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Temperature-Induced Expression of Yeast *FKS2* Is under the Dual Control of Protein Kinase C and Calcineurin

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FKS1 and FKS2 are alternative subunits of the glucan synthase complex, which is responsible for synthesizing 1,3-β-glucan chains, the major structural polymer of the *Saccharomyces cerevisiae* cell wall. Expression of *FKS1* predominates during growth under optimal conditions. In contrast, *FKS2* expression is induced by mating pheromone, high extracellular [Ca^{2+}], growth on poor carbon sources, or in an *fks1* mutant. Induction of *FKS2* expression in response to pheromone, $CaCl_2$, or loss of *FKS1* function requires the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. Therefore, a double mutant in calcineurin (*CNB1*) and *FKS1* is inviable due to a deficiency in *FKS2* expression. To identify novel regulators of *FKS2* expression, we isolated genes whose overexpression obviates the calcineurin requirement for viability of an *fks1* mutant. Two components of the cell integrity signaling pathway controlled by the RHO1 G protein (*MKK1* and *RLM1*) were identified through this screen. This signaling pathway is activated during growth at moderately high temperatures. We demonstrate that calcineurin and the cell integrity pathway function in parallel, through separable promoter elements, to induce *FKS2* expression during growth at 39°C. Because RHO1 also serves as a regulatory subunit of the glucan synthase, our results define a regulatory circuit through which RHO1 controls both the activity of this enzyme complex and the expression of at least one of its components. We show also that *FKS2* induction during growth on poor carbon sources is a response to glucose depletion and is under the control of the SNF1 protein kinase and the MIG1 transcriptional repressor. Finally, we show that *FKS2* expression is induced as cells enter stationary phase through a *SNF1*-, calcineurin-, and cell integrity signaling-independent pathway.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (4, 20). Vegetative proliferation requires that the cell remodels its wall to accommodate growth. The main structural components responsible for the rigidity of the yeast cell wall are 1,3-β-linked glucan polymers with some branches through 1,6-β linkages. The biochemistry of the yeast enzyme complex that catalyzes the synthesis of 1,3-β-glucan chains has been studied extensively (15, 29), and three genes that encode components of this complex have been identified. A pair of closely related genes, *FKS1* and *FKS2*, encode alternative subunits of the 1,3-β-glucan synthase (GS) (8, 15, 28, 36). Either *FKS1* or *FKS2* function is sufficient for GS activity and cell viability. Additionally, the Rho1 GTPase is an essential regulatory subunit of the GS complex, serving to stimulate GS activity in a GTP-dependent manner (9, 35).

A second essential function of RHO1 is to regulate the cell integrity signaling pathway by binding and activating protein kinase C (19, 33), which is encoded by *PKC1* (25). Loss of *PKC1* function results in a cell lysis defect that is attributable to a deficiency in cell wall construction (23, 24, 34). Components on one branch of the *RHO1-PKC1*-regulated signaling pathway comprise a linear mitogen-activated protein kinase (MAPK) activation cascade. These include a MEK kinase (MEKK) homolog (*BCK1* [5, 22]), a redundant pair of MEK homologs (MKK1 and MKK2 [16]), and a MAPK homolog (*MPK1* [21], initially designated *SLT2* [40]). Deletion of any of these components results in cell lysis when cells are cultivated under conditions of mild thermal stress (i.e., 37 to 39°C). Elevated growth temperature, thought to pose a challenge to the cells' ability to construct adequate cell walls, also induces persistent activation of MPK1 (18). The regulatory output of the cell integrity signaling pathway is only now beginning to be explored.

The *FKS1* and *FKS2* genes differ primarily in the manner in which their expression is controlled. Under optimal growth conditions, *FKS1* is the predominantly expressed gene, and its mRNA levels fluctuate periodically through the cell cycle (28, 36). Expression of *FKS2* is low under optimal growth conditions, but expression is induced in response to treatment with mating pheromone, $CaCl_2$, or growth on poor carbon sources or in the absence of *FKS1* function (28). The pathway for induction of *FKS2* expression by pheromone or $CaCl_2$ or in *fks1* mutants requires the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (PP2B [6, 39]). However, *FKS2* induction by poor carbon sources is calcineurin independent.

A double mutant in calcineurin (*CNB1*) and *FKS1* is inviable due to a deficiency in *FKS2* expression (11). To identify novel regulators of *FKS2* gene expression, we have isolated genes whose overexpression obviates the calcineurin requirement for viability of an *fks1* mutant. In this study, we describe the isolation of *MKK1*, a component of the cell integrity signaling pathway (16), and *RLM1*, a putative transcription factor that has also been implicated in cell integrity signaling (7, 42, 43), as positive regulators of *FKS2* expression. We demonstrate that calcineurin and the cell integrity pathway function in parallel, through separable promoter elements, to induce *FKS2* expression under conditions of thermal stress and thereby provide the first clear evidence for a direct target of cell integrity signaling.

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

Strain	Genotype	Source or reference
1783	*MAT***a**[a]	I. Herskowitz
1788	*MAT***a**/*MAT*α[a]	I. Herskowitz
DL1009	*MAT***a**/*MAT*α *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1* (YEp351[*mpk1* T190A, Y192F::HA])[a]	This study
DL251	*MAT***a**/*MAT*α *bck1*Δ::*URA3*/*bck1*Δ::*URA3*[a]	22
GMY63-5D	*MAT***a** *rlm1*Δ::*LEU2*[a]	K. Irie
PGY440	*MAT*α *mpk1*Δ::*TRP1 fks1*Δ::*hisG*[a]	This study
PGY482	*MAT***a** *rlm1*Δ::*LEU2 fks1*Δ::*hisG*[a]	This study
YPH499	*MAT***a**[b]	P. Hieter
MCY3-1D	*MAT***a** *cnb1*Δ::*LEU2*[b]	11
PGY5	*MAT***a** *fks1*Δ::*URA3*[b]	11
PGY220	*MAT***a** *fks1*Δ::*TRP1*[b]	This study
PGY318	*MAT***a** *fks1*Δ::*HIS3 cnb1*Δ::*ADE2* (pGAL[*CNB1*])[b]	This study
MCY1093	*MAT***a**[c]	M. Carlson
MCY1846	*MAT***a** *snf1*Δ*10*[c]	M. Carlson
YM4797	*MAT***a** *trp1-901 tyr1-501 met? can1?*[d]	M. Johnston
YM4807	*MAT***a** *mig1*Δ::*ura3*::*LYS2 met*$^-$ *can1*[rd]	M. Johnston

[a] EG123 background: *leu2-3,112 trp1-1 ura3-52 his can1.*
[b] YPH499 background: *ura3-52 lys2-801 ade2-101 trp-*Δ*63 his3-*Δ*200 leu 2-*Δ*1.*
[c] MCY1093 background: *lys2-801 ura3-52 his4-539.*
[d] Congenic strains.

We demonstrate also that *FKS2* induction in response to glucose depletion is under the control of the SNF1-regulated MIG1 transcriptional repressor (31, 32). Finally, we show that *FKS2* expression is induced strongly as cells enter stationary phase and that this induction is not mediated by any of the known regulatory inputs for *FKS2* expression.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in YEP (1% Bacto-Yeast Extract, 2% Bacto-Peptone) supplemented with 2% glucose. Synthetic minimal medium (SD [37]) supplemented with the appropriate nutrients was used to select for plasmid maintenance. Yeast transformations were carried out by the lithium acetate method (17). *Escherichia coli* DH5α was used for the propagation of all plasmids. *E. coli* cells were cultured in Luria broth medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl) and transformed by standard methods (26).

Isolation of high-copy-number suppressors of a *cnb1*Δ *fks1*Δ mutant. PGY318 was transformed with one of two yeast genomic libraries constructed in high-copy-number *LEU2*-bearing plasmids, either YEp351 (13) or YEp13 (ATCC), and was grown at 25°C on solid synthetic medium lacking leucine and containing glucose. A portion of each transformation was grown on solid synthetic medium lacking leucine and containing galactose to estimate the transformation efficiency. Selection for plasmid loss was carried out by using the uracil biosynthesis antagonist 5-fluoro-orotic acid (5-FOA) (1). An FK506 sensitivity test was done by spotting the cells onto medium containing 2 mg of FK506/ml.

Plasmid construction. Plasmids pLGΔ-178 and pLGΔ-312, which contain the *CYC1-lacZ* fusion genes were described previously (12). Plasmid *FKS2*(−928 to −1)-*lacZ* was constructed by two steps. First, pCZ-*FKS2* was created by inserting a 955-bp PCR-amplified *FKS2* fragment into the *Xba*I/*Bam*HI sites of pCZ4 (2). PCR was carried out with the primer pair 5′-GGCCCGCTCTAGAATCTTCC GATCATCATCATCGGCGCGTTC-3′ (sense) and 5′-TCGGATCCGGTCAT AACTATGACAGTTTAATAAT-3′ (antisense). (The *Xba*I and *Bam*HI sites used for cloning are underlined.) Second, a *Sal*I/*Bam*HI fragment from pCZ-*FKS2* was excised and cloned into the *Xho*I/*Bam*HI sites of pLGΔ-178. To generate plasmid *FKS2*(−706 to −1)-*lacZ*, a 713-bp PCR-amplified *FKS2* fragment was placed in frame into the *Xho*I/*Bam*HI sites of pLGΔ-178, which had been made blunt with Klenow fragment. PCR was carried out with the primer pair 5′-TGGTGATGGGGTGTAGG-3′ (sense) and 5′-CGGACATAACTATG ACAG-3′ (antisense). Plasmid *FKS2*(−706 to −361)-*CYC1-lacZ* was constructed by replacing the 134-bp *Sma*I/*Xho*I fragment of the *CYC1* promoter of pLGΔ-312 with a 345-bp PCR-amplified *FKS2* fragment, resulting in the removal of the *CYC1* upstream activation sequence sites from the promoter region and the fusion of *FKS2* upstream sequence from −706 to −361 to the *CYC1* minimal promoter. PCR was carried out with the primer pair 5′-TGGTGATGGGGTG TAGG-3′ (sense) and 5′-AATACACTCGAGAAGCTTTTATTTTTGTA-3′ (antisense). (The *Xho*I site used for cloning is underlined.) All numbers are given with respect to the translational start codon.

RNA analysis. Strains were grown to a density of 1.5×10^7 to 3×10^7 cells/ml (4.5×10^8 to 6×10^8 cells/ml for stationary-phase cells), collected by centrifugation at 1,500 × *g* for 5 min at 4°C, washed once with 1 ml of ice-cold diethylpyrocarbonate-H_2O, frozen on dry ice, and stored at −70°C. Total RNA was prepared by resuspending the cell pellet in 400 μl of TES solution (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% sodium dodecyl sulfate), adding 400 μl of acidic phenol, vortexing vigorously for 10 s, and incubating for 1 h at 65°C with occasional, brief vortexing. The mixture was placed on ice for 5 min, and after centrifugation at 13,000 × *g* in a microcentrifuge for 5 min, the aqueous phase was removed. The RNA was purified by phenol and chloroform extractions and precipitated with 100% ethanol in the presence of 0.3 M sodium acetate (pH 5.3) at −20°C. RNA was separated on a 1% formaldehyde-agarose gel (1% agarose, 20 mM MOPS [morpholinepropanesulfonic acid], 1 mM EDTA, 5 mM sodium acetate, 2.2 M formaldehyde), transferred to a Hybond-N membrane (Amersham) as described in Maniatis et al. (26), and cross-linked by incubation in a vacuum incubator at 80°C for 2.5 h. Blots were probed with radiolabelled restriction fragments (*ACT1* and *RHO1*) or PCR-amplified fragments (*FKS1* and *FKS2*). The following primer pairs were used to synthesize *FKS1* and *FKS2* fragments, respectively: 5′-ATGAACACTGATCAAC-3′ (sense) and 5′-AATT ACCGTAAATTGG-3′ (antisense) and 5′-ATGTCCTACAACGATCC-3′ (sense) and 5′-GAACCATCTTGATCAGG-3′ (antisense). The probes for *FKS1* and *FKS2* hybridize to a region encoding the divergent N termini of each protein product. RNA levels were quantitated by exposing the membrane to a Fuji phosphorimaging plate and the phosphorimaging data were analyzed by MacBAS 2.0 software. Autoradiographs of membranes were used for figures.

β-Galactosidase assays. Cells were harvested by centrifugation at 3,000 × *g* for 5 min and resuspended in 300 μl of breaking buffer (100 mM Tris-HCl [pH 8.0], 1 mM dithiothreitol, and 20% glycerol). The cells were not to be used immediately, and they were quickly frozen at −70°C and stored at −20°C. An equal volume of glass beads, 2 μl of phenylmethylsulfonyl fluoride (10 mg/ml), and 1 μl of leupeptin (10 mg/ml) were added to this suspension, and cells were broken by vigorous vortexing for 4 min. After removal of the beads and cell debris by centrifugation at 13,000 × *g* for 1 min, the supernatant was further clarified by additional centrifugation for 10 min. All steps were carried out at 4°C. Protein concentrations of cell extracts were measured by the Bradford method (Bio-Rad). β-Galactosidase assays were carried out as previously described (37). Specific activities were given in nanomoles of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) converted per minute per milligram of protein. Data presented are mean values from two or three experiments.

GS assays. Crude extracts were prepared as previously described (18) and stored at −80°C in lysis buffer supplemented with 33% glycerol. GS activity was measured as previously described (29) with the following modifications: uridine diphosphate-[^{3}H]glucose was used as the substrate and α-amylase (1 U/40 μl) was added to reaction mixtures to eliminate [^{3}H]glucose incorporation into glycogen.

RESULTS

Isolation of *MKK1* and *RLM1* as high-copy-number suppressors of a *cnb1*Δ *fks1*Δ mutant. To identify novel regulators of *FKS2* expression, we screened a high-copy-number genomic yeast library for clones that could suppress the synthetic lethality of a *cnb1*Δ *fks1*Δ mutant, presumably by restoring *FKS2*

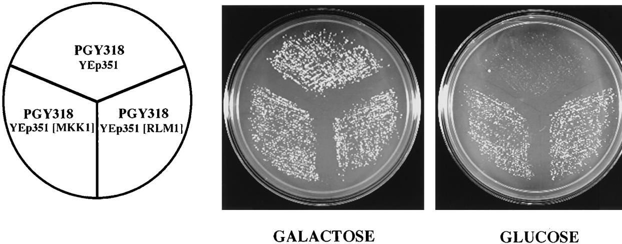


FIG. 1. Suppression of a *cnb1*Δ *fks1*Δ mutant by high-copy-number *MKK1* or *RLM1*. Yeast strain PGY318, transformed with YEp351(*MKK1*), YEp351 (*RLM1*), or YEp351, was streaked onto SD-Leu medium supplemented with 2% glucose or 2% galactose and incubated at 30°C.

expression. We used a *cnb1*Δ *fks1*Δ mutant that carries a plasmid with *CNB1* under the inducible control of the *GAL1-10* promoter (PGY318). This strain grows normally on galactose-containing medium but is inviable on glucose-containing medium. PGY318 was transformed with either of two high-copy-number *S. cerevisiae* genomic libraries, and transformants were screened for the ability to grow on glucose-containing medium. To eliminate galactose regulatory mutants, the *CNB1*-containing plasmid was evicted with 5-FOA (1). A total of 32 5-FOA-resistant transformants were isolated and tested for sensitivity to the calcineurin inhibitor FK506. FK506-sensitive isolates were assumed to carry a *CNB1* gene and were not characterized further. Plasmids from 12 transformants were rescued and back-transformed into PGY318 to confirm that the plasmids allowed this strain to grow on glucose-containing medium. Among 10 plasmids isolated, three subjected to DNA sequence analysis are described here. One of these contained a region of chromosome XV with two complete open reading frames, *MKK1* and *MGE1*. A subclone containing only *MKK1*, a MEK homolog of the cell integrity signaling pathway (16), effectively suppressed the growth defect of PGY318 (Fig. 1). The other two plasmids contained an overlapping region of chromosome XVI that included *RLM1*, which encodes a member of the serum response factor family of transcription factors (38) that has also been implicated in cell integrity signaling (7, 42, 43). A subclone containing only *RLM1* was competent for suppression activity (Fig. 1). The finding that overproduction of either *MKK1* or *RLM1* suppresses the synthetic lethality of a *fks1*Δ *cnb1*Δ mutant suggests that the cell integrity pathway contributes to the expression of *FKS2*.

Additional genetic evidence supports the notion that the cell integrity pathway positively regulates *FKS2* expression. First, as reported previously, an *fks1*Δ *mpk1*Δ double mutant is inviable on glucose-containing medium (11). That result may now be explained by a deficiency in *FKS2* expression, similar to

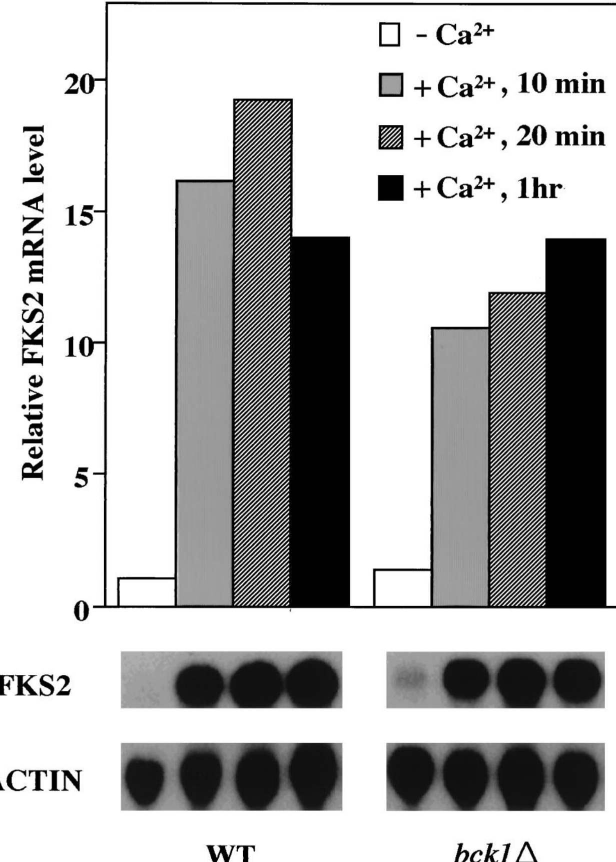


FIG. 3. Calcium induction of *FKS2* gene is independent of cell integrity pathway signaling. Log-phase cultures of wild-type (1788) and *bck1*Δ (DL251) cells were grown at 23°C in the presence or absence of 30mM $CaCl_2$ for the indicated times. Total RNA, isolated after the indicated times, was probed for *FKS2* and *ACT1* mRNAs. *FKS2* mRNA was normalized to *ACT1* mRNA. The *ACT1*-normalized *FKS2* mRNA level from wild-type cells grown in the absence of $CaCl_2$ was arbitrarily designated a value of 1. WT, wild type; ACTIN, *ACT1*.

the synthetic lethality of an *fks1*Δ *cnb1*Δ double mutant. Consistent with this interpretation, we found that expression of *FKS2* from a high-copy-number plasmid suppressed the growth defect of an *fks1*Δ *mpk1*Δ double mutant (Fig. 2). Second, we found that an *fks1*Δ *rlm1*Δ double mutant is inviable on glucose-containing medium and that this defect is suppressed by overexpression of *FKS2* (Fig. 2). Interestingly, both double mutants are viable on galactose-containing medium (see section on glucose starvation below).

Ca^{2+} induction of *FKS2* is not dependent on the cell integrity pathway. Exogenous Ca^{2+} stimulates *FKS2* expression through a calcineurin-dependent pathway (28). We tested the possibility that the cell integrity signaling pathway is also required for Ca^{2+}-dependent *FKS2* expression. A *bck1*Δ mutant, which is defective in the MEKK of the cell integrity pathway and grows normally at room temperature, was used for this experiment. Figure 3 shows that *FKS2* mRNA accumulation was induced rapidly in both wild-type and *bck1*Δ cells grown at 23°C after addition of $CaCl_2$ (30 mM) to the growth medium, indicating that the MAPK branch of the cell integrity pathway is not required for this calcineurin-dependent response.

***FKS2* mRNA accumulates in response to growth at high temperature.** We showed previously that the cell integrity sig-

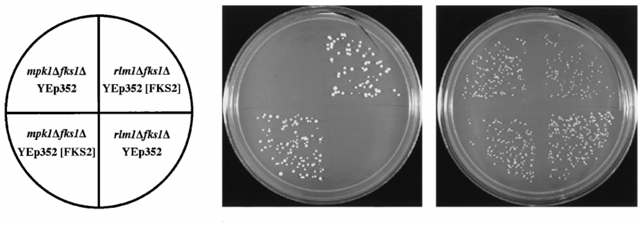


FIG. 2. Suppression of an *mpk1*Δ *fks1*Δ mutant and an *rlm1*Δ *fks1*Δ mutant by high-copy-number *FKS2*. Yeast strains PGY440 and PGY48, transformed with YEp352(*FKS2*) or YEp352, were streaked onto SD-Ura medium supplemented with either 2% glucose or 2% galactose and incubated at 30°C.

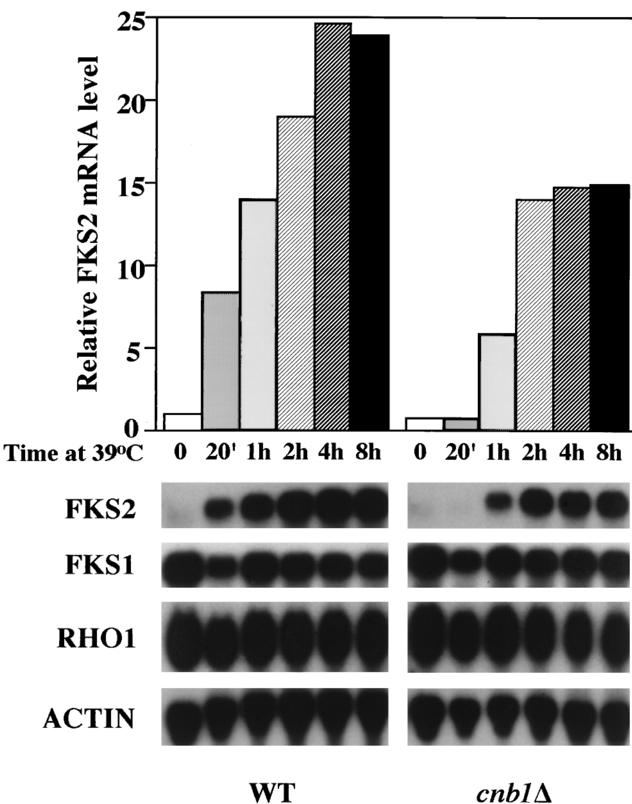


FIG. 4. Temperature-induced accumulation of *FKS2* mRNA. Accumulation of *FKS1*, *FKS2*, and *RHO1* mRNA in response to temperature upshift was examined in wild-type (YPH499) and *cnb1*Δ (MCY3-1D) cells. Cells were grown to an A_{600} of 0.5 to 1 in YEP containing 2% glucose at 23°C. An immediate temperature shift to 39°C was achieved by adding an equal volume of fresh medium prewarmed to 55°C, followed by incubation at 39°C with agitation for the indicated times. Total RNA was probed for *FKS2*, *FKS1*, *RHO1*, and *ACT1* mRNAs. WT, wild type; ACTIN, *ACT1.*

naling pathway is strongly activated in response to growth at moderately high temperatures (i.e., 37 to 39°C [18]). Mutants in cell integrity signaling lyse when cultivated at elevated temperatures due to a deficiency in cell wall construction (18). We have interpreted these findings to indicate that growth under thermal stress poses a challenge to the cells' ability to construct adequate cell walls, to which the cell responds by activation of cell integrity signaling (18). However, the cellular targets that are induced by this signaling have yet to be identified. The observations described above suggested that *FKS2* might be one such target.

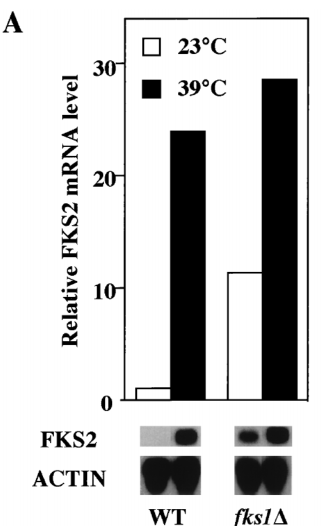
As a first step to determine if the expression of *FKS2* is regulated in response to cell integrity signaling, we examined the effect of growth temperature on the steady-state levels of mRNA from *FKS2*. Figure 4 shows that the level of *FKS2* mRNA, which is barely detectable in cells growing at 23°C, is strongly induced by a shift to growth at 39°C, peaking 2 to 4 h after shift, and persists at the elevated level as long as the cells continue to grow at high temperature (not shown). In contrast, accumulation of neither *FKS1* nor *RHO1* mRNA, which encode the other known components of the GS complex, was induced by growth at high temperature (Fig. 4).

Because FKS1 and FKS2 are catalytic subunits of the GS complex, we examined the effect of growth temperature on GS activity. Wild-type cells shifted from growth at 23°C to growth at 39°C for 6 h strongly induced *FKS2* expression (Fig. 5A), but this induction was accompanied by an increase in GS activity of less than twofold (Fig. 5B). This may be explained by the relatively large contribution of *FKS1* to GS activity (28). In an *fks1*Δ mutant, *FKS2* expression was elevated (relative to wild type) during growth at low temperature, but was further induced (approximately threefold) in response to elevated temperature (Fig. 5A). This induction was accompanied by a fivefold increase in GS activity (Fig. 5B), presumably because all of the GS activity in this mutant is derived from *FKS2*. GS activity in an *FKS2* mutant was unaffected by growth temperature, consistent with the observation that *FKS1* mRNA levels are not influenced by thermal stress (Fig. 4).

Next we examined the mechanism by which *FKS2* expression is induced in response to temperature upshift. Accumulation of *FKS2* mRNA in response to extracellular Ca^{2+} or treatment with mating pheromone is strictly dependent on a calcineurin-mediated pathway (28). Therefore, we examined the effect of increased growth temperature on *FKS2* expression in a calcineurin mutant (*cnb1*Δ [6]). Thermal induction of *FKS2* mRNA accumulation was nearly as strongly induced in this mutant as in the wild type (Fig. 4) but with somewhat delayed kinetics. Most notably, the response observed in the wild-type strain 20 min after shift was absent in the *cnb1*Δ mutant, suggesting that calcineurin may play a role in the early stages of this response to thermal stress.

The cell integrity pathway and calcineurin collaborate to induce *FKS2* expression at high temperature. To determine if the cell integrity signaling pathway is required for thermal induction of *FKS2* mRNA accumulation, we examined a mutant in *MPK1*, the gene encoding the MAPK of this pathway. Because *mpk1* mutants lyse when cultivated at high temperature, we suppressed the growth defect of an *mpk1*Δ mutant by overexpression of a nonactivatable allele of *MPK1* (*mpk1-TAYF* [18]). The basal kinase activity provided by this allele allows survival at otherwise lethal temperatures, but because the mutant kinase cannot be phosphorylated by its activating kinase, it is not subject to regulation (18). Figure 6 shows that *FKS2* mRNA accumulation was induced briefly in the *mpk1* mutant by a shift from growth at 23°C to growth at 39°C, but this induction was transient, with *FKS2* mRNA levels diminishing to nearly the preinduction level by 2 h after shift. Therefore, persistent expression of *FKS2* at elevated temperature requires the MAPK branch of the cell integrity signaling pathway.

To determine if the transient induction of *FKS2* mRNA accumulation observed in the *mpk1* mutant is calcineurin dependent, we pretreated cultures with the calcineurin inhibitor FK506. Wild-type and *mpk1* mutant strains, growing at 23°C, were treated with FK506 (200 ng/ml) for 30 min prior to increasing the temperature to 39°C. As expected, the kinetics of *FKS2* mRNA accumulation in the wild-type strain treated with FK506 (Fig. 6) approximated those observed in the *cnb1*Δ mutant (Fig. 4). Pretreatment of the *mpk1* mutant with FK506 completely blocked *FKS2* mRNA accumulation, indicating that the transient induction observed in this mutant is calcineurin dependent. Therefore, calcineurin collaborates in parallel with the cell integrity signaling pathway to induce the accumulation of *FKS2* mRNA in response to elevated growth temperature. The calcineurin-dependent pathway induces a rapid but transient increase in *FKS2* mRNA, whereas the cell integrity pathway (acting through MPK1) induces a delayed but sustained increase. These pathways appear to function independently of each other because their effects on *FKS2* expression were roughly additive and because both pathways must be blocked to prevent *FKS2* mRNA accumulation completely in response to temperature upshift. This conclusion is



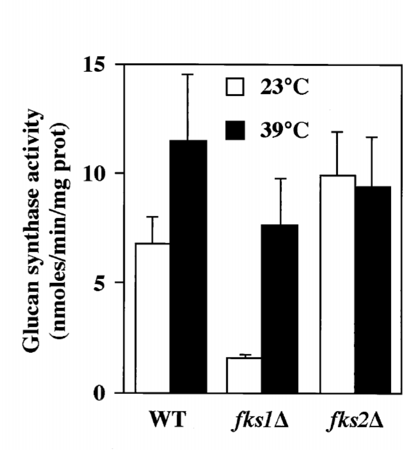


FIG. 5. Effect of growth temperature on GS activity in *fks1*Δ and *fks2*Δ mutants. (A) Cultures of wild-type (YPH499) and *fks1*Δ (PGY5) cells were shifted from log-phase growth at 23 to growth at 39°C. Total RNA, isolated 0 and 6 h after shift, was probed for *FKS2* and *ACT1* mRNA. (B) Log-phase cultures of wild-type (YPH499), *fks1*Δ (PGY5), and *fks2*Δ (PGY220) cells were split and incubated at 23 or 39°C for 6 h. Crude extracts were prepared, and GS activities were determined as described in Materials and Methods. prot, protein; WT, wild type; ACTIN, *ACT1*.

supported by the observation that the cell integrity pathway is not required for the calcineurin-mediated induction of *FKS2* by exogenous Ca^{2+}. Moreover, the calcineurin- and PKC1-responsive sequences in the *FKS2* promoter are separable (see below).

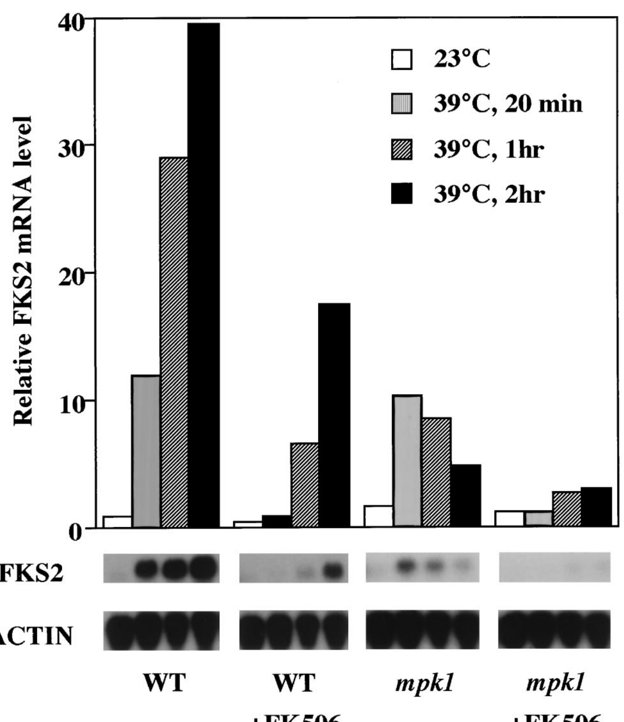


FIG. 6. Effect of an *mpk1* mutation on *FKS2* mRNA accumulation in response to elevated growth temperature. Cell growth and temperature shift were conducted as described in the legend for Fig. 4. Total RNA, isolated at the indicated times from wild type (1788) or an *mpk1* mutant (DL1009), was probed for *FKS2* and *ACT1* mRNA. Cells were pretreated with FK506 by addition of drug to the cultures to a final concentration of 0.2 mg/ml 30 min before temperature shift. WT, wild type; ACTIN, *ACT1*.

Dissecting the *FKS2* promoter. To further define the molecular mechanisms of calcineurin- and cell integrity-dependent control of *FKS2* expression, we created several *FKS2* reporter plasmids. Sequences 5′ to the *FKS2* translational start site, spanning from either nucleotide −706 to −1 or from −928 to −1, were fused to the *E. coli lacZ* gene (encoding β-galactosidase). Table 2 shows that a wild-type strain bearing either of these plasmids induced β-galactosidase activity 10- or 20-fold, respectively, in response to a 24-h shift from growth at 23°C to growth at 39°C. A third reporter plasmid that contains *FKS2* sequences from nucleotide −706 to −360 fused to the basal *CYC1* promoter was also strongly activated by growth at high temperature (Table 2), indicating that the region from −359 to −1 is dispensable for thermal induction of *FKS2* transcription. A strain bearing a control plasmid with no *FKS2* sequences and only the basal *CYC1* promoter expressed similar levels of β-galactosidase activity at both temperatures. We conclude that increased transcription of *FKS2* mRNA, mediated by promoter sequences between nucleotides −928 and −360, accounts for part or all of the observed increase in mRNA accumulation in response to temperature upshift.

We also examined the response of various *FKS2-lacZ* reporter plasmids to exogenous $CaCl_2$. Although the *FKS2* reporter plasmid containing 928 bp of 5′ sequence was induced strongly in response to exogenous Ca^{2+}, which stimulates *FKS2* expression through a calcineurin-dependent pathway (28), the reporter plasmid containing only 706 bp of *FKS2* was unresponsive to this stimulus (Table 2). This indicates that the calcineurin-responsive sequence(s) resides within the 222 bp between nucleotides −928 and −706. Because the reporter that carries *FKS2* sequences from −706 to −360 was stimulated by growth at high temperature, the PKC1-responsive sequences must reside between −360 and −706 and are, therefore, separable from the calcineurin-responsive sequences. These results further support the conclusion that the mechanisms by which calcineurin and cell integrity signaling regulate *FKS2* expression are completely independent.

We next examined the effect of overproducing *MKK1* or *RLM1* from high-copy-number plasmids on *FKS2* expression using the reporter plasmids described above. Overexpression

TABLE 2. Temperature-induced expression of β-galactosidase from *FKS2-lacZ* reporter plasmids[a]

FKS2-lacZ reporter plasmid	β-Galactosidase sp act (nmol/min/mg of protein)		
	23°C	39°C	23°C + Ca^{2+}
-928 FKS2 +1 lacZ	3.3	67.2	51.8
-706 FKS2 +1 lacZ	1.8	17.7	1.9
-706 FKS2 -360 pCYC1 +1 lacZ	1.8	17.1	2.0
pCYC1 +1 lacZ	1.5	2.1	1.9

[a] Wild-type (1788) cells were transformed with the indicated reporter plasmids. Transformants were grown to saturation at 23°C in SD-Ura medium. These cultures were diluted into 20 ml of YEP containing 2% glucose (YEPD) so that subsequent incubation at either 23 or 39°C for 24 h resulted in mid-log-phase cultures (densities of 1.5×10^7 to 3×10^7 cells/ml). For the Ca^{2+} experiment, $CaCl_2$ was added to YEPD cultures to a final concentration of 30 mM, and the mixture was incubated for 24 h at 23°C. Crude extracts were prepared and enzyme activities were determined as described in Materials and Methods.

of *MKK1* or *RLM1* increased the basal level (at 25°C) of β-galactosidase expression from the reporter that contains *FKS2* sequences from −706 to −1 by approximately three- or eightfold, respectively (data not shown). As expected for a signaling component that controls *FKS2* expression through the cell integrity pathway, the region from nucleotide −359 to −1 was dispensable for the effect of *MKK1* overproduction on β-galactosidase expression. Surprisingly, the effect of *RLM1* overproduction was eliminated in the reporter lacking this region (not shown), suggesting that *RLM1* affects *FKS2* expression through a mechanism other than cell integrity signaling. No perfect matches to the consensus DNA binding site for RLM1 (CTA[T/A]$_4$TAG [7]) exist within the 1 kb of sequence 5′ to the *FKS2* translational initiation site, but a lone sequence with a single mismatch to this consensus exists at position −336 (CTAAAGATAG).

The RLM1 transcription factor is not involved in thermal induction of *FKS2* expression. *RLM1* has been proposed to mediate some functions of MPK1 (7, 42, 43). Therefore, even though the cell integrity pathway-responsive sequences in the *FKS2* promoter were separable from the *RLM1*-responsive sequences, we examined an *rlm1*Δ mutant for the ability to accumulate *FKS2* mRNA in response to elevated growth temperature. To eliminate the contribution of the calcineurin-dependent pathway, the *rlm1*Δ strain was pretreated with FK506. Figure 7 shows that this mutant was not impaired for thermal induction of *FKS2*, suggesting that the putative transcription factor does not mediate this MPK1-dependent function. Additionally, the *fks1*Δ *rlm1*Δ double mutant, which fails to grow at 25 to 30°C, is rescued by growth at 38°C (data not shown), consistent with thermal induction of *FKS2* being independent of *RLM1*.

Induction of *FKS2* expression by glucose starvation and entry to stationary phase. Accumulation of *FKS2* mRNA has been reported to increase during growth on nonglucose carbon sources in a calcineurin-independent manner (28). Figure 8A shows that a *bck1*Δ mutant responds similarly to wild type in its accumulation of *FKS2* mRNA in cells growing on galactose, acetate, or glycerol as carbon sources, indicating that this response does not depend on the MAPK branch of the cell integrity pathway. Similarly, an *rlm1*Δ mutant induces *FKS2* expression normally in response to growth on galactose-containing medium (not shown). These results are consistent with the observation that *fks1*Δ *mpk1*Δ and *fks1*Δ *rlm1*Δ double mutants can grow on galactose but not glucose as a carbon source (Fig. 2). To determine if the accumulation of *FKS2* mRNA in response to growth on nonglucose carbon sources might reflect a general response to glucose starvation, we examined *FKS2* mRNA levels in response to a shift from high glucose (2%) to low glucose (0.05%) growth conditions. Within 2 h after shift to low glucose, *FKS2* mRNA levels were induced sevenfold (data not shown), indicating that the absence of glucose rather than the presence of alternate carbon sources is responsible for *FKS2* induction.

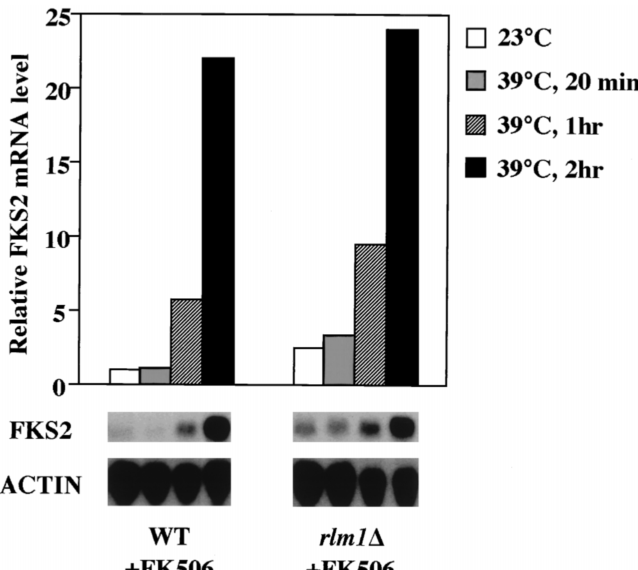
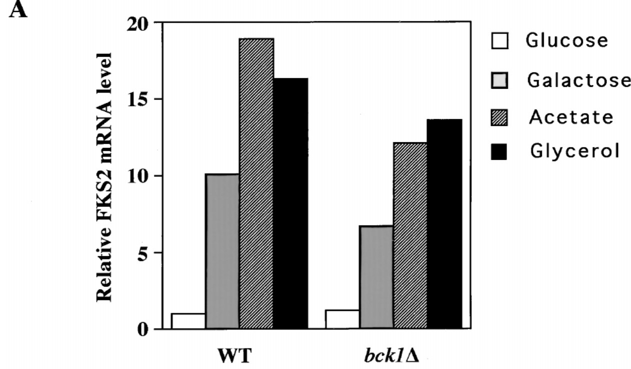


FIG. 7. Transcription factor RLM1 is not required for the temperature-induced expression of *FKS2*. Log-phase cultures of wild-type (1783) and *rlm1*Δ (GMY63-5D) cells, grown at 23°C, were subjected to temperature shift and FK506 treatment as described in the legends for Fig. 4 and 6. Total RNA, isolated at the indicated times after temperature shift, was probed for *FKS2* and *ACT1* mRNAs. WT, wild type; ACTIN, *ACT1*.

A



B

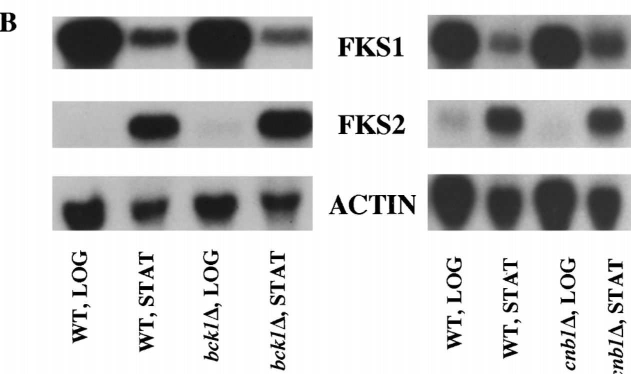


FIG. 8. Induction of *FKS2* expression by poor carbon sources and entry to stationary phase are independent of the cell integrity pathway and calcineurin. (A) Wild-type (1788) and *bck1*Δ (DL251) cells were grown to mid-log phase at 23°C in YEP supplemented with 2% glucose, 2% galactose, 2% glycerol, or 2% sodium acetate. Total RNA was probed for *FKS2* and *ACT1* mRNA. Levels of *FKS2* mRNA are presented relative to that of the wild type grown in YEP supplemented with 2% glucose. (B) Cultures of *bck1*Δ (DL251) and *cnb1*Δ (MCY3-1D) and their isogenic wild-type strains (1788 and YPH499, respectively) were grown in YEP containing 2% glucose. Total RNA, extracted from cultures in log phase (LOG) (1.5×10^7 to 3×10^7 cells/ml) or stationary phase (STAT) (4.5×10^8 to 6×10^8 cells/ml), was probed for *FKS1*, *FKS2*, and *ACT1* mRNAs. WT, wild type; ACTIN, *ACT1.*

Next we examined the effect of nutrient depletion on *FKS2* expression as cells entered stationary phase. Figure 8B shows that although *FKS2* expression is low in cells growing in log phase at 23°C, its mRNA accumulates in cells that have exhausted their glucose supply and entered stationary phase at this temperature. A concomitant decrease in *FKS1* mRNA was observed in stationary-phase cells. Both a calcineurin mutant(*cnb1*Δ) and a cell integrity pathway mutant (*bck1*Δ) behaved similarly to wild-type cells in this response (Fig. 8B), indicating that, like the response to glucose starvation, stationary phase-induced expression of *FKS2* is independent of both signaling pathways.

Because neither calcineurin nor the cell integrity signaling pathway are responsible for starvation-induced expression of *FKS2*, we hypothesized that the glucose derepression pathway controlled by *SNF1* might regulate *FKS2* under these conditions. The protein kinase encoded by the *SNF1* gene is required for derepression of many glucose-repressible genes (3). To determine if induction of *FKS2* mRNA accumulation in response to glucose starvation and entry to stationary phase is dependent on the *SNF1* gene, we tested a *snf1*Δ*10* mutant for transcriptional activation of the *FKS2* reporter plasmids described above. Table 3 shows that the *FKS2* reporter plasmid bearing 928 bp of 5′ sequence was responsive to glucose starvation in *SNF1* cells, but not in an otherwise isogenic *snf1*Δ*10* mutant, indicating that *SNF1*-dependent derepression is responsible for *FKS1* transcriptional induction in response to glucose starvation. In contrast, the reporter with only 706 bp of *FKS2* sequence failed to respond to glucose starvation even in *SNF1* cells, indicating that *SNF1*-dependent sequences reside within the 222-bp region between the endpoints of these reporters.

Mazur et al. (28) identified two putative "carbon source-regulatory sequences" within the *FKS2* promoter region at positions −583 and −489. Our promoter analysis indicates that these sequences are not sufficient for induction of *FKS2* in response to glucose starvation and are probably not involved in this regulation. Rather, the SNF1 protein kinase mediates derepression of glucose-repressible genes by inhibition of the MIG1 transcriptional repressor (41). Examination of the *FKS2* promoter region revealed two consensus MIG1 binding sites (31, 32) at positions −785 and −847. Both of these sites are within the 222-bp region that distinguishes the starvation-responsive *FKS2* reporter from the nonresponsive reporter, strongly suggesting the importance of the MIG1 repressor in starvation-induced expression of *FKS2*. This was supported by the observation that a *mig1*Δ mutant displayed a ninefold greater basal level of expression (on 2% glucose) from the starvation-responsive reporter compared with a congenic *MIG1* strain (Table 3). As anticipated, the *mig1*Δ mutant displayed a less than twofold increase in basal expression from the nonresponsive reporter relative to wild type.

Similar to the effect of glucose starvation, only the reporter plasmid with the longer *FKS2* regulatory sequence was transcriptionally activated upon entry to stationary phase (Table 3). However, unlike the response to glucose starvation, this activation was not *SNF1* dependent. The ability of a *snf1*Δ*10* mutant to derepress *FKS2* expression upon entry to stationary phase reveals the existence of yet another regulatory sequence within the 222-bp region of the *FKS2* promoter between nucleotides −928 and −706.

DISCUSSION

The cell integrity signaling pathway and calcineurin act in parallel to stimulate *FKS2* expression in response to thermal stress. FKS1 and FKS2 are alternative subunits of the GS complex (8, 15, 28, 36). Calcineurin is an important regulator of *FKS2* expression, and thus a double mutant in calcineurin (*CNB1*) and *FKS1* is inviable due to a deficiency in *FKS2* expression (11). To identify additional regulators of *FKS2* expression, dosage-dependent suppressors of a strain bearing deletions in *CNB1* and *FKS1* were isolated. Two of the suppressors isolated through this screen were *MKK1* and *RLM1*, which encode a MEK kinase and a member of the serum response factor family of transcription factors, respectively (16, 42). Both of these genes have been implicated previously in the cell integrity signaling pathway mediated by *RHO1* and *PKC1*. These results suggested that *FKS2* expression is also under the control of cell integrity signaling.

Cell integrity signaling is stimulated strongly by growth at elevated temperatures (e.g., 37 to 39°C) but does not regulate either the well-characterized heat shock response or the expression of stress element (STRE)-controlled stress response genes (18). We found that expression of *FKS2* was stimulated by shift from growth at 23°C to 39°C, though this gene possesses no heat shock elements (30) or STREs (27) within the

TABLE 3. Roles of *SNF1* and *MIG1* in the induction of *FKS2* expression by glucose starvation and entry to stationary phase[a]

FKS2-lacZ reporter plasmid	Strain (gene)	β-Galactosidase sp act (nmol/min/mg of protein)			
		2% glucose	0.05% glucose	Log phase	Stationary phase
-928 FKS2 +1 lacZ	MCY1093 (*SNF1*)	7.9	23.7	7.1	380.4
	MCY1846 (*snf1Δ10*)	14.1	14.7	11.7	368.8
	YM4797 (*MIG1*)	7.5	16.7	ND	ND
	YM4807 (*mig1Δ*)	68.4	96.3	ND	ND
-706 FKS2 +1 lacZ	MCY1093 (*SNF1*)	3.1	3.3	2.5	5.4
	MCY1846 (*snf1Δ10*)	3.7	3.6	4.2	7.5
	YM4797 (*MIG1*)	2.1	2.1	ND	ND
	YM4807 (*mig1Δ*)	3.8	3.8	ND	ND

[a] Log-phase cultures (A_{600} = 0.5 to 1) of *SNF1* (MCY1093), *snf1Δ10* (MCY1846), *MIG1* (YM4797), and *mig1Δ* (YM4807) cells, harboring the indicated reporter plasmids, were grown in YEP containing 2% glucose (YEPD) at 23°C. For glucose starvation, cultures were washed with YEP twice, resuspended in YEP supplemented with 0.05% glucose, and incubated for 3 h at 23°C. Stationary-phase samples were obtained by incubating YEPD cultures at 23°C to an A_{600} of 8 to 11. Samples designated 2% glucose and Log phase were treated identically but were harvested from cultures prior to low glucose and stationary-phase treatments, respectively. Cell extracts were made and enzyme activities measured as described in Materials and Methods.

[b] ND, not determined.

1 kb of sequence 5′ to its translational start site. Similar to the maintenance of cell integrity signaling at high-growth temperature (18), increased expression of *FKS2* was sustained indefinitely while cells continued to grow at elevated temperature. Three lines of evidence indicate that the cell integrity pathway and calcineurin act in parallel to stimulate *FKS2* expression. First, a calcineurin mutant displayed a delayed but sustained induction of *FKS2* expression in response to temperature upshift, whereas a cell integrity signaling mutant (*mpk1*) displayed a rapid but transient induction of *FKS2* in response to this challenge. The contributions of these pathways to *FKS2* expression was roughly additive to that of the wild type. These results indicate that calcineurin mediates the early response to temperature shift and that the cell integrity signaling pathway mediates the persistent maintenance of *FKS2* expression at elevated temperature. Simultaneous inhibition of both pathways completely prevented thermal induction of *FKS2*. Second, induction of *FKS2* expression by exogenous Ca^{2+}, a calcineurin-dependent process, was not impaired in mutants defective in cell integrity signaling. Third, the calcineurin-responsive region of the *FKS2* promoter was separable from the cell integrity signaling-responsive region. The calcineurin-responsive sequences reside in the region between −928 and −706, whereas the cell integrity-responsive sequences reside in the region between −706 and −360. In this regard, it is interesting to note that in mammalian T cells, calcineurin and protein kinase C act synergistically to activate transcription of the interleukin-2 gene (10). Thus, cooperative activity of these signaling molecules may be a common theme.

The involvement of cell integrity signaling in *FKS2* expression indicates that a regulatory circuit for cell wall construction exists in which the RHO1 G protein controls both the activity of GS and the expression of at least one of its components. However, temperature upshift during growth had only a modest effect on GS activity in wild-type cells. This can be explained by the large contribution to GS activity of *FKS1*, whose expression was not influenced by temperature upshift. Only in an *fks1* mutant was an appreciable increase in GS activity observed in response to temperature upshift. These results suggest that *FKS2* is of minor importance as an effector of cell integrity signaling. This conclusion is supported by the absence of a growth defect associated with loss of *FKS2* function (28).

Our results provide the first clear evidence for a direct target of cell integrity signaling. Igual et al. (14) proposed that the cell integrity pathway positively regulates *FKS1*. However, this was based on the observation that *FKS1* mRNA levels were modestly reduced (25 to 80%) in *pkc1* and *mpk1* mutants. Moreover, the most severely impaired signaling mutant tested in that study, a *pkc1Δ* strain maintained in the presence of osmotic support, displayed only a 25 to 35% decrease in the steady-state level of *FKS1* mRNA compared with wild type. Finally, the failure of Igual et al. to observe an increase in *FKS1* mRNA in response to stimulation of cell integrity signaling by temperature upshift of wild-type cells was confirmed in the present study and suggests to us that *FKS1* is not under the control of this signaling pathway. The reported diminution of *FKS1* mRNA levels in cell integrity pathway mutants might be explained by differences in growth rates of these mutants or by other secondary factors.

The RLM1 transcription factor is not required for thermal induction of *FKS2* expression. *RLM1* encodes a member of the serum response factor family of transcription factors that has been implicated in cell integrity signaling (7, 42, 43). A recessive mutation in *RLM1* was isolated as a suppressor of the growth defect associated with expressing a hyperactive *MKK1* allele (42). Recent reports suggest that transcriptional activity of RLM1 is regulated positively in response to phosphorylation by MPK1 (7, 43). However, an *rlm1Δ* mutant does not display a cell integrity defect, suggesting that any role it may have in cell integrity signaling is minor. Additionally, mutation of the only *RLM1*-related yeast gene, designated *SMP1*, also failed to display cell integrity defects alone or in combination with *rlm1* (7, 43).

We found that overexpression of *RLM1* suppressed the synthetic lethality of a *cnb1Δ fks1Δ* double mutant, presumably by mediating increased expression of *FKS2*. This conclusion was supported by the observation that *RLM1* overproduction increased the basal level of *FKS2* expression at room tempera-

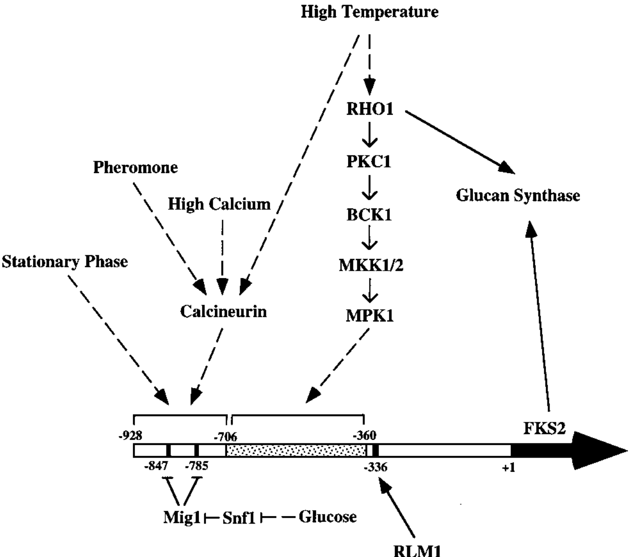


FIG. 9. Known inputs for the regulation of *FKS2* expression.

ture by eightfold. Additionally, an *rlm1*Δ *fks1*Δ double mutant displayed synthetic lethality that was suppressed by overexpression of *FKS2*, further suggesting that *RLM1* normally contributes to *FKS2* expression. However, an *rlm1*Δ mutant was not deficient for induction of *FKS2* expression in response to temperature upshift. Moreover, the only potential RLM1 binding site (7) within the *FKS2* promoter resides within a region that is dispensable for thermal induction of this gene. These results support the conclusion that although RLM1 contributes to *FKS2* expression, it does not mediate the thermal induction of this gene stimulated by cell integrity signaling. Because the inputs to *FKS2* expression from calcineurin, *RLM1*, and cell integrity signaling appear to be independent, the synthetic lethality of each of these inputs with *fks1*Δ suggests that they all contribute significantly to *FKS2* expression in the absence of *FKS1*.

Expression of *FKS2* in response to glucose starvation and entry to stationary phase. Regulation of *FKS2* expression is under the independent control of a variety of signaling pathways. In addition to the roles of the cell integrity pathway, calcineurin, and *RLM1* in the regulation of this gene, *FKS2* induction in response to glucose starvation was found to be under the control of the SNF1 protein kinase. Glucose repression of *FKS2* is apparently mediated by the MIG1 transcriptional repressor at two consensus MIG1 binding sites in the region between residues −706 and −928 of the *FKS2* promoter. Additionally, *FKS2* expression was strongly induced as cells entered stationary phase. However, though the regulatory site for this induction was mapped to the same region as the SNF1- and calcineurin-responsive sequences (−928 to −706), neither a *snf1*Δ mutant nor a *cnb1*Δ mutant were impaired for the stationary-phase response. Therefore, *FKS2* induction in response to entry to stationary phase represents a fifth distinct regulatory input for the control of this gene. A summary of these inputs is presented in Fig. 9.

Expression of *FKS1* and *FKS2* are generally regulated in opposition to each other, with *FKS1* predominating under optimal growth conditions. However, *FKS2* is induced in response to calcium influx, mating pheromone, thermal stress, glucose starvation, entry to stationary phase, and mutation of *FKS1*. Induction of *FKS2* is usually accompanied by a decrease in *FKS1* expression so that the level of GS activity in cells is not appreciably altered in response to these conditions. Why then do cells shift from one form of GS to the other in response to stress? One possibility is that the two forms of GS may be functionally distinct in ways that have yet to be discovered. For example, GS derived from FKS2 may be more stable than that derived from FKS1, thereby enhancing survival in stasis and under conditions of stress.

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C.Z., U.S.J., and P.G.-E. contributed equally to this work.

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