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Mutants of *Saccharomyces cerevisiae* that block intervacuole vesicular traffic and vacuole division and segregation

(lysosome/organelle inheritance/VAC1)

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ABSTRACT **Intervacuole vesicular exchange and the segregation of parental vacuole material into the bud are strikingly impaired in a temperature-sensitive yeast mutant, *vac1-1*. At the nonpermissive temperature, haploid *vac1-1* cells show a pronounced delay in separation of mature buds from the mother cell and accumulate cells with multiple buds. At both the permissive and restrictive temperatures, daughter cells are produced that lack a detectable vacuole or contain a very small vacuole. In zygotes, vacuoles from a *vac1-1* strain are defective as donors, or recipients, of the vesicles of intervacuole vesicular traffic. These defects are specific for the vacuole in that the segregation of nuclear DNA and of mitochondria into the bud appears normal. The isolation of the *vac1-1* mutation is a first step in the genetic characterization of vacuole division and segregation.**

Accurate division and segregation of cytoplasmic organelles is an integral part of cell division (1, 2). This entails coordination of the timing of organelle division with cell division, accurate spatial segregation of organelles into each daughter cell, and maintenance of organelle integrity during division. Structures of very high copy number, such as ribosomes, may rely on diffusion instead of a more specific mechanism for segregation. At the other extreme, the single nucleus of each cell undergoes highly regulated stages of mitosis to faithfully segregate the replicated DNA. The depolymerization of cytoplasmic microtubules, a prerequisite for formation of the nuclear division spindle, also coincides with the fragmentation of the mammalian endoplasmic reticulum (3) and the Golgi apparatus (4, 5) into multiple small vesicles. These organelle-derived vesicles assume a diffuse distribution during M phase and then re-form the characteristic organelle structures during telophase and cytokinesis. Multicopy organelles such as mitochondria and lysosomes must also duplicate their number during cell division. Early studies suggested that there is a single yeast vacuole in unbudded cells, which vesiculates during bud emergence (6).

We have begun a study of the division, segregation, and inheritance of vacuoles (lysosomes) of the yeast *Saccharomyces cerevisiae*. This is a low-copy-number organelle (7). We have not detected a cell-cycle-specific fragmentation of the yeast vacuole as is seen in mammalian Golgi and endoplasmic reticulum. A small vacuole appears in the bud soon after its emergence in S phase (7). The size of this new vacuole increases along with growth of the bud itself, while the parent cell vacuole remains approximately constant in size. Despite this asymmetric growth, the bud vacuole inherits a significant portion of the parental vacuole contents. This phenomenon was explained by the discovery of intervacuole communication mediated by vesicular or tubular structures (hereafter called "vesicular traffic"), occurring between vacuoles of the mother cell and the bud (8). We proposed that vesicles derived from the parental vacuole form the new vacuole in the bud, and we now describe the isolation and phenotype of a mutant, which provides strong support for this postulate.

Newly synthesized proteins are delivered to the vacuole via the endoplasmic reticulum and Golgi (9). This process is presumed to be mediated by vesicles with specific receptor proteins in a manner analogous to the sorting of proteins to lysosomes in mammalian cells. Indeed, vacuole proteins, which are either overproduced, thereby saturating such a sorting receptor system, or which lack the sorting signals, are secreted to the yeast periplasm (10–12). This has been the basis for the isolation of *vps* (*v*acuole *p*rotein *s*orting) mutants (13, 14). Over 50 *vps* complementation groups have been identified, and mutants have been grouped as follows: class A, with normal vacuole morphology; class B, with fragmented or disrupted vacuoles; class C, with no morphologically obvious vacuoles (15). Although most of the *vps* mutants isolated are not temperature sensitive for growth, all the class C complementation groups contain temperature-sensitive alleles.

Mutants in which the primary defect is in vacuole segregation might yield large buds, or even cells, without a vacuole, and substantial amounts of vacuolar protein might be secreted due to the absence of a vacuole. We now report the isolation of six mutants that are defective in vacuole division and segregation. One of these, *vac1-1*, is a temperature-sensitive, conditionally lethal lesion that yields large buds (and even unbudded cells) without vacuoles; yet it has little effect on the division of nuclear contents or on the partitioning of mitochondria between the parent and daughter cell.

MATERIALS AND METHODS

Medium. Yeast was grown in yeast extract/citrate medium (YCM) (pH 4.5) (16). Yeast extract/peptone (YP) (17) contained 2% glucose, 2% glycerol, 2% sucrose, or 3% sodium lactate (pH 5.5).

Mutant Isolation. Mutants that secrete vacuole proteins were isolated (13, 18). The parent strain, SEY6210, was transformed with pCYI-433, a plasmid containing *URA3* and a *PRC–SUC* gene fusion. The fusion protein contains carboxypeptidase Y fused to invertase (11). This fusion protein has invertase activity and is localized to the vacuole. Mutants that mislocalize the fusion protein to the cell surface grow on sucrose and form colonies on YP sucrose plates containing antimycin A (10 μg/ml) and bromcresol purple (32 μg/ml). Spontaneous mutants were isolated. Colonies were screened

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Abbreviations: FITC, fluorescein isothiocyanate; CDCFDA, carboxy-2′,7′-dichlorofluorescein diacetate; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide.

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for temperature-sensitive growth by replica plating on this same medium at 23°C and 37°C. Prior to characterization, mutants were cured of their plasmid and back-crossed five times to DBY1398 *MAT***a**, *ura3-52*, *ade2-1* (kindly provided by D. Botstein, Genentech) or to DBY1398 that had been converted to *MATα* by a plasmid containing a GAL·HO fusion (19).

Labeling Vacuoles. Two contaminants present in fluorescein isothiocyanate dextran (FITC-dextran; Sigma), FITC, and an uncharacterized fluorescein derivative, label vacuoles (20, 21). When cells were labeled with FITC-dextran containing these contaminants, we refer to the labeling molecules as fluorescein derivatives. Cells were grown to an OD_{600} of <0.4, collected by centrifugation, and resuspended at an OD_{600} of <1.0 in YCM containing FITC-dextran (100 mg/ml). Cells were incubated for 15–30 min, collected by centrifugation (14,000 × *g*; 0.5 min), and resuspended in 1.5 times the original volume of YCM. This centrifugation was repeated, and the cells were resuspended in YCM. Vacuoles were also labeled with quinacrine or the *ade2* fluorophore (7). Cells to be labeled with carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA) (Molecular Probes) were collected by centrifugation (14,000 × *g*; 0.5 min), suspended in the original volume of YCM containing 30 μM CDCFDA, incubated with aeration for 25 min, centrifuged, and resuspended in 1/10th the original volume of YCM. All incubations were performed at 23°C or 37°C.

Photography. Cells were immobilized for photography on glass slides pretreated with Concanavalin A (0.5 mg/ml) (Sigma). Photomicrographs were taken through a Zeiss neofluor ×100/1.30 lens with a ×12.5 eyepiece. Kodak T_{max} p3200 film was developed at an ASA of 3200. Fujichrome p1600 was developed at an ASA of 3200. Cells stained with 4′,6-diamidino-2-phenylindole were viewed with excitation at 365-nm and 420-nm bandpass emission. For the other fluorophores, a filter set allowing 450- to 490-nm excitation and 520-nm bandpass emission was used.

Mating Yeast. *ade2* was present in both mating types, but only one type was grown under adenine-limiting conditions to produce the fluorophore. Equal numbers of *MAT***a** and *MATα* cells were mixed and incubated with slow shaking at 23°C for 3–4 hr (8).

RESULTS

To isolate mutants in vacuole segregation, we selected mutants that secrete soluble vacuolar proteins and screened the resulting strains for temperature-sensitive growth. Only a small fraction of *vps* mutants are temperature sensitive for growth, while ≈50% of all mutants that lack a vacuole are temperature sensitive (15). Of 23 temperature-sensitive mutants we isolated, 6 had defects in vacuole division and segregation. One of these, *vac1-1*, which is apparently nonallelic with known class C mutants (L.S.W., unpublished data), was characterized further.

The bud of large, budded *vac1-1* cells often lacks a vacuole or contains an unusually small vacuole (Fig. 1*A*) and many unbudded cells contain no apparent vacuole or an unusually small vacuole (Fig. 1*A*). In contrast, wild-type yeast with a large bud almost always have one or more prominent vacuoles in the bud, and every unbudded yeast contains a vacuole (Fig. 1*B*).

vac1-1 cells were crossed five times successively with a wild-type strain and then sporulated; the tetrads were then dissected and the spores were analyzed. In each tetrad, the mutant phenotype segregated 2:2. The temperature-sensitive growth phenotypes cosegregated with the defect in vacuole segregation (data not shown). The *vac1-1* mutant does not grow on solid medium with YCM (pH 4.5) or YP glycerol or YP lactate (pH 5.5) at 37°C. The mutant can grow on rich

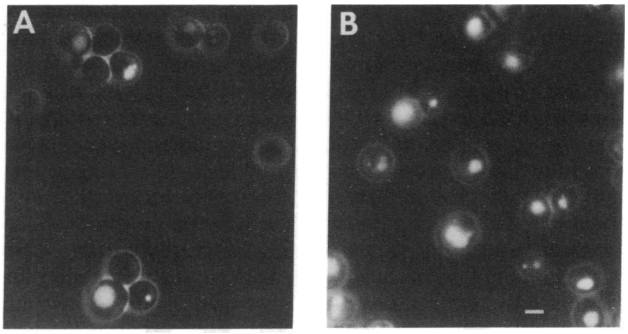

FIG. 1. Defective vacuole segregation in *vac1-1* cells. Both *vac1-1* (*A*) and *VAC* (*B*) yeast were grown for more than 10 generations on YCM (pH 4.5) at 23°C, shifted to 37°C for 3 hr, and labeled with the fluorescein derivatives. (Bar = 1 μm.)

unbuffered medium (yeast extract/peptone/dextrose) at 37°C, but far more slowly. The mutant grows almost as well as wild-type cells on glucose at 23°C.

vac1-1 cells have a much smaller vacuole in the bud than wild-type cells. *VAC1* and *vac1-1* cells were labeled, random fields of cells were photographed, and the sizes of the vacuoles were scored in unbudded cells and in cells with large buds. By analyzing maternal cells, the buds of these cells, and cells without buds as separate populations, it can be seen that the *vac1-1* defect decreases the frequency of vacuoles in buds (Fig. 2*A*) and in cells without buds (Fig. 2*B*). Even when a vacuole is present, it is generally small. This phenotype, which is also observed in cells labeled with quinacrine or CDCFDA (data not shown), is seen at both 23°C and at 37°C. Only in the case of mother cells with large buds was the profile of vacuole size similar in *vac1-1* and *VAC1* (Fig. 2*C*).

Inheritance of the mother cell vacuole contents by the bud may be assayed by accumulating an endogenous fluorophore in the vacuoles of *ade2* cells that have been transiently starved for adenine (7). The fluorescence then serves as a marker of vacuole matrix inheritance during cell growth. Contrary to what was observed in *vac1-1* cells labeled with the fluorescein derivatives, almost none of the buds contained *ade2* fluorophore inherited from mother cell vacuoles (Fig. 3*A*, Fig. 4*A*). This phenotype is also observed at 37°C (see, for example, Fig. 6). These results contrast with wild-type cells (Fig. 3*B*, Fig. 4*A*), where all the large buds contained a vacuole labeled with the *ade2* fluorophore.

Since the buds of *vac1-1*, *ade2* yeast did not inherit the vacuolar *ade2* fluorophore, we labeled these cells with the fluorescein derivatives to determine whether the buds had any vacuoles. The fluorescein derivatives alone appear green when excited with 450- to 490-nm light, while the *ade2* fluorophore appears yellow/orange (8). When cells were labeled with both fluorophores, many of the buds had no detectable vacuoles. Most of the buds that contained a vacuole were labeled with both the *ade2* fluorophore and the fluorescein derivatives. However, some buds contained vacuoles that, although labeled by the fluorescein derivatives, appeared to contain no *ade2* fluorophore (Fig. 5*A*). We exploited the fact that the fluorescence from the fluorescein derivatives is more rapidly bleached than the *ade2* fluorophore (Fig. 5*B*) to observe that the vacuole contained little or no *ade2* fluorophore and, therefore, presumably arose with little inheritance from the maternal cell. Two possibilities for this alternative mode of initiation of a bud vacuole are endocytosis and new synthesis targeted from the Golgi apparatus.

We previously demonstrated intervacuole communication in yeast zygotes and postulated that this traffic is an essential feature of vacuole division and segregation. Intervacuole vesicular traffic may also account for the high degree of

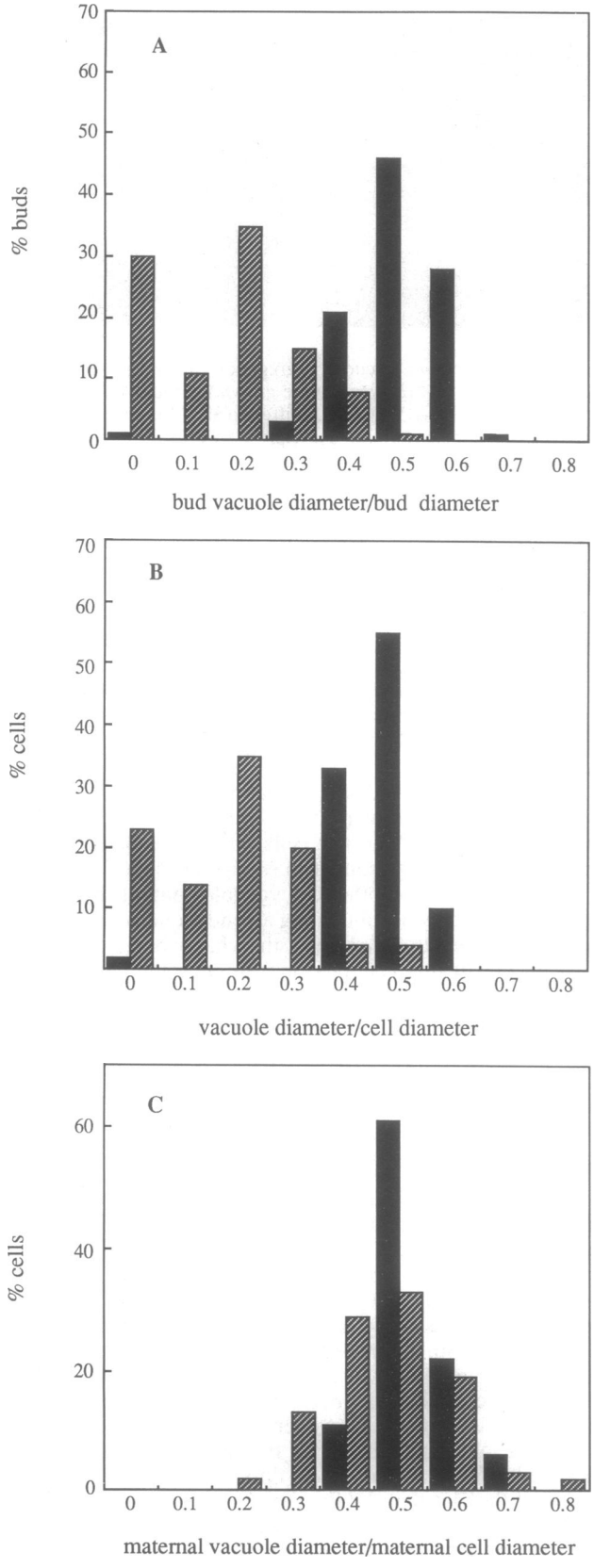

FIG. 2. Quantitation of vacuoles in *vac1-1* and *VAC* yeast labeled with fluorescein derivatives. Random fields of cells, labeled as in Fig. 1, were measured. Vacuole size was recorded as ratio of vacuole diameter/diameter of bud or mother cell. Solid bars, *VAC*; hatched bars, *vac1-1*. Large buds (*A*), unbudded cells (*B*), and mother cells containing large buds (*C*) were scored separately. Numbers of cells measured: *vac1-1* and *VAC* buds, 148 and 72, respectively; *vac1-1* and *VAC* unbudded cells, 51 and 60, respectively; *vac1-1* and *VAC* mother cells containing large buds, 119 and 72, respectively.

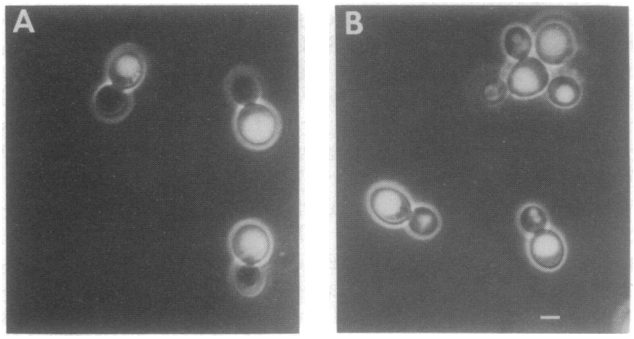

FIG. 3. *vac1-1* is defective in vacuolar inheritance. *vac1-1* (*A*) and *VAC* (*B*) yeast were incubated at 23°C under conditions that produce the endogenous *ade2* fluorophore. Cells were collected by centrifugation and resuspended in YCM (pH 4.5) and grown at 23°C for approximately one doubling and were then photographed. (Bar = 1 μm.)

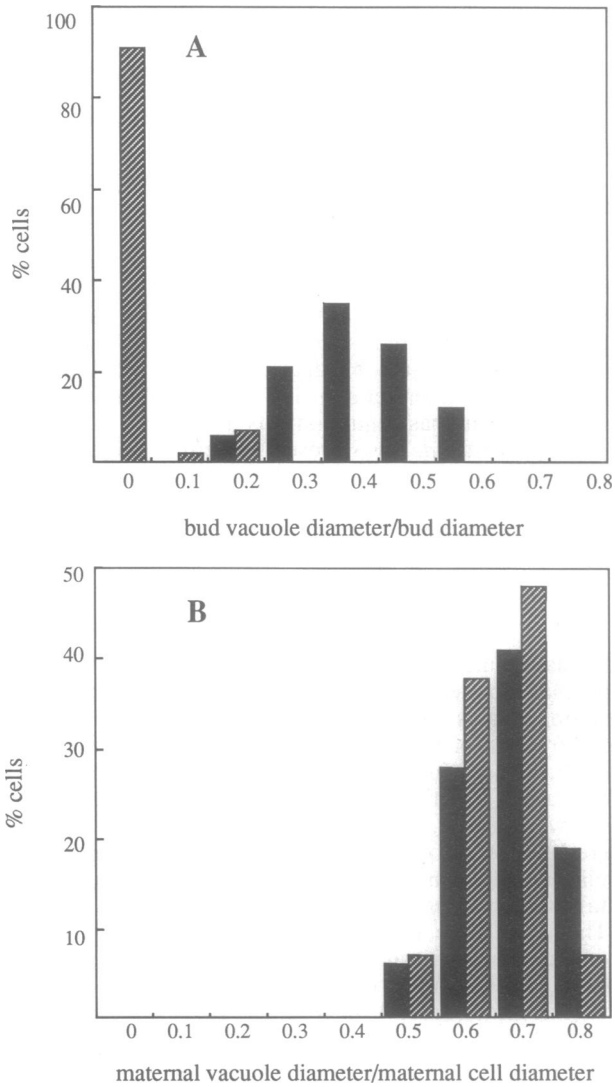

FIG. 4. Quantitation of vacuoles in *vac1-1* and *VAC* yeast labeled with the endogenous *ade2* fluorophore. Photographs of random fields of cells, labeled at 23°C with the endogenous *ade2* fluorophore, were measured. Large buds (*A*) and mother cells containing large buds (*B*) were scored separately. Solid bars, *VAC* yeast; hatched bars, *vac1-1*. The cells scored were as follows: *vac1-1* and *VAC* buds, 42 and 34, respectively; *vac1-1* and *VAC* mother cells, 42 and 32, respectively.

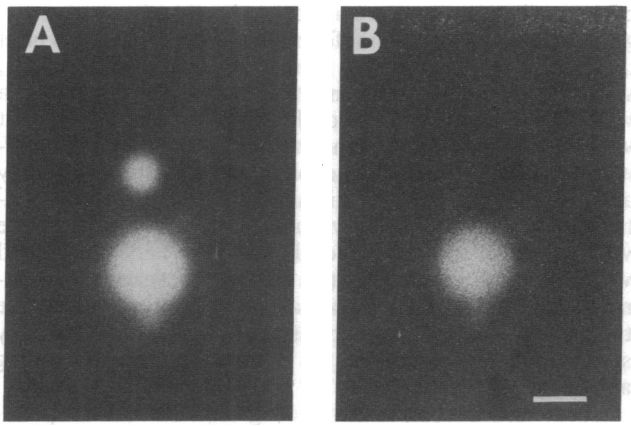

FIG. 5. Some buds that appear to lack an inherited vacuole contain a detectable vacuole when labeled with the fluorescein derivatives. *vac1-1* yeast were incubated at 23°C under conditions that produce the endogenous *ade2* fluorophore, resuspended in YCM (pH 4.5), and allowed to grow at 23°C for approximately one doubling. This population was labeled with the fluorescein derivatives. (*A*) One double-labeled cell was photographed as described in *Materials and Methods*. (*B*) This same cell was exposed to 450- to 490-nm light for 2 min, and then photographed as described. (Bar = 1 μm.)

inheritance of the bud's vacuole contents from the mother cell. To determine whether *vac1-1* affects intervacuole communication, *ade2* cells of mating type **a** or α were grown either under conditions of adenine limitation, to accumulate the *ade2* fluorophore, or with adenine supplementation, and then mated to form zygotes (Table 1). *VAC1* cells initiate the formation of a vacuole in the bud by vesicular traffic from the parental vacuoles when the bud is ≈9% the volume of the zygote (8). When *vac1-1* cells with accumulated fluorophore were mated with *VAC* cells, very few examples of large-budded zygotes with fluor in the bud vacuole were observed. In contrast, when a *VAC1* strain bearing the fluorophore was mated with a *vac1-1* strain, almost all the buds contained fluorescent vacuoles. In these zygotes, although transfer occurs to the bud, it does not proceed to the unlabeled parental vacuole. These results suggest that the failure of vacuole division and segregation in *vac1-1* is due to a defect in intervacuole communication and strengthen the postulate that this traffic is involved in vacuole segregation in haploid cells. Vacuoles from a *vac1-1* strain can neither donate nor receive vesicles.

To determine the specificity of the *vac1-1* mutation, we have analyzed the segregation of mitochondria and of nuclear DNA in these cells. Mitochondria may be observed in living yeast using 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], a dye that accumulates in the mitochondria in response to the electrochemical potential across the inner membrane (22).

Table 1. The *vac1* mutation inhibits vacuoles from either donating or receiving vacuole-derived vesicles in mating yeast

	% large-budded zygotes with fluorescent label in		
Mated strains	Labeled parent only	Labeled parent and bud	Both parents and bud
VAC × *VAC*	4	0	96
vac1 × *vac1*	81	5	14
vac1 × *VAC*	74	0	26
VAC × *vac1*	6	72	23

All strains were *ade2*. The strains listed on the left were grown to produce the *ade2* fluorophore. In those on the right, *ade2* fluorophore production was suppressed. All cultures were mated at 23°C.

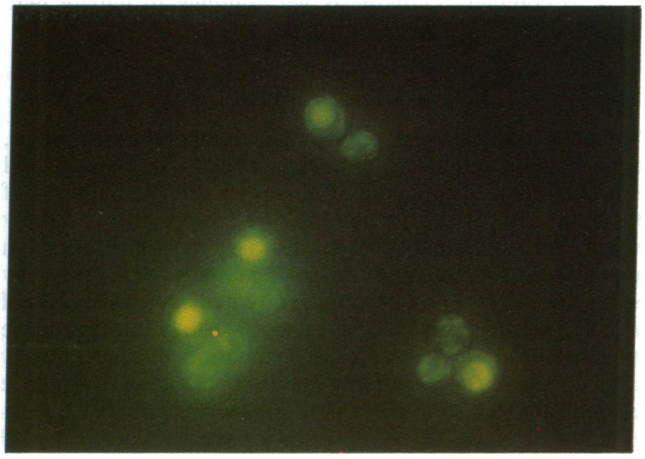

FIG. 6. Mitochondria partition normally in *vac1-1* yeast. *vac1-1*, *ade2* yeast were grown in YCM to stationary phase (to produce the *ade2* fluorophore), then collected by centrifugation, and resuspended in YP glycerol at 37°C. After approximately 4 hr, a significant number of the originally unbudded cells produced a large bud. These buds did not contain vacuoles labeled with the *ade2* fluorophore. Yeast mitochondria were labeled with 50 ng of $DiOC_6(3)$ per ml (Molecular Probes), a membrane-potential dye (22). The $DiOC_6(3)$ fluorescence is green. The *ade2* fluorescence is yellow.

VAC and *vac1-1* cells that were already labeled with the *ade2* fluorophore were labeled with $DiOC_6(3)$. In wild-type yeast, mitochondria can be detected in the bud as soon as the bud is formed (T. Stearns and D. Botstein, personal communication; L.S.W., unpublished observations). Likewise, mitochondria can always be detected in the buds of *vac1-1* cells (Fig. 6), whether or not that bud contains a detectable vacuole. This phenotype is observed at both 23°C and 37°C.

To assess nuclear segregation, *vac1-1* cells from a culture arrested at 37°C were fixed and stained with 4′,6-diamidino-2-phenylindole, a DNA-specific fluorophore (17). Nuclear segregation was normal (Fig. 7). Thus, although *vac1-1* has a defect in vacuole segregation, it segregates these other organelles normally. In addition, the appearance and position of both the nucleus and mitochondria in electron micrographs of *vac1-1* cells is normal (data not shown).

At the nonpermissive temperature, *vac1-1* cells accumulate multiple buds with a corresponding decrease in the percent of cells in S phase (data not shown). It is possible that these multiply budded forms arise because *vac1-1* cells are

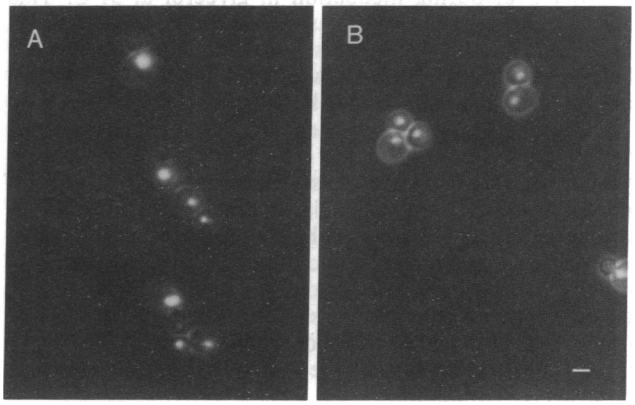

FIG. 7. The nucleus segregates normally in *vac1-1* cells. Mutant and wild-type cells were grown to midlogarithmic phase, shifted to 37°C for 2.5 hr, then fixed and labeled with 4′,6-diamidino-2-phenylindole (Sigma) (17). Both wild-type (*A*) and *vac1-1* (*B*) cells show normal nuclear segregation. (Bar = 1 μm.)

slow to complete cytokinesis yet continue to initiate new rounds of cell growth and division.

DISCUSSION

We have found a means to collect temperature-sensitive, conditionally lethal mutants that affect the division and segregation of the vacuole of *S. cerevisiae*. The hallmark of these mutants is the production of a large bud with a very small, or undetectable, vacuole. This is not a common phenotype; for example, no such mutants were found by screening 40 strains of the currently available collection of *cdc* and *ndc* mutants (L.S.W., unpublished data). One of the mutants, *vac1-1*, is a recessive mutation that specifically affects vacuole segregation without affecting the inheritance of nuclear DNA or of mitochondria. This selectivity suggests that *VAC1* does not affect common elements used by all organelles, but it may affect the vacuole or vacuole/cytoskeleton interactions. This idea is supported by the studies (Table 1) of vacuole-derived vesicular traffic in zygotes. *ade2* fluorophore dye transfer is blocked from the vacuole derived from the cell with the *vac1-1* mutation but occurs normally from the vacuole derived from a wild-type *VAC1* cell. These data suggest that *VAC1* encodes a protein that is not freely diffusible in the cytosol.

Although *vac1-1* is a temperature-sensitive, conditional lethal mutant, the phenotype of abnormal vacuole segregation is present at both the permissive and nonpermissive temperatures. There are at least two examples of *vps* genes that have been removed from *S. cerevisiae* by gene disruption. The resultant phenotype of each gene disruption has been a temperature-sensitive, conditional lethal strain with an altered vacuole phenotype present at both the permissive and restrictive temperatures (ref. 23; L. Banta and S.D.E., unpublished observations). Because *vac1-1* doubles at nearly the wild-type rate at 23°C, it seems likely that the cells that are produced with very tiny vacuoles are viable at the permissive temperature. Perhaps some aspect of vacuole function is not required at 23°C but is required at higher temperatures. By the time these cells have proceeded through the cell cycle, they have once again obtained normal-sized vacuoles in the maternal cell (Figs. 2*C* and 4*B*).

Both the *vac1-1* mutant and *vps* gene disruptions grow poorly on glycerol and other nonfermentable carbon sources at 23°C or 37°C. Dulic and Riezman (23) have shown that *end1* (allelic to *vps11* and *pep5*) grows poorly on glycerol at 23°C even though its mitochondrial function is normal. Mitochondria of *vac1-1* cells can be labeled with $DiOC_6(3)$, a membrane potential dye, during incubation in glycerol at 37°C. This suggests that *vac1-1* has at least partial mitochondrial function. Almost all *vps* mutants show impaired growth on glycerol at 37°C (data not shown), although the biochemical basis of this phenotype is not known.

The *vac1-1* lesion apparently allows cells to continue through multiple rounds of the cell cycle despite the delay in cytokinesis. Most of the cells that eventually do bud off contain a vacuole, although this vacuole inherits little of the parental contents and presumably arises directly from Golgi-derived vesicles or from endocytosis. One interpretation of our data is that *de novo* vacuole biosynthesis becomes the rate-limiting step in cytokinesis in the *vac1-1* mutant at 37°C. Vacuole division and segregation, which normally begin in S phase, and cytokinesis, at the end of M phase, may each require *VAC1* function.

Cytological studies have now defined the process of vacuole division and segregation. It is controlled by the cell cycle, as it begins at a precise time shortly after bud emergence (7). Vacuoles are not found in the bud of zygotes until there is vesicular transfer from a maternal vacuole (8), suggesting that this is the normal means of establishing a vacuole in the bud. Studies of the *vac1-1* mutant reveal that vacuoles can also arise in the bud without significant inheritance from the maternal vacuole, albeit much more slowly and infrequently. Vacuole division and segregation are spatially controlled, as the new vacuole, founded by intervacuole vesicular traffic, is always localized to the bud. As with all vesicular traffic in the cell, this process preserves compartmentation. This traffic appears as vesicles or tubules. These tubular and vesicular structures may be closely related by small kinetic differences in the rates of membrane budding and fusion.

Four class C complementation groups have been previously identified that lack vacuoles and are defective in protein sorting from the Golgi (15). It is intriguing that vacuoles form rapidly when complementing class C mutants mate to form a zygote. However, the *vac1-1* mutation is not in one of these complementation groups (unpublished data). The ability to isolate and characterize *vac* mutants may allow a thorough genetic analysis of their interrelationships, the sequence and possible conserved functional motifs of their proteins, and their subcellular localization.

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