Na^+/H^+ Exchangers, NHE-1 and NHE-3, of Rat Intestine

Expression and Localization

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Abstract

Na-H exchange (NHE) is one of the major non-nutritive Na absorptive pathways of the intestine and kidney. Of the four NHE isoforms that have been cloned, only one, NHE-3, appears to be epithelial specific. We have examined the regional and cellular expression of NHE-3 in the rat intestine. NHE-3 message in the small intestine was more abundant in the villus fractions of the small intestine than in the crypts. Analysis of NHE-3 mRNA distribution in the gut by in situ hybridization demonstrated epithelial cell specificity, as well as expression preferential to villus cells. NHE-1 message, in contrast, was ubiquitous, with slightly greater expression exhibited in the differentating crypt and lower villus cells of the small intestine. Isoform-specific NHE-3 fusion protein antibody identified a 97-kD membrane protein in the upper villus cells of the small intestine, which was exclusively localized in the apical membrane. In contrast, antibody previously developed against the COOH-terminal region of human NHE-1 (McSwine, R. L., G. Babnigg, M. W. Musch, E. B. Chang, and M. L. Villereal, manuscript submitted for publication) identified a 110-kD basolateral membrane protein. These data suggest that unlike NHE-1, which probably serves a "housekeeping" function, NHE-3 may be involved in vectorial Na transport by the intestine. (*J. Clin. Invest.* 1994. 93:106–113.) Key words: vectorial sodium transport • brush border • basolateral membrane • villus • crypt

Introduction

Na-H exchange (NHE)[1] can be identified in virtually all cell types (1, 2), and to date, four distinct NHE isoforms, designated NHE-1, NHE-2, NHE-3, and NHE-4, have been cloned (3–7). The expression of isoforms NHE-2, NHE-3, and NHE-4 are tissue and cell specific (4–7). All four have similar predicted membrane spanning domains, but their cytoplasmic regions, which contain several putative regulatory domains, are markedly different. This may explain the observed differences in function and regulation of Na-H exchange activities of various tissues and cells (8, 9). In contrast to the extensively studied NHE-1, the other members of this family are uncharacterized, and their physiological roles are undefined. Of the four isoforms, only one, NHE-3, appears to be epithelial specific (4, 6). This exchanger is, therefore, a potential candidate for mediating vectorial Na transport, a major function of such cells.

In the intestine, electroneutral Na absorption is predominantly mediated by an amiloride-sensitive luminal membrane Na-H exchanger. Na entry into the cell is favored by the existing Na and proton gradients, Na then being rapidly pumped out by the basolateral membrane Na, K, ATPase. This model predicts that the basolateral membrane NHE isoform NHE-1 is an unlikely candidate for vectorial Na transport, its activation actually being counterproductive to net Na absorption by intestinal epithelial cells. In support of this, luminal application of amiloride analogs inhibit net Na absorption in avian intestine, but when applied serosally, these analogues have no effect on serosal-to-mucosal unidirectional or net Na fluxes (10). These observations suggest that basolateral membrane NHE activity is normally "silent" during net Na absorption, and, based on our knowledge of NHE-1 in other cells, it probably serves cell maintenance functions, activated only in response to conditions such as perturbations in cellular pH or volume (8). By determining the regional and cellular distribution of intestinal NHE-1 and NHE-3, we should gain insight into their potential functional and physiological roles.

Methods

cDNA constructs. The full-length NHE-3 cDNA was cloned from a rat kidney pBR322 library and generously provided to us by G. Shull (University of Cincinnati, Cincinnati, OH) (4). This cDNA, bases 1–3,036, was subcloned into the unique SmaI of pAlter (Promega Biotec, Madison, WI) by blunt end ligation of the 3.16-kb PvuI-NsiI fragment from the original clone. The gel-purified NHE-3 cDNA insert was used as a probe in Northern analysis. The full length NHE-1 cDNA, cloned from a rat heart cDNA library by Orlowski et al. (4), was used as a probe on Northern analysis.

Sequential isolation of enterocytes along the villus to crypt axis. Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 150–250 g were killed with sodium pentobarbitol (100 mg/kg intraperitoneally, as approved by the Animal Care Committee of the University of Chicago), and the small intestine was immediately removed and rinsed with ice-cold PBS (15.2 mM Na_2HPO_4, 4.4 mM KH_2PO_4, pH 7.4, 139 mM NaCl) according to the method of Weiser (11) with modifications by Hoffman and Chang (12). Jejunum (a 20-cm segment immediately distal to the ligament of Treitz) and ileum (a 20-cm segment proximal to the ileo-cecal valve) were removed, rinsed, clamped, filled with 20 mM EDTA in PBS, 1 mM dithiothreitol, and 10% bovine serum, and incubated for 5 min at 37°C

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1. *Abbreviations used in this paper:* BBM, brush border membrane; BLM, pellet containing basolateral, Golgi, and endoplasmic reticulum membranes; GADPH, glyceraldehyde phosphate dehydrogenase; GST, glutathione-S-transferase; NHE, Na-H exchange; SI, sucrose isomaltase.

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with mild agitation to remove the mucus coating the surface. This solution was then discarded and replaced with elution buffer (20 mM EDTA, 1 mM dithiothreitol, and 0.05% β-mercaptoethanol in PBS). The first incubation/rinse step reduced the mucous and noncellular material lining the intestine and improved the yield of RNA from the first fraction. Cells were harvested at intervals of 7 min, 10 min for the last two aliquots. Each fraction was divided. Three quarters was used for RNA extraction and one quarter was reserved for protein isolation, quantitation by Bradford (13), and enzyme assays. The protein fraction was resuspended in PBS and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 6 μg/ml tosylphenylalanine chloromethyl ketone, 1 mM PMSF), immediately processed or quick frozen to −70°C. Verification of gradient was done by determining the sucrase specific activity for each fraction, according to Messer (14).

Preparation of RNA and Northern analysis. The RNA fraction was resuspended in GTC lysis buffer (4 M guanidinium thiocyanate in 100 mM Tris-Cl, pH 7.5, 1% vol/vol β-mercaptoethanol, 0.5% wt/vol sodium lauryl sarcosinate, 0.07% vol/vol Anti-Foam A). The cells were disrupted at high speed by Ultra-Turax (IKA-Works, Inc., Cincinnati, OH) for 60 s and immediately frozen in a dry ice/isopropanol bath. Total RNA was purified by centrifugation through a CsCl cushion. (15). Polyadenylated mRNA was recovered on immobilized oligo(dT) (16, 17). The flow-through fraction, nonpolyadenylated and ribosomal RNA, was included on the gel to serve as control of hybridization specificity. RNA markers (Novagen, Inc., Madison, WI) served as molecular weight size standards. The RNA was transferred to Hybond-N (Amersham Corp., Arlington Heights, IL) by capillary action in 20× SSC (3 M NaCl, 300 mM NaCitrate, pH 7); the RNA crosslinked to the membrane by ultraviolet radiation (Stratalinker; Stratagene, La Jolla, CA). cDNA probes were labeled with $\alpha[^{32}P]$dCTP by Nick translation (Gibco BRL, Gaithersburg, MD) or linearized and random primed (Ambion, Inc., Austin, TX). After removing unincorporated nucleotides (Pharmacia Nick Column; Pharmacia LKB Biotech, Piscataway, NJ), 10^5–10^7 cpm probe/ml hybridization buffer were added to the prehybridized membrane. The blots were prehybridized (5 min to overnight) and hybridized 16–20 h at 55°C in 10 mM EDTA, 200 mM NaH_2PO_4, pH 7, 7% wt/vol SDS, 1% wt/vol BSA and 15% vol/vol deionized formamide (50 μl/cm^2 membrane). Washes, at 65°C, were 15 min in 2× SSC (twice), 30 min in 2× SSC + 0.1% SDS, and 10 min in 0.1× SSPE (18 mM NaCl, 1 mM $NaPO_4$, pH 7.7, and 0.1 mM EDTA) + 0.5% SDS. The final wash time was adjusted as necessary to reduce background. Equal loading was verified by hybridization with glyceraldehyde phosphate dehydrogenase cDNA.

In situ hybridization was carried out as described by Simmons et al. (18), with the following modifications: Male Sprague-Dawley rats were deeply anesthetized by an intraperitoneal injection of 7% chloral hydrate and were then transcardially perfused with a solution of 4% paraformaldehyde (initially at a pH of 6.5, then changed to the same solution at pH 9.5). Tissues were resected and placed in a solution of 15% sucrose and 4% paraformaldehyde overnight. Cryostat sections (12 μm) were mounted on gelatin and poly-L-lysine–coated slides. The slides were treated with a solution of 0.001% proteinase K in 0.1 M Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, and 0.05 M EDTA, pH 8.0, for 30 min at 37°C and then with 0.025% acetic anhydride for 10 min. They were rinsed in 2× SSC and dehydrated in increasing concentrations of ethanol. ^{35}S-labeled sense and antisense cRNA probes of high specific activity were prepared by the procedure of Melton (19).

For NHE-3, the 391 bp $BglII_{1645}$ to $BglII_{2036}$ fragment was subcloned into BamHI cut pGEM7Z. This region encodes amino acids 520–650, a unique stretch in the carboxy cytoplasmic domain. The plasmid pG7N3A has this insert oriented so that the T7 promoter transcribes the sense cRNA from 1,645 to 2,036 (linearized by NsiI, 3′ overhang blunted). Polyermization from the SP6 promoter (when linearized by HindIII) produced the antisense cRNA. Riboprobes for NHE-1 comprised 3′ coding and noncoding bases corresponding to amino acids 639–820 and including 230 bases of 3′ noncoding region (base 3,452 to the AccI site at 2,281).

The tissue sections were hybridized for 16 h at 55°C in a solution of 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1×10^7 dpm/ml of probe. The slides were washed with 4× SSC for 1 h at room temperature, and unhybridized probe was removed by digestion with a solution of 20 μg/ml of ribonuclease A in 0.5 M NaCl, 0.01 M Tris-HCl, pH 8.0, and 0.001 M EDTA for 30 min at 37°C. The slides were further washed with decreasing concentrations of SSC for 1 h with a final wash in 0.1× SSC at 60°C for 30 min and then dehydrated in increasing concentrations of ethanol. Slides were initially exposed to x-ray film (Hyperfilm-βmax; Amersham Corp.) for 4 d to provide an indication of the intensity of the hybridization signal. They were then dipped in Kodak NTB-2 liquid autoradiography emulsion. After 10 d, the slides were developed with D-19 developer and fixed with Kodak rapid fixer. The slides were stained with hematoxylin to enhance histology.

Preparation of membrane proteins and separation of brush border from basolateral membranes. Intestinal cells were harvested either by sequential isolation (described above), or mucosal scraping by glass slide, mixed with ice-cold hypotonic buffer (2 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, 10 mM Tris-Cl, pH 7.4) and protease inhibitors. Cells were disrupted with low speed Ultra Turax for 30 s, and centrifuged for 3 min at 500 *g* to remove nuclei and cell debris. The supernatant was centrifuged at 100,000 *g*, 4°C, for 1 h to pellet membranes. Quantitated proteins were solubilized in Laemmli buffer for electrophoresis.

To separate the apical from basolateral membrane domains, the supernatant after the first 500 *g* spin (above) was centrifuged at 10,000 *g* for 20 min at 4°C to pellet mitochondria. $MgSO_4$ was added to 15 mM, and the cell extract was stirred at 4°C for 15 min before a 10-min spin at 8,000 *g* at 4°C. This pellet (BLM) contained basolateral, Golgi, and endoplasmic reticulum membranes. The final supernatant was centrifuged at 100,000 *g* for 45 min to pellet brush border membranes. The specific activities of Na, K, ATPase (20), a marker for the basolateral domain, and alkaline phosphatase (21), an apical surface marker, were determined on crude homogenate and purified brush border membrane (BBM) and BLM fractions. Relative purity of each domain was determined by measuring and comparing activities of the apical marker enzyme, alkaline phosphatase and the basolateral marker Na, K, ATPase. All samples used showed a minimum eightfold enrichment for alkaline phosphatase in the brush border preparation and an increase of one third for the sodium/potassium pump in BBM proteins.

Proteins were resolved by 7.5% SDS-PAGE and electroeluted to polyvinyldifluoride membrane (Immobilon; Millipore Corp., Medford, MA), in 1× Towbin buffer with 15% methanol (according to manufacturer's recommendations). Either affinity purified NHE-1 antibody (1:300) or the NHE-3 serum antibody (1:800) were used. Immunodetection was by enhanced chemiluminescence (Amersham Corp.) using horseradish peroxidase–conjugated donkey anti-rabbit Ig F(ab′)2 fragment (Amersham Corp.).

Development of antibodies. The production and characterization of polyclonal antibodies to the COOH-terminal cytoplasmic tail of NHE-1 (XB17) is described in detail by McSwine et al. (manuscript submitted for publication). NHE-3 antibody was developed by constructing a glutathione-S-transferase (GST) fusion protein including NHE-3 amino acids 528–648. The $BstYI_{1815}$-$BstYI_{2181}$ fragment was ligated into BamHI cut pGEX-3X generating an in-frame fusion to GST. Sanger dideoxy DNA sequencing confirmed that the fusion was in the correct reading frame.

Results

NHE-3 mRNA is expressed most abundantly in the villus of rat small intestine. Enterocytes from rat jejunum and ileum were sequentially isolated from villus to crypt regions in order to

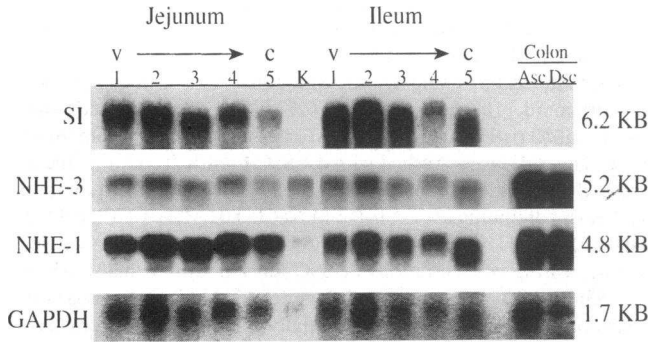

Figure 1. Northern blot analysis of sequentially isolated enterocyte mRNA, kidney (*K*) mRNA, and mucosal scrapings from ascending and descending colon of rat intestine. This figure shows the results of separate hybridizations of the same membrane by four different cDNA probes: SI, NHE-3, NHE-1, and GAPDH. Approximate size of each transcript is indicated in the right column. V→C refers to a villus to crypt gradient of sequentially extracted cells. Kidney mRNA (*K*) was derived from homogenized whole kidney. Colon mRNA from mucosal scrapings also includes nonepithelial RNA. Each lane was loaded with 5 μg of poly A^+ RNA, based on OD_{260}. Variations in loading were normalized by comparison with the constitutive marker GAPDH. This Northern is representative of several similar blots from different animals.

determine axial gradients of NHE-1 and NHE-3 mRNA and protein expression (as described in Methods). Because there is inherent variability from animal to animal, we show in Fig. 1 the results of probing a single representative Northern blot (and thus the same cellular mRNA) with different cDNAs. In this manner, direct comparisons of axial mRNA expression of the NHE isoforms are possible. Messenger RNA from whole kidney (*K*) and mucosal scrapings from ascending and descending portions of the colon are also shown in Fig. 1. The signals are normalized to constitutively expressed GAPDH as shown in Fig. 2.

As shown in the top row of Figs. 1 and 2*A*, sucrase-isomaltase (SI) mRNA is more highly expressed in villus cell fractions. SI is a brush border hydrolase predominantly expressed in mature villus cells with only minimal expression detected in the crypts (12). (SI-specific activity was also measured for each fraction, confirming sequential isolation. Specific activity in fraction 5 was < 1% of that measured in villus fraction 1 [data not shown]). Shown in Fig. 1, *second* NHE-3 mRNA abundance, reflected by a single transcript at 5.2 kb, exhibited highest expression in the upper and midvillus fractions. In the jejunum (Fig. 2 *B, top*), message level in fraction 5, which contains the earliest villus cells and true crypt cells, is significantly lower than in the more mature villus. In the ileum (Fig. 2 *B, bottom*), expression of NHE-3 appears to increase at a later stage of villus differentiation. In contrast, NHE-1 message, appearing as a single 4.8-kb transcript (Fig. 1, *third row*), did not increase in villus cells. In fact, when normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) message (Fig. 2 *C*), NHE-1 message level in the jejunum (*top*) appeared to gradually decrease as the cells matured from crypt to villus, while it remained essentially constitutive from crypt to villus in ileum (Fig. 2 *C, bottom*).

NHE-3 mRNA, by in situ hybridization, is localized exclusively in small intestinal villus cells and the luminal epithelial cells of the colon. Frozen tissue sections of 12 μm thickness were prepared from perfused rat intestine and probed with antisense and sense cRNA from unique regions of the NHE-3 or NHE-1 messages. Both sense and antisense NHE-3 cRNA probes (391 bases long) corresponded to amino acids 520–650, a region with no significant nucleotide homology to other NHE sequences. The top row of Fig. 3 *A* shows representative results when the NHE-3 antisense riboprobe was hybridized to these intestinal sections. In the well-differentiated brush borders of the small intestine (*left*, jejunum and ileum), NHE-3 message was clearly most predominant in villus cells, with little or no expression in the crypt. In the proximal colon (Fig. 3 *A right*), NHE-3 mRNA appeared on surface epithelium; none was found in the crypt or submucosa. The distal colon cross section (Fig. 3 *A, far right*) also showed NHE-3 message clearly localized to luminal epithelial cells. Little or no message was detected in the crypt cells, or underlying substrata. The sense strand probe, second row, showed no specific distribution of silver grains in any of the sections, indicating that antisense NHE-3 cRNA was specific for NHE-3 messenger RNA.

In contrast, NHE-1 (*third row*) was expressed in virtually all of the cells in these tissues, including subepithelial mesenchymal cells as well as smooth muscle cells of the muscularis propria. The hybridization signal generated by antisense NHE-1 cRNA (corresponding to amino acids 639–820 and including 230 bases of 3′ noncoding region) was diffusely distributed in all the tissues analyzed. The pattern of expression in jejunum and ileum epithelial cells, in contrast to NHE-3, appeared somewhat greater in crypt regions, although the distinction between villus and crypt expression of this transcript was unclear. Expression in the proximal and distal colon was more evenly distributed within the mucosa. The corresponding sense strand control for NHE-1 probe specificity (*bottom row*) produced no detectable signal.

In Fig. 3 *B*, the antisense NHE-3 and NHE-1 images from jejunum and ileum have been enlarged to present better detail and resolution of the tissue sections. The generalized distribution of NHE-1 message appeared in stark contrast to the more restricted localization of NHE-3 mRNA to the villus cells. NHE-1 message was abundant in the tightly packed crypt cells (*open arrows, bottom row*), while the corresponding crypt regions (*open arrows, top row*) contained little or no NHE-3 mRNA. The latter was found almost exclusively in villus cells (*solid white arrows, top row*).

NHE-3 protein is limited to epithelial tissue. Polyclonal antibody with specificity for NHE-3 was constructed by a GST transferase protein fusion to NHE-3 cDNA (amino acids 528–648) as described. Specificity of this antibody to a 97-kD protein is illustrated in Fig. 4. Membrane proteins, isolated from representative tissues (kidney, liver, and spleen) were probed with NHE-3 antibody. NHE-3 was detected only in kidney, consistent with the epithelial tissue-specific distribution of NHE-3 mRNA reported by Orlowski et al. (4), unlike the ubiquitous expression pattern of the 110-kD NHE-1.

NHE-3 protein is selectively expressed in the villus. We wished to determine if the pattern of protein expression for NHE-3 reflected the villus-specific pattern of its mRNA. Jejunal and ileal enterocytes were sequentially isolated as described above. To enrich for the integral membrane NHE exchangers, each extract was separated into membrane and soluble protein fractions and 100 μg of purified total membrane proteins were resolved by SDS-PAGE. Fig. 5 *A* indicates the

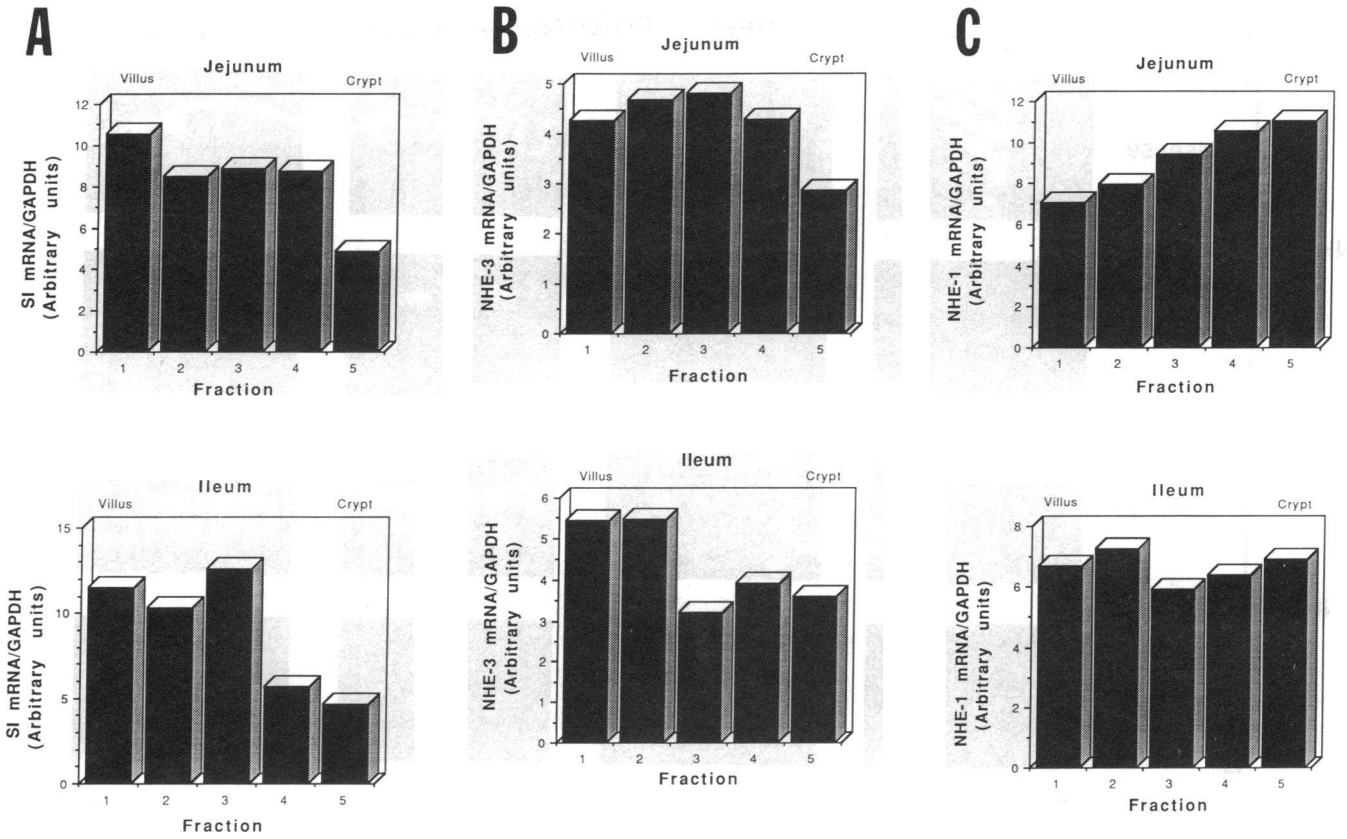

Figure 2. Scanning densitometry of the Northern blot analyses shown in Fig. 1 normalized mRNA levels for SI, NHE-3, and NHE-1 to constitutively expressed GAPDH. Fraction 1 corresponds to villus tip cells, proceeding sequentially through the villus to fraction 5, in which crypt cells are the major component. The top figure in each panel shows the values for jejunum, and the bottom shows values for ileum. Units are arbitrary, derived by dividing the peak area of each band by the corresponding GAPDH band value. (*A*) Sucrase/isomaltase; (*B*) NHE-3; (*C*) NHE-1.

distribution of NHE-3 in sequential crypt to villus fractions, jejunum and ileum. (To have 100 μg of membrane proteins in each lane, mRNA sample fractions 4 and 5 were combined to make the fourth "crypt" lane.) In jejunum, NHE-3 protein was abundant in the villus tip, with none detected beyond midvillus. Similarly, NHE-3 protein expression in ileum was largely confined to villus cell fractions. As shown in Fig. 5 *B*, NHE-1 protein was as abundant in villus as in crypt regions of both jejunum and ileum.

NHE-3 protein is selectively localized in the brush border membrane. Finally, we wished to determine where in the villus cell NHE-1 and NHE-3 exchangers were localized. Membranes were isolated from rat small intestine mucosal scrapings and the two membrane domains were separated as described. In Fig. 6, 100 μg of membranes were resolved by 7.5% SDS-PAGE, electroeluted, and analyzed by immunoblot for cross reactivity with NHE-1 or NHE-3 antibody. The 97-kD NHE-3 protein was detected only in the brush border membrane fractions (*bottom*), none being found in basolateral membrane of either ileum or jejunum. Samples from the same membrane preparations were separately immunoblotted (*top*) with NHE-1 antibody. The 110-kD NHE-1 was detected only in basolateral membrane proteins.

Discussion

This study establishes that NHE-3 expression is specific to mature intestinal villus cells and is localized to their brush border, suggesting it may have a primary role in the absorption of Na from the intestinal lumen. These observations are consistent with the physiological measurements made by Knickelbein et al. (22), who demonstrated apical membrane NHE activity only in villus, and not in crypt cell brush border membrane vesicles. By Northern blot analysis of rat tissue mRNA, we found the NHE-3 5.2-kb message primarily in epithelial tissues, including the ascending and descending colon, ileum, jejunum, and kidney (in order of decreasing abundance). After extended exposure times, NHE-3 transcript was also detected in brain, but no message was found in liver, spleen, muscle, or heart (data not shown). Although this tissue-specific pattern of expression is in agreement with the report of Orlowski et al. (4), it differs from the findings reported by Tse et al. (6) who reported a different distribution pattern. Using NHE-3 cDNA cloned from a rabbit ileal cDNA library, they found NHE-3 expressed in ileum, ascending colon, kidney, and jejunum, with no message detected in distal colon, a tissue that they describe as lacking neutral NaCl absorptive processes. These disparities in tissue expression may be explained by species and/or dietary differences. Nevertheless, the expression of NHE-3 in tissue having sodium absorptive functions is consistent with the hypothesis that it has a major role in vectorial ion transport. The axial (villus-crypt) pattern of NHE-3 expression is similar to that reported for other ion/nutrient transporters, such as the sodium-dependent glucose cotransporter (23), basolateral membrane Glut 2 (24), apical membrane Glut 5 (25), and maturation-dependent brush border hydrolases,

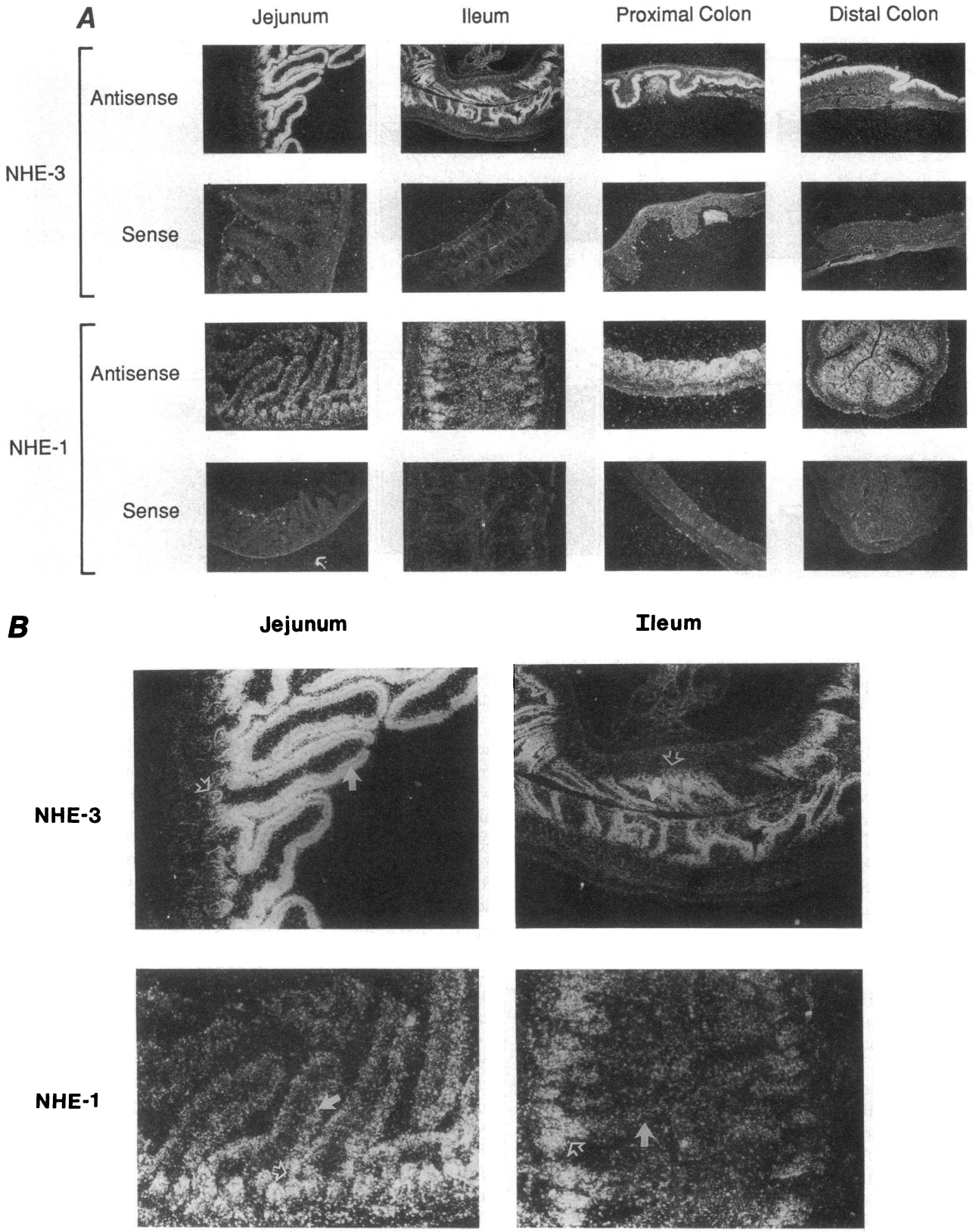

Figure 3. Dark-field photomicrographs showing in situ hybridization of representative 12-μm cryostat sections of rat intestine probed with ^{35}S-labeled cRNA. Detection of RNA appears as aggregates of silver grains over the cell. (*A*) Representative sections from one rat, which were probed with four different internally labeled cRNA probes: antisense and sense NHE-3 and NHE-1. The sections are from left to right: Jejunum, ileum, proximal, and distal colon. NHE-3 antisense cRNA probe results are shown on the top row. Directly below are the control NHE-3 sense strand cRNA hybridizations. The third row shows the results of NHE-1 antisense cRNA hybridization. The corresponding control hybridization by NHE-1 sense strand cRNA appears in the bottom row. (*B*) Enlargements of jejunum and ileum cross sections that were probed with the NHE-3 and NHE-1 antisense probes, respectively. *Solid white arrows,* regions of villus cells; *open arrows,* point out crypt regions.

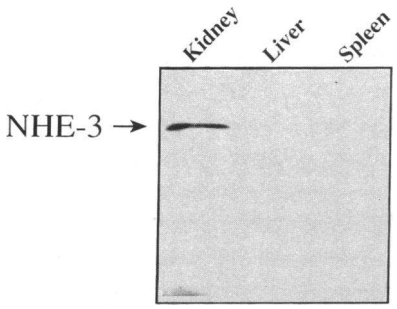

Figure 4. Analysis of NHE-3 protein distribution by immunoblot. Total cellular membrane proteins (100 μg/lane) from homogenized kidney, liver, and spleen were separated by 7.5% SDS-PAGE. Transferred to PVDF membrane, they were then analyzed for cross-reactivity to the NHE-3 fusion protein antibody, crude serum. A single 97-kD band in the kidney was identified by this antibody.

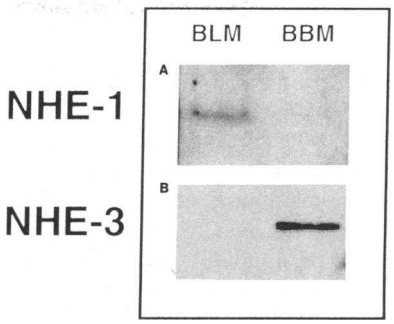

Figure 6. Immunoblot analysis of separated brush border and basolateral membrane domains. (*A*) Membrane proteins: brush border separated from basaolateral, endoplasmic reticulum, and Golgi, were probed with NHE-1 antibody, which crossreacted with a single 110-kD protein. (*B*) NHE-3 antibody cross-reacted with a 97-kD brush border membrane protein. BLM refers to the basolateral membrane protein fraction; BBM to brush border membrane proteins. The two blots shown are representative of several membrane domain separations from both jejunum and ileum.

such as sucrase-isomaltase (12), lactase (26), and mucosal peptidases (27). To our knowledge, the demonstration of NHE-3 expression solely in mature intestinal epithelial cells has not been previously reported and may be the first demonstration of a villus cell specific, non-nutritive transporter. Furthermore, it supports the paradigm that these cells may be generally "absorptive."

We have used two methods to determine that NHE-3 message is most abundant in villus (Na-absorptive) epithelial cells. Epithelial cells were harvested sequentially by the distended sac method, a modification of one originally described by Weiser (11) and mRNA was analyzed by Northern. An extensive study supporting the reliability of this isolation method to successfully separate villus from crypt cells has been reported by Traber et al. (28). The enterocytes are primarily isolated as sheets of cells still attached at tight junctions from villus tip to lower villus, with the first fraction consisting predominantly of villus tip cells. The final sample contains more individual cells, largely undifferentiated crypt cells. The intervening fractions consist of mixtures of these two cells, the proportions changing as the samples descend. As useful as this method is for rapid determination of expression patterns along the crypt-villus axis, it does not provide the precise localization of in situ hybridization. Based on the latter, NHE-3 message is primarily expressed in differentiating and mature villus cells in both jejunum and ileum and, in colon is expressed only in the epithelial cells. This suggests at least two levels of transcriptional regulation of NHE-3. Expression is epithelial specific and, in enterocytes, apparently determined by mechanisms related to the process of terminal differentiation. NHE-3 protein as determined by Western blot analysis was consistently abundant in the villus tip, with none detected in the lower or crypt fractions. If NHE-3 is being synthesized in the differentiating early villus cells, as suggested by the Northern and in situ analyses, the amount of protein present may require more sensitive detection methods. Alternatively, translation may be active only in the mature villus cells, suggesting another level of regulation exists to control synthesis of the protein.

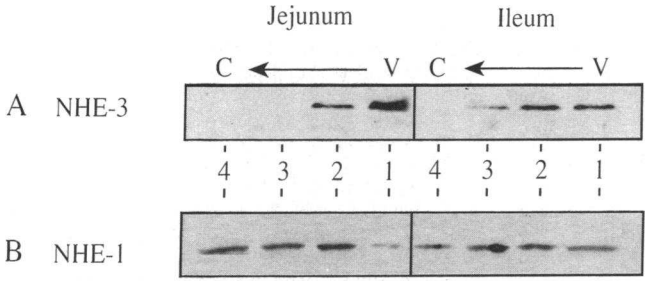

Figure 5. Analysis of regional distribution of NHE-3 and NHE-1 by immunoblot. *A, top row* shows the results of hybridization by NHE-3 antibody to total membrane proteins that were sequentially isolated from rat small intestine; *B, bottom row* was probed with NHE-1 antibody. *C,* crypt cells; *v,* villus. The gradient shown here is arranged in the reverse of that shown in Fig. 1. Fractions numbered 1 (*top and bottom*) correspond to the first villus tip fraction of Fig. 1. Fraction 4 is actually a composite of the lower villus and crypt fractions 4 and 5 of Fig. 1. Combining fractions was necessary to load equal amounts of protein.

As previously shown, apical membrane NHE activity differs from NHE-1 in several other ways. For example, brush border membrane NHE activity appears to be more resistant to amiloride inhibition than NHE-1 (29). In addition, factors that stimulate net intestinal NaCl absorption (e.g., glucocorticoids) act only on apical NHE activities, not basolateral. Yun et al. (30) for example, demonstrated that corticosteroid increases both rabbit ileal NHE-3 mRNA abundance and brush border membrane NHE activity, while having little or no effect on NHE-1 expression. These findings further support the notion that NHE-3 mediates vectorial Na absorption in the intestine.

In contrast to NHE-3, NHE-1 appears to be ubiquitous, and in the intestine, it appears in mesenchymal as well as in epithelial cells. This observation is consistent with its role in cell "housekeeping" functions, activated only under anomalous conditions such as increased acid loads, cell volume changes and growth factor activation (31–33). NHE-1 is allosterically sensitive to intracellular pH, and appears to have a proton modifier site which adjusts the setpoint of activation of this transporter (32, 34). Interestingly, we and others have found that the NHE activity of avian and rabbit intestinal brush border membranes differs from NHE-1 by lacking pH-sensitive allosteric modification of Na-H exchange (29, 36). If this activity is mediated by NHE-3, these observations are consistent with different functional roles of NHE-1 and NHE-3, the latter mainly mediating vectorial Na transport and not pH rectification. Alternatively, it is possible that the above obser-

vations may be a result of measured activity of other apically located NHE isoforms, possibly NHE-2, which has been reported not to be allosterically sensitive to intracellular proton concentrations (5).

In further support of NHE-1 not being involved in vectorial Na absorption by the intestinal epithelium are the findings that it is localized to the basolateral membrane, and it is found in crypt and villus cells. This extends previous observations of Tse et al. (37) who demonstrated by immunofluorescence the basolateral location of NHE-1 in villus cells of rabbit ileum. Activation of basolateral membrane NHE-1 during active Na absorption, therefore, would be counterproductive. In fact, as we and others have previously demonstrated (38–40), the regulation of apical membrane NHE in avian and mammalian intestine is quite different from conditions that activate NHE-1. For example, apical membrane NHE activity appears to be inhibited by activation of protein kinase C (40), as well as Ca^{2+}/calmodulin–dependent (41) and cyclic nucleotide–dependent protein kinases (10), conditions that have been shown to activate NHE-1 (42). The disparity of regulatory responses may, therefore, serve an important teleological function that would ensure that apical and basolateral membrane NHEs are not simultaneously activated, thereby maximizing the efficiency of luminal Na absorption. In support of this, luminal application of NHE-selective analogues of amiloride inhibits net Na absorption in avian intestine, but when applied serosally, these analogs have no effect on serosal-to-mucosal unidirectional or net Na fluxes (10). These observations suggest that basolateral membrane NHE activity, most likely represented by NHE-1, is normally "silent" during net Na absorption.

In conclusion, we demonstrate here significant differences in tissue, regional (intestinal) and cell membrane expression of NHE-3 and NHE-1. In intestine, NHE-3 is only expressed in mature small intestinal villus and colonic surface cells, consistent with the paradigm that these cells have predominantly nutrient and ion absorptive functions. We also demonstrate that NHE-3, in contrast to NHE-1, is exclusively localized to intestinal epithelial cells and is found only in their apical membranes. These observations are consistent with NHE-3 having an important role in vectorial Na absorption by the intestine.

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References

1. Clark, J. D., and L. E. Limbird. 1991. Na^+-H^+ exchanger subtypes: a predictive review. *Am. J. Physiol.* 261:C945–C953.

2. Grinstein, S., and A. Rothstein. 1986. Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Mem. Biol.* 90:1–12.

3. Sardet, C., A. Franchi, and J. Poussegeur. 1989. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell.* 56:271–280.

4. Orlowski, J., R. A. Kandasamy, and G. E. Shull. 1992. Molecular cloning of putative members of the NHE exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat NHE exchanger NHE-1 and two structurally related proteins. *J. Biol. Chem.* 267:9331–9339.

5. Tse, C.-M., A. J. M. Watson, A. I. Ma, J. Pouyssegur, and M. Donowitz. 1991. Cloning and functional expression of a second novel rabbit ileal villus epithelial cell Na^+/H^+ exchanger isoform (NHE-2). *Gastroenterology.* 100:258. (Abstr.)

6. Tse, C.-M., S. R. Brant, M. S. Walker, J. Pouyssegur, and M. Donowitz. 1992. Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na^+/H^+ exchanger isoform (NHE-3). *J. Biol. Chem.* 267:9340–9346.

7. Wang, Z., J. Orlowski, and G. E. Shull. 1993. Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. *J. Biol. Chem.* 268:11925–11928.

8. Aronson, P. S. 1985. Kinetic properties of the plasma membrane Na^+/H^+ exchanger. *Annu. Rev. Physiol.* 47:545–560.

9. Musch, M. W., C. Bookstein, D. L. Arvans, E. J. Cragoe, Jr., M. C. Rao, and E. B. Chang. 1992. Characterization of chicken intestinal brush border membrane Na/H exchange. *Comp Biochem. Physiol.* 103:439–444.

10. Semrad, C. E., and E. B. Chang. 1990. Inhibition of Na/H exchange in avian intestine by atrial natriuretic factor. *J. Clin. Invest.* 86:585–591.

11. Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. *J. Biol. Chem.* 248:2536–2541.

12. Hoffman, L. R., and E. B. Chang. 1991. Determinants of regional sucrase-isomaltase expression in adult rat intestine. *J. Biol. Chem.* 266:21815–21820.

13. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

14. Messer, M., and A. Dahlquist. 1966. A one step ultramicro method for the assay of intestinal disaccharidases. *Anal. Biochem.* 14:376–392.

15. Glisin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry.* 13:2633–2637.

16. Nakazoto, H., and M. Edmonds. 1974. Purification of messenger RNA and heterogeneous nuclear RNA containing poly(a) sequences. *Methods Enzymol.* 29:431–443.

17. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 66:1408–1412.

18. Simmons, D. M., J. L. Arriza, and L. W. Swanson. 1989. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single stranded RNA probes. *J. Histotechnol.* 12:169–181.

19. Melton, D., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

20. Forbush III, B. 1983. Assay of Na, K, -ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulphate buffered with bovine serum albumin. *Anal. Biochem.* 128:159–163.

21. Cox, R. P., and C. M. MacLeod. 1961. Hormonal induction of alkaline phosphatase in human cells in tumor culture. *Nature (Lond.).* 190:85–86.

22. Knickelbein, R. G., P. S. Aronson, and J. W. Dobbins. 1988. Membrane distribution of sodium-hydrogen and chloride-bicarbonate exchangers in crypt and villus cell membranes from rabbit ileum. *J. Clin. Invest.* 82:2158–2163.

23. Takata, K., T. Kasahara, M. Kasahara, O. Ezaki, and H. Hirano. 1992. Immunohistochemical localization of Na^+-dependent glucose transporter in rat jejunum. *Cell Tissue Res.* 267:3–9.

24. Thorens, B., Z. Q. Cheng, D. Brown, and H. F. Lodish. 1990. Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. *Am. J. Physiol.* 259:C279–285.

25. Davidson, N. O., A. M. L. Hausman, C. A. Ifkovits, J. B. Buse, G. W. Gould, C. F. Burant, and G. I. Bell. 1992. Human intestinal glucose transporter expression and localization of GLUT5. *Am. J. Physiol.* 262:C795–800.

26. Zweibaum, A., H. P. Hauri, E. Sterchi, I. Chantret, K. Haffen, J. Bamat, and B. Sordat. 1984. Immunohistological evidence, obtained with monoclonal antibodies, of small intestinal brush border hydrolases in human colon cancers and foetal colons. *Int. J. Cancer.* 34:591–598.

27. Casanova, J. E., Y. Mishumi, Y. Ikehara, A. L. Hubbard, and K. E. Mostov. 1991. Direct apical sorting of rat liver dipeptidylpeptidase IV expressed in Madin-Darby canine kidney cells. *J. Biol. Chem.* 36:24428–24432.

28. Traber, P. G., D. L. Gumucio, and W. Wang. 1991. Isolation of intestinal epithelial cells for the study of differential gene expression along the crypt-villus axis. *Am. J. Physiol.* 260:G895–G903.

29. Knickelbein, R. G., P. S. Aronson, and J. W. Dobbins. 1990. Characterization of Na^+-H^+ exchangers on villus cells in rabbit ileum. *Am. J. Physiol.* 259:G802–G806.

30. Yun, C. H., S. Gurubhagavatula, S. A. Levine, J. L. M. Montgomery, S. R. Brant, M. E. Cohen, E. J. Cragoe, J. Pouyssegur, C.-M. Tse, and M. Donowitz. 1993. Glucocorticoid stimulation of ileal Na^+ absorptive cell brush border Na^+/H^+ exchange and association with an increase in message for NHE-3, an epithelial Na^+/H^+ exchanger isoform. *J. Biol. Chem.* 268:206–211.

31. Rothstein, A. 1989. The Na/H exchange system in cell pH and volume control. *Rev. Physiol. Biochem. Pharmacol.* 112:235–257.

32. Grinstein, S., M. Woodside, C. Sardet, J. Pouyssegur, and D. Rotin. 1992. Activation of the Na^+/H^+ antiporter during cell volume regulation. *J. Biol. Chem.* 267:23823–23828.

33. Sardet, C., L. Counillon, A. Franchi, and J. Pouyssegur. 1990. Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. *Science (Wash. DC)*. 247:723–726.

34. Aronson, P. S., J. Nee, and M. A. Suhm. 1982. Modifier role of internal H^+ with the Na^+/H^+ exchange in renal microvillus membrane vesicles. *Nature (Lond.)* 299:161–163.

35. Wakabayashi, S., P. Fafournoux, C. Sardet, and J. Pouyssegur. 1992. The Na^+/H^+ antiporter cytoplasmic domain mediates growth factor signals and controls "H^+-sensing." *Proc. Natl. Acad. Sci. USA*. 89:2424–2428.

36. Musch, M. W., D. Drabik-Arvans, M. C. Rao, and E. B. Chang. 1992. Bethanechol inhibition of chicken intestinal brush border Na/H exchange: role of protein kinase C and other calcium-dependent processes. *J. Cell Physiol.* 152:362–371.

37. Tse, C-M., A. I. Ma, V. W. Yang, A. J. M. Watson, S. Levine, M. H. Montrose, J. Potter, C. Sardet, J. Pouyssegeur, and M. Donowitz. 1991. Molecular cloning and expression of a cDNA encoding the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger. *EMBO J. (Eur. Mol. Biol. Organ. J.)* 10:1957–1967.

38. Chang, E. B., N. S. Wang, and M. C. Rao. 1985. Phorbol ester stimulation of active anion secretion in intestine. *Am. J. Physiol.* 249:C356–C361.

39. Donowitz, M., H. Cheng, and G. W. G. Sharp. 1986. Effect of phorbol esters on sodium and chloride transport in rat colon. *Am. J. Physiol.* 251:G509–G517.

40. Fonderacaro, J. D., and L. S. Henderson. 1985. Evidence for protein kinase C as a regulator of intestinal ion transport. *Am. J. Physiol.* 249:G422–426.

41. Cohen, M. E., L. Reinlib, A. J. M. Watson, F. Gorelick, K. Rys-Sikora, M. Tse, R. P. Rood, A. J. Czernik, G. W. G. Sharp, and M. Donowitz. 1990. Rabbit ileal villus cell brush border Na^+/H^+ exchange is regulated by Ca^{2+}/calmodulin-dependent protein kinase II, a brush border membrane protein. *Proc. Natl. Acad. Sci. USA* 87:8990–8994.

42. Sundaram, U., R. G. Knickelbein, and J. W. Dobbins. 1991. Mechanism of intestinal secretion: effect of cyclic AMP on rabbit ileal crypt and villus cells. *Proc. Natl. Acad. Sci. USA*. 88:6249–6253.