The EMBO Journal vol.14 no.22 pp.5679–5689, 1995

A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in budding yeast has homology to bacterial signal transduction proteins

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The transcription factors SBF and DSC1/MBF bind SCB and MCB promoter elements, respectively, and are essential for the cell cycle progression of *Saccharomyces cerevisiae* through the control of G_1 cyclin gene expression. We isolated a gene (*BRY1*; *B*acterial *R*esponse regulator in *Y*east) able to activate either MCB or SCB promoter elements on a reporter plasmid which, when overexpressed, can bypass the normally essential requirement for SBF and DSC1/MBF by the stimulation of *CLN1* and *CLN2* expression. In the case of *CLN2* at least, this expression depends upon the MCB and SCB promoter elements. In wild-type yeast, the disruption of *BRY1* has no apparent phenotype, but under conditions where the activities of SBF and DSC1/MBF are reduced, *BRY1* becomes essential. Our data imply the existence of a third pathway affecting cyclin expression. *BRY1* is the same gene as *SKN7* which has significant sequence homology to the receiver domains found in response regulator proteins from the bacterial two-component signal transduction pathways. *SKN7* is thought to affect cell wall structure, and when highly overexpressed we find that *BRY1/SKN7* is lethal perhaps because of perturbations in cell wall biosynthesis. The lethality is partially rescued by genes from the protein kinase C pathway, but genetic data imply that *BRY1/SKN7* and protein kinase C are not in the same pathway. Our results suggest that Bry1/Skn7 can influence the expression of MCB- and SCB-driven gene expression in budding yeast, perhaps including genes involved in cell wall metabolism, via a two-component signal transduction pathway which activates Bry1/Skn7 in response to an unidentified signal.

Keywords: cell wall biosynthesis/G_1 cyclin transcription/two-component signal transduction pathway/yeast cell cycle

Introduction

In yeast, commitment to the cell division cycle occurs in G_1 at a point known as START. The decision whether or not to enter the cell cycle depends on specific environmental conditions such as the presence or absence of appropriate nutrients and mating pheromone. Presumably a variety of different signal transduction pathways assess these growth conditions and converge at START. In *Saccharomyces cerevisiae*, one of the best characterized signal transduction pathways that regulates the cell cycle is the inhibition of START by mating pheromone (for a recent review see Herskowitz, 1995). In response to mating pheromone, a MAP kinase signal transduction pathway is activated which results in the inhibition of Cdc28/Cln1,2 (and perhaps Cdc28/Cln3) protein kinase by the Far1 protein. This specific inhibition of cells at START is essential for the mating response. Similarly, bacteria have signal transduction pathways with which they respond to extracellular stimuli. These pathways, the so-called 'two-component' signal transduction systems, comprise a sensor protein, responsible for signal detection, and the response regulator protein, usually involved in regulating transcription and responsible for the output of the signal (for reviews see Stock *et al.*, 1989; Bourret *et al.*, 1991; Parkinson, 1993). These systems are all related because the various sensors and response regulators from different bacteria have a number of conserved motifs and some highly conserved amino acids. Biochemical and genetic studies have shown that following signal detection, a histidine kinase, the sensor protein, usually located in the cell membrane, autophosphorylates on a histidine residue. The phosphate on the histidine residue is then transferred from the sensor protein to a highly conserved aspartic acid residue within a 120 amino acid 'receiver' domain in the response regulator protein, resulting in the activation or repression of the activity of the protein. So far, at least 100 different two-component signal transduction pathways have been identified in bacteria, which are responsible for detecting a wide range of environmental conditions affecting, for example, cell movement, cell shape, nutrient utilization and the cell cycle. Until very recently, no examples of this type of signal transduction mechanism had been found in eukaryotes.

Recently, several laboratories have identified likely eukaryotic candidates, mainly based on sequence homologies, in *Arabidopsis thaliana*, *Neurospora crassa*, *Dictyostelium discoideum* and *S.cerevisiae* (Brown *et al.*, 1993; Chang *et al.*, 1993; Ota and Varshavsky, 1993; Maeda *et al.*, 1994; Swanson *et al.*, 1994). The best characterized, and currently the only complete, two-component signal transduction pathway is for *S.cerevisiae*, where a potential histidine kinase, Sln1, and its cognate response regulator protein, Ssk1, have been shown to regulate the HOG1 osmo-sensing MAP kinase pathway (Maeda *et al.*, 1994). Only one other potential response regulator protein, Skn7, has been found in *S.cerevisiae* (Brown *et al.*, 1993). The *SKN7* gene was cloned as a high copy suppressor of a mutation, *kre9*Δ, involved in cell wall biosynthesis. It was also found that the overexpression of *SKN7* could weakly suppress the cell lysis defect of a *pkc1*Δ, and that the deletion of the *SKN7* gene was synthetically lethal with a *pkc1*Δ strain (Brown

et al., 1994). This led to the proposal that *SKN7* functions in a signal transduction pathway in parallel to the protein kinase C (PKC) MAP kinase pathway to regulate the growth of the cell wall. No direct link between *SKN7* and gene expression in yeast has been demonstrated to date.

The output from signal transduction pathways frequently involves new gene expression. In the case of yeast, signal transduction pathways affecting START would presumably influence the activation of the cyclin-dependent protein kinase Cdc28. Passage through START occurs by the activation of the cyclin-dependent Cdc28 protein kinase through association with the G_1 cyclins Cln1 and Cln2 (reviewed in Nasmyth, 1993). This activation involves G_1 cyclin expression under the control of the transcription factors SBF and DSC1/MBF (reviewed in Johnston, 1992). SBF, composed of Swi4 and Swi6 (Andrews and Herskowitz, 1989a; Taba *et al.*, 1991), controls genes encoding G_1 cyclins (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). DSC1/MBF, comprising Swi6 and Mbp1 (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1993), primarily regulates S phase genes but is also important for G_1 cyclin expression (Koch *et al.*, 1993). DSC1/MBF recognizes a *cis*-acting MCB element, ACGCGT (Lowndes *et al.*, 1991; MacIntosh *et al.*, 1991; Lowndes *et al.*, 1992b), whilst SBF recognizes a *cis*-acting SCB element, composed of a related sequence, CACGAAAA (Andrews and Herskowitz, 1989a,b; Taba *et al.*, 1991). Indeed, recent data indicate that SBF and DSC1/MBF can bind either SCBs or MCBs, at least *in vitro* (Dirick *et al.*, 1992; Primig *et al.*, 1992). The promoters of the G_1 cyclin genes, *CLN1* and *CLN2*, contain both SCB and MCB elements. At least in the case of *CLN2*, these elements have been shown to be important for expression (Cross *et al.*, 1994; Stuart and Wittenberg, 1994).

The Swi4 and Mbp1 proteins contain the specific DNA binding domains of SBF and DSC1/MBF (Dirick *et al.*, 1992; Primig *et al.*, 1992; Koch *et al.*, 1993). In contrast, the Swi6 protein binds DNA only non-specifically (Koch *et al.*, 1993; Sidorova and Breeden, 1993). Although the deletion of either the *SWI4* or *MBP1* gene is not lethal, the deletion of both genes simultaneously results in inviability (Koch *et al.*, 1993). This lethality is caused by a loss of G_1 cyclin expression because the heterologous expression of G_1 cyclins from a constitutive promoter restores viability. The discovery that the Swi6 protein is present in both transcription factors, and that Swi6 has no specific DNA binding activity, led to the proposal that Swi6 is the target of cell cycle regulation in these complexes (Dirick *et al.*, 1992; Johnston, 1992; Lowndes *et al.*, 1992a). Surprisingly, unlike the deletion of the *SWI4* and *MBP1* genes, the deletion of the *SWI6* gene is not lethal (Nasmyth and Dirick, 1991). In *Schizosaccharomyces pombe*, the Cdc10 protein is thought to have a similar function to Swi6, yet the mutation of $cdc10^+$ is lethal with cells blocked at START (Nurse *et al.*, 1976). Thus, *S.cerevisiae* may contain a Swi6 homologue or an alternative pathway(s) for MCB/SCB-driven gene expression.

Here we describe a genetic screen to identify alternative pathways which could influence the expression of genes regulated by MCB and SCB elements. We describe one gene isolated in this manner, *BRY1* (*B*acterial *R*esponse regulator in *Y*east), which, surprisingly, can completely bypass the requirement of the known G_1 transcription factors SBF and DSC1/MBF. The *BRY1* gene is identical to the previously identified *SKN7* gene encoding a potential response regulator. Our results suggest that *BRY1/SKN7* acts in a two-component signal transduction pathway in late G_1 through the activation of MCB/SCB elements, and that this pathway is completely independent of SBF and DSC1/MBF.

Results

Identification of a novel gene activating MCB and SCB elements

Strains deleted for *SWI6* do not express β-galactosidase from a reporter plasmid (pLGΔ178.3M) containing the *lacZ* gene under the control of MCB elements (Lowndes *et al.*, 1992a). Therefore, we searched for genes which could activate the MCB-dependent expression of the *lacZ* reporter gene in a *swi6*Δ strain. A yeast genomic library on a high copy vector, YEp24, was introduced into strain BY600 (*swi6::TRP1*); blue colonies were picked for further investigation. Plasmids were isolated from the blue colonies and restriction fragment analyses identified three different classes of insert. One of these, as expected, contained the *SWI6* gene. Somewhat surprisingly, a second class contained the *SWI4* gene. Previously, Swi4 had only been shown to bind MCBs *in vitro* (Primig *et al.*, 1992); its isolation here implies that Swi4 can also bind MCBs *in vivo*, at least when expressed from a high copy number plasmid. The third class of insert contained a novel gene which we named *BRY1* (see below). *BRY1* interacted specifically with the MCB elements on the reporter plasmid because mutating the MCB elements to ACtaGT (pLGΔ178.3mut) abolished activation. However, the *BRY1* gene was not specific for MCB elements *per se*, because when YEp24/BRY1 was introduced into the *swi6*Δ strain with a reporter plasmid carrying the *lacZ* gene regulated by four SCB elements from the *HO* gene promoter (pLGΔ178.SCB), blue colonies were also obtained.

The BRY1 gene can bypass the requirement for SBF and DSC1/MBF

Because MCB and SCB elements have a central role in the regulation of *CLN1* and *CLN2*, the involvement of *BRY1* in the control of G_1 cyclin expression was examined. Given the essential function of Swi4 and Swi6 in the activation of *CLN1* and *CLN2* (Nasmyth and Dirick, 1991), we initially explored any interaction between Bry1 and these two transcription factors. Introducing plasmid YEp24/BRY1 into the $swi4^{ts}$ *swi6*Δ strain K2003 (see Materials and methods) suppressed the inviability of this strain at 37°C (Figure 1A). A series of transposon insertions (Sedgwick and Morgan, 1994) which prevented the YEp24/BRY1 activation of MCB and SCB expression from the appropriate reporter plasmids also prevented YEp24/BRY1 from rescuing the lethality of the $swi4^{ts}$ *swi6*Δ strain at 37°C. It was possible that overexpression of the *BRY1* gene was simply stabilizing the temperature-sensitive Swi4 protein rather than providing an alternative transcriptional activity. Thus, we next asked whether *BRY1* could entirely bypass the requirement for *SWI4* and *SWI6*. YEp24/BRY1 was introduced into a diploid strain heterozygous for *swi4*Δ and *swi6*Δ (see Materials and

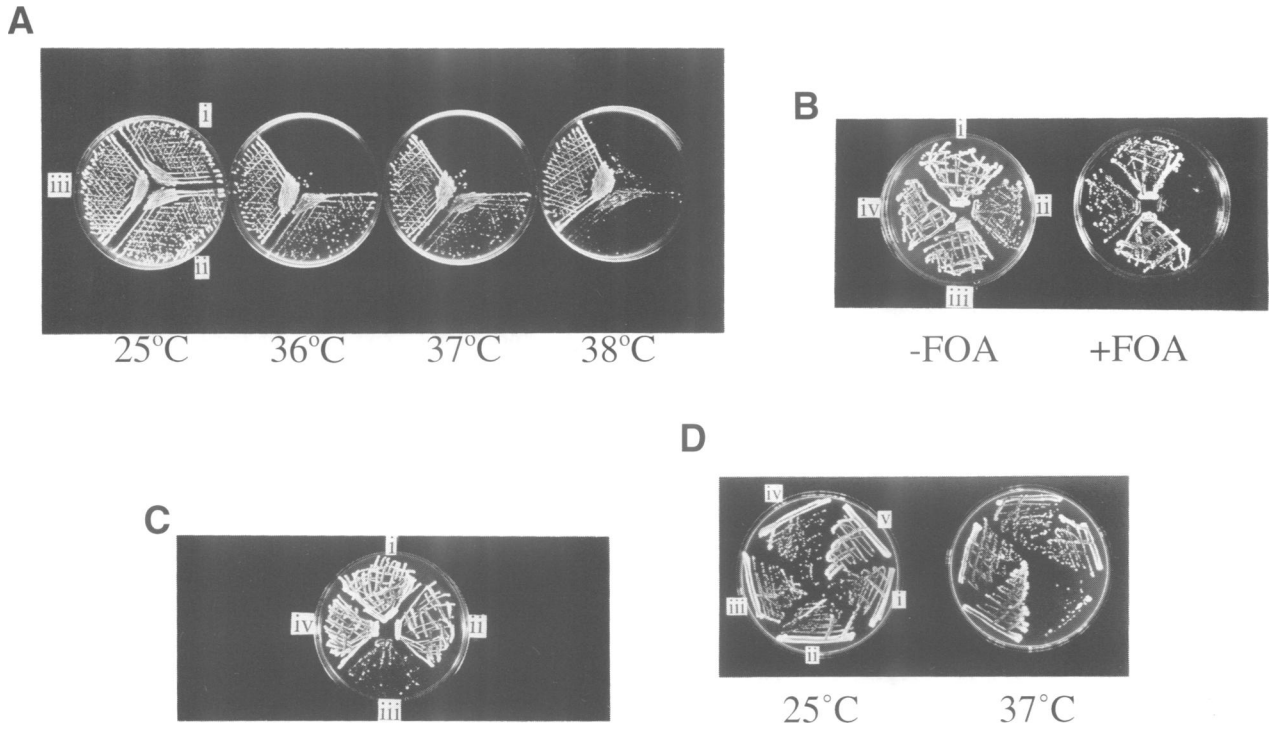

Fig. 1. Bypass of the essential requirement for Swi4/Swi6 and Swi4/Mbp1. (**A**) The *swi4^{ts} swi6*Δ strain plated on YPD agar at 25, 36, 37 and 38°C, carrying (i) vector YEp24, (ii) YEp24/BRY1 or (iii) YEp24/SWI6. (**B**) A representative tetratype tetrad from the dissection of a diploid strain, heterozygous for *swi4*Δ and *swi6*Δ. All spores carry the plasmid YEp24/BRY1 and they are plated on minimal agar plates (–FOA) or FOA plates (+FOA) to select cells which have lost the *URA3* plasmid. Their genotypes are (i) *swi4*Δ *SWI6*, (ii) *swi4*Δ *swi6*Δ, (iii) *SWI4 SWI6* and (iv) *SWI4 swi6*Δ. (**C**) A representative tetratype tetrad from the dissection of a diploid strain heterozygous for *swi4*Δ and *mbp1*Δ. All spores carry YEp24/BRY1-H and they are plated on a minimal agar plate. Their genotypes are (i) *swi4*Δ *MBP1*, (ii) *SWI4 MBP1*, (iii) *swi4*Δ *mbp1*Δ and (iv) *SWI4 mbp1*Δ. (**D**) The *swi4^{ts} swi6*Δ *mbp1*Δ strain plated on YPD agar at 25 and 37°C, carrying (i) pRS313, (ii) a *HIS3* plasmid carrying p*ADH–CLN2* or (iii)–(v) three different pB-BRY1 plasmids (see Materials and methods).

methods), grown under conditions selective for the plasmid, and then plated directly on sporulation medium. Following tetrad dissection, a number of viable spores were obtained that were deleted for both *swi4* and *swi6* and contained YEp24/BRY1. Not all predicted *swi4*Δ *swi6*Δ spores grew, presumably because of plasmid loss or copy number effects in spore outgrowth. The YEp24/BRY1 rescued *swi4*Δ *swi6*Δ double delete cells that did grow were totally dependent on YEp24/BRY1 for viability as they were unable to lose the plasmid on 5-fluoroorotic acid (FOA)-containing medium (Figure 1B). The rescued double delete cells were much elongated and grew more slowly than those cells deleted for either *swi4* or *swi6* alone.

Normally the disruption of both the *SWI4* and *MBP1* genes also results in inviable cells because of a failure to express *CLN1* and *CLN2* (Koch *et al.*, 1993). Therefore we next investigated whether YEp24/BRY1 could suppress this lethality. YEp24/BRY1-H (see Materials and methods) was introduced into a diploid strain heterozygous for *swi4*Δ and *mbp1*Δ, sporulated and dissected as described above. Genetic analyses again showed that overexpression of the *BRY1* gene could suppress the inviability of the *swi4*Δ *mbp1*Δ double mutant (Figure 1C). These YEp24/BRY1-H rescued *swi4*Δ *mbp1*Δ cells were of similar phenotype to the rescued *swi4*Δ *swi6*Δ cells. The rescue of the *swi4*Δ *mbp1*Δ mutant cells by YEp24/BRY1-H appears to be weaker than that of the *swi4*Δ *swi6*Δ mutant cells by YEp24/BRY1. However, the much larger size of the YEp24/BRY1-H plasmid (see Materials and methods) might result in a lower copy number and/or decreased stability which would result in weaker suppression. Indeed, the YEp24/BRY1-H plasmid commonly rearranges in yeast cells.

In each of the rescued double deletion mutants described above, one of the *SWI4*, *SWI6* or *MBP1* genes was still present. Therefore we also constructed a *swi4^{ts} swi6*Δ *mbp1*Δ triple mutant strain and introduced *BRY1* on a high copy plasmid (see legend to Figure 1D). Clearly, overexpression of the *BRY1* gene suppressed the temperature-sensitive growth defect at 37°C of this strain, even in the absence of any of the proteins present in SBF and DSC1/MBF (Figure 1D). Thus, in high copy, Bry1 completely bypasses the need for the known G_1 transcription factors SBF and DSC1/MBF.

Effects of the BRY1 gene on G_1 cyclin expression

One likely explanation for the bypass of DSC1/MBF and SBF by the overexpression of *BRY1* was the restoration of *CLN1* and *CLN2* expression. Thus, Northern blot analyses were performed on the *swi4^{ts} swi6*Δ strain K2003 at 37°C carrying either the YEp24 or the YEp24/BRY1 plasmid (Figure 2A). In the *swi4^{ts} swi6*Δ strain carrying YEp24, the *CLN1* transcript was barely detectable even at 25°C and entirely absent at 37°C. In the presence of YEp24/BRY1, *CLN1* was not only clearly present at 25°C but increased during the incubation at 37°C; it is possible that the absence of SBF at 37°C allowed more ready access to the promoter of the Bry1-dependent transcrip-

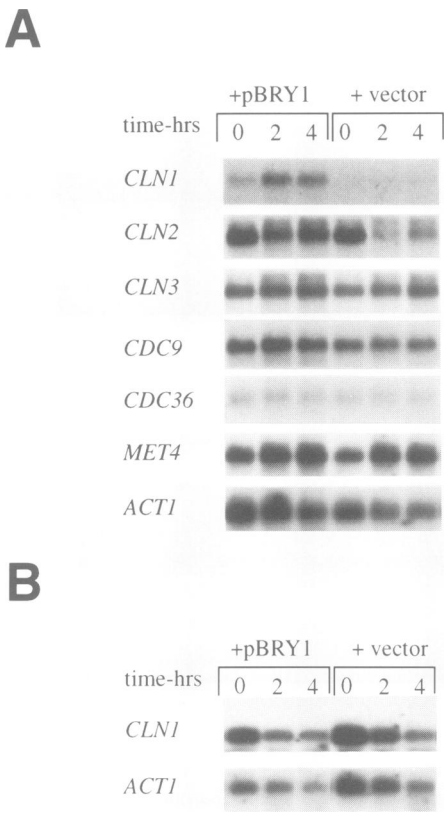

Fig. 2. Effect of *BRY1* on the expression of the *CLN1* and *CLN2* genes. (**A**) Northern blot analysis of RNA isolated from the *swi4*^{ts} *swi6*Δ strain carrying either vector YEp24 or YEp24/BRY1, after 0, 2 and 4 h at 37°C. (**B**) Northern blot analysis of RNA isolated from the wild-type strain W303-1a carrying either vector YEp24 or YEp24/BRY1, after 0, 2 and 4 h at 37°C.

tional activity. The *CLN2* transcript was abundant at 25°C with and without high copy *BRY1*. However, levels declined at 37°C in the presence of YEp24 but were maintained with YEp24/BRY1. Thus, high copy *BRY1* clearly has an effect on *CLN1* and *CLN2* levels in this strain at 37°C in the absence of SBF. Moreover, in the *swi4*Δ *swi6*Δ and *swi4*Δ *mbp1*Δ double delete strains containing YEp24/BRY1, there were also detectable levels of *CLN1* and *CLN2* even though DSC1/MBF and SBF transcription factors have been deleted (data not shown). The *CLN* levels were low but presumably sufficient for viability.

To eliminate the possibility that the overexpression of *BRY1* could be having a general effect on transcription, other transcripts were also examined. Importantly, expression of the G_1 cyclin *CLN3*, which is not regulated by SBF or DSC1/MBF, was unaffected (Figure 2A). Similarly, the expression of the cell cycle-regulated DNA ligase gene *CDC9* and the constitutively expressed cell cycle gene *CDC36* were not affected to any significant degree (Figure 2A). Two further genes involved in different aspects of cellular metabolism, *ACT1* (actin) and *MET4* (methionine biosynthesis), also showed no changes in transcript levels that could be ascribed to YEp24/BRY1 (Figure 2A). Thus, of seven genes examined, only *CLN1* and *CLN2* are significantly affected by high copy *BRY1*.

It was possible that the induction of *CLN1* and *CLN2* expression in the *swi4*^{ts} *swi6*Δ strain on transfer to 37°C could be caused by a general heat shock effect on Bry1, which contains the heat shock factor (HSF) DNA binding domain (see below). Therefore, YEp24 and YEp24/BRY1 plasmids were introduced into the wild-type strain W303-1a and midlog cells shifted from 25 to 37°C for 2 and 4 h. Northern blot analysis of these RNAs revealed that *CLN1* levels were the same in cells containing either YEp24 or YEp24/BRY1 at 25 or 37°C (Figure 2B). In addition, we found no effect of YEp24/BRY1 in the wild-type cells on any other mRNA examined (Figure 2B and data not shown). Thus, YEp24/BRY1 did not activate *CLN1* and *CLN2* expression in response to heat shock. The rescue of the double delete strains without any heat shock is, of course, consistent with this conclusion.

To test if the overexpression of *BRY1* was perhaps partly bypassing DSC1/MBF and SBF by inducing the expression of a G_1 cyclin other than *CLN1*, *CLN2* or *CLN3*, YEp24/BRY1 was introduced into a *cln1*Δ *cln2*Δ *cln3*Δ triple mutant strain kept alive by a galactose-inducible *CLN3* gene. Shifting this strain carrying YEp24/BRY1 from galactose to glucose media demonstrated that YEp24/BRY1 could not bypass the deletion of these three G_1 cyclins (data not shown). However, we cannot rule out the possibility that *BRY1* induces the expression of a cyclin in addition to *CLN1* and *CLN2*, but at a level insufficient to bypass the inviability associated with the deletion of the *CLN1*, *CLN2* and *CLN3* genes.

BRY1 stimulation of *CLN2* expression occurs through the MCB/SCB promoter elements

The *BRY1* gene was originally detected as a specific activator of MCB and SCB elements. Hence, one might expect that the observed induction of *CLN1* and *CLN2* expression by YEp24/BRY1 in the absence of SBF would be specifically through the MCB and SCB elements in their promoters rather than via a non-specific general induction of transcription (see above). To test whether *BRY1* activates *CLN2* expression through SCB and MCB elements, different *CLN2* promoter constructs fused to a *lacZ* reporter gene (Stuart and Wittenberg, 1994) were introduced into a *swi4*Δ strain carrying either a vector pRS313 (Sikorski and Hieter, 1989) or pB-BRY1 (see Materials and methods). The construct containing the entire *CLN2* promoter, pΔ5′728, was fully activated by pB-BRY1 (Figure 3). Stuart and Wittenberg (1994) identified two activating elements responsible for the expression of *CLN2*, namely UAS1 and UAS2. The deletion of UAS2, pΔ5′728se, did not affect activation by pB-BRY1, suggesting that *BRY1* may function through UAS1 which contains the MCB and SCB elements (Figure 3). Consistent with this, the use of construct pΔSCB, in which point mutations are introduced into the SCB elements, resulted in a decrease in *BRY1* stimulation (Figure 3). When both SCB and MCB elements were specifically mutated (pΔSCB/MCB), activation by *BRY1* was abolished (Figure 3). The other constructs examined, pΔ605-505 and pΔ505-400, supported the notion that *BRY1*-dependent *CLN2* expression occurred by the SCB and MCB elements (Figure 3). Thus, as expected from its isolation, *BRY1* clearly acts on the *CLN2* promoter specifically through MCB and SCB elements.

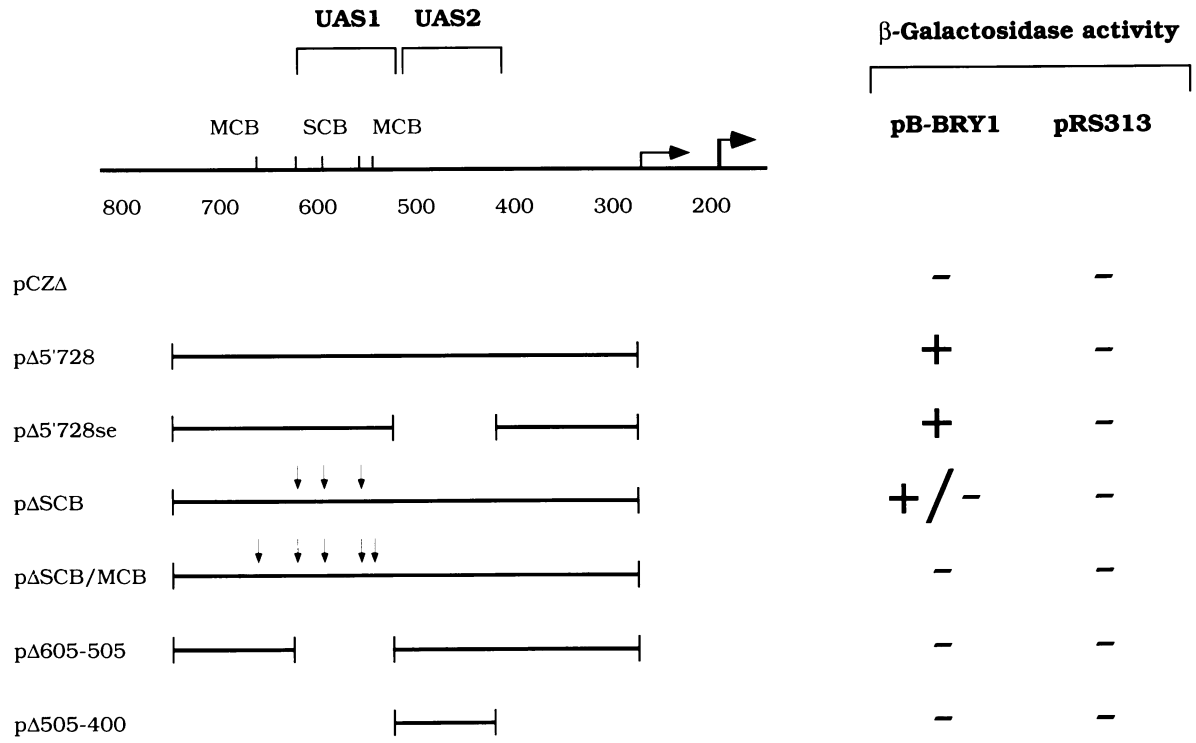

Fig. 3. *BRY1* specifically activates the MCB and SCB elements in the *CLN2* promoter. Filter assays were performed for *lacZ* activity in the *swi4Δ* strain, BY604, carrying the *CLN2* promoter–*lacZ* fusions, indicated on the left of the figure, and either vector pRS313 or pB-BRY1. Qualitative activation (+) and background (–) levels of *lacZ* activity are shown.

Structure of the BRY1 gene

Transposon mutagenesis (see Materials and methods) and DNA sequencing revealed an open reading frame capable of encoding a protein of 622 amino acids with a predicted molecular weight of ~68 kDa. The sequence analysis of *BRY1* revealed that it is identical to the *SKN7* gene isolated recently in an unrelated genetic screen (Brown *et al.*, 1993; see Introduction). Clues to Bry1/Skn7 function come from its striking homology to bacterial response regulator proteins which contain the 120 amino acid 'receiver' domain, typical of two-component signal transduction pathways (amino acid residues 378–497; Figure 4). As noted by Brown *et al.* (1993), all the conserved amino acids, including the important phosphorylatable aspartic acid residue at position 427, are found in the potential receiver domain of *BRY1* (Figure 4). As expected for a transcriptional response regulator protein, Bry1/Skn7 also contains a potential DNA binding domain. Between amino acids 87 and 150, there is homology to the DNA binding domain of eukaryotic heat shock transcription factors (HSFs; Brown *et al.*, 1993). In addition, we have identified homology, not noted by Brown *et al.* (1993), between amino acids 243 and 303 to the region of coiled-coil structure important for protein–protein interaction in the heavy chain of myosin (Figure 4). Interestingly, HSFs also have a domain consisting of a coiled-coil structure adjacent to the DNA binding domain (reviewed in Lis and Wu, 1993); in Bry1/Skn7 this arrangement is conserved.

BRY1 has no role in the heat shock response

To test whether Bry1/Skn7 is an HSF1 homologue, the YEp24/BRY1 plasmid was introduced into an *hsf1* temperature-sensitive mutant strain. However, unlike the

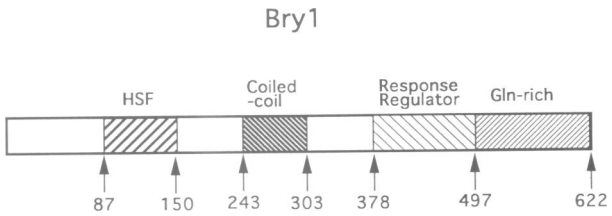

Fig. 4. Structure of the *BRY1/SKN7* gene. The amino acid residues are shown that correspond to the positions of homology to the HSF DNA binding domain, the potential coiled-coil structure, the homology to the bacterial response regulator domain and a region rich in glutamine (Gln) residues that is commonly found in eukaryotic transcription factors.

rescue of the *swi4^{ts} swi6Δ* strain, the YEp24/BRY1 plasmid was unable to restore the viability of the *hsf1* temperature-sensitive strain at 37°C (data not shown). Further, the deletion of the wild-type *BRY1/SKN7* gene did not produce any phenotypes associated with a loss of heat shock gene expression (data not shown). A detailed comparison of the HSF-like DNA binding domain of *BRY1/SKN7* with the amino acid sequences of the budding and fission yeast Hsf proteins, which recognize identical UAS elements, revealed that *BRY1/SKN7* had diverged significantly from the budding and fission yeast Hsf proteins. This might be expected if the Bry1/Skn7 protein recognized a different, but perhaps related, DNA sequence element. Additionally, the Northern blot analyses performed on wild-type W303-1a cells, or *swi4^{ts} swi6Δ* cells containing YEp24/BRY1, revealed no general effect of *BRY1/SKN7* on transcription following heat shock (Figure 2B). Thus, there is no evidence for any role of *BRY1/SKN7* in heat shock.

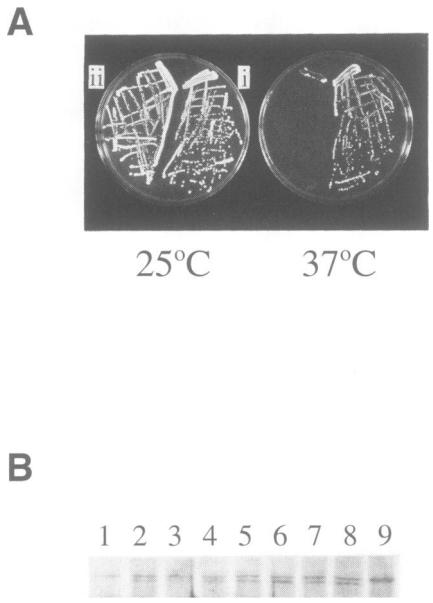

Fig. 5. Effects of the mutation of Asp427 on the function and stability of Bry1/Skn7. (**A**) The *swi4*^{ts} *swi6*Δ strain plated on YPD agar at 25 and 37°C, carrying either (i) pB-BRY1 Asp427 or (ii) pB-BRY1 Asn427. (**B**) Extracts of W303-1a cells containing no plasmid (tracks 1 and 9), three independent transformants with a plasmid carrying HA epitope-tagged wild-type Bry1/Skn7, pB-BRY1HA (tracks 2–4), or four independent transformants with a plasmid carrying HA epitope-tagged Asn427 Bry1/Skn7, pB-BRY1HA Asn427 (tracks 5–8), were made and subsequently Western blotted and probed with 12CA5 antibodies to the HA epitope.

The receiver domain is essential for the bypass of SBF and DSC1/MBF

In bacterial response regulator proteins, the aspartic acid residue occurring at position 427 in Bry1/Skn7 is phosphorylated in response to the appropriate signal and is essential for the activity of the receiver domain (Stock *et al.*, 1989; Bourret *et al.*, 1991; Parkinson, 1993). To test the importance of the receiver domain in the function of Bry1/Skn7, we mutated Asp427 to an asparagine residue (see Materials and methods). Plasmids containing the wild-type or mutated *BRY1/SKN7* gene were then introduced into the *swi4*^{ts} *swi6*Δ strain and their effects on temperature sensitivity tested (Figure 5A). The mutation of Asp427 clearly prevented the *BRY1/SKN7* gene from rescuing the lethality of the *swi4*^{ts} *swi6*Δ cells at 37°C. To ensure that the mutation of Asp427 had not simply rendered the Bry1/Skn7 protein unstable, a single copy of the HA epitope was introduced in-frame between the C-terminal amino acid and the stop codon of the wild-type and Asn427 versions of the *BRY1/SKN7* gene (see Materials and methods). Several plasmids carrying HA-tagged versions of the wild-type *BRY1/SKN7* gene and the Asn427 *BRY1/SKN7* gene were introduced into the *swi4*^{ts} *swi6*Δ strain and shown to have identical effects to the untagged genes at 37°C (data not shown). Western blot analysis was then performed using the 12CA5 (anti-HA) monoclonal antibody on protein extracts of W303-1a containing these plasmids (Figure 5B). The stabilities of both the wild-type and mutant proteins were identical. Thus, Asp427 is essential for the activity of Bry1/Skn7, supporting the existence of a functional receiver domain in Bry1/Skn7.

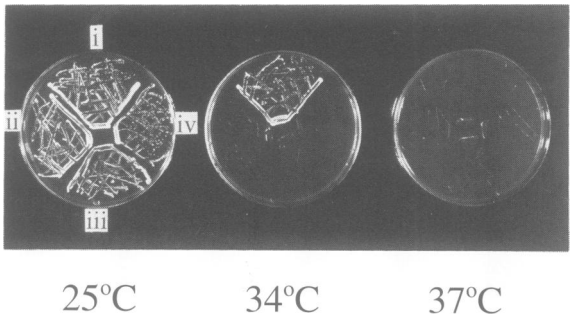

Fig. 6. Effects of disruption of the *BRY1/SKN7* gene. The *swi4*^{ts} *swi6*Δ strain plated on YPD agar at 25, 34 and 37°C, containing (i) an undisrupted *BRY1/SKN7* gene or (ii–iv) three independent disruptions of the *BRY1/SKN7* gene.

Disruption of the BRY1/SKN7 gene

The data above show that in high copy the *BRY1/SKN7* gene can completely bypass the requirement for SBF and DSC1/MBF. However, it was possible that the observed high copy number rescue of the mutants was a non-specific effect caused by the overproduction of *BRY1/SKN7*. To investigate the possible contribution of Bry1/Skn7 itself to G_1 cyclin expression, we disrupted the wild-type *BRY1/SKN7* gene. Disruptants in wild-type haploid cells had no obvious phenotype, as judged by growth rate, cell size or fluorescence-activated cell sorting (FACS) analysis; neither did they have any major effect on *CLN1* and *CLN2* expression (data not shown). Similarly, homozygous diploids deleted for both copies of the *BRY1/SKN7* gene could be obtained with no obvious phenotype, including displaying normal sporulation. In addition, disruption of the *BRY1/SKN7* gene in a haploid was not lethal in combination with the disruption of the *SWI4*, *SWI6* or *MBP1* genes. However, when the *BRY1/SKN7* gene was disrupted in the *swi4*^{ts} *swi6*Δ strain, the lethal temperature was substantially lowered to 34°C (Figure 6). Interestingly, the *swi4*^{ts} *swi6*Δ and the *swi4*^{ts} *swi6*Δ *bry1*Δ strains appear to have similar levels of *CLN* gene expression at 25°C (data not shown), consistent with *BRY1/SKN7* acting on SCB/MCB elements only in the complete absence of Swi4 protein. The above data provide further genetic evidence that the wild-type *BRY1/SKN7* gene indeed has a role to play in a pathway that can function in parallel to SBF and DSC1/MBF. However, only when Swi4/Swi6 activity is limiting does Bry1/Skn7 clearly make a significant contribution to cell viability. The absence of a *bry1*Δ phenotype in wild-type cells is typical of the deletion of many individual genes involved in cyclin expression. Only when genes such as *SWI4* and *MBP1* are deleted together are severe growth defects observed (Koch *et al.*, 1993), suggesting a high degree of functional overlap.

Overexpression of the BRY1/SKN7 gene is lethal

Wild-type strains carrying the YEp24/BRY1 plasmid had slower growth rates than those carrying vector alone. Hence, we investigated the effect of overexpressing the *BRY1/SKN7* gene from a galactose-inducible promoter. Wild-type cells carrying this plasmid, pGAL–BRY1, were viable on glucose-containing medium, but when shifted to galactose-containing medium were inviable (data not

Table I. Suppression of pGAL–BRY1 lethality by the PKC pathway

Plasmid[a]	Glucose[b]	Galactose[b]	Source[c]
CEN–vector	+	–	This study
CEN–*PKC1*	+	±	Levin *et al.* (1990)
CEN–*BCK1*	+	–	Lee and Levin (1992)
CEN–*BCK1–20*	+	–	Lee and Levin (1992)
CEN–*MPK1*	+	±	Lee *et al.* (1993b)
CEN–*BCK2*	+	±	Epstein and Cross (1994)
2μ–*PKC1*	+	±	This study
2μ–*BCK1*	+	±	This study
2μ–*MKK1*	+	±	Irie *et al.* (1993)
2μ–*MKK2*	+	±	Irie *et al.* (1993)
2μ–*MPK1*	+	±	This study
2μ–*PPZ1*	+	–	This study

[a]Plasmids constructed in this study are indicated. Note that the plasmid backbones are not identical except that they were either cloned in a *CEN*- or a 2μ-based vector. For a review of the genes in the PKC pathway, see Errede and Levin (1993). For a description of *PPZ1*, see Lee *et al.* (1993a).
[b]W303-1a containing pGAL–BRY1 and the plasmid indicated were plated on medium with either glucose or galactose as a carbon source. +, good growth; –, no growth; ±, very slow growth.
[c]The sources of the plasmids used in this study are indicated.

shown). FACS and microscopic analyses revealed that cells arrested randomly in the cell cycle, after several generations, as large swollen cells which then lysed. RNA hybridization analysis showed no dramatic effect on *CLN1* and *CLN2* levels (data not shown). This result is perhaps not surprising because, as described above, YEp24/BRY1 only had detectable effects on *CLN1* and *CLN2* expression when the activity of SBF was reduced. Lysis of the cells suggested that the defect associated with the galactose-induced overexpression of *BRY1/SKN7* might indicate a defect in cell wall metabolism. To test this further, strain W303-1a carrying pGAL–BRY1 was grown on galactose medium containing 1 M sorbitol. Plating on this medium weakly suppressed the lethal phenotype. As 1 M sorbitol is known to suppress certain defects in cell wall metabolism such as occur when the *PKC1* gene is deleted (Levin and Bartlett-Heubusch, 1992), this result supports the possibility that cell wall metabolism is defective. Surprisingly, the lethality caused by pGAL-driven overexpression of *BRY1/SKN7* was not affected by the Asp427 mutation. The mutated *BRY1/SKN7* gene killed cells as effectively as the wild-type gene. This is in marked contrast to the effect of YEp24/BRY1 on the rescue of the lethality of the *swi4^{ts} swi6*Δ strain at 37°C, which was sensitive to the Asp427 mutation (Figure 5A).

Interaction between the PKC pathway and BRY1/SKN7

The *S.cerevisiae* PKC homologue, encoded by the *PKC1* gene, regulates a MAP kinase pathway important for cell growth and cell wall synthesis (for a review see Herskowitz, 1995). Indeed, Brown *et al.* (1994) presented data that *SKN7* could weakly suppress a complete deletion of the *PKC1* gene. Thus, we investigated whether pGAL–BRY1 lethality could be suppressed by the overexpression of different genes in the PKC pathway. CEN plasmids carrying these genes were introduced into W303-1a containing pGAL–BRY1 and shifted to galactose medium. Weak suppression was observed by several genes including *PKC1* and *MPK1* (Table I). When the genes were located on high copy number YEp vectors, the suppression was not enhanced to any significant degree. Thus, only a partial suppression of the lethal effect of *BRY1/SKN7* overexpression was possible by overexpressing genes from the PKC pathway.

The deletion of *PKC1* is lethal and, as mentioned above, this can be rescued by growth on medium containing 1 M sorbitol. However, when pGAL–BRY1 was introduced into a *pkc1*Δ strain and transferred to galactose-containing medium, the resulting lethality was not prevented by growth on sorbitol. This is in contrast to the expression of *SKN7* from a 2 μm plasmid which can weakly suppress the phenotypes associated with the *pkc1*Δ strain (Brown *et al.*, 1994). One explanation for this apparent paradox is that the heavy overexpression of *BRY1/SKN7* is having a dominant negative effect, perhaps similar to the loss of *BRY1/SKN7* function, whereas lower levels of overexpression of *BRY1/SKN7* are sufficient to boost the Bry1/Skn7 pathway to a limited extent. Consistent with this suggestion, a *pkc1*Δ *skn7*Δ double delete is inviable even in the presence of 1 M sorbitol (Brown *et al.*, 1994; our unpublished observations). The important point, however, is that the effects of the *pkc1*Δ and the high overexpression or deletion of the *BRY1/SKN7* gene are not epistatic—in fact they are additive, arguing that the effects are exerted through separate pathways. Thus, when overexpressed from the GAL promoter, Bry1/Skn7 might have an effect on cell wall metabolism but it seems not to act through the PKC pathway.

In view of this result, we next investigated whether the PKC pathway had a similar effect to *BRY1/SKN7* on G_1 cyclin expression. YEp24/*PKC1* was unable to suppress either the inviability of the *swi4^{ts} swi6*Δ strain at 37°C or the inviability of the *swi4^{ts} swi6*Δ *bry1*Δ strain at the lower temperature of 34°C (data not shown). Further, unlike YEp24/BRY1, YEp24/*PKC1* was unable to restore the activity of the MCB–*lacZ* or SCB–*lacZ* reporter plasmid in the *swi6*Δ strain. Thus, we can find no evidence that the Pkc1 protein contributes to any of the *BRY1/SKN7* effects on G_1 cyclin expression.

Discussion

In a genetic screen to identify those genes, in addition to *SWI6*, affecting the expression from MCB and SCB elements, two genes were isolated: *SWI4* and *BRY1/SKN7*. The isolation of the *SWI4* gene was not entirely unexpected because *in vitro* experiments have shown that Swi4 can interact with MCB elements (Primig *et al.*, 1992). However, this is the first *in vivo* evidence for such an interaction. The identification of the *BRY1/SKN7* gene, encoding a protein with a two-component receiver domain, was totally unexpected. Moreover, overexpression of the *BRY1/SKN7* gene is able to completely bypass the requirement for the known G_1 transcription factors and this is dependent on the receiver domain. The deletion of the *BRY1/SKN7* gene shows a synthetic interaction with a partially active SBF. Our data suggest that Bry1/Skn7 functions in a two-component signal transduction pathway that acts in late G_1.

The essential function of SBF and DSC1/MBF is probably the expression of *CLN1* and *CLN2*, which are required for START (Nasmyth and Dirick, 1991; Koch *et al.*, 1993). Hence, the most likely explanation for the

ability of the *BRY1/SKN7* gene to bypass the requirement for SBF and DSC1/MBF is that the overproduction of the gene induced *CLN1* and *CLN2* expression through the MCB and/or SCB elements in the cyclin promoters rather than a general effect of *BRY1/SKN7* on transcription. Others have suggested the possible existence of further G_1 transcriptional activities capable of regulating *CLN1* and *CLN2* (Foster *et al.*, 1993; Breeden and Mikesell, 1994). It is conceivable that the Bry1/Skn7-dependent pathway could be one of these. Indeed, there was a demonstrable effect of the overexpression of *BRY1/SKN7* specifically on *CLN* levels in the *swi4^{ts} swi6*Δ strain and specifically through the activation of MCB/SCB elements in *CLN2* promoter fusions to the *lacZ* reporter in a *swi4*Δ strain.

The only DNA binding domain in Bry1/Skn7 has significant homology to the DNA binding domain of Hsf1 (Brown *et al.*, 1993). This has no homology to the DNA binding domains of Swi4 or Mbp1. Further attempts to demonstrate a band shift using a substrate containing MCB elements with the Bry1/Skn7 protein have been unsuccessful (data not shown). Hence the effect of Bry1/Skn7 on the expression from MCB/SCB elements may be indirect, perhaps involving the expression of another transcription factor. One potential candidate for this is the Phd1 protein which has homology to the DNA binding domains of Mbp1 and Swi4 (Gimeno and Fink, 1994). The overexpression of *PHD1* induces pseudohyphal growth in a diploid in response to nitrogen starvation. It is worth noting that the morphology of the *swi4*Δ *swi6*Δ and *swi4*Δ *mbp1*Δ double mutant strains rescued by the overexpression of *BRY1/SKN7* is similar to that of cells where the *PHD1* gene is overexpressed. Given the Hsf1 homology, Bry1/Skn7 may bind a sequence related to heat shock elements, although we can find no evidence to suggest a role for Bry1/Skn7 in the heat shock response. Hsf proteins from yeast to human bind an identical element in the promoters of genes which are regulated by them (Lis and Wu, 1993). A detailed comparison of the amino acid sequences of the DNA binding domains of Hsf proteins from budding and fission yeast with the Hsf-like DNA binding domain of Bry1/Skn7 suggests that the latter has diverged significantly from genuine Hsf proteins. Therefore the Bry1/Skn7 protein may recognize a different, but perhaps related, DNA sequence element.

The *BRY1/SKN7* gene also has striking homology to the receiver domain of response regulator proteins of two-component signal transduction pathways (Brown *et al.*, 1993). All the conserved amino acids, including the essential phosphorylatable aspartic acid residue (Asp427), are found in the potential receiver domain of Bry1/Skn7. The mutation of Asp427 to an asparagine residue completely abolishes the ability of the *BRY1/SKN7* gene to rescue the lethality of the *swi4^{ts} swi6*Δ strain at 37°C. This supports the existence of a functional receiver domain in Bry1/Skn7 and further suggests that the phosphorylation of Asp427 is required for the function of the protein in bypassing the requirement for the G_1 transcription factors. Brown *et al.* (1994) also showed that Asp427 was essential for *SKN7* to bypass the slow growth of a *kre9*Δ strain.

Deletion of the entire *BRY1/SKN7* gene in a wild-type strain resulted in no obvious phenotype nor any effect on *CLN* expression. This can be explained if the Bry1/Skn7 pathway is functionally redundant with other pathways involved in the expression of the same target genes, or if the Bry1/Skn7 pathway is activated only under conditions where SBF is absent or inactive. If the *BRY1/SKN7* gene is functionally redundant, then the effects of deletion would only become obvious when parallel pathways were destroyed or inactive. Many examples of the redundancy of cell cycle pathways have been demonstrated in *S.cerevisiae*, including the genes for the G_1 cyclins and the transcription factors which control their expression (Nasmyth and Dirick, 1991). Consistent with the redundancy of G_1 transcription factors, *bry1*Δ can be combined with *swi4*Δ, *swi6*Δ or *mbp1*Δ without effect. However, these latter three genes are lethal in various combinations (see Introduction); when we exploited this, a *bry1*Δ phenotype was apparent. The deletion of *BRY1/SKN7* in the *swi4^{ts} swi6*Δ strain results in a synthetic lethal phenotype at intermediate temperatures. Thus, Bry1/Skn7 may be rate limiting in a pathway controlling an alternative, or additional, means of expression from MCB/SCB-regulated genes. Moreover, the effects of the pathway on *CLN* expression may only be apparent when SBF activity is compromised in some way, because the effects of *BRY1/SKN7* overexpression were not evident in a wild-type strain. Details of the Bry1/Skn7 signal transduction pathway are not clear, but given the conservation of the receiver domain in Bry1/Skn7, and the importance of Asp427 to the function of the protein, another component is likely to be a histidine kinase sensor (Figure 7). This would presumably respond to a signal, possibly from outside the cell, and Bry1/Skn7 would then be activated by phosphorylation.

When highly overexpressed, *BRY1/SKN7* was lethal in wild-type cells, although this did not appear to involve the overexpression of *CLN1* or *CLN2*. Significantly, the effects of Bry1/Skn7 on *CLN1* and *CLN2* were apparent only when the SBF and DSC1/MBF transcriptional activities were absent or attenuated, perhaps allowing the proposed Bry1/Skn7-controlled transcription factor ready access to the *CLN* promoters. Instead, *BRY1/SKN7* overexpression killed wild-type cells through a defect in cell wall metabolism as the lethality was partially alleviated by 1 M sorbitol or the overexpression of genes from the PKC pathway. Independent evidence for a Bry1/Skn7 role in cell wall metabolism came from the initial observations of Brown *et al.* (1993, 1994). However, the unconditional lethality of pGAL–BRY1 expression in a *pkc1*Δ strain (our data) and the unconditional lethality of *skn7*Δ *pkc1*Δ cells (Brown *et al.*, 1994; our unpublished observations), argue that the Bry1/Skn7 and PKC pathways independently impinge on cell wall metabolism. If Bry1/Skn7 and Pkc1 functioned in the same pathway, then these combinations of gene deletion would have been epistatic and the lethality would have been rescued by 1 M sorbitol. The unconditional lethality of *skn7*Δ *pkc1*Δ cells also suggests that pGAL–BRY1 behaves in a manner similar to a loss of function of the Bry1/Skn7 pathway. The molecular basis of the lethality of the galactose-induced *BRY1/SKN7* is not clear. The overexpression of *BRY1/SKN7* with Asp427 replaced with an asparagine residue is still lethal, questioning whether the receiver domain even has a role in the phenomenon. It is possible that the lethality reflects titration by the highly overexpressed *BRY1/SKN7* of some

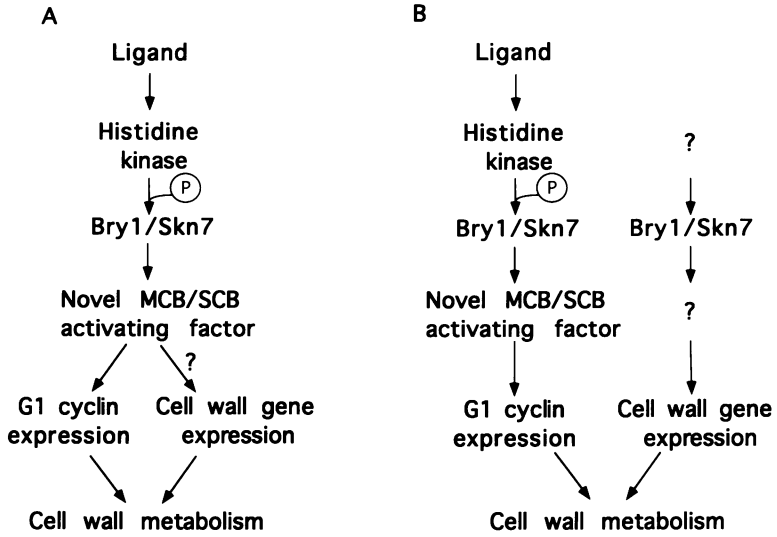

Fig. 7. Alternative models for the Bry1/Skn7 signal transduction pathway(s). Based on the presence of a bacterial response regulator domain in Bry1/Skn7, and that Asp427 is essential for the bypass of SBF and DSC1/MBF, we assume that Bry1/Skn7 is activated by a sensor component, likely to be a histidine kinase. Once activated, Bry1/Skn7 causes the transcription of G_1 cyclin genes and perhaps genes involved in cell wall synthesis. Two alternative models are presented. (**A**) We have presented evidence that Bry1/Skn7 can induce G_1 cyclin expression. However, the direct binding of Bry1/Skn7 to MCB/SCB elements is unlikely because the potential DNA binding domain in Bry1/Skn7 does not have homology to the DNA binding domains of Swi4 and Mbp1. Hence the MCB/SCB activation is probably a secondary consequence of genes controlled directly by Bry1/Skn7. The ability of Bry1/Skn7 to suppress defects in cell wall synthesis could be through the regulation of cell wall gene expression by this same novel MCB/SCB binding transcription factor. Alternatively, the effects on cell wall synthesis may be indirect via the activity of Cdc28–Cln1–Cln2 complexes in bud emergence (Benton *et al.*, 1993; Cvrčková and Nasmyth, 1993; Amon *et al.*, 1994; Schwob *et al.*, 1994). (**B**) The suppression of defects in the PKC pathway by the overexpression of Bry1/Skn7 (Brown *et al.*, 1994) and the lysis phenotype associated with the lethality of high overexpression of Bry1/Skn7 from the galactose promoter are both relatively insensitive to the presence or absence of Asp427. This suggests that Bry1/Skn7 may have an alternative pathway to cell wall gene expression, unlinked to the histidine kinase pathway. The suppression of *kre9*Δ strains sensitive to Asp427 can be explained in this model if the expression of certain cell wall genes is regulated by MCB/SCB elements or, as described above, perhaps via Cdc28–Cln1–Cln2 complexes.

factor, perhaps in the Bry1/Skn7 pathway, important for the viability of the wild-type cells and the *pkc1*Δ mutant.

Interestingly, the high copy suppression of *kre9*Δ by *SKN7* was sensitive to the mutation of Asp427, while the suppression of *pkc1*Δ was not (Brown *et al.*, 1994). By combining our data and the Brown *et al.* (1994) data, we propose that *BRY1/SKN7* has at least two outputs: one pathway leading to the expression of, as yet, unidentified cell wall genes, and another leading to G_1 cyclin expression (Figure 7). Bry1/Skn7 might regulate these different genes by regulating different transcription factors (Figure 7B). Alternatively, both of these outputs could be through a single transcription factor recognizing MCB/SCB elements (Figure 7A), in which case the relative expression of the MCB/SCB binding transcription factor might be sensitive to the phosphorylation of Asp427, explaining the different phenotypes observed. In the former model, the different phenotypes would be explained by one pathway being sensitive to phosphorylation of Asp427, whilst the other was insensitive.

Brown *et al.* (1993) proposed that Skn7 was a transcription factor which could activate the transcription of unidentified cell wall genes. The second of our models provides a possible mechanism for that expression through MCB/SCB elements. A large number of genes are coordinately expressed in late G_1 in *S.cerevisiae* and are regulated by DSC1/MBF and SBF (Johnston and Lowndes, 1992; Kilmartin *et al.*, 1993). These include the G_1 cyclin genes and genes involved in DNA replication and spindle pole body duplication. Because bud emergence occurs at the same time as the initiation of DNA replication and the initiation of spindle pole body duplication, it is not unreasonable to suggest that genes important for cell wall biosynthesis will be regulated at the same time by these transcription factors, perhaps including Bry1/Skn7, as part of the general coordination of events in late G_1 (Johnston, 1992). Regarding the role of Bry1/Skn7 in cyclin expression, our data suggest that this is most apparent when SBF is absent or attenuated. Therefore it is conceivable that, under appropriate conditions, Bry1/Skn7 is activated prior to START, when SBF and DSC1/MBF are absent or inactive. Bry1/Skn7 might then function to boost initial *CLN* levels as part of the events in the SBF–*CLN*–Cdc28 activation cycle. Clearly a number of signal transduction pathways impinge and coordinate passage through START. It has been proposed, for example, that the PKC pathway has a role in growth control. However, at present no evidence exists for any role of this pathway at START. Even if it does have a role to play at START, the evidence presented here and elsewhere indicates that *BRY1/SKN7* and *PKC1* are separate signal transduction pathways. Recently it was shown that high cAMP levels result in G_1 arrest and the inhibition of *CLN1* and *CLN2* transcription, although the mechanism of this regulation is not understood (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). It would be interesting to know whether the *BRY1/SKN7* pathway in any way interacts with the cAMP pathway in the regulation of START.

Materials and methods

Strains, growth conditions and yeast techniques

The haploid yeast strains used in this study were as follows: W303-1a (**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3*), BY600 (**a** ade2 met$^-$ *trp1 leu2 can1 his3 ura3 ho-lacZ swi6::TRP1*), BY604 (α *ade2 met$^-$ trp1 leu2 can1 his3 ura3 ho-lacZ swi4::LEU2*), K2003 (**a** ade2 his3 met$^-$ *leu2 trp1 ura3 swi4^{ts} swi6::TRP1*) and K3294 (**a** ade2-1 met$^-$ *trp1-1 leu2-3,112 can1-100 his3 ura3 ho-lacZ mbp1::URA3*). The heterozygous *swi4Δ swi6Δ* and *swi4Δ mbp1Δ* diploid strains were constructed by crossing BY600 with BY604, and BY604 with K3294, respectively.

YPD and minimal media used for yeast growth, as well as FOA medium for the selection of the loss of the *URA3* gene, have been described previously (Boeke *et al.*, 1984; Sherman *et al.*, 1986). The growth temperature used was 30°C unless otherwise stated. Yeast transformations were performed by a modification of the lithium acetate technique (Gietz and Sugino, 1988). To assay β-galactosidase activity in yeast colonies, transformations were plated directly onto minimal media supplemented with X-gal to a final concentration of 40 μg/ml; the pH was adjusted to 7.0 with 1 M phosphate buffer (pH 7.0). Plasmids were recovered out of yeast, as described by Robzyk and Kassir (1992), for further characterization.

Detection of lacZ activity on filters

Colonies of *swi4Δ* cells, containing the various *CLN2* promoter–*lacZ* fusion constructs and either the control vector pRS313 or pB-BRY1, were first grown on yeast selective medium at 30°C. Then the cells were replicated onto 3 MM Whatman paper and frozen in liquid nitrogen for 30 s. The filters were then soaked in Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, pH 7.0, 135 μl β-mercaptoethanol/50 ml Z buffer) containing X-gal at a final concentration of 1 mg/ml. Following incubation at 30°C for 1 h, the reaction was stopped by soaking the filters briefly in 1 M Na_2CO_3 and then drying at room temperature.

Plasmid constructs and site-directed mutagenesis

The *BRY1/SKN7* and *SWI4* genes were isolated from a *S.cerevisiae* genomic library cloned into the YEp24 vector (kindly provided by D.Botstein). YEp24/BRY1 and YEp24/SWI6 were the original plasmids isolated in the screen described in the text. YEp24/BRY1-H was constructed by transposing a derivative of the bacterial transposon, Tn*1000*, carrying the *HIS3* marker, Tn*HIS3* (Sedgwick and Morgan, 1994), into a non-essential region of YEp24/BRY1. The pB-BRY1 plasmids are described below. To disrupt the *BRY1/SKN7* gene on the chromosome, amino acids 1–471 of Bry1/Skn7 and 230 nucleotides of the promoter region on YEp24/BRY1 were replaced with the *HIS3* gene. Then, a restriction fragment spanning this insertion was used to disrupt the *BRY1/SKN7* gene on the chromosome in various genetic backgrounds. The galactose-inducible *BRY1/SKN7* plasmid, pGAL–BRY1, was constructed by ligating the coding region of *BRY1/SKN7*, with *Bam*HI linkers added by PCR, into the *Bam*HI site of the galactose promoter expression vector pMW20 (kindly provided by M.Walberg).

The *CLN2* promoter–*lacZ* constructs, kindly provided by C.Wittenberg and D.Stuart, have all been described previously (Stuart and Wittenberg, 1994).

Site-directed mutagenesis was performed by firstly subcloning the complete *BRY1/SKN7* gene, including the promoter region, from the primary YEp24/BRY1 clone into Bluescript. Then, Asp427 was mutated to an asparagine residue by site-directed oligonucleotide mutagenesis (Sambrook *et al.*, 1989) of the single-strand DNA. Mutations were confirmed by DNA sequencing. To provide a yeast replication origin and selectable marker, a derivative of the bacterial transposon Tn*1000*, Tn2μ*HIS3*, carrying the 2μ origin and *HIS3* selectable marker (Morgan *et al.*, submitted) was transposed into a non-essential region of Bluescript containing either *BRY1/SKN7* (Asp427) or *BRY1/SKN7* (Asn427). Several different transposon insertion hits in both the wild-type (pB-BRY1) and mutant (pB-BRY1 Asn427) plasmids were tested individually for their ability to rescue the lethality of the *swi4^{ts} swi6Δ* strain at 37°C (Figure 5). All hits in non-essential regions of these plasmids behaved as shown in Figure 5; the wild-type plasmids could rescue the lethality while the mutant version could not. The same transposon insertions in the wild-type pB-BRY1 plasmid were also tested individually for their ability to rescue the lethality of the *swi4^{ts} swi6Δ mbp1Δ* strain at 37°C (Figure 1D). An oligonucleotide was then used to introduce a single copy of the HA epitope in-frame between the C-terminal amino acid and the stop codon of the wild-type and Asn427 versions of the *BRY1/SKN7* gene in Bluescript by site-directed mutagenesis. This was confirmed by DNA sequencing. As described above, Tn2μ*HIS3* was then transposed into the HA epitope-tagged versions of the wild-type and mutated plasmids in non-essential regions. The wild-type HA epitope-tagged *BRY1/SKN7* plasmids (pB-BRY1HA) and mutant HA epitope-tagged *BRY1/SKN7* plasmids (pB-BRY1HA Asn427) were introduced into the *swi4^{ts} swi6Δ* strain and found to have identical phenotypes to the untagged versions of the gene.

DNA sequencing

This was performed using the T7 Sequencing™ kit and instructions provided by Pharmacia P-L Biochemicals Inc. Sequencing gels were prepared using the Sequagel™ Sequencing System and instructions provided by National Diagnostics.

Transposon insertions

Target plasmids were transposed with the desired transposon using a protocol described previously (Sedgwick and Morgan, 1994). The transposons used in this study were Tn*HIS3* (Sedgwick and Morgan, 1994) and Tn2μ*HIS3* (Morgan *et al.*, submitted).

RNA analysis

RNA extraction, Northern blotting and probing have been described previously (White *et al.*, 1986). The *CDC36* gene was used as an internal loading control. Densitometric analysis confirmed that *CLN1* and *CLN2* gene expression is higher in the *swi4^{ts} swi6Δ* strain at 37°C carrying YEp24/BRY1 relative to the vector-only control. No obvious effects of YEp24/BRY1 on *CDC9*, *MET4* or *ACT1* expression were detected in any of the samples.

Western blotting

Approximately 50 μg of total yeast protein isolated, as described previously (Toyn and Johnston, 1994), from the wild-type yeast strain W303-1a carrying three independent versions of the tagged wild-type Bry1/Skn7, and four of the tagged Asn427 Bry1/Skn7, were loaded onto a 10% SDS–polyacrylamide minigel (Hoefer). The gel was transferred to nitrocellulose using an electroblotter (Bio-Rad) and then tagged protein was detected using the 12CA5 (anti-HA) antibody and the ECL chemiluminescence kit supplied by Amersham Life Sciences.

Acknowledgements

We thank our colleagues, particularly Steve Sedgwick and Mark Toone for discussions and help with the figures, and Jonathan Millar, Steve Sedgwick and Mark Toone for reading this manuscript. We would also like to thank Hiro Araki, Joaquin Ariño, Fred Cross, David Levin, Kim Nasmyth, David Stuart and Curt Wittenberg for gifts of strains and plasmids. N.B. was supported partly by an EU Human Capital Mobility Grant (no. ERBCHRXCT930248) and partly by an International Travelling Research Fellowship award from the Wellcome Trust.

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Received on February 28, 1995; revised on July 21, 1995