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Regulation of the Murine αB-Crystallin/Small Heat Shock Protein Gene in Cardiac Muscle

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The murine αB-crystallin/small heat shock protein gene is expressed at high levels in the lens and at lower levels in the heart, skeletal muscle, and numerous other tissues. Previously we have found a skeletal-muscle-preferred enhancer at positions −427 to −259 of the αB-crystallin gene containing at least four *cis*-acting regulatory elements (αBE-1, αBE-2, αBE-3, and MRF, which has an E box). Here we show that in transgenic mice, the αB-crystallin enhancer directs the chloramphenicol acetyltransferase reporter gene driven by the αB-crystallin promoter specifically to myocardiocytes of the heart. The αB-crystallin enhancer was active in conjugation with the herpes simplex virus thymidine kinase promoter/human growth hormone reporter gene in transfected rat myocardiocytes. DNase I footprinting and site-specific mutagenesis experiments showed that αBE-1, αBE-2, αBE-3, MRF, and a novel, heart-specific element called αBE-4 are required for αB-crystallin enhancer activity in transfected myocardiocytes. By contrast, αBE-4 is not utilized for enhancer activity in transfected lens or skeletal muscle cell lines. αBE-4 contains an overlapping heat shock sequence and a reverse CArG box [5′-GG(A/T)$_6$CC-3′]. Electrophoretic mobility shift assays with an antibody to serum response factor and a CArG-box-competing sequence from the c-*fos* promoter indicated that a cardiac-specific protein with DNA-binding and antigenic similarities to serum response factor binds to αBE-4 via the reverse CArG box; electrophoretic mobility shift assays and antibody experiments with anti-USF antiserum and heart nuclear extract also raised the possibility that the MRF E box utilizes USF or an antigenically related protein. We conclude that the activity of the αB-crystallin enhancer in the heart utilizes a reverse CArG box and an E-box-dependent pathway.

Crystallins comprise approximately 90% of the total soluble protein of the transparent eye lens of a vertebrate (8, 77). These proteins include three families of ubiquitously expressed crystallins (α, β, and γ), as well as taxon-specific crystallins found only in certain species (21, 62, 63). α-Crystallins are members of the small heat shock protein family (22) which can act as molecular chaperones (37) and possess autokinase activity (41).

The αB-crystallin/small heat shock protein gene (43) is expressed constitutively at very high levels in the lens and also to a considerable extent in a variety of other tissues, including the heart, skeletal muscle, lung, and brain (4, 26). The murine αB-crystallin gene contains a skeletal-muscle-preferred enhancer (−427 to −259) (25) which also functions to a lesser degree in lens and lung cell lines (25, 32, 35). The αB-crystallin enhancer contains at least four *cis*-acting regulatory elements (αBE-1, αBE-2, αBE-3, and MRF) (32). αBE-1 and αBE-2 are utilized for expression in the lens, skeletal muscle, and lung (32, 35), αBE-3 is important for expression in the lens and skeletal muscle, and MRF is essential for αB expression in skeletal muscle. The MRF contains an E box (18, 29) and binds MyoD family members for its function in transfected C2C12 muscle cells (32). Transgenic mouse experiments have established that sequences between −426 and −164 (which include the enhancer) are required for high-level expression in heart and skeletal muscles, while sequences downstream of −164 (which lack the enhancer) are sufficient for expression in the lens (33).

The molecular basis of cardiac myogenesis is not well understood. Many skeletal-muscle-specific genes are transcriptionally controlled by myogenic factors (basic helix-loop-helix [bHLH] proteins, e.g., MyoD and myogenin) that have not been detected in the heart (9, 45, 59, 60, 67). Thus, it is uncertain whether αB-crystallin gene expression in cardiac muscle requires the same combination of elements that are used in skeletal muscle or a different combination. Indeed, many skeletal- and/or heart muscle-specific genes use a combination of tissue-specific and ubiquitous factors for their regulation (5, 46).

Here we have established that in transgenic mice, the activity of the murine αB-crystallin enhancer in the heart is confined to myocardiocytes. Consequently, we have used a culture system with rat primary myocardiocytes (PMC) (39) to analyze αB-crystallin expression in the heart and have employed DNase I footprinting, electrophoretic mobility shift assays (EMSA), and transient-transfection experiments coupled with site-directed mutagenesis to identify the regulatory elements used by the murine αB-crystallin enhancer in myocardiocytes. The results show that the enhancer activity in myocardiocytes, as in transfected skeletal muscle cells (32), requires αBE-1, αBE-2, αBE-3, and MRF, but unlike in skeletal muscle cells, requires an additional enhancer element, called here αBE-4. αBE-4 contains a sequence, 5′-GGAATCTTCC-3′, that resembles a reverse CArG box [5′-CC(A/T)$_6$GG-3′], a control sequence found in other genes expressed in the heart (50, 52, 61, 65, 79). In addition, we report here that the E-box-containing MRF element of the αB enhancer, which binds MyoD family members in skeletal muscle, is also required for the activity in transfected myocardiocytes, in which it appears to interact with USF or a nuclear factor antigenically related to USF.

MATERIALS AND METHODS

Northern (RNA) blot hybridization. Total RNA isolated from neonatal rat heart, neonatal rat liver, or cultured rat myocardiocytes was fractionated by elec-

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trophoresis through 1.5% agarose–formaldehyde gels, transferred to a Duralon membrane (Stratagene, La Jolla, Calif.), and hybridized to a 230-bp *Hin*dIII-*Bam*HI restriction fragment from exon 3 of the mouse αB-crystallin gene (26). The probe was labeled by using the Prime-A-Gene random prime labeling system (Promega, Madison, Wis.). Prehybridizations were performed at 60°C for 20 min, and hybridizations were carried out at 60°C for 60 min by using QuikHyb (Stratagene) according to the manufacturer's instructions. Membranes were washed twice for 15 min each with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 22°C, twice at 60°C for 15 min each with the same buffer, and autoradiographed. Hybridization to an end-labeled oligodeoxynucleotide complementary to rat 28S rRNA was also performed as previously described (27) to monitor the integrity of RNA, the relative amounts of RNA loaded on the gel, and the efficiency of transfer to Duralon membranes. Membranes were exposed for autoradiography on Kodak XAR5 film at −80°C with an intensifying screen for 4 h.

Primer extension. For primer extension analysis, 2×10^5 cpm of end-labeled primer 8879 (5′-GTGGTGGATGGCGATGTCCAT-3′; complementary to the coding sequence from +46 to +66 of the murine αB-crystallin gene) was incubated with 5 μg of FVB/N mouse heart total RNA for 5 min at 70°C and allowed to cool to room temperature. Extension was carried out at 70°C for 20 min by using thermostable r*Tth* reverse transcriptase (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's instructions. Following ethanol precipitation, samples were resuspended in formamide-dye buffer and analyzed on an 8% polyacrylamide–8 M urea sequencing gel in parallel with a sequence ladder obtained by using unlabeled primer 8879 and an αB-crystallin cDNA clone.

Histochemical staining for CAT. Adult transgenic mice and wild-type littermates were anesthetized with ketamine-xylazine (7 to 8 mg/100 g of body weight) and perfused with saline and subsequently with 2% paraformaldehyde for 5 min. Hearts were removed, fixed in 2% paraformaldehyde, and cryoprotected as described by Donoghue et al. (24). Hearts were sectioned to 5 to 10 μm in a cryostat, mounted on gelatin-coated slides, and stained for chloramphenicol acetyltransferase (CAT) by incubation in a mixture of sodium citrate, potassium ferrocyanide, chloramphenicol, and acetyl coenzyme A for 6 h at room temperature (24). The slides were washed briefly in water and mounted for microscopic examination.

Plasmid constructions and site-directed mutagenesis. 5′ deletion fragments containing the αB-crystallin promoter attached to the CAT reporter gene and wild-type and mutant enhancer-containing plasmids derived from ptkGH (Nichols Institute, Capistrano, Calif.) have been described previously (25). pUCSV2PAP (1) was kindly provided by S. D. Hauschka (University of Washington, Seattle) and was constructed from pSV2PAP (36). For site-directed mutagenesis, a restriction fragment spanning positions −427 to −259 of the murine αB-crystallin gene (with *Hin*dIII linkers at both ends) was cloned into the *Hin*dIII site of bacteriophage M13mp19 and mutations (Mu-9788 to Mu-9790) were introduced into the αBE-4 site of the enhancer by using Amersham's site-directed mutagenesis kit (version 2; Amersham Corp., Arlington Heights, Ill.). Mutated restriction fragments were subcloned into ptkGH as described previously (32). All constructs were confirmed by sequencing the ligation junctions and mutated regions with Sequenase (U.S. Biochemicals Corp., Cleveland, Ohio).

Nuclear extracts, EMSA, and DNase I footprinting. Nuclear extracts were prepared from the hearts of 2-month-old mice and C2C12 myotubes (7) by the method of Dignam et al. (23). The final protein concentrations were 6.6 mg/ml (heart extract) and 6.0 mg/ml (C2C12 extract). Complementary oligodeoxynucleotides were synthesized (ABI model 380A synthesizer) and annealed as described previously (25). Double-stranded oligodeoxynucleotides E1 (−426 to −371), E2 (−370 to −315), E3 (−314 to −258) (25), and MRF (−307 to −282) of the murine αB-crystallin enhancer were used for EMSA and labeled on one strand by using polynucleotide kinase-[γ-^{32}P]ATP. EMSA were performed with 0.5 ng of labeled DNA in a final volume of 25 μl containing the standard buffer (12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 60 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 4% [wt/vol] Ficoll 400), 1 μg of a nonspecific competitor [poly(dI-dC)], and 0.5 to 6 μl of nuclear extract or purified protein. DNase I footprinting was performed with a radiolabeled *Kpn*-*Bam*HI fragment from the αB-crystallin enhancer (−427 to −259) as described earlier (32). The gels were dried and autoradiographed on Kodak XAR5 film at −80°C for 12 h with intensifying screens.

Cell culture, transient-transfection, and enzyme assays. The rabbit N/N1003A lens cell line (64) was propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in 10% CO_2. The murine muscle cell line C2C12 (7) was maintained and transfected as myoblasts by being grown in DMEM plus 20% fetal calf serum (proliferative medium) in 5% CO_2. Myotube differentiation was induced by adding DMEM containing 5% horse serum. All media contained 50 μg of gentamicin per ml. Cells were propagated in 60-mm-diameter plastic dishes. Primary myocardiocyte cultures (PMC) were prepared from 2- to 3-day-old neonatal rats (Sprague-Dawley; Taconic Farms). Excised hearts were trypsinized, and myocardiocytes were purified by using a discontinuous Percoll gradient according to the method of Iwaki et al. (39). Cells were plated on 100-mm-diameter collagen-coated dishes at a density of 2.7×10^6 in plating medium (DMEM-medium 199 [1:4] containing 10% horse serum, 5% fetal calf serum, 100 U of penicillin G per ml, 0.1 mg of streptomycin per ml, and 250 ng of amphotericin B per ml).

For rabbit N/N1003A lens cells and murine C2C12 skeletal muscle cells, 4 μg of test plasmid and 2 μg of internal control plasmid were cotransfected by calcium phosphate precipitation (34) as described previously (25). For PMC, 8 μg of test plasmid and 2 μg of internal control plasmids (placental alkaline phosphatase [36]) were added directly to the medium on the plate. Cells were glycerol shocked after 4 h, and the plating medium was replaced with serum-free maintenance medium (DMEM-medium 199 [4:1] containing 1 M insulin and 250 ng of amphotericin B per ml).

Lens cells (N/N1003A) were harvested after 30 h, muscle cells (C2C12 myotubes) were collected 51 h after glycerol shock, and cells from PMC were harvested 48 h after glycerol shock. (Note that C2C12 cells were transfected as myoblasts and harvested after myotube formation.) Extracts were prepared by the method of Amacher et al. (1). The human growth hormone levels in media were determined with a commercially available radioimmunoassay kit (Nichols Institute). Placental alkaline phosphatase activity was determined as described previously (36), and CAT activity was determined by biphasic assay (57). The transfection data represent the means of three separate experiments, each performed with duplicate plates. At least six myocardiocyte preparations were used in these experiments.

RESULTS

Histochemical staining for CAT in the hearts of transgenic mice. In previous studies of transgenic mice containing an αB-crystallin–CAT reporter transgene (33), we established that the αB-crystallin promoter fragment containing the −426 to +44 sequence gives a very high level of transgene expression in the lens, skeletal muscle, and heart. In order to extend this analysis, we examined the expression of this transgene within the heart by histochemistry. CAT-stained sections of the heart revealed that the expression of this transgene was confined to myocardiocytes (Fig. 1A). Connective tissues and blood vessels were not detectably stained (Fig. 1B). Myocardiocytes from nontransgenic mice did not show any CAT staining (Fig. 1C).

Northern blot hybridization of αB-crystallin RNA in rat myocardiocytes. In order to detect αB-crystallin RNA in PMC, Northern blot hybridizations were performed with total RNAs isolated from primary myocardiocytes cultured for different lengths of time. The results are shown in Fig. 2. A 0.9-kb hybridizing transcript was observed in RNA samples from the neonatal rat heart (Fig. 2, lane 1) and rat PMC (lanes 2 to 5) at each time point examined. While it appears that the level of expression is somewhat higher at the 46-h time point (Fig. 2, lane 3), hybridization to a 28S rRNA probe (lower portion of Fig. 2) as well as examination of the stained gel (data not shown) indicated that an excess of RNA in this lane accounts for the increased signal intensity. The presence of a hybridizing transcript of approximately 0.9 kb in neonatal rat heart RNA (Fig. 2, lane 1) and the absence of such a transcript in neonatal rat liver RNA (lane 6) are consistent with the αB-crystallin expression patterns previously observed in adult rats (38) and 1- to 2-week-old as well as 9- to 11-week-old mice (26).

Determination of the transcription initiation site for the αB-crystallin gene in the heart. On the basis of direct sequencing of αB-crystallin RNA from the mouse lens, the T nucleotide within the sequence 5′-GCTGAAGGA-3′ was designated the lens transcription initiation site (26). Transcription initiation at or near this location was suggested by primer extension experiments with RNAs derived from several nonlenticular tissues, including the heart (26). However, because radiolabeled size markers rather than a sequencing ladder were used to estimate the length of the extended fragment(s), the precise heart initiation site(s) was not established unequivocally. Therefore, we have performed primer extension experiments on RNA derived from the murine heart and electrophoresed the products adjacent to a sequencing ladder (Fig. 3). A major band was detected at +3 (relative to the lens initiation site), and two fainter bands were obtained at +2 and +4. No larger extended products were detected. The smaller bands were presumably incomplete extension products.

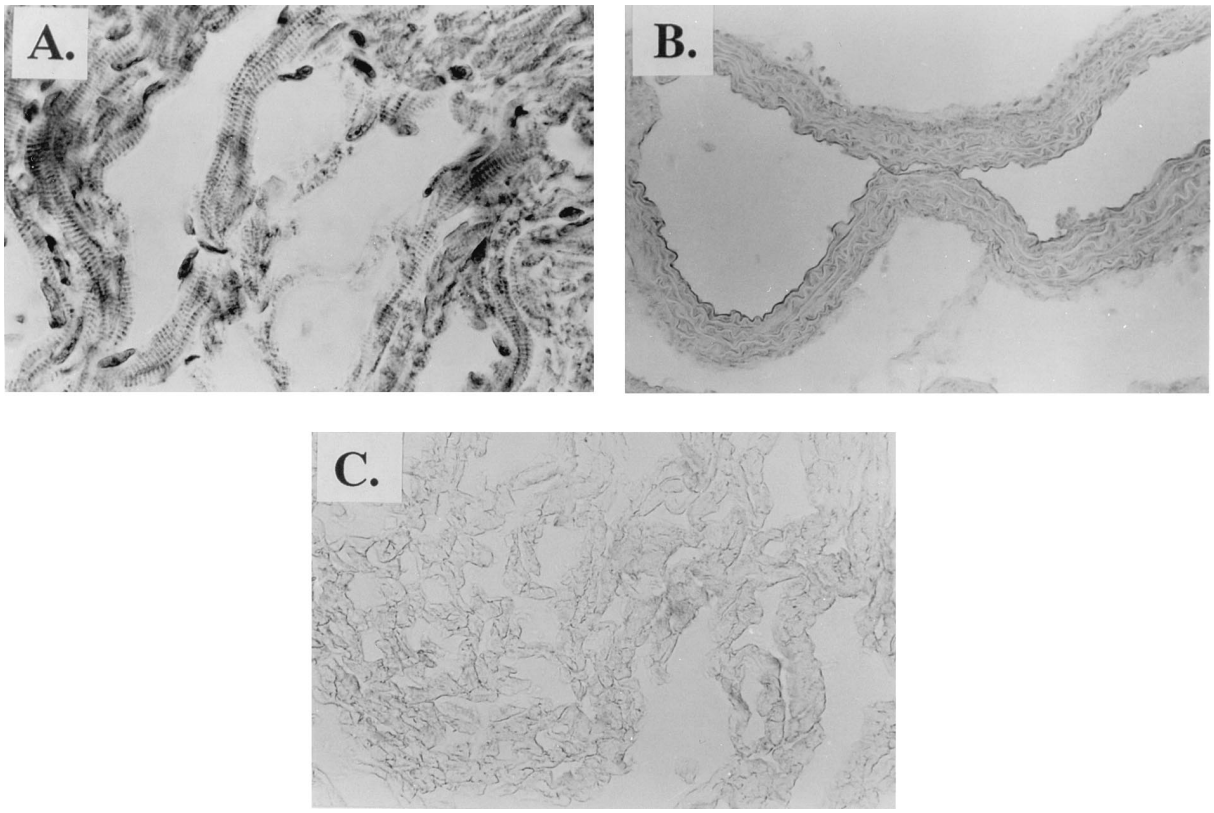

FIG. 1. (A) Demonstration of the homogeneous staining of cardiac myocytes in the heart of F_1 transgenic mouse no. DB140074. Histochemical staining for CAT in a heart section of a transgenic mouse containing the αB426-CAT fragment (33). CAT staining of a heart section showing connective tissue of transgenic mouse no. DB140074 (B) and of a control heart section of nontransgenic mouse no. DB140069 (C).

DNase I footprinting of the αB-crystallin enhancer. DNase I footprinting was performed in order to identify the regions within the αB-crystallin enhancer that bind heart nuclear proteins. Regions protected from DNase I digestion by crude nuclear extracts from the mouse heart are shown in Fig. 4. The previously identified *cis*-acting regulatory elements (αBE-1, αBE-2, αBE-3, and MRF) of the αB-crystallin enhancer were protected from digestion with DNase I (open boxes in Fig. 4). There could be another footprinted region at the 3′ end of the upper strand; it is hard to reach any conclusion for this region as it is very close to the end of the probe. It is, however, definitely separated from the MRF site, as denoted by asterisks (Fig. 4). The footprinted regions obtained with heart nuclear extracts were generally similar to the footprinted regions previously obtained with lens and C2C12 myotube nuclear extracts (32), except that the footprints of the αBE-1, αBE-2, and MRF elements obtained with heart nuclear extracts were more extended in both the 5′ and 3′ directions. In addition, positions −369 to −384 and −389 to −394 of the upper (sense) strand and positions −371 to −388 of the lower (antisense) strand were protected by the heart nuclear extract (solid boxes in Fig. 4). The upper-strand footprints were quite strong, whereas weaker protection of the lower strand was observed (note the disappearance of the faint band near −388 and the decrease in the intensity of the band in the −371 region; compare lane 1 with lanes 2 and 3 for the lower strand in Fig. 4). We named this protected region αBE-4. It contains a sequence resembling a reverse CArG box within the heat shock consensus sequence (Fig. 5). In previous experiments, αBE-4 was not protected by nuclear extracts from lens (αTN4-1) (78), fibroblast (L929)

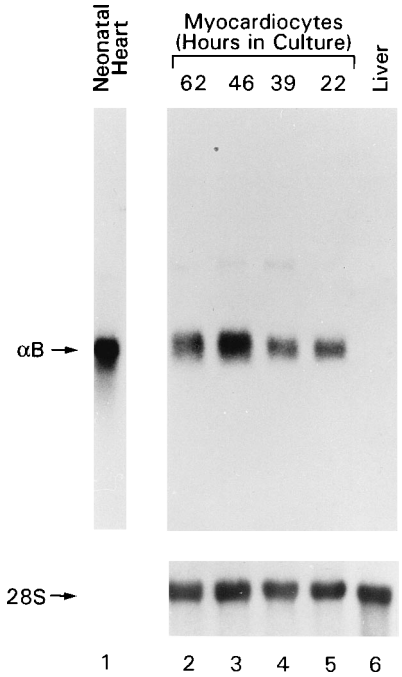

FIG. 2. Northern blot hybridization of total RNA from PMC. RNA was isolated from the specified neonatal rat heart or liver or from primary myocardiocytes of neonatal rats at the indicated times of culture. Ten micrograms of RNA was loaded in each lane (except for lane 1, which contained 2.5 μg of RNA). Membranes were hybridized with the αB-crystallin exon 3 probe. The probe was re-moved from the membrane containing RNA from PMC, and this membrane was rehybridized with the 28S RNA probe, as described in Materials and Methods.

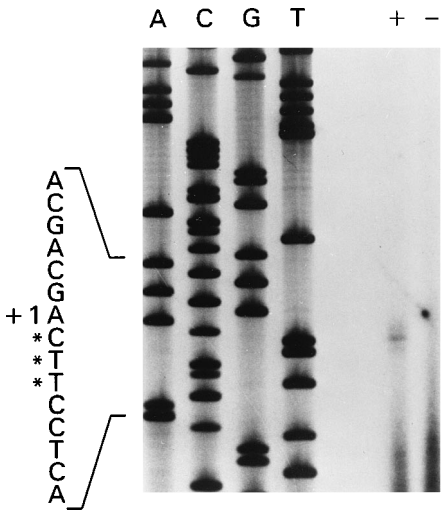

FIG. 3. Primer extension analysis of murine αB-crystallin mRNA in the heart. RNAs were isolated from the hearts of FVB/N mice, and 5 μg of total RNA was included (+) or omitted (−) in primer extension reaction mixtures together with radioactively labeled oligodeoxynucleotide 8879 (complementary to positions +66 to +46). Products were resolved on an 8% polyacrylamide sequencing gel adjacent to a ladder obtained by using the same primer to sequence a cloned fragment of the αB-crystallin gene. This sequence is presented to the left of the ladder, and the sequence positions of primer-extended products are denoted by asterisks. The previously determined transcription initiation site (26) is indicated by +1.

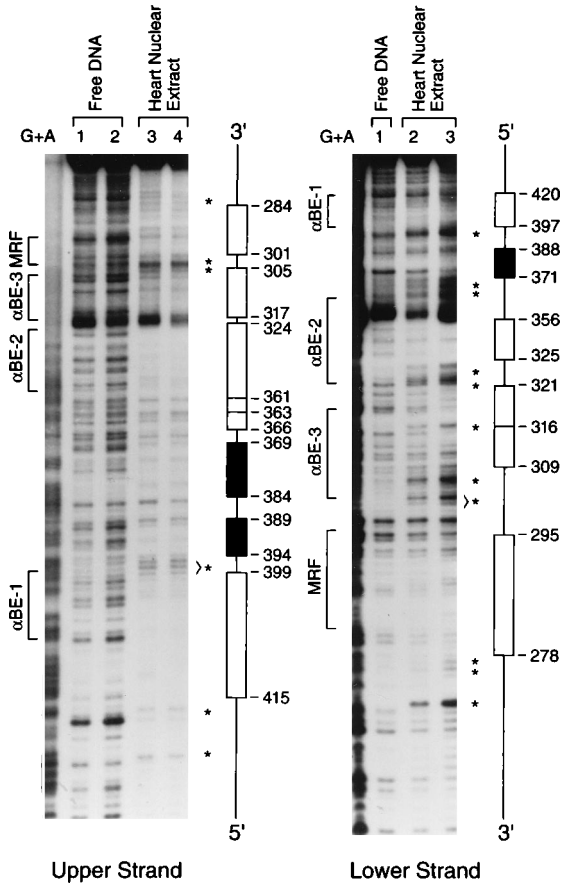

FIG. 4. In vitro DNase I footprinting of the murine αB-crystallin enhancer. Footprinting was performed with crude heart nuclear proteins from 2-month-old FVB/N mice. Upper-strand and lower-strand 5′-end-labeled probes (−427 to −259) were incubated with nuclear proteins (12 μg) for 25 min and digested with DNase I. G+A lanes are Maxam-Gilbert G+A reactions. For the upper strand, lanes 1 and 2 represent duplicate samples and contain free DNA while lanes 3 and 4 contain DNAs incubated with heart nuclear proteins. For the lower strand, lane 1 contains free DNA and lanes 2 and 3 contain DNAs incubated with heart nuclear proteins. Regions protected from DNase I digestion are diagrammed to the right of each gel with their corresponding positions boxed and numbered. Solid boxes represent the DNase I-protected region (newly identified αBE-4) unique to heart cells. Open boxes represent previously identified regulatory elements of the αB-crystallin enhancer used for αB-crystallin expression in the lens, skeletal muscle, and lung. *, DNase I-hypersensitive sites.

(28), or muscle (C2C12) (32) cell lines of mice. The footprinting data are summarized and placed in context within the αB-crystallin enhancer in Fig. 5.

EMSA and immunological tests. The DNase I footprinting data demonstrate that heart nuclear extracts from adult mice contain one or more factors that interact with the αB-crystallin enhancer at the reverse CArG box within the αBE-4 site. It is known that a canonical CArG box can bind the serum response factor (SRF) (10, 73). To test whether the cardiac protein which binds to the reverse CArG box within αBE-4 has antigenic similarity to SRF, a ^{32}P-labeled 56-bp oligodeoxynucleotide, E1, containing the αBE-4 sequence was incubated with heart nuclear extracts from adult mice in the presence or absence of an antibody against murine SRF (generously provided by R. Prywes, Columbia University, New York, N.Y.). The polyclonal antibody was generated against DNA-affinity-purified SRF in a BALB/c mouse (11). Incubation of the SRF antibody with E1 in the absence of heart nuclear extract did not produce a protein-DNA complex (Fig. 6A, lane 2). Preincubation of heart nuclear extract with anti-SRF antibody resulted in the formation of a supershifted complex and a concomitant decrease of another complex (denoted by S and B, respectively; Fig. 6A, lanes 3 and 6), suggesting that complex B (formed between the reverse CArG box in the αB-crystallin enhancer and heart nuclear proteins) contains at least one protein which is antigenically related to SRF. In a control test, preincubation of heart nuclear extract with mouse anti-MyoD monoclonal antibody (kind gift of W. E. Wright, Southwestern Medical Center, Dallas, Tex.) did not affect complex formation (Fig. 6C, lane 4). A fos-serum response element (SRE) oligodeoxynucleotide competed with E1 for complex formation (Fig. 6B, lane 11), consistent with the idea that complex B contains a protein with antigenic similarity to SRF. The ability of the fos-SRE oligodeoxynucleotide to compete with the reverse CArG box oligodeoxynucleotide (E1) for complex formation suggests that SRF itself, a closely related protein, or a modified form of SRF is involved in complex B. This reverse CArG box binding factor appears to be cardiac muscle specific since none of the nuclear proteins tested from other cell types bound to the reverse CArG box. While only one broad band was observed with oligodeoxynucleotide E1 when gels were autoradiographed at −80°C for 12 h (complex B [Fig. 6A, lane 4, and B, lane 9), a shorter exposure (4 h at −80°C) revealed that this broad band actually consisted of two closely migrating complexes (Fig. 6D, lane 2). To determine the number of heart nuclear proteins present in complex B, competition EMSA were performed with oligodeoxynucleotides αBE-1 (−424 to −395) and αBE-4 (−394 to −368). Both αBE-1 (−424 to −395) and αBE-4 (−394 to −368) wild-type oligodeoxynucleotides competed with oligodeoxynucleotide E1 for complex formation (Fig. 6D, lanes 4, 5, 7, and 8). The results of these competition experiments suggest that oligodeoxynucleotide E1 forms a complex with mouse heart nuclear proteins consisting of at least two interacting protein components.

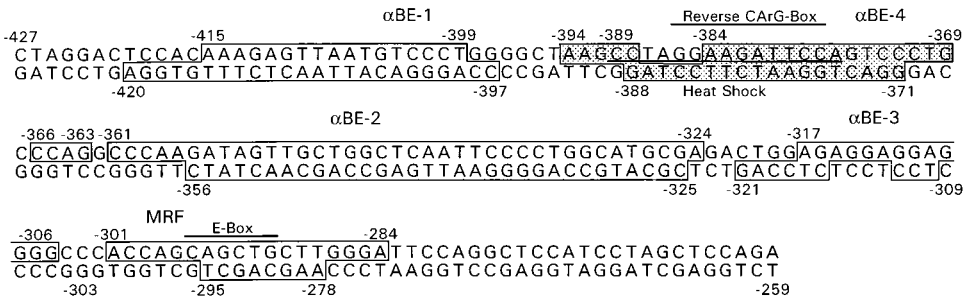

FIG. 5. Summary of DNase I footprinting of the αB-crystallin enhancer. Regions footprinted by heart nuclear proteins which have also been shown (32) to be protected by C2C12 muscle cell nuclear proteins are shown in open boxes. The stippled box indicates the unique region protected by heart nuclear proteins (αBE-4). The underlined region is the heat shock consensus sequence, and the regions of reverse CArG box and E box sequences are overlined.

The DNAse I footprint data also indicated that a heart nuclear protein binds to the MRF E box. Many transcription factors (helix-loop-helix proteins, including USF) are known to interact with E boxes (69). To further characterize the heart nuclear protein which binds to the MRF E box, EMSA were performed by incubating a 26-bp ^{32}P-labeled MRF oligodeoxynucleotide (−307 to −282) and heart nuclear extract at 4°C for 1 h. A slowly migrating complex (denoted by U; Fig. 7A, lane 2) and a rapidly migrating complex were obtained. Complex U was effectively eliminated by a 25-fold molar excess of unlabeled MRF oligodeoxynucleotide (Fig. 7A, lane 3). Competition with an oligodeoxynucleotide containing the adenovirus major late promoter (AdML) USF-binding site (69) also abolished the formation of this complex (Fig. 7A, lanes 4 and 5), suggesting that USF can interact with the MRF E box. An oligodeoxynucleotide containing AdML USF and anti-USF antiserum did not significantly affect the formation of the rapidly moving complex (Fig. 7A, lanes 4, 5, and 7), whereas the oligodeoxynucleotide MRF did compete (Fig. 7A, lane 3). These results suggest that the rapidly migrating complex is specific for MRF but does not involve USF or a USF-related protein. To investigate whether USF or an antigenically related protein is one of the components of complex U, anti-USF antiserum (13) (provided by E. H. Bresnick and G. Felsenfeld,

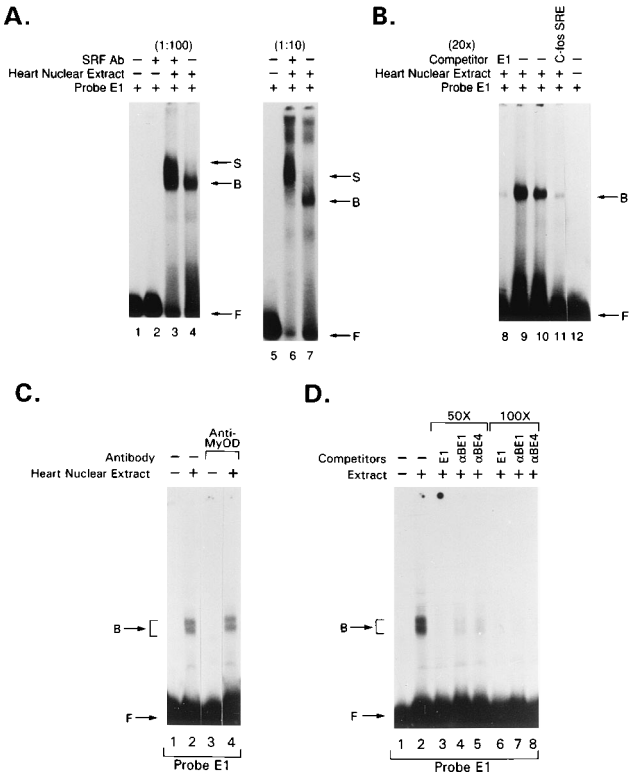

FIG. 6. (A) Autoradiograms of EMSA indicating that the heart nuclear protein which binds to the reverse CArG box is antigenically related to SRF. S, supershifted complex; F, free E1 oligodeoxynucleotide; B, complex formed between heart nuclear proteins and E1 oligodeoxynucleotide; SRF Ab, SRF antibody. (B) Lane 8 contained a 20-fold (20×) molar excess of nonradioactive E1 oligodeoxynucleotide, and lane 11 contained a 20-fold excess of nonradioactive c-fos–SRE double-stranded oligodeoxynucleotide (5′-AATTCCAGGATGTC CATATTAGGACATCTGCA-3′) (72) as competitor DNA. (C) Effect of anti-MyoD antibody on the heart nuclear protein(s) that binds to the reverse CArG box. (D) Oligodeoxynucleotide E1 forms a complex with heart nuclear proteins consisting of at least two interacting components. Competitions were performed with wild-type unlabeled double-stranded oligodeoxynucleotides (αBE-1 [−424 to −395] [lanes 4 and 7] and αBE-4 [−394 to −368] [lanes 5 and 8]).

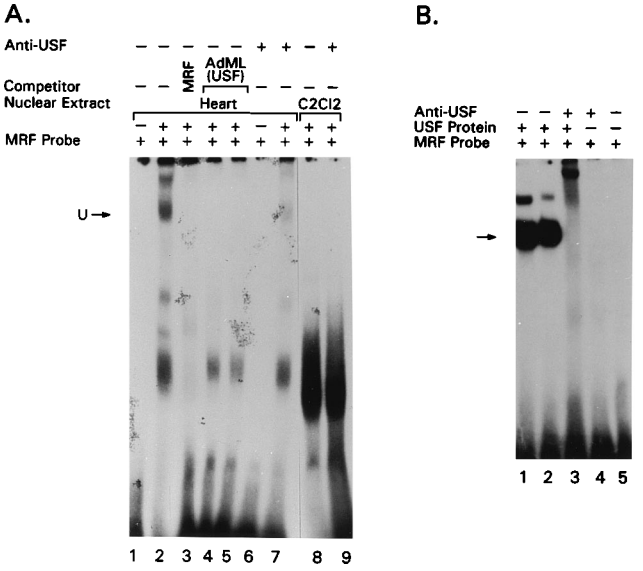

FIG. 7. Autoradiograms of EMSA indicating that the heart nuclear protein which binds to the MRF E box is antigenically related to USF. (A) Complex U is the slowly migrating protein-DNA complex obtained by incubation of the MRF-containing oligodeoxynucleotide (−307 to −282) and heart nuclear proteins at 4°C for 1 h. Lane 3 contained a 25-fold molar excess of nonradioactive MRF oligodeoxynucleotide, and lanes 4 and 5 contain a 25-fold molar excess of AdML USF double-stranded oligodeoxynucleotide 5′-CGGTGTAGGCCACG TGACCGGTG-3′ (69) as competitor DNA. (B) USF can directly interact with the MRF E box. The arrow on the left indicates the position of the complex obtained with MRF oligodeoxynucleotide and purified human USF (12, 13).

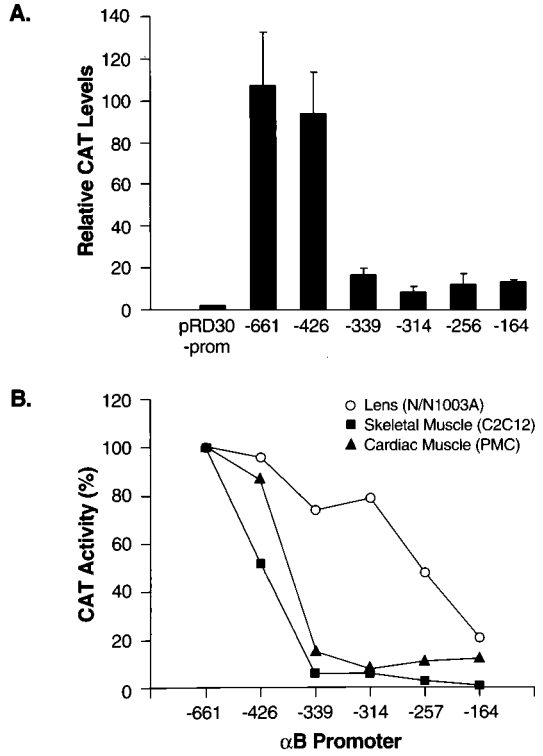

FIG. 8. (A) 5′ deletion analysis of the αB-crystallin promoter in rat myocardiocytes. Progressive 5′ deletion fragments of the murine αB-crystallin gene (all extending to +44) linked to the CAT gene have been described previously (25). Constructs were introduced by transfection as described in Materials and Methods. CAT activity was measured by a biphasic assay (57) and normalized for transfection efficiency by using a cotransfected, internal control plasmid, pUCSV2PAP. CAT levels (averages ± standard deviations) are expressed relative to the levels expressed by the promoterless plasmid (−prom) pRD30. (B) Relative promoter strength of αB-crystallin–CAT constructs. Numbers on the abscissa represent the 5′ endpoints of the αB-crystallin promoter sequence for each construct described previously (25). The CAT activities of 5′ deletion series of αB-crystallin promoter constructs transfected into rabbit lens cells (N/N1003A), murine skeletal-muscle-derived cells (C2C12), and primary rat myocardiocyte cells (this report) are shown. An activity of 100% refers to the activity of the construct −661-CAT when transfected into N/N1003A, C2C12, and PMC cells for each series.

National Institutes of Health, Bethesda, Md.) was used in an EMSA experiment. Incubation of anti-USF antiserum in the absence of heart nuclear extract did not produce any protein-DNA complex (Fig. 7A, lane 6). Preincubation of heart nuclear extract with anti-USF antiserum, however, decreased the formation of complex U (Fig. 7A, lane 7). In contrast, C2C12 myotube nuclear extract did not produce complex U and anti-USF antiserum did not have a significant effect on the rapidly migrating complex (Fig. 7A, lanes 8 and 9, respectively). Purified human USF (provided by E. H. Bresnick) (12) also interacted with the MRF oligodeoxynucleotide (Fig. 7B, lanes 1 and 2). In addition, anti-USF antiserum eliminated the complex formed with purified USF (Fig. 7B, lane 3).

5′ deletion analysis of αB-crystallin promoter in transfected cardiac muscle cells. Transfection tests utilizing PMC were performed with 5′ deletion fragments containing the αB-crystallin promoter linked to the CAT reporter gene in order to identify cardiac muscle regulatory elements. The activity of each construct was expressed relative to that of the promoterless construct pRD30 (Fig. 8A). CAT activity remained essentially the same for constructs containing fragments −661 to +44 (full-length promoter) and −426 to +44 but was reduced fivefold upon a 5′ deletion to −339 (resulting in removal of the reverse CArG box). Further 5′ deletions to −314, −257 (eliminating the entire enhancer), and −164 did not cause significantly greater reductions in promoter activity in transfected PMC. These relative enhancer activities were generally similar to those we reported previously (25, 32) for the same constructs in transfected myoblasts of the skeletal-muscle-derived C2C12 cell line (Fig. 8B).

Site-specific mutations of the αB-crystallin enhancer. We next tested the functional importance of various regulatory elements for enhancer ability in primary myocardiocytes. PMC were transfected with constructs (25) lacking (ptkGH) or containing (pH64, pH63, and pH118) the αB-crystallin enhancer fused to the herpes simplex virus thymidine kinase promoter and human growth hormone (hGH) reporter gene. pH64 contains the sequence from −427 to −259 in the same relative position and orientation as in the normal αB-crystallin gene, while pH63 contains the sequence from −427 to −259 in the reverse orientation; pH118 contains the sequence from −427 to −259 in the normal orientation, but it is placed within the second intron of the hGH gene (25). These transfection experiments revealed that the αB-crystallin enhancer can stimulate the thymidine kinase promoter in an orientation- and position-independent manner (Fig. 9A), indicating that it can function as an enhancer in myocardiocytes. To determine whether the DNase I-footprinted regions of the αB-crystallin enhancer have functional significance in myocardiocytes, we tested site-specific and deletion mutations by transient-transfection experiments. In all cases, mutated enhancers were placed in the correct orientation upstream of the viral thymidine kinase promoter and compared with the wild-type enhancer for the ability to direct expression of the reporter hGH gene in transfected myocardiocytes, C2C12 skeletal muscle cells, and N/N1003A lens cells. Five mutations in the *cis*-acting regulatory elements αBE-1 (MuR), αBE-2 (MuF), αBE-3 (MuK), and MRF (MuP and MuQ) shown earlier (32) to decrease enhancer activity in N/N1003A and αTN4-1 lens cells and C2C12 muscle cells were used. In addition, constructs containing mutations Mu-9788, Mu-9789, and Mu-9790 (Fig. 9B) in the αBE-4 reverse CArG box region were made and transfected into PMC, C2C12, and N/N1003A cells. Figure 9A shows the levels of secreted hGH in culture media for cells transfected with the test plasmid relative to those obtained with cells transfected with the enhancerless vector (ptkGH). The data were normalized for transfection efficiency as described in Materials and Methods. The wild-type enhancer construct (pH64) directed approximately 12-times-higher hGH levels in PMC than did the enhancerless plasmid. All mutations of sequences within αBE-1, αBE-2, αBE-3, and MRF decreased enhancer activity in transfected PMC as they did previously in C2C12 cells (32). In contrast, αBE-4 mutations (Mu-9788, Mu-9789, and Mu-9790) resulted in decreased expression of hGH only in transfected PMC (Fig. 9A). Mutations at the 3′ border of αBE-2 (MuI), at the 5′ border of αBE-3 (MuJ), and at the 5′ border of MRF (MuM) described previously (32) had little effect on enhancer activity in transfected PMC. Thus, αBE-1, αBE-2, αBE-3, and MRF contribute to αB-crystallin transcription in transfected skeletal and cardiac muscle cells, while αBE-4 activity is limited to myocardiocytes. These results are consistent with our DNase I footprinting results and indicate that αBE-4 is a distinct *cis*-acting element for regulating αB-crystallin gene expression in cardiac muscle.

Since mutations in the E box (MuP and MuQ) decreased the αB-crystallin enhancer activity in transfected PMC, we examined whether Id (3), the negative regulator of myogenic factors, inhibited myocardial activation of the αB-crystallin pro-

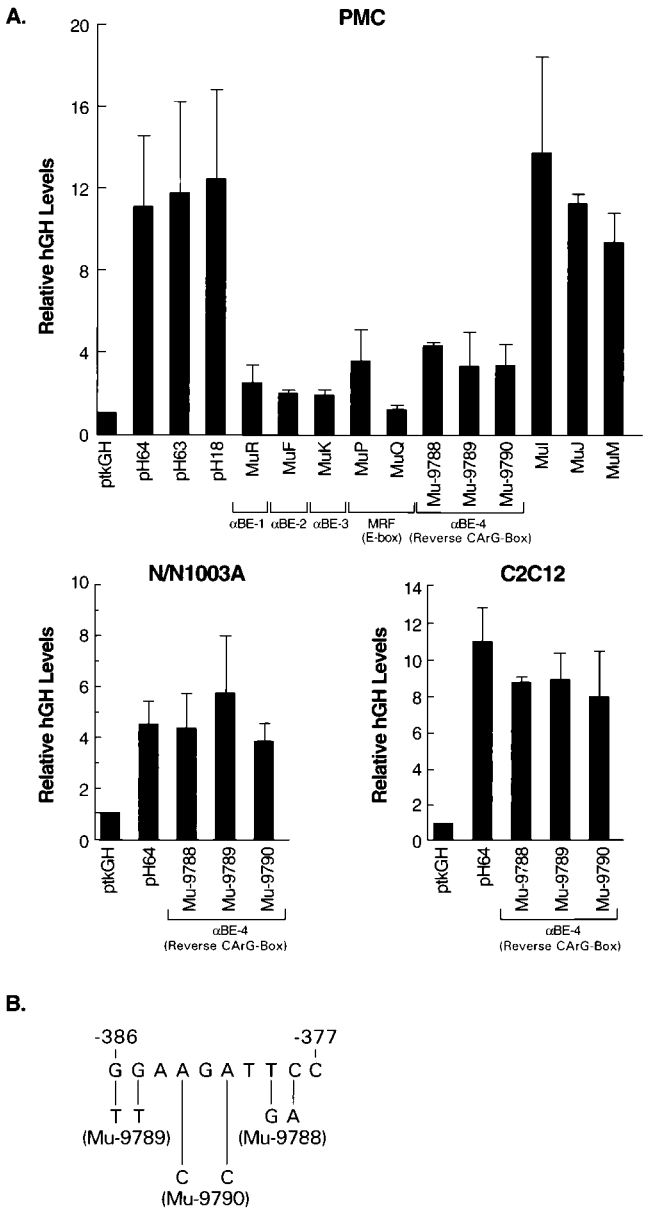

FIG. 9. (A) Transfection of the αB-crystallin enhancer in PMC, C2C12 myoblasts, and N/N1003A lens cells. Wild-type and mutant αB-crystallin enhancer fragments, cloned into ptkGH (25), were used for transfection as described in Materials and Methods. The medium was assayed for hGH and normalized for transfection efficiency. The hGH levels (± standard deviations) presented are relative to the levels produced by the parental enhancerless plasmid (ptkGH). (B) Site-directed mutagenesis of the reverse CArG box within αBE-4. Mutations are indicated below the sequence.

moter. Cotransfection of pH64 (containing the E box) and an Id expression vector, pEMSV-Id (kindly provided by H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, Wash.), did not inhibit hGH gene expression in myocardiocytes (data not shown). Taken together, our studies demonstrate that both cardiac- and skeletal-muscle-specific αB-crystallin enhancer activities employ an E-box-dependent pathway, although the cardiac nuclear protein factors (with antigenic similarity to USF) differ from known myogenic factors.

EMSA competition experiments. EMSA competition experiments were conducted to test whether reductions in enhancer activity by site-specific mutations could be correlated with corresponding losses in the ability to bind nuclear factors from the heart. Double-stranded oligodeoxynucleotides composed of αB-crystallin enhancer sequences between positions −426 and −371 (oligodeoxynucleotide E1, containing the αBE-1 and αBE-4 regions), between positions −370 and −315 (oligodeoxynucleotide E2, containing the αBE-2 region), and between positions −314 and −258 (oligodeoxynucleotide E3, containing parts of the αBE-3 and MRF regions) produced retarded bands after incubation with heart nuclear extract (Fig. 10). Complex formation by oligodeoxynucleotides E1, E2, and E3 was severely reduced by self-competition (Fig. 10 [lanes 3, 8, and 9 for E1; lane 25 for E2; and lanes 15 and 30 for E3]). Mutated versions of oligodeoxynucleotides E1, E2, and E3 containing site-specific mutations that decreased or abolished αB-crystallin enhancer activity in transfection experiments competed poorly for complex formation with the corresponding wild-type oligodeoxynucleotides (Fig. 10 [lanes 4 to 6 and 10 to 12 for E1; lanes 16 to 22 and 31 for E3; and lane 26 for E2]). In contrast, mutations MuI, MuJ, and MuM, which did not alter enhancer activity, competed efficiently (Fig. 10, lanes 27, 28, and 31, respectively). These results provide a positive correlation between protein-DNA complex formation and enhancer activity for the αBE-1, αBE-2, αBE-3, αBE-4, and MRF elements.

DISCUSSION

Previous experiments have shown that expression of the murine αB-crystallin gene in different tissues is controlled by differential use of shared and tissue-specific regulatory elements (32). Transfection experiments have indicated that enhancer elements αBE-1 and αBE-2 are used in the lens, skeletal muscle, and lung; αBE-3 is used in the lens and skeletal muscle; and MRF is used in skeletal muscle and possibly the lung (35) (Fig. 11). While transgenic-mouse experiments have established that the αB-crystallin enhancer as a whole is required for expression in the heart (33), in which αB-crystallin is highly expressed (4, 26), nothing is known about the use of individual regulatory elements of the enhancer in the heart. The present transgenic-mouse experiments have established that heart expression driven by the αB-crystallin enhancer is confined to myocardiocytes, allowing the use of cultured myocardiocytes (39) to identify regulatory elements of the αB-crystallin enhancer in transfection experiments. The results show that αBE-1, αBE-2, αBE-3, MRF, and newly identified αBE-4 all contribute to enhancer activity in transfected myocardiocytes (Fig. 11). The confinement of αB-crystallin enhancer activity to myocardiocytes of the heart fits well with the histochemical localization of αB-crystallin in cardiac muscle fibers (40, 44). αB-crystallin has been localized in the Z bands of myofibrils of skeletal muscle (2) and myocardiocytes (44), in which it has been suggested to have a myofibril-stabilizing role.

The heart-specific αBE-4 regulatory element contains a sequence, 5′-GGAAGATTCC-3′, which is identical to the reverse orientation of the CArG box sequence [5′-CC(A/T)$_6$GG-3′] (49) except for the underlined G. The CArG box sequence constitutes a part of the SRE involved in the regulation of immediate-early genes such as c-*fos* (51, 73, 74) and has been implicated in the regulation of genes in cardiac muscle as well as skeletal muscle (17, 30, 42, 50, 52, 61, 66, 70, 75, 79). CArG boxes are also found in the regulatory regions of a number of genes expressed in nonmuscle cells, for example, the β-actin gene (58). Interestingly, the present results for EMSA using a c-fos–SRE competing sequence and anti-SRF antiserum (11) indicate that the reverse CArG box in αBE-4 interacts with one

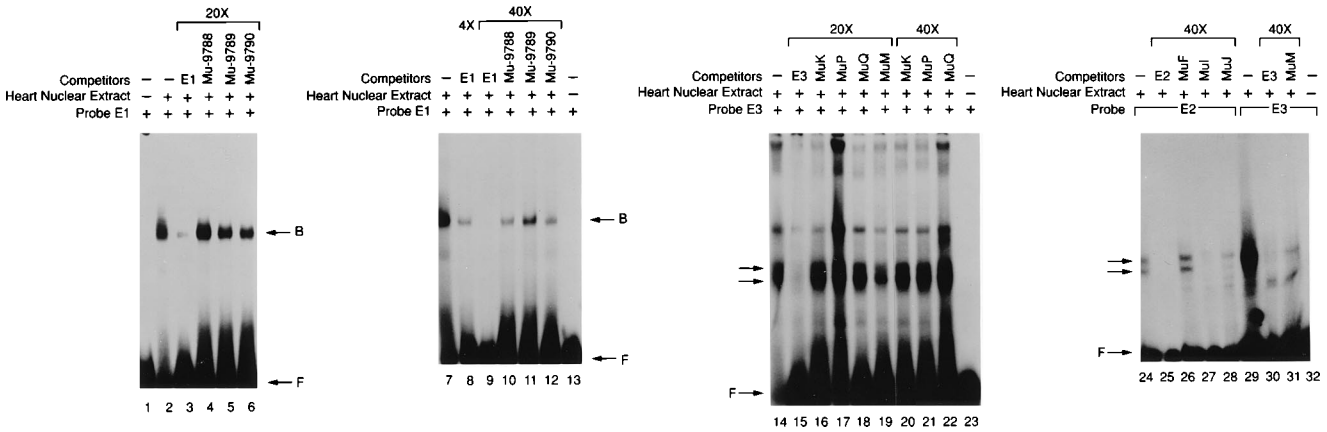

FIG. 10. EMSA and competition analyses of protein-DNA interactions between heart nuclear extracts and oligodeoxynucleotides E1, E2, and E3. The sequences of oligodeoxynucleotides E1, E2, and E3 are shown in Fig. 5. Radioactively labeled oligodeoxynucleotides E1 (−426 to −371), E2 (−370 to −315), and E3 (−314 to −258) were incubated in the presence of heart nuclear extract, and protein-DNA complexed species were resolved by 5% polyacrylamide gel electrophoresis. Competitions were performed with nonradioactive double-stranded oligodeoxynucleotides. Mutants have been described previously (32). Autoradiograms were exposed for 12 h. B and double arrows, complex formed between heart nuclear proteins and oligodeoxynucleotide; F, free oligodeoxynucleotide.

or more myocardiocyte nuclear factors which possess binding and antigenic similarities to SRF. The factor that interacts with the heart reverse CArG box appears to be different from SRF since SRF is present in nuclear extracts of C2C12 myotubes which do not protect αBE-4 from DNase I digestion (32). Of course, it is possible that cardiac-muscle-specific modifications of SRF are necessary for binding to the αBE-4 reverse CArG box within the context of the αB-crystallin enhancer. If this is the case, it is interesting that this protein-DNA interaction occurs with CArG boxes in either orientation, which may have different states of DNA. It is not known whether the binding of nuclear factors to the reverse CArG box requires interaction with other cardiac-muscle-specific factors or cofactors bound elsewhere on the αB-crystallin enhancer. To the best of our knowledge, no cardiac-muscle-specific factor has been found. Clearly, further experiments are necessary to establish unequivocably the identity of the heart nuclear factor that binds to the αBE-4 reverse CArG box and the molecular basis for its specificity.

The present data indicate that the MRF E box is required for the activity of the αB-crystallin enhancer in myocardiocytes. E boxes have been shown to bind many factors (6, 47, 54), as well as those of the MyoD family (59). The absence of down-regulation of αB-crystallin enhancer activity in myocardiocytes cotransfected with an Id-encoding plasmid and the results of EMSA experiments employing MyoD and myogenin antibodies (data not shown) suggest that the αB-crystallin E box does not function in myocardiocytes by binding a bHLH myogenic protein related to the MyoD and myogenin family of transcription factors. Other examples of E boxes utilized in heart cells include the β-myosin heavy-chain gene (71) and the human cardiac α-actin promoter (65). The α-actin E box binds cardiac muscle factors that heterodimerize with the ubiquitous bHLH protein E12 (65). Interestingly, the α-actin E box is not necessary for promoter activation in P19-derived myocardiocytes (48), although it does bind E-type bHLH nuclear proteins from P19 myocardiocytes (68). Another example of E box utilization in the heart is that by the α-myosin heavy-chain gene (53). An E box within the PRE-B element of the α-myosin heavy-chain gene interacts with a cardiac nuclear protein, α-myosin heavy-chain gene binding factor (BF-2). A BF-2-like protein also interacts within regulatory element HF-1a of the cardiac myosin light-chain 2 promoter (56, 79), indicating that both BF-2 and HF-1a are E-box-binding proteins. Like the cardiac protein which binds to the MRF E box in the αB-crystallin enhancer, HF-1a is an E-box-binding protein which cannot be inhibited by Id (31), indicating that it is not a typical myogenic bHLH family member (reviewed in reference 76).

The results of our EMSA experiments raise the possibility that the helix-loop-helix protein, USF, or a related protein interacts with the MRF of the αB-crystallin enhancer in myocardiocytes. USF was initially identified as an upstream stimulatory factor that binds the core sequence CACGTG in the AdML as well as consensus E box sequences (CANNTG) (14, 69). Recent reports have indicated that USF may play an important role in transcription of the chicken αA-crystallin (19) and δ1-crystallin (20) genes. USF has also been shown to interact with and increase transcription of metallothionein I (15) and γ-fibrinogen (16) by involving similar, but not consensus, E box sequences. It has recently been reported (55) that USF interacts with E box elements in the cardiac myosin light-chain 2 promoter, providing evidence that USF may play a role in the regulation of this heart-specific gene. Since MRF

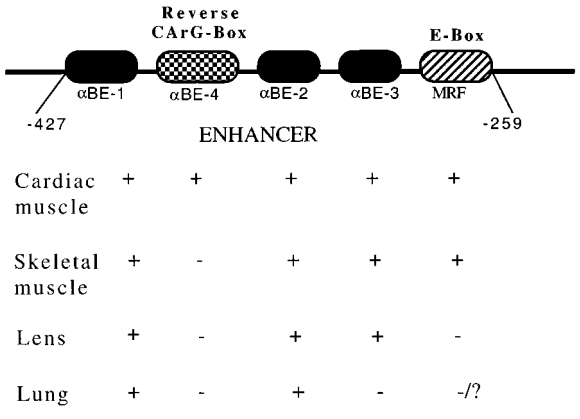

FIG. 11. Regulatory tissue print of the αB-crystallin enhancer. +, activity; −, no activity in cells derived from cardiac muscle (rat primary myocardiocytes), skeletal muscle (murine C2C12 cells), the lens (rabbit N/N1003A and murine αTN4-1 cells), or the lung (murine MLg cells). The question mark for MRF denotes that this element may be utilized by the αB-crystallin enhancer in lung cells through weak binding of nuclear protein (35).

footprints with nuclear extracts from C2C12 cells (32) and its complex does not react with anti-USF antiserum (this study), putative USF interaction with MRF in the heart must involve cardiac-muscle-specific modification of USF or its related protein or collaboration with other cardiac-muscle-specific proteins. Thus, our data add to the growing evidence that E boxes bind proteins that differ from typical myogenic transcription factors and provide evidence that the αB-crystallin enhancer is an example of an E-box-containing regulatory element that is required for activity in the heart. The results of our previous experiments also suggested that the E box is utilized for weak αB-crystallin enhancer activity in transfected lung cells (35), where it may use a different nuclear protein (Fig. 10).

In conclusion, we have demonstrated that several enhancer elements (αBE-1, αBE-2, αBE-3, and MRF) are essential for αB-crystallin expression in both cardiac muscle and skeletal muscle, whereas the newly identified element, αBE-4, is critical for αB-crystallin expression selectively in cardiac muscle. Our results also suggest that the αB-crystallin enhancer uses a reverse CArG box and an E-box-dependent pathway for regulating αB-crystallin gene expression in myocardiocytes. Now it is essential to clone the factors that bind to these cardiac muscle regulatory elements in order to delineate further the molecular basis of αB-crystallin expression in the heart.

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