Characterization of Promoter Elements of the Rabbit Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Gene Required for Expression in Cardiac Muscle Cells

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The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) plays a critical role in the contractile performance of cardiac and slow-twitch skeletal muscle by restoring cytosolic calcium to low resting levels during the contractile cycle. We have previously shown that SERCA2 expression in the heart is altered by a number of pathophysiological stimuli. In an effort to define molecular mechanisms regulating expression of the SERCA2 gene in cardiac muscle cells, deletions of a 1460-bp promoter fragment were generated and inserted into a luciferase reporter plasmid. Promoter constructs were transiently transfected into embryonic cardiocytes and skeletal muscle cell lines Sol 8 and C\(_{12}\)C\(_{12}\) in vitro and injected into adult myocardium in vivo. Results demonstrate that sequences from the transcription start site to \(-284\) are both necessary and sufficient for high-level transcription of the reporter gene in differentiating muscle cells and in fetal cardiocytes in culture. We further demonstrate that this promoter fragment is highly active in vivo when injected into rat hearts, suggesting that the same regulatory elements are functional in vivo as well as in vitro. The region of the gene from \(-284\) to \(-658\) exerts a modest positive effect in cardiocytes and Sol 8 myotubes but exerts a negative effect in C\(_{12}\)C\(_{12}\) fast skeletal muscle cells. This initial analysis of transcriptional regulation of the SERCA2 gene will serve as a foundation for the study of alterations of expression of the gene in pathological conditions. (Circ Res. 1993;73:622-628.)

KEY WORDS • sarcoplasmic reticulum • Ca\(^{2+}\)-ATPase • transcriptional regulation • gene expression

Received December 22, 1992; accepted July 7, 1993.

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thyroid response element exists between −262 and −322 upstream from the transcription start site.

The purpose of this study was to define promoter elements critical for expression of the rabbit SERCA2 gene in cardiac cells, both in vitro and in vivo. To achieve this, deletions of a previously characterized 1460-bp promoter region of the rabbit SERCA2 gene were generated and inserted into a luciferase reporter plasmid. Transient in vitro transfection experiments were performed with isolated embryonic chick cardiocytes as well as fast (C5C12) and slow (Sol 8) skeletal muscle cell lines. In addition, direct DNA injections into adult rat myocardium were performed to analyze cis elements responsible for in vivo regulation.

In the present study, we identify a 284-bp region of the SERCA2 gene as necessary for its transcription in cardiac myocytes and in differentiating skeletal muscle cells. We also define the region from −284 to −658 bp as one that behaves differentially, suppressing transcription of the gene in C5C12 myotubes (fast) but not in Sol 8 myotubes (slow) or embryonic and neonatal cardiocytes. In addition, our direct DNA injections into rat hearts demonstrate that the same promoter elements are functional in vitro and in vivo. This study provides an initial step toward understanding the complex regulation of the SERCA2 gene.

Materials and Methods

Plasmid Construction

A 1460-bp DNA fragment of the 5′ flanking region of the SERCA2 gene was excised from the rabbit genomic clone RbCA3-3α using EcoRI and Sma I endonucleases. This 1460-bp fragment extends to position −1110 upstream from the transcription initiation site and includes +350 bp of the 5′ untranslated region. The DNA fragment was blunt-ended with Klenow enzyme, and BamHI linkers were added. The resulting fragment was ligated in a 5′ to 3′ orientation into the unique BamHI site of the chloramphenicol acetyltransferase (CAT) expression vector pBlCAT3. The resulting plasmid, pD1110, was used as a template for 5′ to 3′ exonuclease digestion with the Erase-a-Base kit (Promega Corp, Madison, Wis). Four promoter fragments beginning at −1110, −658, −284, and −72 were excised with HindIII and BamHI and directionally cloned into unique HindIII and Bgl II sites of the luciferase reporter plasmid pXP2. Constructs are presented schematically in Fig 1. The 5′ end of each construct was verified by dideoxynucleotide sequencing.

Plasmids pSV2CAT, RSVCAT, and RSVLuc containing the strong viral promoters of SV40 and RSV, respectively, were commercially obtained and used as controls.

Cell Culture

Embryonic chick cardiocyte and neonatal rat cardiocyte cultures were established by standard methods. White Leghorn chicken eggs (Spafas Inc, Norwich, Conn) were incubated for 10 days at 37.5°C, and the hearts were removed after decapitation of the embryos. One-day-old neonatal rats were decapitated, and the hearts were removed. Rat and chick cardiocyte cultures were established by identical methods. Five serial digests were performed with 0.1% collagenase (GIBCO-BRL) in Hanks’ buffered saline solution (HBSS containing [g/L] NaCl, 8; KCl, 0.4; KH2PO4, 0.06; NaHCO3, 0.35; Na2HPO4, 0.048; D-glucose, 1.10; and phenol red, 0.01) at 37°C for 15 minutes each with gentle trituration. Cells were then kept at 4°C. To the final digest, trypsin
The cardiocytes grown were plated onto Falcon tissue culture dishes and incubated at 37°C for 90 minutes so that fibroblasts could attach. The unattached cells were collected, counted, and plated at 1.4×10^5 cells per square centimeter on 0.5% gelatin precoated 60-mm (transfection) or 100-mm (RNA analysis) Falcon dishes. The cells were grown to confluence in glutamine-free Dulbecco’s modified Eagle medium (DMEM, GIBCO-BRL) with 10% fetal bovine serum (FBS) at 37°C and 10% CO2. The initial dishes containing predominantly fibroblasts were passaged in DMEM with 10% FBS (GIBCO-BRL). Kanamycin was added at a final concentration of 60 mg/L to all culture media.

The skeletal muscle cell lines C2C12 and Sol 8 were grown and passaged at low densities in DMEM with 20% FBS at 37°C and 10% CO2. At near confluence, the media was changed to DMEM with 5% horse serum to promote myogenesis. Myotubes were evident at 48 to 72 hours of serum deprivation.

**DNA Transfections**

After initial plating, chick and neonatal rat cardiocytes were allowed to grow to 40% to 70% confluence before transfection. These cells were washed with serum-free medium (OptiMEM, GIBCO-BRL) and placed in 3 mL OptiMEM. Ten micrograms of each SERCA2 luciferase test plasmid and 1 μg of pSV2CAT control plasmid were mixed with 30 μg of liposomes (Lipofectin, GIBCO-BRL) in a final volume of 100 μL. The liposome-DNA complex was added dropwise with swirling to duplicate sets of plates. Six hours later, the medium was changed to DMEM with 10% FBS. The cells were harvested 48 to 60 hours later. All transfections and data collection were from preparations that showed greater than 90% initial cell viability, as determined by trypan blue exclusion, spontaneous grouped beating of cardiocytes, and a population of cells that was greater than 95% cardiocytes, as determined by co-staining of parallel cultures with a muscle-specific troponin T antibody and a Hoechst nuclear stain. C2C12 cells and Sol 8 myoblasts were transfected at 50% to 70% confluence with plasmid DNA by the calcium phosphate precipitation methods as described by Gorman et al. Five hours later, cells were washed, and Sol 8 and C2C12 cells were incubated overnight in DMEM/20% FBS and then switched to differentiation medium for 48 to 72 hours. Cultures in which myoblasts had fused to form myotubes were harvested for analysis. All transfections were performed on duplicate plates, and each construct was tested at least three times with two different plasmid preparations.

**Preparation of Cell Extracts and Enzyme Assays**

Transfected cells at the time of harvesting were washed on ice with cold PBS (g/L: NaCl, 8; KCl, 0.2; NaH2PO4, 1.44; and KH2PO4, 0.24; pH 7.4), and 150 μL of lysis buffer (25 mmol/L Tris-phosphate [pH 7.8], 2 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100) was added. Cells were scraped off the dish, and the lysate was centrifuged at 12,000 rpm for 15 minutes (to remove insoluble debris). Ten microliters (5%) of the supernatant was used to perform the luciferase assay (Promega kit) as per kit instructions. Luminescence was measured for 20 seconds with a Berthold LB9501 luminometer. CAT assays were performed on 25% of the supernatant by standard techniques. Reaction mixtures were incubated for 90 minutes at 37°C, and percent CAT conversion was measured directly from the thin-layer chromatographic plate with a betascope (model 603, Betagen, Waltham, Mass).

Luciferase activity is presented as background-subtracted maximum raw luminometer units divided by the control CAT activity to normalize for differences in transfection efficiency. The data are presented as the percentage of activity of a given construct relative to the construct with maximum activity, which is assigned a value of 100%. The fold increase in activity of the test construct relative to the promoterless pXP2 plasmid is obtained for each group by dividing the test construct value by the promoterless construct value.

**DNA Injections Into Myocardium**

Injections of the plasmids into adult female Wistar rat myocardium were performed as previously described. In brief, 175- to 200-g rats were anesthetized with a 4% chloral hydrate intraperitoneal injection (1 mL/100 g), the heart was exteriorized through a left lateral thoracotomy, and 50 μL of a normal saline solution (containing 10 μg of test luciferase plasmid) and 2 μg of control pRSV CAT plasmid with 3% Evans blue were injected through a 27-gauge needle into the left ventricular apex. Adult rats were killed at 5 days, and the hearts were homogenized and assayed for luciferase and CAT activity as previously described. The data are presented as described above for the in vitro experiments.

**RNA Isolation and Analysis**

Total RNA from neonatal cardiocytes, C2C12, and Sol 8 myotubes, and heart muscle was isolated by the guanidine thiocyanate method, and 15 μg of each sample was analyzed by Northern blots, as previously described. A 1.7-kb cDNA fragment (BamH I-3’ end) from SERCA2 cDNA was used; this fragment hybridizes to SERCA2a and SERCA2b at the 4.6-kb position and to SERCA1a and SERCA1b at the 4.0-kb position on Northern blot analysis. The blots were stripped (of radioactive signal) by washing at 70°C in 0.1× standard sodium citrate for 1 hour and probed for α-, β-, and γ-actin to assess the degree of differentiation of the various muscle cells. A 900-bp (Pst I–Pst I) fragment of α-actin cDNA was used as a probe.

Blots were quantified by direct counting of beta emissions on a Betagen 603 betascope or autoradiographed.

**Results**

**Analysis of SERCA1 and SERCA2 mRNA Levels in Cardiac and Skeletal Muscle Cells**

To determine endogenous SERCA1 and SERCA2 gene expression levels, we determined their mRNA levels in cardiac and skeletal muscle cells using Northern blot analysis. The relative expression of SERCA1 and SERCA2 mRNA transcripts in different muscle cell types is shown in Fig. 2. The murine fast skeletal muscle cell line C2C12 equally coexpresses SERCA1 and SERCA2 after 72 hours in differentiation medium. The
cardiac and skeletal muscle cells. Embryonic chick heart fibroblast cultures (derived by differential plating of the embryonic heart digest) express SERCA2 message at a very low level. Beta emissions from blots were directly measured with a betascope and are presented quantitatively in the Table.

The same blots were probed with an α-actin cDNA that hybridizes to α-, β-, and γ-actin to assess the extent of cell differentiation. As expected, the skeletal muscle cell lines, embryonic cardiocytes, and adult myocardium all express a greater proportion of α-actin than β- and γ-actin. The embryonic chick heart fibroblast cultures, passaged twice and consisting of nonbeating cells, predominantly express β- and γ-actin.

Transgenic Transfection Analysis of SERCA2 Promoter Constructs in Embryonic Cardiocytes and Skeletal Muscle Cells In Vitro

To analyze promoter elements responsible for SERCA2 gene expression, a series of transient transfection assays were performed in vitro with fetal cardiocytes and skeletal muscle cell lines C1C12 and Sol 8. Four different deletion constructs of the SERCA2 gene, which encompassed sequences from −1110 to −72 relative to the transcription start site, were used. The pSV2CAT plasmid was cotransfected along with each SERCA2 promoter construct as a standard to correct for differences in transfection efficiency.

The results of transient transfections of embryonic cardiocytes and C1C12 and Sol 8 myotubes are displayed in Fig 3. A SERCA2 promoter fragment extending from −72 to +350 bp, which excludes the putative CCAAT box, is unable to increase luciferase activity significantly above the background obtained with the promoterless pXP2 plasmid. When a 5' flanking region of the gene extending to −284 is included in the construct, luciferase values are increased by 75-fold or more relative to the promoterless construct in cardiocytes and Sol 8 and C1C12 myotubes. The promoter construct −658 produces the highest luciferase activity in embryonic cardiocytes and Sol 8 myotubes. However, this SERCA2 promoter construct was less active in C1C12 myotubes, as evidenced by a 40% decrease in luciferase values. The promoter construct that extends −1110 nucleotides upstream from the transcription start site is significantly less active in all cell types.

Quantification of Northern Analysis of mRNA Levels

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Embryonic Cardiocytes</th>
<th>Embryonic Fibroblasts</th>
<th>Adult Myocardium</th>
<th>Sol 8 Myotubes</th>
<th>C1C12 Myotubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA2</td>
<td>0.40</td>
<td>0.15</td>
<td>57.0</td>
<td>4.80</td>
<td>4.80</td>
</tr>
<tr>
<td>SERCA1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>5.80</td>
<td>4.80</td>
</tr>
<tr>
<td>SERCA2/SERCA1+SERCA2</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.55</td>
<td>0.50</td>
</tr>
<tr>
<td>α-Actin</td>
<td>1.00</td>
<td>0.48</td>
<td>4.40</td>
<td>7.90</td>
<td>4.70</td>
</tr>
<tr>
<td>β-γ-Actin</td>
<td>0.74</td>
<td>1.50</td>
<td>1.31</td>
<td>3.43</td>
<td>2.10</td>
</tr>
<tr>
<td>α-Actin/total actin</td>
<td>0.59</td>
<td>0.27</td>
<td>0.77</td>
<td>0.70</td>
<td>0.69</td>
</tr>
</tbody>
</table>

SERCA indicates sarcoplasmic reticulum Ca$^{2+}$-ATPase. Values are background-subtracted and represent a single measurement. The units are arbitrary. Values for SERCA and actin cannot be compared directly as probes because each were not of identical specific activity.

The blot depicted in Fig 2 was quantified directly by counting beta emissions for 4° on a betascope.
**Analysis of SERCA2 Promoter Constructs In Vivo by Direct Injection of Plasmid DNA Into Adult Rat Myocardium**

To determine whether the same SERCA2 promoter regions are of functional importance in vivo, the same four SERCA2 promoter–luciferase reporter constructs were injected into the adult rat left ventricular apex. Relative promoter activities, normalized for differences in transfection efficiency, are presented in Fig 4. The region of the gene extending to −284 (from the transcription start site) is necessary to produce high luciferase activity, as observed in the in vitro experiments. Promoter constructs extending to −658 and −1110 (from the transcription start site) produce 73% and 62%, respectively, of the luciferase activity of the most active −284 construct. These in vivo DNA injection results are consistent with the in vitro DNA transfection analyses.

The most active SERCA2 promoter construct produced luciferase values (both in vitro and in vivo) that averaged 5% to 15% of the luciferase value obtained with the potent RSV viral promoter (data not shown). Transfection efficiencies were similar in all cell types as estimated by the CAT activity emanating from the control pSV2CAT plasmid.

**Discussion**

The present study was designed to identify the 5′ promoter regions critical for the transcriptional activity of the SERCA2 gene in cardiac muscle cells. To achieve this, we made serial 5′ deletions from the 1460-bp fragment of the rabbit SERCA2 gene that had previously been shown, in stable transfections, to be sufficient to activate transcription of the gene when C2C12 myoblasts differentiate into myotubes.16

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**Fig 3.** Transcriptional activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2)–luciferase constructs in fetal cardiocytes, Sol 8 myotubes, and C\(_2\)C\(_12\) myotubes. The luciferase values are presented as bar graphs showing the percent activity of the most active promoter construct. pXP2 indicates the promoterless luciferase vector. The luciferase values represent the mean±SEM from at least three separate experiments. The pSV2 chlornaphenicol acetyltransferase plasmid was used as internal control for transfection efficiency.

**Fig 4.** Transcriptional activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2)–luciferase constructs injected into adult rat myocardium. The luciferase value for each plasmid represents the mean±SEM from four to six animals. pXP2 indicates the promoterless luciferase vector. The luciferase values are presented as bar graphs showing percent activity of the most active promoter construct. SERCA2–luciferase activity from fetal cardiocytes is shown for comparison.
Using deletion constructs linked to a luciferase reporter plasmid in transient transfection assays, we were able to identify a region of the SERCA2 gene extending from the transcription start site to the -284-bp position as necessary and sufficient to promote transcription of the gene. This is true when the gene is studied in vitro in embryonic cardiocytes, during Sol 8 and CTC12 skeletal muscle myogenesis in vitro, and in adult myocardium in vivo. This region contains several common consensus sequences that are likely of importance. These include a TATAA-like GATAA box at -32 bp, a CCAAT box at -78 bp, and three Sp1 consensus sites (GGGCCG) at -122 bp, -185 bp, and -200 bp. These bind, respectively, the transcription factor II complex, the CTF family of factors, and the zinc finger protein Sp1, which have been previously shown to promote transcription of muscle and nonmuscle eukaryotic genes. Of note MEF-1, MEF-2, and M-CAT consensus sequences, which promote muscle-specific transcription of a number of genes in cardiac and skeletal muscle, are not present between -72 and -284 bp, the region of the SERCA2 gene that is critical for its transcription.

The upstream regions of the gene have differential effects in embryonic cardiocytes and Sol 8 myotubes compared with CTC12 myotubes. The region from -284 to -658 bp reproducibly causes a 40% reduction in transcription in CTC12 myotubes but a modest 15% to 25% increase in transcription in embryonic cardiocytes and Sol 8 myotubes. An explanation for this is not currently available. However, studies of other cardiac genes that are transiently expressed in fetal fast skeletal muscle, such as cardiac troponin T, have also found common and divergent cis-regulatory elements in cardiac versus skeletal muscle cells. During in vivo development of skeletal muscle, the SERCA2 gene expression is selectively downregulated in fast-twitch skeletal muscle but upregulated in slow-twitch muscle. Thus, sequences upstream from the -284 bp region may be responsible for the selective downregulation of this gene in fast-twitch skeletal muscle.

Our results from transient in vitro transfection assays are consistent with the previously reported study of the rat SERCA2 gene by Rohrer et al. Our study and theirs identifies -658 bp of the rabbit gene and -559 bp of the rat gene, respectively, as regions producing high-level transcriptional activity in cardiocytes. However, we did not attempt to isolate the effect of thyroid hormone (T3) on transcription of the rabbit SERCA2 gene. They reported that T3 increased SERCA2 transcription in neonatal cardiocytes in the presence of cotransfected T3 receptor expression vector and identify a minimal thyroid response element between -322 and -262 bp. Thyroid inducibility in the SERCA2 transient transfection assays was dependent on T3 receptor cotransfection. Since we did not cotransfect a T3 receptor in our experiments, it is likely that the transcriptional activity that we measure is not due to thyroid hormone. Given the high degree of conservation of nucleotides of the rat and rabbit genes of the proximal promoter region, it is likely that they share regulatory elements that would include thyroid response elements, the previously mentioned motifs, in addition to as-yet-unidentified elements.

Furthermore, the promoter activity is similar across species, including chicken, rat, and rabbit (data not shown). This supports the claim that these cis elements, particularly in the -284 to +1 region, are important across species for transcription of the SERCA2 gene. However, we cannot entirely eliminate the possibility that the differences in the upstream regions are in part due to species differences. This will require identification of the specific cis and trans elements that are operative in each species. However, this alternative explanation is unlikely to be the major cause of the differences. First, slow and fast skeletal muscle cell lines, each derived from the mouse, show discordance in the -658- to -284-bp region. Second, cardiocytes from different species (embryonic chick versus neonatal rat) produce similar luciferase activity with all constructs tested.

We also demonstrate that the -284-bp region that is required for transcription of the SERCA2 gene in vitro is also necessary and sufficient for transcription of the gene in adult myocardium in vivo. The initial reports of gene transfer into rat heart in vivo by direct injection of plasmid DNA demonstrated that the technique assesses transcription in adult cardiocytes, and not in nonmuscle cells, that is due to the unique ability of the cardiocytes to take up plasmid DNA. The method was subsequently used to demonstrate that the a-myosin heavy chain promoter, when injected in vivo, would behave in a tissue-specific and thyroid hormone–responsive manner, in concordance with previous in vitro studies. Subsequent reports of, for example, the myosin light chain-2 and the tropinin C genes have confirmed a concordance of promoter activity when studied by in vitro transfection and in vivo injection. We likewise have demonstrated in both embryonic cardiocytes in vitro and adult cardiocytes in vivo that a promoter construct extending to -72 bp is inadequate, whereas a promoter construct extending to -284 bp is necessary and sufficient to promote SERCA2 transcription. This concordance confirms that the -72- to -284-bp region contains cis elements critical for the transcription of the SERCA2 gene in both developing and mature cardiocytes.

In conclusion, we have identified a 284-bp proximal promoter region in the SERCA2 gene that is necessary and sufficient to promote transcription in cardiac cells in vitro and in vivo and in differentiating skeletal muscle cells in vitro. The region from -284 to -658 exerts a negative effect in the fast skeletal muscle cell line CTC12 but a positive effect in cardiocytes in vitro and the slow skeletal muscle cell line Sol 8 myotubes. This region may be responsible for the developmental downregulation of this gene in fast skeletal muscle. Future studies will use this report as a basis to define cis-acting elements that influence expression of the SERCA2 gene during in vivo physiological and hormonal stress of the heart and to more specifically define regulatory elements in the -284- to -72-bp region.

Acknowledgments

This study was supported by National Institutes of Health Grants PO1 39303 and PO1 HL-28001 (Dr Periasamy) and Grant HL-46034 (Dr Buttrick). Dr Periasamy is an Established Investigator of the American Heart Association. Dr Fisher was supported by a National Institutes of Health postdoctoral cardiovascular research training program.
thank Drs Norman R, Alpert, Martin M, Lewinter, and Leslie Leinwand for their advice and support of this work. We thank Matt Kaplan for his technical assistance, Junaid Shabbeer for critical reading of the manuscript, and Julie Lovelette and Caryn Miller for secretarial assistance.

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Circ Res. 1993;73:622-628
doi: 10.1161/01.RES.73.4.622

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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