MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 5801–5812
0270-7306/91/125801-12$02.00/0

Vol. 11, No. 12

Isolation and Characterization of *PEP3*, a Gene Required for Vacuolar Biogenesis in *Saccharomyces cerevisiae*

ROBERT A. PRESTON, MORRIS F. MANOLSON, KATHLEEN BECHERER, ELAINE WEIDENHAMMER, DAVID KIRKPATRICK,† ROBIN WRIGHT,‡ AND ELIZABETH W. JONES*

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received 13 May 1991/Accepted 19 August 1991

The *Saccharomyces cerevisiae PEP3* gene was cloned from a wild-type genomic library by complementation of the carboxypeptidase Y deficiency in a *pep3-12* strain. Subclone complementation results localized the *PEP3* gene to a 3.8-kb DNA fragment. The DNA sequence of the fragment was determined; a 2,754-bp open reading frame predicts that the *PEP3* gene product is a hydrophilic, 107-kDa protein that has no significant similarity to any known protein. The *PEP3* predicted protein has a zinc finger ($CX_2CX_{13}CX_2C$) near its C terminus that has spacing and slight sequence similarity to the adenovirus E1a zinc finger. A radiolabeled *PEP3* DNA probe hybridized to an RNA transcript of 3.1 kb in extracts of log-phase and diauxic lag-phase cells. Cells bearing *pep3* deletion/disruption alleles were viable, had decreased levels of protease A, protease B, and carboxypeptidase Y antigens, had decreased repressible alkaline phosphatase activity, and contained very few normal vacuolelike organelles by fluorescence microscopy and electron microscopy but had an abundance of extremely small vesicles that stained with carboxyfluorescein diacetate, were severely inhibited for growth at 37°C, and were incapable of sporulating (as homozygotes). Fractionation of cells expressing a bifunctional *PEP3*::*SUC2* fusion protein indicated that the *PEP3* gene product is present at low abundance in both log-phase and stationary cells and is a vacuolar peripheral membrane protein. Sequence identity established that *PEP3* and *VPS18* (J. S. Robinson, T. R. Graham, and S. D. Emr, Mol. Cell. Biol. 11:5813–5824, 1991) are the same gene.

The vacuole in *Saccharomyces cerevisiae* is a polymorphic organelle that has similarities both to vacuoles in plants and to lysosomes in animal cells. Like lysosomes, yeast vacuoles are relatively acidic organelles that contain a number of proteases and other hydrolases that have relatively broad substrate specificities. Mutants that lack particular vacuolar proteases have been useful for determining the functions of specific enzymes (23, 25). One search for protease-deficient mutants unexpectedly identified 16 genes, mutations in any of which gave rise to deficiencies of two or three different vacuolar hydrolase activities (*PEP* genes) (21). Because *pep* mutations are pleiotropic, the phenotypes of the mutants provide very limited information about the primary functions of the *PEP* gene products. At the time the genes were identified, several general hypotheses were advanced to account for the pleiotropy of the mutations: that (i) these are regulatory mutations, (ii) the mutations cause changes in the structure of the compartments containing the enzymes, or (iii) the mutations alter components of the system that places the enzymes in the compartments (21). Thus, the *PEP* genes appeared to provide access to cellular "capital equipment" common to the expression of more than one vacuolar protease, but it was not clear in biochemical terms what type of equipment that might be. All three of the mechanisms mentioned above (or others not thought of) might be affected by different subsets of *PEP* genes. We are analyzing the primary functions of *PEP* genes to gain a better understanding of the biochemical requirements for vacuolar protease expression in yeast cells.

PEP4 was the first of these genes to have its product defined in biochemical terms. The finding that *PEP4* encodes the vacuolar protease A (PrA) (1, 26, 58) led to clarification of the major role of PrA in the processing of several vacuolar zymogens and hydrolase precursors (24, 26). Information that narrows the range of possible functions of the other *PEP* genes is beginning to emerge with the application of molecular biological methods. For example, the *PEP5* gene product is a low-abundance, peripheral vacuolar membrane protein (59); the predicted sequence of the *PEP7* gene product carries five zinc fingers and three apparent nuclear localization signals (64); and the *PEP12* gene product is a low-abundance integral membrane protein, localized in the vacuolar membrane and perhaps also in a Golgi compartment (4).

The *pep3* mutation causes deficiencies in PrA, PrB, carboxypeptidase Y (CpY), and alkaline phosphatase (ALP) activities and gross abnormalities in vacuolar structure, a phenotype closely resembling that seen in *pep5* mutants (43; see below). We describe here the cloning and analysis of the *PEP3* gene. The DNA sequence of *PEP3* predicts a 918-amino-acid protein that contains a zinc finger near its C-terminal end. Cells that contain *pep3* deletion alleles are viable, but they have gross defects in vacuolar content and morphology. As with the *PEP5* gene, the product of the *PEP3* gene is a low-abundance, peripheral vacuolar membrane protein. The data suggest that the protein resides on the cytoplasmic surface of the vacuolar membrane.

(Parts of this work have been presented elsewhere in preliminary form [44]).

MATERIALS AND METHODS

Strains and media. All strains were derived from X2180-1B (*MATα gal2 SUC2*) or from crosses between isogenic derivatives of that strain and strains congenic to X2180-1B obtained from D. Botstein. The relevant genotypes of strains

* Corresponding author.
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.
‡ Present address: Department of Zoology, University of Washington, Seattle, WA 98195.

used are as follows: BJ2788, *MATa pep3-12 ura3-52 trp1 leu2*; BJ2776, *MATα ura3-52*; BJ2665, *MATa leu2 ura3-52*; BJ3131, *MATa/MATα ura3-52/ura3-52 leu2/leu2*; BJ5591, BJ5592, and BJ5593, *MATa/MATα pep3Δ2::LEU2/pep3Δ2::LEU2 ura3-52/ura3-52 leu2/leu2*; BJ5579, *MATα pep3Δ2::LEU2 ura3-52 leu2 his1*; BJ6607, *MATa pep3Δ2::LEU2 suc2Δ9 ura3-52 leu2 his1*; BJ6608, *MATα pep3Δ2::LEU2 suc2Δ9 ura3-52 leu2*; BJ6617, *MATα suc2Δ9 ura3-52 leu2 his1* [YCp50:*PEP3*::*SUC2*]. Strains BJ5571-BJ5574 are meiotic segregants from a transformant of BJ3131 that was heterozygous for the *pep3Δ2::LEU2* disruption. Bacterial strains HB101 (36) and JM101 (60) were used for plasmid propagation. YEPD and synthetic media for yeast cells were prepared as described previously (51). Unless otherwise specified, glucose was included along with all other ingredients during autoclaving of these media. LB and M9CA media for bacteria have been described elsewhere (36).

Plasmids. The parental plasmids used were YCp50 (31), YEp13 (8), and YIp5 (53). The original complementing plasmids pRPA1 and pRPA3 are derivatives of YCp50. pRPA1-7 is a sequential deletion derivative of pRPA1 made by cleavage with *Cla*I, ligation, amplification in *Escherichia coli*, cleavage with *Sph*I, and ligation. To make pRPA3-4, the *Bgl*II-*Sal*I fragment of pRPA3 was subcloned into the *Bam*HI and *Sal*I sites of YCp50, and the same fragment was subcloned into the *Bam*HI and *Sal*I sites of YIp5 to give the integrating plasmid, YIp5:3SB. To make pRPA3-5, the *Xho*I-*Bam*HI fragment of pRPA3 was subcloned into the *Bam*HI and *Sal*I sites of YCp50. Construction of the *PEP3* gene disruption plasmids as derivatives of pRPA1-7 is described in Results. The plasmid containing the *PEP3*::*SUC2* gene fusion was constructed as follows. The 2.2-kb *Sma*I-*Pvu*II fragment of the *SUC2* gene from pSEYC306 (20) was ligated into the *Sma*I site of the polylinker of the Gene-Scribe Z vector pTZ19U (United States Biochemical) downstream from the 3.8-kb *Sph*I-*Cla*I fragment of pRPA1-7 that had itself been ligated into the *Acc*I-*Sph*I gap of the pTZ19U polylinker. Oligonucleotide-directed mutagenesis of the resulting plasmid (7) was then used to delete the 0.4-kb region between the final sense codon of the *PEP3* open reading frame (ORF) and the codon for the third amino acid of mature invertase, giving an in-frame fusion. The 5.7-kb *Sph*I-*Sac*I fragment containing the fusion was then subcloned into the *CEN* vector YCp50.

Recombinant DNA methods and DNA sequencing. In general, the procedures used for molecular genetics were as described previously (36). Bacterial plasmids were extracted from spheroplasts by using Brij-deoxycholate and purified by CsCl gradient centrifugation (42). Plasmid minipreps were made by an alkaline lysis method (5). Yeast genomic DNA was prepared from spheroplasts (12) by the method of Holm et al. (19). RNA for transcript analysis was prepared from cells grown in YEPD medium at 30°C as described previously (18, 28, 34). DNA fragments for cloning and hybridization analysis were isolated from agarose gels either by using an NA45 DEAE membrane and the protocol of the manufacturer (Schleicher & Schuell) or by electroelution as instructed by the manufacturer (IBI, Inc.). In some cases, DNA fragments were ligated after analysis in low-melting-point agarose gels without prior elution (52). DNA probes for nucleic acid blots were labeled in vitro with [α-^{32}P]dCTP using a random primer extension kit and the protocol of the manufacturer (New England Nuclear). DNA restriction fragments were analyzed on 0.6 to 0.8% agarose gels, RNA was analyzed on 1.2% agarose-formaldehyde gels, and the nucleic acids were blotted to nitrocellulose or Nytran filters for hybridization to labeled probes as described previously (58).

DNA sequencing. The insert in pRPA1-7 or suitable restriction fragments from it were subcloned into Gene-Scribe Z vectors (United States Biochemical), and a set of deletion subclones was generated. Single-stranded DNA templates were prepared according to the Gene-Scribe protocol. Sequencing reactions by the dideoxy-chain termination method (49) were done with a Sequenase kit as instructed by the manufacturer (United States Biochemical). The 3.8-kb *Sph*I-*Sac*I fragment of the *PEP3* clone was sequenced completely on both strands. The GenPept (release 64.3) and SWISS-PROT (release 17.0) data bases were searched, using the FASTA program (35). Hydropathy predictions were done by the method of Kyte and Doolittle (33), and protein secondary structure predictions were made by the method of Chou and Fasman (11). A search for interesting motifs was conducted with the PCGene program (Intelligenetics, Mountain View, Calif.).

Immunoassays and enzyme activity assays. Cell extracts for immunoblots and activity assays were prepared by using a Braun homogenizer as previously described (24, 59). Proteins were analyzed by polyacrylamide gel electrophoresis (PAGE), and immunoblots were made as previously described (59), with visualization by horseradish peroxidase conjugate (Bio-Rad).

Polyclonal antibodies to invertase were raised in rabbits by using antigen prepared from deglycosylated commercial invertase, as described elsewhere (41). Ten milliliters of serum from rabbit 7213 was purified by ammonium sulfate precipitation and preadsorption against whole cells (200 mg [dry weight]) of a strain that carried the *sucΔ9* allele. Aliquots of the preadsorbed serum were then affinity purified on nitrocellulose blots of deglycosylated invertase as described elsewhere (45) and were used at 1:50 dilutions for immunoblotting.

ALP activity was assayed on a nondenaturing gel as described previously (24). Acid phosphatase activity was assayed colorimetrically on whole cells by the method of Toh-E et al. (54), with *p*-nitrophenyl phosphate as the substrate. α-Mannosidase was assayed fluorimetrically as described (32) previously, using 4-methylumbelliferyl-α-D-mannopyranoside as the substrate. Invertase was assayed either on a nondenaturing gel system as described elsewhere (10) (for earlier work that characterized the invertase activity of *pep3* mutants) or, in subsequent quantitative work with the *PEP3*::*SUC2* fusion protein, by enzymatic assay of glucose production as described previously (17).

Biochemical methods. Vacuolar membranes were prepared by osmotic lysis of spheroplasts, followed by flotation of intact vacuoles on Ficoll gradients as described in detail elsewhere (59). Purified vacuolar membranes were extracted with alkaline carbonate essentially as described elsewhere (59), with the following modifications: 0.1 mg of vacuolar membrane protein was extracted with 1 ml of alkaline carbonate, pH 11.5, and the carbonate-soluble proteins were analyzed by sodium dodecyl sulfate (SDS)-PAGE directly, without a trichloroacetic acid precipitation step.

Microscopy techniques. Vacuoles were observed and photographed by fluorescence microscopy as previously described, using 5(6)-carboxydichlorofluorescein diacetate (CDCFDA) as a morphological stain (45). Electron micrographs of embedded thin sections were prepared as previously described (59).

Genetics. Sporulation and tetrad analysis were done as described elsewhere (51). The *pep3* marker was scored by

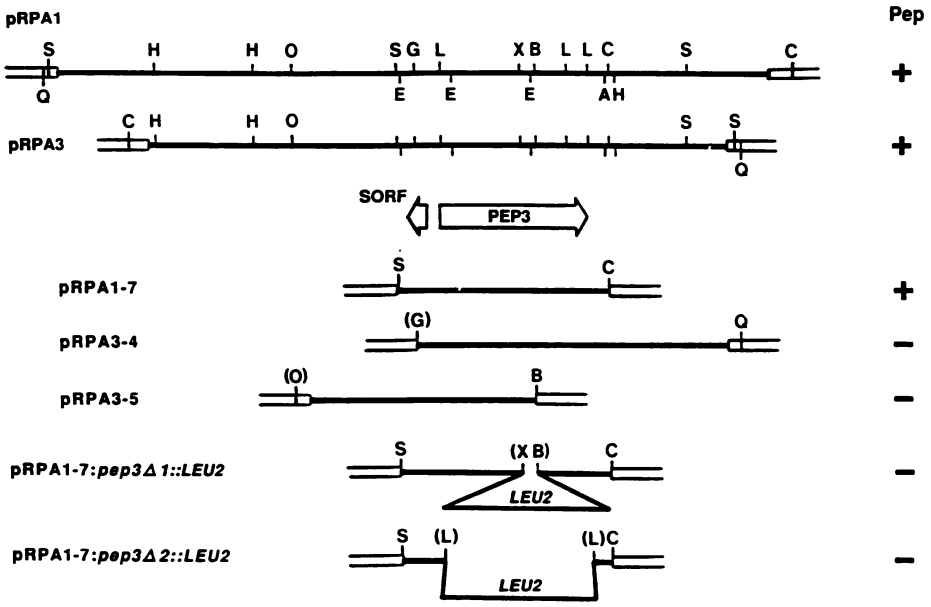

FIG. 1. Restriction maps and complementation results for pRPA1 and related plasmids. Restriction sites: A, *Sac*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; L, *Bcl*I; O, *Xho*I; Q, *Sal*I; S, *Sph*I; X, *Xba*I. Parentheses indicate destroyed sites. Lines are genomic sequences; open bars are vector sequences. See text for details of construction. Relevant ORFs are indicated by wide arrows. *PEP* complementation is based on plate assays of CpY activity.

using a yeast colony overlay assay that detects CpY activity by its ability to cleave the substrate, acetylphenylalanine β-naphthyl ester (21).

Materials. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim Biochemicals. [α-^{32}P]dCTP was from Amersham, [^{35}S]dATP was from New England Nuclear, and CDCFDA was from Molecular Probes. Nitrocellulose HAHY was from Millipore; Nytran, DEAE-nitrocellulose, and nitrocellulose BA85 were from Schleicher & Schuell.

Nucleotide sequence accession number. The GenBank accession number for the *PEP3* DNA sequence is M65144.

RESULTS

Cloning of the *PEP3* gene. Yeast cells bearing the *pep3-12* allele were transformed with a genomic library in the *CEN* vector YCp50 (gift from M. Rose, J. Thomas, and P. Novick), using the $CaCl_2$ method (9); 20,000 Ura$^+$ transformants were screened (59) to identify plasmids that complemented the *pep3* CpY deficiency. Two plasmids were obtained that were able to complement the *pep3* mutation when they were retransformed into yeast cells after amplification in *E. coli*. Restriction digest analysis revealed that the genomic fragments in these two plasmids were identical except for a slight difference in length (Fig. 1, pRPA1 and pRPA3).

Chromosomal integration and genetic mapping of the insert in pRPA3 was used to test for homology between the cloned sequence and the *PEP3* region of chromosome XII. The 5.6-kb *Bgl*II-*Sal*I fragment of pRPA3 was subcloned between the *Bam*HI and *Sal*I sites of the integrating vector, YIp5 (53). The resulting plasmid (YIp5:3SB) was then linearized at the *Bam*HI site in the putative *PEP3* region and used to transform a *pep3-12* recipient to Ura$^+$. The transformation gave a mixture of Pep$^+$ (9 of 48) and Pep$^-$ (39 of 48) transformants. Two transformants of each Pep phenotype were crossed to *PEP3 ura3-52* testers for linkage analysis (Table 1). The results of the crosses with Pep$^+$ transformants showed that the integrated sequence and the *PEP3* locus were tightly linked, since no Pep$^-$ segregants were observed in 37 tetrads. The same conclusion followed from the cross with the Pep$^-$ transformants, since no recombination between the *PEP3* locus and the integrated *URA3* marker was observed in 41 tetrads. We did not pursue an explanation for the occurrence of both the Pep$^+$ and Pep$^-$ classes of transformants among the integrants obtained with the YIp5:3SB plasmid (the insert in YIp5:3SB, when present in a *CEN* vector, did not complement *pep3-12*, as shown below). Consistent with the genetic analysis presented above and with previous mapping data for *PEP3* (40), the insert from pRPA1 hybridized to chromosome XII on orthogonal-field-alternation gel electrophoresis blots (data not shown).

Subclones or deletions of the complementing library plas-

TABLE 1. Integration of YIp5:3SB at the *PEP3* locus

Cross[a]	Genotypes	No. of indicated tetrad types[b]		
		Parental ditype	Nonparental ditype	Tetratype
1	*MAT***a** *pep3-12 ura3-52* Ura$^+$ Pep$^+$ transformant × *MATα PEP3 ura3-52*	37	0	0
2	*MAT***a** *pep3-12 ura3-52* Ura$^+$ Pep$^-$ transformant × *MATα PEP3 ura3-52*	41	0	0

[a] Strains: cross 1, BJ3148 × BJ2776; cross 2, BJ3146 × BJ2776.

[b] Tetrad types in cross 1: parental ditype, 4 Pep$^+$:0 Pep$^-$; nonparental ditype, 2 Pep$^+$:2 Pep$^-$; tetratype, 3 Pep$^+$:1 Pep$^-$. Tetrad types in cross 2: parental ditype, 2 Ura$^+$ Pep$^-$:2 Ura$^-$ Pep$^+$; nonparental ditype, 2 Ura$^+$ Pep$^+$:2 Ura$^-$ Pep$^-$; tetratype, 1 Ura$^+$ Pep$^-$:1 Ura$^+$ Pep$^+$, 1 Ura$^-$ Pep$^-$:1 Ura$^-$ Pep$^+$.

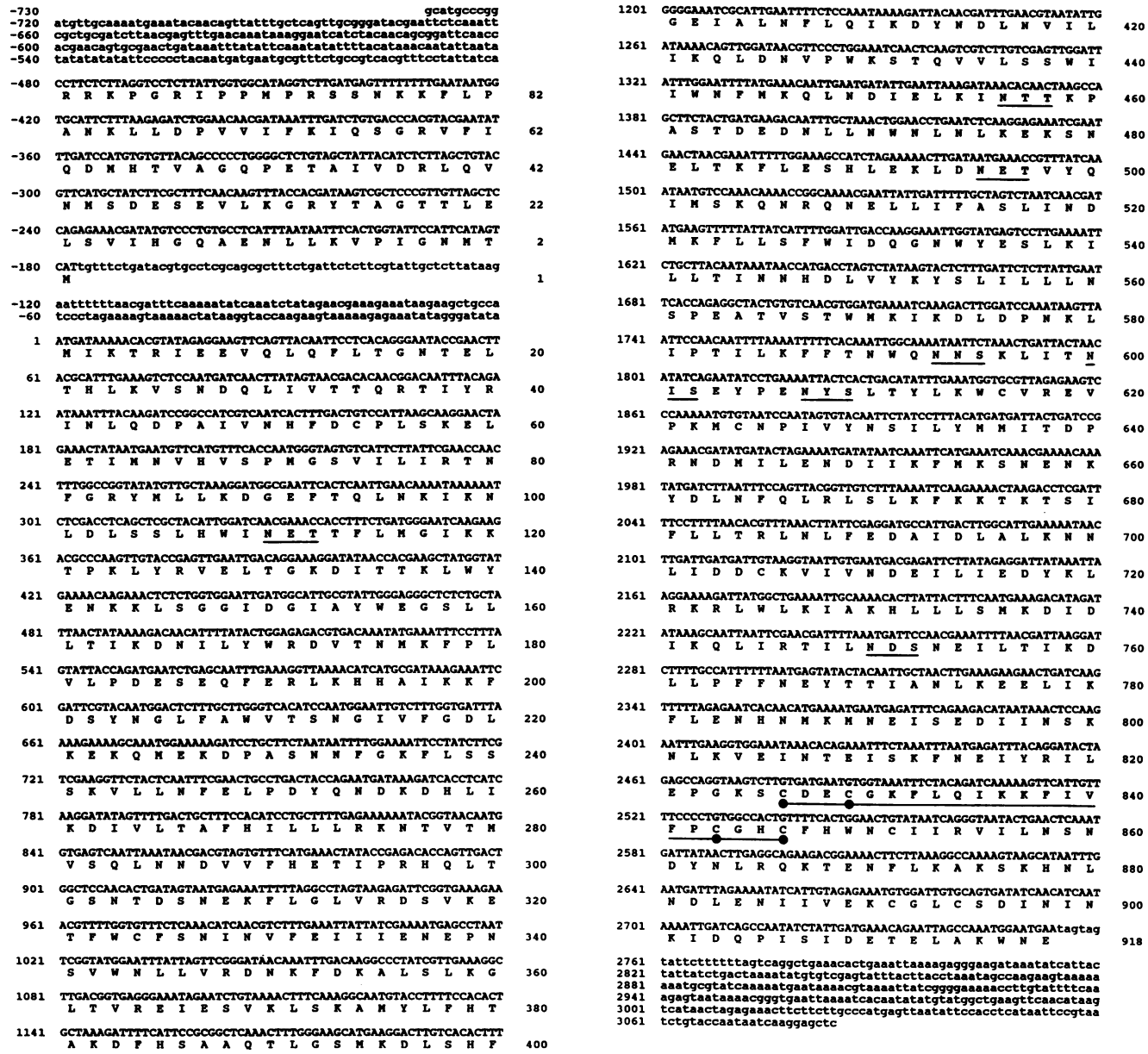

FIG. 2. Sequence of the *PEP3* gene and flanking DNA. The predicted amino acid sequence is shown under the DNA sequence. Potential sites of asparagine-linked glycosylation are underlined. The zinc finger between amino acids 826 and 846 is indicated. The predicted amino acid sequence of the small ORF in the *PEP3* upstream flanking sequence (divergently transcribed) is shown (translated by using the DNA strand complementary to the one depicted here).

mids were constructed to determine the minimal region of the insert necessary for complementation of the CpY deficiency in a *pep3* strain. The 3.8-kb fragment from *Cla*I to *Sph*I retained complementation activity (Fig. 1, pRPA1-7). Further removal of DNA on the left, to the *Bgl*II site (Fig. 1, pRPA3-4), or on the right, to the *Bam*HI site (Fig. 1, pRPA3-5), abolished complementation activity.

Sequence analysis. The DNA sequence of the 3.8-kb insert of pRPA1-7 (Fig. 1) was determined on both strands. The region contains an ORF of 2,754 bp that predicts a 918-amino-acid polypeptide of calculated molecular mass 107,413 Da (Fig. 2). Several much smaller ORFs were found in the sequenced region. The largest of these (303 bp; designated SORF) was located 178 bp upstream from the 2,754-bp ORF in a divergent transcriptional orientation (Fig. 1 and 2). Deletion mutations (see below) established that the 2,754-bp ORF corresponds to the *PEP3* gene. Codon usage in the *PEP3* gene matches the bias against major isoacceptor tRNA species that has been reported for yeast genes with low levels of expression (50). No significant similarities were detected between the *PEP3*-predicted protein and any protein in the NBRF-PIR, SWISS-PROT, or translated GenBank data base.

The predicted *PEP3* protein has no N-terminal hydrophobic signal sequence, but it does have an internal sequence of 19 amino acids (residues 511 to 529) that were identified as a potential membrane-spanning sequence according to an algorithm developed by Klein et al. (29). By using the method

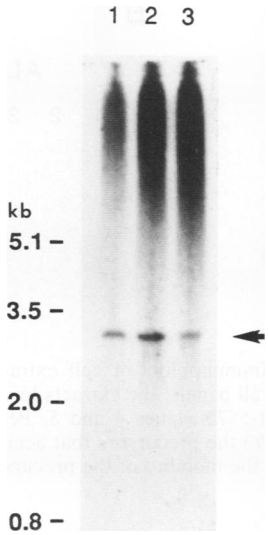

FIG. 3. RNA blot of the transcript of the *PEP3* gene. Total cellular RNA was extracted from cultures of BJ1983 grown to three different densities. Twenty micrograms of each RNA sample was analyzed on an agarose-formaldehyde gel, blotted to nitrocellulose, and probed with the ^{32}P-labeled 2.1-kb *Bgl*II-*Bam*HI fragment from pRPA1-7. Culture densities were 2×10^7 (lane 1), 1×10^8 (lane 2), and 2×10^8 (lane 3) cells per ml.

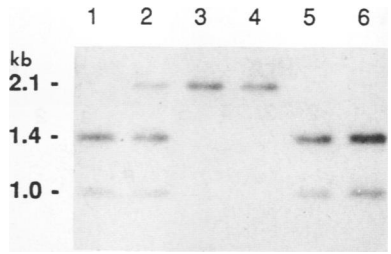

FIG. 4. DNA blot of chromosomal DNA from *PEP3* and *pep3Δ2::LEU2* strains. DNA was extracted, digested with *Eco*RI, analyzed on an agarose gel, blotted to Nytran, and probed with the ^{32}P-labeled 2.1-kb *Bgl*II-*Bam*HI fragment from pRPA1-7. Lanes: 1, the parental diploid (BJ3131); 2, its Leu$^+$ transformant (BJ5570); 3 and 4, Pep$^-$ Leu$^+$ segregants of a tetrad from the transformant (BJ5571-5572); 5 and 6, Pep$^+$ Leu$^-$ segregants from the same tetrad (BJ5573-5574).

of Garnier et al. (16), this 19-amino-acid sequence is predicted to be α-helical, and a helical wheel plot shows that the helix is amphipathic. In known transmembrane proteins, the amino acid sequences adjacent to the transmembrane helical region (on both sides) usually carry a net positive charge; the amino acids immediately adjacent to the helix almost never have a negative charge (6). In the *PEP3* sequence, the net charge of the 15 amino acids on the N-terminal side of the amphipathic helix is −2, and that on the C-terminal side is −1; the two residues adjacent to the helix are both negative (Fig. 2). Thus, the context of the helix is atypical of that seen in known membrane-spanning sequences. Localization results (see below) also suggest that the amphiphilic helix has a function other than that of a transmembrane domain. The predicted *PEP3* protein has seven potential acceptor sites for asparagine-linked glycosidic side chains (Fig. 2), although the evidence suggests that these sites are not glycosylated (see below). There are no noteworthy features in the secondary structure predictions of the *PEP3* protein, as determined by using the method of Chou and Fasman (11).

The most interesting feature of the predicted *PEP3* sequence is a $CX_2CX_{13}CX_2C$ zinc finger in the C-terminal region of the protein, beginning at amino acid residue 826 (Fig. 2). There is almost no similarity between the noncysteine residues in this region and those in any other zinc finger protein in the data bases. Results that address the functional significance of this region are presented in the accompanying report (46).

Transcript analysis. Total cellular RNA was isolated from cultures grown on YEPD to early and late log phase and to a stage several hours past the point of depletion of glucose from the medium. The RNA was fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with the gel-purified 2.1-kb *Bgl*II-*Bam*HI fragment of *PEP3* from pRPA1-7. A 3.1-kb transcript was detected, in reasonable agreement with the 2.75-kb ORF in the *PEP3* sequence (Fig. 3). The transcript was present in cells harvested at all three culture densities tested, possibly being more abundant in late-log-phase cells.

***PEP3* gene disruptions.** To determine the phenotype of the null allele, deletions and disruptions of the *PEP3* gene were constructed by replacing portions of the insert in pRPA1-7 with the *LEU2* gene. For one disruption, the 2.6-kb *Bcl*I fragment of pRPA1-7 (which lies entirely within but includes essentially all of the *PEP3* ORF) was replaced with a 2.8-kb *Bgl*II fragment from YEp13 that contains the *LEU2* gene (Fig. 1). The 6.5-kb *Sph*I-*Sac*I fragment of the resulting plasmid (pRPA1-7:*pep3Δ2::LEU2*) was used to transform BJ3131 to Leu$^+$. Transformants heterozygous for the disruption were identified by using gel transfer DNA hybridization (data not shown). After sporulation, 16 tetrads were dissected. These tetrads had four viable spores and gave the segregation expected from a disruption heterozygote, 2 Pep$^+$ Leu$^-$:2 Pep$^-$ Leu$^+$. Genomic DNA from the diploid transformant, its parent, and segregants of three tetrads was analyzed by gel transfer hybridization (Fig. 4). The labeled 2.1-kb *Bgl*II-*Bam*HI fragment of *PEP3* hybridized to the expected 1.0- and 1.4-kb *Eco*RI fragments of DNA from Pep$^+$ segregants. The 2.1-kb *Eco*RI fragment found in the Pep$^-$ segregants (and the heterozygous diploid) was in accord with the map predicted for the disruption allele. Results consistent with those were obtained by hybridization to genomic DNA cut with *Eco*RV (data not shown). Thus, the Pep$^-$ phenotype in these tetrads was caused by the integrated deletion/disruption allele. This allele was named *pep3Δ2::LEU2*. Similar results were obtained with a different deletion/disruption allele, in which the 258-bp *Xba*I-*Bam*HI portion of *PEP3* was replaced by the *LEU2* gene. Fifty-five tetrads resulting from that construction had viability and marker segregation consistent with the results obtained with the *pep3Δ2::LEU2* disruption allele (data not shown). This allele was named *pep3Δ1::LEU2*.

Effects of disrupted *PEP3* alleles on vacuolar hydrolases. Strains bearing the *pep3Δ1::LEU2* disruption allele contained greatly diminished amounts of the mature-size antigens for the vacuolar luminal proteases PrA, PrB, and CpY. The defects cosegregated in three tetrads examined, one of which is shown in Fig. 5A. Low but detectable amounts of antigens for each protease were present in forms larger than the respective mature-size forms. For CpY, the larger form corresponds in size to that of the p2 precursor form seen in a *pep4-3* strain. The larger form of PrB antigen most evident in Fig. 5 (38 kDa) is smaller than the 40-kDa intermediate that accumulates in a *pep4-3* strain; it may or may not be the

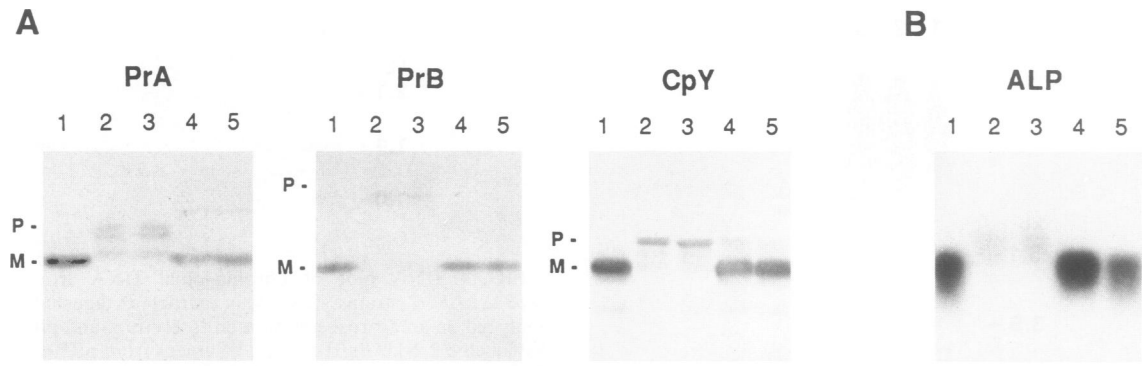

FIG. 5. Expression of vacuolar hydrolases in *pep3Δ2::LEU2* meiotic segregants. (A) Immunoblots of cell extracts probed with the indicated antisera; (B) native polyacrylamide gel of cell extracts stained for ALP activity. In all panels, the extracts loaded were as follows: lane 1, wild-type parental diploid (BJ3131); lanes 2 and 3, Pep^- Leu^+ segregants (BJ5571-5572); lanes 4 and 5, Pep^+ Leu^- segregants (BJ5573-5574). M and P indicate the mobilities of mature-size antigens and (for PrB and CpY) the precursors that accumulated in a *pep4-3* strain (lanes that contained the *pep4-3* samples are not shown). For PrA, P is an estimate of the mobility of the precursor that accumulated in a *pep4-7* mutant.

same size as the 37- to 38-kDa intermediate recently reported (39). In addition to the 38-kDa form of PrB visible in the disruption strains in Fig. 5, trace amounts of 40-kDa and mature-size antigen were visible in the original immunoblots. Three bands somewhat larger than mature PrA migrated as 43-, 47-, and 49-kDa proteins in strains bearing the disruption allele. The largest of these may be the pro-PrA form that accumulates in a *pep4-7* mutant (26), but comparisons with that strain on a single gel were not made.

The activity level of the vacuolar membrane protein, repressible ALP (3, 30), was greatly reduced in strains bearing the *pep3Δ2::LEU2* allele, and this defect cosegregated with the disruption mutation in three tetrads (Fig. 5B). However, the *pep3Δ2::LEU2* allele caused only a slight deficiency in the activity of α-mannosidase, an enzyme thought to be a peripheral membrane protein inside the vacuole (55, 62). Assays of cell extracts of meiotic segregants from the diploid heterozygous for the disruption allele (with the substrate 4-methylumbelliferyl-α-D-mannopyranoside) gave a specific activity for α-mannosidase of 3.2 nmol/min/mg of protein (standard deviation = 0.4, $n = 4$) for Pep^- segregants, compared with 4.1 nmol/min/mg of protein (standard deviation = 0.5, $n = 4$) for Pep^+ segregants.

Vacuolar hydrolases use parts of the secretory pathway for their transport to the vacuole. The viability of strains bearing *pep3* disruptions argues that *PEP3* is not essential for secretion itself (which is essential for growth). We detected no significant deficiencies in the secretion of either invertase or acid phosphatase activities in *pep3-12* mutants (data not shown).

Vacuolar morphology in *pep3* mutants. When cells containing the *pep3Δ2::LEU2* allele were labeled with the vital dye CFCFDA and examined by fluorescence microscopy (45), few vacuoles of normal size could be seen (Fig. 6). Unlike wild-type cells, in which the vacuole resembled a cluster of grapes that filled a substantial fraction of the cell volume, *pep3* mutant cells contained only vestigial remnants of vacuolelike structure. Apart from a few bodies that resembled the smallest of vacuolar vesicles present in wild-type cells, the mutant cells contained a large number (100 to 200) of fluorescent particles visible only as bright points of light. Unlike the relatively slowly moving, larger vesicles present in mutant and wild-type cells, the pointlike particles in the mutant cells exhibited rapid Brownian motion-like movement. Because of that movement, the pointlike particles are not visible in Fig. 6 except as an unresolved background fluorescence. No pointlike particles were detectable in wild-type cells.

Examination of thin sections by electron microscopy showed that *pep3Δ2::LEU2* cells contained few membrane-bound structures that might be interpreted as vacuoles (Fig. 7). The structures present were smaller than the vacuoles typical of wild-type cells; their small number and size

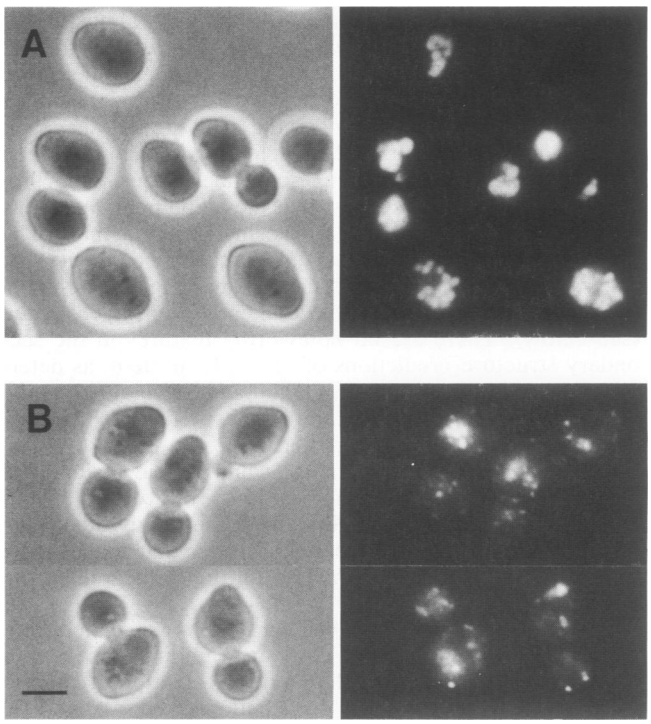

FIG. 6. Phase-contrast (left) and fluorescence (right) micrographs. (A) Wild-type (BJ3131); (B) *pep3Δ2::LEU2* (BJ5592). Vacuoles of cells in exponential growth in YEPD were labeled with CDCFDA, and the cells were photographed by phase-contrast or epifluorescence, using a fluorescein filter set.

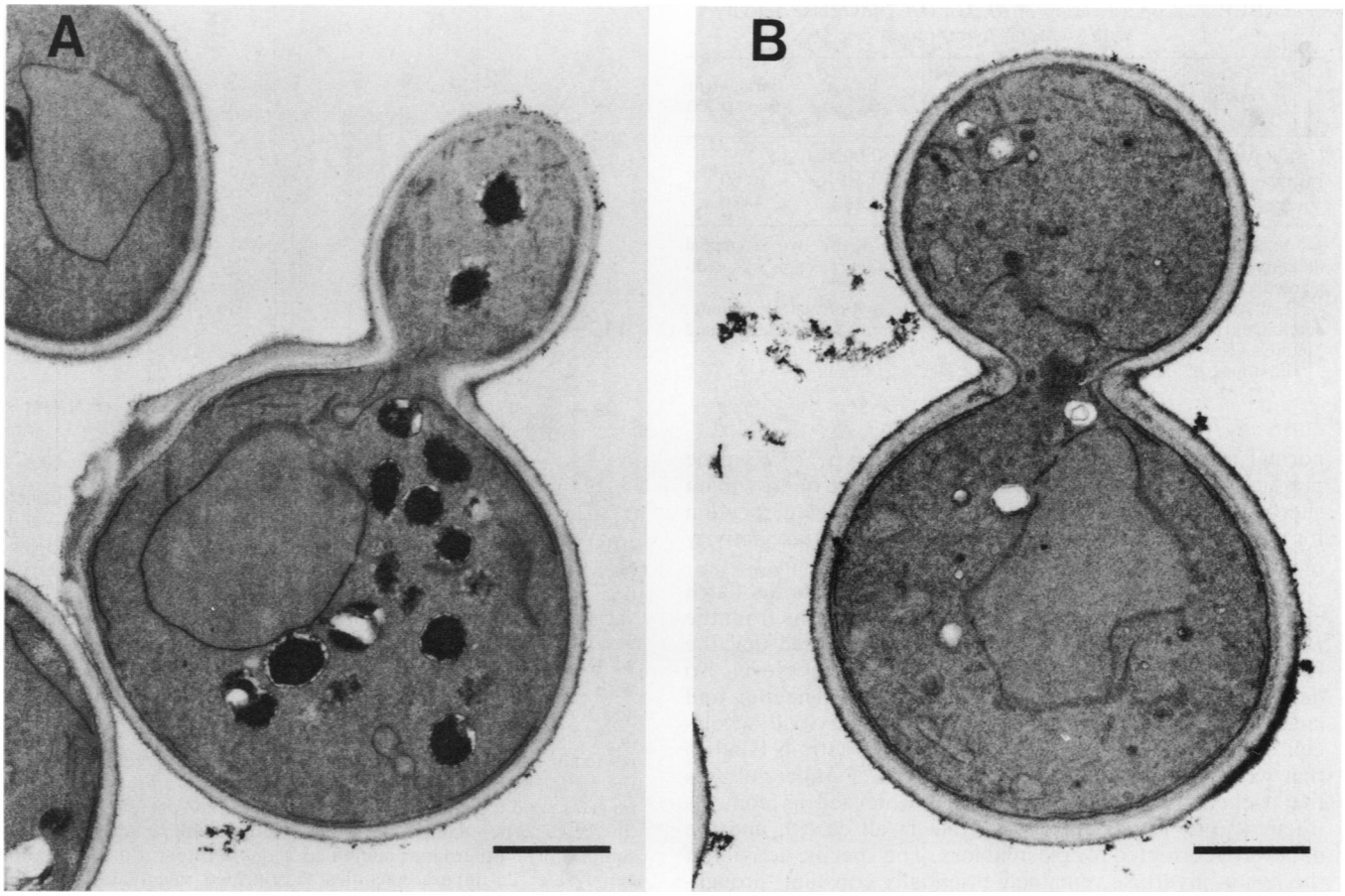

FIG. 7. Thin-section electron micrographs of yeast cells. (A) Wild type (BJ492); (B) *pep3Δ2::LEU2* (BJ5579). Cells growing exponentially in YEPD were rapidly fixed, embedded, and sectioned, and the mounts were stained with lead citrate to visualize vacuoles.

suggest that they are the larger of the structures visualized in *pep3Δ2::LEU2* mutants by CDCFDA staining. No population of vesicles clearly corresponding to the pointlike particles seen by fluorescence was evident by thin-section electron microscopy.

Heat sensitivity of *pep3* mutants. The ability of *pep3* mutants to grow at 37°C depended on unidentified details of the growth assay. In general, using a pronged replicating block for transfers of cells from tetrad masters to the wells of microtiter plates and then to YEPD replica plates, every meiotic segregant bearing a *pep3* allele grew very poorly, or not at all, after 3 days of incubation at 37°C. The extent of the growth defect for any given segregant was quite variable in replicate tests done several weeks or months apart. We could not identify the factor(s) responsible for *pep3* segregants growing either poorly or not at all under ostensibly identical conditions. The severe growth defect at 37°C was equally evident in mutants bearing point mutations or either of the disruption alleles *pep3Δ1::LEU2* and *pep3Δ2::LEU2*.

Homozygous *pep3* diploids do not sporulate. Mutations in the *PEP4* gene cause severe deficiencies in a number of vacuolar hydrolases; the lack of sporulation competence in diploids homozygous for the *pep4* mutation has suggested that vacuolar hydrolases are required for sporulation (65). The same result was obtained when three independent diploids homozygous for *pep3Δ2::LEU2* (BJ5591, BJ5592, and BJ5593) were incubated on sporulation media of several different types, including buffered, acidic acetate medium (38). No asci were detectable in any of these cultures after incubation for 2 weeks at 30°C.

Construction of a functional *PEP3::SUC2* fusion. Several attempts to obtain antiserum to the *PEP3* gene product to study its expression and intracellular localization were unsuccessful. Alternatively, we constructed a *PEP3::SUC2* gene fusion (Materials and Methods) that enabled us to use invertase activity as well as anti-invertase antibodies for similar purposes. The protein predicted from the gene fusion contains all but the first two amino acids of mature invertase linked to the C-terminal glutamic acid predicted by the *PEP3* gene. The protein component of the fusion would have a molecular mass of 167 kDa; asparagine-linked glycosylation of the potential acceptor sites in the fusion could add up to 110 kDa of carbohydrate, depending on the intracellular localization of the fusion protein and the details of such posttranslational modifications.

Yeast strains bearing deletions of the chromosomal *SUC2* and *PEP3* genes were transformed with the fusion gene carried in the *CEN* vector YCp50. Five independent fusion constructions gave transformants (with two independent recipient strains, BJ6607 and BJ6608) that had the same phenotypes: the *PEP3::SUC2* fusion plasmid complemented the CpY deficiencies and the defect in vacuolar morphology caused by chromosomal *pep3Δ2::LEU2* alleles. Thus, the fusion protein retained the function, and probably also the

TABLE 2. Copurification of *PEP3*::*SUC2* invertase activity with vacuolar membranes[a]

Fraction	Protein (mg)	Total activity (U)[b]	Sp act (U/mg)	Enrichment (fold sp act)
Crude lysate	1,000	2.0	0.0020	1
Impure vacuoles[c]	13	1.3	0.10	50
Vacuolar membranes	2.1	0.8	0.362	180

[a] Vacuoles were purified from a spheroplast lysate by two sequential flotations on Ficoll density gradients, and a membrane fraction was sedimented as described in Materials and Methods.

[b] Units are expressed as micromoles of sucrose hydrolyzed per minute, determined by enzymatic assay of glucose as described in Materials and Methods.

[c] First float of vacuoles: SW28 rotor, 100,000 × *g*, 45 min.

normal intracellular location, of the wild-type *PEP3* gene product. All transformants remained incapable of growth on sucrose, indicating an internal, rather than secreted, location for the fusion protein. (The fusion did have invertase activity in cell extracts; see below).

Expression of the *PEP3*::*SUC2* fusion protein in batch cultures. On the basis of codon usage comparisons from the *PEP3* DNA sequence (see above), we anticipated that the fusion protein might be expressed at a very low level. To determine reasonable cultural conditions for detecting and analyzing the fusion, we assayed invertase activity in whole-cell extracts from plasmid-bearing cultures (strain BJ6617) that were grown to various cell densities. Parallel cultures that lacked the fusion plasmid were analyzed as controls (their activity levels were negligible in all cases), and the data were corrected for plasmid loss. The specific activity of the fusion invertase remained essentially constant throughout log-phase growth in YEPD at least until 1.5 h past the point of glucose exhaustion, when the culture had entered the diauxic growth plateau (2×10^8 cell per ml). The specific activity of the fusion invertase in log-phase extracts was 0.006 U/mg of protein. An extract from a culture grown to stationary phase (5×10^8 cell per ml) had a specific activity 80% lower than that of the log-phase extracts. Neither plasmid loss nor proteolytic degradation in vitro appeared to be responsible for the decreased specific activity of stationary-phase extracts (data not shown). An estimate of the relative abundance of the *PEP3*::*SUC2* fusion protein was obtained by comparing its activity with the activity of the cytoplasmic form of wild-type invertase, the latter being assayed in extracts of a *SUC2* strain grown under glucose-repressing conditions. The specific activity of the *PEP3*::*SUC2* fusion protein (log phase) was about 1/10 the activity of the wild-type, cytoplasmic invertase.

Intracellular localization of the *PEP3*::*SUC2* fusion protein. Crude extracts containing the *PEP3*::*SUC2* fusion protein were prepared from log-phase cultures of BJ6617 and centrifuged at 100,000 × *g* for 1 h to sediment membranes; 96% of the fusion invertase activity present in the initial extract was recovered in the sedimented membrane fraction. The soluble and membrane fractions were analyzed by SDS-PAGE and immunoblotted by using affinity-purified antibody to invertase. A single peptide of molecular mass 150 kDa (not present in control extracts prepared from cells lacking the fusion plasmid) was detected in the membrane fraction only (data not shown, but see below). Purified vacuolar membranes were then prepared from a strain (BJ6617) that contained the *PEP3*::*SUC2* fusion protein (Materials and Methods). As shown in Table 2, the invertase activity of the

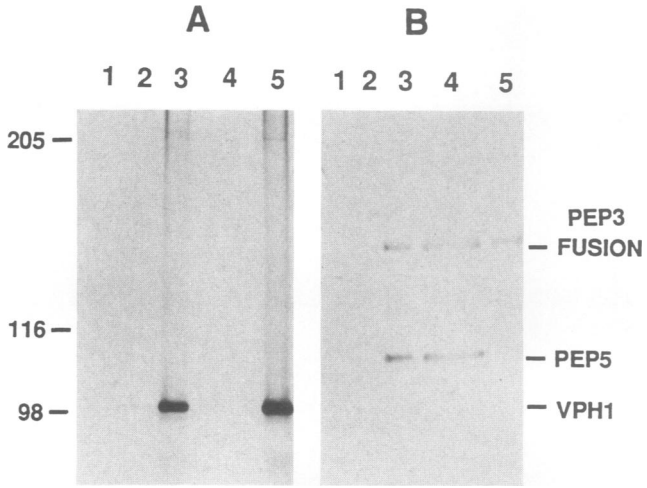

FIG. 8. Immunoblots of fractions taken during vacuolar vesicle purification and extraction by alkaline carbonate. Log-phase cells (BJ6617) expressing the *PEP3*::*SUC2* fusion protein in a wild-type background were spheroplasted and lysed by gentle homogenization. Vacuoles were purified by density gradient flotation, and vacuolar membrane vesicles were isolated and extracted with alkaline carbonate as described in Materials and Methods. Fractions were analyzed by SDS-PAGE, blotted to nitrocellulose, and stained with antibodies as described in Materials and Methods. Panels A and B represent identical sets of samples from a single gel that were probed with different antibodies; panel A was probed with antibodies to the 95-kDa vacuolar integral membrane protein encoded by the *VPH1* gene, and panel B was probed with antibodies to both invertase and the vacuolar peripheral membrane protein encoded by the *PEP5* gene. Samples in lanes 1 contained 70 μg of protein; samples in all other lanes contained 7 μg of protein. Lanes: 1, crude cell lysate; 2, impure vacuoles; 3, purified vacuolar membrane vesicles; 4, carbonate-soluble extract of purified vacuolar membrane vesicles (the increased width of these lanes is an effect of the large amount of carbonate buffer present in the samples loaded); 5, carbonate-insoluble residue from purified vacuolar membrane vesicles. The relevant bands are identified on the right; molecular weight markers migrated as shown at the left (indicated in kilodaltons).

fusion protein was enriched 180-fold in purified vacuolar membrane vesicles, with a recovery of 40% of the initial lysate activity. Fractions taken during successive steps of the vacuole purification were analyzed by SDS-PAGE, and immunoblots were prepared as described above. The *PEP3*::*SUC2* fusion peptide detected with anti-invertase was highly enriched in vacuolar membrane vesicles (Fig. 8).

The data presented above established that the bulk of the *PEP3*::*SUC2* fusion protein is localized at the vacuolar membrane. To test whether the fusion protein was an integral or peripheral membrane protein, vacuolar membrane vesicles were extracted with alkaline carbonate to solubilize peripheral membrane proteins (Materials and Methods). The carbonate-soluble (peripheral protein) and the carbonate-insoluble (integral protein) fractions were analyzed by SDS-PAGE and immunoblotting. Antibodies to the *VPH1* gene product (an integral membrane protein [37]) were used as a control to show that the peripheral membrane fraction was not contaminated with integral membrane proteins. Antibodies to the peripheral membrane protein encoded by the *PEP5* gene (59) were used as a similar control to establish the purity of the integral membrane fraction. The carbonate-soluble and carbonate-insoluble fractions were found to contain the *PEP3*::*SUC2* fusion protein in roughly

equal amounts (Fig. 8). This result suggests that the fusion is probably a peripheral membrane protein that resists complete solubilization by the carbonate method.

The state of glycosylation of the *PEP3*::*SUC2* fusion protein provided an indirect clue to the orientation of the protein on the vacuolar membrane. The fusion protein migrated on polyacrylamide gels as would be expected for a nonglycosylated polypeptide (Fig. 8; see Discussion). In addition, in crude cell extracts, none of the invertase activity of the fusion protein adsorbed to a lectin affinity matrix (concanavalin A–Sepharose 4B) under conditions in which, as a control, 36% of the activity of wild-type, glycosylated invertase did adsorb.

DISCUSSION

Like the other *PEP* genes, *PEP3* was initially identified by the effect of *pep3* mutations on the expression of CpY activity in a yeast colony overlay assay (21). *pep3* (*vpt18 vps18* [46]), *pep5* (*end1* [59]; *vpt11 vpl9 vps11* [48]), and *pep14* (*vpt33 vps33* [47]; *slp1* [64]) are now known to be pleiotropic mutations that cause gross defects in vacuole structure, multiple vacuolar hydrolase deficiencies, and a variety of additional phenotypic abnormalities that may or may not be directly related to vacuolar functions (22, 43). As indicated, the *PEP3*, *PEP5*, and *PEP14* genes have also been identified and given synonymous names in screens for mutants that mislocalize vacuolar hydrolases (2, 48), that have defects in endocytosis (13, 14), or that have abnormal vacuolar pools of amino acids (56). The results reported here begin to address the molecular basis for the pleiotropic *pep3* phenotype.

The *PEP3* DNA sequence contains no striking clues to the function of the *PEP3* gene product, since it lacks informative motifs or similarities to other known proteins. One interesting feature in the predicted protein is a $CX_2CX_{13}CX_2C$ zinc finger in the C-terminal region of the protein (Fig. 2). The results of mutagenizing one of the cysteine codons in the *PEP3* zinc finger show that this region is important for stabilizing the *PEP3* protein at elevated temperatures (46). The spacing of the cysteines in this finger is identical to that of the finger found specifically in the larger of the two proteins encoded in the adenovirus E1a region, and these sequences also share a similar triplet, PG(H/K), adjacent to the upstream CX_2C residues (27). Unfortunately, the role of the zinc finger in the transactivating function of the E1a protein is not known. See the accompanying report by Robinson et al. (46) for further discussion of this region of the *PEP3* protein. One other important aspect of the *PEP3* sequence is its overall hydrophilicity, including the N-terminal region. It seems unlikely that this protein could enter the secretory pathway or insert in membranes as an integral membrane protein, and that accords with the available localization data (see below).

The profile of vacuolar hydrolase expression in strains bearing the *pep3Δ2*::*LEU2* deletion allele is almost identical to that reported for mutants bearing *pep5* deletions (59). Detectable levels of mature-size antigens for PrA, PrB, and CpY do occur in extracts of the deletion mutants. Since the *pep3Δ2*::*LEU2* allele abolishes 95% of the coding region of the gene, it is undoubtedly a null allele. The residual hydrolase expression in these strains (and their vestigial vacuolar structures; see below) cannot be ascribed to a partially functional *PEP3* gene product, as could have been argued with strains previously isolated that contained point mutations (2, 21, 47). The persistent low-level hydrolase expression detected in the deletion mutants suggests that a parallel (but inefficient) mechanism provides a substitute for at least some of the functions of the *PEP3* and *PEP5* gene products in the respective mutants.

Decreased activity of ALP in strains bearing the *pep3Δ2*::*LEU2* allele shows that the expression of vacuolar integral membrane proteins as well as luminal hydrolases is dependent on the *PEP3* gene product. Since we assayed ALP activities, and since activation of ALP is PrA dependent (24, 30), it is possible that the decreased expression of ALP in *pep3* strains is a secondary consequence of the low levels of PrA in the strains.

It is an interesting result that α-mannosidase activity is nearly normal in whole-cell extracts of *pep3* mutants (and of *pep5* [59], *pep7* [64], and *pep12* [4] mutants). α-Mannosidase is thought to be a peripheral, luminal vacuolar membrane protein (55, 62, 63). The fact that none of these *pep* mutations strongly affect its activity suggests that α-mannosidase differs from the luminal proteases and the integral membrane enzyme, ALP, in posttranslational processing or localization mechanisms. One clear difference is that α-mannosidase activity arises independently of *PEP4* (PrA) function, unlike the activity of ALP and the luminal proteases (24). Absence of a signal sequence in the predicted product of the gene that encodes α-mannosidase (61) and lack of glycosylation (62) are among the anomalies (for a vacuolar hydrolase) that confuse the issue of the biosynthesis and localization of α-mannosidase. It has been reported that alleles of *VPS18* (allelic to *PEP3*) that contain point mutations cause secretion of a substantial fraction of the total α-mannosidase activity (47), a result that seemingly requires entry of α-mannosidase into the secretory path without benefit of a signal sequence. Further studies of the biosynthesis and intracellular localization of α-mannosidase in wild-type cells seem to be required for interpreting the meaning of its fate in *pep* mutants (see also below).

The aberrant vacuolar morphology in *pep3* mutants (Fig. 6 and 7) is very similar to that seen in *pep5* (59) and *pep14* (43) mutants. Similar morphologies in other mutant collections have been reported (class C *vpt* mutants [2]; *vam* mutants [56]). The morphologies in these mutants are sufficiently abnormal to raise doubts about the nature of the aberrant vacuoles in these strains. It is possible that the vesicular structures in any or all of these mutants bear no relation at all to the vacuoles of normal cells; the vesicles in mutants bearing *pep3*, *pep5*, or *pep14* mutations do stain with the vital dye CDCFDA, but the vacuolar specificity of that dye has not been rigorously established in these mutant strains (45). On the assumption that the CDCFDA-stained particles in *pep3* mutant cells are indeed vacuolar in some sense, direct visual observation of the hundreds of very minute stained particles in the mutants gives the impression that, collectively, the particles have a large total surface area (possibly even exceeding the surface area of wild-type vacuoles), despite their individual small sizes. The normal α-mannosidase activity levels in these mutants would be consistent with a constancy of vacuolar membrane area, despite an apparent severe reduction in vacuolar volume as judged by phase-contrast microscopy or thin-section electron microscopy.

Since cells bearing *pep3* null alleles are viable, the heat sensitivity conferred by the various *pep3* alleles is not indicative of a thermolabile, essential *PEP3* protein. Instead, loss of the *PEP3* gene product restricts the temperature range for growth of *S. cerevisiae* on YEPD medium. This phenotype is not uncommon for mutants of *S. cerevisiae* that

have a variety of unrelated genetic defects, so it is not very informative concerning the function of the *PEP3* gene product.

The finding that strains bearing deletions of the *PEP3* gene are viable adds *PEP3* to a set of genes that are required for the expression of vacuolar hydrolase activities but are not essential for vegetative growth on YEPD medium. The *PEP4*, *PEP5*, *PEP7*, and *PEP12* genes are other members of this set (1, 4, 15, 58, 59). Since the *PEP* genes sequenced to date are not at all similar to each other, and since these *pep* mutations cause different phenotypes, the encoded proteins are not likely to have entirely redundant functions. A number of strains that bear two different *pep* mutations have been constructed (including the *pep3 pep5* double mutation); the pairwise combinations tested did not result in lethality. The fact that vacuolar functions dependent on these *PEP* gene products are dispensable for growth on standard media does not imply that all vacuolar functions are dispensable for such growth: none of the *pep* mutants is entirely devoid of vesicular structures that may retain some vacuolar functions.

Expression of a functional *PEP3*::*SUC2* gene fusion in *S. cerevisiae* gave information relevant to an eventual determination of the biochemical function of the *PEP3* gene product. A number of conclusions follow from the work presented. The low specific activity and antigen level of the *PEP3*::*SUC2* fusion protein confirmed the prediction based on codon usage that *PEP3* is expressed at low levels. Since the fusion protein copurified with vacuolar membranes and was substantially solubilized by alkaline carbonate treatment, the *PEP3* gene product, by inference, appears to be reasonably identified as a minor peripheral vacuolar membrane component. We think that the incomplete solubilization of the fusion protein by alkaline carbonate treatment (Fig. 8) simply reflects a limitation of the solubilizing ability of the (relatively mild) carbonate treatment. The possibility that some peripheral membrane proteins would be difficult to solubilize with that treatment seems much more likely than other possibilities, such as a single protein being both an integral and peripheral membrane component, or a lipophilic integral membrane protein being partially soluble in a detergent-free carbonate buffer.

Our results suggest that the fusion protein, and presumably also the *PEP3* protein itself, is located on the cytoplasmic rather than the luminal face of the vacuolar membrane. The only well-established biogenetic route to the luminal side of the vacuolar membrane involves passage through the early stages of the secretory pathway with its associated glycosylating enzymes. The *PEP3* sequence lacks appropriate hydrophobic signal sequences that would allow entry into the secretory pathway. Furthermore, the *PEP3*::*SUC2* fusion protein appears not to be glycosylated. On polyacrylamide gels, it shows no evidence of the increased, heterogeneous molecular weight that glycosylation would cause, as seen, for example, with a CpY-invertase fusion (20). In addition, the fusion failed to bind to a lectin that did bind glycosylated invertase. A mechanism for transport to the luminal face of the vacuolar membrane by a path that bypasses the glycosylation system of the secretory path has been reported for the enzyme α-mannosidase (62). Since the half-time for vacuolar entry by that mechanism appeared to be 10 h (62), or about 2 orders of magnitude slower than the rate of entry by the secretory path, it is not clear that the mechanism proposed for α-mannosidase is relevant in a biogenetic (as opposed to degradative) sense. We think that the failure of the *PEP3*::*SUC2* fusion protein to enter the secretory path, evidenced by its lack of glycosylation, provides reasonable, although indirect, evidence pointing toward a location for the *PEP3* protein on the cytoplasmic side of the vacuolar membrane. Clearly, a final answer to the localization of the wild-type *PEP3* product will require a more direct analysis of that product.

As a low-abundance, 107-kDa protein located on the vacuolar membrane, the *PEP3* gene product seems to be required for either establishing or maintaining the gross morphology of the vacuole (Fig. 6 and 7). The low abundance of the gene product rules out a role as a major structural component of vacuolar membranes. Possibly, vesicular biogenetic precursors require the presence of *PEP3* protein as a receptor or part of a specific vacuolar fusion agent in order to fuse together to produce vacuoles of normal size. Alternatively, vacuoles that might arise (de novo) normally in *pep3* mutants could require the *PEP3* gene product for subsequent structural stability. For example, the vacuolar compartment appears to break up and reassemble during the course of the cell cycle (57), and the reassembly (fusion) mechanism could involve the *PEP3* gene product directly or indirectly. This reasoning does not immediately account for the fact that the *pep3* mutation causes secretion of a large fraction of the precursors to PrA and CpY (47, 48). While it is possible that the *PEP3* gene product functions directly in a mechanism for correctly targeting vacuolar proteins, it is at least equally likely that secretion of such proteins from *pep3* cells represents an indirect targeting mechanism overload condition that could result from the simple absence of vacuoles competent to receive any input, whether targeted correctly or not.

ACKNOWLEDGMENTS

We thank Kathleen Conley for skilled technical assistance, Carol Woolford for valuable discussions and assistance, Vicki Nebes for antiserum to invertase, John Hill for assistance with DNA data base searching, Janine Maddock for assistance with sequencing, and Jane S. Robinson for sharing ideas and results prior to publication.

This research was supported by Public Health Service grants GM29713 and DK18090 from the National Institutes of Health to E.W.J., by National Research Service Award GM11329 to R.A.P., by a grant from the Medical Research Council of Canada to M.F.M., by American Cancer Society fellowship PF2946 to R.W., by Public Health Service grant GM35827 to Jasper Rine, and in part by a grant from the Pittsburgh Supercomputing Center through the NIH Division of Research Resources cooperative agreement U41 RR04154.

REFERENCES

1. **Ammerer, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls, and T. H. Stevens.** 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. **6**:2490–2499.
2. **Banta, L. M., J. S. Robinson, D. J. Klionsky, and S. D. Emr.** 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. J. Cell Biol. **107**:1369–1383.
3. **Bauer, H., and E. Sigarlake.** 1975. Localization of alkaline phosphatase in *Saccharomyces cerevisiae* by means of ultrathin frozen sections. J. Ultrastruct. Res. **50**:208–215.
4. **Becherer, K., and E. W. Jones.** Unpublished data.
5. **Birnboim, H., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Boyd, D., and J. Beckwith.** 1990. The role of charged amino acids in the localization of secreted and membrane proteins. Cell **62**:1031–1033.
7. **Brizuela, L., G. Draetta, and D. Beach.** 1987. $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$

protein kinase. EMBO J. 6:3507–3514.
8. **Broach, J. R., J. N. Strathern, and J. B. Hicks.** 1979. Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene **8**:121.
9. **Bruschi, C. V., A. R. Comer, and G. A. Howe.** 1987. Specificity of DNA uptake during whole cell transformation of *S. cerevisiae*. Yeast **3**:131–137.
10. **Carlson, M., B. C. Osmond, and D. Botstein.** 1981. Mutants of yeast defective in sucrose utilization. Genetics **98**:25–40.
11. **Chou, P., and G. Fasman.** 1974. Prediction of protein conformation. Biochemistry **13**:222–245.
12. **Davis, R., M. Thomas, J. Cameron, T. St. John, S. Scherer, and R. Padgett.** 1980. Rapid DNA isolation for enzymatic and hybridization analysis. Methods Enzymol. **65**:404–411.
13. **Dulic, V., and H. Riezman.** 1989. Characterization of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. EMBO J. **8**:1349–1359.
14. **Dulic, V., and H. Riezman.** 1990. *Saccharomyces cerevisiae* mutants lacking a functional vacuole are defective for aspects of the pheromone response. J. Cell Sci. **97**:517–525.
15. **Garlow, S. G.** 1989. Ph.D. thesis. Carnegie Mellon University, Pittsburgh, Pa.
16. **Garnier, J., D. J. Osgusthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
17. **Goldstein, A., and J. O. Lampen.** 1979. β-D-Fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42**:504–511.
18. **Hereford, L., and M. Rosbash.** 1977. Number and distribution of polyadenylated RNA sequences in yeast. Cell **10**:453–462.
19. **Holm, C., D. W. Meeks-Wagner, W. L. Fangman, and D. Botstein.** 1986. A rapid, efficient method for isolating DNA from yeast. Gene **42**:169–173.
20. **Johnson, L. M., V. A. Bankaitis, and S. D. Emr.** 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell **48**:875–885.
21. **Jones, E.** 1977. Proteinase mutants of *Saccharomyces cerevisiae*. Genetics **85**:23–33.
22. **Jones, E.** 1983. Genetic approaches to the study of protease function and proteolysis in *Saccharomyces cerevisiae*, p. 167–203. *In* J. Spencer, D. Spencer, and A. Smith (ed.), Yeast genetics: fundamental and applied aspects. Springer-Verlag, New York.
23. **Jones, E.** 1984. The synthesis and function of proteases in *Saccharomyces cerevisiae*: genetic approaches. Annu. Rev. Genet. **18**:233–270.
24. **Jones, E., G. Zubenko, and R. Parker.** 1982. PEP4 gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. Genetics **102**:665–677.
25. **Jones, E. W.** 1991. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **266**:7963–7966.
26. **Jones, E. W., C. A. Woolford, C. M. Moehle, J. A. Noble, and M. A. Innis.** 1989. Genes, zymogens, and activation cascades of yeast vacuolar proteases, p. 141–147 *In* Cellular proteases and control mechanisms. Alan R. Liss, Inc., New York.
27. **Kimelman, D., J. S. Miller, D. Porter, and B. E. Roberts.** 1985. E1a regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. J. Virol. **53**:399–409.
28. **Kirby, K.** 1965. Isolation and characterization of ribosomal ribonucleic acid. Biochem. J. **96**:266–289.
29. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815**:468–476.
30. **Klionski, D. J., and S. D. Emr.** 1989. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. **8**:2241–2250.
31. **Kuo, C.-L., and J. Campbell.** 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. **3**:1730–1737.
32. **Kuranda, M. J., and P. W. Robbins.** 1987. Cloning and heterologous expression of glycosidase genes from *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **84**:2585–2589.
33. **Kyte, J., and R. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
34. **Larkin, J.** 1985. The *CRY1* gene of *S. cerevisiae*—expression of a yeast ribosomal protein gene. Ph.D. dissertation. Carnegie Mellon University, Pittsburgh, Pa.
35. **Lipman, D., and W. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Manolson, M. F.** Unpublished data.
38. **McCusker, J. H., and J. E. Haber.** 1977. Efficient sporulation of yeast in media buffered near pH 6. J. Bacteriol. **132**:180–185.
39. **Moehle, C. M., C. K. Dixon, and E. W. Jones.** 1989. Processing pathway for protease B of *Saccharomyces cerevisiae*. J. Cell Biol. **108**:309–324.
40. **Mortimer, R. K., and D. Schild.** 1985. Genetic map of *Saccharomyces cerevisiae*, edition 9. Microbiol. Rev. **49**:181–212.
41. **Nebes, V. N., and E. W. Jones.** J. Biol. Chem., in press.
42. **Petes, T., J. Broach, P. Wensink, L. Hereford, G. Fink, and D. Botstein.** 1978. Isolation and analysis of recombinant DNA molecules containing yeast DNA. Gene **4**:37–49.
43. **Preston, R. A.** Unpublished data.
44. **Preston, R. A., D. Kirkpatrick, and E. W. Jones.** 1987. Characterization of *PEP3*, an essential gene required for vacuole biogenesis. Abstr. Meet. Yeast Cell Biol.
45. **Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones.** 1989. Fluorescence microscopy methods for yeast. Methods Cell Biol. **31**:357–435.
46. **Robinson, J. S., T. R. Graham, and S. D. Emr.** 1991. A putative zinc finger protein, *Saccharomyces cerevisiae* Vps18p, affects late Golgi functions required for vacuolar protein sorting and efficient α-factor prohormone maturation. Mol. Cell. Biol. **11**: 5813–5824.
47. **Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr.** 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. **8**:4936–4948.
48. **Rothman, J. H., I. Howald, and T. H. Stevens.** 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. EMBO J. **8**:2057–2065.
49. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
50. **Sharp, P., T. M. F. Tuohy, and K. R. Mosurski.** 1986. Codon usage in yeast: cluster analysis clearly differentiates highly and lowly expressed genes. Nucleic Acids Res. **14**:5125–5143.
51. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Struhl, K.** 1985. A rapid method for creating recombinant DNA molecules. BioTechniques **3**:6.
53. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
54. **Toh-E, A., Y. Ueda, S. Kakimoto, and Y. Oshima.** 1973. Isolation and characterization of acid phosphatase mutants in *Saccharomyces cerevisiae*. J. Bacteriol. **113**:727–738.
55. **Van der Wilden, W., P. Matile, M. Schellenberg, J. Meyer, and A. Weimken.** 1973. Vacuolar membranes: isolation from yeast cells. Z. Naturforsch. **28C**:416–421.
56. **Wada, Y., K. Kitamoto, T. Kanbe, K. Tanaka, and Y. Anraku.** 1990. The *SLP1* gene of *Saccharomyces cerevisiae* is essential for vacuolar morphogenesis and function. Mol. Cell. Biol. **10**:2214–2223.
57. **Wiemken, A., P. Matile, and H. Moor.** 1970. Vacuolar dynamics in synchronously budding yeast. Arch. Microbiol. **70**:89–103.
58. **Woolford, C. A., L. B. Daniels, F. J. Park, E. W. Jones, J. N.**

VanArsdell, and M. A. Innis. 1986. The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. Mol. Cell. Biol. **6**:2500–2510.

59. **Woolford, C. A., C. K. Dixon, M. F. Manolson, R. Wright, and E. W. Jones.** 1990. Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. Genetics **125**:739–752.
60. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33**:103–119.
61. **Yoshihisa, T., and Y. Anraku.** 1989. Nucleotide sequence of *AMS1*, the structure gene of vacuolar α-mannosidase of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **163**: 908–915.
62. **Yoshihisa, T., and Y. Anraku.** 1990. A novel pathway of import of α-mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae*. J. Biol. Chem. **265**:22418–22425.
63. **Yoshihisa, T., Y. Ohsumi, and Y. Anraku.** 1988. Solubilization and purification of α-mannosidase, a marker enzyme of vacuolar membranes in *Saccharomyces cerevisiae*. J. Biol. Chem. **263**: 5158–5163.
64. **Zhang, J.-Q., and E. W. Jones.** Unpublished data.
65. **Zubenko, G. S., and E. W. Jones.** 1981. Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. Genetics **97**:45–64.