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An M-CAT Binding Factor and an RSRF-Related A-Rich Binding Factor Positively Regulate Expression of the α-Cardiac Myosin Heavy-Chain Gene In Vivo

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Cardiac muscle-restricted expression of the α-myosin heavy-chain (α-MHC) gene is regulated by multiple elements in the proximal enhancer/promoter. Within this region, an M-CAT site and an A-rich site were identified as potential regulatory elements. Site-specific mutations in each site, individually, reduced activity from the wild-type promoter by approximately 85% in the adult rat heart, demonstrating that these sites were positive regulatory elements. α-MHC, β-MHC, and chicken cardiac troponin T (cTnT) M-CAT sites interacted with an M-CAT-binding factor (MCBF) from rat heart nuclear extracts that was immunologically related to transcriptional enhancer factor 1, a factor that binds within the simian virus 40 enhancer. The factor that bound the A-rich region (ARF) was antigenically related to the RSRF family of proteins. ARF was distinct from myocyte-specific enhancer factor 2 (MEF-2) on the basis of DNA-binding specificity and developmental expression. Like MEF-2, ARF DNA-binding activity was present in the heart and brain; however, no ARF activity was detected in extracts from skeletal muscle or C2C12 myotubes. MCBF and ARF DNA-binding activities were developmentally regulated with peak levels in the 1- to 2-day neonatal heart. The activity of both factors increased nearly fivefold in adult rat hearts subjected to a pressure overload. By comparison, the levels of α-MHC binding factor 2 did not change during hypertrophy. Binding sites for MCBF and ARF are present in several genes that are upregulated during cardiac hypertrophy. Our results suggest that these factors participate in the alterations in gene expression that occur during cardiac development and hypertrophy.

The α-cardiac myosin heavy-chain (α-MHC) gene is expressed in the heart and in a layer of the lung referred to as the pulmonary myocardium (13, 17, 42). The rat α-MHC gene is developmentally and hormonally regulated at the transcriptional level by a complex interplay of multiple *cis* elements and *trans*-acting factors. Upstream regulatory factors that have been characterized include the product of the *egr*-1 cellular oncogene that stimulates α-MHC transcription through a sequence element located from −1698 to −1283 (11) and α-MHC binding factor 1, which binds a region from −599 to −576 (26) and might act as a negative regulator (44). While these elements may contribute to the overall regulation of the α-MHC gene, several reports taken together indicate that the region from −374 to −39 is sufficient for directing cardiac muscle-restricted expression of the α-MHC gene (4, 12, 34, 44). Within this proximal region, three positive elements have been described. The thyroid regulatory element (TRE; −153 to −133) interacts with thyroid hormone receptors (THRs) to induce α-MHC expression (16, 27). A second factor, α-MHC binding factor 2 (BF-2), is a positive transcription factor that interacts with an E-box element (PRE-B1) and flanking sequences located from −318 to −303 (31). Finally, myocyte-specific enhancer factor 2 (MEF-2) interacts with a site located from −335 to −327 in the α-MHC enhancer (32). The latter two binding activities appear to interact with each other and possibly other elements within the α-MHC regulatory region to enhance transcription (32). In addition, there are two DNA recognition sequences for transcription factor GATA-4 (2) located from −253 to −270.

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Within the region between −374 to −39 is a DNA recognition sequence (5′-CATTCCT-3′; −236 to −241) for M-CAT binding factor (MCBF) in the orientation opposite from that in the chicken cardiac troponin T (cTnT) gene (24). The M-CAT binding site was shown to be an important transcriptional regulator of several muscle-specific genes despite the fact that MCBF has been found in a wide variety of cell types (19, 25). The M-CAT site appears to be involved in mediating the increase in β-MHC expression following α_1-adrenergically induced hypertrophy of cultured cardiomyocytes and this activity is likely due to the interaction of this site with MCBF (19). Recently, two MCBFs were shown to interact with the regulatory regions of rat and rabbit β-MHC genes (38, 39). One of these factors was antigenically related to transcriptional enhancer factor 1 (TEF-1) that binds to the GTIIC motif of the Simian virus 40 enhancer (8). The TEF-1-related factor was found in muscle and nonmuscle tissues. The second binding activity did not interact with TEF-1 antiserum and was found in a skeletal muscle cell line, but not in HeLa cells.

Just downstream from the M-CAT site in the rat α-MHC gene is an element containing seven consecutive A residues (−223 to −217). This site has been reported to interact with cloned MEF-2 and aMEF-2 (46). Since multiple isoforms of MEF-2 and MCBF act as positive regulators of muscle-specific genes, their possible role in the regulation of the rat α-MHC gene was investigated.

In this study, we examine the contribution that the M-CAT and A-rich sites make to the regulation of expression of the α-MHC gene in the adult rat heart. Site-directed mutagenesis experiments demonstrate that both of these sites act as positive elements to control α-MHC expression. The α-MHC M-CAT site interacted with an MCBF that was similar in DNA binding specificity to the factor that interacted with the β-MHC and

TABLE 1. Sequences of the sense-strand oligonucleotides

Oligonucleotide	Sequence[a]	Gene[b]
PRE-E	−248 GGGAGGAGGAATGTGCCCAAGGACTAAAAAAAGGCC −213	
αMCAT	−247 GGAGGAGGAATGTGCCCA −230	
βMCAT	−286 GAGCTGTGGAATGTAAGG −269	β-MHC
A rich	−229 AGGACTAAAAAAAGGCCC −213	
MEF2mut	−340 CAGATTAAAAAAAACTAAGGTAAGGGC −314	
M-CAT1	−101 CGTGTTGCATTCCTCTCTGGATC −83	cTnT
MCKMEF2	−1078 GCTCTAAAAATAACCCTG −1061	MCK
A-richmut	−248 GGGAGGAGGAATGTGCCCAAGGACTGCAGGAAGGCCCTGG −209	
M-CATmut	−248 GGGAGGATTAATGTGCCCAAGGACTAAAA −220	
αCArG	−74 GACTCCAAATTTAGGCAGC −56	
PRE-B1	−318 AGGGCCATGTGGGTAG −303	
EB3	−60 GCAGCAGGCACGTGGAATGAGCT −38	
MCS	TCGCGACTCGAGCCCGGGATCGATGCGGCCGCCACGTG	

[a] Sequences are numbered relative to the start sites of transcription from their respective genes (see Materials and Methods for description). Underlined bases are mutations.

[b] All sequences are derived from the rat α-MHC gene except where indicated; MCS is a synthetic multiple cloning site.

cTnT M-CAT sites. α-MCBF was found to be antigenically related to TEF-1. The A-rich region of the α-MHC gene interacted with an A-rich binding factor (ARF) that was antigenically related to RSRF (36) and MEF-2 (46). However, ARF was distinct from MEF-2 in its DNA-binding specificity and developmental expression. ARF DNA-binding activity was enriched in nuclear extracts from heart and brain. The DNA-binding activities of MCBF and ARF were upregulated during late cardiac development and were differentially increased in the adult rat heart exposed to a pressure overload. Upregulation of MCBF is also consistent with a role for this factor in the induction of the β-MHC gene in response to hypertrophic stimuli (15, 19). Binding sites for MCBF and ARF are present in genes that are induced in response to cardiac hypertrophy (38, 43). Thus, these factors may be important factors in the coordinate gene regulation during that process.

MATERIALS AND METHODS

Oligonucleotides. The sense strand sequences of the double-stranded (ds) oligonucleotides used in this study are shown in Table 1. Proximal regulatory element E (PRE-E) contains sequences from −248 to −213 of the α-MHC enhancer/promoter (23). αMCAT, A-rich, and αCArG oligonucleotides contain sequences from −247 to −230, from −229 to −213, and from −74 to −56 of the α-MHC enhancer/promoter, respectively (23). βMCAT contains sequences from −286 to −269 in the rat β-MHC promoter (43). M-CAT1 contains sequences from −101 to −83 of the chicken cTnT promoter as previously described (25). EB3 contains sequences from −60 to −38 of the α-MHC promoter (23). PRE-B1 (−318 to −303) from the α-MHC promoter has been described previously (31). MCKMEF contains sequences from −1078 to −1061 of the muscle creatine kinase enhancer (40). A-richmut and M-CATmut are oligonucleotides that contain mutations in their binding sites. MEF2mut is an oligonucleotide containing sequences from the α-MHC MEF-2 site (−340 to −314) that have been mutated to contain eight consecutive A residues. MCS is a multiple-cloning-site oligonucleotide used as a nonspecific competitor (31). All oligonucleotides were purchased from Operon Technologies Inc. (Alameda, Calif.).

Site-directed mutagenesis. Site-directed mutagenesis was performed by the rolling-circle PCR method as previously described (14). The scheme for mutating elements from −612 to +420 in the α-MHC gene and the subsequent analysis of the mutant constructs have been described elsewhere (31). All mutations were introduced into the α-MHC enhancer/promoter (3,300 bp) present in the plasmid pSV0MCAT (12). The sequences of the oligonucleotides used to introduce mutations A-richmut and M-CATmut are shown in Table 1, except for that of the antisense oligonucleotide PRE-D (CAAAGGGC CGATGGGCAGATAGAGGAGAGACAGGA). PRE-D was used as the opposite primer in the PCR mutagenesis procedure.

In vivo gene transfer by direct injection. Clones to be assayed for activity were injected directly into the apex of the adult heart as previously described (20, 35). Sprague-Dawley rats (200 to 300 g) were anesthetized with 0.15 ml of a Ketaset–acepromazine (promACE) mixture (10 ml of Ketaset [100 mg/ml] and 2.2 ml of promACE [10 mg/ml]) per 100 g of body weight and were ventilated with a respirator. DNA (80 μg of test DNA and 10 μg of pRSVβgal) was injected into the heart with a 28-gauge needle in a volume of 80 μl of saline. DNA was also injected into soleus muscle (45) of the same animals receiving the cardiac injection as a control. The animals were given penicillin G at 30,000 U/100 g of body weight postoperatively and were allowed to recover for 6 days. The rats were sacrificed by lethal injection of pentobarbital, and the hearts and soleus muscle samples were collected. Heart and soleus muscle extracts 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 Ultrasonics, Danbury, Conn.). The cellular debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. Chloramphenicol acetyltransferase (CAT) assays and β-galactosidase (β-Gal) assays were performed as previously described (9, 31).

Preparation of nuclear extracts. Nuclear extracts were prepared from fetal, neonatal, and adult rat tissues as described previously (31, 32). C2C12 myotube and myoblast extracts were prepared by the method described by Dignam et al. (6).

EMSA and DEPC interference footprinting. Electrophoretic mobility shift assays (EMSAs) were performed as described earlier (31). Unless otherwise indicated, 15 μg of protein extract (3) and 1 μg of poly(dI-dC) · (dI-dC) were used in each assay. The probes used in each experiment are indicated in the figure legends. Diethylpyrocarbonate (DEPC) footprinting was performed essentially as described previously (41). DEPC-treated probe was used in reaction mixtures described for gel shift experiments, except that the reaction mixtures were scaled up 25-fold. Complex isolation and electrophoresis con-

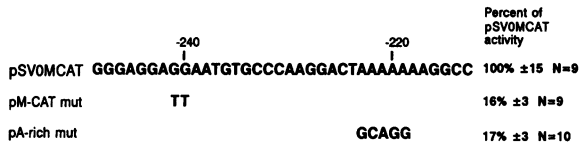

FIG. 1. Mutation of the M-CAT and A-rich binding sites inhibits expression from the α-MHC enhancer/promoter. Site-directed mutations were introduced into the M-CAT and A-rich sites of the α-MHC regulatory region as described in Materials and Methods. The wild-type promoter plasmid was pSV0MCAT (12). The CAT activities from the wild-type and mutant plasmids were assayed in the adult rat heart 6 days after their introduction into the heart by direct injection. pRSVβgal was injected as a control for DNA uptake and was used to normalize the results. Activity is given as the percentage of normalized CAT activity from pSV0MCAT ± SD. The sequence shown is the wild-type sequence of the α-MHC gene in the region of PRE-E. The mutations introduced into each site are indicated. N represents the sample number.

ditions were as described previously (31). Oligonucleotides used as probes were radioactively end labeled as previously described (29). Complementary strands of each oligonucleotide were annealed (18) prior to their use as a probe or competitor.

Immunoprecipitation experiments. Anti-TEF-1 serum was obtained from Charles Ordahl. This immune serum was raised in rabbits against synthetic peptides deduced from the chicken TEF-1 cDNA. Anti-RSRF serum (36) was obtained from Richard Treisman. Immunoprecipitation experiments were performed using these antisera. Five microliters of anti-TEF-1, anti-RSRF, or nonimmune serum was incubated with 10 μg of neonatal nuclear heart protein extract on ice for 30 min. Next, 8 μl of a 1:1 slurry (in water) of protein A agarose beads (Sigma Chemicals, St. Louis, Mo.) was added to these reaction mixtures, which were incubated on ice for an additional 20 min. The reaction mixtures were spun at 4°C at 10,000 rpm in a microcentrifuge (Microspin; Sorvall) for 15 s to pellet the agarose beads. The supernatant was then used in gel shift reactions with either 40 fmol of the M-CAT oligonucleotide or the A-rich oligonucleotide. EMSA conditions were as described previously (8).

Animal model 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, intramuscularly) anesthesia. Coarctation was performed by partial ligation of the aorta between the renal arteries with 2-0 braided silk in a manner similar to methods previously described (1). The animals received penicillin as described above and were allowed to recover for 10 to 11 days. The animals were sacrificed by pentobarbitol overdose, their hearts were harvested, and the atria were removed. The ventricles were weighed and then quick frozen in crushed dry ice and stored at −80°C until use. Two separate groups of eight coarctated hearts or hearts from sham-operated controls were used to prepare two separate extracts.

RESULTS

The M-CAT and A-rich sites in the α-MHC promoter are positive regulatory elements. To determine if the M-CAT and A-rich sites influenced expression of the α-MHC gene in the heart, each site was mutated within the context of the larger α-MHC enhancer/promoter (Fig. 1). The mutants were assayed in vivo by direct injection of constructs into the adult rat heart. Two transversion mutations were introduced into the M-CAT site that changed the core 5′-CATTCCT-3′ sequence to 5′-CATTAAT-3′. Four mutations were introduced into the A-rich region to change the site from 5′-TAAAAAAAGG-3′ to 5′-TGCAGGAAGG-3′. The mutated plasmids, pM-CAT mut and pArichmut, were identical to pSV0MCAT in sequence and spacing of sequence elements, except for these specific alterations. The activity of pSV0MCAT (20,595 ± 3254 cpm/50 μg of protein ± standard deviation [SD] normalized for β-Gal activity) was set at 100%. Compared with the wild-type level, the levels of activity from pM-CATmut and pArichmut were 16% ± 3% and 17% ± 3% (± SD), respectively (Fig. 1). The activity of each construct in nine animals was determined. For each site, the mutations reduced activity nearly 85%, indicating that each site is an important positive regulatory element.

Identification of factors in cardiac nuclear extracts that interact with the M-CAT and A-rich sites. EMSAs were used to characterize factor binding to the M-CAT and A-rich sites. Initial analysis was performed using a ds oligonucleotide that contained α-MHC sequences from −248 to −213. This element has been designated PRE-E and contains both sites. PRE-E was used to probe neonatal rat heart nuclear extracts (nRHNE). Two specific interactions were seen with PRE-E. The fastest migrating complex had DNA-binding characteristics similar to those of MCBF (Fig. 2). A 100-fold-excess of unlabeled PRE-E or the αMCAT or cTnT M-CAT (M-CAT1) site, but not other competitors, competed successfully for MCBF binding (Fig. 2, lanes 3 and 4). The more slowly migrating band that associated with PRE-E was due to the interaction of a cardiac nuclear factor, ARF, with the A-rich sequence in PRE-E. The interaction of ARF with labeled PRE-E was successfully competed for by a 100-fold excess of the following oligonucleotides: PRE-E, A-rich, and a mutated MEF-2 site (MEF2mut) containing eight consecutive A residues. The muscle creatine kinase (MCK) MEF-2 site did not compete with PRE-E for ARF binding, indicating that ARF has DNA-binding properties distinct from those of MEF-2 (32). The α-MHC CArG-like sequence that contains seven consecutive A or T residues (Fig. 2, lane 8) and a ds oligonucleotide containing six consecutive A residues (data not shown) also failed to compete with PRE-E for ARF binding. Therefore, the binding site for ARF appears to require at least seven A residues. The M-CAT sites and MCS ds oligonucleotide did not compete for ARF binding.

The mutations in the M-CAT and A-rich sites in the α-MHC enhancer/promoter that attenuated functional activity (Fig. 1) abolished factor binding (data not shown). These results indicate that both the MCBF and ARF are involved in the transcriptional regulation of the α-MHC gene.

DEPC footprinting was used to define the MCBF- and ARF-binding sites (Fig. 3). With the M-CAT site, modification of the G and A residues in the α-MHC enhancer/promoter from −244 to −235 interfered with factor binding (Fig. 3A and C). DEPC footprinting of the ARF interaction indicated that modification of the sense strand A residues from −222 to −217 or the A residue on the antisense strand at position −224 interfered with factor binding (Fig. B). The footprints are shown schematically in Fig. 3C. The sites for these two factors are separated by 10 bp, indicating that these factors are in close proximity on the same side of the helix.

Tissue distribution of MCBF and ARF DNA-binding activities. We wanted to determine the tissue distribution of MCBF and ARF activity, because muscle-specific and ubiquitous MCBFs have been reported elsewhere (25, 38) and because cloned MEF-2a has been reported to interact with the α-MHC A-rich region (46). Nuclear extracts from various neonatal rat

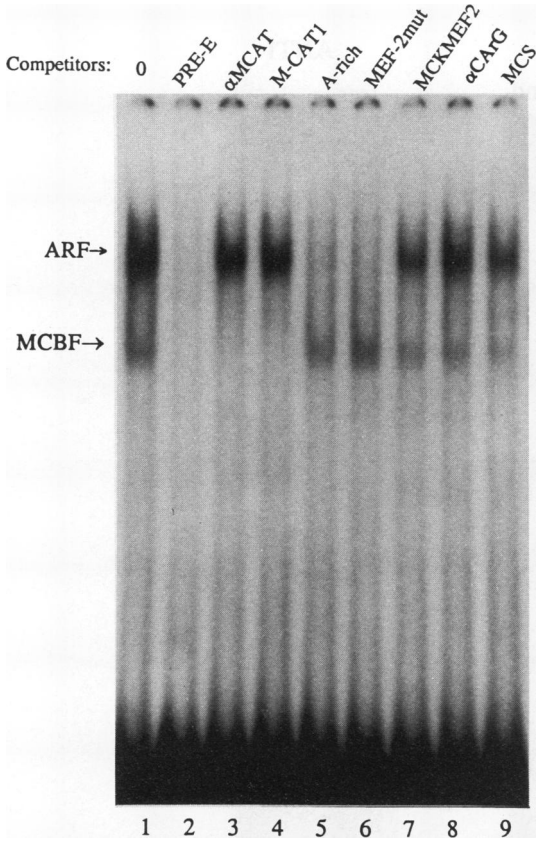

FIG. 2. Factors that bind to the α-MHC M-CAT and A-rich sites and DNA-binding specificities of factors that interact within the α-MHC PRE-E. EMSAs were used to identify DNA-binding factors present in nRHNE that interact with the radioactive PRE-E probe. The two major DNA-binding activities are indicated. The sequences of the probes and competitors are given in Table 1. Each competitor was added at a 100-fold molar excess of the indicated unlabeled ds oligonucleotide. The positions of the complexes representing ARF and MCBF are indicated.

tissues were probed with the A-rich and αMCAT sites (Fig. 4). Factors that interacted with the A-rich site were enriched in nuclear extracts from heart and brain tissue. A faint interaction could be seen in the lung, which presumably represents an ARF-dependent complex (Fig. 4). ARF was not detected in extracts from the neonatal kidney or soleus muscle. A second, faster migrating ARF DNA-binding activity was present in each of the tissues examined, except heart tissue. The two ARF activities in neonatal brain nuclear extract were examined by competition EMSAs and were found to interact in a sequence-specific manner with the A-rich site of the α-MHC promoter (data not shown). Similar experiments with the lung extract demonstrated that all three of the bands observed with that extract (Fig. 4, lane 4) represented sequence-specific interactions (data not shown). Since C2C12 myoblasts and myotubes are often used to represent skeletal muscle, we used PRE-E to examine the levels of ARF in nuclear extracts of myoblasts and myotubes. The results of that experiment (Fig. 4, lanes 11 and 12) indicate that ARF is present at high levels in the myoblast and absent in the myotube, consistent with its absence from soleus muscle extracts (lane 5). The same extracts were probed with the MEF-2 site from the MCK enhancer. MEF-2 was clearly upregulated in myotubes (Fig. 4, lanes 13 and 14). The absence of ARF DNA-binding activity in C2C12 myotube extracts further distinguishes this factor from MEF-2.

With the αMCAT probe, MCBF DNA-binding activity was detected in each of the nuclear extracts from each tissue examined (Fig. 4, lanes 6 to 10). These results were similar to those seen when the cTnT M-CAT site was used as a probe (25) (data not shown). MCBF DNA-binding activity was present in higher amounts in neonatal heart, brain, and lung tissue and to a lesser extent in kidney tissue and skeletal muscle. Two distinct bands were clearly visible when extracts derived from the kidney and lung tissue were probed with the αMCAT probe (Fig. 4, lanes 8 and 9). A weak interaction was observed between the M-CAT site and a factor in C2C12 myoblast nuclear extracts. However, when the myotube extract was probed, a prominent broad band was seen (data not shown).

MCBF and ARF are antigenically related to TEF-1 and RSRF, respectively. The chicken MCBF that associates with the cTnT M-CAT site is antigenically related to TEF-1 (8). To determine if a protein in the M-CAT complex was related to TEF-1, we obtained purified rabbit immunoglobulin G from immune serum produced against synthetic peptides deduced from the sequence of the chicken TEF-1 cDNA (8). nRHNE was probed with the αMCAT site in the presence of nonimmune serum or anti-TEF-1 serum. As seen in Fig. 5, TEF-1-specific antibody but not nonimmune serum dramatically reduced MCBF activity from nRHNE.

The A-rich binding site of ARF is reminiscent of the binding sites for RSRFs and MEF-2 that have been reported to bind to this site (36, 46). Therefore, anti-RSRFC4 serum was obtained and used to determine if ARF was related to the RSRF family. In the presence of anti-RSRF serum, an ARF-dependent complex was not observed from nRHNE, while the complex was present when the EMSA reaction mixture was treated with nonimmune serum (Fig. 5). The anti-RSRFC4 serum reduced MEF-2 binding to the α-MHC MEF-2 site to a similar extent (data not shown). Anti-TEF-1 serum had no effect on ARF, and anti-RSRF serum had no effect on MCBF (data not shown). These results indicate that ARF is related to RSRF and MEF-2 proteins.

MCBF and ARF DNA-binding activities in the heart are developmentally regulated. Because of the disparate expression of MCBF and ARF in the developmentally immature and mature skeletal muscle cells, we wanted to determine if these activities were differentially regulated in the developing rat heart. MCBF and ARF DNA-binding activities were assayed together on PRE-E in EMSAs. Cardiac extracts prepared from hearts at different stages of development were probed (Fig. 6). A faint but detectable MCBF activity was observed in 12-day fetal heart extracts (upon longer exposures of the gel) but was higher at fetal day 18 and reached peak levels in the 1- to 2-day neonatal heart. MCBF DNA-binding activity declined by 13 days postpartum and continued to decline to a steady-state level in the adult heart which was approximately 60% less than peak values. The developmental change in MCBF activity reported here is different from results of previous reports (31, 32). This difference can be directly attributed to different probes used in these experiments (see Discussion).

At fetal days 12 and 18, ARF was undetectable. ARF activity increased dramatically between fetal day 18 and birth, reaching a peak in the 1- to 2-day neonatal rat heart. Levels of ARF activity decreased to extremely low levels in the adult rat heart (Fig. 6).

To control for possible differences in the extract preparation, extracts used in the assay shown in lanes 1 to 6 were probed with a second oligonucleotide binding site. This second

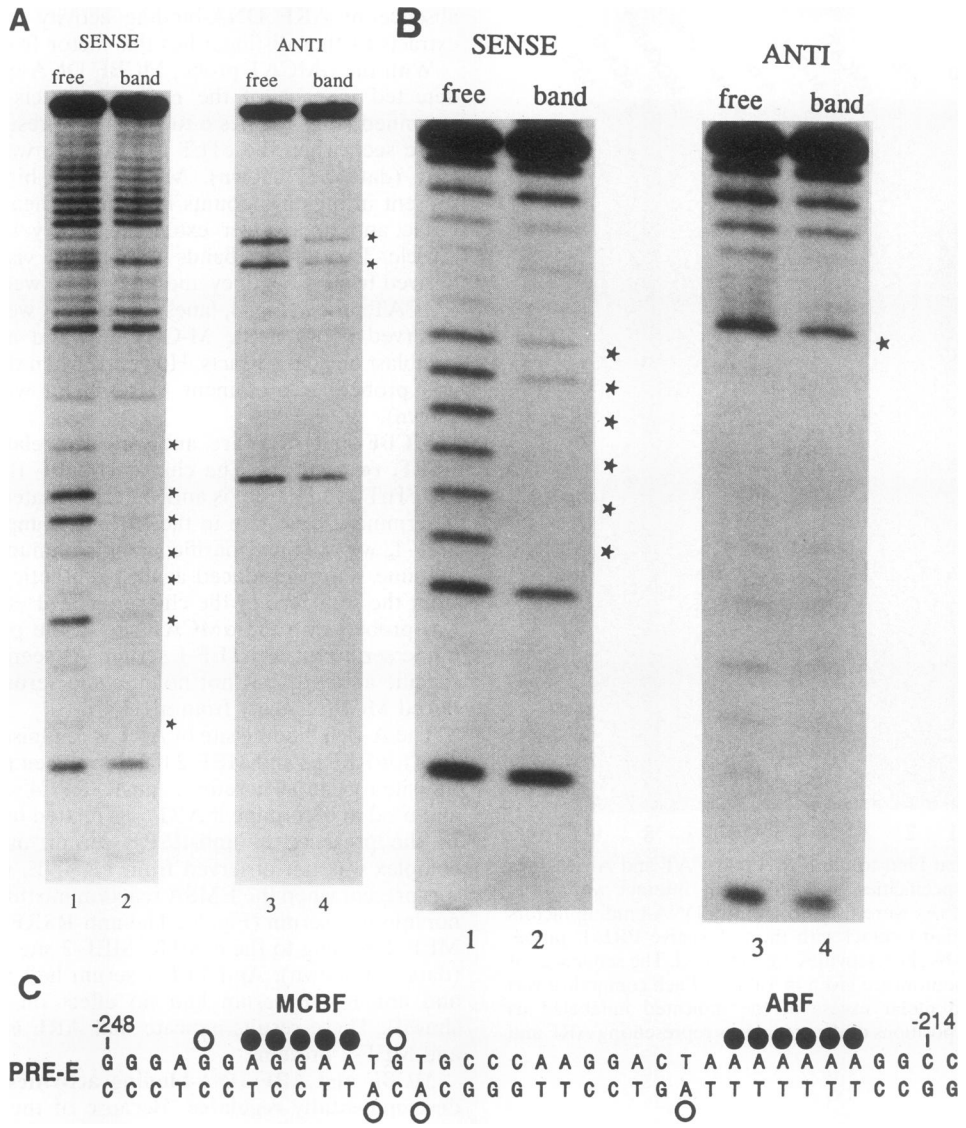

FIG. 3. Identification of MCBF and ARF binding sites in the α-MHC regulatory region. DEPC interference footprinting was carried out as described in Materials and Methods. Free and band lanes indicate the unbound and bound probe, respectively. The stars indicate positions at which modification interferes with factor binding. The sense (lanes 1 and 2) and antisense (lanes 3 and 4) footprints are shown. (A) DEPC footprint of the M-CAT site; (B) DEPC footprint of the A-rich site; (C) schematic representation of the DEPC footprint of the MCBF and ARF on PRE-E. The numbering is relative to the α-MHC transcription start site. The closed and open circles indicate positions at which modification interfered with factor binding more or less severely, respectively.

probe (EB3 [Table 1]) was made to the sequences from −60 to −38 in the α-MHC promoter and contained an E-box element that is distinct from the previously described E-box element that binds α-MHCBF-2 (31). The sequence of EB3 shows a difference from the previously published sequence (23). The EB3-dependent complex (C2) is distinct from BF-2 on the basis of DNA-binding specificity (data not shown), and therefore it is likely that they are not the same factor. C2 is present at low levels in the 12-day fetal extract, gradually increases to a maximum level in the neonatal heart, and then is maintained at a relatively high level in the adult rat heart compared with ARF and MCBF. Therefore, the loss of ARF and MCBF from the adult extracts reflects a real decrease rather than loss due to the experimental procedure.

MCBF and ARF DNA-binding activities are upregulated in the hypertrophied rat heart. Since the levels of MCBF and ARF activity varied during development (Fig. 6), we explored the possibility that the activity of these factors also changed in the heart in response to a hypertrophy stimulus. An acute pressure overload was induced by aortic coarctation as previously described (1, 32). Hearts were harvested 11 days postoperatively, and ventricular tissue was used to prepare nuclear extracts. The hearts had visible left-ventricle hypertrophy. Only hearts that weighed at least 1.4 times as much as the average control heart weight were used to prepare extracts. Sixteen hearts from coarctated and sham-operated control animals were obtained. The hearts from each group were separated into two subgroups of eight hearts, and nuclear extracts were prepared from each subgroup, resulting in two hypertrophied and two control extract preparations. EMSAs were used to

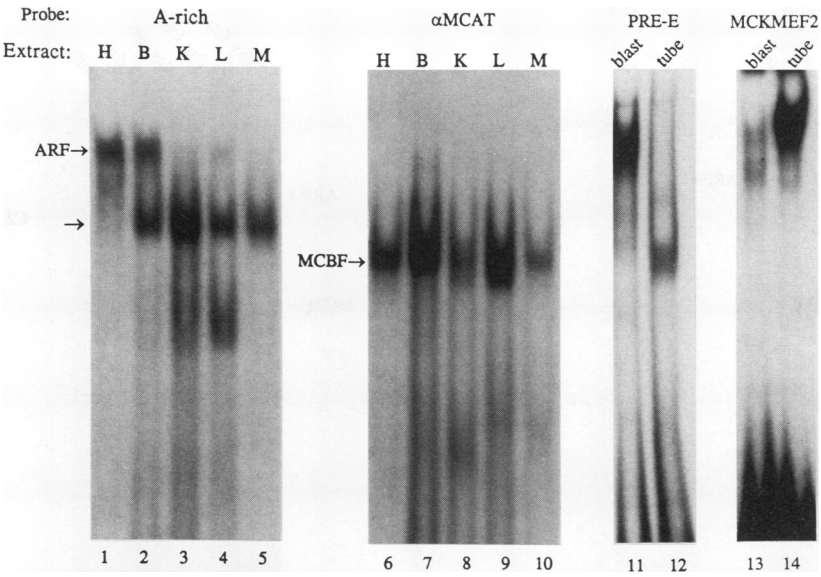

FIG. 4. Tissue distribution of ARF and MCBF DNA-binding activities. DNA-binding activities of ARF and MCBF were examined in nuclear extracts of the indicated neonatal rat tissues and in C2C12 myoblasts and myotubes using EMSAs. The A-rich, αMCAT, PRE-E and MCKMEF2 ds oligonucleotide sites were used as probes as indicated. The tissues examined were heart (H), brain (B), kidney (K), lung (L), and skeletal muscle (M). Blast and tube refer to C2C12 myoblast and myotube extracts, respectively. The positions of the ARF- and MCBF-dependent complexes are indicated by the arrows. The unlabeled arrow represents the position of a second interaction with the A-rich region that is sequence specific.

measure the levels of binding of MCBF and ARF in these extracts using the α- and β-MHC M-CAT and the ARF sites as probes (Fig. 7). The binding site for α-MHCBF-2 (PRE-B1 [Table 1]) was used as a control, since its levels were previously shown to be unaffected by pressure overload (32). In multiple determinations, MCBF and ARF DNA-binding activities increased 4.6- ± 1.2-fold and 5.5 ± 1.6-fold, respectively, above their levels in the sham-operated control heart extracts (measured per milligram of protein) (Fig. 7). The results with the β-MHC M-CAT site were similar to those with the α-MHC M-CAT site (Fig. 7).

DISCUSSION

The M-CAT site. The M-CAT site has been shown to be an important element in the regulated expression of the cTnT and β-MHC genes (8, 24, 43). This site is similar to the GT-IIC motif found in the simian virus 40 enhancer which binds TEF-1. MCBF has been shown to be immunologically related to human TEF-1 (8). MCBF and its DNA-binding site have also been implicated in the upregulation of β-MHC gene expression in response to α_1-adrenergic agonists (19). Therefore, the identification of an M-CAT motif in the α-MHC gene was of interest as a potential regulatory element. Site-specific mutation of the α-MHC M-CAT site resulted in significant loss of activity from the α-MHC enhancer/promoter (Fig. 1), indicating that this site acts as a positive transcriptional regulatory element. The cardiac factor that binds to the α-MHC M-CAT site is recognized by anti-TEF-1 serum prepared against chicken TEF-1, indicating that the factor is TEF-1 or a protein that is antigenically related. This factor is also similar to a previously reported MCBF in its ubiquitous expression.

Our results differ from two recently published studies which demonstrated the presence of a second MCBF activity that appeared to be muscle specific (38, 39). In the present work, we used two different probes and have identified MCBF activity in extracts from the heart and various other neonatal rat tissues, in extracts from hearts at various developmental stages, and extracts of C2C12 myoblasts and myotubes. Only in kidney and liver extracts were more than one band observed. Our results indicate that the M-CAT sites examined in this study formed only one complex, suggesting that there is only one MCBF in the heart and that this factor is TEF-1 related. However, in several experiments using the M-CAT1 oligonucleotide as a probe (data not shown), we observed a different protein-DNA complex that was M-CAT dependent. That complex migrated more rapidly than the complex formed with the αMCAT oligonucleotide, was present in extracts from all tissues examined, did not react with TEF-1 antibody, did not decrease significantly during late development, and was not upregulated during hypertrophy (33). Subsequently, we redesigned the M-CAT1 oligonucleotide so that it retained the sequence of the cTnT M-CAT site but was identical to the α- and β-MHC sites in terms of oligonucleotide length and the position of the M-CAT site within the oligonucleotide. With this redesigned probe, only one complex was observed, and it was TEF-1 dependent. The formation of the TEF-1-independent complex with M-CAT1, while dependent on the M-CAT site, was also dependent on other aspects of the oligonucleotide which remain unidentified. Therefore, it is possible that this complex was an artifact of our in vitro assay conditions.

The rat α- and β-MHC M-CAT sites appear to interact with the same MCBF isoform from the rat heart. Our probe containing the rat β-MHC M-CAT site from −269 to −285 (43) interacted with a factor that was indistinguishable from MCBF on the basis of mobility, DNA-binding specificity in EMSAs, and interaction with anti-TEF-1 serum (33). No other interaction with the β-MHC M-CAT site was observed. This M-CAT site is also conserved in the human β-MHC gene (43). A second rat β-MHC M-CAT site from −196 to 202 also binds TEF-1 (18). The other putative M-CAT site in the rat β-MHC gene, from −110 to −116, has not been examined.

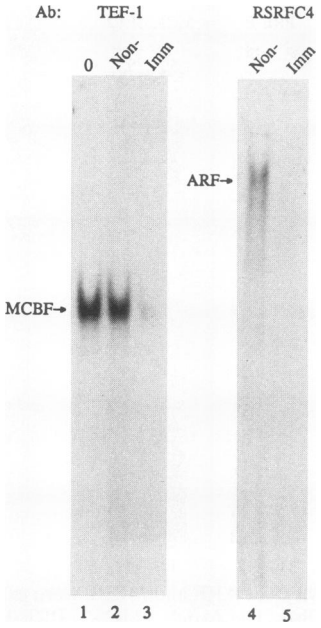

FIG. 5. MCBF and ARF are antigenically related to TEF-1 and RSRF, respectively. Neonatal rat heart nuclear extract was pretreated with either anti-TEF-1 or anti-RSRF serum (imm) or nonimmune serum (non) as described in Materials and Methods. Extracts pretreated with nonimmune or anti-TEF-1 serum were probed with the αMCAT oligonucleotide, while extracts pretreated with anti-RSRF serum were probed with the A-rich oligonucleotide in standard EMSA reactions. 0, no pretreatment. The positions of MCBF and ARF are indicated. Ab, antibody.

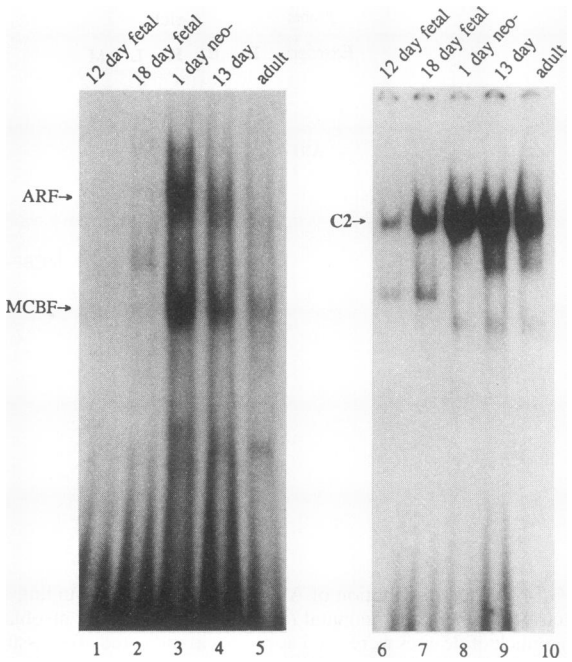

FIG. 6. ARF and MCBF DNA-binding activities are developmentally regulated in the rat heart. EMSAs were used to assay the DNA-binding activities of ARF, MCBF, and an E-box binding protein in cardiac muscle-derived extracts from rat hearts at various developmental stages. ARF and α-MCBF activities were probed with PRE-E. The E-box binding protein (C2) was probed with the EB3 oligonucleotide. The probes are described in Table 1. Equal amounts of protein were used in each lane. neo, neonatal.

Comparison of the DEPC footprint of the α-MHC M-CAT site with the methylation interference footprint of the rabbit β-MHC M-CAT site (38) indicates an analogous footprint pattern. The rat α- and β-MHC, rabbit β-MHC, and cTnT M-CAT sites are all capable of binding a ubiquitous MCBF. The M-CAT core motif that interacts with these sites is degenerate in the last position (5′-CATTCCT/A-3′). The region that footprints is from 1 bp 5′ to 2 bp 3′ to the core (Fig. 3) (38). We mutated each of the footprinted bases outside the core M-CAT site in the α-MHC gene in an unsuccessful attempt to find a combination which would allow the formation of muscle-specific M-CAT-dependent complexes (data not shown).

The A-rich site. The A-rich site located from −223 to −217 in the α-MHC gene was of interest because it interacted with a factor present in cardiac nuclear extracts and because this region had been reported to interact with cloned MEF-2 (46). An α-MHC enhancer/promoter with a mutated A-rich site was only 17% as active as the wild type (Fig. 1), implicating the A-rich region as a positive regulator of the α-MHC gene. The factor in cardiac nuclear extracts (ARF) that bound to this site was distinguishable from previously described MEF-2 activities by its DNA-binding properties (Fig. 2) and by its developmental expression in the C2C12 cell line (Fig. 4) (10). The DNA-binding site for ARF was 5′-TAAAAAAA-3′, and our results indicate that seven or more consecutive A residues are required for ARF binding. While the α-MHC A-rich site might bind cloned MEF-2a at high concentrations (46), we were unable to detect a MEF-2 interaction with this site even when C2C12 myotube extract was used as a protein source (Fig. 4). ARF was similar to MEF-2 in its mobility in nondenaturing gels (Fig. 2B), and both proteins interacted with antibodies to RSRFC4 (Fig. 4).

It is likely that ARF is a member of the RSRF/MEF-2 family of proteins that has a DNA-binding site that is different from those previously reported (36). Previous work has demonstrated that each of the four MEF-2 gene loci generate multiple isoforms by alternate splicing and that members of the MEF-2 family of proteins bind to DNA as homo- and heterodimers (28, 30, 36). Given the potential complexity of combinatorial associations of these various MEF-2 isoforms, it is possible that a specific heterodimer or set of heterodimers represents the ARF DNA-binding activity described here. This complexity may also make it difficult to identify the precise combination that is responsible for ARF activity. Alternatively, it is possible that ARF binding activity represents a unique gene product that contains a domain with epitopes in common with MEF-2 family member RSRFC4.

Expression of MCBF and ARF during cardiac development and hypertrophy. We have measured the DNA-binding activities of MCBF and ARF in the heart during development and hypertrophy. These results give an indication of the relative amount of protein, present in nuclear extracts, that is capable of interacting with a specific DNA-binding site. The levels of activity of both MCBF and ARF increased from very low or undetectable levels at fetal day 12 to peak levels 1 day postpartum. ARF DNA-binding activity was not detectable in the fetal heart, suggesting that it may not be an important regulator in early development. The presence of MCBF in the fetal rat heart suggests that it might play a role in cardiac gene expression in the developing heart. In support of this, the β-MHC gene is expressed at high levels in fetal heart muscle

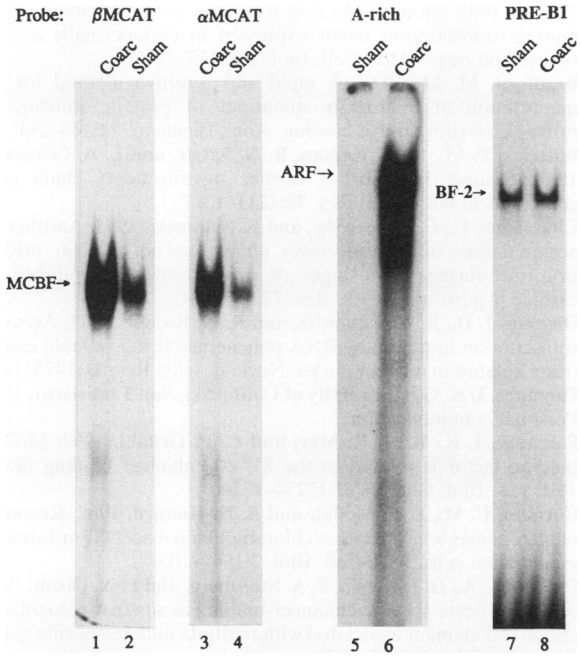

FIG. 7. Levels of α-MCBF and ARF DNA-binding activities are upregulated in the hypertrophied heart. EMSAs were used to compare the levels of MCBF and ARF DNA-binding activities in nuclear extracts from sham-operated control adult rat hearts (sham) and in adult hearts subjected to an acute pressure overload by coarctation of the abdominal aorta (coarc; see Materials and Methods). The probes used are described in Table 1, and the positions of the complexes are indicated. This is a representative experiment, and individual probes were assayed with identical extracts on the same day and run on the same gel. The extracts were also probed with PRE-B1 as a control.

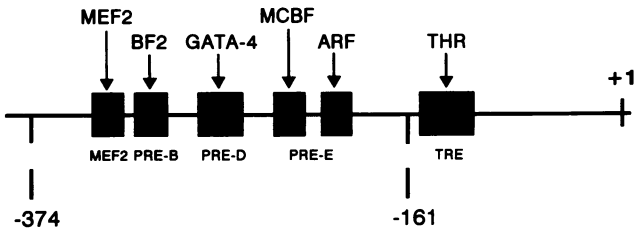

FIG. 8. A schematic representation of the regulatory sites in the proximal regulatory region of the α-myosin heavy-chain gene and the factors with which they interact. Numbering is relative to the transcription start site (+1).

cells (21) and two M-CAT sites have been detected in the β-MHC gene which footprint in vitro (43). An increase to peak levels of MCBF and ARF occurs just prior to the induction of expression of the α-MHC gene in the rat heart (21). Early in neonatal development, the levels of activity of both factors begin to decline, and this continues into adulthood. In the adult animal, ARF is detectable in EMSAs only when large amounts of extract are used and the gels are exposed to film for longer time periods. The loss of MCBF activity is not as dramatic compared with that of ARF, and there is a low but detectable level of MCBF in the adult rat heart. Western blot (immunoblot) analysis of some of our developmental and hypertrophy extracts with the TEF-1 antibody detected a single faint TEF-1-related band only in the neonatal rat heart extract (7).

The results with MCBF and ARF are similar to those previously reported for α-MHC BF-2 and MEF-2 DNA-binding activities in the heart (31, 32). Although the level of each of these factors is lower in the adult than in the neonate, our site-directed mutagenesis data indicate that these sites and the factors that interact with them regulate α-MHC gene expression in the adult heart. The level of these factor in the adult heart must be sufficient for α-MHC expression. The high levels of activity, present in neonatal hearts, suggest the possibility that increased levels of these factors are required to induce expression within the context of normal chromosomal structure. Once the proper conformation of the active gene has been established, lower levels of these factors may be sufficient to maintain expression. The constructs that we injected into the adult heart lacked chromosome-associated proteins and might not require high levels of factors to initiate transcription. In addition, MCBF, ARF, BF-2, and MEF-2 may control the expression of several genes that are active in the neonatal but not the adult heart, requiring high levels of expression at earlier developmental stages. However, not all DNA-binding activities that associate with the α-MHC promoter decrease, as evidenced by the C2 complex (Fig. 6).

In two previous reports, we have shown that the binding activity associated with the cTnT M-CAT site does not change during development (31, 32). This was done on the basis of experiments using the MCAT1 oligonucleotide (27), which, as discussed above, gave results that were different from those using the α-MHC MCAT site. When we repeated the experiment shown in Fig. 6 using the MCAT1 probe, there was no significant difference in the DNA-binding activity that interacted with MCAT1 between the neonate and adult heart extracts. However, the DNA-binding activity associated with the MCAT1 probe did not interact with polyclonal anti-TEF-1 serum, and on that basis it must be considered to be distinct from TEF-1. Therefore, the results presented here indicate that MCBF (TEF-1) DNA-binding activity in the heart is developmentally regulated.

MCBF and ARF DNA-binding activities also increase during cardiac hypertrophy at a time when gene expression in the heart is in a state of flux. This may reflect a requirement for these factors in the expression of several genes. Increased MCBF binding was observed when the α-MHC M-CAT site or the rat β-MHC upstream M-CAT site, previously designated βe2 (43), was used as probe (Fig. 7). Increased MCBF activity in adult hearts induced to hypertrophy by aortic coarctation is consistent with the proposed role of TEF-1 in mediating the increased expression of the β-MHC gene in cultured cardiomyocytes treated with α_1-adrenergic agonists (19). The increase in both MCBF and ARF activities associated with cardiac hypertrophy suggests that these factors play an important role in regulating the expression of genes during this adaptation. There are M-CAT sites in the promoter regions of several genes whose expression is induced during hypertrophy, such as the rat β-MHC gene from −269 to −285 and −110 −116 (43) and the rat α-skeletal actin promoter between the CArG and TATA boxes (47). There are ARF sites in the rat atrial natriuretic factor promoter from −589 to −583 and from −3517 to −3523 (37) that potentially regulate this gene. These genes are all induced in response to pressure overload.

Multiple elements regulate the α-MHC gene in the heart during development and during the onset of cardiac hypertrophy. The work reported here identifies two new elements within the proximal enhancer/promoter that participate in the regulation of the α-MHC gene. This adds to a growing list of densely packed elements and *trans*-acting factors that interact

to give tissue-restricted, developmentally regulated, and hypertrophy-sensitive expression of this gene (Fig. 8). The list of factors from 5′ to 3′ includes (i) MEF-2, (ii) the E-box binding factor BF-2, (iii) GATA-4, (iv) α-MCBF, (v) ARF, and (vi) thyroid hormone receptors. This list is not all inclusive, and several interesting elements remain to be characterized (33). The mechanism by which these factors interact remains to be defined. Promoter reconstitution experiments have thus far demonstrated that the enhancer activity of individual elements is low compared with that of combinations of several elements (33). However, results obtained with short promoter fragments must be interpreted with caution. Recent reports examining the role of MEF-2 in the regulation of the α-MHC gene in the adult rat heart have yielded contradictory results, and the difference appears to depend on the context in which the element is assayed (4, 32). These differences indicate that there are interactions between upstream and downstream regulatory elements.

The developmental expression of the α-MHC gene and its expression during cardiac hypertrophy are of major interest as a model for how the cardiomyocyte responds to its environment and to increased workload by altering its pattern of growth and gene expression. α-MHC mRNA is present in the embryonic cardiac tube but is lost in the ventricle as the chamber forms (22). The α-MHC gene is re-expressed in the neonatal heart and is maintained at high levels throughout the life of the rat (21). The peak in the DNA-binding activities of MEF-2, BF-2, α-MCAT, and ARF corresponds to the time at which the α-MHC gene is re-expressed, indicating a role for these factors in initiating this event (31, 32; see above). Lower levels are apparently sufficient for maintenance of expression in the adult heart.

The level of α-MHC mRNA decreases 3.5-fold during cardiac hypertrophy (5). It is not yet clear if this results from a repression of expression from the α-MHC gene or a decrease in mRNA stability. If gene expression is repressed, it occurs at a time at which the activities of at least two positive regulators of this gene are dramatically increased. These apparently conflicting events could be explained on the basis of the complexity of the regulation of the α-MHC gene as discussed above. Cardiac hypertrophy may cause a decrease in activity of a set of regulatory factors, resulting in repression of the α-MHC gene. However, the work presented here suggests that neither MCBF nor ARF is a member of that set. A second possibility is that critical interactions between factors are disrupted during the onset of cardiac hypertrophy. Identification and characterization of individual elements and how they interact will be critical for understanding the complex regulation of α-MHC gene expression.

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