Journal of Bioenergetics and Biomembranes, Vol. 25, No. 6, 1993

Na^+/H^+ Antiporters, Molecular Devices that Couple the Na^+ and H^+ Circulation in Cells

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Received July 12, 1993; accepted July 15, 1993

Na^+/H^+ antiporters are universal devices involved in the Na^+ and H^+ circulation of both eukaroyotes and prokaryotes, thus playing an essential role in the pH and Na^+ homeostasis of cells. This review focuses on the major impact of the application of molecular biology tools in the study of the antiporters. These tools permit the verification of the role of the antiporters and provide insights into their unique biology. A novel signal transduction to Na^+ involving *nha*R, a positive regulator, controls the expression of *nha*A in *E. coli*. A "pH sensor" regulates the activity of Na^+/H^+ antiporters, both in eukaryotes and prokaryotes. A most intricate signal transduction to pH involving phosphorylation steps controls the activity of *nhe*1 in higher mammals. The identification of Histidine 226 in the "pH sensor" of NhaA is a step forward towards the understanding of the pH regulation of these proteins.

KEY WORDS: Na^+/H^+ antiporters; pH regulation; halotolerance; transport; membranes; membrane proteins.

1. INTRODUCTION

Circulation of H^+ and Na^+ ions is maintained across the cytoplasmic membrane of bacteria, animal and plant cells, and the membranes of various subcellular organelles. The coupling between these ion circulations is catalyzed by the ubiquitous Na^+/H^+ antiporters. A most central role is therefore implied for the Na^+/H^+ antiporters in the H^+ and Na^+ ion circulations of cells.

The physiological roles of the Na^+ and H^+ cycles share common properties in various cells but they also exhibit unique specializations related to the particular environment and metabolism of the organism. In the present review we will explore these unique properties and how they are reflected in the activity, regulation, and molecular architecture of the antiporters. Comprehensive reviews of the Na^+/H^+ antiporters of *Escherichia coli* have recently been published (Padan and Schuldiner, 1992; Schuldiner and Padan, 1992, 1993).

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2. MOLECULAR BIOLOGY TOOLS FOR THE STUDY OF Na^+/H^+ ANTIPORTERS

Both in prokaryotes and eukaryotes application of molecular biology had a major impact on the understanding of the Na^+/H^+ antiporters. Therefore, we will first review the development of this approach for the study of the antiporters.

2.1. Prokaryotes

The molecular biology of the Na^+/H^+ antiporters in prokaryotes was initiated by the study of an *E. coli* mutant which led to a strategy for cloning of antiporter genes by functional complementation. This mutant with increased rather than decreased antiporter activity was isolated based on its resistance to Li^+ (Niiya *et al.*, 1982). Li^+ ions are toxic to *E.coli* cells due mostly to their effect on the cell pyruvate kinase (Umeda *et al.*, 1984). When grown on

0145-479X/93/1200-0647$07.0/0

melibiose, which is symported to the cells with Na^+, the toxicity is augmented due to Li^+ inhibition of the melibiose transporter. An *E. coli* mutant that tolerates Li^+ concentrations otherwise toxic to the wild-type cells has been isolated (Niiya *et al.*, 1982). This mutant harbors at least two mutations responsible for the acquisition of the resistance: one in the *melB* allele (*melBLid*), in which replacement of proline at position 122 with serine brings about a modification in the melibiose transporter such that it can now cotransport the sugar both with Li^+ and Na^+ (Niiya *et al.*, 1982; Yazyu *et al.*, 1985). The second mutation is in an additional locus, which causes an enhanced antiporter activity capable of an increased excretion of the toxic ion (which is also a substrate of the antiporter). We have separated the two mutations and showed that the one which increases antiporter activity (*nha*Aup, previously called *ant*up), maps at 0.3 min on the *E.coli* chromosome and is necessary to confer resistance to toxic levels of Li^+ ions (Goldberg *et al.*, 1987).

Taking advantage of the toxicity of Li^+ ions and the resistance associated with high activity of the antiporter, we cloned the wild-type *nha*A gene (Goldberg *et al.*, 1987; Karpel *et al.*, 1988; Table I). We assumed that when in high copy number (plasmidic + chromosomal) the wild-type *nha*A would increase Na^+/H^+ antiporter activity and thereby confer Li^+ resistance to cells, i.e., an $NhaA^{up}$ phenotype. Scoring this phenotype in cells transformed with plasmids containing DNA inserts covering 15 kb from *car* to *dnaJ* (Mackie, 1980, 1986) that include the wild-type locus affecting the antiporter, yielded a plasmid bearing *nha*A which confers Li^+ resistance.

To study the role of the antiporter and to assess whether *E. coli* has an additional specific Na^+/H^+ antiporter, the strategy was to inactivate the chromosomal gene by replacing most or all of it with a selectable marker (Padan *et al.*, 1989). We constructed Δ*nha*A strains in which either two-thirds (Padan *et al.*, 1989) or the whole gene (Karpel, 1990) was replaced with *kan* without disrupting any of the neighboring genes. The Δ*nha*A strains obtained grow normally in low sodium medium, indicating that, at least under these conditions, *nha*A is not an essential gene. Δ*nha*A, on the other hand, is markedly sensitive to Li^+ and Na^+, and its sensitivity to the latter ion increases with pH (Padan *et al.*, 1989). The Δ*nha*A strain thus permitted study of the involvement of *nha*A in the H^+ and Na^+ circulation of the cell (Padan *et al.*, 1989; Pinner *et al.*, 1993 and Sections 3 and 4). Furthermore, although the level of the Na^+/H^+ antiporter activity in membrane vesicles isolated from Δ*nha*A was reduced to 50% of the wild-type level, a detailed analysis of the remaining antiporter activity in the Δ*nha*A strain revealed an additional Na^+/H^+ antiporter, designated NhaB, with specific properties differing from those displayed by the NhaA protein: (I) the K_m for transport of Li^+ and Na^+ ions of NhaB is different than that of NhaA. (II) The activity of NhaB is practically independent of intracellular pH, while that of NhaA increases dramatically with increasing pH. Hence our results have demonstrated the presence of two different Na^+/H^+ antiporters in *E.coli* (Padan *et al.*, 1989).

Cloning of *nha*B (Pinner *et al.*, 1992b and Table I) made possible the generation of Δ*nha*B and Δ*nha*AΔ*nha*B strains which already supplied further invaluable information about the components involved in the metabolism of Na^+ and H^+ in *E.coli* (Sections 3 and 4). It also showed that there is no active additional specific Na^+/H^+ antiporters in *E.coli* and that the K^+/H^+ nonspecific antiporter (Brey *et al.*, 1978; Plack and Rosen, 1980; Rosen, 1986) is the only remaining activity in Δ*nha*AΔ*nha*B.

The Na^+/H^+ antiporter deletion mutants are also most important for study of structure–function relationship of the antiporters. They permit expression of various plasmidic mutated antiporter genes and study of their phenotype with no background of the wild-type genes (Section 5.5). In summary, the antiporter genes with their respective deletion mutation opened the way to the application of a molecular biology approach studying the properties of antiporters, their physiological role and regulation (Sections 3–5).

2.2. Cloning by Functional Complementation of Antiporter Genes from Prokaryotes and Eukaryotes

Both Δ*nha*A and Δ*nha*AΔ*nha*B are Na^+ and Li^+ sensitive. The sensitivity to Na^+ of the latter strain (> 10 mM) is even higher than that of the former (> 400 mM, pH 7). However, transformation of either of the deletion strains by multicopy plasmid carrying *nha*A renders the transformants resistant to the ions (Padan *et al.*, 1989; Pinner *et al.*, 1993). Therefore, both strains have provided selection vehicles for cloning of genes coding for antiporters by functional complementation. Using this paradigm (with Δ*nha*A; Table I) we have succeeded in cloning three other genes: one from *Salmonella entiritidis*

homologous to *nha*A (Pinner *et al.*, 1992a), the *nha*B gene from *E.coli* (Pinner *et al.*, 1992b) and, in collaboration with the group of T. A. Krulwich, a novel gene from the alkaliphilic *Bacillus firmus* OF4 that codes for a putative antiporter (Ivey *et al.*, 1991). For the cloning of *nha*B the DNA library was prepared from the Δ*nha*A strain in order to prevent recloning of *nha*A (Pinner *et al.*, 1992b).

The higher Na^+ sensitivity of Δ*nha*AΔ*nha*B as compared to Δ*nha*A permits one to apply even a wider range of selection pressures. Most interestingly, this selection yielded genes which, before cloning, when in single copy in the chromosome, do not confer Na^+ resistance and therefore most probably are not engaged in the native Na^+/H^+ antiporter activity of the cells. However, when selected in multicopy plasmids, they restore partial Na^+ resistance and membrane Na^+/H^+ antiporter activity to both Δ*nha*A or Δ*nha*AΔ*nha*B strains (Ivey *et al.*, 1992a and b, 1993 and Table I). One example is *cha*A which maps at 27 min on the *E. coli* chromosome and which is predicted to encode a polytopic membrane protein that possesses sequence similarity to several Ca^{++} binding proteins and the Na^+/Ca^{++} antiporter on one of its predicted hydrophilic loops. The Ca^{+2}/H^+ antiporter activity of membranes from an *E. coli* transformant with this gene is enhanced and pH-independent. Mg^{++} inhibits both the Na^+/H^+ and Ca^{++}/H^+ antiporter activities conferred by the clone. *cha*A is proposed to be the structural gene for a nonspecific antiporter exchanging H^+ with either Ca^{++} or Na^+.

Another example is provided by the alkaliphilic bacterium (*B. firmus* OF4) inserts which restore certain Na^+ resistance to the *E. coli* mutant and some of which even enhance Na^+/H^+ exchange activity. Interestingly, some of these clones show amino acid sequence similarity with putative ion binding sites of various ion transport systems including *cad*C of *Staphylococcus aureus* cadmium resistance plasmid (pI258; Ivey *et al.*, 1992a and b and Table I). The significance of these clones is still not clear.

The Na^+/H^+ antiporter gene of *Enterococcus hirae* has been cloned by an approach similar to the one used for cloning of *E. coli* antiporters (Waser *et al.*, 1992 and Table I): complementation of a Na^+-sensitive mutant defective in both ATP-driven Na^+ extrusion and the Na^+/H^+ antiporter. The gene termed *nap*A enhances Na^+/H^+ antiporter activity and its disruption leads to loss of the exchange activity as measured in whole cells or membrane vesicles.

Lithium is 10 times more toxic than Na^+, on a concentration basis, both to wild-type *E. coli* (Goldberg *et al.*, 1987) and fission yeast (Jia *et al.*, 1992). Since it is usually transported by Na^+ carriers, it provides a screen for cells capable of maintaining low internal sodium or lithium levels without selecting for osmotolerance. Another advantage of Li^+ selection over that of Na^+ is that it can be applied directly to wild-type cells. As shown above, this selection led to cloning of *nha*A (Goldberg *et al.*, 1987). A similar approach yielded the gene *sod*2 from *Schizosaccharomyces pombe* (Jia *et al.*, 1992 and Table I).

It was inferred that multiple copies of the wild-type *sod*2 gene would be sufficient to confer Li^+ resistance to *S. pombe* by increasing the export capacity of the ion. Transformants of *S. pombe* with genomic gene bank scored for Li^+ resistance yielded a strain carrying a recombinant plasmid including *sod*2 (Jia *et al.*, 1992). Overexpressing of *sod*2 increased Na^+ export capacity and conferred Na^+ tolerance (Jia *et al.*, 1992).

2.3. Cloning of the Plasma Membrane Exchanger of Higher Mammals

The use of a powerful and elegant combination of genetic techniques allowed the isolation of a cDNA that codes for a human exchanger (Nhe1; see Sardet *et al.*, 1989 and Table I). The first step included selection of an exchanger-deficient mouse fibroblast cell line. It was based on loading of the cells with Li^+ and their exposure to an acidic pH in a Na^+ and Li^+-free medium. The wild-type exchanger-containing cells rapidly died due to the exchanger-catalyzed H^+ uptake, and cytoplasm acidification. However, exchanger-deficient cells, resistant to the treatment, were thereby isolated (Pouyssegur *et al.*, 1984, 1988). In addition, isolation of mutants overexpressing the exchanger was possible by acid loading cells and by allowing them to recover under conditions in which the exchanger activity is slowed down. Only cells overexpressing the exchanger survive this treatment (Franchi *et al.*, 1986a).

Transfection of the exchanger-deficient mouse cell line with human genomic DNA and selection of cells overexpressing the human exchanger allowed for isolation of a genomic probe that was used for cDNA cloning of Nhe1 (Franchi *et al.*, 1986b; Sardet

Table I. Genes Encoding Na^+/H^+ Antiporters and Related Proteins

Gene	DNA source	Complemented strain	Selection conditions (mM)[a]	Protein structure			Na^+/H^+ antiporter activity			Function
				MW (kDa)	α Helixes	Homology	Cells	Membranes[b]	Purified reconstituted	
*nha*A	*E. coli*	*E.coli* wild type	Li^+, 100, (10)	41.3	11	Low to Na^+-transporters[3]	nd	+ +	+	Structural gene of an *E. coli* Na^+/H^+ antiporter[1-3]
*nha*B	*E. coli* Δ*nha*A	*E. coli* Δ*nha*A	Na^+, 500, pH7.5 (400)	55.5	12	Low to Na^+-transporters[3]	nd	+ +	+	Structural gene of an *E. coli* Na^+/H^+ antiporter[4]
*cha*A	*E. coli* Δ*nha*AΔ*nha*B	*E. coli* Δ*nha*A or Δ*nha*AΔ*nha*B	Na^+, 600, pH 7.5 (400) Na^+, 400, pH 7.5 (20)	39.2	12	Calsequestrin	nd	+	nd	Putative Ca^{++}/H^+ antiporter of *E. coli*[5]
*nha*A/S	*Salmonella entiritidis*	*E. coli* Δ*nha*A	Na^+, 700, pH 6.8 (500)	41.4	11	High to NhaA	nd	+ +	nd	Structural gene of an *S. entiritidis* Na^+/H^+ antiporter[6]
*nha*C	*Bacillus firmus* OF4	*E. coli* Δ*nha*A	Na^+, 700, pH 7.5 (400)	43	10	Low to other antiporters	nd	+	nd	Putative Na^+/H^+ antiporter of *B. firmus* OF4[7]
*cad*C/B	*Bacillus firmus* OF4	*E. coli* Δ*nha*A	Na^+, 700, pH 8.2 (250)	13.9	—	*cad*C of *S. aureus* plasmid pI258	nd	+-	nd	Putative *cad*C of *B.alkalophilus* OF4[8]
napA	*Enterococcus hirae*	*E. hirae* mutant devoid of sodium extrusion capacity	Na^+, 100 (10)	41.5	12	*sod* 2 and low to *kef* C	nd	+	nd	Putative Na^+/H^+ antiporter of *E. hirae*[9]

sod 2	*Schizosaccharomyces pombe*	wild type	Li^+, 40 pH 5 (15)	52.2	12	Low but significant to Nhe family	+ +	nd	nd	Putative Na^+/H^+ antiporter of *S. pombe*[10]
*nhe*1	Higher mammals (rabbit rat, man)	*nhe*1 less mouse fibroblasts	Li^+, 130 pH 5.5	90 (130 including sugars)	12	Nhe family	+ +	nd	nd	Putative Na^+/H^+ antiporter of higher mammals[11,12]
nhe2 nhe3 nhe4	Higher mammals (rabbit, rat, man)	Screening by hybridization		About 90	12	Nhe family	+ +	nd	nd	Putative Na^+/H^+ antiporter of higher mammals[12–14]
nhe	*C. elegans*	Sequence		Sequence not complete	—	Nhe family	nd	nd	nd	Putative Na^+/H^+ antiporter of *C. elegans*[15]
βnhe	*Salmo gardneri*	Screening by hybridization		85	12	Nhe family	+ +	nd	nd	Putative Na^+/H^+ antiporter of trout[16]

[a] The numbers in brackets refer to the respective permissible concentrations of the ion of the complemented strain.
[b] As compared to the control (no plasmid); + + denotes an amplified antiporter activity whereas +, a low enhancement of antiporter activity (< 2) conferred by the plasmidic cloned genes. nd, not determined.

References: [1] Goldberg *et al.*, 1987; [2] Taglicht *et al.*, 1991; [3] Padan and Schuldiner, 1992; [4] Pinner *et al.*, 1992b; [5] Ivey *et al.*, 1993; [6] Pinner *et al.*, 1992a; [7] Ivey *et al.*, 1991; [8] Ivey *et al.*, 1992b; [9] Waser *et al.*, 1992; [10] Jia *et al.*, 1992; [11] Sardet *et al.*, 1989; [12] Wakabayashi *et al.*, 1992a; [13] Tse *et al.*, 1992; [14] Orlowski *et al.*, 1992; [15] Marra *et al.*, 1992; [16] Borgese *et al.*, 1992.

et al., 1990). The nucleotide sequence predicts a protein of 815 amino acids with 12 putative transmembrane helices. As in the case of many other secondary transporters from higher eukaryotes, the exchanger has two distinct domains, a 500 amino acids membrane-bound NH_2-terminus, and an hydrophylic cytoplasmic C-terminal domain of 315 residues.

This cDNA facilitated the isolation, at lower stringency, of several isoforms referred as Nhe 2, 3, 4, and β-Nhe (Tse *et al.*, 1991, 1992; Orlowski *et al.*, 1992; Borgese *et al.*, 1992; Table I). All these forms exhibit 45–70% identity with Nhe-1 and possess a similar hydropathy profile. Yet another member of this family has been fortuitously identified in *Caenorhabditis elegans* (Marra *et al.*, 1992). The highest degree of conservation in the various members of the family is observed in the putative fourth and sixth transmembrane helices.

3. THE ROLE OF THE Na^+/H^+ ANTIPORTERS IN Na^+ EXTRUSION AND HALOTOLERANCE

3.1. Na^+/H^+ Antiporters in Bacteria Living in Low Na^+ (< 900 mM)

The most compelling evidences for the major role of Na^+/H^+ antiporter activity in the Na^+ cycle of bacteria are provided by the work with *E. coli*. Analysis of the phenotype of a Δ*nha*A mutant shows that Na^+ has a specific toxic effect on the cells, and that the sensitivity of the cells to Na^+ is pH-dependent, markedly increasing with increasing pH (Padan *et al.*, 1989); Δ*nha*A cannot adapt to high sodium concentrations which do not affect the wild type (0.7 M NaCl at pH 6.8); the Na^+ sensitivity of Δ*nha*A is pH dependent, increasing at alkaline pH (0.1 M NaCl at pH 8.5). The Δ*nha*A strains also cannot challenge the toxic effects of Li^+ ions (0.1 M), a substrate of the Na^+/H^+ antiporter system. It is concluded that *nha*A is indispensable for adaptation to high salinity, for challenging Li^+ toxicity, and for growth at alkaline pH (in the presence of Na^+)

The Δ*nha*B strain shows no impairement in its ability to adapt to high salt or alkaline pH or in its resistance to Li^+ (Pinner *et al.*, 1993). These findings suggest that NhaA has a capacity high enough to cope, without *nha*B, with the salt and pH stress in *E. coli*. However, in the absence of *nha*A, *nha*B confers certain Na^+ resistance; the double mutant, Δ*nha*AΔ*nha*B, grows very poorly in the presence of Na^+ concentrations as low as 15–20 mM. At concentrations of 100 mM Na^+ (pH 7.5), growth is completely arrested. Yet in the absence of added Na^+ (contaminating levels of 10 mM) it grows at the entire pH range, 6.5–8.4. Analysis of the antiporter activity in membranes prepared from the Δ*nha*AΔ*nha*B strain shows no residual activity of Na^+/H^+ antiporter (Pinner *et al.*, 1993).

Based on these results and on the fact that *nha*B shows a higher affinity for Na^+ than NhaA, we have tentatively suggested that the recurrent theme described for many other transport systems possibly holds also for the systems handling Na^+ and H^+: a low-affinity, high-capacity system (in our case *nha*A) and another high-affinity, low-capacity system (*nha*B) are required to cope with adaptation to a wide range of concentrations (Schuldiner and Padan, 1992). Interestingly, and unlike most other chromosomally encoded mineral transport systems (Silver and Walderhaug, 1992), in the case of the Na^+/H^+ antiporters the high-capacity one (*nha*A) is regulated so that its expression increases significantly under the conditions in which it is essential: high salt, alkaline pH (in the presence of Na^+) (Karpel *et al.*, 1991 and Section 5.4). Nothing is known thus far about the regulation of *nha*B.

Although it confers resistance to Li^+, *nha*A does not increase the limits of pH or salt that wild-type *E. coli* can cope with, suggesting that factors other than *nha*A are limiting in setting the upper limits of tolerance.

Since the Na^+/H^+ antiporters are dependent on $\Delta\tilde{\mu}_{H^+}$ for Na^+ extrusion we termed secondary the Na^+ cycle initiated by them and suggested that this form of Na^+ export is not efficient when $\Delta\tilde{\mu}_{H^+}$ is limiting (Padan and Schuldiner, 1992). Whereas the extreme halophiles and alkaliphiles do not fall into this category (see Sections 3.2 and 4.2), alternative systems initiating primary Na^+ cycles exist in marine and anaerobic bacteria.

In anaerobic bacteria represented by *Propionigenum modestum* there is a primary Na^+ cycle, consisting of a primary Na^+ pump linked to a decarboxylation reaction such as methylmalonyl-CoA decarboxylase (Dimroth, 1987, 1992a), and F_1F_0 type ATPase which can pump H^+ but under physiological conditions uses only Na^+ (Laubinger and Dimroth, 1988a, b, 1992b; Hoffmann *et al.*, 1990). Nevertheless, Na^+/H^+ antiporter activity has been demonstrated in membrane vesicles of *P. modestum*.

Since $\Delta\tilde{\mu}_{Na^+}$ (directed inward) is the energetic currency of this organism, the Na^+/H^+ antiporter would only be able to excrete H^+ in this bacterium at the expense of the $\Delta\tilde{\mu}_{Na^+}$. This can be important for pH homeostasis (Section 4) in an organism that usually grows at neutral or acidic pH. If a $\Delta\tilde{\mu}_{H^+}$ is formed, it could also be utilized for $\Delta\tilde{\mu}_{H^+}$-coupled reactions. However, there is no experimental evidence for these suggestions. There is also no information regarding the antiporters in this or similar bacteria.

Methanogens have primary H^+ pumps linked to electron transport reactions which maintain a primary cycle completed by an H^+/ATPase (Muller *et al.*, 1987; Muller and Gottschalk, 1992; Shonheit, 1992). They also have a secondary Na^+ cycle via a Na^+/H^+ antiporter. The Na^+ uptake limb completing this cycle is a reversible primary Na^+ pump which is not a Na^+/ATPase. Methyl-tetrahydromethanopterin coenzyme M methyltransferase (Becher *et al.*, 1992) and formyl-MFR dehydrogenase (Shonheit, 1992) were shown to operate as reversible Na^+ pumps. Depending on the substrates, $\Delta\tilde{\mu}_{Na^+}$ can be either consumed or generated by these pumps. In the former case the required $\Delta\tilde{\mu}_{Na^+}$ is generated by the Na^+/H^+ antiporter, whereas in the latter $\Delta\tilde{\mu}_{H^+}$ is formed by the antiporter for ATP synthesis via an H^+/ATPase (Shonheit, 1992). The coupling of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{Na^+}$ via the antiporter is thus essential for the adaptation of the bioenergetics of these organisms to various modes of metabolism as well as for pH regulation and Na^+ extrusion.

The involvement of the Na^+/H^+ antiporter of the methanogens and other organisms with primary sodium cycles in H^+ extrusion may be similar to its mode of activity in animal cells (Section 4.4). Interestingly, some of these antiporters are also inhibited by amiloride derivatives (Table II and Section 5.2).

In fermenting anaerobes, like *E. hirae*, ATP is produced by substrate-level phosphorylation. A primary H^+ cycle is initiated by an F_1F_0 type H^+ ATPase which functions to excrete H^+, maintains a $\Delta\tilde{\mu}_{H^+}$, and regulates intracellular pH (Kobayashi, 1985, Shibata *et al.*, 1992). This organism has a Na^+/H^+ antiporter as well as a Na^+-inducible primary Na^+ pump, Na^+/ATPase, similar to the vacuolar type (archaebacterial) H^+/ATPase (Heefner and Harold, 1982; Kakinuma *et al.*, 1991). Both systems have been implied mainly in Na^+ excretion, the Na^+/H^+ antiporter at neutral and acidic pH when $\Delta\tilde{\mu}_{H^+}$ is sufficient, and the Na^+ ATPase at alkaline pH when $\Delta\tilde{\mu}_{H^+}$ is limiting (Kakinuma, 1987a,b; Waser *et al.*, 1992). The reentry routes for Na^+ are not clear in these organisms.

As expected from the Na^+ load (0.5 M) challenging marine bacteria, they have in addition to Na^+/H^+ antiporters an electron transport-linked primary Na^+ pump (Dibrov, 1991; Skulachev, 1988; Tokuda, 1989, 1992; Ken-Dror *et al.*, 1986; Unemoto *et al.*, 1990). The Na^+/H^+ antiporter of *Vibrio alginolyticus* maintains a secondary Na^+ cycle which is observed under conditions in which the Na^+ pump activity is low, i.e. below pH 8 (Tokuda, 1992). Thus, the extrusion of Na^+ against its concentration gradient at acidic pH is performed by the Na^+/H^+ antiporter and inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Na^+ extrusion at alkaline pH is resistant to CCCP and dependent on respiration. Accordingly, growth is inhibited by the uncoupler at acidic pH but not at alkaline pH, and so are all $\Delta\tilde{\mu}_{Na^+}$-requiring processes, i.e. active transport (Skulachev, 1988; Tokuda *et al.*, 1990) and flagellar motility (Hirota *et al.*, 1981).

The pH profile of CCCP-resistant growth does not simply reflect the pH dependence of the primary Na^+ pump. The latter are still functional at acidic pH, and in fact the overacidification caused by H^+ ions driven by the electrical potential generated by the pump accounts for the growth inhibition in the presence of the uncoupler at acidic pH. Whether the primary Na^+ pumps are expressed and active together with the secondary Na^+ cycle and thus have a role at acidic pH in the noninhibited cells is not clear. In mutants defective in the primary Na^+ pumps, the secondary Na^+ cycle is observed even at alkaline pH (Tokuda, 1992).

Since at alkaline pH, $\Delta\Psi$ is the only product of the electron transport-linked primary H^+ pumps, it is implied that the Na^+/H^+ antiporter activity of *V. alginolyticus* is electrogenic (Section 5.3). Provided that the stoichiometry of cation/e^- at the electron transport segment (NADH:quinone oxidoreductase) is identical between the Na^+ pump and the H^+ pump, a secondary Na^+ cycle dependent on electrogenic antiporter will consume more NADH than does the Na^+ pump for generation of the same magnitude of $\Delta\tilde{\mu}_{Na^+}$ at alkaline pH (when $\Delta\Psi = \Delta\tilde{\mu}_{H^+}$). It has been suggested that, for this difference in energy economy, the primary Na^+ pump is widely distributed among marine bacteria (Tokuda and Unemoto, 1985).

It is yet possible that at alkaline pH the Na^+/H^+

antiporter activity of *V. alginolyticus* is important for pH homeostasis (Section 4). However, K^+/H^+ antiporter which is activated at alkaline cytoplasm has been implied in the regulation of cytoplasmic pH in this organism (Nakamura *et al.*, 1984).

3.2. Na^+/H^+ Antiporters in Halophilic Bacteria

It is striking that in the extreme halophiles which can face up to 4.5 M Na^+ the only device known to export Na^+ are the Na^+/H^+ antiporters and no primary Na^+ pumps have as yet been discovered.

The extreme halophiles seem to tolerate high cytoplasmic concentrations of Na^+ (up to 1 M) (Kushner and Kamekura, 1988). It appears therefore that they compromise with a Na^+ gradient lower than 10, which can be easily produced by the Na^+/H^+ antiporter in spite of the heavy Na^+ load imposed on cell energetics. A mutant devoid of the antiporter activity if viable would add to substantiate the role of the Na^+/H^+ antiporter in extreme halophilic bacteria. Study of the characteristics of the antiporter of these organisms is certainly intriguing (Section 5).

3.3. Na^+/H^+ Antiporters Involved in Na^+ Extrusion and Halotolerance in Eukaryotic Microorganisms and Plant Cells

Membrane research of plant cells and eukaryotic microorganisms over the past decade has established that the cytoplasmic membrane of these cells maintain a primary proton cycle initiated by a P-type H^+/ATPase excreting H^+ (for a comprehensive review see Sussman and Harper, 1989). The proton uptake limb of this cycle is formed by solute/proton symporters (Frommer *et al.*, 1993). There are some indications of a primary Na^+ cycle in the extreme halophilic alga *Dunaliella salina* (Katz *et al.*, 1991), and a Na^+/ATPase has recently been cloned from yeast cells (Haro *et al.*, 1991). On the other hand, Na^+/H^+ antiporters and, thus, secondary Na^+ cycles are widely spread in the cytoplasmic membranes of eukaryotic microorganisms as well as plant cells (Mennen *et al.*, 1990; Jacoby, 1993; Hassidim *et al.*, 1990; Cooper *et al.*, 1991).

It has been suggested that the Na^+/H^+ antiporter of the eukaryotic extreme halophilic alga *D. salina* plays a role in adaptation to high salinity and in pH homeostasis (Katz *et al.*, 1991, 1992). However, since these cells maintain relatively high Na^+ gradients ([100 mM] inside when grown in media containing 0.5–4 M NaCl), involvement of a primary Na^+ pump rather than the Na^+/H^+ antiporter has been implied in salt adaptation (Katz *et al.*, 1991).

A Na^+/H^+ antiporter on the plasma membrane in *Sacharomyces cerevisiae* and *Neurospora crassa* has been suggested to be responsible for Na^+ export from the cell (Rodriguez-Navarro and Asensio, 1977; Rodriguez-Navarro and Ortega, 1982; Rodriguez-Navarro *et al.*, 1981; Ortega and Rodriguez-Navarro, 1986).

Based on selection for increased LiCl tolerance in *Schizosaccharomyces pombe*, a gene *sod*2 encoding a putative Na^+/H^+ antiporter has recently been identified (Jia *et al.*, 1992). Disruption of *sod*2 yielded cells incapable of exporting Na^+, hypersensitive to Na^+ (unable to grow above 125 mM) and Li^+ and sensitive to an increase in pH between 3.5 to 7.5 even with no addition of Na^+. These results suggest that the role of *sod*2 in pH homeostasis at increasing pH and in Na^+ extrusion. The increasing sensitivity of the mutants with pH is reminescent of the Δ*nha*A *E. coli* strain (Padan *et al.*, 1989 and Section 3.1). However, the pH sensitivity of Δ*nha*A is detected above pH 7.5 and is Na^+ dependent (Padan *et al.*, 1989). Various levels of amplification of *sod*2 could be selected stepwise, and the degree of such amplification correlated with the level of Na^+ or Li^+ tolerance. Na^+ does not affect *sod*2 transcription (Jia *et al.*, 1992). Although vacuolar storage of various ions in yeast has been reported, there is no report yet for Na^+.

In the cytoplasmic membrane of various plant cells (Braun *et al.*, 1988; Hassidim *et al.*, 1990; Clint and MacRobbie, 1987; Ratner and Jacoby, 1976; Jacoby and Teomi, 1988; Cooper *et al.*, 1991) a primary H^+ cycle and a Na^+/H^+ antiporter-dependent secondary Na^+ cycle have been implicated from measurements in intact cells and isolated membrane vesicles. Similarly, a secondary Na^+ cycle driven by a H^+/ATPase (Blumwald and Poole, 1985; Blumwald, 1987; Blumwald *et al.*, 1987; Barkla and Blumwald, 1992; Barkla *et al.*, 1990) and possibly also H^+/PPase (Rea and Sanders, 1987) has been demonstrated in the plant vacuole.

The transport of Na^+ from the cytosol, via the tonoplast antiporter, and its accumulation in the vacuole of halophytes and salt-tolerant glycophytes is an important mechanism for averting the damaging affects of Na^+ on key biochemical processes in the cytosol. In certain plants the Na^+/H^+ antiporter at the cytoplasmic membrane excretes Na^+ from the

cells and thus acts synergistically with the tonoplast antiporter in maintaining the cytoplasmic Na^+ concentrations (Hassidim *et al.*, 1990; Barkla and Blumwald, 1992; Jacoby, 1993).

It is apparent that study of the antiporters is of paramount importance to the understanding of the mechanism of salt tolerance in plants.

4. THE ROLE OF Na^+/H^+ ANTIPORTERS IN pH HOMEOSTASIS, FROM *E. Coli* TO PLANT AND ANIMAL CELLS

4.1. All Cells Maintain Intracellular pH Constant

A central role assigned to the antiporter in all cells is in the regulation of intracellular pH (pH_{in}). In *E. coli*, pH_{in} has been shown to be clamped at around 7.5–7.8 despite large changes in the extracellular medium pH (Padan *et al.*, 1976, 1981; Zilberstein *et al.*, 1979; Slonczewski *et al.*, 1981; Booth, 1985; Castle *et al.*, 1986a). When the pH of the external medium is rapidly lowered or raised by over one unit, *E. coli* internal pH shifts slightly, then recovers (Slonczewski *et al.*, 1982; Zilberstein *et al.*, 1984). During anaerobic growth, cells maintain a constant internal pH 7.4 at external pH 6.6–7.0 (Kashket, 1983). Many bacteria as well as eukaryotic cells have since been shown to strictly maintain a constant cytoplasmic pH at around neutrality (Sections 4.2–4.5) and Padan *et al.*, 1976, 1981; Booth, 1985; Pouyssegur *et al.*, 1984; Grinstein *et al.*, 1989; Krulwich, 1986; Pan and Macnab, 1990; Slonczewski *et al.*, 1981; Haussinger, 1988).

Relatively small increases in pH_{in} stop cell division and activate expression of specific genes (Bingham *et al.*, 1990) and of regulons (Schuldiner *et al.*, 1986; Padan and Schuldiner, 1987). It is therefore not surprising that both eukaryotic and prokaryotic cells have evolved several pH_{in} regulative mechanisms to eliminate metabolically induced changes in pH_{in} or to counter extreme environmental conditions (Grinstein, 1988; Booth, 1985; Grinstein *et al.*, 1989; Sardet *et al.*, 1989, 1990; Padan *et al.*, 1981, 1989; Slonczewski, 1992; Olson, 1993).

4.2. Na^+/H^+ Antiporters Acidify the Cytoplasm of Neutrophilic Bacteria for pH Homeostasis at Alkaline pH

The mechanism of pH homeostasis in neutrophilic bacteria, including *E. coli*, have proven remarkably elusive (Padan *et al.*, 1981; Padan and Schuldiner, 1992; Booth, 1985; Padan and Schuldiner, 1986, 1987). A well-documented system is that of *Enterococcus hirae* (*Streptococcus faecalis*), in which the proton-translocating ATPase regulates internal pH by excreting H^+ (Kobayashi *et al.*, 1982, 1984, 1985). However, this cell is limited in its pH range of growth in the absence of carbonate (pH 6.5–7.9) (Kobayashi *et al.*, 1985; Kakinuma, 1987a,b). In *E. coli*, *unc* mutants regulate internal pH normally (Kashket, 1981).

We have proposed that Na^+/H^+ antiporters in conjunction with the primary H^+ pumps are responsible for homeostasis of intracellular pH in *E. coli* at alkaline pH (Padan *et al.*, 1976, 1981); the H^+ expelled by the pumps are recycled and acidify the cytoplasm at alkaline pH to maintain the homeostatic value. This suggestion had its most compelling experimental validation in akaliphilic bacteria, in which it was shown that Na^+ ions are required for acidification of the cytoplasm and for growth (Krulwich *et al.*, 1982, 1985; McLaggan *et al.*, 1984 and Section 4.3). In neutrophiles, such as *E. coli*, there is no direct evidence that supports this contention since it is not clearly established that Na^+ is required for growth at alkaline pH. In some alkaliphiles the requirement for Na^+ is not easy to demonstrate either, presumably due to a very high affinity for Na^+ (as low as 0.5 mM), such that the contamination present in most media suffices to support growth (Sugiyama *et al.*, 1985). McMorrow *et al.* (1989) have taken special precautions to reduce Na^+ to very low levels (5–15 μM) and reported a strict requirement for Na^+ (saturable at 100 μM) for growth of *E.coli* at pH 8.5. This range of concentrations of Na^+ required for growth is well within the range of the K_m of the NhaB system (40–70 μM) (Pinner *et al.*, 1992b and Section 5.2).

Another approach to study the involvement of the Na^+/H^+ antiporters in pH homeostasis is the analysis of the phenotypes of the various antiporter mutants and deduction of the role of the antiporters by a comparison to the wild-type phenotype under different conditions pertaining to Na^+ and H^+.

As long as Na^+ is not added to the medium, Δ*nha*AΔ*nha*B, as well as the two respective single mutants, grow at the entire pH range of growth (Padan *et al.*, 1989; Pinner *et al.*, 1993). Hence, the simplistic assumption that cells lacking the antiporters are pH sensitive has been disproved. Since in the wild-type pH homeostasis exists under all tested conditions

(Padan *et al.*, 1976; Slonczewski *et al.*, 1981), one possibility to explain the mutation phenotypes is that although the antiporters are involved in pH regulation in the wild type, in the absence of Na^+, pH homeostasis is not required. Therefore the mutants lacking the antiporters survive also at alkaline pH, as long as Na^+ is not added. On the other hand, assuming that even in the absence of added Na^+, pH homeostasis is an absolute requirement for growth, these results suggest that either both NhaA and NhaB do not participate in pH homeostasis, or that in their absence *kha*A (Brey *et al.*, 1978; Rosen, 1986) or another, as yet unidentified, system regulates intracellular pH under conditions of low Na^+. A mutation, Hit1, which maps in the same area as *nha*B, has recently been implied to affect pH homeostasis, based on its growth inhibitory effect at alkaline pH (Ishikawa *et al.*, 1987; Thelen *et al.*, 1991). Since a deletion in *nha*B grows at the entire pH range like the wild-type strain, we have concluded that Hit1 contains an additional unidentified mutation, or that *hit*1 modifies NhaB so that the aberrant protein inhibits growth at alkaline pH (Pinner *et al.*, 1993). The role of NhaB in pH regulation is therefore still unclear.

As described above, the addition of Na^+ dramatically affects the growth of Δ*nha*A, and its sensitivity to Na^+ intensifies with increasing pH, implying that NhaA is indispensable under these conditions (Padan *et al.*, 1989). NhaB on its own has a limited capacity to confer Na^+ resistance at alkaline pH (Pinner *et al.*, 1993). These results may suggest that the antiporters are involved at the same time in Na^+ extrusion (see Section 3.1) and in pH regulation, and the importance of both or one of these functions increases when Na^+ and pH increase. The increase in the detrimental effect of Na^+ on Δ*nha*A with pH may be explained by either the observed increase in intracellular Na^+ with pH (Pan and Macnab, 1990) and/or an increase in Na^+ toxicity in increasingly alkalinizing cytoplasm. The possibility that Na^+ may comete for H^+ in the active sites of many essential systems have recently been raised (for review see Padan and Schuldiner, 1992).

If there are alternative mechanisms for pH homeostasis operating in the absence of the antiporters (see above), they may be inefficient in the face of increased Na^+ load at alkaline pH. There is also the last possibility that the antiporters are involved only in Na^+ extrusion, and their role becomes more prominent at alkaline pH for the reasons described above.

Recently, *E. coli* has been shown to grow with negligible protonmotive force, in the presence of CCCP both at alkaline pH (Mugikura *et al.*, 1990; Ohyama *et al.*, 1992) and at neutral pH (Kinoshita *et al.*, 1984). Since the Na^+/H^+ antiporter cannot operate and pH homeostasis is not maintained under these conditions, these results were suggested to imply that neither pH homeostasis nor the operation of the Na^+/H^+ antiporters are essential under these conditions. Since the media used in these experiments contained low Na^+, these results corroborate our results with Δ*nha*AΔ*nha*B conducted in the absence of added Na^+ (see above), suggesting that the mutant phenotype is similar to the energy-uncoupled cells. It is thus apparent that in order to further understand the mechanism of pH homeostasis, its relation to the Na^+ cycle, and the role of the Na^+/H^+ antiporters in these processes, we must directly measure the capacity of pH homeostasis and Na^+ circulation, i.e., pH_{in} and $[Na^+]_{in}$, in the wild type, and the antiporter mutants under various loads of Na^+ and pH stress. These experiments are currently being done in our laboratory.

These data together with the parameters regarding the regulation of activity and expression of the antiporters will eventually permit one to construct a model simulating the integrative activity of the antiporters in the H^+ and Na^+ cycles of the cells. An understanding of these cycles will also provide important clues for our comprehension of the process of adaptation to extreme pH and salt environments.

4.3. Na^+/H^+ Antiporters in pH Homeostasis in Alkaliphiles

In the extreme alkaliphiles a low $\Delta\tilde{\mu}_{H^+}$ is maintained across the membrane, its exact value still debatable (Hoffmann and Dimroth, 1991; Krulwich and Ivey, 1990; Krulwich *et al.*, 1990; Krulwich and Guffanti, 1992). This low $\Delta\tilde{\mu}_{H^+}$ is due to the Na^+/H^+ antiporter which generates a secondary Na^+ cycle with a reversed ΔpH (3 units acidic inside). There is compelling evidence which shows that, in addition to Na^+ extrusion, the antiporter activity has a crucial role in homeostasis of cytoplasmic pH of the extreme alkaliphiles; the resulting secondary Na^+ cycle facing values of extracellular pH up to 11 maintains constant intracellular pH at about pH 8.5 (Krulwich and Guffanti, 1992; Krulwich and Ivey, 1992). Accordingly, although growing at neutral pH, mutants devoid of the antiporter activity lost the capacity to

grow above pH 9 (Krulwich *et al.*, 1979; Koyama *et al.*, 1986; Kitada *et al.*, 1989).

Alkaliphilic cells subjected to an upward shift in pH (pH 8.5 ⇒ 10.5) maintain their cytoplasmic pH at 8.5 if adequate Na^+ is present. Sustained maintenance of pH_i below 8.5 is observed in such experiments if Na^+ and a solute that is co-transported with Na^+ are both present. If, on the other hand, Na^+ is not present during the upward pH shift, the pH_i immediately rises to 10.5. Accordingly, most alkaliphilic Bacillus species can easily be shown to require Na^+ for growth (Krulwich *et al.*, 1982). In some other cases the Na^+ requirement for growth can only be demonstrated if special care is taken to reduce the inevitable contaminating Na^+ (Krulwich *et al.*, 1988).

Whether the antiporters functioning in the alkaliphiles have unusual kinetic properties and stoichiometries await purification and functional reconstitution of the antiporters in proteoliposomes (Section 5). Similarly, study of the physiology and regulation of these antiporters await developing of molecular genetic tools in the alkaliphiles. As yet only very limited progress has been achieved (Kudo *et al.*, 1990).

4.4. Na^+/H^+ Antiporters in pH Homeostasis in Eukaryotic Microorganisms and Plants

The Na^+/H^+ antiporter of *Dunaliella* has been implied in pH homeostasis (Katz *et al.*, 1991, 1992). Weak acid-induced intracellular acidification was used to follow the activity of the plasma membrane Na^+/H^+ antiporter *in vivo*. Monitoring the changes in both intracellular pH and Na^+ and the effect of inhibitors, Katz *et al.* (1991, 1992) showed that the Na^+/H^+ antiporter is involved in intracellular pH homeostasis. Intracellular acidification due to addition of weak acids at appropriate external pH elicited a dramatic increase in intracellular Na^+ which then decreased. Whereas Li^+ inhibited the Na^+ influx phase, vanadate inhibited the Na^+ efflux phase, implying the involvement of Na^+/H^+ antiporter activity in the former process and an ATPase (most probably a Na^+–ATPase) in the latter (Katz *et al.*, 1991).

The effect of different growth conditions on the activity of the Na^+/H^+ antiporter in *Dunaliella* has also been investigated (Katz *et al.*, 1992). Adaptation of the algal cells to ammonia at alkaline pH (interpreted as acidification of the cytoplasm) or to high NaCl concentrations increased the Na^+/H^+ exchange activity of the plasma membrane. The enhanced activity was manifested both *in vivo*, by stimulation of Na^+ influx into intact cells in response to internal acidification, and *in vitro*, by a larger ^{22}Na accumulation in plasma membrane vesicles in response to an induced pH gradient. Kinetic analysis showed that the stimulation does not result from a change of the K_m for Na^+ but rather from an increase in the V_{max}. These results suggest that adaptation to ammonia or to high salinity induce overproduction of the plasma membrane Na^+/H^+ antiporter in *Dunaliella* (Katz *et al.*, 1992), expressing the importance of the antiporter in these adaptations.

4.5. Na^+/H^+ Antiporters Alkalinize the Cytoplasm of Animal Cells for pH Homeostasis

Under physiological conditions the antiporter in cells of higher mammals catalyzes net uptake of Na^+ coupled to efflux of cellular H^+. However, *in vivo*, the system never seems to reach equilibrium and the Na^+ gradient directed inwards (5- to 15-fold) is never balanced by a H^+ gradient directed outwards. The reason the antiporter cannot reach equilibrium seems to be the basis of the mechanism of pH regulation: the protein is kinetically blocked above certain pH values, and the dependence on the concentration of internal H^+ is quite steep, with a Hill constant of more than 2. Aronson *et al.* (1982, 1985) observed that acid loading of membrane vesicles from brush border stimulated rather than inhibited Na^+ efflux, an effect opposite to that expected from competition. They proposed the existence of internal "H^+ modifier" sites at which the operation of the exchanger is modulated. In other words, the protein is poised to function only when the intracellular pH is below a certain homeostatic value. When the physiological set point is reached, the activity is switched off so that the pH does not increase further; upon acidification of the cytoplasm the activity rapidly increases to allow for efficient extrusion of H^+.

5. DO THE PROPERTIES OF THE ANTIPORTERS REFLECT THEIR VARIOUS PHYSIOLOGICAL ROLES?

5.1. Amino Acid Sequence Homology

In view of the occurrence of the antiporters in all cells tested and their central roles in various essential physiological processes, it is tempting to look for

common denominators in them but also for unique properties related to their specific functions.

Sequencing of genes permits a comparison of the deduced amino acid sequences of the putative respective encoded proteins. In many cases such analysis had already yielded most significant information regarding conserved sequences important in function, structure, or assembly of proteins (Ames *et al.*, 1990).

The number of genes encoding putative antiporters is increasing (Table I). Yet it should be emphasized that the only way to prove that a certain gene is the structural gene of a particular antiporter is to purify the protein and show that indeed it is responsible for the function and that the amino acid sequence of the isolated protein is, at least in part, identical with that deduced from the nucleotide sequence of the gene. As yet this has been achieved only with *nha*A antiporter of *E. coli* (Taglicht *et al.*, 1991; Schuldiner and Padan, 1992). The realization that the Na^+/H^+ antiporter activity measured in intact cells and membrane vesicles in *E. coli* is a combined activity of two individual antiporters proteins, NhaA and NhaB, further stresses the importance of the purification and the study of each antiporter separately.

Assuming that the genes listed in Table I indeed encode for Na^+/H^+ antiporters, it is striking that they share very limited homology (Padan and Schuldiner, 1992). The Nhe family shows a relatively strong homology between the various members (Nhe1, 2, 3, and 4 from various sources and β-Nhe from trout). The predicted sequence from a *C. elegans* open reading frame is clearly related to the family, and Sod2 and Nap1 display weak homologies. In the case of NhaA, NhaB, and NhaC the homologies are practically nonexistent. The putative "Na^+ box" formed by conserved amino acids identified in many Na^+ symporting transporters (Reizer *et al.*, 1990; Yamato and Anraku, 1992; Padan and Schuldiner, 1992) was found by site-directed mutagenesis of *nha*A to be dispensable (P. Dibrov, E. Padan, and S. Schuldiner, unpublished data). Certain putative Na^+/H^+ antiporters exhibit interesting homologies to ion binding proteins or other transporters; *cha*A possesses in a short hydrophylic loop a strong sequence similarity to Ca^{2+} binding proteins and the Na^+/Ca^+ antiporter (Ivey *et al.*, 1993; Table I and Section 2.2); NapA shows a weak but significant homology to KefC, a putative K^+/H^+ antiporter in *E. coli* (Reizer *et al.*, 1992). Certainly additional antiporter genes are required to look for functionally meaningful conserved domains, if any, in the Na^+/H^+ antiporters.

5.2. Specificity and Sensitivity to Inhibitors

For an antiporter, the role of which is to excrete Na^+ from the cytoplasm, high specificity to the ion is required to avoid excretion of physiologically important ions such as K^+. Indeed, as shown in Table II, most of the antiporters are specific to Na^+ and Li^+, the ion which physiochemically is very similar to Na^+. However, the plasma membrane plant Na^+/H^+ antiporters have been claimed to recognize also K^+ (Jacoby, 1993). As discussed above, it will be possible to test this suggestion only when these plant antiporters are purified in an active state and reconstituted into proteoliposomes; in membranes existence of K^+/H^+ antiporters or Na^+ leaks may complicate interpretations of the results (see above).

The K_m for Na^+ of the various antiporters ranges between 0.01 and 280 mM and probably reflects the adaptation of each protein to its function. Thus, the Nhe family displays high K_ms (20–60 mM), as expected from their role in recycling sodium from a medium in which its concentration is 150 mM. The K_m of the invertebrate antiporters reflect the concentrations of Na^+ in the prawn hemolymph (220 mM) and lobster blood (470 mM). The K_m of NhaA for Na^+ decreases with an increase in pH as expected from a protein that plays its major role at the alkaline pH range, while NhaB seems to have a constant and high affinity at all pH values. Most interestingly, the K_m for Li^+ is usually lower than Na^+ (Padan *et al.*, 1989; Jia *et al.*, 1992). This may be related to its higher toxicity, implying that, compared to Na^+ a lower Li^+ concentration must be maintained for survival.

Specificity to inhibitors may reflect different antiporter structures. Most prokaryotic Na^+/H^+ antiporters are not sensitive to amiloride derivatives (Schuldiner and Padan, 1992 and Table II). However, the archaebacterial one of methanogens and most of the known eukaryotic Na^+/H^+ antiporters are sensitive to these inhibitors (Table II). In accordance with this similarity as discussed above (Section 3.1), the mode of activity of the methanogenic antiporter(s) under certain metabolic conditions resemble the eukaryotic mode of activity excreting H^+ rather than Na^+.

Chemical modification of a specific amino acid type is a powerful tool to identify residues in a protein, important for activity, stability, and/or

Table II. Properties of Na^+/H^+ Antiporters

Organism	Na^+/H^+ antiporter	Experimental system[a]	Stoichiometry (H^+/Na^+) or electro-genicity (+)	pH sensitivity[b]	Expression	Specificity K_m (mM) Na^+	Specificity K_m (mM) Li^+	Inhibitors and unique properties	Function: Na^+ extrusion	Function: pH homeostasis
Prokaryotes										
E. coli	NhaA + NhaB	Cells[1–3]	1.1 (pH 7.4)–1.4 (pH 8.4)[2,3]						+	Acidification[2,15]
		Membranes (RO, IO)[1,4,5,8,9,62]	+	pH_i 6.5 ⇒ pH_i 9[8,9]		40 (pH 6.8)[c,8,9,21] 3.5 (pH 7.7)[c,e]		DEPC[f,12,13,14]		
	NhaA	Membranes[16,18]	+	pH 7.0 ⇒ pH 8.5	Na^+ dependent[10,11] (increases with pH)	0.6 (pH 8.6)[e] 0.1 (pH 8.6)[d]	0.06 (pH 8)[e,16,18]	DEPC[13] Not inhibited by emiloride		
		Pure protein[6]	2[7]	pH 7 ⇒ pH 8.5[6]						
	NhaB (in ΔnhaA cells)	Membranes[16,18]	+	pH independent[18,19]		0.05–0.25[e,18,20,21]	0.86 (pH 8)[e,18]	Inhibited by amiloride[17]	+	Acidification
		Pure proteins[17]	+							
B. alcalophilus		Cells[22,23]	+	pH_i 7 ⇒ pH_i 9[22]		1[c,22,23]	1[c,22,23]		+	Acidification[23]
		Membranes (RO[24], IO[23])				0.7[c,23,24]	+			
		Crude extract reconstituted[23]	+							
B. firmus RAB		Cells, membranes (RO)[22,23]				15–30[c,22–24]			+	Acidification[23]
Bacillus sp.		Membranes (RO)[25]	+	pH_i 7 ⇒ pH_i 9[25]		2–5[c,25]			+	Acidification[25]
B. firmus OF4	NhaC	In *E. coli* membranes (IO)[26]		pH 8 ⇒ pH 8.5[26]				EIPA, 40 μM[26]		
H. Halobium		Membranes (RO)[27,28,30]	2[27]; 1[28]	pH 4 ⇐ pH 7[c,29]		ΔpH, pH dependent[c,30] (30–300)		DCCD[29,30] EEDQ[31] Voltage activated[30] undirectional outward[30]	+	Acidification
		Crude extract reconstituted[32]						DCCD[32]		

Table II. Continued.

Organism	Na^+/H^+ antiporter	Experimental system[a]	Stoichiometry (Na^+/H^+) or electro-genicity (+)	pH sensitivity[b]	Expression	Specificity K_m (mM)		Inhibitors and unique properties	Function	
						Na^+	Li^+		Na^+ extrusion	pH homeostasis
V. alginolyticus		Cells[63]							+	+
E. hirae	Nap1	Cells[33]	+[33]	pH 7.4 ⇐ pH 8.5[33]	Constitutive[33]				+	
		Membranes (IO)[33,34]		pH 7.4 ⇐ pH 8.5[33]		~ 0.010[d,34]				
Methanosarcina-barkeri		cells[36,37]	1.5[35]			+	+	Amiloride, harmaline[35]	+	Acidification and alkalinization
Methanobacterium thermoauto-trophicum		cells[35]				+	+	Amiloride, harmaline[35]	+	
Eukaryotes										
S. pombe	*sod* 2	cells[38]	1	pH 4.5 ⇐ pH 7	Na^+ does not induce	+	+	Not inhibited by amiloride	+	+
Macrobrachium rosenbergu (freshwater prawn)		Membrane vesicles[52]	$2Na^+/H^+$			82[52]		Amiloride[52] (K_i 50 and 1250 μM)		Gastric acidification[52]
Homarus americanes (marine lobster)		Membrane vesicles[52]	$2Na^+/H^+$ $2Na^+/H^+$			280[52] (epithelia) 310[54] (antennal glands)		Amiloride[52] (K_i 9 and 340 μM) (K_i 14 and 1340 μM)		Gastric acidification[52]
Pynocopodia helianthiodes		Membrane vesicles[53]	$2Na^+/H^+$			120		(K_i 28 and 1250 μM)[53]		
D. salina		Intact cells[39,42]			Na^+ ammonia at alkaline pH[39,42] induce					
		Plasma membrane[40,41]	1	pH 7 ⇒ pH 8.3 ⇐ pH 9[41]		20[c,40,41]	0.03[c,40,41]	Amiloride (25 μM)[40,41]	+	+

		Reconstituted partially purified[41]	+				Li^+ (less inhibited)[41]	Amiloride less inhibited[41]		
Plant plasmalemma		Cells[45,46]			Na^+	+	+		+	+?
		Membranes (IO)[43,44]	1		induced (?)[43,44]	+			+	+?
Plant tonoplast		Intact vacuole[49,50,51]			Na^+ and amiloride induced (?)[47,48]					+?
		Membranes (RO)[43,47,48]				4.5[48]		Amiloride[48]		+?
Higher mammal plasma membrane	Nhe family	Intact cells and membrane vesicles[57–60]	1	pH 6 ⇐ pH 7	Gene amplification upon exposure to acid load	20–60 mM	1 mM	DEPC, NEM,[58] EEDQ, amiloride and various derivatives[59]	—	Alkalinization
Mitochondrial	Na^+/H^+	Membranes[55] and proteo-liposomes[56]	1			30	0.5	Mn^+, Ca^{++}, Mg^{++}, DCCD and timolol		
	K^+/H^+		1	Inhibited by acid pH						
Chromaffin granule		Memrane vesicles[61]	1			20–30		Amiloride		

[a] When known, the membrane orientation is mentioned: RO, right side out membrane vesicles; IO, inside out membrane vesicles.

[b] When the pH sensitivity to either the cytoplasmic or the extracellular face of the membrane is established and the effect of ΔpH excluded, it is designated pH_i or pH_o, respectively. The pH effect is described by an arrow in the direction of increase of activity.

[c] Downhill Na^+ efflux from RO vesicles or cells.

[d] ΔpH-driven Na^+ uptake into IO or proteoliposomes.

[e] Activity measured with acridine orange in IO vesicles.

[f] EIPA, ethylisopropylamiloride; DCCD, *N*, *N'*-dicyclohexylcarbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DEPC, diethylpyrocarbonate, NEM *N*-ethyl maleimide.

References: [1] Borbolla and Rosen, 1984; [2] Macnab and Castle, 1987; [3] Pan and Macnab, 1990; [4] Brey *et al.*, 1978; [5] Schuldiner and Fishkes, 1978; [6] Taglicht *et al.*, 1991; [7] Taglicht *et al.*, 1993; [8] Bassilana *et al.*, 1984a; [9] Bassilana *et al.*, 1984b; [10] Karpel *et al.*, 1991: [11] Rahav-Manor *et al.*, 1992; [12] Leblanc *et al.*, 1988; [13] Taglicht, 1992; [14] Damiano *et al.*, 1985; [15] Padan *et al.*, 1976; [16] Karpel, 1990; [17] Pinner, E., Padan, E. and Schuldiner, S. (unpublished results); [18] Padan *et al.*, 1989; [19] Pinner *et al.*, 1993; [20] Pinner *et al.*, 1992b; [21] Schuldiner and Padan, 1992: [22] Garcia *et al.*, 1983; [23] Krulwich, 1983; [24] Krulwich *et al.*, 1992; [25] Kitada and Horikoshi, 1992; [26] Ivey *et al.*, 1991; [27] Lanyi, 1979; [28] Konishi et al., 1992; [29] Murakami and Konishi, 1989; [30] Murakami and Konishi, 1990; [31] Niwano *et al.*, 1991; [32] Konishi and Murakami, 1990; [33] Kakinuma, 1987b; [34] Waser *et al.*, 1992; [35] Shonheit, 1992; [36] Muller and Gottschalk, 1992; [37] Muller *et al.*, 1987; [38] Jia *et al.*, 1992; [39] Katz *et al.*, 1991; [40] Katz *et al.*, 1986; [41] Katz *et al.*, 1989; [42] Katz *et al.*, 1989; [42] Katz *et al.*, 1992; [43] Hassidim *et al.*, 1990; [44] Braun *et al.*, 1988; [45] Mennen *et al.*, 1990; [46] Jacoby, 1993; [47] Barkla and Blumwald, 1992; [48] Barkla and Blumwald, 1991; [49] Fan *et al.*, 1989; [50] Blumwald and Poole, 1985; [51] Gabarino and Dupont, 1988; [52] Ahearn *et al.*, 1990; [53] Ahearn and Franco, 1991, [54] Ahearn and Franco, 1990; [55] Garlid *et al.*, 1991; [56] Li *et al.*, 1990; [57] Aronson, 1985; [58] Grinstein, 1988; [59] Benos, 1988; [60] Wakabayashi *et al.*, 1992a,b; [61] Haigh and Philips, 1989; [62] Reenstra *et al.*, 1980; [63] Unemoto *et al.*, 1990.

assembly into the membrane. In cloned genes, site-directed mutagenesis is then used to identify the individual amino acid(s) involved. DCCD and its derivatives which specifically modify carboxylic acids inhibit the halobacterial antiporter (Murakami and Konishi, 1989 and Table II) implicating the importance of glutamate and/or aspartate in the function or structure of this interesting antiporter which operates against the highest Na^+ load (see Section 3.2).

DCCD does not affect NhaA but DEPC, which specifically modifies histidine residues, inactivates NhaA. It inhibits the pH-dependent Na^+/H^+ antiporter activity in *E. coli* membrane vesicles (Damiano *et al.*, 1985) and the purified NhaA protein (Taglicht, 1992). With the use of site-directed mutagenesis, it was then shown that out of the eight histidines of NhaA only His226 is important; it forms part of the pH sensor of NhaA (see Section 5.5). It will be most interesting to explore the effect of DEPC on the other pH-dependent antiporters.

5.3. The H^+/Na^+ Stoichiometry

5.3.1. Prokaryotes

The issue of whether the *E. coli* antiporter is electrogenic and what is its actual Na^+ to H^+ stoichiometry has been extensively studied over the years since their discovery (Mitchell, 1961; Mitchell and Moyle, 1967; Harold and Papineau, 1972; West and Mitchell, 1974; Schuldiner and Fishkes, 1978; Beck and Rosen., 1979). This property of the antiporter is crucial not only for the understanding of its molecular mechanism but also for the comprehension of its physiological mode of operation; only an electrogenic antiporter can be driven by a $\Delta\Psi$ without a ΔpH. As we have first shown in *E. coli* in bacteria at alkaline pH, the ΔpH is reversed (acid inside) to maintain intracellular pH constant at the homeostatic value, leaving only $\Delta\Psi$ as a driving force (Padan *et al.*, 1976). We have suggested that an electrogenic Na^+/H^+ antiporter can fulfill this role, using $\Delta\Psi$ to excrete Na^+ and acidify the cytoplasm when needed to maintain constant intracellular pH (Padan *et al.*, 1976; Schuldiner and Fishkes, 1978).

Electrogenicity of an antiporter can be demonstrated in three ways: $\Delta\Psi$-dependent turnover of the antiporter; an increase in rate of the antiporter due to an increase in permeability of a counterion such as K^+ in the presence of valinomycin; creation of a $\Delta\Psi$ via the operation of the antiporter. Whereas the first two approaches have been used in studies using membrane vesicles of many bacteria, the only study in which all three techniques were applied is in the case of purified reconstituted NhaA. As shown in Table II, it is most striking that all prokaryotic antiporters tested thus far are electrogenic, reflecting the importance of the antiporters at alkaline pH in many bacteria. Accordingly, it is interesting to note that the role of *nha*A in halotolerance increases with pH (Padan *et al.*, 1989 and Sections 3.1 and 4.2).

Macnab and colleagues studied the antiporter stoichiometry in *E. coli* (Castle *et al.*, 1986a, b; Macnab and Castle, 1987; Pan and Macnab, 1990). In this careful study, steady-state values of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{Na^+}$ were measured under various conditions in endogenously respiring *E. coli* using $^{23}Na^+$- and ^{31}P-NMR spectroscopy. Na^+ extrusion and maintenance of a low intracellular Na^+ concentration were found to correlate with the development and maintenance of $\Delta\tilde{\mu}_{H^+}$. At pH 6.7 a concentration ratio ($[Na^+]_{out}/[Na^+]_{in}$) of about 25 was observed; this was independent of extracellular Na^+ concentrations over the measured range of 4–285 mM, indicating that intracellular Na^+ concentration is not regulated. When the gradients were measured at various pH values, it was found that in the acidic to neutral pH range the Na^+ chemical potential followed the proton chemical potential quite closely, always exceeding it slightly. Above pH 7.4, there was a progressive divergence between the two values. Thus, whereas the ΔpH continued to decrease, reached zero at pH 7.5, and changed signs (pH_{in} becoming more acidic than pH_{out}), ΔpNa [$\Delta pNa = (-\log[Na^+]_{in}/[Na^+]_{out})$] practically leveled off at a value of 25–40 mV, corresponding to a Na^+ concentration gradient of 2.5- to 5-fold at the alkaline pH values. As a consequence, the apparent overall stoichiometry changes from 1.1 at $pH_{out} = 6.5$ to 1.4 at $pH_{out} = 8.5$ (Pan and Macnab, 1990).

It was suggested that this change in apparent overall stoichiometry might reflect a change in the relative rates of two antiporters with different stoichiometries rather than a change in stoichiometry of a single protein (Macnab and Castle, 1987). Our studies indeed show that there are two antiporters and that both of them are electrogenic (Taglicht *et al.*, 1993; E. Pinner, E. Padan, and S. Schuldiner, unpublished results).

Determination of the rates of H^+ and Na^+ fluxes in proteoliposomes bearing pure NhaA permitted the calculation of the H^+/Na^+ stoichiometry of NhaA

(Taglicht *et al.*, 1993). H^+ movements were followed by the pH indicator pyranine, and Na^+ movements by monitoring either $^{22}Na^+$ or changes in fluorescence of a novel sodium indicator, SBFI (sodium-binding benzofuran isophtalate). Some of the problems which we faced in these experiments illustrated important properties of the antiporter: Measurable movements of Na^+ could be detected only under conditions in which the formation of $\Delta\tilde{\mu}_{H^+}$ by the antiporter was prevented or when performed gradients were collapsed. Thus, proteoliposomes loaded with Na^+ lose very small amounts of ion, while a pH gradient is formed and reaches its maximum value as soon as it can be measured. Addition of 10 mM methylamine transiently alkalinizes the internal milieu and allows for exit of some of the Na^+ ions. Addition of three identical aliquots of methylamine is necessary to release most of the internal Na^+. If the generation of $\Delta\tilde{\mu}_{H^+}$ is prevented by doing the experiment in the presence of potassium acetate and valinomycin, half of the internal Na^+ is lost after about 60 s (Taglicht *et al.*, 1993). A stoichiometry of about 2 was estimated for the NhaA antiporter in these experiments.

A stoichiometry of $2H^+/Na^+$ was estimated also in experiments using a thermodynamic rather than a kinetic approach. The size of the $\Delta\tilde{\mu}_{H^+}$ generated by a Na^+ gradient could be predicted from the equilibrium equation for two substrates whose transport is completely coupled:

$$\Delta\tilde{\mu}_{Na^+} = n\Delta\tilde{\mu}_{H^+}$$

where n is the stoichiometry (H^+/Na^+), meaning that

$$\Delta pNa^+ = n\Delta pH + (n-1)\Delta\Psi$$

We measured with Oxonol VI the size of the $\Delta\Psi$ generated at various ΔpNa^+, in the presence of nigericin, which allowed for an electroneutral exchange of K^+ and H^+ and thereby discharges the ΔpH. The magnitude of the $\Delta\Psi$ generated at various pH values (7.1–8.2) was consistent with a stoichiometry of 2 (Taglicht *et al.*, 1993).

Our results indicate that the apparent changes in stoichiometry measured in the intact cell (Castle *et al.*, 1986a, b; Pan and Macnab, 1990) and membrane vesicles (Schuldiner and Fishkes, 1978) at alkaline pH are not due to a change in stoichiometry of NhaA, but rather, as predicted (Macnab and Castle, 1987), to its relative contribution to the Na^+ cycle. We are currently measuring the H^+/Na^+ stoichiometry of purified NhaB, a value which will be most important for the simulation of the integrative activity of the antiporters in *E. coli*.

5.3.2. Stoichiometry in Eukaryotic Na^+/H^+ Antiporters

Thus far, electrogenicity has been found in Na^+/H^+ antiporters of the lower eukaryotes including invertebrates (Table II).

The Na^+/H^+ antiporter of *D. salina* was found to be electroneutral when measured in membrane vesicle (Katz *et al.*, 1986), but electrogenic when the activity of the partially purified antiporter was assayed in proteoliposomes (Katz *et al.*, 1989). This discrepancy is instructive since it may imply that ionic leaks in membrane preparations may preclude the demonstration of the rheogenic property of the antiporter. Proteoliposomes reconstituted with pure protein are tight with respect to ions (Taglicht *et al.*, 1991, 1993) and therefore are preferable for demonstration of electrogenicity. However, none of the eukaryotic antiporters have as yet been purified. The possibility that the antiporter may change its properties in the new matrix cannot be excluded.

All other eukaryotic Na^+/H^+ antiporters, whether localized in organelles or the plasma membrane of yeast plants or animals, appear electroneutral (Table II). It is therefore tempting to suggest that this property of the antiporter reflects different modes of activity in higher mammals as compared to the prokaryotic type. This certainly appears to be the case with the Na^+/H^+ antiporter operating in membranes which are energized by a Na^+/K^+ ATPase. These antiporters utilizing ΔpNa^+ are involved in H^+ extrusion rather than Na^+ extrusion which is conducted by the pumps, and play a major role in pH homeostasis at the acidic to neutral pH range (Section 4.5). Interestingly, the methanogenic antiporter which under certain conditions acquires this eukaryotic mode of operation is sensitive to amiloride-derived inhibitors like the eukaryotic counterparts (Section 5.2). Nevertheless very little homology was found between *sod*2 and *nhe*1, two genes which are supposed to encode electroneutral Na^+/H^+ antiporters (Section 5.1). The former antiporter is also not inhibited by amiloride (Jia *et al.*, 1992 and Table II).

5.4. Regulation of the Antiporter Expression

Since the Na^+/H^+ antiporters couple two major ion cycles which are involved in bioenergetics and

very basic regulatory processes in cells, it is expected that these transport systems will be regulated. The necessity of regulation is also implicit in the example of *E. coli* in which two antiporters NhaA and NhaB are engaged in the Na^+/H^+ antiporter activity of the cell.

Thus far, regulation of expression has only been studied with *nha*A of *E. coli*. In the *nha* gene we have mapped two promoters by primer extension in the 5′ upstream region (Karpel *et al.*, 1991). In addition, a quite extensive putative secondary structure in the RNA has been predicted in the 5′ end of the gene (Karpel *et al.*, 1988), and the first codon in GTG rather than ATG (Taglicht *et al.*, 1991). GTG has been found to mediate initiation of translation in about 8% of the documented *E. coli* proteins (Gold and Stromo, 1987), and it has been suggested that it may be used in mRNA's that are poorly translated. Also the codon usage in *nha*A is typical for poorly expressed proteins (Pinner *et al.*, 1992a). We estimate that under the growth conditions which are standard in our laboratory (L broth adjusted to pH 7.5 in which the Na^+ is replaced with K^+ and the contamination levels of Na^+ are around 10 mM, or minimal salt medium to which sodium is not added), NhaA is a minor component of the membrane [less than 0.2%, or an equivalent of less than 500 copies per cell (Taglicht *et al.*, 1991)]. Expression with an exogenous promoter (*tac*) is much higher when the regulatory sequences of *nha*A are deleted (Taglicht *et al.*, 1991), implying that, at least under some conditions, the upstream region has an inhibitory effect on expression of *nha*A.

We have constructed a chromosomal translation fusion between *nha*A and *lac*Z (*nha*A′–′ *lac*Z) and have found that the levels of expression are very low unless Na^+ or Li^+ are added. Na^+ and Li^+ ions increase expression in a time- and concentration-dependent manner (Karpel *et al.*, 1991): maximum increase is detected when the cells are exposed to 50–100 mM of either ion for a period of 2 h. The effect is specific to the nature of the cation and is not related to a change in osmolarity. Alkaline pH potentiates the effect of the ions. The pattern of regulation of *nha*A thus reflects its role in adaptation to high salinity and alkaline pH in *E.coli* (Padan *et al.*, 1989; Padan and Schuldiner, 1992). This pattern also suggests the possibility of involvement of novel regulatory genes in addition to *nha*A and *nha*B.

A gene downstream of *nha*A, *nha*R [previously known as *ant*O (Henikoff *et al.*, 1988), or 28-kDa protein (Mackie, 1986)], is proposed to play a role in the regulation of *nha*A. In addition to its proximity to *nha*A, and due to the fact that there are no conspicuous consensus sequences of either terminators or promoters between the two genes, it is likely that *nha*R and *nha*A form an operon. Expression with foreign promoters cloned upstream of *nha*A brings about expression of *nha*A as well as *nha*R (Karpel *et al.*, 1988).

A multiple dose of *nha*R enhances the Na^+-dependent induction of the *nha*A′–′ *lac*Z fusion. The fact that the dose level affects the induction by Na^+, but not the basic level of expression, suggests that the Na^+ induction involves *nha*R either directly or indirectly. NhaR exerts its effect in *trans*, as shown in the latter experiments. Furthermore, extracts derived from cells overexpressing *nha*R exhibit DNA binding capacity specific to the upstream sequences of *nha*A, as observed by gel retardation assays (Rahav-Manor *et al.*, 1992). Inactivation of the chromosomal *nha*R by insertion unveils a phenotype of sensitivity to Li^+ higher than that displayed by the wild type (Rahav-Manor *et al.*, 1992). A change of tolerance toward Na^+ in these cells becomes apparent only at pH 8.5, under conditions in which the load seems to be more pronounced, as suggested by the phenotype of the Δ*nha*A strain and by the pattern of regulation of *nha*A. Both phenotypes are corrected by *nha*R in *trans* (Rahav-Manor *et al.*, 1992).

On the basis of the latter results, it is proposed that NhaR is a positive regulator of *nha*A. This suggestion is in accordance with the fact that NhaR belongs to the OxyR-LysR family of positive regulators first described by Henikoff *et al.* (1988) and also studied by Christman *et al.* (1989). All the proteins in this group have in their N-terminus a conserved helix-turn-helix domain which is supposed to bind to DNA. Several of these proteins are involved in the response of the organism to stress, such as, for example, OxyR, which is essential for the resistance of the organism to oxidative stress (Storz *et al.*, 1990).

We therefore suggest that NhaR and its effector *nha*A are the first example of a signal transduction of a specific adaptation to Na^+ and possibly also to alkaline stress, unrelated to stress of osmolarity or ionic strength. Indeed, extending our studies to extreme stress conditions of pH and Na^+, under which the cells do not divide but survive, we see that in the absence of NaCl, the wild type stops growing at pH 8.7 and lyses at pH 9.6. Between pH 8.8–9.6, it survives for periods which depend on the time of exposure, as well as the combination of the pH and Na^+

stresses. The higher the pH, the higher the sensitivity to salt concentration. The reason for the extreme stress caused by the combination of alkaline pH and Na^+ is not clear. However, NhaA and its Na^+-induced regulation via *nha*R are essential for survival under this extreme Na^+/pH stress (Padan *et al.*, 1989; Karpel *et al.*, 1991; Rahav-Manor *et al.*, 1992).

The molecular details of the mechanism by which cells sense environmental adversity and then transduce the stress signal into a change in gene expression is known only for a limited number of responses: MerR, a regulator of mercury resistance in *E. coli*, is activated to induce the expression of mercuric reductase upon binding mercury (Shewchuk *et al.*, 1989; Helmann *et al.*, 1989); OxyR, a regulator of genes involvled in resistance to oxidative stress, transduces an oxidative stress signal to RNA polymerase upon its own oxidation (Storz *et al.*, 1990). For many other environmental stresses including heat, the transcriptional regulator has been characterized, but little is known about how the environmental signal is transmitted to it (Storz *et al.*, 1990; Straus *et al.*, 1987).

A large body of evidence suggests that the immediate signal for induction of *nha*A is the intracellular, rather than the extracellular, level of Na^+ (Padan and Schuldiner, 1992). Thus, the effectiveness of extracellular Na^+ ($[Na^+]_{out}$) in inducing *nha*A increases under conditions at which the intracellular concentration ($[Na^+]_{in}$) rises [at alkaline pH (Pan and Macnab, 1990), in antiporter defective strains (Padan and Schuldiner, 1992), or upon addition of uncouplers (Y. Kotler, A. Rimon, S. Schuldiner and E. Padan, unpublished results)]. In addition, large variations in $[Na^+]_{out}$ have practically no effect on *nha*A induction in strains carrying either multicopy *nha*A or *nha*B (Padan and Schuldiner, 1992; Rahav-Manor *et al.*, 1992) and which display a higher extrusion capability.

Thus, our working hypothesis is that a change in extracellular Na^+, the extracellular signal, is conveyed into the cell as a change in the intracellular Na^+. NhaR itself transduces this information and induces expression of *nha*A. It will be most intriguing to delineate this novel signal transduction pathway which is essential for halotolerance.

Although in eukaryotes regulation of expression of the antiporters has not as yet been demonstrated, there are hints for the existence of such regulation. In the halophilic alga *Dunaliella* an increase in Na^+ concentration in the medium and presence of ammonia which is supposed to acidify the cytoplasm lead to an increased Na^+/H^+ antiporter activity of the cytoplasmic membrane (Katz *et al.*, 1992).

Interestingly, in yeast, although Na^+ does not induce *sod*2, an increase in Li^+ or Na^+ concentration leads to amplification of the *sod*2 gene and the resulting increase in Na^+/H^+ antiporter activity (Jia *et al.*, 1992).

Mouse fibroblast cell lines acid loaded with a NH_4^+ prepulse technique and exposed to a Na^+-containing medium rapidly extrude H^+ to the medium. However, if H^+ efflux is slowed down by lowering the concentration of Na^+ or by adding an amiloride analogue, the cells do not survive unless they overexpress the exchanger or have a mutated exchanger with lower affinity for the amiloride analogue (Franchi *et al.*, 1986a,b; Pouyssegur *et al.*, 1984). The molecular basis of the overexpression has not been characterized.

5.5. Regulation of Activity, the pH sensors

A crucial role in pH homeostasis has been implied for the antiporters in all cells (Section 4). It is thus most significant that one of the most prominent characteristics of the Na^+/H^+ antiporters is a remarkable pH sensitivity of their activity (Table II). In fact, among the many antiporters tested only NhaB is pH independent (Padan *et al.*, 1989; Pinner *et al.*, 1992b and Table II).

In prokaryotes the pH sensitivity of the antiporter has been demonstrated in membrane vesicles (Bassilana *et al.*, 1984a, b; Leblanc *et al.*, 1988) and purified antiporter in proteoliposomes (Taglicht *et al.*, 1991). Since a change in pH of the experimental medium usually results in a change in ΔpH, an effect of the latter parameter must be excluded before a pure pH effect is concluded. To avoid this complication, the pH sensitivity of NhaA has been measured in pure NhaA-proteoliposomes in which the ΔpH was collapsed by the presence of a permeant acid and the $\Delta\Psi$ by the presence of valinomycin and K^+. The effect of pH on efflux of $^{22}Na^+$-loaded proteoliposomes was measured and shown to cause a three orders of magnitude difference in the activity of NhaA between pH 7–8.5. This effect of pH on pure NhaA activity is therefore a purely kinetic effect since no H^+ ion gradient is generated under the conditions tested.

In these experiments, however, it is not possible to determine at which side of the membrane the pH exerts its effect on NhaA. Changing the intravesicular pH of right side out vesicles at various extravesicular pH values, Leblanc and his colleagues (Bassilana

et al., 1984a) deduced that the pH-sensitive site of the *E. coli* Na^+/H^+ antiporter activity of vesicles is at the cytoplasmic face of the membrane. The effect of pH on everted membrane vesicles isolated from Δ*nha*AΔ*nha*B mutant bearing multicopy plasmid with *nha*A corroborate these results (Padan *et al.*, 1989).

In eukaryotes as yet the pH sensitivity of the antiporter has been shown only in membrane vesicles and intact cells. Acidification of the cytoplasm in *D. salina* by application of ammonia increased the Na^+/H^+ antiporter activity (Katz *et al.*, 1991). The effect of pH on the antiporter activity of isolated membrane vesicles corroborated these results (Katz *et al.*, 1989). As described above, the antiporter from higher mammals is regulated by H^+ modifier sites. A wide variety of external signals, including growth factors, hormones, neurotransmitters, lectins, phorbol esters, and sperm, shift the set point of the Nhe antiporters to more alkaline values and thereby allow for a higher activity and eventually increase of intracellular pH (Schuldiner and Rozengurt, 1982; Moolenaar, 1986; Grinstein *et al.*, 1989). It has been proposed that this stimulation is associated with activation of protein kinase activity, and phosphorylation of serine residues on the protein has been demonstrated directly (Sardet *et al.*, 1990, 1991a, b).

Removal of the cytoplasmic hydrophylic C-terminus domain preserved Na^+/H^+ exchange activity while abolishing the exchanger activation in response to external signals (Wakabayashi *et al.*, 1992b). It was suggested that one of the eight serine residues in this domain is phosphorylated, but mutation of each of the residues to alanine did not abolish growth factor activation (Wakabayashi *et al.*, 1992a). It is still possible, however, that phosphorylation at multiple serine residues is required for mitogen activation.

Although phosphorylation plays a key role in mitogen-induced activation, a phosphorylation-independent mechanism has been demonstrated upon activation during cell volume regulation. The effect of hyperosmolarity on the exchanger is also manifested as an alkaline shift in the pH_i dependence of the modifier site. However, this change seems to be induced by a different process (Grinstein *et al.*, 1992).

We have suggested that the steep pH dependence of NhaA defines a "set point" for the activity such that NhaA is practically inactive at pH values below the intracellular homeostatic one (7.6–7.8). When the pH increases, the antiporter is activated so that it can acidify the cytoplasm back to the "resting pH_{in}" in a self-regulated mechanism. This idea of a molecular pH meter and titrator in the same molecule seems to be quite a successful one since it was chosen also by completely different molecules: the animal Na^+/H^+ antiporter (Aronson, 1985) and the nonerythroid Cl/HCO_3 exchanger (Olsnes *et al.*, 1986, 1987; Raley-Susman *et al.*, 1991). Whether the set point of NhaA or the Cl/HCO_3 exchanger is regulated or modulated by physiological factors as is Nhe is not known.

The identification of the "pH sensor" on such pH-regulated proteins and the study of whether this H^+-sensing and the ion-transporting sites are identical, overlapping, or different are most intriguing. It is conceivable that residues involved in pH sensing or H^+ transport undergo protonation at the physiological pH range of activity. Histidines (pK 6.0 in solution) are likely candidates for such a role and they have also been implicated in the mechanism of H^+ transport in the lactose carrier (Padan *et al.*, 1985; Kaback, 1988) in the photosynthetic reaction center (Okamura and Feher, 1992), and in the Na^+/H^+ antiporter activity of *E. coli* (Damiano *et al.*, 1985 and Section 5.2).

We therefore deleted or mutated by site-directed mutagenesis the histidines of NhaA. We found that none of the eight histidines of NhaA are necessary for activity, while His-226 is required for the response of the protein to pH (Gerchman *et al.*, 1993). As revealed by analysis of the Na^+/H^+ antiporter activity of membrane vesicles, the replacement of His-226 by Arg markedly changes the pH dependence of the antiporter. Whereas the activation of the wild-type NhaA occurs between pH 7 and 8, that of H226R antiporter occurs to the same extent but between pH 6.5 and 7.5. Furthermore, while the wild-type antiporter remains fully active at least up to pH 8.5, H226R is reversibly inactivated above pH 7.5, reaching 10–20% of the maximal activity at pH 8.5. We suggest that His-226 is part of a "pH sensor" or modifies it in some way (Gerchman *et al.*, 1993). In any case, it is most likely that other amino acids are involved in this sensor since, albeit abnormally, the mutated protein still reacts to pH. If protonation of His-226 or Arg-226 is involved in the reactivity of the protein to pH, we must conclude that either both or one of them has a pK in the protein which is different from its pH in solution.

Most importantly, the NhaA "pH sensor" is physiologically essential; similar to the wild-type gene, all mutants except H226R confer Na^+ resist-

ance up to pH 8.5 as well as Li^+ resistance. On the other hand, H226R cannot grow at alkaline pH in the presence of Na^+, but is as competent as the wild type at the neutral or acidic pH range (Gerchman *et al.*, 1993).

ACKNOWLEDGMENTS

The research in the authors' laboratory was supported by grants from the US-Israel Binational Science Foundation: Gesellschaft für biotechnologische forschung, GBF; Ministry of Science and Technology; Association Franco-Israelienne pour la Recherche Scientifique et Technologique (AFIRST); and the Moshe Shilo Center for Marine Biogeochemistry.

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