# Alternative Splicing of NHE-1 Mediates Na-Li Countertransport and Associates With Activity Rate

**Gianpaolo Zerbini,[1] Anna Maestroni,[1] Diego Breviario,[2] Ruggero Mangili,[1] and Giorgio Casari[3]**

**Sodium-lithium countertransport (SLC) is an ouabain-insensitive exchange of Na for Li found in the erythrocyte membrane of several mammalian species. Although increased SLC activity is presently the most consistent intermediate phenotype of essential hypertension and diabetic nephropathy in humans, the gene responsible for this membrane transport has not been identified. Because of functional similarities, SLC was suggested to represent an in vitro mode of operation of the Na-H exchanger (NHE). This hypothesis, however, has been long hampered by the total insensitivity of SLC to amiloride, which is an intrinsic inhibitor of the first isoform of NHE, the only NHE isoform detected in human erythrocytes. We describe here the identification in human reticulocytes and erythrocytes of an alternative splicing of NHE lacking the amiloride binding site. Transfection experiments with this spliced variant restore amiloride-insensitive, phloretin-sensitive SLC activity. Expression of both regular and spliced transcripts of NHE is increased in subjects with high SLC activity. Altogether, these findings, by extending to NHE the characteristics of inheritance and predictivity previously attributed to SLC, eventually restore the candidacy of NHE isoform 1 as a gene involved in the pathogenesis of essential hypertension and diabetic nephropathy. *Diabetes* 52:1511–1518, 2003**

Elevated activity of erythrocyte sodium-lithium countertransport (SLC) is presently the most consistent intermediate phenotype of essential hypertension (1,2) and diabetic nephropathy (3,4). Genetic epidemiology studies have suggested that the activity of this membrane transport can be explained by inheritance more than environmental factors when a major gene and polygenic effects are taken into account (5,6). Recent evidence indicates that an increased activity of SLC predicts the development of both essential hypertension (7,8) and diabetic nephropathy (9).

Although SLC is an established property of the erythrocyte of humans and several other animal species, where it mediates a bidirectional one-to-one exchange of Na for Li (10,11), its nature and role in human physiology are still unknown. Studies of Na flux across the membrane have suggested that Na-H exchanger (NHE)—a membrane transporter present in virtually every animal cell involved in regulation of intracellular pH, cell volume, and cell proliferation (12)—may also exchange Li for Na, which has contributed to the hypothesis that SLC may reflect an operational mode of this system (13). In line with this hypothesis, increased NHE activity was subsequently demonstrated in patients affected by essential hypertension (14,15) and diabetic nephropathy (16,17).

A major argument against the common nature of SLC and NHE is, however, represented by their different response to inhibitors. Sensitivity to amiloride, although at different degrees, is an invariable feature of NHE isoforms (18,19), whereas SLC is totally insensitive to this drug (20).

We have recently provided functional evidence that SLC activity might be mediated by an as yet unidentified, amiloride-insensitive NHE isoform (21,22). Although only the amiloride-sensitive first isoform of NHE (NHE-1) has been identified in human erythrocytes (23), the possibility that an otherwise unrecognized, amiloride-insensitive NHE isoform might characterize the membrane of the erythrocyte cannot be ruled out. With the present study, we demonstrate that an alternative splicing of NHE-1 (DNHE-1) mediates SLC and that its expression correlates with SLC activity.

## RESEARCH DESIGN AND METHODS

**RNA extraction.** Human erythrocytes and reticulocytes were separated by double centrifugation through Ficoll-Hypaque (24). Total RNA was extracted from human erythrocytes and reticulocytes and from transfected and non-transfected PS200 cells according to the guanidine thiocyanate method (25), and cDNA was retrotranscribed using SuperScript II Reverse Transcriptase (Gibco, Gaithersburg, MD).

**RT-PCR.** RT-PCR primers were designed along the sequence of NHE-1 (GenBank AC S68616) as follows: NHE-1.1, position 534–556 F (forward), 5′-ACTCTCATTTGGGTAAGGTCGA-3′; NHE-1.2, position 1098–1118 R (reverse), 5′-CCGCAGTGGCAGGAAGTAGC-3′; NHE-1.3, position 1066–1084 F, 5′-CTTCCTGCTGCCGCCCAT-3′; NHE-1.4, position 1656–1676 R, 5′-ATAGG GGCGCATCACCACTC-3′; NHE-1.5, position 1610–1630 F, 5′-GCCGAGCTCT TCCACCTGTC-3′; NHE-1.6, position 2106–2126 R, 5′-GCGCTTCGTCTC TTGCTTTT-3′; NHE-1.7, position 2020–2042 F, 5′-CACTGCCATCATCA CTGTCATC-3′; NHE-1.8, position 2653–2672 R, 5′-CCGAGACATGGTGGGT GAG-3′; NHE-1.9, position 2508–2527 F, 5′-AGCGGCTGCGGTCCTACAA-3′; NHE-1.10, position 3201–3221 R, 5′-TCTGGTGGGGAGGATGCTTC-3′.

**Expression constructs.** Full length NHE-1 (kind gift of J. Pouyssegur and L. Counillon, Universitè de Nice, Nice, France) was cloned into pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA) to obtain pcNHE-1. pcNHE-1 was then mutagenized by replacing the *Bst*ZI–*Csp*45I fragment (spanning nucleotides 715-1281) of NHE-1 DNA with the corresponding fragment of the alternative spliced form, lacking nucleotides 809–1105 and therefore obtaining the expression construct pcDNHE-1.

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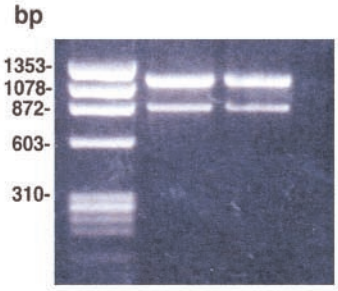
DMA, dimethyl-amiloride; DNHE, alternative splicing of NHE-1; NHE, Na-H exchanger; NHE-1, first isoform of NHE; SLC, sodium-lithium countertransport.

A

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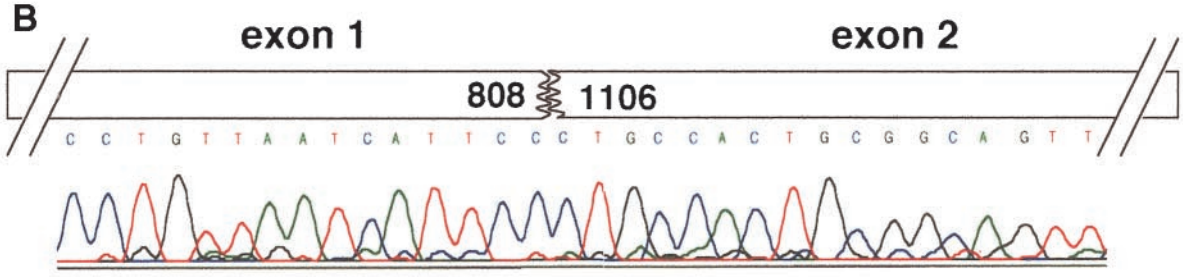


C

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NHE1   CCGGTTTTACTCTCATTTGGGTAAGGTCGAGGCTGGCTCTGGAAGCAGCACC ATGGTTCTGCGGTCTGGCATCTG 600
DNHE1  CCGGTTTTACTCTCATTTGGGTAAGGTCGAGGCTGGCTCTGGAAGCAGCACC ATGGTTCTGCGGTCTGGCATCTG 600
                                                            M  V  L  R  S  G  I  C
NHE1   TGGCCTCTCTCCACATCGGATCTTCCCTTCCTTACTCGTGGTGGTTGCTTTGGTGGGGCTGCTGCCTGTTCTCAG 675
DNHE1  TGGCCTCTCTCCACATCGGATCTTCCCTTCCTTACTCGTGGTGGTTGCTTTGGTGGGGCTGCTGCCTGTTCTCAG 675
        G  L  S  P  H  R  I  F  P  S  L  L  V  V  V  A  L  V  G  L  L  P  V  L  R
NHE1   GAGCCATGGCCTCCAGCTCAGCCCAACTGCCAGCACCATTCGAAGCTCAGAGCCACCACGAGAACGCTCGATTGG 750
DNHE1  GAGCCATGGCCTCCAGCTCAGCCCAACTGCCAGCACCATTCGAAGCTCAGAGCCACCACGAGAACGCTCGATTGG 750
        S  H  G  L  Q  L  S  P  T  A  S  T  I  R  S  S  E  P  P  R  E  R  S  I  G
NHE1   GGATGTCACCACCGCTCCACCGGAGGTCACCCCAGAGAGCCGCCCTGTTAATCATTCCGTCACTGATCATGGCAT 825
DNHE1  GGATGTCACCACCGCTCCACCGGAGGTCACCCCAGAGAGCCGCCCTGTTAATCATTCC---------------- 808
        D  V  T  T  A  P  P  E  V  T  P  E  S  R  P  V  N  H  S  V  T  D  H  G  M
NHE1   GAAGCCGCGCAAGGCCTTTCCAGTCCTGGGCATCGACTACACACACGTGCGCACCCCCTTCGAGATCTCCCTCTG 900
DNHE1  ------------------------------------------------------------------------
        K  P  R  K  A  F  P  V  L  G  I  D  Y  T  H  V  R  T  P  F  E  I  S  L  W
NHE1   GATCCTTCTGGCCTGCCTCATGAAGATAGGTTTCCATGTGATCCCCACTATCTCAAGCATCGTCCCGGAGAGCTG 975
DNHE1  ------------------------------------------------------------------------
        I  L  L  A  C  L  M  K  I  G  F  H  V  I  P  T  I  S  S  I  V  P  E  S  C
NHE1   CCTGCTGATCGTGGTGGGGCTGCTGGTGGGGGGCCTGATCAAGGGTGTAGGCGAGACACCCCCCTTCCTGCAGTC 1050
DNHE1  ------------------------------------------------------------------------
        L  L  I  V  V  G  L  L  V  G  G  L  I  K  G  V  G  E  T  P  P  F  L  Q  S
NHE1   CGACGTCTTCTTCCTCTTCCTGCTGCCGCCCATCATCCTGGATGCGGGCTACTTCCTGCCACTGCGGCAGTTCAC 1125
DNHE1  ------------------------------------------------------CTGCCACTGCGGCAGTTCAC 828
        D  V  F  F  L  F  L  L  P  P  I  I  L  D  A  G  Y  F  L  P  L  R  Q  F  T
NHE1   AGAAAACCTGGGCACCATCCTGATCTTTGCCGTGGTGGGCACGCTGTGGAACGCCTTCTTCCTGGGCGGCCTCAT 1200
DNHE1  AGAAAACCTGGGCACCATCCTGATCTTTGCCGTGGTGGGCACGCTGTGGAACGCCTTCTTCCTGGGCGGCCTCAT 903
        E  N  L  G  T  I  L  I  F  A  V  V  G  T  L  W  N  A  F  F  L  G  G  L  M
NHE1   GTACGCCGTGTGCCTGGTGGGCGGTGAGCAGATCAACAACATCGGCCTCCTGGACAACCTGCTCTTCGGCAGCAT 1275
DNHE1  GTACGCCGTGTGCCTGGTGGGCGGTGAGCAGATCAACAACATCGGCCTCCTGGACAACCTGCTCTTCGGCAGCAT 978
        Y  A  V  C  L  V  G  G  E  Q  I  N  N  I  G  L  L  D  N  L  L  F  G  S  I
NHE1   CATCTCGGCCGTGGACCCCGTGGCGGTTCTGGCTGTCTTTGAGGAAATTCACATCAATGAGCTGCTGCACATCCT 1350
DNHE1  CATCTCGGCCGTGGACCCCGTGGCGGTTCTGGCTGTCTTTGAGGAAATTCACATCAATGAGCTGCTGCACATCCT 1053
        I  S  A  V  D  P  V  A  V  L  A  V  F  E  E  I  H  I  N  E  L  L  H  I  L
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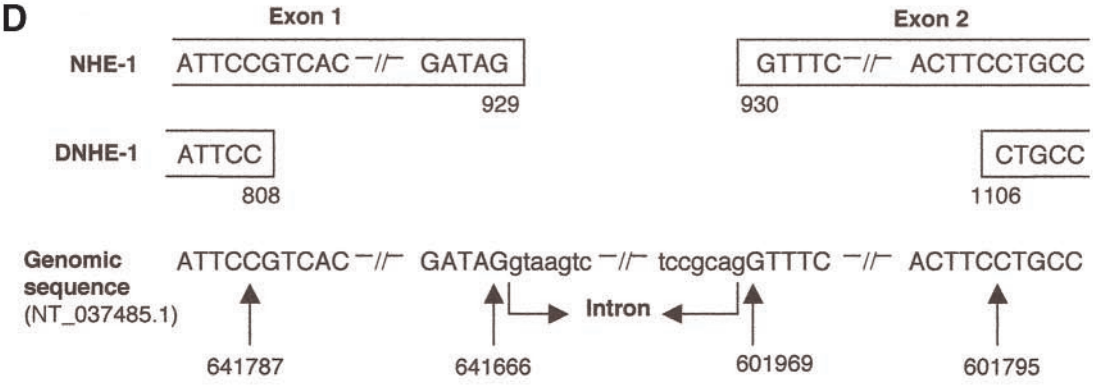
D



**Cell culture and transfection.** An NHE-deficient PS200 hamster fibroblast cell line [a twin clone of the PS120 hamster fibroblast cell line (26), gift of J. Pouyssegur and L. Counillon] was cultured in minimal essential medium supplemented with 10% fetal bovine serum. Before transfection, PS200 cells were trypsinized, seeded at a density of $5 \times 10^5$ cells/dish in 60-mm dishes, and incubated overnight. Transfection was performed according to the calcium phosphate–DNA coprecipitation method (27).

**Cell selection**

***Acid loading.*** Three days after transfection, cells transfected with NHE-1 or DNHE-1 were subject to the proton killing test (28), an acid-loading selection test that allows elimination of those cells that do not express functional NHE. Cells were allowed to load with $NH_4$ during 60-min incubation at 37°C in an $NH_4$-saline medium. External $NH_4$ was then rapidly removed by two washes with an Na-rich medium and the cells were finally incubated in this same medium for 60 min at 37°C. At the end of incubation, the Na-rich medium was replaced by culture medium. This treatment was repeated five times over a period of 2 weeks, and the successfully transfected cells were used for experiments.

***Resistance to geneticin.*** Three days after transfection, cells transfected with DNHE-1 were treated with 400 μg/ml geneticin (Gibco). This treatment was repeated 10 times over a period of 3 weeks, and finally the transfected cells were used for experiments.

**Measurement of Na-driven Li efflux.** Na-driven Li efflux was evaluated as previously described for human skin fibroblasts (29). In brief, Li efflux was measured in PS200 cells, in PS200 cells transfected with NHE-1, and in PS200 cells transfected with DNHE-1 in the presence of ouabain, bumetanide, and 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid to inhibit the Na pump, Na-K cotransport, and the anion exchanger, respectively. Dimethyl-amiloride (DMA, the specific inhibitor of NHE) and/or phloretin (the strongest inhibitor of SLC) were added as appropriate to probe the NHE and SLC, respectively (Fig. 3). Na-driven Li efflux in human erythrocytes was evaluated as previously described (22).

**NHE-1 polyclonal antibody.** A rabbit polyclonal antibody against amino acids 56–70 of human NHE-1 (common to both NHE-1 and DNHE-1) ($NH_2$-SIGDVTTAPPEVTPE-COOH) was generated and purified by absorption in protein A-Sepharose.

**NHE-1 isoform expression pattern in human tissues.** RT-PCR was performed for 40 cycles on 2 μl cDNA (2–10 ng/ml) from six human tissues (brain, leukocyte, heart, liver, kidney, spleen) (OriGene Technologies, Rockville, MD) using primers NHE-1.1 and NHE-1.4, as described above (Fig. 6).

**Semiquantitative RT-PCR.** Total RNA was extracted from whole blood of 10 individuals with low SLC activity (142.5 ± 8.7 μmol · liter$^{-1}$ cells · hour$^{-1}$, mean ± SE, range 90–174) and from 10 individuals with high SLC activity (939.0 ± 58.6 μmol · liter$^{-1}$ cells · hour$^{-1}$, range 768–1,369), and cDNA was retrotranscribed. PCR conditions were optimized for $Mg^{2+}$ concentration, primer concentration, primer annealing temperature, and number of cycles for NHE-1, DNHE-1, and β-actin cDNA amplification. Oligonucleotide positions were, respectively, 721–740 F 5′-CTCAGAGCCACCACGAGAAC-3′ and 1120–1139 R 5′-TGCCCAGGTTTTCTGTGAAC −3′. In the case of β-actin, oligonucleotides 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′, upstream and downstream primers, respectively, were used for amplification of a 646-bp fragment of human β-actin cDNA located between nucleotides 2198 and 3065 of the reported human gene sequence (GenBank AC E00829). Semiquantitative RT-PCR experiments were performed by incorporating α-$^{32}$P-dCTP in two systems: primers 1 and 2 on NHE-1 and primers 3 and 4 on β-actin. Expression of NHE-1 + DNHE-1 was normalized by β-actin values.

**Statistics.** Data are shown as arithmetical mean with standard error. Comparisons between observations were addressed by Student's *t* test. Correlations were sought by simple linear regression. The null hypothesis was rejected for two-tailed *P* values <5% (JMP software for the Apple Macintosh, SAS Institute, NC, USA).

## RESULTS

To search for possible amiloride-insensitive alternative splicing(s) of NHE-1, cDNA prepared with total RNA obtained from human erythrocytes and reticulocytes was amplified using 10 specific primers designed along the entire sequence of NHE-1 (NHE-1.1–NHE-1.10, see RESEARCH DESIGN AND METHODS for details). Amplification of cDNA using NHE-1.1 and NHE-1.4 primers revealed two independent PCR fragments of 1,142 and 845 bp respectively (Fig. 1*A*). Sequence analysis of cloned RT-PCR products confirmed the upper band as NHE-1 and showed an in-frame deletion of 297 nucleotides (nucleotides 809-1105 of NHE-1; Fig. 1*B*, *C*, and *D* and Fig. 2*A*) in the lower band (DNHE-1; Fig. 2*B*). The possibility that this cDNA variant originates from a second gene homologous to NHE-1 or a pseudo-gene was proven to be highly unlikely after database searching using both the canonical NHE-1 or the new DNHE-1 variant sequence as probes. No sequence other than the expected NHE-1 gene was identified in the human genome (data not shown).

DNHE-1 might have originated either directly through the splicing of the GT-TC splice donor-acceptor pair or through a first splicing involving the canonical 5′ GT (of NHE-1) splice site and the noncanonical 3′ TC (of DNHE-1) splice site followed by a second splicing of a smaller intron involving the canonical 5′ GT (of DNHE-1) splice site and the canonical AG (of NHE-1), now as a 3′ splice site (Fig. 1*D*).

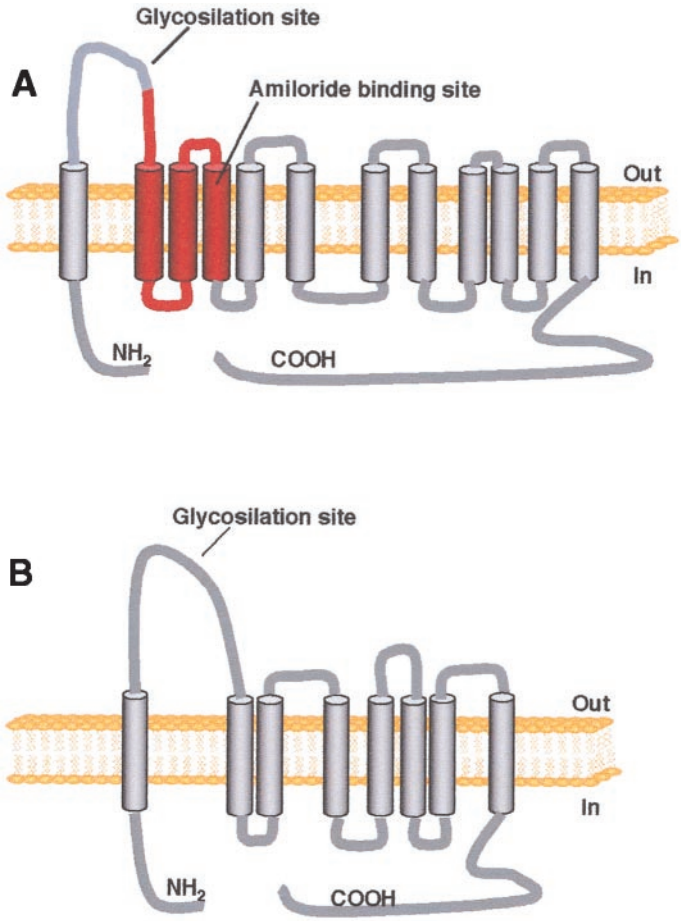



**FIG. 2. NHE-1 and DNHE-1 topology. Schematic representation of NHE-1 (*A*) and DNHE-1 (*B*) topology based on the work of Wakabayashi et al. (30).**

**FIG. 1. *A*: RT-PCR amplification products from human reticulocyte cDNA of two control subjects by primers NHE-1.1 and NHE-1.4 (molecular weights in base pairs are indicated on the left). *B*: Electropherogram of DNHE-1 in the area surrounding the new junction between exon 1 and exon 2. *C*: Sequence alignment of NHE-1 and DNHE-1 forms. The upper band is consistent with full-length NHE-1 and the lower band with a smaller variant of NHE-1 (DNHE-1), characterized by an in-frame skipping of 297 nucleotides. *D*: cDNA sequence of NHE-1 and DNHE-1 aligned to the nucleotide sequence of genomic clone NT_037485.1.**

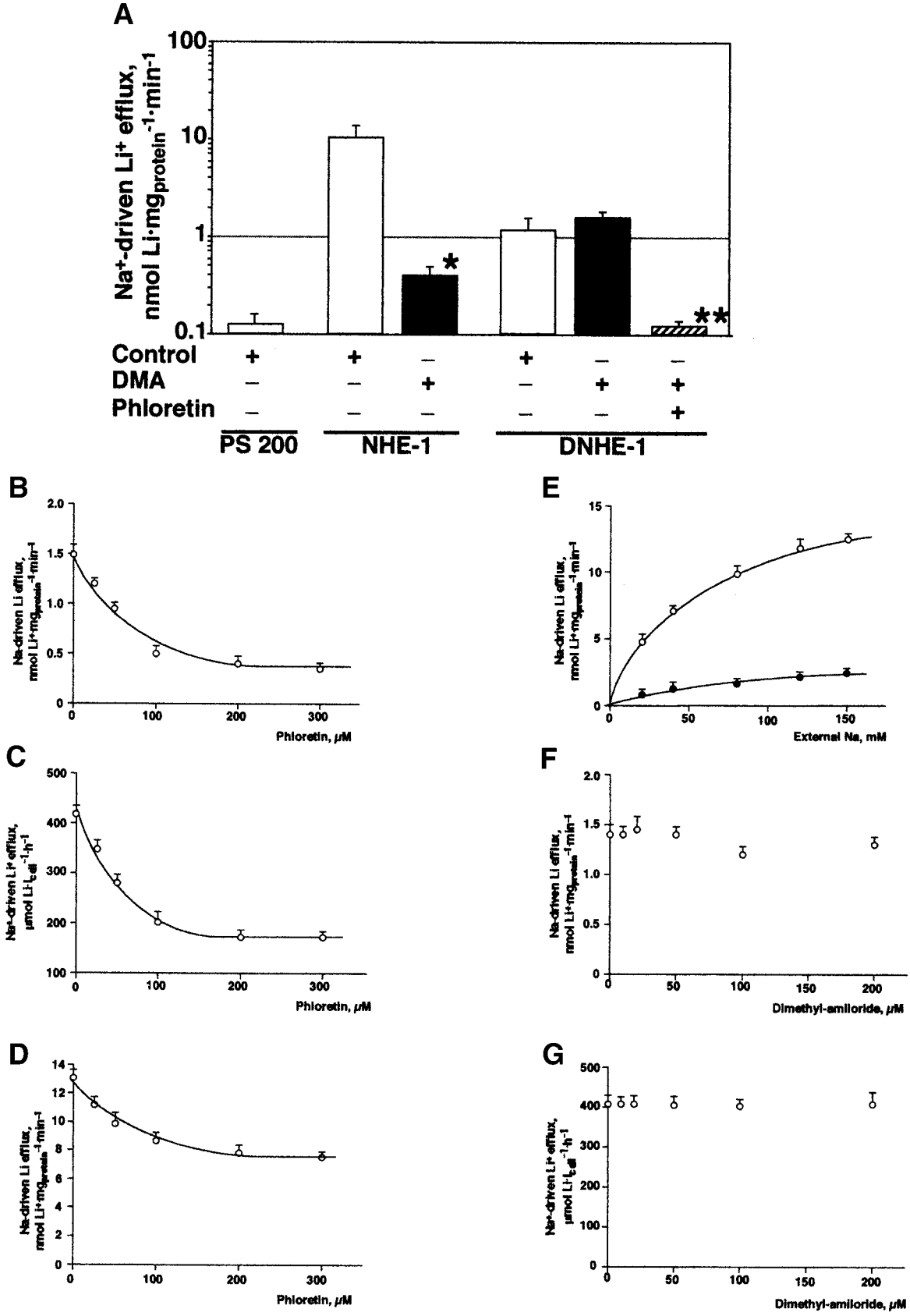



**FIG. 3. Na-driven Li efflux in PS200 cells and in the same cells transfected with either NHE-1 or DNHE-1. *A*: Na-driven Li efflux is virtually absent in nontransfected cells (first column) and is restored after NHE-1 transfection and strongly inhibited by DMA (second and third columns); *$P <$ 0.05. A lower Na-driven Li efflux is also restored after transfection of DNHE-1 (fourth column); this flux appears to be insensitive to DMA and significantly inhibited only by phloretin (fifth and sixth columns); **$P < 0.05$. Data shown are the results of three independent experiments. *B*: Representative experiment showing the titration curve of phloretin's inhibitory effect on Na-driven Li efflux in PS200 cells transfected with**

The open reading frame of the DNHE-1 isoform leads to a predicted 99 amino acid–smaller protein (missing transmembrane domains M2, M3, and M4), which lacks the putative amiloride-binding region (30). The putative topology of DNHE-1 obtained from TMAP, a web-based tool for prediction of transmembrane segments in proteins (31), is shown in Fig. 2*B*. According to this model, DNHE-1 would contain eight transmembrane domains with $NH_2$- and COOH-terminals on the internal side of the membrane. To evaluate the functional activity of this isoform, we inserted full-length (NHE-1) and DNHE-1 transcripts into a pcDNA3 expression plasmid and then transfected the obtained expression constructs pcNHE-1 and pcDNHE-1 into an NHE-deficient PS200 hamster fibroblast cell line (PS200).

NHE transfectants were selected according to the proton killing test by their ability to survive 1-h of acidification induced by $NH_4$ prepulse acid loading (28). As expected, cells transfected with NHE-1 survived the acid-loading selection test. On the contrary, cells transfected with DNHE-1 did not survive this procedure, suggesting that DNHE-1–mediated exchange of Na for H, if any, was insufficient to allow cell survival.

To clarify whether DNHE-1 can mediate SLC activity even in the absence of a significant exchange of Na for H, DNHE-1–transfected cells were selected by their acquired resistance to geneticin (pcDNA3 expression plasmid contains the sequence conferring resistance to geneticin).

As described in Fig. 3*A*, in the presence of specific inhibitors of Na pump, Na-K cotransport, and anion exchanger, exchange of Na for Li in PS200 cells was virtually absent ($0.13 \pm 0.1$ nmol $Li^+ \cdot mg^{-1}$ protein $\cdot$ $min^{-1}$, mean $\pm$ SE, $n = 3$). NHE-1–tranfected cells were instead characterized by an active exchange of Na for Li ($10.8 \pm 1.6$), which was blunted by the addition of DMA, as expected ($0.41 \pm 0.2$, $P < 0.05$, $n = 3$) (19). Finally, DNHE-1–transfected cells expressed a small but significant exchange of Na for Li ($1.21 \pm 0.18$), which was consistent with SLC-mediated transport because it was resistant to DMA ($1.64 \pm 0.08$, NS) but was largely reduced by the addition of phloretin ($0.10 \pm 0.09$, $P < 0.05$, $n = 3$) (11,20). The inhibitory effect of phloretin on Na-driven Li efflux was similar in DNHE-1–transfected cells (Fig. 3*B*) and in human erythrocytes (Fig. 3*C*). Only partial (43%) inhibition of Na-driven Li efflux was exerted by phloretin in NHE-1–transfected cells (Fig. 3*D*), in line with our previous report in human erythrocytes (22). As shown in Fig. 3*E*, $K_m$ for external Na was similar in NHE-1– and DNHE-1–transfected cells. Finally, no inhibitory effect of DMA could be demonstrated on either DNHE-1–transfected cells or erythrocytes (Fig. 3*F*, *G*). The evidence that Na-driven Li efflux in human erythrocytes is completely insensitive to DMA, while confirming previous reports (20), could conflict with our hypothesis that human erythrocytes express both amiloride-sensitive NHE-1 and amiloride-insensitive DNHE-1. However, we have previously demonstrated that in the human erythrocyte, the amiloride-sensitive (NHE-1–mediated) component of Na-driven Li efflux is virtually inactive in resting conditions (as in this case) and can be activated only after osmotic shrinkage (21).

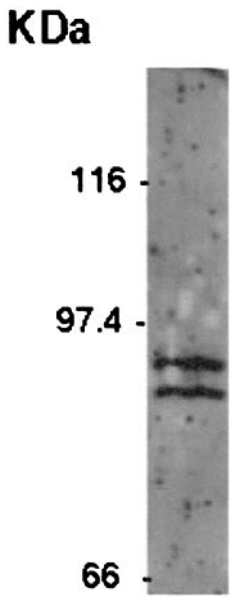



**FIG. 4. Characterization by immunoblotting of NHE-1 and DNHE-1 protein expression in human erythrocytes and reticulocytes. Sequential immunoblotting was performed using an anti-human NHE-1 rabbit polyclonal antibody. (Molecular masses, expressed in kilodaltons, are indicated on the left). Molecular masses calculated from the amino-acidic sequence are as follows: NHE-1 = 90.8 kDa, DNHE-1 = 80 kDa.**

Because DNHE-1 should be transduced into a smaller protein than NHE-1, we examined protein extracts from the erythrocyte membrane and from PS200 cells transfected with either NHE-1 or DNHE-1 using a polyclonal antibody raised against a peptide shared by NHE-1 and its spliced variant. After BLAST analysis on all nonredundant protein databases, the peptide described above showed only nine 100% homologue matches, and all of them were specific for NHE-1. As expected (Fig. 4), two independent bands were identified in human erythrocyte membranes by immunoblotting and were consistent with full-length NHE-1 (upper band) and with the smaller variant due to the predicted alternative splicing (lower band).

As shown in Fig. 5*A*, RT-PCR amplification of cDNA extracted from PS200 cells transfected with NHE-1 displayed a single band consistent with full-length NHE-1, while amplification of cDNA extracted from PS200 cells transfected with DNHE-1 originated a lower single band consistent with the alternative splicing.

In parallel, as shown in Fig. 5*B*, immunoblotting of protein extracted from PS200 cells transfected with NHE-1

**DNHE-1. Vertical bars are the SE of duplicate within-assay observations ($K_i$ = 39.1 μmol/l phloretin). *C*: Representative experiment showing the titration curve of phloretin's inhibitory effect on Na-driven Li efflux in human erythrocytes. Vertical bars are the SE of duplicate within-assay observations ($K_i$ = 35.6 μmol/l phloretin). *D*: Representative experiment showing the titration curve of phloretin's inhibitory effect on Na-driven Li efflux in PS200 cells transfected with NHE-1. Vertical bars are the SE of duplicate within-assay observations ($K_i$ = 48.5 μmol/l phloretin). *E*: Representative experiment showing the activation curve for external Na of Na-driven Li efflux in PS200 cells transfected with NHE-1 (○); $V_{max}$ = 15.9 nmol Li · $mg^{-1}$ protein · $min^{-1}$, $K_m$ = 40.9 mmol/l Na) or with DNHE-1 (●; $V_{max}$ = 2.19 nmol Li · $mg^{-1}$ protein · $min^{-1}$, $K_m$ = 48.6 mmol/l Na). Vertical bars are the SE of duplicate within-assay observations. *F*: Representative experiment showing the titration curve of DMA's inhibitory effect on Na-driven Li efflux in PS200 cells transfected with DNHE-1. Vertical bars are the SE of duplicate within-assay observations. *G*: Representative experiment showing the titration curve of DMA's inhibitory effect on Na-driven Li efflux in human erythrocytes. Vertical bars are the SE of duplicate within-assay observations.**

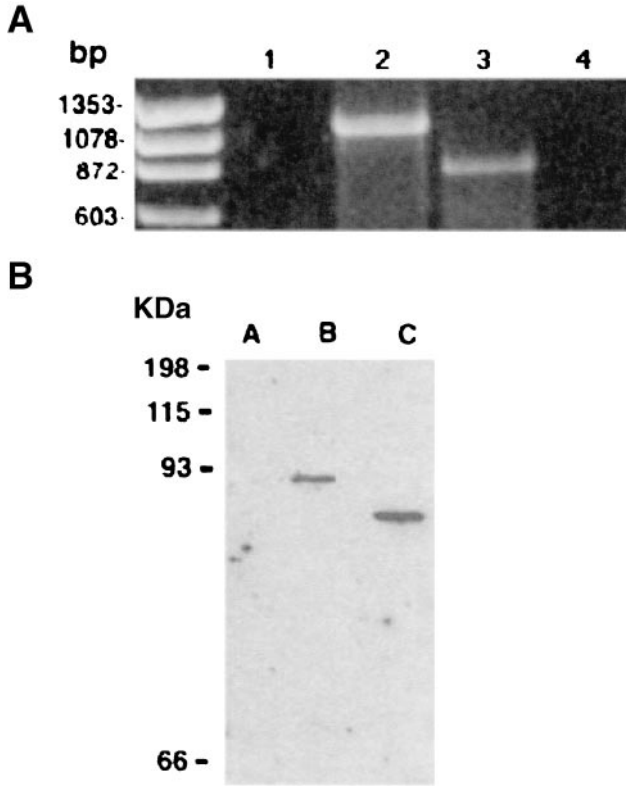



**FIG. 5. *A*: NHE-1 and DNHE-1 RNA expression in transfected cells. RT-PCR amplification products of nontransfected PS200 cells (*lane 1*) and of PS200 cells transfected with NHE-1 (*lane 2*), DNHE-1 (*lane 3*), or nonretrotranscribed mRNA derived from a pool of PS200 cells transfected with NHE-1 and DNHE-1 (*lane 4*). (Base pairs are indicated on the left). *B*: NHE-1 and DNHE-1 protein expression in transfected cells. Characterization by immunoblotting of NHE-1 and DNHE-1 protein expression in nontransfected PS200 cells (*lane 1*) and PS200 cells transfected with NHE-1 (*lane 2*) or DNHE-1 (*lane 3*). (Molecular masses, expressed in kilodaltons, are indicated on the left).**

originated a single band consistent with full-length NHE-1, while immunoblotting of protein extracted from PS200 cells transfected with DNHE-1 originated a lower single band consistent with the alternative splicing. No bands could be detected in nontransfected PS200, thus confirming the specificity of the antibody. Because the methodology we used to extract membrane proteins was proven to disrupt glycosylation (data not shown), neither erythrocytes nor transfected cells showed glycosylated NHE-1.

To clarify whether the spliced variant of NHE-1 is expressed in tissues other than erythroid cells, we performed a PCR using cDNAs from different human tissues with the same primers (NHE-1.1 and NHE-1.4) employed to identify NHE-1 and DNHE-1 in human erythrocytes and reticulocytes. As shown in Fig. 6, the DNHE-1 isoform was detected in kidney and spleen, suggesting that its presence is not restricted to the hematopoietic system.

Finally, as shown in Fig. 7*A*, NHE-1 and DNHE-1 expression levels were correlated to each other, as individuals characterized by low NHE-1 expression showed low DNHE-1 expression and vice versa. For this reason, we performed a semiquantitative RT-PCR aimed at evaluating the sum of the expression levels of NHE-1 and DNHE-1. As described in Fig. 7*B*, individuals with higher SLC activity were also characterized by an increased cumulative expression level of NHE-1 + DNHE-1 normalized for β-actin

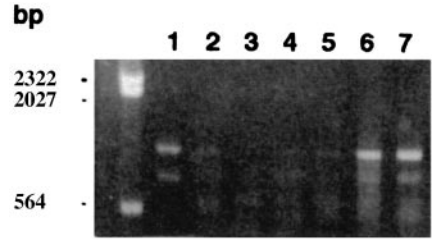



**FIG. 6. RT-PCR amplification products of NHE-1 in different tissues. Amplification was obtained using NHE-1.1 and NHE-1.4 primers (see RESEARCH DESIGN AND METHODS) on the following cDNAs: human erythrocytes and reticulocytes (*lane 1*), brain (*lane 2*), leukocytes (*lane 3*), heart (*lane 4*), liver (*lane 5*), kidney (*lane 6*), and spleen (*lane 7*). (Base pairs are indicated on the left).**

(1.88 ± 0.2 vs. 0.71 ± 0.04 arbitrary units, mean ± SE, $P = 0.0002$) when compared with individuals with low SLC activity.

Together, these findings demonstrate that SLC is mediated by an alternative splicing of NHE-1 and that its activity directly depends upon NHE-1 expression.

## DISCUSSION

The results of the present study demonstrate that SLC is mediated by an amiloride-insensitive splicing of NHE-1 and that its activity correlates with the expression of both NHE-1 and its spliced transcript. As shown above, a very large (~40-kb) intron with canonical splice sites in the relevant gene segment generates NHE-1 mRNA. DNHE-1 is instead the likely result of a splicing event using splice junction sequences in the coding region of NHE-1 involving a GT-TC splice donor-acceptor pair.

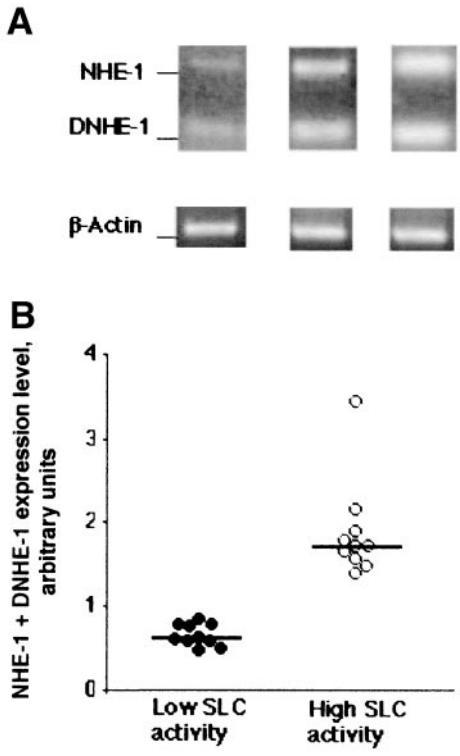



**FIG. 7. *A*: Expression levels of NHE-1 (upper band) and DNHE-1 (lower band) are correlated with each other in different individuals. *B*: Cumulative expression level of NHE-1 and DNHE-1 normalized for β-actin is significantly increased in individuals with high SLC activity when compared with individuals with low SLC activity. *$P = 0.0002$.**

Although the identification of a GT-TG splice pair of the human adenylyl cyclase stimulatory G-protein $G\alpha_s$ has recently been described (32), ours may nonetheless be the first described example of a GT-TC noncanonical splice donor-acceptor pair. The most simple mechanism to explain the origin of DNHE-1 would be that the canonical intron yielding NHE-1 is spliced first, followed by splicing of the noncanonical intron, to yield DNHE-1. If so the NHE-1 expression construct would also generate DNHE-1; however, this is not the case, as described in RESULTS and in Fig. 5, panel A. This result supports the alternative possibility that the splicesome recognizes both the canonical AG (of NHE-1) and, less frequently, the noncanonical TC donor splice site. Such splicing and resplicing mechanisms have been described previously, especially when very large introns are involved (33). The latter mechanism might also be consistent with the evidence (32) that atypical noncanonical sites are often near canonical sites. In our case, canonical NHE-1 and noncanonical DNHE-1 3′ splice sites are not that close (175 bp), but relative to the large size of the intron they become close, which could account for the use of the atypical site.

The hypothesis, based on functional evidence, that SLC could represent an operational mode of NHE has actually long been suggested (13,21,22). Nonetheless, this is the first time that this assumption has been definitively confirmed by a molecular approach.

The significance of the identification of the gene responsible for SLC activity rests in two main arguments: *1*) This result might be of help in perspectively finding a genetic marker useful for the early detection of patients predisposed to the development of essential hypertension and diabetic nephropathy. The molecular dysfunction that underlies the deranged SLC activity should in fact be, at least in principle, a more sensitive marker of disease than the SLC overactivity by itself. *2*) This finding, by translating to NHE-1 the characteristics of inheritance and predictivity previously attributed to SLC, once more designates NHE-1 as a candidate to explain the pathogenesis of essential hypertension and, consequently, diabetic nephropathy.

A few years ago, after genetic linkage analysis, NHE-1 was excluded as the gene responsible for elevated SLC activity and therefore as a candidate gene for essential hypertension (34). Our finding that SLC activity is mediated by DNHE-1 and correlates with the expression of NHE-1 transcripts should nonetheless renew interest in the potential contribution of this gene to the pathogenesis of essential hypertension. At present, indeed, it is not possible to exclude the possibility that recently identified NHE-1 gene single-nucleotide polymorphisms (National Center for Biotechnology Information's Entrez system) might correlate with SLC activity, NHE-1 transcript levels, and hypertension.

As shown by our experiments, DNHE-1, although virtually unable to mediate a significant exchange of Na for H when transfected into NHE-deficient cells, is nonetheless capable of mediating a "remnant" exchange of Na for Li. This finding, although surprising at first sight, is justified by a few pieces of evidence: *1*) previous studies indicate that mutations in the amiloride binding region induce a reduced sensitivity to amiloride paralleled by slower Na transport (35); *2*) functional studies have suggested that SLC is unable, possibly because of an excessive affinity for external H, to exchange Na for H (36,37); and *3*) SLC activity in human erythrocytes has been demonstrated to be 10–50 times less active than NHE (38). Finally, recent evidence (39) suggests that mutations in the fourth transmembrane domain of NHE-1 (a segment that is lost in DNHE-1, as shown in Fig. 2) reduce the affinity of the exchanger for external Na. Interestingly, the same report also indicates that none of the mutations introduced affect the affinity of the exchanger for Li.

In conclusion, we have identified in human erythrocytes and reticulocytes a spliced variant of NHE-1 lacking the nucleotide sequence that specifies for the amiloride-binding region. Once transfected in NHE-lacking cells, this smaller variant restores an amiloride-insensitive, phloretin-sensitive exchange of Na for Li compatible with SLC activity. Finally, expression of both regular and spliced transcripts of NHE-1 is increased in subjects with high SLC activity. Analysis of NHE-1 polymorphisms should clarify the molecular basis of NHE and SLC hyperactivity found in essential hypertension and diabetic nephropathy.

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