Vacuole Partitioning During Meiotic Division in Yeast

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ABSTRACT

We have examined the partitioning of the yeast vacuole during meiotic division. In pulse-chase experiments, vacuoles labeled with the lumenal *ade2* fluorophore or the membrane-specific dye FM 4-64 were not inherited by haploid spores. Instead, these fluorescent markers were excluded from spores and trapped between the spore cell walls and the ascus. Serial optical sections using a confocal microscope confirmed that spores did not inherit detectable amounts of fluorescently labeled vacuoles. Moreover, indirect immunofluorescence studies established that an endogenous vacuolar membrane protein, alkaline phosphatase, and a soluable vacuolar protease, carboxypeptidase Y, were also detected outside spores after meiotic division. Spores that did not inherit *ade2*- or FM 4-64-labeled vacuoles did generate an organelle that could be visualized by subsequent staining with vacuole-specific fluorophores. These data contrast with genetic evidence that a soluble vacuolar protease is inherited by spores. When the partitioning of both types of markers was examined in sporulating cultures, the vacuolar protease activity was inherited by spores while fluorescently labeled vacuoles were largely excluded from spores. Our results indicate that the majority of the diploid vacuole, both soluble contents and membrane-bound components, are excluded from spores formed during meiotic division.

EUKARYOTIC cells contain a variety of membrane-bound organelles that organize the cytoplasm into functional compartments. During cell division, the production of viable progeny requires that these cytoplasmic structures are partitioned into daughter cells. A combination of genetic, cellular and biochemical approaches in a variety of cell types have been used to show that nuclei, the endoplasmic reticulum (ER), mitochondria, lysosomes (vacuoles), and the Golgi apparatus are all inherited by mechanisms that require the transmission of the organelle or an organelle template from the mother cell to the daughter cells during mitosis (ZELIGS and WOLLMAN 1979; STEVENS 1981; BIRKY 1983; WARREN 1985, 1993; LUCOCQ *et al.* 1987; LUCOCQ and WARREN 1987; WEISMAN *et al.* 1987, 1990; MCINTOSH and KOONCE 1989; MCCONNELL *et al.* 1990; SHAW and WICKNER 1991; YAFFE 1991; WARREN and WICKNER 1996). In contrast, analyses of organelle partitioning during meiotic division have focused mainly on the nucleus. These studies indicate that meiosis is considerably more complex than mitosis and that the molecular mechanisms used to segregate nuclear components during these two types of division can differ markedly. More recent studies suggest that the partitioning of cytoplasmic organelles other than the nucleus may differ during meiotic and mitotic division (MIYAKAWA *et al.* 1984). A detailed analysis of the fate of each organelle during meiotic division is an essential first step in understanding special features of the meiotic partitioning process.

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The budding yeast *Saccharomyces cerevisiae* is often used to study meiosis, in part, because all four products of a meiotic division are easily recovered. In diploid yeast cells, two successive rounds of chromosome segregation without nuclear envelope breakdown give rise to a four-lobed nucleus. Subsequently, each nuclear lobe becomes surrounded by a separate plasma membrane and cell wall to form four haploid spores that are retained within the mother cell (ascus) (MOENS 1970; LYNN and MAGEE 1970; MOENS and RAPPORT 1971; GUTH *et al.* 1972; BECKETT *et al.* 1973; BYERS 1981; BRIZA *et al.* 1988, 1990, 1994). Because the spores are formed within the mother cell, the mechanisms used to partition organelles during meiotic division in yeast may be very different than those used during mitotic division (budding). In particular, cytoplasmic organelles that are inherited by spores must be subdivided into at least four fragments that are positioned close to each nuclear lobe before enclosure of each lobe by plasma membrane. This idea is supported by the recent finding that, in yeast, mitochondrial inheritance during meiosis requires elaborate changes in mitochondrial morphology and distribution that do not occur during mitosis (MIYAKAWA *et al.* 1984). These directed mitochondrial movements serve to position part of the mitochondrial network close to each nuclear lobe where it can be included in newly formed spores. Similar changes in the distributions of other cytoplasmic organelles may position them close to the dividing nucleus during meiosis. According to this scenario, any organelle that failed to become perinuclearly localized would be excluded from the spores.

The most prominent organelle in the yeast cytoplasm is the lysosome-like vacuole. In addition to its roles in intracellular protein degradation, amino acid and ion storage, regulation of pH and osmolarity, and degradation of ligands internalized through the endocytic pathway (MESSENGUY *et al.* 1980; PRESTON *et al.* 1989; KLIONSKY *et al.* 1990), the vacuole may also serve as a storage site for deleterious compounds that accumulate during vegetative growth. Genetic analyses indicate that vacuolar protease activities play an important role in sporulation (ZUBENKO *et al.* 1979; MECHLER and WOLF 1981; WOLF and EHMANN 1981; ZUBENKO and JONES 1981; JONES 1984). Both proteinase A (PrA) and proteinase B (PrB) are required for the nitrogen-starvation induced protein degradation that accompanies sporulation. Diploids lacking PrA activity do not undergo meiosis or sporulation. PrB deficient diploid cells sporulate poorly and produce unusually small spores that remain embedded in a dense matrix. These spores are only able to germinate after they are manually dissected and removed from the ascus. This observation suggests that vacuolar PrB (or an enzyme activated by PrB) may aid in spore dispersal by digesting matrix material located between the spore and ascus walls.

Although a vacuole inheritance pathway has been clearly documented during mitotic division in yeast (WEISMAN *et al.* 1987; RAYMOND *et al.* 1990; GOMES DE MESQUITA *et al.* 1991), the fate of the vacuole during sporulation is less clear. Genetic studies suggest that proteases of the diploid vacuole are inherited by haploid spores during meiotic division (ZUBENKO *et al.* 1982). However, inheritance of the vacuolar compartment during sporulation has not been observed cytologically. Early studies using ultraviolet (UV) microscopy of vacuolar S-adenosylmethionine suggested that vacuoles fragment before spore formation (SVIHLA *et al.* 1964). The eventual loss of S-adenosylmethionine from these vacuole fragments, the disappearance of the vacuoles, and the brief appearance of S-adenosylmethionine outside spores was interpreted by the authors as a release of vacuolar contents (SVIHLA *et al.* 1964). These results could also be explained, however, by the complete lysis of vacuoles during sporulation or by the exclusion of diploid vacuoles from newly formed spores. Evidence for the exclusion of vacuoles from spores is provided by an early electron microscopy study that localized vacuoles to the space surrounding mature spores in the ascus (SCHWENCKE *et al.* 1977). Although these observations suggest that part or all of the vacuolar compartment may be excluded from spores, a vacuolar exclusion pathway has not been described during sporulation in yeast. Furthermore, such an exclusion model does not account for the observation that vacuolar protease activity is inherited by spores during meiotic division (ZUBENKO *et al.* 1982).

In this study, we describe alternative fates for vacuolar components during meiotic division in yeast. We show that both lumenal and membrane-associated markers of the diploid vacuole are largely excluded from spores. This bulk exclusion of vacuolar contents does not preclude the transmission of vacuolar protease activity to newly formed spores as described previously (ZUBENKO *et al.* 1982). Although a small amount of diploid vacuolar material may be directly inherited by spores, our results indicate that the majority of the diploid vacuole is not passed on to spores during meiotic division.

MATERIALS AND METHODS

Materials: Glucose, peptone, and yeast extract were obtained from BBL. All other materials used in media preparation were purchased from DIFCO. Adenine Sulfate, supplemental amino acids, Fast Garnet GBC, N-acetyl-DL-phenylalanine β-naphthyl ester, and β-Glucuronidase were purchased from Sigma. 5-fluoroorotic acid (5-FOA) was from American Biorganics, Inc. Zymolyase was purchased from ICN Biomedicals, Inc. Molecular Probes, Inc. supplied N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium, dibromide (FM 4-64).

Yeast strains: The *S. cerevisiae* strains used in this study are listed in Table 1. M2528 (provided by D. STILLMAN) was used in constructing the *ADE2* disruptions in strains AMY13, AMY14, AMY15, AMY19, AMY20, AMY26, AMY27, and AMY28. Disruptions were verified by PCR analysis (HERMAN and EMR 1990).

ADE2 disruptions were made in the strains SEY6210, FY10, and FY17 to construct AMY13, AMY14, and AMY15, respectively. BJ5171 was selected for growth on 5-FOA plates. The *ADE2* gene was then disrupted in the *ura3* cells to produce strain AMY19. X2180-1A was selected for growth on 5-FOA plates, and an *ade2* disruption was made in the *ura3* cells to produce AMY20. JSY117 was produced by mating strains JSY102-2A and JSY104-2C. AMY14 and AMY15 were mated to produce AMY26. AMY27 is a diploid derived from the strains SEY6211 and AMY13. The parent strains of AMY28 are AMY20 and AMY19.

Culture conditions and media: All strains were grown and maintained in liquid YPD media and on YPD plates. For some experiments, cells were grown in YPD supplemented with 800 μg/ml adenine sulfate or on YPD plates containing 16 mg/plate adenine sulfate. To induce sporulation, cells were transferred to sporulation medium containing amino acids at 25% of SMM (supplemented minimal medium) levels (KAISER *et al.* 1994). To suppress synthesis of the *ade2* fluorophore, 800 μg/ml adenine sulfate was added to the sporulation medium each day until sporulation was complete. Alternatively, the cells were patched out on sporulation plates containing supplemental amino acids and in some cases 16 mg/plate adenine sulfate. For all experiments the cultures were grown at 30°. Liquid cultures were grown in Innova 3000 water bath shakers (New Brunswick Scientific) at shaking speeds between 200 and 240 rpms.

Fluorescent labeling of vacuoles and sporulation: Growing *ade2* mutants overnight in YPD was sufficient to load the vacuoles with the *ade2* fluorophore. The cells were then pelleted and resuspended in sporulation media supplemented with amino acids. Adenine sulfate (800 μg/ml) was added to the sporulation culture each day to suppress synthesis of new fluorophore.

The dye FM 4-64 was also used to stain vacuole membranes (VIDA and EMR 1995). Ten milliliter cultures were grown overnight in YPD. In strains containing the *ade2* mutation, adenine sulfate was added to suppress synthesis of the *ade2* fluorophore. The cells were pelleted, resuspended in 2 ml of sporulation media and a stock solution of FM 4-64 in PBS was added to a concentration of 1 μg/ml. The culture was then

TABLE 1

Yeast strains

Strain	Genotype	Source
JSY102-2A	*Mat***a**, *ura3-52, lys2-801, ade2-101*	This laboratory
JSY104-2C	*Matα, ura3-52, ade2-101*	This laboratory
SEY6210	*Matα, ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, lys2-801 suc2-Δ9, GAL*	S. EMR (ROBINSON *et al.* 1988)
SEY6211	*Mat***a**, *ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, ade2, suc2-Δ9, GAL*	S. EMR (ROBINSON *et al.* 1988)
SEY2108	*Matα, ura3-52, leu2-3, 112, prc1::LEU2, suc2Δ9*	S. EMR (BANKAITIS *et al.* 1986)
SEY2109	*Mat***a**, *ura3-52, leu2-3, 112, prc1::LEU2, suc2Δ9*	S. EMR (BANKAITIS *et al.* 1986)
FY10	*Matα, mal, GAL2, ura3-52, leu2Δ1*	F. WINSTON (WINSTON *et al.* 1995)
FY17	*Mat***a**, *mal, GAL2, ura3-52, lys2Δ202*	F. WINSTON (WINSTON *et al.* 1995)[a]
X2180-1A	*Mat***a**,*SUC2, mal, mel, GAL2,* CUP1	YGSC[b]
BJ5171	*Matα, trp1, pep4-3*	E. JONES
DKY6280	*Mat***a**, *ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, pho8::TRP1*	D. KLIONSKY (KLIONSKY and EMR 1989)
DKY6281	*Matα, ura3-52, leu2-3, 112, lys2-801, his3-Δ200, trp1-Δ901, suc2-Δ9, pho8::TRP1*	D. KLIONSKY (CONRADT *et al.* 1994)
AMY13	*Matα, ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, lys2-801 suc2-Δ9, GAL, ade2::URA3*	This work
AMY14	*Matα, mal, GAL2, ura3-52, leu2Δ1, ade2::URA3*	This work
AMY15	*Mat***a**, *mal, GAL2, ura3-52, lys2Δ202, ade2::URA3*	This work
AMY19	*Matα, trp1, ura3*^{r}, *pep4-3, ade2::URA3*	This work
AMY20	*Mat***a**, *SUC2, mal, mel, GAL2, CUP1, ura3*^{r}, *ade2::URA3*	This work
JSY117	*Mat***a**/*α, ura3-52/ura3-52, lys2-801/LYS2, ade2-101/ade2-101*	This laboratory
FY17/FY10	*Mat***a**/*α, mal/mal, GAL2/GAL2, ura3-52/ura3-52, lys2Δ202/LYS2, LEU2/leu2Δ1*	This laboratory
SEY6211/SEY6210	*Mat***a**/*α, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, his3-Δ200/his3-Δ200, trp1-Δ901/trp1-Δ901, ade2/ADE2, suc2-Δ9/suc2-Δ9, GAL/GAL, LYS2/lys2-801*	This laboratory
SEY2109/SEY2108	*Mat***a**/*α, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, prc1::LEU2/prc1::LEU2, suc2Δ9/suc2Δ9*	This laboratory
DKY6280/DKY6281	*Mat***a**/*α, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, his3-Δ200/his3-Δ200, trp1-Δ901/trp1-Δ901, ade2-101/ADE2, suc2-Δ9/suc2-Δ9, LYS2/lys2-801, pho8::TRP1/pho8::TRP1*	This laboratory
X2180-1A/BJ5171	*Mat***a**/*α, TRP1/trp1, PEP4/pep4-3*	This laboratory
AMY26	*Mat***a**/*α, mal/mal, GAL2/GAL2, ura3-52/ura3-52, lys2Δ202/LYS2, ade2::URA3/ade2::URA3, LEU2/leu2Δ1*	This work
AMY27	*Mat***a**/*α, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, his3-Δ200/his3-Δ200, trp1-Δ901/trp1-Δ901, ade2/ade2::URA3, suc2-Δ9/suc2-Δ9, GAL/GAL*	This work
AMY28	*Mat***a**/*α, ura3*^{r}/*ura3*^{r}, *TRP1/trp1, ade2::URA3/ade2::URA3, PEP4/pep4-3*	This work

[a] Same as FY8 but *Mat***a**.
[b] Yeast Genetic Stock Center.
[r] Selected by growth on 5-FOA plates.

incubated for 30 min at 30°. Following incubation, the cells were pelleted to remove the excess dye and resuspended in sporulation media. Additional adenine sulfate (800 μg/ml) was added to cultures of *ade2* mutants. As a precaution against photobleaching of the FM 4-64 dye, the stained cells were sporulated in the dark. FM4-64 remained stable in the sporulating culture for at least 5 days. For time course studies with either the *ade2* fluorophore or the FM 4-64 dye, 3 μl of the sporulating culture were removed at time intervals after the cells were placed in sporulation media.

Immunofluorescence: Indirect immunofluorescence was performed essentially as described by ROBERTS *et al.* (1991). Cultures containing sporulated cells were fixed in 4.4% formaldehyde for 1 hr followed by an overnight incubation in 4% paraformaldehyde, 0.042 N NaOH, 0.25 M KH_2PO_4, and 0.001 M $MgCl_2$. Following fixation, the cells were placed in TE (200 mM Tris-Cl pH 8.0 and 20 mM EDTA) containing 1% β-Mercaptoethanol for 10 min. Cell walls were removed by incubating for 2–3 hr in SPM (1.2 M sorbitol, 50 mM KPO_4 pH 7.3, and 1 mM $MgCl_2$) containing 2% β-glucuronidase and 0.015% zymolyase-100T. After spheroplasting, the cells were washed in 1.2 M sorbitol and incubated in 1.2 M sorbitol containing 2% SDS for 2 min. The cells were then washed three times with 1.2 M sorbitol and finally, resuspended in 0.5–1 ml of 1.2 M sorbitol.

The fixed cells were allowed to settle onto immunofluorescence slides (Polysciences, Inc.) that had been pretreated with 1 mg/ml polylysine, washed with PBS-BSA (110 mM NaCl, 16 mM Na_2HPO_4, and 4 mM KH_2PO_4 at pH 7.3 with 5 mg/ml BSA) and incubated for 30 min in PBS-BSA. Rabbit anti-alkaline phosphatase (Pho8p) (provided by G. PAYNE) was incubated with the cells for a minimum of 16 hr. The

TABLE 2

***ade2* fluorophore distribution during meiosis and sporulation**

Diploid yeast strain	No. of asci analyzed	Percentage of asci with fluor outside of the spores	Percentage of asci with fluor detected in spores
JSY117	376	100	8.2
AMY26	302	100	6.3
AMY27	300	100	3.3
AMY28	302	100	4.3

alkaline phosphatase antibody was affinity purified and preabsorbed with fixed *pho8::TRP1* yeast cells. Cells were washed in PBS-BSA to remove excess antibody and were incubated for at least 2 hr with preabsorbed affinity purified fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). After repeated washes in PBS-BSA, the cells were placed in a mounting medium consisting of 90% glycerol, 10 mM Tris pH 8.0, and 10 mM NaN_3 for microscopic analysis.

For analysis of the localization of the vacuolar protease carboxypeptidase Y (CPY) in asci, fixed cells were incubated with a preabsorbed 20 μg/ml solution of a mouse monoclonal anti-yeast CPY (Molecular Probes, Inc.) for a minimum of 16 hr. Cells were subsequently incubated in preabsorbed affinity purified LRSC-conjugated Goat anti-mouse IgG (10 μg/ml) for 2 hr, washed and mounted as described above.

Microscopy: For time course analysis the cells were viewed with a Zeiss Axioplan equipped with differential interference contrast optics and epifluorescence capabilities. A Zeiss Acroplan-Neofluar 100× (NA 1.3) objective was used to observe the cells. A 450–490 nm bandpass filter was used for both the FM 4-64 and the *ade2* fluorophore fluorescence. Rhodamine, FITC, and 4′,6-diamidino-2-phenylindole (DAPI) fluorescence were visualized using 546, 450–490, and 365 nm bandpass filters, respectively.

For video capture of images, an identical Zeiss Axioplan with a Zeiss Plan-Neofluor 100× objective (NA 1.3) was used. Images were captured at an optivar setting of 1.25 or 1.6. The images were captured with a Zeiss ZVS-47E CCD video camera and ColorSNAP 32+ capture board (Computer Friends, Portland Oregon) with RGB inputs in an Apple Macintosh Quadra700. Adobe Photoshop (Adobe Systems, Mountain View, CA) was used to visualize the images and to assemble and label the figures. Adjustments to brightness and contrast were the only alterations that were used to improve the quality of the image. A Tektronix (Wilsonville, OR) Phaser IIsdx dye-sublimation printer was used to print figures 1–3, 5 and 6.

In some experiments the cells were examined by confocal microscopy with a Bio-Rad MRC-600 (Bio-Rad Microsciences, Cambridge, MA) attached to a Nikon optiphot microscope. A Zeiss 100× plan apochromat objective (NA 1.3) was used for viewing the cells. Confocal microscopy was used to optically serial section the cells. Optical sections were taken at 3 μm intervals.

APE assay for phenotypic lag: APE assays for CPY activity were performed essentially as described in ZUBENKO *et al.* (1982). Tetrads were dissected onto YPD plates and incubated for 2–3 days at 30°. Small portions of the colonies were then restreaked onto YPD plates. Strains that contained the *ade2* mutation were restreaked onto YPD plates containing 16 mg/plate adenine sulfate. APE assays were done on the original colonies. The colonies were then scored as red (R) or white (W). The subclones of the original colonies were allowed to grow for 3 or 4 days and restreaked onto new plates. APE assays were done at intervals of at least every fourth subcloning. The APE assays were continued until a 2:2 segregation of red:white colonies was observed.

RESULTS

A soluble marker of diploid vacuolar contents, the ade2 fluorophore, is excluded from spores during meiotic division: Inheritance of soluble vacuolar contents can be easily visualized in yeast by following the distribution of an endogenous fluorophore that accumulates in *ade2* mutants. When *ade2* strains are grown in limiting amounts of adenine, they produce a stable fluorophore that is localized in the vacuole (SMIRNOV *et al.* 1967; WEISMAN *et al.* 1987; BRUSCHI and CHUBA 1988). Upon reintroduction of high levels of adenine, synthesis of the *ade2* fluorophore is suppressed and the accumulated fluorophore can be used as a marker to follow subsequent changes in vacuolar morphology and distribution. The *ade2* fluorophore has previously been used to follow vacuolar inheritance during budding and the exchange of vacuolar contents in *S. cerevisiae* zygotes (WEISMAN *et al.* 1987, 1990; WEISMAN and WICKNER 1988; SHAW and WICKNER 1991; NICHOLSON *et al.* 1995). To determine how this compartment is partitioned during meiotic division, we followed the *ade2* fluorophore during sporulation in four strain backgrounds. The *ade2/ade2* diploid strains (JSY117, AMY26, AMY27, and AMY28) were grown overnight in low levels of adenine to induce synthesis of the *ade2* fluorophore. The cells were then transferred to sporulation medium and supplemented with adenine sulfate until sporulation occurred. In all four strains, the majority of the *ade2* fluorophore derived from the diploid vacuole was detected outside spores in all the asci examined (at least 300 asci from each strain) (Table 2). In a small percentage of these asci (<10% for each strain) fluorophore was also detected within spores (see below). As shown in Figure 1, *ade2* fluorophore that was not inherited by spores was found trapped in the space between the spore cell walls and the diploid cell membrane.

To rule out the possibility that *ade2* fluorophore was being inherited by spores but its presence was obscured by fluorescent material trapped outside the spore walls, 101 asci from an *ade2/ade2* strain (JSY117) were optically serial sectioned using a confocal microscope. Figure 2 shows a series of 3 μm optical sections through an

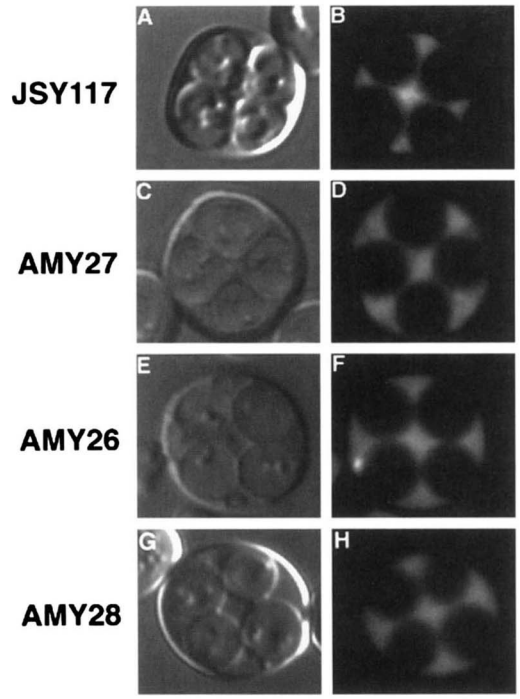

FIGURE 1.—Fluorescent contents of the diploid vacuole are excluded from newly formed spores in four yeast strains. Diploid strains homozygous for the *ade2-101* mutation were grown in YPD medium at 30° to accumulate the vacuolar *ade2* fluorophore. Labeled cells were transferred to sporulation medium and incubated with aeration at 30°. The medium was supplemented daily with adenine sulfate (800 μg/ml) to suppress synthesis of new *ade2* fluorophore. DIC images of asci are shown on the left (A, C, E, and G). The corresponding epifluorescence images (*ade2* fluorophore) are shown on the right (B, D, F, and H).

ascus containing four spores arranged in a tetrahedron (Figure 2, B–I). In progressive optical sections (Figure 2, B–I), the spores appear as dark spaces surrounded by the *ade2* fluorophore. In all 101 of these asci, the majority of the *ade2* fluorophore was found outside of the spores. In one of the asci (not shown), the *ade2* fluorophore was also detected inside spores (see below). Together, these results indicate that a large part of the lumenal contents of the vacuole are not inherited by spores during meiotic division.

We occasionally detected a fluorescent signal within spores (<10% of the total number of asci analyzed in each experiment). In these cases, the number of spores in the ascus that contained the fluorophore varied from one to four. Although the *ade2* fluorophore that accumulates in haploid or diploid vacuoles appears yellow when viewed using a 450–490 nm bandpass filter, the fluorophore within these spores often appeared green (for example see Figure 5). If the sporulated cells were left in culture for several days, the spore vacuoles became filled with the yellow *ade2* fluorophore and the green fluorescence within spores was no longer visible. While we do not rule out the possibility that the green fluorescence represents a small amount of *ade2* fluorophore from the diploid vacuole that is being inherited by some spores, we think it is likely that this green fluorescence represents newly synthesized *ade2* fluorophore in spore vacuoles. We have observed a similar change in vacuolar *ade2* fluorophore color when unlabeled *ade2/ade2* cells are sporulated and left in medium containing low levels of adenine (data not shown). The basis for this change in *ade2* fluorophore color is not known. In the asci containing fluorescent spore vacuoles, the majority of *ade2* fluorophore was still localized outside spores. Thus, even for these asci, most of the *ade2* fluorophore derived from the diploid vacuole does not appear to be included in spores. These results suggest that the majority of diploid vacuolar contents, as followed by the *ade2* fluorophore, are not being inherited by spores.

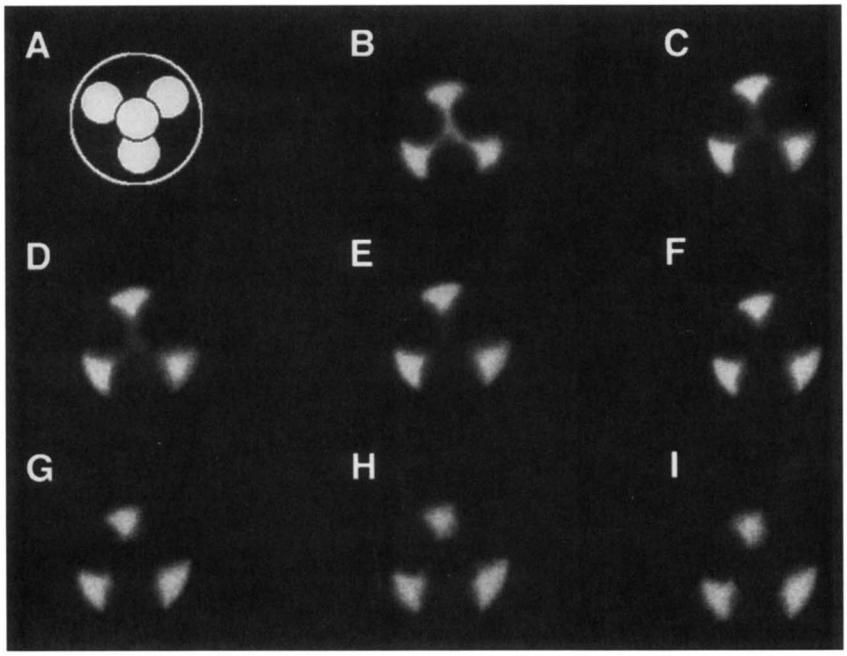

FIGURE 2.—Optical serial sections through an ascus indicate that the fluorescent contents of the diploid vacuole cannot be detected in spores. Confocal microscopy was used to visualize 3 μm serial optical sections through a JSY117 ascus. The cartoon in A depicts the tetrahedral arrangement of four spores within the ascus visualized in B–I. The three spores at the base of the tetrahedron lie in a single plane and appear as dark circles in B surrounded by the excluded *ade2* fluorophore. The fourth spore (located at the top of the tetrahedron) comes into view in subsequent sections (F–I) and appears as a dark circle in the center of the final image (I).

TABLE 3

FM 4-64 dye distribution during meiosis and sporulation

Diploid yeast strain	No. of asci analyzed	Percentage of asci with fluor outside of the spores	Percentage of asci with fluor detected in spores
JSY117	149	100	6.0
FY17/FY10	67	100	0.0
SEY6211/SEY6210	145	100	8.3

A vacuolar membrane-specific dye, FM 4-64, is excluded from spores during meiotic division: To determine whether the vacuolar membrane is also excluded from spores during meiotic division, we analyzed the distribution of the vacuolar membrane-specific dye, FM 4-64 (VIDA and EMR 1995), during sporulation in three diploid strains (JSY117, FY17/FY10, SEY6211/SEY6210). Diploid vacuoles in all three strains were labeled with the FM 4-64 dye and sporulated in medium supplemented with adenine sulfate. As observed for the *ade2* fluorophore (above), the majority of the FM 4-64 dye derived from the diploid vacuole membrane was detected outside spores in all the asci examined (Table 3 and Figure 3F). In a small percentage of these asci (<10% for each strain) the FM 4-64 dye was also detected within spores (see below). As shown in Figure 3F, the FM 4-64 dye that was not inherited by spores was found trapped in the space between the spore cell walls and the diploid cell membrane. Together, the observed changes in the distributions of the soluble *ade2* fluorophore and the membrane-associated FM 4-64 dye during sporulation suggest that the majority of the diploid vacuole is not inherited by spores during meiotic division.

If asci stained with the FM 4-64 dye were left in culture for several days after sporulation was complete, the vacuoles within some of the spores also became stained with the dye (Table 3). It is not clear whether the dye that stains the spore vacuolar membranes is inherited or relocalizes during or after sporulation. However, several observations suggest that labeling of the spore vacuolar membranes by the FM 4-64 dye results from relocalization of the dye. First, the FM 4-64 dye is not detected within spores until after spore walls have formed. Second, as shown in Figure 3F, the area stained with the FM 4-64 dye completely surrounds each of the spores at times before spore vacuole "labeling". Previous studies showed that when cells are exposed to FM 4-64, the dye initially stains the plasma membrane and is then transferred to the vacuole membrane (VIDA and EMR 1995). Based on these observations, it seems possible that FM 4-64 trapped in the ascus could be taken up by spores and transferred to the spore vacuole membrane. Alternatively, FM 4-64 released from dying unsporulated cells in these older sporulation cultures might be taken up by spore vacuoles. Finally, we do not rule out the possibility that FM 4-64 inherited from the diploid vacuole might not be visible in these experiments until a vacuole of some critical size is assembled in the spore. Regardless of the mechanism by which spore vacuoles become labeled with FM 4-64, our data clearly demonstrate that the majority of this dye remains outside newly formed spores after meiotic division.

Temporal changes in vacuolar morphology and distribution during sporulation: To further characterize the vacuole partitioning process, changes in diploid vacuolar morphology during sporulation were studied using both the soluble *ade2* fluorophore and the membrane-specific FM 4-64 dye. Diploid vacuoles (JSY117) were

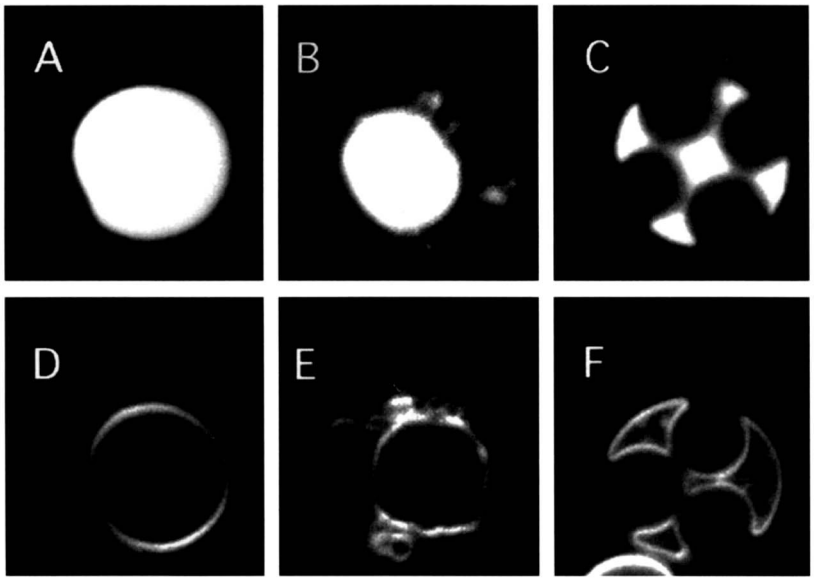

FIGURE 3.—Changes in wild-type vacuole morphology during sporulation. Diploid JSY117 vacuoles were labeled with the *ade2* fluorophore (A–C) or the vacuole membrane-specific dye FM 4-64 (D–F). The labeled cells were subsequently harvested and transferred to sporulation medium supplemented with adenine sulfate. Vacuole morphologies were recorded in samples removed from the cultures every 2–6 hr after transfer into sporulation medium. The three temporally distinct vacuole morphologies are shown: single large vacuoles (A and D), multiple vacuoles (B and E) and sporulated cells with the vacuolar fluorophore excluded (C and F).

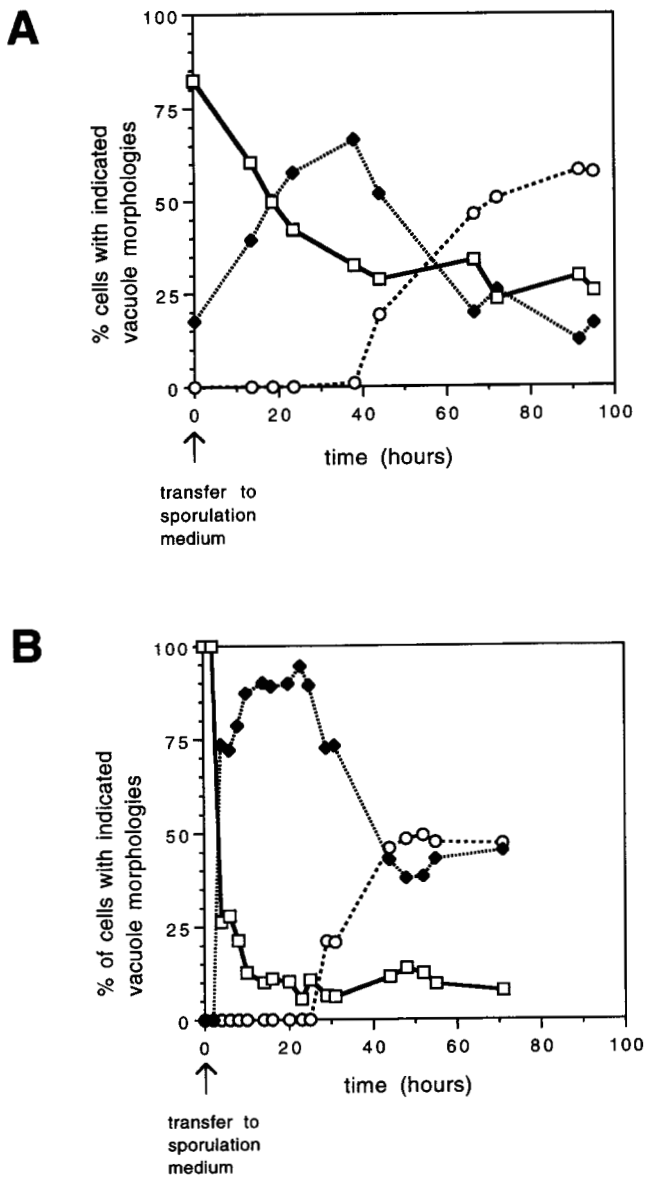

FIGURE 4.—Temporal analysis of vacuole morphology changes during sporulation. Vacuoles in JSY117 cells were labeled with the *ade2* fluorophore (A) or stained with FM 4-64 (B) and transferred into sporulation medium supplemented with adenine sulfate (see MATERIALS AND METHODS). Vacuole morphologies (300 cells/timepoint) were quantified in samples collected at the indicated times after transfer into sporulation medium. □, single vacuole; ◆, multiple vacuoles or large central vacuole surrounded by smaller satellite vacuoles; ○, asci with the *ade2* fluorophore or the FM 4-64 dye excluded from spores.

labeled with the *ade2* fluorophore or the FM 4-64 dye and sporulated in medium supplemented daily with adenine sulfate. Microscopic analysis of vacuolar morphologies was begun as soon as the cells were transferred into sporulation medium and was repeated at intervals until sporulation was complete. In cultures labeled with either the *ade2* fluorophore or FM 4-64, we observed three distinct changes in vacuolar morphology and distribution as sporulation proceeded (Figures 3 and 4). Initially, the majority of the diploid cells in the population contained a single, round vacuole (Figure 3, A and D; represented by the open squares at the zero timepoint in Figure 4, A and B). After ~2 hr, a decline in the percentage of cells containing single vacuoles was accompanied by an increase in the percentage of the cells containing multiple vacuolar compartments (not shown) or a large vacuole surrounded by smaller satellite vacuoles (Figure 3, B and E; compare open squares and closed diamonds in Figure 4, A and B). At later timepoints, the percentage of cells containing multiple or satellite vacuoles decreased (closed diamonds in Figure 4, A and B). This decrease coincided with the appearance of sporulated cells in the culture (Figure 3, C and F; compare closed diamonds and open circles in Figure 4, A and B). As described above, both the *ade2* fluorophore and the FM 4-64 dye were found outside the spores in these asci (Figure 3, C and F). In none of these strains could we observe a distinct intermediate between the multiple vacuolar stage and the stage at which vacuoles are excluded from spores.

Yeast vacuole morphology is sensitive to environmental changes, including variations of carbon source and temperature (PRINGLE *et al.* 1989). To determine whether the changes in vacuole morphology and distribution described above were the result of transferring cells from rich medium to sporulation medium, both of the haploid strains used to construct strain JSY117 (JSY102-2A and JSY104-2C) were grown overnight in YPD, stained with the vacuole membrane-specific dye FM 4-64 and subjected to conditions identical to a sporulation time-course. The vacuole morphologies of these haploid strains were monitored microscopically every several hours for 92 hr. In both haploid strains, the cells initially contained large single vacuoles similar to those observed in diploid JSY117 cells. Unlike JSY117 cells, however, the vacuole morphology in the haploid strains did not change in the course of the experiment (data not shown). This result indicates that the changes in vacuole morphology observed in the JSY117 strain are specific for sporulating cells and are not simply due to changes in growth medium. Our results suggest that the vesiculation and/or fragmentation of the diploid vacuole may play an important role in the partitioning of this organelle during sporulation.

Vacuoles are present in spores that lack a fluorescent vacuole after meiotic division: Previous studies of *vac* mutants have shown that vacuoles can be regenerated in daughter cells that fail to inherit a detectable amount of the organelle during mitosis (WEISMAN *et al.* 1990; SHAW and WICKNER 1991; GOMES DE MESQUITA, personal communication). Since haploid spores inherit very little or none of the diploid vacuole during meiotic division (as visualized with the *ade2* fluorophore and the FM 4-64 dye), we asked whether vacuoles were present in these spores. Figure 5 shows asci from a culture (JSY117) that was stained with the FM 4-64 dye before sporulation. Upon transfer to sporulation medium, the culture was supplemented with adenine sulfate to pre-

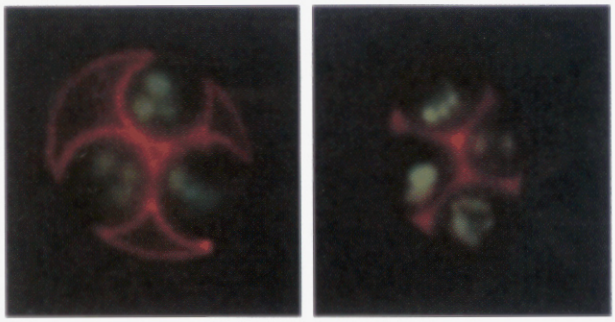

FIGURE 5.—Vacuoles can be detected in spores that fail to inherit fluorescent contents from the diploid vacuole. JSY117 cells stained with the vacuole-specific dye FM 4-64 were sporulated in medium supplemented with adenine sulfate (red membrane-associated fluorescence). After sporulation, the cells were incubated at 30° in low levels of adenine to induce synthesis of the *ade2* fluorophore in the spore vacuoles (green fluorescence).

vent synthesis of the *ade2* fluorophore. Vacuolar membranes labeled with the FM 4-64 dye are not inherited during meiotic division and remain trapped outside spores in the ascus as shown previously (Figure 5, red staining). After sporulation, the asci were left in culture medium until adenine supplies were depleted and the spores began synthesizing the *ade2* fluorophore. This newly synthesized *ade2* fluorophore accumulated in vacuolar-like structures within each spore (Figure 5, green fluorescence). The *ade2* fluorophore within spores initially appeared green but turned yellow upon longer incubations. Thus, vacuoles can apparently be generated in spores by a mechanism that requires little or no inheritance of diploid vacuolar contents.

Endogenous vacuolar proteins are also found outside spores after meiotic division: Failure of spores to inherit contents of the diploid vacuole might be due to the fact that these vacuoles were labeled with fluorescent compounds. To test this possibility, indirect immunofluorescence was used to analyze the distribution of the vacuolar membrane protein, alkaline phosphatase (ALP, encoded by the *PHO8* gene) in asci from *PHO8/PHO8* wild-type (SEY6210/SEY6211) and *pho8Δ/pho8Δ* disrupted (DKY6280/DKY6281) strains. To aid in the localization of the ALP staining, DIC imaging was used to determine the positions of the spores in the asci (Figure 6, A and D) and spore nuclei were localized by DAPI staining (Figure 6, B and E). When asci of the *PHO8/PHO8* strain (JSY117) were stained with anti-ALP antibody, the area between the spores was densely labeled (Figure 6C). In areas that were less densely stained, the signal often appeared somewhat punctate suggesting that smaller vacuolar vesicles might be present. The signal detected outside spores was specific for ALP and was not detected in asci from the diploid strain DKY6280/DKY6281 that does not express the alkaline phosphatase protein (Figure 6F). Indirect immunofluorescence of the vacuole membrane-associated 60 kD subunit of the vacuolar ATPase also resulted

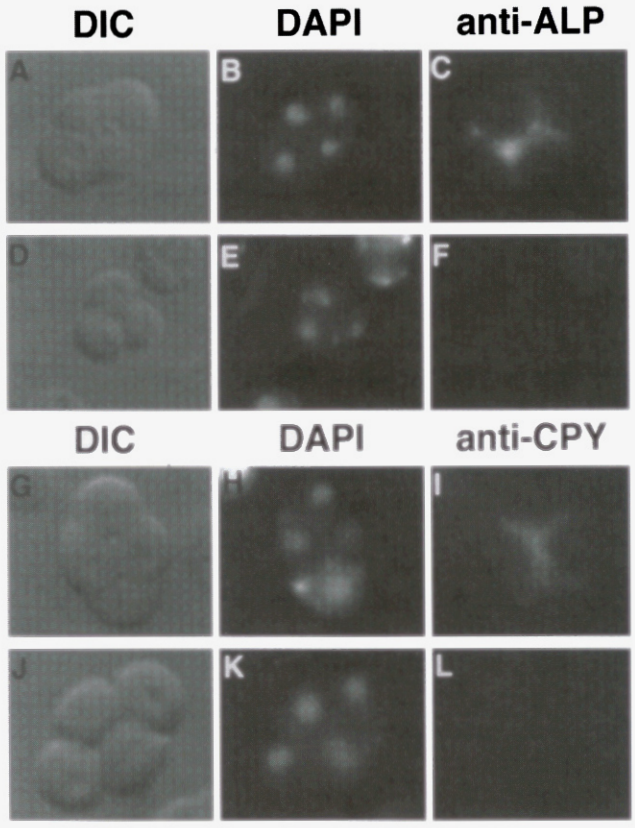

FIGURE 6.—A vacuole membrane protein (alkaline phosphatase) and a soluble vacuole protease (CPY) are localized outside spores. SEY6210/SEY6211 (*PHO8/PHO8, PRC1/PRC1*) (A–C, G–I), DKY6280/DKY6281 (*pho8Δ/pho8Δ*) (D—F), and SEY2108/SEY2109 (*prc1Δ/prc1Δ*) (J–L) diploid cells were sporulated in medium supplemented with adenine sulfate. In A–F, vacuolar membranes in fixed, spheroplasted, and SDS-treated cells were stained by indirect immunofluorescence using affinity purified and adsorbed anti-yeast alkaline phosphatase antibodies as described in MATERIALS AND METHODS. The lumenal vacuolar protease CPY was visualized in G–L by indirect immunofluorescence using preabsorbed monoclonal anti-yeast CPY antibodies (Molecular Probes). Individual asci were visualized by DIC (A, D, G, J), nuclei in the asci were visualized by DAPI staining (B, E, H, K), alkaline phosphatase immunofluorescence was viewed through a FITC filter set (C, F), and CPY immunofluorescence was viewed through a rhodamine filter set (I, L).

in labeling of the area between the spores (data not shown). We also examined the distribution of a soluble vacuolar protease, CPY, in *PRC1/PRC1* wild-type (SEY6210/SEY6211) and *prc1Δ/prc1Δ* (SEY2108/SEY2109) asci. As shown in Figure 6I, a large portion of this protein is also found outside of the spores. These data clearly show that a significant fraction of the diploid vacuole (both soluble and membrane-bound components) is found outside spores at the end of meiotic division.

Although all four spores produced by the SEY6210/SEY6211 strain are capable of producing both alkaline phosphatase and CPY, labeling of the spore vacuoles was seen in <1% of the cells and only with the alkaline phosphatase antibody. When visible, ALP labeling ap-

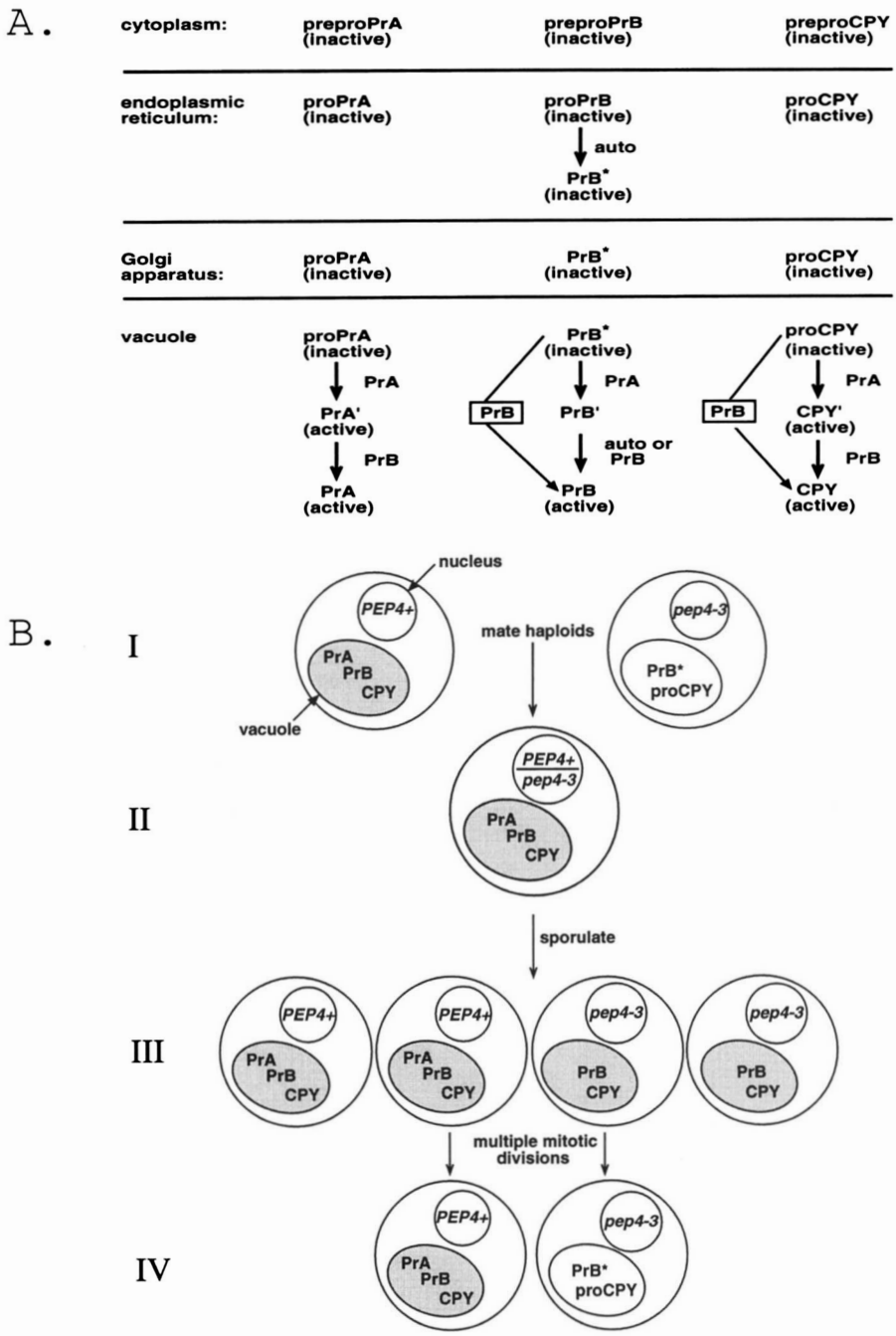

FIGURE 7.—The vacuolar protease cascade and phenotypic lag. (A) Proposed activation cascade for the vacuolar proteases PrA, PrB, and CPY. Initiation of the proteolytic cycle requires activation of PrA. Vacuolar proPrA can be activated autocatalytically (not shown) or proteolytically matured by PrA and PrB acting sequentially. Once active PrA is present, vacuolar PrB* can be activated in a two step reaction by PrA and then PrB or through an independent PrB-catalyzed reaction (indicated by the boxed PrB). This independent PrB-catalyzed reaction is thought to be responsible for the phenotypic lag of CPY activity observed in *pep4* meiotic segregants (see Figure 6B and text). proCPY is proteolytically cleaved by PrA to form CPY′. Further cleavage of CPY′ by PrB generates CPY. In the absence of PrA, proCPY can be converted to CPY by an independent PrB-catalyzed reaction (indicated by the boxed PrB) that is responsible for the phenotypic lag of CPY activity observed in *pep4* meiotic segregants (see Figure 6B and text). Adapted from WOOLFORD *et al.* (1993) and HIRSCH *et al.* (1992). (B) Phenotypic lag of CPY activity in *pep4-3* meiotic segregants. The diagram illustrates how the protease activities described in Figure 6A are expressed by the meiotic progeny of a *PEP4+/pep4-3* diploid. (I) Haploid *PEP4*$^{+}$ and *pep4-3* parental strains, (II) *PEP4+/pep4-3* diploid, (III) colonies initially formed from the haploid *PEP4+* and *pep4-3* meiotic progeny of the diploid in (II) all exhibit vacuolar CPY activity (shaded vacuoles), (IV) after multiple mitotic divisions, colonies derived from the *pep4-3* spores lose CPY activity (unshaded vacuoles).

peared as a ring around the spore vacuole (data not shown). This pattern of staining is consistent with the protein's vacuolar membrane localization (RAYMOND *et al.* 1990, 1992). The variability in spore vacuole labeling is probably due to the fact that primary or secondary antibodies do not always penetrate into the spores. These antibodies do appear to penetrate the ascus wall and membrane since we detect large amounts of ALP and CPY outside spores in the asci. Together with our observations of *ade2* fluorophore and FM 4-64 dye localization in asci, these indirect immunofluorescence studies support our conclusion that a significant portion of the diploid vacuole (both soluble and membrane components) is not inherited by spores.

Exclusion of the *ade2* fluorophore and phenotypic lag occur simultaneously in sporulating yeast cultures: Although the results described above demonstrate that vacuolar fluorophores and vacuolar membrane proteins can be excluded from spores, studies of soluble vacuolar proteases suggested that these proteins may be inherited during sporulation (ZUBENKO *et al.* 1982). Many of the vacuolar proteases, including PrA, PrB, and CPY, are synthesized as inactive zymogens that transit the early part of the secretory pathway (HEMMINGS *et al.* 1981; MECHLER *et al.* 1982; AMMERER *et al.* 1986; WOOLFORD *et al.* 1986; MECHLER *et al.* 1988; MOEHLE *et al.* 1989; JONES 1991). In the Golgi apparatus, these proteins are sorted from secretory proteins and delivered to the vacuole

where they are activated by a proteolytic cascade that begins with PrA (see Figure 7A). PrA (encoded by the *PEP4* gene) (WOOLFORD *et al.* 1986; AMMERER *et al.* 1986) is able to activate itself as well as a number of other vacuolar enzymes including PrB and CPY (JONES *et al.* 1982; ZUBENKO *et al.* 1983; VAN DEN HAZEL *et al.* 1992). Once activated, PrB can mature itself and CPY (Figure 7A, boxed PrB) (HIRSCH *et al.* 1992). Therefore, the activation of CPY can be catalyzed by PrA either directly or indirectly by PrA activated PrB.

Previous studies showed that PrA activity can be detected indirectly using a plate assay that distinguishes between the presence or absence of CPY activity (JONES 1977). In this assay, CPY^+ colonies turn red and CPY^- colonies remain white. Experiments described by ZUBENKO *et al.* (1982) showed that when a *pep4-3/PEP4* heterozygote was sporulated, colonies derived from all four of the spores, including the *pep4-3* mutant spores, initially exhibited CPY activity (Figure 7B). CPY activity was subsequently lost from cells containing the *pep4-3* allele if the colonies were allowed to undergo multiple rounds of mitotic division. This "phenotypic lag" was shown to be dependent on the presence of PrB (ZUBENKO *et al.* 1982), suggesting that the pronounced delay in the loss of active CPY from *pep4-3* mutant spores was due to the inheritance of active PrB from the diploid vacuole (ZUBENKO *et al.* 1982).

Our analysis, together with the work of ZUBENKO *et al.* (1982), suggests that vacuolar components can be differentially localized during yeast sporulation. Vacuolar contents and vacuolar membranes can be excluded from spores as indicated by the partitioning of the soluble *ade2* fluorophore, the soluble CPY protease, the membrane-associated FM 4-64 dye and the membrane-bound alkaline phosphatase protein. Studies using the vacuolar protease cascade as a vacuolar marker suggest that some lumenal contents of the organelle may be inherited by spores (ZUBENKO *et al.* 1982). We used a combination of these assays to determine whether individual sporulating yeast cells could exhibit both exclusion of the *ade2* fluorophore and phenotypic lag (inheritance of vacuolar protease activity). For these studies, we constructed two diploid strains. The first strain was a *pep4-3/PEP4* heterozygote and an *ADE2/ADE2* homozygote (BJ5171/X2180-1A). The second strain was a *pep4-3/PEP4* heterozygote and an *ade2Δ/ade2Δ* homozygote (AMY28). Both strains contained the mutant *pep4-3* allele used in the original phenotypic lag study (Table 4) (ZUBENKO *et al.* 1982). The *pep4-3* allele contains a G to A transition that results in the substitution of a stop codon for TRP at position 39 (WOOLFORD *et al.* 1993). These two strains were sporulated with (AMY28) and without (BJ5171/X2180-1A) loading the *ade2* fluorophore into the diploid vacuole. Once sporulation occurred, the asci from the AMY28 strain were scored for the exclusion of the *ade2* fluorophore. Asci from both strains were then dissected into tetrads, colonies derived from each spore were allowed to grow for 2–3 days and CPY assays were performed on the colonies.

As reported by ZUBENKO *et al.* (1982), all four spore colonies in 100% of the tetrads from the *pep4-3/PEP4 ADE2/ADE2* strain (BJ5171/X2180-1A) initially exhibited wild-type CPY activity (Table 4; initial CPY activity, 71 tetrads, 4R:0W). The initial CPY assay was performed on spore colonies that arose 2–3 days after dissection of the tetrads ($\sim 10^6$ cells/colony). These colonies were continuously restreaked (subcloned) onto YPD plates every 3–4 days. CPY assays were performed at intervals of every two to four subclonings. In our experiments, the phenotypic lag in *pep4-3* cells extended well beyond three successive subclonings (ZUBENKO *et al.* 1982). All of the subcloned spore colonies (including the *pep4-3* mutant colonies) exhibited wild-type CPY activity for at least 3 weeks after dissection of the asci (approximately five subclonings). After 3–6 months of continuous subcloning, most of the *pep4-3* colonies began to express the mutant phenotype and the majority of the tetrads (68 of 71) segregated $2CPY^+$:$2CPY^-$ (2R:2W). In three of the 71 tetrads, three of the four spore colonies continued to test positive for CPY activity after 9 months (Table 4). It is possible that one of the *pep4-3* segregants in each of these three tetrads has been converted to a wild-type *PEP4* allele (gene conversion). Alternatively, one of the *pep4-3* segregants in each of these three tetrads could be exhibiting an extreme case of phenotypic lag.

When the *pep4-3/PEP4 ade2Δ/ade2Δ* strain (AMY28) was sporulated, *ade2* fluorophore from the diploid vacuole was localized outside of the spores in 100% of the asci (Table 2). In <5% of these asci, the *ade2* fluorophore could also be detected in one or more spores. The lack of inheritance of the *ade2* fluorophore in this strain did not prevent phenotypic lag in *pep4-3* meiotic segregants. In the 37 tetrads examined, all of the spore colonies exhibited some degree of CPY activity in the initial assay (2–3 days of growth) (Table 4). Unlike spore colonies derived from the *pep4-3/PEP4 ADE2/ADE2* strain (BJ5171/X2180-1A), however, some of the colonies turned pink instead of red. When these pink colonies were streaked to yield single cells and allowed to grow, the resulting colonies turned red, pink or white in the CPY assay. ZUBENKO *et al.* (1982) reported a similar mixture of red, pink and white sectored subclones of *pep4-3* genotype. These mixed colonies are composed of *pep4-3* mutant cells that contain varying amounts of CPY activity. Once again, the CPY^+ phenotype extended for many generations in *pep4-3* cells derived from strain AMY28. Spore colonies from the AMY28 tetrads began segregating 2:2 for CPY activity after 3 weeks of continuous subcloning. Genotypically *pep4-3* colonies in 30 of the 37 tetrads lost CPY activity after 1 month. The *pep4-3* colonies in four of the tetrads exhibited phenotypic lag for as long as 6 months as described for the *ADE2/ADE2* parent strain (BJ5171/X2180-1A, see above). Finally, *pep4-3* colonies in three of the 37 tetrads analyzed

TABLE 4

Analysis of *ade2* fluorophore partitioning and phenotypic lag during meiotic division

Yeast strain	*PEP4* genotype	*ADE2* genotype	No. of tetrads analyzed	Presence of *ade2* fluorophore[a]	CPY analysis R(CPY⁺):W(CPY⁻) Initial	Final
BJ5171 / X2180-1A	*pep4-3* / *PEP4*	*ADE2* / *ADE2*	71	No	(71)4R:0W[b]	(68)2R:2W (3)3R:1W[c]
AMY28	*pep4-3* / *PEP4*	*ade2:URA3* / *ade2::URA3*	37	Yes	(37)4R:0W	(34)2R:2W (3)3R:1W[c]

[a] In all strains, the *ade2* fluorophore was found outside the spores.
[b] The number of tetrads are shown in parentheses.
[c] These tetrads tested positive for CPY activity after 6 months of continuous culture.

continued to test positive for CPY activity after 6 months. As discussed above, the segregation of wild-type CPY activity in these tetrads could result from gene conversion events or may simply represent extreme cases of phenotypic lag. When identical experiments were performed with the AMY28 strain without first labeling the diploid vacuole with the *ade2* fluorophore, the resulting tetrads also exhibited phenotypic lag (data not shown). The fact that both phenotypic lag (inheritance by *pep4-3* spores) and the exclusion of the *ade2* fluorophore are observed in the AMY28 strain supports the model that vacuolar components can be partitioned differently during meiotic division.

DISCUSSION

We have used a variety of techniques to determine the fate of the diploid vacuole during meiotic division. When diploid cells containing vacuoles labeled with the *ade2* fluorophore or the FM 4-64 dye are sporulated, the majority of the labeled material ends up outside of the spores. The distribution of these compounds should accurately reflect the distribution of diploid vacuolar components in the asci because both the *ade2* fluorophore (a large polymer inside the vacuole) and the vacuole membrane-specific FM 4-64 dye are extremely stable in dividing yeast cells and do not degrade once in the organelle (WEISMAN *et al.* 1987; WEISMAN and WICKNER 1988; SHAW and WICKNER 1991; VIDA and EMR 1995). We also observed that the endogenous vacuolar proteins alkaline phosphatase and CPY are localized outside spores in asci. Alkaline phosphatase is an integral membrane protein of the vacuole and is unlikely to redistribute from this membrane. The fact that all four of these markers are similarly localized after meiotic division argues that both the vacuolar membrane and soluble vacuolar contents are largely excluded from spores.

While our results provide compelling evidence for the exclusion of diploid vacuolar contents from spores, data presented initially by ZUBENKO *et al.* (1982) and repeated here suggest that spores inherit vacuolar protease activity. In this report, we also demonstrate that *pep4-3* spores that fail to inherit the vacuolar *ade2* fluorophore from diploid cells still exhibit vacuolar protease activity. One interpretation of these results is that spores inherit a small portion of the diploid vacuole while the remainder of the organelle is excluded. It is possible that only the CPY plate assay is sufficiently sensitive to detect the inheritance of very small amounts of vacuolar material by spores. For this reason, we do not exclude the possibility that spores inherit small amounts of the *ade2* fluorophore or FM4-64 dye-labeled membranes that we are unable to detect. Further experiments will be required to determine the extent to which vacuolar contents are inherited by spores and to identify the specific cellular compartment that is being inherited. An alternative interpretation of these results is that spores do not inherit any of the diploid vacuole but instead inherit PrA messenger RNA from the diploid cell. The autocatalytic activation of newly synthesized PrA protease in *pep4* spores could initiate the proteolytic cascade in newly generated spore vacuoles and account for the prolonged CPY activity observed in *pep4* spore colonies. Regardless of the mechanism responsible for incorporating vacuolar protease activity in spores, our data clearly show that the the bulk of the diploid vacuole is excluded from spores.

Although the localization of vacuolar components outside spores could result from an active exclusion mechanism, we think it is more likely that the failure to include vacuolar components in spores reflects their distribution in the cell at the time the spore membranes form. Organelles that are inherited by spores, such as mitochondria, are known to undergo dramatic changes in morphology and distribution during meiosis and sporulation. The string-like mitochondrial network condenses in the periphery of the cell and then moves adjacent to the nucleus where it remains throughout meiotic division (MIYAKAWA *et al.* 1984). These specific movements ultimately result in the association of a portion of the mitochondrial network with each nuclear lobe and ensure that mitochondria are inherited by each spore. In order for the vacuole to be inherited by spores, it seems likely that this compartment would also have to undergo changes in morphology and distribu-

tion. The temporal changes in vacuole morphology we describe suggest that vacuoles vesiculate before spore formation. Unlike the mitochondrial network, however, these vacuolar compartments remain distributed throughout the cytoplasm during sporulation. Since the spore membrane is initially laid down very close to each nuclear lobe and includes only a small volume of cytoplasm (LYNN and MAGEE 1970; MOENS 1970; GUTH *et al.* 1972; BECKETT *et al.* 1973), a significant fraction of this vacuolar material would be excluded from spores as observed in our experiments.

Exclusion of vacuolar components from spores formed during meiotic division may serve several important functions. First, the yeast vacuole acts as a storage compartment in both haploid and diploid cells. In the course of cell metabolism, compounds that are not used by or are deleterious to the cell, such as the *ade2* fluorophore, may routinely be localized to the vacuole. Exclusion of diploid vacuolar contents from haploid spores provides a mechanism for purging this undesirable material. Second, the release of vacuolar proteases into the ascus may be necessary for germination of the spores. This idea is supported by the observation that, although diploids homozygous for a mutation in the *PRB1* gene are able to sporulate, the spores remain embedded in a dense matrix and are unable to germinate unless they are manually dissected out of the ascus (ZUBENKO and JONES 1981). The release from the diploid vacuole of PrB or an enzyme activated by PrB may be responsible for dissolving the matrix that surrounds the spores (ZUBENKO and JONES 1981).

Our results indicate that failure to inherit a detectable portion of the diploid vacuole has little affect on spore germination or viability. This is presumably because spores are able to generate a vacuole before germination occurs. This formation of new vacuoles has also been observed in yeast *vac* mutants that fail to partition detectable vacuoles into buds during mitotic division (WEISMAN *et al.* 1990; SHAW and WICKNER 1991). In the case of the *vac2* mutant, mitotic daughter cells that do not inherit vacuoles quickly form vesicles that fuse to form a new organelle before the next round of budding (GOMES DE MESQUITA, personal communication). These daughter cells divide at rates similar to wild-type cells, therefore, the lack of vacuole inheritance does not appear to impair the rate of cell division. Our double labeling experiments suggest that a similar vacuolar generation process could be occurring in spores. Whether formation of new vacuoles in daughter cells or spores represents *de novo* synthesis or requires a template is not clear.

Studies in mammalian cells suggest that organelles linked to the ER by membrane traffic (such as the Golgi apparatus) can be synthesized *de novo* as long as a part of the ER is inherited by daughter cells (ZORN *et al.* 1979; MANIOTIS and SCHLIWA 1991; WARREN and WICKNER 1996). If an analogous regeneration mechanism exists in yeast, it may not be necessary for spores to inherit a vacuole from the diploid cell. Instead, spores could inherit organelles from the secretory pathway or a prevacuolar compartment and use these membranes to generate a new vacuole. Interestingly, a prevacuolar compartment was recently identified in a subset of vacuole protein sorting (*vps*) mutants that fail to target proteins from the Golgi to the vacuole (RAYMOND *et al.* 1992). These class E *vps* mutants accumulate 250–400 nm structures adjacent to the vacuole that contain vacuolar proteins including the 60 kD vacuolar ATPase, proteinase A and CPY (RAYMOND *et al.* 1992; VIDA *et al.* 1993; CEREGHINO *et al.* 1995). This compartment also contains Golgi proteins such as the CPY sorting receptor (Vps10p) and at least one plasma membrane receptor (RAYMOND *et al.* 1992; DAVIS *et al.* 1993; CEREGHINO *et al.* 1995; PIPER *et al.* 1995). Based on these results, it has been proposed that the class E compartment functions as a late endosome through which vacuolar proteins and endocytosed materials transit on their way to the vacuole. The recent observation that the degradation of Vps10p is *PEP4* dependent in class E *vps* mutants indicates that PrA is active in the class E compartment (CEREGHINO *et al.* 1995). In the absence of vacuolar inheritance, it is possible that such prevacuolar compartments are inherited by spores or generated in spores from other inherited membranes. These prevacuolar compartments could then mature into vacuoles in newly formed spores. Since active vacuolar proteases are present in these prevacuolar compartments, this scenario could also account for the persistence of CPY activity in *pep4* meiotic segregants. To test this model, it will be important to evaluate the fate of the prevacuolar compartment in class E *vps* mutants and wild-type cells during meiotic division.

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