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Differential Expression and Function of Two Homologous Subunits of Yeast 1,3-β-D-Glucan Synthase

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1,3-β-D-Glucan is a major structural polymer of yeast and fungal cell walls and is synthesized from UDP-glucose by the multisubunit enzyme 1,3-β-D-glucan synthase. Previous work has shown that the *FKS1* gene encodes a 215-kDa integral membrane protein (Fks1p) which mediates sensitivity to the echinocandin class of antifungal glucan synthase inhibitors and is a subunit of this enzyme. We have cloned and sequenced *FKS2*, a homolog of *FKS1* encoding a 217-kDa integral membrane protein (Fks2p) which is 88% identical to Fks1p. The residual glucan synthase activity present in strains with deletions of *fks1* is (i) immunodepleted by antibodies prepared against *FKS2* peptides, demonstrating that Fks2p is also a component of the enzyme, and (ii) more sensitive to the echinocandin L-733,560, explaining the increased sensitivity of *fks1* null mutants to this drug. Simultaneous disruption of *FKS1* and *FKS2* is lethal, suggesting that Fks1p and Fks2p are alternative subunits with essential overlapping function. Analysis of *FKS1* and *FKS2* expression reveals that transcription of *FKS1* is regulated in the cell cycle and predominates during growth on glucose, while *FKS2* is expressed in the absence of glucose. *FKS2* is essential for sporulation, a process which occurs during nutritional starvation. *FKS2* is induced by the addition of Ca^{2+} to the growth medium, and this induction is completely dependent on the Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase calcineurin. We have previously shown that growth of *fks1* null mutants is highly sensitive to the calcineurin inhibitors FK506 and cyclosporin A. Expression of *FKS2* from the heterologous *ADH1* promoter results in FK506-resistant growth. Thus, the sensitivity of *fks1* mutants to these drugs can be explained by the calcineurin-dependent transcription of *FKS2*. Moreover, *FKS2* is also highly induced in response to pheromone in a calcineurin-dependent manner, suggesting that *FKS2* may also play a role in the remodeling of the cell wall during the mating process.

The cell wall of *Saccharomyces cerevisiae* is essential for the integrity and shape of the cell and is a highly dynamic structure the composition and architecture of which vary widely depending upon the composition of the growth medium and the stage of the cell cycle (41). In addition, when haploid cells encounter pheromone of the opposite mating type, the cells transiently arrest in the G_1 phase of the cell cycle and develop an elongated projection requiring new cell wall synthesis (12). Furthermore, diploid cells which are nutritionally starved undergo meiosis and sporulation, a process requiring the formation of new cell wall around the developing spores (reviewed in reference 42).

An important component of each of these cell wall types is the glucose polymer 1,3-β-D-glucan (10, 38, 41). 1,3-β-D-Glucan synthase (UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyltransferase; EC 2.4.1.34) is a membrane enzyme activated by GTP which has been fractionated into soluble (GTP-binding) and membrane-bound (catalytic) components (39, 53). Members of the echinocandin family of antifungal agents inhibit 1,3-β-D-glucan synthase and have been used to isolate mutations in the *FKS1* gene, which encodes a subunit of this enzyme (18–20). The resistance mutations result in echinocandin-resistant enzyme activity which is associated with the membrane fraction (19). The nucleotide sequence of *FKS1* encodes a 215-kDa polypeptide (Fks1p) predicted to be an integral membrane protein. The residual glucan synthase activity present in *fks1* disruption mutants, the nonessential nature of the gene, and hybridization analysis of yeast chromosomal DNA pointed to the existence of a homologous gene encoding a functionally redundant product (18). We report here the cloning and characterization of this second gene (*FKS2*), present immunochemical evidence that the gene product (Fks2p) is a component of 1,3-β-D-glucan synthase, and demonstrate that it is essential for sporulation. In addition, we show that its expression is repressed by glucose but can be induced in the presence of glucose by Ca^{2+}, or pheromone, in a calcineurin-dependent manner, suggesting an additional, although nonessential, role for the gene in the mating process.

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MATERIALS AND METHODS

Media, microbiological methods, and strains. YPAD, synthetic complete, and dropout media and procedures for mating, sporulation, tetrad analysis, transformation, gene disruption, and determination of antibiotic sensitivity have been described previously (18). YPA-galactose, YPA-glycerol, and YPA-acetate are the same as YPAD except that 2% glucose was replaced with 2% galactose, 2% glycerol, and 2% sodium acetate, respectively. All yeast strains used in this study are derivatives of YFK007 and YFK005 (9) and are listed in Table 1. Halo assays for sensitivity to, and recovery from, α-mating pheromone were as described previously (22). Percent ascus formation was evaluated microscopically. At least 1,000 cells were counted for each cell type. An ascus was counted as one cell. Sensitivity to salts was determined by seeding the strain to be tested in YPAD soft agar overlay (as for the halo assay) and applying antibiotic discs (6-mm diameter) containing 25 μl of 4 M LiCl or 0.1 or 0.2 M $MnSO_4$. Zones of inhibition were measured after 24 h of incubation at 30°C. Mating was assayed with a 1:1 ratio of mating types at a high cell density (all cells physically touching neighboring cells; verified microscopically) on solid YPAD after an incubation period of 5 h at 30°C by determining the formation of prototrophic diploids from auxotrophic haploids. The formation of budding zygotes was verified microscopically. About 50% of the colonies were diploid, and the remainder were haploid, under these conditions.

Cloned DNA and nucleotide sequence analysis. Southern hybridization analysis of yeast genomic DNA from strain YFK007 revealed a 2.5-kb *Pst*I fragment hybridizing to *FKS1* DNA which did not derive from the *FKS1* locus (18).

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

Strain	Genotype	Reference or derivation
YFK005	*MAT*α *ade2-101 his3-*Δ*200 lys2-801 trp1-*Δ*1 ura3-52*	9
YFK007	*MAT***a** *ade2-101 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52*	9
YFK016	YFK005 × YFK007	9
YFK016-28D	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52*	Meiotic segregant
YFK419[a]	*MAT***a**/*MAT*α *ade2-101*/*ade2-101 his3-*Δ*200*/*his3-*Δ*200 leu2-*Δ*1*/*leu2-*Δ*1 lys2-801*/*lys2-801 trp1-*Δ*1*/*trp1-*Δ*1 ura3-52*/*ura3-52*	18
YFK532-7C	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 fks1-1*	62
YFF1864	YFK016-28D *cna1-*Δ*1*::*URA3*	Transformation
YFF1868	YFK016-28D *cna2-*Δ*1*::*LEU2*	Transformation
YFF1872	YFK016-28D *cnb1-*Δ*1*::*LYS2*	Transformation
YFF1930	YFF1864 *cna2-*Δ*1*::*LEU2*	Transformation
YFF2066	YFK016-28D *bar1-*Δ*1*::*ADE2*	Transformation
YFF2127	YFF1872 *cna1-*Δ*1*::*URA3*	Transformation
YFF2218	YFF2127 *cna2-*Δ*1*::*LEU2*	Transformation
YFF2421	YFK419 *fks1-*Δ*1*::*HIS3*/+	Transformation
YFF2714	YFF2421 *fks2-*Δ*1*::*TRP1*/+	Transformation
YFF2714-1A	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 fks2-*Δ*1*::*TRP1*	Meiotic segregant
YFF2714-3C	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 fks1-*Δ*1*::*HIS3*	Meiotic segregant
YFF2714-5B	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 fks2-*Δ*1*::*TRP1*	Meiotic segregant
YFF2714-10B	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 fks2-*Δ*1*::*TRP1*	Meiotic segregant
YFF2716	YFF2421 *fks2-*Δ*1*::*TRP1*/*fks2-*Δ*1*::*TRP1*	Transformation
YFF2936	YFK532-7C *fks1-*Δ*1*::*HIS3*	Transformation
YFF2957	YFK419 *fks2-*Δ*1*::*TRP1*/*fks2-*Δ*1*::*TRP1*	Transformation
YFF2986	YFF2714-1A × YFF2714-5B (*fks2-*Δ*1*::*TRP1*/*fks2-*Δ*1*::*TRP1*)	Mating
YFF2975	YFF2714-1A × YFF2936 (*fks1-*Δ*1*::*HIS3*/+ *fks2-*Δ*1*::*TRP1*/+)	Mating
YFF3030	YFF2975/YCp-*FKS1*	Transformation
YFF3162	YFF2218 *bar1-*Δ*1*::*ADE2*	Transformation

[a] Derived from YFK005 and YFK007.

Further analysis (data not shown) showed that the *Pst*I fragment was shifted in size to 1.7 kb when digested with *Bgl*II. The 1.7-kb *Bgl*II-*Pst*I fragment was cloned by constructing a library from gel-eluted fragments in the size range of 1.5 to 2.0 kb from a *Bgl*II-*Pst*I digest of genomic DNA from strain YFK007 (9) and identifying cross-hybridizing clones from that library by colony hybridization. The chromosomal location of *FKS2* was determined by hybridization of a Chromo-Blot (Clontech) of *S. cerevisiae* YPH80 with the 1.7-kb *Bgl*II-*Pst*I fragment used as the probe. The 1.7-kb *FKS2* fragment from YFK007 was used to screen a lambda library of genomic yeast DNA from strain S288C (Stratagene; catalog no. 951901) by plaque hybridization. A 10-kb *Eco*RI fragment carrying the cross-hybridizing DNA was subcloned from a positive phage. A 7,070-bp *Eco*RI-*Apa*I region was sequenced and contained a single large open reading frame (Fig. 1A). The 10-kb *Eco*RI fragment carrying *FKS2* was inserted into the centromeric *URA3*-selectable yeast shuttle vector YCplac33 (27), giving YCp-*FKS2*, for complementation analysis and into pBluescriptII KS(+) (Stratagene) in both orientations for sequencing. Nested deletions were created by complete digestion with *Bam*HI and partial digestion with *Sau*3AI (26). The sequences of both strands of DNA were determined with Sequenase version 2.0 (U.S. Biochemical Corp.) or by *Taq* cycle sequencing on the model 373A automated DNA sequencer (Applied Biosystems Inc.). Sequence analysis was performed with the computer programs provided by the Genetics Computer Group (25). A deletion/disruption of the cloned DNA (designated *fks2-*Δ*1*::*TRP1*) (Fig. 1A) was constructed by replacing an internal 0.4-kb *Afl*II-*Bbs*I region of the 1.7-kb fragment derived from YFK007 with the 0.8-kb *Pst*I *TRP1* fragment from plasmid pJJ246 (37). A plasmid (YCp-*ADH1-FKS2*) carrying a transcriptional fusion of *FKS2* to the *ADH1* promoter was constructed by replacing the 1.0-kb *Bam*HI-*Eco*47III *FKS2* promoter region of YCp-*FKS2* with the 1.6-kb *Bam*HI-*Hin*dIII *ADH1* promoter fragment from pAAH5 (2).

Northern (RNA) blotting. Strains were grown to a density of 3×10^7 cells per ml (1.5×10^7 cells per ml on YPA-acetate), collected by centrifugation at 2,000 × *g* for 5 min at 4°C, washed once with 1 ml of ice-cold buffer containing 10 mM Tris HCl (pH 8.0) and 1 mM EDTA, frozen on dry ice, and stored at −70°C. Total RNA was prepared as described previously (13) and separated on 1% formaldehyde-agarose gels (64). RNA was transferred to Nytran Plus membranes (Schleicher & Schuell) with the PosiBlot pressure blotter (Stratagene) and UV cross-linked with the Stratalinker UV box (Stratagene). Hybridization was performed as described previously (64). Probes were radiolabeled with [α-^{32}P]dTTP by using a random-primed (Stratagene) or PCR (Bethesda Research Laboratories) DNA labeling kit according to the manufacturer's instructions. Probes were prepared from gel-purified PCR fragments synthesized with the following primers: *ACT1*, 5′-AGGTTGCTGCTTTGGTTATT-3′ (sense) and 5′-TTAGAAACACTTGTGGTGAA-3′ (antisense); *CHS1*, 5′-ATGAGTG ATCAAAATAATCGATCGA-3′ (sense) and 5′-AGGTACGTTATTGTATT GATGTTCC-3′ (antisense); *FKS1*, 5′-CAGAACACTACAGCTGTTTTAACC G-3′ (sense) and 5′-CCATATTGGTCATAGTCTTGTTCC-3′ (antisense); *FKS2*, 5′-GGCATATTAAAGAAGTTACAAAAGG-3′ (sense) and 5′-CCAGTTGGTT TTGTGTATAGATTGG-3′ (antisense); *FUS1*, 5′-GTAGCAACAATAATGCA GACGACAA-3′ (sense) and 5′-CTGAGCCGCCACATTAGAAAAGAGT-3′ (antisense); histone H2A, 5′-ATGTCCGGTGGTAAAGGTGGTAAAG-3′ (sense) and 5′-TTATAATTCTTGAGAAGCCTTGGTA-3′ (antisense); *SUC1*, 5′-CCAACAAGGGTTGGATGAATGACCC-3′) (sense) and 5′-GCTCACTT-GGGACTTCGATCAAACC-3′ (antisense); and *TMP1*, 5′-GATGAAGGT GAATTTAGGCCAGATA-3′ (sense) and 5′-CCTTGTCCAGTATAGTCGT CATCGC-3′ (antisense). RNA levels were quantitated with a PhosphorImager (Molecular Dynamics). The probes for *FKS1* and *FKS2* hybridize to a region encoding the divergent N termini of each protein product and were determined to be gene specific by hybridization to total RNA from strains YFF2936 (*fks1*Δ) and YFF2714-5B (*fks2*Δ) (data not shown).

Glucan synthase. The methods used for membrane preparation and glucan synthase assay have been described previously (18). Protein was determined with the bicinchoninic acid protein assay reagent (Pierce). Detergent extracts of microsomal membranes were prepared by using 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS). Membrane preparations were diluted with disruption buffer (18) to a concentration of 4 mg/ml and collected by ultracentrifugation (105,000 × *g*, 1 h, 4°C). The resulting pellet was resuspended with a 2-ml Dounce homogenizer in an equal volume of solubilization buffer containing 50 mM sodium phosphate (pH 7.5), 100 mM sodium citrate, 100 mM potassium chloride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.2% CHAPS. The mixture was stirred with a magnetic stir bar for 30 to 60 min at 4°C and centrifuged (105,000 × *g*, 1 h, 4°C). The supernatant fluids were collected and frozen at −80°C.

Preparation of antisera and Western blot (immunoblot) analysis. Synthetic peptides based on the amino acid sequences of Fks1p and Fks2p were used to generate polyclonal antisera in rabbits. The antibodies, anti-Fks1p (no. 152), anti-Fks2p (no. 228), and anti-Fks2p (no. 223), were derived from peptides corresponding to amino acids (aa) 1128 to 1140 (EQVNPYAPGLRYE) of Fks1p and 113 to 125 (SGTYPNDQYTPSQ) and 1147 to 1159 (EQIHPYTPGLKYE) of Fks2p, respectively. The peptides were synthesized with a C-terminal amide, an additional Nle residue, and an additional Cys residue at the N terminus, through which the peptide was coupled to bovine thyroglobulin by using *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for Western blotting was performed on microsomal membrane samples (20 μg of protein) which were diluted in 2× SDS sample application buffer (Novex) containing 1% (vol/vol) β-mercaptoethanol and incubated at 65°C for 2 min prior to electrophoresis on 4 to 12% gradient polyacrylamide gels (Novex). Molecular masses were esti-

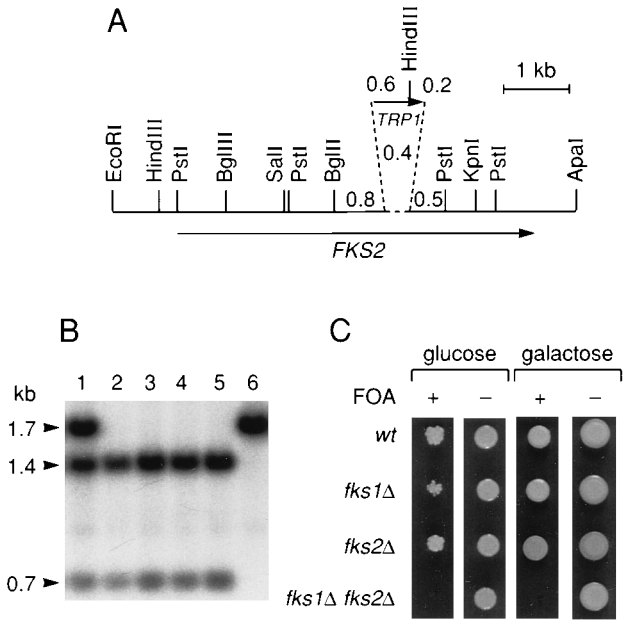

FIG. 1. Disruption of *FKS2*. (A) Diagram of the restriction map of the DNA encoding *FKS2*. The location of the open reading frame is indicated by the arrow beneath the map. The construction of *fks2-Δ1*::*TRP1* is illustrated with the dashed lines indicating the region deleted and marked by the *TRP1* gene. The numbers above the map indicate the size (in kilobases) of each region relevant to interpreting the Southern analysis shown in panel B. (B) Southern blot analysis of *fks2-Δ1*::*TRP1* strains. Genomic DNA was digested with *Bgl*II-*Hin*dIII-*Pst*I, subjected to agarose gel electrophoresis, blotted, and hybridized with a probe prepared from the 1.7-kb *Bgl*II-*Pst*I *FKS2* fragment. Lane 1, heterozygous *fks2-Δ1*::*TRP1* diploid YFF2714. Lanes 2 to 5, His^- Trp^+ haploid segregants YFF2714-1A, -4B, -5B, and -10B, respectively. Lane 6, wild-type YFK007. (C) Disruption of *FKS1* and *FKS2* is synthetically lethal. The *MAT***a** haploid segregants YFF3030-6B (wild type [wt]), -2C (*fks1*Δ), -2A (*fks2*Δ), and -6A (*fks1*Δ *fks2*Δ) containing the YCp-*FKS1* plasmid pFF133 (18) were grown from single cells as colonies on synthetic complete medium containing uracil and either glucose or galactose as the carbon source, transferred to 10 ml of liquid medium, grown to stationary phase, and diluted to 5×10^6 cells per ml. Aliquots (5 μl) were spotted on agar plates containing synthetic complete medium with and without FOA and with glucose or galactose as the carbon source as indicated. The relevant genotypes are indicated to the left of each row.

mated from high-range prestained SDS-PAGE standards (Bio-Rad). Gels were wet transferred to polyvinylidene difluoride membranes, and the blots were probed with anti-FKS antiserum (diluted 1:10,000) and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate. The blots were developed with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium) (Sigma).

Immunodepletion of glucan synthase activity. Anti-Fks2p (no. 228) was affinity purified on a 2-ml Sulfolink column (ImmunoPure Ag/Ab Immobilization Kit 2; Pierce) to which 5.9 mg of the corresponding *FKS2* peptide (aa 113 to 125) was coupled according to the manufacturer's protocols. The antiserum (5 ml diluted 1:1 with phosphate-buffered saline [PBS]) was passed over the column for 1 h at room temperature, washed with PBS, and eluted with 0.1 M glycine, pH 2.2. Fractions (1 ml) were collected into 0.5 ml of 0.4 M dibasic sodium phosphate. The purified antibody was dialyzed against PBS overnight at 4°C. The antibody was incubated with CHAPS-solubilized glucan synthase activity and immunodepleted with attenuated *Staphylococcus aureus* cells (Pansorbin cells; Calbiochem). The cells were removed by centrifugation, and the supernatant fraction was assayed for glucan synthase activity as described above (the assay mixture was incubated for 90 min at 25°C).

Nucleotide sequence accession number. The nucleotide sequence of *FKS2* has been assigned GenBank accession number U16783.

RESULTS

Cloning and sequence of *FKS2*. Southern hybridization analyses of yeast genomic DNA with an *FKS1* probe revealed the presence of a homologous gene (*FKS2*), which we cloned and sequenced (see Materials and Methods). *FKS2* is located on chromosome VII or XV (unresolved in the blot; data not shown), while previous work has shown that *FKS1* is located on chromosome XII (18). *FKS2* encodes a predicted protein (Fks2p) of 1,895 amino acids with a molecular mass of 217 kDa and a pI of 7.0. The amino acid sequence of Fks2p is 88% identical to that of Fks1p and 19 amino acids longer at the N terminus (Fig. 2). Only the N-terminal domains (aa 1 to 133 of Fks1p and 1 to 152 of Fks2p) diverge significantly (48% identity, with several gaps in the alignment), while the remaining 1,743 residues of the two proteins are much the same (91% identity, with no gaps). Although the sequences of the N-terminal domains differ, they have a similar and unusual amino acid composition. Overall the region is highly acidic (pI of 2.71 for Fks1p and 2.62 for Fks2p) compared with the C-terminal domains (pI of 8.14 for Fks1p and 8.06 for Fks2p).

Hydropathy analysis by the method of Sipos and von Heijne (70) predicts that Fks2p, like Fks1p, is an integral membrane protein with 16 transmembrane helices (TMHs) (Fig. 2). The locations of the TMHs are highly conserved between the two proteins. According to the rule of Hartmann et al. (31), the N terminus of Fks2p, like that of Fks1p, is predicted to be cytoplasmic. Fks1p and Fks2p have six consensus N-linked glycosylation sites which are conserved between them (Fig. 2). All of these sites are predicted from the hydropathy analysis to be on the cytoplasmic side of the membrane. In general, the topology of Fks2p, like that of Fks1p (18), is similar to that of a large class of membrane transport proteins.

A search of the National Center for Biotechnology Information databases (10 May 1995) with TBLASTA (1) revealed a yeast homolog (accession number Z49212; nucleotides 16727 to 22084) which is on chromosome XIII upstream of *GGP1* (73) and has 56% identity to Fks1p and Fks2p. Hydropathy analysis showed that the predicted TMHs of the homolog are similar to those of Fks1p and Fks2p except for an additional TMH between TMHs corresponding to TMHs 14 and 15 of Fks1p and Fks2p. The function of this homolog is unknown. Fks1p and Fks2p have no significant homologies to any other open reading frames in the databases.

Anti-Fks2p antibody immunodepletes glucan synthase activity. We have previously shown that *FKS1* is likely to encode a subunit of 1,3-β-D-glucan synthase (18). This conclusion was based primarily on the observation that certain mutations in *FKS1* result in whole-cell resistance to the glucan synthase inhibitor L-733,560, a member of the echinocandin class of antifungal agents (45, 72), as well as in glucan synthase activity which is resistant to L-733,560 in vitro (19, 20). The high degree of similarity between Fks2p and Fks1p indicates that Fks2p is also likely to be a subunit of this enzyme. To test whether Fks2p is associated with glucan synthase, immunodepletion of enzyme activity with anti-Fks2p antibody was investigated. We found that solubilized enzyme can be prepared from microsomal membranes by detergent extraction (see Materials and Methods). Addition of the affinity-purified anti-Fks2p antibody, followed by *S. aureus* cells, to solubilized enzyme prepared from the *fks1-Δ1*::*HIS3* mutant substantially depleted activity from the supernatant fraction after centrifugation (Fig. 3). Similar results were obtained with intact microsomal membranes (data not shown). Addition of antibody or *S. aureus* cells alone resulted in little or no depletion of the enzyme (Fig. 3). The immunodepletion was specifically blocked by preincubation with the peptide used to raise the anti-Fks2p antibody (pfks2-1) and was unaffected with a different peptide (pfks2-2). These results show that Fks2p is physically associated with glucan synthase activity.

Synthetic lethality of *fks1-Δ1*::*HIS3* and *fks2-Δ1*::*TRP1*. A deletion/disruption (*fks2-Δ1*::*TRP1*; see Materials and Meth-

FIG. 2. Amino acid alignment of Fks1p and Fks2p. The amino acid sequences of Fks1p and Fks2p were aligned with the GAP program from the Genetics Computer Group (25). The alignment was edited by hand to maximize identities and similarities. Gaps are marked by periods in the sequence. Residues identical in both sequences are indicated by a vertical line between the two sequences. Similar residues are marked by a colon when the comparison value (28) is greater than or equal to 0.5 and by a period when it is greater than 0 but less than 0.5. The predicted TMHs are overlined and numbered. Conserved potential N-linked glycosylation sites are marked above the appropriate residue with the letter v.

ods and Fig. 1A) was introduced into the *fks1-Δ1*::*HIS3* heterozygous diploid YFF2421 by one-step gene replacement. Southern hybridization analysis of genomic DNA from the Trp^+ transformants confirmed heterozygosity for *fks2-Δ1*::*TRP1* (Fig. 1B). Of 12 asci dissected from the sporulated diploid, 11 yielded one parental-type His^- Trp^- spore, one His^+ Trp^- spore, one His^- Trp^+ spore, and one inviable spore predicted to be His^+ Trp^+. One ascus yielded two viable parental-type spores and two inviable spores also predicted to be His^+ Trp^+. Microscopic observation showed that the inviable spores failed to germinate. The presence of *fks2-Δ1*::*TRP1* in the His^- Trp^+ segregants was confirmed by Southern blot analysis (Fig. 1B), and the absence of Fks2p cross-reacting material was confirmed by Western blot analysis with anti-Fks2p (no. 223 and no. 228) antibodies (see Materials and Methods; data not shown). None of 13 spores predicted to be *fks1-Δ1*::*HIS3 fks2-Δ1*::*TRP1* was viable, strongly supporting the idea that simultaneous disruption of *FKS1* and *FKS2* is lethal.

To eliminate the possibility of these mutations affecting only sporulation or germination, we also checked for synthetic lethality during vegetative growth by testing for the spontaneous loss of a centromeric plasmid carrying the *FKS1* gene and a *URA3* marker. Cultures were grown nonselectively for several generations, diluted, and spotted on plates with glucose or galactose as the carbon source and with or without 5-fluoroorotic acid (FOA). Only cells that have lost the plasmid and are phenotypically Ura^- grow in the presence of FOA. The results (Fig. 1C) show that wild-type, *fks1-Δ1*::*HIS3*, and *fks2-Δ1*::*TRP1* cells readily lost the *FKS1* plasmid and became FOA resistant on plates with either glucose or galactose as the carbon source. In contrast, the *fks1-Δ1*::*HIS3 fks2-Δ1*::*TRP1* double mutant did not give rise to FOA-resistant segregants; that is, these alleles are synthetically lethal during vegetative growth on either carbon source.

Cells carrying the *fks2-Δ1*::*TRP1* allele have a sporulation defect. The growth of haploid *fks2-Δ1*::*TRP1* mutants and homozygous diploids was tested by plating for single colonies on solid rich and synthetic defined media with glucose, galactose, glycerol, or acetate as the sole source of carbon at 23, 30, and 37°C. Growth under these conditions was indistinguishable from that of the wild type. Haploid *fks2-Δ1*::*TRP1* mutants

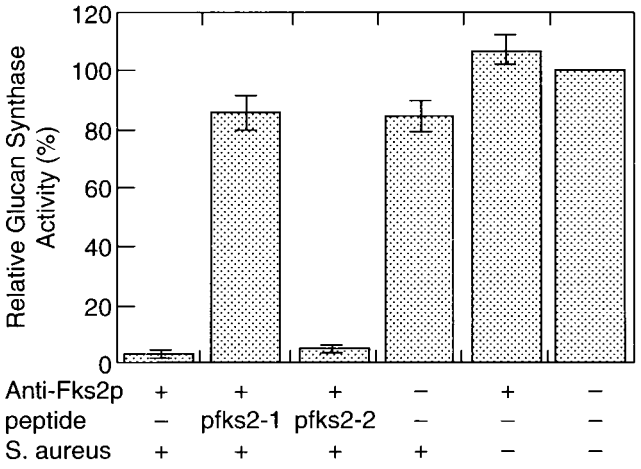

FIG. 3. Immunodepletion of solubilized glucan synthase activity from *fks1-Δ1::HIS3* cells with anti-Fks2p. Affinity-purified anti-Fks2p (no. 228) (5 μl; 0.3 mg/ml) was added to a sample (25 to 40 μl) of the CHAPS extract of microsomal membranes isolated from YFF2936 (*fks1*Δ) and incubated for 30 min at 0°C; in some cases, as indicated, the antibody was preincubated with the homologous peptide pfks2-1 (aa 113 to 125) or the heterologous peptide pfks2-2 (aa 1147 to 1159) (20 μM peptide after addition of the detergent extract) for 30 min at 22°C. The antibody-Fks2p complexes were precipitated by addition of 25 μl of a 10% (wt/vol) suspension of *S. aureus* cells, incubation for 30 min at 0°C, and centrifugation (3,000 × *g* for 4 min). Samples of the supernatant fluid were assayed for glucan synthase activity as described in Materials and Methods. The average glucan synthase activity from three separate experiments with three different preparations of CHAPS membrane extracts is shown. The enzyme activity from supernatant fractions of samples incubated without *S. aureus* cells and antibody was set at 100% and was equivalent to 1.0 to 1.7 nmol of glucose incorporated into glucan per h. Error bars indicate the standard deviation (n = 3).

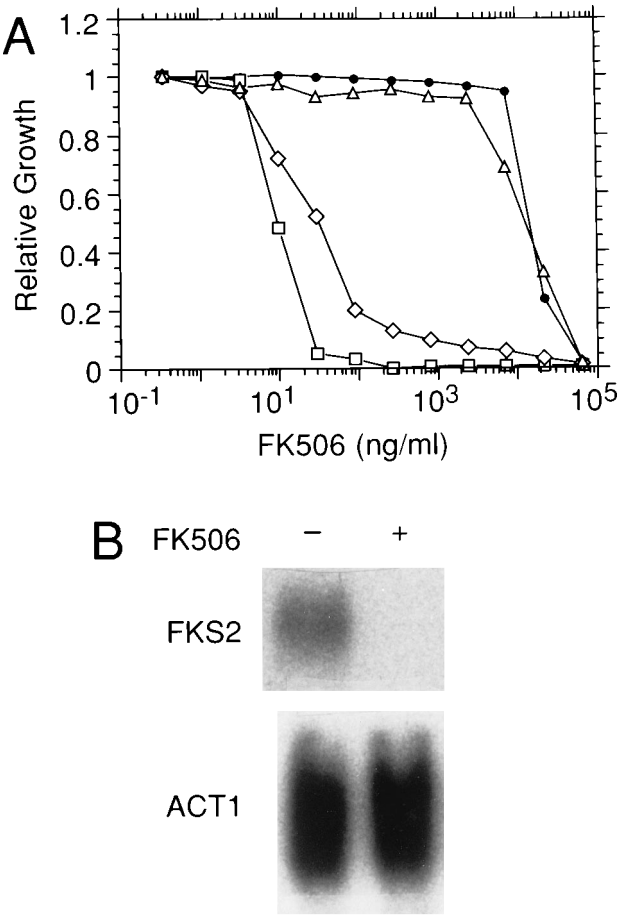

FIG. 4. Transcription of *FKS2* is inhibited by FK506. (A) FK506 sensitivities of the wild type (YFK007) (●) and of YFF2714-3C (*MAT***a** *fks1-Δ1::HIS3*) containing YCplac33 (□), YCp-*FKS2* (◇), and YCp-*ADH1-FKS2* (Δ). Cells were grown on YPAD supplemented with 10 mM $CaCl_2$. Essentially identical results were obtained with YPAD alone (data not shown). (B) Northern blot of total RNA isolated from the cultures without and with FK506 and probed with the 1.7-kb *Bgl*II-*Pst*I *FKS2* fragment and the *ACT1* probe (see Materials and Methods). YFF2714-3C (*MAT***a** *fks1-Δ1::HIS3*) was grown at 30°C in 50 ml of YPAD supplemented with 10 mM $CaCl_2$. When the culture density reached 3×10^7 cells per ml, the culture was split into two aliquots of 25 ml, and 5 μl of a stock solution containing FK506 at 5 mg/ml in ethanol was added to one aliquot to give a final concentration of 1 μg/ml. The cultures with and without drug were incubated further. At 2 h, 20-ml samples were removed for the preparation of total RNA.

have the same sensitivity to LiCl and $MnSO_4$ as the wild type. Haploid *MAT***a** *fks2-Δ1::TRP1* mutants have the same sensitivity to α-factor as the wild type, maintain viability for at least 6 h when arrested with α-factor, form shmoos (cells which have formed mating projections), and recover from arrest normally. *MAT***a** *fks2-Δ1::TRP1* and *MAT*α *fks2-Δ1::TRP1* haploids mate normally to each other and to wild-type haploids of the opposite mating type. However, homozygous *fks2-Δ1::TRP1* diploids heterozygous for *fks1-Δ1::HIS3* (YFF2716) or homozygous for *FKS1* (YFF2957 and YFF2986) failed to sporulate (<0.1% ascus formation). This defect was fully complemented with YCp-*FKS2* (24% ascus formation in YFF2716) and partially complemented with the centromeric *FKS1* plasmid pFF133 (18) (7% ascus formation in YFF2716).

Transcription of *FKS2* is FK506 sensitive. Previous work has shown that null mutations in *FKS1* result in FK506-hypersensitive and Ca^{2+}-responsive growth (18, 21, 62). FK506 is a potent inhibitor of the Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase calcineurin (62). These observations suggested that transcription of *FKS2* might be calcineurin dependent. To test the FK506 sensitivity of *FKS2* transcription, the *fks1-Δ1::HIS3* mutant was grown in YPAD medium in the presence of 10 mM $CaCl_2$. The culture was grown to a density of about 3×10^7 cells per ml and divided in half. FK506 was added to one portion at a final concentration of 1 μg/ml. Previous work has shown that the growth of wild-type cells is unaffected by this concentration of FK506, while the growth of the *fks1* mutant strain is eventually inhibited (62). Logarithmic growth continued for 2 h after the addition of drug, at which time samples were removed for the isolation of total RNA. Northern analysis with the 1.7-kb *FKS2* fragment used as a probe (Fig. 4B) showed that the *FKS2* mRNA was readily detectable in the cells grown without FK506 and was absent with FK506. The levels of actin mRNA were unaffected. To test whether the FK506-sensitive growth of an *fks1* null mutant is due solely to the inhibition of transcription of *FKS2*, this gene was fused to the *ADH1* promoter, which promotes transcription at high levels on glucose-containing media, and expressed from a plasmid. The strain carrying this plasmid was essentially as resistant to FK506 as the wild type (Fig. 4A).

Induction of *FKS2* by Ca^{2+} is calcineurin dependent. Calcineurin is a heterodimer consisting of one catalytic subunit and one regulatory subunit (40). For *S. cerevisiae* two genes (*CNA1* and *CNA2*) encoding alternative catalytic subunits and one gene (*CNB1*) encoding the regulatory subunit have been described. Disruption of these genes has shown that calcineurin is not required for vegetative growth (16, 17, 44, 47, 56). Total RNAs from wild-type, *cna1*Δ, *cna2*Δ, *cna1*Δ *cna2*Δ, and *cnb1*Δ cells grown on glucose with and without 10 mM

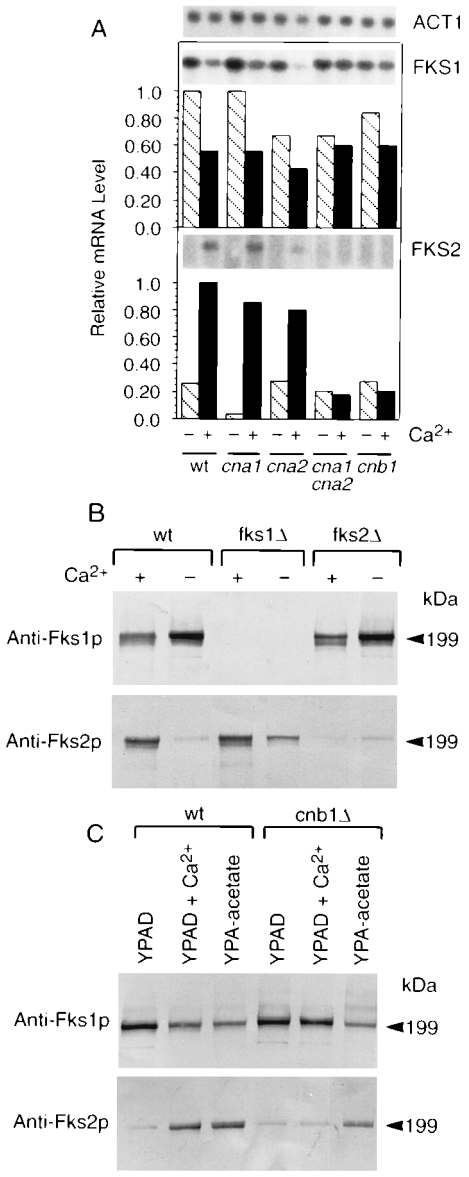

FIG. 5. Expression of *FKS2* is induced by Ca^{2+} in a calcineurin-dependent manner. (A) Northern blots of total RNA isolated from strains YFK016-28D (wild type [wt]), YFF1864 (*cna1*Δ), YFF1868 (*cna2*Δ), YFF1930 (*cna1*Δ *cna2*Δ), and YFF1872 (*cnb1*Δ) grown on YPAD with and without 10 mM $CaCl_2$. The relative mRNA levels shown in the bar graphs under the autoradiograms were calculated by dividing the *FKS1* and *FKS2* mRNA levels by the *ACT1* mRNA level and setting the *ACT1*-normalized *FKS1* mRNA level in the wild type without Ca^{2+} and the normalized *FKS2* mRNA level in the wild type with Ca^{2+} equal to one. (B and C) Western blot analysis of levels of expression of Fks1p and Fks2p. Samples of the membrane fraction (20 μg of protein) prepared from the indicated strains were subjected to electrophoresis on a 4 to 12% gradient polyacrylamide gel and then transferred to polyvinylidene difluoride for Western blot analysis. (B) Membrane proteins from cultures grown in YPAD with and without 10 mM $CaCl_2$ were probed with anti-Fks1p (no. 152) and anti-Fks2p (no. 223), respectively. (C) Membrane proteins from cultures grown in YPAD with and without 10 mM $CaCl_2$ or in YPA-acetate were probed with anti-Fks1p (no. 152) and anti-Fks2p (no. 223), respectively. In all blots the position of the myosin standard (199 kDa) is indicated by the arrowhead.

$CaCl_2$ were prepared and analyzed by Northern blot analysis (Fig. 5A). *ACT1* mRNA levels were determined as a control for the amount of RNA loaded on the gel. The level of *FKS2* mRNA was low in the absence of Ca^{2+} and significantly higher in its presence in the wild type and the *cna1*Δ and *cna2*Δ mutants. The Ca^{2+}-dependent increase did not occur in the *cna1*Δ *cna2*Δ and *cnb1*Δ strains. Thus, induction of *FKS2* transcript levels by Ca^{2+} is calcineurin dependent. The level of *FKS1* mRNA was decreased by the presence of Ca^{2+}, and this decrease was somewhat less pronounced in the strains lacking calcineurin, but the latter effect was not highly reproducible.

Western blot analysis was performed with polyclonal antisera raised against peptides from the deduced amino acid sequences of Fks1p and Fks2p. The specificities of the antisera (and the localization of the *FKS1* and *FKS2* gene products to the microsomal membrane fraction) were demonstrated by Western blot analysis of microsomal membranes isolated from *fks1*-Δ*1*::*HIS3* and *fks2*-Δ*1*::*TRP1* cultures grown on glucose in the presence or absence of 10 mM $CaCl_2$ (Fig. 5B). The anti-Fks1p antiserum is specific for Fks1p, while the anti-Fks2p antiserum shows a low level of cross-reactivity. Both antisera led to the detection of a single polypeptide with a molecular mass of approximately 200 kDa, a size slightly smaller than that predicted for Fks1p (215 kDa) or Fks2p (217 kDa). The binding of each antibody was blocked by preincubation with the corresponding peptide (data not shown).

Likewise, levels of the *FKS2* gene product were shown to be inducible by Ca^{2+} in a calcineurin-dependent fashion. Western blot analysis was performed on membrane fractions prepared from wild-type, *fks1*Δ, and *cnb1*Δ cells grown on glucose with and without 10 mM $CaCl_2$. Fks2p was essentially absent from wild-type cells grown in the absence of Ca^{2+} and was induced in the wild-type and *fks1*Δ cells in the presence of Ca^{2+} (Fig. 5B). The level of Fks2p was very low in *cnb1*Δ cells regardless of the presence or absence of Ca^{2+} (Fig. 5C). Growth in the presence of Ca^{2+} resulted in decreased levels of Fks1p in the wild-type, *fks2*Δ, and *cnb1*Δ strains.

Pheromone induction of *FKS2* mRNA is calcineurin dependent. Calcineurin is required for recovery from mating factor arrest. In addition, *FKS2* possesses a pheromone response element (with the consensus sequence 5′-TGAAACA) (43) at bp −894 from the start codon of the open reading frame and a potential inverted element at bp −326. We therefore tested the effect of α-factor on the levels of *FKS1* and *FKS2* mRNAs (Fig. 6A). The levels of the pheromone-inducible *FUS1* (required for cell fusion) and *CHS1* (chitin synthase 1) mRNAs were determined for comparison. The *FUS1* and *CHS1* promoters each have four pheromone response elements (6, 29). *ACT1* mRNA levels were determined as a control for the amount of RNA loaded on the gel. The *FUS1* and *CHS1* mRNA levels increased significantly within the first 15 min after the addition of pheromone, while *FKS2* was induced after a delay of about 45 to 60 min. This induction is calcineurin dependent (Fig. 6B). The levels of *FKS1* mRNA decrease slowly after the addition of pheromone, and this decrease is not affected by the presence or absence of calcineurin. This decrease is due to cell cycle-dependent transcription of *FKS1*, which is not expressed in G_1 (see below). When cycloheximide was added at the same time as pheromone, the increase in the optical density of the cells exposed to cycloheximide was inhibited strongly, and they failed to form shmoos. Nevertheless, the induction of *FKS2* mRNA, as well as that of *FUS1* and *CHS1* mRNAs, still occurred (data not shown).

Expression of *FKS2* in the absence of glucose is largely calcineurin independent. The *fks1*-Δ*1*::*HIS3* mutant lacking calcineurin fails to grow on glucose but does grow on galactose as the carbon source (data not shown). This result suggested that *FKS2* might be expressed in a calcineurin-independent manner in the absence of glucose. The nucleotide sequence upstream of the *FKS2* open reading frame was examined for

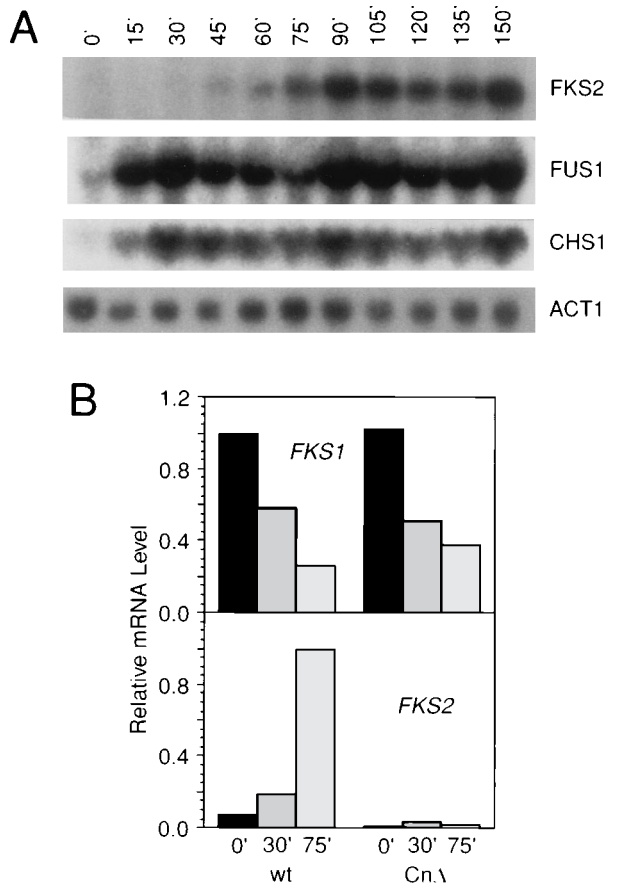

FIG. 6. The level of *FKS2* mRNA is increased by mating pheromone in a calcineurin-dependent manner. (A) Northern blots of total RNA isolated at the indicated times (minutes) after the addition of α-factor (400 ng/ml) and probed for *FKS2*, *FUS1*, *CHS1*, and *ACT1* mRNAs. (B) Pheromone induction of *FKS2* mRNA is calcineurin dependent. Total RNA was isolated from strains YFF2066 (*bar1*Δ) (wild type [wt]) and YFF3162 (*bar1*Δ*cna1*Δ*cna2*Δ*cnb1*Δ) (CnΔ) at 0, 30, and 75 min after the addition of α-factor (400 ng/ml) and was probed for *FKS1*, *FKS2*, and *ACT1* mRNAs. Relative mRNA levels were calculated by dividing the *FKS1* and *FKS2* mRNA levels by the *ACT1* mRNA level and setting the *ACT1*-normalized *FKS1* mRNA level in the wild type at 0 min and the normalized *FKS2* mRNA level in the wild type at 75 min equal to one.

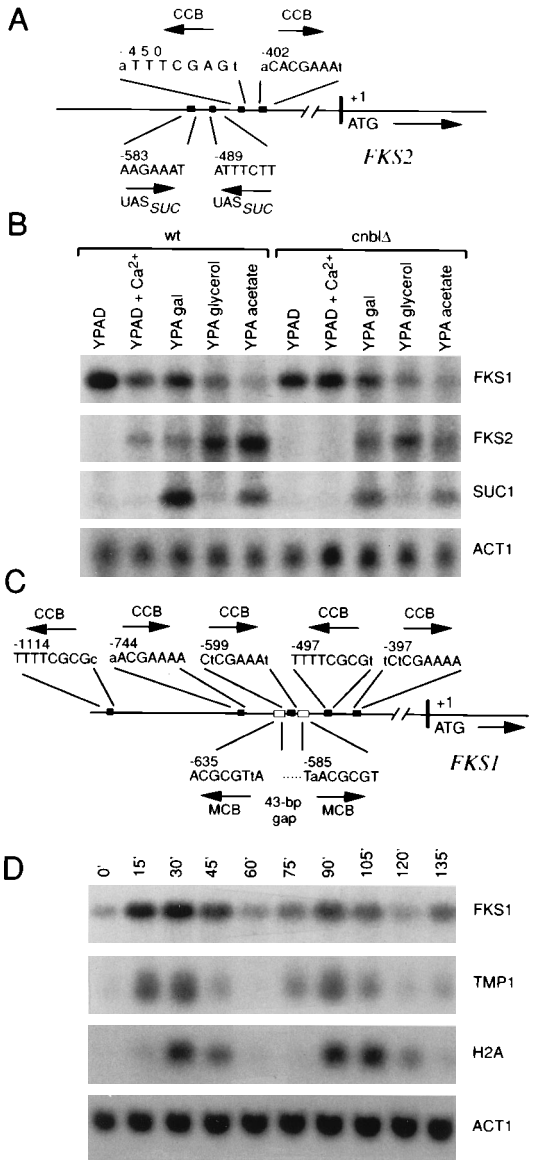

FIG. 7. Carbon source and cell cycle regulation of *FKS1* and *FKS2*. (A) Locations, sequences, and orientations of two CCB and two UAS_{SUC} motifs upstream of *FKS2*. Capital letters in the sequences of the motifs indicate matches to the consensus sequences. For the CCB element, both the stringent (57) and relaxed (60) consensus sequences were used. (B) Expression of *FKS2* in the absence of glucose is largely calcineurin independent. Total RNA was isolated from strains YFK016-28D (wild type [wt]) and YFF1872 (*cnb1*Δ) grown on the indicated media and probed for *FKS1*, *FKS2*, *SUC1*, and *ACT1* mRNAs. (C) Locations, sequences, and orientations of five CCB and two MCB motifs upstream of *FKS1*. (D) *FKS1* mRNA levels are cell cycle dependent. Northern blot analysis of *FKS1*, *TMP1*, histone H2A, and *ACT1* mRNAs was carried out following release from mating pheromone arrest. Strain YFF2066 (*bar1*Δ) was synchronized in G_1 by treatment with 400 ng of α-factor per ml for 150 min at 30°C followed by release into medium without α-factor at 0 min.

the presence of sequence elements potentially involved in activation of carbon source-regulated genes (66). This analysis revealed the presence of two carbon source-regulatory UAS_{SUC} binding motifs with the consensus sequence 5′-AA GAAAT (33, 65) (Fig. 7A). Total RNA was isolated from wild-type and *cnb1*Δ cells grown on glucose, glucose plus Ca^{2+}, galactose, glycerol, or acetate and probed for *FKS1*, *FKS2*, *SUC1*, and *ACT1* mRNAs (Fig. 7B). The levels of *FKS2* mRNA increased progressively, and those of *FKS1* mRNA decreased, when glucose was replaced with galactose, glycerol, or acetate, in that order. The levels of *SUC1* mRNA were high on galactose and acetate but low on glucose and glycerol. The expression of *FKS2* was mostly unaffected by the presence or absence of calcineurin except when cells were grown on glucose plus Ca^{2+}. The levels of Fks1p and Fks2p in wild-type and *cnb1*Δ cells grown on acetate were also measured by Western blot analysis of microsomal membrane preparations (Fig. 5C). In both cases, the levels of Fks2p were increased with a concomitant decrease in those of Fks1p, relative to levels in cells grown on glucose. The relative levels of Fks1p and Fks2p are very similar to those observed in the wild type grown on glucose in the presence of calcium. The presence of Fks2p in cells grown on acetate was unaffected by the presence or absence of calcineurin.

Accumulation of *FKS1* mRNA is periodic during the cell cycle. The nucleotide sequence upstream of the *FKS1* promoter possesses several sequence elements involved in the cell cycle regulation of many genes (Fig. 7C). These include two exact matches to the MCB (*Mlu*I cell cycle box) element, with

TABLE 2. Glucan synthase from mutants grown with and without Ca^{2+}

Strain	Relevant genotype	Ca^{2+}	Glucan synthase sp act[a]	L-733,560 IC_{50} (μM) for glucan synthase[b]
YFK016-28D	Wild type	−	6.6 ± 1.2 (4)	1.9 ± 0.8 (4)
		+	10.2 ± 2.5 (3)	0.6 ± 0.4 (3)
YFF2936	*fks1*Δ	−	1.8 ± 0.9 (6)	0.4 ± 0.03 (2)
		+	8.5 ± 2.2 (6)	0.4 ± 0.2 (2)
YFF2714-5B	*fks2*Δ	−	9.1 ± 1.4 (6)	1.8 ± 0.9 (2)
		+	4.3 ± 2.1 (6)	2.4 ± 0.4 (2)
YFF1872	*cnb1*Δ	−	4.6 ± 0.6 (2)	1.8 ± 0.9 (2)
		+	5.2 ± 0.1 (2)	1.5 ± 0.5 (2)

[a] Glucan synthase was assayed in cells grown on glucose as described previously (18). The specific activity was determined from the rate of accumulation of labeled glucose from UDP-glucose into trichloroacetic acid precipitate over the time interval of 60 to 120 min and is given in units of nanomoles per minute per milligram of protein. Results are expressed as means ± standard deviations, and the number of samples assayed is shown in parentheses.

[b] IC_{50}, 50% inhibitory concentration. Results are expressed as means ± standard deviations, and the number of samples assayed is shown in parentheses.

the consensus sequence 5′-ACGCGTNA (51), and five close matches to the CCB (cell cycle box) element, with the consensus sequence 5′-CACGAAAA (3, 4, 7, 57, 60). To determine whether *FKS1* is cell cycle regulated, α-factor was used to synchronize *bar1*Δ cells. On YPAD, the *FKS1* mRNA level, initially low, was maximal at 15 to 30 min in the first cycle after removal of mating factor (Fig. 7D). The level of *ACT1* message was fairly constant throughout the experiment. The levels of *FKS1* mRNA correlated with those of *TMP1*, an MCB-regulated gene (50). The level of histone H2A mRNA reached a maximum approximately 10 min later (32). The cell cycle regulation of *FKS1* was also found by Ram et al. (63). *FKS2* was induced by pheromone during the arrest stage and decayed to undetectable levels with a half-life of about 30 min after the removal of pheromone (data not shown). When the medium was supplemented with 10 mM $CaCl_2$, *FKS2* was expressed continuously during both the arrest and release stages with no evidence of cell cycle regulation, while *FKS1* was cell cycle regulated in the same manner as in the absence of $CaCl_2$ (data not shown). We were unable to determine whether *FKS2* is cell cycle regulated during growth on YPA-acetate, since growth did not appear to start synchronously after pheromone arrest under these conditions.

Properties of glucan synthase in the disrupted mutants. We prepared extracts from the wild type, the two disruption mutants, and a calcineurin mutant grown on glucose as the carbon source with and without Ca^{2+} and determined the level and drug sensitivity of the glucan synthase activity present in each (Table 2). As shown previously (18), the level of glucan synthase activity in the *fks1*Δ mutant grown in the absence of Ca^{2+} is lower than that in, and the enzyme is more sensitive to L-733,560 than is the enzyme in, the wild type. In the presence of Ca^{2+}, the level of enzyme activity in the *fks1*Δ mutant increased approximately fivefold and sensitivity to the drug remained high, while in the wild type the level of enzyme activity increased slightly and sensitivity to the drug increased threefold. In the *fks2*Δ mutant the level of enzyme activity was about twofold less in the presence of Ca^{2+} than in its absence, while sensitivity to the drug remained low. This is consistent with the finding that the sensitivity of the *fks2*Δ mutant in vivo to L-733,560 is indistinguishable from that of the wild type (data not shown) and indicates that a higher level of drug sensitivity is associated with Fks2p. In the *cnb1*Δ mutant the levels of enzyme activity were similar in the presence or absence of Ca^{2+}, and the drug sensitivity remained low. The levels of glucan synthase activity correlate with the level of Fks1p plus Fks2p as determined by Western blot analysis (compare Table 2 and Fig. 5). This is particularly evident with respect to the effects of Ca^{2+} in the *fks1*Δ and *fks2*Δ mutants.

DISCUSSION

1,3-β-D-Glucan synthase requires at least two subunits for full activity, as demonstrated by the separation of the enzyme activity into soluble (GTP-binding) and membrane (catalytic) fractions (39, 53). Several lines of evidence show that the *FKS* genes encode subunits essential for activity of the membrane fraction of glucan synthase. Fks1p and Fks2p are both integral membrane proteins and, as determined by Western blot analysis, are associated with the membrane fraction of the cell. The levels of glucan synthase activity in vitro correlate with the levels of Fks1p and Fks2p. In particular, disruption of *FKS1* reduces glucan synthase activity, and this reduction can be remedied by the presence of Ca^{2+} in the growth medium with a concomitant elevation in the level of Fks2p. Moreover, solubilized glucan synthase activity from the null mutant can be quantitatively immunodepleted with anti-Fks2p antibody. Other mutations in *FKS1* confer echinocandin resistance in vivo, and this resistance is associated with the membrane fraction of the enzyme in vitro (18, 19). A strain carrying the *fks1*Δ allele is more sensitive to L-733,560 than the wild type, and the residual glucan synthase activity in this strain is more sensitive to the drug in vitro (18). These results suggest that the enzyme containing Fks2p is more sensitive to the drug than that containing Fks1p. Consistent with this idea, we find that growth in the presence of Ca^{2+} increases the drug sensitivity of the enzyme in the wild type, reflecting the increase in the level of Fks2p under these conditions. This increase in sensitivity does not occur in *fks2*Δ or *cnb1*Δ mutants, as expected. An additional mutant allele of *FKS1* (*cwh53*) was isolated in a cell wall mutant screen based on sensitivity to the cell wall perturbant calcofluor white. The mutant cells show a reduction in cell wall glucose of 75%, indicating that they are affected in the synthesis of 1,3-β-D-glucan (63).

The association of the *FKS* gene products with the catalytic activity suggests that these genes may encode catalytic subunits. However, the possibility of other glucan synthase subunits being present in the membrane fraction or the solubilized enzyme cannot be ruled out. The topological similarity of Fks1p and Fks2p to many transporters suggests a possible role for these proteins in transport of the growing glucan polymer across the membrane. Recently we have learned that another group of investigators has solubilized and purified yeast glucan synthase. They obtained peptide sequences and were able to clone two genes, *GSC1* and *GSC2* (GenBank accession numbers D42126 and D42127, respectively), corresponding to *FKS1* and *FKS2*, respectively (35). These workers obtained a monoclonal antibody which inhibited glucan synthase activity as well as an immunoprecipitating antibody (74). Their results showing that these genes encode subunits of 1,3-β-D-glucan synthase are in agreement with our own, and the isolation of a neutralizing monoclonal antibody provides evidence that these subunits may be catalytic.

The synthetic lethality of *fks1*-Δ*1*::*HIS3* and *fks2*-Δ*1*::*TRP1* strongly supports the idea that the subunits encoded by these genes have an essential overlapping function and that 1,3-β-D-glucan is an essential component of the yeast cell wall. It

provides further evidence that the antifungal antibiotics such as the pneumocandins, echinocandins, and papulacandins, which are inhibitors of glucan synthase, can act solely by inhibiting this enzyme (45, 72). Moreover, it appears that in *S. cerevisiae* both forms of the enzyme must be inhibited by L-733,560 to achieve a fungicidal effect.

Since Fks1p and Fks2p seem to be alternative subunits with similar functions, it is interesting that their regulation often appears to be opposite: when the level of one is high, that of the other is low. This is true for carbon source regulation, regulation by external calcium, and regulation by pheromone. This phenomenon may also account for the fact that the redundancy of Fks1p and Fks2p appears to be partial in both directions. Disruption of *FKS1* results in poor growth compared with that of the wild type; thus, *FKS2* only partially compensates for the lack of *FKS1*. However, the growth defect is at least partly due to low levels of expression of *FKS2* under these conditions and is largely eliminated by the addition of Ca^{2+} to the growth medium, which we have shown increases the level of expression of *FKS2*, and by the expression of *FKS2* from the *ADH1* promoter (data not shown). Thus, Fks2p appears to be fully competent to substitute for Fks1p, when it is expressed at a high enough level.

Disruption of *FKS2*, on the other hand, although it does not affect growth, does result in a sporulation defect which is not complemented by the presence of one or two intact chromosomal copies of *FKS1*. The yeast spore wall has been reported to contain glucan (38). Thus, *FKS2* seems to be important for the formation of this apparently essential spore wall polymer. *FKS1* is likely to be poorly expressed on sporulation medium, which contains acetate as the sole source of carbon. The sporulation defect of *fks2*Δ/*fks2*Δ diploids is partially complemented when *FKS1* is expressed from a plasmid. Northern data show that expression of *FKS1* from this plasmid is much higher than that from a chromosomal copy (data not shown). These results imply that, if expressed, Fks1p can substitute functionally for Fks2p in the synthesis of spore wall glucan. It is also important to note that the increase in expression of *FKS2* in the absence of glucose during vegetative growth and its strong induction by mating pheromone indicate that Fks2p plays roles in the formation of glucan under these other conditions as well and does not appear to be solely involved in the formation of spore walls. The cell cycle regulation of *FKS1* suggests that Fks1p may be important for the synthesis of 1,3-β-D-glucan in the bud during growth on glucose. Although we were unable to demonstrate cell cycle regulation of *FKS2*, Fks2p may play a similar role during growth on acetate.

Calcineurin has been shown to regulate the renal Na^+, K^+-ATPase (5) and a plant K^+ channel (48). In *S. cerevisiae*, calcineurin is essential for Na^+, Li^+, and Mn^{2+} tolerance (14, 15, 52, 55, 56, 58). It may also be involved in postranslational modification of the high-affinity K^+ transport system that facilitates better discrimination of K^+ over Na^+ (52). It has also been suggested that calcineurin leads to an increase in Ca^{2+} pump activity (14). Thus, a major role for calcineurin in many cell types is the regulation of cation transport. We tested the *fks2*Δ mutant for sensitivity to LiCl and $MnSO_4$ but did not find any differences from the wild type. Thus, the failure to express *FKS2* is not responsible for the salt sensitivity phenotypes of calcineurin null mutants.

In T cells FK506 and cyclosporin A inhibit the proliferation response to antigen (69). Calcineurin is the target of these drugs in vivo (46, 67). The binding of antigen to the T-cell receptor at the cell surface results in an increase in cytosolic Ca^{2+} and activation of calcineurin, leading to the activation of transcription factors involved in the expression of the interleukin-2 gene (11, 23, 49, 59, 61, 68). This activation is probably due to the direct dephosphorylation of a component of the transcription factor NF-AT (36). The mechanism of inhibition of calcineurin by FK506 and cyclosporin A is highly conserved between mammals and *S. cerevisiae* (22). Previous work has shown that certain mutations in *FKS1* result in Ca^{2+}/calcineurin-dependent growth and hypersensitivity of growth to FK506 and cyclosporin A (8, 21, 24, 62). The Ca^{2+}/calcineurin-dependent expression of *FKS2* and the essentiality of the *FKS1-FKS2* gene pair account for these phenotypes, since in the absence of *FKS1*, the growth of the cells is completely dependent on the expression of *FKS2*. The amelioration of the growth defect of the *fks1-Δ1::HIS3* mutant by the addition of 10 mM $CaCl_2$ to the growth medium is possibly due to an increase in the level of cytosolic Ca^{2+}, which activates calcineurin and the transcription of *FKS2*. Others have shown that elevation of external Ca^{2+} to 10 mM increases cytosolic Ca^{2+} from about 0.1 to 0.3 μM (30), which is in the range activating calcineurin (71). One can speculate that similar to the case for T cells, calcineurin dephosphorylates a yeast transcription factor which directly activates transcription of *FKS2*, or calcineurin might dephosphorylate another cellular component which indirectly activates *FKS2*. Alternatively, transcription of *FKS2* might be regulated by a Ca^{2+}-activated component other than calcineurin. It has been suggested that calcineurin decreases Ca^{2+} sequestration in the vacuole (14); thus, inactivation of calcineurin by mutation might prevent cytosolic Ca^{2+} signalling.

FKS2 is strongly induced by mating pheromone after a delay of about 45 to 60 min, and this induction is calcineurin dependent. The addition of pheromone to yeast haploids leads to an increase in cytosolic Ca^{2+} from 0.1 to 0.6 μM after a delay of about 40 to 50 min, and this increase is restricted to shmoos (34, 54). It is therefore possible that the induction of *FKS2* is due to the activation of calcineurin by the increase in cytosolic Ca^{2+} at this time. Alternatively, calcineurin (see above) may be required for the generation of the pheromone-induced Ca^{2+} signal. *FUS1* and *CHS1*, which are induced within 15 min after the addition of pheromone (Fig. 6A), each have four copies of the pheromone response element in their promoters. Two potential elements are found upstream from *FKS2*. There may be additional sequence motifs in the *FKS2* promoter which are responsive to the Ca^{2+} signal.

If cells are deprived of external Ca^{2+} during prolonged exposure to high levels of mating pheromone, there is a decrease in cell viability (34). This decrease in cell viability correlates with the formation of shmoos. A similar decrease in viability following exposure to high concentrations of pheromone occurs in mutants lacking calcineurin (75). These observations suggest that the failure of cells lacking calcineurin to recover from mating factor arrest (16, 17) may be a consequence of the loss of cell viability due to abnormal synthesis of the cell wall during shmoo formation rather than of a defect in the recovery process per se. It is possible that *FKS2* is induced under these conditions precisely because it is involved in new wall synthesis during this process. However, we found that the ability of the *fks2-Δ1::TRP1* deletion mutant to recover from mating factor arrest is unimpaired, there is no decrease in cell viability upon exposure to pheromone, and shmoos appear to form normally. The nonessential nature of *FKS2* in this process may be due to sufficient expression of *FKS1* under these conditions to compensate for the lack of *FKS2* and suggests that there may be other genes induced by pheromone in a calcineurin-dependent manner which, either in combination with *FKS2* or independently, are essential. It is also relevant that neither calcineurin nor *FKS2* is required for mating. This may be due to the fact

that mating occurs quite rapidly under typical laboratory conditions and the loss in cell viability does not have a chance to occur. Under conditions in which mating is delayed, for instance, when the mating partners are at a great distance, calcineurin may be more important.

In conclusion, the differences in the phenotypes caused by mutations in *FKS1* and *FKS2* and the differences in the regulation of these genes suggest that the cell has two distinct forms of 1,3-β-D-glucan synthase, one containing Fks1p and the other containing Fks2p, and that these two forms have different functions within the cell.

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