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The *ras*-Like Yeast *YPT1* Gene Is Itself Essential for Growth, Sporulation, and Starvation Response

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The *Saccharomyces cerevisiae* gene *YPT1* encodes a protein that exhibits significant homology to the mammalian *ras* proteins. Using gene disruption techniques, we have shown that the intact *YPT1* gene is required for spore viability. Lethality caused by loss of *YPT1* function, unlike that caused by loss of the yeast *ras* homologs *RAS1* and *RAS2* function, is not suppressed by the *bcy1* mutation, suggesting that *YPT1* does not act through the adenylate cyclase regulatory system. A cold-sensitive allele, *ypt1-1*, was constructed. At the nonpermissive temperature, mutants died, exhibiting aberrant nuclear morphology, as well as abnormal distribution of actin and tubulin. The mutant cells died without exhibiting classical cell-cycle-specific arrest; nevertheless, examination of cellular DNA content suggests that the *YPT1* function is required, particularly after S phase. Cells carrying the *ypt1-1* mutation died upon nitrogen starvation even at a temperature permissive for growth; diploid cells homozygous for *ypt1-1* did not sporulate. The *YPT1* gene is thus involved in nutritional regulation of the cell cycle as well as in normal progression through the mitotic cell cycle.

The family of *ras* oncogenes was discovered first in RNA tumor viruses and identified subsequently in a number of human cancers (5). The *ras* oncogenes are derived from normal cellular analogs (8), the study of which should provide a way of understanding the normal and pathological mechanisms of cell growth control. The *ras* genes are a highly evolutionarily conserved gene family in vertebrates (47). More surprisingly, highly homologous genes have been identified in *Drosophila melanogaster* (32) and more recently in *Saccharomyces cerevisiae* (6, 27). *S. cerevisiae* provides the most simple eucaryotic system that can be subjected to genetic analysis to study the function of *ras*-related genes in normal cell growth.

Two close homologs of the *ras* genes, *RAS1* and *RAS2*, were detected in *S. cerevisiae* by nucleic acid homology (6, 27). The presence of either *RAS1* or *RAS2* is essential for cellular growth; therefore they form a complementary set (18, 40). The *RAS1* and *RAS2* genes function by modulation of the adenylate cyclase activity (45), and their proper function is needed for appropriate response to changes in growth conditions (41). Functional homology between the mammalian and yeast *ras* proteins was strongly supported by demonstration of the ability of each product to function in the heterologous cell (7, 17).

A third gene, *YPT1*, previously called *YP2*, was found in *S. cerevisiae* that encodes a protein with a weaker, yet significant, homology to the mammalian *ras* P21 proteins. This gene is located between the actin (*ACT1*) and β-tubulin (*TUB2*) genes, on chromosome VI; it encodes a 206-amino-acid protein and is expressed in growing cells (11). The *YPT1* gene is apparently not a member of the complementary set of *RAS1* and *RAS2* genes (18, 40). Very recently, it has been reported that the *YPT1* gene product binds GTP and has an essential function in spore viability (30).

In this paper we show that the *YPT1* gene plays, by itself, an essential role in both the mitotic and meiotic stages of the *S. cerevisiae* life cycle. We constructed and examined conditional-lethal as well as null mutations. Unlike *RAS1* and RAS2 genes, the YPT1 gene does not seem to work through the adenylate cyclase regulatory system. We further show that the *YPT1* gene is involved both in the organization of the cytoskeleton during vegetative growth and in the nutritional regulation of the cell cycle.

MATERIALS AND METHODS

Strains and plasmids. *S. cerevisiae* strains used in this paper were derived from the family of S288C strains and are listed in Table 1. Strain DBY1739 is a derivative of T16-3B (45). The *bcy1* mutation was monitored in the construction and analysis of DBY1739 and derivatives by three methods: iodine-iodide staining (45); heat shock sensitivity (T. Toda, personal communication); and the original phenotype, suppression of *cyr1* gene (24). All three methods agree.

Escherichia coli strains used were HB101 (*leu pro thr hsdR hsdM recA*); DB6507, a pyrF74::Tn*5* derivative of HB101; and DB4904 (*hsdR hsdM*$^+$ *supA supF nad-7 ung-1*).

Plasmids used were YIp5 (39), which was used as yeast-integrating plasmid; pRB214 (or YCp50), which is YIp5 with *CEN4* and *ARS1* (C. Mann, personal communication; see reference 20 for map) and was used as low-copy-number plasmid; pRB307, which is YIp5 with a 2μm fragment and was used as high-copy-number plasmid (provided by G. Fink); and pRB129, which is YIp5 with a 4.5-kilobase *Bam*HI fragment that contains the *YPT1* gene (41).

Plasmids that contain the *YPT1* gene and were constructed for this study are pRB301 (ND plasmid), a nondisrupting plasmid, and pRB302 (D plasmid), a disrupting plasmid described in Fig. 1; pRB319, which was constructed by insertion of a 1.76-kilobase *Cla*I-*Bam*HI fragment containing the *YPT1* gene (from PRB129) into pRB214 (CEN plasmid); and pRB320, which was constructed by insertion of the above fragment into pRB307 (2μm plasmid).

Yeast media, growth, and genetic methods. Media and methods of mating and tetrad analysis were as described by Sherman et al. (31). For nitrogen starvation experiments, we used 0.17% yeast-nitrogen base without amino acids and

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype/phenotype	Source
DBY1034	*MATa his4-539 lys2-801 ura3-52*	Our laboratory
DBY1035	*MATα his4-539 ade2 ura3-52*	Our laboratory
DBY947	*MATα ade2-101 ura3-52*	Our laboratory
DBY1395	*MATa his4-539 ura3-52*	Our laboratory
DBY1428	*MATα ade2 ura3-52 lys2-801* Cyh^r *tub2-104*	Our laboratory
PNY135.5	*MATa lys2 ura3-52 act1-3*	P. Novick
DBY1739	*MATα lys2 ura3-52 Cyh^r tub2-104 bcy1*	This study
DBY1773	*MATa trp1 cyr1-1*	T. Toda (T43-6c)
DBY1801	*MATa ura3-52 his4-539 lys2-801 ypt1-1*	This study
DBY1802	*MATa ura3-52 his4-539 lys2-801 YPT1*	This study
DBY1803	*MATa ura3-52 his4-539 lys2-801 ypt1-1*	This study
DBY1806	*MATα ura3-52 his4-539 ade2-101 ypt1-1*	This study

ammonium sulfate and containing 2% glucose. Quantitative sporulation in liquid was done as described by Simchen et al. (37).

Disruption of the *YPT1* gene. Diploid *URA3* auxotrophs heterozygous at one of the loci neighboring *YPT1*, *TUB2* or *ACT1*, were transformed with the D or ND plasmid (Fig. 1) (35). Ura$^+$ transformants were selected, purified, and sporulated, and tetrad analysis was performed. The plasmids had been cut with *Nco*I or *Sma*I to direct their integration into the *YPT1* or *URA3* gene. *Nco*I is the only restriction enzyme useful for site direction of the plasmids into the *YPT1* gene, but since it also cuts the *URA3* gene, partial digestion was performed. Ura$^+$ transformants with such partial digests should contain the plasmid in the *YPT1* or the *URA3* gene. We distinguished between these two cases by analyzing the linkage of the Ura$^+$ to the markers in the *YPT1* neighboring genes, *ACT1* and *TUB2*. The *ACT1* and *TUB2* genes were marked with recessive conditional lethal mutations *act1-3*/+ (36) and *tub2-104*/+ (43).

Construction of the cold-sensitive lethal *ypt1-1* mutation. A deletion gap heteroduplex, with the *YPT1* (lacking its 5′ end) present as a single-stranded DNA, was prepared as described by Shortle (33). This heteroduplex was mutagenized with sodium bisulfite as described by Shortle and Botstein (34). After filling in and amplification in a repair-deficient *E. coli* strain (DB4904*ung*$^-$), the mutagenized plasmid was directed to the *YPT1* gene on the yeast chromosome (by *Nco*I partial digestion), and Ura$^+$ transformants of strain DBY1034 were selected. The *YPT1* gene in such transformants is only partially duplicated, and recessive mutations in the complete copy will be phenotypically expressed (36). We screened for a conditional-lethal phenotype and showed that it was due to the integrated by demonstrating linkage of the conditional-lethal and the Ura$^+$ phenotypes. Once such a mutant was found, the mutation was stabilized by excising the plasmid by 5-fluoro-orotic acid selection against the Ura$^+$ phenotype (4). The excision leaves a single copy of the *YPT1* gene in the chromosome, and, depending on the site of recombination between the two partially duplicated copies, it can be either mutated or unmutated.

We found one such mutant, which showed a cold-sensitive lethal phenotype. After excision of the plasmid, the Ura$^-$ segregants contained either an unmutated copy of the *YPT1* gene (DBY1802, which was used as the control cold-resistant strain) or the *ypt1-1* mutation (DBY1801, which exhibits cold sensitivity). We backcrossed it (to eliminate any suppressors) to the wild-type strains (DBY947 and DBY1034) three times to get the strains that we used for most of the following experiments (DBY1803 and DBY1806; Table 1).

Temperature or starvation shift experiments. Cells were grown at 30°C in liquid medium. Exponentially growing cultures (5×10^6 to 1×10^7 cells per ml) or stationary-phase cultures ($>5 \times 10^8$ cells per ml) were shifted to 14°C for temperature shift experiments or washed and suspended in medium without nitrogen for starvation experiments. After various incubation periods, aliquots were removed from cultures and sonicated for 5 s to disrupt cell aggregates. For determination of total cell number and cell morphology, the cells were counted by phase microscopy. To determine viability, the cells were diluted in YEP medium and plated on YEPD medium. Colonies were scored after 3 days at 30°C.

Synchronization of cells on Ficoll gradients was done by centrifugation of harvested exponentially growing cells through a step gradient of Ficoll in water (10, 8, 6, and 4%). The fractions that contained more than 95% unbudded cells were combined, centrifuged, and suspended in YEPD medium. The synchronized cells were incubated and studied as described above.

Immunofluorescence microscopy and photography. Yeast cells were fixed and stained as described by Kilmartin and Adams (19). Affinity-purified anti-yeast-actin and monoclonal anti-tubulin antibodies were kindly provided by J. Kilmartin. Nuclear staining was done with 4′,6′-diamidino-2-phenylindole (DAPI) (48). Microscopy and photography procedures were as described by Novick and Botstein (26).

Fluorescence-activated cell sorter. Cells were fixed, stained, and checked as described by Hutter and Eipel (16). Exponentially growing cells were fixed in 70% ethanol for 1 h at room temperature, treated with 0.1% RNase for 1 h at 37°C and 0.5% pepsin for 5 min at room temperature, and stained with 0.05 mg of propidium iodide per ml overnight at 4°C. Before being run in the flow cytometer, the cells were washed and suspended in 0.2 M Tris buffer (pH 7.5).

RESULTS

***YPT1* gene product is essential for yeast vegetative growth.** We used gene disruption techniques (35) to examine whether *YPT1* is an essential gene for yeast cell viability. Two plasmids were constructed, disrupting (D) and nondisrupting (ND) (Fig. 1A). The disrupting plasmid pRB302 contains an internal (i. e., both 3′ and 5′ ends deleted) fragment of the *YPT1* coding region, while the nondisrupting plasmid pRB301 is deleted only for the 5′ end of the gene. Both

plasmids contain *URA3* as a selectable marker for transformants. If *YPT1* is an essential gene, it is expected that integrative DNA transformation of the D plasmid into the *YPT1* gene will be a lethal event in a haploid strain, since each of the two repeated copies of the gene will be incomplete. Integration of the D plasmid into the *URA3* gene is not expected to be lethal, nor is integration of the ND plasmid into either the *YPT1* or *URA3* gene.

Since the *YPT1* gene is flanked by the *TUB2* (β-tubulin) and *ACT1* (actin) genes, disruptions were performed in diploid strains carrying mutant alleles of these genes. Tetrad analysis of diploids that were transformed with D plasmid directed to the *YPT1* gene revealed a pattern of viability expected for a recessive lethal mutation: two viable spores were found in most of the tetrads (one viable spore was found in the rest). All the living spores were Ura$^-$, indicating

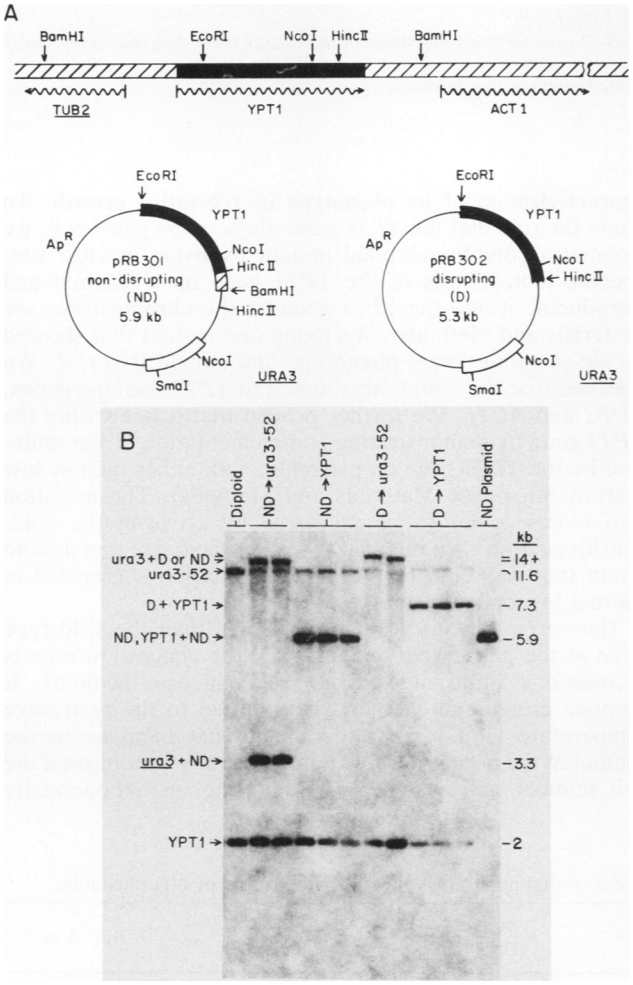

FIG. 1. Structure of disrupting (D; pRB302) and nondisrupting (ND; pRB301) *YPT1* plasmids (A) and the results (B) of gel transfer hybridization of diploids transformed with these plasmids. Yeast DNA extracted (14) from diploid transformants (Table 2), was cut with *Bam*HI. Gel transfers (38) of this DNA were hybridized with ^{32}P-labeled ND plasmid. Abbreviations: Diploid, untransformed *tub2-104*/+ diploid; ND→ura3-52 ND plasmid integrated into the *ura3-52* gene; ND→YPT1, ND plasmid integrated into the *YPT1* gene; D→ura3-52, D plasmid integrated into the *ura3-52* gene; D→YPT1, D plasmid integrated into the *YPT1* gene; kb, kilobases. Symbols: ■, *YPT1* coding sequence; ▨, flanking sequence; ▭, other yeast DNA; ——, vector (pBR322).

that the segregation of the locus containing the disrupted *YPT1* gene (Ura$^+$) is lethal (Table 2). Using the *tub2-104*/+ or *act1-3*/+ diploids, we could show that the lethal event was linked to *TUB2* or *ACT1* (Table 2) and that the integration could occur, as expected, in either of the homologous chromosomes. The site of plasmid integration in these transformants was verified by gel transfer hybridization (Southern) analysis (Fig. 1B).

Transformants in which the D plasmid was integrated into the *URA3* gene, as well as transformants in which the ND plasmid integrated into *YPT1* or *URA3*, generally produced four viable spores per ascus, with no tetrad having fewer than three viable spores (Table 2). The Ura$^+$ phenotype segregated 2:2. These controls show that the presence of the D plasmid itself does not cause lethality, nor does the integration of the ND plasmid into the *YPT1* region. We can conclude from the above experiments that the intact *YPT1* gene is essential for germination of spores or vegetative growth of yeast cells, or both.

The disruption of the *YPT1* gene could cause lethality either because of the requirement of the *YPT1* gene product for the cell growth or because it interrupts in some way the expression of its neighboring essential genes, *TUB2* (25) and *ACT1* (35). To distinguish between these possibilities, we examined the expression of *ACT1* and *TUB2* in transformed diploids. Strains of genotype *tub2-104*/+ and *act1-3*/+ in which *YPT1* was disrupted in the chromosome carrying the wild-type alleles were analyzed. Since *tub2-104* and *act1-3* are recessive mutations (36, 43), it is expected that if *TUB2* and *ACT1* function normally when they are located in *cis* to a *YPT1* disrupted gene, the diploid transformant will show a wild-type phenotype. All the diploids described in Table 2 were tested and all showed normal function of the neighboring genes even when the D or ND plasmid was integrated *cis* to the wild-type copy of the gene. In particular, two *ypt1*-null:*ACT1*/*act1-3* transformants were temperature resistant and all four of the *ypt1*-null:*TUB2*/*tub2-104* transformants were benomyl sensitive and cold resistant, as were the respective parental diploids. These data show that disruption of the *YPT1* gene does not affect the expression of the neighboring *ACT1* and *TUB2* genes and that the *YPT1* gene itself is essential for vaibility.

We monitored, by microscopy, the fate of individual spores that contain a disrupted *YPT1* gene. After 24 to 48 h on rich medium, some of the spores that had failed to form a colony had not budded, some had one large bud, and some had divided once or twice. This result indicates that there can be enough *YPT1* gene product in the cell to allow one or two cell divisions and strongly suggests that *YPT1* function is required for growth of cells, and not just for spore germination.

***YPT1* does not function through the adenylate cyclase regulatory pathway.** Recently it was shown that *RAS1* and *RAS2* genes function as controlling elements of the adenylate cyclase and cyclic AMP-dependent protein kinase pathway (45). *YPT1* is not a member of the complementary set of *RAS1* and *RAS2* genes, since it is essential for viability even when the others are functioning and vice versa. However, like these two genes, the *YPT1* gene shares sequence similarity with the G proteins (15) that function through activation of adenylate cyclase. Therefore, we tested whether the *YPT1* gene is also involved in this pathway, maybe in a different step. We used the *bcy1* mutation, which lies in the structural gene for the regulatory subunit of the cyclic AMP-dependent protein kinase (44) and results in an activated form of this enzyme that normally is cyclic AMP

TABLE 2. Viability and linkage analysis of haploid progeny from diploid strains[a] transformed with D or ND plasmids

Plasmid[b]	No. of tetrads (live: dead spores)				Phenotype[c]			Deduced linkage[e]
	4:0	3:1	2:2	1:3	Ura$^+$/viable	Tub2$^-$/viable[d]	Act1$^-$/viable[d]	
tub2-104/+ diploid								
D	0	0	30	1	0/61	61/61		*TUB2*
D	0	0	11	0	0/22	0/21		*tub2-104*
D	21	1	0	0	32/64	16/32		*ura3-52*
ND	10	0	0	0	16/32	16/16		*TUB2*
ND	31	1	0	0	48/96	0/48		*tub2-104*
ND	9	1	0	0	16/32	9/16		*ura3-52*
act1-3/+ diploid								
D	0	0	17	3	0/37		36/37	*ACT1*
D	0	0	19	0	0/40		1/40	*act1-3*
D	20	1	0	0	32/64		16/32	*ura3-52*
ND	9	1	0	0	16/32		16/16	*ACT1*
ND	8	0	0	0	16/32		0/16	*act1-3*
ND	19	1	0	0	32/64		14/16	*ura3-52*

[a] The diploid strains were DBY1034/DBY1428 (*tub2-104*/+) and PNY135.7/DBY1035 (*act1-3*/+).
[b] Plasmids were directed to the *YPT1* gene by *Nco*I partial digestion or to the *ura3-52* gene by *Sma*I digestion. In most cases more than one independent transformant was used.
[c] For cases in which four spores were viable, eight complete tetrads (32 spores) were tested per transformant.
[d] The Tub2$^-$ phenotype is Ben^r Cs; the Act1$^-$ phenotype is Ts$^-$.
[e] The linkage was deduced from the previous column.

dependent (24). If the *YPT1* gene acts at any point prior to the cyclic AMP-dependent protein kinase, then the *bcy1* mutation should suppress the loss of the *YPT1* function.

The experiment was done by disrupting the *YPT1* gene in a *bcy1*/+ heterozygote and examining the viability of *bcy1* *YPT1*-disrupted haploid segregants. The results (Table 3) show that the disrupting plasmid causes lethality upon integration into the *YPT1* gene (shown by linkage to the *TUB2* gene as above) whether or not the *bcy1* mutation was in the same spore. In the control experiments, we tested *bcy1*/+ transformants in which the nondisrupting plasmid was integrated into the *YPT1* gene or the disrupting plasmid was integrated elsewhere in the genome (probably into the *URA3* gene). In each case, the haploid cells that contained the plasmids (Ura$^+$ phenotype) were viable regardless of the presence of the *bcy1* mutation.

We can conclude that unlike the *RAS1* and *RAS2* genes, the *YPT1* gene is not suppressed by the alteration in activity of the cyclic AMP-dependent protein kinase caused by expression of the *bcy1* mutation. This makes it very likely that the *YPT1* gene product is not involved directly in activation of the cyclic AMP-dependent protein kinase.

Isolation of the cold-sensitive lethal mutation *ypt1-1* and characterization of its phenotype in vegetative growth. To study the role that the *YPT1* gene plays in the yeast cell, we isolated a conditional-lethal mutant by using in vitro site-specific mutagenesis of the *YPT1* gene on a plasmid and introducing it into the *YPT1* gene on the chromosome (see Materials and Methods). We found one mutant that showed a cold-sensitive lethal phenotype and named it *ypt1-1*. We checked that the mutation is linked to *YPT1* flanking genes, *TUB2* and *ACT1*. We further proved that it lies within the *YPT1* gene by demonstrating complementation of the mutation by the *YPT1* gene on plasmids, with either high or low copy number (see Materials and Methods). The mutation *ypt1-1* was found to be recessive by crossing the cold-sensitive strain with the wild type; this gave rise to a diploid strain that was cold resistant. The mutation segregated in normal Mendelian fashion.

The *ypt1-1* mutant grew more slowly than the wild type even at the permissive temperature (the generation time is increased 1.5-fold at 30°C in YEP-glucose medium). It stopped growing altogether when shifted to the restrictive temperature (14°C). To understand what happens to the mutant at the nonpermissive temperature, we monitored the cell number and viability after shifting an exponentially

TABLE 3. Viability and linkage analysis of haploid progeny from *bcy1*/+ diploid strains[a] transformed with D or ND plasmids

Plasmid[b]	No. of tetrads (live: dead spores)					Phenotype (Ura$^+$/viable)	Deduced linkage[c]	Bcy$^-$/Ura$^-$[d]
	4:0	3:1	2:2	1:3	0:4			
D	0	0	23	7	2	3[e]/53	*TUB2*	25/53
D	0	0	10	0	0	0/20	*tub2-104*	9/20
D	18	3	1	0	0	42/81	Not *TUB2*	NT[f]
ND	7	3	0	0	0	16/33	*TUB2*	10/17
ND	6	3	1	1	0	13/26	*tub2-104*	9/13

[a] The diploid strain was DBY1739/DBY1395.
[b] Site direction to the *YPT1* gene was done by digestion of the plasmid DNA with *Nco*I in the presence of 20 mg of ethidium bromide per ml, conditions that were found to favor cutting in the *YPT1* gene in the D and ND plasmids. Direction to the *ura3-52* gene was done by *Sma*I digestion. In most cases, more than one independent transformant was used.
[c] The location of the integrated plasmid was concluded from the linkage between Ura$^+$ and Tub2$^-$ phenotypes as in Table 2 (data not shown).
[d] The Bcy$^-$ phenotype was monitored by iodine-iodide staining.
[e] The 3/53 spores that showed the Ura$^+$ phenotype were found to be diploids heterozygous for the disruption.
[f] NT, Not tested.

growing culture from permissive to restrictive conditions (Fig. 2A and B). Wild-type cells, after a short lag, continued to grow, with a generation time of about 12 h. The *ypt1-1* mutant cells, however, lost viability when they were shifted to the low temperature. After 24 h (about 2 generation times), half or more of the cells were dead; after 48 h, only 10% of the cells were still viable if plated at permissive temperture. The total number of *ypt1-1* cells increased less than twofold in 48 h, showing that most of them arrested and died during the first cell cycle at the nonpermissive temperature. This result was confirmed by monitoring the arrest of individual cells on solid medium (data not shown).

When stationary-phase *ypt1-1* cells were shifted from the permissive to the restrictive temperature, they remained viable (Fig. 2C and D). This suggests that the mutant cells die at the restrictive temperature only if they are growing. The fact that the *ypt1-1* mutant cannot multiply and dies (if already growing) at the nonpermissive temperature confirms our conclusions from the *YPT1* disruption experiments, namely, that the *YPT1* gene is essential not only for spore germination but also for vegetative growth.

In our attempts to understand the function that the *YPT1* gene product plays in mitotic growth, we studied the morphology of the *ypt1-1* mutant cells after they were shifted to the nonpermissive temperature. The cells did not behave like classical cell-division-cycle (*cdc*) mutants (12), since they did not arrest with a unique cell morphology. After 24 h, about 50% of the cells were unbudded and the rest had buds of various sizes, mainly small. After incubation at the low temperature, however, the *ypt1-1* cells were bigger than the wild-type cells, a characteristic shared by many *cdc* mutants.

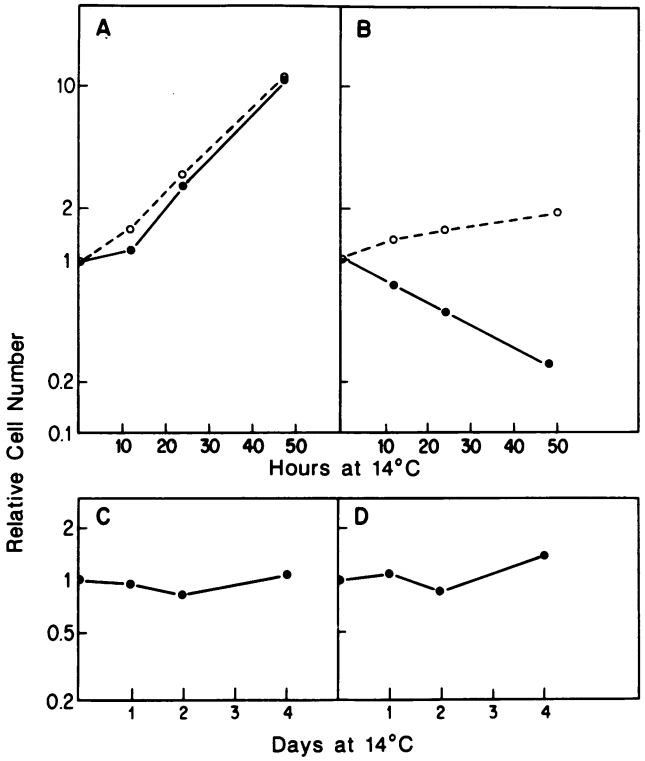

FIG. 2. Behavior of *YPT1* and *ypt1-1* cells at 14°C in vegetative growth medium. Exponentially growing (A, B) and stationary-phase (C,D) cell cultures of *YPT1* (A, C) and *ypt1-1* (B, D) were shifted from 30 to 14°C. Total cell number (○) and viable counts (●) were measured after various incubation periods. The *YPT1* strain was DBY1802, and the *ypt1-1* strain was DBY1801.

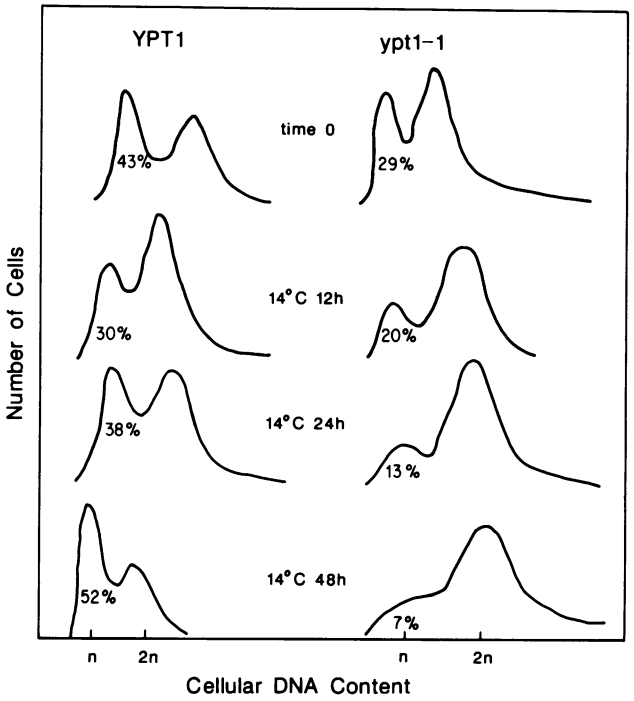

FIG. 3. Measurement of cellular DNA content of *YPT1* and *ypt1-1* cultures at 14°C in vegetative growth medium. *YPT1* (strain DBY1034) and *ypt1-1* (strain DBY1803) cell cultures were shifted from 30 to 14°C. After various incubation periods, their cellular DNA content was measured by fluorescence-activated cell sorter analysis (see Materials and Methods).

Since it appeared that the *ypt1-1* mutant cells arrested during their first cycle at the restrictive temperature but did not show a unique terminal cell morphology phenotype, we studied the fate of the nuclear DNA. We measured the cellular DNA content with a fluorescence-activated cell sorter (Fig. 3). In an exponentially growing wild-type culture, at 30 or 14°C, about 40% of the cells contained an amount of DNA equivalent to one haploid genome and 60% contained twice that amount per cell. In a culture that approaches stationary phase (48 h at 14°C), more cells contained one haploid unit, as expected. An exponentially growing *ypt1-1* culture also contained cells with DNA equivalent to one or two haploid genomes per cell, but upon incubation at the restrictive temperature, an increasing fraction of the culture contained DNA equivalent to two genomes per cell. After 24 h at 14°C, about 90% of the *ypt1-1* cells arrested with two haploid units of DNA per cell. This result suggests that the *YPT1* gene is needed during the cell cycle specifically after most of the nuclear DNA has been replicated.

This conclusion is fortified by another observation. When *ypt1-1* cells were synchronized by isolation of unbudded cells on Ficoll gradients and returned to the growth medium at the nonpermissive temperature, few if any cells proceeded past the stage of having a small bud. After a long incubation, typically 70% of the cells arrested with a small bud less than half the size of the mother; the remaining cells were still unbudded. This result contrasts with the result of simply shifting an asynchronous culture to the nonpermissive tem-

perature, suggesting that the *YPT1* function may act more than just once in the cell cycle.

Intracellular morphology of the *ypt1-1* mutant. Using different cytological procedures, we tried to find whether there are specific intracellular abnormalities in *ypt1-1* mutant cells after they are shifted to the nonpermissive conditions. We found that nuclear staining with the fluorescent DNA stain DAPI was aberrant in these cells (Fig. 4). Wild-type cells at 30 or 14°C or mutant cells at the permissive temperature had a single round, distinct nucleus and faint cytoplasmic staining that corresponds to mitochondrial DNA. After 24 h at 14°C, the DAPI staining in the *ypt1-1* cells was fragmented and distributed widely through the cell.

We studied the microtubules of the mutant by using anti-tubulin immunofluorescence microscopy (Fig. 4). Almost all the wild-type cells at 30 or 14°C and the *ypt1-1* cells at the permissive temperature were stained, most of them in a very small area near or at the nucleus; in cells that were undergoing nuclear division, we could see that the spindle coincided with the nuclear staining through the necks between the mothers and the buds. Many of the *ypt1-1* cells (40 to 50%) that were incubated at 14°C for 24 h were not stained. We do not know whether this reflects a real lack of microtubules in vivo. The microtubules that were stained, however, were aberrant. They were long and sometimes very thick. Often they extended outside the region of the nuclear staining, frequently traversing an entire unbudded cell. We never saw a complete spindle, typical of those seen in cells undergoing nuclear division, in the culture of *ypt1-1* cells that was shifted to the nonpermissive temperature.

We studied the actin staining of the *ypt1-1* mutant by using anti-actin immunofluorescence microscopy. The wild-type cells at 30 or 14°C and the *ypt1-1* mutant at the permissive temperature appeared as described previously by Kilmartin and Adams (19). Unbudded cells were stained with dots and patches, while budded cells were stained asymmetrically, i.e., extensive staining of the bud and faint cables in the mother cell. The *ypt1-1* cells, after 24 h at 14°C were stained but did not show the asymmetry of staining in budded cells (Fig. 5).

It thus seems that after two generation times under the nonpermissive conditions, the *ypt1-1* mutant cells have aberrant nuclear, microtubule, and actin staining. After shorter incubation times of the mutant at the restrictive temperature, we observed a gradual increase of aberrant staining with time.

***YPT1* is essential for entry into the meiosis and sporulation pathway.** To find whether the *YPT1* gene product is essential for meiosis, we constructed a *ypt1-1/ypt1-1* homozygous diploid and checked its ability to form spores. This diploid did not sporulate and died even at temperatures (26 and 30°C) fully permissive for vegetative growth (Table 4). This defect is recessive, since diploids that are heterozygous for the *ypt1-1* mutation sporulate as efficiently as wild-type diploids at 26°C.

Introduction of the *YPT1* gene, on a low- or high-copy-number plasmid, into the *ypt1-1/ypt1-1* homozygous diploid complements not only its inability to grow at low temperatures but also its inability to perform meiosis. These transformants sporulated efficiently and stayed alive (Table 4, experiment 3), showing that the inability to undergo meiosis was due to the *ypt1-1* mutation.

We noticed that at 30°C, but not at 26°C, the *ypt1-1* mutation showed incomplete dominance with respect to the sporulation defect. The efficiency of sporulation in the *ypt1-1/+* heterozygous diploid was only 30% that of the wild-type cells. Under these conditions, the *ypt1*-null/+ heterozygous diploid showed the same reduction in sporulation efficiency (Table 4, experiment 2), suggesting that even at this temperature, *ypt1-1* shows some loss of function.

***YPT1* is essential for proper response to nitrogen starvation.** When yeast haploid or diploid cells are starved for nitrogen under conditions that do not allow sporulation, they stop growing and remain viable almost indefinitely (for a review, see reference 28). We tested whether the *YPT1* gene plays a role in this process. Since the *ypt1-1* mutant was defective in sporulation at temperatures that are permissive for vegetative growth (26 and 30°C) and since sporulation requires the ability to respond to nitrogen starvation signal, we checked whether the *ypt1-1* mutant arrested properly upon nitrogen starvation at 30°C.

When an exponentially growing culture of wild-type cells was shifted from rich medium to medium without nitrogen, the cell number doubled and viability remained constant for days (Fig. 6A). Within 6 h of the shift, 95% of the cells were unbudded and had unreplicated DNA (measured by the fluorescence-activated cell sorter; data not shown). Under the same conditions, the *ypt1-1* mutant also doubled in cell number, and within 6 h 70 to 80% of the cells accumulated as unbudded cells with unreplicated DNA. However, the cells died slowly upon continuing starvation. After 24 h 60 to 70% of the cells were viable, and after 48 h only 10 to 15% were alive (Fig. 6A).

The inability to remain viable upon nitrogen starvation is a recessive phenotype of the *ypt1-1* mutation, since *ypt1-1/ypt1-1* homozygous diploids died upon nitrogen starvation like the *ypt1-1* haploid, but *ypt1-1/+* heterozygous diploids arrested like wild-type diploids. The inability to remain viable is caused by the defect in the *YPT1* gene, since this phenotype of the *ypt1-1* mutant can be complemented by the *YPT1* gene on low- or high-copy-number plasmids (Fig. 6B).

DISCUSSION

We have shown here that the *YPT1* gene is essential for the execution of three options in the life cycle of the budding yeast *S. cerevisiae*: vegetative growth, sporulation, and nitrogen starvation-induced arrest. The need for the *YPT1* gene product in vegetative growth was proven by using both *ypt1*-null and *ypt1-1* conditional-lethal mutations. The lethality of the disruption was shown not to be the result of an effect on either one of its two essential neighbors, *ACT1* or *TUB2*, but to be due to the need for an intact *YPT1* gene. The *ypt1-1* conditional-lethal mutant that we isolated cannot grow vegetatively at low temperatures and is defective in meiosis and nitrogen starvation-induced arrest even at temperatures that are permissive for growth. All these phenotypes can be complemented by the *YPT1* gene alone on a plasmid, showing that the mutation in the *YPT1* gene causes all of the pleiotropic phenotypes noted. Since in some of these experiments we used the *YPT1* gene on high- as well as low-copy-number plasmids, it seems that *YPT1*, unlike its neighbors *ACT1* (10) and *TUB2* (J. H. Thomas, Ph. D. thesis, Massachusetts Institute of Technology, Boston, 1984), can be present in the cell at high copy number without impairing growth.

The inability of the *ypt1-1* mutant to undergo vegetative growth, sporulation, and starvation arrest is probably due to a reduction in the level of *YPT1* gene function in the mutant cells rather than to inappropriate activation of the protein, since all the phenotypes that we observed were recessive to the wild-type *YPT1* gene. This conclusion is supported by

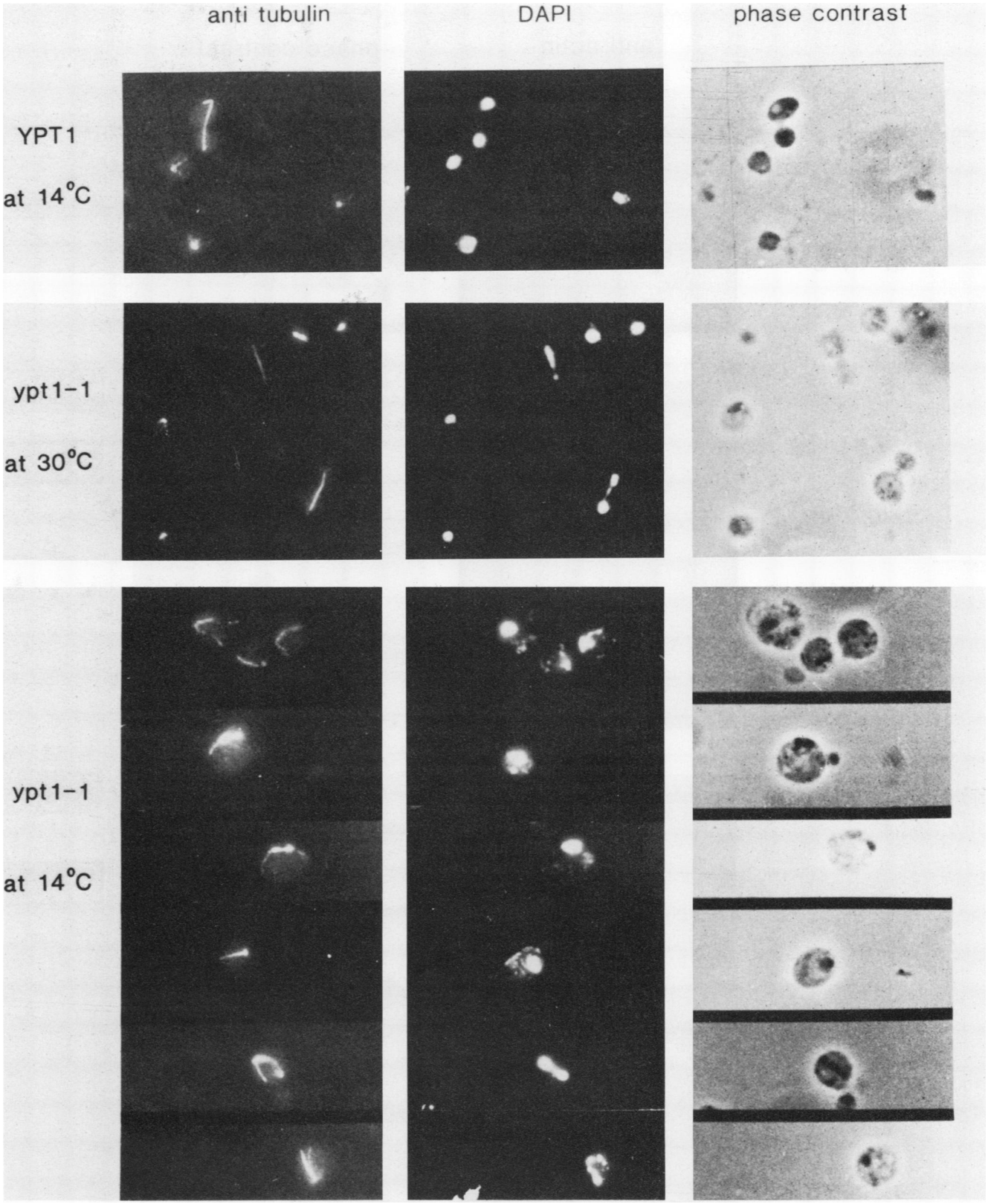

FIG. 4. Nuclear and microtubular staining of *YPT1* and *ypt1-1* cells at 30 and 14°C. Exponentially growing cultures of *YPT1* (strain DBY1802) and *ypt1-1* (strain DBY1801) cells were shifted from 30 to 14°C for 24 h. The cells were fixed and stained for fluorescence microscopy with DAPI for nuclear staining and with anti-tubulin antibodies for microtubular staining (see Materials and Methods). The cell outlines are visible by phase contrast.

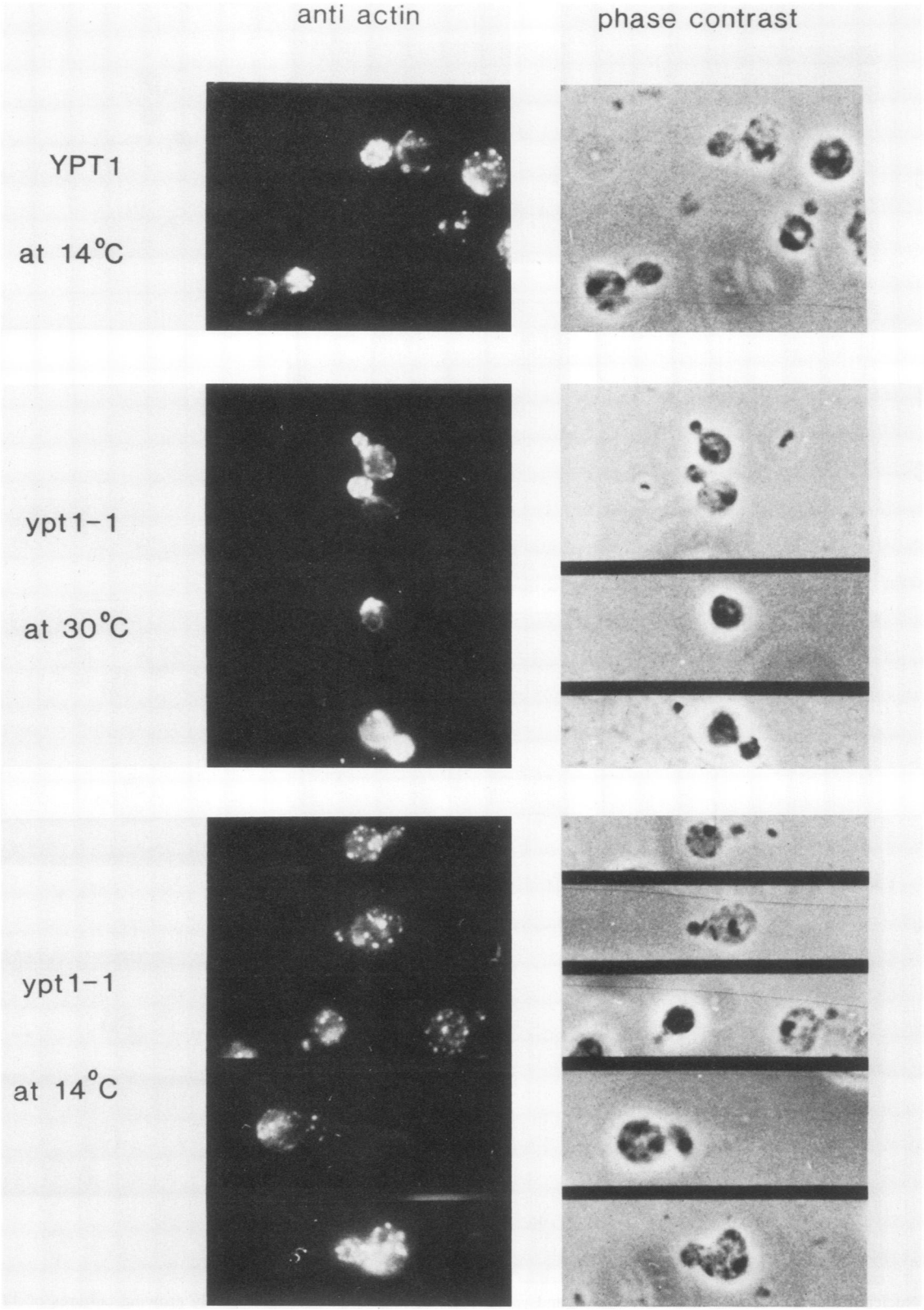

FIG. 5. Staining of actin in *YPT1* and *ypt1-1* cells at 30 and 14°C. Exponentially growing cultures of *YPT1* (strain DBY1802) and *ypt1-1* (strain DBY1801) cells were shifted from 30 to 14°C for 24 h. The cells were fixed and stained for fluorescence microscopy with anti-actin antibodies. The cell outlines are visible by phase contrast.

the similarity in the behavior of *ypt1-1/YPT1* and *ypt1-null/YPT1* heterozygous diploids in sporulation at 30°C.

***YPT1* is implicated in the mitotic cell division cycle.** When an asynchronous, logarithmically growing culture of the *ypt1-1* mutant was shifted from the permissive to the nonpermissive temperature, the cell number doubled and the viability dropped gradually. The mutant did not exhibit a uniform terminal morphology, suggesting that the *YPT1* gene is not required at a single point in the cell cycle (12). This does not mean, however, that the *YPT1* gene is not a cell cycle gene. Important counter-examples include the genes *NDC1* (42) and *TOP2* (13); mutants defective in these genes do not exhibit uniform terminal morphology but are clearly needed at a single stage of the cell cycle.

Several items of data given above suggest that the *YPT1* gene is involved primarily at the post-DNA-replication stage of the mitotic cell cycle. First, and most significant, is the observation that synchronous populations of *ypt1-1* cells do arrest at the small-bud stage. Second, most of the cells, regardless of how arrested, end up with twice the normal amount of DNA, suggesting they all passed through the S period. Third, the loss of viability shown by *ypt1-1* after a shift to the nonpermissive temperature is not observed when the cells are in the stationary phase. This observation suggests strongly that the lethal event is either the result of progress through the cell cycle or the result of mass increase. Last, the mutants grow in size without apparently cycling, a phenotype shared by most cell cycle mutants.

At the morphological level, *ypt1-1* mutants arrested during vegetative growth by a shift to the nonpermissive temperature showed aberrant nuclear staining and aberrant tubulin and actin immunocytology. Since after 24 h more than half of the cells were dead, we do not know which of these aberrant intracellular structures are a direct consequence of *YPT1* protein depletion and cause cell death and which are merely a reflection of cell death. It should be noted, however, that the kind of aberrant nuclear staining observed is uncommon even among cell division cycle mutants and that the tubulin staining, suggesting the possibility of extranuclear polymerization at the expense of spindle formation, is similarly nearly unprecedented. Finally, in many experiments involving shorter incubations, all these features became evident within the first generation time after the shift to the nonpermissive temperature in most of the cells. It should also be emphasized that we never saw normal mitosis in *ypt1-1* cells at the nonpermissive temperature, suggesting that all the aberrant morphologies are the consequence of failure to undergo mitosis promptly after finishing replication of the nuclear DNA.

TABLE 4. Sporulation efficiency

Strain (*MATa*/*MATα*)[a]	% Sporulation	
	26°C	30°C
Expt 1		
YPT1/*YPT1*	87	60
YPT1/*ypt1-1*	95	22
ypt1-1/*YPT1*	70	16
ypt1-1/*ypt1-1*	0	0
Expt 2		
YPT1/*YPT1*	80	63
YPT1/ypt1:null (transformant 1)	80	35
YPT1/*ypt1*:null (transformant 2)	83	20
Expt 3		
ypt1-1/*ypt1-1*	0	
ypt1-1/*ypt1-1* + 2μm plasmid	0	
ypt1-1/*ypt1-1* + *YPT1* on 2μm plasmid	40	
ypt1-1/*ypt1-1* + *YPT1* on CEN plasmid	55	
YPT1/*ypt1-1*	55	

[a] Strains used for these experiments: *YPT1*, DBY1034 (*MATa*) or DBY947 (*MATα*); *ypt1-1*, DBY1803 (*MATa*) or DBY1806 (*MATα*). For experiment 2, the *ypt1*:null diploid strains are described in Table 2, experiment I. For experiment 3, the DBY1803/DBY1806 diploid strain was transformed with 2μm plasmid (pRB307), with the *YPT1* gene on 2μm plasmid (pRB320), and with the *YPT1* gene on the CEN plasmid (pRB319).

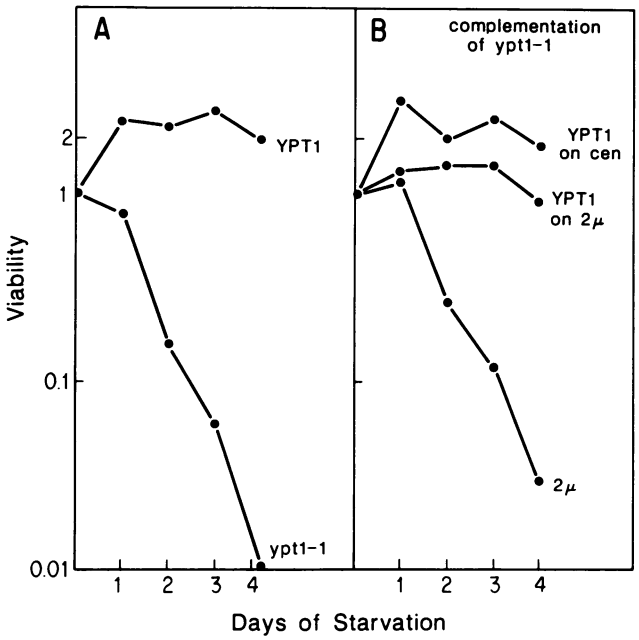

FIG. 6. Viability of *YPT1* and *ypt1-1* cells after nitrogen starvation, with or without complementing plasmids carrying the *YPT1* gene. (A) Exponentially growing cultures of *YPT1* (strain DBY1034) and *ypt1-1* (strain DBY1803) cells were shifted from YEPD to medium lacking nitrogen. Viability was measured after various incubation periods. (B) Complementation of the *ypt1-1* mutation. Strain DBY1803 was transformed with plasmids that either did or did not contain the *YPT1* gene (Table 4; see Materials and Methods).

Recently a paper was published (30) that shows that the *YPT1* gene is essential for growth (independently of our demonstration) and that provides some evidence for cytoskeletal aberrations after many generations of growth during which the *YPT1* gene product is increasingly depleted. Our results with the conditional-lethal *ypt1-1* mutation confirm and extend these results, showing that the effects on the cytoskeleton extend to actin filaments and begin within a generation of loss of *YPT1* function.

***YPT1* has a role in regulation of cell cycle arrest upon nitrogen starvation.** Wild-type cells that are starved for nitrogen complete their cell cycle, arrest at or before "start," and stay viable. These cells are in a physiological state distinct from that of cells that are at or before start in growing cultures. Such arrested cells display more resistance to killing by heat, by cell-wall-degrading enzymes, and by various chemicals than do G1-arrested cells (for a review, see reference 28). The *ypt1-1* mutant cells die instead of arresting, as do some of the mutants implicated in the cyclic AMP-dependent protein kinase pathways: i.e., RAS^{Val-19} (45) and *bcy1* (22). Unlike the RAS^{Val-19} and *bcy1* mutants, which fail to arrest in the G1 phase as unbudded cells, the majority of the *ypt1-1* cells appear to arrest in the G1 phase

but do not remain viable. Therefore, they are different both from wild-type and from *bcyl* and RAS^{Val-19} mutant cells. It is possible that the *ypt1-1* cells do not enter the physiological state characteristic of wild-type cells under nitrogen starvation.

Relationship among the *ypt1-1* phenotypes. The fact that the *ypt1-1* mutant expresses the inability to grow mitotically at the restrictive temperature, while the inability to sporulate and to respond to nitrogen starvation are expressed at the temperature that is permissive for growth, can be explained by two alternative models. The first model envisions two different functions associated with the *YPT1* protein, one that is involved in mitotic growth and the other that is involved in nitrogen starvation-induced arrest and sporulation. The second model supposes that the level of active *YPT1* protein required for sporulation and nitrogen starvation-induced arrest is higher than that needed for mitosis. The level of active *YPT1* protein in the *ypt1-1* mutant cells is probably lower than in wild-type cells even at temperatures that are partially permissive for mitotic growth, as shown by a lower growth rate of the mutant. This lower level might not be sufficient for sporulation and starvation arrest. The fact that the *ypt1*-null/+ heterozygous diploid sporulates less efficiently than the wild type at 30°C suggests that the level of *YPT1* gene product in the cell might be crucial for efficient meiosis and thus might support this model.

Relationship of *YPT1* to the *RAS* genes. The two close *ras* homologs of *S. cerevisiae*, *RAS1* and *RAS2*, like the *YPT1* gene, play an essential role in vegetative growth and are also involved in sporulation and nitrogen starvation-induced arrest. However, there are major differences between the ways in which the *YPT1* and the *RAS* genes operate.

The *RAS1* and *RAS2* genes operate by modulation of the adenylate cyclase activity (45), which determines the intracellular level of cyclic AMP. Cyclic AMP is thought to play an important regulatory role in the yeast cell division cycle as a positive regulator in the transition from the G1 phase to the S phase and as a negative regulator in the switch from the G1 phase to the sporulation pathway (for a review, see reference 23). Two kinds of mutations were found in the *RAS* genes: recessive mutations that cause reduction or inactivation of the gene products and a dominant mutation that results in the activation of the *RAS* protein. Cells that carry the first kind of mutation either lack both *RAS* genes and are unable to grow vegetatively or lack just the *RAS2* gene and have reduced levels of cyclic AMP and undergo premature sporulation (sporulation on rich medium) (41, 45). The second kind of mutation that causes activation of the *RAS2* gene, RAS^{Val-19}, results in the opposite phenotype. Cells that carry this mutation have elevated levels of cyclic AMP and are defective in both sporulation and proper response to nitrogen starvation (45).

We showed that *YPT1* does not have a function similar to *RAS1* and *RAS2*, since an activated protein kinase mutation (*bcy1*) cannot compensate for lack of *YPT1* gene activity, as it does for *RAS1* and *RAS2* inactivation (45). Thus the *YPT1* gene is probably not involved in the activation of adenylate cyclase or the cyclic AMP-dependant protein kinase. Furthermore, unlike mutations in the *RAS1* and *RAS2* genes, a single recessive mutation in the *YPT1* gene causes all the phenotypes: failure to undergo mitosis, failure to sporulate, and failure to arrest upon nitrogen starvation. This suggests that the *YPT1* gene product is involved in a process that is common to these three activities. It may be that *YPT1* is a member of another regulatory system that acts in parallel to the cyclic AMP-driven system to regulate similar events, or it may be that the *YPT1* gene can carry messages in different, otherwise unconnected, regulatory pathways. The idea of *YPT1* as a messenger is, of course, derived from its similarity to other G proteins in DNA sequence (15) and the recent demonstration (30) that the *YPT1* gene product binds GTP in vitro.

Relationship of *YPT1* to *RAS* and G-proteins of other organisms. It was shown recently that in another yeast, *Schizosaccharomyces pombe*, its single known *RAS* homolog, *ras1*, has a quite different role in the life cycle (9). It is not essential for vegetative growth and is not involved in the cyclic AMP pathway. It is essential for mating and is required for efficient sporulation. Together, the results for the *S. cerevisiae* genes *YPT1*, *RAS1*, and *RAS2* and the *S. pombe* gene *ras1* show only that different *ras* homologs are involved in different processes; a common theme, if there is one, must be in the mechanism and not in the result.

Recent studies about the role of *ras* proteins in higher organisms also suggest that they are involved in many different basic systems that control cell proliferation. H-*ras* protein can induce DNA synthesis (21) or differentiation (1) in different cell types. In *Xenopus* oocytes, *ras* protein can induce meiosis (2). Because of their localization in the plasma membrane (49) and their similarity to G proteins (for a review, see reference 3), it was suggested that the *ras* proteins are involved in *trans*-membrane signal transduction through the cyclic AMP pathway (29) or the inositol phosphate turnover (46). The molecular mechanisms by which the different *ras* proteins and their homologs function in different organisms remain to be determined.

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