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A New Serum-Responsive, Cardiac Tissue-Specific Transcription Factor That Recognizes the MEF-2 Site in the Myosin Light Chain-2 Promoter

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We have identified a serum-responsive, cardiac tissue-specific transcription factor, BBF-1, that recognizes an AT-rich sequence (element B), identical to the myocyte enhancer factor (MEF-2) target site, in the cardiac myosin light chain-2 (MLC-2) promoter. Deletion of the element B sequence alone from the cardiac MLC-2 promoter causes, as does that of the MEF-2 site from other promoters and the enhancer of skeletal muscle genes, a marked reduction of transcription. BBF-1 is distinguishable from cardiac MEF-2 on the basis of immunoprecipitation with an antibody which recognizes MEF-2 but not BBF-1. Unlike MEF-2, BBF-1 is present exclusively in nuclear extracts from cardiac muscle cells cultured in a medium containing a high concentration of serum. Removal of serum from culture medium abolishes BBF-1 activity selectively with a concomitant loss of the positive regulatory effect of element B on MLC-2 gene transcription, indicating that there is a correlation between the BBF-1 binding activity and the tissue-specific role of the element B (MEF-2 site) sequence. The loss of element B-mediated activation of transcription is reversed following the refeeding of cells with serum-containing medium. These data demonstrate that cardiac muscle cells contain two distinct protein factors, MEF-2 and BBF-1, which bind to the same target site but that, unlike MEF-2, BBF-1 is serum inducible and cardiac tissue specific. BBF-1 thus appears to be a crucial member of the MEF-2 family of proteins which will serve as an important tool in understanding the regulatory mechanism(s) underlying cardiogenic differentiation.

The expression of cardiac muscle genes during myogenesis is influenced by a plethora of factors, including serum and growth factors (15, 18, 23, 27, 40, 45). Although recent work has established that these agents exert their effects directly, or indirectly, on transcription of target genes, the molecular events involved in production of the cardiac phenotype and differentiation of the cardiac myoblast remain to be elucidated. The expression of cardiac muscle genes is activated by serum and serum growth factors in culture medium (40, 45), while removal of growth factors is obligatory for differentiation and activation of gene transcription in skeletal muscle cells (14). Serum induces an early growth response gene, Egr-1, which can *trans*-activate the cardiac α-myosin heavy chain (α-MHC) gene in a transfection assay (18). In general, serum-induced regulation of muscle-specific genes is mediated by serum response elements and serum response element-related sequences which serve as target sites for binding of serum response factors (SRF) and SRF-related factors (36 and references therein), suggesting that serum and serum growth factors play a pivotal role in control of myogenic differentiation.

The tissue-specific expression of cardiac muscle genes is mediated by several *cis* elements in the respective genes and the cognate *trans*-acting regulatory factors (15, 18, 22, 41, 51, 52). The lack of expression of the chicken cardiac myosin light chain-2 (MLC-2) gene in skeletal muscle cells is due to a negative regulatory mechanism mediated by an upstream promoter element, CSS, and its binding proteins present in skeletal muscle cells (41). Another group of researchers (33, 52) has described an activator element (HF-1) in the rat cardiac tissue-specific MLC-2 promoter which is implicated in cardiac tissue-specific gene transcription. A 47-bp element in the cardiac troponin T (cTnT) gene containing a 10-bp AT-rich sequence, conserved in both chicken and rat cardiac MLC-2 genes, is also involved in cardiac tissue-specific transcription (22). However, proteins that recognize the AT element in the cTnT promoter are present in both muscle and nonmuscle cells.

We have recently described (38) an activator, element A (CCAAAAGTGG), in the cardiac MLC-2 gene similar to the evolutionarily conserved CArG sequence responsible for up-regulation of muscle genes (29, 30). The CArG box sequence, however, interacts with multiple nuclear proteins (19, 26, 29–31), including the ubiquitous SRF which are involved in activation of both skeletal and cardiac muscle gene transcription (2, 5, 19, 26, 31; also see reference 39 for a review). A second AT-rich sequence, element B, in the MLC-2 promoter is indistinguishable from the consensus (C/T)T(A/T)(A/T)AAATA(A/G) sequence present in a number of muscle gene promoters and enhancers which are recognized by the myocyte-specific enhancer binding factor (MEF-2), originally identified in extracts of a skeletal muscle cell line, C2 (17). The MEF-2 DNA site sequence is present, in addition to the muscle creatine kinase (MCK) enhancer (17), in rat cardiac MLC-2 (52), brain and muscle creatine kinases (21) and in human phosphoglycerate mutase gene promoters (32). Deletion of this element from the respective genes causes a drastic reduction in transcription (17, 22, 32, 51). A protein, designated TARP, binds to the AT-rich sequence in the brain creatine kinase gene and is presumably functionally interchangeable with MEF-2 (21). TARP is,

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however, found in both muscle and nonmuscle cell types (7, 21, 22).

Thus, there is no clear evidence for a single element or factor that would account for the existence of a common regulatory pathway for activation of cardiac muscle-specific gene transcription. Neither is there evidence for a cardiac tissue-specific SRF despite the fact that cardiac muscle genes respond to serum-mediated stimulation. The best-characterized muscle-specific regulatory factors to date are members of helix-loop-helix (HLH) proteins of the MyoD family, present in skeletal muscle cells which recognize a consensus E-box sequence found in muscle genes (3, 4, 12, 25; see references 34 and 39 for a review). Myogenin, a member of the MyoD family, is involved in regulation of MEF-2-dependent activation of the skeletal muscle MCK gene (9), suggesting a pivotal role for MEF-2 in the myogenic program. However, many muscle genes, like the cardiac MLC-2 gene, do not contain a functional E-box. Also, MyoD family proteins are not present in cardiac muscle cells as such; other regulatory pathways, independent of the MyoD–E-box requirement, must be operational in transcriptional activation of these genes. In this context, MEF-2 family proteins are potentially important in mechanisms underlying differentiation of these two muscle lineages. Specific MEF-2 isoforms, generated through alternatively spliced RNA, have recently been identified in skeletal and heart muscle cells and are implicated in tissue-specific function of the respective genes (49). In this paper, we describe a transcription factor, BBF-1, present in cardiac muscle cells, which binds with sequence specificity to the AT-rich MEF-2 binding site (element B) in the cardiac MLC-2 gene. BBF-1 activity is present in cardiac muscle nuclear extracts but is absent, or present at a barely detectable level, in skeletal muscle. BBF-1 is distinguishable from MEF-2 on the basis of immunoprecipitation with an antibody, SRF RSRFC4 (36), directed against the AT-rich site-binding protein, which recognizes MEF-2 but not BBF-1. BBF-1 activity disappears in cardiac muscle cells grown in serum-reduced culture medium with a concomitant loss of the positive role of element B in transcription. These studies thus suggest a mechanism whereby the tissue-specific activation of the cardiac MLC-2 gene appears to be the function of a member of the MEF-2 family of proteins, BBF-1, which responds to a serum-mediated signalling pathway in the regulation of cardiac MLC-2 gene expression.

MATERIALS AND METHODS

Cell culture. Heart and skeletal (thigh) muscle tissues excised from 13-day-old chicken embryos were used to prepare primary cell cultures as described previously (41). Dissociated cells, freed of fibroblasts by repeated differential plating, were cultured in growth medium F-10 (10% fetal bovine serum, 10% horse serum, 100 U of penicillin per ml, 100 U of streptomycin per ml). Skeletal muscle cells were grown in medium containing 10% horse serum–2% chicken serum–100 U of penicillin per ml–100 U of streptomycin per ml. Both cell cultures were plated at a density of 1.5×10^6 cells per 100-mm plate for transient transfection. To test the effect of serum concentration on cardiac muscle cells, the cells were cultured with high serum levels (10% FBS and 10% horse serum) for 2 days or as indicated otherwise and then shifted to low serum levels (3% horse serum).

Transfection of cells and CAT assay. Cells were transfected by the calcium phosphate precipitation method (16) as described earlier (41). Twenty micrograms of plasmid DNA per plate was used in each case, unless stated otherwise. Cells were harvested 56 h after transfection to prepare cell extracts for chloramphenicol acetyltransferase (CAT) assay (16). For serum induction, the cells were refed with high- or low-serum-concentration medium 16 h after transfection and then cultured for another 56 h. For normalization of DNA uptake, cotransfection of a plasmid, CMV-gal, containing the β-galactosidase reporter gene (1) was done and data (means ± standard deviations) were presented as percent activity of PSV2CAT, used as a positive control. Multiple plasmid DNA preparations were used for each experiment to ensure reproducibility.

Mutagenesis of the MLC-2 proximal promoter. 5′-deletion and site-directed mutants of the MLC-2 promoter were constructed as described previously (38, 50, 51). The parent plasmid, pLC106CAT (50), which contains a 1.3-kb MLC-2 gene promoter and the 5′-flanking region, was linearized by *Xho*I, digested by *Bal* 31, and terminated at different time points to obtain different lengths of the promoter. The DNA was digested with *Sma*I, and the large fragment of the digestion product was ligated with *Bal* 31 products and transformed into *Escherichia coli* JM109. Site-directed mutants were obtained from polymerase chain reaction products synthesized with oligonucleotide primers containing a *Kpn*I site (CTCGAG) displacing the target sequence and the two 5′ and 3′ distal primers including *Nde*I or *Hin*dIII sites as described previously (51). The recombinants were identified by restriction endonuclease digestion and DNA sequencing analysis.

Preparation of nuclear extracts. The nuclear extracts were prepared essentially according to Dignam et al. (11) and as described previously (41). The protease inhibitors leupeptin (1 μg/ml) and pepstatin (1 μg/ml) were used routinely. The extracts usually contained 4 to 6 mg of protein per ml and remained stable in liquid nitrogen for several months. To compare the serum effect on cardiac nuclear proteins, the extracts were prepared from myocytes cultured for 5 days, 2 days with high serum levels and 3 days with low serum levels for low-serum extracts and 5 days continuously with high serum levels for high-serum extracts.

Gel shift assay. Double-stranded DNA fragments obtained by renaturation of chemically synthesized oligonucleotides were radio-labeled at the 5′ end by polynucleotide kinase and [^{32}P]ATP and used for the binding assay. DNA fragments (5,000 cpm) and nuclear proteins (10 μg) were incubated in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (pH 7.9)–50 mM KCl–5 mM $MgCl_2$–0.5 mM EDTA–1 mM dithiothreitol–12.5% glycerol at 4°C for 90 min and were separated on the 8% polyacrylamide gel at 4°C as described earlier (41, 51). For competition, the unlabeled DNA in 100-fold excess was added to the reaction mixture before the addition of nuclear extracts. For immunoprecipitation assay, 0.5 μl of preimmune serum or antiserum of RSRFC4 was preincubated with 10 μg of nuclear extracts for 30 min at 4°C before incubation with the DNA probes as analyzed, as described above.

RESULTS

The AT-rich element B is a cardiac-tissue specific activator of MLC-2 gene transcription. In previous studies (38, 51), we have demonstrated that the AT-rich elements A and B (Fig. 1) are muscle-specific activators as these two elements caused stimulation of transcription following transfection of primary cardiac muscle cells but not of fibroblasts and brain cells. Element A is similar to the CArG box sequence (19,

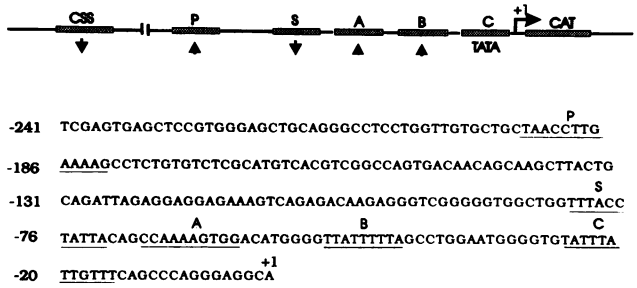


FIG. 1. Partial nucleotide sequence of the chicken cardiac MLC-2 gene promoter. Multiple *cis*-acting DNA elements, A, B, C, P, S, and CSS, are indicated (see text). +1 denotes the transcription start site. Arrowheads pointing upward denote positive roles and arrowheads pointing downward denote negative roles in transcriptional regulation.

30), and element B is indistinguishable from the MEF-2 site sequence in the MCK enhancer (17), both implicated in tissue-specific activation of transcription. To examine whether the regulatory role of element B is restricted to cardiac muscle tissue, 5′-deletion and site-specific mutants of the MLC-2 promoter fused to the CAT coding sequence were transfected simultaneously into primary cardiac and skeletal muscle cells in culture. The results shown in Fig. 2 confirmed that plasmid pLCΔ31CAT, containing element C (TATA box) alone, produced basal-level promoter activity, approximately equal in cardiac and in skeletal muscle cells, whereas pLCΔ53CAT, which includes element B (MEF-2 site), showed a fivefold increase in activity in cardiac cells but not in skeletal muscle cells. pLCΔ72CAT, which includes element A containing the CArG-like sequence (CCAAAAGTGG), caused a further 3.5-fold increase in CAT expression, but in both cardiac and skeletal muscle

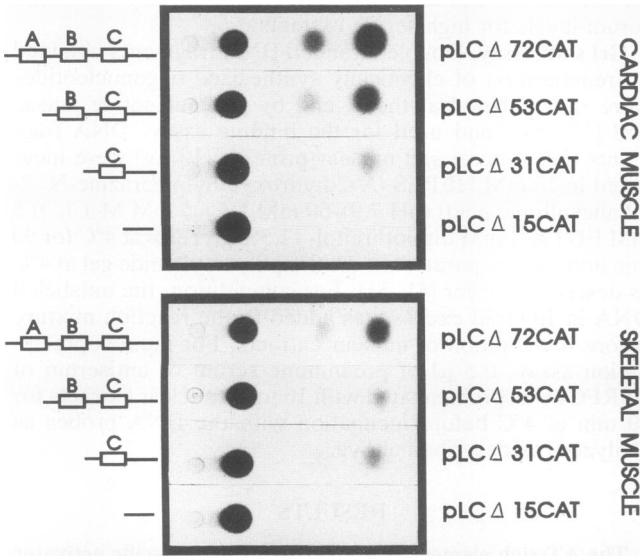
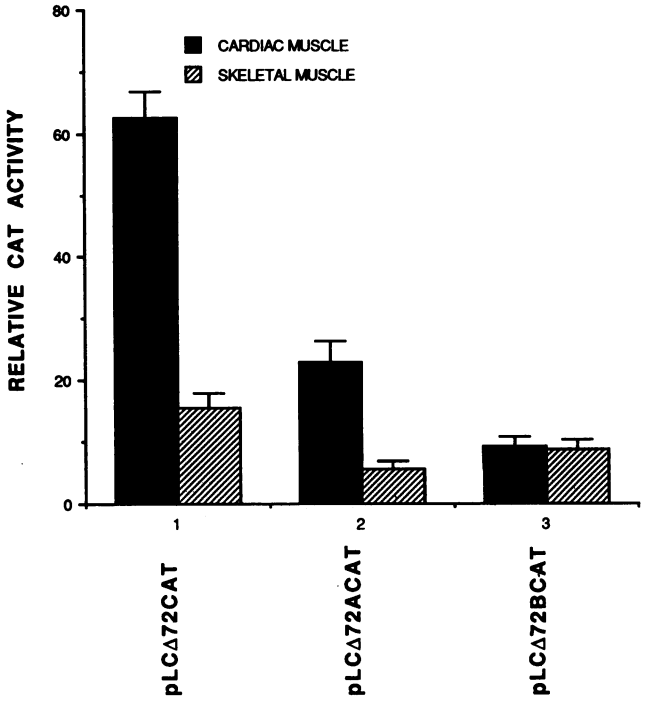


FIG. 2. Comparison of the promoter activities of 5′-deletion mutants of the MLC-2 promoter in primary cardiac and skeletal muscle cells. The sequentially 5′-deleted MLC-2 promoters fused to the CAT coding sequence were used to measure promoter activity in primary cardiac and skeletal muscle cells in culture as described in Materials and Methods. A, element A, containing the CArG-like sequence; B, element B (MEF-2 site); C, element C (TATA box).



A B
pLCΔ72CAT: TACAGCCAAAAGTGGACATGGGGTTATTTTTAGCCT
pLCΔ72ACAT: TACAGCCCTCGAGGGACATGGGGTTATTTTTAGCCT
pLCΔ72BCAT: TACAGCCAAAAGTGGACATGGGGTGGATCCTAGCCT

FIG. 3. Comparison of MLC-2 promoter activity of site-specific mutants in cardiac and skeletal muscle cells. Deletion/substitution mutants of elements A and B were constructed by using the parent plasmid, pLCΔ72CAT, as described in Materials and Methods and transfected into primary cardiac and skeletal cells. CAT activity is expressed as a percentage of activity of pSV2CAT, used as a positive control, following normalization for DNA uptake as described in Materials and Methods. Each bar represents a mean value ± the standard deviation for 3 to 5 independent experiments. A and B, elements A and B, respectively.

cells, suggesting that element B-mediated activation of transcription is cardiac tissue specific whereas element A activates transcription in both types of muscle cells, consistent with the expected role of the CArG box sequence (29–31). We also made site-specific mutations in A and B sequences (see Materials and Methods) and tested for activity in cardiac and skeletal muscle cells. Data summarized in Fig. 3 indicated that the mutation of the element A sequence (pLCΔ72ACAT) caused a reduction in transcription levels in both kinds of cells as expected and yet the differential expression in cardiac and skeletal muscle cells was maintained. However, when the element B sequence was mutated (pLCΔ72BCAT), the reduction in transcription obliterated the differential expression in the two cell populations. Several precautions were taken (see Materials and Methods and references 38 and 41) to ensure reproducibility. The results were normalized for DNA uptake following cotransfection with plasmid CMV-gal containing the β-galactosidase coding sequence (1). Previous studies (33, 52) have identified a 28-bp sequence element (HF-1) in the rat cardiac MLC-2 gene which was implicated in cardiac tissue-specific activation of transcription on the basis of transcription in primary cardiac cells and in a soleus muscle cell line (Sol-8). Our studies have delineated at least two functional elements (A

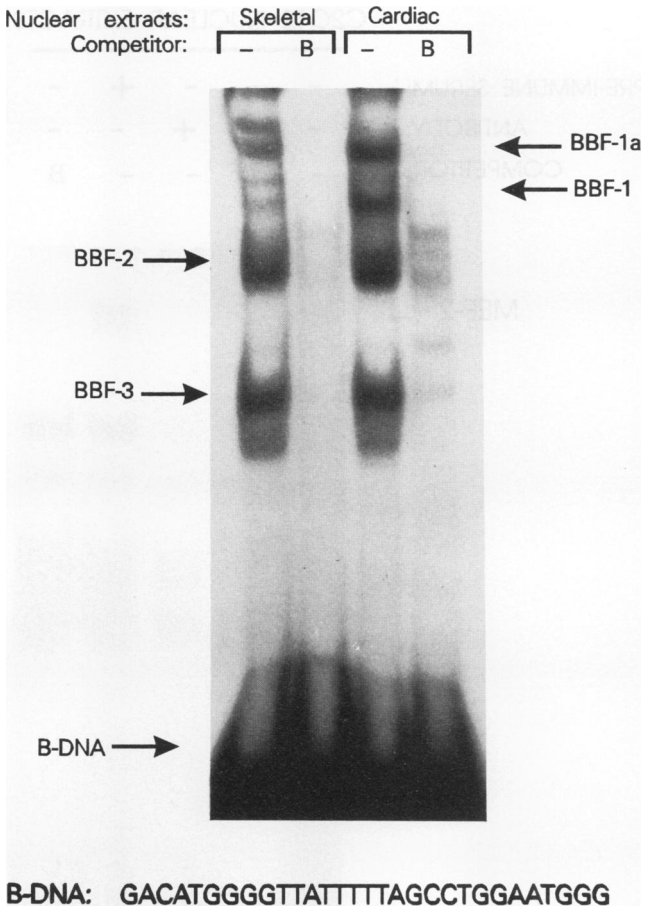


FIG. 4. Mobility shift assay with cardiac and skeletal muscle nuclear proteins. Nuclear extracts were prepared from cardiac and skeletal muscle tissues and analyzed for binding to a synthetic 30-bp oligonucleotide (−57 to −28) containing element B as described in Materials and Methods. The gel shift assay was performed without (−) or with a 100-fold molar excess of oligonucleotide B as competitor. The protein-DNA complexes are shown as BBF-1a, BBF-1, BBF-2, and BBF-3.

and B) within the HF-1-like segment in the chicken cardiac MLC-2 gene, one of which (element A), the CArG-like sequence, activates transcription in both cardiac and skeletal muscle cells, while the other MEF-2-binding site, element B, is cardiac tissue specific consistent with the activity of HF-1B in rat MLC-2 (33, 52). We have recently demonstrated that these two elements are recognized by different transcription factors and that the DNA-protein complexes produced in a gel shift assay are not blocked by each other (38, 51).

A cardiac tissue-specific transcription factor (BBF-1) recognizes element B. To examine whether the cardiac tissue-specific role of element B (MEF-2 site) is mediated through its interaction with a cardiac tissue-specific DNA-binding protein(s), nuclear extracts were prepared from both cardiac and skeletal muscle tissues and tested in a gel shift assay for sequence-specific binding to a 30-bp-long chemically synthesized oligonucleotide encompassing the sequence from −24 to −53 containing element B (MEF-2 site). At least four protein-DNA complexes, designated BBF-1a, BBF-1, BBF-2, and BBF-3, were formed with nuclear extracts from cardiac muscle (Fig. 4). This was consistent with the complex formation pattern with the DNA probe containing the MEF-2 site and the adjoining sequence with the rat cardiac myocyte extracts reported recently (32). One of the four complexes, BBF-1, was abundant in the cardiac extracts but was absent, or present only at a barely detectable level, in skeletal muscle tissue. When extracts were prepared from chicken skeletal primary cells in culture, BBF-1 was totally absent (see below). The extracts from cardiac and skeletal muscle tissues were prepared in parallel from the same embryos. The possibility that the lack of BBF-1 activity in skeletal muscle extracts was due to nonspecific inactivation of DNA-binding proteins was discredited by the fact that the intensities of BBF-2 and BBF-3 were unchanged.

Since the element B sequence is identical to the MEF-2 binding site of the MCK enhancer which produces a characteristic slow-moving complex with MEF-2 from the skeletal muscle cell line C2 (17), we asked whether BBF-1a, or BBF-1, is MEF-2. An antibody, RSRFC4 (a kind gift of R. Treisman), directed against the common carboxyl terminus of known serum-responsive DNA-binding proteins, which also recognizes MEF-2 (see below; 36, 49) was used in a gel shift assay. When preincubated with the cardiac nuclear extracts, the antibody eliminated the BBF-1a activity (designated MEF-2 from here on) (Fig. 5) without affecting BBF-1 or other element B-protein complexes. In a reciprocal experiment, nuclear extracts from skeletal muscle cell line C2C12 and the MEF-2 binding site DNA probe of the MCK enhancer were used (Fig. 6). The antibody inactivated MEF-2, which was also inhibited by element B DNA, demonstrating that MEF-2 and BBF-1a share the same DNA recognition site and are immunologically identical proteins. While only MEF-2 of the skeletal muscle C2C12 extracts was inhibited by element B DNA, both MEF-2 and BBF-1 of the cardiac extracts were inhibited effectively by MEF-2 DNA of the skeletal MCK enhancer (Fig. 7). DNAs containing the MyoD binding site (E-box) and the negative element S of the cardiac MLC-2 gene (51) (Fig. 1) were ineffective as competitors. Thus, the cardiac proteins contain at least two MEF-2-like factors with strict MEF-2 site sequence dependence, but one of the proteins, BBF-1, is different from MEF-2 on the basis of its lack of recognition by the antibody RSRFC4. We further examined the sequence requirements for BBF-1 and MEF-2 binding to element B by using DNA probes with mutations in the core element B (ΔB), 5′-flanking (5′ΔB), and 3′-flanking (3′ΔB) regions (Fig. 8). Clearly, both MEF-2 and BBF-1 require the core element B sequence as 5′ΔB and 3′ΔB, but not ΔB, inhibited the binding effectively. BBF-2, on the other hand, requires a sequence outside the mutated sequences, since it was inhibited by all three competitors. The binding properties of BBF-3 are presently unclear as it was only partially affected by the competitors.

Serum induces the expression of BBF-1 activity. It is well recognized that serum and serum growth factors regulate the expression of both cardiac and skeletal muscle genes (18, 40, 45). A conserved DNA binding site, $CC(A/T)_6GG$ (element A in cardiac MLC-2), the CArG box, is present in many muscle-specific and growth factor-regulated genes and binds to an SRF to activate both skeletal and cardiac muscle gene transcription (2, 5, 19, 26, 31, 44). It is believed that the SRF family consists of multiple proteins, which also recognize the AT-rich MEF-2 binding site (36, 49). MEF-2 appears in cultured skeletal muscle cells accompanying differentiation of the skeletal myoblast into myocytes due to serum deprivation (17). To ascertain whether the cardiac tissue-specific BBF-1 activity responds to modulation in serum concentra-

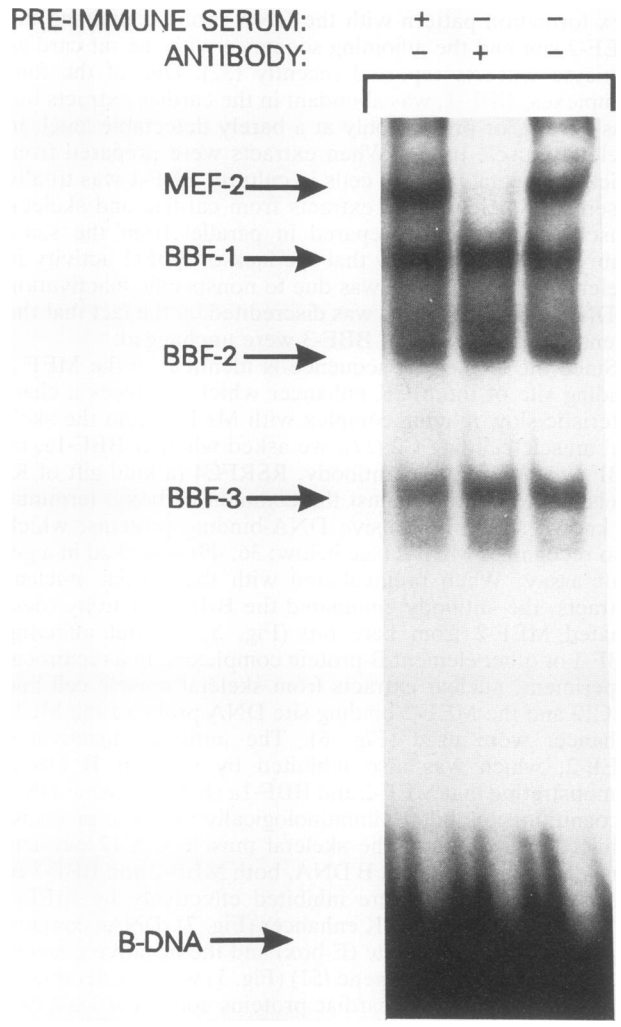


FIG. 5. Effect of an antibody, RSRFC4, on B-DNA and protein complex formation in a gel shift assay. A quantity of 10 μg of nuclear proteins from cardiac tissue was incubated with 0.5 μg of antiserum or preimmune serum of RSRFC4 for 30 min at 4°C or with neither, and the mixture was analyzed by gel shift assay with B-DNA as a probe as shown in Fig. 4.

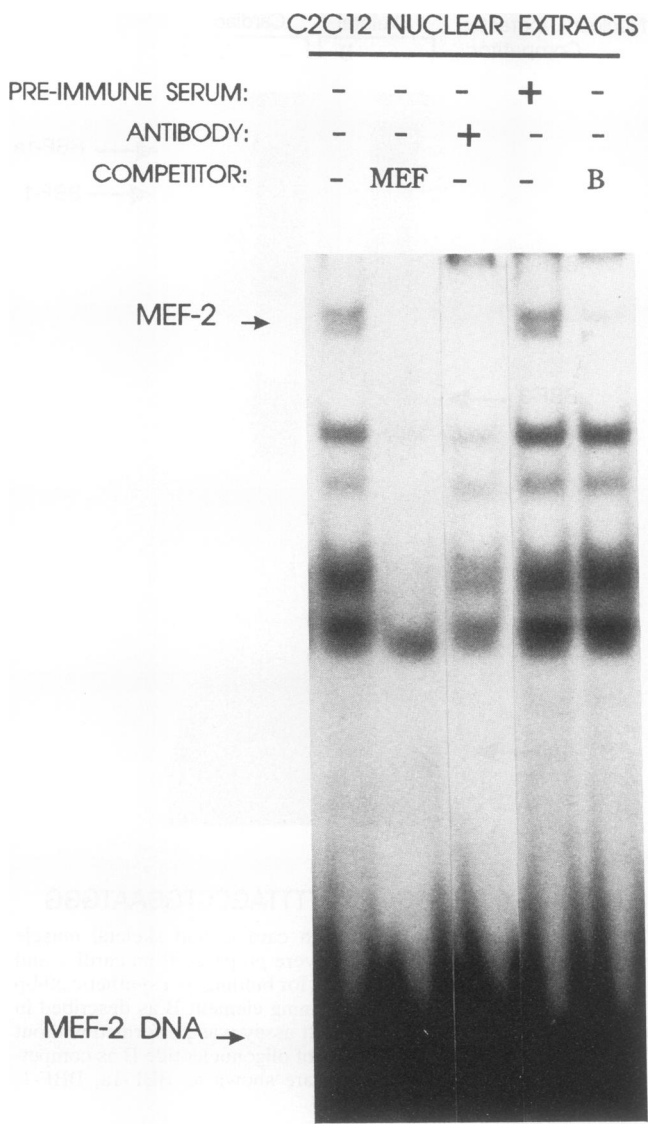


FIG. 6. Gel shift analysis of MEF-2 from C2C12 cells with RSRFC4 antibody. The antiserum of RSFRC4 was tested by preincubation with the nuclear extracts prepared from C2C12 myocytes in a gel shift assay with a probe containing the MCK MEF-2 site. The competitors used were MEF-2 and element B DNAs.

tion in culture medium, we tested BBF-1 binding to MLC-2 element B (MEF-2 site) and the MCK enhancer MEF-2 site as DNA probes in serum-stimulated and serum-deprived cardiac cells in culture. For this purpose, nuclear extracts were prepared from primary cardiac cells cultured in medium with high serum levels for 2 days, washed, and then switched to low-serum medium for 2 to 3 days or kept in high-serum medium continuously (see Materials and Methods). Three defined complexes were formed with element B and nuclear extracts prepared from the cardiac muscle cells cultured continuously in high-serum medium (Fig. 9). However, in extracts from the low-serum medium the slow-moving complex (BBF-1) (see arrow in Fig. 9) disappeared. MEF-2 site DNA produced only two complexes, one of which (see arrow) was also serum dependent (Fig. 9). Interestingly, the MEF-2 binding activity appeared to be at a minimum discernible level in both extracts cultured for 4 to 5 days. To confirm whether the continuously cultured cells are deprived of MEF-2, the extract from cells cultured for 5 days in high-serum medium (Fig. 10, I) was compared with extracts from cells cultured for 2 days in high-serum medium (Fig. 10, II) for binding to element B with and without preincubation with RSRF antibody, as before. Clearly, 2-day-cultured cells contain MEF-2 (BBF-1a) activity which was immunoprecipitable with the antibody. BBF-1 activity remained the same in both extracts. Although the loss of MEF-2 in prolonged cultures is of potential interest and would require further investigation, our data, presented in Fig. 9, nevertheless demonstrated unequivocally that BBF-1 activity is serum inducible.

BBF-1 target site (element B) is a serum response element. If BBF-1 is serum inducible, then its target sequence (element B or the MEF-2 site) must function in a serum-responsive manner. To demonstrate this, we transfected the mutant MLC-2–CAT recombinants into cardiac muscle cells after cells were grown in serum-containing medium for 24 h and then shifted to low-serum medium for the next 3 days. As

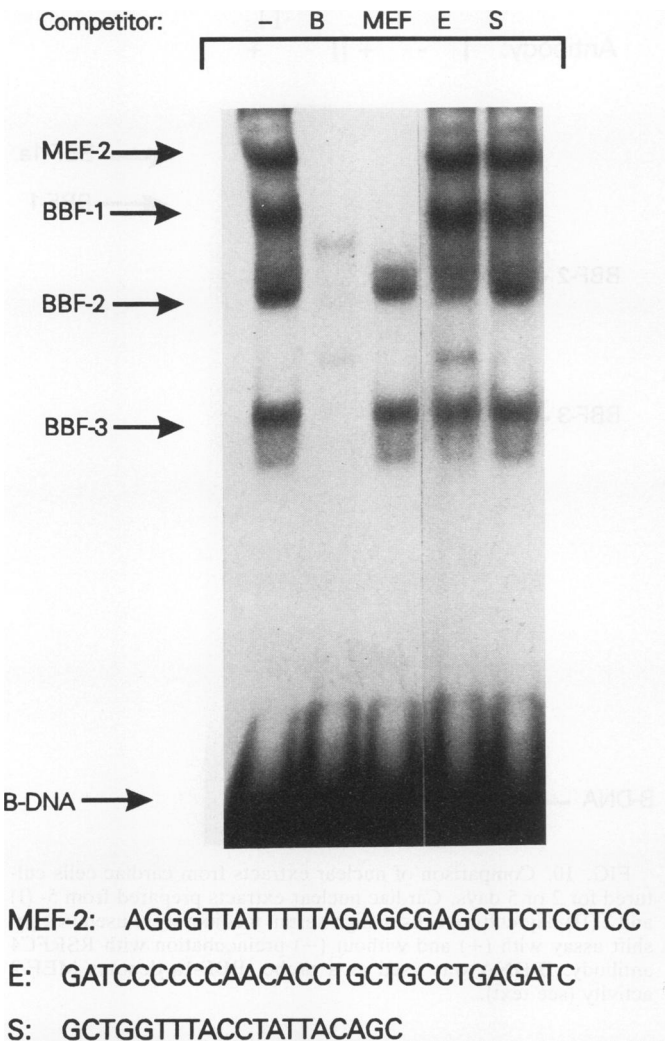


FIG. 7. Analysis of MEF-2 and BBF-1 by DNA competition. The specific binding of cardiac nuclear protein with element B was analyzed by a gel mobility shift assay using different oligonucleotides as competitors and nuclear extracts prepared from cardiac cells. The assay was performed in the absence of competitors (–) or with a 100-fold molar excess of B oligonucleotides, MEF-2 site (MEF), E-box (E), and S-element (S) DNA probes as competitors.

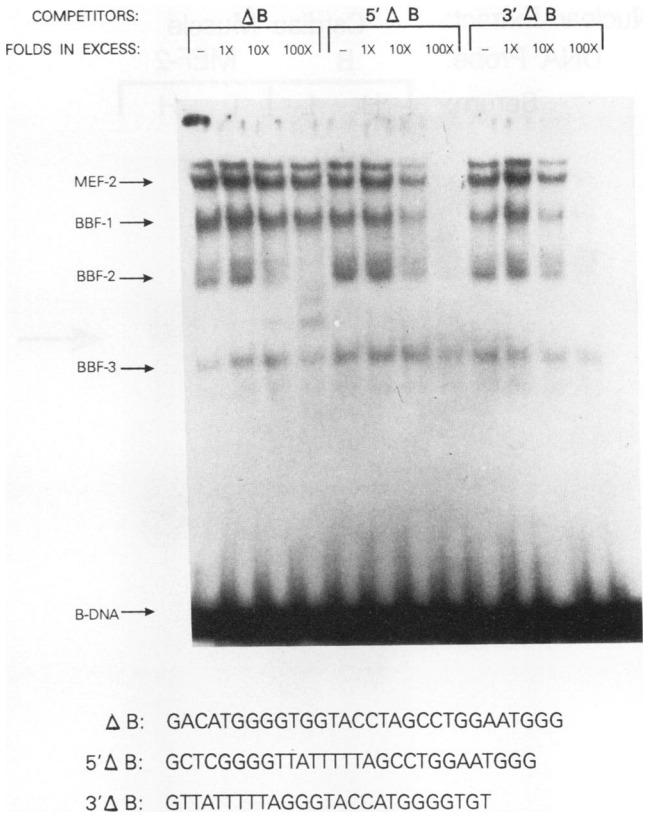


FIG. 8. Sequence requirements for MEF-2 and BBF-1 complex formation. Element B DNA and cardiac MEF-2 and BBF-1 complex formation was tested with DNAs containing a mutated core sequence of element B (ΔB) or a mutated 5′-flanking sequence (5′ΔB) and 3′-flanking (3′ΔB) DNAs as competitors used in 1, 10, and 100× molar excesses in a gel shift assay as described before. The sequences of mutated DNA probes are shown below.

shown in Fig. 11, the promoter activity of plasmid pLCΔ53CAT containing element B in cardiac muscle cells in low-serum medium was reduced significantly while the level of pLCΔ31CAT, the basal promoter, appeared to be insensitive to the serum shift. This was consistent with the disappearance of BBF-1 binding activity in extracts from cardiac muscle cells cultured in low-serum medium. These results also discredit the possibility that the loss of MEF-2 activity observed earlier in 5-day-old cardiac cell culture (Fig. 9 and 10) was due to fibroblasts or nonmyocytes which can overgrow myocytes. We have previously demonstrated that the cardiac MLC-2 promoter is totally inactive in fibroblasts and nonmuscle cells (51). The cultures used here promote MLC-2 expression optimally. More importantly, refeeding of the same cardiac muscle cells with high-serum medium after the shift to low-serum medium for 2 to 3 days allowed recovery of the promoter activity in pLCΔ53CAT, documenting that 5-day cultures contain predominantly myocytes. The serum-responsive regulation of MLC-2 promoter activity mediated by BBF-1, as is the case with SRF (36, 37, 44), is also reversible. Taken together, these results demonstrate that the AT-rich counterpart of the MEF-2 site in the cardiac MLC-2 gene promoter (element B) is recognized by at least two factors, MEF-2 and BBF-1. BBF-1 is different immunologically from MEF-2, and it displays tissue specificity and serum responsiveness.

DISCUSSION

In this report, we have presented evidence for the existence of a cardiac tissue-specific and serum-responsive nuclear factor, BBF-1, which mediates the activation of cardiac MLC-2 gene transcription through its interaction with an AT-rich MEF-2 site promoter sequence, element B. Deletion of this sequence, like that of the MEF-2 binding site from other promoters and enhancers of skeletal muscle genes (17, 21), caused a marked reduction of transcriptional activity, suggesting that element B (or the MEF-2 site) plays a pivotal role in both skeletal and cardiac muscle genes. Indeed, a recent report (32) suggests that a single MEF-2 binding site in the muscle-specific subunit of the human phosphoglycerate mutase gene accounts for the enhanced expression of the gene in both cardiac and skeletal muscle cells. Despite the potential of the MEF-2 site sequence as a regulator common to both muscle lineages, there are conflicting reports on the

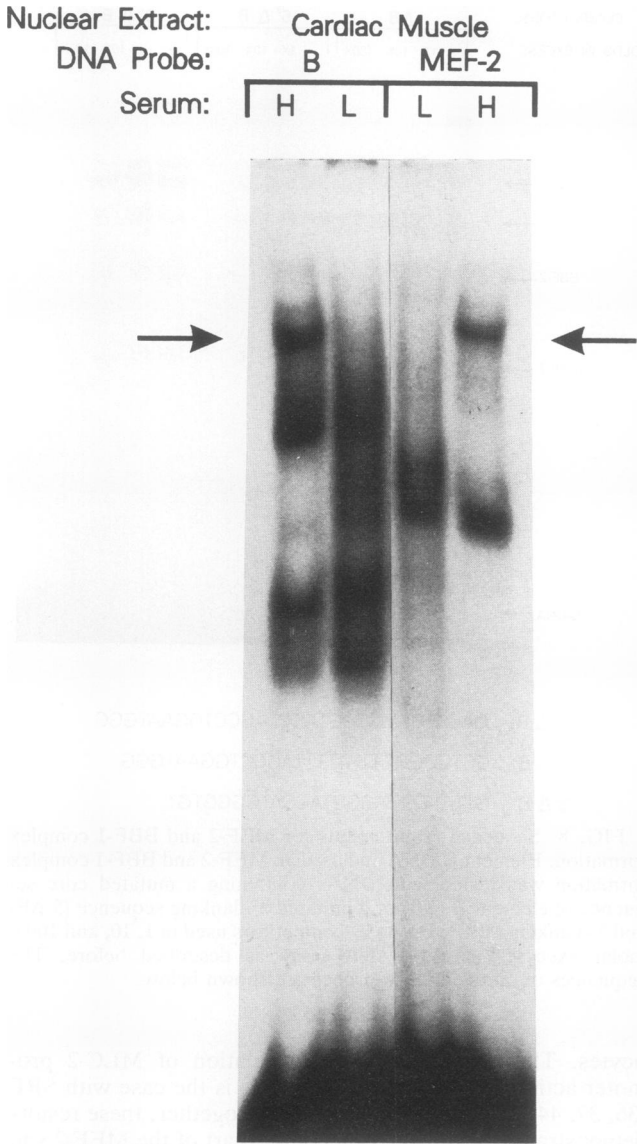


FIG. 9. Serum responsiveness of BBF-1 binding to element B and MEF-2 site DNAs. Cardiac muscle nuclear extracts isolated from primary cells cultured in medium with high levels of serum for 5 days (H) or shifted to low-serum medium after 2 days with high-serum medium (L) were used in a gel mobility shift assay with probes containing element B or MCK MEF-2 site DNAs. Arrows indicate the complex(es) which disappeared in the serum-cultured cells.

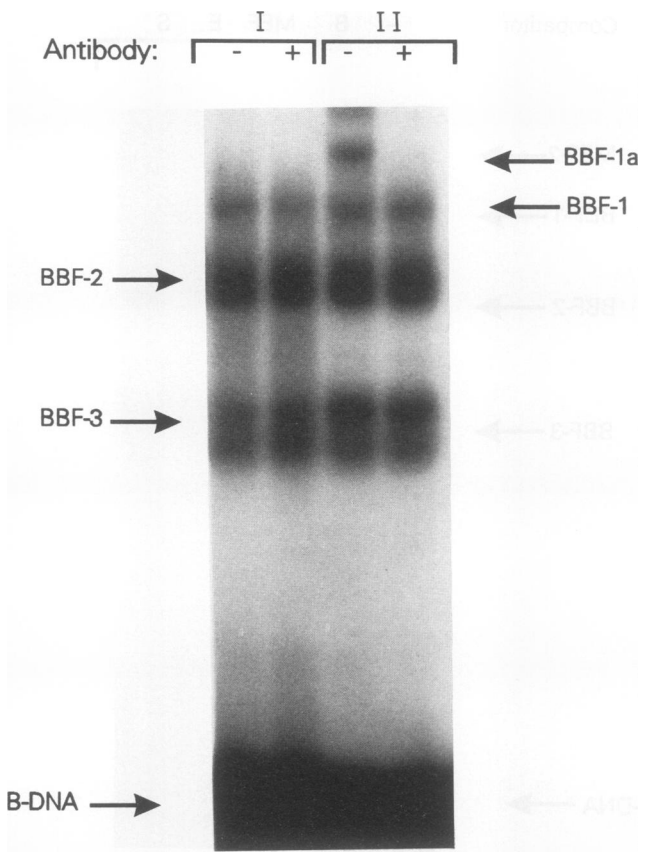


FIG. 10. Comparison of nuclear extracts from cardiac cells cultured for 2 or 5 days. Cardiac nuclear extracts prepared from 5- (I) and 2 (II)-day-culture cells in high-serum medium were used in a gel shift assay with (+) and without (−) preincubation with RSRFC4 antibody. B-DNA was used as a probe. BBF-1a denotes MEF-2 activity (see text).

muscle specificity of protein factors which recognize that sequence. For example, TARP, a non-muscle-specific factor, recognizes the MEF-2 site as well as the AT-rich segments in the brain and muscle creatine kinase promoter and enhancer, respectively (21), and the ubiquitous SRF-related proteins (RSRF) recognize sequences homologous to SRF, TARP, and MEF-2 binding sites (36).

MEF-2 was originally identified as a muscle-specific factor in the skeletal muscle cell line C2 during the transition of the serum-deprived myoblast to myocytes (17). The MEF-2 activity is inhibited by serum in culture medium. Subsequent studies (7, 20, 21) did not confirm the skeletal muscle specificity of protein interactions with the MEF-2 site. It is now believed that both ubiquitous and muscle-specific nuclear factors which bind to the MEF-2 site but with distinct sequence specificities exist (9). Our results here demonstrated that cardiac muscle cells contain at least two MEF-2-like transcription factors, MEF-2 and BBF-1, which both bind to the MEF-2 site in the cardiac MLC-2 promoter (element B), and that one, BBF-1, is cardiac tissue specific and the other, MEF-2, is not. Furthermore, antibodies raised against RSRF recognize MEF-2 but not BBF-1. There is a clear correlation between the appearance of a BBF-1–element B complex and the tissue-specific activity of element B. Taken together, this evidence suggests that cardiac MEF-2 and BBF-1 are members of the MEF-2 family of proteins which have a muscle-specific function but that, unlike the ubiquitous MEF-2, BBF-1 is responsible for cardiac tissue specificity. The potential of element B in cardiac tissue-specific function is also supported by the existence of a 28-bp sequence (HF-1) in rat cardiac MLC-2 (52) which contains within it the core 9-bp-long element B sequence and of a 47-bp segment in the cTnT promoter (22) which shares a distinct homology with element B, both of which are implicated in cardiac tissue-specific transcription.

We have recently reported (41) that the lack of expression of cardiac MLC-2 in skeletal muscle is due to a negative regulatory mechanism which involves an upstream repressor

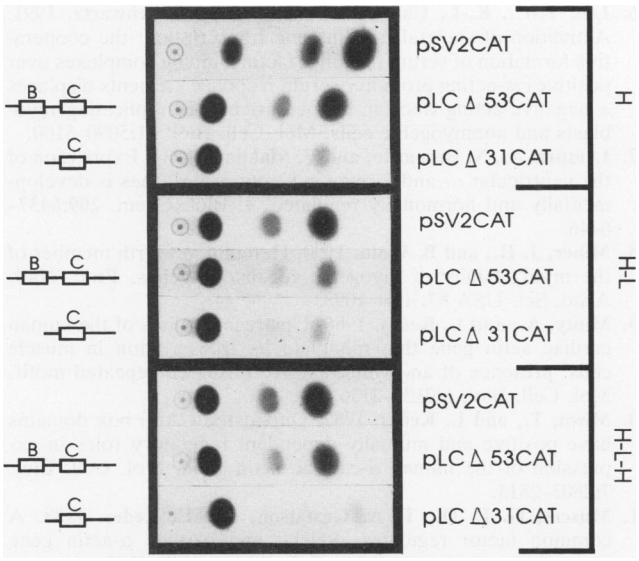


FIG. 11. Serum responsiveness of element B-mediated activation of MLC-2 promoter function. The promoter activity of 5'-deletion mutants containing element C alone (pLCΔ31CAT) and elements B and C (pLCΔ53CAT) was analyzed by transient transfection in cardiac cells grown continuously in high-serum medium for 5 days (H) or shifted to low-serum medium after 2 days in high-serum medium (H-L); some H-L cells were refed with high-serum medium (H-L-H) as described in Materials and Methods.

element, CSS, and its binding proteins, CSFs, present in skeletal muscle. Our data here do not exclude the possibility that element B is not involved in the regulation of cardiac MLC-2 in skeletal muscle. Indeed, the skeletal nuclear proteins recognize element B with sequence specificity. Furthermore, we have observed (unpublished results) that common protein factors might be involved in interactions with these two elements. In early chicken embryonic development, cardiac MLCs are present in both cardiac and skeletal muscle tissues (46), and one could envisage a situation in which the expression of cardiac MLC-2 in early embryonic muscles and its repression in skeletal muscle in later stages of development are functions of a developmental program involving differential syntheses, or activities, of BBFs and CSFs. Conceptually, the developmental changes in MLC-2 expression may be achieved via a combinatorial or preferential use of the positive and negative transcription factors, and we anticipate that such variations in expression and/or utilization of CSFs and BBFs can be experimentally demonstrated. These regulatory sequences and their cognate transcription factors will thus serve as powerful tools for delineating the mechanisms underlying the development and differentiation of cardiac and skeletal muscle lineages (14).

It is well established that the requirements of serum in culture media are different for optimal expression of cardiac tissue- and skeletal muscle-specific genes (6, 8, 13, 14, 24, 35, 40, 42, 43, 45, 47). While reduction of serum levels is required for conversion of the skeletal muscle myoblast to myocytes and activation of skeletal genes, a higher level of serum is needed for optimal expression of cardiac muscle genes (18, 45). It is also recognized that activation of the skeletal myogenic program is dependent upon the expression of MyoD and/or MyoD-related genes (3, 6, 10, 12, 25, 28, 34, 48) following serum withdrawal and through an interaction of MyoD with the target sequence (E-box) in the respective genes. Muscle-specific genes which lack the E-box are presumably regulated through induction of intermediate regulatory factors induced by MyoD or members of the MyoD family. The activation of the MCK gene through MEF-2 is regulated by myogenin, a member of the MyoD family, through a mechanism which requires withdrawal of mitogens from the medium (9), suggesting a pivotal role for MEF-2 in the differentiation pathway of myogenic cells. Mitogenic signals apparently impair regulatory interaction by suppression of MyoD-myogenin expression, and this leads to a block in the skeletal myogenic program.

MyoD family genes are, however, not expressed in cardiac muscle cells. The physiological expression of cardiac muscle genes, which, unlike skeletal genes, are activated by mitogens, might involve factors distinct from the members of the MyoD gene family. No such regulatory factor has so far been isolated. The appearance of BBF-1 due to serum leads to a high level of transcription of cardiac MLC-2 in primary cardiac cells and, conversely, the lack of serum causes its disappearance and a concomitant loss of the promoter activity. We believe that the loss of MLC-2 promoter activity due to serum deprivation is attributable to the disappearance of BBF-1 binding activity. BBF-1 thus appears to be a crucial protein that responds to signals emanating from a cardiac tissue-specific factor(s) functionally analogous to MyoD family proteins. Element B is a potential target site for SRF-related proteins which bind to AT-rich sequences in a number of growth-factor-inducible and muscle-specific genes, including the cardiac MLC-2 gene (36). The serum inducibility of these sites suggests that, like BBF-1, they may also be linked to the extracellular signalling pathway. The notion that accessory proteins participate in SRF interactions has been invoked previously to explain the function of SRF and SRF-related target sites in serum-inducible promoters (36, 37). Such factors might also be involved in functional expression of element B-protein complexes. BBF-1 thus appears to be an important member of the MEF-2 family which would serve as an important tool to advance our understanding of the regulatory mechanisms in cardiogenic differentiation.

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