Identification of a Mitochondrial Na$^+$/H$^+$ Exchanger*

(Received for publication, October 28, 1998)

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The electroneutral exchange of protons for Na$^+$ and K$^+$ across the mitochondrial inner membrane contributes to organellar volume and Ca$^{2+}$ homeostasis. The molecular nature of these transporters remains unknown. In this report, we characterize a novel gene (YDR456w; renamed NHA2) in Saccharomyces cerevisiae whose deduced protein sequence is homologous to members of the mammalian Na$^+$/H$^+$ exchanger gene family. Fluorescence microscopy showed that a Nha2-green fluorescent protein chimera colocalizes with 4',6-diamidino-2-phenylindole staining of mitochondrial DNA. These data indicate that Nha2 and NHE6 encode homologous Na$^+$/H$^+$ exchangers and suggest they may be important for mitochondrial function in lower and higher eukaryotes, respectively.

Electroneutral monovalent cation/proton antiporters or exchangers are important components of mitochondrial volume and Ca$^{2+}$ homeostasis. In mammals, the latter is achieved by an integrated cycle of distinct Ca$^{2+}$ uniport and Na$^+$/H$^+$ and Na$^+$/Ca$^{2+}$ antiport pathways located in the inner membrane (1–6). Matrix Ca$^{2+}$ influences dehydrogenases of the tricarboxylic acid cycle (7, 8), F$_{1}$F$_{0}$-ATPase (9), and adenine nucleotide translocase (10, 11) and has been proposed as a mechanism for coordinating energy (ATP) production with cellular requirements (7, 8, 12). Although mitochondrial Ca$^{2+}$ levels depend on Na$^+$/H$^+$ exchangers and the inner membrane Na$^+$ and H$^+$ gradients, the transporters have not been identified at the molecular level.

Functional studies suggest there are at least two distinct types of monovalent cation/proton exchangers in mammalian mitochondria. One of these preferentially mediates the exchange of matrix Na$^+$ for intermembrane H$^+$ generated by respiration (i.e. Na$^+$-selective Na$^+$/H$^+$ exchanger (NHE1)) (1, 13). This mechanism also transports Li$^+$ (and possibly NH$_4^+$) at a much lower rate and is inhibited by benzamil derivatives of amiloride (14–17). It is constitutively active in respiring mitochondria and is primarily responsible for establishing the [Na$^+$]/[H$^+$] gradient ([Na$^+$]$_{m}$ < [H$^+$]$_{i}$) that allows Na$^+$-dependent extrusion of matrix Ca$^{2+}$ (18). The other monovalent cation/H$^+$ exchanger is latent, transports all alkali cations (i.e. Li$^+$, Na$^+$, K$^+$, Rb$^+$, and Cs$^+$) at similar rates, and is antagonized by quinine, dicyclohexylcarbodiimide, and propranolol (19–21). Since K$^+$ is the predominant intracellular alkali cation, it is usually referred to as a K$^+$/H$^+$ exchanger. In isolated mitochondria, it is active only upon depletion of matrix Mg$^{2+}$ and simultaneous elevation of matrix pH or upon hypotonic swelling (19, 21, 22). It is postulated to play a role in organellar volume homeostasis (23). Mitochondria from Saccharomyces cerevisiae possess a similar nonselective K$^+$/H$^+$ exchanger, although the yeast transporter can be activated without depletion of matrix Mg$^{2+}$ (24). However, functional studies of yeast mitochondria have not revealed any Na$^+$-selective NHE activity (25).

In addition to their role in mitochondria, mammalian NHEs participate in a wide array of other essential cellular processes, including control of intracellular pH (pH$_{i}$), maintenance of cellular volume, and reabsorption of Na$^+$ across renal, intestinal, and other epithelia (26, 27). Activation of NHE activity also facilitates growth factor-induced proliferation of certain cell types (27) and is associated with events leading to apoptosis (28–30). To date, five NHE isoforms (NHE1 to NHE5) have been identified that exhibit considerable differences in their primary structures (34–60% identity), tissue distribution, membrane localization, biochemical and pharmacological properties, and responsiveness to various stimuli (31, 32). While characterization of some of these isoforms is incomplete, the existing data do not support a direct role for any of them in mitochondrial function.

To identify mitochondrial type NHEs, we have focused our attention on S. cerevisiae because the sequence of the entire genome is known and because of the ease with which this organism can be manipulated genetically and analyzed functionally. In addition, yeast are facultative aerobes that can proliferate in the absence of aerobic respiration, which permits in vivo studies of mutations that disrupt mitochondrial function. To date, a S. cerevisiae gene (NHA1) conferring tolerance to increasing concentrations of external NaCl has been isolated (33), and its protein product was found to be homologous (40% amino acid identity) to the cell surface Na$^+$-H$^+$ antiporter Sod2

1 The abbreviations used are: NHE, Na$^+$-H$^+$ exchanger; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; GFP, green fluorescent protein; TES, 2-[(hydroxy-1,1-bis(hydroxymethyl)ethyl] amino) ethanesulfonic acid.
of Schizosaccharomyces pombe, which is essential for Na+ excretion (34). Further examination of the *S. cerevisiae* genome has revealed 165 unique open reading frames (YDR456w, YJL094c, YJR106w, YAL022c) whose encoded protein sequences show similarity to Na+/H+ exchangers. YDR456w is of particular interest, since PSORT analysis (35) predicts that its protein product would be situated in the mitochondrial inner membrane due to potential targeting signals at its N terminus. To test this hypothesis, we isolated the YDR456w gene (renamed NHA2) and show that its protein product is localized to yeast mitochondria. We also characterized its function in isolated mitochondria and demonstrated its physiological role by comparing wild-type yeast with an iso- enic strain lacking this gene. Yeast NHA2 also has strong sequence similarity to a partial cDNA (KIAA0267) that was recently cloned from a human bone marrow cell line.2 We report the complete primary structure and tissue distribution of this novel human isoform (renamed NHE6) and show that it is also localized exclusively in mitochondria.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carrier-free, 25 NaCl (range of specific activity, 300–900 mCi/mg; concentration, 5 mCi/mL) and 15 NaCl (range of specific activity, 2 mCi/mL; concentration, 1 mCi/mL) were purchased from Radiochemical Centre (Amersham, Buckinghamshire, England). 1,2,3,4-2H4-D-glucose (98 atom %, specific activity 1.1 mCi/mg), 1,2,3,4-2H4-NaCl (1 atom %, specific activity 1.7 mCi/mg), and EDTA-Na2 (98 atom %, specific activity 1.1 mCi/mg) were purchased from NEN Life Sciences Products (Mississauga, Ontario, Canada). Benzamid, quinine, rotenone, and phenylmethylsulfonyl fluoride were purchased from Sigma. α-Minimal essential medium, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were purchased from Life Technologies (Burlington, Ontario). Cell culture dishes and flasks were purchased from Becton Dickinson and/or Corning (Montréal, Québec, Canada). All other chemicals and reagents used in these experiments were purchased from BDH Inc. (St. Laurent, Québec, Canada) or Fisher and were of the highest grade available.

**Strains and Media**—The *S. cerevisiae* strains used in this study are SEY6210 (MATa, leu2–3, 112, ura3–52, his3–200, lys2–801, trp1–200) [kindly provided by Dr. Marc Lessier, McGill University] (36) and L4244 [kindly provided by Dr. Gerald R. Fink, Whitehead Institute, Cambridge, MA]. A rich medium (YPD) containing 1% Bacto-peptone, 2% Bacto-tryptone, and 2% dextrose or a minimal medium (SD) containing 0.67% yeast nitrogen base and 1% dextrose supplemented with required amino acids was used for standard growth of *S. cerevisiae*. To analyze mitochondrial function, 2% of galactose, glycerol, succinate, or lactate was substituted as the carbon source.

**Measurement of Yeast Survival in the Stationary Phase**—Wild type and the corresponding Δnha2 strains were inoculated into 10 ml of YPD medium and grown at 30°C in a shaking platform incubator. Incubation for 2, 10, and 20 days, optical densities of the cultures were determined by absorbance at 600 nm. Aliquots of equivalent optical densities were plated on YPD plates and cultured for a further 2 days.

**Molecular Cloning**—The anonymous open reading frame YDR456w in *S. cerevisiae* corresponding to gene D9461.40 (renamed NHA2) was cloned by polymerase chain reaction (PCR) based on the known sequence (GenBank™ accession number U33007). The sequence identity and fidelity was confirmed by DNA sequencing.

A partial human cDNA (KIAA0267) showing homology to members of the NHE gene family was generously provided by Dr. T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). To obtain the full-length sequence, we extended the partial cDNA sequence by 5′-rapid amplification of its cDNA end (5′-RACE) (37) using Marathon-Ready™ cDNAs from human kidney (CLONTECH, Palo Alto, CA) as a template. For the initial PCR, the 5′-oligonucleotide primer was API, which corresponded to a sequence within the 5′-adaptor (CLONTECH), and the 3′-primer (5′-GCTTCTCAGACGGCAGCTCCCGCC-3′) was derived from the 5′-sequence of KIAA0267. Five μl of the initial 25 μl PCR product was reamplified with a nested 5′-adaptor primer AP2 (CLONTECH) and a nested 3′-primer (5′-GCTTCTCAGACGGCAGCTCCCGCC-3′). The PCR products were resolved by electrophoresis on agarose gels, isolated, subcloned into the pGEM-T vector (Promega, Madison, WI), and analyzed by DNA sequencing. Then, a NotI–Nol fragment was isolated from one of the 5′-RACE products containing the missing sequence and ligated to the partial cDNA to obtain the full-length sequence.

**Mammalian Cell Culture**—HeLa cells were maintained in complete α-minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mm of NaHCO3 (pH 7.4) and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Northern Blotting**—A human poly(A+) RNA blot was purchased from CLONTECH and hybridized with a 0.6-kilobase pair Clal–EcoRI fragment isolated from the 3′-end of the cDNA that exhibited little sequence identity to the other known mammalian NHE isoforms. The probe was radiolabeled with [32P]dCTP by the random primer method (Pharmacia Biotech Inc.) and hybridized at 68°C for 1 h in the Express HybTM buffer (CLONTECH). The blot was washed twice in 2× SSC, 0.05% SDS at room temperature for 30 min each followed by a high stringency wash in 0.1× SSC, 0.1% SDS at 50°C for 40 min. The radioactive signals were analyzed using a PhosphorImager (Molecular Dynamics).

**Gene Disruption in Saccharomyces cerevisiae**—Creation of null alleles in yeast was accomplished using the PCR-based gene disruption technique (38, 39). This was accomplished using the pBM2983 plasmid carrying a gene replacement cassette, which encompasses a promoterless green fluorescent protein (GFP) open reading frame from the jellyfish *Aequorea victoria* and a tandemly located HIS3 marker with its own promoter (kindly provided by Dr. J. H. Hegemann, Justus-Liebig-Universität Giessen, Germany) (39). GFP emits green fluorescence (sharp peak at ~505 nm) when excited between 450 and 490 nm and can be followed in both living and formaldehyde-fixed cells. The presence of GFP in the replacement cassette is obtained by checking the regulation of the endogenous NHA2 promoter, although this was not undertaken in the current study. The replacement gene was amplified by PCR using the following primers. Primer 1 (5′-oligonucleotide) contains 45 nucleotides immediately 5′ of the ATG start codon of NHA2 followed by 19 nucleotides of the GFP coding region beginning at the ATG start codon (5′-TCAGAGGATATTCCTATCGCTGCGTACACACAAATCGGCGCCTTGTTCA-3′); primer 2 (3′-oligonucleotide) contains 45 nucleotides immediately 3′ of the stop codon of NHA2 followed by 19 nucleotides of the HIS3 coding region beginning 154 bp downstream of the HIS3 stop codon (5′-TTAATATATATATTAGAAAAAAGAAACCATACATTTAAGGCGCCTTGTTCA-3′). The PCR fragment was transformed into the SEY6210 haploid yeast strain by the lithium acetate/single-stranded DNA/polyethylene glycol method (40) and grown on histidine-deficient medium. Homologous recombinants were selected by using the HIS3 auxotrophic marker and expression of GFP (under the control of the NHA2 promoter). Deletion of NHA2 was confirmed by PCR analysis of genomic DNA using specific primers to an upstream region of the NHA2 promoter and an internal sequence of GFP.

**Construction of Na+/H+ Exchanger-Green Fluorescent Protein Chimera**—The PCR fragment corresponding to the entire open reading frame of *S. cerevisiae* NHA2, but excluding the termination codon, was digested with XhoI and CfoI and cloned into the corresponding site in the yeast expression vector pGFP-C-FUS (kindly provided by Dr. Hege- mann) (39). This resulted in an NHA2-GFP fusion construct under control of the methionine-repressible MET25 promoter (pGFP-C-FUS-NHA2). To construct a human NHE6-GFP chimera, an XhoI site was introduced by PCR at the C-terminal end of the NHE6 open reading frame just before the stop codon. A NotI–XhoI fragment containing the entire NHE6 coding sequence was then fused in frame with the XhoI–EcoRI fragment of cDNA encoding a modified GFP exhibiting enhanced fluorescence (GFP-cycle 3) (41), and then subcloned into the mammalian expression vector pIREs-neo (CLONTECH).

**Subcellular Localization**—The pGFP-C-FUS control plasmid and pGFP-C-FUS-NHA2 fusion construct were transformed into SEY6210 haploid strain and ΔURA1 colonies were selected on SD plates supplemented with Leu, His, Tryp, and Lys. Transformed cells were inoculated into SD medium devoid of uracil and methionine to maintain selection and to induce high levels of expression from the MET25 promoter, respectively. To stain mitochondria in living cells, exponentially growing cells were incubated in SD medium plus 2.5 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 min at 30°C, followed by fixation by incubation for 30 min in SD medium containing 3.7% formaldehyde. Under these conditions, mitochondrial DNA is preferentially stained compared with nuclear DNA (42). The transformed cells were examined by fluorescence microscopy (Nikon) with the appropriate filter sets (filter set UV-1A for DAPI: excitation 365 ± 10 nm, emission 400 nm; filter set B-2A for GFP: excitation 450–490 nm, emission 520 nm).

HeLa cells were transiently transfected with the human NHE6-GFP fusion construct by the calcium phosphate method (43). Following transfection, cells were kept for 24 h at 37°C and then incubated at a
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reduced temperature (30 °C) for another 36 h to enhance the fluorescence signal of GFP, as described previously (44). Mitochondria were selectively stained by incubating the cells with 25 nM of the Mitotracker Red CM-H₂XRos™ dye (Molecular Probes, Eugene, OR) for 20 min at 37 °C. This dye is not fluorescent until it enters metabolically active cells, where it is oxidized to the fluorescent probe and sequestered into the mitochondria. The results were confirmed by confocal laser microscopy using a Zeiss LSM 410 inverted microscope. The Mitotracker Red CM-H₂XRos™ dye was excited with a helium/neon (543-nm) laser and was imaged on a photomultiplier after passage through FT560 and LP590 filter sets. The NHE6-GFP was imaged by exciting the sample with a 488-nm line from an argon/krypton laser, and the resulting fluorescence was collected on a photomultiplier after passage through FT510 and BP515–540 filter sets. All images were line averaged and printed on a Kodak XLS8300 high resolution (300 dots per inch) printer. Optical sections were taken using a × 63, 1.4 NA objective (optical thickness, ~1.0 μm).

Measurement of 22Na⁺ Influx into Isolated Mitochondria—Mitochondria from S. cerevisiae wild-type and Δnha2 strains, which were grown on 2% glycerol-containing medium, were isolated by differential centrifugation as described by Yaffe (45). The mitochondrial pellet was resuspended in 0.25 m sucrose, 2 mM Tris-HCl (pH 7.4) in a concentration of 50 μg of original cells. NHE activity was assessed by measuring 22Na⁺ fluxes as described previously (14). Briefly, mitochondria (75 μg) were treated with 4 μg/ml rotenone in 300 μl of buffer containing 100 mM mannitol, 45 mM sodium acetate, 20 mM TES (pH 7.2), and 1 mM EGTA for 6 min at room temperature. The mitochondrial suspension was incubated with 1.5 μCi of 22Na⁺ for 15 s, immediately diluted in 5 ml of ice-cold stop solution (0.1 mM LiCl), filtered through a 0.3-μm Millipore filter (Millipore Corp., Bedford, MA), and washed three times with 5 ml of ice-cold stop solution. Each experiment was replicated 3–5 times from the same mitochondrial preparation.

RESULTS

Subcellular Localization of S. cerevisiae Nha2—Analysis of the primary structure of S. cerevisiae Nha2 using PSORT (35), which estimates the subcellular localization of proteins in eukaryotic cells based on known targeting motifs, predicted that it would be localized in the mitochondrial inner membrane due to targeting signals at its N terminus. These signals are highly degenerate residues (20–30) capable of forming a positively charged amphiphilic helix, which direct the protein to the mitochondrial matrix, followed by one or more hydrophobic α-helical segments that arrest the protein in the membrane (46). To test the hypothesis that Nha2 is targeted to mitochondria, we amplified the NHA2 gene by PCR from S. cerevisiae genomic DNA and inserted it into the green fluorescent protein fusion vector pGFP-C-FUS (39) in the proper open reading frame to create an NHA2-GFP fusion gene (GFP at the C terminus of Nha2) under the control of the methionine-regulated MET25 promoter. The sequence of NHA2 was verified to ensure that mutations were not introduced during gene amplification. This approach has been used successfully for studying the expression and subcellular localization of other genes in budding yeast (39). The pGFP-C-FUS-NHA2 fusion plasmid and the pGFP-C-FUS control plasmid were transformed into the yeast strain SEY6210 and maintained in medium lacking uracil (to select for transfectants) and methionine (to induce high levels of expression from the MET25 promoter). GFP fluorescence was monitored in formaldehyde-fixed yeast cells using fluorescence microscopy. Mitochondria were detected using the fluorescent DNA-binding dye, DAPI, under conditions that stain yeast mitochondria more intensely than nuclear DNA (42). As shown in Fig. 1A, DAPI staining was observed at discrete sites along the periphery of a representative cell, which are presumed to be mitochondria, and these co-localized with the fluorescence signal of the Nha2-GFP fusion protein (Fig. 1B). By contrast, expression of unfused GFP, which lacks membrane targeting motifs, showed a more diffuse green fluorescence throughout the cytoplasm (Fig. 1C). These data are consistent with the localization of Nha2-GFP to mitochondria.

A

B

C

Fig. 1. Localization of a Nha2-GFP chimera to mitochondria in transformed yeast cells. A, exponentially growing cells were incubated in SD medium plus DAPI for 30 min at 30 °C, followed by fixation (42). Under these conditions, mitochondrial DNA is preferentially stained compared with nuclear DNA. A representative cell is shown where mitochondrial DNA appears as dots or bands along the cell periphery. B, a wild-type strain (SEY6210) was transformed with the NHA2-GFP fusion construct, and cells were grown in SD medium lacking uracil and methionine, fixed, and examined by fluorescence microscopy. C, a wild-type strain (SEY6210) was transformed with a GFP control plasmid and processed as indicated above. The scale bar in C represents 2 μm.

Functional Characterization of S. cerevisiae Nha2 Protein—To define the function of yeast Nha2, the entire open reading frame of NHA2 was replaced with a cassette containing the HIS3 auxotrophic marker. Gene disruptants were selected by their ability to grow on histidine-deficient medium, and deletion of NHA2 was confirmed by PCR analysis (data not shown). As illustrated in Fig. 2, mitochondria isolated from the wild-type strain displayed measurable acid-induced 22Na⁺ influx that was inhibited by 100 μM benzamil, an amiloride analogue that preferentially antagonizes mitochondrial NHE activity (14–17) compared with plasma membrane NHEs (47, 48). 22Na⁺ influx was also inhibited by Li⁺ (100 μM), which antagonized Na⁺ transport (data not shown). By comparison, benzamil-inhibitable 22Na⁺ influx was absent in the Δnha2
mitochondria. These data support the notion that Nha2 is a functional Na⁺/H⁺ exchanger in yeast mitochondria.

To assess the physiological consequences of deleting the NHA2 gene on mitochondrial function, both the wild-type SEY6210 and mutant Δnha2 strains were subjected to different growth conditions that rely primarily on aerobic metabolism for energy (45, 49). The Δnha2 cells showed slightly retarded growth on nonfermentable carbon sources such as galactose when compared with wild-type, indicating that mitochondrial function was compromised (Fig. 3). Similar results were also obtained when cells were grown on succinate or lactate as the carbon source (data not shown). More drastic effects were observed for long term survival of the Δnha2 cells in the stationary phase (49), which was considerably reduced at day 10 under conditions (nutrient depletion) that had no noticeable effect on the viability of the wild-type strain (Fig. 4). However, a few Δnha2 cells retained wild-type growth (i.e. large colony size), suggesting that secondary mechanisms exist that modulate the mutant phenotype. A further reduction in survival of the Δnha2 cells was observed at day 20 of the stationary phase (data not shown).

**Similarity of Nha2 with Other Na⁺/H⁺ Exchangers—**An extensive search (50) of sequences in the GenBank™ database identified putative NHE homologues in *Caenorhabditis elegans* (F57C7.2) and *Homo sapiens* (KIAA0267) with strong amino acid identity (27 and 30%, respectively) to *S. cerevisiae* Nha2. *C. elegans* F57C7.2 and *H. sapiens* KIAA0267 exhibited even higher identity (34%) to each other. By comparison, *H. sapiens* KIAA0267 showed lower amino acid identity (~20–24%) to other mammalian NHE isoforms (NHE1–NHE5). The closer relatedness of *C. elegans* F57C7.2 and *H. sapiens* KIAA0267 to *S. cerevisiae* Nha2 suggested that they may fulfill a common function.

Further examination of the human KIAA0267 cDNA indicated that the 5'-region seemingly lacked nucleotide sequence containing the initiation methionine codon, although a potential in-frame AUG start site is present 67 nucleotides from the extreme 5'-end. To determine if other possible start codons exist upstream, we extended the sequence by 5'-RACE (37) using nested oligonucleotide primers and a single-stranded cDNA library generated from human kidney mRNA as a template. One of the generated PCR products (Fig. 5A) contained 175 bp of overlapping sequence with the 5'-region of KIAA0267 and an additional 44 nucleotides, which encoded three amino acids starting from an AUG in frame with the putative open reading frame. This AUG codon is preceded by an in-frame TAG stop codon at nucleotide position 24, thereby making it a likely site for translation initiation. Preferred purine nucleotides are also present at positions −6, −3, and +4 relative to the apparent translation initiation codon (AGGAGA CAUGG), placing it in a favorable context for initiation by eukaryotic ribosomes that recognize the optimal consensus sequence GCC(A/G)GCCAUGG, as defined by Kozak (51).

The combined nucleotide sequence of KIAA0267 (renamed NHE6) contains 35 nucleotides of 5'-untranslated sequence, a 2010-nucleotide open reading frame that ends with a TAA stop codon, and 2407 nucleotides of 3'-untranslated sequence. The cDNA did not terminate with a poly(A)⁺ tract, although it does contain a potential polyadenylation signal near the 3'-end of the cDNA at position 4442 (AAUAAA), which is similar to the classical hexamer signal (AAUAAA) (52, 53). Nevertheless, it is possible that part of the 3'-untranslated region of the mRNA is missing from the cDNA. The primary sequence deduced from the cDNA is 669 amino acids in length and has a calculated Mr of 74,163 (Fig. 5B).

Hydropathy analysis (54) of NHE6 indicates 12 hydrophilic membrane-spanning segments within the N-terminal region that may form α-helices and a hydrophilic region in the C terminus that may reside in the matrix (Fig. 5C), similar to the topologies predicted for other NHEs.

A comparison of the primary structures of human NHE6, *C. elegans* F57C7.2, and *S. cerevisiae* Nha2 with that of human plasma membrane NHE1 is presented in Fig. 5B. The alignment shows that the greatest similarity occurs in the membrane-spanning segments, whereas the putative hydrophilic tails are more divergent. PSORT analysis predicted that hu-
man NHE6 also contains a putative mitochondrial inner membrane targeting signal at its N terminus. By contrast, the *C. elegans* F57C7.2 sequence was predicted to localize to the plasma membrane, although from the alignment shown in Fig. 5B, it is also possible that F57C7.2 is a partial sequence lacking the mitochondrial targeting signal at its N terminus.

### Tissue Distribution of Human NHE6

If NHE6 is a mitochondrial protein, one would expect it to be broadly distributed in various tissues.

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**Fig. 5.** Primary structure and predicted membrane topology of the human Na⁺/H⁺ exchanger NHE6 isoform. A, the extreme 5'-nucleotide and deduced N-terminal amino acid sequence of human NHE6 is shown, as determined by 5'-RACE. The 3'-nested oligonucleotide primers P1 and P2 are indicated by the *underline* arrows. B, comparison of the amino acid sequences of human NHE1 (Hu_NHE1), human NHE6 (Hu_NHE6), *C. elegans* F57C7.2 (Ce_F57C7.2), and *S. cerevisiae* Nha2 (Sc_NHA2) (GenBank™ accession numbers J03163/M81768, D87743/AF030409, Z69646, and U33007, respectively) was rendered using the CLUSTAL W multiple sequence alignment algorithm (69). Gaps (indicated by *periods*) were introduced in the sequence to maintain the alignment. Positions containing identical residues are shaded in *black*, while conservative amino acid differences are shaded in *gray*. Predicted membrane-spanning segments of NHE6 are numbered 1–12 and indicated by an *overline*.

C, a hydrophobicity plot determined by the algorithm of Kyte and Doolittle (window of 11 amino acids) (54) and corresponding model of the transmembrane organization of the NHE6 protein are shown. A putative mitochondrial matrix targeting signal at the N terminus (a positively charged amphiphilic helix) is illustrated in the model.
among mammalian tissues. To test this hypothesis, a Northern blot containing poly(A+) RNA isolated from several adult human tissues was hybridized at high stringency with an isoform-specific, 32P-labeled NHE6 probe (0.6-kilobase pair ClaI–EcoRI fragment) (see “Experimental Procedures” for details). The signals were detected using a Phosphorlmager. The positions and sizes (in kilobases) of the RNA markers are shown on the left.

**Subcellular Localization of Human NHE6**—To determine if NHE6 is targeted to mitochondria as predicted, dual labeling experiments were carried out in HeLa cells using a mitochondrion-specific marker, MitoTracker Red CM-H2XRosTM, and an isoform-specific NHE6 probe (0.6-kilobase pair). The signals were overlaid (Fig. 7). This pattern represents the staining with the relative abundance of mitochondria in these tissues (56).

**DISCUSSION**

BLAST analysis of the deduced protein sequences from three uncharacterized genes, one from *S. cerevisiae* (i.e. YDR456w or NHA2), one from humans (i.e. KIAA0287 or NHE6), and the other from *C. elegans* (i.e. F57C7.2), identifies them as homologues of the Na+/H+ exchanger gene family. Moreover, these three sequences exhibit higher amino acid identity to each other (27–34%) than they do for other NHE isoforms within their respective species, suggesting that they may share a common function. In the case of *S. cerevisiae* Nha2 and human NHE6, PSORT analysis predicts that their extreme N-terminal regions contain targeting signals that direct proteins to the mitochondrial inner membrane. Accordingly, we tested this prediction by fluorescence microscopy and found that they were indeed localized to mitochondria. Furthermore, we found a wide distribution and high expression of human NHE6 mRNA in tissues with abundant mitochondria (i.e. brain, skeletal muscle, heart), consistent with a role for this isoform in mitochondrial function. It is noteworthy that pathological changes resulting from mitochondrial dysfunction are also manifested predominantly in these same tissues (56). The absence of a putative mitochondrial targeting motif in the F57C7.2 protein of *C. elegans* may indicate a different cellular role for this NHE homologue or simply reflect incomplete sequence at its N terminus.

While the functional properties of the mammalian Na+-selective NHE are well described (14–17), there is little information concerning its structural features. Using subcellular fractionation and gel electrophoresis, Garlid et al. (13) identified a major 59-kDa protein in beef heart submitochondrial particles that was associated with Na+-selective NHE activity. This value approximates the molecular mass calculated for human NHE6 (~74 kDa), particularly since hydrophobic membrane proteins tend to migrate faster in polyacrylamide gels relative to other proteins of the same mass, which results in an underestimation of their true size. Proteolytic removal of the matrix targeting signal upon insertion into the inner membrane may also contribute to an observed smaller mass of the mature protein, as has been reported for other similarly targeted mitochondrial proteins (46). However, whether the 59-kDa protein and NHE6 are the same protein remains uncertain. The yeast Nha2 also has a molecular mass in this range (~70 kDa). By contrast, the mammalian nonselective K+/H+ (Na+/H+) exchanger has been tentatively identified as an 82-kDa protein based on radioisotope labeling of rat liver mitoplast extracts with [3H]dicyclohexylcarbodiimide, which irreversibly inhibits the transporter (20).

Predictions of secondary structure indicate that NHE6 and Nha2 have the same membrane topology as other NHEs, with 12 α-helical hydrophobic membrane-spanning segments in the N terminus followed by a hydrophilic region at the C terminus. The N terminus contains a single putative N-linked glycosylation site (Asn 369-Leu-Ser) in the predicted extracellular loop between membrane-spanning segments 9 and 10, which is therefore, its significance is obscure. The cytoplasmic region of NHE6 contains putative recognition sites (60) for phosphorylation by cyclic nucleotide-dependent protein kinases (i.e. 524RRTT, 570RCLT, and 643RGT) and casein kinase II (i.e. 512SDQE, 527TKAE, 601TYGD, and 622SSED). Whether protein kinases regulate NHE6 activity is unknown; nevertheless, this remains a distinct possibility, since cAMP-dependent protein kinase I and II (61) and casein kinase II (62) are located at the inner membrane/matrix space of mitochondria. By comparison, plasma membrane NHEs (e.g. NHE1, NHE2, and NHE3) are highly regulated phosphoproteins that contain multiple consensus phosphorylation sites recognized by various protein kinases in their C termini, some of which are known to be phosphorylated (63).

The chromosomal copy of NHA2 present in *S. cerevisiae* was deleted by insertion of the HIS3 auxotrophic marker into its open reading frame. Comparison of 25Na+ uptake into the acidified matrix of intact mitochondria isolated from the wild-type and the isogenic NHA2 gene-disrupted strains indicated that Nha2 is capable of mediating Na+/H+ exchange that is inhibited by benzamil and Li+. Benzamil has been shown to
preferentially inhibit the mammalian mitochondrial Na\(^+\)-selective NHE at the concentration (0.1 mM) used in this study (16), whereas it is a poor inhibitor of plasma membrane NHEs. This suggests that yeast Nha2 has pharmacological properties similar to those of its mammalian homologue.

These pharmacological data seem to contradict an earlier study that reported that mitochondria from S. cerevisiae have a nonselective K\(^+\)/H\(^+\) (Na\(^+\)/H\(^+\)) exchanger but lack a discernible Na\(^+\)-selective NHE (25). However, the low signal-to-noise ratio for measuring Nha2 activity may have caused it to go undetected in the earlier study, because we found that it was difficult to measure in our mitochondrial preparations. Indeed, benzamil-inhibitable Nha2 activity (1 nmol/min/mg protein) was only detected in three of five of our mitochondrial preparations. Our preliminary attempts to determine if Nha2 activity is sensitive to quinine, an inhibitor of the nonselective cation/H\(^+\) exchanger, were inconclusive. By comparison, Na\(^+\)-selective NHE activity in heart mitochondria is about 100-fold higher (110–160 nmol/min/mg protein), which facilitates pharmacological and kinetic measurements (2, 16). Attempts to measure matrix pH (as an assay of Na\(^+\)/H\(^+\) exchange activity) using the membrane-permeant dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester were unsuccessful. Others have also reported that yeast mitochondria do not accumulate fluorescent indicators when present as membrane-permeant esters (64), perhaps because they lack the esterases required for cleavage of the acetoxymethylester group. We are currently manipulating the assay conditions and improving the mitochondrial preparation in an attempt to increase the specific rates of transport by Nha2. At present, we cannot determine if Nha2 functions as a Na\(^+\)-selective NHE or as a nonselective cation/H\(^+\) exchanger. It is worth noting that, aside from Nha1 (located at the cell surface) (33) and Nha2, S. cerevisiae contains three additional anonymous open reading frames (YJL094c, YJR106w, and YAL022c) that share weak similarity to Na\(^+\)/H\(^+\) exchangers. Of these, YJL094c also shows closest homology to a glutathione-regulated K\(^+\) efflux transporter (KefC) from Escherichia coli (65), although it does not appear to contain a putative mitochondrial targeting signal at its N terminus. Determination of their transport properties and cellular location may clarify the role of Nha2.

Additional evidence supporting a role for Nha2 in organellar Na\(^+\) transport was provided by a very recent study that showed that deletion of NHA2 (referred to as NHX1 in this study) decreases the Na\(^+\) tolerance of a yeast strain expressing a mutant plasma membrane H\(^+\)-ATPase (66). It was speculated that Nha2 regulated the sequestration of Na\(^+\) into an intracellular acidic compartment, possibly vacuoles, although localization to mitochondria was not considered.

In the present study, initial characterization showed that yeast cell growth on fermentable carbon sources (i.e. glucose) was unaffected by the loss of NHA2 (data not shown). Under these growth conditions, yeast derive their energy primarily from glycolysis rather than respiration. The tolerance of \(\Delta nha2\) cells to 100 mM concentrations of external cations (i.e. Li\(^+\), Na\(^+\), K\(^+\), or Cu\(^{2+}\)) was also unaltered when grown on regular YPD medium (data not shown). However, further analysis of the \(\Delta nha2\) cells revealed that mitochondrial function was compromised. The growth of \(\Delta nha2\) cells on nonfermentable carbon

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**Fig. 7.** Localization of a human NHE6-GFP chimera in transfected HeLa cells. An expression vector containing the entire coding sequence of human NHE6 fused to a modified GFP (41) was transiently transfected into HeLa cells. Following transfection, cells were kept for 24 h at 37 °C and then incubated at 30 °C for another 36 h to enhance the fluorescence signal of GFP, as described previously (44). The results were analyzed by laser-scanning confocal microscopy using a Zeiss LSM 410 inverted microscope with the appropriate filter sets (for details, see “Experimental Procedures”). Optical sections were taken using a 63, 1.4 NA objective (optical thickness, ~1.0 μm). A representative binucleate HeLa cell is shown. A, mitochondria were selectively stained by incubating live cells with 25 μM of MitoTracker Red CM-H2XRos \(^{TM}\).

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(Molecular Probes) for 20 min at 37 °C and then imaged. B, the fluorescence signal of NHE6-GFP was localized to organelar structures matching the distribution of mitochondria as revealed by MitoTracker Red staining. C, overlay of scans generated by the MitoTracker Red (panel A) and NHE6-GFP (panel B) fluorescence, with the yellow indicating co-localization of the signals. The scale bar in C represents 20 μm.
sources was retarded when compared with the parental strain, suggesting that energy production from aerobic metabolism was reduced.

To further examine mitochondrial function, we investigated the ability of these yeast strains to survive in the nonproliferative (i.e., stationary) phase of the cell cycle upon nutrient depletion (49). When exponentially growing cells exhaust their supply of glucose, they adapt to respiratory metabolism using nonfermentable carbon sources generated by glycolysis. At this stage, the cells resume growth by using the energy from oxidative phosphorylation, but at a greatly reduced rate. The cells eventually enter the stationary phase upon carbon starvation (around day 6 of growth). During this transition, wild-type yeasts undergo numerous biological adaptations that allow them to survive over several weeks without added nutrients and quickly resume growth when nutrients become available. As demonstrated in this study, both wild-type SEY6210 and isogenic Δnha2 cells survived equally well in glucose-rich medium during the early stages (day 2) of cell growth, but, when sampled during the stationary phase (day 10), the survival of the Δnha2 cells was dramatically reduced, with the vast majority of the remaining cells exhibiting a petite phenotype. By contrast, the wild-type cells showed no detectable loss of viability. Interestingly, a few Δnha2 cells did retain wild-type growth (large colony size) in the absence of glucose, suggesting that the defect caused by the loss of Nha2 activity may be either weakly penetrating or that disruptants can spontaneously develop a suppressor activity that reduces severity of the gene loss. These explanations would also account for the modest reduction in growth of Δnha2 on nonfermentable carbon sources. Indeed, we have recently observed a variation in the strength of the null mutant phenotype with different disruptions. Using another yeast strain, L4244, more severe effects on mitochondrial function were obtained following homologous disruption of the NHA2 gene. Unlike the parental strain, the L4244Δnha2 cells showed retarded growth on regular YPD medium and no growth on nonfermentable carbon sources (data not shown). These data suggest that the SEY6210 strain contains a second-growth on nonfermentable carbon sources (data not shown). Indeed, we showed retarded growth on regular YPD medium and no growth on nonfermentable carbon sources (data not shown).

In summary, we have characterized a novel Na+/H+ exchanger isofrm (Nha2) found in yeast, which is targeted to mitochondria, mediates Na+/H+ exchange, and is important for mitochondrial function. Furthermore, we have also demonstrated that a related gene product (which we named NHE6) in humans is also localized in mitochondria. We propose that both Nha2 and NHE6 are important for the regulation of intramitochondrial Na+ and H+ levels. The yeast system should facilitate further studies on the mechanisms and functional importance of this transporter in eukaryotic cells.

Acknowledgment—We thank Dr. J. Hanrhan (McGill University) for useful discussions and for critically reviewing the manuscript.

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J. Biol. Chem. 1998, 273:6951-6959.
doi: 10.1074/jbc.273.12.6951

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