Transcriptional Analysis of the Human Cardiac Calsequestrin Gene in Cardiac and Skeletal Myocytes*

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Calsequestrin is the main calcium-binding protein inside the sarcoplasmic reticulum of striated muscle. In mammals, the cardiac calsequestrin gene (casq2) mainly expresses in cardiac muscle and to a minor extent in slow-twitch skeletal muscle and it is not expressed in non-muscle tissues. This work is the first study on the transcriptional regulation of the casq2 gene in cardiac and skeletal muscle cells. The sequence of the casq2 genes proximal promoter (180 bp) of mammals and avians is highly conserved and contains one TATA box, one CArG box, one E-box, and one myocyte enhancer factor 2 (MEF-2) site. We cloned the human casq2 gene 5’-regulatory region into a luciferase reporter expression vector. By functional assays we showed that a construct containing the first 288 bp of promoter was up-regulated during myogenic differentiation of SO8 cells and had higher transcriptional activity compared with longer constructs. In neonatal rat cardiac myocytes, the larger construct containing 3.2 kb showed the highest transcriptional activity, demonstrating that the first 288 bp are sufficient to confer muscle specificity, whereas distal sequences may act as a cardiac-specific enhancer. Electrophoretic mobility shift assay studies demonstrated that the proximal MEF-2 and CArG box sequences were capable of binding MEF-2 and serum response factor, respectively, whereas the E-box did not show binding properties. Functional studies demonstrated that site-directed mutagenesis of the proximal MEF-2 and CArG box sites significantly decreased the transcription of the gene in cardiac and skeletal muscle cells, indicating that they are important to drive cardiac and skeletal muscle-specific transcription of the casq2 gene.

The sarcoplasmic reticulum (SR) is an intracellular organelle present in striated muscle cells, which has a key role on the regulation of the calcium concentrations during muscle contraction and relaxation (1). The SR is the main storage site of intracellular calcium, storing Ca\textsuperscript{2+} up to 20 mM while maintaining the free SR Ca\textsuperscript{2+} concentration at ~1 mM (2, 3). The SR storage capacity enables the muscle cell to continuously contract without diminishing the Ca\textsuperscript{2+} available for each contraction-relaxation cycle. Calsequestrin (CASQ) is the most abundant protein in the lumen of the SR; it has a high capacity to bind Ca\textsuperscript{2+} (40–50 mol of Ca\textsuperscript{2+}/mol of CASQ) with a moderate affinity (K\textsubscript{d} ~ 1 mM) and prevents the precipitation of Ca\textsuperscript{2+} inside the SR. It is a highly acidic protein with over 50 Ca\textsuperscript{2+} binding sites, which are formed by the clustering of two or more negatively charged residues. The molecular weight of the CASQ monomer is 40 kDa and is capable of polymerizing in response to increasing Ca\textsuperscript{2+} concentrations (>1 mM) (3). CASQ localizes near the ryanodine receptor and attaches to it, via direct interaction or through anchoring by triadin and junctin. It has been suggested that CASQ has a regulatory role in SR Ca\textsuperscript{2+} release, by inhibiting the ryanodine receptor through interactions via triadin/junctin at high luminal Ca\textsuperscript{2+} concentrations (1–2 mM) (4), although the exact mechanism and physiological role of this inhibition has not been established. Thus, in recent years it has become clearer that CASQ has a role beyond its capability to buffer Ca\textsuperscript{2+}.

In mammals two CASQ isoforms have been described, each one encoded by a different gene. In humans, the casq1 gene is located in chromosome 1q21, and encodes for the CASQ1 isoform, and the casq2 gene, located in chromosome 1p23, which encodes for the CASQ2 isoform. The adult fast-twitch skeletal muscle expresses exclusively the casq1 gene, whereas slow-twitch skeletal muscle expresses mainly the casq1 gene (~75% of total) and to a minor extent the casq2 gene (~25% of total). Cardiac muscle expresses exclusively the casq2 gene. Neither of the CASQ isoforms are present in non-muscle tissues nor in smooth muscle (5). Both human isoforms share a high nucleotide and amino acid homology, 84 and 80%, respectively (5). At this time there are no studies that point to differences in the physiological role of both CASQ isoforms, thus their roles shall be considered equivalent. The rabbit CASQ1 and the dog CASQ2 have been crystallized (6, 7); results of crystallization studies showed that the casq monomer was found to be constituted by three almost identical domains (I, II, and III) similar to that of Escherichia coli thioredoxin domain. It was found that CASQ polymerizes in response to rising concentrations of Ca\textsuperscript{2+} in the lumen of the SR, to form a homotetrameric complex (10 μM to 1 mM) and at higher concentrations of polymers, in concentrations higher than 10 mM the CASQ polymer dissociates from the Ca\textsuperscript{2+} releasing channel (8).

In the heart, the casq2 gene expresses during fetal development and continues to adult life. In fast-twitch skeletal muscle,
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CASQ2 is the predominant isoform during the fetal period, and during neonatal life. Afterward, there is a switch to CASQ1 gene expression, making the CASQ1 isoform the only one found in adult muscle. Although, in slow-twitch skeletal muscle there is also a switch on CASQ isoform expression during development, in the adult life the casq2 gene is still expressed (9). The CASQ2 mRNA levels have been measured in several heart pathological states such as cardiac hypertrophy, dilated cardiomyopathy, and heart failure, showing no changes on its expression level (10). Interestingly, in transgenic mice that overexpress CASQ2, it was observed that the mice develop cardiac hypertrophy and heart failure, with a typical fetal phenotype, associated with a higher Ca²⁺ storage capacity of the SR, as well as an impaired Ca²⁺ release, which leads to a diminished contractility (11). Another pathological state where CASQ2 is involved is the catecholaminergic polymorphic ventricular tachycardia (CPVT), where several mutations in the casq2 gene are present, inserting stop codons in the first exon of the gene, thus nullifying the expression of the casq2 gene. These patients have a morphologically normal heart but are susceptible to develop ventricular arrhythmias when exercise or another stressing event are present (12–15). Taken together, these findings are suggestive that the transcription of the casq2 gene is finely controlled during skeletal and cardiac muscle development, as well as in cardiac pathologies. Although the casq2 gene of rabbit and mouse have already been cloned (16, 17), there are no studies regarding transcriptional regulation of the casq2 gene.

The identification of cardiac-specific transcriptional regulatory elements and transcription factors controlling gene expression have been studied in vitro using the 5'-regulatory regions from contractile genes (α-myosin heavy chain, cardiac troponin I, ventricular myosin light chain 2), atrial natriuretic factor and brain natriuretic peptide (18–22). The studies have shown that GATA4, MEF-2, Nkx2.5, HAND1, HAND2, and serum response factor (SRF) are major regulatory factors responsible for cardiac-specific gene expression.

In this work, we report the first attempt to understand the molecular basis of tissue specificity of the casq2 gene. We cloned 3.2 kb of 5'-regulatory region of the human casq2 gene, generated deletion constructs and performed functional analyses. We performed targeted mutagenesis and DNA-protein binding analysis in cardiac myocytes and in the skeletal muscle cell line Sol8. We identified proximal and distal regulatory elements important for the expression of skeletal myotubes and neonatal cardiac myocytes.

Our efforts were directed to a highly conserved region among species located in the first 180 bp of the 5'-regulatory region of the casq2 gene, which contains one MEF-2, one E-box, and one CArG box putative binding sites. The results obtained in this work suggest that the proximal promoter is necessary and sufficient for cardiac and skeletal muscle expression, and that MEF-2 and SRF transcription factors participate for the tissue-specific expression of the casq2 gene.

### EXPERIMENTAL PROCEDURES

**Materials**—All restriction enzymes were acquired from Invitrogen and New England Biolabs. [γ-32P]ATP and [α-32P]dCTP were acquired from PerkinElmer Life Sciences. All DNA oligonucleotides were synthesized in the Molecular Biology Unit from the Instituto de Fisiología Celular, UNAM, México. Human heart total RNA was acquired from BD Biosciences.

**DNA Cloning**—A 3276-bp Stu1-Stu1 human genomic fragment from clone RP11-485H18 (BACPAC Resources), which contains 3102 bp of the 5'-regulatory region of the human casq2 gene and 176 bp of the 5'-nontranslated sequence of exon 1, was subcloned into a pGL3-basic plasmid previously cut with restriction enzymes NcoI, AflII, Stu1, using standard techniques. Five more constructs were generated by digestion with restriction enzymes NcoI, AflII, Pvull, Xhol, and BlpI generating fragments of 2322, 1169, 754, 254 bp, respectively.

**Oligonucleotides sequences for PCR and EMSA**

| Oligonucleotides | PCR              |
|------------------|------------------|
| GAPDH forward    | 5′-GGAGAACCCTCCCAATGAGATGAC-3′ |
| GAPDH reverse    | 5′-TGGATCTCCGCTTTGAACTG-3′    |
| CASQ2 forward    | 5′-AGCTTCTAGATGAGTTGAC-3′    |
| CASQ2 reverse    | 5′-GATCTCTAGATGAGTTGAC-3′    |
| MEF-2C forward   | 5′-AGCTTCTAGATGAGTTGAC-3′    |
| MEF-2C reverse   | 5′-GATCTCTAGATGAGTTGAC-3′    |
| Myogenin forward | 5′-GATACTGACCCTTCTCACTG-3′    |
| Myogenin reverse | 5′-GATCTGACCCTTCTCACTG-3′    |
| SRF forward      | 5′-GATTGCAGACTGCTCTCACTG-3′    |
| SRF reverse      | 5′-GATTGCAGACTGCTCTCACTG-3′    |

**Transfection and Reporter Assays**—The pGL3-hcasq2 constructs (0.8 μg) and the pRL-CMV (0.05 μg) were co-transfected into cells grown in 12-well plates using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. After
2 h the transfection mixture was replaced by DMEM with 3% HS to induce differentiation of Sol8 cells to myotubes; or by DMEM with 10% fetal bovine serum to maintain the myoblast phenotype, as well for C3H10T1/2 and neonatal rat cardiomyocytes. The positive control in all experiments was the pGL3-promoter, which contains the proximal 202 bp of the SV40 promoter. Following 48 h incubation at 37 °C and 5% CO2, the cells were lysed and assayed for firefly and Renilla luciferase, using the dual luciferase assay reagent kit (Promega) in a multiwell plate counter Wallac Victor2 luminometer (PerkinElmer Life Sciences). The results were normalized with the Renilla luciferase activity.

Stably transfected Sol8 cell lines were generated by co-transfecting the pGL3-hcasq2 constructs containing 288 and 3102 bp of the 5'-regulatory region of the human casq2 gene with pCDNA3.1, containing the resistance gene to the selecting antibiotic G418. The cells were grown in DMEM supplemented with 10% fetal bovine serum and 500 μg/ml G418, and selected after 1 week of growth. Afterward, colonies selected were tested for luciferase activity. The number of copies of the hcasq2/Luc fragment integrated into genomic DNA was verified by dot-blot analysis using a DNA probe for luciferase. The colonies selected were grown on 24-well plates until they reached 70% confluence; at that time the cells were induced to differentiate by replacing the media with differentiation media. Cells were lysed on a time course from myoblasts to 5-day myotubes; cell extracts were assayed for luciferase activity. The results were normalized dividing the luciferase activity by the protein concentration.

Real Time RT-PCR—Total RNA was extracted from Sol8 myoblasts and myotubes daily up to day 5 of muscle differentiation, with Trizol reagent (Invitrogen). RT was performed with 2 μg of total RNA using SuperScript III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer’s instructions, cDNA was diluted 15 times, and 9.2 μl/reaction were used for qPCR with SYBR Green ER qPCR Supermix for iCycler (Invitrogen). Results were analyzed according to the method suggested by Pfaffl (26), using GAPDH as loading control. Primers sequences are indicated on Table 1.

Mutagenesis—Site-directed mutagenesis of the proximal MEF-2, E-box, and CArG box sequences was conducted using the QuikChange site-directed mutagenesis kit (Stratagene) as indicated by manufacturer’s instructions, the MEF-2, E-box, and CArG box mutated oligonucleotides used are listed in Table 1. Mutation of the above elements was confirmed by DNA sequencing of the plasmids obtained.

Electrophoretic Mobility Shift Assay Studies—Nuclear extracts from Sol8 myoblasts and myotubes, and from neonatal rat cardiomyocytes were prepared as previously described (21). The sequence of the double-stranded DNA oligonucleotides were the same ones that were used for site-directed mutagenesis; consensus oligonucleotides for MEF-2 and CArG box used as specific competitors were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (see Table 1). Binding reactions were performed by preincubating at room temperature in 1 × Binding Buffer (Tris-HCl, 50 mM; glycerol, 20%; NaCl, 250 mM; MgCl₂, 5 mM; EDTA, 2.5 mM; dithiothreitol, 1 mM; phenyl-
methylsulfonyl fluoride, 1 mM), 10 μg of nuclear extract, and 1 mg of poly(dI-dC), 100-fold molar excess unlabeled competitor when needed, and γ-32P-labeled double-stranded DNA oligonucleotides. Supershift antibodies for MEF-2 (sc-313x) and SRF (sc-335x) were purchased from Santa Cruz Biotechnology.

Sequence Analysis—Sequence analysis was performed with MacVector 6.5.3 (Accelrys), BLAST (NCBI), and MathInspector (Genomatix).

Statistical Analysis—Values are expressed as means of at least 3 independent experiments ± S.E. Mean values were compared by analysis of variance (SPSS 11) applying the Bonferroni method for multiple comparisons. p values <0.05 were considered significant.

RESULTS

Characterization of the Human casq2 Gene 5′-Regulatory Region—The human genomic clones (RP11-485H8 and RP5-929G5) were analyzed in silico with DNA analysis software (NCBI Blast, Genomatix suite and MacVector) to determine the complete human casq2 gene structure and its 5′-regulatory region. The analyses revealed that both clones overlap and contain the complete casq2 gene, integrated with 11 exons and interrupted by 10 introns that span over 68 kb of the genome (Fig. 1A).

To determine the transcription initiation site of the human casq2 gene, primer extension analysis of human CASQ2 mRNA was performed using human heart poly(A) RNA. Two main extension products (358 and 348 bp) were obtained, mapping two transcription initiation sites at positions 241 and 251 bp upstream from the ATG translation initiation codon (Fig. 1B).

The 5′-regulatory region was analyzed with the MacVector program and Genomatix suite, to identify possible binding sites for transcription factors. In this analysis we identified a highly conserved DNA homology region among several species including the chimp (100%), mouse (95%), rat (98%), cow (93%), and chicken (87%), located between −30 to −140 bp that contains one imperfect TATA box, one MEF-2 site, one E-box, and one CArG box (Fig. 2).
Transcriptional Activity of the hcasq2 Gene 5′-Regulatory Region in Cardiac and Skeletal Muscle Cells—Six deletion constructs in pGL3-basic containing 3102, 2148, 1095, 580, 288, and 71 bp of the human casq2 gene 5′-regulatory region and 176 bp of 5′-nontranslated sequence of exon 1 were generated and used for transfection experiments, to identify the basal promoter region and upstream 5′-regulatory sequences of the human casq2 gene in neonatal rat cardiac myocytes and Sol8 skeletal muscle cells. The transcriptional activity of the different constructs was determined by transiently transfecting them into neonatal rat cardiomyocytes, the skeletal muscle cell line Sol8, and C3H10T1/2 cells. The results obtained by transient transfection in neonatal rat cardiac myocytes showed that the −288-bp construct had a transcriptional activity similar to the one observed with the pGL3-promoter construct (202 bp of the SV40 proximal promoter), which has been described as a strong promoter in muscle. The transcriptional activity observed with the −288-bp construct was 2-fold higher of that observed with the −71-bp construct, which contains only a TATA box, but no other regulatory elements. The −580 and −1095-bp constructs exhibited 2-fold higher activity compared with the −288-bp construct, the −2148 bp was 3.5-fold higher and the −3102-bp construct had the highest activity, increasing over 3.7-fold relative to the −288-bp construct (Fig. 3A). The promoter-less pGL3-basic plasmid showed only background firefly luciferase activity (Fig. 3A). In 2-day Sol8 myotubes, the highest transcriptional activity was observed with the −288-bp construct, showed 12-fold more activity than the −71-bp construct, and was similar to that observed with the SV40 promoter construct. The −3102-bp construct had 65% less activity compared with the −288-bp construct. The other intermediate length constructs showed similar activity than the −3102-bp construct. The −71-bp construct activity in myotubes was almost equal to the activity observed on undifferentiated myoblasts with all of the constructs, reflecting basal promoter activity. The pGL3-basic construct had only background activity. (Fig. 3B). In C3H10T1/2 fibroblasts, all the casq2 gene constructs showed only basal transcriptional activity, similar to the activity of the promoter less construct, whereas the SV40 promoter showed strong transcriptional activity (Fig. 3C).
To analyze the transcriptional activity of the \textit{casq2} gene regulatory region constructs during the course of myogenic differentiation, we generated stable transfectant Sol8 cell lines, which integrated into two cell genome of the chimeric \textit{casq2}/Luc constructs, of which, one contains 288 bp and the other contains 3.1 kb of the 5′-regulatory region. The resulting stable transfectant cell lines were induced to differentiate by serum withdrawal, and the luciferase activity was determined daily during 5 days of myogenic differentiation. The results indicated that both constructs have a very similar pattern of activation, showing increases in the activity level since day 1 of differentiation (Fig. 4A). The pattern of activation of the \textit{casq2} gene constructs was very similar to the pattern of increase of the endogenous level of \textit{casq2} mRNA expression determined by real time RT-PCR (Fig. 4B). The myogenin, MEF2C, and SRF mRNA levels were also quantified by real time RT-PCR. Myogenin mRNA increased since day 1, whereas MEF2C and SRF mRNA increased after 3 days of myogenic differentiation (Fig. 4C).

\textbf{The Proximal MEF-2 and CArG Box Sites Are Functional in Cardiac Myocytes—}Due to the results obtained by transcriptional activation of the chimeric \textit{casq2} gene constructs, which suggest an important role played by the first 288 bp of the 5′-regulatory region in cardiac and skeletal muscle myocytes, we determined the DNA-protein binding capabilities of the putative binding sites (MEF-2, E-box, and CArG box) present in this region. Because MEF-2, basic helix-loop-helix, and SRF transcription factors are present in Sol8 myotubes and have been demonstrated to participate in the regulation of several muscle genes, we decided to perform electrophoretic mobility shift assay studies using synthetic double-stranded DNA oligonucleotides and specific antibodies for MEF2 and SRF for supershift assays (see “Experimental Procedures”).

The results showed that the proximal \textit{casq2} gene MEF-2 site was capable of specifically binding a protein present in nuclear extracts of neonatal rat cardiomyocytes, which was of the same size to the one observed with a previously tested MEF-2 consensus oligonucleotide (Fig. 5A). The complex was not competed by the addition of 50–100-fold molar excess of the mutated \textit{casq2} MEF-2 oligonucleotide or the mutated consensus MEF-2 oligonucleotide. The protein contained in the complex was supershifted using a MEF-2 antibody (Fig. 5A). Nuclear extracts of Sol8 myoblasts and myotubes were also tested; both were capable of binding specifically the \textit{casq2} gene MEF-2 oligonucleotide, the signal obtained with myoblasts had
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FIGURE 5. DNA binding capabilities of the MEF-2 site and CARG box located on the first 140 bp. A, a double-stranded DNA oligonucleotide containing the casq2 gene MEF-2 site was incubated with nuclear extracts of neonatal rat cardiomyocytes. The DNA sequences of the oligonucleotides used are indicated in Table 1. The 32P-labeled wild type casq2 MEF2 oligonucleotide formed several DNA-protein complexes with nuclear extracts from cardiomyocytes (lane 1). To test specificity of the complexes observed, 50- and 100-fold molar excesses of the non-labeled wild type casq2 MEF2 oligonucleotide (lanes 2 and 3), consensus MEF-2 site (lanes 4 and 5), mutated casq2 MEF-2 site (lanes 6 and 7), and a mutated consensus MEF-2 site (lanes 8 and 9) were used. The complex formed was also incubated with a MEF-2 antibody (lane 10). B, the same experiment was done with nuclear extracts from Sol8 myoblasts (lanes 1 and 2) and 5-day myotubes (lanes 3–12). The myoblast-formed complexes (lane 1) were competed with a 100-fold excess casq2 MEF2-2 oligonucleotide (lane 2). The myotube complex (lane 3) was competed with 50- and 100-fold excess of unlabeled casq2 gene MEF-2 site (lanes 4 and 5), MEF-2 consensus (lanes 6 and 7), casq2 gene mutated MEF-2 (lanes 8 and 9), and consensus mutated MEF-2 (lanes 10 and 11). The complex was preincubated with a MEF-2 antibody (lane 13). C, the 32P-labeled wild type casq2 gene CARG box oligonucleotide formed several DNA-protein complexes with nuclear extracts from cardiomyocytes (lane 1). To test specificity of the complexes observed 50- and 100-fold molar excesses of the non-labeled wild type casq2 CARG box oligonucleotide (lanes 2 and 3), consensus CARG box site (lanes 4 and 5), and 100-fold mutated casq2 CARG box site (lane 6) were used. The competitor formed was also preincubated with a SRF antibody (lane 7). D, the same experiment was done with nuclear extracts from Sol8 myoblasts (lanes 1 and 2) and 5-day myotubes (lanes 3–12). The myoblast-formed complex (lane 1) was competed with a 100-fold excess casq2 gene CARG box oligonucleotide (lane 2). The myotube complex (lane 3) was competed with 50- and 100-fold excess of casq2 CARG box site unlabeled (lanes 4 and 5), CARG box consensus (lanes 6 and 7), casq2 gene mutated CARG box (lanes 8 and 9) consensus mutated CARG box (lanes 10 and 11). The complex was preincubated with a SRF antibody (lane 12). Arrows indicate specific complex positions and supershifted complex. Mb, myoblasts.

A lesser intensity than the one observed with myotubes (Fig. 5B). The complex formed was of similar apparent size to the one observed in cardiac cells. The complex observed with Sol8 nuclear extracts was also supershifted in the presence of the MEF-2 antibody (Fig. 5B).

The proximal casq2 gene CARG box was also assayed to test its capabilities of binding the transcription factors present in nuclear extracts of cardiomyocytes as well as Sol8 myoblasts and myotubes. The CARG box oligonucleotide formed a complex when incubated with cardiomyocytes nuclear extracts (Fig. 5C). This complex was specifically competed with the addition of the wild type CARG box casq2 (50–100-fold molar excess) unlabeled probe, or a previously tested consensus CARG box oligonucleotide, whereas it did not change by the addition of the mutated casq2 gene CARG box oligonucleotide. The protein present in this complex was identified by supershift assay, using a SRF-specific antibody (Fig. 5C). We also assayed the binding capabilities with Sol8 myoblasts and myotube nuclear extracts. The results showed a complex of similar apparent size to the one observed in cardiomyocytes; it also was a specific complex and was supershifted with a SRF antibody. The complex produced by myoblast nuclear extracts was of lesser intensity than the one observed with myotubes (Fig. 5D).

The proximal casq2 E-box site was also assayed to determine its binding properties. When incubated with cardiomyocytes or Sol8 myoblasts and myotubes nuclear extracts, no DNA-protein complex was observed. The same nuclear extracts were assayed with a consensus E-box sequence (5’-CANNNTG-3’) and a specific complex was observed (data not shown).

Functional Properties of the MEF-2 and CARG Box Sites—To further understand the role of the MEF-2, E-box, and CARG box sites in the regulation of the human casq2 gene, we performed site-directed mutagenesis as described under
We performed the same experiment in Sol8 myoblasts (white bars) and myotubes (black bars) (Fig. 6B). The results showed that in myoblasts the level of transcriptional activity of all the casq2 gene constructs was low and minimally affected by the mutation of the MEF2 CArG box sites. In myotubes the activity of the −288-bp construct is high, we observed a similar behavior to the one seen in cardiomyocytes, with a reduction of 70% in activity with mMEF-2 construct, a 60% reduction with the mCArG box construct, and an 80% reduction when both the double mutated mMEF-2 and mCArG box construct were assayed. Both cardiomyocytes and Sol8 myotubes, when transfected with the −288-bp construct containing the mutated MEF-2 and CArG box sites, exhibited very low transcriptional activity, quite similar to that observed with the −71-bp construct, which has only basal transcriptional activity.

**DISCUSSION**

Calsequestrin has a major role to maintain the calcium homeostasis in the striated muscle cells, also the data regarding its specific tissue expression and absence of changes on its expression associated with pathological conditions, evidences the existence of a fine regulation of its expression. The results presented in this work are the first attempt to understand the transcriptional regulation of the expression of the casq2 gene.

The DNA sequence analysis of the human casq2 gene 5′-regulatory region, compared with the regulatory region of this gene in other species (chimp, mouse, rat, cow, dog, and chicken), reveals that the only region that has a high homology among them is contained within the −30 to −140-bp region upstream from the main transcription initiation site. Upstream from this region only a few small isolated sequences (20–30-bp long) display homology among mammalian casq2 genes, but not with the chicken casq gene (data not shown). This observation is also relevant when one considers that avians only have one casq gene, which is expressed in fast- and slow-twitch skeletal and cardiac muscles. Also, this highly conserved proximal promoter region contains one MEF2, one E-box, and one CArG box putative DNA that are of relevance for gene expression in skeletal and cardiac muscle cells.

The functional results we report demonstrate that the first 288 bp of the human casq2 gene 5′-regulatory region are capable of directing the expression of the casq2/Luc constructs in neonatal rat cardiomyocytes and myotubes of the mouse Sol8 cell line; but not in C3H10T1/2 fibroblasts. The results also showed that the −288-bp casq2 gene construct drive a similar expression in Sol8 myotubes and in neonatal rat cardiomyocytes. The construct containing 3.1 kb of 5′-regulatory region increased transcriptional activity relative to the 288-bp construct (4-fold) in cardiac myocytes. In contrast, in Sol8 myotubes the 288 bp had the higher transcriptional activity, which is similar to the activity of the SV40 promoter construct, but constructs containing longer sequences showed 50–60% less transcriptional activity. The above results agree with the casq2 mRNA and protein tissue expression pattern, where CASQ2 has been shown to be abundantly expressed in cardiac muscle, but only to a minor extent in slow-twitch skeletal muscle (17, 27). These results also demonstrate that the first 288 bp are necessary and sufficient to confer a striated muscle-specific

“Experimental Procedures” of the sites previously tested by electrophoretic mobility shift assay.

The four mutated constructs containing the mutations of the MEF-2 site (mMEF-2), E-box (mE-box), CArG box (mCArG box), and MEF-2 and CArG box (mMEF2 + mCArG-box), were transiently transfected into Sol8 myoblasts (white bars) and assayed 48 h later, and into myoblasts induced to differentiate for 48 h with DMEM supplemented with 3% HS (black bars) and assayed 48 h later. Results are expressed in percentage change of the activity observed with the wild type −288 bp construct. Mean ± S.E.
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expression, and that DNA elements located between 1.1 and 2.14 kb may function as an enhancer with specific activity in cardiac myocytes, and may have a negative role in the regulation of transcription in skeletal muscle. The results obtained with the stable transfected Sol8 cell lines showed a similar pattern of induction of transcriptional activity of the −288- and −3102-bp casq2 gene constructs during skeletal muscle differentiation, which also followed a similar pattern to the level of expression of endogenous casq2 mRNA. Interestingly, the expression of MEF-2C and SRF mRNA was induced during Sol8 muscle differentiation increasing from myoblasts to myotubes.

By DNA sequence analysis of the highly conserved casq2 gene proximal 5′-regulatory region, we identify three putative binding sites for transcription factors relevant in the regulation of cardiac and skeletal muscle-specific genes (MEF-2, E-box, and CArG box). The proximal putative MEF-2 and E-box elements share high homology with the consensus sequences of cardiac and skeletal muscle-specific genes (MEF-2, E-box, CArG box). The proximal putative MEF-2 and E-box elements share high homology with the consensus sequences for these sites (17). Although, the CArG box sequence present in the casq2 gene (CC(A/T)GG), is not a canonical site (CC(A/T)6GG), previously a CArG box site with the same configuration of the one present in the casq2 gene regulatory region has been described and demonstrated to be functional in the cardiac α-myosin heavy chain gene (21). Therefore, we further analyzed the role of these muscle regulatory elements on the regulation of the casq2 gene. The results demonstrate that the MEF-2 and CArG box sites are capable of binding the MEF-2 and SRF, respectively; whereas the E-box site did not bind any protein present in the nuclear extracts of cardiomyocytes, C3H10T1/2 cells, as well as Sol8 myoblasts and myotubes.

Because of these results, we decided to focus our work on analyzing the function of the proximal MEF-2, E-box, and CArG box sites, to understand their role in the tissue-specific regulation of the casq2 gene. We generated mutants for these three sites by site-directed mutagenesis. The functional studies demonstrate that the MEF-2 and CArG box mutations decrease the transcriptional activity of the −288-bp human casq2 gene construct, 70 and 40%, respectively. The DNA protein binding studies confirmed that the mutated sites were unable to bind MEF-2 and SRF. However, mutation of the putative E-box site did not change the transcriptional activity confirming the results showing the absence of protein binding. The mutation of both the MEF-2 site and CArG box on the same construct decreases the transcriptional activity to basal level on both cell types (cardiac, skeletal), similar to that observed with the −71-bp construct, which is devoid of both elements.

The results indicate that MEF-2 and SRF have a very important role in regulating the expression of the casq2 gene in skeletal and cardiac muscle. Mutation of both sites leaves only basal promoter activity, which may be directed by the TATA-like box present in this region. We must also consider that DNA elements present upstream from −288 to −580 bp and from −1095 to −3.1 kb have a significant role in regulating the expression of the casq2 gene in cardiac muscle, where these regions show an increase of the activity compared with the −288-bp construct. DNA sequence analysis of the −288 to −3.1 kb region shows the presence of several putative MEF-2, E-box, NFAT, and GATA-4 binding sites (data not shown); the transcription factors that bind to these sites have been mentioned in the regulation of several cardiac and skeletal muscle genes. The presence of several E-box sites in this region may play an important role, because previously it has been reported by DNA microarray analysis that the casq2 gene is regulated in skeletal myotubes by myogenin (28).

In this study, we show that the transcription factors MEF-2 and SRF play a significant role in the regulation of the casq2 gene. However, it is difficult to assume that these are the only factors involved in its regulation because it is well documented that both MEF-2 and SRF are activated via Ca2+-regulated pathways, like calcineurin for MEF-2 (29–31) and CaM kinase II for SRF (32, 33). When this data is considered along with previous reports that show no changes on the expression of CASQ2 in cardiac pathological states, which imply abnormal intracellular Ca2+ concentrations, it suggests that non-Ca2+-regulated pathways may be involved in the regulation of the expression of the casq2 gene (2, 10). Therefore, we believe that other transcriptional mechanisms must be involved in the regulation of the expression of casq2 gene, and have to be studied in more detail.

The transcriptional regulation of the casq2 gene is similar to previous publications on cardiac-specific genes like the cardiac troponin C, cardiac α-actin, and cardiac α-myosin heavy chain genes. The regulatory mechanisms for these genes consist of a basal promoter with the proximal regulatory region activating the transcription in the adult slow-twitch skeletal muscle and cardiac muscle, being sufficient to have striated muscle-specific expression (21). Also in genes with cardiac-specific expression, in the distal regulatory region are enhancers that confer cardiac expression (21, 34, 35). Recently, it was proposed that a larger enhanceosome complex mediates the cardiac-specific expression, where several transcription factors like GATA4, SRF, MEF-2, NFAT, and HAND2, interact with each other through indirect mechanisms that involve transcriptional scaffolding molecules. The co-activator p300/CBP has been proposed as one of the molecules involved in the formation of the cardiac-specific enhanceosome (29, 36, 37).

The MEF-2 and SRF factors belong to the MADS family of transcription factors, and are capable of interacting with a large number of transcription factors and cofactors through the MADS DNA binding domain. MEF-2 has been mentioned several times as regulating the transcription of cardiac, skeletal, and smooth muscle genes (38, 39). The SRF factor has been particularly involved in the regulation of smooth muscle genes, although it has also been associated with some cardiac and slow-twitch skeletal muscle genes (40–42). Both factors have the possibility of acting as positive or negative regulators when they bind to DNA, which is dependent of the recruitment of positive or negative transcriptional co-regulators. In recent years the myocardin family of transcription factors, especially myocardin-related transcription factors A/B, have been associated with the regulation of cardiac genes (43–46), by interactions with the SRF, potentiating the effect of these factors. Thus, it is possible that some of these cofactors contribute with SRF to regulate expression of the casq2 gene. The MEF-2 factor has been mentioned to interact with basic
helix-loop-helix transcription factors (47, 48), even when the E-box site present on the first 140 bp did not show binding capabilities, it is still possible that basic helix-loop-helix factors acting as cofactors of MEF-2 and are involved in regulating the casq2 gene expression.

In summary, in this work we have demonstrated that the 5′-regulatory region of the human casq2 gene has a high homology with other species on the first 180 bp, where only one MEF-2 and one CArG box binding sites are present. These sites are functional and interact with MEF-2 and SRF transcription factors present in cardiac myocytes and Sol8 myotubes. Mutations of the MEF2 or CArG box sites leads to a diminished transcriptional activity in neonatal rat cardiomyocytes and Sol8 myotubes in culture. Remarkably, when both sites are mutated simultaneously the transcriptional activity of the −288-bp construct decreases to basal level, indicating that the MEF2 and CArG box sites are necessary for transcription of the casq2 gene. We demonstrated that the first 288 bp of the 5′-regulatory region of the casq2 gene are necessary and sufficient to regulate the expression in skeletal myotubes and cardiomyocytes, but not in non-muscle cells. The results suggest that, the regions between −288 and −580 bp and from −1095 to −3102 bp have a role as cardiac-specific enhancers, whereas in skeletal muscle do not play a positive role, but possibly a negative regulatory role. Further studies are still needed on the distal regulatory elements to have a more complete understanding of the muscle-specific transcriptional regulation of the casq2 gene.

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