The Function of Chitin Synthases 2 and 3 in the *Saccharomyces cerevisiae* Cell Cycle

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Abstract. The morphology of three *Saccharomyces cerevisiae* strains, all lacking chitin synthase 1 (Chs1) and two of them deficient in either Chs3 (*cal*^{R}*1* mutation) or Chs2 was observed by light and electron microscopy. Cells deficient in Chs2 showed clumpy growth and aberrant shape and size. Their septa were very thick; the primary septum was absent. Staining with WGA-gold complexes revealed a diffuse distribution of chitin in the septum, whereas chitin was normally located at the neck between mother cell and bud and in the wall of mother cells. Strains deficient in Chs3 exhibited minor abnormalities in budding pattern and shape. Their septa were thin and trilaminar. Staining for chitin revealed a thin line of the polysaccharide along the primary septum; no chitin was present elsewhere in the wall. Therefore, Chs2 is specific for primary septum formation, whereas Chs3 is responsible for chitin in the ring at bud emergence and in the cell wall. Chs3 is also required for chitin synthesized in the presence of α-pheromone or deposited in the cell wall of *cdc* mutants at nonpermissive temperature, and for chitosan in spore walls. Genetic evidence indicated that a mutant lacking all three chitin synthases was inviable; this was confirmed by constructing a triple mutant rescued by a plasmid carrying a *CHS2* gene under control of a *GAL1* promoter. Transfer of the mutant from galactose to glucose resulted in cell division arrest followed by cell death. We conclude that some chitin synthesis is essential for viability of yeast cells.

CHITIN is an important structural polysaccharide of fungal cell walls. Its synthesis constitutes a model for morphogenesis and for the definition of potential targets in antifungal chemotherapy (11, 12). In *Saccharomyces cerevisiae*, synthesis of this polymer occurs in different phases of the life cycle. During the vegetative phase, chitin is formed in two stages of the cell cycle: first, a ring of chitin appears at the base of an emerging bud; later, at cytokinesis, chitin is laid down centripetally from the ring simultaneously with the invagination of the plasma membrane, until the chitin primary septum separates mother and daughter cell (11).

Chitin is also involved in the yeast sexual phase. During the process of mating, as a response to the appropriate pheromone, large amounts of chitin are laid down in the cell wall of the growing shmoo (32). Finally, the deacetylated analog of chitin, chitosan, has been recently identified in the spore cell wall (3).

The enzymatic synthesis of chitin in yeast has been extensively studied (8). Chitin synthase 1 (Chs1)[1] was the first chitin synthase to be detected and characterized (23). Cloning and disruption of its structural gene showed that this synthase is not required for septum formation (6), whereas recent evidence indicates that Chs1 has a repair function during cell separation (13). Another candidate for primary septum formation, chitin synthase 2 (Chs2) was later identified (31) and its structural gene, *CHS2*, was cloned (38) and sequenced (37). Disruption of *CHS2* resulted in spores that could germinate but did not give rise to colonies (38). The few abnormal cells formed appeared to lack a septum. Based on this apparently conclusive evidence, it was concluded that Chs2 is essential for septum formation and cell division (38). Recently, however, Bulawa and Osmond (5) reported that in some genetic backgrounds or under certain growth conditions, cells defective in both Chs1 and Chs2 can grow and divide. These cells had a normal chitin content and contained a chitin synthase that, in contrast with Chs1 and Chs2, did not require partial proteolysis for activation (chitin synthase 3; 5). The properties of this activity coincide with

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1. *Abbreviations used in this paper*: Chs1-3, chitin synthase 1-3; PEG, polyethylene glycol.

those previously described by Orlean for "chitin synthase II" (26).

These results reopened the question of which synthase is actually responsible for primary septum formation. The discovery that Calcofluor-resistant mutants *cal^{R}1* are defective in Chs3 activity (43), together with the newly observed viability of *chs2* mutants (5), provided the possibility of investigating the function of each of the two synthases by examining cells lacking one or the other. As a result of such a study, we report herein that Chs2 is specifically required for primary septum formation, whereas Chs3 is involved in chitin synthesis in all other locations and circumstances. We also present evidence that a defect in both synthases is lethal.

Materials and Methods

Strains and Culture Conditions

The strains of *S. cerevisiae* used in this study are listed in Table I. Genetic crosses were performed with standard methodology (35). Tetrads were dissected on minimal medium (2% glucose, 0.7% Difco yeast nitrogen base without amino acids, 2% agar) plus necessary supplements, to allow growth of mutants in the *CHS2* gene (5). With our strains, spores containing such mutations do not give rise to colonies on rich medium (38). Liquid cultures were grown in minimal medium plus supplements, buffered with succinate at pH 5.8 (13). Galactose was substituted for glucose when cells containing plasmid pAS4 were used, except where stated otherwise. *cdc* mutants and *cdc, cal^{R}1* double mutants were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). Calcofluor resistance was determined either by replica plating on YEPD plates containing 10 mg/ml Calcofluor white or by growing in liquid YEPD medium with or without 1 mg/ml Calcofluor white. Calcofluor white was a gift of American Cyanamid Co. (Bound Brook, NJ).

Plasmid Construction and Transformation

To place *CHS2* under the control of the *GAL1* promoter, plasmid pAS4 was constructed. pAS3, the source of *CHS2*, was obtained by digestion with EcoRV and SmaI and subsequent recircularization of a modified (37) pGEM3Z (Promega Biotec, Madison, WI) carrying *CHS2*. This manipulation was necessary to remove inconveniently located XbaI and EcoRI sites. The construct still carried 1 kb of DNA upstream of the initiation codon. It was thought that the presence of this DNA in the final construct might interfere with the *GAL1* promoter. Therefore an EcoRI site 140 bp upstream was chosen as the optimal 5′ end for use in this construction. The presence of two EcoRI sites within the coding sequence necessitated the following indirect approach. pAS3 was cut with EcoRI in one reaction and with PstI and XbaI in another. An EcoRI fragment representing positions −140 to +1,688 and a PstI/XbaI fragment representing positions +579 to +3,295 were isolated by electrophoresis in Seaplaque low gelling temperature agarose (FMC, Rockland, ME). The EcoRI fragment was purified from the gel with Geneclean (Bio101, Vista, CA) and further digested with PstI. The 719-bp EcoRI/PstI fragment (−140 to +579) was then isolated in a gel as above. The two fragments were mixed with an EcoRI/XbaI fragment from pYES2.0 (Invitrogen, San Diego, CA) and ligated in-gel according to Struhl (42). The ligation mixture was used to transform *Escherichia coli* AG1 cells (Stratagene Inc., La Jolla, CA). Five minipreps with the proper restriction pattern were used to transform the diploid ECY36 to Ura$^+$ by the lithium acetate procedure (22). One of each of the ensuing yeast transformants was grown in minimal medium with either glucose or galactose as the carbon source. Chitin synthase activity was measured in digitonin-treated cells (17). All five showed approximately wild-type levels in the glucose-grown cultures whereas the galactose-grown cultures had from four to 20 times the wild-type activity (data not shown). Transformant ASY2, which had the widest range of activity, was chosen for sporulation and for dissection on galactose minimal medium.

To construct pAS5, a plasmid harboring *CHS1* and *TRP1*, the XmaI/SalI fragment from pMS1 (6), containing *CHS1*, was inserted into the vector YEp352 (18), which had been cut with the same enzymes. The resulting plasmid, pAS1, was digested with SacI and SalI; the *CHS1*-containing fragment was inserted into the centromere plasmid pRS314 (36), which had also been cut with SacI and SalI.

Table I. S. cerevisiae Strains Used in This Study

Strain	Genotype	Source
ECY33-16C	*MATα chs1-23 cal^{R}1 his4*	This study
ECY33-18A	*MATa chs1-23 cal^{R}1 ura3-52 leu2-3,112 trp1-1*	This study
ECY19Δ2-5B	*MATα chs1-23 chs2::LEU2 ura3-52 leu2-3,112 trp1-1*	This study
ECY36-3A*	*MATα chs1-23 ura3-52 leu2-3,112 trp1-1*	This study
ECY36-3C*	*MATa chs1-23 chs2::LEU2 ura3-52 leu2-3,112 trp1-1*	Same tetrad as ECY36-3A
ECY36-3D*	*MATa chs1-23 cal^{R}1 ura3-52, leu2-3,112 trp1-1*	Same tetrad as ECY36-3A; and this study
ASY2	*MATa/MATα chs1-23/chs1-23 chs2::LEU2/CHS2 cal^{R}1/CAL1 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1* pAS4	
ASY2-24A‡	*MATa chs1-23 chs2::LEU2 ura3-52 leu2,3-112 trp1-1* pAS4	This study
ASY2-24B‡	*MATα chs1-23 chs2::LEU2 cal^{R}1 ura3-52 leu2,3-112 trp1-1* pAS4	Same tetrad as ASY2-24A
ASY2-24C‡	*MATα chs1-23 cal^{R}1 ura3-52 leu2,3-112 trp1-1* pAS4	Same tetrad as ASY2-24A
ASY2-24D‡	*MATa chs1-23 ura3-52 leu2,3-112 trp1-1* pAS4	Same tetrad as ASY2-24A
EMY12-1C	*MATa cdc 24-1 cal1::URA3*	This study
EMY13-2C	*MATα cdc3 cal1::URA3*	This study
H182-6-3	*MATa cdc24-1 tyr1 arg4 thr4 ade1 his7 trp1 gal1*	L. H. Hartwell
H102-3-1	*MATα cdc3*	L. H. Hartwell

* Segregant from cross ECY33-18A X ECY19Δ2-5B (cross ECY36).
‡ Segregant from sporulation of ASY2.

Light and Fluorescence Microscopy

Cells were observed under phase contrast in a Zeiss Universal microscope (Oberkochen, Germany) or by differential interference contrast in a Zeiss ICM 405 microscope. For fluorescence observations with either DAPI or Calcofluor white, the Zeiss Universal microscope was used, equipped with a G365 excitation filter, an FT 395 beam splitter, and an LP 420 barrier filter. Staining was carried out as described, for DAPI (44), Calcofluor (9), or Trypan blue (17).

EM

Yeast cells were prepared for EM in two ways. Cells were fixed in 3% glutaraldehyde for 1 h at room temperature, followed by 30 min in 1% OsO_4 at 4°C. Both fixatives were in 0.1 M sodium phosphate buffer, pH 6.8. After dehydration in graded ethanol solutions, and two changes of propylene oxide, the cells were embedded in EM bed-812 (Electron Microscopy Sciences, Ft. Washington, PA). Alternatively, cells were rapidly frozen by spraying into liquid propane cooled with liquid nitrogen, essentially as described by Horowitz et al. (20). Frozen cells were freeze substituted by gradual warmup from anhydrous methanol containing 2% OsO_4. The dehydrated cells were embedded as above.

WGA-gold probes were prepared as follows: 13-nm colloidal gold was prepared by the method of Slot and Geuze (41). Approximately 0.8 mg of WGA (Pharmacia LKB Biotechnology, Piscataway, NJ) in 0.1 M K_2CO_3 was added to 10 ml of gold sol at pH 10.1 with stirring. After 30 min on ice, filtered 1% polyethylene glycol (PEG) (20,000 mol wt) was added to make the gold probe 0.1% with respect to PEG. The probe was diluted with an equal volume of 10 mM Tris-HCl buffer, pH 9.0, before centrifugation. The probe was washed once with the Tris buffer supplemented with 0.05% PEG and suspended in a small volume of the same buffer, followed by filtration through a 0.45-μm filter for storage at 4°C.

For chitin labeling, the stock WGA-gold probe was diluted 1:400 with 10 mM Tris-HCl, pH 7.0, containing 0.15 M NaCl, 0.05% PEG, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Thin sections on nickel grids, prestained with aqueous solutions of uranyl acetate and lead citrate, were floated on drops of the WGA-gold probe on a rocking table for 45–60 min and rinsed twice for 15 min each on the dilution buffer followed by a jet rinse with deionized water. The gold-labeled sections were examined in an electron microscope (model 410; Philips Electronic Instruments, Inc., Mahwah, NJ).

As a control, sections from each yeast sample were exposed to WGA-gold probe containing 50 mM *N,N',N''*-triacetylchitotriose, to block WGA-binding sites. There was no label present on the wall in these controls. We have previously reported other controls (25) and the variable, nonspecific staining of vacuole contents with WGA probes (27).

Cell Wall Preparation and Chitin Determination

Cell walls were prepared and chitin determined as previously described (27).

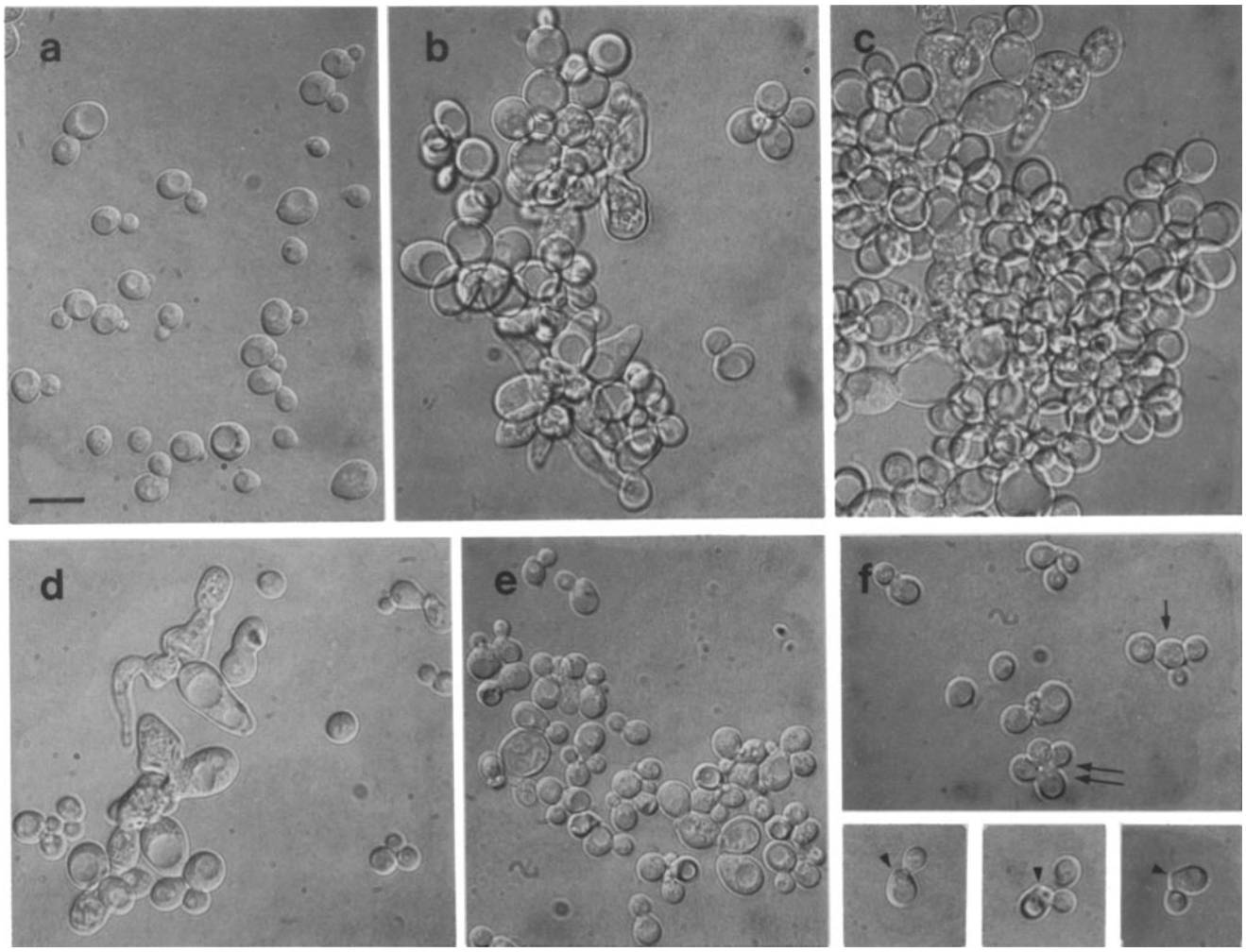

Figure 1. Morphology of different strains viewed by differential interference contrast. *a*, ECY36-3A (*chs1 CHS2 CAL1*); *b*, a relatively small aggregate and some free cells of ECY36-3C (*chs1 chs2::LEU2 CAL1*); *c*, part of a large aggregate of ECY36-3C; *d*, ECY36-3C cells after sonication; *e*, cells of ECY36-3D (*chs1 CHS2 cal^{R}1*) forming loose aggregates; *f*, abnormal budding pattern (*arrow*) and multiple budding (*double arrow*) in ECY36-3D; *insets*, cells of the same strain that exhibit protuberances near the bud (*arrowheads*). Bar, 10 μm.

Chitin Synthase Preparation and Assay

Cells were disrupted with glass beads and membranes isolated essentially as described by Orlean (26). Chitin synthase activity in the membrane preparation was measured with or without trypsin treatment, as previously described (31). Protein was assayed according to Lowry et al. (24).

Results

Morphology of Cells Deficient in Chs2 and Chs3

For comparison of morphology, both by light and electron microscopy, three strains were used, ECY36-3A (*chs1 CHS2 CAL1*), ECY36-3C (*chs1 chs2::LEU2 CAL1*), and ECY36-3D (*chs1 CHS2 cal^{R}1*), all belonging to the same tetrad (Table I). The fourth spore of the tetrad, with the expected genotype *chs1 chs2::LEU2 cal^{R}1*, did not germinate, presumably because a mutation in both *CHS2* and *CAL1* is lethal (see below). To prevent lysis of daughter cells caused by lack of Chs1, all strains were grown in buffered minimal medium (13). Cells defective in Chs2 did grow, although more slowly than those with an intact *CHS2* gene (generation time ~3 h, compared to ~2 h for ECY36-3A). As reported by Bulawa and Osmond (5), the cells grew in clumps of variable size (Fig. 1, *b* and *c*), although some free cells were observed (Fig. 1 *b*). Most cells were larger than those of a wild-type strain and many had elongated and bizarre shapes; observation of these abnormal cells was easier after sonication, that resulted in some reduction of the clumps (Fig. 1 *d*). Several cells appeared granulated and were probably lysed. In fact, Trypan blue stained a small but significant percentage of cells, mostly those exhibiting aberrant shapes (results not shown).

Cells defective in Chs3 (*cal^{R}1* mutants; 43) were much more similar to wild-type cells. However, some abnormalities were observed: the cells showed some tendency to clumping (Fig. 1 *e*), although much less so than the *chs2*::

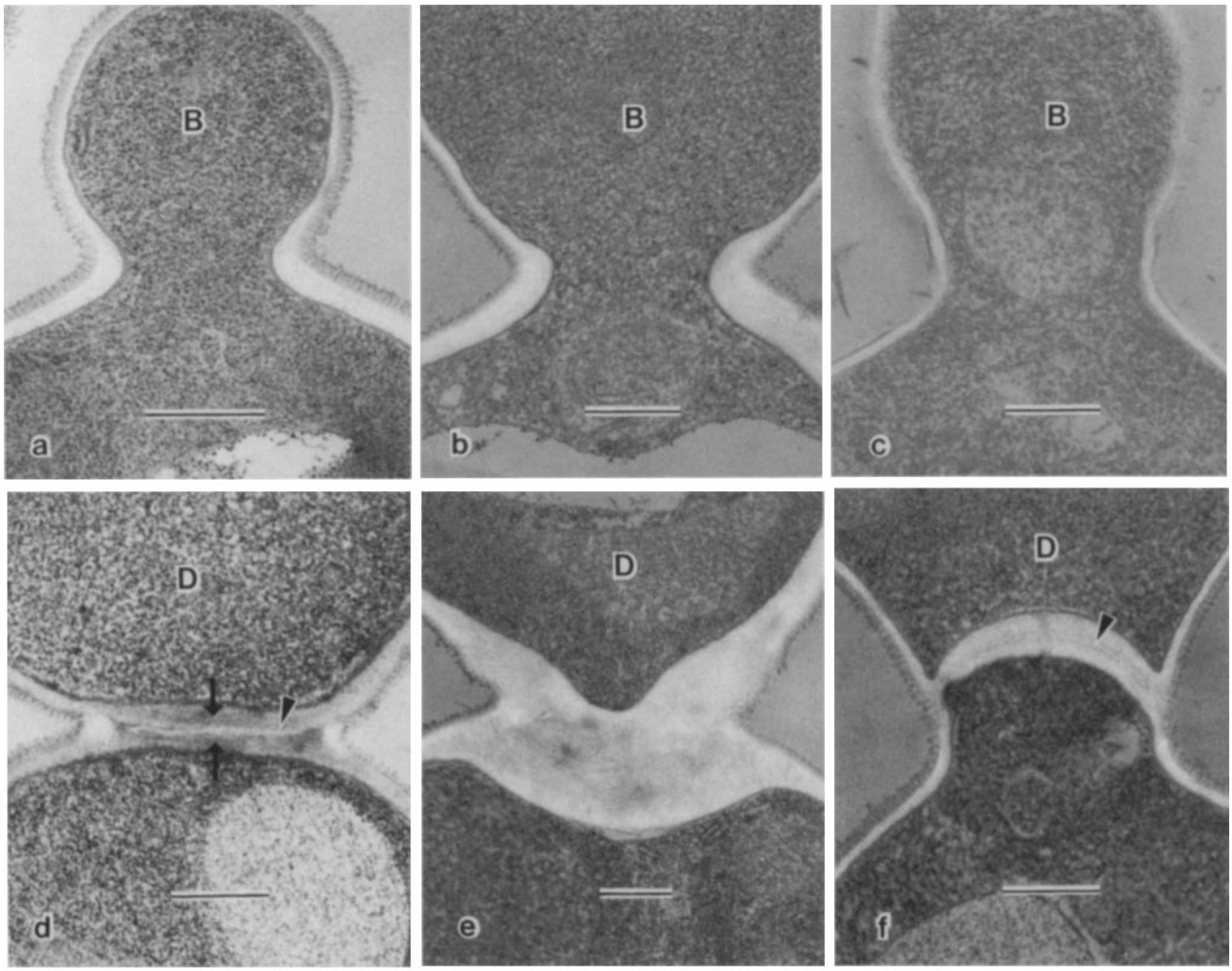

Figure 2. Neck region at early budding and after septum formation. Freeze-substituted, unlabeled cells. *a* and *d*, wild-type cells show a thickening of the wall at the base of the bud that is due to the chitin ring. The thin septum of chitin is electron transparent (*d*; *arrowhead*); *b* and *e*, Chs2$^-$ cells have a wall similar in appearance to wild type in the bud stage. They do not form a well-defined septum, but close the neck with a mass of unoriented filaments; *c* and *f*, Chs3$^-$ cells lack the thickening of the wall at the bud base and form more tubular necks, but structure of the complete septum is otherwise similar to the wild type. Arrowhead points to the primary septum and arrows to secondary septa. *B*, bud; *D*, daughter cell. Bars, 0.5 μm.

LEU2 strain. Aggregates were readily disrupted by light sonication. Often an unusual budding pattern, including multiple budding from the same cell, was seen (Fig. 1 *f*). One feature that frequently appeared was a protuberance next to a bud (insets below Fig. 1 *f*; see also following section on ultrastructure). In *cal*^{R}*1* segregants from different crosses, i.e., with slightly different genetic background, these features varied somewhat; for example, some strains showed more aggregation than others. However, the traits added up to define a characteristic phenotype. During examination of segregants, five of 46 strains were classified by morphological examination as wild type in both *CHS2* and *CAL1*, 25 as *cal*^{R}*1*, and 16 as *chs2*::*LEU2*. Subsequent determinations of nutritional markers and of Calcofluor resistance confirmed the initial categorization in every case.

Cells deficient in Chs2 showed a bright fluorescence over the whole surface after staining with Calcofluor (5), whereas *cal*^{R}*1* cells were quite dim (29). Some of the *cal*^{R}*1* cells, however, showed diffuse and rather intense fluorescence. By simultaneous staining with Calcofluor and Trypan blue the fluorescent cells were also found to be permeable to the blue dye (results not shown). We have observed in other cases that lysed cells become fluorescent in the presence of Calcofluor. Thus, occasional lysis is another characteristic of *cal*^{R}*1* cells.

All strains shown in Fig. 2 are defective in Chs1. As previously reported (13), the only morphological change so far observed in *chs1* strains with respect to wild type is the lysis of daughter cells in unbuffered medium. The presence of a normal *CHS1* gene in *chs2* cells did not result in a phenotype different from *chs1 chs2* strains (5). As for *cal*^{R}*1* cells, this mutation was originally isolated in a *CHS1* background (29). Those cells have the same appearance as the double mutant *cal*^{R}*1 chs1*, except for the already mentioned lysis of daughter cells during growth at low pH in the latter.

Ultrastructure of Septal Region in Cells Deficient in Chs2 and Chs3

To detect changes in septum structure in the different strains, observations were made by EM of the neck region between mother and daughter cell, both at bud emergence and after septum formation. A thickening of the cell wall at the base of the bud, present in wild-type cells (Fig. 2 *a*; 27) was also observed in the *chs2*::*LEU2* strain (Fig. 2 *b*) but appears to be absent in the *cal*^{R}*1* strain (Fig. 2 *c*). Cells deficient in Chs2 (Fig. 2 *e*) present an extremely thick septum of amorphous aspect, devoid of the trilaminar structure shown by wild-type cells (Fig. 2 *d*), in which the primary septum is sandwiched between two secondary septa (11, 27). Those thick septa hold together different cells (Fig. 3, *b* and *c*) and are responsible for the large aggregates shown in Fig. 1, *b* and *c*. Pockets of cytoplasm were often trapped inside the septa (Fig. 3 *b*). These may result from the irregular growth of the septal material (Fig. 3 *a*) that may not coalesce simultaneously through the thickness of the septum.

In contrast to the Chs2$^-$ cells, those deficient in Chs3 (*cal*^{R}*1*) have septa with a clearly visible trilaminar structure (Fig. 2 *f*). However, these septa are of uniform thickness throughout, whereas those of wild-type cells thicken as they reach the cell wall (Fig. 2 *d*), in correspondence with the thickening observed at bud emergence. Other abnormalities observed in the *cal*^{R}*1* septa are the elongated aspect of the channel between mother and daughter cells and, as in Chs2$^-$ cells, the occasional presence of cytoplasm inclusions between primary and secondary septum.

Furthermore, *cal*^{R}*1* cells often exhibit protuberances (Fig. 4 *a*) that probably correspond to those observed in light microscopy (Fig. 1 *f*; insets). It is likely that these protuberances are bud scars (see chitin localization below). Their different aspect when compared with normal bud scars (Fig.

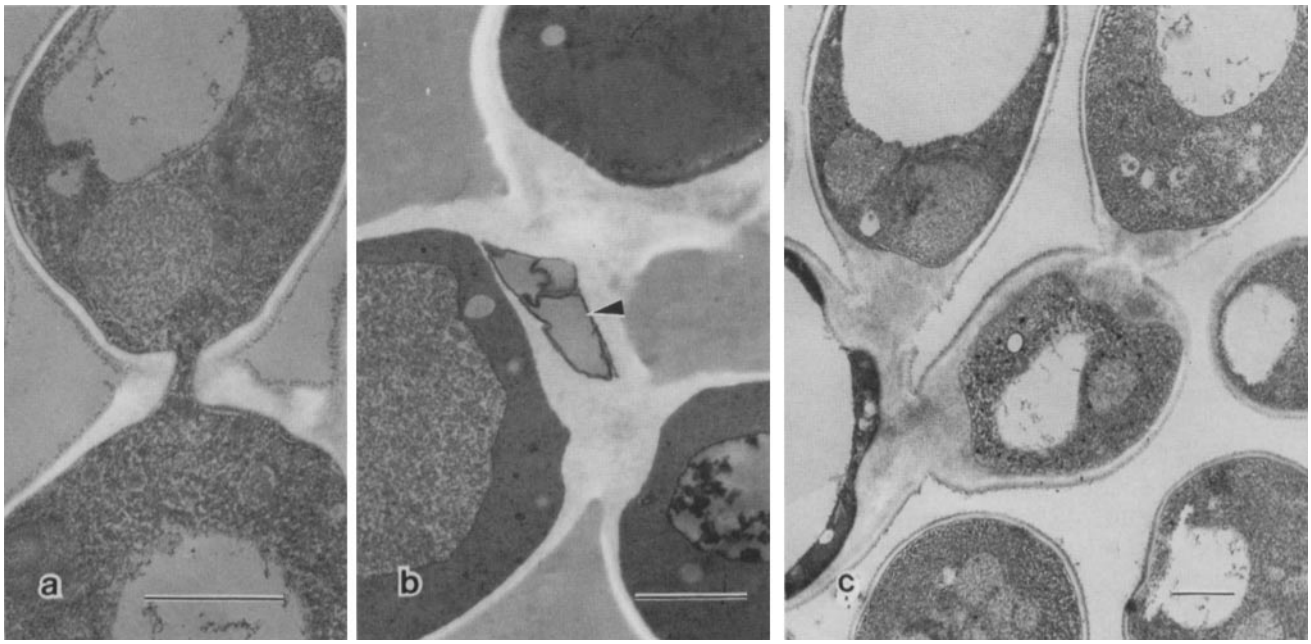

Figure 3. Chs2$^-$ septa. Cells were fixed by freeze substitution and are unlabeled. *a*, shows constriction of the channel between mother cell and bud caused by proliferation of wall material in the neck region; *b*, cytoplasm is occasionally trapped within the septal area and degenerates, forming lacunae (*arrowhead*); *c*; daughter cells do not separate readily and clumps of cells are common. Bars, 1 μm.

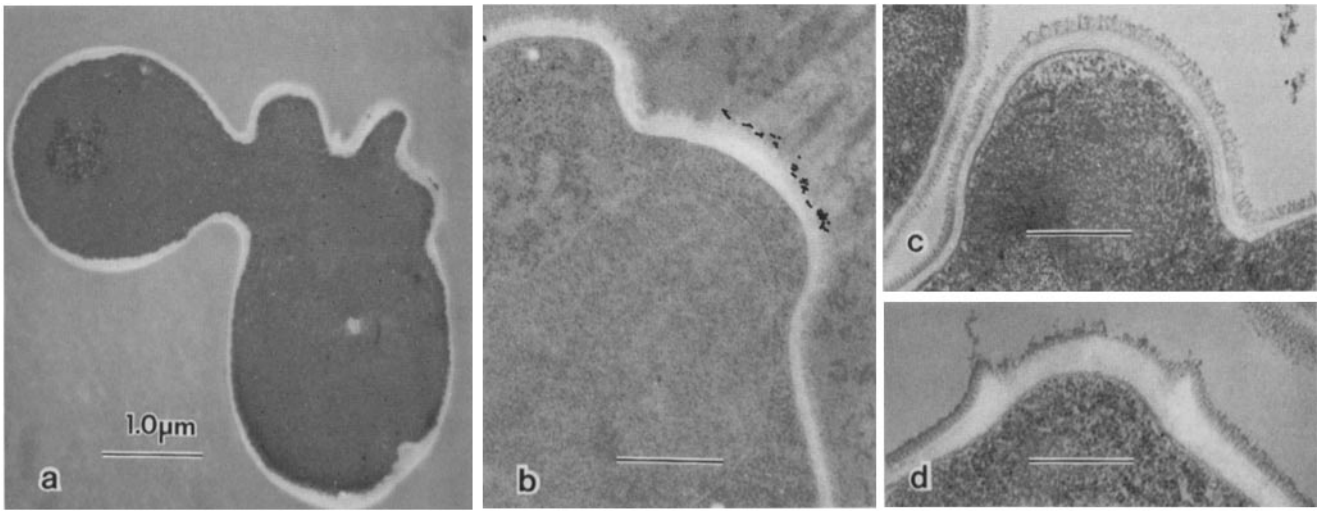

Figure 4. Chs3⁻ bud scars. *a* and *b* are conventionally fixed and WGA-gold labeled. *c* and *d* are freeze substituted and unlabeled. *a*, Low-magnification view of budding cell with protuberances adjacent to the bud; *b*, similar structure showing surface label of WGA-gold. Approximately one-third of profiles recognizable as bud scars are not labeled, as is the case in *a*; *c*, Chs3⁻ bud scar compared to *d*, wild-type bud scar. Bars, 0.5 μm.

4, *c* and *d*) may be explained by the different morphology of the septum, especially with regard to the length of the channel between mother and daughter cell.

Localization of Chitin in the Different Mutants

To determine the distribution of chitin in walls and septa, sections were stained with WGA–colloidal gold (25, 27). In previous observations (27), chitin was detected with this method in a ring at the base of the emerging bud and, later in the cell cycle, in the primary septum. Some chitin also appears to be present all over the cell wall (19, 25). These findings were confirmed with the strain harboring wild-type *CHS2* and *CAL1* (Fig. 5, *a* and *d*), except that in these cells the amount of chitin in the wall appears to be greater than observed previously in other strains (25, 27). Because of this feature, it can be clearly seen that the chitin is present in the wall of the mother cell but not in that of the bud (Fig. 5 *a*). Chitin does, however, appear in the daughter cell wall after septum formation (Fig. 5 *d*). Some label was occasionally observed in large buds still connected to the mother cell by a channel.

The distribution of chitin in cells containing the *CHS2* null mutation that had not yet formed a septum was similar to that of wild-type cells (Fig. 5 *b*), except that the label was heavier, in accordance with the higher chitin content of these cells (Table II). In the septa, chitin was distributed in an irregular fashion (Fig. 5 *e*) and there was no indication of a primary septum line, as in wild-type cells. The density of label in the septum, very heavy in Fig. 5 *e*, was comparable to that of the cell wall but varied from cell to cell.

The chitin localization in *cal*^{R}*1* cells was quite different from that of both wild-type and Chs2 cells. The label was strictly limited to the primary septum line (Fig. 5, *c* and *f*). No colloidal gold was observed at the base of the bud or in other regions of the cell wall, except for a few grains often seen at the top of the protuberances mentioned in the preceding section (Fig. 4 *b*). This confirms the hypothesis that the protuberances are, in fact, bud scars. Again, these results are in consonance with the low chitin content of *cal*^{R}*1* cell walls (Table II; 29). These findings clearly indicate that Chs2 is specifically involved in the formation of the primary septum, whereas Chs3 provides the chitin found in the cell wall, both in the budding ring and elsewhere.

Chs3 and the Deposition of Chitin in cdc Mutants

The finding that in the vegetative cell cycle Chs3 is required for the formation of chitin at all locations except for the primary septum suggested that this synthase may be involved in other circumstances in which chitin is laid down in the cell wall. It has already been shown that the deposition of chitin in the cell wall triggered by α-factor does not occur in *cal*^{R}*1* mutants (29), thereby implicating Chs3 in that case as well. It has also been found that chitin is laid down in the cell wall of *cdc* mutants incubated at a nonpermissive temperature (27, 40). To ascertain whether the formation of chitin was catalyzed by Chs3 also in this instance, double mutants containing both the *cal*^{R}*1* and a *cdc* mutation were obtained by appropriate crosses. The *cdc* mutations used for this experiment were *cdc24* and *cdc3*, which are those that yielded the highest accumulation of chitin in previous experiments (27). Both double mutants, *cdc24 cal*^{R}*1* and *cdc3 cal*^{R}*1*, stained very poorly with Calcofluor at the permissive temperature, as expected (Fig. 6, *b* and *h*). The fluorescence was not increased by shifting the cells to 37°C (Fig. 6, *e* and *k*). It may be concluded that also in this case Chs3 is required for chitin formation.

Requirement of Chitin Synthesis for Viability

Previous results have shown that double mutants lacking both Chs1 and Chs2 (5) or Chs1 and Chs3 (29, 43) are viable. It was of interest to find out whether a triple mutant lacking all three chitin synthases would be viable. Two approaches were used to obtain this information. In the first, a *chs1 chs2::LEU2* strain (ECY19Δ2-5B) was mated to a *chs1 cal*^{R}*1* strain (ECY33-18A). The resulting diploid was sporulated and tetrads were dissected on minimal medium

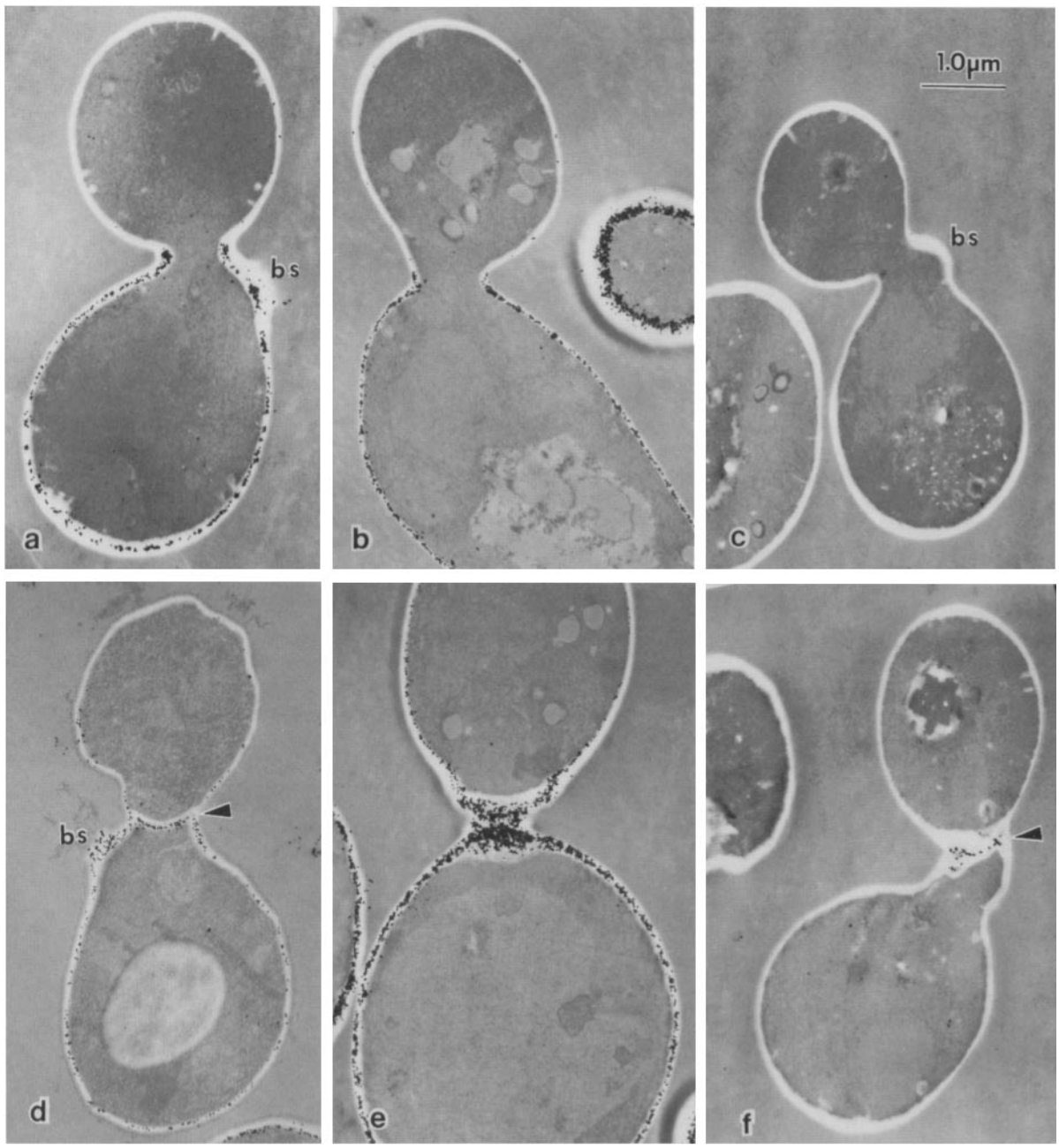

Figure 5. WGA-gold label of chitin. Cells are conventionally fixed. *a* and *d*, wild type. Wall of emerging bud is initially unlabeled but becomes labeled either late in bud formation or after septum formation. *b* and *e*, $Chs2^-$ walls of mother cells are heavily labeled, whereas the wall of the emerging bud shows very light label that increases after septum formation. The thick septal area is as heavily labeled as the mother cell wall. *c* and *f*, $Chs3^-$ cells show virtually no label in the wall. Only the septum is labeled. *bs*, bud scar. All micrographs are the same magnification.

(5). Dissections were also performed on minimal medium containing 1 M sorbitol to mitigate any potential osmotic defect that the triple mutant could exhibit. The results with and without sorbitol were similar. Spores containing the *CHS2* null mutation did give rise to colonies on these media, albeit more slowly, but germination was relatively poor with our strains. Analysis of the results showed weak linkage between the *CHS2* and the *CAL1* genes, an indication that both genes are on the same chromosome. Since *CAL1* has been assigned to chromosome II by chromosome blots (43) it may be concluded that *CHS2* also is on this chromosome. This result is corroborated by the observation that sequences found in the upstream region of *CHS2* (37) are identical to those of the gene *SCO1* (34), which has been assigned to chromosome II (33).

Of the 161 surviving spores examined none was *cal^{R}1 chs2::LEU2*, whereas random assortment would have resulted in about one quarter of the spores with this genotype if the genes were unlinked. Since the linkage between the two genes is weak, we conclude that the lack of recombinants

Table II. Chitin Content of Different Strains

Strain	Chitin (percentage of cell wall dry weight)
ECY36-3A (*chs1 CHS2 CAL1*)	7.1
ECY36-3C (*chs1 chs2::LEU2 CAL1*)	12.3
ECY36-3D (*chs1 CHS2 cal^{R}1*)	0.6

results from lethality of the *cal^{R}1 chs2::LEU2* combination. The poor germination of *chs2* spores cannot explain the results (60 *chs2::LEU2* colonies were obtained). It is of particular interest to note that, of 17 three-spore tetrads that could be inferred as being a tetratype, the missing spore in each case was the *cal^{R}1 chs2::LEU2* recombinant.

Another, more direct approach to the viability question was to construct a triple mutant in *CHS1*, *CHS2*, and *CAL1* that was rescued by the presence of a plasmid containing the *CHS2* gene under the control of the *GAL1* promoter (pAS4, see Materials and Methods). This strain was obtained by transforming the diploid resulting from crossECY36 (Table I) with pAS4, followed by sporulation and tetrad dissection on galactose-containing medium. One of the colonies from a tetrad obtained in the dissection was a leucine prototroph and was Calcofluor resistant, i.e., contained both the *chs2::LEU2* and the *cal^{R}1* mutations (strain ASY2-24B, Table I). After growth in galactose-containing minimal medium, cells of this strain were transferred to both galactose- or glucose-containing minimal medium. Changes in morphology and in several parameters were followed during three doublings of the absorbance and again checked after 24 h. The galactose-cultured cells displayed the typical morphology of *cal^{R}1* mutants, as expected (Fig. 7 *a*), although with a somewhat higher proportion of abnormal cells. These may be cells that lost the plasmid and grew for some time, until uracil became limiting (the plasmid also supplies the *URA3* gene). Even after 24 h, several hours after reaching stationary phase, the cells appeared to be essentially normal (Fig. 7 *b*). On the other hand, when cells were shifted to glucose, thereby stopping expression of Chs2, aberrant changes were soon observed. At the first doubling of turbidity, most cells were in the form of a mother cell with two buds, the second one of which appeared to emerge from the junction between the other two cells (Fig. 7 *c*). At this stage, staining with DAPI showed an abnormal DNA segregation (Fig. 8). The group of three cells contained two or three nuclei, that were found in one or two of the cells, or at the junction between them (Fig. 8 *f*). At the second doubling in absorbance, one of the three cells, presumably the original mother cell, had swelled considerably and contained a large vacuole. The cells also started to aggregate in clumps (Fig. 7 *d*). At the third doubling, the cells were extremely large, 20 to 30 times the normal volume, with the vacuole occupying most of the intracellular space. The daughter cells had almost disappeared in many cases (Fig. 7 *e*). From here on, the absorbance did not increase further; after 24 h it was essentially unchanged whereas the absorbance of galactose-grown cells had increased 5.6-fold above the last value shown in Fig. 9 *A*. At this point, most cells of the glucose culture were granulated and apparently lysed (Fig. 7 *f*), and were permeable to Trypan blue. In the control culture (Fig. 7 *b*), very few cells were stained even after 24 h (results not shown).

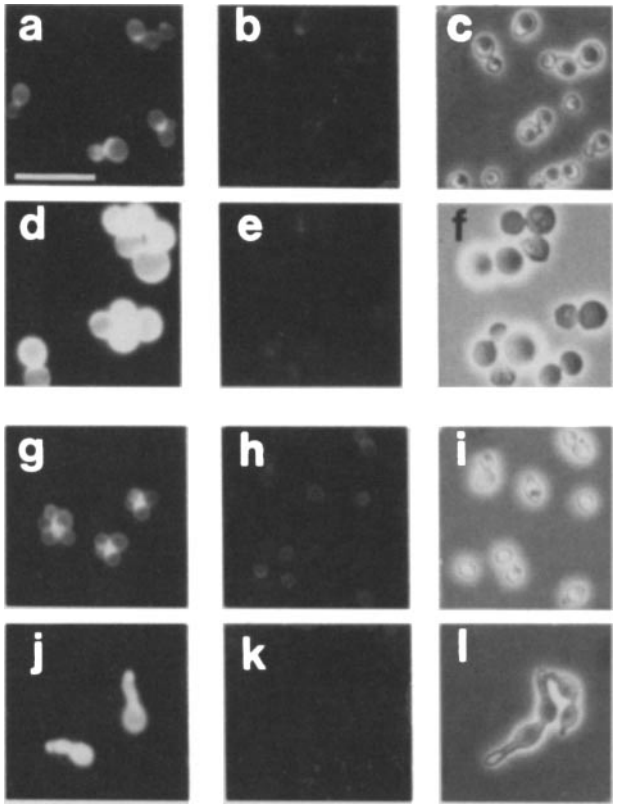

Figure 6. Staining with Calcofluor of *cdc* mutants and *cdc cal^{R}1* double mutants grown at permissive and nonpermissive temperature. *a* and *d*, H182-6-3 (*cdc24*); *b*, *c*, *e*, and *f*, EMY12-1C (*cdc24 cal^{R}1*). *a*, *b*, and *c*, 25°C; *d*, *e*, and *f*, 37°C. *c* and *f* are the phase-contrast images of *b* and *e*, respectively. *g* and *j*, H102-3-1 (*cdc3*); *h*, *i*, *k*, and *l*, EMY13-2C (*cdc3 cal^{R}1*). *g*, *h*, and *i*, 25°C; *j*, *k*, and *l*, 37°C. *i* and *l* are the phase-contrast images of *h* and *k*, respectively. Bar, 20 μm.

In both cultures, the turbidity increased exponentially (Fig. 9, *A* and *B*). The number of cells increased in parallel with turbidity in the galactose culture (Fig. 9 *A*) but remained stationary in glucose (Fig. 9 *B*). In this experiment, cells were counted as the number of units present after light sonication. For example, each tri-celled group of Fig. 7 *c* was counted as one cell. Samples were also plated on YEP-Galactose plates to test for viability. The number of colony-forming units increased exponentially in galactose culture, but declined rapidly in the glucose culture (Fig. 9, *A* and *B*). After two doublings of turbidity, only 1% of the cells gave rise to colonies. The decrease in viability might have been partially due to increased sensitivity of the cells to the sonic treatment that was applied to break up clumps for counting and plating. However, only a moderate increase in stainability with Trypan blue (from ~10 to 20%) was caused by sonication of cells that had undergone three doublings in absorbance (as in Fig. 7 *e*).

The specific activity of chitin synthase 2 remained high during growth in galactose (Fig. 9 *C*). The changes probably reflect the variability in activity of different preparations. In glucose, however, the specific activity declined more rapidly than expected from simple dilution, indicating a rapid turnover of the enzyme (Fig. 9 *C*). The percentage of chitin in

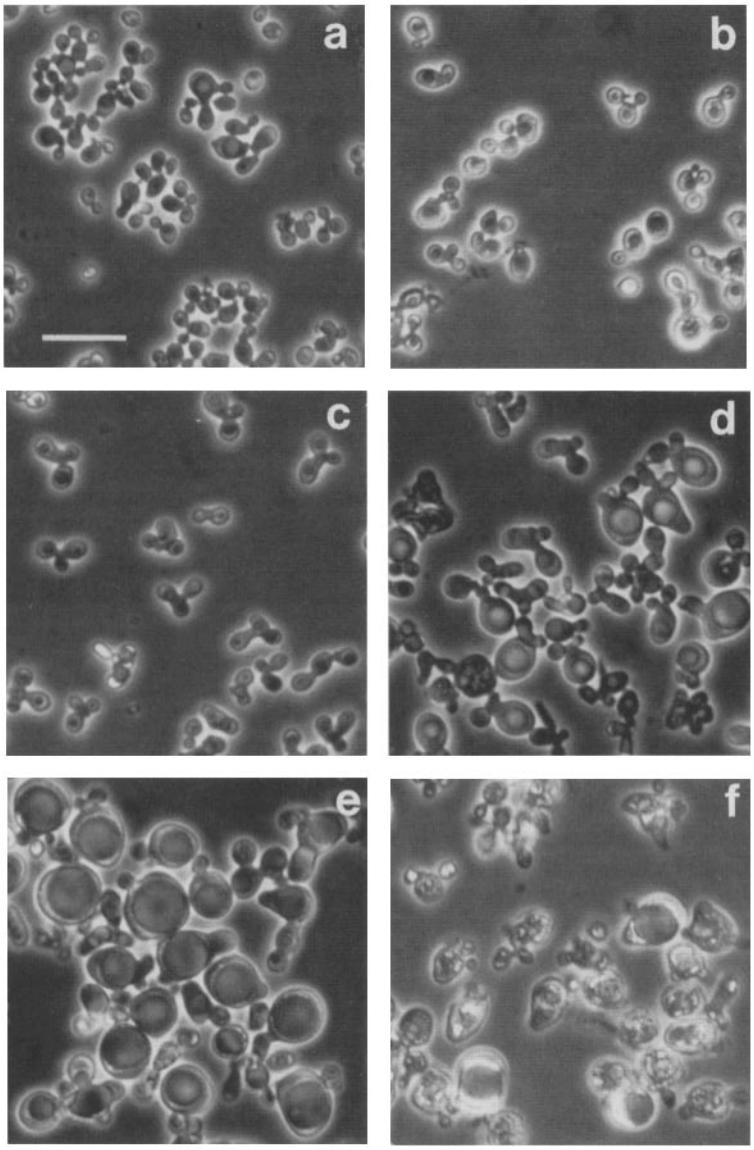

Figure 7. Morphology of strain ASY2-24B (*chs1 chs2::LEU2 cal^{R}1* pAS4), after growth on galactose or glucose. *a* and *b*, cells growing in galactose at logarithmic (*a*) and stationary (*b*; 24 h) phase. *c–f*, cells transferred to glucose medium at zero time (see Fig. 9) after one doubling (*c*), two doublings (*d*), and three doublings (*e*) in absorbance. The absorbance did not increase further after this point. *f*, 24 h after transfer to glucose. Bar, 20 μm.

the cell wall remained essentially constant in galactose but decreased in glucose (Fig. 9 *D*). Because of the very low initial chitin content of this strain, quantitative estimations were difficult and these results have only indicative value.

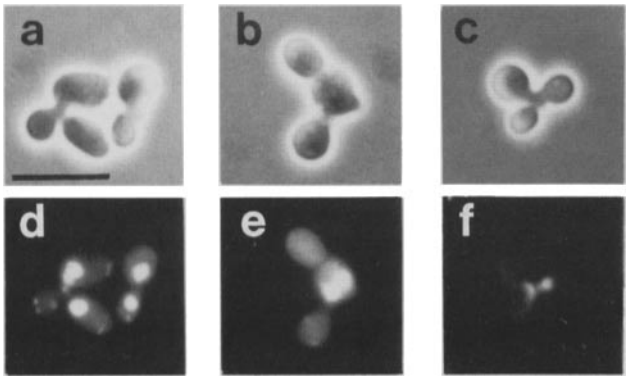

Figure 8. DAPI staining of cells of strain ASY2-24B after growth in glucose until the absorbance had doubled (corresponds to Fig. 7 *c*). *a*, *b*, and *c*, phase contrast; *d*, *e*, and *f*, corresponding fluorescence photographs, in the same order. Bar, 10 μm.

Addition of 1 M sorbitol to the growth medium as osmotic protector did not prevent the cell division arrest or the morphological changes observed in the glucose-containing cultures, although the swelling of cells was somewhat reduced. However, lysis in the 24-h cultures was largely prevented and survival rates after plating the cells on sorbitol-supplemented YEP-Galactose were strikingly improved: 29% of the cells gave rise to colonies, as compared to 4% of the cells grown in glucose without sorbitol. Even the latter number was surprisingly high, compared to <1% after about 9 h (Fig. 9 *B*). To investigate the meaning of this result, cells from colonies obtained after 24-h growth in osmotically unprotected glucose medium were further studied. These cells grew equally well in galactose or in glucose medium, with a morphology typical of *cal^{R}1* mutants (which they are). This behavior, however, does not appear to result from the presence of a suppressor that would enable the cells to grow in the absence of chitin synthesis. Extracts were found to contain chitin synthase activity, with the characteristics of Chs2, at a level somewhat lower but comparable to that of wild-type strains (results not shown). Thus, the abnormal behavior of the surviving cells probably results from integration of *CHS2* from

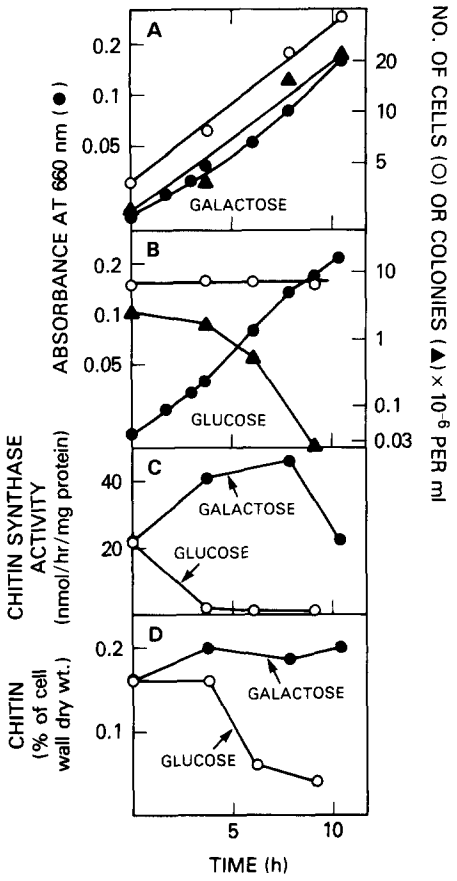

Figure 9. Changes in absorbance, cell number, and viability, chitin synthase activity and chitin content of ASY2-24B cells growing on galactose and glucose. A logarithmic-phase culture in galactose was centrifuged under sterile conditions. Equal portions of the sedimented cells were suspended in minimal medium with either galactose or glucose as carbon source. At time zero, incubation of the cultures at 30°C was started. *A* and *B*, absorbance, cell number, and colony-forming units with galactose (*A*) and glucose (*B*). Note that the right hand ordinate scale in *B* is different from that in *A*, to accommodate the decrease in colony-forming units of glucose-grown cells. *C*, activity of Chs2. For each time point, cells were harvested by filtration; membranes were prepared and chitin synthase activity measured as described in Materials and Methods. The activity measured in the presence of Co^{2+} is represented. The initial activity was approximately three times that of a wild-type strain. With Mg^{2+}, the values were lower but parallel to those shown. *D*, changes in chitin content of cell walls. Cell walls were obtained after centrifugation of the same extracts used to prepare membranes in *C* and then purified as described (26). Chitin was assayed as indicated in Materials and Methods.

plasmid pAS4 into the genome, with concomitant loss of *GAL1* promoter control.

As controls for the above experiments, the other three components of the tetrad to which ASY2-24B belonged were also subjected to the shift from galactose to glucose. After transfer to glucose, ASY2-24C (*chs1 CHS2 cal^{R}1* pAS4) and ASY2-24D (*chs1 CHS2 CAL1* pAS4) maintained their Calcofluor-resistant and wild-type phenotype, respectively, whereas ASY2-24A (*chs1 chs2 CAL1* pAS4) acquired the appearance of a *chs2* mutant (Fig. 1, *b* and *c*), as expected (results not shown).

All strains used in this study were defective in Chs1. To ascertain whether Chs1 could substitute for the absence of Chs2 and Chs3, the following experiment was carried out. A centromere plasmid (pAS5; see Materials and Methods) was constructed which harbored both the *CHS1* and the *TRP1* genes. Transformation of strain ASY2-24B to prototrophy with pAS5 resulted in cells with greatly enhanced chitin synthase activity, as expected. When these cells were transferred from galactose to glucose medium they underwent the same changes as those shown in Fig. 7 for the untransformed ASY2-24B cells. After 24 h, most cells were lysed as in the untransformed strains (results not shown). It may be concluded that Chs1 cannot rescue the cells from the simultaneous Chs2 and Chs3 defect.

In summary, the deprivation of both Chs2 and Chs3 results in the cessation of cell division followed by cell death, in confirmation of the genetic results.

Discussion

Function of Chs2 and Chs3

In confirmation of our previous findings (38), the results of this study show that the specific function of Chs2 is the synthesis of chitin in the primary septum. Of the three chitin synthase activities, the *chs1 cal^{R}1* mutant only has Chs2, yet it exhibits a trilaminar septum to which WGA binds along the primary septum line. No labeling is seen elsewhere in the cell wall, in particular, at the base of an emerging bud. Conversely, mutants lacking Chs2 present very thick septa, with scattered labeling by WGA, similar to that of the adjacent cell wall and without indication of a primary septum. The thick septa may be the equivalent of what in wild-type cells are secondary septa: these are normally laid down onto the primary septum and perpendicular to the mother-daughter cell axis (11; Fig. 2 *d*). The lack of the primary septum in *chs2* mutants apparently leads to a lateral deposition of the secondary septa, 90° to the usual direction (Fig. 3 *a*), finally resulting in the formation of an amorphous and thick cross wall. A similar interpretation was given earlier for the formation of aberrant septa, that resemble those of Fig. 2 *b*, in the presence of polyoxin D (2). The cells defective in Chs2, although viable, are severely impaired in growth, larger than normal cells and often of aberrant shape. They also form large aggregates in which the cells are held together by the thick septa. This feature is probably ascribable to the lack of a chitin primary septum. In wild-type strains, cell separation occurs along the chitin line, with the help of a chitinase (13, 14, 16) that hydrolyzes part of the polysaccharide. Inhibition of this chitinase (30) or disruption of its structural gene (Kuranda and Robbins, personal communication) results in the formation of large cell aggregates. In the *chs2* mutants it is the substrate, chitin, rather than the enzyme, that is missing at the appropriate location, but the outcome is the same, i.e., lack of cell separation.

Whereas the action of Chs2 is strictly limited to the primary septum, that of Chs3 appears to extend to all other locations where chitin is found in the cell, i.e., both in the ring at the neck between mother and daughter cell and all around the wall. The labeling with WGA in those areas is totally absent in *cal^{R}1* mutants, which are deficient in Chs3. From the data on chitin content (Table II; 29) it is clear that only a small percentage of the polysaccharide is present in the pri-

mary septum and that most of the remaining chitin is synthesized through the agency of Chs3. Despite this fact, the absence of Chs3 activity results in relatively modest changes in the growth and general morphology of the cells. Clearly, the absence of the chitin ring does not prevent budding. A similar conclusion was reached earlier, in a study on the effect of polyoxin D, an inhibitor of all three chitin synthases, on yeast growth (9). There are, however, abnormalities in the budding pattern and in the morphology of the septal region as well as of the corresponding bud scars. These abnormalities may be explained by the absence of a chitin ring at early budding.

The relatively high level of chitin in the cell walls of the wild-type strain used in the present study led to the observation that little or no chitin is present in the bud cell wall. Deposition of chitin in the wall appears to be part of the process of final maturation of the daughter cell. The WGA label was observed most often in walls of daughter cells that had already been separated by a septum from the mother cells, although occasionally a large bud still connected to the mother cell showed some labeling. This asymmetrical distribution of chitin is most striking in *chs2* cells, because of the heavier label in those cells. The reason for this higher chitin content is not clear. It is possible that the absence of Chs2 somehow triggers an increase in the expression or activation of Chs3, but there may be a trivial explanation based on the abnormal growth of the Chs2-deficient strains.

The finding that Chs3 is responsible for the deposition of chitin all over the cell wall suggested that it may be similarly involved in other circumstances in which chitin formation in the cell wall is observed. It has already been reported that no chitin is laid down in the wall in response to α-factor treatment of *cal^{R}1* (Chs3$^-$) mutants (29). We (27) and others (40) have also observed that incubation of several *cdc* mutants at the nonpermissive temperature leads to generalized deposition of chitin in the wall. Simultaneous presence of a *cal^{R}1* and a *cdc24* or *cdc3* mutation in the same cell abolished the increase in Calcofluor staining of the cells at 37°C. It may be concluded that Chs3 is also involved in chitin synthesis in this case. In an earlier report, we suggested that the abnormal synthesis of chitin in the *cdc* mutants may be caused by a generalized activation of chitin synthase due to unbalanced conditions created by cell cycle arrest. The finding that there is deposition of chitin in the wall of the daughter cell as a normal component of a maturation process suggests a different explanation: the extra chitin may come from successive "maturation cycles" in a cell that is unable to divide further at the nonpermissive temperature.

The deacetylated analog of chitin, chitosan, has been found to form a layer in the spore cell wall (3). In other fungi, chitosan has been postulated to arise in a two-step process, the first step being chitin formation and the second, deacetylation of the polysaccharide (15). We reported previously a sporulation defect in homozygous *cal^{R}1* diploids (29). Observations by EM (results not shown) reveal that such spores lack the two outermost layers of the cell wall, which include the chitosan layer. A similar result has been reported by Briza et al. (4) for mutant *dit101*. Based on a comparison of restriction maps, the corresponding gene, *DIT101*, appears to be identical to *CAL1* (C. Bulawa, personal communication). This indicates that Chs3 is also required for chitosan formation in the spore wall.

From these and previous findings (13), it follows that each chitin synthase is endowed with specific functions. It is, therefore, both incorrect and misleading to assert that "chitin synthase I and chitin synthase II are not required for chitin synthesis in vivo in *S. cerevisiae*" (5). Each chitin synthase is indeed required for the formation of chitin at a certain location and with a certain function. In the absence of each synthase the corresponding chitin will not be made, with consequent cell abnormalities, such as lysis of daughter cells for Chs1$^-$ strains, aberrant septa, aggregation, and stunted growth in Chs2$^-$ strains, and morphological changes in vegetative growth as well as defective spores in Chs3$^-$ strains.

Requirement of Chitin Synthesis for Viability

To understand morphogenetic problems, such as the formation of septum and cell wall, it is not necessary to know whether the different chitin synthases or chitin synthesis itself are required for viability. However, this question is of general biological interest and is of paramount importance for assessing the possibility that chitin synthesis inhibitors may serve as antifungal agents. Clearly, either Chs2 (43) or Chs3 (5) is sufficient to support viability in the absence of the other two synthases. To investigate whether a triple mutant would be viable we first crossed two strains, both with *chs1* mutations and each one with a mutation either in *CHS2* or *CAL1*. No triple mutants were isolated from the cross, an indication that such a combination would be lethal. From previous results (5, 38), however, it seemed possible that while spores with the triple mutation could be unable to germinate, vegetative cells with the same genotype, if they could be obtained, might perhaps be viable. Such triple mutants were constructed in the presence of a plasmid carrying *CHS2* under the control of a *GAL1* promoter. Expression of Chs2 and synthesis of chitin were shut off by transferring the cells from galactose to glucose. The result was cessation of cell division and abnormal growth of the cells, followed by cell death and lysis. Thus, chitin is required even for the formation of abnormal septa, as those of *chs2* cells, and is also necessary for viability, at least in the strains that we have studied. We conclude that an inhibitor of both Chs2 and Chs3 would be a potential antifungal agent. Polyoxin D is such an inhibitor (26, 31), and it is active against certain fungi (21). In *S. cerevisiae* and *Candida albicans* it is a poor inhibitor of growth, probably because of permeability problems (1, 2).

The fact that Chs3 participates in generalized chitin synthesis in the yeast cell wall suggests that it may be analogous to a corresponding synthase in vegetative hyphae of filamentous fungi. Because those fungi that have chitin in their cell wall also contain this polysaccharide in the septa (10), it seems likely that they possess an enzyme analogous to Chs2. Availability of the sequence of the *CHS2* (37) and *CAL1* (43) genes should facilitate identification of the corresponding putative genes in filamentous fungi.

The present study and others reported previously (5, 13, 38, 43) have elucidated the different functions of Chs1, Chs2, and Chs3. At the same time they have uncovered the need for independent regulation of the three activities, so that each one will play its role at the appropriate time in the cell cycle and at the required location. We know very little about this regulation, except that Chs1 and Chs2 are in a zymogenic

form, thereby requiring activation of some sort; it is also clear that the *CAL1* product interacts somehow with those of other CAL genes, because Chs3 is defective in mutants of all four *cal*^{R}*1* complementation groups (43). The lack of an increased chitin deposition in the cell wall in the presence of Calcofluor in *cal*^{R}*1* mutants indicates that the brightener exerts its effect on Chs3, although the mechanism of this action is unknown. We previously reported (13) that Congo red, which appears to act in the same way as Calcofluor (28), partially prevents the lysis of daughter cells in *chs1* mutants growing in unbuffered medium. This effect was attributed to Chs2, the only other chitin synthase known at the time. It is now clear that Chs3 is responsible for the diminished lysis, probably by increasing the overall synthesis of chitin in the cell wall under the stimulation of Congo red.

The results of the experiment with the triple mutant harboring the GAL1-CHS2 plasmid suggest that Chs2 has a rapid turnover. This may be important for its regulation and deserves further study; similar experiments can also be done to determine the turnover of Chs1 and Chs3. Finally, it is known that the location of septa, although not necessarily their formation (39), is dependent on the existence of a microfilament ring (7) that is absent in *cdc* mutants 3, 10, 11, and 12. It is to be hoped that further studies of the interrelationships between the proteins encoded by all the genes that have been identified as participants in these processes may provide new avenues for the understanding of the regulation of chitin synthesis and septum formation.

We thank T. E. Olszewski and M. Nwulia for valuable technical assistance. We also thank A. Meyers and R. Sikorski for plasmids and W. B. Jakoby, R. Myerowitz, and A. Robbins for useful criticism. We are also indebted to R. Baum for pointing out to us the identity between upstream sequences of *CHS2* and reading frame sequences of *SCO1*.

M. H. Valdivieso was the recipient of a fellowship from the Ministerio de Educación y Ciencia, Madrid, Spain. M. H. Valdivieso and A. Duran received partial support from Research Project Bio 88-0234 of the Comisión Interministerial de Ciencia y Technología and from Glaxo S. A., Madrid, Spain.

Received for publication 8 February 1991 and in revised form 15 March 1991.

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