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Regulation of the NCX1 Gene in the Normal and Hypertrophied Myocardium

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REGULATION OF THE NCX1 GENE IN THE NORMAL AND HYPERTROPHIED MYOCARDIUM

by

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ABSTRACT

The sodium-calcium exchanger (NCX1) plays a major role in calcium efflux, and therefore, in calcium homeostasis in the heart. The exchanger mRNA and protein is rapidly upregulated within one hour of pressure overload. In this work, the exchanger has been shown to be upregulated by persistent pressure overload for at least one to two weeks. The 5' end of the NCX1 gene has been characterized in order to examine transcriptional regulation of the cardiac promoter.

Four feline NCX1 isoforms of the 5' untranslated region have been cloned: one from brain (Br1), one from kidney (K1), and two from heart (H1 and H2). Each isoform is expressed in a tissue specific manner. Seven unique clones of the alternatively spliced portion of the NCX1 intracytoplasmic loop have been identified in libraries from feline heart, brain, liver and kidney. These also exhibit tissue specific expression. Three are unique to brain. Three clones are expressed in kidney; one of these three is exclusively expressed in fetal kidney. Two isoforms are expressed in heart: one is unique to heart, and the other is predominately expressed in kidney. Both cardiac exchanger loop isoforms are upregulated for at least one to two weeks under continuous pressure overload.

The 5'-end of the feline NCX1 gene has been cloned. The four tissue specific 5'untranslated regions are encoded by three alternatively spliced exons that have been mapped to nonoverlapping genomic clones. Exons K1 and Br1 lie on the same 13 Kb genomic clone separated by approximately 1 Kb. H1 has been mapped to a separate upstream 15 Kb genomic clone. Exon 2, the first common exon which encodes the translational start site, is found on a separate downnstream 17 Kb genomic clone. The K1 and Br1 isoforms each have unique promoters. The two heart isoforms, H1 and H2, share a single cardiac promoter. Primer extension has been used to map three transcriptional start sites for the cardiac promoter at 131, 139, and 143 bp 5' of the AUG. These sites have been confirmed by S1 nuclease protection assays.

A DNA fragment containing 2000 bp of cardiac 5' flanking region, exon H1, and 67 bp of intron drives expression in cardiocytes, but not mouse L cells. Through deletion analysis, a fragment of the 5' flanking region from -184 to +200 has been determined to be the minimal promoter sufficient to drive expression and alpha-adrenergic induction in cardiocytes. At least one enhancer has been detected between -2000 and 1054, and another between +22 and +112. One or more negative elements exist between -1054 and -184. Further study will lead to identification of specific positive and negative elements involved in transcriptional control of the NCX1 gene.

CHAPTER I
INTRODUCTION

The Sodium-Calcium Exchanger

The sodium-calcium exchangers are antiport proteins that exchange sodium for calcium across extracellular membranes. There are three general types of sodium-calcium exchangers: the retinal rod exchanger, the mitochondrial exchanger, and NCX.

The retinal rod exchanger is specifically expressed only in the retinal rod. This exchanger cotransports a potassium with each calcium ion with a stoichiometry of 4 $Na^+/(Ca^{++}+K^+)$. This exchanger has very little amino acid homology with NCX (1). The mitochondrial exchanger has not been well characterized.

NCX is involved in the regulation of intracellular calcium in a variety of cell types including heart, brain, kidney, smooth muscle, lung and spleen (2-6). Very low levels of NCX expression can be detected ubiquitously by polymerase chain reaction. In each reaction cycle, this exchanger exchanges three sodium atoms for one calcium atom and is therefore electrogenic. This antiporter is often involved in the net efflux of calcium, driven by a sodium gradient established through the sodium-potassium ATPase (7-9).

Regulators of the NCX include intracellular calcium and sodium. Both calcium and sodium bind to specific transport binding sites as well as separate regulatory sites. Binding of these ions to the respective regulatory sites modulates transition of NCX between active and inactive states. Adenosine triphosphate (ATP) also regulates NCX, increasing its activity (10-13). The mechanism of this regulation is not well understood. One theory is that ATP directly

affects exchanger activity through phosphorylation of the enzyme via a protein kinase C dependent pathway (14). Another theory suggests that ATP increases exchanger activity indirectly through modulation of the surrounding phospholipid bilayer through synthesis of phosphatidylinositol [4,5]-bisphosphate(15).

The first cDNA for NCX, NCX1, was cloned by screening a canine cardiac lambda gt11 library with anti-exchanger antibody(16). This clone encoded a nine hundred seventy amino acid protein. The amino terminal thirty-two amino acids are believed to comprise a signal sequence cleaved from the mature protein. NCX1 is one of only a few transport proteins containing signal sequences. The function of this signal sequence is unknown. The exchanger protein is glycosylated extracellularly at position N-9, but this glycosylation does not seem to alter exchanger activity when it is expressed in oocytes (17).

On western analysis NCX1 can be identified as a one hundred twenty kDa protein. By hydropathy analysis, the mature protein is predicted to consist of eleven hydrophobic transmembrane segments with a five hundred twenty residue intracellular loop between transmembrane segments five and six (16, Figure 1-1). Several important regional domains have been identified. Amino acids 180-202 encode portions of transmembrane segments four and five. This region exhibits 43% identity with a similarly located region of the Na^+/K^+ ATPase. Within this region is a nine base pair sequence, beginning at amino acid 194, that is conserved with 61% identity in many ATPases and is important for ion binding (16).

The NCX1 intracellular loop is not necessary for activity but does contains a number of domains important for regulation of the

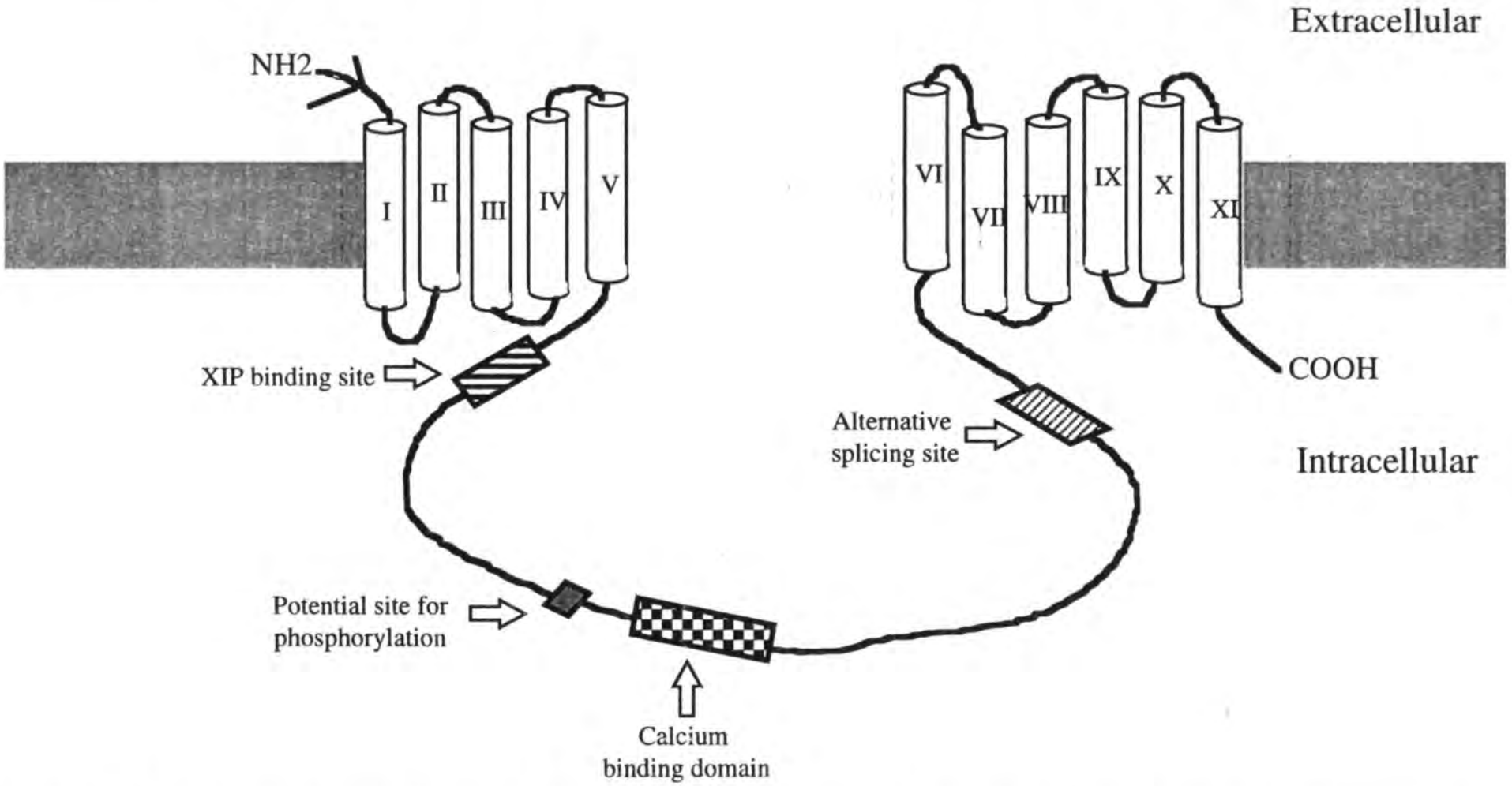


Figure 1-1 Schematic illustration of the NCX1 protein structure. Based on hydropathy analysis, the protein structure contains eleven putative transmembrane regions with a 520 amino acid intracellular loop between transmembrane regions 5 and 6. Several important regulatory regions have been identified within the putative loop. These include sites for regulation by intracellular calcium and exchanger inhibitory protein (XIP). A region formed by alternative splicing and a potential site for phosphorylation have also been identified.

exchanger. Deletion of this loop region produces a functional exchanger molecule but eliminates regulation of the protein by such factors as sodium, calcium, and ATP (18). Within the intracellular loop, amino acids 219-238 form a basic, hydrophobic region that resembles the calmodulin binding domains of a number of proteins regulated by calcium. NCX1 activity is not regulated by calmodulin; however, a synthetic peptide termed the exchanger inhibitory peptide (XIP) binds to this region and does regulate exchanger activity. It is postulated that this region is involved in the autoregulation of the exchanger, inhibiting ion transport through binding to another region of the exchanger protein (19,20).

Amino acids 570 to 645 at the carboxyl end of this loop are encoded by six exons, exons A through F, which can be alternatively spliced to form a number of isoforms (Figure 1-2). Each splice variant must begin with either exon A or B, which are mutually exclusive, and may contain any combination of the remaining cassette type exons (21). Sixteen different isoforms have been shown to be expressed out of thirty-two possible combinations. The isoforms that have been identified are expressed in a tissue specified manner (22). It is of interest to tie differences in the inclusion or exclusion of particular exons to differences in sensitivity to certain regulators, localization within the cell, or other protein-protein interactions. This may provide information as to the significance of the developmental and tissue specific pattern of expression of the loop isoforms.

Recently cDNA from two additional NCX genes have been cloned: NCX2 and NCX3. NCX1 and NCX2 share 68% identity, NCX2

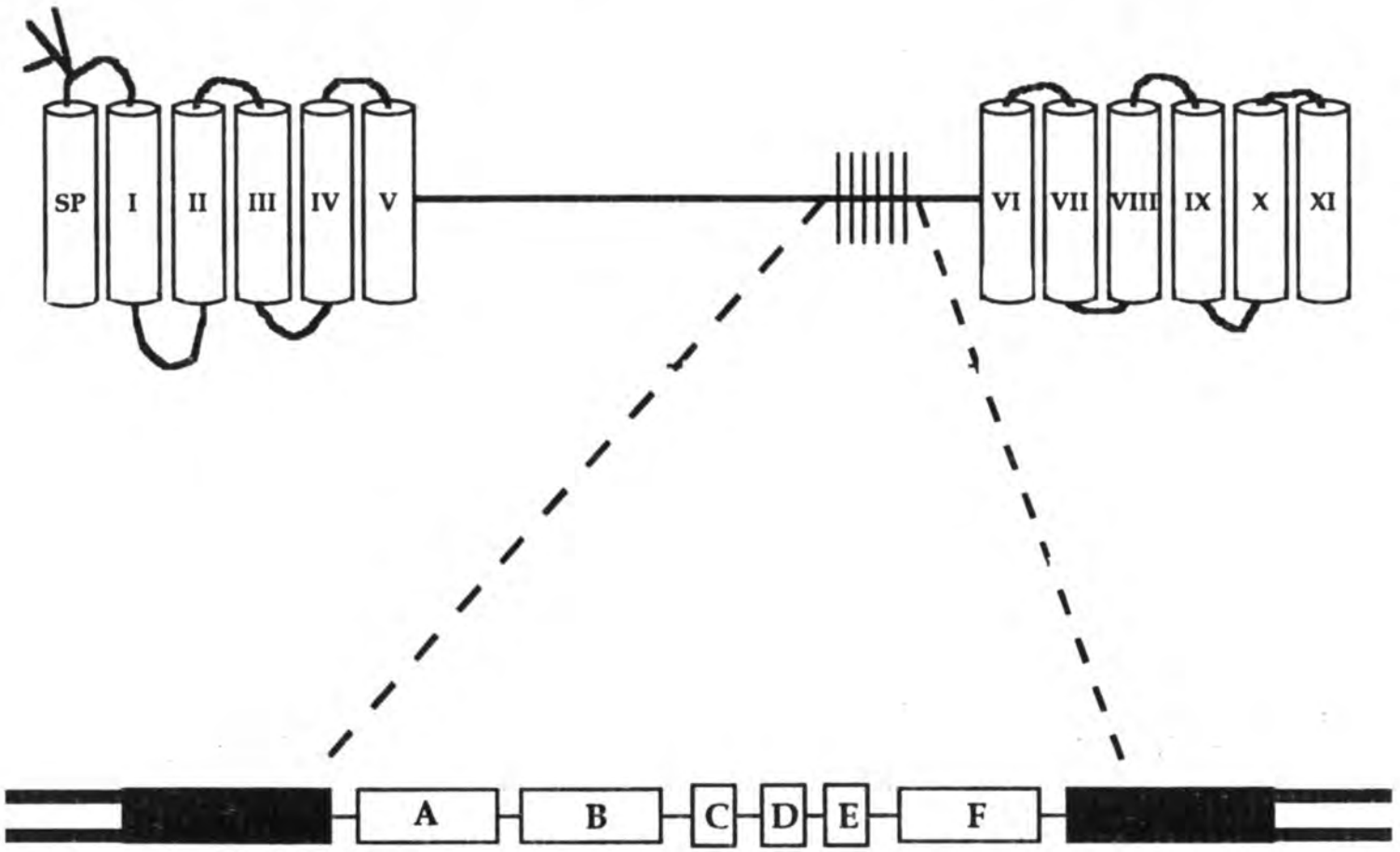


Figure 1-2 Schematic illustration of exons encoding the variable region of the NCX1 intracytoplasmic loop. This region consists of six exons, A-F. Exons A and B are mutually exclusive. Exons C-F function as cassette exons. (21)

and NCX3 share 73% identity, and NCX1 and NCX3 share 75% identity at the amino acid level. Hydropathy plot analysis predicts that all three share the same basic structure: eleven transmembrane segments with a large cytoplasmic loop. The NCX genes are dispersed with NCX1, 2 and 3 being mapped to chromosomes 17, 7, and 12 respectively (23-24). Similarly, transcripts from each gene vary in size. NCX1 produces a 7.2 kb transcript, NCX2 produces a 5 kb transcript and NCX3 produces 6 kb transcript. Expression of NCX2 and NCX3 is largely restricted to brain and skeletal muscle.

The Exchanger and Calcium Homeostasis

The importance of the sodium-calcium exchanger in understanding heart function is underscored by its role in calcium homeostasis. As in most cells, calcium serves as a second messenger in the cardiocyte, carrying signals from the cell membrane to the interior. Calcium is also a key component for excitation contraction coupling. Before contraction, the cardiac cell membrane is depolarized by the opening of fast sodium channels and, later, slow calcium channels. The entrance of calcium into the cardiocyte by these slow calcium channels as well as the sodium-calcium exchanger induce the sarcoplasmic reticulum to release its calcium stores into the cytoplasm (25-26). As calcium enters the cytoplasm, it complexes with troponin C allowing myosin heads to contact and move the actin filaments resulting in sarcomeric shortening--contraction. The contraction continues until calcium leaves the cytoplasm, primarily through the sarcoplasmic reticulum calcium ATPase which moves approximately 80 percent of the calcium into

the sarcoplasmic reticulum, and the exchanger which moves approximately 20 percent of the calcium across the cell membrane. A plasmalemmal calcium pump also moves calcium out of the cell but has been shown to play only a minor role in this regard (25-26). While only approximately 20 percent of the cytoplasmic calcium exits via the exchanger, cardiocytes which lack a functional exchanger are unable to relax showing the importance the exchanger plays in the normal functioning myocardium (27).

Changes in exchanger activity may affect the availability of cytoplasmic calcium which can be stored in the sarcoplasmic reticulum for later release. For example, less exchanger activity means less calcium will leave the cardiocyte and, hence, more will be taken into the sarcoplasmic reticulum. Therefore, changes in exchanger activity can regulate the calcium level stored in the sarcoplasmic reticulum, and thus the level released into the cytoplasm; it follows that the level of exchanger activity affects heart contractility. The attempt at short and long term regulation of calcium stores through modulation of the exchanger may allow for adaptive and maladaptive changes as the heart responds to increased exercise and stress.

The sodium calcium exchanger may play a role in the pathogenesis of pressure overload hypertrophy and heart failure. Both overload hypertrophy and heart failure have been associated with alterations in calcium handling which include slowing of calcium transients and retarded diastolic calcium decline (28-30). These changes are coincident with diminished calcium accumulation in the sarcoplasmic reticulum (with decreased expression and activity of

both the SR calcium ATPase and its regulatory protein, phospholamban) and increased exchanger activity (31-33). Thus, in overload hypertrophy and heart failure some of the calcium uptake by the sarcoplasmic reticulum shifts to calcium extrusion by the exchanger. These findings suggest that exchanger mRNA may be differentially expressed in response to pressure overload. This hypothesis was tested using a model of early right ventricular hypertrophy.

The Exchanger In Early Hypertrophy

In order to identify genes that are differentially expressed in normal cardiocytes versus those adapting to pressure overload, a feline *in vivo* model of right ventricular pressure overload was established by Dr. John Rozich under the direction of Dr. George Cooper. In this model the exchanger is acutely sensitive to changes in pressure induced load; one hour of pressure overload produced a two-to-four fold increase in the level of exchanger mRNA in the RV when compared to the levels in the LV control. Westerns showed a significant increase in exchanger protein in the membrane in response to pressure overload (34-35).

The sequence of the exchanger 3' untranslated region suggested that exchanger mRNA may be regulated by message stability. A portion consists of an AU-rich region containing a Shaw-Kamen sequence (AUUUA). This Shaw-Kamen sequence has been reported in the mRNA of low abundance, high turnover proteins, such as lymphokines. cytokines, and immediate early genes, that are regulated by message stability. This region of the exchanger 3'UTR is

well conserved across species with 100% identity between feline and canine sequences and 88% between human and bovine (16, 36-38).

Message half-life analysis has been carried out in Dr. Menick's laboratory by Usha Thacker using a neonatal rat cell model. Cardiocytes were either untreated or treated with veratridine for four hours. Actinomycin D was then added and RNA isolated at various time points. The mRNA half-life of untreated versus veratridine treated cardiocytes were 6 and 5.4 hours respectively implicating a transcriptional mechanism for the regulation of the cardiac exchanger (35). Thus study of the cardiac promoter may yield valuable insights concerning transcriptional response to cardiac load.

The Exchanger and Transcriptional Control

The exchanger is critical to calcium homeostasis in the heart. Its cardiac expression and rapid load induced upregulation make it an excellent candidate for the study of transcriptional control in the heart. Very little is known about *cis*-elements and transactivation factors involved in cardiac development or in adaptation of the heart to disease states. There have recently been great advances in the understanding of skeletal muscle development. A family of helix - loop-helix proteins which can induce fibroblasts to differentiate into skeletal muscle has been discovered (39-40). This family of transcription factors, called the Myo D family, has greatly increased our understanding of the mechanisms controlling skeletal muscle commitment and differentiation. There are significant similarities between cardiac and skeletal muscle. For instance, both tissues arise

from mesoderm, share many of the same contractile proteins, and are often multinucleated. These and other similarities have led to the proposition that regulation of cardiac differentiation could also occur through MyoD-like factors. Expectation for similar mechanisms was reinforced by the discovery that expression of many contractile proteins present in both cardiac and skeletal muscle require similar *cis*-elements such as M-CAT, CArG, and AT-rich MEF-2 elements, and that transcription factors that bind these elements are present in both heart and skeletal muscle (41-42). But despite the similarities found between cardiac and skeletal muscle, mice homozygous for inactivated forms of these MyoD proteins have normal cardiac phenotype.

The factors controlling cardiac differentiation may be very different from those involved with skeletal muscle differentiation. This might be expected due to the significant differences in the development of skeletal and cardiac muscle. While both are derived from mesoderm, skeletal muscle arises largely from the somites and lateral splanchnic mesoderm which associates with the limb buds, while the heart arises from the specialized cardiogenic region of mesoderm located caudal to the procordial plate in the trilaminar embryo (43-45). The cells that give rise to the cardiogenic area are somehow designated as early as the blastocyst stage of development while the progenitor cells for skeletal muscle appear to be designated much later. Also there are differences in the formation of the multinucleated cell type: skeletal myocytes fuse to form syncytia while cardiocytes do not. Instead they become multinucleated through DNA duplication without cell division and then aggregate as

discrete cells that communicate through gap junctions, forming a functional but not structural syncytium. (46). All of these chronologic and qualitative differences support the existance of unique developmental cascades in the two tissues, and there is much interest in uncovering *cis*-regulatory elements or combinations of elements that are uniquely active in cardiac expression. Unfortunately, relatively little is known about elements specific for cardiac transcription.

Several *cis*-regulatory elements have been described which are involved with cardiac specific gene expression. The MyoD analogues eHand and dHand have recently been discovered in cardiac tissue. These proteins are important in looping of the heart during early stages of development. Also, the GATA motif has been found in the proximal promoters of several cardiac specific genes. This motif is bound by GATA-4, a transcription factor expressed in the embryonic heart, the gut, but not in skeletal muscle (47-48). Mutation of the GATA motif decreases expression of the cardiac specific alpha-myosin heavy chain gene by up to 88 percent (49). The early expression of GATA-4 in the embryonic heart indicates a possible role in cardiocyte commitment and differentiation. Another *cis*-regulatory element is the 28 bp HF-1 shown to be critical for cardiac specific expression of the rat myosin light chain-2 gene. Significantly, HF-1 is also implicated as a mediator of alpha-adrenergic induction of this gene in rat neonatal cardiocytes (50). Alpha-adrenergic stimulation of primary neonatal cardiocyte culture is a well established model for hypertrophy. HF-1 contains binding sites for several regulatory factors, and it is unclear whether induction occurs

through the same factors that mediate basal cardiac specific transcription. Studying the promoter involved in cardiac expression of the sodium-calcium exchanger will enhance our understanding of the molecular mechanisms governing both cardiac gene expression and the induction of genes in response to pressure overload.

CHAPTER 2

CHARACTERIZATION OF THE NCX1 ISOFORMS

INTRODUCTION

The sodium-calcium exchanger is involved in calcium homeostasis in numerous and diverse cell types. Interestingly, the calcium ion is involved in a variety of cell processes and signaling pathways, some virtually ubiquitous and others highly specialized. In the kidney, the exchanger is involved in calcium reabsorption from the renal tubules, impacting extracellular calcium stores. Thus the renal exchanger plays a role in regulating systemic calcium levels (51-52). In the heart the exchanger is the primary mechanism of calcium efflux; thus influencing the amount of calcium that remains in the cytoplasm to be stored in the sarcoplasmic reticulum (27). By regulating intracellular calcium levels the exchanger impacts cardiac contractility on a beat to beat basis. The exchanger is also involved in the contraction of skeletal and smooth muscle, and in neuronal neurotransmitter reuptake (4, 53, 54). With the heterogeneity of calcium handling in different cell types, qualitative and quantitative differences in exchanger expression across cell types may exist to cope with the differing demands.

Cardiac NCX1 transcripts have already been shown to be regulated developmentally (55). In the adult heart, approximately eighty percent of the calcium involved in excitation-contraction coupling is supplied to and removed from the cytoplasm by the sarcoplasmic reticulum. In the immature fetal heart, relatively little calcium is controlled by the sarcoplasmic reticulum. The fetal heart

relies more heavily on the exchanger to remove calcium from the cytoplasm in diastole. Levels of exchanger expression are highest at birth, but decrease shortly after birth as the capacity of the sarcoplasmic reticulum increases and cardiocytes rely more heavily on intracellular calcium stores for the changes in calcium levels required for contraction and relaxation (38,55,56). There is little information about the role of splice variation of the exchanger in development.

There is also evidence for species differences in exchanger function and expression. Several studies have established species differences in the function of the sarcoplasmic reticulum (57-59). Because the exchanger and sarcoplasmic reticulum work together in regulating calcium homeostasis, these studies indirectly support the possibility of species differences in exchanger function. Sham et al. have recently used fluorescent measurement and whole-cell patch clamp technique to study cardiac myocytes isolated from different species to directly determine whether species differences in exchanger activity do exist. Their studies show that the rate at which calcium was extruded by the exchanger, exchanger current densities, and regulation of exchanger activity by calcium varied between rodents and larger mammals such as humans (60). The mechanisms that generate this variability remain to be determined.

Nature has employed numerous mechanisms to generate cell-type, developmental, and species related diversity as seen in the exchanger. For instance, variation in the level of expression of genes may be achieved through the presence of more than one gene copy, the presence of more than one promoter within a single gene, or

variation in untranslated regions which alter message stability. Diversity of function can result from splice variation which alters protein activity or localization.

Many of these mechanisms have been employed to regulate NCX. Three distinct NCX genes, each with unique patterns of expression, have been found in mammals: NCX1, NCX2 and NCX3. While NCX1 is found ubiquitously with most marked expression in the heart, brain and kidney; NCX2 and NCX3 are primarily expressed in the brain and skeletal muscle (23,24).

In addition three unique NCX1 5' untranslated region isoforms have been identified in rat (61). Each of these isoforms has a specific tissue distribution. A region of alternative splicing has also been identified within the translated portion of NCX1. This region is 110 nucleotides long and encodes a portion of the intracellular loop of the exchanger (21). This loop is not necessary for exchanger function but is involved in its regulation by calcium, sodium, ATP, and exchanger inhibitory peptide (18-20).

This variable region of the intracellular loop has been cloned in rabbit. As described in Chapter 1, it is formed by alternative splicing of 6 exons, exons A-F (Figure 1-2). Exons A and B are mutually exclusive--inclusion of one of the two is necessary in order for the sequence to remain in frame. Exons C through F are cassette exons; inclusion or exclusion of these in any combination results in an in frame message. Sixteen tissue selective isoforms have been identified (21-22).

Much of this work on 5' untranslated regions and loop isoforms has been done in rat and rabbit, the genetics of which varies

significantly from that of humans. In addition, if differences in isoforms expressed in the heart are found, they will perhaps be tested in animal models that best simulate human cardiac physiology. Such models often involve larger mammals. The focus here is first to examine the 5' untranslated region and loop isoforms present in feline adult tissues. Next, the isoforms will be compared to what has been uncovered in rat and rabbit. Finally, variation in expression of loop isoforms expressed in the feline fetus and adult will be explored.

EXPERIMENTAL PROCEDURES

Isolation of RNA

Tissue samples were dissected and immediately frozen in liquid nitrogen. Total RNA was then isolated from the tissue by the method of Chomczynski and Sacchi, 1987 (62). Briefly, frozen tissue was homogenized for 20 to 30 seconds in 10 µl of 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, and 0.5% laurylksarcosine. 1 µl of 2M sodium acetate, 10 ml of water saturated phenol, and 2 mL of chloroform:isoamyl alcohol were added, and the solution was kept on ice for 20 minutes with occasional vortexing. Phases were separated by centrifugation at 12000 X *g* for 20 minutes at 4°C. The RNA was precipitated from the aqueous layer by addition of an equal volume to isopropyl alcohol. The sample was centrifuged at 12000- X *g* for 30 minutes at 4°C. The pellet was washed with 70% ethanol before being resuspended in diethyl pyrocarbonate treated water. Samples were stored at -70°C. Feline mRNA from heart, brain, and kidney was isolated using the FAST TRACK kit from Stratagene.

Northern Analysis

For Northern analysis, 1.2 µg of mRNA was electrophoresed on each lane of a 1% agarose-formaldehyde gel and transferred by downward capillary method to a Duralon membrane to which it was UV-crosslinked. The membrane was hybridized to ^{32}P-dCTP labeled

PCR probes generated as described in results. (Primer pairs used to generate the probes were as depicted in figure 2-3.) Membranes were then washed to a stringency of 1 x SSC, 0.1% SDS at 42°C, and developed on XOMAT AR film (66).

5' Rapid Amplification of cDNA Ends (RACE) and Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

RACE was carried out as previously described (63-65). One microgram of total RNA isolated from feline brain, kidney, and heart were annealed to an antisense NCX1 gene specific primer at position +797 to +781 (5'-TTGTAAAACAGCAGCCT-3') and reverse transcribed for 45 minutes at 42°C using 1 µl Superscript II reverse transcriptase (Gibco) in a 25 ml reaction mix containing 0.05 M Tris-Hcl, pH 8.5, 0.03M Kcl, 8 mM $MgCl_2$, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, 1 mM dithiothreitol, and 2.5 pM primer. The RNA was then degraded by addition of 1 µl RNASE H and incubation for 10 minutes at 55°C. The cDNA was purified using the GlassMAX DNA purification system (Gibco) and then tailed with dCTP at 37°C for 15 minutes using terminal dipeptidyl transferase (Promega). The tailed cDNA was amplified by polymerase chain reaction (30 cycles of 94°C, 50°C and 72°C each for 1 minute) using a second antisense NCX1 gene specific primer located at position +207 to +224 (5'-CTCTGGCAATTTTGTCTC-3') and an anchor primer (5' GGCCACGCGTC GACTAGTACGGGIIGGGIIGGGIIG-3', Gibco). Products were subjected to southern analysis (described below) and hybridized to a ^{32}P dCTP (NEN, 3000 Ci/mmole) labeled PCR probe generated from the region -26 to +59 (sense primer 5'-ACACTTGGAGGTCTAC-3', and antisense

primer 5'-CAACGGCTAACAGATGACATC-3'). Products that hybridized to the probe were ligated into the PT7 T-vector (Novagen) and transformed into competent NovaBlue cells (Novagen). Minipreps were prepared from the colonies and screened by Southern hybridization as described above. Positives were sequenced by either the dideoxy chain termination method using Sequenase 2.0 (USB) or cycle sequencing using the Ampli-Cycle Sequencing kit (Perkin Elmer) as specified by the manufacturer using an annealing temperature of 55°C.

For RT-PCR, the reverse transcription, RNAse treatment, and purification of cDNA was carried out as described for RACE, but using different primers. The antisense primer 5'-CTTTGCTTGCAAATGTGTCTGGTAC-3' (bases 2581-2557) was used for reverse transcription. Five µl of the resultant cDNA was amplified as desribed above using sense primer 5'-TTGAGGACACTTGTGGAGA-3' (bases 1781-1799) and antisense primer 5'-ACGGTACTCTTGAATT-3' (2195-2180). 20 µl of each reaction was electrophoresed on a 1.5% agarose gel. Positives of interest were ligated into the PT7 Blue vector (Novagen) and transformed into NovaBlue cells (Novagen). Minipreps were prepared from the colonies and sequenced as described for RACE products.

Screening of the cDNA Library

An oligonucleotide (dT) primed cDNA library was constructed in vector Lambda Zap using feline cardiac mRNA and MMLV reverse transcriptase (Stratagene). The library was screened using standard techniques (66). The library was plated on 10 150 mm plates at

30,000 plaques/ plate. Duplicate lifts from each plate were hybridized to a ^{32}P-dCTP labeled PCR probe generated from -26 to +224 (sense primer, 5'-ACACTTGGAGGTCTAC-3', and antisense primer, 5'-CTCTGGCAATTTTGTCTC-3'). Filters underwent four 20 minute washes at 42°C: the first two washes in 2 x SSC/.1% and the last two in 0.2 x SSC/ .1% SDS. The filters were then exposed to XOMAT AR film (Dupont) overnight. Plaques that produced signals on both of the duplicate lifts were isolated and purified to homogeneity through three to four additional rounds of screening using the same probes and wash parameters. Clones were sequenced as described for 5'RACE experiments.

Southern Analysis

cDNA was run on a 2.0% agarose gel, and transferred by downward capillary method onto Duralon membrane. The membrane was then hybridized to the same ^{32}P-dCTP labeled PCR probes used for library screening, washed to a stringency of 0.2 x SSC/ 0.1 % SDS, and exposed to XOMAT AR film overnight (66).

RESULTS

Cloning the Feline NCX1 5' Untranslated Regions

The 5' rapid amplification of cDNA ends (5'-RACE) was utilized to determine whether the 5'-UTR of the feline NCX1 gene demonstrated similar tissue specific heterogeneity to that identified in the rat (61). After two rounds of amplification four major products are clearly present from brain, two from heart, and two from kidney (Fig. 2-1). RACE was repeated at least three times from different tissue samples and separate RNA isolations, each time yielding similar results.

The 5'-RACE experiments were designed so that positive clones should contain NCX1 sequence from -34 to +250 bp. This allowed for the screening of positive clones by Southern blot. Interestingly, all four major products from the brain showed positive hybridization in the Southern blot, and sequencing revealed that each had the correct NCX1 sequence from +250 to -34 but unique 5'-ends.

Each of these unique 5'-UTR sequences were used to generate PCR probes, and Northern blot analysis revealed that only the 350 bp 5'-RACE cDNA clone is present at a detectable level in the brain. This clone, Br1, contained 139 bp 5' of -34, has a very high GC content (Figure 2-2), and has a 65% homology with the rat brain 5'-UTR.

Both products found in kidney were positive by Southern blot and have the correct NCX1 sequence from +250 to -34 where they diverge into a unique sequence. Again only one, K1, was positive in

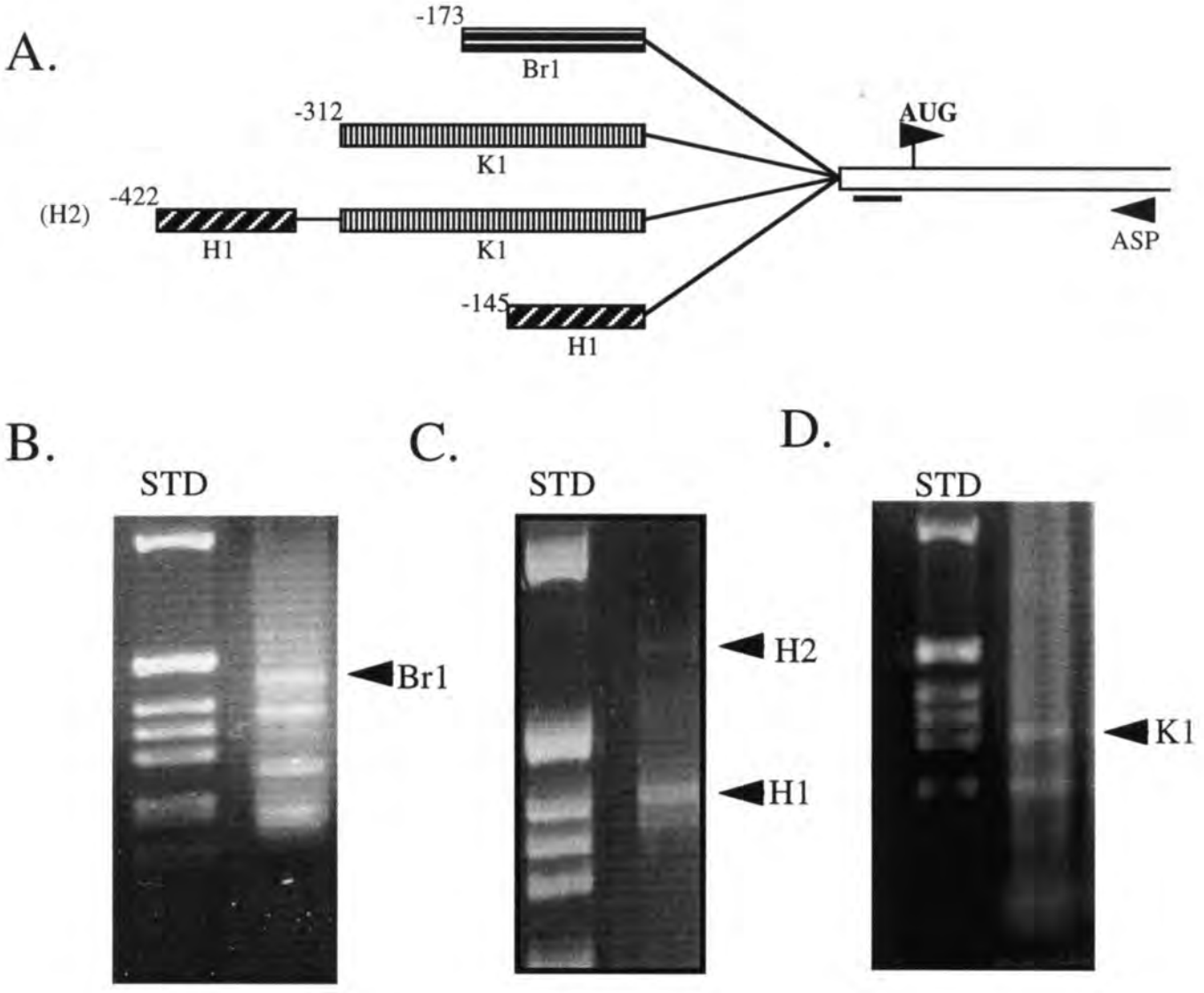


Figure 2-1 5'RACE amplification of NCX1 transcripts in feline brain, heart, and kidney. The antisense 3'-primer (ASP) used for reverse transcription corresponds to positions +781- +797 of the feline NCX1 cDNA. The cDNA was amplified using a second antisense primer, which bound the cDNA at position 250, and an anchor primer. Race reactions were run on agarose gels and stained with ethidium bromide. The products are shown below a diagram depicting the four unique 5'UTRs spliced to the common sequence of the feline NCX1 mRNA in brain (B), heart (H), and kidney (K).

A. H1

ACAAGCATCT CAGGTCCTCT CTTTCTGGTA AATTCGGAGC AGCCATCATA
CGGGGTCTTT TCTCACATCC AGCCCATGCG GACCGAGCAG CCCAGACTTG
ACGGAGGTAG gttgggacag ttggaagtct accattgtac aac**atg**ctgc

B. K1/ H2

GTGAGC TGCAATCTTT GAGAGAAACA GCCACACGGA GAATCCTTCA
GAGATCTTGG ACTGGGGACC ACCGCGCCCC GCACCCCCAT GCCCTTAGCT
GCTTGTGTCA GCAGCTTCTA GCCACGGTAC TGCTGTGCAC GTCTACATGT
AGGAAAAACA CAATGAGGGG GTAGGAGAAA ATCATTGATG CAACCCCACA
TCCCTTTGCT AAACGAGAGC TTCCTTCAGC ACAAGGGATA GAAGCGGAAG
AGAGATACAT CTGGAGTAAC AATTTCAGAG gttgggacag ttggaagtct
accattgtac aac**atg**ctgc

C. BR1

AGGCCTG CCCGCCGCCG CCCGCTTTGT GCTTCCCCCA GAAGATGGGA
CGACCTCTTC CCGGTCGGGA GCTATTTAAA AGGAGGGAGC CGCCCGTTGC
CTGCTAGCGC CGGAACGGGG GAAGGATCGA GCCACACTCT CCCGAACCAG
gttgggacag ttggaagtct accattgtac aac**atg**ctgc

Figure 2-2 The sequence of the NCX1 5'UTRs identified in feline heart, brain,and kidney. The translational start site is indicated by (**atg**). Upstream ATGs are underlined. A.) H1 contains a single alternative upstream ATG that is out of frame and terminates with stop codon eleven nucleotides 3' of the translational start. B.) The K1 sequence contains four upstream ATGs, each out of frame and terminated by a downstream stop codon prior to the translational start. C.) The Br1 5'UTR contains an out of frame ATG followed by an early stop codon as well.

the kidney Northern analysis. This clone contained 278 bp 5' of -34. K1 contains an out of frame initiation codon within a nucleotide environment showing reasonable conformity to the Kozak consensus sequence (figure 2-2). A polypeptide initiated at this site would precede 17 amino acids before terminating at a stop codon. The presence of alternative upstream initiation sites has been demonstrated to reduce the efficiency of mRNA translation (67,68). This suggests that NCX1 isoforms containing K1 may be under some level of translational control. Surprisingly, the feline kidney 5'-UTR shows no sequence homology within the rat kidney clone (61).

In heart, both 5' RACE products were positive on Southern analysis. Extensive sequencing of numerous clones showed that the products consisted of two separate isotypes, H1 and H2. The H1 product contained 110 bases 5' of -34 splice site (Figure 2-2). This isoform shows significant homology to rat H1 and bovine P17 clones, 68% and 85% respectively. The H2 isoform consisted of the entirety (278 bp) of K1 sequence 5' of -34 and then continued 5' to include the entire 110 bp of H1.

Northern analysis indicates that both H1 and K1 are present in the heart (Table 2-1). Importantly, 5'-RACE with primers within the K1 sequence always gave a product which contained all or a substantial portion of the H1 sequence. Therefore, in the normal heart K1 is only expressed as the H2 5'-UTR and never detected alone. The existence of two feline cardiac 5'-UTR isoforms differing only by the splicing in of intervening sequence (K1) is consistent with reports concerning bovine cardiac NCX1 (36) but differs from

		Total RNA		
		Brain	Heart	Kidney
PROBE	Br1	++		
	H1	+	+++	
	K1		++	+
	C	+	+++	+

Table 2-1 Tissue distribution of the unique 5' untranslated region isoforms of NCX1. Northern blots containing 15 micrograms of brain, heart, or kidney total RNA were probed with ^{32}P-labeled probes specific for regions K1, Br1, H1, or common coding region (C). The results are summarized in this table. The number of (+) signs indicates the relative abundance as judged by the level of band intensity on northern blot; comparisons are valid between RNA samples probed with the same probe, but not between probes.

what has been found in rat (61) as is the existence of an out of frame upstream AUG.

Extensive screening a feline cardiac cDNA library resulted in confirmation of H1 and H2 and uncovered no new alternative 5'-UTRs. The library yielded a ratio of three clones containing H1 for one containing H2 (out of 23 positive clones). While not quantitative the relative number of H1 clones compared to H2 suggests that H1 may be the relatively more abundant cardiac isoform. This was further substantiated by Northern analysis.

The Tissue Distribution of the feline 5' Untranslated Region Isoforms

Northern analysis indicates a tissue selective expression of the isoforms (Table 2-1). Comparing expression levels using a common probe generated from the open reading frame of NCX1, the exchanger appears to be most abundant in the heart, less abundant in the brain, and still less in the kidney (6,16). The Br1 5'-UTR is specifically expressed only in the brain, while the other 5'-UTR exons show more promiscuity. The H1 5'-UTR (H1) is expressed in both heart and brain. The K1 5'-UTR sequence is expressed in both kidney and heart. Band intensity indicates that in heart the K1 sequence (H2) is expressed at very low levels compared with H1, again suggesting that H1 is the predominant cardiac isoform.

RT-PCR of the Loop Region

Uncovering the splice variation in the NCX1 5' untranslated region lead to interest in possible splice variation within the coding region of the gene. Attention was directed towards the previously

identified region of alternative splicing within the intracellular loop. RT-PCR was used to examine tissue specific expression of the feline NCX1 loop isoforms. Figure 2-3 shows the strategy used to amplify the loop region from brain, heart and kidney total RNA isolated from adult cat tissues. For each tissue, the RT-PCR reaction was repeated at least three times using RNA isolated from different tissue samples. Figure 2-4 shows the results of a typical RT-PCR experiment. Multiple clones from each tissue were subcloned and sequenced to verify the exon content of each amplified loop region.

The data are summarized and compared with data previously reported in rat and rabbit in Table 2-2. The number and size of the products agreed with previous reports in each tissue except heart. In brain three products consisting of ACD, ADF and AD were seen as previously reported in rat. In kidney both feline products, BDF and BD, were previously reported in rat by Lytton (61).

In feline heart two products were visible. In the literature, only one product consisting of ACDEF has been consistently reported in rat and rabbit heart, however, there is one report of a rarely expressed isoform ACDE from rat heart (16,21,22).

Of the two products found in feline heart, the larger and more abundant heart product was of the size which agreed with previous reports. Sequencing the heart products showed that the larger heart product consisted of exons A,C,D,E and F. This isoform has been previously reported in the works of Lytton (61), Shultz (21), and Philipson (22). The second smaller product was identical to one of the kidney isoforms, consisting of only exons B and D.

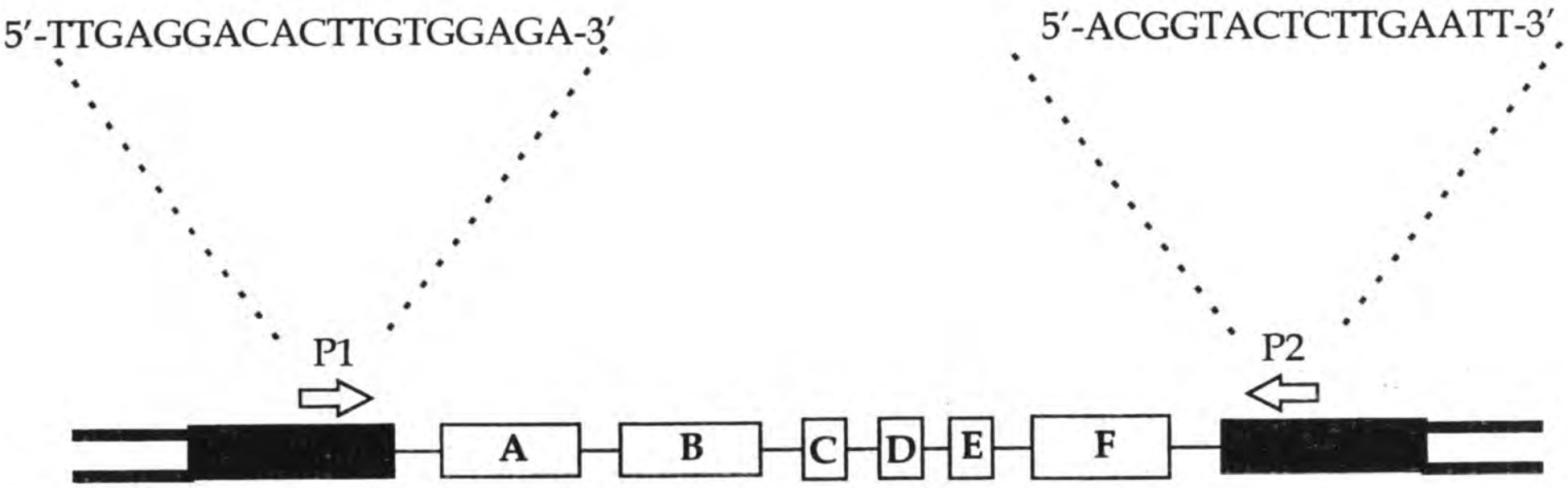


Figure 2-3 Strategy for reverse-transcribed polymerase chain reaction amplification of the variable region of the NCX1 intracytoplasmic loop. Primers P1 (sense) and P2 (antisense) flanking the alternatively spliced region were used to amplify cDNA from feline tissues.

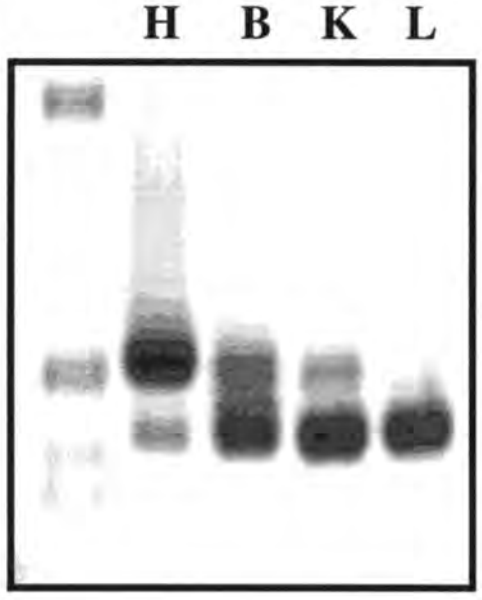


Figure 2-4 Products of reverse-transcribed polymerase chain reaction amplification of the variable region of the NCX1 intracytoplasmic loop in adult feline tissues. Total RNA from adult feline heart (H), brain (B), kidney (K), and liver (L) were reverse-transcribed. The resultant cDNA was amplified using the primer pair illustrated in Figure 2-5, separated on an agarose gel, and visualized by ethidium bromide staining.

Tissue / Isoform	Brain	Heart	Kidney	Liver
ACDEF		*		
ACDE		-		
ACD	*			
AD	*			
ADF	*			
BCD			-	
BD		+	*	*
BDF			*	

* Feline isoforms previously reported in rat

+ Feline isoforms not previously reported in rat

- Isoforms previously reported in rat, not detected in cat

Table 2-2 Summary of NCX1 splicing isoforms encoding the intracellular loop. Products of reverse-transcribed polymerase chain reaction (Figure 2-5) were subcloned and sequenced. Here, the feline loop isoforms identified are presented and compared with loop isoforms previously described in rat.

The relative intensity of this smaller product was much lower than the intensity of the larger one, and of the fifteen clones sequenced only four consisted of this smaller clone, suggesting that the ACDEF isoform is the predominant splice variant in the normal adult heart.

Interestingly the BD product is one of the major products expressed in feline kidney tissue, and has been identified in rat and rabbit kidney (21,61). The rare isoform consisting of ACDE, reportedly derived from rat heart (22), was not seen in this feline work.

To determine whether the rat heart truly lacked expression of B containing NCX1 loop isoforms, or if the presence of the B isoform was not detected by others due to a less sensitive RT-PCR protocol, northern hybridization of rat cardiac total RNA was carried out using the above method. No exchanger mRNA was detected even after prolonged exposure. These results agree with those previously reported showing expression only of isoforms containing exon A in rat heart.

This is the first report of expression of exon B containing NCX1 splice variants in cardiocytes. The pattern of distribution of previous clones has led to the hypothesis that exon B expression may be limited to non-excitable tissues. These feline results suggest that the hypothesis is true for rodents but not larger mammals. The significance of expression of this isoform in the feline heart and not in rat or rabbit heart is not known, however this may be related to the differences in exchanger regulation across different species. More recently, northern analysis by others in our laboratory have

indicated that exon A and B are both expressed in human heart tissue as well, suggesting that studies concerning the regulation of NCX1 expression in the cat may be in some respects more applicable to the human than those studies in the rat.

RT-PCR Analysis of Embryonic and Adult Tissues

The NCX1 has been shown to be developmentally regulated in the heart, with levels being lower at adulthood than at neonatal stages. The decreasing expression of the NCX1 is coincident with an increase in the levels of calcium stored in and released from the sarcoplasmic reticulum. It seems that as the heart develops, it shifts to a different mode of calcium handling.

No one has explored whether the down-regulation of the exchanger with maturity is accompanied by a switch in isoforms expressed. To determine if the expression of NCX1 splice variants is developmentally regulated in the heart and/ or other tissues, reverse transcriptase-polymerase chain reaction using the same primers described above was executed using total RNA isolated from the heart, brain, and kidney of fetal and adult cats. The experiment was repeated at least twice for each tissue type using RNA isolated from the tissues of different animals. Each repetition yielded identical results. Products from a typical experiment are shown in Figure 2-5. The products from the fetal tissue were subcloned sequenced and compared with adult products from the same tissue type.

No developmental isoform differences were detected in brain or heart tissue. Fetal and adult heart samples shared the same pattern of two products reported above in adult heart. Similarly, the

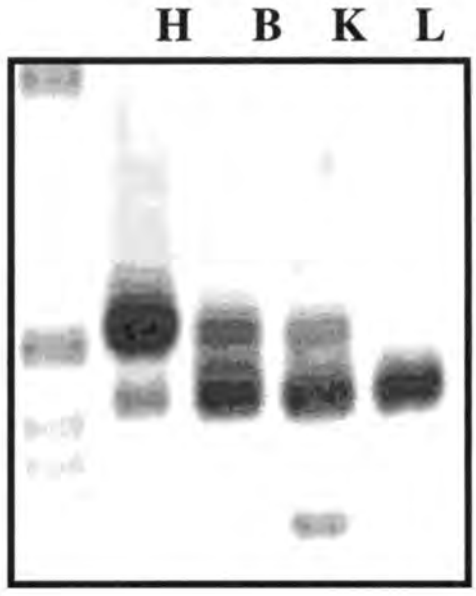


Figure 2-5 Products of reverse-transcribed polymerase chain reaction amplification of the variable region of the NCX1 intracytoplasmic loop in fetal feline tissues. RT-PCR of total RNA from adult feline heart (H), brain (B), kidney (K), and liver (L) was carried out as for adult tissues. Products from fetal heart, brain, and liver were identical to their adult counterparts. Two fetal kidney products corresponded to the two adult kidney isoforms. A third novel kidney product was identified. It was much smaller than the other products and would require an alternative splicing pathway, that differs from that presented in Figure 2-1, for its production.

same pattern of three bands were present in fetal and adult brain samples. Sequencing confirmed the identity of the fetal bands to be those of their adult counterparts.

The expression of NCX1 splice variants in kidney fetal tissue differed from that of the adult. Both adult and fetal kidney samples contained the two splice variants reported above, namely BD and BDF. However, there was one smaller variant which was exclusive to the fetal kidney sample. This product was too small to be formed within the splice paradigm featured in Figure 1-1. The sequence of this variant revealed only the most 5' twenty-six bases of exon B, with none of exons A,C, D, E or F, nor the first one hundred one base pairs of the next exon being expressed (Figure 2-6). Neither the splice donor or splice acceptor sites had been previously reported.

While this smaller product does not follow previously published patterns of splice variation in this region, there are facts that support the possibility that this is a true transcript normally expressed in the fetal kidney. Notably, the intensity of the product is similar to that of the other isoforms and despite the unconventional nature of the splice variant, the sequence does remain in frame. In addition, this band was not detected in any of the other fetal or adult tissues. This small band appeared without fail in each of the fetal kidney RT-PCR experiments which were carried out using RNA isolated from different kidneys.

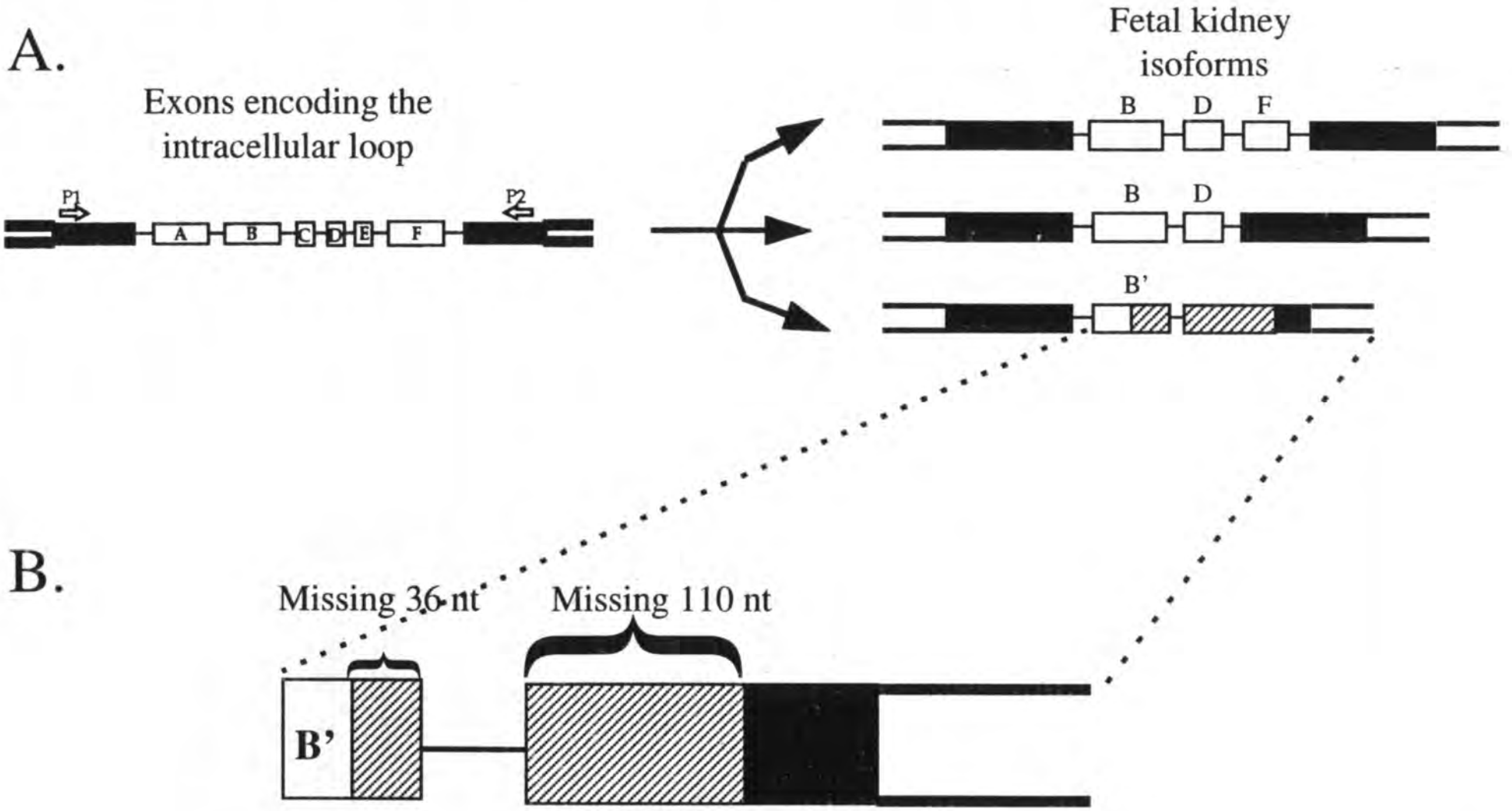


Figure 2-6 Diagram illustrating the fetal kidney reverse-transcribed polymerase chain reaction products. A.) Comparison of the three fetal kidney products identified. B.) Illustration of sequence of the smallest fetal isoform which is missing a portion of exon B and a portion of the exon distal to the variable region. (nt=nucleotides).

DISCUSSION

In this study 5' RACE and RT-PCR have been successfully used to clone splice variants of the NCX1 gene and to determine how these isoforms are distributed in fetal and adult brain, kidney and heart. The results are very interesting. Although the distribution of the NCX1 gene is very wide, the expression of the exchanger isoforms appears to be highly regulated, with specific isoforms selectively expressed in particular tissues.

The NCX1 gene contains three 5'untranslated regions (UTR), which will be shown in chapter four to represent separate exons with expression driven by three separate promoters. Alternative splicing of these three 5' UTR exons produces at least four different 5' termini for NCX1 transcripts in brain, heart and kidney. The Br1 5' UTR is expressed predominantly in the brain but is not detectable (by Northern analysis) in the heart or kidney. Unlike the rat heart in which only a single species of 5' UTR is expressed, we have discovered two forms in the feline heart. The first isoform contains the H1 exon spliced to the common acceptor site (-34) at the beginning of the common exon containing the translational start (exon 2). This is the predominant transcript found in the heart. The transcript is derived by an alternative splice receptor in the K1 exon which results in the inclusion of the entire K1 exon between the H1

exon and exon 2. Because they share the identical most 5' exon, both of these transcripts probably are regulated by the same promoter.

The existence of two feline cardiac 5' untranslated isoforms differing only by the splicing of intervening sequence (K1 exon) is consistent with what was reported for the bovine cardiac NCX1 p17 clone (36). The bovine p13 and 5' end of the bovine p17 clone are identical and have 85% homology with the feline H1 sequence. Interestingly, the p17 clone contains a 56 bp insert, which, although not homologous with the feline K1 sequence, has significant homology with the rat kidney 5' UTR sequence (61). The K1 sequence was always found downstream from exon H1 and never detected alone. In addition, transfection of neonatal cardiocytes with the kidney promoter-luciferase fusion construct never gave luciferase expression above background levels. Therefore, K1 expression in the heart is not regulated by the kidney promoter. Only one 5' UTR appears to be expressed in kidney; one in which the K1 exon is spliced directly to the common receptor site at the beginning (-34) of exon 2.

The purpose of variability in the loop region is unclear. Deletion of this loop resulted in an exchanger that was still functional, but lacked regulation by such factors as calcium and ATP (18). More recently additional mutagenesis has narrowed the region of the loop required for regulation by calcium to a span of 130 nucleotides (18-20). This region contains the sequence formed by alternative splicing of exons A through F (21). It is thought that loop splice variation in this region may explain some of the tissue and species differences seen in studies of exchanger activity.

The mechanism by which sequences in this region affect regulation of exchanger activity by calcium and other factors is not clear. It is not known whether regulatory factors such as calcium bind directly to amino acids encoded by the exons in this region or whether those amino acids facilitate direct interaction with other cytoskeletal or cytoplasmic proteins which can be involved in modulation by these factors.

Some insight may be gained by comparison with the similar splice variation of the troponin-T gene. The amino terminal portion of this gene is formed by a splicing paradigm similar to that of the exchanger loop (21). The amino terminal is formed by alternative splicing of seven exons with two being mutually exclusive and the remaining acting as cassette exons (69). In this case, the variability in the region allows for differences in troponin protein-protein interactions, directly effecting interaction with tropomyosin (70).

There is growing evidence that the exchanger is in contact with the cytoskeleton. Li et al. reported in 1993 that the exchanger binds to the peripheral membrane protein ankyrin (71). Ankyrin can link integral membrane proteins, such as the exchanger, to other proteins that make up the cytoskeleton. The regulation of the exchanger by ATP is postulated to be indirectly due to the ability of ATP to affect membrane components altering interactions with cytoskeletal elements (15). These issues are still being debated.

The feline exchanger loop isoforms show similar patterns of variability seen previously in rodents. One exception is the presence of two isoforms, one containing the B exon, in heart. It is very significant that the human heart also expresses both exon A and B

containing isoforms. Further study of this dichotomy in isoform expression between rodents and larger mammals may provide insight into the differences in exchanger function seen between the two groups.

Another exception is the presence of a novel NCX1 loop isoform in the fetal kidney not seen in any other adult or fetal tissue tested. Not much is known about differences in exchanger regulation in the fetal and adult kidney. Study of exchanger activity in kidney development may provide insight into the significance of this finding.

CHAPTER 3
THE SODIUM-CALCIUM EXCHANGER IN CARDIAC HYPERTROPHY

INTRODUCTION

Cardiocyte pressure overload hypertrophy is characterized by a complex set of morphological, biochemical, genetic and pathophysiological changes that result in increased muscle mass. Changes in gene expression begin within hours of the initial change in load and occur in a pattern distinct for pressure overload hypertrophy (72-74).

First, a rise in expression of immediate early genes such as c-fos, and c-myc occurs. Increased message for these proteins can be detected within minutes of pressure overload (75-76). The significance of the upregulation of these immediate early genes to hypertrophy of cardiac tissue is unknown. Increased expression of immediate early genes is a fairly ubiquitous response, occurring in reaction to a multitude of stimuli. This first response usually precedes and may actually induce a later upregulation of genes which may be more directly involved in adaptation to the initiating stimulus (75). It is not clear whether the distinct pattern of upregulation of several immediate early genes, or the environment in which they are upregulated is the key to generating the subsequent specific gene responses to a given stimuli.

Within two hours of increased load, there is a general increase in the expression of messenger RNA encoding other functional proteins (75) . This is characterized by qualitative as well as quantitative changes in gene transcription. Some isoforms return to

a relative level of expression most associated with the fetal stage of development. Hence this pattern of gene expression has been termed a return to the fetal gene program (75). One example is the relative changes in isoforms of the myosin heavy chain in response to load. In fetal rodents β-myosin heavy chain is most abundant, but after birth it is largely replaced by the α isoform. Pressure overload hypertrophy induces increased expression of β myosin heavy chain mRNA and protein while not affecting α myosin heavy chain expression (77). Other examples of the return to the fetal pattern of expression include the re-expression of skeletal α actin in the adult heart and re-expression of atrial natriuretic factor in the ventricles (75).

The changes in proteins of the sarcoplasmic reticulum in response to pressure overload are particularly relevant to the regulation of the cardiac sodium calcium exchanger (NCX1). The hypertrophied heart expresses less of the cardiac isoform of the sarcoplamic reticular calcium ATPase (SERCA2a) which is responsible for pumping calcium into the sarcoplasmic reticulum (78-79). While the sarcoplasmic reticulum in the healthy heart controls 80% of the calcium involved in contraction, the hypertrophied heart has a less active sarcoplasmic reticulum. Subsequently there is a greater demand on flux of calcium across the plasma membrane to provide the changes in cytoplamic calcium levels needed for contraction and relaxation (80-81). This implies a need for upregulation of the cardiac exchanger, NCX1, which functions as the primary mechanism through which calcium is extruded from cardiocytes.

There is another argument for the necessity of upregulation of NCX1 during increased cardiac load. For all cell types, as cells begin to grow, there is an influx of sodium followed by an influx of calcium. In the cardiocyte, early hypertrophic stimulus would lead to the influx of these cations disturbing the ion balance. Increased NCX1 function would help to restore the proper ion balance during the growth process (82-83).

Before my arrival in this lab, studies were carried out to determine if the NCX1 mRNA was in fact increased by pressure overload using a feline model of acute right ventricular overload established by Dr. John Rozich under direction of Dr. George Cooper (84). A balloon catheter was inserted into the pulmonary artery and carefully inflated to maintain a two to four fold rise in right ventricular (RV) pressure for one to four hours. This was carried out under condition of anesthesia which prevented any perturbations in catecholamines, pH, heart rate, or systemic blood pressure. As a result, the left ventricle (LV) could serve as a same animal control. After maintaining pressure overload for the allotted time, the animal was sacrificed and the right and left ventricular free walls were used for isolation of RNA.

Northern analysis of RNA isolated from the right and left ventricles was used to initially identify differentially expressed genes. Using this model, there was a rapid upregulation of NCX1 in response to load. As shown in figure 3-1 within one hour the level of NCX1 mRNA more than doubled and remained elevated for at least four hours (34).

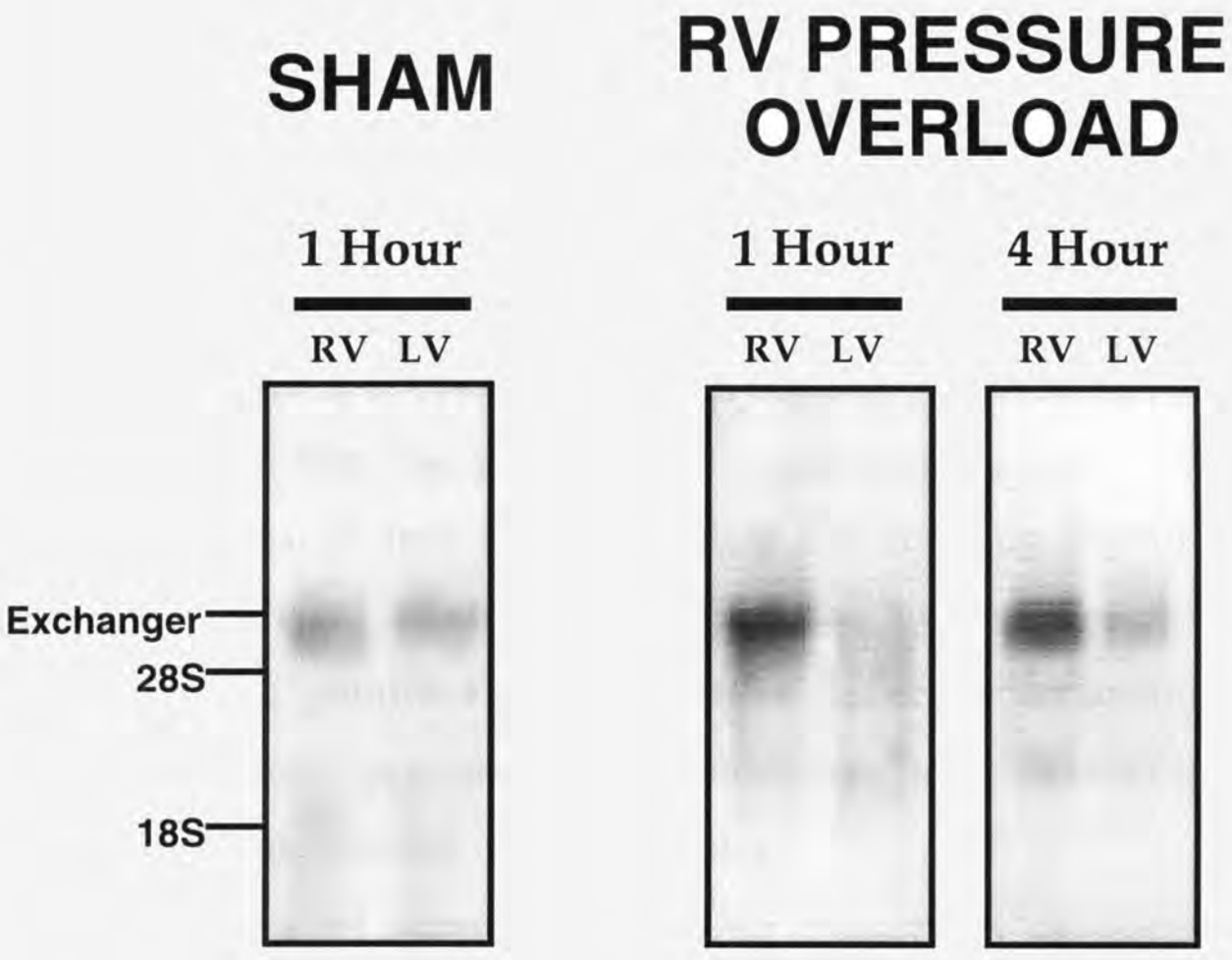


Figure 3-1 Northern analysis of the sodium-calcium exchanger after one and four hours of right ventricular pressure overload. 15 micrograms of total RNA isolated from the right (RV) and left (LV) ventricles of sham-operated, one hour, and four hour pressure overload cats was was subjected to northern analysis using a ^{32}P-dCTP labeled exchanger probe. There is an increase in exchanger mRNA at both one and four hours in the pressure over-loaded ventricles compared with the left ventricles. The sham-operated cat shows no increased exchanger mRNA in the right ventricle. Equal amounts were loaded on each lane as verified by subsequent measurement of 28s rRNA.

To examine the effect of load on exchanger protein levels, cats were subjected to forty-eight hours of right ventricular pressure overload through surgical banding of the pulmonary artery. Microsomal fractions were prepared from tissue of the right and left ventricular free walls. Equal quantities of each sample were subjected to western analysis using NCX specific polyclonal antibody The western analysis showed that forty-eight hours of pressure overload induced a significant increase in exchanger protein, as shown in figure 3-2 (34).

The fact that the exchanger is rapidly upregulated early in hypertrophy makes it a good candidate for studying signaling pathways involved in compensatory cardiac hypertrophy. Exploring those signaling pathways may lead to a better understanding of why cardiocytes under prolonged load conditions may eventually undergo decompensation leading to heart failure.

A number of stimuli can produce changes in cardiocytes similar to those resulting from pressure overload. These changes include the return to the fetal pattern of gene expression, upregulation of protein synthesis, and increased cell mass. Stimuli falling into this group include alpha adrenergic agonists such as phenylephrine (85-87), passive stretch (88), endothelin I (89), phorbol esters (90), and veratridine (34). Each of these stimuli activate a number of pathways. Which of these pathways are important in the hypertrophic response and which are simply epiphenomona is not known.

The goal of this chapter is to further characterize the upregulation of the exchanger. The first focus is to determine

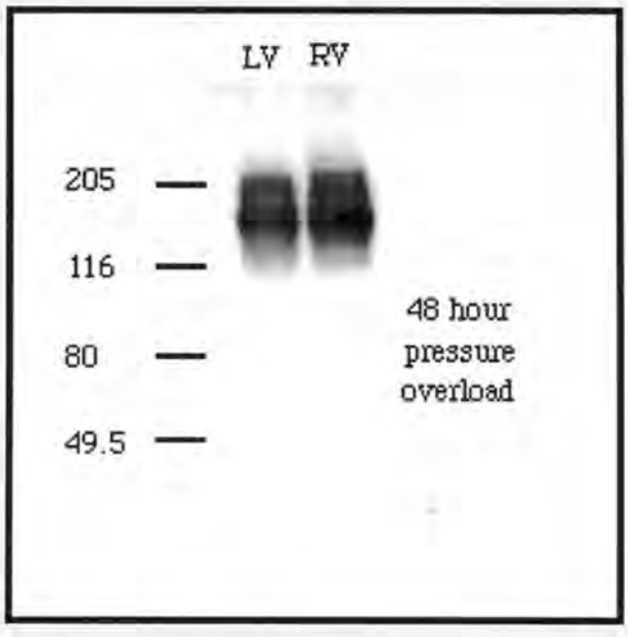


Figure 3-2 Western analysis of the sodium-calcium exchanger after forty-eight hours of pressure overload. Twenty micrograms of microsomal protein were isolated from the left and right ventricles of a cat subjected to forty-eight hours of right ventricular pressure overload. Protein samples were separated on a 7.5% polyacrylamide gel, tranferred to PVDF membrane, and hybridized to anti-exchanger antibody. More exchanger protein was detected in the right ventricle compared to the left.

whether the upregulation of the NCX is simply a short term response to load. For instance, the upregulation may simply be an immediate reaction to cation imbalances that are corrected quickly allowing the exchanger to return to basal levels. On the other hand, this upregulation may be a long term response that has significant consequences in the growth and adaptation to load. Second, the regulation of the NCX by other stimuli of hypertrophy will be explored and experiments will be described which may help explain part of the mechanism by which the NCX1 is upregulated and proved a cell model in which to study transcriptional control of NCX1.

Finally, NCX1 isoform variations in pressure overload will be explored. RT-PCR will be employed to determine if there is any deviation under conditions of pressure overload from the pattern of isoform expression seen in normal feline myocardium. (See Chapter 2). Northern analysis will be used to compare levels of isoform splice variants present if more than one is expressed in the pressure overloaded tissue.

EXPERIMENTAL PROCEDURES

Cell Culture

Primary cardiocytes were obtained from day 2-3 neonatal rats by previously published methods (91). Briefly, ventricular myocardium was isolated from 40-50 neonatal rats. The tissue was finely minced, rinsed, and placed in a cellstir apparatus. A mixture of 2.4 units/ml partially purified trypsin, 2.7 units/ml chymotrypsin, and 0.94 units/ml elastase in calcium and magnesium free Hank's salt solution buffered with 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4 was repeatedly added to the cellstir for six 20 minute incubations at 37°C to dissociate the cardiocytes. After each incubation, dissociated cells were centrifuged at 500 X *g* and resuspended in Eagles Modified Eagle's Medium (MEM) supplemented with 10% newborn calf serum. In order to enrich for cardiocytes, the cells were pooled and subjected to differential plating for 90 min. Cardiocytes were plated at a density of 1 x 10^5 cardiocytes/cm^2. The cardiocytes were maintained overnight in MEM supplemented with 10% newborn calf serum, 2 mM L-glutamine, 10 μg/ml human transferrin, and 0.25mM Acorbic Acid. The next day the cells were switched to serum-free maintenance medium.

Isolation of Total RNA

Feline tissue was dissected and immediately frozen in liquid nitrogen. Total RNA was then isolated from the tissue by the method of Chomczynski and Sacchi, 1987 (62). Essentially, frozen tissue was homogenized for 20 to 30 seconds in 10 ml of 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, and 0.5% laurylksarcosine. 1 ml of 2M sodium acetate, 10 ml of water saturated phenol, and 2 mL of chloroform:isoamyl alcohol were added, and the solution was kept on ice for 20 min with occasional vortexing. Phases were separated by centrifugation at 12000 X *g* for 20 min at 4°C. The RNA was precipitated from the aqueous layer by addition of an equal volume to isopropyl alcohol. The sample was centrifuged at 12000- X *g* for 30 min at 4°C. The pellet was washed with 70% ethanol before being resuspended in diethyl pyrocarbonate treated water. Samples were stored at -70°C. Feline mRNA from heart, brain, and kidney was isolated using the FAST TRACK kit from Stratagene. Total RNA was isolated from rat neonatal cardiocytes using RNAzol as directed by the manufacturer (Cinna Biotex).

Northern Analysis

Total RNA or mRNA (as specified) was electrophoresed on a 1% agarose-formaldehyde gel and transferred by downward capillary method to a duralon membrane to which it was UV-crosslinked. The membrane was hybridized to ^{32}P-dCTP labeled PCR probes generated as described in results. Membranes were then washed to a stringency of 1 x SSC, 0.1% SDS at 42°C (66).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out as previously described (63-65 and Chapter 2). One microgram of total RNA isolated from feline brain, kidney, and heart were annealed to the antisense NCX1 gene specific primer 5'-CTTTGCTTGCAAATGTGTCTGGTAC-3' (bases 2581-2557) and reverse transcribed for 45 min at 42°C using 1 µl Superscript II reverse transcriptase (Gibco) in a 25 ml reaction mix containing 0.05 M Tris-Hcl, pH 8.5, 0.03M Kcl, 8 mM $MgCl_2$, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, 1 mM dithiothreitol, and 2.5 pM primer. The RNA was then degraded by addition of 1 µl RNASE H and incubation for 10 min at 55°C. The cDNA was purified using the GlassMAX DNA purification system (Gibco). Five ml of the purified cDNA was amplified by polymerase chain reaction (30 cycles of 94°C, 50°C and 72°C each for 1 min) using a second antisense NCX1 gene specific primer 5'-ACGGTACTCTTGAATT-3' (bases 2195-2180) and a sense primer 5'-TTGAGGACACTTGTGGAGA-3' (bases 1781-1799). Twenty µl of each reaction was electrophoresed on a 1.5% agarose gel. Products of interest were ligated into the PT7 T-vector (Novagen) and transformed into competent NovaBlue cells (Novagen). Minipreps of cDNA were prepared from the colonies. Clones were sequenced by either the dideoxy chain termination method using Sequenase 2.0 (USB) or cycle sequencing using the Ampli-Cycle Sequencing kit (Perkin Elmer) as specified by the manufacturer using an annealing temperature of 55°C.

Western Analysis

Cells were harvested in ice-cold phosphate buffered saline. The samples were centrifuged at 3000 x g for 5 minutes. The pellet was disolved in buffer containing 50mM Tris at pH 7.4, 50 mM Sodium Fluoride, 100 mM Sodium Chloride, 1 mM Sodium Vanadate, 1mM Dithiothreitol, 200μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin and 10 μg/ml aprotinin and centrifuged 15 minutes at 12,000 x g. Next 10 μg of protein from each sample was electrophoresed on a 7.5% SDS-polyacrylamide gel. The protein was electroeluted onto an Immobilon membrane (Millipore). The membrane was blocked with 10% nonfat milk and 1% Tween 20 for 1 hour and incubated with anti-exchanger polyclonal antibody (provided by Dr. John Reeves) in 1% nonfat milk. The membrane was washed and then incubated with protein A (Sigma). The blots were developed with enhanced chemiluminescence detection (Amersham), exposed to XOMAT AR film and developed (66).

RESULTS

Determination of the Time Course of Exchanger Message Upregulation

In order to examine whether the upregulation of the exchanger message was a long term event, northern analysis was carried out using RNA isolated from the ventricles of cats subjected to one week and two weeks of right ventricular pressure overload. Fifteen micrograms of total RNA from each ventricle was hybridized to a ^{32}P-dCTP labeled NCX1 probe specific for the open reading frame. This probe was generated by polymerase chain reaction of nucleotides 2257 to 3185. A probe for GapDH, a housekeeping gene, was used to normalize for loading differences.

Representative data from these experiments is depicted in Figure 3-3. The data show significant elevation of exchanger in each pressure overloaded right ventricle compared with the same animal control. The upregulation was consistently at least two fold; however, variation in exchanger expression between cats makes more detailed comparison of levels between different time points difficult. The trend shown here, approximately two fold increases in the right ventricle, has been duplicated with other specimens at each time point. In summary, the rapid upregulation of the exchanger in response to pressure overload appears not to be a transient phenomenon but is actually maintained for at least one to two weeks.

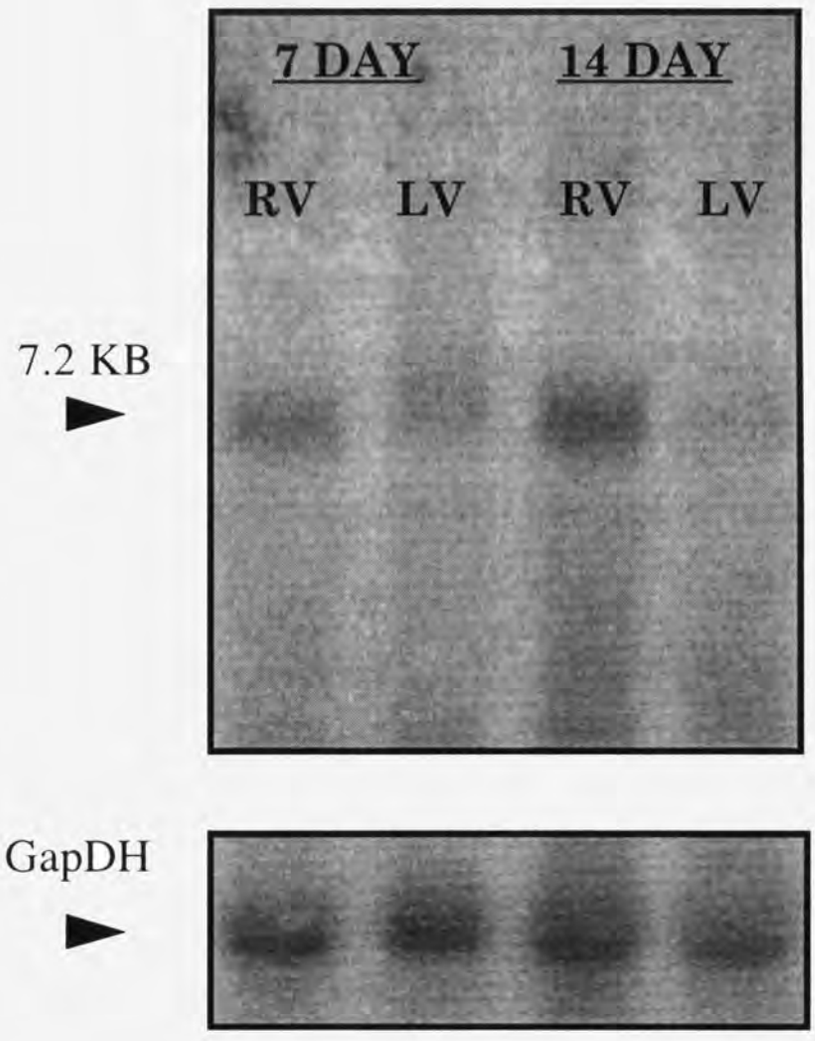


Figure 3-3 Northern analysis of the sodium-calcium exchanger after one and two weeks of right ventricular pressure overload. The pulmonary arteries of cats were surgically banded for one to two weeks, producing right ventricular pressure overload. The cats were then sacrificed and 15 micrograms of total RNA from each ventricle was subjected to northern analysis using a 32P-labeled exchanger probe. There is an increase in exchanger mRNA in the right ventricle compared to the left at each time point. Quantity of RNA in each lane was normalized with a probe for GapDH, a housekeeping gene.

Effect of Other Hypertrophic Stimulators on Exchanger mRNA Expression

Based on the upregulation of the exchanger by pressure overload, and the role the exchanger may play in the changing calcium homeostasis associated with pressure overload it was postulated that other mediators known to produce similar patterns of gene expression to those seen in pressure overload hypertrophy may also affect exchanger levels. This would suggest that the exchanger was regulated by a similar pathway to other proteins upregulated in hypertrophy. This would also provide a cell model of cardiac hypertrophy in which to explore transcriptional control. To test the effects of other stimuli, phenylephrine, an α-adrenergic agonist, was used to stimulate exchanger expression in a rat neonatal cell model. Cardiocytes were plated at a density of 1 x 10^5 cardiocytes/cm^2 , allowed to attach overnight, and placed on serum free media the next morning.

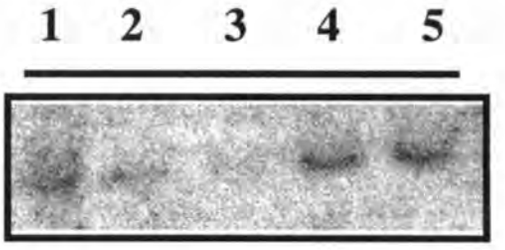
On day three, the cells were treated. The experimental cells were treated with 100 μM phenylephrine for one hour. Beating has been shown to induce the hypertrophic response. In fact, cardiocytes plated in high density, which leads to strong rhythmic beating, has been used by researchers as a cell model for hypertrophy. Therefore three controls were used: untreated cells, cells treated with 50 μM potassium chloride, and cells treated with 10 μM verapamil. The latter two controls are quiescent controls; potassium chloride stops beating through affects on membrane depolarization, and verapamil

blocks calcium channels preventing the calcium influx necessary for contraction.

After treatment, the cells were harvested. Ten micrograms of total RNA from each sample was subjected to northern analysis, including hybridization to the NCX1 probe described above. As shown in Figure 3-4 the exchanger was upregulated at least two fold by phenylephrine treatment when compared to nontreated control, and several fold when compared with quiescent cardiocytes. Similar experiments in which cardiocytes were treated for longer time periods indicate that the exchanger is upregulated as long as 8 hours. Others have found that veratridine stimulation of rat neonatal cardiocytes produces similar results; two to four hours of veratridine treatment causes a similar increase in exchanger message levels (34).

The effects of passive stretch on exchanger mRNA levels was tested using adult feline cardiocytes. These experiments were carried out in conjunction with Mary Barnes and Dr. Robert Kent. Adult cardiocytes were prepared by Mary Barnes. The cells were plated on laminin coated, 2 mm thick polyurethane membranes by Dr. Kent. The cardiocytes were stretched ten percent past their resting length. Total RNA was isolated from stretched and non-stretched cardiocytes, and 15 μg of each total RNA sample was used for northern analysis and probed for NCX1. A probe for a ribosomal subunit (P59) was used for normalization. As demonstrated in Figure 3-5 the exchanger mRNA levels increased more than two fold in response to one and four hours of stretch.

A.



1. Control
2. KCl
3. Verapamil
4. Phenylephrine-1 hr
5. Phenylephrine-2hr

B.

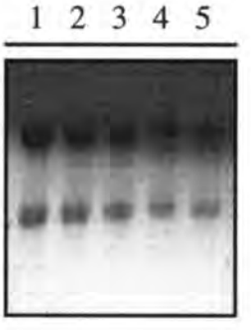


Figure 3-4 The effect of one to two hours of phenylephrine stimulation on NCX1 mRNA. A.) Rat neonatal cardiocytes were treated with phenylephrine for one to two hours. Untreated, verapamil, treated, and KCl treated cells served as controls. Total RNA was isolated from each set of cells and subjected to northern analysis using 32P-labeled exchanger cDNA. B.) Prior to transfer, the RNAwas stained with ethidium bromide. Differences in staining reflect variation in the quantity of RNA loaded in each sample.

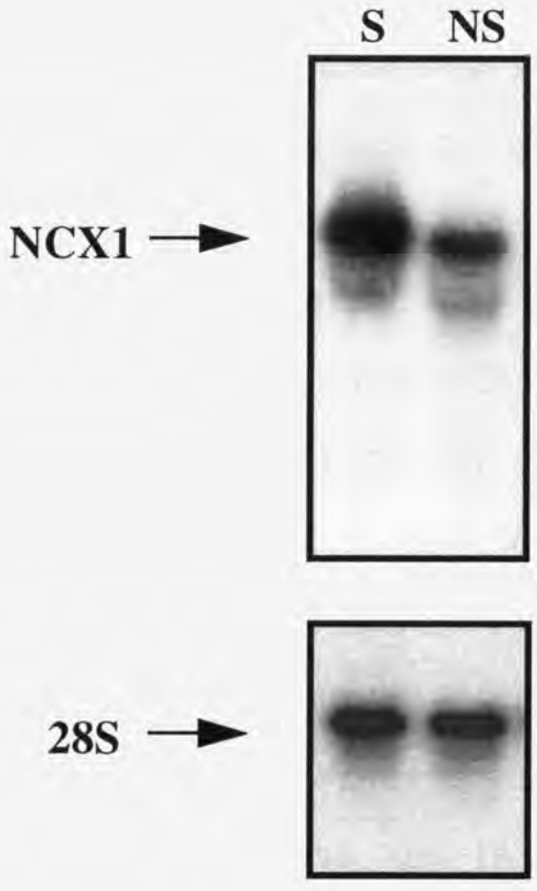


Figure 3-5 The effect of passive stretch on NCX1 mRNA. Cardiocytes were unstretched (NS) or stretched by 10 % (S). Northern blot hybridization using a 32P-labled NCX1 probe was performed on total RNA isolated from each sample. The blot was hybridized to a probe for the 28S ribosomal subunit to normalize for differences in loading.

Analysis of Loop Isoforms In Pressure Overload vs. Normal Feline Myocardium

While it is evident the exchanger message is upregulated in response to pressure overload, it is not known whether there are qualitative changes in the number or ratio of exchanger isoforms expressed. To assess differences in isoform distribution resulting from pressure overload, reverse transcriptase-polymerase chain reaction experiments were carried out using cats subjected to one hour and one week of right ventricular pressure overload. The protocol was as described for similar experiments in chapter two in which normal adult and fetal tissues were used. The results are depicted in Figure 3-6. Only bands corresponding to the two loop splice variants seen in the normal heart (see chapter 2) were seen after one hour (data not shown) and one week of pressure overload.

Northern analysis of one and two week pulmonary banded cats was carried out to determine how the two cardiac loop isoforms are regulated in response to load. Fifteen micrograms of total RNA was isolated from each ventricle and separated on a formaldehyde-agarose gel. The RNA was then transferred to a membrane and hybridized to a ^{32}P-labeled probe specific for exon A or B. As described in chapter 2, in the heart, exon A is found only in the larger splice variant (ACDEF) while exon B is found only in the smaller variant (BD). The results of a typical experiment are shown in figure 3-7. Both splice variants are increased in response to one to two weeks of pressure over load.

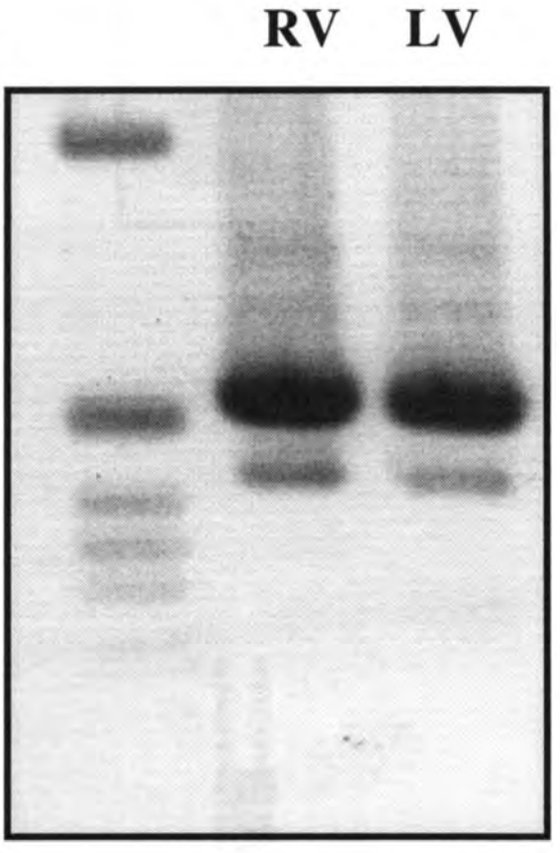


Figure 3-6 Products of reverse-transcribed polymerase chain reaction amplification of the variable region of the exchanger cytoplasmic loop in pressure overload. Amplification of NCX1 cDNA from right (RV) and left (LV) ventricles of a one week right ventricular pressure overloaded cat was carried out as for adult tissues (Chapter 2). No differences in the expression of loop isoforms expressed was detected between LV and RV.

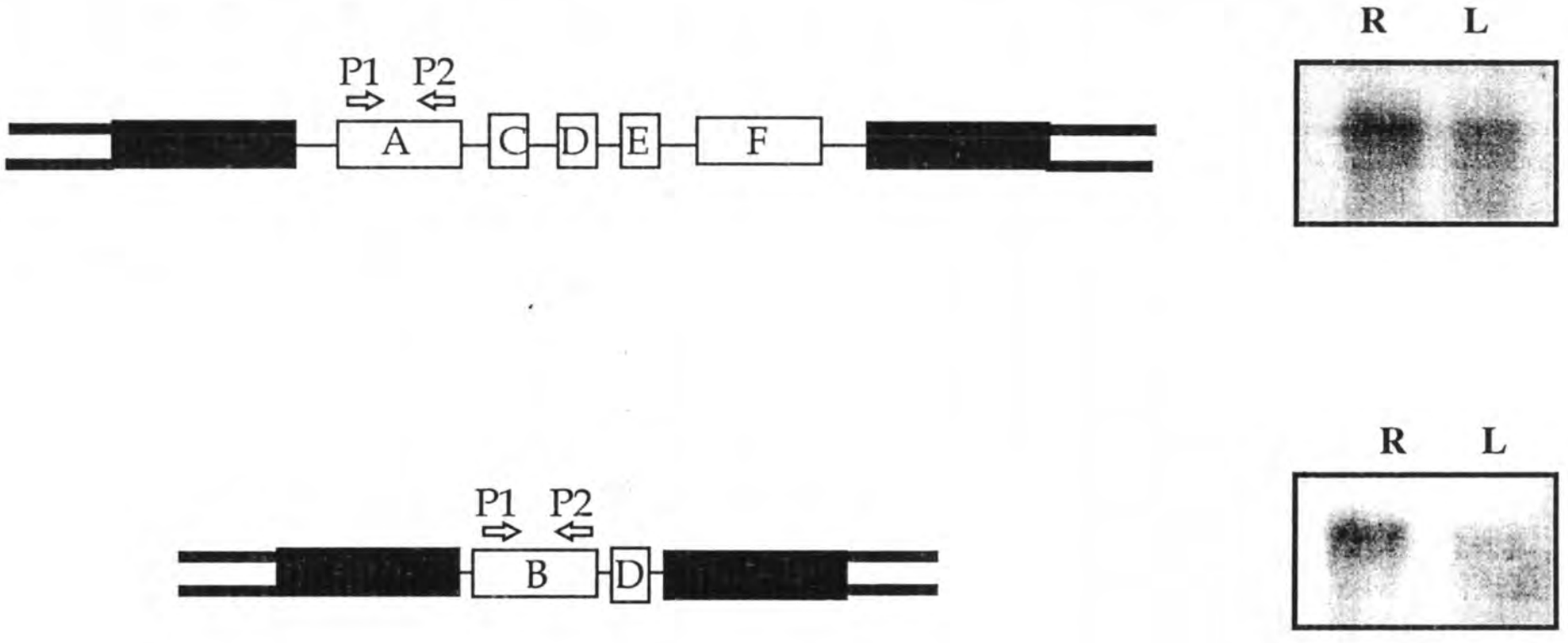


Figure 3-7 Northern analysis of the cardiac expression of NCX1 exons A and B after one week of right ventricular pressure overload. The pulmonary arteries of the cats were surgically banded for one week producing increased right ventricular pressure. Each cat was then sacrificed and 15 micrograms of total RNA from each ventricle was subjected to northern analysis using 32P-labeled cDNA probes specific for exons A and B.

DISCUSSION

This study has explored several aspects of the regulation of sodium calcium exchanger gene expression in cardiac hypertrophy. Upregulation of the exchanger is rapid but until recently had only been shown to exist for up to four hours. This allowed for the possibility that the upregulation may be a shock response which, like immediate early gene upregulation, is seen only in the first few hours after load is imposed.

In this work, Northern analysis has shown that the upregulation of the exchanger mRNA is sustained for at least one to two weeks. Within two weeks, an overloaded ventricle has fully adapted its mass to the changes in load. Hence, the upregulation of the exchanger is coexistent with the changes in the heart involved with hypertrophic growth. This does suggest that the upregulation of NCX1 may be important in long term adaptation of the cardiocyte to the load conditions. In more recent studies, human hearts that are in failure have been shown to express significantly more NCX1 mRNA and protein. This increased NCX1 gene expression in failing hearts was significant and comparable whether the hearts were damaged by ischemia or cardiomyopathy (33). Thus the rapid upregulation of the exchanger appears to be maintained throughout the process of hypertrophic adaptation and into maladaption of the myocardium, and appears to be a general response to a variety of cardiac insults.

The potential role of the exchanger in heart failure is consistent

with earlier studies that have implicated abnormal calcium regulation in the pathogenesis of heart failure. For instance, prolongation of calcium currents and impaired cardiocyte relaxation have been associated with both cardiac hypertrophy and heart failure (30-32). In addition to the studies presented here, others have shown the exchanger to be upregulated in pressure overload hypertrophy and heart failure, coincident with down-regulation of SERCA 2a (32-34,78-81,92,93). It is clear that changes in calcium homeostasis occur in hypertrophy and failure, and that the exchanger, as the primary mechanism for calcium efflux, can have a major impact on calcium handling. It is presumed that the increased expression of the exchanger may be a compensatory mechanism responding to the decreased function of proteins of the sarcoplasmic reticulum.. More studies are required to determine the impact to the changes of NCX1 expression on calcium handling.

The exchanger is upregulated by other factors which simulate the pressure overload pattern of gene expression. For example, the exchanger is upregulated by phenylephrine stimulation of neonatal cardiocytes, which is a very well characterized cell model for pressure overload hypertrophy. Phenylephrine has been shown to drive increases in exchanger mRNA and protein (85-87). Similarly, passive stretch and veratridine stimulate increases in exchanger mRNA (88, 34, this work).

Interestingly, the exchanger mRNA is not upregulated by insulin, a hormone that causes an increase in protein synthesis in the cardiocyte (94). Thus, not all anabolic stimuli upregulate the

exchanger. This further underscores the specificity of the exchanger response.

Each of the agents which turn on the hypertrophic gene pattern act through a number of distinct signaling pathways. Some of these pathways may not be directly related to hypertrophy. The direct effectors of these agents are very different indicating that pathways intersect distally to produce some shared effects. The data suggests that the exchanger, like atrial natriuretic factor and many contractile proteins, is stimulated by components common to the transduction pathways of these agents--components which are most likely involved in the production of the hypertrophic response which they all share.

Several signaling molecules common to the signaling pathways of each stimuli have been explored as components of hypertrophic signaling pathway. Protein kinase C is turned on downstream of each of the above stimuli and has been implicated in transcriptional activation of several markers for hypertrophy including the β myosin heavy chain gene (95). Similarly, the activation of mitogen activated protein (MAP) kinase has been implicated in turning on the hypertrophic gene responses (96,97).

Most of this work has been done with upregulation of contractile proteins as an endpoint. In order to better understand signaling pathways involved in hypertrophy, it may be useful to determine what role these and other signaling components play in the upregulation of non-contractile proteins, especially proteins that have other functional significance in the heart. The rapid and sustained upregulation of the exchanger in response to load makes it

an excellent marker for hypertrophy for these studies, and the functional significance of the exchanger in the heart is better defined than for other non-contractile protein markers such as immediate early genes and atrial natriuretic factor.

Hypertrophy, like development, does not change the pattern of NCX1 isoform expression seen in heart. The two loop isoforms seen in the normal heart are still seen in the heart after one hour or even one week of pressure overload. The quantity of both isoforms are increased in response to load conditions. Preliminary studies suggest that the BD containing isoform may increase more dramatically than the ACDEF isoform. Additional Northern analysis or S1 protection studies are necessary to confirm that the level of increase of BD is significantly different.

The role of the primary sodium and secondary calcium flux into the loaded cardiocyte in the upregulation of the exchanger is not clear. Veratridine functions as a fast sodium channel agonists--cardiocytes treated with veratridine experience an internal flux of sodium followed by calcium just as pressure overloaded cardiocytes do. Others have shown that the veratridine does stimulate increases in exchanger mRNA, and other messages upregulated in pressure overloaded (34). This suggests that the influx of sodium is enough to start the process which turns on the gene program seen in pressure overload. How important is the influx of calcium? Cells treated with either veratridine or phenylephrine in media lacking calcium have diminished or undetectable upregulation of the exchanger. It appears that calcium influx is necessary for the upregulation of the exchanger by these stimuli (34).

In conclusion, the rapid, long-term, upregulation of the exchanger specific to pressure overload type hypertrophic stimuli makes the exchanger an excellent candidate gene for the study of the more distal signaling molecules involved in the hypertrophic response. Particularly, study of the NCX1 exchanger cardiac promoter may provide insight into the transcription factors that regulate the changes in gene expression characteristic to pressure overload hypertrophy.

CHAPTER IV
CLONING OF THE CARDIAC, KIDNEY, AND BRAIN PROMOTERS OF THE FELINE NCX1 GENE

INTRODUCTION

The Na-Ca exchanger is an integral membrane protein involved in the maintenance of calcium homeostasis in a number of cell types. Under physiological conditions, the exchanger functions as an antiporter moving sodium and calcium in opposite directions across the cell membrane. The exchanger electrogenically catalyzes the exchange of one intracellular Ca^{2+} for three extracellular Na^{+} ions (7-9). NCX1 is predominantly expressed in cardiocytes, neurons and cells of the renal tubules. However, it is expressed at lower levels in a variety of other cell types including skeletal muscle, smooth muscle, lung and spleen (2-6).

The cDNA for the NCX1 Na-Ca exchanger was originally cloned from canine heart (16) and more recently has been cloned from other tissues such as brain and kidney, and other species including the cow (36), rat (37), human (38) and cat (99). It shows high homology across species and tissues and the predicted primary structure of NCX1 includes a 32 amino acid NH_2-terminal signal sequence followed by eleven putative transmembrane domains (16).

Between domains five and six lies a large intracytoplasmic loop which is involved in regulation of the exchanger (18). Near the C-terminal end of the loop is a variable domain of 110 amino acids which is generated by the tissue specific alternative splicing of six NCX1 exons (21). Sixteen tissue specific isoforms have been identified (19). In addition, four distinct 5'-untranslated regions

have been identified each with a unique tissue distribution (21, 100, and chapter 2).

Regulation of exchanger isoforms is made even more complex by the discovery of two additional NCX genes in mammals. In contrast to the more ubiquitous expression of NCX1, the NCX2 and NCX3 genes are expressed only in brain and skeletal muscle (23-24). NCX1 expression appears to be regulated by three tissue specific promoters which could play a role in determining the resulting splice variants in the large regulatory intracytoplasmic loop (21 and Chapter 2).

Exchange activity in cardiocytes is regulated by several factors. It is activated by cytosolic Ca^{2+} and Mg-ATP (10) and inhibited by a peptide called XIP (11), by cytosolic Na (12) and ATP depletion (13). A high affinity Ca^{2+} binding domain has been identified in the large cytoplasmic loop (residues 371-508) which is believed to be responsible for calcium regulation (19). A recent study has demonstrated that the exchanger is phosphorylated via a protein kinase C dependent pathway and that NCX1 phosphorylation coincides with upregulation of exchanger activity (14).

In addition, the exchanger is regulated at the transcriptional level in cardiac hypertrophy. The NCX1 mRNA is upregulated for at least two weeks in response to pressure overload (see Chapter 3). Other researchers have shown upregulation of exchanger message in end-stage heart failure (33). Study of the exchanger promoter may yield insight into the factors involved in cardiac gene regulation during hypertrophy and failure. This chapter features the molecular cloning of the cardiac, brain and kidney promoters, the exons encoding their

respective 5' untranslated regions, and the first open reading frame exon of the feline NCX1 gene. In addition, the NCX1 cardiac promoter will be explored revealing regions required for cardiac expression and upregulation.

EXPERIMENTAL PROCEDURES

Screening of Genomic Libraries

A cat genomic library in Lambda Fix II vector purchased from Stratagene. The library was screened using standard techniques (66). The library was plated on 10 150 mm plates at 30,000 plaques/ plate. Duplicate lifts from each plate were hybridized to the ^{32}P-dCTP labeled PCR probes. This library was screened using the same ^{32}P-dCTP labeled PCR probes used for Northern analysis of 5' untranslated region isoform distribution in chapter 1. Filters underwent four 20 minute washes at 42°C: the first two washes in 2 x SSC/.1% and the last two in 0.2 x SSC/ 0.1% SDS. The filters were then exposed to XOMAT AR film (Dupont) overnight. Plaques that produced signals on both of the duplicate lifts were isolated and purified to homogeneity through three to four additional rounds of screening using the same probes and wash parameters.

Lambda DNA was isolated from plate lysates using The Lambda Quick Kit (Stratagene). To map the clones, DNA from each clone was digested with restriction enzymes, run on a 0.7% agarose gel, and transferred by downward capillary method onto Duralon membrane. The membrane was then hybridized to the same probes used for library screening, washed to a stringency of 0.2 x SSC/ 0.1% SDS, and exposed to XOMAT AR film overnight. To subclone fragments containing exons, genomic clones were then digested with EcoR1 (Promega) and ligated into the pBluescript SK vector (Stratagene)

using T4 ligase (Promega). Subclones containing exons were identified through colony hybridization to the appropriate probe. Both strands of each positive subclone were sequenced in the regions of the exons to determine exon-intron boundaries and confirm the sequence of each exon. Positives were sequenced by either the dideoxy chain termination method using Sequenase 2.0 (USB) or cycle sequencing using the Ampli-Cycle Sequencing kit (Perkin Elmer) as specified by the manufacturer using an annealing temperature of 55°C.

Isolation of mRNA

Feline brain, kidney, and heart tissue were dissected and immediately frozen in liquid nitrogen. Feline mRNA from heart, brain, and kidney was isolated using the FAST TRACK kit from Stratagene. Samples were stored at -70^{0}C.

Primer Extension

10 µg of mRNA was reversed transcribed at 42°C for 1 h in a 20 µl reaction consisting of 1 x reverse transcriptase buffer, 0.2 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 0.02 mM dCTP, 4 mM DTT, 200 units of M-MLV reverse transcriptase (Gibco) and 6.5 µl ^{32}P dCTP. The reaction was heat inactivated at 90°C. After addition of 10 µl of Sequencing stop buffer (USB) each sample was heated at 95°C for 3 minutes and run on a 7 M urea, 8% polyacrylamide sequencing gel. In order to determine the size of primer extension products a sequencing reaction was loaded on the gel and run simultaneously. Sequencing was carried out as described for 5'RACE experiments

(Chapter 2). The gel was then dried and exposed to XOMAT AR film for 3 to 6 h.

S1 Nuclease Protection

S1 protection was carried out according to previously published methods (101). A 57 bp end labeled oligo was coprecipitated with 30 mg of cardiac total RNA or yeast tRNA. The pelleted nucleic acids were dissolved in 80% formamide, 0.4 M Pipes, pH 6.4, 0.4 M NaCl, 1 mM EDTA, denatured 10 min. at 90 degrees, and annealed for 3 h at 55-60°C. The DNA-RNA hybrids were subjected to S1 nuclease digestion in 300 ml of 0.05 M ammonium acetate, pH 4.5, 0.25 M NaCl, 0.01 M zinc chloride containing 6.25 mg of denatured salmon sperm DNA. 200-300 units of S1 nuclease was added and samples were incubated 30 min at 37°C. Reactions were stopped by the addition of 3 µl of 5 mM EDTA, extracted with phenol/chloroform, and precipitated with ethanol. S1 products were resuspended in 10 µl of dH_20 and analyzed by polyacrylamide gel electrophoresis as with primer extension reactions.

Promoter Reporter Plasmid Constructs

Digestion of the genomic subclone containing exon H1 with Sac I (New England Biolabs) liberated a 2200 bp fragment encompassing the H1 exon and 2000 bp of 5' flanking region. This fragment was cloned into the SacI site of the PGL2 Basic vector in both forward and reverse orientations. Deletion mutants were generated by a PCR mutagenesis strategy (102). First a region was deleted from the genomic subclone containing exon H1 by amplification with XL Taq

Polymerase (Perkin Elmer) using a 3' sense primer and 5' antisense primer lying on either side of the region to be deleted (described in results). The amplification reaction mix consisted of 1x XL buffer, 1.1 mM $Mg(OAc)_2$, 200 mM each dATP, dGTP, dTTP, and dCTP, 400 nM each primer, 50 ng of pBH1, and 4 units of rTth DNA Polymerase, XL. The reaction underwent 12 cycles of 92°C 1 min, 55°C 1 min, and 68°C 3 min. The template was then digested using DPN I. The PCR product was blunt ended using Pfu polymerase (Stratagene), circularized using T4 ligase (Promega) and transformed into DH5α cells (Gibco). Minipreps were made and desired fragments were released from pBluescript by Sac I digestion, ligated into the SacI site of the PGL2 Basic vector (Promega) and confirmed through sequence analysis. Sequencing was carried out as described above.

Cell Culture

Primary cardiocytes were obtained from day 2-3 neonatal rats by previously published methods (91). Briefly ventricular myocardium was isolated from 40-50 neonatal rats. The tissue was finely minced, rinsed, and placed in a cellstir apparatus. A mixture of 2.4 units/ml partially purified trypsin, 2.7 units/ml chymotrypsin, and 0.94 units/ml elastase in calcium and magnesium free Hank's salt solution buffered with 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4 was repeatedly added to the cellstir for six 20 minute incubations at 37°C to dissociate the cardiocytes. After each incubation, dissociated cells were centrifuged at 500 X *g* and resuspended in Eagles Modified Eagle's Medium (MEM) supplemented with 10% newborn calf serum. In order to

enrich for cardiocytes, the cells were pooled and subjected to differential plating for 90 min. Cardiocytes were plated at a density of 1 x 10^5 cardiocytes/cm^2 for future mRNA isolation or 1 x 10^6 cardiocytes/60 mm dish for transfection. The cardiocytes were maintained overnight in MEM supplemented with 10% newborn calf serum. The next day the cells were switched to serum-free maintenance medium. Cells treated with phenylephrine prior to RNA extraction were treated for 2 h with 20 mg/ml after 24 h of serum free media. Mouse L cells were grown and passaged at low density in Dulbecco's MEM supplemented with 10% fetal calf serum. They were plated at a density of 1 x 10^6 cells/60mm dish 24 h prior to transfection.

Transfections

One day prior to transfection, all cell types were plated at a density of 1 x 10^6/60 mm dishes. The following day the cardiocytes were placed in maintenance media supplemented with 4% horse serum 1-4 h prior to transfection while Mouse L cells were placed in DMEM supplemented with 4% horse serum.

All transfections were carried out in triplicate using 16 μg of NCX/luciferase construct cotransfected with 8 μg of cytomegalovirus promoter driven β-galactosidase expression plasmid. Cells were transfected by a modified calcium phosphate precipitation technique (103). Cells were incubated with the DNA-calcium phosphate precipitate for 16 h, then washed and kept on either maintenance media (cardiocytes) or DMEM supplemented with 10% horse serum

(Mouse L cells). Transfected cardiocytes were treated with 20 μg/ml phenylephrine immediately after being placed on maintenance medium. After 48 h, all transfected cells were washed twice with phosphate buffered saline and scraped in 400 μl 1 x Reporter Lysis Buffer (Promega). Lysates were quick frozen and stored at -70°C until they were assayed for luciferase and β-galactosidase activities.

To determine luciferase activity 5 μl of crude lysate was added to 50 μl of luciferin mix containing 75 mM luciferin, 5.5 mM ATP, 10 mM $MgSo_4$, 100 mM Tricine, pH8, and 2 mM EDTA. Light emission was measured using n Auto Lumat LB 953 luminometer. The β-galactosidase activity was determined by addition of 100 μl of crude lysate to 200 μl of a mix containing 0.1 M phosphate buffer, pH 7.3, 2 mg/ml *o*-nitrophenyl-b-D-galactopyranoside, 75 mM 2-mercaptoethanol, and 1.5 mM $MgCl_2$. The reaction was allowed to proceed 2-5 h at 37°C before it was terminated by the addition of 500 μl of 0.5 M $NaCO_3$. Absorbance was then measured spectrophotometrically at a wavelength of 410.

RESULTS

Genomic Clones

In order to determine the structure of the 5'-end of the exchanger gene and isolate its cardiac promoter region, a feline Lambda Fix II genomic library was screened using PCR probes generated from the open reading frame and each of the unique 5'-UTR regions. Screening with a probe generated from +152 to +484 yielded three clones (P1, P2, and P3) approximately 17 Kb in size, each containing a 1.8 Kb exon encoding nucleotides -34 to +1826 (Figure 4-1). Screening with probes generated from the K1 5'-UTR yielded one 18 Kb clone (M1) which encoded both K1 and Br1 sequences. Screening with probes from the H1 5'-UTR yielded three identical clones approximately 15 Kb in length.

To further characterize the clones, each was mapped through restriction analysis. EcoR1 fragments of the genomic clones were subcloned into pBluescript. These subclones were then restricted and subjected to Southern analysis which in combination with PCR between exons and sequencing were used to determine the arrangement of exons, distances between exons and the ends of the clones, as well as exon-intron boundaries.

The data were combined to prepare the map shown in Figure 4-1. The 1.8 Kb exon 2 lies alone near the center of the P clones which contain at least 6 Kb of intron upstream and at least 6 Kb of intron downstream. The M clone does not overlap with any of the P

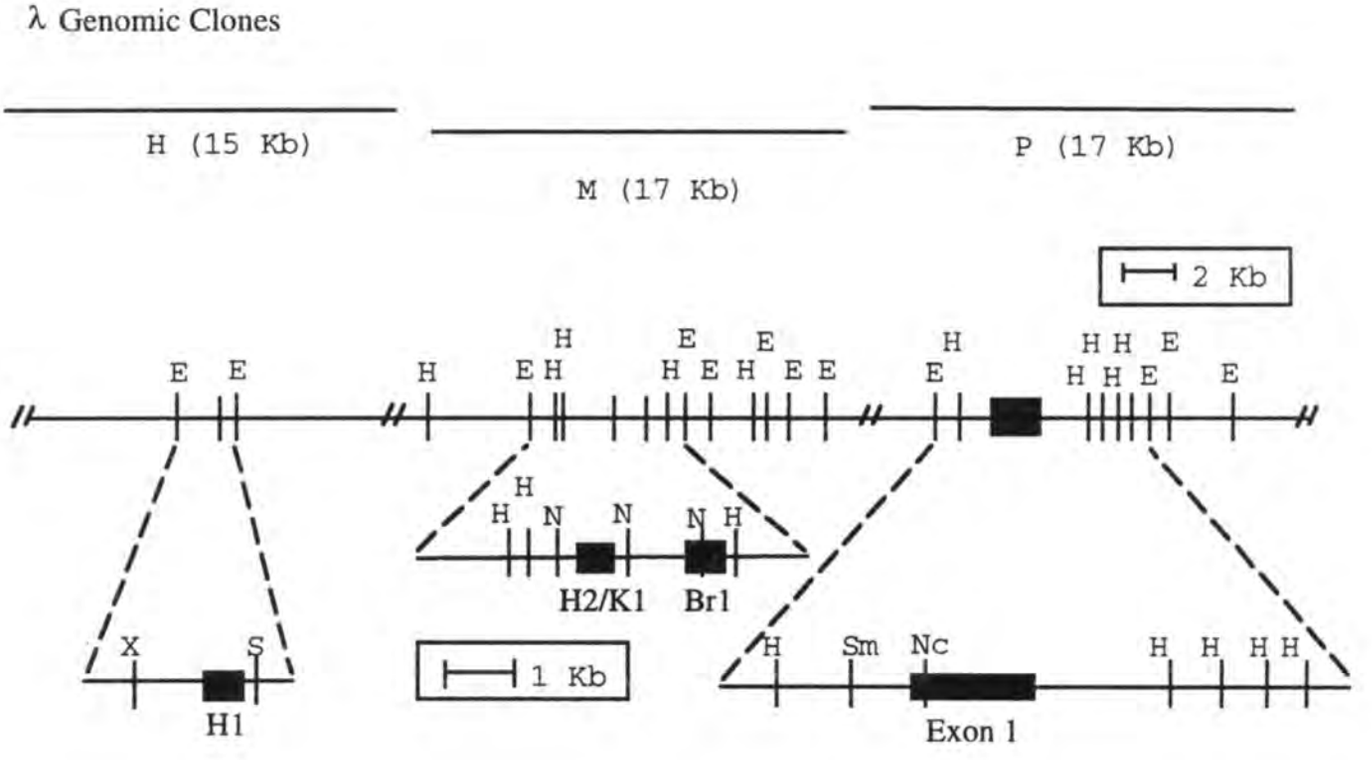


Figure 4-1 Structure of the 5'-end of the feline NCX1 gene. The three nonoverlapping genomic fragments encoding the NCX1 5'UTR exons and the first open reading frame exon are shown at the top. A partial restriction map of these clones is shown, with closed boxes depicting the exons with noncoding regions H1, K1, and Br1 and the first exon encoding translated regions. Introns as well as flanking regions are represented by interconnecting solid lines. Restriction sites for EcoRI (E), HindIII (H), NcoI (Nc), NheI (N), SalI (S), SmaI (Sm), and XhoI (X) are indicated.

clones but encodes both K1 and Br1 exons. K1 lies 1 Kb upstream from Br1 with 6 Kb of intron upstream and 6 Kb downstream of the pair. The R clones contain only the H1 5'-UTR encoded as one exon located 3 Kb from the 3'-end. There is no overlap between the R and M clones. At least 10 Kb of intron lie between the 1.8 Kb exon 2 and Br1 and greater than 10 Kb between K1 and H1. Hence, the 5'-UTR exons are spread over at least 25 Kb of genomic sequence. Each exon conforms in sequence with the cDNA clones (Fig. 4-2). Each exon ends with GT and all except K1 and the 1.8 Kb exon 2 begin with AG.K1 and the exon 2 begin with AC.

Identification of Transcriptional Start Sites

The start sites of the cardiac isoforms have been mapped by primer extension. Three transcriptional start sites were identified in heart corresponding to 110, 118 and at 122 bp 5' of the AUG codon (Figure 4-3) using an antisense oligonucleotide corresponding to 51-71 nucleotides 5' of the AUG codon. Primer extension with a second oligonucleotide yielded products corresponding to the same initiation sites. These were verified through S1 protection using an antisense end labeled oligonucleotide spanning -142 to -88. S1 digestion produced protected fragment 40, 36, and 22 nucleotides in length, confirming sites identified through primer extension. Control primer extension and S1 protection reaction using yeast tRNA as template showed no products.

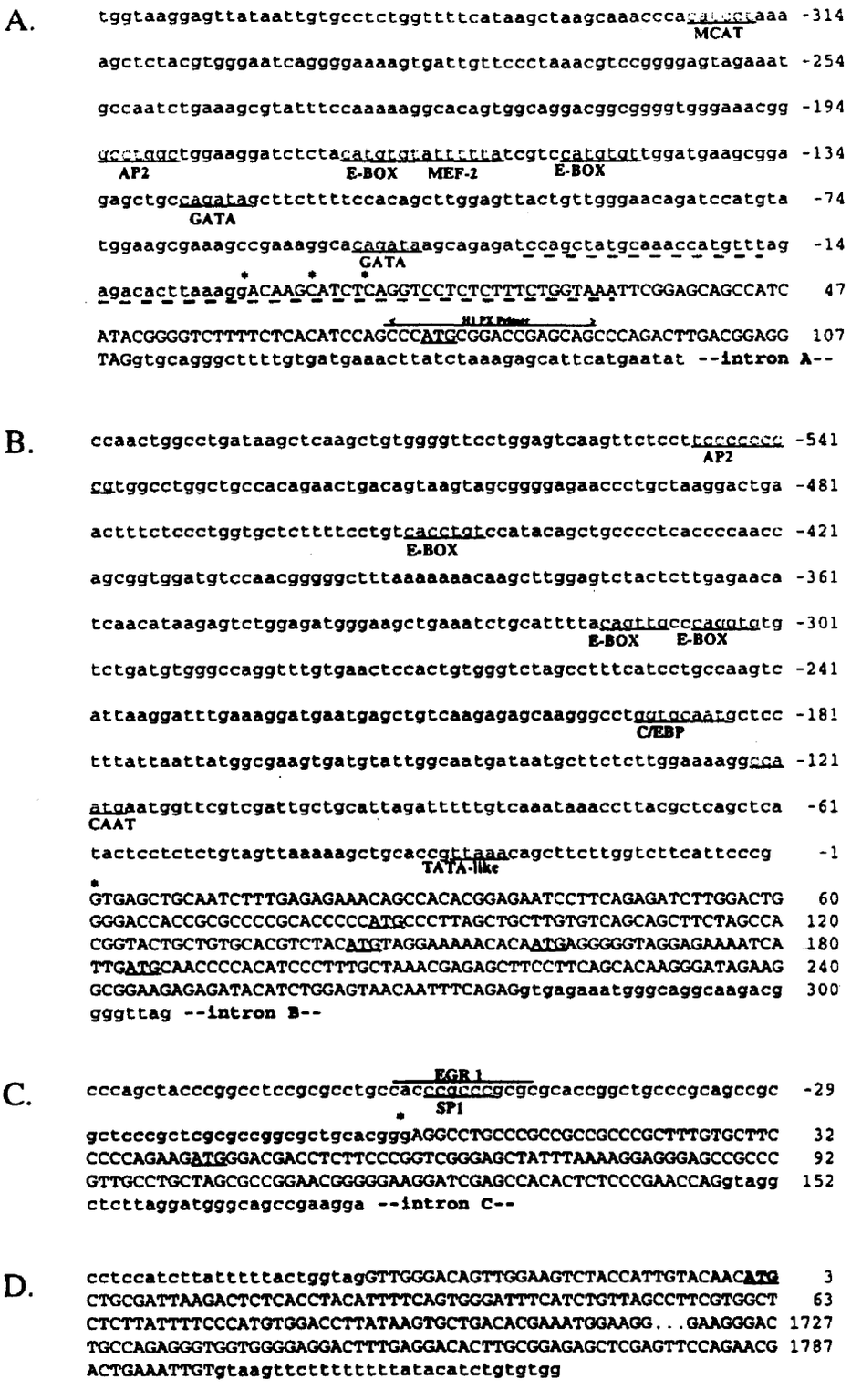


Figure 4-2 DNA sequence of the first four exons and immediate flanking regions of the H1, K1, and Br1 exons. Exons are uppercase, dashed line is the site of the probe for S1 mapping, and (*) denotes transcriptional start sites. A, B, and C are genomic sequence of H1, K1 and Br1 respectively, numbered from the transcriptional start. D is the genomic sequence of exon 2 numbered from the translational start (**ATG**). (...) represents bases 113-1719.

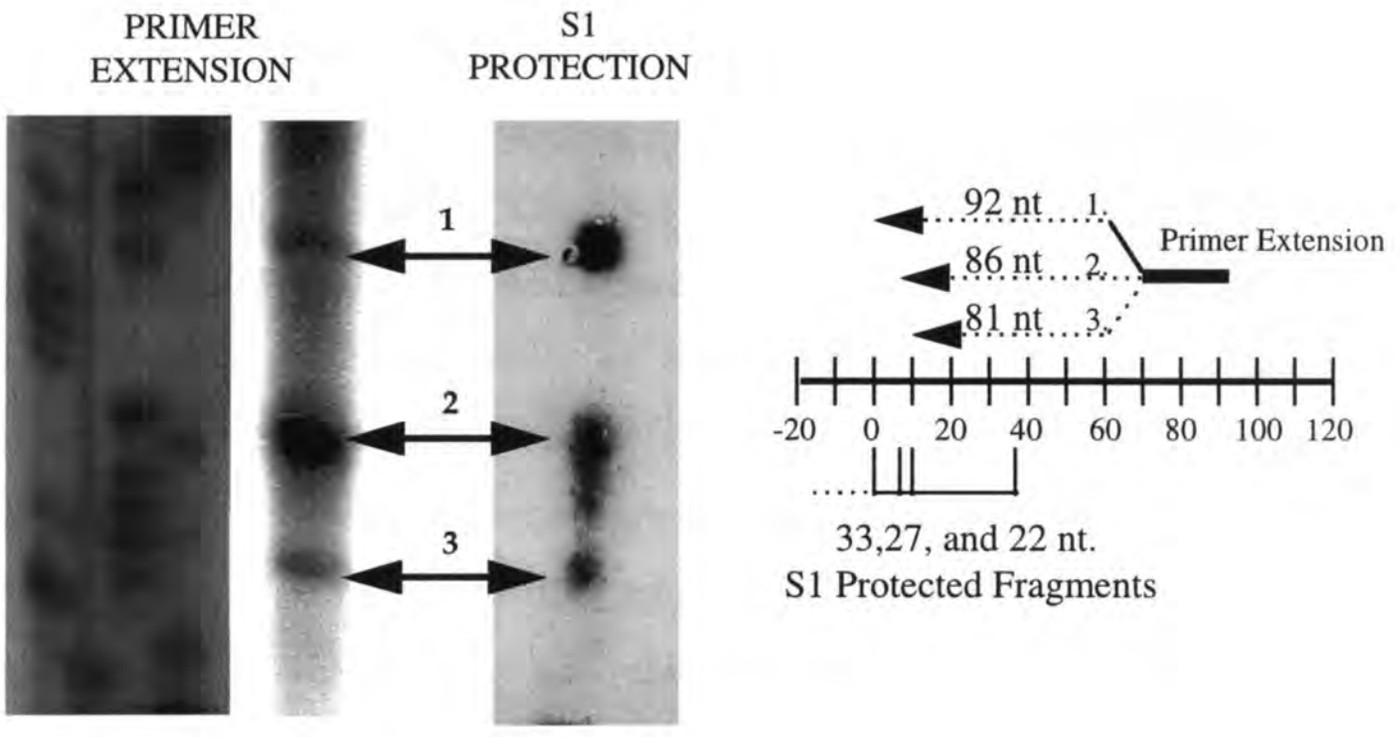


Figure 4-3 High resolution mapping of heart isoform transcriptional start sites. Shown are the results from the primer extension mapping of the 5'-end of the heart NCX1 transcripts. The primer used is shown in Figure 4-2 and begins 71 nucleotides 5' of the translational start site. Three distinct products were observed 92, 86, and 81 nucleotides long. The multiple start sites were confirmed by S1nuclease mapping. 5'-labeled oligonucleotide probe (position shown in Figure 4-2) was annealed to 30 micrograms of total cardiac RNA and then digested with S1 nuclease. The positions of the major protected fragments are indicated. The results of S1 nuclease protection and primer extension are summarized in the schematic diagram, with nucleotides numbered in reference to the transcriptional start of exon H1.

5' Upstream Regions

The sequence surrounding the cardiac transcriptional start site contains no apparent TATAA sequence or CCAAT box. As in other promoters lacking a TATAA box, there are several SP1 sites. These SP1 sites are found overlapping -208, -212 bp upstream of the first transcriptional start site. There are two CANNTG motifs (E boxes) at positions -173 and -155 which are potential target sites for the helix-loop-helix (HLH) family of transcription factors (Figure 4-2). A single M-CAT consensus element is present at position -325. M-CAT elements govern myoD independent cardiac transcription of several cardiac genes including cardiac troponin T (104) and C (105), b myosin heavy chain (106) and skeletal α-actin (107). The promoter region contains consensus sequence for two GATA boxes at position -49 and -124. Several cardiac specific genes such as myosin light chain 1A, IV and b myosin heavy chain (108, 109) contain conserved GATA binding motifs. The GATA elements in atrial natriuretic peptide (110) and a myosin heavy chain (111) genes have been shown to be critical for cardiac expression.

Sequencing the 5' flanking region upstream from the K1 exon revealed a TATAA like motif at -30 and a CAAT box at -133 (Fig. 4-2). A consensus site for C/EBP was found at -204. Further upstream are putative sites for P2, AP2, GATA-1, and two E boxes. In order to identify consensus sequences possibly involved in transcriptional regulation of the brain isoform, we have sequenced a portion of the 5' flanking region. Sequencing proved difficult presumably due to a GC content of over 75% in the region. No TATAA or CAAT boxes were evident. We have identified two overlapping Sp1 sites at -54 and -

53 which are common in housekeeping genes (Figure 4-2). An *EGR-1* consensus site begins at -54, encompassing both Sp1 sites. At position -7 there is an E box, which is the consensus sequence for the helix loop helix family of transcription factors. Members of the helix loop helix transcription factor family have been implicated in the regulation of other neuronal genes. An AP2 site has also been identified at +13.

Transfection Experiments

Transient transfection of freshly isolated rat neonatal cardiocytes confirmed the identity of the cardiac promoter. A fragment containing the exon H1 and 2 Kb of 5'-flanking sequence was cloned in both the forward and reverse (pH1-2.0) directions into the PGL2 basic vector (Promega). As shown in Figure 4-4 the forward construct reproducibly drove levels of luciferase expression 24-55 times that of the PGL2 basic vector alone. The reverse construct had no activity over vector alone. In addition, 1 Kb of the kidney 5'-flanking sequence was also ligated into the PGL2 basic vector. The kidney promoter did not drive luciferase expression significantly above background levels when transfected into neonatal cardiocytes (data not shown). The chimeric luciferase gene construct containing either the cardiac or kidney promoters was transfected into fibroblast mouse L cells. Luciferase activity in the transfected L cell extract was just above that of the pGL2-Basic vector alone.

We have previously shown that in the intact heart the levels of sodium calcium exchanger mRNA and protein are significantly elevated in early hypertrophy produced by pressure overload. This

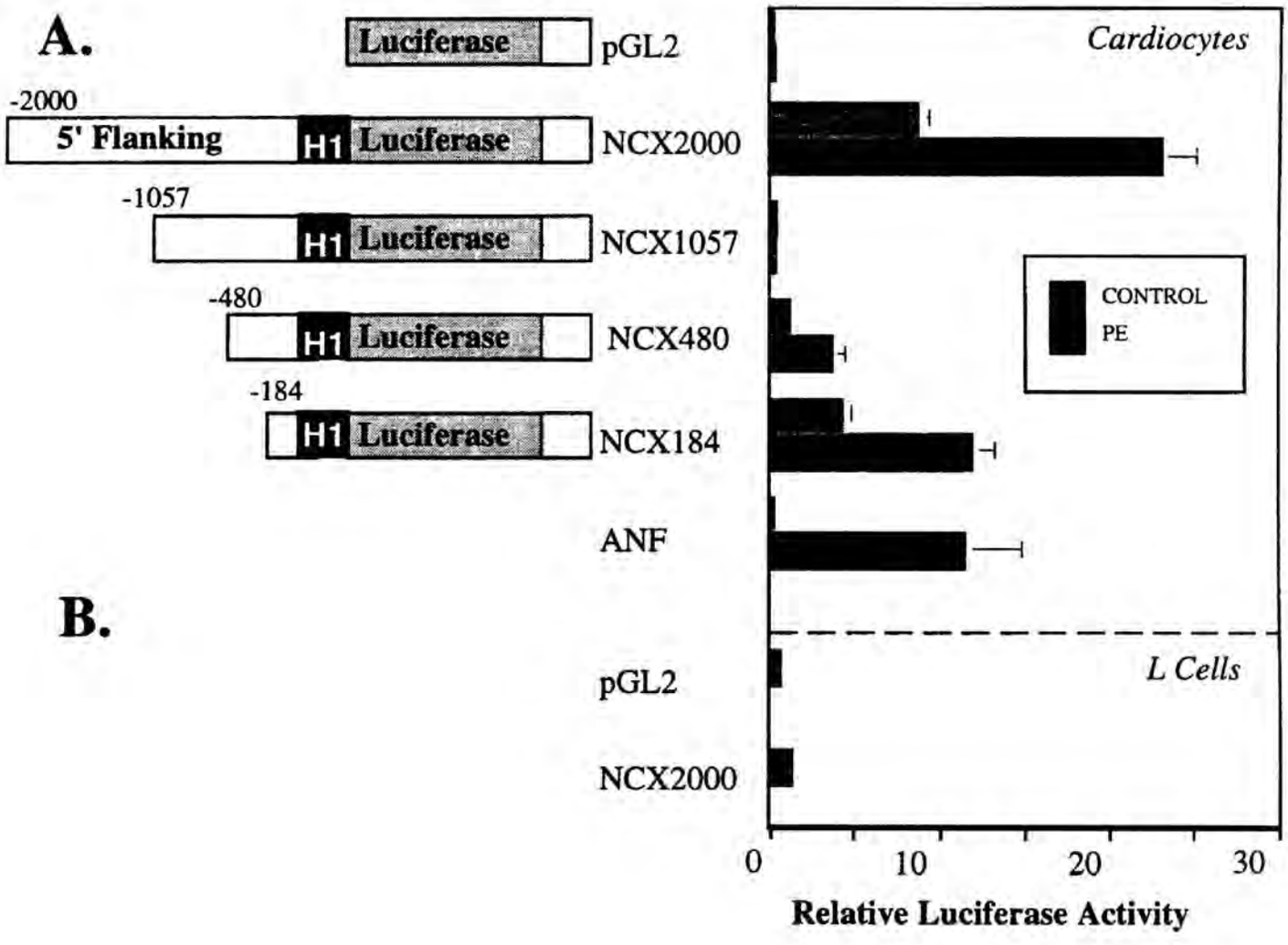


Figure 4-4 Expression of NCX1 H1-luciferase chimeric constructs in neonatal rat cardiomyocytes (A) and mouse L Cells (B). A series of constructs containing various lengths of the NCX1 heart promoter were fused to the luciferase gene (pBL2). The constructs were individually transfected into neonatal rat cardiomyocytes (A) or mouse L cells (B). All cells were cotransfected with a cytomegalovirus promoter-driven beta-galactosidase fusion vector to normalize for transfection efficiency. 48 hours after transfection, cells were harvested, and luciferase activity was determined and normalized as described in "Experimental Procedures." Values are the mean from at least three experiments. (PE= phenylephrine).

increase in mRNA is at least two fold and occurs within one hour of pressure overload indicating regulation of the exchanger in the manner of an immediate early gene (34). $Alpha_1$-adrenergic stimulated neonatal cardiomyocytes have several structural, morphological and genetic markers of hypertrophy (85-87) and provide a good starting point for our investigation of candidate cis-elements involved in the hypertrophic upregulation of NCX1.

In chapter 2, Northern analysis was carried out using total RNA isolated from neonatal cardiocytes that were untreated or treated with 100 μM phenylephrine for 2 h. The data show at least a two-fold increase in exchanger mRNA. To determine if the rise in mRNA seen under phenylephrine stimulation is transcriptionally regulated by elements within the pH1-2.0 construct, we treated cells transiently transfected with pH1-2.0 with 100 μM phenylephrine for 48 h. All dishes were cotransfected with a constant amount of cytomegalovirus-β-galactosidase vector as a control for transfection efficiency. Phenylephrine treatment induced a 1.5 to 3-fold rise in luciferase activity over that of untreated transfected cells, consistent with data from Northern analysis.

Interestingly, cell density was an important factor in the transfection experiments. All data reported above were derived from cardiocytes plated at 1.0 x 10^6/60 mm plate. At this density, there is very little cell contact and spontaneous beating is rare. Several experiments included pH2000 transfection of cells plated at higher densities (data not shown). When cells were plated at densities high enough to produce rhythmic, spontaneous beating, the basal level of activity of pH2000 transfected cardiocytes was

measured 1.5 to 2-fold greater than in transfecting of less densely plated cells. However, in these experiments the level of phenylephrine induction decreased and in several of these experiments was undetectable. If beating of control cells was inhibited by 10 mm verapamil basal activity decreased to levels just below that of the more sparsely plated cells, and phenylephrine upregulation was restored.

To further delineate the promoter regions directing cardiac NCX1 gene transcription and phenylephrine induced upregulation, luciferase reporter constructs containing shorter portions of the cardiac 5'-flanking region were created through a PCR based method (see Experimental Procedures). Clones containing 1057 bp (pH1057), 480 bp (pH480) and 184 bp (pH184) of 5'-flanking region fused to the luciferase reporter gene were generated and transfected into rat neonatal cardiocytes. In all experiments, transfection efficiency was monitored by cotranfection of a cytomegalovirus-β-galactosidase vector, and the luciferase activity was normalized to β-galactosidase activity. The deletion of -2000 to -1057 bp yields a dramatic decrease in basal promoter activity. The pH1250 construct had luciferase activity just above that of the control promoterless plasmid pGL2-Basic. Constructs truncated at -480 (pH500) or -184 (pH250) bp yield luciferase activity at 15 and >50% of the pH2000 construct. Both pH184 and pH480 construct promoter activities were up-regulated at least 2-fold by α_1-adrenergic treatment.

All of the constructs presented thus far include exon H1 and the first 67 bp of intron in addition to portions of the 5' flanking region. Constructs containing 2000, 1054, and 184 bp of 5' flanking

region were created in which a major portion of exon H1 (bases 22 to 112 or bases 17 to 112) was deleted. When transfected into cardiocytes, each of these constructs had less than 10 percent of control activity; activity levels very similar to those of the promoterless pGL2. These data suggest that a positive regulatory element necessary for expression in cardiocytes is present in that region. This small region does not contain previously identified regulatory elements suggesting the presence of one or more novel regulatory sequences.

In summary, a DNA fragment containing -184 to +200 bp of the feline ncx1 cardiac promoter is sufficient to drive cardiac expression and α_1-adrenergic induction. There appears to be at least one enhancer between bases +22 and +112 that may represent a new cardiac regulatory element. There also appears to be at least one other enhancer between -2000 and -1057. And finally, one or more negative regulatory elements may exist between -1057 and -184 bp.

DISCUSSION

The existence of two feline cardiac 5' isoforms differing only by the splicing of intervening sequence (K1 exon) is consistent with what was reported for the bovine cardiac NCX1 p17 clone (36). The K1 sequence was always found in cDNA clones that also included H1 and was never detected alone in any of our cardiac clones. Transfection of neonatal cardiocytes with the kidney promoter-luciferase fusion construct never gave luciferase expression above background levels, confirming that K1 expression in the heart is not regulated by the kidney promoter.

Several other genes have multiple promoters and transcripts that undergo complex alternative splicing events that are regulated in a tissue-specific or developmental manner. In some cases the alternative promoters control transcription of the same protein but diverge only in the 5'UTR sequences, such as the human mineral cortical receptor gene (113) and *lck* gene (114). The parathyroid hormone receptor gene (115) is regulated by two promoters, one tissue-specific promoter and the second controlling ubiquitous expression. The Pem homeobox gene is regulated by either an androgen dependent or androgen independent promoter in a tissue-specific manner (116). In some cases, the alternative splicing events alter both the 5' UTR and coding regions. That is what is occurring with the NCX1 gene (21,61, this work). Importantly, the two alternatively spliced 5' UTR species found in the heart yield two loop

isoforms of the exchanger (Chapter 2). Therefore unlike rodent (21,61), the cat expresses two distinct NCX1 isoforms in cardiac tissue. The cytoplasmic loop has been demonstrated to contain domains involved with intracellular calcium regulation and exchanger inhibitor peptide interaction. Given the diverse roles that the exchanger plays in kidney, brain and heart, it is believed that its regulation would differ between tissues. Thus, the variable region may play a role in the tissue specific regulation (21,61), or possibly in subcellular targeting of the exchanger.

The NCX1 brain and heart promoters have several features common to a number of housekeeping genes including a high GC content, a high frequency of the dinucleotide CpG, the absence of TATA and CAAT boxes and both have two potential binding sites (GC boxes) for Sp1. Only the kidney promoter contains canonical CAAT and TATA boxes. This is consistent with the finding that the brain and heart isoforms are expressed in several tissues (61). Although not conclusive, 5' RACE products and transfections of the kidney promoter support the premise that kidney promoter expression is tissue specific. The kidney promoter has several consensus sequences including two E boxes, a C/EBP, P2, AP2, and GATA-1 site. Only 88 bp have been obtained of the brain promoter sequence presumably because of extensive secondary structure. This proximal end of the brain promoter has a very high GC content (>75%) and two Sp1 elements. As discussed above although the heart promoter has several features of a housekeeping gene, it also has several elements which have been demonstrated to be required or important for

cardiac expression of other genes. These included a GATA element, two E boxes and a M-CAT element.

To begin to characterize the NCX1 cardiac promoter, a DNA fragment containing 2000 bases of the 5' flanking region and 122 bases of the H1 exon Plus 80 bp of the first intron was fused to the luciferase reporter gene and transfected into rat neonatal cardiocytes. Deletion analysis demonstrates that there may be one or more enhancer elements between -1057 and -480 and between -184 and +112. The first 184 bp of the 5' flanking region contain all that is requisite for cardiac directed expression and α_1-adrenergic stimulation of the NCX1 gene. This region contains the two GATA elements, two SP1 sites, a single AP2 element and two E boxes. Importantly, the M-CAT element at -380 is not required for basal expression or α_1-adrenergic stimulation. This is clearly different from what has been reported for both the β-myosin heavy chain (βMHC) (95) and skeletal α-actin (117). The α_1-adrenergic induction of βMHC requires M-CAT and possible AP-1 elements, and the induction of the skeletal α-actin promoter also requires M-CAT as well as CArG and SP-1 elements. In the future the specific elements required for cardiac expression and the upregulation of the NCX1 gene in cardiac hypertrophy may be identified.

CHAPTER 4

DISCUSSION

When the heart is subjected to increased load, a complex set of morphological and molecular changes take place. Grossly, the heart will enlarge when subjected to increased load. At the molecular level, the individual cardiocytes increase protein synthesis as they hypertrophy. This is an adaptive response of the cell, optimizing the ability of the cell to function under the more strenuous demands. However, when the increased load is prolonged, it is clear that some of the changes that occur are maladaptive, leading to decreased contractility and heart failure (72-73).

Gene regulation during the hypertrophic response is characterized by a pattern of qualitative and quantitative changes in transcription and translation that has been described as a return to a fetal gene program. It is exemplified by increased expression of beta myosin heavy chain in rodents, increased expression of alpha-actin and atrial natriuretic factor. It is also characterized by changes in genes expressed in the sarcoplasmic reticulum (73-73,79).

In fact alterations in calcium homeostasis have been implicated in load induced cardiac decompensation. Significant abnormalities in calcium handling occur in cardiac hypertrophy and failure, including prolongation of calcium currents and decreased capacity to restore low cytoplasmic calcium levels during diastole (30-32). Intracellular calcium levels regulate contraction and relaxation of the cardiocyte. The cytoplasmic calcium level is controlled predominately by the reuptake into the sarcoplasmic reticulum (SR) throughout the SR

Ca^{2+}-ATPase, and also by extrusion of calcium across the cell membrane through the NCX1 (80-81).

These changes coincide with altered expression of proteins involved with calcium handling. The SR Ca^{2+}-ATPase is down-regulated at the mRNA and protein levels in hypertrophy and in failure, decreasing the reuptake of calcium by the SR (78-79). This could produce the slowing of calcium transients and impaired diastolic calcium decline. The exchanger mRNA and protein are increased in hypertrophy and failure (34,33,93,99); this allows increased extrusion of calcium across the cell membrane, thereby limiting calcium overload during diastole. Thus the upregulation of the exchanger may be a compensatory response to decreased SR calcium uptake. The upregulation of the exchanger is maintained throughout the one to two weeks during which the heart hypertrophies in adaptation to the stimulus. This is a return to the pattern of expression seen in the early stages of development. The level of exchanger expression is initially relatively high but decreases with maturation from embryo to adult, while SR Ca^{2+}-ATPase levels increase from initially low levels (55-56).

Very little is known about the signaling pathways that lead to the cardiac expression and hypertrophic induction of genes. Much of the information that is known about transcriptional control in the heart in normal and load conditions is from the study of contractile proteins. Exceptions include ANF and immediate early genes; however, the function of these proteins in the heart is unclear (74-77). The exchanger is a noncontractile protein with a clear function in the cardiocyte. The discovery that this protein was rapidly

upregulated at the transcriptional level in response to pressure overload makes it an excellent subject for the study of transcriptional control. Characterization of the NCX1 gene can provide greater insight into gene regulation in the normal and hypertrophied heart.

The 5' end of the feline NCX1 gene has been cloned. Four unique 5' untranslated regions formed by the alternative splicing of three exons have been mapped to three nonoverlapping genomic clones. The 5' UTR exons are spread out over at least twenty-five kilobases of genomic sequence. Two different 5' UTRs were identified in heart, one of which consists of a unique cardiac exon (H1), and the other includes the exon encoding the kidney 5' UTR spliced downstream of H1. Both cardiac isoforms share the same 5' flanking sequence.

The cDNAs encoding the intracytoplasmic loop of feline NCX1 in heart, brain, and kidney have also been cloned. Two isoforms are expressed in feline heart, one previously cloned in rat heart (exons A,C,D,E, and F) and one found predominately in the kidney (B and D). Both cardiac isoforms are upregulated by pressure overload. The previous studies utilizing rat and rabbit hearts have shown only isoforms containing exon A in the heart. This is the first report of expression of exon B in an excitable tissue (22). Further studies by Dr. Guangmao Cheng have shown both isoforms to be present in pig, cow, dog, and human. The functional significance of the isoforms of the exchanger loop has not been determined, however the expression of exon B in the hearts of certain mammals may be involved in the differences in exchanger function across species. Also regulation of

the two isoforms by pressure overload may vary. Both isoforms are upregulated, however preliminary evidence suggests the BD isoform which is less abundant in the normal myocardium, exhibits a greater increase in response to load.

In order to begin characterizing the cardiac promoter, cardiac promoter-luciferase constructs were transfected into rat neonatal cardiocytes. The largest construct, consisting of bases -2000 to +200, drove expression and alpha-adrenergic induction in rat neonatal cardiocytes. By deletional analysis, a minimal cardiac promoter for cardiac expression and induction consisting of -184 to +200 has been identified. This region contains a number of elements important in the cardiac expression of other genes. These include two GATA elements, two E-boxes, a MEF-2 site, and a CArG site. Analysis of shorter constructs has uncovered other regions containing enhancers or negative elements (Chapter 4).

Since my departure from the laboratory, efforts have been underway to identify which elements are important regulators of exchanger transcription. Site directed mutagenesis has shown the 5' E-box, 3' GATA element and the CArG box to be required for expression in cardiocytes. These elements have already been shown to drive cardiac expression of contractile proteins including cardiac troponin, myosin heavy chain, and alpha skeletal actin (95,105-109,117). In addition a novel 15 bp element required for cardiac expression has been identified within the proximal region from +22 to +112. This element is also involved in regulating the load induced upregulation of the exchanger. Thus the cardiac promoter is fertile ground for the study of transcriptional control in the heart.

Experiments are underway to further characterize these elements, to define additional elements, and to determine the trans-acting factors which associate with them. In the future, regions of the promoter which are integral to in vitro regulation may be tested in transgenic mice. In addition to verifying that in vitro data is applicable *in vivo*, these studies allow exploration of regulation in development and under pressure overload in the presence of physiologically appropriate system. Unraveling the signaling pathways involved in exchanger expression and upregulation in the heart will add to our understanding of the mechanisms of altered gene expression in hypertrophy and heart failure.

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