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Coordinated regulation of gene expression by the cell cycle transcription factor SWI4 and the protein kinase C MAP kinase pathway for yeast cell integrity

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Specific transcription in late G_1, mediated by the transcription factors SBF (Swi4p–Swi6p) and MBF (Mbp1p–Swi6p), is crucial for cell cycle progression in budding yeast. In order to better understand the G_1/S transition, we initiated a search for conditional mutations synthetic lethal with *swi4Δ*. One of the isolated mutants, *rsf8swi4Δ*, showed a growth defect due to cell lysis. *rsf8* is allelic to *PKC1*, encoding a protein kinase C homologue which controls cell integrity. In the presence of the *rsf8*/(*pkc1-8*) mutation, a functional SBF but not MBF is required for viability. Importantly, *swi4Δ* and *swi6Δ* strains are hypersensitive to calcofluor white and SDS, indicating that they possess a weakened cell wall. Overexpression or ectopic expression of *CLN* did not suppress the *pkc1-8swi4Δ* mutant phenotype, thus SBF must control cell integrity independently, rather than acting through *CLN* expression. We found that at least six genes involved in cell wall biosynthesis are periodically expressed at the G_1/S phase boundary. In all six cases, cell cycle-regulated expression is due mainly to Swi4p. Finally, we found that the *PKC1* MAP kinase pathway is a positive regulator of five of these cell wall genes, these genes being novel targets of regulation by this pathway. We suggest that SBF and the *PKC1* MAP kinase pathway act in concert to maintain cell integrity during bud formation.

Keywords: cell cycle/cell wall/MAP kinase/PKC/*SWI4*

Introduction

In the yeast *Saccharomyces cerevisiae*, the major control in the cell cycle occurs in late G_1, at a point called Start (reviewed in Cross, 1995). Execution of Start requires activation of the Cdc28 kinase through its association with G_1-specific cyclins encoded by the *CLN1*, *CLN2* and *CLN3* genes (Richardson *et al.*, 1989; Cross, 1990). Although any one of the three Clns is sufficient for Start, recent work suggests that they are not equivalent. Cln1p and Cln2p differ from Cln3p in their structure and regulation and carry out different functions in the initiation of the cell cycle (Tyers *et al.*, 1993). The major function of the Cln3–Cdc28 kinase is to trigger a burst of late G_1-specific gene transcription, including *CLN1* and *CLN2* (Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). As a result, there is an abrupt accumulation of Cln1–Cdc28 and Cln2–Cdc28 kinase activity, which in turn trigger the early events of the cell cycle: DNA replication, spindle pole body (SPB) duplication and polarized cell surface growth leading to bud emergence. Thus, the cell cycle regulation of *CLN1* and *CLN2* gene expression is a crucial step in initiation of the cell cycle.

The Cln3–Cdc28-dependent activation of gene expression in late G_1 is mediated by the related transcription factors SBF and DSC1/MBF (reviewed in Koch and Nasmyth, 1994; Breeden, 1995). Both are heterodimeric, consisting of a common regulatory protein, Swi6p (Breeden and Nasmyth, 1987a), and one of two highly homologous specific DNA binding protein: Swi4p in SBF (Andrews and Herskowitz, 1989a) and Mbp1p in MBF (Koch *et al.*, 1993). The importance of this late G_1-specific transcriptional system is emphasized by the lethality of the *swi4mbp1* and *swi4swi6* double mutants. SBF was characterized as the binding activity on the SCB element (CACGAAA) (Andrews and Herskowitz, 1989b), controlling the periodic expression in late G_1 of *HO*, *CLN1*, *CLN2* and *PCL1* (Breeden and Nasmyth, 1987b; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). DSC1/MBF was identified as the factor recognizing the MCB element (ACGCGT) (Lowndes *et al.*, 1991) and is responsible for cell cycle regulation in late G_1 of the *CLB5* and *CLB6* genes (Epstein and Cross, 1992; Koch *et al.*, 1993) and of many DNA synthesis genes (Johnston and Lowndes, 1992). However, SBF and MBF have partially redundant functions. Thus, SBF is able to bind MCBs *in vitro* and, conversely, MBF recognizes the SCB element. Consistent with this, SBF-dependent regulation of the *CLN1* gene is mediated by MCB-like rather than SCB elements (Breeden, 1995). Moreover, in the absence of SBF, MBF partially substitutes for it and activates *CLN1* and *CLN2* gene expression (Koch *et al.*, 1993). Thus, more work is required to better understand the SCB and MCB system and also to further characterize periodic gene expression in late G_1, since recently at least one additional regulatory mechanism has been implicated (Cross *et al.*, 1994; Stuart and Wittenberg, 1994).

Commitment to the cell cycle and cell growth in yeast are influenced by external stimuli and several signal transduction pathways mediating the cellular response to these stimuli have been characterized (Thevelein, 1994; Levin and Errede, 1995; Herskowitz, 1995; Schulz *et al.*, 1995), including the *PKC1* pathway (Levin *et al.*, 1994). The *S.cerevisiae PKC1* gene encodes a homologue of the α, β and γ isoforms of mammalian protein kinase C. In mammalian cells, protein kinase C plays a crucial role in the regulation of growth, proliferation and differentiation in response to extracellular signals (Dekker and Parker,

1994). In yeast, the *PKC1* gene is essential for cell growth (Levin *et al.*, 1990). The growth defect in *pkc1* mutants results from cell lysis that can be reversed by osmostabilizing agents (Levin and Barlett-Heubusch, 1992; Paravicini *et al.*, 1992) suggesting a defect in cell wall structure. In fact, the cell wall in *pkc1*Δ cells is markedly thinner than in the wild-type in both the glucan and the mannoprotein layers and contains reduced amounts of these compounds (Levin *et al.*, 1994; Roemer *et al.*, 1994). Recently, Pkc1p has been shown to act upstream of a MAP kinase cascade. Pkc1p regulates the activity of the MAP kinase kinase kinase homologue Bck1p (Lee and Levin, 1992), which controls the redundant pair of MAP kinase kinases Mkk1p and Mkk2p (Irie *et al.*, 1993), which in turn regulate the MAP kinase Mpk1p (Torres *et al.*, 1991; Lee *et al.*, 1993). Mutations in *BCK1*, *MPK1* or in both *MKK1* and *MKK2* also result in cell lysis, but only at high temperature. Because of the more severe defect in *pkc1* mutants (lysis at all temperatures) it has been proposed that *PKC1* controls a bifurcated pathway with the MAP kinase module in one branch (Lee and Levin, 1992). Besides the major role in ensuring integrity of the cell, the *PKC1* pathway has been also implicated in polarized growth (Costigan *et al.*, 1992; Mazzoni *et al.*, 1993), as well as in more direct aspects of growth control, like nutrient sensing (Costigan and Snyder, 1994) and DNA replication (Huang and Symington, 1994). A possible nexus of the *PKC1* pathway with cell cycle regulation has been suggested by the observations that an *mpk1* mutation accentuates the division defect at the G_1/S transition of *cdc28* mutants (Mazzoni *et al.*, 1993) and that *BCK2*, a suppressor of *pkc1* mutants, is a positive regulator of *CLN2* expression (Di Como *et al.*, 1995).

The crucial issues concerning MAP kinase pathways are the signals activating them and their outputs. Common targets for MAP kinase cascades are transcription factors, which, of course, leads to alterations in gene expression. Recently, a genetic screen has identified the transcription factor Rlm1p as a putative downstream effector of Mpk1p (Watanabe *et al.*, 1995). In addition, some of the signals that activate the *PKC1* MAP kinase pathway have been characterized (Kamada *et al.*, 1995; Zarzov *et al.*, 1996). However, in spite of these advances, the molecular basis of the regulation of cell integrity by *PKC1* remains unclear.

In order to further understand the role of Swi4p and to help elaborate the connections between growth control and Start, we searched for genes that functionally interact with *SWI4*. Here we report a synthetic lethality between mutations in the components of SBF and the *PKC1* MAP kinase pathway. We demonstrate that at least six genes involved in cell wall biosynthesis are coordinately expressed at G_1/S and that this cell cycle regulation is due mainly to SBF. In addition, we show that five of these genes are also regulated by the *PKC1*-activated MAP kinase. We conclude that SBF and the *PKC1* MAP kinase pathway act in parallel to control cell integrity in budding yeast.

Results

The rsf8 mutation is synthetic lethal with swi4Δ

We isolated conditional lethal mutants unable to grow at 37°C in a *swi4*Δ background. To identify those potentially interacting with *SWI4*, the mutants rescued by re-introduction of the *SWI4* gene were selected for further study (our manuscript in preparation). One of these mutations, *rsf8* (requiring swi four), is described here.

Table I. Synthetic interaction between *rsf8* and *swi4*Δ

Spore genotype	No. of spores	Growth at 37°C
SWI4	53	51 + 2 –
swi4	38	21 + 17 –

The *rsf8swi4*Δ double mutant was crossed with a congenic wild-type (W303-1A).

Backcrossing *rsf8swi4*Δ to the parental *swi4*Δ strain, BY604, indicated that the thermosensitive phenotype was due to a single recessive mutation. The *rsf8swi4*Δ strain was also crossed to the wild-type strain W303-1A. Analysis of the dissected tetrads demonstrated a clear synthetic interaction between *rsf8* and *swi4*Δ. Whereas in the *swi4*Δ spore clones the conditional phenotype segregates 2:2, in the presence of wild-type *SWI4*, only two out of 53 segregants retained their temperature sensitivity (Table I). Thus, the conditional lethal phenotype is only apparent in a *swi4*Δ background.

The rsf8swi4Δ strain has a cell lysis phenotype at 37°C

When exponentially growing cells of the *rsf8swi4*Δ double mutant were shifted from 25 to 37°C a first cycle arrest occurred with little increase in cell number (data not shown). Morphological observation revealed a high proportion (80%) of budded cells, mainly cells with a tiny bud. More striking was the loss of the normal smooth cell shape, the cells becoming rather shrunken and irregular (Figure 1A). Continued incubation at 37°C resulted in massive lysis and accumulation of cell ghosts and debris. This lethality can be suppressed by growing the cells in the presence of 1 M sorbitol (data not shown). FACS analysis indicated that prior to lysis at 37°C the bulk of cells accumulate with a 2C DNA content (Figure 1B). In summary, the *rsf8swi4*Δ mutant shows a defect in cell growth which results in a complete arrest in the first cycle, mainly at an early stage of budding, with a loss of cell integrity.

rsf8 is allelic to PKC1

The phenotype of the *rsf8swi4*Δ mutant resembles some of the reported characteristics of mutation in *PKC1*, namely cell lysis and arrest at an early stage of budding (Levin and Barlett-Hensbusch, 1992; Paravicini *et al.*, 1992). To explore this further, the *rsf8swi4*Δ mutant was transformed with centromeric and multicopy plasmids containing the *PKC1* gene. Both plasmids rescued the conditional phenotype of *rsf8swi4*Δ (Figure 2). The centromeric *PKC1* plasmid completely restores growth at 37°C, with a doubling time identical to the parental strain BY604 (data not shown). Furthermore, all the morphological defects associated with the *rsf8* mutation were also corrected by the centromeric *PKC1* plasmid (Figure 1A). In a parallel experiment to identify the *RSF8* gene, a centromeric genomic library was introduced into the

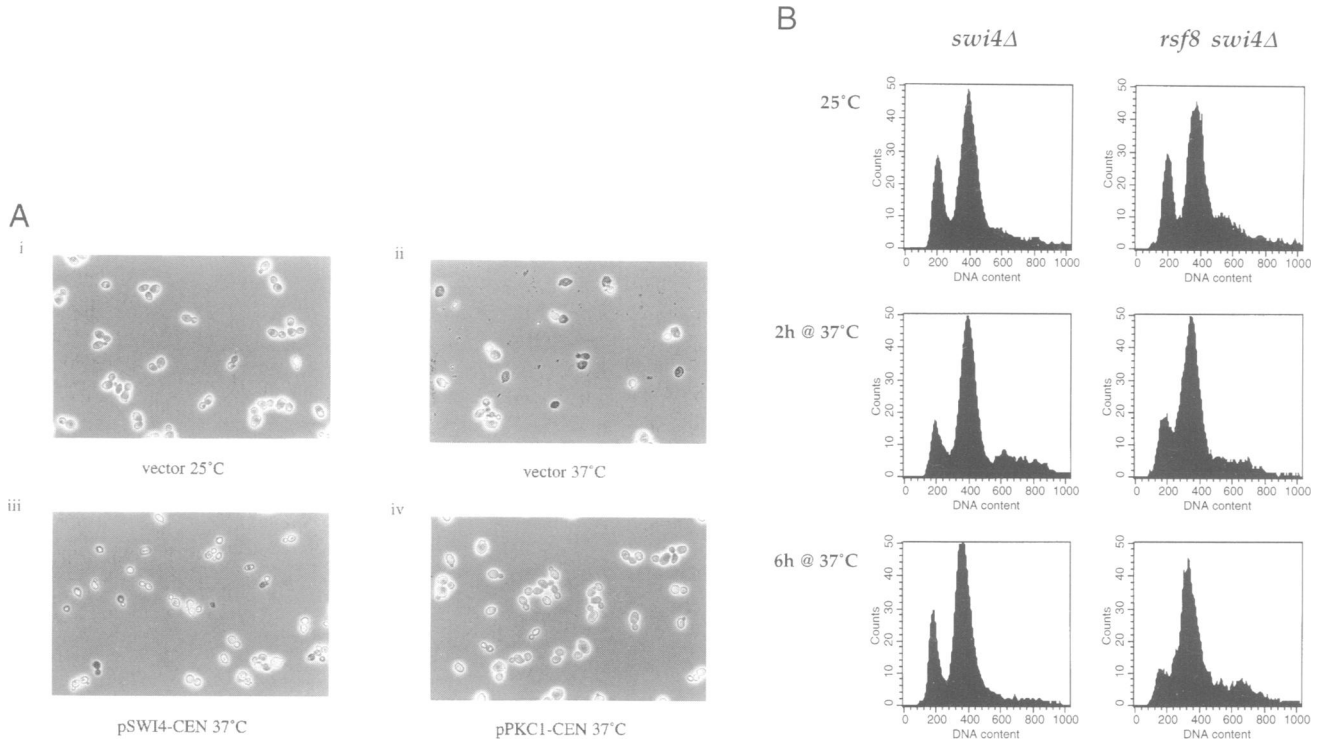

Fig. 1. Characterization of the *rsf8swi4Δ* mutant. (**A**) Cell morphology. The *rsf8swi4Δ* strain transformed with vector pRS314 (i and ii) or with centromeric plasmids containing either the *SWI4* (iii) or the *PKC1* gene (iv) was grown to mid-log phase in YPD at 25°C (i) and then transferred to 37°C for 6 h (ii–iv). (**B**) DNA flow cytometric analysis. A mid-log culture of the *rsf8swi4Δ* strain was shifted from 25 to 37°C and at the indicated times cells were collected and processed for measurement of the DNA content.

rsf8swi4Δ strain and five plasmids that suppressed the thermosensitivity were recovered. All of them contained the *PKC1* gene.

To determine whether *rsf8* is a conditional allele of *PKC1*, the *rsf8swi4Δ* strain was crossed with a *pkc1Δ::HIS3* deletion mutant. Analysis of the progeny revealed that the *rsf8* temperature sensitivity always segregated in opposition to *pkc1Δ* (Table II). Thus, the 42 *swi4his3* spores which did not contain the *HIS3*-disrupted *pkc1* gene were all temperature sensitive and must have contained *rsf8*. This confirms the allelism of *PKC1* and *rsf8*, which henceforth will be referred to as *pkc1-8*.

The cross in Table II also confirmed the synthetic lethality of the *swi4* and *pkc1* mutations. In 48 tetrads dissected on rich medium supplemented with sorbitol, no *swi4Δpkc1Δ* segregant was recovered. It was possible to infer the *swi4Δpkc1Δ* genotype in 31 spores; microscopic observation of some of them indicated that spores germinated but arrested with one elongated bud. Hence, deletion of both *swi4* and *pkc1* results in a synthetic lethality that, in marked contrast to the *pkc1Δ* mutation alone, cannot be rescued by the presence of an osmotic stabilizer.

pkc1-8swi4Δ is suppressed by SBF but not by G_1 cyclins

The temperature sensitivity of *pkc1-8swi4Δ* is suppressed by both multicopy and centromeric plasmids containing the *SWI4* gene (Figure 2), but neither plasmid completely restores the wild-type characteristics. The growth rate at the restrictive temperature is slower, with a doubling time of 7 h, compared with 2 h in a wild-type control. In addition, normal cellular morphology is not completely restored, the rather shrunken irregular shape being retained, although cell lysis is avoided (Figure 1A).

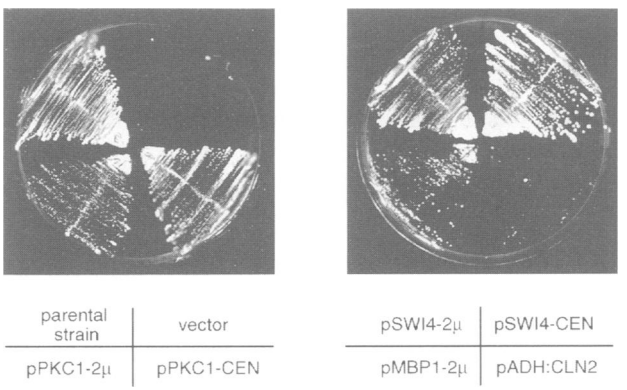

Fig. 2. Suppression analysis of the temperature-sensitive growth defect of the *rsf8swi4Δ* mutant. The parental strain BY604 (*swi4Δ*) and the *rsf8swi4Δ* strain transformed with a control vector, a centromeric or multicopy plasmid containing either the *SWI4* or *PKC1* gene, a multicopy plasmid bearing the *MBP1* gene or a multicopy plasmid with the *CLN2* gene under the control of the *S.pombe adh* promoter were streaked onto minimal medium. Plates were incubated for 3 days at 37°C.

Swi4p forms, together with Swi6p, the heterodimeric transcription factor SBF. In order to determine whether the *SWI4* interaction with *PKC1* is dependent on the whole SBF, a genetic cross between *pkc1-8swi4Δ* and *swi6Δ* was carried out. The thermosensitivity segregated 2:2 in the presence of either *swi4Δ* or *swi6Δ* spore clones, but all the *SWI4SWI6* segregants grew at 37°C (Table III). These results confirm that, like *swi4Δ*, *swi6Δ* is synthetic lethal

Table II. *rsf8* is allelic to *PKC1*

Spore genotype	No. of spores	Growth at 37°C
SWI4 his3	39	38 + 1 –
SWI4 pkc1::HIS3	33	–[a]
swi4 his3	42	0 + 42 –
swi4 pkc1::HIS3	0	–

The *rsf8swi4Δ* double mutant was crossed with a *pkc1::HIS3* deletion strain. All tetrads were dissected on agar plates containing 1 M sorbitol to allow growth of spore clones containing the *pkc1* deletion. [a]Analysis of the temperature-sensitive growth defect was done on YPD plates; because of the absence of an osmotic stabilizer all the *pkc1Δ* spore clones failed to grow.

Table III. Synthetic interaction between *pkc1-8* and *swi6Δ*

Spore genotype	No. of spores	Growth at 37°C
SWI4 SWI6	41	41 + 0 –
SWI4 swi6	37	21 + 16 –
swi4 SWI6	38	19 + 19 –
swi4 swi6	0	–

The *pkc1-8swi4Δ* double mutant was crossed with a congenic *swi6Δ* strain (BY600).

with *pkc1-8*. Swi4p is the DNA binding component of SBF and when overexpressed it can activate *HO* transcription in the absence of Swi6p (Breeden and Nasmyth, 1987b). In agreement with this, a multicopy plasmid containing the *SWI4* gene rescued a *pkc1-8swi6Δ* mutant (data not shown). In contrast, overexpression of Swi6p could not bypass the absence of Swi4p in the *pkc1-8swi4Δ* strain. These observations are consistent with a functional SBF being essential in cells bearing the *pkc1-8* mutation.

Next, we explored the interaction between *pkc1-8* and the *MBP1* gene, which encodes a protein closely related to Swi4p and also involved in transcriptional control at the G_1/S boundary. Although overexpression of *MBP1* rescued the *pkc1-8swi4Δ* temperature sensitivity, suppression was much weaker than that observed with *SWI4* (Figure 2). Moreover, genetic analysis of a cross between *pkc1-8swi4Δ* and CY19 (*mbp1Δ*) did not reveal any interaction between *mbp1Δ* and *pkc1-8* (data not shown).

SBF regulates transcription of the G_1 cyclin genes *CLN1* and *CLN2*, so it was possible that the phenotype of *pkc1-8swi4Δ* could be due to a defect in Cln function. However, multicopy plasmids containing *CLN1* or *CLN2* genes did not rescue the *pkc1-8swi4Δ* defect (data not shown). Moreover, ectopic expression of *CLN2* under the control of the *Schizosaccharomyces pombe adh* promoter also failed to suppress the thermosensitivity of *pkc1-8swi4Δ* (Figure 2). The same result was obtained with an ectopically expressed dominant allele of *CLN2*, *CLN2-4* (kindly provided by C.Wittenberg), coding for a truncated protein lacking the C-terminus (data not shown). We also tested the effect of multicopy plasmids containing other cyclins genes, like *PCL2*, *CLB5* and *CLB6*, and a chimeric

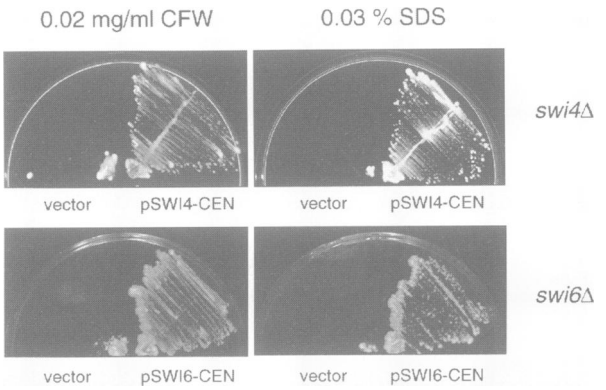

Fig. 3. Hypersensitivity of *swi4Δ* and *swi6Δ* mutants to CFW and SDS. BY604 (*swi4Δ*) transformed with vector pRS314 or a centromeric plasmid containing the *SWI4* gene and BY600 (*swi6Δ*) transformed with vector pRS313 or a centromeric plasmid containing the *SWI6* gene were streaked onto YPD medium supplemented with either 0.02 mg/ml CFW or 0.03% SDS. Plates were incubated at 25°C for 3–4 days.

GAL1:CLB5 gene. None of them suppressed the *pkc1-8swi4Δ* lethality (data not shown). This is an important result which indicates that the phenotype of the *pkc1-8swi4Δ* mutation is not caused by a defect in *CLN* activity. Thus, the synthetic interaction between *swi4Δ* or *swi6Δ* and *pkc1* mutations is probably related to a defect in expression of some other gene(s) regulated by SBF.

swi4Δ and swi6Δ mutants are sensitive to cell wall stresses

The synthetic interaction of *SWI4* and *SWI6* with *PKC1* described above indicates that SBF is involved to some degree in cell wall metabolism. To further investigate this potential new role for SBF, we examined the cell wall integrity of a *swi4Δ* mutant. Sensitivity to compounds such as SDS and calcofluor white (CFW) has proved to be a powerful tool in revealing cell wall defects in yeast mutants (Ram *et al.*, 1994; Shimizu *et al.*, 1994). Accordingly, we examined the sensitivity of a *swi4Δ* strain to these compounds. As shown in Figure 3, *swi4Δ* cells were unable to grow on YPD medium supplemented with 0.03% SDS or 0.02 mg/ml CFW, whereas the *SWI4* control grew readily under these conditions. In both cases the *swi4Δ* mutant showed a 4-fold increased sensitivity compared with the wild-type. Note that disruption of *SWI4* in an unrelated genetic background (strain CG378) gave essentially the same results (data not shown), so that the cell wall defects were not strain specific.

Similar experiments were carried out with a *swi6Δ* mutant. Consistent with the synthetic lethality between *swi6Δ* and *pkc1*, the *swi6Δ* cells showed the same hypersensitivity to SDS and CFW as *swi4Δ* cells (Figure 3). The increased sensitivity of the *swi4Δ* and *swi6Δ* strains to SDS and CFW confirms a role for SBF in cell wall assembly.

Finally, the effect of the *mbp1* mutation was also tested. In two different backgrounds, W303 and CG378, loss of *mbp1* function resulted in no increase in sensitivity to CFW or SDS (data not shown). Thus, as opposed to *swi4* and *swi6*, a weakened cell wall structure is not apparently associated with *mbp1* mutation.

Table IV. Cell cycle regulatory elements in the promoters of cell wall genes

Gene	SCB	MCB
(1-3)-β-glucan		
FKS1	–396, –496, –599	(–436), –583, –637
FKS2	–390, –439	(–907), (–982)
GNS1	–101	
GAS1	–308, –352, (–749) 749	(–343)
KNR4	–129	
(1-6)-β-glucan		
SKN1	–209	
Chitin		
CHS1		(–293), (–283), (–271)
CHS2	–68	(–554)
CHS3/CSD2	–242	–267
Mannoproteins/glycosylation		
ALG1	–338	(–115)
ALG7	–102, –126	
GAA1		–20
MNN1	–61, –498	(–366)
OCH1	–283	(–260)
OST2	–550	–295
OST3		–31
PMI40	–172	(–139)
PMT1	–90	
SEC59	–35	
STT3	–156	
VAN2/VRG4	–146, –310	–267

SCB, C(A/G/T)CGAAA; MCB, ACGCGT (numbers in parentheses ACGCG).

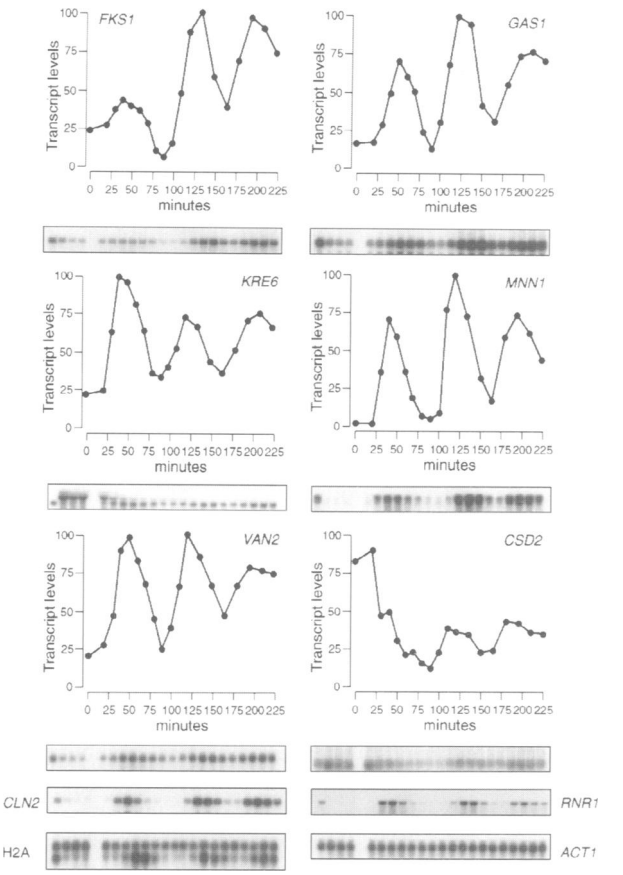

Fig. 4. Cell cycle-regulated expression of genes involved in cell wall biosynthesis. The expression of some cell wall genes in wild-type cells synchronized with α-factor was investigated by Northern analysis. mRNA levels during the α-factor-induced arrest were monitored at 0, 80, 160 and 240 min after addition of the pheromone to cycling cells (first four lanes in the autoradiograph). After removing the α-factor, cells were resuspended in fresh medium and samples were taken at 20 min and then at 10 min intervals up to 120 min and thereafter at 15 min intervals. In the case of cell wall genes, the amounts of the different transcripts were quantitated relative to *ACT1* as internal control and are graphically displayed above the corresponding autoradiograph. In each case, the maximum value is referred to as 100 (note that in the case of *CSD2* the maximum value occurs during the α-factor holding). The zero time point in the graph corresponds to the 240 min α-factor holding sample. In the case of *MNN1*, two different transcripts that behaved similarly were detected; values here and in Figures 6, 7 and 9 are the total amount of both transcripts. In the case of *KRE6*, the α-factor-induced transcript was excluded from the quantification. These RNA preparations have been previously used in figure 8 in Toone *et al.* (1995), where the budding profile for the synchronous culture is shown.

SWI4-dependent cell cycle regulation of cell wall gene transcription

SBF regulates gene expression in late G_1 by its binding to a specific promoter sequence called the SCB element. In order to understand the role of *SWI4* in cell wall metabolism we searched the EMBL database for genes containing SCB elements in their promoter. The yeast cell wall consists of (1-3)-β-glucan, (1-6)-β-glucan, chitin and mannoproteins (reviewed in Klis, 1994; Cid *et al.*, 1995). Interestingly, the search revealed that many genes involved in the synthesis of these components contain one or more cell cycle regulatory elements in their promoter (Table IV). Not only were SCB elements detected, but, significantly, many genes contain MCB elements as well. These latter elements are the binding site for the related transcription factor DSC1/MBF and are also responsible for cell cycle-regulated expression in late G_1. The occurrence of both of these elements in the promoters of genes encoding cell wall components is a striking coincidence.

The data in Table IV strongly suggest that transcription of at least some genes involved in cell wall metabolism is coordinately regulated under cell cycle control at G_1/S, as occurs for DNA synthesis genes. To test this hypothesis, the pattern of expression of a number of these genes was analysed in α-factor-synchronized cultures. We observed that transcription of *FKS1*, *GAS1*, *KRE6*, *MNN1*, *VAN2* and *CSD2* is clearly cell cycle regulated, peaking near the G_1/S boundary (Figure 4). *FKS1* codes for a subunit of the (1-3)-β-glucan synthase (Douglas *et al.*, 1994). *GAS1* encodes a GPI-anchored membrane protein (Nuoffer *et al.*, 1991) involved in the synthesis of (1-3)-β-glucan (Ram *et al.*, 1995). Recently, others have made similar observations on cell cycle regulation for both *FKS1* and *GAS1* (Ram *et al.*, 1995). *KRE6* codes for a key protein in the synthesis of (1-6)-β-glucan (Roemer *et al.*, 1994). Curiously, *KRE6* expression is strongly activated in the presence of the mating pheromone (Figure 4), but the α-factor-induced transcript is considerably bigger than the non-induced mRNA. At least two genes involved in different aspects of mannosylation of proteins, *MNN1* (Yip *et al.*, 1994) and *VAN2* (Kanik-Ennulat *et al.*, 1995), are also periodically expressed. The *CSD2* gene encodes chitin synthase III, the enzyme responsible for the synthesis of most of the chitin in the cell wall (Bulawa, 1993). It is

known that *CSD2* is periodically expressed, but the exact timing of this expression compared with known cell cycle-regulated genes has not been completely defined (Pammer *et al.*, 1992). Expression of *CSD2* was not apparent in the first cycle after release, perhaps because of activation of the gene in the presence of α-factor; however, in the next two cycles a fluctuation of 3-fold was detected. Maximum expression of all these genes occurs near the G_1/S transition, although the precise timing of expression between them differs slightly. Thus, whereas *KRE6*, *MNN1*, *VAN2* and *CSD2* expression occurs coincidentally with *CLN2* and *RNR1* (genes known to be expressed in late G_1), *FKS1* and *GAS1* peaks occur slightly later, between *CLN2/RNR1* and H2A. The *FKS1* transcript peak in the first cycle after release from α-factor block is clearly lower than the peaks in the subsequent two cell cycles. This does not occur with the other genes tested, including the three cell cycle-regulated control transcripts (*CLN2*, *RNR1* and histone H2A), and is of unknown significance at present. The periodic expression of cell wall genes was also observed in wild-type cells synchronized by an independent means, namely elutriation (data not shwon).

To determine whether these genes could be controlled by SBF, the consequences of the mutation or overexpression of *SWI4* on *FKS1*, *GAS1*, *KRE6*, *MNN1*, *VAN2* and *CSD2* transcription were analysed in asynchronous exponential cultures. No significant difference in transcript levels between *swi4*Δ and isogenic wild-type strains was detected and overexpression of *SWI4* had no effect (data not shown). The same result was observed with *swi6*Δ and *mbp1*Δ mutants. Furthermore, when a *swi4*^{ts}*swi6*Δ*mbp1*Δ triple mutant was shifted to 37°C no significant changes in level of the different transcripts could be detected (data not shown). DNA synthesis genes are regulated by the transcription factor DSC1/MBF and it has been shown that loss of DSC1 function severely perturbs cell cycle regulation of these genes but has no apparent effect on transcript levels in mid-log phase (Lowndes *et al.*, 1992; Koch *et al.*, 1993). We therefore suspected that the consequences of *swi4* mutation might only be detectable in synchronized cultures. Accordingly, cell wall gene transcription was investigated in a culture of a *swi4*Δ strain synchronized by elutriation. In sharp contrast to *RNR1*, a gene whose cell cycle regulation is not dependent on *SWI4*, and to *CLN2*, which has lower but still periodic expression (Cross *et al.*, 1994; Stuart and Wittenberg, 1994), the cell cycle regulation of cell wall genes was severely perturbed in the *swi4*Δ mutant (Figure 5). Periodicity of *FKS1* expression was almost entirely abolished, although a slight 2-fold increase in late G_1 was still detected. The cell cycle regulation of *MNN1*, *KRE6* and *CSD2* gene expression was not only severely reduced, but also the timing was changed, peaking in early G_1. In the case of *GAS1* and *VAN2*, no fluctuations through the cell cycle were detected. Thus, Swi4p coordinates the cell cycle-regulated expression of at least *FKS1*, *GAS1*, *KRE6*, *MNN1*, *VAN2* and *CSD2* and the high level of constitutive expression in its absence must be due to another transcription factor.

pkc1 mutants show reduced transcription of cell wall genes

Transcription factors have been shown to be common targets for MAP kinases and we have shown that inactivation of the *PKC1* MAP kinase pathway is lethal in the absence of Swi4p. Since Swi4p regulates genes involved in cell wall metabolism, a strong possibility is that the *PKC1* MAP kinase pathway might control similar or the same genes through a still uncharacterized transcription factor. To test this hypothesis, the effect of the *pkc1-8* conditional allele on cell wall gene transcription was analysed by shifting a culture of a *pkc1-8* strain to 37°C. A severe reduction in *FKS1*, *MNN1* and *CSD2* transcription was reproducibly detected at the restrictive temperature (Figure 6). In contrast, *VAN2* expression was not affected and only a slight effect was detected in the expression of *GAS1* and *KRE6* (data not shown). Importantly, there was no decrease in the level of two control transcripts, *TMP1*, which is cell cycle regulated in a very similar manner to the cell wall genes (Storms *et al.*, 1984; our unpublished observations), and H2A. This result ruled out the possibility that the reduced transcription of some cell wall genes was an indirect effect on all late G_1/early S expressed genes caused by a perturbation of the cell cycle.

Essentially the same result was obtained when cell wall gene expression was analysed in the *pkc1-8swi4*Δ double mutant (data not shown), consistent with *swi4* mutation having no apparent effect on the level of transcripts in asynchronous cultures.

The effect of a *pkc1* null mutation was also tested. Reduced expression of *FKS1* was consistently detected. However, somewhat surprisingly, expression was reduced only 25–35% compared with the 70–80% decrease in the

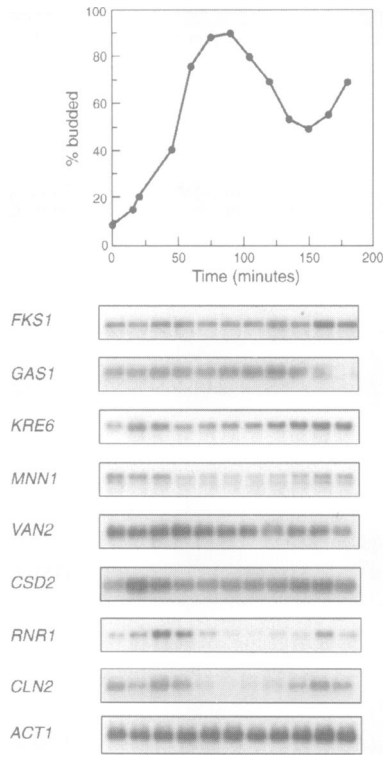

Fig. 5. Effect of mutating *SWI4* on the periodic expression of cell wall genes. Small G_1 daughter cells from a *swi4*Δ strain were isolated by centrifugal elutriation and incubated in YPD. Samples were removed at 15 min intervals for monitoring budding index and for Northern analysis. The first lane of each autoradiograph corresponds to cycling cells before synchronization. The *ACT1* transcript is shown as a loading control.

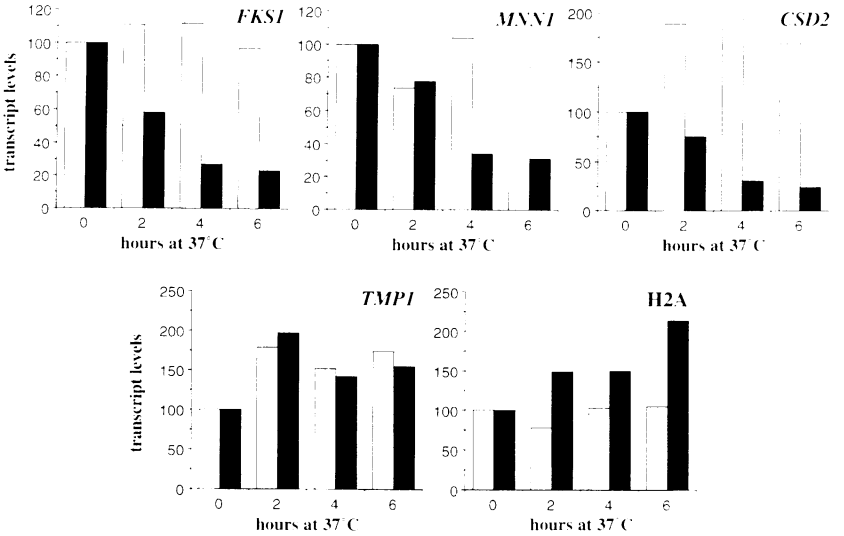

Fig. 6. Effect of mutation of *PKC1* on the expression of cell wall genes. Mid-log cultures of congenic wild-type (W303-1a; open bars) and *pkc1-8* strains (JC6-3A; black bars) were shifted from 25 to 37°C. Transcription of the indicated genes was analysed at 0, 2, 4 and 6 h after the shift by Northern analysis. Transcript levels were quantitated relative to actin as internal control. In each case, the transcript level at 25°C is referred to as 100.

pkc1-8 allele (data not shown). In the case of *MNN1* and *CSD2* genes, the effect of the *pkc1*Δ mutation was even weaker, with a slight 15% decrease in the level of transcript (data not shown). This observation could be a consequence of the different culture conditions used. The *PKC1* pathway is activated at high temperature and this activation is prevented by the presence of an osmotic stabilizer (Kamada *et al.*, 1995). It is possible that the consequences of the loss of *PKC1* function on gene expression would be more dramatic at 37°C (conditions used to analyse the effect of the thermosensitive *pkc1-8* allele) than at 25°C in the presence of sorbitol (conditions used for growing the *pkc1* null mutant).

PKC1 and SWI4 have separable effects on cell wall gene expression

The above data indicate that Pkc1p provides the bulk expression of some cell wall genes, since Pkc1p loss is clearly evident in mid-log phase cultures. In contrast, the effect of Swi4p is cell cycle specific and only apparent in synchronous cultures, suggesting that in fact the two genes have separable roles in cell wall gene expression. To confirm this we synchronized a *SWI4pkc1-8* strain. To ensure inactivation of Pkc1p, cells were shifted to 37°C prior to release from the α-factor block. One hour later, when the cells had assumed a morphology consistent with loss of Pkc1p function, the pheromone was removed and trancription of cell wall genes was analysed. There was clear periodic expression peaking at the G_1/S boundary of all the genes examined (Figure 7). Only one cycle was followed, as the cells had a generation time of some 7 h and synchrony was rapidly lost. Thus, Pkc1p function is not required for the cell cycle regulation of these genes. We assume the cell cycle regulation in the *pkc1-8* strain to be *SWI4* dependent, but we were unable to synchronize a *pkc1-8swi4*Δ strain to test this. Taken together with the results described above, this shows *SWI4* and *PKC1* to have distinct roles in cell wall gene expression.

We wished to further demonstrate the separable effects of Swi4p and Pkc1p on the regulation of the cell wall

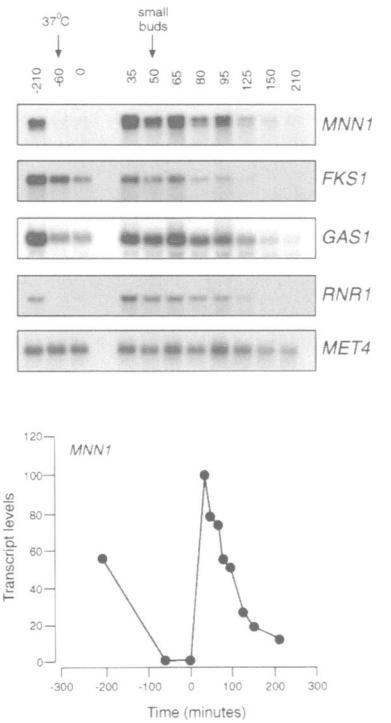

Fig. 7. Periodic expression of cell wall genes in a *pkc1-8* mutant. The expression of some cell wall genes in *pkc1-8* cells synchronized with α-factor was investigated by Northern analysis. mRNA levels were monitored at the indicated times. The zero time point corresponds to the sample taken just before removing the pheromone. At 150 min after addition of α-factor the temperature was raised to 37°C for the rest of the experiment. The time of appearance of small buds is indicated. Per cent budded cells increased from 5% at release from α-factor block to 66% at 80 min, remaining at that level for the rest of the experiment. The *RNR1* transcript is shown as an internal control for late G_1/S cell cycle-regulated genes and the *MET4* transcript is shown as a loading control. Quantitated *MNN1* transcript relative to *MET4* is graphically displayed.

genes and, in particular, to begin identifying the sequences mediating *PKC1*-dependent expression. Accordingly, different fragments from the *FKS1* upstream region were

Table V. Analysis of *FKS1* upstream regulatory sequences by using heterologous gene fusions

	β-Galactosidase specific activity[a]				
	pF712-82:*lacZ*[b]	pF390-82:*lacZ*[b]	pF712-380:*lacZ*[b]	SCB:*lacZ*[c]	MCB:*lacZ*[d]
W303-1A (wild-type)	195 ± 15	3.8 ± 1.6	17 000 ± 5000	220 ± 30	288 ± 22
BY604 (*swi4Δ*)	48 ± 8	3.5 ± 1.7	4300 ± 1300	20 ± 6	140 ± 40
JC6-3a (*pkc1-8*)	8 ± 3	1.6 ± 0.6	2900 ± 400	170 ± 30	255 ± 11
JC800 (*swi4Δ pkc1-8*)	1.9 ± 0.5	1.6 ± 0.7	420 ± 170	10.0 ± 1.5	n.d.

[a]Samples were collected after 4 h incubation at 37°C and enzymatic activity assayed in crude extracts. β-Galactosidase activity is expressed in U/mg protein, where 1 U is defined by $A_{420}\times10^3$/min. Values are derived from assays of at least three different extracts.
[b]Vector pLGL containing different fragments from the *FKS1* promoter (see Materials and methods).
[c]Vector pLGL containing a promoter fragment from the *HO* gene (from –507 to –367) with three SCB elements.
[d]Vector pLGL containing a synthetic oligonucleotide with three MCB elements.

inserted into a *lacZ* reporter construct and the effects of *swi4* and *pkc1* mutations on expression were examined. When a fragment encompassing sequences from –712 to –82 was used (plasmid pF712-82:*lacZ*), the effect of both the *swi4Δ* and *pkc1-8* mutations was more dramatically manifested than in the Northern analysis described above. In asynchronous cells the loss of Swi4p caused a 4-fold decrease in expression from the reporter, whereas the *pkc1-8* mutation led to a 25-fold reduction (Table V). Interestingly, the *pkc1-8swi4Δ* double mutant showed additive effects of the two mutations, transcription being almost entirely abolished. The activating region in this fragment was located primarily in the upstream half, since sequences from –390 to –82 (plasmid pF390-82:*lacZ*) promoted little expression from the reporter. Indeed, this region appears to contain a powerful repressor element, since the upstream half of the promoter in isolation (plasmid pF712-380:*lacZ*) stimulated β-galactosidase levels in the wild-type strain 100-fold greater than those observed with plasmid pF712-82:*lacZ*. Moreover, this region from –712 to –380 confers both *SWI4*- and *PKC1*-dependent regulation, expression being reduced 4-fold in the *swi4Δ* strain and 6-fold in the *pkc1-8* mutant. Again, the effects of the two mutations are additive, as transcription is reduced 40 times in the double mutant. Thus, the regulatory elements mediating *SWI4*- and *PKC1*-dependent expression are located in a region from –712 to –380 of the *FKS1* gene. In the case of *SWI4*, these are almost certainly the SCB and MCB elements identified in this region (Table IV). However, for *PKC1*-dependent expression sequences other than SCB and MCB elements are clearly necessary, since *pkc1* mutation had no significant effect on expression from SCB– or MCB–reporter constructs (Table V). This, together with the additivity of the *swi4Δ* and *pkc1-8* mutations, is consistent with *SWI4* and *PKC1* having separable roles in cell wall gene expression.

swi4Δ and mpk1Δ are synthetic lethal

Pkc1p acts through a bifurcated pathway comprising a MAP kinase cascade in one branch. Both single copy and multicopy plasmids containing genes encoding components of this kinase cascade (*BCK1*, *MKK1*, *MKK2* and *MPK1)* rescued the *pkc1-8swi4Δ* mutant, suggesting that the likely reason for the synthetic lethality between *swi4* and *pkc1* is inactivation of the *PKC1*-regulated MAP kinase. To investigate this possibility, we explored a possible interaction between *SWI4* and *MPK1*. We found that overexpression of *SWI4* rescued the temperature-sensitive cell lysis defect of a *mpk1Δ* strain (Figure 8). In addition, tetrad analysis of a diploid heterozygous for both *swi4Δ* and *mpk1Δ* indicated that disruption of *swi4* is lethal with *mpk1Δ* (Table VI). In our strain background *mpk1* deletions are not completely temperature sensitive at 37°C, but grow weakly. Thus, of 48 *mpk1Δ* spore clones, 43 grew at 37°C and only five failed to grow. In contrast, none of the 32 *swi4Δmpk1Δ* spores grew at 37°C. These results demonstrate a synthetic interaction between *swi4Δ* and *mpk1Δ*.

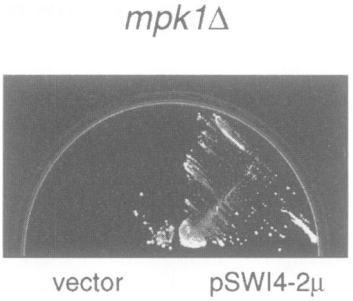

Fig. 8. Suppression of the temperature-sensitive growth defect of the *mpk1Δ* mutant by overexpression of *SWI4*. The *mpk1Δ* strain transformed with YEp24 or a multicopy plasmid containing the *SWI4* gene was streaked onto YPD medium and incubated at 37°C for 3 days.

Table VI. Synthetic interaction between *swi4Δ* and *mpk1Δ*

Spore genotype	No. of spores	Growth at 37°C
SWI4 MPK1	44	44 +
		0 –
swi4 MPK1	43	43 +
		0 –
SWI4 mpk1	48	43 +
		5 –
swi4 mpk1	32	0 +
		32 –

The *pkc1-8swi4Δ* double mutant was crossed with a *mpk1Δ* strain. All tetrads were dissected on agar plates containing 1 M sorbitol. Note that *mpk1Δ* strains in the genetic background we used do grow at 37°C, albeit rather weakly.

Mpk1p positively regulates cell wall gene transcription

The previous result prompted us to analyse whether the regulation of cell wall gene expression by *PKC1* is also dependent on the MAP kinase cascade by measuring the

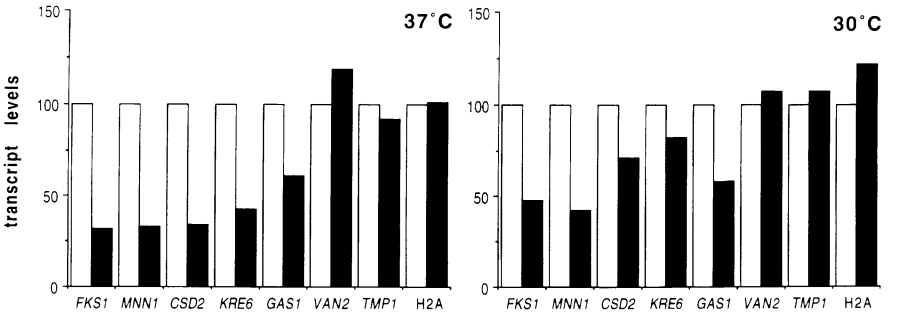

Fig. 9. Regulation of cell wall gene expression by the Mpk1p MAP kinase. Mid-log cultures in YPD of a *mpk1*Δ strain transformed with a centromeric plasmid containing the *MPK1* gene (open bars) or a control vector (black bars) were split in two and incubated at 30 or 37°C. After 6 h, transcript levels of the indicated genes were determined by Northern analysis and quantitated relative to actin as internal control. Values of the wild-type strain are referred to as 100.

transcript levels of some cell wall genes in a *mpk1*Δ strain. Disruption of *MPK1* reproducibly led to a strong reduction in the expression of *FKS1*, *MNN1* and *CSD2* at 37°C (Figure 9), as was observed with the *pkc1-8* mutant. In addition to these genes, expression of *GAS1* and *KRE6* was also significantly compromised. The fact that the *pkc1-8* mutation only slightly affects these two genes could be due to a residual Mpk1p kinase activity in the *pkc1-8* strain compared with the total absence of activity in the *mpk1* null mutant. In contrast to these results, the loss of Mpk1p function, as occurs with the *pkc1-8* mutation, had no effect on the level of the *VAN2* transcript or on the control transcripts *TMP1* and H2A. Decreased expression of the same five genes was also reproducibly observed at 30°C (Figure 9). However, the reduction was less severe than at 37°C, an observation consistent with the temperature sensitivity of the cell lysis defect in the *mpk1*Δ strain. These results complement those obtained with the *pkc1* mutants and demonstrate that the *PKC1*-regulated MAP kinase cascade is a positive regulator of the expression of, at least, five genes (*FKS1*, *MNN1*, *CSD2*, *KRE6* and *GAS1*) involved in cell wall biosynthesis.

Discussion

A synthetic interaction between SWI4 and PKC1 reveals a role for SBF in cell wall biosynthesis

We have started a search for genes functionally interacting with *SWI4*. The characterization of *rsf8* (now *pkc1-8*), one of the mutants isolated, has revealed a synthetic lethal interaction between the cell cycle transcription factor SBF (Swi4p–Swi6p) and the *PKC1* MAP kinase pathway. The major known function of the *PKC1* pathway is the control of cell wall assembly. The detected genetic interaction of *SWI4* and *SWI6* with the *PKC1* pathway suggests a role for SBF in cell wall assembly in yeast. This idea is strongly supported by the additive effects of *swi4*Δ and *pkc1-8* on cell integrity. At 37°C, the *pkc1-8* mutation alone displays no cell lysis, whereas the *pkc1-8swi4*Δ double mutant shows essentially 100% lysis. Moreover, treatments affecting cell wall structure are more harmful in the absence of *SWI4* or *SWI6*. Calcofluor white is a dye that blocks chitin polymerization, resulting in a weakened cell wall, and SDS induces lysis of cells with fragile cell walls. The hypersensitivity of *swi4*Δ and *swi6*Δ strains to both compounds clearly suggests that loss of SBF function leads to a weakened wall. We conclude that SBF has a function in yeast morphogenesis, controlling some aspect of cell wall biosynthesis.

To date, the known function of SBF is transcriptional regulation of the *HO* gene and the cyclin genes *CLN1* and *CLN2*. We considered the possibility that the effect of SBF in cell wall integrity was indirect, through its control of *CLN1* and *CLN2*. Cln–Cdc28 kinase activity is already known to have a role in bud emergence (Benton *et al.*, 1993; Cvrckova and Nasmyth, 1993). However, neither high copy *CLN1* or *CLN2* nor ectopic expression of *CLN2* was able to rescue the *pkc1-8swi4*Δ double mutant, ruling out an indirect effect of Swi4p through *CLN* expression. The synthetic lethality between *swi4* or *swi6* and *pkc1* has therefore revealed a novel role for SBF in cell wall metabolism, presumably through the control of new target genes.

Coordinated cell cycle regulation of cell wall genes by Swi4p

A computer search for genes containing SCB elements in their promoters revealed a number of genes encoding proteins involved in cell wall biosynthesis. Importantly, we showed that expression of at least some of these genes is regulated in the cell cycle, maximum transcription occurring in late G_1. This timing is coincident with the expression of *CLN2* and *RNR1* genes and slightly prior to expression of histone H2A. However, in contrast to *CLN2* and *RNR1*, most of the cell wall genes we studied have a relatively high constitutive expression throughout the cycle, probably because cell wall biosynthesis is not totally restricted to one stage of the cycle. In addition, the peaks in cell wall gene expression are broader, spreading into early S phase, and the precise timing of expression between genes varies slightly. This slight difference could be due to the different stability of the transcripts or different kinetics in the activation depending on the context of each promoter and on interactions with other regulatory mechanisms.

The cell wall genes we examined are not only expressed at approximately the same time in the cell cycle, but they are also coordinately regulated by the same transcription factor. In all six cases, cell cycle regulation is severely perturbed in a *swi4*Δ strain, indicating that the periodic expression is due specifically to SBF. This result seems to preclude a major role for DSC1/MBF in regulating cell wall genes. In agreement with this, overexpression of *MBP1* only weakly suppresses the *pkc1-8swi4*Δ strain and

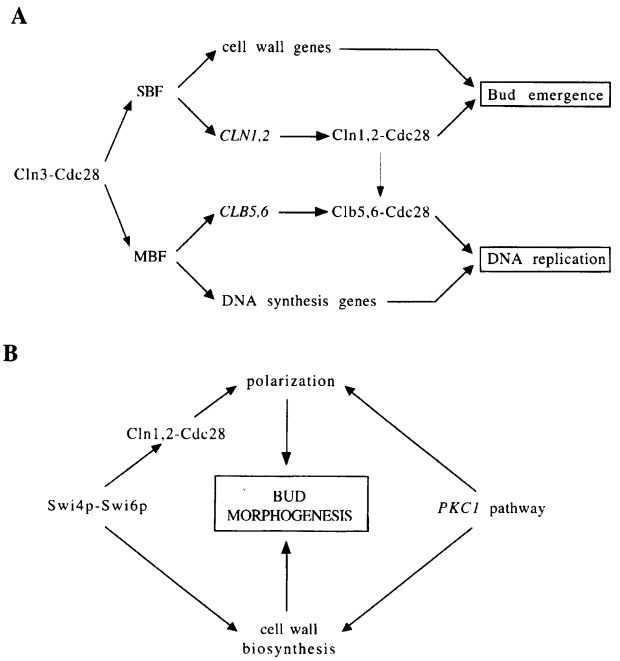

Fig. 10. (**A**) The Start-specific transcription program. The first step in initiating a new cell cycle is activation of the Cln3–Cdc28 kinase, which in turn triggers a burst of new gene expression in late G_1. This expression is mediated by the transcription factors SBF and DSC1/MBF and provides gene products necessary for performance of the different Start-related events. SBF is the primary regulator of genes involved in bud emergence, both through controlling expression of *CLN1* and *CLN2* and at least six genes involved in cell wall biosynthesis. On the other hand, DSC1/MBF is the predominant transcription factor controlling genes involved in DNA replication, regulating the expression of *CLB5* and *CLB6*, as well as most of the DNA synthesis genes. (**B**) Control of bud morphogenesis in yeast. Bud development depends upon two simultaneous processes: activation of cell wall biosynthesis and polarization of growth to the bud. Coordination of both processes relies on the use of common regulatory pathways: the *PKC1* pathway and the transcription factor SBF activate transcription of genes involved in cell wall biosynthesis, as well as control the establishment of polarized growth, in the case of SBF indirectly through activation of *CLN1* and *CLN2* gene expression.

*mbp1*Δ, as opposed to *swi4*Δ and *swi6*Δ, showed neither synthetic interaction with *pkc1* mutations nor hypersensitivity to calcofluor white or SDS. In summary, the primary transcription factor in coordinating periodic expression of cell wall genes is SBF and DSC1/MBF has a minor role, if any.

This result further elaborates the late G_1-specific transcription programme (Figure 10A) and could help explain coordination of events in late G_1. Of these disparate events, Start, initiation of S phase, spindle pole body duplication and bud emergence, the first three were already known to involve cell cycle regulation of gene expression (Johnston and Lowndes, 1992; Kilmartin *et al.*, 1993; Koch and Nasmyth, 1994). We have now described the occurrence of cell cycle-regulated genes involved in cell wall biosynthesis. It therefore seems likely that periodic expression of appropriate genes plays a crucial role in coordination of events in late G_1, which may be physiologically advantageous if not essential for viability.

The PKC1 MAP kinase pathway regulates genes involved in cell wall biosynthesis

The phenotype of mutants in the *PKC1* MAP kinase pathway indicates that this pathway is involved in the maintenance of yeast cell integrity (Levin *et al.*, 1994). However, the molecular basis of the regulation of cell integrity remains unclear. Because transcription factors are common targets for eukaryotic MAP kinase cascades, a plausible hypothesis is that the *PKC1* MAP kinase pathway could affect cell wall metabolism through control of appropriate genes. Indeed, we have found that a number of genes involved in different aspects of cell wall biosynthesis are positively controlled by *PKC1*. They are *FKS1*, *GAS1*, *KRE6*, *CSD2* and *MNN1*, which code for key proteins in the biosynthesis of all the components of the cell wall, i.e. (1-3)-β-glucan (*FKS1* and *GAS1*), (1-6)-β-glucan (*KRE6*), chitin (*CSD2*) and *N*- and *O*-linked mannoproteins (*MNN1*). The defective transcription of these genes may well be at least one of the causes of the weakened cell wall in *pkc1* and *mpk1* mutants. Certainly, the (1-3)-β-glucan is responsible for the mechanical strength of the wall (Klis, 1994) and cells with a reduced activity in the synthesis and/or assembly of this component will have a fragile cell wall that could lead to the loss of cell integrity.

This is the first time that regulation of gene transcription by the *PKC1* MAP kinase pathway has been clearly demonstrated, with the possible exception of the *PTP2* gene (Huang and Symington, 1995). Presumably, Mpk1p phosphorylates a transcription factor which in turn activates expression of these cell wall genes. Recently, the transcription factor Rlm1p was suggested to act downstream of Mpk1p (Watanabe *et al.*, 1995). However, in contrast to *mpk1*Δ, *rlm1*Δ cells grow normally at any temperature without any apparent defect in cell integrity, suggesting that another still unknown transcription factor is responsible for the Mpk1p-dependent expression of cell wall genes.

In an initial attempt to understand the regulation of transcription by the *PKC1* pathway, we have identified a UAS mediating this regulation between –712 and –380 in the *FKS1* gene. Work is in progress to further characterize the sequences and the transcription factor involved in *PKC1*-dependent expression of cell wall genes.

The role of the *PKC1* pathway in cell wall metabolism is surely not limited to the regulation of the genes reported here. Recently, Shimizu *et al.* (1994) showed that there is a slight, 2-fold increase in the level of the (1-3)-β-endoglucanase Bgl2p in a *hpo2/pkc1* mutant. They suggested that *PKC1* negatively regulates expression of this endoglucanase. In addition, the *PKC1* pathway could regulate cell wall biosynthesis at levels other than transcriptional control. For instance, cell wall components must be delivered to the bud through the secretory pathway during polarized growth. It has been shown that a mutant in *mpk1* failed to establish polarization and accumulated secretory vesicles in the cytoplasm (Mazzoni *et al.*, 1993). This defect in polarization must also affect, indirectly, cell wall assembly.

Implications of the regulation of cell wall gene expression by SBF and PKC1 on yeast morphogenesis

Our data indicate a dual regulation of cell wall gene expression with different and separable functions for the *PKC1* MAP kinase pathway and SBF in the control of cell integrity. Mutations in the *PKC1* MAP kinase pathway

severely affect the mid-log transcript levels of some cell wall genes, but the genes are still periodically expressed. On the other hand, the *swi4* mutation severely perturbs the periodic expression of cell wall genes, but without significantly affecting the overall level of transcription in mid-log cells. The predominant role for *PKC1* was also clearly evident in the dramatic reduction in β-galactosidase expression from the *FKS1*–reporter construct in the *pkc1-8* mutant strain. We therefore propose that the *PKC1* MAP kinase pathway is the major regulator of cell wall biosynthesis, being responsible for the constitutive expression of some cell wall genes across the entire cell cycle, whereas SBF coordinates cell wall biosynthesis with cell cycle modulating cell wall gene expression through the cell cycle. Our data does not rule out the possibility that Swi4p could be a target of the *PKC1* pathway; however, other major inputs on Swi4p and other major outputs for the *PKC1* pathway would be necessary to explain the synthetic lethality between *swi4* and *pkc1* mutations and their different roles in gene expression.

The timing of maximum expression of the cell wall genes we analysed occurred in late G_1 and is coincident with the early stages of budding. This is the most crucial period in the cell cycle regarding the maintenance of cell integrity, since the cell wall necessarily must undergo extensive remodelling to enable bud expansion (Levin *et al.*, 1994). Periodic expression of cell wall genes in late G_1/early S may help to successfully traverse this stage. This notion is supported by the sensitivity of *swi4*Δ and *swi6*Δ strains to CFW and SDS. The absence of periodic expression of cell wall genes in these mutants presumably reduces the supply and/or normal stoichiometry of cell wall components during bud emergence, leading to defects in cell wall integrity at this critical stage of the cell cycle. Hence, the regulation of cell wall genes is an example where periodic expression fulfils an important physiological function.

The proposed dual regulation of cell wall genes might explain the synthetic lethality between *swi4*Δ or *swi6*Δ and mutants in the *PKC1* MAP kinase pathway. The lysis phenotype of the *pkc1-8swi4*Δ mutant at 37°C would be due to reduced expression of the various cell wall genes controlled by each of the two pathways. Indeed, an additive effect of the two pathways is evident in the *FKS1*–reporter constructs. Whilst none of the *SWI4*- and *PKC1*-regulated genes we have identified is essential, reduced expression of a large group of genes involved in cell wall construction might have cumulative effects on cell wall structure. This could result in compromised cell integrity and the lysis we observed with the *pkc1-8swi4*Δ strain.

Our results provide new insights into the regulation of bud morphogenesis. Bud growth occurs as the result of two combined events. First, an increased synthesis and assembly of cell wall components and, second, polarization of growth by rearrangement of the cytoskeleton and the secretory machinery to specifically deliver cell wall constituents to the bud. It is already known that Cln–Cdc28 activity (Lew and Reed, 1993) and the *PKC1* MAP kinase pathway (Costigan and Snyder, 1992; Mazzoni *et al.*, 1993) are required for the establishment of polarization and our data show that SBF and the *PKC1* MAP kinase pathway positively regulate expression of cell wall genes (Figure 10B). Thus, by using common mechanisms in the regulation of both events, cells could coordinate polarization and cell wall biosynthesis during bud morphogenesis.

Materials and methods

Strains and growth conditions

The haploid yeast strains used in this study were as follows: W303-1A (MATa, *ade2, trp1, leu2, his3, ura3, can1*), CG378 (MATa, *ade5, trp1, leu2, ura3, can1*), BY604 (MATα, *ade2, trp1, leu2, his3, ura3, met⁻, can1, ho-lacZ, swi4::LEU2*), BY600 (MATα, *ade2, trp1, leu2, his3, ura3, met⁻, can1, ho-lacZ, swi6::TRP1*), CY19 (MATα, *ade2, trp1, leu2, his3, ura3, met⁻, can1, ho-lacZ, mbp1::URA3*), JC800 (MATα, *ade2, trp1, leu2, his3, ura3, met⁻, can1, ho-lacZ, swi4::LEU2, rsf8/pkc1-8*), JC6-3a (MATa, *ade2, trp1, leu2, his3, ura3, can1, rsf8*), GPY1115 (MATa, *ade2, trp1, leu2, his3, ura3, pkc1::HIS3*) (Paravicini *et al.*, 1992) and DL454 (MATa, *trp1, leu2, his4, ura3, can1, mpk1::TRP1*) (Lee *et al.*, 1993). Disruption of the chromosomal copy of *SWI4* in CG378 was carried out using plasmid Bd194 kindly provided by L.Breeden (Breeden and Mikesell, 1991).

Cells were grown in YPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose) or, for diploid or plasmid selection, in synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate amino acids at 40 μg/ml. In the case of the *pkc1*Δ strain media were supplemented with 1 M sorbitol. For testing cell wall defects CFW (Sigma) or SDS was used at concentrations from 0.002 to 0.04 mg/ml or 0.001 to 0.04% respectively in YPD medium. Unless otherwise stated the growth temperature used was 25°C.

Plasmid construction

Oligonucleotides 1 (5′-ATGTCTCGAGCGGGCAATCAGAATCTGT-3′), 2 (5′-GAGAGGTTAACGTCCTCTAACTATCAG-3′), 3 (5′-CTA-GTCTCGAGAATTTTCATCGATCCA-3′) and 4 (5′-GGAACGAATG-GATCGATGAAAA-3′) were used to amplify different DNA fragments from the 5′ flank of the *FKS1* gene. Fragments (digested with *Xho*I and *Hin*cII in the case of DNA amplified with oligonucleotides 1 and 2 and 3 and 2 and digested with *Cla*I, filled in with Klenow and digested with *Xho*I for the fragment amplified with oligonucleotides 1 and 4) were cloned in plasmid pLGL (containing a *CYC1* minimal promoter fused to the *lacZ* coding region) cleaved with *Xho*I and *Sma*I. The resulting plasmids, pF712-82:*lacZ*, pF390-82:*lacZ* and pF712-380:*lacZ*, contain *FKS1* gene sequences from −712 to −82, from −390 to −82 and from −712 to −380 respectively. Two different clones from each construct were checked in the expression analysis.

Cell synchronization techniques

Synchronization of MATa wild-type cells (strain CG378) was obtained by release from α-factor-induced cell cycle arrest. Cells were grown in YPD to a density of 4.5×10^6 cells/ml at 25°C. Synthetic α-factor peptide was added to a final concentration of 3.5 μg/ml and the cells were incubated for a further 4 h. The α-factor was removed by rapid filtration and washing and the cells were resuspended in fresh medium and incubated at 25°C. In the case of synchronization of MATa *pkc1-8* cells (strain JC6-3a), α-factor peptide to a final concentration of 3 μg/ml was added to cells grown in YPD at 25°C to a density of 2×10^6 cells/ml. After 150 min the culture was shifted to 37°C and incubated for a further 60 min. The α-factor was removed and cells incubated in fresh YPD at 37°C. Synchronization of *swi4*Δ cells was carried out by centrifugal elutriation. Strain BY604 was grown in YPD to 5×10^6 cells/ml at 30°C. A 4 l culture was cooled to 4°C and loaded at a flow rate of 100 ml/min into a 40 ml chamber in a Beckman JE-5.0 rotor running at 4800 r.p.m. in a Beckman J-6M/E centrifuge. Once the culture was loaded, the flow rate was gradually increased to ~170 ml/min, at which point 250 ml samples were collected. After being checked microscopically for uniformity of size, the first six samples were pooled to give a culture with a cell density of 6×10^5 cells/ml and incubation was continued at 30°C.

RNA analysis

Total RNA was extracted from cells as described previously (White *et al.*, 1986). A 5 μg sample of total RNA was denatured with glyoxal, separated on a 1.2% agarose gel and transferred to a GeneScreen hybridization membrane (Dupont, NEN Research Products, Boston, MA). Blots were probed with restriction fragments (*ACT1, CLN2*, H2A, *RNR1* and *MET4*) or PCR fragments (*FKS1, GAS1, KRE6, MNN1, VRG4*

and *CSD2*) internal to the genes concerned. In the case of *FKS1*, the probe extended from −34 to +410, a region with low identity to the highly homologous *FKS2* gene. Levels of hybridization were quantified by densitometry of appropriately exposed autoradiographs using a Personal Densitometer PD-130 (Molecular Dynamics) and were normalized to actin or *MET4* as loading control.

Analysis of the cellular DNA content by flow cytometry

Samples containing ~10^7 cells were fixed in 70% ethanol, treated successively with RNase and pepsin and stained with 50 μg/ml propidium iodide essentially as described in Corliss and White (1981). DNA content was then analysed with a Becton Dickinson FACScan and CELLQuest software.

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