Cloning of a Third Mammalian Na\(^+\)-Ca\(^{2+}\) Exchanger, NCX3

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NCX3 is the third isoform of a mammalian Na\(^+\)-Ca\(^{2+}\) exchanger to be cloned. NCX3 was identified from rat brain cDNA by polymerase chain reaction (PCR) using degenerate primers derived from the sequences of two conserved regions of NCX1 and NCX2. The NCX3 PCR product was used to isolate two overlapping clones totalling 4.8 kilobases (kb) from a rat brain cDNA library. The overlapping clones were sequenced and joined at a unique Bsp106I restriction enzyme site to form a full-length cDNA clone. The NCX3 cDNA clone has an open reading frame of 2.8 kb encoding a protein of 927 amino acids. At the amino acid level, NCX3 shares 73% identity with NCX1 and 75% identity with NCX2 and is predicted to share the same membrane topology as NCX1 and NCX2. Following addition of a poly(A)\(^+\) tail to the NCX3 clone, exchanger activity could be expressed in Xenopus oocytes. NCX3 was also expressed in the mammalian BHK cell line. NCX3 transcripts are 6 kb in size and are highly restricted to brain and skeletal muscle. Linkage analysis in the mouse indicated that the NCX family of genes is dispersed, since the NCX1, NCX2, and NCX3 genes mapped to mouse chromosomes 17, 7, and 12, respectively.

Several different isoforms of the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX)\* have been cloned. Two mammalian isoforms, NCX1 (Nicoll et al., 1990) and NCX2 (Li et al., 1994), represent the products of distinct genes. NCX1 is predominantly expressed in heart, where it plays a major role in excitation-contraction coupling, but is also abundant in a variety of other tissues. Extensive alternative splicing generates tissue-specific variants of NCX1 (Gabellini et al., 1995; Kofuji et al., 1994; Lee et al., 1994). In contrast, expression of NCX2 seems to be restricted to brain and skeletal muscle (Li et al., 1994). Two nonmammalian NCX isoforms have also been cloned: one from Drosophila (Valdivia et al., 1995) and another from squid (He et al., 1996).

The NCX1 protein has been studied in considerable detail. NCX1 contains 11 putative transmembrane segments (Nicoll et al., 1990) preceded by a cleaved signal peptide (Durkin et al., 1991; Hryshko et al., 1993). There is a long, intracellular loop (sometimes called loop f) between transmembrane segments 5 and 6. NCX1 is inhibited by a peptide called XIP (Li et al., 1991). The amino acid sequence of XIP corresponds to a 20-amino acid segment of NCX1 in loop f called the endogenous XIP region. NCX1 has two internal repeats called the \(\alpha\)- and \(\beta\)-repeats.\* Two groups of three acidic amino acid residues in the Ca\(^{2+}\)-binding domain appear to be directly involved in ion binding (Levitsky et al., 1994; Matsuoka et al., 1993, 1995). The binding site for regulatory Ca\(^{2+}\) has been determined to be in loop f and is comprised of \(\beta\)-1 and residues between \(\beta\)-1 and \(\beta\)-2. Two nonmammalian NCX-type isoforms were sought by PCR using degenerate oligonucleotide primers derived from two highly conserved regions of NCX1 and NCX2. A unique exchanger clone from a rat brain cDNA library was isolated. This clone is designated as NCX3 and has been sequenced and expressed. We examined the tissue distribution of NCX transcripts and determined the chromosomal localization of the NCX genes. An analysis of the three mammalian NCX protein sequences is also presented.

EXPERIMENTAL PROCEDURES

Cloning NCX3—PCR with degenerate primers was used to identify a fragment of NCX3. The primers were derived from the amino acid sequences of conserved regions of NCX1 and NCX2. The forward primer was a 17-mer with 4-fold degeneracy (5′-GCIGGTAYAAATGGT-3′) based on a portion of proposed transmembrane segment 3. The reverse primer was a 19-mer with 6-fold degeneracy (5′-GTAACRTYTTARTA-3′) based on a portion of the endogenous XIP region (Li et al., 1991). cDNA from rat brain stem was used as template for the PCR reaction. The PCR reaction was carried out for 35 cycles (94°C, 30 s; 42°C, 30 s; 72°C, 120 s), and the PCR product was cloned into the vector pCRII (Invitrogen). After transformation, four plasmids with the described sequence of NCX3. In initial experiments, other primer pairs only amplified either NCX1 or NCX2.

Probe synthesized from the cloned PCR product was used to screen a rat brain stem λZAPII cDNA library (Stratagene). After screening about 1.8 × 10\(^6\) plaques, two overlapping clones, p15 and p20 (Fig. 1), were isolated. The full-length clone, pIII, was constructed from p15 and p20 as described under “Results.”

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\* The abbreviations used are: NCX, Na\(^+\)-Ca\(^{2+}\) exchanger; BHK, baby hamster kidney; XIP, exchanger inhibitory peptide; UTR, untranslated region; MOPS, 3-N-morpholino-propanesulfonic acid; 5′-RACE, 5′-rapid amplification of cDNA ends; PCR, polymerase chain reaction; RFIV, restriction fragment length variants; CM, centimorgans; bp, base pair(s); kb, kilobase(s); GSP, gene-specific primer.

\* E. Schwarz and S. Benzer, personal communication.
Expressing NCX3 Activity—Oocytes were prepared, and exchanger activity (as described previously by Longoni et al., 1988). The plasmid vector containing NCX3 was linearized with the restriction enzyme EcoRI, and synthesis of cRNA was performed with the T7 message mMessage mMachine (Ambion). Unincorporated nucleotides were removed with Chromaspin-100 columns (Clontech). cRNA or water (46 nl) was injected into each oocyte. Twelve days after injection, the oocytes were loaded with Na+ in the presence of nystatin, washed, and then incubated in medium containing Ca2+ and either Na+ (to measure Na+-dependent Ca2+ uptake) or K+ (to measure K+-dependent Ca2+ uptake). All solutions were as described previously (Longoni et al., 1988).

To express NCX3 in BHK cells, a 3640-bp NheI/BamHI fragment of NCX3 was subcloned into the Smal site of the transfer vector pNUT (Pantaloni et al., 1988). In this vector, NCX3 is under the control of the metallothionein I promoter. The resultant recombinant plasmid was transfected into BHK cells by the calcium phosphate method (Gorman et al., 1982). Twenty-four h after transfection, methotrexate (2.5 μM) was added to the culture medium and cells were incubated with this selection medium for 10 days. Methotrexate-resistant colonies were picked and screened for exchanger activity by Ca2+-uptake assay.

Fig. 1. Construction of NCX3 clones. Overlapping clones p20 and p15 were isolated from a rat brain cDNA library. Clone pII1 consists of the SacI-Bsp106I fragment of p20 ligated to the Bsp106I-3′-end of p15. pII/MC has the 3′-UTR, including the poly(A) tail, from the Na+-glucose cotransporter (Hediger et al., 1987) ligated into the BglII site of pII. The asterisk indicates the position of the stop codon.

RESULTS

Cloning of NCX3—The NCX3 isoform of the Na+-Ca2+ Exchanger—We searched for previously undescribed exchanger cDNAs by PCR amplification of rat brain cDNA using degenerate oligonucleotide primers to two highly conserved regions of the exchanger. The primers were designed to hybridize to the nucleotide sequences of transmembrane segment 3 in the α1 motif and to the endogenous XIP motif (Li et al., 1991). A PCR product of the appropriate size (~350 bp) was detected, subcloned into the pCRII vector, and sequenced. The sequence of one clone, which we designate NCX3, was previously undescribed but showed similarity to both NCX1 and NCX2. To obtain the full coding region of NCX3, the NCX3 PCR product was used to probe a rat brain cDNA library. Two overlapping clones, p15 and p20, were isolated. A full-length cDNA clone was constructed by ligating the 1.5-kb SacI-Bsp106I fragment from p20 into the HinclI-Bsp106I site of p15 (Fig. 1). The resulting clone, pIII, contained the initiating methionine, the entire coding region, and the 3′-UTR from p15 but terminated 750 bp from the 5′-UTR of p20.

NCX3 DNA Sequence—The combined sequence of clones p20 and p15 (Fig. 2) is 4855 bases long. Both the 5′- and 3′-UTRs are extensive, with 833 nucleotides in the 5′-UTR and 1236 nucleotides in the 3′-UTR. The NCX3 cDNA clone, like those reported for NCX1 and NCX2, does not contain a poly-
adenylation site or poly(A) tail. However, the NCX3 mRNA is approximately 1000 bases longer than the cDNA clone (see below) and may contain a poly(A) tail that is not included in the clone.

The NCX3 exchanger clone contains an open reading frame of 2784 nucleotides, starting at base 834 and ending at base 3617, which encodes for a protein of 927 amino acids. The sequence around the initiating methionine conforms well to the consensus sequence described by Kozak (1994) with six identities in 13 bases.

The 5'-UTR of NCX3 contains two distinguishing characteristics. First, there are 17 ATG triplets in the region upstream of the initiating methionine (Fig. 3B). None of these is likely to serve as an initiation codon for NCX3, as there are stop codons in each reading frame between the ATG at position 834 and the next nearest upstream ATG at position 672. Also, the longest potential coding region is only 111 bp.

The second characteristic of the 5'-UTR is an extremely GC-rich region (Fig. 3A). Between bases 520 and 720, the %GC content averages nearly 80%.

According to the ribosome scanning model of translation initiation, the first ATG in a mRNA is almost always the translation initiation site. The further downstream an ATG, the less likely it is to serve as an initiation site (Kozak, 1994). Also, GC-rich regions are capable of forming secondary structures which could impede the progress of the ribosome. Hence, the 5'-UTR of NCX3 is not conducive for translation efficiency. This suggests that translation of NCX3 is a highly regulated event.

We further investigated the 5'-UTR of NCX3 by 5'-RACE analysis. cDNA synthesis was followed by two rounds of PCR amplification using a nested pair of primers (Fig. 3B). A single RACE product of about 220 bp was observed (Fig. 3C). The PCR product was subcloned into a plasmid vector, and three individual plasmids were sequenced. The 5'-ends were all within three bases of nucleotide 734 (Fig. 3B). These results suggest that, in brain tissue, a single 5'-UTR exists for NCX3. The 5'-RACE products ended just downstream from the site where GC content rises. This may be indicative of strong secondary structure, as mentioned above, although further analysis of the 5'-UTR of NCX3 is necessary.

NCX3 Protein Sequence—The translation of the NCX3 open reading frame is shown in Fig. 4. As predicted for NCX1 and NCX2, NCX3 has 12 potential a-helical transmembrane segments. The first putative transmembrane segment is designated as a signal peptide by analogy to NCX1, which has been shown to have a cleaved signal peptide sequence (Durkin et al., 1991; Hryshko et al., 1993; Nicoll et al., 1990). A potential signal peptidase recognition site, AEA (Perlman and Halvorson, 1983), is located at amino acid 30. If this site is used in vivo, then cleavage could occur following the second alanine. Thus, the amino terminus is modelled to be extracellular, the carboxyl terminus to be intracellular, and there is a long cytoplasmic loop between putative transmembrane segments 5 and 6.

NCX3 contains 5 potential asparagine-linked glycosylation sites. Two (Asn-45, Asn-67) are in the amino-terminal, putative extracellular loop. Two more (Asn-130, Asn-135) are in the putative intracellular loop between transmembrane segments 1 and 2, and the fifth (Asn-823) is in the putative extracellular loop between transmembrane segments 8 and 9. By analogy to NCX1, where it has been demonstrated that only the first from clones p20 and p15, respectively, used to generate pIII and pIII/MC are in bold. These sequences have been submitted to GenBank under accession number U53420.
potential N-linked glycosylation site is used (Hryshko et al., 1993), it is predicted that only the first site in NCX3 is glycosylated.

NCX3 contains four consensus phosphorylation sites. Thr-113 is contained within consensus sites for either Ca\(^{2+}\)/calmodulin-dependent kinase or cAMP-dependent kinase. Thr-267 is within a consensus site for either cAMP-dependent kinase or calmodulin-dependent kinase; PKC, protein kinase C; TyrK, tyrosine kinase. *, site which is conserved in NCX1 and/or NCX2.

As further confirmation that NCX3 expresses exchange activity, we also expressed the NCX3 protein in BHK cells. Cells stably transformed with vector containing the NCX3 coding region expressed 12-fold more Na\(^{+}\)/Ca\(^{2+}\) exchange activity compared to cells transformed with vector alone (Fig. 6A), albeit at a reduced level compared to cRNA injected into oocytes expressing NCX1. The maximum level expressed by NCX3 is about 20% of the level seen for NCX1 expression in oocytes. It also takes much longer for the oocyte to begin expressing NCX3. We normally observe peak levels of NCX1 expression at 3–5 days after cRNA injection. NCX3 expression peaks at 7 or more days post-injection.

Tissue Specificity of NCX3—The expression of NCX1, NCX2, and NCX3 was investigated by Northern blot analysis to directly compare transcript sizes and tissue specificity. A Northern blot with poly(A)\(^+\) RNA from adult rat brain, heart, lung, ileum, liver, and pancreas was sequentially probed for NCX2, NCX1, and NCX3. The expression of NCX1, NCX2, and NCX3 is highest in ileum, liver, and pancreas (Fig. 7).
NCX1, and then NCX3 expression. Each NCX-specific DNA probe was derived from the large intracellular loop of the protein, where the NCX clones differ most. For NCX1, a 7-kb transcript was detected (Fig. 7). The strongest hybridization signal was observed in heart followed by spleen, with weaker signals in all other tissues except liver and pancreas. After a prolonged exposure time (3 days; not shown), a very weak hybridization signal could be detected in skeletal muscle (Fig. 7). No hybridization signals were seen in the other tissues even after a prolonged exposure.

Chromosomal Localization of NCX Genes—The NCX genes were mapped in mouse by linkage analysis of RFLVs. A survey of restriction enzymes using Southern hybridization revealed informative RFLVs for each of the three genes (Table I). In each case, C57BL/6J × M. spretus F1 hybrid animals exhibited all of the parental bands. The RFLVs were then scored in backcross progeny and compared with the segregation pattern of over 300 other markers spanning the mouse genome which were typed in this cross (Warden et al., 1993). Linkage analysis revealed significant linkage for each of the three NCX genes (Fig. 8). The NCX1 gene was located in the distal region of chromosome 17, 8.2 cM distal (with respect to the centromere) to marker D17Mit41 and 4.8 cM proximal to D17Ucla2. NCX2 was located in the proximal region of mouse chromosome 7, tightly linked to the gene for apolipoprotein E (ApoE) (zero recombinations out of 66 meioses tested). NCX3 was located in the middle region of mouse chromosome 12, tightly linked to the gene for apolipoprotein E (ApoE) (zero recombinations out of 66 meioses tested). Each of the above linkages was highly significant, as all lod scores exceeded 10 (a lod score is a statistical measure of whether a gene is linked to a particular genetic marker. A lod score of ≥3 is generally considered to be strong evidence of linkage). No other significant linkages were observed. We conclude that the NCX genes are part of a dispersed gene family.

**DISCUSSION**

**Activity of NCX Proteins**—We have described here the cloning and sequencing of a third, distinct, Na⁺-Ca²⁺ exchanger,
NCX3. The NCX3 cDNA, like those for NCX1 and NCX2, has been expressed in *Xenopus* oocytes and in BHK cells. A Na\(^+\) gradient-dependent \(^{45}\)Ca\(^{2+}\) uptake can be measured, thus verifying that NCX3 encodes a Na\(^+\)-Ca\(^{2+}\) exchanger. With the relatively low levels of activity expressed by NCX3, complete characterization has not yet been possible. It will be of much interest to determine if NCX3 shares the same regulatory properties which have been described for NCX1 and NCX2.

**Sequence Comparison of the NCX Proteins—** Overall, the NCXs share a considerable amount of sequence identity. When the putative leader peptides (see below) and potential alternative splice sites are discounted, the percent identity is 68% between NCX1 and NCX2, 75% between NCX1 and NCX3, and 75% between NCX2 and NCX3.

Each of the exchangers can be modelled to contain 12 hydrophobic segments. The first hydrophobic segments in each of NCX2 and NCX3 are likely to be cleaved leader peptides as has been demonstrated for NCX1 (Durkin et al., 1991; Hryshko et al., 1993). The amino acid sequences in this region are highly divergent (Fig. 9). Each of the remaining 11 hydrophobic segments is modelled to be a transmembrane helix. A long intracellular loop is located between putative transmembrane segment 5 and 6.

Within the transmembrane segments, the identity between the exchangers exceeds 75% except for transmembrane segment 11. The most highly conserved transmembrane segments are 2, 6, and 8. Transmembrane segments 2 and 8 are part of the \(\alpha\)-repeats and have been shown to be functionally important in NCX1. The last putative transmembrane segment, 11, is the least conserved with only about 60% identity between the exchangers. This suggests that putative transmembrane segment 11 is not involved in ion transport. Perhaps transmembrane segment 11 plays a structural role in the exchanger.

In NCX1, the intracellular loop has been shown to serve a regulatory role (Levitsky et al., 1994; Matsuoka et al., 1993, 1995). The loop is involved in inactivation of the exchanger and in binding regulatory, but not transported, Ca\(^{2+}\). The intracellular loops of the exchangers contain several interesting conserved motifs.

The first area of sequence conservation is in the amino-terminal end of the loop, in what is designated the endogenous XIP (Fig. 9). The endogenous XIP region may be involved in...
The second area of sequence conservation in the cytoplasmic loop is at amino acids 354–382 of NCX1 (Fig. 9). In this stretch of 29 residues, only four amino acids vary, and the amino acid substitutions are chemically similar to the residues in NCX1. No functional role has yet been attributed to this segment.

A third region of conservation is in the β-repeats (Fig. 9). Each repeat consists of a stretch of 76 amino acid residues. The β-1, β-2 repeats of NCX1 are 33% identical to each other. Those of NCX2 and NCX3 are 36% and 32% identical, respectively. The regions between the β-repeats are highly variable in both amino acid sequence and length.

Because the β-repeats are found in the long intracellular loop, it is likely that they are involved in exchanger regulation. Indeed, the Ca\(^{2+}\)-regulatory site of NCX1 (Levitsky et al., 1994; Matsuoka et al., 1995) contains all of β-1 and the stretch of amino acid residues between β-1 and β-2 (Figs. 4 and 9). Binding regulatory Ca\(^{2+}\) requires the presence of two triads of acidic residues. One of the triads is at the carboxyl end of β-1 and the other is just before β-2.

Thus, β-1 is involved in Ca\(^{2+}\) regulation, and the extent of similarity between β-1 and β-2 suggests that β-2 is also involved in regulation. However, the Ca\(^{2+}\) binding motif of β-1 is not fully conserved in β-2. Perhaps β-2 plays a different regulatory role, for example, via phosphorylation (see below).

Another interesting area in the long intracellular loop is in the variable region of NCX1 where alternative splicing occurs (Koju et al., 1994; Lee et al., 1994). In this area, both NCX2 and NCX3 have a deletion of 37 amino acids relative to NCX1.

The fourth conserved area in the cytoplasmic loop is at the carboxyl end of the loop, from residue 708 of NCX1 to the 6th transmembrane segment. Like the Ca\(^{2+}\)-binding domain, this region also contains two groups of acidic residues; one spans residues 710–718 including four glutamate residues, and one starting at residue 756, where there is a string of at least six consecutive acidic residues. No role has yet been attributed to this conserved region of the exchangers.

**Consensus Phosphorylation Sites in the Exchangers**—In at least one neural tissue (squid axon (DiPolo and Beauge, 1994)) and in smooth muscle (Iwamoto et al., 1995), there is evidence of regulation of the exchanger by phosphorylation. Also, NCX2 can be inhibited by treatment with a tyrosine kinase inhibitor when expressed in CHO cells (Condrescu et al., 1996). Each of the exchangers contains several consensus sites for phosphorylation by different protein kinases. The location of some of the phosphorylation consensus sites make them potentially interesting. In NCX3, the residue Thr-113 is located in the cytoplasmic loop connecting transmembrane segments 1 and 2. Transmembrane segment 2 is a part of the Ca\(^{2+}\)-binding domain, this region also contains two groups of acidic residues; one spans residues 710–718 including four glutamate residues, and one starting at residue 756, where there is a string of at least six consecutive acidic residues. No role has yet been attributed to this conserved region of the exchangers.

### Fig. 9. Comparison of NCX1, NCX2, and NCX3 amino acid sequences.

The complete amino acid sequence of NCX1 is shown. Identities between NCX1 and NCX2 or NCX3 are indicated with dots and alignment gaps with dashes. Potential transmembrane segments are underlined. The α- and β-repeats and endogenous XIP regions are highlighted. Amino acid numbers are those of NCX1 with the initiating methionine of NCX1 being number 1.

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3 S. Matsuoka, unpublished observations.

4 Z. He, unpublished observations.
Cloning of NCX3

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