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Molecular cloning and expression of a cDNA encoding the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger

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A cDNA clone encoding a rabbit ileal villus cell Na^+/H^+ exchanger was isolated and its complete nucleotide sequence was determined. The cDNA is 4 kb long and contains 322 bp of 5'-untranslated region, 2451 bp of open reading frame and 1163 bp of 3'-untranslated area, with 70%, 91% and 40% identity to the human sequence, respectively. Amino acid sequence deduced from the longest open reading frame indicated a protein of 816 residues (predicted M_r 90 716) which exhibits 95% amino acid identity to the human Na^+/H^+ exchanger. The two putative glycosylation sites in the human Na^+/H^+ exchanger are conserved in this protein, suggesting that it is a glycoprotein. Stable transfection of the cDNA into an Na^+/H^+ exchanger deficient fibroblast cell line, established Na^+/H^+ exchange. The Na^+/H^+ exchanger was stimulated by serum and a phorbol ester but not by 8-Br-cAMP. In Northern blot analysis, the cDNA hybridized to a 4.8 kb message in rabbit ileal villus cells, kidney cortex, kidney medulla, adrenal gland, brain and descending colon and to a 5.2 kb message in cultured human colonic cancer cell lines, HT29-18 and Caco-2. In immunoblotting, a polyclonal antibody raised against a fusion protein of β-galactosidase and the C-terminal 158 amino acids of the human Na^+/H^+ exchanger identified a rabbit ileal basolateral membrane protein of 94 kd and only weakly interacted with the ileal brush border membrane. In immunocytochemical studies using ileal villus and crypt epithelial cells, the same antibody identified basolateral and not brush border epitopes. Restriction analysis of genomic DNA with a 462 bp *PstI*–*AccI* fragment of the rabbit Na^+/H^+ exchanger strongly suggests the existence of closely related Na^+/H^+ exchanger genes. The near identity of the basolateral Na^+/H^+ exchanger and the human Na^+/H^+ exchanger plus the ubiquitous expression of this message suggests that the ileal basolateral Na^+/H^+ exchanger is the 'housekeeping' Na^+/H^+ exchanger.

Key words: Na^+/H^+ exchanger/intestine/transfection

Introduction

All mammalian cells have Na^+/H^+ exchangers (Grinstein *et al.*, 1989). Identified functions for Na^+/H^+ exchangers include regulation of intracellular pH, in particular recovery from an acid load, maintenance of cellular volume in response to an osmotic load, transcellular Na^+ absorption in epithelial cells, and as a target for growth factors (Grinstein *et al.*, 1989; Pouyssegur, 1985). Na^+/H^+ exchangers do not carry out all these functions in each cell type. For instance, cell shrinkage due to increased extracellular osmolarity activates Na^+/H^+ exchangers in thymocytes, lymphocytes, erythrocytes and necturus gallbladder. However, this stimulus fails to activate Na^+/H^+ exchange in the opossum kidney (OK) cell line, P19 embryonal carcinoma cells, and in the human colon cancer cell line, Caco-2 (Bierman *et al.*, 1987; Watson *et al.*, 1991; Montrose *et al.*, 1988). What allows an individual Na^+/H^+ exchanger to assume a specific function is unknown.

It is unknown whether there are multiple forms of Na^+/H^+ exchangers. Evidence suggesting multiple isoforms comes from differences in amiloride sensitivity and differences in regulation. In the LLC-PK_1/Cl_4, a porcine kidney cell line, Haggerty *et al.* (Haggerty *et al.*, 1988a,b) demonstrated that the apical and basolateral plasma membranes have different forms of Na^+/H^+ exchange based on their sensitivity to the diuretic amiloride analogue, ethylisopropylamiloride, with the basolateral membrane form being more sensitive. Ileal villus epithelial cells carry out intestinal Na^+ absorption. Knickelbein *et al.* (Knickelbein *et al.*, 1988a,b) demonstrated by plasma membrane vesicle studies, that rabbit ileal villus epithelial cells have Na^+/H^+ exchangers on their apical and basolateral membranes, with a 36-fold difference in sensitivity to amiloride inhibition (amiloride K_i was 216 μM for the apical membrane and 6 μM for the basolateral membrane). As a comparison, the fibroblast Na^+/H^+ exchanger has an amiloride K_i of ~3–6 μM (Pouyssegur *et al.*, 1986). In terms of regulation, protein kinase C stimulates the Na^+/H^+ exchange activity in fibroblasts (Grinstein *et al.*, 1989; Vincentini and Villereal, 1985) but inhibits the ileal epithelial cell apical membrane exchanger (Rood *et al.*, 1988; Cohen *et al.*, 1991). These differences in amiloride sensitivity and regulatory mechanisms most likely indicate different isoforms of the protein. However, it is also possible that there is only one exchanger, with the difference in amiloride sensitivity being due to a variation in lipid composition of the membranes, and the difference in regulation being due to separate proteins (probably kinases), which are involved in regulation.

Recently, Sardet *et al.* (Sardet *et al.*, 1989, 1990) cloned by genetic complementation a cDNA encoding the human growth factor activated Na^+/H^+ exchanger. The cDNA is

4 kb long and the longest open reading frame encodes a protein of 815 amino acids. It is not known whether the expression of this gene is tissue-specifc and whether the size of message is the same in each tissue. In the present studies, we cloned a 4 kb cDNA encoding the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger. This cDNA encodes a protein of 816 amino acids and is highly homologous to the human Na^+/H^+ exchanger. This protein is called NHE-1. Stable transfection of this clone into a fibroblast deficient in Na^+/H^+ exchange activity established serum and phorbol ester stimulatable Na^+/H^+ exchange activity. This cDNA identified a 4.8 kb message on Northern blot analysis of poly(A)$^+$ RNAs isolated from multiple rabbit tissues. Furthermore, Southern blot analysis of rabbit genomic DNA with a rabbit Na^+/H^+ exchanger cDNA fragment suggests the existence of other closely related Na^+/H^+ exchanger genes.

Results

Northern blot analysis of rabbit ileal villus mRNA using a human Na^+/H^+ exchanger cDNA probe

In order to demonstrate the expression of a Na^+/H^+ exchanger in the rabbit ileal villus cells and to establish conditions for cross hybridization, rabbit ileal villus cell mRNA was analyzed by Northern blotting (Sambrook *et al.*, 1989). A 1.9 kb *Bam*HI−*Bam*HI fragment of the human growth factor activated Na^+/H^+ exchanger cDNA (encoding amino acid residues 109−746) (Sardet *et al.*, 1989, 1990) was used to probe rabbit ileal villus cell mRNA under high and low stringency conditions. This cDNA hybridized to a single 4.8 kb message under both conditions (Figure 1). No additional message was identified even after longer exposure (data not shown). The message size in rabbit ileal villus cells is smaller than the 5.6 kb message of human RNA obtained from A431 cells as reported by Sardet *et al.* (Sardet *et al.*, 1989). It is possible that the difference in size of poly(A)$^+$ RNA identified by the probe between man and rabbit arose from the use of different standards, different RNA denaturation in the Northern blot analyses and/or species variation. However, analysis of mRNA from Caco-2 cells (a human colonic carcinoma cell line) with the same probe under parallel conditions consistently revealed an apparently larger mRNA of 5.2 kb (C.M.Tse and M.Donowitz, unpublished results) (see also Figure 2). Therefore, there appears to be species variation in the size of message coding for human and rabbit Na^+/H^+ exchangers.

Isolation of a cDNA encoding a rabbit ileal villus cell Na^+/H^+ exchanger

The 1.9 kb *Bam*HI−*Bam*HI fragment of the human Na^+/H^+ exchanger cDNA was used as a probe to screen a cDNA library constructed in λ_{ZAP} from poly(A)$^+$ RNA isolated from rabbit ileal villus cells. Screening was performed under high stringency hybridization but low stringency washing conditions (see Materials and methods). Of 10^6 plaques, 20 clones scored positive and were plaque purified. cDNA inserts were rescued with helper phage R408 into Bluescript (Stratagene). The size of rescued inserts ranged from 0.7 to 4 kb. Of the 20 positive clones obtained, 16 were later found to be independent and four were redundant. Southern analyses of rescued cDNA inserts

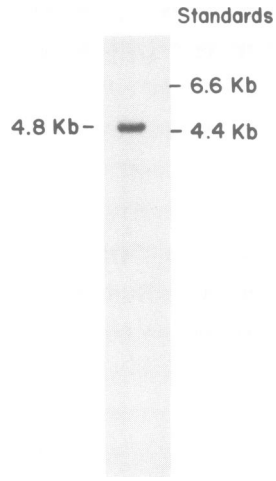

Fig. 1. Expression of the Na^+/H^+ exchanger in rabbit ileal villus cells. A *Bam*HI−*Bam*HI cDNA fragment of the human Na^+/H^+ exchanger (encoding amino acid residues 109−746) was used to probe, by Northern blot analysis, rabbit ileal villus cell poly(A$^+$) RNA (2 μg per lane) under high stringency hybridization conditions at 42°C in 50% formamide, 4×SSC, 4×Denhardt's solution, 1% SDS and high stringency washing conditions at 65°C in 0.1×SSC, 0.1% SDS or under low stringency hybridization condition at 42°C in 30% formamide, 4×SSC, 1% SDS and low stringency washing conditions at 42°C in 1×SSC, 0.1% SDS (data not shown). DNA size standards are shown in the right margin. Size of the message is shown in the left margin.

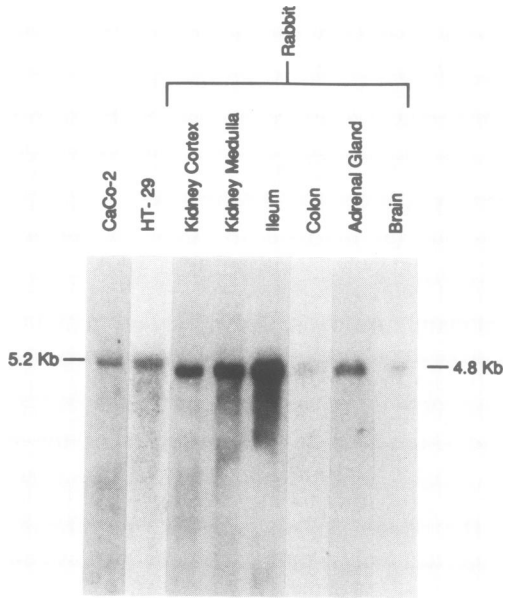

Fig. 2. Tissue distribution of the rabbit ileal villus cell Na^+/H^+ exchanger message. The Northern blot analysis of poly(A)$^+$ RNA isolated from rabbit kidney cortex, kidney medulla, ileal villus cells, descending colon, adrenal gland, brain and two cultured human colonic cancer cell lines, HT29-18 and Caco-2. The blot was probed with a full-length cDNA insert of R6 under high stringency hybridization and washing conditions (see Materials and methods). Size of messages are shown at the margins. Each lane contained 2−5 μg of poly(A$^+$) RNA.

revealed that all cDNA clones hybridized to the human cDNA probe used in Figure 1 (data not shown). The longest cDNA clone (4 kb), called R6, was further characterized by Northern blot analysis with the rabbit ileal villus cell

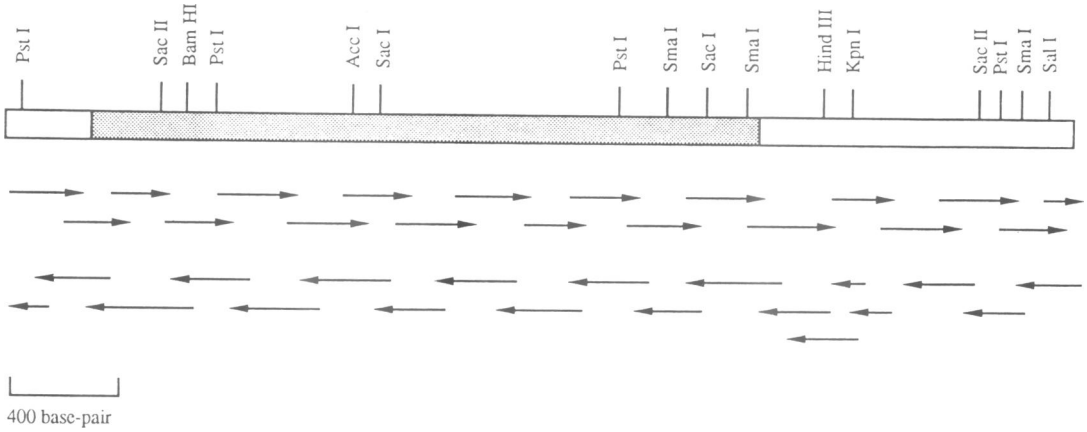

Fig. 3. Restriction map and sequencing strategy for a rabbit ileal villus cell Na^+/H^+ exchanger cDNA (R6). The shaded area represents the open reading frame and the open areas represent the untranslated sequences. Restriction sites relevant to the sequencing strategy are shown. Arrows indicate the direction and extent of sequencing.

Fig. 4. Nucleotide sequence of the rabbit ileal villus cell Na^+/H^+ exchanger cDNA (R6) and predicted amino acid sequence derived from R6. Nucleotides are numbered at the right of the sequence with respect to the putative translation initiation site. Amino acids are numbered above the sequence. The in-frame upstream mini-cistron and the polyadenylation signal are underlined. Additional poly(A) sequence (begining at nucleotide 3937), $TGAT(A)_{29}$, was obtained by end-sequencing of another independent clone, R13. This sequence is available from the EMBL database (accession number X59935).

mRNA. As shown in Figure 2, this clone hybridized to a 4.8 kb message on the Northern blot under high stringency conditions. This was the same message size that was recognized in Northern blot analysis of rabbit ileal villus cell poly(A)$^+$ RNA by the human Na^+/H^+ exchanger cDNA probe (Figure 1). Thus, this strongly suggests that this 4 kb cDNA clone encodes a rabbit ileal villus cell Na^+/H^+ exchanger.

Nucleotide sequence and deduced amino acid sequence of a rabbit ileal villus cell Na^+/H^+ exchanger

The clone R6 was completely sequenced in both strands by the dideoxy sequencing method (Sanger *et al.*, 1977). Figure 3 shows the strategy for sequencing and the partial restriction map of this clone. Figure 4 displays the complete nucleotide sequence of R6. This cDNA is 3936 nucleotides

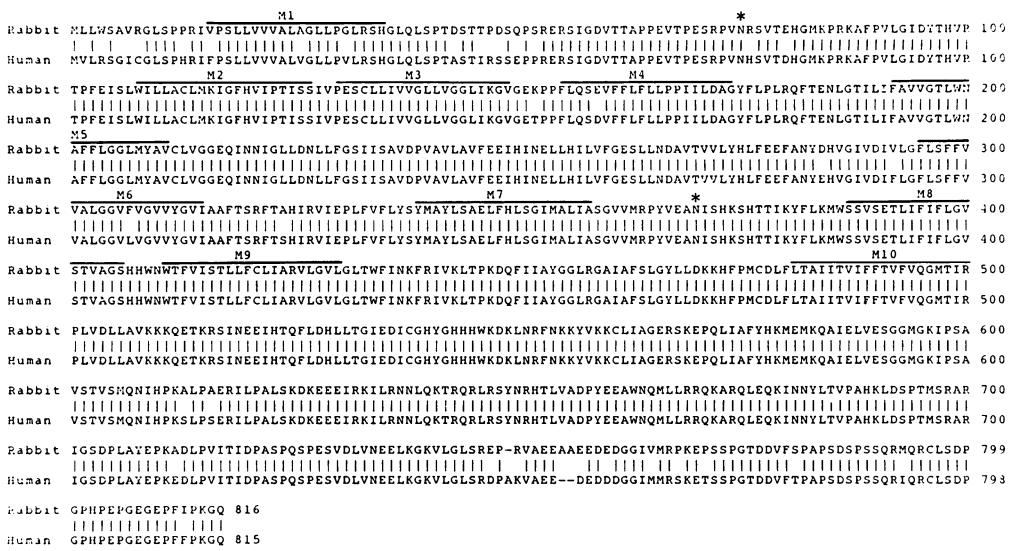

Fig. 5. Alignment of the amino acid sequences of the human growth factor activated Na^+/H^+ exchanger and the rabbit ileal villus cell Na^+/H^+ exchanger. Amino acids are indicated by their single-letter abbreviations. Two gaps were introduced in order to maintain the alignment of the two sequences. The 10 putative membrane spanning domains are overlined. Potential N-linked glycosylation sites (Asn75 and Asn370) are marked with *. The sequence data for the human growth factor activated Na^+/H^+ exchanger are taken from Sardet *et al.* (Sardet *et al.*, 1989, 1990). Amino acid numbers are shown on the right.

in length and contains 322 bp of 5′-untranslated region, 2451 bp of a long open reading frame and 1163 bp of 3′-untranslated region, corresponding to 70%, 91% and 40% identity, respectively to the human Na^+/H^+ exchanger sequence reported by Sardet *et al* (Sardet *et al.*, 1989). The ATG triplet at the beginning of this open reading frame is in good context for initiation of translation (A/GCCATGG) (Kozak, 1987). In the 5′ non-coding region, there is a 4 amino acid mini-cistron upstream from the putative initiator methionine, beginning at nucleotide −120 and terminating at nucleotide −108. Although the 3′-untranslated sequence does not contain a poly(A) tract, a consensus polyadenylation signal (AATAAA) is present 20 nucleotides from the 3′ end. In fact, a poly(A) tail was found in another independent, but overlapping, clone (R13), by sequencing the two ends of this cDNA clone. This poly(A) tail is located 19 bp downstream of the consensus polyadenylation signal. Amino acid sequence deduced from the open reading frame revealed a protein of 816 amino acid residues with a predicted M_r of 90 716. Apparently, this is 78 amino acids shorter than the human Na^+/H^+ exchanger previously described by Sardet *et al.* (Sardet *et al.*, 1989) However, on sequencing an independent cDNA clone isolated from a human small intestinal library (kindly provided in λgt11 by Dr Yvonne Edwards), we confirmed the presence of an additional G at position 2442 of the published human Na^+/H^+ exchanger sequence (Sardet *et al.*, 1989, 1990). This shifts the reading frame to a stop codon 3 bp downstream. The new stop codon of the human sequence aligned exactly with the position of the stop codon of the rabbit sequence. Therefore, the correct sequence of the human Na^+/H^+ exchanger cDNA is (from nucleotide 2437) AAG(Lys)GGG(Gly)CAG(Gln)TAA(new stop codon) [the additional G at position 2442 of the published human Na^+/H^+ exchanger sequence has been confirmed by Sardet *et al.* (Sardet *et al.*, 1990)]. This human protein encodes 815 residues instead of 894 as previously reported. Figure 5 compares the amino acid sequence of the rabbit ileal villus cell Na^+/H^+ exchanger with that of the human Na^+/H^+ exchanger. In order to maintain alignment, two gaps are introduced into the putative cytoplasmic tail. These two proteins are highly similar to each other and exhibit 95% amino acid identity.

Table I

Cell line	*n*	Serum ΔpH	Time
NHE-1/PS120/5	4	0.23 ± 2.02	400 s
PS122	3	0.34 ± 0.06	400 s
p*		NS	

Results are of two cloned Na^+/H^+ exchangers in the PS120 fibroblast cell line, which is derived from the Chinese hamster lung fibroblast line CCL39 and is deficient in endogenous Na^+/H^+ exchangers. NHE-1/5 is derived from the rabbit ileal villus cell and PS122 is derived from the human fibroblast. *n* refers to the number of coverslips studied. ΔpH is in reference to the change from the steady state pH following NH_4Cl to a new steady state in the presence of serum, with the minimal drift in control cells studied the same day subtracted. Time refers to the number of seconds required to reach a new steady state following serum. There was no significant difference in ΔpH between the two clones (unpaired *t* test).

Stable expression of R6 cDNA in a Na^+/H^+ exchanger deficient fibroblast

To show that R6 encoded a cDNA which functions as a Na^+/H^+ exchanger, the coding sequence of R6 was subcloned into a mammalian Na^+/H^+ expression vector, pMAM-neo, followed by transfection into the mammalian Na^+/H^+ exchanger deficient fibroblast cell line, PS120, and then doubly selected by geneticin exposure and acid loading as previously described (Sardet *et al.*, 1989, 1990). One clone called NHE-1/PS120/5 was used for detailed studies of extracellular regulation of Na^+/H^+ exchange.

Na^+/H^+ exchange was measured in NHE-1/PS120/5 as Na^+-dependent alkalinization of cells, which were

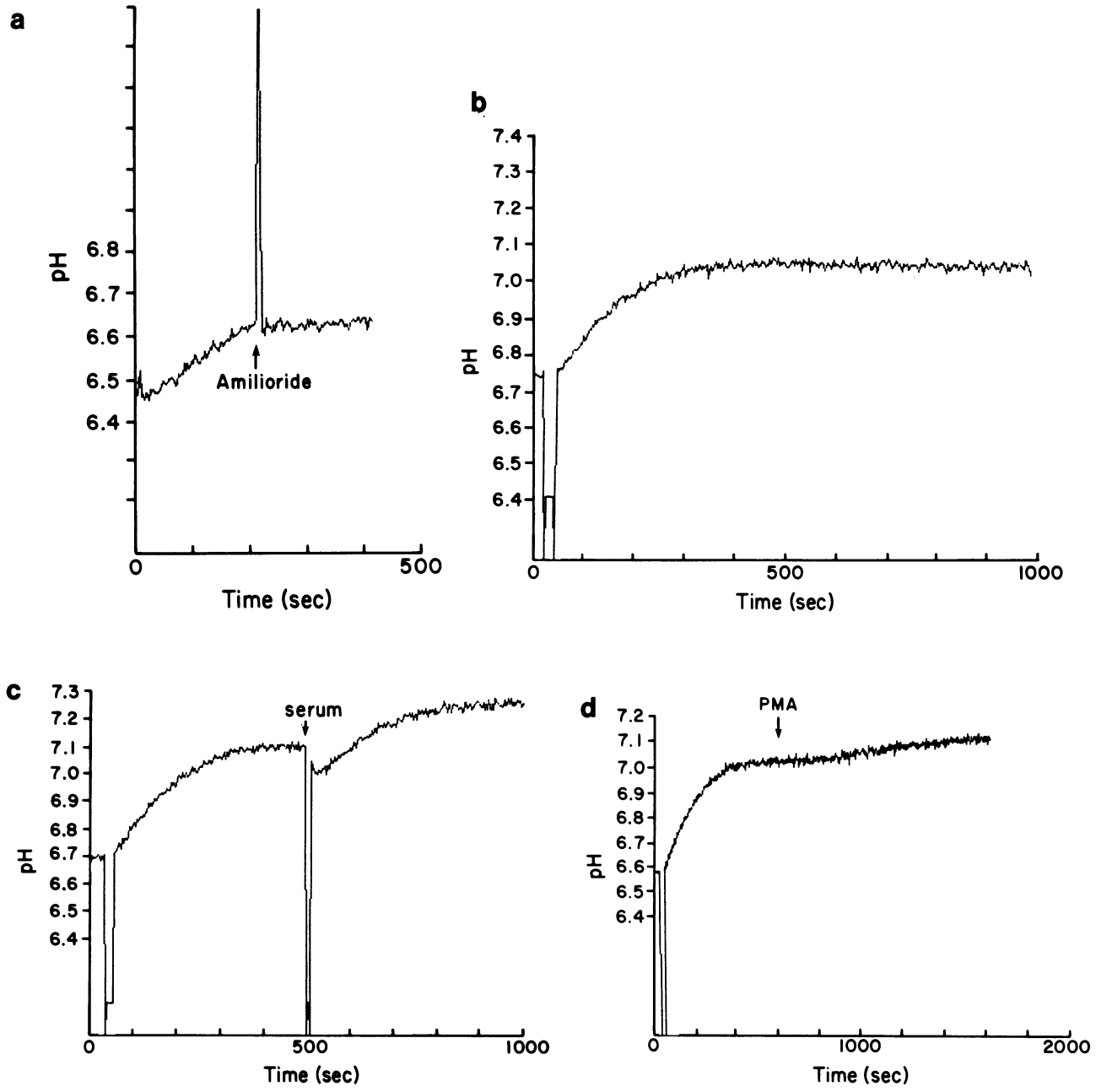

Fig. 6. Na^+/H^+ exchange activity of the cell line NHE-1/PS120/5 (**a**) Amiloride inhibition of Na^+ dependent cellular alkalinization in NHE-1/PS120/5. The cells were acidified in 40 mM NH_4Cl for 15 min, washed free of NH_4Cl and Na^+ medium (130 mM) added. Initial pH after acidification was 6.48. Addition of 300 μM amiloride at the (↑) inhibited Na^+/H^+ exchange. (**b**) Achievement of steady state pH in NHE-1/PS120/5 following acidification in 40 mM NH_4Cl. Steady state pH is constant for at least 450 s at 7.06. (**c**) Stimulation of Na^+/H^+ exchanger by serum. 10% serum increased Na^+ dependent cellular alkalinization in NHE-1/PS120/5. The cells were grown to 70–80% confluency on glass coverslips and acidified with 40 mM NH_4Cl for 15 min. The cells were then placed in Na medium (130 mM) and the pH allowed to come to steady state. Serum was then added at the (↓) after the steady state pH was reached. The pH decreased immediately after the addition of serum and then increased to a new steady state pH, which was higher than the initial pH. (**d**) Stimulation of Na^+/H^+ exchange by PMA. PMA (1 μM) increased Na^+ dependent cellular alkalinization in NHE-1/PS120/5. Cells were grown to 70–80% confluency on glass coverslips and acidified with 40 mM NH_4Cl for 15 min. The cells were then placed in Na medium (130 mM) and the pH allowed to come to steady state. pH at steady state was 7.01. PMA (1 μM) was then added at 600 s at the (↓), and a new steady state pH of 7.10 was attained.

previously acidified by 15 min exposure to 40 mM NH_4Cl. As shown by a typical experiment in Figure 6a, exposure to 300 μM amiloride totally inhibited the Na^+-dependent alkalinization. In contrast to the results shown in Figure 6a, which demonstrate the presence of a Na^+/H^+ exchanger, non-transfected PS120 cells showed no Na^+ dependent alkalinization (data not shown). In NH_4Cl acidified cells, following exposure to 130 mM Na, these stably transfected cells reached a steady state pH of 6.99 ± 0.03, $n = 15$ (Figure 6b). When these cells were then exposed to 10% fetal calf serum (Figure 6c, Table I), or to the phorbol ester, PMA (10^{-6} M) (Figure 6d), a further intracellular alkalinization occurred, which required Na, indicating further stimulation of Na^+/H^+ exchange. With initial exposure to serum, pH decreased (Figure 6c); this has been shown previously to result from activation of the hexose

monophosphate shunt (Stanton and Seifter, 1988) and is not a direct serum effect on the BCECF, since addition of serum to free BCECF does not alter the fluorescence ratio. In contrast to the effect of serum, exposure to 1 mM 8-Br-cAMP did not alter intracellular pH (data not shown).

The magnitude and regulation of Na^+/H^+ exchange in this stably transfected rabbit ileal villus cell Na^+/H^+ exchanger (NHE-1/PS120/5) was compared with regulation of the human fibroblast Na^+/H^+ exchanger, also stably transfected into PS120 (called PS122). Cells with the human fibroblast Na^+/H^+ exchanger had similar resting pH (7.01 ± 0.02, after NH_4Cl exposure) and responded to serum with a slightly, but not significantly larger increase in intracellular pH (0.34 ± 0.06) than NHE-1/PS120/5 (Table I).

Tissue distribution of the rabbit ileal Na^+/H^+ exchanger mRNA

$Poly(A)^+$ RNA was prepared from rabbit kidney cortex, kidney medulla, ileal villus cells, descending colon, adrenal gland and brain. $Poly(A)^+$ RNA was also prepared from two cultured human colon cancer cell lines, HT29−18 (grown in glucose-containing medium) and Caco-2. In Northern blot analysis, the R6 full-length cDNA hybridized under high stringency conditions to a 4.8 kb message in all rabbit tissues and to a 5.2 kb message in two cultured human colonic cancer cell lines (Figure 2).

Cellular localization of an ileal Na^+/H^+ exchanger by immunoblotting and immunocytochemistry

Western blot analysis was performed on rabbit ileal villus cell brush border and basolateral membrane preparations using polyclonal antibody (RPI-c28), as previously described (Sambrook *et al.*, 1989). The polyclonal antibodies were raised against a fusion protein composed of the putative C-terminal 158 amino acids in the cytoplasmic domain of the human Na^+/H^+ exchanger (amino acids 658−815) linked to β-galactosidase at its N-terminus. The antibodies were affinity-purified by incubating the antiserum with a nitrocellulose sheet coated with the fusion protein, and the reacting antibodies were eluted with glycine at 0.1 M (pH 2.2). As shown in Figure 7, these antibodies detected a diffuse-staining band of 94 kd in the ileal villus cell basolateral membrane preparation. A similar but much fainter band was identified in the brush border membrane preparation. It is possible that the faint band recognized by the antibodies in the brush border preparation resulted from contamination by basolateral membranes or that there was weak cross-reactivity of these antibodies with the villus cell brush border membrane Na^+/H^+ exchanger.

The same antibody used in the immunoblotting studies (RPI-c28) was used to study the ileal Na^+/H^+ exchanger distribution in ileal villus and crypt cells by immunocytochemistry.

The Na^+/H^+ exchanger antibody bound to the basolateral regions of the villus epithelial cells and to the crypt epithelial cells and not to the brush border membrane (Figure 8). This was confirmed to be specific binding, as replacement of the Na^+/H^+ exchanger antibody with non-immune rabbit serum produced minimal fluorescent labeling (data not shown).

Southern blot analysis of rabbit genomic DNA with a cDNA probe derived from R6

Southern blot hybridization of rabbit genomic DNA was performed to determine whether there were multiple Na^+/H^+ exchanger genes. A *Pst*I−*Acc*I fragment of the rabbit Na^+/H^+ exchanger cDNA probe was used to probe *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Pst*I restriction digests of rabbit genomic DNA under high stringency hybridization conditions. Washing was performed under either low stringency (Figure 9, left) or high stringency conditions (Figure 9, right) (see Materials and methods). This cDNA probe was derived from a part of the coding region which represents the membrane spanning domains 4, 5, 6 and the extracellular hydrophilic loop b as defined by Sardet *et al.* (Sardet *et al.*, 1989) (amino acid residues 157−310); is 462 nucleotide long (nucleotides 471−932); and is not cleaved by the restriction enzymes used in the Southern blot analysis. As shown in Figure 9 (left panel), a single major band with multiple faint bands were observed under low stringency washing conditions (1×SSC, 0.1% SDS at 42°C) in DNA samples digested with various restriction enzymes. Four faint bands were found in samples digested with *Eco*RI and *Bam*HI, three with *Hin*dIII, two with *Kpn*I and one with *Pst*I. Some of these faint bands were washed away under high stringency washing conditions (0.1×SSC, 0.1% SDS at 65°C) (Figure 9, right panel). Thus, in addition to the single major band, three faint bands were observed in samples digested with *Eco*RI and one with *Bam*HI, *Hin*dIII, *Kpn*I and *Pst*I. Furthermore, these faint bands that were washed away under conditions of high stringency washing, were not seen when the entire R6 cDNA was used as the probe (data not shown). Thus, these results suggest that at least some of these faint bands labeled by the 462 bp probe are due to weak hybridization to other closely related genes.

Discussion

There is very little information on the molecular properties of Na^+/H^+ exchangers. By DCCD (*N,N'*-dicyclohexylcarbodiimide) covalent labeling, a 65 kd protein has been proposed as a candidate for the rat (Friedrich *et al.*, 1986) and a 100 kd protein for the rabbit renal Na^+/H^+ exchanger (Igarashi and Aronson, 1987). By purification of an amiloride-binding protein, a 25 kd protein was shown to exhibit properties consistent with the rabbit renal Na^+/H^+ exchanger (Huot *et al.*, 1989). By inverse correlation between the amount of phosphorylation and the rate of Na^+/H^+ exchange, 116 and 77 kd proteins were proposed as candidate rabbit ileal villus cell Na^+/H^+ exchangers (Donowitz *et al.*, 1984; Sharp *et al.*, 1987). However, none of these is conclusive. Inhibition by DCCD or amiloride is not specific to Na^+/H^+ exchange (Grinstein *et al.*, 1989); and the correlation between the amount of phosphorylation and the rate of exchange is circumstantial.

The major step in identification of the mammalian Na^+/H^+ exchanger came from the expression cloning by Sardet *et al.* (Sardet *et al.*, 1989). They isolated a cDNA which encoded a human Na^+/H^+ exchanger of 815 amino acid residues with M_r of 90 762 (Sardet *et al.*, 1989, 1990). This protein is proposed to have two domains: 10−12 membrane spanning domains [12 by Kyte−Doolittle (Kyte and Doolittle, 1982) and 10 by Engelman (Engelman

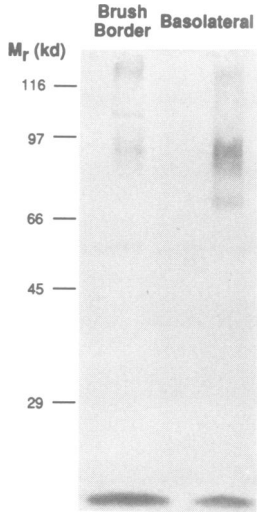

Fig. 7. Immunoblot localization of intestinal Na^+/H^+ exchangers. Ileal villus cell highly purified brush border (left lane) and basolateral membranes (right lane) (100 μg per lane) were separated on 10% SDS–PAGE and transferred to a nitrocellulose sheet. The nitrocellulose was incubated for 4 h at 23°C with antibody RPI-c28 (see text) at a dilution of 1 in 200. The sheet was washed 3 times and incubated for 1 h at 23°C with a goat anti-rabbit IgG–alkaline phosphatase conjugate. Bands were detected as indicated in Materials and methods. Numbers in the left margin indicate molecular weight standards (Sigma no. MW-SDS- 200), including β-galactosidase, phosphorylase B, bovine serum albumin, egg albumin and carbonic anhydrase.

et al., 1986) paradigms], which make up the amino-terminal part of the protein and a large cytoplasmic portion at the carboxy end. The membrane spanning domains, with predicted α-helical structures, are believed to participate in the exchange function, whereas the cytoplasmic portion of the protein is thought to be involved in the regulation of the exchange. Protein kinases, including cAMP dependent kinase, CaM kinase II and protein kinase C, regulate some Na^+/H^+ exchangers (Grinstein *et al.*, 1989; Pouyssegur, 1985; Vincentini and Villereal, 1985; Rood *et al.*, 1988; Cohen *et al.*, 1991; Emmer *et al.*, 1989; Weinman *et al.*, 1989). Consistent with this, the C-terminal cytoplasmic tail contains several potential phosphorylation sites for these kinases. Recently, Sardet *et al.* (Sardet *et al.*, 1990) showed that the human Na^+/H^+ exchanger is a phosphoprotein.

Rabbit ileal villus epithelial cells are Na^+ absorptive cells whereas the crypt cells secrete Cl^- (Welsh *et al.*, 1982). Crypt cells do not have a Na^+/H^+ exchanger on their apical membrane, based on transport studies using plasma membrane vesicles, but have one on their basolateral membrane (Knickelbein *et al.*, 1985). In contrast, villus cells appear to have separate Na^+/H^+ exchangers on their apical and basolateral membranes, which are differentiated by 36-fold sensitivity to inhibition by amiloride (Knickelbein *et al.*, 1988a,b). Functionally, the two exchangers are different. The apical membrane Na^+/H^+ exchanger, coupled with a Cl^-/HCO_3^- exchanger, is responsible for the neutral NaCl absorptive process (Knickelbein *et al.*, 1985); whereas the basolateral membrane Na^+/H^+ exchanger is thought to be a 'housekeeper', performing functions like pH regulation (Grinstein *et al.*, 1989). Therefore, it is likely that in ileal villus epithelial cells, there are two isoforms of Na^+/H^+ exchanger which are distinct in terms of function, pharmacology and localization.

In order to understand the molecular evolution of the Na^+/H^+ exchanger and as the first step to delineate the different forms of the exchanger in ileal villus cells, a 1.9 kb *Bam*HI–*Bam*HI fragment of the human Na^+/H^+ exchanger cDNA (Sardet *et al.*, 1989) was used to identify its rabbit homologue. After screening 10^6 plaques from a rabbit ileal villus cell cDNA library with the human cDNA probe, a 4 kb cDNA clone (R6) was obtained. The longest open reading frame of this cDNA encodes a protein of 816 residues with a predicted M_r of 90 716. This protein is very similar to the human Na^+/H^+ exchanger, exhibiting 95% amino acid identity. The two putative glycosylation sites in the human Na^+/H^+ exchanger (Asn75 and Asn370) are also conserved in the rabbit Na^+/H^+ exchanger, suggesting that the rabbit protein is also a glycoprotein. This is supported by the diffuse nature of the band by immunostaining of the rabbit basolateral Na^+/H^+ exchanger in the Western blot (Figure 7). It has been reported by Sardet *et al.* (Sardet *et al.*, 1990) that the human Na^+/H^+ exchanger is a 110 kd glycoprotein. Recently, they demonstrated that mitogenic activation of the human Na^+/H^+ exchanger resulted in phosphorylation on its serine residues (Sardet *et al.*, 1990). Likewise, the rabbit Na^+/H^+ exchanger is likely to be regulated by phosphorylation by protein kinases, as several potential phosphorylation sites, especially for protein kinase C and calmodulin kinases, are also conserved.

The cloned rabbit ileal protein carries out Na^+/H^+ exchange. We have stably expressed R6 cDNA in the Na^+/H^+ exchanger deficient fibroblast cell line, PS120 (clone NHE-1/PS120/5). Expression of the cDNA in these cells restored their ability to demonstrate amiloride sensitive, Na^+ dependent alkalinization. This Na^+/H^+ exchanger is on the basolateral membrane of the ileal villus Na^+ absorbing cell, based on immunoblotting and immunocytochemical localization studies, and based on the latter is present on the crypt epithelial cell basolateral membrane but is not present on the brush border of ileal villus Na^+ absorbing cells or crypt epithelial cells. Furthermore, the same antibody used in these ileal studies was used in confocal microscopy studies to show that NHE-1 is present on the basolateral and not apical membranes of Caco-2 and HT-29 cells (C.Huet, J.Pouyssegur and D.Louvard, unpublished observations).

In Northern blot analysis, the rabbit ileal villus cell Na^+/H^+ exchanger cDNA hybridized to a 4.8 kb message in rabbit ileal villus cells, kidney cortex, kidney medulla, adrenal gland, brain and descending colon and to a 5.2 kb message in cultured human colon cancer cell lines, HT29-18 and Caco-2. The difference in message size appears to represent a species variation. However, the amino acid sequences, as deduced from the longest open reading frames from human and rabbit Na^+/H^+ exchanger cDNAs, are very similar to each other. The difference in apparent size of message therefore probably reflects a difference in length in the 5′ and/or 3′ non-coding regions, which are more divergent than the coding region between the two species. The amino acid sequences of human and rabbit Na^+/H^+ exchangers aligned perfectly except for two gaps in the putative 3′ cytoplasmic tail (Figure 5). This probably also represents a species difference of the exchangers at the protein level. Furthermore, the difference in size of the

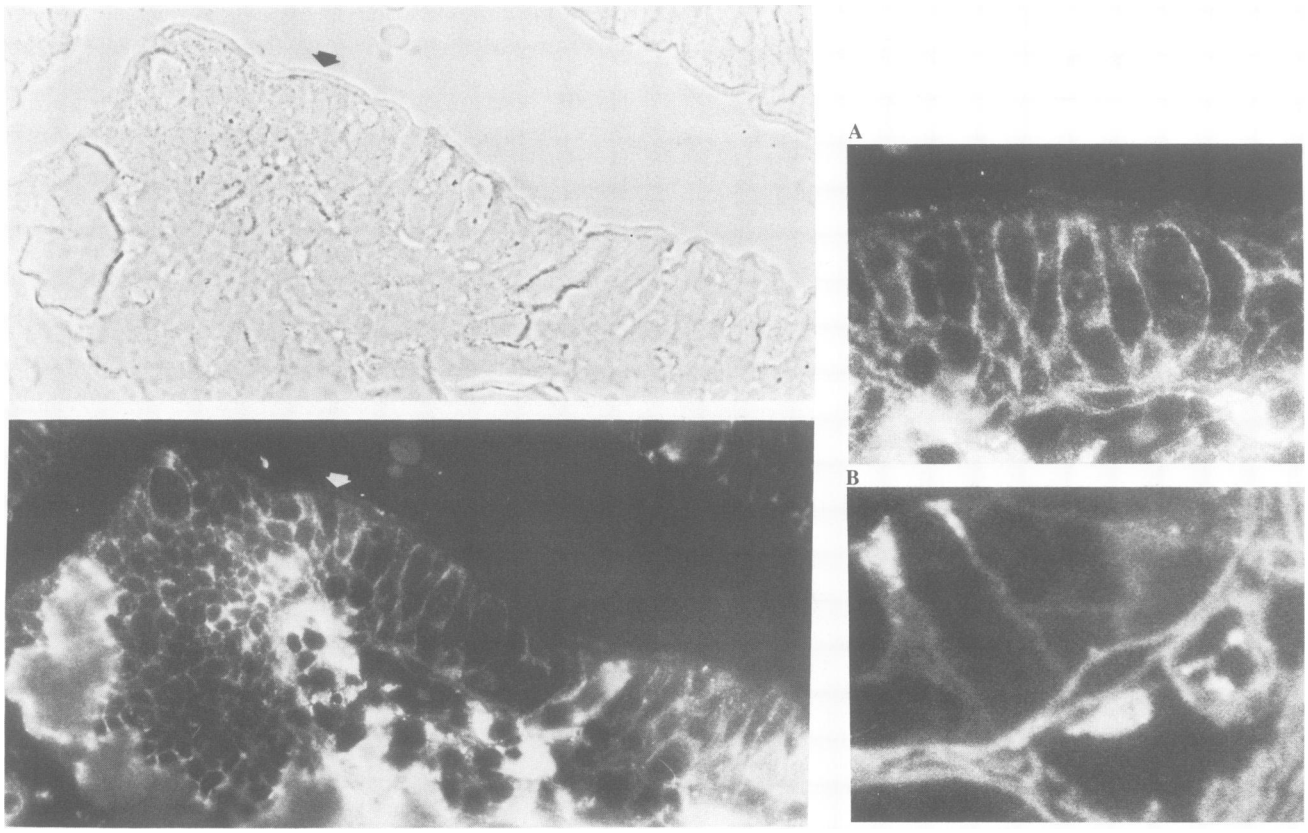

Fig. 8. Immunocytochemical localization of ileal villus cell Na^+/H^+ exchange activity to the basolateral membrane. Rabbit distal ileum was fixed in paraformaldehyde-lysine-periodate-phosphate buffer and 0.5 μm frozen sections were made. The ileal mucosa was exposed for 30 min to a 1:50 dilution of antibody RPI-c28 in 0.25% Triton X-100; and binding of this antibody was detected by 15 min exposure to a 1:50 dilution of goat anti-rabbit IgG antibody, labelled with fluorescein. The villus areas were examined with phase contrast (**above**) and fluorescence microscopy (**below**) at a magnification of 1325×. An arrow marks the brush border, which is not recognized by the antibody. Insert **A** is a magnification of the area below the arrow in Figure 8 to demonstrate lack of brush border labelling. Insert **B** is the base of the crypt area of the same section shown in Figure 8 and shows similar basolateral and not brush border staining.

Caco-2 cell Na^+/H^+ exchanger (data not shown) and the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger identified on the immunoblot with the polyclonal antibody, RPI-c28, might represent a difference in glycosylation, a post-translational event.

The stable transfection of the rabbit ileal basolateral Na^+/H^+ exchanger into PS120 cells has allowed production of a cell line, which should be useful for study of extracellular regulation of this Na^+/H^+ exchanger. In fact, these studies represent the initial characterization of regulation of the ileal basolateral Na^+/H^+ exchanger, with it being stimulated by serum and phorbol ester but not by cAMP. These results are consistent with the NHE-1 amino acid sequence described here, in which there are putative protein kinase sequences for protein kinase C but not for cAMP dependent protein kinase.

It is very likely that the basolateral membrane Na^+/H^+ exchanger encoded by R6 is the housekeeper Na^+/H^+ exchanger cloned by Sardet and Pouyssegur. This is supported by (i) the high degree of homology along the whole amino acid sequence between this clone and the human growth factor activated Na^+/H^+ exchanger; (ii) the ubiquitous expression of this message; and (iii) the high stringency Northern analysis of Caco-2 cells, indicating the presence of a similar message in a cell which does not carry out transcellular Na^+ absorption (Watson *et al.*, 1991).

What accounts for the two apparent different isoforms of the Na^+/H^+ exchanger in the rabbit ileal villus cells? It is possible that the heterogeneity arises from post-translational modification of the same protein; from the expression of two related proteins from two different genes (Birnbaum, 1989); or from the same gene via alternate transcription or splicing (Brosius *et al.*, 1989; Kudrycki and Shull, 1989). Upon analyzing the 15 remaining independent clones by their terminal sequences and by extensive restriction mapping (data not shown), no clone different from R6 was identified. Thus, it is less likely that there are additional isoforms of ileal villus cell Na^+/H^+ exchangers resulting from alternate transcription or splicing, although it is possible that the abundance of the second isoform is so low that it was not detected during library screening or that the difference between these two isoforms is so small that it was not revealed by restriction mapping. Northern blot analysis of the rabbit ileal villus cell poly(A)$^+$ RNA probed with R6 under low stringency hybridization conditions did not reveal any additional message. However, it is possible that the second species is in low abundance or is similar in size to the first. Thus, it was not possible based on the available data to exclude the presence of a second gene. Therefore, Southern analysis of rabbit genomic DNA was performed to determine whether there were any related Na^+/H^+ exchanger gene(s).

In order to obtain a simple pattern, we probed the genomic Southern blot with a single, small cDNA restriction fragment

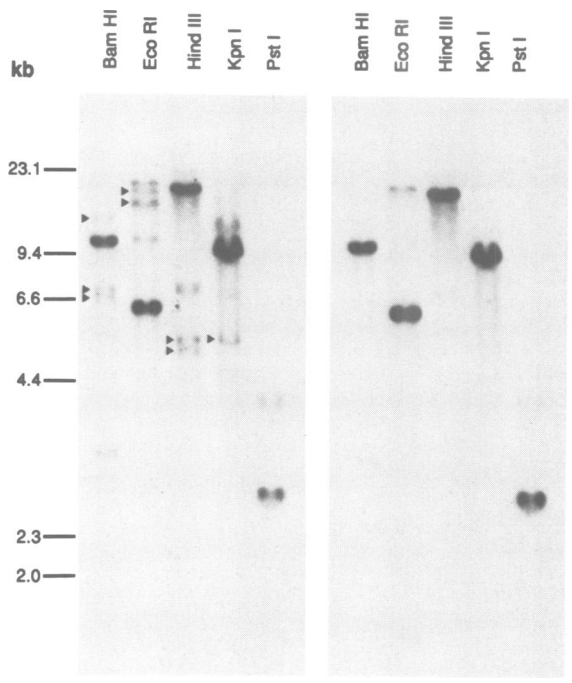

Fig. 9. Genomic Southern blot hybridization analysis of the rabbit Na^+/H^+ exchanger gene. Rabbit genomic DNA was isolated from whole blood as described (Sambrook *et al.*, 1989). It was digested in parallel with restriction enzymes *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (land 3), *Kpn*I (lane 4) and *Pst*I (lane 5) and was analyzed by Southern blot hybridization using the 462 bp *Pst*I−*Acc*I fragment (encoding amino acids 157−310) of the rabbit ileal villus cell Na^+/H^+ exchanger cDNA as the probe. The genomic Southern blot was hybridized with the probe under high stringency hybridization conditions (see Materials and methods) for 20 h and was washed with low stringency condition (1×SSC, 0.1% SDS at 42°C) and the blot was exposed for three days (left panel). After the exposure, the blot was then rewashed under high stringency conditions (0.1×SSC, 0.1% SDS at 65°C) and exposed for seven days (right panel). In each of the lanes, a single major band with several faint bands was identified. The bands that disappeared after the high stringency washing are marked with ▶. The positions and sizes (in kb) of the DNA standards are indicated in the left margin.

of R6. This *Pst*I−*Acc*I restriction cDNA fragment is 462 bp long and encodes part of the membrane spanning domains 4, 5, 6 and the extracellular hydrophilic loop b (Sardet *et al.*, 1989). Intron−exon boundaries are usually located between the putative membrane spanning hydrophobic segments in membrane spanning proteins like murine band 3 protein (Kopito *et al.*, 1987) and rat brain glucose transporter (William and Birnbaum, 1989). If this is a general phenomenon, the probe that we used would be encoded by at most three exons. Genomic organization of the human growth factor activated Na^+/H^+ exchanger gene have revealed that the membrane spanning regions 4, 5, 6 and the extracellular loop b is encoded by two exons (G.Pages and J.Pouyssegur, unpublished results). It is most likely that the 462 bp *Pst*I−*Acc*I fragment derived from R6 is also encoded by two exons. Furthermore, the *Pst*I−*Acc*I cDNA restriction fragment does not contain the *Pst*I, *Eco*RI, *Kpn*I, *Hin*dIII and *Bam*HI restriction sites. Thus, the number of bands identified by this probe will be at most two. The fact that more than two bands were identified by this probe under high stringency hybridization conditions but low stringency washing conditions and that some of these faint bands were washed away under high stringency washing conditions strongly suggests that some of these faint bands resulted from weak hybridization with other closely related DNA sequences. In fact, some of the faint bands which persisted after the high stringency washing could possibly also be due to cross hybridization to other related sequences. This view is further supported by the observation that the faint bands that were washed away under high stringency conditions were not seen when the entire R6 cDNA was used as the probe. These data strongly raised the possibility of the presence of other Na^+/H^+ exchanger genes or closely related genes.

In conclusion, we have isolated and functionally expressed in fibroblasts a rabbit ileal epithelial cell basolateral membrane Na^+/H^+ exchanger cDNA, which probably is the same housekeeper Na^+/H^+ exchanger found in humans. Southern blot analysis of rabbit genomic DNA with the *Pst*I−*Acc*I fragment of the rabbit Na^+/H^+ exchanger cDNA provides the first evidence suggesting the presence of other Na^+/H^+ exchanger genes or closely related genes. In fact, a second Na^+/H^+ exchanger (called NHE-2) from rabbit ileal villus cells has now been cloned, sequenced and expressed. NHE-2 has a similar hydrophobicity plot to NHE-1 and is also predicted to have 10 membrane spanning domains and a long cytoplasmic C-terminus. It exhibits overall 50% amino acid identity with NHE-1 and is likely to be a phosphoprotein as, like NHE-1, it has several potential phosphorylation consensus sequences in the putative cytoplasmic C-terminus (C.M.Tse, J.Pouyssegur and M.Donowitz, in preparation). We anticipate that this cDNA probe will be useful in further elucidating different isoforms of the Na^+/H^+ exchanger.

Materials and methods

RNA isolation

Total RNA from rabbit tissues was isolated by a commercially available kit, RNA_{ZOL}, which utilizes a modification of the one-step procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA was also isolated from two cultured human colon cancer cell lines−uncloned Caco-2 and the clonal line HT29-18 grown in a glucose containing medium (Huet *et al.*, 1987). Poly(A)$^+$ RNA was purified from total RNA by two passages of affinity chromatography on oligo(dT)−cellulose (Aviv and Leder, 1972).

Northern blot hybridization analysis

Two to five μg of poly(A)$^+$ RNA was denatured with glyoxal, size-fractionated by 1% agarose gel electrophoresis and transferred to a nylon filter (Hybond-N, Amersham) by capillary blotting (Sambrook *et al.*, 1989). The membrane was prehybridized overnight at 42°C in 50% formamide (high stringency conditions) or 30% formamide (low stringency conditions), 4×SSC (1×SSC = 150 mM NaCl, 15 mM sodium citrate), 5×Denhardt's solution (1×Denhardt's = 0.02% each of Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 1% SDS, 50 μg of denatured salmon sperm DNA/ml buffered with 50 mM sodium phosphate at pH 7 (Sambrook *et al.*, 1989). Hybridization was carried out for 24−36 h in the same solution containing 10^6 c.p.m./ml of denatured ^{32}P-labeled probe (the *Bam*HI−*Bam*HI restriction fragment of the human Na^+/H^+ exchanger cDNA (Sardet *et al.*, 1989) or the full-length rabbit Na^+/H^+ exchanger cDNA (R6) as described below), which was labeled with a random primer labeling kit (Pharmacia) (Feinberg and Vogelstein, 1983). The blot was washed sequentially once each for 30 min at room temperature in 2×SSC, 0.1% SDS; and 1×SSC, 0.1% SDS. A final wash was performed twice for 15 min at 42°C with 1×SSC, 0.1% SDS (low stringency washing conditions) or at 65°C with 0.1×SSC, 0.1% SDS (high stringency washing conditions). The blot was then analyzed by autoradiography.

cDNA library

Rabbit ileal villus cells were obtained by lightly scraping ileal mucosa with a glass slide (Weiser, 1973). Poly(A)$^+$ RNA was obtained as described

above. A cDNA library was constructed in λ_{ZAP} by Stratagene Inc. (La Jolla, CA) using the rabbit ileal villus cell poly(A)$^+$ RNA. cDNA inserts were size fractionated to >0.5 kb before being ligated to the *Eco*RI cloning site of the arms of the λ_{ZAP} vector. 3.2×10^6 primary independent clones were obtained, 10^6 of which were amplified to a titre of 3.5×10^{10} p.f.u./ml.

cDNA cloning

10^6 phage from the amplified rabbit ileal villus cell cDNA library were plated at a density of 250 000 plaques per 22×22 cm^2 culture dish, transferred in duplicate to nylon filters (Hybond-N, Amersham) and screened with a ^{32}P-labeled 1.9 kb *Bam*HI−*Bam*HI fragment of the human Na$^+$/H$^+$ cDNA (Sardet *et al.*, 1989) (encoding amino acid residues 109−746) using high stringency hybridization conditions at 42°C in 50% formamide, 4×SSC, 5×Denhardt's solution, 1% SDS, but low stringency washing conditions at 42°C in 1×SSC, 0.1% SDS. Positive clones were plaque purified after three rounds of screening. Bluescript plasmids containing cDNA inserts were excised *in vivo* from the λ_{ZAP} vector by co-infecting the XL-1 Blue cells with the helper phage R408. Plasmid DNA was isolated by alkaline lysis (Sambrook *et al.*, 1989) and digested with *Eco*RI to release the cDNA inserts. The cDNA inserts were characterized by Southern blot and/or Northern blot hybridization analysis (Sambrook *et al.*, 1989).

cDNA sequencing

The longest cDNA clone (R6), which hybridized to a 4.8 kb message on Northern blot analysis of rabbit ileal mucosal cDNA, was sequenced on both strands. Progressive unidirectional deletions of the R6 cDNA insert were obtained using the ExoIII/mung bean nuclease kit (Stratagene Inc.). Deletion plasmids were purified by alkaline lysis and were used as double-stranded templates for sequencing by the dideoxy termination procedure (Sanger *et al.*, 1977), using the Sequenase kit (USB Corp.).

Genomic Southern blot hybridizaton analysis

Rabbit genomic DNA was isolated from whole blood as described (Sambrook *et al.*, 1989). Genomic DNA (10 μg) was digested in parallel reactions with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Pst*I. The digested samples were size fractionated by electrophoresis through 0.7% agarose and were transferred to a nylon filter (Hybond-N, Amersham) (Sambrook *et al.*, 1989). The filter was prehybridized overnight at 42°C under high stringency conditions in 50% formamide, 4×SSC, 5×Denhardt's solution, 1% SDS. Hybridization was carried out for 20 h at the same temperature in the same solution containing 10^6 c.p.m./ml of the denatured ^{32}P-labeled 462 bp *Pst*I−*Acc*I cDNA fragment of R6 (nucleotides 471−932, encoding amino acids 157−310). The blot was washed under low stringency washing conditions (15 min at 42°C, 1×SSC, 0.1% SDS) and analyzed by autoradiography. Following autoradiography, the filter was further washed for 30 min under high stringency conditions (15 min at 65°C in 0.1×SSC, 0.1% SDS) and then again analyzed by autoradiography.

Stable expression of the R6 cDNA in Na$^+$/H$^+$ exchanger deficient fibroblasts

The Chinese hamster lung fibroblast line CCL39 Na$^+$/H$^+$ exchanger deficient derivative PS120 (Pouyssegur *et al.*, 1984) was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. To express the R6 cDNA in PS120 cells, the R6 cDNA (nucleotides −109 to 3514) was subcloned into the mammalian expression vector, pMAM-neo (Clontech). Most of the 5′ untranslated sequences (−323 to −109) were removed with *Mae*I before ligation to the expression vector because the 5′ untranslated sequence may have a negative effect on the expression of the cDNA (J.Pouyssegur and C.Sardet, personal communication). Blunt ends were created using Klenow polymerase. Part of the 3′-untranslated sequence (3515 to end) was removed by *Sal*I creating a sticky end for unidirectional subcloning of the R6 into the *Nhe*I−*Sal*I sites of the expression vector. The transcription of the insert is under control of the Rous Sarcoma virus LTR enhancer linked to the dexamethasone inducible MMTV-LTR promoter and confers resistance to the antibiotic geneticin (G418). The pMAM-neo R6 construct was transfected into Na$^+$/H$^+$ exchanger deficient cells (PS120) by calcium phosphate precipitation (Kingston, 1989). Cells near confluency were split 1:15 the day before transfection and 1.5×10^6 cells were transfected using 125 mM $CaCl_2$, 140 mM NaCl, 25 mM HEPES, 0.75 mM Na_2HPO_4, pH 7.05 and 20 μg of DNA per 10 cm dish. After 6 h of DNA incubation, the cells were exposed to 10% DMSO for 3 min. Starting 48 h after transfection, the cells were selected by growth in the antibiotic G418 (800 μg/ml). After 3 weeks of growth in G418, single G418 resistant clones were further selected by acid loading and subsequent recovery in Na medium as described (Sardet *et al.*, 1989). This was repeated 3 times with a 48 h incubation period between sequential acid loadings.

Before analysis, the clones were grown for 24 h both with and without 1 μM dexamethasone. Cells which survived this double (G418/acid) selection were then trypsinized and analyzed for the presence of Na$^+$/H$^+$ exchange using the fluorescent pH sensitive probe, 2′7′-bis (2-carboxyethyl)-5,6 carboxy-fluorescein (BCECF) by measuring the Na$^+$ dependent, amiloride sensitive intracellular pH recovery after an acute acid load with NH_4Cl, as we have described (Watson *et al.*, 1991). Screening of 10 separate clones yielded seven with functional Na$^+$/H$^+$ exchange activity. All clones tested had similar levels of Na$^+$/H$^+$ exchange activity; dexamethasone did not significantly alter the Na$^+$/H$^+$ exchanger activity, unlike in similar clones which did not have truncation of the 5′-end before subcloning into pMAM-neo (Sardet *et al.*, 1989). A single clone called NHE-1/PS120/5 was used for the studies of regulation of Na$^+$/H$^+$ exchange. Parallel regulation of Na$^+$/H$^+$ exchange was studied in a stable cell line called PS122, a derivative of PS120 established using the above techniques with c-28, a cDNA for a human Na$^+$/H$^+$ exchanger (Sardet *et al.*, 1989). PS122 is inducible with dexamethasone to produce a 12× increase in Na$^+$/H$^+$ exchange activity (A.Franchi, C.Sardet and J.Pouyssegur, unpublished observations). For comparison with NHE-1/PS120/5, PS122 was not exposed to dexamethasone in these studies.

Regulation of Na$^+$/H$^+$ exchange

Cells were grown to 70−80% confluency on glass coverslips mounted on plastic frames and then serum starved for 17 h to arrest growth. The medium was then removed and the cells washed twice with 3 ml of Na medium (containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $NaPO_4$, 25 mM glucose, 20 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin). The cells were then loaded with 2.5 μM BCECF in Na medium for 60−90 min. After 15 min of acid loading, the cells were washed three times with TMA medium (containing 130 mM TMA, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $NaPO_4$, 25 mM glucose, 20 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin) to remove the extracellular dye and the frame was then mounted at 45° in a glass cuvette containing TMA medium for fluorescence determination. The cuvette was placed in the fluorometer and a baseline was obtained. The cells were then removed to a cuvette containing Na medium and pH recovery measured. After steady state was reached, serum (10% final concentration) was added and the cells were allowed to come to a new steady state. Fluorescence was measured in an SLM spectrofluorometer (SPF 500C, SLM, Urbana, II) equipped with a stir cuvette maintained at 37°C. Excitation wavelengths for BCECF were altered between 440 ± 1 and 500 ± 1 nm and emission was monitored at 530 ± 10 nm. A CompuAdd 286 computer regulated the fluorometer and collected, analyzed and stored the data. Autofluorescence was determined daily using a cuvette with cells which were not loaded with BCECF. Autofluorescence-corrected fluorescence ratio values (500/440) were calculated and graphically presented every 3 s during the experiment. The pH of the medium was continuously monitored by a mini pH electrode (MI-506; Microelectrodes, Inc., Londonderry, NJ) immersed in the cuvette but placed out of the light path. At the end of the experiment, 100 μM digitonin was used to permeablize the cells to release all intracellular dye, after which calibration was performed using nitric acid (Watson *et al.*, 1991). The external pH was simultaneously recorded during the stepwise nitric acid acidification. The fluorescence ratio (500/440) was calibrated by nigericin titration to yield the initial pH after acidification and the final pH after recovery. Presence of Na$^+$/H$^+$ exchange was defined as Na-dependent cell alkalinization after an acid load and was shown to be amiloride sensitive.

Cellular localization of an ileal Na$^+$/H$^+$ exchanger by immunoblotting and immunocytochemical analysis

Immunoblotting. Highly purified rabbit ileal villus cell brush border membranes and basolateral membranes, prepared as previously described (Rood *et al.*, 1988; Cohen,M.E. *et al.*, 1991), were separated on 10% SDS−PAGE and then were transferred to nitrocellulose for 12 h (150 mA) according to the method of Towbin (Towbin *et al.*, 1979). Following transfer, the nitrocellulose was incubated for 1 h at 23°C in incubation buffer (150 mM NaCl, 50 mM Tris, pH 7.0, 7.7 mM NaN_3 plus 0.5% (v/v) polyoxyethylene sorbitan monolaurate). The nitrocellulose was then incubated with an affinity purified polyclonal antibody, RPI-c28 (Sardet *et al.*, 1990), at a dilution of 1 in 200 for 4 h at 23°C. The nitrocellulose was then washed for 15 min with three changes of wash buffer (150 mM NaCl, 50 mM Tris, 7.7 mM NaN_3, 12 mM Na desoxycholate) and then incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (Gibco BRC, Gaithersberg, MD) antibody at a dilution of 1 in 200 for 1 h at 23°C. The nitrocellulose was then washed again as described above. Alkaline phosphatase conjugate

was then detected by incubation in a solution containing 146 mM Nitro Blue Tetrazoleum, 138 μM 5-bromo-4-chloro-3-indoyl phosphate (*p*-toluidine salt), 50 mM $NaCO_3$, pH 9.5 and 1% (v/v) *N,N*-dimethylformamide for 10 min at 23°C.

Immunocytochemistry. A segment of rabbit distal ileum was removed, washed in PBS and cut open along the mesenteric border. After fixation in 2% paraformaldehyde, 0.1 M lysine, 0.01 M sodium periodate, 0.05 M phosphate buffer as described (McLean and Nalkane, 1974) for 2 h, the tissue was washed in PBS, cut into 1–2 mm^2 pieces and placed in a cryoprotectant solution (PVP-sucrose) for 6 h at 4°C. Tissue pieces were mounted on Reichert specimen nails and then placed in liquid nitrogen. Thin (0.5 μm) frozen sections were then made such that the villi were cut in a sagittal plane, allowing visualization of the apical and basolateral regions of the villus epithelial cells. After quenching with 0.25% NH_4Cl and preabsorbing with 0.2% gelatin, the sections were incubated for 30 min with a 1:50 dilution of the affinity purified polyclonal antibody RPI-c28 to the Na^+/H^+ antiporter in 0.25% Triton X-100. Binding of this antibody was detected with a fluorescein-labeled affinity purified goat antibody to rabbit IgG (Kirkgaard & Perry Lab, Inc., Gaithersburg, MD) (1:50 dilution for 15 min in 0.25% Triton X-100). The sections were examined with phase contrast and fluorescent microscopy using a Zeiss Axioplan fluorescence microscope (1325× magnification). Parallel sections were incubated with a 1:50 dilution of nonimmune rabbit serum in place of the Na^+/H^+ antiporter antibody, to differentiate between specific and non-specific binding.

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