Molecular Biology of the Cell
Vol. 10, 3389–3400, October 1999

Association of the Cell Cycle Transcription Factor Mbp1 with the Skn7 Response Regulator in Budding Yeast

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Submitted January 26, 1999; Accepted July 23, 1999
Monitoring Editor: Thomas D. Fox

We previously isolated the *SKN7* gene in a screen designed to isolate new components of the G1-S cell cycle transcription machinery in budding yeast. We have now found that Skn7 associates with Mbp1, the DNA-binding component of the G1-S transcription factor DSC1/MBF. *SKN7* and *MBP1* show several genetic interactions. Skn7 overexpression is lethal and is suppressed by a mutation in *MBP1*. Similarly, high overexpression of Mbp1 is lethal and can be suppressed by *skn7* mutations. *SKN7* is also required for *MBP1* function in a mutant compromised for G1-specific transcription. Gel-retardation assays indicate that Skn7 is not an integral part of MBF. However, a physical interaction between Skn7 and Mbp1 was detected using two-hybrid assays and GST pulldowns. Thus, Skn7 and Mbp1 seem to form a transcription factor independent of MBF. Genetic data suggest that this new transcription factor could be involved in the bud-emergence process.

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, entry into the cell cycle is controlled at a point in late G1, called START, when the environmental conditions are assessed. Passage through START commits the cell to a new cell cycle and triggers the initial events, namely DNA replication, spindle pole body duplication, and bud emergence. START depends on the activation of two G1/cdk complexes, Cln1-Cdc28 and Cln2-Cdc28 (reviewed by Nasmyth, 1993). The transcription of the two genes *CLN1* and *CLN2*, and of many other genes acting in late G1, is dependent on the accumulation of Cln3, a third G1 cyclin. When a threshold concentration of the Cln3-Cdc28 kinase complex is reached, a burst of late G1-specific gene transcription occurs, including *CLN1* and *CLN2* (Dirick *et al.*, 1995; Stuart and Wittenberg, 1995).

The Cln3-Cdc28–dependent transcription of late G1-specific genes is mediated by two related heterodimeric transcription factors, SBF and DSC1/MBF (reviewed by Mendenhall and Hodge, 1998). They each contain a related DNA-binding protein, Swi4 for SBF and Mbp1 for MBF, and the same regulatory protein, Swi6. Although SBF and MBF bind to specific DNA sequences, the SCB element (CACGAAA) and the MCB element (ACGCGT), respectively, their functions are partially redundant (Koch *et al.*, 1993).

In addition to *CLN1* and *CLN2*, it has recently been shown that a large group of genes are expressed in late G1 (Cho *et al.*, 1998; Spellman *et al.*, 1998). These genes code for proteins necessary for the correct execution of START-dependent functions. For example, most DNA replication genes are under the control of MBF (Johnston and Lowndes, 1992). Similarly, the coordinated expression at START, or immediately after, of a number of genes involved in cell wall biosynthesis has been shown to be dependent on SBF (Igual *et al.*, 1996); likewise, genes involved in budding and morphogenesis are also expressed in late G1 (Cho *et al.*, 1998; Spellman *et al.*, 1998). Coordinated cell cycle–dependent transcription through the SCB and MCB elements is thus an important means used by the cell to achieve coordination of the early events of the cell cycle (Johnston, 1992).

Combinations of mutations that inactivate both transcription factors, such as *swi4 swi6* and *swi4 mbp1*, are lethal (Nasmyth and Dirick, 1991; Koch *et al.*, 1993). However, surprisingly, *SWI6* is not an essential gene, suggesting additional complexity in the G1 transcriptional machinery. In view of this fact, we screened for additional activators of MCB- and SCB-dependent transcription and identified one gene, *SKN7/BRY1* (Morgan *et al.*, 1995b). On a high-copy-number plasmid, *SKN7* bypasses the essential requirement

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for SBF and MBF, restoring *CLN1* and *CLN2* transcription through their SCB and MCB promoter elements (Morgan *et al.*, 1995b). Skn7, therefore, can activate the expression of late G1-specific genes. However, the means by which it does so is not clear.

The Skn7 protein interacts with the small GTPase Rho1 (Alberts *et al.*, 1998), suggesting that it might be partly controlled by Rho1. In agreement with this idea, mutations in *SKN7* and *PKC1*, one of the known Rho1 effectors (Nonaka *et al.*, 1995), are synthetically lethal (Brown *et al.*, 1994; Morgan *et al.*, 1995b). In budding yeast, the PKC MAPK pathway controls cell wall gene expression (Igual *et al.*, 1996), perhaps through direct regulation of Swi4 (Madden *et al.*, 1997). Given its role in G1 transcription, *SKN7* could itself be a transcriptional activator of cell wall genes (Brown *et al.*, 1993, 1994; Morgan *et al.*, 1995b), although direct experimental evidence in support of this idea is lacking. In addition, *SKN7* functions in the oxidative stress response (Krems *et al.*, 1996; Morgan *et al.*, 1997), acting as a transcription factor in cooperation with Yap1 in the regulation of at least *TRX2* (Morgan *et al.*, 1997). However, any connections between the Skn7-Yap1 interaction, the oxidative stress response, and the G1 transcription program remain obscure.

The *SKN7* protein shows in its C-terminal homology to the response regulators of bacterial two-component signal transduction systems (Brown *et al.*, 1993; Morgan *et al.*, 1995b). Skn7 has indeed been shown to form a two-component system in yeast with the histidine kinase Sln1 (Ketala *et al.*, 1998; Li *et al.*, 1998). In prokaryotes, these signaling pathways affect many aspects of cell physiology, including the cell cycle (Quon *et al.*, 1996). They comprise a sensor and a response regulator; stimulation of the sensor leads to a phosphotransfer to a conserved aspartate residue of the response regulator, usually a transcription factor (for reviews, see Bourret *et al.*, 1991; Parkinson, 1993). The *SKN7* and *SLN1* proteins, therefore, may be part of a two-component system in yeast that regulates late G1 gene expression and cell wall gene expression in response to an as-yet-unidentified signal.

The *SKN7* DNA-binding domain shows homology to the heat-shock-factor DNA-binding domain (Brown *et al.*, 1993; Morgan *et al.*, 1995b), and it is thus unlikely that Skn7 binds SCB and MCB elements directly. This suggests that for any G1 transcriptional role, Skn7 would require association with a partner protein. Here we provide genetic and biochemical evidence that the partner for Skn7 in G1-regulated transcription is *MBP1*, the DNA-binding component of the MBF transcription factor. We show that Skn7 and Mbp1 associate, both in vivo and in vitro, and that in the absence of Swi6, Skn7 is necessary for Mbp1-dependent transcription. Finally, genetic data suggest that the Skn7/Mbp1 complex has a role in bud emergence.

MATERIALS AND METHODS

Strains and Growth Conditions

The haploid strains used in this study were as follows: W303-1a (**a** *ade2-1 trp1-1 can1-100 leu2-3112 his3-11 ura3*), K2003 (**a** *ade2 his3 met leu2 trp1 ura3 swi4*^{ts} *swi6*::TRP1), K3294 (**a** *ade2-1 met trp1-1 leu2-3112 can1-100 his3 ura3 ho-lacZ mbp1::URA3*), GPY1115 (**a**, *ade2 trp1 leu2 his3 ura3 pkc1*::*HIS3*) (Paravicini *et al.*, 1992), DJTD2-16D (α *cdc42-1 leu2 ura3 his4 trp1 gal2*), CG379 (α *his7-2 leu2-3 trp1-289 ura3-52*), and YN166 (*trp1 ade2 leu2 ura3 GAL1-URA3 GAL1-lacZ*). The *skn7*Δ, *swi6*Δ, and *swi4*Δ strains used in this study are isogenic strains in the W303 background. The *mbp1/bor2* mutants used in this study were those isolated in the W303 background.

Yeast Techniques

Cells were grown in YEPD (1% yeast extract, 2% bactopeptone, 2% glucose) or, for diploid or plasmid selection, in synthetic minimal medium (0.67% yeast nitrogen base [YNB], 2% glucose or galactose) supplemented with the appropriate amino acids at 40 μg/ml. The growth temperature was 30°C, unless otherwise stated. Yeast transformations were performed by a modification of the lithium acetate procedure (Gietz and Sugino, 1988).

Assays for β-galactosidase activity were performed on midlog-phase cells as described previously (Guarente, 1983). Activities are given in OD units at 420 nm·min^{-1}·mg^{-1} protein. Values represent the average of four independent experiments. FACS analysis was carried out as described previously (Igual *et al.*, 1996).

Plasmid Constructs

SKN7 Plasmids YEp24/*SKN7* and pAB36 (pMW20/*SKN7*) have been described previously (Morgan *et al.*, 1995b), as have pBAM1 (YCplac33/*SKN7*) and pBAM2 (YCplac33/*skn7D427N*) (Morgan *et al.*, 1997). pAB56 was created by inserting a 2.9-kilobase *Pvu*II-*Sph*I fragment containing the *GAL-SKN7* fusion into YEplac112 (Gietz and Sugino, 1988). pAB52 was created by ligating the coding region of *SKN7*, with *Bam*HI (5′) and *Spe*I (3′) linkers added by PCR, into pT7linktag (a kind gift from N. Jones, Imperial Cancer Research Fund, London, United Kingdom) so that *SKN7* is under the control of the T7 promoter. pAB53 was constructed by ligating the coding region of *SKN7*, with *Bam*HI (5′) and *Spe*I (3′) linkers added by PCR, into pGEX-KG (Pharmacia, Uppsala, Sweden) so that Skn7 can be expressed in *Escherichia coli* as a fusion with GST. pAB61, pAB63, and pAB64 are deletions of pAB53 in which the fusion protein is truncated after residues 247, 473, and 311, respectively. pAB65 is a fusion between GST and residues 381–623 of *SKN7*, including the receiver domain. The *skn7ΔHR* allele, encoding a protein deleted from residues 238–261, was amplified by PCR from the pGAD*skn7ΔHR* plasmid and inserted into the *Eco*RI site of pGEX-KG in frame with the GST, thus creating pAB81. pAB93 and pAB96 were constructed as follows: the *skn7ΔHR* allele was cut by *Pst*I and *Sal*I from the pGAD*skn7ΔHR* plasmid and inserted into pB-*BRY1*/*SKN7* (Morgan *et al.*, 1995b) digested with *Pst*I and *Sal*I. A *Sac*I/*Sal*I fragment from the resulting plasmid was then ligated into either YEplac195 or YCplac33, thus placing the *skn7ΔHR* under the control of the *SKN7* promoter, on a 2-μm or a centromeric plasmid to give pAB93 and pAB96, respectively. For pAB97 and pAB98, the *SKN7* promoter was amplified by PCR with *Kpn*I and *Eco*RI linkers added on both ends. The *SKN7* ORF was amplified by PCR from residues 151–623 (*skn7ΔDBD*), i.e., excluding the DNA-binding domain, with an in-frame ATG codon and an *Eco*RI site added in 5′ and a *Spe*I site added in 3′. The two PCR fragments were then cloned into Bluescript so that *skn7ΔDBD* was put under the control of the *SKN7* promoter. The p*SKN7-skn7ΔDBD* construction was then moved as a *Sac*I/*Kpn*I fragment into YEplac195 and YCplac33, creating pAB97 and pAB98, respectively. All four plasmids, i.e., pAB93, pAB96, pAB97, and pAB98, were checked by Western blotting for production of a protein of the expected size in a W303 *skn7*Δ background.

MBP1 Plasmids The *MBP1* ORF was amplified by PCR, with *Bam*HI sites added at either end of the gene, and then cloned into pEMBLyex4 (Murray, 1987) to create p*GAL-MBP1*. pAB48 was made by subcloning a *Sac*I/*Sal*I fragment containing the *MBP1* gene and its upstream sequences from pCK13 (a kind gift from K. Nasmyth, Institute of Molecular Pathology, Vienna, Austria) into YEplac195. pAB59 was constructed by ligating the coding region of *MBP1*, with

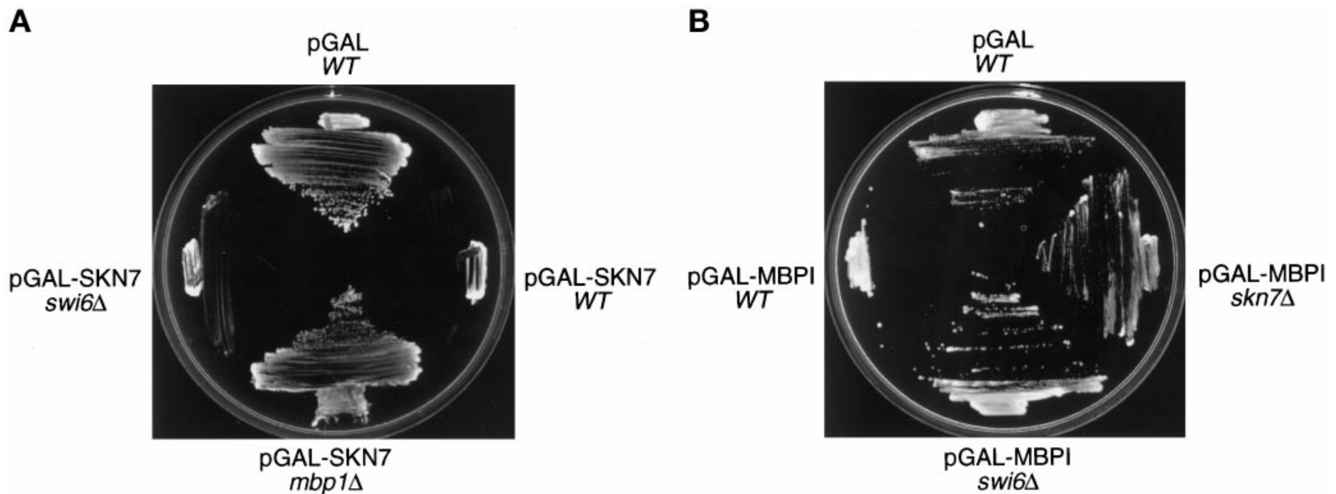

Figure 1. The genetic interactions between *SKN7* and *MBP1*. (A) *mbp1* mutations confer resistance to p*GAL-SKN7*–dependent lethality. Derivatives of the wild-type strain W303 (WT) were transformed with either the pMW20 empty vector (pGAL) or the p*GAL-SKN7* construct. Transformants were streaked on galactose-containing minimal agar plates and incubated at 30°C. (B) *skn7* mutations confer resistance to p*GAL-MBP1*–dependent lethality. Derivatives of the wild-type strain W303 were transformed with either the pEMBLyex4 empty vector (pGAL) or the p*GAL-MBP1* construct. Transformants were streaked on minimal agar plates containing galactose and lacking leucine and incubated at 30°C.

*Bam*HI (5′) and *Spe*I (3′) linkers added by PCR, into pT7linktag so that *MBP1* is under the control of the T7 promoter. For the two-hybrid experiments, an *Nco*I-*Bgl*II fragment, corresponding to residues 215–833, was cloned in frame into pAS1-CYH_2 (Harper *et al.*, 1993), thus creating pAB75.

The plasmids expressing *SWI4* and *SWI6* under the control of the T7 promoter have already been described, as has the *GAL-CDC42A118* plasmid (Ziman *et al.*, 1991). The 2-μm–based plasmids carrying *CLB5* and *CLB6* come from laboratory stocks.

Isolation of Mutants Resistant to GAL-SKN7–dependent Lethality

The W303-1A and CG379 strains were transformed with pAB36, a centromeric plasmid carrying *GAL-SKN7*. Transformants were grown in liquid glucose minimal medium at 25°C until midlog phase (~5×10^6 cells/ml). They were then plated onto minimal medium, with galactose as the carbon source, and incubated at 25°C. Spontaneous mutants appeared at a frequency of ~10^{-7}. These were crossed to the wild-type strain of the opposite mating type. If the growth phenotype on galactose was due to a plasmid rearrangement, this would be apparent in the diploid, so only those mutants complemented by the wild type were studied further.

GST-pulldown Assays

GST-pulldown assays were performed essentially as described by Siegmund and Nasmyth (1996), except that after binding of the in vitro synthesized protein to the GST fusion protein, four washes were performed at 500 mM NaCl. Pellets were washed another two times in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA before being boiled in SDS-PAGE sample buffer and loaded on a SDS-polyacrylamide gel. After migration, the gel was fixed, dried, and subjected to autoradiography at −70°C.

Gel Mobility Shift Assays

Protein extracts from log-phase cultures grown at 30°C in YEPD were prepared and gel-retardation assays were performed as previously described (Lowndes *et al.*, 1991). The 3xMCB probe has already been described (Lowndes *et al.*, 1991).

RNA Analysis

Total RNA was isolated from yeast strains grown under the conditions described (Morgan *et al.*, 1995b). Northern hybridization techniques have also been described previously (Morgan *et al.*, 1995b).

RESULTS

Isolation of Mutants Resistant to GAL-SKN7

We wished to explore further the interaction of Skn7 with the G1 transcription machinery and to determine its cell cycle role. We previously showed that overexpression of *SKN7* from the *GAL* promoter is lethal (Morgan *et al.*, 1995b) (Figure 1A). Microscopic analysis showed that cultures of cells overexpressing *SKN7* accumulate swollen, unbudded cells with a single nucleus (Figure 2A). FACS analysis showed an accumulation of cells with a 1C DNA content, although a significant fraction of the population had initiated S phase and showed a 2C DNA content (Figure 2B).

Because mutations conferring resistance to *GAL-SKN7* expression might affect proteins interacting in some way with *SKN7*, spontaneous mutants resistant to *SKN7* overexpression were isolated (see MATERIALS AND METHODS). Twenty-two recessive mutants were thus identified, and these fell into two complementation groups, *bor1* and *bor2* (for *BRY1/SKN7* overexpression resistant). We recovered 12 *bor1* mutants and 10 *bor2* mutants, which suggests that our screen was saturated.

At this time, we became aware that high overexpression of *MBP1* was also lethal (see below). Because both *MBP1* and *SKN7* act through SCB and MCB elements (see INTRODUCTION), we tested whether the *MBP1* gene was required for

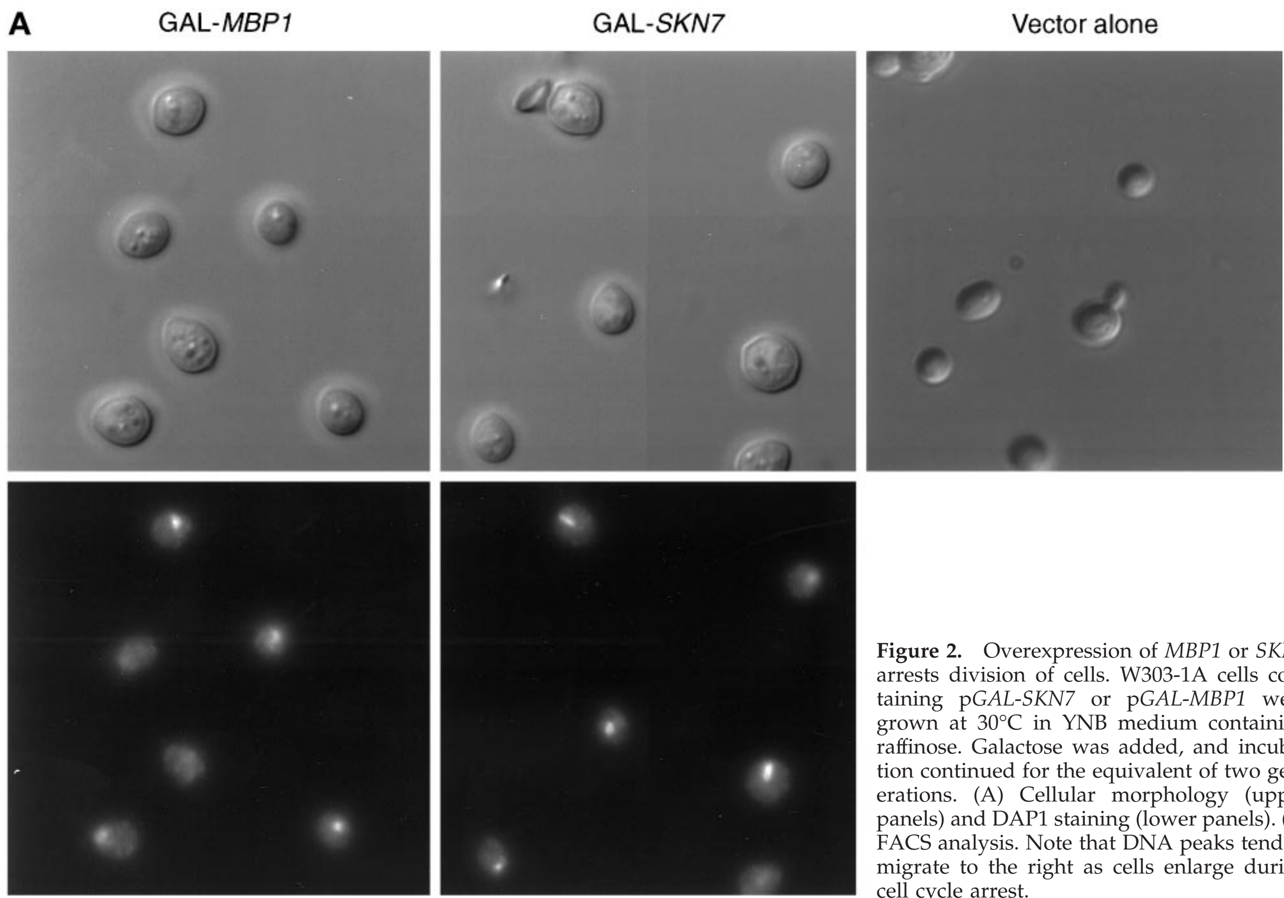

Figure 2. Overexpression of *MBP1* or *SKN7* arrests division of cells. W303-1A cells containing p*GAL-SKN7* or p*GAL-MBP1* were grown at 30°C in YNB medium containing raffinose. Galactose was added, and incubation continued for the equivalent of two generations. (A) Cellular morphology (upper panels) and DAP1 staining (lower panels). (B) FACS analysis. Note that DNA peaks tend to migrate to the right as cells enlarge during cell cycle arrest.

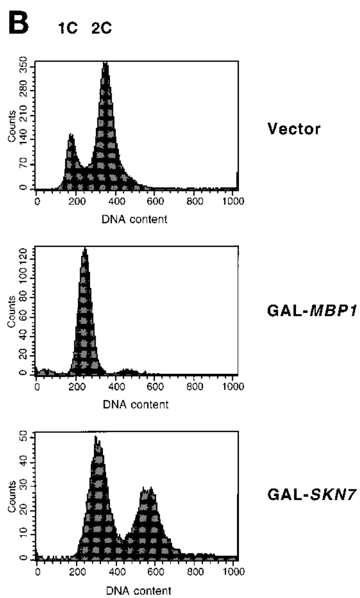

Figure 2. (cont).

GAL-SKN7–dependent lethality. Indeed, *mbp1*Δ cells containing pMW20-*SKN7* (p*GAL-SKN7*) (Morgan *et al.*, 1995b) were able to grow in the presence of galactose (Figure 1A). Reintroduction of *MBP1* into the *mbp1*Δ p*GAL-SKN7* strain restored sensitivity to galactose (our unpublished results), confirming that resistance to *GAL-SKN7* is conferred by the *mbp1* mutation. We checked by Northern blotting that *SKN7* transcription is not regulated by *MBP1* and that *MBP1* expression is not under the control of *SKN7* (our unpublished results).

We then determined whether the *mbp1*Δ mutation belonged to either the *bor1* or the *bor2* complementation group. Diploids of *mbp1*Δ with mutants from the *bor2* complementation group retained resistance to *GAL-SKN7* expression. Moreover, when a *mbp1*Δ/*bor2* diploid was sporulated, all of the spores were resistant to *GAL-SKN7*; hence, *MBP1* and *BOR2* are allelic. We also showed that *BOR1* was allelic to *GAL3*, so the *bor1* mutants were not studied further.

The Mbp1 protein interacts with Swi6 in the MBF transcription factor, and it is also homologous to Swi4 (Koch *et al.*, 1993). Therefore, we tested whether *swi4* and *swi6* mutants would be resistant to *SKN7* overexpression. Importantly, neither a *swi6*Δ mutant (Figure 1A) nor a *swi4*Δ mutant (our unpublished results) containing the *GAL-SKN7* plasmid was able to grow on galactose. The genetic interaction of *Skn7* with components of the G1 transcriptional machinery was specific for *MBP1*.

Overexpression of MBP1 Is Lethal and Is Suppressed by skn7Δ

The *MBP1* gene was inserted into the pEMBLyex4 2-μm–based vector under control of the *GAL* promoter. This plasmid also bears the *leu2-d* allele, so that its copy number can be boosted by selecting for leucine, thus achieving even higher levels of expression. Wild-type W303 containing the pEMBLyex4/*MBP1* plasmid (p*GAL-MBP1*) does not grow on minimal medium containing galactose but lacking leucine (Figure 1B). High overexpression of *MBP1* is thus toxic in yeast. Under similar conditions, neither *SWI4* nor *SWI6* overexpression is lethal (our unpublished results). As with Skn7 overexpression, cells overexpressing *MBP1* arrested as large, round, unbudded cells with a single nucleus (Figure 2A). However, in contrast to *SKN7* overexpression, these cells showed little evidence of any initiation of S phase, but they arrested with a clear 1C peak of DNA (Figure 2B).

Given the interactions between *MBP1* and *SWI4* and *SWI6*, we tested whether *swi4*Δ and *swi6*Δ mutants, as well as *skn7*Δ, would be resistant to *GAL-MBP1*. These three mutants containing p*GAL-MBP1* were tested for growth on minimal medium containing galactose and lacking leucine. The *swi4*Δ p*GAL-MBP1* did not grow on galactose (our unpublished results), whereas both *swi6*Δ p*GAL-MBP1* and *skn7*Δ p*GAL-MBP1* grew under these conditions (Figure 1B). The *swi6*Δ and *skn7*Δ mutations, therefore, are able to confer resistance to *MBP1* overexpression, indicating that both Swi6 and Skn7 participate in this Mbp1-induced lethality.

SKN7 Is Necessary for Suppression of a swi4^{ts} swi6Δ Strain by MBP1

The above data suggest that Mbp1 and Skn7 might interact. Because Mbp1 is part of the MBF transcription factor, we explored whether Skn7 could be part of MBF. Initially, we addressed this by a genetic experiment. In a *swi6*Δ mutant, no MBF activity can be detected, suggesting that Mbp1 requires Swi6 to bind DNA, at least in vitro (Dirick *et al.*, 1992; Lowndes *et al.*, 1992). However, a high-copy-number plasmid carrying *MBP1* is able to rescue the temperature sensitivity of the *swi4*^{ts} *swi6*Δ mutant (Figure 3A). Mbp1, therefore, might associate with a transcriptional activator other than Swi6 to suppress the double mutant. To test whether this is Skn7, we transformed *MBP1* on a high-copy-number plasmid into an isogenic *swi4*^{ts} *swi6*Δ *skn7*Δ strain. The *MBP1* plasmid is totally unable to suppress the temperature sensitivity of this strain (Figure 3A). Reintroduction of a centromeric plasmid carrying *SKN7* restores the ability to grow at 37°C, but the *skn7ΔDBD* allele, which lacks the DNA-binding domain, is unable to do so (our unpublished results). Thus, Skn7 function is required for *MBP1* to suppress *swi4*^{ts} *swi6*Δ, consistent with a Skn7 and Mbp1 association.

The suppression of the *swi4*^{ts} *swi6*Δ strain by *MBPI* must entail increased cyclin expression. This, of course, is also the basis of suppression of this strain by high-copy-number *SKN7* (Morgan *et al.*, 1995b). The Skn7 requirement for Mbp1 suppression, therefore, should be reflected in G1 cyclin levels, and Northern hybridization confirmed this (Figure 3B). We introduced high-copy-number *MBP1* into the isogenic *swi4*^{ts} *swi6*Δ and *swi4*^{ts} *swi6*Δ *skn7*Δ strain mentioned above. As controls, we also used high-copy-number *SWI4* and the empty vector. The resulting strains were grown to midlog phase and transferred to 37°C, and the transcript levels of *CLN1* and *CLN2* were examined. In the presence of *SKN7*, both *SWI4* and *MBP1* led to abundant *CLN1* expression at 37°C. In the absence of *SKN7*, high-copy-number *SW14* still stimulated *CLN1* levels but, importantly, high-copy-number *MBP1* failed to stimulate *CLN1* expression. Smaller but reproducible effects on *CLN2* expression were also seen (Figure 3B).

As control transcripts, we examined *CDC9* and *CDC36*, which were unaffected by the *skn7*Δ mutation. However, there was a slight effect on *CLN3* levels. This was not apparent when *MET4* was used as a loading control, which had no effect on relative levels of other transcripts (our unpublished results). Therefore, the apparent effect on *CLN3* in Figure 3B may not be significant. However, it is intriguing that Skn7 affects the function of the Mcm1 transcription factor (Yu *et al.*, 1995) and, in turn, Mcm1 functions in the regulation of *CLN3* expression (McInerny *et al.*, 1997).

In summary, the physiological consequences of the Skn7-Mbp1 interaction can be visualized. Ablation of *SKN7* results in a marked decrease in *CLN1* transcript levels in the presence of high-copy-number *MBP1*. This is sufficient to explain the *SKN7* requirement for the *MBP1* suppression of a *swi4*^{ts} *swi6*Δ strain. The minor effects on *CLN2* may also contribute to this effect.

Skn7 Is Not Part of the Core MBF

As currently characterized, MBF consists of Mbp1 and Swi6 (see INTRODUCTION). To investigate whether Skn7 is part of MBF, we performed gel-retardation experiments using as a probe a synthetic oligonucleotide containing 3xMCB sites (Lowndes *et al.*, 1991). In cell extracts from wild-type cells, a retarded complex could be seen (Lowndes *et al.*, 1991), which, as expected for MBF, was absent in a *swi6*Δ strain, was much reduced in *mbp1 (bor2)* (Figure 4A), and was competed away by an excess of cold MCB probe (Figure 4B, lanes 2 and 3). It was also supershifted by antibodies against Swi6 (Figure 4B, lane 10). However, this complex was clearly not supershifted by the addition of antibodies directed against Skn7 (Figure 4B, lanes 7–9). It is important to note that the lowest concentration of Skn7 antibodies used in this experiment has previously been shown to supershift a promoter complex containing Skn7 (Morgan *et al.*, 1997). The retarded band was also still present in a *skn7*Δ strain and was of the usual mobility (Figure 4A). Moreover, the MBF in the *skn7*Δ extracts could still be supershifted by Swi6 antibodies (Figure 4B, lane11). Our results thus strongly suggest that Skn7 is not part of the core MBF that binds simple MCB repeats and that MBF is also clearly not dependent on Skn7.

Two-Hybrid Interaction between MBP1 and SKN7

The genetic data described above suggest a direct interaction between Skn7 and Mbp1. To address this, the *MBP1* gene was cloned in frame with the *GAL4* DNA-binding domain and tested for interaction in the two-hybrid system with *SKN7* (Table 1). As expected, *MBP1* interacted strongly with the control *SWI6*. Significantly, *MBP1* also interacted strongly with *SKN7*. Because both proteins are transcriptional activators, the entire *SWI6* and *SKN7* genes, rather than fusions to the *GAL4* activation domain, were used in

this study. On the other hand, there was no interaction between *MBP1* and an unrelated fission yeast gene, *psh1*$^{+}$ (Millar, personal communication); similarly, there was no interaction between *SKN7* and *SWI6* (our unpublished results) or the *Schizosaccharomyces pombe* gene *crk1*$^{+}$ (Table 1). These data clearly support some form of physical interaction between Skn7 and Mbp1.

In Vitro Interaction between Mbp1 and Skn7

We used GST-pulldown experiments to determine whether the interaction between *MBP1* and *SKN7* was direct or whether it required ancillary proteins. Once again, we used Swi6 as a control and, as expected, in vitro synthesized Mbp1 was retained by a GST-Swi6 fusion protein but not by GST alone (Figure 5A, lanes 11 and 5). More importantly, Mbp1 also clearly bound to a GST-Skn7 fusion protein (Figure 5A, lane 7). Note that in vitro synthesized Mbp1 runs as two bands, with the faster-migrating band being most probably a C-terminal truncation, because it is not retained by GST-Swi6 (Figure 5A, compare lanes 1 and 11). Both forms, however, are bound by GST-Skn7. The Mbp1-Skn7 association is a strong interaction, for it is stable in up to 1 M salt, like the Mbp1/Swi6 complex (our unpublished results). On the other hand, Swi4 shows only a weak interaction with GST-Skn7, although it is strongly retained by GST-Swi6 (Figure 5A, lanes 9 and 13). Emphasizing the specificity of the Mbp1-Skn7 association, no interaction could be detected between in vitro synthesized Skn7 and GST-Swi6 (Figure 5A, lane 12). In the reverse experiment, in vitro synthesized Swi6 also was not retained by GST-Skn7 (Figure 5A, lane 10). The Skn7 protein thus interacts directly with Mbp1 but not with Swi6 and only weakly with Swi4 (see DISCUSSION).

Attempts at coimmunoprecipitation were only partially successful, i.e., a weak but irreproducible signal was detected (our unpublished results). Possibly the proportion of Skn7 complexed with Mbp1 at any one time is too low for such experiments (see DISCUSSION).

The Receiver Domain and the HR Region in the Skn7 Protein Are Required for the Interaction with Mbp1

In a preliminary attempt to identify the region(s) of the Skn7 protein necessary for the interaction with Mbp1, deletions were made in the GST-Skn7 fusion protein (Figure 5B). These deletions were then tested for their ability to bind Mbp1 (Figure 5C). A deletion starting from the C terminus of the protein, removing the glutamine-rich region and a small part of the receiver domain (pAB63), was still able to bind Mbp1 to the same extent as the full protein (Figure 5C, compare lanes 3 and 1). On the other hand, when the receiver domain was completely deleted (pAB64), the interaction was substantially reduced (Figure 5C, lane 4). It was further reduced when the coiled-coil region was deleted (Figure 5C, lane 2). However, a fusion between GST and the receiver domain (residues 381–623, pAB65) only bound Mbp1 very weakly, if at all (Figure 5C, lane 5). Thus, although the receiver domain is involved in the interaction, other parts of the protein are involved as well, probably including the coiled-coil domain.

A short region in the Skn7 protein, designated HR and encompassing part of the coiled-coil domain, has been shown

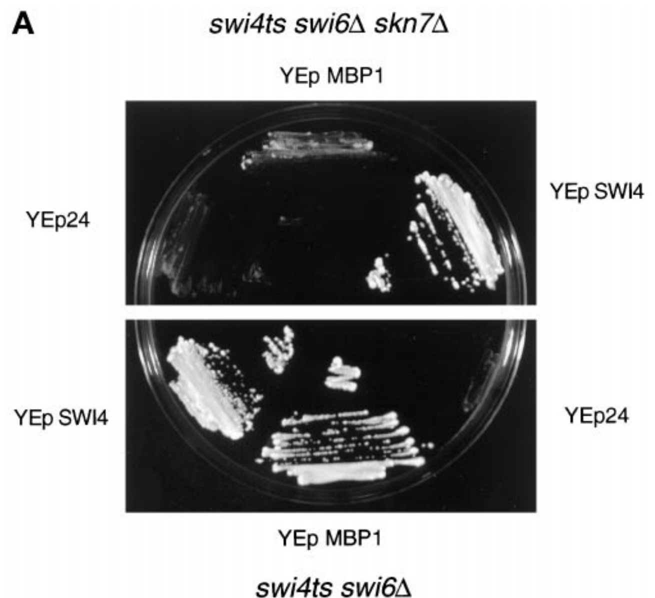

Figure 3. SKN7 function is required for *MBP1* suppression of the *swi4*^{ts} *swi6*Δ mutant. (A) *Skn7* is necessary for suppression of *swi4*^{ts} *swi6*Δ by high-copy-number *MBP1*. The *swi4*^{ts} *swi6*Δ and *swi4*^{ts} *swi6*Δ *skn7*Δ strains were transformed with YEp24 or a multicopy plasmid carrying either *SWI4* or *MBP1* and streaked on YEPD agar plates at 37°C. (B) Skn7 is required for increased cyclin levels in Mbp1 suppression of a *swi4*^{ts} *swi6*Δ strain. The appropriate strains (see text) were grown to midlog phase in YNB glucose at 25°C, transferred to 37°C for the times shown, and sampled, and total RNA was extracted and a Northern blot was prepared (Morgan *et al.*, 1995b). Probes were internal fragments of the genes concerned, and *RPB4* was used as a loading control. This experiment was repeated, and an average of the data points is presented above with error bars, but raw data are shown for only one experiment. For the two sets of data to be directly comparable, levels are presented relative to the level at 0 time of strain K2003 containing vector alone. This value was arbitrarily set at 1. Note that where no error bar is shown, the difference between the two samples concerned was insignificant and the graphing package used was unable to draw the error bars. Quantitation was by phosphorimager.

to mediate interactions between Skn7 and Rho1 (Alberts *et al.*, 1998). The HR region lies between residues 238 and 261 at the beginning of the coiled-coil domain (Figure 5B), and this might account for the apparent role of the coiled-coil domain in the interaction with Mbp1. Indeed, deletion of the HR sequences almost abolished the binding to Mbp1 (Figure 5C, lane 6). In addition, a construct lacking the HR region (a kind gift from R. Treisman) was almost inert in a two-hybrid interaction with Mbp1 (Table 1). Therefore, at least the HR region of the coiled-coil domain and the receiver domain are required for interaction of Skn7 with Mbp1. Consistent with this finding, the HR region and the receiver domain are required for the in vivo function of Skn7. A *swi4*^{ts} *swi6*Δ was not suppressed by *SKN7* lacking either the HR region or the receiver domain (Alberts *et al.*, 1998).

SKN7 and MBP1 Are Involved in Bud Emergence

The genetic data described above regarding rescue of the double mutant *swi4*^{ts} *swi6*Δ imply a role for Skn7 and Mbp1

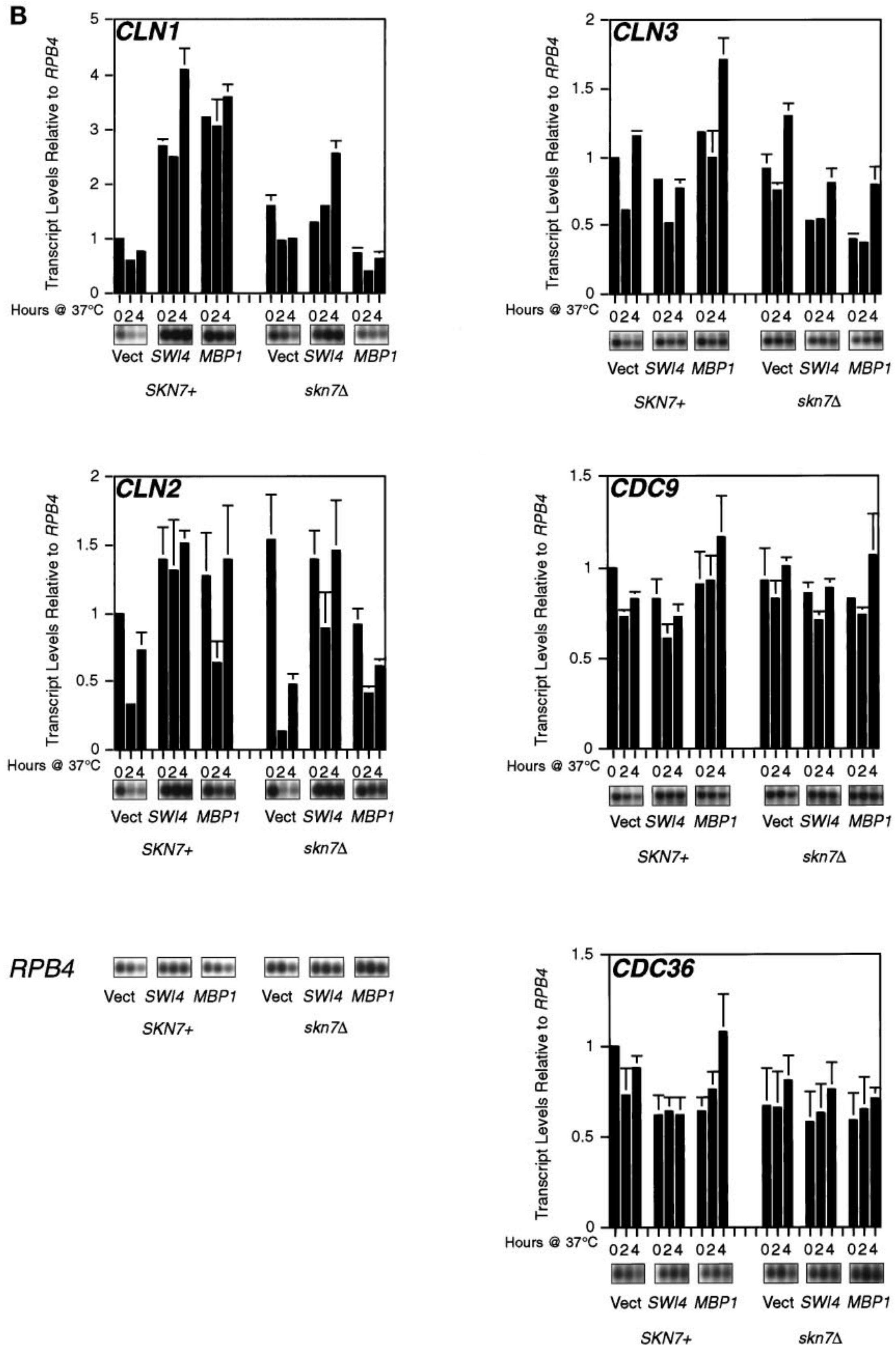

Figure 3 (cont).

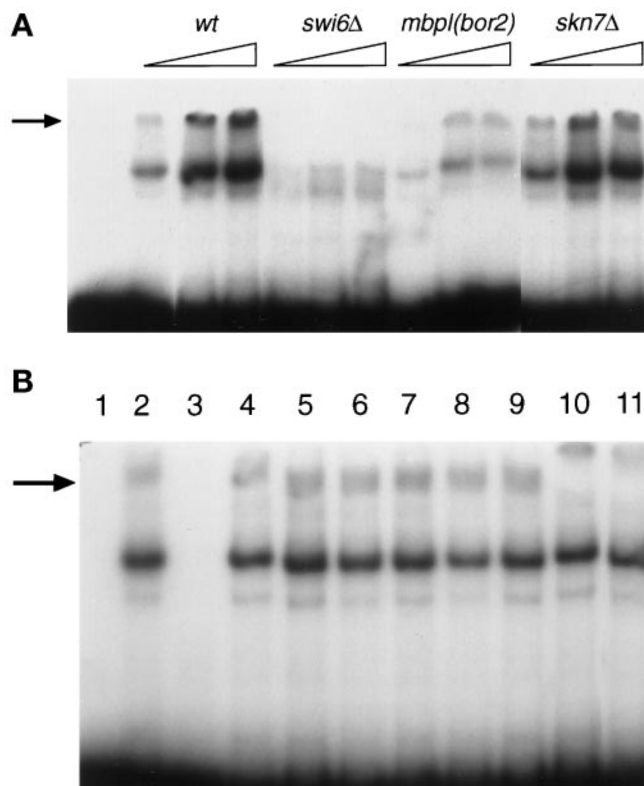

Figure 4. Skn7 is not part of the core MBF. MBF was analyzed by gel retardation with a radioactively labeled 3xMCB probe (Lowndes *et al.*, 1991). (A) Deletion of *SKN7* does not affect MBF. Increasing amounts of crude extract were assayed from the strains shown. Note that the *mbp1* mutation used is a point mutation (an allele of *bor2*) rather than a deletion. (B) Antibodies to Skn7 do not supershift MBF. Lane 1, free probe; lanes 2–10, wild-type (W303) crude extract; lane 2, no competitor; lane 3, competition with a 100-fold excess of the cold probe; lanes 4–6, preimmune serum (1:100, 1:200, 1:500); lanes 7–9, anti-Skn7 antibodies (1:100, 1:200, 1:500); lane 10, anti-Swi6 antibodies (1:200); lane 11, W303 *skn7*Δ crude extracts with anti-Swi6 antibodies (1:200).

in cyclin expression, although it is far from clear whether this is of physiological relevance in a normal cell cycle (see DISCUSSION). Therefore, we investigated other events of G1 in which Skn7 and Mbp1 could participate. Some cell wall genes have SCB and MCB elements in their promoters and are under cell cycle control (Igual *et al.*, 1996). Previous data, especially the finding that *skn7*Δ and *pkc1*Δ are synthetically lethal, suggested that *SKN7* may have a role in cell wall metabolism (Brown *et al.*, 1993, 1994; Morgan *et al.*, 1995b; see INTRODUCTION). Similarly, even though *mbp1* and *pkc1* mutations are not synthetically lethal (Igual *et al.*, 1996), we found that strains deleted for both genes display a synthetic enhancement of spore lethality: 77% of the double-mutant spores were dead compared with 30 and 7% for *pkc1*Δ and *mbp1*Δ single-mutant spores, respectively. This finding might suggest a role for Mbp1 in cell wall metabolism, but we could find no obvious cell wall defects in *mbp1/bor2* mutants or in *skn7*Δ strains, and we could find no effect of *SKN7* and *MBP1* on expression of the cell wall genes that we examined (our unpublished results).

Table 1. *SKN7* and *MBP1* interact in a two-hybrid assay

	MBP1	$crk1^+$
SWI6	4.62 ± 0.23	0.23 ± 0.01
SKN7	4.39 ± 0.99	0.15 ± 0.06
skn7ΔHR	0.49 ± 0.1	0.19 ± 0.1
$psh1^+$	0.31 ± 0.01	NT[a]

β-Galactosidase activities are given in OD units at 420 $nm \cdot min^{-1} \cdot mg^{-1}$ protein. Values represent the average of four independent experiments.

[a] NT, not tested.

Another key event in G1 is bud emergence. Significantly, the *GAL-SKN7*–induced lethality can be rescued by a 2-μm plasmid carrying either *CLN1* or *CLN2* but not by high-copy-number *CLB5* or *CLB6* (Figure 6A). This suggests that *SKN7* and *MBP1* could be involved in the budding process, because *CLN1* and *CLN2* promote bud emergence, partly through actin cytoskeleton reorganization (Benton *et al.*, 1993; Cvrckova and Nasmyth, 1993; Lew and Reed, 1993). Therefore, we looked for genetic interactions between *SKN7* and genes involved in bud emergence, particularly *CDC42*, which encodes a small GTPase that plays a central role in actin polarization (Pringle *et al.*, 1995). When a 2-μm–based plasmid carrying *SKN7* was introduced into the *cdc42-1* mutant, the restrictive temperature of this mutant was decreased from 37 to 34°C (Figure 6B). The *skn7ΔHR* and *skn7D427N* alleles showed the same effect (Alberts *et al.*, 1998). On the other hand, *cdc42-1* was not affected by the *skn7ΔDBD* allele (our unpublished results), indicating that the effect of high-copy-number *SKN7* on *cdc42-1* growth is likely to be transcriptional. Note that the Skn7 DNA-binding domain is known to be functional (Morgan *et al.*, 1997). However, neither *CDC42* nor *CDC24* expression is controlled by *SKN7* (our unpublished results). As with *cdc42-1*, we found that the restrictive temperature of a *bem1*Δ mutant, another mutant defective in actin polarization, is also decreased by a high-copy-number plasmid carrying *SKN7* (our unpublished results). When a dominant-negative allele of *CDC42*, *CDC42A118*, is expressed from the *GAL* promoter, growth of wild-type strains is almost abolished on galactose (Ziman *et al.*, 1991). Mutations in *SKN7* or *MBP1* suppressed the *CDC42A118* lethality on galactose, whereas *swi6*Δ had no effect (Figure 6C). Moreover, introducing 2-μm–based *SKN7* or *MBP1* plasmids into W303 carrying *GAL-CDC42A118* completely eliminated any residual growth on galactose. These results strongly suggest that the G1 function of *SKN7* and *MBP1* lies in bud emergence.

DISCUSSION

We initially isolated *SKN7* in a genetic screen designed to identify new genes involved in late G1 transcription (Morgan *et al.*, 1995b). Although Skn7 stimulated *CLN2* expression through MCB and SCB promoter elements, the heat-shock-factor DNA-binding domain meant that it was unlikely to bind these elements directly. Skn7 overexpression was found to be lethal, and we have now exploited this

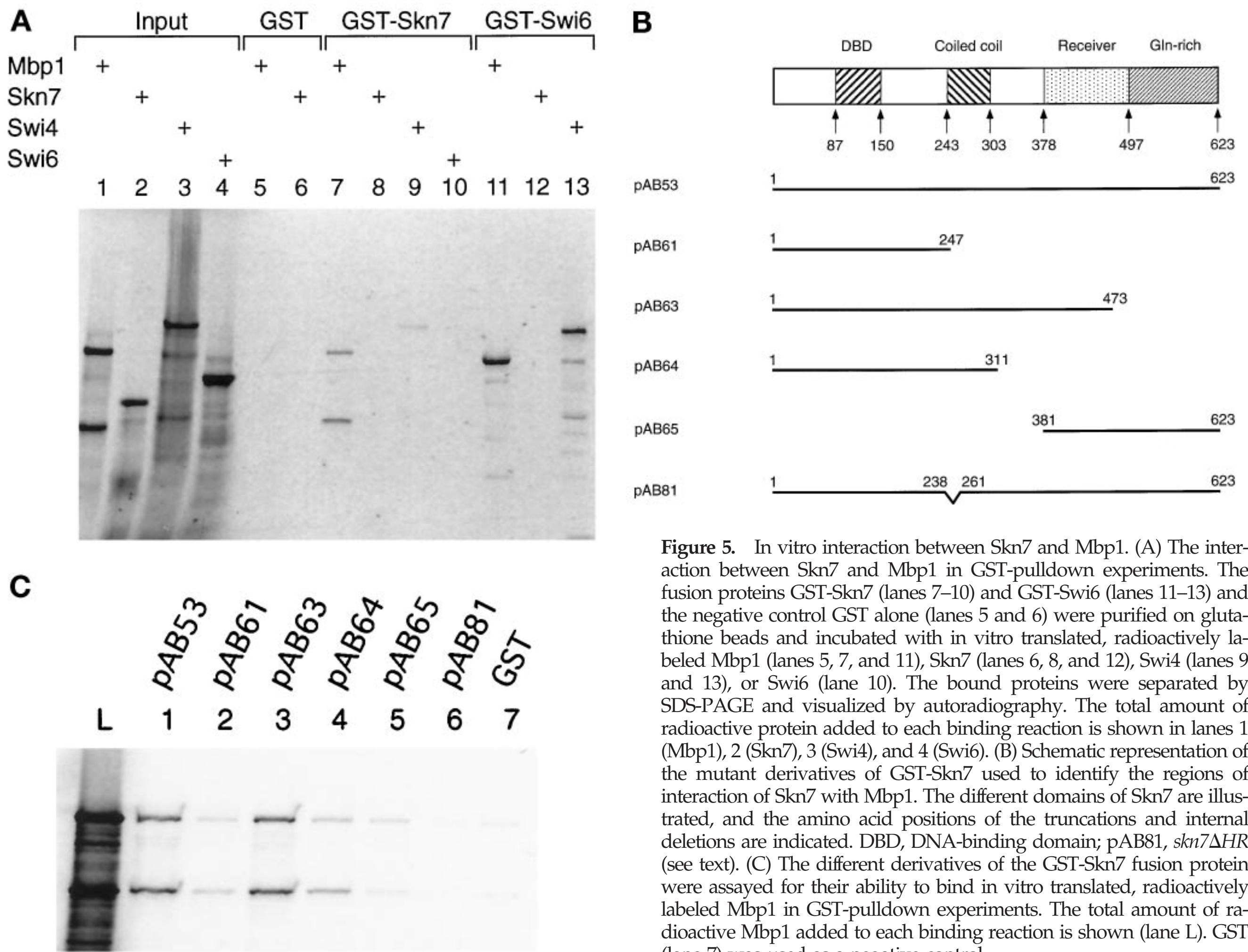

Figure 5. In vitro interaction between Skn7 and Mbp1. (A) The interaction between Skn7 and Mbp1 in GST-pulldown experiments. The fusion proteins GST-Skn7 (lanes 7–10) and GST-Swi6 (lanes 11–13) and the negative control GST alone (lanes 5 and 6) were purified on glutathione beads and incubated with in vitro translated, radioactively labeled Mbp1 (lanes 5, 7, and 11), Skn7 (lanes 6, 8, and 12), Swi4 (lanes 9 and 13), or Swi6 (lane 10). The bound proteins were separated by SDS-PAGE and visualized by autoradiography. The total amount of radioactive protein added to each binding reaction is shown in lanes 1 (Mbp1), 2 (Skn7), 3 (Swi4), and 4 (Swi6). (B) Schematic representation of the mutant derivatives of GST-Skn7 used to identify the regions of interaction of Skn7 with Mbp1. The different domains of Skn7 are illustrated, and the amino acid positions of the truncations and internal deletions are indicated. DBD, DNA-binding domain; pAB81, *skn7ΔHR* (see text). (C) The different derivatives of the GST-Skn7 fusion protein were assayed for their ability to bind in vitro translated, radioactively labeled Mbp1 in GST-pulldown experiments. The total amount of radioactive Mbp1 added to each binding reaction is shown (lane L). GST (lane 7) was used as a negative control.

finding to further investigate Skn7 interaction with the G1 transcription apparatus. Here we have isolated mutants resistant to overexpression of *SKN7* and found that they mapped to the *MBP1* gene. In turn, high overexpression of *MBP1* is lethal and deletion of *SKN7* relieves this lethality. These genetic data suggested that Skn7 and Mbp1 might physically interact. We have demonstrated this interaction: first, the two genes interact in the two-hybrid system; second, an in vitro synthesized Mbp1 protein is retained by a GST-Skn7 fusion protein. The failure to detect coimmunoprecipitation of Skn7 with Mbp1 is disappointing. However, there is also good evidence for a direct association of Skn7 with Yap1 (Morgan *et al.*, 1997) and Rho1 (Alberts *et al.*, 1998) and possibly also Sln1-Ypd1 (Li *et al.*, 1998). In none of these cases has coimmunoprecipitation been demonstrated. Possibly only a small proportion of the total cellular Skn7 associates with any one of these proteins at one time, making coimmunoprecipitation difficult to detect.

We could not detect any interaction between Skn7 and Swi6 in GST-pulldown assays. This is in agreement with our genetic results, which showed that a *SWI6* deletion does not suppress the Skn7-induced lethality. On the other hand, Swi4 did bind weakly to GST-Skn7. This may simply be the result of the high level of homology between Swi4 and Mbp1 (Koch *et al.*, 1993), because we could detect no genetic evidence for a Swi4-Skn7 interaction in vivo. Thus, Skn7 interacts specifically with Mbp1 and is therefore directly associated with the G1 transcriptional machinery.

Gel-retardation experiments suggested that Skn7 is not a component of MBF. MBF is not supershifted by polyclonal antibodies directed against Skn7, and we could not detect Skn7, by Western blotting, in an affinity-purified MBF fraction (our unpublished results). However, Skn7 may still be an MBF-associated factor that is not necessary for DNA binding but only for transcription activation. Such a protein has already been described in fission yeast. In *S. pombe*, the Rep2 protein binds to Res2, an Mbp1 homologue, and can be immunoprecipitated with the Res2/Cdc10 complex, an MBF-like transcription factor (Nakashima *et al.*, 1995). Rep2 is a transactivator that is absolutely required for Res2/Cdc10 activity, because the Res2/Cdc10 transcription factor is inactive in *rep2*$^-$ cells (Nakashima *et al.*, 1995; Baum *et al.*, 1997), although the Res2/Cdc10 complex can bind MCB elements on its own (Zhu *et al.*, 1994, 1997; Baum *et al.*, 1997).

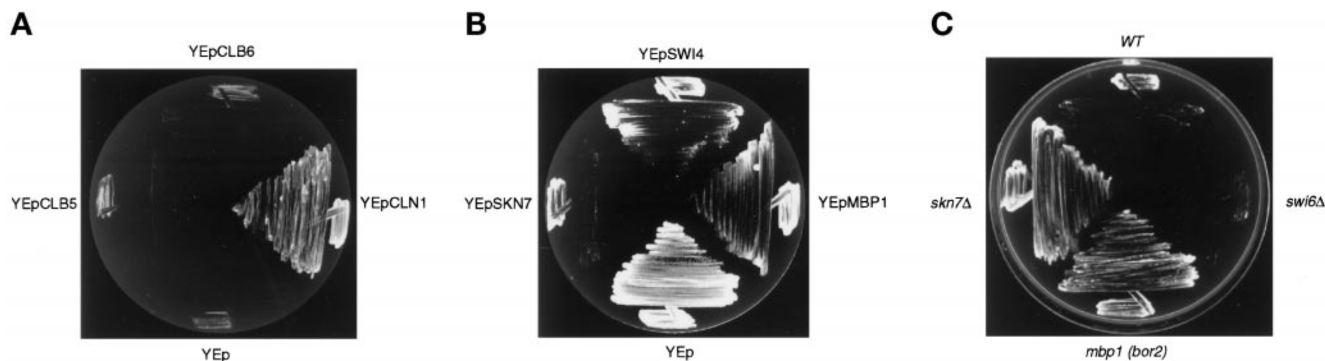

Figure 6. Genetic interactions between *SKN7*, *MBP1*, and genes involved in bud emergence. (A) p*GAL-SKN7*–dependent lethality is suppressed by high-copy-number *CLN1*. W303-1A containing pAB56 (2-μm, *TRP1*, *GAL-SKN7*) was transformed with YEp24 or a multicopy plasmid carrying *CLN1*, *CLB5*, or *CLB6*. Transformants were streaked on galactose-containing minimal agar plates and incubated at 30°C. (B) The temperature sensitivity of a *cdc42-1* mutant is enhanced by high-copy-number *SKN7*. A *cdc42-1* strain was transformed with the plasmids shown and streaked onto YEPD agar plates at 34°C. (C) p*GAL-CDC42A118*–dependent lethality is suppressed by *skn7* and *mbp1* mutations. The p*GAL-CDC42A118* plasmid was transformed into W303, W303 *swi6*Δ, W303 *skn7*Δ, and W303 *mbp1*/*bor2*. The transformants were streaked onto galactose-containing minimal plates at 30°C.

Just as Rep2 binds only to Res2 (Nakashima *et al.*, 1995), Skn7 binds only to Mbp1. Moreover, Skn7 is a transcriptional activator (Brown *et al.*, 1994; Morgan *et al.*, 1995b, 1997) essential for the suppression by *MBP1* of a mutant with crippled SBF activity (*swi4*^{ts} *swi6*Δ). Thus, Skn7 could be a functional analogue of Rep2 in budding yeast. However, *swi4* and *skn7* mutations are not synthetically lethal (Morgan *et al.*, 1995b), indicating that MBF is still active in *skn7*Δ cells. Moreover, only *mbp1* mutations, but not *swi6* mutations, are able to confer resistance to Skn7 overexpression, strongly suggesting that MBF activity is not affected by accumulation of the Skn7 protein in the cell. It is unlikely, therefore, that MBF activity is controlled by Skn7.

Nonetheless, our genetic data clearly support the notion that Skn7 and Mbp1 form a functional transcription factor. High-copy-number *MBP1* suppresses the temperature sensitivity of a *swi4*^{ts} *swi6*Δ strain largely through activation of *CLN1* and, to a lesser extent, *CLN2*, transcription. It is not surprising that there should be differences between *CLN1* and *CLN2* expression in these experiments, because *CLN2* regulation is known to depend on SCB promoter elements and *CLN1* regulation depends on MCB elements (Partridge *et al.*, 1997). However, Mbp1 is known not to bind MCB elements on its own, MBF-binding activity being undetectable in *swi6* mutants (Dirick *et al.*, 1992; Lowndes *et al.*, 1992). Importantly, the high-copy-number *MBP1* suppression is dependent on *SKN7*, because a *swi4*^{ts} *swi6*Δ *skn7*Δ strain transformed with *MBP1* on a high-copy-number plasmid does not grow at 37°C. Moreover, the suppression is dependent on the presence of a functional DNA-binding domain in the Skn7 protein. In addition, whereas overexpression of Skn7 results in *MBP1*-mediated lethality, a wild-type strain carrying a p*GAL-skn7*Δ*DBD* plasmid grows normally on galactose (our unpublished results). The Skn7 DNA-binding domain, therefore, is clearly required for the functional interaction with Mbp1. So the two transcription factors probably cooperate to control *CLN1* expression.

Note that the studies implicating *MBP1* and *SKN7* in the control of *CLN* gene expression were performed in strains with crippled SBF activity (Koch *et al.*, 1993; Morgan *et al.*, 1995b) and that deletions of *skn7* or *mbp1* in wild-type cells have no effect on G1 cyclin expression (Koch *et al.*, 1993; Morgan *et al.*, 1995b). The Skn7/Mbp1 transcription factor may be only a minor component of *CLN1* regulation normally, e.g., binding the promoter only weakly and being readily displaced by SBF. Only under particular conditions in which SBF activity is reduced or not associated with *CLN* promoters might Skn7/Mbp1 control of *CLN1* expression be physiologically significant. One such situation could be after oxidative stress (Morgan *et al.*, 1997), but even under these conditions we could detect no novel species forming on the *CLN1* or *CLN2* promoters in band shifts (our unpublished results). Presumably, to detect the binding of Skn7/Mbp1 to DNA will require identification of more prominent physiological targets or determining more precisely the role of the Skn7-Mbp1 interaction in *CLN1* expression.

Skn7 is a signaling protein involved in a wide range of processes that are apparently unrelated to one another, e.g., oxidative stress response, *CLN* expression, and maintenance of cell wall integrity (see INTRODUCTION). The nature of the signal involved remains obscure, but we note that two-component systems have only been found in cell wall–containing eukaryotes (reviewed by Morgan *et al.*, 1995a). Moreover, Skn7 interacts with Rho1 (Alberts *et al.*, 1998). Interestingly, Rho1 controls the PKC cascade (Nonaka *et al.*, 1995), and its activity has recently been suggested to be responsive to cell wall integrity (Bickle *et al.*, 1998). Finally, *skn7* mutants are hypersensitive only to hydrogen peroxide and not to diamide (Krems *et al.*, 1996; Morgan *et al.*, 1997), and only the former is known to induce some form of cell wall damage. It is thus quite a strong possibility that Skn7 is part of a signal transduction pathway that is somehow regulated by cell wall integrity. According to the nature of the stress, Skn7 would then associate with a partner, e.g., Yap1 (Morgan *et al.*, 1997) or Mbp1 (this work), to direct transcription of the appropriate targets. In this respect, it is noteworthy that the response regulator and the HR domain are both required for the interaction with Mbp1.

We could find no evidence for an effect of *skn7*Δ or *mbp1*Δ on cell wall structure per se. On the other hand, Skn7/Mbp1

may be involved in actin organization and/or bud emergence. Genetic interactions were detected between *SKN7* and *CDC42*, which is directly involved in actin polarization (Pringle *et al.*, 1995), and also with *BEM1*, another gene involved in actin polarization. In addition, overexpression of *MBP1* or *SKN7* prevented bud emergence, although in the case of *SKN7* S phase was initiated in at least part of the population. Possibly, *MBP1* overexpression interfered with normal expression of the MCB-regulated genes required for S phase as well as those required for bud emergence, whereas *SKN7* overexpression only affected genes required for budding. Taken together, these data strongly suggest that Skn7/Mbp1 controls expression of at least one gene involved in bud emergence and/or actin organization.

ACKNOWLEDGMENTS

We thank all of our colleagues in the Division of Yeast Genetics, particularly J.-C. Igual and J.B.A. Millar for helpful advice and discussion. We are especially indebted to G. Banks and A. Spanos, who made the first observation that overexpression of Mbp1 is lethal. We are grateful to A. Alberts and R. Treisman for communicating results before publication. We thank R. Treisman, N. Lowndes, K. Nasmyth, J. Pringle, and D. Johnson for sending plasmids and strains. N.B. was supported by an International Travelling Research Fellowship Award from the Wellcome Trust.

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