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A Ubiquitous Factor (HF-1a) and a Distinct Muscle Factor (HF-1b/MEF-2) Form an E-Box-Independent Pathway for Cardiac Muscle Gene Expression

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Recent studies have identified a conserved 28-bp element (HF-1) within the rat cardiac MLC-2 gene which confers cardiac muscle-specific and inducible expression during myocardial cell hypertrophy. Utilizing a combination of independent experimental approaches, this study characterizes two cardiac nuclear factors which bind to HF-1, a ubiquitous factor (HF-1a), and an A+T-rich binding factor (HF-1b) which is preferentially expressed in differentiated cardiac and skeletal muscle cells. The HF-1a binding site is located in a core region of the 28-bp conserved element, immediately upstream from the A+T-rich HF-1b site, which is homologous to the MEF-2 site found in a number of muscle genes. By a number of separate criteria (gel mobility shift, competition, and mutagenesis studies), HF-1b and MEF-2 appear to be indistinguishable and thus are either identical or closely related muscle factors. Transient assays of luciferase reporter genes containing point mutations throughout the 28-bp HF-1 regulatory element document the importance of both the HF-1a and HF-1b sites in transient assays in ventricular muscle cells. In the native 250-bp MLC-2 promoter fragment, mutations in the single E box had little effect on cardiac muscle specificity, while point mutations in either the HF-1a or HF-1b binding site significantly reduced promoter activity, underscoring the importance of both the HF-1a and HF-1b sites in the transcriptional activation of this cardiac muscle gene. Thus, this study provides evidence that a novel, ubiquitous factor (HF-1a) and a muscle factor (HF-1b/MEF-2) can form a novel, E-box-independent pathway for muscle-specific expression in ventricular cardiac muscle cells.

The myocardium plays a central role in the maintenance of blood flow and oxygenation in avian and mammalian species. The normal development of the heart requires the stage-dependent activation of a panel of muscle-specific genes as well as a regionally restricted pattern of gene expression that accompanies the formation of distinct atrial and ventricular chambers within the myocardium. At present, relatively little is known regarding the regulatory networks which control the cardiac muscle gene program. In vertebrate species, cardiac and skeletal muscle are the major striated muscle subtypes, sharing several metabolic, mechanical, and electrophysiological properties that are due to the coexpression of a subset of muscle-specific genes. Recent studies in skeletal muscle have uncovered a host of helix-loop-helix myogenic determination genes (MyoD, myogenin, myf-5, and MRF-4/myf-6/herculin) (7, 8, 16, 21, 39, 43, 51, 57, 63) which can dominantly activate the skeletal muscle gene program in permissive cell types (62) by binding to E-box consensus sites (CANNTG) within muscle-specific genes (37, 46, 47). Although MyoD and the other myogenic determination genes are not expressed outside of skeletal muscle, their helix-loop-helix partner (E-12/E-47) and counterregulator (Id) are expressed in cardiac muscle (23, 48). In addition, recent studies have suggested an importance of E-box-dependent pathways in the expression of the cardiac α-actin gene in both skeletal and cardiac muscle cells (54, 55). These considerations have led to the concept that similar molecular paradigms, employing E-box-dependent pathways, might mediate cardiac and skeletal muscle-specific expression (50).

To elucidate the molecular pathways which control the cardiac gene program and to critically examine its relationship to the well-established E-box-dependent pathways for skeletal muscle-specific expression, our laboratory has used the rat cardiac myosin light chain-2 (MLC-2) gene as a model system (13, 30, 31, 38, 53). In both the rat and mouse, the cardiac MLC-2 gene encodes the regulatory, phosphorylatable myosin light chain that is a highly abundant mRNA in both cardiac and slow-twitch skeletal muscle (53). Transient assays in cultured myocardial cells (66) and parallel studies in transgenic mice (53) have identified a 250-bp fragment of the MLC-2 5′ flanking region which can confer cardiac muscle-specific expression. Within this MLC-2 promoter fragment lies a 28-bp conserved regulatory element (HF-1), which confers muscle specificity in transient assays in primary ventricular muscle cells (66). Although the HF-1 sequence contains homology to known regulatory elements, including CArG/serum response element (SRE) (44), AP-2 (33, 45), and MEF-2 (27), the precise cardiac muscle factors which bind to the HF-1 region and which govern muscle specificity in the ventricular cell context are unknown.

Using a combination of independent experimental approaches, this study characterizes two cardiac nuclear factors which bind to HF-1, a ubiquitous factor (HF-1a), and an A+T-rich binding factor (HF-1b) which is preferentially expressed in differentiated cardiac and skeletal muscle cells. The HF-1a binding site is located in a core region of the 28-bp conserved element, immediately upstream from the A+T-rich HF-1b site, which is homologous to the MEF-2 site found in a subset of muscle genes. By a number of

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separate criteria (gel mobility shift, competition, and mutagenesis studies), HF-1b and MEF-2 appear to be indistinguishable and thus are either identical or closely related muscle factors. Transient assays of luciferase reporter genes containing point mutations throughout the 28-bp HF-1 regulatory element document the importance of both the HF-1a and HF-1b sites in transient assays in ventricular muscle cells. In the native 250-bp MLC-2 promoter fragment, mutations in the single E box had little effect on cardiac muscle specificity, while point mutations in either the HF-1a or HF-1b binding site significantly reduced promoter activity, underscoring the importance of both the HF-1a and HF-1b sites in the transcriptional activation of this cardiac muscle gene. Thus, the current study provides evidence that a novel, ubiquitous factor (HF-1a) and a muscle factor (HF-1b) can form a novel, E-box-independent pathway for muscle-specific expression in ventricular cardiac muscle cells.

MATERIALS AND METHODS

Cell culture. Cultured neonatal rat myocardial cells were prepared as previously described (34, 58). Myocytes were dispersed from the ventricles of 1- to 2-day-old Sprague-Dawley rats by digestion with collagenase II (Worthington) and pancreatin (GIBCO) at 37°C, and the cell suspensions were purified on a discontinuous Percoll (Pharmacia LKB Biotechnology Inc.) gradient, resulting in >95% purity (myocytes/nonmyocytes), as assessed by immunocytofluorescence with MLC antisera (34). The myocytes were plated in 6-cm culture dishes (Falcon) at a density of 2.75×10^6 cells in 5 ml of 4:1 Dulbecco's modified Eagle medium (DMEM)-medium 199 (GIBCO) supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (ampicillin [34 μg/ml] and gentamicin [3 μg/ml]). Sol 8 myoblasts, derived from mouse soleus muscle, were grown in DMEM with 20% fetal calf serum and antibiotics (penicillin [200 U/ml] and streptomycin [200 μg/ml]) as previously described (66). Conversion of the myoblasts into myotubes was induced by changing confluent myoblast cultures to DMEM with 5% horse serum and antibiotics. HeLa, CV-1, and NRK-49F cell lines were grown in DMEM with 10% fetal calf serum and antibiotics, respectively.

Preparation of extracts. Total extracts of myocardial, CV-1, NRK-49F, and HeLa cells were prepared by lysis of 5×10^7 cells in 500 μl of EBC buffer (50 mM Tris-Cl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin, and 0.15 U of aprotinin per ml for 5 min at 4°C. The lysate was harvested by scraping and then centrifuged in a microfuge at maximum speed for 10 min. The supernatant was stored at −70°C until use. Nuclear extracts from Sol 8 myotubes were prepared by a modification of the method of Dignam et al. (18). The cultured cells (approximately 2×10^8 cells) were washed twice with cold phosphate-buffered saline (PBS) and harvested by scraping in cold PBS. The cells were centrifuged at 4,000 rpm for 10 min, and the cell pellet was resuspended in 5 ml of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT]) and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 4,000 rpm for 10 min, and the pellet was resuspended in 5 ml of buffer A and rehomogenized. The homogenate was centrifuged at 4,000 rpm for 10 min, and the nuclear pellet was resuspended in 3 ml of extraction buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.55 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 μg of antipain per ml, 1 μg of leupeptin per ml, 10 μg of benzamidine per ml, 1 μg of chymostatin per ml, 1 μg of pepstatin per ml, 0.15 U of aprotinin per ml, 0.5 mM PMSF, 0.5 mM DTT) and homogenized. The extract was centrifuged in a microfuge at maximal speed for 30 min, and the supernatant was dialyzed against 1 liter of dialysis buffer (40 mM KCl, 15 mM HEPES [pH 7.9], 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% glycerol) for 4 h. The dialysate was centrifuged in a microfuge for 10 min, and the supernatant was stored at −70°C prior to use. For the preparation of nuclear extracts from neonatal rat hearts, 100 ventricles from neonatal rats were removed, trisected, and washed three to four times with PBS. The heart tissues were homogenized in 10 ml of buffer A with a Dounce homogenizer. The homogenate was centrifuged at 6,000 rpm for 15 min, and the pellet was resuspended in 10 ml of buffer A and rehomogenized. The homogenate was centrifuged at 6,000 rpm for 15 min, and the nuclear pellet was resuspended in 5 ml of extraction buffer and homogenized. The subsequent steps followed the procedure for the preparation of nuclear extracts from Sol 8 myotubes as described above.

Plasmid constructs and transfection by calcium phosphate precipitation. Oligonucleotides were synthesized on a Gene Assembler DNA synthesizer (Pharmacia LKB Biotechnology Inc.) and gel purified. A single copy of the wild-type or mutated HF-1 element was inserted into *Hin*dIII-*Sst*I site of the thymidine kinase (TK)-luciferase vector PT109 (66). The mutant MLC-2–luciferase vectors were constructed by subcloning the *Ava*II-*Eco*RI fragment of the MLC-2 5′ flanking region into a pBST phagemid vector. The fragment was mutated by the Olsen-Eckstein method of site-directed mutagenesis (49), using an Amersham in vitro mutagenesis kit. MLC-2 promoters bearing specific point mutations were recovered as *Eco*RI-*Hin*dIII fragments and blunt ended by filling the recess termini with Klenow enzyme. The expression vector pSVOALΔ5′ (17) was restricted with *Hin*dIII, blunt ended with Klenow enzyme as described above, and ligated to the mutated *Eco*RI-*Hin*dIII fragment. The orientation of each promoter was verified by double-stranded DNA sequencing. For transient assays, transfection of the vectors was performed by a modification of the calcium phosphate precipitation method as previously described (11). Each 60-mm culture dish of myocardial cells was transfected with 10 μg of a luciferase reporter vector and 2 μg of a cytomegalovirus–β-galactosidase expression vector (12). The luciferase and β-galactosidase assays were performed by a previously described method (17, 52).

Gel mobility shift assay. For analytical gel mobility shift assays, 10 μg of nuclear extract or 35 μg of total cellular extract was preincubated with 4 μl of 5× binding buffer (200 mM KCl, 75 mM HEPES [pH 7.9], 5 mM EDTA, 2.5 mM DTT, 25 mM $MgCl_2$, 25% glycerol) and 4 μg of poly(dI-dC) in a final volume of 18 μl for 20 min at room temperature. The DNA fragment was end labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP, 10,000 to 20,000 cpm of the labeled probe (2 μl) was added to the assay mixture, and the mixture was incubated at room temperature for an additional 20 min. The reaction mixture was separated by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 13 V/cm per gel at room temperature. For the competition studies, a 300-fold molar excess of unlabeled competitor DNA fragment was added during preincubation of the reaction mixture. For the gel mobility shift assays with serum response factor (SRF) antibodies, 3 μl of the affinity-purified antibody (a generous gift of Michael Greenberg's laboratory) was used in each reaction. The specificity of the

antibody was tested in competition assays utilizing an equal volume of antibody and nonspecific competitor (TrpE fragment) or specific competitor (TrpE-SRF fusion protein).

Methylation interference assay. Methylation interference studies were performed according to the procedure described by Sturm et al. (59). A synthetic oligonucleotide of the sense or antisense strand of the HF-1 element (GCCAA AAGTGGTCATGGGGTTATTTTTA) was end labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP and reannealed to a 10-fold molar excess of the unlabeled complementary oligonucleotide. The labeled fragment was purified through Sephadex G-50 and was treated with dimethyl sulfate. The methylated probe was used for the preparative gel mobility shift DNA binding assays. The DNA binding reaction was scaled up fivefold by using 10^5 cpm of probe and 50 μg of nuclear extract from neonatal rat hearts in a total volume of 50 μl. The reaction mixture was then separated by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE as described above. The free and bound probes from four reaction mixtures were excised from the gel and electroeluted. The eluted DNAs were extracted with an equal volume of phenol-chloroform and precipitated with ethanol. They were then cleaved in 100 μl of 1 M piperidine at 90°C for 30 min, and the piperidine was removed by repeated addition of water and lyophilization three times. The bound and free DNA fragments were then separated by electrophoresis and flanked by respective Maxam-Gilbert G and A+G sequencing reaction on a 12% sequencing gel.

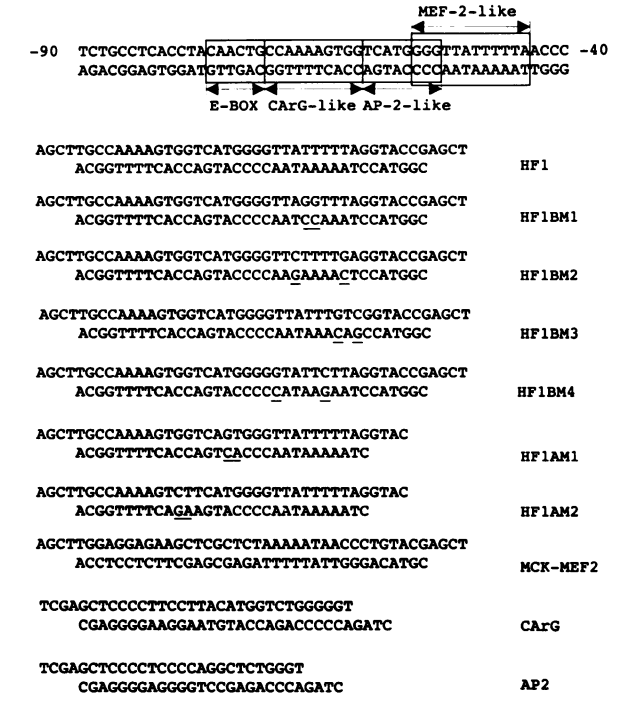

FIG. 1. Putative regulatory elements within a proximal region of the rat cardiac MLC-2 gene and competitor DNA sequences used in gel mobility shift assays. Putative *cis* elements within the proximal promoter region of the cardiac MLC-2 gene (−40 to −90) are delineated by the squares and arrows. The underlines indicate the nucleotides that are mutated in duplex oligonucleotides containing the HF-1 sequence. A MEF-2 motif [CTAAAAATAACCCT], CArG motif [CCTTACATGG] (29), and AP-2 motif [CCCCAGGC] are located within muscle creatine kinase (MCK)–MEF-2, CArG, and AP-2 competitor sequences, respectively.

RESULTS

Identification of two cardiac myocyte factors, distinct from SRF or AP-2, that bind to the conserved HF-1 element in the rat cardiac MLC-2 gene. Previous studies have led to the identification of a conserved 28-bp element (HF-1) in the rat MLC-2 5′ flanking region which is sufficient to confer both cardiac muscle-specific and inducible expression to a neutral promoter in transient assays of myocardial cells (66). The HF-1 element contains sequences which are homologous to *cis* regulatory elements that regulate skeletal muscle specificity or inducibility following agonist stimulation (Fig. 1 shows a diagram of putative regulatory elements within HF-1). A consensus SRE/CArG box, which is required for serum inducibility of the c-*fos* gene (60) and the skeletal muscle-specific expression of the cardiac α-actin gene (44), is found at the 5′ boundary of the conserved HF-1 sequence. Within the core of the HF-1 element lies an AP-2-like motif (33, 45) which mediates inducible expression following activation of protein kinase A or protein kinase C (33). At the 3′ boundary of the HF-1 sequence lies an A+T-rich region, which is homologous to the MEF-2 site in the creatine kinase enhancer (27).

To characterize cardiac muscle factors which bind to HF-1, gel mobility shift assays were carried out, using cardiac extracts and a radiolabeled HF-1 fragment of the MLC-2 promoter as a probe. Two specific DNA protein complexes of different mobilities (designated HF-1a and HF-1b) were detected in nuclear extracts derived from intact heart (data not shown) or cardiomyocyte extracts (Fig. 2). Formation of both the HF-1a and HF-1b complexes were effectively inhibited by incubation with an excess of unlabeled HF-1 fragment, documenting the specificity of binding. Bands of faster mobility than HF-1a or HF-1b were infrequently observed but were of much lower abundance and were not effectively inhibited by the addition of the HF-1 competitor fragment, indicating the nonspecific nature of these complexes. The addition of an excess of unlabeled fragments containing a CArG box from the human cardiac α-actin promoter (44) or a consensus AP-2 *cis* regulatory element (33, 45) did not effectively abolish out either the HF-1a or HF-1b complexes (Fig. 2). Gel mobility shift assays using a radiolabeled CArG probe derived from the human cardiac α-actin promoter documented that cardiac muscle cells contain SRF/CArG-binding factors. Preincubation with SRF antibodies retarded the mobility of the factor binding to the radiolabeled CArG probe (SRF) but had no effect on the HF-1a and HF-1b complexes observed with the radiolabeled HF-1 probe. The specificity of the antibody effect was documented by the addition of a TrpE-SRF fusion protein, which eliminated the supershifting effect of the antibody. Addition of the TrpE protein alone did not effectively inhibit the effect of the SRF antibody, providing further evidence of specificity (Fig. 2). Thus, while cardiac muscle cells have CArG binding activity, and HF-1 contains a consensus SRE/CArG box, the two factors which bind to HF-1 appear to be distinct from SRF. Similarly, gel mobility shift competition studies with a consensus AP-2 site indicate that AP-2 cannot account for the HF-1a or HF-1b binding activities in cardiac muscle cells (Fig. 2).

The A+T-rich region of HF-1 binds a cardiac muscle factor (HF-1b) similar to the skeletal muscle factor, MEF-2. To determine whether either the HF-1a or HF-1b factor recognized the A+T-rich region of HF-1, gel mobility shift studies were performed with a radiolabeled HF-1 probe and a

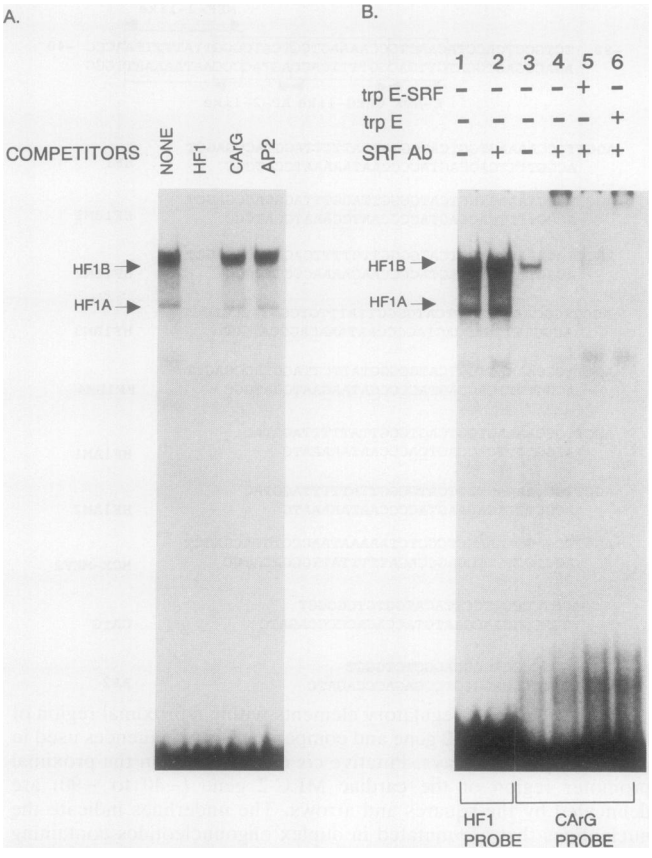

FIG. 2. Identification of two myocardial cell factors that interact with the HF-1 element. (A) Gel mobility shift assays using cardiac myocyte extracts, a radiolabeled HF-1 probe, and CArG and AP-2 competitor sequences. The HF-1 probe was end labeled with ^{32}P and analyzed by gel mobility shift assays in the absence or presence of a 300-fold molar excess of each indicated unlabeled competitor DNA. (B) Effects of the addition of an immunopurified SRF antibody. Gel mobility shift assays were performed with an end-labeled HF-1 probe (indicated at the bottom) in the absence or presence of an SRF antibody (lanes 1 and 2). The presence of a CArG-binding factor in myocardial cell extracts was confirmed by using a radiolabeled CArG probe (29) (indicated at the bottom) in the absence or presence of an SRF antibody (lanes 3 and 4). The specificity of the antibody effect was confirmed by the addition of an excess of TrpE or TrpE-SRF fusion protein to compete for binding to the SRF antibody (lanes 5 and 6).

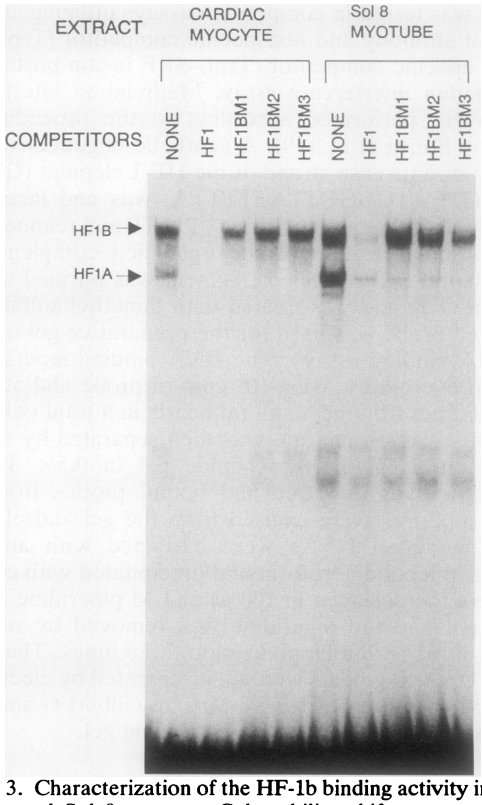

FIG. 3. Characterization of the HF-1b binding activity in cardiac myocyte and Sol 8 extracts. Gel mobility shift assays were performed with a radiolabeled HF-1 probe in the absence or presence of a 300-fold molar excess of either wild-type and mutant HF-1 competitor DNAs, myocardial cell extracts, or nuclear extracts of Sol 8 myotubes, as indicated. The sequences of the mutant DNA competitors are displayed in Fig. 1.

competitor duplex oligonucleotide containing the A+T-rich site. The addition of an excess of the nonradiolabeled A+T-rich duplex oligonucleotides successfully inhibited the formation of the slower-migrating complex, HF-1b, but had no effect on the faster-migrating complex, HF-1a, thereby indicating that the HF-1b factor was binding to the A+T-rich region of HF-1 (data not shown). To establish the boundaries of the HF-1b binding site, a series of duplex oligonucleotides containing the 28-bp HF-1 element with point mutations in the A+T-rich sequence was used in gel mobility shift competition studies (see Fig. 1 for the precise sequences of the mutant competitor oligonucleotides). As displayed in Fig. 3 and 4, the four oligonucleotides containing mutations in the A+T-rich region of HF-1 failed to compete effectively for binding to the HF-1b factor while retaining their ability to compete for binding to the HF-1a factor. Gel mobility shift assays with a radiolabeled HF-1 probe and Sol 8 myotube extracts revealed two specific DNA-protein complexes identical in mobility to the HF-1a and HF-1b complexes found in studies with cardiac muscle cell extracts (Fig. 3). As observed in studies using cardiac muscle cell extracts, the HF-1b binding activity in Sol 8 skeletal myotubes did not effectively bind to the HF-1 oligonucleotide competitors which contained mutations in the A+T-rich region. On the other hand, these mutant HF-1 competitor DNAs were effective competitors for the HF-1a binding activity in Sol 8 myotubes, consistent with the prior results with cardiac cell extracts. Thus, the HF-1b binding activity in cardiac and Sol 8 skeletal muscle myotubes appears to be indistinguishable on the basis of both mobility and studies using mutant A+T competitor binding sites. These studies indicate that terminally differentiated cardiac muscle cells and Sol 8 muscle myotubes express similar HF-1 binding factors.

Interestingly, the mutations which abolished the binding of the HF-1b factor conform to previously established contact points for the skeletal muscle factor, MEF-2, which binds to the A+T-rich sequences in the creatine kinase enhancer (27). Previous studies have documented that MEF-2 is upregulated during the conversion of C2 myoblasts to myotubes. To directly compare the HF-1b binding activity in cardiac cells with the well-characterized MEF-2 factor in C2 skeletal muscle cells, a series of gel mobility shift assays was carried out, using C2 myoblast and myotube extracts and a radiolabeled probe containing the MEF-2 site in the mouse muscle creatine kinase enhancer. In gel mobility shift

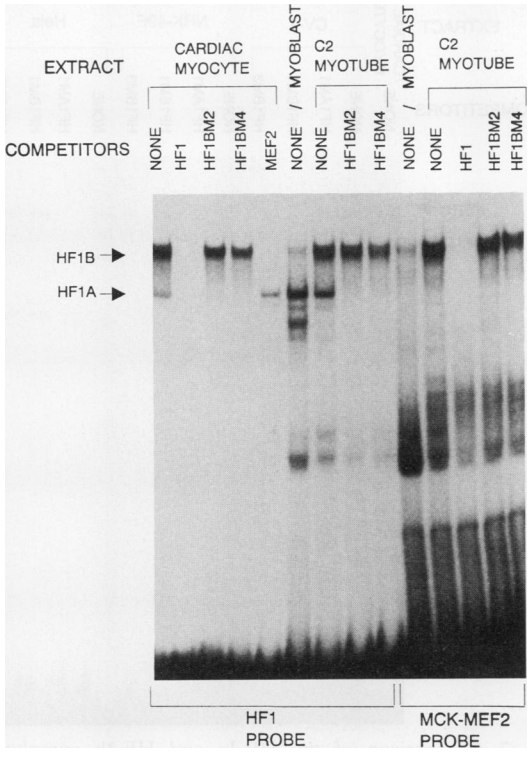

FIG. 4. Comparison of HF-1b and MEF-2 complexes in myocardial cells and C2 muscle cells. HF-1b and MEF-2 probes (indicated at the bottom) were end labeled and used in gel mobility shift assays in the absence or presence of a 300-fold molar excess of each unlabeled competitor DNA, using extracts from myocardial cells, nuclear extracts from C2 myoblasts, or C2 myotubes, as indicated. Equivalent amounts of C2 myoblasts and C2 myotubes extract were used.

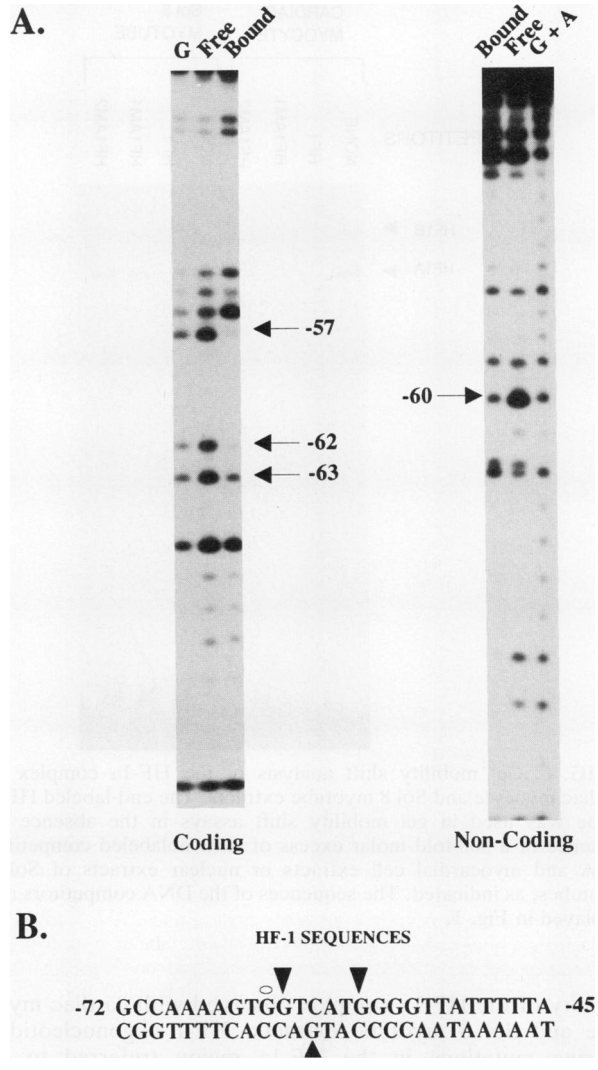

FIG. 5. Methylation interference analysis of the HF-1a complex. (A) Methylation interference analysis of both coding and noncoding strands that form the HF-1a complex. The arrows indicate the positions of the nucleotides which are less methylated following binding to the factor. (B) Summary of methylation interference data. ▼ and ○ denote strong and weak interference, respectively.

assays using a radiolabeled HF-1 probe and either cardiac muscle extracts or C2 myotube extracts, addition of an excess of nonradiolabeled MEF-2 binding sites effectively inhibited the formation of the HF-1b complex but had no effect on the HF-1a complex (Fig. 4). Similarly, duplex oligonucleotides containing mutations in the A+T-rich region of HF-1 were ineffective competitors for binding to the HF-1b factors in both the myocardial cell extracts and C2 myotube extracts (Fig. 4). In agreement with previous studies by Gossett et al. (27), the binding activity to a radiolabeled MEF-2 probe was significantly upregulated during the conversion of C2 myoblasts to myotubes. Similarly, the HF-1b binding activity to a radiolabeled HF-1 probe was significantly upregulated during the conversion of C2 myoblasts to myotubes, consistent with previous studies of MEF-2 (27), and the relative mobilities of the HF-1b and MEF-2 complexes were identical in studies using DNA probes of similar length (Fig. 4). In contrast, the binding activity to the HF-1a site was slightly greater with C2 myoblasts than with C2 myotube extracts, documenting specificity for the increase in the HF-1b factor during the conversion of myoblasts to myotubes. The low level of HF-1b/MEF2 activity in myoblasts may also reflect contamination with differentiated myotubes or may indicate the presence of ubiquitous A+T-rich binding factors that have been noted by a number of investigators. Taken together, these studies indicate that the HF-1b factor and MEF-2 in cardiac and skeletal muscle cells are indistinguishable by several independent criteria, including relative mobility, competition studies, upregulation during conversion of myoblasts to myotubes, and effects of point mutations in the A+T-rich DNA binding sites.

Characterization of the HF-1a complex. To characterize the HF-1a binding factors in cardiac muscle cells, a methylation interference assay was employed with an HF-1 probe which was partially modified at the guanine residues by dimethyl sulfate. Since the HF-1a factor was more abundant with whole heart extracts than with cardiac myocyte extracts (data not shown), extracts of intact neonatal hearts were used to isolate the HF-1a binding complex by preparative gel mobility shift studies. As shown in Fig. 5, methylation of guanines at positions −57 and −62 on the sense strand and at −60 on the antisense strand interfered markedly with the binding activity to the HF-1a factor, whereas methylation of guanine at position −63 on the sense strand had a relatively weak effect on the binding activity of HF-1a. To confirm the

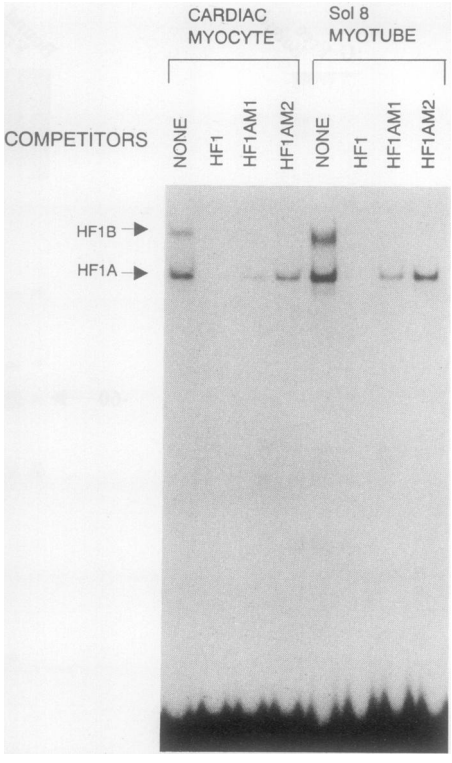

FIG. 6. Gel mobility shift analysis of the HF-1a complex in cardiac myocyte and Sol 8 myotube extracts. The end-labeled HF-1 probe was used in gel mobility shift assays in the absence or presence of a 300-fold molar excess of each unlabeled competitor DNA and myocardial cell extracts or nuclear extracts of Sol 8 myotubes, as indicated. The sequences of the DNA competitors are displayed in Fig. 1.

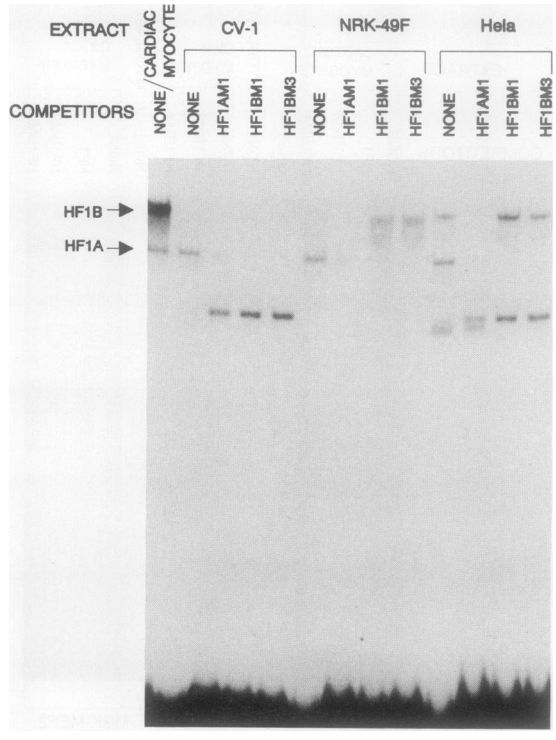

FIG. 7. Comparison of the HF-1a and HF-1b complexes in cardiac muscle cells and nonmuscle cell lines. The HF-1 probe was end labeled, and gel mobility shift assays were performed with equal amounts of extracts from cardiac myocytes, CV-1 cells, NRK-49F cells, and HeLa cells, as indicated. The identities of HF-1a and HF-1b complexes in nonmuscle cell lines were confirmed by incubation of the extracts in the presence of a 300-fold molar excess of each unlabeled HF-1 mutant DNA. The new bands visualized in the competition experiment are not consistently observed in all experiments and appear to be nonspecific.

identity of the HF-1a complex observed with cardiac myocyte and Sol 8 myotube extracts, HF-1 oligonucleotides bearing mutations in the HF-1a region (referred to as HF1AM1 and HF1AM2; see Fig. 1 for precise locations of mutations) were synthesized and used in subsequent competition experiments. In gel mobility shift assays, the addition of an excess of nonradiolabeled HF-1 sequences which contain mutations in the HF-1a site did not effectively inhibit the formation of the HF-1a complex. The HF-1b binding activity was effectively abolished by the addition of an excess of the HF-1a mutant oligonucleotides (Fig. 6), documenting specificity of the competition studies with the HF-1a mutant oligonucleotides. Thus, the faster component of the HF-1 binding activity (HF-1a) in cardiac muscle cells can be accounted for by a novel factor that binds to a GGTCATG sequence at the core of the HF-1 element, which is immediately adjacent to the A+T-rich HF-1b site.

Distribution and relative abundance of HF-1a and HF-1b binding activities in nonmuscle cells. To determine whether the HF-1a and HF-1b binding factors were specific for muscle cells, gel mobility shift assays were performed with extracts from a variety of nonmuscle cell lines, including HeLa, CV-1, and NRK-49F fibroblasts. To confirm the identity of the complexes in nonmuscle cells, various mutants of HF-1 were used as competitor DNAs. To allow an estimate of the relative abundance of the factors which bind to HF-1a and HF-1b, equivalent amounts of protein were used in the various extracts. As shown in Fig. 7, similar levels of HF-1a binding activity were observed in extracts from all of the cell lines examined. With all extracts examined, the mobility of the HF-1a complex was identical, and the mutant HF-1a oligonucleotides were ineffective competitors, similar to results obtained with cardiac muscle cell extracts. On the other hand, the HF-1b binding activity was barely detectable in a nonmuscle cell line (CV-1) and was expressed at relatively low levels in HeLa and NRK-49F fibroblasts, similar to the levels observed in undifferentiated myoblasts (>10:1, cardiac or Sol 8 myotubes:myoblasts or HeLa). These results suggest that HF-1a is a widely expressed (perhaps ubiquitous) factor, while HF-1b is preferentially expressed in differentiated cardiac and skeletal muscle cells.

HF-1a and HF-1b are both required for maximal HF-1 activity in transient assays in cultured myocardial cells. To directly assess the role of the HF-1a and HF-1b binding factors in the regulated expression of the MLC-2 gene in cardiac cells, a number of HF-1 elements which contained mutations in the HF-1a or HF-1b site were subsequently fused to a minimal TK-luciferase vector and assayed in transient expression studies with primary neonatal rat ventricular myocardial cells (Fig. 8). As previously described, a single copy of the 28-bp element HF-1 can confer cardiac muscle-specific expression to this neutral promoter. As displayed in Fig. 8, mutations within HF-1a or HF-1b significantly reduced expression of the luciferase reporter to 35 to 45% of the wild-type activity. Each of these mutations

	HF1 SEQUENCES	LUCIFERASE ACTIVITY	GEL SHIFT HF1A	GEL SHIFT HF1B
PT109-HF1	GCCAAAAGTGGTCATGGGGTTATTTTTA	100	+	+
HF1AM1	GCCAAAAGTGGTCAGTGGGTTATTTTTA	37±4.0	–	+
HF1AM2	GCCAAAAGTCTTCATGGGGTTATTTTTA	49±5.4	–	+
HF1BM1	GCCAAAAGTGGTCATGGGGTTAGGTTTA	39±1.3	+	–
HF1BM2	GCCAAAAGTGGTCATGGGGTTCTTTTGA	33±3.8	+	–
HF1BM3	GCCAAAAGTGGTCATGGGGTTATTTGTC	44±6.0	+	–
PT109		27±2.3	–	–

FIG. 8. Effects of mutations in the HF-1a and HF-1b regions on the expression of a TK-luciferase reporter gene. Synthetic duplex oligonucleotides containing wild-type or mutant HF-1 sequences were inserted into the TK-luciferase vector PT109. The sequences of the wild-type promoter HF-1 and its mutants are displayed (underlined nucleotides indicate mutations). The activities of the mutants were compared with that of the wild type in transient expression assays in neonatal rat myocardial cells. The efficiency of transfection was controlled by cotransfection with a cytomegalovirus–β-galactosidase vector. The luciferase activity of the mutants was normalized to the wild-type activity, which was assigned a value of 100%. Results represent the means ± standard errors of the means of three experiments with three separate plasmid preparations. The effects of the various mutations on HF-1a and HF-1b binding activities (as assessed by gel mobility shift assays) are summarized at the right.

had a significant inhibitory effect on the binding of either the HF-1a or HF-1b factor, as indicated in the aforementioned studies. These studies indicate that HF-1a and HF-1b are both required to maintain the maximal activity of HF-1 in conferring cardiac muscle specificity and that a single copy of either site is insufficient to confer specificity. Since a single copy of either the HF-1a or HF-1b site had a negligible effect on cardiac muscle-specific expression of the TK-luciferase construct, it is apparent that both the HF-1a and HF-1b sites are required for maximal expression in cardiac muscle cells.

Relative importance of the HF-1a/HF-1b and E-box sites within the native 250-bp MLC-2 promoter fragment. Previous studies have documented that the 250-bp fragment of the MLC-2 promoter region is sufficient to confer both cardiac muscle specificity and inducible in transient assays and cultured myocardial cells, as well as in vivo studies in transgenic mice which harbor the MLC-2 luciferase fusion genes (53). To evaluate the relative importance of the single E-box site versus the HF-1a/HF-1b sites within this 250-bp MLC-2 promoter fragment, mutant MLC-2 promoters containing point mutations which abolish binding to each one of these sites were generated. Subsequently, the mutant MLC-2 fragments were placed upstream of the luciferase reporter gene in the construct PSVOALΔ5′. As displayed in Fig. 9, mutations in the single E-box site of the 250-bp MLC-2 promoter fragment had little effect on transcriptional activity in the myocardial cell context. However, mutations in the HF-1a or HF-1b site markedly crippled promoter activity in transient assays in cultured ventricular myocardial cells (5- to 20-fold decrease in activity). These studies document that the 250-bp MLC-2 promoter fragment confers cardiac muscle specificity through an E-box-independent pathway. Furthermore, it is apparent that both the HF-1a and HF-1b sites are absolutely required for maximal MLC-2 promoter activity in the cardiac cell context. These studies indicate that a ubiquitously expressed factor (HF-1a) and a muscle factor (HF-1b/MEF-2) forms an E-box-independent pathway to achieve cardiac muscle-specific expression.

DISCUSSION

A distinct muscle factor (HF-1b/MEF-2) mediates cardiac muscle-specific expression. Cardiac and skeletal muscle are the major striated muscle types in avian and mammalian species. In skeletal muscle, a great deal of progress has been made in the isolation and characterization of myogenic determination genes (7, 8, 16, 21, 39, 51, 57, 62, 63), the identification of muscle-specific regulatory elements (4, 6, 9,

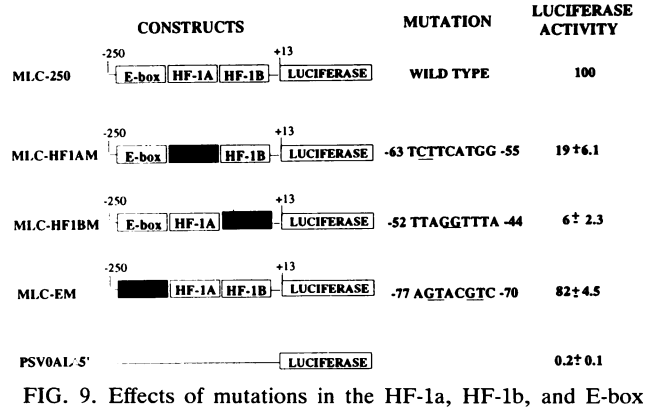

FIG. 9. Effects of mutations in the HF-1a, HF-1b, and E-box regions on the expression of the 250-bp MLC-2–luciferase reporter gene in cardiac muscle cells. The 250-bp MLC-2–luciferase promoter containing wild-type sequence or its mutants were inserted upstream of the luciferase reporter gene vector, PSVOALΔ5′. The mutations introduced to the wild-type promoter are indicated by the underlines, and the corresponding *cis* elements are indicated by black boxes. Activities of the mutants were compared with that of the wild type in transient expression assays in neonatal rat myocardial cells. The efficiency of transfection was controlled by cotransfection with a cytomegalovirus-β-galactosidase vector. Luciferase activities of the mutants were normalized to the wild-type activity, which was assigned a value of 100%. Result represent the means ± standard errors of the means of three experiments with three separate plasmid preparations.

19, 27, 41, 44), the characterization of their corresponding *trans*-acting factors (9, 27, 36, 42), and the elucidation of the potential role of several of these *cis* elements and *trans*-acting factors in skeletal myogenesis during embryonic development (56). In contrast, relative little is known concerning the program which controls cardiac muscle genes during the normal growth and development of the heart. Further understanding of the molecular mechanisms which regulate muscle genes during cardiac growth and development will be required to unravel the mechanisms responsible for the diversity of congenital heart defects which are found in human biology and medicine (22), as well as to interpret the increasing number of studies using transgenic models of abnormal cardiovascular growth and development (2, 14, 24, 25, 28, 35, 64).

Utilizing a combination of experimental approaches, this study identifies two cardiac nuclear factors (HF-1a and HF-1b) which bind to adjacent sequences within HF-1 to confer cardiac muscle-specific expression of the MLC-2 gene. Although the 28-bp HF-1 element contains a consensus CArG box (44) and an AP-2 like motif (33, 45) in its core region, neither CArG-binding factor/SRF nor AP-2 can account for the HF-1 binding activity in cardiac nuclear extracts, as assessed by gel mobility shift assays with competitor DNAs and antibodies directed against SRF. However, the A+T-rich sequence within HF-1, which is homologous to the MEF-2 binding site in the creatine kinase enhancer (27), binds a factor that is similar to MEF-2 in skeletal muscle cells by a number of criteria, including gel mobility shift assays with competitor duplex oligonucleotides, point mutational analyses throughout the A+T-rich region, upregulation during the conversion of skeletal muscle myoblasts to myotubes, and comparison with gel mobility shift complexes obtained with skeletal extracts from C2 myotubes and a fragment of the creatine kinase enhancer containing the authentic MEF-2 site (27). Although these studies indicate that MEF-2 and HF-1b are indistinguishable by currently available assays, a precise determination of whether HF-1b in cardiac muscle cells and MEF-2 in skeletal muscle cells are identical proteins will await molecular cloning of these respective A+T-rich binding factors from cardiac and skeletal muscle tissues.

Recent studies of other cardiac genes suggest a particular importance of A+T-rich binding sites, such as MEF-2, in the control of cardiac muscle specificity. Transient assays of creatine kinase enhancer (TK-chloramphenicol acetyltransferase [CAT]) constructs have demonstrated that the MEF-2 site is required for maximal transcriptional activity in cultured myocardial cells (20, 22a). Similarly, an A+T-rich binding site in the 5′ flanking region of the chick cardiac troponin T gene is required to maintain cardiac muscle-specific expression in chicken embryonic cells (32). Gel mobility shift assays using this A+T-rich element as a competitor suggest that the HF-1b site and the cardiac muscle-specific element in the troponin T gene are bound by similar or closely related factors (65a). Thus, there is a growing body of evidence that the A+T-rich binding sites, like HF-1b/MEF-2, may be an important component of the regulatory pathway that confers cardiac muscle specificity. However, it should be noted that studies of all three genes (MLC-2, troponin T, and creatine kinase) have suggested that the A+T-rich site alone is insufficient to confer cardiac muscle specificity. In the case of the troponin T gene, a skeletal muscle regulatory element (M-CAT) is also required for muscle-specific expression in cardiac cells (40). Furthermore, a single copy of the HF-1b site is unable to confer the degree of cardiac muscle specificity observed with the intact 28-bp HF-1 element. Thus, it is clear that MEF-2, while being a critical component of the regulatory program for cardiac muscle gene expression, must interact with other factors to confer muscle-specific expression.

A ubiquitous factor (HF-1a) and a muscle factor (HF-1b/MEF-2) are both required for cardiac muscle-specific expression. The inability of HF-1b sites alone to confer cardiac muscle specificity implies the presence of other critical regulatory sites within the HF-1 element. In this regard, gel mobility shift studies clearly indicate the presence of another cardiac muscle factor which can bind to the HF-1 conserved element. By methylation interference, the binding site of this factor (HF-1a) is located within the core of the HF-1 sequence (GGTCATG), which lies immediately adjacent to HF-1b. Interestingly, the HF-1b site corresponds to a consensus half site for the retinoic acid receptor response element (26), which usually consists of two palindromic (GGTCA-TGACC) repeats found in many retinoic acid-responsive genes, including the rat growth hormone gene (26). However, by a number of criteria, including competition studies with bona fide retinoic acid response elements, gel mobility shift studies with in vitro-translated α-retinoic acid receptors, and supershift studies using antibodies directed against retinoic acid receptors, the HF-1a factor appears to be distinct from known retinoic acid receptors (67). In addition, retinoic acid does not appear to influence the expression of the HF-1 TK-luciferase construct in cultured ventricular muscle cell studies (65a). Thus, HF-1a, while binding to sequences which are part of a consensus retinoic acid response element, appears to be a novel transcriptional factor.

The tissue distribution of HF-1a binding activity indicates that it is not muscle specific but rather is ubiquitously expressed at relatively uniform levels in all cell types examined (CV-1, HeLa, etc.). Despite its widespread expression, binding to the HF-1a site is required to maintain the ability of HF-1 to confer cardiac muscle specificity. The HF-1a and HF-1b sites are immediately adjacent to one another and do not display apparent overlap. Their close proximity and functional synergism imply that protein-protein interaction between these factors, as well as protein-DNA interactions, might be important for their functional effect. However, the binding of HF-1a and HF-1b to their respective target sequences within HF-1 does not appear to require protein-protein interactions between the factors, since point mutations in either the HF-1a or HF-1b site do not prevent the binding of its respective partner.

There is ample precedent for the interaction between ubiquitous and tissue-restricted transcriptional factors to control differentiated cell phenotypes. The Pit-1 POU domain factor can synergistically interact with Oct-1 to control the expression of pituitary specific genes, such as growth hormone (61). Likewise, the hepatocyte-specific factor HNF-1 can interact with Oct-1 to regulate the activity of the hepatitis B viral promoter (65). While the molecular natures of the HF-1a and HF-1b binding factors are unknown, it will become of future interest to determine the nature of their interaction. The widespread expression of HF-1a raises the possibility that it might be involved in conferring tissue specificity in other cell types through interactions with other tissue-specific factors that are analogous to HF-1b/MEF-2 binding factors in muscle cells.

E-box-independent pathway for cardiac muscle gene expression. The recent identification of a host of myogenic determination genes, including MyoD, myf-5, myogenin, and

myf-6/MRF-4/herculin (7, 8, 16, 21, 39, 43, 51, 57, 62, 63), has led to a wealth of information on the mechanisms which dictate the muscle-specific expression of contractile protein and other muscle genes during skeletal myogenesis. The molecular paradigms which control muscle specificity require the formation of a heterodimer, consisting of a muscle-specific myogenic determination factor and a ubiquitous helix-loop-helix partner E-12/E-47 (46, 47). These heterodimers subsequently bind to CANNTG sequences (E-box sites) found in many muscle-specific genes (5, 36, 37, 46, 47), thereby activating their expression. Similar paradigms which require E-box sites have been found to be important in the regulated expression of the chymotrypsin gene in pancreatic exocrine cells (48) and immunoglobulin genes (10, 47). The ubiquitous expression of E-12/E-47 (48), as well its negatively regulatory counterpart, Id (3, 23), has given rise to the concept that E-box-dependent pathways may represent a paradigm for the control of tissue-specific genes in a wide variety of cells, including those outside of the muscle cell lineage (50, 62). Although MyoD and the other myogenic determination genes are not expressed in cardiac muscle (56), E/12-E-47 (48) and Id (23) are found in cardiac muscle cells, raising the possibility that a cardiac muscle factor, analogous to MyoD, may act to orchestrate cardiac muscle gene expression. Further support for this notion has been provided by Bader and coworkers, who have found E-box-binding factors in chick cardiac progenitor cells (1). Recently, Sartorelli et al. have provided evidence that an E-box site in a proximal segment of the cardiac α-actin promoter is required for expression in both cardiac (54) and skeletal (55) muscle. Thus, circumstantial evidence suggest that E-box-dependent pathways might be important in cardiac, as well as skeletal, muscle specificity.

The results of the current study provide evidence that cardiac muscle specificity can be mediated through E-box-independent pathways. The 250-bp MLC-2 promoter fragment, which confers cardiac muscle- and ventricular cell-specific expression in transgenic mice (53), contains a single CANNTG site. However, site-directed mutation of this E-box site has little effect on MLC-2 promoter activity in the cardiac cell context, as assessed by transient assays in cultured ventricular cells. On the other hand, point mutations in either the HF-1a or HF-1b site have a profound effect on MLC-2 promoter activity, reducing activity to 5 to 20% of wild-type levels. Thus, in the MLC-2 gene, the HF-1 site appears to represent a new paradigm to achieve muscle-specific expression through an E-box-independent pathway (15). Future studies will be directed toward characterizing the molecular nature of the HF-1a and HF-1b binding factors to allow a precise determination of the mechanisms by which these factors can specify the ventricular cell phenotype.

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