A novel E box/AT-rich element is required for muscle-specific expression of the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2) gene

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ABSTRACT

The cardiac/slow twitch sarcoplasmic reticulum (SR) Ca²⁺-ATPase gene (SERCA2) encodes a calcium transport pump whose expression is regulated in a tissue- and development-specific manner. Previously we have identified two distinct positive regulatory regions (bp –284 to –72 and –1815 to –1105) as important for SERCA2 promoter activity. Here we demonstrate that the SERCA2 distal promoter region functions like an enhancer by activating a heterologous promoter (TK) in a muscle cell-specific manner. Through deletion analysis a core enhancer region was delimited to the –1467 to –1105 bp fragment. We identified the E box/AT-rich element located at –1115 bp as critical for maximal enhancer activity. Gel mobility shift studies revealed that this E box/AT-rich element specifically binds a protein which is induced during Sol8 myogenesis. This region includes two other cis-acting elements, CArG and MCAT, which also bind specific nuclear protein complexes from Sol8 myotubes. Mutagenesis of each of these sites resulted in decreased SERCA/TK–CAT promoter activity. Based on these data, we propose that the E box/AT-rich element may contribute along with CArG and MCAT elements to the overall activation and regulation of the SERCA2 gene promoter.

INTRODUCTION

The rabbit cardiac/slow twitch muscle sarcoplasmic reticulum (SR) Ca²⁺-ATPase gene (SERCA2) encodes a calcium transport pump, the function of which is to transport calcium from the cytoplasm into the SR lumen (reviewed in 1). The calcium ATPase protein plays a critical role in regulation of the contraction/relaxation cycle of cardiac, skeletal and smooth muscles. The SR Ca²⁺-ATPase is a transmembrane protein of 997 amino acids (M, 109 763) which is a member of a highly conserved family of isoenzymes (reviewed in 2). Three principle isoenzymes of this family (SERCA 1, 2 and 3) have been cloned and their alternative splice variants have been identified (3–9).

The SERCA family of genes is expressed in many diverse tissues and respond to complex developmental, tissue-specific and pathophysiological conditions (5,9–13). The SERCA1 gene encodes two alternatively spliced isoforms, SERCA1a (adult) and SERCA1b (fetal), which are expressed exclusively in fast twitch skeletal muscle (3–5). The SERCA2 gene also encodes two alternatively spliced isoforms, SERCA2a and SERCA2b, which diverge in their C-termini (SERCA2a, 4 amino acids; SERCA2b, 49 amino acids) (6–8). SERCA2a is the predominant isoform expressed in the heart and slow twitch skeletal muscle (9). The SERCA2a isoform is also transiently expressed in fetal fast twitch skeletal muscle, but is replaced by the SERCA1a isoform in adult muscle. The SERCA2b isoform is expressed in multiple tissues, including smooth muscle and most non-muscle tissues (6–8). The SERCA3 gene also encodes two isoforms that are found primarily in endothelial and epithelial cells (10,11).

The SERCA2a isoform is abundantly expressed in cardiac muscle, both in the atrium and the ventricle (9). The earliest expression of SERCA2a mRNA can be traced to the heart tube of the 10 day post-coitum rat embryo (11). During heart development the SERCA2 level increases gradually from fetal to adult stages (9). SERCA2a mRNA expression can be altered by a variety of neurohormonal and mechanical stimuli that alter muscle function (13–17). Previous work from our laboratory and others has demonstrated that expression of SERCA2a mRNA levels are altered during cardiac hypertrophy. SERCA2a mRNA levels were shown to be increased in response to thyroid hormone (T3)-induced cardiac hypertrophy (13–15). In contrast, decreased SERCA2 expression and protein levels were observed in hypothyroidic hearts (13–15) and in heart muscle subjected to pressure overload following constriction of the pulmonary artery (13,16,17). Recent studies have shown that the SERCA2 promoter contains three conserved thyroid response elements which were shown to be essential for up-regulation of the SERCA2 promoter by T3 (18).

The goal of our research has been to perform a detailed analysis of the SERCA2 promoter and identify cis-regulatory elements important for SERCA2 promoter regulation in muscle cells. We have previously shown that the rabbit SERCA2 gene promoter
extending between bp –1815 and +350 is highly active in Sol8 muscle cells but not in fibroblasts (19). Using deletion mapping analyses we have identified two distinct positive regulatory regions: a proximal region located between bp –284 and –72 (20) and a distal region at bp –1815 to –1105 (19). We demonstrated that the SERCA2 proximal region extending from –284 to –72 was highly active in skeletal muscle cell lines (20) and functions like an enhancer element when linked to a heterologous promoter–reporter system (TK–CAT) (19). This region was shown to be regulated by seven Sp1 elements that work synergistically to promote SERCA2 expression (19).

The goal of the present study was to define the important regulatory elements within the distal positive regulatory region extending from bp –1815 to –1105. In this study we demonstrate that the 710 bp region functions like an enhancer and activates the heterologous (TK) promoter in a muscle-specific manner. This region includes several known cis-acting elements, including CarG box, MCAT, A/T-rich (Mef-2-like) and E box sequences. We demonstrate in this study that a novel E box/AT-rich element is critical for muscle-specific enhancer function in addition to the known cis-acting sequences (CarG and MCAT elements). Furthermore, we show that the E box/AT-rich element binds a unique protein complex which is induced during Sol8 myogenesis.

MATERIALS AND METHODS

Cell culture

Sol8, a mouse soleus Muscle cell line (21) (ATCC no. CRL2174), and NIH 3T3 fibroblasts (ATCC no. CRL1658) were maintained in Dulbecco’s Modified Eagles medium (DMEM) with 10% fetal bovine serum and 1% antibiotic/antimycotic supplement (Life Technologies). Sol8 myoblasts were induced to differentiate by switching to a medium supplemented with 5% horse serum. Sol8 myoblasts are induced to differentiate by bovine serum and 1% antibiotic/antimycotic supplement (Life Technologies). Sol8 myoblasts are induced to differentiate by switching to a medium supplemented with 5% horse serum. Myoblasts were incubated with DNA for 5 h, followed by a glycerol shock step and replacement of 5% horse serum medium. Cells were harvested 48–72 h after transfection, washed twice with phosphate-buffered saline, resuspended in 100 µl 250 mM Tris, pH 7.5, and lysed through three freeze–thaw cycles.

Plasmid construct

The –1815 to –1105 SERCA1/TK–CAT construct was produced through ligation of a 710 bp fragment (Sol8–EcoRI) of the SERCA2 promoter region to the heterologous promoter (TK) linked to the CAT reporter gene in pBLCAT5 (22; Fig. 1). Serial deletions of this fragment were performed using appropriate restriction enzymes and ligation back into the pBLCAT5 vector (Fig. 2). Constructs pBLCAT5, pBLCAT6 and pSV2CAT were used as positive and negative controls in transfection analysis (22,23). pMSVbgal was used to standardize for transfection efficiency. Mutations to the CarG, MCAT and E box consensus elements were generated through the Altered Site mutagenesis kit (Promega) or through PCR-generated mutations. Mutagenic oligonucleotides were as follows: CarG-mut, GGCTCATAGATAAGTACCCCTAAC-\textless b a s e s \textgreater; MCAT-mut, CCAAT-TTGCTCAACAGTTTGGCCATTCATCATGCTGCTC-\textless b a s e s \textgreater; E box-mut, GAAATTCTTGTTTCCGAGGAAATAG); TEF1a and TEF1b expression vectors (24) were provided by Dr C.P. Ordahl.

DNA transfections and CAT assays

SERCA2 promoter construct (20 µg) containing the CAT reporter gene was co-transfected with pMSVbgal (5 µg) into Sol8 and NIH 3T3 cells by the calcium phosphate co-precipitation method (25). For DNA transfections Sol8 cells were seeded at 5 × 10^5 cells/10 cm dish and calcium phosphate–DNA precipitates were added 24 h later. Myoblasts were incubated with DNA for 5 h, followed by a glycerol shock step and replacement of 5% horse serum medium. Cells were harvested 48–72 h after transfection, washed twice with phosphate-buffered saline, resuspended in 100 µl 250 mM Tris, pH 7.5, and lysed through three freeze–thaw cycles. β-Galactosidase activity was assayed according to established procedures and normalized for transfection efficiency as determined by β-galactosidase expression. Data represent the average of three or more independent transfection experiments run in duplicate. CAT activity was assayed according to established procedures and normalized for transfection efficiency as determined by β-galactosidase expression. Data represent the average of three or more independent transfection experiments run in duplicate. CAT activity is represented as the relative CAT activity as compared with the promoterless control pBLCAT6 or the enhancerless TK–CAT vector, pBLCAT5, as appropriate (22).

Preparation of nuclear extracts

Briefly, monolayer cells were washed with PBS, scraped into 50 ml conical tubes and centrifuged at 1800 r.p.m. for 10 min. Cells were resuspended first in 5 packed cell vol. hypotonic buffer (10 mM HEPES, pH 7.9, 15 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 1 mM DTT) and incubated for 15 min at 4°C. When cells had swollen 2-fold they were transferred to a type B homogenizer and given 10 strokes to disrupt the cell

Figure 1. Sequence analysis of the distal enhancer region (bp –1467 to –1105) in the SERCA2 promoter. The consensus sequences for the GATA, CarG, MCAT, CRE and E box/AT-rich elements are boxed.
Figure 2. The SERCA2 distal promoter region (bp –1815 to –1105) activates the TK promoter in Sol8 muscle cells. A representative CAT assay is shown in the upper right panel. The 710 bp fragment was linked both 5′ (sense and antisense) and 3′ (antisense) to the TK–CAT reporter. The CAT values represent the averages of at least three separate transient transfection experiments in Sol8 myotubes. CAT data are represented as the fold activity over the pBLCAT5 construct.

RESULTS

The SERCA2 distal promoter region (bp –1815 to –1105) functions like an enhancer and activates the TK promoter in a muscle-specific manner

Our recent work identified that the SERCA2 promoter contains two important regulatory regions, a proximal promoter region from bp –284 to –72 and a distal region between bp –1815 and –1105 (19, 20). The distal promoter region contains the consensus sequences for several known muscle-specific regulatory elements, including CArG, MCA T, E box and A/T-rich regions (Fig. 1). These elements are clustered within the region from bp –1467 to –1105.

To determine if the upstream regulatory region acts as a muscle-specific enhancer we cloned the 710 bp distal promoter fragment from bp –1815 to –1105 in the sense and antisense orientations upstream of the TK promoter or 3′ of the CAT gene in the pBLCAT5 reporter vector. The TK/SERCA promoter constructs and control vectors (pBLCAT5 and pSV2CAT) were transiently transfected into Sol8 and NIH 3T3 cell lines (Fig. 2). CAT activity was determined 72 h following transfection. The CAT activity represents the fold activity of the TK/SERCA

[α-32P]dATP (3000 Ci/mmol) and Klenow enzyme. The DNase I mapping assay contained 5 fmol (~10 000 c.p.m.) end-labeled fragment in 50 µl 2× binding buffer. Sol8 nuclear extracts were added in increasing concentrations of 100 and 200 µg and the binding reaction was carried out for 10 min at room temperature and for an additional 10 min on ice. An aliquot of 50 µl DNase I (Worthington) at a concentration of 5 µg/ml in 10 mM Tris–HCl, pH 8.0, 10 mM MgCl2 and 1 mM CaCl2 was added to the binding mix and incubated for 30 s at room temperature. The reaction was terminated by addition of 100 µl stop solution (200 mM NaCl, 30 mM EDTA and 1% SDS). Samples were phenol extracted and ethanol precipitated. The samples were then heat denatured and loaded onto a 6% sequencing gel. The A+G ladders were generated by the Maxam–Gilbert chemical sequencing method.

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| Gel mobility shift assays |

RNA extracts from the Sol8 myoblasts and myotubes, NIH 3T3 fibroblasts and HeLa cells were prepared as described above. Gel mobility shift assays were performed by incubating 3′-end-labeled SERCA2 fragment E122 (bp –1815 to –1105) or double-stranded oligonucleotides (5′-TGTGAACACAAA TGA-3′) with nuclear extracts (4 µg) for 30 min at room temperature in 30 µl binding buffer [20 mM HEPES–KOH, pH 7.9, 0.2 mM EDTA, 0.2 mM PMSF, 100 mM KCl] and 2 µg poly(dI)·poly(dC)]. Competitions were performed using the MCK E box sequence (27). The binding reactions were immediately loaded onto a 6% native polyacrylamide gel containing 0.5× TBE. Electrophoresis was carried out at 4°C for 3–6 h at 100 V. The gel was subsequently dried and autoradiographed.

DNAse I footprinting

DNase I footprinting was performed on the distal enhancer region (bp –1467 to –1105) using Sol8 nuclear extracts as previously described (19). The DNA fragments from bp –1467 to –1226 (Xbal–Stu restriction fragment) and bp –1227 to –1105 (Xbal–BamHI restriction fragment) (Fig. 1) were 3′-end-labeled using [α-32P]dATP (3000 Ci/mmol) and Klenow enzyme. The DNase I mapping assay contained 5 fmol (~10 000 c.p.m.) end-labeled fragment in 50 µl 2× binding buffer. Sol8 nuclear extracts were added in increasing concentrations of 100 and 200 µg and the binding reaction was carried out for 10 min at room temperature and for an additional 10 min on ice. An aliquot of 50 µl DNase I (Worthington) at a concentration of 5 µg/ml in 10 mM Tris–HCl, pH 8.0, 10 mM MgCl2 and 1 mM CaCl2 was added to the binding mix and incubated for 30 s at room temperature. The reaction was terminated by addition of 100 µl stop solution (200 mM NaCl, 30 mM EDTA and 1% SDS). Samples were phenol extracted and ethanol precipitated. The samples were then heat denatured and loaded onto a 6% sequencing gel. The A+G ladders were generated by the Maxam–Gilbert chemical sequencing method.

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construct divided by the activity of the basal (TK) promoter obtained from the pBLCAT5 control vector (Fig. 2). Inclusion of the 710 bp fragment in the TK–CAT test vector (in sense and antisense orientations 3′ of the TK promoter) increased CAT activity significantly (4- to 6-fold) in Sol8 cells. However, the same fragment did not increase TK promoter activity in NIH 3T3 fibroblast cells (data not shown), suggesting that the distal regulatory elements function in a muscle-specific manner. Interestingly, when the same promoter fragment was placed 3′ of the CAT reporter gene there was no further increase in TK promoter activity, indicating that the position of these elements with respect to the core promoter is important.

**DNase I footprinting analyses reveal nuclear protein binding to CArG, MCAT and E box/A/T-rich sequences in the distal enhancer region**

The 710 bp enhancer region contains several known consensus sequences for cis elements, including CArG (28), MCAT (29,30), GATA (31), CRE (32), AT-rich/Mef-2 (33) and E box (27), which have been shown to be important transcriptional regulators for other muscle-specific genes. To identify which of these sequences interact with muscle nuclear proteins, DNase I footprinting was performed using two separate labeled DNA fragments from the 710 bp region (bp –1467 to –1226 and –1227 to –1105) incubated with nuclear extract from Sol8 myotubes (Fig. 3A and B). DNase I footprinting of the bp –1467 to –1226 region revealed strong protection of a region containing overlapping MCAT/CRE elements at position –1400 (Fig. 3A). In addition, a CArG box element located at position –1431 within this fragment was completely protected. However, we did not see footprinting of the consensus sequences for GATA, at positions –1464, –1458 and –1257.

DNase I footprinting of a second fragment extending from bp –1227 to –1105 demonstrated that a single A/T-rich sequence was protected. This A/T-rich region overlaps with an E box consensus sequence (CAAA TGAA TT) located between bp –1115 and –1105 (Fig. 3B). Protection of this region introduced a hypersensitive site at the cytosine residue (position –1115).

**Mutations to the CArG, MCAT and E box elements decrease promoter activity in muscle cells**

To determine the functional relevance of the CArG, MCAT and E box/AT-rich elements, site-directed mutagenesis was used to introduce mutations to the core binding sites of these sequences within the 710 bp TK–CAT construct (described in Fig. 2). Mutations to the CArG box at position –1331 (CCAAA TTTGG to GGA TCCTTGG) in the context of the 710 bp TK–CAT vector produced a 40% loss of promoter activity (Fig. 4 A). Similarly, site-directed mutagenesis of the MCAT element (CA TTCTT to CA TGGTT) in the 710 bp TK–CAT vector produced a 60% loss of CAT activity (Fig. 4A). In addition, the E box consensus sequence CAAA TG located at position –1115 was mutated to GGAA TG in both the TK–CAT construct resulting in a 40% loss of promoter activity (Fig. 4A).

**Deletion mapping of the 710 bp element shows that the region from bp –1467 to –1105 containing the E box/AT-rich element is essential for muscle-specific enhancer activity**

To precisely map the region responsible for activation of the TK promoter in muscle cells, deletions of the 710 bp upstream region were made and the fragments cloned in front of the TK–CAT sequence. SERCA2 deletion constructs were transiently transfected and assayed for CAT activity. Deletion mapping of the 710 bp element shows that the region from bp –1467 to –1105 containing the E box/AT-rich element is essential for muscle-specific enhancer activity.
Figure 4. Mutagenesis of CArG, MCAT and E box consensus sites in the distal enhancer. (A) (Left panel) Diagram showing mutations to individual consensus sites CArG, MCAT and E box in the 710 bp TK–CAT vector. (Right panel) Promoter activity of mutant and wild-type constructs in Sol8 myotubes. CAT activity is represented as the fold SERCA2–CAT activity over pBLCAT5 relative to the wild-type 710 bp TK–CAT construct. (B) Mutations to the E box consensus in the context of the SERCA2–CAT (~1815 to +350) construct.

Figure 5. Dissection of the enhancer activity in the SERCA2 distal promoter (bp ~1815 to ~1105). Deletion fragments of the ~1815 to ~1105 bp SERCA2 distal promoter were subcloned into the TK–CAT vector pBLCAT5 as shown. pBLCAT6 and pBLCAT5 were used as controls (constructs 1 and 2). CAT values are represented as the CAT activity relative to that of the 710 bp TK–CAT (construct 3). The deletion construct (pCATS/1227–1105) includes the E box/AT-rich region.

Gel shift analysis shows that the E box/AT-rich region binds a muscle-specific protein that is induced during myogenesis

Our deletion mapping analyses of the 710 bp enhancer region identified the region between bp –1227 and –1105 as containing an important cis-acting element. To determine if this region is a target for specific transcription factors from muscle cells, gel shift analysis was performed on the 122 bp fragment, bp –1227 to –1105, with an oligo corresponding to the E box/AT-rich sequence. As shown in Figure 6A, a specific DNA–protein complex was observed only with nuclear extract from differentiated Sol8 myotubes. Surprisingly, nuclear extracts from Sol8 myoblasts (undifferentiated muscle cells) did not show any specific protein binding, suggesting that this protein is induced during myogenesis. In addition, nuclear extracts from HeLa cells and NIH 3T3 fibroblasts did not show any specific protein binding. Specific protein binding from Sol8 myotubes could be competed away with a 100-fold excess of cold homologous probe. In order to delineate the region of protein binding within the 122 bp fragment, DNA competitions were performed with restriction fragments obtained from the 5′- (bp –1227 to –1158) and 3′-halves (bp –1159 to –1105) of the 122 bp fragment (Fig. 6B). The 5′-half failed to compete even at 200× molar excess, whereas the 3′-half effectively competed protein binding at 50× molar excess. The 3′-half contains the consensus site for an E box,
The results presented in this paper demonstrate that a novel E box/AT-rich element (CAAAATGAAAAAT) is essential for muscle-specific activation of the SERCA2 promoter. DNase I footprinting of this region showed that muscle-specific nuclear proteins bind to the E box/AT-rich region. A mutation to the E box decreased promoter activity by 50% in a 710 bp TK–CAT construct and 75% in the SERCA2–CAT construct. Further deletion mapping demonstrated that the E box/AT-rich sequence is essential for muscle-specific activation of the distal promoter region. Additionally, the E box/AT-rich sequence can activate the TK promoter alone. Gel mobility shift analyses showed that the E box/AT-rich region binds to a specific protein complex only from Sol8 myotube nuclear extracts. However, this protein binding is not competed away by the MCK E box (27), suggesting that this element does not bind myogenin or related proteins. Furthermore, the protein binding pattern to the E box/AT-rich sequence is quite different from that of the MCK E box. In conclusion, our data suggest that the E box/AT-rich sequence binds a novel protein complex that is induced during myogenesis. Future studies will aim to determine the biochemical nature of this protein complex.

In addition to the E box/AT-rich element, we have found that the CArG box and MCAT elements are important for enhancer activity of the distal region. DNase I footprinting analyses revealed that these three elements, namely the E box/AT-rich sequence, CArG and MCAT bind specific nuclear protein complexes from Sol8 myotubes. Further, mutations to each of these sites individually result in a significant loss in total SERCA2/TK–CAT promoter activity (Fig. 4A). The data presented here suggest that all three cis-acting elements are important and may play a role in overall activation and regulation of the SERCA2 gene promoter in the muscle environment.

In this study we identified an MCAT element in the distal enhancer that was protected by nuclear proteins from Sol8 myotubes. A point mutation to the MCAT core element decreased promoter activity, further suggesting that this element is important for SERCA2 transcription. MCAT sequences have been demonstrated to bind the transcription factor TEF1 or TEF1-related factors (24) and are important for muscle-specific expression of cardiac troponin T (29), bMHC (30,34) and skeletal muscle actin (35,36). To test if overexpression of TEF1 factors increased SERCA2 promoter activity, we co-transfected the 710 bp TK–CAT construct with TEF1 expression vectors. Overexpression of TEF1a but not TEF1b up-regulated the 7K promoter 4-fold in Sol8 muscle cells (data not shown). This data further demonstrates that the TEF1 transcription factor may also play an important role in SERCA2 gene regulation.

During muscle development the coordinate induction of muscle-specific genes is regulated by both tissue-specific and ubiquitous nuclear transcription factors. The MyoD family of transcription factors (MyoD, myogenin, myf5 and MRF4/herculin/myf-6) are known to bind to the consensus E box site (CANNTG) and activate several muscle genes (37,38). It has been demonstrated that forced expression of these transcription factors alone is sufficient to induce a skeletal muscle phenotype in non-muscle (fibroblast) cells (38). However, not all muscle-specific promoters contain the ideal E box element and require direct myogenin binding for activation. It has been shown that expression of cardiac TNT (29), bMHC (30) and cardiac MLC-2 (39) genes in muscle cells does not require MyoD, but rather depends on other transcription factors. The bMHC
gene, which is expressed both in cardiac and slow twitch skeletal muscle (similar to SERCA2), requires both MCAT sequences and an adjacent E box motif (which does not bind MyoD) for full activation of the proximal enhancer (30). Additionally, the cardiac troponin T gene promoter, which is expressed in cardiac and fetal skeletal muscle, requires a MCAT/TEF-1 element for transcriptional activation, instead of MyoD (29). Finally, cardiac myosin light chain-2, which is abundant in both cardiac and slow twitch muscle, is activated through an E box/MyoD-independent pathway by a ubiquitous factor HF-1a and MEF-2, which bind to an AT-rich sequence with homology to a CArG box (39). Furthermore, several recent studies suggest that transcriptional activation of muscle-specific genes requires multiple cis-acting elements, both muscle-specific and ubiquitous, such as CArG, SP1, MEF-2 and AP-1 (36,39–44).

In conclusion, the data presented here suggests that a novel E box/AT-rich element together with MCAT and CArG box elements function as transcriptional activators in the distal promoter region of the SERCA2 gene. Future experiments will attempt to characterize the nature of protein that binds to the AT-rich element and its interaction with other transcription factors.

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