Cardiac-Specific Expression and Hypertrophic Upregulation of the Feline Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger Gene H1-Promoter in a Transgenic Mouse Model

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Abstract—The NCX1 gene contains three promoters (H1, K1, and Br1), and as a result of alternative promoter usage and alternative splicing, there are multiple tissue-specific variants of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. We have proposed that for NCX1, the H1 promoter regulates expression in the heart, the K1 promoter regulates expression in the kidney, and the Br1 promoter regulates expression in the brain as well as low-level ubiquitous expression. Here, using a transgenic mouse model, we test the role of the DNA region including −1831 to 67 bp of intron 1, encompassing exon H1 of the feline NCX1 gene (NCX1H1). The NCX1H1 promoter was sufficient for driving the normal spatiotemporal pattern of NCX1 expression in cardiac development. The luciferase reporter gene was expressed in a heart-restricted pattern both in early embryos (embryonic days 8 to 14) and in later embryos (after embryonic day 14), when NCX1 is also expressed in other tissues. In the adult, no luciferase activity was detected in the kidney, liver, spleen, uterus, or skeletal muscle; minimal activity was detected in the brain; and very high levels of luciferase expression were detected in the heart. Transverse aortic constriction–operated mice showed significantly increased left ventricular mass after 7 days. In addition, there was a 2-fold upregulation of NCX1H1 promoter activity in the left ventricle in animals after 7 days of pressure overload compared with both control and sham-operated animals. This work demonstrates that the NCX1H1 promoter directs cardiac-specific expression of the exchanger in both the embryo and adult and is also sufficient for the upregulation of NCX1 in response to pressure overload. (Circ Res. 2002;90:158-164.)

Key Words: Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger ■ pressure-overload hypertrophy ■ transgenic mice

Cardiac muscle contracts in response to the rise in intracellular Ca\textsuperscript{2+}, which is released from the sarcoplasmic reticulum (SR) and from influx across the sarcolemma, primarily through voltage-sensitive channels. SR Ca\textsuperscript{2+}-ATPase (SERCA) recycles Ca\textsuperscript{2+} from the cytosol into the lumen of the SR, and the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX) mediates the movement of cytosolic Ca\textsuperscript{2+} across the sarcolemma to the extracellular space. The exchanger transfers ≈20% of the cellular Ca\textsuperscript{2+} during a contraction-relaxation cycle, with the remainder being accumulated again in the SR via SERCA, although this varies between species and during development.\textsuperscript{1,2} Both SR Ca\textsuperscript{2+} uptake and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange appear to be independently capable of mediating cardiac relaxation. Importantly, alterations in any of the activities associated with this complex process cause a corresponding change in the amount of Ca\textsuperscript{2+} released from the SR and, thereby, affect the force of cardiac contraction.\textsuperscript{3} In addition to its role in Ca\textsuperscript{2+} efflux, the exchanger has been reported by Leblanc and Hume\textsuperscript{4} to play a role in cardiac Ca\textsuperscript{2+} influx during cardiac excitation, contributing to the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release mechanism.

The exchanger is regulated at the transcriptional level in cardiac hypertrophy, ischemia, and failure. There is rapid upregulation of NCX1 (an NCX isoform) mRNA in response to pressure overload.\textsuperscript{5-7} The exchanger is also upregulated in end-stage heart failure,\textsuperscript{8-11} but exchanger levels drop in ischemia.\textsuperscript{12} Hasenfuss et al\textsuperscript{13} have demonstrated that variations in the concentration of the NCX protein among myocytes from failing human hearts are inversely related to variations in the frequency-dependent increase in diastolic Ca\textsuperscript{2+}, which results in diastolic dysfunction. Therefore, the upregulation of the exchanger has been proposed as a compensatory mechanism for the decrease in SERCA level. The increase in exchanger activity would increase Ca\textsuperscript{2+} extrusion and act to preserve low diastolic Ca\textsuperscript{2+} levels. However, others have proposed that any change in either the abundance or activity of the exchanger or SERCA would lead to an imbalance in Ca\textsuperscript{2+} homeostasis.\textsuperscript{14} When the ratio of
SERCA to NCX is decreased, peak systolic Ca$^{2+}$ is blunted because forward-mode exchanger activity will lower [Ca$^{2+}$].

Furthermore, the resulting lower levels of systolic Ca$^{2+}$ coupled with the prolonged action potential duration in the failing cardiocytes would increase the level of reverse-mode exchanger activity in the late phases of the action potential. This would slow the decay of the Ca$^{2+}$ transient, contribute to an increased [Ca$^{2+}$], and cause diastolic dysfunction.\(^{14-16}\) In failing rabbit hearts, local SR Ca$^{2+}$ release elevates local [Ca$^{2+}$], which stimulates Ca$^{2+}$ extrusion via the exchanger.\(^{17}\) This Ca$^{2+}$ extrusion produces a transient inward depolarizing current that can produce a delayed afterdepolarization and brings the diastolic membrane potential closer to the threshold to trigger an inappropriately timed action potential. With the increased expression of the exchanger resulting in an increased activity, any given amount of SR Ca$^{2+}$ release will result in a greater transient inward depolarizing current and an increased probability that a triggered arrhythmia will result.\(^{18}\) Although it is still controversial, there is growing evidence of an important role of the exchanger in altered excitation-contraction coupling and arrhythmogenesis in the context of cardiac hypertrophy and failure. But, unfortunately, very little is known about the genetic elements and transcription factors that regulate NCX1 expression.

The NCX1 gene contains three promoters and multiple 5′–untranslated region (UTR) exons upstream from the coding region. As a result of alternative promoter usage and the resulting alternative splicing, there are multiple tissue-specific variants of NCX.\(^{19-23}\) We have shown that a construct containing only 184 bases of the 5′-flanking region, the H1 exon, and 67 bases of the first intron is sufficient for cardiac-directed expression and α-adenrenergic stimulation of the luciferase reporter gene.\(^{23}\) Furthermore, the feline NCX1 heart promoter did not drive expression in mouse L cells, human CHO cells, or 293 cells. The NCX1 gene has also been cloned in both the rat\(^{24}\) and human,\(^{25}\) with each having three promoters with a structure identical to that of the feline NCX1 gene.

On the basis of results from 5′ rapid amplification of cDNA ends (RACE) together with genomic mapping and transfection data, we proposed that the promoter 5′ to the H1 exon was cardiac specific and activated by GATA-4 and serum response factor. An alternative model proposes that the NCX1 gene is driven by a multipartite promoter in which no single transcription start can be called tissue specific.\(^{23}\) In this model, the Br1 promoter drives ubiquitous expression of NCX1, and the H1 and K1 promoters serve as auxiliary promoters augmenting expression in various tissues. In the present study, we have produced transgenic mouse lines containing a luciferase reporter driven by the H1 promoter of the feline NCX1 gene to directly test the role of the NCX1H1 promoter. Is the NCX1H1 promoter responsible for cardiac-specific expression in the adult myocardium and also for expression during development? Is it sufficient for upregulation in response to pressure overload?

The present study shows for the first time that the DNA region, including –1831 to 67 bp of intron 1, encompassing all of exon H1 is responsible for heart-specific expression of the exchanger in both the embryo and adult and is also responsible for the upregulation of NCX1 in response to pressure overload.

### Materials and Methods

#### Production of Transgenic Mouse Lines

A 2-kb feline NCX1 promoter construct containing 1831 bp of the 5′-flanking region, the first cardiac-specific exon (H1), and the following 67 bp of the adjacent first intron were introduced into the multiple cloning site of the firefly luciferase reporter vector pGL2 (Promega) as described.\(^{18}\) The resulting construct, pNCX1H1-luc, was digested with Kpn1 and BamHI to release a linearized 4.8-kb NCX promoter-luciferase reporter construct. After gel purification, the DNA was dried and redissolved in injection buffer (10 mmol/L Tris, pH 7.4, and 0.1 mmol/L EDTA). The constructs were verified by double-stranded sequencing performed at the Biotechnology Resource Facility of the Medical University of South Carolina. DNA sequence analyses were performed with the use of the Mac Vector 6.5 software package (Oxford Molecular Group). Pronuclear microinjection in the FVB/N mouse strain was performed at the Transgenic Core Facility of the University of Cincinnati, Cincinnati, Ohio (Dr Jon Neumann). Genomic DNA was isolated from tail-clip biopsies with the QIAamp Kit (Qiagen). Founder mice were identified by polymerase chain reaction (PCR) and confirmed by genomic Southern blots. PCR screening was performed with a primer pair specific for a 700-bp fragment of the firefly luciferase gene (lac2 sense, 652-GCACTGATAATGAATCTCTCCTGG-674; lac4 antisense, 1346-CTTACTGGGACGAAGACGAAC-1368) and a control primer pair specific for a 384-bp fragment of the murine thyroid-stimulating hormone β gene (TSH β1 sense, TCCCTAAA-GATGCGATTAG; TSH β2 antisense, GTAACTCACTCAT-GAAGT; Southern blots were performed according to standard techniques and probed with a 400-bp fragment of the feline NCX1H1 promoter. Transgenic lines were maintained by mating transgenic offspring with normal FVB/N mice from Taconic Labs (German-town, NY).

#### Preparation of Luciferase cDNA Probe

A 776-bp EcoRI-ClaI fragment from the gEM-Luc vector from Promega was cloned into p-BluescriptSK from Stratagene. The p-BSLuci-1 plasmid DNA containing the luciferase insert was isolated from a single clone and was checked by use of restriction enzyme mapping and sequencing. A sense probe was made with the use of T3 polymerase on Clal-digested pBSLuci-1 plasmid, and an antisense probe was made with the use of T7 polymerase on EcoRI-digested pBSLuci-1 plasmid.

#### In Situ Hybridization

The sense and antisense \(^{35}S\)-labeled cDNA probes were transcribed from both the cloned luciferase (see above) and the PCR-generated NCX1 exon 2 probes, as described in detail elsewhere.\(^{26}\) The sense probe was used as a negative control, and there was no nonspecific hybridization with either the cloned luciferase or the PCR-generated NCX1 exon 2 probes. Additionally, there was no nonspecific hybridization with the antisense cloned luciferase probe when transgenic-negative embryos were used, indicating that both antisense probes are specific. Radioactive in situ hybridization was performed on frozen 10-μm sections on at least 14 transgenic-positive and 8 transgenic-negative mouse embryos at E8.5 to E16.5 (where E indicates embryonic day) by using the techniques previously described.\(^{27}\) Embryos were photographed in both light field and dark field on an Olympus AX70 microscope.

#### Northern Blot Analysis

Total RNA was isolated from ventricles, as described,\(^{28}\) and fractionated by electrophoresis in a denaturing formaldehyde/agarose gel. It was transferred to a nylon membrane and hybridized with a PCR-generated NCX1 exon 2 or GAPDH probe.
Western Blotting
Mouse ventricular tissue was homogenized, and protein extract was subjected to PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-NCX1 (R3F1, generous gift of Dr Ken Philipson, UCLA, Los Angeles, Calif) or anti-GAPDH (6C5, RD1).

Pressure-Overload Mouse Model
Acute left ventricular (LV) pressure overload was created by microsurgical transverse aortic constriction (TAC), as described previously.29,30 Briefly, transgenic FVB/N mice carrying the NCX1H1-luc construct 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 6-0 prolene suture around the vessel over a blunted 27-gauge needle. Two and 7 days after surgery, the heart was removed from deeply anesthetized animals. LV and right ventricular (RV) weight was determined after microdissection, and tissue samples of the heart and other organs were snap-frozen in liquid nitrogen. The body weight at the end of the study was used for indexing purposes. Sham-operated control animals underwent an identical protocol except that the 6-0 prolene suture was passed around the aorta but not tied and was then removed. All animal experimentation was performed in accordance with National Institutes of Health Guidelines, and protocols were approved by the Institutional Animal Use and Care Committee of the Medical University of South Carolina.

Luciferase Activity in Mouse Tissue
To quantify NCX promoter expression, samples of heart, brain, liver, kidney, uterus, spleen, and skeletal muscle tissue were ground in a mortar under liquid nitrogen. The resulting powder was solubilized in 1X reporter lysis buffer (Promega). After they were frozen in liquid nitrogen and thawed, the lysates were cleared by centrifugation, and the supernatant was used for the following assays: Luciferase activity was measured by using the luciferase assay system (Promega) according to the manufacturer’s protocol on a luminometer (Autolumat 953, Berthold). For normalization, protein concentration of the tissue lysate was determined with a modified Lowry protein assay kit (Bio-Rad).

Statistical Analyses
Multiple comparisons of experimental groups were performed with factorial ANOVA and the Scheffé F post hoc test with the use of StatView 5.0 software (SAS Institute). A value of $P<0.05$ was considered to be significant. All data are shown as mean±SEM.

Results
Production of Transgenic Mice
The linearized NCX1H1-luc DNA construct that contains the feline H1 promoter fused to a luciferase reporter gene was introduced into the germline of FVB/N mice by pronuclear microinjection. PCR screening and Southern blot analysis confirmation yielded five putative founder animals. Two F0 animals underwent an identical protocol except that the 6-0 prolene suture was tied around the vessel over a blunted 27-gauge needle. Two and 7 days after surgery, the heart was removed from deeply anesthetized animals. LV and right ventricular (RV) weight was determined after microdissection, and tissue samples of the heart and other organs were snap-frozen in liquid nitrogen. The body weight at the end of the study was used for indexing purposes. Sham-operated control animals underwent an identical protocol except that the 6-0 prolene suture was passed around the aorta but not tied and was then removed. All animal experimentation was performed in accordance with National Institutes of Health Guidelines, and protocols were approved by the Institutional Animal Use and Care Committee of the Medical University of South Carolina.

Expression of the Transgene in Adult Mice
In the adult mouse, the NCX1 gene is expressed at high levels in the heart, kidney cortex, and parts of the brain and at low levels elsewhere. To verify that the H1 promoter directs cardiac-specific expression in vivo, various tissues of 6- to 12-week-old transgenic and nontransgenic littermate controls were tested for luciferase activity. In both lines expressing the transgene, no luciferase activity was detected in the kidney, liver, spleen, uterus, or skeletal muscle, but very high levels of luciferase expression were detected in the heart (Table). Interestingly, luciferase activity just above the background level was detected in the brain of the line 72 transgenic animals. As expected, no luciferase activity was detected in any tissue from the nontransgenic littersmates. Luciferase activity was slightly higher in the LV compared with the RV, and the atria expressed levels very similar to those found in the ventricles (Table).

Developmental Expression
On the basis of the finding that the DNA region from −1831 to 67 bp of intron 1 of the feline NCX1 gene directs cardiac-specific expression in the adult mouse, we wanted to determine whether it also directs the spatiotemporal pattern of NCX1 expression during development. We have previously shown that endogenous NCX1 is expressed in the cardiogenic plate by E7.75 to E8.0, which is before the first heartbeat. Importantly, NCX1 is initially expressed in a heart-restricted pattern within the early mouse embryo.26 Therefore, to determine the role of the promoter region 5′ from the H1 exon (NCX1H1) in development, we studied the pattern and onset of expression of NCX1H1-luc transgene within the developing mouse embryonic heart by the use of in situ hybridization.

The luciferase reporter gene did not differ between the two lines, indicating a reciprocal activation of the transgenes in their respective chromosomal insertion sites.
To determine whether this is also true protein levels are increased with pressure overload in a feline. We have previously demonstrated that NCX1 transcript and expression is present. NCX1 indicates probing with antisense endogenous NCX; lucf, probing with antisense luciferase. Bar hybridization analysis of both NCX1 mRNA expression (E) and NCX1H1-luc expression (F and I), note that only the endogenous NCX1 expression is present. NCX1 indicates probing with antisense endogenous NCX; lucf, probing with antisense luciferase. Bar=293 μm (A through F); bar=118 μm (G through I).

Figures 1F and 1I; n=8/8). However, endogenous NCX1 was normally expressed within all embryos (see Figures 1A, 1B, and 1G and Figures 2A and 2B). Expression of NCX1H1-luc and endogenous NCX1 was present only within hearts of the E8.5 and E9.0 (see Figure 1) transgenic-positive embryos, indicating that the cloned NCX1H1-luc promoter is sufficient and capable of driving the normal spatiotemporal pattern of NCX1 expression within the early mouse embryo. Note that the pattern of NCX1H1-luc expression is identical to that previously described for endogenous NCX1. Importantly, at later fetal stages (E14.0 and older), when the normal heart-restricted pattern of the endogenous NCX1 is no longer present, the NCX1H1-luc cDNA expression remains heart specific (Figure 2). Although endogenous NCX1 is still highly expressed within the heart, expression is also now detectable within the neural tube, smooth muscle of the esophagus, and bronchus. This is similar to our recent findings in which a lacZ reporter was knocked into the endogenous NCX1 locus. However, NCX1H1-luc cDNA expression remains heart specific. Luciferase expression is restricted to only the cardiomyocytes within both ventricles and atria. It is absent from the endocardial cushion tissues of the future valves, indicating not only that the cloned NCX1H1-luc promoter is expressed in a heart-specific manner but also that it is sufficient and capable of driving the normal spatiotemporal pattern of NCX1 expression within the embryonic mouse heart itself. These results indicate that the 1831-bp feline H1 promoter construct contains the cis-regulatory elements necessary for responding to the appropriate developmental cues and directs cardiac-specific expression in embryonic mice.

Response of the Transgene to Pressure Overload

We have previously demonstrated that NCX1 transcript and protein levels are increased with pressure overload in a feline model of hypertrophy. To determine whether this is also true in the mouse, transgenic mice expressing the feline NCX1H1-luc promoter/reporter construct were subjected to acute cardiac LV pressure overload by microsurgical TAC. To control for the surgical trauma, sham-operated littermates underwent the same anesthesia and thoracotomy protocol without TAC. Animals from both the sham-operated and TAC groups were euthanized 2 days and 7 days after surgery, and findings in these animals were compared with those in transgenic control animals without surgical intervention. Doppler echocardiography was used to determine the transconstriction pressure gradient. The pressure gradient across the constriction averaged 42 mm Hg in TAC-treated animals. LV mass indexed to body weight was significantly increased after 7 days of pressure overload. No significant change was detected after 7 days in the sham-operated mice or in the groups of mice euthanized after 2 days of pressure overload (Figure 3).

We first examined whether the endogenous exchanger was upregulated with pressure overload. Northern analysis of NCX1 mRNA in the LV revealed both the full-length 7-kb transcript and the 1.8-kb band that we and others have reported. Unlike the transcript found in the cat, the 1.8-kb transcript was more abundant in the mouse. Importantly, transcript levels of both the 7-kb and 1.8-kb species increased significantly in hearts after 48 hours of pressure overload compared with the control condition (Figure 4A). NCX1 transcript levels were still slightly above control levels after TAC for 7 days. Changes in the levels of NCX1 protein after pressure overload were measured by immunoblot analysis. The mature exchanger protein appears as two bands with molecular masses of 120 and 160 kDa when ventricular extracts were run under nonreducing SDS-PAGE. Figure 4B demonstrates a clear increase in exchanger protein in mice after 7 days of banding compared with control or sham-operated mice. This is consistent with the findings recently reported by Wang et al in mice subjected to thoracic aortic banding.

To quantify the response of the feline NCX1H1-luc promoter/reporter transgene, protein extracts were prepared from frozen LV and RV tissue samples from control, sham-operated, and TAC-operated transgenic mice. Luciferase activity was assayed and normalized for protein content (Figure 5). Parallelng the development of hypertrophy (as demonstrated by LV/body weight ratio, Figure 3), TAC-operated mice, compared with control and sham-operated
mice, showed an ~2-fold upregulation of NCX1H1 promoter activity in the LV after 7 days of LV pressure overload. There was no significant upregulation in the mice that were studied after 2 days of LV pressure overload. Comparison with the RV samples demonstrated that after 7 days of pressure overload, NCX1H1 promoter upregulation occurs specifically in the LV. Importantly, this corresponds to the level of NCX1 mRNA and protein upregulation (~2-fold) that we and others have observed in other models of pressure-overload hypertrophy.5–7

Discussion

The present study has analyzed the regulation of the feline NCX1 gene in transgenic mice. Our results indicate that the 1831-bp NCX1 heart promoter directs cardiac-specific expression of the exchanger in development and regulates its expression in the normal and hypertrophic heart. NCX is encoded by a small multigene family. NCX1 is expressed at low levels in almost all cell types but is most abundant in the heart, the kidney cortex, and brain. The expression of two other genes, NCX2 and NCX3, is restricted primarily to the brain and skeletal muscle, respectively. At least 15 splice variants of NCX1 have been identified, and they are expressed in a tissue-specific manner. The splice variants differ in a small domain at the C-terminus of the large cytoplasmic loop.5-7-RACE has also revealed that NCX1 has three distinct 5'-UTRs selectively expressed in the heart, kidney, and brain. We were the first to demonstrate that the NCX1 gene contains three promoter regions, which regulate the expression of three different 5'-UTRs that are spliced to a common core (exon 2) containing the protein coding sequence.19 We proposed that these alternative promoters orchestrated the tissue-specific expression pattern of NCX1. The 5'-most promoter, H1, drove the expression in neonatal cardiocytes but not in mouse L cells.19 Therefore, we and others have proposed that the promoter 5' from the H1 exon drove the expression in the heart, the promoter 5' from the K1 exon drove the expression found exclusively in the kidney, and the promoter 5' from the

Figure 2. Spatiotemporal detection of endogenous NCX1 and NCX1H1-luc reporter cDNA expression within the fetal mouse cardiovascular system. Transverse serially sectioned transgenic-positive E14.5 fetus is shown after radioactive in situ hybridization analysis of both NCX1 mRNA expression (A and B) and NCX1H1-luc expression (C and D). A and B, Endogenous NCX1 expression is strongly present throughout the fetal heart (except within the valvular cushion tissues, indicated by asterisk) and is also present within the neural tube (nt), smooth muscle of the esophagus (o), and bronchus (b) leading to the lungs. C and D, NCX1H1-luc reporter cDNA is expressed only within the fetal heart. Also note that (similar to the endogenous NCX1 expression) the NCX1H1-luc reporter cDNA is absent within the valvular cushion tissues (indicated by asterisk). rv indicates right ventricle; lv, left ventricle; NCX1, probing with antisense endogenous NCX; and lucf, probing with antisense luciferase. Bar=293 μm.

Figure 3. Effect of LV pressure overload by TAC on LV hypertrophy. Results of LV weight/body weight ratios are compared for TAC-treated animals vs sham-operated and control animals. *P<0.01 vs all other group means.

Figure 4. Pressure overload–induced increases in NCX1 transcript and protein levels. A, Northern blot analysis of total RNA extracted from control LVs and 48-hour TAC-treated LVs hybridized with a probe specific for NCX1 exon 2 and GAPDH. The GAPDH mRNA levels were used as an internal standard to demonstrate equal loading for each RNA sample. B, Total protein (30 μg) from control, 7-day (7D) sham-operated, and 7D TAC-treated mouse hearts subjected to PAGE and immunoblotted with anti-NCX1 (R3F1).37 Equal protein loading in each of the lanes is demonstrated by immunoblot analysis using anti-GAPDH. Northern and Western blot data are representative of 5 independent experiments.
Figure 5. Effect of LV pressure overload by TAC on NCX1H1 promoter activity. Animals were euthanized 2 days and 7 days after surgery. Luciferase activities in LV and RV extracts normalized for protein concentration are shown for TAC-treated animals vs sham-operated and control animals. *P<0.01 vs all other groups.

Br1 exon regulated the expression in the brain and also regulated the expression at low levels in a wide range of tissues.19,24,35 In contrast, Scheller et al25 presented evidence that no single transcript start site can be called tissue specific. They propose that the H1 and K1 promoters act as auxiliary promoters, with each or both augmenting the ubiquitous Br1 promoter in specific tissues.

The present study demonstrates conclusively that the feline NCX1 heart promoter does not augment the expression of the ubiquitous promoter (Br1) in a variety of tissues but directs cardiac-specific expression of the exchanger. In both transgenic lines, luciferase was expressed at high levels only in the heart. It is expressed in both the atria and the ventricles. Luciferase activity just above the background level was detected in the brain. Importantly, this was >800-fold lower than the activity observed in the heart. Luciferase activity was not detected in any other tissue. In addition, the heart promoter was sufficient and capable of driving the normal spatiotemporal pattern of NCX1 expression in cardiac development. Luciferase was expressed only within the ventricles and atria of the E8.5 and E9.0 transgenic animals. At E16, when the endogenous exchanger is beginning to be expressed in the neural tube and smooth muscle of the esophagus and bronchus, the heart promoter still drives the expression in a heart-restricted manner.

Although the data presented in the present study do not rule out the possibility of low-level cardiac expression of the exchanger driven by the Br1 promoter, they demonstrate conclusively that the feline NCX1 heart promoter drives cardiac-restricted expression of the exchanger. It is possible that NCX1 transcripts with a Br1 initiation site are expressed at low levels in the feline heart, but we have not detected any with either 5'-RACE19 or reverse-transcription PCR (data not shown).

Comparison of rat,24,35 human,25,36 and feline19 gene sequences in this region revealed a >90% sequence identity from −250 to −50 bp as well as significant similarity between the respective H1 exons. This may explain why the feline NCX1 heart promoter directs the correct spatiotemporal pattern of exchanger expression in the mouse. These studies complement and extend the data obtained with the use of NCX1-cardiac promoter constructs to drive reporter gene expression in vitro transient assays.19,25 Deletion analysis revealed that a minimal cardiac promoter fragment from −184 to 172 bp is sufficient for cardiac expression and α-adrenergic stimulation. Mutational analysis revealed that both the CArG element at −80 bp and the GATA element at −50 bp were required for expression in neonatal rat cardiomyocytes.35 Gel-mobility supershift analysis demonstrated that the serum response factor binds to the CArG element and that 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 element in the first intron (94 to 114 bp), which is essential for phenylephrine upregulation but bears no homology to any known transcription element.23

Although our earlier study demonstrated that the 1831-bp heart promoter mediates α-adrenergic stimulation,23 it remained to be seen whether it also mediated upregulation in response to hemodynamic load. In vitro transfection of neonatal cardiomyocytes is an extremely valuable system to identify and begin to elucidate the role of specific cis elements, which mediate changes in expression. However, the significance of the 1831-bp NCX1 heart promoter and the elements identified by transfection need to be confirmed in adult cardiomyocytes in vivo. The transgenic mouse model used in the present study has allowed us to demonstrate that the NCX1 heart promoter mediates the hypertrophy-induced upregulation of exchanger expression. Luciferase activity is upregulated by nearly 2-fold after 7 days of aortic banding. This corresponds to the level of upregulation of the endogenous exchanger after 1 week of pressure overload.5 Therefore, the DNA region including −1831 to 67 bp of intron 1, encompassing all of exon H1, is sufficient for directing cardiac expression and upregulation of the exchanger in response to pressure overload.

Importantly, the present study has demonstrated the feasibility of using transgenic lines with the NCX1 promoter to examine the relative importance and role of CArG, GATA, the E-box, and the novel element in mediating the expression of NCX1 in the developing heart and in the normal and hypertrophic heart.

Finally, the present study demonstrates the utility of the NCX1 heart promoter in the expression and analysis of other transgenes, in which early cardiac-restricted expression is a necessity.

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