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Research Article

Activation of cAMP-dependent protein kinase A inhibits the renal proximal tubule brush border membrane Na(+)-H+ exchanger by a process involving participation of a regulatory cofactor (NHE-RF) that is distinct from the transporter itself. Recent studies from this laboratory reported a partial amino acid sequence of this putative cofactor (Weinman, E. J., D. H. Steplock, and S. Shenolikar. 1993. *J. Clin. Invest.* 92:1781-1786). The present experiments detail the structure of the NHE-RF protein as determined from molecular cloning studies. A codon-biased oligonucleotide probe to a portion of the amino acid sequence of the putative cofactor was used to isolate a 1.9-kb cDNA from a rabbit renal library. The encoded protein is 358 amino acids in length and is rich in proline residues. Search of existing data bases indicates that NHE-RF is a unique protein. Using a reticulocyte lysate, the cDNA translated a product of approximately 44 kD, which was recognized by an affinity-purified polyclonal antibody to NHE-RF. Potential phosphorylation sites for protein kinase A are present. The mRNA for the protein is expressed in kidney, proximal small intestine, and liver. Reverse transcription/PCR studies in the kidney indicate the presence of mRNA for NHE-RF in several distinct nephron segments including the proximal tubule.

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Characterization of a Protein Cofactor That Mediates Protein Kinase A Regulation of the Renal Brush Border Membrane $\text{Na}^+ - \text{H}^+$ Exchanger

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Abstract

Activation of cAMP-dependent protein kinase A inhibits the renal proximal tubule brush border membrane $\text{Na}^+ - \text{H}^+$ exchanger by a process involving participation of a regulatory cofactor (NHE-RF) that is distinct from the transporter itself. Recent studies from this laboratory reported a partial amino acid sequence of this putative cofactor (Weinman, E. J., D. H. Steplock, and S. Shenolikar. 1993. *J. Clin. Invest.* 92:1781–1786). The present experiments detail the structure of the NHE-RF protein as determined from molecular cloning studies. A codon-biased oligonucleotide probe to a portion of the amino acid sequence of the putative cofactor was used to isolate a 1.9-kb cDNA from a rabbit renal library. The encoded protein is 358 amino acids in length and is rich in proline residues. Search of existing data bases indicates that NHE-RF is a unique protein. Using a reticulocyte lysate, the cDNA translated a product of ~ 44 kD, which was recognized by an affinity-purified polyclonal antibody to NHE-RF. Potential phosphorylation sites for protein kinase A are present. The mRNA for the protein is expressed in kidney, proximal small intestine, and liver. Reverse transcription/PCR studies in the kidney indicate the presence of mRNA for NHE-RF in several distinct nephron segments including the proximal tubule. (*J. Clin. Invest.* 1995. 95:2143–2149.) Key words: renal electrolyte transport • sodium hydrogen exchange • molecular cloning • cAMP-dependent protein kinase

Introduction

Activation of cAMP-dependent protein kinase A (PKA)¹ inhibits the activity of the renal brush border membrane (BBM) $\text{Na}^+ - \text{H}^+$ exchanger (1–4). In vitro studies involving solubilized membranes and a reconstitution assay have suggested that the inhibitory effect of PKA on the activity of the transporter requires participation of another intermediary protein; a regulatory cofactor (NHE-RF) (5–7). A partial amino acid sequence

of this putative cofactor, from which an affinity-purified polyclonal antibody to an artificial peptide representing NHE-RF was developed, has been obtained (7). This antibody immunoprecipitated a single protein from renal brush border membranes and decreased the inhibitory effect of PKA on the activity of the reconstituted $\text{Na}^+ - \text{H}^+$ exchanger. The immunoprecipitated product could be renatured and co-reconstituted with other brush border membrane proteins to restore the inhibitory effect of PKA.

The present report details the structure of the NHE-RF protein as determined from molecular cloning studies. In addition, the tissue distribution of the mRNA of the protein in rabbit organs and in the cells of rabbit kidney was examined. The cloning strategy involved use of a codon-biased oligonucleotide probe to a portion of the amino acid sequence of the putative NHE-RF. The probe allowed isolation of a 1.9-kb cDNA from a rabbit renal library. The encoded protein is 358 amino acids in length and is rich in proline residues. The cDNA is unique in nucleotide and predicted amino acid sequence. Potential phosphorylation sites for PKA are present. The mRNA for the protein is expressed in kidney, proximal small intestine, and liver. Reverse transcription/(RT)-PCR studies in the kidney indicate the presence of the mRNA for NHE-RF in several distinct nephron segments including the proximal tubule.

Methods

An antisense oligonucleotide probe corresponding to amino acids 2–10 of the cyanogen bromide fragment of NHE-RF was used to screen a rabbit kidney library (7). The sequence of the oligonucleotide probe was 5'-GTTGAAICCGTAICCGTTIGGICCTT-3'. Cloning was performed using an oligo-dT rabbit kidney cDNA library (Stratagene Inc., La Jolla, CA) constructed in the lambda ZAP II vector. The library had an average insert size of 1 kb and underwent one round of amplification before use. The host strain for screening and amplification was XL1-Blue *Escherichia coli*. The cloning strategy followed the manufacturer's recommendations. Initially, ~ 1 million recombinants were screened and positive colonies were plaque purified by additional screenings. R408 helper phage was used to excise and circularize the pBluescript SK M13(-) phagmid containing the DNA insert. This derived plasmid was transformed and propagated in XL1-Blue. The plasmid DNA was then extracted for sequencing and restriction analysis using a rapid purification protocol (Qiagen, Inc., Chatsworth, CA). The isolated cDNA was sequenced in both directions using sequential oligonucleotide primers and nested deletions generated from both the T7 and T3 ends by ExoIII and S1 nucleases. Nucleotide and amino acid sequence analyses were performed using DNASIS/PROSIS (Hitachi America, Ltd., Brisbane, CA). The GCG software package (Genetics Computer Group, Inc., Madison, WI) was used to perform sequence comparisons and multiple alignments.

Total RNA was extracted using an improved modification of the guanidinium isothiocyanate/acid-phenol method (8). Poly(A)⁺ RNA was isolated by absorption to oligo-dT cellulose in a binding buffer containing 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and

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1. Abbreviations used in this paper: BBM, brush border membrane; NHE-RF, regulatory cofactor of $\text{Na}^+ - \text{H}^+$ exchanger; PKA, protein kinase A; RT, reverse transcription.

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0.1% SDS. The column was washed in the same buffer. Poly(A)⁺ RNA was eluted from the oligo-dT cellulose with 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA at 65°C, followed by overnight precipitation at -20°C in a solution containing 1/10 vol 3 M sodium acetate (pH 5.2) and 2.5 vol of absolute ethanol. For Northern RNA hybridization analysis, RNA was separated using 1% agarose/2.2 M formaldehyde gel electrophoresis and transferred in 3 M NaCl/0.3 M sodium citrate (pH 7.0) to nitrocellulose paper. The blots were baked at 80°C for 2 h. Prehybridization was performed in a buffer containing 0.001% BSA (BSA Fraction V; Sigma Chemical Co., St. Louis, MO), 0.001% Ficoll (Type 400; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), 0.001% polyvinylpyrrolidone, 0.75 M NaCl, 0.05 M NaH₂PO₄ (pH 7.0), 1.0 mM EDTA (pH 8.0) supplemented with 0.75 M NaCl, 75 mM sodium citrate (pH 7.0), 0.1% SDS, 10% dextran sulfate, and denatured, fragmented salmon sperm DNA (0.2 mg/ml). Hybridization was performed at 42°C using the isolated cDNA or the oligonucleotide probe labeled by the random hexamer priming method using α-[³²P]dCTP, or end labeled using γ-[³²P]ATP. The filters were hybridized for 16 h at 42°C. After hybridization, the filters were washed twice for 15 min at 42°C in 5× SSC and 0.1% SDS. The filters were then sequentially washed at room temperature for 15 min in solutions containing 0.1% SDS with decreasing concentrations of salt (2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC) until background counts were reduced. The washed filters were exposed to x-ray film at -70°C overnight. Loading of the RNA gels was quantitated by ethidium bromide staining and/or hybridization using cDNA probes to actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In vitro translation studies were performed using a cell-free reticulocyte lysate system following the manufacturer's recommendations (Stratagene Inc.) (9). The lysate media contained [³⁵S]methionine. The polypeptide product was immunoprecipitated by addition of a PBS solution containing 10 mM Na₂HPO₄/1.8 mM KH₂PO₄ (pH 7.0), 137 mM NaCl, 3 mM KCl, and protein A Sepharose beads cross linked to an affinity-purified antipeptide antibody to NHE-RF (7). After rocking for 2 h at room temperature, the protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) were collected by centrifugation and washed four times with PBS. The bound protein was released by heating in SDS sample buffer and resolved by autoradiography of 15% SDS-PAGE gels.

RT/PCR was performed modified from the protocols of Chen and co-workers and validated initially using rabbit kidney RNA (10, 11). To determine the distribution of the mRNA of the regulatory protein in defined nephron segments, the glomerulus, convoluted and straight portions of the proximal tubule, thin and thick ascending limbs of Henle's loop, and cortical and inner medullary collecting ducts were dissected in PBS containing 0.1% BSA and 10 mM vanadyl ribonucleoside complex. Single defined nephron segments were transferred to RT/PCR tubes containing PBS using pulled microhematocrit tubes coated with a 0.1% BSA solution. The nephron segments were washed several times with PBS to remove the vanadyl ribonucleoside complex and resuspended in 7 μl of a solution containing 2% Triton X-100, 1 U/μl RNase inhibitor (Perkin-Elmer Corp., Norwalk, CT), and 5 mM 1,4-DTT. Reverse transcription was performed using the reverse transcription system of Gibco BRL (Gaithersburg, MD) using random primers. The final concentrations were 1 U/μl SuperScript II RT (a cloned Moloney murine leukemia virus reverse transcriptase) (GIBCO-BRL, Gaithersburg, MD), 2.5 mM MgCl₂, 0.5 mM of each dNTP, 12 mM DTT, and 25 ng/μl of primers in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 μg/ml BSA, 0.35× PBS, 0.35 U/ml RNase inhibitor, and 0.7% Triton X-100. Reverse transcriptase was omitted from control tubes. The reaction was performed for 50 min at 42°C and then terminated at 70°C for 15 min. Before the addition of the PCR reaction mixture, 2 U/μl *E. coli* RNase was added to each tube and incubated for 20 min at 37°C. PCR was performed in a solution containing 2.5 mM MgCl₂, 0.1 mM dNTP, 0.1 μM of each specific primer, 0.05 U/μl Taq DNA polymerase, 50 mM KCl, 0.1 μg/ml BSA, 0.14% Triton X-100, 0.07× PBS, and 20 mM Tris-HCl (pH 8.4) using a Thermocycler (480; Perkin-Elmer Cetus Instruments). After denaturation for 3 min at 94°C, the

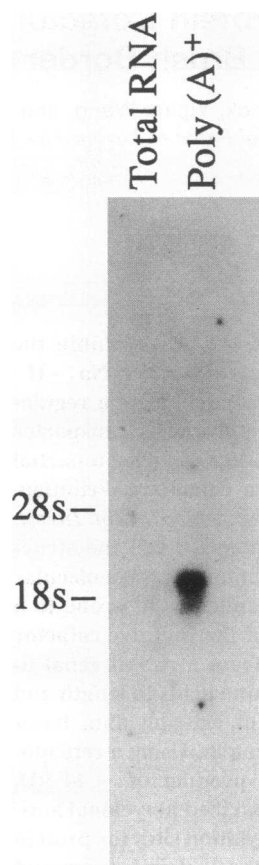


Figure 1. Northern hybridization analysis of rabbit kidney total and poly(A)⁺ RNA using the antisense oligonucleotide probe to amino acids 2–10 of the cyanogen bromide fragment of NHE-RF (7). 28S and 18S markers are shown. The size of the mRNA is estimated to be 1.9 kb.

samples were maintained at 80°C and the Taq polymerase was added to each tube. The products were amplified using 40 cycles at 95°C for 1 min for melting, and 65°C for 3 min for annealing and extending. At the end of the reaction, the samples were cooled and maintained at 4°C. 10 μl of the PCR product was size fractionated using 2% NuSieve:1% SeaKem agarose gel electrophoresis (FMC Corp., Rockland, ME) in 0.5× Tris-Borate EDTA electrophoresis buffer and stained with ethidium bromide. For Southern blotting, the PCR product was transferred to nitrocellulose and hybridized with the isolated cDNA multiprimed labeled with α-[³²P]dCTP. The PCR product was excised from the gel and purified using chloroform-phenol extraction and subsequently cloned into pCR II vector using the TA cloning kit (Invitrogen, San Diego, CA). Sequences were determined using a DNA sequencer (373A; Applied Biosystems, Inc., Foster City, CA).

Results

Fig. 1 is a Northern blot of rabbit renal poly(A)⁺ RNA probed with the oligonucleotide probe. The oligonucleotide probe hybridized to a single mRNA of 1.9 kb. The probe was then used to screen a rabbit kidney cDNA library. Initially, 1 million colonies were screened. Three positive colonies were isolated and sequenced. Two isolates contained inserts that proved to be fragments of the largest isolate of 1.9 kb. The complete nucleotide sequence of the 1.9-kb clone is shown in Fig. 2. The longest open reading frame predicts a protein of 358 amino acids in length. There are two regions corresponding to amino acids 19–26 and amino acids 159–166 that are 100% homologous to the sequence of the isolated cyanogen bromide peptide

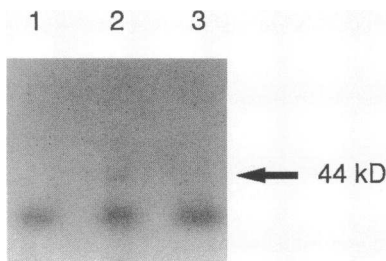


Figure 3. Cell-free translation of the cDNA in a reticulocyte lysate. The product was immunoprecipitated using an affinity-purified antipeptide antibody to NHE-RF. Shown is an autoradiograph of a 15% SDS-PAGE gel of the immu-

noprecipitated product. Control lane contains no promoter. Lanes 2 and 3 represent the T7 and T3 promoter elements, respectively.

taining [³⁵S]methionine. The product of the reaction was immunoprecipitated using an affinity-purified polyclonal antibody to an artificial peptide representing the same amino acids used to develop the oligonucleotide probe (7). Fig. 3 is an autoradiograph of the immunoprecipitated ³⁵S product of the isolated cDNA. The size of the product recognized by the antibody is > 44 kD as estimated by SDS-PAGE and is close to that previously reported (12).

Fig. 4 is a Northern hybridization analysis using poly(A)⁺ RNA from the rabbit kidney cortex. Fig. 5 is a Northern hybridization analysis using total RNA from various tissues of the rabbit. Equal loading of the lanes was verified by ethidium bromide staining and by hybridization using a cDNA probe to GAPDH. The cDNA to NHE-RF hybridized to a single mRNA of 1.9 kb in the kidney, small intestine, and liver. All other organs tested were negative. The nephron distribution of the mRNA of the putative regulatory cofactor. RT-PCR was used to determine the nephron distribution of the mRNA of the putative regulatory cofactor. RT-PCR was performed initially using rabbit kidney RNA and oligonucleotide or random primers for reverse transcription. For the PCR reaction, the oligonucleotide sense primer, 5'-GAAGCGCAGCAGCAGACGGGC-3', and anti-sense primer, 5'-ACTGGAGAATCGGGCGGAGAGG-3', were used. These primers spanned the stop codon region of the cDNA. The RT-PCR product was of the appropriate size of ~ 121 bp; it was hybridized to the isolated cDNA using South-

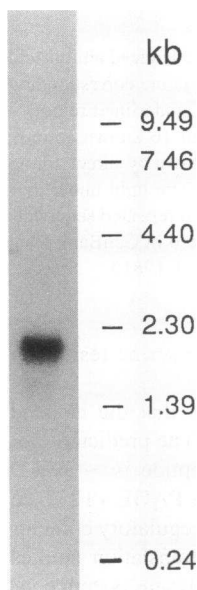


Figure 4. Northern hybridization analysis of rabbit kidney poly(A)⁺ RNA using the cloned cDNA as the probe.

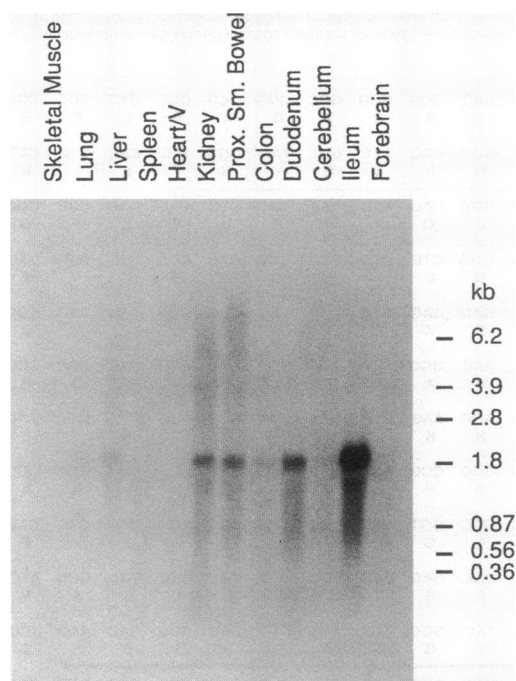


Figure 5. Northern hybridization analysis of RNA from various tissues from the rabbit using the cloned cDNA as the probe.

ern hybridization techniques; and it contained the exact predicted sequence as confirmed by direct analysis. Fig. 6 is an ethidium bromide stain of the RT-PCR product indicating generation of a product of appropriate molecular weight. Also shown is the lack of a product in the absence of RT. For the study of tubules, distinct microdissected tubule segments were pooled for RT-PCR. The product was identified by size and by Southern blotting using the isolated cDNA. Each type of individual dissected nephron segment was studied two or more times in tubules obtained from different animals. As summarized in Table I, the mRNA for the regulatory cofactor was detected in the glomerulus; the S₁, S₂, and S₃ portions of the proximal tubule; the thick ascending limb of Henle's loop; and the cortical collecting duct. It was not detected in the thin descending limb of Henle's loop or the medullary collecting duct. To insure that the failure to detect the mRNA for NHE-RF in the thin descending limb and in the medullary collecting duct was not the consequence of technical considerations, RT-PCR was performed in these nephron segments using primers to GAPDH. A product of appropriate size was detected in both nephron segments (data not shown).

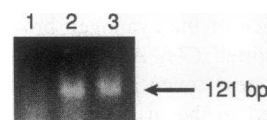


Figure 6. Representative agarose gel of RT-PCR product of NHE-RF using rabbit kidney RNA. The results in the absence (lane 1) or presence (lane 2) of reverse transcriptase are shown. Lane 3 is the S₁ segment of the proximal convoluted tubule as a representative example of the product obtained from tubules containing the mRNA for NHE-RF.

from either parent NHE-RF protein or the 21-kD peptide fragment suggests also that they shared a blocked NH₂-terminal end. It is likely that the 21-kD peptide represents the primary sequence between residues 2 and 157. By contrast, the 31-kD peptide contained all the [³²P]phosphate incorporated into NHE-RF by PKA and probably represents a more COOH-terminal fragment between residues 158 and 346 in the NHE-RF protein.

Northern blot hybridization analysis was used to determine the size of the mRNA and its tissue distribution. The cDNA identified a single message in rabbit kidney of a size identical to that seen using the oligonucleotide probe. A message of the same size was identified also in small intestine. Western immunoblot analysis of rabbit renal and small intestinal brush border membranes using an antibody to NHE-RF identified a protein of the same molecular weight in both tissues (7). As determined immunologically, protein expression per milligram of total protein was somewhat greater in the small intestine than kidney. These results appear consistent with the greater abundance of the mRNA for NHE-RF in small intestine as compared to kidney. It is of interest that liver contained significant amounts of NHE-RF mRNA. The significance of this finding is unknown.

RT-PCR was used to identify more precisely the nephron segments that expressed the mRNA for the regulatory cofactor. These experiments were important since prior efforts using the antipeptide antibody and immunocytochemistry to localize the protein to specific cells in the kidney were unsuccessful. The RT-PCR procedure was validated using total RNA from kidney. The RT-PCR product was of the correct predicted size, hybridized to the cloned cDNA using Southern blotting procedures, and was the precise nucleotide sequence encompassed by the PCR primers. The RT-PCR product from tubule segments was of the same size as that obtained using total rabbit kidney RNA, and the identity of this product was confirmed by Southern hybridization with the cloned cDNA. By this analysis, the glomerulus, the proximal tubule, the thick ascending limb, and the cortical collecting duct contained NHE-RF mRNA. No RT-PCR product was found in the thin descending limb or medullary collecting duct.

Hydropathy analysis gives no indication of transmembrane structures in the NHE-RF protein (Fig. 8). This predicted structure correlates well with Western immunoblot studies that indicate the presence of the protein in BBM preparations from rabbit kidney, rabbit small intestine, LLCPK₁ cells, and in the plasma membrane of OK cells (7). In all these tissues, the protein was detected also in significant abundance in the cytosolic fraction. As NHE-RF exists in both soluble and membrane-bound forms, it could be speculated that, in addition to its phosphorylation by PKA, the distribution of this cofactor within the cell may play a role in hormonal control of Na⁺-H⁺ exchange. Moreover, the duplicated domains may represent sites of interaction of NHE-RF with the transmembrane protein that represents the antiporter. Selective deletion of each of these domains and potential sites of phosphorylation by PKA will help to define the functional role of individual structural elements in the control of Na⁺-H⁺ exchange activity.

The present studies define the structure of a unique protein expressed in tissues that have Na⁺-H⁺ exchange activity that is inhibited by cAMP. To date, the evidence that indicates that NHE-RF is required for expression of the inhibitory effect of

PKA on the Na⁺-H⁺ exchanger derives solely from in vitro studies using BBM from the kidney of rabbit (5-7). Recently, Levine et al. transfected PS120 cells, Chinese hamster lung fibroblast cells lacking endogenous Na⁺-H⁺ exchange activity, with either NHE-1, NHE-2, or NHE-3. The transfected cells displayed Na⁺-H⁺ exchange activity as determined by a functional assay but did not demonstrate regulation of Na⁺-H⁺ exchange activity by cAMP (16). These experiments would appear to indicate that none of the three isoforms tested is regulated by PKA under the conditions of study. Although metabolic labeling and increased incorporation of [³²P]phosphate into expressed NHE proteins following cAMP stimulation was not examined, these results are consistent with the hypothesis that PKA regulation of the BBM exchanger requires a regulatory cofactor distinct from the exchanger itself. Preliminary studies in our laboratory using Western immunoblot analysis indicate that PS120 cells do not contain the putative regulatory protein. This may provide an explanation for the inability of PKA to regulate the activity of the transfected NHE isoforms. By contrast, Yamaji et al. have reported in abstract form the expression of NHE-3 in xenopus oocytes (17). In this expression system, cAMP did inhibit Na⁺-H⁺ exchange activity. This predicts that amphibian oocytes contain a regulatory factor that is functionally similar to NHE-RF. It is also possible that PKA affects the activity of the Na⁺-H⁺ exchange activity in oocytes by a mechanism different from that identified in rabbit kidney proximal tubule cells (14). The expression of recombinant NHE-RF protein should facilitate future studies examining the expression of this protein in other cells and species as well as establishing the physiologic role of NHE-RF in mammalian cells.

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References

1. Kahn, A. M., G. M. Dolson, M. K. Hise, S. C. Bennett, and E. J. Weinman. 1985. Parathyroid hormone and dibutyryl cyclic AMP inhibit Na⁺-H⁺ countertransport in brush border membrane vesicles isolated from a suspension of rabbit proximal tubules. *Am. J. Physiol.* 248 (*Renal Fluid Electrolyte Physiol.* 17):F212-F218.
2. Dolson, G. M., M. K. Hise, and E. J. Weinman. 1985. Relationship among parathyroid hormone, CAMP, and calcium on proximal tubule sodium transport. *Am. J. Physiol.* 249 (*Renal Fluid Electrolyte Physiol.* 18):F409-F416.
3. Weinman, E. J., S. Shenolikar, and A. M. Kahn. 1987. CAMP-associated inhibition of Na⁺-H⁺ exchanger in rabbit kidney brush-border membranes. *Am. J. Physiol.* 252 (*Renal Fluid Electrolyte Physiol.* 21):F19-F25.
4. Weinman, E. J., W. P. Dubinsky, and S. Shenolikar. 1988. Reconstitution of cAMP-dependent protein kinase regulated renal Na⁺-H⁺ exchanger. *J. Membr. Biol.* 101:11-18.
5. Weinman, E. J., W. P. Dubinsky, Q. Dinh, D. Steplock, and S. Shenolikar. 1989. The effect of limited trypsin digestion on the renal Na⁺-H⁺ exchanger and its regulation by CAMP dependent protein kinase. *J. Membr. Biol.* 109:233-241.
6. Weinman, E. J., D. Steplock, G. Bui, N. Yuan, and S. Shenolikar. 1990. Regulation of the renal Na⁺-H⁺ exchanger by CAMP dependent protein kinase. *Am. J. Physiol.* 258 (*Renal Fluid Electrolyte Physiol.* 27):F1254-F1258.

7. Weinman, E. J., D. Steplock, and S. Shenolikar. 1993. CAMP-mediated inhibition of the renal brush border membrane Na⁺-H⁺ exchanger requires a dissociable phosphoprotein cofactor. *J. Clin. Invest.* 92:1781-1786.
8. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294-5299.
9. Rothman, R. E., D. W. Andrews, M. C. Calayag, and V. R. Lingappa. 1993. Construction of defined polytopic integral transmembrane proteins. *J. Biol. Chem.* 268:17-20.
10. Chen, D., V. Magnuson, S. Hill, C. Arnaud, B. Steffensen, and R. J. Klebe. 1992. Regulation of integrin gene expression by substrate adherence. *J. Biol. Chem.* 267:23502-23506.
11. Chen, D., V. L. Magnuson, B. Steffensen, and R. J. Klebe. 1993. Use of stock solutions to simplify mRNA quantitation by reverse transcription PCR assays. *PCR Meth. Applications.* 2:351-353.
12. Morell, G., D. Steplock, S. Shenolikar, and E. J. Weinman. 1990. Identification of a putative Na⁺-H⁺ exchanger regulatory co-factor in rabbit renal apical membranes. *Am. J. Physiol.* 259(Renal Fluid Electrolyte Physiol. 28):F867-F871.
13. Weinman, E. J., W. Dubinsky, and S. Shenolikar. 1989. Regulation of the renal Na⁺-H⁺ exchanger by protein phosphorylation. *Kidney Int.* 36:519-525.
14. Weinman, E. J., and S. Shenolikar. 1993. Regulation of the renal brush border membrane Na⁺-H⁺ exchanger. *Annu. Rev. Physiol.* 55:289-304.
15. Adams, J. A., and S. S. Taylor. 1993. Phosphorylation of peptide substrates for the substrates for the catalytic subunit of cAMP-dependent protein kinase. *J. Biol. Chem.* 268:7747-7752.
16. Levine, S. A., M. H. Marshall, C. M. Tse, and M. Donowitz. 1993. Kinetics and regulation of three cloned mammalian Na⁺/H⁺ exchangers stably expressed in a fibroblast cell line. *J. Biol. Chem.* 268:25527-25535.
17. Yamaji, Y., M. Amemiya, R. J. Alpern, and O. W. Moe. 1993. Protein kinase A (PKA) and a calcium independent protein kinase phosphorylate the cytoplasmic domain of Na/H exchanger-3 (NHE-3) in vitro. *J. Am. Soc. Nephrol.* 24:851. (Abstr.).