Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 15400–15405, December 1998
Cell Biology

Differential regulation of transcription: Repression by unactivated mitogen-activated protein kinase Kss1 requires the Dig1 and Dig2 proteins

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Communicated by Michael S. Levine, University of California, Berkeley, CA, October 21, 1998 (received for review September 29, 1998)

ABSTRACT **Kss1, a yeast mitogen-activated protein kinase (MAPK), in its unphosphorylated (unactivated) state binds directly to and represses Ste12, a transcription factor necessary for expression of genes whose promoters contain filamentous response elements (FREs) and genes whose promoters contain pheromone response elements (PREs). Herein we show that two nuclear proteins, Dig1 and Dig2, are required cofactors in Kss1-imposed repression. Dig1 and Dig2 cooperate with Kss1 to repress Ste12 action at FREs and regulate invasive growth in a naturally invasive strain. Kss1-imposed Dig-dependent repression of Ste12 also occurs at PREs. However, maintenance of repression at PREs is more dependent on Dig1 and/or Dig2 and less dependent on Kss1 than repression at FREs. In addition, derepression at PREs is more dependent on MAPK-mediated phosphorylation than is derepression at FREs. Differential utilization of two types of MAPK-mediated regulation (binding-imposed repression and phosphorylation-dependent activation), in combination with distinct Ste12-containing complexes, contributes to the mechanisms by which separate extracellular stimuli that use the same MAPK cascade can elicit two different transcriptional responses.**

Cells respond to developmental cues and environmental stimuli by altering gene expression. Often, transmission of different upstream signals involves common components yet elicits distinct (but appropriate) outcomes. Numerous agonists activate mitogen-activated protein kinase (MAPK) cascades (1), which stimulate a relatively limited set of transcription factors (2, 3) yet evoke diverse responses. In *Drosophila melanogaster*, the *rolled* MAPK functions downstream of several receptor-tyrosine kinases that specify different cell fates (4, 5). Even in the same cell type, the same MAPK cascade can be recruited for distinct purposes, for example, mitogenesis or differentiation in mammalian cell lines (6, 7). How specificity from signal to cellular response is maintained in such cases is not well understood. Differences in signal strength, duration, or frequency can lead to distinct outputs (6, 8). Also, transcriptional regulators that are tissue-specific, or modulated by parallel pathways, can influence which genes are controlled by signal-dependent activation of more ubiquitous transcription factors (2, 3, 5). Most likely, multiple mechanisms are required to achieve the requisite fidelity.

In the yeast *Saccharomyces cerevisiae*, two developmental options—mating and invasive (filamentous) growth—are regulated by the same MAPK cascade (9). Elements of this cascade include the MAPK kinase, Ste7, and its target MAPKs, Kss1 and Fus3 (9, 10). The Ste12 transcription factor (11), and its associated negative regulators, Dig1 and Dig2 (also called Rst1 and Rst2) (12, 13), act downstream of the MAPKs in both developmental processes. Despite this overlap in key regulatory components, mating and invasive growth are discrete end points. Cells exposed to mating pheromone cease growth and prepare to fuse with an appropriate nearby partner (for review, see ref. 14), whereas cells undergoing invasive growth continue to divide, forming filaments that can adhere tightly to and penetrate beneath an agar substratum (9, 15). Moreover, Ste12-regulated filamentation response elements [FREs (16)] are constitutively expressed in haploids, yet Ste12-regulated pheromone response elements [PREs (14)] in many mating-specific genes, such as *FUS1*, are not expressed in the absence of pheromone stimulation. Conversely, pheromone stimulation does not hyperactivate FREs (17).

The Ste12 DNA-binding domain is a distant relative of the homeodomain (18). Ste12 forms homooligomers to regulate genes, such as *FUS1*, forms heterooligomers with a MADS-box [a conserved DNA-binding and dimerization domain shared by a variety of transcription factors from different kingdoms (including yeast Mcm1, plant Agamous, plant Deficiens, and mammalian serum-response factor)] transcription factor, Mcm1, to regulate *STE2*, and associates with both Mcm1 and another homeodomain protein, Matα1, to regulate *MFα1* (19, 20). All three classes of elements are pheromone-inducible. At FREs, Ste12 forms heterooligomers with the TEA/ATTS-family [a group of transcription factors from different kingdoms that share a conserved DNA-binding motif (including mammalian TEF-1, yeast Tec1, and *Aspergillus nidulans* AbaA/mold AbaA, mammalian TEF-1, yeast Tec1, *D. melanogaster* scalloped)] transcription factor, Tec1 (16, 21). Thus, combinatorial interactions of Ste12 with other regulatory proteins may confer some of the specificity for mating versus invasive growth responses.

Differential interactions of the MAPKs with Ste12-containing complexes may provide additional specificity. Kss1 and Fus3 are positive regulators of Ste12 function at pheromone-induced promoters. Furthermore, their catalytic (phosphotransferase) activity is essential for this purpose (22, 23), presumably because they phosphorylate Ste12 (24) and/or Dig1 and Dig2 (12, 13). In contrast, at FREs, the MAPKs control Ste12 function by an unexpected and previously unknown mode of protein kinase-mediated regulation (25, 26). Unphosphorylated Kss1 binds directly to Ste12 and potently represses FRE-driven transcription (27). Fus3 binds much less strongly to Ste12 (27) and is, correspondingly, a weaker repressor (26). Phosphorylation of Kss1 by Ste7 weakens Kss1–Ste12 binding, consequently relieves Kss1-imposed repression, and simultaneously activates Kss1 catalytic activity (27). Activated Kss1 has a positive function at FREs that may

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Abbreviations: MAPK, mitogen-activated protein kinase; FRE, filamentous response element; PRE, pheromone response element.

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correspond to its positive role at pheromone-inducible promoters (25, 26). Whether unphosphorylated Kss1 plays a role in repressing PRE-containing promoters had not heretofore been examined.

Although direct binding of Kss1 to Ste12 is required for Kss1-imposed repression at FREs (27), it seemed likely that Kss1, which contains no sequence features outside of its protein kinase domain, might require the help of cofactors to repress Ste12. Herein we show that Dig1 and Dig2, two nuclear proteins that bind directly to both Kss1 and Ste12 (12), are required for Kss1-imposed repression of Ste12. We also demonstrate that Kss1-imposed Dig-dependent repression of Ste12 occurs at pheromone-inducible elements. These findings suggest a model that accounts for much of the regulatory specificity observed in mating and invasive growth.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Procedures. Growth media and culture conditions were as described (28). Table 1 lists the yeast strains used in this work. The *dig1-Δ1::HIS3* and *ste12Δ::LEU2* (12), *ste7Δ::URA3* (26), and *kss1Δ::hisG* and *fus3–6::LEU2* alleles (23) are described in the citations given. The *dig2-Δ1::URA3* allele was generated by the PCR using plasmid pRS306 (29) as template and primers 103-KO1 and 103-KO2 (12). These alleles were introduced by the method of one-step gene replacement (30). JCY600 and JCY300 were derived from JCY100. JCY500 was derived from JCY300. JCY501 is a spontaneous Ura$^-$ and 5-fluoroorotic acid-resistant derivative of JCY500. YLB507 and JCY512 were derived from JCY501. YDM200 and YDM600 (gifts from Doreen Ma, this laboratory) were derived from YPH499. JCY101 and JCY502 were derived from JCY100 and JCY501, respectively, by *HO*-induced mating-type switching (31). JCY102 and YLB503 were generated by mating the appropriate haploid progenitors (see Table 1). All gene replacements were confirmed by Southern blot hybridization and/or PCR analysis of genomic DNA. Genotype was further confirmed by complementation of the mutant phenotype by introduction of the corresponding wild-type gene on a *CEN* plasmid.

Reporter Genes. Reporter (*lacZ*) constructs used in this study are representative of genes expressed during mating and invasive growth. Their level of expression correlates well with the phenotypic effects of manipulations that alter mating proficiency and invasiveness, respectively (26, 27). YEpU-FTyZ and YEpL-FTyZ contain *FRE*^{Ty1}*-lacZ* on a 2-μm DNA plasmid (26, 27). Plasmid pJD11 contains eight tandem PREs (dimeric Ste12-binding sites) (32). To construct YEpU-*FUS1Z* (gift from Iain Cheeseman, this laboratory), the *FUS1* promoter was amplified by PCR using primers IMC-FUS-1 (5′-GCGCGCGCTAGCTGCAG-GATCGCCCTTTTTGACG-3′) and IMC-FUS-2 (5′-GCGCGCGGATCCTGCTACCATTTTGATTTTAGAAAC-3′). The resulting fragment was digested with *Bam*HI and *Nhe*I and used to replace the *Bam*HI–*Nhe*I fragment (containing the *CYC1* promoter) of plasmid pLG669-Z (33). The *URA3* gene was excised from YEpU-*FUS1Z* by digesting with *Sse*8387I and *Xma*I and replaced with the *TRP1* gene on a *Pst*I–*Xma*I fragment excised from pJJ281 (34), to yield YEpT-*FUS1Z*. Reporter gene expression was quantitated as described (26).

Other Plasmids. Plasmids YEpU [YEplac195 (35)], YEp-*KSS1*, and YEp*GAL-KSS1* (23), and p*GAL-DIG1* (12) are described in the citations given. YCpU, YCpU-*KSS1*, YCpU-*kss1(Y24F)*, YCpU-*kss1(AEF)*, YCpU-*kss1(Δloop)*, and YCpU-*kss1(Y231C)* are described elsewhere (27). p*DIG1* carries a genomic DNA fragment containing *DIG1* in a low-copy (*CEN*) vector harboring the *LEU2* gene (36). To construct YCpLG (a *CEN*-based, *LEU2*-marked, galactose-inducible expression vector), a 0.68-kb *Eco*RI–*Bam*HI fragment containing the bidirectional *GAL1/10* promoter was excised from plasmid pMTL4 (gift from Stephen Johnston, Univ. of Texas Southwestern Medical Center, Dallas, TX) and inserted into the corresponding sites of YCplac111 (35). A *Bam*HI–*Sph*I fragment carrying the entire *KSS1* coding sequence was excised from YEp*GAL-KSS1* and inserted into YCpLG, yielding YCpLG-*KSS1*.

Bioassays for Pheromone Response and Invasive Growth. Bioassays for pheromone response were performed as described (28). For assessment of diploid pseudohyphal development, synthetic low ammonia/dextrose plates, supplemented with histidine and uracil as required, were prepared as described elsewhere (37). Diploid strains were transformed with YCplac22 and YCplac111 (35), as required, to confer prototrophy for growth on tryptophan and leucine, respectively, because these compounds can serve as nitrogen sources for *S. cerevisiae*. In the original assay for invasion (9), the agar plate was held under a stream of running water to remove noninvasive cells. Two classes of cells remain after washing: those that have penetrated the agar and those adhering to cells of the first class [because some of the genes involved in a

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

Strain	Relevant genotype	Source or ref(s).
Haploid strains derived from the Σ1278b lineage		
JCY100	*MAT***a** *his3Δ::hisG leu2Δ::hisG trp1Δ::hisG ura3-52*	25, 26
JCY101	*MATα* otherwise isogenic to JCY100	This work
JCY107	JCY100 *ste7Δ::ura3*	26
JCY137	JCY100 *ste7Δ::ura3 kss1Δ::hisG fus3Δ::TRP1*	26
JCY300	JCY100 *dig2-Δ1::URA3*	This work
JCY500	JCY100 *dig1-Δ1::HIS3 dig2-Δ1::URA3*	This work
JCY501	JCY100 *dig1-Δ1::HIS3 dig2-Δ1::ura3*	This work
JCY502	*MATα* otherwise isogenic to JCY501	This work
YLB507	JCY100 *ste7Δ::URA3 dig1-Δ1::HIS3 dig2-Δ1::ura3*	This work
JCY512	JCY100 *dig1-Δ1::HIS3 dig2-Δ1::ura3 ste12Δ::LEU2*	This work
JCY600	JCY100 *ste12Δ::LEU2*	This work
Diploid strains derived from the Σ1278b lineage		
JCY102	*MAT***a**/*MATα* (JCY100 × JCY101)	This work
YLB503	*MAT***a**/*MATα dig1-Δ1::HIS3*/*dig1-Δ1::HIS3* *dig2-Δ1::ura3*/*dig2-Δ1::ura3* (JCY501 × JCY502)	This work
Haploid strains derived from the S288C lineage		
YPH499	*MAT*a *ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ63 ura3-52*	29
JCY5	YPH499 *dig1-Δ1::HIS3 dig2-Δ1::TRP1*	12
YDM200	YPH499 *fus3-6::LEU2*	This work
YDM230	YPH499 *kss1Δ::hisG fus3-6::LEU2*	23
YDM600	YPH499 *kss1Δ::hisG*	This work

cell–cell adhesion process termed flocculation also function in invasive growth (38–40)]. Rubbing the agar plate with a latex-gloved finger removes the superficially adherent cells, leaving behind only those that have penetrated the agar. This latter step permits assessment of agar penetration *per se*.

Other Methods. Metabolic labeling, immunoprecipitation, and immunostaining of Kss1 have been described (23, 27). Kss1 expression levels were determined by immunoblotting of serial dilutions using known quantities of Kss1 as a standard.

RESULTS

Dig1 and Dig2 Regulate Invasive Growth in Naturally Filamentous Strains. Invasive growth is typical of strains of *S. cerevisiae* isolated from the wild (39) and in pathogenic strains recovered from immunocompromised patients (41). This capacity has been lost, however, in many common laboratory stocks, due to several incompletely characterized genetic changes (39). Deletion of both *DIG1* and *DIG2* confers invasiveness to otherwise noninvasive strains (12, 13). Whether *DIG1* and *DIG2* regulate invasive growth in normally invasive strains had not heretofore been examined. To determine whether Dig1 and Dig2 regulate invasive growth in strains of the Σ1278b lineage normally used to study filamentation, a *dig1*Δ/*dig1*Δ *dig2*Δ/*dig2*Δ homozygous diploid (YLB503) was constructed in this background. Compared with its otherwise isogenic parental diploid, the strain lacking Dig1 and Dig2 displayed a hyperfilamentous phenotype. This enhancement was most obvious after 2 to 3 days of growth on low nitrogen medium, when the wild-type cells were only just beginning to extend filaments (pseudohyphae) (Fig. 1 *Ab* versus *Aa*). In addition, the *dig1*Δ/*dig1*Δ *dig2*Δ/*dig2*Δ diploid was able to form pseudohyphae in the absence of a nitrogen limitation signal, unlike the wild-type cells (Fig. 1 *Ad* versus *Ac*). To determine whether the absence of Dig1 and Dig2 resulted in deregulation of Ste12/Tec1-dependent transcription, we used the FRE from the Ty1 transposon to drive expression of a reporter gene (*lacZ*). As shown in Fig. 1*B*, FRE-driven expression increased by almost 20-fold in the absence of Dig1 and Dig2.

To determine the role of Dig1 and Dig2 in haploid invasive growth, an isogenic set of haploid strains of the Σ1278b lineage was constructed: JCY501 (*MAT***a** *dig1*Δ *dig2*Δ), YLB507 (*MAT***a** *ste7*Δ *dig1*Δ *dig2*Δ), and JCY512 (*MAT***a** *dig1*Δ *dig2*Δ *ste12*Δ). The properties of these strains were compared with the wild-type parental strain and to otherwise isogenic *ste7*Δ and *ste7*Δ *kss1*Δ *fus3*Δ strains. As previously observed (26), the inability of a Ste7-deficient cell to grow invasively (Fig. 2*A*) or to support FRE-driven transcription (Fig. 2*B*) was completely alleviated when both Kss1 and Fus3 were also removed. Informatively, both of these same phenotypes of the *ste7*Δ cells were also reversed by elimination of Dig1 and Dig2 (Fig. 2). In the *dig1*Δ *dig2*Δ cells, both invasiveness (Fig. 2*A*) and FRE-driven transcription (Fig. 2*B*) were elevated compared with either wild-type cells or the *ste7*Δ *kss1*Δ *fus3*Δ strain. Hyperinvasiveness of cells lacking Dig1 and Dig2 was most clearly manifested by using a modification of the standard invasive growth assay that achieves a more stringent test for agar penetration of the adherent cells (Fig. 2 *Ac*). The *dig1*Δ *dig2*Δ cells also exhibited efficient agar penetration on synthetic medium (data not shown), a condition in which wild-type cells of the Σ1278b lineage show only a weak invasive growth response (9). The behavior of Dig1- and Dig2-deficient cells was not significantly altered by the absence Ste7 (Fig. 2), as expected if Dig1 and Dig2, like Kss1, act downstream of Ste7. In contrast, the enhanced invasiveness and enhanced FRE-driven transcription of Dig1- and Dig2-deficient Σ1278b cells was totally dependent on the presence of Ste12 (Fig. 2), indicating that these phenotypes are due largely or solely to derepression of Ste12.

***DIG1* or *DIG2* Function Is Required for Kss1-Imposed Repression at FREs.** Repression of Ste12-dependent FRE-driven

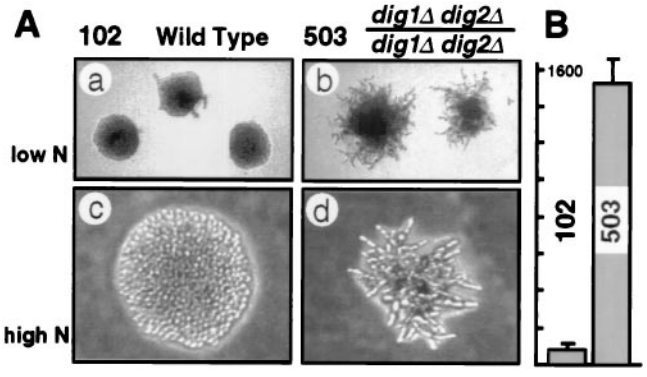

FIG. 1. Dig1 and Dig2 regulate filamentous growth in naturally invasive diploids. (*A*) Loss of *DIG1* and *DIG2* enhance diploid pseudohyphal development. Strain JCY102 (*MAT***a**/*MAT*α *DIG*$^+$) (*a* and *c*) and its otherwise isogenic derivative, YLB503 (*MAT***a**/MATα *dig1*Δ/*dig1*Δ *dig2*Δ/*dig2*Δ) (*b and d*), were assayed for filament formation on plates containing either low nitrogen (low N) (*a* and *b*) or high nitrogen (high N) (*c* and *d*). Representative colonies, photographed after 3 days (low N) or 1 day (high N) of growth at 30°C, are shown. (*B*) Expression of the *FRE*$^{Ty1\text{-}lacZ}$ reporter. The strains described in *A* were transformed with plasmid YEpU-FTyZ and grown on low nitrogen plates for 48 hr at 30°C, and β-galactosidase specific activity (nmol per min per mg of protein) was measured. Values represent the average of measurements, made in duplicate, on protein extracts prepared from at least three transformants of each strain. Error bars indicate the standard deviation. Comparable results were obtained on high nitrogen plates (data not shown).

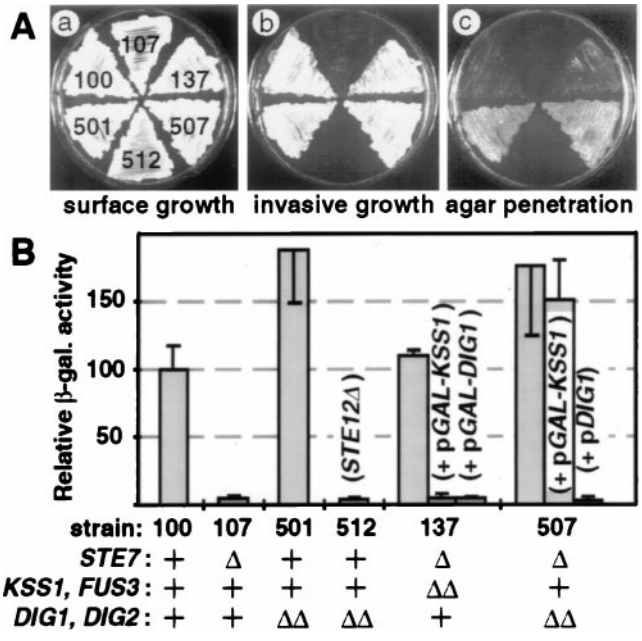

FIG. 2. Dig1 and Dig2 cooperate with Kss1 to inhibit invasive growth. (*A*) Loss of *DIG1* and *DIG2* enhance haploid invasive growth. Strain JCY100 (*MAT***a** *STE*$^+$ *DIG*$^+$) and its otherwise isogenic derivatives, JCY107 (*ste7*Δ), JCY137 (*ste7*Δ *kss1*Δ *fus3*Δ), JCY501 (*dig1*Δ *dig2*Δ), JCY512 (*dig1*Δ *dig2*Δ *ste12*Δ), and YLB507 (*ste7*Δ *dig1*Δ *dig2*Δ), were streaked onto YPD plates and assayed for surface growth (*a*), invasive growth (*b*), and agar penetration (*c*) after 3 days at 30°C. (*B*) Effect of Kss1 or Dig1 overproduction on expression of the *FRE*$^{Ty1\text{-}lacZ}$ reporter: Dig1 and Dig2 are required for Kss1-imposed repression. The strains described in *A* were transformed with plasmid YEpU-FTyZ or YEpL-FTyZ. JCY137 was also transformed with either YCpLG-*KSS1* (p*GAL-KSS1*) or p*GAL-DIG1*, for overproduction of Kss1 or Dig1, respectively, from the *GAL1* promoter. YLB507 was also transformed with YCpLG-*KSS1* (p*GAL-KSS1*) or p*DIG1*, for overproduction of Kss1 or endogenous-level expression of Dig1, respectively. Strains were grown on plates containing 2% galactose and 0.2% sucrose as the carbon source for 24 hr at 30°C, and β-galactosidase specific activity was measured as in Fig. 1. Values are normalized to that observed for JCY100 (11, 300 nmol per min per mg of protein).

transcription observed in a Ste7-deficient cell is reversed by removing both Kss1 and Fus3 (Dig1 and Dig2 still present) or by removing both Dig1 and Dig2 (Kss1 and Fus3 still present), suggesting that the MAPKs and the Dig proteins operate interdependently to repress Ste12. To gain further insight into this relationship, the consequences of overproducing Kss1 or Dig1 in these strains was examined. Expression of *KSS1* from its own promoter (27) or overexpression from the *GAL1* promoter (Fig. 2*B*) sufficed to repress FRE-driven transcription in strain JCY137 (*MAT***a** *ste7*Δ *kss1*Δ *fus3*Δ), which contains Dig1 and Dig2. Overexpression of *DIG1* from the *GAL1* promoter also repressed FRE-driven transcription in this same strain (Fig. 2*B*), indicating that, when overproduced, Dig1 is capable of repressing Ste12 even in the absence of the MAPKs (and Ste7). In contrast, overexpression of *KSS1* from the *GAL1* promoter had little or no repressive effect in the Dig1- and Dig2-deficient strain JCY507 (*MAT***a** *ste7*Δ *dig1*Δ *dig2*Δ), whereas expression of *DIG1* from its own promoter fully restored repression of FRE-driven transcription in this strain (Fig. 2*B*). Thus, in the absence of Dig1 and Dig2, Kss1 is unable to effectively repress Ste12.

Kss1-Imposed Repression Occurs at Pheromone-Induced Genes. To determine whether MAPK-imposed repression also occurs at pheromone-inducible promoters, basal and pheromone-induced transcription driven by the *FUS1* promoter was examined (using a *FUS1–lacZ* reporter) in a wild-type strain and isogenic cells lacking either Kss1 or Fus3 or both (Fig. 3). As expected, there was essentially no expression of this reporter in cells lacking both MAPKs (data not shown). *FUS1–lacZ* expression in unstimulated cells lacking Fus3 was not significantly different from wild-type cells. In marked contrast, *FUS1–lacZ* expression was reproducibly increased (approximately 7-fold compared with wild-type) in unstimulated cells lacking Kss1. This result was observed in two different strain backgrounds: normally noninvasive S288C (Fig. 3) and normally invasive Σ1278b (data not shown). Hence, Kss1 plays a role in maintaining low basal-level expression of pheromone-inducible genes.

To examine the mechanism of Kss1-dependent repression of basal *FUS1* expression, a series of *kss1* alleles, expressed from the endogenous *KSS1* promoter on a low-copy (*CEN*) vector, was introduced into a strain lacking Kss1 (Fig. 3). Vector alone had no effect. However, repression was restored by plasmid-borne wild-type Kss1, by an unactivatable Kss1 mutant ($Kss1_{AEF}$ that cannot be phosphorylated by Ste7), and by a catalytically inactive variant ($Kss1_{Y24F}$). Hence, neither phosphorylation of Kss1 by Ste7 nor the resultant activity of Kss1 is required for repression. In contrast, two derivatives ($Kss1_{\Delta loop}$ and $Kss1_{Y231C}$) that we have shown (27) exhibit decreased binding to Ste12 (but undiminished binding to Ste7, Dig1, or Dig2) displayed a correspondingly reduced ability to repress Ste12 function at the *FUS1* promoter (Fig. 3). Hence, binding of Kss1 to Ste12 is required for Kss1 to effectively repress Ste12 function at pheromone-inducible promoters, as demonstrated for FREs (25, 27).

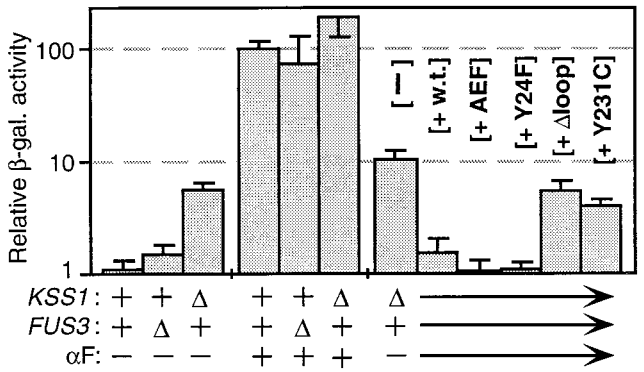

FIG. 3. Unphosphorylated Kss1 represses Ste12 action at a pheromone-inducible promoter. Strain YPH499 (*MAT***a**) and its otherwise isogenic derivatives, YDM600 (*kss1*Δ) and YDM200 (*fus3*Δ), were transformed with plasmid YEpU-*FUS1Z*, grown to midexponential phase, and incubated in the absence (−) and presence (+) of 1 μM α-factor mating pheromone (αF) for 90 min, and β-galactosidase specific activity was measured. Also, strain YDM600 was cotransformed with YEpT-*FUS1Z* and empty vector YCpU (−), YCpU-*KSS1* (w.t.), YCpU-*kss1(AEF)*, YCpU-*kss1(Y24F)*, YCpU-*kss1(*Δ*loop)*, or YCpU-*kss1(Y231C)*; streaked on plates selective for plasmid maintenance; and grown for 48 hr at 30°C. β-Galactosidase-specific activity was then measured. Values are normalized to that observed for YPH499 treated with pheromone (250 nmol per min per mg of protein).

Kss1 Overproduction Inhibits Pheromone-Induced Transcription and Mating. Upon pheromone stimulation, the transcriptional response of cells containing only Kss1, or only Fus3, was not significantly different from that of cells containing both

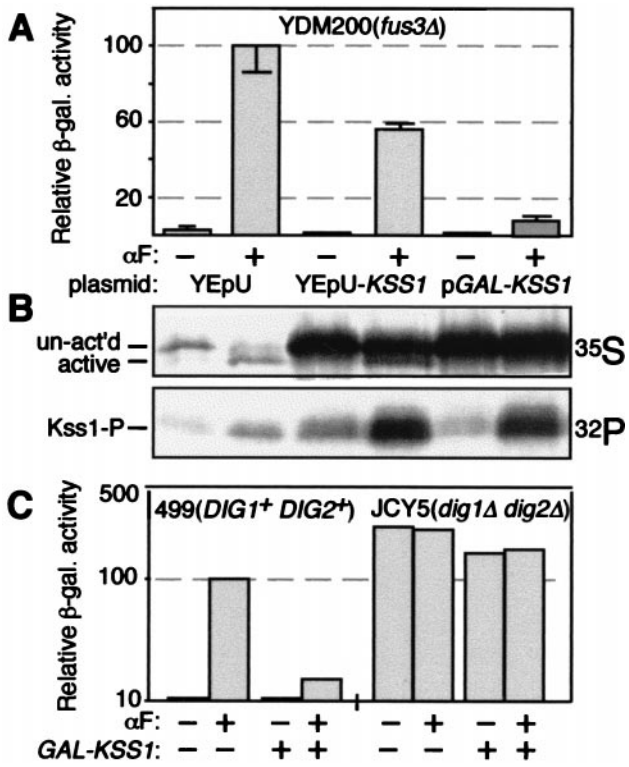

FIG. 4. Kss1 overproduction inhibits pheromone-induced transcription and Dig1 and Dig2 are required for this Kss1-imposed repression. (*A*) Strain YDM200 (*MAT***a** *fus3*Δ) was cotransformed with plasmid pJD11 containing a PRE-driven *lacZ* reporter and an empty vector, YEpU (bars 1 and 2), a multicopy plasmid, YEp-*KSS1* (bars 3 and 4), or a *GAL*-driven multicopy plasmid, YEp*GAL-KSS1* (bars 5 and 6); grown to midexponential phase in medium containing 2% galactose and 0.2% sucrose; and incubated in the absence (−) and presence (+) of 6 μM α-factor mating pheromone (αF) for 2 hr. β-Galactosidase specific activity was then measured. Values are normalized to that observed for the pheromone-induced control cells (point 2; 4,500 nmol per min per mg of protein). (*B*) The strains described in *A* were labeled with either ^{35}S (*Upper*) or ^{32}P (*Lower*), incubated with or without 6 μM α-factor for 15 min, lysed, subjected to immunoprecipitation with polyclonal anti-Kss1 antiserum, resolved by SDS/PAGE, and analyzed by fluorography, all as described elsewhere (23). (*C*) Strain YPH499 (*MAT***a** *DIG*$^+$) and its otherwise isogenic derivative, JCY5 (*dig1*Δ *dig2*Δ), were cotransformed with YEpU-*FUS1Z* and either an empty vector YCpLG (−) or YCpLG-*KSS1* (*GAL-KSS1*) (+), grown to midexponential phase in medium containing 2% galactose and 0.2% sucrose, and incubated in the absence (−) and presence (+) of 1 μM α-factor for 90 min. β-Galactosidase specific activity was then measured. The values for vector-containing YPH499 not treated with pheromone (1.5 relative units for both points), which should lie below the abscissa, are shown as a bar just above the line to increase the clarity of presentation. Values are normalized to that observed for vector-containing YPH499 treated with pheromone (198 nmol per min per mg of protein). Standard deviations (data not shown) were less than 10% of the mean in all cases.

MAPKs (Fig. 3). When Kss1 was present at its endogenous level [approximately 5,000 molecules per cell (28)], stimulation with mating pheromone induced the expression of the pheromone-regulated reporter by approximately 100-fold (Fig. 4*A*). In contrast, when *KSS1* was overexpressed approximately 20-fold from its own promoter on a multicopy plasmid, pheromone-induced expression was reduced by ~50% (Fig. 4*A*). Correspondingly, mating efficiency was reduced by ~75% (data not shown). When Kss1 was overproduced approximately 100-fold from the strong *GAL1* promoter, pheromone-induced expression was reduced by ~95% (Fig. 4*A*), and mating efficiency was comparably reduced (data not shown). To rule out the possibility that this effect was caused by any sort of competition between overproduced Kss1 and endogenous Fus3, these experiments were performed in a *fus3*Δ strain (Fig. 4*A*); comparable results were obtained in a *FUS3*$^+$ strain (data not shown and Fig. 4*C*).

The level and state of phosphorylation of Kss1 were monitored by metabolic labeling and immunoprecipitation (Fig. 4*B*). Kss1 migrates as a doublet on polyacrylamide gels, where the faster-migrating species is activated Kss1 (23). In naive cells, endogenous Kss1 was mostly unactivated, and a prominent shift was seen upon pheromone stimulation (Fig. 4*B Upper*). Overproduction of Kss1 elevated the absolute amount of activated Kss1, as indicated by the increase in both ^{35}S-label (Fig. 4*B Upper*) and ^{32}P-label (Fig. 4*B Lower*) associated with the faster-migrating band. However, the increase in activated Kss1 was only 3- to 5-fold, despite 20- to 100-fold overproduction of total Kss1 (Fig. 4*B*). Ste7 [<1,000 molecules per cell (28)] is presumably limiting under these circumstances; indeed, cooverproduction of Ste7 partially reversed the effects of Kss1 overproduction (data not shown). Because overproduction primarily increased the amount of unactivated Kss1, the ability of unactivated Kss1 to repress Ste12 (25, 27) presumably accounts for the inhibition of pheromone-induced transcription observed.

***DIG1* or *DIG2* Function Is Required for Kss1-Imposed Repression at Pheromone-Inducible Genes.** The effect of Kss1 overproduction on pheromone-induced transcription was compared in the presence or absence of Dig1 and Dig2 (Fig. 4*C*). As found by others (13), *FUS1–lacZ* expression was significantly derepressed in a strain lacking Dig1 and Dig2. Kss1 overproduction substantially repressed pheromone-induced transcription in the wild-type strain but was ineffective at repressing pheromone-induced transcription in the Dig-deficient strain (Fig. 4*C*). Immunoblot analysis using specific anti-Kss1 antiserum (23) indicated that Kss1 was overproduced to equivalent levels (~50-fold) in both the wild-type and Dig-deficient cells (data not shown). These data indicate that, as in the case of FREs (Fig. 2), Dig1 and Dig2 are required for effective Kss1-imposed repression at pheromone-inducible promoters.

DISCUSSION

The regulation of gene expression by protein kinase-mediated phosphorylation is well established (2, 3). Repression imposed by direct binding of a protein kinase, so far demonstrated only in yeast (25, 27), is likely to be found in other protein kinase signaling pathways that act as developmental switches. In this study, we compared MAPK-imposed repression of the Ste12 transcription factor at genes involved in mating and invasive growth and revealed a function for the Dig1 and Dig2 proteins as cofactors in this process. We also further examined the role of Dig1 and Dig2 in regulating invasive growth.

We showed herein that deletion of both *KSS1* and *FUS3* or deletion of both *DIG1* and *DIG2* was sufficient to derepress Ste12, indicating that the Dig1 and Dig2 proteins and the Kss1 and Fus3 MAPKs act cooperatively to repress Ste12 action at FREs. Absence of Dig1 and Dig2 conferred a markedly hyperinvasive phenotype, including abolishing the requirement for a nitrogen-limitation signal in diploids. Conversely, when overproduced, Dig1 was able to repress FREs even in the absence of the MAPKs. In contrast, substantial overproduction of Kss1 had no markedly repressive effect in the absence of the Dig proteins. These data suggest that the Dig proteins directly repress Ste12, whereas Kss1 acts primarily to potentiate Dig-mediated repression, perhaps by stabilizing Dig–Ste12 interaction by virtue of its ability to bind to both proteins (12, 13, 25, 27) (Fig. 5*A*). The mechanism(s) by which Dig1 and Dig2 repress Ste12 [e.g., short-range masking or squelching versus long-range or active repression (42, 43)] remains to be determined. Dig1 and Dig2 are homologous to each other but not to other repressors in current databases.

Dig1 and Dig2 regulate Ste12 function in naturally filamentous and invasive *S. cerevisiae*. At least one prominent fungal pathogen of humans, *Candida albicans*, contains a Ste12 homolog (Cph1) important for hyphal formation and virulence (44). Moreover, a small region of Ste12 sufficient for its interaction with Dig1 and Dig2 in the two-hybrid assay is conserved between Ste12 and Cph1 (11). Also, a Ste7 homolog and a Kss1 homolog have been shown to regulate hyphal development in *C. albicans* (45–47). Hence, Dig homologs, in cooperation with MAPKs, may regulate dimorphism, filamentation, and invasiveness in other fungi.

Having established that Dig1 and Dig2 are required for Kss1-imposed repression of Ste12 at FREs, we sought to gain

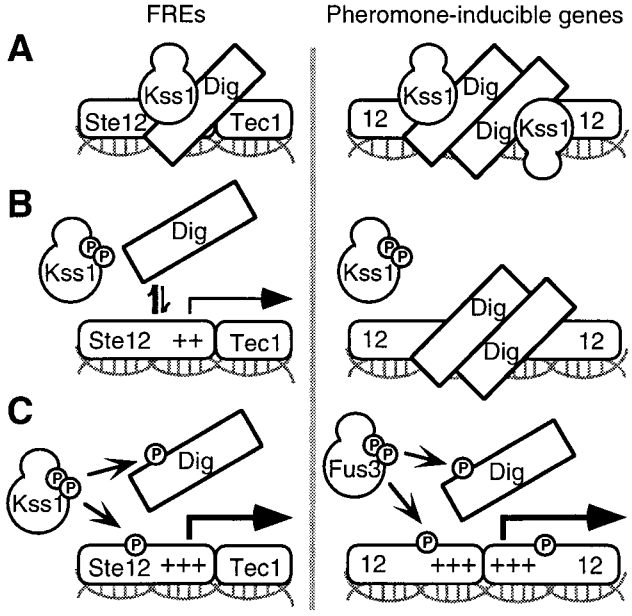

FIG. 5. Model for differential control of FREs and PREs by MAPK- and Dig1/2-mediated regulation. (*A*) At both elements, unphosphorylated MAPK [principally Kss1 (25–27)] binds directly to Ste12 and to Dig1 (and Dig2), thereby stabilizing Dig1/2–Ste12 complexes and potentiating Dig-mediated repression of Ste12. (*B*) At FREs, phosphorylation of Kss1 by Ste7, in response to upstream signals, weakens its association with Ste12, consequently promoting dissociation of Dig proteins from Ste12–Tec1 complexes, permitting substantial derepression (27). Ste7-dependent phosphorylation of Kss1 may also attenuate its binding to Ste12 at pheromone-inducible promoters, but this event is not sufficient for effective derepression, presumably because Dig proteins are bound more stably to Ste12–Ste12 homooligomers. (*C*) At FREs, phosphorylated (activated) Kss1 reinforces the transition, presumably by phosphorylating Dig1/2 (12, 13) and/or Ste12 and/or Tec1 (25, 27). Phosphorylation of these targets may prevent their reassociation, stimulate the transactivator activity of Ste12 and/or Tec1, or both. However, the level of Kss1 activation that results from the signals promoting invasive growth are insufficient to achieve derepression at PRE-bound complexes. In contrast, when more fully activated by pheromone, the MAPKs [principally Fus3 (25, 48)] derepress pheromone-inducible promoters, again presumably by phosphorylating Dig1/2 and Ste12 (11, 12, 13, 24). FRE-bound complexes are apparently insensitive to Fus3 action, perhaps because Fus3 cannot gain access to these complexes or phosphorylate them appropriately.

insight into the potential role of both regulators in the control of Ste12 function at pheromone-inducible promoters. Fundamentally, both elements (FREs and PREs) are not expressed until derepressed in response to a signal transmitted by the MAPK cascade. Mechanistically, we found two key similarities in regulation. (*i*) Dig function is absolutely required for repression at both types of elements. (*ii*) Kss1-imposed repression, mediated by direct Kss1-Ste12 binding, operates at both elements. However, with regard to the latter, there is a significant difference in degree. As shown herein, in the absence of Kss1, expression of *FUS1* increases about 7-fold above its normal basal level but only to ~7% of its pheromone-induced level. In contrast, expression of FREs in the absence of a signal (in *ste7*Δ cells) is substantially elevated, to ~60% of its normally derepressed level, by removing Kss1 alone and fully derepressed by removing both MAPKs (26). Therefore, at PREs, Dig binding to Ste12 is presumably less dependent on Kss1 (Fig. 5*B*).

At PREs, absence of both MAPKs prevents pheromone-induced derepression, whereas at FREs, absence of both MAPKs results in constitutive derepression. The most likely explanation for this apparent paradox relies on the demonstrated ability of the activated MAPKs to phosphorylate both Ste12 and the Dig proteins (12, 13, 25, 27) (Fig. 5*C*). Full derepression at pheromone-induced promoters presumably requires two steps: Ste7-dependent phosphorylation of the MAPKs and subsequent phosphorylation of substrates (Ste12 and Dig1) by the activated MAPKs. We have shown that derepression of FREs requires the first step but not the second (26, 27). In principle, then, the level of activated Kss1 could be set so that mating genes are not expressed even though FREs are expressed.

When the MAPK pools are more fully activated in response to pheromone stimulation, both classes of elements are derepressed (but the mating program exerts some dominance over the invasive growth program). Thus, flux through the MAPK cascade may dictate the response as follows: low signal, FREs on and PREs off; high flux, both on. Additional mechanisms also contribute to response specificity. Genetic data suggest that Fus3-mediated phosphorylation can promote derepression at pheromone-inducible promoters but not at FREs, whereas Kss1-mediated phosphorylation can act at both classes of element (L.B., unpublished observations). In haploids, Fus3 may not be efficiently activated by invasive-growth promoting signals. Also, competition between Fus3 and Kss1 for their activator, Ste7, and/or for the Ste5 scaffold protein may prevent full activation of Kss1 by pheromone, so that FREs are not hyperinduced in response to pheromone (25, 48). In diploids, which display robust filamentation, genes required for mating (including *FUS3* and *STE5*) are not expressed, and Ste12 expression is reduced (10, 19).

The difference in sensitivity between FREs and PREs to MAPK-imposed and Dig-dependent repression presumably lies in the architecture and stability of the nucleoprotein structures (49) formed around Ste12-Tec1 heterooligomers and around Ste12–Ste12 homooligomers, respectively. The interactions occurring in these structures may be extensive. It has been shown, for example, that the Dig proteins can associate with themselves, with Ste12, and with the MAPKs (12, 13). Hence, for instance, the Ste12–Dig complexes bound at the multiple PREs found in the *FUS1* promoter (50) may assemble into a higher-order structure that is more stable in the absence of the MAPKs than the complex formed at the single Ste12-binding site in the Ty1 FRE (Fig. 5).

Many of the hallmarks of the transcriptional controls manifest in the mating and invasive growth responses of yeast are also displayed in the responses of animal cells to growth factors and differentiation inducers, including repression and combinatorial regulation, threshold responses to a graded signal, and discrimination between distinct signals transduced by overlapping components (5, 6, 43, 51, 52, 53). Hopefully, molecular mechanisms for these processes revealed by study of experimentally accessible model organisms, such as yeast, will continue to be generally applicable.

We thank Gerald Fink, Stephen Johnston, and especially Doreen Ma and Iain Cheeseman for generous gifts of research materials. This work was supported by Special Fellow Award 3754-98 from the Leukemia Society of America (to L.B.), by National Institutes of Health–National Cancer Institute Postdoctoral Traineeship CA09041 (to J.G.C.), by National Institutes of Health–National Research Service Award Postdoctoral Fellowship GM19474 (to J.X.Z.-S.), by National Institutes of Health Research Grant GM21841 (to J.T.), and by resources provided by the Berkeley campus Cancer Research Laboratory.

1. Cobb, M. H., Boulton, T. G. & Robbins, D. J. (1991) *Cell Regul.* **2,** 965–978.
2. Treisman, R. (1996) *Curr. Opin. Cell Biol.* **8,** 205–215.
3. Wasylyk, B., Hagman, J. & Gutierrez-Hartmann, A. (1998) *Trends Biochem. Sci.* **23,** 213–216.
4. Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., III, Zipursky, S. L. & Hafen, E. (1994) *Cell* **76,** 875–888.
5. Kumar, J. & Moses, K. (1997) *Genes Dev.* **11,** 2023–2028.
6. Marshall, C. J. (1995) *Cell* **80,** 179–185.
7. Sale, E. M., Atkinson, P. G. P. & Sale, G. J. (1995) *EMBO J.* **14,** 674–684.
8. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W. & Stork, P. J. (1998) *Nature (London)* **392,** 622–626.
9. Roberts, R. L. & Fink, G. R. (1994) *Genes Dev.* **8,** 2974–2985.
10. Liu, H., Styles, C. A. & Fink, G. R. (1993) *Science* **262,** 1741–1744.
11. Pi, H., Chien, C. & Fields, S. (1997) *Mol. Cell. Biol.* **17,** 6410–6418.
12. Cook, J. G., Bardwell, L., Kron, S. J. & Thorner, J. (1996) *Genes Dev.* **10,** 2831–2848.
13. Tedford, K., Kim, S., Sa, D., Stevens, K. & Tyers, M. (1997) *Curr. Biol.* **7,** 228–238.
14. Bardwell, L., Cook, J. G., Inouye, C. J. & Thorner, J. (1994) *Dev. Biol.* **166,** 363–379.
15. Kron, S. J., Styles, C. A. & Fink, G. R. (1994) *Mol. Biol. Cell* **5,** 1003–1022.
16. Madhani, H. D. & Fink, G. R. (1997) *Science* **275,** 1314–1317.
17. Mösch, H.-U., Roberts, R. L. & Fink, G. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5352–5356.
18. Yuan, Y. O. & Fields, S. (1991) *Mol. Cell. Biol.* **11,** 5910–5918.
19. Dolan, J. W. & Fields, S. (1991) *Biochim. Biophys. Acta* **1088,** 155–169.
20. Yuan, Y. O., Stroke, I. L. & Fields, S. (1993) *Genes Dev.* **7,** 1584–1597.
21. Baur, M., Esch, R. & Errede, B. (1997) *Mol. Cell. Biol.* **17,** 4330–4337.
22. Elion, E. A., Brill, J. A. & Fink, G. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9392–9396.
23. Ma, D., Cook, J. G. & Thorner, J. (1995) *Mol. Biol. Cell* **6,** 889–909.
24. Hung, W., Olson, K., Breitkreutz, A. & Sadowski, I. (1997) *Eur. J. Biochem.* **245,** 241–251.
25. Madhani, H. D., Styles, C. A. & Fink, G. R. (1997) *Cell* **91,** 673–684.
26. Cook, J. G., Bardwell, L. & Thorner, J. (1997) *Nature (London)* **390,** 85–88.
27. Bardwell, L., Cook, J. G., Voora, D., Baggott, D. M., Martinez, A. R. & Thorner, J. (1998) *Genes Dev.* **12,** 2887–2898.
28. Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R. & Thorner, J. (1996) *Mol. Cell. Biol.* **16,** 3637–3650.
29. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122,** 19–27.
30. Rothstein, R. (1991) *Methods Enzymol.* **194,** 281–301.
31. Herskowitz, I. & Jensen, R. E. (1991) *Methods Enzymol.* **194,** 132–146.
32. Davis, J. L., Kunisawa, R. & Thorner, J. (1992) *Mol. Cell. Biol.* **12,** 1879–1892.
33. Guarante, L. & Ptashne, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2199–2203.
34. Jones, J. S. & Prakash, L. (1990) *Yeast* **6,** 363–366.
35. Gietz, R. D. & Sugino, A. (1988) *Gene* **74,** 527–534.
36. Cook, J. G. (1996) Ph.D. thesis (Univ. of California, Berkeley).
37. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992) *Cell* **68,** 1077–1090.
38. Lambrechts, M. G., Bauer, F. F., Marmur, J. & Pretorius, I. S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8419–8424.
39. Liu, H., Styles, C. A. & Fink, G. R. (1996) *Genetics* **144,** 967–978.
40. Lo, W. & Dranginis, A. (1998) *Mol. Biol. Cell* **9,** 161–171.
41. McCusker, J. H., Clemons, K. V., Stevens, D. A. & Davis, R. W. (1994) *Infect. Immun.* **62,** 5447–5455.
42. Johnson, A. D. (1995) *Cell* **81,** 655–658.
43. Gray, S. & Levine, M. (1996) *Curr. Opin. Cell Biol.* **8,** 358–364.
44. Lo, H. J., Köhler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997) *Cell* **90,** 939–949.
45. Köhler, J. R. & Fink, G. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13223–13228.
46. Leberer, E., Harcus, D., Broadbent, I. A., Clark, K. L., Dignard, D., Ziegelbauer, K., Schmidt, A., Gow, N. A. R., Brown, A. J. P. & Thomas, D. Y. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13217–13222.
47. Csank, C., Schroppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D. Y. & Whiteway, M. (1998) *Infect. Immun.* **66,** 2713–2721.
48. Madhani, H. D. & Fink, G. R. (1998) *Trends Genet.* **14,** 151–155.
49. Echols, H. (1986) *Science* **233,** 1050–1056.
50. Hagen, D. C., McCaffrey, G. & Sprague, G. F., Jr. (1991) *Mol. Cell. Biol.* **11,** 2952–2961.
51. Ferrell, J. E., Jr. (1996) *Trends Biochem. Sci.* **21,** 460–466.
52. Hill, C. S. & Treisman, R. (1995) *Cell* **80,** 199–211.
53. Rusch, J. & Levine, M. (1996) *Curr. Opin. Genet. Dev.* **6,** 416–423.