Molecular Cloning of NHE3 from LLC-PK1 Cells and Localization in Pig Kidney

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Abstract. LLC-PK1 cells, an established line from pig kidney, express basolateral and apical Na+/H+ exchangers that can be distinguished by their differing sensitivities to the amiloride analog N-ethyl-N-isopropylamiloride (EIPA). It has been shown previously that the basolateral exchanger is encoded by NHE1. In the present study, a combination of reverse transcription-PCR, 5′ RACE, and genomic library screening was used to clone the coding region of the porcine NHE3 gene. There was significant homology between the LLC-PK1 sequence and the previously reported rabbit and rat NHE3 genes, with nucleotide and deduced amino acid identities of 87 and 85% in rabbit, and 85 and 87% in rat, respectively. To study expression patterns, Northern analysis was carried out using an NHE3 cDNA to probe poly(A)+ RNA isolated from LLC-PK1 cells, and from pig kidney cortex. In all three cases, a major transcript of 6.1 kb was detected along with two minor transcripts of 4.7 and 3.8 kb. In situ hybridization with two different NHE3 probes gave intense labeling of the distal convoluted tubule in pig kidney but (unexpectedly) no detectable labeling of the proximal tubule. These studies suggest that there are marked species differences in NHE3 expression in the distal nephron.

Na+/H+ exchangers are integral membrane proteins present in the plasma membrane of almost all vertebrate cells. Under normal physiologic conditions, they mediate an electroneutral exchange of one extracellular Na+ for an intracellular H+ (1). In mammals, molecular cloning studies have shown that Na+/H+ exchangers are encoded by a multigene family. cDNA encoding six members of the family (NHE1, -2, -3, -4, -5, and -6) have been isolated (2–7). At the protein level, NHE1, -2, -3, and -4 are predicted to have similar secondary structures consisting of an amino-terminal domain with 10 to 12 membrane-spanning segments, followed by a long carboxy-terminal hydrophilic region that is presumably cytoplasmic. Overall, the deduced amino acid sequences are 41 to 60% identical in the membrane-associated domain, and 25 to 31% identical in the hydrophilic region.

The various NHE isoforms are known to be expressed in a tissue-specific manner, and presumably have different physiologic functions. The NHE1 exchanger, whose gene was originally isolated by Sardet et al. using genetic complementation (2), is expressed in all tissues including the kidney (8,9). It is highly sensitive to amiloride (10) and is phosphorylated on serine residues in response to growth factors and mitogens (11). Indirect immunofluorescence studies have revealed NHE1 to be located in the basolateral membrane of all renal tubular epithelial cells with the exception of intercalated cells (12). NHE2, cloned from rabbit ileum by low-stringency cDNA library screening, is intermediate in its sensitivity to amiloride (3). It is expressed in kidney, ileum, and adrenal gland. The localization of NHE2 in kidney remains controversial. NHE2 has been localized to the distal convoluted tubule using in situ hybridization (13). Others have reported expression in the luminal membrane of the proximal convoluted tubule (14) and medullary thick ascending limb (15). Studies in inner medullary collecting duct cells have suggested that NHE2 may play a role in volume regulation, and may be expressed in the basolateral membrane (16). NHE3, which was isolated from rat kidney and rabbit ileum, has proved to be relatively resistant to amiloride (10,17). Its expression has been detected only in intestine and kidney. In immunolocalization studies, Biemesderfer et al. have identified it on the microvillar membrane of the brush border of rabbit proximal tubule (18), and Amemiya et al. have detected it in the apical membrane of the proximal tubule as well as the medullary and cortical thick ascending limbs in rat (19). NHE4 was cloned from rat stomach (5), is extremely resistant to amiloride and ethylisopropylamiloride (20), and is expressed in the basolateral membrane of the early proximal tubule, ascending limb of Henle, and distal tubule (21).

Because of the clear physiologic importance of Na+/H+
exchange in the kidney, the LLC-PK₁ cell line has become an actively studied model system. These cells, isolated from pig kidney by Hull et al. (22), form a polarized epithelium in culture and display many of the properties of the proximal tubule (23). We have reported previously that LLC-PK₁ cells express two different Na⁺/H⁺ exchangers that can be distinguished by their differing sensitivities to the amiloride analogue N-ethyl-N-isopropylamiloride (EIPA) (24). One form, relatively sensitive to EIPA (IC₅₀ = 0.03 μM), is expressed in rapidly growing cells and becomes restricted to the basolateral surface at confluence; this exchanger has been identified as NHE1 (25). The second form, highly resistant to EIPA (IC₅₀ = 13 μM), is not expressed by rapidly growing cells but appears on the apical surface at confluence. The aim of the present study was to identify the second Na⁺/H⁺ exchanger.

Materials and Methods

Cell Culture

To aid in cloning, we used an LLC-PK₁ line (PKE20) with a three-to fivefold elevated activity of the apical Na⁺/H⁺ exchanger (26). PKE20 was selected from the parent LLC-PK₁/Cl₂ cell line by its ability to survive acid-loading in the presence of EIPA (11); it has remained stable in culture for up to 20 passages. LLC-PK₁/Cl₂ and PKE20 were maintained in α-minimum essential medium supplemented with 10% fetal calf serum under a 5% CO₂ atmosphere at 37°C. Subculturing was performed at 5- to 7-d intervals by trypsinization with ATP using T4 polynucleotide kinase. First strand cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) primed by oligo(dT)₁₇ as described by the manufacturer. Reverse transcription (RT)-PCR was carried out in a reaction volume of 50 μl as described previously (25,27). The reaction mixture was heated for 5 min at 94°C in a thermal cycler (Perkin Elmer, Norwalk, CT) and then cooled to 4°C. Tag DNA polymerase (2.5 U) was added, and the mixture was overlaid with mineral oil. Forty cycles of RT-PCR were performed, each consisting of denaturation for 1 min at 94°C, annealing of oligonucleotides for 1 min at 50°C, and elongation for 3 min at 72°C. A final elongation of 15 min was performed at 72°C. Every RT-PCR included a reagent control that contained all components except primers and minus corresponding to the antisense sequence. Two additional oligonucleotides were designed based on the published rabbit NHE3 sequence (4). The DNA sequence of these oligonucleotides was: 1369 GACGTGCACCTCCTCGAA; 2 and 1626 CCAGGCCGAACTGACGTCGACTAG. The products of this PCR then served as a template for a second nested PCR reaction using the universal anchor oligonucleotide (CTACTACTAGCCGTCGACTAG) and a third sequence-specific oligonucleotide (180–, CCAGGCCGAACTGACGTCGACTAG).

Detection of RACE Products

RACE products were size-fractionated by agarose gel electrophoresis, transferred to a nylon filter, and fixed by ultraviolet transillumination. An internal sequence-specific oligonucleotide (121+, GTTCCTTCAATGGGCAACCA) was radiolabeled with [γ-³²P]dATP using T4 polynucleotide kinase. Filters were prehybridized for 24 h in Church-Gilbert (0.5 M sodium phosphate, pH 7.2, 1 mM ethylenediaminetetra-acetic acid, 7% sodium dodecyl sulfate [SDS], and 1% bovine serum albumin [BSA]) at 55°C, then hybridized for 24 h at 55°C in the same medium that contained 100 μg of denatured salmon sperm DNA/ml, and 10⁶ cpm/ml ³²P-labeled probe. Filters were washed 5 min in 2× SSC at room temperature, 5 min in 0.5× SSC that contained 0.1% SDS at 55°C, and exposed to film.

Genomic Library Screening

The first 20 nucleotides of the coding region were obtained by screening a commercially available porcine genomic library in the bacteriophage EMBL3 SP6/T7 (Clontech Laboratories, Palo, Alto, CA). The library was screened using the plaque-lift method. A ³²P-labeled probe was generated by random primer extension in the presence of [α-³²P]dCTP using as template the 5’ RACE product (nt 21 to 180) (28). Duplicate nylon filters were prepared from the plated genomic library. Filters were prehybridized in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0]), 5× Denhardt’s solution, 0.1% SDS, and 100 μg of denatured salmon sperm DNA/ml at 55°C; hybridized for 24 h in the same medium plus 10⁶ cpm/ml ³²P-labeled probe; washed in 2× SSC at room temperature and in 2× SSC, 0.5% SDS at 55°C for 30 min; and exposed to film. Phage that gave positive signals on duplicate filters were plaque-purified through secondary and tertiary screens. Tertiary positives were purified using a modification of the method of Yamamoto et al. (29). Purified phage was digested with Sall and BamHI, size-fractionated by agarose gel electrophoresis, transferred to a nylon filter, and fixed by ultraviolet transillumination. The filter was then probed in the same manner as the genomic library.

Northern Analysis

Poly(A)⁺ RNA was purified from LLC-PK₁/Cl₂ and PKE20 cells grown to confluence in 175-cm² plastic flasks, and from pig kidney nucleotides are enclosed within parentheses. The three RT-PCR products were subcloned and sequenced as described previously (25,27).

5’ Rapid Amplification of cDNA Ends

5’ rapid amplification of cDNA ends (RACE) was carried out using a commercially available kit as described by the manufacturer (Life Technologies). Briefly, first-strand cDNA was synthesized with a sequence-specific primer, 339–, AACAGACACAGCTTCTCA. After RNase H removal of the RNA template, the first-strand cDNA was purified using GLASSMAX DNA isolation spin cartridges. A homopolymeric tail was added using terminal deoxynucleotidyltransferase and dCTP. Thirty-five cycles of PCR were performed, each consisting of denaturation for 1 min at 94°C, annealing of oligonucleotides for 30 s at 57°C, and elongation for 2 min at 72°C, using the 5’ RACE anchor oligonucleotide (CTACTACTAGCCGTCGACTAG), and a second sequence-specific oligonucleotide (218–, CTTCGCGACAGTGGAAGC). The products of this PCR then served as a template for a second nested PCR reaction using the universal anchor oligonucleotide (CTACTACTAGCCGTCGACTAG) and a third sequence-specific oligonucleotide (180–, CCAGGCCGAACTGACGTCGACTAG).

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Northern Analysis

Poly(A)⁺ RNA was purified from LLC-PK₁/Cl₂ and PKE20 cells grown to confluence in 175-cm² plastic flasks, and from pig kidney
cortex. In each case, the RNA was size-fractionated by denaturing agarose gel electrophoresis and transferred to a nylon filter. A 1085-bp cDNA (nt 1369 to 2496) encoding most of the cytoplasmic domain of the exchanger was radiolabeled using random primer extension as described above (Genomic library screening). Filters were prehybridized in 6× SSC, 5× Denhardt’s solution, 0.1% SDS containing 50% formamide, and 100 μg of denatured salmon sperm DNA/ml at 42°C; hybridized for 24 h in the same medium plus 106 cpm/ml 32P-labeled probe; washed in 2× SSC at room temperature for 30 min and in 0.1× SSC, 0.1% SDS at 65°C for 30 min; and exposed to film. In comparing NHE3 transcript expression between PKE20 and LLC-PK1/C14, an actin probe was used as an RNA loading control.

Genomic Southern Analysis

One microgram of genomic DNA from LLC-PK1/C14 and PKE20 was digested with either HindIII or EcoRI, size-fractionated on a 0.6% agarose gel, transferred to a nylon filter, and fixed by ultraviolet transillumination. The filter was prehybridized, hybridized, and probed as described above (Northern analysis). The probe for genomic Southern analysis spanned nucleotides 541 to 1626. The filter was then washed in 2× SSC at room temperature for 30 min; in 2× SSC, 0.1% SDS at 55°C for 30 min; and exposed to film.

Generation of Fusion Protein and Polyclonal Antiserum

A porcine NHE3 fusion protein was generated by subcloning a PCR product that was amplified from cloned PKE20 NHE3 into p-mal-cII (New England Biolabs, Beverly, MA). The following oligonucleotides were used in the PCR: 1561TTTCTTCCTCCATAGCTTCCACTCTTTCCTCCCTCAGGAGG-3’ (sense) and 1770CTTCTCCTCCATAGCTTCCACTCTTTCCTCCCTCAGGAGG-3’ (antisense). The integrity of the cloning site and the NHE3 cDNA was verified by DNA sequencing. For purification of the fusion protein, transformed bacteria (Escherichia coli strain DH5α) were grown in Luria-Bertani medium, and fusion protein synthesis was induced by incubation with 0.3 mM IPTG for 2 h. A sonicated cell lysate was prepared and passed over an amylase affinity column as described by the manufacturer (New England Biolabs), and the fusion proteins were eluted with 10 mM maltose. Purified fusion proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and quantified based on Coomassie Blue staining relative to known concentrations of BSA. Polyclonal antiserum were raised in three female rabbits. One hundred micrograms of the protein was injected subcutaneously into each animal at monthly intervals, and immune sera were collected. Because antisera from all animals gave the identical result, only one was chosen for further study. This antiserum was then purified over a column of bacterial lysate coupled to cyanoan bro-mide-activated Sepharose-4B to remove antibodies directed against contaminating Escherichia coli proteins and the maltose-binding protein. Purification was verified by enzyme-linked immunosorbent assay (ELISA).

ELISA

Indirect ELISA was carried out as described previously (30). Serial dilutions of antiserum (before and after passage over the bacterial lysate column) were incubated with a 1:1000 dilution of both the maltose-binding protein and the fusion protein. Detection was performed with a horseradish peroxidase-conjugated goat anti-rabbit IgG. If antibodies to the maltose-binding protein portion of the construct, as well as other contaminants, were successfully removed by passage over the bacterial lysate column, then the purified antiserum should react only with the fusion protein and not the maltose-binding protein alone.

Western Analysis

Microsomes were prepared from LLC-PK1 and PKE20 as described previously (20). The pro tease inhibitors phenylmethylsulfonylfluoride (40 mg/ml), pepstatin (0.7 mg/ml), ethylenediaminetetraacetic acid (1 mM), and leupeptin (0.5 mg/ml) were present in all solutions. Protein concentration was determined by Lowry assay with BSA as the standard. Membranes were separated by 7.5% SDS-PAGE under reducing conditions and electroblotted onto polyvinylidene difluoride (Millipore Immobilon-P, Bedford, MA) filters. To verify the specificity of the polyclonal antiserum for NHE3, Western analysis was also carried out, using a polyvinylidene difluoride filter to which solubilized LAP1 cells (a mouse cell line that is deficient in Na+/H+ exchange activity) transfected separately with rabbit NHE1, -2, -3, and -4 as described previously (18) had been transferred (a generous gift of Dr. Peter Aronson and Dr. Daniel Biemesderfer). Immunoblots were blocked for 4 h with Blotto and incubated overnight with a 1:1500 dilution of purified antiserum. After further washing with Blotto, filters were incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG, and then developed using enhanced chemiluminescence Western blot detection system as recommended by the manufacturer (Pierce, Rockford, IL). Filters were then exposed to Kodak X-Omat AR film.

Preparation of Riboprobes for In Situ Hybridization

Riboprobes were prepared from two partial-length cDNA encoding porcine NHE3. The first probe comprised the predicted cytoplasmic domain (nucleotides 1369 to 2494), and the second comprised a portion of the membrane-associated domain (nucleotides 765 to 1616). Each cDNA was subcloned into the EcoRI site of the pBluescript KS+ plasmid. RNA probes were synthesized and labeled by in vitro transcription using digoxigenin-11-dUTP. Sense and antisense probes were generated using either T3 or T7 polymerase (as determined by the orientation of the cDNA), after linearization of the plasmid. RNA probes for in situ hybridization were shortened by alkali hydrolysis to an average length of 200 bases.

In Situ Hybridization and Immunocytochemistry

Cryostat sections (5 to 7 μm thick) were thawed onto silanized glass slides and processed as described previously (31). Hybridization was performed at 40°C for 16 to 18 h. Slides were washed once at 47°C for 30 min in 0.1× SSC containing 50% formamide, twice for 30 min in 0.1× SSC containing 50% formamide, and twice for 30 min in 0.1× SSC containing 50% formamide. The slides were then rinsed twice in 0.5× SSC at room temperature for 10 min, once in 0.2× SSC for 10 min, and twice in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). Blocking of nonspecific antibody binding sites was performed by incubation with blocking medium (2% normal sheep serum, 0.5% BSA, 3% Triton X-100 in buffer 1). To identify specific nephron segments that express NHE3, combined in situ hybridization and immunohistochemistry with antibodies specific to defined nephron segments were performed on the same section. To label thick ascending limb cells, a commercially available goat anti-human Tam-m–Horsfall antibody was used (Organon Technika, Durham, NC). To identify connecting tubular cells, a guinea pig anti-rabbit Na+/Ca2+ exchanger antibody was used (30). After removal of the blocking medium, a mixture of sheep anti-digoxigenin alkaline phosphate conjugate and one of the specific antibodies was diluted in blocking medium. This mixture was incubated with the sections for 2 h at room
temperature in a moisture chamber, followed by 16 h at 4°C. Slides were then washed twice for 15 min in buffer I. The primary antibodies were detected using the biotin-streptavidin Texas Red detection system. Afterward, message was detected using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate system as detailed previously (31).

Materials

Chemicals were molecular biology grade from Sigma Chemical Co. (St. Louis, MO), Life Technologies, or U.S. Biochemical Corp. (Cleveland, OH). Solutions for use with RNA were treated with diethylpyrocarbonate and autoclaved before use. All restriction endonucleases and DNA-modifying enzymes were from New England Biolabs or Promega (Madison, WI). PCR reagents were from Perkin Elmer. 32P-labeled nucleotides were from Amersham. Escherichia coli strain DH5α (Life Technologies) was maintained as frozen stocks, and grown on Luria-Bertani plates.

Results

Molecular Cloning

As described above, the object of this study was to clone and characterize a cDNA encoding the apical Na+/H+ exchanger of LLC-PK1 cells. For three reasons, this seemed likely to be the NHE3 isoform. First, measurements on differentiated LLC-PK1/Cl− epithelia have given IC50 values for the inhibition of apical Na+/H+ exchange by EIPA (10 to 15 μM) that agree well with published values for NHE3 (17). Second, NHE3 protein has been detected on the brush border of the proximal tubule in immunolocalization studies in rabbit (18) and rat kidney (19), and LLC-PK1 cells are thought to be of proximal tubular origin (23). Third, immunoblotting of LLC-PK1 luminal membranes with polyclonal antisera directed against cytoplasmic epitopes of rat NHE3 labeled 90- and 95-kD proteins was obtained by screening a commercially available porcine kidney genomic library in the bacteriophage EMBL3 SP6/T7 (Clontech Laboratories) using the RACE product as probe. Where the five DNA fragments overlapped, their sequences were identical, consistent with the notion that they originated from the same transcript. Overlapping sequence was obtained for both strands of all fragments. Taken together, the four clones spanned nucleotides −116 to 2496, numbered according to the rabbit sequence (4). The DNA sequence of the coding region of the LLC-PK1 Na+/H+ exchanger was highly conserved when compared to rabbit and rat, with 90 and 87% nucleotide identity in the membrane-associated domain, and 84 and 82% identity in the cytoplasmic domain. The deduced amino acid sequence was also highly conserved compared to rabbit and rat, with 89% identity in the membrane-associated domain, and 81 and 84% identity in the cytoplasmic domain. A comparison of the deduced amino acid sequence of the porcine and rabbit exchangers is shown in Figure 1. The single, long open reading frame is predicted to encode a protein of 846 amino acids. Phylogenetic tree reconstruction confirms that this gene is a member of the NHE3 subfamily (data not shown).

Expression of an NHE3 Transcript in Pig Kidney and LLC-PK1 Cells

To determine the size of the NHE3 transcript, Northern analysis was performed. A 1085-nt cDNA (nt 1369 to 2496) encoding most of the cytoplasmic domain of NHE3 was chosen as the probe since there is a relatively low degree of nucleotide identity (25 to 31%) between members of the NHE gene family in this region. Poly(A)+ RNA from pig kidney cortex, as well as...
as confluent LLC-PK₁/Cl₄ and PKE20 cells, was hybridized to the cDNA probe and washed at high stringency. As shown in Figure 2A, a transcript of 6.5 kb was detected in pig kidney cortex. In PKE20 cells, a major transcript of 6.5 kb and a minor transcript of 4.4 kb were detected after a 1-d exposure (Figure 2B, lane 1), whereas in LLC-PK₁/Cl₄ (lane 2) a 6-d exposure was required to detect the same two transcripts. In addition, the relative abundance of the two NHE3-related transcripts was different in the two cell lines. The 6.5-kb transcript predominated in PKE20, whereas the 6.5- and 4.4-kb transcripts were present in similar amounts in LLC-PK₁/Cl₄. The relative abundance of the NHE3-related transcripts in LLC-PK₁/Cl₄ and PKE20 could not be quantified due to the much longer exposure required to visualize the transcript in LLC-PK₁/Cl₄. It appeared, however, that transcript levels were increased much more than the three- to fivefold increase in apical amiloride-resistant Na⁺/H⁺ exchange activity. An actin probe was used to verify that an equal amount of poly(A)⁺ RNA was loaded into each lane.

Genomic Analysis of LLC-PK₁/Cl₄ and PKE20

Given the marked increase in NHE3 transcript levels between PKE20 and LLC-PK₁/Cl₄, genomic Southern analysis was carried out to determine whether the NHE3 gene had undergone duplication during the selection process that was used to isolate PKE20 (acid-loading in the presence of EIPA).

Although apical amiloride-resistant Na⁺/H⁺ exchange activity in PKE20 is increased three- to fivefold, basolateral Na⁺/H⁺ exchange activity, which has been shown to be the result of NHE1 expression (25), is the same as in LLC-PK₁/Cl₄. Genomic DNA was isolated from both cell lines, restricted with EcoRI, size-fractionated by agarose gel electrophoresis, and transferred to a nylon membrane. Southern analysis performed with an NHE3-specific probe detected a 9.3-kb band (Figure 3). There was a slight increase in the intensity of hybridization to PKE20 DNA (Figure 3, lane 2) compared with LLC-PK₁/Cl₄ (Figure 3, lane 1). To quantify this difference, the autoradiograph was scanned and densitometric analysis was performed using NIH Image 1.61. There was a 20% increase in optical density in PKE20 compared with LLC-PK₁/Cl₄.

Antibody Generation and Western Analysis

To further demonstrate that the cloned cDNA encoded NHE3, polyclonal antisera were generated against a fusion protein that contained a portion of the porcine NHE3 cytoplasmic domain fused to the maltose-binding protein. The specificity of the affinity-purified antisera for NHE3 was assessed by Western analysis. Plasma membranes were isolated from mouse fibroblasts devoid of endogenous Na⁺/H⁺ exchange activity that were transfected with either full-length rabbit NHE1, -2, -3, or -4 cDNA. As can be seen in Figure 4A, the affinity-purified antisera identified a protein in fibroblasts transfected only with NHE3 (lane 3), and not in those transfected with NHE1, -2, or -4 (lanes 1, 2, and 4). Plasma

![Figure 2](image-url)  
Figure 2. Northern analysis. (A) Pig kidney cortex. Poly(A)⁺ RNA isolated from pig kidney cortex was probed with an NHE3 cDNA as described in Materials and Methods. The sizes of the RNA markers are shown on the left. (B) LLC-PK₁/Cl₄ and PKE20. Poly(A)⁺ RNA from PKE20 (lane 1) and LLC-PK₁/Cl₄ (lane 2) was analyzed by Northern analysis as in Panel A.

![Figure 3](image-url)  
Figure 3. Genomic Southern analysis. EcoRI-restricted genomic DNA from LLC-PK₁/Cl₄ (lane 1) and PKE20 (lane 2) was analyzed by Southern analysis as described in Materials and Methods. DNA size markers are shown to the left.
membranes from rabbit kidney cortex served as a positive control (lane 5). We next investigated whether the marked increase in NHE3 transcript that was detected in PKE20 compared with LLC-PK1/Cl4 was reflected by a similar increase in the transcribed protein. Western analysis was then carried out on plasma membranes isolated from PKE20 and LLC-PK1/Cl4 (Figure 4B). Equal amounts of protein (100 μg) were loaded in each lane as determined by Lowry assay, and verified by Coomassie staining of duplicate aliquots (data not shown). An 87-kD protein was detected in both cell lines, and more of the protein was detected in PKE20 (lane 2) compared with LLC-PK1/Cl4 (lane 1). Densitometric analysis was again performed using NIH Image 1.61, and a 2.5-fold increase in NHE3 was noted in PKE20. The polyclonal antibody has not worked in immunofluorescence studies under conditions tested to date.

In Situ Hybridization and Immunohistochemical Studies

Regional expression of the NHE3 transcript was studied in pig kidney by means of in situ hybridization. Sense and antisense riboprobes were prepared from two partial-length LLC-PK1 NHE3 cDNA. The first (nt 1369 to 2496) encoded the predicted cytoplasmic domain, and the second (nt 765 to 1616) encoded a portion of the membrane-associated domain. Labeling was identical for both antisense probes. Therefore, results from only the first cRNA probe are shown. There was an excellent signal-to-background ratio and an absence of nonspecific staining when an adequate time for signal development was used (15 to 20 h for enzyme-substrate reaction). Negative controls using sense cRNA probes showed no staining (data not shown).

The antisense NHE3 cRNA probe primarily labeled the distal convoluted tubule. A transition between a thick ascending limb (marked by the letter T) and the distal convoluted tubule (marked by the letter D) is illustrated in Figure 5. The macula densa is shown between the arrowheads. Although only the distal convoluted tubule is labeled in this section, faint labeling was often observed in the thick ascending limb in other sections (data not shown). Labeling was of sufficient intensity to allow further examination of the proximal and distal extent of the distal tubular expression of NHE3. Low-power microscopy of double labeling with an antibody directed against Tamm–Horsfall protein (Figure 6A), and NHE3 in situ hybridization (Figure 6B) indicated that there was no overlap between the two labels. A transition between thick ascending limb and distal convoluted tubule is shown on the right side of the figure, where one portion of the tubular lumen is stained by the anti-Tamm–Horsfall antibody and the other portion by the NHE3 riboprobe. Low-power microscopy of double labeling with an antibody to the Na+/Ca2+ exchanger a marker for the connecting tubule (30) (Figure 7A), and NHE3 in situ hybridization (Figure 7B) showed no overlap between the two labels. NHE3 staining was visualized in the distal convoluted tubule but not in the connecting tubule.

Discussion

For reasons described above, we anticipated that the apical Na+/H+ exchanger of LLC-PK1 cells was encoded by NHE3. Therefore, to facilitate its molecular cloning, we used a cell line (PKE20) that displays a three- to fivefold elevated activity of the apical amiloride-resistant Na+/H+ exchanger when compared to the parent LLC-PK1/Cl4 cells. PKE20 was selected from the parent line based on its ability to survive acid loading in the presence of EIPA. The cells were not mutagenized before selection.

We used a combination of RT-PCR, 5’ RACE, and library screening to clone a cDNA encoding NHE3 from LLC-PK1 cells. As expected, there was a high degree of sequence con-
ervation between the pig sequence and previously published rabbit and rat NHE3 sequences at both the DNA and protein levels. Phylogenetic tree reconstruction confirmed that the cloned cDNA was a member of the NHE3 subfamily. In addition, a fusion protein antibody directed against a portion of porcine NHE3 recognized an 87-kD protein only in LAP cells transfected with NHE3 and not LAP cells transfected with NHE1, -2, or -4, confirming that the cloned cDNA was indeed a member of the NHE3 family.

Northern analysis in pig kidney cortex revealed the expression of a 6.5-kb transcript. However, when NHE3 expression was compared between PKE20 and the parent line LLC-PK1/Cl4, qualitative and quantitative differences in expression were noted. As in pig kidney, a transcript of 6.5 kb was detected in both PKE20 and LLC-PK1/Cl4. In LLC-PK1/Cl4, a transcript of 4.4 kb was also detected and appeared to be the predominant transcript. In addition, the increase in NHE3 transcript expression appeared to exceed the three- to fivefold increase in transport activity. The increase could not be accurately quantified due to the large difference in exposure time required to obtain a suitable autoradiographic signal (PKE20, 1 d; LLC-PK1/Cl4, 6 d). Southern analysis with the same probe showed only a minor increase in hybridization to PKE20 genomic DNA, indicating that little, if any, of the increased transcript expression was due to gene duplication. Whole-cell NHE3 protein expression was increased 2.5-fold, similar to the three- to fivefold increase in transport activity.

An unexpected finding concerned the localization of the NHE3 transcript in pig kidney. There was every reason to predict NHE3 expression in the proximal tubule, given previous immunolocalization studies in rabbit (18) and rat (19). In fact, no signal was detected in this location in pig kidney, perhaps because the level of expression of NHE3 in this segment is below the threshold of detection for in situ hybridization. By contrast, a strong hybridization signal was seen in the distal convoluted tubule of pig kidney.

The distal convoluted tubule reabsorbs approximately 5 to 7% of the filtered load of sodium (33). Published data point to dramatic differences in distal tubular bicarbonate absorption, depending on the species. On one hand, Iino and Burg (34) measured little or no absorption of bicarbonate in the distal tubule during in vivo microperfusion studies of rabbit kidney.

Figure 6. Double labeling with an antibody directed against Tamm–Horsfall protein. (A) Staining with an anti-Tamm–Horsfall antibody. (B) In situ hybridization with an NHE3 riboprobe. A distal nephron profile shows NHE3 mRNA along half of a sectioned transition between the cortical thick ascending limb and the distal convoluted tubule (line). Magnification, ×330.

Figure 7. Double labeling with an antibody directed against the Na+/Ca2+ exchanger. (A) Staining with an anti-Na+/Ca2+ exchanger antibody. (B) In situ hybridization with an NHE3 riboprobe. There is no overlap between NHE3 mRNA-positive and Na+/Ca2+ exchanger-immunoreactive segments. Magnification, ×330.
On the other hand, Wang and coworkers (35) observed bicarbonate absorption in the early distal convoluted tubule of the rat and found that it was significantly inhibited when 100 μM EIPA was added to the perfusate, suggesting that it may be mediated by a luminal Na⁺/H⁺ exchanger, possibly NHE2 (13). Furthermore, Good (36) reported that cortical thick ascending limbs of rat kidney reabsorb bicarbonate against both a voltage and a concentration gradient. This process required carbonic anhydrase activity and sodium transport but was not dependent on transepithelial voltage, suggesting that it was mediated by an apical membrane Na⁺/H⁺ exchanger. Immunolocalization studies have revealed NHE3 expression in this segment in rat (19). Taken together with the hybridization data from the present study, these results suggest that there is a marked species difference in distal tubular Na⁺/H⁺ exchanger expression.

In summary, the present results indicate that NHE3 is expressed in porcine kidney cortex, PKE20, and LLC-PK₁/C14 cells. In situ hybridization with an NHE3 probe detected expression in the distal convoluted tubule. Expression was not detected in the proximal tubule probably because expression there was below the threshold for the nonisotopic technique that was used.

Acknowledgments
This research was supported by National Institutes of Health Grant RO1-DK47904. Dr. Reilly is the recipient of a Department of Veterans Affairs Research Associate Career Award.

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