

## Characterization of Yeast Vps33p, a Protein Required for Vacuolar Protein Sorting and Vacuole Biogenesis

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Received 27 March 1990/Accepted 15 June 1990

***vps33* mutants mis-sort and secrete multiple vacuolar hydrolases and exhibit extreme defects in vacuolar morphology. Toward a molecular understanding of the role of the *VPS33* gene in vacuole biogenesis, we have cloned this gene from a yeast genomic library by complementation of a temperature-sensitive *vps33* mutation. Gene disruption demonstrated that *VPS33* was not essential but was required for growth at high temperatures. At the permissive temperature, *vps33* null mutants exhibited defects in vacuolar protein localization and vacuole morphology similar to those seen in most of the original mutant alleles. Sequence analysis revealed a putative open reading frame sufficient to encode a protein of 691 amino acids. Hydropathy analysis indicated that the deduced product of the *VPS33* gene is generally hydrophilic, contains no obvious signal sequence or transmembrane domains, and is therefore unlikely to enter the secretory pathway. Polyclonal antisera raised against TrpE-Vps33 fusion proteins recognized a protein in yeast cells of the expected molecular weight, ~75,000. In cell fractionation studies, Vps33p behaved as a cytosolic protein. The predicted *VPS33* gene product possessed sequence similarity with a number of ATPases and ATP-binding proteins specifically in their ATP-binding domains. One *vps33* temperature-sensitive mutant contained a missense mutation near this region of sequence similarity; the mutation resulted in a Leu-646 → Pro substitution in Vps33p. This temperature-sensitive mutant strain contained normal vacuoles at the permissive temperature but lacked vacuoles specifically in the bud at the nonpermissive temperature. Our data suggest that Vps33p acts in the cytoplasm to facilitate Golgi-to-vacuole protein delivery. We propose that as a consequence of the *vps33* protein-sorting defects, abnormalities in vacuolar morphology and vacuole assembly result.**

Eucaryotic cells contain a number of distinct membrane-enclosed organelles whose function and morphology are defined largely by the unique set of proteins residing within them. Thus, mutations that disrupt the accurate sorting of proteins to their correct intracellular locations might also affect the assembly and morphology of the target organelle. Conversely, abnormalities in organelle biogenesis may be manifest as protein trafficking or delivery defects.

In the yeast *Saccharomyces cerevisiae*, proteins destined for the lysosomelike vacuole traverse the secretory pathway en route to the vacuole. Soluble vacuolar proteins such as carboxypeptidase Y (CPY) and proteinase A (PrA) are synthesized as inactive precursors; upon delivery to the vacuole, these proenzymes undergo proteolytic processing to generate the mature active enzymes (14, 15, 26, 49). We and others have recently isolated a number of mutants that exhibit defects in the proper localization and processing of multiple vacuolar hydrolases (1, 40, 42, 43). These vacuolar protein sorting, or *vps*, mutants define more than 40 complementation groups. Morphological studies using both light and electron microscopy have revealed at least three distinct groups of *vps* mutants (2). The majority of the *vps* mutants (called class A *vps* mutants) contain morphologically wild-type (wt) vacuoles. A second class of mutants, class B, is characterized by an altered morphology in which the vacuole is highly fragmented. Mutants in four *vps* complementation groups exhibit the most extreme abnormalities in vacuole morphology. Electron microscopic analysis has revealed that these cells accumulate many small vesicles but lack any compartment that resembles a wt vacuole. This morphology has been designated class C (2).

The class C *vps* mutants exhibit extreme defects in the sorting and processing of soluble vacuolar proteins. In these strains, the Golgi-modified forms of CPY, PrA, and proteinase B accumulate and are secreted. Little or no mature forms of these vacuolar enzymes can be detected in these mutants. Unlike other *vps* mutants, these class C strains also mislocalize as much as 50% of the activity of a vacuolar membrane enzyme,  $\alpha$ -mannosidase (40). The class C *vps* mutants exhibit other genetically linked phenotypic defects, including temperature-sensitive (Ts) growth and sensitivity to osmotic stress. However, nonvacuolar functions such as secretion and microtubule assembly, as well as nuclear and mitochondrial structure, appear to be unaffected in these strains (2, 40).

In an effort to understand the molecular basis for the phenotypic defects exhibited by the class C *vps* mutants, we have examined in more detail the seven mutants comprising one of these class C complementation groups, *vps33*. We report here the cloning of the *VPS33* gene. This gene is not essential but is required for growth at 37°C. The predicted *VPS33* gene product is a relatively hydrophilic protein of ~75 kilodaltons (kDa) that shares sequence similarity with a number of ATP-binding proteins. Cell fractionation studies indicate that Vps33p is soluble. We suggest that the *VPS33* gene product acts in the cytoplasm to facilitate Golgi-to-vacuole trafficking, thereby promoting vacuole assembly.

### MATERIALS AND METHODS

**Strains and media.** *Escherichia coli* MC1061 [ $F^-$  *araD139*  $\Delta$ (*araABOIC-leu*)7679  $\Delta$ *lacX74 galU galK rpsL hsdR strA*] (4), JM101 [*supE thi*  $\Delta$ (*lac-proAB*)] ( $F'$  *traD36 proAB lacI<sup>q</sup> Z M15*) (27), and DH1 [ $F^-$  *endA1 hsdR17* ( $rK^- mK^+$ ) *supE44 thi-1 recA1 gyrA96 relA1?*] (13) were used for cloning and TrpE-Vps33 fusion protein production. *E. coli* JF1754 (*hsdR*

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*metB leuB hisB lac gal*) (25) was used in screening plasmids pLB33-313 and pLB33-317. *S. cerevisiae* parental strains were SEY6210 (*MAT $\alpha$  ura3-52 leu2-3,112 his3- $\Delta$ 200 trp1- $\Delta$ 901 lys2-801 suc2- $\Delta$ 9*) and SEY6211 (*MAT $\alpha$  ura3-52 leu2-3,112 his3- $\Delta$ 200 trp1- $\Delta$ 901 ade2-101 suc2- $\Delta$ 9*) (40). *vps33* mutant strains SEY33-2, SEY33-4, and SEY33-5 are isogenic with SEY6210 and carry the *vps33-2*, *vps33-4*, and *vps33-5* mutations, respectively. SEY33-7, carrying the *vps33-7* allele, is isogenic with SEY6211. LB317 carries a *vps33* null mutation and was derived from SEY6210 (see below). Bacterial strains were grown on standard media (28). Yeast strains were grown on standard yeast extract-peptone-dextrose (YPD) or synthetic dextrose (SD) (synthetic minimal, supplemented as necessary) medium (47).

**Materials.** Restriction enzymes, T4 DNA ligase, Klenow enzyme, and polyacrylamide gel electrophoresis supplies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Mung bean nuclease and exonuclease III were the products of New England Biolabs (Beverly, Mass.). 5-Fluoroorotic acid was purchased from PCR, Inc. (Gainesville, Fla.). Isopropyl- $\beta$ -D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal), and Sequenase DNA sequencing kit were purchased from United States Biochemical Corp. (Cleveland, Ohio). Deoxynucleotides and Miniprep Kit Plus were the products of Pharmacia, Inc. (Piscataway, N.J.). Tran<sup>35</sup>S label was the product of ICN Radiochemicals (Irvine, Calif.). Multiprime DNA labeling system, [ $\alpha$ -<sup>35</sup>S]dATP, and [ $\alpha$ -<sup>32</sup>P]dCTP were purchased from Amersham Corp. (Arlington Heights, Ill.). Nitrocellulose and the Elutrap Electro-Separation Chamber were the products of Schleicher & Schuell, Inc. (Keene, N.H.) and were used as instructed by the manufacturer. Autofluor was purchased from National Diagnostics (Manville, N.J.), Freund complete and incomplete adjuvants were purchased from GIBCO Laboratories (Grand Island, N.Y.), and 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CD CFDA) was purchased from Molecular Probes, Inc. (Eugene, Ore.). Antiserum against CPY was from Klionsky et al. (19). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or other standard sources.

**Genetic and DNA manipulations.** Standard recombinant DNA techniques were performed as described previously (23). Genetic crosses, sporulation of diploids, and dissection of tetrads were performed according to Sherman et al. (47). Yeast transformations were done by the lithium acetate method of Ito et al. (16) in the presence of 10  $\mu$ g of denatured salmon sperm carrier DNA.

Chromosomal DNA was isolated and Southern analysis was performed (23), using DNA fragments containing either the *HIS3* gene or the *VPS33* gene as a probe. Northern (RNA) analysis (23) was carried out by using the 1.1-kilobase (kb) *Cla*I-*Sma*I *VPS33* fragment as a probe, and total RNA was isolated as described by Eakle et al. (9). DNA probes were labeled by using the Amersham Multiprime DNA labeling system.

**Vectors and plasmid constructions.** *E. coli* plasmids pBluescript KS (+) and (-) are described in the Stratagene (La Jolla, Calif.) catalog. The yeast-integrating vector pPHY110 (*TRP1*, selectable marker) and the *E. coli*-yeast shuttle vectors pPHYC16 and pPHYC18 (*CEN4 ARS1 URA3*) will be described elsewhere (P. Herman, unpublished data). The *E. coli*-yeast shuttle vector pSEY18 is a derivative of pSEY8 (11) in which the pUC8 polylinker has been replaced with the polylinker of pUC18. The *HIS3* gene was isolated as a 1.75-kb *Bam*HI fragment from a plasmid kindly provided by E. Phizicky. The pATH vectors used in the TrpE-Vps33

fusion protein production have been previously described (6).

The 3.7-kb *Sall*-*Xba*I fragment of *VPS33* (see Fig. 2B) was subcloned into the pBluescript KS (+) and (-) vectors to generate pLB33-211 and pLB33-212, respectively. Plasmid pLB33-102, used in the integrative mapping experiment, was constructed by cloning the 5.3-kb *Sall*-*Pvu*II fragment shown in Fig. 2B into the *Sall*-*Sma*I sites of the yeast-integrating vector pPHY110. Construction of pLB33-313, used in the disruption of *VPS33*, was achieved by digesting plasmid pLB33-212 with *Eco*RI to remove the region shown in Fig. 2C, gel purifying the resulting linear DNA away from the two *Eco*RI fragments, blunting the ends with Klenow enzyme, and inserting the *HIS3* gene as a blunted *Bam*HI fragment. Recombinant plasmids were selected directly for the presence of the *HIS3* gene in the *hisB* bacterial strain JF1754 (25) on M63 minimal medium lacking histidine (28). In a similar manner, the 2.1-kb *Hind*III-*Sma*I fragment indicated in Fig. 2C was removed and replaced with the *HIS3* gene to generate plasmid pLB33-317. Plasmid pLB33-221, carrying the *VPS33* gene on a multicopy (2  $\mu$ m) vector, was constructed by excising the *Sall*-*Xba*I fragment from pLB33-21 (see below) as a *Sall*-*Sac*I fragment (using the *Sac*I site in the polylinker) and ligating it to the *Sall*-*Sac*I sites of the 2  $\mu$ m vector pSEY18. A truncated form of *VPS33*, encoding all but the carboxy-terminal 56 amino acids (aa) of Vps33p, was constructed by subcloning the 2.7-kb *Pst*I fragment (Fig. 2C) into the *Pst*I site of pBluescript KS (-) and then moving this fragment (as a *Bam*HI-*Sall*I fragment) into the corresponding sites in the *E. coli*-yeast shuttle vector pPHYC18 to construct pLB33-304. Plasmid pLB33-162 was constructed by cloning the 3.7-kb *Sall*-*Xba*I fragment into the *E. coli*-yeast shuttle vector pPHYC16.

**Cloning and sequence analysis of VPS33.** Yeast strain SEY33-2 was grown in YPD to an optical density at 600 nm of 0.5 to 1.0 and transformed at room temperature as described previously (16) with a yeast genomic library constructed by Rose et al. (41). The library contains inserts from a partial *Sau*3A digestion of *S. cerevisiae* genomic DNA carried on the *E. coli*-yeast shuttle vector YCp50 (*CEN4 ARS1 URA3*). Transformants were selected on SD lacking uracil at 25°C and replicated to YPD plates, which were incubated at the restrictive temperature, 37°C. Temperature-resistant transformants were picked, restreaked, and cured of the plasmid by incubation on 5-fluoroorotic acid plates (3). Complementing plasmid DNA (pLB33-6) was isolated and propagated in the bacterial strain MC1061. Plasmid DNA was transformed into SEY33-2, SEY33-5, and SEY33-7, and transformants were tested for temperature sensitivity (growth on YPD at 37°C), osmotic sensitivity (growth on YPD containing 1.5 M NaCl) (2), vacuolar protein sorting (40), and vacuole morphology (see below). The plasmid DNA was also subjected to restriction enzyme analysis. The restriction fragments shown in Fig. 2B were isolated from pLB33-6 and subcloned into the appropriate restriction sites within the polylinker of the *E. coli*-yeast shuttle vector pPHYC18 to generate plasmids pLB33-20 through pLB33-24. Plasmid pLB33-21 was digested with *Sall*I and *Kpn*I, and exonuclease III was used (50) to generate deletions from the *Sall*I end of the insert. The ends were blunted by using mung bean nuclease and Klenow enzyme and religated to generate deletion plasmids pLB33-41 and pLB33-42. The approximate endpoints of these deletions were determined by restriction analysis.

Two sets of nested deletion templates for sequencing were generated by deleting from the *Sall*I end of the insert in

pLB33-212 and the *XbaI* end of the insert in pLB33-211, using exonuclease III (50). Deletions were also generated from the internal *SmaI* site in pLB33-211, and additional templates were constructed by cloning the 0.5-kb *EcoRI* fragment, the 1.5-kb *ClaI-XbaI* fragment, the 0.4-kb *PstI-XbaI* fragment, the 2.7-kb *PstI* fragment, and the 1.4-kb *HindIII* fragment (see Fig. 2C) into pBluescript KS (-). Single-stranded template DNA was prepared from these constructs (50) after infection with the helper bacteriophage VCSM13 (Stratagene), and synthetic primers corresponding to the T3 or T7 promoter sequence were prepared by DNA synthesizer. The sequence of both strands of *VPS33* was determined by the dideoxy-chain termination method (46).

DNA sequence and hydropathy analyses (21) were done by using a program written by K. Eakle (unpublished). A DNA homology search was performed by using the TFASTA algorithm, the GENEMBL data bank, and the University of Wisconsin Genetics Computer Group sequence analysis software package. Additional homology searches using the GenBank, EMBL, NBRF, and SWISS-PROT protein data bases and the FASTA program (36) were done on BIONET.

**Mapping and rescue of the *vps33-4(Ts)* allele.** Recombinational rescue analysis was carried out on SEY33-4 carrying plasmid pLB33-23 or pLB33-24, described above. Additional plasmids were constructed by cloning the 2.1-kb *SmaI-PvuII* fragment, the 2.3-kb *SphI-PvuII* fragment, and the 0.7-kb *HindIII-SmaI* fragment (see Fig. 2B and C) into the appropriate restriction sites of pSEY18. In each case, the plasmid was transformed into SEY33-4 and the frequency of temperature-resistant recombinants was scored.

The *vps33-4(Ts)* mutation was rescued by using double-stranded gap repair (33) as follows. The complete *VPS33* gene was subcloned as a 5.3-kb *PvuII-SalI* fragment into the *E. coli*-yeast shuttle vector pPHYC16, and the resulting plasmid (pLB33-160) was digested with *XbaI* and *SmaI* to generate a gapped linear plasmid. The linear plasmid was gel purified away from the 0.5-kb *SmaI-XbaI* fragment. SEY33-4 was transformed with this plasmid, and Ura<sup>+</sup> transformants were scored for temperature sensitivity. Plasmid DNA (designated pLB33-161) carrying the rescued *vps33-4(Ts)* allele was purified. The *SmaI-XbaI* fragment carrying the mutation was isolated and subcloned into pBluescript KS (-). Double-stranded DNA was isolated by using the Pharmacia Miniprep Kit Plus and sequenced. The *VPS33* gene carrying the *vps33-4(Ts)* mutation was excised from pLB33-161 as a *SalI-SacI* fragment (using the *SacI* site in the polylinker) and cloned into the 2  $\mu$ m vector pSEY18 to generate pLB33-271.

**Antibody production.** *trpE-VPS33* gene fusions were constructed by using the two fragments of the *VPS33* gene depicted in Fig. 2C. The 0.7-kb *HindIII-SmaI* fragment was isolated from pLB33-21, blunted by using Klenow enzyme, and ligated to the blunted *SalI* site of the pATH3 vector to generate an in-frame fusion encoding aa 379 to 608 of Vps33p. The 0.5-kb *EcoRI* fragment was isolated, blunted as described above, and ligated into the *SmaI* site of the pATH2 vector to generate an in-frame fusion encoding aa 140 to 303 of Vps33p. Individual transformants were purified, and the presence and orientation of the *VPS33* sequence was confirmed by restriction analysis. Bacterial strains carrying candidate fusion constructs were assayed for the production of fusion protein (48). Whole-cell lysates and insoluble fractions were prepared basically as described by Kleid et al. (18) (except that 2% Triton X-100 was used rather than 0.2% Nonidet P-40) and subjected to sodium

dodecyl sulfate-9% polyacrylamide gel electrophoresis (22). Inducible fusion proteins of the expected molecular sizes (62 and 55 kDa) were the predominant bands visualized in the insoluble fractions upon Coomassie staining. The bands containing the fusion proteins were excised from Coomassie-stained preparative gels and electroluted from the gel slices by using a Schleicher & Schuell Electro-Separation Chamber. Approximately 175  $\mu$ g of each purified fusion protein, emulsified with Freund complete adjuvant, was injected intramuscularly and subcutaneously into a young male New Zealand White rabbit. After 4 weeks, the rabbit was boosted with 25  $\mu$ g of each fusion protein in an emulsion with Freund incomplete adjuvant. Antisera were screened by immunoprecipitation. A titration curve was generated for these antisera to determine the amount needed for a quantitative recovery of overproduced levels of Vps33p.

**Immunoprecipitation.** Cells were grown in SD medium (47) supplemented with the appropriate amino acids. Whole cells (1.5 U at an optical density at 600 nm of 1.0) were collected by centrifugation. The cells were resuspended in 0.5 ml of the same medium, and 125  $\mu$ Ci of Tran<sup>35</sup>S label was added. The cells were labeled for 20 min with shaking at the appropriate temperature. A 30-min chase was initiated by the addition of methionine to a final concentration of 4 mM. The chase was terminated, and cells were lysed and subjected to a single round of immunoprecipitation, using anti-serum specific for Vps33p or CPY as described previously (9, 19).

**Cell fractionation.** Cells were grown in SD medium (47) as described above. Six units of cells at an optical density at 600 nm of 1.0 were harvested by centrifugation and spheroplasted in the same medium as described previously (40). Spheroplasts were labeled and chased as described above. All subsequent manipulations were performed at 4°C. Labeled spheroplasts were pelleted at 1,200  $\times$  g for 2 min. The spheroplasts were suspended in 1 ml of lysis buffer (50 mM Tris chloride [pH 7.5], 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M leupeptin, 1 mM sodium azide, 0.125 mg of  $\alpha_2$ -macroglobulin per ml) with various concentrations of KCl as indicated and vortexed for 30 s. In some experiments, the lysis buffer also contained 0.3 M mannitol or 0.8 M sorbitol for partial osmotic support (29, 45). Unlysed spheroplasts were removed by centrifugation at 500  $\times$  g for 2 min and subjected to a second round of lysis. Unlysed spheroplasts were removed as before, and the lysates were pooled and immediately centrifuged at 100,000  $\times$  g for 90 min. The pellet was rinsed gently with 1 ml of lysis buffer and suspended in 1 ml of 0.5% sodium dodecyl sulfate. Supernatant and pellet fractions were precipitated with trichloroacetic acid (5% final concentration) and immunoprecipitated.

**Fluorescent labeling of cells and microscopy.** Cells were grown in YPD, labeled with CDCFDA or fluorescein isothiocyanate (FITC), and observed as described previously (2, 37, 38).

**GenBank accession number.** The GenBank accession number for the sequence reported in this paper is M34638.

## RESULTS

**Cloning and genetic analysis of the *VPS33* gene.** The *VPS33* gene was cloned by complementation of the *vps33-2(Ts)* mutation (in the Ura3<sup>-</sup> strain SEY33-2) with a yeast genomic library carried on the single-copy vector YCp50 (41). Among 11,000 Ura<sup>+</sup> transformants, 7 were temperature resistant. Upon curing of these transformants of the plasmid, five

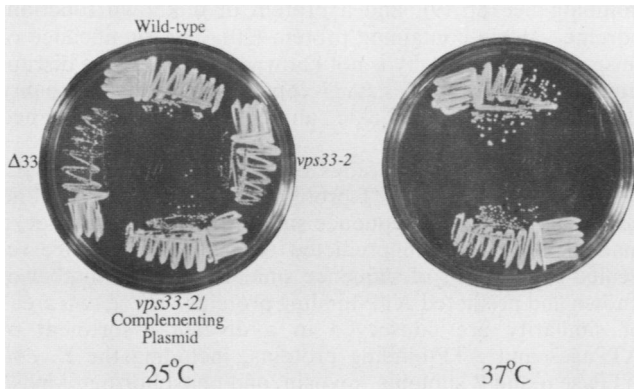


FIG. 1. Complementation of *vps33-2(Ts)* by the cloned *VPS33* gene. The parental strain SEY6210, the SEY33-2 mutant strain without and with the complementing plasmid pLB33-21, and the isogenic *vps33* null mutant LBY317 were streaked on YPD solid media and incubated at 25 or 37°C, as indicated.

remained temperature resistant, indicating that the temperature resistance was not dependent on the presence of the plasmid. The remaining two transformants were temperature sensitive when cured, suggesting that the temperature-resistant phenotype was indeed conferred by the plasmid. Restriction analysis of the purified plasmid from these two transformants revealed that the two complementing plasmids contained identical inserts. Retransformation of SEY33-2 and two other *vps33* strains complemented that this plasmid, designated pLB33-6, complemented all of the phenotypes associated with the *vps33* mutations, including temperature sensitivity (Fig. 1), mislocalization of vacuolar proteins (see Fig. 5), osmotic sensitivity, and the defect in vacuole biogenesis (data not shown).

A restriction map of the 12-kb insert in pLB33-6 is shown in Fig. 2A. Subcloning and complementation analysis (Fig. 2B) indicated that the complementing activity resided on a 3.7-kb *SalI-XbaI* fragment. Removal of as much as 1,000 base pairs (bp) from the *SalI* end, using exonuclease III, did not destroy the ability to complement.

To confirm that this complementing ability was not due to suppression by another gene, we carried out integrative

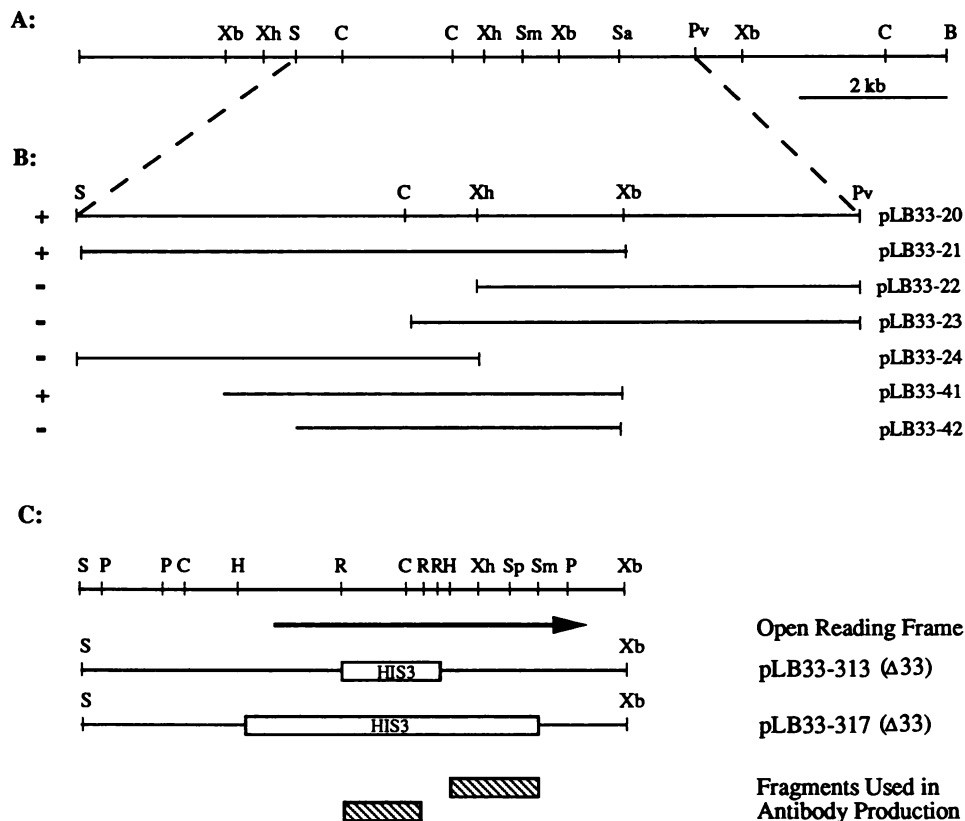


FIG. 2. (A) Restriction map of the entire 12-kb *VPS33* complementing clone. Restriction site abbreviations: B, *Bam*HI; C, *Cla*I; Pv, *Pvu*II; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I. (B) Complementation analysis of the *VPS33* subclones. Restriction fragments were cloned into pPHYC18 (*CEN4 ARS1 URA3*) to generate pLB33-20, pLB33-21, pLB33-22, pLB33-23, and pLB33-24. Plasmids pLB33-41 and pLB33-42 were the result of exonuclease III deletions from the *SalI* end of the complementing fragment in pLB33-21 to yield the endpoints shown. The plasmids were transformed into SEY33-7, and Ura<sup>+</sup> transformants were tested for growth at 37°C. Vacuole morphology, as assessed by staining with CDCFDA (see Materials and Methods), was also scored. Symbols: +, the transformants were temperature resistant and exhibited class A (wt) vacuole morphology; -, the transformants had a temperature-sensitive class C phenotype. (C) Fragments used for antibody production and gene disruption. The position of the ORF as determined by sequencing is shown. Plasmids pLB33-313 and pLB33-317, in which the *HIS3* gene replaced the indicated portions of *VPS33*, were used to generate *vps33* null mutants as described in the text. ▨, Fragments used to generate in-frame *trpE-VPS33* gene fusions. The encoded hybrid proteins were used in antibody production as described in Materials and Methods. Additional restriction site abbreviations: H, *Hind*III; P, *Pst*I; R, *Eco*RI; Sp, *Sph*I.

mapping studies with the clone. The complementing 5.3-kb *SalI-PvuII* fragment (Fig. 2B) was cloned into the integrating vector pPHYI10, which carries the selectable marker *TRP1*. The resulting plasmid was linearized at the internal *XhoI* site to facilitate homologous recombination (32) and was transformed into a *Trp1*<sup>-</sup> strain harboring the *vps33-7*(Ts) mutation. *Trp1*<sup>+</sup> temperature-resistant transformants were crossed to the *Trp1*<sup>-</sup> *VPS33*<sup>+</sup> parental strain SEY6210. Tetrad analysis of the sporulated diploids showed the expected 2:2 *Trp1*<sup>+</sup>:*Trp1*<sup>-</sup> segregation pattern; all four spores in each of the 12 asci analyzed were temperature resistant and exhibited wt vacuolar morphology. Further analysis of 190 random spores also failed to uncover any temperature-sensitive segregants, whereas the *Trp1*<sup>+</sup>:*Trp1*<sup>-</sup> phenotype segregated 97:93. These observations demonstrated that the complementing clone is tightly linked to the *vps33* mutant locus.

The complementing *SalI-XbaI* fragment was also cloned into the 2  $\mu$ m (multicopy) vector pSEY18 to generate the plasmid pLB33-221. Yeast transformants harboring this plasmid overproduced the *VPS33* gene product ~20-fold (see below). Transformation of SEY33-7 or wt strains with pLB33-221 resulted in a *Vps33*<sup>+</sup> phenotype, indicating that severalfold overproduction of the *VPS33* gene product is not detrimental to otherwise wt cells. We also transformed pLB33-221 into a representative allele of each of the other three class C complementation groups, *vps11*, *-16*, and *-18*. In no case did the plasmid suppress any of the defects associated with these mutations; thus, overproduction of *Vps33p* appears unable to rescue any of these phenotypically similar mutants.

***VPS33* sequence analysis.** The nucleotide sequence of *VPS33* was determined by sequencing a series of nested exonuclease III-generated deletion templates by the dideoxynucleotide-chain termination method (46). The DNA sequence (Fig. 3) contains a single long open reading frame (ORF) of 2,073 bp, with the potential to encode a protein of 691 aa. Examination of the 5' noncoding sequences revealed a possible transcription initiation sequence, TATATTAA (31), at position -157 relative to the putative translational initiation codon. A consensus sequence for transcription termination, TAG...TAGT...TTTT (31) (underlined in Fig. 3), is found 147 bp downstream of the ORF stop codon TAA. Northern analysis was performed, using an internal fragment of *VPS33* as a probe to identify the *VPS33* transcript. Consistent with the ORF size predicted from the DNA sequence, a single RNA species of 2.2 kb was detected (data not shown).

The sequence of the predicted *VPS33* gene product was subjected to hydropathy analysis according to the model of Kyte and Doolittle (21). The predicted *Vps33* polypeptide is hydrophilic (26% charged amino acids), with no apparent hydrophobic domains that could span a membrane or function as a signal sequence (data not shown). A search for sequence homology using the GENEMBL data bank and the TFASTA algorithm (36) revealed that the 3' end of the *VPS33* gene (nucleotides 2307 to 2695) is identical to the sequence upstream of the yeast *COX8* gene, which encodes the cytochrome *c* oxidase subunit VIII. Furthermore, the published sequence of the *COX8* gene contains a short ORF 5' of the *COX8* coding region which corresponds to the last 55 codons of the *VPS33* ORF (35). From these results, we conclude that the *VPS33* gene lies next to the *COX8* gene in the yeast genome. Sequence analysis also revealed an ORF 5' of the *VPS33* gene. An interesting sequence similarity was noted between this ORF and three other proteins: the mammalian *N*-ethylmaleimide-sensitive factor (56), its yeast

homolog Sec18p (9), and a protein of unknown function, porcine valosin-containing protein (20). The significance of this sequence similarity is not known; however, gene disruption of the ORF 5' of *VPS33* appears to result in lethality (L. M. Banta, K. A. Eakle, and S. D. Emr, unpublished observations).

Further homology searches using the GenBank, EMBL, NBRF, and SWISS-PROT protein data bases uncovered no additional significant sequence similarities (36). However, a manual search of the predicted amino acid sequence revealed two regions of sequence similarity with a number of known and predicted ATP-binding proteins. These two areas of similarity are conserved in a diverse assortment of ATPases and ATP-binding proteins, including the *E. coli* ATPase  $\alpha$  and  $\beta$  subunits, myosin, and phosphofructokinase (Fig. 4), and a consensus sequence has been determined (53). The two domains are often separated by 60 to 100 aa, and some proteins (e.g., myosin and phosphofructokinase) contain only one of the two regions. In *Vps33p*, the two regions are located at residues 480 to 498 and 661 to 679 (Fig. 3).

**Disruption of *VPS33*.** Disruption of the *VPS33* locus was accomplished by using the one-step gene disruption technique (44) with two different constructions. In the first, the 700-bp *EcoRI* fragment shown in Fig. 2C was replaced with the *HIS3* gene. This disrupted *VPS33* construction was digested with *SalI* and *XbaI* and used to transform the parental *his3- $\Delta$ 200* diploid SEY6210/SEY6211. Two different resulting *His*<sup>+</sup> transformants were subjected to tetrad analysis. In each of the 10 asci examined, all spores were viable but a segregation pattern of 2 temperature-sensitive *His*<sup>+</sup>:2 temperature-resistant *His*<sup>-</sup> spores was observed. Microscopic examination of the temperature-sensitive segregants streaked on YPD revealed that the cells doubled at most two times at 37°C. A second construction replaced the entire 2.1-kb *HindIII-SmaI* fragment (indicated in Fig. 2C) with the *HIS3* gene. This construction deleted all but the carboxy-terminal 250 bp of the putative *VPS33* ORF. Transformation of the haploid strain SEY6210 with the linear *SalI-XbaI* fragment carrying this construction yielded temperature-sensitive *His*<sup>+</sup> transformants. The temperature-sensitive phenotype of one such transformant, LBY317, is shown in Fig. 1. In liquid media, this strain doubles 1.5 times at 37°C and exhibits a terminal arrest phenotype in which, after 24 h at 37°C, approximately 50% of the cells contain a medium or large bud and an additional 20% contain two or more buds. Southern analysis of the chromosomal DNA from these disrupted strains confirmed the removal of the *VPS33* sequences and the integration of the *HIS3* gene at the *VPS33* locus as expected (data not shown). From these results, we conclude that *VPS33* is not essential at low temperatures but is required for growth at 37°C.

As expected, disruption of the *VPS33* gene also led to the other phenotypic defects associated with the original mutant alleles. Strains harboring a disrupted *VPS33* gene exhibited extreme defects in vacuole morphology and in vacuole protein localization and processing at the permissive temperature (Fig. 5). The *vps33* null mutant strain LBY317 was radioactively labeled at 25°C for 20 min and chased for 30 min before immunoprecipitation with antiserum specific for the vacuolar protease CPY. CPY was present almost exclusively in the precursor form in these cells (Fig. 5, lane 2), whereas in wt cells labeled under identical conditions, 100% of the CPY was present in the mature vacuolar form (lane 1). Previous characterization of *vps33* mutants has demonstrated that most (>70%) of the precursor CPY is secreted from the cell (40). The *Vps*<sup>-</sup> defect exhibited by the null

1	GTCAAGAAC	GGCGTGGCT	GCCATAATGG	AAGATTAGA	TGTCGCAAA	GTGGAATTAC	GTCATTTGA	GAAAGCTTTT	AAAGGAATTG	CTAGGGGCAT	100
101	TACTCCAGAA	ATGCTCTCTT	ATTATGMAAG	GTTTGCCTTA	AGAAGGGGTT	CATCTTCGTA	AGCTGTGCA	TAGTCAATT	TTTTCCTTG	TGTCGCTAAT	200
201	AATAGTAGAT	AGAAATTATA	CTGAACCTCG	GTCATTITGT	ATAATATATT	AATCACTTCA	CACGAACATA	CATAAATAAA	ATATCATAAA	GGTACGAAA	300
301	TTGGAAGTAC	TTATATGTTA	ATTAGTTAAA	AGATAGAAAA	TTGAGAAAG	GAAAGAAAAG	CTGATATTGC	CCATCTCCAA	CTTTATCAAA	TCATTTCAG	400
401	ATG AAT AGA TTT TGG AAT ACT AAG AAA TTT TCA TTA ACA AAT GCC GAT GGA CTA TGT GCT ACC TTA AAT GAG ATA TCT CAA AAT GAT GAA	Met Asn Arg Phe Trp Asn Thr Lys Lys Phe Ser Leu Thr Asn Ala Asp Gly Leu Cys Ala Thr Leu Asn Glu Ile Ser Gln Asn Asp Glu	490								
1			30								
491	GTT CTT GTG GTT CAA CCA AGT GTA TTG CCA GTA CTC AAT AGT TTG CTA ACT TTC CAA GAT TTG ACT CAA TCA ACT CCT GTA AGG AAA ATT	Val Leu Val Val Gln Pro Ser Val Leu Pro Val Leu Asn Ser Leu Leu Thr Phe Gln Asp Leu Thr Gln Ser Thr Pro Val Arg Lys Ile	580								
31			60								
581	ACG TTA CTC GAT GAT CAG CTA AGT GAC GAT TTA CCG AGT GCC TTA GGC AGC GTT CCG CAA ATG GAT CTT ATT TTT CTT ATT GAT GTC AGA	Thr Leu Leu Asp Asp Gln Leu Ser Asp Asp Leu Pro Ser Ala Leu Gly Ser Val Pro Gln Met Asp Leu Ile Phe Leu Ile Asp Val Arg	670								
61			90								
671	ACA TCT CTC CGA CTC CCT CCA CAA CTG CTT GAT GCT GCT CAA AAG CAC AAT TTA TCA TCT TTG CAT ATA ATA TAC TGT CGA TGG AAA CCG	Thr Ser Leu Arg Leu Pro Pro Gln Leu Leu Asp Ala Ala Gln Lys His Asn Leu Ser Ser Leu His Ile Ile Tyr Cys Arg Trp Lys Pro	760								
91			120								
761	CT TTC CAA AAT ACT TTG GAG GAT ACA GAG CAA TGG CAA AAG GAT GGT TTC GAT TTG AAT TCA AAA AAA ACA CAT TTC CCT AAC GTC ATT	er Phe Gln Asn Thr Leu Glu Asp Thr Glu Gln Trp Gln Lys Asp Gly Phe Asp Leu Asn Ser Lys Lys Thr His Phe Pro Asn Val Ile	850								
121			150								
851	AA TCT CAG TTA AAG GAG CTA TCG AAC GAA TAT ACC CTT TAC CCT TGG GAT CTC TTG CCC TTC CCA CAG ATT GAT GAA AAT GTT CTA TTG	lu Ser Gln Leu Lys Glu Leu Ser Asn Glu Tyr Thr Leu Tyr Pro Trp Asp Leu Pro Phe Pro Gln Ile Asp Glu Asn Val Leu Leu	940								
151			180								
941	ACT CAT TCC CTT TAT AAC ATG GAA AAT GTA AAC ATG TAT TAT CCC AAC TTA CGT TCT TTG CAG AGT GCC ACA GAG TCA ATA CTG GTT GAT	Thr His Ser Leu Tyr Asn Met Glu Asn Val Asn Met Tyr Tyr Pro Asn Leu Arg Ser Leu Gln Ser Leu Ile Leu Val Thr Leu Asp	1030								
181			210								
1031	GAT ATG GTC AAT TCG TTG CAG AGC TTG ATT TTT GAA ACT AAT AGT ATC ATA ACA AAT GTT GTG TCG ATA GGT AAT CTG TCT AAG AGA TGT	Asp Met Val Asn Ser Leu Gln Ser Leu Ile Phe Glu Thr Asn Ser Ile Ile Thr Asn Val Val Ser Ile Gly Asn Leu Ser Lys Arg Cys	1120								
211			240								
1121	AGC CAT CTT TTG AAG AAA CGA ATC GAT GAG CAT CAA ACA GAG AAT GAT TTA TTC ATC AAG GGT ACG CTT TAT GGT GAA CGA ACC AAC TGT	Ser His Leu Leu Lys Lys Arg Ile Asp Glu His Gln Thr Tyr Pro Asn Leu Phe Ile Lys Gly Thr Leu Tyr Gln Thr Lys Leu Ser	1210								
241			270								
1211	GGA CTA GAA ATG GAC TTG ATT ATC TTG GAA AGG AAT ACC GAT CCT ATA ACG CCA TTG TTG ACA CAA CTT ACG TAT GCA GGA ATA CTA GAT	Gly Leu Glu Met Asp Leu Ile Ile Leu Glu Arg Asn Thr Asp Pro Ile Thr Pro Leu Leu Thr Gln Leu Thr Tyr Ala Gly Ile Leu Asp	1300								
271			300								
1301	GAT CTA TAT GAA TTC AAT TCT GGC ATA AAG ATA AAG GAG AAA GAC ATG AAC TTC AAT TAT AAG GAA GAT AAA ATA TGG AAT GAT TTG AAA	Asp Leu Tyr Glu Phe Asn Ser Gly Ile Lys Ile Lys Glu Ser Asp Met Asn Phe Asn Tyr Lys Glu Asp Lys Ile Trp Asn Asp Leu Ser	1390								
301			330								
1391	TTT TTA AAT TTT GGG TCG ATT GGG CCG CAG TTA AAT AAA TTG GCA AAG GAA CTA CAA ACG CAA TAT GAT ACA AGG CAT AAA GCC GAG AGC	Phe Leu Asn Phe Gly Ser Ile Gly Pro Gln Leu Asn Lys Leu Ala Lys Glu Leu Gln Thr Gln Tyr Asp Thr Arg His Lys Ala Glu Ser	1480								
331			360								
1481	GTA CAT GAA ATC AAA GAA TTC GTT GAT TCC TTA GGT TCT TTG CAA CAA AGG CAA GCT TTT TTG AAA AAT CAC ACA ACC TTA TCA TCC GAC	Val His Glu Ile Lys Glu Phe Val Asp Ser Leu Gly Ser Leu Gln Gln Arg Gln Ala Phe Leu Lys Asn His Thr Thr Leu Ser Ser Asp	1570								
361			390								
1571	GTT TTG AAA GTG GTA GAG ACT GAA GAG TAC GGA TCT TTC AAT AAA ATC TTA GAG TTA GAG CTG GAA ATT TTG ATG GGA AAT ACA CTT AAT	Val Leu Lys Val Val Glu Thr Glu Glu Tyr Ser Phe Asn Lys Ile Leu Glu Leu Glu Ile Leu Met Glu Leu Thr Leu Asn	1660								
391			420								
1661	AAC GAC ATT GAA GAT ATT ATA CTC GAG TTG CAG TAC CAG TAC GAG GTT GAT CAA AAG AAG ATT CTC AGA TTA ATC TGT TTA TTG TCT CTT	Asn Asp Ile Glu Asp Ile Ile Leu Glu Leu Gln Tyr Gln Tyr Glu Val Asp Gln Lys Lys Ile Leu Arg Leu Ile Cys Leu Leu Ser Leu	1750								
421			450								
1751	TGT AAA AAT TCA CTT CGA GAA AAG GAT TAT GAA TAT CTA AGA ACC TTT ATG ATC GAC TCT TGG GGC ATT GAA AAA TGC TTT CAA CTT GAA	Cys Lys Asn Ser Leu Arg Glu Lys Asp Tyr Leu Arg Thr Phe Met Ile Leu Ser Thr Phe Met Ile Leu Ser Lys Phe Gln Leu Glu	1840								
451			480								
1841	<u>TCA TTG GCT GAG TTA GGA TTT TTC ACT AGC AAA ACG GGA AAA ACT GAT TTG CAT ATT ACA ACA AGT AAG TCA ACA AGA TTA CAG AAA GAA</u>	<u>Ser Leu Ala Glu Leu Gly Phe Phe Thr Ser Lys Thr Gly Lys Thr Asp Leu His</u> Ile Thr Thr Ser Lys Ser Thr Arg Leu Gln Lys Glu	1930								
481			510								
1931	TAC CGT TAT ATT TCA CAA TGG TTC AAT ACA GTA CCC ATA GAA GAC GAG CAT GCT GCC GAT AAA ATC ACA AAT GAG AAC GAT GAC TTC TCG	Tyr Arg Tyr Ile Ser Gln Trp Phe Asn Thr Val Pro Ile Glu Asp Glu His Ala Ala Asp Lys Ile Thr Asn Glu Asn Asp Asp Phe Ser	2020								
511			540								
2021	GAA GCC ACT TTT GCT TAC AGT GGT GTA GTG CCC TTG ACA ATG AGA CTG GTT CAG ATG TTA TAT GAT AGG TCT ATC TTG TTC CAT AAT TAT	Glu Ala Thr Phe Ala Tyr Ser Gly Val Val Pro Leu Thr Met Arg Leu Val Gln Met Leu Tyr Asp Arg Ser Ile Leu Phe His Asn Tyr	2110								
541			570								
2111	TCC TCG CAG CAG CCT TTT ATA CTG TCA AGA GAA CCT AGA GTT TCT CAA ACG GAG GAT TTA ATT GAA CAG TTA TAT GGA GAC TCA CAT GCG	Ser Ser Gln Gln Pro Phe Ile Leu Ser Arg Glu Pro Arg Val Ser Gln Thr Glu Asp Leu Ile Glu Gln Leu Tyr Gly Asp Ser His Ala	2200								
571			600								
2201	ATC GAA GAG AGT ATA TGG GTC CCG GGA ACC ATT ACA AAA AAG ATC AAT GCA ACG ATC AAG AGC AAT AAT AGA CCG TCC ATA GAC GGA TCT	Ile Glu Glu Ser Ile Trp Val Pro Gly Thr Ile Thr Lys Lys Ile Leu Ser Phe Met Ile Leu Ser Ile Lys Ser Asn Asn Arg Arg Ser Ile His Ser	2290								
601			630								
2291	AAT GGG ACA TTT CAT GCT GCA GAG GAT ATT GCA CTC GTA GTA TTC CTC GGA GGT GTA ACA ATG GGT GAA ATA GCT ATA ATG AAG CAT TTG	Asn Gly Thr Phe His Ala Ala Glu Asp Ile Ala Leu Val Val Phe Leu Gly Gly Val Thr Met Gly Glu Ile Ala Ile Met Lys His Leu	2380								
631			660								
2381	<u>CAA AAA ATA CTA GGT AAA AAA GGT ATC AAT AAA AGG TTT ATC ATC ATC GCC GAT GGC TTG ATC AAT GGC ACA AGG ATC ATG AAC TCT ATA</u>	<u>Gln Lys Ile Leu Gly Lys Lys Gly Ile Asn Lys Arg Phe Ile Ile Ile Ala Asp Gly</u> Leu Ile Asn Gly Thr Arg Ile Met Asn Ser Ile	2470								
661			690								
2471	TCT TAA	TTATTATATG	ATAGATTGT	TAATTTTTG	TATATGCAA	TGTCCTTTT	TTCCACCAAC	GGTTGCAAC	AATCATACGA	GAGAAGTGT	2566
691	Ser END	691									
2567	CGGTGTTAC	GGAAAAGCTA	GGGGACTAAG	AAAAATTGAA	AATAAAGGCT	GACAGCAGTA	GAAACCAATTG	TGCTGGCTTA	GTGATTATA	AGAATGGTTA	2666
2667	ATTAGTTTTG	TATCCTTTAA	TTTTCTAGA	2695							

FIG. 3. Sequence of VPS33. The nucleotide sequence and the deduced amino acid sequence of the ORF are shown. The putative transcription initiation and termination sequences are underlined. The regions which share sequence similarity with ATP-binding proteins are boxed. The T marked with an asterisk is replaced with a C in vps33-4(Ts).

mutant was completely complemented when the wt VPS33 gene was present on a single-copy vector (lane 3).

**Identification of Vps33p.** To identify Vps33p, we overproduced the VPS33 gene product as two different TrpE-Vps33 hybrid proteins in *E. coli* and raised antisera against the fusion proteins. Two coding fragments of the VPS33 gene, from HindIII-SmaI and from EcoRI-EcoRI, were cloned into the appropriate pATH vectors (6) to generate in-frame fusions to the trpE gene of *E. coli*. These fragments (Fig. 2C) encode 230 and 164 aa, respectively, of Vps33p. When these constructions were expressed in *E. coli*, hybrid proteins of

the expected molecular weights were overproduced upon induction. Both overexpressed proteins were isolated and injected into a single rabbit as described in Materials and Methods. The resulting antisera were used in immunoprecipitation experiments to detect Vps33p. The antibodies recognized a single major species of the predicted molecular weight, ~75,000, in radioactively labeled wild-type cells (Fig. 6, lane 1). This protein was ~20-fold more abundant when the VPS33 gene was present on a multicopy plasmid (lane 2) and was not detected in the vps33 null mutant (lane 5) or by the preimmune serum (lane 6). Pulse-chase analysis

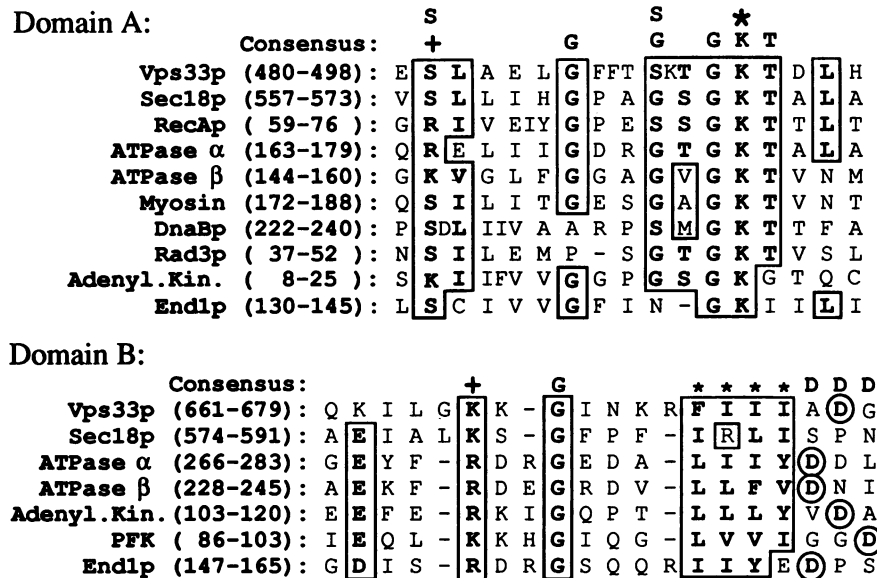


FIG. 4. Comparison of the predicted Vps33p sequence with known and predicted ATP-binding proteins. The deduced amino acid sequence of Vps33p shares similarity with the proteins listed through the two domains shown. Residues that are highly conserved at a given position are shown in boldface and boxed, and a consensus sequence is indicated above each domain. The significance of the starred lysine in domain A is discussed in the text. In domain B, asterisks denote hydrophobic residues; this region is followed by an aspartic acid in one of the next three positions in each of the proteins listed (53). The sequences shown are taken from Walker et al. (53) (*E. coli* ATPase  $\alpha$  and  $\beta$  subunits, adenylate kinase [Adenyl. Kin.], rabbit myosin, phosphofructokinase [PFK], and RecAp), Naumovski and Friedberg (30) (DnaBp and Rad3p), or Dulic and Riezman (8) (End1p). By analogy to the *N*-ethylmaleimide-sensitive factor of mammalian cells, with which it is functionally equivalent (56), the yeast Sec18p (9) is also predicted to bind ATP.

suggests that the turnover rate for Vps33p is relatively slow; after a 30- or 90-min chase, there was no significant difference in the amount of the protein produced from a multicopy plasmid. Comparison with CPY indicates that the *VPS33* gene product is present at very low levels in wt yeast cells. By densitometric quantitation, we estimate that the production of Vps33p from the multicopy plasmid is comparable to the level of expression of CPY from the chromosomal locus (0.05 to 0.1% of the total cell protein). Cells were also labeled in the presence of tunicamycin, an inhibitor of N-linked glycosylation (10). The size of Vps33p was unaffected by the drug (Fig. 6, lanes 3 and 4), indicating that none of the seven potential sites for N-linked carbohydrate addition (24) (Asn-X-Ser/Thr) is utilized.

The subcellular localization of Vps33p was examined by cellular fractionation and immunoprecipitation studies.

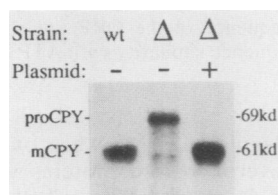


FIG. 5. Processing of CPY in a *vps33* null mutant in the presence and absence of the cloned *VPS33* gene. Whole cells were labeled with  $\text{Tran}^{35}\text{S}$  label for 20 min at 25°C, chased for 30 min by the addition of 4 mM methionine, and subjected to immunoprecipitation with CPY-specific antisera. The migration positions of precursor (proCPY) and mature (mCPY) forms are indicated. The isogenic strains used were as follows: lane 1, *VPS*<sup>+</sup> (SEY6210); lane 2, *vps33* null mutant (LBY317); and lane 3, *vps33* null mutant carrying the cloned *VPS33* gene on a single-copy plasmid [LBY317(pLB33-162)].

Wild-type cells carrying *VPS33* on a multicopy plasmid were converted to spheroplasts, radioactively labeled, and osmotically lysed under a variety of conditions before being subjected to centrifugation at 100,000  $\times g$  for 90 min. These centrifugation conditions were sufficient to pellet >95% of the vacuolar membrane protein alkaline phosphatase, whereas the cytosolic marker enzyme glyceraldehyde 3-

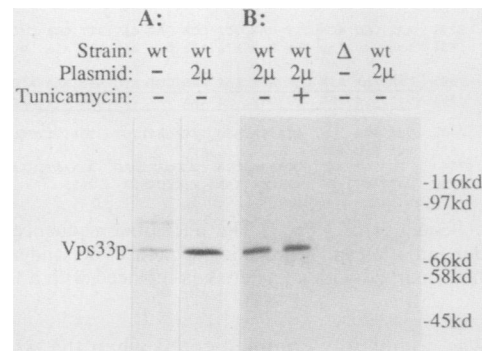


FIG. 6. Identification of the *VPS33* gene product. Spheroplasts (A) or whole cells (B) were labeled and chased as described in the legend to Fig. 5. Strains used were parental strain SEY6210 (lane 1), SEY6210 carrying *VPS33* on the multicopy plasmid pLB33-221 (lanes 2, 3, 4, and 6), or the *vps33* null mutant LBY317 (lane 5). Tunicamycin was added to a 20- $\mu\text{g}/\text{ml}$  final concentration as indicated 15 min before the addition of the radioactive label. Immunoprecipitations were performed by using the preimmune (lane 6) or Vps33p immune (lanes 1 to 5) sera. The migration positions for molecular size standards are indicated in kilodaltons (kd). In panel A, the exposure time for lane 1 was twice as long as for lane 2.



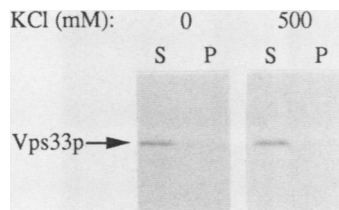


FIG. 7. Subcellular fractionation of Vps33p. Strain SEY6210 carrying *VPS33* on the multicopy plasmid pLB33-221 was enzymatically converted to spheroplasts. Spheroplasts were labeled at 30°C for 20 min and chased for 30 min by the addition of 4 mM methionine. Labeled spheroplasts were pelleted and osmotically lysed as described in Materials and Methods in lysis buffer containing the indicated concentration of KCl. Two representative conditions are shown; other concentrations of KCl (100 mM, 250 mM, and 1 M) gave identical results. Unlysed cells were removed by centrifugation at  $500 \times g$ , and the resulting lysate was centrifuged at  $100,000 \times g$  for 90 min at 4°C. The supernatant (S) and pellet (P) fractions were processed as described in Materials and Methods and immunoprecipitated with antiserum to Vps33p.

phosphate dehydrogenase was found exclusively in the supernatant fraction (data not shown). The results of two representative experiments are shown in Fig. 7. Vps33p was found primarily in the supernatant fraction; this solubility was not affected by varying the salt concentration of the lysis buffer from 0 to 1 M KCl or by varying the pH of the lysis buffer (7.5 to 6.5 or 8.0) (44) (data not shown). These results suggest that the *VPS33* gene product resides in the cytoplasm and is not tightly associated with any membrane fraction. Vps33p expressed from the chromosomal *VPS33* locus, although considerably more difficult to detect, also behaved as a soluble cytosolic protein in cell fractionation studies (data not shown). These observations do not, however, exclude the possibility that Vps33p associates weakly or transiently with some subcellular membrane or complex. By immunofluorescence studies, we have detected a weak background fluorescence that may be due to cytoplasmic staining (data not shown).

***vps33* mutant strain SEY33-4 exhibits a bud vacuole defect.** Wild-type vacuoles can be visualized in live cells by using various dyes, including CDCFDA (38) and FITC (37), that accumulate in this organelle. As previously mentioned, most of the class C *vps* mutants exhibit extreme defects in vacuole morphology in that they contain no compartments that resemble wt vacuoles. Instead, they accumulate many small vesicles and other membranous structures (2). When these class C *vps* mutants are stained with CDCFDA or FITC, only small speckles of fluorescence are detected. Two *vps33* mutant strains, SEY33-4 and SEY33-5, however, do not exhibit these gross abnormalities in their vacuole morphologies. Instead, they contain vacuoles that stain like those in wt cells. Unlike the other, more extreme *vps33* mutants, these strains also are not sensitive to osmotic stress. Furthermore, SEY33-4 and SEY33-5 are only moderately defective in vacuole protein localization, missorting ~40% of the soluble vacuolar protease CPY. One of these strains, however, is temperature sensitive for growth. This strain, SEY33-4, was found to contain morphologically wt vacuoles, as visualized by using either CDCFDA or FITC, in both the mother cell and the bud at 25°C, the permissive temperature (Fig. 8A and B). However, when the strain was shifted to the nonpermissive temperature (37°C) for 2 h, budding cells were observed in which the bud did not appear to have any stain (Fig. 8C and D). Incubation for longer

times at 37°C resulted in fewer buds with detectable vacuole staining (Fig. 8E to J). By 6 h after the shift to 37°C, only a small fraction of the buds had vacuole fluorescence, and some unbudded cells exhibited a typical class C phenotype in that little or no vacuole fluorescence was visible (Fig. 8J). The wt parental strain SEY6210 is shown for comparison in Fig. 8K and L. Under identical staining conditions, bud vacuole fluorescence was clearly visible in these cells at 25°C (Fig. 8K) or after 6 h at 37°C (Fig. 8L). The extent of the bud vacuole staining defect in SEY33-4 was quantitated (Fig. 8). When SEY33-4 was grown at 25°C, 80% of the buds examined contained vacuole fluorescence. After 6 h at 37°C, only 14% of the buds exhibited vacuole staining. The vacuole protein processing phenotype of SEY33-4 was also exacerbated by exposure to 37°C. After 6 h at 37°C, ~70% of the CPY accumulated in the precursor form, as compared with 40% at 25°C (Fig. 9B; compare lanes 2 and 6). The temperature-sensitive mutation and the bud vacuole defect in SEY33-4 are genetically linked to the vacuole protein processing defect (2, 40), and each of the defects is completely complemented by the cloned *VPS33* gene.

**The *vps33-4*(Ts) mutation results in a Leu-646 → Pro change.** The temperature-sensitive mutation in SEY33-4 was mapped by using a strategy of recombinational rescue. Initially, SEY33-4 was transformed with the noncomplementing plasmid pLB33-23 or pLB33-24 (Fig. 2B); transformants streaked on YPD plates were incubated at 37°C, and the presence or absence of temperature-resistant colonies was scored. Many temperature-resistant recombinants were obtained from SEY33-4 harboring pLB33-23, indicating that the temperature-sensitive mutation likely lies 3' of the *ClaI* site within the ORF. Various fragments containing this region of the *VPS33* gene were subcloned into the multicopy vector pSEY18, and SEY33-4 transformants carrying these plasmids were again scored for the frequency at which temperature-resistant revertants arose. The results of this analysis (data not shown) suggested that the temperature-sensitive mutation mapped to the 250-bp region between the *SmaI* site and the end of the ORF (Fig. 2C). The chromosomal mutation was recovered by transforming SEY33-4 with a replicating plasmid carrying *VPS33* containing a double-stranded gap between the *SmaI* and *XbaI* sites (33). Sequencing of the *SmaI-XbaI* fragment from the rescued plasmid revealed that a single base pair change within the ORF, resulting in a leucine-to-proline change at residue 646, was responsible for the temperature sensitivity. The predicted amino acid sequences of the wt *VPS33* gene product and the mutant Vps33p carrying the Leu-646 → Pro alteration were subjected to Chou-Fasman analysis (5). The presence of proline at position 646 is predicted to cause a structural change in the *VPS33* gene product, inserting a turn in a region that is expected to form a random coil in the wt protein.

The nature of the temperature sensitivity was examined by retransforming various wt and *vps33* strains with the *vps33* gene carrying the rescued temperature-sensitive mutation on single and multicopy vectors. Transformation of SEY33-4 with a second copy of the temperature sensitive mutant allele resulted in a strain which grew slightly better at 37°C than did the original mutant. A null mutant carrying the *vps33-4* mutant allele on a single-copy vector had a growth rate comparable to that of the original mutant allele. Both mutants exhibited wt growth at 37°C when the temperature-sensitive allele was present on a multicopy vector. Transformation of the wt strain SEY6210 with either the single-copy or the multicopy construction did not affect its ability to



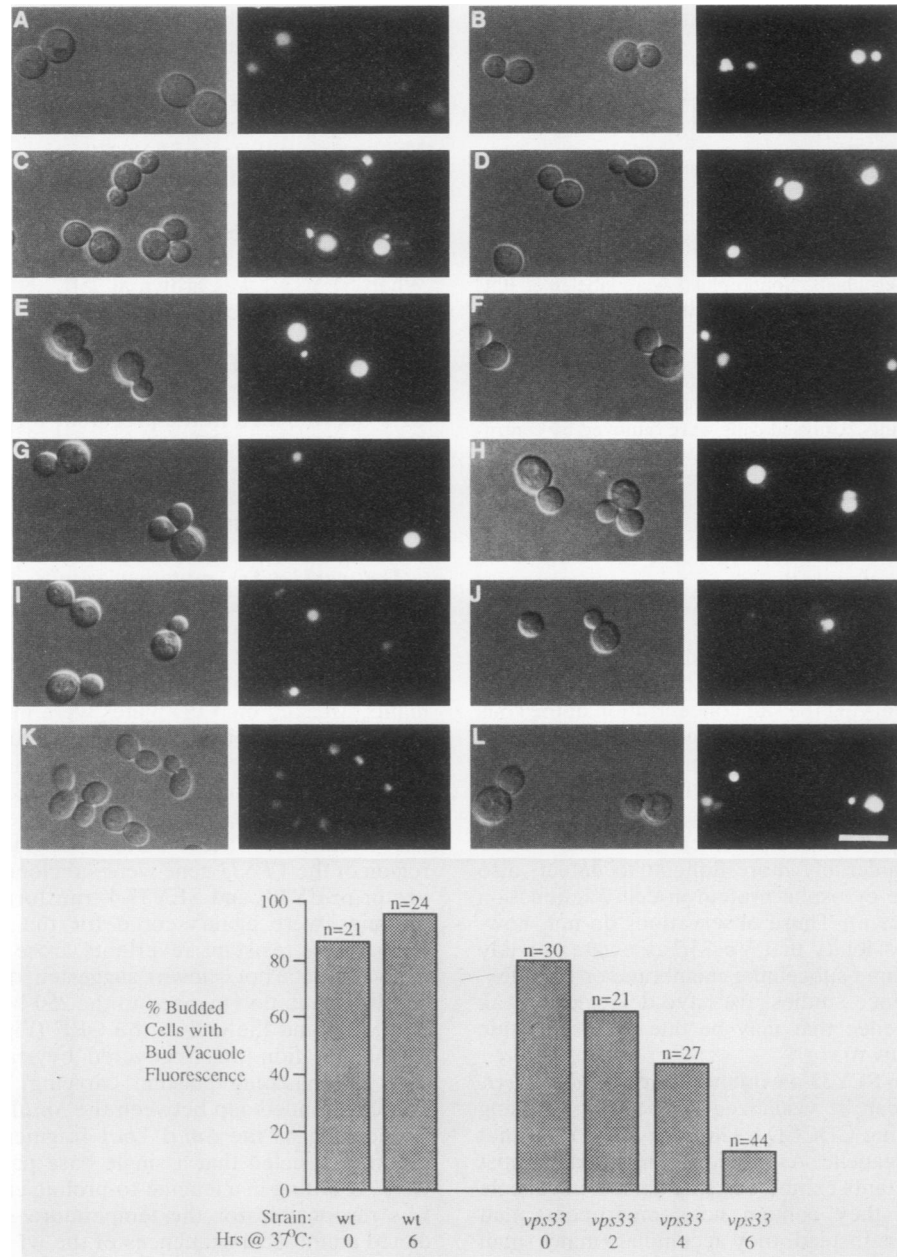


FIG. 8. Mother and bud vacuole staining in SEY33-4. SEY33-4 was grown in YPD at 25°C (A and B) and shifted to 37°C for 2 h (C and D), 4 h (E and F), or 6 h (G to J) before harvesting. Also shown for comparison is the parental strain SEY6210 grown at 25°C (K) and shifted to 37°C for 6 h (L). Cells were stained with FITC as described in Materials and Methods and observed by using Nomarski (left) or fluorescence (right) optics. The number of buds exhibiting vacuole fluorescence was quantitated and expressed as a percentage of the total number of large budded cells (n). Cells were grown at 25°C, incubated at 37°C for the indicated lengths of time, and stained with FITC as described above. Bar, 10  $\mu$ m.

grow at 37°C (Fig. 9A). Thus, the mutant allele *vps33-4*(Ts) is recessive to the wt allele even when overproduced. Furthermore, strains carrying the *vps33-4* mutant allele appeared to exhibit a dosage-dependent growth phenotype; overexpression of the mutant allele resulted in complementation of the temperature-sensitive defect of the null mutant or SEY33-4. However, although the strains carrying multiple copies of *vps33-4* were temperature resistant, they still exhibited a partial *Vps*<sup>-</sup> phenotype. Immunoprecipitation experiments indicated that 15% of the CPY accumulated in the precursor

form in SEY33-4 carrying the temperature-sensitive allele on a multicopy plasmid (Fig. 9B). Together, our observations suggest that the *vps33-4* mutant allele encodes a partially functional protein and that multiple copies of this protein can compensate for its diminished activity. The partial function of this *Vps33-4p* is unlikely to be a result of inactivation of the 45 aa following the changed residue, since a 3' truncation of the *VPS33* gene, lacking the coding sequence for the carboxy-terminal 56 aa, is unable to complement a *vps33* null mutation (data not shown).

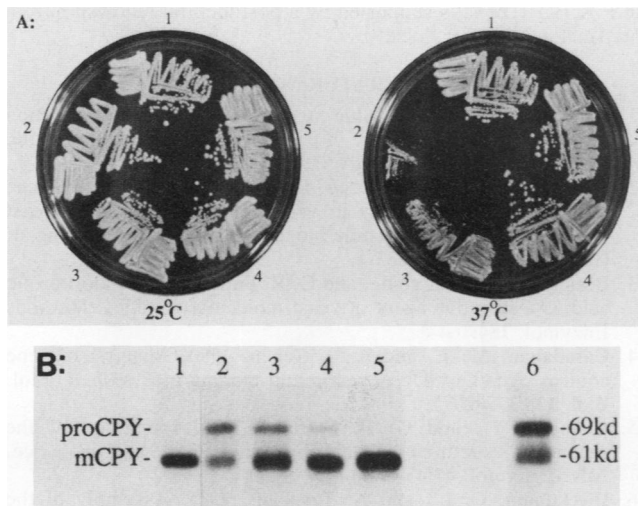


FIG. 9. Rescue of the SEY33-4 phenotypic defects by overexpression of the mutant allele. (A) The indicated strains were streaked on YPD solid medium and incubated at 25 or 37°C. 1, Parental strain SEY6210; 2, SEY33-4; 3, SEY33-4 carrying the plasmid pLB33-161 [*vps33-4*(Ts) allele on a single-copy vector]; 4, SEY33-4 carrying the plasmid pLB33-271 [*vps33-4*(Ts) allele on a multicopy vector]; 5, SEY6210 carrying pLB33-271. (B) Whole cells were labeled and chased at 25°C (lanes 1 to 5) or 37°C (lane 6) and subjected to immunoprecipitation with CPY-specific antisera as described in the legend to Fig. 5. Lanes 1 to 5 contain the strains listed above; for lane 6, SEY33-4 was grown at 25°C and shifted to 37°C for 5 h before radioactive labeling. Wild-type cells grown and labeled under these conditions contain CPY exclusively in the mature form (data not shown). The migration positions of precursor (proCPY) and mature (mCPY) forms are shown.

## DISCUSSION

*VPS33*, a gene required for vacuole protein sorting and normal vacuole formation, has been cloned and sequenced. Disruption of *VPS33* leads to a temperature-sensitive growth phenotype (Fig. 1), indicating that the gene is essential for growth only at elevated temperatures. At the permissive temperature, the null mutants are extremely defective in vacuolar protein processing and sorting (Fig. 5) and lack any structure resembling a wt vacuole. These phenotypes are comparable to those exhibited by most of the original *vps33* mutants, implying that many of the originally isolated alleles may be null (complete loss of function) mutations. The temperature sensitivity of the null mutants likely reflects a requirement at 37°C for one or more vacuolar functions that cannot be provided by whatever vacuole remnants remain in these mutant cells.

The phenotypes exhibited by *vps33* mutants resemble those of the recently described mutant *slp1* (17). This mutant was originally selected for its sensitivity to lysine. The strain contains smaller than normal vacuolar pools of lysine, arginine, and histidine and is characterized by pleiotropic defects, including lowered activities of soluble vacuolar enzymes such as CPY and proteinase A and an aberrant morphology in which many small vesicles but no large vacuoles are observed. Complementation analysis revealed that the *slp1* and *vps33* mutants define a single complementation group (L. Banta, S. Emr, Y. Wada, and Y. Anraku, unpublished observations). Comparison of the restriction maps and sequences of our *VPS33* clone and the recently cloned *SLP1* gene (52) has demonstrated that the two genes are identical.

Antibodies raised against a TrpE-Vps33 fusion protein recognize a relatively rare protein in yeast cells (<0.01% of cell protein) of ~75 kDa (Fig. 6). In cell fractionation experiments, Vps33p behaves as a soluble cytoplasmic protein under a variety of lysis conditions (Fig. 7). The hydrophilic nature of the predicted amino acid sequence and the apparent lack of N-linked glycosylation (Fig. 6) are consistent with a cytoplasmic location for Vps33p.

The predicted amino acid sequence of Vps33p possesses two regions of similarity with a number of ATPases and ATP-binding proteins (Fig. 4). Several studies suggest that these domains are directly involved in nucleotide binding or enzyme function. The conserved lysine residue in adenylate kinase, marked with an asterisk in Fig. 4, Domain A, has been shown by affinity labeling and other experiments to interact with ATP (7, 12, 51), and mutagenesis of the corresponding lysine in the *E. coli* ATPase  $\alpha$  or  $\beta$  subunit impaired apparent ATP-binding and catalytic activity (34, 39). A truncated form of *VPS33*, in which the coding sequence for aa 636 to 691 (including the second region of ATP-binding protein homology) has been deleted, is unable to complement the temperature-sensitive or class C defects associated with a *vps33* null mutation. This observation is consistent with the idea that the region of sequence similarity between Vps33p and the family of ATP-binding proteins is functionally significant. A second class C *vps* mutant, *vps11*, falls into the same complementation group as the mutant *end1* (8, 40). The *END1* gene has been cloned and sequenced (8). This gene also shares sequence similarity with *VPS33* and the family of ATP-binding protein genes throughout the two conserved domains. This observation raises the interesting possibility that ATP binding (and perhaps ATP hydrolysis) plays a role in vacuolar protein sorting and vacuole biogenesis. Further experiments, such as ATP-binding studies and affinity labeling, will be necessary to determine whether Vps33p does indeed bind ATP.

One *vps33* mutant strain, SEY33-4, exhibits a particularly interesting temperature-conditional defect in vacuolar protein sorting and vacuole inheritance. At the permissive temperature, this temperature-sensitive strain mislocalizes 40% of the CPY but contains vacuoles that are apparently normal by a number of criteria. These vacuoles exhibit wt staining with a variety of dyes, including quinacrine, a compound that accumulates in vacuoles in a pH-dependent manner (2, 54; L. Banta, unpublished observations). Unlike the more defective class C strains, SEY33-4 does not mislocalize the vacuolar membrane enzyme  $\alpha$ -mannosidase to the cell surface and is able to process 60% of the CPY to the mature form, indicating that the vacuoles in these cells are able to function as acceptor compartments for at least some vacuolar proteins. Upon incubation of SEY33-4 at 37°C, budding cells appear to lack vacuole staining selectively in the bud (Fig. 8) and the vacuolar protein processing defect is exacerbated (Fig. 9). The temperature-sensitive defect in this strain is due to a single base pair substitution which results in a leucine-to-proline change at position 646 in the *VPS33* gene product. This mutation is predicted to cause a structural change in the carboxy terminus of the protein.

The bud vacuole defect and increase in protein missorting observed in SEY33-4 at the nonpermissive temperature suggest a number of possible roles for the wt Vps33p in vacuole biogenesis. For example, Vps33p may be required for (i) vacuole segregation to the bud during cell division (55), (ii) maintenance of normal vacuolar structure, or (iii) sorting and delivery of vacuolar proteins between the Golgi and the vacuole. For the following reasons, we favor a model

in which Vps33p acts primarily in Golgi-to-vacuolar protein transport. The observation that Vps33p fractionates as a cytosolic protein suggests that this protein is not a structural component of the vacuole itself. In addition, the existence of SEY33-4 and a second *vps33* strain, SEY33-5, that mislocalize CPY yet contain apparently normal vacuoles at 25°C is inconsistent with a structural function for Vps33p in maintaining vacuole integrity. A primary role for Vps33p in vacuole inheritance also seems unlikely, given that SEY33-4 and SEY33-5 exhibit apparently normal vacuole segregation patterns during cell division at the permissive temperature. These observations suggest that the primary defect in these mutants lies in Golgi-to-vacuole trafficking. If Vps33p were directly involved in vacuole inheritance or vacuolar structure, mutants that clearly affect vacuole protein localization would also be expected to exhibit morphological abnormalities in the vacuole.

The simplest explanation for the extreme abnormalities in vacuole morphology exhibited by most of the *vps33* mutants is that as a consequence of a primary defect in Golgi-to-vacuole protein transport, proteins necessary for vacuolar inheritance or structural stability are mislocalized. In strong *vps33* mutants, which exhibit partial missorting of a vacuolar membrane component as well as almost completely aberrant targeting of soluble hydrolases, vacuole assembly is severely impaired. The less defective mutant SEY33-4 appears to contain a partially functional Vps33p. The residual activity is sufficient to permit some Golgi-to-vacuole protein transport and thus the formation of nearly normal vacuoles. The lack of a vacuole in the bud at 37°C may reflect a more complete loss of function if, for example, the mutant protein is less stable at high temperatures. We cannot exclude, however, the possibility that the *VPS33* gene product is bifunctional, with roles in both vacuole protein targeting and vacuole maintenance or segregation to emerging buds. In this scenario, the protein encoded by the *vps33-4* allele might be defective in only the former function at 25°C but both functions at 37°C.

A possible role for Vps33p in Golgi-to-vacuole sorting is suggested by a recent observation that the protein is phosphorylated in vivo (P. Herman, J. Stack, and S. Emr, unpublished observations). Perhaps Vps33p undergoes a cycle of phosphorylation-dephosphorylation, which in turn regulates its activity in promoting Golgi-to-vacuole protein transport. Since this transport is assumed to be vesicle mediated, the *VPS33* gene product could act at the level of vesicle formation, transport, or fusion with the target organelle. The accumulation of many small vesicles in *vps33* mutant cells (2) suggests that the defect in these strains may occur after vesicle formation. Using the probes developed in this study in conjunction with an in vitro assay for protein transport from the Golgi to the vacuole (T. A. Vida, T. R. Graham, and S. D. Emr, submitted for publication), we hope to determine the step(s) at which Vps33p acts as well as the significance of the observed phosphorylation.

#### ACKNOWLEDGMENTS

We thank Raffi Aroian for the YCp50 library DNA, Dan Klionsky for many helpful discussions, and Elliot Altman, Editte Gharaikhanian, and Bruce Horazdovsky for critically reading the manuscript. We also thank Yoh Wada and Yasuhiro Anraku for communicating results prior to publication.

This work was supported by Public Health Service grant GM-32703 from the National Institutes of Health (to S.D.E.). Partial support was also provided by General Electric Co. (to L.M.B.) and the Natural Sciences and Engineering Research Council of Canada

(to P.K.H.) T.A.V. is supported by a postdoctoral fellowship from the American Cancer Society.

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