Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3938–3942, May 1993
Physiology

Molecular cloning, sequencing, tissue distribution, and functional expression of a Na^+/H^+ exchanger (NHE-2)

JAMES F. COLLINS*†, TATSUYA HONDA*, SUSAN KNOBEL*, NADA M. BULUS*, JON CONARY‡, RAY DUBOIS‡, AND FAYEZ K. GHISHAN*†§

Departments of *Pediatrics, †Molecular Physiology and Biophysics, and ‡Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232

Communicated by Charles R. Park, January 25, 1993

ABSTRACT **The present studies demonstrate cloning, sequencing, tissue distribution, and functional expression of a Na^+/H^+ exchanger which was isolated from a rat intestinal cDNA library. The cloned cDNA recognizes two transcripts in poly(A)$^+$ RNA from the stomach, jejunum, ileum, liver, large intestine, and uterus. Based on deduced amino acid sequences, this clone shares sequence homology with the other known Na^+/H^+ exchanger isoforms (NHE-1, NHE-3, and NHE-4) except for its 5′ end. Overall, the protein exhibits 47.8%, 41.2%, and 56.2% amino acid sequence identity to NHE-1, NHE-3, and NHE-4, respectively. The hydropathy profile of the predicted protein shows 10 transmembrane domains, suggesting a protein with transport characteristics. The tissue distribution differs from that of the other Na^+/H^+ exchanger isoforms. The cDNA hybridizes to two closely related transcripts in the mRNA of these tissues, which suggests that the predominant transcript of this clone is alternatively spliced. Transfection of this cDNA into Na^+/H^+ exchanger-deficient mutant fibroblasts (PS120 cells) results in functional Na^+/H^+ exchange activity. These data suggest that we have cloned a member of the Na^+/H^+ exchanger family with tissue-specific expression. We suggest the designation of NHE-2 for this Na^+/H^+ exchanger.**

The Na^+/H^+ exchangers belong to a family of transporters which catalyze the electroneutral exchange of extracellular Na^+ for intracellular H^+ (1). The presence of a Na^+/H^+ exchanger was originally shown in plasma membrane vesicles of the kidney (2). Since then, the presence of Na^+/H^+ exchange activity has been described in a variety of mammalian biological systems (3). Studies utilizing membrane vesicles have defined kinetically distinct intestinal apical and basolateral membrane Na^+/H^+ exchangers (4, 5). Pouyssegur's group (6) has cloned a cDNA which encodes the growth factor-activated Na^+/H^+ exchanger. This cDNA, designated NHE-1, recognizes a transcript of 5.6 kb in human tissue poly(A)$^+$ RNA (6). Orlowski *et al.* (7) have identified three closely related Na^+/H^+ exchangers in rat tissues, NHE-1, NHE-3, and NHE-4, by screening cDNA libraries from the stomach, colon, brain, and spleen. Similarly, Tse *et al.* (8, 9) have identified two isoforms, NHE-1 and NHE-3, in rabbit by screening an ileal cDNA library. These isoforms exhibit differences in sequence homology, tissue expression, and molecular mass (7–9).

To identify other Na^+/H^+ exchanger isoforms, we screened rat intestinal cDNA libraries by using the 1.9-kb *Bam*HI–*Bam*HI fragment of human NHE-1 cDNA as a probe.¶

EXPERIMENTAL PROCEDURES

cDNA Library Screening. Two cDNA libraries were screened with the 1.9-kb *Bam*HI–*Bam*HI fragment from the human fibroblast Na^+/H^+ exchanger cDNA. The first library was prepared from rat ileum and was a gift from Raymond DuBois of Vanderbilt University. The second library was prepared from the entire small intestine and was a gift from Lawrence Chan of Baylor Medical School. Both libraries were cloned into λZAP II vector (Stratagene). Positive plaques were purified by an *in vivo* excision procedure utilizing *Escherichia coli* XL1-Blue and R408 helper phage (Stratagene).

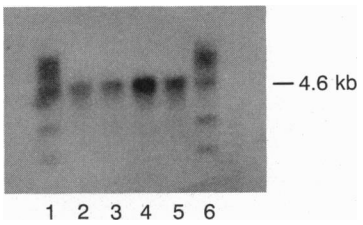

FIG. 1. Northern blot analysis of mRNA from the jejunum and ileum of 3- and 6-week-old rats. Poly(A)$^+$ RNA (5 μg per lane) from jejunum and ileum was analyzed by Northern blot hybridization using the *Bam*HI–*Bam*HI NHE-1 cDNA fragment. Lanes 2 and 4, ileal poly(A)$^+$ RNA from 3- and 6-week-old rats, respectively; lanes 3 and 5, jejunal poly(A)$^+$ RNA from 3- and 6-week-old rats, respectively; lanes 1 and 6, RNA standards.

cDNA Cloning and Sequencing. Sequencing of the positive cDNA clones was carried out by the dideoxy chain-termination technique (10). Both strands of the cDNAs were sequenced by using vector-derived primers and synthetic oligonucleotides derived from the cDNA sequence.

Sequence Homology and Protein Structure Analysis. Hydrophobicity and potential membrane-associated helical regions were predicted with the algorithms of Kyte and Doolittle (11). The computer programs PC-GENE (Intelligenetics) and DNASIS were used to analyze nucleotide and amino acid homology and protein structure.

RNA Isolation. Poly(A)$^+$ RNA was isolated from various rat tissues with a commercially available kit, Fast Track (Invitrogen), which uses guanidinium isothiocyanate-containing lysis buffer and oligo(dT)-cellulose affinity chromatography.

Northern Blot Analysis. Five micrograms of poly(A)$^+$ RNA from each of the tissues was denatured with formaldehyde, size-fractionated by agarose gel electrophoresis, and transferred onto a nylon membrane for Northern blot hybridization (12).

Cell Culture and Transfection. The Na^+/H^+ exchanger-deficient PS120 fibroblast cell line (a gift from J. Pouyssegur, Centre de Biochimie, Nice, France) and the corresponding

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

§To whom reprint requests should be addressed at: Department of Pediatrics, Division of Gastroenterology/Nutrition, 21st and Garland Avenues, S-4322 Medical Center North, Nashville, TN 37232-2576.

¶The sequence reported in this paper has been deposited in the GenBank database (accession no. L11004).

transfectants were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). For transfection, PS120 fibroblasts were trypsinized, seeded at 5×10^5 cells per 10-cm plate, and incubated overnight in 10 ml of growth medium. Twenty micrograms of an expression plasmid (pCMV4) containing cloned H7 cDNA was mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2× BBS (1× is 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid/1.5 mM Na_2HPO_4/280 mM NaCl) and left at 22°C for 20 min. The calcium phosphate/DNA suspension (1 ml) was added dropwise to the plate of cells and the mixture was swirled gently and incubated for 24 hr at 37°C under 5% CO_2. The medium was removed and cells were rinsed twice with growth medium, refed, and incubated for 24 hr at 37°C under 5% CO_2. The cells were split and incubated for an additional 24 hr before selection for transport studies and stable transformation (13).

Measurement of Na^+/H^+ Exchange Activity. ^{22}Na uptake in confluent monolayers was initiated in 122 mM choline chloride buffer (pH 7.4) containing 0.1 mM $^{22}NaCl$, 1 mM ouabain, and 25 μM amiloride. Uptake was stopped at various time points with ice-cold choline buffer containing 1 mM ouabain and 1 mM amiloride. 3-*O*-Methylglucose equi-

```
                                                                                                                CGGCACGAGGTGTCGTTCTCCTAATGTAGAGAGCACCT  - 38
GAGCAGAAAGAAGGCTGTGGTGAAGAGGAGAGAAATGCTGAGTGCACACACACAGGCAAAGACGTTGTACTGGTATTGCCACAAAGAGGAGCAGGGGCACACATCGGGGCACACACGACACATACGC  -165
ACAAATCTCATCATTCTCTCCCATTTCTGCACAGCATTTTAGAAAATGTAATAAAGGTATAGAAGCCCCCACATTGTGCCATGGAAGCAACATGTATTTAAAACACTTGATTTCTGGCTTAGAATTC  -292
AGTTTATGCCTCAGTAATGTCTCCTGCATACACATAAATAGGTAAGTGTAAACTTTTCAAAAGTGGGCCATTTTGCAATATGGGAGCCAAGTATAGTGATCTAGGGGATTCTGAAGTTATATCCATT  -419
GTTGCTATGGGAACTAATACCCCTTTTCTTTGACTCCTGCCTAACCCAAGCTCTGTGTTTCTCTTAACCAGGTTTCCATCTCTATCACAAGTTGCCCACGATTGTGCCGGAGAGCTGTCTTCTCATA  -546

ATG GTT GGG CTT CTA CTA GGT GGG ATT ATC TTC GGT GTC GAT GAG AAG TCT CCT CCT GCA ATG AAA ACG GAT GTA TTT TTC CTA TAC CTC CTG CCA    96
Met Val Gly Leu Leu Leu Gly Gly Ile Ile Phe Gly Val Asp Glu Lys Ser Pro Pro Ala Met Lys Thr Asp Val Phe Phe Leu Tyr Leu Leu Pro
                                10                                  20                                  30

CCC ATT GTT CTG GAT GCT GGC TAC TTC ATG CCT ACT CGA CCT TTC TTT GAG AAC TTG GGC ACC ATT TTC TGG TAT GCT GTG GTA GGC ACA CTG TGG   192
Pro Ile Val Leu Asp Ala Gly Tyr Phe Met Pro Thr Arg Pro Phe Phe Glu Asn Leu Gly Thr Ile Phe Trp Tyr Ala Val Val Gly Thr Leu Trp
                            42                                  52                                  62

AAT TCC ATT GGC ATT GGG CTG TCT TTG TTC GGC ATC TGC CAG ATT GAG GCG TTT GGC CTC AGC GAC ATC ACC TTG CTC CAG AAC CTG CTC TTC GGC   288
Asn Ser Ile Gly Ile Gly Leu Ser Leu Phe Gly Ile Cys Gln Ile Glu Ala Phe Gly Leu Ser Asp Ile Thr Leu Leu Gln Asn Leu Leu Phe Gly
                        74                                  84                                  94

AGC CTG ATC TCA GCT GTG GAC CCT GTG GCT GTG CTT GCT GTC TTT GAG AAC ATT CAT GTC AAT GAA CAG CTC TAT ATC CTG GTT TTT GGC GAG TCT   384
Ser Leu Ile Ser Ala Val Asp Pro Val Ala Val Leu Ala Val Phe Glu Asn Ile His Val Asn Glu Gln Leu Tyr Ile Leu Val Phe Gly Glu Ser
                    106                                 116                                 126

CTG CTC AAC GAT GCA GTC ACA GTG GTC CTG TAC AAC TTG TTC AAA TCC TTC TGC CAG ATG AAG ACC ATC CAG ACC GTA GAC GTG TTT GCT GGC ATC   480
Leu Leu Asn Asp Ala Val Thr Val Val Leu Tyr Asn Leu Phe Lys Ser Phe Cys Gln Met Lys Thr Ile Gln Thr Val Asp Val Phe Ala Gly Ile
                138                                 148                                 158

GCT AAC TTC TTC GTA GTG GGG ATT GGC GGG GTG CTG ATT GGT ATC CTG CTG GGA TTC ATA GCA GCG TTC ACC ACC CGT TTC ACA CAC AAC ATC CGG   576
Ala Asn Phe Phe Val Val Gly Ile Gly Gly Val Leu Ile Gly Ile Leu Leu Gly Phe Ile Ala Ala Phe Thr Thr Arg Phe Thr His Asn Ile Arg
                170                                 180                                 190

GTC ATT GAG CCG CTC TTT GTC TTC CTG TAC AGC TAC TTG TCC TAC ATC ACT GCT GAA ATG TTC CAC CTT TCA GGC ATC ATG GCG ATC ACA GCC TGT   672
Val Ile Glu Pro Leu Phe Val Phe Leu Tyr Ser Tyr Leu Ser Tyr Ile Thr Ala Glu Met Phe His Leu Ser Gly Ile Met Ala Ile Thr Ala Cys
                202                                 212                                 222

GCC ATG ACG ATG AAC AAG TAT GTG GAA GAG AAC GTG TCC CAG AAG TCC TAC ACG ACC ATC AAG TAC TTC ATG AAG ATG CTG AGC AGC GTG AGT GAG   768
Ala Met Thr Met Asn Lys Tyr Val Glu Glu Asn Val Ser Gln Lys Ser Tyr Thr Thr Ile Lys Tyr Phe Met Lys Met Leu Ser Ser Val Ser Glu
                234                                 244                                 254

ACC CTC ATC TTC ATC TTC ATG GGC GTG TCC ACC GTT GGG AAG AAC CAT GAG TGG AAC TGG GCT TTC GTC TGC TTC ACC CTG GCC TTC TGC CTG ATC   864
Thr Leu Ile Phe Ile Phe Met Gly Val Ser Thr Val Gly Lys Asn His Glu Trp Asn Trp Ala Phe Val Cys Phe Thr Leu Ala Phe Cys Leu Ile
                266                                 276                                 286

TGG CGA GCA CTG GGT GTC TTT GTC CTG ACT CAG GTG ATT AAT TGG TTC CGG ACC ATC CCA CTG ACC TTC AAG GAC CAG TTC ATC ATT GCC TAT GGA   960
Trp Arg Ala Leu Gly Val Phe Val Leu Thr Gln Val Ile Asn Trp Phe Arg Thr Ile Pro Leu Thr Phe Lys Asp Gln Phe Ile Ile Ala Tyr Gly
                298                                 308                                 318

GGG CTC CGC GGG GCC ATC TGC TTT GCA CTG GTG TTC CTC CTC CCT GCT ACT GTG TTT CCC CGA AAG AAG CTG TTC ATC ACA GCT GCC ATC GTC GTT  1056
Gly Leu Arg Gly Ala Ile Cys Phe Ala Leu Val Phe Leu Leu Pro Ala Thr Val Phe Pro Arg Lys Lys Leu Phe Ile Thr Ala Ala Ile Val Val
                330                                 340                                 350

ATA TTC TTC ACT GTC TTC ATC CTG GGA ATA ACC ATC CGG CCA CTG GTG GAA TTT CTT GAT GTT AAG AGA TCC AAT AAG AAG CAG CAA GCT GTC AGT  1152
Ile Phe Phe Thr Val Phe Ile Leu Gly Ile Thr Ile Arg Pro Leu Val Glu Phe Leu Asp Val Lys Arg Ser Asn Lys Lys Gln Gln Ala Val Ser
                362                                 372                                 382

GAA GAG ATC CAC TGT CGG TTT TTT GAT CAT GTG AAG ACT GGG ATT GAA GAT GTT TGT GGA CAC TGG GGT CAC AAC TTC TGG AGA GAC AAG TTT AAG  1248
Glu Glu Ile His Cys Arg Phe Phe Asp His Val Lys Thr Gly Ile Glu Asp Val Cys Gly His Trp Gly His Asn Phe Trp Arg Asp Lys Phe Lys
                394                                 404                                 414

AAA TTT GAT GAC AAA TAC CTT CGG AAG CTT CTG ATT CGG GAA AAC CAA CCC AAG TCT AGC ATT GTG TCC TTA TAT AAA AAG CTT GAA ATA AAA CAT  1344
Lys Phe Asp Asp Lys Tyr Leu Arg Lys Leu Leu Ile Arg Glu Asn Gln Pro Lys Ser Ser Ile Val Ser Leu Tyr Lys Lys Leu Glu Ile Lys His
                426                                 436                                 446

GCC ATA GAG ATG GCA GAG ACA GGG ATG ATA AGC ACT GTC CCC TCA TTT GCA TCT CTC AAC GAC TGC CGT GAA GAG AAA ATA AGG AAG CTC ACG CCA  1440
Ala Ile Glu Met Ala Glu Thr Gly Met Ile Ser Thr Val Pro Ser Phe Ala Ser Leu Asn Asp Cys Arg Glu Glu Lys Ile Arg Lys Leu Thr Pro
                458                                 468                                 478

GGA GAA ATG GAT GAA ATT AGA GAG ATA TTG TCA AGG AAT CTC TAT CAA ATC CGC CAG CGC ACA TTG TCC TAC AAC AGA CAC AAC CTG ACG GCA GAC  1536
Gly Glu Met Asp Glu Ile Arg Glu Ile Leu Ser Arg Asn Leu Tyr Gln Ile Arg Gln Arg Thr Leu Ser Tyr Asn Arg His Asn Leu Thr Ala Asp
                490                                 500                                 510

ACA AGT GAG AGG CAA GCT AAG GAG ATC TTG ATT CGC CGA CGA CAC AGT CTT CGG·GAA AGT CTT AGG AAG GAC AAC AGC TTA AAC CGC GAG CGC AGG  1632
Thr Ser Glu Arg Gln Ala Lys Glu Ile Leu Ile Arg Arg Arg His Ser Leu Arg Glu Ser Leu Arg Lys Asp Asn Ser Leu Asn Arg Glu Arg Arg
                522                                 532                                 542

GCT TCT ACT TCA ACC TCC CGA TAT TTA TCC TTA CCT AAA AAC ACA AAG CTT CCC GAG AAG TTG CAG AAG AAG AAT AAG GTT TCA AAT GCA GAT GGT  1728
Ala Ser Thr Ser Thr Ser Arg Tyr Leu Ser Leu Pro Lys Asn Thr Lys Leu Pro Glu Lys Leu Gln Lys Lys Asn Lys Val Ser Asn Ala Asp Gly
                554                                 564                                 574

AAT AGC AGC GAC TCG GAC ATG GAT GGA ACC ACT GTA CTC AAT TTG CAG CCC AGG GCC AGG CGC TTC TTG CCA GAT CAG TTC TCA AAG AAA GCC TCA  1824
Asn Ser Ser Asp Ser Asp Met Asp Gly Thr Thr Val Leu Asn Leu Gln Pro Arg Ala Arg Arg Phe Leu Pro Asp Gln Phe Ser Lys Lys Ala Ser
                586                                 596                                 606

CCA GCC TAC AAA ATG GAA TGG AAG AAT GAG GTG GAC GTG GGT TCT GCG CGA GCC CCC CCC AGC GTC ACT CCA GCG CCT CGC AGT AAA GAG GGG GGC  1920
Pro Ala Tyr Lys Met Glu Trp Lys Asn Glu Val Asp Val Gly Ser Ala Arg Ala Pro Pro Ser Val Thr Pro Ala Pro Arg Ser Lys Glu Gly Gly
                618                                 628                                 638

ACC CAG ACA CCA GGG GTC TTG AGG CAG CCC CTG CTC TCC AAA GAC CAA CGG TTT GGC CGG GGC AGG GAA GAC AGT TTG ACT GAA GAT GGT CCA CCC  2016
Thr Gln Thr Pro Gly Val Leu Arg Gln Pro Leu Leu Ser Lys Asp Gln Arg Phe Gly Arg Gly Arg Glu Asp Ser Leu Thr Glu Asp Val Pro Pro
                650                                 660                                 670

AAG CCC CCA CCA AGG TTG GTG CGA CGA GCA TCA GAA CCC GGA AAT AGG AAA GGC CGC CTT GGC AAC GAA AAG CCA TGA                        2094
Lys Pro Pro Pro Arg Leu Val Arg Arg Ala Ser Glu Pro Gly Asn Arg Lys Gly Arg Leu Gly Asn Glu Lys Pro
                682                                 692

TATGAAGCCAAAGCAGACTTGTGGTGAATGGGCAAGCATGGTTGTGACTTGTTACTCAAACCCAATCGACCGTGGAATCCACTTAAAGTCCTCTATCCAGAGGCATTCTTTGGGAAGTAGAGTCAAG  2221
CCACTGATTCATGGGAGGAGCGCTGCTTATGTATCCCAAGAAGAGGAAGGCAGAGGAAGGAAGTCCCACAAAATGACTTTTGTGACTTTTGGCTAGTAAATGTATTCTTTCAAGGCCTGAAAGGAAA  2348
AAAAAATGTGTGCCTTTATCGAACTTGAATGACAGAACTTGAAAATTTTAACGCACCTTTGTTGGTGAAAATTTTAATATATTACAAATATGCCTCAGATTTATTTATGAAAAAAATGTGTTTATCT  2475
GTAGCGATCTTTTTTTTCCCAAGGGAATGAAAACGTTGGCAAGAACATTTTTTCCAAGGGGGTAAAGGTCAGCTCCTAGAGGTGGGTCTTAAGATCCAGATCCCACCATCCCATCCTGAGCTGATGG  2602
ACCACTTGGTGAGTAGAACACTGTCCCCAGCAGAGGCCTTGCGTGACGGTATTAGGGCACAGGTTGGAATGTATAGGCAGATGAAATGCCCTGACTCTCAGAGTGTGGGTGGGAAGTCCCTGTACAA  2729
CTTTGAAACAAGGAGGTTCAGACTGCTCTCAGTCCATTCCTGTGTTTTCCATGGCTTTGGAGTAACATGTAATCTCCACCCCCCTCCCTTACCCCCGCCTCTGTCCCAATTTTTATCTAGGTACAGA  2856
AAGATTCACTTGCACAAAATCAGCCCATAGTGTTTATAGACTTGGTGGCAGCTTCCGAGAAGTCCTACCAGTGAGGACCTCTTGTGGCCAGTGTGAGGGCAGCCACTAGTGACCGCCTGGGTTTTAT  2983
GTGTGTGGTGATGGGAGGAAAGGGAGGTGGTTCCTTTCATTAGGGATCCATGGCCACCTGCTTTAATAAAATTGTCCTGCAGTCCCTTCAGAGCTGCGAGCTGGGAAGGGTCAACGTTTATACGAAC  3110
CGCTTTCCAGTGTTAAAGAAAATGGTGGGTCTTTGGGTTTGACTTCGAATGAGGAAGCACTTTGGAGAAACCACCCACTTTTTGCACTAGTTTTTAATTTGTTACCTCTTCACCTTTGATGATGTAG  3237
TGACTCAAATTTGTCATTAAAAAAATTAGATTCCATTCTCTGGAATACTTTTTAGGAGTATGGCAGAGAGGAAACAGTGGGACCAAATCATTAAAGATTCATATTTAAACCAATGCCTCTCAAGCTG  3364
CAGATTTATTATTAAAGATTCATATTTAAACAAAAAAAAAAAAAAAAA                                                                            3413
```

FIG. 2. Nucleotide and deduced amino acid sequence of clone H7. Nucleotide residues (right) and amino acids (below sequence) are numbered with respect to the initiation (ATG) codon. Three N-linked glycosylation sites are mentioned in the text, but only the conserved site is underlined here.

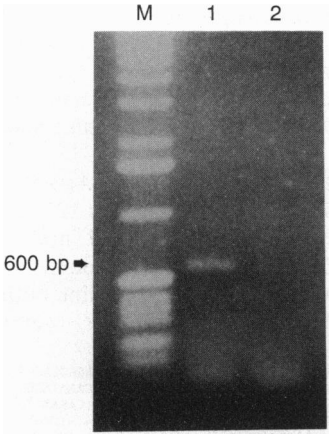

FIG. 3. PCR amplification from rat ileal first-strand cDNA of a DNA fragment homologous to the 5′ end of clone H7. The primers used for PCR were 5′-CTCCTAATGTAGAGAGCACC-3′ and 5′-TTCGGTGTCGATGAGAAGTC-3′, which were derived from the H7 sequence. Lane M, DNA "ladder" (size markers); lane 1, amplification of rat ileal cDNA shows a single band of 600 bp; lane 2, control with no template.

librium uptake was used to determine intracellular water space (14).

Measurement of Intracellular pH. pH dependence of the Na^+/H^+ exchanger was determined by recovery from an acid load with NH_4Cl. Intracellular pH was estimated from the distribution of [7-^{14}C]benzoic acid (15). [^{3}H]Mannitol was used as an extracellular marker.

Reverse Transcriptase–Polymerase Chain Reaction (PCR). We used thermostable *Taq* DNA polymerase and oligonucleotide primers for PCR, as shown in *Results*. PCR was performed with a programmable thermal controller (MJ Research, Watertown, MA). TET-z reverse transcriptase (Amersham) was used for first-strand cDNA synthesis. PCR was repeated for 45 cycles as follows: 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2 min. PCR products were subsequently ligated into PCR 2000 vector (Invitrogen) and the nucleotide sequence was determined as described earlier.

RESULTS

Northern Blot Analysis of Poly(A)$^+$ RNA from Rat Ileum and Jejunum with the *Bam*HI–*Bam*HI Fragment of Human NHE-1 cDNA. To determine the feasibility of using the human

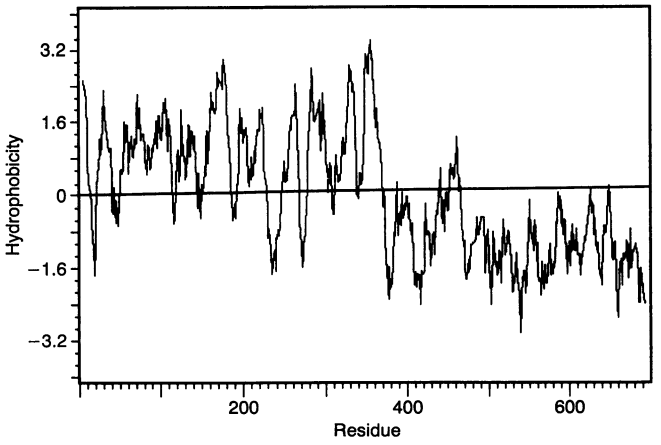

FIG. 4. Hydropathy plot of the predicted rat H7 Na^+/H^+ exchanger. Hydropathy indexes were calculated by the method of Kyte and Doolittle (11). Positive values represent hydrophobicity and negative values represent hydrophilicity. Ten predicted membrane-spanning domains are apparent.

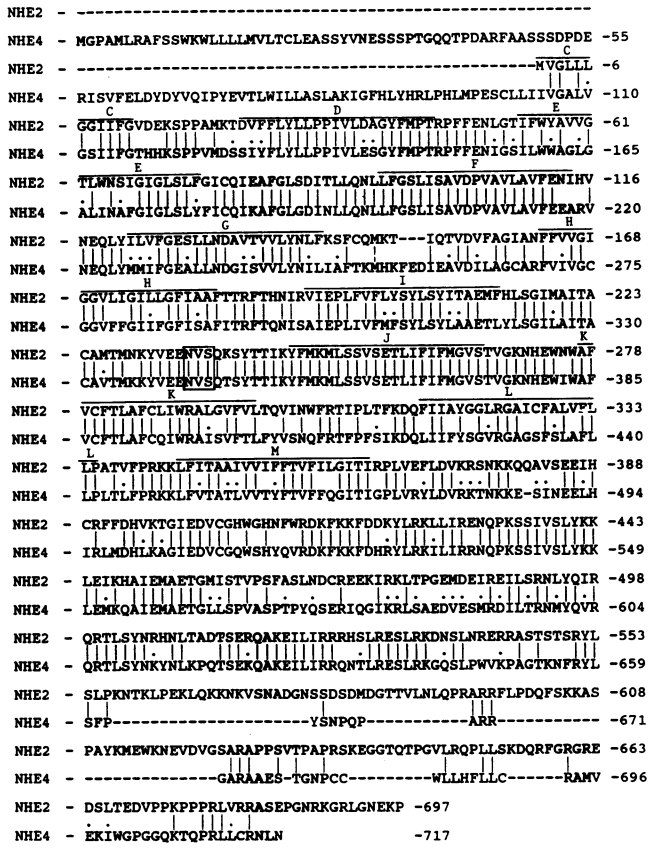

FIG. 5. Alignment of the amino acid sequences of rat NHE-4 and clone H7. Ten putative membrane-spanning domains are overlined (the 5′ end is missing the hydrophobic A and B domains present in the other isoforms). Overlined region K is thought to not span the membrane. Vertical bars indicate identity and colons indicate similarity. The boxed amino acids indicate the conserved N-glycosylation site.

NHE-1 cDNA to identify rat cDNAs coding for Na^+/H^+ exchangers, we probed rat ileum and jejunum poly(A)$^+$ RNA from weanling and adolescent rats with the *Bam*HI–*Bam*HI cDNA fragment of human NHE-1. A single 4.6-kb transcript was identified (Fig. 1).

Analysis of Rat cDNAs Encoding Na^+/H^+ Exchangers. To identify cDNAs encoding Na^+/H^+ exchanger-related proteins, we screened ileal and total small intestinal cDNA libraries under low-stringency conditions (7) with the 1.9-kb *Bam*HI–*Bam*HI cDNA fragment from human NHE-1 cDNA. Screening of the ileal library identified several positive clones. C_{12b} was the largest of these clones, 3.4 kb. C_{12b} was sequenced completely on both strands. The other clones were smaller but showed 100% sequence homology to C_{12b}.

Screening of the total small intestinal library with the *Bam*HI–*Bam*HI fragment of human NHE-1 cDNA resulted in several positive clones, the largest of which, H7, was 4.0 kb. This clone was sequenced completely on both strands and was shown to have sequence identity with C_{12b}. Clone H7 has a 546-nucleotide 5′ untranslated region, 1322 nucleotides of 3′ untranslated region, and an open reading frame of 2091 nucleotides encoding a protein of 697 amino acid residues with a calculated molecular weight of 79,063 (Fig. 2). H7 has overall nucleotide sequence homology of 58.3%, 59.6%, and 66.2% with NHE-1, NHE-3, and NHE-4, respectively. The overall amino acid sequence identity of the deduced H7 sequence is 47.8%, 41.2%, and 56.2% with NHE-1, NHE-3, and NHE-4, respectively. The nucleotide sequence at the 5′ end of H7 does not show close sequence homology to the other known Na^+/H^+ exchangers. To provide evidence that

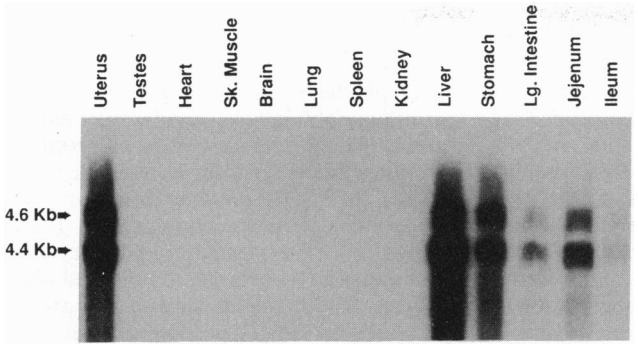

FIG. 6. Northern blot analysis of mRNAs from various rat tissues. Poly(A)$^+$ RNA (5 μg per lane) from various rat tissues was analyzed by Northern blot hybridization with H7 cDNA as a probe. The autoradiogram was exposed for 2.5 hr. Positions and sizes of the hybridization bands are shown at left. A positive signal was detected on the autoradiogram from the ileal sample but did not reproduce well in this photograph.

this 5′ portion of the cDNA belonged to the same transcript and was not a cloning artifact, we amplified a DNA fragment from rat ileum first-strand cDNA by PCR using primers based on the 5′ region of clone H7. We identified a single band of the expected size, 600 bp, which showed sequence identity to the 5′ end of the H7 clone (Fig. 3). This indicates that it belongs to the same transcript. Furthermore, we used *Eco*RI to release the 5′ end of H7. We then radiolabeled this fragment and used it as a probe for Northern blot analysis. This 5′-end probe of 600 nucleotides recognized the same mRNA transcripts in various tissues as did the entire H7 clone. The 5′ end of the other isoforms of the Na^+/H^+ exchanger cDNAs encodes two domains which appear to exhibit hydrophobic properties of membrane-associated surface or globular domains, which are predicted to not cross the lipid bilayer (7). The 3′ untranslated sequence terminates with a poly(A) tract. The apparent translation initiation codon is preceded by a purine nucleotide in position −3, placing it in good context for initiation by eukaryotic ribosomes (16). Moreover, the translation initiation site is also preceded by an in-frame stop codon at nucleotide −63.

Hydropathy Plot and Amino Acid Analysis of the H7 Clone. The hydrophobicity plot of H7 was carried out with the Kyte–Doolittle algorithm using a window of 11 amino acids (12). The H7 protein is predicted to have 10 transmembrane domains (Fig. 4). Orlowski *et al.* (7) have proposed the alphabetical designations A–M to represent the 13 hydrophobic regions within the N terminus of rat NHE-1, NHE-3, and NHE-4, but they have predicted that only 10 of these are transmembrane domains. The N-terminal region of the predicted H7 protein contains 11 hydrophobic domains, and the C-terminal region contains extensively hydrophilic domains which most likely are located on the cytoplasmic side of the membrane. Comparison of the hydrophobic, N-terminal domains of NHE-1, -3, and -4 reveals a high degree of sequence identity with H7 except that H7 lacks the first two hydrophobic domains (A and B). These two domains are predicted to exhibit the hydrophobic properties of membrane-associated surface or globular domains rather than properties of transmembrane regions (7).

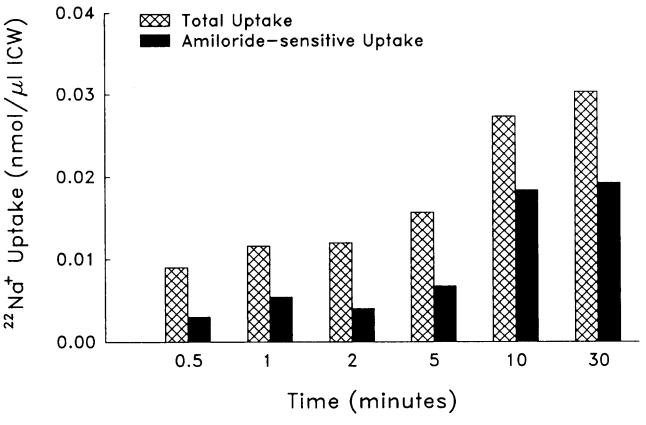

FIG. 7. Amiloride-sensitive Na^+ uptake in PS120 cells transfected with clone H7. PS120 cells, lacking Na^+/H^+ exchange activity, were transfected by a calcium phosphate technique with pCMV-4 plasmid containing H7 cDNA. Following acid selection with 50 mM NH_4Cl, surviving cells were removed and amiloride-sensitive Na^+ uptake was determined. ICW, intracellular water.

H7 was most highly homologous to NHE-4 at the amino acid level. Comparison between H7 and NHE-4 (Fig. 5) shows 56.2% amino acid sequence identity. H7 contains three potential N-linked glycosylation sites with the consensus sequence Asn-Xaa-Ser/Thr. The site at Asn^{235} is conserved among all reported Na^+/H^+ exchangers. There are seven potential protein kinase C sites which are located within the cytoplasmic domain and which have the consensus sequence (Ser/Thr)-Xaa-(Arg/Lys). There are also five potential cAMP- and cGMP-dependent protein kinase phosphorylation sites within the cytoplasmic domain, at Thr^{479}, Ser^{528}, Ser^{546}, Ser^{608}, and Ser^{683}. The consensus sequence for this kinase is $(Arg/Lys)_2$-Xaa-(Ser/Thr).

Northern Blot Analysis and Tissue Distribution of H7 mRNA. Two transcripts (4.6 and 4.4 kb) were recognized in poly(A)$^+$ RNA from large intestine, jejunum, ileum, stomach, liver, and uterus (Fig. 6). The message level was lower in the ileum than in the jejunum. This tissue distribution is different from that recognized with NHE-1, NHE-3, and NHE-4. The hybridization to two transcripts in these tissues suggests an alternatively spliced gene product.

Expression of H7 in Na^+/H^+ Exchanger-Deficient Cells. To demonstrate that clone H7 encoded a polypeptide with Na^+/H^+ exchange function, we used a calcium phosphate precipitation technique to transfect PS120 mutant cells with the expression vector pCMV4 containing H7 cDNA. Following acid selection, intracellular pH determination and ^{22}Na uptake were determined. These measurements were compared with those for control cells which were treated with calcium phosphate without the expression plasmid and for cells transfected with the expression vector without H7 cDNA. Cells transfected with H7 cDNA showed amiloride-sensitive ^{22}Na uptake (Fig. 7) and recovered from an acid load (Fig. 8), unlike the control cells.

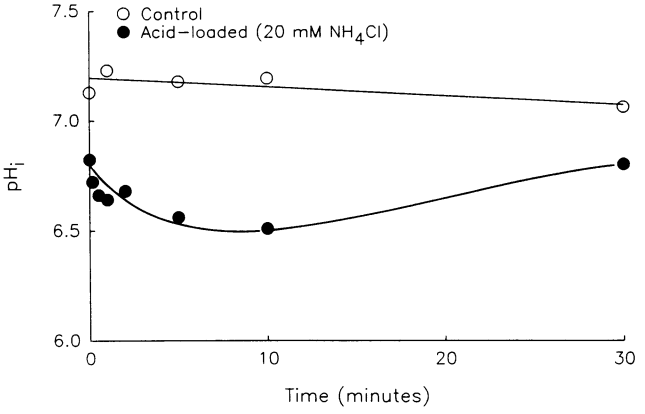

FIG. 8. Recovery of intracellular pH in PS120 cells stably transfected with clone H7. Stably transfected PS120 cells were loaded with either choline buffer (○) or acid-loaded with 20 mM NH_4Cl (●). Intracellular pH (pH_i) was measured from [^{14}C]benzoic acid with [^3H]mannitol as a marker for extracellular space.

DISCUSSION

This study demonstrates the cloning, sequencing, tissue distribution, and functional expression of a Na^+/H^+ exchanger which was isolated from a rat intestinal cDNA library. This cDNA, H7, shows high nucleotide sequence homology to all reported Na^+/H^+ exchangers. The tissue distribution and the sequence of this cDNA clone are different from the three other identified rat clones NHE-1, NHE-3, and NHE-4 (7–9). H7 cDNA has a 58.3%, 59.6%, and 66.2% identity at the nucleotide level and 47.8%, 41%, and 56.2% identity at the amino acid level with NHE-1, NHE-3, and NHE-4, respectively. H7 also shows 47.8% and 57.2% identity at the nucleotide and amino acid level with the human NHE-1. Highest homologies occur within the N-terminal region where the putative transmembrane regions are located. The H7 deduced amino acid sequence shows complete identity to a clone of Shull and coworkers, except for the fact that their clone has another 116 amino acids at the 5′ end of the open reading frame (G. Shull, personal communication). The tissue distribution of clone H7 is different from that of the reported Na^+/H^+ exchangers NHE-1, -3, and -4. Our clone recognizes poly(A)$^+$ RNA transcripts in the jejunum, large intestine, and stomach, as well as the liver and uterus. This distribution suggests a relative functional role in the gastrointestinal tract. We cannot detect any message in the kidney as seen with NHE-1, NHE-3, and NHE-4. A strong hybridization signal is seen in the liver, unlike NHE-3 and NHE-4.

The 5′ end of our cDNA clone does not share sequence homology with the other Na^+/H^+ exchangers, and this may suggest that the predominant transcript of this clone is alternatively spliced. This finding is supported by the observation that the 5′-end fragment of the H7 cDNA recognizes mRNA transcripts in various tissues that are similar to the mRNAs recognized by the full-length clone. Moreover, we used the reverse transcriptase–PCR technique to amplify a DNA fragment of the expected size from rat ileal cDNA by using primers from the 5′ end of H7 and from within the open reading frame. Sequencing of this fragment showed complete identity to the 5′ end of H7. This suggests that the divergent sequence at the 5′ end of H7 is not due to a cloning artifact of the cDNA library. The facts that two transcripts are recognized on the Northern blot and that G. Shull (personal communication) has isolated a similar clone with 100 extra amino acids in the open reading frame suggest possible alternative splicing. Therefore, the H7 cDNA is most likely an alternatively spliced transcript which varies at the 5′ end of the open reading frame from the other NHE isoforms.

The findings of amiloride-sensitive Na^+ uptake suggest that the H7 protein is functional despite lacking the first two hydrophobic domains which are present in other reported Na^+/H^+ exchangers. The biological function of the polypeptide encoded by H7 was determined by expression in PS120 cells, which are mutant Chinese hamster lung fibroblasts that lack Na^+/H^+ exchange activity.

The above findings, along with the hydropathy plot showing 10 possible transmembrane domains, and the close homology of H7 with the other human, rat, and rabbit Na^+/H^+ exchangers, suggest that we have cloned and expressed another member of the Na^+/H^+ exchanger family. The N and C termini of the H7 deduced amino acid sequence are divergent from those of the known Na^+/H^+ exchanger isoforms. The C terminus contains the regulatory domains which include potential phosphorylation sites. H7 contains seven potential phosphorylation sites for protein kinase C and five potential sites for cAMP- and cGMP-dependent phosphorylation within this C-terminal region. NHE-1 does not contain any potential cAMP-dependent phosphorylation sites, whereas NHE-3 and NHE-4 do contain potential phosphorylation sites for cAMP-dependent kinase and protein kinase C. The N terminus of the H7 predicted amino acid sequence has the properties required to catalyze amiloride-sensitive Na^+/H^+ exchange and the features which confer specificity of expression of the protein. The first hydrophobic domain in NHE-1 and NHE-3 is predicted to contain a peptide signal, suggesting that this domain might be cleaved off in the mature protein (9). This domain is absent in the protein encoded by H7 cDNA.

The physiological significance and the cellular localization of this exchanger are not known. However, studies utilizing antibodies against this and related proteins may shed light on this important family of exchangers.

Note Added in Proof. Subsequent Northern blot analysis with NHE-2 cDNA shows hybridization to the following additional rat tissues: kidney, spleen, lung, brain, and testis.

This study was funded by National Institutes of Health Grant DK41274.

1. Aronson, P. S. (1985) *Annu. Rev. Physiol.* **47,** 545–560.
2. Murer, H., Hopfer, U. & Kinne, R. (1976) *Biochem. J.* **155,** 597–604.
3. Pouyssegur, J. (1985) *Trends Biochem. Sci.* **10,** 453–455.
4. Kikuchi, K., Abumrad, N. & Ghishan, F. K. (1988) *Gastroenterology* **95,** 388–393.
5. Acra, S., Dykes, W., Nylander, W. & Ghishan, F. K. (1993) *Am. J. Physiol.* **264,** G45–G50.
6. Sardet, C., Franchi, A. & Pouyssegur, J. (1989) *Cell* **56,** 271–280.
7. Orlowski, J., Kandasamy, R. & Shull, G. (1992) *J. Biol. Chem.* **267,** 9331–9339.
8. Tse, C. M., Ma, A. I., Yang, V. W., Watson, A. J. M., Levine, S., Montrose, M. H., Potter, J., Sardet, C., Pouyssegur, J. & Donowitz, M. (1991) *EMBO J.* **10,** 1957–1967.
9. Tse, C. M., Brant, S. R., Walker, M. S., Pouyssegur, J. & Donowitz, M. (1992) *J. Biol. Chem.* **267,** 9340–9346.
10. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
11. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
12. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY), pp. 7.43–7.50.
13. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **1,** 2745–2752.
14. Kletzien, R. F., Pariza, M. W., Becker, J. & Potter, V. R. (1975) *Anal. Biochem.* **68,** 537–544.
15. L'Allemain, G., Paris, S. & Pouyssegur, J. (1984) *J. Biol. Chem.* **259,** 5809–5815.
16. Kozak, M. (1986) *Cell* **44,** 283–292.