



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 β NHE from trout red blood cells: 69% and 58% respectively. Two potential N-linked glycosylation sites at Asn⁵⁶ and Asn³⁵¹ were identified. Three potential protein kinase C phosphorylation sites at the cytoplasmic tail were identified at Ser⁴⁹⁴, Thr⁷²⁶ and Ser⁷⁴⁷. RT-PCR revealed the expression of the XI-NHE in *Xenopus* heart, reticulocytes and skeletal muscle. © 1997 Elsevier Science B.V. All rights reserved.

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 β NHE, [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; XI-NHE, X. *lae-vis*-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 β NHE 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 β NHE 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 $[\gamma^{-32}P]ATP$, and probed to 250 000 independent lysis plaques. Hybridization was performed at 42°C in a buffer containing $6 \times SSC$, $1 \times Denhardt's, 0.05\%$ sodiumpyrophosphate and yeast tRNA (100 μ g/ml). Washings were done at 40°C in a buffer containing $6 \times$ SSC and 0.05% sodiumpyrophosphate for 20-30 min. Eight positive phage clones were further purified through three rounds of consecutive screenings. Purified $\lambda gt10$ DNA from these clones was amplified by PCR using λ -phage specific primers with *Eco*RI restriction sites (Primer λ 1 5'-GAATTCAGCAAGTTCAGCCTGGT-TAAG-3' and Primer $\lambda 2$ 5'-GAATTCTTATGAG-TATTTCTTCCAGGG-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 μ g 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 XI-NHEspecific 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 XI-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 XI-NHE cDNA predicts a protein of 782 amino acids with a calculated M_r of 87814. Fig. 1 displays the alignment of the complete XI-NHE sequence to the human NHE-1 sequence. Overall, the XI-NHE exhibits 69% amino acid identity with NHE-1 and 58% identity to β NHE (not shown), respectively. The hydrophobicity plot of XI-NHE is very similar to those of the human NHE-1 and the trout β NHE 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 XI-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 XI-NHE (at position 243) as well as in the whole NHE-family [17]. Like other NHEs, XI-NHE is likely a glycoprotein and potential N-linked glycosylation sites were found at positions Asn⁵⁶ and Asn³⁵¹. These corresponds to the positions Asn⁷⁵ and Asn³⁷⁰ in the NHE-1 sequence. Recently it was demonstrated by

XL-NHE: NHE-1:	M	V	L	R	s	G	I	С	G	L	s	Р	Н	R	I	F	Р	s	M L	G L	к V	K V	v v	5 23
XL-NHE: NHE-1:	F A	L L	N V	Y G	C L	G L	Y P	r V	F L	I R	L S	A H	L G	I L	P Q	I L	V S	F P	S T	E A	s s	s T	Y I	28 46
XL-NHE: NHE-1:	L R	L S	N S	P E	V P	T P	L R	A E	S R	R S	T I	S G	T D	I V	P T	L T	M A	I P	S P	N E	M V	N T	R P	51 69
XL-NHE: NHE-1:	H E	D 5	s R	T P	N V	H N	S H	Q S	G V	G T	G D	H H	H G	G K	K P	K R	K K	A A	F F	P P	V V	$_{\rm L}^{\rm L}$	D G	74 92
XL-NHE: NHE-1:	L I I	0	Y Y	T T	R H	I V	R R	M T	P P	F F	E E	I I	A S	L L	W W	I	L L	L L	A A	s C	L L	M M	K	97 115
XL-NHE: NHE-1:		3	F F	H H	V	I	P P	T T	L I	S S	N S	V I	v v	<u>Р</u> Р	E E	S S	C C	L L	L L	I	V V	V V	G G	120 138
XL-NHE: NHE-1:	<u>L</u> L	<u>[.</u> [V V	G G	G G	L L	I	K K	A G	V V	G G	E E	T T	P P	P P	V F	L L	N Q	S S	D D	V V	F	F	143 161
XL-NHE: NHE-1:	L L	F	L L	L L	P P	P P	I	I I	L	D D	A A	G G	Y Y	F F	L L	P P	L L	R R	P Q	F F	S T	E	N N	166 184
XL-NHE: NHE-1:	L (L (5	T T	I	L L	M	F	A A	V V	V V	G G	T T	L L	W	N N	A	F F	F F	L L	G G	S G	L L	L M	189 207
XL-NHE: NHE-1:	F F	A A	v v	c c	Q L	I V	G G	G G	E E	D Q	L I	R N	N N	V I	G G	L L	L L	A D	N N	L L	L L	F F	G G	212 230
XL-NHE: NHE-1:	<u>S</u>	ľ Ľ	I I	S S	A A	V V	D D	P P	V V	A A	V V	L L	A A	V V	F F	E E	E E	I I	H H	I I	N N	E E	L L	235 253
XL-NHE: NHE-1:	L I L I	H	I	L L	V V	F F	G G	E	S S	L L	L L	N N	D D	A A	V V	T T	V V	V V	L	Y Y	H H	L L	F	258 276
XL-NHE: NHE-1:	E E	E	Y F	A A	s N	L Y	E E	Q H	I V	T G	F I	R V	D D	I I	S F	L L	G G	F F	L L	S	F F	L F	V	281 299
XL-NHE: NHE-1:	V V	ð ð	L L	G G	G G	V V	F L	V	G G	L V	V V	Y Y	G G	I V	I	A A	A A	F F	T T	S S	R R	F F	$_{\mathrm{T}}^{\mathrm{T}}$	304 322
XL-NHE: NHE-1:	S I S I	ł	I	R R	V V	I I	E E	P P	L L	F F	V V	F F	L L	Y Y	S S	Y Y	M M	A A	Y Y	L L	<u>S</u>	A A	E	327 345
XL-NHE: NHE-1:	L	F	H H	L L	S S	G G	I	M M	A A	L L	I	A A	T S	G G	V V	V V	M M	R P	P P	Y Y	v v	E E	A A	350 368
XL-NHE: NHE-1:	N I N I	C	s s	H H	K K	s s	H H	T T	T T	I I	K K	Y Y	F F	Ĺ L	K K	<u>M</u> M	W W	s s	s s	V V	S S	E E	T	373 391
XL-NHE: NHE-1:	L L	[[F F	I I	F F	L L	G G	V V	S S	T T	V V	A	G G	P S	H H	K H	W W	N N	W W	P T	Y F	V V	I	396 414
XL-NHE: NHE-1:	s s		L L	I L	F F	c c	F L	V I	S A	R R	V V	L L	G G	V V	L L	G G	L L	Q T	W W	F F	L I	N N	K K	419 437
XL-NHE: NHE-1:	F I F I	२ २	I	V V	K K	L L	T T	P P	K K	D D	Q	F	I I	I I	A A	Y Y	G G	G G	L L	R R	G G	A A	I I	442 460

Fig. 1.

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 X1-NHE at positions Ser⁴⁹⁴, Thr⁷²⁶ and Ser⁷⁴⁷ respectively. Although we observed an increase in XI-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 in-

XL-NHE: NHE-1:	A A	F F	S	L L	G Ģ	Y Y	L L	L L	D D	S K	N K	H H	F F	P P	R M	c'	E D	M L	F F	L	I T	A A	I	464 483	
XL-NHE: NHE-1:	I	T T	V V	I	F F	F F	T T	V V	F	V V	0	G G	M M	T T	I	R R	P P	L L	V V	E D	L L	L L	A A	487 506	
XL-NHE: NHE-1:	v v	K K	K K	K K	Q Q	E E	S T	K K	R R	S S	I I	N N	E E	E E	I I	H H	S T	E Q	F F	L L	D D	H H	L L	510 529	
XL-NHE: NHE-1:	L L	T T	G G	I	E E	D D	I	C C	G G	H H	Y Y	G G	H H	H H	H H	W W	K K	D D	K K	L L	N N	R R	F F	533 552	
XL-NHE: NHE-1:	N N	K K	T K	Y Y	V V	K K	K K	C C	L L	I I	A A	G G	E E	R R	S S	T K	E E	P P	Q Q	L L	I	A A	F F	556 575	
XL-NHE: NHE-1:	Y Y	H H	K K	M M	E E	M M	K K	Q Q	A A	I I	E E	L L	V V	E E	S S	G G	G G	I M	G G	K K	I I	P P	s s	579 598	
XL-NHE: NHE-1:	T A	V V	S S	T T	V V	S S	M M	Q Q	N N	I I	Q H	P P	K K	P S	K L	L P	A S	E E	R R	F I	I L	P P	T A	602 621	
XL-NHE: NHE-1:	L L	S S	K K	G D	K K	E E	E E	E E	I I	R R	K K	I	L L	R R	T N	N N	L L	Q Q	K K	T T	R R	Q Q	R R	625 644	
XL-NHE: NHE-1:	L L	R R	S S	Y Y	N N	R R	H H	T T	L L	V V	A A	D D	P P	Y Y	E E	D E	A A	W W	N N	Q Q	M M	$_{\rm L}^{\rm L}$	L L	648 667	
XL-NHE: NHE-1:	R R	R R	Q Q	K K	V A	H R	Q Q	M L	E E	Q Q	R K	V I	T N	N N	Y Y	L L	T T	V V	P P	A A	H M	K K	L L	671 690	
XL-NHE: NHE-1:	D D	S S	P P	Т	M M	S S	R R	A A	R R	V I	G G	S S	D D	P P	L L	A A	Y Y	E E	P P	K K	P E	D	S L	694 713	
XL-NHE: NHE-1:	E P	N V	1 -	Р -	т -	I	T T	I I	D D	P P	A A	S S	- P	- Q	s	P P	E E	S S	V V	D D	T L	V V	N N	714 733	
XL-NHE: NHE-1:	E E	T E	Q L	E K	E -	E -	E -	G G	I -	T K	M V	TL	V G	R L	E S	P R	P D	S P	P A	G K	A V	D A	D E	737 752	
XL-NHE: NHE-1:	V E	F D	I E	P D	Q D	E D	S G	P G	N I	S M	Q M	R R	L S	Q K	R E	C T	L S	S S	D P	P G	G T	P D	H D	760 775	
XL-NHE: NHE-1:	P V	E F	E T	I P	Q A	P P	K S	I D	P S	L P	K S	K S	K Q	L R	I	D Q	F R	V C	K L	G S	L D	Ρ	G	782 798	
XL-NHE: NHE-1:	Р	н	Ρ	Е	P	G	Е	G	Е	Р	F	F	P	К	G	Q								814	

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 (XI-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.

vestigations. Subsequently, we analyzed the expression of the XI-NHE in heart, reticulocytes, and skeletal muscle by RT-PCR of cDNA using XI-NHEspecific primers. The resulting PCR products (468 bp, Fig. 2) were subcloned and identified as XI-NHE-specific fragments by direct sequencing. Thus, the XI-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 XI-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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