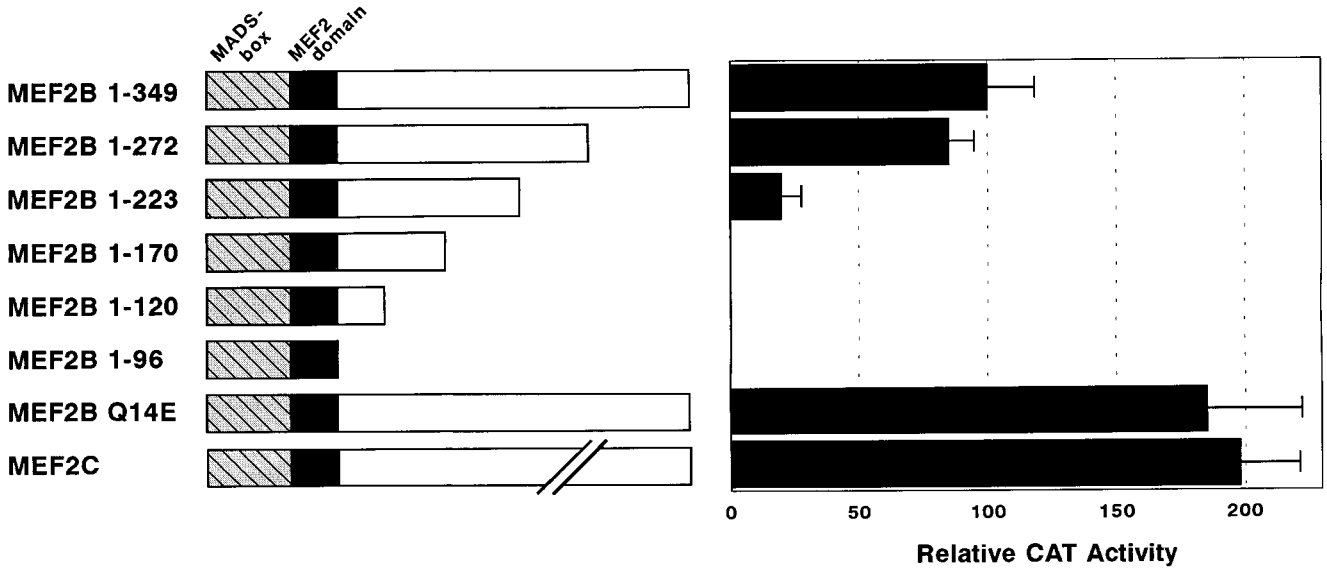


FIG. 6. Transcriptional activity of MEF2B deletion mutants. A series of progressive C-terminal deletion mutants of MEF2B were created and tested for their abilities to transactivate the MEF2-dependent reporter gene pE102MEF2x2CAT in transiently transfected 10T1/2 cells. The number of amino acids in each deletion mutant is indicated to the left of each construct. Transcriptional activities are expressed relative to the level of transactivation observed with full-length MEF2B. Values represent the averages from three independent experiments; errors bars represent the standard error of the mean.

high level of transactivation was observed when all three factors, Myo-bHLH, E12, and MEF2B, were combined (lane 7). This activation occurs because MEF2B contains a strong transactivation domain and is recruited to the promoter by its interaction with the heterodimer formed between the bHLH regions of myogenin and E12. Transactivation was dependent on the E boxes in the reporter gene and was not observed with the thymidine kinase promoter alone (not shown). These re-

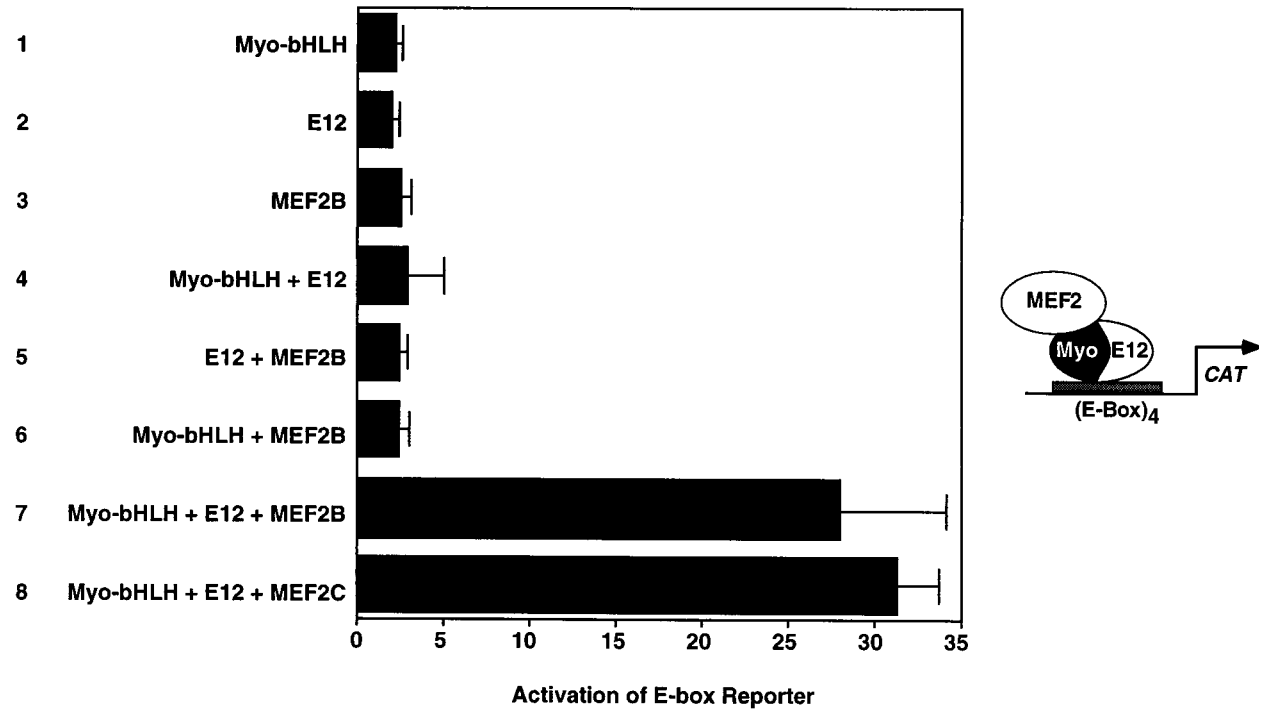


FIG. 7. MEF2B interacts with the Myo-bHLH–E12 heterodimer to activate transcription. 10T1/2 cells were transiently transfected with the E-box-dependent reporter gene 4R-tkCAT and expression vectors encoding wild-type MEF2B or MEF2C, a mutant myogenic bHLH factor, and a mutant E12 factor, as indicated. The mutant myogenic bHLH construct and mutant E12 construct lack transactivation domains, yet the MEF2B and -C factors have transactivation domains that are recruited by interaction to activate the CAT reporter. CAT activity in cell extracts was determined as described in Materials and Methods. Values are expressed as the fold induction of CAT activity above that observed with each reporter gene alone. Values represent the averages from three independent experiments; errors bars represent the standard error of the mean. The diagram to the right illustrates how transcriptional activation of the reporter gene via protein-protein interactions may occur.

sults demonstrate that MEF2B is capable of interacting with the heterodimer formed between myogenin and E12, as was demonstrated for the other three MEF2 proteins (32).

DISCUSSION

We characterized the structure and expression of the mouse *Mef2b* gene and analyzed the DNA binding and transcriptional activities of the MEF2B protein. Like the other MEF2 factors, MEF2B was expressed in myogenic lineages during embryogenesis and acted as a potent transactivator through the MEF2 binding site. Our results contrast with those of previous studies of human MEF2B, which reported that this member of the MEF2 family did not bind the same DNA sequence bound by MEF2A, -C, or -D (41, 50). The basis for these different results is unclear.

Our results show that the C-terminal region of MEF2B is required for transcriptional activation. Within the C-terminal region of MEF2B, we detected two protein motifs that are conserved in the four vertebrate MEF2 factors. Both of these motifs contain consensus sequences for protein phosphorylation and are within regions of the C terminus that the deletion analyses indicated were important for MEF2 function. We are presently investigating the potential role of phosphorylation of these motifs in MEF2 function.

Mef2b gene expression, like that of *Mef2c* (6), is an early marker for the cardiac muscle lineage. Cardiac progenitor cells arise from the anterior lateral plate mesoderm around 7.5 d.p.c. in the mouse (43). *Mef2b* and *-c* are expressed in the cardiac mesoderm prior to the formation of a primitive heart tube. The expression of these two genes precedes that of contractile protein genes such as myosin and cardiac actin (26). Thus, either MEF2B, MEF2C, or both of these proteins may be involved at least in the differentiation of cardiac muscle if not in determination of this cell lineage. The onset of expression of the *Mef2* genes coincides with that of *csx/Nkx-2.5* (19, 23), a homeobox gene that plays an important role in cardiac muscle development (27). Since MADS box and homeodomain proteins are known to interact (reviewed in reference 39), it will be interesting to determine if the MEF2 proteins and Csx/Nkx-2.5 interact to regulate cardiac muscle gene expression. GATA4 is also expressed concomitantly with MEF2B in the early cardiac muscle lineage (12).

Mef2b gene transcripts were also detected in an overlapping pattern with those of *Mef2c* during skeletal muscle development. *Mef2c* mRNAs are first detectable between 8.5 and 9.0 d.p.c. in rostral myotomes (6), and those of *Mef2b* are expressed in myotomes at 9.0 d.p.c. *Mef2b* and *-c* gene transcripts are first detected in muscle cells in the limb buds and branchial arches at 11.5 d.p.c. As myogenesis proceeds, *Mef2b* mRNAs are expressed at the highest level at the ends of developing myotubes and myofibers. *Mef2c* mRNAs also show this restricted expression pattern in fetal muscle (25). This pattern may represent the preferential fusion of myoblasts with the ends of developing myofibers. The expression patterns of *Mef2b* in cardiac and skeletal muscles are summarized in Fig. 8 and are compared with those of the other *Mef2* genes and representative muscle-specific marker genes.

We note that *Mef2b* does not appear to be expressed at any time or place at which other *Mef2* genes are not expressed. This raises the possibility that its functions are difficult to define by targeted inactivation of *Mef2b* in mice. The spatial and temporal expression pattern of *Mef2b* in myogenic and neurogenic lineages is almost identical to that of *Mef2c* (6, 24), suggesting that at least these two genes have redundant functions or are capable of compensating for each other. Indeed, *Mef2b*-null mice are viable and do not exhibit obvious defects at birth (35). Whether *Mef2b*-null mice show later phenotypes and whether the functions of *Mef2b* overlap with those of other *Mef2* genes is currently under investigation.

The expression level of *Mef2b* in smooth muscle lineages is below the level of sensitivity of in situ hybridization after a 14-day exposure of hybridized slides to emulsion. This lower level of sensitivity may be due in part to the shorter gene-specific probe for *Mef2b* compared to probes used to detect other *Mef2* mRNAs (6). Interestingly, in balloon-injured adult rat carotid arteries, *Mef2b* mRNA and protein are up regulated within smooth muscle cells of the neointima, which are induced to proliferate and dedifferentiate as a result of injury (7). Noninjured vessels show very low levels of MEF2B protein expression, which suggests that MEF2B is involved in smooth muscle cell proliferation (7).

The *Mef2b* gene mapped to the central region of mouse chromosome 8 and is unlinked to other *Mef2* genes. The four vertebrate *Mef2* genes, *Mef2a*, *-b*, *-c*, and *-d*, map to different chromosomes and belong to evolutionarily conserved linkage groups on mouse chromosomes 7, 8, 13, and 3 and human chromosomes 19, 15, 5, and 1, respectively (15, 30). We have compared our interspecific map of chromosome 8 with a composite mouse linkage map that reports the map locations of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at Jackson Laboratory, Bar Harbor, Maine). *Mef2b* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).

MEF2 DNA binding sites have been identified in a large array of skeletal and cardiac muscle-specific structural genes, suggesting that MEF2 proteins activate markers of terminal muscle differentiation (reviewed in reference 39). In this regard, MEF2A, -C, and -D proteins have previously been shown to synergize with myogenic bHLH factors to regulate skeletal muscle-specific gene expression (17a, 32). We show here that MEF2B is also capable of interacting with myogenin, suggesting that it too controls muscle gene expression. This notion is further supported by the appearance of MEF2B transcripts in development at a time when the myogenic bHLH proteins are present in the myotome during the differentiation of the skeletal muscle cell lineage.

We have previously shown that MEF2 factors lack myogenic activity on their own but that they are able to synergize with the myogenic bHLH factors to activate muscle gene expression (32). This synergy appears to be mediated by direct protein-protein interactions between MEF2 factors and heterodimers formed between myogenic bHLH factors and E proteins (32). In this manner, MEF2 factors may regulate muscle gene expression by association with the myogenic bHLH proteins bound to E-box sites in the control regions of contractile protein genes. MEF2 factors have also been shown to regulate the expression of a number of contractile genes directly through the MEF2 site (reviewed in reference 39), suggesting that MEF2 factors are part of a complex regulatory network that directs muscle-specific gene expression.

The role of MEF2 in muscle formation has been demonstrated most clearly in *D. melanogaster*, which contains a single *Mef2* gene, *D-mef2* (21, 37). Inactivation of *D-mef2* results in embryos lacking differentiated skeletal, cardiac, and visceral muscle (1, 22, 42). However, myoblasts from these lineages are correctly positioned and specified, which indicates that *D-mef2* acts at a relatively late step in the developmental pathways leading to the formation of differentiated muscle cells. *nautilus*, the single *Drosophila* myogenic bHLH gene, is expressed at the

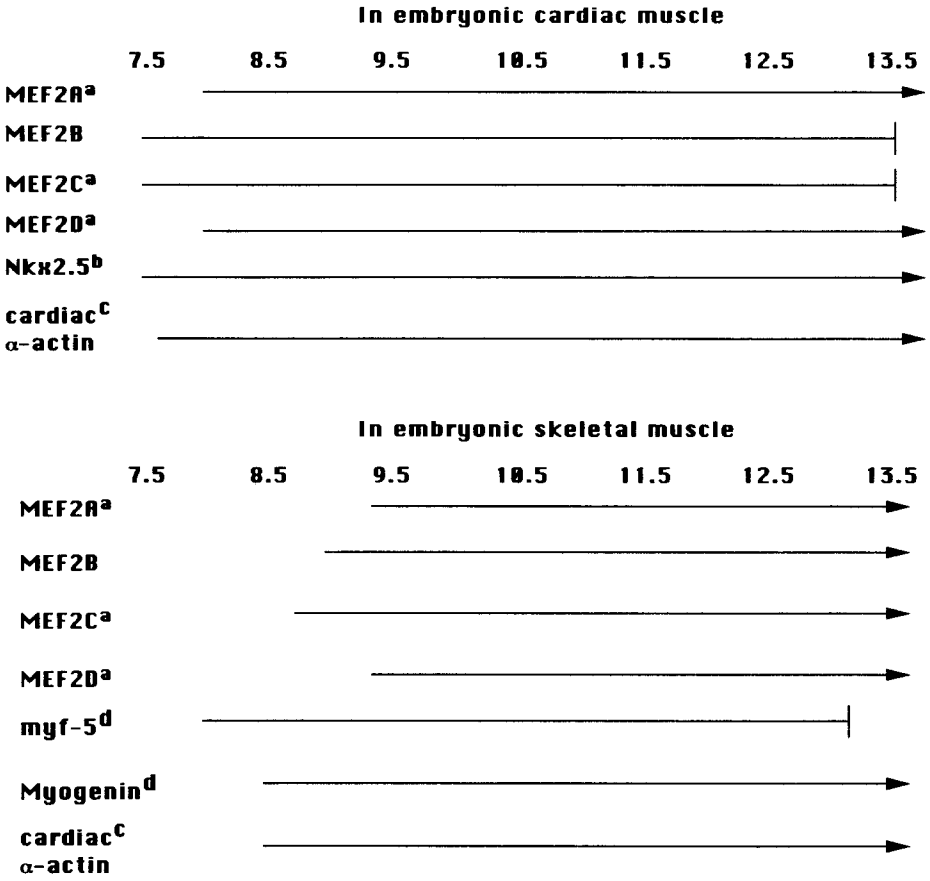


FIG. 8. Temporal patterns of *Mef2b* gene expression relative to those of other *Mef2* genes and other lineage markers in cardiac and skeletal muscle. Expression patterns of each gene during mouse embryogenesis (d.p.c.) are indicated by lines. Short vertical lines indicate that the level of mRNA falls below the level of sensitivity of the in situ technique. Sources for data not shown in this study are references 6 (a), 19 and 23 (b), 26 (c), and 35 (d).

correct time and place in the skeletal muscle lineage of *D-mef2* mutant embryos, but muscle structural genes are not expressed. Likewise, the homeobox genes *tinman* and *bagpipe*, which are required for the formation of cardiac and visceral muscle, are both expressed correctly in mutant embryos (22). Our current view is that MEF2 factors play a similar role in regulating the differentiation of skeletal, cardiac, and visceral muscle in higher organisms but that they probably do not play a role in specifying these cell lineages.

Transcription factors often belong to small families that have homology in their DNA binding and dimerization domains but diverge in other regions. This divergence allows related factors, which recognize the same DNA binding site, to activate different sets of target genes as a consequence of different protein-protein interactions mediated by their divergent regions. Now that the four vertebrate MEF2 factors have been cloned and characterized, it should be possible to determine whether they possess novel functions and regulate unique sets of target genes.

ACKNOWLEDGMENTS

We thank M. Perry for helpful comments on the paper, A. Tizenor for assistance with graphics, T. Davis for editorial assistance, and D. J. Gilbert for excellent technical assistance. We are grateful to Ron Prywes for antibodies to MEF2B and -D. Gary E. Lyons thanks Bruce K. Micales for excellent technical assistance.

This work was supported by grants from NIH, the Muscular Dystrophy Association, and the Human Science Frontiers Program to Eric Olson and in part by the National Cancer Institute, DHHS, under contract with ABL to Neal Copeland and Nancy Jenkins. Jeffery Molkentin was supported by an NIH postdoctoral fellowship. James Martin was supported by an NIH training grant. Anthony Firulli was supported by a postdoctoral fellowship from the Muscular Dystrophy Association, and Brian Black was supported by a postdoctoral fellowship from the American Cancer Society. Gary E. Lyons was supported by a grant from the Muscular Dystrophy Association.

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