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Short sequence-paper

Cloning and sequencing of the cDNA encoding for a Na^+/H^+ exchanger from *Xenopus laevis* oocytes (Xl-NHE) [1]

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Abstract

We have cloned and sequenced the cDNA for a Na^+/H^+ exchanger (NHE) from *Xenopus laevis* oocytes. This cDNA contains an open reading frame encoding a protein of 782 amino acids with 12 putative transmembrane domains and a long cytoplasmic tail. The protein exhibits a strong homology at the amino acid level to the human NHE-1 as well as to the βNHE from trout red blood cells: 69% and 58% respectively. Two potential N-linked glycosylation sites at Asn^{56} and Asn^{351} were identified. Three potential protein kinase C phosphorylation sites at the cytoplasmic tail were identified at Ser^{494}, Thr^{726} and Ser^{747}. RT-PCR revealed the expression of the Xl-NHE in *Xenopus* heart, reticulocytes and skeletal muscle.

Keywords: Na^+/H^+ exchange; *X. laevis*; Oocyte; Cloning; cDNA

The Na^+/H^+ exchangers (NHEs) belong to a family of plasma membrane proteins which catalyze the electroneutral exchange of extracellular Na^+-ions for intracellular H^+-ions. They mediate regulation of intracellular pH as well as the maintenance of cell volume. In epithelial cells such as intestine and renal tubule cells, Na^+/H^+ exchangers are also involved in Na^+ absorption. NHEs have been found in vertebrate cells [1], invertebrate cells [2], yeast [3] and in prokaryotes [4]. Existence of multiple isoforms of NHEs was initially suggested based on pharmacological, kinetic and immunological studies, and up to now five mammalian isoforms, referred to NHE-1 to NHE-5 [1,5–8], and one isoform from nucleated trout red blood cells, termed βNHE, [9] have been cloned. Under basal conditions the net direction of the cation exchange process is dictated by the prevailing concentration gradients for Na^+-ions and H^+-ions. The rate of transport is primarily controlled by intracellular H^+-ions which are not only transported but also mediate allosteric activation of the NHEs. In addition, the activity of NHE-1 is modulated by a variety of hormones and growth factors which can induce a persistent rise in cytoplasmic pH [10]. It is generally accepted that the phospholipase C-PKC pathway constitutes one route for NHE activation, with the notable exception of the NHE-3, which is inhibited by phorbol ester activated PKC [11]. In

Abbreviations: NHE, Na^+/H^+ exchanger; Xl-NHE, *X. laevis*-Na^+/H^+ exchanger; PKA, protein kinase A; PKC, protein kinase C; kb, kilobases; SSC, sodium chloride/sodium citrate; RT-PCR, reverse transcription-polymerase chain reaction

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[1] The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank and DDBJ NucleotideSequence Database under the Accession number Y08635.

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contrast, the data on regulation of mammalian NHEs by PKA-dependent phosphorylation are rather variable and only the βNHE from trout red blood cells is reported to be activated by agents causing an increase in intracellular cAMP concentration [12]. Recently, we characterized the regulation of the endogenous Na^+/H^+ exchanger in *X. laevis* oocytes and the data indicated that it is stimulated by both PKC- and/or PKA-dependent mechanisms, thus resembling the regulation of the βNHE of trout red cells [13]. We report here the isolation and characterization of a cDNA clone encoding a Na^+/H^+ exchanger from *X. laevis* oocytes termed Xl-NHE.

Total RNA was prepared from *X. laevis* oocytes by the method using guanidinethiocyanate [14] and poly(A)$^+$ RNA was isolated using Dynabeads mRNA Direct Kit (Dynal, Oslo, Norway) as described by the manufacturer. A 'Dumont stage VI' *X. laevis* oocyte cDNA library was screened with a mixture of six different oligonucleotides corresponding to the highly conserved amino acid regions between the transmembrane domains IV and Vb of the human NHE-1 (P1 sense 1110-1129, P2 sense 1216-1235, P3 sense 1452-1471, P4 antisense 1559-1578, P5 antisense 1190-1209 and P6 antisense 1375-1399). The oligonucleotides were labelled at their 5′-ends with T4 polynucleotidekinase and [γ-^{32}P]ATP, and probed to 250 000 independent lysis plaques. Hybridization was performed at 42°C in a buffer containing 6 × SSC, 1 × Denhardt's, 0.05% sodiumpyrophosphate and yeast tRNA (100 μg/ml). Washings were done at 40°C in a buffer containing 6 × SSC and 0.05% sodiumpyrophosphate for 20–30 min. Eight positive phage clones were further purified through three rounds of consecutive screenings. Purified λgt10 DNA from these clones was amplified by PCR using λ-phage specific primers with *Eco*RI restriction sites (Primer λ1 5′-GAATTCAGCAAGTTCAGCCTGGTTAAG-3′ and Primer λ2 5′-GAATTCTTATGAGTATTTCTTCCAGGG-3′). The PCR cycles consisted of 30 s denaturation at 95°C, 30 s annealing at 62°C, and 90 s extension at 72°C for a total of 35 cycles. Inserts > 2.2 kb were subcloned into the *Eco*RI sites of the pBluescript SK$^+$ vector (Stratagene). To study tissue-specific expression 2 μg of total RNA from *Xenopus* heart, muscle and reticulocytes were reverse-transcribed using Superscript™ reverse transcriptase (Gibco, BRL). The synthetized cDNAs were subjected to PCR amplification using Xl-NHE-specific primers (conditions, see above). The resulting fragments were subcloned into the *Eco*RI sites of the pBluescript SK$^+$ vector and identified by direct sequencing. DNA sequencing was performed on both strands by the dideoxy chain termination technique using an automatic sequencer (Applied Biosystems).

Of 250 000 plaques screened, eight positives were obtained and one was > 3 kb of length. The sequence of this clone contains 3004 bp with 499 bp 5′-untranslated region, 2346 bp of an open reading frame, and 159 bp of 3′-untranslated region. Four out-of-frame minicistrons were identified in the 5′-untranslated region at positions −319, −206, −136, and −49 respectively. No polyadenylation signals could be identified in the 3′-untranslated region.

The designated ATG initiation codon of Xl-NHE (ATAATGG) is in agreement with Kozak's consensus sequence [15] for translation initiation (A/GCCATGG). There was no other in-frame initiation codon further upstream or downstream in the cDNA which fits better. The amino acid sequence deduced from the longest open reading frame of Xl-NHE cDNA predicts a protein of 782 amino acids with a calculated M_r of 87 814. Fig. 1 displays the alignment of the complete Xl-NHE sequence to the human NHE-1 sequence. Overall, the Xl-NHE exhibits 69% amino acid identity with NHE-1 and 58% identity to βNHE (not shown), respectively. The hydrophobicity plot of Xl-NHE is very similar to those of the human NHE-1 and the trout βNHE and predicts 12 putative transmembrane domains according to Kyte and Doolittle [16] and a long cytoplasmic tail. The N-terminus represents a highly conserved domain and is responsible for cation exchange, whereas the C-terminus is more divergent among NHEs. The first transmembrane domains and cytoplasmic loops of the Xl-NHE and NHE-1 are completely different between the two species, suggesting that the conservation of this domain is not required for the antiport-function. The essential glutamate for sodium binding and transport is conserved in Xl-NHE (at position 243) as well as in the whole NHE-family [17]. Like other NHEs, Xl-NHE is likely a glycoprotein and potential N-linked glycosylation sites were found at positions Asn^{56} and Asn^{351}. These corresponds to the positions Asn^{75} and Asn^{370} in the NHE-1 sequence. Recently it was demonstrated by

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XL-NHE:                                                M G K K V    5
NHE-1:   M V L R S G I C G L S P H R I F P S L L V V V   23

XL-NHE:   F L N Y C G Y I F I L A L I P I V F S E S S Y   28
NHE-1:    A L V G L L P V L R S H G L Q L S P T A S T I   46

XL-NHE:   L L N P V T L A S R T S T I P L M I S N M N R   51
NHE-1:    R S S E P P R E R S I G D V T T A P P E V T P   69

XL-NHE:   H D S T N H S Q G G G H H G K K K A F P V L D   74
NHE-1:    E S R P V N H S V T D H G K P R K A F P V L G   92

XL-NHE:   L D Y T R I R M P F E I A L W I L L A S L M K   97
NHE-1:    I D Y T H V R T P F E I S L W I L L A C L M K  115

XL-NHE:   L G F H V I P T L S N V V P E S C L L I V V G  120
NHE-1:    I G F H V I P T I S S I V P E S C L L I V V G  138

XL-NHE:   L L V G G L I K A V G E T P P V L N S D V F F  143
NHE-1:    L L V G G L I K G V G E T P P F L Q S D V F F  161

XL-NHE:   L F L L P P I I L D A G Y F L P L R P F S E N  166
NHE-1:    L F L L P P I I L D A G Y F L P L R Q F T E N  184

XL-NHE:   L G T I L M F A V V G T L W N A F F L G S L L  189
NHE-1:    L G T I L I F A V V G T L W N A F F L G G L M  207

XL-NHE:   F A V C Q I G G E D L R N V G L L A N L L F G  212
NHE-1:    F A V C L V G G E Q I N N I G L L D N L L F G  230

XL-NHE:   S I I S A V D P V A V L A V F E E I H I N E L  235
NHE-1:    S I I S A V D P V A V L A V F E E I H I N E L  253

XL-NHE:   L H I L V F G E S L L N D A V T V V L Y H L F  258
NHE-1:    L H I L V F G E S L L N D A V T V V L Y H L F  276

XL-NHE:   E E Y A S L E Q I T F R D I S L G F L S F L V  281
NHE-1:    E E F A N Y E H V G I V D I F L G F L S F F V  299

XL-NHE:   V A L G G V F V G L V Y G I I A A F T S R F T  304
NHE-1:    V A L G G V L V G V V Y G V I A A F T S R F T  322

XL-NHE:   S H I R V I E P L F V F L Y S Y M A Y L S A E  327
NHE-1:    S H I R V I E P L F V F L Y S Y M A Y L S A E  345

XL-NHE:   L F H L S G I M A L I A T G V V M R P Y V E A  350
NHE-1:    L F H L S G I M A L I A S G V V M P P Y V E A  368

XL-NHE:   N I S H K S H T T I K Y F L K M W S S V S E T  373
NHE-1:    N I S H K S H T T I K Y F L K M W S S V S E T  391

XL-NHE:   L I F I F L G V S T V A G P H K W N W P Y V I  396
NHE-1:    L I F I F L G V S T V A G S H H W N W T F V I  414

XL-NHE:   S T L I F C F V S R V L G V L G L Q W F L N K  419
NHE-1:    S T L L F C L I A R V L G V L G L T W F I N K  437

XL-NHE:   F R I V K L T P K D Q F I I A Y G G L R G A I  442
NHE-1:    F R I V K L T P K D Q F I I A Y G G L R G A I  460
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Fig. 1.

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XL-NHE:   A F S L G Y L L D S N H F P R - E M F L I A I  464
NHE-1:    A F S L G Y L L D K K H F P M C D L F L T A I  483

XL-NHE:   I T V I F F T V F V Q G M T I R P L V E L L A  487
NHE-1:    I T V I F F T V F V Q G M T I R P L V D L L A  506

XL-NHE:   V K K K Q E S K R S I N E E I H S E F L D H L  510
NHE-1:    V K K K Q E T K R S I N E E I H T Q F L D H L  529

XL-NHE:   L T G I E D I C G H Y G H H H W K D K L N R F  533
NHE-1:    L T G I E D I C G H Y G H H H W K D K L N R F  552

XL-NHE:   N K T Y V K K C L I A G E R S T E P Q L I A F  556
NHE-1:    N K K Y V K K C L I A G E R S K E P Q L I A F  575

XL-NHE:   Y H K M E M K Q A I E L V E S G G I G K I P S  579
NHE-1:    Y H K M E M K Q A I E L V E S G G M G K I P S  598

XL-NHE:   T V S T V S M Q N I Q P K P K L A E R F I P T  602
NHE-1:    A V S T V S M Q N I H P K S L P S E R I L P A  621

XL-NHE:   L S K G K E E E I R K I L R T N L Q K T R Q R  625
NHE-1:    L S K D K E E E I R K I L R N N L Q K T R Q R  644

XL-NHE:   L R S Y N R H T L V A D P Y E D A W N Q M L L  648
NHE-1:    L R S Y N R H T L V A D P Y E E A W N Q M L L  667

XL-NHE:   R R Q K V H Q M E Q R V T N Y L T V P A H K L  671
NHE-1:    R R Q K A R Q L E Q K I N N Y L T V P A M K L  690

XL-NHE:   D S P T M S R A R V G S D P L A Y E P K P D S  694
NHE-1:    D S P T M S R A R I G S D P L A Y E P K E D L  713

XL-NHE:   E N I P T I T I D P A S - - - P E S V D T V N  714
NHE-1:    P V - - - I T I D P A S P Q S P E S V D L V N  733

XL-NHE:   E T Q E E E E G I T M T V R E P P S P G A D D  737
NHE-1:    E E L K - - - G - K V L G L S R D P A K V A E  752

XL-NHE:   V F I P Q E S P N S Q R L Q R C L S D P G P H  760
NHE-1:    E D E D D D D G G I M M R S K E T S S P G T D D  775

XL-NHE:   P E E I Q P K I P L K K K L I D F V K G L       782
NHE-1:    V F T P A P S D S P S S Q R I Q R C L S D P G   798

XL-NHE:
NHE-1:    P H P E P G E G E P F F P K G Q                814
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Fig. 1. Alignment of the amino acid sequences of the human growth factor-activatable Na^+/H^+ exchanger (NHE-1) and the *Xenopus* oocyte Na^+/H^+ exchanger (Xl-NHE). The 12 putative membrane-spanning domains are underlined. The N-linked glycosylation sites are in bold letters. The potential PKC-consensus sites are in italics.

immunoblot analysis and *N*-glycosidase F treatment that only the first of these two sites is indeed glycosylated in NHE-1 [18]. In vivo, protein kinase C exhibits a preference for the phosphorylation of Ser or Thr residues close to a C-terminal basic residue [19]. Three of such putative phosphorylation sides were identified in the primary structure of Xl-NHE at positions Ser^{494}, Thr^{726} and Ser^{747} respectively. Although we observed an increase in Xl-NHE activity after treatment of oocytes with forskolin or cAMP [13], the primary structure of the C-terminal domain does not contain typical sites for phosphorylation by PKA (RRXS) [20]. This suggests that Xl-NHE stimulation via cAMP-dependent pathways is not due to a direct phosphorylation of the protein by PKA. This notion as well as more detailed characterization of regulatory pathways for Xl-NHE require further investigations. Subsequently, we analyzed the expression of the Xl-NHE in heart, reticulocytes, and skeletal muscle by RT-PCR of cDNA using Xl-NHE-specific primers. The resulting PCR products (468 bp, Fig. 2) were subcloned and identified as Xl-NHE-specific fragments by direct sequencing. Thus, the Xl-NHE cloned from oocytes is also expressed in these cells and tissues.

In conclusion, we have determined the complete nucleotide sequence of the cDNA coding for the *X. laevis* Na^+/H^+ exchanger. Further experiments regarding the transport kinetics as well as regulatory pathways for Xl-NHE, are necessary because pH regulation is an important process during oocyte maturation and fertilization [21].

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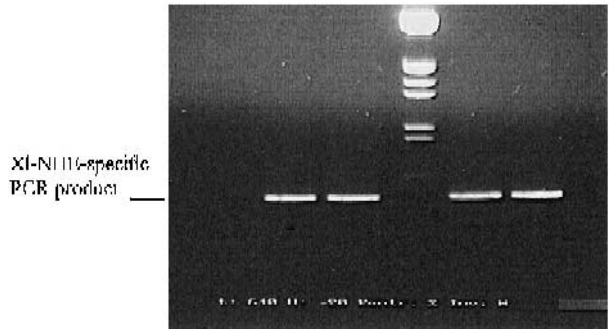

Fig. 2. RT-PCR from *Xenopus* heart (lane 1), reticulocytes (lane 2), skeletal muscle (lane 4) and ovary (lane 5). Lane 3: λ*Hin*dIII/*Eco*RI marker (from left to right).

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