The Nha1 antiporter of *Saccharomyces cerevisiae* mediates sodium and potassium efflux

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The *NHA1* gene of *Saccharomyces cerevisiae*, transcribed into a 3.5 kb mRNA, encodes a protein mediating Na⁺ and K⁺ efflux through the plasma membrane that is required for alkali cation tolerance at acidic pH. Deletion of the gene in a wild-type strain resulted in higher sensitivity to both K⁺ and Na⁺ at acidic pH. Measurements of cation loss in strains carrying deleted or overexpressed alleles of *NHA1* demonstrated its role in K⁺ and Na⁺ efflux. In addition, high K⁺ and Na⁺ efflux observed upon alkalization of the cytoplasm implies a role of Nha1p in the regulation of intracellular pH. Moreover, the overexpression of *ENA1* and *NHA1* genes in an *ena1-4Δ-nha1Δ* strain showed that the Nha1 alkali-cation antiporter is responsible for growth on high concentrations of KCl and NaCl at acidic pH, and Ena alkali-cation ATPases are necessary at higher pH values. Both systems have a complementary action to maintain the intracellular steady-state concentration of K⁺ and Na⁺.

Keywords: K⁺/H⁺ antiporter, Na⁺/H⁺ antiporter, salt tolerance, yeast

INTRODUCTION

The most abundant cation in *Saccharomyces cerevisiae* cells, as well as in most other eukaryotic and prokaryotic organisms, is K⁺, which is involved in numerous enzymic activities. In contrast, a high intracellular concentration of Na⁺ ions is toxic for most organisms, including yeast. Thus, organisms which lack the capacity to escape the environmental stress of increased NaCl concentrations, for example plants and fungi, must possess very efficient mechanisms for controlling the optimal intracellular concentrations of this cation. As K⁺ is capable of preventing most intracellular Na⁺ inhibitory effects, it seems clear that the intracellular K⁺/Na⁺ ratio, which is proportional to net uptakes, i.e. differences between influx and efflux, plays a crucial role in the maintenance of cellular ionic homeostasis and reducing Na⁺ toxicity (Haro *et al.*, 1991; Gómez *et al.*, 1996; Rios *et al.*, 1997).

An extensive study of potassium uptake in *Sacch. cerevisiae* revealed at least two genes which are thought to be components of a complex K⁺ transport system or independent potassium transporters: *TRK1* and *TRK2* (Ko & Gaber, 1991; Ramos *et al.*, 1994). In addition, a

K⁺ uptake channel has been detected in *Sacch. cerevisiae* by patch-clamp experiments (Bertl *et al.*, 1995), but not yet genetically characterized. Less is known about potassium efflux, which is also an important component of ionic homeostasis. In *Sacch. cerevisiae*, about two-thirds of the K⁺ taken up is returned to the external medium (Rodríguez-Navarro & Ramos, 1984; Ortega & Rodríguez-Navarro, 1985). In depolarized *Sacch. cerevisiae*, potassium efflux is mediated by a channel encoded by a gene identified in the yeast sequencing programme (André, 1995) and characterized as *TOK1* (Ketchum *et al.*, 1995) or *YCK1* (Zhou *et al.*, 1995) by patch-clamp analyses in *Xenopus* oocytes and yeast, respectively. In addition, several reports have described in *Sacch. cerevisiae* an active K⁺ efflux mediated by a K⁺/H⁺ antiporter (Ortega & Rodríguez-Navarro, 1985; Peña & Ramírez, 1991; Camarasa *et al.*, 1996; Ramírez *et al.*, 1996), but the gene(s) responsible has not yet been identified. Finally, it must be noted that the *ENA1* gene involved in Na⁺ efflux (see below) could also be involved in K⁺ efflux, as has been suggested after characterization of *ena1-4* mutants (Haro *et al.*, 1991) and in a biochemical study of Ena1p phosphorylation (Benito *et al.*, 1997).

Concerning Na⁺, under non-stressed conditions, the amount of Na⁺ entering cells is very low. Upon salt

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stress this cation is believed to be taken up with low affinity by systems involved in transport of K^+ and other ions. To prevent toxic effects, Na^+ must be in turn efficiently extruded. The main system eliminating Na^+ ions from *Sacch. cerevisiae* cells is a Na^+ ATPase encoded by the *ENA1* gene allelic to *PMR2* (Haro *et al.*, 1991; Wieland *et al.*, 1995). In most *Sacch. cerevisiae* strains, *ENA1* is the first unit of a tandem array of four or five genes, depending on the strain (Garcia-deblás *et al.*, 1993; Wieland *et al.*, 1995). *ENA2*, *ENA3* and *ENA4* are expressed constitutively at low levels and *ENA1* is induced by Na^+ , Li^+ and high pH values (Garcia-deblás *et al.*, 1993).

Considering that *Ena1p* is active mainly at alkaline pH values, a putative Na^+/H^+ antiporter active at acidic pH has been postulated (Rodríguez-Navarro & Ortega, 1982). This hypothesis was strengthened by two other observations: (i) the small residual Na^+ efflux observed in *ena1-4* mutants (Haro *et al.*, 1991) and (ii) the characterization of Na^+/H^+ antiporters in other yeasts. In *Schizosaccharomyces pombe*, the efflux of intracellular Na^+ is mediated by a Na^+/H^+ antiporter encoded by the *sod2* gene (Jia *et al.*, 1992). This protein shows some similarity to the human and bacterial Na^+/H^+ antiporters, and extensive homology to the product of the *Zygosaccharomyces rouxii* *Z-SOD2* gene (Watanabe *et al.*, 1995). In both yeasts, these antiporters are probably major systems for eliminating toxic Na^+ cations from cells as the Na^+ tolerance observed depends on the level of *sod2* (*Z-SOD2*) expression. Recently, we have selected a Na^+ -resistant clone containing a truncated allele of the ORF YLR138w. Since the corresponding putative protein was highly similar to *Schiz. pombe* and *Z. rouxii* antiporters, we proposed that the cloned locus encoded a putative Na^+/H^+ antiporter and designated it *NHA1* (Prior *et al.*, 1996).

This paper describes a functional study of the *NHA1* gene. In the first part, we report the cloning of the complete *NHA1* gene, characterization of its mRNA and attempts to increase its expression. In the second part, Western blots using a tagged *Nha1p* suggest that the protein is localized in the plasma membrane. In addition, the phenotypes of different strains with disrupted or overexpressed *NHA1* were determined and evidence for a pH-dependent role of *Nha1p* in Na^+ and K^+ tolerance is presented. Finally, results of Na^+ and K^+ efflux measurements demonstrate that, as previously assumed, the *NHA1* gene product is not only a Na^+/H^+ antiporter but is also an efficient K^+/H^+ antiporter.

METHODS

Strains and media. All *Sacch. cerevisiae* strains used in this work were derivatives of W303.1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100*) and W303.1B (*MATα*) (Wallis *et al.*, 1989): G19 (*MATα*) containing the deletion *ena1Δ::HIS3::ena4Δ* (Bañuelos *et al.*, 1995); B31 (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2*) derived from G19 by substituting the 1.0 kb *SnaBI-HincII* fragment of *NHA1* by the *LEU2* gene (a gift from A. Rodríguez-Navarro, Polytechnic University of Madrid, Spain); and C25 (*MATα*-

Fig. 1. Restriction map of the *NHA1* locus in chromosome XII of *Sacch. cerevisiae* and in the pCS1 plasmid. Arrows with numbers correspond to oligonucleotides used for PCR amplifications (see Methods).

nha1Δ::LEU2), obtained by crossing strains W303.1A and B31. In all experiments, strains were transformed either with appropriate 'empty' vector or with one of the plasmids described below. The strains were routinely grown in YNB minimal medium (2% glucose, 0.7% Bacto yeast nitrogen base without amino acids and nutritional requirements), or in complex YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. The K^+ - and Na^+ -free minimal liquid medium used in this study was arginine phosphate medium (AP) (Rodríguez-Navarro & Ramos, 1984) supplemented with KCl and NaCl as indicated.

Plasmids. To construct all the plasmids listed below we used the original pCS1 and two DNA fragments obtained by PCR. The complete *NHA1* ORF was amplified using primers 1 (5' GCTCTAGATATTATGGCTATCTG 3') and 2 (5' GCTC-TAGAAATTACTTATTGAGACCAAG 3'), and a DNA fragment corresponding to the last 2098 nucleotides of the truncated ORF was obtained with primers 3 (5' GGTTA-TGTAGGCAGAATCAC 3') and 4 (5' CCATCGATCTAA-GGAATCTTGAAC 3') (Fig. 1). Both DNA fragments were first cloned in pBluescript (Short *et al.*, 1988) and sequenced. The plasmids were as follows: pSCMCY, complete *NHA1* gene with its own promoter and *CYC1* terminator in YEp352 (Hill *et al.*, 1986); pCSCY, truncated *NHA1* gene with its own promoter and *CYC1* terminator in YEp352; pBS2, complete *NHA1* gene with its own promoter and *CYC1* terminator in a single-copy vector derived from p416ADH (Mumberg *et al.*, 1995); pMA52, complete *NHA1* gene with *PGK1* promoter and *PGK1* terminator in pYPGE15 (Brunelli & Pall, 1993); pMAADH426, complete *NHA1* gene with *ADH1* promoter and *CYC1* terminator in p426ADH (Mumberg *et al.*, 1995); pMAGPD426, complete *NHA1* gene with *GPD1* promoter and *CYC1* terminator in p426GPD (Mumberg *et al.*, 1995). For epitope tagging of *NHA1*, a *SmaI* site was introduced by PCR before its stop codon using oligonucleotides 3 and 5 (5' TATAGCATGCTAATCCCGGCAACTCCTCTCGGTG-GCGCAGG 3') for the truncated version and oligonucleotides 3 and 6 (5' TATAGCATGCTAATCCCGGGACCAAGCG-TTTTGTAGTCGC 3') for the complete *NHA1* version (Fig. 1). The following plasmids were then constructed: pCSEmyc, (truncated *NHA1* gene with its own promoter and *c-myc* epitope in its C terminus in YEpmc181 (Reisdorf *et al.*, 1993); pCSMEmyc, complete *NHA1* gene with its own promoter and *c-myc* epitope in its C terminus in YEpmc181; pGB34, *ENA1* gene with its own promoter in a single-copy vector, YCp50 (Haro *et al.*, 1993).

Salt tolerance determination. The growth capacity of yeast strains in the presence of NaCl or KCl was tested on solid YPD or YNB media inoculated with serial 10-fold dilutions of saturated cultures. Growth was recorded after 4 d. At the concentrations reported in Tables 1 and 2, no appreciable growth was observed. When growth was tested at pH 6.6 or 7.0, media were supplemented with 20 mM HEPES. To attain pH 5.5, HCl was added to the media, and for pH 3.6 tartaric acid was used after autoclaving.

Cation loading of cells. For slow K⁺ or Na⁺ loading, cells were grown overnight in AP medium pH 5.5 supplemented with the concentrations of KCl and NaCl. For rapid Na⁺ loading, cells grown in AP were transferred to 10 mM MES buffer adjusted to pH 5.5 with Ca(OH)₂ containing 2% glucose, 0.1 mM MgCl₂, 1 mM KCl and 50 mM NaCl and incubated in this medium for 10 min at 30 °C.

Cation contents and loss. Samples of cells were withdrawn from the incubation mixtures at various time intervals, cells were collected on Millipore membrane filters, rapidly washed with a 20 mM MgCl₂ solution, acid-extracted and analysed by atomic emission spectrophotometry as described by Rodríguez-Navarro & Ramos (1984) and Haro *et al.* (1991). Measurements of Na⁺ or K⁺ loss were carried out in incubation buffer consisting of either 10 mM MES adjusted to pH 5.5 with Ca(OH)₂, or 10 mM TAPS [N-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid] adjusted to pH 8.0 with Ca(OH)₂. In both cases buffers contained 0.1 mM MgCl₂ and 2% glucose and were supplemented with KCl, RbCl or NH₄Cl as indicated. Results are reported as the means of at least four independent experiments. The standard errors of the means were 20% lower than the corresponding mean.

DNA and RNA manipulation. Manipulation of nucleic acids was done by standard protocols (Sambrook *et al.*, 1989) or, where appropriate, following the manufacturer's instructions. For Northern blots, total RNA was extracted as described by Carlson & Botstein (1982) and the probe was prepared as an *in vitro* run-off transcript (Quillet *et al.*, 1989).

Western blot analysis. Yeast cells in the exponential phase of growth were harvested by centrifugation. Extracts from whole cells and plasma membranes were prepared and immunoblotting performed as described previously (Matejcková & Sychrová, 1997). The primary antibody, monoclonal anti-c-myc 9E10 (1:250, Santa Cruz Biotechnology) was detected by chemiluminescence (ECL, Amersham) using HRP-conjugated anti-mouse IgG secondary antibody (1:2500, Sigma).

RESULTS

Cloning of the complete *NHA1* gene and its expression in different vectors

The original plasmid (pCS1) selected by complementation of Na⁺ sensitivity of the strain HS100-3C (Prior *et al.*, 1996) contained a truncated allele of *NHA1* in which the 3' terminal 294 bp of the coding sequence were missing, according to the GenBank/EMBL nucleotide sequence database (Fig. 1). In addition, the insert was a chimera, containing two short fragments from other chromosomes at both ends of the *NHA1* insert. To study gene expression and to establish the role of the gene product, we amplified by PCR both truncated and complete *NHA1* ORFs encoding proteins of 888 and 985

amino acid residues, respectively. The two DNA fragments were cloned with the *NHA1*'s own putative promoter region (0.7 kb) in the multicopy vector YEp352 (see Methods, plasmids pCSCY and pCSMCY). Both plasmids were transformed into the Na⁺-sensitive B31 strain (*ena1-4Δ nha1Δ*). As expected, overexpression of Nha1 proteins in B31 brought about a significant decrease in Na⁺ sensitivity.

Next, the DNA fragment corresponding to the complete *NHA1* ORF was cloned in a series of vectors differing in promoters and/or copy number. Table 1 summarizes the Na⁺ tolerances of the B31 strain transformed with different plasmids harbouring the complete *NHA1* ORF. Expression of the *NHA1* gene behind strong promoters (*PGK1*, *ADH1*, *GPD1*) did not cause higher Na⁺ tolerance than that observed for *NHA1* expression behind its own promoter, even though the amount of *NHA1* mRNA in cells transformed with pMAADH426 was very high (Fig. 2a, lane 4). In the case of pMAGPD426, a plasmid with a very strong promoter (*GPD1*), the Na⁺ tolerance was only slightly higher but the growth of transformed cells was very slow, even in media without NaCl. Such an effect could result from overloading of the secretory pathway. A similar effect was observed during overexpression of the *CAN1* gene (encoding a plasma membrane permease for basic amino acids), where the cells were full of mislocalized Can1p but their growth and transport activity were very low (H. Sychrová, unpublished data). In conclusion, the highest Na⁺ tolerance was observed in a strain transformed with a multicopy vector harbouring the truncated version of the *NHA1* gene behind its own promoter.

Transcription of *NHA1*

NHA1 transcripts were visualized on Northern blots with a ³²P-riboprobe since our previous attempts using a DNA probe were unsuccessful. Fig. 2(a) shows the levels of mRNA corresponding to the *NHA1* gene, prepared from exponentially growing cells. As expected, no significant mRNA signal was detected in B31 in which the *NHA1* allele had been deleted (lane 2). In all other strains (lanes 1, 3 and 4), *NHA1* transcripts with an approximate size of 3.5 kb were detected. The expression of the *NHA1* gene under the control of its own promoter was insensitive to the addition of NaCl to the culture medium (data not shown).

Western blot analyses

To visualize the product of *NHA1* in *Sacch. cerevisiae*, we tagged Nha1p with the c-myc epitope. In plasmids pCSEmyc and pCSMEmyc, the sequence encoding the epitope (EQKLISEEDLN) was attached to the 3' end of the gene behind the last codon of truncated (888 aa) and complete (985 aa) versions of *NHA1*, respectively. The attachment of the epitope did not influence the protein function: the expression of tagged *NHA1* versions in *Sacch. cerevisiae* resulted in the same level of salt tolerance as in cells transformed with plasmids con-

Table 1. NaCl tolerance on YNB plates of *Sacch. cerevisiae* B31 transformed with different plasmids containing the complete *NHA1* ORF

At the concentrations reported, no appreciable growth was observed.

Plasmid	Copy number*	Promoter	Terminator	NaCl tolerance (mM)
YEp352	MC	—	—	< 400
pCSMCY	MC	<i>NHA1</i>	<i>CYC1</i>	1000
pMA52	MC	<i>PGK1</i>	<i>PGK1</i>	600
pMAADH426	MC	<i>ADH1</i>	<i>CYC1</i>	900
pMAGPD426	MC	<i>GPD1</i>	<i>CYC1</i>	400
pBS2	SC	<i>NHA1</i>	<i>CYC1</i>	700

* MC, multicopy; SC, single copy.

Fig. 2. Northern blot analysis of the *NHA1* transcripts in *Sacch. cerevisiae* with a 32 P-labelled riboprobe. Total RNA was extracted from exponentially growing cells of wild-type W303.1A (lane 1), and the B31 strain (*ena1-4Δ nha1Δ*) transformed with YEp352 (lane 2), pCSCY (lane 3) or pMA426ADH (lane 4). (a) RNA samples (60 µg in lanes 1, 2 and 3, 20 µg in lane 4) were run in agarose/formaldehyde gel, transferred to nitrocellulose membrane and hybridized with the *NHA1* probe. The probe corresponding to the last 2000 nt of the gene was obtained by *in vitro* run-off transcription from *NHA1* cloned in pBluescript and linearized by *HpaI*. The positions of the 3.5 and 1.8 kb rRNAs are indicated. (b) Ethidium-bromide-stained rRNAs on the membrane were visualized by UV light as a control.

taining original, non-tagged *NHA1* genes (data not shown).

The amount of complete and truncated *c-myc*-tagged Nha1 transporters in the cells was estimated using Western blots. Immunoreactive signals were first detected in total protein extracts of G19 strain containing either pCSEmyc, pCSMEmyc or 'empty' YEpmc181. As a control, protein extracts from G19 cells transformed with the pCAmc181 plasmid (Matejcková & Sychrová, 1997) containing a *c-myc* epitope-tagged version of the plasma membrane amino-acid permease CAN1 were also prepared. Fig. 3(a) shows that both tagged Nha1 proteins have an apparent molecular mass corresponding approximately to those calculated from deduced primary structures (109 and 98 kDa, respectively). No immunoreactive signal was detected in

Fig. 3. Immunodetection of *c-myc*-tagged complete Nha1p (lane 1), truncated Nha1p (lane 2) and Can1p (lane 3). (a) Total cell extracts (10 µl per lane); (b) isolated plasma membranes (10 µg total protein per lane).

extracts from G19 cells transformed with 'empty' YEpmc181 vector (data not shown). The amount of both truncated and complete Nha1 proteins in total cell extracts was much smaller than the amount of Can1p expressed in the same vector. If Western blot analysis was performed with purified plasma membranes, immunoreactive signals with similar relative intensities were detected (Fig. 3b), indicating that at least an important part of protein was localized in the plasma membrane. The smaller quantity of Nha1 protein in comparison with Can1p is probably due to the rather weak *NHA1* promoter, resulting in low expression. Moreover, on both types of Western blots, the signal corresponding to truncated protein was much weaker than the signal of the complete Nha1p, which suggests lower stability of the truncated version.

Role of the *NHA1* gene in Na⁺ and K⁺ tolerance

To assess the benefits of the *NHA1* gene for alkali cation tolerance, the growth of W303.1A-derived strains with different combinations of alleles *ena1-4Δ/ENA1-4* and *nha1Δ/NHA1* was tested on plates of YPD medium at

Table 2. Na⁺ and K⁺ tolerance of *Sacch. cerevisiae* strains on YPD plates at different pH values

At the concentrations reported, no appreciable growth was observed.

Strain	NaCl (mM)			KCl (mM)			
	pH ...	3·6	5·5	7·0	3·6	5·5	7·0
W303.1A (<i>ENA1-4 NHA1</i>)		500	1500	1300	1800	1800	1300
C25 (<i>ENA1-4 nha1Δ</i>)		400	1500	1300	400	900	1300
G19 (<i>ena1-4Δ NHA1</i>)		350	300	50	1800	1800	500
B31 (<i>ena1-4Δ nha1Δ</i>)		200	150	40	350	600	400
B31(pCSMCY)		700	700	50	1800	1800	500
B31(pCSCY)		900	900	50	1800	1800	500
B31(pGB34)		400	1500	1300	350	1000	1300

Fig. 4. Growth of strains expressing different combinations of *NHA1* and *ENA* genes in the presence of KCl at different pH values. Serial 10-fold dilutions of saturated cultures were spotted onto YPD medium at pH 5·5, 6·6 or 7·0 supplemented with KCl as indicated.

different pH values and increasing concentrations of NaCl and KCl. These strains were also transformed with plasmids containing either complete *NHA1* (pCSMCY), or truncated *NHA1* (pCSCY) or *ENA1* (pGB34).

Regarding the function of *NHA1* in Na⁺ tolerance, at pH 7·0 *NHA1* showed a very slight effect, which was only observed in the absence of the *ENA* genes. Overexpression of Nha1p did not show a significant effect at this pH [B31(pCSMCY) and B31(pCSCY) in Table 2]. At pH 5·5, the increase in tolerance caused by the presence of *NHA1* was very clear in the absence of *ENA* genes, which encode a more effective system at this pH. Overexpression of Nha1p increased the Na⁺ tolerance above the level observed for the chromosomal *NHA1* copy [Table 2, B31(pCSMCY) vs G19]. At pH 3·6, the results were similar to those described for pH 5·5, except that the lower activity of Ena ATPases, probably reflecting the loss of expression of *ENA1* (Garcia-deblás *et al.*, 1993), left a major role to Nha1p. At this pH the Na⁺ tolerance of the strain lacking the *NHA1* gene (C25) was lower than the wild-type (400 mM and 500 mM NaCl, respectively, Table 2) and overexpression of *NHA1* (either complete or truncated)

increased the tolerance of the *ENA1-4 nha1Δ* strain over the limit of the wild-type. Interestingly, the truncated version of *NHA1* conferred on cells higher Na⁺ tolerance than the complete gene (700 and 900 mM NaCl, respectively, Table 2).

Unlike the findings for Na⁺ tolerance, where the role of Nha1p seemed to be less important compared to that of Ena ATPases, K⁺ tolerance showed a distinctive function of Nha1p (Table 2). At pH 7·0, the effect of *NHA1* presence was negligible, thus confirming that Nha1p as an electroneutral cation/proton antiporter does not function at this pH. Conversely, at pH 5·5, and especially at pH 3·6, the function of Nha1p in K⁺ tolerance was evident. At both acidic pH values, Ena ATPases had no effect in the presence of Nha1p (W303.1A vs G19) and the absence of the *NHA1* gene decreased considerably the K⁺ tolerance, both in the presence (C25) or in the absence (B31) of *ENA* genes (Table 2). Interestingly, contrary to Na⁺ tolerance, overexpression of Nha1p did not improve K⁺ tolerance above the level of the wild-type, suggesting that factors other than K⁺ were already limiting growth when *NHA1* was normally expressed. Comparing in more detail the effects of *NHA1* and *ENA* genes at pH values ranging from 5·5 to 7·0, Fig. 4 shows

Fig. 5. Changes in intracellular concentrations of K^+ and Na^+ in *Sacch. cerevisiae* strains B31, G19 and B31 expressing either complete or truncated *NHA1* (plasmids pCSMCY and pCSCY, respectively). Cells were grown in AP medium pH 5.5 containing 1 mM KCl and the indicated concentrations of NaCl, harvested at OD₅₅₀ 0.15–0.2 and the internal K^+ (□) and Na^+ (■) concentrations determined.

that Nha1p had a greater effect on K^+ tolerance up to pH 6.6, but at higher pH values, Ena ATPases were more important (see *ENA1 nha1* vs *ena1 NHA1*).

In summary, the results demonstrate the complementary functions of Ena ATPases and the Nha1 antiporter both for Na^+ and especially K^+ tolerances in cells growing at different pH values. The Ena ATPases are effective at moderately acidic or alkaline pH values, whereas at acidic pH values ionic homeostasis depends mainly on the activity of Nha1p.

Intracellular K^+/Na^+ ratio

As it is likely that the positive role of Nha1p in Na^+ tolerance is associated with maintenance of low intracellular concentrations of Na^+ , we estimated the influence of external Na^+ concentration on the K^+/Na^+ ratio in cells of strains with deleted or overexpressed *NHA1* and with the *ena1-4Δ* background to avoid the Na^+ efflux mediated by Na^+ ATPases. In the absence of NaCl in the growth medium, the internal concentration of K^+ ions was almost constant in all strains tested including 'K⁺-sensitive' B31, independent of the external concentration of KCl ranging in our experiments from 1 mM to 0.5 M (data not shown). To measure changes in the K^+/Na^+ ratio, cells were grown in liquid AP media pH 5.5 containing a constant low concentration of K^+ (1 mM) and increasing concentrations of sodium (0–100 mM).

If the cells were grown in the presence of NaCl, the total intracellular concentration of Na^+ plus K^+ was similar to the internal concentration of K^+ in the absence of NaCl (Fig. 5). With increasing Na^+ concentration in the media, a progressively larger part of the internal K^+ was

replaced by Na^+ (Fig. 5). The K^+/Na^+ ratio in the cells depended not only on external Na^+ concentration but also on the presence of Nha1p. Strains overexpressing *NHA1* could preserve higher K^+/Na^+ ratios in the presence of higher concentrations of external Na^+ than strains B31 and G19, and again, an advantageous difference for the truncated allele was observed [cf. Fig. 5, B31(pCSCY) vs B31(pCSMCY)]. We conclude that the function of Nha1p influences the intracellular K^+/Na^+ ratio, which in turn plays a very important role in Na^+ tolerance. In our conditions, similar to that shown by Camacho *et al.* (1981), the K^+/Na^+ ratio must be higher than 1:2 to guarantee normal growth of cells in the presence of Na^+ .

Nha1p mediates Na^+ efflux

The high intracellular concentrations of Na^+ in the B31 mutant accompanied by its defective growth suggested that Nha1p might mediate an efflux of Na^+ , similar to that observed for the homologous Sod2p in *Schiz. pombe*. To test this possibility we measured Na^+ loss in strains with an *ena1-4Δ* background (to avoid the Na^+ efflux mediated by Na^+ ATPases) harbouring either a chromosomal copy of *NHA1* (G19) or multicopy vectors with truncated [B31(pCSCY)] and complete [B31(pCSMCY)] *NHA1* versions, respectively. As a negative control, the B31 strain lacking *NHA1* was used.

To preload the cells of these four strains with equivalent amounts of intracellular Na^+ , cells were grown in the presence of different NaCl concentrations [20 mM for B31; 40 mM for G19; 60 mM for B31(pCSMCY); 90 mM for B31(pCSCY)] to obtain an initial intracellular K^+/Na^+ ratio of about 1:1 (approx. 300 nmol mg^{-1} of each). Na^+ -preloaded cells were resuspended in a Na^+ -free incubation buffer containing 50 mM KCl to prevent Na^+ uptake and the loss of Na^+ was followed over 20 min (Fig. 6). In accordance with observed differences in Na^+ tolerance, B31 strains overexpressing *NHA1* genes showed higher Na^+ efflux than the G19 strain harbouring only one copy of *NHA1*. In the strain lacking both *ENA1-4* and *NHA1* (B31), no significant loss of Na^+ was observed (Fig. 6). We also detected a difference in the initial rate of efflux mediated by truncated and complete Nha1 proteins, respectively. Slightly higher initial Na^+ loss observed in cells with truncated Nha1 protein could explain their increased Na^+ tolerance.

Further, we found that the initial rate of Na^+ efflux in strains overexpressing *NHA1* genes decreased with time and the efflux almost stopped at a certain intracellular concentration (approx. 120 nmol mg^{-1}). This could be due to an accumulation of a portion of the intracellular Na^+ in vacuoles via the recently described Na^+/H^+ exchanger Nhx1p (Nass *et al.*, 1997). To eliminate the possible distortion of our results brought about by intracellular sequestration of Na^+ , we loaded the cells very rapidly (incubation with NaCl for 10 min), transferred them to a Na^+ -free incubation buffer and the Na^+ loss was measured. Under these conditions, the Na^+

Fig. 6. Na⁺ loss from Na⁺-loaded cells at pH 5.5. Cells of B31 (○), G19 (●), B31(pCSMCY) (▽) and B31(pCSCY) (▼) were Na⁺-loaded by growing them in AP medium pH 5.5 with 1 mM KCl and 20 mM NaCl (B31), 40 mM NaCl (G19), 60 mM NaCl [B31(pCSMCY)] or 90 mM NaCl [B31(pCSCY)]. Na⁺-loaded cells were transferred to incubation buffer and the internal content of Na⁺ was followed for 20 min.

efflux from cells overexpressing Nha1p was continuous, leaving almost no Na⁺ ions in the cells within 20 min (data not shown).

The tests described above were performed in the absence of external Na⁺, thus leaving unanswered the question of whether Nha1p can mediate Na⁺ efflux against a concentration gradient. To address this question, we

measured the net Na⁺ loss in B31(pCSMCY) cells suspended in a buffer pH 5.5 containing a Na⁺ concentration similar to the initial internal Na⁺ concentration and a significant Na⁺ loss at an initial rate of about 5 nmol mg⁻¹ min⁻¹ was found (data not shown). This observation is in accordance with the putative Na⁺/H⁺ antiport mechanism, in which Na⁺ efflux is driven by ΔpH.

Nha1p mediates K⁺ efflux

In addition to Na⁺ extrusion, K⁺ extrusion is also an important component of ionic homeostasis in cells. In *Sacch. cerevisiae*, two-thirds of the K⁺ taken up by growing cells is returned to the external medium (Rodríguez-Navarro & Ramos, 1984; Ortega & Rodríguez-Navarro, 1985). Several reports suggested that K⁺ efflux could be mediated by a K⁺/H⁺ antiporter (Ortega & Rodríguez-Navarro, 1985; Peña & Ramírez, 1991; Camarasa *et al.*, 1996; Ramírez *et al.*, 1996). Consistent with this notion and with the defective growth of *NHA1*-deficient strains (C25 and B31) in the presence of high concentrations of KCl, we studied the K⁺ loss from strains containing either no *NHA1* (B31) or the chromosomal *NHA1* gene (G19) to confirm the role of Nha1p in K⁺ efflux. Cells of both strains were grown at pH 5.5 in the presence of 3 mM KCl, harvested, resuspended in incubation buffer containing 200 mM RbCl, and the K⁺ loss from cells followed. Cells harbouring functional Nha1p (strain G19) lost K⁺ at an initial rate of about 2 nmol mg⁻¹ min⁻¹, whereas cells lacking the *NHA1* gene did not show any appreciable K⁺ loss.

In cells grown in a medium containing NaCl and with approximately the same initial intracellular K⁺ and Na⁺

Fig. 7. K⁺ and Na⁺ loss from cells of B31 and B31(pCSMCY). Cells of B31 (□, ■) and B31(pCSMCY) (○, ●) were Na⁺-loaded by growing them in AP medium pH 5.5; for B31 the medium contained 1 mM KCl and 20 mM NaCl, and for B31(pCSMCY) 1 mM KCl and 60 mM NaCl. Cells were then transferred to incubation buffer pH 5.5 (a) or pH 8.0 without (b) or supplemented with (c) 20 mM NH₄Cl, and changes in the internal K⁺ (■, ●) and Na⁺ (□, ○) contents were followed for 20 min.

content (approx. 300 nmol mg⁻¹ of each), simultaneous measurements of the loss of both cations at pH 5.5 (in the presence of 20 mM RbCl to inhibit Na⁺ and K⁺ influx) showed (Fig. 7a) that the Na⁺ loss was only slightly higher than the K⁺ loss, indicating that Nha1p is able to mediate at acidic pH the efflux of both cations with comparable affinity.

As the efflux of Na⁺ is inhibited by permeable organic acids, which decrease the internal pH (Rodríguez-Navarro *et al.*, 1981), the Nha1p function could be limited by the concomitant intracellular pH decrease, and similarly its activity could also be influenced by an increase in the cytosolic pH. In a first experiment, we measured Na⁺ and K⁺ losses in an alkaline incubation buffer (pH 8.0), and found that the increase in the external pH activated the effluxes, which were almost identical for both cations (Fig. 7b) (it is worth mentioning that these experiments were performed in the absence of external Na⁺ and K⁺, so the Δ pH was not necessary to drive the process). In a second experiment, we measured the Na⁺ and K⁺ loss from cells resuspended in an alkaline buffer containing 20 mM NH₄Cl (to increase the intracellular pH). Interestingly, as shown in Fig. 7(c), the alkalization of the intracellular pH resulted in a very high efflux of both ions in the strain overexpressing *NHA1*. These conditions might also permit detection of another marginal system involved in K⁺ loss as the internal K⁺ concentration decreased very slowly in the strain lacking *NHA1* (B31, Fig. 7c).

Based on these results, we conclude that not only is the product of the *Sacch. cerevisiae* *NHA1* gene involved in Na⁺ efflux as predicted from its deduced primary sequence, but also that it plays an important role in K⁺ loss from cells.

DISCUSSION

The major goal of the present work was to characterize the function(s) of the product of the *Sacch. cerevisiae* *NHA1* gene. Our present results show that the product of *NHA1* is located in the plasma membrane and probably exchanges cytoplasmic K⁺ and Na⁺ with external H⁺.

The phenotype of increased salt tolerance obtained upon cloning the gene was enhanced by a truncation of the *NHA1* ORF (Prior *et al.*, 1996). The ability of a C-terminal truncation to increase protein activity has already been observed for other plasma membrane proteins (Grauslund *et al.*, 1995). In the case of Nha1p, the truncation of 97 amino acids at 446 aa from the last putative transmembrane stretch, lowers the amount of protein present in the cells as indicated by Western blot analysis, perhaps as a consequence of elimination of some stabilizing region(s). Regarding these results, we are at present trying to characterize the function of the Nha1p C-terminal region in more detail.

Detection of tagged Nha1p in the isolated plasma membranes, together with measurements of K⁺ and Na⁺

losses from cells with functional Nha1p, suggest that Nha1p is located in the plasma membrane of *Sacch. cerevisiae*.

Although Nha1p has high amino acid sequence similarity with a Na⁺/H⁺ antiporter of *Schiz. pombe*, our results indicate that its role is more complex. Nha1p is not only involved in NaCl tolerance by mediating the efflux of Na⁺ like its *Schiz. pombe* homologue, but also plays an important part in K⁺ efflux and KCl tolerance at acidic pH values. Comparing the effects of the lack and overexpression of Nha1p on NaCl and KCl tolerance (Table 2), it can be proposed that the function of this protein is more important for K⁺ tolerance than Na⁺ tolerance. Because high KCl is not a natural stress, it can be deduced that the major function of Nha1p is related to K⁺ homeostasis, by maintaining the steady-state level of K⁺. Through this activity, Nha1p could participate in regulation of intracellular pH, cell volume and osmoregulation. The finding of an antiport system mediating exchange of both K⁺ and Na⁺ with H⁺ is new in the physiology of yeasts. In *Schiz. pombe*, a role of Sod2p in K⁺ efflux has never been shown and the only systems characterized in yeasts as mediating K⁺ efflux are the Tok1p channel of *Sacch. cerevisiae* (Ketchum *et al.*, 1995) and the Ena2 alkali-cation ATPase of *Schwanniomyces occidentalis* (Bañuelos & Rodríguez-Navarro, 1998).

As we observed that Na⁺ and K⁺ effluxes mediated by Nha1p are activated when the internal pH increases, we regard it as likely that the *NHA1* antiporter has dual functions. At acid external pH, Nha1p participates in regulation of the internal concentrations of alkali cations using the high gradient of protons across the plasma membrane as a driving force to eliminate (when necessary) toxic amounts of Na⁺ or an excess of K⁺ from cells. When cytoplasmic pH increases, possibly when the external pH is in the upper limit for the growth of *Sacch. cerevisiae*, Nha1p could act as a short-term safety valve to contribute to the buffering of cytosolic pH by using the outward gradient of K⁺ (or Na⁺) which can drive in some protons.

The function of Nha1p cannot be considered redundant with the function of the Ena ATPases, which probably also exchange Na⁺ and K⁺ for H⁺ (Benito *et al.*, 1997), but complementary. The requirement for two different systems (an alkali-cation/proton antiporter and an alkali-cation ATPase) for regulation of cellular alkali cation concentration may be connected with the natural environment of *Sacch. cerevisiae* cells in which acid pH values prevail, although growth at near neutral pH is also possible. In the fission yeast *Schiz. pombe*, which grows more strictly at acid pH, only a proton antiport mechanism for Na⁺ efflux has been demonstrated (Jia *et al.*, 1992) (nothing being known about K⁺ homeostasis and efflux in this yeast). On the other hand, *Schwan. occidentalis* is able to grow at alkaline pH and it possesses two different types of alkaline-pH-inducible Na⁺ ATPases, mediating efflux of both Na⁺ and K⁺. In *Schwan. occidentalis* a Na⁺/H⁺ antiporter probably does not exist as no efflux of Na⁺ was detected in a strain

with an *enaΔ* background at acid pH values (Bañuelos & Rodríguez-Navarro, 1998).

In *Sacch. cerevisiae*, the Nha1p function can be coordinated not only with Ena1p but also with the function of a Na⁺/H⁺ exchanger (encoded by the *NHX1* gene) which mediates the active sequestration of Na⁺ in cells, probably in vacuoles (Nass *et al.*, 1997). As was shown, this Na⁺ sequestration is enhanced when the intracellular pH decreases. Hence it would be interesting to study whether, upon exposure to NaCl, the toxic high concentration of Na⁺ is eliminated from the cytoplasm by cooperation of three systems, two of which are associated with the plasma membrane, a Na⁺ ATPase (Ena1p) and a Na⁺/H⁺ antiporter (Nha1p), and the third one located in the vacuole (Nhx1p). The action of Nha1p would import protons, resulting in a drop in intracellular pH which could trigger the sequestering activity of Nhx1p. The decrease in intracellular pH caused by the alkali cation/proton antiport activity of Nha1p could also play an important role in triggering other processes, for example response to osmotic shock and/or cell volume control. Thus our future studies will be focused on the general role of Nha1p in cell physiology.

The existence of at least two different mechanisms involved in yeast salt tolerance and their distribution in different yeast species according to their special needs during environmental stress may be an important feature of adaptation to salt stress. Therefore, the characterization of yeast Na⁺ and K⁺ transporting systems may be significant for the urgent biotechnological challenge of equipping crop plants with the capacity to grow in the world's increasing areas of acid and salted soils (Serrano *et al.*, 1997).

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