Identification of a Contractile-responsive Element in the Cardiac α-Myosin Heavy Chain Gene*

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The mechanisms by which the cardiac-specific α-myosin heavy chain (α-MHC) gene responds to contractile activity was studied in cultured cardiomyocytes and in vivo. Deletion analysis of the α-MHC promoter transiently transfected into neonatal rat cardiomyocytes localized the contractile-responsive element within −80 to −40 base pairs of the transcriptional start site. Mutational analysis of an E-box motif at position −47 showed that it was necessary for the contractile response both in cultured cardiomyocytes and in the intact heart. Competition gel mobility shift experiments indicated that the protein-DNA complex formed within the −39 to −59 base pair region could be competed by the E-box element at −309 of the α-MHC gene and that base substitutions within an E-box motif at −47 eliminated the protein-DNA complex. To identify the contractile-responsive nuclear protein, antibodies specific for E1/2, a E-box binding basic-helix-loop-helix (bHLH) protein, and antibodies recognizing upstream stimulatory factor (USF), a widely expressed bHLH-leucine zipper transcription factor, were studied for their ability to inhibit cardiomyocyte nuclear protein binding to the E-box motif at −47. Anti-USF antibody abolished formation of the protein-DNA complex, thus identifying the protein as antigenically related to USF and demonstrating that bHLH-leucine zipper proteins are involved in the contractile-induced expression of the cardiac α-MHC gene.

The cardiac myocyte responds directly to mechanical stimuli such as load, stretch, or contractility by changes in cell mass and by alterations in specific gene expression resulting in changes in contractile function (1–5). In the pressure-overloaded rat heart, expression of the myosin heavy chain (MHC) genes are altered such that the β-MHC isoform appears denovo while the α-MHC isoform is decreased (6, 7). Hemodynamic unloading of the heart as occurs in the heterotopically transplanted isograft results in a decrease in α-MHC expression which is mediated by a decrease in promoter activity of this gene (3, 8). The mechanism by which the hemodynamic or contractile stimulus is transduced to the nucleus remains unclear. Stretch-induced alterations in plasma membrane-associated ion channels, phospholipases, G proteins, and their associated cytoplasmic second messengers, including cAMP, inositol phosphates, calcium, and diacylglycerol leading to the induction of protein kinase cascades are potential signaling pathways (9–11). Alternatively, stretch-activated release of various autocrine factors may promote immediate-early gene expression and/or growth factor gene induction which in turn may induce cardiac-specific gene transcription (9, 11, 12).

Based on consensus sequence, mutational analysis and nuclear protein binding activity, the identities of several regulatory elements have been delineated in the α-MHC gene. Elements sufficient for both high levels of expression and cardiac myocyte-restricted expression have been localized to the proximal 5′-flanking region of the gene from −380 to −40 bp of the transcriptional start site. These DNA elements include a site that binds the myocyte-specific enhancer-binding factor-2 at position −327/−337 (13, 14), an E-box sequence at position −308/−313 (15), and M-CAT and A-rich motifs at positions −236/−242 and −217/−223, respectively (16). Recently, two sites located at −258/−269 that interact with the transcription factor, GATA-4, have been shown to be necessary and sufficient to impart cardiac myocyte-restricted expression of the α-MHC gene (17). Responsiveness of the α-MHC promoter to activators of the cyclic-AMP/protein kinase A signaling pathway has been mapped to an M-CAT/E-box hybrid element (18).

We have shown that the contractile activity per se of cultured neonatal rat cardiac myocytes modulates the expression of the MHC genes (1, 5, 19). In the present study we used deletion analysis of the 5′-flanking region of the α-MHC gene to identify DNA elements that determine responsiveness of the promoter to contractile activity, and have identified putative transcription factors that mediate this response.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Neonatal Rat Cardiac Myocytes—Ventricular myocytes were isolated from hearts of 2-day-old rat pups by collagen digestion as described previously (5, 19). Cells were plated at approximately 1.5 × 10^5/cm^2 on collagen-coated culture plates or flasks in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1, DMEM/F-12, Life Technologies, Inc.) containing insulin (120 IU/liter), transferrin (5 mg/liter), and selenium (5 μg/liter) (ITS) plus L-triiodothyronine (10⁻¹⁰ M), L-glutamine, and antibiotics. Media were changed daily. In selected culture dishes, spontaneous contractile activity (present 18 h after plating) was prevented by the addition of verapamil (10⁻⁵ M) or 2,3-butanedione monoxime (BDM, 5 mM) to the culture medium.

For the preparation of nuclear extracts, cardiomyocytes were plated onto collagen-coated 75-cm² tissue culture flasks and maintained in DMEM/F-12 medium containing ITS plus T3 for 48 h prior to harvest.

Transfection Studies—Plasmid DNA was introduced into the cultured cells 18 h after plating onto six-well dishes and immediately following removal of the nonadherent cells. Plasmids containing 5′-flanking regions of the α-MHC gene were co-transfected with a constitutively active RSV (Rous sarcoma virus long terminal repeat) β-galac-
tosidase plasmid (pRSVZ, ATCC, Rockville, MD) using the lipofection method of transfer. Cells in each 35-mm well were exposed for 6 h to DMEM/F-12-PC-1 (2:1) medium containing Lipofectin reagent (Life Technologies, Inc.), 2.5 μg of α-MHC promoter/luciferase reporter plasmid, and 0.25 μg of pRSVZ. After lipofection, the cells were washed twice in Hank’s buffered salt solution and maintained in DMEM/F-12 supplemented with ITS plus other reagents as indicated with daily medium changes. Luciferase and β-galactosidase were analyzed after 48–72 h by lysis of the cells in 300 μl of lysis buffer (Promega, Madison, WI). Luciferase activity was determined by the addition of 10 μl of lysis to 100 μl of luciferin reagent and measured as light production using a Turner Designs model 20 luminometer. β-Galactosidase activity was measured by 10–150 μl of lysis and compared with a standard curve of purified β-galactosidase (Promega) from 0.1 to 0.5 milliunits. Luciferase activity is expressed as a function of β-galactosidase activity in the same volume of cell lystate (luciferase luminescence units/β-galactosidase). To determine efficiency of transfection, myocyte cultures were stained for β-galactosidase activity 48 h after lipofection with pRSVZ. Cells were fixed in solution containing 2% paraformaldehyde, 5 mM EGTA, 2 mM MgCl₂, 0.1 M Pipes, pH 7.3, and stained by standard methods.

Plasmid Constructs—The α-MHC promoter constructs were generated from plasmid pSVMOCAT (20) generously provided by Dr. B. E. Markham (Ann Arbor, MI). The 5′-flanking region of the α-MHC gene was restricted using NheI, BglII, EcoRI, and BglII to generate fragments terminating at positions −2560, −1660, −1036, and −195, respectively, and at the same HindIII site at +421 of the transcriptional start site. Fragments were ligated into a promoterless firefly luciferase expression plasmid (pLUC) that has been described previously (8). The sequence identity of all clones was verified by DNA sequence analysis. A second series of promoter constructs was generated that terminated at the downstream position +32 and extended upstream to positions −388, −163, −80, and −40. These sequences were generated using polymerase chain reaction to amplify the regions from the plasmid pSVMOCAT. Sense primers contained a BglII site and antisense primers contained a NcoI site to facilitate subcloning into the multiple cloning region of pLUC. These α-MHC promoter constructs were made that contained the entire genomic region between transcriptional and translational start sites and included the sequences from −2560 to +1036 and from −1660 and +1036. Polymerase chain reaction methodology was used to amply the region from +421 to +1036 from rat genomic DNA (Clontech, Palo Alto, CA). The sense primer contained sequences from +421 and antisense primer contained the first ATG of the α-MHC coding sequence. This fragment was ligated to previously constructed plasmids containing sequences from −2560 to +421 and −1660 to +421. Proper orientation and ligation were ascertained by automated DNA sequence analysis (Applied Biosystems 373A sequencer). All plasmids were purified by ion exchange chromatography (QiAGEN, Inc., Chatsworth, CA) and examined by agarose gel electrophoresis with ethidium bromide staining.

Mutagenesis of the α-MHC Promoter—The −388/HM plasmid contains a C to A transversion position −50 within the context of the −388 to −32 bp region of the gene. An antisense primer containing the base substitution at position −50 and the EcoG71I site at −21 and a sense primer containing sequences from −388 to −368 and a BglII site were synthesized and used for polymerase chain reaction amplification of the −388 to −21 sequence. The wild type −388 to −21 sequence in the −388/+32 luciferase expression plasmid was excised using BglII and EcoG71I and replaced by the amplified fragment containing the mutation to generate the −388/HM plasmid. The sequence was verified by automated DNA sequence analysis.

Direct Shift Assay—The direct shift assays of the sequence strands of each deoxyadenosine used for EMSA is shown in Table I. Both sense and antisense oligonucleotides contained an AGCCT sequence at their 5′ ends and were synthesized by Appligene, Inc. (Pleasanton, CA). The sense strands were end-labeled using T4 polynucleotide kinase and α-32P-ATP (3000 Ci/mmol) (DuPont NEN) to a specific radioactivity of 1.5 × 10⁷ cpm/μg. The labeled oligonucleotides were separated from free label over Sephadex G-200 spin columns, and annealed to a 50-fold molar excess of nonradioabeled complementary oligonucleotide. Reactions (20 μl) contained 5–10 μg of nuclear extract protein, 1 μg of poly(dI-dC), in 12 mM Hepes-NaOH pH 7.9, 60 mM KCl, 4 mM Tris-HCl, 0.6 mM EDTA, 0.6 mM dithiothreitol, 12% glycerol, 0.6 mM phenylmethylsulfonyl fluoride. Reactions were preincubated at room temperature for 15 min with incubation with 20–40 fmol of labeled DNA (40,000 dpm) for 30 min. In competition experiments, competitor oligonucleotides were added at 100-fold excess of the labeled DNA. In gel shift assays with antibodies to E12/E47 and USF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the binding reaction was completed prior to incubation with the antibody for 60 min on ice. The complexed products were resolved in 6% polyacrylamide gels which were subsequently dried and exposed to x-ray film.

Nuclear Extract Preparation—Cardiomyocytes from approximately 15 neonatal rat hearts plated on two 75-cm² flasks (T75) were lysed and pooled for a single sample of nuclear extract. Myocytes (>90% of total cardiac cells) were harvested in DMEM/F-12 plus ITS and α-triiodothyronine (10−6 M) for 48 h prior to harvest. Typical yields from two T75 flasks were 100 μg of nuclear protein. Myocytes were Dounce homogenized in ice-cold buffer containing 0.3 M sucrose, 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 50 μM each of antipain, leupeptin, benzamidine, and aprotinin. Nondet 5–40 was added to 0.4% final concentration. Nuclear material were recovered by centrifugation at 3000 × g for 10 min at 4°C and washed once with homogenization buffer without detergent. Nuclear material was extracted as described by Sierra et al. (21) except that protease inhibitors were added at 50 μg/ml. Protein concentration in the final extract was determined by Lowry assay, aliquoted, and stored at −80°C for single 50-μl aliquots.

RESULTS

DNA Transfection into Cultured Myocytes—The efficiency of DNA transfer by lipofection into the neonatal cardiomyocytes was assessed using β-galactosidase reporter plasmid (pRSVZ) and by staining the cells 48 h after transfection. From 16 separate experiments, the transfection efficiency was estimated at 6 ± 1% of the myocytes.

Identification of a Contractile Responsive Region by Deletion Analysis of the α-MHC Promoter—To identify the region of the α-MHC gene that is both necessary and sufficient for contractile responsiveness, the activities of deletion mutants of the 5′-flanking sequences were determined by transient transfection of neonatal cardiac myocytes. Fig. 1A compares the activities of promoter constructs terminating at positions −2560, −1660, −612, and −195 with a common downstream site at +421 in spontaneously contracting cardiomyocytes and in noncontracting verapamil-treated cultures. All promoter deletions were contractile-responsive in 2.5–4-fold greater activity in contracting myocytes compared with noncontracting cells.

To determine contractile responsiveness of the proximal region of the promoter, a series of promoter constructs were made that terminated at position +32 and extended upstream to positions −388, −163, −80, and −40. Fig. 1B shows that contractile responsiveness of the proximal region of the promoter, a series of promoter constructs were made that terminated at position +32 and extended upstream to positions −388, −163, −80, and −40. Fig. 1B shows that...
bp is necessary to mediate the contractile response of the
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B promoter constructs except recombinant plasmid p40 (2). All promoter constructs except those containing sequences upstream of the transcriptional start site were contractile-responsive, while constructs containing sequences upstream but terminating at position 2.5–3-fold in response to contractile activity (data not shown). These results indicate the absence of contractile-responsive elements downstream of the transcriptional start site.

Effects of Contractile-arresting Agents on α-MHC Promoter Activity—The spontaneous contractions characteristic of the neonatal cardiac myocytes plated at high density can be prevented by culturing in medium containing 10 μM verapamil, an inhibitor of calcium influx across the sarcolemma (23). Studies with the fluorescent indicator, Fura-2, indicated that verapamil treatment of cardiomyocytes eliminated phasic levels of intracellular [Ca2+] but maintained diastolic [Ca2+] at approximately 200 nm. An alternative agent that reduces contractile activity in cardiomyocytes is BDM (24). At 5 mM concentration, BDM prevented tension development in myocytes by inhibiting actomyosin cross-bridge formation while having no effect on intracellular Ca2+ transients.

α-MHC promoter activities were measured by transient transfection of cardiomyocyte cultures treated either with 10 μM verapamil or 5 mM BDM and compared with untreated spontaneously contracting cultures. Activities of three promoter constructs containing sequences from positions −2560 to +421 (p2560), −612 to +421 (p612), and −388 to +32 (p388) are expressed as a percent of their activity in contracting cultures (Table II). Verapamil and BDM had similar effects on α-MHC promoter activity, significantly decreasing activity by 2.5–4.5-fold compared with spontaneously contracting cultures (p < 0.01).

To ascertain that culture conditions used in these experiments did not alter the expression of the control plasmid, β-galactosidase activity was measured in both contracting and noncontracting myocytes in defined medium in the absence or presence of the agents used in this study. β-Galactosidase activity measured per mg of myocyte protein was not significantly altered by myocyte contractile activity. Activity of the promoterless luciferase reporter plasmid, pLUC, was measured in every culture condition. Its activity was significantly lower than any of the α-MHC promoter constructs studied and was not altered by contractile activity or by addition of any agent studied.

Identification of a Contractile-responsive Cis-regulatory Element—Sequence analysis of the −80 to −40 bp region revealed a consensus E-box motif at position −74/−80, a CArG element at −60/−70 (18), an E-box at −47/−52 and an M-CAT site on the opposing DNA strand at position −42/−48 (18). Table I shows the contractile-responsive region from −83 to −40 to indicate the arrangement of these DNA elements. Also listed are the oligonucleotide sequences used for electrophoretic mobility shift analysis to determine cardiomyocyte nuclear protein binding to these cis-acting elements. The HME oligomer contains the E-box and overlapping M-CAT elements at −42/−52.

Fig. 1. Contractile-mediated activation of α-MHC promoter constructs in transiently transfected cardiomyocytes. Activities of recombinant plasmids containing deletions in the 5’-flanking region of the α-MHC gene were measured in cultured myocytes after 48 h of spontaneous contractile activity or contractile arrest using 10 μM verapamil. Promoter constructs contained common 3’ sites at either +421 (A) or +32 (B) and extended upstream of the transcriptional start site to the positions indicated. The α-MHC promoter/luciferase expression plasmids were co-transfected with pRSVZ and the activities expressed as luminescence units normalized for β-galactosidase activity (Luc/β-gal). All values are percent of the activity of the largest promoter construct in contracting myocyte cultures: p2560 in A and p388 in B. n = 3 separate determinations using three to four different myocyte preparations. p < 0.01, contracting versus noncontracting activities for all promoter constructs except recombinant plasmid p40 (B).

The first intron. Activities of all promoter constructs were induced 2.5–3-fold in response to contractile activity (data not shown). These results indicate the absence of contractile-responsive elements downstream of the transcriptional start site.

To determine whether contractile-responsive elements exist downstream of the transcriptional start site, two constructs were made that contained the entire genomic region between the transcriptional and translational start sites and extending to −2560 nd −1660 bp upstream of the transcriptional start site. Transient transfection analysis of these two constructs was compared with promoter constructs containing the same sequences upstream but terminating at position +421 within

**Table II**

Comparison of the effects of contractile arrest using verapamil or BDM on α-MHC promoter activity

| Plasmid | Untreated | Verapamil* | BDM* |
|---------|-----------|------------|------|
| p2560   | 100       | 0.39 ± 0.06| 0.44 ± 0.12|
| p612    | 100       | 0.27 ± 0.08| 0.43 ± 0.09|
| p388    | 100       | 0.47 ± 0.05| 0.21 ± 0.10|

*Mean ± S.E., percent activity of the same plasmid in untreated spontaneously contracting myocyte cultures. n = 3 separate determinations using two different myocyte preparations. p < 0.01, all verapamil and BDM values versus their respective plasmid untreated group.

2. A. M. Samarel and K. Byron, unpublished data.
E74 oligomer contains the E-box motif at –74. Probe E318 contains the E-box at –309/–314 (15); probe MCT241 contains the M-CAT element at position –237/–243 (16), and the TRE135 oligomer contains the thyroid hormone-responsive element located at position –142/–157 (25) of the α-MHC promoter.

Fig. 2 shows EMSA using contracting myocyte nuclear extracts with the ds oligonucleotide probes listed in Table I. The HME probe that contains both consensus E-box and M-CAT elements shows one prominent retarded band (B1) and a minor broad band (B2). The single protein-DNA complex formed with the E74 probe containing a consensus E-box motif corresponds to the higher molecular weight band, B1, seen with the HME probe. Binding to the E-box sequence at position –309 (probe E318) retards a band corresponding to B1 and a second band in the region of B2. Two protein-DNA complexes are observed binding to the M-CAT element at –237 of α-MHC (probe MCT241) as has been described previously (26, 27).

Competition experiments were used to identify the nuclear proteins bound to the HME and E74 DNA probes. Fifty- and 100-fold molar excess unlabeled competitor DNA sequences were used to determine specificity of competition. A ds oligonucleotide containing the α-MHC thyroid hormone response element (TRE135) was used as nonspecific competitor DNA. Fig. 3 (lane 1) shows the retarded bands bound to the ds HME oligonucleotide probe. Competition with 100-fold excess of the E318 ds oligonucleotide probe (lane 2) completely eliminated the B1 complex. To determine whether nuclear protein bound to the consensus M-CAT sequence within the HME probe, 100-fold excess of a known M-CAT sequence (MCT241) was added to the binding reaction. MCT241 did not compete for binding to either the prominent B1 protein-DNA complex or the minor B2 complex (lane 3). The TRE135 sequence did not compete for any of the HME-protein complexes (lane 4). Since the HME B1 complex is retarded similarly to the complex retarded by the E-box at –309 (E318) and is competed by the E318 ds oligonucleotide but not by the M-CAT sequence (MCT241), the proteins contained within the B1 complex appear to be E-box- and not M-CAT-binding proteins.

The protein complex bound to the E-box motif at –74 was retarded similarly to the B1 protein-DNA complex of the HME probe (Fig. 3, lane 5) and was competed by 100-fold excess of the HME oligomer (lane 6). Similarly, 100-fold excess of the E74 oligomer effectively competed B1 binding to the labeled HME probe (lanes 7 and 8). The relative quantity of B1 complex bound to E-box −74 was consistently less than the protein-DNA complex bound to E-box at position −47 (lanes 5 versus 7), suggesting differences in binding affinities, protein oligomerization, or that the E-box motifs bind distinct nuclear proteins.

Mutational Analysis of the HME Sequence—To determine which nucleotides within the HME sequence were essential for protein-DNA complex formation, four separate transversion mutations were introduced into the consensus E-box and M-CAT elements (Fig. 4). Gel shifts using ds oligonucleotides containing these base substitutions are shown in Fig. 4. Lane 1 shows the standard gel shift analysis using the wild type HME sequence. Lane 2, which corresponds to mutation (2), shows that substitution of the conserved T in the E-box motif did not inhibit formation of the B1 protein-DNA complex, whereas the C to A transversion at position −50 (mutation 3) abolished B1 binding. The G to T base substitution at position −53 (mutation 4) which is outside the consensus E-box sequence CANNTG had no effect on B1 complex formation. To determine whether nuclear protein binding to the M-CAT consensus sequence at −42/−48 was involved in B1 complex formation, a ds oligomer was synthesized containing three base substitutions at positions −43 to −45 (mutation 5). Gel shift analysis showed that the mutant M-CAT oligomer did not alter B1 protein-DNA complex formation (lane 5), suggesting that binding to this M-CAT sequence did not occur in these cardiomyocyte nuclear extracts.

E-box-47 Is Necessary for Contractile Responsiveness of the α-MHC Promoter—To determine if the E-box element at position −47/−52 was necessary for the contractile-mediated activation of the α-MHC promoter, the T to C transversion at −50 that abolished B1 complex formation (Fig. 4) was introduced into the 5′-flanking region of the gene for analysis in cultured cardiomyocytes and in the intact animal. The base substitution at position −50 was introduced into the context of a larger promoter region that included −388 to +32 bp of the transcriptional start site, generating the α-MHC promoter/luciferase reporter plasmid, −388αHME. Transient transfection analysis of the mutant construct showed that the E-box element was necessary for the contractile-mediated activation of the promoter in neonatal rat cardiomyocytes. Activity of the mutant
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-59
1 HME  5′CAGCAAGCAGCTGGGatGAGC 3′
2 δT-48 C
3 δC-50 A
4 δG-53 T
-39
5 δAAT-43-45 CCG

Fig. 4. Mutational analysis of the E-box and M-CAT sequences within the HME oligomer probe. Gel shift conditions are described under “Experimental Procedures.” The wild type HME sequence is shown in lane 1 with the E-box motif underlined and the M-CAT element overlined. Base mutations introduced into the HME probe are shown in lowercase letters, and the substituted bases are indicated for each of the four mutant oligomer probes listed as mutants 2 to 4. The gel shift analysis shows the major retarded protein-DNA complex B1 with the wild type HME probe (lane 1) and with the mutant probes in lanes 2-5.

−388δHME luciferase reporter plasmid in contracting cardiomyocytes was 37 ± 4% of the activity of the wild type promoter (−388wt) in contracting myocytes and the level of activity was the same as the wild type promoter in noncontracting myocyte cultures (48 ± 3%) (Table III). Activities of the wild type and mutant promoter constructs in noncontracting myocytes were the same (48 ± 3 versus 33 ± 7%), suggesting that the E-box element is necessary for the contractile-mediated activation of the promoter and not the basal activity of the promoter.

To determine whether the E-box element at −47 is necessary for promoter activity in the contracting myocardium in vivo, the same wild type (−388wt) and mutant (−388δHME) promoter/luciferase reporter plasmids used in cultured myocytes were injected directly into the ventricular myocardium of normal animals. Three days after DNA injection, luciferase analysis of the injected ventricular tissue showed that the activity of the mutant promoter was 38 ± 6% of the wild type promoter (p < 0.01) (Table III). These data in cell culture and in the intact working heart suggest that the E-box element at position −47 is required to enhance α-MHC promoter activity in the contracting cardiac myocyte.

HME-binding Protein Is Antigenically Related to USF—Several distinct families of nuclear proteins bind to DNA sequences containing the core CANNTG element or E-box motif, including those comprising the basic-helix-loop-helix (bHLH) proteins such as the myogenic factors and E12/E47 (28, 29) and the bHLH-leucine zipper (bHLHZ) proteins such as upstream stimulatory factor (USF) (30). The E12/E47 nuclear proteins are widely expressed heterodimerization partners of cell-specific bHLH proteins such as MyoD (28), allowing for genespecific regulation. Similarly, USF is a ubiquitously expressed protein but has been shown to be important in cell-specific gene regulation (31, 32). To identify the proteins which form the −47 E-box B1 complex, antibodies produced against E12/E47 and USF were used in gel shift experiments (Santa Cruz Biotechnology).

The protein-DNA complex B1 that forms in the presence of cardiomyocyte nuclear extracts and the HME probe is shown in Fig. 5, lane 1. Formation of the B1 complex was not altered by including 2 μg of rabbit IgG (lane 2) or 1 μg E12/E47 antibody (lane 3) in the binding reaction. However, USF antibody blocked the formation of the B1 complex (lane 4). This blocking ability of the USF antibody has been observed previously with other E-box sequences (31–33). To ascertain that the blocking effect of the USF antibody was specific to the B1 complex, myocyte nuclear extracts were incubated with the MCT241 probe containing the α-MHC M-CAT element at position −237 (lane 5). Addition of USF antibodies to the DNA/nuclear extract binding reaction did not affect protein binding to the M-CAT motif (lane 6). These data indicate that the protein(s) that form the B1 complex is USF or is antigenically related to USF and that it is distinct from the E12/E47 proteins.

DISCUSSION

The importance of cardiomyocyte contractile activity in maintaining protein synthesis and cardiac mass has been well documented in the intact heart and in cultured myocytes (1–3, 19). Hemodynamic load and mechanical stimuli have also been shown to modulate the expression of cardiac-specific proteins (3, 5, 34–36). We reported that the effect of contractile activity on myosin heavy chain mRNA expression in cultured cardiomyocytes was independent of serum effects (19), suggesting that the contractile stimulus per se influenced phenotype. Studies of cultured cardiac myocytes subjected to metabolic inhibition and subsequently allowed to recover showed that only when contractile function was re-established did the expression of the cardiac myosin light chain-2 gene return to control levels and the myofilaments reorganize (37).

To study the mechanisms by which a contractile/mechanical stimulus could regulate specific gene transcription, we used primary cultures of neonatal rat cardiomyocytes in which spontaneous contractile activity could be prevented by verapamil or BDM (23, 24). When myocyte contractile activity was prevented using either agent, α-MHC promoter activity was significantly decreased. Deletional analysis of the α-MHC promoter showed that the contractile-responsive region resided within −80 to −40 bp of the transcriptional start site. Sequence analysis of this region identified four consensus elements, in-

| TABLE III | Mutation in the E-box motif at position −47 of the α-MHC promoter inhibits contractile-mediated activation of the promoter |
|------------|---------------------------------------------------------------|
|            | Cultured cardiomyocytes | In vivo contracting |
|            | Noncontracting | Contracting |
| −388wt     | 48 ± 3* | 100 |
| −388δHME   | 33 ± 7* | 37 ± 4* |
|            | 38 ± 6* |

* p < 0.01 versus −388wt in contracting cardiomyocytes.
* p < 0.01 versus −388wt in vivo.
...vivo element in a gene coding for a sarcomeric protein that is independent of the activation of immediate-early genes (11, 12).

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REFERENCES

1. McDermott, P., Daood, M., and Klein, I. (1985) Am. J. Physiol. 249, H763–H769
2. Cooper, G., IV, Kent, R. I., Uboh, C. E., Thompson, E. W., and Marino, T. A. (1989) J. Clin. Invest. 75, 1403–1414
3. Klein, I., Ojamaa, K., Samarel, A. M., and Hong, C. (1992) J. Clin. Invest. 91, 68–73
4. Nagai, R., Zarain-Herzberg, A., Brandl, C. J., Fujiij, J., Tada, M., MacLennan, D. H., Alipe, N. R., and Periasamy, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86. 2966–2970
5. Samarel, A. M., and Engelmann, G. L. (1991) Am. J. Physiol. 261, H1077–H1107
6. Izumo, S., Lompre, A., Matsuoka, R., Keren, G., Schwartz, K., Nadal-Ginard, B., and Mahdavi, V. (1987) J. Clin. Invest. 79, 970–977
7. Irimura, S. I., Matsuoka, R., Hiratsuka, E., Limura, M., Nakashima, T., Nishioka, T., Furutani, Y., and Takao, A. (1991) Am. J. Physiol. 261, H73–H79
8. Ojamaa, K., Petrie, J., Bakman, C., Tong, C., and Klein, I. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6347–6351
9. Sadoshima, J., Takekoshi, T., Hahn, L., and Izumo, S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9095–9099
10. Kent, R. L., Hoober, J. K., and Cooper, G., IV (1989) Proc. Natl. Acad. Sci. U. S. A. 86. 8905–8909
11. Samarel, A. M., and Engelmann, G. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6094–6098
12. Komuro, I., Kaida, I., Shibazaki, Y., Kurabayashi, M., Kato, Y., Hoh, E., Takaku, F., and Yazzky, Y. (1991) J. Biol. Chem. 266, 3995–3998
13. Molkentin, J. D., and Markham, B. E. (1993) J. Biol. Chem. 268, 19152–19150
14. Adolph, E. A., Subramaniam, A., Cserjesi, P., Olson, E. N., and Robbins, J. (1993) J. Biol. Chem. 268, 5349–5352
15. Molkentin, J. D., Brogan, R. S., and Markham, B. E. (1993) J. Biol. Chem. 268, 2620–2629
16. Molkentin, J. D., and Markham, B. E. (1994) Mol. Cell. Biol. 14, 5056–5060
17. Molkentin, J. D., Kalvakolanu, D. V., and Markham, B. E. (1994) Mol. Cell. Biol. 14, 4947–4957
18. Gupta, M. P., Gupta, M., and Zak, R. (1994) J. Biol. Chem. 269, 29677–29687
19. Oi, M., Ojamaa, K., Elettheriades, E. G., Klein, I., and Samarel, A. M. (1994) J. Physiol. 476, C580–C592
20. Gustafson, T. A., Markham, B. E., Bahl, J. J., and Morkin, E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3122–3126
21. Sierra, F., Tian, J. M., and Schibler, U. (1993) Gene: Transcription: A Practical Approach (Barnes, D. B., and Higgins, S. J., eds) pp. 131–134, IRL Press, New York
22. Ojamaa, K., and Klein, I. (1993) Endocrinology 132, 2001–2006
23. Braunwald, E. (1982) N. Engl. J. Med. 307, 1618–1627
24. Backx, P. H., Gao, W. D., Azam-Backx, M. D., and Marban, E. (1994) J. Physiol. Lond. 476, 487–500
25. Tsika, R. W., Bahl, J. J., Leinwand, L. A., and Morkin, E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 379–383
26. Mar, J. H., and Ordahl, C. P. (1990) Mol. Cell. Biol. 10, 4271–4283
27. Shimizu, N., Smith, G., and Izumo, S. (1993) Nucleic Acids Res. 21, 4103–4106
28. Blackwell, T. K., and Weintraub, H. (1990) Science 250, 1104–1110
29. French B. A., Chow, K. L., Olson, E. N., and Schwartz, R. J. (1991) Mol. Cell. Biol. 11, 2439–2450
30. Ferre-D’Amare, A. R., Pogonoc, P., Roeder, R. G., and Burley, S. K. (1994) EMBO J. 13, 180–189
31. Bresnick, E. H., and Felsenfeld, G. (1993) J. Biol. Chem. 268, 18824–18834
32. Kozlowski, M. T., Gan, L., Yenuth, J. M., Sawadogo, M., and Klein, W. H. (1993) Dev. Biol. 148, 625–630
33. Jackson, S. M., Guitierrez-Hartmann, A., and Hoffer, J. P. (1995) Mol. Endocrinol. 9, 278–291
34. Samarel, A. M., Spraga, M. L., Maloney, V., Kamal, S. A., and Engelmann, G. L. (1992) Am. J. Physiol. 263, C642–C652
35. Sharp, W. W., Terracio, L., Borg, T. K., and Samarel, A. M. (1993) Circ. Res. 73, 172–183
36. Klein, I., Daood, M., and Whiteside, T. (1985) J. Cell. Physiol. 124, 49–53
37. Barry, W. H., Hamilton, C. A., and Knollton, K. U. (1995) J. Mol. Carbohydr. 27, 551–561
38. Weintraub, H., Davis, R. L., Tapposs, S., J. Thayer, M. J., Krause, M., Benesora, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y., and Lassar, A. B. (1981) Science 210, 733–739
39. Murre, C., McCaw, P. S., and Baltimore, D. (1989) Cell 56, 777–783
40. Navankasattusas, S., Sawadogo, M., Bilens, M., Dang, C. V., and Chien, K. R. (1994) Mol. Cell. Biol. 14, 7311–7320
41. Nelson, C., Shen, L. P., Melster, A., Fodor, E., and Rutter, W. J. (1990) Gene & Dev. 4, 1035–1044
42. Ferre-D’Amare, A. R., Prendergast, G. G., Ziff, E. B., and Burley, S. K. (1993) Nature 363, 8–45
43. Chiang, C. M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993) EMBO J. 12, 2749–2752
44. Mitsui, K., Shirakata, M., and Paterson, B. M. (1993) J. Biol. Chem. 268, 24415–24420

FIG. 5. The HME E-box-binding protein is antigenically related to USF. Cardiomyocyte nuclear extracts were incubated with the HME probe, which included a novel E-box motif at position 47. E-box binding proteins including the basic helix-loop-helix (bHLH) family of proteins are involved in muscle-specific gene expression (15, 29) and in establishing diverse cell lineages (38, 39). E-box-binding proteins have been shown to modulate expression of several cardiac genes, including α- and β-MHC and cardiac α-actin (15, 29), and the bHLH2 protein, USF, has been shown to regulate expression of the cardiac ventricular myosin light chain-2 gene (40). In the present study, gel mobility shift analysis showed that the nuclear protein complexed to the contractile-responsive region from −60 to −40 bp was an E-box binding protein(s). Although a consensus M-CAT sequence overlaps with this E-box motif (18), we could not detect TEF-1 binding to this region of the promoter. Anti-USF antibodies prevented protein binding to the 47 E-box element significantly reduced α-MHC promoter activity via a protein kinase signaling pathway (5, 18, 19). Such a mechanism may explain the process by which changes in myocyte contractile activity regulates specific gene transcription.

The importance of this putative contractile response element in α-MHC promoter activity in the intact rat heart was evaluated by DNA transfer directly into the ventricular myocardium. In the normal hemodynamically contracting rat heart, mutation of the −47 3 bp E-box element significantly reduced α-MHC promoter activity compared with the wild type promoter. These data are consistent with the idea that a contractile-respon...
