# Cloning, expression, and characterization of the trout cardiac Na+/Ca2+ exchanger

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# Cloning, expression, and characterization of the trout cardiac $Na^+/Ca^{2+}$ exchanger

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**Xue, Xiao-Hua, Larry V. Hryshko, Debora A. Nicoll, Kenneth D. Philipson, and Glen F. Tibbits.** Cloning, expression, and characterization of the trout cardiac $Na^+/Ca^{2+}$ exchanger. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C693–C700, 1999.—Isoform 1 of the cardiac $Na^+/Ca^{2+}$ exchanger (NCX1) is an important regulator of cytosolic $Ca^{2+}$ concentration in contraction and relaxation. Studies with trout heart sarcolemmal vesicles have shown NCX to have a high level of activity at 7°C, and this unique property is likely due to differences in protein structure. In this study, we describe the cloning of an NCX (NCX-TR1) from a Lambda ZAP II cDNA library constructed from rainbow trout (*Oncorhynchus mykiss*) heart RNA. The NCX-TR1 cDNA has an open reading frame that codes for a protein of 968 amino acids with a deduced molecular mass of 108 kDa. A hydropathy plot indicates the protein contains 12 hydrophobic segments (of which the first is predicted to be a cleaved leader peptide) and a large cytoplasmic loop. By analogy to NCX1, NCX-TR1 is predicted to have nine transmembrane segments. The sequences demonstrated to be the exchanger inhibitory peptide site and the regulatory $Ca^{2+}$ binding site in the cytoplasmic loop of mammalian NCX1 are almost completely conserved in NCX-TR1. NCX-TR1 cRNA was injected into *Xenopus* oocytes, and after 3–4 days currents were measured by the giant excised patch technique. NCX-TR1 currents measured at ~23°C demonstrated $Na^+$-dependent inactivation and $Ca^{2+}$-dependent activation in a manner qualitatively similar to that for NCX1 currents.

teleosts; myocardium; contractility; sodium/calcium exchange

THE SODIUM/CALCIUM EXCHANGER (NCX) is an integral membrane protein that plays an important role in the regulation of cytosolic $Ca^{2+}$ concentration in many cell types. NCX transports $Ca^{2+}$ across the membrane with a stoichiometry of three $Na^+$ to one $Ca^{2+}$ and is therefore electrogenic (32). The role of the NCX in excitation-contraction (E-C) coupling in the mammalian heart has been studied extensively. Its function as the prime mechanism of $Ca^{2+}$ extrusion from the cardiomyocyte and therefore an important component of relaxation is well documented (1, 2, 4). More controversial roles for NCX as a pathway for depolarization-induced $Ca^{2+}$ influx (18) and/or $Na^+$ current-induced $Ca^{2+}$ influx (16) have also been proposed.

The NCX in the lower vertebrate heart has not been studied in great detail. Despite this, there is considerable circumstantial evidence from teleost and amphibian hearts that NCX plays a critical role in E-C coupling in these species. Electron micrographs of the teleost heart reveal both a scarcity of sarcoplasmic reticulum (SR) compared with mammal hearts and an absence of transverse tubules (34). Furthermore, the density of the SR $Ca^{2+}$ release channel in the hearts of teleosts is substantially lower than that in the hearts of mammals (37), and ryanodine does not reduce the contractile force of the teleost ventricle under most physiological conditions (8). In addition, the surface-to-volume ratio is higher in teleost myocytes than in mammalian myocytes, implying that a greater rise in cytosolic intracellular $Ca^{2+}$ concentration for a given $Ca^{2+}$ influx occurs. Thus it has been suggested that, in the teleost heart, transsarcolemmal $Ca^{2+}$ transport is sufficient to support contraction (38) and that reverse-mode NCX activity may contribute to the $Ca^{2+}$ influx during E-C coupling. Furthermore, in the absence of a substantial SR in teleosts, it is likely that the NCX is the primary means of reducing cytosolic $Ca^{2+}$ (38).

One prominent aspect of cardiac function in the rainbow trout (*Oncorhynchus mykiss*) is its ability to maintain adequate contractility under hypothermic conditions that are cardioplegic to mammals. It has been suggested that this capability requires at least some of the proteins involved in E-C coupling to have evolved differently in these species. Studies of $Na^+/Ca^{2+}$ exchange in trout heart sarcolemmal vesicles have demonstrated properties of this protein that are both unique and common to isoform 1 of the mammalian NCX (NCX1) (39). Immunoblots of trout sarcolemma (SL) probed with polyclonal antibodies raised against the canine NCX1 have a pattern of banding that is the same as that of immunoblots of mammalian SL, indicating similarities in antigenicity and molecular weight. Northern blots of trout mRNA, probed with a fragment of the canine NCX1 cDNA, demonstrate comparable transcript sizes (~7 kb), and this suggests substantial conservation of transcript sequence (39). Other similarities of the NCX1 from these divergent species include similarities of electrogenicity and stimulation by chymotrypsin treatment. However, despite these striking similarities of trout and mammalian

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NCX, the differences are also remarkable. Most notable is that reducing the temperature from 21 to 7°C results in a canine NCX1 activity <10% of the initial level, whereas in trout the activity remains >75% (39). This behavior is observed not only in the native membranes but also when both canine and trout NCX proteins are reconstituted into asolectin vesicles. These data strongly suggest that the differential temperature dependencies in the mammalian and teleost NCX are due to important differences in the primary structures of these isoforms. The cloning of the trout cardiac NCX (NCX-TR1) described here represents the first and crucial step in providing a mechanistic explanation of these important physiological differences.

## MATERIALS AND METHODS

*Screening of cDNA library.* PCR was conducted with a pair of degenerate primers to amplify a fragment of NCX from a trout heart Lambda ZAP II cDNA library that we have constructed and described in detail previously (25). The primers were designed by using the highly conserved amino acid sequences of putative transmembrane segments 1 and 3 in NCX1 and NCX2. The sense primer was a 17-mer with 8-fold degeneracy (5′-GCIATGGTNTAYATGTT-3′), and the antisense primer was a 17-mer with 16-fold degeneracy (5′-AYRAACATRTTRAAIGC-3′). The PCR was performed under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 10 s, 50°C for 30 s, and 72°C for 30 s, and then 72°C for 10 min. The PCR product was subcloned into pCR-Script SK(+) (Stratagene, La Jolla, CA). After the sequence was identified as trout heart exchanger on the basis of sequence similarity with NCX1, this fragment was labeled and used as a probe to screen the trout heart cDNA library by using enhanced chemiluminescence random prime labeling and detection systems (Amersham Canada, Oakville, ON, Canada). A full-length clone (T6–1) was isolated, and both strands of the cDNA were sequenced by the dideoxynucleotide chain termination method using ABI's AmpliTaq dye terminator cycle sequencing.

*Expression of trout NCX in Xenopus oocytes.* T6–1 was subcloned from the original pBluescript into a modified vector, in which the multiple cloning site of pBluescript was removed and only *Bam*H I and *Hin*d III sites were left (27). The 3′-untranslated region of T6–1 was replaced with that of the $Na^+$-glucose cotransporter clone, which contains a poly(A)$^+$ tail (24). The *Hin*d III site at nucleotide 2947 of T6–1 was removed by silent mutation with the Quickchange site-directed mutagenesis kit (Stratagene). The mutation was made in a 550-bp cassette generated by *Aat* II digestion. T6–1 was linearized with *Hin*d III, and cRNA was synthesized with the T3 mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Oocytes were prepared as described by Longoni et al. (21). They were injected with 5 nl of cRNA, and exchange activity was measured 3–4 days after injection as $Na^+$ gradient-dependent $^{45}Ca^{2+}$ uptake (28) or as exchange current (see below).

*Assay of $Na^+/Ca^{2+}$ exchange activity.* Outward $Na^+/Ca^{2+}$ exchange currents were measured by the giant excised patch technique, as described previously (31). Borosilicate glass pipettes were pulled and polished to a final inner diameter of ≈20–30 μm and coated with a Parafilm-mineral oil mixture. The vitellin layer was removed, and oocytes were placed in a solution containing (in mM) 100 KOH, 100 MES, 20 HEPES, 5 EGTA, and 5 $MgCl_2$; the pH was maintained at 7.0 at room temperature with MES. Gigaohm seals were formed via suction, and membrane patches (inside-out configuration) were excised by movements of the pipette tip. A computer-controlled, 20-channel solution switcher was used for rapid solution changes. Axon Instruments hardware and software were used for data acquisition and analysis. The pipette solution contained (in mM) 100 *N*-methyl-D-glucamine-MES, 30 HEPES, 30 tetraethylammonium (TEA) hydroxide, 16 sulfamic acid, 8 $CaCO_3$, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, and 0.1 flufenamic acid; the pH was maintained at 7.0 with MES. Outward $Na^+/Ca^{2+}$ exchange currents were activated by switching from intracellular $Li^+$ ($Li_i^+$)- to intracellular $Na^+$ ($Na_i^+$)-based bath solutions containing (in mM) 100 $Na^+$ or $Li^+$ aspartate, 20 MOPS, 20 TEA hydroxide, 20 CsOH, 10 EGTA, 0–7.3 $CaCO_3$, and 1.0–1.13 $Mg(OH)_2$; the pH was maintained at 7.0 with MES or LiOH. $Mg^{2+}$ and $Ca^{2+}$ were adjusted to yield free concentrations of 1.0 mM and 0 or 1 μM, respectively, with MAXC software (3). All experiments were conducted at room temperature (22–23°C).

## RESULTS

*Cloning of trout heart NCX.* To obtain a probe for library screening, we conducted PCR amplification on trout heart cDNA. Degenerate primers were designed on the basis of conserved amino acid sequences of putative transmembrane segments 1 and 3 of the mammalian exchangers NCX1 and NCX2. A PCR product of the appropriate size (~290 bp) was isolated, subcloned into pCR-Script SK(+) and sequenced. Sequence analysis suggested that this cDNA fragment coded for a protein with a high degree of conservation compared with NCX1 and NCX2. The PCR product was used to screen a trout heart cDNA library. After secondary screening, we obtained several positive clones. Partial sequencing indicated one of the clones, T6–1, contained full-length cDNA, and the cDNA was subsequently sequenced in both directions. The complete nucleotide and deduced amino acid sequences of T6–1 are shown in Fig. 1.

*The structure of trout heart NCX.* The T6–1 clone has a nucleotide sequence of 3302 bp with an open reading frame of 2904 bp from base 72 to 2975 encoding a protein of 968 amino acids with a deduced molecular size of 108 kDa, designated NCX-TR1. The nucleotide sequence around the start site, AATCATGA (start codon underlined), is similar to the sequence of the Kozak consensus initiation site (14). No poly(A)$^+$ tail is shown in the 3′-untranslated region. The result of a hydropathy analysis of the deduced amino acids of NCX-TR1 is shown in Fig. 2, which was determined by a modification of the method of Kyte and Doolittle (15).

Like the hydropathy plot of mammalian NCX1, the NCX-TR1 hydropathy plot showed 12 hydrophobic segments and a long hydrophilic region forming an intracellular loop. The first hydrophobic segment is designated a signal peptide by analogy to NCX1. The cleavage site would be between amino acids 32 and 33 (30). Recent evidence (29) indicates that NCX1 has nine transmembrane segments and that two of the hydrophobic segments do not span the membrane. It is likely that NCX1 and NCX-TR1 have similar topological arrangements.

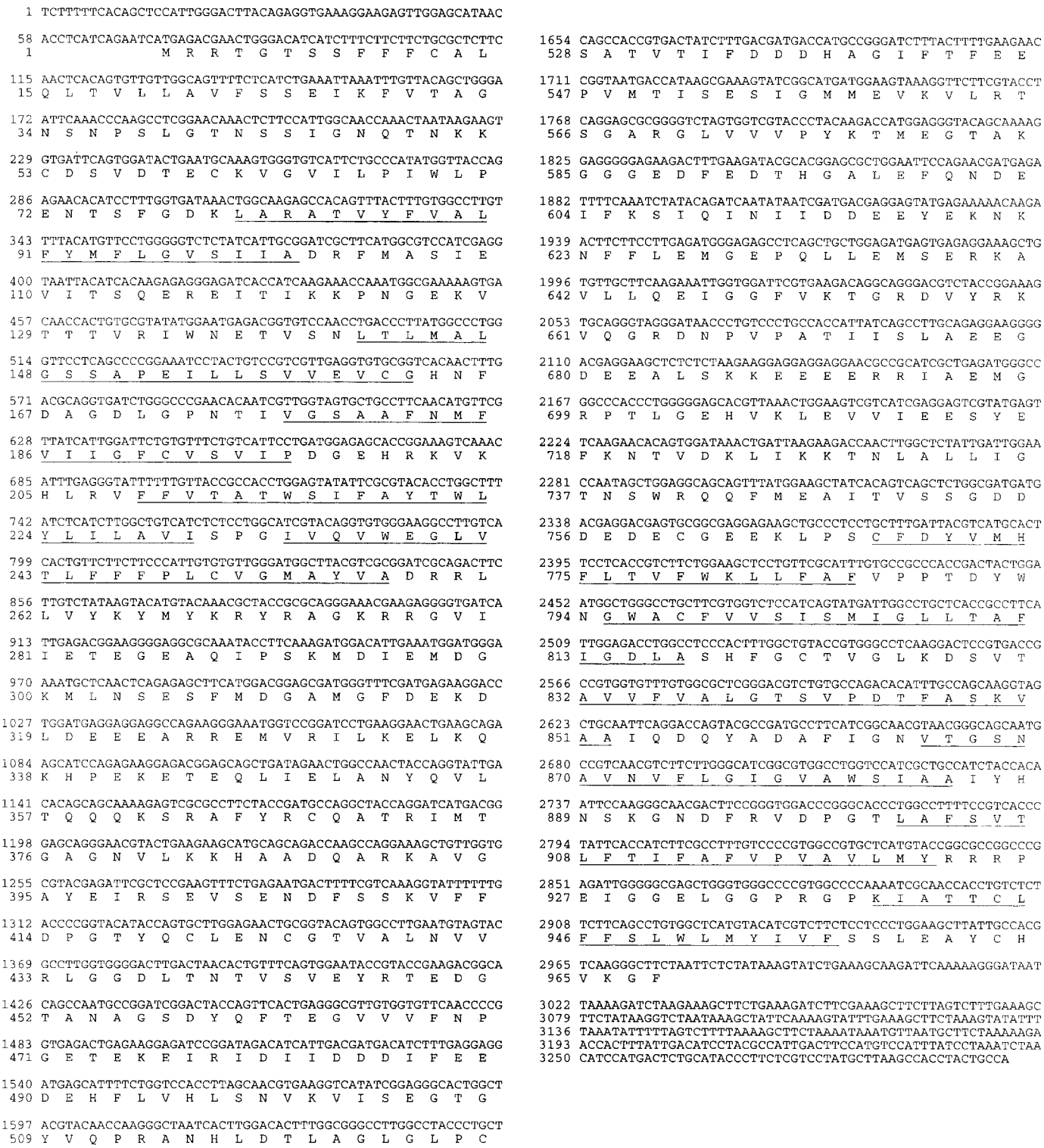

Fig. 1. Nucleotide and deduced amino acid sequences of trout heart $Na^+/Ca^{2+}$ exchanger (NCX). Hydrophobic segments are underlined.

NCX-TR1 has six potential *N*-linked glycosylation sites. Glycosylation occurs at amino acid 40 (asparagine), or the ninth residue after the predicted site for cleavage of the leader peptide, of the mammalian NCX1 (10). Assuming NCX-TR1 undergoes glycosylation similar to that of NCX1, asparagine 42 (residue 10 postcleavage) would be glycosylated; however, this requires experimental confirmation. NCX-TR1 contains 10 potential protein kinase C (PKC) sites, two $Ca^{2+}$/calmodulin-dependent kinase sites, and two tyrosine kinase sites, all shown in Fig. 3.

The sequence comparison of NCX-TR1 and dog NCX1 (28) shows ~75% identity at the amino acid level (Fig.

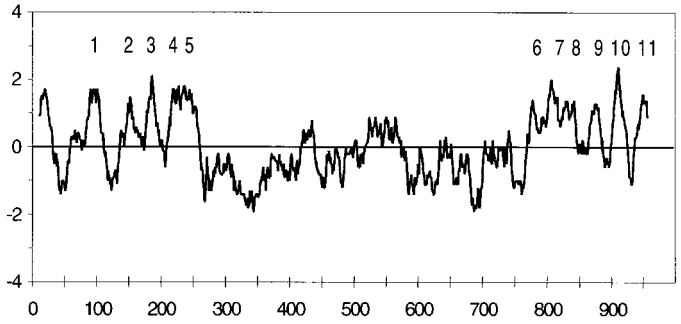


Fig. 2. Hydropathy plot of NCX-TR1. Hydrophobicity is indicated by positive numbers, and hydrophilicity is indicated by negative numbers. Hydrophobic regions are numbered 1–11. The first hydrophobic region is not numbered as it is designated a signal peptide (see text).

3) and 69% identity at the nucleotide level. The hydrophobic segments (especially the α-repeats) are highly conserved. The α-repeats (α1 and α2) are in hydrophobic segments 2–3 and 8–9, respectively, which are involved in the ion binding and translocation of the exchanger (27). The sequences in the vicinity of the two groups of three acidic amino acids are identical and appear to be directly involved in $Ca^{2+}$ binding, thus regulating exchanger activity (19, 23). The exchanger inhibitory peptide (XIP) site associated with $Na^+$-dependent inactivation of the exchanger (20) is also highly conserved. The high level of conservation of these regions suggests that the NCX-TR1 is also regulated by a similar modulation mechanism (7). The most divergent sequence is in the $NH_2$ terminus of the protein. Other sequences with lower levels of homology are scattered in various regions of the intracellular loop besides the region where alternative splicing occurs (13, 17).

The frog cardiac NCX contains a novel exon of 27 bp inserted in the alternative splicing region, which completes a consensus ATP and GTP binding sequence, GXXXXGKS, and it is speculated that this sequence may influence cAMP regulation of exchange activity (12, 36). NCX-TR1, like the mammalian NCX1, does not have this nine-amino acid exon product in the corresponding region, although trout are evolutionarily closer to frogs. A phylogenetic tree is shown in Fig. 4, in which the position of NCX-TR1 based on percent amino acid identity can be seen relative to those of the various mammalian NCX1, NCX2, and NCX3 clones, as well as those of NCX from squid, *Drosophila*, and frogs.

*Functional expression of NCX-TR1 in Xenopus oocytes.* To confirm that a functional trout exchanger had been cloned, NCX-TR1 cRNA was synthesized in vitro and injected into *Xenopus* oocytes. After 3 days of incubation, expression was assessed by measuring $^{45}Ca^{2+}$ uptake. The uptake of $Ca^{2+}$ in the presence of an outwardly directed $Na^+$ gradient was on the average more than an order of magnitude greater than that in the absence of a $Na^+$ gradient (data not shown).

*Exchange current of the NCX-TR1.* Figure 5 shows outward $Na^+/Ca^{2+}$ exchange currents for the NCX-TR1 exchanger expressed in *Xenopus* oocytes. Currents were activated by the application of 100 mM $Na^+$ to the cytoplasmic surface of an excised patch of oocyte membrane. The pipette contained 8 mM $Ca^{2+}$ (extracellular surface). As indicated on the overlapping current traces, records were obtained in the presence or absence of regulatory $Ca^{2+}$ (1 μM) at the cytoplasmic surface. Outward $Na^+/Ca^{2+}$ exchange currents show characteristics similar to those of other mammalian exchangers. That is, both peak and steady-state outward currents were larger in the presence of regulatory $Ca^{2+}$, demonstrating positive regulation of exchange current by intracellular $Ca^{2+}$ ($Ca_i^{2+}$). Also, in response to $Na_i^+$ application, the current peaks and then slowly decays, indicative of $Na_i^+$-dependent inactivation (7). The results shown are representative of those obtained with three additional patches.

Figure 6 shows the current-voltage (*I-V*) relationship for NCX-TR1. Outward $Na^+/Ca^{2+}$ exchange currents were activated by applying 100 mM $Na_i^+$ to the cytoplasmic surface of the patch in exchange for 8 mM pipette $Ca^{2+}$. A series of 10-mV voltage steps from a holding potential of 0 mV were applied from −100 to 100 mV for 20 ms, with a return to the holding potential after each step. This voltage clamp protocol was applied in the presence or absence of $Na^+$ to allow leak subtraction. The graph illustrates the *I-V* relationship obtained from an α-chymotrypsin-treated patch. After treatment of the cytoplasmic surface of the patch with α-chymotrypsin (1 mg/ml) for 1–2 min, the exchange currents were no longer regulated by cytoplasmic $Ca^{2+}$ (data not shown), as has been demonstrated for the NCX1 (7). The *I-V* relationship obtained for NCX-TR1 is similar to that for NCX1.

## DISCUSSION

We have cloned and expressed the trout heart NCX, NCX-TR1. The hydropathy plot is similar to that for mammalian NCX1 and predicts 12 hydrophobic segments and a large hydrophilic domain. The mammalian NCX1 has a cleavable signal peptide that is removed from the protein during biosynthesis in the endoplasmic reticulum (10, 28). A potential cleavage site of NCX-TR1 is predicted to exist between amino acids 32 and 33 on the basis of the sequence homology between mammalian NCX1 and NCX-TR1 and the criteria of Nielsen et al. (30). Thus the topology of NCX-TR1 is not different from that proposed for NCX1, which is now modeled to have nine transmembrane segments (29).

A sequence comparison, including the cleaved leader peptide, showed ~75% identity at the amino acid level to the dog NCX1 and 61 and 66% identity to rat NCX2 and NCX3, respectively. Like all NCXs, NCX-TR1 has the most divergent sequence at the $NH_2$ terminus (40). Sequence identity becomes very high (85%) within the putative transmembrane segments, consistent with their functional significance for ion translocation. The last putative transmembrane segment is the least well conserved transmembrane domain (26). Overall, the amino acid sequence of the intracellular loop is 73% identical to that of NCX1 and, as expected, is less conserved than those of the transmembrane segments.

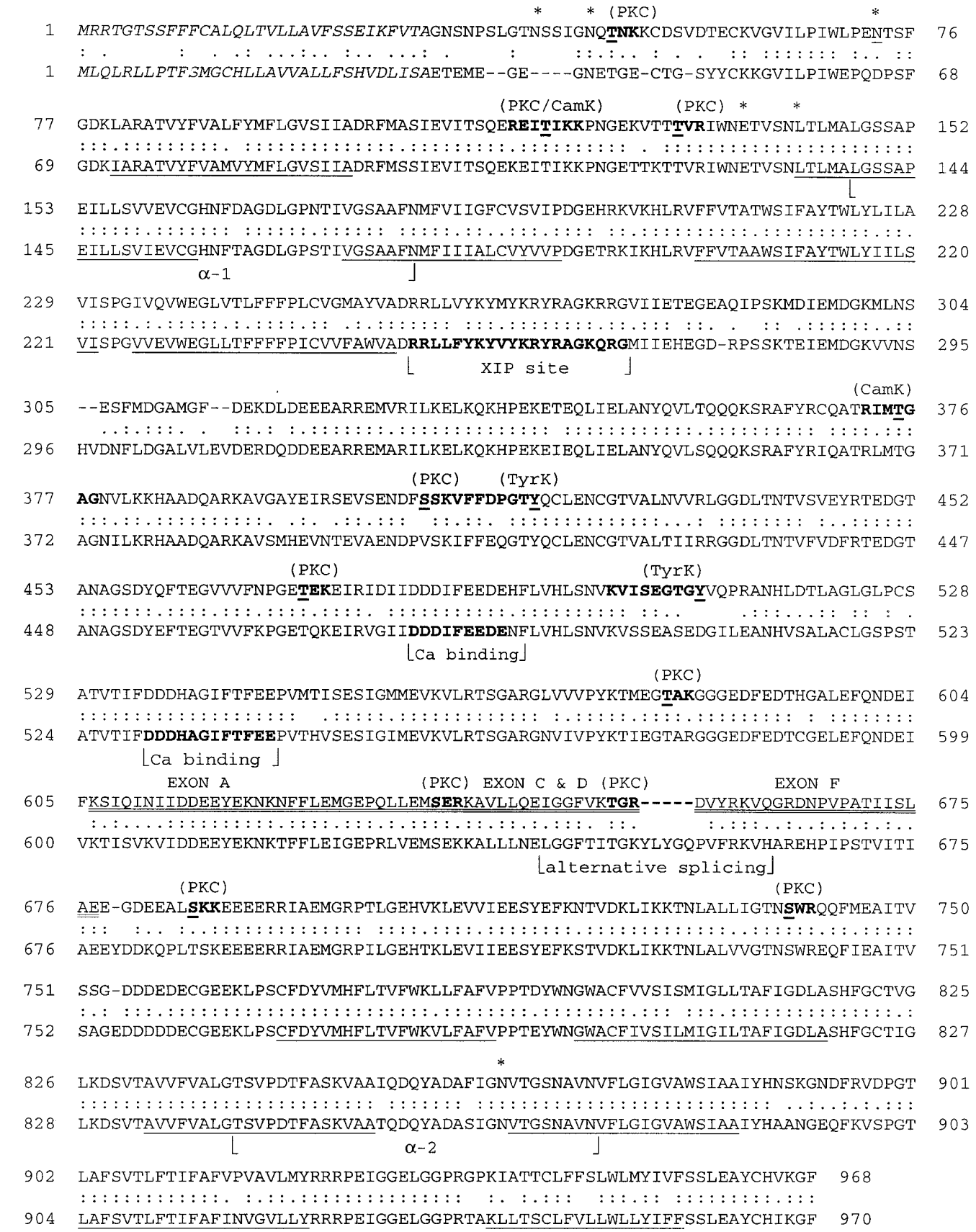


Fig. 3. Amino acid sequence comparison of NCX-TR1 (*upper sequence*) and canine NCX1 (*lower sequence*). Signal sequence is italicized. Two dots indicate identity, and 1 dot indicates conservative amino acid substitution. The 11 hydrophobic segments of NCX1 are underlined. The α-repeats, exchanger inhibitory peptide (XIP) site, $Ca^{2+}$ regulatory binding sites, and alternative splicing sites of NCX1 are also shown. *Potential *N*-linked glycosylation sites of the type NXS/T in NCX-TR1. Potential phosphorylation sites for protein kinase C (PKC), $Ca^{2+}$/calmodulin-dependent kinase (CamK), and tyrosine kinase (TyrK) are indicated with bold letters and the residues to be phosphorylated are underlined. Exons determined from NCX1 are double underlined.

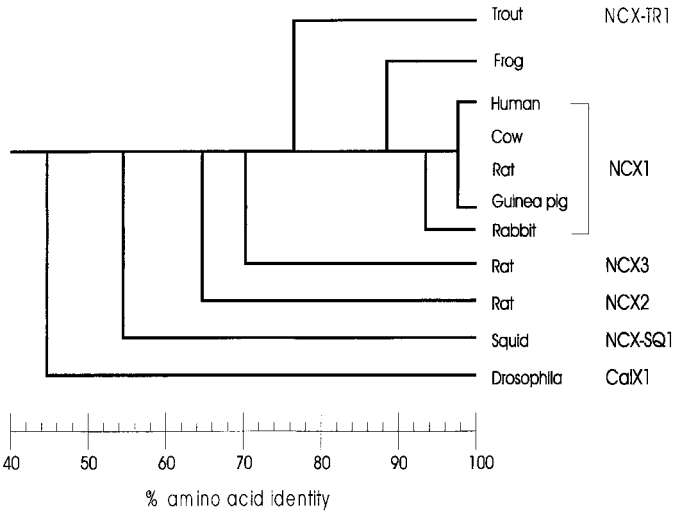


Fig. 4. Phylogeny of NCX. Tree was constructed from percent amino acid sequence identities of all published full-length NCX clones in comparison with dog NCX1.1. Amino acid sequence derived from accession number was first analyzed with Signal P V1.1, which uses algorithms based on criteria of Nielsen et al. (30) to predict cleavage sites (CS) for leader peptide. Remaining sequence was compared with dog NCX1.1 with pairwise sequence alignment tool of Baylor College of Medicine search launcher. Data used in construction of tree are as follows (species/NCX isoform/accession no./cleavage site): dog/NCX1.1/M57523/32, human/NCX1.1/M91368/35, guinea pig/NCX1.1/U04955/32, cow/NCX1.1/L06438/32, rat/NCX1.1/Q01728/32, rabbit/NCX1.3/U52665/32, frog-A/NCX1/X90839/16, frog-B/NCX1/X90838, rat/NCX3/U53420/30, rat/NCX2/U081411/20, squid/NCX-SQ1/U93214/26, *Drosophila*/CalX1/AF009897/20. Frog-A is from genomic DNA, and frog-B is from cardiac cDNA. Full-length clone used in comparison was "constructed" by using genomic section coding for amino acids 1–403 and cDNA section coding for amino acids 404–963.

However, those regions within the loop with known functional importance are well conserved. The endogenous XIP site, consisting of 20 amino acids at the $NH_2$ terminus of the loop, exhibits a high degree of conservation. There is strong evidence that the XIP site of NCX1 is involved in $Na^+$-dependent inactivation (20). The regulatory $Ca^{2+}$ binding domains characterized by three

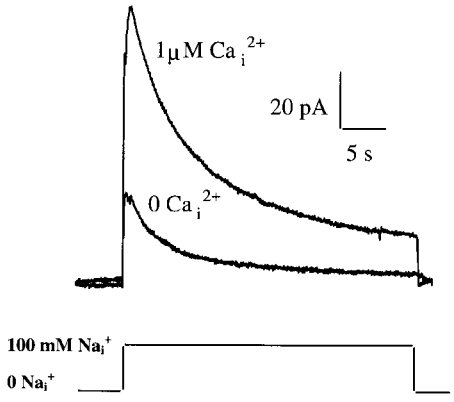


Fig. 5. Representative outward $Na^+/Ca^{2+}$ exchange currents obtained from excised oocyte membrane patch expressing NCX-TR1. Currents were activated by replacing 100 mM $Li^+$ with 100 mM $Na^+$ on cytoplasmic surface of patch. Pipette $Ca^{2+}$ (transported) concentration was 8 mM, and currents were activated in presence or absence of regulatory $Ca^{2+}$ (1 μM) on cytoplasmic surface. $Na_i^+$ and $Ca_i^{2+}$, intracellular $Na^+$ and $Ca^{2+}$, respectively.

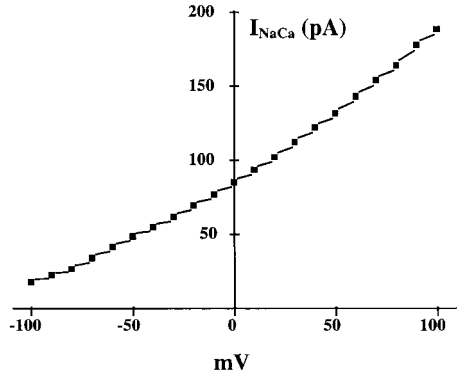


Fig. 6. Leak-subtracted current-voltage relationship for NCX1-TR1. In response to 10-mV voltage steps (−100 to 100 mV) from 0-mV holding potential, outward currents obtained in presence of 100 mM bath $Li^+$ were subtracted from those obtained in presence of 100 mM bath $Na^+$. Voltage was returned to 0 mV between each 20-ms voltage step. To avoid alterations associated with current inactivation, patch was treated with 1 mg/ml α-chymotrypsin for ~1 min. $I_{NaCa}$, $Na^+/Ca^{2+}$ exchange current.

consecutive aspartic acid residues have been found to be highly conserved in the NCX family (6) and are completely conserved in NCX-TR1.

The high degree of conservation of the known regulatory components of the intracellular loop is reflected in the NCX-TR1 giant excised patch current records shown in Fig. 5. The current decay or inactivation seen after peak current requires the presence of the XIP site in mammalian NCX1 (22). The modulation of the NCX current by regulatory $Ca^{2+}$ seen in both NCX1 and NCX-TR1 is dependent on the presence of the $Ca^{2+}$ binding sites of the cytoplasmic loop (23). The regulation of the NCX-TR1 current by $Ca^{2+}$ is positive, making it similar to NCX1 and the opposite of the cloned *Drosophila melanogaster* NCX, CalX, which is characterized by reduced exchange current magnitude in response to increasing cytoplasmic $Ca^{2+}$ (9).

A regulatory role for phosphorylation of the exchanger has been described for squid axons (5) and vascular smooth muscle cells (35). The frog NCX has also been reported to be modulated by cAMP (36). Recently, Iwamoto et al. (11) examined the phosphorylation regulation of mammalian NCX and found that the activities of both NCX1 and NCX3 but not NCX2, when expressed in CCL-39 fibroblasts, are stimulated by a pathway involving PKC. Their results also suggested that the large cytoplasmic loop is required for stimulation, although it may not directly involve phosphorylation. Each of the exchangers contains several consensus sites for phosphorylation by different protein kinases. Potential sites for phosphorylation by PKC, $Ca^{2+}$/calmodulin-dependent kinase, and tyrosine kinases are shown in Fig. 3. NCX-TR1 has one potential tyrosine kinase site, KVISEGTGY (Tyr-509), which is unique in the exchanger family. This site is located next to one of the regulatory $Ca^{2+}$ binding domains, suggesting that phosphorylation may play a role in the $Ca^{2+}$ regulation of exchange activity. Residue Thr-119 in NCX-TR1 could be a potential phosphorylation site of PKC or calmodulin-dependent kinase and is also pres-

ent in NCX3. Because this site is located near the α1 repeat, phosphorylation of this residue may be involved in modulating ion transport (26).

The gene coding for NCX is characterized by a cluster of six exons (A-F) coding for a variable region in the COOH terminus of the large intracellular loop (13). Alternative splicing of these exons generates multiple tissue-specific variants of NCX (17). Exons A and B are mutually exclusive and are used in conjunction with the other four exons (C-F) to produce all NCX isoforms. On the basis of these findings, NCX-TR1 uses exons A and C and D and F (Fig. 3). Compared with NCX1.1 cDNA, which is composed of exons A and C-F (33), NCX-TR1 cDNA apparently lacks exon E, and this accounts for the five-amino acid deletion in the alternate-splicing region.

This initial characterization of NCX-TR1 is an important first step in understanding how the teleost heart functions normally over a range of temperatures (4–15°C) that is debilitating to the mammalian heart. Furthermore, this represents the first nonmammalian heart-specific full-length NCX clone to be expressed and characterized. Although the full-length clones NCX-SQ1 and CalX for squid and *Drosophila* NCXs, respectively, have been expressed, neither is heart specific. The published NCX clone of the amphibian *Xenopus laevis* (12, 36) represents the splicing of frog heart cDNA clones with a genomic clone, with only 93% identity in the 178-amino acid overlap region in the cytoplasmic loop.

Thus knowledge of the sequence and biophysical properties of NCX-TR1 can contribute to our understanding of the evolution of the NCX (as the teleosts are ~400 million years removed from mammalian species) and how proteins in general can evolve to function under hypothermic conditions.

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The NCX-TR1 cDNA sequence described in this paper has been granted accession number AF175313.

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