Multiple muscle-specific regulatory elements are associated with a DNase I hypersensitive site of the cardiac β-myosin heavy-chain gene

Weei-Yuarn HUANG*, Jin-Jer CHEN†, N.-L. SHIH† and Choong-Chin LIEW*[1]

*Laboratory for Molecular Cardiology, Departments of Clinical Biochemistry and Medicine, University of Toronto, Toronto, Ontario, Canada, and †Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan

Using nuclei isolated from neonatal cardiomyocytes, we have mapped the DNase I hypersensitive sites (DHSs) residing within the 5′-upstream regions of the hamster cardiac myosin heavy-chain (MyHC) gene. Two cardiac-specific DHSs within the 5 kb upstream region of the cardiac MyHC gene were identified. One of the DHSs was mapped to the −2.3 kb (β-2.3 kb) region and the other to the proximal promoter region. We further localized the β-2.3 kb site to a range of 250 bp. Multiple, conserved, muscle regulatory motifs were found within the β-2.3 kb site, consisting of three E-boxes, one AP-2 site, one CArG motif, one CT/ACCC box and one myocyte-specific enhancer factor-2 site. This cluster of regulatory elements is strikingly similar to a cluster found in the enhancer of the mouse muscle creatine kinase gene (−1256 to −1050). The specific interaction of the motifs within the β-2.3 kb site and the cardiac nuclear proteins was demonstrated using gel mobility-shift assays and footprinting analysis. In addition, transfection analysis revealed a significant increase in chloramphenicol acetyltransferase activity when the β-2.3 kb site was linked to a heterologous promoter. These results suggest that previously undefined regulatory elements of the β-MyHC gene may be associated with the β-2.3 kb site.

INTRODUCTION

There exists in vertebrates a subset of muscle-specific genes that are expressed in both cardiac and skeletal muscle. Transient transfection assays and/or transgenic mouse studies have identified distinct cardiac and skeletal elements within the 5′-flanking regions of the myosin light chain-2 [1], cardiac troponin T [2], cardiac troponin C [3] and cardiac α actin genes [4]. Such studies may elucidate the molecular pathways that control the cardiac gene programme and its relationship to the E-box-dependent pathways characterized in many skeletal-muscle genes (for review see [5]).

Myosin heavy chains (MyHCs) in mammalian hearts are expressed in two isoforms: α-MyHC and β-MyHC. The cardiac α- and β-MyHC genes are tandemly aligned in the genome (i.e. β-α) and are separated by a 4 kb intergenic region (for review see [6]). The cardiac β-MyHC gene is expressed in a tissue- and developmentally specific manner, and is regulated primarily at the transcriptional level [1,7]. In the cardiac ventricle of small mammals, such as the mouse, the β-MyHC transcripts predominate during embryonic and fetal development. At about the time of birth, a switch in cardiac MyHC gene expression takes place, characterized by the down-regulation of β-MyHC gene expression, together with the concomitant induction of the α-isoform. The adult slow-twitch skeletal muscles continue to express the β-MyHC gene. Regulation of these genes is also influenced by pathological processes, e.g. cardiac hypertrophy causes the re-expression of the β-MyHC gene in the ventricles of small mammals [8]. The developmental expression pattern of the small mammal β-MyHC gene is different from that of the human β-MyHC gene, where it is expressed both in fetal and adult heart ventricles [9].

Using *in vitro* transient transfection assays, the promoter region of the cardiac β-MyHC gene has been studied in various species, and a number of potential regulatory elements within 300 bp of the transcriptional start site have been identified [10–13]. However, the results from transgenic studies suggest that other, as yet undefined, elements may reside further upstream of the β-MyHC gene [14–16]. Furthermore, Sadoshima et al. [17] found that the stretch-response element of the rat cardiac β-MyHC gene lies beyond −628 bp of the 5′-flanking region, providing more evidence for the presence of far-upstream regulatory elements.

Recently we characterized and completely sequenced the hamster cardiac α- and β-MyHC genes [18,19]. One of the cosmid clones that we isolated contains 12 kb of upstream sequence from the transcriptional start site of the β-MyHC gene. Since regions of chromatin that are hypersensitive to nuclease digestion are often believed to be nucleosome-free (thereby allowing resident DNA access to sequence-specific DNA binding factors [20]), we endeavoured to map the DNase I hypersensitive sites (DHSs) within the 5′-flanking region of the cardiac β-MyHC gene. In this report we describe the characterization of one of the DHSs, the β-2.3 kb site.

MATERIALS AND METHODS

Preparation of nuclei from cardiomyocytes, fibroblasts and baby hamster kidney (BHK) cells

Neonatal hamster cardiomyocytes were prepared, with minor modifications, as described previously for neonatal rat cardiomyocytes [21]. One-day-old neonatal hamster hearts were excised and minced in 1 × PBS, and the cells were dispersed by successive additions of 1 × PBS containing 0.125 % (w/v) trypsin and stirred at 37 °C. The cells were then collected in F10 medium + 20 % (v/v) fetal-bovine serum and preplated for 45 min to remove

Abbreviations used: AP, activator protein; CAT, chloramphenicol acetyltransferase; BHK, baby hamster kidney; DHS, DNase I hypersensitive site; MCK, mouse muscle creatine kinase; MEF-2, myocyte-specific enhancer factor 2; MyHC, myosin heavy chain; TK, thymidine kinase.

[1]To whom correspondence should be addressed at: The Cardiac Gene Unit, 100 College Street, Banting Institute, Room 418, University of Toronto, Toronto, Ontario M5G 1L5, Canada.

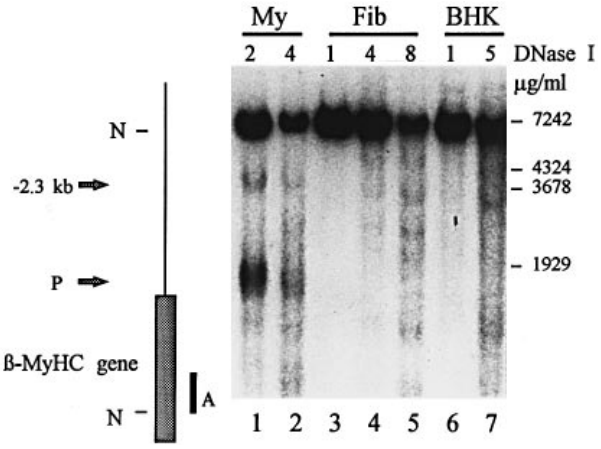


Figure 1 DHSs of the cardiac β-MyHC gene

The position of probe A is shown. DHSs are indicated by arrowheads. The positions of the two *Nco*I (N) restriction sites in the β-MyHC gene are −5269 and +1717 respectively. Isolation of nuclei from cardiomyocytes (My, lanes 1 and 2), fibroblasts (Fib, lanes 3–5) or BHK cells (lanes 6 and 7), and treatment with various concentrations of DNase I, as indicated, was conducted according to the procedure described in the Materials and methods section. Purified genomic DNAs (15 µg) were digested with *Nco*I, electrophoresed on a 1% agarose gel, and subjected to Southern blot hybridization with probe A. The DHSs identified are indicated by the arrows, −2.3 kb and P (proximal promoter) respectively. The DNA marker used is lambda DNA digested by *Bst*EII.

fibroblasts. The enriched cardiomyocytes were removed and replated at a density of 1×10^7/100-mm-diameter dish with 0.1 mM bromodeoxyuridine to prevent proliferation of non-myocytes. After 36 h, the cells were harvested by treatment with 0.05% trypsin/EDTA, washed three times with 1×PBS and resuspended with nuclear buffer containing 60 mM KCl, 15 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris/HCl (pH 7.5), 0.5 mM dithiothreitol, 0.1 mM PMSF, 300 mM sucrose and 0.5% (v/v) Nonidet P40. After incubation at 4 °C for 10 min, nuclei were collected by centrifugation at 1000 ***g*** (4 °C for 10 min), washed three times with nuclear buffer (without Nonidet P40) and re-suspended at a density of 1×10^7/ml. The isolation of nuclei from cardiac fibroblasts (harvested from the preplating plates) and BHK cells was carried out as described for the cardiomyocytes. Aliquots of nuclei were then treated with DNase I (Pharmacia) at concentrations ranging from 1 to 10 µg/ml at 37 °C for 2 min in the presence of 5 mM $MgCl_2$. The nuclei were then disrupted in a 1 M NaCl solution containing proteinase K (200 µg/ml) and 0.25% (w/v) SDS and incubated overnight at 37 °C. Genomic DNA was then extracted three times with phenol and chloroform and precipitated with sodium acetate and ethanol.

Indirect-end-labelling method [22]

The method used for detecting the DHSs of the cardiac β-MyHC gene was performed as described previously [23]. The probe used in this study was amplified by PCR, using cosmid DNA containing the 5′-upstream region of the hamster β-MyHC gene as template. The amplified PCR fragment was subcloned into the TA vector (Invitrogen) and subsequently verified by dideoxy-DNA sequencing using an automated DNA sequencer (Pharmacia). The position of probe A (β-MyHC gene) adjacent to the *Nco*I restriction site is indicated in Figure 1. The genomic DNA isolated as described above was digested by *Nco*I and fractionated in 1% (w/v) agarose gel. The gel was then transferred to nitrocellulose and hybridized with the probes, which were prepared by random-primer labelling. The nitrocellulose was then washed in 0.1×SSC containing 0.1% SDS at 65 °C for 20–45 min and exposed to photographic film with an intensifying screen at −80 °C for 2–7 days.

Gel mobility-shift assay

Nuclear extracts of neonatal hamster cardiomyocytes and C2C12 myotubes were prepared as described by Andrews and Faller [24]. The extracts were used in gel-shift assay experiments with a ^{32}P-labelled 85 bp DNA fragment (*Acc*I–*Pst*I), derived from the β-2.3 kb site. The assay was carried out as described previously [25]. The nucleotide sequences of the CT/ACCC box (derived from the β-2.3 kb site) and the myocyte-specific enhancer factor 2 (MEF-2) binding site [derived from the mouse muscle creatine kinase (MCK) gene] [26] are 5′-GAAGCGGCCCTCCCTCCA-GTCCCTGT-3′ and 5′-CTCGCTCTAAAAATAACCCTGT-3′ respectively.

Methylation interference analysis

End-labelled DNA (i.e. the 85 bp DNA fragment used in gel mobility-shift assays) was methylated with dimethyl sulphate, as described by Baldwin [27], and used in a binding reaction that was scaled up 5-fold. After electrophoresis and autoradiography of the wet gel, bands were excised and electroeluted, and the DNA was cleaved by piperidine. The cleavage products were separated on denaturing 8% (w/v) polyacrylamide gels and exposed overnight at −80 °C with an intensifying screen.

Cell culture and transfection experiments

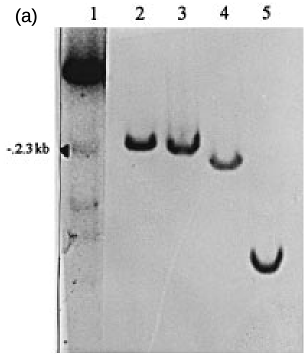
The β-2.3 kb site (−2477 to −2240) was amplified by PCR and subcloned into the chloramphenicol acetyltransferase (CAT) vector containing a minimal thymidine kinase (TK) promoter. The orientation and nucleotide sequence of the insert was verified by automatic DNA sequence (Pharmacia).

The C2C12 cell line was grown in Dulbecco's modified Eagle's medium with 10% fetal-bovine serum. After confluence, the cells were induced to differentiation by switching the culture medium to Dulbecco's modified Eagle's medium with 2% (v/v) horse serum. The cells were grown for 4 days, then the CAT constructs (15 µg) and pRSV β-galactosidase control plasmid (10 µg) were introduced into cells using the calcium phosphate precipitation method [28]. After 6 h, the cells were washed with 1×PBS and fresh medium was added. After another 48 h, the cells were assayed for CAT and β-galactosidase activities [29,30]. For each CAT assay equal amounts of protein were assayed (100 µg), and the reactions were allowed to proceed for 2 h. The CAT assay results were quantified using the Bio-Rad phosphorimager, and the mean for four transfections was plotted after adjusting for β-galactosidase activity. The CAT assays were repeated with at least three separate preparations of cells. Cardiofibroblasts were derived from the preplating plates of primary cardiomyocyte cultures, and maintained in F10 medium with 10% fetal-bovine serum.

RESULTS

Cardiac-specific DHSs of the cardiac β-MyHC gene

The position of probe A on the β-MyHC gene is shown in Figure 1. Since only 50–60% of cells in the neonatal heart are cardiomyocytes, the DHS pattern of enriched cardiomyocytes was compared with that of another major cell type in the myocardium, the cardiofibroblasts. Using probe A, two strong DHSs were detected in the cardiomyocytes (Figure 1, lanes 1 and 2) but not



(b)

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(1) Cardiac ß-MyHC gene (ß-2.3 kb site)

(-2508)  ctggtagtaattttagcaaactttccgctggtctcca ctggcgtgtt (M)
(-2823)  ....g..............ct.....t...aa..a.cg.......... (H)

         cacagCAAATGgctcctgtttgggcctgaCATTTGatcaggttggtga (M)
         ..............c.......t...t.....c..........g...... (H)
             E-box                      E-box
         cagagcccagacaaag cagcacaatgcctaGGGCTGGGggagtaggg (M)
         .....tg.........c.g........   c..gga......g.cc. (H)
                                        AP-2
         aggcagatcaccttcctgccac  ccagaCCTTATTTGGtctacccca (M)
         g................tt...tt.ag.g.................ttg (H)
                                      CArG box   AccI
         gtgaagcggCCCTCCCTCcagtccctgtTTATTTATAgCACTT Gctc (M)
         cc..............t.....c.t..c..............t..t...t (H)
              CT/ACCC                 MEF-2    E-Box
         tgtcccctttgtcttgtctgctgtcactctgcag (M) (-2240)
         gc..t.....a.....ctat.ac.tgtagca..c (H) (-2554)
                                      PstI


(2) Mouse MCK gene

(-1256)  ccactacgggtctaggctgcCCATGTAAGGaggcaaGGCCTGGGgaca
                                CArG box      AP-2
         cccgagatgcctggttataattaacccagaCATGTGgctgccccccc
                                          E-box
         cccccaaCACCTGctgcctgagcctcaCCCCCACCCCggtgcctgggt
              E-box                     CT/ACCC
         cttaggctctgtacaccatggaggagaagctcgctCTAAAAATAACCc
                                                MEF-2
         tgtccctggtggatc (-1050)
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Figure 2 Defining the region of the β-2.3 kb site

(**a**) The isolation of adult ventricular nuclei was performed as described previously [31]. Lane 1, genomic DNA (25 μg) from adult heart nuclei treated with 3 μg/ml DNase I was digested with *Nco*I and run in parallel with DNA markers (lanes 2–5). Subsequent Southern blot analysis with probe A was performed as described above. The -2.3 kb site is indicated by an arrowhead. Lanes 2–5 represent 25 μg of genomic DNA digested by *Sph*I, *Pst*I, *Sac*I + *Nco*I and *Pst*I + *Nco*I respectively. The size of DNA fragments from lanes 2–5 are 4177 bp, 4032 bp, 3783 bp and 2015 bp respectively. (**b**) Nucleotide sequence of the β-2.3 kb site (1) and the enhancer of the mouse MCK gene (2) are listed for comparison. In order to maximize alignment of the hamster (M) and human (H) nt sequences, some gaps were created.

in the cardiofibroblasts (lanes 3–5) or BHK cells (lanes 6 and 7). Based on the size of DNA fragments detected in the gel and on the position of probe A, these two DHSs were mapped to the -2.3 kb and the -0.2 kb regions (P, proximal promoter) respectively. Although weaker in intensity, the -2.3 kb site can also be detected in adult heart ventricles (see Figure 2a, lane 1).

Identification of DNA binding activity in the β-2.3 kb site

Hamster genomic DNA digested with a variety of enzymes was electrophoresed alongside the DNase I-treated sample to provide accurate molecular size markers (Figure 2a). Based on the DNA markers, the size of the β-2.3 kb site was estimated at between 4177 and 4032 bp (i.e. -2460 bp to -2315 bp relative to the transcription-initiation site of the β-MyHC gene). Since the size range of most DHSs is about 200 bp [20,32], we defined this -2.3 kb site to a range of 250 bp (-2508 bp to -2258 bp) to include most of the potential regulatory elements residing within this region.

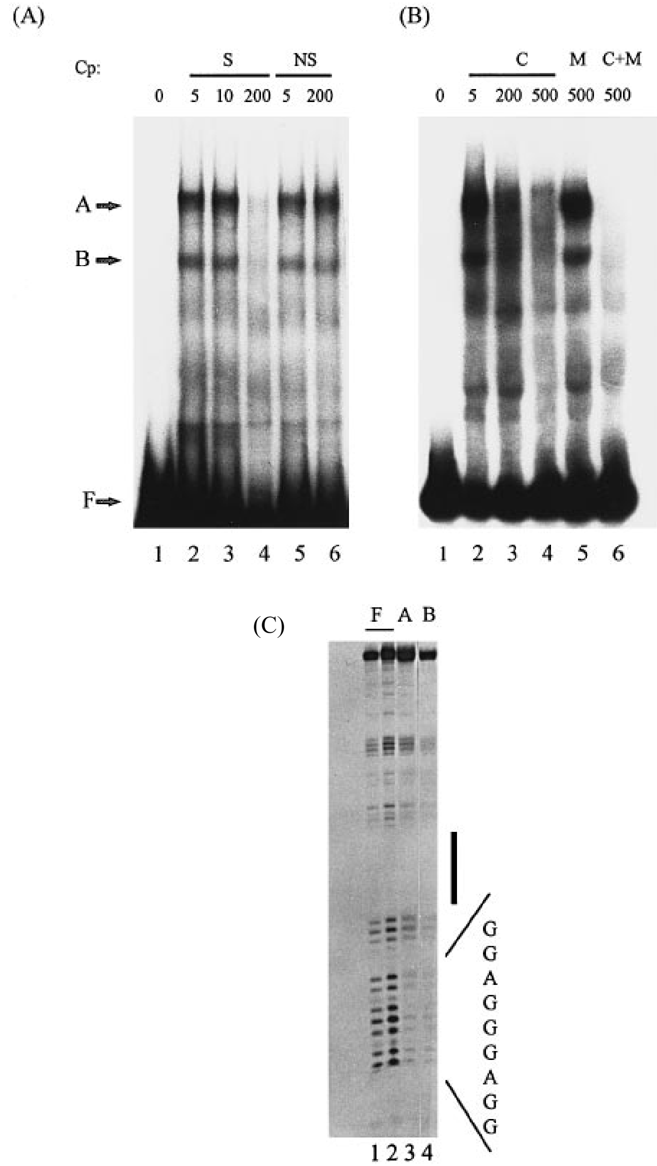

Figure 3 DNA binding activity in the β-2.3 kb site

(**A**) and (**B**) From lanes 2–6, 10 μg nuclear extracts from neonatal hamster heart were incubated with the end-labelled 85 bp DNA fragment (a subregion of the β-2.3 kb site derived by digestion with *Acc*I and *Pst*I restriction enzymes, see Figure 2b) at room temperature for 15 min in the presence of non-radioactive specific (S) or non-specific (NS) competitors (Cp) added at the molar ratios shown. Retarded migration of complexes A and B which represent DNA-binding activity are indicated. (**B**) Competition analysis using CT/ACCC box [(**C**) (lanes 2 to 4)], or MEF-2 oligomers (M, lane 5) as competitors. In lane 6 both CT/ACCC box (500 M) and MEF-2 competitors (500 M) were added together. (**A**) and (**B**) Lane 1, free probe. (**C**) Protein–DNA contacts in complexes A (lane 3) and B (lane 4), were revealed by methylation interference analysis (see the Materials and methods section). Lanes 1 and 2 are free DNA (F). The protected residues (5′-GGAGGGAGG-3′, antisense strand) in complexes A and B are shown on the right. The vertical bar indicates the MEF-2 site.

Multiple regulatory motifs, which have been shown to be important for muscle-specific gene expression, were identified within the 250 bp region of the β-2.3 kb site (Figure 2b). These include three E-boxes, one acivator protein (AP)-2 site, one

CArG motif, one CT/ACCC box and one MEF-2 site. Except for the AP-2 site and the E-boxes adjacent to the MEF-2 site, other sites are conserved across species. Although this AP-2 site is not conserved in human, its nt sequences remain quite similar to the consensus binding sites (SSSNKGGG: S, G/C; K, G/T; N, A/C/G/T) for AP-2 factor [33]. Whether this nt discrepancy may contribute to the different developmental expression patterns between the human and the hamster remains to be determined.

We compared the entire nt sequences of the β-2.3 kb site (250 bp) in the human [9,34] and the hamster [18], using the GeneStream align program [35]. We found that the identity between these two species is almost 80% (79.8%; global alignment score, 618). However, the identities of the nt sequences 200 bp upstream and downstream of this site are only 58.8% (score, 157) and 64.4% (score, 233) respectively. The lower score found in the adjacent regions of the β-2.3 kb site is due to multiple gap regions that were created to obtain maximal alignment between the human and hamster nt sequences.

Of particular interest is an 85 bp subregion of this -2.3 kb site, containing a MEF-2 site juxtaposed with an E-box motif and a CT/ACCC box [36]. The proximity of an MEF-2 site and an E-box motif within the enhancers or promoters of numerous muscle-specific genes is well documented [37,38]. In addition, in the enhancers of a few muscle-specific genes, the DNA spacing between the MEF-2 site and CT/ACCC box is a multiple of a DNA helix turn (10.5 bp) [37]. In our case, a 20–22 bp spacing was found between the two core motifs. The enhancer of the mouse MCK gene (-1256 to -1050) [26], containing regulatory elements similar to those identified within the β-2.3 kb site, is shown in Figure 2(b).

We performed gel mobility-shift assays using this 85 bp fragment as a probe to determine whether specific DNA–protein complexes were formed with binding sites within this region. As shown in Figure 3(A), two specific complexes were identified (lane 2). The complexes could be competed away by cold probe (lane 4) but not by a large excess of an unrelated DNA competitor (lanes 5 and 6). Furthermore, when two oligomers (one derived from the CT/ACCC box of the β-2.3 kb site and the other from the MEF-2 binding site of the mouse MCK gene) were added together as a competitor (both at 500 molar excess), these two complexes were completely competed away (Figure 3B, lane 6). However, neither of these two oligomers alone was sufficient to eliminate binding to the 85 bp DNA fragment (Figure 3B, lanes 2–5).

The specific protein–DNA interactions in complexes A and B were demonstrated by methylation interference analysis with the 85 bp fragment. Comparing lane 3 (Figure 3C, complex A) and lane 1 (free DNA) reveals a protected region corresponding to the position of the CT/ACCC box within the 85 bp fragment. The overall lower intensity found in lane 4 (complex B) is because of loading of a lesser amount of DNA. With exposure of the film long enough for the level of intensity in lane 4 to be similar to that of free DNA in lane 1, the protected region found in the complex A was also observed (results not shown). We were unable to demonstrate any footprinting within the MEF-2 binding site due to weaker DMS reaction of 'A' residues within this region [27].

β-2.3 kb site is not a typical enhancer in transient transfection assays

To investigate the function of the β-2.3 kb site, two chimeric CAT constructs linking this DHS (-2477 to -2245) in both orientations to the TK promoter were constructed and then transfected into C2C12 myotubes [39]. We chose to study this cell line for the following reasons. (1) The cardiac β-MyHC gene is expressed when this cell line differentiates into myotubes [10], (2) the C2C12 cell line has been shown to express MyoD and myogenin, which bind to the E-box motif [40], and MyoD and MEF-2 have been shown to be capable of activating gene expression synergically [41], and (3) the β-2.3 kb site can interact with specific C2C12 nuclear proteins in gel mobility-shift assays (results not shown). As shown in Table 1 and Figure 4, when the β-2.3 kb site linked to the TK promoter in a positive orientation, there was a significant increase ($P < 0.01$) in CAT expression within the C2C12 myotubes. However, such a substantial increase in CAT expression was not observed when this site is linked to the TK promoter in a negative orientation (see the Discussion).

Table 1 Relative CAT activity of the β-2.3 kb site in TK promoter construct

Plasmids as indicated were transfected into C2C12 myotubes or cardiofibroblasts (Fib). Relative CAT activities (mean ± S.D.) were calculated relative to the TK CAT construct, and expressed as percentage TK CAT activity. Three independent transfection experiments were performed, and the data were normalized with pRSV-β-gal activity. Student's t test was used to assess differences between transfections of reporter genes into C2C12 cells and cardiofibroblasts. The CAT activity in transfection of E+TK into C2C12 myotubes is significantly higher than the activity in the cardiofibroblasts ($P < 0.01$). E+TK, the insert (β-2.3 kb site) of positive orientation in TK CAT plasmid; E−TK, the insert of negative orientation.

	Relative activity (%)	
CAT constructs	C2C12	Fib
E+TK/TK	312.0 ± 24.5	92.0 ± 20
E−TK/TK	136.7 ± 27.4	112.7 ± 14.8

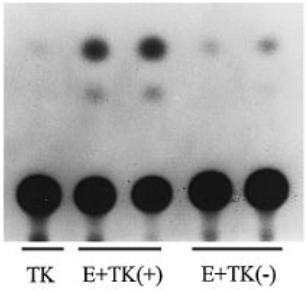

Figure 4 CAT assay in C2C12 cells

A representative CAT assay after transfection of each plasmid, as indicated, into the C2C12 myotubes. The CAT reaction mixture was extracted with ethyl acetate and chromatographed on silica-gel thin layers in 5% (v/v) methanol/95% (v/v) chloroform as described previously [28]. E+TK(+), insert (β-2.3 kb site) of positive orientation; E+TK(−), insert of negative orientation in TK CAT plasmid.

DISCUSSION

In this report we characterize the β-2.3 kb site of the cardiac β-MyHC gene and describe an array of muscle-specific regulatory motifs found to be associated with this site. Using gel mobility-shift assays and footprinting analysis, we further demonstrated the specific DNA-binding activity of this site. When this site was

linked to a TK promoter, the expression of reporter gene increased significantly. These results suggest that the β-2.3 kb site may contain previously undefined *cis*-acting elements of the cardiac β-MyHC gene.

A plausible interpretation of the observation that both the CT/ACCC box factor and MEF-2 are required to completely eliminate the complexes A and B (Figure 3B, lane 6), would be based on the assumption that both factors cannot simultaneously bind to the 85 bp probe (because of the proximity between the CT/ACCC box and MEF-2 binding sites; see Figure 2b). When the CT/ACCC box and MEF-2 binding motif of the β-2.3 kb site was used alone as a probe in gel mobility-shift assays, the intensity of the retarded complexes was similar to that of lane 2 and lane 4 respectively in Figure 3(B) (results not shown), indicating that there is abundant CT/ACCC factor, but not very much MEF-2 present in our cardiac nuclear extracts. Thus abundant CT/ACCC factor could occupy a large amount of the probe, resulting in the complex A and B observed in Figure 3, and little competition by the MEF-2 binding site in lane 5 of Figure 3(B) (however, note that the background in lane 5 is still much lower than that of lane 2). Not until in a large excess of CT/ACCC box binding motif was present as a competitor (Figure 3B, lane 4), was the binding activity of MEF-2 observed (Figure 3B, lane 4).

The *in vivo* function of the β-2.3 kb site has yet to be determined. In transient transfection assays (Table 1 and Figure 4), it appears to lack typical enhancer function, which, by definition, is the ability to cause high level expression of the reporter gene in both orientations. Numerous studies have shown that DHSs are associated with sequences that function in processes such as transcription, replication, recombination or chromosome segregation (for a review see [32]). Additional sites for which no function is readily apparent in transient transfection assays have also been identified, and genetic assessment of such sites often yields interesting results. For example, two recent studies demonstrated that some DHSs within the human CD2 or β-globin gene locus control region have a role in establishing an open chromatin structure, despite the fact that they do not act as typical enhancers [43,44]. Thus, by analogy, one possible role of the β-2.3 kb site is that it functions in the establishment, maintenance, or both, of an open chromatin domain that confers position-independent transgene expression. Such a possibility can be tested in transgenic studies, or by using gene targeting technique to delete this DHS in mouse embryonic stem cells [45].

Significantly, an MEF-2 binding site was identified within the β-2.3 kb site. The MEF proteins, members of the MADS box family, which includes serum response factor (SRF, $p67^{SRF}$), are involved in the regulation of muscle gene expression (for a review see [46]). Loss-of-function mutations in the single *mef*2 gene in *Drosophila* prevent differentiation of somatic, cardiac and visceral muscle cells, although the specification and early development of these tissues appears to be unaffected [47–48]. In addition, the cardiac cell of the mutant embryos do not express MyHC [47], indicating that MEF-2 is required for the expression of the MyHC gene.

Recent studies have demonstrated that there is a close link between the MyoD and MEF-2 families. MEF-2A and MyoD physically interact through the MADS domain of MEF-2A and three amino acids within the basic helix-loop-helix (bHLH) motif of MyoD, and together can activate skeletal-muscle gene expression [38,41]. The proximity of the MEF-2 binding site and an E-box motif (binding site for MyoD family) in the β-2.3 kb site identified in this study provides another example supporting the interplay between the MyoD family and MEF-2 that establishes the myogenic program.

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