

A fourth human MEF2 transcription factor, hMEF2D, is an early marker of the myogenic lineage

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SUMMARY

The transition from multipotent mesodermal precursor to committed myoblast and its differentiation into a mature myocyte involve molecular events that enable the cell to activate muscle-specific genes. Among the participants in this process is the myocyte-specific enhancer factor 2 (MEF2) family of tissue-restricted transcription factors. These factors, which share a highly conserved DNA-binding domain including a MADS box, are essential for the expression of multiple muscle genes with cognate target MEF2 sites in *cis*. We report here a new human MEF2 factor, hMEF2D, which is unique among the members of this family in that it is present not only in myotubes but also in undifferentiated myoblasts, even before the appearance of myogenin. hMEF2D comprises several alternatively spliced products of a single gene, one of which is the human homolog of the *Xenopus* SRF-related factor SL-1. Like its relatives, cloned hMEF2D is capable of activating transcription via sequence-specific

binding to the MEF2 site, recapitulating endogenous tissue-specific MEF2 activity. Indeed, while MEF2D mRNAs are ubiquitous, the protein is highly restricted to those cell types that contain this activity, implicating posttranscriptional mechanisms in the regulation of MEF2D expression. Alternative splicing may be important in this process: two alternative MEF2D domains, at least one of which is specifically included during myogenic differentiation, also correlate precisely with endogenous MEF2 activity. These findings provide compelling evidence that MEF2D is an integral link in the regulatory network for muscle gene expression. Its presence in undifferentiated myoblasts further suggests that it may be a mediator of commitment in the myogenic lineage.

Key words: MEF2, myogenesis, commitment, transcription factors, muscle-specific gene regulation

INTRODUCTION

Lineage determination, commitment and differentiation are critical events in the development of multicellular organisms, from the most primitive to the most complex. Individual tissue types are understood to arise during embryogenesis through the activation of specific programs of gene expression. In muscle, a detailed but still incomplete picture of these mechanisms has emerged through the elucidation of tissue-specific factors that regulate muscle gene expression. The myogenic basic-helix-loop-helix (bHLH) proteins, including MyoD, myogenin, Myf5, and MRF4, have been the paradigm for such lineage-determining factors (see Weintraub et al., 1991). The myogenic bHLH proteins, however, do not account for all aspects of muscle gene regulation. There are circumstances in which their activity is neither necessary nor sufficient for expression of the full skeletal muscle phenotype (Hopwood and Gurdon, 1990; Michelson et al., 1990; Miller, 1990; Peterson et al., 1990; Chen et al., 1992). Furthermore, tissue-specific bHLH factors have not been found in cardiac muscle where many

of the same striated contractile protein genes are transcribed (Olson, 1993; and authors' unpublished observations). In fact, the *cis* control sequences for some skeletal and cardiac muscle-specific genes lack functional bHLH binding sites altogether (Thompson et al., 1991; Navankasattusas et al., 1992).

Taken together, these findings point to the participation of regulatory factors outside the bHLH group in myogenic development. The myocyte-specific enhancer factor 2 (MEF2) has been the subject of considerable interest in this regard. This DNA-binding activity has been found to be restricted to muscle cells, encompassing the skeletal, cardiac, and smooth lineages, and to brain (Gossett et al., 1989; Cserjesi and Olson, 1991; Yu et al., 1992; Leifer et al., 1993), although conflicting data indicating a more widespread tissue distribution of MEF2 DNA binding also exist (Horlick et al., 1990; Pollock and Treisman, 1991; Chambers et al., 1992; see Discussion). Transactivation by MEF2, however, mediated by this specific DNA binding, has thus far been demonstrated only in the muscle lineages and not in a variety of non-muscle cells (Gossett et al., 1989;

Yu et al., 1992). Furthermore, MEF2 target binding sequences fitting the consensus (C/T)T(A/t)(A/T)AAATA (A/G) are present in many, if not all, muscle gene regulatory regions (Cserjesi and Olson, 1991). The muscle creatine kinase and myosin light chain 1_f/3_f enhancers and the promoters for myosin light chain 2, cardiac troponin T, and muscle-specific phosphoglycerate mutase depend absolutely on MEF2 sites for skeletal and cardiac muscle-specific activity (Gossett et al., 1989; Wentworth et al., 1991; Braun et al., 1989; Zhu et al., 1991; Navankasattusas et al., 1992; Lee et al., 1992; Ianello et al., 1991; Nakatsuji et al., 1992). Moreover, transcription of the myogenin gene itself also depends on a MEF2 site in its promoter, and the same may be true for MEF2 sites in the MRF4 and MyoD promoters (Edmondson et al., 1992; Tapscott et al., 1992). MEF2 activity, therefore, is evidently an integral link in the regulatory network that maintains the differentiated muscle phenotype, and may even participate more fundamentally in the induction of myogenesis.

Factors responsible for MEF2 activity *in vivo* have recently been cloned. Multiple alternatively spliced isoforms are the products of at least three human genes, and *Xenopus* and mouse homologs have also been identified (Pollock and Treisman, 1991; Yu et al., 1992; Chambers et al., 1992; McDermott et al., 1993; Leifer et al., 1993; Martin et al., 1993; Table 1 shows a proposed consistent MEF2 nomenclature, and these designations will be used in this report). The MEF2 genes are members of the MADS gene family, which also includes an array of homeotic genes and other transcription factors in yeast, plants, and animals, all of which share a highly conserved DNA-binding protein domain, the MADS box (see Yu et al., 1992; Bradley et al., 1993; and references therein). Among the MEF2 proteins examined thus far, MEF2A and MEF2C are specific to differentiated skeletal (and cardiac for MEF2A) muscle and nerve cells (Yu et al., 1992; McDermott et al., 1993). The presence of MEF2A coincides with the earliest expression of sarcomeric proteins during myogenesis *in vitro*, whereas MEF2C appears only later. Thus, while MEF2A and MEF2C have comparable DNA binding and transactivation capabilities, the timing of their expression, at least in cultured cells, suggests that MEF2A may participate in the induction and MEF2C in the maintenance of the differentiated state.

Table 1. Proposed MEF2 Nomenclature

Human gene*	Products†	Previously published designations‡
<i>hMEF2A</i>	hMEF2A	RSRFC4, MEF2, SL-2 (<i>Xenopus</i>) RSRFC9, aMEF2, a*MEF2
<i>hMEF2B</i>	hMEF2B	RSRFR2, xMEF2
<i>hMEF2C</i>	hMEF2C	hMEF2C
<i>hMEF2D</i>	hMEF2D§	SL-1 (<i>Xenopus</i>)

**h* refers to human; A-D simply represent the order in which the corresponding cDNAs have been reported.

†*h* refers again specifically to the human factors, but generic designations, e.g. "MEF2A," will be used where appropriate; multiple alternatively spliced gene products are designated collectively unless otherwise specified.

‡From Pollock and Treisman (1991), Yu et al. (1992), Chambers et al. (1992), Leifer et al. (1993), McDermott et al. (1993); Martin et al. (1993).

§This report.

We have now identified the products of yet a fourth human MEF2 gene, *hMEF2D*. This gene, the apparent human homolog of the recently reported *Xenopus* SRF-related SL-1 (Chambers et al., 1992), is expressed as multiple alternatively spliced isoforms which all exhibit MEF2 site-specific DNA binding and transactivation. MEF2D is also restricted to muscle cell lineages; however, in contrast to MEF2A and MEF2C, it is present even in undifferentiated myoblasts, and remarkably before the appearance of myogenin. MEF2D, therefore, may participate in the earliest commitment events leading to myogenesis.

MATERIALS AND METHODS

Library screening and DNA sequencing

An adult human cardiac ventricle ZAPII cDNA library (1 × 10⁶ recombinants; Stratagene, LaJolla, CA) was screened at low stringency with a probe comprising the 233 bp *Bgl*II-*Xho*I fragment of the hMEF2B cDNA (formerly xMEF2; Yu et al., 1992) that includes all but the 7 N-terminal codons of the MADS/MEF2 domain, labeled to a specific activity of 10⁸-10⁹ cts/minute per µg. Isolation, subcloning, and sequence analysis of positive cDNAs was accomplished as previously described (Yu et al., 1992).

Construction of expression plasmids

The full-length MEF2D δ b coding sequence (nt 196-1919; see Fig. 1) was obtained by polymerase chain reaction (PCR) and ligated into the *Eco*RI site of the pMT2 eukaryotic vector (Kaufman et al., 1989) to create pMT2/hMEF2D δ b. pMT2/hMEF2D δ ab was derived from this by deleting the 5' sequences between the pMT2 *Pst*I site and the hMEF2D *Pst*I site at nt 659/660 and replacing them with a corresponding hMEF2D δ ab partial cDNA fragment bounded by the *Pst*I sites at nt 82/83 and 659/660. pMT2/hMEF2D δ o δ was also derived from pMT2/hMEF2D δ b by replacing the 3' sequences between the *Sac*I sites at nt 652/653 and nt 1226/1227 with the corresponding hMEF2D δ o δ partial cDNA fragment. pMT2/hMEF2A and pMT2/hMEF2C were as used previously (Yu et al., 1992; McDermott et al., 1993).

Cell culture, transient expression and gel shift assay

Cells were grown, transfected and analyzed for chloramphenicol acetyl-transferase (CAT) activity, controlled for transfection efficiency, as previously described (Yu et al., 1992). L₆E₉ (Nadalinard, 1978) and C2C12 (Yaffe and Saxel, 1977) myoblasts were grown conventionally in medium containing 20% fetal bovine serum and differentiated in 2% horse serum. Primary cardiocytes from neonatal rats were cultured according to Chien et al. (1985). Whole cell extracts from untransfected and transfected cultures for gel shift assay (and for western blots; see below) were prepared as described (McDermott et al., 1993). Radiolabeled double-stranded MEF2 probe and unlabeled MEF2, MEF2mt4, and MEF2mt6 competitors were those used previously (Cserjesi and Olson, 1991; Yu et al., 1992; 5' 3')

MEF2	CGCTCTAAAATAACCCT
MEF2mt4	CGCTCTATAAATAACCCT
MEF2mt6	CGCTCTAAACATAACCCT

The 20 µl *in vitro* DNA binding reaction contained: 7-12 µg of cell extract protein preincubated with 1 µl pre- or immune serum (37°C, 15 minutes); 1 × buffer (20 mM Hepes, pH7.6, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol); nonspecific competitor DNA (1 µg poly-dIdC and 50 ng random single-stranded oligo); and, after 10 minutes prebinding at 0°C, 10 fmole probe

with or without 1 pmole of competitor. This mixture was incubated for 20 minutes at room temperature before electrophoresis in non-denaturing 5% polyacrylamide gel and detection by autoradiography.

RNA analyses

The MEF2D-specific *Bgl*III-*Bam*HI cDNA fragment (nt 1066-1621, Fig. 1) was radiolabeled and used to probe poly(A)⁺ RNA blots at high stringency, as previously described (Yu et al., 1992). Reverse transcriptase (RT)-PCR analysis of alternatively spliced MEF2D mRNAs was also performed as previously described, using the gene-specific synthetic single-stranded oligodeoxynucleotide primers and Southern blot probes specified in Figs 5 and 6. Products from the RT-PCR amplification of human skeletal muscle RNA per Fig. 6 were also subcloned and sequenced, leading to the identification of the alternative a sequence (Fig. 1).

Immunocytochemistry

Rabbit polyclonal antiserum specific for MEF2D (D) was raised against a pGEX (Amrad, Melbourne, Australia) bacterial fusion protein containing the C-terminal aa 269-521, according to standard procedures. Anti-MEF2A-specific antiserum (A) was paradoxically obtained from a rabbit injected with a pET-His (Novagen, Madison, WI) fusion protein containing the hMEF2D aa 291-477 internal peptide which apparently presented epitopes in tertiary conformation that more accurately reflect the native MEF2A protein. The respective specificities of these antisera were determined in control gel supershift, western, and immunofluorescence assays (not shown). Monoclonal anti-myogenin (MG; Funk and Wright, 1992) and anti-sarcomeric myosin heavy chain (MF20; Bader et al., 1982) antibodies were gifts of W. Wright and D. Bader, respectively. Western blotting and indirect immunocytofluorescence analyses were performed as described (McDermott et al., 1993; Yu et al., 1992).

RESULTS

Sequence analysis of hMEF2D cDNAs predicts a highly conserved genomic organization among three members of the MEF2 family

We isolated three overlapping partial cDNA clones corresponding to alternatively spliced isoforms of a fourth MEF2 gene in a low-stringency screen of a human cardiac ventricle library, using as a probe the MADS/MEF2 fragment of the hMEF2B cDNA (xMEF2, Yu et al., 1992; see Materials and Methods). The composite sequences and diagrammatic representation of these hMEF2D cDNAs are shown in Fig. 1. The alternative domains *a* and *b* encompass nt 434-591 (aa 87-132) and nt 1051-1071 (aa

286-292), respectively. Note that throughout this report, alternatively spliced hMEF2D isoforms will be designated using *a* and *b*, as well as \emptyset to indicate skipping of the alternative domain in that position, e.g. hMEF2D*ab* includes *a* and *b*, while hMEF2D $\emptyset b$ includes *b* but excludes *a*. The original cloned cDNAs corresponded to hMEF2D $\emptyset b$ and hMEF2D*a* \emptyset . The existence in vivo of isoforms hMEF2D*ab*

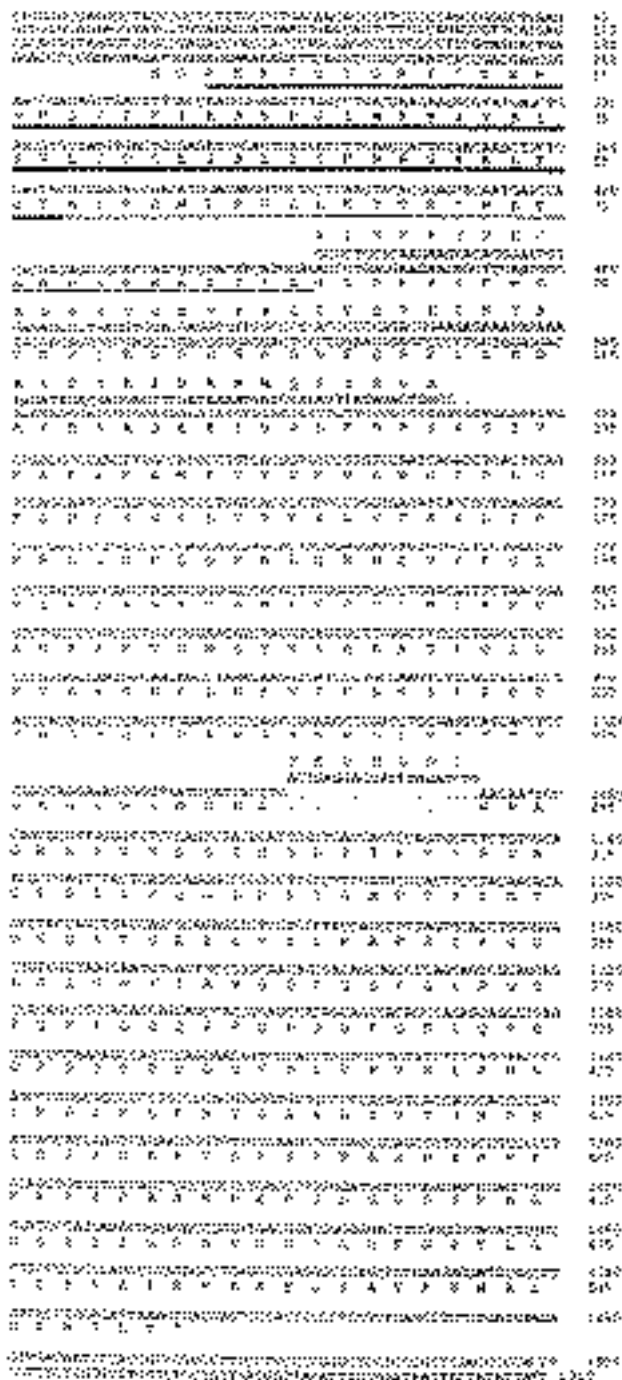
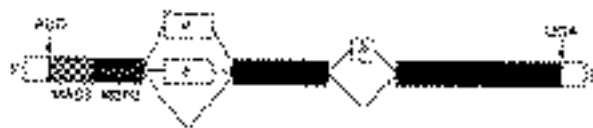


Fig. 1. hMEF2D cDNA and predicted amino acid sequences. The composite sequence was compiled from multiple cDNA isolates and is numbered continuously at the right. Included are the alternatively spliced domains comprising nt positions 434-591 (domain *a* below, and domain *a* one codon shorter, above), and 1051-1071 (domain *b* above, and exclusion of this sequence below). The highly conserved MADS box (bold underlined) and MEF2 domain (underlined) are indicated. The corresponding hMEF2D mRNA isoforms are represented in the diagram at the bottom, including the MADS (checked) and MEF2 (cross-hatched) domains and alternatively spliced exons *a*, *a*, and *b*. (Note that nt 1228 appeared as a C rather than T in one cDNA, but the T preserves the serine codon conserved in both hMEF2A and hMEF2C.)



and hMEF2D $\phi\phi$ was confirmed by RT-PCR analysis (not shown). An additional alternatively spliced sequence, *a*, was also identified by RT-PCR amplification of skeletal muscle RNA. The longest open reading frame is 521 codons (hMEF2D*ab*, calculated M_r 56 \times 10³), with an initiation codon preceded by an in-frame stop codon (nt 178-180). The 3' untranslated sequence appears to be incomplete, lacking both a canonical polyadenylation signal and poly(A) tail.

hMEF2D, like the other three MEF2s, has an N terminus comprising highly conserved MEF2 (underline) and MADS (bold underline) domains, implicated in DNA binding in other proteins of this family (Pollock and Treisman, 1991; authors' unpublished observations). The amino acid sequence of this region is identical to that of hMEF2A at 81/86, hMEF2B at 76/86, and hMEF2C at 81/86 positions, sharing their predicted amphipathic α -helical secondary structure (aa 20-48 and 60-69; not shown; McDermott et al., 1993). The hMEF2D MADS domain also has a richly basic segment (aa 3-31) expected to mediate sequence-specific DNA binding (Pollock and Treisman, 1991; Sharrocks et al., 1993). hMEF2D is approx. 75% similar overall, including conservative substitutions, to both hMEF2A and hMEF2C and, like these two proteins, is entirely dissimilar to hMEF2B outside the MADS/MEF2 domain (cf. Yu et al., 1992; McDermott et al., 1993). Like hMEF2A, hMEF2D also has a long series of consecutive glutamine and proline residues (aa 365-404); glutamine- and proline-rich domains in other transcription factors have been shown to participate in transactivation (Courey and Tjian, 1988; Mermod et al., 1989). In addition to these sequence homologies, the conserved positions of certain alternatively spliced domains among hMEF2A, hMEF2C, and hMEF2D indicate that the corresponding genes, descendants of a common ancestor, almost certainly share a similar exon/intron organization. hMEF2D*ab* is evidently the human homolog of the recently reported *Xenopus* SRF-related factor SL-1 (Chambers et al., 1992). The two proteins are identical in the N-terminal 103 residues and show 85% amino acid similarity overall, with the principal difference being the more extensive glutamine- and proline-rich domain in hMEF2D. Also of interest, the hMEF2D nucleotide sequence from position 749 through 1033 is exactly the complement of the human genome project expressed sequence tag EST00076, and thus is evidently the source of this previously unidentified brain-derived cDNA fragment (Adams et al., 1991).

Cloned hMEF2D exhibits DNA binding and transactivation properties indistinguishable from those of hMEF2A and hMEF2C, recapitulating endogenous MEF2 activity

Sequence-specific binding of the MEF2 DNA site and MEF2 site-dependent activation of gene transcription are characteristic features of MEF2 activity. Given the strong sequence conservation between hMEF2D and the previously examined hMEF2A and hMEF2C, particularly in the MADS/MEF2 DNA binding domain, we predicted that hMEF2D would behave similarly. We tested the DNA binding activity of hMEF2D*ab* in extracts of pMT2/hMEF2D*ab*-transfected COS cells using a gel shift assay (Fig. 2). The radiolabeled MEF2 site probe was bound

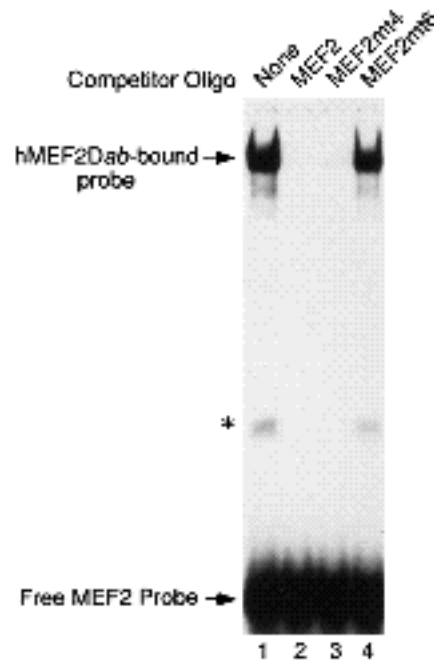


Fig. 2. Cloned hMEF2D recapitulates endogenous MEF2 DNA-binding. An extract of COS cells transiently transfected with pMT2/hMEF2D*ab* was analyzed by gel shift assay, using the radiolabeled MEF2 site probe and unlabeled MEF2, MEF2mt4, and MEF2mt6 competitor oligos (see Materials and Methods). The mobility of the probe was retarded (lane 1) in a binding complex formed in this extract, as compared with that of the free probe (*, non-specific band). Extracts of COS cells transfected instead with the empty pMT2 vector as a control showed no binding (see Fig. 7B, lane 1). Binding of the probe was efficiently competed with a 100-fold molar excess of MEF2 (lane 2), as well as with MEF2mt4 (lane 3), but not by MEF2mt6 (lane 4).

by hMEF2D*ab* in these extracts, but not by extracts of control-transfected COS cells, which have no endogenous MEF2 activity (see Fig. 7B). Moreover, this binding showed the same sequence-specificity as the endogenous muscle MEF2 since excess unlabeled MEF2 site DNA, as well as MEF2mt4, competed effectively for binding, while MEF2mt6 did not (binding site point mutants MEF2mt4 and MEF2mt6 were shown previously to distinguish between muscle-specific and non-specific binding activities, respectively; Cserjesi and Olson, 1991). The same results were also obtained using *in vitro* translated hMEF2D*ab* as well as hMEF2D $\phi\phi$, indicating no detectable difference in binding between alternative isoforms (not shown).

We next examined whether cloned MEF2D could confer MEF2 site-dependent transcription regulation in cotransfection assays combining pMT2/MEF2D expression vectors with a panel of reporter constructs in non-muscle cells devoid of endogenous MEF2 activity. The reporter genes comprised the CAT coding sequences linked to the basal embryonic myosin heavy chain promoter, with or without duplicated intact or mutated MEF2 binding sites (Fig. 3A), or linked to the complete embryonic myosin heavy chain promoter (-175 to +1) which contains a single native MEF2 binding site, as used previously (Yu et al., 1992).

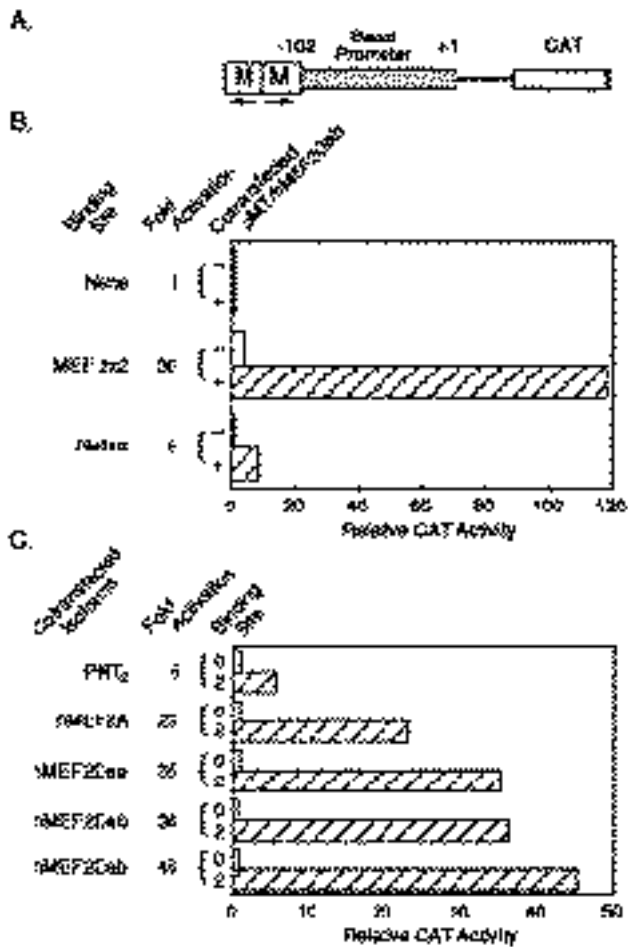


Fig. 3. Cloned hMEF2D isoforms recapitulate endogenous MEF2 transactivation. (A) Diagram of the prototypical reporter gene for these cotransfection experiments showing duplicate MEF2 binding sites and the basal embryonic myosin heavy chain gene promoter (-102 to +1) linked to the bacterial CAT coding sequence. (B) Cotransfection of HeLa cells with pMT2/hMEF2D δb and reporter constructs produced relative CAT activities, corrected for transfection efficiency, as graphed. The reporter genes were those including duplicate MEF2 binding sites (MEF2x2, pE102CAT-MEF2x2) or no sites (None, pE102CAT). Also tested was a reporter gene with the full-length myosin promoter (-175 to +1), containing the single native MEF2 site alone (Native, pE175CAT). (C) The transactivation efficiency of hMEF2D δb was similarly compared to those of hMEF2D δa and hMEF2D δc , as well as to hMEF2A, using the pE102CAT (0) and pE102CAT-MEF2x2 (2) reporter genes.

hMEF2D δb produced marked transactivation that was absolutely dependent on the presence of intact MEF2 sites in the reporter gene (Fig. 3B). In addition, hMEF2D δb specifically transactivated the native embryonic myosin heavy chain promoter (-175 to +1), although less vigorously (Fig. 3B). We also compared the transactivation capacities of hMEF2D δb , hMEF2D δa , and hMEF2D δc and found that all were effective transcription factors, comparable as well to hMEF2A, indicating that particular alternative domains are not essential for this activity, within the limits of the assay (Fig. 3C).

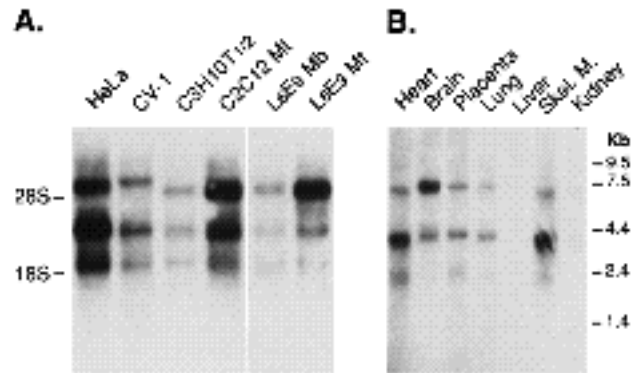


Fig. 4. MEF2D mRNAs are ubiquitous, but particular transcripts are more abundant in skeletal muscle, heart, or brain. Poly(A)⁺ RNA blots from a variety of established muscle and non-muscle cell lines (A; Mb, myoblasts; Mt, myotubes; equivalent amounts of RNA per lane, data not shown) and from different adult human tissues (B; Skel. M., skeletal muscle) were probed with a radiolabeled MEF2D-specific cDNA fragment (*Bgl*III-*Bam*HI, nt 1066-1621 in Fig. 1) at high stringency, revealing three major transcripts from both primate and rodent sources.

MEF2D mRNAs are ubiquitous, but certain alternative isoforms are restricted to those tissues with endogenous MEF2 activity

MEF2A mRNAs are ubiquitously expressed, while MEF2B and MEF2C transcripts are largely restricted to muscle and brain tissues; moreover, MEF2A and MEF2C are subject to highly tissue-specific splicing events that correlate with the tissue-specific accumulation of the corresponding protein factors (Yu et al., 1992; Leifer et al., 1993; McDermott et al., 1993). *Xenopus* SL-1 RNAs were shown by in situ analysis to be restricted to the somitic mesoderm during early development, but distributed widely in the adult (Chambers et al., 1992). We determined the distribution of MEF2D-related transcripts in northern blots of poly(A)⁺ RNAs from a variety of cultured cell lines and adult human tissues, using a MEF2D-specific cDNA fragment as a probe (Fig. 4A,B). Major transcripts of approx. 7 and 4 kb were present in all cell types, but more abundant in differentiated myotubes, HeLa cells, skeletal and cardiac muscle, and brain; a minor approx. 2.5 kb transcript was also detected. The MEF2D mRNAs from both primate and rodent sources are substantially longer than the cloned cDNAs, which presumably lack much of the untranslated sequences, as was inferred for all the other human MEF2s and *Xenopus* homolog (Pollock and Treisman, 1991; Yu et al., 1992; Chambers et al., 1992; Leifer et al., 1993; McDermott et al., 1993; Martin et al., 1993). The variety of sizes is most likely the result of alternative RNA processing outside the coding sequences, and there are certain tissue-specific differences in relative abundance among them (cf. heart and skeletal muscle with brain). As in the case of hMEF2A, transcriptional regulation of hMEF2D cannot, alone, account for the tissue-specificity of endogenous MEF2 activity as originally described (Gossett et al., 1989; Yu et al., 1992; see Discussion).

Given the conservation of certain alternatively spliced coding domains among hMEF2A, hMEF2C, and hMEF2D,

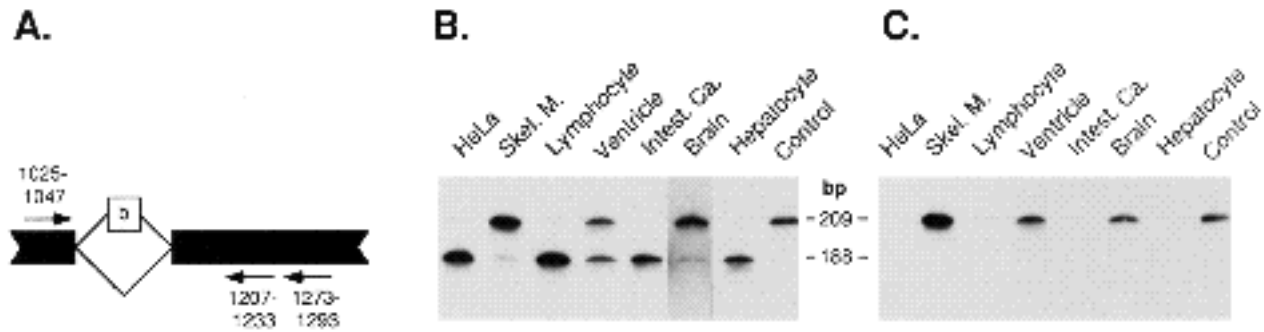


Fig. 5. Tissue-restricted expression of the alternatively spliced *b* domain. Equal amounts of total RNA from each of several human cell types including HeLa, adult skeletal muscle (Skel. M.), Jurkat T lymphocytes, adult cardiac ventricle, CaCo colon carcinoma (Intest. Ca.), fetal brain, and HepG2 hepatocytes, were subjected to RT-PCR analysis. (A) Diagram of the pertinent region of hMEF2D per Fig. 1. Oligodeoxynucleotide primers used for reverse transcription (antisense nt 1273-1293) and PCR (sense nt 1025-1047 and antisense nt 1207-1233) are indicated (arrows). (B) A Southern blot of the products was probed with a radiolabeled oligodeoxynucleotide (antisense nt 1089-1118) hybridizing to the constant region just 3' to the *b* sequence. Products corresponding to inclusion (209 bp) or exclusion (188 bp) of the alternative *b* domain differ by precisely the 21 bp length of that domain (Control, amplification product using the hMEF2D δ b cDNA as a template; the 209 nt product was detected only faintly in HeLa, lymphocytes, CaCo, and HepG2 cells on long exposure). (C) The same blot was also probed with a *b*-specific oligodeoxynucleotide (antisense nt 1048-1074), confirming that the 209 nt product includes the alternative sequence.

we sought to determine whether regulated hMEF2D splicing might correlate with the presence of MEF2 DNA binding and transactivation in specific tissues. In hMEF2A, incorporation of a sequence encoding the peptide SEEEELEL occurred only in skeletal and cardiac muscle and brain (Yu et al., 1992). Splicing of the corresponding alternative domain *b* encoding TEDHLDL in hMEF2D was analyzed by RT-PCR amplification of RNAs from a variety of human cell types (Fig. 5A). The longer product, representing inclusion of *b*, was largely restricted to skeletal muscle, cardiac ventricle, and brain, while the shorter product excluding it was detected ubiquitously (Fig. 5B,C). Inclusion of the *b* domain in hMEF2D, therefore, correlates precisely with endogenous MEF2 activity, as seen for hMEF2A; of note, the corresponding alternative domain in hMEF2C was restricted to skeletal muscle only (McDermott et al., 1993).

We used an analogous strategy to analyze the splicing of the hMEF2D *a/a* domain (Fig. 6A). The corresponding

exons in hMEF2A were alternatively spliced in a mutually exclusive but apparently unregulated fashion (Yu et al., 1992). For hMEF2D, in contrast, we found that while transcripts containing *a* were ubiquitous, those containing *a* instead were largely limited to skeletal muscle (Fig. 6B; *Xmn*I-resistant products corresponding to *a* were verified by subcloning and sequencing; data not shown). The product representing exclusion of both *a* and *a*, as found in one of the original cDNA clones, was faintly detected in all cells; inclusion of both *a* and *a* in the same transcript was not detected (not shown). We also applied this analysis to RNAs isolated from undifferentiated rat skeletal myoblasts and progressively more mature myotubes and found that splicing to incorporate *a* is specifically induced during myogenesis (Fig. 6C).

Against a background of ubiquitous MEF2D transcripts, therefore, particular alternatively spliced isoforms show striking correlations with endogenous MEF2 DNA binding and transactivation, activities that are restricted to muscle

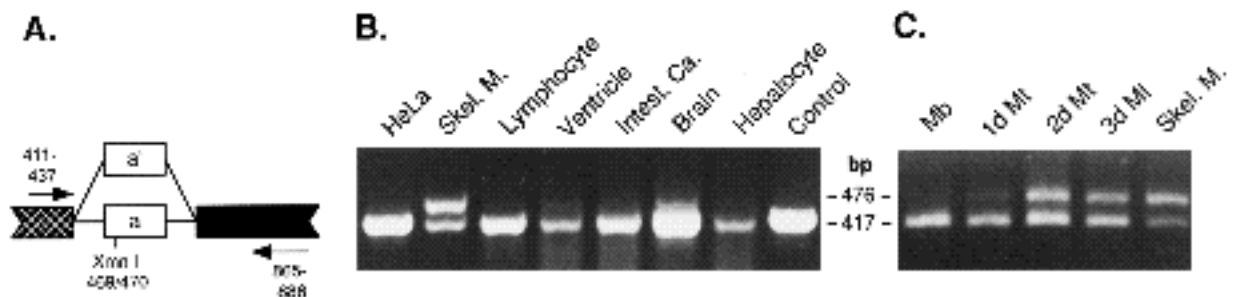


Fig. 6. Induction of tissue-specific alternative splicing of the *a/a* domain during myogenesis. (A) The same reverse transcribed RNAs as in Fig. 5 were PCR amplified using primers (sense nt 411-437 and antisense nt 865-886) flanking the *a/a* domain. Products containing *a* were resolved from those containing *a* by cleavage at the *Xmn*I site unique to the latter, and complete digestion was verified in multiple repetitions. (B) The resulting products were separated in ethidium bromide-stained agarose gel, showing bands corresponding to *a* (417 bp after *Xmn*I cleavage; the remaining 59 bp fragment is not shown) and *a* (476 bp). (C) The same RT-PCR analysis was carried out with equivalent amounts of RNA prepared from rat L₆E₉ myoblasts (Mb) and myotubes (Mt) in differentiation medium for 1, 2 and 3 days (d). The human sequence-based primers sufficed for the rodent MEF2D.

and brain. These findings provide compelling evidence that tissue-specific alternative domains may play a key role in the regulation of MEF2 activity *in vivo*. However, it is also clear that these alternative protein domains are not themselves essential for hMEF2D to function as a transcription factor, since all isoforms tested, including hMEF2D $\emptyset\emptyset$, were equally effective in cotransfection experiments (see above). Whether they might be important in tissue-specific post-translational events such as protein-protein interactions remains to be determined (see Discussion).

MEF2D protein is expressed prior to myogenic differentiation, in contrast to other MEF2 factors

Despite the ubiquitous distribution of MEF2D at the RNA level, we suspected that MEF2D protein, like other members of the MEF2 family, would be restricted to those differentiated cell types that manifest the characteristic MEF2 DNA binding and transactivation activity. We used a MEF2D-specific antiserum, raised to a C-terminal fragment common to all the hMEF2D alternative isoforms, to assay for MEF2D protein in a variety of cell types using several techniques.

Remarkably, western blotting with α D antiserum detected MEF2D not only in differentiated cardiocytes and skeletal myotubes, but also abundantly in *undifferentiated* skeletal myoblasts where MEF2A and MEF2C are entirely absent (Fig. 7A; Yu et al., 1992; McDermott et al., 1993). The pre-differentiated state of these myoblasts was confirmed by the absence of myogenin, which first appears only in myotubes in culture and which, potentially, could be induced by MEF2D (Fig. 7A; Edmondson et al., 1992).

We also detected functional endogenous MEF2D protein in extracts of undifferentiated skeletal myoblasts by gel shift assay with the same α D antiserum (Fig. 7B). The amount of MEF2 protein:DNA complex in these myoblasts was low, relative to that seen in myotubes, but virtually all of it was supershifted by this antiserum (lanes 4 and 5). This indicates that the MEF2 DNA binding complex in myoblasts comprises MEF2D alone, or stable hetero-oligomers which include MEF2D. In myotubes, MEF2D was seen to account for the uppermost (slowest-migrating) portion of the broad binding complex (lanes 8 and 9), consistent with the migration of cloned hMEF2D expressed in transfected COS cell extracts (lanes 1-3) or translated *in vitro* (not shown). Supershift by α A antiserum showed that MEF2A protein forms the lower portion of the complex (lanes 8 and 10), also consistent with the somewhat faster migration of cloned hMEF2A (lanes 12 and 13). Together, MEF2D and MEF2A appear to account for all of the MEF2 DNA binding activity in myotubes 48 hours after serum withdrawal (lanes 8 and 11). MEF2C does not appear until two days later (McDermott et al., 1993; examination of MEF2B awaits specific antiserum).

These findings were reinforced by immunocytofluorescence analysis. In addition to documenting that MEF2D is a nuclear protein, these studies showed it to be present uniformly in all growing myoblasts (Fig. 8C) and stationary myocytes (Fig. 8D) devoid of sarcomeric myosin heavy chain, as well as in differentiated skeletal myotubes with myosin heavy chain (Fig. 8E,F; the intensity varied somewhat among myotube nuclei, probably reflecting different levels, but all stained). MEF2D protein is notably

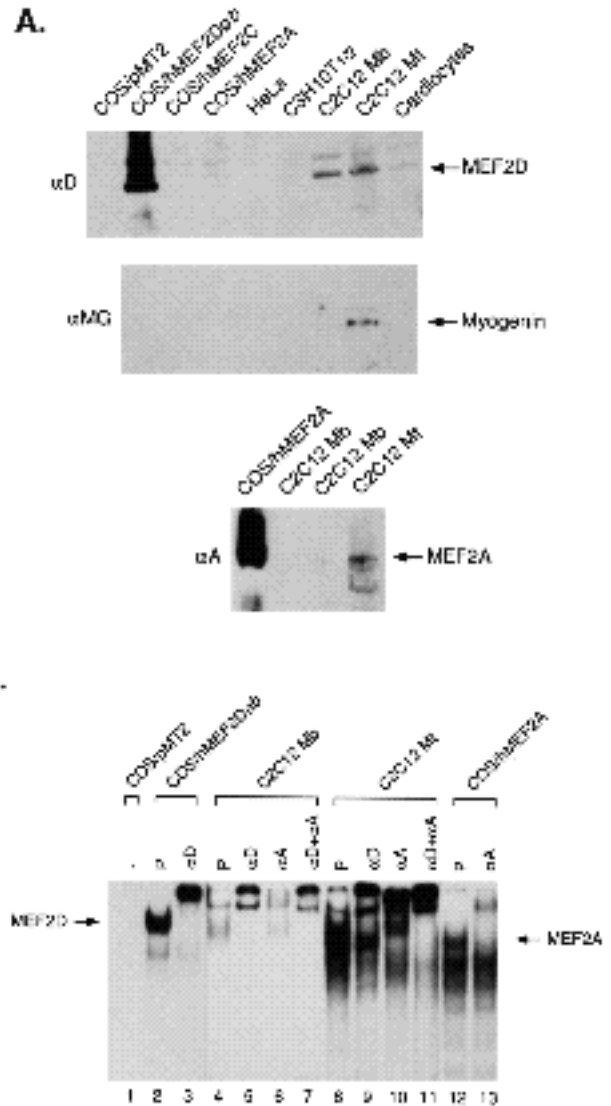


Fig. 7. Immunodetection of MEF2D in advance of myogenin in myoblast and myotube whole cell extracts. (A) Specific α D antiserum (1:800) was used in western blot analysis to detect MEF2D protein in mouse C2C12 myoblasts (Mb) and myotubes (Mt), as well as rat neonatal cardiocytes. Controls include COS cells (COS) transfected with either empty pMT2 vector, pMT2/hMEF2D $\emptyset\emptyset$, pMT2/hMEF2C, or pMT2/hMEF2A, as well as HeLa cells and mouse C3H10T1/2 fibroblasts. α D antibody cross-reacts with a nonspecific higher M_r band, but this is found in all tested cell types and has no bearing on the immunofluorescence analysis in Fig. 8. Myogenin and hMEF2A were detected in some of these extracts using monoclonal MG (1:4) and polyclonal α A (1:800) antibodies, respectively. (B) Gel shift assay using the same C2C12 extracts and COS-transfected controls, preincubated with either preimmune serum (P), α D, α A, or both α D and α A, shows specific 'supershifts' (i.e. further retardation) of the corresponding protein-DNA complexes. The MEF2 probe is the same as in Fig. 2 (unbound probe is not shown). Faster-migrating COS/hMEF2A species not supershifted by α A (lanes 12 and 13) are presumably truncated products lacking the specific C-terminal epitopes recognized by α A, but retaining the DNA binding domain.

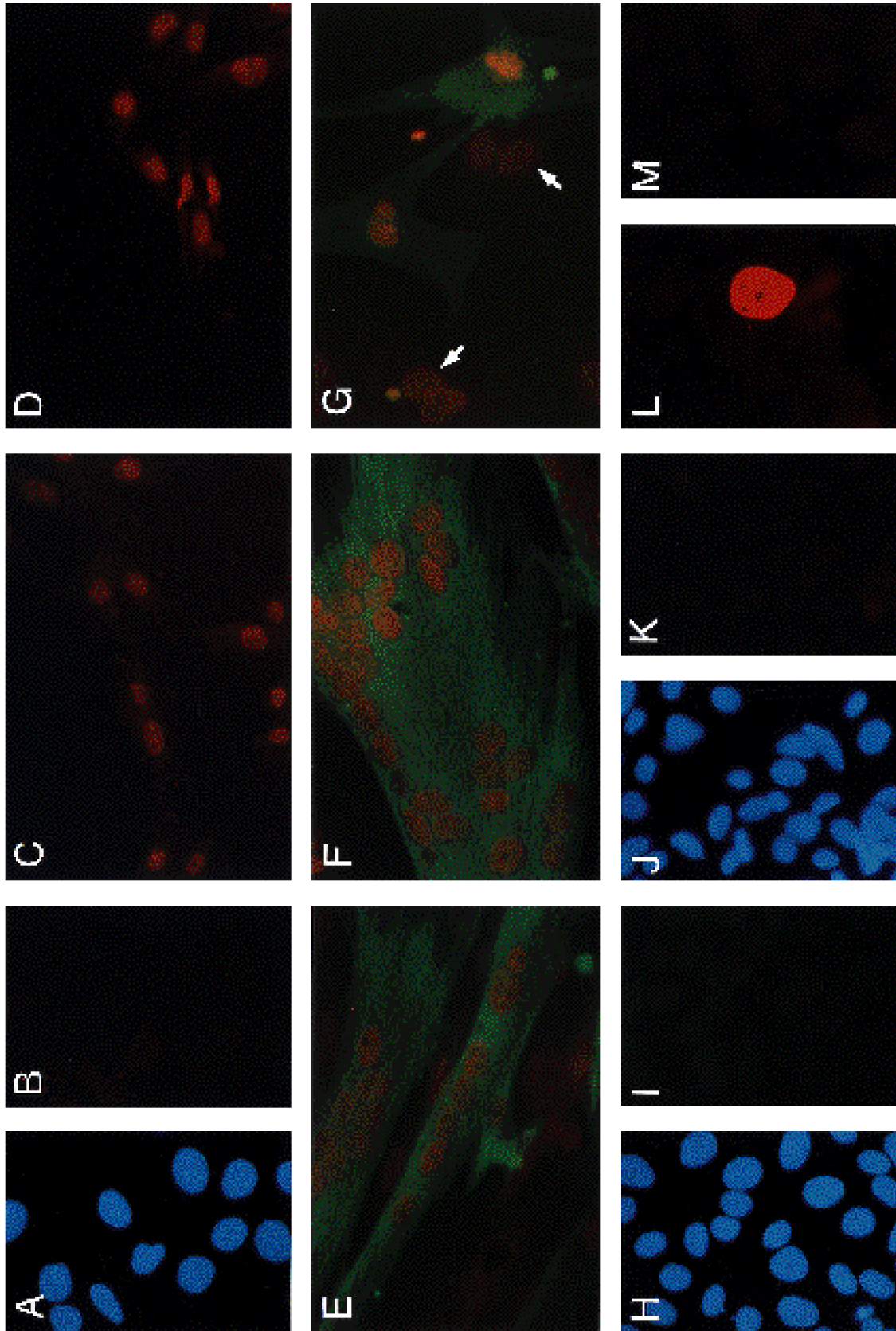


Fig. 8. Immunocytofluorescence shows nuclear MEF2D in undifferentiated myoblasts as well as in myotubes and cardiocytes. Logarithmically growing C2C12 myoblasts (C) and myocytes and myotubes in differentiation medium for 1 (D), 2 (E), or 3 (F) days were fixed and double-stained with α D antiserum (1:500, detected indirectly with Texas Red-conjugated goat anti-rabbit IgG) and MEF20 anti-sarcomeric myosin heavy chain monoclonal antibody (1:1; detected indirectly with fluorescein-conjugated goat anti-mouse

IgG). Primary neonatal rat cardiocytes (G) were identically double-stained (arrows show presumptive 'cardioblasts' with nuclear MEF2D but no cytoplasmic sarcomeric myosin), as were C3H10T1/2 fibroblasts (B), HeLa cells (I), NIH3T3 cells (K), and COS cells transiently transfected either with hMEF2D δ b (L; single positive transfectant among several cells in field) or hMEF2A (M; negative control). C3H10T1/2 (A), HeLa (H), and NIH3T3 (J) nuclei were visualized using Hoechst 33258 dye.

absent, however, in a variety of non-muscle cell types (Fig. 8I,K,L), including C3H10T1/2 fibroblasts (Fig. 8B) which are pluripotent but *uncommitted* precursors of the myogenic and other mesodermal lineages. MEF2D is also expressed in primary myocardial cultures (Fig. 8G), not only in differentiated cardiocytes containing sarcomeric myosin heavy chain, but also in apparently undifferentiated cells which, by analogy to the skeletal myogenic cells (above), may represent 'cardioblasts' (arrows). The possibility that these are, instead, heart fibroblasts cannot be absolutely excluded at present, although the fact that this antiserum does not stain any tested fibroblast cell line or connective tissue weighs against this (Fig. 8B,K; D. Laheru and V. Mahdavi, unpublished observations). Preliminary studies indicate that MEF2D protein is also expressed in particular regions in the brain (not shown). Thus, MEF2D is an early marker of the myogenic, and possibly cardiac and neural, cell lineages, expressed well before other members of the MEF2 family. Its unique position raises the intriguing possibility that it may be a mediator of commitment during development.

DISCUSSION

MEF2 was originally characterized in myogenic cells as a muscle-specific activity that binds DNA and up-regulates transcription via an A/T-rich sequence motif which has subsequently been identified in the promoters or enhancers of multiple skeletal and cardiac muscle genes (Gossett et al., 1989; Cserjesi and Olson, 1991). In previous work with cloned hMEF2A, hMEF2B, and hMEF2C, we found a strict correlation between the presence of endogenous MEF2 protein and MEF2 activity in a cell type-restricted distribution encompassing not only skeletal muscle but also cardiac and smooth muscle and certain neural cells; a variety of cells outside these lineages, in contrast, showed no MEF2 protein and no corresponding activity (Yu et al., 1992; Leifer et al., 1993; McDermott et al., 1993). The results reported here for MEF2D maintain this correlation and suggest that MEF2D may produce detectable DNA binding (Fig. 7B; Pollock and Treisman, 1992) and limited transactivation (Gossett et al., 1989; Yu et al., 1992) in skeletal myoblasts prior to differentiation. However, there remains a discrepancy between the original characterization of MEF2 and our findings on the one hand, and work from other laboratories on the other. Horlick et al. (1990) reported a ubiquitous TA-rich binding activity that recognized MEF2 sites in the brain and muscle creatine kinase genes. Pollock and Treisman (1991) and Chambers et al. (1992) have detected MEF2 DNA binding activity in certain non-muscle, non-neural cell types where we have found neither substantial DNA binding, transactivation, nor endogenous MEF2 protein. It has not yet been possible to reconcile these contradictory results.

hMEF2D is part of a multigene family of transcription factors and may participate in diverse tissue-specific regulatory programs

hMEF2D, apparently the human homolog of the recently identified *Xenopus* SRF-related factor, SL-1 (Chambers et al., 1992), resembles the previously cloned hMEF2A, hMEF2B, and hMEF2C in that it contains an N-terminal

MADS box and the adjacent, highly conserved MEF2 domain. It also has sequence homologies with hMEF2A and hMEF2C outside this region, as well as certain conserved alternative splicing patterns. hMEF2D exhibits sequence-specific DNA binding properties indistinguishable from those of the other members of this family (note, however, that DNA binding by hMEF2B could be demonstrated *in vitro* only when its C-terminus was truncated; Pollock and Treisman, 1991). Furthermore, like all of these factors, hMEF2D mediates MEF2 site-dependent transcriptional up-regulation, recapitulating endogenous tissue-specific MEF2 transactivation.

The human MEF2 gene family, therefore, comprises a minimum of four members, a number that is consistent with genomic Southern blot analyses (not shown). This family has been wholly or partially conserved in other mammalian, amphibian, and fish species as well (Chambers et al., 1992; Martin et al., 1993; R.E. Breitbart, unpublished observations). Despite this evolutionary persistence, there is considerable functional redundancy among the MEF2 proteins, at least in the experimental contexts in which they have been tested. *Drosophila*, in contrast, appears to have a single MEF2 gene (H. Nguyen and B. Nadal-Ginard, unpublished observations). These features are reminiscent of the myogenic bHLH factor gene family, which also has a single member in *Drosophila* (Michelson et al., 1990) and multiple members in vertebrates which all share key activities; however, the bHLH factors are distinguished in part by their individual patterns of expression during development (Sassoon et al., 1989). To the extent that the myogenic bHLH family may be taken as a paradigm, it is expected that different MEF2 factors will also be expressed in distinct spatial and temporal patterns in the early embryo and participate in specific gene regulatory programs (see below). Moreover, the four MEF2 genes together may encode a repertoire of as many as fifteen functional protein isoforms as a result of alternative splicing. Combined with the possibility for heterodimerization among them (Pollock and Treisman, 1991), the potential for regulatory diversity by MEF2 becomes very large.

Posttranscriptional mechanisms regulate cell-specific expression of MEF2D

Chambers et al. (1992) have shown that transcripts of the *Xenopus* homologs of hMEF2D and hMEF2A are highly restricted to the somitic mesoderm and subsequently the myotomes in early development, appearing later in heart, brain and other adult tissues (Chambers et al., 1992). Thus, in the early embryo, transcriptional regulation of these genes (or, formally, the stability of their RNAs) represents a critical level of control on MEF2 expression. Later in development, however, posttranscriptional mechanisms may supervene. We detect MEF2D and MEF2A transcripts in many cell types where they are apparently not translated, or the corresponding proteins are selectively degraded. Our immunochemical analyses show definitively that these proteins are specific to particular lineages, correlating precisely with functional MEF2 activity (see Results; and Yu et al., 1992).

It is also likely that alternative RNA splicing, which figures prominently in hMEF2D as it does in other members

of the family, is an important posttranscriptional regulatory mechanism in the generation of tissue-specific MEF2 activity (Yu et al., 1992; McDermott et al., 1993). A variety of hMEF2D isoforms arises as a result of highly specific RNA splicing events. Two separate domains of the hMEF2D protein are encoded by putative exons (*a* and *b*) which are incorporated only in cell types that accumulate MEF2D protein and exhibit endogenous MEF2 activity, i.e. DNA binding and transactivation. This strict correlation, and the conservation of alternative splicing patterns among the MEF2s, provide compelling evidence that these domains are important for the expression of MEF2 activity *in vivo*. The participation of the other isoforms in this activity, however, is not excluded by the present data. Whether all or just some of the MEF2D proteins in these cells contain these domains cannot be determined with available antibodies. In either case, the inclusion of these specific domains is associated with the production of stable proteins, presumably due to either increased translational efficiency or a direct effect on protein stability. In addition, these alternative domains may mediate the interaction of MEF2D with other regulatory proteins. It must be re-emphasized, however, that within the limits of our assays, tissue-specific alternative domains are not essential for the activity of the cloned hMEF2D. And if cofactors are required, they must be available even in the non-muscle cells which, when transfected with hMEF2D, support activation of MEF2-dependent reporter genes.

MEF2D is an early marker of the myogenic lineage

The cell culture model of skeletal myogenesis relies on the withdrawal of serum growth factors from committed myoblasts for the induction of differentiation (see Olson, 1992). This process is characterized by activation of myogenic bHLH factors and expression of sarcomeric and other markers of the muscle phenotype, as well as by cell cycle arrest and myotube fusion. Differentiation also involves the appearance and presumed participation of MEF2A initially, and of MEF2C somewhat later (Yu et al., 1992; McDermott et al., 1993; Martin et al., 1993; MEF2B protein not yet investigated). The results reported here demonstrate that MEF2D, while also found in myotubes, is sharply distinguished from MEF2A and MEF2C by virtue of its presence in growing myoblasts. Remarkably, MEF2D is expressed in the myogenic lineage even before the appearance of myogenin. Furthermore, it is also detected in certain undifferentiated cells from heart which, by extrapolation, may be 'cardioblasts.' MEF2D, therefore, is likely to be an integral component of the regulatory mechanisms that govern commitment in the myogenic and, perhaps, cardiogenic lineages, independent of differentiation. The protein is present uniformly in myoblast nuclei and is responsible for limited but reproducible MEF2 site-specific DNA binding in myoblast extracts. It is probably also responsible for the modest activation of MEF2-dependent transcription detected previously in these cells (Gossett et al., 1989; Yu et al., 1992). Endogenous MEF2D, however, is apparently insufficient by itself to activate native chromosomal target genes in myoblasts, not unlike MyoD in these same cells. There may be a simply quantitative basis for this, but a more attractive model would invoke a require-

ment for specific posttranslational modification and/or interaction, direct or indirect, with auxiliary factors induced (or inhibitory factors lost) during differentiation. The other MEF2 proteins, with which MEF2D likely heterodimerizes, are obvious candidates. So is myogenin, as there is increasing evidence that MEF2 and bHLH factors potentiate each other (Funk and Wright, 1992; S. Kaushal and B. Nadal-Ginard, unpublished data). In addition, members of the *paired*-like homeodomain family or the ets domain family could also be cofactors for MEF2 as they are for other MADS proteins (Grüneberg et al., 1992; Cserjesi et al., 1992; Dalton and Treisman, 1992).

It is tempting to speculate that certain alternatively spliced hMEF2D domains may be necessary for important post-translational modifications or interactions with coregulators. For example, the *b* peptide in hMEF2D and its *Xenopus* homolog, and the corresponding peptides in hMEF2A and hMEF2C, all contain potential sites for casein kinase II serine/threonine phosphorylation (Kennelly and Krebs, 1991). That the cloned isoforms both with and without these domains were equally effective in the experimental context remains a significant caveat here. However, alternative domains could mediate protein-protein interactions with other myogenic factors *in vivo* during the transition to the differentiated phenotype. And to the extent that this transition requires a timely molecular 'switch', alternative RNA splicing is potentially a rapid and plastic mechanism for the expression of new gene products in advance of new gene transcription; thus, it may be an integral part of the myogenic signal transduction pathway responsive to growth factor withdrawal.

MEF2D occupies the primary position in a temporal hierarchy of MEF2 expression in cultured myogenic cells, followed in succession by MEF2A and MEF2C. Moreover, the *Xenopus* MEF2D homolog precedes MEF2A in the somitic mesoderm of the early embryo, at least at the RNA level (Chambers et al., 1992). MEF2D may be pre-eminent, as well, in a functional hierarchy among these factors in skeletal muscle development. MEF2D is present even before myogenin, which itself depends on MEF2 activity for expression (Edmondson et al., 1992). And MEF2D might contribute to the regulation of MyoD in myoblasts, since the MyoD promoter contains a putative MEF2 site (Tapscott et al., 1992). A number of findings strongly suggest that MEF2 activity both induces and is induced by bHLH myogenic factors (Cserjesi and Olson, 1991; Yu et al., 1992; S. Kaushal, B. Nadal-Ginard and V. Mahdavi, unpublished data). Thus, the MEF2 and bHLH factors must operate together to govern skeletal myogenesis. While a role for MEF2D in the committed precursors of the cardiac and neural lineages remains to be investigated, it is likely that in these, too, MEF2 will act in conjunction with lineage-specific factors functionally comparable to the myogenic bHLH proteins.

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Note added in proof

The GenBank accession numbers for the reported sequences are L16794 and L16795.