Molecular Analysis of the Yeast VPS3 Gene and the Role of Its Product in Vacuolar Protein Sorting and Vacuolar Segregation during the Cell Cycle

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Abstract. *vps3* mutants of the yeast *Saccharomyces cerevisiae* are impaired in the sorting of newly synthesized soluble vacuolar proteins and in the acidification of the vacuole (Rothman, J. H., and T. H. Stevens. *Cell.* 47:1041–1051; Rothman, J. H., C. T. Yamashiro, C. K. Raymond, P. M. Kane, and T. H. Stevens. 1989. *J. Cell Biol.* 109:93–100). The *VPS3* gene, which was cloned using a novel selection procedure, encodes a low abundance, hydrophilic protein of 117 kD that most likely resides in the cytoplasm. Yeast strains bearing a deletion of the *VPS3* gene (*vps3-Δ1*) are viable, yet their growth rate is significantly reduced relative to wild-type cells. Temperature shift experiments with strains carrying a temperature conditional *vps3* allele demonstrate that cells rapidly lose the capacity to sort the vacuolar protein carboxypeptidase Y upon loss of *VPS3* function. Vacuolar morphology was examined in wild-type and *vps3-Δ1* yeast strains by fluorescence microscopy. The vacuoles in wild-type yeast cells are morphologically complex, and they appear to be actively partitioned between mother cells and buds during an early phase of bud growth. Vacuolar morphology in *vps3-Δ1* mutants is significantly altered from the wild-type pattern, and the vacuolar segregation process seen in wild-type strains is defective in these mutants. With the exception of a vacuolar acidification defect, the phenotypes of *vps3-Δ1* strains are significantly different from those of mutants lacking the vacuolar proton-translocating ATPase. These data demonstrate that the acidification defect in *vps3-Δ1* cells is not the primary cause of the pleiotropic defects in vacuolar function observed in these mutants.

EUKARYOTIC cells possess several distinct membrane-bounded intracellular organelles, each of which is composed of unique proteins and other cellular components. It is of interest to understand how these structures are assembled, maintained, and partitioned during cell growth and cell division. The vacuole of the budding yeast *Saccharomyces cerevisiae* is one such organelle that has become the focus of genetic and biochemical investigation. Yeast vacuoles are similar in many respects to lysosomes in mammalian cells and vacuoles in plant cells (Rothman and Stevens, 1988). The vacuole occupies a significant fraction of the overall volume in a yeast cell and is thought to mediate many diverse processes such as intracellular protein degradation, amino acid and ion storage, pH and osmotic regulation, and degradation of internalized ligands (Rothman and Stevens, 1988; Jones, 1984). The interior of the vacuole is maintained at an acidic pH by a membrane-associated proton-translocating ATPase (H^+-ATPase) (Preston, et al., 1989; Kane, et al., 1989). The vacuole contains many hydrolytic enzymes some of which are soluble in the lumen of the organelle while others are integral membrane proteins (Rothman and Stevens, 1988; Roberts et al., 1989; Klionsky and Emr 1989). All resident vacuolar proteins studied to date enter the secretory pathway, receive carbohydrate modifications characteristic of passage through the ER and Golgi apparatus, and are subsequently sorted away from nonvacuolar proteins within or shortly after transport through the Golgi apparatus (Stevens et al., 1982; Moehle et al., 1988; Roberts et al., 1989; Klionsky and Emr, 1989). In certain well-characterized vacuolar proteins, separate *cis*-acting signals have been identified that direct entry into the ER and sorting to the vacuole (Valls et al., 1987; Johnson et al., 1987; Klionsky et al., 1988). In one case, the vacuolar targeting signal has been localized to a few contiguous amino acid residues (Valls et al., 1987, 1990; Johnson et al., 1987; Rothman et al., 1989*b*). Several vacuolar hydrolases traverse the secretory pathway as inactive precursors and are proteolytically processed to the active species upon delivery to the vacuole by a process that requires the activity of proteinase A (PrA),[1] the product of the *PEP4* gene (Jones et al., 1982;

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1. *Abbreviations used in this paper*: CDCFDA, 2',7'dichlorocarboxyfluorescein diacetate; CPY, carboxypeptidase Y; ORF, open reading frame; PrA, proteinase A.

Ammerer et al., 1986; Woolford et al., 1986; Mechler et al., 1987).

While the biosynthesis of many vacuolar proteins has been relatively well characterized, other aspects of vacuolar biology are poorly understood. Little is known about the components that recognize vacuolar targeting information, the manner in which sorting occurs, the processes whereby vacuoles are assembled and maintained, or the mechanism of organelle segregation during cell division. Several laboratories have isolated mutants that secrete newly synthesized soluble vacuolar proteins instead of efficiently localizing them to the vacuole. Vacuolar protein sorting (*vps*) mutants were isolated by selection for secretion of the vacuolar hydrolase carboxypeptidase Y (CPY) (Rothman and Stevens, 1986; Rothman et al., 1989*a*; Bankaitis et al., 1986; Robinson et al., 1988). Most of the *pep* mutants, isolated in a screen for mutants defective in CPY activity (Jones, 1977), also missort soluble vacuolar proteins (Rothman et al., 1989*a*). Complementation analysis between these mutants has shown that at least 49 genes are required for the proper sorting of vacuolar proteins (Rothman et al., 1989*a*; Robinson et al., 1988). Further phenotypic characterization has revealed that certain of these mutants are also defective in vacuolar acidification, organelle assembly, and/or protease maturation (Rothman et al., 1989*a*,*c*; Rothman and Stevens, 1986; Banta et al., 1988). Analysis of these mutants should ultimately identify the molecular components and mechanisms necessary for the biogenesis of vacuoles.

The mechanism by which vacuoles are segregated to daughter cells during cell division is also not well understood. Separate genetic and morphological studies have provided evidence that daughter cells receive vacuolar material from mother cells before cell division (Zubenko et al., 1982; Weisman et al., 1987; Weisman and Wickner, 1988). While it has been proposed that small transport vesicles might mediate this transfer process (Weisman and Wickner, 1988), the mechanism underlying vacuolar segregation remains unclear. In fact, even the issue of what constitutes normal vacuolar morphology has been the subject of recent controversy (Weisman et al., 1987; Pringle et al., 1990).

Previous reports have shown that *vps3* cells secrete roughly 90% of newly synthesized CPY as well as substantial amounts of PrA, and the remaining intracellular CPY and PrA molecules accumulate primarily as unprocessed zymogens (Rothman and Stevens, 1986; Rothman et al., 1989*c*; unpublished results). The *vps3* mutant was referred to as the *vpl3* mutant in these earlier reports, and the name was changed in a consolidation of *vpl* (Rothman and Stevens, 1986) and *vpt* (Bankaitis et al., 1986) mutant nomenclature (Robinson et al., 1988; Rothman et al., 1989*a*). *vps3* mutant cells also have defects in vacuolar acidification. Vacuolar membranes purified from *vps3* mutants retain only 5–10% of the wild-type vacuolar H^+-ATPase activity, and they exhibit a corresponding deficiency in at least two subunits of this multi-subunit enzyme complex (Rothman et al., 1989*c*; Kane et al., 1989). These subunits are present at wild-type levels in total cell extracts, suggesting that the vacuolar H^+-ATPase complex is incorrectly assembled onto the vacuolar membrane in *vps3* mutants (Rothman et al., 1989*c*). Here we report on the characterization of the *VPS3* gene, the *VPS3* gene product (Vps3p), and the phenotypes of *vps3* mutants in greater detail. Our studies indicate that Vps3p is a cytoplasmic protein whose function is likely to be intimately involved in vacuolar protein sorting. We also show that the vacuole in wild-type cells appears to be actively segregated between mother and daughter cells during cell division, and *vps3* mutants are defective in this process.

Materials and Methods

Strains, Growth Conditions, and Materials

The genotypes of the *S. cerevisiae* strains used in this study are shown in Table I. The *vps3-Δ1* mutation was referred to as the *vpl3-Δ1* mutation in a previous report (Rothman et al., 1989*c*). The SF838-1D *pho8-Δ1* strain carries a substitution of the *LEU2* gene within the *PHO8* gene. Recombinant strains were constructed by single step gene replacement or integration (Rothstein, 1983), and the lithium acetate transformation procedure was used (Ito et al., 1983) to introduce DNA fragments or plasmids into yeast cells. Spheroplast transformation (Sherman et al., 1982) was used in the cloning of the *VPS3* gene. The *Escherichia coli* strain used in this study was MC1061 (F⁻ *hsdR hsdM*⁺ *araD139* (*araABOIC-leu*)*7679* (*lac*)*X74 galU galK rpsL*; Casadaban and Cohen, 1980). The growth media for yeast were rich medium (YPD) containing 1% Bacto yeast extract, 2% Bacto peptone (Difco Laboratories Inc., Detroit, MI), and 2% glucose or minimal media (SD) containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories Inc.) and 2% glucose (Sherman et al., 1982) as specified, and the latter medium was supplemented with the appropriate nutrients (Stevens et al., 1986). Strains were grown at 30°C unless otherwise indicated. The negative selection, which must be conducted on Leu^+ yeast strains, was performed on supplemented SD plates lacking leucine and containing 1 mM CBZ·pheF₃leu and 0.03 mM F₃leu. CBZ·pheF₃leu was synthesized from CBZ·phenylalanine and F₃leu by a standard peptide coupling procedure (Paul and Anderson, 1960).

F₃leu was from Fairfield Chemical Co. (Blythwood, SC). Restriction endonucleases and DNA modifying enzymes were from Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), or New England Biolabs (Beverly, MA). Glusulase was from DuPont Co. (Boston, MA). Carrier free $H_2{}^{35}SO_4$ and zymolyase 100T were from ICN Biomedicals Inc. (Irvine, CA). [^{32}P]dCTP and [^{35}S]dATP were from New England Nuclear (Boston, MA), [^{125}I]protein A was from Amersham Corp. (Arlington Heights, IL), and nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). 2',7'dichlorocarboxyfluorescein diacetate (CDCFDA) was from Molecular Probes (Eugene, OR). Multiwell test slides were from Flow Laboratories, Inc. (McLean, VA). CPY antibody has been described previously (Rothman and Stevens, 1986), affinity-purified rabbit antiyeast alkaline phosphatase (Pho8p) antibody will be described elsewhere (Dr. G. Pohlig and Dr. T. Stevens, unpublished observations), alkaline phosphatase conjugated goat anti-rabbit IgG was from Promega Biotec (Madison, WI), alkaline phosphatase color development reagents, used as recommended, were from Bio-Rad Laboratories (Richmond, CA), and IgG Sorb was from the Enzyme Center (Boston, MA). 37% formaldehyde was from J. T. Baker Chemical Co. (Phillipsburg, NJ), urea was from International Biotechnologies, Inc. (New Haven, CT), SDS was from BDH Biochemicals Ltd. (Poole, UK), and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cloning, Sequencing, and DNA Manipulations

DNA manipulations were performed by routine procedures (Maniatis et al., 1982). The *VPS3* gene was cloned by transforming mutants *vps3-2* or *vps3-10* (Table I) to Ura^+ with yeast genomic clone banks carried in the centromere vector YCp50 (Rose et al., 1987) or the multicopy vector YEp24 (Carlson and Botstein, 1982). Roughly 1×10^5 Ura^+ transformants derived from each library were pooled and replated on CBZ·pheF₃leu plates at a density of 1×10^5 cells/plate. Survivors arose at a frequency of 1×10^2 colonies/plate. 50 were tested for secretion of CPY by the immunoblot procedure (Rothman et al., 1986). Vps^+ colonies were observed at a frequency of 10–100%. Certain plasmids from independent Vps^+ isolates shared common restriction fragments, and these plasmids complemented the *vps3* defect. One of these complementing plasmids isolated from the YEp24 clone bank, pVPS3, was characterized further. An integrating plasmid was built from pVPS3 by ligating two of the four Eco RI restriction fragments from this plasmid together to form a separate, smaller plasmid. The subcloning removed the 2 μm-based yeast origin of replication but retained

Table I. Strains Used in this Study

Strains	Genotype	Source or reference
SF838-1D	*MATα, ade6, his4-519, leu2-3, leu2-112, ura3-52, pep4-3, gal*	Rothman and Stevens, 1986
JHRY20-2C	*MATa, his3-Δ200, leu2-3, leu2-112, ura3-52*	Rothman et al., 1986
SEY6211	*MATa, ade2-101, his3-Δ200, leu2-3, leu2-112,trp1-Δ901, ura3-52, suc2-Δ9*	Robinson et al., 1988
SF838-9D	*MATα, ade6, his4-519, leu2-3,leu2-112, lys2, pep4-3, gal*	Rothman et al., 1989*a*
SF838-9DR2L1m1006	*MATa, his4-519, ura3-52, lys2, pep4-3, gal, vps3-2*	Rothman and Stevens, 1986
SF838-9DR2L1m1062	same as above except *vps3-10*	Rothman and Stevens, 1986
SF838-9Dm300	same as SF838-9D except *vps3-28*	Rothman et al., 1989*a*
SF838-9Dm301	same as SF838-9D except *vps3-29*	Rothman et al., 1989*a*
CRY1	diploid from cross of JHRY20-2C and SF838-9D	This study
SF838-1D *vps3-Δ1*	same as SF838-1D except *vps3-Δ1::LEU2*	Rothman et al., 1989*c*
SF838-1D *vps3-Δ2*	same as SF838-1D except *vps3-Δ2::LEU2*	This study
SF838-1D *pho8-Δ1*	same as SF838-1D except *pho8-Δ1::LEU2*	This study
JHRY20-2C *vps3-Δ1*	same as JHRY20-2C except *vps3-Δ1::LEU2*	This study
JHRY20-2C *vps3-Δ2*	same as JHRY20-2C except *vps3-Δ2::LEU2*	This study
JHRY20-2C *vps3^{ts}*	same as JHRY20-2C *vps3-Δ2* except *vps3-Δ2::vps3^{ts},URA3* (see Materials and Methods)	This study
SEY6211 *vps3-Δ1*	same as SEY6211 except *vps3-Δ1::LEU2*	This study

the bacterial origin of replication, the bacterial and yeast selectable markers Amp^R and *URA3*, respectively, and a large portion of the *VPS3* complementing region. This plasmid was digested at a unique Bgl II site within the putative *VPS3* insert and used to transform JHRY20-2C to Ura$^+$. Mapping experiments were performed by mating this Ura$^+$ strain to *vps3-28* and *vps3-29* mutants, selecting for His$^+$ diploids, sporulating, and tetrad analysis. *VPS3* complementing subclones were constructed by subcloning the 4.2-kb Bam HI fragment (see Fig. 2) into the Bam HI site of YCp50 (pCKR68), and the 3.9-kb Bam HI–Sna BI fragment into the Bam HI-Sma I site of the same vector (pCKR76).

Restriction fragments from the 3.6-kb Hind III–Bam HI region containing the *VPS3* gene were subcloned into phage M13 mp18 and mp19 for DNA sequence determination by the dideoxy chain termination method (Sanger et al., 1977). Sequencing reaction products were polymerized by modified T7 polymerase (U.S. Biochemicals, Cleveland, OH), primed by a universal M13 primer or with oligonucleotides complementary to *VPS3* DNA. Oligonucleotides were synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA). All sequences were determined on both strands, and all restriction sites used in subcloning were sequenced using overlapping fragments. The *VPS3* DNA and predicted protein sequence were analyzed using the FASTA and TFASTA sequence comparison programs (Pearson and Lipman, 1988).

The *VPS3* deletion and insertion plasmids were built by subcloning the 4.2-kb Bam HI fragment into the Bam HI site of a pUC12 vector in which the Hind III and Pst I had been removed (pCKR65). The *VPS3* deletion allele (*vps3-Δ1*) was constructed by digestion of pCKR65 with Hind III and Sna BI blunt ending with T4 polymerase, and insertion of *LEU2* gene carried on a 2.1-kb Hpa I fragment. The insertion allele (*vps3-Δ2*) was made by digestion of pCKR65 with Pst I, blunt ending, and insertion of the same *LEU2* fragment. The deletion and insertion mutations were introduced into yeast strains by transformation with Bam HI–digested plasmids and selection for Leu$^+$ cells. Haploid transformants were screened for a Vps$^-$ phenotype, and homologous integration in Vps$^-$ cells was confirmed by Southern analysis. Yeast genomic DNA for Southern blotting was prepared by the method of Nasmyth and Reed (1980). Southern blots were probed with a 1.1-kb Bam HI–Hind III fragment from the 5′ region of the *VPS3* gene which had been gel purified and radiolabeled with a kit from Amersham Corp. Hybridizations and autoradiography were performed by routine procedures (Strauss, 1987).

The proteins used as antigens for the production of antibodies (see below) were expressed in *E. coli* from a series of protein expression plasmids with unique polylinker cloning sites in three separate reading frames downstream of an inducible promoter and a translational start codon. Plasmids pEXP1, pEXP2, and pEXP3 were constructed from pHSe5, which contains the T4 lysozyme gene under the control of the TAC promoter (Muchmore et al., 1989). The pUC19 polylinker was cloned into the Eco RI–Hind III sites within the T4 lysozyme coding sequence (pCKR101). pEXP2 was created from pCKR101 by cloning an 8-bp Eco RI linker into the Sna BI site positioned 24 nucleotides downstream of the T4 lysozyme ATG start codon, digestion of the resulting plasmid with Eco RI, and religation. This placed the polylinker eight codons downstream of the ATG start site. pEXP1 was made by digesting pCKR101 with Sna BI and Eco RI, blunt ending, and religation. pEXP3 was built from pEXP2 by digesting with Sac I, blunt ending, and religation. Two plasmids that lead to the expression of a portion of the Vps3p protein were made. A 900-bp Pst I–Bgl II fragment from the middle of the Vps3p open reading frame (ORF) (see Fig. 2) and a 750-bp Pst I–Hind III from the same region were cloned into the Sma I site in pEXP2 (pCKR114 and pCKR115, respectively).

A temperature-sensitive allele of the *VPS3* gene was generated by in vitro hydroxylamine mutagenesis of plasmid pCKR68 (Schauer et al., 1985). The mutagenized plasmid DNA was used to transform a *vps3-Δ1* strain to Ura$^+$. 2,000 transformant colonies were screened for a Vps$^+$ phenotype at 30°C and a Vps$^-$ phenotype at 37°C. Eight putative conditional transformants were isolated, and one was characterized further. Subcloning of the putative *vps3^{ts}* allele revealed that the conditional phenotype was linked to the *VPS3* gene. The conditional allele carried on a 4.2-kb Bam HI fragment was subcloned into the Bam HI site of the integrating vector YIp5 (Botstein et al., 1979) which was lacking the Sal I site (pCKR75). pCKR75 was digested at the Sal I site within the *VPS3* gene and used to transform JHRY20-2C *vps3-Δ2* to Ura$^+$ (JHRY20-2C *vps3^{ts}*; Table I). The genomic integrants analyzed displayed the same conditional phenotype as the original isolate. Further analysis has suggested that the *ts* mutation maps to the 3′ end of the *VPS3* gene.

Fluorescence Microscopy

Vacuolar vital staining was performed on log-phase yeast cultures at a density of 1 × 10^7 cells/ml in supplemented SD broth buffered to pH 5.0 with 50 mM citrate and containing 10 μM CDCFDA. Cultures were shaken at room temperature for ~30 min before observation. Double labeling with calcofluor was achieved by addition of 1 μg/ml calcofluor to the labeling media. Vacuoles were labeled with endogenous *ade2* fluorophore, produced in *ade2* yeast strains under conditions of limiting adenine, by the methods described by Weisman et al. (1987). *ade2* pigment synthesis was depressed by 10-fold dilution of these cells into YEPD broth supplemented with 200 μg/ml adenine. All labeled cells were applied directly to Con A–coated slides (Pringle et al., 1990) and viewed or photographed using an Axioplan photomicroscope (Carl Zeiss Inc., Thornwood, NY) and TMAX-400 film (Eastman Kodak Co., Rochester, NY). Calcofluor was viewed through a DAPI filter set (Zeiss 487702; Carl Zeiss Inc.), CDCFDA through an FITC filter set (Zeiss 487709; Carl Zeiss Inc.) and *ade2* pigment through a rhodamine filter set (Zeiss 487715; Carl Zeiss Inc.).

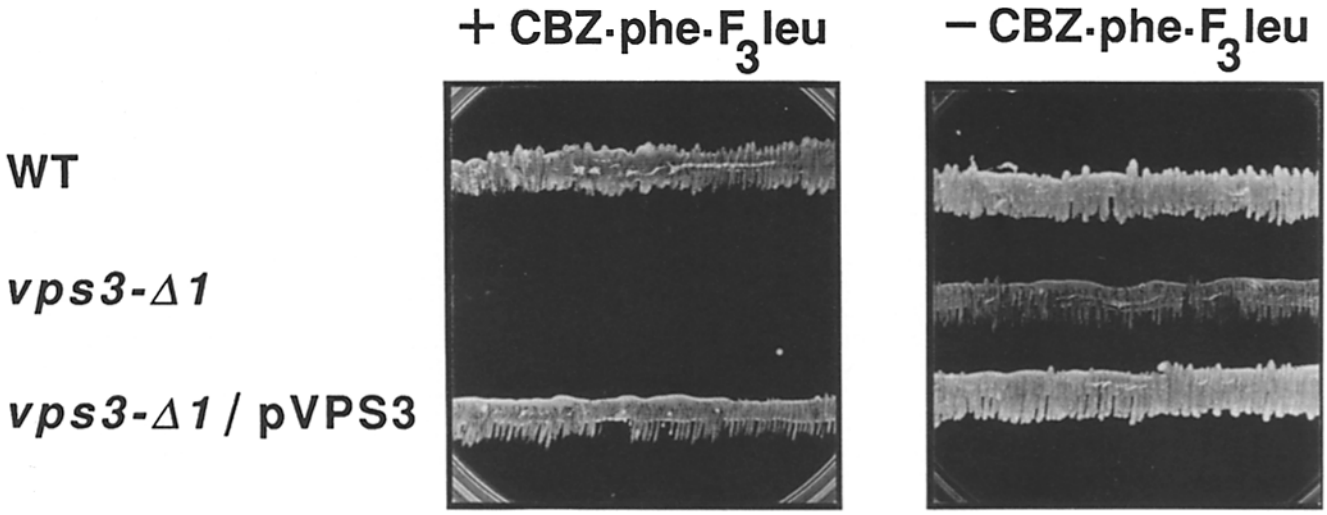


Figure 1. CBZ·phe·F_3leu selects against vps mutants. Isogenic haploid strains of the indicated genotype were streaked onto appropriately supplemented SD medium alone (−CBZ·phe·F_3leu) or the same SD medium containing 1 mM CBZ·phe·F_3leu and 0.03 mM F_3leu (+CBZ·phe·F_3leu). The plates were incubated at 30°C for 3 d. The strains were SF838-1D (*WT*), SF838-1D *vps3-Δ1* (*vps3Δ*), and SF838-1D *vps3-Δ1*/pVPS3 (*vps3Δ*/pVPS3).

For immunofluorescence, yeast cells were grown in YEPD broth to a density of 1×10^7 cells/ml at 30°C and fixed by the addition of formaldehyde to 4%. After 1 h at 30°C, the cells were harvested and resuspended in fixative containing 4% formaldehyde, 100 mM KPO_4, pH 6.5, and 1 mM $MgCl_2$ at a density of 1×10^8 cells/ml. The suspension was gently shaken for 12 h at 25°C. The cells were then harvested and resuspended in 200 mM Tris, pH 8.0, 20 mM EDTA, and 1% β-mercaptoethanol for 10 min at 30°C. After two washes with 1.2 M sorbitol, 50 mM KPO_4, pH 7.3, and 1 mM $MgCl_2$, the cells were resuspended at 1×10^8 cells/ml in the same buffer containing 150 μg/ml Zymolase 100T and 25 μl/ml glusulase and shaken at 30°C for 30 min. All subsequent steps were performed at room temperature. Spheroplasts were washed three times in 1.2 M sorbitol, resuspended at a density of 5×10^8 cells/ml in 4% SDS and 1.2 M sorbitol for 5 min, washed three more times in 1.2 M sorbitol, and resuspended at a density of 1×10^8 cells/ml in 1.2 M sorbitol. 40 μl of the cell suspension was applied to a polylysine-coated multi-well slide for 10 min. The wells were washed three times with PBS (Pringle et al., 1990) containing 5 mg/ml BSA (PBS-BSA) and incubated with the same solution in a humid chamber for 30 min. A 10-μl aliquot of a 1:10 dilution of adsorbed primary antibody (see below) in PBS-BSA was applied to each well for 1 h. Each well was then washed nine times with PBS-BSA. The wells were then treated in an identical manner as above with goat anti-rabbit IgG, then rabbit anti-goat IgG, and finally FITC-conjugated goat anti-rabbit IgG. All secondary antibodies were purchased as commercially available 1 mg/ml stock solutions (Jackson ImmunoResearch, West Grove, PA), and they were used at 1:500 dilutions in PBS-BSA. After the final washes, the cells were mounted in medium containing 4',6'-diamidino-2-phenyl-indole (DAPI) for visualization of cell nuclei (Pringle et al., 1990). Photomicrographs were made as described for vital staining above.

The specificity of labeling in immunofluorescence experiments was improved by adsorbing affinity-purified rabbit anti-Pho8p antibodies to fixed yeast cells. 1 l of a *pho8-Δ1* strain (Table I), grown to a density of 1×10^7 cells/ml, was fixed, spheroplasted, and treated with SDS as described above. The spheroplasts were incubated in 20 ml PBS-BSA for 30 min at room temperature two times and harvested as two separate pellets. One pellet was resuspended in 500 μl PBS-BSA, 10 μl of 1 mg/ml affinity-purified rabbit anti-Pho8p antibody was added, and the suspension was shaken for 1 h. The cells were then removed by centrifugation, the supernatant was transferred to the second cell pellet, and the procedure was repeated. Both pellets were subsequently washed with 500 μl PBS-BSA which was pooled with the adsorbed antibody. Unlike the starting affinity-purified antibody, the adsorbed antibody gave negligible staining in *pho8-Δ1* cells.

Antibodies, Immunoblotting, and Immunoprecipitation

The Vps3p antigen was expressed in *E. coli* from plasmid pCKR114, purified, and used to immunize rabbits as described by Roberts et al. (1989). Rabbit anti-Vps3p antibodies were affinity purified against protein expressed from plasmid pCKR115 as described by Hicke and Schekman (1989) except that antibody bound to the column was washed with 1 M NaCl, 0.1 M $NaHCO_3$, pH 8.5, and 1 M NaCl, 0.1 M Na/H·OAc, pH 4.0 before elution, antibody binding and elution were performed twice in succession, and the final eluted antibody was resuspended in PBS containing 5 mg/ml BSA. Immunoblots of total yeast protein extracts (prepared as described by Rothman et al., 1989*c*), subcellular fractions (prepared as described by Gould et al., 1988), or yeast vacuoles (prepared as described by Kane et al., 1989) were performed by the method of Rothman et al. (1989*c*). The blots were probed with a 1:1,000 dilution of rabbit anti-Vps3p antibody followed by either alkaline phosphatase–conjugated goat anti-rabbit IgG or [^{125}I]protein A. Immune complexes were visualized according to the manufacturers' suggestions.

For CPY immunoprecipitations, *vps3^{ts}* cultures were grown in supplemented SD containing 50 μM sulfate at 30°C. At various times before labeling, aliquots were shifted to 37°C. The strains were then harvested, resuspended in 0.5 ml of sulfate-free SD containing 50 mM KH_2PO_4, pH 5.7, and 0.5 mg/ml BSA at a density of 1×10^7 cells/ml, and 200 μCi of $H_2{}^{35}SO_4$ was added. After 30 min of labeling, the cells were chased with 10 mM Na_2SO_4, 0.4 mM cysteine, and 0.3 mM methionine for 30 min. The cells were maintained at 37°C or 30°C throughout the pulse and chase as specified. The chase was terminated by the addition of 10 mM sodium azide, the cells were converted to spheroplasts (Stevens et al., 1986), the periplasmic and medium fractions were pooled to give the extracellular fraction (Valls et al., 1987), the spheroplasts were boiled for 2 min in 1% SDS, and both intracellular and extracellular fractions were adjusted to 1 ml in PBS, 0.1% SDS, 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 10% IgG Sorb (prepared as suggested by the manufacturer) final concentrations. CPY was immunoprecipitated as described by Stevens et al. (1986). The IgG Sorb-immune complexes were washed twice in 10 mM Tris, pH 8.0, 0.1% Triton X-100, 0.1% SDS, and 0.1 mM EDTA, and the samples were analyzed by SDS-PAGE and fluorography as described previously (Stevens et al., 1986). The amount of radiolabeled CPY in each lane was quantified directly using an Ambis Radioanalytic Imaging System (Ambis Systems, Inc., San Diego, CA). CPY and Vps3p were immunoprecipitated from the intracellular fraction of $H_2{}^{35}SO_4$-labeled wild-type strain SF838-1D and quantified by the same procedures as above. Percent of total cell protein was calculated from the counts incorporated into CPY divided by the total incorporated counts ×100.

Results

Isolation and Sequence of the VPS3 Gene

We have described previously a selection procedure for ob-

taining *vps* mutants in yeast (Rothman and Stevens, 1986). The selection permitted leucine-independent growth of a *vps* leucine auxotroph by utilizing extracellular CPY activity to release leucine from the CPY specific substrate *N*-carbobenzoxy-L-phenylalanyl-L-leucine (CBZ·pheleu) (Rothman and Stevens, 1986). A negative selection against *vps* mutants was devised by exploiting the observation that the leucine analog D,L-5,5,5-trifluoroleucine (F_3leu) is toxic to Leu$^+$ yeast cells in the absence of exogenous leucine (Bussey and Umbarger, 1970). The compound *N*-carbobenzoxy-L-phenylalanyl-D, L-5,5,5-trifluoroleucine (CBZ·pheF_3leu) inhibited the growth of a *vps3* mutant relative to the isogenic wild-type strain (Fig. 1). The observed growth inhibition was typical of most *vps* mutants (data not shown). Growth was restored in a *vps3* mutant which harbored the corresponding *VPS3* gene on a plasmid (Fig. 1 and see below). However, the selection had certain limitations. *vps* mutants insensitive to CBZ·pheF_3leu but still Vps$^-$ arose at a frequency of 1×10^{-4}–1×10^{-5}. Furthermore, F_3leu liberated by Vps$^-$ cells poisoned adjacent Vps$^+$ cells at high plating densities (>10^6 cells/plate). Finally, growth of some *vps* mutants was only partially inhibited by the selection (data not shown). Thus, the selection was ideal for high frequency events that required a low plating density, such as the cloning of a gene from a genomic library.

The *VPS3* gene was cloned from wild-type yeast genomic clone banks contained in the centromere vector YCp50 (Rose et al., 1987) or in the multicopy, 2 μm-based vector YEp24 (Carlson and Botstein, 1982) by complementation of the CBZ·pheF_3leu growth inhibition of *vps3* mutants. Complementing plasmids were isolated from Vps$^+$ survivors of the negative selection. All complementing plasmids shared characteristic restriction fragments, and the 10.5-kb complementing region from a YEp24 isolate, pVPS3, is shown in Fig. 2 *A*. To test whether the complementing sequences encompassed the *VPS3* genetic locus, a *URA3*-marked integrating plasmid was constructed from pVPS3. This plasmid was digested at a unique restriction site within the complementing region and used to transform a *VPS3 ura3* strain to Ura$^+$. A representative transformant was crossed to two different *vps3 ura3* mutants. Diploids from these crosses were sporulated and tetrads were dissected and analyzed. The Ura$^+$ marker segregated 2:2 away from the *vps3* defect in all 29 four spore tetrads analyzed, indicating that the cloned sequences map to the *VPS3* locus. The position of the *VPS3* gene within the pVPS3 genomic insert was determined by subcloning and transposon mutagenesis (Huisman et al., 1987). Transposon insertions which destroyed *VPS3* complementation were clustered in a 3.6-kb region. This transposition system also permitted the direction of transcription and translation of the *VPS3* gene to be determined (Fig. 2 *A*). A 3.9-kb Bam HI–Sna BI fragment encompassing the 3.6-kb cluster of insertions complemented the *vps3* defect when subcloned into either single copy or multicopy shuttle vectors, suggesting that the entire *VPS3* gene was localized to this region (Fig. 2 *B*).

The DNA sequence of 3.6 kb within this region was determined (Figs. 2 *C* and 3). It was found to contain a single, long ORF of 3,033 nucleotides which would encode a hydrophilic protein of 1,011 residues with a predicted molecular mass of ~117 kD. Neither the *VPS3* sequence nor the translated ORF had significant homology to other nucleic acid (GENBANK and EMBL) or protein sequences (SWISS PROT and PIR) available. Furthermore, there were no significant hydrophobic regions suggestive of membrane spanning domains, and the amino terminus of the protein did not appear to encode an ER signal sequence. This analysis suggested that the putative *VPS3* gene product is a soluble cytoplasmic protein.

Construction and Characterization of vps3 Deletion Mutants

A null mutation of the *VPS3* gene was created by replacing the entire *VPS3* coding sequence with the *LEU2* gene. This removed 224 nucleotides 5′ of the Vps3p start codon through

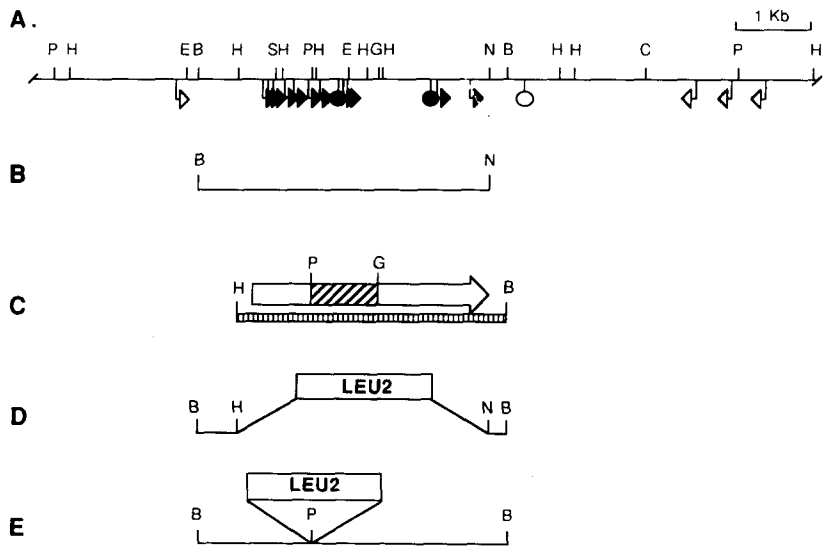
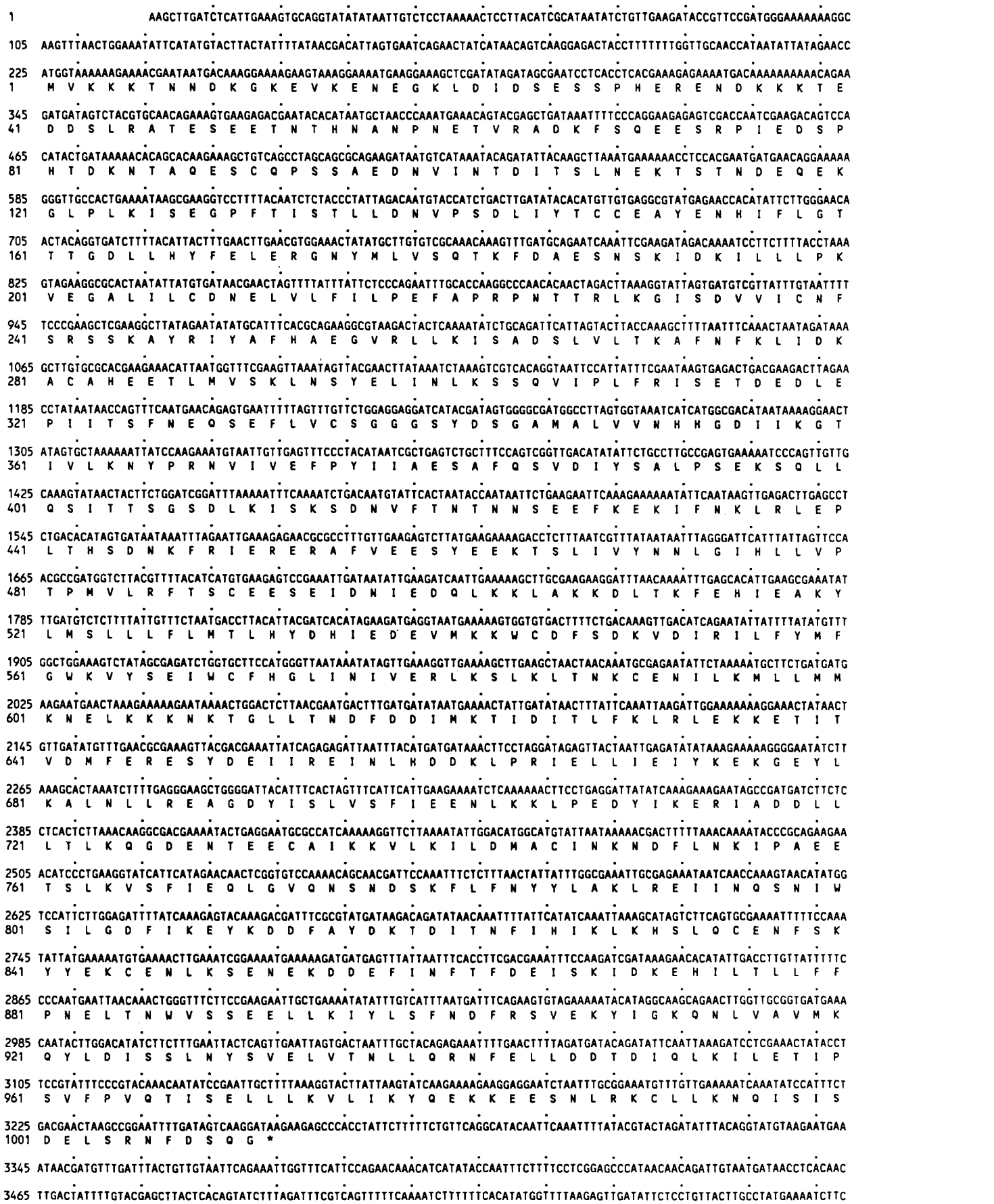


Figure 2. Physical map of the *VPS3* gene, and DNA manipulations used in this study. (*A*) The 10.5-kb genomic DNA insert in pVPS3. The restriction map of this region is shown above the line, and the results of transposon mutagenesis are shown below the line. Filled symbols designate transposon insertions that disrupt *VPS3* complementing activity, and open symbols represent insertions that do not affect complementation. Arrows indicate the orientation of insertions that express LacZ activity in yeast and thereby define the location and direction of translation of expressed ORFs. Round symbols denote insertions that do not express LacZ activity. *B*, Bam HI; *C*, Cla I; *E*, Eco RI; *G*, Bgl II; *H*, Hind III; *N*, Sna BI; *P*, Pst I; *S*, Sal I. (*B*) The smallest subclone that complements the *vps3-Δ1* mutation is the 3.9-Kb Bam HI–Sna BI fragment. (*C*) The 3.6-Kb Hind III–Bam HI segment was sequenced (▥). The location and direction of the Vps3p ORF are represented by the large arrow (⇨). The region used to generate rabbit anti-Vps3p antibody is designated by diagonal bars within the arrow (▨). (*D* and *E*) the LEU2 gene substitution deletion (*vps3-Δ1*) and insertion (*vps3-Δ2*) constructs, respectively.



1 AAGCTTGATCTCATTGAAAGTGCAGGTATATATAATTGTCTCCTAAAAACTCCTTACATCGCATAATATCTGTTGAAGATACCGTTCCGATGGGAAAAAAAGGC

105 AAGTTTAACTGGAAATATTCATATGTACTTACTATTTTATAACGACATTAGTGAATCAGAACTATCATAACAGTCAAGGAGACTACCTTTTTTTGGTTGCAACCATAATATTATAGAACC

225 ATGGTAAAAAAGAAAACGAATAATGACAAAGGAAAAGAAGTAAAGGAAAATGAAGGAAAGCTCGATATAGATAGCGAATCCTCACCTCACGAAAGAGAAAATGACAAAAAAAAAACAGAA
1 M V K K K T N N D K G K E V K E N E G K L D I D S E S S P H E R E N D K K K T E

345 GATGATAGTCTACGTGCAACAGAAAGTGAAGAGACGAATACACATAATGCTAACCCAAATGAAACAGTACGAGCTGATAAATTTTCCCAGGAAGAGAGTCGACCAATCGAAGACAGTCCA
41 D D S L R A T E S E E T N T H N A N P N E T V R A D K F S Q E E S R P I E D S P

465 CATACTGATAAAAACACAGCACAAGAAAGCTGTCAGCCTAGCAGCGCAGAAGATAATGTCATAAATACAGATATTACAAGCTTAAATGAAAAAACCTCCACGAATGATGAACAGGAAAAA
81 H T D K N T A Q E S C Q P S S A E D N V I N T D I T S L N E K T S T N D E Q E K

585 GGGTTGCCACTGAAAATAAGCGAAGGTCCTTTTACAATCTCTACCCTATTAGACAATGTACCATCTGACTTGATATACACATGTTGTGAGGCGTATGAGAACCACATATTCTTGGGAACA
121 G L P L K I S E G P F T I S T L L D N V P S D L I Y T C C E A Y E N H I F L G T

705 ACTACAGGTGATCTTTTACATTACTTTGAACTTGAACGTGGAAACTATATGCTTGTGTCGCAAACAAAGTTTGATGCAGAATCAAATTCGAAGATAGACAAAATCCTTCTTTTACCTAAA
161 T T G D L L H Y F E L E R G N Y M L V S Q T K F D A E S N S K I D K I L L L P K

825 GTAGAAGGCGCACTAATATTATGTGATAACGAACTAGTTTTATTTATTCTCCCAGAATTTGCACCAAGGCCCAACACAACTAGACTTAAAGGTATTAGTGATGTCGTTATTTGTAATTTT
201 V E G A L I L C D N E L V L F I L P E F A P R P N T T R L K G I S D V V I C N F

945 TCCCGAAGCTCGAAGGCTTATAGAATATATGCATTTCACGCAGAAGGCGTAAGACTACTCAAAATATCTGCAGATTCATTAGTACTTACCAAAGCTTTTAATTTCAAACTAATAGATAAA
241 S R S S K A Y R I Y A F H A E G V R L L K I S A D S L V L T K A F N F K L I D K

1065 GCTTGTGCGCACGAAGAAACATTAATGGTTTCGAAGTTAAATAGTTACGAACTTATAAATCTAAAGTCGTCACAGGTAATTCCATTATTTCGAATAAGTGAGACTGACGAAGACTTAGAA
281 A C A H E E T L M V S K L N S Y E L I N L K S S Q V I P L F R I S E T D E D L E

1185 CCTATAATAACCAGTTTCAATGAACAGAGTGAATTTTTAGTTTGTTCTGGAGGAGGATCATACGATAGTGGGGCGATGGCCTTAGTGGTAAATCATCATGGCGACATAATAAAAGGAACT
321 P I I T S F N E Q S E F L V C S G G G S Y D S G A M A L V V N H H G D I I K G T

1305 ATAGTGCTAAAAAATTATCCAAGAAATGTAATTGTTGAGTTTCCCTACATAATCGCTGAGTCTGCTTTCCAGTCGGTTGACATATATTCTGCCTTGCCGAGTGAAAAATCCCAGTTGTTG
361 I V L K N Y P R N V I V E F P Y I I A E S A F Q S V D I Y S A L P S E K S Q L L

1425 CAAAGTATAACTACTTCTGGATCGGATTTAAAAATTTCAAAATCTGACAATGTATTCACTAATACCAATAATTCTGAAGAATTCAAAGAAAAAATATTCAATAAGTTGAGACTTGAGCCT
401 Q S I T T S G S D L K I S K S D N V F T N T N N S E E F K E K I F N K L R L E P

1545 CTGACACATAGTGATAATAAATTTAGAATTGAAAGAGAACGCGCCTTTGTTGAAGAGTCTTATGAAGAAAAGACCTCTTTAATCGTTTATAATAATTTAGGGATTCATTTATTAGTTCCA
441 L T H S D N K F R I E R E R A F V E E S Y E E K T S L I V Y N N L G I H L L V P

1665 ACGCCGATGGTCTTACGTTTTACATCATGTGAAGAGTCCGAAATTGATAATATTGAAGATCAATTGAAAAAGCTTGCGAAGAAGGATTTAACAAAATTTGAGCACATTGAAGCGAAATAT
481 T P M V L R F T S C E E S E I D N I E D Q L K K L A K K D L T K F E H I E A K Y

1785 TTGATGTCTCTTTTATTGTTTCTAATGACCTTACATTACGATCACATAGAAGATGAGGTAATGAAAAAGTGGTGTGACTTTTCTGACAAAGTTGACATCAGAATATTATTTTATATGTTT
521 L M S L L L F L M T L H Y D H I E D E V M K K W C D F S D K V D I R I L F Y M F

1905 GGCTGGAAAGTCTATAGCGAGATCTGGTGCTTCCATGGGTTAATAAATATAGTTGAAAGGTTGAAAAGCTTGAAGCTAACTAACAAATGCGAGAATATTCTAAAAATGCTTCTGATGATG
561 G W K V Y S E I W C F H G L I N I V E R L K S L K L T N K C E N I L K M L L M M

2025 AAGAATGAACTAAAGAAAAAGAATAAAACTGGACTCTTAACGAATGACTTTGATGATATAATGAAAACTATTGATATAACTTTATTCAAATTAAGATTGGAAAAAAAGGAAACTATAACT
601 K N E L K K K N K T G L L T N D F D D I M K T I D I T L F K L R L E K K E T I T

2145 GTTGATATGTTTGAACGCGAAAGTTACGACGAAATTATCAGAGAGATTAATTTACATGATGATAAACTTCCTAGGATAGAGTTACTAATTGAGATATATAAAGAAAAAGGGGAATATCTT
641 V D M F E R E S Y D E I I R E I N L H D D K L P R I E L L I E I Y K E K G E Y L

2265 AAAGCACTAAATCTTTTGAGGGAAGCTGGGGATTACATTTCACTAGTTTCATTCATTGAAGAAAATCTCAAAAAACTTCCTGAGGATTATATCAAAGAAAGAATAGCCGATGATCTTCTC
681 K A L N L L R E A G D Y I S L V S F I E E N L K K L P E D Y I K E R I A D D L L

2385 CTCACTCTTAAACAAGGCGACGAAAATACTGAGGAATGCGCCATCAAAAAGGTTCTTAAAATATTGGACATGGCATGTATTAATAAAAACGACTTTTTAAACAAAATACCCGCAGAAGAA
721 L T L K Q G D E N T E E C A I K K V L K I L D M A C I N K N D F L N K I P A E E

2505 ACATCCCTGAAGGTATCATTCATAGAACAACTCGGTGTCCAAAACAGCAACGATTCCAAATTTCTCTTTAACTATTATTTGGCGAAATTGCGAGAAATAATCAACCAAAGTAACATATGG
761 T S L K V S F I E Q L G V Q N S N D S K F L F N Y Y L A K L R E I I N Q S N I W

2625 TCCATTCTTGGAGATTTTATCAAAGAGTACAAAGACGATTTCGCGTATGATAAGACAGATATAACAAATTTTATTCATATCAAATTAAAGCATAGTCTTCAGTGCGAAAATTTTTCCAAA
801 S I L G D F I K E Y K D D F A Y D K T D I T N F I H I K L K H S L Q C E N F S K

2745 TATTATGAAAAATGTGAAAACTTGAAATCGGAAAATGAAAAAGATGATGAGTTTATTAATTTCACCTTCGACGAAATTTCCAAGATCGATAAAGAACACATATTGACCTTGTTATTTTTC
841 Y Y E K C E N L K S E N E K D D E F I N F T F D E I S K I D K E H I L T L L F F

2865 CCCAATGAATTAACAAACTGGGTTTCTTCCGAAGAATTGCTGAAAATATATTTGTCATTTAATGATTTCAGAAGTGTAGAAAAATACATAGGCAAGCAGAACTTGGTTGCGGTGATGAAA
881 P N E L T N W V S S E E L L K I Y L S F N D F R S V E K Y I G K Q N L V A V M K

2985 CAATACTTGGACATATCTTCTTTGAATTACTCAGTTGAATTAGTGACTAATTTGCTACAGAGAAATTTTGAACTTTTAGATGATACAGATATTCAATTAAAGATCCTCGAAACTATACCT
921 Q Y L D I S S L N Y S V E L V T N L L Q R N F E L L D D T D I Q L K I L E T I P

3105 TCCGTATTTCCCGTACAAACAATATCCGAATTGCTTTTAAAGGTACTTATTAAGTATCAAGAAAAGAAGGAGGAATCTAATTTGCGGAAATGTTTGTTGAAAAATCAAATATCCATTTCT
961 S V F P V Q T I S E L L L K V L I K Y Q E K K E E S N L R K C L L K N Q I S I S

3225 GACGAACTAAGCCGGAATTTTGATAGTCAAGGATAAGAAGAGCCCACCTATTCTTTTTCTGTTCAGGCATACAATTCAAATTTTATACGTACTAGATATTTACAGGTATGTAAGAATGAA
1001 D E L S R N F D S Q G *

3345 ATAACGATGTTTGATTTACTGTTGTAATTCAGAAATTGGTTTCATTCCAGAACAAACATCATATACCAATTTCTTTTCCTCGGAGCCCATAACAACAGATTGTAATGATAACCTCACAAC

3465 TTGACTATTTTGTACGAGCTTACTCACAGTATCTTTAGATTTCGTCAGTTTTTCAAAATCTTTTTTCACATATGGTTTTAAGAGTTGATATTCTCCTGTTACTTGCCTATGAAAATCTTC

3585 TGGGTCTTCTTCAATAAGTAAATTCTGCAATTGAGGCTCTTTGGCAAAAATCAAGGAGGATCC

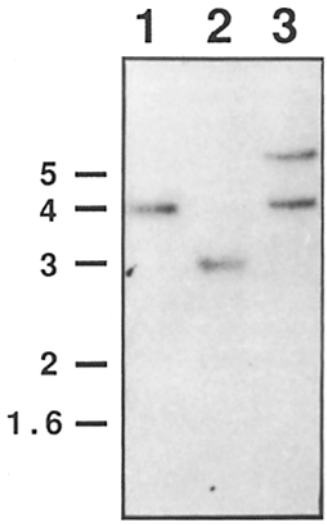


Figure 4. Southern analysis of *VPS3*, *vps3-Δ1*, and *vps3^{ts}* strains. Genomic DNA from isogenic strains SF838-1D (lane *1*), SF838-1D *vps3-Δ1* (lane *2*), and SF838-1D *vps3^{ts}* (lane *3*) was digested with Bam HI, separated by electrophoresis, blotted, and probed with a radiolabeled 1.1-kb Bam HI–Sal I fragment from the 5′ end of the *VPS3* gene. The *vps3^{ts}* strain carries a tandem duplication of the *VPS3* gene separated by the YIp5 integrating plasmid. One copy is the *vps3-Δ2* insertion allele (the upper, 6.3-kb band) and one copy is the *vps3^{ts}* allele (the lower, 4.2-kb band), which migrates with the wild-type *VPS3* gene. Strains of the same genotypes in a JHRY20-2C genetic background gave identical patterns of hybridizing fragments as those shown.

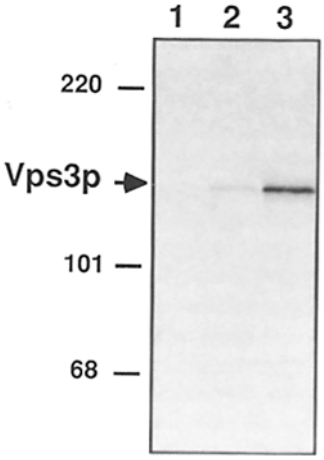


Figure 5. Identification of the Vps3p protein. 50 μg of total protein extracts from isogenic *vps3-Δ1* (lane *1*), wild-type (lane 2), and Vps3p overproducing (lane *3*) strains were electrophoresed on an 8% SDS-PAGE gel and blotted onto nitrocellulose. The blots were probed with affinity-purified rabbit anti-Vps3p antibody followed by alkaline phosphatase–conjugated goat anti-rabbit IgG antibody. All strains were in an SF838-1D genetic background.

52 nucleotides 3′ of the stop codon (designated *vps3-Δ1*). A fragment containing the gene substitution was used to construct heterozygous *VPS3/vps3-Δ1* diploids by single step gene replacement into diploid strain CRY1 (Rothstein, 1983; Table I). Three such diploids were used for tetrad analysis. Approximately 80% of the tetrads dissected yielded four viable spores. The 48 complete tetrads analyzed invariably grew into two large and two small colonies which were Leu$^-$Vps$^+$ and Leu$^+$Vps$^-$, respectively. From this analysis we conclude that while the *VPS3* gene does appear to be required for wild-type growth rates, it is not an essential gene. The gene substitution construct was subsequently used to delete the *VPS3* gene from haploid strains (SF838-1D, JHRY20-2C; Table I). Southern blots of Bam HI–digested genomic DNA from a wild-type haploid and an isogenic *vps3-Δ1* strain were probed with a labeled fragment from a region 5′ of the *VPS3* gene (Fig. 4). This blot and others not shown using different restriction enzymes confirmed that legitimate integration of the *vps3-Δ1* fragment had taken place at the *VPS3* locus. Southern blot analysis of the other strains used in these studies verified that all manipulations of the *VPS3* locus yielded the predicted hybridizing genomic restriction fragments (data not shown).

Haploid *vps3-Δ1* mutants exhibited growth characteristics different than wild-type cells. The doubling time of *vps3-Δ1* cells during log phase growth in YEPD broth at 30°C was 1.5 times that of isogenic wild-type strains. In synthetic media, the difference in doubling rates increased to greater than twofold. Furthermore, the *vps3-Δ1* mutants grew poorly at high temperatures, forming minute colonies on YEPD plates incubated at 37°C for several days. The mutant strains were also sensitive to low pH. While wild-type cells formed colonies rapidly on YEPD plates adjusted to pH 3.0, only marginal growth of the mutant was observed. These data imply that the Vps3p facilities the growth of wild-type cells under a wide range of conditions. The CPY sorting defect in *vps3-Δ1* strains was comparable to the sorting defect of other *vps3* mutants reported previously (Rothman and Stevens, 1986). The sorting of the vacuolar membrane proteins dipeptidyl aminopeptidase B (DPAP B; Roberts et al., 1989) and alkaline phosphatase (Klionsky and Emr, 1989) to the vacuole appears to be normal in *vps3-Δ1* cells. Neither protein is found on the cell surface (data not shown). The vacuolar acidification defect in *vps3-Δ1* mutants was described in an earlier report (Rothman et al., 1989*c*).

A disruption of the *VPS3* gene by an insertion of the *LEU2* gene was also constructed (designated *vps3-Δ2*; Fig. 2 *E*). However, the NH_2-terminal 264 codons of the*VPS3* gene remain intact in this construct. While the CPY missorting defect of the *vps3-Δ2* insertion mutant is as severe as the *vps3-Δ1* deletion mutant (data not shown), the growth rate of the insertion mutant is comparable to wild-type cells. Two phenotypic characteristics of *vps3* mutants, defective acidification of the vacuole (Rothman et al., 1989*c*) and peculiar vacuolar morphology, (see below) were observed in both the deletion and the insertion mutants, though perhaps to a lesser extent in the latter (data not shown). Thus, it is possible that expression of the NH_2-terminal 264 amino acids of the *VPS3* gene is sufficient to encode some Vps3p function. Alternatively, the *vps3-Δ1* deletion, which extends slightly outside of the Vps3 ORF, may interfere with the expression of an adjacent gene thus resulting in a more severe phenotype. This possibility seems unlikely since the 3.9-kb Bam HI–Sna BI fragment shown in Fig. 2 *B* completely complemented all of the vacuolar defects in *vps3-Δ1* mutants, yet it contained very little DNA flanking the *VPS3* gene. In addition, several *vps3* alleles isolated from a collection of spontaneous Vps$^-$ mutants had phenotypic characteristics seemingly identical to the deletion mutant (data not shown).

Figure 3. Nucleotide and amino acid sequence of the *VPS3* gene. A 3.6-kb region defined by Hind III and Bam HI sites was sequenced as described in Materials and Methods. The predicted amino acid sequence of the Vps3p protein is depicted in the one letter code. These sequence data are available from EMBL/GenBank/DDBJ under accession number M33314.

The VPS3 Gene Encodes a Nonabundant Protein

The protein product of the *VPS3* gene, Vps3p, was detected using antibodies directed against a portion of the protein. Roughly 1 kb of the *VPS3* ORF (Fig. 2 *C*) was expressed in *Escherichia coli* as a hybrid protein which was then purified and used to raise antisera in rabbits; Vps3p-specific antibodies were subsequently affinity purified. A protein of ~140 kD was recognized in immunoblots of wild-type extracts (Fig. 5). This protein was absent in *vps3-Δ1* extracts and increased in abundance in extracts of wild-type cells that carried the *VPS3* gene on a multicopy plasmid (pVPS3). These data established that the 140-kD protein was Vps3p. This and other blots suggested that Vps3p was expressed at low levels in wild-type cells. To examine this further, Vps3p and CPY were immunoprecipitated from $H_2{}^{35}SO_4$–radiolabeled wild-type cell extracts, and the counts in each were quantified. Given that CPY constitutes roughly 0.1% of total cell protein (T. H. Stevens, unpublished observations), that the counts associated with Vps3p were slightly <5% of the counts in CPY, and adjusting for the fact that there are about three times more sulfur atoms in Vps3p than in CPY, we estimate that Vps3p constitutes 0.001% of total cell protein. In subcellular fractionation experiments, roughly 50% of the Vps3p was sedimentable when osmotically lysed spheroplasts were centrifuged at 10,000 *g*; under these same conditions, a soluble cytoplasmic protein (phosphoglycerate kinase) was found exclusively in the supernatant and a membrane protein (DPAP B) sedimented quantitatively (data not shown). Vps3p was not detected in samples of purified vacuolar membranes. Indirect immunofluorescence using Vps3p antibody in wild-type cells failed to produce a specific signal; however, the Vps3p product was readily detected in Vps3p overproducing cells as a cytoplasmically diffuse, nuclear and vacuole-excluded signal (data not shown). While the possibility that Vps3p resides in a distinct subcellular compartment cannot be rigorously excluded, the hydrophilic nature of the putative Vps3p amino acid sequence taken together with the fractionation and immunofluorescence data are most consistent with an interpretation that Vps3p is a cytoplasmic protein which forms stable associations with intracellular membranes, cytoskeleton, or some other sedimentable subcellular structure.

A vps3 Temperature Conditional Mutant Rapidly Loses the Capacity to Sort CPY at the Nonpermissive Temperature

Mutations were generated in the *VPS3* gene by in vitro mutagenesis and a temperature conditional allele of the gene was isolated. The *vps3ts* mutant allele was integrated into a *vps3-Δ2* disruption strain by tandem integration (JHRY20-2C *vps3-Δ2*; Table I). Southern blots demonstrated that a single copy of the *vps3ts* construct had integrated at the *VPS3* locus (Fig. 4 and data not shown). Yeast strains carrying this mutant allele were viable at both the permissive and nonpermissive temperatures, but they were conditionally defective for the sorting of CPY. Upon shift from the permissive to the nonpermissive temperature, it was possible to monitor the time required for the CPY missorting defect to occur. We reasoned that if the appearance of the sorting defect was rapid, occurring in only a fraction of a cell generation, it would suggest that the *VPS3* gene encodes a function that is in some way intimately required for vacuolar protein sorting. If the sorting defect took more than one cell generation to become apparent, this would imply that the function of the gene might be more distantly removed from the actual process of protein sorting.

Sorting of newly synthesized CPY was examined in the *vps3ts* mutant and the isogenic wild-type strain. They were initially grown, pulsed with $H_2{}^{35}SO_4$, and chased entirely at the permissive (30°C) or the nonpermissive temperature (37°C). The wild-type strain secreted <10% of the protein at either growth temperature (data not shown). By contrast, the temperature-sensitive mutant secreted 82% of the total labeled CPY at 37°C, while only 17% was extracellular at

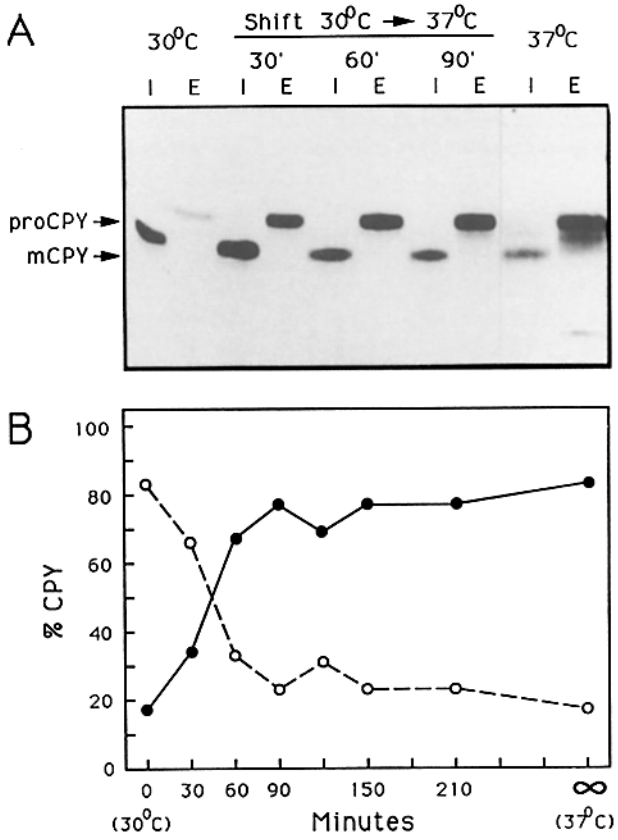


Figure 6. A temperature conditional *vps3ts* mutant rapidly loses the capacity to sort CPY upon shift to the nonpermissive temperature. (*A*) Strain JHRY20-2C *vps3ts* was grown at 30°C and aliquots were shifted to 37°C at various times. Shifted cells were labeled at 37°C for 30 min with $H_2{}^{35}SO_4$ and chased at 37°C for 30 min as described in Materials and Methods. CPY was immunoprecipitated from intracellular (*I*) and extracellular (*E*) fractions, and the immunoprecipitates were analyzed on 8% SDS-PAGE gels followed by fluorography. The 30°C sample was from cultures maintained at 30°C throughout the growth, pulse, and chase procedure. The 37°C sample was from a culture grown for several generations at 37°C before a pulse and chase at 37°C. The time of the shift was computed as the sum of the preshift time plus the 30-min pulse. (*B*) Quantitation of CPY in intracellular (○) and extracellular (●) fractions as a function of time (min). The amount of CPY was determined by direct counting of CPY-associated ^{35}S in each fraction (see Materials and Methods). The 0-min point was from cells treated exclusively at 30°C, and the ∞ symbol represents cells treated exclusively at 37°C.

30°C (Fig. 6). The intracellular CPY was in the mature form while the extracellular CPY was in the unprocessed proCPY form (Fig. 6 *a*), demonstrating that the extracellular CPY had been secreted rather than released by cell lysis (Rothman and Stevens, 1986). Thus, there was a pronounced difference in the CPY sorting capacity of the *vps3*^{ts} mutant at the two different growth temperatures.

The *vps3*^{ts} mutant rapidly lost the ability to sort CPY upon shift to the nonpermissive temperature. We defined time zero in this experiment as the time when the chase period was initiated after labeling. Accordingly, a culture that was shifted to 37°C for 30 min before the 30-min pulse, 30-min chase regimen was defined as having been at the nonpermissive temperature for 1 h and so on. This was a reasonable definition since newly synthesized CPY reaches its ultimate destination with a half time of ~5 min (Hasilik and Tanner, 1978). Therefore, by the time the chase was begun most of the labeled CPY had already been routed to its final destination. By these criteria the defect in sorting upon shift to the nonpermissive temperature was evident at the earliest time points and was essentially complete after a 90-min shift (Fig. 6 *b*). The doubling time of the *vps3*^{ts} mutant under these conditions was roughly 4 h. Thus, the onset of the CPY missorting defect occurred in much less than the *vps3*^{ts} cell generation time.

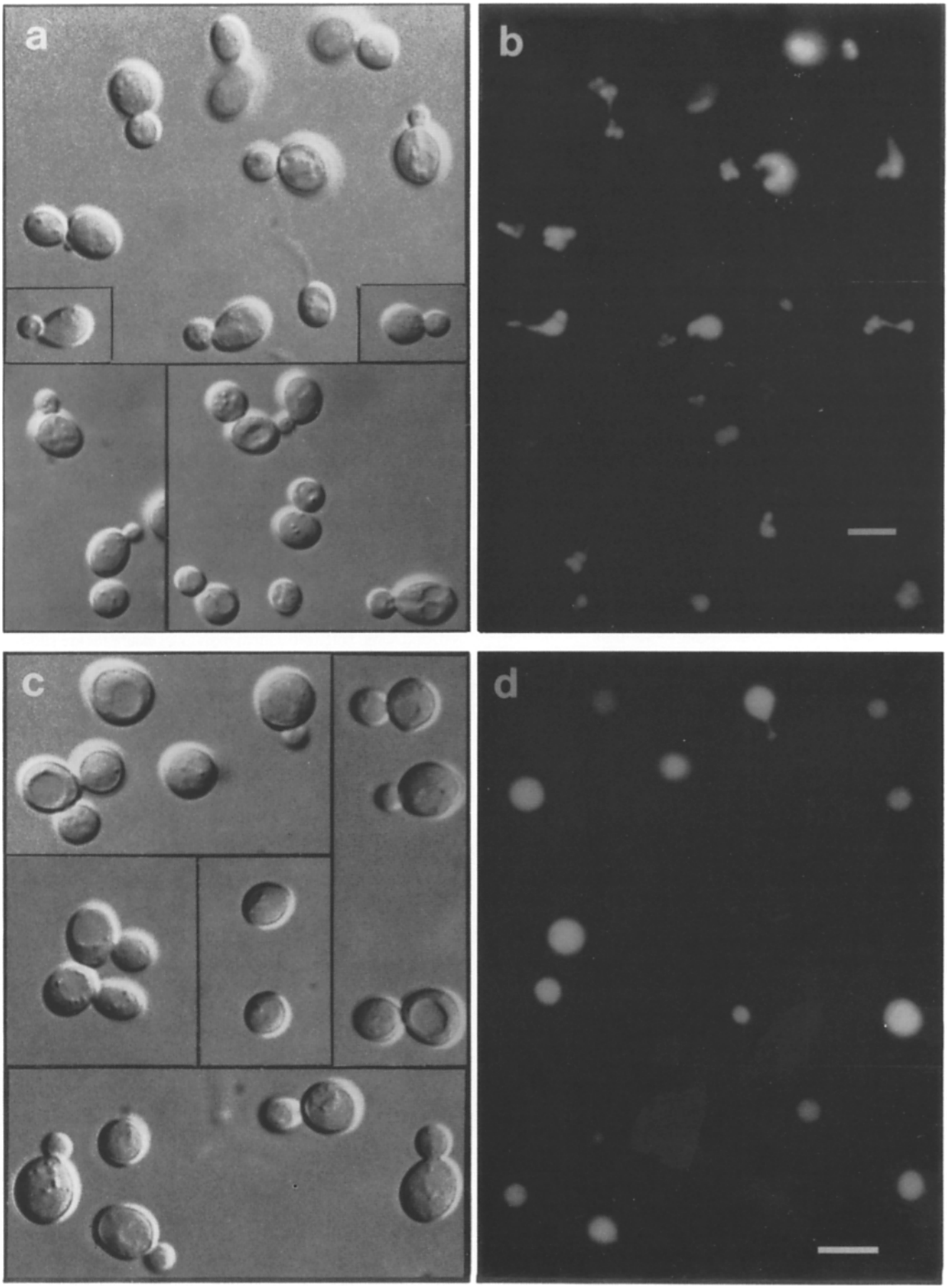
The Vacuolar Morphology in vps3-Δ1 Cells Is Aberrant

Given that *vps3-Δ1* mutants missort soluble vacuolar proteins and fail to acidify their vacuoles, we were interested to know whether the assembly and/or morphology of vacuoles were also perturbed in the mutant strains. To perform this study, vacuolar morphology was characterized in asynchronous cultures of wild-type cells (SF838-1D, JHRY20-2C) and isogenic *vps3-Δ1* mutants and then compared. Two techniques were used to label vacuolar structures with fluorescent probes for subsequent examination by fluorescence microscopy. Vacuoles were stained in live cells using the vacuolar vital stain CDCFDA (Fig. 7); this nonfluorescent compound is hydrolyzed in yeast cells to the fluorescent molecule 2',7'carboxyfluorescein, which accumulates specifically in the lumen of the vacuole (Pringle et al., 1990). This vital stain was particularly useful because it allowed cells to be examined in the same growth medium in which they were labeled. Indirect immunofluorescence was also used to view vacuolar membranes in fixed cells (Fig. 8). Existing immunofluorescence procedures (Kilmartin and Adams, 1984; Pringle et al., 1990) were modified in order to achieve an optimal combination of morphological preservation and satisfactory antibody penetration. Cells were fixed for longer periods of time, and treatment with an ionic detergent was used (Roberts et al., 1990). Vacuolar membranes were identified with antibodies that recognize alkaline phosphatase (Pho8p), a vacuolar membrane protein encoded by the *PHO8* gene (Kaneko et al., 1987; Klionsky and Emr, 1989).

The vacuoles in wild-type cells generally appeared as irregularly shaped, morphologically complex organelles composed of several small, membrane-enclosed compartments (Figs. 7, *a* and *b*; 8, *a* and *c*). These structures were usually clustered in one region of the cell, and it was not possible to discern whether these compartments were physically separated by cytoplasm or joined by interconnecting membrane channels. Photographs fail to record the entire extent of vacuolar material in cells because they are limited to a single focal plane. The morphology of the yeast vacuole was significantly influenced by environmental factors. When yeast cells were exposed to an energy poison, deprived of a carbon source, or merely centrifuged, it was observed that vacuoles tended to appear as single, spherical structures (Pringle et al., 1990; Makarow and Nevalainen, 1987; our unpublished results). Throughout this study, cultures were minimally perturbed before observation or fixation, and the combined approaches of vital staining and immunofluorescence provided essentially the same view of vacuolar structures. In addition, both wild-type strains gave similar results. Our results are also consistent with other published observations on vacuolar morphology (Wiemken et al., 1970; Pringle et al., 1990; Preston et al., 1989).

Microscopy allowed examination of individual cells at various stages in the cell cycle, and thus it was possible to reconstruct probable changes in vacuolar morphology as wild-type yeast cells bud and divide. The most surprising discovery, revealed clearly by both microscopic techniques, concerns the segregation of vacuolar material into growing buds in a certain phase of the cell cycle. Shortly after bud emergence, through a later stage when the nucleus migrates to the neck of the bud and nuclear division begins, there was a high probability of observing continuous vacuolar structures extending from the mother cell through the bud neck and into the bud (Figs. 7 *b* and 8 *c*, Table II). These mother cell to bud vacuolar networks were designated as "segregation structures." The morphology of these segregation structures varied, and some fairly prominent individual examples are shown. In other cases, the structure appeared as a thin line or a string of smaller round compartments which extended from vacuoles in the mother cell into the bud. Vacuolar segregation structures were seen in both wild-type strains used in this study as well as in all wild-type strains we have examined thus far. Quantitation of vacuolar morphology as a function of bud enlargement using indirect immunofluorescence is shown in Table II. While apparent segregation structures were observed during all phases of bud growth, they were observed most frequently in cells where the ratio of bud diameter to mother cell diameter ranged from 0.3 to 0.6 (77% of these cells had segregation structures). Mitochondrial segregation, monitored by the appearance of mitochondrial DNA in the bud, appears to occur in the same portion of the cell cycle, perhaps lagging slightly behind vacuolar segregation (Table II). However, since the mitochondrial organelle extends beyond the mitochondrial DNA (Pringle et al., 1990), the number of buds containing mitochondria must be regarded as a minimum estimate. The vacuoles in mother cells and buds rarely appeared connected in cells where mitotic events had commenced, suggesting that the initiation of nuclear division marks the end of the vacuolar segregation process (Fig. 8, *b* and *c*; Table II). Mitotic cells were defined as those large budded cells in which the nucleus had migrated into the neck of the bud, the nuclei were dividing, or there were separate nuclei in the mother and the bud. Of 72 mitotic cells quantified, only 13 (18%) had apparent segregation structures; only 4 of 45 cells undergoing or beyond the stage of mitosis and 0 of 26 cells with separate nuclei in the mother and bud had apparent segregation structures.

When the vacuoles in *vps3-Δ1* cells were examined by the


a
b
c
d

same procedures, three significant differences from the vacuoles in the isogenic wild-type cells were observed. First, there was a difference in vacuolar morphology. Whereas wild-type vacuoles assume a variety of convoluted shapes, the vacuoles in *vps3-Δ1* cells, if present, were almost always nearly perfect spheres (Fig. 7, *c* and *d*, Fig. 8, *d* and *f*). While the relative volume occupied by vacuoles in wild-type cells appeared more or less uniform, the size of the vacuoles in *vps3-Δ1* cells varied. In some *vps3-Δ1* cells the vacuole seemed to occupy almost the entire cell, while in other cells the vacuoles were a small fraction of the total cell volume. Live mutant cells were double labeled with CDCFDA and calcofluor. Calcofluor stained bud scars and thus allowed determination of cell "age", that is, the number of budding cycles a mother cell has been through (Pringle et al., 1990). The ratio of vacuole diameter to cell diameter was measured in 40 cells with only one bud scar and in 40 cells that had at least three visible bud scars. In going from the former class of cells to the latter class of cells, the average vacuole diameter to cell diameter ratio increased from 0.42 to 0.66 and the average cell size increased slightly from 1.3 to 1.5 arbitrary units. These data may have interesting implications (see Discussion). Unfortunately, the nonuniform vacuolar morphology in wild-type cells precluded a similar comparative study.

The second unique characteristic of vacuoles in *vps3-Δ1* mutants was the paucity of vacuolar material in developing buds and the virtual absence of vacuolar segregation structures in these cells (Figs. 7 *d* and 8 *f*, Table III). In wild-type cultures, vacuoles were observed in 99% of the buds of cells where the ratio of the bud to mother cell diameter exceeds 0.6 (Figs. 7 *b* and 8 *c*, Table II). Only 26% of the mutant buds in this stage of bud enlargement had detectable vacuoles (Table III). Where discernable vacuoles were present in *vps3-Δ1* buds, they were very small. Examples of these minute bud vacuoles are visible in Figs. 7 *d* and 8 *f*. With regard to vacuolar segregation structures in *vps3-Δ1* mutants, quantitation of immunofluorescence experiments revealed these structures in only 7% of the small-budded cells where the ratio of bud to mother cell diameter was 0.3–0.6 (Table III); by contrast, 77% of wild-type cells in this stage of bud growth had segregation structures. Even when present, the segregation structures in the mutant were less prominent than those observed in wild-type cells (Fig. 7 *d*). Mitochondrial segregation and nuclear division into the buds of *vps3-Δ1* cells appears to proceed normally (Table III), although the latter may be partially delayed with respect to bud growth. This suggests that the defect in these mutants is specific to vacuolar segregation.

The third significant difference between the vacuoles in wild-type cells and *vps3-Δ1* cells was that not all *vps3-Δ1* cells had detectable vacuoles. Vital staining revealed that in ~15–30% of the *vps3-Δ1* cells, CDCFDA failed to label a specific subcellular structure (Fig. 7 *d*, Table IV). Double labeling with CDCFDA and calcofluor revealed that the majority of mutant cells lacking apparent vacuoles were daughter cells (which lack bud scars; Table IV). The percentage of CDCFDA-labeled daughter cells in SF838-1D *vps3-Δ1* cultures (41%) and JHRY20-2C *vps3-Δ1* cultures (67%) was reproducibly different, suggesting that genetic background can influence the extent of the vacuole deficient phenotype in *vps3-Δ1* mutants. By contrast, almost all mother cells in the mutant populations, and comparable percentages of all cells in the wild-type populations of cells possessed detectable vacuoles. Thus, vacuolar deficiencies were specific to *vps3-Δ1* daughter cells. These vacuole deficient cells were likely to be live cells; when fresh cultures of wild-type cells or *vps3-Δ1* mutants from either strain were plated and examined several hours later, >90% of the plated cells had formed micro-colonies (data not shown). Significant differences in the percentage of colony forming units between wild-type and mutant cells were not detected. Furthermore, the percentage of mother cells versus daughter cells in the wild-type and mutant strains was roughly the same (Table IV). If a large number of daughter cells were inviable, they would be expected to constitute a greater proportion of the overall cell population. Immunofluorescence experiments yielded similar results. Those *vps3-Δ1* cells that had visible vacuoles also had prominent labeling of the organelle by alkaline phosphatase antibody (Fig. 8 *f*). Once again, however, there was a subclass of cells that was deficient in vacuolar structures when viewed by Nomarski optics (Fig. 8 *d*). These same cells had a variety of labeling patterns. Some did not label, others had punctate labeling throughout the cell, some had specific labeling of a very small organelle, and some had a combination of specific and punctate labeling (Fig. 8 *f*; data not shown). A plausible explanation is that cells which had diffuse alkaline phosphatase staining were daughter cells in which nascent vacuolar material had failed to assemble into a coherent structure. However, it is not possible to differentiate between mother and daughter cells in immunofluorescence experiments since bud scars are lost when cells are spheroplasted (Pringle et al., 1990).

The vacuolar segregation defect in *vps3-Δ1* cells is consistent with the observation that many daughter cells do not appear to possess coherent vacuoles. The fact that nearly all *vps3-Δ1* mother cells have vacuoles suggests that vacuole assembly does occur in daughter cells that go on to divide, and this assembly event must occur during an early portion of the cell cycle. Incidentally, vacuolar assembly does not appear to be a prerequisite to budding since 5% of the fixed budding cells quantified in Table III did not appear to have a coherent vacuole but rather had punctate labeling throughout the cell characteristic of many unbudded mutant cells. Given that vacuolar assembly does proceed, the question arises as to whether these vacuoles are assembled from material contributed by the mother cell or whether the vacuoles are assembled by de novo synthesis. Previous reports have demonstrated that the endogenous, fluorescent pigment produced in *ade2* yeast strains accumulates in vacuoles and is segregated

Figure 7. Vacuolar morphology in live cells of wild-type and *vps3-Δ1* strains. Vacuoles in living cells were stained with the vacuolar vital stain CDCFDA, and the stained cells were photographed by Nomarski optics (*a* and *c*) and by epifluorescence through an FITC filter set (*b* and *d*). The wild-type strain was JHR20-2C (*a* and *b*). The *vps3-Δ1* strain was JHRY20-2C *vps3-Δ1* (*c* and *d*). Bar, 5 μm.

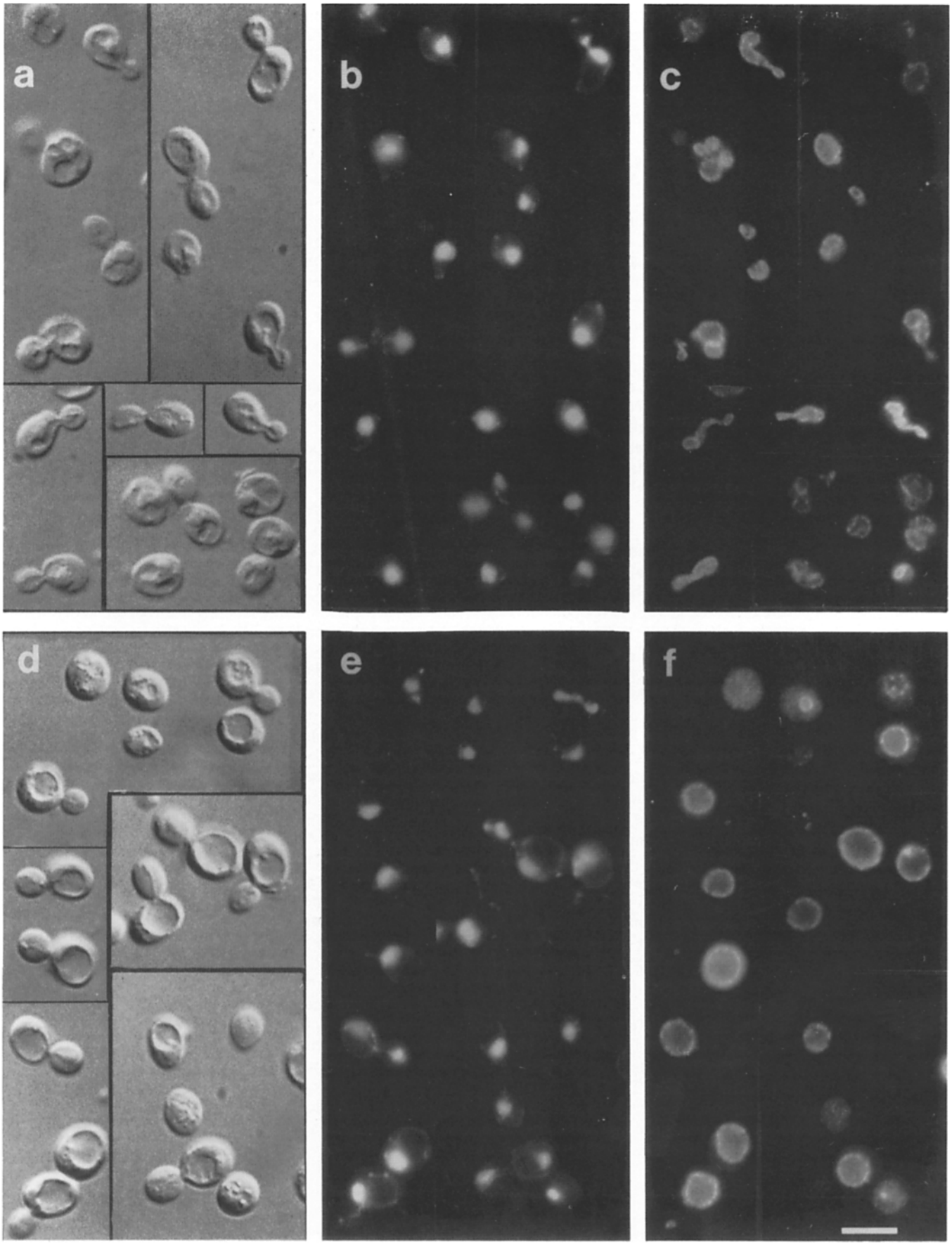

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Table II. Segregation of Organelles into Buds in Wild-type Cells

Bud/ mother cell*	Vacuoles‡			Nuclei§		Mitochondria‖	
	NV	S	VB	PM	M	NB	B
0–0.19	27	1	0	28	0	27	1
0.20–0.29	19	8	0	27	0	24	3
0.30–0.39	10	18	1	29	0	22	7
0.40–0.49	4	36	3	42	1	20	23
0.50–0.59	1	38	9	40	8	10	38
0.60–0.69	0	16	30	14	32	1	45
0.70–1.00	1	6	25	1	31	0	32

Wild-type strains were fixed and labeled by indirect immunofluorescence as described in Materials and Methods. 125 cells of SF838-1D and 125 cells of JHRY20-2C were quantified, and the sum of these data is shown. The numbers represent the number of cells scored in each category (see below).

* The ratio of bud diameter to mother cell diameter.

‡ Vacuoles were scored by alkaline phosphatase indirect immunofluorescence. *NV*, No vacuole detected in the bud; *S*, vacuolar segregation structure extending into the bud; *VB*, independent vacuoles in the mother cell and the bud.

§ Nuclei were scored by DAPI staining. *PM*, Premitotic nuclei, defined by apparent position in the mid-body of the mother cell; *M*, mitotic nuclei, defined by apparent migration to the neck of the bud, mitosis, or completion of nuclear division.

‖ Mitochondria were scored by DAPI staining of mitochondrial DNA. *NB*, No mitochondrial DNA detected in the bud; *B*, mitochondrial DNA detected in bud.

Table III. Segregation of Organelles into Buds in vps3-Δ1 Cells

Bud/ mother cell*	Vacuoles‡			Nuclei§		Mitochondria‖	
	NV	S	VB	PM	M	NB	B
0–0.19	23	1	0	24	0	24	0
0.20–0.29	29	0	0	29	0	24	5
0.30–0.39	25	3	1	29	0	15	4
0.40–0.49	35	2	5	41	1	10	32
0.50–0.59	32	3	4	31	8	5	34
0.60–0.69	38	0	13	25	26	2	49
0.70–1.00	26	0	10	6	30	2	34

*‡§‖ See footnotes for Table II. 125 cells of SF838-1D *vps3-Δ1* and 125 cells of JHRY20-2C *vps3-Δ1* were quantified, and the sum of these data is shown. The numbers represent the number of cells scored in each category.

to the vacuoles of progeny cells after synthesis of the fluorescent compound has been suppressed (Weisman et al., 1987). The vacuolar segregation of *ade2* pigment was examined in the *ade2* yeast strain SEY6211 (Table I) and an isogenic *vps3-Δ1* mutant. After growth of these strains in limiting adenine, which induced labeling of the vacuoles with *ade2* fluorophore, the cells were diluted into fresh media containing excess adenine, thereby suppressing *ade2* fluorophore synthesis, and they were incubated for 4 h. The cells were then labeled with CDCFDA and examined by fluorescence microscopy. Large buds in wild-type cells or *vps3-Δ1* mutants that contained CDCFDA-labeled vacuoles were scored for the simultaneous presence of *ade2* fluorophore in the same compartment. Of 119 wild-type buds investigated, 111 (93%) were also labeled with *ade2* fluorophore. By contrast, only 14 of 114 (12%) of the rare *vps3-Δ1* buds that had discernable vacuoles by CDCFDA labeling also had detectable labeling with *ade2* pigment. Since the vacuoles in *vps3-Δ1* buds are often quite small, a control for the efficiency of vacuolar labeling by *ade2* fluorophore was to conduct the same experiment on *vp3-Δ1* cells grown continuously under limiting adenine conditions. Greater than 90% of the large buds containing CDCFDA-labeled vacuoles also contained *ade2* fluorophore under these conditions. These data suggest that the mother cell contributes very little if any vacuolar material to many of the vacuoles which arise in the buds and daughter cells of *vps3-Δ1* mutants.

Discussion

Vacuolar protein sorting and vacuolar biogenesis in yeast is a complex problem. Characterization of genes that effect these processes will help unravel some of the complexity of these phenomena. A negative selection against *vps* mutants was devised which facilitates the cloning of *VPS* genes from a yeast genomic library. This selection was used to clone the *VPS3* gene as well as several other *VPS* genes (Raymond, C. K., unpublished observations). Sequencing of *VPS3* revealed an ORF with the potential to encode a hydrophilic protein with a predicted molecular mass of 117 kD. Antibodies were used to demonstrate that the *VPS3* gene product, Vps3p, is expressed as a low abundance protein with an apparent molecular mass of ~140 kD by gel electrophoresis. The discrepancy between the predicted and apparent molecular weights is not uncommon (Sorger and Pelham, 1987; Novick et al., 1989), and the possibility of posttranslational modifications has not been pursued. Immunofluorescence and subcellular fractionation experiments suggest that Vps3p is neither a nuclear nor a vacuole-associated protein, and the data support the interpretation that Vps3p is a cytoplasmic protein that stably binds to, and may function in association with, intracellular membranes or cytoskeletal elements.

Mutations in the *VPS3* gene were constructed in vitro and introduced into the yeast genome. Haploid cells that suffered a complete deletion of the *VPS3* gene were viable, somewhat slow-growing cells with many characteristics in common with spontaneous mutants of *vps3*. Strains bearing an insertion mutation partway through the *VPS3* gene had some phenotypic characteristics intermediate between wild-type cells and the complete deletion mutant. One hypothesis is that the truncated gene retains partial function.

vps3 mutants are defective in vacuolar protein sorting, vacuolar acidification, and processing of the zymogen form of PrA to its mature form (Rothman and Stevens, 1986; Roth-

Figure 8. Vacuolar membrane morphology in fixed cells of wild-type and *vps3-Δ1* strains. Vacuolar membranes in fixed, spheroplasted, and SDS-treated cells were stained by indirect immunofluorescence with affinity-purified and adsorbed rabbit antiyeast alkaline phosphatase antibodies as described in Materials and Methods. Cells were photographed through Nomarski optics (*a* and *d*), nuclei and mitochondrial DNA were visualized by DAPI staining (*b* and *e*), and alkaline phosphatase immunofluorescence was viewed through an FITC filter set (*c* and *f*). The wild-type strain was SF838-1D (*a–c*) and the *vps3-Δ1* strain was SF838-1D *vps3-Δ1* (*d–f*). Bar, 5 μm.

Table IV. Percentage of Mother Cells and Daughter Cells with Vacuoles in Wild-type and vps3-Δ1 Strains

Strain	% Mother cells	% Daughter cells	% CDCFDA-labeled mother cells	% CDCFDA-labeled daughter cells
SF838-1D	55	45	94	97
SF838-1D *vps3-Δ1*	54	46	93	41
JHRY20-2C	52	48	100	95
JHRY20-2C *vps3-Δ1*	52	48	95	67

The indicated strains were double labeled with CDCFDA and calcofluor as described in Materials and Methods. The percentages of mother cells and daughter cells were determined by counting the number of cells with bud scars in a total population of at least 110 cells chosen at random. The percentages of cells with CDCFDA-labeled vacuoles were determined from a population of at least 120 mother cells or 375 daughter cells for each of the strains listed above.

man et al., 1989*c*). The latter two phenotypes were unique to 2 of 19 *vps* complementation groups examined. The specific activity of the vacuolar H^+-ATPase was greatly reduced in vacuoles purified from *vps3* strains, and the acidification defect was proposed to be the cause of the missorting and processing phenotypes. However, this does not appear to be the case. The gene encoding a major subunit of the vacuolar H^+-ATPase, the 60-kD subunit, was cloned in this laboratory, and the gene (*VAT2*) was deleted from yeast strains (Rothman et al., 1989*b*; Yamashiro et al., 1990). The vacuoles in these *vat2-Δ1* strains were no longer acidified, yet vacuolar zymogen activation was normal, protein sorting was only slightly perturbed, and vacuolar morphology was very similar to that of wild-type cells. These data indicate that the acidification defect in *vps3* mutants is not the primary cause of the mutant phenotypes.

Studies with a temperature conditional allele of *VPS3* suggest that Vps3p may be directly involved in protein sorting. Vacuolar protein sorting is nearly normal at the permissive temperature, but *vps3*^{ts} cells rapidly lose the capacity to sort CPY upon shift to the nonpermissive temperature. Three other temperature-sensitive *vps* mutants (the mutations were in *VPS* genes other than *VPS3*) were characterized in this manner, and all of them required several cell generations at the nonpermissive temperature before the sorting defect became evident (Rothman et al., 1989*a*; Rothman, 1988). These data are consistent with a model in which certain gene products, such as Vps3p, participate directly in protein sorting while other gene products influence sorting through less direct mechanisms. The *vps3*^{ts} mutant will also be useful in determining whether Vps3p plays a role in the maintenance as well as in the assembly of vacuolar structures. Using temperature shift experiments, it should be possible to determine if preexisting vacuolar H^+-ATPase complexes and/or wild-type vacuolar morphology are stable in the absence of Vps3p function.

The two independent microscopic techniques used to examine vacuoles in wild-type and *vps3-Δ1* cells yielded consistent results. The organelle in wild-type cells generally appeared to be composed of several, irregularly shaped vacuolar compartments. Other studies on vacuolar morphology in wild-type yeast cells are consistent with the observations presented here (Wiemken et al., 1970; Pringle et al., 1990; Preston et al., 1989), and these data appear different than the previously reported finding that yeast cells possess single, major vacuoles throughout the cell cycle (Weisman et al., 1987). The differences may be due to the observation that the morphology of the vacuole is easily perturbed, especially by energy deprivation, and under these conditions it readily changes into a single, spherical organelle (Pringle et al., 1990; Raymond, C. K., unpublished observations). It is tempting to speculate that the vacuole maintains its unusual, convoluted morphology in growing cells through attachment to cytoskeletal elements, most notably actin. The same conditions that cause collapse of the actin network in yeast cells (Pringle et al., 1990; Novick et al., 1989) also lead to rearrangement of the vacuole into a spherical organelle (Raymond, C. K., unpublished observations). Microtubule structures, on the other hand, are not visibly altered by energy deprivation. The observed rearrangement of a complex vacuole into a single sphere may involve coalescence of several independent vacuolar compartments mediated by vacuolar membrane fusion. Alternatively, if a complex vacuole is an interconnected vacuolar network, then rearrangement may simply involve a change in shape.

Previous evidence had suggested that daughter cells receive vacuolar material from mother cells (Zubenko et al., 1982; Weisman et al., 1987; Weisman and Wickner, 1988). Our observations suggest that the transfer of vacuolar contents may occur through a continuous network of vacuolar structures which extends from the mother cell into the newly forming bud. The appearance of these segregation structures is generally constrained to a part of the cell cycle shortly after bud emergence but before migration of the nucleus into the neck of the bud. This proposed mechanism of vacuolar segregation is an alternative to previous suggestions that vacuolar transfer is mediated by small transport vesicles (Weisman et al., 1987; Weisman and Wickner, 1988; Weisman et al., 1990). Intervacuolar exchange of vacuolar material may therefore be accomplished by a combination of continuous vacuolar structures and vacuolar transport vesicles. At least two plausible models can be envisioned to account for segregation structures. Vacuoles may extend into nascent daughter cells by active translocation of the organelle along a cytoskeletal track or other intracellular structure that leads into the bud. Another possibility is that fusion of new vacuolar material with the vacuolar structures in mother cells may be spatially constrained to a region of the organelle facing the bud such that all new synthesis leads to the polarized growth of vacuoles into the bud.

The vacuoles in *vps3-Δ1* cells differed from the wild-type organelle in several important respects. The vacuoles in *vps3-Δ1* cells almost always assumed a simple, spherical shape. Furthermore, the relative cellular volume occupied by the vacuole varied greatly. Some cells had little or no detectable vacuoles while other cells had vacuoles that occupied the majority of the cytoplasmic compartment. Finally, segregation structures were rarely observed in the mutant cell population. The aberrant vacuolar morphology in *vps3-Δ1* cells may be a general consequence of the vacuolar protein sorting defect in this mutant. If this were the case, we would expect all *vps* mutants to exhibit abnormal vacuoles similar to those in *vps3-Δ1* cells. This is not the case. Mutants representing 26 different *vps* complementation groups have been examined by immunofluorescence proce-

dures, and only 4 of them, including *vps3* mutants, exhibited defects in vacuolar segregation (C. K. Raymond, I. Howald, and T. H. Stevens, unpublished). Segregation defective strains bearing mutations in these three other *vps* complementation groups also had an overall vacuole morphology similar to that seen in *vps3-Δ1* cells. Another mutant with vacuolar segregation defects was recently described in the literature (Weisman et al., 1990). The mutation was named *vacl-1*, and the mutant strain had many characteristics in common with the segregation defective *vps* mutants. Whether the *vacl-1* mutation is allelic to any of the *vps* mutations is not yet known.

All of the aberrant morphological characteristics of vacuoles in *vps3-Δ1* cells are potentially interrelated. The characteristic spherical shape of the vacuole and the defect in segregation reflect the possibility that the organelle is detached from the structural elements that normally determine shape and segregation of vacuoles in wild-type cells. The lack of normal segregation might give rise to two effects. Mother cells, unable to segregate vacuoles to the bud, would continue to accumulate vacuolar material to the point where the organelle assumed a disproportionate amount of cell volume. Measurement of cell diameter and vacuolar diameter in populations of *vps3-Δ1* mother cells with either one bud scar or greater than three bud scars supports this possibility. Assuming that the cells and vacuoles are simple spheres, the data demonstrated that as *vps3-Δ1* cells "aged," the relative vacuolar volume increased from 7 to 29% of total cell volume. On the other hand, an apparent consequence of the vacuolar segregation defect in *vps3-Δ1* cells is that many daughter cells are born with little or no vacuolar compartment. In a wild-type population, the maternal cell contributes a substantial fraction of its lumenal vacuolar material to daughter cells (Weisman et al., 1987; this study). By contrast, the maternal cell contribution to daughter cell vacuoles in *vps3-Δ1* populations was marginal, at best. Similar observations have been made with the segregation defective *vacl-1* mutant (Weisman et al., 1990). These data raise the possibility that the vacuoles which eventually appear in *vps3-Δ1* daughter cells arise by de novo synthesis from accumulated but unassembled vacuolar constituents synthesized in independent daughter cells. The presence of accumulated but unassembled vacuolar membrane constituents is suggested by the punctate labeling pattern seen within certain *vps3-Δ1* cells by immunofluorescence. An alternate possibility is that the mother cell contributes a small, and in many cases undetectable, vacuolar compartment to the growing bud. This tiny vacuole would then serve as an assembly point for the daughter vacuole, yet vacuole assembly is delayed in the mutant. Vacuolar assembly must eventually proceed as almost all cells that go through a budding cycle have a coherent vacuole.

Our data have demonstrated that the product of the *VPS3* gene is required for sorting and processing of soluble vacuolar proteins, integrity of vacuolar morphology, efficient segregation of vacuolar material into the bud during the cell cycle, acidification of the vacuolar lumen, and assembly of the vacuolar H^+-ATPase. How might Vps3p function be required for these many diverse aspects of vacuolar assembly and function? One model is that Vps3p may be involved in the assembly of (some) peripheral membrane proteins onto Golgi apparatus-derived transport vesicles that are destined to fuse with the vacuole. This is based on the assumption that vacuolar protein sorting, acidification, and morphology, including segregation structures, are dependent on the activities of peripheral membrane proteins. For example, peripheral membrane proteins may assist in the sorting, packaging, and delivery of newly synthesized soluble vacuolar proteins from the main secretory pathway to the vacuole, and these same activities may effect processing in some manner. Peripheral membrane proteins would likely be involved in the presumed attachment of the vacuolar membrane to the cytoskeletal network in the cell. Vacuolar acidification is already known to be dependent on the peripheral membrane proteins which constitute a major portion of the vacuolar H^+-ATPase (Kane et al., 1989; C. T. Yamashiro and T. H. Stevens; unpublished observations). Given this model, the assembly of these peripheral membrane proteins would not occur in the absence of Vps3p function. This would account for the observed defects in sorting, acidification, and vacuolar morphology observed in *vps3-Δ1* mutants. Future experiments will be aimed at clarifying the role that the *VPS3* gene product plays in the assembly and maintenance of this dynamic organelle.

We would like to thank Chris Roberts, Nick Davis, and members of the Stevens' lab for insightful discussions and critical reading of the manuscript; Gaby Pohlig for affinity-purified rabbit anti-Pho8p antibody and a plasmid for the construction of *pho8-Δ1* yeast strains; Harrison Howard and Sean Poston for excellent assistance with microscopy and photography; Peter Lockhart for synthesizing oligonucleotides; Drs. W. R. Pearson and D. J. Lipman for sequence analysis software; and Drs. Vivian MacKay, Rob Preston, and Lois Weisman for helpful discussions.

This work was supported by a National Science Foundation graduate fellowship to C. K. Raymond, by National Institutes of Health predoctoral traineeships to C. K. Raymond and J. H. Rothman, and by a grant from the National Institutes of Health (GM32448) and an American Cancer Society Faculty Research Award to T. H. Stevens.

Received for publication 26 January 1990 and in revised form 21 April 1990.

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