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Effect of Calcofluor White on Chitin Synthases from *Saccharomyces cerevisiae*

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The growths of *Saccharomyces cerevisiae* wild-type strain and another strain containing a disrupted structural gene for chitin synthase (*chs1*::*URA3*), defective in chitin synthase 1 (Chs1) but showing a new chitin synthase activity (Chs2), were affected by Calcofluor. To be effective, the interaction of Calcofluor with growing cells had to occur at around pH 6. Treatment of growing cells from these strains with the fluorochrome led to an increase in the total levels of Chs1 and Chs2 activities measured on permeabilized cells. During treatment, basal levels (activities expressed in the absence of exogenous proteolytic activation) of Chs1 and Chs2 increased nine- and fourfold, respectively, through a mechanism dependent on protein synthesis, since the effect was abolished by cycloheximide. During α-factor treatment, both Chs1 and Chs2 levels increased; however, as opposed to what occurred during the mitotic cell cycle, there was no further increase in Chs1 or Chs2 activities by Calcofluor treatment.

Recent studies have shown that Calcofluor and another dye, Congo red, induce abnormally thick septa between mother and daughter cells of *Saccharomyces cerevisiae* (13, 19) and enhance the rate of chitin polymerization in several systems (13). However, all attempts to demonstrate in vitro activation of chitin synthase failed (13); furthermore, in vitro inhibition of chitin synthase by Calcofluor has been reported (13, 17).

The enzymatic synthesis of chitin in yeasts has been studied by characterizing the enzyme now called chitin synthase 1 (Chs1). However, a new chitin synthase, chitin synthase 2 (Chs2), has been very recently described in *S. cerevisiae chs1*::*URA3* which contains the disrupted structural gene for Chs1 (2, 11, 15).

In this study, we investigated the effect of Calcofluor on Chs1 and Chs2, zymogenic enzymes that require proteolytic activation. We showed that during vegetative growth of *S. cerevisiae*, Calcofluor increased the levels of the preactivated enzymes and activated both synthases. We also showed that cycloheximide abolished the above-mentioned activations. The levels of Chs1 and Chs2 rising in response to the α-factor were not further increased by Calcofluor.

MATERIALS AND METHODS

Chemicals. Calcofluor White M 2R New (disodium salt of 2,2'-(1,2-ethenediyl)bis[5-[[4-[bis(2-hydroxyethyl)amino]-6-(phenylamino)-1,3,5-triazin-2-yl]amino]-benzenesulfonicacid) and the benzidine derivative Congo red were from American Cyanamid Co., Bound Brook, N. J., and Serva Feinbiochemica, Heidelberg, Federal Republic of Germany, respectively. α-Factor, UDP-*N*-acetylglucosamine, *N*-acetylglucosamine, trypsin, trypsin inhibitor, cycloheximide, and digitonin were from Sigma Chemical Co., St. Louis, Mo. UDP-[U-^{14}C]*N*-acetylglucosamine (346 mCi/mmol) and [U-^{14}C]glucose (295 mCi/mmol) were from Amersham International plc, Amersham, U.K. Other reagents were of analytical grade.

Strains and growth conditions. *S. cerevisiae* X2180-1A and its parental diploid X2180 were obtained from the Yeast Genetic Stock Center. The **a** *his chs1*::*URA3* strain was kindly supplied by E. Cabib (National Institutes of Health, Bethesda, Md.); this strain was mated to an α Cal^R *ura3* strain resistant to the fluorochrome Calcofluor (14). The diploid sporulated, and an α *chs1*::*URA3* strain was selected by tetrad analysis. **a** and α strains deficient in Chs1 were crossed to obtain the corresponding homozygous diploid. Genetic crosses were performed as described elsewhere (18).

Cells were grown in YED medium containing 1% yeast extract (Difco Laboratories, Detroit, Mich.) and 1% glucose or in defined medium containing 0.7% yeast nitrogen base (Difco) and 2% glucose. Minimal medium buffered at either pH 6.5 or 4.0 was prepared in 250 mM potassium biphthalate-NaOH. For solid media, 1.5% agar (Difco) was added. Growth was monitored either by measuring the A_{600} of cultures in a Coleman Junior II spectrophotometer or by counting the number of cells.

Chitin synthase assay. Chitin synthase assays were performed on digitonin-permeabilized cells as described previously (6). *S. cerevisiae* log-phase cells (wet weight; 0.1 g) were suspended in 1 ml of 25 mM MES (morpholineethanesulfonic acid [pH 6.0])–1% digitonin, incubated for 15 min at 30°C, washed twice with the same buffer without digitonin by centrifugation at 15,000 × *g* for 10 min, and, finally, resuspended in 1 ml of 25 mM MES (pH 6.0)–33% glycerol.

Chitin synthase activity was always measured without trypsin (basal activity) or with the addition of different amounts of trypsin to assess the potential activity (maximal activity) (4, 5). To assay Chs1, *S. cerevisiae* X2180-1A cells (5 to 20 μl) were added to 50 mM phosphate (pH 6.3)–20 mM $MgSO_4$–40 mM *N*-acetylglucosamine–2.5 mM UDP-[U-^{14}C]*N*-acetylglucosamine (400,000 cpm/μmol) in a final volume of 40 μl. To assay Chs2, *S. cerevisiae chs1*::*URA3* cells were used, and the assay was performed essentially as described earlier (15). The assay mixture for Chs2 was similar to that for Chs1, except for the buffer (50 mM Tris hydrochloride [pH 8.0]) and the divalent cation (10 mM $CoCl_2$) used. Incubation was usually for 60 min at 30°C for Chs1 and 120 min for Chs2. Under these conditions, the rates of reactions were linear. Radioactivity from the tri-

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chloroacetic acid-insoluble material was determined after filtration and washing (8). We defined 1 U of enzyme as the amount that catalyzed the incorporation of 1 μmol of *N*-acetylglucosamine per min into chitin.

Other methods. Protein was determined as described previously (10). Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid-insoluble material (12) from cultures supplemented with [U-^{14}C]glucose. Cell wall chitin was determined by digestion with chitinase after alkali extraction as previously described (12). Fluorescence was observed with a Nikon Optiphot microscope with an epifluorescent attachment and an HBO 100W/2 light source as described elsewhere (13).

RESULTS AND DISCUSSION

Effect of Calcofluor on *S. cerevisiae* growth. The addition of Calcofluor (200 μg/ml) to a growing culture of *S. cerevisiae* in YED medium resulted in a complete arrest of growth (Fig. 1A), whereas almost no effect was observed with the growth in minimal medium. When the initial pH value of YED medium was adjusted to 4.0, Calcofluor did not affect growth (Fig. 1A). The results suggested that the pH could be important for Calcofluor action. To verify this hypothesis, buffered minimal medium at pH 6.5 or 4.0 was used to grow *S. cerevisiae* under the same conditions described above. At pH 4.0, growth was unaffected by Calcofluor, whereas at pH 6.5, inhibition of growth took place, as with the case of growth in YED medium (Fig. 1B). During growth in YED medium, the initial pH value (6.5) remained practically constant with time, probably because of the buffering capacity of the yeast extract component, and Calcofluor exerted its action. In minimal medium, the pH decreased to 2.7, and Calcofluor was not effective. Therefore, the interaction of Calcofluor with growing *S. cerevisiae* cells requires a pH value above 4.0 and close to 6.

The growth of the *S. cerevisiae* diploid strain homozygous for a disrupted structural gene for Chs1 was also affected by Calcofluor and Congo red; in fact, this strain seemed to be more susceptible to the drugs than the wild type was (Fig. 2). Haploid strains behaved in the same fashion, except that their degree of susceptibility to Calcofluor was higher than that of the corresponding isogenic diploids. After an 8-h culture in the presence of Calcofluor, no growth was detected in either *chs1::URA3* or wild-type haploid cultures; however, there was always a small percentage of surviving cells that recovered and that started growing again later. In the absence of Calcofluor, the *chs1::URA3* and wild-type strains grew up to A_{600} values of 0.5 and 0.7, respectively.

Chains of cells, resulting from budding without complete separation between mother and daughter cells, were absent in cultures of *chs1::URA3* supplemented with Calcofluor but not in the wild-type cultures (Fig. 3). Phase-contrast and fluorescence microscopy revealed an accumulation of fluorescence not only at the level of septa (as previously described for the wild type) but sometimes also around the cell surface of mother or daughter cells for the *chs1::URA3* strain (Fig. 3C).

It is important to notice that the effect of Calcofluor on *S. cerevisiae* growth clearly depended on the size of the inoculum (results not shown), as has been described for *Geotrichum candidum* (13).

Effect of Calcofluor on chitin synthases. (i) Growing cells. Calcofluor or Congo red (100 μg/ml) was added to *S. cerevisiae* X2180-1A (A_{600} = 0.1) growing in YED medium. Samples from control and treated cultures were withdrawn at different time intervals, and chitin synthases were determined. A similar experiment was performed with *S. cerevisiae chs1::URA3*, except that 25 μg of Calcofluor per ml of culture was added. During the treatment, only 10 and 11.5% of the wild-type and *chs1::URA3* cells, respectively, were lysed. Values from strain X2180-1A represented Chs1 levels, since under these conditions, Chs2 accounted for about 10% of the final value (15; Table 1), and those from the *chs1::URA3* strain corresponded to Chs2 levels.

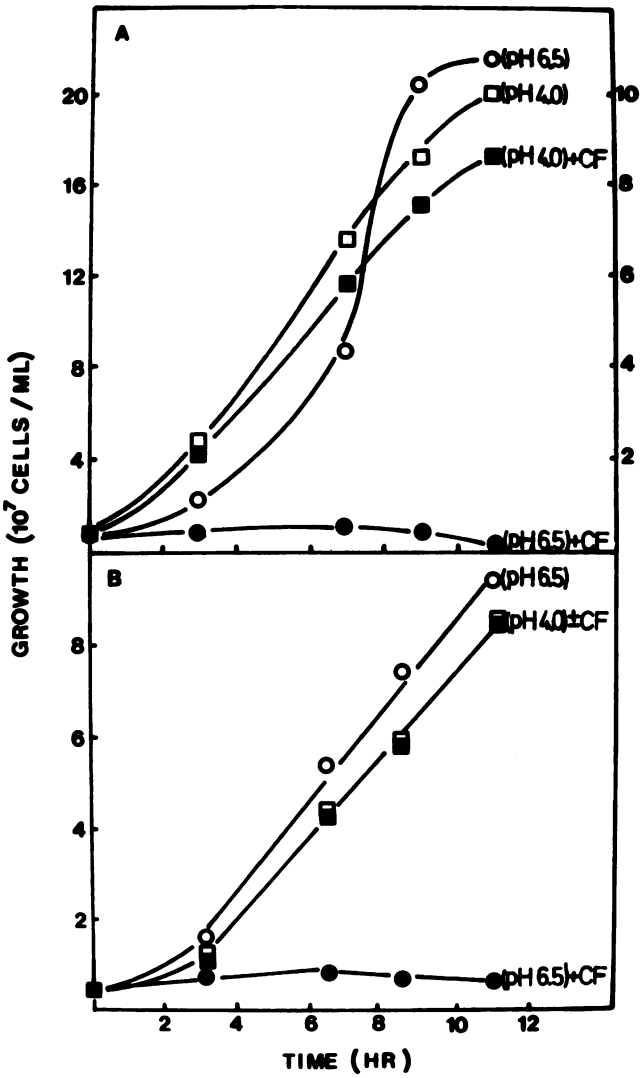

FIG. 1. Growth of *S. cerevisiae* X2180-1A in YED medium (A) or minimal medium (B) buffered at different pH values and supplemented with or without 200 μg of Calcofluor (CF) per ml. The numbers on the right-side ordinate represent the values of growth in YED medium buffered at pH 4.0.

Chs2 behaved as a zymogen, as recently described (15) (Table 1, no addition). Treatment of growing X2180-1A cells with Calcofluor induced a clearly time-dependent increase (up to about ninefold) in the level of Chs1 basal activity (expressed in the absence of trypsin activation) (Table 1, no addition and with Calcofluor only). Congo red exerted a similar effect on strain X2180-1A (Table 1). The level of Chs2 basal activity also increased (up to fourfold) after Calcofluor treatment of *chs1::URA3* cells. In addition, Chs1 and Chs2 maximal activities also increased after 90 min of Calcofluor

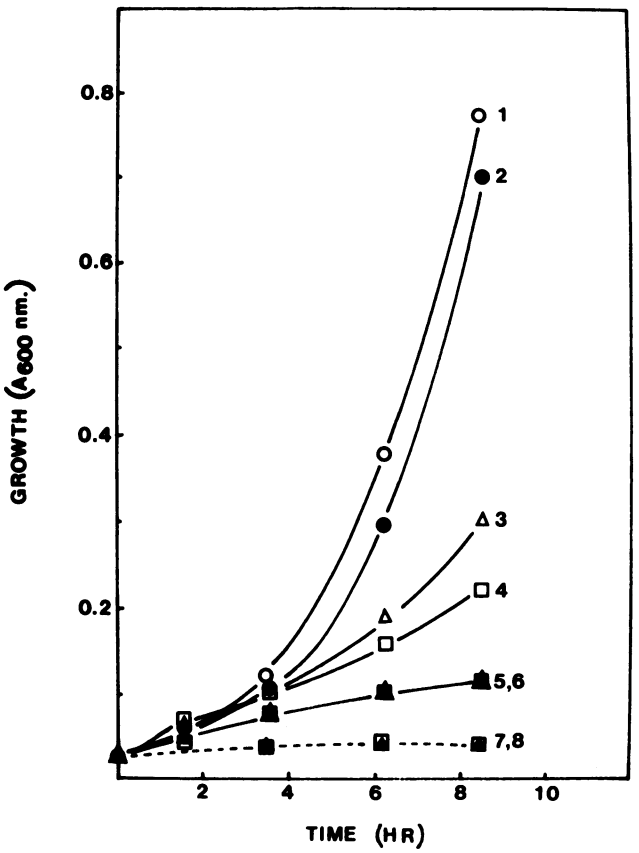

FIG. 2. Growth of several *S. cerevisiae* strains in the presence or absence of Calcofluor. Curves: 1, 3, and 4, *S. cerevisiae* X2180; 2, 5, 6, and 8 (▲), *S. cerevisiae chs1::URA3*; 7 (□), *S. cerevisiae* X2180-1A. Symbols: ○, ●, control cultures; △, ▲, cultures treated with 25 μg of Calcofluor per ml; □, ■, cultures treated with 75 μg of Calcofluor per ml.

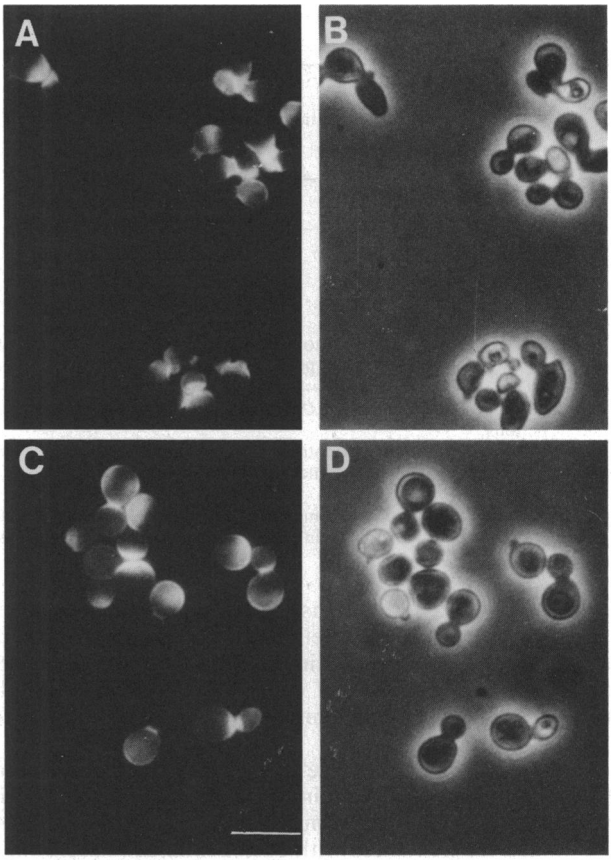

FIG. 3. Fluorescence and phase-contrast micrographs of *S. cerevisiae* X2180-1A and the *chs1::URA3* strain treated with Calcofluor. Cells from the wild-type (A and B) or the *chs1::URA3* (C and D) strains were grown on YED medium in the presence of Calcofluor (50 μg/ml) and observed under a phase-contrast (B and D) or a fluorescence (A and D) microscope after 3 h. The fluorescence micrographs were taken with the same lenses and exposure times. The bar corresponds to 10 μm.

treatment and remained constant after 3 h. Under these conditions, Calcofluor- and Congo red-treated cells accumulated 2.1- and 2.7-fold more chitin, respectively, than did the wild-type control cells, whereas Calcofluor-treated *chs1::URA3* cells accumulated 1.6-fold more chitin than did the control cells (measured as radioactivity incorporated from [^{14}C]glucose into chitin per total radioactivity incorporated).

As already described (3, 11), basal and maximal activity values from log-phase cultures growing in the absence of Calcofluor did not change during the time course of the experiment (Table 1, no addition). Addition of Calcofluor immediately before the permeabilization of cells resulted in Chs1 basal activity values similar to that of control; however, the level of Chs1 maximal activity was slightly but consistently higher (12.1 mU/10^9 cells) than that of the control. No increase of Chs1 and Chs2 activities was observed in chitin synthase assays performed in cell extracts, but inhibition of these activities was observed (results not shown). Mechanical breakage of the cells probably allowed Calcofluor interaction with the internal side of the membranes causing irreversible inhibition of the synthases (13).

(ii) α-factor-treated cells. Since treatment of a *S. cerevisiae* cells with the pheromone α-factor induces the synthesis of chitin (16), it seemed obvious to determine whether Calcofluor would affect chitin synthesis in α-factor-treated cells. To answer this question, cells of the X2180-1A and a *chs1::URA3* strains (A_{600} = 0.1) were incubated in YED medium supplemented with α-factor (final concentration, 5 μM), and after 90 min, Calcofluor (50 μg/ml) was added to the appropriate cultures. At 2 h later, cells from control and Calcofluor-treated cultures were harvested, and chitin synthases were determined. Under these conditions, no lysis due to Calcofluor was observed, and 85 to 92% of the cells presented the well-known polar elongation called shmoos (9). It was found that (i) Chs1 basal and maximal activity levels were dramatically increased by α-factor treatment as has been already described (16) (compare control values from Tables 1 and 2); (ii) Chs2 levels were also increased by α-factor treatment, although to a lesser extent than Chs1 levels; and (iii) Calcofluor did not enhance Chs1 and Chs2 levels, as opposed to what occurred during the mitotic cell cycle. The more obvious reason for this last observation is that the highest activation has already been achieved by α-factor treatment. However, under the same experimental conditions, pheromone-treated wild-type cells accumulated 1.4-fold more chitin in the presence of Calcofluor than did the control cells (the chitin contents were 8.1 and 11.6% of the cell wall, respectively).

It is clear from Tables 1 and 2 that Chs1, despite being dispensable for growth (2), responds in vivo to Calcofluor and to α-factor. A recent report (11) indicates that the level of Chs2 is not modified by α-factor treatment. However, our

TABLE 1. Effects of Calcofluor, Congo red, and cycloheximide on chitin synthases in growing cells of *S. cerevisiae* X2180-1A and *S. cerevisiae chs1::URA3*

Addition to culture (incubation time [min])[a]	Chitin synthase activity (mU/10^9 cells)[b]			
	X2180-1A		*chs1::URA3*	
	Basal	Maximal	Basal	Maximal
No addition (0, 180)	1.1 ± 0.06	9.5 ± 1.5	0.10 ± 0.02	0.48 ± 0.06
Calcofluor (90)	5.0 ± 0.6	23.5 ± 0.6	0.31 ± 0.03	0.68 ± 0.08
Calcofluor (150)	7.4 ± 0.5	21.6 ± 0.7	0.36 ± 0.04	0.69 ± 0.1
Calcofluor (180)	9.8 ± 0.5	22.3 ± 1.5	0.40 ± 0.03	0.68 ± 0.1
Congo red (180)	12.5 ± 0.5	24.0 ± 1.2	N.D.[c]	N.D.
Cycloheximide (60, 120)	1.1 ± 0.1	9.7 ± 0.8	0.18 ± 0.03	0.43 ± 0.02
Cycloheximide (120) + Calcofluor during last 90 min[d]	0.9 ± 0.07	14.1 ± 1.5	0.18 ± 0.04	0.50 ± 0.02
Calcofluor (150) + cycloheximide during last 60 min[e]	4.5 ± 0.4	19.5 ± 0.8	0.32 ± 0.03	0.51 ± 0.03

[a] Cultures of X2180-1A and *chs1::URA3* were grown on Calcofluor (100 and 25 μg/ml, respectively), Congo red (100 μg/ml), or cycloheximide (100 μg/ml) for different periods.
[b] Values from X2180-1A are essentially Chs1 levels, whereas those from *chs1::URA3* are Chs2 levels. Activity was measured without trypsin (basal) or with trypsin (maximal). For more experimental conditions, see text.
[c] N.D., Not determined.
[d] After cells were grown for 30 min with cycloheximide, Calcofluor was added to the culture, and cells were harvested after 90 min.
[e] After cells were grown for 90 min with Calcofluor, cycloheximide was added to the culture, and cells were harvested 60 min later.

results show that both Chs2 basal and maximal levels increase in a *chs1::URA3* cells after pheromone treatment. Our use of a method for chitin synthase assay more conservative (permeabilization of cells) than the one used by Orlean (mechanical homogenization; 11) may be a reason for that discrepancy.

Abolition by cycloheximide of the effect of Calcofluor on chitin synthases. To determine how Calcofluor exerted its action, growing cultures from X2180-1A or the *chs1::URA3* strain were supplemented with cycloheximide (100 μg/ml) to inhibit protein synthesis; after 30 min, cultures of both strains were split into two portions, and Calcofluor (100 μg/ml) was added to one of each. After 90 min, cells were harvested, and chitin synthases were determined as described above. Under these conditions, protein synthesis was completely inhibited and growth was totally arrested (results not shown). The increase in Chs1 and Chs2 basal activities promoted by Calcofluor was abolished by cycloheximide (Table 1, with cycloheximide plus Calcofluor), whereas maximal activities still remained slightly activated by the fluorochrome. In a different experiment, cycloheximide was added after 90 min of Calcofluor treatment, and the cultures were harvested 60 min later. Under these conditions, the Chs1 and Chs2 basal activities (Table 1, with Calcofluor plus cycloheximide) were similar to those present at the time of cycloheximide addition (Table 1, with Calcofluor [90 min]). Therefore, cycloheximide, under the conditions of this experiment, also abolished the increase of basal activity due to Calcofluor. Maximal activities in treated cells also remained unchanged or were somewhat reduced compared with those of the controls. Examination of X2180-1A cells growing in the presence of both cycloheximide and Calcofluor by fluorescence microscopy revealed that the typical enhanced fluorescence caused by Calcofluor at the level of septa was considerably reduced in the presence of cycloheximide (Fig. 4).

On the basis of the above-mentioned results, we propose that the increase in basal activities due to Calcofluor occurs by conversion of a significant portion of the zymogen to an active form throughout the action of a hypothetical activating factor, not yet found, implicated in the physiological activation of chitin synthase (5, 15). In the presence of cycloheximide, the effect (or synthesis) of the presumptive activator would be abolished and the conversion of the zymogen to active form would not take place.

Crystallization in some polysaccharides (1, 7) can limit the rate of polymerization. Therefore, Calcofluor, by binding to

TABLE 2. Effect of Calcofluor on chitin synthases in α-factor-treated cells of *S. cerevisiae* X2180-1A and *S. cerevisiae chs1::URA3*[a]

Organism and type of enzyme activity	Chitin synthase activity (mU/10^9 cells)		
	−CAL	+CAL	Ratio (+CAL/−CAL)
X2180-1A			
Basal	40.3 ± 5.2	34.0 ± 4.0	0.84
Maximal	148.5 ± 10.3	110.0 ± 12.5	0.74
chs1::URA3			
Basal	0.37 ± 0.04	0.36 ± 0.05	0.97
Maximal	0.96 ± 0.12	0.71 ± 0.10	0.74

[a] For experimental details, see text. −CAL, Control culture; +CAL, Calcofluor-treated culture.

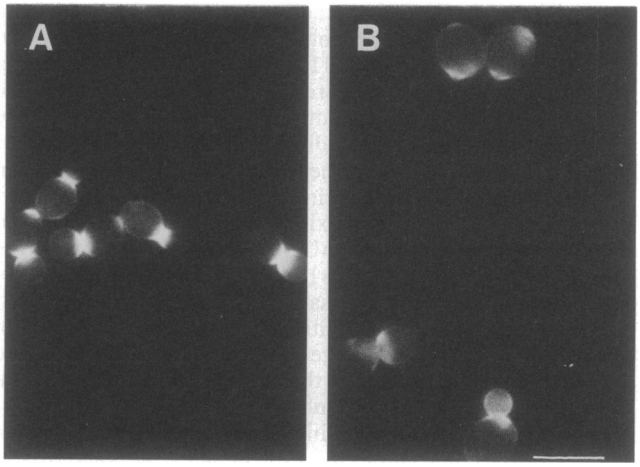

FIG. 4. Fluorescence micrographs of *S. cerevisiae* X2180-1A treated with Calcofluor in the absence (A) or presence (B) of cycloheximide. Micrographs were taken with the same lenses and exposure times. The bar corresponds to 10 μm.

the growing chitin microfibrils, would uncouple the two processes and activation of chitin synthase might occur. These processes may explain the increase in maximal activities observed even during cycloheximide treatment or during addition of Calcofluor immediately before the permeabilization of cells.

In conclusion, (i) the levels of Chs1 and Chs2 activities in **a** cells increased during α-factor treatment while chitin synthesis was activated, and (ii) the activation of chitin synthesis in growing cells by Calcofluor treatment ran parallel to the increase in both Chs1 and Chs2 activities accomplished in part, and probably in both cases, by a protein synthesis-dependent conversion of zymogen to an active form of enzyme. Why does Chs1 respond to the fluorochrome when it is not required for chitin synthesis in vivo? Yeast Chs1 may be, as already discussed elsewhere (15), a vestige of a fungal chitin synthase evolutionary ancestor having a role not yet found and being nonfunctional under normal conditions. However, under some circumstances, Chs1 activity may respond to a stimulus, as with the case of Calcofluor treatment.

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