The Role of GATA, CArG, E-box, and a Novel Element in the Regulation of Cardiac Expression of the Na⁺-Ca²⁺ Exchanger Gene*

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The cardiac Na⁺-Ca²⁺ exchanger (NCX1) is the principal Ca²⁺ efflux mechanism in cardiocytes. The exchanger is up-regulated in both cardiac hypertrophy and failure. In this report, we identify the cis-acting elements that control cardiac expression and α-adrenergic up-regulation of the exchanger gene. Deletion analysis revealed that a minimal cardiac promoter fragment from −184 to +172 is sufficient for cardiac expression and α-adrenergic stimulation. Mutational analysis revealed that both the CArG element at −80 and the GATA element at −50 were required for cardiac expression. Gel mobility shift assay supershift analysis demonstrated that the serum response factor binds to the CArG element and GATA-4 binds to the GATA element. Point mutations in the −172 E-box demonstrated that it was required for α-adrenergic induction. In addition, deletion analysis revealed one or more enhancer elements in the first intron (+103 to +134) that are essential for phenylephrine up-regulation but bear no homology to any known transcription element. Therefore, this work demonstrates that SRF and GATA-4 are critical for NCX1 expression in neonatal cardiomyocytes and that the −172 E-box in addition to a novel enhancer element(s) are required for phenylephrine up-regulation of NCX1 and may mediate its hypertrophic up-regulation.

The Na⁺-Ca²⁺ exchanger (NCX1) catalyzes the electrogenic exchange of one intracellular calcium ion for three extracellular sodium ions across the plasma membrane in many mammalian cells. Transport is reversible and can facilitate calcium entry, which in the heart is capable of triggering calcium release from the sarcoplasmic reticulum (1). The exchanger is most abundant in the heart, where it regulates Ca²⁺ fluxes across the sarcolemma and serves a critical role in the maintenance of the cellular calcium balance for excitation-contraction coupling.

Na⁺-Ca²⁺ exchanger activity in cardiomyocytes is regulated by several factors. It is activated by cytosolic Ca²⁺ and MgATP (2) and inhibited by cytosolic sodium (3) and ATP depletion (4). A high affinity Ca²⁺-binding domain has been identified in the large cytoplasmic loop (residues 371–508) that is believed to be responsible for calcium regulation (5). It is also inhibited by the exchanger inhibitory peptide, which corresponds to a 20-amino acid segment at the N terminus of the large cytoplasmic loop (6). A recent study has demonstrated that the exchanger is phosphorylated via a protein kinase C-dependent pathway and that NCX1 phosphorylation appears to coincide with up-regulation of exchanger activity (7).

In addition, the exchanger is regulated at the transcriptional level in cardiac hypertrophy, ischemia, and failure. In the feline model of acute right ventricular hypertrophy, NCX1 message levels are rapidly up-regulated following pressure overload (8, 9). An increase in NCX1 mRNA expression is also observed in cultured cardiac myocytes following α-adrenergic stimulation by phenylephrine or exposure to verapamil. Importantly, the exchanger is also up-regulated at both the message and protein levels in end-stage heart failure (10). Very little is known about the genetic elements and transcription factors that regulate NCX1 expression. Identification of the factors involved in NCX1 up-regulation is important to unraveling the sequence of molecular events that initiate hypertrophic growth. Furthermore, it may provide insight into the basis of the development of decompensated heart failure.

The feline (11), human (12, 13), and rat (13, 14) NCX1 genes have recently been cloned. The NCX1 gene is unusual in that it contains three promoters and multiple 5′-untranslated region exons upstream of the coding region. As a result of alternative promoter usage and the resulting alternative splicing, there are multiple tissue-specific variants of the Na⁺-Ca²⁺ exchanger (11, 15–17). The feline cardiac minimal promoter (−184 to +172) is responsive to α-adrenergic stimulation and sufficient to drive expression of a reporter gene in neonatal cardiomyocytes but not mouse L cells (18). Analysis of the DNA sequence of the feline cardiac basal NCX1 promoter revealed a number of elements that may be involved in regulation and are conserved in the rat promoter (14). There are two CANNTG motifs (E-boxes) at positions −172 and −153 that are potential target sites for the basic helix-loop-helix family of transcription factors. E-box-binding proteins have been demonstrated to mediate the cardiac expression of several genes including the ventricular myosin light chain 2 (19), cardiac α-actin (20), and α- and β-myosin heavy chain (21). This region also contains consensus sequence for two GATA boxes at positions −125 and −50. Several cardiac specific genes such as myosin light chain IA, myosin light chain IV, and β-myosin heavy chain (22, 23) contain conserved GATA binding motifs. The GATA elements in the atrial natriuretic peptide (24) and α-myosin heavy chain (25) gene have been shown to be critical for cardiac expression. This region also contains a single MEF-2 element at position −166. A MEF-2-like motif appears to be required for cardiac-specific expression of the rat cardiac troponin T gene. There are six Nkx-2.5 binding sites in the first 1831 bases of the NCX promoter including one in the first 250 bases. The cardiogenic
homeodomain factor Nkx-2.5 has been shown to be expressed in early cardiac cell progenitors and plays an important role in cardiac development. A single CARG element is present at position −80. A CARG element (CC(A/T)6GG) is also present in the 5′-flanking region of the cardiac α-actin, skeletal α-actin, β-actin, α-myosin heavy chain, cardiac myosin light chain 2, and troponin T genes (25–29). The CARG elements of skeletal and cardiac α-actin are very homologous to the serum response element and serve as a binding site for the nuclear serum response factor (SRF). In the present study, we perform a detailed analysis of the NCX1 promoter elements important for neonatal cardiomyocyte expression and α-adrenergic induction. We present three main findings. First, expression of NCX1 in neonatal cardiomyocytes requires both the CARG element at −80 to −71 and the GATA element at −50 to −45. Second, electrophoretic mobility shift analysis revealed specific DNA–protein complexes for both of these elements. SRF is one of the factors binding to the CARG element, and GATA-4 binds to the GATA element. Third, mutagenesis and deletion analysis revealed a E-Box at position −172, and an additional enhancer element or elements in the first exon-intron boundary (+103 to +134), which are essential for α-adrenergic induction of the exchanger. This region shows no homology to any of the known transcription elements.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes and DNA-modifying enzymes were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). QuikChange site-directed mutagenesis and luciferase reporter kits were from Stratagene (La Jolla, CA). Eagle’s minimum essential medium (MEM), Hanks’ balanced salt solution, horse serum, and newborn calf serum were all from Life Technologies, Inc. Antibodies for supershift assays were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). The AmpliCycle sequencing kit was obtained from Perkin-Elmer (Foster City, CA). All common reagents were of the highest quality and were purchased from either Fisher or Sigma.

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**RESULTS**

In our initial characterization of the NCX1 cardiac promoter (11), we demonstrated that a construct containing the first 250 bp of the 5′-flanking region, the H1 exon, and 67 bp of the first intron is sufficient for cardiac-directed expression and α-adrenergic stimulation of the luciferase reporter gene. We have since shown that a construct containing only 184 bases of the 5′-flanking region has the same activity as the 250-bp construct (18). This is also in agreement with what has been reported for the rat NCX1 minimal promoter (14). There are consensus sequences for a number of potential DNA-binding factors in the NCX1 cardiac minimal promoter (Fig. 1). There are two potential binding sites for the GATA family of zinc-fingered transcription factors (A/TGATAA/G) and two CANTG motifs (E-boxes) that are potential target sites for the basic helix-loop-helix family of transcription factors. This region also contains a single MEF-2 element, a CARG element, and a binding site for the cardiogenic homeodomain factor Nkx-2.5. It is of interest to note that sequence of both GATA elements, the CARG element, MEF element, and the −153 E-box are perfectly conserved in both the feline and rat NCX1 promoters (14). Using the full-length (1831-bp) construct we introduced site-specific point mutations into these three elements, and the activity of each of the mutants was compared with the wild-type full-length construct to determine its contribution to cardiac specific expression (Fig. 2). Each NCX1 mutant promoter construct was transfected in triplicate in at least three independent neonatal cardiomyocyte preparations. Point mutations within the −166 MEF-2 and −10 Nkx-2.5 elements resulted in

**Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from neonatal rat ventricles as described (35). Briefly, 20 hearts from 1–4-day-old neonatal rats were digested in ice-cold phosphate-buffered saline. 5 ml of NE1 buffer (250 mM sucrose, 15 mM Tris-HCl (pH 7.9), 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 25 mM KCl, and 2 mM MgCl2) was added to the tissue, which was immediately homogenized and filtered through two layers of cheese cloth. Nonidet P40 was added to the homogenate to a final concentration of 0.5%. Following five more strokes with a Dounce homogenizer, the homogenate was centrifuged at 1000 × g for 10 min at 4°C. Nuclei were then washed with 5 ml of NE1 buffer and centrifuged as above. The pellet was resuspended in 1 packed cell volume of NE1 buffer containing 350 mM KCl followed by another wash with a homogenizer. The homogenate was centrifuged at 12,000 × g for 5 min at 4°C to eliminate the large cell debris fraction. The nuclei were washed again with 1 ml of NE1 buffer and centrifuged at 80,000 × g for 90 min at 4°C. The supernatant was dialedyzed for 1 h to overnight at 4°C against dialyzing buffer (50 mM KCl, 4 mM MgCl2, 20 mM KPO4 (pH 7.4), 1 mM β-mercaptoethanol, 20% glycerol). After enriching for DNA-binding proteins on heparin-Sepharose CL-6B, the supernatant was stored at −80°C. Nuclear extract (5 μg) was incubated in the presence of 50 μg/ml poly(dG-dC) in binding buffer (50 mM NaCl, 0.1 mM EDTA, 20 mM Hepes (pH 7.9), 0.5 mM dithiothreitol, 10% glycerol). After 20 min of incubation at room temperature, the samples were loaded on 6% polyacrylamide gels and electrophoresed in 0.5% TBE at 10 V/cm of gel at 4°C. The gels were dried and exposed to an x-ray film. For oligonucleotide competition or antibody supershift assays, nonradioactive oligonucleotides or antibodies were added to the reaction mixture and incubated for 10 min prior to the addition of the radiolabeled probe (for a complete listing of the oligonucleotide probes used in these studies, see Table I).
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response to increased contractile activity. Moreover, this E-box has been shown to bind a cardiomyocyte nuclear protein antigenically related to upstream stimulatory factor 1 (USF-1) (34). Gel supershifts were performed to determine if USF-1 is a part of the NCX1 –172 E-box binding complex. Incubation of the DNA-nuclear protein complex with 2 μg of USF-1 antibody did not result in a supershift (Fig. 3B). In addition, the formation of this complex was not altered by incubation with antibodies against either of the widely expressed basic helix-loop-helix factors E12 or E47 (Fig. 3B). Therefore, the NCX1 –172 E-box element-nuclear protein complex does not include detectable amounts of E12, E47 or USF-1.

Incubation of nuclear extracts from neonatal rat hearts with the \(^{32}\)P-labeled oligonucleotide for the NCX1 –80 CArG element resulted in the formation of two specific protein-DNA complexes (Fig. 4). Competition with a 100-fold excess of the unlabeled 43-mer probe or a shorter 22-mer containing the NCX1 CArG sequence completely eliminated both of these complexes, whereas competition with a 100-fold excess of unlabeled mutant CArG sequence did not affect the binding of the probe to either of these complexes. In addition, incubation with non-specific competitor DNA (100-fold E-box element) did not compete for binding to either complex. The ubiquitous SRF, which recognizes a CArG element in the cardiac α-actin promoter (35), may be involved in the cardiac specific transcription of NCX1. To determine whether SRF actually binds to the NCX1 CArG element, a supershift assay was performed with anti-SRF polyclonal antibody. Incubation of the DNA-nuclear protein complex with 2 μg of SRF antibody showed a definitive supershift, demonstrating that at least one of the components is SRF or is antigenically related to SRF (Fig. 4, lane 6).

Incubation of nuclear extracts from neonatal cardiomyocytes with a \(^{32}\)P-labeled GATA probe (–68 to –29), revealed a single protein-DNA complex (Fig. 5). Competition with 100-fold excess of unlabeled mutant GATA sequence or nonspecific competitor DNA (E-box (–172)) did not affect the complex; however, competition with a 100-fold molar excess of an unlabeled GATA probe completely eliminated the complex (Fig. 5A). Both GATA-4 and GATA-6 are expressed in the adult heart. To determine if GATA-4 and/or GATA-6 was present in the NCX1 –50 GATA sequence-specific interaction, GATA-4 and GATA-6 antibodies were incubated with the nuclear protein-DNA complex and examined by gel shift analysis. The GATA-4 antibody clearly supershifted the –50 GATA complex (Fig. 5B). No supershift was detected with the GATA-6 antibody (data not shown), indicating that GATA-4 but not GATA-6 interacts with this site.

Identification of Elements Responsive to α-Adrenergic Stimulation—In order to identify elements mediating α-adrenergic up-regulation, constructs containing mutations in the putative elements in the minimal promoter were transfected into neonatal rat cardiomyocytes treated with phenylephrine. Phenylephrine treatment induced an approximately 2-fold increase in luciferase activity over that of untreated (11) or verapamil-treated cardiomyocytes transfected with the 1831-bp full-length wild-type NCX1 luciferase construct (Fig. 6). Fig. 6 demonstrates that mutations in the –10 Nkx, –166 MEF, –153 E-box, and –125 GATA elements did not affect α-adrenergic stimulation. Each shows a 1.8–2-fold induction of luciferase activity over that of untreated (11) or verapamil-treated cardiomyocytes treated with phenylephrine for 16 h.

Fig. 1. Partial nucleotide sequence of the cardiac NCX1 promoter sequence. Shown are the H1 exon (uppercase) along with a 361 bp of 5′-flanking sequence and the first 67 nucleotides of the first intron. The cardiac minimal promoter (–184 to +172) is contained within this sequence. Numbering is relative to the transcriptional start site represented by the asterisk. Putative cis-acting regulatory elements are underlined. The region contains two E-box elements, two GATA elements, a MEF-2 recognition sequence, a CArG element, and one Nkx2.5 element. The sequence for the first four exons and the three promoters of the feline NCX1 gene has been previously published (11) and has been submitted to GenBank™ with accession numbers U67072–U67075.

Tissue. Oligonucleotide probes were generated for the 5′-flanking sequence and the first 67 nucleotides of the first intron (Fig. 1). The region contains two E-box elements, two GATA elements, a MEF-2 recognition sequence, a CArG element, and one Nkx2.5 element. The sequence for the first four exons and the three promoters of the feline NCX1 gene has been previously published (11) and has been submitted to GenBank™ with accession numbers U67072–U67075.

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20-fold lower than wild type) mutant constructs. However, an NCX1 construct with the 2172 E-box sequence mutated yielded only a 1.2-fold increase in luciferase activity when treated with phenylephrine (Fig. 6). This is significantly less induction than what is seen in the wild type NCX1 construct. Therefore, the NCX1 2172 E-Box element appears to be required for α-adrenergic up-regulation. This is similar to the α–MHC promoter in which an E-box element was demonstrated to be responsible for up-regulation in response to increased contractile activity (34).

Each of the above constructs contained the entire first exon (H1) and 67 bases of the first intron. Constructs in which luciferase was fused at position +22 of the H1 exon had only 20% of control activity. More importantly, these constructs did not show any up-regulation in response to phenylephrine treatment (data not shown). Therefore, in addition to the -172 E-box, one or more elements within the H1 exon or the first 67 bases of intron 1 appear to be required for α-adrenergic stimulation of the NCX1 gene. A series of deletions were made to identify the region responsible for α-adrenergic stimulation. Deletion of the last 13 bases of the first exon and the first 67 bases of intron sequence (D94–172) also resulted in a construct with low activity and insensitivity to PE stimulation, indicating that the elements responsible for α-adrenergic stimulation are located in this region (Fig. 7).

Analysis of the first intron sequence revealed a single GATA element at +135. Since GATA-4 has been recently demonstrated to play an important role in the hypertrophic responsiveness of both β-MHC and angiotensin IIα-receptor promoters (36, 37), mutations were introduced into the consensus +135 GATA element (Fig. 7). PE

### Table 1

**Oligonucleotide probes**

| Probe                     | Sequence                                                                 |
|---------------------------|--------------------------------------------------------------------------|
| E-box 151 (−189), 43-mer  | 5′-GATCGCGTGGAGGATCTAGCTACATGTTTATGGGCTCAT-3′                            |
| E-box 151 (−189), 43-mer  | Mutant GT A                                                               |
| GATA 29 (−68), 44-mer     | 5′-GATCCGGAACGAAAGCCGACAGATAAGCATGCTAT-3′                                |
| CARG 55 (−93), 43-mer     | Mutant CARG                                                               |
| CARG, 22-mer              | 5′-GATCGGGAACAGATAAGCATGCTATGGAAGGAAAGGCGAGAAAG-3′                       |
| MEF2, 20-mer              | 5′-GATCTCGATTTTTATCGTC-3′                                                |
| Novel element             |                                                                          |
| Region +89 to +128, 44-mer| 5′-GATCCGAGCTGTTGAAAGCAGACATGCTATGGAAGGAAAGGCGAGAAAG-3′                  |
| Region +89 to +128        | Mutant ACGT                                                               |
| Region +103 to +117, 19-mer| 5′-GATCGGAGCTGTTGAAAGCAGACATGCTATGGAAGGAAAGGCGAGAAAG-3′                  |

**Figure 2**

Effects of mutations of transcriptional elements on expression of the NCX1 gene. Upper panel, a diagram of the promoter-proximal sequences and the mutated bases (below) for each transcriptional element. Point mutations were created in the full-length, 1831-bp NCX1 promoter-luciferase construct. Lower panel, relative luciferase values for wild type and mutant constructs transfected into neonatal rat cardiomyocytes (n = 3 separate experiments performed in triplicate). Individual constructs were cotransfected with a cytomegalovirus promoter-driven β-galactosidase fusion vector to normalize transfection efficiency. The relative luciferase values were then normalized to the wild-type NCX1 construct, and the average was reported as a percentage of the wild-type value.
treatment stimulated reporter gene expression approximately 2-fold, indicating that the +134 GATA element was not required for α-adrenergic up-regulation. A deletion from +94 to +119 had low activity and was recalcitrant to PE stimulation, but the smaller deletion from +94 to +103 was still responsive to PE stimulation. In order to further define this element or elements present in the first intron. A 100-fold molar excess of unlabeled CARG probe to verify that the interactions were specific to the CARG element. The labeled probes were 43-mers, and the unlabeled competitors were 20-, 22-, 43-, or 44-mers. Supershift analysis was performed by incubating the reaction mixture with 2 μg of SRF antibody.

FIG. 3. Gel mobility shift assay for E-box −172. Neonatal rat heart extract (20 μg) was probed with 32P-labeled double-stranded oligonucleotides containing the NCX1 −172 E-box sequence and its immediate flanking sequence. A 100-fold molar excess of unlabeled −172 E-box probe was used as a specific competitor to the labeled probe. Extracts were also probed using a molar excess of −166 MEFP2, −51 GATA, and mutant −172 E-box probe to verify that the interactions were specific to the E-box element. All probes and oligonucleotides used for competition experiments are listed in Table I. The labeled probes were 44-mers, and the unlabeled competitors were 20-, 22-, 43-, or 44-mers. Supershift analysis was performed by incubating the reaction mixture with 2 μg of USF-1, E12, or E47 antibody.

FIG. 4. Gel mobility shift assay for CARG. Neonatal rat heart extract (20 μg) was probed with 32P-labeled double-stranded oligonucleotides containing the NCX CARG sequence and its immediate flanking sequence. A 100-fold molar excess of unlabeled CARG probe was used as a specific competitor to the labeled probe. Extracts were also probed using a molar excess of −172 E-box probe and mutant CARG probe to verify that the interactions were specific to the CARG element. The labeled probes were 43-mers, and the unlabeled competitors were either 22- or 43-mers. Supershift analysis was performed using 2 μg of SRF antibody.

FIG. 5. Gel mobility shift assay for GATA −50. Neonatal rat heart extract (20 μg) was probed with 32P-labeled double-stranded oligonucleotides containing the NCX GATA −50 sequence and its immediate flanking sequence. A 100-fold molar excess of unlabeled −50 GATA probe was used as a specific competitor to the labeled probe. Extracts were also probed using a molar excess of −172 E-box probe and mutant −50 GATA probe to verify that the interactions were specific to the −50 GATA element. The labeled probes were 44-mers, and the unlabeled competitors were either 43- or 44-mers (see Table I). Supershift analysis was performed by incubating the reaction mixture with 4 μg of GATA-4 antibody.

FIG. 6. Effects of adrenergic stimulation on the expression of NCX1 H1-luciferase chimeric point mutation constructs. Point mutations were created in the putative cis-elements of the feline NCX1 cardiac 1831-bp promoter, the first exon, and the first 67 bp of the first intron fused to the luciferase gene in the pGL2 vector. All transfections of NCX1 deletion and point mutants were performed in neonatal rat cardiomyocytes. Individual constructs were co-transfected with a cytomegalovirus promoter-driven β-galactosidase fusion vector to normalize transfection efficiency. Data are shown as fold induction of the PE-treated transfections over the reporter activity of each construct without PE treatment. Averages shown are for at least three independent transfection experiments performed in triplicate. Cells were treated with 100 μM phenylephrine (PE) 24 h after transfection. Control cells were treated with 10 μM verapamil 24 h after transfection to inhibit any spontaneous contractile activity. *, p < 0.05 versus activity of NCX1 1831 for each mutant construct. S.E. bars are shown.
whether elements in this region bind nuclear factors, electrophoretic mobility shift assays were performed using neonatal heart nuclear extracts with a double-stranded oligonucleotide probe corresponding to +89 through +128 containing the NCX1 cardiac novel element region (Fig. 8). Four protein-DNA complexes were observed binding to the cardiac novel element region probe. Competition experiments using a 100-fold molar excess of the unlabeled competitor DNA sequences were used to determine specificity of the complexes. Incubation with cold cardiac novel element region probe competed away all but the band 4 protein-DNA complex. Competition with the mutant cardiac novel element region oligonucleotide, NCXm109–112, containing the four point mutations between positions +106 and +112 did not affect B1, B2, or B3 protein-DNA complexes, but the B4 complex was slightly diminished. Competition with the shorter (19-mer) oligonucleotide containing only +103–117 wild type sequence, eliminated the B1, B2, and B3 complexes but had less of an affect on B4. Therefore, the B1, B2, and B3 protein-DNA complexes appear to be specific for the 103–117 region. In addition, the 4-base point mutation further defines the element by demonstrating that a portion of it lies between positions +109 and +112.

**DISCUSSION**

We have used promoter-reporter gene constructs to identify the elements regulating cardiac expression and mediating α-adrenenergic up-regulation of the NCX1 gene. Here, we demonstrate that NCX1 expression in neonatal rat ventricular myocytes requires at least two DNA sequence elements, CArG and GATA. The CArG element (CCATGTATGG) present at −80 bp diverges from the canonical CArG sequence CC(AT)₆GG but, nevertheless, is required for cardiac expression of the NCX1 gene. In addition, we have demonstrated that the SRF is a part of the complex binding to the NCX1 CArG element, suggesting that SRF is required for basal NCX1 expression. CArG boxes are present in the proximal promoters of many muscle genes and have been shown to be involved in skeletal as well as cardiac muscle-specific regulation (38–40).

Although the NCX1 CArG element is required for basal activity, it does not appear to mediate α-adrenenergic stimulated expression. This is similar to what has been identified in the human cardiac α-actin promoter and mouse skeletal α-actin promoter (41). Only recently has it been determined how the ubiquitous SRF factor, which mediates transcriptional activation of serum-responsive genes, could regulate muscle-specific genes. Interactions between SRF and other nuclear factors appear to provide mechanisms by which SRF could provide tissue-specific transcriptional activity. SAP-1, Elk-1, and Phox-1 have been demonstrated to potentiate the transcriptional activity of SRF on the c-fos promoter (42). Chen and Schwartz (43) have shown that the cardiogenic homeodomain factor, Nkx-2.5, interacts with SRF to synergistically trans-activate the cardiac α-actin promoter. This trans-activation is dependent on an intact serum response element and not on the Nkx-2.5 element. Interaction of SRF with Nkx-2.5 may also be important in the cardiac regulation of the NCX1 promoter. However, we showed earlier that the −10 Nkx-2.5 element does not appear to be required for NCX1 expression in neonatal rat cardiomyocyte (Fig. 2), and this element is not preserved in the rat NCX1 promoter (14). This interaction, if present in the NCX1 promoter, must be mediated via the CArG and not the
Nkx-2.5 element, similar to what was found for the cardiac α-actin gene. Importantly, Nkx-2.5 and SRF have recently been shown to regulate the cardiac α-actin promoter through combinatorial interactions with GATA-4 (44). Although these studies have not focused on transcription factor interactions, our experiments here demonstrate that, in addition to SRF, cardiac expression of NCX1 is also regulated by a GATA-4 factor. We are currently exploring the interactions of Nkx-2.5, SRF, and GATA-4 factors in the co-activation of the NCX1 promoter.

GATA elements have an important role in the transcriptional regulation of several cardiac specific genes including α-myosin heavy chain (25), cardiac troponin (45), myosin light chain 1/3 (46), and the β-type natriuretic peptide (24). Although GATA elements do not play a role in the basal cardiac expression of the β-myosin heavy chain or angiotensin II type 1a receptor, a GATA binding site is requisite for the induction of these genes in in vitro hemodynamic pressure overload (36, 37). GATA-4 plays a critical role in the cardiac expression of NCX1 but is not required for α-adrenergic up-regulation. With the exception of the −172 E-box, none of the consensus sequences in the +184 minimal promoter appeared to mediate the α-adrenergic stimulation. A series of deletion constructs indicated that a 32-bp region spanning the first exon-intron boundary (+103 to +134) contains one or more additional elements requisite for α-adrenergic stimulated up-regulation. Interestingly, this region contains no consensus binding motifs for known transcription factors. Comparison of the rat (14), human (13), and feline (11) gene sequences in this region reveals 100% homology between 59 bases +104 to +114 and that 17 out of the 21 bases in the region from +94 to +114 are identical. Although the findings of this work by no means exclude the existence of other α-adrenergic responsive elements elsewhere in the NCX1 gene, they demonstrate clearly that the −172 E-Box and the region between +103 to +134 are both requisite for α-adrenergic stimulation in the context of the NCX1 promoter-luciferase construct. α-adrenergic stimulation has been demonstrated to activate signaling pathways that result in cardiac hypertrophy. Although this study demonstrates that this region mediates α-adrenergic stimulation, it remains to be seen whether it also mediates up-regulation in response to hemodynamic load. It is important to note that these studies were carried out in neonatal cardiocytes. In vitro transfection of neonatal cardiocytes is an extremely valuable system to identify and begin to elucidate the role of specific cis-elements that mediate changes in expression. But the significance of the elements identified here and whether they mediate basal cardiac expression and/or hypertrophic induced up-regulation needs to be confirmed in adult cardiomyocytes in vivo. Transgenic lines with the NCX1 promoter should permit us to examine the relative importance and role of CaR/G, GATA, the E-box, and the novel element in mediating the expression of NCX1 during development and in the normal and hypertrophic heart.

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