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CHS5, a Gene Involved in Chitin Synthesis and Mating in *Saccharomyces cerevisiae*

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The *CHS5* locus of *Saccharomyces cerevisiae* is important for wild-type levels of chitin synthase III activity. *chs5* cells have reduced levels of this activity. To further understand the role of *CHS5* in yeast, the *CHS5* gene was cloned by complementation of the Calcofluor resistance phenotype of a *chs5* mutant. Transformation of the mutant with a plasmid carrying *CHS5* restored Calcofluor sensitivity, wild-type cell wall chitin levels, and chitin synthase III activity levels. DNA sequence analysis reveals that *CHS5* encodes a unique polypeptide of 671 amino acids with a molecular mass of 73,642 Da. The predicted sequence shows a heptapeptide repeated 10 times, a carboxy-terminal lysine-rich tail, and some similarity to neurofilament proteins. The effects of deletion of *CHS5* indicate that it is not essential for yeast cell growth; however, it is important for mating. Deletion of *CHS3*, the presumptive structural gene for chitin synthase III activity, results in a modest decrease in mating efficiency, whereas *chs5*Δ cells exhibit a much stronger mating defect. However, *chs5* cells produce more chitin than *chs3* mutants, indicating that *CHS5* plays a role in other processes besides chitin synthesis. Analysis of mating mixtures of *chs5* cells reveals that cells agglutinate and make contact but fail to undergo cell fusion. The *chs5* mating defect can be partially rescued by *FUS1* and/or *FUS2*, two genes which have been implicated previously in cell fusion, but not by *FUS3*. In addition, mating efficiency is much lower in *fus1 fus2* × *chs5* than in *fus1 fus2* × wild type crosses. Our results indicate that Chs5p plays an important role in the cell fusion step of mating.

The polysaccharide chitin is an important structural component of the cell walls of many fungi. In vegetative cells of *Saccharomyces cerevisiae*, it accounts for only a small percentage of the cell wall dry weight and is mostly present in the bud scar, a craterlike structure found on the surface of the mother cell after cell separation (3, 12). The formation of the bud scar during the budding cycle occurs in several steps (15). Before the bud emerges, a chitin ring is laid down at the base of the growing bud. The deposition of chitin at this location continues as the bud grows to near the size of the mother cell. The activity responsible for this synthesis, chitin synthase III (CSIII) (9, 61), requires the action of several genes. One of them is *CHS3* (7, 11, 41, 61), which probably encodes the catalytic component for CSIII or a subunit thereof. Others are *CHS4*, *CHS5*, and *CHS6* (see below). At cytokinesis, a new deposition of chitin occurs in a centripetal fashion in the furrow of the invaginating plasma membrane. Thus, a thin electron-translucent disk structure, the so-called primary septum, is formed (15, 54) by the action of a different chitin synthase activity, chitin synthase II (CSII), encoded by the *CHS2* gene (56). Finally, the two cells separate in an asymmetrical fashion so that most of the chitin remains in the mother cell as a bud scar. Cell separation requires the partial degradation of chitin by the action of a periplasmic chitinase (36) as well as the function of another different activity, chitin synthase I (CSI), encoded by the *CHS1* gene (10). The latter counterbalances the action of the chitinase and therefore functions as a safety mechanism in the final process of cell separation (13, 14). Outside the bud scar, the cell wall of the mother cell contains a small amount of uniformly dispersed chitin that is synthesized by CSIII (44, 54) and involved in the linkage with β(1,3)glucan (34).

Synthesis of chitin in budding yeasts is not restricted to the mitotic cycle. During conjugation, haploid cells of complementary mating types form projections (shmoos) toward each other, under the stimulus of the corresponding sexual pheromones. This is an example of asymmetric polarization in which new cell wall growth and membrane deposition occur to enable cells to fuse and eventually mate (for a review see reference 57). During this process, chitin is synthesized, and its deposition occurs mainly at the subapical portion of the shmoo, as can be readily observed after Calcofluor staining (52). CSIII is the activity responsible for chitin synthesis in the mating projections (44, 54), although, paradoxically, *CHS1* transcription is highly induced by pheromone treatment (1) whereas *CHS3* transcription is not (17). In addition to its role in chitin synthesis during vegetative growth and mating, *CHS3* also participates in sporulation. During sporulation, a layer of chitosan, a deacetylated derivative of chitin, is formed in the ascospore walls (5). A mutation causing defects in the chitosan and the dityrosine-rich layers of the spore wall maps in the *CHS3* locus (41). This result suggests that chitosan is made by synthesis of chitin mediated by CSIII activity, followed by deacetylation.

Although this picture assigns a role to each chitin synthase during the life cycle of *S. cerevisiae*, it does not address the nature of the regulatory mechanisms for the deposition of chitin in the cell wall at each morphogenetic event. Recent studies suggest that both transcriptional and posttranslational regulatory systems are involved in this regulation (17, 18). In contrast with CSI and CSII, for which only the genes putatively encoding the catalytic subunits have been described, several

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genes have been identified as being involved in CSIII activity. In addition to *CHS3*, at least three additional genes, *CHS4*, *CHS5*, and *CHS6* (7, 11, 44), have been implicated both in Calcofluor resistance and in chitin synthesis mediated specifically by CSIII activity. The function of these genes has not been clearly established (for reviews see references 8 and 20).

Here we report the characterization of the *CHS5* gene. *CHS5* encodes a unique protein of 671 residues that displays some similarity to neurofilament proteins. *CHS5* is not essential for vegetative growth, but its disruption results in Calcofluor resistance and diminished chitin formation, as expected for a specific defect in CSIII. Surprisingly, the null mutants also manifest striking defects in shmoo morphology, cell fusion, and mating. Although these defects may be due in part to the decrease in chitin synthesis, as shown by results with *chs3* mutants, the mating defect of *chs5* mutants is more severe than that of *chs3* mutants and implicates the *CHS5* gene in other cell processes besides CSIII activity.

MATERIALS AND METHODS

Strains, media, and microbiological methods. The yeast strains used in this study are listed in Table 1. Yeast cells were grown in yeast extract-peptone-dextrose (YEPD), yeast-peptone-glycerol, or minimal medium (55). *Escherichia coli* DH5α and MV1190 were grown in Luria-Bertani medium containing 50 μg of ampicillin per ml. Calcofluor white was kindly provided by S. Silverman, American Cyanamid Co., Pearl River, N.Y. Calcofluor resistance was tested by a plate assay as previously described (61).

Yeast strains were mated quantitatively according to the following procedure. A total of 2×10^7 log-phase cells from each parental strain carrying complementary auxotrophic markers were mixed in 5 ml of YEPD and incubated for 5 h at 28°C with vigorous shaking in 50-ml flasks. After this period, agglutinated cells were sonicated by a 1-s pulse at an intensity of 0.5 with a MSE Soniprep, model 150. Finally, appropriate dilutions of mating mixtures were plated on YEPD and selective-medium plates. Some experiments involving the wild type and the *chs5* and *chs3* mutants were also performed on solid medium at different cell densities, as described previously (16). The effect of Calcofluor on mating was determined as follows. Mixed complementary strains were grown for 1.5 h. The fluorochrome was then added to a final concentration of 50 μg per ml, and incubation was continued for 3.5 h. In all cases, the frequency of diploid formation was estimated as the number of diploids (colonies on selective medium) formed per the total number of cells (colonies on YEPD). Each datum is the average of at least three independent experiments in which at least 100 diploids were recovered, except in those cases where the yield was lower than 10% of the control value. Cytoduction tests were carried out as described previously (31). L839 [*rho*^{0}] and YB25 [*rho*^{0}] strains were produced by treatment with 10 μg of ethidium bromide per ml (29). Lack of respiration-competent mitochondria was confirmed by testing the ability to grow on media with glycerol as the carbon source. For sporulation analysis, cells were treated as described previously (50). Ascus formation was detected by phase-contrast microscopy.

Synchronization of cultures and treatment with α-factor. For synchronization of cultures in G_1, cells were incubated for 3 days at 28°C in 500 ml of YEPD medium, followed by centrifugation at $350 \times g$ for 4 min. The pellet was discarded, and the supernatant was centrifuged at $4{,}000 \times g$ for 5 min in order to collect the small unbudded cells. Cells were resuspended in 100 ml of fresh YEPD medium and incubated for 2 h at 28°C. α-Factor (kindly provided by C. Wittenberg, Scripps Clinic and Research Foundation, La Jolla, Calif.) was added to a final concentration of 200 ng per ml. Cells were incubated for another 2 h and finally collected by centrifugation. Under these conditions, at least 80% of the treated cells had begun to form shmoos, while untreated cells remained unbudded.

Recombinant DNA manipulations. Transformation of *E. coli*, plasmid preparation, restriction mapping, DNA ligations, Southern blotting, and other DNA manipulations were performed by standard techniques (48). Transformation of yeast cells was carried out by the lithium acetate procedure (32). Yeast genomic DNA for Southern blots was prepared as described previously (58). DNA fragments to be used as probes were labeled by a random priming procedure with Klenow enzyme and purified on Sepharose CL-6B columns (28).

Plasmid and strain constructions. Gene replacement was performed as described previously (46, 47). The *chs5*Δ*1* disruption was constructed by replacing the 2.5-kb *Sal*I-*Bgl*II fragment containing the *CHS5* coding region (from nucleotide −182 to nucleotide 340 downstream of the stop codon) with the *LEU2* gene so that plasmid pSR14 was generated. By digestion of pSR14 with *Sph*I and *Xba*I, a 3.1-kb fragment containing the *LEU2* gene marker and the flanking regions of *CHS5* was prepared. This was used to transform strain L839, giving rise to strain YB25; correct substitution at the *CHS5* locus was determined by Southern gel blot analysis and a test of Calcofluor resistance. The *chs5*Δ*1* construct was also transformed into a diploid strain (YPA17), and the resulting *chs5*Δ*1*/*CHS5* heterozygote was sporulated. Tetrad analysis revealed 2:2 segregation of the *LEU2* marker; the marker always cosegregated with Calcofluor resistance. The *chs5*Δ*2* disruption was constructed by replacing the 2-kb *Sal*I-*Stu*I fragment, containing the *CHS5* coding region minus the last 55 codons corresponding to the carboxy-terminal region, with the *ADE2* gene so that pSR19 was produced. By digestion of pSR19 with *Sph*I and *Bgl*II, a 2.8-kb fragment, containing the *ADE2* gene and flanking regions from *CHS5*, was prepared. This was used to transform strain α131-20, generating strain YB26. The *chs3*Δ*1* disruption was constructed by using pHV8*::URA3*, in which the 2.8-kb *Xho*I-*Kpn*I fragment, containing the *CHS3* open reading frame minus the last 230 codons corresponding to the carboxy-terminal region, was replaced by *URA3*. A 4.2-kb *Cla*I-*Pvu*II fragment from this plasmid was used to transform strains L839, YB25, α131-20, and YB26, giving rise to YB31, YB33, YB32, and YB34, respectively. In this fashion, a set of *chs3*, *chs5*, and *chs3 chs5* isogenic strains was constructed. *chs3*Δ*2* was constructed by ligating a *Sal*I-*Kpn*I DNA fragment containing the *ADE2* gene into the unique *Xho*I and *Kpn*I sites of *CHS3*, generating plasmid pHV139; a 4.7-kb *Cla*I-*Pvu*II fragment was used to transform α131-20, giving rise to HVY246. *chs3*Δ*3* was constructed by replacing the DNA sequence between nucleotide −76 (*Xho*I site) and nucleotide 2314 (*Pst*I site), leaving 393 codons corresponding to the carboxy-terminal region of the *CHS3* product, with a 3.5-kb *Xho*I-*Pst*I fragment containing *LEU2*. This plasmid, pTC4 (23), was digested with *Mlu*I, and the 4.6-kb fragment was used to transform L839, generating strain HVY244. *chs1*Δ

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant characteristics	Source
α131-20	*MAT*α *ade2 ura3 leu1 cyh2 can1*	F. del Rey[b]
L839	*MAT***a** *ura3 his1 leu2*	F. del Rey
ECY18-4B	*MAT***a** *ura3 leu2 trp1 chs1*	E. Cabib[c]
K5-5A	*MAT*α *his4 ade2 can1 kar1*	T. Benitez[d]
HV23	*MAT*α *ura3 leu2 chs5*[a]	Our laboratory
HV23 V-4A	*MAT*α *ura3 leu2 trp1 chs5*	This study
HV23 IV-5A	*MAT***a** *ura3 leu2 trp1 his4 chs1 chs5*	This study
HV23 IV-5B	*MAT***a** *ura3 leu2 trp1 chs1*	This study
YB25	L839 *chs5*Δ*1*	This study
YB26	α131-20, *chs5*Δ*2*	This study
YB27	L839 × α131-20	This study
YB28	L839 × YB26	This study
YB30	YB25 × YB26	This study
YB31	L839 *chs3*Δ*1*	This study
YB32	α131-20 *chs3*Δ*1*	This study
YB33	YB25 *chs3*Δ*1*	This study
YB34	YB26 *chs3*Δ*1*	This study
YB37	YB31 × YB32	This study
YB40	YB33 × YB34	This study
AP1	*MAT***a**/*MAT*α *ade1*/*ADE1 ade2*/*ade2 ura1*/*URA1 his7*/*HIS7 lys2*/*LYS2 tyr1*/*TYR1 gal1*/*GAL1 ura3*/*URA3 cyh2*/*CYH2 can1*/*CAN1 leu1*/*LEU1*	F. del Rey
X2180-2N	*MAT***a**/*MAT*α *SUC2*/*SUC2 mal*/*mal mel*/*mel gal2*/*gal2 CUP1*/*CUP1*	F. del Rey
YPA17	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2*	F. del Rey
L4773	**a** *fus1-1 fus2-3 leu2 ura3*	G. R. Fink[e]
L4769	α *fus1-1 fus2-3 leu2 ura3*	G. R. Fink
HVY269	L839 *chs1*Δ	This study
HVY280	α131-20 *chs1*Δ	This study
HVY244	L839 *chs3*Δ*3*	This study
HVY246	α131-20 *chs3*Δ*2*	This study
HVY270	L839 *chs1*Δ*chs3*Δ*3*	This study
HVY271	α131-20 *chs1*Δ*chs3*Δ*2*	This study
15Daub	**a** *ura3*Δ*ns leu2 ade1 his2 trp1 bar1*Δ	S. I. Reed[f]
CWY271	15Daub *fus3::LEU2*	C. Wittenberg[f]
HVY260	15Daub *chs5*Δ*3*	This study
HVY309	15Daub *chs3*Δ*3*	This study
HVY268	15Daub *kss1::URA3*	This study
HVY272	15Daub *fus3::LEU2 kss1::URA3*	This study

[a] The *chs5* mutation was initially designated *cal3* (44).
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[c] National Institutes of Health, Bethesda, Md.
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[e] Whitehead Institute.
[f] Scripps Clinic and Research Foundation.

was constructed as follows: the 1.2-kb *Hin*dIII *URA3* fragment was cloned between a *Hin*dIII site, created by PCR at nucleotide +227, and the site at nucleotide +2720 of *CHS1*, generating pHV149. In this way, the *CHS1* open reading frame, except for the first 75 and the last 275 codons, was replaced by *URA3*. Digestion of plasmid pHV149 with *Bam*HI and *Bgl*II gave rise to a 2.4-kb fragment that was used to transform L839, α131-20, HVY244, and HVY246 to obtain HVY269, HVY280, HVY270, and HVY271, respectively. In this way, a set of *chs1*, *chs3*, and *chs1 chs3* isogenic strains was produced. The *chs5Δ3* disruption was constructed by cloning the *ADE1* gene, as a *Kpn*I-*Spe*I fragment, into the corresponding sites of *CHS5*, generating plasmid pHV143; the *CHS5* open reading frame, except for the first 73 codons, was removed in that way. This plasmid was digested with *Ava*II, and the 2.9-kb fragment containing the disruption was used to transform strain 15Daub, generating strain HVY260. A 15Daub *chs3Δ3* strain (HVY309) was constructed with the 4.6-kb *Mlu*I fragment from plasmid pTC4 (see above). pBC65 (22) was used as a source for a *kss1::URA3* disruption. 15Daub and CWY271 were transformed with the *Eco*RI-*Sph*I fragment that carried the *KSS1* disruption, generating HVY268 and HVY272. pBC65 was kindly provided by C. Wittenberg. In this fashion, a set of *bar1 chs5*, *bar1 chs3*, *bar1 fus3*, *bar1 kss1*, and *bar1 fus3 kss1* isogenic strains was produced.

Plasmids containing *FUS1* or *FUS2* in YCp50 were kindly supplied by G. Fink, Whitehead Institute, Boston, Mass. pHV151 was constructed by cloning *FUS2* as an *Eco*RI-*Kpn*I fragment into the corresponding sites of the polylinker of vector YEp352. *FUS1* was then introduced into this plasmid as a *Xho*I-*Xba*I fragment. pHV151 was used to transform strains L839, α131-20, YB25, and YB26. The pHV155 plasmid was obtained by cloning *FUS3*, as an *Eco*RI-*Bam*HI fragment, into the corresponding sites of the polylinker of YEp352 and used to transform strains YB25 and YB26.

RNA analysis, DNA sequencing, and *CHS5* mapping. RNA was prepared by a slight modification of a procedure described previously (42). Poly(A)$^+$ RNA was purified by affinity chromatography with oligo(dT)-cellulose (2), and Northern blot analysis was performed exactly as described previously (17). The probe for *CHS5* was the 3-kb *Sal*I-*Xba*I fragment of plasmid pSR6 (see Fig. 1A). Primer extension analysis was carried out essentially as described previously (48) with a synthetic oligonucleotide (5′-CGCCAATGAGGCATCCAACTTACCTAC-3′) complementary to nucleotide positions +28 to +54 of the *CHS5* coding sequence. The entire sequence of both strands of *CHS5* was determined by the dideoxy method of Sanger et al. (49) with single-stranded DNA purified as described previously (48). Separation of *S. cerevisiae* chromosomes was achieved by alternating-field gel electrophoresis (19) with a Bio-Rad CHEF DR-II system. *CHS5* was located on chromosome XII by using a 3-kb *Sal*I-*Xba*I *CHS5* probe to hybridize a yeast chromosomal blot from *S. cerevisiae* YNN295. Precise mapping of *CHS5* was achieved by using a digoxigenin-labeled *CHS5* probe against a collection of overlapping recombinant clones containing inserts from the yeast genome (gene mapping kit ATCC 77284). *CHS5* hybridized to lambda clones 70036, 70235, and 70313, which contain overlapping inserts derived from the right arm of chromosome XII, close to *CDC3*.

Other methods. Cell extracts were prepared and CSIII activity assays were performed essentially as reported previously (17) under conditions where only this activity was measured. The amount of chitin was determined in at least three independent experiments as described previously (10). Protein amounts were measured by the method of Lowry et al. (39). Light microscopy was performed with a Zeiss Axiophot microscope by phase-contrast or differential-interference-contrast microscopy. Calcofluor staining was performed as described previously (43). For Hoechst staining, cells were fixed in 3.7% formaldehyde for 40 min at room temperature and then mounted in 70% glycerol with 2% *n*-propyl gallate and 0.25 μg of Hoechst 33258 solution per ml. Fluorescence microscopy was performed with a Zeiss Axiophot microscope. Cells were prepared for electron microscopy as follows. Cells were fixed first in glutaraldehyde and then in OsO_4. After dehydration in graded acetone solutions, samples were embedded in Spurr resin (EMSCOPE, Ashford, Kent, England). Thin sections were stained with aqueous solutions of uranyl acetate and lead citrate and then observed in a Zeiss EM-902 microscope.

Nucleotide sequence accession number. The nucleotide sequence of the DNA fragment cloned in pSR6 is available from EMBL and GenBank under accession no. Z49198.

RESULTS

Cloning of *CHS5*. *CHS5* was cloned by complementation of the Calcofluor resistance phenotype of a *chs5* mutant previously named *cal3* (11, 44). A Calcofluor-resistant, *chs5 ura3 leu2* strain (HV23) was transformed with a yeast genomic library constructed in the centromere plasmid YCp50, which carries the *URA3* marker (45). A single transformant in which simultaneous loss of uracil prototrophy and sensitivity to Calcofluor were observed after growth under nonselective conditions was isolated. DNA from this transformant was amplified in *E. coli*, and a single plasmid (pSR1) (Fig. 1A) was isolated and used again to transform the original mutant strain. The

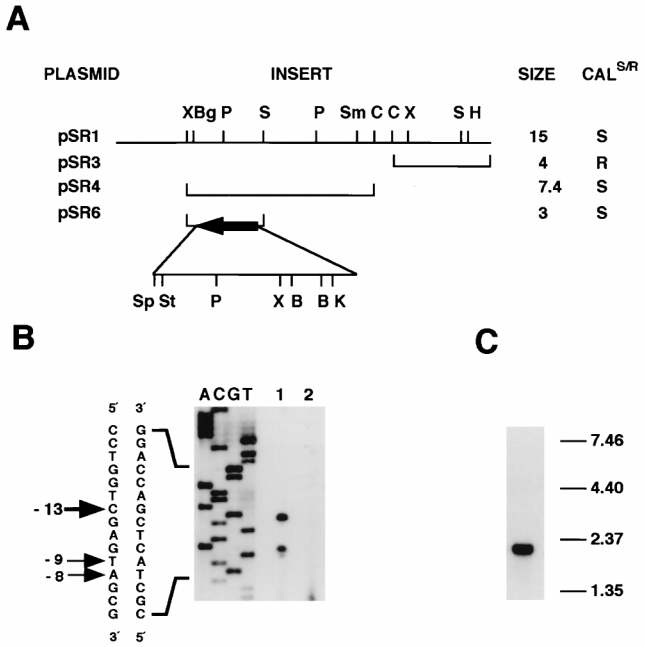

FIG. 1. (A) Restriction map and subcloning diagram of the DNA region containing the *CHS5* open reading frame. The arrow indicates the position of *CHS5* and the direction of transcription. Relevant restriction sites are depicted. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Spe*I; St, *Stu*I; X, *Xba*I. Sizes are given in kilobases. The sensitivity (S) or resistance (R) to Calcofluor (Cal) of the corresponding transformant is indicated. Plasmids were constructed in vector YCp50. (B) Mapping of transcription initiation sites by primer extension. A synthetic oligonucleotide complementary to the sense strand of *CHS5* (see Materials and Methods) was labeled at the 5′ end and annealed at 30°C to 8 μg of poly(A)$^+$ mRNA from *S. cerevisiae* X2180-2N exponentially growing cells. The primer was elongated with reverse transcriptase and the extended products (lane 1) were resolved in a sequencing gel next to a sequencing ladder of the noncoding strand (lanes A, C, G, and T); results of a control reaction, in which poly(A)$^+$ mRNA was replaced by yeast tRNA, are shown in lane 2. (C) Northern blot. The 3-kb *Sal*I-*Xba*I fragment of pSR6 was used as the probe. Positions and sizes (kilobases) of the RNA markers are indicated on the right.

15-kb DNA insert cloned in pSR1 was subcloned, and the minimal sequence necessary for restoring sensitivity to Calcofluor was localized to the 3-kb *Sal*I-*Xba*I insert of pSR6 (Fig. 1A). Data presented below indicate that the cloned DNA fragment corresponds to the *CHS5* locus.

The *chs5* phenotype is pleiotropic, including resistance to Calcofluor, absence of thick septa in the presence of Calcofluor (44), and reduction of cell wall chitin content to about one-fourth of the wild-type value. This reduction of chitin synthesis was specifically ascribed to a defect in CSIII activity (Table 2). The availability of a plasmid containing the coding sequence of *CHS5* allowed us to test whether all these defects were due to a single mutation. Indeed, transformation of strain HV23 IV-5A (*chs1 chs5*) with a centromere plasmid carrying *CHS5* (pSR6) restored all the wild-type characteristics (Table 2).

Disruption of *CHS5*. A *chs5* null allele, *chs5Δ1*, was constructed in which *CHS5* coding sequences were replaced by *LEU2*. A DNA fragment containing this allele was transformed into a diploid strain to replace one copy of *CHS5* by *chs5Δ1* (see Materials and Methods). The resulting heterozygote was sporulated; four viable progeny were produced, and the *LEU2* marker segregated 2:2. Thus, *CHS5* is not essential for *S. cerevisiae* vegetative growth. Crosses between a haploid *chs5Δ1* strain and a wild type demonstrated that leucine pro-

TABLE 2. Characterization of *chs5* mutants and effects of transformation with a centromeric plasmid carrying *CHS5*

Strain	Plasmid	Calcofluor resistance[a]	Thick septa[b]	Chitin (%)[c]	Activity (10^{-2} mU/mg of protein)[d]	
					CSIII	CSII
HV23 IV-5B (*chs1 CHS5*)		−	+	100	3.4	7.4
HV23 IV-5A (*chs1 chs5*)	Vector	+	−	29	0.2	8.7
HV23 IV-5A	pSR6	−	+	103	2.5	10.0
L839 (*CHS5*)		−	+	100	ND	ND
YB25 (*chs5Δ1*)	Vector	+	−	25	ND	ND
YB25	pSR6	−	+	88	ND	ND

[a] Ability (+) or inability (−) to grow in the presence of 1-mg/ml Calcofluor (plate assay).
[b] Presence (+) or absence (−) of anomalous thick septa between mother and daughter cells during growth in the presence of 50-μg/ml Calcofluor.
[c] Level of chitin as a percentage of the level present in the wild type.
[d] Chitin synthase activities were measured as described previously (17). CSIII values represent activity without trypsin treatment. ND, not determined.

totrophy and Calcofluor resistance cosegregated in the 10 tetrads that were analyzed; thus, *chs5Δ1* (null) strains are resistant to Calcofluor. Finally, to determine if the correct gene had been cloned, strains YB25 (*chs5Δ1*) and HV23 (*chs5*) were mated. This produced a diploid resistant to Calcofluor, and after sporulation, each of the 13 tetrads analyzed produced four Calcofluor-resistant colonies. Therefore, the cloned gene corresponds to the *CHS5* locus. Analysis of *chs5Δ1* cells revealed that the phenotypes of the null strains were very similar to those of the original *chs5* mutant (i.e., the level of cell wall chitin was reduced to 25% of that in the isogenic wild-type strain, and CSIII activity was specifically absent). *chs5Δ1* cells carrying plasmid pSR6 have wild-type levels of chitin and are able to form thick septa when grown in the presence of Calcofluor (Table 2).

***CHS5* encodes a unique open reading frame.** Sequence analysis of the DNA fragment cloned in pSR6 revealed a single open reading frame of 671 codons that encodes a protein with a predicted molecular mass of 73,642 Da (Fig. 2). The nucleotide sequence data are available from EMBL GenBank (see above) and correspond exactly to the YLR330w sequence, as recently released by the Yeast Genome Sequencing Project. To confirm that this open reading frame was *CHS5*, an internal *Bam*HI-*Bam*HI fragment was removed from plasmid pSR6 (Fig. 1) and then religated so that the *CHS5* pieces were put back in frame. This produced a truncated gene in which 83 internal codons were missing. The resulting plasmid did not complement either the resistance to Calcofluor or the lack of chitin in HV23 (*chs5*) cells (results not shown).

The promoter region of *CHS5* does not contain a perfect match to the consensus TATA element. However, there are two TATA-like sequences at positions −319 and −234 from the predicted initiation ATG. By primer extension, it was determined that the main initiation point for *CHS5* transcription is located at the cytosine residue 13 bases upstream from the predicted start of translation, within the consensus sequence TCGA located at nucleotides −14 to −11 (Fig. 1B). Two of the minor initiation points for *CHS5* transcription were located at nucleotides −9 and −8. These initiation points define presumptive 5′ nontranslated leader sequences smaller than those normally found in *S. cerevisiae* mRNAs (20 to 60 nucleotides).

A

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TTTAGCTTGCATGTTACGTTTCCGTTTTAGAACCTGGTCGAGTAGCGAATAATG
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B

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  1  M S S V D V L L T V G K L D A S L A L L T T Q D H
 26  H V I E F P T V L L P E N V K A G S I I K M Q V S
 51  Q N L E E E K K Q R N H F K S I Q A K I L E K Y G
 76  T H K P E S P V L K I V N V T Q T S C V L A W D P
101  L K L G S A K L K S L I L Y R K G I R S M V I P N
126  P F K V T T T K I S G L S V D T P Y E F Q L K L I
151  T T S G T L W S E K V I L R T H K M T D M S G I T
176  V C L G P L D P L K E I S D L Q I S Q C L S H I G
201  A R P L Q R H V A I D T T H F V C N D L D N E E S
226  N E E L I R A K H N N I P I V R P E W V R A C E V
251  E K R I V G V R G F Y L D A D Q S I L K N Y T F P
276  P V N E E E L S Y S K E N E P V A E V A D E N K M
301  P E D T T D V E Q V A S P N D N E S N P S E A K E
326  Q G E K S G H E T A P V S P V E D P L H A S T A L
351  E N E T T I E T V N P S V R S L K S E P V G T P N
376  I E E N K A D S S A E A V V E E P N E A V A E S S
401  P N E E A T G Q K S E D T D T H S N E Q A D N G F
426  V Q T E E V A E N N I T T E S A G E N N E P A D D
451  A A M E F G R P E A E I E T P E V N E S I E D A N
476  E P A E D S N E P V E D S N K P V K D S N K P V E
501  D S N K P V E D S N K P V E D S N K P V E D A N E
526  P V E D T S E P V E D A G E P V Q E T N E F T T D
551  I A S P R H Q E E D I E L E A E P K D A T E S V A
576  V E P S N E D V K P E E K G S E A E D D I N N V S
601  K E A A S G E S T T H Q K T E A S A S L E S S A V
626  T E E Q E T T E A E V N T D D V L S T K E A K K N
651  T G N S N S N K K K N K K N K K K G K K K
                • • •   • •   • • •   • • •
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FIG. 2. (A) Nucleotide sequence upstream of the start codon of *CHS5*. (B) Predicted amino acid sequence of Chs5p. Heptads are underlined. Lysine residues at the carboxy-terminal end are indicated (•).

No TGAAACA pheromone response elements are found upstream of the *CHS5* open reading frame.

The putative amino acid sequence of Chs5p contains two interesting features (Fig. 2). One is a consecutive repeat (10 times) of the heptapeptide (E/K)DSN(E/K)PV, located between amino acids 472 and 541. The other is a carboxyterminal sequence, KKKNKKNKKKGKKK, very rich in lysine residues, which presumably confers a highly hydrophilic character on that portion of the protein. The hydropathic profile and charge distribution of the amino acid sequence reveal two other domains: an amino-terminal domain (residues 1 to 250), slightly hydrophobic and neutral in charge, and a second domain (residues 250 to 671), hydrophilic and acidic except in the carboxy-terminal end.

Sequence similarity searches of databases showed that the Chs5 protein is unique. The sequence was distinct from those of all of the proteins encoded by chitin synthase genes. Chs5p has neither a presumptive transmembrane domain nor an obvious signal sequence. However, Chs5p shows weak homology to several cytoskeletal proteins, including protein H from human neurofilaments (20% identity and 70% similarity over 401 amino acids) (38), protein H from mouse neurofilaments (33), and human myosin heavy chain (26).

Expression of *CHS5* mRNA. Northern blot analysis of poly(A)$^+$ RNA from vegetative cells, with the 3-kb *Sal*I-*Xba*I fragment of pSR6 as the probe, revealed a single transcript of 2.1 kb corresponding to the *CHS5* gene (Fig. 1C). This size is similar to that predicted from the open reading frame (2,013 bp) and is in agreement with the existence of a short leader sequence in the mRNA, as inferred from the primer extension experiment. In a Northern blot experiment to study the expression of different chitin synthase genes during α-factor treatment, *CHS5* transcription was found to be essentially constant, similar to *CHS3* transcription (Fig. 7 in reference 17 and results not shown). Control experiments that detected *FUS1* or *CHS1* transcripts revealed a significant and transient induction

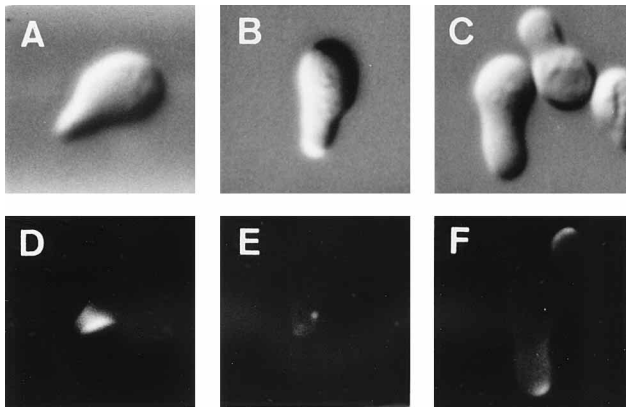

FIG. 3. Micrographs of shmoos from wild-type (15Daub) (A and D), *chs3*Δ*3* (HVY309) (B and E), and *chs5*Δ*3* (HVY260) (C and F) strains. **a** cells from the different strains were incubated with α-factor (200 ng/ml) for 2.5 h and observed by interference-contrast microscopy (A to C) or fluorescence microscopy (Calcofluor staining) (D to F). In both wild-type and mutant strains the frequency of shmoo formation was approximately 90%. Shmoo projections in more than 90% of both *chs3* and *chs5* mutants are broader and rounder than those in the wild-type strain. Calcofluor staining is reduced in all *chs3*Δ*3* and *chs5*Δ*3* shmoos. Due to the intensity of fluorescence at the bases of wild-type shmoos (C), the fluorescence at the tips of the shmoos cannot be seen in the picture. The times of exposure for micrographs E and F were, respectively, eight and four times longer than that for micrograph D; other photographic conditions were identical in all cases.

TABLE 3. Synthesis of chitin in response to α-factor treatment in different *S. cerevisiae* strains

Expt[a] and strain (relevant genotype)	α-Factor[b]	Amt of cell wall chitin[c] (% of wild-type level)	Fold increase
A			
15Daub (*bar1*Δ)	−	0.187 (100.0)	
	+	0.388 (207.5)	2.1
HVY309 (*bar1 chs3*Δ*3*)	−	0.023 (12.5)	
	+	0.030 (16.3)	1.3
HVY260 (*bar1*Δ *chs5*Δ*3*)	−	0.042 (22.6)	
	+	0.128 (68.5)	3.0
B			
CWY271 (*bar1*Δ *fus3*)	−	0.200 (108)	
	+	0.420 (227)	2.1
HVY268 (*bar1*Δ *kss1*)	−	0.182 (97)	
	+	0.363 (194)	2.0
HVY272 (*bar1*Δ *fus3 kss1*)	−	0.116 (62)	
	+	0.131 (70)	1.1

[a] In experiment A, cells were collected after 2 h of incubation with the pheromone. In experiment B, cells were arrested in G_1 by growing them to stationary phase. They were then refed and treated with α-factor for 2 h (see Materials and Methods).

[b] α-factor was added to a final concentration of 200 ng/ml.

[c] Values are micromoles of GlcNAc per 10^9 cells. Chitin was measured as described previously (10).

of these messages (17). The level of *CHS5* transcript in mRNA from cells that had been incubated for 10 h in sporulation medium was also examined; the level of *CHS5* mRNA did not change relative to the level of *CHS5* mRNA from cells grown in rich medium (results not shown). In contrast, *CHS3* mRNA levels are induced approximately sevenfold relative to the level in vegetative cells (Fig. 7 in reference 17).

Role of *CHS5* in mating. Since CSIII is responsible for chitin synthesis during mating, we analyzed the role of *CHS5* in this process. *MAT***a** strains carrying a *chs5*Δ*3* or *chs3*Δ*3* disruption were constructed in a *bar1*Δ strain to enhance the response to the α-factor pheromone. Treatment with this pheromone mimics the initial steps of mating and promotes the production of shmoos, during which synthesis of cell wall chitin is activated and a projection grows from one edge of the cell (52). In both wild-type and mutant strains, the frequency of shmoo formation was approximately 90%. However, in more than 90% of *chs5*Δ*3* and *chs3*Δ*3* shmoos, the projections were much broader and rounder than their wild-type counterparts (Fig. 3A to C). Approximately 50% of *chs5* shmoos exhibited a "peanut" morphology similar to that described for *spa2* and *pea2* mutants, which are defective in shmoo morphology and mating (16).

Chitin synthesis and staining was also examined in the wild-type, *chs3*Δ*3*, and *chs5*Δ*3* strains. In the wild type, fluorescence was mainly located at the base of the shmoo, even though some fluorescence can also be observed, under the microscope, at the tip of the projection. As expected from previous results (61), induction of chitin synthesis after α-factor treatment was almost absent in the *chs3*Δ*3* strain (Table 3; Fig. 3E). In the *chs5*Δ*3* strain, the Calcofluor fluorescence at the base of the shmoo projection was reduced compared to that in the wild type (Fig. 3D and F). However, the pheromone-stimulated increase in chitin content in *chs5*Δ*3* shmoos was proportionally as large as that in the isogenic wild-type strain (Table 3).

The mating efficiency of *chs5* null mutants was examined by a liquid-medium (see Materials and Methods) assay that allowed us to obtain quantitative and reproducible results. When only one of the parent strains carried a *chs5* null allele, mating efficiency was approximately 40% of the wild-type value. However, when the *chs5* null allele was present in both strains, the mating efficiency decreased to 15% of the wild-type value (Table 4). The mating efficiency of *chs5* cells was restored to wild-type levels when plasmid pSR4 or pSR6, both of which contain *CHS5*, was introduced into these strains (Table 5). Transformation of these strains with pSR12 (pSR6 lacking the *Bam*HI-*Bam*HI internal fragment of *CHS5*) did not complement the mating defect (results not shown), indicating that the defect is indeed due to the absence of *CHS5* function. Mating tests were conducted at high or low cell densities and in liquid or solid medium (see Materials and Methods). The percentage of mating, relative to the wild-type control, was the same under all conditions tested (results not shown).

Chitin synthesis is required for a normal rate of mating. Since *chs5*Δ cells are defective in mating and have reduced chitin levels in shmoos, we investigated whether the loss of other genes involved in chitin synthesis affects the conjugation process. Previous studies have shown that induction of chitin synthesis by α-factor does not occur in *chs3*Δ cells (44). However, it remained unclear whether chitin synthesis, and specifically the action of the *CHS1* and *CHS3* gene products, has a function in mating. Therefore, the mating efficiencies of isogenic *chs1*Δ, *chs3*Δ, and *chs1*Δ *chs3*Δ strains were measured (Table 4). Analysis of *chs1*Δ cells revealed that *CHS1* is not required for mating in either rich medium or minimal medium (minimal-medium results not shown). However, the loss of *CHS3* reduced mating by 50%. The defect in mating in *chs3*Δ mutants was not significantly reduced by the additional disruption of *CHS1.*

chs1 mutants exhibit a partial lytic phenotype when grown in

TABLE 4. Diploid formation in isogenic *S. cerevisiae* strains defective in different chitin synthesis genes, and effect of Calcofluor on diploid formation[a]

Cross	Calcofluor	No. of colonies scored in medium		Ratio (%)[b]
		YEPD	Minimal	
L839 × α131-20 (wt × wt)	−	3,838	971	25.3 (100)
	+	1,890	30	1.6 (6.5)
HVY269 × HVY280 (*chs1* × *chs1*)	−	3,226	1,019	31.6 (125)
HVY244 × HVY246 (*chs3* × *chs3*)	−	3,840	457	11.9 (47)
	+	3,918	286	7.3 (29)
HVY270 × HVY271 (*chs1 chs3* × *chs1 chs3*)	−	3,684	374	10.1 (40)
YB25 × YB26 (*chs5* × *chs5*)	−	8,052	306	3.8 (15)
YB33 × YB34 (*chs3 chs5* × *chs3 chs5*)	−	3,250	140	4.3 (17)

[a] Calcofluor was added to mating mixtures to a final concentration of 50 μg/ml (see Materials and Methods). The actual, directly comparable numbers of colonies scored are included in the appropriate lines so that the effect of Calcofluor can be directly observed. wt, wild type.

[b] Ratio of the number of colonies scored in minimal medium to the number scored in YEPD medium, multiplied by 100. The results are from a representative experiment. The numbers in parentheses are the frequencies of diploid formation as percentages of the value for wild-type strains in the absence of Calcofluor.

TABLE 5. Complementation of the *chs5* mating defect by different genes

Cross[a]	Plasmid type[b]	% Diploids (mean ± SD)[c]	Shmoo fluorescence[d]
L839 × α131-20 (wt × wt)	M (vector)	100	+
	C (*FUS1*)	87 ± 4	+
	C (*FUS2*)	98 ± 3	+
	M (*FUS1 FUS2*)	83 ± 4	+
YB25 × YB26 (*chs5* × *chs5*)	M (vector)	15 ± 2	±
	C (*CHS5*)	116 ± 2	+
	M (*FUS3*)	7 ± 0.3	±
	C (*FUS1*)	39 ± 3	±
	C (*FUS2*)	35 ± 3	±
	M (*FUS1 FUS2*)	48 ± 2	±
L4773 × L4769 (*fus1 fus2* × *fus1 fus2*)	None	<1[e]	+
	C or M (*CHS5*)	<1[e]	+
L4773 × α131-20 (*fus1 fus2* × wt)	None	18 ± 2	
L4773 × YB26 (*fus1 fus2* × *chs5*)	None	0.2 ± 0.04	

[a] wt, wild type.

[b] C, centromeric; M, multicopy. The plasmids used were pSR6 or pSR4 (*CHS5*), pSB245 (*FUS1*) (59), pSB265 (*FUS2*) (59), pHV151 (*FUS1 FUS2*), and pHV155 (*FUS3*).

[c] Values are averages for at least three experiments.

[d] Level of shmoo fluorescence (+, wild-type level; ±, *chs5* mutant level) observed after Calcofluor staining.

[e] No sporulating clones were detected among the colonies recovered on YEPD after the mating mixtures were plated.

minimal medium, but this defect is strain dependent (14). In the case of the HVY269 × HVY280 cross, only strain HVY280 showed such a lytic phenotype. Therefore, in order to further analyze the role of *CHS1* in mating, the efficiency of zygote formation was quantified in a cross in which both *chs1* strains showed such a lytic phenotype (i.e., ECY18-4B × HVY280). Neither the efficiency of zygote formation nor the ratio of mature zygotes to prezygotes was significantly different from that found in the isogenic wild-type cross (Table 6).

Calcofluor has been shown to interfere with chitin synthesis during the yeast vegetative cycle (43); however, its effect on mating has not been reported. Wild-type and isogenic *chs3*Δ strains were mated in the presence or absence of a sublethal concentration of Calcofluor (see Materials and Methods), and the mating efficiencies were measured. As indicated in Table 4, for wild-type cells, Calcofluor had a modest effect on vegetative growth (as assayed by colonies grown on YEPD) but had a drastic effect on diploid formation (6.5% that for the wild type in the absence of Calcofluor). As expected, the relative effect of Calcofluor on the mating efficiency of *chs3*Δ strains (less than twofold inhibition) was considerably smaller than its effect on the mating of wild-type cells.

Chitin synthesis in response to α-factor requires either *FUS3* or *KSS1* genes. We investigated how synthesis of chitin is regulated by the genes involved in the pheromone signal transduction pathway. In *S. cerevisiae*, transduction of this signal occurs through a mitogen-activated protein (MAP) kinase cascade that results in the expression of several genes involved in the transduction pathway itself, in shmoo formation, and in cell fusion (for reviews, see references 26 and 58). *FUS3* and *KSS1* are redundant MAP kinases for transduction of the α-factor signal. A *fus3 kss1* double mutant is α-factor resistant because it does not transduce the signal. However, each one of these genes plays a specific role in the mating process. *FUS3* function is essential in order for the cells to arrest in the G_1 phase of the cell cycle, and *KSS1* is required for desensitization (22, 27). We analyzed chitin synthesis in *fus3*Δ, *kss1*Δ, and *fus3*Δ *kss1*Δ cells which had been synchronized in G_1, by being grown to stationary phase and refed (see Materials and Methods), and then treated with the pheromone. Unlike wild-type, *kss1*Δ, or *fus3*Δ cells, which exhibit a twofold increase in chitin levels after pheromone treatment, *fus3*Δ *kss1*Δ double mutant cells treated in a similar fashion did not exhibit an increase in chitin (Table 3).

***CHS5* is involved in cell fusion.** Since conjugation is less efficient in *chs5*Δ than in *chs3*Δ mutants, we decided to characterize further the mating defects of these mutants in order to elucidate which step(s) of the mating process was affected. We first determined, by phase-contrast microscopy, the percentage of mating structures (bilobated plus trilobated zygotes) formed in liquid medium relative to the total number of cells. As shown in Table 6, these values for *chs3*Δ, *chs5*Δ, and *chs3*Δ *chs5*Δ mutants were approximately 60% those for the wild type and the *chs1*Δ mutant. However, observation of *chs5*Δ and *chs3*Δ *chs5*Δ mating mixtures revealed that most of the mating partners were in close contact but still had cell walls separating them (Fig. 4). As shown in Table 6, the ratio of trilobated to bilobated zygotes in strains carrying a *chs5*Δ mutation is reduced to approximately one-fifth that for *chs1*, *chs3*, or wild-type cells, indicating that the *chs* mutant cells have a defect in the ability to form mature zygotes. The results did not change even when the time of mating was increased to 24 h.

It was important to ascertain if the cell pairs (bilobated structures) could be considered prezygotes as defined by Trueheart et al. (59), i.e., if the cell wall that lies between the pair of cells fails to be degraded so that the nuclei do not fuse and remain located on opposite sides of the structure. Hoechst staining showed that 88% of the bilobated zygotes from

TABLE 6. Efficiency of zygote formation in different *S. cerevisiae chs* mutants[a]

Cross[b]	Mean zygote formation ± SD (%)[c]	% (mean ± SD)		Ratio[d]
		Mature zygotes	Pre-zygotes	
L839 × α 131-20 (wt × wt)	25 ± 2 (100)	61 ± 5	39 ± 5	1.5
ECY18-4B × HVY280[e] (*chs1* × *chs1*)	23 ± 2 (92)	62 ± 3	38 ± 3	1.6
HVY244 × HVY246 (*chs3* × *chs3*)	16 ± 1 (64)	51 ± 1	49 ± 1	1.1
YB25 × YB26 (*chs5* × *chs5*)	15 ± 0.5 (60)	16 ± 2	84 ± 2	0.2
YB25 (pHV151) × YB26 (pHV151)[f]	17 ± 1 (62)	50 ± 1	50 ± 1	1.0
YB33 × YB34 (*chs3 chs5* × *chs3 chs5*)	17 ± 1 (62)	21 ± 2	79 ± 2	0.3

[a] All counts were performed after the mating mixtures were incubated for 5 h.
[b] wt, wild type.
[c] Values are the ratios of the number of bilobated plus trilobated structures to the total number of cells, multiplied by 100. Values are averages for at least three experiments. In all cases, a minimum of 1,000 cells was counted in each experiment. Numbers in parentheses are the percentages of the values obtained for the wild-type strains.
[d] Values are the ratios of the number of trilobated to the number of bilobated structures. In all cases, a minimum of 100 zygotes was counted in each experiment.
[e] Strains that manifest the *chs1* phenotype (13) were grown and mated in minimal medium. The values are given relative to those for the corresponding isogenic wild-type strains.
[f] pHV151 is a multicopy plasmid containing *FUS1* and *FUS2* (see Materials and Methods).

TABLE 7. Frequency of cytoduction in *chs5* crosses

*MAT***a** ([*rho*^{0}]) × *MAT*α ([*rho*$^+$])	Diploids (%)[a]	Cytoductants (%)[b]	Cytoductants/ diploids
L839 (wt[c]) × α131-20 (wt)	100	0	0
L839 (wt) × YB26 (*chs5*Δ*2*)	30	ND[d]	ND
L839 (wt) × K5-5A (*kar1*)	4	7	1.6
YB25 (*chs5*Δ*1*) × α131-20 (wt)	3	0	0
YB25 (*chs5*Δ*1*) × YB26 (*chs5*Δ*2*)	0.4	0	0
YB25 (*chs5*Δ*1*) × K5-5A (*kar1*)	0.14	0.5	3.5

[a] Colonies produced on uracil-supplemented minimal medium plates with glycerol as the carbon source.
[b] The number of cytoductants was obtained as the difference between the number of colonies produced on minimal medium plates with glycerol as the carbon source, supplemented with uracil, leucine (when L839 strain was involved in the cross), and histidine (diploids plus cytoductants), and the number of colonies grown on plates supplemented with only uracil (diploids).
[c] wt, wild type.
[d] ND, not determined.

*chs5*Δ*1* × *chs5*Δ*2* crosses were binucleate, with each nucleus located in its respective half of the zygote (Fig. 4A and B), and only 11% were uninucleate. By contrast, in crosses with the wild type, 33% of the bilobated structures were binucleate and 66% were uninucleate. Electron micrographs suggested that cell walls from cell pairs failed to degrade at the site of fusion (Fig. 4C).

A genetic test was also used to determine whether *chs5* null mutants could still fuse their cytoplasms properly. If mating cells fuse their cytoplasms but not their nuclei through a partial cell fusion, then the binucleate condition is not maintained in subsequent cell divisions and haploid progeny called cytoductants are produced. A *chs5*Δ × *chs5*Δ mating was performed, and the rate of diploid formation and the cytoductant production frequency were determined. For these studies, one *chs5*Δ strain lacked functional mitochondria (i.e., was [*rho*^{0}]) in order to assess cytoplasmic mixing and cytoductant formation. As shown in Table 7, in addition to a low rate of diploid formation, cytoductants were not observed. Control matings with the *kar1* mutation (21), which unilaterally blocks nuclear fusion but does not prevent cytoplasmic mixing, produced cytoductants at a high frequency. The enhancement of the mating defect when strain YB25 (*chs5*Δ*1*) [*rho*^{0}] was involved in the cross was striking (Table 7). It can be concluded that the mating defect in *chs5*Δ × *chs5*Δ crosses does not occur at the level of nuclear fusion.

***FUS1*, *FUS2*, and *CHS5* perform related functions during mating.** A defect in cell fusion similar to that of *chs5*Δ mutants was described previously for *fus1 fus2* (59) and for *fus3* (24) mutants. *FUS1* and *FUS2* each have a role in the initial steps of zygote formation (25, 59, 60), but their biochemical functions remain unknown. Their expression is dependent on the activation of the pheromone signal transduction pathway involved in pheromone response (57). *FUS3* encodes one of the MAP kinase homologs involved in the transmission of the signal and therefore acts prior to *FUS1* and *FUS2*. In order to investigate whether *CHS5* is functionally related to *FUS1*, *FUS2*, or *FUS3*, we carried out several complementation experiments (Table 5). The presence of centromeric plasmids containing either *FUS1* or *FUS2* improved the mating efficiency of *chs5*Δ cells, but the *CHS5* gene, also in a centromeric plasmid, did not alleviate the strong *fus1 fus2* mating defect. The mating efficiency of a wild-type strain did not improve when it was transformed with the same plasmids.

It has been suggested that *FUS1* and *FUS2* are not sequentially ordered but act in parallel fusion pathways (25). To check if both genes were necessary to suppress the *chs5*Δ mating defect, we transformed a *chs5*Δ strain with a multicopy plasmid carrying both *FUS1* and *FUS2* (pHV151) (see Materials and Methods). Mating efficiency increased to approximately 50% of the wild-type control value. *CHS5* in a multicopy plasmid had no effect on a *fus1 fus2* strain. We also transformed a *chs5*Δ strain with the *FUS3* gene in a multicopy plasmid (pHV155) and observed no improvement in mating efficiency.

fus1 and *fus2* mutants exhibit normal levels of fluorescence at the base of the shmoo projection when they are treated with α-factor and stained with Calcofluor, suggesting that chitin synthesis induced by the pheromone is not affected in these mutants. When *chs5*Δ was transformed with *FUS1* and/or *FUS2* plasmids, or vice versa, and treated with mating pheromone, the chitin level, as visualized by Calcofluor fluorescence, did not change compared to the chitin levels for the respective control strains (Table 5). Moreover, when *chs5*Δ mutants were transformed with *FUS1* and *FUS2*, they formed zygotes (bilobated plus trilobated structures) at 62% of the frequency of zygote formation for wild-type control cells, a value that was similar to those for *chs3*Δ, *chs5*Δ, and *chs3*Δ *chs5*Δ mutants. However, the ratio of trilobated to bilobated forms in these cells rose to 1.0, a value higher than that for the *chs5*Δ mutant and similar to that for the *chs3*Δ mutant.

In order to confirm that *CHS5* is functionally related to *FUS1* and *FUS2*, we analyzed the frequency of diploid formation in *fus1 fus2* × wild type and *fus1 fus2* × *chs5*Δ matings. As shown in Table 5, a *fus1 fus2* × wild type mating produced an 18% frequency of diploid formation, whereas a *fus1 fus2* × *chs5*Δ mating produced only a 0.2% frequency of diploid formation. In addition, no trilobated zygotes could be observed in *fus1 fus2* × *chs5*Δ mating mixtures.

Overexpression of *SPA2*, a gene involved in pheromone-induced morphogenesis and efficient mating (31), did not suppress either shmoo morphology is or the chitin defect in a *chs5*

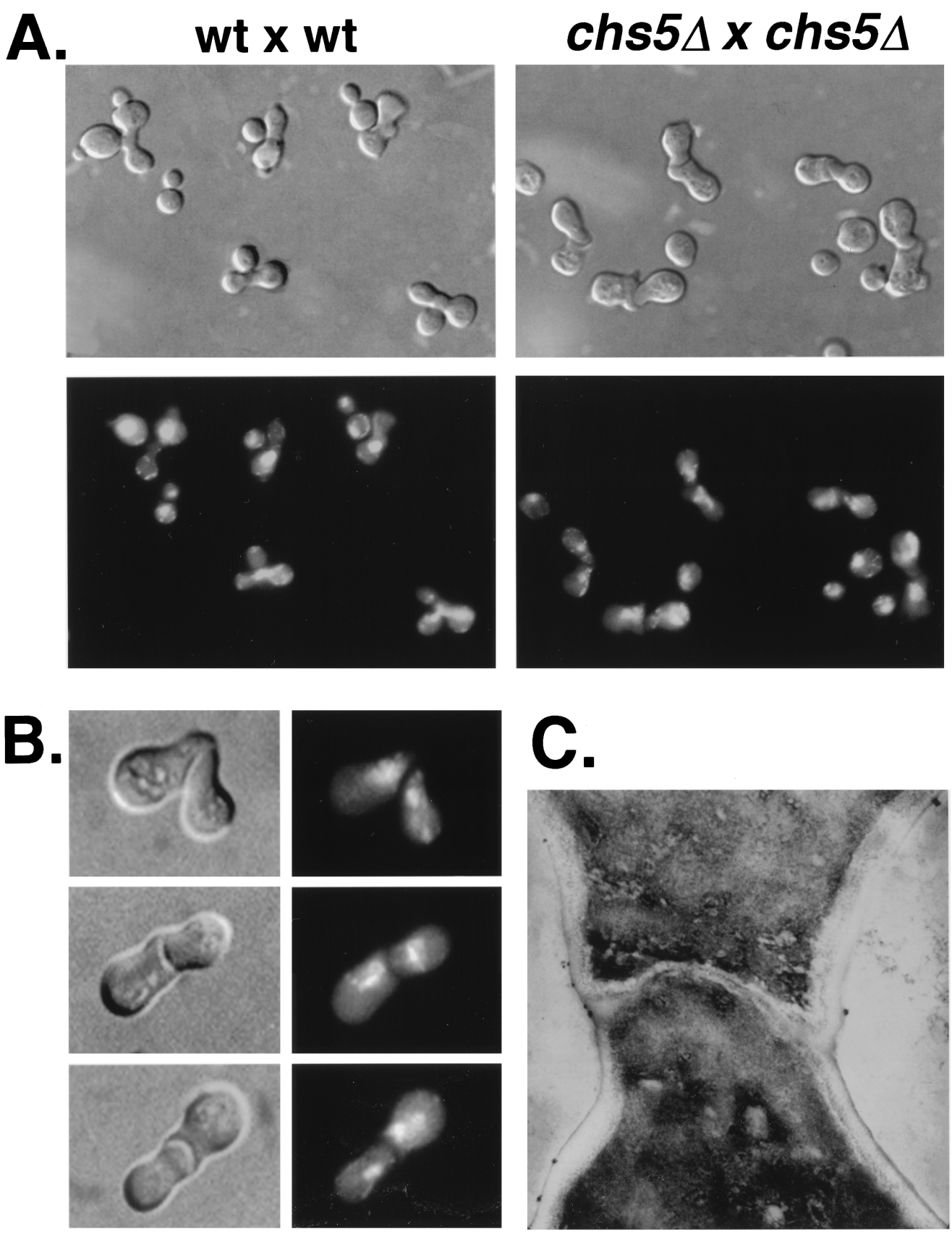

FIG. 4. (A) Differential-interference-contrast and fluorescence (Hoechst staining) micrographs of zygote formation in matings of wild-type (wt) (L839 × α131-20) and isogenic *chs5* null mutant (YB25 × YB26) strains. (B) Phase-contrast and fluorescence (Hoechst staining) micrographs of prezygotes in a mating of *chs5*Δ*3* (YB25 × YB26) strains. All micrographs were taken after 5 h of mating. (C) Electron micrograph of the interparental junction of a YB25 (**a** *chs5*Δ*1*) × YB26 (α *chs5*Δ*2*) prezygote after 5 h of mating (magnification, ×32,000).

null mutant. In addition, *CHS5* overexpression did not suppress the *spa2* defect in shmoo formation (results not shown).

Role of Chs5p in sporulation. In addition to its role in mating, CSIII has also been implicated in the synthesis of the chitosan layer of the ascospore wall (41). As shown by electron-microscopic analysis, ascospores from a *chs3*/*chs3* diploid mutant lack the chitosan layer and the outermost dityrosine-rich layer, which are present in wild-type ascospore walls. These asci are very sensitive to lytic enzymes, as expected for strains with these defects (41). To study the role of *CHS5* in sporulation, isogenic diploid strains carrying heterozygous or homozygous *chs5*Δ disruption alleles (YB28 and YB30, respectively) were tested for their ability to sporulate compared to the isogenic wild-type strain (YB27), in which the sporulation process is fast, synchronous, and efficient (50). *chs5* null diploids were able to sporulate, suggesting that *CHS5* is not essential for this process. There was a delay in ascus formation in the *chs5*Δ/*chs5*Δ culture, because the percentage of asci formed after 24 h was lower (22%) than those in the *chs5*Δ/*CHS5* and *CHS5*/*CHS5* cultures (52 and 56%, respectively). However, after 44 h, the sporulation efficiencies were quite similar: 67, 73, and 74% for *chs5*Δ/*chs5*Δ, *chs5*Δ/*CHS5*, and *CHS5*/*CHS5* cells, respectively. Under the same conditions, homozygous *chs3*Δ/*chs3*Δ (YB37) and *chs3*Δ *chs5*Δ/*chs3*Δ *chs5*Δ (YB40) isogenic diploids also sporulated, but the sporulation efficiency in the case of YB40 decreased to approximately 25%.

By electron microscopy, it was found that ascospores from *chs5*Δ/*chs5*Δ cells (strain YB30) contained outermost layers the same as those described for the wild type, although the distinction between the dityrosine-rich and the chitosan layers was not as clear (results not shown). Under the conditions in which *chs3* ascospores were susceptible to glusulase treatment, *chs5* ascospores were very resistant, similar to wild-type cells (results not shown). Curiously, the fluorescence due to the presence of the ascospore dityrosine-rich layer (6) was not evident in *chs3* and *chs3 chs5* ascospores but was present in *chs5* ascospores. However, the fluorescence intensity was considerably reduced in *chs5*Δ ascospores compared to that in wild-type ascospores (results not shown).

DISCUSSION

Role of *CHS5* in chitin synthesis during the yeast life cycle. We have identified a gene, *CHS5*, required for normal levels of chitin synthesis in *S. cerevisiae*. Mutations in this gene cause resistance to Calcofluor and reduce the amount of cell wall chitin to one-fourth of the control value by specifically interfering with the function of CSIII. This defect has little or no apparent effect on the budding cycle.

The existence of several genes involved in CSIII activity is well established, but little is known about their specific functions (8, 20). Transformation of a wild-type strain or a *chs3 chs5* double mutant with multicopy plasmids carrying *CHS3* and *CHS5* did not increase the level of CSIII activity measured in vitro (results not shown). Perhaps coordinate overexpression of additional components, including *CHS4* (8), is needed to increase CSIII activity. The *chs3*Δ *chs5*Δ double mutant shows a phenotype more similar to that of the *chs3*Δ mutant than to that of the *chs5*Δ mutant, i.e., a stronger reduction in cell wall chitin (10% of the control value) and of chitin restricted to the primary septa observed by wheat germ agglutinin-fluorescein staining (23). This result is in agreement with the hypothesis that Chs3p is the CSIII catalytic component (or a subunit thereof) and that Chs5p plays a regulatory role in CSIII activity. The level of either *CHS3* mRNA or hemagglutinin epitope-tagged Chs3p in a *chs5* disruptant is not different than the level in the wild-type isogenic strain (23). Therefore, Chs5p does not seem to be either a transcriptional or a translational effector of *CHS3*.

Upon incubation with α-factor, chitin synthesis in *chs5*Δ strains is induced to a level which is about one-third of the wild-type value. However, because the percent increase in chitin upon α-factor treatment is similar in *chs5*Δ and wild-type strains, it can be concluded that the reduced chitin level in pheromone-treated cells is not due to a defect in the induction mechanism but rather to a lower basal level. Thus, the mechanism of activation of chitin synthesis during mating does not depend on *CHS5*. Because some chitin synthesis occurs during budding or mating in the absence of Chs5p, it can be concluded that a gene or genes are taking over, at least in part, the function of *CHS5* in chitin synthesis.

Based on electron-microscopic observations, chitosan synthesis in *chs5*Δ ascospores is apparently not affected, although the appearance of the dityrosine-rich–chitosan bilayer is slightly different from its appearance in wild-type ascospores. In contrast, in *chs3*Δ ascospores the bilayer cannot be observed at all (41). In addition, wild-type and *chs5*Δ ascospores are resistant to lytic enzyme treatment, in contrast to *chs3*Δ ascospores. It can be concluded that *CHS5* is not as important as *CHS3* for chitosan synthesis in the spore wall. The natural fluorescence emitted by the dityrosine-rich layer is reduced in *chs5*Δ disruptant ascospores, although not as much as it is in *chs3*Δ ones. Thus, the presence of some chitin probably allows the deposition of some dityrosine-rich components.

Role of chitin in mating. Because chitin synthesis occurs during mating, it was of interest that the lack of *CHS5* in both mating partners reduced mating efficiency. Shmoo morphology is altered in *chs3*Δ and *chs5*Δ null mutants. Is this alteration due to the defect in chitin synthesis? Clearly, genes other than those involved in chitin synthesis are required for normal pheromone-induced cell morphology (16, 31, 57), but we suggest that misshapen shmoos in *chs3*Δ mutants are a consequence of the defect in chitin synthesis.

Could the *chs5*Δ mating defect be attributed to the reduction in chitin level, or is another function affected? To answer this question, the first problem addressed was the role of the different chitin synthases in mating. CSI (i.e., the *CHS1* gene product), whose mRNA is strongly induced after pheromone treatment (1, 17), was found to have no role in mating, as *chs1*Δ cells mated at frequencies similar to those for wild-type cells. Furthermore, the efficiency of zygote formation in matings involving *chs1* cells that exhibit the lytic phenotype in minimal medium was not significantly different from that in matings of the wild-type isogenic strain. Therefore, the possibility that CSI might act in mating as a repairing enzyme, as it does in the mitotic cycle at the time of cytokinesis, can be ruled out. It remains unexplained why transcriptional activation of *CHS1* occurs at the time of mating. CSIII, whose absence (due to *chs3* mutation) leads to a lack of chitin at the base of the shmoo projection (44), is required for a normal rate of mating, as *chs3*Δ cells exhibit a modest defect in mating efficiency. Assuming that *CHS3* is the structural gene for the CSIII catalytic subunit, it follows that the mating impairment in *chs3* null mutant pairs is due solely to the defect in chitin. Additionally, the efficiency of zygote formation in partners carrying any mutation that affects chitin synthesis is reduced to approximately 60% of the wild-type value.

Mating is strongly inhibited when wild-type cells, but not *chs3*Δ cells, are mated in the presence of sublethal concentrations of Calcofluor. The inhibition of mating by Calcofluor, a substance that binds to chitin, altering its crystalline structure and strongly affecting its synthesis in vivo (43), further indi-

cates the role of this polysaccharide in the mating process. However, it is possible that Calcofluor may exert some other indirect deleterious actions that result from its binding to chitin. Thus, the fact that Calcofluor inhibits mating more efficiently than a *chs3*Δ mutation may be due to the interference of this compound with enzymes that participate in the assembly or remodeling of other cell wall components. Consistent with this possibility, mutations in genes involved in the synthesis of other cell wall polymers lead to some reduction in mating efficiency (our unpublished results).

These different results indicate that there is a correlation among lack or alteration of chitin, a defect in shmoo morphology, and impaired conjugation efficiency. Therefore, it is likely that part of the mating defect in *chs5* null mutants is a consequence of their defect in chitin synthesis.

The fact that the *fus3*Δ and *kss1*Δ mutants but not the *fus3*Δ *kss1*Δ mutant are able to activate chitin synthesis in response to α-factor indicates that transduction of the pheromone response signal by either *FUS3* or *KSS1* is essential for this activation.

Role of *CHS5* in mating. The mating defect in *chs5* null mutant pairs is stronger than that observed in strains lacking *CHS3*, even though chitin levels, upon α-factor treatment, are lower in *chs3*Δ than in *chs5*Δ mutants (Tables 4 and 6). Furthermore, microscopic observations clearly indicate that the mating defect in *chs3*Δ mutant strains is qualitatively different from the defect described for *chs5*Δ strains. Therefore, the defects in chitin synthesis and shmoo morphogenesis in *chs5*Δ mutants may be responsible for a certain reduction in the rate of mating, but there must be another function affected in *chs5*Δ mutants that accounts for the additional mating defect.

Several lines of evidence indicate that the *chs5*Δ mating defect that is independent of chitin occurs at a very early step of cell fusion, as described for *fus1 fus2* or *fus3* mutants (24, 25, 59, 60). First, differential interference and phase-contrast microscopy showed a greater number of cell pairs in *chs5*Δ mutants than in the wild type, even after a prolonged period of mating, indicating that the defect is not a consequence of a slow mating process. Second, under the same conditions, Hoechst staining showed a greater number of binucleate zygotes in *chs5*Δ mutants than in the wild type. Third, cytoduction experiments demonstrated that there was no transfer of cytoplasmic elements between *chs5* mating pairs in the absence of nuclear fusion. Fourth, electron-microscopic observations detected a blockage at the level of the prezygote, as defined by Trueheart et al. (59), before cell wall degradation and plasma membrane fusion occur between mating partners. Finally, *CHS5* has been isolated in a genetic screening designed to isolate genes involved in cell fusion. *FUS1*, *FUS2*, *FUS3*, and *SPA2* were isolated in the same screening (4).

The *chs5* mating defect was partially suppressed by centromeric plasmids carrying either *FUS1* or *FUS2*, and the suppression was slightly better with a multicopy plasmid carrying both genes. Conversely, *CHS5* did not alleviate the *fus1 fus2* mating defect, and therefore, *CHS5* and *FUS1* and/or *FUS2* do not act in partially redundant pathways. Since transformation with *FUS3* did not improve mating in *chs5*Δ mutants, we propose that *CHS5* is downstream of *FUS3* and upstream of *FUS1* and/or *FUS2*. Furthermore, partial suppression of the *chs5*Δ mating defect by *FUS1* and *FUS2* is accompanied by an increase in the rate of mature-zygote formation to values similar to those for the *chs3* null mutant but not by suppression of the chitin synthesis defect, measured as the level of Calcofluor fluorescence observed at the time of shmoo formation. The fact that the mating efficiency in a *fus1 fus2* × *chs5* cross was much lower than in a *fus1 fus2* × wild type mating indicates that the functions of these genes are related, since *fus1 fus2* mutants display a strong mating defect only in bilateral crosses (59). Therefore, we speculate that one part of the mating defect in *chs5*Δ mutants is related to the role of chitin in mating while another part is related to an independent function that is specifically required for cell fusion and dependent on *FUS1* and/or *FUS2*. However, the mating and cell wall integrity pathways may be coordinately regulated, as suggested by the observation that the Slt2 kinase pathway, which participates in maintaining cell wall integrity, is activated during polarized growth in mating (27, 63).

Mating efficiency is reduced when *chs5* strains lack mitochondrial DNA (i.e., are [rho^0]). Sena et al. reported that conjugation is severely compromised when both mating partners are respiration incompetent (53). In our case, the strong [rho^0] enhancement of the *chs5*Δ mating defect can probably be explained as the nonspecific consequence of the sum of two conditions that negatively affect conjugation. On the other hand, it is also possible that Chs5p is important for the correct function of some proteins required for mating in respiration-compromised cells. If the latter is the case, this would imply that *CHS5* is involved in one more function required for a normal rate of mating.

Function of Chs5p. Chs5p is a novel yeast protein that displays a modest homology to cytoskeletal proteins such as neurofilament proteins and myosin heavy chains. The homology extends over a long stretch of the sequence (about 400 amino acids), and no precise motifs have been identified. Spa2p (31) and Fus2p (25) also show some similarity to myosins, including the yeast myosin-like protein Mlp1p (35). The presence of internal repeats, similar to those described for Chs5p, has also been reported for Spa2p and Mlp1p, although no clear function has been assigned to them. Myosins and intermediate filament proteins display a coiled-coil structure along these repeated heptads; however, the Chs5p heptads contain an internal proline that does not allow the formation of such a coiled-coil structure. Therefore, Chs5p and neurofilament proteins probably do not perform equivalent functions. The Chs5p lysine-rich carboxy-terminal tail constitutes a rather basic and hydrophilic domain. Similar basic regions have been described as actin-binding domains in villin (30) and S1 myosin (62). A multicopy plasmid carrying the construction *Sal*I-*Stu*I (in which the codons corresponding to the last 56 amino acids of Chs5p, including the lysine-rich tail, are missing) is able to complement the *chs5* mutant Calcofluor resistance phenotype, indicating that this region is not essential for the Chs5p role in chitin synthesis. Finally, it has recently been reported that Chs5p contains small blocks of homology with Sec16p (51), a part of the transport vesicle coat structure.

In contrast to what occurs with several other genes involved in mating, *CHS5* expression does not depend on α-factor treatment, which is consistent with the lack of pheromone response elements in the *CHS5* promoter. The constitutive expression of Chs5p suggests that it has a general function in both budding and mating, but it is certainly more important for the latter process.

Based on the results discussed above, Chs5p may be considered a posttranslational regulator of CSIII. In fact, Chs5p localizes in cytoplasmic patches and may target *CHS3* to polarized growth sites during vegetative growth and in response to α-factor (51). Therefore, it is tempting to speculate that it may be part of the machinery required to deliver material to polar growth sites. Mating projections constitute a model for polar growth, and it is quite apparent that shmoo formation, pairing of complementary cells, and cell fusion require the transport of many different biosynthetic and degradative prod-

ucts. In the case of chitin synthase, it has been proposed that specific vesicles, the chitosomes, carry the enzyme to the plasma membrane (37). A similar hypothesis could be appropriate in our case. It is possible that Chs5p is required to assemble Chs3p not only at the right place but also in the appropriate way so that it can be functional. Other components, including the products of *CHS4* and *CHS6*, are also important for CSIII activity and may be involved in the transport and/or activation of Chs3p (7, 44).

We can speculate that Chs5p associates with the outside of vesicles, allowing them to interact with the transport machinery. In this way, Chs5p might also be involved in the delivery of other components implicated in cell wall synthesis and/or modification. Experiments to ascertain whether the function of the 1,3-β-D-glucan synthase activity involved in mating (Fks2p [40]) is affected in *chs5*Δ mutants are in progress. It is also possible that Chs5p plays a role in delivering other proteins whose functions are not primarily related to cell wall synthesis, for example, Fus1p and Fus2p. Why the defect in *CHS5* is critical for mating and not for budding remains unknown. Curiously, polarized growth is also more dependent on Spa2p in shmoos than in mitotic cells. Therefore, it is possible that proteins with functions related to those of Chs5p and/or Spa2p are present in vegetative cells but are absent or reduced in mating cells.

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