THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 16, Issue of June 5, pp. 11917-11924, 1993
Printed in U.S.A.

Cloning and Expression of a Rabbit cDNA Encoding a Serum-activated Ethylisopropylamiloride-resistant Epithelial Na^+/H^+ Exchanger Isoform (NHE-2)*

(Received for publication, December 22, 1992)

Chung-Ming Tse‡, Susan A. Levine, C. H. Chris Yun, Marshall H. Montrose, Peter J. Little§, Jacques Pouyssegur¶, and Mark Donowitz

From the Departments of Physiology and Medicine, Division of Gastroenterology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and the ¶Center de Biochimie-Center National de la Recherche Scientifique, Parc Valrose, 06034 Nice, France

A unique Na^+/H^+ exchanger isoform, NHE-2, was cloned and characterized. NHE-2 is a protein of 809 amino acids with a calculated size of 90,787. It exhibits overall amino acid identity of 50, 44, and 60% with other cloned mammalian Na^+/H^+ exchangers NHE-1, NHE-3, and NHE-4, respectively. Northern blot analysis of poly(A^+) RNA isolated from rabbit ileum, kidney cortex, and kidney medulla using NHE-2 cDNA as a probe revealed messages of 5.2, 4.2, and 3.2 kilobases with relative abundance (in descending order) kidney medulla > kidney cortex > ileum. More detailed tissue distribution of message was performed by ribonuclease protection assay. NHE-2 was predominantly expressed in kidney, intestine, and adrenal gland with a small amount in skeletal muscle and trachea. Stable expression of NHE-2 in PS120 fibroblasts confirmed that NHE-2 is a functional Na^+/H^+ exchanger which is defined by amiloride-sensitive Na^+-dependent alkalinization of acid-loaded cells. NHE-2 has the same K_i for amiloride inhibition as NHE-1 (1 μM) but is 25-fold more resistant to ethylisopropylamiloride inhibition than is NHE-1 (500 *versus* 20 nM). Like NHE-1, NHE-2 can be activated by serum. Expression of NHE-2 in a polarized human intestinal epithelial cell line, Caco-2 cells, results in functional expression of NHE-2 in the apical membrane. Thus, we conclude that NHE-2 is a candidate to be an apical membrane Na^+/H^+ exchanger in intestinal and renal epithelial cells.

Na^+/H^+ exchangers (NHEs)[1] are plasma membrane proteins which catalyze the exchange of extracellular Na^+ for intracellular H^+ in an electroneutral mode with a stoichiometry of 1:1 and are found in all mammalian cells (for reviews, see Refs. 1–3). They are responsible for regulation of intracellular pH (pH_i) and maintenance of cellular volume. In epithelial cells, Na^+/H^+ exchangers are also involved in transepithelial Na^+ absorption (4).

The first cloned human Na^+/H^+ exchanger was reported by Sardet and Pouyssegur (5, 6). This cloned Na^+/H^+ exchanger is now called NHE-1. It is a glycoprotein of 815 amino acids and can be stimulated by growth factors via phosphorylation of the protein (6). Using a *Bam*HI-*Bam*HI fragment of human NHE-1 cDNA, we isolated a 4-kb cDNA encoding a rabbit NHE-1. The deduced protein has 816 amino acids and exhibits 95% identity to the human homologue (7).

Existence of multiple isoforms of Na^+/H^+ exchangers was initially suggested based on pharmacological, kinetic, and immunological studies of Na^+/H^+ exchangers in various cell types, tissues, and species (for review, see Ref. 8). For instance, in epithelial cells where Na^+/H^+ exchangers are found in both apical and basolateral membranes, the apical membrane Na^+/H^+ exchanger, which is involved in transcellular Na^+ absorption, is more resistant to amiloride and ethylisopropylamiloride (EIPA) inhibition than its basolateral counterpart (9, 10), and the cultured hippocampal neuron Na^+/H^+ exchanger is not inhibited by either amiloride or its 5′-amino-substituted analogues (11). Using antibodies against the cytoplasmic domain of NHE-1, we found that NHE-1 has a polarized distribution in ileal epithelial cells, localizing to the basolateral membrane of both ileal villus and crypt epithelial cells (7). In addition, Pouyssegur, Huet, and Louvard[2] found that NHE-1 is found only on the basolateral membrane of Caco-2 cells. Similarly, using antibodies against an extracellular domain of the cloned porcine NHE-1, it was found that porcine NHE-1 also localizes to the basolateral membrane of LLC-PK_1/Cl_4 cells (12). This evidence suggests that the apical membrane Na^+/H^+ exchanger is molecularly distinct from the basolateral isoform. We previously established the existence of a mammalian Na^+/H^+ exchanger gene family by genomic Southern blot analysis (7) and by cloning and sequencing an additional putative member of the Na^+/H^+ exchanger gene family in rabbit, NHE-3 (13). NHE-3 was also cloned by

* This work was supported in part by National Institutes of Health Grants R01DK26523, R01DK42457, R29DK43778, and P01DK44484, Institutional Training Grant T32DK07632, and National Research Service Awards F32DK08820 and F32DK08672. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L13733.

‡ To whom correspondence should be addressed: Dept. of Medicine, GI Division, The Johns Hopkins University School of Medicine, 918 Ross Research Bldg., 720 Rutland Ave., Baltimore, MD 21205–2195. Tel.: 410-955-9685; Fax: 410-955-9677.

§ Current address: Baker Medical Research Inst., Alfred Hospital, Melboure 3181, Australia.

[1] The abbreviations used are: NHE, Na^+/H^+ exchanger; EIPA, ethylisopropylamiloride; bp, base pair; kb, kilobase(s); PIPES, 1,4-piperazinediethanesulfonic acid; SSC, sodium chloride/sodium citrate; FBS, fetal bovine serum; BCECF, 2′7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; TMA, tetramethylammonium.

[2] J. Pouyssegur, L. Huet, and D. Louvard, personal communication.

Orlowski *et al.* (14), who identified an additional isoform called NHE-4 from rat. Among these gene family members, NHE-1 has been characterized in most detail (3, 5, 6, 15). Functional properties of both NHE-3 and NHE-4 have not been reported. In this paper, we report the cloning, sequencing, and expression of a unique rabbit Na^+/H^+ exchanger isoform, called NHE-2. NHE-2 is an EIPA-resistant Na^+/H^+ exchanger isoform and can be stimulated by serum. When it is expressed in a polarized intestinal epithelial cell, Caco-2, its functional activity is found on the apical membrane.

EXPERIMENTAL PROCEDURES

cDNA Cloning

We previously obtained a 4-kb cDNA encoding the rabbit NHE-1 and showed by genomic Southern blot analysis that the 462-bp *Pst*I-*Acc*I restricted fragment of rabbit NHE-1 (encoding amino acids 157–310) contained sequences conserved among the gene members of the Na^+/H^+ exchanger gene family. Therefore, we rescreened the rabbit ileal villus cell cDNA library in λ_{ZAP} using this 462-bp cDNA probe under conditions of differential hybridization. 400,000 phages were plated at a low density of 100,000 plaque-forming units/22 × 22-cm^2 culture dish and were transferred to nylon filters in triplicate; one of which was screened under high stringency conditions (hybridized at 42 °C in 50% formamide, 4 × SSC, 5 × Denhardt, 1% SDS and washed at 62 °C in 1 × SSC and 0.1% SDS) and the remaining two were screened under low stringency conditions (hybridized at 42 °C in 35% formamide, 4 × SSC, 5 × Denhardt, 1% SDS and washed at 42 °C in 1 × SSC and 0.1% SDS). Those clones that did not hybridize with the probe under high stringency conditions but hybridized and appeared in duplicate filters under low stringency conditions were scored positive. Two such clones, called RA1 and RA5, were identified. These two clones were plaque-purified, and their inserts were rescued into pBluescript SK by *in vivo* excision by co-infecting the XL1-blue cells with the helper phage R408, as described previously (7). Restriction digestion with *Eco*RI showed that RA1 is 2.9 kb and RA5 is 1.1 kb. End terminal sequencing of these two clones showed that they were overlapping and had homology with, but were not identical to, NHE-1. RA5 (Fig. 1) was then further sequenced completely in both strands, confirming that this clone encoded another isoform Na^+/H^+ exchanger (now called NHE-2). To obtain the full-length NHE-2 cDNA, RA5 was used to rescreen the ileal villus cell cDNA library under high stringency conditions (hybridized at 42 °C in 50% formamide, 4 × SSC, 5 × Denhardt and at a final wash at 62 °C in 0.1 × SSC, 0.1% SDS).

RNA Isolation

Intestinal mucosa from rabbit duodenum, jejunum, ileum, ascending colon, and descending colon was obtained by light scraping of the corresponding tissues on ice with a glass slide (16). Total RNA from various rabbit tissues, cultured cells (rabbit skin fibroblasts, a lymphoblast cell line, TP-3), and the above intestinal mucosa were isolated by a commercially available kit, RNA_{ZOL} (Tel/Test Inc.). Poly(A)$^+$ RNA from rabbit ileal mucosa, kidney cortex, and kidney medulla was purified from the corresponding total RNA by two passages of affinity chromatography on oligo(dT)-cellulose.

Northern Blot Analysis

2 μg of poly(A)$^+$ RNA from rabbit ileal villus mucosa, kidney cortex, and kidney medulla was denatured with glyoxal, size-fractionated by 1% agarose gel electrophoresis, and transferred to a nylon filter by capillary blotting (17). The membranes were prehybridized for 2 h under high stringency conditions, as described above for library screening. Hybridization was carried out for 20 h in the same solution containing 10^6 cpm/ml of denatured ^{32}P-labeled RA14 cDNA as described in Fig. 1. RA14 cDNA was labeled by the random-primed method. Washing was also performed under high stringency conditions with a final wash at 62 °C, 0.1 × SSC, 0.1 × SDS. The blot was analyzed by autoradiography using Kodak XAR film.

Ribonuclease Protection Assay

Tissue distribution of rabbit NHE-2 message was determined by ribonuclease protection assay with the commercially available ribonuclease protection assay kit (Ambion). NHE-2 antisense cRNA probe (223 bp corresponding to nucleotides 1773–1995 and amino acid residues 592–665) was transcribed *in vitro* from pBluescript using T7 RNA polymerase and radioactive [^{32}P]CTP (800 Ci/mmol), as described by Melton *et al.* (18). After the transcription reaction, the DNA template was removed by digestion with RQ1 RNase-free DNase (Promega). ^{32}P-Labeled NHE-2 antisense cRNA probe was purified by a centrifugation-Sephadex column. For ribonuclease protection assay, labeled antisense cRNA probes were hybridized overnight at 42 °C with 30 μg of total RNA isolated from multiple rabbit tissues or the cultured cell lines. Hybridization solution contained 80% formamide, 40 mM PIPES, pH 6.4/400 mM NaOAc, 1 mM EDTA. Following hybridization, the hybridization mixtures were treated with 0.01 unit RNase A and 20 units of RNase T1 at 37 °C for 30 min to degrade single-stranded unhybridized probe. Labeled probes that hybridized to their complementary RNA, and thus were "protected" from RNase digestion, were precipitated with ethanol and resuspended in DNA loading buffer. Half of each sample (which represents 15 μg of the total RNA initially added) was separated on a 6% denaturing polyacrylamide gel, and the gel was analyzed by autoradiography.

cDNA Sequencing

Sequencing of cDNA clones was performed on both strands by Sanger's dideoxy termination procedure. Progressive unidirectional deletion clones were obtained by the method of exonuclease III/mung bean nuclease digestion. Deletion plasmids were purified by the alkaline lysis method and used as double-stranded templates for sequencing.

Cell Culture

PS120 (a Na^+/H^+ exchanger-deficient derivative of the Chinese hamster lung fibroblast cell line CCL39) and Caco-2 cells (a polarized human colonic carcinoma cell line) (control and transfected cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM $NaHCO_3$, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 1% nonessential amino acids (only for Caco-2 cells), pH 7.4, in a 5% CO_2, 95% air incubator at 37 °C. G418 (400 μg/ml) was used to maintain selection pressure in transfected cells and was added immediately after subculturing.

Stable Transfection

PS120 Cells—RA11 cDNA which contains the entire coding sequence of NHE-2 (Fig. 1) was subcloned into pMAM-neo (Clontech) by blunt-ended ligation into the blunted *Nhe*I site of the pMAM-neo, and the resulting construct is called NHE-2/pMAM-neo. The NHE-2/pMAM-neo construct was transfected into PS120 cells by the method of $CaPO_4$ precipitation as described previously (7). Briefly, cells were split 1:20 the day before transfection and were transfected at approximately 30% confluency using 125 mM $CaCl_2$, 140 mM NaCl, 25 mM HEPES, 0.75 mM $NaHPO_4$, pH 7.05, and 5 μg of DNA/10-cm dish. After 16 h of DNA incubation, cells were shocked by 10% dimethyl sulfoxide for 3 min. 48 h after transfection, cells were subjected to double selection by growth in G418 (200 μg/ml) and acid loading with subsequent recovery in Na^+-containing medium. Stable clones that survived the double selection were individually selected, and five such clones were analyzed for Na^+/H^+ exchange activity using the pH-sensitive dye, 2′7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF), as described below. One of these clones, called NHE-2/PS120/2, was selected for further characterization.

Caco-2 Cells—Caco-2 cells were grown to 30% confluency on collagen coated (rat tail collagen) 10-cm Petri dishes and were transfected with the NHE-2/pMAM-neo construct by $CaPO_4$ precipitation as described above for PS120 cells. 48 h after transfection, cells were selected in complete medium containing G418 (400 μg/ml). Stable clones which exhibited G418 resistance after 3 weeks of G418 selection were pooled and expanded into a mixed cell population, NHE-2/Caco-2 cells.

Measurement of Na^+/H^+ Exchange Activity: Fluorescence Measurement with BCECF

NHE-2/PS120/2—Cells were grown to 70–80% confluency on glass cover slips and then serum starved for 17 h to arrest growth. The culture medium was then removed and the cells washed twice with 3 ml of Na^+ medium (containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $NaPO_4$, 25 mM glucose, 20 mM HEPES, pH 7.4). The cells were then loaded with the acetoxymethyl ester of BCECF

(5 μM) in Na^+ medium for 60–90 min at 22 °C. The cells were washed three times with TMA medium (containing 130 mM TMA-Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM TMA-PO_4, 25 mM glucose, 20 mM HEPES, pH 7.4) to remove the extracellular dye, and the coverslip was mounted at 45° in a perfusion cuvette which allowed separate perfusion of apical and basolateral chambers (17). The basolateral chamber was filled with TMA-Cl medium but not perfused, whereas the apical chamber was perfused with TMA-Cl medium at a rate of 10 volume changes/min (1 ml/min). A heating block maintained the temperature at 37 °C. The cells were pulsed with 40 mM NH_4Cl for 3–5 min, depending on the degree of acidification desired. Removal of NH_4Cl and perfusion with TMA-Cl resulted in stable acidification of the cells. The cuvette was then perfused with Na^+ medium, and pH recovery was measured. Na^+/H^+ exchange was defined as sodium-dependent amiloride-sensitive cell alkalinization after the acid load. When the effect of fetal bovine serum (FBS) on Na^+/H^+ exchange was studied, cells were acidified and were allowed to reach a steady state. 10% FBS (final concentration) was then added and allowed to come to a new steady state. Fluorescence was measured in an SLM spectrofluorometer (SPF500C, SLM, Urbana, IL). Excitation wavelengths for BCECF were alternated between 440 ± 1 and 500 ± 1 nm, and emission was monitored at 530 ± 10 nm. A CompuAdd 286 computer regulated the fluorometer and collected, analyzed, and stored the data. Autofluorescence was determined daily from cells not loaded with BCECF. Autofluorescence-corrected fluorescence ratio values (500/440) were calculated and graphically presented every 3 s during the experiment. The pH of the medium was continuously monitored from the efflux port of the cuvette by a mini pH electrode (MI-506: Microelectrodes, Inc, Londonderry, NJ). A calibration curve for intracellular BCECF was constructed by equilibrating cells in pH clamp medium with 10 μM nigericin and titrating pH with additions of HNO_3. At the end of each experiment, cells were perfused with pH clamp medium, pH 7.40, with 10 μM nigericin, and a single point calibration was used to normalize the data to this calibration curve.

Caco-2 Cells—Both untransfected Caco-2 cells and NHE-2/Caco-2 cells were seeded on Cyclopore membranes® (Falcon, Beckton Dickinson, Oxnard, CA) glued over an aperture in a plastic coverslip and grown for 14 days (approximately 10 days postconfluence). The method of BCECF-loading and the mounting of cells grown on this permeable filter in a cuvette is as described above for PS120 cells. Both apical and basolateral surfaces were perfused independently.

$^{22}Na^+$ Uptake Studies

NHE-2/PS120/2 were grown to near confluency in 24-well plates and were serum-starved to arrest growth for 17–24 h. Cells were then washed two times with 1 ml of TMA medium and then incubated in 1 ml of TMA medium containing 30 mM NH_4Cl for 20 min at room temperature. Acidification was induced by aspiration of the NH_4Cl solution and rapidly washing cells with 1 ml of TMA medium. $^{22}Na^+$ uptake was measured at 22 °C for 2 min by adding 0.5 ml of incubation medium to the cells (2 mM NaCl, 128 mM TMA-Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM K_2PO_4, 25 mM glucose, 20 mM HEPES, pH 7.4, 1 mM ouabain, and 1 μCi/ml of $^{22}NaCl$) in the presence and the absence of various concentrations of amiloride or EIPA as indicated. The Na^+ uptake rate was linear with time for at least 5 min (data not shown). At the end of the incubation, cells were rapidly washed five times with 1 ml of ice-cold 0.1 M $MgCl_2$. Intracellular $^{22}Na^+$ then was released by lysing the cells with 0.5 ml of 0.1 M HNO_3, and radioactivity was measured with a γ counter.

RESULTS

Cloning and Sequencing a Composite cDNA Encoding a Rabbit Na^+/H^+ Exchanger Isoform NHE-2—To identify further members of the mammalian Na^+/H^+ exchanger gene family, we rescreened a rabbit ileal villus cell cDNA library in λ_{ZAP} using the *PstI-AccI* cDNA fragment of rabbit NHE-1 under differential hybridization conditions of high and low stringency as described under "Experimental Procedures." Of 400,000 plaques screened, two positive clones were obtained. These two clones were initially characterized by sequencing both ends of the cDNA inserts. One of these two clones, called RA5 which is 1.1 kb (Fig. 1), was completely sequenced in both strands, and its amino acid sequence was deduced based on its homology with NHE-1. It has 60% amino acid identity with NHE-1. Therefore, this suggests that RA5 represents a partial cDNA encoding a distinct isoform of Na^+/H^+ exchanger, which is called NHE-2. In order to obtain a full-length NHE-2 cDNA, RA5 was used to rescreen the same ileal villus cell cDNA library under high stringency conditions. More than 20 positive clones were obtained. Two of these clones, RA11 and RA14 (Fig. 1), were sequenced completely in both strands and gave the complete sequence of rabbit NHE-2.

Fig. 2 displays the composite nucleotide and amino acid sequences of rabbit NHE-2. The composite sequence contains 3877 bp with 115 bp of 5′-untranslated region, 2430 bp of an open reading frame, and 1332 bp of 3′-untranslated region. Mini-cistrons were identified in the 5′-untranslated regions of rabbit NHE-1 and NHE-3 cDNAs (7, 13, 14); however, this was not found in the corresponding region of rabbit NHE-2 cDNA. Instead, an in-frame stop codon is found 55 nucleotides upstream of the putative initiation codon. In the 1332-bp 3′-untranslated region sequenced, two potential polyadenylation signals were identified (AATAAA at nucleotides 3604–3609 and ATTAAA at nucleotides 3676–3681), and a polyadenylation tail was found 19 bp downstream of the second polyadenylation signal, suggesting that the second polyadenylation signal was being used in the mRNA from which RA14 was transcribed. In another independent partial cDNA clone, called RA6, an additional 69 bp in the 3′-untranslated sequence was identified (Figs. 1 and 2), further suggesting that multiple polyadenylation signals were used.

The amino acid sequence deduced from the longest open reading frame of NHE-2 cDNA revealed a protein of 809 amino acids with a calculated M_r of 90787. The designated ATG initiation codon of NHE-2 (TCCATGC) was not in a good agreement with the Kozak's consensus sequence (20) for translation initiation (A/GCCATGG). However, there was no other in-frame initiation codon found further upstream in the cDNA. The next ATG triplet is 127 bp downstream (encoding Met^{43}), and the sequence GCCATGG containing the Met^{43} fits well with the Kozak consensus sequence. This ATG triplet is less likely to be the initiation codon, because the predicted topology of that deduced protein lacks the first putative membrane-spanning domain which is present in NHE-1, NHE-3, and NHE-4 (Fig. 3*A*).

By sequencing multiple overlapping cDNA clones, it was found that there were base substitutions at $C^{819} \rightarrow A$; $T^{1038,1050,1083,1828} \rightarrow C$ and $C^{1100} \rightarrow T$. Only the substitution of $T^{1828} \rightarrow C$ led to a change in deduced amino acids because of codon degeneracy (tyrosine 610 was substituted by histidine).

The hydrophobicity plot of rabbit NHE-2 is very similar to those of rabbit NHE-1 and NHE-3 and rat NHE-4 (data not shown) and is predicted to have 12 membrane-spanning domains and a long cytoplasmic tail (5, 7, 13–15). Fig. 3*A* compares the amino acid sequences of the putative membrane-spanning domains of rabbit NHE-1, NHE-2, and NHE-3 and rat NHE-4 (rabbit NHE-1, NHE-2, and NHE-3 and rat NHE-4 contain 816, 809, 832, and 717 amino acids, respectively). Structure-function analysis of NHE-1 has suggested that the membrane-spanning domains are responsible for ion exchange, whereas the cytoplasmic domain is the regulatory part of the molecule (3, 15). The N terminus is the more conserved domain, whereas the C terminus is more divergent among NHEs and becomes even more divergent towards the C terminus. Surprisingly, the cytoplasmic domain of NHE-2 exhibits high homology with that of rat NHE-4. Fig. 3*B* shows the amino acid alignment of the most highly homologous region in the putative cytoplasmic domain of rabbit NHE-2 (amino acids 481–680) with that of rat NHE-4 (amino acids 473–671). This homologous region has 60% amino acid iden-

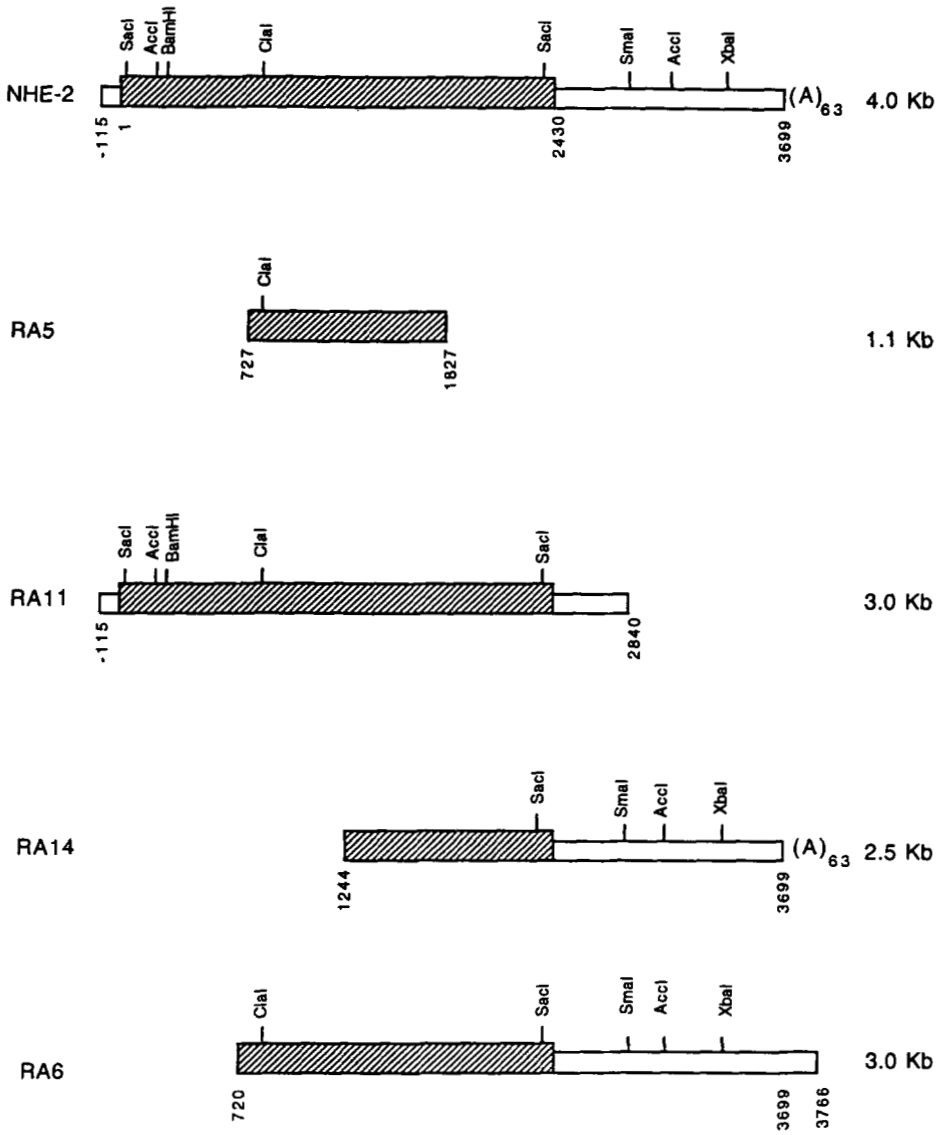

FIG. 1. **Diagrammatic representation of rabbit NHE-2 partial cDNA clones.** The protein coding region is represented by the *hatched area* and the noncoding regions by *open bars*. The composite NHE-2 cDNA is 3877 bp long with an open reading frame of 2430 bp. Nucleotide numbers are indicated on the bottom of partial cDNA clones, the sizes of which are indicated at the *right*. Also shown are some restriction sites on NHE-2 cDNA.

tity between the two isoforms. Overall, rabbit NHE-2 exhibits 50 and 44% amino acid identity with NHE-1 and NHE-3, respectively. It exhibits 60% amino acid identity with rat NHE-4, despite the comparison being made across species. Fig. 3*C* shows a phylogenetic tree generated by comparison and alignment of these four NHE amino acid sequences. This tree shows that rabbit NHE-2 and rat NHE-4 are more closely related (*i.e.* scored higher percent in amino acid identity) to each other than to rabbit NHE-1 and that rabbit NHE-3 is the least related isoform.

Like other NHEs, NHE-2 is likely a glycoprotein as three potential *N*-linked glycosylation sites were found at Asn^{350}, Asn^{623}, and Asn^{685}. One of these sites is conserved among these gene family members; that is, Asn^{370} in NHE-1, Asn^{350} in NHE-2, Asn^{325} in NHE-3, and Asn^{342} in rat NHE-4. This asparagine is located on a putative extracellular loop between membrane spanning domains 7 and 8.

Northern Blot Analysis and Tissue Distribution of NHE-2 Message—In order to determine the size of NHE-2 message and to quantitate the relative amount of NHE-2 message expression in intestine and kidney, poly(A)$^+$ RNA was isolated from rabbit ileum, kidney cortex, and kidney medulla and analyzed by Northern blot analysis using RA14 cDNA (Fig. 1) as the probe. As shown in Fig. 4*A*, RA14 cDNA hybridized to messages of 5.2, 4.2, and 3.2 kb in ileum and kidney. The amount of NHE-2 message decreases in the order kidney medulla > kidney cortex > ileum. 5.2 kb is the most abundant message in all three.

Ribonuclease protection assay (18) was used to define in detail the magnitude of message distribution among various rabbit tissues. As shown in Fig. 4*B*, the NHE-2 message is expressed predominantly in kidney, intestine, adrenal gland, and minimally in skeletal muscle and trachea. Consistent with the Northern blot analysis (Fig. 4*A*), NHE-2 message decreases in the order kidney medulla > kidney cortex > ileum. NHE-2 message is expressed most in kidney medulla and ascending colon, followed equally by kidney cortex and adrenal gland, then, in decreasing order, descending colon > jejunum > ileum > duodenum > skeletal muscle and least in trachea. It is absent from brain, heart, liver, the cultured rabbit lymphoblast cell line, TP-3, and cultured rabbit skin fibroblasts.

Functional Characterization of NHE-2 cDNA—To establish that NHE-2 cDNA encoded a functional Na^+/H^+ exchanger, the NHE-2/pMAM-neo construct was transfected into PS120 by $CaPO_4$ precipitation (7). PS120 cells were derived from Chinese hamster lung fibroblasts and had been selected to be deficient in all endogenous Na^+/H^+ exchange activity (21), which was confirmed by us (data not shown). One of these stably transfected clones, called NHE-2/PS120/2, was used

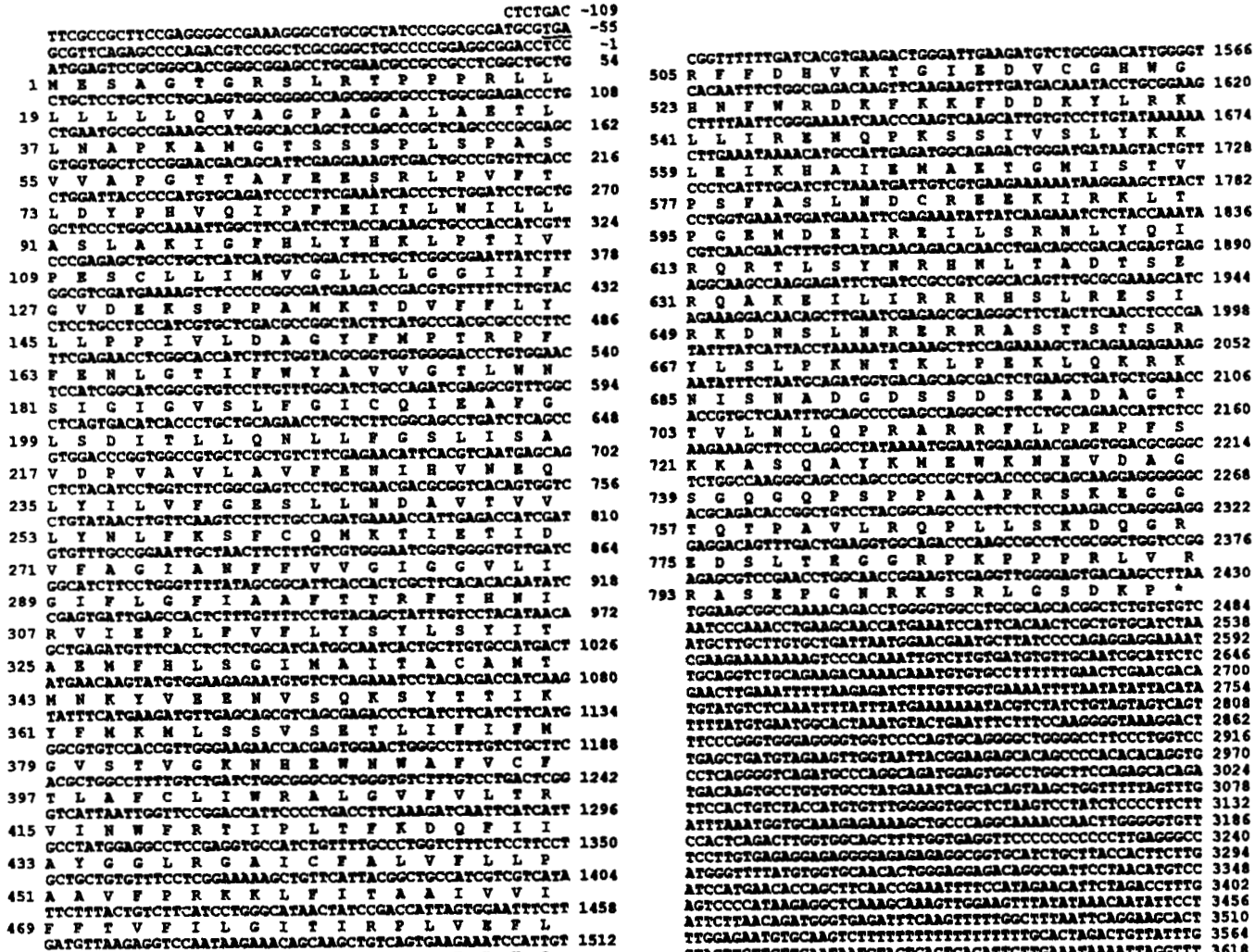

FIG. 2. **Nucleotide sequence of rabbit NHE-2 and the deduced amino acid sequence of the protein.** Nucleotides are numbered at the *right* of the sequence with respect to the putative translation initiation site. Amino acids are numbered at the *left* of the sequence and are represented by their single letter abbreviations. The in-frame upstream stop codon and the two putative polyadenylation signals are *underlined*. * indicates the stop codon of the longest open reading frame. Base pair variations were found at positions: $C^{819} \rightarrow A$; $T^{1038,1050,1083,1828} \rightarrow C$; $C^{1100} \rightarrow T$. In the partial cDNA clone, RA6 (see Fig. 1), instead of the poly(A) tract found starting at nucleotides 3700, the sequence extends further downstream.

for detailed functional characterization. Na^+/H^+ exchange activity in NHE-2/PS120/2 was measured by fluorescence measurement with the pH sensitive dye, BCECF, and was defined as amiloride-sensitive Na^+-dependent alkalinization of acid-loaded cells. As shown in Fig. 5*A*, NHE-2/PS120/2 cells, which were acidified previously by exposure to 40 mM NH_4Cl, remained acidified at a constant pH_i in TMA medium. Addition of Na^+ medium (130 mM) allowed the cells to recover from the acid load. This Na^+-dependent pH_i recovery was totally inhibited by addition of 1 mM amiloride, suggesting that NHE-2 was a functional Na^+/H^+ exchanger.

Epithelial apical Na^+/H^+ exchangers are known to be resistant to amiloride and its 5-amino-substituted analogues (8, 9). Thus, the inhibition of NHE-2 by amiloride and EIPA was further characterized. Fig. 5*B* shows the concentration dependence for amiloride and EIPA inhibition of the initial rate (studied at 2 min with Na^+ uptake rate linear for at least 5 min) of $^{22}Na^+$ (2 mM) uptake into acid loaded NHE-2/PS120/2 cells. The K_i values for amiloride and EIPA were 1 μM and 500 nM, respectively.

Serum has been shown to activate NHE-1 (7). In order to study whether NHE-2 could also be regulated by serum, the effect of serum on NHE-2 was studied at steady-state pH_i. That is, NHE-2/PS120/2 cells were acidified and allowed to recover in Na^+ medium until a steady-state pH_i was attained. 10% fetal bovine serum was added at this steady-state pH_i. As shown in Fig. 5*C*, addition of fetal bovine serum to NHE-2/PS120/2 cells at steady-state pH_i (pH 7.45) induced further intracellular alkalinization to a new steady-state pH_i of 7.58, suggesting that NHE-2 was stimulated by serum.

Expression of NHE-2 in a Polarized Intestinal Epithelial Cell Line Caco-2—Caco-2 cells have been shown to lack any Na^+/H^+ exchange activity on their apical membrane (22). Na^+/H^+ exchange activity is restricted to the Caco-2 basolateral surface and is known to be NHE-1[2]. Thus, Caco-2 cells provide a useful system to study whether NHE-2, when expressed in polarized intestinal epithelial cells, could be functionally expressed in the apical membrane. NHE-2/pMAM-neo construct was transfected into Caco-2 cells, under the

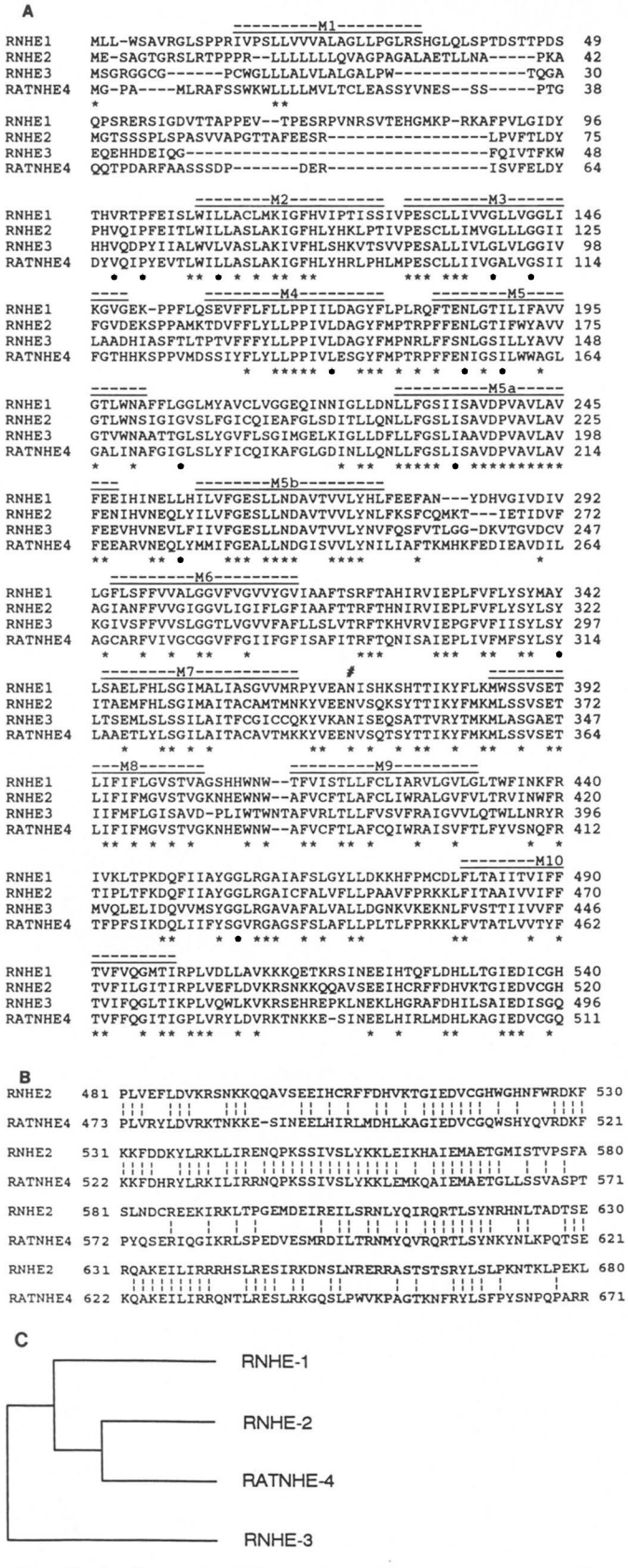

FIG. 3. *A*, alignment of the amino acid sequences of putative membrane spanning domains of rabbit NHE-1 (*RNHE1*, amino acids 1–540), rabbit NHE-2 (*RNHE2*, amino acids 1–520), rabbit NHE-3 (*RNHE3*, amino acids 1–496) and rat NHE-4 (*RATNHE4*, amino acids 1–511). Rabbit NHE-1 and NHE-3 sequence were obtained from Tse *et al.* (7, 13). Rat NHE-4 sequence was from Orlowski (1992). The entire rabbit NHE-1, rabbit NHE-2, rabbit NHE-3, and rat NHE-4 have 816, 809, 832, and 717 amino acids, respectively.

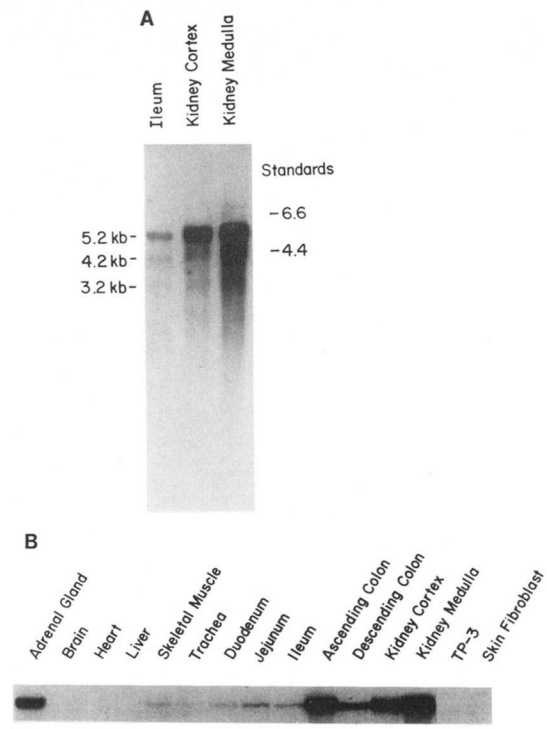

FIG. 4. **Northern blot analysis and tissue distribution of NHE-2 message.** *A*, Northern analysis. RA14 (see Fig. 1) was used as a probe to determine the expression and the size of the NHE-2 transcript by Northern analysis. 2 μg of poly(A)$^+$ RNA was used per each sample. DNA size standards are shown in the *right margin* and the size of the message is shown in the *left margin*. *B*, ribonuclease protection assay. Total RNA (30 μg) isolated from multiple rabbit tissues and cultured rabbit cells, as shown on the *top* of the figure, were hybridized overnight at 42 °C to a 223-bp antisense NHE-2 ^{32}P-cRNA probe which corresponded to nucleotides 1773–1995 under the conditions of 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaOAc, 1 mM EDTA. After hybridization, hybridization mixtures were treated with 0.01 unit of RNase A and 20 units of RNase T1 at 37 °C for 30 min. Labeled probes that had annealed to their complementary RNA were protected from RNase digestion and were ethanol-precipitated and resuspended in DNA loading buffer. Half of each sample (representing 15 μg of the total RNA initially added) was analyzed by 6% denatured polyacrylamide gel electrophoresis. The gel was then analyzed by autoradiography.

same conditions as described for PS120 cells, and transfectants were selected only by G418 resistance. Na^+/H^+ exchange activity was measured fluorometrically in cells loaded with BCECF and mounted in a chamber which allows separate apical and basolateral membrane perfusion (19). As reported previously (22) and as is shown in Fig. 6*A*, endogenous Na^+/H^+ exchange activity is restricted to the basolateral membrane of untransfected Caco-2 cells. Similarly, Caco-2 cells transfected with NHE-1 also showed only basolataeral membrane

Amino acids are indicated by their single letter abbreviation. Membrane-spanning domains are indicated by ===== (M1–M10, M5a, and M5b) using the definition for NHE-1 (5, 13, 15). The conserved *N*-linked glycosylation site is indicated by "#". Identical amino acids are represented by "*". Amino acid numbers are shown on the *right*. *B*, homologous region in the cytoplasmic domain of rabbit NHE-2 (*RNHE2*) and rat NHE-4 (*RATNHE4*). Alignment was made between amino acids 481–680 of rabbit NHE-2 and amino acids 473–671 in rat NHE-4. This area exhibited 60% amino acid identity between rabbit NHE-2 and rat NHE-4. In contrast, this area is not conserved among other NHEs. "¦" represents amino acid identity. *C*, phylogenetic tree generated by amino acid comparison among NHEs. This tree, which is generated using the program Clustal (PC gene; IntelliGenetics), shows that rabbit NHE-2 and rat NHE-4 are more closely related to each other than to rabbit NHE-1 and that NHE-3 is the least related isoform.

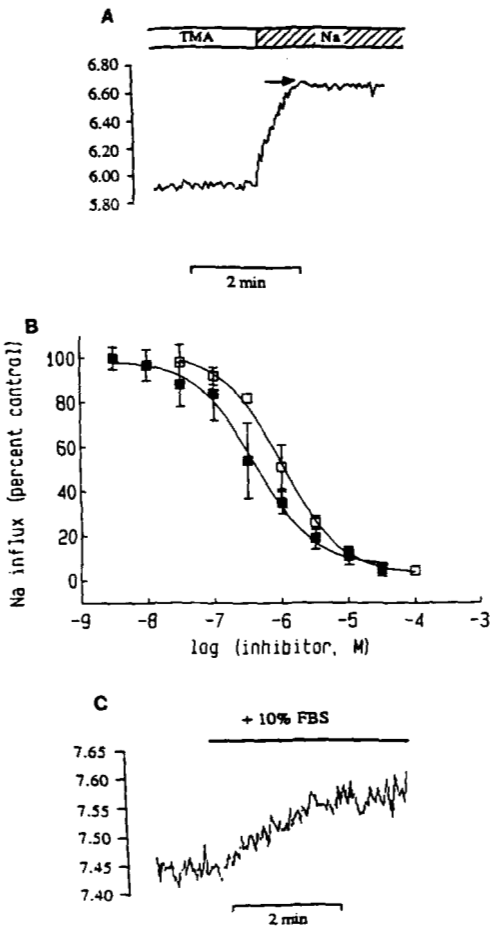

FIG. 5. **Functional characterization of NHE-2.** *A*, NHE2/PS120/2 was loaded with BCECF and studied spectrofluorometrically to measure pH_i. Cells were acidified by NH_4Cl prepulse. In the presence of Na^+, but not TMA^+ (both at 130 mM), cells were able to recover from the acid load. At the time indicated by an *arrow*, 1 mM amiloride was added and the rate of pH_i recovery was reduced almost to 0. *B*, concentration response curves for inhibition of $^{22}Na^+$ uptake by amiloride (□) and EIPA (■). Results are presented as mean ± S.E. of three experiments. The curves are nonlinear least squares fits of the data assuming a single binding site for the inhibition. From these curves, the IC_{50} were calculated to be 0.5 and 1 μM for EIPA and amiloride, respectively. *C*, regulation of rabbit NHE-2 by fetal bovine serum (*FBS*). NHE-2/PS120/2 cells loaded with BCECF were acidified by NH_4Cl prepulse and then allowed to recover to steady-state pH_i in "Na^+ medium." As shown here, the steady-state pH_i reached 7.45 in NHE2/PS120/2. At the time indicated in the figure by the *horizontal solid line*, perfusion was changed to Na^+ medium containing 10% FBS. FBS causes further alkalinization to a new steady-state pH_i of 7.58. This represents a typical trace from five identical experiments.

Na^+/H^+ exchange activity and no apical membrane Na^+/H^+ exchange activity (data not shown). In contrast, Caco-2 cells stably transfected with NHE-2 cDNA exhibited both apical and basolateral membrane Na^+/H^+ exchange activity (Fig. 6*B*), suggesting the presence of functional NHE-2 in the apical membrane.

DISCUSSION

Four members of the mammalian Na^+/H^+ exchanger gene family have been identified at a molecular level (5, 7, 13, 14). NHE-1 is the housekeeping Na^+/H^+ exchanger which is expressed ubiquitously in tissues and cells and is the basolateral Na^+/H^+ exchanger found in both intestine and kidney epithelial cells (7, 12). Unlike NHE-1, expression of NHE-3 and NHE-4 showed tissue specificity. NHE-3 is expressed only in intestine, kidney, and stomach (13, 14). NHE-4 is highly expressed in stomach and intestine and to lesser amounts in kidney, brain, uterus, and skeletal muscle (14). In this study, we demonstrated that expression of NHE-2 was also tissue-specific, being present predominantly in kidney, intestine, and adrenal gland and to a lesser amount in skeletal muscle and trachea. Thus, message expression of NHE-2 is not limited to intestine and kidney, and the functional significance of the differential epithelial message expression (kidney medulla > kidney cortex; ascending colon > descending colon > jejunum > ileum > duodenum) is not yet known.

At the protein level, rabbit NHE-2 is more related to rat NHE-4 than to the other identified NHE isoforms. They exhibited overall 60% amino acid identity which would be expected even to be higher if rabbit NHE-4 protein sequence were available for comparison. The putative cytoplasmic C terminus was the least conserved area among NHE-1, NHE-2, and NHE-3. The cytoplasmic C terminus of NHE-1 had been shown to contain sites for protein kinase phosphorylation, to be responsible for kinase and growth factor regulation, and to interact with the pH sensor. Since each Na^+/H^+ exchanger is likely to be regulated differently by protein kinases, the divergence in the C terminus may explain the specificity of each protein for kinase regulation. Of note, this area was highly homologous between NHE-2 and NHE-4 (Fig. 4*B*). The conservation of this area between NHE-2 and NHE-4 raises the possibility that these two Na^+/H^+ exchangers might be regulated in a similar fashion and by similar mechanisms.

All NHEs identified to date are similar in size, ranging from 81 kDa (NHE-4) to 93 kDa (NHE-3). They have similar hydrophobicity plots and thus have similar predicted secondary structure of 12 membrane-spanning domains. All are likely to be glycoproteins and are likely to be substrates for protein kinases (3). At least for NHE-1, it has been shown that it is a glycoprotein and can be phosphorylated *in vivo* (6). Whether there are splice variants for each isoform is not known. In the present study, it was found that NHE-2 cDNA hybridized to three different sizes of message; 5.2, 4.2, and 3.2 kb. It is possible that the different sizes of message arose from additional alternate splice variants of NHE-2 that have not been identified or from messages with different length in the 3′-untranslated region, since the 3′-untranslated sequences contain multiple potential polyadenylation signals. Furthermore, we have identified a partial cDNA clone (RA6, Figs. 1 and 2) which gives additional 3′-untranslated region sequences, suggesting that multiple polyadenylation signals were used. Similarly, NHE-1 and NHE-4 have been shown to have multiple message sizes (14, 23), although their identities are not known. Interestingly, NHE-3 only has a single size message (13, 14).

Upon sequencing multiple overlapping cDNA clones, it was found that there were base substitutions in the NHE-2 cDNA. These substitutions presumably arose from cDNAs synthesized from mRNA templates transcribed from different alleles of the NHE-2 gene. That is, the NHE-2 gene probably exhibits allelic polymorphism. Such allelic polymorphism has been demonstrated for NHE-3 (24).

Expression of NHE-2 cDNA in PS120 cells suggested that NHE-2 is a functional Na^+/H^+ exchanger and could be stimulated by serum. Interestingly, the steady-state pH_i reached in NHE-2/PS120/2, after recovery from acid load, was 7.45 (Fig. 5*C*). Under identical conditions, we previously reported that the steady-state pH_i reached in NHE-1/PS120/5 (PS120 cells stably transfected with NHE-1) was only 7.0, a difference

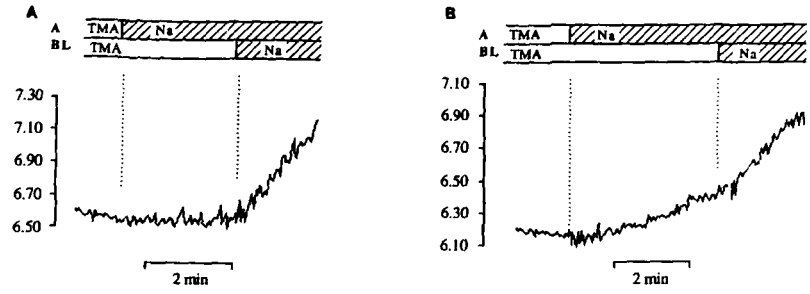

FIG. 6. **Apical expression of functional NHE-2 in Caco-2 cells.** *A*, nontransfected Caco-2 cells have only basolateral Na^+/H^+ exchange, as demonstrated in this experiment that Na^+ added to the apical surface of acidified cells caused no change in pH_i, whereas addition of Na^+ to the basolateral surface resulted in prompt alkalinization. *B*, NHE2/PS120/2 cells have both apical and basolateral Na^+/H^+ exchange activity. When Na^+ was added to the apical surface of acidified cells, there was a gradual increase in pH_i; subsequent addition of Na^+ to the basolateral surface caused a further rapid increase in pH_i.

of 0.45 unit of pH_i. There appears to be multiple determinants of the steady-state pH_i or "set-point," one of which is the amount of Na^+/H^+ exchange activity expressed in transfected PS120 cells. In this regard, the V_{max} of Na^+/H^+ exchange in NHE-2/PS120/2 is five times than that of NHE-1/PS120/5.[3]

5-Amino-substituted amiloride analogues are known to be more potent inhibitors of Na^+/H^+ exchangers than amiloride, and most epithelial apical Na^+/H^+ exchangers are more resistant to the 5-amino-substituted amiloride than to amiloride (8). In order to determine whether NHE-2 had characteristics of an apical epithelial Na^+/H^+ exchanger, the sensitivity of NHE-2 to EIPA and amiloride inhibition was determined. It was found that the K_i for EIPA inhibition was 500 nM. Compared with NHE-1 which has a K_i for EIPA of 20 nM (3), NHE-2 is 25-fold more resistant to EIPA inhibition than NHE-1. Interestingly, there was no difference of amiloride sensitivity between NHE-1 and NHE-2, the K_i for both being 1 μM.

In conclusion, we have cloned, sequenced, and expressed a unique Na^+/H^+ exchanger isoform, NHE-2. Since, NHE-2 is resistant to EIPA and can be expressed in the apical membrane of a polarized intestinal epithelial cell, Caco-2, NHE-2 is likely to be an apical membrane Na^+/H^+ exchanger isoform. With the recognition that there are at least four Na^+/H^+ exchanger isoforms, all of which are expressed in intestine and kidney, it is anticipated that there might be multiple apical and/or basolateral Na^+/H^+ exchanger isoforms.

Acknowledgment—We thank Dr. D. Jefferson (Tufts University, School of Medicine) for providing us with rabbit skin fibroblasts.

Note Added in Proof—After our paper was accepted for publication, we were asked to compare the sequence of rabbit NHE-2 with that of a protein cloned by Dr. Gary Shull from the rat, as described in the accompanying paper (Wang, Z., Orlowski, J., and Schull, G. E. (1993) *J. Biol. Chem.* **268**, 11925-11928.

[3] S. Levine, M. Montrose, M. Donowitz, and C. M. Tse, unpublished result.

REFERENCES

1. Aronson, P. S. (1985) *Annu. Rev. Physiol.* **47**, 545-560
2. Grinstein, S., Rutin, D., and Mason, M. J. (1989) *Biochim. Biophys. Acta* **988**, 73-97
3. Wakabayashi, S., Sardet, C., Fafournoux, P., Counillon, L., Meloche, S., Pages, G., and Pouyssegur, J. (1992) *Rev. Physiol. Biochem. Pharmacol.* **119**, 157-186
4. Knickelbein, R. G., Aronson, P. S., Atherton, W., and Dobbins, J. W. (1983) *Am. J. Physiol.* **245**, G504-G510
5. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) *Cell* **56**, 271-280
6. Sardet, C., Counillion, L., Franchi, A., and Pouyssegur, J. (1990) *Science* **247**, 723-726
7. Tse, C. M., Ma, A. I., Yang, V. W., Watson, A. J. M., Levine, S., Montrose, M. H., Potter, J., Sardet, C., Pouyssegur, J., and Donowitz, M. (1991) *EMBO J.* **10**, 1957-1967
8. Clark, J. D., and Limbird, L. L. (1991) *Am. J. Physiol.* **261**, C945-C953
9. Haggerty, C., Agarwal, N., Reilly, R. F., Adelberg, E. A., and Slayman, C. W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6797-6801
10. Knickelbein, R. G., Aronson, P. S., and Dobbins, J. W. (1990) *Am. J. Physiol.* **259**, G802-G806
11. Raley-Susman, K. M., Cragoe, E. J., Jr., Sapolsky, R. M., and Kopito, R. R. (1991) *J. Biol. Chem.* **266**, 2739-2745
12. Reilly, R. F., Hildebrant, F., Biemesderfer, D., Sardet, C., Pouyssegur, J., Aronson, P. S., Slayman, C. W., and Igarashi, P. (1991) *Am. J. Physiol.* **261**, F1088-F1094
13. Tse, C. M., Brant, S. R., Walker, M. S., Pouyssegur, J., and Donowitz, M. (1992) *J. Biol. Chem.* **267**, 9340-9346
14. Orlowski, J., Kandasamy, R. A., and Shull. G. E. (1992) *J. Biol. Chem.* **267**, 9331-9339
15. Wakabayashi, S., Fafournoux, P., Sardet, C., and Pouyssegur, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2424-2428
16. Weiser, M. M. (1973) *J. Biol. Chem.* **248**, 2536-2541
17. Maniatis, T., Fritsch, E., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 7.43-7.50, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035-7056
19. Krayer-Pawlowska, D., Helmle-Kolb, C., Montrose, M. H., Krapf, R., and Murer, H. (1991) *J. Membr. Biol.* **120**, 173-183
20. Kozak, M. (1988) *Nucleic Acids Res.* **15**, 8125-8148
21. Pouyssegur, J., Franchi, A., L'Allemain, G., and Paris, S. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4833-4837
22. Watson, A. J., Levine, S., Donowitz, M., and Montrose, M. M. (1991) *Am. J. Physiol.* **261**, G229-G238
23. Hildebrandt, F., Pizzonia, J. H., Reilly, R. F., Reboucas, N. A., Sardet, C., Pouyssegur J., Slayman, C. W., Aronson, P. S., and Igarashi, P. (1991) *Biochim. Biophys. Acta* **1129**, 105-108
24. Brant, S. R., Bernstein, M., Wasmuth, J. J., Taylor, E. W., McPherson, J. D., Li, X., Walker, S., Pouyssegur, J., Donowitz, M., Tse, C. M., and Jabs-Wang, E. (1993) *Genomics* **15**, in press