Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4047–4051, May 1992
Biochemistry

Myocardial activation of the human cardiac α-actin promoter by helix–loop–helix proteins

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Communicated by Harold Weintraub, January 22, 1992 (received for review November 26, 1991)

ABSTRACT **The cardiac α-actin gene is expressed in both heart and skeletal muscle. In skeletal myogenic cells, the 177-base-pair promoter of the human cardiac α-actin (HCA) gene requires three transcription factors for activation: Sp1, serum response factor (SRF), and MyoD. However, MyoD is undetectable in heart. To search for a functional equivalent of MyoD, we analyzed the transcriptional regulation of the HCA promoter in primary cultures of rat cardiac myocytes. The same DNA sequence elements recognized by SRF, Sp1, and MyoD and required for HCA transcription in skeletal muscle cells were also found to be necessary for expression in cardiomyocytes. Overexpression of Id, a negative regulator of basic helix–loop–helix proteins, selectively attenuated expression of the HCA promoter. Cardiomyocyte nuclei contain a protein complex that specifically interacts with the same required sequence (E box) in the HCA promoter that is bound by MyoD in skeletal myogenic cells. Furthermore, these complexes contain a peptide that is a member of the E2A family of basic helix–loop–helix proteins. Cardiomyocyte nuclei appear to be enriched for a protein that can bind to the E-box site as dimers with the E12 protein. These results suggest that a member of the basic helix–loop–helix family, together with SRF and Sp1, activates the HCA promoter in heart. Alternative strategies for myocardial transcription of HCA are discussed.**

MyoD and other myogenic determination factors of the basic helix–loop–helix (bHLH) protein family play a pivotal role in inducing and maintaining the skeletal muscle phenotype (reviewed in ref. 1). Molecular mechanisms activating the myogenic program have been partially clarified. The bHLH myogenic determination factors appear to regulate their own expression (2–4) and act as transcriptional regulators through direct interaction with a specific DNA sequence, the E box (5, 6). Myogenic bHLH proteins greatly increase their affinity for this target sequence by forming heterodimers with bHLH products of the E2A gene, expressed in many cell types. In contrast, heterodimerization with Id, a bHLH protein lacking the characteristic DNA-binding domain, specifically reduces the ability of MyoD and its homologues to bind DNA and to activate transcription. Down-regulation of Id during development is thought to trigger myogenesis (7). The human cardiac α-actin (HCA) promoter is expressed in both heart and skeletal muscle, two embryologically distinct forms of striated muscle. We have previously shown (8) that in skeletal myogenic cells, activation of the HCA promoter requires MyoD as well as two ubiquitous transcription factors, Sp1 and serum response factor (SRF). Since the heart contains neither MyoD nor any related myogenic transcription factors, it is unclear how genes common to both tissues are activated in the heart. Here we report that the same DNA sequences are required for HCA promoter expression in both skeletal and myocardial cell types, including a bHLH-binding E box and binding sites for Sp1 and SRF. Cardiac nuclear factors corresponding to SRF and Sp1 interact with these sequences. We also present several lines of evidence that the element binding MyoD in skeletal muscle cells is bound by a related protein or proteins in cardiac myocytes. Our results suggest that cardiac myocytes contain a factor analogous to MyoD that, with SRF and Sp1, regulates myocardial expression of the cardiac α-actin gene.

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MATERIALS AND METHODS

Plasmid Constructs. The HCA–chloramphenicol acetyltransferase (CAT) and β-actin–CAT chimeras used in this study have been described (8). The Id cDNA expression vector is described in ref. 7. Plasmids pEMSV-CAT and pEMSV were a gift of A. Lassar (Hutchinson Cancer Center, Seattle). To generate pGEX-E12, the plasmid pE12 (9) was cleaved with *Xho* I restriction endonuclease, filled-in with phage T4 DNA polymerase, and cleaved with *Eco*RI. The E12 fragment (encoding amino acids 158–439) was ligated into *Sma* I/*Eco*RI-digested pGEX-2T vector (Pharmacia), and the correct reading frame between the glutathione *S*-transferase (GST) and E12 cDNAs was confirmed by DNA sequencing. Induction of the pGEX-E12 vector and purification of GST–E12 protein were performed as indicated (10).

Cell Culture and Transfections. The myogenic C2 cell line was cultured as described (8). Primary cardiomyocytes were isolated and cultured as described (11). Transfection was done by the calcium phosphate method (8). For both cardiomyocytes and C2 cells, 30 μg of the Id cDNA expression vector or pEMSV vehicle were cotransfected with 4 μg of the −263 HCA–CAT construct. CAT assays were performed as described (8). Transfection experiments were repeated at least twice using two independently prepared plasmids and included duplicate or triplicate samples. Variability between independent experiments was 10–20%. CAT activity was quantitated by densitometry on an Ambis radioanalytic imaging system.

Nuclear Extracts, Antiserum, Gel Electrophoretic Mobility-Shift Assay, and DNA Footprinting. The sequences of the oligonucleotides (5′ to 3′, top strands) employed in mobility-shift assays are the following.

HCA −70/−40	GCCCCCGGCTGCT CCAACTGAC CCTGTCCAT
HCA −70/−40 μA	GCCCCCaGCTCGT CCAACTGAC CCTGTCCAT
HCA −70/−40 μB	GCCCCCGGCTGCT CCAACTaAC CCTGTCCAT
HCA−70/−40 μ6	GCCCCCGGCTGCT tggtCctga CCTGTCCAT
CArG1	AATTCGAAGGGGACCAAATAAGGCAAGGTGGA
Sp1	CCGGGCCCCCACCCCTGCCCCCG

Abbreviations: HCA, human cardiac α-actin; SRF, serum response factor; bHLH, basic helix–loop–helix; CAT, chloramphenicol acetyltransferase; NMC, nonmuscle cardiac; GST, glutathione *S*-transferase.
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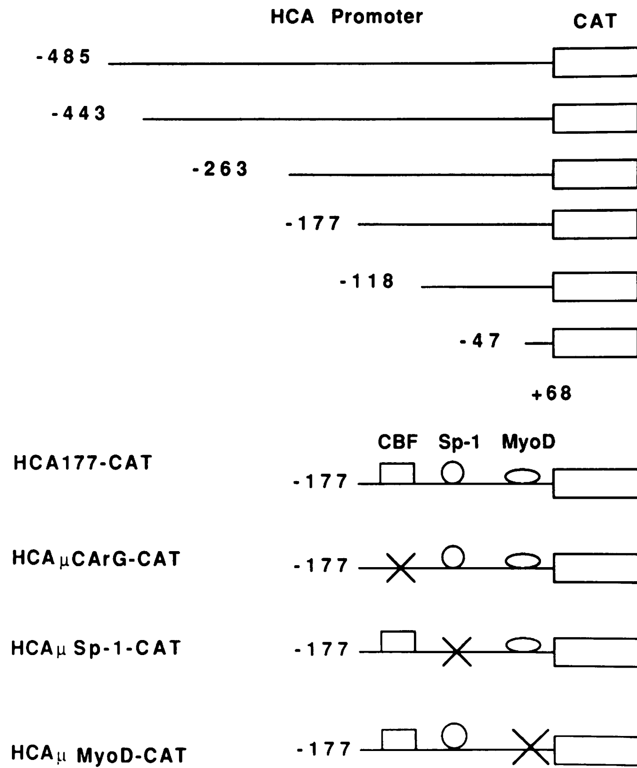

FIG. 1. HCA promoter deletion series. Numbers at left represent the promoter nucleotides included in each construct and refer to the start of transcription at position +1. Every construct terminates at position +68 and is fused to the CAT reporter gene. One of these constructs (−177 HCA–CAT) is also shown at the bottom along with three mutant constructs. Binding sites for CBF (*CArG-binding factor*, here referred to as SRF); Sp1, and MyoD are indicated. An X at the DNA binding sites symbolizes nucleotide mutations interrupting the binding site.

The underlined bases indicate the E box. The black dots are located over base pairs contacted by MyoD in *in vitro* footprinting experiments (8). The underlined lowercase letters indicate nucleotide mutations made either excluding (μA) or including (μB, μ6) the MyoD binding site. Nuclear extracts from the C2 cell line, primary cardiomyocytes, and nonmuscle cardiac (NMC) cells were prepared as described (12). The antiserum against E12/E47 has been described (6). Binding reactions and mobility-shift assays were performed essentially as described (8) except that electrophoresis was conducted at 4°C for 5 hr at 200 V. Methylation interference footprint assays were performed as previously reported (8). The mixing experiments reported in Fig. 4 were conducted by incubating recombinant purified GST-E12 (1 μg) or GST (1 μg) protein with 10 μg of C2, NMC, and cardiomyocyte nuclear extracts for 15 min at room temperature. Radiolabeled HCA −70/−40 oligonucleotide was then added for an additional 15 min and the reaction was analyzed as described for the mobility-shift assay. Protein concentrations were determined with the Bio-Rad protein assay kit.

RESULTS

Myocardial Activation of the HCA Promoter. A summary of the HCA–CAT constructs transfected into primary neonatal rat cardiomyocytes is given in Fig. 1. The construct −485 HCA–CAT sustained maximal CAT activity, whereas the truncated forms −263 HCA–CAT and −177 HCA–CAT retained 50% and 30% of maximal activity (Fig. 2*A*). These relative activities were generally similar to those previously reported for the same constructs in the skeletal muscle-derived C2 cell line (Fig. 2*B*). None of the HCA–CAT constructs was expressed in selectively plated NMC cells (data not shown). Constructs bearing mutations in the MyoD (−177 μMyoD), Spl (−177 μSpl), or SRF (−177 μCArG) binding sites were expressed at levels comparable to that of the basal promoter containing just the TATAA box (−47 HCA–CAT) and at least 4-fold less than that of the intact −177 HCA–CAT construct and ≈16-fold less than the full-length −485 HCA–CAT construct (Fig. 2*A*). We cannot explain why, at these low levels of expression, the activity of −177 μCArG in cardiomyocytes was less than that of the other site-specific mutants and even less than that of the −47 construct. Nevertheless, these findings suggest that cardio-

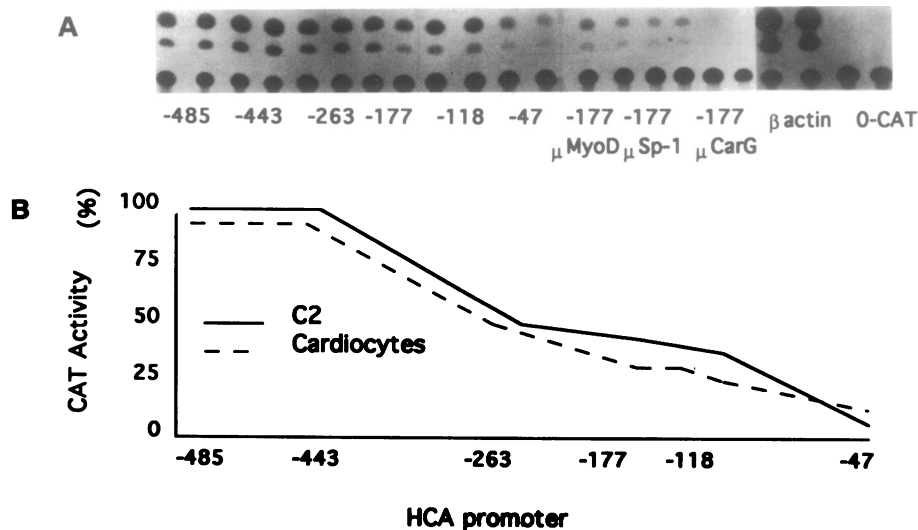

FIG. 2. (*A*) Expression of HCA–CAT constructs in neonatal rat cardiomyocytes. Numbers refer to the length of the HCA promoter sequence of each construct as described in Fig. 1. The structures of the three −177 mutants (μMyoD, μSpl and μCArG) are also shown in Fig. 1. The ubiquitously expressed promoter of the β-actin gene and the backbone of the plasmid (0-CAT) employed to clone the HCA promoter series fused to the CAT gene are included as positive and negative controls, respectively. Each construct was transfected in duplicate. (*B*) Relative transcriptional strength of HCA promoter constructs. CAT activities of 5′-deletion series of HCA promoter in skeletal muscle-derived C2 cell line (as reported in ref. 13) and primary neonatal rat cardiomyocytes (this study) are plotted against promoter length; 100% activity refers to the construct −485 HCA-CAT when transfected into C2 cells or in cardiomyocytes.

Table 1. Id trans-represses the HCA promoter in myocardial and skeletal muscle cells

Exp.	Cells	Reporter	% CAT conversion* − Id	% CAT conversion* + Id	Repression factor (−/+)†
1	Cardiomyocytes	−263 HCA–CAT	17.2, 13.6	8.2, 5.0	2.33
		β-Actin–CAT	100	70	1.43
2	Cardiomyocytes	−263 HCA–CAT	30, 41, 41	9, 12, 15.8	3.04
		pEMSV–CAT	100	103	0.97
3	C2 myocytes	−263 HCA–CAT	8, 18.9	2.7, 2	5.7
		β-Actin–CAT	100	101	0.99

Experiments were performed twice, once in duplicate and once in triplicate, with primary cardiomyocytes and once, in duplicate, with C2 cells.

*Values represent the relative conversion of [^{14}C]chloramphenicol to acetylated forms after incubation with lysates of separately transfected cells. Values from either β-actin–CAT- or pEMSV-CAT-transfected cells were set at 100%. Cells were transfected with CAT reporter genes and either pEMSV plasmid (− Id) or pEMSV-Id (+ Id).

†Ratio of arithmetic means of conversion values from the −Id and + Id transfected cells.

myocyte nuclear transcription factors necessary for HCA–CAT expression interacted with all three sites, including the E-box domain.

The Negative Regulator Id Inhibits Myocardial Activation of the HCA Promoter. If members of the bHLH family were involved in controlling the HCA promoter in heart, we would anticipate that overexpression of the trans-dominant negative regulator Id should repress the activation of the HCA promoter in cardiomyocytes. As seen in Table 1, transient cotransfection of the −263 HCA–CAT construct (containing the single E box at position −50) with an Id expression vector reproducibly inhibited CAT gene expression in cardiomyocytes and in C2 myocytes. In contrast, cotransfection of the Id expression vector did not significantly reduce expression of the CAT gene under control of the Moloney murine sarcoma virus long terminal repeat (pEMSV-CAT in Table 1) or β-actin promoter. The expression vehicle alone (pEMSV) did not affect HCA promoter activity.

E2A Gene Products Interact with Functional Domains of the HCA Promoter. The functional results reported above suggest that bHLH proteins regulate the transcriptional activity of the HCA promoter in myocardial cells. Furthermore, two of the DNA motifs required for activation of the HCA promoter in cardiomyocytes are binding sites for Sp1 and SRF (8) (Fig. 2*A*). Mobility-shift assays were performed to determine whether these elements are bound by cardiomyo-

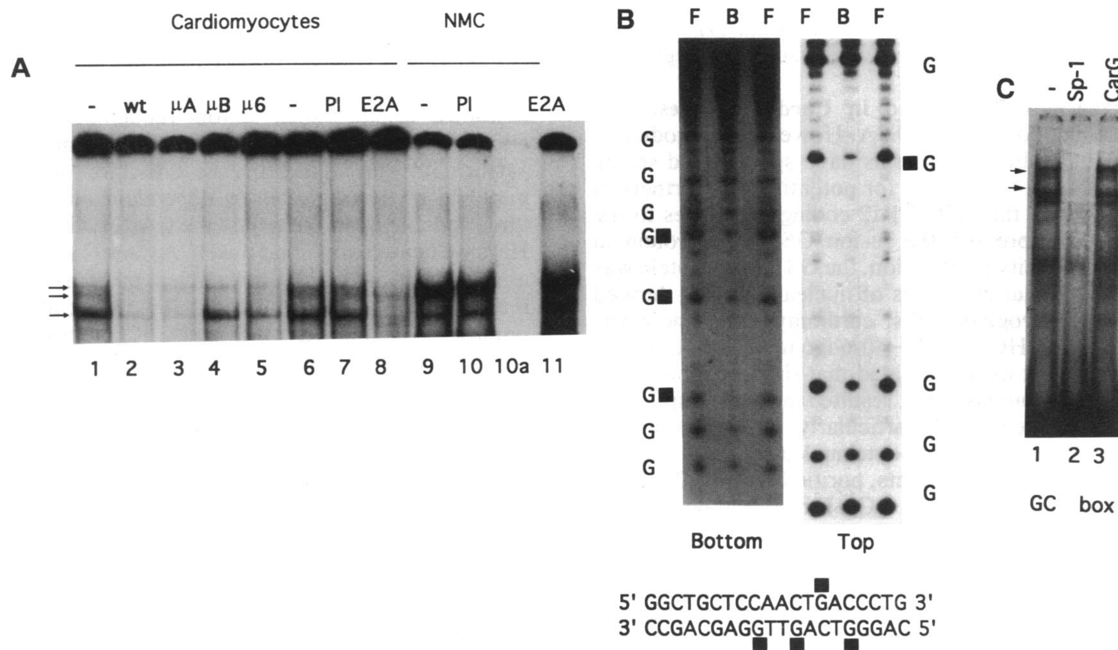

FIG. 3. HCA promoter forms specific complexes with nuclear proteins of cardiomyocytes. (*A*) The three arrows point to a doublet (slowest migrating complex) and a single complex (fastest migrating band) observed when primary cardiomyocyte extract was mixed with the radiolabeled HCA −70/−40 oligomer (lanes 1–8). Competition experiments with wild-type (wt) and mutant (μA, μB, μ6; see *Materials and Methods* for sequences) oligonucleotides are represented in lanes 2–5. NMC cell nuclear extract formed two complexes with the HCA −70/−40 oligomer (lanes 9–11). Lane 10a was not loaded. PI and E2A indicate that a preimmune serum or the E12/E47 antisera was added to the reaction mixture. (*B*) Methylation interference analysis of the interaction of the HCA −70/−40 oligonucleotide with cardiomyocyte nuclear protein. The fastest migrating band (lower band in *A*) was isolated and investigated for nucleotide contacts. Solid squares indicate guanine (G) residues whose methylation interfered with the binding of the cardiac factors. Lanes F and B, free and bound oligomers, respectively. A summary of the interference pattern observed on the top and bottom strands of the HCA −70/−40 sequence is presented below the autoradiogram. (*C*) The oligonucleotide spanning nucleotides −87 to −65 of the HCA promoter, containing the Sp1 binding site, was added to cardiomyocyte nuclear extract and a mobility-shift assay was performed. The Sp1 oligonucleotide has been shown to interact with genuine Sp1 protein (8, 13). Arrows point to specific complexes (lane 1) whose formation was abolished by a 100-fold molar excess of nonradiolabeled Sp1 oligonucleotide (lane 2) but not by the CArG1 oligonucleotide (lane 3).

cyte nuclear proteins. The cardiomyocyte extracts engendered two complexes when incubated with the HCA −70/−40 oligomer. A close inspection of the slowest migrating complex reveals a doublet (Fig. 3*A*, lane 1). An excess of the HCA −70/−40 "wild-type" and μA oligomers prevented the formation of the retarded complexes (lanes 2 and 3). Oligomers with mutations in the E box [μ6, which is responsible for reduced function in the −177 HCA μMyoD–CAT construct (see Fig. 2*A*), and μB] were unable to compete efficiently for protein binding (lanes 4 and 5). The NMC cell extract displayed a gel retardation pattern similar to that observed with cardiomyocyte nuclear extracts (Fig. 3*A*, lane 9; see below). Preincubation of the cardiomyocyte nuclear extract with the E2A antiserum resulted in the destabilization of the specific retarded complexes, whereas a preimmune serum did not affect complex formation (Fig. 3*A*, lanes 7 and 8). The NMC cell extract behaved differently, since neither the preimmune serum nor the E2A antiserum modified the electrophoretic pattern (lanes 10 and 11). Experiments conducted with the HCA −70/−40 oligomer and C2 nuclear extract showed that the E2A antiserum prevented formation of specific retarded complexes (data not shown).

To analyze the contacts made by the cardiac proteins on the HCA −70/−40 sequence, we performed a methylation interference assay. The methylated guanine residues that prevented the myocardial factors from binding were restricted to the CAACTGAC sequence (Fig. 3*B*). The G·C box located between the CArG1 box and the E box was bound by cardiomyocyte nuclear extract factor(s), and an oligonucleotide containing an Sp1 binding site competed specifically with the G·C box (Fig. 3*C*, lanes 1–3). In addition, the CArG1 oligomer is contacted by a protein present in the cardiac extract that is antigenically related to SRF (data not shown). Thus both functional and DNA binding data argue for an involvement of Sp1 and SRF (or related factors) in regulating the HCA promoter in heart.

E12 Partners Are Enriched in Cardiomyocytes. Homodimers of E12 do not bind DNA. However, heterodimers of E12 with other bHLH proteins have specific and strong affinity for DNA (14). To look for potential E12 partners in the heart we fused the bHLH E12 coding sequences to the GST gene and expressed the fusion GST–E12 protein in bacteria. After affinity purification, the GST–E12 protein was incubated with equal quantities of nuclear extracts derived from either C2 myogenic cells, cardiomyocytes, or NMC cells and with the HCA −70/−40 oligonucleotide (HCA E box). Mobility-shift assay revealed that the inclusion of the GST–E12 protein, but not GST, resulted in the appearance of an additional shifted band particularly prominent in the reactions containing cardiomyocyte nuclear extracts (Fig. 4). In the absence of nuclear extracts, purified GST–E12 protein (1 μg) failed to interact with the HCA E box, but the addition of GST–MyoD (35 ng) to GST–E12 (35 ng) produced a detectable and specific band shift with the HCA E box (data not shown). We interpret the GST–E12-dependent shift as resulting from dimerization between bacterially produced E12 and bHLH partners, possibly E47 or related proteins (9, 15), present in the nuclear extracts. The GST–E12-dependent shift was present but barely detectable with C2, NMC (Fig. 4) COS (kidney), DBT (astrocytes), and Huh 7 and Alexandra (liver) nuclear extracts (data not shown). The intensity of the GST–E12-dependent shift with cardiomyocyte nuclear extracts suggests that proteins heterodimerizing with E12 are enriched in these cells.

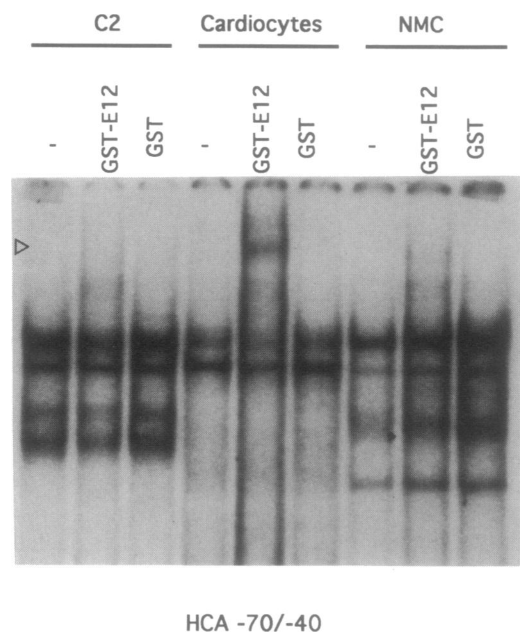

FIG. 4. Nuclear factors heterodimerizing with the GST–E12 protein are enriched in the heart. Nuclear extracts from the C2 cell line, from cardiomyocytes, and from NMC cells were incubated with bacterially expressed GST–E12 or GST protein or in the absence of recombinant protein (−) before the radiolabeled HCA −70/−40 oligomer was added and the complexes were subjected to mobility-shift assay. Arrowhead indicates a band shift particularly evident when the GST–E12 protein was combined with cardiocyte nuclear extract.

DISCUSSION

Most muscle-specific genes are transcriptionally controlled in skeletal muscle by the myogenic determinant bHLH proteins, of which MyoD and myogenin are examples. Many of these genes are also expressed in heart, but the heart contains no myogenic bHLH proteins. The identity of the putative myocardial transcription factor(s) is unknown, nor is it known whether such factors recognize the same DNA elements as their counterparts in skeletal muscle.

Our data show that selective activation of the HCA promoter in myocardial cells is dependent on the integrity of three specific DNA elements: the CArG1, G·C, and E boxes. Here we demonstrate that the HCA CArG1 and G·C elements are bound by myocardial cell nuclear proteins that are similar if not identical to SRF and Sp1. In support of this observation, Sp1 (16) and SRF (V.S. and L.K., unpublished results) have been detected in the heart. A more important purpose of this study was to identify an E-box-binding factor in myocardial cells analogous to MyoD. The existence of such a factor is implied by our finding that Id inhibits HCA promoter transcription. By use of methylation interference and mobility-shift assays, we have further shown that the E box is bound by a complex of myocardial nuclear proteins recognized by antiserum against E2A proteins. Moreover, cardiac myocyte nuclear protein forms a specific HCA promoter-binding complex with E12, which by itself is incapable of binding DNA. Taken together, these data provide evidence that myocardium expresses a bHLH protein involved in the regulation of cardiac actin gene expression. The similar electrophoretic mobilities of the complexes observed with extracts from cardiomyocytes, C2 cells (data not shown), and NMC cells (Fig. 3*A*) contrasts with the difference of the susceptibility of these complexes to E2A antiserum. This, and the subtle but reproducible differences we observe between C2 and myocardiocyte nuclear extracts, suggests that the myocardial complex may contain a cell-type-specific component. Our data are consistent with the following possibilities. Heterodimerization between tissue-specific and non-tissue-specific bHLH proteins seems to be a general feature of tissue-specific gene regulation by these factors.

Examples of this are found in muscle (17), B lymphocytes (18), T lymphoblasts (19), pancreas (20), and developing neural tissue (21). E12/E47 heterodimers or E47 homodimers bind DNA and specifically recognize the E box of the muscle creatine kinase enhancer (18). Both of these proteins are expressed in a wide range of cell types. However, such complexes fail to activate transcription in nonmyogenic cells. By analogy with other cell types, an argument can be made for a cardiac-specific bHLH protein that interacts with E2 proteins to drive myocardial expression of the HCA promoter. Another explanation is that the relative abundance of a non-tissue-specific factor in myocardium determines tissue-specific expression of cardiac actin. We found that cardiac myocyte nuclear extracts were relatively enriched in protein capable of complexing with E12 and conferring DNA binding specificity. Nuclear extracts from all other cell types tested, including fibroblasts, C2 myogenic cells, and hepatocytes, had significantly lower concentrations of this protein. The selective activation of liver-specific genes is thought to be due to the high concentration of a non-tissue-specific factor in liver cells (22–24). In addition, the relative activities of ubiquitously expressed Fos and Jun proteins determine the cell-specific activation of the proliferin promoter (25). It is therefore possible that uneven distribution of generally expressed activators, including the bHLH and homeodomain proteins (26), conveys the selective activation of certain genes and contributes to cell-fate decisions.

We thank S. Tapscott, A. Lassar, and H. Weintraub for providing pEMSV-Id and pEMSV-CAT. We thank A. Voronova, D. Baltimore, and C. Murre for E12/47 antiserum. S. Tapscott, K. Webster, B. Wold, and members of the Kedes laboratory provided thoughtful comments. This work was supported in part by grants from the National Institutes of Health, the American Heart Association–Los Angeles Affiliate, and the Muscular Dystrophy Association (to L.K.) and from the Department of Veterans Affairs (to N.H.B.). V.S. is supported by the George Meany Neuromuscular Disease Research Fellowship of the Muscular Dystrophy Association.

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