J. Biochem. 131, 821–831 (2002)

# Structurally and Functionally Conserved Domains in the Diverse Hydrophilic Carboxy-Terminal Halves of Various Yeast and Fungal $Na^+/H^+$ Antiporters (Nha1p)[1]

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Received March 18, 2002; accepted April 4, 2002

**Genes encoding the $Na^+/H^+$ antiporter (Nha1p) from *Candida tropicalis* (C.t.), *Hansenula anomala* (H.a.) (also named *Pichia anomala*), and *Aspergillus nidulans* (A.n.) were cloned, and the nucleotide sequences were determined. The deduced primary sequences revealed highly conserved hydrophobic regions and rather diverse hydrophilic regions. Among the seven known Nha1p sequences, *Schizosaccharomyces pombe* (S.p.) Nha1p is exceptional in lacking the hydrophilic region. Within the diverse hydrophilic regions, we found six conserved regions (C1–C6). Expression of C.t. Nha1p in *Saccharomyces cerevisiae* (S.c.) cells lacking *NHA1* and *ENA1* ($Na^+$-ATPase) complemented the salinity-sensitive phenotype, suggesting that C.t. Nha1p is functionally related to S.c. Nha1p. Expression of various truncated forms of the C-terminal half of S.c. and C.t. Nha1p showed essentially the same phenotype for both species: deletion of the C4–C6 region caused cell growth to be more resistant to high salinity than the wild type, suggesting an inhibitory function of these domains on the antiporter activity. However, complete loss of C1–C6 caused a severe growth defect under conditions of high salinity, suggesting a defect in antiporter activity. The ΔC2–C6 form of C.t. Nha1p, containing only C1, restored the retarded cell growth at high salinity more than the control vector alone, but to a value lower than the wild type. These results suggest an essential role for C1 and an activating role of the C2–C3 region in the functional expression of Nha1. High expression of the ΔC2–C6 form of S.c. Nha1p was toxic for yeast cells, although low expression was not, suggesting that the overexpression of C1 is toxic. The results in this study suggest that the diverse hydrophilic region of yeast and fungal Nha1p has six conserved domains with conserved functions in terms of expression of Nha1p activity.**

**Key words: conserved domains, hydrophilic tail, $Na^+/H^+$ antiporter, salinity resistance.**

$Na^+/H^+$ antiporters have been found in the cytoplasmic membranes of a wide variety of organisms (*1–4*). Some isoforms of this antiporter have been also found in the membranes of endocytic vacuoles (*5–7*). These antiporters exchange $Na^+$ inside of cells for $H^+$ outside of cells in bacteria, while the direction of the exchange is reversed in mammalian cells (*1, 4*). This ion-exchange activity is important for maintaining the homeostasis of $Na^+$ and $H^+$ within living cells. Cloning and sequencing of the antiporter genes from various organisms have revealed that antiporters contain a highly hydrophobic primary sequence with a structure composed of 10 to 12 transmembrane domains (*1–7*). While most bacterial antiporters have this type of transmembranous structure alone (*4*), mammalian antiporters also have a long hydrophilic region in the carboxy terminal half of the molecule (*1*). Based on molecular genetic approaches, the hydrophobic membrane domains of various antiporters have been found to play an essential role in ion transport (*1–4*). Extensive studies have also revealed that the hydrophilic region functions in regulation of the antiporter in response to intracellular signaling of various growth stimuli (*1*). Several novel proteins interacting with this region have also been reported (*1, 8–12*).

The antiporter located in the cytoplasmic membrane of yeasts and fungi has been named Nha1p (*13*). Another type of antiporter found in the endomembrane, named Nhx1p, has been also reported (*6*). *NHA1* was originally cloned from *S. pombe* (S.p.) as a product of *SOD2* (*14, 15*). A defect in *SOD2* causes sensitivity to $Li^+$ and to high concentrations of $Na^+$ because of the loss of $Na^+/H^+$ antiporter activity. In *S. cerevisiae* (S.c.), Nha1p has been shown to transport $K^+$ as well as $Na^+$ and $Li^+$ (*16*). The deduced pri-

[1] The present study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, to H.K. The study was partly supported by a grant from CREST (Core Research for Evolutional Science and Technology) of Japan Science Technology Corporation to H.K.

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mary sequence of *S. pombe* Nha1p (S.p. Nha1p, Sod2p) has the typical structure of a 12-transmembrane protein but is missing the long hydrophilic region observed in mammalian antiporters (*1*). However, the Nha1p sequences determined for *Zygosaccharomyces rouxii* (Z.r. Nha1p, Zsod2p) (*17*) and S.c. Nha1p (*13*) do contain the hydrophilic region. These observations raised the question of whether the yeast and fungal Nha1s are diverse in terms of the hydrophilic tail. Although Z.r. and S.c. both have hydrophilic tails, the primary sequences are not conserved. This observation raised another question, whether the hydrophilic regions of various yeast and fungal Nha1ps have diverse structures in general and play different roles depending on species, analogous to the different functions of the structurally diverse hydrophilic tail regions in the isoforms of mammalian antiporters (*1*).

Here, we have cloned genes encoding Nha1p from *Hansenula anomala* (H.a.; also named *Pichia anomala*), *Candida tropicalis* (C.t.), and *Aspergillus nidulans* (A.n.) and compared the sequences of the deduced primary structures with those reported previously. We found that all three Nha1p sequences described here contain hydrophilic tails, leading to the conclusion that the lack of a hydrophilic tail in S.p. Nha1p is exceptional. We also found that although the overall structures of the hydrophilic tails are diverse, six conserved regions exist and probably have essentially similar functions in $Na^+/H^+$ antiporter activity.

## MATERIALS AND METHODS

*E. coli, Yeast Strains, and Cell Culture*—*Escherichia coli* JM109 (*18*) and K802 (*19*) were used for propagation of plasmid and phage DNA, respectively. *E. coli* cells were cultured in L broth at 37°C with an appropriate antibiotic for selection of transformants, as described previously (*20*). S.c. W303-1B (MATα *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100*) (*21*), and G19 (MATα) containing the deletion of the *ENA1* gene (*ena1*Δ::*HIS3*::*ena4*Δ) were a kind gift from A. Rodriguez-Navarro (*21*). SK5 (*ena1*Δ::*HIS3*::*ena4*Δ, *nha1*Δ::*LEU2*) lacking *NHA1* was constructed in this study by substituting the 3.9-kb *Xba*I–*Nde*I fragment of *NHA1* containing an insertion of the *LEU2* gene for the wild-type *NHA1* sequence in G19. Yeast cells were cultured in a rich medium (YPD) (*22*) containing 1% yeast extract, 2% peptone, and 2% glucose or a minimal medium (SD) (*22*) containing 0.67% yeast nitrogen base and 2% glucose supplemented with required amino acids at 30°C. For agar plates, agar (1.5%) was added to these media.

*Cloning of NHA1 Genes from Genomic Libraries of C.t., H.a., and A.n.*—λ phage libraries of genomic DNA from C.t. pK233 (*23*) and H.a. (*24*), and a genomic cosmid library from each of eight chromosomes of A.n. were described previously (*25*). *E. coli* K802 harboring transfected phages or cosmids was cultured on L broth plates, and plaques or cosmids were transferred to nylon membranes as described previously (*26*). Phages or cosmid DNA on the membrane filters were subjected to hybridization as described (*26*) with a DNA probe prepared by amplification of a portion of the genes encoding *NHA1* in C.t., H.a., and A.n. For the screening, the most conserved region in the Nha1p sequences of S.p. Sod2p (*14*), Z.r. ZSod2p (*17*), and S.c. Nha1p (*13*) was identified in transmembrane domains 6 and 11 (Fig. 1). The conserved sequences in S.c. were chemically synthesized as the primers for PCR (*27*), and DNA segments were amplified from C.t., H.a., and A.n. by PCR using these primers as described previously (*28*). Total genomic DNA from these organisms was prepared for the template as described previously (*26*). The amplified DNA (about 600 bp) was cloned into pBR322, and this DNA fragment, labeled with $^{32}P$ (Amersham) as described previously (*26*), was used as the probe DNA for screening the library. Candidate clones were selected based on this hybridization. The inserted DNA was recovered, and a physical map of restriction endonulease digestion sites was constructed using restriction endonucleases from Toyobo and Takara. By using this map, the entire nucleotide sequences of the coding regions of C.t., H.a., and A.n. NHA were determined. Upstream from the translation start site, regions up to –400, –70, and –500 bp were determined for C.t., H.a., and A.n. NHA, respectively. Downstream from the translation stop signal, regions up to +500, +150, and +200 bp were determined for C.t., H.a., and A.n. NHA, respectively.

*Expression Vectors of Various Forms of NHA1 from S.c. and C.t.*—DNA fragments corresponding to the entire region and the truncated forms of S.c. and C.t. Nha1p were amplified by PCR, using DNA polymerase (KOD polymerase) purchased from Toyobo and Takara, with pBluescript II KS$^+$ (*29*) containing cDNA of S.c. *NHA1* derived from cosmid 2A16 and the cloned C.t. *NHA1* DNA as the templates. The following oligonucleotides were synthesized by Pharmacia Biotech for use as PCR primers: S.c. Nha1p, 5′-TACTGGTACCATGGCTATCTGGGAGCAA-3′ (PSK6) (underlined sequence for *Kpn*I site) and 5′-TCTGCATGCCCTTATTGAGACCA-3′ (PSK31); S.c. Nha1p (ΔC4–C6), PSK6 and 5′-TGTGCATGCGGAGAGATGAAGAT-3′ (PSK29) (underlined sequence for *Sph*I site); S.c. Nha1p (ΔC2–C6), PSK6 and 5′-TCTGCATGCTGGTTAACGTTATC-3′ (PSK32) (underlined sequence for *Sph*I site); S.c. Nha1p (ΔC1–C6), PSK6 and 5′-ATGAGCATGCCTGAAGAACCATGAAC-3′ (PSK36) (underlined sequence for *Sph*I site); C.t. Nha1p, 5′-GGAATTCATGGCTTGGAGTCAG-3′ (CT1) (underlined sequence for *Eco*RI) and 5′-ACATGCATGCACACTTCTTCTTCGTCGTC-3′ (CT18) (underlined sequence for *Sph*I); C.t. Nha1p (ΔC4–C6), CT1 and 5′-ACATGCATGCATGAATTCTTGAGTTCATTG-3′ (CT13) (underlined sequence for *Sph*I site); C.t. Nha1p (ΔC2–C6), CT1 and 5′-ACATGCATGCTGGCCATTCTGTTCAAACG-3′ (CT10) (underlined sequence for *Sph*I); C.t. Nha1p (ΔC1–C6), CT1 and 5′-ACATGCATGCCAGAAGATCCATGGAC-3′ (CT9) (underlined sequence for *Sph*I site). All amplified DNAs were first integrated between the unique restriction sites *Kpn*I or *Eco*RI and *Sph*I in the cloning vector pUC18, and the sequences of the amplified regions were confirmed by DNA sequencing. Then *Kpn*I–*Sph*I or *Eco*RI–*Sph*I DNA fragments of various forms of S.c. or C.t. Nha1p, respectively, from the derivatives of pUC18 were integrated into an expression vector of the yeast high-copy type, pKT10. For addition of the FLAG-tag sequence, these fragments were joined together with a *Sph*I–*Sal*I fragment encoding the FLAG epitope sequence (DYKDDDK) and inserted into pKT10 (*30*). The wild-type and truncated genes lie under the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GAP promoter). For inducible expression of Nha1p, the coding sequences of truncated S.c. and C.t. Nha1p (Nha ΔC2–C6) from derivative plasmids of pKT10 were amplified and inserted between *Eco*RI

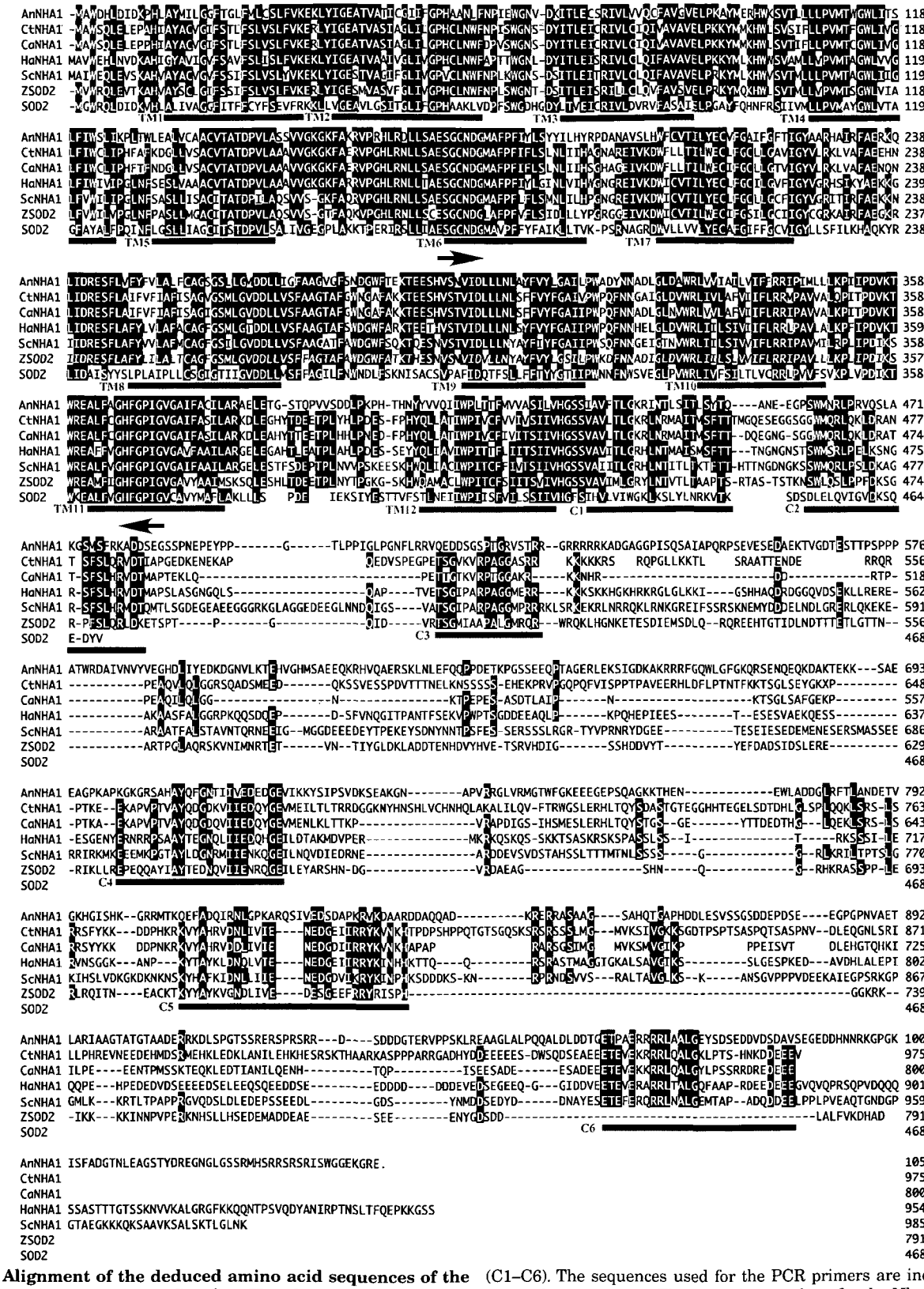



Fig. 1. **Alignment of the deduced amino acid sequences of the $Na^+/H^+$ antiporters of A.n., C.t., C.a., H.a., S.c., Z.r., and S.p.** Residues identical in 50% or more of the aligned sequences are shaded. Proposed transmembrane (TM) sequences are underlined under the aligned sequences (TM1–12). Conserved regions in the hydrophilic carboxy-terminal halves are underlined under the aligned sequences (C1–C6). The sequences used for the PCR primers are indicated between the two arrows. The accession numbers for the Nha1p in GenBank-DDBJ are as following: C.t., AB073975; H.a., AB073976; A.n., AB073977; C.a., AF375984; S.c., Z73310; S.p. *sod2*, Z11736; Z.r., D43629.

and *Sph*I sites in the expression vector YPN1 (*31*) with a GAL promoter. For a lower level of expression of Nha1, the promoter for the GAPDH gene in the pKT10 derivative plasmids was replaced by the native promoter region that extends to a *Bam*HI site located 700 bp upstream of the transcriptional initiation site of S.c. NHA1.

*Immunological Detection of the Wild-Type and Truncated Forms of Nha1*—SK5 cells transformed with various plasmids were cultured in SD medium at 30°C up to the logarithmic phase of growth. Harvested cells were washed twice with cold distilled water and suspended in 50 ml of breaking buffer (50 mM Tris-HCl [pH 7.6], 0.3 M sucrose, 5 mM EDTA, one tablet of protease inhibitor cocktail [Roche Diagnostics]). Fractionation of subcellular structures followed the published procedure (*32*). Cells were disrupted by mixing vigorously with a vortex mixer in the presence of glass beads. After removing undisrupted cells by centrifugation (400 ×*g* for 5 min), the supernatant fraction was subjected again to centrifugation for 15 min at 13,000 ×*g*. The precipitated fraction, designated P13, contained vacuoles, mitochondria, ER, and nuclei (*32*). The supernatant fraction was further centrifuged for 60 min at 100,000 ×*g*. The resultant precipitate was defined as the cytoplasmic membrane fraction (P100). A 30-μg aliquot of this fraction was subjected to SDS–polyacrylamide gel electrophoresis on a 10% acrylamide gel, and the separated proteins were transferred to a membrane filter (GHVP, Millipore) (*33*). The transferred proteins were treated with an anti-FLAG monoclonal antibody (Sigma), and the reactive bands were visualized by the ECL chemiluminescence method, as described previously (Amersham Pharmachia) (*34*).

*DNA Manipulation, Sequencing, and Transformation*—Manipulation of DNA, including subcloning, digestion, ligation of DNA, and preparation of plasmid DNA, was performed by previously published methods (*26*). T4 DNA ligase and restriction endonucleases were purchased from Toyobo and Takara. Nucleotide sequences of cloned DNA were determined by dideoxy-chain termination methods (*35*) with an automated ABI377 sequencer (Applied Biosystems). Transformation of *E. coli* by electroporation (*26*) and yeast by the lithium acetate method (*36*) followed published procedures. Cells transformed by an expression vector were selected based on resistance to an appropriate antibiotic for *E. coli* or auxotrophic markers for yeast.

*Measurement of Intracellular $Na^+$ and $K^+$*—Yeast cells were cultured in 25 ml of SD medium containing various concentrations of NaCl at 30°C up to the logarithmic phase of growth ($OD_{600}$ = 0.4). Harvested cells were trapped on membrane filters (0.45 μm, Advantec) and washed with 10 mM $MgCl_2$ solution containing sorbitol to maintain isotonicity. Cells on the filter were soaked in distilled water and boiled for 15 min, cell debris was removed by centrifugation, and the supernatants were subjected to optical emission spectrophotometry (Perkin Elmer, Optima 3000XL) as described previously (*37*). The total amount of protein in the cell extracts after boiling was measured by the method by Lowry *et al.* (*38*).

## RESULTS

*Cloning and Sequencing of NHA1s from H.a., A.n., and C.t.*—For the structural comparison of a wide variety of yeast and fungal Nha1p, we selected H.a., and A.n. and C.t., which are phylogenetically proximal and distal, respectively, to S.c. and S.p. Based on the reported nucleotide sequences of Nha1p from S.p. (*14*), S.c. (*13*), and Z.r. (*17*), highly conserved sequences within putative transmembrane domains 6 and 11 were selected for the primer sequence in PCR (Fig. 1). By using these primers synthesized based on the sequence of S.c. and genomic DNA prepared from H.a., A.n., C.t., DNA fragments encoding the conserved sequences were amplified and cloned. The amplified sequences had the expected size of about 600 bp, and the amino acid sequences deduced from the DNA sequence of the cloned fragments were found to be highly conserved (Fig. 1). By using these DNA sequences as screening probes, 3 out of $2.4 \times 10^5$ candidate phage clones of C.t. *NHA1* and 1 out of $4.0 \times 10^5$ candidate phage clones of H.a. *NHA1* were selected from the genomic DNA library of each species. For A.n., 12 candidate clones were selected from the cosmid library of genomic DNA derived from chromosome 4, and 2 candidate clones were selected from the chromosome 5 library. We constructed a physical map of cleavage sites of several restriction endonucleases and determined partial nucleotide sequences for each candidate clone of H.a. and C.t. and clones of chromosome 4 from A.n. The results indicated that 3 clones from C.t. were derived from the same gene, as were 12 clones from A.n. Translational initiation sites were estimated from the locations of the characteristic sequences described by Kozak (*39*) and by comparison of the reading frames of S.c. and Z.r. *NHA1*. Based on the determined nucleotide sequences, the primary structures of Nha1p from the three species were deduced and compared with other Nha1p sequences reported previously (Fig. 1). All three Nha1ps contained similar hydrophobic N-terminal halves and hydrophilic C-terminal halves to the S.c. and Z.r. Nha1ps (Fig. 2). The N-

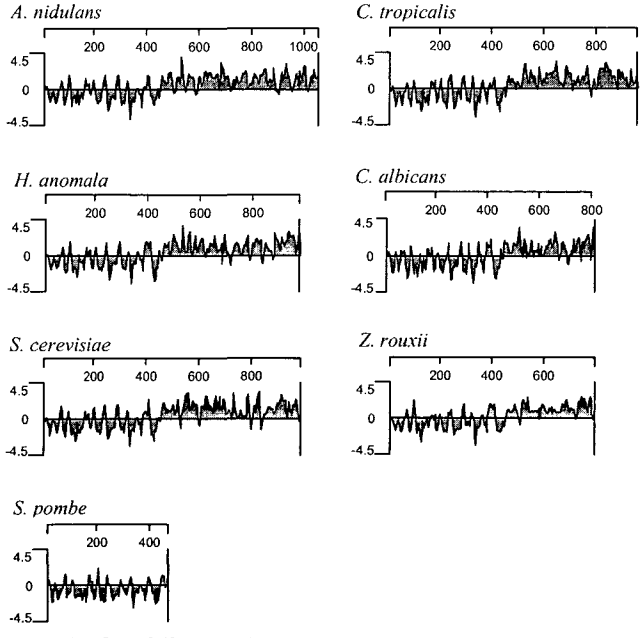



Fig. 2. **Hydrophilicity plot of the $Na^+/H^+$ antiporters (Nha1p) of A.n., C.t., H.a., S.c., Z.r., C.a., and S.p.** Average hydrophilicity values were determined for spans of nine residues using the method of Kyte and Doolittle (*47*). Vertical and horizontal scales indicate hydrophilicity values and residue numbers, respectively.

terminal halves are highly conserved among seven species (identities in the amino acid sequences: C.t., 70.4%; A.n., 58.9%; H.a., 75.8% to S.c. Nha1p) and 12 transmembrane domains were found for every Nha1p (Fig. 1). In contrast, the overall structure of the hydrophilic C-terminal halves is not conserved (identities in the amino acid sequences: C.t., 24.1%; A.n., 16.6%; H.a., 30.5% to S.c. Nha1p) and their lengths varied (Fig. 1). However, based on the seven Nha1p sequences we found that six short regions within the C-terminal halves are relatively well conserved (Fig. 1). We surveyed a protein database and found that none of these sequences was similar to motif sequences reported previously. The H.a. *NHA1* DNA sequence from the 25th to 94th residues is out of a reading frame and contained GT and AG sequence at the 5′ and 3′ ends, suggesting that this sequence is a putative intron like that found for S.p. *NHA1*. A.n. and C.t. *NHA1* did not have this sequence (data not shown).

*Complementation of the Salinity-Sensitive Phenotype of SK5 by C.t. NHA1*—Among the three Nha1ps, we selected C.t. Nha1p because it is phylogenetically distant from S.c. and compared the functional relationship of C.t. Nha1p to S.c. Nha1p. We expressed C.t. or S.c. Nha1p with a high-copy-type expression vector (pKT10) in the mutant strain SK5 of S.c., which lacks *NHA1* and *ENA1* (Fig. 3). At lower salinity of up to 100 mM NaCl, cells with the control vector alone grew on the plates. However, at higher salinity of above 300 mM NaCl, the cells transformed with *NHA1* from both species grew while the control did not. These results suggest that C.t. and S.c. Nha1p transported intracellular $Na^+$ to the outside of the cells, thus reducing the internal $Na^+$ concentration. At 500 mM NaCl, the colony sizes of C.t. *NHA1* transformants were bigger than those for S.c. *NHA1* transformants. In liquid culture, a significant delay of cell growth was found for S.c. *NHA1* while transformants of C.t. *NHA1* adapted to the higher salinity more quickly (Fig. 4). These results suggest that C.t. *NHA1* transformants recovered salinity resistance more efficiently than S.c. *NHA1* transformants.

The ion specificity and pH-dependent activity of C.t. Nha1p were tested based on salinity resistance. As shown in Fig. 5, salinity resistance was observed for $K^+$ as well as for $Na^+$ and $Li^+$, suggesting that C.t. Nha1p transports $K^+$ as does S.c. Nha1. Since $K^+$ is not a substrate ion for S.p. Nha1p, C.t., and S.c. Nha1p may have a closer structure in terms of ion specificity. Salinity resistance was higher in acidic conditions than at neutral pH for the ions tested, as it was for S.p. Nha1p (*14*). These results suggest that C.t. Nha1p plays a role in the regulation of pH and $Na^+$ under acidic pH conditions.

*Functional Comparison of the C-Terminal Regions Conserved between S.c. and C.t. Nha1ps*—Since the overall functions of S.c and C.t. Nha1p were found to be similar at

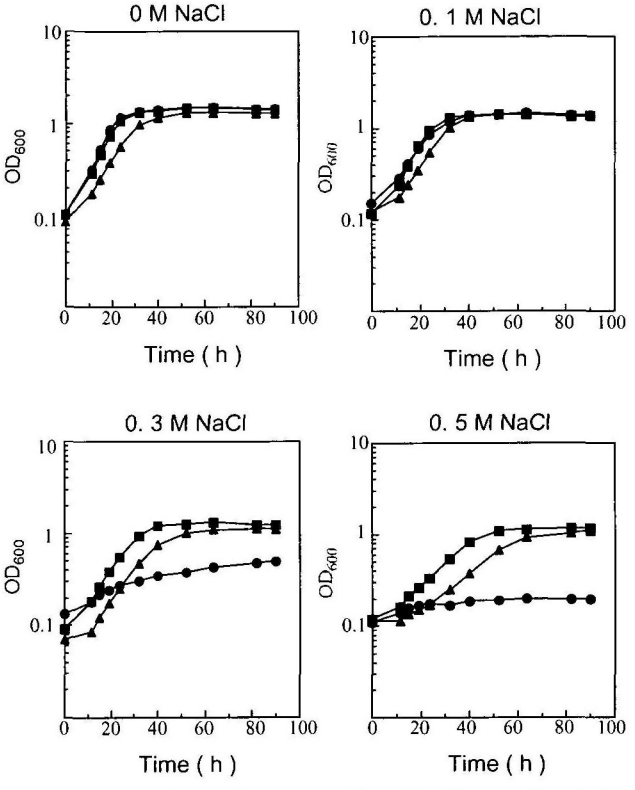
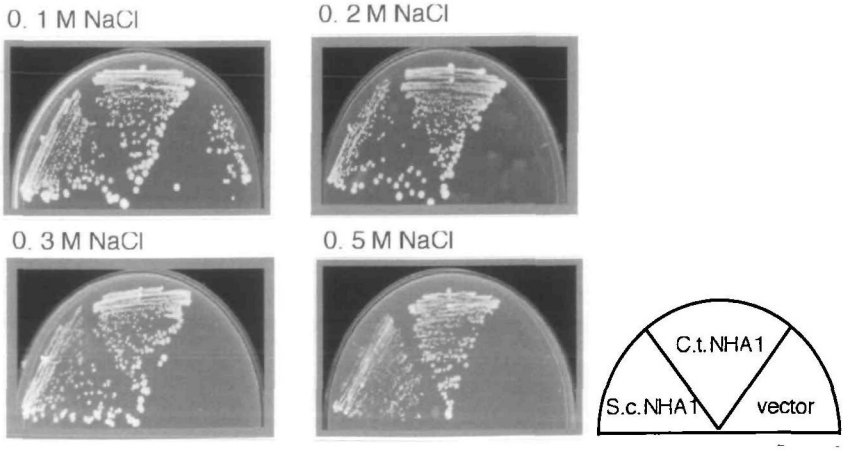



Fig. 4. **Growth of transformant cells with C.t. and S.c. *NHA1* in the presence of various concentrations of NaCl.** SK5 cells transformed with S.c. and C.t. *NHA1* with pKT10 were inoculated in 3 ml of SD medium containing various concentrations of NaCl as shown and cultured at 30°C with vigorous shaking. Growth of cells was monitored by measuring turbidity of the culture at 600 nm with a spectrophotometer. Circle, SK5; triangle, S.c. *NHA1*; square, C.t. *NHA1*.

Fig. 3. **Complementation of the salinity-sensitive phenotype of SK5 by C.t. *NHA1*.** SK5 cells transformed with the expression vector (pKT10) of S.c. or C.t. *NHA1* were spotted on SD medium containing the various concentrations of NaCl indicated on the panel. Plates were incubated at 30°C for 4 days.



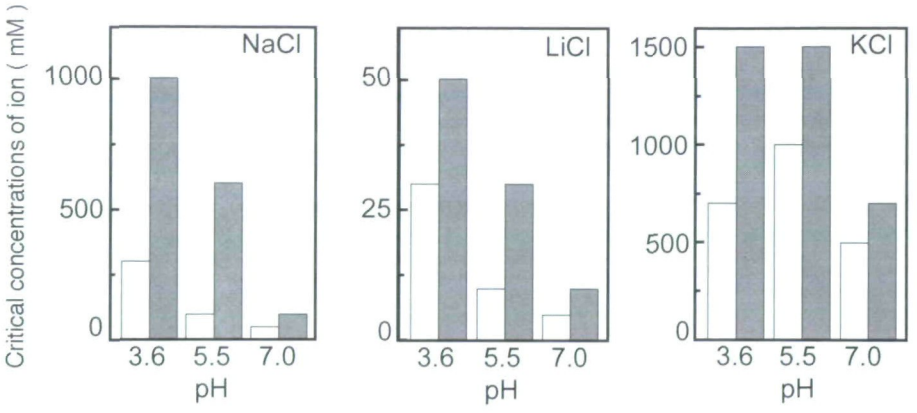



Fig. 5. **Ion specificities and pH dependency of C.t. Nha1p.** SK5 cells transformed with C.t. *NHA1* with pKT10 were incubated on SD medium plates containing various concentrations of NaCl, LiCl, or KCl for 4 days at 30°C. Critical concentrations of the ions that caused no growth of cells after incubation for days are indicated on the axis at various pH conditions. Tested concentrations of ions are as follows: NaCl, 50, 100, 200, 300, 500, 600, 800, and 1,000 mM; LiCl, 2, 5, 10, 30, 50, 100, 200 and 300 mM; KCl, 500, 700, 900, 1,500, 1,800, and 2,000 mM. Open and closed bars indicate cells transformed with vector alone and C.t. NHA1, respectively.

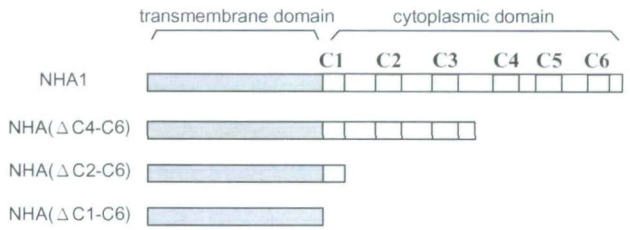



Fig. 6. **Schematic illustration of portions of *NHA1* integrated into the expression vectors.** Portions of S.c. and C.t. *NHA1* integrated into the expression vector pKT10 are shown. The total residues are 985 and 975 for S.c. and C.t. Nha1p, respectively. The transmembrane domains, shown by shadowed boxes, are residues between 1 and 433, and 1 and 432, for S.c. and C.t., respectively. The truncated forms contain the following residues: S.c. Nha1p ΔC4–C6, 1–647; ΔC2–C6, 1–449; ΔC1–C6, 1–447; C.t. Nha1 ΔC4–C6, 1–596; ΔC2–C6, 1–447; ΔC1–C6, 1–432. The conserved regions (C1–C6) are as follows. S.c. C1, residues 434–454; C2, 465–487; C3, 526–540; C4, 694–717; C5, 787–815; C6, 919–944. C.t. C1, 433–453; C2, 465–487; C3, 511–525; C4, 653–676; C5, 777–805; C6, 948–974.

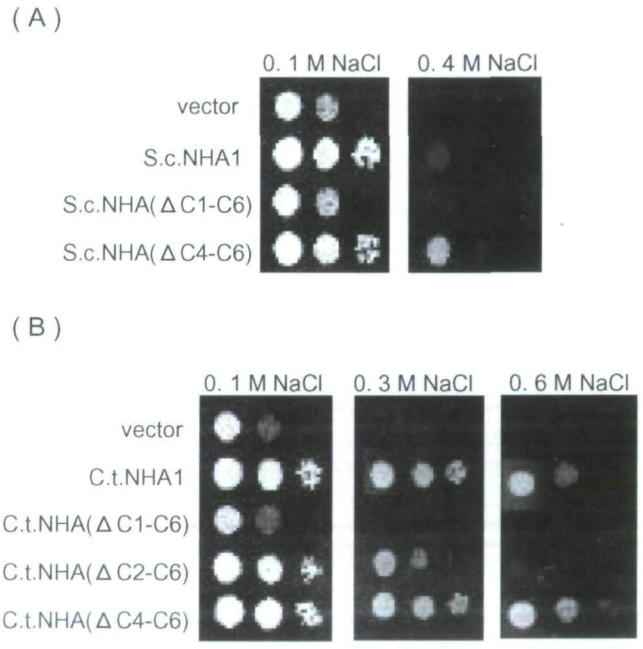



Fig. 7. **Complementation of salinity-sensitive SK5 by various truncated forms of S.c. and C.t. *NHA1*.** Cultures of SK5 cells transformed with various truncated forms of S.c. and C.t. *NHA1* were diluted serially and spotted on SD plates containing 0.1 M, 0.4 M, or 0.6 M NaCl. After incubation for 40 h at 30°C numbers of viable cells were counted. C1 to C6 correspond to the regions shown in Figs. 1 and 6.

least in terms of sensitivity to salinity, we analyzed whether the C-terminal structurally diverse regions are functionally similar. To test this, we again selected S.c. and C.t. Nha1p as a model because of the distant relationship of these species on the phylogenetic tree. Various forms of Nha1 with truncations in the C-terminal conserved regions were integrated into a high-copy–type expression vector (pKT10) and expressed in mutant S.c. cells (SK5) (Fig. 6). As shown in Figs. 7 and 8, SK5 cells transformed with the truncated form of Nha1 (ΔC4–C6) showed higher salinity resistance than the wild types of both S.c. and C.t. Nha1p. Since essentially the same effects were caused by the truncation (ΔC4–C6) for both Nha1ps, we thought that the high salinity resistance was due to the conserved regions, and that the C4–C6 region has an inhibitory effect on the $Na^+/H^+$ antiporter activity. C.t. Nha1p with a truncation of the C2–C6 region showed lower salinity resistance than the wild type but higher than the vector alone (Figs. 7 and 8). This result suggests that the C2–C3 region of C.t. Nha1p contributes to the activation of the $Na^+/H^+$ antiporter activity. Surprisingly, this truncation in S.c. Nha1p caused the transformed cells to die. This toxic effect was confirmed by conditional expression of the truncated form of Nha1 under the control of the GAL promoter (Fig. 9). In the presence of galactose to induce the gene, no viable transformed cells were observed, while the transformed cells were viable in the absence of galactose (Fig. 9). We tested whether excess production of the truncated form caused the lethality. For this test, DNA encoding the truncated form was integrated into the expression vector pKT10 under the transcriptional control of the original promoter region of S.c. Nha1p, so that the expression level of Nha1p became lower than it was under the GAP promoter in the original pKT10 vector. The cells transformed with this vector grew on the plates and showed a similar phenotype to the truncated form (ΔC2–C6) of C.t. Nha1p (data not shown). Finally, SK5 cells transformed by C.t. or S.c. *NHA1* without the entire hydrophilic C-terminal tail (Figs. 7 and 8) were able to grow in the absence of NaCl, unlike the S.c. Nha1p with the truncation of the C2–C6 region. However, the transformants of

truncated NHA1s (ΔC1–C6) for both S.c. and C.t. did not grow at 300 mM NaCl, basically the same result as obtained for the control vector alone (Figs. 7 and 8). These results suggest that the activity of the truncated forms of the $Na^+/H^+$ antiporter was very low. Thus the C1 region may be essential for the expression of the transporter activities of both S.c. and C.t. Nha1p. The results also suggest that excess expression of the C1 region of S.c. Nha1p is toxic.

*Expression of Nha1p in the Transformed Cells*—The expression of the various forms of S.c. and C.t Nha1p shown in Fig. 6 was analyzed using a specific antibody against the FLAG sequence fused to the C-terminal end of each Nha1p. All FLAG-tagged Nha1p showed essentially the same phenotype of cell growth and salinity tolerance as Nha1p without the tag (data not shown). Expression was detected only for two cases, the wild-type Nha1p and a truncated Nha1p (ΔC1–C6) of S.c., but not for any type of C.t. Nha1p nor for S.c. Nha1p with the C4–C6 truncation (Fig. 10). These results suggest that the expression of C.t. Nha1p and the truncated form (ΔC4–C6) of S.c. Nha1p is much lower than those of wild-type S.c. Nha1p and the truncated form (ΔC2–C6). It should be noted that S.c. Nha1p and the truncated form (ΔC1–C6) were detected only by the highly sensitive chemiluminescence method but not by the colorimetric method, which has lower sensitivity. We tested the expression of the cytoplasmic region (C5–C6) without the transmembrane region of S.c. Nha1p with the tag sequence carried on pKT10 under the GAP promoter and were able to detect this form by the colorimetric method (data not shown). These results suggest that the expression of membrane-bound forms of Nha1p in general are very low. The reason for the lower expression for C.t. Nha1p and the truncated form (ΔC4–C6) is not clear at present. Although the truncated form (ΔC4–C6) was not detected, the salinity resistance of cells expressing this form increased. Therefore, the increase in resistance is due not to increased expression but to enhanced antiporter activity.

Subcellular localization of S.c. Nha1p and the ΔC1–C6 truncated form was further analyzed by fractionation of yeast cells. The wild-type Nha1p was found in the cytoplasmic membrane fraction (P100) and also the nuclear and endocytic membrane fraction (P13) (Fig. 10). The truncated form was also found in the cytoplasmic membrane (P100), but much more protein was detected in the endocytic membrane fraction (P13). The relative amount of the protein in the cytoplasmic membrane fraction (P100) was significantly lower for the truncated form than for the wild type.

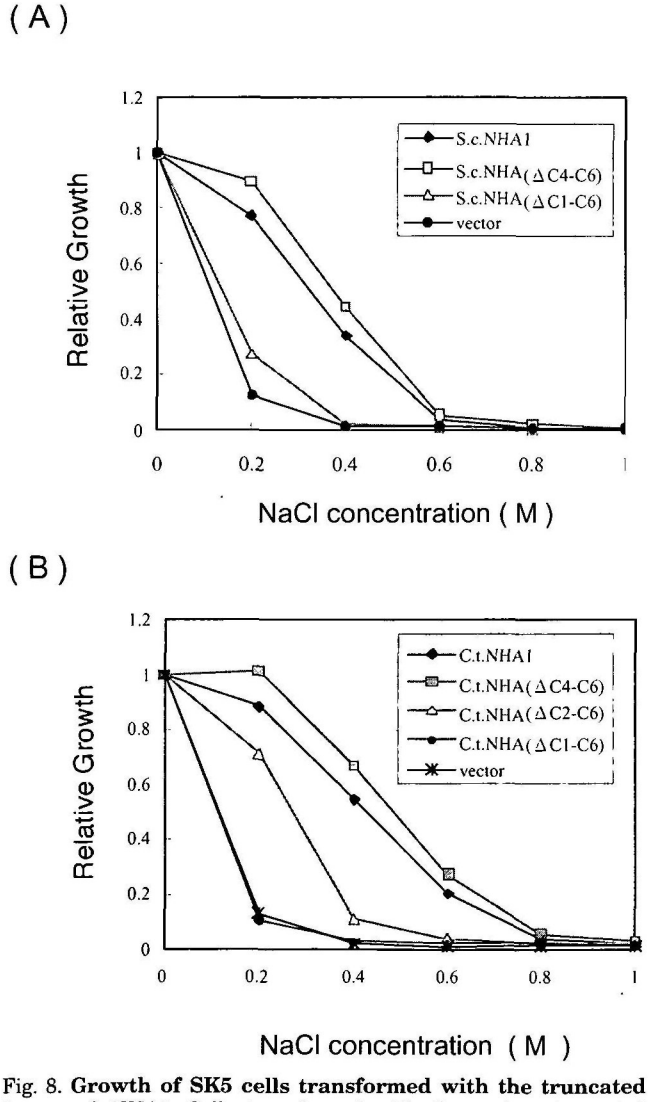



Fig. 8. **Growth of SK5 cells transformed with the truncated forms of *NHA1*.** Cells transformed with the various truncated forms as described in the Fig. 7 legend were cultured in SD medium with vigorous shaking at 30°C for 2 days. Growth of cells was estimated by measuring turbidity at 600 nm. The turbidity value after 2-day culture without NaCl was taken as 1.0.

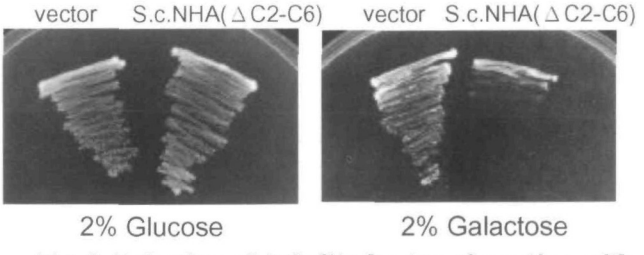



Fig. 9. **Induction of lethality by transformation with S.c. *NHA1* (ΔC2–C6).** SK5 cells transformed with expression vectors carrying S.c. or C.t. *NHA1* under the transcriptional control of the GAL promoter were inoculated onto SD medium plates containing 2% galactose or 2% glucose as an uninduced control. Plates were incubated at 30°C for 4 days.

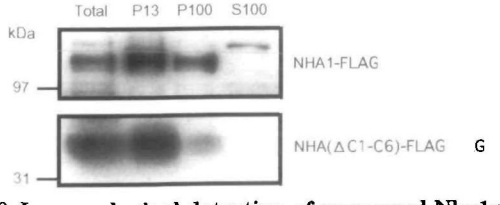



Fig. 10. **Immunological detection of expressed Nha1p in SK5.** SK5 cells transformed with various forms of Nha1p with the FLAG sequence at the C-terminal end were cultured in SD medium and harvested. Harvested cells were fractionated as described in Materials and Methods. Aliquots of the P13, P100, and S100 fractions equivalent to 30 μg of total fraction were subjected to SDS–polyacrylamide gel electrophoresis and blotted onto a GVHP filter. Nha1p was detected using anti-FLAG antibodies and chemiluminescence methods.

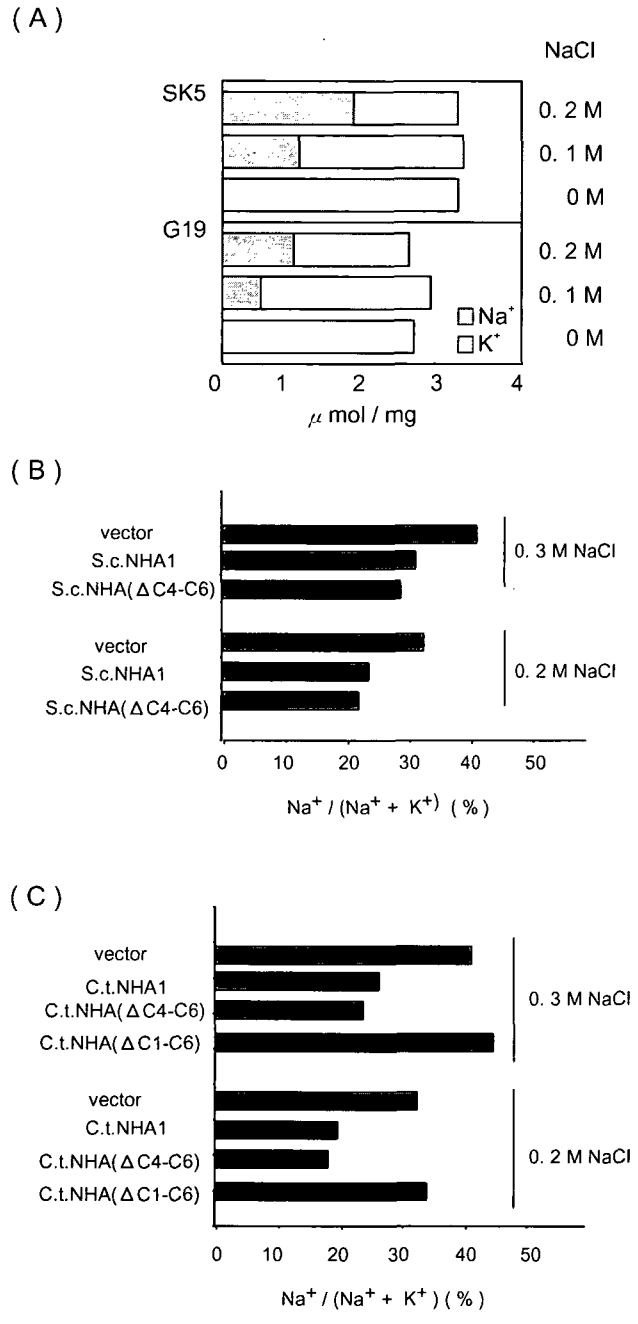



Fig. 11. **Intracellular $Na^+$ and $K^+$ concentrations in the transformants of various *NHA1s*.** (A) Yeast G19 or SK5 cells were grown in SD medium containing 0.1 or 0.2 M NaCl. Intracellular $Na^+$ or $K^+$ was measured by optical emission spectrophotometry. Concentrations of the ions were normalized against the total protein of the cells. (B) SK5 cells transformed with S.c Nha1, S.c. *NHA1* (ΔC4–C6), or control vector alone were grown in SD medium containing 0.2 or 0.3 M NaCl and intracellular ion concentrations were measured. Concentrations of $Na^+$ relative to total concentrations of $Na^+$ and $K^+$ are shown as percentages. (C) SK5 cells transformed with C.t. *NHA1*, truncated forms (ΔC4–C6 and ΔC1–C6), and the control vector alone were grown in SD medium containing 0.2 or 0.3 M NaCl, and intracellular concentrations of the ions were measured. Concentrations of $Na^+$ relative to total concentration of $Na^+$ and $K^+$ are shown as percentages.

*Intracellular $Na^+$ and $K^+$ Concentrations in the Various NHA1 Transformants*—Intracellular $Na^+$ concentrations were determined in order to estimate the correlation between Nha1p activity and salinity sensitivity. Intracellular $Na^+$ concentrations for G19 lacking *ENA1* were compared with those for SK5 in the presence or absence of NaCl (Fig. 11). Since total concentrations of $Na^+$ and $K^+$ have been reported to be kept constant (*37*), $K^+$ concentrations as well as $Na^+$ concentrations were measured. As shown in Fig. 11, intracellular $Na^+$ concentrations changed depending on the NaCl concentrations outside of the cells, but total concentrations of intracellular $Na^+$ and $K^+$ were kept constant as expected. For SK5 lacking both *NHA1* and *ENA1*, intracellular $Na^+$ concentrations were higher than those for the cells lacking *ENA1* alone. These results support the idea that lack of *NHA1* increases the intracellular $Na^+$ concentration because of lack of $Na^+/H^+$ exchange activity. Cells transformed with the wild-type and the ΔC4–C6 of S.c. Nha1p (Fig. 6) showed lower $Na^+$ concentrations than those of the control vector alone. This mutant Nha1p reproducibly showed slightly lower intracellular $Na^+$ concentrations than the wild type. C.t. Nha1p lacking C4–C6 showed lower $Na^+$ concentrations than the control vector but also slightly lower concentrations the wild type, as was seen for S.c. Nha1p. S.c. and C.t. Nha1p lacking the entire hydrophilic region showed $Na^+$ concentrations as high as the control.

The intracellular concentration of $Na^+$ in cells transformed by various forms of Nha1p from S.c. and C.t. corresponded well with their phenotype in terms of salinity-resistant growth. The transformants that showed salinity-sensitive cell growth showed higher intracellular $Na^+$ concentrations, while the salinity-resistant cells showed lower $Na^+$ concentrations (Fig. 11). Thus, the differences in growth depending on the salinity conditions are due to the Nha1 activity in the transformants.

## DISCUSSION

We cloned genes encoding the $Na^+/H^+$ antiporter Nha1p from three different yeast and fungal species, H.a., C.t., and A.n. The amino acid sequences deduced from the determined nucleotide sequences of these genes revealed that the sequences contain a highly hydrophobic membrane domain and a hydrophilic C-terminal domain. Our first question in this study was whether the hydrophilic C-terminal region found in Z.r. and S.c. is generally present in yeast and fungal species. During the preparation of this paper, the Nha1p sequence from *Candida albicans* (C.a.) was reported (*40*). The sequence of Nha1p is now known from seven species, including the three species in this study, C.a., and three species reported previously, and the C-terminal hydrophilic sequence is found in all but S.p. (Sod2p or S.p. Nha1p). The sequences of the hydrophobic N-terminal regions that may be transmembrane domains were highly conserved among these Nha1ps including S.p., supporting the notion that S.p. Nha1p belongs to a family of Nha1p proteins. On the other hand, the hydrophilic C-terminal region is not conserved and is even missing in S.p. In S.p. Nha1p, Asp-241, Asp-266, and Asp-267 have been reported to be essential for the transport of $Na^+$ and $Li^+$ but not for $K^+$ (*41*). Functionally essential Asp residues are conserved among the seven Nha1ps and have been also found

in the $Na^+/H^+$ antiporters of *E. coli* (NhaA) and *Vibrio alginolyticus* (*42, 43*). Therefore, we support the idea that the amino terminal region of Nha1ps may form an essential pathway in the membrane for ion transport (*1, 40*). Although the Nha1p sequences of C.t. and C.a. are highly conserved, the sequences in the C-terminal hydrophilic regions of the other species are very diverse. The presence of an isoform of Z.r. Nha1p (Zsod2p) has been reported for Z.r. (Zsod22p) (*44*), and a sequence homologous to this isoform has been described in the sequence database of S.p. (tentatively named Sod22p). A hydrophilic C-terminal domain is observed in the Sod22p sequence. Currently, it can be suggested that the C-terminal hydrophilic region has an important but non-essential function for the expression of Nha1p activity, because most of these phylogenetically distant species had the hydrophilic regions, with the one exception of S.p. (Sod2p). Although in the present study we found only one *NHA1* each from H.a., C.t., and A.n., another isoform might exist, because two isoforms have been found in S.p. and Z.r. We found two positive clones on chromosome 5 of A.n. in a hybridization assay, which might represent two isoforms.

The second purpose of this study was to learn whether the structurally diverse hydrophilic regions are also functionally diverse. The three sequences of Nha1p newly determined in this study, together with the previous four sequences, enabled us to search for homologous sequences in small parts of the diverse C-terminal domain. We found that six regions in the C-terminal domain are relatively conserved (C1 to C6). We have not yet found any sequences that are significantly homologous to these sequences by searching databases of protein sequences. We analyzed the functional conservation of these homologous sequences between S.c. and C.t., which are the most phylogenetically distant among the seven species. Truncation of the C4–C6 region caused an increase in salinity resistance, suggesting enhancement of $Na^+/H^+$ antiporter activity and a potential inhibitory role of the C4–C6 region on this activity. As shown here, although the overall sequences of the C-terminal region are diverse, the phenotype caused by the truncation was similar for both species. Therefore, the putative mechanism by which Nha1p activity is enhanced may be based on the conserved structures C4, C5, and/or C6. We found that truncation of the sequence between residue 910 and the C-terminus of C.t. Nha1p causes the same enhancement of salinity resistance as the truncation of the C4–C6 region (data not shown). This result suggests that the C6 region has an important role. Kinclova *et al.* recently reported (*45*) that truncation between residues 916 and the C-terminal end of S.c. causes an enhancement of salinity resistance to high concentrations of NaCl in culture medium. This observation is essentially the same as our observation for C.t. Nha1p. Therefore, the enhancing mechanism may be ascribed to C6. Truncation of the C2–C6 region resulted in lower salinity resistance than seen in the wild type, suggesting a decrease in the $Na^+/H^+$ antiporter activity and an activating role for the C2–C3 region in the transport activity.

Two possible explanations can be considered for the enhancement or decrease of salinity resistance caused by the truncations of the C4–C6 or C2–C3 regions, respectively. One is alteration of the expression level of the truncated peptides compared to the wild type; the other is alteration of transport activities. Levels of both truncated forms were too low to detect immunologically in this study, although we could detect wild-type S.c. Nha1p (Fig. 10). These results suggest that the enhancement by the truncation of the C4–C6 region is due to activation of the transporter activity and that the decrease induced by truncation of the C2–C3 region is based on decreased expression level. However, a more precise quantitation of expressed Nha1p may be required to draw a firm conclusion.

An important finding in this study is that the complete loss of the C-terminal hydrophilic domain (C1–C6) caused extensive loss of the salinity resistance for both S.c. and C.t. Nha1p, suggesting the loss of $Na^+/H^+$ antiporter activity. We observed that the ΔC2–C6 form of C.t. Nha1p showed higher salinity resistance than the ΔC1–C6 form. Based on these results, we believe that the extensive loss of the antiporter activity accompanying the loss of the entire hydrophilic region of Nha1p can be attributed solely to the loss of the C1 region rather than to the loss of the entire C-terminal region. Analysis of a truncated form of the C-terminal region of S.c. Nha1p has recently been reported elsewhere (*45*), in which truncation from the C-terminal end up to a position within C2 but not including C1 was studied. Expression of the truncated form of C1–C6 was found in the total extracts of transformed cells, but significantly lower levels of the protein were detected in the membrane fraction (P100, Fig. 10) compared to the wild-type Nha1p. These results suggest that the targeting of the truncated form to the membrane was affected and that the C1 region has an essential role in directing Nha1p to the membrane surface. Since we could not detect the truncated form of C2–C6 (ΔC2–C6) of S.c and C.t. Nha1p by immunological assay, a more precise analysis of the expression of the truncated form of ΔC2–C6 is needed to obtain a firm conclusion about its location. It should be noted that the C1 sequence exists even in S.p. Nha1p, which also suggests an essential role for C1 in the functional expression of Nha1p.

Despite the necessity of C1, the addition of C1 to the membrane domain of S.c. Nha1p was unexpectedly lethal to the transformed cells. This effect was not observed for C.t. Nha1p nor even for S.c. Nha1p expressed at low levels. We observed that every form of C.t. Nha1p was expressed in SK5 at a lower level than S.c. Nha1p, as detected by immunological analyses. Taken together, these observations suggest that an excess of the C1 region is toxic for cell growth. In the wild-type Nha1p this toxic effect of the C1 region may be masked to certain extent. We observed that SK5 cells transformed by C.t. *NHA1* adapted to high salinity conditions more quickly than those transformed by S.c. *NHA1*. This difference could be due to the difference of expression levels of Nha1p in the two species. Overexpression of Nha1p might be also toxic because of the toxic effect of the C1 region. The mechanism of the putative functional role of the C1 region in transporting Nha1p to the membrane surface is also of interest, and we are currently studying this.

Simon *et al.* recently reported (*46*) that the residues between 800 and 948 in S.c. Nha1p have an important role in the cell cycle and in $K^+$ transport but not in $Na^+$ transport. This domain is a diverse region between C5 and C6, and its functions are specific for S.c. Nha1. Our results and this observation suggest that the C-terminal halves in yeast and fungal Nha1p are comprised of conserved regions

with conserved regulatory roles and non-conserved regions outside of C1 to C6 with species-specific functions that are mostly not identified at present.

The authors thank Dr. Hiroshi Murakami and Dr. Hajime Muraguchi for their helpful discussion and encouragement during this study.

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