Identification of Weight-bearing-responsive Elements in the Skeletal Muscle Sarco(endo)plasmic Reticulum Ca\(^{2+}\) ATPase (SERCA1) Gene*

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The skeletal muscle sarco(endo)plasmic reticular calcium ATPase (SERCA1) gene is transactivated as early as 2 days after the removal of weight-bearing (Peters, D. G., Mitchell-Felton, H., and Kandarian, S. C. (1999) Am. J. Physiol. 276, C1218-C1225), but the transcriptional mechanisms are elusive. Here, the rat SERCA1 5′ flank and promoter region (−3636 to +172 base pairs) was comprehensively examined using in vivo somatic gene transfer into rat soleus muscles (n = 804) to identify region(s) that are both necessary and sufficient for sensitivity to weight-bearing. In all, 40 different SERCA1 reporter plasmids were constructed and tested. Several different regions of the SERCA1 5′ flank were sufficient to confer a transcriptional response to 7 days of muscle unloading when placed upstream of a heterologous promoter. Two of these regions were analyzed further because they were necessary for the unloading response of −3636 to +172, as demonstrated using internal deletion constructs. Deletion analysis of these regions (−1373 to −1158 and −330 to +172) suggested that unloading responsiveness corresponded to CACC sites and E-boxes. Mutagenesis of cis-elements in the first region showed that a specific CACC box (−1262) was involved in SERCA1 transactivation and a nearby E-box (−1248) was also implicated. Constructs containing trimemized CACC sites and E-boxes showed that the presence of both elements is required to activate transcription. This is the first identification of specific cis-elements required for the regulation of a Ca\(^{2+}\) handling gene by changes in muscle loading condition.

The sarco(endo)plasmic reticulum is the major organelle that regulates the Ca\(^{2+}\) signaling associated with a multitude of cellular processes (1, 2). The sarco(endo)plasmic reticular Ca\(^{2+}\) ATPase (SERCA)\(^1\) proteins translocate Ca\(^{2+}\) from the cytosol to the sarcoplasmic reticulum or endoplasmic reticulum lumen, thereby reducing intracellular Ca\(^{2+}\) and refilling the sarcoplasmic/endoplasmic reticular Ca\(^{2+}\) stores. The cloning, expression, and functional characterization of three SERCA genes and their splicing variants have been described (3–12).

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\(^1\) The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase; NFAT, nuclear factor of activated T cells; PCR, polymerase chain reaction; RLU, relative light units.

At least one of the SERCA gene products is expressed in every mammalian tissue studied, emphasizing the fundamental role of this protein in cellular function. Products of the SERCA1 gene are the predominant isoforms expressed in skeletal muscle (SERCA1a adult, SERCA1b neonatal) (4, 9), and one product of the SERCA2 gene is the predominant isoform expressed in cardiac myocytes (SERCA2a (3)). The alternative splicing product of SERCA2 (SERCA2b (3)) and the products of the SERCA3 gene (3, 11) are ubiquitously expressed in muscle and non-muscle tissue but in much lower levels. SERCA expression is much higher in striated muscle than in smooth muscle or non-muscle to accommodate the large calcium fluxes associated with excitation-contraction coupling.

Contractile activity has a marked effect on SERCA expression in striated muscle. Increased contractile activity leads to decreases in SERCA1 and SERCA2a expression in skeletal (13, 14) and cardiac (15, 16) muscle, respectively, whereas decreased contractile activity leads to increases in expression (17–19). SERCA1 expression is particularly sensitive to the reduction of contractile activity due to rat soleus muscle unloading, with significant increases by 2 days and 7-fold increases by 7 days (19). Moreover, the increase in expression is due to increases in SERCA1 gene transcription (19). The up-regulation of SERCA1 with unloading is counterintuitive as it occurs in a background of overall decreases in total protein and mRNA synthesis (20). This suggests that the up-regulation of SERCA1 is an active and highly regulated process. However, the functional significance and the molecular mechanisms of selective gene activation are unknown.

In previous work we isolated the rat 5′ flank and promoter region of SERCA1 to −3636 base pairs (21). As a first step in elucidating the transcriptional regulation of SERCA1 by unloading, the sequence from −3636 to +172 was examined using in vivo somatic gene transfer to identify the region(s) both necessary and sufficient for weight-bearing sensitivity. Comprehensive promoter analysis revealed two regions of SERCA1 that were sufficient to confer unloading responsiveness and which were necessary for the unloading sensitivity of the −3636 to +172 construct. Mutagenesis and further deletion analysis of these regions identified a CACC box/E-box interaction, which was required for transcriptional activation with unloading. This is the first demonstration of specific cis-elements required for the in vivo regulation of a Ca\(^{2+}\) handling gene by changes in muscle loading activity.

EXPERIMENTAL PROCEDURES

Animals—Female Wistar rats (Bantam and Kingman, n = 402) 8 weeks of age (−200–225 g) were used for all experiments. Twenty-four hours after plasmid DNA was injected into soleus muscles, the hind limbs of one-half of the rats were removed from weight-bearing for 7 days as previously detailed (19).

In Vivo DNA Injections—In vivo injections were carried out as pre-
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Table I
Sequence of PCR primers used to make many of the constructs in this study

| Construct name          | Sense primer                          | Antisense primer                          |
|-------------------------|---------------------------------------|-------------------------------------------|
| pGL3-promoter constructs|                                       |                                           |
| SERCA1 (−3636 to −3174)-SV40 | TTTGACAGGCCTGGCGCGGGCCTGATCATTGTGTTTG | GGGGTAGATCTACAGGTTACAGGGGTAGAAGG |
| SERCA1 (−3174 to −2874)-SV40 | TTAGGGCTCAGCTCATTAATGATTGGTCTGCG | GAACAGATGTCCTACAGGGGTAGAAGG |
| SERCA1 (−2874 to −2652)-SV40 | GTCGATTGAGGAGGGGAAGTGGTGATTCG | GGATACGTTTACAGGGGTAGAAGG |
| SERCA1 (−1373 to −1158)-SV40 | CCAACACGGTGTGGTCTGACATTGTCCTCCTC | CACCAACGGTGTGGTCTGACATTGTCCTCCTC |
| SERCA1 (−1373 to −1248)-SV40 | CTTCATGCGGTGGTCTGACATTGTCCTCCTC | CTTCATGCGGTGGTCTGACATTGTCCTCCTC |
| SERCA1 (−962 to −612)-SV40 | AATTGAGACGGTGTCACTGCTACAGTTG | CTCTGGAAGATCTGTAAGCAAGAGGCAG |
| SERCA1 (−330 to −103)-SV40 | TCGTAC | TTGTTGACTGAGGATTTCTGCTGAC |
| SERCA1 (−3636 to −330)-SV40 | TTTGACAGGCCTGGCGCGGGCCTGATCATTGTGTTTG | GGGGTAGATCTACAGGTTACAGGGGTAGAAGG |
| SERCA1 (−3636 to −103)-SV40 | TTTGACAGGCCTGGCGCGGGCCTGATCATTGTGTTTG | GGGGTAGATCTACAGGTTACAGGGGTAGAAGG |
| SERCA1 (−330 to −21)-SV40 | TACCTAC | TTGTTGACTGAGGATTTCTGCTGAC |
| SERCA1 (−330 to +27)-pGL3 | TACCTAC | TTGTTGACTGAGGATTTCTGCTGAC |
| Internal deletion constructs |                                       |                                           |
| SERCA1 (−3636 Δ−883 to −2542)-pGL3 | TGTGGTGGGAAGGGCAAGAGGGCATC | AGGCAAGGCGTACCGGGTGAGAAGAC |
| SERCA1 (−3636 Δ−1403 to −1158)-pGL3 | GCCACTCCCACCCGTCTTGGTTTCTTT | CTTCCTGACGATCATGACAGATGACAGATG |
| SERCA1 (−3636 Δ−962 to −612)-pGL3 | CCAGGCGCTTGGACACTAAATCCCTTGTTT | GATCGTC |
| SERCA1 (−3636 Δ−330 to −103)-pGL3 | TCTCTCTTGACCCCTCCCTGCTGGTTTGC | CCGGGCATCGTTACAGTCTCCTCCCCTGCG |
| Mutagenesis constructs |                                       |                                           |
| SERCA1 (mNFAT −1367)-SV40 | GGCGGTTCTCCATCTGCAATGTCCCTGGTG | GCCTGCAGCTCAATAGGAGTCCCTCAATAGGAG |
| SERCA1 (mCACCC −1305)-SV40 | CGCTGGCTTGTGTCATCAAAAGATACCTCT | GCAGCCCAACCTGGTGAGTGAGGAG |
| SERCA1 (mCCAAAT −1287)-SV40 | GGTTGAAATATAAGGAATGACACCTTTTCTTCT | CAGGGCGGAGATCTTACCCCTGCTGAC |
| SERCA1 (mCCAC −1272)-SV40 | CCAATCGTGATGGTGCCAACCTCTGCTT | GGCTGTCG |
| SERCA1 (mCCAC −1262)-SV40 | CAACTCGTGATGGTGCCAACCTCTGCTTG | TCTTGAACCTG |
| SERCA1 (mE-Box −1248)-SV40 | HGGGGAGGGGCCCTGCAAGACTGAGTCTCGAC | ACTGGTACCTCCTGACG |
| SERCA1 (mNFI −1198)-SV40 | GGGTTTGGGGCTGCTGATGCTGCTGCTG | CTGGTCG |
| SERCA1 (mSp1 −1171)-SV40 | ACCAAGGTCAG | TCGCTG |

vously detailed (21). In brief, plasmid DNA was isolated using the endotoxin-free Megaprep kit (Qiagen). The DNA was ethanol-precipitated overnight and resuspended to the appropriate concentration in a 25% sucrose-phosphate-buffered saline solution such that 50 μl of plasmid DNA (50, 75, or 125 μg) was injected per soleus muscle. Luciferase activity was determined on 20 μl of the supernatant fraction as previously detailed (21), so that data reflect activity per whole muscle. Where indicated, luciferase activity was normalized to DNA uptake, as determined by Southern blot analysis (21). Briefly, total muscle DNA was isolated and digested with HaeIII. Control and unloaded DNA (10 μg/lane) was run on a gel with a pGL3-basic DNA standard curve and transferred to membrane, and plasmid DNA was detected with a luciferase-specific random primed probe.

Plasmid Construction—SERCA1 (−3636)-pGL3, SERCA1 (−962)-pGL3, SERCA1 (−612)-pGL3, and SERCA1 (−330)-pGL3 were constructed from rat genomic DNA as previously detailed (21). These constructs contain SERCA1 5’ flank and promoter sequence that was ligated upstream of the luciferase gene in pGL3-basic. Additional 5’ deletion constructs in the present study were made from SERCA1 (−3636)-pGL3 using the Erase-a-Base kit (Promega). The clones obtained were subjected to restriction analyses and sequencing to determine the exact length.

Fragments of the SERCA1 5’ flank were amplified by PCR and ligated upstream of the SV40 minimal promoter in the luciferase reporter plasmid pGL3-promoter (Promega). Primers were designed with restriction enzyme sites added to their 5’ end (MluI, BglII, or XhoI). PCR protocol was 25–32 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min; then 72 °C for 20 min. SERCA1 (−1314 to −1158)-SV40 and SERCA1 (−1248 to −1158)-SV40 were created by digesting SERCA1 (−1373 to −1158)-SV40 with EcoRI and PvuII and recircularizing the large fragment. Internal deletion constructs of SERCA1 (−3636)-pGL3 were created using inverse PCR and components from the Advantage Genomic PCR kit (CLONTECH) following instructions from the manufacturer. The Quik-Change mutagenesis kit (Stratagene) was used to mutate specific bases in SERCA1 (−1373 to −1158)-SV40 according to the manufacturer. All clones were checked by sequencing. The construct names and the primers used to create them are listed in Table I.

Sense and antisense oligonucleotides were designed with a trimerization sequence added to make double-stranded oligonucleotides. The sense primer was designed to contain the oligonucleotide sequence of the probe (shown in Table I). The annealed double-stranded oligonucleotides contained BglII and HindIII ends and were easily ligated into the pGL3 promoter. The sequence of each oligonucleotide strand is shown in Table II.

Statistics—An unpaired Student’s t test was used to determine statistical significance between control and unloaded groups at p < 0.05.
fold activation) was evident in all constructs except SERCA1 muscles, expression of the SERCA1-pGL3 reporter constructs performed with the constructs depicted in Fig. 2. In control units, soleus muscles (control intervention of hind limb unloading altered plasmid uptake in size of the constructs (Ref. 21 and data not shown) nor the activity (21). Southern blot analysis revealed that neither the with respect to the efficiency of plasmid uptake and luciferase activity were contained in this construct.

Initial in vivo deletion analysis of the SERCA1 5’ flank was performed with the constructs depicted in Fig. 2. In control muscles, expression of the SERCA1-pGL3 reporter constructs decreased with serial deletions. Unloading responsiveness (i.e. fold activation) was evident in all constructs except SERCA1 (−3174)-pGL3, SERCA1 (−2874)-pGL3, SERCA1 (−1158)-pGL3, SERCA1 (−962)-pGL3, SERCA1 (−612)-pGL3, and SERCA1 (−103)-pGL3. The most obvious loss of unloading responsiveness occurred upon deletion of the region between −1373 and −1158. Therefore, this region was of interest and was selected for further study. Unloading responsiveness was not observed with the deletion of DNA between −1158 and −612. However, injection of the SERCA1 (−330)-pGL3 construct resulted in a return of the unloading response, which was lost again with injection of SERCA1 (−103)-pGL3. The return of unloading responsiveness was unexpected, so the SERCA1 (−330)-pGL3 injection was repeated with newly amplified DNA and a different set of muscles. The same unloading response was observed in the second experiment. Thus, the sequence between −330 and +172 was sufficient in isolation to confer unloading responsiveness, so it was a second region designated for further study. We also observed loss of unloading responsiveness in SERCA1 (−3174)-pGL3 and SERCA1 (−2874)-pGL3 compared with SERCA1 (−3636)-pGL3, although the decrease in fold activation was due to increased control values as opposed to decreased unloading values. These regions were also tested further. Taken together these data suggest that there are multiple regions in the SERCA1 promoter-enhancer that can confer unloading responsiveness. The intricacy of the unloading response in this figure is likely due to the comprehensiveness of the promoter analysis as well as the complexity of the in vivo physiological phenomena under study.

RESULTS

Previous work in this laboratory showed that 75 µg was an optimal amount of SERCA1 plasmid to inject in soleus muscles with respect to the efficiency of plasmid uptake and luciferase activity (21). Southern blot analysis revealed that neither the size of the constructs (Ref. 21 and data not shown) nor the intervention of hind limb unloading altered plasmid uptake in soleus muscles (control = 378 ± 40 densitometric absorbance units, n = 108; unloaded = 440 ± 47 absorbance units, n = 109; p = 0.32). To determine whether different amounts of injected DNA affected the extent of transactivation with muscle unloading, constructs were created containing SERCA1 (−3636)-pGL3 normalized to plasmid uptake was increased 7-fold after 7 days of unloading (Fig. 1). Since the extent of increase in luciferase activity was similar at the different quantities tested, the amount of DNA injected did not influence the magnitude of the unloading response. The increase in luciferase activity was the same as the unloading-induced activation of the endogenous SERCA1 gene (19), suggesting that elements sufficient for unloading sensitivity were contained in this construct.

To test regions of SERCA1 for sufficiency in activating transcription with unloading, constructs were created containing SERCA1 fragments upstream of the heterologous SV40 promoter (Fig. 3A). SERCA1 (−1373 to −1158)-SV40 and SERCA1 (−962 to −612)-SV40 resulted in the strongest response with 5.0- and 5.7-fold activation, respectively, over control muscles. The activation of SERCA1 (−962 to −612)-SV40 was unexpected since neither SERCA1 (−962)-pGL3 nor SERCA1 (−612)-pGL3 showed unloading responsiveness (Fig. 2). This finding emphasized the context specificity of the promoter analysis. Other fragments of the SERCA1 5’ flank showed either modest increases or no change in luciferase activity when examined in isolation. Next, we sought to determine whether the regions that were sufficient for unloading responsiveness were required in the context of SERCA1 (−3636)-pGL3. To do this, inverse PCR was used to delete a specific region from the −3636 to +172 parent construct, thereby creating internal deletion constructs (Fig. 3B). Removal of sequence between −1403 and −1158 abolished the unloading response, indicating that this region is required for unloading induced transcriptional activation. Deletion of other sequences did not result in a loss of unloading sensitivity. These data reveal that one obvious region of the SERCA1 5’ flank containing elements both necessary and sufficient for unloading responsiveness was between −1373 and −1158. The other observation made from this experiment was that sequences involved in high level expression and unloading sensitivity are distinct and, furthermore, that the sequence required for high level expression is not confined to the 5’ end of the construct (Fig. 3B). Data from Fig. 3, A and B were re-plotted as fold activation for comparative purposes, since heterologous and wild type promoters were used to help visualize that −1373 to −1158 was both necessary and sufficient for transcriptional activation with 7 days of muscle unloading (Fig. 3C).

A more extensive analysis of SERCA1 (−1373 to −1158)-
SV40 showed that removal of the sequence from −1373 to −1314 did not alter the magnitude of transcriptional activation, but the removal of the region between −1373 and −1248 decreased the response from 5.0- to 2.2-fold. Thus, sequence between −1314 and −1248 is involved in unloading sensitivity (Fig. 4). However, the sequence from −1373 to −1248 was not sufficient, in isolation, to up-regulate activity, suggesting that an interaction between −1314 to −1248 and −1248 to −1158 exists that confers unloading responsiveness. This interaction may involve the E-box at −1248, which is deleted in the constructs showing only a 2-fold activation. Thus, the E-box (−1248) may be necessary for weight-bearing responsiveness.

Mutagenesis of SERCA1 (−1373 to −1158)-SV40 was then performed to target cis-elements required for unloading induced transactivation (Fig. 5). Consensus cis-elements were identified using MatInspector (Version 2.2). Mutation of the CACC box at position −1262 abolished the unloading response, indicating that this CACC site is involved in the up-regulation of SERCA1 (−1373 to −1158)-SV40. Disruption of the NFAT (−1367) and CACC (−1272) sites did not change transcriptional activation, whereas mutation of CACC (−1305) and CCAAT (−1287) sites show moderate decreases in the unloading response. Surprisingly, with the exception of the E-box at −1248, mutation of the known elements between −1248 and −1158, the NF-I (−1198) and Sp1 (−1171) sites, did not decrease the unloading response. Since an interaction was predicted from the results of Fig. 4, we explored the possibility that the E-box (−1248) is interacting with the CACC (−1262) site.

To test if a CACC/E-box interaction was sufficient to increase transcription, constructs containing trimerized CACC sites and E-boxes upstream of SV40 were examined in control and 7-day unloaded soleus muscles (Fig. 6). Since weight-bearing sensitivity was abolished with the mutation of the CACC box at −1262, the first step was to examine this cis-element. Injection of SERCA1 (CACC)-SV40, a trimerized construct containing the CACC (−1262) site, resulted in no significant change in luciferase activity, supporting the hypothesis that an interaction of CACC (−1262) with a second cis-element is required for transcriptional up-regulation. Examination of SERCA1 (CACC/E-box)-SV40, containing CACC (−1262) and E-box (−1248), resulted in a 9.0-fold increase over control values. Thus, the combination of CACC (−1262) and E-box (−1248) is sufficient to transduce an unloading response, suggesting communication between trans-factors binding these elements. A multimerized construct containing CACC (−1272), CACC (−1262), and E-box (−1248) had a 4.6-fold increase in luciferase activity with unloading. However, the decrease in fold activation was due to a greater increase in the control versus the unloaded luciferase activity, suggesting that the CACC box at −1272 may be important for general high level transcription of SERCA1. This is consistent with the overall decrease in luciferase activity in both control and unloaded muscles with mutagenesis of the CACC box at −1272 base pairs (Fig. 5).

The sequence between −330 to +172 was further examined because 1) SERCA1 (−330)-pGL3 shows a 9.3-fold increase with unloading, 2) the sequence is in close proximity to the basal promoter, and 3) the sequence contains multiple CACC and E-boxes, which were found to be important in −1373 to −1158. Further interest in the −330 region was spurred by the finding that SERCA1 (−3636 to −330)-SV40 could not activate transcription with unloading (Fig. 7), suggesting that the SERCA1 (−3636)-pGL3 construct requires the sequence from −330 to +172 for transactivation. Since the sequence between −330 and −103 was not necessary for unloading responsiveness (Figs. 3B and 7), it was particularly interesting to find that luciferase activity was increased in SERCA1 (−3636 to −103)-SV40 with unloading. In addition, SERCA1 (−330 to −103)-SV40 (Figs. 3A and 7) and SERCA1 (−103)-pGL3 (Figs. 2 and 7) were not sufficient to activate transcription, whereas SERCA1 (−330)-pGL3 was sufficient (Figs. 2 and 7). The results indicate that sequence between −330 and +172 was required for the unloading response seen with SERCA1 (−3636)-pGL3, but that required elements were found in both −330 to −103 and −103 to +172, regions that contain both CACC sites and E-boxes in high numbers. To delineate the minimal required sequence for activation, further deletion analysis was performed. SERCA1 (−330 to +27)-pGL3 was sufficient to up-regulate transcription, whereas SERCA1 (−330 to −21)-SV40 was not, suggesting that the sequence from −21 to +27 contributes to transducing the unloading signal. It is of interest that the spacing of the E-box and CACC site between −21 and +27 is similar to the CACC site and E-box in −1373 to −1158. Given the density of CACC and E-boxes in −330 to +172, the necessity of this sequence for unloading sensitivity of SERCA1 (−3636)-pGL3, and the requirement for the sequence between −21 and +27, which contains a CACC site and an E-box, it is likely that these elements are critical to transducing transactivation in response to the removal of weight-bearing in the soleus muscle.
Identification of SERCA Weight-bearing-responsive Elements

Intracellular Ca\(^{2+}\) regulates the signaling associated with diverse cellular processes such as excitation-contraction coupling, cell proliferation, differentiation, and death (2). SERCA1, by translocating Ca\(^{2+}\) from the cytosol to the lumen of the sarcoplasmic reticulum, is a critical protein in the regulation of skeletal muscle Ca\(^{2+}\) homeostasis. The SERCA1 gene is highly mutable, with mRNA expression and transcription being up-regulated 7-fold by 7 days of unloading in rat soleus muscles (19). Given the central role that SERCA1 plays in maintaining Ca\(^{2+}\) homeostasis, it is surprising how little is known about the transcriptional regulation of the gene. Thus, the focus of the present study was to identify elements involved in the transcriptional activation of the SERCA1 gene by muscle inactivity due to unloading. We found that there were specific CACC sites and E-boxes that were required for unloading-induced transactivation.

The rat SERCA1 5’ flank contains cis-elements between –3636 and +172 that are known to be important in regulating muscle-specific genes. These include 21 CACC sites (CACC), 17 myogenic E-boxes (CAGNTG), 2 M-CAT sites (GGAATG), 4 NFAT sites (WGGAANH), 1 Sp1 site (GGCGG), 10 Sp1-like sites (GGAGG), and 12 TRE half-sites (RGGTSA). Interestingly, there are no MEF2 sites (YYAWWWWWTAR), MEF3 sites (SSTTACGTTTWC), or CarG boxes (CCWWWWWWGCG) previously shown to be important for transcription of some muscle genes (22–27). Given the relatively high number of CACC sites and E-boxes, it is not surprising that these two elements were found to be necessary for transcriptional regulation of the SERCA1 gene with unloading. There is precedence in the literature for the regulatory pairing of CACC and MEF2 sites necessary for transcription of many muscle genes, including rat myosin light chain 2 slow (28), human myoglobin (24), rat muscle creatine kinase (29), mouse troponin C slow (25), and human \(\beta\)-enolase (30), but the regulatory pairing of CACC sites and E-boxes is less well defined (31, 32).

FIG. 3. Identification of regions of the SERCA1 5’ flank both necessary and sufficient for unloading sensitivity. A, a fragment of the SERCA1 5’ flank (black box) was ligated upstream of the heterologous SV40 promoter to test each fragment for sufficiency of unloading. B, the indicated regions of the SERCA1 5’ flank were removed from the SERCA1 (–3636)-pGL3 parent construct to test their necessity for unloading sensitivity in the context of SERCA1 (–3636)-pGL3. The lack of an exact match between the removed nucleotides in B and their corresponding fragment in A is because the PCR primers used to create deletion constructs had stringent criteria that could not always be met. Control (black bars) and 7-day-unloaded (gray bars) values are as plotted. C, data from A and B re-plotted as fold activation to emphasize that the region between –1373 and –1158 is both necessary and sufficient for the unloading response. Fold activation is the unloaded divided by the control value. n = 8–16 muscles per group. Data are the means \(\pm\) S.E.

FIG. 4. Deletion analysis of the SERCA1 region from –1373 to –1158. The cis-elements present in each construct are as indicated. The E-boxes at –1314 and –1248 were disrupted in the creation of the different deletion constructs. Constructs were injected into control (black bars) or 7-day-unloaded (gray bars) soleus muscles. Fold activation is the unloaded divided by the control value. n = 8–16 muscles/group. Data are the means \(\pm\) S.E.

DISCUSSION

The transactivator(s) that binds CACC sites for functional activation has not been definitively determined, although there are a number of candidates including the myocyte nuclear factor, a member of the winged helix family of proteins (33), and CBP40 (34). In addition, members of the Sp1/kruppel-like factor family interact with CACC sites (35), although the affinity of binding is lower relative to consensus Sp1 sites (27). Recently a negative regulator, BERF1, was characterized as a CACC-binding protein, and a second CACC-binding protein, which may be an activating factor, was identified with a protein-DNA binding assay (36). The proteins that interact with E-boxes have been well characterized and consist of the myogenic regulatory factors (MRFs) MyoD, myogenin, myf-5, and MRF4. They are basic helix-loop-helix proteins that dimerize to drive the muscle developmental program. However, their role in adult muscle is not yet fully defined, and there are indications that other proteins may interact with E-boxes in adult muscle to direct transcription (37). Functional E-boxes are required for expression of many genes in adult tissue, including muscle creatine kinase (38), myosin light chain 1/3 fast (39), myosin heavy chain IIB (40), cardiac \(\alpha\)-actin (27), and slow skeletal troponin I (31, 32). Studies with cardiac troponin C have shown that overexpression of MyoD can stimulate transcription even in the absence of E-boxes (25). This can be explained by the fact that MyoDE12 (or myogenin/
E12) and MEF2 proteins trimerize and activate transcription in a synergistic and co-operative manner (41, 42), and this trimer can function when only one cis-element (MEF2 site or E-box) is present (42). Given that SERCA1 contains no MEF2 sites, and requires an E-box and CACC site for unloading induced transcription, it is possible that a protein complex binding to SERCA1 contains a myogenic regulatory factor, a CACC box-binding protein, and MEF2. Crude nuclear extracts from control and unloaded soleus muscles showed no difference in the pattern or intensity of protein-DNA binding complexes when incubated with individual consensus CACC or E-box sequences. Studies detailing the protein biochemistry involved in binding to the SERCA1 CACC-E box sequence revealed here is the next logical series of experiments and will be addressed next.

The involvement of the CACC site at −1 to +5 and the E-box at −17 to −12 in the transcriptional regulation of SERCA1 is interesting because of the importance of the proximal promoter in the regulation of SERCA genes. Work done with rabbit SERCA2 revealed that transcriptional activation in C2C12 cells was dependent upon intact Sp1 and Sp1-like sites between −284 and +1 (43). The human SERCA3 gene also contains seven Sp1 and Sp1-like sites in the same region that have been suggested as being critical to transcriptional activation (12). Thus, transcription of both SERCA2 and SERCA3 is dependent upon GC-rich regions in the proximal promoter. The rat SERCA1 proximal promoter contains no Sp1 sites, and although it contains three of the related GC-rich CACC sites, the difference in the GC content of rat SERCA1 promoter (62.4%) versus the rabbit SERCA2 (81.6%) and human SERCA3 (81.2%) promoters suggests that transcriptional regulation of SERCA1 is distinct. This idea is further supported by the observation that thyroid stimulation (44, 45) and contractile activity (14, 17, 19, 46) differentially target SERCA1 and SERCA2a in skeletal muscle.

Promoter analysis of the SERCA1 and SERCA2 gene in skeletal (47, 48) and cardiac muscle (49–51), respectively, has identified an important role of thyroid hormone (T3). Examination of the rat SERCA1 5′ flank and promoter region (−962 to +41) in COS cells showed that treatment with T3 activated SERCA1 transcription (47). Within this sequence three regions were identified that contain TRE sites capable of binding T3 receptors and subsequently activating transcription when the receptor was bound to T3. However, unloading does not appear to be regulated by T3, since the TREs do not tract with unloading responsiveness.

Besides revealing the elements involved in the unloading
response of SERCA1, the present study also uncovered information about high level expression in control soleus muscles. Many regions of the SERCA1 gene were required for high level expression within the context of SERCA1 (~3636)-pGL3, including the sequence between ~3636 to ~2652 (Fig. 2), −1403 to −1158, and −962 to −612 (Fig. 3B). Simonides et al. (47) performed promoter analysis of rat SERCA1 (~962 to +41) in COS because the promoter constructs exhibited low expression levels when transfected into L6 muscle cells. However, a subsequent study by the same group (48) using the SERCA1 promoter from −2658 to +91 was performed in L6 muscle cells, where expression was detectable, indicating that sequence between −2658 and −962 contributes to high level SERCA1 expression. Thus, our work and that of others supports the finding that regulation of high level expression of SERCA1 is complex and requires a sequence more than 1 kilobase from the transcription start site.

The present study is a comprehensive in vivo analysis of the transcriptional regulation of the rat SERCA1 gene by muscle unloading. We identified specific CACC site-E-box pairings that were necessary and sufficient for the unloading responsiveness of the SERCA1 (~3636)-pGL3 construct. Not only was the pairing of the CACC site at −1262 and E-box at −1248 necessary and sufficient to confer transactivation with unloading, but a CACC and E-box pairing near the transcription start site was also found to be important. The identification of transcription factors that interact to increase transcription of SERCA1 will be critical to understand the regulation of this fundamental calcium-handling gene. It has been suggested that the regulation of calcium-handling genes is co-regulated (52). This is evidenced in our model by increased expression of the L-type calcium channel, the ryanodine receptor, and calsequestrin as well as SERCA1 with unloading (46, 53). Thus, understanding the regulation of SERCA1 may also illuminate regulation of other calcium-handling genes by contractile activity.

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