

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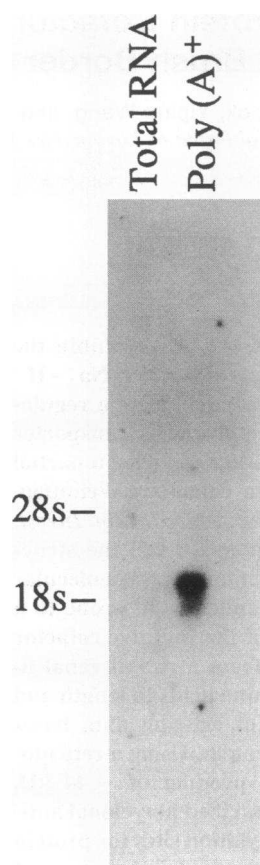
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1. Abbreviations used in this paper: BBM, brush border membrane; NHE-RF, regulatory cofactor of  $\text{Na}^+/\text{H}^+$  exchanger; PKA, protein kinase A; RT, reverse transcription.

0.1% SDS. The column was washed in the same buffer. Poly(A)<sup>+</sup> 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 NaHPO<sub>4</sub> (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  $\alpha$ -[<sup>32</sup>P]dCTP, or end labeled using  $\gamma$ -[<sup>32</sup>P]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 [<sup>35</sup>S]methionine. The polypeptide product was immunoprecipitated by addition of a PBS solution containing 10 mM Na<sub>2</sub>HPO<sub>4</sub>/1.8 mM KH<sub>2</sub>PO<sub>4</sub> (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  $\mu$ l of a solution containing 2% Triton X-100, 1 U/ $\mu$ l 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/ $\mu$ l SuperScript II RT (a cloned Moloney murine leukemia virus reverse transcriptase) (GIBCO-BRL, Gaithersburg, MD), 2.5 mM MgCl<sub>2</sub>, 0.5 mM of each dNTP, 12 mM DTT, and 25 ng/ $\mu$ l of primers in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1  $\mu$ g/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/ $\mu$ l *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 MgCl<sub>2</sub>, 0.1 mM dNTP, 0.1  $\mu$ M of each specific primer, 0.05 U/ $\mu$ l Taq DNA polymerase, 50 mM KCl, 0.1  $\mu$ g/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)<sup>+</sup> 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  $\mu$ l 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  $\alpha$ -[<sup>32</sup>P]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)<sup>+</sup> 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

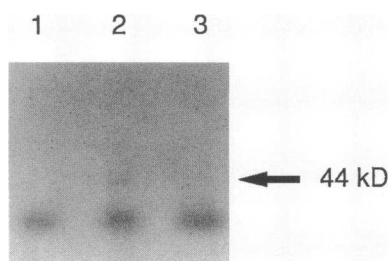
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																-1
ATG	AGC	GCG	GAC	GCG	GCG	GCC	GGG	GCG	CCC	CTG	CCC	CGG	CTC	TGC	TGC	48
M	S	A	D	A	A	A	G	A	P	L	P	R	L	C	C	16
CTG	GAG	AAG	GGG	CCG	AAC	GGC	TAC	GGC	TTC	CAC	CTG	CAT	GGG	GAG	AAG	96
L	E	K	G	P	N	G	Y	G	F	H	L	H	G	E	K	32
GGC	AAG	GTG	GGC	CAG	TAC	ATC	CGG	CTG	GTG	GAG	CCC	GGC	TCT	CCG	GCC	144
G	K	V	G	G	Y	I	R	L	V	E	P	G	S	P	A	48
GAG	AAG	GCG	GGG	CTG	CTG	GCC	GGG	GAC	CGG	CTG	GTG	GAG	GTG	AAC	GGC	192
E	K	A	G	L	L	A	G	D	R	L	V	E	V	N	G	64
GAG	AAC	GTG	GAG	AAG	GAG	ACC	CAC	CAG	CAG	GTG	GTG	AGC	CGC	ATC	CGC	240
E	N	V	E	K	E	T	H	Q	Q	V	V	S	R	I	R	80
GCC	GCG	CTC	AAC	GCC	GTG	CGC	CTG	CTG	GTG	GTC	GAC	CCC	GAC	ACG	GAC	288
A	A	L	N	A	V	R	L	L	V	V	D	P	D	T	D	96
GAG	CAG	TTC	AGG	AAG	CTC	GGA	GTC	CAG	ATC	CGG	GGG	GAG	CTG	CTA	CGC	336
E	Q	F	R	K	L	G	V	Q	I	R	G	E	L	L	R	112
GCC	CAG	GCG	GGG	CCG	GAG	CAG	GCC	GGG	CCG	CCG	GCC	GCC	CCC	GGC	GAG	384
A	Q	A	G	P	E	Q	A	G	P	P	A	A	P	G	E	128
CAG	GGC	CCG	GCC	GGT	GAG	AAC	GAG	CCG	CGC	GAG	GTC	GAG	AAG	AGC	CAC	432
Q	G	A	G	E	E	N	E	P	R	E	V	E	K	S	H	144
CCG	GAG	CGG	CGC	GAG	CTC	CGG	CCT	CGG	CTC	TGC	GCC	ATG	AAG	AAG	GGC	480
P	E	R	R	E	L	R	P	R	L	C	A	M	K	K	G	160
CCC	AAC	GGC	TAC	GGC	TTC	AAC	CTG	CAC	AGC	GAC	AAG	TCC	AGG	CCG	GGC	528
P	N	G	Y	G	F	N	L	H	S	D	K	S	R	P	G	176
CAG	TTC	ATC	CGC	GCC	GTG	GAC	CCA	GAC	TCC	CCG	GCT	GAG	GCC	TCG	GGG	576
Q	F	I	R	A	V	D	P	D	S	P	A	E	A	S	G	192
CTC	CGG	GAG	CAG	GAC	CGC	ATC	GTG	GAG	GTG	AAC	GGG	GTC	TGC	GTG	GAG	624
L	R	E	Q	D	R	I	V	E	V	N	G	V	C	V	E	208
GGC	AAG	CAG	CAT	GGG	GAC	GTG	GTG	ACT	GCC	ATC	AAG	GCC	GGC	GGG	GAT	672
G	K	Q	H	G	D	V	V	T	A	I	K	A	G	G	D	224
GAG	GCC	AAG	CTG	CTG	GTG	GTG	GAC	AAG	GAG	ACC	GAC	GAC	TTC	TTC	AAG	720
E	A	K	L	L	V	V	D	K	E	T	D	D	F	F	K	240
AAA	TGC	AAA	GTG	GTC	CCA	TCT	TCT	GAG	CAC	CTC	AAC	GGT	CCC	TTG	CCT	768
K	C	K	V	V	P	S	S	E	H	L	N	G	P	L	P	256
GAG	CCC	TTC	ACC	AAT	GGG	GAG	ATA	CAG	AAG	AAC	AAC	CCT	GAG	ACC	CTG	816
E	P	F	T	N	G	E	I	Q	K	N	N	P	E	T	L	272
GCC	CCA	GCG	GCC	TCG	GAG	AGC	CCC	AGG	CCA	GCC	CTG	GCA	AGA	TCC	GCC	864
A	P	A	A	S	E	S	P	R	A	L	L	A	R	S	A	288
TCC	AGT	GAC	ACC	AGC	GAG	GAG	CTG	GCT	TCC	CAA	GAC	AGC	CCC	AAG	AAA	912
S	S	D	T	S	E	E	L	A	S	Q	D	S	P	K	K	304
GAG	GAC	TCC	ACG	GCC	CCC	TCC	TCG	ACC	TCC	TCC	TCC	TCC	GAC	CCC	ATC	960
E	D	S	T	A	P	S	S	T	S	S	S	S	D	P	I	320
CTG	GAC	TTC	AGC	ATC	TCG	CTG	GCC	GTG	GCC	AAG	GAG	AGG	GCC	CAC	CAG	1008
L	D	F	S	I	S	A	V	A	K	E	R	A	A	H	Q	336
AAG	CGC	AGC	AGC	AGA	CGG	GCC	CCC	CAA	ATG	GAC	TGG	AGC	GAG	AAA	AAG	1056
K	R	S	S	R	R	A	P	Q	M	D	W	S	E	K	K	352
GAG	CTT	TTC	AGC	AAC	CTC	TGA	gcg	ccc	cgg	ccg	ccc	acc	ccc	cgt	cac	1104
E	L	F	S	N	L	.										368
ctcctctcgccccgattctcattccccagccgccacccctcttcacctctctcgcgccaccttcacacccctggcgccccacccacctcactctctc cgcccgcttcacagtcacccggcgccacccctcactcactctctcgcgccgcttcctcaalcacccctcgatctacagatcagcaccagtgccc cgcttcctggccalggacttttttagagaactgtgcacttcctgctcgcaggaaggcaaatgctttctgacctaccccatcaaacagcagcagactctgccc tctgctcccgctggctgtgcttgggacggcaccgggacgtttagcgaggaagggtggccaccagggcagtggtctcttcctcacaacccacaaag ggacaggggactttggcgagccacagctcactcgcggggcgctcgctgctgacagctgtggctggttcgacctctgtttttgtgatttttttaag ggtgcacalcagtttagagagacgttcattcttgaftaacgtgatttt																1207
																1309
																1413
																1514
																1626
																1676

Figure 2. Nucleotide and deduced amino acid sequences of the 1.9-kb clone corresponding to NHE-RF. The heavy underlined region corresponding to the first 16 known amino acids determined previously by direct amino acid sequence analysis. The light underline indicates the 8 amino acid repeated sequence. This sequence is registered in GenBank Data Bank, accession number U19815.

used to construct the oligonucleotide probe. The sequence starting at residue 157 and extending to amino acid 172 is 100% homologous to the amino acid sequence of the cyanogen bromide peptide fragment originally reported (7). As predicted from the cDNA, the sequence of the next seven amino acids contains four "mismatches" compared to the original amino acid sequence. Two of the "errors" were alternate calls near the end of the amino acid sequence determination, a region where confidence in the sequencing is low. Two potential phos-

phorylation sites for PKA are predicted. The serine residue at position No. 339 is probably preferred.

Search of computer data banks indicates that the isolated cDNA represents a newly described protein. The predicted molecular mass of the 358-amino acid polypeptide is ~ 39 kD and is smaller than that estimated by SDS-PAGE (12). To establish that the cDNA encodes the putative regulatory cofactor and to determine the size of the product, translation studies were performed using a cell-free reticulocyte lysate system con-

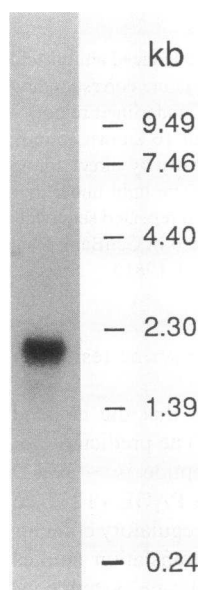


**Figure 3.** Cell-free translation of the cDNA in a reticulocyte lysate. The product was immunoprecipitated using an affinity-purified antipeptide antibody to NHE-RF. Shown is an autoradiograph of a 15% SDS-PAGE gel of the immu-

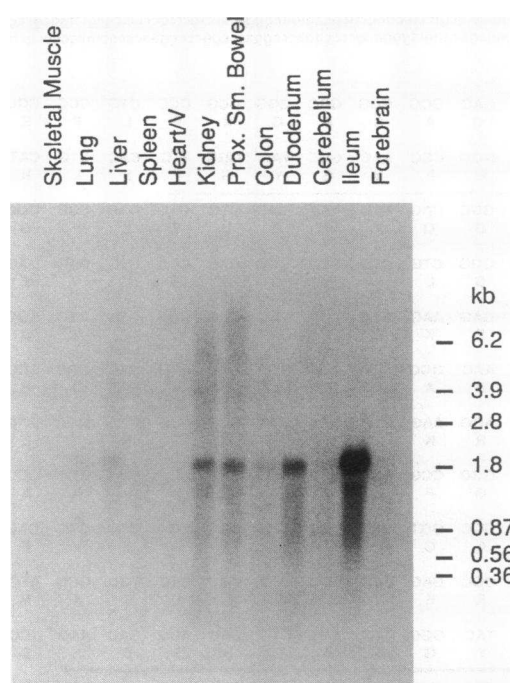
noprecipitated product. Control lane contains no promoter. Lanes 2 and 3 represent the T7 and T3 promoter elements, respectively.

taining [ $^{35}\text{S}$ ]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  $^{35}\text{S}$  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. 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  $\sim 121$  bp; it was hybridized to the isolated cDNA using South-

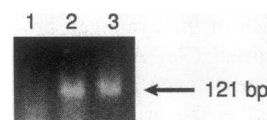


**Figure 4.** Northern hybridization analysis of rabbit kidney poly(A) $^{+}$  RNA using the cloned cDNA as the probe.



**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  $S_1$ ,  $S_2$ , and  $S_3$  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  $S_1$  segment of the proximal convoluted tubule as a representative example of the product obtained from tubules containing the mRNA for NHE-RF.

**Table I. Detection of mRNA for NHE-RF in Nephron Segments of the Rabbit Nephron Using RT-PCR**

Nephron segment	NHE-RF mRNA
Glomerulus	Detected
Proximal tubule-S <sub>1</sub>	Detected
Proximal tubule-S <sub>2</sub>	Detected
Proximal tubule-S <sub>3</sub>	Detected
Thin descending limb	Not detected
Thick ascending limb	Detected
Cortical collecting duct	Detected
Medullary collecting duct	Not detected

## Discussion

As summarized in the introduction and in recent publications from this laboratory, the inhibitory effect of PKA on the renal brush border  $\text{Na}^+ - \text{H}^+$  exchanger appears to involve participation of an additional protein cofactor (13, 14). This cofactor, named NHE-RF, is a thermolabile, trypsin-sensitive substrate for PKA that is distinct and dissociable from the  $\text{Na}^+ - \text{H}^+$  exchange transporter itself. In prior studies from this laboratory, NHE-RF had been purified using column chromatography, and a partial amino acid sequence was obtained (7). This amino acid sequence information permitted construction of an oligonucleotide probe, biased toward codon usage in the rabbit, and containing four inosines in the highly variant third base positions. The oligonucleotide probe identified a single mRNA in rabbit kidney cortex using Northern hybridization analysis. Screening of a rabbit kidney cDNA library with the oligonucleotide probe resulted in isolation of a 1.9-kb cDNA. Computer search of existing data bases indicated that the cDNA encodes a protein not previously described.

The finding that size of the mRNA and the cloned cDNA were the same suggests that a full-length clone was isolated. The cDNA encodes a protein consisting of 358 amino acids with a calculated molecular mass of 38.6 kD. This predicted size is less than that suggested from prior studies using column chromatography and autoradiography of SDS-PAGE gels containing the cofactor phosphorylated by PKA (4–7, 12). To clarify this issue, cell-free translation studies were performed. These experiments indicated that the cDNA encodes a single protein recognized by the affinity-purified polyclonal antibody to the protein with an apparent molecular mass of 44 kD as determined by slab gel electrophoresis. This estimated size is more consistent with that determined previously (12). Translation of the cDNA indicates that there are relatively few hydrophobic residues. There is a single tryptophan, three tyrosine, and nine phenylalanine residues. By contrast, the protein is relatively enriched in charged residues, with 55 acidic and 48 basic amino acids. The predicted amino acid sequence of NHE-RF indicates a sequence, residues 160–181, closely following a methionine at position 157 that matched precisely the NH<sub>2</sub>-terminal sequence obtained from the purified cyanogen bromide peptide fragment of NHE-RF (7). This finding confirms that the appropriate cDNA had been isolated. Further analysis of this amino acid sequence identified a second sequence, residues 21–42, that shared 75% identity with the NH<sub>2</sub>-terminal peptide

#### DUPLICATION OF AN INTERNAL SEQUENCE IN NHE-RF

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11                                     59
Domain I L-PRLLCCLEKGPNGYGFHLHGEGKVGQYIRLVEPGSPAEEKAGLLAGDRLL
          : : : : : : : : : : : : : : : : : : : : : : : : : : :
Domain II LRPRLLCAMKKGPNGYGFNLHSDKSRGPQIRAVDPDSPAEEASGLREQDRIR
          150                                     200

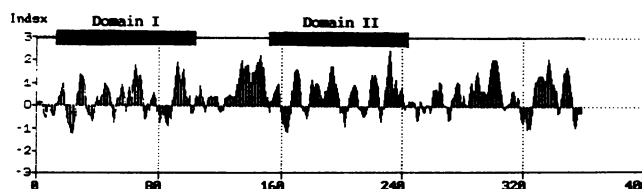
60                                     101
Domain I VEVNGENVE-KETHQQVVSRIIRAALN-AVRLLVDPDPTDEQFRK
          : : : : : : : : : : : : : : : : : : : : : : : : : :
Domain II VEVNGVCVEGKQ-HGDVVTAIKAGGDEA-KLLVVDKETDEFFPK
          201                                     241

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**Figure 7.** Comparison of the predicted amino acid sequences of the NHE-RF cDNA indicating homology between amino acids 11–101 (*Domain I*) and amino acids 150–241 (*Domain II*).

sequence of the 31-kD phosphopeptide that was used to develop the oligonucleotide probe and the anti-peptide antibody described in this and prior studies (7). In fact, the homologous sequence exceeded the 21 amino acids that were sequenced (Fig. 7). The two domains encompassed by the residues 11–101 and 150–241 showed 74% overall homology suggesting duplication of this domain. The homology between these domains predicts considerable conservation of the secondary structure of domains I and II. Collectively, domains I and II make up nearly 70% of the NHE-RF protein (Fig. 8).

The extreme COOH-terminal sequence of NHE-RF that extends beyond domain II contains the majority of the serine and threonine residues and may include the sites phosphorylated by PKA. The optimal consensus motif for PKA has been defined as RR/KXYS\*H, where \* indicates the phosphorylated serine residue (15). In the NHE-RF protein, only two serine residues and none of the threonine residues are preceded by NH<sub>2</sub>-terminal basic residues that are essential determinants for substrate phosphorylation by PKA. This is consistent with the suggestion that NHE-RF is phosphorylated by PKA exclusively on serine residues. However, since there is only one basic residue preceding each of the two serines, neither of the two potential phosphorylation sites appears to represent an idealized substrate for PKA. The predicted NHE-RF protein sequence contained three methionines at positions 1, 157, and 346. This information provided an explanation for the observation that cyanogen bromide cleavage of the native NHE-RF resulted in only two major peptide fragments, of apparent molecular mass 21 kD and 31 kD. The 21-kD cyanogen bromide peptide obtained by hydrolysis of the PKA-phosphorylated NHE-RF contained no [<sup>32</sup>P]-phosphate, and repeated efforts to sequence this NH<sub>2</sub>-terminal peptide were unsuccessful. Attempts to determine the NH<sub>2</sub>-terminal sequence of the intact NHE-RF protein were also unsuccessful. These results are consistent with the location of this 21-kD cyanogen bromide peptide fragment at the NH<sub>2</sub>-terminal end of NHE-RF. The failure to obtain amino acid sequences



**Figure 8.** Hydropathy analysis of NHE-RF indicating homologous domains.

from either parent NHE-RF protein or the 21-kD peptide fragment suggests also that they shared a blocked NH<sub>2</sub>-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 [<sup>32</sup>P]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, LLC-PK<sub>1</sub> 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<sup>+</sup>-H<sup>+</sup> 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<sup>+</sup>-H<sup>+</sup> exchange activity.

The present studies define the structure of a unique protein expressed in tissues that have Na<sup>+</sup>-H<sup>+</sup> 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<sup>+</sup>-H<sup>+</sup> 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<sup>+</sup>-H<sup>+</sup> exchange activity, with either NHE-1, NHE-2, or NHE-3. The transfected cells displayed Na<sup>+</sup>-H<sup>+</sup> exchange activity as determined by a functional assay but did not demonstrate regulation of Na<sup>+</sup>-H<sup>+</sup> 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 [<sup>32</sup>P]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<sup>+</sup>-H<sup>+</sup> 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<sup>+</sup>-H<sup>+</sup> 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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