The Basic Helix-Loop-Helix Protein Upstream Stimulating Factor Regulates the Cardiac Ventricular Myosin Light-Chain 2 Gene via Independent *cis* Regulatory Elements

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Previous studies have documented that 250 bp of the rat cardiac ventricular myosin light-chain 2 (MLC-2v) promoter is sufficient to confer cardiac muscle-specific expression on a luciferase reporter gene in both transgenic mice and primary cultured neonatal rat myocardial cells. Utilizing ligation-mediated PCR to perform *in vivo* dimethyl sulfate footprinting, the present study has identified protein-DNA interaction within the position from -176 to -165. This region, identified as MLE1, contains a core sequence, CACGTG, which conforms to the consensus E-box site and is identical to the upstream stimulating factor (USF)-binding site of the adenovirus major late promoter. Transient assays of luciferase reporter genes containing point mutations of the site demonstrate the importance of this *cis* regulatory element in the transcriptional activation of this cardiac muscle gene in ventricular muscle cells. The protein complex that occupies this site is capable of binding to HF-1a and PRE B sites which are known to be required for cardiac muscle-specific expression of rat MLC-2v and α -myosin heavy-chain genes, respectively. This study provides direct evidence that USF, a member of the basic helix-loop-helix leucine zipper family, binds to MLE1, HF-1a, and PRE B sites and suggests that it is a component of protein complexes that may coordinately control the expression of MLC-2v and α -myosin heavy-chain genes. The current study also provides evidence that USF can positively and negatively regulate the MLC-2v gene via independent *cis* regulatory elements.

The myocardium plays a central role in the maintenance of blood flow during vertebrate growth and development. The normal development of the heart requires the formation of distinct atrial and ventricular chambers, which allow the myocardium to handle variations in volume, pressure, and blood flow between the various regions of the heart. Presumably, the distinct properties of various cardiac muscle cell lineages are due, in large part, to the selective activation of various subsets of cardiac muscle genes during the course of cardiogenesis. At the present time, relatively little is known concerning the positional and/or molecular cues which guide mesodermal progenitor cells into the cardiac muscle lineage and ultimately the pathways which lead to the regional specification of cardiac muscle cells.

Since a number of muscle genes are coexpressed in both cardiac and skeletal muscle, the view that similar mechanisms would be operative to maintain muscle specificity in these distinct striated muscle subtypes has been proposed. Given the central role of a family of basic helix-loop-helix (HLH) myogenic determination genes (the *MyoD*, *myf-5*, myogenin, and *myf-6/MRF4/herculin* genes) in skeletal myogenesis, a number of laboratories have examined the potential role of basic HLH proteins in the control of cardiac myogenesis. Cardiac muscle

cells express the ubiquitous basic HLH protein E12/E47 and are permissive for the actions of MyoD (21, 33). Furthermore, a number of cardiac promoters have been documented to acquire an intact E-box site to maintain optimal activity in transient assays in cultured cardiac muscle cells (35). The known myogenic determination genes are not expressed in the heart, and knockouts of these HLH genes have not resulted in any gross defects in cardiogenesis (49). As a result, several laboratories have focused on isolating new members of the MyoD family which might display restricted expression to cardiac muscle lineages. In this regard, a number of new basic HLH proteins which are expressed early during cardiac development have been isolated (9, 30). However, a direct role for these or any other member of the basic HLH protein family in the regulation of the cardiac muscle genes has yet to be demonstrated. Although early skeletal muscle-fibroblast cell fusion studies predicted the presence of molecules which would dominantly transmit the myogenic phenotype, analogous studies employing heterokaryons between cardiac myocytes and fibroblasts have recently revealed a lack of dominance of the cardiac muscle phenotype (20). Accordingly, the precise role of basic HLH proteins in the regulation of the cardiac muscle gene program is currently unclear.

Recent studies have established the rat cardiac ventricular myosin light-chain 2 (MLC-2v) gene as a model system for the identification of the molecular signaling pathways which control the muscle gene program during cardiac growth and development. A 250-bp MLC-2v promoter fragment can confer ventricle-specific expression in the embryonic myocardium and can restrict expression of a *lacZ* reporter gene to the

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ventricular segment of the looped heart tube at day 8.5 of murine embryogenesis (48). Within this MLC-2 promoter fragment, three conserved regulatory elements (HF-1a, HF-1b/MEF-2, and HF-3) are required for the maintenance of the ventricular muscle specificity in transient assays and in transgenic mice (28, 37).

A mutation in a consensus E-box site, which lies just upstream of HF-1, is without significant effect in transient assays, although a mild negative regulatory effect was observed in transgenic animals which harbor this mutant MLC-2v promoter construct (28). In transient assays, the MLC-2v promoter is not downregulated by cotransfection with an *Id* expression vector that negatively regulates the activity of E-box-dependent promoters in the cardiac and skeletal muscles (21). Taken together, there is increasing evidence that cardiac muscle specificity for the MLC-2v promoter does not rely on a single site and requires combinatorial interactions between a variety of regulatory elements located within the 250-bp MLC-2v promoter fragment. Although clearly required for the maintenance of cardiac muscle specificity, the HF-1b/MEF-2 site alone does not appear to be sufficient to confer cardiac muscle specificity, underscoring the importance of identifying other potential regulatory sites in the MLC-2v promoter that may confer specificity (37).

Accordingly, the present study was designed to examine the potential regulatory role of other *cis* regulatory elements which might lie within the 250-bp MLC-2v promoter. The experimental approach involved *in vivo* footprinting to identify sites outside of HF-1a, HF-1b/MEF-2, or HF-3 which were occupied by cardiac nuclear factors and to subsequently utilize gel shift and transient assays to characterize the factor(s) and its potential importance in the regulation of the MLC-2v gene.

By employing ligation-mediated PCR (LMPCR) to perform *in vivo* dimethyl sulfate footprinting, a novel regulatory element, referred to as MLE1, was identified between -176 and -165 in the MLC-2v promoter. This site contains a consensus upstream stimulating factor (USF)-binding site (CACGTG) and is required for the maximal activity of the MLC-2v promoter in transient expression assays in primary cultured neonatal cardiac myocytes. The nuclear protein complex that binds to this site appeared to be ubiquitous, as assessed by gel mobility shift assays with extracts from various noncardiac cell types. USF antibodies supershifted the endogenous MLE1-binding activity in cardiac nuclear extracts, and *in vitro*-translated USF protein binds to the MLE site in a sequence-specific manner. Interestingly, USF also appears to bind to the HF-1a site in the MLC-2v promoter and the PRE B site in the α -myosin heavy-chain (α -MHC) promoter, which are required for the maintenance of muscle specificity in transient assays in ventricular cardiac muscle cells (35, 37). Cotransfection studies with an MLC-2v reporter construct containing point mutations in either the MLE1 or HF-1a site indicate that USF can positively and negatively regulate the MLC-2v promoter activity through these independent sites. Accordingly, the present study has identified the transcriptional factor USF as a nuclear factor that binds to MLE1, HF-1a, and PRE B sites in cardiac muscle genes and provides evidence that USF may play a role in the regulation of the MLC-2v gene.

MATERIALS AND METHODS

Cell culture and DNA transfections. Cultured neonatal rat myocardial cells were prepared as previously described (51). RMo myoblasts (32) (derived from a rat embryonic muscle) and NRK-49F (normal rat kidney fibroblast) were grown in Dulbecco's modified Eagle's medium (DMEM) with fetal calf

serum (20 and 10%, respectively) and antibiotics (200 U of penicillin per ml and 200 μ g of streptomycin per ml). For transient assays, myocardial cells were transfected by using a modification of the calcium phosphate precipitation method, as previously described (13). Each 60-mm-diameter culture dish of 1.5×10^6 myocardial cells was transfected with 4 μ g of a luciferase reporter plasmid, 8 μ g of expression plasmid (with or without USF cDNA), and 1 μ g of cytomegalovirus β -galactosidase plasmid (14). The luciferase and β -galactosidase assays were performed by using a previously described method (6, 47).

***In vivo* footprinting.** *In vivo* footprinting was performed by using the modified method of Mueller and Wold (25, 35a, 55). *In vivo* methylation of cultured myocardial cells with dimethyl sulfate was performed for 2 min, and the genomic DNA isolation and the guanine- or adenine-specific cleavage of methylated DNA were performed essentially as described by Strauss et al. (55). LMPCR was done with the following set of primers: for the coding strand, (i) TCTTCCCTGGGGTTA AAAATA, (ii) AATAACCCCATGACCACCTTTTGGCAG, and (iii) GGCAGTTGTAGGTGAGGCAGAGGCC; for the noncoding strand, (i) AATGGCAGGACCCAGAGCACA, (ii) CAGAGCATCGTTCCCAGGCCAG, and (iii) CCAGGCCCACTGTCTCTTTAACC.

Preparation of nuclear extracts. For preparation of nuclear extracts, 90×10^6 cells were washed twice with ice-cold phosphate-buffered saline and resuspended in 0.4 ml of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9], 10 mM KCl, 0.1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) on ice for 15 min. Nonidet P-40 was added to a final concentration of 0.25% and mixed briefly. The nuclei were pelleted in a microcentrifuge for 4 min at 4°C and resuspended in 50 μ l of extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 2 μ g of leupeptin per ml, 2 μ g of pepstatin per ml, and 2 μ g of aprotinin per ml) on ice for 15 min with occasional mixing. The nuclear extract was centrifuged for 5 min at 4°C, and the supernatant was stored at -70°C. The full-length Max protein was purified as previously described (27).

Gel mobility shift assay. The gel mobility shift assays were performed as previously described (37). The following oligonucleotides were used as probes and competitors, as indicated in the figures (only the sense strand sequences are shown here): AAGGCATTTTTGGGTCTCACGTGTCCACCCAG GCG (MLE1), AAGGCATTTTTGGGTCTCACTTTTCCAC CCAGGCG (MLE1mt), AAGGCATTTTTGGGTCTAACC TTTCCACCCAGGCG (MLE1mt1), AAGGCATTTTTGGG TCTCAATTGTCCACCCAGGCG (MLE1mt2), TTCGAAG TGGTCATGGGGTTACGTG (HF1A), TTCGAAGTGGT AATGGGTTACGTG (HF1Amt), ATCCGCAGGGCCAT GTGGGTAGCGG (PRE B), ATCCGCAGGGCAATCTGG GTAGCGG (PRE Bmt), CGGTGTAGGCCACGTGACCG GGTG (AdML), and AGCTTGGGGCACGTGCCCCAAG CA (PMB). For the competition studies, a 300-fold molar excess of unlabeled competitor was used, unless otherwise specified in the figure legends. For the gel mobility shift assays with antibodies, 0.5 μ l of USF (53), 1 μ l of a 1:5 dilution of USF-2 (53), and 1 μ l of Myc (19) antibodies were used.

***In vitro* translation.** Plasmid d12 containing the full-length cDNA encoding the 43-kDa human USF (22) was translated *in vitro* by using a TNT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase.

Plasmid constructions. The expression plasmid pBJUSF1 was constructed by subcloning full-length cDNA encoding human USF from plasmid d12 into an *Eco*RI site of mamma-

FIG. 1. In vivo identification of DNA-protein interaction by guanine or adenine LMPCR in vivo footprinting. ○, positions of nucleotides which are methylation protected following binding to the nuclear factors; ●, hypersensitive site. Lanes: N, in vitro-methylated protein-free cardiac myocyte DNA; C, in vivo-methylated cardiac myocyte DNA.

lian expression vector pBJ1, a derivative of plasmid pcD-SRα (56). The expression of USF was directed by the simian virus 40 early promoter and the R-U5' segment of the long terminal repeat of human T-cell leukemia virus type I. The mutant MLC-2v-luciferase vectors were constructed by subcloning the *Ava*II-*Eco*RI fragment containing the MLC-2v 5' flanking region into a pSELECT-1 phagemid vector. The fragment was mutated by using an altered-site in vitro mutagenesis system (Promega). The MLE1 site CACGTG was converted to CAATTG, and HF-1a site (37) GGTCATGGG was mutated to GAGATCTGG. The mutated fragment was then subcloned into the *Xho*I-*Sma*I site of luciferase reporter gene vector pXP1 (39). The mutation and orientation of each promoter were verified by double-stranded DNA sequencing.

RESULTS

Identification and characterization of the *cis* regulatory element in the rat cardiac MLC-2v gene. Previous studies have documented that the 250-bp fragment of the MLC-2 promoter region is sufficient to confer both cardiac muscle-specific and inducible expression in cultured myocardial cells and in transgenic mice (23, 29). Within this relatively short MLC-2v promoter fragment, a number of conserved *cis* regulatory elements that act positively and negatively to regulate the ventricular chamber-restricted expression of a luciferase reporter gene in transgenic mice have been identified. In separate studies, the cardiac factors which occupy these sites have also been determined. To identify the locations of other potential DNA-protein interactions within this promoter region in vivo, LMPCR was utilized to perform in vivo dimethyl sulfate footprinting. As shown in Fig. 1, in vivo footprinting reveals protein occupancy of the CACGTG motif (referred to hereafter as MLE1 at positions -173 to -168) in cultured myocardial cells. The protection of guanine residues was shown at positions -170 and -168 on the coding strand and at positions -173 and -171 on the noncoding strand. The hypersensitivity of the guanine residue was observed at position -157 on the noncoding strand. Interestingly, the CACGTG motif is identical to the core sequence of the major late transcription factor (or USF)-binding site in the major late promoter of adenovirus (10, 50) and conforms to the consensus E-box site CANNTG. On the basis of the contact points

TABLE 1. Effect of mutations in MLE1 site on expression of 250-bp MLC-2v luciferase reporter gene in cardiac muscle cells^a

Promoter	MLE1 sequence ^b	Luciferase activity (%)
PXP-WT	TCTCACGTGTCC	100
PXP-MLE1mt1	TCTA <u>AC</u> GTGTCC	56 ± 5.2
PXP-MLE1mt2	TCTCA <u>AT</u> GTGTCC	31 ± 1.4
PXP1		0.1 ± 1.0

^a The 250-bp MLC-2v luciferase promoter (PXP) containing the wild type (WT) or MLE1 mutant was transfected into neonatal rat myocardial cells in transient expression assays. The efficiency of transfection was controlled by cotransfection with a cytomegalovirus β-galactosidase vector, and the luciferase activity was normalized to the β-galactosidase activity. The activities of the mutants were compared with the wild-type activity, which was assigned a value of 100%. Results are the means ± standard errors of the means for three experiments with three separate plasmid preparations.

^b The MLE1 sequences from positions -176 to -165 are shown; nucleotides different from those in the wild-type sequence are underlined.

identified in the in vivo footprinting studies, mutations in the MLE1 site were introduced in the context of the 250-bp fragment of the MLC-2v promoter. Subsequently, this construct was assayed in transient expression studies with primary neonatal rat ventricular myocardial cells. As shown in Table 1, mutations in the MLE1 site in the MLC-2v-luciferase fusion genes significantly inhibited transcriptional activity by two- to threefold in cultured cells. Accordingly, the present studies indicated that the MLE1 site is required to maintain the maximum activity of the 250-bp MLC-2v promoter and that the corresponding cardiac factor which occupies this site is likely to be a positive regulator of MLC-2v promoter activity in cardiac cells.

Characterization of nuclear factors that bind to MLE1. To characterize the cardiac nuclear factors which bind to MLE1, gel mobility shift assays were performed with myocardial nuclear extracts and a radiolabeled MLE1 double-strand oligonucleotide of the MLC-2v promoter as a probe. A major DNA-protein complex which was effectively inhibited by an excess of unlabeled MLE1 fragment was identified (Fig. 2). Formation of the complex was not effectively inhibited by incubation with competitor duplex oligonucleotides bearing mutations in the MLE1 site which were designed on the basis of the DNA-protein contact points elucidated in the in vivo footprinting (Fig. 2). Since the MLE1 site had a core sequence which was also present in the binding site of the USF (AdML) and in the recognition site of Myc-Max heterodimers (PMB) (3, 4, 43), competition experiments with both oligonucleotides were performed. As shown in Fig. 2A, both competitor duplex oligonucleotides AdML and PMB effectively abolished the MLE1-binding activity. To determine whether the MLE1-binding factor was specific for cardiac muscle cells, gel mobility shift assays were carried out with nuclear extracts of noncardiac muscle cell lines. With all nonmuscle cell extracts examined, including RMo myoblasts and NRK-49F fibroblasts, the mobility of the MLE1 complex was identical. Similar to the results obtained with myocardial cells, the duplex oligonucleotide containing a mutation of the MLE1 site did not effectively compete for binding to the MLE1 factor (Fig. 2).

Interestingly, previous studies have reported that USF, or the major late transcription factor, can bind to a CCCCCGT GACC motif in the rat γ-fibrinogen promoter (16), which is almost identical to the opposite orientation of the HF-1a site (CCCCATGACC) in the rat MLC-2v promoter (37). Gel shift and methylation interference analyses have suggested that similar cardiac nuclear factors may bind to the HF-1a and PRE

FIG. 2. Characterization of nuclear factors that interact with MLE1 element. (A) Gel mobility shift assays employing cardiac myocyte nuclear extract. The MLE1 probe was end labeled with ^{32}P and analyzed by gel mobility shift assays in the presence or absence of a 300-fold molar excess of each indicated unlabeled competitor DNA. (B) Comparison of MLE1 complexes in cardiac and noncardiac muscle cells. Arrows indicate specific DNA-protein complexes.

B sites of the rat MLC-2v and α -MHC promoters, respectively; the latter site contains a consensus E-box motif (37). To determine whether HF-1a- and PRE B-binding factors were related to the MLE1 complex, competition studies were performed with radiolabeled HF-1a and PRE B probes. The formation of the more slowly migrating HF-1a and PRE B complexes was effectively inhibited by the addition of unlabeled probe, AdML, or MLE1 competitor oligonucleotides, and the oligonucleotides which contained mutations in the HF-1a, PRE B, or MLE1 sites were ineffective competitors (Fig. 3). Infrequently, a band with faster mobility that was observed with some nuclear extract preparations was more prominent with the PRE B probe than with the HF-1a probe and was not effectively inhibited by incubation with an excess of unlabeled AdML or MLE1 duplex oligonucleotide (Fig. 3). To compare the affinities of the nuclear factor for the MLE1 and HF-1a sites of the MLC-2v promoter, competition studies employing gel mobility shift assays were performed. The DNA-protein complex formed with a labeled MLE1 probe was effectively inhibited by the addition of a 200-fold molar excess of unlabeled MLE1 oligonucleotide, whereas a remnant of the

FIG. 3. Gel mobility analysis of the HF-1a and PRE B complexes in cardiac myocytes nuclear extract. The end-labeled HF-1a probe and PRE B probe were utilized in gel mobility shift assays with myocardial cell nuclear extracts in the presence or absence of a 300-fold molar excess of each indicated unlabeled competitor DNA. Arrows indicate specific DNA-protein complexes.

complex with labeled MLE1 still remained after incubation with a 400-fold excess of the HF-1a competitor oligonucleotide (Fig. 4). This result suggested that the binding affinity of the cardiac nuclear factors for the MLE1 was severalfold higher than that for the HF-1a site.

USF binds to MLE1 and HF-1a elements in the rat MLC-2v promoter and the PRE B element in the rat α -MHC gene. The ability of the USF-binding site AdML to abolish the DNA-protein complex formation with the labeled oligonucleotides

FIG. 4. Comparison of affinities of MLE1 and HF-1a for the ability to interact with nuclear factors in cardiac myocytes. Gel mobility shift assays were performed with a radiolabeled MLE1 probe in the absence of competitor DNA (lane 0) or presence of molar excess (fold) of MLE1 or HF-1a competitor DNA, respectively, indicated above each lane. The arrow indicates the specific DNA-protein complex.

FIG. 5. Analysis of in vitro-translated USF-binding activity to MLE1. The MLE1 probe was end labeled and analyzed by gel mobility shift assays in the absence (lanes labeled none) or presence of each indicated unlabeled competitor DNA, using cardiac (c.) myocyte nuclear extract, rabbit reticulocyte lysate (control), or in vitro-translated full-length USF. The arrow indicates the specific DNA-protein complex.

corresponding to the MLE1, HF-1a, or PRE B site suggested that USF can interact with MLE1, HF-1a, and PRE B *cis* regulatory elements of the rat MLC-2v and α -MHC promoters. To directly demonstrate that USF can bind to these sites, a full-length cDNA encoding human USF was transcribed in vitro with T7 RNA polymerase and translated in vitro in a rabbit reticulocyte lysate system. As shown in Fig. 5 and 6, the in vitro-translated USF can interact specifically with radiolabeled MLE1, HF-1a, and PRE B probes, while incubation with the reticulocyte lysate control did not result in the formation of a site-specific complex. The addition of an excess of the nonradiolabeled duplex oligonucleotide AdML, MLE1, HF-1a, or PRE B inhibited the binding of in vitro-translated USF to the MLE1, HF-1a, or PRE B probe, documenting specificity of the complex formation. The oligonucleotides bearing mutations in the MLE1, HF-1a, or PRE B site did not compete for the specific interaction of USF with any of these probes. The specific DNA-protein complexes with in vitro-translated USF were similar in mobility to the complexes found in studies with cardiac muscle cell extracts. To determine whether USF is a component of MLE1, HF-1a, and PRE B complexes in cardiac myocyte nuclear extracts, gel mobility shift assays were performed with antibodies directed against USF. Preincubation with USF antibodies supershifted the mobility of MLE1, HF-1a, PRE B, and AdML complexes but had no effect on the complex of another basic HLH leucine zipper, Max, and the PMB probe. In addition, nonimmune serum did not affect the mobility of the MLE1 complex, therefore documenting the specificity of the results with the USF antibodies. Although Myc-Max heterodimers have also been shown to recognize the same core sequence of MLE1 and AdML, Myc antibodies did not retard the mobility or inhibit the formation of the MLE1 complexes (Fig. 7). Taken together, these studies indicate that USF is the predominant endogenous cardiac factor which occupies the MLE1 *cis* regulatory element in cardiac extracts and that it can also bind to the HF-1a element in a sequence-specific manner, albeit with a lower affinity. Interestingly, the antibody specific for USF2 can also supershift the mobility of

FIG. 6. Analysis of in vitro-translated USF-binding activity to HF-1a and PRE B. The HF-1a probe and PRE B probe were end labeled and analyzed by gel mobility shift assays in the absence (lanes labeled none) or presence of a 300 fold-molar excess of each unlabeled competitor DNA, using cardiac (c.) myocyte nuclear extract, rabbit reticulocyte lysate (control), or in vitro-translated full-length USF. The arrow indicates the specific DNA-protein complex.

MLE1, HF-1a, and PRE B complexes in cardiac myocyte nuclear extracts (Fig. 8). Since USF2 can form a heterodimer with USF, this study suggests that heterodimers of USF and USF2 can occupy those sites.

Positive and negative regulation of MLC-2v promoter activity by USF. The finding of two discrete USF-binding sites raises the question of whether USF would act through these sites to regulate the expression of the MLC-2v gene in cardiac cells. To determine whether USF can regulate the activity of the MLC-2v promoter in cardiac myocytes, the full-length USF cDNA under the control of the R-U5 segment human T-cell leukemia virus long terminal repeat and simian virus 40 early promoter (56) was cotransfected into myocardial cells with a wild-type, MLE1 mutant, or HF-1a mutant MLC-2v 250-bp promoter. Overexpression of USF did not result in the increased expression of the wild-type MLC-2v 250-bp luciferase reporter gene. However, mutations in the HF-1a site resulted in a 2.5- to 4.7-fold induction of the luciferase reporter gene in cotransfection studies with the USF expression vector, whereas the expression of the MLC luciferase reporter gene containing a mutation in the MLE1 site was repressed 1.9- to 5.5-fold by overexpression of USF. These results suggested the possibility that USF positively regulates MLC-2v promoter activity through the MLE1 site and can act as negative regulator via the HF-1a site. In this manner, the dual positive and negative regulatory effects of USF overexpression would obscure any net effect on MLC-2 promoter activity in studies with the wild-type promoter. As displayed in Fig. 9, cotransfection of the USF expression construct with the MLC-2 luciferase reporter containing a double mutation in both the HF-1a and MLE1 sites or a Rous sarcoma virus luciferase reporter

FIG. 7. Characterization of MLE1, HF-1a, and PRE B complexes in cardiac myocyte nuclear extract with rabbit antiserum against USF. The gel mobility shift assays were performed with radiolabeled AdML, MLE1, HF-1a, or PRE B probes (indicated at the bottom) in the presence (+) or absence (–) of antiserum against USF, nonimmune serum, or monoclonal antibody against Myc.

displayed little effect on luciferase activity. Since USF binds to the MLE1 site with higher affinity than to the HF-1a site, one might expect that low levels of the USF expression plasmid would activate the expression of wild-type MLC-2v promoters. However, a titration with lower amounts of the USF expression plasmid and a fixed level of the wild-type MLC-2v reporter plasmid did not result in the increased expression of the wild-type MLC-2v 250-bp luciferase reporter gene (data not shown). Thus, USF apparently is capable of transactivating MLC-2v expression through the MLE1 site and repressing MLC-2v promoter activity via the HF-1a site, but the mechanisms by which it may contribute to cardiac muscle specificity of the MLC-2v promoter may require modification through heterodimerization or phosphorylation or may require concomitant upregulation of other transcription factors.

DISCUSSION

USF and positive regulation of the MLC-2v gene via the MLE1 site. Cardiac and skeletal muscles are the major striated muscle subtypes in vertebrate species and coexpress a number of muscle-specific genes. Utilizing a variety of experimental approaches, studies of skeletal muscle have identified a number of muscle-specific regulatory elements (1, 26, 34, 41, 54) and transcriptional factors which control and confer muscle specificity. Although studies of the myogenic determination genes (7, 8, 17, 18, 45, 57), including the *MyoD*, *myf-5*, myogenin, and *myf-6/MRF4/herculin* genes, have led to a great deal of information on the molecular paradigms which control muscle specificity during skeletal myogenesis, relatively little is known regarding the pathways which dictate cardiac muscle-specific expression and normal development of the heart. Further understanding of the molecular mechanisms which

FIG. 8. Analysis of MLE1, HF-1a, and PRE B complexes in cardiac myocyte nuclear extract with rabbit antiserum against USF-2. The MLE1, HF-1a, and PRE B complexes in myocardial cell nuclear extract were analyzed by gel mobility shift assays using end-labeled MLE1, HF-1a, and PRE B probes (indicated at the bottom) in the presence (+) or absence (–) of rabbit antiserum against USF-2 or nonimmune serum.

regulate cardiac muscle genes will require characterization of the regulatory elements and corresponding *trans*-acting factors which are responsible for the control of the cardiac muscle gene program.

In this regard, the MLC-2v gene has served as a model system for identifying the pathways which lead to cardiac myogenesis and the restricted expression of cardiac muscle

FIG. 9. Effect of overexpression of USF on expression of 250-bp MLC-2v luciferase reporter gene in cardiac muscle cells. The 250-bp MLC-2v luciferase promoter containing the wild-type (W.T.) or mutant sequence was cotransfected with the USF expression construct or expression vector (pBJ1). The efficiency of transfection was controlled by cotransfection with a cytomegalovirus β -galactosidase construct, and the luciferase activity was normalized to the β -galactosidase activity. The results are representative of three experiments with three separate plasmid preparations. RSVluc, Rous sarcoma virus luciferase.

genes to particular chambers of the heart during cardiogenesis (15, 28, 29). The MLC-2v gene is the regulatory light chain that is expressed as an abundant contractile protein in the ventricular chamber of the heart, as well as in slow-twitch skeletal muscles (29). Within the heart, MLC-2v expression becomes restricted to the ventricular segment of the primitive linear heart tube, suggesting that positional specification of MLC-2v expression is a relatively early event during patterning of the mammalian heart tube (40). In independent lines of transgenic mice, a 250-bp MLC-2v promoter can confer ventricular specificity to the expression of a luciferase reporter gene (29), and the maintenance of specificity appears to be dependent on two adjacent positive regulatory elements (HF-1a and HF-1b/MEF-2), as well as a novel negative regulatory element (HF-3) that acts to suppress expression in other muscle subtypes (i.e., skeletal and smooth muscles) (28). Of these cardiac muscle regulatory elements, the HF-1b/MEF-2 site clearly play an important role in cardiac specificity. A number of cardiac and skeletal muscle promoters require an intact MEF-2 site for the maintenance of muscle specificity, and cotransfection studies have documented their ability to *trans* activate muscle promoters. The MEF-2A and MEF-2C proteins are members of the RSRF protein family and are expressed early during the course of murine cardiogenesis. However, MEF-2 sites are relatively weak enhancers, and recent studies have suggested an interaction with additional ubiquitously expressed transcriptional factors to exert their effects on the muscle gene program (31, 59).

In this regard, the current study has characterized a *cis* regulatory element, MLE1, and the corresponding cardiac nuclear factors which bind to this sequence and which also regulate the activity of the 250-bp MLC-2v promoter. By *in vivo* footprinting, the protein-DNA interaction has been shown to occur at a consensus (CACGTG) E-box sequence of MLE1 located at -173 to -168. In the context of the MLC-2v promoter, there appears to be a specific requirement for the CG nucleotides between the CA and TG nucleotides. Point mutations in those nucleotides which maintain the consensus CANNTG E-box site result nevertheless in a decrease in MLC-2v promoter activity and eliminate the binding of cardiac nuclear factors. The consensus E-box is a core motif for many sequences that bind to transcriptional factors of the basic HLH-leucine zipper family (2, 3, 5, 53) and basic HLH family (24, 36, 38), which include the *MyoD* family of myogenic determination genes. The results of the current study show that USF, a member of the basic HLH leucine zipper protein family, binds to the MLE1 site in the MLC-2v promoter. Cotransfection studies with an MLC-2v promoter construct that eliminates the negative regulatory HF-1a site suggest that USF can positively regulate MLC-2v promoter activity in cardiac cells and thus may be important in the regulated expression of the MLC-2v gene. This effect requires an intact MLE-1 site, as an additional mutation in the MLE1 site eliminates the *trans* activation by USF overexpression.

Although the USF-binding site was originally identified as a promoter element from the major late promoter of adenovirus 2, several promoters and enhancers of cellular genes have been reported to have USF-binding sites that are required for efficient expression *in vitro* and *in vivo*. USF has been shown to bind to the consensus E-box sequences and activate the expression of class I alcohol dehydrogenase (42), type I plasminogen activator inhibitor (46), immunoglobulin λ 2-chain promoter (12), and p53 tumor suppressor (44) genes. In addition, USF has also been shown to bind to and stimulate the transcription of γ -fibrinogen (16) and metallothionein I (11) through similar, but not consensus E-box, sequences. By gel

mobility shift assay and methylation interference, USF was shown to interact with GACCCCGTGACC of the γ -fibrinogen promoter and GGGCGCGTGACT of the metallothionein I promoters, respectively. The present study suggests that the basic HLH protein USF may also play a role in the regulation of the cardiac muscle gene program, raising the question of its potential interactions with more tissue-restricted transcriptional factors in the context of the MLC-2v 250-bp promoter. It will be of interest to determine if USF-1 and its cofactor might serve as novel partners for more tissue-restricted factors (HF-1b and MEF-2/RSRF) in the maintenance of cardiac muscle specificity in the expression of a panel of cardiac muscle genes. Several other transcription factors that required heterodimer formation have also been observed to control tissue-specific and developmentally regulated genes. They include MyoD/E2a (36) and retinoic acid receptor/retinoid X receptor (58), which regulate muscle-specific genes and the developmental effects of retinoic acid *in vivo*, respectively. Interestingly, USF-2 (52), which can dimerize to USF, was shown to be a component of MLE1, HF-1a, and PRE B complexes by gel mobility shift assay with rabbit antiserum specific against USF-2. Whether USF forms heterodimers to USF-2 or to an as yet unidentified related transcription factor to activate the transcription of native MLC-2v remains to be determined.

USF and negative regulation of the MLC-2v gene via the HF-1a site. In addition to binding to the CACGTG in the 250-bp MLC-2v promoter fragment, USF can also bind to the HF-1a site in a sequence-specific manner, albeit with a severalfold decrease in relative affinity compared with that to the MLE1 site. By utilizing antibodies directed against USF, a component of the endogenous HF-1a-binding activity can be supershifted, although not all HF-1a-binding activity is removed following incubation with the USF antibodies. The binding of USF to the HF-1a site appears to be sequence specific, as duplex oligonucleotides which contain point mutations in the HF-1a site are poor competitors for USF binding. Interestingly, cotransfection studies with an MLC-2 reporter gene which does not contain the positive USF regulatory site MLE1 suggest that USF may serve as a negative regulator of MLC-2v gene expression. This effect is site specific, as it is eliminated by point mutations in the HF-1a site. While the basis for this negative regulatory effect is not clear, one possibility is that USF may act to displace the binding of positively acting transcriptional factors which can occupy the HF-1a site. In this regard, recent studies have suggested that a YB-1 complex is a component of endogenous HF-1a-binding activity within cardiac muscle cells. Cotransfection of an EFl α /YB-1 expression vector leads to the *trans* activation of a 250-bp MLC-2v luciferase reporter gene, and this activation is dependent on a cardiac muscle cell context (60). Studies are currently in progress to determine if the negative regulatory effect of USF acts through displacement of the YB-1/p30 complex on the HF-1a site in the 250-bp MLC-2v promoter.

USF is widely expressed, and the mechanisms by which it may contribute to cardiac muscle specificity of the MLC-2v promoter are not completely clear. In contrast, skeletal muscle specificity appears to be dominantly conferred, and the specificity for the myogenic pathway in skeletal versus cardiac muscles can be readily explained by the skeletal muscle-specific expression of these dominantly acting myogenic determination genes, such as the *myf-5*, *MyoD*, myogenin, and *MRF4*/heryculin/*myf-6* genes. To date, a functional equivalent of these genes has not been found in the cardiac cell context, and recent studies of cardiac fibroblast heterokaryons suggest that the cardiac phenotype may indeed be recessive and that divergent pathways upregulate a regulatory gene which is coexpressed in

cardiac and skeletal muscles. The results of this study provide additional evidence that factors which are not themselves restricted in their expression to the cardiac muscle can also play important roles in regulating the activity of a small MLC-2v promoter fragment which is sufficient to confer cardiac muscle- and chamber-restricted expression in vivo in transgenic mice. It will be of interest to determine directly whether the MLE1 site and USF contribute to either the cardiac muscle-specific or inducible expression of the MLC-2v gene during cardiac growth and hypertrophy.

Recent studies of the α -MHC promoter have found that a sequence which is similar to the HF-1a site is important in the maintenance of maximal activity of α -MHC reporter gene constructs in transient assays of cardiac muscle cells. Mutations in the PRE B site result in a marked decrease in activity of the α -MHC promoter, in a manner analogous to the role of HF-1a in the context of the MLC-2v 250-bp promoter. An AT-rich MEF-2 site is located in close proximity to the PRE B site, which resembles the adjacent HF-1a and HF-1b/MEF-2 sites in the MLC-2v promoter (35). The current study raises the issue of whether there is coordinate regulation of the MLC-2v and α -MHC genes via an HF-1-dependent paradigm. Since the present study provides evidence that USF can also bind in a sequence-specific manner to the PRE B site in the α -MHC promoter, this study suggests the possibility not only that there may be coordinated positive regulation via HF-1a-like sites in these two promoters but also that USF may serve as a negative regulator of these two genes in the context of these two promoters.

Future studies to directly assess the role of the importance of the negative regulation of the cardiac muscle gene program by USF should be of interest. This negative regulation could be very important in restricting expression in regions of the heart that do not express the MLC-2v gene, such as the atrial and other segments. Evidence that the MLC-2v gene is positionally specified early during heart tube formation has accumulated, and studies of transgenic animals suggest that both positive and negative regulatory pathways are required to restrict its expression to the ventricular segment of the heart tube. Future studies should be of interest to determine the relative role of USF versus that of other recently discovered negative regulators (HF-3) of the cardiac muscle gene program.

USF and combinatorial regulation of the cardiac muscle specificity of the MLC-2v gene. An increasing body of evidence suggests that the cardiac and chamber-specific expression of the MLC-2v gene may be due to combinatorial regulation by a number of transcriptional factors which are not restricted in their expression to the heart or a particular chamber (atrium versus ventricle). The identification of the MLE1 site now brings the total number of independent *cis* regulatory elements which regulate the 250-bp MLC-2v promoter to four, including a combination of both positive and negative regulatory elements that appear to be operative in cells in transient assays and also in transgenic mice. Interestingly, none of the factors which have been characterized to date displays restricted expression to cardiac muscle cells. In addition, increasing evidence suggests that ubiquitously expressed factors can also play an important role in tissue-restricted expression of a number of muscle genes. Accordingly, it will be interesting to explore the role of USF in the cardiac and chamber-restricted expression of the MLC-2v gene in future studies.

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