MEF2 proteins, including MEF2A, are expressed in both muscle and non-muscle cells

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ABSTRACT

The MEF2 proteins are involved in regulation of many muscle specific genes. Although MEF2 RNAs encoding the MEF2A and MEF2D isoforms are ubiquitously expressed, the presence of MEF2 proteins in non-muscle cell types has been controversial. Here we use a well-characterised antibody in conjunction with DNA binding studies to provide evidence that members of the MEF2 family are widely expressed in the nuclei of cultured cells and are competent to bind DNA. The data show that non-muscle MEF2 complexes contain MEF2A, and that another MEF2 protein, probably MEF2D, is also present. These results suggest that MEF2 proteins fulfil functions in addition to muscle-specific gene expression.

INTRODUCTION

Muscle-specific gene expression provides a good model system for the study of both differentiation and tissue-specific gene expression at the molecular level. Several different promoter elements have been identified as important for muscle-specific gene transcription. Some bind complexes containing muscle-specific transcription factors: for example, the E-box (CANNTG) binds members of the myogenic family of bHLH (basic-helix-loop-helix) proteins typified by MyoD (1,2). In contrast, other sequences in muscle-specific promoters bind ubiquitous factors whose activity can at least in some cases be modified according to the promoter context. An example is the CArG box (CC[A/T]₆GG), which binds the ubiquitous transcription factor SRF (serum response factor; 3), the prototype of the MADS family of transcription factors (4). In muscle-specific promoters, the CArG box acts as a strong constitutive promoter element (5–8); in contrast, in immediate-early gene promoters, such as that of *c-fos*, the CArG box functions as a growth factor-inducible promoter element, the SRE (serum response element) and has only low basal activity (7,9). Thus, the involvement of a transcription factor in muscle-specific transcription need not imply that the factor is expressed exclusively in muscle cells.

The MEF2 (myocyte-specific enhancer binding factor 2) transcription factor was first identified as a protein that could bind an AT-rich sequence present in the muscle-specific enhancer of the muscle creatine kinase (MCK) gene (10,11). MEF2 binding

activity is increased upon myocyte differentiation, and muscle-specific enhancer activity requires the integrity of both MEF2 and E-box elements (12,13). Examination of several muscle-specific promoters defined a MEF2 site consensus sequence, YTAATAATAACYYY (10,11). Molecular cloning experiments have identified four MEF2 genes including MEF2A (RSRFC4/SL2), MEF2B (RSRFR2), MEF2C and MEF2D (SL1) which, like SRF, are MADS box proteins (14–22). Analysis of the binding specificity of MEF2A and MEF2D indicates that their true binding consensus sequence is CTA(T/A)₄TAG (14,21), which encompasses all known muscle-specific gene MEF2 sites and is distinct from that of SRF (CC[A/T]₆GG; 23). This differential DNA binding specificity is determined by residues in the N-terminal basic part of the MADS box (14,24,25). Antisera against recombinant MEF2A, C and D react with MEF2 proteins in muscle cell extracts (14–22) and DNA binding and chemical interference studies indicate that MEF2A and MEF2D interact with the MCK MEF2 site in a manner identical to that of muscle cell MEF2 (10,14).

Although the importance of MEF2 proteins for activation of muscle-specific genes is clear, several findings suggest that MEF2 proteins may also play a role in non-muscle gene expression. In mammals, MEF2A and MEF2D RNAs are expressed in many tissues, while MEF2C RNAs appear restricted to muscle and brain (14–18); in *Xenopus*, expression of MEF2A and MEF2D RNAs is restricted to somitic mesoderm in the embryo, although ubiquitous in the adult (21,22). Furthermore, consensus MEF2 binding sites are found in genes whose transcription is not restricted to muscle. For example a MEF2 consensus site which forms part of the brain creatine kinase (CKB) gene promoter can functionally substitute for a MEF2 site present in the muscle-specific MCK enhancer (12,13,26). Moreover, in non-muscle cells MEF2 sites from two immediately early gene promoters, *c-jun* and N10, have been shown to act as promoter elements, and their activity is abolished by mutations that block MEF2 binding (14,27). In gel mobility-shift assays both mammalian and amphibian non-muscle cell extracts form complexes with either N10 or MCK MEF2 site probes that are recognised by MEF2A antiserum (14,21,28,29).

Despite the findings reviewed above, the issue of whether expression of MEF2 proteins is itself muscle-restricted has remained contentious. For example, some reports have suggested that non-muscle factors binding the MCK MEF2 probe in gel

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mobility-shift assays are immunologically distinct from muscle MEF2 (15) and possess a distinct binding specificity (11). To resolve this apparent controversy we have investigated MEF2 expression using a well characterised MEF2 antibody. We demonstrate that members of the MEF2 family are present in substantial amounts in nuclei of both muscle and non-muscle cells, and that these proteins are competent to bind DNA. The binding specificity of these factors is identical to that of recombinant MEF2A or MEF2D.

MATERIALS AND METHODS

Plasmids and recombinant proteins

pET.MEF2A is a derivative of pET15b that expresses an N-terminally His6-tagged fusion protein containing the entire MEF2A open reading frame (cDNA RSRFC4; 14). pET.MEF2D encodes an N-terminally His6-tagged MEF2D fusion protein containing mouse MEF2D codons 261–521, constructed by insertion of an appropriate PCR-generated fragment into pET15b. The MEF2C cDNA was a generous gift from Peter Rigby. pET.MEF2C encodes an N-terminally His6-tagged MEF2C fusion protein containing human MEF2C codons 245–465. The appropriate fragment of MEF2C cDNA was isolated from a Namalwa (B cell) cDNA library by the PCR using primers 5'-GTTATCGATCTCGAGATGAATTTAGGAATGAATAACCGT-3' and 5'-CGCTCTAGAGGATCCAAAACTAGTAAGTAATAATCTGA-3'. Proteins were purified by Nickel chelate chromatography according to the supplier's protocol (Novagen).

Cell extracts

Cells were cultured in DME containing 10% fetal calf serum. For differentiation of C2C12 myoblasts, the cells were transferred to medium containing 2% horse serum for 48 h. Cell extracts were prepared as described previously (29). For dephosphorylation, 25–100 µg extract protein (2–10 µl) was treated with 5–20 U Calf intestine alkaline phosphatase (Boehringer) for 90 min at 37°C in 20 µl 50 mM Tris-HCl pH 8.5, 10 mM MgCl₂, 0.1 mM ZnCl₂, 0.1% NP-40. For phosphatase inhibition, K₂HPO₄ (10 mM), micro-cystin LR (1 µM) and EGTA (20 mM) were added. Reactions were terminated by addition of phosphatase inhibitors and 4× SDS-PAGE sample buffer, and boiled for 2 min.

Gel mobility-shift assays and binding site selections

Binding reactions (20 µl) contained 15–30 µg of whole cell extract in (10 mM HEPES buffer, 0.2% Triton X-100, 150 mM KCl, 10% glycerol, 1 mg/ml ovalbumin, 3 mM spermidine, 5 mM EGTA, 5 mM EDTA and protease inhibitors as above), 2 µg pUC12 cut with *MspI* as a non-specific competitor DNA and 0.2 ng of labelled double-stranded probe. Complexes were allowed to form for 30–45 min at room temperature and were resolved on 5% 40:1 acrylamide-bisacrylamide non-denaturing gel in 0.5× Tris-borate-EDTA. For competition experiments 300-fold of wild-type or mutant unlabelled oligonucleotide was pre-incubated with the extract for 20 min before the addition of the labelled probe. For antibody supershift experiments, extracts were pre-incubated with antibody for 20 min prior to addition of the labelled probe.

Figure 1. (A) Affinity purification of MEF2A antibody. Extracts (120 µg, except SAOS, 40 µg and C2C12 myotubes, 35 µg) prepared from the indicated cells were fractionated by 8% SDS-PAGE, transferred to immobilon membranes, and used for immunoblotting with crude MEF2 antiserum (lanes 1 and 2), once affinity-purified MEF2 antibody (lanes 3 and 4) and twice affinity-purified MEF2 antibody (lanes 5–14). MEF2 proteins are indicated. The asterisk indicates a non-specific cross-reacting polypeptide that does not react with affinity purified MEF2 antibody. (B) Heterogeneity of MEF2 polypeptides is due to phosphorylation. Whole cell extracts from the indicated cells were incubated in phosphatase buffer alone (lanes 1, 4 and 7), treated with alkaline phosphatase (lanes 2, 5 and 8) or treated with alkaline phosphatase in the presence of phosphatase inhibitors (lanes 3, 6 and 9), before fractionation by 8% SDS-PAGE. MEF2 proteins were detected by immunoblotting with twice purified MEF2 antibody.

Oligonucleotides with *XbaI* compatible 5' ends (14) were as follows. The MEF2 core consensus sequence is underlined, and mutations are shown in *italic*:

N10WT:	AGGAAA <u>ACTATTTATAGATCAAAT</u>
N10M1:	AGGAAA <u>ACCATTATGGATCAAAT</u>
N10M2:	AGGAAA <u>ACGATTATCGATCAAAT</u>
N10M3:	AGGAAA <u>GTATTTATACATCAAAT</u>
N10M4:	AGGAAA <u>ACTTTTAAAGATCAAAT</u>
MCK1:	ACTCGCTCTAAAAATAACCCCTGTC
MCK2:	CGCTCTAAAAATAACCCCT (11)
MCK2-M6:	CGCTCTAAACATAACCCCT (11)

Site selections with whole cell extracts were performed essentially as described (23) using 1 µl of once affinity-purified MEF2 antibody, and 2 µl (20–25 µg) whole cell extract. Oligonucleotides were cloned and sequenced following five rounds of site selection.

Figure 2. Detection of MEF2 proteins by immunofluorescence microscopy. (A) NIH3T3 cells were fixed and stained using either pre-immune serum (frame 1), crude MEF2 antibody (frame 2) or twice affinity-purified MEF2 antibody (frame 3; this antibody is used at an equivalent dilution to the crude antibody as judged by MEF2A ELISA). Removal of cytoplasmic immunoreactivity correlates with depletion of the 80 kDa species in Figure 1A. (B) Detection of MEF2 proteins in various cell types. Cells were fixed and stained using twice affinity-purified MEF2 antibody as follows: HeLa, frames 1 and 6; SAOS-2, frames 2 and 7; C3H10T1/2 frames 3 and 7; C2C12 myoblasts, frames 4 and 8; C2C12 myotubes, frames 5 and 10. Controls with pre-immune serum are shown in frames 1–5.

Antibodies

MEF2 antibody (14) was raised against a GST fusion protein containing MEF2A codons 272–497 (from MEF2A cDNA RSRFC4; 14). MEF2 affinity columns were prepared by coupling 5–10 mg purified proteins to 1 ml CNBr-activated Sephadex (Pharmacia) in 0.1 M NaHCO₃, 0.5 M NaCl pH 8.3, according to the manufacturer's protocol. Crude anti-MEF2 immunoglobulins, recovered from 5 ml serum by addition of 0.82 vol saturated ammonium sulphate and EDTA to 5 mM, was redissolved in 12 ml TBS/0.1% NP-40 and loaded onto the affinity column. MEF2 antibody was eluted with 100 mM sodium citrate pH 2.5, neutralised with Tris base and recovered by ammonium sulphate precipitation. A MEF2A-specific anti-peptide serum, Ab115, was a gift from Dr Bernardo Nadal-Ginard (15).

Immunofluorescence and immunoblotting

SDS–PAGE and immunoblotting was by standard methods. For immunofluorescence, cells were plated on coverslips, fixed in 3.7% formaldehyde/PBS for 10 min at room temperature, and washed three times with PBS and once with PBS/0.1% Tween 20. Staining was with either crude pre-immune or immune antibody at 1:1000 dilution, or with twice affinity-purified MEF2 antibody at 1:200 dilution (equivalent reactivity in MEF2A ELISA), in PBS containing 0.5% NP-40 and 10% donkey serum. Following three washes with PBS, MEF2 was detected with Texas Red-conjugated donkey anti-mouse immunoglobulin (Jackson

Laboratories, West Grove, PA) at 1:200 dilution followed by three washes with PBS/0.1% Tween 20.

RESULTS

MEF2 proteins can be detected in a variety of cell lines

We previously raised a polyclonal antiserum against a GST fusion protein containing the 250 C-terminal amino acids of human MEF2A. In gel mobility-shift assays, the antiserum recognises MEF2 complexes in both muscle and non-muscle cell extracts (14). To characterise this serum further, we prepared an immunoglobulin fraction by caprylic acid precipitation and used it to assay whole cell extracts of C2C12 myotubes and HeLa cells for MEF2 proteins by immunoblotting (Fig. 1A). The antiserum reacts with a number of polypeptides predominantly in the 70–80 kDa range, and a well-defined species of ~80 kDa (Fig. 1A, lanes 1 and 2). No reactivity was observed with pre-immune serum (data not shown). We next prepared a MEF2A-Sepharose affinity column and used it for affinity purification of a crude MEF2 immunoglobulin fraction. Following a single round of affinity purification [MEF2(1×)], reactivity against the 80 kDa polypeptide was greatly reduced, and after a second round it was undetectable (Fig. 1A, compare lanes 3 and 4, 14 and 5, with lanes 1 and 2). In contrast, affinity purification had little effect on the reactivity of the antiserum against the majority of polypeptide species between 70 and 80 kDa. These results indicate that the 80 kDa polypeptide most likely represents a non-specific reactivity of the

antibody, while the 70–80 kDa species represent polypeptides antigenically related to MEF2A.

We used the twice affinity-purified serum [MEF2(2x)] to assess a number of muscle and non-muscle cell lines for the presence of MEF2 polypeptides. All the non-muscle cell lines showed a similar heterogeneous pattern of immunoreactive polypeptides, although at lower levels than those found in myoblasts or myotubes (Fig. 1A, lanes 5–14). The multiplicity of immunoreactive polypeptides observed in the immunoblotting assays is consistent either with the presence of multiple MEF2 isoforms or heterogeneity arising from post-translational modifications. Since radiolabelling experiments indicated that in NIH3T3 cells, MEF2-reactive material appears extensively phosphorylated (E.D. and R.T., unpublished observations), we tested whether phosphatase treatment of cell extracts would reduce the heterogeneity in MEF2-reactive polypeptides observed in the immunoblotting experiments. Following treatment of C2C12 myotube, HeLa or mouse C3H10T1/2 extracts with alkaline phosphatase, only a single broad band of MEF2-reactive polypeptide was detectable by immunoblotting (Fig. 1B, compare lanes 2, 5 and 8 with 1, 4 and 7). We conclude that a limited number of extensively phosphorylated MEF2A-related polypeptides are present in both muscle and non-muscle cell lines.

MEF2 proteins are nuclear in muscle and non-muscle cells

We next used immunofluorescence microscopy to examine the subcellular localisation of MEF2 proteins in muscle and non-muscle cell lines (Fig. 2). The crude MEF2 antibody reacted with both nuclear and cytoplasmic material in NIH3T3 cells; in contrast, the twice affinity-purified anti-MEF2A antibody generated an exclusively nuclear signal (Fig. 2A). The cytoplasmic signal observed with the crude MEF2 antibody may therefore be due to the 80 kDa non-specific polypeptide. The affinity-purified anti-MEF2 antibody gave a strong nuclear signal with a variety of other non-muscle cell lines including HeLa, 10T1/2 and SaoS-2, and as well as both C2C12 myoblasts and C2C12 myotubes (Fig. 2B). These results demonstrate that the MEF2A-like polypeptides detected in Western blots of whole cell extracts are localised in the cell nucleus.

Generation of a MEF2A-specific antibody

Four different MEF2 gene products have been characterised (14–18,20,21). Although the C terminal fragment of MEF2A used in our immunisations does not include the highly homologous DNA binding domain common to all MEF2 family members, this region does show significant primary sequence homology to MEF2C and MEF2D. We therefore next investigated the extent to which the MEF2 antiserum cross-reacts with these other MEF2 proteins. The C terminal regions of the MEF2C and MEF2D proteins were expressed in *E.coli* and a range of serial dilutions of each protein was used in immunoblotting experiments to evaluate the extent of cross-reactivity of the once affinity-purified MEF2 antibody. The MEF2 antibody reacted with MEF2D ~3-fold less efficiently than with MEF2A (Fig. 3A, lanes 2), and with MEF2C >10-fold less efficiently than with MEF2A (Fig. 3A, lanes 3). Thus, the MEF2 proteins detected by immunoblotting of whole cell extracts may represent MEF2A, MEF2C or MEF2D. Similar results were obtained using the twice affinity-purified antibody (data not shown).

Figure 3. (A) Purification of a MEF2A-specific antibody. Three-fold serial dilutions recombinant His-tagged MEF2A (lanes 1 and 4), MEF2D[261–521] (lanes 2 and 5) and MEF2C[245–465] (lanes 3 and 6) were fractionated by 12% SDS-PAGE, transferred to immobilon membrane and probed with once affinity-purified MEF2 antibody (top panel) or once affinity-purified, MEF2D and MEF2C-depleted antibody (anti-MEF2A; lower panel). (B) MEF2A is present in non-muscle cells. Extracts from HeLa (100 µg; lanes 1, 4 and 7) C2C12 myotube (30 µg; lanes 2, 5 and 8) and NIH3T3 cells (100 µg; lanes 3, 6 and 9) were fractionated by 8% SDS-PAGE, transferred to immobilon membranes, and used for immunoblotting with once affinity-purified MEF2 antibody (lanes 1–3), anti-MEF2(A+C) lanes (3–6) or anti-MEF2A (lanes 7–9). The polypeptide indicated by the asterisk indicates the non-specific species that is removed by subsequent MEF2A affinity purification.

To determine whether the MEF2 polypeptides contain MEF2A, we generated a MEF2A-specific antibody. The crude MEF2 antibody was purified by a single round of affinity purification

Figure 4. Gel mobility-shift analysis of MEF2 with the N10 MEF2 site. (A) MEF2A in both muscle and non-muscle cell extracts binds the N10 MEF2 site probe. Binding reactions with extracts from the indicated cells were incubated with no added antibody (lanes 1, 6, 11, 16 and 21) pre-immune serum (PI; lanes 2, 7, 12, 17 and 22), once affinity-purified MEF2 antibody (lanes 3, 8, 13, 18 and 23), anti-MEF2(A+C) (lanes 4, 9, 14, 19 and 24), or anti-MEF2A (lanes 5, 10, 15, 20 and 25). Lanes 1–5, C2C12 myoblasts (30 μ g); lanes 6–10, C2C12 myotubes (10 μ g); lanes 11–15, C3H10T1/2 (30 μ g); lanes 16–20, NIH3T3 (25 μ g); lanes 21–25, HeLa (15 μ g); lanes 26–30, SAOS-2 (10 μ g). (B) Binding competition analysis. Extracts from the indicated cells were used for mobility-shift analysis; reactions were either mock-incubated (lanes 1, 8 and 15) or incubated with the competitor oligonucleotides N10WT (lanes 2, 9 and 16), M1 (lanes 3, 10 and 17), M2, (lanes 4, 11 and 18), M3 (lanes 5, 12 and 19), M4 (lanes 6, 13 and 20) MCK1 (lanes 7, 14 and 21), before probe addition and subsequent gel electrophoresis. Lanes 1–7, C2C12 myotubes (10 μ g); lanes 8–14, HeLa (15 μ g); lanes 15–21, NIH3T3 (25 μ g).

and then depleted of MEF2D reactivity by passage through a MEF2D affinity column. Antibody present in the MEF2D column flow-through reacted with MEF2A and cross-reacted only with MEF2C in immunoblotting experiments (data not shown) and therefore was designated anti-MEF2(A+C). This material was then further purified by passage through a MEF2C column. The flow-through from this column was tested for reactivity against MEF2A, MEF2C and MEF2D by immunoblotting and found to be specific for MEF2A (Fig. 2A, compare lane 4 with 5 and 6). We shall refer to this material as anti-MEF2A.

The variously purified antibody fractions were used to detect MEF2 proteins in whole cell extracts by immunoblotting.

Interestingly, anti-MEF2A detected essentially the same pattern of polypeptides in HeLa, C2C12 myotubes and NIH3T3 cells as the anti-MEF2 and anti-MEF2(A+C) fractions (Fig. 3B, compare lanes 1–3, 4–6 and 7–9). (Note that since the starting material used to generate anti-MEF2A was purified by a single round of MEF2A affinity chromatography, both the anti-MEF2(A+C) and anti-MEF2A fractions retain cross reactivity with the 80 kDa polypeptide described above; this is lost after a second round of MEF2A affinity purification). These findings suggest that either MEF2A is the predominant isoform present in the cell lines tested or that the anti-MEF2 antibody has the greatest affinity for MEF2A.

Figure 5. Complexes formed with the MCK MEF2 site in non-muscle cells contain MEF2A. (A) MEF2A in both muscle and non-muscle cell extracts binds the MCK MEF2 site. As probe the MCK2 oligonucleotide was used. Binding reactions containing proteins from the indicated cells were incubated with either no additions (lanes 1, 6, 11, 16 and 21), MCK2 competitor oligonucleotide (lanes 2, 7, 12, 17 and 23), MCK2m6 competitor oligonucleotide (lanes 3, 8, 13, 18 and 24), pre-immune serum (lanes 4, 9, 14, 19 and 25), anti-MEF2A (lanes 5, 10, 15, 20 and 26) or MEF2A specific antipeptide antiserum Ab115 (lanes 6, 11, 16, 21 and 27) before probe addition and subsequent gel electrophoresis.

Non-muscle MEF2 proteins bind DNA

The results above demonstrate that MEF2 proteins are widely expressed and that expression of MEF2A is not restricted to muscle cells. These data are consistent with previous reports of MEF2-like DNA binding activities in a wide range of cell types (12–14,27). We used gel mobility-shift analysis to analyse these binding activities in detail, using the purified antibody to test for the presence of MEF2A.

For the first experiments we used a DNA probe from the N10 nuclear receptor gene promoter which conforms to the MEF2A binding site consensus sequence determined by *in vitro* binding site selections (23). In all the extracts including C2C12 myoblasts and myotubes, NIH3T3 fibroblasts, C3H10T1/2 fibroblasts, HeLa and SAOS-2 cells, a well-resolved low mobility complex was detectable (Fig. 4A, lanes 1, 6, 11, 16, 21 and 26), which co-migrated with that formed by MEF2A (data not shown; see 14). This complex reacted quantitatively with the anti-MEF2 antibody (Fig. 4A, lanes 3, 8, 13, 18, 23 and 28). We next used the isoform-specific antibodies to assess the composition of this MEF2 complex. In each extract, the MEF2 complex reacted with both the anti-MEF2(A+C) and the anti-MEF2A antibodies. Thus, all these lines thus contain MEF2A in a state competent to bind DNA. However the presence of MEF2 complexes that react with neither the anti-MEF2(A+C) nor the anti-MEF2A antibody indicates that at least some of the MEF2 complexes contain neither MEF2A nor MEF2C; they most likely represent MEF2D homodimers (see Discussion).

MEF2 binding activities from muscle and non-muscle cells exhibit the same DNA sequence specificity

The results described in the previous section show that MEF2 complexes that contain MEF2A are present in non-muscle cells.

We next tested whether the binding specificity of these complexes is in any way different from that of MEF2 as defined by our previous studies. Complexes formed between C2C12 myotube, HeLa or NIH3T3 extracts and the N10 probe were challenged with excess unlabelled oligonucleotides containing the intact N10 MEF2 site or mutant N10 oligonucleotides containing changes at conserved or invariant positions of the MEF2 consensus sequence. In each case, complex formation was entirely abolished by competitors containing either the unlabelled N10 site, or the prototype MEF2 site from the MCK enhancer (Fig. 4B, lanes 2, 7, 9, 14, 16 and 17). As previously observed, three N10 oligonucleotide derivatives containing mutations at invariant positions of the MEF2 consensus site (competitors M1, M2 and M4) failed to compete with the N10 probe for complex formation, while an oligonucleotide containing transversions at the highly conserved edges of the consensus sequence (competitor M3) competed only very weakly [Fig. 4B, lanes 3–6 (C2C12 myotube extract), 10–13 (HeLa extract), 17–20 (NIH3T3 extract)]. According to this analysis the sequence specificity of both muscle and non-muscle MEF2 complexes is identical and indistinguishable from that of recombinant MEF2A (14).

Many of the initial gel mobility-shift studies of MEF2 utilised probes derived from the MCK enhancer, which contains a non-consensus MEF2 binding site (10). It has been reported that non-muscle complexes formed by this probe do not contain MEF2, based on their ability to bind a mutant derivative of the MCK site, MCKm6 (11). To address this issue in more detail, we used an MCK probe to investigate our different cell extracts. The probe formed a specific complex with proteins from both C2C12 myoblasts and myotubes, C3H10T1/2 cells, C3H10T1/2 aza-myoblasts and NIH3T3 cells (Fig. 5, lanes 1, 2, 6, 7, 11, 12, 16, 17, 22 and 23). Two experiments identified this complex as authentic MEF2. First, its formation was not affected by excess mutated MCK oligonucleotide MCKm6 (Fig. 5, compare lanes 1,

6, 11, 16 and 22 with lanes 3, 7, 10, 17 and 23). Second, the MCK complexes exhibited a similar pattern of immunoreactivity with both our anti-MEF2A antibody (Fig. 5, lanes 5, 10, 15, 20 and 26) and an antibody raised against a MEF2A synthetic peptide (15; Fig. 5, lanes 21 and 26).

Anti-MEF2 recognises only proteins that bind the MEF2 consensus

To demonstrate unambiguously that the non-muscle MEF2 proteins detected by our antibody represent *bona fide* MEF2 species rather than crossreacting proteins, we performed a binding site selection experiment (23). In this assay, antibody is used to immunoprecipitate proteins from cell extracts, and the immune complexes are then incubated with a pool of random sequence oligonucleotides; the oligonucleotides are then recovered from the immunoprecipitate using the PCR and used for further rounds of selection. Enrichment of the oligonucleotide pool for MEF2 sites in this assay will therefore indicate that the proteins recognised by the antibody represent authentic MEF2. Whole cell extracts of NIH3T3 fibroblasts and C2C12 myotubes were incubated with a random sequence oligonucleotide pool; DNA associated with MEF2-reactive protein was recovered by immune-precipitation using once affinity purified antibody and amplified using the PCR. After four rounds of selection, the selected DNA was subcloned and sequenced. The results are shown in Figure 6. The consensus binding site derived using both the fibroblast and myotube cell extracts are identical, and essentially indistinguishable from results previously obtained for recombinant MEF2A or MEF2D (14,21). Thus, muscle and non-muscle cell extracts contain proteins that both react with the anti-MEF2 antibody and bind DNA with identical sequence specificity to recombinant MEF2A or MEF2D protein. We conclude that these proteins represent authentic MEF2.

DISCUSSION

In this work we used a well characterised MEF2 antibody in conjunction with DNA binding studies to investigate the nature of proteins interacting with the MEF2 consensus sequence in muscle and non-muscle cells. We found that MEF2 proteins were easily detectable by immunoblotting in the nuclei of a variety of non-muscle cell lines and were competent to bind DNA. The amount of MEF2 protein was ~5-fold increased in C2C12 myotubes compared with C2C12 myoblasts, and the amount of MEF2 specific DNA binding activity was increased in parallel. In both muscle and non-muscle cell lines MEF2 proteins are heavily phosphorylated.

We used affinity chromatography to generate an antibody specific for MEF2A, which cross-reacted with the MEF2A-related proteins MEF2C and MEF2D ~1000-fold less efficiently. DNA binding studies of extracts prepared from both non-muscle cell lines and C2C12 myoblasts and C2C12 myotubes revealed that approximately half of the MEF2 complexes detectable in gel mobility-shift experiments contained MEF2A. Moreover, these complexes also reacted with an anti-MEF2A-specific serum previously reported to react only with muscle cell extracts (15). MEF2A can heterodimerise with both MEF2B (14) and MEF2D (18); since the DNA binding and dimerisation domains of the individual MEF2 proteins are virtually identical, it is likely that each can heterodimerise with other family members. The MEF2A

NIH3T3 MEF2

Sequences:																			
A	54	67	4	-	-	124	1	8	53	102	-	124	-	84	53	25			
G	37	22	25	-	-	-	-	-	-	-	-	-	124	-	1	6			
C	7	1	-	124	-	-	-	-	-	-	-	-	-	34	18	51			
T	15	34	95	-	124	-	123	116	71	22	124	-	-	6	52	42			

Consensus:																			
(C) T/G	C	T	A	T	T	T/A	A/T	T	A	G	A/C (G)								
	C	T	A	T	T	T	A	T	A	G	47								
				T	T	A	A				45								
				T	T	T	T				21								

C2C12 myotube MEF2

Sequences:																			
A	45	46	7	-	-	105	-	9	37	77	-	106	-	61	32	24			
G	33	23	32	-	-	-	-	-	-	-	-	-	106	2	8	16			
C	10	2	1	106	-	-	1	-	-	-	-	-	-	36	23	34			
T	16	32	64	-	106	-	105	97	69	29	106	-	-	7	44	31			

Consensus :												
(C)	T/G	C	T	A	T	T/A	T/A	T/A	T	A	G	A/C (G)
		C	T	A	T	T	T	A	T	A	G	47
					T	T	A	A				45
					T	T	T	T				21
					T	A	T	T				2

Recombinant MEF2A (Ref. 14)

Consensus :												
(C)	T/G	C	T	A	T	T/A	T/A	T/A	T	A	G	A/C (G)
	C	T	A	T	T	T	A	A	T	A	G	28
				T	T	T	A	A				6
				T	T	T	T	T				13
				T	A	T	T	T				4
				T	A	T	A	A				3
				T	T	A	T	T				1
				T	A	A	T	T				1

Figure 6. The MEF2 antibody recovers proteins that bind DNA with MEF2 specificity. The once affinity purified MEF2 antibody was used in a binding site selection experiment with whole cell extracts from C2C12 myotubes (25 µg) or NIH3T3 cells (20 µg). Sequences of oligonucleotides present following five rounds of selection are summarised, with the frequency of central AT quartets summarised below, and compared with the consensus derived using *in vitro* translated MEF2A (14).

reactive material that we observe in the DNA binding assays may therefore represent heterodimers rather than MEF2A homodimers. If this is the case, the reactivity of MEF2 complexes with the MEF2A antibody will somewhat overestimate the proportion of MEF2 protein in the cell that is MEF2A. The substantial amount of anti-MEF2(A+C) non-reactive material observed in our experiments is likely to represent MEF2D homodimers, in agreement with a recent report (29).

Previous studies of MEF2-like activities in non-muscle cells have led to conflicting findings concerning the DNA binding specificity of the complexes. Studies of complexes formed on the mouse MCK MEF2 site led to the proposal that non-muscle cells contain activities with a different binding specificity but similar mobility to that of authentic MEF2 (11,15). We have not observed such complexes in our experiments, and the complexes that we detect fail to bind a mutated MCK MEF2 site previously used to distinguish these MEF2-like complexes from authentic MEF2 (11). Moreover, when our affinity-purified MEF2 antiserum is used in binding site selection experiments with either muscle or non-muscle extracts as a source of protein, the binding sites selected have identical specificity to that previously determined for recombinant MEF2 proteins (14,21). Our results are in full agreement with studies utilising a MEF2 consensus sequence from the rat CKB promoter; this probe detected a factor, TARP, with similar binding properties to MEF2, that was not muscle-restricted (13). It is likely that the discrepancy between our results

and previous studies arises from the different probes and binding conditions used, and from the fact that the mouse MCK sequence is a relatively low affinity MEF2 site variant [note that the rabbit MCK MEF2 site matches exactly the MEF2 consensus site derived *in vitro* (31)].

Studies on the expression of MEF2 RNAs have shown that MEF2A and MEF2D RNAs are widely expressed (14,15,18,20); in contrast, MEF2C RNA is apparently restricted to muscle and brain, while some splice variants of MEF2A and MEF2D are muscle-specific (15,16,18,20). The apparent discrepancy between expression at the RNA and protein levels has led to the proposal that expression of at least some MEF2 isoforms may be controlled at the post-transcriptional level. In contrast, our results and those of others (29) suggest that translational control of MEF2A protein expression is not a major control mechanism. Moreover, we have been able to recover cDNAs representing both the ubiquitous and muscle-specific mRNA isoforms of both MEF2C and MEF2D from a Namalwa B cell cDNA library (E.D. and R.T., unpublished results), suggesting that post-transcriptional control of MEF2 expression, if it occurs, is not absolute.

At present the function of MEF2 proteins in non-muscle cells remains unclear. MEF2 sites contribute to activity of the CKB (12) and c-jun promoters (27) and MEF2 sites function as weakly serum-inducible promoter elements in transfection assays (14,29). However, one group has reported that MEF2 proteins have some capability to induce myogenic conversion of C3H10T1/2 cells (32). In view of the abundant MEF2 DNA binding activity that we can detect in C3H10T1/2 cells, this finding is inconsistent with a simple model that MEF2 expression is sufficient to induce conversion. Perhaps negative regulators of MEF2 are titrated out by MEF2 overexpression; alternatively, it remains possible that the different results may reflect cell line differences. The MEF2 relative SRF has targets in both muscle and non-muscle cells, and appears to mediate either growth factor-inducible or constitutive transcriptional activation according to promoter context (7,8,33). If MEF2 proteins exhibit similar context dependent regulatory properties, it will be interesting to identify tissue-specific cofactors that might mediate such effects.

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REFERENCES

- Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y., and Lassar, A. (1991) *Science*, **251**, 761–766.
- Weintraub, H. (1993) *Cell*, **75**, 1241–1244.
- Norman, C., Runswick, M., Pollock, R. and Treisman, R. (1988) *Cell*, **55**, 989–1003.
- Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. and Sommer, H. (1990) *Science*, **250**, 931–936.
- Walsh, K. (1989) *Mol. Cell. Biol.*, **9**, 2191–2201.
- Minty, A. and Kedes, L. (1986) *Mol. Cell. Biol.*, **6**, 2125–2136.
- Taylor, M., Treisman, R., Garrett, N. and Mohun, T. (1989) *Development*, **106**, 67–78.
- Sartorelli, V., Webster, K. A. and Kedes, L. (1990) *Genes Dev.*, **4**, 1811–1822.
- Treisman, R. (1990) *Semin. Cancer Biol.*, **1**, 47–58.
- Gossett, L. A., Kelvin, D. J., Sternberg, E. A. and Olson, E. N. (1989) *Mol. Cell. Biol.*, **9**, 5022–5033.
- Cserjesi, P. and Olson, E. N. (1991) *Mol. Cell. Biol.*, **11**, 4854–4862.
- Hobson, G. M., Molloy, G. R. and Benfield, P. A. (1990) *Mol. Cell. Biol.*, **10**, 6533–6543.
- Horlick, R. A., Hobson, G. M., Patterson, J. H., Mitchell, M. T. and Benfield, P. A. (1990) *Mol. Cell. Biol.*, **10**, 4826–4836.
- Pollock, R. and Treisman, R. (1991) *Genes Dev.*, **5**, 2327–2341.
- Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V. and Nadal-Ginard, B. (1992) *Genes Dev.*, **6**, 1783–1798.
- Martin, J. F., Schwarz, J. J. and Olson, E. N. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5282–5286.
- Leifer, D., Krainc, D., Yu, Y. T., McDermott, J., Breitbart, R. E., Heng, J., Neve, R. L., Kosofsky, B., Nadal-Ginard, B. and Lipton, S. A. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1546–1550.
- Martin, J. F., Miano, J. M., Hustad, C. M., Copeland, N. G., Jenkins, N. A. and Olson, E. N. (1994) *Mol. Cell. Biol.*, **14**, 1647–1656.
- Leifer, D., Golden, J. and Kowall, N. W. (1994) *Neuroscience*, **63**, 1067–1079.
- Breitbart, R. E., Liang, C. S., Smoot, L. B., Laheru, D. A., Mahdavi, V. and Nadal-Ginard, B. (1993) *Development*, **118**, 1095–1106.
- Chambers, A. E., Kotecha, S., Towers, N. and Mohun, T. J. (1992) *EMBO J.*, **11**, 4981–4991.
- Chambers, A. E., Logan, M., Kotecha, S., Towers, N., Sparrow, D. and Mohun, T. J. (1994) *Genes Dev.*, **8**, 1324–1334.
- Pollock, R. and Treisman, R. (1990) *Nucleic Acids Res.*, **18**, 6197–6204.
- Sharrocks, A. D., von Hesler, F. and Shaw, P. E. (1993) *Nucleic Acids Res.*, **21**, 215–221.
- Nurrish, S. J. and Treisman, R. (1995) *Mol. Cell. Biol.*, **15**, 4076–4085.
- Mitchell, M. T. and Benfield, P. A. (1990) *J. Biol. Chem.*, **265**, 8259–8267.
- Han, T. H., Lamph, W. W. and Prywes, R. (1992) *Mol. Cell. Biol.*, **12**, 4472–4477.
- Buchberger, A., Ragge, K. and Arnold, H. H. (1994) *J. Biol. Chem.*, **269**, 17289–17296.
- Han, T. H. and Prywes, R. (1995) *Mol. Cell. Biol.*, **15**, 2907–2915.
- Marais, R., Wynne, J. and Treisman, R. (1993) *Cell*, **73**, 381–393.
- Yi, T. M., Walsh, K. and Schimmel, P. (1991) *Nucleic Acids Res.*, **19**, 3027–3033.
- Kaushal, S., Schneider, J. W., Nadal-Ginard, B. and Mahdavi, V. (1994) *Science*, **266**, 1236–1240.
- Boxer, L. M., Prywes, R., Roeder, R. G. and Kedes, L. (1989) *Mol. Cell. Biol.*, **9**, 515–522.