Molecular Cloning of a Fourth Member of the Potassium-dependent Sodium-Calcium Exchanger Gene Family, NCKX4*

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We report here the identification and characterization of a fourth member of the potassium-dependent sodium-calcium exchanger gene family, NCKX4 (gene SLC24A4), which mapped to the chromosomal region 14q32. Human NCKX4 encoded a protein of 605 amino acids that displayed a high level of sequence identity to previously described family members, rod NCKX1 (gene SLC24A1), cone/neuronal NCKX2 (gene SLC24A2), and ubiquitous NCKX3 (gene SLC24A3), in the hydrophobic regions surrounding the α-repeat sequences thought to form the ion-binding pocket used for transport. The protein product of the NCKX4 gene shared the highest level of amino acid identity, as well as an almost identical arrangement of exon boundaries, with NCKX3, indicating that these two genes have arisen from a recent duplication. NCKX4 transcripts were expressed in all brain regions, aorta, lung, and thymus, as well as at a lower level in many other tissues. The NCKX4 protein demonstrated potassium-dependent sodium calcium exchanger activity when assayed in transfected HEK293 cells using digital imaging of fura-2 fluorescence. The discovery of NCKX4, as far as can be ascertained from the current version of the human genome sequence, completes the mammalian potassium-dependent sodium-calcium exchanger gene family.

Sodium-calcium exchange is an important determinant of intracellular Ca2+ control. Detailed structural and functional studies have revealed an exchanger gene superfamily comprising two arms: the potassium-independent sodium-calcium exchangers (NCX) and potassium-dependent sodium-calcium exchangers (NCKX) (1–3). The protein products of these two family members share sequence similarity in two internally homologous, hydrophobic, domains commonly referred to as the α-repeat (4). NCKX proteins are thought to catalyze the extrusion of one intracellular Ca2+ ion in exchange for three or four extracellular Na+ ions (1, 5, 6). On the other hand, NCKX proteins are thought to transport one intracellular Ca2+ and one K+ ion in exchange for four extracellular Na+ ions (7–10). Three NCKX genes (NCKX1 (SLC8A1), NCKX2 (SLC8A2), and NCKX3 (SLC8A3)) whose protein products share a high degree of sequence identity, especially within the transmembrane spanning domains, have been cloned (11–13). NCKX1 is widely distributed in many different mammalian tissues and cell types and is driven by three tissue-specific promoters (14–17), whereas NCKX2 and NCKX3 are only expressed in brain and skeletal muscle (12, 13). The functional role of the NCKX family is best exemplified by the much studied mammalian cardiac NCX1, which plays a crucial role in the relaxation process of heart muscle by extruding the Ca2+ that enters at the beginning of systole. The physiological role(s) of NCKX1 and of the other NCKX family members in tissues other than the heart has recently attracted considerable attention (1). Three genes of the NCKX family have also been cloned. NCKX1 (SLC24A1) was initially characterized in retinal rod outer segments and first cloned from bovine retina (18). NCKX1 was originally thought to be expressed only in photoreceptors where the protein plays a unique role in the visual transduction process (7). More recent molecular evidence, however, has confirmed the expression of NCKX1 in cells of hematopoietic origin (19). The second family member, NCKX2, was initially cloned from rat brain (20) and then subsequently from chicken and human retina (21). The NCKX2 gene (SLC24A2) has an unusually long second exon that contains the translational start site and codes for more than half of the protein, a property also found in the NCKX1, NCKX1, and NCKX3 genes. The NCKX2 protein has a high degree of sequence identity with NCKX1 in the hydrophobic regions and shares significant functional similarity as well (9, 22). NCKX2 is expressed mainly in neurons of both the central and the peripheral nervous system, where the physiological role of the expressed protein is currently being investigated. More recently, the third member of the NCKX family (NCKX3 (SLC24A3)) was cloned from human and mouse brain and human skeletal muscle (23). Although NCKX3 is expressed in brain, it is also present in many other tissues at significant levels and is thus the most widely expressed NCKX family member to date. NCKX3 is more distantly related to NCKX1 and NCKX2 than these genes are to one another, as is seen in the somewhat lower deduced amino acid sequence identity and in the absence of the unusually long exon 2 sequence.

As noted previously (23), analysis of NCKX transcript distribution using probes from regions of sequence conservation under conditions of reduced stringency revealed evidence for another mammalian member of the NCKX family. Based on the data of the human genome project (24, 25), a putative NCKX4 gene was located on chromosome 14 and tentatively assigned...
the name SLC24A4 (23). In this study, we have characterized this fourth member of the potassium-dependent sodium-calcium exchanger gene family. We demonstrate that NCKX4 transcripts are particularly prominent in brain and aorta and that the NCKX4 protein indeed displays potassium-dependent sodium-calcium exchange activity when expressed in HEK293 cells.

**EXPERIMENTAL PROCEDURES**

Common chemicals reagents were purchased from Fisher, Sigma, or BDH and were of analytical grade or better. Molecular cloning procedures followed standard protocols (26, 27) or those provided by the reagent supplier unless noted otherwise. Fluorescent dideoxynucleotide cycle sequencing was done at the University of Calgary Core DNA Service Facility. DNA contig assembly and sequence analysis were done in MacVector (Accelrys Inc., Madison, WI). BLAST (28) searches of the sequence data bases were run at the National Center for Biotechnology Information website (Bethesda, MD), at the Sanger Center web site (Cambridge, UK), or at the Ensemble Genome Server of the European Bioinformatics Institute (Hinxton, UK).

**Identification and Cloning of Human NCKX4 cDNA—**BLAST search of the draft human genome sequence using the human NCKX3 cDNA revealed a putative new NCKX member on chromosome 14. GRAIL (compbio.ornl.gov/Grail-1.3/) was used to screen the region surrounding this putative gene exons. The predicted exons were aligned with the human NCKX3 sequence and used in a BLAST search of the human EST data base, which identified EST clone AW337854 (among others) that mapped to UniGene Cluster Hs.177386. This gene was designated as SLC24A4 (NCKX4). The primers were designed according to the assembled virtual sequence for NCKX4 to amplify the cDNA in three overlapping segments: (i) the 5' end (although incomplete, as assessed by comparison to NCKX3 sequence), 5'-F (ATT CAC GAG TTC CCC ACCA GAT) and 5'-R (CTT CAC ATT GTA CTT CAT GAT CAG); (ii) the central portion, cyto-F (GCC TCC GTG TAC TAC ACC TTC) and cyto-R (GTG ATG AAG GTG ACC ATG AAG A); and (iii) the 3' region to a point downstream of the putative coding sequence, 3'-F (GTG ATG ATG GTG GAC GAG ATT ATG) and 3'-R (AC AGC AGA AGC GCA GTG TCA). Marathon-Ready human brain cDNA (Clontech-BI Biosciences, Palo Alto, CA) was used for PCR with Expand High Fidelity polymerase (Roche Molecular Biochemicals). PCR products were gel-purified and cloned into the EcoRI site of pBluescript SK(-) (Stratagene, La Jolla, CA), and at least four independent clones for each product were sequenced on both strands using overlapping primers. 5'-RACE was used to identify the 5' end of the human NCKX4 transcript, essentially according to published methods (29, 30). Briefly, a PCR was performed using an NCKX4-specific primer, G1 (GCA TAG AAC ATA TAC AGA GCA CCA AGG A), based on the sequence obtained as described above, and the adapter primer, AP1, from the Marathon kit. The predicted PCR fragment and a 3' end PCR fragment using G2 (GCC CCA AGG ATG TGC TCC ACC AGC AC) and the AP2 Marathon adapter primer. The second round PCR products were gel-purified, cloned, and sequenced. A second contig was then assembled from the various clones and aligned with human genome and EST data base entries. Because the EST clone AW337854 contained a plausible polyadenylation signal followed by what appeared to be a poly(A) tract, sequences further 3' to this clone were not considered. Three different classes of cDNA were assembled from these data and have been deposited in the GenBank™ with accession numbers AF520704, AF520705, and AF520706.

**Isolation of Mouse NCKX4 cDNA—**The completed human NCKX4 cDNA sequence was used to search mouse genome and EST databases, and a virtual cDNA was assembled based on the sequences thus identified. Primers designed to bracket the predicted coding sequence were used to amplify a fragment by reverse transcription-PCR from mouse (strain C57BL/6J) cerebellum poly(A) mRNA. The product was gel-purified and sequenced directly on both strands using overlapping primers at the Sequencing Core Facility of the Samuel Lunenfeld Research Institute (Toronto, Canada). Additional primers were designed to resolve an ambiguity arising from alternatively spliced exon 10. The mouse sequence was submitted to the GenBank™ under accession number AE156046.

**Genomic Structure and Chromosomal Location of the Human NCKX4 Gene—**A BLAST search of the human genomic sequence with the NCKX4 cDNA revealed a greater than 99% match on chromosome region 14q32. Comparison of the cDNA sequence with the genomic sequence and a consideration of consensus exon-intron boundaries provided the exon structure of the NCKX4 gene.

**Northern Blot Analysis—**10-µg samples of RNA, isolated from various rat tissues by the guanidinium isothiocyanate/cesium centrifugation method, were separated on 1% agarose-formaldehyde gels and transferred to nylon membranes by capillary diffusion overnight. The UV cross-linked membranes were hybridized with a digoxigenin-labeled antisense riboprobe according to the instructions of the manufacturer (Roche Molecular Biochemicals) as described previously (31). The probe used was a fragment isolated by reverse transcription-PCR from mouse brain that corresponded to the human NCKX4 cDNA sequence from nucleotides 680–1340 (essentially corresponding to the unique central cytoplasmic loop of the encoded protein).

**In Situ Hybridization—**In situ hybridization was performed essentially as described previously (20) on parasagittal sections of mouse brain (purchased paraffin-embedded, sectioned, and mounted from Novex, San Diego, CA). Hybridization was performed in the presence of DIG-dUTP, followed by what appeared to be a poly(A) tract, sequences further 3' down the molecule were digested, fixed in 4% paraformaldehyde/phosphate-buffered saline for 2 h, equilibrated with sucrose solution for cryoprotection, and frozen in OCT, and 10-µm transverse cryosections were prepared. The aorta sections were post-fixed in 4% paraformaldehyde/phosphate-buffered saline. The brain sections were dewaxed in xylene and then hydrated through a graded ethanol-water series. Both brain and aorta sections were then processed as described previously (20).

**NCKX4 Expression Construct—**A construct encoding the full-length coding sequence of NCKX4, suitable for functional expression studies, was obtained by combining PCR fragments together. In addition to the three PCR products derived from the cDNAs from brains (nucleotides 217–723), central region (nucleotides 589–1349), and 3' end (nucleotides 889–1902) of the coding sequence, obtained as described above, an additional fragment was obtained using primers 5'E-F (CTC AAA GGT CCG AGG AGG CG) and 5'E-R (AC ACA GCA GAC CCC ACC AGT), based on the sequence of the longest 5'-RACE-derived clone (nucleotides 1–508). Each fragment used had been subcloned in the EcoRI site of pBluescript SK(-) and corresponded to the NCKX4 consensus, as determined by DNA sequencing. The Kpn1 (multiple cloning site) to Stul (nucleotide 665) fragment from the 5' end clone and the Stul to SacI (nucleotide 913) fragment from the central region clone were ligated into the 3' end clone, previously opened by digestion with Kpn1 (multiple cloning site) and SacI, to create a clone spanning nucleotides 217–1902. An AatII (nucleotide 476) to Apal (multiple cloning site) fragment from the latter clone, together with a Kpn1 (multiple cloning site) to AatIII fragment from the 5'-RACE clone were ligated into Kpn1 and Apal digested pCDNA3.1 (+) (Invitrogen) to create the final expression construct.

**Analysis of NCKX4 Function by Ca²⁺ Imaging in HEK293 Cells—**The pCDAO3.1-NCKX4 expression construct DNA described above was purified using Qiagen (Mississauga, Canada) columns. HEK293 cells grown on coverslips were transfected with this construct or with vector DNA alone using a standard Ca²⁺-phosphate precipitation protocol and analyzed using fluorescent digital imaging with the ImageMaster system (Pharmacia-LKB International, as modified (20). In brief, transfected cells were loaded with fura-2-AM (Molecular Probes Inc., Eugene, OR) and mounted in a perfusion chamber on the stage of a Zeiss Axiovert microscope, and the ratio of fluorescence observed with excitation at 340 or 380 nm was followed with time. The cells were initially perfused with Na⁺-containing K⁺-free solution (145 mM NaCl, 10 mM d-glucose, 0.1 mM CaCl₂, 10 mM Heps-trimethylammonium, pH 7.4) for at least 5 min, followed by alternating changes to solutions in which the NaCl was substituted with either 145 or 140 mM LiCl and 5 mM KCl.

**RESULTS**

During the cloning of the K⁻-dependent Na⁺/Ca²⁺ exchanger NCKX1 from rat eye, Northern blot analysis of mRNA from different rat tissues, using a probe derived from conserved sequence and conditions of reduced stringency, suggested the presence of further NCKX family member(s) not previously characterized (32). A search of the human genome draft sequence (24, 25) revealed a candidate on chromosome region 14q32, which was denoted NCKX4 (23). Using a combination of bioinformatic tools, PCR cloning, and 5'-RACE analysis, as described in detail under “Experimental Procedures,” we were able to complete cDNAs corresponding to the NCKX4 transcript from both human and mouse brain. The encoded proteins were 95% identical, and so further experi-
Molecular Cloning of NCKX4

The NCKX4 cDNA sequence encodes a protein of 605 amino acids, with 11 transmembrane segments predicted by hydropathy analysis (Fig. 1). This suggests a topology analogous to that predicted for other NCKX family members (18, 20, 23), including a putative N-terminal signal peptide (M0), followed by a glycosylated extracellular loop, a cluster of five hydrophobic transmembrane spanning segments, a larger intracellular loop containing a site of diversity caused by potential alternative splicing and several putative phosphorylation sites, and finally a second cluster of five predicted transmembrane segments identified by hydropathy profile generated using the Goldman-Engelman-Steitz hydrophobicity scale and a window of 19 amino acids predicted to be deleted in some transcripts as the downstream common sequence (Fig. 3), which would produce a protein that lacked the M0 hydrophobic putative signal sequence. The structure of the human NCKX4 gene, SLC24A4, on chromosome region 14q32 is shown in Fig. 4. Comparison of the NCKX4 cDNA with the human genome sequence allowed the intron-exon structure of the gene to be constructed. The gene consists of 18 exons spread over 174 kb. The two unique sequences identified by 5’-RACE with flanking primers, suggesting a conserved alternative splicing process. Although not analyzed in a carefully quantitative manner, the relative intensity of the amplified product bands suggested that the smaller species (i.e., lacking the region encoding the 19 amino acids) was the most abundant in human and rat brain, whereas the longer species was the most abundant in mouse cerebellum (data not shown).

The 5’-RACE analysis gave rise to multiple independently isolated clones that corresponded to two different species. Each shared the common 3’ sequence corresponding to authentic NCKX4 but had unique 5’ ends, one of 55 nucleotides and the other of 103 nucleotides, suggesting alternatively spliced first exons (Fig. 3). Based on the number of independent clones isolated for each species, the longer one predominated. Translation of this species beginning at the first Met codon results in the protein shown in Fig. 1, although there are no stop codons present in the short upstream 5’-untranslated sequence. The shorter clone lacks an initiator codon in its unique sequence but could be translated starting at the first Met codon in the downstream common sequence (Fig. 3), which would produce a protein that lacked the M0 hydrophobic putative signal sequence.

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The nucleotide sequences are shown for the two classes of human NCKX4 5'-RACE products isolated. One class corresponded to alternate exon 1B spliced as indicated to common exon 2 (GenBank™ accession numbers AF520704 and AF520705). These clones encode the longer NCKX4 protein, the first part of which is shown in single-letter amino acid code below the nucleotide sequence. The other class corresponded to alternate exon 1A spliced as indicated to common exon 2 (GenBank™ accession number AF520706). These clones encode a protein with a downstream start site, as indicated.

FIG. 3. Comparison of human NCKX4 5'-RACE products. The nucleotide sequences are shown for the two classes of human NCKX4 5'-RACE products isolated. One class corresponded to alternate exon 1B spliced as indicated to common exon 2 (GenBank™ accession numbers AF520704 and AF520705). These clones encode the longer NCKX4 protein, the first part of which is shown in single-letter amino acid code below the nucleotide sequence. The other class corresponded to alternate exon 1A spliced as indicated to common exon 2 (GenBank™ accession number AF520706). These clones encode a protein with a downstream start site, as indicated.

FIG. 4. Genomic organization of the human NCKX4 gene (SLC24A4). Exons were identified by BLAST search of human NCKX4 cDNA against the human genome sequence. This region corresponds to parts of sequence contig NT_026437.8 located on chromosome region 14q32. The positions of the exons within the gene are indicated by sequentially numbered vertical bars, with the first two alternatively spliced exons denoted 1A and 1B (see Fig. 3). The size in base pairs and arrangement of these exons within mRNA are illustrated, with the coding region shown as a filled box. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon. The sequences of the leading and trailing ends of the introns flanking each exon are listed at the bottom of the figure, together with the identity and size of each exon.
kb of sequence corresponds to a contiguous extension of the 3′-untranslated region that is part of exon 17.

The tissue distribution of NCKX4 expression was analyzed by Northern blot of RNA samples from various rat tissues using the mouse NCKX4 probe at high stringency (Fig. 5). Major transcripts of about 10 kb were found to be abundantly expressed in all regions of the brain examined and in aorta, lung, and thymus. In addition, a lower level of expression was found in a variety of other tissues such as heart, stomach, small intestine, spleen, lymph node, skeletal muscle, kidney, and adrenal gland. Liver, on the other hand, was essentially negative. This tissue distribution pattern is more similar to the ubiquitous distribution of NCKX3 (23) than it is to the restricted distribution of NCKX1 and NCKX2 in eye and brain, respectively (20, 32). The major 10-kb NCKX4 transcript appeared to represent a doublet of bands, perhaps most clearly seen in the midbrain or lung samples. The relative level of each band was tissue-dependent, although the mechanism leading to the size difference is not known. Smaller transcripts of about 4–4.5 and 2.5 kb were evident in all tested tissues except liver. The 4.5-kb transcript matches the size of the cDNA sequence we obtained and may arise through alternate polyadenylation signal usage. The origin of the 2.5-kb transcript is not clear, but it may arise via a similar mechanism.

Because NCKX4 transcripts were particularly abundant in brain, the spatial pattern of their distribution was further studied by in situ hybridization, as shown in Fig. 6. NCKX4 transcripts were abundant in almost all regions of brain, consistent with the Northern blotting results. In neocortex, neurons through layers II–VI were strongly and uniformly labeled. Expression was especially robust in the pyramidal cells of the hippocampus, particularly in CA1, CA3, and dentate gyrus neurons. In cerebellum, NCKX4 transcripts were present at a moderate level in Purkinje cells, at a low level in the densely packed cell bodies of the granular layer, and at an even lower level in stellate cells of the molecular layer. Expression was evident in all regions of thalamus, but was particular high in the anterodorsal nucleus. The expression level was also high in the glomerula and granule cell layers of olfactory bulb and throughout the striatum.

Outside the brain, NCKX4 transcripts were particularly abundant in aorta and noticeably less so in stomach and small intestine, despite the fact that all of these tissues are rich in smooth muscle. In situ hybridization of rat aorta clearly revealed abundant NCKX4 transcripts present in all of the smooth muscle layers (Fig. 7). Unfortunately, the endothelial cell layer was not sufficiently well preserved in our sections to confirm the presence of transcripts at that site. Thus, the difference in the level of NCKX4 mRNA between the smooth muscle cells in aorta and in stomach or small intestine is likely due to differences in the specialized smooth muscle subtypes of these different tissues.

The ability of the protein encoded by the NCKX4 gene to function as a Na+/Ca2+ exchanger was tested by transfecting HEK293 cells with a construct encoding the protein illustrated in Fig. 1. Digital imaging of fura-2 fluorescence was used to examine Na+-dependent Ca2+ flux in control and in NCKX4-
transfected cells (Fig. 8). Fura-2-loaded transfected cells on coverslips were mounted in a perfusion device on the microscope stage at room temperature. The cells were first perfused with a medium containing 145 mM NaCl and lacking K⁺ to raise the Na⁺ level within the cells. The NaCl in the perfusate was then replaced with LiCl (still in the absence of K⁺) to reverse the Na⁺ gradient across the plasma membrane. In the NCKX4-transfected cells, the Na⁺ to Li⁺ solution switch elicited only a small increase in the fura-2 fluorescence. A subsequent perfusion switch to medium containing 5 mM KCl and 140 mM LiCl caused a pronounced increase in fura-2 fluorescence, whereas switching back to a 145 mM NaCl-containing medium caused a very rapid reduction in fluorescence. This K⁺-dependent Na⁺/Ca²⁺ exchange activity could be observed repeatedly by repeated perfusion switches. In control cells transfected with vector alone, the same perfusion switches did not elicit any increase in the fura-2 fluorescent signal.

DISCUSSION

In this study we have described the cloning and characterization of a cDNA encoding the fourth and presumably last member of the K⁺-dependent Na⁺/Ca²⁺ exchanger family. Like the other three members, retinal rod NCKX1, neuronal/cone NCKX2, and ubiquitous NCKX3, NCKX4 clearly demonstrated K⁺-dependent Na⁺/Ca²⁺ exchange activity when measured with the fluorescent Ca²⁺ dye, fura-2, in transfected HEK293 cells. Although we did not formally determine whether K⁺ was actually transported together with Ca²⁺ in exchange for Na⁺, there was a clear K⁺ requirement for Ca²⁺ uptake. As we have observed previously for rat NCKX2 (9, 20), we presume that the small amount of K⁺-independent Ca²⁺ flux reflects contaminating K⁺ present during the assay, possibly leaking from the cells during the initial K⁺-free incubation period. The high sequence identity within the hydrophobic regions between NCKX4 and the other family members (Fig. 2) also suggests a similar transport stoichiometry is likely, because this region is known to confer the unique ion transport properties of the NCKX family (33).

Hydropathy analysis supports a proposed transmembrane topology for NCKX4 that is largely similar to those proposed previously for other family members, particularly NCKX3 (18, 20, 23). These models predict an N-terminal signal peptide, M0, a glycosylated extracellular loop, an N-terminal hydrophobic region comprising five transmembrane spans, a larger cytoplasmic loop, and finally a second, C-terminal cluster of hydrophobic transmembrane spans. Experimental tests of the originally predicted topology for the cardiac Na⁺/Ca²⁺ exchanger, NCX1, have provided evidence for a revised model where the C-terminal hydrophobic region is composed of four helical transmembrane segments and a pore-like re-entrant loop structure that extends into the membrane (34, 35). In the NCKX family, the original models for NCKX1 and NCKX2 suggested six transmembrane spans in the C terminus of the protein, although the first of these was only barely above the prediction threshold (18, 20). The model proposed for NCKX4 and NCKX3 (23), on the other hand, suggests only five spans. Although the latter arrangement preserves the opposite orientation of the two α-repeats, as currently modeled for NCX1, it places the C terminus of the protein on the outside, rather than the inside, of the cell. Data from our laboratory using epitope tagging suggest that the NCKX2 C terminus does indeed have an extracellular orientation (39). In addition, experimental evidence indicates that the Escherichia coli YRBG protein, which is distantly related to mammalian Na⁺/Ca²⁺ exchangers but closer to the NCX branch than to the NCKX branch, contains two clusters of five transmembrane spans and an extracellular C terminus (36). These data suggest the intriguing hypothesis that the NCKX family of Na⁺/Ca²⁺ exchangers may feature a different membrane organization than the NCX family.

Like other family members, the NCKX4 protein contains an N-terminal hydrophobic region, denoted M0, followed by a potential cleavage site for a signal peptidase, suggesting that M0 may act as a signal peptide to facilitate incorporation of the exchanger protein into the membrane. Indeed, previous studies on the NCX1 molecule have indicated that, although not essential, M0 does play a role in optimal functional expression (37). Similarly, activity of NCKX3 in HEK293 cells was reduced when a clone lacking the M0 region was expressed. Because in vitro transcription and translation did not reveal a significant difference in the efficiency of protein expression between longer and shorter clones, the difference in activity presumably reflected the efficiency or stability of membrane insertion and trafficking (23). Isolation of the shorter NCKX3 clone lacking M0 was most likely a technical artifact resulting from the very high GC content of the 5’ end of the transcript. In the present study we also isolated two different NCKX4 clones, one with and one without an encoded M0 region. But, surprisingly, in this case the two species appeared to result from an alternative splicing event. In our functional assay with NCKX4, we only tested the construct encoding the longer protein species. In parallel with the observations on NCX1 and NCKX3 expression, however, we would predict that the shorter species lacking M0 would be expressed at a lower level. The physiological significance of differential expression efficiency as a consequence of alternatively spliced inclusion or exclusion of the M0 sequence for NCKX4 is currently unclear.

The human NCKX4 gene is spread out over about 174 kb on human chromosome 14. Interestingly, the exon boundary sites within the encoded protein are (with the exception of the exon1/exon 2 boundary) identical to those of the NCKX3 gene, although the spacing of exons along the gene is different (23). Similarly, NCX1 and NCKX2 genes also share virtually identical exon boundaries, whereas the two pairs have arrangements quite different from one another. This pattern of similarity/dissimilarity seen for exon boundaries is also evident in the amino acid identity between proteins, which is about 60%
within each pair (NCKX1/NCKX2 and NCKX3/NCKX4) and only 35% between pairs. These comparisons suggest that the four members of the NCKX branch of the Na+/Ca2+ exchanger gene superfamily have arisen from two distant, sequential, symmetrical duplication events. In contrast, the three members of the NCX branch of the family all share about 70% amino acid identity in their protein products. Additionally, the exon boundaries of the three NCX genes are identical, except that the long coding exon 2 found in NCX1 and NCX3 is split into three exons in NCX2. These observations suggest that much more closely spaced asymmetrical duplication events resulted in the three members of the NCX branch of the Na+/Ca2+ exchanger gene superfamily.

The expression pattern of NCKX4 transcripts in different rat tissues is much more similar to that of NCKX3 (23) than to those of NCKX1 or NCKX2 (32). Both NCKX3 and NCKX4 were expressed in all regions of brain and also in many other tissues, whereas NCKX1 expression was limited to brain and eye (20, 21). Notably, NCKX4 was found at particularly high levels in aorta, lung, and thymus but at lower levels in stomach and intestine. This is distinct from NCKX3 distribution, which was high in all tissues rich in smooth muscle, not just the vascular subset. Our NCKX4 probe also recognized an mRNA species of about 2.5 kb in many tissues that was particularly strong in small intestine. Although this band would, in principle, be long enough to code for the full-length NCKX4 protein, its identity is currently unknown. The NCKX4 gene has two sites of alternative splicing, and as a consequence of this mechanism several different transcripts appear to be expressed. Whether these alternatively spliced species are expressed in a tissue-specific manner requires further investigation.

NCKX4 transcripts were widely expressed in most regions of mouse brain, including hippocampus, neocortex, thalamus, striatum, and olfactory bulb. This spatial distribution was similar to the broad expression pattern of NCX1 (38) or NCKX2 (20) but strikingly different from that of NCKX3 (23). In cerebellum, NCKX4 was strongly expressed in Purkinje cells as well as in the granular layer, a pattern that is similar to that of NCX1 but dissimilar from those of NCKX2 and NCKX3, which were almost exclusively found in stellate and basket cells of the molecular layer. In neocortex, NCKX4 was uniformly and abundantly expressed from layer II to layer VI, whereas NCX1, NCX2, and especially NCKX3 all had more lamellar distribution. Transcripts for all four exchangers (NCX1, NCX2, NCKX3, and NCKX4) were present in hippocampal pyramidal cells, although with subtly different distributions between CA1, CA3, and dentate gyrus neurons. In the thalamus, each exchanger had a characteristic regional distribution with intensities in different nuclei. Striatum expressed high levels of NCKX2 and NCKX4 but very low levels of NCX1 and NCKX3. A detailed comparison of the distribution of transcripts for NCX and NCKX family members in rat brain is currently underway in our laboratory.

The potassium requirement for NCX of rod outer segments was originally thought to be a unique adaptation to the unusual ionic environment of the vertebrate eye. Since then, however, the identification of NCKX2 in brain and of NCKX3 and now NCKX4 in brain and many other mammalian tissues indicates a much more widespread role for the NCX family in intracellular Ca2+ handling than previously anticipated. The high level of expression of NCKX4 in aorta reported here suggests that NCKX family members may be critical for the control of Ca2+ levels in vascular smooth muscle, a completely unexplored area. Our data further suggest that different types of neurons feature a remarkable variation in the concentration of the NCX transcripts, wherein some neuronal types may contain all four NCX gene products, whereas others contain only some or none at all. Unfortunately, the individual physiological roles of each NCKX family member are still largely unknown, which precludes further speculation on their possible roles beyond the obvious proposition that their distribution may be dictated by the subcellular complexities of neuronal Ca2+ signaling. It seems likely that detailed studies of the unique intracellular Ca2+ handling requirements of specific tissues and cell types, combined with knowledge of the kinetic, thermodynamic, or regulatory differences in the function of the different exchanger proteins, may provide important clues. It is possible that a requirement for independent modes of gene regulation or differential control over subcellular distribution are also important factors. It is expected that gene knock-out experiments in mice or other model organisms will be essential in moving toward a complete understanding of the physiological roles of all individual NCKX family members.

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