Regulation of Ncx1 Expression

**IDENTIFICATION OF REGULATORY ELEMENTS MEDIATING CARDIAC-SPECIFIC EXPRESSION AND UP-REGULATION**

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The Na\(^{+}\)–Ca\(^{2+}\) exchanger (NCX1) is up-regulated in hypertrophy and is often found up-regulated in end-stage heart failure. Studies have shown that the change in its expression contributes to contractile dysfunction. We have previously shown that the 1831-bp Ncx1 H1 (1831Ncx1) promoter directs cardiac-specific expression of the exchanger in both development and in the adult, and is sufficient for the up-regulation of Ncx1 in response to pressure overload. Here, we utilized adenoviral mediated gene transfer and transgenics to identify minimal regions and response elements that mediate Ncx1 expression in the heart. We demonstrate that the proximal 184 bp of the Ncx1 H1 (184Ncx1) promoter is sufficient for expression of reporter genes in adult cardiomyocytes and for the correct spatiotemporal pattern of Ncx1 expression in development but not for up-regulation in response to pressure overload. Mutational analysis revealed that both the −80 CArG and the −50 GATA elements were required for expression in isolated adult cardiomyocytes. Chromatin immuno-precipitation assays in adult cardiocytes demonstrate that SRF and GATA4 are associated with the proximal region of the endogenous Ncx1 promoter. Transgenic lines were established for the 1831Ncx1 promoter-luciferase containing mutations in the −80 CArG or −50 GATA element. No luciferase activity was detected during development, in the adult, or after pressure overload in any of the −80 CArG transgenic lines. The Ncx1 −50 GATA mutant promoter was sufficient for driving the normal spatiotemporal pattern of Ncx1 expression in development and for up-regulation in response to pressure overload but importantly, expression was no longer cardiac restricted. This work is the first in vivo study that demonstrates which cis elements are important for Ncx1 regulation.

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The Na\(^{+}\)–Ca\(^{2+}\) exchanger (NCX) is one of the essential regulators of Ca\(^{2+}\) homeostasis within cardiomyocytes and is an important regulator of contractility. The exchanger catalyzes the electrogenic exchange of Ca\(^{2+}\) and Na\(^{+}\) across the plasma membrane in either the Ca\(^{2+}\)-influx or Ca\(^{2+}\)-efflux mode. The NCX family includes three mammalian exchanger genes (Ncx1, Ncx2, and Ncx3) with very similar functional properties (1). Ncx1 is highly expressed in the heart and there is a rapid up-regulation of Ncx1 in response to pressure overload (2–4). The exchanger is often found up-regulated in end-stage heart failure and many studies have shown that the change in Ncx1 expression contributes to the pathophysiological phenotype (5, 6). Therefore, the up-regulation of the exchanger has been proposed to play an important role in altered excitation-contraction coupling and arrhythmogenesis in the context of cardiac hypertrophy and failure.

There are multiple tissue-specific variants of Ncx1 resulting from alternative promoter usage (H1, K1, and Br1) and alternative splicing (3, 7, 8). The H1 promoter directs cardiac-specific expression and contains many of the cis elements that have been demonstrated to be important in both regulation of cardiac expression and induction in response to α-adrenergic stimulation. Our previous study in neonatal rat cardiomyocytes showed that a construct containing only 184 bases of the 5′-flanking region, the H1 exon, and 67 bp of the first intron is not only sufficient for cardiac-directed expression but also for α-adrenergic stimulation of the luciferase reporter gene. The mutational analysis revealed that both the CArG box at −80 and the GATA element at −50 were required for expression in rat neonatal cardiomyocyte but were not required for α-adrenergic induction (9). In contrast to what we found in neonatal cardiomyocytes, the −80 CArG element mediates a part of the α-adrenergic stimulated up-regulation and is required for Ncx1 up-regulation in response to p38 stimulation in isolated adult cardiomyocytes (10).

In the present study we examine regulation of exchanger expression in the adult heart. We first characterized the role that each of the cis elements plays in Ncx1 expression in adult feline cardiomyocytes and demonstrated that both the −80

4 The abbreviations used are: NCX, Na\(^{+}\)–Ca\(^{2+}\) exchanger; SRF, serum response factor; ANF, atrial natriuretic factor; m.o.i., multiplicity of infection; GFP, green fluorescent protein; PE, phenylephrine; TAC, transverse aortic constriction; LV, left ventricle; BW, body weight; E, embryonic day.
GATA and −50 CARG were important for expression in adult cardiomyocytes. Both GATA4, which binds to GATA elements, and the serum response factor (SRF), which binds to the CARG box (CC(A/T)nGG), play essential roles in cardiac development and regulating hypertrophic growth in the adult heart. Numerous cardiac-expressed genes in addition to the exchanger are regulated by both GATA4 and SRF including ANF, brain natriuretic peptide, α-myosin heavy chain, and β-myosin heavy chain. SRF has been demonstrated to stimulate the expression of cardiac muscle genes in association with a variety of cofactors including LIM domain proteins (11) and with transcription factors such as GATA4 and Nkx2.5 (12–14). GATA4 has been shown to mediate inducible expression of several cardiac genes both in in vitro studies in response to phenylephrine, isoproterenol, and endothelin-1 (15, 16) and in vivo studies in response to pressure overload (17, 18).

The GATA4 and SRF consensus binding regions shown to be important in Ncx1 expression were further characterized utilizing transgenic mouse lines containing reporter genes driven by mutant Ncx1 promoters to determine the role they play in Ncx1 expression in cardiac development and in response to pressure overload in the adult heart. The −50 GATA mutant promoter is still able to drive transgenic expression in both development and in the adult heart and is still capable of being responsive to pressure overload induced up-regulation but the loss of the −50 GATA element results in some non-cardiac-restricted expression of the Ncx1 promoter. The −80 CARG element is critical for transgene expression and response to pressure overload. Lastly, the Ncx1 minimal (184 bp) promoter containing both the −80 CARG and −50 GATA elements is sufficient for driving the normal spatiotemporal pattern of Ncx1 expression in cardiac development but is not sufficient for pressure overload induced up-regulation indicating that at least one additional element is required for hypertrophic regulation of the exchanger.

**EXPERIMENTAL PROCEDURES**

*Rat Neonatal and Feline Adult Cardiomyocytes Isolation and Culture*—Primary neonatal cardiomyocytes were obtained from 2–4-day-old neonatal rats and cultured by the method described previously (9). Adult feline cardiomyocytes were isolated via a hanging heart preparation using enzymatic digestion described previously (9). Adult feline cardiomyocytes were isolated from 2–4-day-old neonatal rats and cultured by the method described previously (9). Adult feline cardiomyocytes were isolated from 2–4-day-old neonatal rats and cultured by the method described previously (9).

**Construction of Adenovirus**—Adenoviral constructs were made using the AdEasy system (Stratagene). The Ncx1 promoter-luciferase constructs were cloned into the promoterless pAdTrack vector as described (10). Viruses were plaque purified, amplified, and titers determined by the Gazes Adenoviral Core. Cardiomyocytes were infected on day 1 in culture by adding titrated adenovirus to the culture medium at a different multiplicity of infection (m.o.i.). After an infection of 8 h the media was changed. When more than one adenoviral construct was used to infect cells, experiments were carried out to ensure there was no competition for infection between the constructs at the multiplicity of infections used. Adult and neonatal cardiomyocytes infected with m.o.i.s of 1 resulted in the infection and gene transfer to greater than 85% of the plated cells based on GFP expression.

**Chromatin Immunoprecipitation Assay**—Adult cardiomyocytes were stimulated with 10 mM phenylephrine (PE) and/or infected with GATA4 or SRF adenovirus (m.o.i. = 1). Forty-eight hours after infection and/or stimulation, cells were treated with 1% (v/v) formaldehyde for 20 min at room temperature with slow rocking. Chromatin immunoprecipitation assay was performed as described in the manufacturer’s manual (Upstate) with some modifications. Cells were washed twice with ice-cold phosphate-buffered saline and collected by centrifugation at 10,000 × g for 2 min. The cell pellet was suspended in lysis buffer and incubated in ice for 20 min. The cell lysate was sonicated 10 times for 10 s each and the cell debris spun down. The sample was pre-cleared and the immunoprecipitation antibody added to the supernatant and incubated overnight at 4 °C. After immunoprecipitation, the eluted protein-DNA complexes were de-cross-linked by heating at 65 °C for 4 h. The DNA was ethanol precipitated and the DNA was suspended in 50 μl of 10 mm Tris buffer. The feline Ncx1 proximal promoter was PCR amplified from the immunoprecipitated and non-immunoprecipitated chromatin using the following primers: sense −152 to −131 (5′-GTG- TGGATGAGACGAGAG-3′) and antisense −14 to −34 (5′-AACATGTTTTGCATAGCTGCA-3′).

**Production of Transgenic Mouse Lines**—The Ncx1 promoter-driven luciferase transgenic mice were produced as described previously (3). Briefly, the 1831-bp full-length feline Ncx1 promoter construct or its mutants (−50 GATA and −80 CarG) were introduced into the multiple cloning site of the luciferase reporter vector pGL2 (Promega, Madison, WI). The 184Ncx1 promoter construct contained the first 184 bases of the feline promoter, the H1 exon, and 67 bases of the first intron fused to the β-galactosidase reporter gene. The resulting constructs were digested, and gel-purified. Pronuclear microinjection in the FVB/N mouse strain was performed at the Transgenic Core Facility of the Medical University of South Carolina. Founder mice were identified by PCR and confirmed by genomic Southern blots. Multitransgenic lines for each construct were maintained by mating transgenic offspring with normal FVB/N mice from Taconic Labs (Germantown, NY).

**Histological Analysis**—Embryo dissection, tissue isolation, fixation, and processing for lacZ staining (n = 23) were carried out as described (20). Similarly, all lacZ stained embryonic, newborn and adult samples were then dehydrated, embedded in paraffin, and sectioned at 6 μm thickness for hematoxylin and eosin staining and microscopic analysis according to standard procedures (20). In situ hybridization was carried out as described (3).

**Pressure Overload Mouse Model**—Left ventricular pressure overload was created by microsurgical transverse aortic constriction (TAC) as described previously (3). Briefly, transgenic FVB/N mice carrying the Ncx1H1-luc construct or mutants were anesthetized with ketamine (50 mg/kg) and xylazine (2.5 mg/kg) and respiration was artificially controlled. The transverse aorta was constricted by tying a suture around the vessel over a 28-gauge or a 30-gauge blunted needle causing complete occlusion of the aorta. The
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FIGURE 1. Effect of α-adrenergic stimulation on the expression of 1831Ncx1 full-length and 184Ncx1 minimal NCX1 promoter–luciferase constructs in adult feline cardiomyocytes (A) or rat neonatal cardiomyocytes (B). Isolated feline adult cardiomyocytes or rat neonatal cardiomyocytes were infected with either the 1831Ncx1 or 184Ncx1 promoter–luciferase adenoviruses (m.o.i. 1.5). Twelve hours after infection medium was changed and cells were incubated in the presence or absence of 10 μmol/liter PE for 48 h. Cells were lysed in reporter buffer and luciferase activity was measured and normalized to GFP levels. Luciferase activities in relative light units (RLU) for each of the constructs are shown as a ratio of the untreated 1831Ncx1 constructs activity. *, p < 0.0001 when compared with untreated control.

needle was withdrawn, resulting in a severely stenotic aortic lumen. Two and 7 days after surgery, animals were sacrificed by removal of the heart in deep anesthesia.

Echocardiography and Doppler echocardiography were performed on mice before banding (baseline echo) and just prior to sacrifice (final echo). A 15-MHz transducer (Sonos 5500, Agilent Technologies, Andover, MA) was placed on a layer of acoustic coupling gel applied to the hemithorax. Measurements of LV dimension and posterior wall thickness were made at end systole and end diastole according to the leading edge convention of the American Society of Echocardiography. Three to six beats were averaged for each measurement. Fractional shortening and LV mass were calculated as we have described previously (21) with standard equations. Standard Doppler techniques were used to measure the velocity (V) across the transverse aortic constriction. Maximum peak pressure gradient was then calculated using a modified Bernoulli equation as 4V². Left and right ventricular weights were determined after microdissection and tissue samples of the heart and other organs were snap-frozen in liquid nitrogen. The mouse body weight (BW) at the end of the study was used for indexing purposes. All animal experimentation was performed in accordance with National Institutes of Health Guidelines, and protocols were approved by the Animal Care Committee of the Medical University of South Carolina.

Luciferase and β-Galactosidase Activity and GFP Assays—To quantitate Ncx1-promoter reporter gene expression, samples of heart tissue were ground in a mortar under liquid nitrogen and assayed for luciferase activity as previously described (3). β-Galactosidase activity was measured using the Galacto-Light and Galacto-Light Plus Systems (AB Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. Luciferase activity and GFP fluorescence from adenoviral infected neonatal and adult cardiomyocytes were assayed as described (10).

Statistical Analyses—Multiple comparisons of experimental groups were done with factorial analysis of variance and the Scheffe F post hoc test. A value of p < 0.05 was considered to be significant. All data are shown as mean ± S.E.

RESULTS

The 184Ncx1 Minimal Promoter Is Sufficient for Expression in Adult Cardiomyocytes but Not for Up-regulation—Fig. 1 demonstrates that the Ncx1 minimal promoter is sufficient to direct expression of the luciferase reporter gene in both neonatal and adult cardiomyocytes. The 184Ncx1 promoter drives reporter gene expression at levels 3–4-fold greater than the full-length Ncx1 (1831Ncx1) promoter in both neonatal and adult cardiomyocytes (Fig. 1, A and B) because of the deletion of putative repressor elements distal to the minimal 184-bp promoter construct. The 1831Ncx1 promoter is up-regulated in response to α-adrenergic stimulation in both adult and neonatal cardiomyocytes. However, the 184Ncx1 promoter is up-regulated in response to α-adrenergic stimulation in neonatal cardiomyocytes but is recalcitrant to α-adrenergic stimulation in adult cardiomyocytes (compare Fig. 1, A to B). These data demonstrate that α-adrenergic-stimulated up-regulation of Ncx1 is different in the neonatal cardiomyocyte when compared with the adult cell.

To test whether the Ncx1 minimal promoter contains sufficient DNA regulatory elements to direct cardiac-specific expression, we established two independent 184Ncx1-β-galactosidase transgenic mouse lines. Moderate levels of β-galactosidase were detected in the hearts, and only the hearts, of both

5 L. Xu and D. R. Menick, unpublished data.
lines expressing the transgene. The 184Ncx1-driven β-galactosidase expression was initially weakly detectable in E8.5 embryos (not shown) and robustly detected in E9.5 (Fig. 2A) embryos. Significantly, heart-restricted lacZ expression was present in 100% of the transgenic embryos (n = 14) and was absent from all the non-transgenic embryos examined (n = 31). When compared with the endogenous Ncx1 mRNA and Ncx1lacZ knock-in reporter expression patterns (20, 22), the lacZ spatiotemporal patterns were very similar (Fig. 2). Tissue distribution of luciferase activity in adult transgenic mice. Reporter activity was restricted to the cardiomyocytes and absent from the adjacent endothelial and endocardial cushion cells (not shown). However, when older E14 (not shown) and newborn (Fig. 2, C and D) hearts were examined, a less prevalent and “patchy” lacZ expression was detected within sub-populations of the cardiomyocytes within the ventricles and atria of both transgenic lines, when compared with the endogenous Ncx1 mRNA (23) and Ncx1lacZ knock-in reporter expression patterns (20). The decreased prevalence and mosaic pattern of expression observed at E14.5 (not shown) and newborn stages persisted into the adult (data not shown). Additionally in these later stages, lacZ reporter activity could be detected in the sinus venosus, pulmonary and caval vein region (Fig. 2D). Importantly, no β-galactosidase activity was detected in the kidney, liver, spleen, brain, or skeletal muscle of the embryonic, newborn, or adult transgenic animals (Table 1). These data reveal that the 184Ncx1 minimal promoter retains the necessary enhancer elements to drive initial early cardiomyocyte-specific reporter expression, but that subsequently undefined enhancer elements required for ubiquitous cardiomyocyte lacZ reporter activity have been deleted.

Ncx1 transcript and protein levels are increased in response to pressure overload in the feline model of hypertrophy (2) and this up-regulation is mediated by the Ncx1 H1 promoter (3). To determine whether the minimal Ncx1 promoter is sufficient for the up-regulation of the exchanger in response to pressure overload hypertrophy, transgenic mice expressing either the 184Ncx1-β-galactosidase, or the wild-type 1831Ncx1-luciferase were subjected to cardiac LV pressure overload by microsurgical TAC. Sham-operated littermates underwent the same anesthesia and thoracotomy protocol without TAC to control for the effect of anesthesia and surgical trauma. The mice from both sham-operated and TAC groups from each transgenic line were euthanized 2 and 7 days after surgery. TAC with either the 28- or 30-gauge needles resulted in a pressure gradient greater than 90 mm Hg. However, 7 days after TAC, LV/BW increased by 31% using a 28-gauge needle and increased by 81% using a 30-gauge needle (Fig. 3, A and B). Protein extracts were prepared from LV tissue samples from control, sham-operated, and TAC-operated transgenic mice to quantify the response of the wild-type and mutant Ncx1 promoter reporter transgene. Reporter gene activity was assayed and normalized for protein content. In the 1831Ncx1 mice, the 28-gauge was sufficient to induce a significant hypertrophic stimulus and increase in luciferase activity. As previously reported (3), endogenous Ncx1 protein levels are up-regulated after 7 days of pressure overload (not shown) and the wild-type full-length 1831Ncx1 promoter driven reporter gene activity showed a 2-fold up-regulation after 7 days of pressure overload (Fig. 3C). Preliminary studies in the 184Ncx1 mice did not produce an increase in β-galacto-

### TABLE 1

| Tissue             | Luciferase activity 1831Ncx1 | Luciferase activity Ncx1 +50 GATA | β-Galactosidase activity, a |
|--------------------|-----------------------------|----------------------------------|-----------------------------|
|                    | 1831Ncx1 -50 GATA | Ncx1 -80 CarG | 184Ncx1                   | 184Ncx1                     |
| Left ventricle     | 37,964                    | 2003                | 6.7                        | 1899                      |
| Brain              | 0                         | 0                   | 0                          | 0                         |
| Kidney             | 0                         | 17.1                | 3.9                        | 0                         |
| Lung               | 0                         | 9.5                 | 0                          | 0                         |
| Liver              | 0                         | 0                   | 0                          | 0                         |
| Spleen             | 0                         | 0                   | 0                          | 0                         |
| Skeletal muscle    | 0                         | 37.5                | 6.2                        | 0                         |
| Uterus             | 0                         | 60                  | 0                          | 0                         |
| Aorta              | 0                         | 15.2                | 0                          | 0                         |

a All values shown are for the luciferase of β-galactosidase activity of the transgenic mouse tissue minus background value of same tissue in non-transgenic mouse.

b RLU, relative light units.
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FIGURE 3. Effect of LV pressure overload by TAC on LV hypertrophy and Ncx1 promoter activity. LV mass normalized for body weight (LV/BW) for transverse aortic arch-banded 183Ncx1 mice (A) and 184Ncx1 (B) for both non-banded controls and banded mice. LV/BW was substantially higher in all banded groups when compared with sham or nonbanded controls. *, p < 0.001 versus control. The effect of TAC on 183Ncx1 and 184Ncx1 promoter-reporter gene activity was also measured. Animals were euthanized 7 days after surgery. Luciferase activities of 1831Ncx1 (C) or β-galactosidase (D) activities of 184Ncx1 from LV extracts normalized for protein concentration are shown for TAC-treated and sham and control animals. *, p < 0.001 when compared with control.

FIGURE 4. Alignment of the nucleotide sequences of human, feline, and rat 184Ncx1 minimal promoter. Feline Ncx1 (GenBank™ accession numbers U67072–67075), human NCX1 (GenBank accession number AC007877), and rat Ncx1 (GenBank accession number U95137) were used. Sequences, which are identical with the feline promoter, are highlighted in gray, promoter elements are underlined.

Effect of LV pressure overload by TAC on LV hypertrophy and Ncx1 promoter activity. To be certain that this was not a false negative response (i.e., insufficient hypertrophic stimulus), we used a 30-gauge needle for TAC in the 184Ncx1 mice. Despite the robust increase in LV mass (81% increase in LV/BW) after 7 days of TAC, there was no up-regulation of the 184Ncx1-β-galactosidase activity when compared with controls after 7 days of TAC (Fig. 3D). Clearly one or more cis elements responsible for Ncx1 up-regulation are distal to the minimal 184-bp promoter construct.

Role of −50 GATA and −80 CArG Elements in the Regulation of Ncx1—The NCX1 minimal promoter contains several consensus sequences for a number of potential DNA binding factors that have been shown to be important in regulating NCX1 expression in neonatal cardiomyocytes and have also been shown to be important in regulating the cardiac expression of ANF, brain natriuretic peptide, β-myosin heavy chain, and skeletal α-actin (24). These include two GATA elements, two E-Box elements, a CArG element, and a binding site for Nkx 2.5 (Fig. 4). It is important to note that the first 184 bp of the human (25) and feline (8) Ncx1 H1 promoters have 97% identity and the rat (26) and feline sequences have 92% identity. Using the 183Ncx1 construct, we introduced site-specific point mutations into each of these elements, and the activity in adult cardiomyocytes of each of these adenoviral constructs was compared with the wild-type construct to determine its contribution to expression. Ablation of the −172 E-Box, −153 E-Box, −10 NKE, and the novel element at +103 resulted in >50% of the reporter activity seen in the wild-type promoter-luciferase construct (Fig. 5A). Mutation of the −123 GATA or −50 GATA element resulted in 35 and 29% of wild-type promoter activity, respectively. A point mutation disrupting the −80 CArG element resulted in luciferase activity of less than 20% of the control level. Clearly the CArG and GATA elements are important to basal Ncx1 expression in adult cardiomyocytes.

Next we examined whether any of these elements played a role in the α-adrenergic stimulated up-regulation of Ncx1 in adult cells. After 48 h of PE treatment there is a 3-fold or greater up-regulation of reporter gene activity in adult cardiomyocytes infected with either the −50 GATA or −80 CArG Ncx1 promoter adenovirus (Fig. 5B). Although we have shown that the −80 CArG element is critical for mitogen-activated protein kinase p38-stimulated up-regulation of Ncx1 (10), neither the −50 GATA nor −80 CArG element is critical for α-adrenergic up-regulation in neonatal or adult cardiomyocytes. Similar to what we observed in neonates, the −153 E-box element, the −123 GATA element, and the −10 NKE were not required for α-adrenergic-stimulated up-regulation in adult cardiomyocytes (data not shown). Interestingly, two of the elements in the minimal promoter play a very different role in Ncx1 up-regula-
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overexpression results in the significant repression of PE stimulation of the Ncx1 promoter. GATA4 overexpression alone in adult cardiomyocytes has very little effect on the ANF promoter activity. But interestingly, GATA4 overexpression represses the PE-stimulated induction of ANF promoter/reporter gene expression. Contrary to what we see with the ANF promoter, overexpression of GATA4 has no significant effect on basal or PE-stimulated Ncx1 promoter activity.

SRF and GATA4 Bind to the Endogenous Ncx1 Promoter in Adult Cardiomyocytes—In earlier work we demonstrated using electrophoretic mobility shift assay and supershift that GATA4 and SRF bind to the −50 GATA element and the −80 CArG element, respectively (9, 14). To determine whether GATA4 and SRF can interact with the endogenous Ncx1 promoter in adult cardiomyocytes, we performed chromatin immunoprecipitation assays. The sheared chromatin was immunoprecipitated with antibodies specific for GATA4 or SRF. PCR primers specific for the proximal region of the Ncx1 promoter −132 to −22 were used to amplify the resulting DNA fragments. Both GATA4 and SRF antibodies specifically pulled down DNA fragments containing the proximal portion of the Ncx1 promoter corresponding to the −80 CArG and −50 GATA elements in both control cells as well as PE-stimulated cardiomyocytes (Fig. 7). Overexpression of GATA4 or SRF did not significantly alter the level of Ncx1 proximal promoter DNA fragments pulled down in control and PE-stimulated cells. As a positive control, 5% of the input chromatin was used for PCR (Fig. 7, input). The specificity of the chromatin immunoprecipitation assay was controlled by running negative controls lacking the precipitating antibody (Fig. 7, No Ab). Overall our data suggest that SRF and GATA4 directly interact with the Ncx1 proximal promoter and are important for regulation of expression of the Ncx1 gene. There is no dramatic difference in levels of GATA or SRF bind-

tion in adult versus neonatal cardiomyocytes. Mutation of either the −172 E-box or novel element at +103 completely eliminated any α-adrenergic-stimulated up-regulation of the exchanger in neonatal cardiomyocytes (9), whereas Fig. 5B shows that in adult cells mutations in either of these elements show a greater than 2-fold up-regulation in response to PE treatment.

Overexpression of SRF and GATA4 in Adult Cardiomyocytes Does Not Result in Ncx1 Up-regulation—Because both SRF and GATA4 have been demonstrated to be key regulators of cardiac genes, we next investigated whether overexpression of either SRF or GATA4 would be sufficient for up-regulation of Ncx1 in adult cardiomyocytes. Adult cardiomyocytes were co-infected with adenovirus expressing SRF or GATA4 and the 1831Ncx1 promoter-luciferase construct. We also used an adenovirus containing an ANF promoter-luciferase reporter construct as a positive control (638ANF/luciferase). Fig. 6 shows that overexpression of SRF results in the dra-

![Figure 5](image-url)

**FIGURE 5.** Effect of Ncx1 promoter transcriptional element mutations on basal and adrenergic stimulation of Ncx1 promoter-driven reporter gene expression in adult feline cardiomyocytes. A, adult feline cardiomyocytes were infected with the wild-type or mutant Ncx1 promoter-luciferase reporter adenoviruses (m.o.i. 1.5). Cells were then incubated for 48 h and lysed in reporter buffer. Luciferase activity was determined relative to GFP. All values are averages from four separate cell isolation experiments performed in triplicate. *, p < 0.0002 when compared with wild-type promoter, and #, p < 0.007 when compared with wild type promoter. B, effects of adrenergic stimulation expression on wild-type or mutant Ncx1 promoter-driven luciferase adenoviruses in adult cardiomyocytes. Cells were infected with either Ncx1 promoter wild-type or mutant adenoviruses (m.o.i. 1.5). Twelve hours after infection, media was changed and cells were incubated in the presence or absence of 10 μmol/liter PE for 48 h. Cells were lysed in reporter buffer and luciferase activity was measured and normalized to GFP levels. Averages are shown from four different cell isolations performed in triplicate. *, p < 0.0001 PE stimulated versus same construct non-stimulated.

mastic induction of ANF promoter-driven reporter gene expression but it did not result in any change in 1831Ncx1 reporter gene expression. Both the ANF and Ncx1 promoters are up-regulated by PE stimulation. The ANF promoter is synergistically activated by PE stimulation when SRF is overexpressed. Unexpectedly, SRF overexpression results in the significant repression of PE stimulation of the Ncx1 promoter. GATA4 overexpression alone in adult cardiomyocytes has very little effect on the ANF promoter activity. But interestingly, GATA4 overexpression represses the PE-stimulated induction of ANF promoter/reporter gene expression. Contrary to what we see with the ANF promoter, overexpression of GATA4 has no significant effect on basal or PE-stimulated Ncx1 promoter activity.
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FIGURE 6. The Ncx1 promoter is not responsive to overexpression of SRF or GATA4. Adult feline cardiomyocytes were infected with the 638ANF promoter-luciferase adenovirus (A) or the 1831Ncx1 promoter-luciferase reporter adenoviruses (B) (m.o.i. 1.5). After 8 h, the media was changed and cells were infected with either SRF or GATA4 adenovirus (m.o.i. = 1) as indicated. Twelve hours after infection media was changed and cells were incubated in the presence or absence of 10 μmol/liter PE for 48 h. Cells were lysed in reporter buffer and luciferase activity was measured and normalized to GFP levels. Averages are shown from three different cell isolations preformed in duplicate. *, p < 0.0001 versus control. †, p < 0.0003 PE-treated versus PE-treated + SRF overexpression.

FIGURE 7. Binding of transcription factors to the endogenous Ncx1 promoter. Chromatin immunoprecipitation assays were performed with formaldehyde cross-linked chromatin isolated from feline adult cardiomyocytes. Where indicated, adult cardiomyocytes were infected with the indicated adenovirus at a m.o.i. of 1. Some cultures were also stimulated with PE (10 μmol/liter) for 48 h. The PCR results using various immunoprecipitated samples are shown. Immunoprecipitations were performed without primary antibody (No Ab) as a negative control, with anti-GATA4 antibody (left) or anti-serum response factor antibody (right). Input DNA is also shown as a control. Similar results were observed in a total of four independent experiments.

Expression of Ncx1 −80 CArG and Ncx1 −50 GATA Transgenic Mice—Because both the −80 CArG and −50 GATA elements play an important role in Ncx1 gene expression in neonatal and adult cardiomyocytes, we assessed their roles in Ncx1 expression in the adult heart. Two independent stable transgenic lines carrying the full-length Ncx1 promoter containing point mutations in the −50 GATA element and three lines containing a mutation of the −80 CArG element were established. Each of the lines expressing the −50 GATA mutant transgene had the highest level of reporter gene expression in the ventricles. When luciferase activity was assayed and normalized for protein content the −50 GATA transgenic ventricles had only 5–10% of the activity compared with the wild-type transgenic mice. But because expression in both −50 GATA transgenic lines was variiegated, the level of luciferase expression does not directly reflect promoter activity. Similar to what we saw in the wild-type transgenic mice, no luciferase activity was detected in the liver, spleen, or brain. But unlike what is seen for the wild type, we do see very low levels of expression in the kidney and lung and higher levels in the uterus, skeletal muscle, and aorta (Table 1). Therefore, it appears that the −50 GATA element is important for directing the cardiac-restricted expression pattern of the exchanger. Interestingly, no luciferase activity was detected in the heart or in any of the tissues assayed from the three −80 CArG transgenic lines (Table 1) clearly indicating that it is critical to Ncx1 expression in both the developing and adult heart.

Developmental Expression of Ncx1 −80 CArG and Ncx1 −50 GATA Transgenic Mice—Because the −50 GATA element is important in cardiac-specific expression, we examined what role this element plays in the spatiotemporal pattern of Ncx1 expression in cardiac development. The Ncx1 −50 GATA-luciferase expression was detected by in situ hybridization within 100% of the transgenic embryos (n = 16) and was absent from all the non-transgenic embryos examined (n = 4). However, in Ncx1 −50 GATA hearts there was only patchy expression within subpopulations of the cardiomyocytes within the ventricles at E9.5 and similarly patchy expression within subpopulations of the cardiomyocytes within the hearts of E14.5 transgenic embryos, when compared with the endogenous Ncx1 mRNA (Fig. 8, A–D). There was no expression of luc
or endogenous Ncx1 in extracardiac tissues. Expression of luc was reduced relative to endogenous Ncx1 and was only present within subpopulations of the cardiomyocytes within the primitive common trabeculated ventricle and was absent from the truncus arteriosus, sinus venous, and atrial chamber of the heart (Fig. 8B). Similarly, luc reporter expression was mosaic in the E14.5 Ncx1−50 GATA fetal ventricle when compared with the robust endogenous Ncx1 mRNA patterns (Fig. 8, C and D), and was absent from the atria. Unlike what we found for endogenous Ncx1 mRNA expression, the Ncx1−50 GATA luc was also expressed within the aorta and trachea (Fig. 8D, arrowhead), however, neither the endogenous nor Ncx1−50 GATA cDNAs were expressed within the endocardially derived aortic and pulmonary valves. This again indicates that loss of the GATA element within the Ncx1 promoter results in non-cardiac-restricted expression of the Ncx1 promoter during development. The expression pattern is similar to what we saw in the adult where low levels of reporter gene expression were detected in smooth and skeletal muscle tissue.

Although we saw no expression of the reporter gene in the adult heart for the −80 CArG transgenic mouse, we tested for transient expression during cardiac development. Radioactive in situ hybridization analyses are shown for both Ncx1 (A) and luc (B) mRNA expression in sagittal serial sections of the transgenic-positive E9.5 Ncx1−50 GATA embryo. Expression of luc is reduced and is only present within a subpopulation of the cardiomyocytes that routinely express endogenous Ncx1 and is absent from the truncus arteriosus, sinus venous, and atrial chamber of the heart (indicated by arrows in B). Similarly, luc reporter expression is mosaic in the E14.5 Ncx1 (GATA−50) fetal ventricle when compared with endogenous Ncx1 mRNA patterns (C and D), and is absent from the atria. Note that there is also extra cardiac expression within the aorta and trachea (indicated by arrowhead in D). mRNA expression for both Ncx1 (E) and luc (F) in sagittal serial sections of the E9.5 and E14.5 (G and H) transgenic-positive Ncx1 (CarG−80) embryos show that only endogenous Ncx1 expression is detectable within the embryonic and fetal cardiomyocytes and that there is no detectable endogenous Ncx1 or luc reporter expression within extracardiac tissues in either embryonic age analyzed.

FIGURE 8. Temporal-spatial detection of endogenous Ncx1, Ncx1−50 GATA, and Ncx1−80 CArG-luciferase (luc) reporter cDNA expression within the mouse cardiovascular system in utero. Radioactive in situ hybridization analysis are shown for both Ncx1 (A) and luc (B) mRNA expression in sagittal serial sections of the transgenic-positive E9.5 Ncx1−50 GATA embryo. Expression of luc is reduced and is only present within a subpopulation of the cardiomyocytes that routinely express endogenous Ncx1 and is absent from the truncus arteriosus, sinus venous, and atrial chamber of the heart (indicated by arrows in B). Similarly, luc reporter expression is mosaic in the E14.5 Ncx1 (GATA−50) fetal ventricle when compared with endogenous Ncx1 mRNA patterns (C and D), and is absent from the atria. Note that there is also extra cardiac expression within the aorta and trachea (indicated by arrowhead in D). mRNA expression for both Ncx1 (E) and luc (F) in sagittal serial sections of the E9.5 and E14.5 (G and H) transgenic-positive Ncx1 (CarG−80) embryos show that only endogenous Ncx1 expression is detectable within the embryonic and fetal cardiomyocytes and that there is no detectable endogenous Ncx1 or luc reporter expression within extracardiac tissues in either embryonic age analyzed.

E14.5 Ncx1−50 GATA fetal ventricle when compared with the robust endogenous Ncx1 mRNA patterns (Fig. 8, C and D), and was absent from the atria. Unlike what we found for endogenous Ncx1 mRNA expression, the Ncx1−50 GATA luc was also expressed within the aorta and trachea (Fig. 8D, arrowhead), however, neither the endogenous nor Ncx1−50 GATA cDNAs were expressed within the endocardially derived aortic and pulmonary valves. This again indicates that loss of the GATA element within the Ncx1 promoter results in non-cardiac-restricted expression of the Ncx1 promoter during development. The expression pattern is similar to what we saw in the adult where low levels of reporter gene expression were detected in smooth and skeletal muscle tissue.

Although we saw no expression of the reporter gene in the adult heart for the −80 CArG transgenic mouse, we tested for transient expression during cardiac development. Radioactive in situ hybridization analyses are shown for both Ncx1 mRNA and Ncx1−80 CArG luciferase expression in sagittal serial sections of the transgenic-positive E9.5 embryo (Fig. 8, E and F). Only endogenous Ncx1 expression is present within the embryonic heart and there is no detectable Ncx1−80 CArG-luciferase within either the embryonic heart or entire rest of the embryo. The same results are found in sagittal serial sections of the transgenic-positive E14.5 embryo (Fig. 8, G and H). There is no detectable Ncx1−80 CArG-luciferase within the entire fetus. Clearly the CArG element is critical for the Ncx1 promoter activity in both the adult and developing heart.

Response of Ncx1 Mutant Promoter-Reporter Transgenes to Pressure Overload—To determine whether the −50 GATA element plays a role in mediating the up-regulation of the exchanger in response to pressure overload hypertrophy, transgenic mice expressing the Ncx1−50 GATA-luciferase promoter was subjected to acute cardiac LV pressure overload by microsurgical TAC using a 30-gauge needle as described previously. Protein extracts were prepared from LV tissue samples from control and TAC-operated transgenic mice to quantify the response of the wild-type and mutant Ncx1−promoter luciferase transgene. There was no change in luciferase activity in the sham operated −50 GATA transgenic mice (data not shown). Luciferase activity in the Ncx1−50 GATA transgene was increased 2-fold following 2 days of TAC (data not shown) and was 3-fold greater than control in response to 7-day TAC (Fig. 9). Therefore the mutation of the −50 GATA element appears to result in a slightly more robust up-regulation of reporter gene activity in response to pressure overload than we see in the wild-type Ncx1 transgenic mice (see Fig. 3).

The Ncx1−80 CArG transgenic mice were also banded for 2 and 7 days using a 28-gauge needle. As with the non-banded controls, there is no detectable reporter gene expression after 2 (data not shown) and 7 days (Fig. 9).

DISCUSSION

Several major findings come out of this study. One of the most significant is that the proximal 184-bp segment of the Ncx1 H1 promoter is sufficient to direct the correct spatiotemporal pattern of Ncx1 expression in cardiac development and cardiac-restricted expression in the adult. One difference that was evident between the minimal and full-length Ncx1 pro-
Regulation of Ncx1 Expression

What appears to be affected is the probability of the expression of the reporter gene in any particular cardiac myocyte not the level of expression. This probability of expression may be highest in specialized cardiomyocytes that make up the conduction system (data not shown), although we have not yet confirmed this using a specific marker against specialized conduction system cardiomyocytes. Additionally, most of the cells that express the transgene appear to be subendocardial rather than subepicardial. Therefore, it appears that the Ncx1 minimal promoter is regulated in a manner consistent with the binary model for transcription that postulates that promoter/enhancer elements increase the probability that a promoter will be on or off rather than governing the level of expression (29, 30).

Although the 184Ncx1 promoter has sufficient regulatory elements to direct the cardiomyocyte-restricted expression at the proper time in development, it is missing some of the elements that contribute to the complexity of regulation mediating the level of Ncx1 expression in response to cellular stimuli. This is true even when the 184Ncx1 transgenic mouse is subject to more robust hypertrophic stimuli using a 30-gauge needle to set our TAC. Placement of the TAC causes an immediate increase in the pressure gradient and results in a stepwise increase in the load-based stimulus to hypertrophy. Importantly, the hypertrophic response is progressive and not complete until at least 14 days after TAC placement. In our laboratories the standard response to TAC using a 28-gauge needle is an LV/BW increase by 30 and 50% for 7 and 14 days after TAC, respectively. Also, the standard response to TAC using 30-gauge needle is an LV/BW increase by 80 and 100% for 7 and 14 days after TAC, respectively.

Our data are consistent with the interpretation that one or more regulatory element(s) distal to ~184 are required for the pressure overload-induced up-regulation of the Ncx1 gene. Clearly, this does not exclude any of the regulatory elements present in the proximal 184 bases of the Ncx1 promoter as not being required for up-regulation but it does demonstrate that they are not sufficient. We are currently working on identifying the distal elements that contribute in mediating the pressure overload-induced up-regulation of the 1831Ncx1 promoter.

Despite the loss of regulation by pressure overload, the temporal and tissue-restricted regulation of expression by such a small region of Ncx1 promoter is remarkable and highlights the importance of the handful of elements present. This work has allowed us to identify some of the elements within the minimal promoter that are critical for regulating Ncx1 gene expression. Ncx1 expression in adult cardiomyocytes and during cardiac

FIGURE 9: Effect of LV pressure overload by TAC on 1831Ncx1 - 50 GATA and 1831Ncx1 - 80 CArG mutant promoter activity in transgenic mice. LV mass normalized for body weight (LV/BW) for transverse aortic arch-banded 1831Ncx1 - 50 GATA mice (A) and 1831Ncx1 - 80 CArG (B) for both non-banded controls and banded mice. LV/BW was substantially higher in all banded groups when compared with sham or nonbanded controls. *, p < 0.001 versus control. Animals were euthanized 7 days after surgery. Luciferase activities of 1831Ncx1 - 50 GATA mice (C) or from 1831Ncx1 - 80 CArG mouse (D) LV extracts normalized for protein concentration are shown for TAC-treated and control animals. *, p < 0.0001 when compared with control.
development and in the adult heart requires two promoter elements within the minimal promoter, the −80 CArG and −50 GATA.

GATA factors have been shown to play an important role in early cardiac development and GATA4 regulates expression of cardiac-specific genes including troponin C and α-myosin heavy chain (13). GATA4 is expressed at high levels in the heart and has been shown to be required for several physiological and adaptive responses in cardiomyocytes (31). We have demonstrated using electrophoretic mobility shift assay that GATA-4 interacts with the −50 GATA site in vitro (9). Based on the present results, ablation of the −50 GATA element results in a less robust reporter gene expression in adult cardiomyocytes. Interestingly, it does not affect the temporal pattern of gene expression in cardiac development in vivo, but the ablation of the −50 GATA element results in the loss of cardiac-restricted expression of the reporter gene. During development, expression is evident in the aorta and trachea. In the adult we see low but significant levels of reporter gene expression in both smooth and skeletal muscles. It is important to note that there are two GATA elements in the NCX1 minimal promoter and the −125 GATA element may play a more substantial role in regulation in the absence of the −50 GATA element. Mutation of either GATA element results in a significant drop in reporter gene expression in neonatal (9) and adult cardiomyocytes so both elements may be important in regulating the level of expression. Importantly, we have demonstrated that GATA4 binds to the −50 GATA element by electrophoretic mobility shift assay (9).

Although GATA4 has been shown to be a key regulator of transcriptional up-regulation of BNP, endothelin, β-myosin heavy chain, and angiotensin type I receptor genes during pressure overload (32), our data demonstrates that the role of GATA4 in NCX1 regulation is primarily to mediate cardiac-restricted expression and not up-regulation. Ablation of the −50 GATA element did not inhibit up-regulation in response to PE stimulation in neonatal or adult cells or in response to TAC in the adult heart. In this work we show for the first time that GATA4 is associated with the proximal portion of the endogenous NCX1 promoter but significantly, GATA4 association does not appear to be dramatically altered by PE stimulation or GATA4 overexpression. Although this work does not rule out a role for GATA4 in NCX1 up-regulation, it clearly demonstrates that the −50 GATA element is not required and NCX1 up-regulation is not associated with changes with GATA4 binding.

Point mutations within the −80 CARG element reduced basal expression of reporter gene expression in adult cells to about 18% of wild-type levels, which is higher than the 3% detected in neonatal cells (9). Mutation of the −80 CARG element had an even greater impact on reporter gene expression in the transgenic mice studies. We detected no luciferase activity in the hearts from any of the three founder lines for the −80 CARG mutant. Although both results are consistent with the CARG element being required for normal levels of NCX1 expression, it is possible that the more dramatic phenotype of the transgenic mice could be the result of “position of integration site” effects from the surrounding chromatin environment. Importantly, none of the three independent lines had any reporter gene expression in cardiomyocytes. This argues against the loss of expression being due to only positional effects. Our finding is consistent with the earlier report that the knock-out of endogenous SRF in isolated neonatal mouse cardiomyocytes resulted in significantly lowered levels of endogenous NCX1 mRNA (33). It is possible that expression of the reporter gene is mosaic and therefore not detectable in the homogenized tissue extract, but we did not detect any expression from individual cardiomyocytes in our in situ analysis of the −80 CARG transgenic embryonic heart. The most reasonable explanation is that there are subtle differences in the regulation of cardiomyocytes in an intact heart versus isolated in culture. Importantly, it is clear that both models support the −80 CARG element as being critical for expression.

The CARG box sequence (CC(A/T)GG) is the core sequence of the serum response element and has been demonstrated to be important for the muscle restrictive expression of several genes. These include ANF, other Ca2⁺ transport proteins such as SERCA2, and contractile proteins such as skeletal α-actin, β-myosin heavy chain, cardiac α-actin, myosin light chain-2, and dystrophin (33). SRF has been shown to be required for the induction of several of the genes up-regulated in cardiac hypertrophy (33). Overexpression of SRF results in the up-regulation of ANF in adult cardiomyocytes as we have previously shown (14), but has no effect on NCX1 expression. Overexpression of SRF at higher levels leads to the inhibition of NCX1 promoter activity (data not shown). This may be due to excess SRF competing for other transcription factors and enhancers required for NCX1 expression. Furthermore, overexpression of SRF results in the repression of PE-stimulated NCX1 expression versus the synergistic up-regulation that is seen with the ANF promoter.

Our data are different from that reported for isolated neonatal mouse cardiomyocytes (33) in which NCX1 is induced by the overexpression of SRF. This may be another example of how cardiac gene regulation is often very different in neonate when compared with the adult. Whereas the precise mechanisms by which SRF regulates gene expression are complex the difference we observed resulting from SRF overexpression on ANF versus NCX1 may be in part due to the fact that the ANF promoter contains 2 CARG boxes, whereas NCX1 has only one and for this reason the co-factors interacting with SRF in each case are different. This is currently under investigation. Earlier work from our laboratory has demonstrated that SRF interacts with Nkx2.5 in the regulation of the NCX1 promoter (14). Previously, using in vitro electrophoretic mobility shift assays we demonstrated that SRF binds to the −80 CARG box (9). This present study demonstrates that SRF is bound to the endogenous proximal NCX1 promoter in adult cardiocytes. There does not appear to be any changes in SRF binding with α-adrenergic-stimulated up-regulation but we are currently examining if there are any changes in co-factors associated with SRF and the CARG element. The fact that the −80 CARG mutation in the 1831NCX1 promoter still drives a low level of reporter gene expression in adult cardiomyocytes has allowed us to demonstrate that a major portion of α-adrenergic-stimulated NCX1 up-regulation is mediated by p38 via the −80 CARG element (10). Our work demonstrating that the −80 CARG box to which
Regulation of \( \text{Ncx1} \) Expression

SRF binds is critical for \( \text{Ncx1} \) expression in the developing and adult heart is complementary to recent work using a loss of function approach that demonstrated that the presence of SRF is required for \( \text{Ncx1} \) expression (33).

In this work we have identified domains and \( \text{cis} \) elements that are important for \( \text{Ncx1} \) gene expression and up-regulation in response to pressure overload. Remarkably, the first 184 bp are sufficient for the correct spatiotemporal pattern of \( \text{Ncx1} \) expression in cardiac development but not for up-regulation in response to pressure overload. Our work along with that of many others demonstrates that there are common themes such as the requirement of GATA4 and SRF for the regulation of many of the genes up-regulated in response to pressure overload.

Regulation of \( \text{Ncx1} \) expression in cardiac development but not for up-regulation in the adult heart is complementary to recent work using a loss of function approach that demonstrated that the presence of SRF is required for up-regulation in the adult heart.

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