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The *RSF1* Gene Regulates Septum Formation in *Saccharomyces cerevisiae*

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Septum formation in the mitotic cell cycle of the budding yeast *Saccharomyces cerevisiae* occurs by conversion of the chitin ring, laid down at bud formation, into the primary septum. We show here that under certain conditions this septation is dependent on the newly identified *RSF1* gene. However, cells harboring the *rsf1-1* mutation accumulated in a postcytokinesis state, with delayed conversion of the chitin-rich annulus into the primary septum. This *rsf1-1*-mediated inhibition of septum formation only occurred under conditions of biosynthetic stress and was correlated with biosynthetically mediated inhibition of the cell-cycle regulatory step START. The *RSF1* gene is distinct from the *CHS2* chitin synthase gene that is responsible for septation, and thus *RSF1* most likely encodes a regulator of chitin synthesis. We hypothesize that *RSF1* activity facilitates septum formation during times of biosynthetic stress, to allow efficient septation even under these conditions.

Cell proliferation by the yeast *Saccharomyces cerevisiae* occurs through bud formation. Several morphological events in bud development have been well characterized. Bud development begins with a localized evagination of the cell wall and underlying plasma membrane. At this time (12) a ring of chitin is laid down in the cell wall at the site of bud emergence, the area that becomes the neck of the bud (3). Final resolution of the bud into an independent cell entails formation of a chitin-rich primary septum between the mother cell and the new daughter cell (the former bud). During septum formation the enzyme chitin synthase 2 synthesizes chitin and deposits it in an orderly fashion (23, 26) to form a central plate that fills in the original chitin ring laid down at bud emergence (reviewed in reference 4). Selective hydrolysis by chitinase (7) then leads to cell separation, which may be followed by repair of the septum by another enzyme, chitin synthase 1 (5).

Bud development is coordinated with other events in the cell cycle. The final stages of bud development depend on the performance of nuclear division, the yeast equivalent of mitosis; cells blocked at or before nuclear division do not form the septum (6). Similarly, the beginning of bud development depends on performance of START, the central regulatory step in the cell cycle; cells blocked in the performance of START do not initiate bud formation (10).

The performance of START, and indirectly therefore the development of the bud, is responsive to the biosynthetic status of the cell (24, 32). In contrast, the rest of the cell cycle after START, including the completion of bud development to produce an independent daughter cell, is relatively unaffected by overall biosynthetic status (16). Even under conditions of severe nutritional stress such as starvation, the final steps of bud development, including septum formation, take place without delay. How the large-scale structural alterations during bud development escape biosynthetic inhibition is not understood.

Here we describe experiments showing that septum formation can indeed be influenced by the biosynthetic status of the cell and inhibited by inhibition of biosynthetic capacity. However, this inhibition only occurs in cells harboring the newly identified *rsf1-1* mutation. Under conditions of biosynthetic insufficiency these mutant cells are inhibited in septum formation, although nuclear DNA is segregated to the bud and cytokinesis is completed. In wild-type cells, these effects of biosynthetic insufficiency on septum formation are not seen. The *rsf1-1* mutant phenotype indicates that septum formation, perhaps through modulation of the activity of chitin synthase 2, can be influenced by biosynthetic status but that these effects are compensated by activity of the previously unidentified gene *RSF1* (regulation of septum formation).

MATERIALS AND METHODS

Strains. *S. cerevisiae* VIIA (*MAT***a** *ade2 ura1 lys2 tyr1* His^- Met^-) (28), which was found (see Results) to harbor the *rsf1-1* mutation, was a gift from P. Venkov. Strain VIIA was derived from parent strains that each exhibit cell wall alterations (28, 31). Segregants harboring the *rsf1-1* mutation were generated by successive backcrosses to the wild-type strain GR2 (17). Transformable *rsf1-1* derivatives were products of crosses with strain 21R (*MAT***a** *ade1 leu2-3,112 ura3-52*) (19), obtained from J. E. Hopper. A multicopy *CHS2* plasmid related to pSS2X (26) was obtained from S. J. Silverman. This plasmid contains the *Hin*dIII-*Xba*I fragment that spans the *CHS2* gene (26) inserted in the multicopy *URA3* vector YEp352 (13). Plasmids YCp50-1C and YCp50-2C, which contain nested fragments derived from the left arm of chromosome I (33), were obtained from Y. Steensma, and cloned regions of chromosome I encompassing the *FUN12*, *FUN19*, *FUN20*, *FUN21*, and *FUN22* genes were kindly provided by B. Diehl. Yeast cells were transformed with plasmid DNA by the method of Hinnen et al. (14).

Growth conditions. Cells were grown at 23°C in YNB minimal medium (16) containing glucose (2%) and supplemented with ammonium sulfate and auxotrophic requirements. Starvation was imposed by transfer of cells by centrifugation to medium lacking either a nitrogen source or histidine. The *rsf1-1* mutation was identified in genetic

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segregants by the budded morphology of cells after starvation in liquid culture. Genetic analysis was performed as described previously (22).

Assessment of cellular parameters. For Formalin-fixed and sonicated samples (9), cell concentration was determined by using an electronic particle counter (Coulter Electronics), and cell morphology was assessed by using a phase-contrast microscope. Cells were stained for DNA with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI; 34) and for chitin with the fluorescent dye Calcofluor White M2R (3, 15). To determine the status of the septum, Calcofluor-stained cells were viewed by fluorescence microscopy after immobilization in agar. To assess the septation status of each budded cell, only immobilized budded cells that were oriented so that the bud and mother cell were in different focal planes were scored. This angled orientation of budded cells allowed unambiguous recognition of an incomplete annular chitin ring compared with a completed septum. At least 50 to 100 cells were assessed for each determination.

Identification of precytokinesis cells. The budded fractions were determined for Formalin-fixed cells before and after treatment to digest the cell wall. Digestion of the cell wall was accomplished with Glusulase, a crude enzyme preparation derived from snail intestinal juice (Sigma Chemical Co., St. Louis, Mo.; product G-2887). Glusulase treatment separates cells that have already completed cytokinesis (25). The precytokinesis subpopulation remains budded after this digestion and was quantified as follows. Let B equal the fraction of the total population that is budded, and let W equal the fraction of the total population that is budded but postcytokinesis. After treatment with Glusulase to separate cells in the W fraction, which have already undergone cytokinesis, the total number of cells increases by the factor $(1 + W)$. Therefore the fraction of the total population after Glusulase treatment that remains budded (G) is equal to $(B - W)/(1 + W)$. Rearrangement shows that W is equal to $(B - G)/(1 + G)$. The fraction of cells in the budded subpopulation that are separated into two cells by Glusulase treatment is W/B. The fraction of the budded subpopulation that is precytokinesis is $(B - W)/B$, which simplifies upon substitution for W to $[G(1 + B)]/[B(1 + G)]$.

RESULTS

Recognition of the *rsf1* mutant phenotype. The phenotype produced by the *rsf1-1* mutation was first observed in our studies of *S. cerevisiae* VIIA (28). This strain exhibited an unusual and unexpected morphological response to histidine starvation. Starvation of a histidine auxotroph for histidine leads to cessation of proliferation, with approximately 90% of the population arrested as unbudded cells (unpublished results). However, cultures of cells of mutant strain VIIA still contained significant proportions (74%) of budded cells long after histidine withdrawal, even though cell proliferation had ceased (data not shown). These mutant cells thus exhibited an aberrant response to starvation, ceasing proliferation as budded cells.

The recessive *rsf1-1* mutation identifies a new gene on chromosome I. The genetic segregation of the phenotype of budded-cell arrest described above was determined through successive backcrosses of strain VIIA and mutant segregants to the wild-type strain GR2 and its isogenic derivatives and through further backcrosses of mutant segregants to the transformable strain 21R. In each successive genetic cross the budded-cell mutant phenotype segregated 2:2, as the product of a single mutation (data not shown). The mutation causing such budded-cell arrest upon starvation was named *rsf1-1*.

These crosses showed also that the *rsf1-1* mutation is recessive. Diploid strains heterozygous for the *rsf1-1* mutation responded to starvation conditions and ceased proliferation with the usual low proportions of budded cells. The *rsf1-1* mutant phenotype could be expressed in diploid cells, however; the characteristic budded-cell arrest of *rsf1-1* mutant haploid cells was also manifested by diploids homozygous for the *rsf1-1* mutation (data not shown). The recessive nature of the *rsf1-1* mutation indicates that the mutation causes loss of function of the *RFS1* gene product.

During these crosses, genetic linkage was noted between the *rsf1-1* mutation and an *ade1* mutation on chromosome I. Further genetic analysis localized *rsf1-1* to the left arm of chromosome I (*rsf1-1* × *ade1*, 11 PD:1 NPD:19 TT = 42 cM; *rsf1-1* × *cys1*, 6 PD:0 NPD:8 TT = 29 cM). No other mutation conferring a related phenotype has been genetically mapped to this area of chromosome I. Cloned DNA fragments of the left arm of chromosome I (20, 33), which correspond to the genetic locale for *rsf1-1*, were tested for complementation of the *rsf1-1* phenotype. Mutant cells transformed with these fragments still expressed the *rsf1-1* phenotype (data not shown), indicating that *RSF1* is distinct from the *MAK16*, *LTE1*, *FUN12*, *FUN19*, *FUN20*, *FUN21*, and *FUN22* genes. These transformation results demonstrate that the *rsf1-1* mutation defines a previously unreported gene.

Mutant cells complete nuclear division and cytokinesis. The position in the cell cycle affected by the *rsf1-1* mutation was determined by assessment of nuclear morphology. Cells were stained with the fluorescent dye DAPI (34), and the morphologies of DAPI-stained nuclei were categorized for cells upon starvation for nitrogen. Cultures of proliferating wild-type and *rsf1-1* mutant cells (at time zero) contained similar proportions of cells in each of the nuclear morphology categories (Fig. 1). However, by 24 or 48 h after imposition of starvation conditions the *rsf1-1* mutant population contained increased proportions of binucleate budded cells in which one of the nuclei was localized to the bud (Fig. 1D and 2B). This finding indicated that the *rsf1-1* mutant cells underwent nuclear division and nuclear segregation. To determine whether the budded *rsf1-1* mutant cells had already performed cytokinesis, mutant and wild-type cells were subjected to 24 h of nitrogen starvation and then fixed by the addition of Formalin, sonicated, and treated with a lytic enzyme preparation (Glusulase; see Materials and Methods) to break cell attachments due only to cell wall material (25). The fraction of the *rsf1-1* mutant population that remained budded was decreased by this digestion from 82% to 14%. These data indicate that 73% of the budded *rsf1-1* mutant cells had completed cytokinesis but not cell separation. (See Materials and Methods for the derivation of this value.) The *rsf1-1* mutation thus affects the cell wall changes that result in cell separation.

A cell that remains budded after cytokinesis is in reality two cells in the G1 part of the cell cycle. Thus the Glusulase treatment showed that the nitrogen-starved *rsf1-1* mutant cells became blocked with respect to the nuclear cycle in G1, which is the usual response to nitrogen starvation (18). By impairing cell separation, the *rsf1-1* mutation keeps the two products of cytokinesis together with a budded-cell morphology.

Mutant cells do not form the primary septum. Cell separation after cytokinesis can be resolved into two stages. First there is conversion of the chitin-rich annulus, laid down at

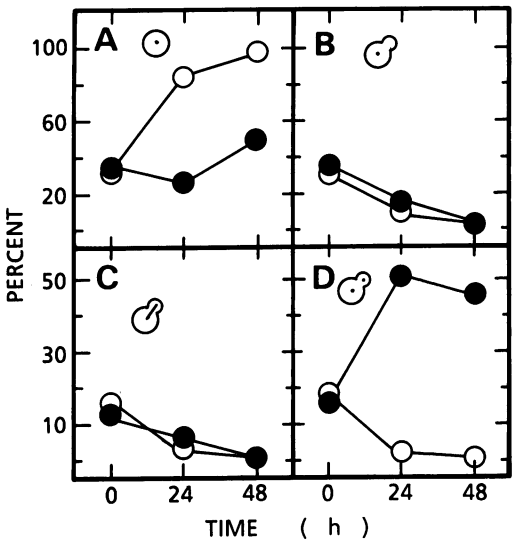

FIG. 1. Cell cycle progress during nitrogen starvation. Proliferating wild-type (○) and *rsf1-1* mutant (●) cells were transferred to medium of the same composition but without nitrogen. Samples removed during further incubation were stained with DAPI to reveal nuclear configuration. By cell morphology and nuclear morphology cells were grouped into four classes represented by the following: A, an unbudded cell with a single nucleus; B, a budded cell with a single nucleus; C, a budded cell with nuclear material extended within the neck between the mother cell and bud; and D, a budded cell with two distinct nuclei. Results are expressed as percentages of the total number of cells (200 to 400 cells were scored per sample).

bud formation, into a primary septum between the plasma membranes of the original cell and the former bud. Septum formation is then followed by controlled hydrolysis of this structure to allow disengagement and cell separation. To determine where in this complex process the *rsf1-1* mutant cells become impaired, the status of the septum was assessed in starved *rsf1-1* mutant cells.

The septum was visualized in mutant and wild-type cells by using the fluorescent staining agent Calcofluor. This dye binds specifically to chitin in the cell wall (3; Fig. 2C and D) and thus allows detection of both the chitin-rich annulus and the primary septum. The proportions of the budded cells that showed either a chitin annulus or a septum were determined microscopically as described in Materials and Methods. Of the annulus-containing cells, however, only those that had already completed cytokinesis were of interest here, because of the postcytokinesis block imposed by the *rsf1-1* mutation. Parallel samples were therefore fixed and digested with Glusulase to quantify in each case (as described in Materials and Methods) the (uninteresting) fraction of the budded cells that had not yet completed cytokinesis. This value was then used to calculate, from the fraction of budded cells that contained an annulus, the size of the subpopulation of interest: those budded cells that had already completed cytokinesis but that still contained only the chitin-rich annulus and so had not yet formed the septum.

Upon transfer of *rsf1-1* mutant cells to nitrogen-free medium the proportion of the budded population that had completed cytokinesis but not septation increased to 80%, whereas for wild-type cells treated in the same way this proportion decreased to negligible levels (Fig. 3C). Therefore the *rsf1-1* mutation impairs conversion of the chitin annulus into the primary septum.

Delayed septation in *rsf1-1* mutant cells. Mutant cells also went through an eightfold increase in cell concentration upon transfer to nitrogen-free medium (Fig. 4A). Therefore the *rsf1-1* mutation, by preventing conversion of the final bud to an independently counted cell, affected only the increase in cell concentration contributed by cell division during the final cell cycle. In fact, after prolonged incubation in nitrogen-free medium, mutant cells did slowly undergo septum formation to accumulate ultimately as populations of unbudded cells (Fig. 4B).

Biosynthetic inhibition impairs septation in *rsf1-1* mutant cells. Impaired septation in *rsf1-1* mutant cells was originally noted upon starvation for histidine or nitrogen. Cells starved in this way uniformly cease proliferation in the G_1 phase of the cell cycle, at the regulatory step START (10, 16). This relationship suggested that other procedures that bring about a cessation of cell proliferation at START may similarly interfere with septum formation in *rsf1-1* mutant cells. Therefore, certain inhibitors that have been shown to cause rapid arrest of proliferation by blocking the performance of START (27) were tested for effects on septum formation in *rsf1-1* mutant cells.

Treatment of *rsf1-1* mutant cells with the START inhibitor *o*-phenanthroline (17) resulted in rapid arrest of cell proliferation, but with significant numbers of cells remaining budded. Other START inhibitors (1, 27) gave similar results (data not shown). Analysis of DAPI-stained cells indicated that *o*-phenanthroline, like starvation, also inhibits septum formation in *rsf1-1* mutant cells. The first-cycle inhibition of START by *o*-phenanthroline (17) was paralleled by first-cycle effects on septum formation (data not shown). *o*-Phenanthroline also significantly decreases rRNA synthesis (17, 30), suggesting that in *rsf1-1* mutant cells septation may be inhibited by *o*-phenanthroline through concomitant biosynthetic inhibition.

A revealing response was that of *rsf1-1* mutant cells to another START inhibitor, the yeast mating pheromone α-factor. This agent did not inhibit septation in *rsf1-1* mutant cells but instead caused these mutant cells to undergo a prompt unbudded-cell arrest (data not shown), just as it does for *MAT***a** wild-type cells (2). The yeast mating pheromone is also the only inhibitor of those tested that blocks START in wild-type cells with little inhibition of biosynthetic activity (27, 29). The impaired septation in *rsf1-1* mutant cells is therefore correlated with situations that cause nutritional or biosynthetic limitation. This correlation suggests that in *rsf1-1* mutant cells the formation of the primary septum is affected by biosynthetic status.

Mutant cells were examined while proliferating under conditions that slow the rate of protein synthesis. Cycloheximide at low concentrations decreases the ongoing rate of protein synthesis to proportionally lower rates and leads to slowed but continued cell proliferation (8, 11, 21). During the proliferation of wild-type cells in medium containing cycloheximide (0.1 μg/ml), the proportion of binucleate cells in the budded-cell population remained unchanged at approximately 35% of the budded subpopulation. This value was consistent with the proportions of binucleate cells in an entire population (zero-time values in Fig. 1 and 3), signifying that in wild-type cells these inhibitory growth conditions of cycloheximide did not differentially inhibit septum formation. In contrast, proliferation of *rsf1-1* mutant cells in medium containing this cycloheximide concentration increased the proportion of binucleate cells in the budded-cell subpopulation from 33% to 67%. Thus a mildly inhibitory

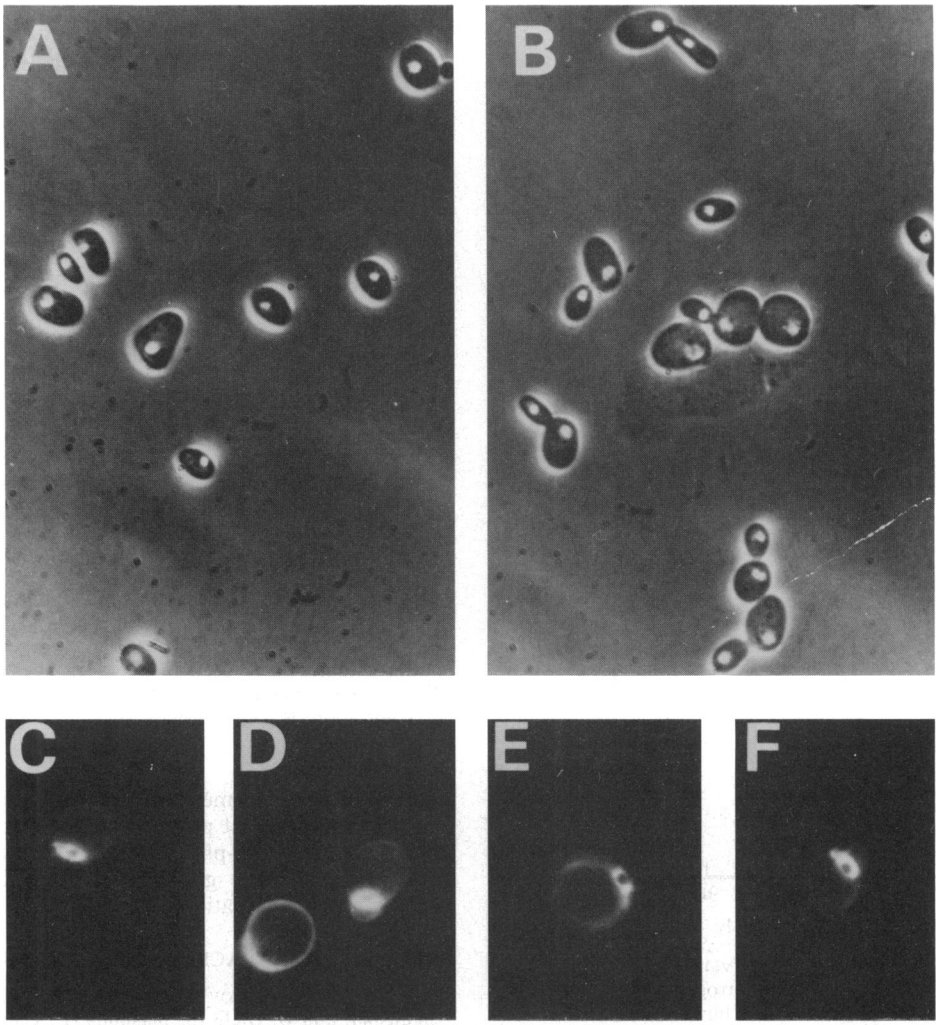

FIG. 2. Nitrogen-starved cells. After 48 h in nitrogen-free medium, cells were stained with DAPI for nuclear material (A, wild type; B, *rsf1-1* mutant) or with Calcofluor for chitin (C and D, wild-type; E and F, *rsf1-1* mutant). The status of the primary septum was unambiguously assessed by inspecting Calcofluor-stained cells in all focal planes.

concentration of cycloheximide also delays septation in *rsf1-1* mutant cells.

DISCUSSION

We report here the phenotype produced by the recessive mutation *rsf1-1*, which causes *S. cerevisiae* cells under biosynthetic limitation to undergo a markedly delayed cell septation. This observation suggests that under these conditions the *rsf1-1* mutation impairs chitin accumulation at the septum. This defect in chitin accumulation during septation does not simply result from a defective chitin synthase enzyme. Chitin synthase 2 is the enzyme that forms the chitin-rich primary septum; disruption of the *CHS2* gene that encodes chitin synthase 2 blocks septum formation (26). However, the cloned *CHS2* gene (kindly provided by S. J. Silverman) did not suppress the *rsf1-1* impairment in septum formation (unpublished data), indicating that *rsf1-1* is not a mutant allele of *CHS2*. These findings suggest instead that the *RSF1* gene modulates chitin synthase 2 activity and that the *rsf1-1* mutation affects the regulation of chitin synthesis during times of biosynthetic stress.

Conditions impairing the formation of the chitin-rich primary septum in *rsf1-1* mutant cells had little effect on the formation of the chitin-rich annulus at bud emergence. An analogous dissociation of synthesis of the annulus from that of the septum was also noted for the aberrant cells produced upon the germination of spores carrying a disrupted *CHS2* gene (26). Staining of these aberrant cells showed that septa were not formed but also indicated the presence of chitin at constrictions that could represent mother cell-bud junctions. One suggested interpretation of this observation was that the chitin annulus is synthesized by an enzyme other than chitin synthase 2. Our results showing that the *rsf1-1* mutation affects only septum formation support this interpretation.

Biosynthetically responsive septation. Our results showed that without wild-type *RSF1* gene product activity septum formation is impaired whenever overall biosynthetic activity is impaired. In wild-type cells, however, this regulation of septation by biosynthetic activity is cryptic: wild-type cells do not accumulate at septation, which is the distinctive phenotype of *rsf1-1* mutant cells. These biosynthetic effects suggest that the wild-type *RSF1* gene product facilitates septum formation under conditions of biosynthetic limita-

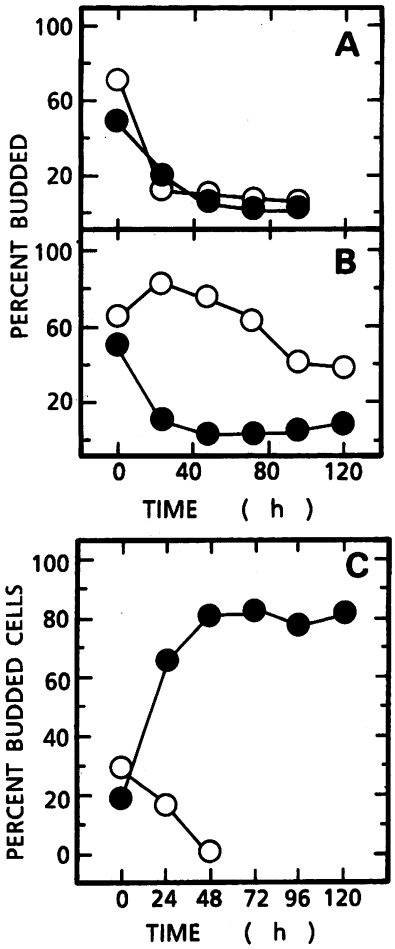

FIG. 3. Cytokinesis upon nitrogen starvation. Wild-type (A) and *rsf1-1* mutant (B) cells were starved for nitrogen as described in the legend to Fig. 1, and the proportions of budded cells were determined before (○) and after (●) Glusulase treatment to digest the cell wall. This treatment converts a postcytokinesis budded cell into two unbudded cells. The proportion of postcytokinesis cells exhibiting impaired septation (C) within wild-type (○) and *rsf1-1* mutant (●) populations was determined after starvation for nitrogen. Cell morphology was scored before and after treatment with Glusulase, and the values for Calcofluor-stained budded cells with an incomplete septum were used as described in Materials and Methods to derive the proportions of budded cells between cytokinesis and septum formation.

tion. This sort of compensatory activity in times of biosynthetic insufficiency would ensure prompt and proper septum formation before the cessation of proliferation.

These findings suggest that two very different activities in the yeast cell cycle, the activation of chitin synthase 2 for primary septum formation and performance of the regulatory step START to initiate the cell cycle, are similarly responsive to biosynthetic activity. Not only did situations that affect START also affect septation in *rsf1-1* mutant cells, but there was temporal correspondence between the onset of those effects for both START and septation. This correspondence was most strikingly evident upon starvation for nitrogen, which allows cells to go through more than one cell cycle before proliferation ceases (18); it was only during the final mitotic cycle that START arrest and the *rsf1-1* mutant phenotype of impaired septum formation were observed.

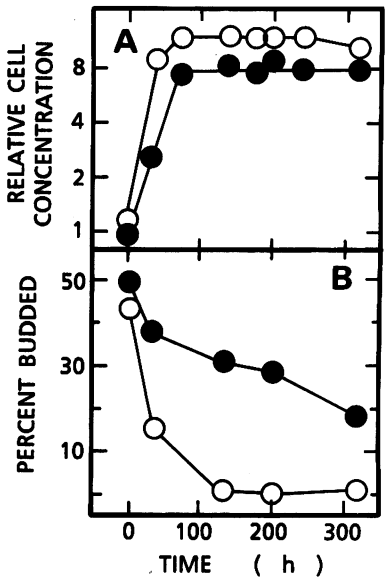

FIG. 4. Delayed septation by *rsf1-1* mutant cells during long-term incubation in nitrogen-free medium. Wild-type (○) and *rsf1-1* mutant (●) cells were incubated in nitrogen-free medium; large initial volumes were used to avoid changes in cell concentration from evaporation over the extended incubation period. A, Cell concentration; B, cell morphology.

The similar impairment of septation caused by different inhibitors that affect protein synthesis (cycloheximide) and rRNA synthesis (*o*-phenanthroline) suggests that in *rsf1-1* mutant cells some general consequence of biosynthetic activity affects septation.

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