MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 2907–2915 Vol. 15, No. 6
0270-7306/95/$04.00+0

Regulatory Role of MEF2D in Serum Induction of the c-*jun* Promoter

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Received 22 December 1994/Returned for modification 3 February 1995/Accepted 27 February 1995

Serum induction of c-*jun* expression in HeLa cells requires a MEF2 site at −59 in the c-*jun* promoter. MEF2 sites, found in many muscle-specific enhancers, are bound by a family of transcription factors, MEF2A through -D, which are related to serum response factor in their DNA binding domains. We have found that MEF2D is the predominant protein in HeLa cells that binds to the c-*jun* MEF2 site. Serum induction of a MEF2 reporter gene was not observed in a line of NIH 3T3 cells which contain low MEF2 site binding activity. Transfection of MEF2D into NIH 3T3 cells reconstituted serum induction, demonstrating that MEF2D is required for the serum response. Deletion analysis of MEF2D showed that its DNA binding domain, when fused to a heterologous transcriptional activation domain, was sufficient for serum induction of a MEF2 reporter gene. This is the domain homologous to that in the serum response factor which is required for serum induction of the c-*fos* serum response element, suggesting that serum regulation of c-*fos* and c-*jun* may share a common mechanism.

Transcription of cellular immediate-early genes is rapidly induced by treatment of cells with serum and many different growth factors (reviewed in reference 13). The promoters of many of these genes, such as c-*fos*, contain a serum response element (SRE) which is required for regulation by many of the inducing agents (reviewed in reference 34). Some immediate-early genes, however, do not appear to contain SRE regulatory sequences and therefore must be regulated by other elements. One such gene that we have examined is the c-*jun* proto-oncogene (12). Serum induction of c-*jun* mRNA peaks within 30 min of treatment and returns to basal levels after 2 to 4 h (18, 29). Induction of c-*jun* occurs primarily at the transcriptional level, as shown by nuclear run-on assays (27, 29).

The c-*jun* promoter region between −117 and −50 contains binding sites for the transcription factors Sp1, CTF, AP-1, and MEF2 (1, 11). The MEF2 site was found to mediate serum induction of a reporter gene, and the MEF2 binding sequence at −59 in the c-*jun* promoter was found to be required for serum and epidermal growth factor (EGF) induction of the c-*jun* promoter (11, 25). The MEF2 site was originally defined as being a required element in the enhancers of several muscle-specific genes and is bound by a family of factors termed MEF2A, -B, -C, and -D (also termed RSRFs) (2, 3, 10, 15, 22–25, 38).

The MEF2 family of factors have common DNA binding domains located at the amino termini of the proteins. The domain is similar to that of MADS box proteins, a family of DNA-binding proteins comprising serum response factor (SRF), the yeast regulatory proteins MCM1 and ARG80, and several plant proteins (reviewed in reference 33). The consensus DNA binding sequences of MEF2 factors, CTA(A/T)$_4$TAG, are found in the regulatory regions of many muscle-specific genes and at least two growth factor-inducible genes (c-*jun* and *nur77*/NFGI-B/N10) (25). The MEF2 site has been shown to be functionally important for the regulation of several muscle-specific genes (2, 10, 15, 36, 39). Notably, in transgenic mice, mutation of the MEF2 site in the myogenin promoter abolished expression of a reporter gene in a subset of myogenic precursors, demonstrating the importance of this site for precise cell-type-specific expression (6, 37). MEF2 site binding activity is also induced during differentiation of skeletal myoblasts in culture and fibroblasts induced to myogenic conversion by MyoD family members (7, 10, 19). In *Xenopus laevis*, MyoD also induced MEF2A and -D expression, and ectopic expression of *Xenopus* MEF2D was able to stimulate cardiac muscle-specific gene expression in cultured blastula animal pole extracts (4). These results suggest that MEF2 proteins act downstream of MyoD-like factors to drive muscle differentiation.

Expression of all of the MEF2 family members and certain specific splice variants is found in muscle cells. Expression of MEF2A and -B mRNA, however, has been found in other cells types (25, 38), and MEF2C expression was found in cortical neurons as well as differentiated muscle cells (23, 24). MEF2D expression appears to be the most ubiquitous (3, 22). MEF2D is expressed earliest in undifferentiated myoblasts, while the other family members and a splice variant of MEF2D are induced by differentiation (3, 4, 22). Although MEF2 mRNAs are found in many different cell types, MEF2 protein expression has been reported to be restricted to muscle-specific cell lines and nervous tissue, suggesting posttranscriptional regulatory mechanisms (3, 38). Several groups, however, have found MEF2 site binding activity in other cell types (11, 16, 25). The reasons for this contradiction are unclear.

The role of MEF2 proteins in growth factor regulation of non-muscle-specific genes has not been previously investigated. It has been unclear which, if any, of these proteins are expressed in nonmuscle cells and how they are involved in gene regulation. Each of the MEF2 family members can activate transcription from a reporter gene containing MEF2 sites such that it is also not known whether there are functional differences among these factors (3, 22–24, 38).

We previously found, using gel mobility shift assays, that there are factors in HeLa cell nuclear extracts which specifically bind to the c-*jun* MEF2 site (12). We sought to determine whether the factors in HeLa cell nuclear extracts are related to the cloned MEF2 genes and whether MEF2 proteins are responsible for growth factor regulation of c-*jun*. In this report, we show that one of the MEF2 factors, MEF2D, is the major

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molecule in HeLa cell nuclear extracts binding to the sequence at −59 in the c-*jun* promoter. We further show that MEF2D is sufficient to reconstitute serum regulation of the c-*jun* MEF2 site in cells containing low MEF2 activity. This assay has allowed us to determine the domains of MEF2D required for serum regulation of c-*jun* expression.

MATERIALS AND METHODS

Plasmids. Details of the construction of the plasmids described below will be provided upon request. In addition, all junction sequences were confirmed by DNA sequencing. The reporter plasmid p0FLuc contains positions −53 to +42 of the c-*fos* promoter upstream of the firefly luciferase gene (17). The specific MEF2 or GAL4 binding sites were cloned into p0FLuc at the −53 position. pMEF2FLuc was generated by cloning a double-stranded oligonucleotide (MEF2-WT; TCGAGGGCTATTTTTAGGGCC) spanning the c-*jun* MEF2 site with *Xho*I ends into the *Xho*I site of p0FLuc. The reporter plasmid pG1FLuc contains a single GAL4 binding site in p0FLuc and was as described previously (17). The reporter plasmid pJLuc contains positions −225 to +150 of the c-*jun* promoter upstream of the firefly luciferase gene (17). The c-*jun* promoter fragment carrying point mutations at the MEF2 site was taken from pJSX (11) to make pJSXLuc.

MEF2A expression plasmid pCGNC4 and MEF2B expression plasmid pCGNR2 were generated by subcloning the full human cDNA sequences from pT7C4 and pT7R2, respectively, downstream of a cytomegalovirus (CMV) promoter in pCGN (31). pT7C4 and pT7R2 were kind gifts from Richard Treisman and were as described previously (25). The expression plasmids pCMVMEF2C, pCMVMEF2D, and pCMVMEF2DVP16 encode full-length mouse MEF2C, full-length mouse MEF2D, and the DNA binding domain of MEF2D (amino acids 1 to 92) fused to the transcriptional activation domain of VP16 (amino acids 412 to 490), respectively, under the control of a CMV promoter and were kindly provided by James Martin and Eric Olson (22, 23). pCMVMEF2D contains MEF2D's alternative exons 1a and 2 (22). pCMVMEF2DT encodes amino acids 1 to 92 of MEF2D. pCMVβgal encodes the β-galactosidase gene under the control of a CMV promoter. The expression plasmid for GAL4-VP16, pCGVP16, contains GAL4's DNA binding domain (amino acids 1 to 147) fused to VP16 (amino acids 412 to 490) under the control of a CMV promoter. The expression plasmid pCGMEF2D encodes GAL4's DNA binding domain fused to full-length MEF2D. The construct pCGMEF2DC′ encodes GAL4's DNA binding domain fused to amino acids 93 to 514 of MEF2D.

Transfections and luciferase assays. HeLa and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% newborn calf serum. One 10-cm plate was transfected by the calcium phosphate coprecipitation method (30) with 10 μg of reporter plasmid, 3 to 6 μg of pCMVβgal as an internal control, and 0.1 to 5.0 μg of expression plasmid as indicated. About 24 h after transfection, the cells were split onto three 6-cm plates and serum starved in 0.5% calf serum for 30 h. Two plates were starved, and the third plate was used to prepare cell extracts for gel mobility shift assays or immunoblots. Cell extracts for those assays were prepared as described previously (32). The starved cells were either untreated or treated with 100 ng of murine EGF (Collaborative Research) per ml or 20% bovine calf serum for 2 h. Preparation of cell extracts, β-galactosidase assays, and luciferase assays using the Promega luciferase assay system were performed according to the manufacturer's protocol and as described previously (17). All luciferase values were normalized to the β-galactosidase values resulting from expression of the internal control plasmid, pCMVβgal.

Antibodies and immunoprecipitations. Regions of human MEF2A (codons 272 to 491; without the alternative exon of amino acids 289 to 296), human MEF2B (codons 234 to 365), and mouse MEF2D (codons 292 to 514) were fused downstream of a polyhistidine tag in plasmid pRSET (Invitrogen), and the fusion proteins were purified on nickel-agarose columns according to the manufacturer's protocols. Rabbits were immunized three to four times at 4-week intervals with 100 μg of fusion protein in Freund's adjuvant. Antisera were tested by immunoblotting and gel mobility shift assays.

For ^{35}S labelling, cells were grown overnight in 4 ml including 3.6 ml of DME lacking methionine and cysteine, 0.4 ml of regular DME with 0.2% calf serum, and 400 μCi of [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-label; ICN) per 10-cm plate. For ^{32}P labelling, cells were incubated for 3 h in 2 ml of DME lacking phosphate and 200 μCi of $^{32}P_i$ (Amersham) per 10-cm plate. Cells were induced with 10% calf serum or EGF (100 ng/ml) for different time intervals before being lysed with radioimmunoprecipitation assay (RIPA) buffer (0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Nonidet P-40, 2 mM EDTA, 150 mM NaCl, 10 mM Tris [pH 7.4], 0.5 mM phenylmethylsulfonyl fluoride). Cell lysates (250 μl) were incubated with 4 μl of preimmune serum or immune serum in the presence of excess amounts (2.5 μg) of various competitors for 1 h at 4°C. They were further incubated for 30 min after addition of 50 μl of a 50% slurry of protein A-Sepharose beads (RepliGen). The beads were then washed two times with RIPA buffer, two times with RIPA buffer with 0.5 M NaCl, and once with RIPA buffer. The beads were then boiled in 1× SDS-polyacrylamide gel electrophoresis sample buffer, and the eluted proteins were resolved on an SDS–8% polyacrylamide gel.

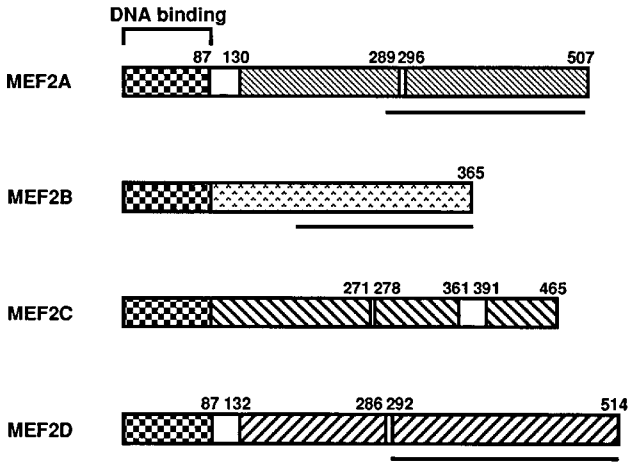

FIG. 1. Structures of MEF2 proteins. The conserved and unique domains of the MEF2 family members are indicated. The checkered boxes at the amino termini represent the conserved DNA binding domains which are similar to MADS box sequences. The alternative exons are shown as empty boxes. The bars underneath the unique domains indicate the regions used to raise specific antisera.

Gel mobility shift assays. In vitro transcriptions and translations using the Promega rabbit reticulocyte lysate system were performed according to the manufacturer's protocols. pT7C4, pT7R2, pCMVMEF2C, and pCMVMEF2D were used for MEF2A, -B, -C, and -D, respectively. Nuclear extracts were prepared and gel mobility shift assays were performed as described previously (26). The DNA binding reaction mixtures contained 1 μg of herring sperm DNA and 1 ng of ^{32}P-labelled probe. Competitor oligonucleotides (50 ng) were added as indicated. For antibody supershift experiments, binding reaction mixtures were incubated with 0.1 to 1 μl of preimmune or immune serum for 2 h before addition of the probe.

RESULTS

MEF2D accounts for MEF2 site binding activity in HeLa cells. We previously found an activity in HeLa cell nuclear extracts that bound to the c-*jun* MEF2 site in gel mobility shift assays (11). We were interested in identifying this activity to understand how serum-induced expression of c-*jun* is regulated through the MEF2 site in HeLa cells. To determine whether the HeLa cell activity corresponded to MEF2 family members, we generated specific antisera to MEF2A, -B, and -D. These sera were generated against unique segments in the C-terminal portion of each protein (Fig. 1). We tested the antisera's ability to supershift in vitro-translated MEF2 proteins. While the DNA binding by the in vitro-translated MEF2 proteins did not give sharp bands in gel mobility shift assays, the binding activity was supershifted by the specific sera, demonstrating the specificity of the sera (Fig. 2). The in vitro-translated proteins may not entirely start at the correct codon or may be partially degraded.

In vitro-translated MEF2A and -C bound specifically to the c-*jun* MEF2 site, as shown by its competition by excess MEF2 oligonucleotide (Fig. 2A). In separate experiments, a mutated MEF2 site (M1; see Fig. 4A) did not compete for binding (data not shown). The anti-MEF2A serum was able to completely supershift the binding of in vitro-translated MEF2A (Fig. 2A, lanes 1 to 3). The anti-MEF2A serum was also able to supershift binding by MEF2C (lanes 4 to 6). While strongly diverged, there are stretches of significant similarity of MEF2A and MEF2C outside the DNA binding domain such that this may account for the ability of the anti-MEF2A serum to cross-react (23, 24). The anti-MEF2A serum did not cross-react with

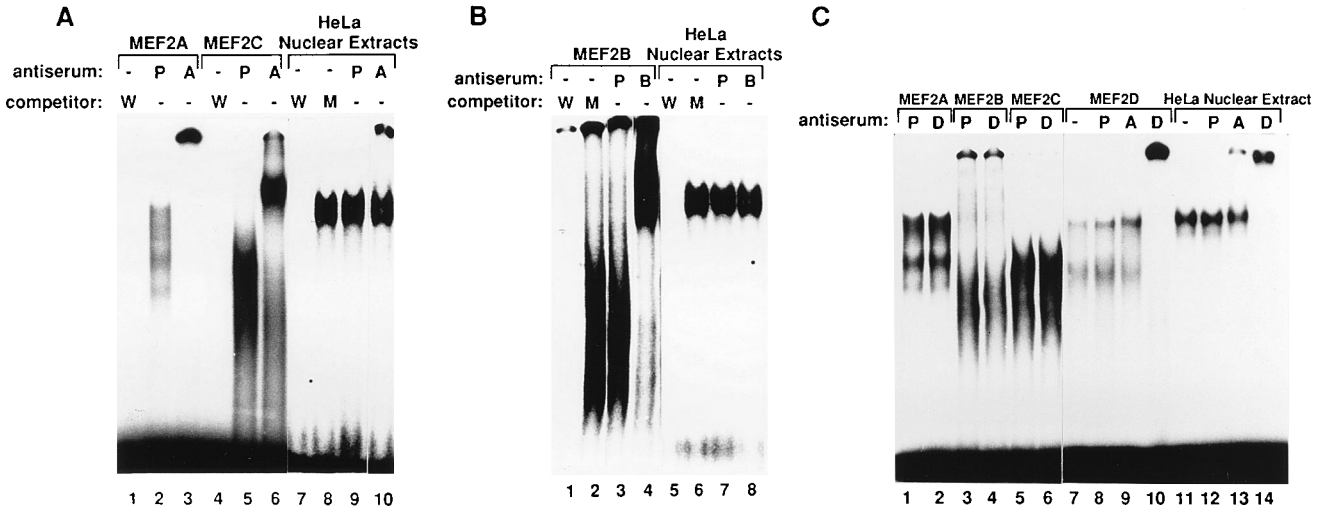

FIG. 2. MEF2D is the major molecule in HeLa cells binding to the c-*jun* MEF2 site. In vitro-translated MEF2 proteins or HeLa nuclear extracts, as indicated, were preincubated with nothing (−) or with preimmune (P), anti-MEF2A (A), anti-MEF2B (B), or anti-MEF2D (D) serum (0.1 μl). A ^{32}P-labelled 21-bp oligonucleotide containing sequence spanning the c-*jun* MEF2 site was used as a probe. Where indicated, 50-fold molar excesses of oligonucleotides were included as competitors. W, wild-type oligonucleotide identical to the probe; M, mutant oligonucleotide M1 (see Fig. 4A).

MEF2B or -D (Fig. 2C and data not shown). Incubation of the anti-MEF2A serum with HeLa nuclear extract only partially shifted the binding to the MEF2 site, while preimmune sera had no effect (Fig. 2A, lanes 9 and 10). We estimate that 10 to 20% of the signal was shifted. Tenfold more antiserum did not cause a greater amount to shift, while tenfold less serum was able to completely shift the in vitro-translated binding activity (data not shown). These results suggested that MEF2A and/or -C are minor components of the MEF2 site binding activity in HeLa cells. Since MEF2A mRNA is expressed in HeLa cells whereas MEF2C mRNA is not (24, 38), it is likely that this low portion of activity is due to MEF2A. We next tested the anti-MEF2B serum, which was able to completely supershift binding by in vitro-translated MEF2B but had no effect on binding by HeLa cell nuclear extract (Fig. 2B). Tenfold more anti-MEF2B serum also did not shift the binding activity (data not shown). The binding by the in vitro-translated proteins, in particular MEF2B, was diffuse, perhaps because of premature termination or degradation of the proteins. Nevertheless, the binding activities could be supershifted by the cognate antisera and specifically competed for by excess unlabelled MEF2 site oligonucleotide.

The last family member to be cloned was MEF2D (3, 23). The anti-MEF2D serum was able to completely supershift DNA binding by in vitro-translated MEF2D but not MEF2A, -B, or -C (Fig. 2C, lanes 2, 4, 6, and 10). The anti-MEF2A serum, however, had no effect on binding by MEF2D (lane 9). Incubation of the anti-MEF2A and -D sera with HeLa cell nuclear extract resulted in only a slight supershift by the anti-MEF2A serum but a complete supershift by the anti-MEF2D serum (lanes 13 and 14). These results suggest that the major MEF2 site binding activity in HeLa cells is due to MEF2D. It is unclear why there is not a stronger band unshifted by the anti-MEF2D serum in lane 14 which would correspond to MEF2A. Since MEF2A and -D can form heterodimers in vitro (22), it is possible that nearly all of the MEF2A is in complexes with the more abundant MEF2D.

As additional evidence that the HeLa cell factor is MEF2D, we have also purified the MEF2 site binding activity from HeLa cells by DNA affinity chromatography. The amino acid sequence of the first 10 amino acids was determined to be identical to that of MEF2D, though the sequence of this region differs by only one amino acid from that of MEF2A, -B, and -C (1a).

Levels of MEF2 DNA binding activity in different cell lines. We compared c-*jun* MEF2 site binding activity in several other cell lines, since MEF2 protein expression has been reported to be restricted to muscle and neural cells (3, 38). Equivalent amounts of nuclear extracts prepared from each cell line were used in a gel mobility shift assay. The MEF2D antiserum almost completely supershifted c-*jun* MEF2 site binding activity from HeLa cells (a human cervical carcinoma line), Namalwa cells (a human B-cell lymphoma line), and Sol8 cells (a mouse myoblast line), showing that MEF2D is the major molecule binding to the site in these cells (Fig. 3A). The binding levels were similar in HeLa cells and the myoblast line Sol8 such that differences in levels of binding cannot explain previous reports of lower binding activity in HeLa cells than in muscle cell lines (3). We also found slightly lower binding activity in extracts from C2C12 mouse myoblasts (data not shown). These results show that MEF2D protein expression is not restricted to muscle-specific cell lines. Nevertheless, MEF2 DNA binding activity is greatly induced in myoblasts during differentiation to myotubes (10) such the DNA binding activity in HeLa cells is likely to be much lower than that in differentiated myotubes.

The MEF2 site binding activity from NIH 3T3 cells was significantly lower than those of the other cell lines (Fig. 3A). We compared binding of another transcription factor, SRF, to confirm that this difference was not due to problems with the extracts. SRF binding activities to its specific site were similar with all of the extracts (Fig. 3B). The low MEF2 activity in NIH 3T3 cells is consistent with our previous observation that serum induction of c-*jun* in these cells is lower than in HeLa cells (unpublished results). Since Pollack and Treisman (25) found similar MEF2 site binding activity in NIH 3T3 and HeLa cells, the low activity that we found in NIH 3T3 cells may be unique to our isolate of this line. This isolate of NIH 3T3 cells, however, proved useful for assaying the function of MEF2 proteins in vivo (see below).

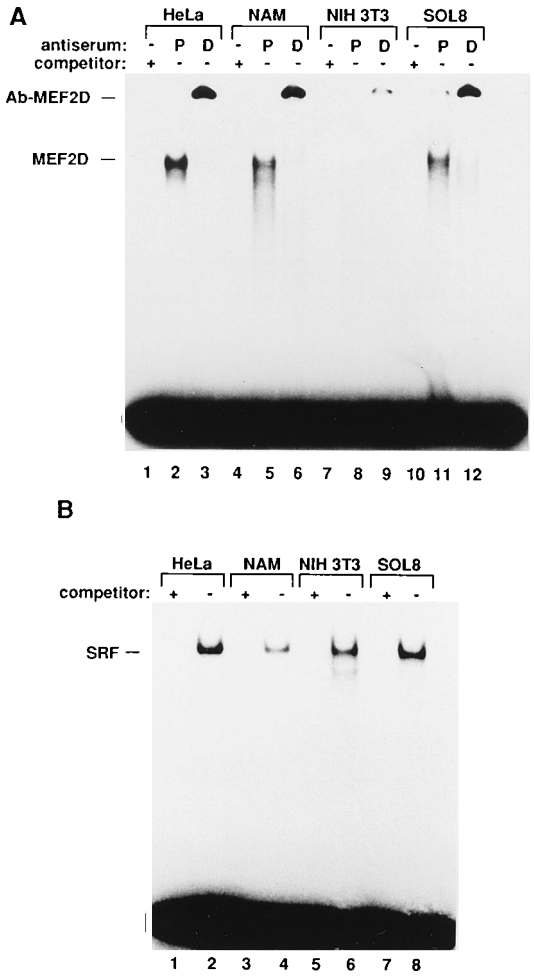

FIG. 3. MEF2D DNA binding activity in different cell lines. (A) MEF2 DNA binding assays were performed as for Fig. 2. Nuclear extracts of the indicated cell lines were preincubated with nothing (−) or with preimmune (P) or anti-MEF2D (D) serum. Excess MEF2 oligonucleotide was included for competition as indicated. The positions of the MEF2D-DNA and the antibody bound MEF2D-DNA complexes are indicated. (B) SRF DNA binding activity was assayed in the indicated cells lines, using a labelled oligonucleotide (XGL) containing an SRF binding site (21). Where indicated, excess XGL oligonucleotide was included as a competitor. The position of the SRF complexes is indicated.

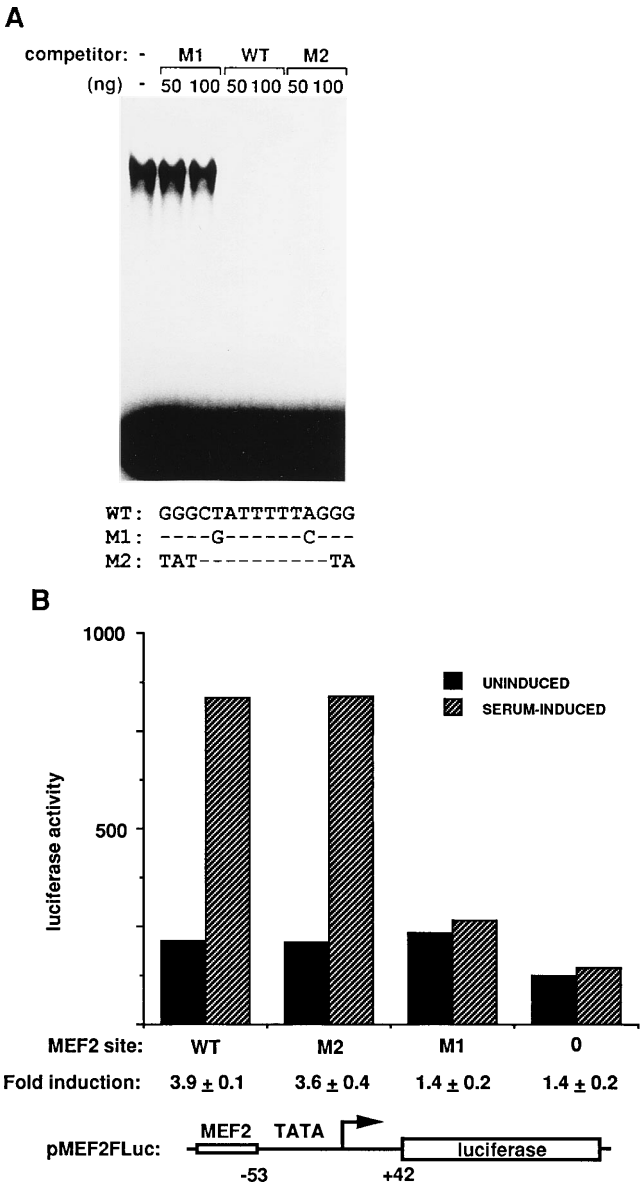

FIG. 4. Correlation of c-*jun* MEF2 site binding activity and serum regulation. (A) MEF2 site binding activity in HeLa nuclear extract was assayed as described for Fig. 2. Excess amounts (50- or 100-fold) of the indicated oligonucleotides were included as competitors. The sequences of the wild-type (WT) and mutant oligonucleotides are indicated at the bottom. (B) HeLa cells were transiently transfected with a reporter plasmid containing the indicated MEF2 sites or lacking any upstream site (0) and an internal control plasmid, pCMVβgal. A diagram of the wild-type reporter plasmid, pMEF2FLuc, is shown at the bottom. The transfected cells were serum starved and treated with or without serum for 2 h before preparing cell lysates for luciferase and β-galactosidase assays. The luciferase values were normalized to expression of the internal control plasmid, pCMVβgal. A representative experiment is shown. The absolute levels of luciferase expression varied depending on the transfection efficiency; however, the fold serum induction was reproducible. The average fold inductions ± standard errors of the means are shown below the graph for two experiments.

Mutation of the MEF2 binding site. We made mutations in the c-*jun* MEF2 site to correlate binding of MEF2D to the site with the ability of the site to mediate serum induction. We made two mutants in the MEF2 site (Fig. 4A). One (M1) consisted of a double point mutation in the c-*jun* MEF2 site that was previously found to abolish MEF2A binding (25) and which reduced EGF induction in the context of a c-*jun* promoter–chloramphenicol acetyltransferase (CAT) reporter gene (11). The second mutant (M2) altered the bases flanking the MEF2 consensus site. We tested these sites for MEF2D binding in HeLa nuclear extracts by competition in a gel mobility shift assay (Fig. 4A). The wild-type c-*jun* MEF2 site and the M2 mutant efficiently competed for MEF2D binding whereas the M1 mutant did not, demonstrating that the M1 mutations abolish binding whereas the M2 mutations had little or no effect.

We next tested these mutants for serum induction of the expression of a reporter gene in HeLa cells. Single MEF2 sites were cloned upstream of a minimal c-*fos* promoter and a luciferase reporter gene. The wild-type c-*jun* MEF2 site (in pMEF2FLuc) mediated about a fourfold serum induction of expression, while a reporter gene lacking a MEF2 site (p0FLuc) was uninduced (Fig. 4B). The M2 mutations did not affect induction, while the M1 mutations nearly abolished serum induction. Thus, this preliminary analysis of c-*jun* MEF2

site mutations suggests that there is a good correlation between MEF2D binding and the ability to mediate serum induction of a reporter gene.

The SRE of the c-*fos* promoter is at least partially regulated by a factor termed $p62^{TCF}$ that binds adjacent to SRF on the 5′ side of the SRE (reviewed in reference 35). Gel mobility shift assays suggest that TCF does not bind MEF2 proteins (reference 25 and data not shown). The observation that mutation of the MEF2 flanking sequences had no effect on serum induction also argues against a model in which regulatory proteins bind to DNA adjacent to MEF2D.

The fold induction of the MEF2 reporter gene is much lower than the fold induction of endogenous c-*jun* mRNA, for which the basal levels are near zero (11). We found that a longer reporter gene with −225 to +150 of c-*jun* fused to the bacterial CAT gene was also induced only about fourfold (11). Part of the difference can be accounted for by the stability of c-*jun* mRNA compared with CAT or luciferase mRNAs and proteins. c-*jun* mRNA has a short half-life due to destabilizing sequence elements in the 3′ untranslated region (5). This keeps the basal levels of c-*jun* mRNA low. In contrast, the CAT and luciferase mRNAs and proteins accumulate to higher levels during the transient transfection process prior to serum induction. We have observed a similar effect with c-*fos* induction whereby c-*fos* promoter–CAT reporter genes are induced to much less an extent than endogenous c-*fos* (8). Nevertheless, it is possible that c-*jun* sequences outside of −225 to +150 further contribute to serum induction of the gene.

Characterization of MEF2D in induced cells. Since the c-*jun* MEF2 site is sufficient for serum and EGF regulation of a reporter gene, we investigated whether MEF2D is regulated by these agents. We first tested whether DNA binding by MEF2D to the c-*jun* MEF2 site is regulated by serum or EGF. Nuclear extracts were prepared from HeLa cells, which were serum starved and treated with serum for different time periods. As shown in Fig. 5A, no changes in MEF2 site binding activity were observed. The same results were observed for HeLa cells treated with EGF (data not shown). These results suggest that MEF2D DNA binding activity is constitutive.

We next tested whether MEF2D is posttranslationally modified in response to serum or EGF treatment. MEF2D was immunoprecipitated from HeLa cells that were grown overnight in the presence of [^{35}S]methionine and [^{35}S]cysteine and treated with or without serum for different times. A 65 kDa band was immunoprecipitated that was specifically competed for by an excess of bacterially expressed MEF2D but not MEF2A (Fig. 5B). This band migrates at roughly the expected molecular weight for MEF2D. Other bands were detected, but these may be due to cross-reactivity with the sera. They may also be MEF2D-associated proteins; however, this is less likely since the cell lysates were precipitated under relatively stringent conditions. The intensity or mobility of the 65-kDa band was not changed with serum treatment, suggesting that MEF2D is not modified in response to serum induction (Fig. 5B). We obtained similar results with EGF treatment of HeLa cells (data not shown). Furthermore, we have metabolically labelled HeLa cells with $^{32}P_i$ and immunoprecipitated MEF2D. While phosphorylated MEF2D was detected, there was no significant change in levels following serum or EGF treatment (data not shown). However, our analysis cannot rule out changes at specific phosphorylation sites.

MEF2D reconstitutes serum regulation in NIH 3T3 cells. Since there were no simple biochemical changes in MEF2D following serum stimulation, we sought to demonstrate in vivo that it is involved in serum regulation of gene expression. Since the NIH 3T3 cells that we have used have low MEF2 site

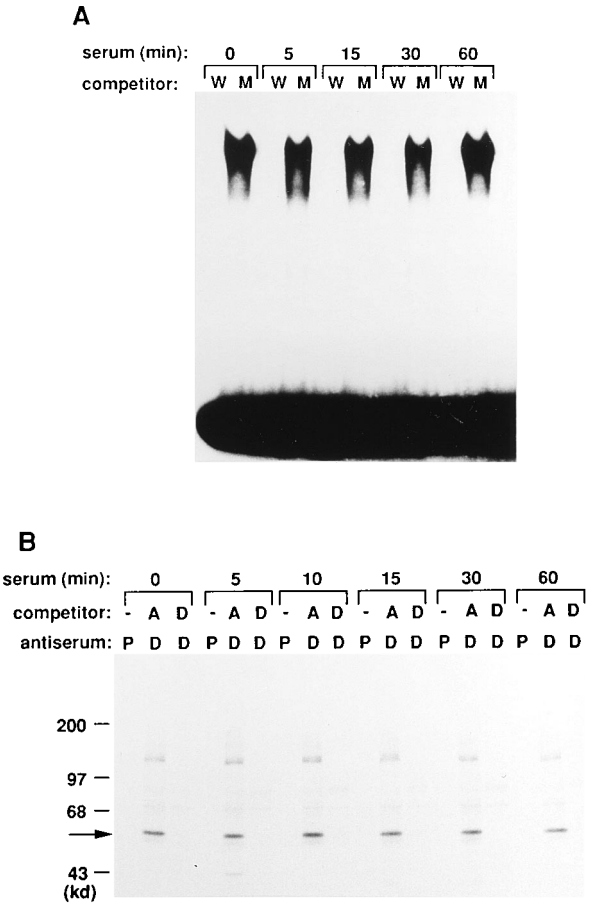

FIG. 5. Constitutive MEF2D DNA binding activity in HeLa cells. (A) HeLa cells were serum starved and treated with serum for the indicated times, and nuclear extracts were isolated. MEF2 DNA binding activity in the extracts was assayed as described for Fig. 2. Excess wild-type (W) or mutant M1 (M) oligonucleotide was included as competitor as indicated. (B) ^{35}S-labelled HeLa cells were serum starved and treated with serum for the indicated times. Immunoprecipitations from the cell lysates were performed with preimmune (P) or anti-MEF2D (D) serum. Excess amounts of bacterially expressed MEF2A or -D proteins (the same as those used to raise the antisera) were added as competitors to demonstrate the specificity of immunoprecipitation. The positions of molecular weight markers are indicated, and the arrow indicates the expected position of MEF2D.

binding activity (Fig. 3A), we tested these cells for serum regulation of a reporter gene containing a single c-*jun* MEF2 site (pMEF2FLuc). Expression from this reporter was not induced by serum in NIH 3T3 cells, in contrast to HeLa cells (compare Fig. 6A and 4B). Pollack and Treisman (25) previously found good serum induction of a MEF2 reporter gene in NIH 3T3 cells; however as mentioned above, their isolate of NIH 3T3 cells contained significant MEF2 site DNA binding activity. We overexpressed MEF2D in our line of NIH 3T3 cells to determine whether it would reconstitute serum regulation. Under control of a CMV promoter in pCMVMEF2D, increasing amounts of MEF2D caused serum induction of the specific reporter gene. There was no effect on a similar reporter gene lacking the MEF2 site (p0FLuc) (Fig. 6A). Maximal serum induction of three- to fourfold was typically observed with 1 to 3 μg of pCMVMEF2D. Higher amounts of pCMVMEF2D led to a strong increase in expression of the MEF2 reporter gene in uninduced cells such that lower induction was seen. It therefore appears that there is a window of

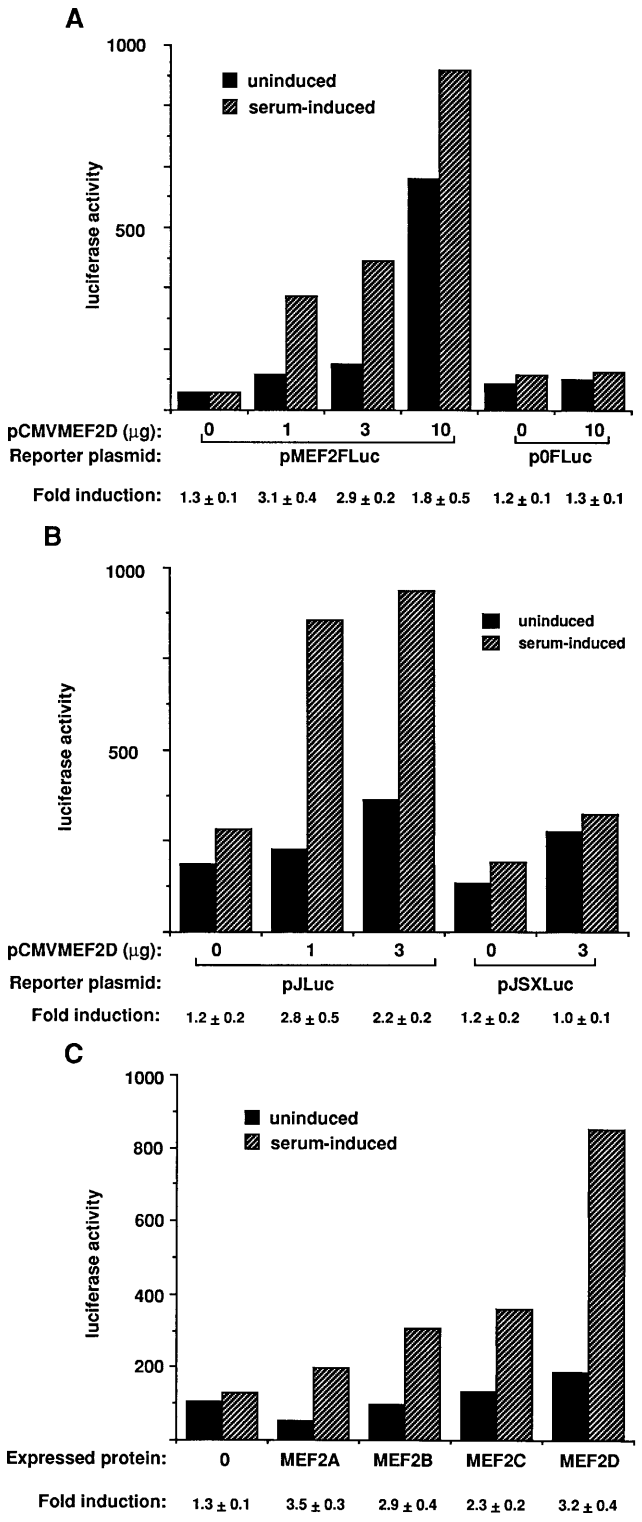

FIG. 6. Effect of MEF2 expression on serum regulation of the c-*jun* MEF2 site in NIH 3T3 cells. (A and B) The indicated amounts of the MEF2D expression plasmid, pCMVMEF2D, the indicated reporter plasmids, and an internal control plasmid, pCMVβgal, were transiently transfected into NIH 3T3 cells. The reporter construct pMEF2FLuc was as described for Fig. 4B. pJLuc contains −225 to +150 of the c-*jun* gene upstream of luciferase sequences. pJSXLuc is similar to pJLuc except for point mutations in the c-*jun* MEF2 site. The transfected cells were serum starved and treated with or without serum for 2 h before preparing cell lysates for luciferase and β-galactosidase assays. The relative luciferase levels were normalized to the levels of β-galactosidase expression. (C) Expression plasmids (3 μg) for the indicated proteins and the reporter plasmid, pMEF2FLuc, were transiently transfected into NIH 3T3 cells as described above. The average fold serum inductions ± standard errors of the means from three or more experiments are indicated.

appropriate levels of MEF2D for serum regulation. Higher levels resulted in constitutively activated expression. Potential mechanisms are proposed in Discussion. These and subsequent transfection experiments were all repeated at least three times, and representative results are shown. The absolute luciferase levels varied depending on the transfection efficiencies. The amount of MEF2D for peak induction varied between 1 and 3 μg, presumably also dependent on the transfection efficiency. Nevertheless, the average fold serum inductions with the indicated amounts of MEF2D expression plasmid are indicated below the graph along with the standard errors of the means.

As further evidence that MEF2D is required for regulation of the c-*jun* promoter, we tested the effect of MEF2D overexpression in the more natural context of a c-*jun* promoter reporter gene, pJLuc, containing −225 to +150 of the c-*jun* promoter sequence upstream of the luciferase coding sequence. The expression from this reporter gene was not serum inducible in NIH 3T3 cells (Fig. 6B), although we have previously found that these sequences are sufficient for serum induction in HeLa cells (11). When pCMVMEF2D was transfected with pJLuc into NIH 3T3 cells, expression from the reporter gene was induced in response to serum treatment (Fig. 6B). However, MEF2D overexpression did not cause serum induction of a similar reporter gene, pJSXLuc, with point mutations in the c-*jun* MEF2 site. These point mutations abolish MEF2D binding and are identical to the mutations in M1 (Fig. 4A).

We tested the other MEF2 family members in NIH 3T3 cells to determine whether MEF2D is unique in its ability to reconstitute serum regulation of pMEF2FLuc. MEF2A, -B, and -C were all able to cause serum-inducible expression of the reporter gene (Fig. 6C). Titration of these family members gave results similar to those for MEF2D in that higher levels caused higher basal levels (data not shown). Maximal activation was typically lower than with MEF2D, but it is difficult to quantitatively compare the effects since the levels of MEF2 expression vary. Using a gel mobility shift assay of extracts from transfected cells, we found that MEF2A and -B were expressed much more poorly than MEF2C and -D (data not shown). The positive effect of each family member on serum-induced expression nevertheless suggests that each has some capacity to mediate serum induction.

Domains of MEF2D required for serum regulation. Since the MEF2 family members are similar only in their DNA binding domains, the results presented above suggest that the MEF2 DNA binding domain is key to its role in serum regulation. We therefore tested whether MEF2D's DNA binding domain was sufficient to mediate serum induction. Deletion of MEF2D's C terminus (in pCMVMEF2DT) abolished its ability to activate transcription, presumably by deleting its transcriptional activation domain (Fig. 7A). This result is consistent with the mapping of MEF2D's transcriptional activation domain by Martin et al. (22) in mouse 10T1/2 cells, although we have not proven here that the truncated protein is made stably. We next replaced MEF2D's transcriptional activation domain with the transcriptional activation domain of the herpesvirus VP16 protein. The MEF2D-VP16 fusion protein caused serum-induced expression when transfected with

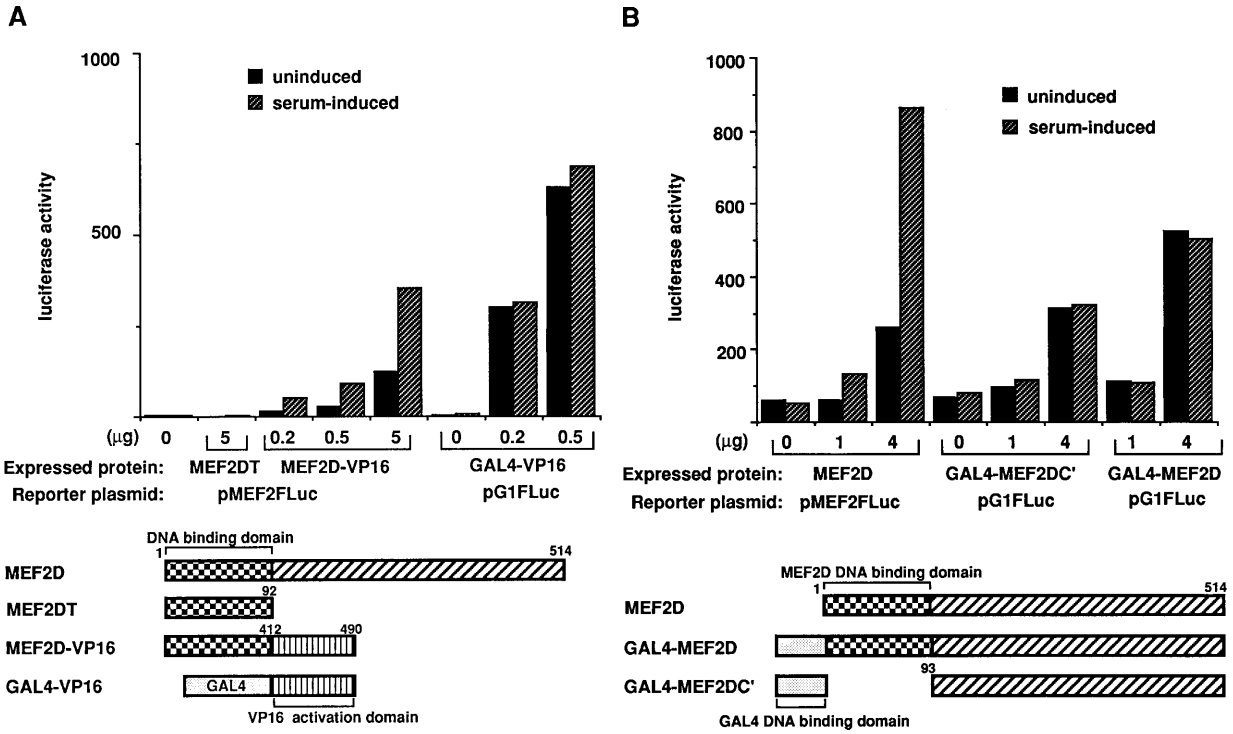

FIG. 7. Domains of MEF2D required for serum regulation. The indicated amounts of each expression plasmid, the reporter plasmids, and the internal control plasmid, pCMVβgal, were transiently transfected into NIH 3T3 cells. The transfected cells were serum starved and treated with or without serum for 2 h before assaying for relative luciferase levels. Diagrams of the expressed proteins are shown at the bottom. The reporter plasmid pG1Fluc is identical to pMEF2FLuc except for containing a single GAL4 binding site in place of the MEF2 site. The experiment in panel A was repeated three times. Peak serum induction with MEF2D-VP16, 3.8 fold ± 0.4 (standard error of the mean), was found in different experiments with 0.5 to 1 μg of expression plasmid. Serum induction with GAL4-VP16 was never greater than 1.1-fold. No discernible activation was found with MEF2DT. The experiment in panel B was repeated twice. Serum induction with various amounts of GAL4-MEF2DC′ and GAL4-MEF2D was never greater than 1.3-fold, compared with 3-fold for MEF2D.

pMEF2FLuc into NIH 3T3 cells (Fig. 7A). As a control, we found that GAL4-VP16, containing VP16's transcriptional activation domain fused to GAL4's DNA binding domain, caused constitutive expression from a reporter gene containing a GAL4 binding site (pG1FLuc). This control shows that serum regulates neither the VP16 transcriptional activation domain nor expression from a CMV promoter. These results show that MEF2D's DNA binding domain is the specific region of MEF2D required for serum regulation of the MEF2 site.

We also tested whether MEF2D's C-terminal transcriptional activation domain was regulated by serum. We constructed GAL4-MEF2D fusions containing either full-length MEF2D (GAL4-MEF2D) or MEF2D's C-terminal domain (GAL4-MEF2DC′) (Fig. 7B, bottom). Both of these caused constitutive, serum-independent expression of the pG1FLuc reporter gene, while MEF2D caused serum-induced expression from pMEF2FLuc (Fig. 7B). This result suggests that MEF2D's transcriptional activation domain is not under serum control and is consistent with our conclusion that MEF2D's DNA binding domain is critical for regulation. The observation that GAL4-MEF2D, containing MEF2D's DNA binding domain, is not regulated suggests that MEF2D's DNA binding domain must be bound directly to DNA to mediate regulation. Possible mechanisms for this regulation will be discussed below.

DISCUSSION

We previously found that a MEF2 site at −59 in the c-*jun* promoter is responsible for serum and EGF induction of the promoter in HeLa cells (11). In this study, we used specific antibodies to different MEF2 family members to show that MEF2D is the major molecule in HeLa cells binding to the c-*jun* MEF2 site. We further found that our line of NIH 3T3 cells contains low MEF2 DNA binding activity and exhibits poor serum induction of a c-*jun* promoter reporter construct and of a reporter gene containing a single MEF2 site. Transfection of MEF2D was sufficient to reconstitute serum regulation of both of these reporter genes. Other MEF2 family proteins were also capable of mediating serum regulation such that there does not appear to be specificity for this function within this family of factors. In mapping the domains of MEF2D required for serum regulation, we found that the DNA binding domain of MEF2D, when fused to a heterologous transcriptional activation domain, was sufficient for serum regulation of the c-*jun* MEF2 site. Previous studies have demonstrated a role for MEF2 proteins in regulating muscle-specific gene expression (3, 22–24, 38). The results described here demonstrate that MEF2 proteins, in particular MEF2D, also have a role in regulating serum-induced gene expression in nonmuscle cells.

Expression of MEF2D in HeLa cells. We found that MEF2D was the major molecule binding to the c-*jun* MEF2 site in HeLa cervical carcinoma, Namalwa B-cell lymphoma, and Sol8 myoblast cells. A small amount of MEF2A was also found in HeLa cells, as shown by gel mobility shift assays with the different antisera. Given that different MEF2 proteins have been shown to form heterodimers (22, 25), potential heterodimer formation between MEF2A and MEF2D may contribute to the fine control of c-*jun* transcription. MEF2D is

expressed earlier in muscle differentiation than the other MEF2 family members (3, 4, 22). Our finding of expression in nonmuscle cells suggests that its expression and function are more ubiquitous.

The question of the cell type and tissue distribution of MEF2 site binding activities has been controversial (3, 11, 16, 25, 38). Given that some previous reports demonstrated that the expression of MEF2 proteins is restricted to muscle-specific cell lines and nervous tissues (3, 38), it is surprising that our gel mobility shift assays detected significant MEF2D activity in several nonmuscle cell lines. This discrepancy may be because the immunoblotting and immunocytochemical techniques used in previous reports were not sensitive enough to detect the relatively low levels in these cells. Binding to the MEF2 site is strongly induced during differentiation of myoblasts to myotubes in culture, with relatively low binding activity detected in myoblasts (10). Since we found similar MEF2 site binding activity in HeLa cells and myoblasts, the level in HeLa cells would correspond to the low levels defined by previous studies.

Serum regulation of expression by MEF2 proteins. We used NIH 3T3 cells, which contain low MEF2 site binding activity, to show that transfection of MEF2D can cause serum induction of a reporter gene. This finding demonstrates that MEF2D is critical for serum induction through the MEF2 site. The other MEF2 family members were also able to mediate serum induction in NIH 3T3 cells. We were not able to accurately quantitate their relative abilities to stimulate serum induction because of the variable expression of each family member following transfection.

Since the MEF2 family members are expressed differentially during muscle differentiation, it is possible that their products have different transcriptional functions. In *X. laevis*, in fact, ectopic expression of MEF2D stimulated cardiac muscle-specific myosin light-chain gene expression whereas MEF2A had no effect (4). In our study, all of the MEF2 proteins were similarly able to mediate serum induction of the c-*jun* MEF2 site. However, since our preliminary analysis suggests that MEF2D is the major MEF2 site-binding molecule in nonmuscle cells, it is likely that MEF2D is the most important MEF2 family member for the induction of growth factor-regulated genes.

While transfection of MEF2D caused serum stimulation of the reporter gene, high levels of MEF2D expression increased transcription in uninduced cells as well as in serum-treated cells. One possible explanation is that serum regulation is mediated by the level of MEF2D binding to the MEF2 site. In uninduced cells, MEF2D would have low DNA binding activity or another factor may bind the site. Serum stimulation would either increase MEF2D binding or lower another factor's DNA binding. Overexpression of MEF2D in uninduced cells may similarly push the balance of binding toward MEF2D occupation of the MEF2 site. This model is tempered by our results that MEF2D DNA binding activity is unchanged in extracts of serum-treated cells and because we have not detected any other factors in HeLa cells which bind to the MEF2 site at significant levels. An alternative explanation that we prefer is that MEF2D is under negative control in uninduced cells. Overexpression of MEF2D could result in titration away of an inhibitor, resulting in increased expression from the MEF2 site.

Rozek and Pfeifer (28) did in vivo footprinting of the c-*jun* promoter in HeLa cells and did not detect occupancy of the MEF2 site. However, since the MEF2 site is A/T rich, it was not well probed by dimethyl sulfate or DNase I and thus protection may have been missed. In addition, footprinting in serum-treated cells was not tested.

Domains of MEF2D required for serum regulation. The DNA binding domain of MEF2D was sufficient for serum regulation of the c-*jun* MEF2 site when fused to VP16's transcriptional activation domain. When MEF2D was fused downstream to GAL4's DNA binding domain, it constitutively activated the expression of a reporter gene containing a GAL4 binding site. This finding suggests that MEF2D's DNA binding domain is subject to regulation only when it is bound directly to DNA. This may be because serum induction involves regulation of MEF2D's ability to occupy the MEF2 site or because MEF2D's DNA binding domain is in the proper conformation to bind regulatory proteins only when it is bound to DNA. Arguing against the former possibility, MEF2D's DNA binding activity does not appear to be regulated by serum, as discussed above.

MEF2D's DNA binding domain spans the conserved MADS box, which is also found in SRF. SRF is involved in serum induction of c-*fos* via the SRE in the c-*fos* promoter (reviewed in reference 34). We have found that SRF's DNA binding domain (including the MADS box) is also sufficient for serum regulation when fused to VP16's transcriptional activation domain (17). Thus, it is possible that there is a conserved function of the MADS box responsible for serum regulation through both MEF2D and SRF.

One mechanism for serum regulation of SRF involves its formation of a ternary complex of the SRE, SRF, and $p62^{TCF}$. TCF is encoded by several Ets-related factors (Elk-1, SAP1, and ERP/Net) and can bind to the 5′ end of the SRE only in conjunction with SRF (reviewed in references 9, 20, and 35). Transcriptional activation by TCF is activated by serum-induced mitogen-activated protein kinase phosphorylation (reviewed in reference 35). TCF, however, binds to SRF's DNA binding domain but does not bind with MEF2 proteins (reference 25 and data not shown). We have also not detected any MEF2D-complexing proteins in gel mobility shift assays of HeLa nuclear extracts (unpublished data). Further, mutation of sequences flanking the MEF-2 site had no effect on expression (Fig. 4), suggesting that there is not a TCF-like protein involved in MEF2 regulation.

There is a second TCF-independent mechanism for serum regulation of the SRE (14, 17). This second pathway also requires SRF's DNA binding domain but is unaffected by mutations that abolish TCF binding. This pathway could be conserved with a MEF2D regulatory pathway. A possible mechanism of regulation by this pathway could involve a complexing protein(s) which binds to the conserved MADS box.

In summary, two models for MEF2 regulation are proposed. In the first, MEF2D DNA binding to the MEF2 site is regulated by serum either by direct regulation of MEF2D activity or by regulation of another factor which precludes MEF2D binding to the site. While there is evidence against this model, further work is required to demonstrate that MEF2D constitutively occupies the c-*jun* MEF2 site in vivo. A second model for MEF2 regulation involves complexing of factors to MEF2D's DNA binding domain when MEF2D is bound to the MEF2 site. These factors would inhibit transcriptional activation in uninduced cells and/or stimulate activation in serum-treated cells. Further work to identify these putative factors may be important to understanding how the signals at the cell surface lead to the transcriptional activation of c-*jun*.

ACKNOWLEDGMENTS

We thank Richard Treisman for providing RSRFC4 and R2 (MEF2A and -B) clones and James Martin and Eric Olson for providing clones for MEF2C and -D.

This work was supported by Basic Research grant 1-FY94-0606 from the March of Dimes Birth Defect Foundation to R.P.

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