A Truncated Form of the Pho80 Cyclin Redirects the Pho85 Kinase to Disrupt Vacuole Inheritance in *S. cerevisiae*

Teresa A. Nicolson,* Lois S. Weisman, Gregory S. Payne, and William T. Wickner*

Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, California 90024-1570; and *Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844

Abstract. Partitioning of the vacuole during cell division in *Saccharomyces cerevisiae* begins during early S phase and ends in late G2 phase before the yeast nucleus migrates into the bud neck. We have isolated and characterized a new mutant, *vac5-1*, which is defective in vacuole segregation. Cells with the *vac5-1* mutation can form large buds without vacuoles. The VAC5 gene was cloned and is identical to PHO80. PHO80 encodes a cyclin which acts in a complex with a cdc-like kinase, PHO85, as a negative regulator of two transcription factors (PHO2 and PHO4) that govern the expression of metabolic phosphatases. The vacuole inheritance defect in *vac5-1* cells is dependent on the presence of the Pho85 kinase and its targets Pho4p and Pho2p. As with other alleles of PHO80, phosphatase levels are elevated in *vac5-1* mutants. A suppressor, the COOH-terminal half of the Gal11 transcription factor, rescues the *vac5-1* phenotype of defective vacuole inheritance without altering the *vac5-1* phenotype of elevated phosphatase levels. In addition, neither maximal nor minimal levels of expression of the inducible "PHO" system phosphatases causes a vacuole inheritance defect. Though *vac5-1* is recessive, *pho80Δ* or *pho85Δ* strains do not show a defect in vacuole inheritance, suggesting that *vac5-1* is not a complete loss-of-function allele. Sequence analysis shows that the *vac5-1* allele encodes a truncated form of the Pho80 cyclin and overexpression of *vac5-1* in *pho80Δ* cells causes a vacuole inheritance defect. We conclude that the *vac5-1* allele directs the Pho85 kinase to regulate, via transcription factors Pho4 and Pho2, genes that affect vacuole inheritance but which are not known to be under normal PHO pathway control.

During each cell cycle, cells must divide and partition their cytoplasmic organelles between daughter cells. Organelles are not made by de novo synthesis, and cytological analyses have shown that organelle segregation is not merely a random process (Warren, 1993). During mitosis in mammalian cells, a number of organelles, including the nuclear envelope, ER, and Golgi, vesiculate and disperse throughout the cytoplasm, later to reassemble in the newly formed daughter cells (Zeligs and Wollman, 1979; Lipsky and Pagano, 1985: Lucocq et al., 1987). Other organelles such as endosomes cluster at the spindle pole bodies and may segregate by co-migration with this structure (Zeligs and Wollman, 1979; Kaplan et al., 1992). Although cytology has formed the foundation of organelle inheritance studies, little is known about the molecular basis of these events.

Yeast mutants have been described which are defective in the inheritance of either nuclei, mitochondria, or the vacuole (the yeast equivalent of the mammalian lysosome) (Yaffe, 1991). Segregation of each organelle in *S. cerevisiae* occurs at a different time during the cell cycle, and so may be mediated by distinct proteins. The yeast nuclear membrane, unlike the mammalian nuclear membrane, does not vesiculate during cell division. Nuclear migration into the bud is dependent on a properly assembled and oriented spindle apparatus. Nuclear migration into the bud neck is blocked by mutations in some of the components of the spindle including cytoskeletal elements such as beta tubulin (Sullivan and Huffaker, 1992), actin (Palmer et al., 1992), the actin-related proteins Act5p (Muhua et al., 1994) and Act3p (Clark and Meyer, 1994), the cytoskeletal associated protein Bik1p (Berlin et al., 1990), and the motor protein, cytoplasmic dynein, Dyn1p (Eshel et al., 1993). Other cytoskeletal elements are important for the segregation of other organelles. A mitochondrial inheritance gene, MDM1, encodes an essential intermediate filament protein which is believed to provide a cytoskeletal track for the transfer of both nuclei and mitochondria into the bud (McConnell and Yaffe, 1992, 1993). In addition, two genes encoding mitochondrial outer membrane proteins, MDM10 and MMM1, are essential for normal mitochondrial morphology and inheritance (Sogo and Yaffe, 1994; Burgess et al., 1994).

Address all correspondence to W. Wickner, Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755-3844. Tel.: (603) 650-1701. Fax: (603) 650-1353.

The current address of L. S. Weisman is Department of Biochemistry, University of Iowa, Iowa City, IA 52242.

Several mutants have been isolated and characterized which are defective in vacuole inheritance. The maternal vacuole normally forms membranous tubules or a series of connected vesicles, termed "segregation structures", which project into the bud during early S phase (Weisman and Wickner, 1988). Mutants defective in this process, termed "vac" mutants (Weisman et al., 1990; Shaw and Wickner, 1991), have buds which receive little or no maternal vesicular material. A collection of twelve vacuolar protein sorting mutants, the "class D" vps group which includes *vac1-1*, missort vacuolar enzymes and are defective in vacuole segregation during cell division (Raymond et al., 1992; Robinson et al., 1980). However, due to their protein sorting defect, it is difficult to assign a direct role to these proteins in vacuole inheritance. In contrast, *vac2-1*, which was isolated from a temperature sensitive collection of yeast mutants, is defective in vacuole inheritance while sorting proteins to the vacuole in a normal fashion (Shaw and Wickner, 1991). Thus, Vac2p is more likely to play a direct role in vacuole inheritance. *vac3-1* and *vac4-1*, isolated during the same screen, mislocalized vacuolar proteins and were not studied further.

We now report the isolation of *vac5-1*, a mutation that results in defective partitioning of vacuolar material to the bud and which, like *vac2-1*, does not disturb sorting of newly made proteins from the Golgi to the vacuole. We find that *vac5-1* is a mutant allele of PHO80, a cyclin which regulates phosphate metabolism (Toh-e and Shimauchi, 1986; Madden et al., 1988; Kaffman et al., 1994). PHO80 acts by forming a complex with PHO85, which encodes a Cdc28-like cyclin-dependent kinase (Uesono et al., 1987; Toh-e et al., 1988; Kaffman et al., 1994). Together, they repress the activity of the transcription factors Pho4 and Pho2 which govern the expression of phosphatase genes (for a review see Johnston and Carlson, 1992). Though we find that the *vac5-1* cyclin requires the presence of its partner kinase Pho85 and downstream targets Pho4p and Pho2p to cause the vacuole inheritance defect, deletion mutants in PHO85, PHO80, PHO4, or PHO2 exhibit normal vacuole inheritance. We also concurrently isolated the 3′ end of GAL11 as a suppressor of the *vac5-1* vacuole inheritance phenotype, but find that it does not affect the derepression of phosphatases by *vac5-1*. Sequence analysis of the *vac5-1* allele reveals a nonsense mutation in the last third of the gene which gives rise to a truncation of the protein. In addition, overexpression of the *vac5-1* allele in a pho80Δ background causes defective vacuole segregation. These findings suggest that the *vac5-1* allele guides Pho85p to redirect Pho4p and Pho2p to a different set of downstream genes.

Materials and Methods

Strains, Genetic Methods, and Media

Yeast strains used in this study are listed in Table IV. Mating, sporulation, and tetrad analysis were done as described by Rose et al. (1990). Mutagenesis was performed with the yeast strain GPY1100 followed by four successive backcrosses to the strains GPY1100 and DBY1398. For double mutant analysis, an additional cross was done with SEY6210. Strains were grown on yeast extract/peptone/dextrose (YEPD)[1] alone or in the presence of KCl (0.9 M in liquid medium or 1.5 M in agar plates) or in the presence of citrate phosphate (YEPD + 27 mM citric acid + 45 mM K_2HPO_4, pH 5.5). Phosphate-depleted YEPD was made according to Rubin (1976). Transformants were plated onto minimal medium without uracil (0.67% yeast nitrogen base and 2% dextrose supplemented with amino acids and nitrogenous bases). Cells transformed with pYES2 plasmids were grown in yeast extract/peptone/2% galactose.

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; YEPD, yeast extract/peptone/dextrose.

Mutagenesis and Separation of Cells

1-ml aliquots of stationary phase yeast cultures (2.4×10^8 cells per ml) were exposed to EMS for 1 h (Rose et al., 1990). 30% of the mutagenized cells were subsequently able to form colonies. The mutation rates in lys2 or lys5 in these populations of mutagenized cells were 3×10^2 fold higher than in a control culture that was not exposed to EMS. Aliquots (200 μl) of cells from four separate mutagenized cultures were innoculated into 200 ml of YEPD and grown for 17 h at 23°C. The cultures were then incubated at 37°C for an additional 3 h. During this time the population of cells doubled twice. Approximately 3×10^8 cells from each culture were harvested by centrifugation and resuspended in 0.5 ml YEPD. The cells were layered onto 12 ml of 90% Percoll (Pharmacia LKB Biotechnology, Piscataway, NJ), 1.1% yeast extract (Difco, Detroit, MI), 0.6% glucose in [14 × 76 mm] "quick seal" polyallomer tubes (Beckman Instrs., Fullerton, CA). Gradients were formed by centrifugation for 15 min, 19,000 rpm (24,700 *g*) in a Ti70.1 rotor at 23°C. The bottom of each tube was punctured and 100-μl fractions were collected, spread onto YEPD plates, and the plates were incubated at 23°C.

Microscopy

Vacuoles were visualized with three different vital fluorophores. For labeling vacuoles with dichlorocarboxyfluorescein diacetate (Pringle et al., 1989; Weisman et al., 1990), 1 ml of culture grown in YEPD to an OD_{600} of 0.05–0.5 was collected by centrifugation (10,000 *g*, 15 s) and suspended in 1 ml YEPD + 50 mM citrate phosphate, pH 5.5, and mixed with 10 μl of 10 mM dichlorocarboxyfluorescein. Cells were incubated for 12 min at 37°C, collected by centrifugation (10,000 *g*, 15 s), and examined. For labeling vacuoles with fluorescein isothiocyanate (FITC) (Preston et al., 1987; Gomes de Mesquita et al., 1991), cells were grown, harvested, and resuspended in 1 ml YEPD + 50 mM citrate phosphate, pH 5.5, as described above and 10 μl of 4 mg/ml fluorescein isothiocyanate was added. Cells were incubated for 10 min at 37°C, collected by centrifugation (10,000 *g*, 15 s), and examined. The endogenous *ade2* fluorophore was used for determining the degree of inheritance of vacuole contents (Weisman and Wickner, 1988). Cells were incubated in YEPD for 2–3 d to allow accumulation of the endogenous *ade2* fluorophore, and then transferred to fresh YEPD media containing 160 μg/ml adenine and allowed to undergo one to two doublings. Since *vac5-1* cells were defective in accumulation of the *ade2* fluorophore, these cultures were grown for one or two extra days to increase the intensity of the dye to wild-type levels.

Whole Cell Blots of CPY Secretion and Analysis of CPY Processing

Yeast patches on YEPD plates were grown for 3 d at 30°C. Nitrocellulose filters were laid on the patches and incubated for 7 h at 37°C. The filters were then washed in water and immunoblotted according to Burnette (1981) with primary antibody directed against carboxypeptidase Y (a gift from Randy Schekman) followed by secondary antibody coupled to horseradish peroxidase (HRP, Bio-Rad Laboratories, Hercules, CA). Antibodies were visualized using epichemiluminescence (ECL) for HRP (Amersham, UK).

Immunoprecipitation of ^{35}S pulse-labeled CPY was performed as described by Seeger and Payne (1992).

Isolation and Cloning of VAC5 and Manipulation of DNA

Stationary phase *vac5-1* cultures were transformed according to Gietz et al. (1992) with either a CEN based library (a gift from Mark Rose) or a 2 micron multicopy library (a gift from David Botstein). Cells were plated onto selective media (SD-ura). Approximately 2×10^4 transformant colonies were twice replicated onto YPD + 1.5 M KCl. Transformants which grew on high salt were further analyzed by FITC staining and were cured of plasmid DNA to check for reversion to wild-type inheritance. Plasmid

curing was done by serial growth to stationary phase in nonselective medium, and then assaying growth on selective medium.

Linkage analysis of clones ABC2 and ABC10 was performed by integrating a linear fragment of each DNA linked to URA3 marker DNA into the genome of a *vac5-1 ura3-52* strain. A ClaI–HindIII fragment of ABC10 linked to a URA3 marker cosegregated with the *vac5-1* phenotype in 18 tetrads. Integrated DNA from ABC2 did not cosegregate with the *vac5* locus. Complimenting fragments of both ABC2 and ABC10 were subcloned into pBluescriptII KS(+) for sequencing by the dideoxynucleotide method of Sanger (1981). The sequence of a 150–200 base pair region of the XbaI fragment of ABC10 was contained in PHO80 (Genbank accession number X07464). The sequence of a region of a BamHI–HindIII fragment of ABC2 was part of GAL11 (Genbank accession number M22481).

Primers directed against the 5′ and 3′ ends of the open reading frame of PHO80 were used in a polymerase chain reaction with UlTma DNA polymerase (Roche) to amplify DNA from the genomic DNA (Rose et al., 1990) of three *vac5-1* segregants. The three *vac5-1* DNA fragments were subcloned into pBluescript II KS (−) and submitted for sequencing (ACGT, Inc., Northbrook, IL).

Restriction sites at the ends of the primers were used to subclone *vac5-1* or PHO80 DNA fragments into a 2 micron yeast expression vector, pYES2, which contains the GAL1 promoter (Invitrogen, San Diego, CA).

Phosphatase Assays

External acid phosphatase activity was assayed as described previously (Bergman, 1986). After growth overnight in YEPD, cells (0.1 OD_{600} U) were centrifuged and the cell pellet resuspended in 0.5 ml of 0.25 M NaOAc, pH 4.0, with 1 mg para-nitrophenylphosphate. Samples were incubated at 37°C for 10 min, and then 0.12 ml of 25% TCA followed by 0.6 ml saturated Na_2CO_3 were added to each sample. Internal alkaline phosphatase was assayed as described previously (Hayashi and Oshima, 1991). The absorbance was measured at 420 nm. One unit of activity corresponds to .0072 μm para-nitrophenol generated in 5 min at 37°C.

Results

Isolation and Characterization of vac5

Ohsumi et al. (1993) observed that two yeast mutants that appear to lack a vacuole, *vam5* and *vam8*, are abnormally dense. This suggested that yeast strains defective in vacuole segregation (*vac* mutants), which have reduced vacuole volume, may also be denser than wild-type strains. As with *vam* mutants, we found that *vac1* and *vac2* cells were ~0.01 g/ml more dense than the corresponding wild-type strain (data not shown). To enrich for additional *vac* mutants, mutagenized cells were fractionated on Percoll gradients. Five hundred and fifty colonies from the densest fractions were screened using FITC microscopy for their pattern of vacuole inheritance. Eleven strains had a defect in vacuole partitioning and further studies were undertaken on one strain carrying the recessive mutation *vac5-1*.

Vacuole inheritance defects can be observed in living cells using either exogenous or endogenous dyes which are specific for the vacuole (Weisman et al., 1987). The original mutant was backcrossed once to its parent strain, GPY1100, and then three times to DBY1398, a strain with an *ade2* mutation which blocks the adenine biosynthetic pathway. When grown in limiting amounts of adenine, cells with this mutation accumulate an endogenous red fluorescent dye in the vacuole. The *ade2* fluorophore is a more specific indicator of vacuole inheritance than exogenous dyes since it can only be transferred to the daughter cell in budding yeast via vacuolar segregation structures (Weisman et al., 1987). To monitor vacuole inheritance, *vac5-1* cells were grown to stationary phase to accumulate the *ade2* fluorophore, and then transferred to fresh medium containing adenine. Through feedback inhibition of the blocked biosynthetic pathway, adenine prevents the further accumulation of the dye. However, the fluorophore is stable and can be used to track vacuole inheritance over several generations (Weisman et al., 1987). Several budding *vac5-1* cells stained with the endogenous *ade2* fluorophore are shown in Fig. 1 *B*. *ade2* fluorophore was not transferred into 53% of the buds of *vac5-1* mutant cells (Table I), whereas cells from a wild-type sister segregant did not show any defects in transfer of the fluorophore (Fig. 1 *C*, Table I). As with *vac1-1* and *vac2-1*, *vac5-1* cells appear to generate a vacuole by some means other than the inheritance pathway. Only a very minor population of *vac5-1* cells appear to completely lack a normal vacuole. Cells which do not inherit any maternal vacuolar material presumably generate a new organelle by de novo synthesis (Weisman et al., 1990; de Mesquita, G., personal communication). It should be noted that the *vac5-1* cells are slightly defective in accumulating the fluorophore and differences between wild-type and mutant cells can be readily seen on a YPD plate, where *vac5-1 ade2* cells appear pink whereas VAC5 *ade2* cells are red. Mid-log phase *vac5-1* cells stained with the vital dye fluorescein isothiocyanate (FITC) also show little vacuolar material in the bud (Fig. 4 *A*). In addition, staining with DAPI (4′,6-diamidino-2-phenylindole), a DNA specific dye (Sherman et al., 1986), reveals that the *vac5-1* mutation does not interfere with the inheritance of mitochondria or nuclei (data not shown). Thus *vac5-1* cells are specifically defective in inheritance of the vacuole.

Retention and Processing of Carboxypeptidase Y Is Normal in vac5-1 Cells

A number of vacuolar protein sorting mutants (*vps* "class D" mutants) are defective in vacuole inheritance (Raymond et al., 1992). The *vac1-1* strain (*vac1-1* is allelic to *vps19*) is one such mutant. However, it is unclear whether the primary defect in *vac1-1* cells is the secretion of vacuolar proteins or the defective vacuole inheritance. It is more likely that vacuole inheritance mutants like *vac2-1*, which do not secrete vacuolar proteins such as carboxypeptidase Y (CPY) or proteinase A (Shaw and Wickner, 1991), have a primary defect in the vacuole inheritance process.

To determine whether *vac5-1* cells secrete CPY, cells were grown in patches on YPD plates, and then overlaid with a nitrocellulose filter for 7 h at 37°C. Filters were then incubated with CPY antibody and assayed for antibody binding. Fig. 2 *A* shows a filter overlay of the two parental strains, GPY1100 (patches 1 and 2, a and α strains, respectively) and DBY1398 (patches 15 and 16, a and α strains). Duplicate patches of *vac1-1* (patches 3 and 4) and *vac2-1* cells (patches 5 and 6) show a striking difference in the levels of secreted CPY. Two different tetrads derived from crossing *vac5-1* to the DBY parental strain (patches 7–10 and 11–14) are shown in the third and fourth row on the filter in Fig. 2. In general, there are no dramatic differences in levels of CPY secretion between cells from the *vac5-1* spores (patches 8, 10, 12, and 13) and those from the VAC5 spores (patches 7, 9, 11, and 14) or parental

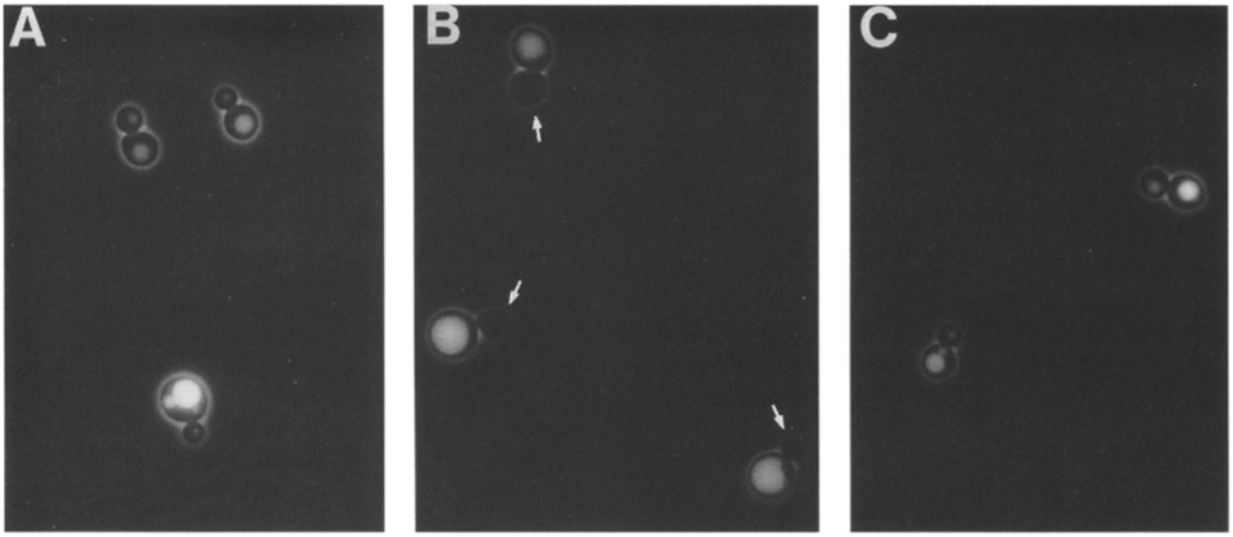

Figure 1. Vacuole segregation of *vac5-1* and wild-type strains in the presence or absence of phosphate, assayed with the *ade2* fluorophore. *vac5-1* (*A* and *B*) and wild-type sister spore (*C*) cultures were grown in YEPD medium to stationary phase (allowing the *ade2* fluorophore to accumulate), then shifted into phosphate-depleted YEPD (*A*) or phosphate-depleted YEPD + 10 mM phosphate (*B* and *C*). Bar, 15 μm.

strains (patches 1, 2, 15, and 16). In contrast to *vac1-1*, the level of CPY secretion caused by *vac5-1* is comparable to the levels seen with *vac2-1*. Moreover, as with *vac2-1* cells, the kinetics of processing CPY from the precursor polypeptide to the mature form is normal in *vac5-1* cells (Fig. 2 *B*).

Identification of vac5-1 as an Allele of PHO80

The yeast vacuole serves as a storage compartment for metabolites such as amino acids, inorganic ions, and polyphosphates and participates in pH buffering and osmoregulation (Klionsky et al., 1990). Mutants in vacuole inheritance, which produce buds without vacuoles, may be sensitive to high osmolarity. We therefore tested the ability of *vac5-1* cells to grow on plates of YPD + 1.5 M KCl (Fig. 3 *A*). Growth of vac5-1 cells was severely inhibited on these high salt plates (Fig. 3 *A*). Over fifty tetrads were examined from a cross between the *vac5-1* strain and a wild-type strain. In each case, growth on high salt segregated 2:2 and each spore unable to grow on high salt was also defective in vacuole inheritance as judged by FITC staining.

Table I. The vac5-1 Mutation Causes a Defect in Inheritance of the Vacuolar ade2 Fluorophore

	Percent of medium and large buds containing the *ade2* fluorophore	
Strain	+phosphate	−phosphate
VAC	100 (0)	99 (0.5)
vac5-1	47 (3.5)	97 (2)

Strains were grown in YEPD until stationary phase to allow the accumulation of the *ade2* fluorophore. They were shifted into fresh phosphate-depleted YEPD with, or without, readdition of 10 mM phosphate and allowed to double before scoring. The presence of *ade2* fluoresence was scored in >50 buds and the numbers shown are the average values of three independent experiments and the standard deviation is indicated in parentheses.

Cloning by rescue of growth on high salt plates yielded five plasmids from two separate libraries (Rose et al., 1987; Carlson and Botstein, 1982). Many colonies able to grow on high salt were revertants, suggesting that *vac5-1* is a single point mutation. Two single copy plasmids, designated ABC10 and ABC16, and one multicopy clone, designated YE15, contained inserts which differed in length but exhibited similar restriction patterns and proved to contain the same gene(s) by Southern analysis (data not shown). The other two single copy clones, ABC2 and ABC15, contained inserts which shared a common restriction pattern. To determine whether either insert class represents the VAC5 locus, the URA3 gene was targeted to the chromosomal position of the inserts by homologous recombination (Rothstein, 1991). Linkage analysis, using the resulting strains crossed to the *vac5-1* strain, demonstrated that ABC10 is linked to the VAC5 locus. The other clone, ABC2, appears to be a single copy suppressor.

Complementation of the high salt sensitivity of *vac5-1* cells was obtained with the ABC10 clone (Fig. 3 *A*) and a 2.5-kb subclone, pABC10SB (Fig. 3 *B*). This fragment also restored wild-type vacuole inheritance (Fig. 4 *B*). Removal of a 500-bp XbaI fragment from within the 2.5-kb subclone abolished both the ability to rescue defective growth on high salt and the inheritance defect (Figs. 3 *A* and 4 *D*). A summary of the complementation results is shown in Fig. 3 *B*. The XbaI fragment was sequenced and the sequence was found to be identical to a portion of the *S. cerevisiae* PHO80 gene. In further functional support for this identity, the ABC10 plasmid restores normal regulation to the expression of phosphatases in a pho80Δ strain and a clone containing the PHO80 gene, YEp351-80BP (Madden et al., 1990), suppresses the defective vacuole inheritance in *vac5-1* cells (data not shown). The ABC2

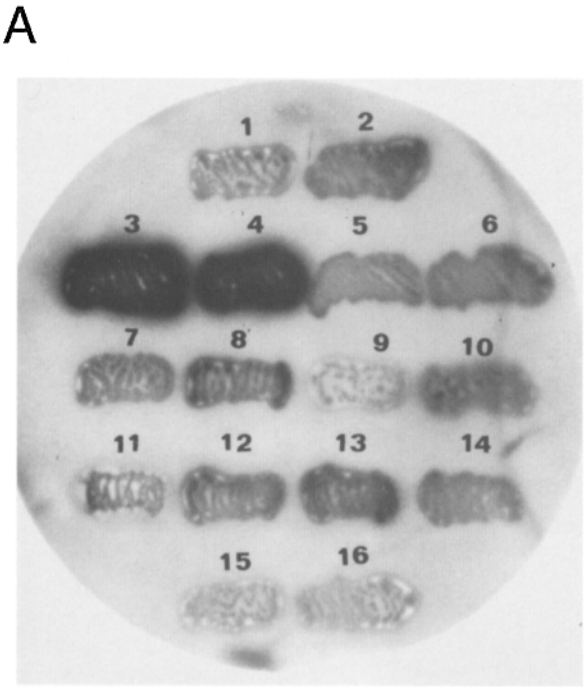

Figure 2. Secretion and processing of vacuolar CPY in wild-type and *vac* strains. (*A*) Secretion of CPY is normal in *vac5-1* cells. Patches of cells were grown for several days on YEPD agar and then overlaid with a nitrocellulose filter for 7 h at 37°C. The filter was incubated with CPY antiserum and developed using ECL for HRP-conjugated secondary antibody. Wild-type parental strains DBY1398 and GPY1100 (both a and α) are indicated by 1, 2, 15, and 16, respectively. Duplicate patches of *vac1-1* (3 and 4) and *vac2-1* (5 and 6) were analyzed along with two complete tetrads of *vac5-1* backcrossed to DBY1398 (7–10 and 11–14). *vac5-1* patches are numbered 8, 10, 12, and 13. Wild-type sister patches are numbered 7, 9, 11, and 14. (*B*) Processing of CPY is normal in *vac5-1* cells. A *vac5-1* and wild-type sister spore strain were metabolically labeled with a 5-min pulse of [^{35}S]methionine and CPY was immunoprecipitated from cell lysates at various times up to 90 min.

clone was also reduced to a 2.5-kb complementing fragment, ABC2′, (Figs. 3 *A* and 4 *C*) and sequenced; it contains ~2 kb of the 3′ half of GAL11. Gal11p is a global transcription factor, involved in the regulation of many genes (Sakurai et al., 1993). Although the promoter region of GAL11 is not present, the ABC2′ clone can partially rescue the growth defect of a gal11Δ strain on galactose (data not shown).

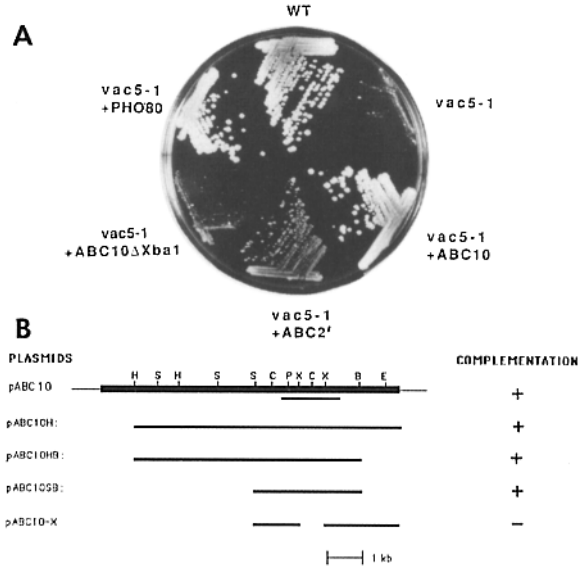

Figure 3. Rescue of the *vac5-1* high salt growth defect by YCp50 plasmid clones ABC10 and ABC2, and complementation analysis of ABC10. (*A*) *vac5-1* alone or *vac5-1* containing single copy vector library clones of ABC2′, ABC10, and ABC10 missing a small XbaI fragment were streaked on YEPD agar + 1.5 M KCl. A wild-type sister segregant and a *vac5-1* strain containing a single copy of PHO80 (gift of L. Bergman) were also included for comparison. (*B*) Summary of the structure and complementation properties of various VAC5-containing plasmids. The thin line indicates the region corresponding to the ORF of PHO80. X=XbaI. (ABC10ΔXba1 is the same plasmid as pABC10-X.) The sequence of the XbaI–XbaI fragment was determined.

To determine the mutation within the *vac5-1* allele, the PHO80 open reading frame was amplified using genomic DNA template from three different segregants of *vac5-1* and Ultma DNA polymerase with exonuclease proofreading activity (Roche). Each fragment was then sequenced and revealed a C to T transition at base position +600 (Fig. 5). This change results in a stop codon which abolishes translation of the COOH-terminal third of the open reading frame. No additional mutations were found within the open reading frame.

Double Mutant Analysis with pho Disruption Strains

All the genes of the inducible phosphate pathway have been cloned and sequenced (Johnston and Carlson, 1992) and disruptions of all the PHO genes are available. A disruption of PHO80 does not result in defective vacuole inheritance, suggesting that *vac5-1* is not a loss-of-function allele. Cells with disruptions of any of the remaining PHO genes, including PHO85, PHO2, and PHO4, or a double disruption of both PHO4 and PHO2, also exhibit wild-type inheritance of the vacuole (data not shown). Double mutant analysis of *vac5-1* in combination with *pho85Δ*, *pho4Δ*, or *pho2Δ* was performed to assess whether the *vac5-1* vacuole inheritance defect depends on the presence of the Cdc28-like Pho85 kinase, and its targets, the Pho4 and Pho2 transcription factors. The *vac5-1* strain was crossed to strains carrying disruptions of individual PHO pathway genes. Over a dozen tetrads from each cross were scored for vacuole inheritance and phosphatase activity in

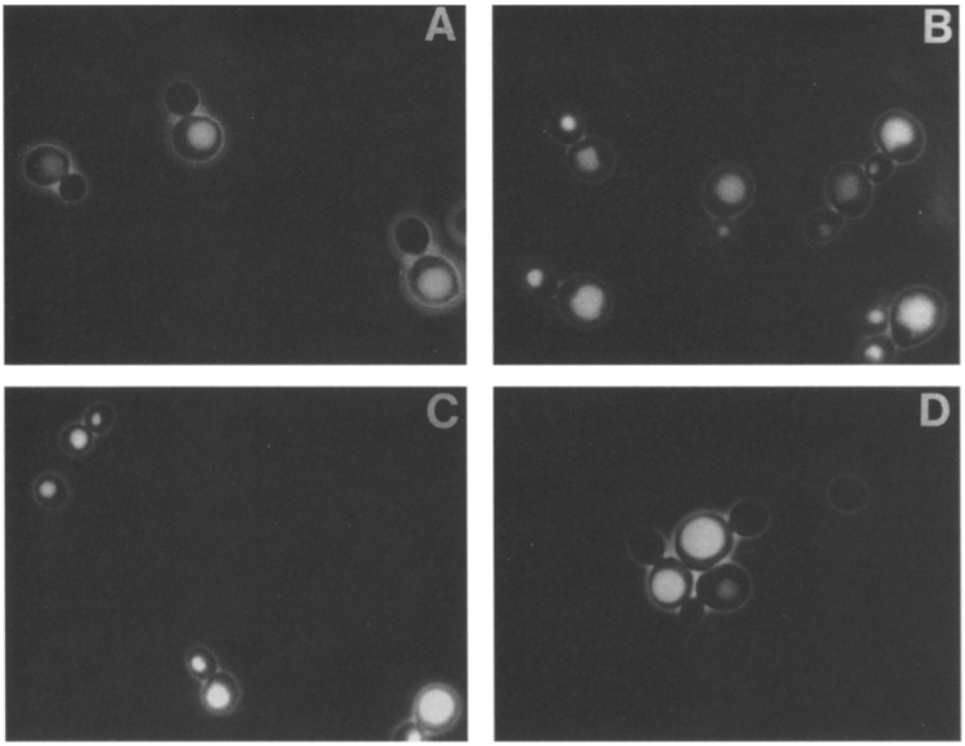

Figure 4. Rescue of the *vac5-1* vacuole inheritance defect by single copy plasmids containing either PHO80 or the 3′ end of GAL11. Cultures of *vac5-1* + YCp50 plasmid control (*A*), *vac5-1* + pABC10SB (*B*), *vac5-1* + pABC2′ (*C*), and *vac5-1* + pABC10SB with a deletion of the XbaI fragment (*D*) were grown to mid-log phase in YEPD + 50 mM citrate phosphate medium, pH 5.5, and stained with a vacuole specific vital dye, FITC. Bar, 10 μm.

YEPD medium (Table II). Each gene was identified by a nutritional marker integrated in or near the coding region. The majority of tetrads exhibited a phenotypic pattern of three wild-type segregants: 1 vacuole inheritance mutant segregant, indicating that they were tetratype tetrads (*phoΔ vac5-1; phoΔ* VAC5; PHO *vac5-1*; PHO VAC5). One third of the tetrads exhibited a 4:0 or 2:2 pattern, indicating that they were parental ditypes (*phoΔ VAC5; phoΔ* VAC5; PHO *vac5-1; PHO vac5-1*) or nonparental ditypes (*phoΔ vac5-1; phoΔ vac5-1*; PHO VAC5; PHO VAC5). Thus, haploid cells containing both the *pho85Δ* and the *vac5-1* allele exhibit wild-type vacuole inheritance, as do *pho4Δ, vac5-1* and *pho2Δ, vac5-1* double mutants. These results show that the effect of *vac5-1* on vacuole inheritance is mediated through the PHO genes encoding the key regulatory proteins of the PHO pathway. In contrast, the vacuole inheritance defect does not require the upstream negative regulator encoded by PHO81. Tetrads derived from a cross of *pho81Δ* and *vac5-1* strains showed a 2:2 segregation of the *vac5-1* phenotype (Table II). The

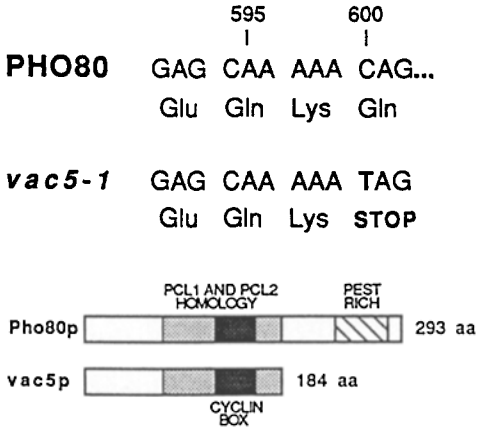

Figure 5. A nonsense mutation within the *vac5-1* allele results in a truncation of Pho80p. A C to T transition at base position +600 creates a stop codon in the COOH-terminal third of the open reading frame of the *vac5-1* allele. The truncation results in removal of the COOH-terminal domain of the protein which is rich in PEST residues (Pro, Glu, Ser, Thr, and Gln) and basic amino acids.

Table II. The vac5-1 Inheritance Defect Is Dependent on the Presence of PHO85, PHO4, and PHO2

Tetratype tetrad	Vacuole inheritance	PHO5 activity
		U
VAC5 PHO85	wild-type	0.9
VAC5 *pho85Δ*	wild-type	4.9
vac5-1 PHO85	defective	7.2
vac5-1 pho85Δ	wild-type	5.7
VAC5 PHO4	wild-type	1.5
VAC5 *pho4Δ*	wild-type	1.2
vac5-1 PHO4	defective	6.1
vac5-1 pho4Δ	wild-type	1.8
VAC5 PHO2	wild-type	1.0
VAC5 *pho2Δ*	wild-type	0.7
vac5-1 PHO2	defective	6.1
vac5-1 pho2Δ	wild-type	1.1
VAC PHO81	wild-type	1.2
VAC5 *pho81Δ*	wild-type	0.8
vac5-1 PHO81	defective	4.7
vac5-1 pho81Δ	defective	5.8

The genotype of each tetrad was determined by the presence of an integrated nutritional marker within, or near, the gene of interest. Tetrads were scored for vacuole inheritance by labeling mid-log phase cultures with FITC. The levels of Pho5p activity of cells grown in YEPD were measured as described in Materials and Methods.

vacuole inheritance defect is also independent of a downstream phosphatase, PHO8, which resides in the vacuole; *pho8Δ, vac5-1* double mutants still exhibit buds without vacuoles (data not shown).

Phosphate Regulation of the vac5-1 Allele

When inorganic phosphate is abundant, expression of the inducible phosphatases is inhibited through the action of the Pho80/Pho85 cyclin-dependent kinase complex on the transcription factors Pho4 and Pho2 (Ueda et al., 1975; Okada and Toh-e, 1992; Kaffman et al., 1994). When phosphate levels are low, the Pho80/Pho85 complex is inactivated by Pho81p (Ueda et al., 1975; Yoshida et al., 1989; Schneider et al., 1994), allowing Pho4p and Pho2p to induce the expression of the genes PHO5, PHO8, PHO10, and PHO11 which encode scavenger phosphatases localized in the periplasm or the vacuole (Johnston and Carlson, 1992). The *vac5-1* allele, like other *pho80* mutants, causes constitutive expression of phosphatases (Table II and Fig. 6). Levels of phosphatase activity remain high in *vac5-1* cells regardless of the phosphate concentration in the medium. Strikingly, the COOH-terminal Gal11p suppressor, which rescues the inheritance defect and salt sensitivity, does not correct the constitutive levels of phosphatase expression (Fig. 6). However, the level of phosphate has a dramatic effect on the inheritance defect, salt sensitivity, and *ade2* fluor accumulation in *vac5-1* cells. To observe segregation of vacuoles in the presence or absence of phosphate, cells were grown in YEPD for 3 d to allow accumulation of the *ade2* fluorophore, and then shifted to fresh, phosphate-depleted YEPD media with, or without, the readdition of 10 mM phosphate. Cells were allowed to double, and then medium and large buds were scored for fluorophore content. In the absence of phosphate, mutant cells (Fig. 1 *A*) displayed normal vacuole inheritance, as seen for sister segregant wild-type cells (Table I). Furthermore, if cells were grown to stationary phase in the absence of phosphate, wild-type and mutant cells had indistinguishable *ade2* fluorophore accumulation (Table III). In addition, Pho81p, which normally participates in phosphate regulation of the PHO pathway, is not required for the phosphate regulation of the *vac5-1* vacuole inheritance defect. As with *vac5-1* cells, *vac5-1 pho81Δ*double mutants display the vacuole inheritance defect only in high phosphate medium (data not shown). Salt sensitivity of *vac5-1* cells was tested by growing cells overnight in phosphate-depleted YPD with, or without, the readdition of either 10 mM phosphate or 0.9 M KCl. Cell death was scored by staining with FITC (dead cells show a bright uniform yellow stain and have a shriveled or shrunken appearance). In medium containing 0.9 M KCl but no phosphate, VAC5 and *vac5-1* strains showed comparable salt sensitivity (Table III). In contrast, *vac5-1* efects in vacuole inheritance, *ade2* accumulation, and salt sensitivity are all seen in the presence of 10 mM phosphate (Fig. 1 *B*, *vac5-1*, + P_i; 1C, VAC5, + P_i; see Tables I and III).

Overexpression of the vac5-1 Allele in pho80Δ Cells

Disruption of the PHO80 gene does not affect vacuole segregation, which suggests that the Pho80 cyclin does not

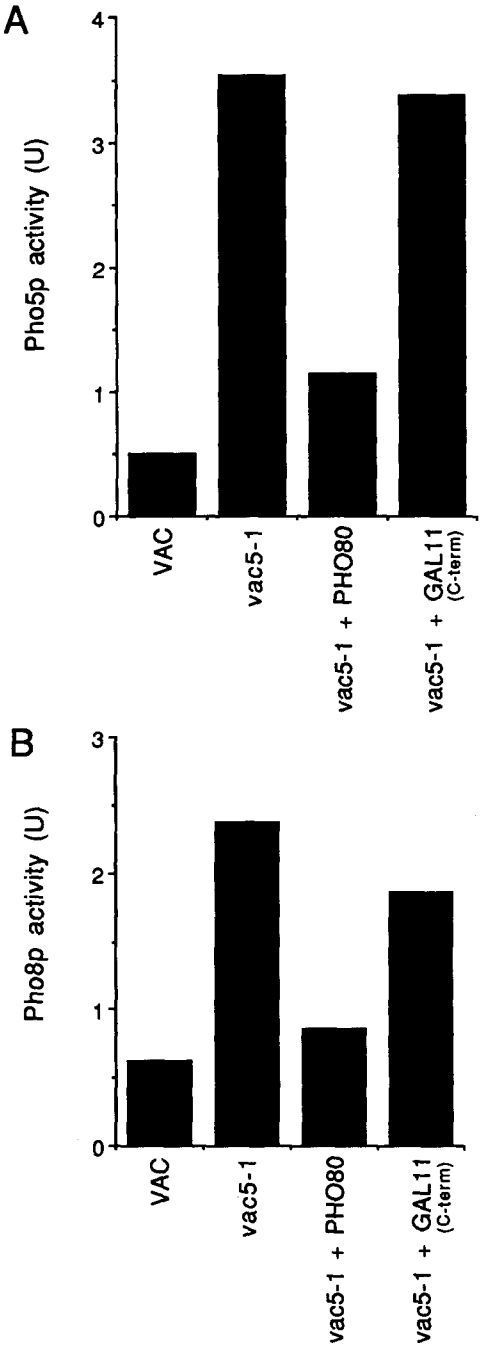

Figure 6. Rescue of constitutive expression of external acid phosphatase or vacuolar alkaline phosphatase in *vac5-1* cells by PHO80 (pABC10SB) but not by the 3′ fragment of GAL11 (pABC2′). (*A*) Cells were grown in YEPD medium to mid-log phase. Samples (0.1 U of OD_{600}) were sedimented (microfuge, 10 min) and resuspended in 0.25 M NaOAc, pH 4.0, with 1 mg/ml para-nitrophenylphosphate (pNNP) and assayed for phosphatase activity (Materials and Methods). (*B*) Cells were grown in YEPD medium to mid-log phase. Cells were lysed with glass beads, and then 8 μg lysate (~0.1 U of OD_{600}) was incubated in 25 mM Tris-Cl, pH 9.0, 1 mM $MgCl_2$, with 1 mg/ml pNNP and assayed for phosphatase activity as above.

Table III. Summary of Regulation of vac5-1 Phenotype by Inorganic Phosphate

	VAC		*vac5-1*	
Phenotype	+phosphate	−phosphate	+phosphate	−phosphate
Vacuole inheritance	normal	normal	defective	normal
Regulation of PHO5	normal	normal	defective	normal
ade2 fluor accumulation	normal	normal	defective	normal
Growth in high salt*	+	+	−	+

Wild-type cells grown ± phosphate in the absence of KCl exhibited <10% cell death; wild-type cells grown in the absence of phosphate + 0.9 M KCl exhibited <15% cell death and in 10 mM phosphate + 0.9 M KCl exhibited <20% cell death. Mutant cells grown ± phosphate in the absence of KCl exhibited <15% cell death; mutant cells grown in the absence of phosphate + 0.9 M KCl exhibited <25% cell death and in 10 mM phosphate + 0.9 M KCl exhibited >85% cell death.
*Cell were grown overnight in phosphate-depleted YEPD with, or without, the readdition of 10 mM phosphate or 0.9 M KCl and scored for cell death using FITC.

normally participate in regulation of vacuole inheritance. To test whether the *vac5-1* allele behaves as a gain-of-function mutation, the *vac5-1* gene was overexpressed in *pho80Δ* cells and the phenotype was examined microscopically. PCR fragments obtained from *vac5-1* genomic DNA or the ABC10 plasmid containing only the open reading frame were subcloned into a 2 micron GAL vector, pYES2 (see Materials and Methods). *pho80Δ* cells were transformed with plasmid alone or plasmid containing the *vac5-1* allele or PHO80 and grown in rich medium with galactose. The vacuoles of mid-log phase cells were stained with FITC. *pho80Δ* cells containing plasmid only or plasmid containing the PHO80 gene retained their original phenotype of wild-type inheritance (Fig. 7, *A* and *B*). In contrast, *pho80Δ* cells transformed with plasmid containing the *vac5-1* gene had the identical phenotype as *vac5-1* cells, exhibiting buds without vacuoles (Fig. 7 *C*). Wild-type and *vac5-1* cells were transformed in parallel with plasmid containing *vac5-1* and did not show any phenotypic differences (data not shown).

Discussion

In a screen for new mutants which affect vacuole inheritance, we have isolated an allele of the cyclin PHO80, a regulatory protein involved in phosphate metabolism. Surprisingly, though the *vac5-1* lesion is recessive, a disruption of the PHO80 gene does not cause a vacuole segregation defect. Other recessive alleles of PHO80 also do not affect vacuole inheritance (unpublished observations), suggesting that the cyclin encoded by *vac5-1* has acquired new properties. Indeed, the *vac5-1* allele contains a nonsense mutation in the last third of the structural gene which results in a truncated form of the cyclin. It has been previously shown that truncations in other cyclins such as CLN2 and CLN3 result in hyperactive proteins (Nash et al., 1988; Hadwiger et al., 1989). The CLN2-1, CLN3-1, and CLN3-2 mutations result in removal of the COOH-terminal third of each cyclin which are rich in PEST residues (Pro, Glu, Ser, Thr, and Gln). These residues are believed to be critical for proteolysis and removal of the CLN2 or CLN3 PEST regions confers stability to otherwise unstable cyclins (Tyers et al., 1992; Salama et al., 1994). The COOH-terminal third of Pho80p is also rich in Pro, Glu, Ser, Thr, Gln, and basic residues (Madden et al., 1988, 1990). This study suggests that the PEST-rich domain of Pho80p is also important for regulation of Pho80p activity in that removal of this domain results in a phenotype not seen with other alleles of PHO80. A gain-of-function model is also supported by the observation that overexpression of the *vac5-1* gene in a normally wild-type *pho80Δ* strain results in a defect in vacuole inheritance. Since the vacuole segregation defect in *vac5-1* cells requires the Pho80-dependent Pho85 kinase, the recessive character of the *vac5-1* mutation may reflect efficient competition for the Pho85p kinase by the normal Pho80p cyclin. In the absence of normal Pho80p, the *vac5-1* trun-

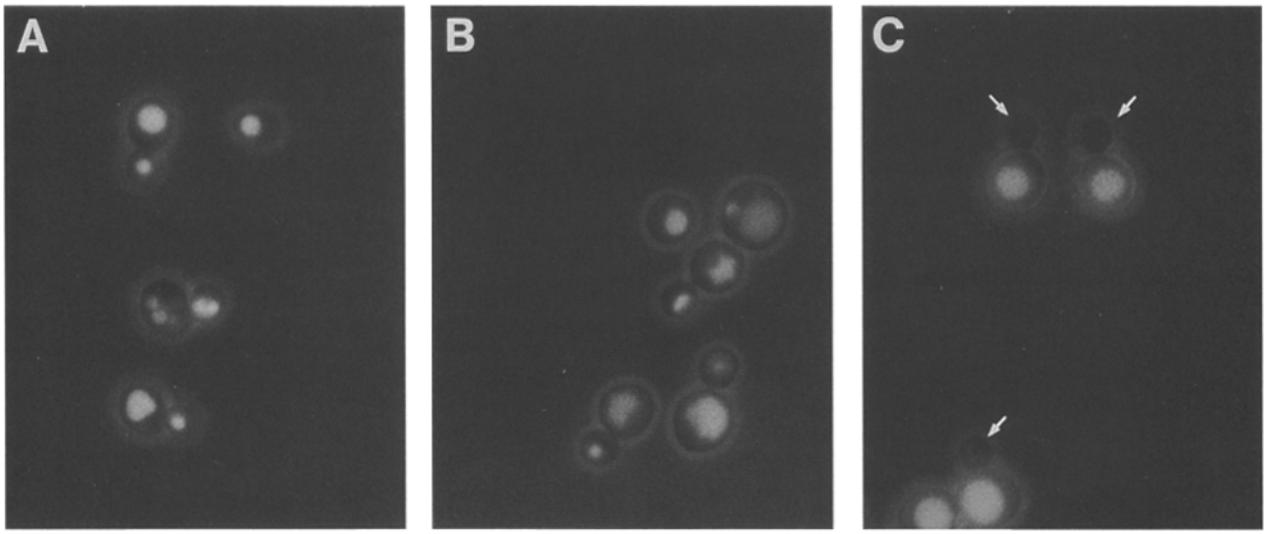

Figure 7. Overexpression of the *vac5-1* allele in *pho80Δ* cells causes defective vacuole inheritance. Cultures of *pho80Δ* + pYES2 plasmid control (*A*), *pho80Δ* + pYES2/PHO80 (*B*), and *pho80Δ* + pYES2/*vac5-1* (*C*) were grown in YEP + galactose and stained with FITC.

Table IV. Yeast Strains Used in This Study

Strains	Genotype	Source
DBY1398	MATα ade2-102 ura3-52	David Botstein
GPY1100	MATα leu2-3,112 ura3-52 his4-519 trp1 can1 gal2	Payne et al. (1987)
SEY6210	MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-901	Robinson et al. (1988)
TN.2B	MATa vac5-1 ura3-52	This work
TN.3A	MATa vac5-1 ura3-52 ade2-102	This work
TN.3B	MATα vac5-1::URA3 ura3-52 his3-Δ200	This work
TN.3B-85	MATa vac5-1::URA3 pho85Δ::ADE2 ura3-52 his3-Δ200 ade2-102	This work
TN.3B-81	MATa vac5-1::URA3 pho81Δ::HIS3 ura3-52 his3-Δ200 ade2-102	This work
TN.3B-4	MATa vac5-1::URA3 pho4Δ::HIS3 ura3-52 his3-Δ200	This work
TN.3B-2	MATa vac5-1::URA3 pho2Δ::HIS3 ura3-52 his3-Δ200	This work
LWY148	MATa vac1-1 ura3-52 ade2-102	Weisman et al. (1990)
JSY103	MATa vac2-1 ura3-52 ade2-102 lys2-801	Shaw and Wickner (1991)
Δ80-hA	MATα pho80Δ::LEU2 ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 leu2-Δ1	Madden et al. (1990)
Δ85-HT	MATa pho85Δ::HIS3 ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1	Madden et al. (1990)
NBD80-1D	MATa pho80Δ::HIS3 ura3-1,2 ade2-102 his3-532 leu2-3 trp1-289	Yasuji Oshima
NBD85A-1	MATa pho85Δ::ADE2 ura3-1,2 ade2-102 his3-532 leu2-3 trp1-289	Yasuji Oshima
NBD4-1	MATa pho4Δ::HIS3 pho3-1 ura3-1,2 ade2-102 his3-532 leu2-3 trp1-289	Ogawa and Oshima (1990)
NBD2-1B	MATa pho2Δ::HIS3 pho3-1 ura3-1,2 ade2-102 his3-532 leu2-3 trp1-289	Bunya et al. (1990)
NBD24-1A	MATa pho2Δ::HIS3 pho4Δ::HIS3 pho3-1 ura3-1,2 ade2-102 his3-532 leu2-3 trp1-289	Yasuji Oshima
NBD81-6D	MATa pho81Δ::HIS3 pho3-1 ura3-1,2 ade2-102 his3-532 leu2-3,112 trp1-289	Bunya et al. (1991)

cated cyclin appears to affect vacuole inheritance by regulating the expression of genes not normally under regulation by the phosphate-sensitive signal transduction pathway.

The *vac5-1* mutant displays a defect in both vacuole inheritance and a defect in the negative regulation of the PHO pathway. However, several lines of evidence suggest that the expression of the downstream metabolic phosphatases is not involved in the vacuole segregation defect caused by the *vac5-1* lesion. First, the Gal11p (COOH terminus) suppressor rescues the vacuole inheritance defect yet does not suppress the constitutive expression of phosphatases. Second, maximal upregulation of the PHO pathway in *pho80Δ* or *pho85Δ* strains or downregulation in *pho4Δ* strains does not affect vacuole inheritance. Third, though the *vac5-1* vacuole inheritance defect is under phosphate regulation, expression of downstream phosphatases remains constitutive at all levels of phosphate in *vac5-1* cells.

vac5-1 cells only display defective vacuole segregation if grown in high phosphate medium. Genetic evidence suggests that Pho81p is the major phosphate sensor of the PHO system and is responsible for repressing the activity of the Pho80p/Pho85p complex (Lemire et al., 1985; Ueda et al., 1975). However, PHO81 is not required for the expression of the *vac5-1* vacuole inheritance defect or for its regulation by phosphate, suggesting that there are additional phosphate-responsive proteins. Recent results from analysis of Pho81p are consistent with this idea (Schneider et al., 1994).

Genetic analysis of double mutants reveals that the *vac5-1* cyclin exerts its effects via the cyclin-dependent kinase Pho85 and transcription factors Pho4 and Pho2. The absence of any of these components causes wild-type segregation of the vacuole in a *vac5-1* background. However, these regulatory genes of the PHO pathway do not appear to be normally involved in a unique or indispensable fashion in vacuole inheritance, since disruptions of any of the regulatory genes PHO81, PHO80, PHO85, PHO4, and PHO2 do not affect vacuole inheritance. The requirement for the Pho85 kinase suggests that a phosphorylation event is necessary for the effect of vac5p and the requirement for the transcription factors Pho4 and Pho2 suggests that the mutant cyclin is affecting the regulation of gene expression. The vac5p cyclin may therefore direct Pho85p to alter the activity or specificity of the transcription factors Pho4 and Pho2 such that they affect the expression of a set of vacuole inheritance genes. The concurrent isolation of a suppressor which is also a transcription factor, Gal11p, supports the idea that *vac5-1* is affecting transcription.

It is possible that the vac5p/Pho85p complex phosphorylates Pho4p and Pho2p in a new manner, thus altering their activity or specificity. Pho4p has a basal level of phosphorylation at two sites and appears to be phosphorylated by the Pho80p/Pho85p complex at four sites (Kaffman et al., 1994). Perhaps the truncated cyclin causes an altered pattern of phosphorylation such that Pho4p and Pho2p are still able to induce expression of their usual target genes, but also affect new genes. Misregulation via *vac5-1* may cause Pho4p and Pho2p to become more promiscuous. It is worth noting that Pho2p has already been shown to have a more general role in transcription. It is involved in the expression of TRP4, HIS4, HO, ADE1, and genes involved in adenine biosynthesis and sporulation (Braus et al., 1989; Arndt et al., 1987; Berben et al., 1988; Brazas and Stillman, 1993). Moreover, it can act as either a repressor or an activator. This complex behavior of Pho2p could play a major role in the phenotype caused by the *vac5-1* mutation. Furthermore, Pho4p forms homodimers (Ogawa and Oshima, 1990) and interacts with Pho2p (Hirst et al., 1994). The vac5p/Pho85p complex may have an effect on the formation, and thus the function, of such oligomers.

It may be possible to look for new targets of Pho4p and Pho2p by the use of an in vitro assay which reflects the in vivo process of inheritance (Conradt et al., 1992; Haas et al., 1994). This assay measures homotypic vacuole fusion which occurs in late G2 phase when vesicular material delivered via segregation structures reassembles into a new vacuole inside the bud (Conradt et al., 1992). Recent results show a complex pattern in which normal assay con-

centrations of *vac5-1* cytosol are defective in promoting vacuole-to-vacuole fusion while, at lower concentrations, *vac5-1* cytosol appears to be more active than wild-type cytosol (unpublished observations). Fractionation of this activity may lead to the identification of the new gene targets of Pho4p and Pho2p.

Our results suggest that *vac5-1* represents a unique allele of PHO80. Further biochemical analysis may distinguish between the various models for *vac5-1* effects on vacuole inheritance. However, our current observations indicate that *vac5-1* is a gain-of-function allele and the truncated cyclin appears to alter the activity and possibly the specificity of its partner kinase. Recent results demonstrate that the Pho85 kinase interacts with other cyclins and has a role in progression through G1 phase (Espinoza et al., 1994; Measday et al., 1994). During cell division, the Pho85 kinase may coordinate phosphate metabolism with cell cycle progression. Although the Pho80 cyclin does not appear to be involved in cell cycle events, our results show that a truncated form of the cyclin via its interaction with the Pho85 kinase, can interfere or alter a cell cycle–regulated event, vacuole inheritance. Of further interest is the ability of a COOH-terminal portion of Gal11p to suppress the vacuole inheritance defect. Only this fragment, and not full-length GAL11, can rescue the defect (unpublished observations). Gal11p is known to be part of the transcriptional holoenzyme complex (Kim et al., 1994) and acts as a global transcriptional activator (Sakurai et al., 1993; Long et al., 1991). The COOH-terminal portion of Gal11p appears to have a dominant effect on the putative misregulation of transcription caused by *vac5-1* since the strains in this study have wild-type chromosomal GAL11. Studies of its role in the holoenzyme and the various domains of GAL11 are underway in several laboratories. Our study offers another possible avenue for exploring the biological effects of Gal11p and potential cross-talk between the GAL and PHO pathways.

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