A conserved GATA motif in a tissue-specific DNase I hypersensitive site of the cardiac α-myosin heavy chain gene

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Transgenic analysis has indicated that far upstream regulatory elements of the cardiac α-myosin heavy chain (MyHC) gene are required for appropriate transgene expression [Subramaniam, Gulick, Neumann, Knotts and Robbins (1993) J. Biol. Chem. **268**, 4331–4336]. In an attempt to identify these as-yet-undefined regulatory elements, we mapped the DNase I hypersensitive sites (DHSs) in the 4 kb upstream region of the hamster cardiac α-MyHC gene. When using nuclei isolated from late-gestational and adult heart ventricles, a strong DHS was identified in the −1.9 kb region (α−1.9 kb site). It cannot be detected in kidney, liver or cardiofibroblast nuclei. Within this site, we found a conserved GATA-motif that interacts specifically with GATA-binding factors in nuclear extracts of cardiomyocytes at various developmental stages. These data provide further evidence to support the role of GATA factors in the regulation of cardiac α-MyHC gene expression.

INTRODUCTION

The α-myosin heavy chain (MyHC) gene is expressed in the cardiac tube of the developing fetal rodent heart at between 7.5 and 8 days *post coitum*. Its expression diminishes as the ventricular chambers form and remains at low levels until just after birth [1,2]. The α-MyHC gene is developmentally and hormonally regulated at the transcriptional level by a complex interplay of multiple regulatory elements. In addition to the well-established thyroid hormone (T_3) response elements [3,4], DNA-binding sites (located within the proximal promoter) for myocyte-specific enhancer factor 2 (MEF-2) [5], myogenin factors [6], muscle-specific 5′-CATTCCT-3′-motif (M-CAT) binding factor (TEF-1) [7], and GATA-4 [8] can all influence the expression of the α-MyHC gene. Transgenic mice with mutations in several DNA-binding motifs within the α-MyHC gene proximal promoter region have recently been generated. A murine α-MyHC/CAT gene construct containing 4 kb of the α-MyHC upstream region is sufficient to drive the developmental- and tissue-specific expression of the reporter gene [9]. While mutations in T_3 response elements reduce the expression of the α-MyHC/CAT constructs significantly, the mutation in the MEF-2 DNA-binding site does not affect the transgene's expression [10,11].

Regulation of the α-MyHC gene by GATA-4 was demonstrated by directly injecting α-MyHC/CAT constructs containing mutations in GATA sites into adult rat myocardium, resulting in an 88% decrease in CAT activity [8]. GATA-4 is a cell-specific transcription factor belonging to a group of related zinc-finger proteins that recognize the consensus GATA motif [12]. Its mRNA is abundantly expressed in adult heart, and some expression is also found in gonads, gut epithelium and yolk sac endoderm [12,13]. Using hybridization *in situ* and immunohistochemistry, Heikinheimo et al. [14] found that GATA-4 mRNA and protein are expressed in progenitor cells associated with the cardiac development of the mouse embryo. In addition to regulating the α-MyHC gene, GATA-4 also regulates the B-type natriuretic peptide [15] and cardiac troponin C genes [16].

It has been suggested that upstream elements outside the proximal promoter region of the α-MyHC gene also play a role in its expression. In one transgenic study, an α-MyHC/CAT construct containing the 4 kb upstream region was shown to respond both to hyperthyroid and to hypothyroid stimulation. However, when its distal 2.8 kb region was deleted, the construct could only partially be suppressed in response to hypothyroid stimulation [10]. The GArC (G, A-rich, C) motif located between nt −908 and −869 of the human α-MyHC gene was initially characterized by Mably et al. [17] using DNase I footprinting analysis. However, the significance *in vivo* of the GArC motif remains to be determined.

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 4 kb of upstream sequence from the transcriptional start site of the α-MyHC gene. Regions of chromatin that are hypersensitive to nuclease digestion are often believed to be free of nucleosomes. This allows access of crucial resident *cis*-acting sequences to sequence-specific *trans*-acting factors [20]. We therefore endeavoured to map the DNase I hypersensitive sites (DHSs) within the 5′ flanking region of the cardiac α-MyHC gene.

MATERIALS AND METHODS

Isolation of nuclei and treatment with DNase I

Nuclei were isolated from freshly excised kidney, liver and heart ventricle of adult (3-month-old), neonatal (1-day-old) and fetal (late gestation) Syrian hamsters as previously described [21]. The nuclei were suspended in a buffer containing 15 mM Tris (pH 7.5), 60 mM NaCl, 0.5 mM spermidine and 0.15 mM spermine, and treated with DNase I (Pharmacia) at concentrations ranging from 1 μg 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

Abbreviations used: CAT, chloramphenicol acetyltransferase; DHS, DNase I hypersensitive site; MEF-2, myocyte-specific enhancer factor 2; MyHC, myosin heavy chain.

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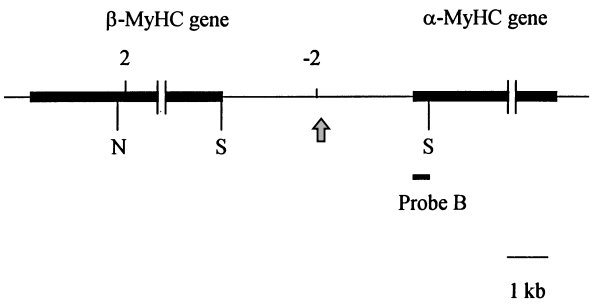


	α-1.9 kb Site	α–MyHC Promoter
Fetal	+	---
Adult	+	+ / ---

Figure 1 Structure of the hamster cardiac α- and β-MyHC genes

The position of probe B is shown as indicated. The DHS is indicated by an arrow. The two *Sac*I (S) sites in the cardiac α-MyHC gene are located at −3915 and +415 respectively. The inset Table summarizes the appearance of the DHSs identified in this study at various developmental stages of the α-MyHC gene. 2, 2 kb downstream of transcription initiation site; −2, 2 kb upstream of transcription initiation site.

0.25% (v/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.

Preparation of hamster cardiomyocytes and fibroblasts

Neonatal hamster cardiomyocytes were prepared, with minor modifications, as previously described [22]. Heart ventricles from 1-day-old hamsters were excised and minced in PBS, and the cells were dispersed by successive additions of 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 fibroblasts. The enriched cardiomyocytes were removed and replated at a density of 1×10^7/100-mm dish with 0.1 mM bromodeoxyuridine to prevent proliferation of non-myocytes. Later (36 h), the cells were harvested by treatment with 0.05% trypsin/EDTA, washed three times with 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 to disrupt cells, nuclei were collected by centrifugation at 1000 ***g*** for 10 min at 4 °C, washed three times with nuclear buffer (without Nonidet P40) and resuspended at a concentration of 1×10^7/ml. The isolation of nuclei from cardiac fibroblasts (harvested from the preplating plates) was carried out as described for the cardiomyocytes. The digestion of nuclei with DNase I (from 1 μg/ml to 10 μg/ml) and purification of genomic DNA were performed as described under 'Isolation of nuclei and treatment of DNase I'.

Indirect end-labelling method [23]

The position of probe B adjacent to the *Sac*I restriction site is indicated in Figure 1. This probe was amplified by PCR, using the cosmid DNA containing the 5′-upstream region of the hamster α-MyHC gene as template. The subcloning of the amplified PCR fragment and subsequent verification of DNA sequencing were performed as previously described [24]. The genomic DNA isolated as described under 'Isolation of nuclei and treatment of DNase I' was then digested by *Sac*I and fractionated on 1.3% (w/v) agarose gel. The gel was then transferred to nitrocellulose and hybridized with the probe, which was prepared by random primer labelling. The nitrocellulose was then washed in 0.1 × SSC and 0.1% SDS at 65 °C for 20–45 min and exposed to film with an intensifying screen at −80 °C for 2–7 days.

Gel mobility-shift assays

The assay was carried out as described previously [17]. Nuclear extracts of hamster cardiomyocytes, cardiofibroblasts and C2C12 myotubes were prepared as described by Andrews and Faller [25]. The preparation of ventricular nuclear extracts from late-gestation fetal and adult hamsters was performed as described previously [17]. DNA–protein binding reactions were carried out for 15 min at room temperature, in the presence of 10000 c.p.m. of the ^{32}P-labelled probe, 10 μg of nuclear extract, 2 μg of poly(dI-dC) and various unlabelled competitors (as described in Figure 4). The reaction mixtures were subjected to electrophoresis on 4% (w/v) polyacrylamide gels with recirculation of the buffer between chambers, and the apparatus was cooled by water. The nucleotide sequence of the probe and the various competitors used are listed below for clarification: (1) probe (GATA motif in the α − 1.9 kb site), 5′-GTCCTGAAGGACATGATAAGGGACTGAGAACAT-3′; (2) GATA-2 binding site [26], 5′-CAACGTGCAGCCGGAGATAAGGCCAGGCCCGAA-3′; (3) MEF-2 binding site [27], 5′-TTCAGTCCCTGTTTATTTATAGCACTTGGTC-3′; (4) CT/ACCC box [28], 5′-GAAGCGGCCCTCCCTCCAGTCCCTGT-3′.

RESULTS

Tissue- and developmentally-specific DHSs of the cardiac α-MyHC gene

The structure of the tandemly linked β- and α-MyHC genes with the position probe B is shown in Figure 1. Using probe B, and hybridizing with genomic DNA from adult and neonatal heart ventricles, a strong cardiac-specific DHS was detected within the 4 kb upstream regulatory region of the α-MyHC gene (Figure 2A, lanes 5–10). This site was not found in the genomic DNA extracted from kidney nuclei (Figure 2A, lanes 1–3) or liver nuclei (Figure 2A, lanes 11–13). Based on the size of the DNA fragments detected in the gel, and on the position of probe B, as shown in Figure 1, this DHS was mapped to −1.9 kb (α − 1.9 kb site). Since the cardiac α-MyHC gene is not expressed in ventricles of the late-gestational fetus [1], we compared the DHS pattern of the cardiac α-MyHC gene at the adult and late-gestational fetal stages. As shown in Figure 2(B), the α − 1.9 kb site was also detected in the late-gestation fetal ventricle (Figure 2B, lanes 1–3). A very weak DHS mapped to the proximal promoter region was barely detected in adult heart ventricles (Figure 2A, lanes 5–7; Figure 2B, lanes 6 and 7). The diffuse band detected around the promoter region is probably due to the relatively low concentration of agarose (1.3%) used in this study.

Defining the region of the α − 1.9 kb site

Since the heart is constituted by a heterogeneous population of cells, we examined whether the α − 1.9 kb site is specific to cardiomyocytes. As shown in Figure 3(A), the α − 1.9 kb site is positive only in the cardiomyocytes (lanes 3 and 4), and could not be

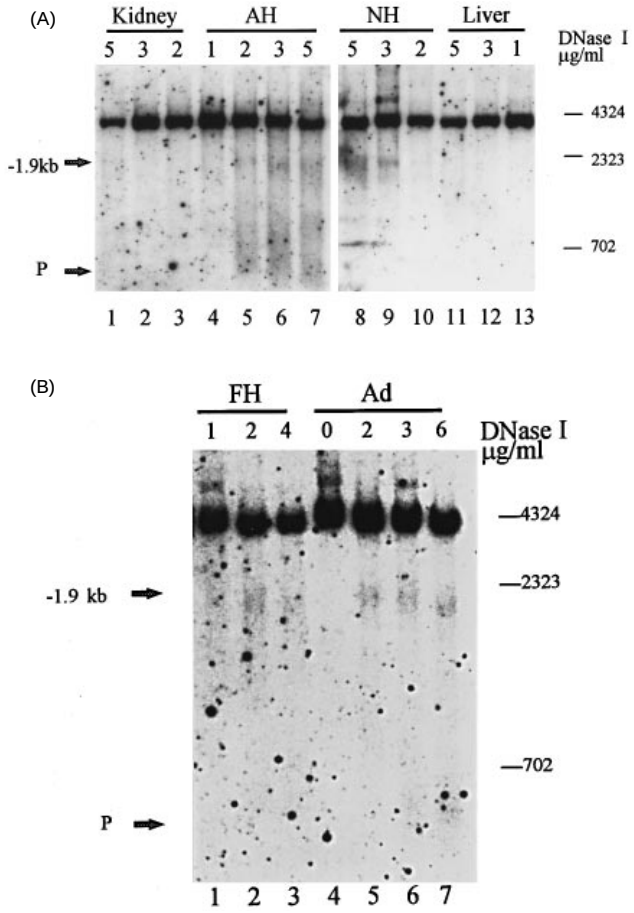
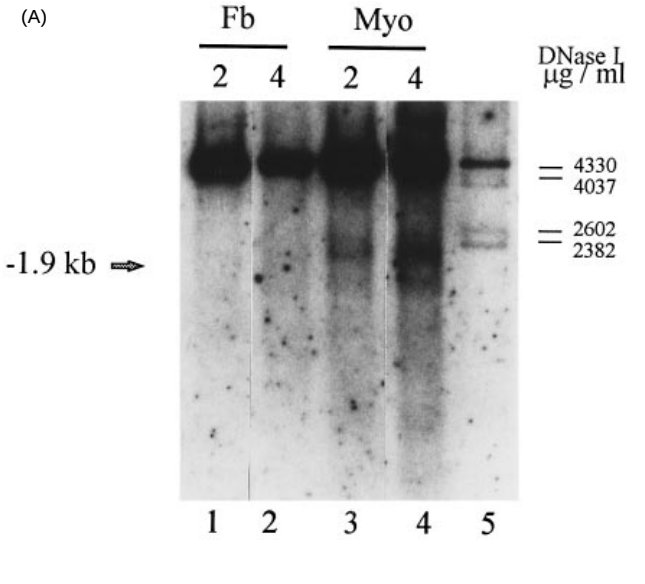


Figure 2 DHSs of the cardiac α-MyHC gene

Genomic DNA (25 μg in each lane) purified from nuclei of various tissues treated with increasing concentrations of DNase I, as indicated, were digested with *Sac*I, fractionated on 1.3% agarose gels and subjected to Southern blot analysis with probe B. (**A**) AH, adult heart ventricle; NH, neonatal heart ventricle. (**B**) FH, fetal heart ventricle; Ad, adult heart ventricle. Lambda DNA digested by *Bst*EII was used as a DNA marker (indicated on the right); P, promoter.



(B)

(A) Hamster cardiac α-MyHC gene (α-1.9 kb site)

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-1972
acagagtatggtctttgtcacttggacttgacccaggctgacccaa

tgttctcagTCCCTTATCATGTCCTtcaggaccttgaaaccaggca
          GATA motif
gtgacatattaggccacaggctaaccctgtgacttgcacaaggtga

ccttccagggacctagctgcagacaggtggcttgcatcctctgaga

acaatcatttggcatagtcacctgcagatgggaatacaaggttggc

tcaggtcccttcaagagaact
                -1722
```

(A) 5'-ggtcctga**AGGACATGATAAGGGA**ctgagaac-3'
(B) 5'-agctcctg**GGGACATGATAAGGAG**ctgagaac-3'
(C) 5'-caccataa**GGGACATGATAAGGGA**gccagca-3'

(A) Hamster GATA motif; (B) Human GATA motif

(C) GATA-1 binding site in the Human ß-globin gene 3' enhancer

Figure 3 Defining the region of the α−1.9 kb site (A), and listing the nucleotide sequence of this site (B)

(**A**) Genomic DNA (25 μg) from cardiofibroblast (Fb, lanes 1 and 2), and neonatal cardiomyocyte (Myo, lanes 3 and 4) nuclei, treated with DNase I as indicated, were digested by *Sac*I and run in parallel with DNA markers (lane 5). These markers were generated by digested genomic DNA with: lane 1, *Sac*I (4330 bp); lane 2, *Apa*I (4037 bp); lane 3, *Sma*I (2602 bp); lane 4, *Apa*I + *Sma*I (2382 bp). (**B**) For comparison, the nucleotide sequences of the human GATA-binding site within the corresponding region of the hamster α−1.9 kb site, and the GATA-1-binding site in the human β-globin gene 3′-enhancer are also provided.

observed in the nuclei from cardiofibroblasts, indicating that the α−1.9 kb site is myocyte-specific.

To further localize the α−1.9 kb site, several hamster genomic DNA markers were run in parallel with this DHS. According to the DNA markers (Figure 3A, lane 5), the upper limit of the α−1.9 kb site was estimated to be approx. 2382 bp. Since the size range of most DHSs is approx. 200 bp [20], we defined the α−1.9 kb site to a range of 250 bp (−1972 bp to −1722 bp) to include all of the potential regulatory elements residing within this region.

A conserved GATA motif residing within the α−1.9 kb site

As shown in Figure 3(B), an evolutionarily conserved GATA motif residing on the antiparallel strand was identified within the α−1.9 kb site. The core motif and flanking sequences of this GATA-binding site are almost identical with those of a well-established GATA-binding site located within the human β-globin gene 3′-enhancer [29] (Figure 3B). The specific GATA-binding activity in the neonatal (1-day-old) hamster cardiomyocyte nuclei extracts was revealed by gel mobility-shift assay, as shown in Figure 4(A). This DNA-binding activity can be competed away by the unlabelled probe (lane 3) and the GATA-2 binding site (lane 4) derived from preproendothelin-1 gene [26], but it cannot be competed away by the CT/ACCC box [28] (lane 5, C/G rich). When the MEF-2 oligomer was added in large molar ratios, a slight decrease in GATA-binding activity was observed (lane 6). This decrease was most probably due to the MEF-2 oligomer used in this study containing a short stretch of DNA sequence (ttatag) similar to the consensus GATA-binding site (A/TGATAA/G). A band-shift (lane 9) was also observed in the nuclear extracts of the C2C12 myotubes. Since the band-shift could easily be competed away by non-specific competitors

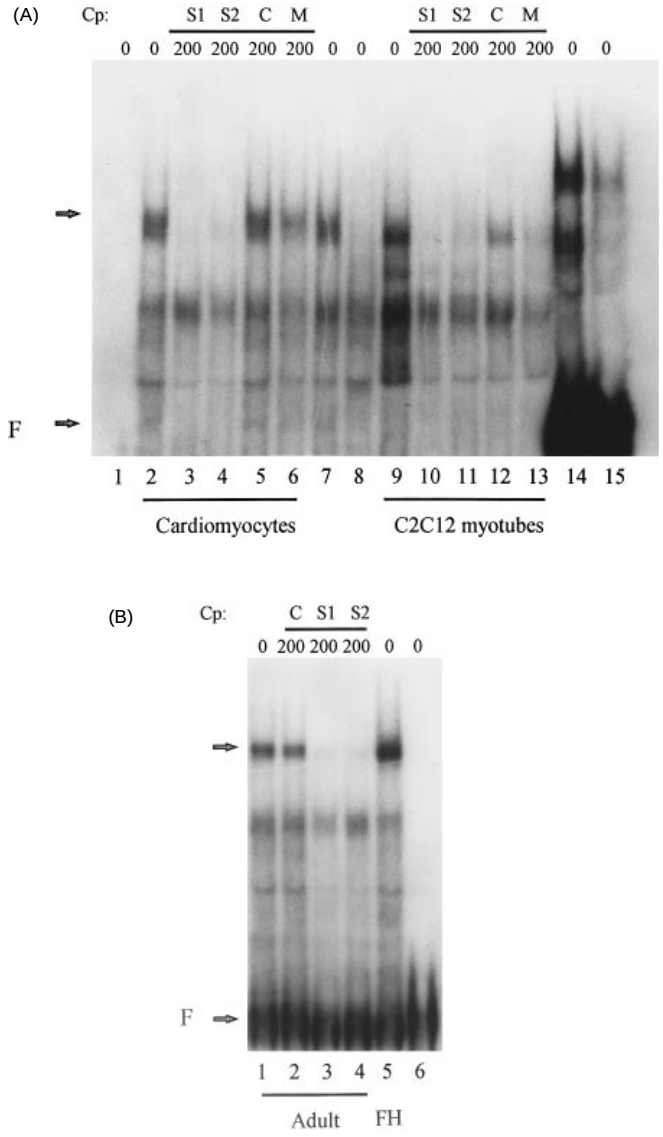

Figure 4 A conserved GATA motif residing within the $\alpha-1.9$ kb site

(**A**) Nuclear extracts from neonatal cardiomyocytes (lanes 2–7), cardiofibroblasts (lane 8) or C2C12 myotubes (lanes 9–13) were incubated with an end-labelled GATA-binding site (for the nucleotide sequence see the Materials and methods section) in the presence of various competitors (Cp), as indicated: S1, cold probe; S2, GATA-2; C, CT/ACCC box; M, MEF-2. Lane 1, free probe. The retard migration complexes detected in cardiomyocytes (lane 2) and C2C12 myotubes (lane 9) are indicated by an arrow. In order to show the free probe (F), the DNA-binding reaction carried out in lane 2 (cardiomyocytes) and lane 9 (C2C12 myotubes) were loaded into lanes 14 and 15 respectively 30 min later than the other lanes. (**B**) Nuclear extracts from adult heart ventricles (lanes 1–4) or from late-gestational fetal ventricles (FH, lane 5) were incubated with the above-described GATA probe and various competitors as indicated: S1, unlabelled probe; S2, GATA-2; C, CT/ACCC box. Lane 6, free probe.

(lanes 12 and 13), the DNA-binding activity observed might be due to non-specific protein–DNA interaction or to a related GATA factor binding to this site. Finally, we further examined the interaction of this GATA motif with ventricular nuclear extracts isolated from cardiac tissues at various developmental stages. As shown in Figure 4(B), the GATA DNA-binding activity could be observed from the late-gestation fetal stage (Figure 4B, lane 5) to the adult heart stage (Figure 4B, lanes 1 and 2). This result is consistent with the expression pattern of GATA factors in Northern blot analysis [13].

DISCUSSION

In this study, we have identified a strong tissue-specific DHS ($\alpha-1.9$ kb site) in the cardiac α-MyHC gene. Within this site a conserved GATA motif was identified, the nucleotide sequence of which is almost identical with the GATA-1 binding site in the human β-globin gene 3′-enhancer (Figure 3B). Since the DHS is often considered to be an 'open' chromatin structure that allows transcription factors to bind to their DNA-binding site, we determined the specific GATA DNA-binding activity within this site using a gel mobility-shift assay. The GATA DNA-binding activity can be detected only in the nuclear extracts from cardiomyocytes; it cannot be detected in C2C12 myotubes, nor in cardiofibroblasts (Figure 4A).

The role of the $\alpha-1.9$ kb site *in vivo* remains to be determined. Transgenic analysis [10] has indicated that an undefined regulatory element is located in the distal 2.8 kb segment of the 4 kb upstream region of the cardiac α-MyHC gene (i.e. the intergenic region between the β- and α-MyHC genes; see Figure 1). Since the $\alpha-1.9$ kb site is located in this distal 2.8 kb segment, this DHS might be associated with the candidate regulatory element. However, so far no upstream regulatory elements have been identified in transient transfection assays; therefore, a more detailed genetic assessment of this site in the context of chromatin structure is required.

The biological significance of the $\alpha-1.9$ kb site is demonstrated by its association with a conserved GATA motif and provides further evidence that GATA-4 plays a role in the regulation of cardiac α-MyHC gene expression [8,13]. Recently, GATA-5 and -6 were isolated in a chicken embryo cDNA library [30]. The temporal and spatial patterns of GATA-4, -5 and -6 expression support a role for these factors in the regulation of cardiac differentiation, analogous to the established role of transcription factor GATA-1 in the regulation of haematopoiesis [31,32]. However, it should be noted that the $\alpha-1.9$ kb site is also located at the 3′-end of the cardiac β-MyHC gene. Whether the conserved GATA motif within the $\alpha-1.9$ kb site also plays a role in the regulation of the β-MyHC gene remains to be tested.

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