Multiple Methods of Visualizing the Yeast Vacuole Permit Evaluation of Its Morphology and Inheritance during the Cell Cycle

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Abstract. The vacuole of the yeast *Saccharomyces cerevisiae* was visualized with three unrelated fluorescent dyes: FITC-dextran, quinacrine, and an endogenous fluorophore produced in *ade2* yeast. FITC-dextran, which enters cells by endocytosis, had been previously developed as a vital stain for yeast vacuoles. Quinacrine, which diffuses across membranes and accumulates in acidic compartments in mammalian cells, can also be used as a marker for yeast vacuoles. *ade2* yeast accumulate an endogenous fluorophore in their vacuoles. Using these stains, yeast were examined for vacuole morphology throughout the cell division cycle. In both the parent cell and the bud, a single vacuole was the most common morphology at every stage. Two or more vacuoles could also be found in the mother cell or in the bud; however, this morphology was not correlated with any stage of the cell division cycle. Even small buds (in early S phase) often contained a small vacuole. By the time the bud was half the diameter of the mother cell, it almost always bore a vacuole. This picture of vacuole division and segregation differs from what is seen with synchronized cultures. In *ade2* yeast, the bud usually inherits a substantial portion of its vacuole contents from the mother cell. We propose that vacuolar segregation is accomplished by vesicular traffic between the parent cell and the bud.

CELL division requires the partitioning of each cytoplasmic organelle into the daughter cells. This process has been studied for the Golgi complex, endoplasmic reticulum (Warren, 1985), peroxisomes (Lazarow and Fujiki, 1985), mitochondria, and chloroplasts (Birky, 1983). Several specific questions can be asked about this partitioning. How does each organelle divide? How is this division coordinated with the cell cycle? How are organelles segregated into the daughter cells, and what mechanism assures that each cell receives the proper proportion of the organelles?

The vacuole (a lysosome-like organelle) of *Saccharomyces cervisiae* is particularly well suited to the study of organelle division and segregation. Because of its low density, it has been isolated in high purity (Wiemken, 1975). Many marker enzymes of the vacuole are known (Jones, 1984), some of which have been purified and their genes cloned (Stevens et al., 1986; Woolford et al., 1986; Ammerer et al., 1986). It is often the largest organelle of the cell and is readily visualized by Nomarski or phase-contrast optics, especially in the G_1 portion of the cell cycle.

Earlier observations of yeast vacuoles with phase-contrast microscopy and electron microscopy showed that there is a single, large vacuole during G_1 (Wiemken et al., 1970; Severs et al., 1976). These studies, which were performed on synchronized cells, suggested that the vacuole fragments into many small vacuoles during bud emergence in S phase. Later in the cycle, some vacuole fragments are thought to migrate into the bud and, upon completion of mitosis and cytokinesis, the vacuoles in both the bud and the mother fuse to form a single large vacuole in each.

It is well known that the vacuole, as a primary storage organelle, changes its contents in response to physiological conditions (Messenguy et al., 1980; Wiemken, 1980). Conditions in which the vacuole and surrounding cytoplasm have comparable refractive indices may make even a larger vacuole difficult to visualize by light microscopy. Furthermore, small vacuoles may not be resolved by this technique, and no firm guidelines exist for distinguishing small vacuoles from other organelles such as secretory vesicles or other vesicular components of the secretory pathway. We have therefore labeled vacuoles with fluorescent dyes in order to study their structure throughout the cell cycle. FITC-dextran enters the cell via endocytosis and concentrates in the vacuole (Makarow, 1985). Quinacrine, which has been previously used as a fluorescent stain for lysosomes (Allison and Young, 1964), crosses membranes by diffusion and concentrates in the acidic vacuole compartment. Using these labeling techniques on asynchronous cultures of *S. cerevisiae*, we observe that a major large vacuole is the most prevalent morphology at all stages of the cell division cycle. In the course of these studies, we also observed that the vacuole in *ade2*

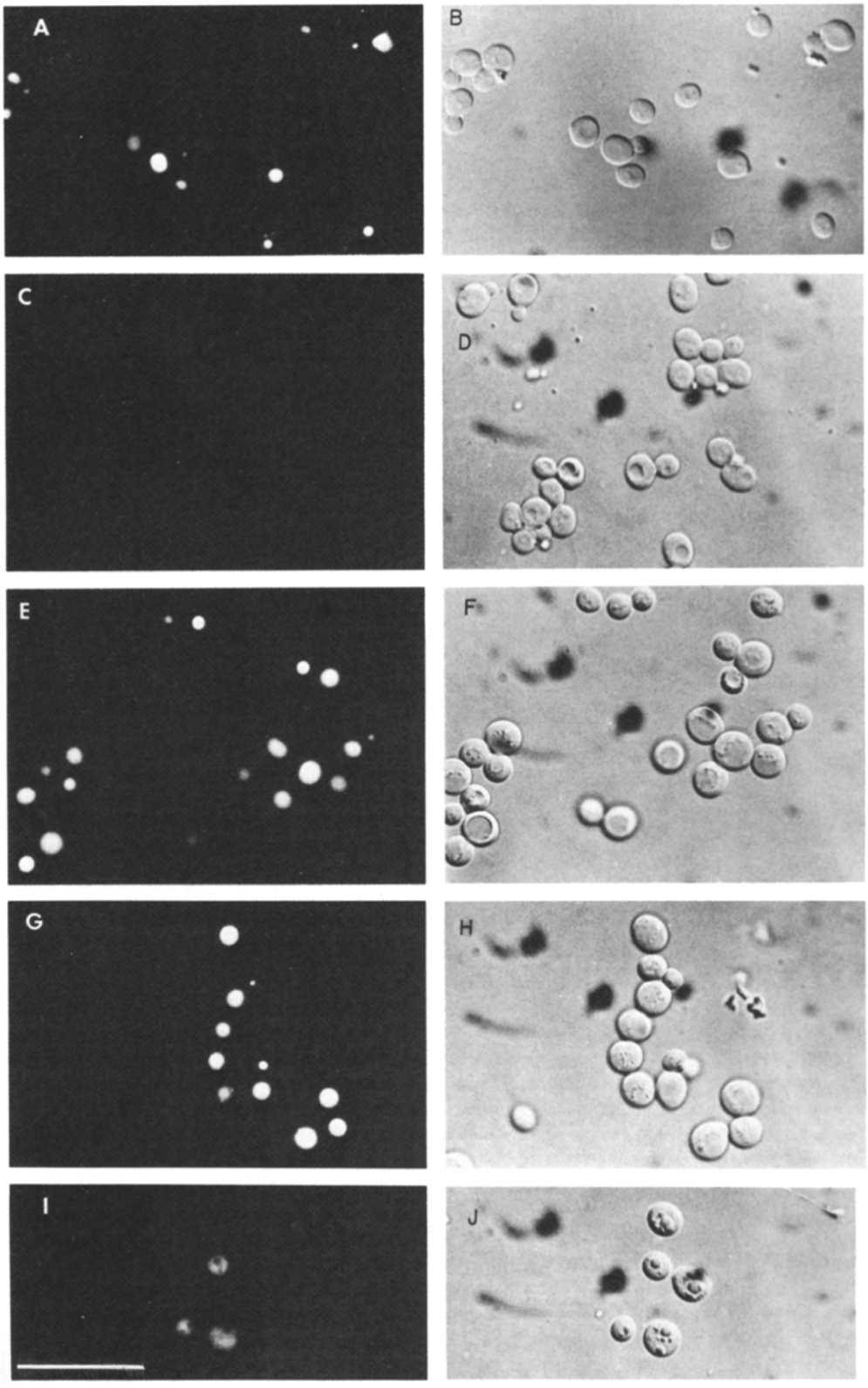

Figure 1. The effect of ammonium acetate on quinacrine and *ade2* labeling of vacuoles and the effect of 5% toluene on *ade2* labeling. Yeast were labeled with quinacrine either in the absence (*A* and *B*) or presence (*C* and *D*) of 200 mM ammonium acetate. *ade2* yeast grown under conditions where they produce the endogenous fluor were also photographed in the absence (*E* and *F*) or presence (*G* and *H*) of ammonium acetate. (*I* and *J*) *ade2* yeast treated with 5% toluene at 23°C for 1–5 min. Bar, 7.6 μm.

strains of yeast is fluorescent, providing an endogenous label. This fluorophore is stable, allowing a study on the inheritance of vacuolar contents. The observations reported here demonstrate that a significant fraction of the bud vacuole is usually inherited from the mother cell.

Materials and Methods

Cell Strains and Growth Conditions

Wild-type *S. cerevisiae*, strain X2180-1A, was used for all studies except those involving *ade2* mutants, in which case DBY 1398, *a, ade2-1, ura3-52*

(Thomas and Botstein, 1986), obtained from Dr. D. Botstein, was used. DBY 1398 was grown on complete SD media (Sherman et al., 1974) with limiting amounts of adenine (12 μg/ml) to induce production of the *ade2* fluorescent dye. The vacuoles become fluorescent in the late logarithmic phase of growth. For all studies on X2180-1A, cells were grown in YPD to mid-log phase (for a minimum of 12 generations). Cells to be stained with quinacrine were grown in YPD plus 50 mM NaH_2PO_4, pH 7.6. In studies of the kinetics of FITC-dextran release, the cells were grown in SD media without methionine or leucine but containing 50 mM NaH_2PO_4, pH 5.0.

Observation of Cells

Quinacrine was added to a final concentration of 170–200 μM to 1-ml aliquots of cells grown in YPD buffered at pH 7.6. Cells were incubated at 30°C for 5 min, collected by centrifugation (30 s, 23°C; microfuge from Brinkmann Instruments Co., Westbury, NY), and resuspended in 30 μl of YPD with 50 mM NaH_2PO_4, pH 7.6 and 1% low-melting agarose (FMC Corp., Rockland, ME). Alternatively, cells could be grown in unbuffered media, collected by centrifugation, and resuspended in buffered media for labeling with quinacrine.

FITC-Dextran Labeling

FITC-dextran labeling was performed as described by Makarow (1985) with some modification. Cells were grown in YPD media with the pH adjusted to 5.0. Cultures (OD_{600} = 2; 10 ml) were centrifuged (30 s, 23°C; clinical centrifuge) and the cell pellets were suspended in 1.0 ml of 50 mM NaH_2PO_4, pH 5.0. They were again centrifuged (30 s at 23°C in a microfuge). This procedure was repeated two more times. The cells were finally suspended in 0.1 ml YPD media containing 50 mM NaH_2PO_4, pH 5.0, and 100 mg/ml 70S FITC-dextran (Sigma Chemical Co., St. Louis, MO). The cells were incubated at 37°C for 45 min, then centrifuged for 30 s at 4°C in a microfuge and resuspended in 1 ml of YPD (pH 5.0) media at 4°C. This centrifugation and suspension was repeated several times in order to remove all of the free FITC-dextran. The cells were kept on ice and 2-μl aliquots were examined.

A Zeiss microscope equipped with filters for Nomarski optics and for epifluorecence was used. Cells stained with either quinacrine, FITC-dextran, or bearing the endogenous *ade2* pigment were observed by exciting with 450–490-nm light and viewing emitted light with a 520-nm cut-off filter. Photographs were taken with a Nikon F3 camera loaded with Kodak Tri-X pan film, and were taken within 10 min of slide preparation.

Data were collected as photographs of random fields. Each field was photographed both with Nomarski optics and epifluorescence. The ratio of the diameter of the bud to diameter of the mother cell was determined from Nomarski photographs.

Results

Visualization of Yeast Vacuoles

Yeast vacuoles were observed by four independent methods: Nomarski optics, quinacrine labeling, FITC-dextran labeling, and labeling with the endogenous *ade2* fluorophore. The latter three methods allow visualization of vacuoles by fluorescence light microscopy. Many of the vacuoles visible by any of the fluorescent methods were not visible by Nomarski optics. Furthermore, when unlabeled yeast were viewed with Nomarski optics, many of the cells did not appear to contain any vacuoles. In contrast, almost all of the vacuoles visible by Nomarski optics are stained by the three fluorescence methods (data not shown). For this reason, only the fluorescence data were quantified.

Before using these techniques for examining the morphologies of yeast vacuoles, we sought to explore the basis of quinacrine and *ade2* dye localization to the vacuole. Yeast stained with quinacrine (Fig. 1 *A*, fluorescence microscopy; Fig. 1 *B*, Nomarski microscopy of the same field of cells) or *ade2* yeast grown to produce the endogenous fluor (Fig. 1, *E* and *F*) were treated with ammonium acetate or sodium

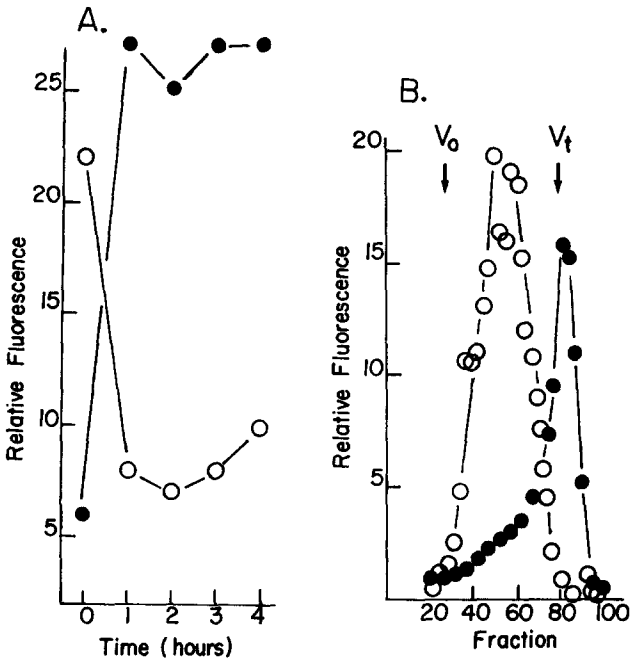

Figure 2. FITC-dextran release from cells. Cells were labeled as described in Materials and Methods except that SD media (pH 5.0) was prepared without methionine or leucine and was supplemented with 100 mg/ml 70S FITC-dextran. This medium was chosen because YPD medium exhibits more background fluorescence than SD medium. After labeling, the cells were suspended in this medium and kept at either 0 or 37°C. At the indicated times, 0.9-ml aliquots were removed and centrifuged in a microfuge for 3 min at 4°C. The supernatants were collected and 0.1 ml of 1.0 N NaOH, 10% β-mercaptoethanol was added. The cell pellets were suspended in 0.9 ml SD media without methionine or leucine (pH 5.0) and mixed with 0.1 ml of 1.0 N NaOH, 10% β-mercaptoethanol. Cells were lysed by incubating at 37°C for 15 min. The lysates were centrifuged (5 min, 23°C) in a microfuge and the supernatants were collected. Relative fluorescence of each sample was measured on an Aminco fluorimeter with the excitation wavelength set at 490 nm, the emission wavelength set at 550 nm, and a band width of 5 nm. To measure the size of the FITC-dextran, 0.5 ml of 50 mg/ml FITC-dextran in 0.1 N NaOH, 1% β-mercaptoethanol was layered on a Biogel A15M column in 100 mM Tris-HCl, pH 8.0, buffer. Fluor which had been released into the culture medium was also analyzed by filtration through the same column. (*A*) The open circles indicate the relative fluorescence of FITC-dextran recovered from the cell pellet, and solid circles indicate relative fluorescence recovered from the media. (*B*) Gel filtration of FITC-dextran on a Biogel A15M column. (*Open circles*) Elution profile of 70S FITC-dextran dissolved in 0.1 N NaOH, 1% β-mercaptoethanol; (*solid circles*) elution profile of FITC-dextran released from labeled cells during incubation at 37°C for 4 h.

acetate. Ammonium acetate but not sodium acetate rapidly raises the pH of mammalian lysozomes or yeast vacuoles (Poole and Ohkuma, 1981; Makarow and Nevalainen, 1987). Quinacrine-stained cells treated with ammonium acetate had no visible fluorescence (Fig. 1 *C*). This loss of fluorescence supports the hypothesis that quinacrine, a weak base, diffuses freely through membranes and concentrates in acidic compartments. As expected, sodium acetate had no effect on quinacrine-stained cells (data not shown). The staining of FITC-dextran-treated cells (data not shown;

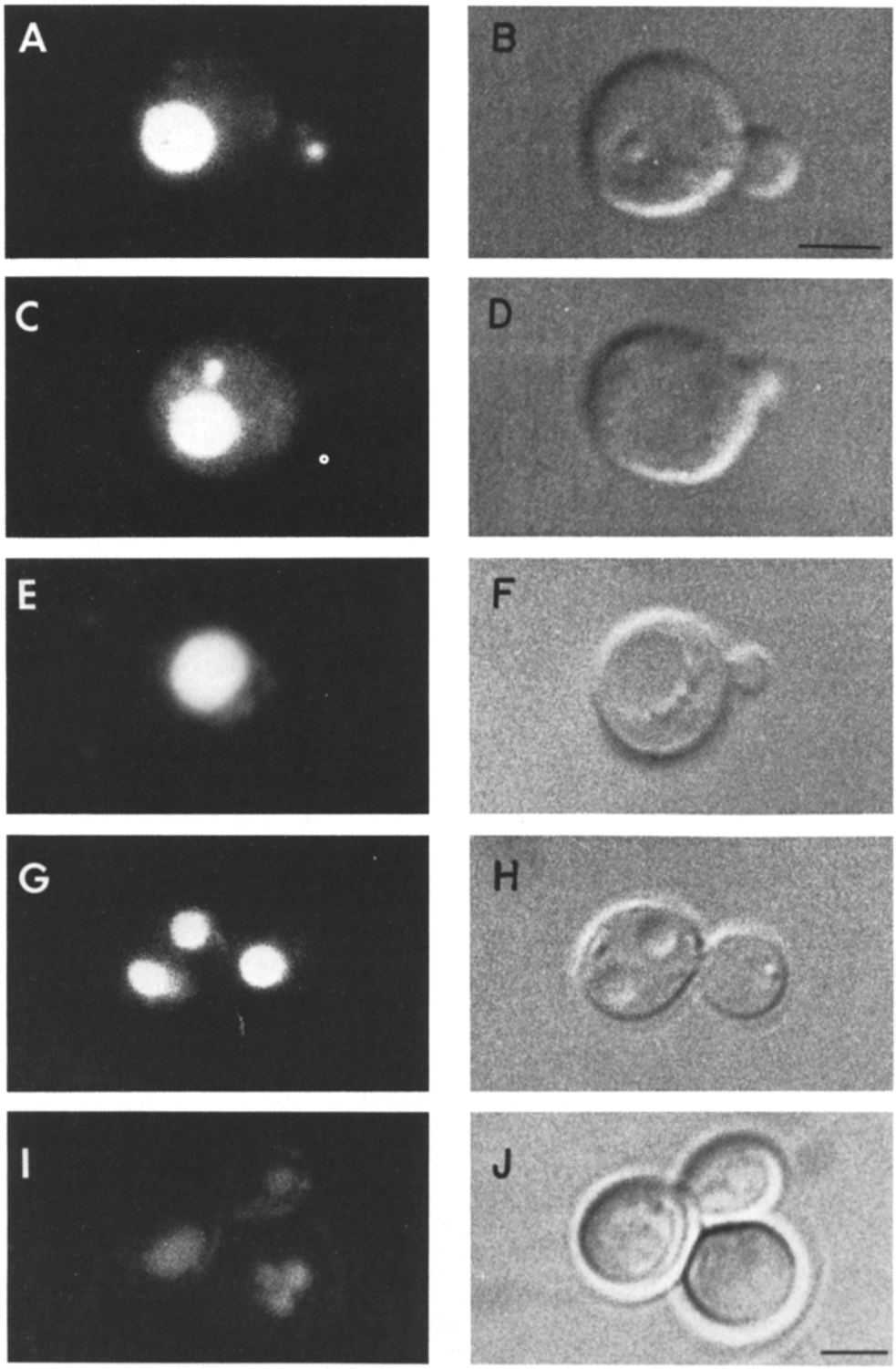

Figure 3. Yeast vacuole morphologies. (*A*, *C*, and *G*) Cells stained with quinacrine. The cell in *E* contains the *ade2* endogenous pigment, and the cells in *I* are stained with FITC-dextran. (*B*, *D*, *F*, *H*, and *J*) Nomarski photomicrographs of the corresponding fields. Examples of vacuolar morphology: (*A*) a single vacuole in the mother cell and a single vacuole in the bud: (*C*) one major vacuole plus one small vacuole in the mother cell and no vacuole in the bud; (*E*) one major vacuole plus many small vacuoles in the mother cell and no vacuoles in the bud; (*G*) two equal vacuoles in the mother cell and one vacuole in the bud; (*I*) (*cell to lower right*) a cluster of vacuoles. The bar in *B* indicates 1 μm for *A–D*; the bar in *J* indicates 1 μm for *E–J*.

Makarow and Nevalainen, 1987) and *ade2* cells (Fig. 1 *G*) was not adversely affected by ammonium acetate. We assume that the metabolic intermediate which accumulates in *ade2* cells crosses the vacuole membranes and polymerizes to form the red dye and a fluorescent compound. However, the stable localization of the fluorophore in the vacuole in the presence of ammonium acetate shows that the retention of this polymer within the vacuole does not require a pH gradient. To test whether the *ade2* polymer is precipitated within the vacuole, yeast cell membranes were permeabilized with 5% toluene. In this case the dye diffuses freely through the cell and is (presumably) retained by the cell wall (Fig. 1, *I* and *J*). The dye is also soluble after lysis of yeast cells (data not shown).

While FITC-dextran has previously been shown to label the vacuole via endocytosis (Makarow, 1985), this fluorescent label is rapidly lost from cells at temperatures above 4°C. Total fluorescence intensity was measured in cell ly-

sates (Fig. 2 *A*, *open circles*) or in the culture medium (solid circles). The loss of cellular fluorescence was time dependent (Fig. 2 *A*) and the fluorescence was quantitatively recovered from the media. This released fluorescent material had a smaller molecular weight than the FITC-dextran, as determined by a shift in the elution profile on a Biogel A15m column (Fig. 2 *B*). For this reason, FITC-dextran could not be used to study the inheritance of vacuole contents (see below) and cells labeled with FITC-dextran were only observed for 10 min after labeling.

Analysis of Vacuole Morphology throughout the Cell Division Cycle

Using the three fluorescent stains described, we have examined the number and size of vacuoles in an exponentially growing population of cells. Vacuolar morphologies were classified into five categories: one vacuole (Fig. 3 *A*, one vacuole in mother cell and one vacuole in bud), one major plus one small vacuole (Fig. 3 *C*, mother cell), one major plus many small vacuoles (Fig. 3 *E*, mother cell), two equal vacuoles (Fig. 3 *G*, mother cell), or a cluster of many vacuoles (Fig. 3 *I*, cell in lower right corner). To simplify presentation of the data, these catagories were condensed into two groups: yeast containing one major vacuole and those containing two or more vacuoles of approximately equal size. The cell cycle stages of individual yeast cells were determined by measuring the ratio of diameter of the bud to the diameter of the mother cell. For each cell, the vacuole number and morphology were scored in both the mother cell and the bud. Some of the small "vacuoles" may in fact represent endosomes (Makarow and Nevalainen, 1987); our current studies do not distinguish these compartments.

One major vacuole was observed in about two-thirds of the cells (Table I). In this table only vacuoles in the mother cell are recorded. Although some variation in vacuolar morphology was seen through the cell cycle with both staining methods, in only one case, quinacrine staining of cells with a ratio of bud-to-parent diameters of 0.21–0.40, was deviation from the mean statistically significant (P less than 0.05 by the chi-square test). Vacuolar morphology observed in *ade2* yeast (Table II) was very different from that observed with FITC-dextran and quinacrine. With the *ade2* endogenous fluorophore, almost all yeast cells contain a single large vacuole. In contrast, when *ade2* yeast are grown in high levels of adenine to suppress formation of adenine precursors, one major vacuole was observed in about two-thirds of the cells (data not shown). Thus, both the endogenous fluorescence and the altered vacuolar morphology observed in *ade2* cells are related to induction of the adenine biosynthetic enzymes.

Table III shows the vacuolar morphology in the bud. By all staining methods, small buds often had no visible vacuole, though vacuoles were detected in buds as small as one-tenth the parental cell diameter. With increasing bud size, the proportion of buds with vacuoles rose sharply. A single major vacuole was the predominant morphology at each stage of bud growth.

The data were evaluated to determine whether the vacuolar morphology in mother cells is related to the vacuolar morphology in the bud. We began by computing the frequency of each bud vacuolar morphology at each stage of the cell division cycle when the connected mother cell contains one vacuole. These frequencies were compared with those found in the whole data base; i.e., when the constraint of a single maternal vacuole was not imposed. If the vacuolar morphologies of the mother cells and buds are independent, then the ratios of these frequencies would be 1. Where sufficient examples had been scored for meaningful comparisons, the ratio only varied from 0.76 to 1.33 (Table IV). The same analysis was performed for the case where multiple vacuoles occur in the mother (Table V) or where the bud vacuolar morphology is held constant while that of the mother cell is examined (Table VI). In no case were the vacuolar morphologies in the bud or mother dependent on each other. When

Table I. Mother Cells Containing One Major Vacuole

Bud diameter/ mother cell diameter	Quinacrine		FITC-dextran		Both Stains	
	n (cells)	%	*n (cells)*	%	*n (cells)*	%
No bud observed	78/101	77	87/152	57	165/253	65
0.10–0.20	5/11	45	13/26	50	18/37	49
0.21–0.40	25/50	50	13/31	42	38/81	47
0.41–0.60	40/63	63	30/52	58	70/115	61
0.61–0.80	74/103	72	19/29	66	93/132	70
0.81–1.0	27/32	84	5/8	62	32/40	80
Total cells	249/360	69	167/298	56		

Table II. Vacuole Morphology in ade2 Yeast

Bud diameter/ mother cell diameter	One major vacuole			
	Mother cell		Bud	
	n (cells)	%	*n (cells)*	%
No bud observed	111/112	99	–	
0.10–0.20*	–		–	
0.21–0.40	19/20	95	11/20	55‡
0.41–0.60	59/60	98	54/60	90
0.61–0.80	28/30	93	29/30	97
0.81–1.0	23/25	92	24/25	96

* No yeast scored in this category.
‡ Eight of the other nine buds contained no vacuole. The ninth bud contained more than one major vacuole.

Table III. Vacuole Morphology in Bud

Bud diameter/ mother cell diameter	Quinacrine		FITC-dextran	
	n (cells)	%	*n (cells)*	%
No vacuole in bud				
0.10–0.20	9/11	82	25/26	96
0.21–0.40	21/50	42	13/31	42
0.41–0.60	4/63	6	6/52	12
0.61–0.80	1/103	1	0/29	
0.81–1.0	0/25		0/8	
One major vacuole in bud				
0.10–0.20	2/11	18	1/26	4
0.21–0.40	29/50	58	13/31	42
0.41–0.60	47/63	75	32/52	62
0.61–0.80	88/103	85	23/29	79
0.81–1.0	27/32	84	7/8	88

*Table IV. Vacuolar Morphology in Bud When Mother Cell Contains One Major Vacuole**

Bud diameter/mother cell diameter	Frequency of buds with indicated vacuole structure among cells with one major vacuole in mother cell: Frequency of buds with indicated vacuole structure among all cells — No vacuole	One major vacuole	Two or more vacuoles of equal size
0.10 - 0.20‡	–	–	–
0.21 - 0.40	1.33	0.76	§
0.41 - 0.60	§	1.04	0.79
0.61 - 0.80	§	1.00	0.86
0.81 - 1.0	§	1.06	§

* Analysis of vacuoles stained with quinacrine.
‡ Less than 20 cells in this size category.
§ Less than four buds with this morphology linked to mother cell containing one major vacuole.

*Table V. Vacuolar Morphology in Bud When Mother Cell Contains Multiple Vacuoles**

Bud diameter/mother cell diameter	Frequency of buds with indicated vacuole structure among cells with multiple vacuoles in mother cell: Frequency of buds with indicated vacuole structure among all cells — No vacuole	One major vacuole	Two or more vacuoles of equal size
0.10–0.20‡	–	–	–
0.21–0.40	0.67	1.24	§
0.41–0.60	§	0.93	1.37
0.61–0.80‡	§	0.97	1.27
0.81–1.0	–	–	–

* Analysis of vacuoles stained with quinacrine.
‡ Less than 20 cells in this size category.
§ Less than four buds with this morphology linked to mother cell containing multiple vacuoles.

the analyses performed above were applied to the original five categories, the same results were obtained (data not shown).

Inheritance of Vacuolar Constituents

Since both quinacrine and FITC-dextran are not stably retained within the vacuole, we turned to the *ade2* fluorophore as a label for studying vacuole inheritance. When *ade2* yeast are grown into stationary phase their vacuoles become highly fluorescent. When these cells are then inoculated into fresh medium containing high levels of adenine, synthesis of the *ade2* intermediate is suppressed (Jones and Fink, 1982) and neither the red dye nor the fluorescent polymer is degraded (Fig. 4 *A*). The fluorescence intensity of individual cells from such a culture were recorded by means of the fluorescence-activated cell sorter. As the cells grow, three distinct populations of cells are detectable (Fig. 4 *B*); faintly fluorescent cells (peak I), highly fluorescent cells (peak III), and cells of intermediate fluorescence (peak II). Wild-type yeast have a low level of endogenous fluorescence and are

*Table VI. Vacuolar Morphology in Mother Cell When Bud Contains One Major Vacuole**

Bud diameter/mother cell diameter	Frequency of mother cells with indicated vacuole structure among cells with a major vacuole in the bud: Frequency of mother cells with indicated vacuole structure among all cells — One major vacuole	Two or more vacuoles of equal size
0.10–0.20‡	–	–
0.21–0.40	0.76	1.24
0.41–0.60	1.04	0.93
0.61–0.80	1.01	0.96
0.81–1.0	1.05	0.71

* Analysis of vacuoles stained with quinacrine.
‡ Less than 20 cells in this size category.

sorted to peak I. The number of highly fluorescent cells remains the same (Fig. 4 *C*, *solid circles*), but the number of cells with intermediate fluorescence (*open circles*) and low-level fluorescence (*open triangles*) increases with time. We postulate that the cells with intermediate fluorescence are the daughter cells of the highly fluorescent yeast. The low level fluorescent cells are daughters of the intermediate level cells and all subsequent daughters. Thus the low level fluorescent cells would eventually increase most rapidly. Typical cells with intermediate fluorescence are indicated by vertical arrows in Fig. 4,*D* and *E*. Three lines of evidence indicate that the highly fluorescent cells are dividing: (*a*) most highly fluorescent cells contain buds (Fig. 4,*D* and *E*); (*b*) while the culture initially contains largely highly fluorescent cells, a population of intermediate fluorescent cells appears and increases; (*c*) the light-scattering profile plus the fluorescence profile recorded for each individual cell from the cell sorter indicates that the highly fluorescent cells start budding almost synchronously (data not shown).

These data are consistent with a portion of the daughter vacuole being inherited from the mother vacuole. In Fig. 4 *B*, the fluorescence intensity in the intermediate class of cells is ~1/16 the intensity measured in the highly fluorescent cells. In most experiments of this type we observed that the intermediate class had 1/32 the fluorescence of the highly fluorescent cells, and observed a total range of inheritance of 1/16–1/64 (data not shown). Note that the vacuole of the intermediate class of cells is only 1/2–1/4 the diameter of the large, swollen vacuole seen in the highly fluorescent cells (Fig. 4, *D* and *E*), corresponding to 1/8–1/64 the vacuole volume. Thus, the concentration of *ade2* fluorophore in the vacuole of parent and daughter cells may be quite similar. The loss of only 1/16–1/64 the vacuolar fluorophore with each cell division cycle is consistent with the persistence for many generations of highly fluorescent but dividing cells. The asymmetry of this division indicates that the mechanisms of vacuole division need not always partition half of the mother cell vacuole contents to the daughter.

Discussion

We have studied vacuolar morphology through the cell cycle with asynchronous cultures. The vacuole was observed with

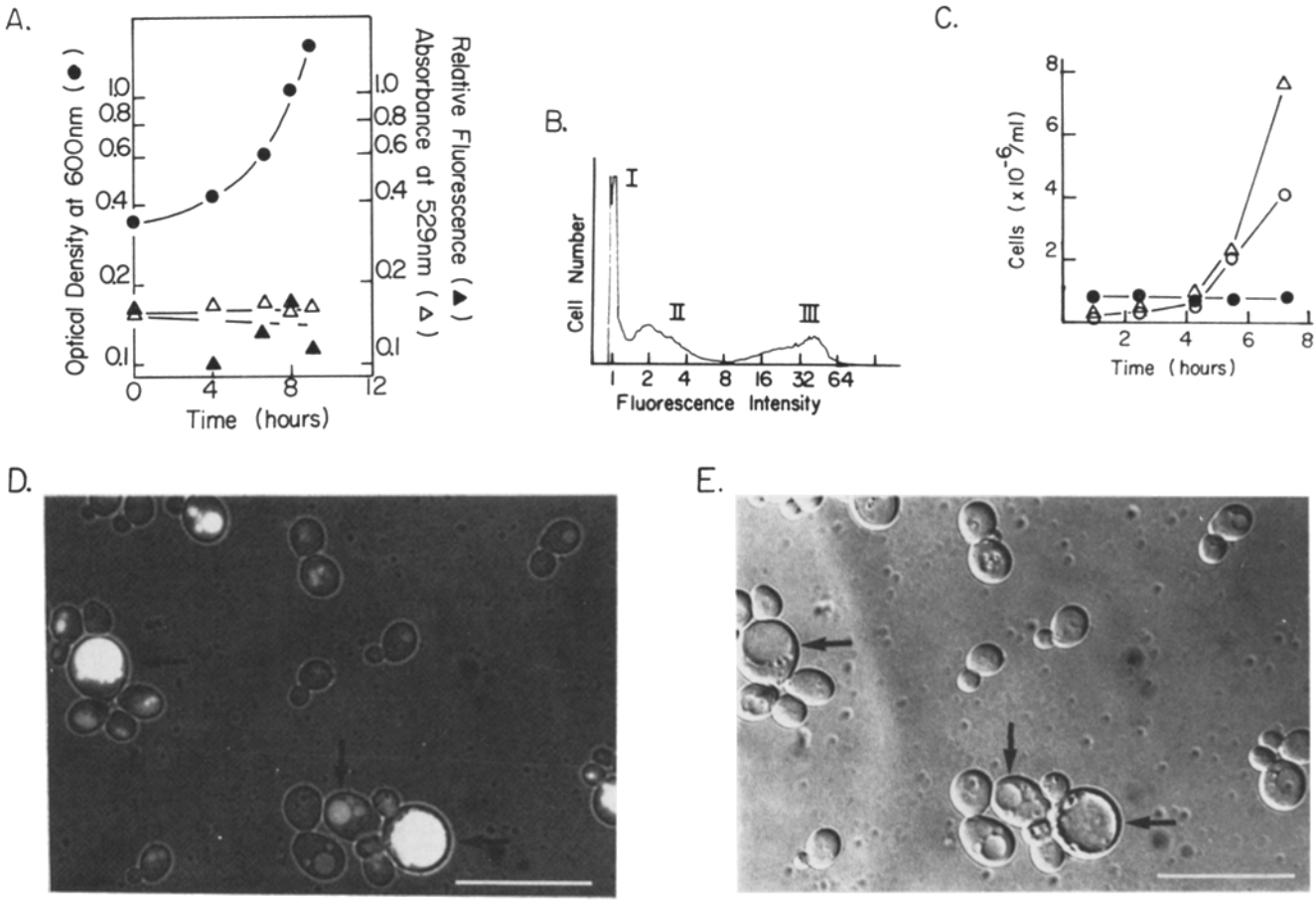

Figure 4. Analysis of fluorescent *ade2* yeast. (*A*) *ade2* yeast were grown for 48 h in YPD to allow production of fluorescent vacuoles. A 5-ml aliquot of cells was added to 500 ml of YPD containing 200 μg/ml adenine to shut off synthesis of the *ade2* intermediate. At the times indicated, cell concentration was determined by optical density at 600 nm (*solid circles*). The cells were harvested from 25-ml aliquots, resuspended in 10% SDS, and heated at 95°C for 5 min. Absorbance at 529 nm, the maximal absorption for the *ade2* polymer in 10% SDS (data not shown), was determined (*open triangles*). The relative fluorescence emitted at 434 nm by extracts excited at 365 nm was also determined (solid triangles). (*B*) *ade2* yeast were grown in YPD for 7 d to allow production of highly fluorescent vacuoles. A 0.2-ml aliquot of cells was added to 20 ml of YPD containing 200 μg/ml adenine to shut off synthesis of the *ade2* intermediate. After growth had resumed (7.25 h after dilution into media with adenine), cells were analyzed by an EPICS V Cell sorter (Coulter Electronics Inc., Hialeah, FL). Peaks, III, II, and I correspond to highly fluorescent cells, cells of intermediate fluorescence, and nonfluorescent cells, respectively. This scan corresponds to the 4.25-h time point in *C*. (*C*) Cell number was determined with a Coulter counter, and cells were analyzed on the cell sorter. These assays were begun 3 h after dilution into media with adenine, a time when the cells emerged from stationary phase and growth resumed. The number of cells per ml in each category was calculated from the total cell number, and the percent of cells under each peak. The cell categories (as illustrated in *B*) are as follows: I, nonfluorescent cells (*open triangles*); II, cells of intermediate fluorescence (*open circles*); and III, highly fluorescent cells (*solid circles*). (*D*) Fluorescent photomicrograph of cells after 1.5 doublings. The field is faintly backlit so that an outline of the yeasts can be seen. The horizontal arrows indicate the highly fluorescent cells. The vertical arrow is an example of a cell with intermediate fluorescence. Bar, 5.7 μm. (*E*) Nomarski optics of the field shown above. Bar, 5.7 μm.

each of three fluorescent dyes: FITC-dextran, which had been developed previously (Makarow, 1985), and two new vacuolar markers, quinacrine and an endogenous fluorophore produced in *ade2* yeast. The relative merits of these three methods are discussed below. Whether assayed with quinacrine or FITC-dextran, the mother vacuole persists throughout the cell division cycle. Approximately two-thirds of the yeast at every stage in the cell cycle contain a major vacuole, with the other third containing two or more vacuoles of equal size. Although some increase in multiple vacuoles was seen in early S phase, in only one case, quinacrine labeling of cells with a ratio of bud diameter to mother cell diameter of 0.21–0.40, was the increase statistically significant.

Previous studies of cell cycle specific changes in vacuolar morphology have used synchronized cultures of *S. cerevisiae*. In these studies, the single, large vacuole seen in G_1 divided into smaller vacuoles early in the budding cycle (Wiemken et al., 1970; Severs et al., 1976). These cell cultures were synchronized either by gradient centrifugation at low temperature or by cycles of starvation. We have also examined synchronized cultures, staining the vacuoles with FITC-dextran or quinacrine. In experiments where X2180-1A was released from alpha factor arrest, the single vacuoles

found in the arrested cells were replaced by fragmented vacuoles as the cells began to bud (Weisman, L., unpublished observations). We conclude that the differences between the vacuole structures reported here and those in earlier studies reflect the differences in physiology between exponentially growing yeast and those which have been synchronized.

Our studies indicate that vacuolar morphology is essentially cell cycle independent. However, these methods cannot determine whether rapid vacuole fission, followed shortly by fusion, is an important step in forming the bud vacuole. It is also important to note that these studies were done with only one strain of *S. cerevisiae*. Alterations in the physiological state of a culture, caused by the techniques used for synchronization, can clearly have important effects on vacuole morphology. We have also recently observed other conditions which affect vacuolar morphology. Yeast treated with drugs which disrupt microtubules and a yeast strain with a mutation in the beta tubulin gene have fragmented vacuoles (Guthrie, B., and W. Wickner, manuscript submitted for publication), suggesting that the vacuole may interact with cytoplasmic microtubules.

We have used the stable vacuolar fluorophore in *ade2* cells to study vacuolar inheritance. Buds attached to fluorescent mother cells always have fluorescent vacuoles (>200 scored), demonstrating that the soluble contents of vacuoles are inherited. Before this study, inheritance had been strongly implied by the observation that pro-carboxypeptidase Y was still activated to the mature form in newly sporulated *PEP4* yeast (Jones et al., 1982). However, only catalytic amounts of proteinase A, the *PEP4* protease, need be inherited for the persistence of pro-carboxypeptidase Y activation. Vacuolar inheritance has also been shown in another species of yeast (Svihla and Schlenk, 1960). In experiments where *ade2* dye distribution was monitored, we noted that the concentration of the dye in parent and daughter cell vacuoles was similar. Since, in asynchronous cultures in exponential growth, we do not observe a consistent, cell cycle-related maternal vacuole fragmentation, we speculate that there is a continuous budding of small vesicles from the vacuole, leaving the major vacuole structure intact while providing "seed" material to the bud. Of course, we do not know whether such traffic might proceed directly or via Golgi, endosomes, or other organelles. Experiments that demonstrate rapid and efficient vesicular traffic between separate Golgi stacks in the same cell (Dunphy and Rothman, 1985) provide a precedent for this proposal.

In previous studies, yeast vacuoles have been visualized by electron microscopy, phase-contrast and Nomarski light microscopy, and fluorescence microscopy with FITC-dextran. In this paper, quinacrine and the *ade2* fluorophore are presented as additional ways to observe vacuoles. Each of these techniques labels or detects vacuoles by a different principle. Nomarski microscopy detects the difference in refractive index between the vacuole and the surrounding cytoplasm. FITC-dextran enters the vacuole by endocytosis, while quinacrine is a weak base which diffuses across membranes and concentrates in the acidic compartments. The *ade2* fluorophore is produced within the cell and, once trapped within the vacuole, remains there independent of the vacuolar pH. Each of these labeling techniques has limitations, depending on the desired application. With freeze-fracture electron microscopy and phase-contrast and Nomarski light microscopy, it may be difficult to distinguish small vacuoles from other small vesicular structures because no specific vacuolar marker is present. In addition, while large vacuoles are often readily visualized by phase-contrast microscopy or Nomarski optics, we find that many small vacuoles are missed by these techniques. Furthermore, many large vacuoles identified by fluorescence techniques could not be seen by Nomarski optics or phase-contrast microscopy. FITC-dextran labeling of vacuoles also has some disadvantages. The most important problem is that the fluorescence disappears rapidly from cells at room temperature. At least part of this loss may be due to degradation of the dextran and release of smaller FITC-dextran oligomers. Consequently, FITC-dextran cannot be used as a permanent marker for vacuoles. This paper describes two new techniques for staining the vacuole with fluorescent dyes. Both of these techniques differ from FITC-dextran labeling in that endocytosis is not required. Quinacrine, a weak base, is lysosomotropic and has previously been used to stain mammalian lysosomes (Allison and Young, 1964). The yeast probably do not metabolize quinacrine and its concentration in the vacuole is high enough that bleaching by the excitation light source does not interfere with photographing a field of cells. However, when cells are resuspended in media without quinacrine, the dye eventually diffuses out of the vacuole (data not shown). Thus, quinacrine is not suitable for inheritance studies. Also, mutants which do not acidify their vacuoles would not be stained by this method. Some of the smaller structures detected by quinacrine and FITC-dextran staining may be endosomes. Makarow and Nevalainen (1987) have recently identified an intermediate compartment in FITC-dextran localization to the vacuole.

The *ade2* endogenous dye has only been partially characterized (Smirnov et al., 1967). The *ade2* defect is a block in adenine biosynthesis such that 4-aminoimidazole ribotide accumulates (Jones and Fink, 1982). A derivative of this colorless compound concentrates in the vacuole. It then undergoes polymerization and oxidation, producing a red color. Initially, we intended to use this red dye for light microscopy studies, but then observed that the polymers were highly flourescent compounds with broad excitation and emission spectra. Unlike quinacrine and FITC-dextran, the dye persists within the vacuole. The main disadvantage of *ade2* staining of vacuoles is that the cells only produce the dye in low concentrations of adenine, yet this compound is required for their growth. Also some of the intermediates produced by the *ade2* strain are toxic to the cell (Dorfman, 1969). Yeast strains with the *ade2*, *ade12* double mutations produce the *ade2* dye constitutively, but grow very poorly.

The advantage of using three separate dyes in this study is that dye-specific effects on vacuolar morphology can be detected. One such effect was observed: with the *ade2* endogenous dye, almost all yeast contained a single major vacuole. This was shown to be related to the accumulation of a metabolic precursor of adenine. In contrast, only two-thirds of the mother cell vacuoles had that morphology with the other two methods of staining.

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