Myocyte-specific Enhancer-binding Factor (MEF-2) Regulates α-Cardiac Myosin Heavy Chain Gene Expression in Vitro and in Vivo*

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A myocyte-specific enhancer-binding factor (MEF-2) DNA binding site was identified in the rat α-myosin heavy chain (MHC) gene adjacent to the E-box binding site for α-MHC binding factor-2 (BF-2). Mutation of the MEF-2 site, within the context of the full-length promoter, reduced activity by 85 and 80% in neonatal cardiomyocytes and the adult heart, respectively. Mutation of the BF-2 site reduced activity approximately 70% in both models. A MEF-2/BF-2 double mutant gave significantly less activity than the BF-2 mutant but not the MEF-2 mutant, suggesting the possibility that BF-2 and MEF-2 interact. Mutations in MEF-2, which decreased functional activity, also abolished MEF-2 DNA binding activity. MEF-2 DNA binding activity was present in the developing heart, reached a peak in the late fetal and early neonatal stages, and then declined to low levels in the adult heart. The adult levels were sufficient to support α-MHC gene expression. MEF-2 activity was increased 2–3-fold in the adult heart subjected to a pressure or volume overload. Two working models are proposed as possible explanations of the antithetic relationship between MEF-2 levels and α-MHC gene expression.

A complete understanding of cardiac muscle growth and development as well as hypertrophy requires detailed information on the regulation of cardiac gene expression. Several transcription factors that regulate gene expression in the heart were first described in skeletal muscle cells indicating that cardiac and skeletal muscle cells share some regulatory factors (Mar and Ordahl, 1990; Gupta et al., 1991; Parmacek et al., 1992; Yu et al., 1992; Molkentin et al., 1993; Sartorelli et al., 1990). One such factor is myocyte-specific enhancer-binding factor-2 (MEF-2).1 Potential MEF-2 binding sites have been identified in several genes that are expressed in the heart, including the muscle creatine kinase (MCK) (Sternberg et al., 1988), myosin light chain-2 (MLC-2) (Navankasattusas et al., 1992), and rat atrial natriuretic factor (ANF) genes (Seidman et al., 1988), suggesting that MEF-2 might influence gene expression in the heart. A putative MEF-2 DNA binding site is located from −335 to −327 in the rat α-myosin heavy chain (α-MHC) gene (Mahdavi et al., 1984). Complementary DNAs encoding MEF-2 have been cloned (Yu et al., 1992). Two distinct genes encode MEF-2 proteins. One gene gives rise to several alternatively spliced forms, some of which are homologous to human serum response factor-related clones (Yu et al., 1992; Pollack and Treisman, 1991). MEF-2 isoforms that bind DNA are present in the heart, skeletal muscle, and brain and are distinguished from the other serum-response factor related proteins by the presence of an additional exon.

The α-MHC and MLC-2 genes both contain putative sites for MEF-2 binding. In the MLC-2 promoter, the HF-1 regulatory region binds two distinct factors; HF-1A and HF-1B (Navankasattusas et al., 1992). HF-1B and MEF-2 DNA binding activities are similar if not identical. A second regulatory element common to the MLC-2 and α-MHC genes is HF-1A/BF2. The factors that bind these sites are E-box binding proteins that are similar if not identical (Molkentin et al., 1993). The binding sites for HF-1A/BF2 in their respective genes are adjacent to MEF-2 sites (see below). The proximity of these sites is reminiscent of an important feature of the paradigm for MEF-2 regulatory activity in skeletal muscle cells where MEF-2 interacts cooperatively with members of the MyoD family (Gossott et al., 1989; Olson, 1990; Cserjesi and Olson, 1991). Thus, a possibility exists that an interaction between these elements could be a mechanism for regulating gene expression in cardiomyocytes.

During adult cardiac hypertrophy, the fetal isoforms of several proteins are expressed (reviewed by Nadal-Ginard and Mahdavi (1989)). Many of these fetal proteins are skeletal muscle isoforms. This observation has led to the hypothesis that a fetal program is re-expressed in the heart during the onset of cardiac hypertrophy (Sheer and Morkin, 1984; Hirzel et al., 1983; Izumo et al., 1987; Mulvagh et al., 1987; Schwartz et al., 1986; Simpson et al., 1989; Nadal-Ginard and Mahdavi, 1989). Since the expression of some skeletal muscle protein isoforms increases during hypertrophy, it follows that the factors that regulate these skeletal muscle genes must increase, become more active, and/or that genes must become more accessible to regulatory factors. Therefore, skeletal muscle regulatory factors, such as MEF-2, are likely candidates as a final target of intracellular signalling pathways that trigger the hypertrophic response.

In this study, we report that MEF-2 positively regulates transcription of the α-MHC gene in the heart. Site-directed mutagenesis experiments revealed that the integrity of the MEF-2 binding site was essential for high level expression of the α-MHC gene in primary neonatal cardiomyocytes and in...
the adult heart. A MEF-2-dependent DNA binding activity in nuclear extracts from heart, brain, and the myogenic cell line C2C12 was characterized by electrophoretic mobility shift assays (EMSA) and DEPC-interference footprinting. EMSAs of the combined MEF-2 and BF-2 binding sites failed to detect any positive cooperative interaction. The levels of MEF-2 DNA binding activity in the heart were high in early development but dropped during maturation to barely detectable levels in the adult animal. The adult level differentially increased 2-3-fold in response to cardiac hypertrophy induced by volume overload and pressure overload, respectively. These changes are inconsistent with the hypothesis that absolute levels of soluble MEF-2 regulate α-MHC gene expression. To explain this apparent contradiction, we propose two possible working models. The first suggests that the ratio of MEF-2 and BF-2 in the cell is the important parameter in regulating the α-MHC gene. The second suggests the possibility that MEF-2 and BF-2 associate with the nuclear matrix as part of their regulatory role.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides**—MCKMEF, MLCMEF, α-MHCMEF, and ANF-MEF represent the MEF-2 sites in MCK, MLCL-2, α-MHC, and ANF genes, respectively. The promoter/enhancer location and sequences of these sites are from -1073 to -968 in MCK, CCCCCTTTCTTTCCAAAATACCCCTGTG (Sternberg et al., 1988); from -56 to -39 in MLC-2, GGTTATTTTTATACAAACCCCA (Zhu et al., 1992); from -345 to -322 in α-MHC, CCTCTAGATGAAAAACTACATTAAGG (Mahdavi et al., 1984); and from -705 to -686 in ANF, AGGGTAAAAATAAGCTTTAGTGC (Seidman et al., 1988). The mutant MCKMEF primer was from -340 to -310, TGGGTAAGGTCCATTGTZGGTAGGGGAGGTGG. The antisense primer was from -341 to -373, AAGGCTGAGTTGAACACACTGGGTAAGGGTCAC, in the α-MHC promoter. The mutagenic primer was from -310 to -340, TAGG, oligonucleotides contain sequences from -72 to -39 and from -340 to -300 in the MLC-2 and β-MHC genes, respectively. The sequences presented are of the sense strand, but both strands were synthesized and annealed to regenerate the sites. The multiple cloning site, PRE B and PRE B1, and M-CAT oligonucleotides have been described previously (Molkentin et al., 1993). The oligonucleotides were purchased from Operon Technologies Inc. (Alameda, CA).

**Site-directed Mutagenesis of MEF-2**—The MEF-2 site in pSVOMCAT (Gustafson et al., 1987a) was mutated by rolling circle polymerase chain reaction mutation technique (Hemsley et al., 1989) as previously described (Molkentin et al., 1993). The mutation primer was from -340 to -310, CAGATGAAAATAACATGATGAGG GCCATG (mutated bases underlined). MEF-2 was also mutated in conjunction with the E-box binding site for BF-2. The mutagenic primer for the double mutant was from -340 to -294, CAGATGAAAATCAACTAGGTCCATGTGAGGTTGG. The antisense primer was from -341 to -373, AAGGCTGAGTTGAAACACACTGGGTAAGGGTCAC, in the α-MHC promoter. The identity of each construct was confirmed by DNA sequencing across the entire insert and ligation sites.

**Cell Culture, Transfection, and CAT Assay**—The conditions for isolation and culturing of cardiomyocytes, and C2C12 myoblasts as described (Dignam et al., 1983) with minor modifications (Molkentin et al., 1993). Adult rats were sacrificed by sodium pentobarbital overdose, hearts excised, and excess connective tissue and large vessels were removed. Neonatal and fetal rats were lightly anesthetized with CO2 gas and sacrificed. Hearts were homogenized in ice and stored at -80°C.

Nuclear extracts were prepared from heart and brain tissue, cultured cardiomyocytes, and C2C12 myoblasts as described (Dignam et al., 1983) with minor modifications (Molkentin et al., 1993). Unless otherwise indicated, 12 μg of protein extract and 2 μg of poly(dI-dC)-(dI-dC) were used in each assay. The probes used in each experiment are indicated in the figure legends. Diethyl pyrocarbonate (DEPC) footprinting was performed essentially as described (Sturm et al., 1987). A DEPC-treated probe was used in reactions described for gel shift experiments except that the reactions were scaled up 25-fold. Complex isolation and electrophoresis conditions are described in Molkentin et al. (1993). In Vivo Transient Expression Assays—Clones to be assayed for activity were injected directly into the apex of the adult heart as previously described (Lin et al., 1989; Ojamaa and Klein, 1991). Sprague-Dawley rats (200–300 g) were anesthetized with 0.15 ml/100 g body weight of a ketaset, ACE promazine mixture (Ketaset (100 mg/ml) 10 ml and proACE (10 mg/ml) 2.2 ml) and ventilated with a respirator. The injection of DNA, 80 μg of test DNA and 10 μg of pRSVβgal, into the heart was performed with a 28-gauge needle in a volume of 80 μl of saline. DNA was also injected into soleus muscle (Wolff et al., 1990) of the same animals receiving the cardiac injection. The animals were given penicillin G at 30,000 units/100 g body weight postoperatively and allowed to recover for 6 days. Rats were sacrificed 7 days postinjection by lethal injection of pentobarbital, and the hearts and soleus muscle samples were collected. Hearts and soleus samples were prepared by mincing the tissue in nuclear lysis buffer followed by two 10-s bursts of sonication using a Branson Sonifier 250 with a microtip probe at a setting of 1 (Branson Ultrasouncers, Danbury, CT). The cellular debris was pelleted by centrifugation at 10,000 × g for 10 min at 4°C. Assays were performed as described above.

**Animal Models of Cardiac Hypertrophy**—Pressure overload was induced by aortic coarctation. This procedure was carried out on male (250 g) Sprague-Dawley rats under telazol (40 mg/kg intramuscular) anesthesia. Coarctation was performed by partial ligation of the aorta between the renal arteries with 2-0 braided silk similar to the method previously described (Anversa et al., 1975). The animals received penicillin as described above and were allowed to recover for 10–11 days. Animals were sacrificed by pentobarbital overdose and hearts were harvested. Hearts were weighed and then quick frozen in crushed dry ice and stored at -80°C until use. Two separate groups of 8 coarctated hearts or hearts from sham-operated controls were used to prepare two separate extracts.
RESULTS

Site-directed Mutagenesis of the α-MHC MEF-2 Site—Site-directed mutants were constructed to determine the role of the MEF-2 site in the regulation of the rat α-MHC gene. Mutations in the MEF-2 site alone (pSVOMMFEF-2 mut) or in combination with a mutated PRE B (pSVOMMBEF-2 mut) site were made in the plasmid pSVOMCAT. The mutant plasmids were assayed for activity in cultured cardiomyocytes (Fig. 1). Mutation of the MEF-2 site reduced activity from the α-MHC promoter by 85%. The dramatic loss of activity resulting from the MEF-2 mutation suggests that this site stimulates expression of the α-MHC gene. Mutation of the PRE B site was previously shown to reduce activity of the α-MHC phospho-myosin enhancer by approximately 70% (Molkentin et al., 1993). The data for the PRE B mutant is updated here for reference, with a larger sample number. A MEF-2, PRE B double mutant (MBF-2 mut) reduced activity 86% from that of pSVOMCAT. The activity of the double mutant was not significantly different than pSVOMMEF-2 mut. None of the constructs were active in C2C12 myotubes (data not shown).

The MEF-2 Site Regulates α-MHC Gene Expression in the Adult Rat Heart—It was also of interest to determine whether MEF-2 maintained a regulatory role in the adult rat heart. The site-directed mutant constructs were assayed in vivo by direct injection into the adult rat heart. pSVOMCAT and site-directed mutants were injected into the apex of the heart and into the soleus muscle of adult rats. The activity from 80 μg of pSVOMCAT injected into the rat heart was 20,595 ± 3,254 cpm (n = 9) per 50 μg of protein ± S.D. normalized for β-galactosidase activity (Fig. 1). This activity was set at 100%. Mutation of the MEF-2 site reduced activity to 21 ± 6% (n = 9) of wild type. Mutation of the PRE B site gave 33 ± 5% (n = 8) of the wild type activity. Mutation of both the MEF-2 and PRE B sites resulted in an activity of 14 ± 2% (n = 7) of the wild type enhancer/promoter. The data for all of the site-directed mutants in the adult heart were analogous to those in the primary neonatal cardiomyocytes. These results establish that the MEF-2- and BP-2 sites participate in stimulation of α-MHC transcription in the adult rat heart. These constructs were not active in soleus muscle.

MEF-2 DNA Binding Activity in the Rat Heart—EMSA were used to determine MEF-2 DNA binding activity in nuclear extracts from the heart. Oligonucleotides were used to reconstruct the MEF-2 binding site from the MCK enhancer, the MLC-2 HF-1 region, α-MHC, and ANF enhancer/promoters. Neonatal rat heart nuclear extracts (nRHNE) and C2C12 myotube nuclear extracts were probed with each site (Fig. 2). An apparent MEF-2 interaction was seen with each binding site when either extract was used. With each probe and either extract, at least two bands were observed. The most slowly migrating band corresponded to the interaction referred to as MEF-2 by other investigators (Gossett et al., 1989). In nRHNE, an additional protein interaction was found to migrate somewhat more rapidly than the putative MEF-2 band. In contrast, C2C12 myotube extracts gave only a minor secondary band which did not have the same mobility as the second band from the rat heart. A third band, that migrated more rapidly than the two major bands was nonspecific. The MEF-2 bands from nRHNE and C2C12 myotube extracts (Fig. 2) comigrate suggesting that the protein complexes which were responsible for the bands were similar. The specific activities of the probes used in this experiment were similar. Thus, the difference in intensity of the MEF-2 bands was obtained with different probes reflects a difference in the affinity of MEF-2 for the different sites. The results demonstrated that the hierarchy of affinity of MEF-2 from the heart or skeletal muscle cells for the various sites was as follows: MCK > MLC-2 > α-MHC > ANF.

Competition experiments (data not shown) demonstrated that the MCK, MLC-2, and α-MHC MEF-2 sites were capable of competing for factor binding with each of the other sites. Factor binding was competed by the three different MEF-2 sites to a similar extent, with the unlabeled MCK MEF-2 site appearing to be the best competitor. Both the upper and lower bands were competed effectively by each competitor. The ANF MEF-2 site was less effective in competitions than the other sites.

DEPC Interference Footprinting of the MEF-2 Interactions—DEPC interference footprinting revealed that a protein or protein complex from nRHNE associated with the MCK MEF-2 DNA binding site in a manner identical to that found in skeletal muscle (Fig. 3A). The footprint was obtained from the upper band seen in Fig. 2. The protected sequences are shown schematically in Fig. 3D. The cardiac MEF-2 DNA binding activity was indistinguishable from that in C2C12 myotube extract by DEPC interference footprinting (data not shown). Subsequently, the MEF-2 interaction with putative sites in the α-MHC and MLC-2 genes were examined (Fig. 3, B and C). The interactions with the α-MHC and MLC-2 MEF-2 sites were similar to the footprint of the MCK MEF-2 site. These results suggest that the factor(s) that interact with the MCK, α-MHC, and MLC-2 MEF-2 sites are closely related. DEPC footprinting was also performed to determine the nature of the protein-DNA interaction resulting in the second, faster migrating, band. With each probe, the footprint of the second band was identical to the more slowly migrating band (data not shown).

We found a MEF-2-dependent interaction in skeletal muscle, heart, and brain not in the liver or kidney (data not shown). The DEPC footprint of both bands obtained from neonatal brain nuclear extract were identical to those obtained from adult rat heart.
MEF-2 DNA binding activity in nuclear extracts from the neonatal rat heart and C2C12 myotubes. The level of MEF-2 DNA binding activity was determined by EMSA. Oligonucleotides containing the MEF-2 site from the MCK enhancer, the MLC-2 HF-1 region, the α-MHC and ANF enhancer/promoters are described under "Experimental Procedures." MEF-2 and MLC-2 are abbreviated MEF and MLC, respectively, for convenience. The specific activities of the probes given as cpm/pmol × 10^-4 were as follows: MCK MEF, 1.00; MLC MEF, 0.94; α-MHC MEF, 0.89; and ANF MEF, 1.56. An equal number of counts were added per reaction. The upper arrow indicates the band corresponding to the MEF-2-dependent interaction. The lower arrow indicates the position of a second significant interaction in heart nuclear extracts.

Tained with nRHNE and C2C12 nuclear extract (data not shown).

MEF-2 and BF-2 Interactions with MFB-2 and HF-1—The MEF-2 and BF-2 binding sites are separated by 10 base pairs in MFB-2 and overlap by 1 base pair in the MLC-2 HF-1 region (Fig. 4A). The proximity of the binding sites suggested the possibility that the two factors interact. To address the possibility of a positive cooperative interaction, MFB-2 or HF-1 were used to probe cardiomyocyte and C2C12 myotube nuclear extracts. In the EMSA, the formation of a single protein-DNA complex is favored since there is an excess of free probe. A complex dependent upon the binding of both BF-2 and MEF-2 would be seen only if there was a positive cooperative interaction. In preliminary experiments, we could not see a MEF-2-dependent interaction with the MBF-2 probe. In an effort to visualize a MEF-2-dependent band, the amount of cardiomyocyte extract was increased 3-fold. The C2C12 extract was titrated to give a level of BF-2 binding similar to that of the cardiomyocyte extract.

With cardiomyocyte nuclear extract (Fig. 4B), three specific bands were observed with the MBF-2 and HF-1 probes. The strongest band (Fig. 4B, band B) migrated at a position similar to the BF-2/PRE B1 and HF-1A/HF-1a complexes. This band was competed by PRE B1. A barely visible band (Fig. 4B, band A) migrated similar to the MEF-2 complex and was competed by the α-MHC MEF-2 site (lanes 3 and 11). The presence of the band was confirmed by longer exposures of the film (data not shown). This band was more pronounced with the HF-1 probe possibly due to the higher specific activity of that probe. A third band migrated to a position between bands A and B and was competed by MEF-2 but not by PRE B1. The observation that no band competed with both the BF-2 and MEF-2 sites indicated that no MEF-2 and BF-2-dependent complex was formed. Thus, no positive cooperative interaction could be detected between MEF-2 and BF-2 in cardiomyocyte extract.

Three bands with mobility and competition characteristics similar to those seen with cardiomyocyte extract were observed when C2C12 myotube nuclear extract was probed with MBF-2 or HF-1 (Fig. 4C). At protein concentrations which gave roughly equivalent BF-2 DNA binding activity, the MEF-2 dependent bands were stronger with C2C12 than with cardiomyocyte extracts. These results indicate that the level of MEF-2 DNA binding activity, relative to BF-2 activity, is greater in C2C12 myotubes than in cultured neonatal cardiomyocytes (compare Fig. 4, B and C, lanes 1 and 9). It also confirms that the combined site oligonucleotides are capable of interacting with MEF-2. As with cardiomyocyte extract, a MEF-2/BF-2 dependent complex was not detected with C2C12 myotube extract.

Visual inspection of Fig. 4 suggests that the MEF-2 interactions with MBF-2 and HF-1 are less than the MEF-2 interaction on the α-MHC MEF-2 or HF-1b site with either extract. However, when the MEF-2 bands from the individual sites and the combined sites were cut out and counted directly, and the specific activities of the probes were taken into account, the amount of MEF-2 binding to the individual and combined sites was similar. The difference in the intensities (Fig. 4, B and C) is exaggerated because the exposure was not in the linear range for MEF-2.

MEF-2 DNA Binding Activity Is Developmentally Regulated in the Rat and Chicken Heart—Initial attempts to find MEF-2 DNA binding activity in the adult rat heart were unsuccessful although it was readily detectable in nRHNE. MEF-2 DNA binding activity was finally demonstrated but only when much greater amounts of adult rat heart nuclear extract were used. These results led us to ask whether MEF-2 DNA binding activity is regulated during rat cardiac development. Chicken heart extracts were also assayed for MEF-2 binding since a putative MEF-2 DNA binding site was present in the chicken MLC-2 promoter. To determine the level of MEF-2 DNA binding activity in the heart during development, EMSAs were performed using the MCK MEF-2 site and cardiac extracts from various developmental stages.

In the rat, a significant MEF-2 interaction was seen in the 12-day fetal heart which increased noticeably in the 18-day fetal heart and peaked in the neonatal rat heart (Fig. 5A). MEF-2 binding activity was decreased at 13 days postpartum and was nearly absent in the normal adult heart. The faster migrating band seen in Fig. 2 with nRHNE was seen only with the nuclear extract prepared from the 1-day neonatal rat heart (Fig. 5A, lane 3). In the embryonic chicken heart (Fig. 5B), MEF-2 DNA binding activity was measurable at 4 days and was dramatically increased by day 11. This was followed by a gradual decrease from 11 to day 16 and then a second peak was seen at day 19. From day 19 in ovo, to the adult chicken, there was a dramatic loss of MEF-2 DNA binding activity. A faint band migrating more rapidly than MEF-2 could be seen in the in ovo chicken heart but was not present after hatching.

To control for possible variations in the extract preparation, we assayed several extracts for the level of M-CAT binding factor-associated DNA binding activity since we have previously shown that this activity is similar in the neonatal and adult rat heart (Molkentin et al., 1993). The levels of M-CAT binding factor in the rat and chicken heart were fairly constant over the time when MEF-2 DNA binding activity drops.
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**Fig. 3.** DEPC footprint analysis of MEF-2 binding. DEPC footprinting was performed as described under “Experimental Procedures.” The footprints shown were obtained using neonatal rat heart nuclear extract. Both the sense and antisense (anti) strands are shown. Footprint of cardiac-MEF-2 DNA binding activity on the MCK MEF-2 (MCKMEF) site (A); the α-MHC MEF-2 (α-MHCMEF) site (B); and the MLC-2 MEF-2 (MLCMEF) site (C). D, schematic representation of the cardiac MEF-2 DEPC footprints. The closed and open circles represent strong and weak interference, respectively.

MEF-2 DNA Binding Activity Is Increased in the Adult Heart Subjected to Increased Workload—EMSAs were used to measure the level of MEF-2 and BF-2 DNA binding activity in adult rat hearts subjected to either a pressure or volume overload. These experiments were done with pooled hearts (8 hearts per pool) since this was the minimum number of adult hearts required to prepare nuclear extracts of consistent quality.

The volume overload model was the RRM rat maintained on a high salt diet (Lombard et al., 1989). Controls for the volume overloaded model were RRM rats maintained on a low salt diet. The former rats had mean arterial blood pressure values of over 200 mm Hg while the control animals had near normal pressures (110 mm Hg). The coarctation pressure overload model was produced by partial ligation of the abdominal aorta (Anversa et al., 1975). While blood pressure measurements were not made in these animals, measurements were made on a second group which underwent the identical procedure. In those animals, the elevated mean arterial pressure...
that mutation of the MEF-2 site, within the context of 3300 base pairs of the α-MHC gene, decreased expression of that enhancer/promoter by 85% in cultured cardiomyocytes and by 80% in the adult rat heart (Fig. 1). Thus, the MEF-2 site is involved in positive regulation of transcription of the rat α-MHC enhancer/promoter in cardiomyocytes. This result is consistent with several studies which demonstrate that MEF-2 is a positive regulatory element in striated and smooth muscle (Cserjesi and Olson, 1991; Gossett et al., 1989; Horlick and Benfield, 1989; Sternberg et al., 1988; Yu et al., 1992; Lee et al., 1992). A recent report demonstrated that mutation of the MEF-2 site in a mouse α-MHC transgene increased transcription in the ventricle and also permitted etopic expression in the aorta (Adolph et al., 1993). These results suggest that within the context of the mouse α-MHC transgene, the MEF-2 site acts as a negative regulatory element which controls cardiac-specific expression (Adolph et al., 1993). The difference in MEF-2 activity in this model as compared to others could be due to the promoter context or a different MEF-2 binding activity, such as a different MEF-2 isoform. The organization of the mouse and rat α-MHC enhancer/promoter regions are somewhat different suggesting that these genes are not regulated in an identical fashion. One example of this is that the mouse MEF-2 site lacks the closely associated BF-2 E-box site which is present in the rat α-MHC gene (Gulick et al., 1991; Molkentin et al., 1993). The difference in MEF-2 activity cannot be directly attributed to the transgenic model since the MEF-2 site in the MLC-2 gene acts as a positive regulator in both cultured cardiomyocytes and in the hearts of transgenic mice (Lee et al., 1992).

At least three different MEF-2 isoforms are expressed in the heart (Yu et al., 1992). Our results confirm and extend these previous results by demonstrating that MEF-2 interacts with binding sites on several genes expressed in the heart. Two distinct complexes (Fig. 2) formed when nRHNE was probed with the MEF-2 sites. The upper band, corresponded to MEF-2 in both EMSA mobility (Fig. 2) and in DNA-binding properties (Fig. 3). The faster migrating complex gave a footprint identical to the upper MEF-2-dependent band and was indistinguishable based on competition EMSA. It probably represents one of the multiple isoforms of MEF-2 (Yu et al., 1992). The mutation in the MEF-2 site which decreased activity from the α-MHC gene also abolished the binding of both complexes (data not shown). The correlation between loss of activity and loss of factor binding indicates that MEF-2 acts as positive transcriptional regulatory element in the α-MHC promoter.

**Discusison**

**MEF-2 Positively Regulates the Expression of the Rat α-MHC Gene**—The work presented in this report demonstrates that proximal to the coarctation was 189 ± 6 mm Hg at 2 weeks after coarctation (Rusch et al., 1992). Sham-operated animals were used as controls. Hearts from the experimental groups were visibly hypertrophied. Only hearts that weighed at least 1.4 times that of the average control heart weight were used to prepare extract. Two ventricular extracts were prepared from each heart type (16 total). MEF-2 DNA binding activity was increased 2.8 ± 0.69-fold in the RRM rat and 2.0 ± 0.68-fold in the coarctated rat over their respective controls (fold increase ± S.D.). The values for MEF-2 DNA binding activity were calculated on a per mg of protein basis from 14 separate determinations with two different extract preparations. These values were obtained by cutting out EMSA bands and counting them in a liquid scintillation counter.

BF-2 levels did not vary under identical conditions.

**Fig. 4. Interaction of MEF-2 and BF-2 with their combined sites in the α-MHC MBF-2 and MLC-2 HF-1 (MLCHF1) regions.** A, the binding sites for MEF-2 and BF-2 are shown in the α-MHC MBF-2 and MLC-2 HF-1 regions. Potential physical interaction between MEF-2 and BF-2 were probed by EMSA using MBF-2 or HF-1 as described under "Experimental Procedures." Nuclear extract from cultured neonatal rat heart cardiomyocytes (B) or from C2C12 myotubes (C) were used where indicated. Lanes 5–8 in B and C represent interactions with the indicated single site.
Results is that MEF-2 and BF-2 interact in some fashion. However, the experiments reported here do not definitively establish this possibility. It is also likely that these sites interact with other elements.

**Developmental and Overload Induced Changes in MEF-2 DNA Binding Activity**—The absolute level of MEF-2 DNA binding activity varies greatly during development of the rat and chicken heart. The significance of the developmental change in MEF-2 activity on α-MHC gene expression is not immediately obvious. There is a discordance between absolute levels of MEF-2 and α-MHC gene expression. The level of MEF-2 DNA binding activity is near its peak in the neonatal animal (Fig. 5) at a time when the expression of the α-MHC gene is being up-regulated (Lompere et al., 1984). The dramatic decline in MEF-2 DNA binding activity to very low levels in the adult heart occurs at a time when α-MHC gene expression is high. However, the low levels in the adult are sufficient for α-MHC expression (Fig. 1). Furthermore, MEF-2 activity levels increase during hypertrophy at a time when transcription from the α-MHC gene is repressed (Chassagne et al., 1993). One obvious explanation of these results is that MEF-2 levels in the heart are always sufficient for α-MHC gene expression. Our results do not rule out this possibility. Functionally saturating levels of MEF-2 have been reported in cardiac, smooth and skeletal muscle cells (Yu et al., 1992). However, this conclusion was based on experiments in primary neonatal cardiomyocytes which contain much more MEF-2 activity than adult cardiac muscle cells (Fig. 5). Changes in absolute MEF-2 levels in the heart may be important for the regulation of genes which have low affinity MEF-2 sites (i.e. ANF in Fig. 2).

Interestingly, BF-2 DNA binding activity parallels that of MEF-2. BF-2 levels peak in the late fetal to early neonatal stages and then drop off precipitously⁵ (Molkentin et al.,

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**Fig. 5.** MEF-2 DNA binding activity in the developing rat and chicken heart. Levels of MEF-2 DNA binding activity were determined by EMSA as described under “Experimental Procedures.” Equal amounts (12 μg of protein) of cellular or nuclear extracts from rat (A) or chicken (B) were probed with radiolabeled MCK MEF-2 binding site. C, extracts giving maximum MEF-2 activity and adult heart extracts from rat and chicken were probed with a radiolabeled binding site for M-CAT binding factor.
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Thus, in the adult rat heart a high level of α-MHC gene expression is maintained at a time when the level of two positive transcription factors is low. Absolute levels of MEF-2 and BF-2 might always be sufficient and alterations in these levels may not, by themselves, influence the expression of the α-MHC gene during cardiac development or hypertrophy. If α-MHC gene expression is dependent upon absolute levels of soluble MEF-2 and BF-2, then maintenance of high levels of expression occurs during a period when there is a dramatic decrease in the levels of these two positive transcription factors. This point is somewhat counter intuitive.

Models for MEF-2 and BF-2 Activity—The parallel between MEF-2 and BF-2 activity in the heart could indicate that the ratio of MEF-2 to BF-2, rather than absolute levels, might serve to maintain high levels of expression. The MFB-2 region might act as a sensing mechanism. The sensing mechanism would detect the ratio of MEF-2 to BF-2 binding and the appropriate ratio would permit high levels of α-MHC expression. Any change that alters the ratio of binding in the sensing region such as a mutation that decreased or increased affinity of a site or increased or decreased the MEF-2 to BF-2 ratio would be expected to result in a decrease in expression from the α-MHC gene. This model could also explain the hypertrophy data. During hypertrophy, the MEF-2 to BF-2 ratio increases resulting in a cellular environment that is not favorable for α-MHC gene expression. Since the cDNAs for MEF-2 have been isolated and cloned into expression vectors, it should be possible to test this model. The effects of exogenous MEF-2 would most likely be seen in the adult rat where MEF-2 levels are significantly lower than in the neonatal heart.

A second possibility is that MEF-2 and BF-2 are active, or more active in the regulation of the α-MHC gene when they are associated with the nuclear matrix. Association with the nuclear matrix has been shown to be important for the activity of several transcriptional regulatory proteins (Bidwell et al., 1993; Dwoertzky et al., 1992; Landers and Spelsberg, 1992). Thus, the second model suggests that the localization of MEF-2 and BF-2 to the nuclear matrix is important for their interaction with the α-MHC promoter. This is consistent with the observation that thyroid hormone receptor, a major regulator α-MHC expression, is active in association with the nuclear matrix (Kumara-Siri et al., 1986). Interestingly, the MEF-2 binding site contains a core motif (ATTA) of the nuclear matrix anchorage site (Boulikas, 1992). The loss of MEF-2 and BF-2 DNA binding activity in our soluble nuclear extracts could indicate that these proteins are associating with the nuclear matrix at a time when α-MHC expression was increasing to peak levels. The increase of MEF-2 activity in the soluble nuclear extract from hypertrophied hearts could correspond to a loss of MEF-2 association with the matrix and thereby correlate with a loss of α-MHC gene expression. This model is also testable.

Both models discussed above could account for data on the regulation of the MLC-2 gene (Zhu et al., 1991; Navankasattus et al., 1992) in the developing and adult rat heart. Difference in the expression of the α-MHC and MLC-2 genes during cardiac hypertrophy indicate that distinct regulatory events influence expression during this process. These differences are undoubtedly due to the influence of a complex series of events which do not depend solely upon MEF-2 and BF-2.

Cardiac-specific Expression—The experiments reported here do not directly address the role of MEF-2 and BF-2 in directing cardiac-specific expression. However, other work has implicated the MEF-2 site in cardiac-specific control of the rat MLC-2 and mouse α-MHC gene in transgenic animals (Lee et al., 1992; Adolph et al., 1993). In the former study, the MEF-2 site acted as a positive regulator that interacts with a BF-2 binding site to target expression of an MLC-2 gene to the heart. In the later study, the MEF-2 site acted as a negative element and loss of binding to the α-MHC transgenic increased expression in the ventricle and permitted high levels of ectopic expression in the aorta. Our results are consistent with the MLC-2 results (Lee et al., 1992).

The results in Fig. 5 demonstrate that the ratio of MEF-2 to BF-2 DNA binding activity is much greater in skeletal muscle cells than in the heart which, according to our sensing mechanism model, should inhibit expression of the α-MHC gene. While it is unlikely that this simplistic model accounts for most aspects of cardiac-specific expression, it is interesting to speculate that it plays a role in this process.

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