Molecular Cloning of a Third Member of the Potassium-dependent Sodium-Calcium Exchanger Gene Family, NCKX3*

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We describe here the identification and characterization of a novel member of the family of K+-dependent Na+/Ca2+ exchangers, NCKX3 (gene SLC24A3). Human NCKX3 encodes a protein of 644 amino acids that displayed a high level of sequence identity to the other family members, rod NCKX1 and cone/neuronal NCKX2, in the hydrophobic regions surrounding the “α-repeat” sequences thought to form the ion-binding pocket for transport. Outside of these regions NCKX3 showed no significant identity to other known proteins. As anticipated from this sequence similarity, NCKX3 displayed Na+/Ca2+ exchange activity when assayed in heterologous expression systems, using digital imaging of fura-2 fluorescence, electrophysiology, or radioactive 45Ca2+ uptake. The N-terminal region of NCKX3, although not essential for expression, increased functional activity at least 10-fold and may represent a cleavable signal sequence. NCKX3 transcripts were most abundant in brain, with highest levels found in selected thalamic nuclei, in hippocampal CA1 neurons, and in layer IV of the cerebral cortex. Many other tissues also expressed NCKX3 at lower levels, especially aorta, uterus, and intestine, which are rich in smooth muscle. The discovery of NCKX3 thus expands the K+-dependent Na+/Ca2+ exchanger family and suggests this class of transporter has a more widespread role in cellular Ca2+ handling than previously appreciated.

Plasma membrane Na+/Ca2+ exchangers are an important component of intracellular Ca2+ homeostasis and have been extensively studied in various cell systems (1). Na+/Ca2+ exchangers are encoded by a protein superfamily present in organisms ranging from bacteria to man (2). All the members of this family share sequence similarity in two hydrophobic and internally homologous domains, commonly referred to as α-repeats (3). Two groups within the Na+/Ca2+ exchanger superfamily have been characterized so far in considerable detail and consist of structurally and functionally distinct proteins. Na+/Ca2+ exchangers (NCX) are thought to catalyze the extrusion of one intracellular Ca2+ ion in exchange for three extracellular Na+ ions (Ref. 1 but see Ref. 4). Na+/Ca2+ + K+ exchangers (NCKX), on the other hand, are thought to transport one intracellular Ca2+ and one K+ ion in exchange for four extracellular Na+ ions (5).

The NCX family of exchangers is best exemplified by the mammalian cardiac Na+/Ca2+ exchanger, NCX1, first cloned from canine heart (6), which plays a crucial role in the relaxation process of heart muscle by extruding the Ca2+ that enters at the beginning of systole. NCX1 is also expressed in a variety of other tissues (7–9) suggesting an important role in the physiological processes of different cell types. The tissue-specific expression pattern of NCX1 has been demonstrated to be under the control of a multipartite promoter (10–12). Moreover, a complex pattern of alternative splicing in the large intracellular loop of NCX1 generates isoforms of the protein, which are also expressed in a highly tissue-specific way (7–9). Functional studies have revealed complex regulatory mechanisms of the NCX1 protein, some of which differ between alternatively spliced isoforms (2, 13). The NCX protein family contains two other members, products of genes NCX2 and NCKX3, whose expression is restricted largely to brain and skeletal muscle (9, 14, 15). All three NCX proteins share a high degree of sequence identity, especially within the transmembrane spanning domains, and are believed to share the same overall topology, modeled to have two clusters of hydrophobic membrane-spanning helices separated by a large hydrophilic, intracellular loop. The hydrophobic domains are thought to pack together in the membrane, forming the ion translocation pathway (16). Indeed, the three NCX proteins show very similar functional properties when assayed in heterologous expression systems (17, 18).

The second major group in the Na+/Ca2+ exchanger superfamily is exemplified by the Na+/Ca2++K+ exchanger from retinal rod outer segments, NCKX1. NCKX1 was first cloned from bovine retina (19) and, more recently, from other species including dolphin, rat, buffalo, and man (20–22). This protein

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‡‡†§¶** The abbreviations used are: NCX, Na+/Ca2+ exchanger; EST, expressed sequence tags; kb, kilobase pair; MOPS, 3-(N-morpholino)propane-sulfonic acid; NCKX, Na+/Ca2++K+ exchanger; PCR, polymerase chain reaction; RT-PCR, reverse transcription-coupled polymerase chain reaction.

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plays a critical role in the visual transduction process of the mammalian retina (5). In darkness, the cyclic nucleotide-gated ion channels of the outer segment are largely open, and both Na\(^+\) and Ca\(^{2+}\) ions flow in. Ca\(^{2+}\) homeostasis must still be maintained under these conditions of membrane depolarization and reduced sodium gradient, and NCKX1 is the principal means by which Ca\(^{2+}\) is extruded from rod outer segments. Such a function could not be achieved by a molecule operating with the 3:1 Na\(^+\)/Ca\(^{2+}\) stoichiometry of NCX1, and indeed it has been demonstrated that NCKX1 couples the entry of four Na\(^+\) ions in exchange for the exit of one Ca\(^{2+}\) and one K\(^+\) (23, 24).

Whereas NCX1 proteins from mammalian species are over 90% identical in their amino acid sequences, NCKX1 orthologs display relatively low sequence identities of around 60%, largely due to differences in the two large hydrophilic loops, an extracellular one near the N terminus and a cytoplasmic one near the center of the molecule. NCKX1 has been modeled to have a similar arrangement of transmembrane-spanning segments as NCX1, although actual amino acid sequence similarity is very limited and restricted only to the two \(\alpha\)-repeats, as mentioned above. The cloning of NCKX1 from rat eye (20) also revealed the presence of alternatively spliced isoforms in this species, which differ by the arrangement of four exons at the N terminus of the large intracellular loop. Interestingly, the equivalent region of bovine NCKX1, but apparently not the alternatively spliced region in rat NCKX1, is responsible for producing a functionally silent protein when expressed in heterologous systems (20, 21).

Functional measurements have pointed toward the presence of K\(^-\)-dependent Na\(^+\)/Ca\(^{2+}\) exchangers in tissues other than eye (25, 26). Molecular evidence has recently confirmed expression of NCKX1 in cells of hematopoietic origin (27) and identified a second K\(^-\)-dependent Na\(^+\)/Ca\(^{2+}\) exchanger (NCKX2) in brain neurons and cone photoreceptors (28, 29). Moreover, sequence analysis of the genomes of model organisms, such as Drosophila and Caenorhabditis elegans, has revealed several new hypothetical proteins with similarity to the NCKX family (23, 24).

The molecular cloning of the NCKX3 cDNA—the first member of this gene family to be cloned—will provide new insights into the physiology and pharmacology of the NCX1 family of Ca\(^{2+}\) exchangers. It is also expected to further our understanding of the evolution of the vertebrate vision system and the encoding proteins that make up the intracellular Ca\(^{2+}\) homeostatic system. Here, we describe the cloning and characterization of the murine NCKX3 gene.

### EXPERIMENTAL PROCEDURES

**cDNA Manipulation and Sequence Analysis**—Common chemical reagents were purchased from Fisher, Sigma, or BDH and were of “molecular biology grade” or better. All molecular procedures followed standard protocols (32, 33), or those provided by the reagent suppliers, unless noted otherwise. DNA sequencing was done at local core facilities of the participating institutions, using either Li-Cor or Applied Biosystems automated fluorescent sequencing equipment. Full-length NCKX3 cDNA was sequenced on both strands. DNA copy assembly was done with either MacVector (Genetics Computer Group, Inc., Madison, WI) or Sequencer (Gene Codes, Ann Arbor, MI), and sequence analysis was done in MacVector or via web interface at the Expasy molecular biology server. BLAST (34) searches of the sequence data bases were run at the National Center for Biotechnology Information web site, at the Sanger Center web site, or at the Ensembl genome server of the European Bioinformatics Institute.

**Identification and Cloning of the Human, Mouse, and Rat Brain NCKX3 cDNAs**—BLAST searches of the human EST data bases identified two sequences (R21117 and T06656) with similarity to \(\alpha\)-repeats conserved among members of the Na\(^+\)/Ca\(^{2+}\) exchanger superfamily (3). Corresponding cDNA clones were purchased from Research Genetics (Huntsville, AL), and their inserts sequenced completely. The sequence information was used to design primer pairs for PCR amplification of cDNA prepared from human total brain RNA (CLONTECH, Palo Alto, CA). These products were subsequently used to screen a number of cDNA libraries from human brain (CLONTECH; generous gifts of K. Petrukhin and B. Soares). These screenings eventually yielded three sets of mouse brain EST clones, to which a composite transmembrane region (GenBank accession number AF169257). The completed transcript was used to re-screen EST data bases of the genome projects, which identified a set of mouse brain ESTs with a high degree of sequence identity to the human NCKX3 coding sequence. Primers based on the mouse EST sequences were used to amplify the NCKX3 transcript from mouse brain poly(A\(^+\)) mRNA by RT-PCR (using Expand Reverse Transcriptase followed by the Expand Long Template PCR System, both from Roche Molecular Biochemicals). The roughly 2.5-kilobase pair (kb) product was subcloned into the mammalian expression vector, pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Six clones with the correct orientation were pooled, and the sequence from a large scale DNA preparation was determined. This sequence has been submitted to the GenBank under accession number AF314821. Rat NCKX3 was amplified by RT-PCR from brain RNA using High Fidelity Taq polymerase (Roche Molecular Biochemicals), and primers were chosen from regions of nucleotide identity between human and mouse NCKX3 cDNAs, lying near the N terminus and just downstream from the C terminus of the deduced coding region. The amplification product was subcloned into pBluescript II SK(--) (Stratagene, La Jolla, CA) and sequenced.

**Cloning of the Transcription Initiation Region from the Murine NCKX3 Gene**—A mouse embryonic stem cell genomic library in the vector Lambda Fix II (Stratagene) was probed with mouse NCKX3 cDNA. A 5-kb ClaI restriction fragment that hybridized to a probe containing only exon 1 sequences was then subcloned from a positive lambda clone into the mammalian expression vector pBluescript II SK(--) (Stratagene and Southern blotting, and the fragment containing exon 1 was sequenced and deposited in the GenBank database (accession number AF090158).

**Cloning of the Full-length NCKX3 cDNA from Human Skeletal Muscle**—Human EST R21117 was identified as a candidate for a new NCKX protein by data base searches using the amino acid sequence of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger NCX1 and comparison to other known K\(^-\)-dependent and -independent Na\(^+\)/Ca\(^{2+}\) exchanger isoforms. The NCKX3 “long clone” was subsequently isolated from Human Skeletal Muscle cDNA libraries (5′-Stretch, a generous gift of Dr. Ira Kurtz, and 5′-Stretch Plus from CLONTECH, Palo Alto, CA) using a recursive PCR-based screening approach that combined primers from the vector with primers based initially on the R21117 sequence and subsequently on sequence from the 5′-end of isolated clones. The \(\lambda\) DNA of the positive full-length clone was digested with KpnI and SacI, and the fragment containing the full-length cDNA was subcloned into pBlueScript II SK(--) (Stratagene and Southern blotting, and the fragment containing the full-length cDNA was subcloned into pBluescript II SK(--)). This clone was sequenced entirely, and the data were deposited in GenBank with accession number AF288087.

**Structure and Chromosomal Location of the Human NCKX3 Gene**—BLAST searches of the human high throughput genomic sequence identified several PAC clones that contained sequence stretches showing >99% match with portions of human NCKX3 cDNA. Analysis of these data eventually provided the exon structure of the NCKX3 gene.
Similarly, assignment of the NCKX3 transcript to a Unigene cluster, and to contiguous sequenced stretches of the draft genome sequence, determined its chromosomal location.

Mapping the 5’-End of the Marine NCKX3 Transcript by RNase Protection Assay—Mouse brain poly(A)+ mRNA was isolated from fresh or frozen tissue using TRIzol reagent (Life Technologies, Inc.), followed by DNase I digestion and further isolation with RNase A Midi columns (Qiagen, Valencia, CA), and poly(A) Spin columns (New England Diabs, Beverly, MA). Three riboprobes were constructed based on murine NCKX3 sequences. Probe 1 was prepared by RT-PCR from mouse brain mRNA, beginning 16 nucleotides upstream of the initiation codon and extending into exon 2 (both from the murine NCKX3 genomic clone (see Fig. 3) inserted between the EcoRI and BamHI sites of pBluescript II SK (-)). Digestion by XhoI and transcription from the T7 polymerase promoter produced a 261-nucleotide-long probe that was expected, when fully protected by NCKX3 mRNA, to result in a fragment 184 nucleotides in length. Probes 2 and 3 were both prepared from a BglII to FseI fragment derived from the murine NCKX3 genomic clone (see Fig. 3) inserted in the Smal site of pBluescript II SK (-). Digestion with XhoI (cutting within the insert) or XbaI, followed by transcription from the T7 polymerase promoter, yielded probe 2 (456 nucleotides) and probe 3 (590 nucleotides). Full-length protection of these probes is expected to result in fragments of 383 and 496 nucleotides, respectively. Radiolabeled riboprobes were prepared using the MAXscript T7 kit from Ambion (Austin, TX), in the presence of [35S]methionine (Amersham Pharmacia Bio- tech), in the presence of either 0.1% Triton X-100 or canine pancreatic microsomes with or without 50 μM of the competitive glycosylation inhibitor peptide, acetyl-Asn-Tyr-Thr-amide. Following an incubation of 90 min at 30 °C, the products were resolved on an SDS-polyacrylamide gel (35), dried, and detected by autoradiography using Biomax MR film (Eastman Kodak Co.).

Tissue-specific Distribution of the NCKX3 Transcript—Distribution of the human NCKX3 transcript was studied using commercially available Multiple Tissue Northern and Multiple Tissue Expression Array filters (CLONTECH) with [32P]-labeled probes corresponding to the entire clone (Northern blots), or encompassing nucleotides 405–620 of the human skeletal muscle “long” clone (generated by PCR (dot blot array)). Hybridization and washing of the membrane was carried out under highly stringent conditions, following the manufacturer’s protocol. Hybridization and washing at high stringency were carried out according to instructions of the manufacturer (Roche Molecular Biochemicals) as described previously (36). In situ hybridization was performed on parasagittal sections of mouse brain using base-hydrolyzed digoxigenin-labeled NCKX3 probes, as described previously (29). The antisense probe spanned BglII (356) to XbaI (2193), whereas the sense control probe covered the entire clone (nucleotides 1–2382). Analysis of NCKX3 Function by Ca2+-dependent Na+/Ca2+ Exchangers—Comparison of the well-characterized mammalian cardiac Na+/Ca2+ exchanger, NCX1, to the sequence data bases of simple model organisms has revealed a surprisingly complex family of related sequences (2, 3, 29). In a further effort to characterize mammalian counterparts to these putative molecules, the human EST data base was screened with a cDNA fragment corresponding to the conserved amino acid sequence of the α-repeat regions of the Na+/Ca2+ exchanger (3). Initially, two entries were identified independently and simultaneously in the Toronto and Los Angeles laboratories (GenBankTM accession Numbers R21117 and T06656) that led to isolation of the corresponding cDNA clones from human fetal brain and skeletal muscle libraries, respectively. Comparison of the deduced amino acid sequence of this novel gene (Figs. 1 and 2) revealed a significant level of similarity to the two known members of the K+-dependent Na+/Ca2+ exchanger family as follows: NCKX1, first identified in retinal rod outer segments (19), and NCKX2, originally identified in rat brain and then in chicken and human retinal cone and ganglion cells (28, 29). Thus, the novel gene presented here encodes the third member of the K+-dependent Na+/Ca2+ exchanger gene family and will be referred to as NCKX3 (gene SLC24A3).

The human skeletal muscle clone (HuNCKX3-L, for “long”) extended 254 nucleotides further 5’ than the human fetal brain clone (HuNCKX3-S, for “short”), but otherwise these clones were identical aside from several, presumably allelic, single nucleotide polymorphisms. The 5’-ends of both clones were found within contiguous genomic DNA sequence of what appears to be the first exon of the human gene (Fig. 3). 5’-Rapid amplification of cDNA ends (RACE) experiments using human brain mRNA were partially to extend past the end of the 5’UTR. As the sequence is very GC-rich, the difference in length between brain and skeletal muscle clones could be due to technical reasons or might indicate that transcription starts at different sites in these different tissues. Mapping of the potential transcriptional start was performed using the mouse genomic clone and mouse brain mRNA (see below), suggesting that the longer clone most likely corresponds to authentic NCKX3 transcripts.
Structure of the Human NCKX3 Gene and Mapping to Its Chromosomal Location—Comparison of the NCKX3 cDNA sequences with the HTGS data base allowed the intron-exon structure of the gene to be reconstructed (Figs. 1 and 3). Human NCKX3 consists of 17 exons spanning over 500 kilobase pairs. The first three exons are spread out over more than half of this distance, followed by two clusters comprising exons 4–5 and 7–17, which are more conventionally spaced. Surprisingly, the unusually long first coding exon found as a conserved feature in NCX1, NCX3, NCKX1, and NCKX2 genes (analyzed by BLAST search; also see Refs. 37 and 38) is not present in NCKX3. Also, unlike its family members, there is no evidence for alternative splicing of NCKX3 among available clones isolated from human cDNA libraries or by RT-PCR analysis of mouse or rat tissues. However, the last exon of NCKX3, encoding the C-terminal end of the protein and all of the 3′-untranslated region, is the longest in the gene, in line with the gene structure of other members of the Na⁺/Ca²⁺ exchanger gene superfamily.

The sequence of the brain and skeletal muscle NCKX3 cDNA clones overlapped in the 3′-untranslated region with more than 20 different EST entries, originating from various human tissue libraries. The 3′-end of the transcript was found to correspond in the human genome to position 30,641 of GenBank™ accession number AL121761, and the nearest sequence-tagged sites were Em:G25896, Em:T03762, and Em:L30728. A representative EST entry that contains the 3′-end of this transcript, including a poly(A) tail (GenBank™ accession number F10455; data base EST entry 132788), belongs to the UniGene EST cluster Hs.12321, which has been mapped to the interval D20S182–D20S106 on chromosome 20p13. This gene has been given the designation SLC24A3.
members of the K+-dependent Na+/Ca2+ exchanger family have also been mapped, NCKX1 (gene SLC24A1) to chromosome 15q22 (38) and NCKX2 (gene SLC24A2) to chromosome 9q22.1–22.3.

Identification of Rodent NCKX3 cDNA and the Putative Promoter Region of the Mouse SLC24A3 Gene—Searches using completed human NCKX3 cDNA identified a set of mouse ESTs from which primers were designed to amplify the mouse NCKX3 cDNA by RT-PCR from brain poly(A) mRNA. Similar primers, based on identity between the human and mouse cDNA sequences, were also used to isolate the rat brain NCKX3. We were unable to amplify products from either mouse or rat mRNA using primers upstream of the presumed initiating methionine residue of human NCKX3, however, and only clones that extended to include the Arg residue at position 20 of the human sequence were obtained (Fig. 1). Nevertheless, 5'-rapid amplification of cDNA ends experiments (data not shown) as well as EST entries (Fig. 4) indicated that the mouse NCKX3 transcript encodes the same N-terminal protein region as the human one. The sequences of the resulting deduced proteins from human, mouse, and rat are approximately 95% identical, as shown in Fig. 1.

The mouse NCKX3 cDNA was used as a probe to isolate a genomic clone, which was found to contain the sequence corresponding to the first exon of the mouse SLC24A3 gene (Fig. 4). The 5'-end of the mouse NCKX3 transcript was mapped using probes derived from cDNA and genomic sequence, as shown in Fig. 4. Probes 1 and 2 were both protected to their full lengths by brain mRNA (184 and 383 nucleotides, respectively), whereas the majority of probe 3 was protected to a point just upstream of the NcoI site that delineates probe 2 (although a small amount of a full-length protected fragment of 496 nucleotides is also visible). These data indicate that transcription of the mouse NCKX3 gene likely initiates close to position 325 of Fig. 4B, which would produce a transcript analogous to the HuNCKX3-L clone. The abrupt loss of cross-species sequence identity close to this site is also consistent with transcription initiation in this region of the gene. The 5'-flanking sequence is very GC-rich and contains no obvious TATA box. There are, however, numerous Sp-1-like sites, CACC box sites, as well as an AP-2 site at 311 and a CArG site at 208, consistent with a ubiquitously expressed gene.

In Vitro Translation of the Cloned NCKX3 cDNAs—Neither the human nor mouse NCKX3 transcripts contain in-frame stop codons upstream of the presumed initiating methionine residue of human NCKX3, however, and only clones that extended to include the Arg residue at position 20 of the human sequence were obtained (Fig. 1). Nevertheless, 5'-rapid amplification of cDNA ends experiments (data not shown) as well as EST entries (Fig. 4) indicated that the mouse NCKX3 transcript encodes the same N-terminal protein region as the human one. The sequences of the resulting deduced proteins from human, mouse, and rat are approximately 95% identical, as shown in Fig. 1. The only areas with clustered differences are at the immediate N terminus and in a repetitive acidic sequence found in the central, presumptive cytoplasmic, loop.

The mouse NCKX3 cDNA was used as a probe to isolate a genomic clone, which was found to contain the sequence corresponding to the first exon of the mouse SLC24A3 gene (Fig. 4). The 5'-end of the mouse NCKX3 transcript was mapped using probes derived from cDNA and genomic sequence, as shown in Fig. 4. Probes 1 and 2 were both protected to their full lengths by brain mRNA (184 and 383 nucleotides, respectively), whereas the majority of probe 3 was protected to a point just upstream of the NcoI site that delineates probe 2 (although a small amount of a full-length protected fragment of 496 nucleotides is also visible). These data indicate that transcription of the mouse NCKX3 gene likely initiates close to position 325 of Fig. 4B, which would produce a transcript analogous to the HuNCKX3-L clone. The abrupt loss of cross-species sequence identity close to this site is also consistent with transcription initiation in this region of the gene. The 5'-flanking sequence is very GC-rich and contains no obvious TATA box. There are, however, numerous Sp-1-like sites, CACC box sites, as well as an AP-2 site at 311 and a CArG site at 208, consistent with a ubiquitously expressed gene.
demonstrating a high degree of preference for the upstream methionine as the starting point. An NCKX3 protein synthesized from this site would also contain a hydrophobic region analogous to the M0 region of other \( \text{Na}^{+}/\text{Ca}^{2+} \) exchanger family members. Synthesis of NCKX3 in the presence of canine pancreatic microsomes resulted in an upward shift in the mobility of the product, especially evident with the HuNCKX3-L clone, consistent with the attachment of oligosaccharide chains to the two glycosylation site motifs present in the extracellular loop between M0 and M1. Synthesis in the presence of the competitive glycosylation inhibitor, acetyl-Asn-Tyr-Thr-amide, prevented appearance of the reduced mobility species characteristic of a glycosylated polypeptide and revealed a more rapidly migrating polypeptide instead, suggestive of signal peptide processing and consistent with the assignment of the M0 region. The HuNCKX3-S protein product of 64 kDa appeared to be too short to start from the first methionine codon in its cDNA sequence, suggesting initiation from a more downstream site.

**FIG. 3. Genomic organization of human NCKX3 (SLC24A3).** Exons were identified by BLAST search of human NCKX3 cDNA against the Human Genome draft sequence of overlapping BAC clones, shown as thick lines with accompanying GenBank™ accession numbers at the top of the figure. This region corresponds to part of sequence contig NT_019671 located on chromosome 20 at p11. The position of each exon within the Gene is indicated by numbered vertical bars below the BAC clones (exons and short introns not drawn to scale). The size in base pairs and arrangement of the Exons within the mRNA is illustrated, with the NCKX3 coding region shown as a filled bar and the untranslated regions as open boxes. The leading and trailing sequence of each intron is listed at the bottom of the figure. The sequence preceding exon 1 corresponds to genomic sequence found upstream of the HuNCKX3-L clone and may not represent true flanking sequence, as the transcriptional starting point has not been mapped in human tissues.
Molecular Cloning of NCKX3

Fig. 4. RNase protection analysis of the 5′-end of the mouse NCKX3 transcript. mRNA from mouse brain was hybridized with one of three different probes and subjected to RNase protection analysis. A, protected fragments were resolved on 5% polyacrylamide/urea gels. Lane labels are as follows: B1, B2, and B3, 1 μg of mouse brain poly(A)+ mRNA hybridized to Probes 1, 2, and 3, respectively; t1, tRNA control hybridized for Probe 1; P, untreated probes (Probe 1 in gel on left, Probes 2 and 3 combined in gel on right). The size of the intact probes, as well as the size of the protected fragments observed are indicated in nucleotides (nt). B, sequence from the mouse NCKX3 gene including exon 1 (GenBank TM accession number AF314822), joined to cDNA sequence for the first part of exon 2 (GenBank TM accession number AF314821). The position of the intron that separates these two sequences is indicated as are putative sites of signal peptide cleavage (SPase?). Since NCKX3 has no such large exon in the region corresponding to the NCX1 exon 2 product itself, as the sequences are too divergent, the observed transcript likely due to cross-reaction with the NCX1 mRNA hybridized to the probe, a similar scenario cannot be the explanation, nor is the observed transcript likely due to cross-reaction with the NCX1 exon 2 product itself, as the sequences are too divergent, and the tissue-specific expression patterns quite different (40).

Since this product was glycosylated (albeit weakly), a start site just before the two potential glycosylation sites is predicted.

Tissue Distribution of NCKX3 Gene Expression—Northern blotting data of NCKX3 distribution is shown for RNA from different rat and mouse tissues and from various human brain regions, in Fig. 6. The major NCKX3 transcript was about 4.5 kb in length and was abundant in various brain regions, as well as several tissues rich in smooth muscle (aorta, intestine, and lung). Lower levels were found in a variety of other tissues, whereas kidney and liver were essentially negative. This pattern of size and distribution clearly distinguishes NCKX3 from the other two known members of the SLC24 gene family, NCX1 (6 kb), and NCX2 (11 kb), whose expression is almost exclusively restricted to eye and brain (20). Larger transcripts of somewhat variable size were evident in all rat tissues but not in mouse or human. Possibly these species represent cross-reaction of the NCKX3 probe with as yet uncharacterized NCKX family members. The 1.8-kb band is almost exclusively restricted to eye and brain (20). A, autoradiograph of SDS-polyacrylamide gel. B, amino acid sequence for the N-terminal region of HuNCKX3, indicating the anticipated size for polypeptides initiating at each methionine residue. The positions of the hydrophobic transmembrane spans M0 and M1 are indicated as are putative sites of signal peptide cleavage (SPase?) and glycosylation (CHO).

Fig. 5. In vitro translation of cloned NCKX3. cDNA clones encoding short and long versions of human NCKX3 (HuNCKX3-S and HuNCKX3-L, respectively) were transcribed and translated in vitro, either in the presence of 0.01% Triton X-100 (Triton), canine pancreatic microsomes (μsomes), or microsomes and 50 μM competitive glycosylation inhibitory peptide, acetyl-Asn-Tyr-Thr-amide (μs+NT). A, autoradiograph of SDS-polyacrylamide gel. Filled circles indicate size of polypeptide produced without modification (i.e. in the presence of Triton alone). Open circles indicate position of polypeptide modified by glycosylation. The triangle indicates a HuNCKX3-L polypeptide that may represent a product of signal peptidase cleavage. B, amino acid sequence for the N-terminal region of HuNCKX3, indicating the anticipated size for polypeptides initiating at each methionine residue. The positions of the hydrophobic transmembrane spans M0 and M1 are indicated as are putative sites of signal peptide cleavage (SPase?) and glycosylation (CHO).
of NCKX3, as shown in Fig. 10. A significant outward current, larger than that in control transfected cells. Inclusion of 1 mM Mg

Fig. 6. Northern blot analysis of NCKX3 tissue expression. Samples (10 µg of total RNA from rat (A), or mouse (B), or 2 µg of poly(A)+ mRNA from human brain regions (C)) isolated from the indicated tissues were separated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized at high stringency with probes from either mouse NCKX3 (A and B) or human NCKX3 (C). Br, brain; CC, cerebral cortex; Ch, cerebellum; BS, brainstem (includes pons and medulla); MB, midbrain (includes thalamus and hypothalamus); M, medulla; SC, spinal cord; Co, cortex, occipital lobe; Cf, cortex, frontal lobe; Ct, cortex temporal lobe; P, putamen; Sk, skeletal muscle (diaphragm in A, leg in B); Ht, heart; Ao, thoracic aorta; St, stomach; SI, small intestine; Li, large intestine; Lu, lung; Li, liver; Th, thymus; Sp, spleen; LN, lymph node; Ad, adrenal; Kc, kidney cortex; Te, testis.

Spatial Distribution of the NCKX3 Transcript in Mouse Brain—The high level of NCKX3 expression in brain prompted an examination of regional distribution using in situ hybridization, as illustrated in Fig. 8. This analysis revealed a unique neuronal staining pattern. NCKX3 transcripts appeared most abundant in the cerebral cortex, where there was a striking pattern within discrete nuclei. Expression levels were also high in the hippocampus, especially in the CA1 neurons. Within the cortex, a clearly laminar pattern was evident, with higher expression in NCKX3 in large neurons of layer IV than in other layers. In cerebellum, NCKX3 appeared to be restricted to stellate cells of the molecular layer. These data on NCKX3 expression in mouse brain are consistent with the regional distribution in human brain, inferred from the dot blot data of Fig. 7 and the Northern blot data of Fig. 6C.

Analysis of NCKX3 Function—The ability of the NCKX3 protein to function as a Na/Ca2+ exchanger was tested by transfecting HEK293 cells with various constructs and measuring Na+-dependent Ca2+ fluxes, as illustrated in Fig. 9. When fura-2-loaded cells were perfused with Li+, to reverse the Na+ gradient across the plasma membrane, a large rise in intracellular [Ca2+]i (evident as an increase in the 340:380 ratio of the dye) was observed only in the presence of extracellular K+, consistent with the operation of a K+-dependent Na+/Ca2+ exchanger. Cells transfected with each of the three NCKX3 cDNA clones (HuNCKX3-L, HuNCKX3-S, and MuNCKX3) corresponded to the short human clone) demonstrated this behavior, whereas the cells transfected with vector alone showed no significant rise in [Ca2+]i. The rate of change and the magnitude of the fura-2 response were consistently greater for the longer human cDNA clone than for either shorter one (see Fig. 9B). Thus, while the M0 region of NCKX3 seemed not to be essential for functional expression, as previously documented for NCX1 (41–43), it appeared as though the level of expression and/or the efficiency of plasma membrane delivery was much greater for the complete exchanger molecule than for the N-terminal truncated one, hence resulting in more robust function.

Whole-cell patch clamp data from HEK293 cells were also consistent with the K+-dependent Na+/Ca2+ exchange function of NCKX3, as shown in Fig. 10. A significant outward current, carried by Na+ movement, was observed only when cells transfected with HuNCKX3-L were perfused with medium containing both K+ and Ca2+ and not with either ion alone or with control transfected cells. Inclusion of 1 mM Mg2+ in the perfusate significantly reduced the magnitude of the currents observed, consistent with competition of Mg2+ at the external Ca2+ transport site, as observed with NCX1 (44). Interestingly, we were unable to observe currents with either shorter version of NCKX3 (data not shown).

Uptake experiments using 45Ca2+ were also performed on Na+-loaded HEK293 cells, as illustrated in Fig. 11. Na+ gra-
dient-driven Ca\textsuperscript{2+} uptake was observed for cells transfected with HuNCKX3-L in medium containing K\textsuperscript{+} but not choline. In contrast, Ca\textsuperscript{2+} uptake into NCX1-transfected cells was much larger and was relatively insensitive to the presence or absence of K\textsuperscript{+}. These data again confirm that NCKX3 functions as a K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. As with the electrophysiological measurements, cells transfected with the HuNCKX3-S clone did not demonstrate any uptake above control levels. Since HuNCKX3-S did display function, albeit much less than HuNCKX3-L, when measured using digital fluorescent Ca\textsuperscript{2+} imaging, it seems likely that lack of function in the other assays indicates a level of expression too low to detect. These observations also suggest that the digital fluorescent Ca\textsuperscript{2+}-imaging technique is at least an order of magnitude more sensitive than other assays.

DISCUSSION

In this study we have described the cloning and characterization of a cDNA encoding a novel, third, member of the family of K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers, NCKX3. Like the other two members, rod NCKX1 and cone/neuronal NCKX2, NCKX3 demonstrated K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity when measured with the fluorescent Ca\textsuperscript{2+} dye fura2, with ion currents, or with \textsuperscript{45}Ca\textsuperscript{2+} uptake. Although we did not formally determine whether K\textsuperscript{+} was actually transported together with Na\textsuperscript{+} and Ca\textsuperscript{2+}, there was an absolute requirement for K\textsuperscript{+} in all of the functional assays. That NCKX3 can be measured electrically, with charge moving in the same direction as Na\textsuperscript{+}, also places limits on the ionic stoichiometry of transport. Combined with the high degree of amino acid sequence similarity to NCKX1, which has been clearly established to transport 4 Na\textsuperscript{+} in exchange for 1 K\textsuperscript{+} and 1Ca\textsuperscript{2+} (23, 24), it seems likely that NCKX3 has a similar stoichiometry.

Previous studies with the cardiac Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, NCX1, had implicated the so-called \(\alpha\)-repeat regions of the molecule in forming the binding pocket for ion translocation (45, 46). Functional studies on a deletion mutant of bovine NCKX1 and a \textit{C. elegans} paralog (cNCKX) have suggested similar regions also specify the transport sites needed for K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (30). A comparison of the sequences between NCKX3 and these molecules (Fig. 2) shows the high sequence similarity anticipated within the \(\alpha\)-repeats. NCKX3 is, however, significantly more divergent in these regions than the NCKX1/NCKX2/cNCKX trio. Intriguingly, these positions of divergence often correspond to identities or similarities with NCX1. Nevertheless, NCKX3 is clearly K\textsuperscript{+}-dependent, in contrast to NCX1, and thus this new sequence...
information places further limits on the amino acids likely to contribute to the ionic specificity of the K+-dependent class of Na+/Ca2+ exchangers.

Hydropathy analysis as well as our in vitro translation experiments support a proposed transmembrane topology for NCKX3 that is largely similar to those proposed previously for other family members (Fig. 1). The NCKX3 protein begins with a functionally dispensable region that may encode a putative "signal" peptide, in a manner analogous to that reported for NCX1 (41–43). The presence of this region in the expressed protein significantly improved the level of functional expression of NCKX3 (Fig. 9). Thus, whereas all cDNA constructs demonstrated K+-dependent Na+/Ca2+ exchange activity when assayed using fur-2 fluorescent digital imaging, only the HuNCKX3-L clone expressed activity above background in either electrophysiological or 45Ca2+ uptake assays. Since in vitro transcription and translation experiments (Fig. 5) did not reveal a significant difference in the efficiency of protein expression, the longer NCKX3 protein species containing the N-terminal hydrophobic M0 sequence may be targeted or delivered to the plasma membrane more efficiently than the protein lacking this sequence.

The processed NCKX3 protein consists of a short, glycosylated, extracellular loop at the N terminus followed by a cluster of five hydrophobic, putative transmembrane, segments, a long hydrophilic loop, and finally, a second cluster of hydrophobic regions. The long central loop, which is presumed to be cytoplasmic, is composed of four helical transmembrane segments and a pore-like re-entrant loop structure that extends into the membrane (45, 47, 48). Hydropathy analysis of NCKX3 suggests a somewhat different topology, with five transmembrane spans separating the central cytoplasmic loop from the protein C terminus, which would reside on the extracellular side of the membrane. The validity of such a model, and the notion that the K+-dependent Na+/Ca2+ exchangers (NCKX) may have a topology different from those of the K+-independent (NCX) family, will need to be tested.

The exon boundaries for human NCKX3 are indicated in Fig. 1, and their location in genomic sequence on chromosome 20 is shown in Fig. 3. The following two features of the NCKX3 gene are striking: (i) the distance over which the exons, especially the first three, are spaced; (ii) the arrangement of exons in comparison to the genomic structure of other Na+/Ca2+ exchanger genes. The NCX1 (SLC8A1), NCX3 (SLC8A3), NCKX1 (SLC24A1), and NCKX2 (SLC24A2) genes all share an unusually large second exon that extends from just before the initiating methionine codon into the central cytoplasmic loop (analyzed by BLAST search; also see Refs. 37 and 38). In contrast, the corresponding sequence of NCKX3 is split into 10 separate exons. Furthermore, the arrangement of exons encoding the C-terminal hydrophobic domain is specific for NCKX3 and differentiates it from either the arrangement conserved between
NCKX1 and NCKX2 or the distinct arrangement conserved between NCX1 and NCKX3. These differences suggest that, despite the sequence similarity within the α-repeat regions, NCKX3 must have its origins in a very ancient gene duplication event and subsequent divergence from other NCKX and NCX family members.

The pattern of expression of NCKX3 transcripts in different tissues was examined in rat and mouse by Northern blot and in human by Multiple Tissue Expression Array dot blot analyses (Fig. 6). From these data it is clear that NCKX3 was most abundantly expressed in various brain regions. Regional distribution was examined in more detail in parasagittal sections of mouse brain using in situ hybridization (Fig. 8), which revealed a very specific pattern. The highest levels of NCKX3 mRNA were found in neurons arranged in distinct nuclei of the thalamus, followed by the pyramidal CA1 neurons of the hippocampus, and large neurons of cortical layer IV. This pattern is quite different from published reports of NCX1 and NCX2 distribution in rat brain (29, 49). Transcripts for both of these exchangers were also abundant in cortex but lacked the laminar pattern evident for NCKX3. All three exchangers were also abundant in cortex but lacked the laminar expression largely overlapped in the cerebellar molecular layer. In contrast, NCKX3 was essentially absent from the striatum, where NCX2 was very abundant, and from the septal nuclei, where NCX1 was highly expressed.

A unique feature of NCKX3 expression in comparison to other K⁺-dependent Na⁺/Ca²⁺ exchangers was the presence of transcripts in many tissues other than brain, although at lower levels. The precise pattern appeared to vary between human and rodent, although part of this may have been due to the manner in which RNA loading was normalized (to a fixed quantity of total RNA in the case of rodent and to a varying amount of poly(A)⁺ mRNA, normalized to several control transcripts, in the case of the Multiple Tissue Expression Array), or due to the difference in the probes used. The tissues that had the next most abundant level of NCKX3 mRNA, after brain, were those generally rich in smooth muscle, such as aorta, uterus, and intestine. Many other tissues expressed lower levels, but only liver and kidney consistently appeared to be essentially negative for NCKX3. This rather ubiquitous pattern of expression matches more closely that of NCX1 than it does any of the other family members, which are thought to have quite restricted tissue expression patterns, NCX1 in eye, NCKX2 in brain neurons (including eye), and NCX2 and NCKX3 in brain and skeletal muscle. The selective presence of NCKX3 in specific unique cell types of the vascular wall (smooth muscle, endothelial cells, or innervating neurons) has yet to be examined. To our knowledge, however, there have been no reports of K⁺-dependent Na⁺/Ca²⁺ exchange activity in these tissues or cell types.

At present the physiological consequences of the differential tissue-specific expression patterns for different Na⁺/Ca²⁺ exchanger family members are unclear. The situation in brain, where at least four different Na⁺/Ca²⁺ exchanger gene products (NCX1, NCX2, NCKX2, and NCKX3)³ are present at high levels, is particularly intriguing. It seems likely that unique Ca²⁺-handling requirements of specific tissues or cell types, combined with kinetic, thermodynamic, or regulatory differences in the function of these exchangers, will provide important clues. The difference in transport stoichiometry between NCKX and NCX families provides an opportunity for NCKX-type exchangers to maintain Ca²⁺ homeostasis in environments where the Na⁺ gradient and/or the membrane potential are lower than normal. Although this argument was first used to justify the expression of NCX1 in rod photoreceptors, there may be situations in other neurons where similar conditions exist. Another possible role for different exchanger gene products might be in the transport of Mg²⁺ instead of Ca²⁺ (50). We have not investigated whether NCKX3 might transport Mg²⁺. It is noteworthy, however, that 1 mM Mg²⁺ significantly inhibits NCKX3 activity (Fig. 10), although under these conditions it had no significant effect on the amplitude of either NCX1 or NCKX2 currents.⁴ These observations suggest that Mg²⁺ interacts more selectively with the transport site of NCKX3 than it does with other family members.

The eventual answer to the question of unique physiological roles for the different Na⁺/Ca²⁺ exchanger family members will require the development of selective pharmacological blockers and/or of recombinant gene knock-out animals. The recent developments of an agent relatively selective for inhibition of NCX-type Na⁺/Ca²⁺ exchangers, KB-R7943 (51), and mice lacking NCX1 (52), will help in this effort, but additional selective molecular tools are eagerly anticipated. Most notably, planning complex gene knock-out experiments will be greatly facilitated by the completion of the mouse genome sequence (due in 2002) which will set a unique stage for genetic dissection of mammalian Ca²⁺ homeostasis.

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REFERENCES

1. Blaustein, M. P., and Lederer, W. J. (1999) Physiol. Rev. 79, 763–854
2. Philipson, K. D., and Nicoll, D. A. (2000) Annu. Rev. Physiol. 62, 111–133
3. We are thankful to Frans van Echten (University of Basel) for the use of his laboratory for some of these studies.
4. H. Dong and J. Lytton, unpublished observations.
31. Haug-Collet, K., Pearson, B., Webel, R., Szerencsei, R. T., Winkfein, R. J., Schnetkamp, P. P. M., and Colley, N. J. (1999) J. Cell Biol. 147, 659–670
32. Ausman, P. M., Brest, R., Kingdom, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (2001) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. Wu, K. D., and Lytton, J. (1993) Am. J. Physiol. 264, C335–C341
37. Kraev, A., Chumakov, I., and Carafoli, E. (1996) Genomics 37, 105–112
38. Tucker, J. E., Winkfein, R. J., Murthy, S. K., Friedman, J. S., Walter, M. A., Demetrick, D. J., and Schnetkamp, P. P. M. (1998) Hum. Genet. 103, 411–414
39. Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870
40. Li, X. F., and Lytton, J. J. (1999) J. Biol. Chem. 274, 8153–8160
41. Sahin-Toth, M., Andjelkovic, I., Frank, J. S., Winkfein, R. J., Demetrick, D. J., and Schnetkamp, P. P. M. (1991) J. Biol. Chem. 266, 22975–22982
42. Iwamoto, T., Nakamura, T., Pan, Y., Uehara, A., Imanaga, I., and Shigekawa, M. (1999) J. Biol. Chem. 274, 910–917
43. Iwamoto, T., Uehara, A., Imanaga, I., and Shigekawa, M. (1999) J. Biol. Chem. 274, 38571–38580
44. Iwamoto, T., Nakamura, T. Y., Pan, Y., Uehara, A., Imanaga, I., and Shigekawa, M. (1999) PERS LETT. 446, 264–268
45. Marlier, L. N., Zhang, T., Tang, J., and Grayson, D. R. (1993) Brain Res. Brain Res. 20, 21–39
46. Tashiro, M., Kuniishi, M., Iwamoto, T., Shigekawa, M., and Kurihara, S. (2000) Pflugers Arch. 439, 819–827
47. Iwamoto, T., Watanabe, T., and Shigekawa, M. (1996) J. Biol. Chem. 271, 23291–23297
48. Kikuchi, K., Kubo, K., Kuro, O. M., Yao, A., Iwamoto, T., Yanaka, N., Kita, S., Nishida, S., Kojima, T., Toyoda, Y., Ono, K., Imahie, H., Ota, K., Ikeda, S., Kohno, M., Imae, Y., Nishida, A., and Kuro, O. M. (2000) J. Biol. Chem. 275, 6987–6987
49. Prinsen, C. F., Szerencsei, R. T., and Schnetkamp, P. P. M. (2000) J. Neurosci. 20, 1424–1434
50. Tsukada, T., Nishida, Y., Kikutani, T., and Hori, T. (1998) J. Biol. Chem. 273, 4115–4126
51. Szerencsei, R. T., Nishida, Y., T. and Hori, T. (1998) J. Biol. Chem. 273, 4115–4126
Molecular Cloning of a Third Member of the Potassium-dependent Sodium-Calcium Exchanger Gene Family, NCKX3
Alexander Kraev, Beate D. Quednau, Stephen Leach, Xiao-Fang Li, Hui Dong, Robert Winkfein, Marco Perizzolo, Xinjiang Cai, Ruomei Yang, Kenneth D. Philipson and Jonathan Lytton

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Molecular cloning of a third member of the potassium-dependent sodium-calcium exchanger gene family, NCKX3.
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On line 4, “9q22.1–22.3” should read “9p22.1–22.3.”

Myeloid ELF-1-like factor is a potent activator of interleukin-8 expression in hematopoietic cells.
Cyrus V. Hedvat, JinJuan Yao, Robert A. Sokolic, and Stephen D. Nimer

Under “Experimental Procedures,” subheading “Plasmid Construction”: In the sentence beginning “PEA3 was cloned by reverse transcription...,” the second primer sequence was printed incorrectly. The sentence should read: “PEA3 was cloned by reverse transcription PCR from K562 cells using the Superscript one-step reverse transcription PCR system (Invitrogen) and the following primers: 5’-ATGGAGCGGAGGATGAAAGC and 5’-AGGGCAACTGGTAGGACAGT.”

A chaperone pathway in protein disaggregation: Hsp26 ALTERS THE NATURE OF PROTEIN AGGREGATES TO FACILITATE REACTIVATION BY Hsp104.
Anil G. Cashikar, Martin Duennwald, and Susan L. Lindquist

In the asterisk footnote to the title, the following should be added as the first line: This work was supported by National Institutes of Health Grant R37 GM25874 (to S. L. L.).

Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking.
Maite Arniges, José M. Fernández-Fernández, Nadine Albrecht, Michael Schaefer, and Miguel A. Valverde

The GenBank™ accession numbers DQ59644 and DQ59645 should be DQ059644 and DQ059645. These sentences should read: “We also identified three new splice variants, named TRPV4-C, TRPV4-D, and TRPV4-E variants (Fig. 1A), affecting the cytoplasmic N-terminal region. TRPV4-C lacks exon 5 (number DQ059644; Δ237–284 amino acids), TRPV4-D presents a short deletion inside exon 2 (number DQ059645; Δ27–61 amino acids), and TRPV4-E (number DQ059646; Δ237–284 and Δ384–444 amino acids) is produced by a double alternative splicing lacking exons 5 and 7.”

Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation.
Serge Grégoire, Annie M. Tremblay, Lin Xiao, Qian Yang, Kewei Ma, Jianyun Nie, Zixu Mao, Zhenguo Wu, Vincent Giguère, and Xiang-Jiao Yang

In the Abstract, line 8, “presented” should be “present.” Line 19, “identified” should be “identify.” Line 20, “revealed” should be “reveal.”