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The human *SLC8A3* gene and the tissue-specific Na^+/Ca^{2+} exchanger 3 isoforms☆

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Abstract

We have identified the human gene for member 3 of Solute Carrier family 8 (*SLC8A3*) by bioinformatic analysis of human genomic sequences. The gene is located on chromosome 14q24.2, and spans a region of about 150 kb. The full-length DNA complementary to RNA encoding the Na^+/Ca^{2+} exchanger isoform 3 (NCX3), amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from the human neuroblastoma SH-SY5Y RNA, includes seven exons and encodes a protein of about 100 kDa. RT-PCR analysis was performed in different tissues to determine the exon composition in the region encoding the large intracellular loop of the protein. The region underwent modifications by alternative tissue-specific splicing. NCX3.2, including exon 4 but not exon 5, was found in human brain and in the neuroblastoma cell line. In human skeletal muscle two additional isoforms were identified: NCX3.3, including exons 4 and 5, and a truncated isoform (NCX3.4) produced by the skipping of both exons 3 and 4. The skipping causes a frame shift downstream of the exon 2 sequence. The new coding sequence of 25 amino acids terminates with a stop codon in exon 6. The NCX3.4 isoform (68 kDa) is truncated in the C-terminal portion of the domain first found in *Drosophila* Na^+/Ca^{2+} exchanger domain (Calxβ) and lacks the C-terminal hydrophobic segments.

Keywords: Na^+/Ca^{2+} exchanger 3 (NCX3); Splicing; Brain; Skeletal muscles; Calxβ domain; Truncation

1. Introduction

The Na^+/Ca^{2+} exchanger of the plasma membrane (NCX) is a reversible transporter controlling Ca^{2+} homeostasis. The forward Na^+/Ca^{2+} exchange activity extrudes one Ca^{2+} against three extra cellular Na^+, using the Na^+ gradient and the electrical potential across the plasma membrane as an energy source (Philipson, 1999; Gabellini et al., 2000).

Three genes, *Slc8a1*, *2* and *3* encoding the highly conserved NCX1, 2, 3 isoforms have been identified as members of the solute carrier gene family 8 and assigned to mouse chromosomes 17, 7 and 12, respectively (Nicoll et al., 1996). The human *SLC8A1* gene, encoding the cardiac type isoform 1 (NCX1) maps on chromosome 2p22.1 (NM_021097, Kraev et al. 1996); the human *SLC8A2* gene, encoding the isoform 2 (KIAA1087) on chromosomes 19q13.2 (/BO29010 Kikuno et al., 1999); and the *SCL8A3* gene, encoding the isoform 3 (NCX3) characterized in the present work on 14q24.2 (AF508982).

The *SLC8A* genes are differently expressed in tissues: isoform 1 is mostly expressed in heart, but at a lower level also in a variety of other cell types. Isoforms 2 and 3 are expressed mostly in brain and muscle (Komuro et al., 1992; Kofuji et al., 1992; Lee et al., 1994; Nicoll et al., 1996).

To elucidate the complete structure of the human *SLC8A3* gene and the tissue-specific alternative splicing operative in brain and skeletal muscle we used a combination of bioinformatic tools and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The work has led to the identification of the neuronal type human NCX3 isoform and of two additional isoforms expressed in human skeletal muscle.

Abbreviations: NCX3, Na^+/Ca^{2+} exchanger 3; TM, trans-membrane; Calxβ, domain first found in *Drosophila* Na^+/Ca^{2+} exchanger; EST, expressed sequence tag; cDNA, DNA complementary to RNA; bp, base pairs; kb, kilo bases; RT-PCR, reverse transcriptase-polymerase chain reaction; kDa, kilo Daltons

☆ The cDNA sequences have been deposited to GenBank under the accession numbers AF508982, AF510501, AF510502 and AF510503.

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2. Materials and methods

2.1. Prediction of the SCL8A3 gene structure

The genomic region of chromosome 14q24.2 was analyzed with UCSC Genome Browser (http://genome.ucsc.edu/), NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and NIX (http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/).

2.2. Cloning and sequencing of the NCX3 cDNA

Total RNA was extracted from SH-SY5Y cells by GeneElute™ Mammalian total RNA Kit (Sigma Chemical Co., St Louis, MO, USA). The DNA complementary to RNA (cDNA) synthesis was performed by the avian myeloblastosis virus reverse transcriptase and the random priming examer (Promega, Madison, WI, USA). PCR amplification of the 2.7 kb NCX3 cDNA was performed with a primer overlapping the ATG start codon (5′-TCGTGTATGGCGTGGTTAAGG) and with a primer matching downstream of the stop codon: 5′-TTAGAACCCCTTGATGTAGCAATA. The exon 1 was amplified by RT-PCR on the RNA of SH-SY5Y cells with a primer matching from position +6 (5′-TGTCTCCCAGCTGATAGGGGT) used in combination with a primer matching from base 66 of the SLC8A3 exon 2 (5′-CCATACACGAGACTTAGC). The reaction was performed with Taq polymerase (Roche Molecular Biochemicals) in the presence of 8% dimethylsulfoxide. The annealing temperature was set close to the Tm of the primers (63°C).

2.3. 3′ UTR region

The 3′ end of the *SLC8A3* messenger RNA (mRNA) was defined by alignment with the partial sequence of the rat *slc8a3* 3′ untranslated region (UTR) and with the available expressed sequence tags (ESTs) sequences extending the 3′ end of the transcript. Computer programs for the prediction of polyadenylation sites (BCM POLYAH and NIX GENSCAN polyA, FGenes polyA and GRAIL polyA) were used to predict the polyadenylation signal.

2.4. SLC8A3 splicing in tissues

For the RT-PCR analysis the human skeletal muscle PolyA$^+$ mRNA (Clontech, Palo Alto, CA) was reverse transcribed from a (oligodT) primer (Promega), as described in Section 2.2. The human skeletal muscle cDNA, the SH-SY5Y cells cDNA and the human whole brain quick-clone cDNA (Clontech) were used to amplify the DNA segments overlapping the spliced region. PCR was performed using an oligonucleotide in the vicinity of the 3′ end of the exon 2 (5′-GACAGTAGAAGGGACAGCCA) with an oligonucleotide matching exon 6 (5′-CTAGTTTG-GGGTGTTCACCC), and the Taq polymerase. The amplified DNA segments were separated by electrophoresis on 6% acrylamide/50% urea denaturing gels and stained by ethidium bromide.

2.5. DNA sequencing

The PCR products amplified by the Taq polymerase were cloned in the pCR vector (Invitrogen, Groningen, The Netherlands) and further analyzed by DNA sequencing (CRIBI sequencing service, Padova, Italy).

3. Results

3.1. The SCL8A3 gene

The human genomic DNA region including the complete *SLC8A3* gene was identified by similarity search (BLAST Human Genome) with the corresponding rat cDNA sequence (Nicoll et al., 1996), and with a partial genomic sequence, which had been previously assigned to human isoform 2 (Kraev et al., 1996). The alignment of the predicted coding sequence with that of *Rattus norvegicus Slc8a1* (AF109163), *Slc8a2* (NM_078619) and *Slc8a3* (U53420) clearly indicated high homology to rat isoform 3.

The human *SCL8A3* gene was identified within the genomic region 14q24.2 (sequence fragments AL160191.3 and AL135747.4, contig NT_010028.7, chr14:68875999–69023425). It includes nine exons, numbered 1–9. It should be noticed that exons 2–5 correspond to homologous exons 2–5 of the *SLC8A1* gene (Kraev et al., 1996); whereas the *SLC8A3* gene exons 6–9 correspond to homologous NCX1 exons 9–12. Thus, *SLC8A3* gene lacks the alternatively spliced exons 6–8.

The sequence and the boundaries of exons 2–10 (now 2–7) were already known (Kraev et al., 1996) and the mutual exclusiveness of exons 3 and 4 was previously demonstrated (also termed A and B, Kofuji et al., 1994). The sequence of the remainder exons (1, 8 and 9) was predicted by the alignment of cDNA sequences with the highly similar rat isoform 3 and by the consensus for splice sites.

The predicted human NCX3 protein is encoded by eight exons (2–9). The coding sequence specifies 928 or 927 amino acids, depending on the inclusion of exons 3 or 4. The human *SLC8A3* coding sequence exhibits 93% nucleotide conservation, and 97% amino acid identity with the orthologous rat sequence.

3.2. Sequence analysis of the SLC8A3 cDNA

Successful amplification of the exon 1 was performed with the cDNA from SH-SY5Y cells, a neuroblastoma cell line that expresses high levels of NCX3 protein and was used for the analysis of the *SLC8A3* proximal promoter region (Gabellini et al., 2002). The segment (752 bp) was amplified starting from nucleotide 6 of the exon 1 and 66 nucleotides downstream of the 5′ of the exon 2. Since exon 1, and the promoter region are located in a GC rich region, the yield of the amplification was low. Probably, the amplification of this segment from total human brain and human skeletal muscle cDNAs failed because the amount of *SLC8A3* cDNA in

Table 1
Intron-exon boundaries of the human *SLC8A3* gene

Intron			Exon			Intron	Exons size (bp)
	CGGCGCTGTC	…	1	…	AGAGGAAACG	gtccagtttg	692
tcttttctag	GTCTCTGGCC	…	2	…	ATGAAACTGT	gtaagtaacc	1846
ccatccacag	CAAAACAATT	…	3	…	TTCAGAAAG	gtgtagtacc	107
gtccctacag	GAAAACCATA	…	4	…	GGAATATCAG	gtgtgagatt	104
ttgcaaacag	CGCTCCTGT		5		TATCTCCAG	gtaagaggtg	18
tttgttgcag	ATGTGACAGA	…	6	…	TGAGTTCAAG	gtcaggcaaa	125
ttcttaacag	ACTACGGTGG	…	7	…	GTCAGTGCAG	gtgagaagtg	100
ttgccttcag	CAGGGGATGA	…	8	…	TCTGTCCCAG	gtgagagtga	276
ccttttccag	ATACGTTTGC	…	9	…	TCTCTACATA		2107

tissues is lower than in the neuroblastoma cell lines. The 5′ exon 1 sequence of the *SLC8A3* gene (Table 1) is more extended than that reported previously from the rat cDNA. Only the 3′ portion of exon 1 (228 bp) displays a significant similarity to the corresponding region of the rat sequence (Fig. 1).

The whole *SLC8A3* coding sequence was amplified by RT-PCR from the RNA of the human neuroblastoma SH-SY5Y cell line. The amplified segment (2766 bp) was cloned in the pCR vector and further analyzed by DNA sequencing. The coding sequence matched perfectly that predicted by the computational analysis, except the small exon 5 was absent. It consists of six exons: 2, 4, 6, 7, 8, 9 (Fig. 1). As in the case of the *SLC8A1*, the ATG start codon is close (63 bp) to the 5′ end of exon 2. This exon encodes the largest portion of the NCX3 protein, whereas the remainder coding exons (3–8) are much smaller (Table 1). The coding sequence terminates with a stop codon located at base 375 of exon 9. The sequence continues with a long 3′ UTR (1733 bp) as established by the alignment of genomic DNA and human EST sequences: N50099 (99.7% identity) AW022249 (98.7%); AW903279 (100%), and cDNAs AF086064 (99.7%) and AL359938 (99.8%). The polyadenylation signal (AATAAA) was found by NIX polyA program 23 nucleotides upstream of the putative polyA site.

The alignment of the deduced amino acids sequence of human NCX3 with that of human NCX1 and NCX2 showed 68% and 71% identities respectively, whereas that of NCX2 and NCX1 showed 65% identities (Fig. 2). The amino acid sequence of the human NCX3 protein was analyzed by Simple Modular Architecture Research Tools (SMART) computer programs. The Signal program predicted an N-terminal signal peptide (residues 1–31), similar to that of NCX1 (Hryshko et al., 1993). The TMHMM2 program predicted ten trans-membrane (TM) domains. The five N-terminal TM domains are clustered (amino acids 74–95, 133–155, 170–189, 202–224, 229–251). The large hydrophilic loop is located from positions 252–744. Five potential TM domains are predicted at the C-terminus of the protein (745–767, 782–801, 821–843, 853–875 and 896–918).

3.3. Alternative splicing

The alternative splicing in the SCL8A3 region encoding the C-terminal portion of the large intracellular loop was analyzed by RT-PCR on the cDNA from SH-SY5Y cells, human skeletal muscle and whole brain (Fig. 3). The amplification of the cDNAs from human brain and neuroblastoma cells produced only one band of 267 bp (Fig. 3,

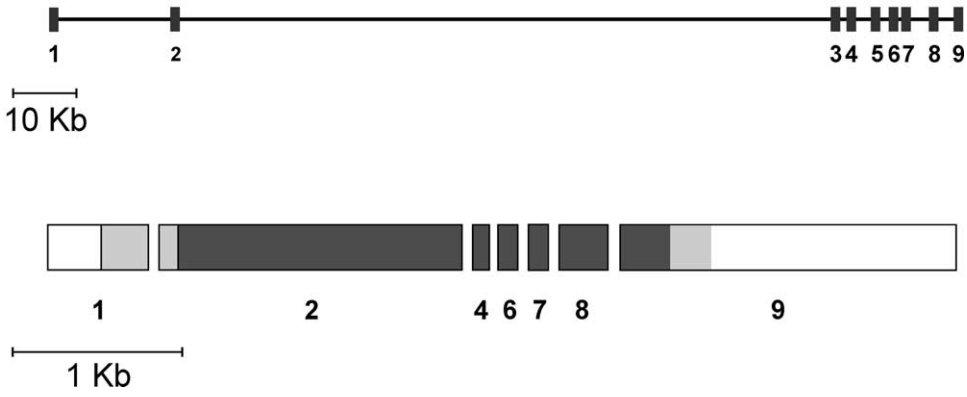

Fig. 1. The SLC8A3 gene and the NCX3.2 cDNA. The exon/intron organization of the gene is shown on the top panel. The spliced mRNA produced in brain and in the SH-SY5Y neuroblastoma cells is shown below. The coding region is shown in dark gray and the portions of the 5′ and 3′ UTR that are homologous to the corresponding murine sequences are shown in light gray.

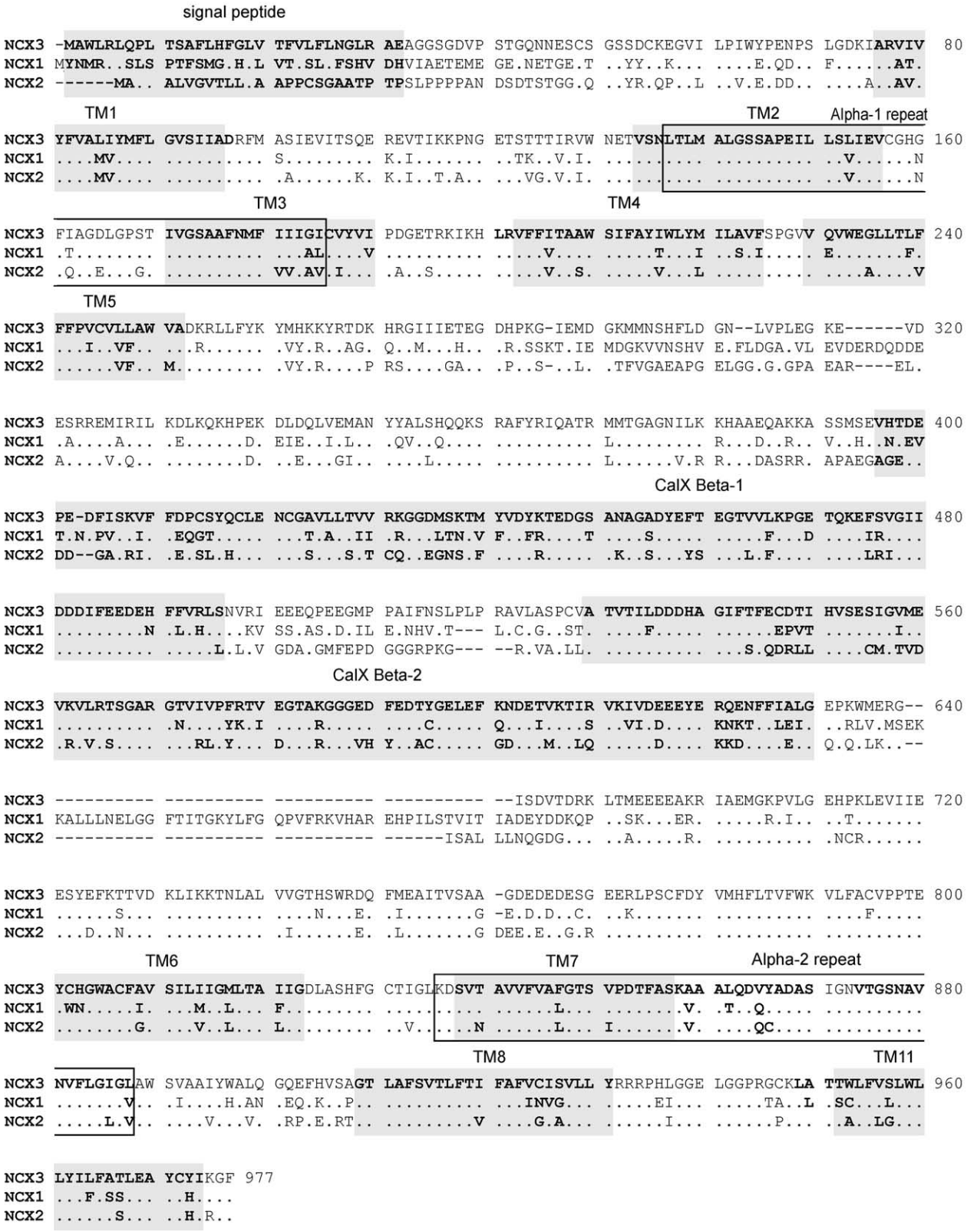

Fig. 2. Alignment of the human SLC8A coding sequences. The amino acid sequence of the human NCX3.2 isoform is aligned with the human NCX1.1 and human NCX2 sequences. The positions of the predicted signal peptide, of the putative TM regions and of the Calxβ-1 and Calxβ-2 domains are shown in bold and shaded. The regions corresponding to the α-1 and α-2 repeats are boxed. Numbering refers to the alignment positions, dots indicate identities, and dashes indicate gaps.

lanes A and C). The DNA sequence of several clones of the fragment was identical: all of them included exon 4. Thus, the NCX3 isoform expressed in brain has the same composition of the NCX3 isoform expressed in the human neuroblastoma cell line (AF510501, Fig. 2), corresponding to the murine isoform named NCX3.2. The alternative splicing pattern of the NCX3 cDNA from human skeletal muscle is more complex. Three DNA fragments were amplified from this tissue (Fig. 3, lane B): the most abundant DNA band corresponded to the NCX3.2 isoform including exon 4 (267 bp), whereas the largest fragment (285 bp) corresponded to the NCX3.3 isoform including the exon 4 and the small exon 5 (AF510502). The sequence of the smallest DNA fragment (164 bp) revealed a NCX3 isoform lacking both exons 3 and 4 (Fig. 3). We propose to name this newly identified isoform NCX3.4 (AF510503). The skipping of both exons 3 and 4 caused a frame shift downstream of the exon 2. The NCX3.4 sequence encodes 620 amino acids, of which 595 are encoded by exons 2 and 25 by the different frame in the exon 6. The NCX3.4 isoform has a predicted molecular weight of about 68 kDa; its domain composition is similar to a truncated NCX1 isoform previously described (Gabellini et al., 1995, 1996). No NCX3 isoform containing exon 3 was found in the human cDNA analyzed.

The sequence analysis performed by SMART similarity search with PFAM (protein families database) domains indicated that the sequence encoded by exons 3 and 4 (amino acids 595–630) overlaps the region encoding the second Calx-β domain (amino acids 519–619). Only 8 of the 25 amino acids specified by the different frame of the exon 6 reconstruct a portion of the second Calx-β domain, the remaining C-terminal sequence of isoform 3.4 includes several positively charged residues (Fig. 4).

4. Discussion

The human NCX3 isoform exhibits greater similarity with NCX2 than with NCX1, whereas the sequences of NCX2 and NCX1 are less similar suggesting that the *SLC8A3* gene could have originated by duplication of the *SLC8A1* gene. This is supported by their common intron/exon organization and by the different organization of the *SLC8A2* gene. For instance, the large exon 2 sequence (1.8 kb) common to the *SLC8A1* and *SLC8A3* genes is interrupted in *SLC8A2*. In contrast to the large degree of conservation in the coding sequence, no significant homology was found between the structure of the *SLC8A1* and *SLC8A3* exon 1. This could reflect a different regulation of transcription initiation: the expression of *SLC8A3* gene is restricted to excitable cells, whereas the *SLC8A1* gene is strongly expressed in heart, and also, albeit at lower levels, in many other cell types (Komuro et al., 1992; Kofuji et al., 1992; Lee et al., 1994; Nicoll et al., 1996).

The highly conserved primary structure of the exchanger isoforms indicates a similar membrane topology. All sequences contain 12 hydrophobic segments, the most N-terminal of which is a cleavable signal sequence (Hryshko et al., 1993). According to general consensus, the N-terminal portion of the mature NCX1 proteins protrudes from the outer side of the plasma membrane, then crosses the membrane five times and continues with a large cytosolic

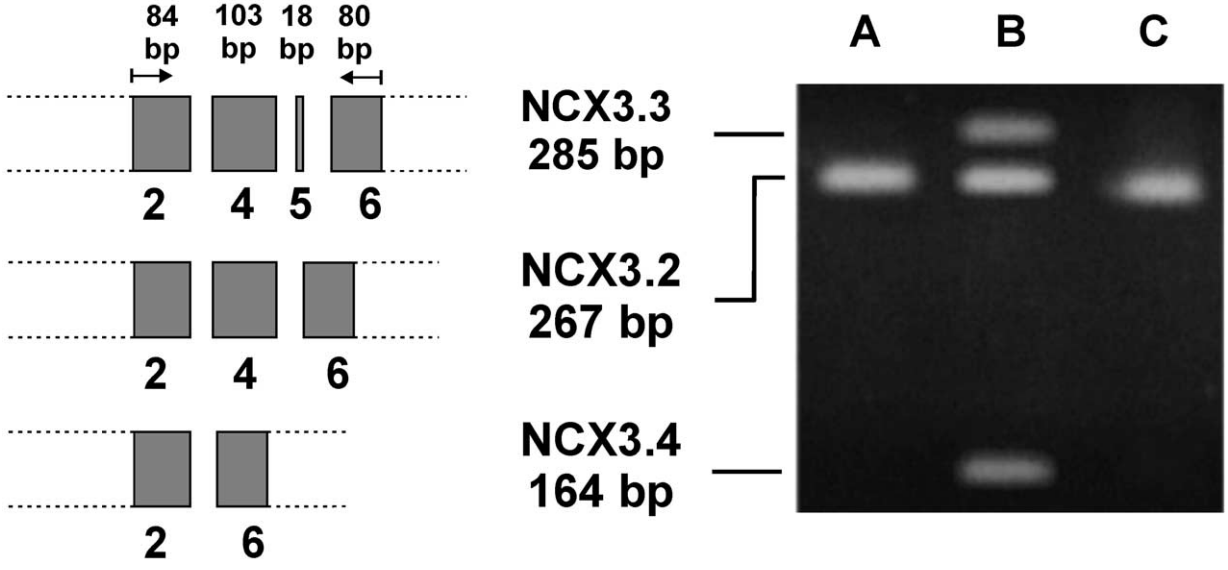

Fig. 3. Analysis of the spliced NCX3 isoforms. The ethidium bromide staining of RT-PCR products from SH-SY5Y human neuroblastoma cells (A); human skeletal muscle (B); and human brain (C), separated by electrophoresis on a denaturing acrylamide gel are shown, along with a schematic representation of the exons included in isoforms NCX3.3, NCX3.2 and NCX3.4. The position of the primers and the length of the amplified exon sequences (bp) are also indicated.

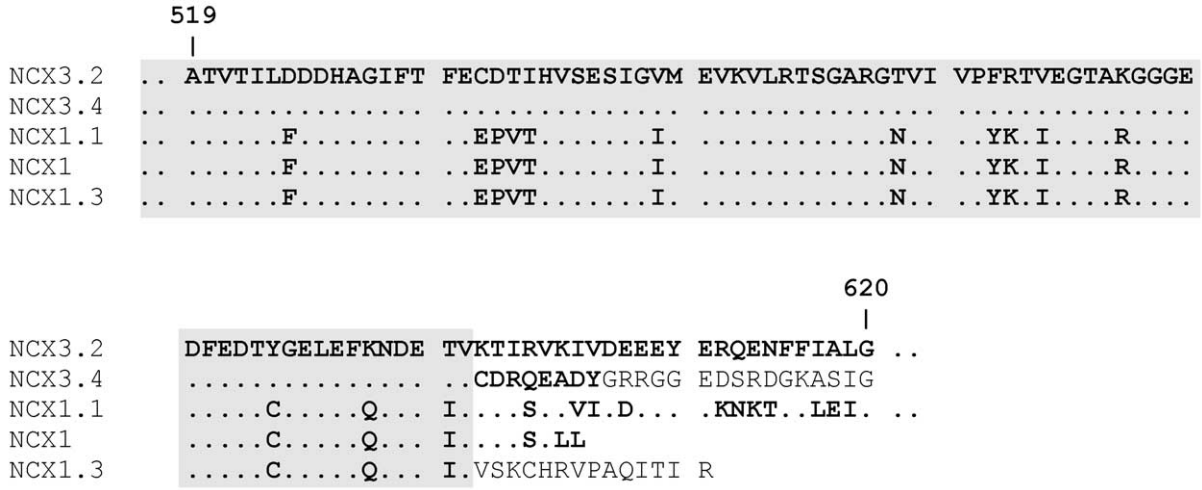

Fig. 4. Alignment of the C-terminal sequence of Calxβ-2 domain of NCX3.2 and of the truncated NCX1 isoforms. The C-terminal sequence (564–619) of Calxβ-2 domain of the NCX3.2 is aligned with the corresponding sequences of the truncated isoforms: NCX3.4, NCX1 (Gabellini et al., 1996), NCX1.3 (Van Eylen et al., 2001) and with the full length NCX1.1. The sequence of the Calxβ-2 domain is in bold; the portion of Calxβ-2 domain encoded by exon 2 is bold and shaded. Dots indicate identities.

loop (Nicoll et al., 1990; Porzig et al., 1993; Cook et al., 1998). The membrane topology of the C-terminal hydrophobic segments is less well established. The original model postulated six TM domains downstream of the large hydrophilic loop (Nicoll et al., 1990), however, the hydrophobic segment between amino acids 722–741, which would correspond to the first TM of the C-terminal hydrophobic block, was not predicted as a membrane spanning segment in the human NCX3 sequence. This agrees with a revised topological model, which postulates only four TM segments in the C-terminal region (Nicoll et al., 1999), however, according to this model, also the segment from amino acids 821–843 of the human sequence would not span the membrane, in contrast with what predicted by TMHMM2 program.

Alternative splicing of primary transcripts involving several small exons, modifies the C-terminal portion of the large intracellular loop which includes several regulatory domains, i.e. the exchanger inhibitory peptide (XIP, Li et al., 1991), the XIP binding peptide (Hale et al., 1997) and two Calxβ domains (Schwarz and Benzer, 1997). For NCX1 these are exons 3–8 (Kofuji et al., 1994; Lee et al., 1994; Furman et al., 1993) and for NCX3 exons 3–5 (Quednau et al., 1997). No splicing variants of NCX2 have been reported as yet. The most abundant NCX3 isoform expressed in human brain includes exon 4 and lacks exon 5. In contrast to NCX1, all NCX3 isoforms lack exons 6–8, corresponding to the C-terminal portion of the large cytosolic loop. This region is the site of alternative tissue-specific splicing, and is likely to be involved in the regulation of the NCX activity (Matsuoka et al., 1993; Condrescu et al., 1995). The new spliced isoform NCX3.4, produced by the skipping of exons 3 and 4 in human skeletal muscle, had not been found previously in any NCX transcript. Exon skipping results in the deletion of about 1/3 of the coding sequence, that terminates in a different frame of exon 6, and generate a novel short NCX3 isoform that is truncated in the C-terminal portion of the Calxβ-2 domain. A similar NCX1 protein truncated after exon 2, was composed of the five N-terminal TM segments and about 2/3 of the large cytosolic loop and retained the ion transport activity (Gabellini et al., 1995). Other groups have also found that the C-terminal portion of the NCX1 protein is not essential for ion transport activity (Li and Lytton, 1999; Van Eylen et al., 2001). Since full-length exchangers exhibit intramolecular homology between the N- and C-terminal hydrophobic regions termed α repeats (Fig. 3; Schwarz and Benzer, 1997), active truncated NCX isoforms could be dimers (Gabellini et al., 1996).

The frame shift caused by the skipping of exon 4 produced a NCX3 protein truncated in the Calxβ-2 domain. Eight of the 25 amino acids specified by the exon 6, including three negatively charged residues, could reconstruct a portion of the Calxβ-2 domain. The Calxβ motifs partially overlap the high affinity Ca^{2+} binding sites identified in the NCX1 protein (Matsuoka et al., 1995), however, they also overlap the region required for the inhibition of the NCX1 protein by elevated intracellular Na^{+} (Matsuoka et al., 1993). Tissue-specific splicing modifying this domain could confer regulatory properties that could respond to specific requirement of Ca^{2+} homeostasis.

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