Previous reports of Na/Ca exchanger gene 1 (NCX1) expression have revealed a major RNA transcript of 7 kilobase pairs (kb), minor transcripts of ~13 and ~4 kb, and a relatively abundant 1.8-kb RNA band. In the present report we demonstrate that the 1.8-kb message, which has a tissue and subcellular distribution matching that of full-length NCX1 but is not polyadenylated, corresponds to a perfectly circularized exon 2 species. The circular transcript contained the normal NCX1 start codon, a new stop codon introduced as a consequence of circularization, and encoded a protein corresponding to the NH2-terminal portion of NCX1, terminating just after amino acid 600 in the cytoplasmic loop. A linear version of the circular transcript was prepared and transfected into HEK-293 cells. A protein, matching the predicted size of ~70 kDa, was expressed, and the transfected cells possessed Na/Ca exchange activity. Although in native tissue we could not detect a protein corresponding exactly to that predicted from the circular transcript, a prominent band of slightly shorter size, possibly representing further proteolytic processing of circular transcript protein, was observed in membranes from LLC-MK2 cells and rat kidney.

The sodium-calcium exchanger (NCX) plays an important role in the regulation of intracellular Ca\(^{2+}\) levels in a broad number of tissues (1). Molecular studies of the Na/Ca exchanger have revealed that NCX1 is the predominant Na/Ca exchanger gene and is expressed in almost every tissue but at a particularly high level in heart, brain, and kidney (2). Studies on the organization of the human NCX1 gene have revealed that it comprises at least 14 exons spread out over more than 200 kb of genomic DNA (3, 4). Several reports have also identified two regions of alternative splicing in NCX1 transcripts from various tissues and animal species.

The first site of alternative splicing is in the 5'-untranslated region of the NCX1 message and involves exons referred to as 1a (or 1-Br), 1b, 1c (or 1-Kc), 1d (or 1-Ht), and 1e (3, 5). The use of tissue-specific promoters and splicing patterns involving these exons gives rise to at least three different transcripts, each with a unique exon 1 sequence at the 5’-end (3, 4, 6–8). Studies in rat using an RNase protection assay have demonstrated that heart expresses primarily NCX1 transcripts possessing exon 1-Ht, kidney expresses transcripts with exon 1-Kc, whereas NCX1 transcripts expressed elsewhere contain primarily exon 1-Br (5). It is thought that this pattern of splicing may allow independent and selective regulation of NCX1 expression in different tissues.

The second region of alternative splicing encodes a stretch of amino acids near the carboxyl terminus of the central intracellular loop of the NCX1 protein. At this site, six different exons (exons 3–8) are arranged in tissue-specific patterns (6, 9, 10). Heart expresses NCX1 transcripts containing exons 3, 5, 6, 7, and 8, brain expresses transcripts with exons 3, 6, and sometimes 8, and most other tissues express transcripts possessing exons 4 and 6 (and sometimes also 8). The functional consequences of this structural heterogeneity are still uncertain (11, 12).

Lying between these two sites of alternative splicing is an unusually long exon 2 sequence (1,832 bp coding for 600 amino acids in human NCX1). Exon 2 encodes the amino-terminal half of the NCX1 protein, including the initiating methionine, the first set of hydrophobic transmembrane segments, and most of the central cytoplasmic regulatory loop. Further alternative splicing, leading to a range of deletions among the carboxyl-terminal transmembrane segments of NCX1, was proposed based on studies in which a 6-kb canine cardiac NCX1 cDNA was expressed in HEK-293 cells (13). Nucleotides 3198, 2821, 2620, and 1845 (based on the coordinates of GenBank accession M57523 (14)) were identified as potential splice donor sites. None of these sites, however, is close to the exon boundaries identified in the human NCX1 gene (4).

Studies of NCX1 expression have all revealed a major transcript of about 7 kb which is expressed abundantly in many tissues (2, 6, 14–18). In addition to this major 7-kb transcript, less abundant transcripts of ~13 and ~4 kb and an abundant RNA band of 1.8 kb have also been reported (6, 15–18). Although present in many different tissues, the origin of these NCX1 transcripts has not been described.

In this report, we describe studies that demonstrate that the 1.8-kb mRNA corresponds to a circularized NCX1 exon 2 transcript encoding a truncated NCX1 protein. The distribution and possible functional role of this transcript are also investigated.

**EXPERIMENTAL PROCEDURES**

All molecular procedures were performed essentially according to standard protocols (19, 20) or the directions of reagent manufacturers, unless indicated otherwise. Chemicals were of the highest quality analytical grade available and were obtained from either Fisher, BDH, or...
RNA Isolation and Northern Blot Analysis—Total RNA preparations from whole tissues were isolated using the GTC-CeCl centrifugation method and from cultured cells using the GTC acid-phenol extraction protocol. Poly(A)^+ mRNA preparations were isolated from total RNA by passage through an oligo(dT) column. To isolate nuclear RNA, nuclei were prepared by hypotonic detergent lysis from LLC-MK2 cells. In brief, cells were collected by centrifugation, washed several times with a phosphate-buffered saline, resuspended in 5 x the packed cell volume of hypotonic buffer (10 mM HEPES/KOH, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 10 mM KCl), chilled on ice for 15 min, and then lysed by the addition of 0.6% (v/v) Nonidet P-40, followed by gentle mixing and three strokes in a tight-fitting Dounce homogenizer.

Nuclei were pelleted from this extract at 1,000 g for 3 min, and RNA was isolated using GTC acid-phenol extraction. Samples of RNA were separated on 1% agarose/formaldehyde gels and transferred into a nylon membrane by capillary diffusion overnight. The UV cross-linked membranes were hybridized with antisense digoxigenin UTP-labeled riboprobes according to the directions of the manufacturer (Boehringer Mannheim) as described previously (21). Probe A was derived from the 5'-untranslated region of exon 1-C (nucleotides 375 to 384) of the canine cardiac NCX1 (6). Probe B was prepared from nucleotides 2269 to 2720 of rat kidney F1 clone as described (6). Probe C was derived from nucleotides 1669 to 2071 of the rat kidney F1 clone (spanning exons 20, 4, 6, and 9–12). Probe D was derived from nucleotides 2269 to 2720 of rat kidney NCX1 (spanning most of exon 11 and part of exon 12), as described previously (17).

Inverse Polymerase Chain Reaction—The schematic description of the inverse PCR (IPCR) protocol is illustrated in Fig. 3B. 5 μg of RNA from LLC-MK2 cells was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) using the gene-specific primer GP1 (GTTTTGTTGTCCTTCTCATT, antisense, nucleotides 346–365; numbering is based on the published canine cardiac NCX1 (14), GenBank accession number M36119). The cDNA was converted to second strand essentially as described (21) and then purified, phosphorylated, and circularized by ligation in dilute solution. These circles were amplified using the primer pair IPCR-2 (ATGGGCTCTTTATTAGTAAGT- CATCAC, sense, nucleotides 289–311) and IPCR-3 (AGACAGTCGC- CAC[A,G]AAATACAC, antisense, nucleotides 229–250), which lie upstream from GSTP1 and face away from one another. Amplified products were gel purified, subcloned, and sequenced with the AmpliTaq FS kit (Perkin-Elmer). Fluorescently labeled sequencing reactions were analyzed at the University of Calgary Core DNA Service Facility.

Reverse Transcription-coupled Polymerase Chain Reaction (RT-PCR)—2 μg of total RNA from LLC-MK2 cells was reverse transcribed using either GP1 as described above or oligo(dT). The cDNA was then amplified using different pairs of primers based on the exon 2 region. The design of primers is shown in Fig. 4B. The first primer pair, C1 and C2, is one another and contain both exons 1 and 2, as well as 5' and 3' UTR regions. GAGTGAGAGAACATTGCATCATC, sense, nucleotides 1638–1659). The second (C2 and C2') and third (C3 and C3') primer pairs face toward each other (C2, TTGGTGGACAGAAATGGAGAGCA, sense, nucleotides 92–114; C2', TCCACCAACACAGAGGAGTA, antisense, nucleotides 662–683; C3, GTGATCCTCTCCCTGTTGTTTG, sense, nucleotides 659–680; C2', GACCTCCAGTGTCCTCAATC, antisense, nucleotides 1669–1690). The amplified products were gel purified, subcloned, and sequenced.

Isolation of the Circular Exon 2 Transcript from Total RNA of LLC-MK2 Cells—To isolate the circular exon 2 transcript, we designed a 16-nucleotide, 5'-end biotin-labeled, antisense oligonucleotide (circular oligonucleotide: AGAAGCTA ACAATTTC) which bridged the circularized 3' - and 5'-ends of exon 2 (18 nucleotides from each end). As a control, we also prepared a similar oligonucleotide (exon 1/2 oligonucleotide, AGAAGCTA AGTTTTGA), which spanned the junction of the 3'-end of exon 1 and the 5'-end of exon 2 and was designed to isolate the full-length NCX1 transcript. Total RNA isolated from LLC-MK2 cells (30 μg) was hybridized with 1.5 μM biotin-labeled circular oligonucleotide or 15 μM exon 1/2 oligonucleotide, 10 μg of yeast tRNA, and 10 μM of Poly(A) as carrier in 300 μl of hybridization buffer (0.5 x SSC, 0.5 mg/ml yeast Poly(A), 100 pg/ml primer C1, 1 μg/ml EDTA, pH 7.5), cooled slowly to room temperature, and then incubated at 37 °C for 30 min. The samples were then diluted to 2 ml with binding buffer and passed over an Immuno-Pure immobilized monomeric avidin column (Pierce). The procedure followed the instructions from the manufacturer except that phosphate-saline buffer was replaced by binding buffer. The biotin-oligonucleotide-hybridized RNA was eluted in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) containing 2 μg biotin. 10 μg of yeast tRNA was added, and the sample was precipitated with cold ethanol. The precipitated samples were then analyzed by Northern blot with probe B to detect the isolated transcripts.

Expression Constructs—The full-length NCX1 construct was prepared by amplifying oligo(dT)-primed reverse transcribed LLC-MK2 cell total RNA with a pair of primers containing Bam HI restriction sites at their 5'-ends (Pr-1, CCGCGATCCACGTGGGATTAAGTTT- TC, sense, nucleotides −3 to 20, and Pr-2, CCGGATCCCCCTTTGA- AGCTTTTATTGGGTTCC, antisense, nucleotides 2786–2809) using the Expand High Fidelity PCR system from Boehringer Mannheim. A linearized (Bam HI) version of the circular NCX1 exon 2 transcript was amplified from LLC-MK2 cell total RNA, reverse transcribed with GSP1 (described above), using a pair of primers (Pr-1, described above, and Pr-3, CCGG- GATCCGTTACCGACCTCCTAAGTG, antisense, nucleotides −24 through −6), which face away from each other. The amplified products were gel purified and cloned into the Bam HI site in pCDNA3.1+ (Invitrogen, Inc.). The resulting constructs were confirmed by sequencing. There were three differences between the full-length and truncated proteins, presumably as a consequence of PCR errors: Glu (full-length) for Gly (truncated) at amino acid 39 (counting the initiator Met as 1); Val for Leu-219, and Ser for Phe-482.

Expression in HEK-293 Cells—Transfection of cDNA expression constructs into HEK-293 cells was performed using a standard calcium-phosphate precipitation protocol. BES buffer essentially as described previously (22, 23). The circular construct cloned in the reverse orientation in the pCDNA3.1 vector was used as a control. Protein expression in crude microsome preparations was analyzed by immunoblotting with the C2C12 monoclonal antibody at 1:1,000 dilution. Calcium transport into transfected HEK cells was analyzed by Fura-2 fluorescent ratio digital imaging essentially as described previously (22). In brief, 2 days after transfection, cells grown on coverslips were loaded by incubation in 5 μM Fura-2/AM, 0.1% pluronic F-127, in serum-free Dulbecco’s modified Eagle’s medium buffered with 25 mM Tris-HEPES for 20–30 min at room temperature. The coverslips were mounted in a temperature-controlled perfusion chamber (Warner Instruments), maintained at 37 °C, on the stage of a Zeiss Axiosvert135 microscope; cells were continually perfused with solutions containing 10 mM Tris-HEPES, pH 7.4, 11 mM glucose, 0.5 mM CaCl2, and 145 mM NaCl or LiCl. The 340 nm/380 nm excitation ratio of Fura-2 fluorescence was measured using the ImageMaster System from Photon Technology International.

Immunoblotting in Native Tissues—Crude microsome preparations and immunoblotting were performed essentially as described previously (22). In brief, frozen or frozen tissue from rabbit heart, rat heart, or kidney was homogenized with a Polytron in ice-cold sucrose buffer containing a mixture of protease inhibitors (Boehringer Mannheim). LLC-MK2 cells were first swollen hypotontically on ice and then lysed with a Dounce homogenizer in buffer containing protease inhibitors. The cell homogenates were centrifuged, first at 8,000 x g for 20 min to remove nuclei, mitochondria, and debris and then at 100,000 x g for 60 min to pellet a crude fraction containing plasma membranes, endoplasmic reticulum, and other microsomes. These crude microsomal fractions were separated on SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and analyzed by immunoblotting using SuperSignal Plus ECL reagents from Pierce. The monoclonal antibody C2C12 recognizes an epitope between amino acids 372 and 535 (26) in the central cytoplasmic loop of the exchanger. The monoclonal antibody 6H2, which was a generous gift from Robert Reilly, University of Colorado, recognizes an epitope within the first extracellular 40 amino acids of the mature exchanger protein.

RESULTS

Several different groups have reported a relatively abundant NCX1 transcript of ~1.8 kb in size (15, 16, 18), although in previous studies we had not observed this species (6, 8, 17). Fig. 1 shows that we were able to confirm the existence of the 1.8-kb transcript when Northern blots were analyzed with a probe spanning most of the NCX1 coding region (probe B). Indeed, we found that this transcript is present in virtually the same tissue and species tested. The abundance of the 1.8-kb band appeared to correlate roughly with the abundance of the major 7-kb full-length NCX1 transcript, although the precise ratio varied among animal species. A particularly high amount of 1.8-kb transcript was observed in RNA from monkey tissues and in LLC-MK2 cells, a cell line derived from monkey kidney.

Sigma, unless indicated otherwise. Nucleic acid and protein amino acid sequence analysis was performed with the MacVector software package (Oxford Molecular Group) and by connection to the National Center for Biotechnology Information at the National Institutes of Health (www.ncbi.nlm.nih.gov).
To characterize the nature of the 1.8-kb NCX1 transcript, we performed the experiments shown in Fig. 2. First, a Northern blot of total RNA from rat kidney was analyzed with three different probes: A (from exon 1-Kc), B (spanning most of the coding region, exons 2, 4, 6, and 9–12), and C (spanning most of exon 11 and part of exon 12). As seen in Fig. 2A, the 1.8-kb NCX1 transcript was detected only with probe B, although 7- and ~13-kb transcripts were clearly evident with all three probes.

Next, total RNA isolated from LLC-MK2 cells was passed over an oligo(dT) column to isolate poly(A)⁺ mRNA. Northern blot analysis of the total RNA, poly (A)⁺ mRNA, and flow-through fractions revealed that the 1.8-kb NCX1 transcript was present quantitatively in the column flow-through, suggesting that it was not polyadenylated (see Fig. 2B). Note that the poly(A)⁺ mRNA lane contained about six times the relative amount of material compared with the other lanes. A band of ~4.5 kb in size was also visible in the poly(A)⁺ mRNA, as described previously (6).

Finally, cellular localization of the 1.8-kb transcript was examined. Total RNA was isolated either from a preparation of detergent-extracted LLC-MK2 cell nuclei or from an equivalent number of whole cells and analyzed by Northern blot. The majority of both 1.8- and 7-kb NCX1 transcripts were present in the cytoplasm and not in the nucleus (see Fig. 2C).

The coincidence of size and the pattern of hybridization with different probes led us to hypothesize that the 1.8-kb NCX1 transcript originated from the unusually long exon 2 sequence. We examined this issue further with a combination of IPCR and RT-PCR studies. Initially, we used the technique of IPCR to define the 5’-untranslated region of NCX1 transcripts expressed in the LLC-MK2 cell line. Following this procedure, as shown in lane 1 of Fig. 3A, two major product bands were detected: one of ~500 bp and one of ~1.8 kb. Subcloning and sequencing revealed that the 500-bp band extended back past the beginning of exon 2, ending in unique sequence that we presume to be the NCX1 exon 1 used in LLC-MK2 cells. The sequence of the 1.8-kb fragment also extended to the 5’-end of exon 2 but then continued directly with sequence from the 3’-end of exon 2 (based on the published exon boundaries (3, 4)). It is noteworthy that the 5’-end of the human NCX1 cDNA reported by Komuro et al. (15) has a structure virtually identical to the LLC-MK2 1.8-kb band, with sequence from the end of exon 2 appearing in the 5’-untranslated region upstream of position ~33.

Analysis of individual clones for both the 500-bp and 1.8-kb bands indicated that some clones were missing a TAG triplet at the 5’-end of exon 2. This difference may arise from the presence of two closely spaced splice acceptor sites at this location (3, 4) and is consistent with our previous observation of splicing heterogeneity at the same location in the rat NCX1 gene (5).

When reverse transcriptase was omitted from the protocol, no bands were detected, as shown in lane 3 of Fig. 3A, indicating that the amplified products did not arise from genomic DNA contamination. Moreover, when the ligation step of the IPCR protocol was omitted, we still were able to detect the 1.8-kb band, but not the 500-bp product (Fig. 3A, lane 2). These results suggested the possibility that the 1.8-kb fragment arose from a circularized exon 2 transcript, as illustrated in the schematic of Fig. 3B.

To confirm the circular nature of the exon 2 transcript, we performed RT-PCR on LLC-MK2 cell RNA using either a gene-specific primer from exon 2 (GSP1) or oligo(dT) to prime the reverse transcription reaction. The cDNA products were then amplified with different pairs of primers from within exon 2, as shown in panel B of Fig. 4. The first pair of primers (C1 and C1’) face away from one another and are therefore expected to amplify only a circular template. The other primer sets (C2 and C2’, C3 and C3’) face toward one another and will thus amplify both linear and circular templates. However, because both of these primer sets have at least one member downstream from the GSP1 reverse transcription priming site, products would...
only be expected from a linear template if it were primed with oligo(dT). As shown in Fig. 4A, products were in fact seen from GSP1-primed cDNA for all three primer sets, whereas oligo(dT)-primed cDNA yielded bands only with the primer pairs designed for a linear template. Sequencing confirmed the identity of bands, as illustrated schematically in Fig. 4B.

The IPCR and RT-PCR experiments thus demonstrated the presence of a circularized NCX1 exon 2 transcript of 1.8 kb in length which was not polyadenylated. The 1.8-kb transcript observed on Northern blots was not polyadenylated and was only detected with a probe containing exon 2 sequence. To demonstrate directly that the 1.8-kb transcript corresponded to a circularized exon 2, we used oligonucleotide affinity chromatography. Biotinylated oligonucleotides spanning the junction between the ends of circular exon 2 (circular oligonucleotide) or the 3' end of exon 1 and the 5' end of exon 2 (exon 1/2 oligonucleotide) were hybridized with total RNA from LLC-MK2 cells. The hybridized samples were then passed over an avidin column, washed, eluted, and analyzed by Northern blot, as shown in Fig. 5. It is evident from these data that hybridization with the circular oligonucleotide selectively enriched for the 1.8-kb transcript, whereas hybridization with the exon 1/2 oligonucleotide selectively enriched for the full-length 7-kb transcript.

The circular NCX1 exon 2 transcript contained the normal NH2-terminal start codon for full-length NCX1 and a new stop codon introduced as a consequence of the circularization and thus encoded a protein of 602 amino acids (Fig. 6). Constructs expressing the full-length LLC-MK2 cell NCX1, or a linear version of the circular exon 2 transcript encoding the truncated protein, were prepared by high fidelity PCR. The deduced amino acid sequence from these cDNAs is shown in Fig. 6. The full-length monkey kidney NCX1 molecule contained 934 amino acids and was greater than 99% identical to human cardiac NCX1 except in the region of alternative splicing, where human cardiac NCX1 contained exons 3, 5, 6, 7, and 8 (isofrom NCX1.1; alternatively spliced exons previously referred to as A, C, D, E, and F), whereas LLC-MK2 cell NCX1 contains only exons 4 and 6 (exons B and D, isofrom NCX1.3). The truncated protein extends to amino acid 600 of full-length LLC-MK2 cell NCX1, plus two more amino acids (Arg and Phe).

Both truncated and full-length constructs were transfected into HEK-293 cells. As seen in the left panel of Fig. 7A, immunoblots using the C2C12 antibody showed a strong band at 70 kDa for the truncated construct, which was absent from control-transfected cells. The full-length construct generated a broad band at 110 kDa, representing the complete NCX1.3 protein, and a minor band at ~50–60 kDa which probably represents a proteolytic degradation product. When the protein samples were run in the absence of reducing agent, as shown in the right panel of Fig. 7A, in addition to the truncated protein product of 70 kDa, a major band of ~140 kDa and a minor band at ~250 kDa were also observed. These aggregates suggested the possibility that truncated NCX1 protein formed disulfide-linked homomeric dimers in the membrane. In the absence of β-mercaptoethanol in the sample buffer, the full-length NCX1.3 ran as a ~220-kDa band in addition to the 110-kDa band, which suggested that it too might also be capable of forming dimers in the membrane.

Fluorescent calcium ratio imaging with Fura-2 was used to examine the transport function of the expressed NCX1 proteins, as illustrated in Fig. 7B. Cells grown on coverslips were transfected, loaded with Fura-2, and then mounted in a perfusion device on a microscope stage and maintained at 37 °C. The cells were first perfused in a medium containing 145 mM sodium chloride and then switched to a medium containing 145 mM lithium chloride. This maneuver reverses the sodium gradient and removes sodium competition at the outwardly facing calcium binding sites and therefore favors calcium entry through an NCX molecule. In cells transfected with a nonexpressing control construct, the medium switch did not elicit any change in Fura-2 fluorescence. In transfected cells expressing the truncated NCX1 protein, the medium switch elicited a significant increase in Fura-2 fluorescence, indicating calcium entry. In paired experiments, however, the change in Fura-2 fluorescence was not as rapid nor as large as that seen for cells expressing the full-length NCX1.3 protein.

Having demonstrated that the truncated construct gave rise to a functional Na/Ca exchange protein when expressed in HEK cells, we investigated the possibility that the circular NCX1 exon 2 transcript could be translated into protein in native tissues. Microsomal fractions isolated from rabbit heart,
NCX protein, the C2C12 antibody also recognized bands of respectively, with either antibody. In addition to the full-length NCX1.1 and NCX1.3 were evident at 120 kDa and 110 kDa, when expressed in HEK cells. In microsomes isolated from rabbit heart or from cultured LLC-MK2 cells, full-length NCX1.3 protein and the 70-kDa truncated NCX protein were observed in microsomes from rat kidney but not rat heart (data not shown). The ~60-kDa band recognized by 6H2 but not by C2C12 thus corresponds to an NCX1 polypeptide extending from the NH2 terminus to the cytosolic loop but terminating before the complete C2C12 epitope (amino acids 372–525). The relative abundance of the ~60-kDa and ~110-kDa NCX1 protein species is comparable to the ratio of 1.8-kb to 7-kb transcripts found in the LLC-MK2 cells (see Fig. 2, B and C).

**DISCUSSION**

In this manuscript we have used IPCR, RT-PCR, and oligonucleotide affinity chromatography to demonstrate that the previously described 1.8-kb NCX1 transcript of unknown origin corresponds to a circularized exon 2 species. The 1.8-kb transcript is expressed ubiquitously, with an abundance that correlates with the abundance of full-length 7-kb NCX1 transcript in all of the tissues and animal species we have tested. Although the 1.8-kb transcript is found in the cytoplasm, it is not polyadenylated. We believe that the inconsistent reporting of this species in earlier literature can be explained by the requirements for a probe to include exon 2 sequence and for the analysis to be of total RNA rather than poly(A)− mRNA.

The appearance of circular transcripts is not unique to NCX1. Eukaryotic circular RNA was first reported in the human ets-1 gene (30) and subsequently in the mouse testis-determining gene, sry (31), the rat cytochrome P450 2C24 gene (32), and the human cytochrome P-450 2C18 gene (33). The functional role(s) for these circular transcripts remain(s) uncertain. Some of the circular transcripts contains several exons joined together in an order different from that present in genomic DNA, whereas others contain a single exon joined head to tail. None of the reported circular transcripts is polyadenylated, and all are present in cytoplasm. Only the circular transcript from the testis-determining gene, sry, is like NCX1...
in that it contains a single exon joined exactly head to tail, with the normal protein start codon and a new stop codon. It seems likely, however, that the mechanism that generates the circular species is entirely different for sry compared with NCX1. In the mouse sry gene, two large inverted repeats flank the circularized exon and are required for its excision from a large linear RNA precursor molecule (31, 34, 35). No similar inverted repeat structures are evident in the NCX1 gene in proximity to exon 2. Instead, we believe that the unusual length of NCX1 exon 2 (1.8 kb) may account for the appearance of this perfectly circularized exon and are required for its excision from a large linear RNA precursor molecule (31, 34, 35). No similar inverted repeat structures are evident in the NCX1 gene in proximity to exon 2. Instead, we believe that the unusual length of NCX1 exon 2 (1.8 kb) may account for the appearance of this perfectly
Circular NCX1 Transcript

Presumably the 5'-end of exon 2, because of its length, can become arranged in a three-dimensional space to lie in proximity to the 3'-end of the same exon. During processing, the splicing event that would normally join the 3'-end of exon 2 to the 5'-end of the subsequent exon instead joins it to its own 5'-end, in an exact head-to-tail arrangement. Circular transcripts of a number of different genes have been found to accumulate in the cytoplasm, suggesting a biological role for these molecules beyond the splicing process. The circular NCX1 transcript encodes a 602-amino acid protein. Internal initiation of translation has been observed in mammalian cells (36), so it is possible that a protein product could be made from the circular transcript, although the efficiency of translation may be affected by the circular structure as well as by the absence of a cap (37) or polyA tail (38). Moreover, Chen and Sarnow (39) have demonstrated that circular transcripts can be translated into protein.

The finding that a truncated protein lacking the COOH-terminal hydrophobic region of NCX1 was functional suggests several possible models. First, it is possible that the COOH-terminal part of NCX1 is not essential for transport. This seems unlikely, however, because mutagenesis studies have shown that key residues within the two repeats are highly conserved among all known classes of Na/Ca exchangers and have been hypothesized to come together in space to form the ion binding pocket required for membrane transport (40). Analysis by mutation of key amino acids within the two α repeats has confirmed the importance of each repeat region in transport function and reinforced the idea of symmetry between the two halves of the NCX1 molecule (41).

The truncated NCX protein encoded by the circular exon 2 transcript contains the first 600 amino acids found in the full-length protein, plus two extra amino acids resulting from circularization, and terminates at the site of alternative splicing in the cytoplasmic domain. This protein thus lacks the COOH-terminal hydrophobic domain and therefore the second of the two α repeats. It was thus of some surprise to find that the truncated NCX1 protein was nevertheless capable of sodium-calcium exchange function. Recent studies from Carafoli’s group had also identified shorter NCX1 transcripts encoding truncated proteins when NCX1 was expressed in HEK cells (13). The shortened NCX1 RNA species found in these studies arose from cDNA constructs in transfected cells and did not correspond to known exon boundaries. The encoded protein was nevertheless very similar in structure to our truncated NCX1 protein and, like the molecule reported here, was capable of transport function (42).

The normal NCX1 protein comprises a cleaved signal sequence followed by a short glycosylated extracellular region, a domain of five hydrophobic transmembrane segments, a long cytoplasmic loop, and a final region of six transmembrane segments (14). Two segments of amino acid sequence, one from the center of each hydrophobic region, have been recognized as similar and are thought to have arisen from an ancient gene duplication event. These regions, the α repeats, are highly conserved among all known classes of Na/Ca exchangers and seem unlikely, however, because mutagenesis studies have shown that key residues within the two α repeats are highly conserved among all known classes of Na/Ca exchangers and have been hypothesized to come together in space to form the ion binding pocket required for membrane transport (40). Analysis by mutation of key amino acids within the two α repeats has confirmed the importance of each repeat region in transport function and reinforced the idea of symmetry between the two halves of the NCX1 molecule (41).

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The finding that a truncated protein lacking the COOH-terminal hydrophobic region of NCX1 was functional suggests several possible models. First, it is possible that the COOH-terminal part of NCX1 is not essential for transport. This seems unlikely, however, because mutagenesis studies have shown that key residues within the second α repeat are essential for functional activity (41). Second, given the pseudo-symmetrical arrangement of the molecule, it seems more plausible that two NH2-terminal halves may come together as a dimer in the membrane and form an ion binding and transport pocket composed of two α1 repeats instead of the normal α1/α2 structure. Our observation of higher order structures on SDS gels run in the absence of reducing agent is consistent with such an arrangement. Although we could also observe higher order structures for full-length NCX1 in transfected HEK cells, we found no evidence for coassembly of full-length and truncated NCX proteins into heterodimers when the two molecules were cotransfected in the HEK cells (data not shown).
The truncated NCX1 protein was expressed in HEK cells at a level similar to full-length NCX1.3 yet had a dramatically lower transport activity. This may reflect the relative inefficiency of an α1/α2 transport pocket compared with the normal α1/α2 pocket. Alternatively, it may be the result of a reduction in efficiency of surface expression and/or protein stability due to the requirements for intermolecular interaction between two separate truncated protein halves compared with an intramolecular interaction between the two halves of a full-length NCX1 protein. Indeed, we have found that the truncated NCX molecule disappears rapidly from transfected HEK cell microsomes, with a half-life of roughly 2 h at room temperature. In contrast, full-length NCX1 is completely stable under these conditions (data not shown).

We searched for the presence of truncated NCX1 protein that might have originated from a circular transcript using the C2C12 and 6H2 antibodies to blot microsomes isolated from rabbit heart, LLC-MK2 cells, rat heart, and rat kidney. Although no band was observed which corresponded exactly in both length and antigenicity to the product from the circular transcript construct, a very strong ~60-kDa band was detected in LLC-MK2 cells. In contrast to the truncated NCX1 protein produced in HEK cells which ran at 70 kDa on SDS gels and was recognized by both 6H2 and C2C12 antibodies, the 60-kDa LLC-MK2 protein was recognized only by the 6H2 antibody and not the C2C12 antibody. It is thus possible that the 60-kDa band in LLC-MK2 cells was derived from protein synthesis off the circular transcript followed by proteolytic processing in the cytosolic loop which shortened the protein and removed part of the epitope essential for recognition by the C2C12 antibody. Strikingly, the LLC-MK2 cell 60-kDa band was much more abundant than the full-length 110-kDa NCX1.3 band. Indeed, the relative abundance of 60-kDa and 110-kDa proteins closely matched that of 1.8-kb and 7-kb mRNA transcripts in these cells (see Fig. 2, B and C). A similar 60-kDa band was also observed, although at lower abundance, in microsomes isolated from rat kidney but not rat or rabbit heart. The abundance of truncated protein found in different tissues thus does not match the abundance of 1.8-kb transcript, suggesting tissue-specific regulation of transcription and/or protein stability.

In summary, we have demonstrated the presence of a circularized NCX1 exon 2 transcript corresponding to a previously described 1.8-kb NCX1 RNA band of unknown origin. The tissue and cellular distribution of the circular transcript match those of the full-length linear NCX1 transcript. The circular RNA encodes a truncated NCX1 protein that expresses Na/Ca exchange function when expressed in HEK cells. We have identified a protein of similar size in membrane fractions from rat kidney and LLC-MK2 cells, possibly suggesting that the protein product of the circular transcript plays a special role in kidney cells.

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