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A *Mef2* Gene That Generates a Muscle-Specific Isoform via Alternative mRNA Splicing

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Members of the myocyte-specific enhancer-binding factor 2 (MEF2) family of transcription factors bind a conserved A/T-rich sequence in the control regions of numerous muscle-specific genes. Mammalian MEF2 proteins have been shown previously to be encoded by three genes, *Mef2*, *xMef2*, and *Mef2c*, each of which gives rise to multiple alternatively spliced transcripts. We describe the cloning of a new member of the MEF2 family from mice, termed MEF2D, which shares extensive homology with other MEF2 proteins but is the product of a separate gene. MEF2D binds to and activates transcription through the MEF2 site and forms heterodimers with other members of the MEF2 family. Deletion mutations show that the carboxyl terminus of MEF2D is required for efficient transactivation. MEF2D transcripts are widely expressed, but alternative splicing of MEF2D transcripts gives rise to a muscle-specific isoform which is induced during myoblast differentiation. The mouse *Mef2*, *Mef2c*, and *Mef2d* genes map to chromosomes 7, 13, and 3, respectively. The complexity of the MEF2 family of regulatory proteins provides the potential for fine-tuning of transcriptional responses as a consequence of combinatorial interactions among multiple MEF2 isoforms encoded by the four *Mef2* genes.

Many transcription factors belong to families in which different members bind the same DNA sequence and share homology within their DNA-binding and dimerization domains, but they diverge in other domains. The combinatorial control afforded by heterodimeric interactions among related factors that bind the same target site allows for fine-tuning of transcriptional responses and provides opportunities for positive and negative control. This type of complexity is exemplified by members of the myocyte-specific enhancer-binding factor 2 (MEF2) family, which bind as homo- and heterodimers to the DNA consensus sequence $CTA(A/T)_4TAG/A$, found in the control regions of numerous muscle-specific and growth factor-inducible genes (1, 8, 10, 15, 19, 21, 24, 29, 34–37, 39, 48, 50, 51). Three *Mef2* genes have been identified in mammals, each of which is subject to alternative splicing, giving rise to proteins that are highly homologous in their DNA-binding and dimerization domains (28, 31, 32, 40, 48).

MEF2 proteins belong to the MADS box family of transcription factors, named for MCM1, which regulates mating type-specific genes in yeast cells, *Agamous* and *Deficiens*, which function as homeotic genes in plants, and serum response factor, which regulates muscle-specific and serum-inducible gene expression (reviewed in references 40 and 43). Members of the MADS family share homology within a 56-amino-acid domain, the MADS box, which mediates DNA binding and dimerization. An additional 29-amino-acid sequence immediately adjacent to the MADS box, called the MEF2 domain, is also highly conserved among MEF2 proteins but is absent from other MADS box proteins (28, 31, 32, 40, 48).

Each of the MEF2 proteins cloned thus far appears to be subject to complex forms of regulation at multiple levels. The *Mef2* and *xMef2* (also known as *Mef2A* and *Mef2B*) genes are transcribed in a wide range of cell types. Alternative mRNA splicing of MEF2A gives rise to muscle-specific isoforms (40, 48). In contrast, MEF2C mRNA expression is primarily restricted to skeletal muscle, brain, and spleen (28, 31, 32). MEF2C mRNA also can be induced in nonmuscle cells by forced expression of the muscle-specific helix-loop-helix protein myogenin, consistent with the notion that the *Mef2C* gene may be a target for transcriptional activation by myogenic helix-loop-helix proteins (31). There is also evidence that expression of MEF2 mRNAs does not parallel expression of MEF2 proteins, suggesting that posttranscriptional mechanisms regulate MEF2 accumulation (48).

Here we describe the isolation of cDNA clones encoding a new member of the MEF2 family from mice, termed MEF2D, which shares extensive amino acid homology with other members of the MEF2 family within the MADS box and MEF2 domain but diverges outside of this region. MEF2D is closely related to the *Xenopus* MEF2 protein SL-1 (2) and may be its mammalian homolog. MEF2D binds the MEF2 consensus sequence with high affinity and is a potent transcriptional activator. Transcripts for MEF2D are widely expressed, but alternative splicing gives rise to a muscle-specific isoform. Gene mapping showed that the *Mef2a*, *Mef2c*, and *Mef2d* genes are located on mouse chromosomes 7, 13, and 3, respectively.

MATERIALS AND METHODS

Library screening. MEF2D cDNAs were isolated by screening an oligo(dT)-primed cDNA library in λZAP prepared from poly(A)$^+$ mRNA from adult mouse cardiac muscle (Stratagene). Screening was performed under conditions of reduced stringency as described previously (10). As a probe, we used an *Nco*I-*Hpa*I fragment of the human MEF2 cDNA RSRFC4 (a gift from Richard Treisman, Imperial Cancer Research Fund Laboratories, London, England). Positive clones were plaque purified and subcloned into Bluescript II (Stratagene) for

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DNA sequencing. Nested deletions were generated from the 5′ and 3′ ends by digestion with exonuclease III-mung bean nuclease, and both strands of the cDNAs were sequenced by using the Sequenase version 2 sequencing kit (United States Biochemical) or with an automated DNA sequencer (Applied Biosystems). Since the first six cDNAs encoding MEF2D were truncated at the 3′ end, PCR was used to obtain cDNAs encoding the 3′ end of the MEF2D transcript. An aliquot of a C2 myotube cDNA library (7) was screened by PCR (13), using T7 and T3 primers and MEF2D-specific primers. Several products were cloned by using the CloneAmp system (Bethesda Research Laboratories) and sequenced. The longest 3′ clone, which encoded MEF2D codons 137 to 374, was then used to screen a 13-day mouse embryonic cDNA library (Novagen). Multiple clones were isolated from this library, and three were characterized and found to contain the 3′ end of MEF2D. The sequences of the MEF2D-specific primers were as follows: M2D R1, 5′ TCA GGC GCT ATG GGT CAT CTG 3′; and M2D R2, 5′ CUA CUA CUA CUA GGC CCC CAA CTT TGC CAT GC 3′.

PCR cloning of an alternative exon. To screen for alternative exons immediately downstream of the MEF2 domain, an aliquot of the C2 myotube library was screened by PCR as previously described (13). After amplification, the PCR products were extracted with chloroform and ethanol precipitated. An aliquot of the amplified products was digested with *Xmn*I and analyzed by agarose gel electrophoresis. Undigested products were cloned by using the CloneAmp system. Sequences of primers used for amplification were as follows: primer 2, CAU CAU CAU CAU AAG GGA TGA TGT CAC CAG GG; and primer 3, CUA CUA CUA CUA CCA TGG GGA GGA AAA AGA TT.

Construction of plasmids. Full-length MEF2D isoforms were subcloned into the *Hin*dIII-*Eco*RI site of pcDNAI/A (Invitrogen, San Diego, Calif.). To create truncation mutant (TM) 375, a *Sac*I-*Eco*RI fragment of an independently cloned truncated cDNA was subcloned into the MEF2D1a2 pcD NAI/A expression vector. TM 354 was generated by subcloning an *Hin*dIII-*Pst*I fragment from the full-length MEF2D1a2 into the *Hin*dIII-*Nsi*I site of pcDNAI/A vector. Full-length pcDNA MEF2D1a2, which contains an internal *Nsi*I site, was digested with *Nsi*I and religated to generate TM 284. An independently cloned truncated MEF2D cDNA was cloned into the *Bam*HI-*Eco*RI site of pcDNAI/A to create TM 252. TM 153 was generated by PCR amplification using primers 2 and 3 and MEF2D1a2 as the template. The PCR fragment was subcloned into the pAmp1 vector by using the CloneAmp system (Bethesda Research Laboratories) and subsequently cloned into the *Hin*dIII-*Eco*RI site of pcDNAI/A.

RNA analysis. Total cellular RNA was isolated from cell lines and mouse tissues as described previously (12). A poly(A)$^+$ mRNA blot containing mouse tissue mRNAs was purchased from Clontech. For Northern (RNA) analysis, a restriction fragment of the MEF2D cDNA encompassing codons 137 to 374 was labeled with ^{32}P by random priming and hybridized at high stringency to RNAs fixed to nylon. To confirm equivalent loading of RNAs, a 417-bp human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Ambion, Austin, Tex.) was used as a probe. Under the hybridization and wash conditions, the MEF2D probe did not cross-hybridize with known members of the MEF2 family.

Reverse transcription PCR analysis of alternate exons 1a and 1b. To potentially identify alternate exons immediately 3′ to the MEF2 domain, as have been described for other MEF2 gene products (28, 31, 32, 40), 1 μg of total cellular RNA was used to synthesize cDNA by using reverse transcriptase (Bethesda Research Laboratories) and 500 ng of primer 3. cDNA synthesis was performed according to the manufacturer's instructions. PCR amplification was performed with 1 μl of cDNA and 100 ng of primers 1 and 2. The conditions used were 93°C for 30 s, 60°C for 25 s, and 72°C for 30 s for 30 cycles. After amplification, 1/10 of the PCR reaction was fractionated on a 1.5% agarose gel and transferred to a nylon membrane (Nytran; Amersham) under denaturing conditions. Nylon membranes were then probed with oligonucleotides A, B, and C. Oligonucleotide hybridization buffer was as detailed by Sambrook et al. (41). The sequences of oligonucleotides were as follows: primer 1, CUA CUA CUA CUA CAA GCT GTT CCA GTA TGC CAG; oligonucleotide A, AAG TAC CGG CGG GCC AGT GAG GA; oligonucleotide B, CCC CGA GGT GGA CGA GGC GTT TG; and oligonucleotide C, TTC AGC AAT CCA AGT AGC.

In vitro transcription and translation and gel mobility shift assays. For in vitro transcription and translation, MEF2D cDNAs were subcloned into pcDNAI/A (Invitrogen) and translated by using the TNT rabbit reticulocyte lysate in vitro translation system (Promega) in the presence or absence of [^{35}S]methionine for analysis of MEF2D proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or gel mobility shift, respectively. Gel mobility shift assays were performed as described previously (31), using an end-labeled oligonucleotide probe encompassing the MEF2 site from the muscle creatine kinase (MCK) enhancer (15). Assay mixtures contained 10,000 cpm of ^{32}P-labeled probe per sample. Sequence specificity of DNA binding was assessed by competition experiments with a 100-fold molar excess of the MEF2 site oligonucleotide or an oligonucleotide containing a mutant MEF2 site (mutant 6), which does not bind MEF2 (8). The sequences of sense strands of the oligonucleotides were as follows: MCK MEF2 site, GATCGCTCTAAAAATAACCC TGTCG; and mutant 6, GATCGCTGTAAACATAACCCTG TCG.

Transfections. Transient transfections were performed as described previously (12), using the reporter genes MHCemb/MEF2x2 and MHCemb/MEF2mtx2 (a gift from V. Mahdavi and B. Nadal-Ginard) (48). Transfections included 1 μg of a β-galactosidase reporter plasmid under control of the Rous sarcoma virus promoter as an internal control for transfection efficiency. Forty-eight hours following transfection, cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed, using aliquots of cell extracts normalized to β-galactosidase activity generated by the Rous sarcoma virus-*lacZ* reference plasmid. Transfections were performed at least three times with comparable results.

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described previously (5). A total of 205 N_2 mice were used to map three *Mef2* loci (see text below for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (25). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). Probes were labeled with [α-^{32}P] dCTP, using a random-priming labeling kit (Amersham) or a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, 65°C. The *Mef2a* probe, a human cDNA (48), detected major fragments of 10.0 kb in C57BL/6J (B) DNA and 8.4 kb in *M. spretus* (S) DNA following digestion with *Bgl*II. The *Mef2c* probe, a 225-bp *Eco*RI fragment of mouse cDNA (31), detected *Bgl*I fragments of >23 and 18.0 kb (B) and 15.0 kb (S). The *Mef2d*

probe, a 250-bp fragment of mouse MEF2D cDNA, detected major *Bgl*II fragments of 9.8 (B) and 5.3 (S) kb. Under the hybridization conditions used, none of the MEF2 probes cross-hybridized with other *Mef2* genes. The presence or absence of *M. spretus*-specific fragments was monitored in backcrossed mice.

Descriptions of the probes and restriction fragment length polymorphisms (RFLPs) for the loci used to position the *Mef* loci in the interspecific backcross have been reported; these include *Snrpn*, *Igf1r*, *Fes*, *Ntrk3*, and *Tyr*, chromosome 7 (13, 27, 45); *Il-9*, *Ntrk2*, *Nec-1*, and *Rasa*, chromosome 13 (4, 45); and *Fgg*, *Ntrk1*, and *Ngfb*, chromosome 3 (45). Recombination distances were calculated as described previously (16) by using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Isolation of MEF2D cDNA clones. To search for additional members of the MEF2 family, we screened a mouse cardiac muscle cDNA library under conditions of reduced stringency with a portion of the RSRFC4 (40) (MEF2A) cDNA encompassing the MADS box and MEF2 domain. Among the positive clones were six cDNAs that appeared to encode a novel MEF2-like protein (Fig. 1A). The mammalian MEF2 proteins identified previously have recently been redesignated MEF2A, MEF2B, and MEF2C (35a). We therefore named this new member of the family MEF2D. Because the cDNAs initially isolated were truncated at the 3′ end, we used a combination of PCR and conventional rescreening of a C2 myotube and a 13-day mouse embryonic cDNA library to obtain overlapping cDNAs. Comparison of these overlapping cDNAs encoding MEF2D revealed an uninterrupted open reading frame of 514 amino acids corresponding to a protein with an M_r of 55,000 (pI = 6.9). The predicted structure of the MEF2D protein compared with structures of other MEF2 proteins is depicted in Fig. 1B. Within the MADS box and MEF2 domain, MEF2D was identical to the *Xenopus* MEF2 protein SL-1 (2) and differed at only a few positions from mouse MEF2A, human MEF2B (40, 48), and mouse MEF2C (31) (Fig. 1C).

Over their entire lengths, the MEF2D and SL-1 proteins showed 73% homology, whereas there was substantially less homology between MEF2D and other MEF2 proteins outside the MADS and MEF2 domains. The region of MEF2D between the MEF2 domain and the carboxyl terminus was extraordinarily rich in serines, threonines, and prolines. Near the carboxyl terminus of MEF2D is a 40-amino-acid stretch of prolines and glutamines that resembles activation domains in other transcription factors (6, 33, 42). There were multiple consensus sites for phosphorylation within MEF2D, including sites for casein kinase II at residues 60 and 172, protein kinase C at residues 70, 121, 133, 227, 231, 265, 274, 443, and 512, calmodulin-dependent protein kinase II at residues 20, 121, 133, 180, 201, and 274, and protein kinase A at residues 121, 133, 180, 201, and 231 and many potential sites for the proline-dependent kinases (26).

A polyadenylation signal and poly(A) tail were identified approximately 1,500 nucleotides 3′ of the termination codon of the MEF2D cDNA (data not shown). Given the large sizes of the MEF2D transcripts (see below), it is likely that MEF2D transcripts contain long 5′ untranslated regions.

Several of the MEF2D cDNAs isolated lacked a 21-nucleotide region in frame at codons 286 to 292 (amino acids TGDHLDL), representing an alternatively spliced mRNA. This alternatively spliced exon was similar in position to exons identified in MEF2A and MEF2C cDNAs (31, 32, 40, 48). We refer to this alternate exon as exon 2 and to transcripts lacking this exon as Δ (see below). Other members of the MEF2 family are also subject to alternative splicing immediately 3′ of the MEF2 domain (31, 32, 40, 48). To determine whether MEF2D transcripts encode alternative exons at this position, we performed PCR on DNA from the C2 myotube cDNA library, using oligonucleotide primers flanking this region. All of our previously isolated cDNAs contained an *Xmn*I site within the region that would potentially encode this alternate exon. Therefore, following PCR, we digested the PCR products with *Xmn*I and analyzed them by electrophoresis on a 1% agarose gel. A large proportion of the PCR products were uncut by *Xmn*I. These 520-bp products were cloned and sequenced and found to encode an alternate 45-amino-acid exon, which is one codon shorter than the corresponding region of the original MEF2D isoform (Fig. 1A). We refer to the 46- and 45-amino-acid exons as 1a and 1b, respectively. Exon 1b shows 85% homology to alternate exon C4 of MEF2A (48). We did not detect any cDNAs lacking both alternate exons 1a and 1b, as has been reported for other MEF2 transcripts (31, 48). Only exons 1 and 2 have been identified in SL-1 (2).

MEF2D is expressed ubiquitously but has tissue-restricted splice variants. The pattern of MEF2D mRNA expression was determined by Northern analysis of RNA from tissues of adult mice, using a fragment of the MEF2D cDNA encompassing codons 137 to 374 (Fig. 2A). This cDNA probe hybridizes with all potential splice products of MEF2D but not with other MEF2 transcripts. In the tissues examined, two predominant MEF2D transcripts of ~3.5 and 6.5 kb were detected at the highest levels in skeletal muscle, heart, kidney, and brain (Fig. 2A). A minor transcript of ~2.5 kb was also observed in some cell types. SL-1 transcripts show a similar pattern of expression in adult *Xenopus* tissues (2). MEF2D transcripts were also expressed in C2 myoblasts and myotubes as well as in 10T1/2 and 3T3 fibroblasts, A7r5 smooth muscle cells, rat aortic smooth muscle cells, a glioblastoma cell line, and the PC12 pheochromocytoma cell line (Fig. 2B).

To analyze the tissue distribution of transcripts containing alternative exons 1a and 1b, reverse transcription PCR analysis was performed (Fig. 3). Amplification of this region of the MEF2D transcript from adult mouse tissues and cell lines demonstrated that exons 1a and 1b were expressed in a tissue-restricted manner. C2 myoblasts, PC12 cells, and adult kidney, stomach, uterus, and brain primarily expressed transcripts containing exon 1a. The alternative expression pattern was seen in C2 myotubes and skeletal muscle, which expressed high levels of transcripts containing exon 1b. Transcripts containing exons 1a and 1b were present at approximately equal levels in adult cardiac muscle. From this analysis, we conclude that exon 1b is expressed predominantly in muscle tissues, while exon 1a is widely expressed.

MEF2D binds the MCK MEF2 site. The in vitro translation product of MEF2D obtained following transcription and translation of MEF2D mRNA showed an M_r of ~60,000 (Fig. 4A). DNA-binding activity of MEF2D was tested in gel mobility shift assays using the in vitro translation product and a labeled oligonucleotide probe encompassing the MEF2 site from the MCK enhancer. Like other MEF2 proteins, MEF2D bound the MCK MEF2 site avidly (Fig. 4B). DNA binding by MEF2D was sequence specific and was competed for by an excess of unlabeled MEF2 site but not by a mutant of the MEF2 site that failed to bind MEF2 (Fig. 4B). The mobility-shifted complex formed with the in vitro translation product of MEF2D showed the same electrophoretic mobility as the MEF2 complex

A.

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CCTTCGCTGTTTCCCGTCGGAGCTGCGGCTTCGCGTAACGAGGATTCGGCGGACCGGGCCGAGGCTCCGGGCGCCGTGACACCCCGTCCCCCCACGGGCTGGAGGCTGTGCATAGGTGTT   120

CTGCAGACCATGAACTGATCACTAGTCCCCAGACATTCATGAGCACAGTGTGAGGCTCCTGATCACCACCCAGCAGCCCCTTTCCTCTCTGGCACTAAGGACCCCCGGAGAAGATGGGGA   240/2
                                                                                                                    M  G

GGAAAAAGATTCAGATCCAGCGAATCACTGATGAACGGAACCGCCAGGTGACCTTCACCAAGCGGAAGTTTGGACTGATGAAGAAGGCCTACGAGCTGAGTGTGCTGTGCGACTGCGAGA   360/42
R  K  K  I  Q  I  Q  R  I  T  D  E  R  N  R  Q  V  T  F  T  K  R  K  F  G  L  M  K  K  A  Y  E  L  S  V  L  C  D  C  E

TCGCGCTCATCATCTTCAACCACTCCAACAAGCTGTTCCAGTATGCCAGCACCGACATGGACAAGGTGCTGCTCAAGTACACCGAGTACAACGAGCCACACGAGAGCCGCACCAATGCTG   480/82
I  A  L  I  I  F  N  H  S  N  K  L  F  Q  Y  A  S  T  D  M  D  K  V  L  L  K  Y  T  E  Y  N  E  P  H  E  S  R  T  N  A

                  A  L  H  N  N  D  R  E  C  E  S  P  E  V  D  E  A  F  A  L  T  P  Q  T  E  E  K  Y  K  K  I  D  E  E  F  D
               GCGCTGCACAACAACGACAGGGAGTGTGAGAGCCCCGAGGTGGACGAGGCGTTTGCCCTGACCCCCCAGACGGAAGAGAAGTATAAAAAAGATAGACGAGGAGTTTGATA   600/122
ACATCATCGAGACCCTGAGGAAGAAGGGTTTCAACGGCTGTGACAGCCCAGAGCCGGATGGGGAGGACTCACTGGAGCAGAGCCCCCTGCTGGAGGACAAGTACCGGCGGGCCAGTGAGG
D  I  I  E  T  L  R  K  K  G  F  N  G  C  D  S  P  E  P  D  G  E  D  S  L  E  Q  S  P  L  L  E  D  K  Y  R  R  A  S  E

K  M  M  Q  S  Y  R  L  A
AGATGATGCAGAGTTATAGACTGGCC...
AGCTGGATGGGCTCTTCAGGCGCTATGGGTCATCTGTTCCGGCCCCCAACTTTGCCATGCCTGTCACAGTGCCCGTGTCCAATCAGAGCTCCATGCAGTTCAGCAATCCAAGTAGCTCTC   720/162
E  L  D  G  L  F  R  R  Y  G  S  S  V  P  A  P  N  F  A  M  P  V  T  V  P  V  S  N  Q  S  S  M  Q  F  S  N  P  S  S  S

TGGTCACTCCTTCCCTGGTGACATCATCCCTTACGGACCCACGGCTCCTGTCCCCCCAGCAGCCAGCACTACAGAGAAACAGTGTTTCTCCAGGCTTGCCCCAGCGGCCTGCTAGTGCAG   840/202
L  V  T  P  S  L  V  T  S  S  L  T  D  P  R  L  L  S  P  Q  Q  P  A  L  Q  R  N  S  V  S  P  G  L  P  Q  R  P  A  S  A

GAGCCATGCTGGGTGGAGACCTCAACAGTGCTAATGGAGCCTGCCCCAGCCCCGTTGGGAATGGCTATGTCAGTGCCCGAGCTTCCCCTGGCCTCCTCCCTGTGGCCAATGGCAACAGCC   960/242
G  A  M  L  G  G  D  L  N  S  A  N  G  A  C  P  S  P  V  G  N  G  Y  V  S  A  R  A  S  P  G  L  L  P  V  A  N  G  N  S

TAAACAAAGTCATCCCTGCCAAGTCTCCGCCCCCACCCACCCACAACACCCAGCTTGGAGCCCCCAGCCGCAAGCCTGATCTGCGGGTCATCACTTCCCAGGGAGGCAAAGGGTTAATGC   1080/282
L  N  K  V  I  P  A  K  S  P  P  P  P  T  H  N  T  Q  L  G  A  P  S  R  K  P  D  L  R  V  I  T  S  Q  G  G  K  G  L  M

      ACTGGGGACCATTTAGATCTG
ATCATTTG.....................AACAATGCCCAGCGCCTTGGGGTCTCCCAGTCTACCCACTCGCTCACCACCCCAGTGGTTTCCGTGGCAACACCAAGTTTACTCAGCCAGG   1200/322
H  H  L  T  G  D  H  L  D  L  N  N  A  Q  R  L  G  V  S  Q  S  T  H  S  L  T  T  P  V  V  S  V  A  T  P  S  L  L  S  Q

GCCTCCCCTTCTCCTCCATGCCCACTGCCTACAACACAGATTACCAGCTGCCCAGTGCAGAGCTATCCTCCTTACCAGCCTTCAGTTCACCTGCAGGGCTGGCACTAGGCAATGTCACCG   1320/362
G  L  P  F  S  S  M  P  T  A  Y  N  T  D  Y  Q  L  P  S  A  E  L  S  S  L  P  A  F  S  S  P  A  G  L  A  L  G  N  V  T

CCTGGCAGCAGCCCCAGCCGCCCCAGCAGCCACAACCGCCACAACCGCCACAGTCACAGCCACAGCCACCACAGCCACAGCCACAGCAGCCACCTCAGCAACAGCCCCACTTGGTCCCCG   1440/402
A  W  Q  Q  P  Q  P  P  Q  Q  P  Q  P  P  Q  P  P  Q  S  Q  P  Q  P  P  Q  P  Q  P  Q  Q  P  P  Q  Q  Q  P  H  L  V  P

TTTCTCTCAGCAACCTCATCCCTGGCAGCCCCTTGCCTCACGTGGGTGCTGCTCTCACAGTCACTACCCACCCCCACATCAGCATCAAGTCAGAACCAGTGTCCCCAAGTCGTGAACGCA   1560/442
V  S  L  S  N  L  I  P  G  S  P  L  P  H  V  G  A  A  L  T  V  T  T  H  P  H  I  S  I  K  S  E  P  V  S  P  S  R  E  R

GCCCTGCACCTCCTCCACCAGCTGTGTTCCCAGCTGCCCGCCCTGAGCCTGGCGAAGGTCTCAGCAGCCCAGCTGGAGGATCCTATGAGACCGGGGACCGGGATGATGGACGGGGGGACT   1680/482
S  P  A  P  P  P  P  A  V  F  P  A  A  R  P  E  P  G  E  G  L  S  S  P  A  G  G  S  Y  E  T  G  D  R  D  D  G  R  G  D

TTGGGCCCACACTAGGCCTGCTGCGCCCAGCCCCAGAGCCTGAGGCTGAGGGCTCAGCTGTGAAGAGGATGCGGCTGGATACTTGGACATTAAAGTGATGGTTCCCACTC   1790/514
F  G  P  T  L  G  L  L  R  P  A  P  E  P  E  A  E  G  S  A  V  K  R  M  R  L  D  T  W  T  L  K
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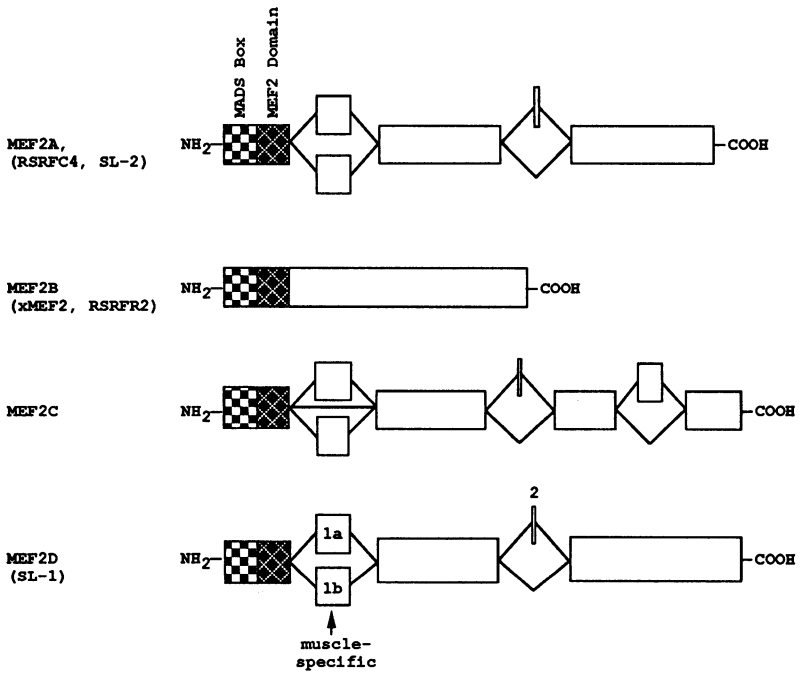
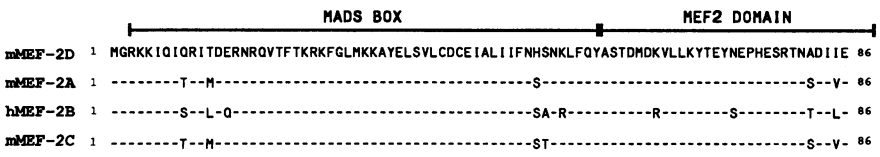
B.



C.



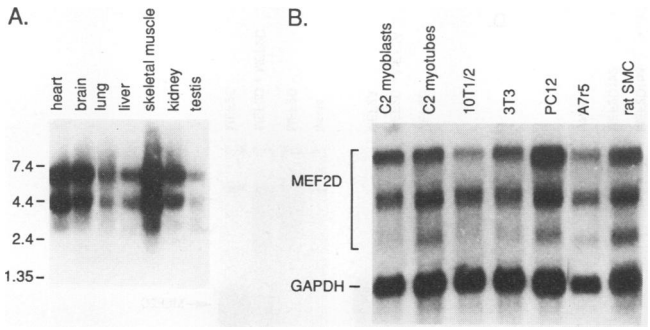


FIG. 2. Expression patterns of MEF2D. (A) Two micrograms of poly(A)$^+$ mRNA from the indicated tissues from adult mice was analyzed by Northern blotting, using a fragment of MEF2D cDNA containing codons 137 to 374 as a probe under high-stringency conditions. Hybridization with a GAPDH probe confirmed equivalent amounts of RNA in each lane (data not shown). Sizes are indicated in kilobases. (B) Twenty micrograms of total RNA from the indicated cell lines was analyzed by Northern blotting, using probes for MEF2D and GAPDH. Three MEF2D transcripts of 6.5, 3.5, and 2.5 kb are detected.

observed with nuclear extracts from C2 myotubes (Fig. 4B). The four MEF2D isoforms appeared to bind the MCK MEF2 site with similar avidity (Fig. 4C).

MEF2D heterodimerizes with other MEF2 gene products. To determine whether MEF2D could heterodimerize with other MEF2 gene products, we cotranslated MEF2D with truncation mutants of MEF2A and MEF2C and performed gel mobility shift assays using a labeled MEF2 site as a probe. As shown in Fig. 4D, DNA-protein complexes with mobilities intermediate between those of MEF2D and truncated MEF2A or MEF2C were observed, consistent with the conclusion that MEF2D dimerizes with these other MEF2 gene products. Since it was necessary to test for heterodimerization by using truncated MEF2 proteins, it is conceivable, though seemingly unlikely, that the dimerization properties of the proteins could be altered by C-terminal deletions.

Transactivation by MEF2D. The potential of MEF2D to activate transcription through the MEF2 site was tested by transient transfection of 10T1/2 cells with a MEF2D expression vector and reporter genes containing multimerized MEF2 sites upstream of the embryonic myosin heavy-chain promoter (MHCemb/MEF2x2). Expression of this reporter was dramatically increased in the presence of MEF2D (Fig. 5). Transactivation by MEF2D was dependent on the MEF2 sites and was not observed with the myosin heavy-chain promoter linked to a mutant MEF2 site (MHCemb/MEF2mtx2).

MEF2D proteins containing alternative exons 1a and 1b were equally efficient in activating transcription through the MEF2 site (Fig. 6A). Similarly, alternative exon 2 near the carboxyl terminus did not significantly influence the transcriptional activity of MEF2D with the reporter plasmid noted above.

We also compared a series of MEF2D deletion mutants for

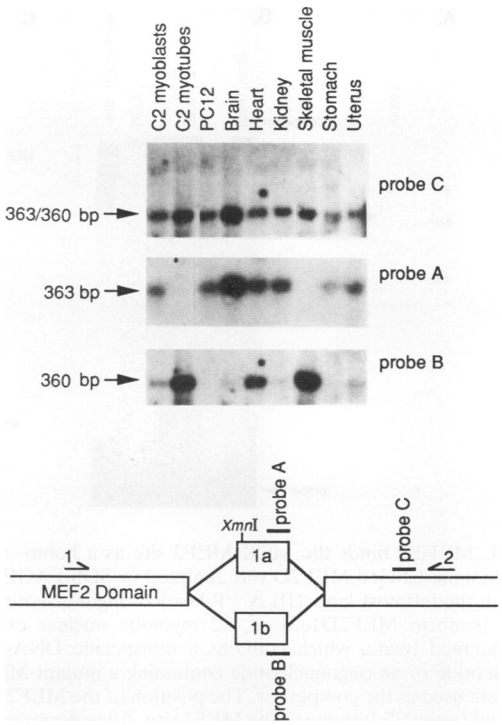


FIG. 3. Detection of a muscle-specific exon in MEF2D by reverse transcription PCR. One microgram of total RNA from the indicated cell lines and tissues was analyzed by reverse transcription PCR. cDNA was synthesized by using primer 2. Amplification of cDNA was then performed with primers 1 and 2. PCR products were detected by Southern blot analysis using oligonucleotide probes A, B, and C, which hybridize to the regions shown in the diagram. Probe C was used to identify total amplified products. Probes A and B hybridized to exons 1a and 1b, which were contained in PCR products of 363 and 360 bp, respectively.

their relative abilities to activate expression of MHCemb/MEF2x2. Deletion of the carboxyl terminus led to a dramatic decrease in transcriptional activity, without affecting DNA binding (Fig. 6), suggesting that this region contains a transcription activation domain. TM 375, which deletes most of the proline- and glutamine-rich region of MEF2D, has approximately one-half of the transcriptional activation potential of wild-type MEF2D1a2. Removal of another 21 amino acids, producing TM 354, completely deletes the proline/glutamine-rich domain and results in a loss of the majority of the transcriptional activity of MEF2D1a2. Transactivating potential is completely lost when all residues C terminal to amino acid 153 are deleted (Fig. 6B).

Chromosomal mapping of mouse *Mef2* genes. The murine chromosomal locations of three *Mef2* genes, *Mef2a*, *Mef2c*, and *Mef2d*, were determined by using an interspecific backcross mapping panel derived from crosses of (C57BL/6J × *M.*

FIG. 1. Open reading frame and structure of MEF2D. (A) Nucleotide sequence and deduced open reading frame of mouse MEF2D. Amino acids 286 to 292 are encoded by an alternate exon (designated 2) present in some cDNAs. Amino acids and nucleotides above codons 87 to 132 correspond to the sequence of alternate exon 1b. Dots within the sequence denote nucleotides that are absent from certain splice variants. The MADS and MEF2 domains are underlined with thick and thin lines, respectively. (B) Schematic representation of MEF2 proteins described to date. The MADS (checkered) and MEF2 (cross-hatched) domains are indicated at the amino terminus of each protein. Alternative exons are shown. Sources of amino acid sequences from which the structures were derived are MEF2A (RSRFC4) (48), MEF2B (RSRFR2) (48), and MEF2C (31, 32). (C) Homology among mammalian MEF2 proteins within the MADS and MEF2 domains. The mouse MEF2A (mMEF2A) sequence is from reference 30a, the human MEF2B (hMEF2B) sequence is from references 40 and 48, and the mouse MEF2C (mMEF2C) sequence is from reference 31. Amino acid identities with MEF2D are indicated by dashes.

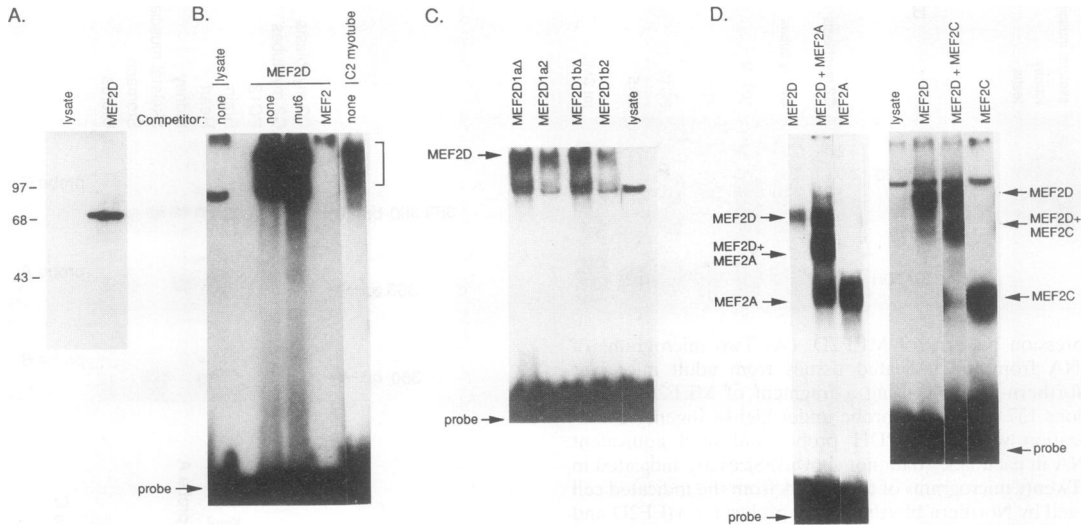


FIG. 4. MEF2D binds the MCK MEF2 site as a homo- or heterodimer. (A) MEF2D mRNA was transcribed and translated in vitro, and [^{35}S]methionine-labeled MEF2D was analyzed by SDS-PAGE. Molecular weight markers are indicated to the left in thousands. Unprogrammed lysate is in the leftmost lane. (B) A ^{32}P-labeled oligonucleotide probe encompassing the MCK MEF2 site was incubated with in vitro-translated MEF2D (isoform MEF2D1a2) or C2 myotube nuclear extracts, and DNA-protein complexes were analyzed by gel mobility shift assay. Unprogrammed lysate, which contains a nonspecific DNA-binding activity, is in the leftmost lane. A 100-fold excess of unlabeled MEF2 oligonucleotide or an oligonucleotide containing a mutant MEF2 site (mutant 6 [mut6]) that binds ubiquitous nuclear factors but does not bind MEF2 were used as the competitor. The position of the MEF2 complex is indicated by a bracket. (C) The four isoforms of MEF2D were translated in vitro and tested for binding to the MEF2 site. All isoforms appeared to bind with comparable affinities. Unprogrammed lysate is in the rightmost lane. (D) MEF2D deletion mutant TM 252 (derived from isoform MEF2D1a) was translated alone or in the presence of a MEF2A or MEF2C deletion mutant containing the first 144 or 117 residues, respectively, of these proteins. A truncated form of MEF2D was used because the full-length protein gave rise to a complex that comigrated with the endogenous reticulocyte lysate binding activity when mixed with MEF2A or MEF2C truncation mutants. We have observed no differences in DNA-binding or dimerization of full-length MEF2D or MEF2D truncation mutant TM 252. Translation reactions were tested for DNA-binding activity by using the MEF2 site as a probe. Positions of the different MEF2-DNA complexes are indicated beside each autoradiograph.

spretus)F_1 with C57BL/6J mice. cDNAs representing each of the loci were used as probes in Southern blot hybridization analysis of C57BL/6J and *M. spretus* genomic DNAs that were separately digested with several different restriction enzymes to identify informative RFLPs useful for gene mapping (see Materials and Methods). The strain distribution pattern of each RFLP in the interspecific backcross was then determined by monitoring the presence or absence of RFLPs specific for *M. spretus* in backcross mice. Each of these genes mapped to a single chromosome location. The mapping results (Fig. 7) assigned the three loci to three different mouse autosomes, indicating that the *Mef2* genes have become well dispersed during evolution.

Mef2a mapped to the central region of mouse chromosome 7 and did not recombine with *Igflr* in 163 mice typed in common. This finding suggests the two loci are within 1.8 centimorgans (cM) of each other (upper 95% confidence interval). *Mef2c* mapped toward the distal region of mouse chromosome 13, 1.1 cM distal of *Nec-1* and 0.6 cM proximal of *Rasa*. *Mef2d* mapped to the central region of mouse chromosome 3 and did not recombine with *Ntrk1* in 179 mice typed in common, suggesting the two loci are within 1.7 cM (upper confidence limit).

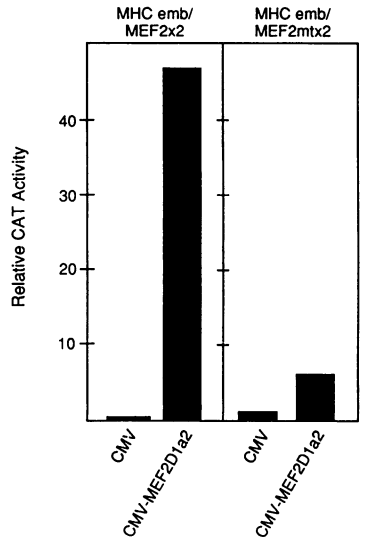


FIG. 5. MEF2D is a transcriptional activator. 10T1/2 cells were transiently transfected with 5 μg of the indicated reporter genes in the presence or absence of 10 μg of the MEF2D expression vector (isoform MEF2D1a2) or the vector alone. Following transfection, CAT activities were determined as described in Materials and Methods. Values are expressed relative to the level of CAT expression from MHCemb/MEF2x2 with the empty expression vector, which was set at 1. A representative experiment is shown. Comparable results were obtained in several independent experiments. CMV, cytomegalovirus.

DISCUSSION

MEF2 was originally described as a muscle-specific DNA-binding activity that recognizes an A/T-rich element within the control regions of numerous muscle-specific genes (15). Recently, three different genes, designated *Mef2a*, *Mef2b*, and *Mef2c*, have been shown to encode proteins with DNA-binding

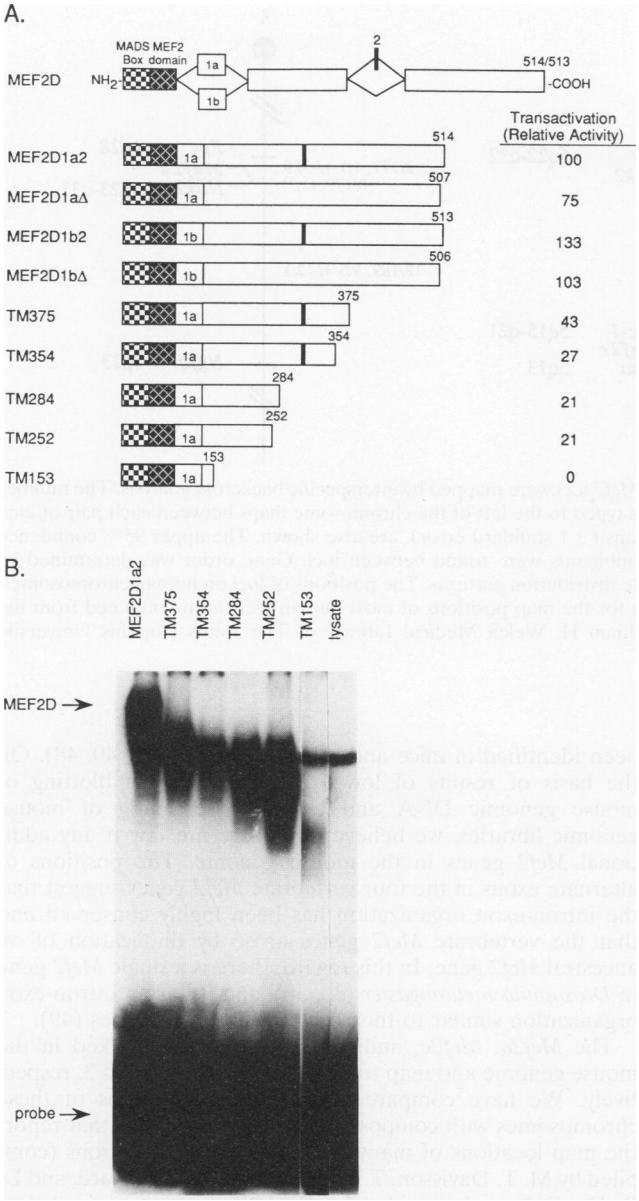


FIG. 6. The C terminus of MEF2D is required for efficient transcriptional activation. (A) 10T1/2 cells were transfected with 5 μg of MHCemb/MEF2x2 and 10 μg of expression vectors encoding each MEF2D isoform or MEF2D truncation mutants derived from MEF2D1a2. CAT activities were determined as for Fig. 5. Values are expressed relative to the level of expression with full-length MEF2D1a2. The results represent averages of at least three independent transfections. The number of amino acids contained within each deletion mutant is indicated at the upper right of each diagram, and the name of the mutant is on the left. (B) The ability of MEF2D truncation mutants to bind DNA was examined by gel mobility shift assay. In vitro-translated MEF2D truncation mutants were incubated with a ^{32}P-labeled MEF2 site and fractionated on a native acrylamide gel. The arrow denotes the full-length MEF2D1a2 complex. Rabbit reticulocyte lysate is in the rightmost lane.

specificities identical to that of MEF2 (28, 31, 32, 40, 48). These *Mef2* gene products share extensive amino acid homology within their DNA-binding domains and belong to the MADS family of transcriptional regulators. We have cloned a fourth member of the MEF2 family of transcription factors from mice, MEF2D, that is highly homologous to other MEF2 proteins in the MADS and adjacent MEF2 domains but contains a unique carboxyl terminus. Among the *Mef2* gene products identified to date, MEF2D shows greatest homology to the *Xenopus* MEF2 protein SL-1 (2) and is likely to represent the mammalian homolog of SL-1.

MEF2 proteins are potent transactivators. Like other MEF2 proteins, MEF2D binds the MEF2 consensus sequence [CTA(A/T)$_4$TAG/A] and activates transcription through that site. Deletion mutations indicate that the C terminus of MEF2D is required for efficient transactivation and that mutants lacking this region, but containing the MADS and MEF2 domains, retain the ability to bind DNA. These transcriptionally inactive mutants may be useful for dissecting the functions of MEF2 in cells because they would be predicted to function in a dominant negative manner by competing with wild-type proteins for binding to MEF2 sites associated with muscle target genes. Consistent with the importance of the carboxyl terminus of MEF2D for transactivation, recent experiments have demonstrated that this region functions as a transactivation domain when fused to the DNA-binding domain of yeast GAL4 (30a).

A common property of MADS proteins is their ability to cooperate with other transcriptional regulators to control gene expression (20, 44). Serum response factor, for example, can recruit Ets and homeodomain proteins to the serum response element, resulting in combinatorial control of transcription (9, 18; reviewed in reference 46). Given the structural similarity between serum response factor and members of the MEF2 family, it is tempting to speculate that MEF2 proteins may also regulate gene expression through combinatorial mechanisms. The recent demonstration that MEF2 and myogenic basic helix-loop-helix proteins associate on DNA raises the possibility that these two classes of myogenic transcription factors collaborate to induce muscle-specific transcription (14). That certain MEF2 sites also bind the mesodermal homeodomain protein MHox suggests additional possibilities for cross-talk between MEF2 proteins and other regulators (7).

There are several isoforms of MEF2D that arise by alternative mRNA splicing. Alternative splicing of exons immediately C terminal to the MEF2 domain yields a ubiquitous and a muscle-specific isoform (designated exons 1a and 1b, respectively). Closer to the C terminus, a second alternative exon (designated exon 2) is present in some transcripts and excluded from others. The different MEF2D isoforms showed similar DNA-binding properties and comparable transcriptional activities when tested for their abilities to activate transcription through tandem copies of the MEF2 site; whether they might differentially regulate native muscle target genes remains to be determined.

Consistent with previous studies, which showed that MEF2 proteins bind DNA as dimers (32, 40), MEF2D can heterodimerize with MEF2A and MEF2C. Assuming that all of the alternative isoforms encoded by the four *Mef2* genes are able to homo- and heterodimerize, there is the potential for greater than 100 combinations of MEF2 dimers. Thus, this family of regulators provides enormous possibilities for combinatorial control of gene expression.

MEF2 mRNAs are expressed more widely than MEF2 proteins. MEF2D transcripts are expressed in a wide range of cell and tissue types and are present at the highest levels in skeletal muscle, heart, kidney, and brain. The presence of MEF2D mRNA in undifferentiated myoblasts as well as nonmuscle cells, which have been reported to contain little or no MEF2 DNA-binding activity (15), suggests that posttranscrip-

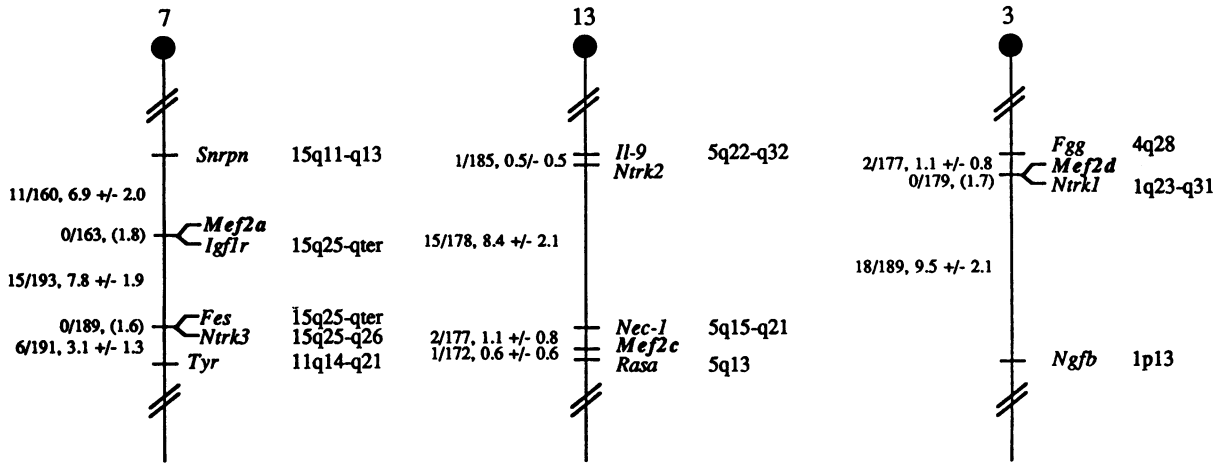


FIG. 7. Chromosomal locations of three *Mef2* loci in the mouse genome. *Mef2* loci were mapped by interspecific backcross analysis. The number of recombinant N_2 animals is presented over the total number of N_2 animals typed to the left of the chromosome maps between each pair of loci. The recombination frequencies, expressed as genetic distance in centimorgans (± 1 standard error), are also shown. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants were found between loci. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. The positions of loci on human chromosomes, where known, are shown to the right of the chromosome maps. References for the map positions of most human loci can be obtained from the genome data base of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, Md.).

tional mechanisms contribute to the regulation of MEF2D expression during myogenesis. It remains unclear why MEF2 mRNA in nonmuscle cells does not give rise to MEF2 DNA-binding activity. However, it is intriguing that alternate exon 1b, which is specific to skeletal muscle and heart, correlates with MEF2 DNA-binding activity.

It should be pointed out that there is disagreement about the cell type distribution of MEF2 DNA-binding activity. Some reports have shown MEF2 binding and transcriptional activity to be highly enriched in skeletal muscle and heart (7, 8, 10, 21, 29, 31, 32, 34–37, 48, 50, 51), consistent with the initial description of MEF2 (15). Others have found MEF2 DNA-binding activity to be ubiquitous (1, 19, 22, 23, 40). The basis for this discrepancy between MEF2 mRNA and DNA-binding activities remains unexplained.

Perhaps the best evidence for muscle-specific expression of MEF2 has come from studies of *Xenopus laevis*, in which transcripts for the MEF2D and MEF2A homologs, SL-1 and SL-2, respectively, are expressed specifically in the somitic mesoderm of early embryos and myotomes of tadpoles (2). During later development, the SL-1 and SL-2 transcripts become expressed ubiquitously. Recent studies have shown that the mouse MEF2 genes show similar restriction to the early somite myotomes, as well as the heart, and later become expressed more widely (11). The localized expression of MEF2 transcripts to the somite myotome strongly suggests that members of the MEF2 family may participate in the early events associated with skeletal muscle determination or differentiation, a hypothesis supported by the finding that an MEF2 site in the promoter of the mouse myogenin gene is required for activation of myogenin gene transcription in somites of mouse embryos (3, 47). It will be interesting to determine whether the pattern of MEF2 mRNA expression in the embryo correlates with MEF2 DNA-binding and transcriptional activities and whether tissue-specific alternative splicing gives rise to specific MEF2 isoforms in early muscle cell precursors.

The *Mef2a*, *-c*, and *-d* genes map to different chromosomes. With the cloning of MEF2D, four separate *Mef2* genes have been identified in mice and humans (28, 30a–32, 40, 48). On the basis of results of low-stringency Southern blotting of mouse genomic DNA and extensive screening of mouse genomic libraries, we believe that there are few if any additional *Mef2* genes in the mouse genome. The positions of alternate exons in the four vertebrate *Mef2* genes suggest that the intron-exon organization has been highly conserved and that the vertebrate *Mef2* genes arose by duplication of an ancestral *Mef2* gene. In this regard, there is a single *Mef2* gene in *Drosophila melanogaster* (30, 38), and it has an intron-exon organization similar to those of the vertebrate genes (49).

The *Mef2a*, *Mef2c*, and *Mef2d* genes are unlinked in the mouse genome and map to chromosomes 7, 13, and 3, respectively. We have compared our interspecific maps of these chromosomes with composite mouse linkage maps that report the map locations of many uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle and provided by GBASE, a computerized data base maintained at The Jackson Laboratory, Bar Harbor, Maine). The *Mef2* genes mapped in regions of the composite map that contain mouse mutations (data not shown). For example, *Mef2a* is near several mutations, including hepatic fusion (*hf*), a recessive mutation associated with a developmental anomaly in which the left and central lobes of the liver are fused (17). *Mef2c* is in the vicinity of furless (*fs*), a recessive mutation associated with reduced viability prior to weaning and loss of the coat in adult mice (17). *Mef2d* is also near several mutations, including spastic (*spa*), a recessive neurological mutation in which mice display tremors and stiffness of posture (17). As detailed developmental and adult expression patterns of the *Mef2* genes are established, it will be possible to evaluate whether any of the mouse mutations are candidates for alterations in a *Mef2* locus.

The human chromosomal locations of the *Mef2* loci can be predicted on the basis of known mouse-human linkage homologies (Fig. 7). *Igflr* has been assigned to human chromosome 15q25-qter. The tight linkage between *Igflr* and *Mef2a* in the mouse suggests that the human homolog of *Mef2a* will reside

on 15q as well (Fig. 7). Similarly, *Mef2c* is likely to map to human chromosome 5q, and *Mef2d* is likely to map to 1q (Fig. 7). It is interesting that each of the *Mef2* genes mapped in the mouse is in the vicinity of a neurotrophin receptor (*Ntrk*) gene. Perhaps this represents an ancient homology unit that has, during the course of evolution, become dispersed to several different mammalian autosomes. This notion can be explored further by confirming the human chromosomal locations of *Ntrk3* and the *Mef2* genes.

Given the diversity and distinct expression patterns of MEF2 proteins, it is likely that members of this family act at multiple stages of development to control gene expression and that subtle differences among the different MEF2 proteins allow them to confer specialized patterns of regulation to their target genes. The challenge for the future will be to define the specific functions of individual MEF2 proteins and to determine how these proteins interact with other components of the transcriptional machinery to coordinate the expression of their target genes. Gene targeting of individual members of the MEF2 family in cultured cells and transgenic mice should shed light on these issues.

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ADDENDUM

After submission of the manuscript, Breitbart et al. reported the isolation of cDNAs for human MEF2D (1a).

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