Copyright © 1992 by the Genetics Society of America

Genes Required for Vacuolar Acidity in *Saccharomyces cerevisiae*

Robert A. Preston, Pamela S. Reinagel[1] and Elizabeth W. Jones

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

Manuscript received December 17, 1991
Accepted for publication March 21, 1992

ABSTRACT

Mutations that cause loss of acidity in the vacuole (lysosome) of *Saccharomyces cerevisiae* were identified by screening colonies labeled with the fluorescent, pH-sensitive, vacuolar labeling agent, 6-carboxyfluorescein. Thirty nine vacuolar pH (Vph$^-$) mutants were identified. Four of these contained mutant alleles of the previously described *PEP3*, *PEP5*, *PEP6* and *PEP7* genes. The remaining mutants defined eight complementation groups of *vph* mutations. No alleles of the *VAT2* or *TFP1* genes (known to encode subunits of the vacuolar H$^+$-ATPase) were identified in the Vph$^-$ screen. Strains bearing mutations in any of six of the *VPH* genes failed to grow on medium buffered at neutral pH; otherwise, none of the *vph* mutations caused notable growth inhibition on standard yeast media. Expression of the vacuolar protease, carboxypeptidase Y, was defective in strains bearing *vph4* mutations but was apparently normal in strains bearing any of the other *vph* mutations. Defects in vacuolar morphology at the light microscope level were evident in all Vph$^-$ mutants. Strains that contained representative mutant alleles of the 17 previously described *PEP* genes were assayed for vacuolar pH; mutations in seven of the *PEP* genes (including *PEP3*, *PEP5*, *PEP6* and *PEP7*) caused loss of vacuolar acidity.

THE vacuole in fungal cells is an organelle that bears resemblances both to mammalian lysosomes and the "central vacuoles" of higher plant cells. Thus, although the fungal vacuole contains a typically lysosomal complement of proteases and other hydrolytic enzymes (MATILE and WIEMKEN 1967; WIEMKEN, SCHELLENBERG and URECH 1979), it also contains substantial pools of free amino acids, inorganic ions, and other metabolites suggestive of the contents of plant vacuoles (MATILE 1978; WIEMKEN 1980). The physiological functions of these polymorphic organelles are not well understood. Because of the advantages offered by the methods of classical and molecular genetics in *Saccharomyces cerevisiae*, analysis of vacuolar structure and function in this organism provides an efficient approach to a deeper understanding of lysosomal biology in general.

Recent studies that focus on the biogenesis of the vacuole (*e.g.*, JONES *et al.* 1989; ROBINSON *et al.* 1988; ROTHMAN, HOWALD and STEVENS 1989; MOEHLE, DIXON and JONES 1989; MECHLER *et al.* 1988) only tangentially address the functions of this lysosome-like organelle. Studies of mutants of *S. cerevisiae* that lack particular vacuolar hydrolases directly address only the digestive functions of the vacuole (WOLF and EHMANN 1979, 1981; ZUBENKO and JONES 1981). Vacuoles are thought to participate in regulatory mechanisms concerned with amino acid and inorganic ion metabolism (WIEMKEN1980; KITAMOTO *et al.* 1988; EILAM, LAVI and GROSSOWICZ 1985; ANRAKU 1987), with osmotic and turgor pressure (MATILE 1978), and possibly with storage of nitrogen or other reserve materials (MATILE 1978; COOPER 1982). If components of the vacuole were indeed required for those (or other) functions, then the phenotypes of mutants that lack those specific components would provide the most convincing evidence concerning the role of the vacuole in performing those functions. We have devised an indirect screen in order to identify such mutants despite our lack of knowledge about their complete phenotype. The gene products and vacuolar functions identified using this screen would form part of an emerging, complete molecular catalog of the vacuole.

To identify vacuole-specific mutations in a way that would permit the detection of various types of gene products, we devised a screen that exploits the acidity of the vacuole. We developed a vacuolar pH assay based on a pH-sensitive fluorescent probe, and we confirmed that the vacuole in wild type yeast is mildly acidic, at pH 6.2 (PRESTON, MURPHY and JONES 1989). Using the same pH-sensitive fluorescent probe, we have now identified mutants that have nonacidic vacuoles. The rationale is that any mutation that impairs the steady-state pH gradient across the vacuolar membrane should cause the vacuolar pH to become neutral, in equilibrium with the cytoplasm. Very likely, some of the mutations identified by this strategy will be those that impair subunits of the vacuolar proton-pumping ATPase (review: FORGAC 1989). We hoped,

[1] Current address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138.

however, that the method would also detect mutations that affect the proton-permeability of a variety of vacuolar membrane components. If so, vacuolar pH (*vph*) mutations would provide access to novel components of the vacuole.

An initial round of screening for mutants that have nonacidic vacuoles identified eight new complementation groups of nuclear mutations. The corresponding loci were named *VPH1*, *VPH2* and *VPH4–VPH9*. The results reported here describe the isolation and initial characterization of these mutants. Since some Vph$^-$ isolates proved to have mutations in previously known *PEP* genes (JONES 1977), we also measured vacuolar pH in representatives from the *PEP* mutant collection. The results reveal a limited overlap between the *VPH* and *PEP* complementation groups.

MATERIALS AND METHODS

Strains, media and buffers: Mutants were obtained from the parental strains BJ1983, *MATα trp1* and BJ2665, *Mata leu2 ura3-52* (derivatives of X2180). Growth media YPD, YPG and SC have been described elsewhere (SHERMAN, FINK and HICKS, 1986). When YPD medium was buffered with phosphate or HEPES, as specified, the buffers were autoclaved separately from other ingredients and added aseptically to give the final concentrations specified. MHG buffer contained 50 mM HEPES, 50 mM MES, 50 mM KCl, 50 mM NaCl and 2% glucose, adjusted to pH 6.5 with NaOH.

6-Carboxyfluorescein diacetate (CFDA) indicator plates contained 2% glucose, 50 mM citric acid (adjusted to pH 3.0 with NaOH), 1% agarose (Seakem "ME") and 20 μM CFDA. The CFDA plates were made by dissolving glucose and citric acid at the stated concentrations and adjusting to pH 3.0. Agarose was then added and dissolved by minimal boiling in a microwave oven, after which the mixture was rapidly cooled to 50° in a cold water bath while stirring with a thermometer. CFDA was added by dilution from a 20 mM stock solution (in dimethylsulfoxide) and plates (Fisher, cat. no. 8-757-12, 100 × 15 mm) were poured immediately (30 ml per plate) and allowed to solidify rapidly by evaporative cooling, without lids (excessive heating hydrolyzed the agarose). Plates were used immediately or stored at 4° up to 3 days, after which partial hydrolysis of CFDA caused an unacceptable amount of background fluorescence. Prepared as described, fresh plates had no visually detectable background fluorescence under the conditions used to screen for mutants (see below).

Mutagenesis: Cultures of the parental strains were grown to stationary phase in YPD. Cells (2×10^9) were harvested by centrifugation and resuspended in 2 ml of 2.4% (w/v) ethyl methanesulfonate (EMS), 0.1 M sodium phosphate, pH 7. The cell suspension was incubated with gentle agitation for 2 hr at 30° and then diluted with 100 volumes of 5% (w/v) sodium thiosulfate to quench the mutagen. Cells were harvested by centrifugation, resuspended in 100 ml YPD plus 15% glycerol, and frozen immediately at −70°. Viability on YPD after mutagenesis was 10% for BJ1983 and 20% for BJ2665.

Genetics techniques: Tetrad analysis (using microtiter wells and a pronged replicator), complementation tests, and other routine yeast genetics operations were done as previously described (SHERMAN, FINK and HICKS 1986; SHERMAN 1991). In some cases, it was possible to follow the Vph$^-$ phenotype in tetrad replicas by performing fluorescence ratio assays on whole CFDA replica-plates (see below, and RESULTS). However, Vph$^-$ scores obtained in that way frequently were ambiguous; Vph$^-$ scores were always confirmed by fluorescence ratio microscopy, usually by examining samples of colonies that had been labeled on a CFDA replica-plate for 20–30 min and then resuspended in MHG buffer on a microscope slide. In some cases, nonuniform labeling of colonies on CFDA replica-plates gave ambiguous fluorescence ratio results; in those cases, the segregants were labeled and washed using liquid culture techniques described in detail elsewhere (PRESTON, MURPHY and JONES 1989).

Carboxypeptidase Y (CpY) activity was assayed using a colony overlay assay in which cleavage of *N*-acetyl-DL-phenylalanine-β-naphthyl ester by CpY is detected with fast garnet GBC salt, as previously described (JONES 1991a).

Vacuolar labeling with 6-carboxyfluorescein (6-CF): Yeast vacuoles were labeled by incubating cells with the membrane-permeant (nonfluorescent) diacetate derivative of 6-CF, CFDA, as described elsewhere, for broth cultures (PRESTON, MURPHY and JONES 1989), or with modifications, as specified, for replica-plates containing CFDA (see RESULTS). Under the conditions employed, CFDA enters yeast cells by diffusion and is hydrolyzed (presumably by various nonspecific esterase activities), releasing the doubly anionic, fluorescent 6-CF. Due to its hydrophilicity, 6-CF is temporarily trapped inside membrane-bounded compartments in which it forms. (Conveniently, leakage is negligible when labeled cells are kept on ice.) During and after incubation in CFDA, the intracellular distribution of fluorescence reflects competing rates of entry of nonfluorescent CFDA, hydrolysis and leakage of fluorescent 6-CF to and from all intracellular compartments, and from the cell as a whole. In practice, yeast vacuoles become much more fluorescent than other compartments; presumably that results from relatively higher levels of nonspecific esterase activities in vacuoles.

Fluorescence instrumentation for pH assays: Fluorometry of 6-CF solutions was done as described elsewhere (PRESTON, MURPHY and JONES 1989). Microscopic pH assays were done with an Axiophot (Zeiss) microscope equipped for epifluorescence with accessory excitation and emission filters, as described elsewhere (PRESTON, MURPHY and JONES 1989; PRINGLE *et al.* 1989). Quantitative pH assays were done with a FACS 440 dual laser flow cytometer (Becton Dickinson) as previously described (PRESTON, MURPHY and JONES 1989). Screening of replica-plates of mutagenized colonies was done by a modification of the microscopic assay, as described below (RESULTS). For the replica-plate screen, the assembly that contains the mercury vapor lamp and the two-position fluorescence filter set holder (taken from a Zeiss standard epifluorescence microscope) was removed from the microscope and set up on a ring stand. The 450- and 495-nm excitation filters and the corresponding dichroic mirrors in the filter holder were manually switched back and forth in the same way that they were used for microscopy, but the excitation beam that diverged from the bottom of the filter holder was aimed at a CFDA replica-plate located 10–20 cm below the filter holder. The illuminated plate was viewed through 520-nm long-pass emission filter "sunglasses" made from safety goggles that were fitted with the barrier filters from two Zeiss fluorescein filter sets. As in the microscopic assay, excitation intensities were normalized to establish a "null point" emission ratio while viewing labeled wild-type colonies (see RESULTS).

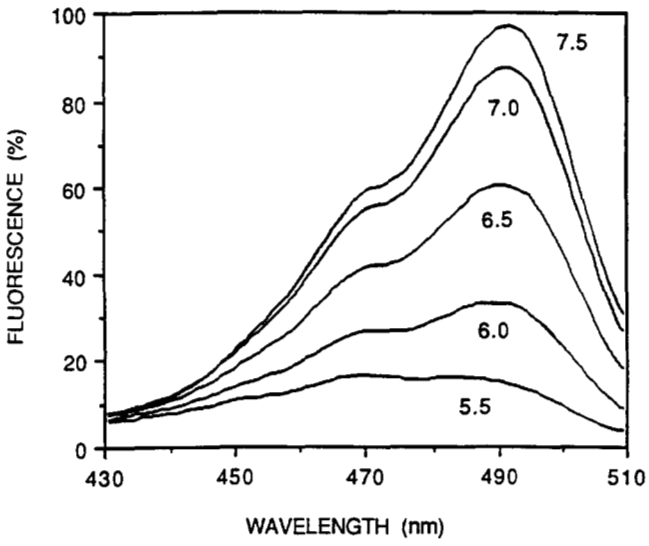

FIGURE 1.—Excitation spectra of 6-CF at different pH values. Solutions of 1 μM 6-CF buffered at the pH values indicated on the curves were scanned over the excitation range indicated on the abscissa. The relative fluorescence emission at 530 nm was recorded continuously.

RESULTS

Principles of fluorescence pH assay: Mutants that contained nonacidic vacuoles (Vph^- mutants) were identified using a null point fluorescence ratio pH assay. Since assays similar in principle might be useful in other genetic screens (depending on the availability of suitable fluorescent indicators), we describe the method in some detail here. The assay relies on the fact that the fluorescence of 6-CF (a vacuole-specific vital dye: see MATERIALS AND METHODS) is affected by pH differently at different excitation wavelengths (because acid dissociation alters the excitation spectrum of the dye). For example, a change in pH from 6 to 7 increases the fluorescence induced by 450-nm light (deep blue) by only 50%, but it more than doubles the fluorescence induced by 490-nm light (aquamarine) (Figure 1). Essentially, the ratio of the fluorescence intensities obtained with two different excitation wavelengths serves to report the pH of a solution of 6-CF (note that the same emission color, yellow, is observed regardless of the excitation wavelength, because pH has almost no effect on the shape of the 6-CF *emission* spectrum).

Fluorescence intensity ratios observed with experimental samples can be interpreted by comparison with a standard curve prepared by measuring intensity ratios observed with solutions of 6-CF buffered at known pH values. However, the following simplified calibration method was more appropriate for the purposes of genetic screening. The fluorescence ratio seen while viewing wild-type cells (comparing the intensities resulting from alternating 450- and 495-nm excitations) was used as a reference ratio against which the ratios of presumptive mutants were compared. For ease of comparison, the ratio observed with wild-type cells was normalized to 1.0 by suitably attenuating the intensity of the 495-nm excitation beam. That is, prior to scoring candidate mutants, the 495-nm excitation intensity was decreased to exactly counterbalance its relatively stronger excitatory effect (see Figure 1; the wild-type response resembles the curve for pH 6), so wild-type vacuoles appeared to be equally bright at either excitation wavelength. Under those conditions ("null point" ratio for wild type), the emission seen while viewing nonacidic mutants would increase abruptly as the excitation was switched from 450 to 495 nm, in accord with the expected increase in emission ratio. (Calculations from the spectra and visual tests with buffered 6-CF samples showed that, under the null point condition described, the brightness of neutral pH samples increased 60% as the excitation was switched from 450 to 495 nm, an amount that was easily detected by eye.)

Replica-plate screen for acidification mutants: A primary screen for Vph^- mutants was done by applying the ratio assay described above to whole replica-plates of colonies labeled with 6-CF. Mutagenized cells were plated on YPD to give 100 colonies per plate. After growth at 30° for 3 days, the colonies were replicated to CFDA indictor plates (MATERIALS AND METHODS). The replicas were incubated at ambient temperature in a dark room for 10–20 min to allow uptake and hydrolysis of CFDA, then they were alternately illuminated (from above) with 450- and 495-nm excitation light, by manually switching the excitation filters at about two cycles per second. With the excitation intensities set for seeing a null point ratio with wild-type colonies (see above), a few of the replicated colonies abruptly increased in brightness as the excitation was switched from 450 to 495 nm. Cells from these colonies were examined by fluorescence ratio microscopy to identify Vph^- mutants.

Of the 5500 colonies (EMS mutagenized; 10% survival) examined in the primary screen 294 presumptive mutants were identified. Microscopic examination revealed that most of these simply had abnormally high levels of cytoplasmic labeling that had masked the fluorescence of acidic vacuoles during the primary screen; those isolates were discarded. Thirty-nine isolates contained vacuoles (or vacuole-like entities: see below) that were uniformly nonacidic by ratio microscopy. These isolates were screened for vacuolar CpY activity to detect *pep* mutations, because we suspected there would be some overlap between the set of genes identified by the Vph^- phenotype and the *PEP* genes, which had been previously identified by CpY deficiencies (see below). Five of the Vph^- isolates proved to be deficient in CpY activity. Complementation tests indicated that the Vph^- and CpY^- phenotypes of four of these mutants were due to new mutant alleles of

TABLE 1

***vph* complementation groups**

Mutation[a]	Isolates	CPY activity[b]	Vacuolar morphology[c]	Growth at neutral pH[d]
vph1	16	+	D	+
vph2	1	+	C	−
vph4	4	−	V	−
vph5	1	+	C	−
vph6	1	+	D	−
vph7	1	+	D	−
vph8	2	+	C	−
vph9	1	+	D	+

[a] An isolate initially named *vph3* proved to be allelic to *pep6*.

[b] Carboxypeptidase Y, assayed by cleavage of *N*-acetyl-phenylalanine β-naphthyl ester in a colony overlay assay, diagnostic for *pep* mutations.

[c] D, dispersed; C, coalesced; V, vestigial (see text).

[d] Ability to grow on YPD medium buffered at pH 7 with 0.1 M sodium phosphate.

the *PEP3*, *PEP5*, *PEP6* and *PEP7* genes. The fifth CpY$^-$, Vph$^-$ isolate identified a gene (subsequently named *VPH4*) that was not represented in the set of known *PEP* genes.

Intercrosses among 28 of the Vph$^-$ isolates were made, and the resulting diploids were tested for complementation of the Vph$^-$ phenotype. The results established eight *VPH* complementation groups. Most of the mutants (16) fell into the single group named *VPH1* (Table 1). Eleven of the remaining mutants established seven additional groups, five of which are represented by single isolates. The last of these 28 mutants contained a semidominant mutation and was not assigned to a complementation group (see below). Seven of the original 39 Vph$^-$ isolates (all with normal CpY activity) have yet to be analyzed by complementation tests. The complementation groups were established by sequential elimination: we did not test every possible combination of all 28 isolates. In particular, since mutations in the *VPH1* gene caused unusually bright labeling by CFDA, all isolates with that phenotype were immediately tested for complementation with strains bearing the *vph1-1* allele. To distinguish between noncomplementation and allelic dominance in these tests, the parental strains were also crossed to wild type and the resulting diploids tested for the Vph$^-$ phenotype. Sixteen of the 17 isolates that labeled unusually brightly with CFDA contained recessive mutations that did not complement *vph1-1*. The other isolate had a semidominant allele that conferred the Vph$^-$ phenotype, so it was not assigned to a complementation group. For Vph$^-$ isolates that were assigned to groups other than the *VPH1* group, the successful complementation testing implies that the relevant *vph* mutations were all recessive. Representatives of the eight *VPH* complementation groups were crossed to wild type, the diploids were allowed to sporulate, and tetrads were dissected and analyzed (at least 12 tetrads in each case). In each case, 2:2 segregation of the Vph$^-$ character in the meiotic progeny established that single nuclear mutations were responsible for the Vph$^-$ phenotypes. These mutations identify eight different *VPH* genes, unless intragenic complementation were responsible for any complementation group.

The vacuolar pH of meiotic segregants (and of diploids, in dominance or complementation tests), was scored routinely by fluorescence ratio microscopy. To determine whether small differences in vacuolar pH, undetectable by a visual assay, might be present in meiotic segregants, we used flow cytometry to analyze two tetrads from a *vph1* × wild-type cross. The assay by flow cytometry uses 6-CF fluorescence in the same manner as the microscopic assay, but it provides calibrated photometric measurements of emission intensities that permit quantitative estimates of vacuolar pH (PRESTON, MURPHY and JONES 1989). The vacuolar pH (6.9) in all four Vph$^-$ segregants tested was essentially identical, as was the vacuolar pH (6.2) in all four wild-type segregants (data from one of these tetrads is shown in Figure 2). That result indicated that vacuolar pH was not very sensitive to minor differences in genetic background in the parental strains.

Segregants bearing the various *vph* mutations grew as well, or nearly as well, as wild-type segregants on standard yeast media (YPD, YPG or SC) at both 30° and 37°. In general, under laboratory conditions employed for the routine handling of strains, Vph$^-$ mutants appeared to be quite healthy. The only notable defects in the mutants (apart from their nonacidic vacuoles) were, in some, an inability to grow on medium buffered at neutral pH, and, in all, aberrant vacuolar morphologies.

As indicated (Table 1), the *vph2*, *vph4*, *vph5*, *vph6*, *vph7* and *vph8* mutations resulted in growth inhibition on YPD medium buffered at neutral pH with 0.1 M sodium phosphate. Similar inhibition occurred with 0.1 M potassium phosphate or HEPES buffers, but no inhibition occurred with unbuffered YPD (adjusted to pH 7 with NaOH), or with YPD containing 0.1 M NaCl or KCl. Thus, the inhibitory effect depended on buffering at neutral pH rather than on any specific ion or osmotic condition.

In conjunction with scoring the segregants' Vph$^-$ phenotype by ratio microscopy, abnormalities in vacuolar morphologies were evident in members of all complementation groups (Table 1). The most extreme example occurred in *vph4* segregants, which appeared to have very few 6-CF-labeled vesicular structures recognizable as "vacuoles." Instead, these segregants contained on the order of 100 or more bright pinpoints of fluorescence randomly distributed in the cytoplasm, along with one or two larger vesicles that resembled very small vacuoles. That same mor-

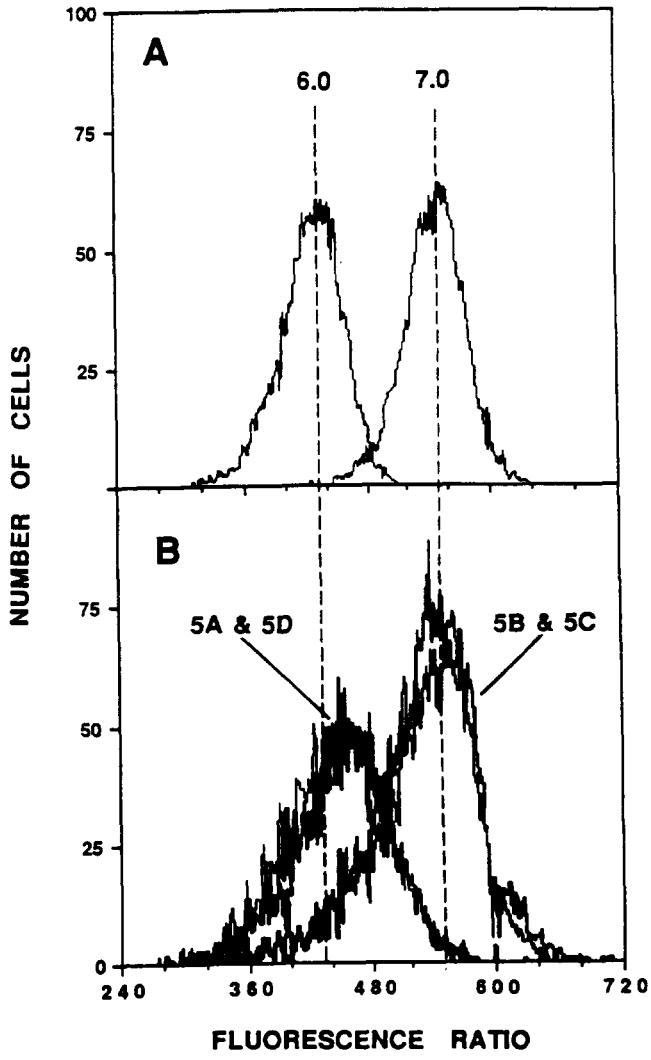

FIGURE 2.—Fluorescence ratio histograms of cells analyzed by dual excitation flow cytometry. Cells were labeled with 6-CF and analyzed by flow cytometry as described (MATERIALS AND METHODS). Abscissa is the relative fluorescence ratio (emission with 488-nm excitation/emission with 458-nm excitation) expressed in units of channel numbers of a multichannel pulse analyzer. For each histogram, 10,000 cells were analyzed. (A) Calibration histograms obtained by analyzing wild-type cells in buffers with the indicated pH values. The buffers contained ionophores that equilibrated the pH of labeled vacuoles to the pH of the external buffer. (B) Histograms for the four segregants of a tetrad (#5) from a backcross in which the *vph1-1* and *VPH1* alleles were segregating. 5A and 5D indicate segregants scored Vph^+ by ratio microscopy; 5B and 5C are Vph^- segregants. (The 5A and 5D histograms are essentially coincident except for random noise.)

phological phenotype ("vestigial vacuole") is seen also in strains bearing the *pep3*, *pep5* and *pep14* mutations (WOOLFORD *et al.* 1990; PRESTON *et al.* 1991). The morphological abnormalities evident in members of the other *vph* complementation groups could be described as apparent aberrations in the cell-cycle-dependent state of dispersion of the vacuole. In wild-type cells, the vacuole appeared to cycle between a dispersed state (10–30 small vacuoles clustered like grapes) and a coalesced state (a single, very large vacuole), in synchrony with the cell division, or budding cycle, as previously described (HARTWELL 1970; WIEMKEN, MATILE and MOOR 1970). Cells bearing the *vph2*, *vph5* or *vph8* mutations contained vacuoles in the coalesced state, primarily, regardless of the position of the cell in the cell cycle. Cells bearing the *vph1*, *vph6*, *vph7* or *vph9* mutations, on the other hand, contained vacuoles that appeared to be highly dispersed throughout the cell cycle.

TABLE 2

***pep* mutants that have nonacidic vacuoles**

Group	Character
1	Cells contain "vestigial vacuoles" consisting of one or two very small vacuoles and a large number (>100) of pointlike fluorescent bodies (like *vph4* mutants) *pep3*, *pep5*, *pep14*
2	Cells contain a single, spherical (coalescent) vacuole having a diameter ⅓–⅔ of the whole cell diameter (like *vph2*, *vph5*, and *vph8* mutants) *pep6*, *pep7*, *pep12*
3	Vacuolar morphology appears to be normal *pep21*

Strains bearing one of the 17 *pep* mutations were screened by fluorescence ratio microscopy (MATERIALS AND METHODS). The 10 *pep* mutations not listed in the table did not cause a detectable defect in vacuolar acidity.

As indicated (Table 1), the CpY activities for mutants in all but the *vph4* complementation group are normal. However, as noted above, four other Vph^- isolates that were CpY^- were excluded from consideration as "*vph*" mutants by historical circumstance, because they identified genes that were already known as *PEP* genes. We further explored the relationship between the *vph* and *pep* complementation groups by measuring the vacuolar pH in 17 strains, each containing a mutation in one of the 17 known *PEP* genes. Seven of these strains had nonacidic vacuoles as well as defects in vacuolar morphology that resembled those seen in *vph* mutants (Table 2). As described previously, the acidification defect in *pep12* strains differed from that seen in all other mutants in having a delayed expression in the microscopic pH assay (PRESTON, MURPHY and JONES 1989). We did not analyze tetrads to establish cosegregation of the Vph^- and Pep^- (CpY^-) phenotypes of *pep* mutants. In the case of the *pep3*, *pep5*, *pep6*, and *pep7* mutations, simultaneous noncomplementation of both phenotypes in tests of mutants that were obtained in independently designed Vph^- and Pep^- screens suggests that single genes determine both phenotypes. For the *pep3*, *pep5*, *pep7* and *pep12* mutations, the Vph^- and Pep^- characters resulted from constructed deletion/disruption alleles, again suggesting single-gene causality.

DISCUSSION

The vacuole is acidified by a single proton translocase that contains eight different subunits, based on

biochemical results (UCHIDA, OHSUMI and ANRAKU 1985; KANE, YAMASHIRO and STEVENS 1989) and on molecular genetic analysis of the *VAT2* and *TFP1* (*VMA1*) genes (YAMASHIRO *et al.* 1990; HIRATA *et al.* 1990; KANE *et al.* 1990). *VAT2* and *TFP1* encode the ATP-binding subunits of the proton translocase, and a constructed deletion of either one causes loss of vacuolar acidity, as measured by 6-CF fluorescence ratio methods (YAMASHIRO *et al.* 1990; R. A. PRESTON, unpublished results). Somewhat surprisingly, none of the *vph* mutations described here is an allele of *VAT2* or *TFP1*, based on the results of complementation tests (data not shown). However, we now know that *VPH1* encodes a 94-kD, integral membrane protein that is a subunit of the vacuolar proton pump (MANOLSON *et al.* 1992), so mutant alleles of *VAT2* and/or *TFP1* may yet surface in a more extensive screen for Vph$^-$ mutants. Apparently, however, mutations in structural genes for the eight (or so) subunits of the vacuolar proton pump itself account for only a fraction of all *vph* mutations. The number of genes known to cause the Vph$^-$ phenotype already exceeds estimates of the number of different subunits in the proton translocase. Also, although the numbers are small yet, the distribution of Vph$^-$ isolates among eight *vph* complementation groups (Table 1) suggests that a substantial number of additional *VPH* genes remain to be identified. It is encouraging that the Vph$^-$ screen identified new alleles of the *PEP3*, *PEP5*, *PEP6* and *PEP7* genes: those genes are known to be required for the expression of several vacuolar hydrolases, and the *PEP3* and *PEP5* genes are now known to encode peripheral components of the vacuolar membrane (WOOLFORD *et al.* 1990; PRESTON *et al.* 1991). Our results indicate, therefore, that loss of vacuolar acidity is a useful marker that serves to identify more than one functional class of vacuole-related genes.

The isolation of 39 Vph$^-$ mutants comprising at least eight *vph* complementation groups (and up to seven *pep* complementation groups) demonstrates that mutations that result in loss of vacuolar acidity are not necessarily lethal or difficult to identify. The viability of *vph* mutants [and of cells bearing null alleles of VAT2 and TFP1: YAMASHIRO *et al.* (1990) and HIRATA *et al.* (1990)] might suggest that neither acidity itself, nor functions that depend on energy transduction by the vacuolar proton gradient, are essential for growth in a laboratory environment. We discuss the energy transduction question first, then consider issues concerning vacuolar acidity *per se*. A number of amino acid (ANRAKU 1987) and ionic (OHSUMI and ANRAKU 1983; EILAM, LAVI and GROSSOWICZ 1985) transporters in the vacuolar membrane are thought to be energized by a proton gradient, and these presumably would not be functional in *vph* mutants. Two potential complications weaken that argument. First, essential transport activities might well occur in more than one form, energized independently, so forms not dependent on a proton gradient could account for the viability of Vph$^-$ mutants. Second, a loss of vacuolar acidity actually eliminates only one component of the electrochemical proton gradient across the vacuolar membrane. It is an assumption, based on analogies to energy transduction mechanisms in various cell types and organelles, that ATP-dependent proton transport entirely accounts for vacuolar membrane energization. In principle, yeast cells could have an ability to produce an electrical potential across the vacuolar membrane by active transport of ions other than protons. If so, depending on the magnitude of the electrical potential, protons in the vacuole of a *vph* mutant (at neutral pH) would still be at a high electrochemical potential relative to the (neutral pH) cytoplasm, and thereby able to drive whatever essential transporters they normally drive. [It might be relevant, here, that the pH of the vacuole in wild-type yeast, 6.2, is considerably higher than lysosomal pH values in other organisms (PRESTON, MURPHY and JONES 1989); despite that pH difference, values for the proton *electrochemical* gradient in yeast vacuoles and other lysosomes might not differ at all if yeast vacuoles could produce a membrane potential higher than that produced by lysosomes by mechanisms other than proton active transport.] Because of those complications, the viability of *vph* mutants, in itself, provides a rather weak argument for the dispensability of vacuolar membrane transport functions.

In mammalian cells, a low lysosomal pH (pH 5 or less) is thought to be necessary for the activities of many acid hydrolases, for various receptor-ligand equilibria, and possibly for the maturation of lysosomal zymogens. As mentioned above, the vacuolar pH (6.2) in wild-type yeast is not very acidic in comparison with pH values of 5 or less reported for mammalian lysosomes. That, in itself, suggests that acidity may not be as important in yeast vacuoles as in other types of lysosomes. Including *VAT2*, *TFP1*, a subset of the *PEP* genes, and all the *VPH* genes, a total of 17 different genes are now known to be required for normal acidity in yeast vacuoles. The viability (and vigor, generally) of cells bearing mutations in those genes strongly suggests that there is no essential vacuolar function that depends on the acidity in wild-type vacuoles. The question remains whether acidity, *per se* (rather than protons as entities that transduce energy, as discussed above), is necessary or beneficial for any vacuolar function. The *vph* mutants should be useful for answering that question on a case-by-case basis. The only case we address here concerns the hypothesis that an acidic vacuolar pH is necessary for

the maturation of vacuolar zymogens, as discussed below.

Of the 17 genes now known to be required for vacuolar acidity, nine (*VAT2*, *TFP1* and the *VPH* genes except *VPH4*) are dispensable for the synthesis of active CpY, based on results presented here and elsewhere (YAMASHIRO *et al.* 1990; R. A. PRESTON, unpublished results). Since, in wild type cells, maturation of CpY zymogen depends on protease A (PrA) (HEMMINGS *et al.* 1981; WOOLFORD *et al.* 1986; AMMERER *et al.* 1986), the presence of CpY activity implies the presence of active PrA. PrA, itself (the product of the *PEP4* gene) is an aspartyl protease that requires activation through an autocatalytic cleavage of its zymogen (JONES *et al.* 1989; JONES 1991b), and it has been thought that the activation depends on vacuolar acidity, by analogy to the maturation of pepsin, another aspartyl protease. The fact that mutations in any of nine different genes caused loss of vacuolar acidity but failed to block activation of PrA (by inference from normal CpY activities) might indicate that the maturation of PrA does not depend on vacuolar acidity. An alternative interpretation is possible, however, because of interactions between the processing pathways for the zymogens of PrA, CpY, and protease B (PrB) [see JONES *et al.* (1989) and JONES (1991b)]. That is, there are reasons to think that the mechanism of activation of PrA in wild-type cells (the postulated acidity-dependent mechanism) can be bypassed by either of two alternative mechanisms in the nonacidic vacuoles of Vph$^-$ mutants, one of these PrB-dependent and the other PrA-dependent (but not autocatalytic). A compelling test of the acidity dependence of the postulated mechanism in wild-type cells requires analysis of the activation of the precursor to PrA in strains that lack PrA and PrB activity completely, for example in strains bearing null alleles of the *PEP4* and *PRB1* genes and an inducible copy of the *PEP4* gene, and that test has not been done.

It was a remarkable and unexpected finding that many (but not all) *vph* mutations result in growth inhibition in medium buffered at neutral pH. During the course of this study, YAMASHIRO *et al.* (1990) independently discovered that disruptions of the *VAT2* gene also confer sensitivity to a buffered, neutral pH medium. The fact that *vph1* and *vph9* mutations fail to cause such sensitivity indicates that loss of vacuolar acidity, *per se*, cannot be the sole cause of the observed pH sensitivity. Indeed, since *VPH1* encodes a subunit of the vacuolar proton pump, loss of vacuolar proton translocating activity, *per se*, is insufficient to cause the pH sensitivity. (As with at least three independently isolated, induced mutations examined in this study, a null allele of *VPH1* constructed from the cloned gene also failed to cause sensitivity to neutral pH medium; M. F. MANOLSON, unpublished results). A determination of the epistasis relationship between *vph1* and *vat2* mutations might help to decipher the meaning of these different pH-sensitivity phenotypes.

It might be significant that the screen for Vph$^-$ mutants failed to identify mutations that completely eliminate all vacuolar structure, as defined by 6-CF labeling. If present among the 5500 colonies screened, such mutants would have been included among candidate Vph$^-$ isolates from CFDA replica-plates as a result of labeling of their (neutral pH) cytoplasm, which occurred to some extent in all replicated colonies. Despite a conscientious search for these mutants during secondary screening by microscopy, none was identified. Possibly these mutations exist but are rare compared with those that cause the Vph$^-$ phenotype. A more interesting possibility is that total loss of vacuolar structure is a lethal event. If so, it might seem that suitable hypomorphs would serve to identify the corresponding essential genes. However, it is not clear that "suitable" hypomorphs would be identifiable. Quite possibly, vacuolar pH (and/or CpY activities) would not be detectably abnormal for any alleles that were sufficiently leaky to confer viability.

We thank R. F. MURPHY, G. M. LAROCCA, M. F. MANOLSON, and members of the E.W.J. laboratory for assistance. This study was supported by grants from the National Institutes of Health, GM11329 (R.A.P.), GM29713 and DK18090 (E.W.J.), GM32508 (R. F. MURPHY), and the National Science Foundation, DCB-8351364 (R. F. MURPHY).

LITERATURE CITED

AMMERER, G., C. P. HUNTER, J. H. ROTHMAN, G. C. SAARI, L. A. VALLS and T. H. STEVENS, 1986 *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. **6:** 2490–2499.

ANRAKU, Y., 1987 pp. 255–265 in *Plant Vacuoles*, edited by B. MARIN. Plenum, New York.

COOPER, T. G., 1982 Nitrogen metabolism in *Saccharomyces cerevisiae*, pp. 39–100 in *The Molecular Biology of the Yeast Saccharomyces*, edited by J. N. STRATHERN, E. W. JONES and J. R. BROACH. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

EILAM, Y., H. LAVI and N. GROSSOWICZ, 1985 Cytoplasmic Ca^{2+} homeostasis maintained by a vacuolar Ca^{2+} transport system in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. **131:** 623–629.

FORGAC, M., 1989 Structure and function of vacuolar class of ATP-driven proton pumps. Physiol. Rev. **69:** 765–796.

HARTWELL, L. H., 1970 Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. J. Bacteriol. **104:** 1280–1285.

HEMMINGS, B. A., G. S. ZUBENKO, A. HASILIK and E. W. JONES, 1981 Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78:** 435–439.

HIRATA, R., Y. OHSUMI, A. NAKANO, H. KAWASAKI, K. SUZUKI and Y. ANRAKU, 1990 Molecular structure of a gene, VMA1, encoding the catalytic subunit of H^+-translocating adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. **265:** 6726–6733.

JONES, E. W., 1984 The synthesis and function of proteases in *Saccharomyces cerevisiae*: genetic approaches. Annu. Rev. Genet. **18:** 233–270.

JONES, E. W., 1991a Tackling the protease problem in *Saccharomyces cerevisiae*. Methods Enzymol. **194:** 428–453.

JONES, E. W., 1991b Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **266:** 7963–7966.

JONES, E. W., C. A. WOOLFORD, C. M. MOEHLE, J. A. NOBLE and M. A. INNIS, 1989 Genes, zymogens, and activation cascades of yeast vacuolar proteases, pp. 141–147 in *Cellular Proteases and Control Mechanisms*, edited by T.E. HUGLI. Alan R. Liss, New York.

KANE, P. M., C. T. YAMASHIRO and T. H. STEVENS, 1989 Biochemical characterization of the yeast vacuolar H^+-ATPase. J. Biol. Chem. **264:** 19236–19244.

KANE, P. M., C. T. YAMASHIRO, D. F. WOLCZYK, N. NEFF, M. GOEBLE and T. H. STEVENS, 1990 Protein splicing converts the yeast TFP1 gene product to the 69 kD subunit of the vacuolar H^+-adenosine triphosphatase. Science **250:** 651–657.

KITAMOTO, K., K. YOSHIZAWA, Y. OHSUMI and Y. ANRAKU, 1988 Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. J. Bacteriol. **170:** 2683–2686.

MANOLSON, M. F., D. PROTEAU, R. A. PRESTON, A. STENBIT, B. T. ROBERTS, M. A. HOYT, D. PREUSS, J. MULHOLLAND, D. BOTSTEIN and E. W. JONES, 1992 The *VPH1* gene encodes a 95 kDa integral membrane polypeptide required for *in vivo* assembly and activity of the yeast vacuolar H^+-ATPase. J. Biol. Chem. (in press).

MATILE, P., 1978 Biochemistry and function of vacuoles. Annu. Rev. Plant Physiol. **29:** 193–213.

MATILE, P., and A. WIEMKEN, 1967 The vacuole as the lysosome of the yeast cell. Arch. Mikrobiol. **56:** 148–155.

MECHLER, B., H. H. HIRSCH, H. MUELLER and D. H. WOLF, 1988 Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. EMBO J. **7:** 1705–1710.

MOEHLE, C. M., C. K. DIXON and E. W. JONES, 1989 Processing pathway for protease B of *Saccharomyces cerevisiae*. J. Cell Biol. **108:** 309–324.

OHSUMI, Y., and Y. ANRAKU, 1983 Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. **258:** 5614–5617.

PRESTON, R. A., R. F. MURPHY and E. W. JONES, 1989 Assay of vacuolar pH in yeast and identification of acidification-defective mutants. Proc. Natl. Acad. Sci. USA **86:** 7027–7031.

PRESTON, R. A., M. F. MANOLSON, K. BECHERER, E. WEIDENHAMMER, D. KIRKPATRICK, R. WRIGHT and E. W. JONES, 1991 Isolation and characterization of *PEP3*, a gene required for vacuolar biogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 5801–5812.

PRINGLE, J. R., R. A. PRESTON, A. E. M. ADAMS, T. STEARNS, D. G. DRUBIN, B. K. HAARER and E. W. JONES, 1989 Fluorescence microscopy methods for yeast. Methods Cell Biol. **31:** 357–435.

ROBINSON, J. S., D. J. KLIONSKY, L. M. BANTA and S. D. EMR, 1988 Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. **8:** 4936–4948.

ROTHMAN, J. H., I. HOWALD and T. H. STEVENS, 1989 Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. EMBO J. **8:** 2057–2065.

SHERMAN, F., 1991 Getting started with yeast. Methods Enzymol. **194:** 3–21.

SHERMAN, F., G. R. FINK and J. B. HICKS, 1986 *Methods in Yeast Genetics (A Laboratory Manual)*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

UCHIDA, E., Y. OHSUMI and Y. ANRAKU, 1985 Purification and properties of H^+-translocating, Mg^{2+}-adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. **260:** 1090–1095.

WIEMKEN, A., 1980 Compartmentation and control of amino acid utilization in yeast, pp. 225–237 in *Cell Compartmentation and Metabolic Channeling*, edited by L. NOVER, N. LYNEN and K. MOTHES. Elsevier, Amsterdam.

WIEMKEN, A., P. MATILE and H. MOOR, 1970 Vacuolar dynamics in synchronously budding yeast. Arch. Microbiol. **70:** 89–103.

WIEMKEN, A., M. SCHELLENBERG and K. URECH, 1979 Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Mikrobiol. **123:** 23–35.

WOLF, D., and C. EHMANN, 1979 Studies on a proteinase B mutant of yeast. Eur. J. Biochem. **98:** 375–384.

WOLF, D., and C. EHMANN, 1981 Carboxypeptidase S- and carboxypeptidase Y-deficient mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **147:** 418–426.

WOOLFORD, C. A., L. B. DANIELS, F. J. PARK, E. W. JONES, J. N. VANARSDELL and M. A. INNIS, 1986 The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. Mol. Cell. Biol. **6:** 2500–2510.

WOOLFORD, C. A., C. K. DIXON, M. F. MANOLSON, R. WRIGHT and E. W. JONES, 1990 Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. Genetics **125:** 739–752.

YAMASHIRO, C. T., P. M. KANE, D. F. WOLCZYK, R. A. PRESTON and T. H. STEVENS, 1990 Role of vacuolar acidification in protein sorting and zymogen activation: a genetic analysis of the yeast vacuolar proton-translocating ATPase. Mol. Cell. Biol. **10:** 3737–3749.

ZUBENKO, G. S., and E. W. JONES, 1981 Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. Genetics **97:** 45–64.

Communicating editor: M. CARLSON