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Saccharomyces cerevisiae expression of exogenous vacuolar ATPase subunits B

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The precise function of subunit B of the vacuolar H^+-ATPase class is unknown, but it is essential for proton pumping. We have previously reported the DNA sequence and predicted protein sequence of the vacuolar ATPase subunit B for *Candida tropicalis* (Gu, H.H., Gallagher, M.J., Rupkey, S. and Dean, G.E. (1990) Nucleic Acids Res. 18, 7446). When the *Candida* gene was expressed in a *Saccharomyce cerevisiae* Δvat2 mutant from which the homologous gene had been deleted, viability and vacuolar acidification was restored to apparently wild-type levels. The predicted identity between these two proteins is 90%. We have searched for vacuolar ATPase subunits B from other species that might show a difference in function, when expressed in yeast, relative to the endogenous gene. We have cloned an apparently full-length 1.8-kb bovine subunit B cDNA from adrenal medulla that is about 1 kb shorter than the previously reported bovine brain cDNA (Puopolo, K., Kumamoto, C., Adachi, I., Magner, R. and Forgac, M. (1992) J. Biol. Chem. 267, 3696–3706; Nelson, R.D., Guo, X.L., Masood, K., Brown, D., Kalkbrenner, M. and Gluck, S. (1992) Proc. Natl. Acad. Sci. USA 89, 3541–3545), but nearly identical throughout the coding nucleotide and protein sequences; it is only 74% identical to the *Saccharomyces* subunit B protein sequence. Upon expression of this cDNA in two different Δvat2 deletion strains, the bovine cDNA restored function only partially, as judged by both viability at high pH and vacuolar acidification. Current work is aimed at determining which regions of the bovine protein require alteration in order to fully restore the Δvat2 strain to wild-type acidification, with the eventual goal of identifying interactive residues between subunit B and other proteins required for pump function.

Introduction

Vacuolar proton-pumping adenosine triphosphatases (H^+-ATPases) serve to acidify certain intracellular compartments in eukaryotic cells, including secretory vesicles, coated vesicles, lysosomes, the *trans*-Golgi network and the vacuoles of fungi and plants (for review, see Ref. 4). These proton pumps generate an electrochemical gradient for protons across the membrane which provides the driving force for active transport of a variety of substances. In the chromaffin granule, the actively transported species are catecholamines.

All of the H^+-ATPases purified to date are multisubunit proteins, composed of subunits with approximate molecular masses of 16 (subunit c), 31 (E), 39 (D), 42 (C), 60 (B) 70 (A) and one which has been variously placed at 100–140 kDa. From biochemical studies, it has been estimated that there are equimolar amounts of the 60 and 70 kDa subunits, and that each of these is in 3:1 ratio with the others [5], but how these subunits are arranged in the intact enzyme is unknown. Current models of the proton pump have been constructed largely by analogy with the mitochondrial F_1F_0 ATPase [6] on a number of grounds: (1) there exists sequence homology between vacuolar subunits A, B and c and mitochondrial subunits β, α and c, respectively [7–9]; (2) it has been demonstrated that vacuolar subunit A (similar to mitochondrial subunit β) is almost certainly the ATP hydrolytic subunit [10,11] and (3) the morphology of the vacuolar ATPase shares the ball-and-stalk appearance of the mitochondrial pump [12,13]. The precise function of subunit B of the vacuolar H^+-ATPase (similar to the mitochondrial pump subunit α) is unknown but it appears to be

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The sequence data reported in this paper have been deposited in the GenBank/EMBL Data Bank under the accession numbers X58385 (Bovine Vacuolar ATPase subunit B) and X54875 (*C. tropicalis* subunit B).

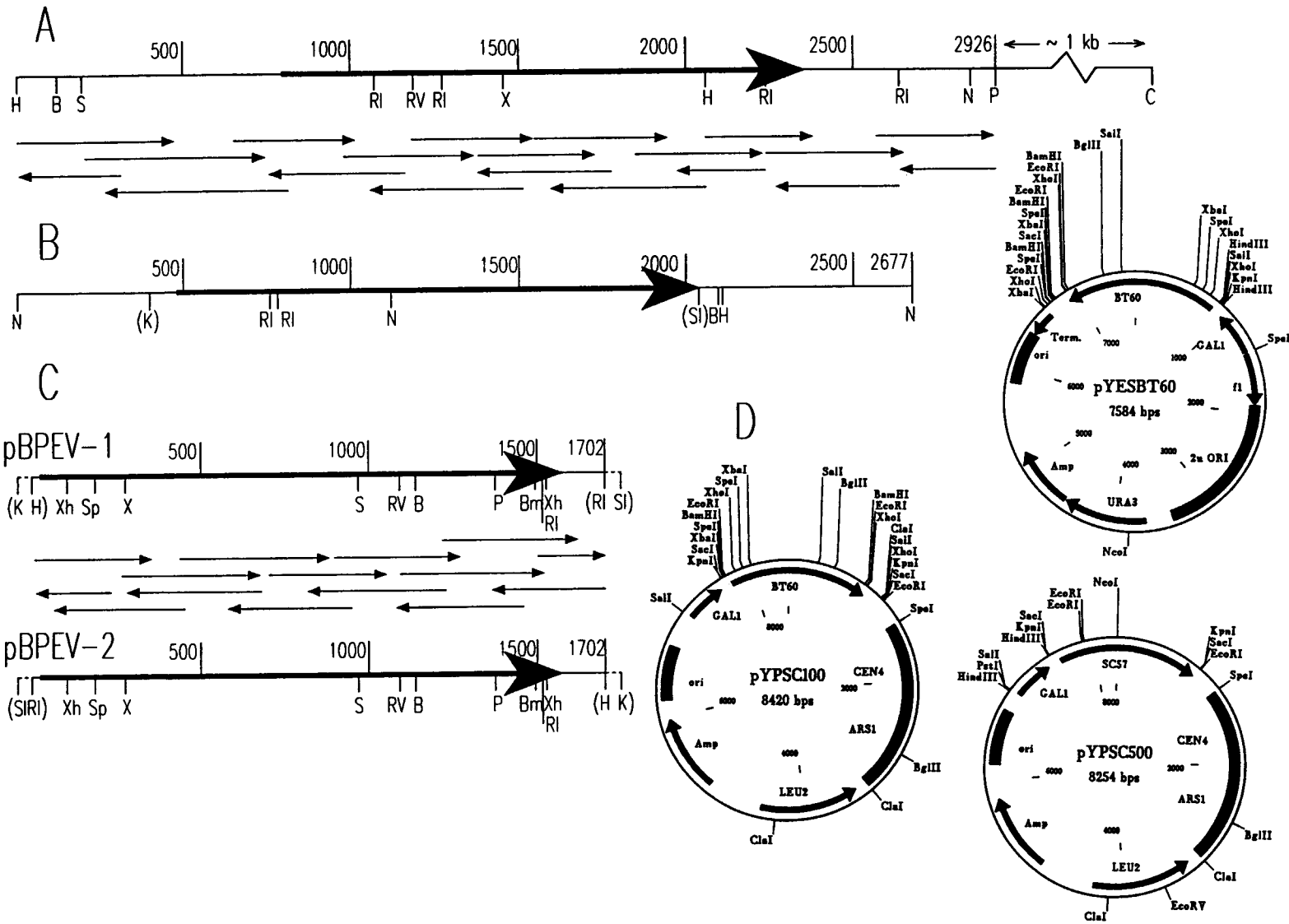

Fig. 1. Restriction maps of the subunit B genes/cDNAs. (A) *C. tropicalis* subunit B gene. The DNA sequence from the *Hin*dIII site at position 1 to the *Pst*I site at position 2926 was determined in both directions; a *Cla*I site approx. 1 kb downstream used for sub-cloning is indicated. Nested deletions were generated with exonuclease III/Mung Bean nuclease digestion. (B) *S. cerevisiae* VAT2 gene [30]; also indicated in parentheses are PCR-generated restriction sites used for sub-cloning. (C) Bovine subunit B cDNA. pBEV-1 and pBEV-2 are clones from two independent PCR reactions in opposite orientations in Bluescript SK(−); the restriction sites shown in parentheses are in the vector polylinker sequence. For each gene or cDNA, the dark arrow indicates the subunit B open-reading frame. Shown also are the sequencing strategies followed for the *C. tropicalis* gene and bovine PCR-generated cDNAs. (D) Selected plasmids described in the text. BT60 refers to the *Bos taurus* (cow) subunit B and SC57 refers to *S. cerevisiae* subunit B. Restriction sites indicated are as follows: B, *Bgl*II; Bm, *Bam*HI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; K, *Kpn*I; S, *Sal*I; SI, *Sac*I; Sp, *Spe*I X, *Xba*I; Xh, *Xho*I.

essential for, and is thought to regulate, proton pumping. This is the subunit for which the most linear sequence data exists, and because all of the subunit B structural data indicate that the protein's linear structure is highly conserved from fungi to mammals, we were curious to know whether these subunits might be exchangeable. Our motive for asking such a question is as follows.

A potentially powerful method for investigating protein–protein interactions in a multi-subunit protein such as the vacuolar ATPase is the isolation of enzyme assembly mutants which are either completely ineffective in proton-pumping or have marginal activity; such mutants may be used to generate further inter-genic mutants (suppressor mutants) that have restored proton pumping. This approach is hampered by the labor-intensive and time-consuming nature of mutant isolation and characterization which must be done prior to the subsequent reverse mutant selection of suppressors. In the case of the vacuolar proton pump, this is made more difficult by the lack of a powerful selection for the initial mutants, for although it is now known that yeast strains which have suffered mutations or deletions in several of the vacuolar ATPase subunit genes show very poor growth at neutral pH [14,15], this is not a particularly convenient method for creating the large numbers of mutations required for this type of analysis. We felt that if we could identify cDNAs or genes encoding vacuolar subunits from one species that only partially restored function when expressed in a *Saccharomyces* strain from which the homologous gene had been deleted, we might be in a better position to isolate such suppressor mutants, as well as begin a process of domain exchanges between the two proteins, and thereby locate the sites in subunit B which make contact with sites in other subunits in the protein. As we believe we have demonstrated here, a significant start in that direction has been made.

Materials and Methods

Materials. DNA modification and restriction enzymes were from New England Biolabs, Gibco-BRL, or Boehringer-Mannheim. Sequenase sequencing kits were purchased from US Biochemical. Blotting media were from either Schleicher & Schuell (nitrocellulose), MSI (Magna2000), or DuPont (GeneScreen Plus). All radiochemicals were from NEN. Oligonucleotide synthesis reagents were from ABN. All other reagents were reagent grade or better.

Strains and vectors used. Bacterial strain: XL1-Blue: F′recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac$^-$ (F′ = proAB lacIqZΔ(M15) Tn10 tet^r); BL21 (DE3), which has the T7 polymerase gene integrated into its genome with an inducible Lac Z promoter, was used to express genes with a T7 promoter. *Saccharomyces cerevisiae* strains: SF838-5Aα (strain No. 63): MATa ade6 gal2 leu2-3,112 ura3-52 (T. Stevens); SF838-5Aα/vat2-Δ1::LEU2 (strain No. 2110): MATα ade6 gal2 leu2-3,112 ura3-52 vat2-Δ1::LEU2 (T. Stevens, constructed by C. Yamashiro); SF838-5Aα/vat2-Δ::URA3 (strain No. 2111): MATα ade6 gal2 leu2-3,112 ura3-52 vat2-Δ2::URA3 (T. Stevens, constructed by C. Yamashiro); *Candida tropicalis* ATCC No. 750 (from the American Type Culture Collection) was used for generating genomic libraries, and for DNA and RNA isolation. Vectors: YCp50 (ATCC No. 37419), pYES2 (Pharmacia) and YCp*lac*111 and YEp*lac*181 (courtesy of R.D. Gietz [16]).

Gene / cDNA cloning, sequencing and sequence analysis. For the purposes of cloning the *Candida tropicalis* subunit B gene, two oligonucleotides, skewed to match the codon usage of *Saccharomyces cerevisiae*, were designed that would hybridize (each in the sense direction) with the *Arabidopsis thaliana* gene sequence [17] between nucleotides 520 and 568 (GD992: GGTCTACCACACAACGAAATTGCTGCACAAATTTGCAGACAGGCCGGT) and 982 and 1035 (GD994: GGGTCTATCACGCAAATACCTATCCTGACAATGCCCAACGACGATATTACCCAT). These and various other deoxyoligonucleotides used for DNA sequencing were synthesized using a Pharmacia Gene Assembler. The oligonucleotides were used sequentially to screen a *Candida tropicalis* genomic library (with an average insert size of 10 kb of *Sau*3A partially digested genomic DNA cloned into the unique *Bam*HI site of pAB109), which was kindly provided by Dr. Jack Loper [18]. Standard hybridization [19] conditions were used. Successful clones were used to generate restriction maps. Restriction fragments of the cloned genes were sub-cloned into Bluescript plasmids and the DNA sequences of a part of the insert DNA was determined. All DNA sequencing was performed by the dideoxy method as described [19] on double-stranded plasmid DNA. Deletions were obtained with the Stratagene *Exo*III/mung bean deletion kit; both strands were sequenced in their entirety. The sequencing strategy used is diagrammed on the abbreviated restriction map shown in Fig. 1A, compared to the *S. cerevisiae* VAT2 gene (Fig. 1B). Computer analysis was performed using DNANALYZ (Gregory Wernke, University of Cincinnati), and Clone and Align software from Scientific and Educational Software. This DNA sequence has been given the EMBL accession number X54875.

A Lambda ZAP library carrying cDNA prepared from bovine chromaffin cell mRNA in the 1.5–4.5-kb size range and containing approx. 10^6 independent recombinants [20] was used for cloning the bovine subunit B cDNA. Degenerate oligonucleotides designed to match the nucleotide sequence of the *Arabidopsis thaliana* vacuolar ATPase subunit B at nucleotide positions 520–544 (GD2354: GGGGCGCC-

NCA[C/T]AA[C/T]GA[A/G]AT[C/T]GC[C/T]GC-NC, in the sense orientation) and nucleotide positions 1012–1039 (GD2357:TGCGATCGGTGAT[A/G]TC [A/G]TC[A/G]TTNGGCAT, in the antisense orientation; N = all four deoxynucleotide triphosphates), but skewed to mammalian codon usage were used in PCR reactions to amplify bovine subunit B cDNA; the template in each reaction was 10 ng of purified Lambda ZAP library DNA. The PCR reaction products were sub-cloned into Bluescript SK(−) at the *Eco*RV site and the DNA sequence of several individual products were determined. The amplified DNA was sub-cloned and its sequence determined to resemble that of subunit B cDNAs or genes from other species. Exactly matched sense and anti-sense oligonucleotides were then designed from this sequence and used in the PCR reaction with the same template DNA, pairing the sense oligonucleotide with an oligonucleotide in the vector predicted to lie downstream of the 3′-end of the cDNA and the anti-sense oligonucleotide with another primer predicted to lie upstream of the 5′-end of the cDNA. The longest reaction products from each reaction were sub-cloned and the DNA sequences determined for each cDNA. PCR attempts to extend the cDNA sequence beyond the 5′-end reported below were unsuccessful. Finally, primers were designed to correspond to the 5′ and 3′ ends of the known sequence and three separate PCR reactions were run with these primers. The 1.65-kb products of each reaction were sub-cloned into the *Eco*RV site of Bluescript SK(−) and the DNA sequences determined for representative clones from all three by di-deoxy sequencing [19] of double-stranded plasmid DNA from synthetic primers oriented in both directions. The sequencing strategy employed is detailed on the abbreviated restriction map shown in Fig. 1C. Deletions were obtained using Stratagene's *Exo*III/mung bean nuclease kits or by restriction digestions and subsequent ligations. In some cases, internal oligonucleotide sequencing primers were synthesized. Computer analysis was performed using DNANALYZ (Gregory Wernke, University of Cincinnati) and Clone and Align software from Scientific and Educational Software. The consensus sequence matches the published bovine brain subunit B cDNA sequence [2], except in the following few locations. (1) There are an additional 11 residues at the 5′-end of the cDNA reported here which is missing in the sequence presented previously [2]. (2) The DNA sequence in the vicinity of the C-terminus of the protein reads TCTGCGAAGCATTAG (where the stop codon is the TAG indicated here by underlining), causing the C-terminus to read ...P-R-D-S-A-K-H. This agrees with the predicted protein sequence reported in Ref. 3, whereas the sequence in the previous report was TCTGCGAACAGTTAG which would give an amino-acid sequence ... P-R-D-S-A-N-S. (3) Finally, the first A in the poly(A) tract of our clone is found at position 1675 in the previously published sequence (which is missing a poly(A) stretch). Two discrepancies were observed among the three DNA sequences. In one of them (pBEV-1), a C was substituted for a T at nucleotide position 563, resulting in a silent mutation; this clone was oriented in the SK(−) vector as shown in Fig. 1C. In the second, an A was substituted for G at position 846, resulting in a change from alanine to threonine in the predicted peptide sequence. One clone (pBPEV-2) displayed the consensus sequence in its entirety and was used in subsequent experiments; it is oriented in the SK(−) vector in the opposite orientation from pBPEV-1, as shown in Fig. 1C. This DNA sequence has been given the EMBL accession number X58385.

Northern blot analysis. Total RNA, or for some cases, poly(A)-selected RNA, was isolated from flash-frozen adrenal medullae, *Candida tropicalis*, or *Saccharomyces cerevisiae* as described [19]. RNA was denatured in 50.7% formamide and separated by electrophoresis through 1% agarose gels in 0.22 M formaldehyde. Capillary transfer to GeneScreen Plus membrane was performed for 12 h in a buffer containing 0.025 M $NaPO_4$ (pH 6.5). The membrane was baked at 65°C for 2 h, pre-hybridized under standard conditions [19], and then probed by incubation in the same buffer containing DNA probe at approx. 10^6 cpm/ml, 10^8 cpm/μg. Probes were either labeled with [^{32}P]dATP by random primer labeling or PCR amplification [19].

Yeast expression and DNA and RNA preparations and manipulations. These were carried out essentially as described [19].

Chromaffin granule membranes. Chromaffin granule membranes were isolated by density gradient centrifugation of bovine adrenal medulla homogenates and hypotonic lysis of the granule pellets, essentially as described [21], dissolved in Laemmli SDS sample buffer at approx. 2 mg/ml, and the proteins separated by SDS-PAGE (20 μg/well). Nitrocellulose replicas of these gels were made by electrophoretic transfer, blocked with Blotto and probed with the anti-subunit B antisera at 1:100 dilution [22].

Isolation of vacuolar vesicles. Isolation of vacuolar vesicles from wild-type and mutant *Saccharomyces cerevisiae* cells were similar to that described [23]. Briefly, mid-log yeast cells were collected by centrifugation, washed twice with distilled water and re-suspended in Buffer I (1 M sorbital, 10 mM Mes-Tris (pH 6.9), 5 mM $MgCl_2$, 1 mM DTT), and incubated in the presence of Zymolyase and glusulase. The spheroplasts were collected by centrifugation, washed twice with Buffer I, re-suspended in ice-cold Buffer II (10 mM Mes-Tris (pH 6.9), 0.1 mM $MgCl_2$, 12% Ficoll-400) and homogenized. The unbroken cells and cell debris were pelleted and the supernatant was layered onto

the top of Buffer II and centrifuged in an SW28 rotor. The white floating layer containing the vacuoles was collected, suspended in Buffer II and transferred to a new centrifuge tube to which was added a layer of Buffer III (10 mM Mes-Tris (pH 6.9), 0.5 mM $MgCl_2$, 8% Ficoll-400). After centrifugation as before, the vacuoles contained in the white layer on top were converted to vacuolar vesicles by hypotonic lysis. The vacuolar vesicles were pelleted, re-suspended in Buffer IV, snap-frozen in liquid nitrogen and stored at −80°C.

Quinacrine fluorescence assay. The fluorescent dye quinacrine was used to label the acidic vacuoles. The procedure used was similar to that described [24–26]. Cells were grown to fresh saturation in YEPD, pelleted by spinning at 3000 rpm for 2 min, re-suspended in YEPD buffered at pH 7.6 containing 50 μM quinacrine, and incubated at 30°C for 10 min. Cells were then pelleted and washed with and re-suspended in cold YEPD (pH 7.6), kept on ice and examined under a microscope within an hour. A Zeiss Microscope II was used to examine the cells and take phase contrast and fluorescent photos. For fluorescent micrographs, a set of filters was used with excitation bandpass of 450–490 nm and emission bandpass of 515–545 nm. Kodak p3200 BW film was used. Phase contrast photos were taken with automatic exposure, and fluorescent photos taken with 10-s exposure time.

Proton transport assay. Proton transport of vacuolar vesicles was determined by measuring the quenching of acridine orange fluorescence similar to that described [27]. The vacuolar vesicles prepared as described above were thawed on ice, diluted (500 μg/ml final) in assay buffer (20 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 μM acridine orange), and reactions were started by adding 250 μM MgATP. Nigericin (10 μM) was used to assess the degree of acidification at the end of reaction. The fluorescence measurements were performed on a SPEX DM 300CN, with excitation wavelength of 493 nm and emission wavelength of 530 nm. Inhibitor concentrations: Na_3N, 500 μM; vanadate, 50 μM; bafilomycin, 50 nM.

Antibody preparation and immunoblot analysis. Fusion constructs between the *lacZ* gene in pUR289 [28,29] and the complete bovine cDNA copy of subunit B were created and used to produce large quantities of the fusion protein in XL1-Blue cells. This fusion protein was then used to raise rabbit polyclonal antisera in New Zealand white male rabbits [22]. The antisera were subsequently pre-adsorbed three times with acetone powders [22] of Bluescript bacterial cells carrying pUR289 grown in the presence of 10 mM IPTG to induce the synthesis of the β-galactosidase protein.

Western blot analysis. In vitro translation products and whole cell lysates of bacteria expressing genes of interest were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were blocked with 10% milk, 0.1% Tween-20 in TBS (Tris-buffered saline solution: 50 mM Tris-HCl (pH 7.4), 137 mM NaCl) or PBS (phosphate-buffered saline solution), washed once in TBS (or PBS) plus 0.1% Tween-20 and once in TBS, incubated with primary antibodies diluted 1:100 in TBS, for 4 h at room temperature. The filters were then washed 4-times with 0.1% Tween-20 in TBS and incubated with horseradish peroxidase-labeled goat-anti-mouse antibody (Sigma, 1:1000 dilution) for 2 h at 4°C. Finally, the filters were washed once with 0.1% Tween-20, twice with 0.3% Tween-20, and again twice with 0.1% Tween-20, all in

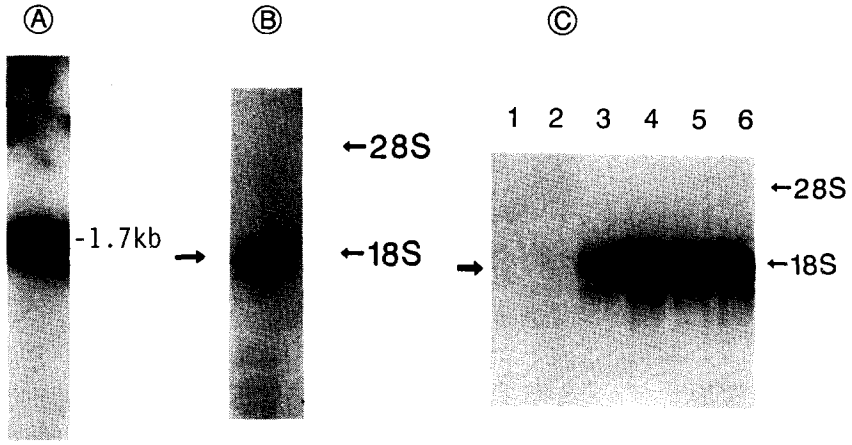

Fig. 2. Northern blots. RNA was separated by formaldehyde agarose gel electrophoresis, transferred to GeneScreen Plus and probed. (A) Total *C. tropicalis* RNA probed with a PCR radio-labeled DNA fragment derived from nucleotide 550 through 2059 of the gene. (B) Poly(A)-selected bovine adrenal medulla RNA probed with a DNA fragment corresponding to nucleotides 785–917. (C) Total RNA from *Saccharomyces cerevisiae* strain 2110 with and without bovine subunit B cDNA, probed with the same fragment as in Panel B. Lane 1, strain No. 2110 (vat2::LEU2); lane 2, strain No. 2110 carrying pYES2; lane 3, strain No. 2110 carrying pYESBT60 in YEPD; lane 4, strain No. 2110 carrying pYESBT60 in YEPD plus 0.2% galactose; lane 5, strain No. 2110 carrying pYESBT60 in YEPD plus 0.5% galactose; lane 6, strain No. 2110 carrying pYESBT60 in YEPD plus 1.0% galactose.

TBS. The signals were detected by incubation with [^{125}I]goat-anti-rabbit IgG (NEN, 2–10 μCi/μg, 0.5 μCi/blot) and subsequent autoradiography.

Results

Cloning and characterization of the C. tropicalis subunit B gene

We began this project as a search for homologous genes from other organisms that could be used to complement the *Saccharomyces* Δvat2 (subunit B deletion) strain less completely than the native gene. At the time we began, cDNA or gene sequences for three of the B subunits of the vacuolar ATPase were known, these being from *Arabidopsis thaliana* [17], *Neurospora crassa* [8] and *Saccharomyces cerevisiae* [30]. Oligonucleotides were synthesized that corresponded to two regions of the protein sequences which were identical among the three and used to probe whole genomic Southern blots of DNA from *C. tropicalis*. Single strongly hybridizing bands were observed in all digests (data not shown), indicating that there was a single *C. tropicalis* subunit B gene. Screens of a *Candida* genomic library in pAB107 yielded two plasmids carrying hybridizing DNA; both carried the 2644-bp fragment whose sequence has been previously reported [1]. From this nucleotide sequence, a protein sequence was predicted with a calculated molecular mass of 57135 Da which was 90% identical to that of the *S. cerevisiae* VAT2 gene product. As expected from the previously known linear structures for subunit B of other species, most of the differences between the *S. cerevisiae* and *C. tropicalis* predicted gene products were at the extreme N- and C-termini of the proteins. Northern blot analysis indicated that the mRNA transcript is 1.7 kb in length (Fig. 2A).

Substitution of the *C. tropicalis* subunit B gene in a Δvat2 mutant of *S. cerevisiae*. To verify that the cloned gene was in fact the *S. cerevisiae* VAT2 homologue, a 3.7 kb *Sal*I–*Cla*I fragment carrying the entire gene with its own promoter was sub-cloned into the single-copy vector, YCp50, between the unique *Sal*I and *Cla*I sites in the vector, resulting in plasmid pHG103Y, and this plasmid was used to transform *Saccharomyces* strain SF838–5Aα/vat2-Δ1::LEU2 (2110), constructed by Carl Yamashiro by insertion of the LEU2 gene between the *Eco*RI sites in the VAT2 gene, resulting in the complete deletion of 18 nucleotides of the gene in addition to the LEU2 insertion [15]. Knowing that this strain could not grow at pH 7.6, we were interested in determining the extent to which the *C. tropicalis* gene would restore function. As illustrated in Fig. 3, the transformed Δvat2 strain is indistinguishable at pH 7.6 from the wild-type parent carrying the YCp50 vector, growing much better than the vat2-Δ1::LEU2 strain, and growth curves of the parent and vat2-Δ1::LEU2 carrying pHG103Y in YEPD buffered to pH 7.6 were indistinguishable (data not shown). To confirm that the effect was specific for the open reading frame predicted to encode the *C. tropicalis* VAT2 homologue, the YCp50-borne gene was disrupted by cleaving the plasmid at the unique *Xba*I site and filling in the

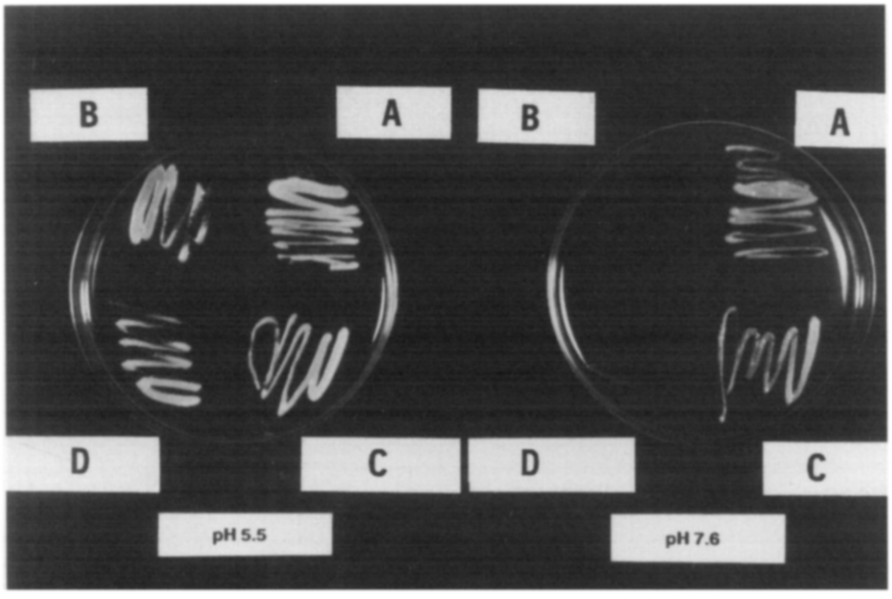

Fig. 3. Growth of wild-type and mutant yeast strains with *Candida* subunit B gene at pH 7.6 and pH 5.5. (A) The wild-type parent strain grows equally well at pH 5.5 (plate on the left) and pH 7.6 (plate on the right). (B) Strain No. 2110 (*vat2::LEU2*), however, grows extremely poorly at pH 7.6. (C) Upon transformation of this strain with plasmid pHG103Y (YCp50 carrying the *Candida* subunit B gene), growth at pH 7.6 is restored. (D) Strain No. 2110 with the plasmid pHG103Yd, carrying the disrupted *Candida* subunit B gene, shows growth similar to that of strain No. 2110.

restriction site with Klenow fragment, resulting in plasmid pHG103Yd, thereby altering the reading frame. vat2-Δ1::LEU2 cells transformed with this plasmid appear to share the growth defect of the original vat2-Δ1::LEU2 mutant.

As a first test in examining whether the restoration of high pH growth was due to acidification of the vacuole, quinacrine fluorescence was used. Quinacrine is a fluorescent weak base which partitions into acidic spaces within the yeast cell; strong vacuolar fluorescence indicates that the compartment is at a low pH [25]. The vat2Δ1::LEU2 mutant carrying the *Candida* gene showed fluorescence that was comparable to that of wild-type *Saccharomyces*, while the disrupted version of the gene did not fluoresce (data not shown), indicating that the *Candida* subunit B was indeed capable of replacing the *Saccharomyces* subunit B to produce a functional proton pump.

To further demonstrate the acidification properties of the vacuoles from each of the above strains, the rate of acridine orange uptake into isolated vacuoles was determined. Acridine orange is another fluorescent weak base which accumulates in acidic compartments with a concomitant decrease in total fluorescence. Measurement of ATP-dependent acridine orange fluorescence quenching is commonly used to examine ATPase-mediated proton pumping into a vesicular lumen. Vacuolar vesicles were isolated from a randomly chosen example of each of the above strains and assayed for vacuolar proton pump activity. This acidification was not inhibited by vanadate, an inhibitor of the P-type ATP-dependent ion pumps, nor by oligomycin, azide, or efrapeptin, agents known to inhibit the F_1F_0 ATPase; it was, however, inhibited by bafilomycin A_1 [31], a specific inhibitor of all known vacuolar ATPases. To estimate the degree of acidification obtained in the vacuole, nigericin was added, permitting the exchange of potassium or sodium for protons and thereby abolishing the proton gradient. The wild-type *Saccharomyces* strain and the vat2Δ1::LEU2 mutant carrying the *Candida* subunit B gene were indistinguishable in these experiments, indicating that proton pumping was comparable in both, while the strains in which either one or both non-functional genes were present appeared to be completely incapable of acidification (data not shown).

Characterization of the bovine 60-kDa cDNA

As it became clear that the *Candida* subunit B was capable of apparently completely replacing the endogenous *Saccharomyces* subunit B, we turned to another organism which might show incomplete replacement. The bovine adrenal gland is composed predominantly of chromaffin cells which are rich in chromaffin granules, carrying a well-studied V-ATPase. From an adrenal medulla lambda-ZAP library, a cDNA was

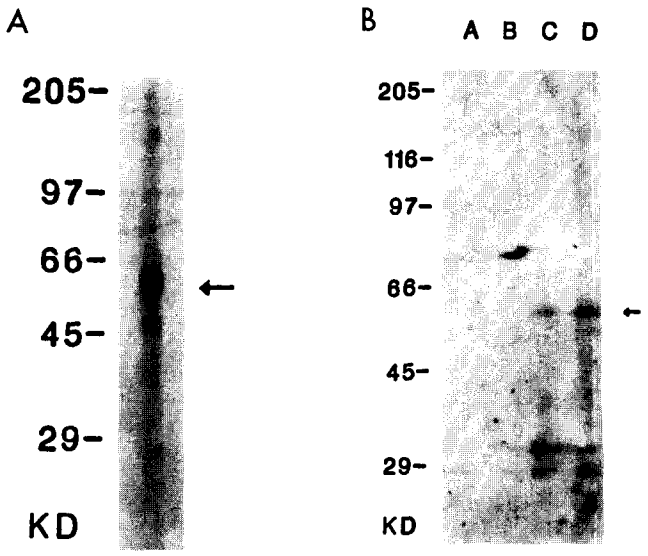

Fig. 4. Western blots with antiserum to bovine Subunit B. (Panel A) Chromaffin granule membrane protein, 4 μg per lane. (Panel B) Whole cell yeast lysates, 10 μg protein per lane. Lane A, Wild-type parent (strain No. 63); lane B, *vat2::LEU2* deletion mutant (strain No. 2110); lane C, strain No. 2110 carrying BT60YES2, no galactose added; lane D, strain No. 2110 carrying BT60YES2, 0.2% galactose.

cloned which apparently encoded the chromaffin granule V-ATPase subunit B, as described below. This bovine cDNA is predicted to encode a polypeptide of 511 residues with a calculated molecular mass of 56511 Da which is 73.9% identical with the *S. cerevisiae* peptide sequence. To verify that the corresponding mRNA is expressed in the adrenal medulla and to determine its size, Northern blots of poly(A)-selected RNA from bovine adrenal medulla were probed at high stringency with portions of the cDNA corresponding to nucleotides 785–917, as shown in Fig. 2B. A single band of approx. 1.8 kb was observed to hybridize with the probe. Furthermore, antisera raised to the complete coding region for subunit B reacted with a protein of approx. 60 kDa in chromaffin granule membrane extracts (Fig. 4A), indicating that the encoded protein is almost certainly a subunit of the chromaffin granule ATPase.

Complementation of vat2 deletion mutants of S. cerevisiae by the bovine subunit B cDNA

To determine whether the bovine cDNA could complement a subunit-B-deficient yeast strain (and to demonstrate unequivocally that the protein encoded by the cDNA in fact functioned as a part of the vacuolar ATPase), a *Kpn*I–*Sac*I fragment carrying the full-length bovine subunit B cDNA was cut from pBPEV-1 (Fig. 1C) and sub-cloned between the *Kpn*I and *Sac*I sites of the multi-copy 2μ vector pYES2 under the control of the galactose-inducible promoter, GAL1/GAL10, and this plasmid (pYESBT60, Fig. 1D) used to transform strain SF838–5Aα/vat2-Δ1::LEU2 (No.

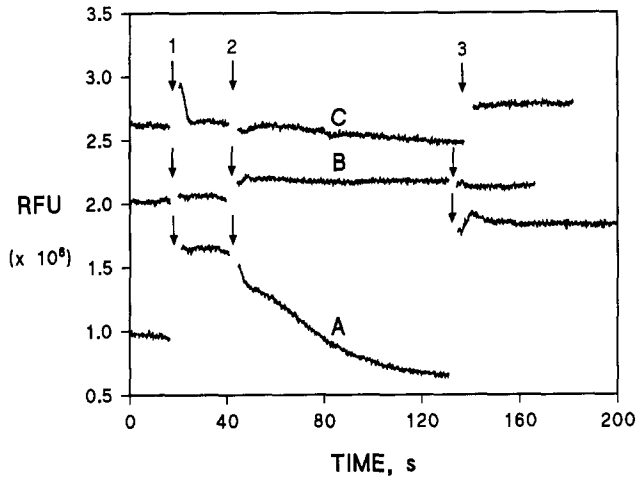

Fig. 5. Acridine orange fluorescence quenching. Vacuolar vesicles were isolated from wild-type and mutants, and diluted into 50 μM acridine orange (500 μg protein/ml final), indicated by the arrow at 1. The solution was brought to 250 μM MgATP to initiate proton pumping (arrow No. 2) and the proton gradient dissipated by the addition of nigericin to 10 μM (arrow No. 3). Bovine subunit B cDNA in *Saccharomyces*. (A) Wild-type parent strain No. 63; (B) strain No. 2110 carrying pYES2; (C) *vat2* strain No. 2110 carrying PYESBT60. Although the fluorescence quenching is relatively mild, the rise in fluorescence seen upon addition of nigericin in trace C is indicative of an acidic compartment.

2110), the same strain used with the *Candida* gene. Growth of five out of five transformed cells displayed very poor growth at pH 7.6 in the presence of galactose concentrations ranging from 0.1% to 1.0%, but growth that was reproducibly intermediate between the parent strain No. 63 on the one hand and either strain No. 2110 or No. 2110 carrying the pYES2 vector. Northern blots of these transformants with probes derived from the bovine cDNA indicated that RNA expression of the bovine cDNA had occurred (Fig. 2C), and Western blots of lysates from these cells indicated that the protein was being expressed (Fig. 4B). This indicated that the bovine cDNA was being expressed and permitted the assembly of vacuolar ATPases that were less effective in proton pumping than either the wild-type *Saccharomyces* subunit B or the protein encoded by the *Candida* subunit B gene.

To assess whether this was indeed true, we tested the proton pumping of vacuoles from the transformed deletion mutants. Acridine orange fluorescence quenching by isolated vacuoles was used. As shown in Fig. 5, vacuoles from the deletion mutant did not display the ATP-induced fluorescence quenching seen in vacuoles derived from wild-type cells, while the mutant cells carrying pYESBT60 showed an intermediate level of fluorescence quench. Fluorescence quenching by both wild-type cells and the transformed mutant was completely blocked by bafilomycin and was insensitive to both 1 mM azide and 1 mM vanadate (data not shown).

We were, however, concerned that the high copy number of the pYES2-derived plasmid might have had a crippling effect upon the cells, that the promoter differences might adversely affect relative protein copy number such that the pump was not effectively assembled, or that, although unlikely, a recombination event between the residual *Saccharomyces* gene and the bovine cDNA might have occurred, resulting in a poorly functional chimeric protein. To address these possibilities, a *Spe*I–*Sac*I fragment from the pYES vector carrying the GAL1/GAL10 promoter was ligated upstream of a *Sac*I–*Kpn*I fragment carrying the bovine subunit B cDNA derived from pBPEV-2 (Fig. 1C) and this ligated fragment then sub-cloned into the CEN4-containing vector YCp111 [16] cut at its unique *Xba*I and *Kpn*I restriction sites, resulting in plasmid pYPSC100, Fig. 1D. This plasmid has a *Kpn*I site 61 nucleotides upstream of the start codon and a *Sac*I site 134 nucleotides downstream of the translation termination codon. The plasmid was used to transform SF838-5Aα/vat2-Δ2::URA3 (strain No. 2111) [15], a deletion mutant created by inserting the URA3 gene between the *Hin*dIII restriction sites which flank the VAT2 gene; this has resulted in the deletion of approx. 2.2 kb of the genome, including the entire VAT2 gene. All of the transformants had growth properties at pH 7.6 which were indistinguishable from those of pYESBT60 (Fig. 6). At the same time, PCR amplification of the *Saccharomyces* VAT2 gene with primers that placed a *Kpn*I site 83 nucleotides upstream of the start codon and a *Sac*I site 8 nucleotides downstream of the termination codon enabled us to clone the *Saccharomyces*

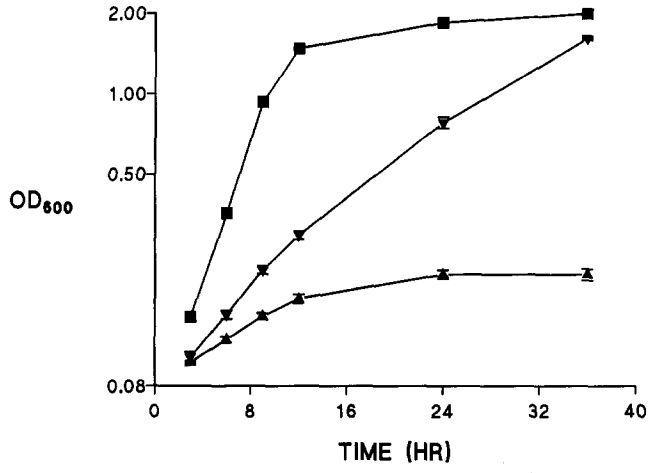

Fig. 6. Growth curves of *S. cerevisiae vat2* deletion strain No. 2111 carrying the bovine subunit B cDNA in YCp111Gal1. ■, Wild-type strain No. 63; this curve matches that obtained with strain No. 2111 carrying pYPSC500; ▲, strain No. 2111 carrying YCp111Gal1 (identical to the growth seen with strain No. 2111 alone); ▼, strain No. 2111 carrying pYPSC100. Data are shown for the standard errors of the mean for three experiments, three independent samples per experiment.

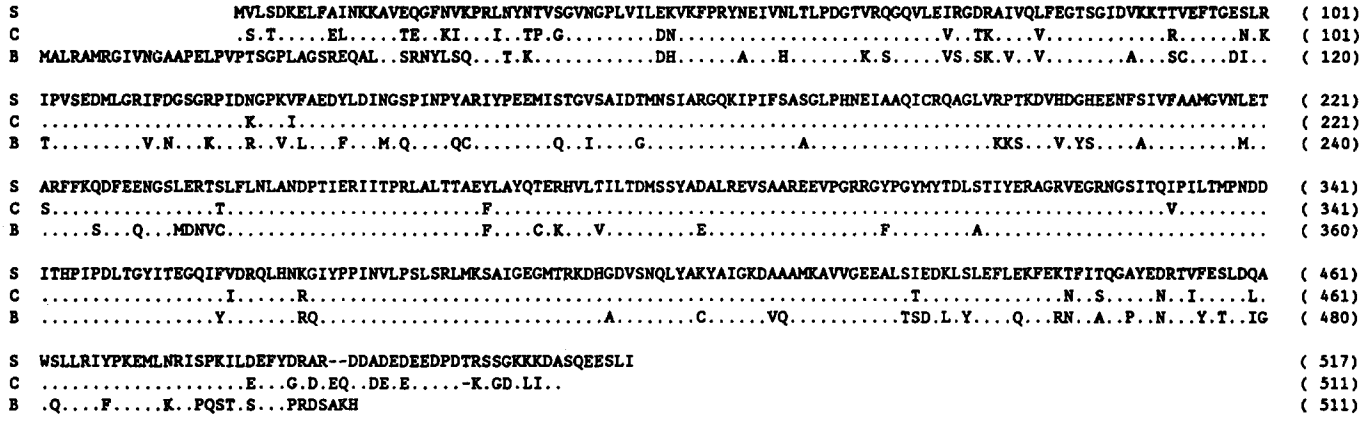

Fig. 7. Comparison of linear protein structures of the vacuolar ATPase subunits B used. S, *Saccharomyces cerevisiae*; C, *Candida tropicalis*; B, *Bos taurus* (cow). The entire *S. cerevisiae* amino-acid sequence is shown in single letter code; in other sequences, identical amino acids are replaced by periods and gaps by hyphens.

VAT2 gene into the same YCp111 vector downstream of the GAL1/GAL10 promoter in a context that was very similar to the bovine cDNA, resulting in plasmid pYPSC500; these cells behaved in all respects precisely like wild-type cells in growth at pH 7.6, as long as the concentration of galactose was above 0.1%. These results confirm that the bovine cDNA was capable of complementing a yeast mutant which had been deleted for the homologous gene, resulting either in the assembly of poorly functional vacuolar proton pumps, or the assembly of a decreased number of functional pumps. From the fact that the growth curves of the transformed vat2 deletion strains were independent of the galactose concentration above 0.1%, we tentatively conclude that the former explanation is the more likely.

Discussion

We have isolated the *C. tropicalis* gene and a bovine adrenal medulla cDNA encoding the vacuolar ATPase subunit B from each species. The *C. tropicalis* gene appears to be present in only a single copy in the genome and apparently gives rise to a single mRNA species as judged by Northern blotting. The bovine subunit B gene has been previously demonstrated to be present as two different genes [2], encoding what have been termed the 'brain' and 'kidney' forms [3] whose protein sequences differ primarily only at the extreme N- and C-termini. The 'brain' form appears to be present in brain as both 3.2-kb and 2.0-kb forms [2]. Our cDNA corresponds to the brain form, but appears to be present only as the shorter species in this tissue.

A comparison of the bovine peptide sequence with those of human, *S. cerevisiae*, *C. tropicalis*, *Arabidopsis thaliana* and *Neurospora crassa* show that the sequences have identities of 82.4%, 73.2%, 72.8%, 72.2% and 71.8%, respectively, indicating that the sequence of this protein is highly conserved, as has been previously noted [32]. There is, in addition, identity of the subunit B sequences with an *Archaebacterial* ATPase subunit at a level of about 49% [33], with the F_1 α-subunit [30], about 25.3%, and with the *FliI* gene product from *S. typhimurium* [34] at about 23.4%. It was this relatively invariant sequence that prompted us to try to complement the yeast vat2 deletion mutants with our subunit B clones from *Candida tropicalis* and bovine adrenal medulla. From the results presented here, it is clear that the *C. tropicalis* subunit B gene, even with its own promoter, is capable of restoring vacuolar acidification to near normal levels in the vat2-Δ1::LEU2 mutant. In contrast, the bovine cDNA appears to be capable of only very weak complementation, whether the cDNA is present in a single copy (pYPSC100) or multiple copies (pYESBT60) and whether galactose-dependent transcription induction is weak or strong. Concerned that there might be something unique about the structure of the *Saccharomyces* gene in and upstream of the N-terminus, we have placed silent *Cla*I restriction sites in the identical locations in the *Saccharomyces* gene (corresponding to amino acid No. 86) and bovine cDNA (amino acid No. 105), permitting us to replace the bovine *Sac*I–*Cla*I fragment in pYPSC100 (carrying the N-terminal domain and upstream nucleotide sequence) with the analogous *Sac*I–*Cla*I *Saccharomyces* domain and find that such a replacement is indistinguishable from pYPSC100 upon complementation of the *vat2* strain for growth at pH 7.6 (data not shown). Such a result removes any doubt that the DNA upstream of the translational start site might have some untoward effects on bovine subunit B complementation of the mutants.

The strong complementation of the vat2-Δ1 strain by the *Candida* subunit B gene, in addition to the work of Kane et al. [35], indicates that there are sufficient copies of each of the other vacuolar pump subunits

present in the vat2-Δ1 cells to permit assembly of the intact structure. Why should the bovine subunit B protein not replace the *Saccharomyces* subunit B, while the *Candida* subunit functions apparently perfectly well? Possible reasons for this are: (1) insufficient synthesis or rapid degradation of the bovine subunit B results in sufficiently few copies of the bovine subunit B that only a small number of intact V-ATPase complexes are produced, or (2) the hybrid enzyme is less catalytically active than the endogenous one, or (3) the bovine subunit B structure is sufficiently different from the endogenous protein that assembly is slow or imperfect, or that the assembled hybrid complex is unstable. None of these explanations are mutually exclusive.

Although further analysis is needed, we consider the first explanation unlikely because expression of the bovine subunit B was independent of galactose concentration in both BT60YES2 and pYPSC100, which would leave reasons 2 and 3 to explain the differences. We consider it most likely that the bovine subunit B structure does not permit the proper contacts between itself and other *Saccharomyces* V-ATPase subunits to be made, resulting in either fewer intact assemblies or less catalytically active assemblies, or both. The number of changes one might need to make in the bovine subunit B structure to allow it to function in the context of a *Saccharomyces* pump are probably reasonably small. The most glaring differences exist at the extreme N- and C-termini of the subunits B, as shown in Fig. 7, where the sequences are effectively totally dissimilar, and we are in the process of replacing the *Saccharomyces* subunit B termini with the equivalent bovine domains. If, however, these chimeric proteins do not alter function significantly (as preliminary results suggest), then we may restrict our comparisons to residues 15–485 for both *Saccharomyces* and the *Candida* proteins and residues 34–504 for the bovine subunit. In these regions, there are 32 differences between the *Saccharomyces* and *Candida* subunits and 94 between *Saccharomyces* and bovine. If one ignores the differences at positions seen between *Saccharomyces* and bovine which occur at the same positions between *Saccharomyces* and *Candida*, the number drops to 72. And if one further ignores conservative replacements, the number of significant differences between *Saccharomyces* and bovine subunits B drops to about half that value. Analysis of mutant or chimeric subunits may permit us to begin to discern the subunit–subunit interactions which exist in the proton pump and allow its assembly and function.

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