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EFI_A/YB-1 Is a Component of Cardiac HF-1A Binding Activity and Positively Regulates Transcription of the Myosin Light-Chain 2v Gene

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Transient assays in cultured ventricular muscle cells and studies in transgenic mice have identified two adjacent regulatory elements (HF-1a and HF-1b/MEF-2) as required to maintain ventricular chamber-specific expression of the myosin light-chain 2v (MLC-2v) gene. A rat neonatal heart cDNA library was screened with an HF-1a binding site, resulting in the isolation of EFI_A, the rat homolog of human YB-1. Purified recombinant EFI_A/YB-1 protein binds to the HF-1a site in a sequence-specific manner and contacts a subset of the HF-1a contact points made by the cardiac nuclear factor(s). The HF-1a sequence contains AGTGG, which is highly homologous to the inverted CCAAT core of the EFI_A/YB-1 binding sites and is found to be essential for binding of the recombinant EFI_A/YB-1. Antiserum against *Xenopus* YB-3 (100% identical in the DNA binding domain and 89% identical in overall amino acid sequence to rat EFI_A) can specifically abolish a component of the endogenous HF-1a complex in the rat cardiac myocyte nuclear extracts. In cotransfection assays, EFI_A/YB-1 increased 250-bp MLC-2v promoter activity by 3.4-fold specifically in the cardiac cell context and in an HF-1a site-dependent manner. EFI_A/YB-1 complexes with an unknown protein in cardiac myocyte nuclear extracts to form the endogenous HF-1a binding activity. Immunocoprecipitation revealed that EFI_A/YB-1 has a major associated protein of ~30 kDa (p30) in cardiac muscle cells. This study suggests that EFI_A/YB-1, together with the partner p30, binds to the HF-1a site and, in conjunction with HF-1b/MEF-2, mediates ventricular chamber-specific expression of the MLC-2v gene.

The normal development of the heart requires the formation of atrial and ventricular chambers that display distinct electrophysiological, morphological, contractile, and biochemical properties. Presumably, the distinct properties of atrial, ventricular, and conduction system cells arise during the selective activation of various subsets of the cardiac muscle gene programs at critical stages of cardiac development. The known MyoD family of myogenic factors are absent in the heart, and knockouts of these factors have no effect on the development of a normal heart (43, 51, 65). The possibility exists that the temporally and spatially distinct pattern of expression of a panel of cardiac muscle genes during cardiogenesis arises through unique combinatorial pathways, as opposed to the actions of a subset of dominantly acting myogenic determination genes, as has been found for skeletal myogenesis (3, 4, 12, 14, 30, 36, 42, 50, 57, 69). It is becoming increasingly clear that cardiac and skeletal muscle development may be controlled by divergent and, to a certain extent, overlapping pathways (15, 29). Understanding of the developmental regulation of genetic markers of cardiac chamber formation and specification at a molecular level should provide a foundation from which to analyze the complex process of cardiogenesis (7, 8, 32, 33).

In this regard, the cardiac myosin light-chain 2v (MLC-2v) gene has served as a model system with which to identify the pathways which lead to cardiac myogenesis and restrict expression of cardiac muscle genes to particular chambers of the heart during cardiogenesis (8, 28, 29). The MLC-2v gene encodes 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 muscle (29). Within the heart, MLC-2v expression becomes restricted to the ventricular segment in 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 (41). In independent lines of transgenic mice, a 250-bp MLC-2v promoter can confer ventricular specificity to 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 muscle) (28). Studies in transient assays document that a single copy of the 28-bp HF-1 element, which contains both HF-1a and HF-1b sites, is sufficient to confer cardiac cell-specific expression in transient assays in primary cultures of ventricular muscle cells (73). In studies using transgenic mice or transient assays in cardiac cells, both the HF-1a and HF-1b sites are required to maintain cardiac and ventricular cell-specific expression (28, 40a, 73). Recent studies have identified two factors which can bind in a sequence-specific manner to the HF-1b/MEF-2 site, a novel C_2H_2 zinc finger protein (74) and a member of the RSRF family of transcriptional factors (MEF-2) (71), and both are capable of transactivation in cotransfection studies with the 250-bp MLC-2v luciferase constructs (74, 75). Previous studies using gel shift assays have suggested that the factors which occupy the neighboring HF-1a site are ubiquitously expressed (40a), but the molecular identity of these factors has been unknown.

In this study, using a combination of independent approaches, we found that a component of endogenous HF-1a binding activity within cardiac muscle cells contains the rat YB-1 homolog, EFI_A (44). Cotransfection of an EFI_A/YB-1

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expression construct leads to the transactivation of a 250-bp MLC-2v luciferase reporter gene, and this activation is dependent on the cardiac muscle cell context. As suggested by antibody supershift and immunocoprecipitation studies, this component of endogenous HF-1a binding activity in cardiac muscle cells may consist of EFI_A/YB-1 complexed with a 30-kDa cofactor (p30) by direct protein-protein interaction. The YB-1-associated protein in COS1 cells has a faster mobility (28 kDa). Recent studies have demonstrated that the HF-1a site can be occupied by another transcription factor, upstream stimulating factor (USF), which serves as a negative regulator of MLC-2v expression via the HF-1a site (40). In antibody supershift assays, neither the YB-3 antibody nor the USF antibody alone could completely abolish the endogenous HF-1a binding activity in cardiac nuclear extract. However, when both antibodies were added to the gel shift reaction, the HF-1a activity was completely abolished. The remaining bands of the HF-1a activity when only one antibody was used were almost identical in mobility. Taken together, these results suggest that there are two independent and mutually exclusive binding activities that bind to the HF-1a sequence and that a YB-1-containing complex functions as a positive regulator and USF most likely functions as a negative regulator on this site (40). Since a number of cardiac muscle genes are dependent on transcriptional factors which are widely expressed (TEF-1 [17, 26, 34, 35], BF-2 [37, 38], CArG-box-binding factor/serum response factor [56], etc.), in concert with tissue-restricted factors, it will become of interest to determine if YB-1 and its cofactor can serve as a novel partner for tissue-restricted factors (HF-1b and MEF-2/RSRF) in the maintenance of cardiac specificity of a panel of cardiac muscle genes.

MATERIALS AND METHODS

Binding site screening and cloning. Southwestern (DNA-protein) screening of a rat cardiac cDNA expression library was performed by a modification of previously described procedures (64, 74). The HF-1a probe (sense, AATTCGC CAAAAGTGGTCATGGGG; antisense, GAATTCCCCATGACCACTTTTG GC) contained the sequence predicted by methylation interference assays with neonatal rat heart nuclear extracts (40a), with an addition of an *Eco*RI site at both the 5′ and 3′ ends. One-tenth microgram of each of the single-stranded oligonucleotides was end labeled with 50 μl of [γ-^{32}P]ATP (6,000 Ci/mmol; Du Pont) by 15 μl of T4 polynucleotide kinase (10 U/μl) separately in a volume of 150 μl of buffer solution. The labeled oligonucleotides were mixed and boiled for 10 min and then reannealed by gradually cooling to room temperature, and 1 μl of 500 mM ATP and 30 μl of T4 DNA ligase (10 U/μl) were then added to the annealing mix. After incubation for 12 h at 16°C, the concatemerized HF-1a probe was extracted with phenol-chloroform and chloroform. The probe was purified of free nucleotides by spinning through a Sephadex G-50 column, filtered through a 0.22 μm-pore-size filter, rinsed with 500 μl of 1× binding buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol [DTT]), and stored on ice before use.

The rat neonatal heart λgt11 cDNA library was plated out at 15×10^3 plaques per 15-cm-diameter plate. The plates were prewarmed to 42°C before plating and incubated at 42°C for 4 h (64). Nitrocellulose membranes (Schleicher & Schuell) were presoaked in 10 mM isopropylthiogalactopyranoside (IPTG), air dried, and laid on the plates. The plates were incubated at 37°C for 8 h. After lifting and air drying for 15 min, a second set of IPTG-impregnated membranes was incubated with the plates for 2 h as the secondary lifts. The membranes were stored at 4°C until further use. The membranes were blocked in prehybridization buffer (5% milk in 1× binding buffer) at 4°C for 1 h. Membranes were placed into binding buffer with 0.25% milk, incubated for 30 min, and subsequently changed to hybridization buffer (0.25% milk, 1× binding buffer, HF-1a probe) supplemented with sonicated salmon sperm DNA for 4 h. The membranes were washed three times in binding buffer with 0.25% milk, air dried, and exposed to X-ray film at −80°C for 12 to 15 h. Positive signals which were duplicated in both lifts were picked and purified to homogeneity by subjecting them to the second and third rounds of screening with the same probe. λ phage DNAs were isolated, and the inserts were cut out and subcloned into pRSET vectors (Invitrogen), resulting in an in-frame fusion with a fusion peptide as with the LacZ in the λgt11 vector. The fusion peptide contains a polyhistidine tag for affinity purification and a T7 phage gene 10 leader peptide tag which can be recognized by a monoclonal T7 tag antibody (Novagen). The inserts were sequenced with Sequenase 2.0 (U.S. Biochemical) by the dideoxynucleotide technique as previously described (29).

Fusion protein expression and purification. pRSET (Invitrogen) constructs harboring polyhistidine-EFI_A/YB-1 fusion proteins were transformed into *Escherichia coli* BL21(DE3) (59), and the cells were grown to an optical density of 1.0 in LB-ampicillin medium and diluted 1:100 in LB-ampicillin medium. When the optical density reached 0.6 to 1.0, IPTG was added to a final concentration of 0.5 mM, and the culture was shaken for 4 h for induction of the fusion proteins. Affinity purification was performed with an Ni^+ column as outlined in the protocol for the X-press protein purification system (Invitrogen). The full-length EFI_A/YB-1 protein seemed to be toxic to the bacterial cells, since the sense construct resulted in tiny colonies on the plates, and remarkably slowed cell growth in liquid media, resulting in a relatively low recovery of full-length EFI_A/YB-1 protein. The relatively slow growth rate may arise from the fact that EFI_A/YB-1 contains a DNA binding domain with 50% homology with that of the major cold shock protein CS7.4 (19, 66). The eluted peaks (F2 and F3) were concentrated fivefold on Centricon columns (Amicon) and changed to the nuclear extract buffer solution (buffer B) for use in gel shift analysis. To express larger quantities of the EFI_A/YB-1 protein, the amino-terminal third region containing the DNA binding domain was truncated, and the truncated EFI_A/YB-1 protein (ΔYB-1) resulted in more than 1 mg of recombinant protein in a 100-ml culture. This recombinant protein (ΔYB-1) was used to generate antibodies against EFI_A/YB-1.

Cell culture. Rat neonatal ventricular myocytes were isolated as previously described (27, 58). The hearts were isolated from 2-day-old neonatal rats, and the ventricular portion was cut and dispersed by collagenase II (Worthington) and pancreatin (GIBCO) digestions at 37°C. The cell suspension was applied to a discontinuous Percoll (Pharmacia LKB Biotechnology Inc.) gradient. The middle layer of cells contained 95% cardiac myocytes (27). Myocytes were cultured in plating medium (Dulbecco's modified Eagle medium [DMEM], high glucose, 10% horse serum, and 5% fetal bovine serum, supplemented with antibiotics and L-glutamine). COS1 cells were grown in DMEM with 10% fetal bovine serum as previously described (40a).

Cell extract preparation. Cardiac nuclear extracts were prepared from confluent ventricular myocytes cultured in plating medium for 2 days after preparation (20×10^6 cells per 15-cm-diameter dish). Cells were washed twice with cold phosphate-buffered saline (PBS), scraped in 1 ml of cold PBS, transferred to Eppendorf tubes, and spun at 2,000 rpm for 4 min at 40°C. Cell pellets were then resuspended with 400 μl of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. After addition of 10 μl of 10% Nonidet P-40, the cell suspension was vortexed. The cell lysate was spun at 6,000 rpm at 4°C for 4 min to pellet the nuclei. The pellet was resuspended in 50 μl of buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 0.2 μg of aprotinin per ml) and incubated on ice for 15 min with frequent vortexing at top speed to elute the nuclear proteins. The nuclei were spun at top speed for 5 min, and the supernatant was stored at 70°C prior to gel mobility shift assays.

Gel mobility shift assays. For DNA binding assays with cardiac myocyte nuclear extracts, 6 μg of the nuclear extract described above was incubated with 4 μl of 5× binding buffer (200 mM KC1, 75 mM HEPES [pH 7.9], 5 mM EDTA, 2.5 mM DTT, 25 mM $MgCl_2$, 25% glycerol), 2 μg of poly(dI-dC) · poly(dI-dC), and 6 μg of bovine serum albumin (BSA) in a total of 19 μl on ice for 30 min (unless otherwise indicated). The oligonucleotides were purified in urea gel (55) and annealed at high concentration. The double-stranded probes with 5′ overhang were end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on Sephadex G-50 spin columns. After addition of 0.1 ng (~10,000 to 20,000 cpm) of the labeled probes (1 μl), the mixture was incubated for 30 min on ice (unless otherwise indicated in the figure legends). The binding reaction mixtures were run on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA at 12.5 V/cm of gel at 4°C for 2.5 h. For DNA binding assays using the bacterially expressed and affinity-purified recombinant protein, the same concentrations of binding buffer, BSA, and probes were used, with slightly less poly(dI-dC) · poly(dI-dC) (0.5 μg). About 0.1 μg of the bacterially expressed EFI_A/YB-1 was used for each binding reaction. Competitor probes were purified and annealed in a similar manner, usually in 500-fold weight excess over the radiolabeled probe. For antibody supershift assays, 2 μl of the YB-3 antiserum (polyclonal; a kind gift from Wanda F. Reynolds) or preimmune serum (a kind gift from Wanda F. Reynolds) was used; 0.5 μl of the USF antiserum (a kind gift from Michele Sawadogo) was used. In the dual-antibody supershift assay, the preimmune was used to supplement the reaction mixtures so that each tube contained equal amount of serum whether it contained preimmune alone, postimmune alone, or both. For the monoclonal antibody against the polyhistidine epitope tag (Novagen), 0.5 μg was used for each reaction. The antibodies were preincubated with the nuclear extracts or recombinant protein at room temperature or on ice for 30 min prior to the addition of probes.

DEPC interference footprinting. Diethyl pyrocarbonate (DEPC) interference footprinting was performed according as described by Sturm et al. (60). Either the sense or antisense strand of the HF-1a probe was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled single-stranded oligonucleotides were modified by DEPC and annealed with 10-fold excess of the unlabeled complementary strand. Double-stranded HF-1a oligonucleotide probe was pu-

rified on a 5% nondenaturing acrylamide gel and used for gel shift assays. The gel shifts with purified recombinant EFI_A/YB-1 or cardiac myocyte nuclear extract were performed as described in this report but were scaled up by 15- to 20-fold. The EFI_A/YB-1-bound, nuclear HF-1a-bound, and free HF-1a probes were recovered by electroelution and extracted of protein by phenol-chloroform and ethanol precipitated with 10 μg of yeast tRNA as carrier. The oligonucleotide probes were treated with 100 μl of fresh 1.0 M piperidine at 90°C for 30 min. Piperidine was removed by lyophilization five times. The cleaved oligonucleotide probes were separated on a 12% sequencing gel, with all lanes loaded with the same amount of radioactivity. G+A (Maxam-Gilbert) reactions were performed as molecular weight markers.

Cotransfection and luciferase and β-Gal assays. Cardiac myocytes were prepared by a Percoll gradient technique and plated in gelatin-coated 6-cm-diameter plates at 10^6 cells per plate in plating medium. After overnight incubation, cardiac myocytes attached to the plates, and the medium was switched to 4% horse serum for 3 to 5 h prior to transfection. Cotransfection of the 250-bp MLC-2 (MLC250) promoter-luciferase reporter construct and the EFI_A/YB-1 expression construct in cardiac myocytes was performed by calcium phosphate precipitation as described by Chen and Okayama (6). For each triplicate of 6-cm-diameter plates, 3.2 μg of pCMV-β-gal, 16 μg of pSVOAΔLU5-250MLC2v, and 32 μg of pCB-YB-1 (or 32 μg of pCB vector alone) were mixed in a polypropylene tube and brought to 720 μl by addition of double-distilled H_2O. Each reaction mixture included 80 μl of 2.5 M $CaCl_2$ and 800 μl of 2× BBS [280 mM NaCl, 50 mM *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 1.5 mM Na_2HPO_4 (pH 6.74)]. After incubation at room temperature for 40 min, 500 μl of the mixture was added to each of the three 6-cm-diameter plates, which were incubated at 3% CO_2 at 37°C for 16 h. A fine layer of precipitate was observed. Cells was washed once with serum-containing medium and once with maintenance medium and incubated in maintenance medium (DMEM supplemented with antibiotics and L-glutamine) for 48 h in 10% CO_2 at 37°C prior to harvesting for luciferase and β-galactosidase (β-Gal) assays. The cells were washed with ice-cold 1× PBS twice and lysed in 400 μl of Triton lysis buffer (0.1 M KH_2PO_4 [pH 7.9], 0.5% Triton X-100, 1 mM DTT) on ice for 30 min. Ten microliters of the lysates was used to measure luciferase activity, and 50 μl of the lysates was used to measure β-Gal activity. The luciferase assay buffer was 27 ml of KTME buffer [100 mM *N*-tris(hydroxymethyl)methylglycine (Tricine; pH 7.8), 10 mM $MgSO_4$, 2 mM EDTA, 1 mM DTT], 27.5 ml of 1 M DTT, 200 μl of 10 mM D-luciferin (Analytical Luminescence Laboratory), and 300 μl of 0.2 M ATP. Luciferase activity was measured by a luminometer with a 2-s assay time. Each plate was measured three times for accuracy. The β-Gal assay buffer was 18.1 ml of 0.1 M sodium phosphate buffer (pH 7.3), 6.6 ml of 10-mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside), and 300 μl of 100× Mg buffer (587 μl of H_2O, 100 μl of 1.M $MgCl_2$, 313 μl of β-mercaptoethanol). Each reaction assay contained 250 μl of the β-Gal assay buffer with 50 μl of the cell lysate, which was incubated at 37°C for 30 min or longer until the appearance of yellow color. β-Gal activity was obtained by measuring the optical density at 410 nm, using Triton lysis buffer as a blank. Each plate was measured twice for accuracy. The luciferase activity was normalized to the β-Gal activity to account for variations in the efficiency of transfection as previously described (73).

Immunoprecipitation and preparation of antisera. Cardiac myocytes (5×10^6) or 40 to 50% confluent COS1 cells in 10-cm-diameter plates were washed with 1× PBS twice and incubated in DMEM without methionine and supplemented with 63 μl of [^{35}S]Met (500 μCi; EXPRE^{35}S^{35}S Protein Labeling Mix; Du Pont) at 37°C overnight and for 4 h, respectively. Cells were then washed twice in 1× PBS and lysed in 1 ml of high-salt lysis buffer (500 mM NaCl, 1% Nonidet P-40, 50 mM Tris [pH 8.0]). Cell lysates were precleared by normal mouse serum and fixed *Staphylococcus aureus* Cowan 1 cells (CalBiochem). One-half milliliter of lysate was incubated with 0.1 μg of purified EFI_A/YB-1 or 1 μg of purified ΔYB-1 fused with a short T7 tag on ice for 1 h; 1 μg of monoclonal antibody against the epitope tag (Novagen) was added to the mixture, which was then incubated for 1 h on ice; 50 μl of washed 10% protein A-Sepharose beads was added, and the mixture was incubated on ice for 30 min. The beads were pelleted for 1 min and washed five times in 500 μl of wash buffer, and the proteins were eluted by heating in 50 μl of Laemmli's buffer at 80°C for 10 min. Negative controls in the absence of either the EFI_A/YB-1 protein or antibody were included to ensure that the associated proteins were specific to EFI_A/YB-1. The resultant immunoprecipitants were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel, dried, and exposed to X-ray film. For immunoprecipitation using an antiserum against EFI_A/YB-1, 10 μl of preimmune and immune sera was added to radiolabeled precleared lysate and incubated with gentle agitation at 4°C for 2 h before the addition of 50 μl of protein A-Sepharose beads.

To generate EFI_A/YB-1 fusion protein antibodies, rabbits were primed with 500 μg of purified ΔYB-1 fusion protein mixed in complete Freund's adjuvant followed by a first boost with 250 μg of ΔYB-1 3 weeks later. The first bleed was taken 2 weeks after the first boost. Later boosts were repeated every 3 weeks, and bleedings were done 2 weeks after each boost with 250 μg of ΔYB-1 fusion protein (23).

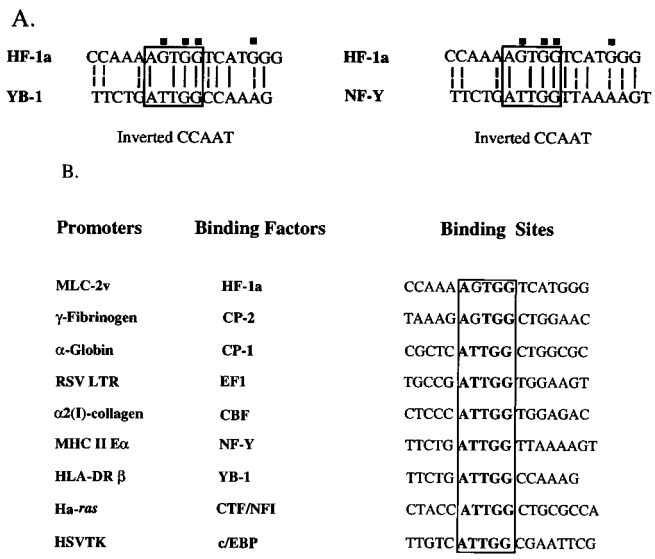

FIG. 1. The HF-1a site contains a degenerate inverted CCAAT box and is similar to the Y-box motif. (A) Sequence comparisons between the HF-1a element and the human and murine Y boxes in the MHCII genes HLA-DRβ and Eα, respectively. The solid dots represent the methylation interference assay-predicted HF-1a region that interacts with the HF-1a binding activity (37, 40a). There is only a one-base difference in the inverted CCAAT core. The flanking sequence also showed significant extent of similarity particularly 3′ to the core. The solid lines represent identical bases, and dashed lines represent similar bases, e.g., purine to purine and pyrimidine to pyrimidine. (B) Sequence comparison between the HF-1a element and the inverted CCAAT-containing sequences that YB-1 and/or a YB-1-containing complex EFI can recognize (16). A high-affinity EFI_A/YB-1 binding site in the γ-fibrinogen gene promoter CP2 (16) has exactly the same sequence variation in the inverted CCAAT core as in HF-1a: AGTGG. The flanking sequences outside the inverted CCAAT, often required for efficient binding, lack a clear consensus among all of the Y-box binding sequences as reported by others. Promoter sequence information was compiled from Faber et al. (16) and Didier et al. (13). RSV LTR, Rous sarcoma virus long terminal repeat; HSVTK, herpes simplex virus thymidine kinase.

RESULTS

Isolation of an HF-1a binding factor by Southwestern screening of a cardiac cDNA expression library. To isolate the cardiac muscle factor which displayed sequence-specific binding to the HF-1a cardiac regulatory element, we used Southwestern screening of a rat cardiac cDNA expression library with a concatenated HF-1a probe, in a manner analogous to previously described methods (64, 74). Screening of 2 million plaques yielded nine independent cDNA clones encoding overlapping regions of EFI_A, the rat homolog of the human Y-box-binding protein that binds to the class II major histocompatibility complex (MHCII) gene promoters (13, 44). All of the nine overlapping clones included the cold shock domain, which serves as a DNA binding domain of EFI_A (44). The longest full-length cDNA clone was sequenced in its entirety on both strands, revealing a nucleotide sequence which was identical to the published sequence of the rat YB-1 homolog, EFI_A (44).

EFI_A/YB-1 displays sequence-specific binding to the HF-1a cardiac regulatory element and interacts with a major subset of the contact points made by the nuclear HF-1a binding activity. The core HF-1a site contains an inverted CCAAT-like sequence within the methylation interference assay-predicted region (37, 40a) with a one-base-pair mismatch (Fig. 1B). In addition, the flanking sequences immediately 3′ to this inverted CCAAT site display a higher degree of sequence similarity with a consensus Y-box sequence in the human HLA-DRβ promoter and murine MHCII Eα gene (16) (Fig. 1A).

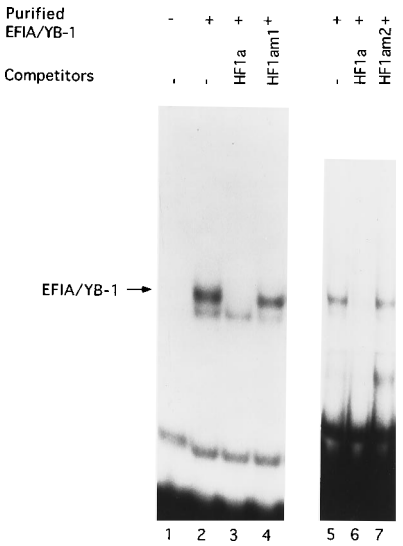

FIG. 2. Rat EFI_A/YB-1 binds to the HF-1a site specifically. The binding of EFI_A/YB-1 to the HF-1a double-stranded probe can be specifically competed for by HF-1a itself (lanes 3 and 6) but not by mutant HF-1a probes HF-1am1 (lane 4) and HF-1am2 (lane 7).

YB-1 binds with high affinity to the CP2 site in the γ-fibrinogen gene promoter, which has exactly the same sequence in the inverted CCAAT motif as in HF-1a, AGTGG (9, 16), consistent with the finding that EFI_A/YB-1 can bind to the HF-1a element. Accordingly, a series of experiments, including gel shift and DEPC interference footprinting, was designed to test whether the rat YB-1 homolog was capable of specifically recognizing the HF-1a site in the MLC-2v gene. The EFI_A/YB-1 protein was expressed in *E. coli* by the pET system as a fusion protein containing a polyhistidine sequence at the N terminus, which can bind to the metal ion Ni^+. The fusion protein has only a 33-amino-acid fusion peptide which included a 12-amino-acid epitope tag (T7 tag) derived from a bacteriophage T7 protein. The fusion protein was induced and affinity purified on an Ni^+ column (Invitrogen) and used in gel shift assays with an HF-1a probe. As displayed in Fig. 2, the YB-1 fusion protein binds to the HF-1a probe in a sequence-specific manner. Wild-type unlabeled HF-1a probe can compete for binding (lanes 3 and 6), while mutant HF-1a probes HF-1am1 and HF-1am2 (Table 1) cannot (lanes 4 and 7). The unlabeled double-stranded competitor probes were at 500-fold excess. To ensure reproducibility, the gel shift assays were repeated with independent preparations of the recombinant protein. As a negative control, we used bacterial extracts derived from the antisense EFI_A/YB-1 construct, which did not display detectable binding activity (data not shown). In addition, the monoclonal

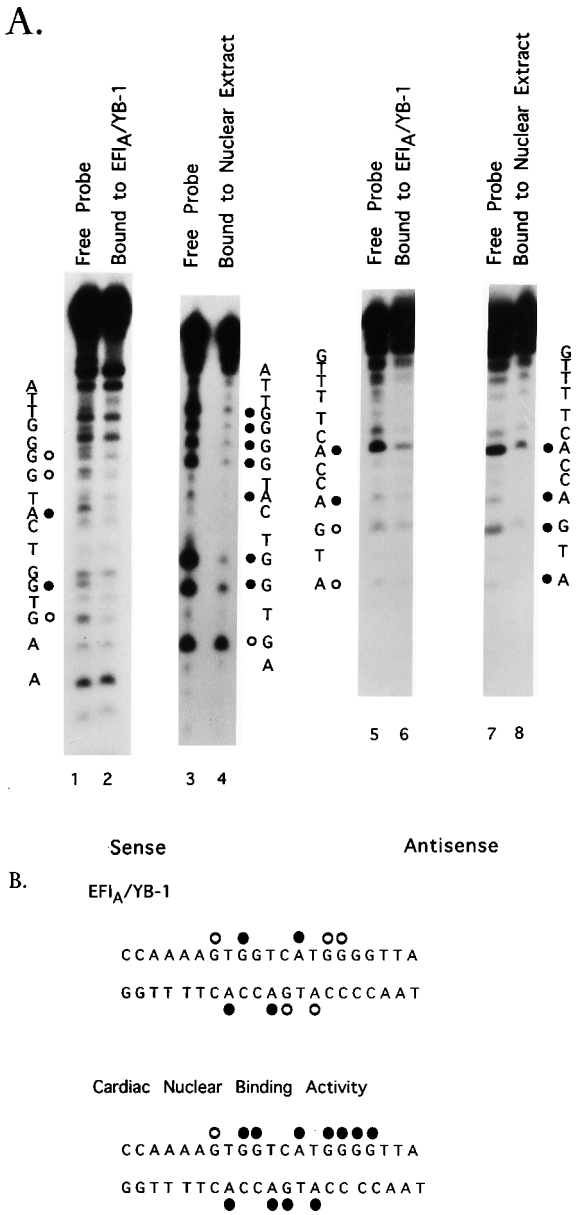

FIG. 3. (A) DEPC interference footprinting demonstrates specific protein-DNA interactions between EFI_A/YB-1 and the HF-1a duplex sequence. Equal amounts of radioactivity were loaded in the free and bound probe lanes. (B) Comparison of the contact points by the purified recombinant EFI_A/YB-1 and the endogenous nuclear factor(s) HF-1a revealed that EFI_A/YB-1 accounts for a major subset of contact points mainly localized at the 5′ portion of the HF-1a sequence. The promoter region shown here was from −71 to −51 (73) of the MLC-2v gene. Closed circles represent strong contact points; open circles represent weak contact points.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
HF-1a	CCAAAAGTGGTCATGGGGTTA
	GGTTTTCACCAGTACCCCAAT
HF-1am1	AGCTTGCCAAAAGTGGTCAGTGGGTTATTTTTAGGTAC
	ACGGTTTTCACCAGTCACCCAATAAAAATC
HF-1am2	CCAAAAGTCTTCATGGGGTTATTTTTAGGTAC
	GGTTTTCAGAAGTACCCCAATAAAAATCCATG
OCT1	TGTCGAAATGCAAATCACTAGAA
	ACAGCTTACGTTTAGTGATCTTT

antibody against the T7 epitope tag supershifted the YB-1/HF-1a complex (SSC1 in lane 2 of Fig. 4 can be better seen in a longer exposure), thereby confirming that the visualized complex contained the YB-1 fusion protein. These data demonstrate that YB-1 can specifically bind to a double-stranded HF-1a probe.

To further test the specificity of the EFI_A/YB-1 binding and to determine its contact points in the HF-1a sequence, DEPC interference footprinting was performed with an end-labeled duplex HF-1a probe and either purified recombinant EFI_A/

YB-1 or cardiac myocyte nuclear extracts. As shown in Fig. 3, a major subset of the contact points found with the endogenous HF-1a binding activity in cardiac nuclear extracts were also occupied by the recombinant EFI_A/YB-1. The closed circles represent strong contact points, and open circles represent weak contact points. The region with strong contact points includes the inverted CCAAT-like sequence on both strands and extends three more bases 3′ to the inverted CCAAT-like sequence. It should be noted that this is the same flanking region where the HF-1a and the Y-box motifs display the most similarity (Fig. 1A), suggesting that this sequence homology is responsible for the binding of EFI_A/YB-1. Both HF-1am1 and HF-1am2 have mutations within the contact point regions as assessed by the DEPC footprinting, confirming that the nucleotides which have been mutated are essential for binding. The DEPC footprints of the HF-1a binding activity in the cardiac myocyte nuclear extracts are generally consistent with the methylation interference footprinting data published previously (37, 40a) except that one more G (at −56) was found to be more strongly protected. It is possible that DEPC interference, which modifies G and A nucleotides with an ethyl group, is more sensitive than the methylation interference assays because of the larger size of the modifying group. Comparison of the HF-1a contact points by EFI_A/YB-1 with those by the nuclear HF-1a binding activity suggests that YB-1 may not be the sole component of the HF-1a binding activity, raising the possibility that some other cofactor(s) contributes directly or indirectly to the remaining contact points (Fig. 3B).

Since YB-1 can bind to single-stranded DNA as well as double-stranded DNA (24, 67, 68) and oligonucleotide probes have single-stranded DNA character, it became important to determine if YB-1 can bind to the HF-1a sequence in a double-stranded fragment excised from a plasmid vector. We have found that the end-labeled 250-bp fragment of the MLC-2v promoter specifically binds to the purified recombinant YB-1 protein through the HF-1a element (data not shown). In addition, we have directly compared the binding of the single-stranded sense and antisense HF-1a probes with that of the double-stranded HF-1a probe. Interestingly, YB-1 does not bind to the antisense strand and binds to the sense strand with a different mobility, which is clearly not seen in our gel shift experiments using a double-stranded probe (data not shown). In conclusion, we are looking at the double-stranded DNA binding activity of the YB-1 protein.

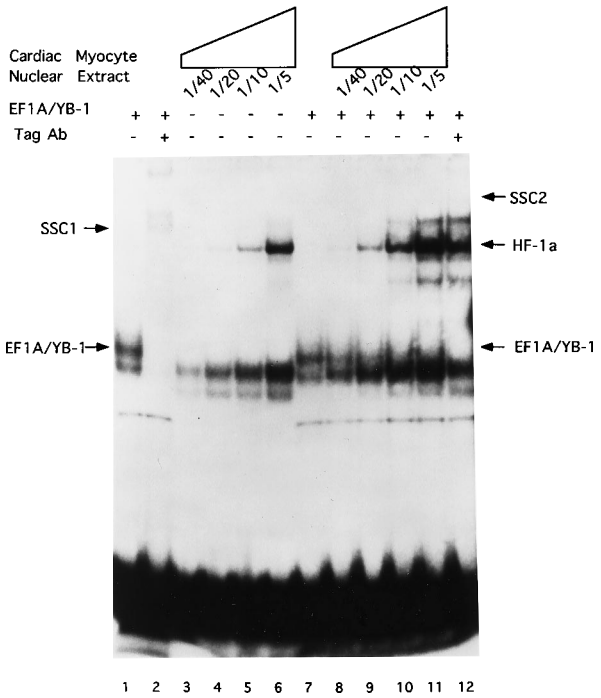

FIG. 4. EFI_A/YB-1 is a component of the endogenous HF-1a binding complex. A constant amount of the bacterially expressed and affinity-purified EFI_A/YB-1 protein was preincubated with increasing amounts of cardiac myocyte nuclear extracts diluted 1/40, 1/20, 1/10, and 1/5 from the nuclear extract preparation (lanes 8 to 11). Lane 7 is the purified EFI_A/YB-1 protein alone. Lanes 3 to 6 are increasing amounts of nuclear extracts alone. The EFI_A/YB-1 band decreased as the nuclear extracts amount increases, while the HF-1a band became more intense (lanes 7 to 11). At each concentration of the nuclear extracts, the HF-1a band is stronger with the preincubation with YB-1 than without the preincubation: lane 8 > lane 3, lane 9 > lane 4, lane 10 > lane 5, lane 11 > lane 6. The monoclonal antibody (Ab) against the T7 epitope tag, which recognizes only the epitope-tagged recombinant EFI_A/YB-1 and supershifts the EFI_A/YB-1 band (lane 2), can take away the increased portion of the HF-1a activity and reduce the HF-1a activity back to the same intensity as the nuclear extract alone (lane 12, which has YB-1 and 1/5-diluted nuclear extracts, has the same HF-1a activity left as lane 6, which has only the 1/5-diluted nuclear extracts). Supershifted complexes SSC1 and SSC2 are visible in a longer exposure (not shown).

EFI_A/YB-1 is a component of the endogenous HF-1a binding activity in cardiac nuclear extracts and directly associates with a 30-kDa protein. Although the previously mentioned studies suggest that EFI_A/YB-1 may account for the HF-1a binding factor in cardiac muscle cells, the mobility of the bacterially expressed EFI_A/YB-1 appeared to be faster than that of the endogenous HF-1a binding activity in gel shift assays using cardiac myocyte nuclear extracts (40a). Accordingly, the possibility exists that the endogenous HF-1a binding activity represents a complex of EFI_A/YB-1 with another protein cofactor(s). To determine if EFI_A/YB-1 was capable of forming a complex with other cardiac nuclear factors, we performed a series of experiments which used bacterially expressed EFI_A/YB-1 protein in mixing experiments with nuclear extracts derived from cardiac muscle cells. In the initial studies, a constant amount of EFI_A/YB-1 protein was preincubated with increasing amounts of nuclear extract. As displayed in Fig. 4, the intensity of the HF-1a band increased in intensity (lanes 8 to 11), while that of the bacterially expressed EFI_A/YB-1 band, with faster mobility, decreased with the addition of increasing amounts of cardiac nuclear extract (lanes 7 to 11). This result supported the possibility that the faster-migrating EFI_A/YB-1 complex was titrated out by some factor(s) in the cardiac nuclear extract, resulting in the formation of a larger complex that comigrated with the endogenous HF-1a band. In the control experiments, increasing amounts of cardiac nuclear extract were added to each gel shift reaction without the bacterial EFI_A/YB-1 protein (lanes 3 to 6). At each concentration of nuclear extract examined, the endogenous HF-1a band was considerably weaker in the control than in the preincubated mixtures (lanes 8 to 11 have stronger HF-1a bands than lanes 3 to 6). This result suggests the possibility that the addition of the bacterial EFI_A/YB-1 protein increases the intensity of the endogenous HF-1a band and thereby is participating in complex formation and that the concomitant decrease in the EFI_A/YB-1 band is not simply due to competition by the endogenous HF-1a-binding proteins. In addition, it should be noted that the mobility of the shifted YB-1 complex appeared identical to the mobility of the endogenous HF-1a binding activity. There is a formal possibility that the effect of EFI_A/YB-1 on increasing the HF-1a binding activity in gel shift assays was due to an indirect effect of the recombinant EFI_A/YB-1 to titrate out a factor which inhibits binding of the HF-1a factor. In this case, the recombinant EFI_A/YB-1 should not be present in the increased HF-1a band after preincubation. To test this possibil-

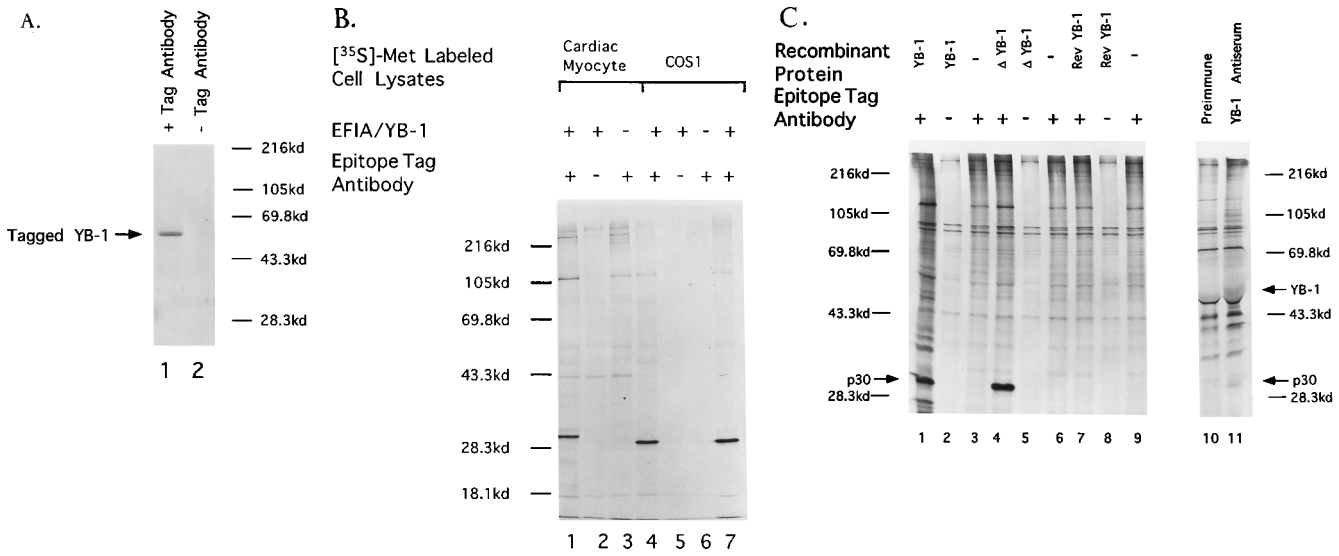

FIG. 5. EFI_A/YB-1 directly associates with a 30-kDa protein in the cardiac myocytes. (A) The monoclonal antibody against the T7 epitope tag can immunoprecipitate the in vitro-translated [^{35}S]methionine-labeled tagged EFI_A/YB-1. (B) Immunocoprecipitation. The cardiac myocytes were labeled with [^{35}S]methionine overnight. After preclearing with normal mouse serum, cell lysates were preincubated with the Ni^+ column affinity-purified EFI_A/YB-1 protein on ice for 1 h. A monoclonal antibody against the T7 epitope tag was used to immunoprecipitate the unlabeled bacterially expressed EFI_A/YB-1 protein and any associated protein(s). The precipitated complex was eluted by Laemmli's buffer and separated on an SDS–10% polyacrylamide gel. Lane 1 shows the EFI_A/YB-1-associated protein (30 kDa) in cardiac myocyte extracts; lane 4 shows the EFI_A/YB-1-associated protein (28 kDa) in the COS1 cell extracts. The same EFI_A/YB-1-associated protein was immunoprecipitated with the truncated YB-1 (ΔYB-1) without the DNA binding domain (lane 7). Lanes 2, 3, 5, and 6 were controls in which either the tag antibody or the EFI_A/YB-1 protein was omitted. None of these control lanes have the associated protein. (C) Immunocoprecipitation. Lanes 1 and 4 show that the same associated protein, p30, is coprecipitated by either the full-length YB-1 or the truncated YB-1 without the DNA binding domain. Lane 7 shows that no associated protein was found when the bacterial extract from the antisense construct of EFI_A/YB-1 is used, documenting the specificity of the interaction: the cofactor was not coprecipitated because of a nonspecific interaction with the 33-amino-acid epitope tag. Lane 11 shows that an antiserum against EFI_A/YB-1 can coprecipitate a 30-kDa protein from cardiac myocytes as well as the endogenous YB-1 protein (~52 kDa). Both the YB-1 and p30 bands were unique to immune serum and absent from the preimmune control. In fact, YB-1 and p30 bands have comparable intensities, suggesting a 1:1 stoichiometry.

ity, antibody supershift assays were performed. A monoclonal antibody against the T7 tag, which recognizes only the epitope-tagged recombinant EFI_A/YB-1 (lane 2), took away the increased portion of the HF-1a binding activity which was observed following the preincubation of nuclear extracts with purified recombinant EFI_A/YB-1, as evidenced by the reversal of the intensity of the HF-1a band (lane 12) to the level seen in assays using the nuclear extracts alone at the same concentration (lane 6). As a negative control, bacterial extracts derived from cells harboring the antisense YB-1 expression construct were preincubated with cardiac nuclear extract and did not increase the intensity of the HF-1a binding activity. Supershifted complexes SSC1 and SSC2 are visible in a longer exposure (not shown). The nuclear factor titration experiment was repeated multiple times with different preparations of recombinant EFI_A/YB-1 and cardiac myocyte nuclear extract, documenting the reproducibility of this result.

To independently assess whether EFI_A/YB-1 can complex with nuclear factors and to identify their relative sizes, neonatal rat ventricular myocytes and COS cells were metabolically labeled with [^{35}S]methionine and incubated with purified EFI_A/YB-1 protein which contained an epitope tag. To recover the EFI_A/YB-1 protein, the mixture was immunoprecipitated with a monoclonal antibody directed against the epitope tag. There was a single major protein (~30 kDa) which specifically associated with the EFI_A/YB-1 (Fig. 5B, lane 1). The apparent molecular mass of the associated protein from the cardiac myocytes was distinct from that of the associated protein from COS cells (~28 kDa) (lane 4). The same p28 protein was coprecipitated from COS1 cells by using a truncated EFI_A/YB-1 protein without the DNA binding domain (lane 7). Figure 5C shows that the full-length YB-1 and the truncated YB-1 (ΔYB-1) associated with the same major protein (lanes 1 and 4) in cardiac myocytes. Thus, in both cardiac myocytes and COS1 cells, the truncated YB-1 can associate with p30/p28, thereby indicating that interaction appears to be independent of the DNA binding domain of EFI_A/YB-1. As a negative control, we used bacterial extracts transformed with a T7-tagged pET expression construct harboring the EFI_A/YB-1 cDNA in the antisense orientation. Immunoprecipitation gave no evidence of a 30-kDa associated protein (lane 7). Thus, the cofactor does not interact with the 33-amino-acid T7 epitope tag and specifically interacts with EFI_A/YB-1. Figure 5A shows that the monoclonal antibody against the epitope tag can immunoprecipitate the in vitro-translated epitope-tagged EFI_A/YB-1. This antibody does not cross-react with any bacterial protein, as assessed by Western blotting (immunoblotting), and recognizes only the epitope-tagged EFI_A/YB-1 (data not shown). An antiserum against EFI_A/YB-1 can specifically coprecipitate a protein of the same molecular mass (30 kDa) with the 52-kDa endogenous EFI_A/YB-1 from cardiac myocytes (Fig. 5C, lane 11), suggesting a direct physical association between the p30 cofactor and EFI_A/YB-1 in vivo. Both YB-1 and p30 were not present in the preimmune control (lane 10).

Antiserum against *Xenopus* YB-3 can specifically abolish a component of the HF-1a binding activity in rat cardiac myocyte nuclear extracts. If EFI_A/YB-1 is a component of the endogenous HF-1a binding activity in cardiac muscle cells, then antibodies recognizing EFI_A/YB-1 should specifically supershift or remove the endogenous HF-1a binding factor(s) in gel shift assays. As revealed in Fig. 6A, antiserum to a *Xenopus* YB-3–glutathione *S*-transferase fusion protein (a kind gift from Wanda F. Reynolds) abolished a major component of the endogenous HF-1a binding activity in cardiac myocytes (lane

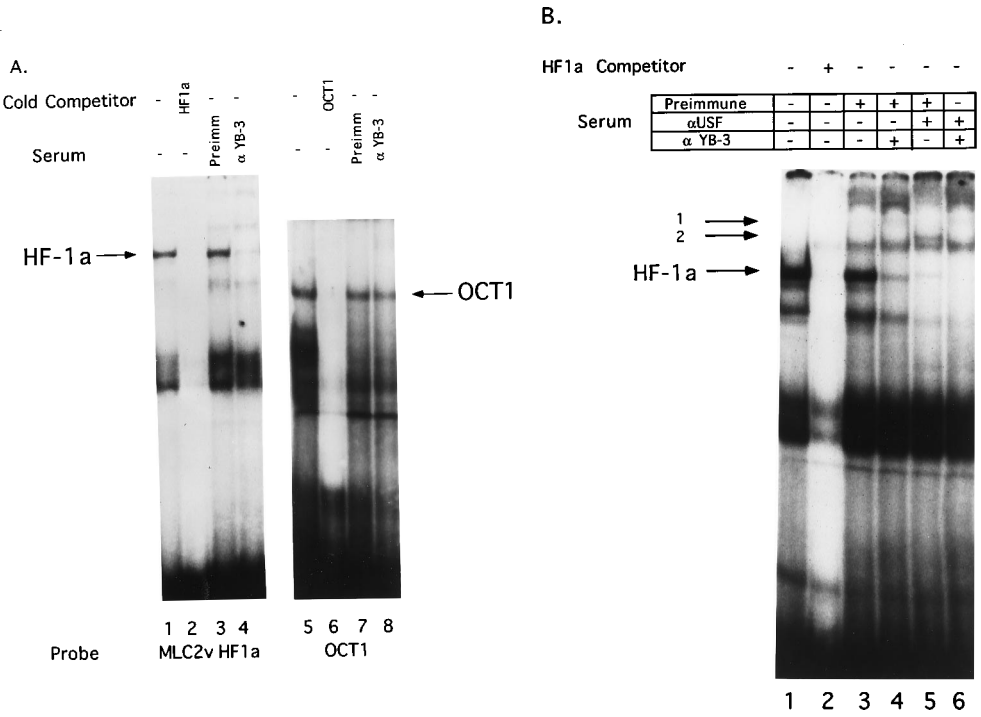

FIG. 6. EFI_A/YB-1 is a major component of the endogenous HF-1a binding activity. (A) Antiserum against the *Xenopus* YB-3 (100% identical in the DNA binding domain and 89% identical in overall amino acid sequence to EFI_A/YB-1 [10]) abolished the majority of the HF-1a binding activity (lane 4), while the preimmune serum did not (lane 3). The same serum has no effect on the OCT1 binding activity (lane 8). (B) Neither a YB-3 antiserum (αYB-3) nor a USF antiserum (αUSF) can completely abolish the HF-1a activity (lanes 4 and 5). Simultaneous addition of both antisera completely abolished the HF-1a activity. Note the different mobilities in the remaining HF-1a binding activities in lanes 4 and 5. Lane 3 has 2.5 μl of preimmune serum. Lane 4 has 2 μl of YB-3 antiserum and 0.5 μl of preimmune serum. Lane 5 has 0.5 μl of USF antiserum and 2 μl of preimmune serum. Lane 6 has 2 μl YB-3 antiserum and 0.5 μl of USF antiserum. Arrow 1 indicates a supershifted band observed when the YB-3 antiserum is added. Arrow 2 indicates a supershifted band observed when the USF antiserum was used. The supershifted bands are better seen in longer exposures. The film in panel B was exposed longer than that in panel A so that the remaining bands after the addition of either the YB-3 or USF antiserum were easier to see.

4). The preimmune control did not affect the HF-1a binding activity (lane 3). It should be noted that the *Xenopus* YB-3 is 100% identical in the DNA binding domain and 89% identical in overall amino acid sequence to the rat EFI_A/YB-1 (10). Only EFI_A has been isolated from rat cells so far. The YB-3 antiserum can recognize the bacterially expressed rat EFI_A/YB-1 in Western blotting and gel mobility shift assays (not shown). The same antiserum did not affect an OCT1 binding activity detected with a consensus OCT1 sequence, documenting the specificity of the antiserum. Since the YB-3 antibody will most likely cross-react with other Y-box factors, we cannot exclude the possibility that other Y-box factors also interact with HF-1a if they exist in the rat cardiac myocyte. But a YB-3 homolog has not been found in rat cells. However, the YB-3 antiserum did not completely remove all of the HF-1a binding activity, consistent with the presence of other nuclear factors which can bind to this HF-1a site independently. Recently, our laboratory provided evidence that the transcription factor USF can bind to an HF-1a probe and that the antiserum against USF can partially supershift a component of the endogenous HF-1a binding activity in cardiac myocyte nuclear extracts, but it cannot remove all of the endogenous HF-1a binding activity (40). As shown in Fig. 6B, both the YB-3 and USF antisera can remove a component of the HF-1a binding activity, but neither removed all of the HF-1a binding activity when they were added alone (lanes 4 and 5). When both were added in the same reaction, all of the HF-1a activity was abolished (lane 6). In fact, the remaining bands in lanes 4 and 5 have distinct mobilities, further supporting the notion that the YB-1 complex and the USF complex are two independent complexes and both antisera are specific. Each antiserum was used at the saturating level, and preimmune serum was supplemented so that each reaction mixture contained the same amount of total serum. These conditions ensure that the removal of HF-1a binding activity by addition of both antisera is not due to a titration effect with more serum. Since the core of the YB-1/cofactor binding site overlaps with the USF binding site in the HF-1a probe, we propose that the bindings of the YB-1/cofactor complex and USF are mutually exclusive. A doublet band migrates in the middle of the gel, which is usually not effectively competed for by the HF-1a probe itself. The DEPC interference assay did not show any specific contact points from these bands.

EFI_A/YB-1 transactivates the 250-bp MLC-2v promoter in cardiac myocytes. To study the functional role of EFI_A/YB-1 in regulating the MLC-2v promoter, cotransfection assays were performed with an EFI_A/YB-1 expression construct driven by the cytomegalovirus (CMV) promoter in a pCB vector (1, 46) and the MLC250-luciferase reporter. Cotransfection with the EFI_A/YB-1 expression construct resulted in a ~3.4-fold-higher luciferase activity than with the pCB vector alone in ventricular myocytes (Fig. 7). Activation of the MLC-250 promoter was reproducibly observed in four independent cotransfection experiments with four different preparations of the EFI_A/YB-1 expression construct. Each cotransfection reaction mixture contained three separate cell plates, and each plate was assayed in triplicate. Cotransfection of CMV–β-Gal was performed as an internal control, and the luciferase activity was

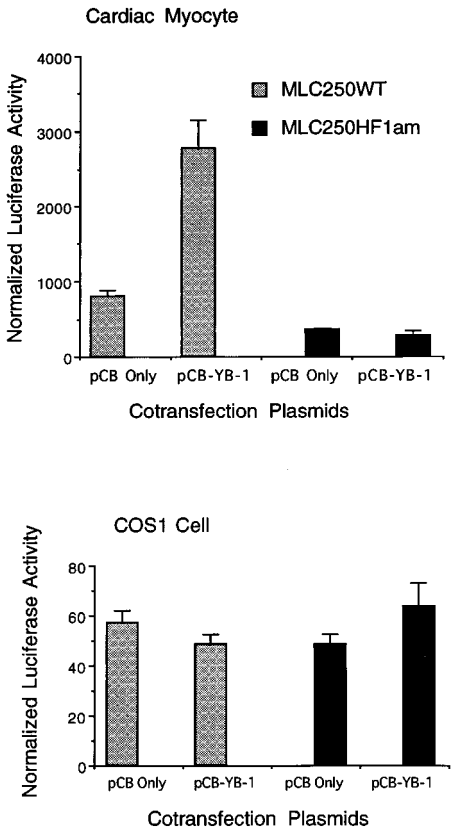

FIG. 7. YB-1 can activate the 250-bp MLC-2v promoter only in the cardiac myocytes through binding to the HF-1a site. For each independent pCB-EFI$_A$/YB-1 preparation, three plates with identical cell numbers were transfected under the same conditions with either the pCB-EFI$_A$/YB-1 or the pCB vector alone, and luciferase and β-Gal activities were measured to obtain the normalized luciferase activity from each plate. The normalized luciferase activities from each of three plates were averaged. Fold activation was obtained by dividing this normalized and later averaged luciferase activity of the pCB-YB-1-transfected cells by that of the pCB vector-only-transfected cells. Fold activation was then averaged from the results of different plasmid preparations. Four CsCl preparations of the YB-1 expression construct were repeated in cotransfection in cardiac myocytes with the wild-type MLC250-luciferase reporter. Three preparations of the YB-1 construct were repeated in the rest of the transfection experiments.

normalized to the β-Gal activity for each plate to correct for the efficiency of transfection. A point mutation in the HF-1a site of the MLC-2v promoter (HF-1am2) eliminated transactivation by the pCB-EFI$_A$/YB-1 construct (repeated with three independent preparations of the EFI$_A$/YB-1 expression construct). This mutant construct, which contains a two-base-pair point mutation, displayed significantly less activity than the wild-type construct (40a) but can be induced by the cotransfection of an expression construct of HF-1b (74), a zinc finger transcription factor which can bind to the HF-1b element in the MLC-2v promoter. It should be noted that the CMV promoter was slightly up-regulated by cotransfection with the YB-1 vector, but to a lesser extent than the MLC-250 promoter in the cardiac myocytes. Since this CMV promoter contains NF-1 CCAAT binding sites (5) which can be recognized by YB-1 with lower affinity (16), it is conceivable that the extent of transactivation of the MLC-2v promoter by EFI$_A$/YB-1 might be underestimated because of the inducibility of the CMV promoter upon overexpression of EFI$_A$/YB-1. Interestingly, the up-regulation of the MLC250- luciferase reporter by EFI$_A$/YB-1 was specific for the cardiac context. Cotransfection studies in a noncardiac cell line (COS1) with three independent preparations of an EFI$_A$/YB-1 expression construct provided no evidence of EFI$_A$/YB-1 transactivation at various concentrations of the YB-1 expression construct or at the same concentration of the YB-1 expression construct as that used in the cardiac myocyte transfection assays (Fig. 7). This result suggests that the activation of the MLC250 promoter may require cardiac factors that are absent in COS cells, e.g., HF-1b, MEF-2/RSRF, and/or a combinatorial code formed with other DNA-binding factors that are restricted to the cardiac cell context. Alternatively, it is also possible that the presence of potentially distinct cofactors, such as p30 (cardiac) versus p28 (COS1), might contribute to the cell context specificity of the functional effect of EFI$_A$/YB-1 on MLC-2v promoter activity. The absence of activation in the COS1 cell context supports the cell type specificity of the action of EFI$_A$/YB-1 on the MLC-2v promoter and suggests that EFI$_A$/YB-1 is not simply acting on the translation of the reporter gene, a function that has been reported for another Y-box family member, FRGY2, which localizes in the cytoplasm and can affect the translation of mRNA (2, 49). By using an EFI$_A$/YB-1 antiserum, EFI$_A$/YB-1 was found to be localized in the cardiac myocyte nucleus (75), suggesting a nuclear function of EFI$_A$/YB-1 similar to that of the *Xenopus* homolog FRGY1 in *Xenopus* cells (49).

DISCUSSION

EFI$_A$/YB-1 is a component of the cardiac HF-1a binding activity and regulates MLC-2v promoter activity. Y-box factors are composed of a conserved family of nucleic acid-binding proteins that can serve as transcriptional regulators and, in vertebrate oocytes, can sequester mRNA from translation (2, 49, 61, 62, 67, 68). The first report of the importance of Y-box-binding proteins in transcriptional regulation was provided by Didier et al., who identified YB-1 as the factor that binds to the Y box, an important regulatory element containing an inverted CCAAT motif in the promoters of a number of human MHCII genes (13). Human YB-1 and a related protein were also isolated by Southwestern screening with a CCAAT-containing site in the epidermal growth factor promoter (54). Subsequently, members of the YB-1 family have been shown to positively regulate a number of eukaryotic promoters, including the Rous sarcoma virus long terminal repeat (16, 44), *Xenopus* heat shock protein 70 (61), human MHCII gene (72), and herpes simplex virus thymidine kinase (61) promoters. YB-1 has also been proposed to regulate genes involved in DNA synthesis and cell growth, such as proliferating cell nuclear antigen/cyclin (53, 63), thymidine kinase (31, 53), and DNA polymerase α (47, 53). Although Y-box factors are ubiquitously expressed in the adult tissues, there is increasing evidence that they may also play a role in the maintenance of tissue-specific expression, as members of the Y-box factor family have been implicated in the control of germ cell-specific gene expression in *Xenopus laevis* (68) and liver-specific gene expression in chickens (21). In addition, recent studies have implicated an important role for a Y box in the inducible expression of the human thrombospondin I gene following serum stimulation (18) in coordination with a serum response element. An NF-Y site and a p67 serum response factor binding site (CArG) are both required for the promoter activity of the β-actin gene. The Y-box element and its binding factors are implicated in the alpha/beta interferon response of the mouse (2-5) oligodenylate synthase gene (70) and tumor necrosis factor alpha enhancement of gamma interferon-induced expression of the MHCII genes, such as HLA-DRA, in astrocytes (45). YB-1 mRNA itself can be induced by interleukin 2 in

T-helper lymphocytes (53). An increasing body of evidence suggests that Y-box factors may serve as highly conserved eukaryotic transcriptional regulators in a variety of cell contexts and may be involved in tissue-specific and inducible expression.

The present study provides evidence that the rat homolog of YB-1 (EFI_A) can specifically bind to the HF-1a cardiac regulatory element in the MLC-2v gene and can positively regulate the transcription from a 250-bp MLC-2v promoter which can confer ventricular cell-specific expression in both cultured myocardial cells and transgenic mice (29, 73). Southwestern screening of a cardiac cDNA expression library resulted in the isolation of several independent clones which were identical to the rat EFI_A/YB-1; the HF-1a site contains an inverted CCAAT-like sequence, and the flanking sequences display a high degree of similarity with the consensus Y-box site found in the MHCII promoter region. EFI_A/YB-1 displays sequence-specific binding to HF-1a and requires a major subset of contact points by the endogenous HF-1a binding activity in cardiac muscle cells. Furthermore, antiserum against the highly homologous YB-3 (with 100% amino acid sequence identity in the DNA binding domain and 89% overall amino acid sequence identity) (10) effectively removes the endogenous HF-1a complex in gel shift assays employing cardiac nuclear extracts. Finally, cotransfection studies demonstrate that EFI_A/YB-1 can not only bind to this HF-1a site in a sequence-specific manner but also transactivate MLC250-luciferase reporter genes. This up-regulation appears to require an intact HF-1a site, again providing evidence for the specificity of the functional effects of EFI_A/YB-1 in the regulated expression of the MLC-2v gene. Since the HF-1a site has been previously shown to be required for the maintenance of cardiac and ventricular cell-specific expression of the MLC-2v gene in both cultured cells and in transgenic mice, these studies provide evidence for the importance of Y-box-binding factors in the regulation of the cardiac muscle gene program. To our knowledge, this is the first direct evidence that Y-box-binding factors can play a positive regulatory role in the muscle gene program. Recent studies by Gore et al. have suggested that YB-1 can bind in a sequence-specific manner to the thyroid hormone response element in the β-myosin heavy-chain promoter region and may mediate the thyroid hormone response (20). An M-CAT/Y-box motif from the rat phosphoglycerate mutase subunit M promoter is required for basal activity and can confer skeletal muscle specificity to a heterologous promoter (52). Our results add to be an increasing body of evidence that EFI_A/YB-1 may be involved in the control of the muscle gene program. These studies extend the potential regulatory importance of this highly conserved nucleic acid-binding factor family to the control of the terminally differentiated phenotype during cardiac myogenesis.

A major component of the endogenous HF-1a binding activity in cardiac muscle cells may consist of a YB-1/p30 complex. In this study, three independent lines of evidence suggest that a major component of endogenous HF-1a binding activity in cardiac muscle cells consists of a complex of the rat homolog of YB-1 and a nuclear factor of approximately 30 kDa (p30). First, antibodies recognizing EFI_A/YB-1 can specifically abolish a component of the HF-1a complex in gel shift assays using cardiac nuclear extracts. Second, the mobility of the YB-1/HF1a complex is faster than that of the endogenous HF-1a binding activity. Mixing experiments with cardiac nuclear extracts document that the EFI_A/YB-1 protein can form a complex, with an endogenous cardiac nuclear factor, that comigrates with the endogenous HF-1a complex in cardiac muscle extracts. Finally, immunoprecipitation studies suggest that YB-1 has a major associated protein, p30.

Our studies using a purified, truncated YB-1 protein have localized the domain in YB-1 which interacts with p30 to a fragment downstream from the DNA binding cold shock domain, providing further evidence of specificity for the YB-1/p30 interaction. This region contains nine alternately positively and negatively charged stretches of amino acids and has been termed a charge zipper (44). The charge zipper has been proposed to mediate protein-protein interactions and was later shown by mutational analysis to be required for protein-protein complex formation (62). In preliminary studies, our laboratory has shown that the YB-1/p30 complex formation is independent of α-adrenergic agonist and serum stimulation of cardiac muscle cells. However, it is currently unclear whether the complex is quantitatively regulated following stimulation (75).

Interestingly, previous studies by Qasba et al. (48) suggested that two fractions in chicken heart nuclear extracts can bind to the conserved A element in the chick myosin light-chain 2 promoter which includes the HF-1a site and have estimated that one of these components is a protein of ~50 kDa (similar to the size of EFI_A/YB-1 by SDS-PAGE). Binding of this factor to HF-1a can be efficiently competed for by a CCAAT sequence from murine sarcoma virus long terminal repeat. In gel shift assays, the other fraction has a faster mobility than the ~50-kDa protein (48). These results parallel our findings that a CCAAT-binding factor YB-1 binds to HF-1a and the notion that a 30-kDa protein may be the partner of YB-1 in binding to the HF-1a site.

The finding of a partner for YB-1 suggests the possibility of the formation of a ternary complex at the HF-1 cardiac regulatory element in the MLC-2v promoter region. At present, the precise function of the p30 partner is unclear. One possibility is that it serves as a transcriptional activating factor or accessory protein, which is incapable of binding directly to the HF-1a site or binds to HF-1a weakly. In this manner, p30 may interact with YB-1 to form a stable complex on the HF-1a site, or it may bridge YB-1 to HF-1b/MEF-2-binding proteins to form a supercomplex. Obviously, further direct analysis of the role of p30 in the regulation of MLC-2v expression awaits its molecular characterization. Cloning of p30 by the yeast two-hybrid system using a rat heart library is currently in progress. Although it has been suggested that the EFI_A has a partner that facilitates the binding of EFI_A to the EFI element in the Rous sarcoma virus long terminal repeat (16, 44), this is the first report that EFI_A/YB-1 physically associates with a single major protein in different cell types.

HF-1a/HF-1b is a paradigm for cardiac muscle gene regulation. Previous studies have documented that the conserved 28-bp element (HF-1) can confer cardiac muscle-specific expression in transient assays in cultured myocardial cells. In addition, the HF-1 element has been implicated in the inducible expression of the MLC-2v gene during myocardial cell hypertrophy (8). Two distinct regulatory elements are found within the 28-bp element (HF-1a and HF-1b), and both of these sites are required for the maintenance of cardiac and ventricular chamber-specific expression of the MLC-2v luciferase fusion genes in transgenic mice (28). The HF-1b site consists of an AT-rich element which corresponds to a consensus binding site for MEF-2/RSRF. MEF-2 can bind to this AT-rich element and may be an important regulator of the MLC-2v gene in the ventricular cell context during cardiac growth and development (71). In addition, our laboratory has isolated a novel member of the C_2H_2 zinc finger protein family which can bind in a sequence-specific manner to the HF-1b site (74). This factor has a high degree of homology to SP-1 within its zinc fingers and is more widely divergent outside of this

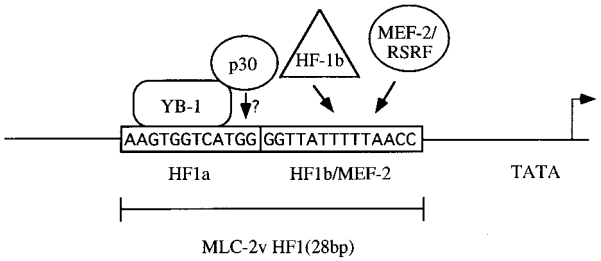

FIG. 8. An E-box-independent pathway involving the YB-1 complex and the AT-rich MEF-2-binding factors is the primary pathway for cardiac-specific and inducible expression of the MLC-2v gene.

DNA binding domain. HF-1b is tissue restricted in its expression and is expressed predominantly in brain, skeletal muscle, and cardiac muscle. While the HF-1b site clearly is important for the maintenance of cardiac muscle specificity, it is also clearly not sufficient to maintain cardiac cell-specific expression and requires an intact adjacent HF-1a site (Fig. 8).

The paradigm for the maintenance of cardiac muscle specificity via interactions between ubiquitously expressed factors (YB-1) and tissue-restricted factors (MEF-2/HF-1b) is paralleled in a number of muscle regulatory elements in other cardiac and skeletal muscle promoters. The cooperation between elements which bind ubiquitous factors and tissue-restricted factors has been found in the troponin T gene (M-CAT and MEF-2) (34, 35), rat aldolase A (MAF/MEF-3 and MEF-2) (25), mouse troponin C (MEF-3 and MEF-2) (26), the mouse myogenin promoter (MEF-3 and MEF-2) (25), and the alpha myosin heavy chain (BF-2 and MEF-2) (37, 38). In each case, these sequences are located in relatively close proximity (less than 60 bp), suggesting that protein-protein interactions are involved in the cooperation. The cooperativity between the HF-1a- and HF-1b-binding factors will be investigated in future studies. Since the report of the HF-1a binding activity (40a), several groups have suggested that an HF-1a-binding factor(s) also binds to some regulatory elements in different promoters, such as the alpha myosin heavy-chain (22, 37) and cardiac α-actin (39) promoters, as judged from gel shift competition assays. Accordingly, the identification of the HF-1a-binding factor(s) may improve our understanding of the transcriptional regulation of a panel of cardiac muscle-specific genes. Interestingly, Gupta et al. (22) recently characterized the binding factors that interact with an E-box/M-CAT hybrid motif, which can be competed for by the HF-1a sequence, by UV cross-linking. They found a 28-kDa and a 25-kDa protein, in addition to a wide band at 53 kDa whose size corresponds to that of the M-CAT-binding factor TEF-1. It should be noted that YB-1 and M-CAT have similar mobilities on an SDS-polyacrylamide gel (52 to 55 kDa). These data are not inconsistent with our findings that one of the HF-1a-binding components contains a 52-kDa YB-1 and an approximately 30-kDa cofactor. Cloning of the 30-kDa protein will be required to address this issue in detail.

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