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Expression of a Novel Sodium-Hydrogen Exchanger in the Gastrointestinal Tract and Kidney

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Abstract. We have recently cloned a novel Na^+/H^+ exchanger (NHE-2) from rat small intestinal cDNA library. The present report characterizes the expression of (NHE-2) mRNA in the rat small intestine, stomach, and kidney using Northern blot analysis and *in situ* hybridization techniques. NHE-2 mRNA was detected in the surface epithelium of the stomach, villus but not crypt epithelial cells of the small intestine and in the distal convoluted, but not in the proximal tubules of the kidney. This distribution suggests a functional role in the transport of sodium across the apical membrane of the gastrointestinal tract and the kidney.

Key words: Sodium-hydrogen exchanger — Gastrointestinal tract — Kidney

Introduction

Na^+/H^+ exchangers belong to a family of exchangers that mediate the electroneutral exchange of Na^+ and H^+ [1]. Four isoforms have been cloned from rat cDNA libraries [13, 21] and three were cloned from rabbit cDNA libraries [17, 18, 19]. These cDNA libraries were screened utilizing a 1.9 kb fragment of the human fibroblast Na^+/H^+ exchanger (NHE-1) [15]. The function of NHE-1 appears to be in the regulation of intracellular pH [20]. We have recently cloned a novel rat Na^+/H^+ exchanger that appears to be expressed in the epithelia of the gastrointestinal tract and the kidney [6]. This exchanger is unique as it shows no homology at its 5′ end to the other Na^+/H^+ isoforms. The cDNA is 4 kb long and has a 546-nucleotide 5′ untranslated region, 1322 nucleotide of 3′ untranslated region, and an open reading frame of 2091 nucleotides encoding a protein of 697 amino acid residue with a calculated molecular weight of 79,063. The protein is predicted to have ten transmembrane domains, but it lacks the first two hydrophobic domains, which are present in a similar NHE-2, cloned from rat stomach cDNA library [21]. Moreover, our NHE-2 recognized two transcripts at 4.4 and 4.6 kb, which suggests that the predominant transcript of our clone is alternatively spliced. To determine the expression of the NHE-2 mRNA in the gastrointestinal and kidney we employed the techniques of Northern blot analysis and *in situ* hybridization.

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Materials and Methods

Generation of Antisense RNA Fragments

For generation of RNA probes, T3 RNA polymerase was used to generate sense probes and T7 RNA polymerase was used to generate antisense probes from the linearized pBluescript^{R}SK(−) plasmid [2]. Probes were generated in the presence of [α-^{35}S] UTP and 5×10^5 cpm/per μl of reaction solution were used for hybridization.

Tissue Preparation for In Situ Hybridization

Adult male rats weighing 250 g were killed by cervical dislocation. The stomach, proximal jejunum and kidneys were quickly excised, flushed with ice-cold saline solution and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and 7 μm sections were cut.

Prior to hybridization, secretions were deparaffinized, refixed in paraformaldehyde and treated with proteinase K (20 μg/ml), washed with phosphate buffered saline, refixed in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% v/v). Finally, sections were dehydrated to 100% ethanol.

Sense and antisense RNA was hybridized to the sections at 50–55° C for approximately 18 hr. Following hybridization sections were washed at 50° C in 5× SSC ± 10 mM β-mercaptoethanol for 30 min.

This was followed by a wash in 50% formamide, 2 × SSC, and 100 mM β-mercaptoethanol for 30 min. Following additional washes in (mM) 10 Tris, 5 EDTA, 500 NaCl, sections were treated with RNAase (10 μg/ml) for 37° C, for 30 min, followed by another wash in the same solution (37° C). Sections were then washed twice in 2 × SSC; and twice in 0.1× SSC (50° C). Slides were then dehydrated with graded ethanols containing 300 mM ammonium acetate.

For detection of the hybridized probe, slides were dipped in photoemulsion (Ilford K5, Knutsford, England) diluted 1:1 with 2% glycerol/water and exposed for 7 days at 4° C. Following development in Kodak D19, slides were taken using a Zeiss Axioskop using both bright and dark field optics.

Tissue Preparation for Fractionation of Rat Enterocytes, Poly(A)$^+$ RNA Preparation and Northern Blot Analysis

Enterocytes were successively eluted from the jejunal crypt-villus axis in five fractions according to the technique originally described by Weiser [23]. The intestinal tissue remaining after elution was designated "lamina propria" and represents nonepithelial elements of the jejunum. Measurement of alkaline phosphatase activity in each cell fraction confirmed that the eluted cells portrayed a typical crypt-villus gradient of differentiation. Total RNA from each epithelial fraction was isolated by centrifugation through a cesium chloride cushion as described by Chirgwin et al. [5]. Oligo(dT)-selected RNA was separated by 1% agarose/formaldehyde gel electrophoresis. Poly(A)$^+$ RNA was also prepared by a commercially available kit, Fast Track (Invitrogen). This method uses a lysis buffer containing RNAse and protein degraders and oligo (dT)-cellulose affinity chromatography five μg of poly(A)$^+$ RNA was fractionated by denaturing agarose gel electrophoresis and transferred onto a nitrocellulose membrane [14]. The poly(A)$^+$ RNA was fractionated on a 1.2% I.D.NA agarose 1× MOPS gel (FMC Bioproducts). The gels were electrophoresed at 33 volts for 12 hr. Membranes were uV cross-linked and prehybridized for 30 min in a Scotlab Cassette at 65° C in the manufacturer's suggested buffer (Scotlab, Shelton, CT). To generate labeled probes, the cDNA was digested with the restriction endonuclease $EcoR_1$ (Promega, Madison, WI) resulting in a 600 bp fragment from the 5′ end. This fragment was gel purified on a 1% agarose 1× TAE gel and random prime labeled with α^{32} PdCTP using The Redivue Kit from Amersham. This 5′ fragment does not show homology to the other NHE isoforms. Hybridization was carried out in the same buffer for 1 hr at 65° C. Post hybridization washes were performed in 0.1× SSC, 0.1% SDS at 65° C.

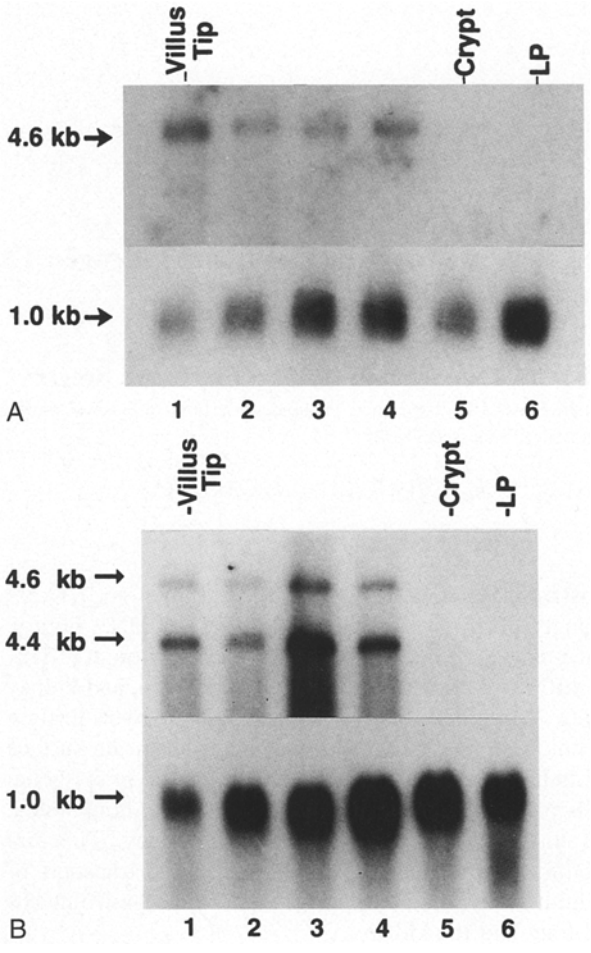

Fig. 1. Differential expression of NHE-2 mRNA in freshly isolated enterocytes from villus-crypt axis. Each lane was loaded with 5 μg of poly(A)$^+$ RNA isolated from rat enterocytes. Following Northern blotting, the nitrocellulose filters were exposed to [^{32}P] dCTP and [^{32}P] dATP labelled NHE-2 and 1B15 cDNA probes and autoradiograms were prepared lane 1–5 indicate poly(A)$^+$ RNA prepared from villus tip to crypt cells sequentially, lane 6 is poly(A)$^+$ RNA from lamina propria (*LP*). Figure 1A depicts Northern blot of RNA prepared by the Chirgwin method. Figure 1B depicts Northern blot of RNA prepared by the Fast Track method (Invirogen).

Results

Northern Blot Analysis of Poly(A)$^+$ RNA from Epithelial Cells Harvested from the Crypt-Villus Axis

Figure 1A depicts Northern blot analysis utilizing two probes, NHE-2 and 1B15 a probe we have used previously for constitutive gene expression (1B15 encodes the rat cyclophilin protein) [3]. The poly(A)$^+$ RNA was prepared according to the original method of Chirgwin utilizing centrifugation through a cesium chloride cushion. As seen, NHE-2 hybridized to a single 4.6 kb transcript in fractions 1–4 representing epithelial cells from tip and mid villus cells. There was no hybridization from fractions 5 and 6 representing cells from the crypt and lamina propria respectively. The constitutive probe 1B15 hybridized to cells from villus tip, crypt and lamina propria. Figure 1B depicts Northern blot analysis utilizing poly(A)$^+$ RNA prepared by the Fast Track kit. As seen, two transcripts were recognized at 4.4 and 4.6 kb in fractions 1–4 representing epithelial cells from tip and mid villus cells. No hybridization was seen from fractions 5 and 6 representing cells from crypt and lamina propria cells. The presence of two transcripts was similar to our previously published Northern blot analysis of various tissue of the rats [6], utilizing poly(A)$^+$ RNA prepared by the Fast Track kit. Scanning densitometry of the Northern blots using a Gel scan XL system showed a threefold increase in mRNA level in fraction 1

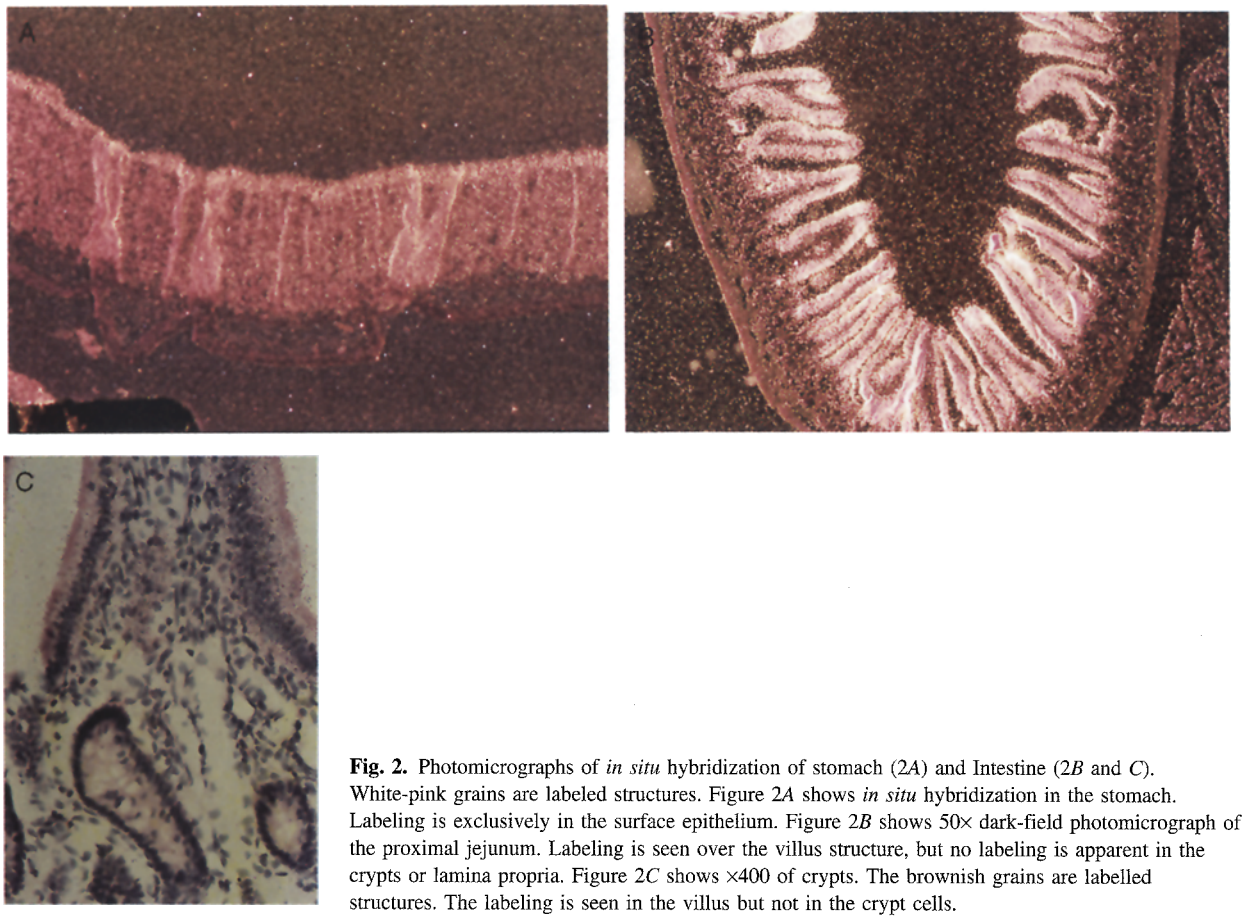

Fig. 2. Photomicrographs of *in situ* hybridization of stomach (2*A*) and Intestine (2*B* and *C*). White-pink grains are labeled structures. Figure 2*A* shows *in situ* hybridization in the stomach. Labeling is exclusively in the surface epithelium. Figure 2*B* shows 50× dark-field photomicrograph of the proximal jejunum. Labeling is seen over the villus structure, but no labeling is apparent in the crypts or lamina propria. Figure 2*C* shows ×400 of crypts. The brownish grains are labelled structures. The labeling is seen in the villus but not in the crypt cells.

compared to fraction 4 in both Figure 1*A* (4.6 kb) and *B* (4.4 and 4.6 kb transcripts).

In Situ Hybridization

Figure 2a–c depict *in situ* hybridization of stomach, and jejunum. Hybridization is seen at the surface epithelium of the stomach and along the villus axis but not in the crypts of the lamina propria. *In situ* hybridization with sense RNA showed minimal or no hybridization (Fig. 3*A* and *B*). Figure 4*A* and *B* depicts *in situ* hybridization of the kidney. As seen, hybridization is present in distinct areas of the kidney cortex and specifically only in the distal convoluted tubules. No hybridization is seen in the glomeruli or in the proximal tubules. *In situ* hybridization with sense RNA showed no hybridization (*data not shown*).

Discussion

The present studies are the first to demonstrate the expression of NHE-2 in the stomach, along the intestinal crypt-villus axis and in the kidney tubules. The presence of Na^+/H^+ exchange at the basolateral membrane of the parietal cell of the stomach has been documented [11] by membrane vesicle studies. The presence of NHE-2,3 and 4 in the stomach was documented by Northern blot analysis [2, 9], however, this is the first demonstration of localization of NHE-2 at the surface of the epithelium in the stomach. In the intestine, the enterocytes constitute 90% of the cells covering the surface of the villus and are renewed continuously every 4–5 days. The cells at the tip of the villus are primarily absorptive, differentiated cells, whereas the cells at the crypts are primarily secretory. NHE-2 is expressed in villus but not crypt cells. This is shown clearly by Northern blot analysis and *in situ* hybridization, both of which show no evidence for the presence of NHE-2 in the crypt whereas the highest intensity for NHE-2 mRNA was found at the tip of the villus as depicted by the scanning densitometry measurements. On the other hand, NHE-1 has a ubiquitous distribution in all mammalian cells suggesting its role as a housekeeping exchanger [1]. NHE-1 has been localized to the basolateral membranes of the enterocytes suggesting again that NHE-1 is a housekeeping exchanger [19, 20]. The NHE-1 message was expressed along the crypt-villus axis, but with greater expression in the differentiating crypt and lower villus cells of the small intestine [4]. NHE-3 appears to be epithelia specific and is ex-

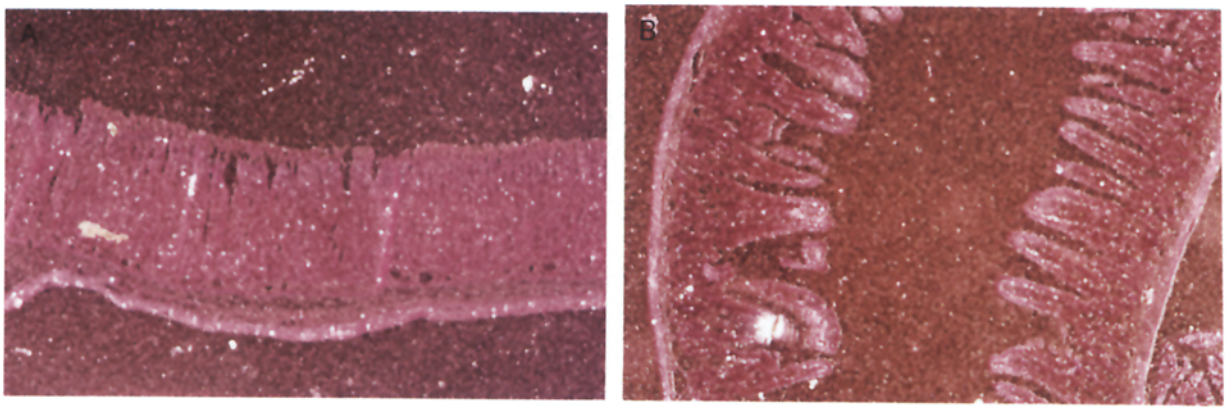

Fig. 3. Photomicromicrophs of *In Situ* hybridization of stomach and small intestine utilizing sense RNA. Figure 3*A* and *B* shows micrograph of stomach and small intestine. No hybridization is seen.

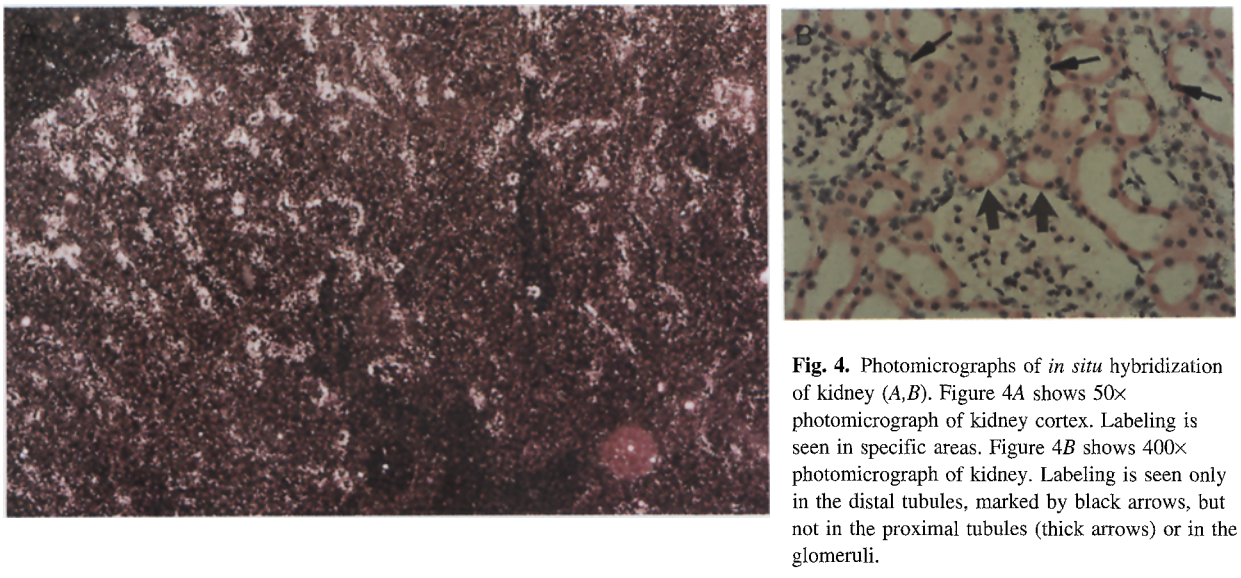

Fig. 4. Photomicrographs of *in situ* hybridization of kidney (*A,B*). Figure 4*A* shows 50× photomicrograph of kidney cortex. Labeling is seen in specific areas. Figure 4*B* shows 400× photomicrograph of kidney. Labeling is seen only in the distal tubules, marked by black arrows, but not in the proximal tubules (thick arrows) or in the glomeruli.

pressed in the ileum, colon and kidney cortex and to a lesser extent in the medulla [17]. NHE-4 has been described in tissues of the rat, however, no function or localization has been ascribed to NHE-4 [21].

The expression of NHE-2 in the distal tubules suggest a functional role in the transepithelial net Na^+ transport and net H^+ secretion. In vivo perfusion studies in the rat suggest that the distal tubule reabsorbs approximately 6.8% of filtered load of Na^+ [10]. At least 50% of this load is thought to be secondary to parallel operation of the apical Na^+/H^+ and Cl/HCO_3^- exchangers [9]. In the distal medullary thick ascending tubule of the rat and mouse an apical Na^+/H^+ exchanger has been described [16, 22]. A distal cortical thick ascending limb Na^+/H^+ exchanger has also been described in the rat and mouse [7, 8]. Na^+/H^+ exchange activity has also been described in the proximal tubules [12] of the kidney. NHE-2 appears to be the predominant isoform in the cortical thick ascending limb. Our studies do not show any evidence for NHE-2 in the proximal tubules or in the glomeruli, suggesting that other isoforms such as NHE-3 might be the predominant form present in the proximal tubules and in the medullary thick ascending limb. An interesting and unexpected finding relates to the effect of method of preparation of mRNA on the expression of NHE-2. Our attention was drawn to this observation when we probed a blot in which the RNA was prepared by the Chirgwin method [5]. A single transcript was observed at 4.6 kb. This observation was in contrast to our previously published data utilizing the Fast Track kit in which two transcripts were recognized at 4.6 and 4.4 kb. Therefore, we carried out several experiments in which we prepared mRNA utilizing the two methods from villus-crypt cells by dividing the eluted cells equally between the two methods. Animals were killed by cervical dislocation without any anesthetic to avoid possible confounding factors, so that the only variable was the method of mRNA preparation. A possible ex-

planation for this finding may include components of the reagents used which degrade certain messages in the Chirgwin method. Regardless of the cause of this finding, investigators need to be aware of this potentially important technical problem which can complicate interpretation of Northern blots.

In summary, we have demonstrated a unique cellular distribution for NHE-2 in the gastrointestinal tract and the kidney.

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