Determination of the Consensus Binding Site for MEF2 Expressed in Muscle and Brain Reveals Tissue-specific Sequence Constraints

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The myocyte-specific enhancer factor-2 (MEF2) proteins are expressed in the three major types of muscle (skeletal, cardiac, and smooth) and function as transcriptional activators of muscle-specific and growth factor-regulated genes through binding to a canonical A/T-rich cis-element. Although MEF2 proteins are also expressed in brain, MEF2-regulated muscle-specific gene products are not detected in this tissue. To gain insight into the regulation of MEF2 function in vivo, we have selected its optimal DNA targets from a library of degenerate oligonucleotides using anti-MEF2A antibodies and cell extracts from skeletal muscle, heart, and brain. The consensus binding site in these three tissues contains an indistinguishable core motif, 5′-CT(a/t)a(t)AAATAG-3′. However, the optimal target for MEF2 expressed in the brain shows additional sequence constraints (5′-TGTTACT(a/r)a(t)AAATAGA(a/t)-3′) that are not observed in the sequences selected with skeletal and cardiac muscle extracts. Thus, differences in DNA binding preferences of MEF2 proteins in muscle and brain may contribute to tissue-specific gene expression during myogenesis and neurogenesis.

In recent years, two families of transcriptional regulators involved in controlling myogenesis have been identified: the MyoD basic helix-loop-helix (bHLH) factors (1, 2) and the MADS box-containing proteins named myocyte-specific enhancer factor-2 (MEF2) or related to serum response factor (RSRF) (3–11). Whereas the bHLH family members appear to be restricted to skeletal muscle, MEF2 proteins have been implicated in transcriptional activation in the three types of muscle (skeletal, cardiac, and smooth). MEF2 proteins are encoded in mammals by four genes, MEF2a, -b, -c, and -d, which display a complex pattern of alternative splicing (3–8, 10, 11). In contrast, a single mef2 gene has been detected in Drosophila (12, 13), which is required in the formation of the three major muscle types in embryos (14).

MEF2 factors activate transcription via binding to a consensus A/T-rich cis-element widely found in the control regions of muscle-specific and growth factor-induced genes (3, 15). Although MEF2 proteins are predominantly expressed in muscle and brain (4–7, 15, 16), MEF2-regulated muscle-specific gene products are not detected in the nervous system. Thus, we reasoned that differences in binding requirements in muscle and brain may contribute to tissue specificity of MEF2 function. To test this hypothesis, we have determined the consensus binding site for MEF2 expressed in skeletal muscle, heart, and brain by CASTing (Cyclical Amplification and Selection of Targets) (17), a technique that has proven useful to elucidate mechanisms of tissue-specific regulation of gene expression (18). Analysis of these optimal DNA targets revealed brain-specific sequence constraints that might be relevant for MEF2 function.

MATERIALS AND METHODS

Cell Culture and Preparation of Protein Extracts—C2C12 skeletal myoblasts (American Type Culture Collection) were grown at subconfluent densities in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum. For myotube formation, cells were grown to 70–80% confluence and then transferred to 2% heat-inactivated horse serum in cold phosphate-buffered saline, frozen in liquid nitrogen, and stored at −80°C until homogenization. Preparation of whole cell extracts in 10 mM HEPES-KOH, 0.5 mM dithiothreitol, 1 mM EDTA, 10% (v/v) glycerol, 1 μg/ml leupeptin (pH 7.8) was carried out as previously described (19). Protein concentration was determined using the modified Bradford assay (BioRad).

 Antibodies—The following rabbit polyclonal anti-MEF2 antibodies were used in this study: anti-MEF2A (9), anti-MEF2C (7), and anti-MEF2D (5). Their specificity for the corresponding MEF2 isoforms has been demonstrated by Western immunoblot and indirect immunofluorescence analyses on COS cells transiently transfected with MEF2 expression vectors.

 Gel Retardation Assays—For electrophoretic mobility shift assays, a double-stranded synthetic oligonucleotide containing a canonical MEF2-binding site (5′-TCAGATTAGAAAAATCGACCTG-3′, MEF2 site underlined) was end-labeled with T4 polynucleotide kinase. Whole cell extracts (10–20 μg of protein) were preincubated for 5 min at room temperature with 50 μg/ml of each poly(dI:dC) and unrelated single-stranded oligonucleotide in binding buffer (20 mM HEPES-KOH, 0.1 mM KCl, 1 mM dithiothreitol, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mg/ml bovine serum albumin (pH 7.8)). Then, 10 fmol of probe, simultaneously with preimmune or immune serum where indicated, was added for a final reaction volume of 20 μl. Incubation was continued for 20 min at 37°C and the samples were electrophoresed at 4°C in 4% native polyacrylamide gels (80:1, acrylamide/bisacrylamide ratio) in 0.5 × TBE (0.045 M Tris borate, 0.001 M EDTA (pH 7.5)). Gels were dried and exposed to x-ray film.

 CASTing—The procedure for CASTing was essentially as described previously (18, 20). In brief, 15 μg of a double-stranded oligonucleotide containing a degrading core of 40 base pairs flanked by defined sequences with restriction sites (5′-GC-SalI-HindII-XbaI-NcoI-EcoR1-BamHI-XhoI-GG-3′) was incubated with whole cell extract and 5 μg of sonicated calf thymus DNA in 20 μl of binding buffer (see above). After
MEF2 Optimal Targets in Muscle and Brain

Fig. 1. MEF2A is the predominant MEF2 DNA binding activity present in muscle and brain. Electrophoretic mobility shift assays were performed using a MEF2 oligonucleotide probe and the indicated extracts and sera (PI, preimmune serum; Imm, immune serum). C2C12 myotubes were used as the source of skeletal muscle. Heart and brain extracts were prepared from 16-day-old mouse embryos. Only the retarded nucleoprotein complexes are shown.

RESULTS AND DISCUSSION

To characterize the MEF2 DNA binding activities present in muscle and brain, we performed electrophoretic mobility supershift assays with a radiolabeled MEF2 oligonucleotide and polyclonal antibodies specific for MEF2A (9), MEF2C (7), and MEF2D (5). Whereas preimmune sera had no effect in these assays, anti-MEF2A antibodies quantitatively supershifted the nucleoprotein complexes detected in extracts from C2C12 myotubes (skeletal muscle) and embryonic heart and brain (Fig. 1). In contrast, anti-MEF2C and anti-MEF2D antibodies only partially supershifted these complexes. Given that MEF2 proteins are assembled in homo- and heterodimers (3, 10, 16), the results of these supershift assays suggest that MEF2A-containing complexes are predominant in skeletal muscle, heart, and brain.

We next sought to determine the optimal targets for MEF2 in muscle and brain tissues using the CASTing technique (17). MEF2 optimal binding sites were selected from a pool of oligonucleotides containing a degenerate core using crude cell extracts and anti-MEF2 antibodies to isolate the specific MEF2-DNA complexes. The selected sequences were then PCR-amplified and mixed with fresh extract, and the process was repeated eight times to enrich for optimal binding sites. Finally, the selected sequences were cloned and individual clones were sequenced. Since MEF2A appears to be the major MEF2 DNA binding activity in these tissues (Fig. 1), anti-MEF2A antibodies were used in this study. However, this procedure should enable the isolation of MEF2-DNA complexes involving both MEF2A homodimers and MEF2A-containing heterodimers. Fig. 2 shows the sequence of individual oligonucleotides selected with skeletal muscle, heart, and brain extracts, which were aligned by the A/T-rich motif they contained and grouped according to the sequence of the central quartet of A/T bases. An analysis of the relative frequency of bases at each position generated the MEF2 consensus binding site shown in Fig. 3. The three tissues analyzed show an indistinguishable core motif, 5′-CT(A/T)(a/t)AAAATAG-3′ (positions 1–10), which conforms to previously identified MEF2 sites contained within skeletal and cardiac muscle-specific control regions that have been shown to be important for expression of the corresponding genes (15). For example, the majority of oligonucleotides bearing the central quartet TAAA (Fig. 2) conform to the MEF2 site found in the regulatory regions of the muscle-specific genes desmin, AMP deaminase, and Xenopus MyoD (5′-CTATA-AATA(G/C)-3′) (15, 21). Furthermore, some selected oligonucleotides correspond to the recently identified MEF2 site in the gax homeobox gene (C144c, C10, C53b, and rC133; Fig. 2A) (22).

The MEF2 consensus binding site reported here also conforms to the optimal MEF2 target determined previously employing truncated derivatives of cloned RSFR2 (MEF2B), RSRC4 (MEF2A), and SL1 (Xenopus MEF2D) (3, 23). However, the present study using crude extracts as a source for MEF2 reveals a preference for the sequence AATA (TTTA; 34, 47, and 35% for RSFR2, RSRFC4, and SL1, respectively), with only 20–22% of the selected oligonucleotides containing the sequence AATA. These differences might be due to the use of different MEF2 isoforms and/or truncated proteins in these previous studies. Formation of MEF2 heterodimeric complexes and/or complexes containing MEF2 and other regulatory proteins in crude extracts, which are unlikely to be present in the preparations of cloned MEF2 proteins, could also account for these differences.

The optimal target for MEF2 expressed in the brain shows sequence constraints in the bases flanking the MEF2 core motif that are not observed in skeletal and cardiac muscle (Fig. 3, positions −5 to −1 and 1 to 2). Whereas 50% of the oligonucleotides selected with brain extracts contain the sequence TGTT at positions −5 to −2 and A(A/T) at 1 to 2, only 3% of the oligonucleotides selected with skeletal and cardiac muscle show these sequence constraints. These observations are intriguing, and several potential explanations may be considered. The complex pattern of alternative splicing of MEF2 transcripts (3–8, 10, 11) may generate tissue-specific MEF2A homo- and heterodimeric complexes with different DNA binding requirements. In this respect, expression of brain-specific MEF2C isoforms (6, 7) might account for the extended binding preferences of MEF2 in this tissue. CASTing would also enable the identification of extended binding requirements resulting from the cooperative interaction of MEF2 with a brain-specific regulatory protein. This protein could either modify the DNA binding specificity of MEF2 or bind itself to DNA, thus contributing to the extended binding preferences detected in brain. Similar types of interactions have been previously suggested by CASTing for myogenin, which allowed the coselection of binding sites for myogenin and nuclear factor 1, MEF2, and COMP1 in crude myotube extracts (18). Recently, cooperative interactions of MEF2A with MyoD and myogenin have been demonstrated in skeletal muscle cells (9). Other examples of multi-component complexes involving myogenic transcription factors include heterodimerization of myogenic bHLH factors with the ubiquitous bHLH E proteins (24–27) and with the cell cycle regulators pRb (28) and p107 (29).

In summary, we have identified sequence constraints in the optimal targets for MEF2 expressed in the brain that are not
**A. MEF2 OPTIMAL BINDING SITES**  
**SKELETAL MUSCLE (C2C12 MYOTUBES) (N = 104)**

![Sequence alignment of the selected MEF2-binding sites recovered after CASTing in skeletal muscle (A), heart (B), and brain (C). Preparation of extracts was as in Fig. 1. The number of sequences reported in each CASTing experiment is indicated (N). Sequences are aligned according to their core A/T-rich motif and arranged according to the central quartet of bases. Nucleotides within the PCR flanking sequences are shown in lowercase. N indicates sequence ambiguities. The sequence R47 selected with brain extract was found in six independent clones.](image)

**B. MEF2 OPTIMAL BINDING SITES**  
**CARDIAC MUSCLE (N = 90)**

![Sequence alignment of the selected MEF2-binding sites recovered after CASTing in skeletal muscle (A), heart (B), and brain (C). Preparation of extracts was as in Fig. 1. The number of sequences reported in each CASTing experiment is indicated (N). Sequences are aligned according to their core A/T-rich motif and arranged according to the central quartet of bases. Nucleotides within the PCR flanking sequences are shown in lowercase. N indicates sequence ambiguities. The sequence R47 selected with brain extract was found in six independent clones.](image)
observed in skeletal and cardiac muscle. Thus, differences in the DNA binding specificities of MEF2 proteins might be a mechanism by which these factors differentially regulate gene expression during myogenesis and neurogenesis. Elucidation of the molecular basis for these tissue-specific binding preferences should reveal important clues to understand the role of MEF2 in the production of muscle and neuronal phenotypes.

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