Cellular Differentiation in Response to Nutrient Availability: The Repressor of Meiosis, Rme1p, Positively Regulates Invasive Growth in *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the transition from a nutrient-rich to a nutrient-limited growth medium typically leads to the implementation of a cellular adaptation program that results in invasive growth and/or the formation of pseudohyphae. Complete depletion of essential nutrients, on the other hand, leads either to entry into a nonbudding, metabolically quiescent state referred to as G_0 in haploid strains or to meiosis and sporulation in diploids. Entry into meiosis is repressed by the transcriptional regulator Rme1p, a zinc-finger-containing DNA-binding protein. In this article, we show that Rme1p positively regulates invasive growth and starch metabolism in both haploid and diploid strains by directly modifying the transcription of the *FLO11* (also known as *MUC1*) and *STA2* genes, which encode a cell wall-associated protein essential for invasive growth and a starch-degrading glucoamylase, respectively. Genetic evidence suggests that Rme1p functions independently of identified signaling modules that regulate invasive growth and of other transcription factors that regulate *FLO11* and that the activation of *FLO11* is dependent on the presence of a promoter sequence that shows significant homology to identified Rme1p response elements (RREs). The data suggest that Rme1p functions as a central switch between different cellular differentiation pathways.

In many unicellular organisms, nutrient-rich environments support the rapid growth and multiplication of single cells, leading to an exponential increase in cell numbers. When essential nutrients become limiting or cannot be efficiently utilized, growth rate is reduced, and organisms use specific strategies to adapt to the changed environment. In some nonmotile species, in particular in numerous species of yeast, including *Saccharomyces cerevisiae* and *Candida albicans*, reduced availability of nitrogen and carbon sources may initiate a morphological differentiation process that is characterized by a dimorphic switch from an ovoid to an elongated cell shape. Cells stay attached to each other after budding, forming hyphae-like structures in a process that is also referred to as pseudohyphal differentiation. Under the same conditions, cells may also grow invasively into the growth substrate, a phenotype referred to as "invasive growth" (Madhani and Fink 1998; Bauer and Pretorius 2001; Gancedo 2001; Gagiano *et al.* 2002). It has been suggested that these adaptations allow yeast cells to grow toward or into nutrient-rich environments (Gimeno *et al.* 1992).

While the shift from a rich to a limited supply of nutrients may lead to a change in growth patterns, a complete depletion of any of several essential nutrients may lead to a different set of adaptations. In haploid yeast, cells arrest in the G_1 phase of the cell cycle and enter a quiescent phase referred to as G_0. Diploid yeast strains, on the other hand, can initiate meiosis to form ascospores (Kron and Gow 1995). Meiosis is favored by the absence of nitrogen and, in addition, requires the absence of glucose and the presence of a nonfermentable carbon source.

Meiosis is a tightly regulated process and several transcriptional regulators play key roles in controlling the sequential expression of sets of genes (Vershon and Pierce 2000). Entry into meiosis is inhibited by Rme1p (*R*egulator of *me*iosis), a three-zinc-finger motif-containing DNA-binding protein (Covitz and Mitchell 1993), which can exert positive or negative effects on gene expression. Rme1p represses the transcription of the *IME1* gene, which is pivotal to the induction of early meiosis-specific genes (Kassir *et al.* 1988; Covitz and Mitchell 1993). The protein has been shown to directly bind to two binding sites, Rme1p response elements (RREs), within the *IME1* promoter (Covitz and Mitchell 1993; Shimizu *et al.* 1997). In addition to repressing *IME1*, Rme1p has been shown to positively regulate the *CLN2* gene (Toone *et al.* 1995; Frenz *et al.* 2001), which encodes a G_1 cyclin and controls cell cycle

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progression through the initializing phase of a new cell division cycle (Cross 1995). Thus, Rme1p appears to be able to promote mitosis by inducing *CLN2* transcription and to prevent meiosis by repressing *IME1* (Toone *et al.* 1995). It has been suggested that repression and activation by Rme1p are due to the exclusion of other factors from the promoter and that this exclusion can occur at large distances from the RRE (Shimizu *et al.* 1997, 1998; Blumental-Perry *et al.* 2002). Some evidence suggests that the Rme1p-dependent exclusion of transcription factors may be linked to chromatin condensation (Covitz *et al.* 1994). Additional data suggest that Rme1p interacts with the yeast Mediator complex, required for various aspects of transcriptional regulation, and in particular with the subunits Rgr1p and Sin4p (Blumental-Perry *et al.* 2002).

In haploid yeast, *RME1* is constitutively expressed at relatively high levels. In these cells, nutrient depletion leads to a further induction of *RME1* expression to ensure that haploids will not initiate meiosis under any circumstances (Shimizu *et al.* 1997). Compared to haploid strains, the expression of *RME1* is repressed 10- to 20-fold in diploid strains by the *MAT***a**/α heterodimeric repressor (Mitchell and Herskowitz 1986). However, expression in both haploid and diploid strains is cell cycle dependent, with an observed increase in expression at the M/G_1 boundary of the cell cycle (Frenz *et al.* 2001). The data suggest that Rme1p may contribute to some unknown cellular functions in diploid strains (Frenz *et al.* 2001).

Invasive and pseudohyphal growth are controlled by a network of signaling modules and transcription factors that respond to the limited availability of nutrients (Gagiano *et al.* 2002). Signaling modules include the nutrient-dependent mitogen-activated protein (MAP) kinase cascade (Liu *et al.* 1993; Mösch *et al.* 1996; Madhani *et al.* 1997) and the cAMP-protein kinase A (PKA) pathway (Ward *et al.* 1995; Robertson and Fink 1998; Pan and Heitman 1999). Some evidence also implicates G_1 cyclins in the regulation of this cellular adaptation (Oehlen and Cross 1998; Loeb *et al.* 1999). Deletions of *CLN1* and/or *CLN2* result in a decrease in invasive growth, with the deletion of *CLN2* leading to a less severe reduction.

All of the signaling pathways appear to converge on the promoter of the *FLO11* (also known as *MUC1*) gene, the expression of which is essential for invasive growth and pseudohyphal differentiation to occur (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Gagiano *et al.* 1999b; Rupp *et al.* 1999). *FLO11* encodes a glycosyl-phosphatidylinositol-anchored cell wall protein and is coregulated with the *STA2* gene, which encodes a starch-degrading glucoamylase (Gagiano *et al.* 1999a,b).

The promoters of *FLO11* and *STA2* are 97% identical and represent some of the largest promoters identified in *S. cerevisiae*. Indeed, sequences >2.5 kb upstream of the ATG translation start site have been shown to be required for proper regulation (Gagiano *et al.* 1999a; Rupp *et al.* 1999). The extensive size of the promoters appears to correlate with the complexity of the transcriptional control, since numerous regulators have been associated with *FLO11* and *STA2* expression. Transcription of *FLO11* and/or *STA2* has been shown to be negatively affected by the products of the *NRG1*, *NRG2* (Kuchin *et al.* 2002), *SFL1* (Robertson and Fink 1998; Pan and Heitman 2002), and *SOK2* (Ward *et al.* 1995; Pan and Heitman 2000) genes, while the *FLO8*, *MSN1*, *MSS11*, *PHD1*, *STE12*, and *TEC1* genes have all been shown to encode activating proteins (Gagiano *et al.* 1999a,b, 2003; Rupp *et al.* 1999; Pan and Heitman 2000; Köhler *et al.* 2002).

Here we show that *RME1* acts as a central switch between nutrient-induced cellular differentiation pathways. The data demonstrate that Rme1p activates invasive growth and starch degradation in haploid cells by inducing *FLO11* and *STA2*. We furthermore show that the promoter of *FLO11* contains a functional RRE and that mutations within this site render Rme1p incapable of exerting its effect. The activity of Rme1p appears independent of the identified signaling pathways that regulate invasive growth, including the cAMP-PKA pathway, the nutrient-sensing MAP kinase cascade, and the G_1 cyclins, as well as of other transcriptional regulators that affect *FLO11* and *STA2* transcription. The data therefore suggest the existence of an additional pathway that controls cellular adaptation to the nutritional status of the environment and that Rme1p may act as a central regulatory element of this pathway.

MATERIALS AND METHODS

Strains and culture composition: The yeast strains used in this study are listed in Table 1. Strains were cultivated at 30° using standard YPED medium prior to transformation or synthetic minimal medium lacking the appropriate amino acids for plasmid/knockout selection (Sherman *et al.* 1991). Yeast strains were transformed using the lithium acetate method according to Ausubel *et al.* (1994). The YPED medium was supplemented with 300 mg/liter geneticin (Sigma-Aldrich, St. Louis) for the selection of geneticin-resistant transformants. Media used for starch degradation, invasive growth, and β-galactosidase assays contained 2% starch (SCS), 3% glycerol and 3% ethanol (SCGE and SLAGE), 2% glucose (SCD and SLAD), or 0.1% glucose (SCLD X-gal) as carbon source. The SCS, SCGE, SCD, and SCLD X-gal media contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit), whereas SLAD and SLAGE media contained 50 μM ammonium sulfate as the sole nitrogen source and 0.17% YNB without ammonium sulfate and amino acids (Difco). The SCLD X-gal medium contained 40 mg/liter X-gal (Sigma-Aldrich) and was prepared according to Ausubel *et al.* (1994). Solid media contained 2% agar (Difco).

Plasmid DNA was amplified with *Escherichia coli* strain DH5α (GIBCO BRL/Life Technologies, Rockville, MD), which was cultivated in Luria-Bertani broth at 37°. Bacterial transformations and plasmid isolation were performed according to the procedures described by Sambrook *et al.* (1989).

Plasmid construction and recombinant DNA techniques: All

TABLE 1

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

Strain	Relevant genotype	Source or reference
ISP15	*MAT***a** *his3 leu2 thr1 trp1 ura3 STA2*	This laboratory
ISP15*cln1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 cln1*Δ::*HIS3*	This study
ISP15*cln2*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 cln2*Δ::*LEU2*	This study
ISP15*rme1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 rme1*Δ::*URA3*	This study
ISP15*flo11*Δ::*lacZ*	*MAT***a** *his3 leu2 thr1 trp1 STA2 flo11*Δ::*lacZ-HIS3*	This study
ISP15*flo11*Δ::*lacZrme1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 flo11*Δ::*lacZ-HIS3 rme1*Δ::*URA3*	This study
ISP15*flo11*Δ::*lacZ*RREmut	*MAT***a** *his3 leu2 thr1 trp1 STA2 flo11*Δ::*lacZ-HIS3*	This study
ISP15*sta2*Δ::*lacZ*	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3*	This study
ISP15*sta2*Δ::*lacZnrg1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3 nrg1*Δ::*kanMX4*	This study
ISP15*sta2*Δ::*lacZnrg2*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3 nrg2*Δ::*kanMX4*	This study
ISP15*sta2*Δ::*lacZrme1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3 rme1*Δ::*URA3*	This study
ISP15*sta2*Δ::*lacZsfl1*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3 sfl1*Δ::*kanMX4*	This study
ISP15*sta2*Δ::*lacZsok2*Δ	*MAT***a** *his3 leu2 thr1 trp1 STA2 sta2*Δ::*lacZ-HIS3 sok2*Δ::*kanMX4*	This study
L5366h	*MAT***a** *ura3*	Radcliffe *et al.* (1997)
L5624h	*ura3 ste20*Δ	Radcliffe *et al.* (1997)
L5625h	*ura3 ste11*Δ	Radcliffe *et al.* (1997)
L5626h	*ura3 ste7*Δ	Radcliffe *et al.* (1997)
L5627h	*ura3 ste12*Δ	Radcliffe *et al.* (1997)
YHUM271[a]	*MAT***a** *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG*	H.-U. Mösch
YHUM272[a]	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG*	H.-U. Mösch
Σ1278b*flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3*	This study
Σ1278b*flo11*Δ::*lacZ*RREmut	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3*	This study
Σ1278b*flo11*Δ::*lacZflo8*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 flo8*Δ::*LEU2*	This study
Σ1278b*flo11*Δ::*lacZgpa2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 gpa2*Δ::*LEU2*	This study
Σ1278b*flo11*Δ::*lacZmsn1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 msn1*Δ::*URA3*	This study
Σ1278b*flo11*Δ::*lacZmss11*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 mss11*Δ::*LEU2*	This study
Σ1278b*flo11*Δ::*lacZnrg1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 nrg1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZnrg2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 nrg2*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZphd1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 phd1*Δ::*LEU2*	This study
Σ1278b*flo11*Δ::*lacZras2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 ras2*Δ::*LEU2*	This study
Σ1278b*flo11*Δ::*lacZrme1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 rme1*Δ::*URA3*	This study
Σ1278b*flo11*Δ::*lacZsfl1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 sfl1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZsok2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 sok2*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZste12*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 ste12*Δ::*URA3*	This study
Σ1278b*flo11*Δ::*lacZtec1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 tec1*Δ::*LEU2*	This study
BY4742[b]	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	EUROSCARF
BY4742*nrg1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 nrg1*Δ::*kanMX4*	EUROSCARF
BY4742*nrg2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 nrg2*Δ::*kanMX4*	EUROSCARF
BY4742*sfl1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sfl1*Δ::*kanMX4*	EUROSCARF
BY4742*sok2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sok1*Δ::*kanMX4*	EUROSCARF
BY4742*rme1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 rme1*Δ::*kanMX4*	EUROSCARF
2NΣ1278*flo11*Δ::*lacZ*	*MAT***a**/α *ura3-52/URA3 trp1*Δ::*hisG/trp1*Δ::*hisG leu2*Δ::*hisG/ leu2*Δ::*hisG his3*Δ::*hisG/his3*Δ::*hisG flo11*Δ::*lacZ-HIS3/FLO11*	This study
2NΣ1278*flo11*Δ::*lacZrme1*Δ/ *rme1*Δ	*MAT***a**/α *ura3-52/ura3-52 trp1*Δ::*hisG/trp1*Δ::*hisG leu2*Δ::*hisG/ leu2*Δ::*hisG his3*Δ::*hisG/his3*Δ::*hisG flo11*Δ::*lacZ-HIS3/FLO11 rme1*Δ::*URA3/rme1*Δ::*kanMX4*	This study

EUROSCARF, European *Saccharomyces cerevisiae* Archive for Functional Analysis.
[a] YHUM271 (10560-4A) and YHUM272 (10560-6B) are both from the Σ1278b background.
[b] BY4742 is from the S288C genetic background (see Brachmann *et al.* 1998).

TABLE 2

Plasmids used in this study

Plasmid	Relevant genotype	Source or reference
YEp24	2μ *URA3*	Botstein *et al.* (1979)
YEpLac112	2μ *TRP1*	Gietz and Sugino (1988)
YEpLac181	2μ *LEU2*	Gietz and Sugino (1988)
YEpLac195	2μ *URA3*	Gietz and Sugino (1988)
YDp-L	*LEU2*	Berben *et al.* (1991)
YDp-U	*URA3*	Berben *et al.* (1991)
YDp-H	*HIS3*	Berben *et al.* (1991)
PJJ252	*LEU2*	Jones and Prakash (1990)
YEpLac112-FLO8	2μ *TRP1 FLO8*	Gagiano *et al.* (1999a)
YEpLac181-FLO8	2μ *LEU2 FLO8*	Gagiano *et al.* (1999a)
YEpLac112-MSN1	2μ *TRP1 MSN1*	Gagiano *et al.* (1999b)
YEpLac112-MSS11	2μ *TRP1 MSS11*	Gagiano *et al.* (1999b)
YEpLac112-PHD1	2μ *TRP1 PHD1*	This study
YEpLac112-RME1	2μ *TRP1 RME1*	This study
YEpLac181-RME1	2μ *LEU2 RME1*	This study
YEpLac195-RME1	2μ *URA3 RME1*	This study
YEpLac112-TEC1	2μ *TRP3 TEC1*	This study
YEp24-MSS12	2μ *URA3* genomic library fragment	This laboratory
YCpLac22-RAS2^{val19}	*CEN4 TRP1 RAS2^{val19}*	Gagiano *et al.* (1999b)
PSPORT1		Invitrogen Life Technologies
PSPORT-TEC1	2055-bp *TEC1* gene in pSPORT1	This study
pPMUC1-lacZ	*CEN4 URA3* P_{FLO11} fused to *lacZ*	Gagiano *et al.* (1999a)
pPSTA2-lacZ	*CEN4 URA3* P_{STA2} fused to *lacZ*	Gagiano *et al.* (1999a)
pGEM-T		Promega
pGEM-T-PMUC1-lacZ-HIS3	430 nucleotides of P_{FLO11} fused to *lacZ HIS3*	This study
pGEM-T-PSTA2-lacZ-HIS3	430 nucleotides of P_{STA2} fused to *lacZ HIS3*	This study
pΔcln1	*cln1*Δ::*HIS3*	B. Futcher
pΔcln2	*cln2*Δ::*LEU2*	B. Futcher
pΔflo8	*flo8*Δ::*URA3*	Gagiano *et al.* (1999a)
pΔgpa2	*gpa2*Δ::*LEU2*	This study
pΔmsn1	*msn1*Δ::*URA3*	Gagiano *et al.* (1999b)
pMSS11-Δ	*mss11*Δ::*LEU2*	Webber *et al.* (1997)
pΔphd1	*phd1*Δ::*LEU2*	This study
pΔras2	*ras2*Δ::*LEU2*	This study
pΔste12	*ste12*Δ::*URA3*	Gagiano *et al.* (1999b)
pΔtec1	*tec1*Δ::*LEU2*	This study

the plasmids, constructs, and primers used in this investigation are listed in Tables 2 and 3. *RME1* was isolated from a genomic library (plasmid YEp24-MSS12) as a 1622-bp *Hpa*I-*Sph*I fragment and was subcloned into the *Hpa*I-*Sph*I sites of the YEpLac plasmids (Gietz and Sugino 1988) to generate YEplac112-RME1, YEplac181-RME1, and YEplac195-RME1. To construct the disruption cassette pΔgpa2, a 1774-bp *Spe*I-*Nru*I fragment from pUC118-GPA2 (kindly provided by J. Winderickx), was replaced with the *Sma*I-*Nhe*I fragment containing the *LEU2* marker of pJJ252 (Jones and Prakash 1990). The episomal plasmid YEpLac112-PHD1 and the disruption cassette pΔphd1 were constructed by digesting a 2792-bp *PHD1* PCR product with *Bam*HI-*Hin*dIII and cloning the obtained fragment into the corresponding sites of YEplac112 and subsequently a 2214-bp *Xba*I-*Bgl*II fragment of the resulting YEplac112-PHD1 was replaced with *LEU2* (*Xba*I-*Bam*HI) of pJJ252. For the disruption of *RAS2*, pΔras2 was constructed by replacing the 428-bp *Bal*I-*Pst*I fragment of YCplac22-RAS2 (Gagiano *et al.* 1999b) with the *LEU2*-containing *Sma*I-*Pst*I fragment of YDp-L (Berben *et al.* 1991). YEplac112-TEC1 and pΔtec1 were constructed by cloning a PCR-amplified *TEC1* fragment, containing primer-generated *Eco*RI sites, into the corresponding *Eco*RI sites of YEplac112 and pSPORT1 (Invitrogen Life Technologies). The resulting pSPORT-TEC1 plasmid was digested with *Xba*I, blunt-ended, and redigested with *Nhe*I, to replace 975 bp of the *TEC1* open reading frame (ORF) with *LEU2* (*Sma*I-*Nhe*I) of YDp-L. The disruption constructs *cln1*Δ::*HIS3* and *cln2*Δ::*LEU2* were supplied by B. Futcher. An *rme1*Δ::*URA3* disruption cassette was generated with RME1-DISR-F and RME1-DISR-R. Both primers contain 48 nucleotides homologous to upstream and downstream sequences of the *RME1* ORF and 20 nucleotides homologous to flanking regions of the *URA3*-gene of YEp24 (Botstein *et al.* 1979). The construction of the additional disruption cassettes used in this study is described in Gagiano *et al.* (1999a,b).

Reporter cassettes were constructed to determine *FLO11* and *STA2* expression. P_{FLO11}-*lacZ* and P_{STA2}-*lacZ* were isolated from pPMUC1-lacZ and pPSTA2-lacZ (Gagiano *et al.* 1999a) as *Xba*I-*Nco*I fragments, with 461 nucleotides of the respective promoters fused to *lacZ*, and ligated to the *Spe*I-*Nco*I sites of pGEM-T (Promega, Madison, WI). The resulting constructs were digested with *Nco*I, blunt-ended, and ligated to the *HIS3*

TABLE 3

Primers used in this study

Name	Sequence
Fp-CLN1	5′-CCATAGCATGGAACTTGCCG-3′
Rp-CLN1	5′-CGGTCCCGTGAACACTTGAT-3′
Fp-CLN2	5′-CCTCCGCACTTTTACCCTGA-3′
Rp-CLN2	5′-TTCGCCGGTTGAGTGTATCG-3′
Fp-FLO8	5′-CTTTCCCACCCAATCTTAGGCACCT-3′
Rp-FLO8	5′-CCGGAACAAACCTTTAGCAATTGCG-3′
Fp-GPA2	5′-AGGCTAAGGAAACGGGTAAC-3′
Rp-GPA2	5′-TTGTCTCTTTCTTGGGTGGC-3′
Fp-MSN1	5′-CACCTACAAAGCGTTGATGG-3′
Rp-MSN1	5′-GTTGTTGGCTGACTTCTGAG-3′
Fp-MSS11	5′-GATGCCATAACCGACTAGAC-3′
Rp-MSS11	5′-ACAGGGCGCAATCAGCTACC-3′
Fp-NRG1	5′-CAGACGGGCACAGGGACCTA-3′
Rp-NRG1	5′-CTTGGCCGAGGATATGGCAC-3′
Fp-NRG2	5′-TAACACGTGGCTACACCGGC-3′
Rp-NRG2	5′-CTGAGTGGCGCACCGTACAC-3′
Fp-PHD1	5′-GGCCTATCCACGCCAATTTA-3′
Rp-PHD1	5′-TCGAGCTTTGAGCGCAGAGT-3′
Fp-RAS2	5′-AGTGGGTGGTGTGGCTAATC-3′
Rp-RAS2	5′-CATCGTCGTCTTCTTCCTCG-3′
Fp-RME1	5′-GTTTGGACAGGGATAGTGGGT-3′
Rp-RME1	5′-CGTGGTGCCATATTCACGACA-3′
Fp-SFL1	5′-CTCGGAATCGGCCAGCTTGG-3′
Rp-SFL1	5′-GCGATTGGGATGTTCACGGG-3′
Fp-SOK2	5′-GCTACGTCACCTTCGCAGCG-3′
Rp-SOK2	5′-GTGACGCCTACAGAGGGCTG-3′
Fp-STE12	5′-CACAGCATTTCTTTTCGGAG-3′
Rp-STE12	5′-AATCTCGCTTTTTCTGGTGG-3′
Fp-TEC1	5′-CCGGAATTCAAACAAGCTGAGCTGGACTCC-3′[a]
Rp-TEC1	5′-CCGGAATTCGCATGGCGCTAGAGAACTTTC-3′[a]
Fp-PFLO11$_{BstEII}$	5′-TCCGTTCTCTTCTGATGAGGTAACC-3′
Rp-PFLO11-lacZ-pGEM-T	5′-AATAACCCATGATATCTAGGCACATTAAGGTTAGCGTGGGGGGACGCGAATAATA TAAGCGCCAGGGTTTTCCCAGTCAC-3′[b]
Rp-PSTA2-lacZ-pGEM-T	5′-TGGCAACAAGTTGACACAGGATGAGAAAGTGAAAAGAACTGCAAACGTGGTTGGGC TGGAGCCAGGGTTTTCCCAGTCAC-3′[b]
RME1-DISR-F	5′-GTGTCAACGCATTGGAACTGACATTGTTCTTATCCTATAAGTCATACAGGCCTGACT GCGTTAGCAATT-3′[c]
RME1-DISR-R	5′-GAGTTTCATGGGGTACATTTTTAATGCCTCAACTATTTGGTATTGTTCCCGTGGAAT TCTCATGTTTG-3′[c]
Fp-PFLO11-RREmut	5′-GGTATGGAGTTTT**A**TA**TT**A**T**AAAACTTTAGGAATACCGGATTGTGTGCCT-3′[d]
Rp-FLO11 (+4.0 kb)	5′-GCGACTGCAGAACCAGAAGC-3′

[a] Underlined text represents *Eco*RI sites used for the cloning of *TEC1*.

[b] Underlined nucleotides are homologous to pGEM-T sequence located downstream of the *lacZ* gene (see MATERIALS AND METHODS); the remaining primer sequence is homologous to nucleotide stretches in the ORFs of either *FLO11* or *STA2*.

[c] Underlined nucleotides are homologous to areas flanking the *URA3* gene of YEp24; the remaining primer sequence is homologous to stretches immediately up- (DISR-F) or downstream (DISR-R) of the *RME1* ORF.

[d] Boldface type represents nucleotides used to mutate the RRE of P_{FLO11} (see MATERIALS AND METHODS).

gene (*Bam*HI digested and blunt-ended) from YDp-H (BERBEN *et al.* 1991). The integration cassettes were PCR amplified with Fp-PFLO11$_{BstEII}$, which binds ~430 bp upstream of *FLO11/STA2* ATGs, in combination with Rp-PFLO11-lacZ-pGEM-T and Rp-PSTA2-lacZ-pGEM-T, consisting of 60-nucleotide *FLO11*- and *STA2*-specific sequences and 20 nucleotides of pGEM-T situated immediately 3′ of the reporter cassettes.

Yeast strain construction: The wild-type yeast used to construct recombinant strains is from the ISP15 and Σ1278b genetic backgrounds. The laboratory strain, ISP15, carries the *STA2* gene, which encodes a glucoamylase (LAMBRECHTS *et al.* 1996; GAGIANO *et al.* 1999a,b, 2003). Expression of *STA2* allows growth on media containing starch as the sole carbon source. L5366h and YHUM272 are Σ1278b derivative strains and were kindly provided by P. Sudberry and H.-U. Mösch, respectively.

The PCR-amplified P_{FLO11}-*lacZ* and P_{STA2}-*lacZ* integration cassettes were transformed into ISP15 and Σ1278b (YHUM272) to generate ISP15*flo11*Δ::*lacZ*, ISP15*sta2*Δ::*lacZ*, and Σ1278b-*flo11*Δ::*lacZ*. Integration into the native loci of *FLO11* and *STA2* was confirmed through Southern blot analysis and subsequent sequencing. All additional gene disruptions were obtained

through the one-step gene replacement method (Ausubel *et al.* 1994) in wild-type ISP15 and YHUM272 and in the newly constructed *lacZ* reporter strains. The knockout cassettes for *NRG1*, *NRG2*, *RME1*, *SFL1*, and *SOK2* were obtained through PCR amplification of the corresponding disrupted genes of the mutants from the BY4742 (Brachmann *et al.* 1998) mutant collection supplied by European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF).

The diploid strain 2NΣ1278*flo11*Δ::*lacZ* is derived from a cross between the two Σ1278b derivatives Σ1278b*flo11*Δ::*lacZ* (YHUM272) and YHUM271 (kindly provided by H.-U. Mösch). The strain carries one functional *FLO11* allele, while the second allele is replaced with the *lacZ* gene under control of the native *FLO11* promoter. The *RME1* alleles of 2NΣ1278-*flo11*Δ::*lacZ* were deleted with the two cassettes, *rme1*Δ::*URA3* and *rme1*Δ::*kanMX4*, to generate the recombinant diploid 2NΣ1278*flo11*Δ::*lacZrme1*Δ/*rme1*Δ.

Site-directed mutagenesis: The genomic DNA of ISP15-*flo11*Δ::*lacZ* and Σ1278b*flo11*Δ::*lacZ* served as templates for the site-directed mutagenesis of the putative RRE. Primer Fp-PFLO11-RREmut (Table 3) was used to convert the GTACCA CAAAA nucleotide sequence to ATATTATAAAA. The subsequent PCR amplification of the RRE mutagenized P_{FLO11}-*lacZ-HIS3* cassettes was performed with primers Fp-PFLO11-RREmut and Rp-FLO11 (+4.0 kb). The mutated *lacZ* reporter cassettes were reintroduced into wild-type ISP15 and Σ1278b (YHUM272) to generate ISP15*flo11*Δ::*lacZ*RREmut and Σ1278b *flo11*Δ::*lacZ*RREmut. The desired nucleotide changes were confirmed through sequence analysis.

Invasive growth, starch utilization, and β-galactosidase assays: The invasive growth and starch utilization plate assays were performed as described previously by Gagiano *et al.* (1999a,b). Transformed strains for the β-galactosidase assays were allowed to grow for 5 days when 5 ml of SCD liquid medium was inoculated to serve as starter cultures. The precultures were grown overnight and 5 ml SCD medium was freshly inoculated to an optical density at 600 nm (OD_{600}) of ~0.05, while 5 ml SCGE medium was inoculated to an OD_{600} of ~0.15. To ensure that the cells were in the logarithmic growth phase, the SCD cultures were assayed at an OD_{600} of between 1.0 and 1.5. Due to the slow generation time observed for Σ1278b strains grown in SCGE medium, the cultures were incubated for 24 hr to ensure that an OD_{600} of at least 0.8 was reached before the cells were harvested and assayed. Three independent transformants were assayed and the differences in β-galactosidase values never exceeded 15%. β-Galactosidase activity is expressed as Miller units (Ausubel *et al.* 1994) and the data represent the average of three independent experiments.

RESULTS

***RME1* affects invasive growth and starch degradation:** *RME1* was isolated from a 2μ-based *S. cerevisiae* genomic library, which was transformed into the starch-degrading ISP15 strain. Transformants were screened for enhanced ability to grow on starch as sole carbon source, a phenotype that suggests increased expression of the *STA2* glucoamylase-encoding gene (Lambrechts *et al.* 1996). As can be seen in Figure 1A, multiple copies of *RME1* resulted in more efficient starch degradation on starch-containing media. Inversely, the deletion of *RME1* led to a decrease in starch utilization. Since starch degradation and invasive growth are coregulated phenotypes, we assessed whether the absence or the increased copy number of *RME1* would have a similar effect on invasive growth. Compared to the wild-type strain transformed with the 2μ-control plasmid, the 2μ-*RME1*-transformed strain invaded the agar more effectively, whereas the *rme1*Δ mutant exhibited a reduced invasiveness (Figure 1B). To assess whether these effects may be an indirect consequence of changes in growth rate, we assessed growth rate in various growth media and under growth conditions. No differences could be observed between the different strains (data not shown).

To verify that multiple *RME1* copies and deletion of *RME1* led to similar phenotypes in nonstarch-degrading strains, the effect of *RME1* on invasive growth was also assessed in the Σ1278b genetic background. This strain was chosen because it is most commonly used for the genetic analysis of pseudohyphal differentiation and invasive growth. The data confirm the observations made in the ISP15 genetic background: Multiple copies of *RME1* led to increased invasiveness, whereas the deletion resulted in a significant decrease in invasive growth (Figure 1C). Again, no growth defects could be observed for any of the strains (data not shown).

Since *FLO11* has a well-documented role in cell-substrate adhesion and invasion (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Pan and Heitman 2000), we decided to assess whether Rme1p requires Flo11p to enhance invasive growth. Figure 1D shows that 2μ-*RME1* was no longer able to induce invasive growth in a strain deleted for *FLO11*, even after a prolonged incubation period of 12 days.

***RME1* regulates the transcription of *FLO11* and *STA2*:** Since Rme1p acts as a transcriptional regulator, we assessed whether *RME1* copy number directly affects the transcription of *STA2* and *FLO11*. For this purpose, we replaced the chromosomal ORFs of these genes with the β-galactosidase-encoding *lacZ* gene. Figure 1, E and F, shows that the presence of 2μ-*RME1* leads to increased *lacZ* activity in the three strains, ISP15*sta2*Δ::*lacZ*, ISP15 *flo11*Δ::*lacZ*, and Σ1278b*flo11*Δ::*lacZ*. We also compared the effect of *RME1* in fermentable and nonfermentable carbon sources, since both *FLO11* and *STA2* are subjected to glucose repression. The expression levels conferred by the *FLO11* promoter in the ISP15 strain were always 7- to 10-fold lower than those conferred by the *STA2* promoter, and both genes showed lower expression in glucose (SCD) than in glycerol-ethanol (SCGE) medium (Figure 1, E and F), confirming previously published information (Gagiano *et al.* 1999b). The presence of 2μ-*RME1* induced both promoters, P_{FLO11} and P_{STA2}, 5- to 10-fold under both conditions. The deletion of *RME1*, on the other hand, decreased the expression levels of all reporter genes by ~30% in both strains. These data correlate well with the phenotypes observed on plates (Figure 1, A–C), as well as with the reported reduction of *CLN2* expression levels in an *RME1* deletion strain (Toone *et al.* 1995; Frenz *et al.* 2001). It should also be noted that low expression levels of *FLO11* in the

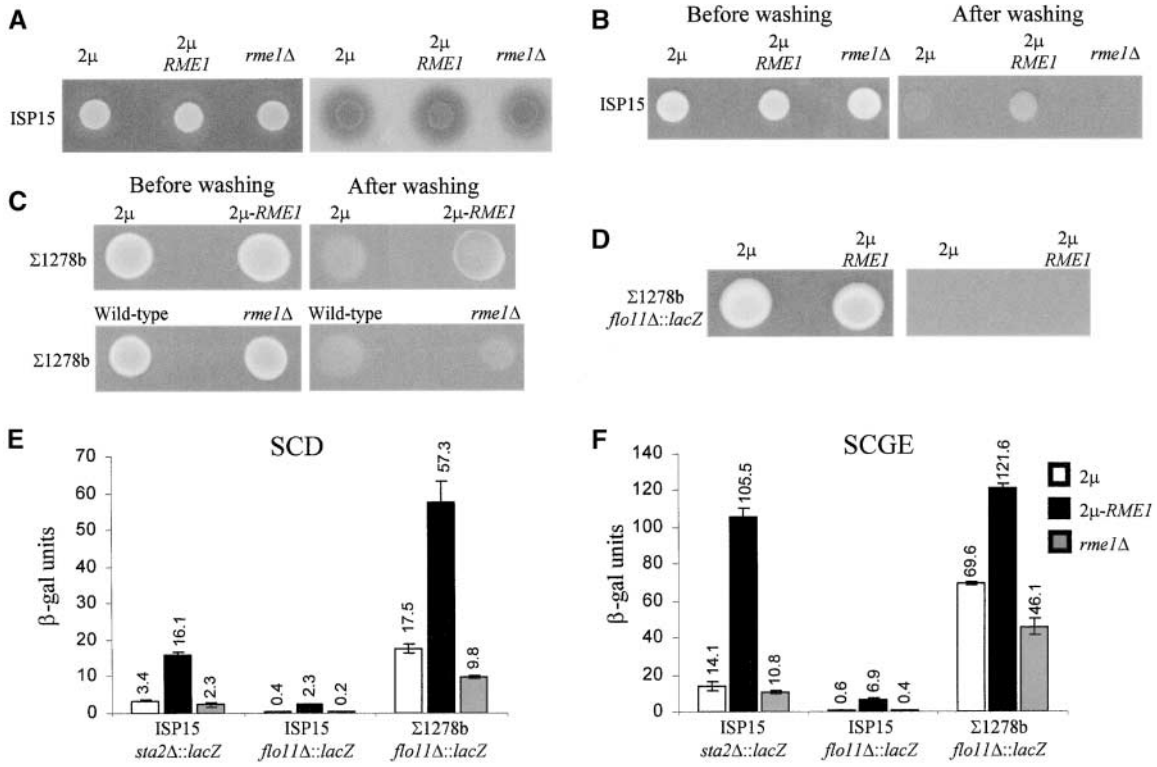

FIGURE 1.—*RME1* regulates starch degradation and invasive growth. (A) Starch degradation phenotypes of ISP15 wild-type strains transformed with YEplac112 (2μ), YEplac112-RME1 (2μ-*RME1*), and *rme1*Δ on starch-containing SCS medium. Multiple copies of *RME1* increase starch degradation, while the *rme1*Δ strain shows a reduction in phenotype. The halos surrounding the colonies reflect Sta2p glucoamylase activity. (B) The same strains as in A on SCD medium. (C) Invasive growth phenotypes of strains Σ1278b (YHUM272) transformed with YEplac112 and YEplac112-*RME1* and Σ1278b*rme1*Δ carrying YEplac112 on SCD medium. As for ISP15, the strain overexpressing *RME1* shows increased invasiveness, while the *rme1*Δ strain shows a significant reduction. (D) Induction of invasive growth by YEplac112-RME1 is blocked in *flo11*Δ strain (Σ1278b*flo11*Δ::*lacZ*) on SCD medium. (E and F) *RME1* regulates *STA2* (ISP15) and *FLO11* (ISP15 and Σ1278b) expression in SCD (E) and SCGE (F) liquid cultures. The genomic ORFs of *STA2* and *FLO11* were replaced with *lacZ* in the wild-type strains and the *RME1* deletions were created in the newly constructed reporter strains. β-Galactosidase activity is expressed in Miller units (AUSUBEL *et al.* 1994).

ISP15 strain make the interpretation of the effects of *RME1* deletion on *FLO11* expression rather difficult, although repeated experiments always yielded similar data.

The data clearly show that multiple copies and deletion of *RME1* result in similar phenotypes and transcriptional changes for both *STA2* and *FLO11*, independently of the genetic background of the strain and of the nature of the carbon source.

Rme1p acts independently of signaling modules that regulate invasive growth: We assessed whether the regulation of *FLO11* by *RME1* would be affected by the hyperactive allele of *RAS2* or the deletion of signaling modules that regulate invasive growth. For this purpose, the 2μ-*RME1* plasmid was transformed into strains with deletions or mutations in genes that affect cAMP-dependent signaling (*RAS2*^{val19}, *gpa2*Δ, *ras2*Δ) or the nutrient-regulated MAP kinase cascade (*ras2*Δ, *ste7*Δ, *ste11*Δ, *ste12*Δ, *ste20*Δ). The experiments were conducted in the haploid Σ1278b genetic background. The data presented in Table 4 show that the deletion of either *RAS2* or *GPA2* did not affect the ability of 2μ-*RME1* to induce P_{FLO11}-*lacZ* transcription. Both deletions (*gpa2*Δ and *ras2*Δ) resulted in a decrease in basal reporter gene-encoded activity in SCD and in SCGE, but the level of induction conferred by the 2μ-*RME1* plasmid was always comparable to, or slightly higher than, that observed in the wild type. The same was true in the reverse situation, when the effects of the hyperactive *RAS2*^{val19} mutation were assessed in both wild-type and *rme1*Δ genetic backgrounds. The increase in transcription was almost identical in both strains, *i.e.*, 7.8- and 8.3-fold in SCD (Figure 2) and 3-fold in SCGE (results not shown).

Similarly, multiple copies of *RME1* were able to activate invasive growth in the absence of elements of the invasive growth-regulating MAP kinase cascade (Figure 3). As reported previously (MÖSCH *et al.* 1996; GAGIANO *et al.* 1999b), deletion of the different *STE* genes resulted in reduced invasive growth, with the strains *ste20*Δ and *ste11*Δ showing the severest phenotypes. Multiple copies of *RME1* were able to restore the invasive growth phenotype in all mutants tested.

Rme1p induces invasive growth and starch degradation independently of Cln1p and Cln2p: We investigated

TABLE 4

Expression of P_{FLO11}-*lacZ* in Σ1278b mutant strains

	Mean β-galactosidase activity (Miller units ±SD)					
	Glucose repressed (SCD)			Nonfermentable (SCGE)		
Relevant genotype	2μ	2μ-*RME1*	Ratio	2μ	2μ-*RME1*	Ratio
Σ1278b*flo11*Δ::*lacZ*	17.2 ± 1.5	54.9 ± 6.9	3.2	63.0 ± 3.1	85.6 ± 2.8	1.4
*gpa2*Δ	5.8 ± 0.3	35.8 ± 2.9	6.2	38.3 ± 1.3	80.8 ± 5.6	2.1
*ras2*Δ	7.7 ± 0.0	51.2 ± 2.3	6.6	22.5 ± 3.4	58.0 ± 1.3	2.6
*flo8*Δ	1.2 ± 0.1	3.1 ± 0.1	2.7	1.7 ± 0.2	22.4 ± 1.9	13.5
*msn1*Δ	2.7 ± 0.3	15.6 ± 1.5	5.9	9.2 ± 0.6	50.0 ± 3.7	5.4
*mss11*Δ	1.4 ± 0.1	3.4 ± 0.2	2.5	1.1 ± 0.2	20.5 ± 1.8	18.7
*phd1*Δ	7.6 ± 0.7	29.3 ± 1.5	3.8	59.7 ± 4.7	81.9 ± 3.4	1.4
*ste12*Δ	3.1 ± 0.1	16.5 ± 1.1	5.4	38.8 ± 1.9	80.6 ± 1.6	2.1
*tec1*Δ	2.2 ± 0.1	13.4 ± 1.9	6.0	26.4 ± 1.1	64.2 ± 7.9	2.4

The listed mutants were generated in Σ1278b*flo11*Δ::*lacZ* in which the open reading frame of *FLO11* is replaced with the *lacZ*-gene. Strains were transformed with YEplac112 and YEpLac112-RME1 and were incubated for 5 days at 30° prior to inoculation into 5 ml SCD media lacking tryptophan. The overnight-grown SCD precultures were subsequently used for inoculation of 5 ml SCD (2% glucose) and SCGE (3% glycerol and ethanol) liquid media (see MATERIALS AND METHODS). Cells were harvested at OD_{600} ~1.0 and β-galactosidase assays were performed according to AUSUBEL *et al.* (1994). The average β-galactosidase activity for at least three transformants is presented and the ratio refers to 2μ-*RME1*-induced activity relative to the control plasmid in a given strain under the growth condition tested. The experiment was performed in triplicate.

whether the effect of Rme1p on *FLO11* was dependent on the presence of Cln1p or Cln2p, since

1. Rme1p is known to control *CLN2* expression (TOONE *et al.* 1995; FRENZ *et al.* 2001).
2. G_1 cyclins regulate invasive growth (LOEB *et al.* 1999; OEHLEN and CROSS 1998).
3. Deletion of *RME1* causes a 30% reduction in *FLO11* promoter activity (Figure 1E), similar to the reduction observed by TOONE *et al.* (1995) for *CLN2* mRNA in an *rme1*Δ strain.

For this purpose, we generated strains deleted for *CLN1*, *CLN2*, or both in the ISP15 genetic background. The *cln1*Δ*cln2*Δ double mutant showed clear growth defects and was excluded from the analysis. In accordance with the results of LOEB *et al.* (1999), the *cln1*Δ strain showed the severest defect in invasive growth, while the *cln2*Δ strain also displayed a clear reduction (Figure 4). The presence of 2μ-*RME1* in both the *cln1*Δ and the *cln2*Δ strains strongly enhanced the level of

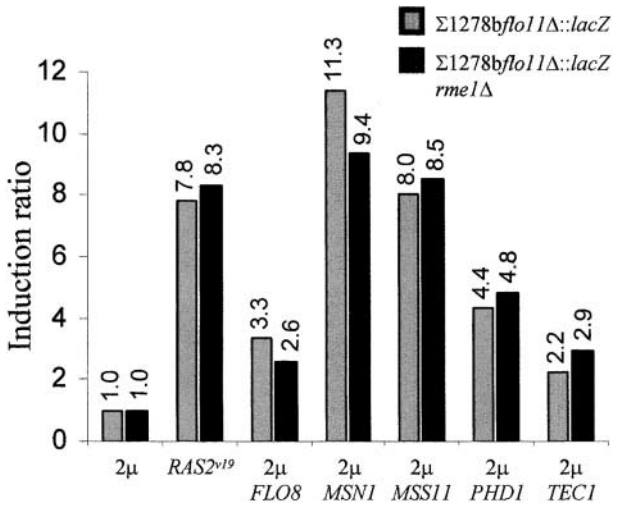

FIGURE 2.—*RME1* deletion does not affect the ability of other transcriptional activators to induce *FLO11*. Histogram representing the induction ratios obtained for Σ1278b*flo11*Δ::*lacZ* and Σ1278b*flo11*Δ::*lacZrme1*Δ transformed with YCplac22-RAS2^{val19} and YEplac112 without insert or with *FLO8*, *MSN1*, *MSS11*, *PHD1*, and *TEC1*. The absolute SCD β-galactosidase values were normalized to the YEplac112 control to obtain the induction ratios for every construct in each strain.

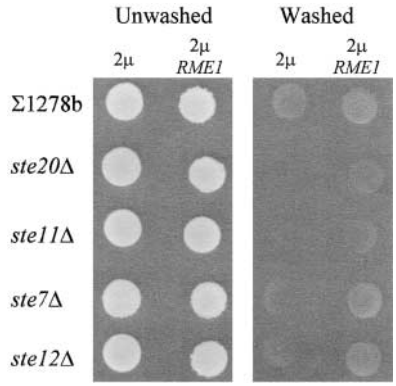

FIGURE 3.—Assessment of the effect of Rme1p in strains with MAPK gene deletions. L5366h (Σ1278b), L5624h (*ste20*Δ), L5625h (*ste11*Δ), L5626h (*ste7*Δ), and L5627h (*ste12*Δ) were transformed with YEplac195 and YEplac195-RME1 and grown on SLAD for 5 days at 30° before washing. *RME1* overexpression results in increased invasiveness in all cases.

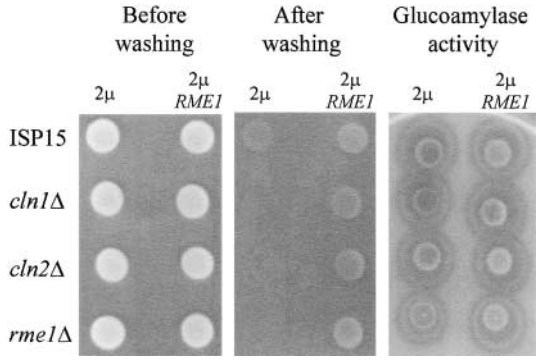

FIGURE 4.—Rme1p induces invasive growth and starch degradation independently of G_1 cyclins. Wild-type ISP15, *cln1*Δ, *cln2*Δ, and *rme1*Δ were transformed with YEplac112 and YEplac112-RME1 and grown on SLAD and SCS (Sta2p activity).

invasion. When tested on starch-containing SCS plates, the deletion of the cyclin genes did not lead to changes in starch degradation, and the presence of 2μ-*RME1* resulted in similar increases in the wild-type and the two cyclin-mutated strains.

Rme1p does not require other transcriptional regulators: Several transcription factors have been shown to activate *FLO11* expression (RUPP *et al.* 1999; GAGIANO *et al.* 1999a). To assess whether Rme1p would require the presence of these factors, we transformed the Σ1278b*flo11*Δ::*lacZ* strain and the isogenic mutants *flo8*Δ, *msn1*Δ, *mss11*Δ, *phd1*Δ, *ste12*Δ, and *tec1*Δ with 2μ-*RME1*. The effects on transcription of *FLO11* were assessed in both fermentable and nonfermentable carbon sources. Basal levels of *lacZ*-encoded β-galactosidase activity in the wild-type strain grown on glucose-containing medium (SCD) were severely affected by deletions of *FLO8*, *MSN1*, *MSS11*, *STE12*, and *TEC1* (Table 4), with expression levels being reduced at least 6-fold. As reported previously, the deletion of *PHD1* did not affect *FLO11* expression to the same extent, but resulted in a still significant reduction of 65% of reporter gene-encoded activity. On nonfermentable carbon sources, however, only deletions of *FLO8*, *MSN1*, and *MSS11* resulted in a similarly severe decrease in *lacZ* expression, suggesting that the presence of *STE12* and *TEC1* may not be required to the same extent under glucose-derepressed conditions. This corroborates data published by RUPP *et al.* (1999) that showed that the *FLO11* expression levels of *ste12*Δ and *tec1*Δ strains were close to wild-type levels in postdiauxic shift cultures, but significantly reduced during exponential growth on glucose. However, under both glucose-repressed and -derepressed conditions and in all the mutants, 2μ-*RME1* was able to increase β-galactosidase activity significantly. Interestingly, the deletion of the two genes that affect basal transcription levels most severely, *MSS11* and *FLO8*, also resulted in the lowest 2μ-*RME1*-dependent induction in SCD. However, in SCGE the induction ratios are the highest for *mss11*Δ (18.7-fold) and *flo8*Δ (13.5-fold), which is probably due to the very low basal *lacZ* transcription levels.

In the reverse situation, all 2μ plasmids carrying the genes of the different factors were able to activate transcription by the same induction factor in the wild-type and *rme1*Δ strains (Figure 2). In all cases, the expression data also correlated well with the invasive growth phenotype of each strain (data not shown).

We also assessed the effect of the deletions on the *STA2* reporter system in the ISP15 strain. An excellent correlation between starch degradation phenotypes and P_{STA2}-*lacZ* expression could be observed (data not shown). Furthermore, the ISP15 *STA2* data also correlate well with the *FLO11* data obtained in the Σ1278b*flo11*Δ::*lacZ* strain, again demonstrating the coregulated nature of the two genes and the validity of the data for different genetic backgrounds.

The effect of the deletion of genes that have been shown to negatively affect *FLO11* and/or *STA2* expression is presented in Table 5. Deletions of *NRG1*, *NRG2*, and *SOK2* result in a slight (*nrg1*Δ) to a 2- and 3-fold increase (*nrg2*Δ and *sok2*Δ, respectively) in P_{FLO11}-*lacZ* expression in SCD medium. The most significant effect is observed with the *sfl1*Δ strain, which shows a 25-fold increase in basal reporter gene activity. As observed for the transcriptional activators described above, none of the deletions appeared to affect the ability of 2μ-*RME1* to induce *lacZ* expression, although the level of induction in the *sfl1*Δ is reduced to 1.2- and 1.1-fold in SCD and SCGE, respectively. However, this may be due to the very high basal level of expression in this strain, which may not allow for further increases in expression levels.

The hypothesis that Rme1p acts independently of the repressor Sfl1p is supported by the data obtained for *STA2* expression in the ISP15 strain. In this case, the deletion of *SFL1* did not derepress the *STA2* gene to the same extent, and 2μ-*RME1* was able to induce transcription significantly by a factor of 4.4. Another important difference between the two strains can be observed in the response to the deletion of *NRG2*. Indeed, the deletion appears not to affect *STA2* expression significantly in ISP15, contrarily to the effect on *FLO11* expression observed in the Σ1278b*flo11*Δ::*lacZ* strain. Nrg2p also appears to mediate glucose repression, since the deletion of *NRG2* leads to a twofold increase in *lacZ* expression in SCD, but no induction can be observed in SCGE medium.

Rme1p induces *FLO11* expression via an Rme1p response element: Sequence analysis of P_{FLO11} and P_{STA2} revealed the presence of a putative RRE, GTACCACA AAA, at positions −1427 and −1314, respectively (Figure 5). The only difference between this sequence and the previously identified RREs in the promoters of *IME1* and *CLN2* is a T to A substitution in position 6 of the consensus sequence in P_{FLO11} and P_{STA2}. To assess the role

TABLE 5

Expression of P_{FLO11} and P_{STA2} in repressor mutants

	Mean β-galactosidase activity (Miller units ±SD)					
	Glucose repressed (SCD)			Nonfermentable (SCGE)		
Relevant genotype	2μ	2μ-*RME1*	Ratio	2μ	2μ-*RME1*	Ratio
Σ1278b*flo11*Δ::*lacZ*	16.2 ± 2.1	55.8 ± 2.8	3.5	68.4 ± 3.0	133.5 ± 3.5	2.0
*nrg1*Δ	22.5 ± 2.3	88.6 ± 3.9	3.9	97.3 ± 6.9	202.3 ± 6.5	2.1
*nrg2*Δ	39.3 ± 2.7	104.7 ± 7.6	2.7	57.1 ± 5.8	149.4 ± 11.1	2.6
*sfl1*Δ	405.4 ± 4.9	474.2 ± 9.8	1.2	226.9 ± 14.0	243.5 ± 12.6	1.1
*sok2*Δ	48.9 ± 5.1	210.6 ± 6.0	4.3	119.9 ± 9.5	176.7 ± 9.6	1.5
ISP15*sta2*Δ::*lacZ*	3.8 ± 1.2	18.1 ± 0.6	4.8	14.5 ± 3.0	110.5 ± 10.6	7.6
*nrg1*Δ	12.7 ± 1.3	45.1 ± 0.04	3.6	123.3 ± 11.1	243.3 ± 14.1	2.0
*nrg2*Δ	3.3 ± 0.6	16.7 ± 2.7	5.1	15.3 ± 1.5	101.4 ± 5.8	6.6
*sfl1*Δ	9.7 ± 0.1	42.6 ± 5.1	4.4	88.3 ± 8.3	222.9 ± 14.1	2.5
*sok2*Δ	48.0 ± 7.6	103.3 ± 10.7	2.2	77.7 ± 9.3	264.9 ± 11.7	3.4

of this putative RRE, we mutated the GTACCACAAAA nucleotide stretch to ATATTATAAAA in the *FLO11* promoters of ISP15*flo11*Δ::*lacZ* and Σ1278b*flo11*Δ::*lacZ*, since the guanine and cytosine nucleotides had been shown to be required for Rme1p-DNA interaction (Shimizu *et al.* 1998, 2001). Figure 6A shows that 2μ-*RME1* was no longer able to properly activate the P_{FLO11}-*lacZ* with the mutated RRE. In strain ISP15*flo11*Δ::*lacZ*, the 2μ-*RME1* plasmid resulted in the production of β-galactosidase, as indicated by the dark color of the colony, whereas the strain with the RRE mutation exhibited very little activity.

The values of β-galactosidase activity indicated a 30% reduction in activity of the *FLO11* promoter when the RRE sequence was mutated in both the ISP15 and the Σ1278b reporter strains (Figure 6, B and C). This reduction is similar to the reduction observed in the *RME1*-deleted Σ1278b strain (Figure 6C). The RRE mutations also significantly reduced the ability of 2μ-*RME1* to induce the reporter gene. However, transcriptional activation by multiple copies of *RME1* was not entirely abolished, since the 2μ-*RME1* plasmid still resulted in a twofold increase in β-galactosidase activity, compared to the eightfold increase observed in the wild-type ISP15 strain.

Promoters with Rme1p Response Elements	Sequences
P_{FLO11} (-1 427 to -1 417)	GTACCACAAAA
P_{STA2} (-1 314 to -1 304)	GTACCACAAAA
P_{IME1} (-2 040 to -2 030)	GTACCTCAAGA
P_{IME1} (-1 959 to -1 949)	GTACCTCAAAA
P_{CLN2} (-683 to -673)	GAACCTCAGTA
P_{CLN2} (-563 to -553)	GAACCTCAAAA
RRE consensus	GWACCWCARDA
Mutated P_{FLO11} RRE	ATATTATAAAA

Figure 5.—Rme1p response elements in the promoters of *FLO11*, *STA2*, *IME1*, and *CLN2*. W, A or T; R, A or G; D, A or G or T.

To further verify whether RREmut specifically affected *RME1*-dependent activation, Σ1278b reporter strains were transformed with 2μ-*FLO8* and 2μ-*RME1* plasmids. Figure 6C shows that the mutated promoter was fully activated by Flo8p, in terms of both absolute β-galactosidase units and induction ratio. Reporter gene-encoded activity increased 3.1- and 3.4-fold in the presence of 2μ-*FLO8* in wild-type and RRE-mutated strains, respectively, while the corresponding values for 2μ-*RME1* are 3 and 1.2. Similar data were obtained when multiple copies of *MSN1*, *MSS11*, *PHD1*, and *TEC1* were assessed in the RRE mutant strain (results not shown).

The very slight residual induction of *lacZ* activity by the 2μ-*RME1* plasmid in both the ISP15 and Σ1278b RREmut reporter strains (Figure 6, B and C) may suggest that the promoter of *FLO11* contains a second RRE. Both the promoters of *IME1* and *CLN2* contain two Rme1p response elements each (Shimizu *et al.* 1997, 2001; Frenz *et al.* 2001). However, no other sequence with significant homology to the identified RREs could be identified in the *FLO11* and *STA2* promoters.

Effects of Rme1p in diploid strains: *RME1* expression is strongly repressed in diploid cells (Mitchell and Herskowitz 1986). We nevertheless assessed whether *RME1* affected invasive growth similarly in diploid and haploid cells. The isogenic diploid strains 2NΣ1278*flo11*Δ::*lacZ* (2N) and 2NΣ1278*flo11*Δ::*lacZrme1*Δ/*rme1*Δ (2N*rme1*Δ) transformed with the 2μ-control and 2μ-*RME1* plasmids were tested for their ability to invade different growth substrates. No difference in invasive growth could be observed between the wild-type strain and the *RME1*-

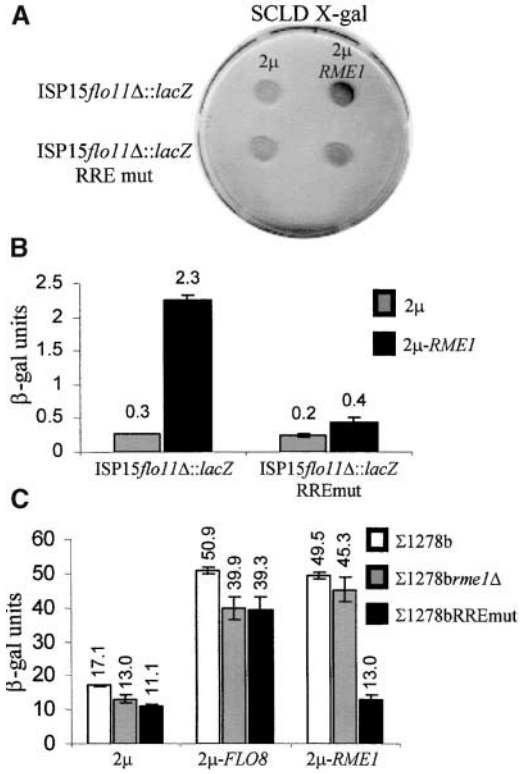

FIGURE 6.—Rme1p requires the P_{FLO11} RRE to induce *lacZ* expression. (A) ISP15*flo11*Δ::*lacZ* and ISP15*flo11*Δ::*lacZ*RREmut were transformed with YEplac195 and YEplac195-RME1 and grown on SCLD (0.1% glucose) supplemented with X-gal, for 12 days. The dark color of the colony formed by strain ISP15*flo11*Δ::*lacZ* transformed with YEplac195-RME1 is indicative of *lacZ* expression. The strain carrying the mutation in the putative RRE does not show a similar induction when transformed with the same plasmid. (B) β-Galactosidase activity of the ISP15 strains used in A measured after growth in SCD (see MATERIALS AND METHODS). (C) β-Galactosidase activity of strains Σ1278b*flo11*Δ::*lacZ*, Σ1278b*flo11*Δ::*lacZrme1*Δ, and Σ1278b*flo11*Δ::*lacZ*RREmut transformed with YEplac181, YEplac181-FLO8, and YEplac181-RME1 and grown in SCD as described in MATERIALS AND METHODS.

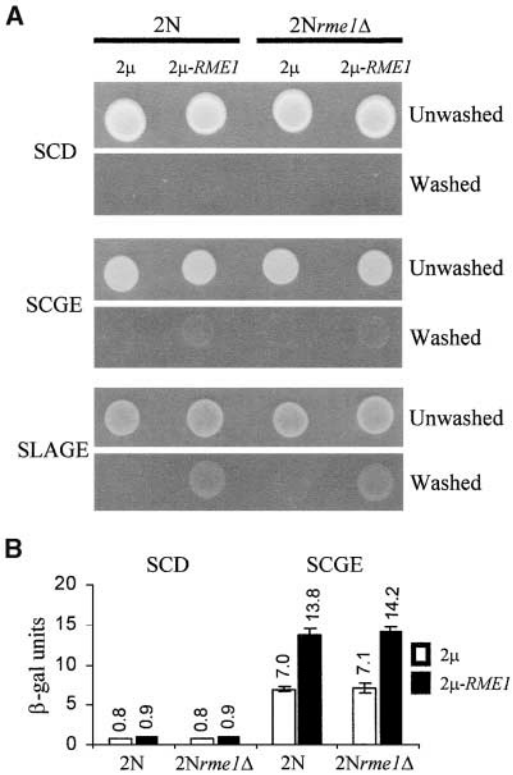

FIGURE 7.—Effect of *RME1* in diploid strains. (A) Strains 2N (*MAT***a**/α *URA3/ura3-52 flo11*Δ::*lacZ-HIS3/FLO11*) and 2N*rme1*Δ (*MAT***a**/α *flo11*Δ::*lacZ-HIS3/FLO11 rme1*Δ::*URA3/rme1*Δ::*kanMX4*) bearing either YEplac195 or YEplac195-RME1 were spotted onto SCD, SCGE, and SLAGE and allowed to grow for 5 days at 30° before washing. 2μ-*RME1* is able to induce invasion on nonfermentable carbon sources, but not on glucose-containing SCD. Deletion of *RME1* does not lead to a visible difference in invasive growth when compared to the wild type. (B) β-Galactosidase activity on SCD and SCGE media of the diploid strains 2NΣ1278*flo11*Δ::*lacZ* (2N) and 2NΣ1278*flo11*Δ::*lacZrme1*Δ/*rme1*Δ (2N*rme1*Δ) transformed with YEplac181 and YEplac181-RME1 (see Table 1 for relevant genotypes).

deleted strain (Figure 7A). In the presence of 2μ-*RME1*, the wild-type and *RME1*-mutant strains presented no observable phenotypes when grown on SCD medium. However, a significant increased invasiveness is exhibited when both strains were grown on nonfermentable carbon sources, with the strongest increase being observed on nitrogen-limited SLAGE medium. We also assessed whether *RME1* affected the formation of pseudohyphae in the diploid strains. The only significant difference was that elongated cells and pseudohyphae formation could be observed 48 hr after spotting on the SLAD medium in the 2μ-*RME1* transformed strain, whereas both the wild type and the disrupted strain required an additional 24 hr before elongated cells could be observed. However, total cell elongation as well as the final length of individual filaments appeared unaffected. The *rme1*Δ/*rme1*Δ strain formed pseudohyphae with an efficiency similar to that of wild type.

To quantify the effect of multiple copies of *RME1* on *FLO11* transcription in the diploid background, strains 2NΣ1278*flo11*Δ::*lacZ* and 2NΣ1278*flo11*Δ::*lacZrme1*Δ/*rme1*Δ, which both still contain one functional copy of *FLO11*, were also tested for β-galactosidase activity. The *lacZ* expression levels were the same for the diploid wild-type reporter and the *rme1*Δ/*rme1*Δ strains in both SCD and SCGE (Figure 7B). In the strains transformed with multiple copies of *RME1*, on the other hand, induction was dependent on the growth substrate, contrary to the situation in the haploid Σ1278b strain (Table 4). Indeed, the 2μ-*RME1*-transformed diploids showed virtually no *lacZ* induction when grown in SCD, while a twofold induction above wild-type level was observed when 2μ-*RME1*-transformed diploids were grown in SCGE medium. Rme1p therefore is able to induce *FLO11* expression in diploid strains in the presence of nonfermentable carbon sources such as glycerol and ethanol (Figure 7B) and

to increase invasive growth under conditions of nitrogen limitation (Figure 7A).

DISCUSSION

Rme1p controls nutrient-dependent cellular differentiation: Our data provide evidence that Rme1p acts as a genetic switch between nutrient-controlled growth forms of *S. cerevisiae* and, in particular, induces invasive growth while repressing meiosis in haploid cells:

1. Multiple copies of *RME1* significantly enhance *FLO11* and *STA2* transcription as well as the associated phenotypes invasive growth and starch degradation.
2. Deletion of *RME1* leads to a 30% reduction in the transcription of *FLO11* and *STA2*, which is also reflected in the associated phenotypes.
3. A specific sequence within the promoters of *FLO11* and *STA2* confers Rme1p responsiveness.
4. The mutation of this promoter element leads to a reduction in basal transcription levels similar to that resulting from the deletion of *RME1*.

Previously, the ability of Rme1p to activate *CLN2* expression, coupled with the cell cycle-dependent expression of *RME1*, has been taken as evidence for the involvement of this protein in the regulation of mitosis (Toone *et al.* 1995). Taken together, these and our data suggest that Rme1p plays a general role as a transcriptional regulator of genes that are central to the control of nutrient-dependent cellular growth forms, *i.e.,* unicellular mitotic growth, invasive and pseudohyphal growth, and spore formation.

Haploid *vs.* diploid strains: Our data clearly indicate that Rme1p enhances invasive growth in haploid strains by activating the expression of *FLO11*. In diploid strains, however, deletion of *RME1* did not reduce invasion or *FLO11* transcription under any of the conditions tested here. These data suggest that Rme1p may not be relevant for the regulation of invasion and pseudohyphal differentiation in diploids. However, multiple copies of *RME1* activated invasion and *FLO11* expression in diploids in a nutrient-dependent manner, requiring the absence of glucose and being enhanced by low levels of available nitrogen. These observations may indicate that Rme1p does play a role in the regulation of invasion in diploid cells, but that the specific conditions required to monitor these phenotypes may not have been tested here. Alternatively, the data may be explained by the fact that the **a**1α2 repressor in heterozygous *MAT***a**/*MAT*α diploid strains strongly represses *RME1* transcription (Mitchell and Herskowitz 1986). *FLO11* itself is also repressed by the same repressor, and it is therefore possible that the induction of *FLO11* observed in the diploid 2μ-*RME*-transformed strain may be due to **a**1α2 titration. However, this scenario would not explain the fact that induction in diploids appears to be dependent on specific growth conditions.

Conditions promoting sporulation in diploid strains and invasive growth in haploid strains are very similar, but for one essential difference: sporulation is favored by the complete depletion of nitrogen sources, whereas invasion requires that nitrogen sources be present, at least in limited amounts. Rme1p could therefore be specifically required to favor invasion and inhibit sporulation in haploids and diploids under conditions when the risk of wrongly activating the sporulation pathway is highest. This hypothesis is strengthened by data of Gasch *et al.* (2000), indicating that *RME1* expression is induced in response to nitrogen limitation.

Recent evidence shows that *RME1* is expressed in a cell cycle-dependent manner in both haploid and diploid cells, peaking in late M/G_1 of the mitotic cell cycle (Frenz *et al.* 2001). On the basis of these results, the authors suggested that Rme1p is linked to the promotion of cell cycle progression in both cell types and may be required for the proper regulation of alternative developmental pathways.

Rme1p regulates *FLO11* transcription via an RRE: Rme1p acts directly via an RRE sequence in the promoter of the *FLO11* gene. As in the case of the RREs in P_{IME1} (Covitz and Mitchell 1993) and P_{CLN2} (Toone *et al.* 1995), the *FLO11* and *STA2* RREs are situated far upstream of the ATG translation start codons, in positions −1427 and −1314, respectively. Mutations within the *FLO11* RRE significantly reduced, but did not completely eliminate, the ability of multiple copies of *RME1* to activate transcription. This might suggest the presence of a second RRE in the promoter of *FLO11*, resembling the situation in P_{CLN2} and P_{IME1}. However, careful scanning did not reveal the presence of a second consensus sequence in the 3.5-kb sequence of P_{FLO11} and P_{STA2}.

The RRE is situated in an area that was pinpointed as being essential for the regulation of *FLO11* by several groups (Rupp *et al.* 1999; Gagiano *et al.* 1999a; Pan and Heitman 2002). In particular, Pan and Heitman (2002) showed that Flo8p acts in close proximity to the identified RRE. Furthermore, Kobayashi *et al.* (1999) proposed that Flo8p might act via a sequence that contains the RRE in the promoters of *STA1* (*STA2* homolog) and *FLO11*. It therefore is highly significant that the mutations in the RRE did not affect the ability of Flo8p to activate *FLO11*, indicating that the presence or absence of Rme1p on the *FLO11* promoter does not affect Flo8p activity.

Rme1p acts independently of known signaling mechanisms and of transcriptional regulators of invasive growth: Rme1p acts independently of the invasive growth-regulating signaling pathways, the cAMP/PKA pathway, and the invasive growth-modulating MAPK pathway. It also does not require the G_1 cyclins. In fact, the deletion of *CLN1* or *CLN2* has no effect on the ability of Rme1p to induce invasive growth.

The data also show that other transcriptional regula-

tors of *FLO11* and *STA2* were not affected by Rme1p. Indeed, all factors investigated were still able to confer similar levels of induction or repression in an *rme1*Δ and in a wild-type strain when present on a multiple copy plasmid. Similarly, 2μ-*RME1* has led to increased *FLO11* expression in strains deleted for any of these factors.

Possible mechanism of Rme1p-dependent regulation of *FLO11*: It is unclear how Rme1p interacts with other elements that regulate invasive and pseudohyphal growth and which signal is responsible for this regulation. A possible link between *RME1* and invasive growth may be established through the further investigation of factors that regulate *RME1* transcription. For example, Swi5p has been shown to regulate *RME1* expression (Toone *et al.* 1995) and has recently also been implicated in the regulation of *FLO11* (Pan and Heitman 2000).

It has been suggested that Rme1p acts by excluding other factors from promoters (Covitz *et al.* 1994; Shimizu *et al.* 1997). Since this exclusion may occur at sites that are situated at significant distances from the RRE, it has been hypothesized that this effect may be chromatin dependent (Covitz *et al.* 1994). The activation of *FLO11* transcription by Rme1p therefore may be due to the exclusion of one or several transcriptional repressors. We investigated whether the effect of *RME1* is dependent on the exclusion of specific or general repressor proteins that regulate *FLO11* transcription, including Sok2p (Pan and Heitman 2000), Sfl1p (Robertson and Fink 1998; Conlan and Tzamarias 2001; Pan and Heitman 2002), Nrg1p, or Nrg2p (Kuchin *et al.* 2002). Our results show that transformants carrying 2μ-*RME1* resulted in elevated P_{FLO11}-*lacZ* and P_{STA2}-*lacZ* expression in strains deleted for any of these repressor genes. Similarly, strains lacking the functional activators Flo8p, Msn1p, Mss11p, Phd1p, Ste12p, and Tec1p also exhibited higher levels of reporter gene activity in the presence of 2μ-*RME1*.

A role for Rme1p in lifting general repression appears the most likely hypothesis and would also best fit other, previously described regulatory roles of the protein. In this regard the Tup1p-Ssn6p general corepressor complex (Conlan and Tzamarias 2001) is a possible candidate for Rme1p-related function. Although it was shown that Rme1p and the Tup1p-Ssn6p repressor complex act independently to repress *IME1* transcription (Mizuno *et al.* 1998), the possibility remains that these proteins interact functionally to regulate *FLO11* transcription, since Rme1p seems to play an activating rather than a repressive role in this context. Other potential proteins involved in Rme1p activity include components of RNA polymerase II holoenzyme, since Rgr1p and Sin4p have been shown to be required for *RME1*-dependent repression of *IME1* (Covitz *et al.* 1994; Blumental-Perry *et al.* 2002), and Sin4p has also been implicated in *FLO11* repression (Conlan and Tzamarias 2001).

Taken together, our data suggest that Rme1p controls cellular adaptation to the nutritional status of the environment and may act as the central regulatory element of a new, previously unidentified pathway. Other proteins, in particular Sok2p, have also been implicated in similar multiple regulatory roles, including repression of meiosis, activation of mitosis, and control of invasive and pseudohyphal differentiation (Shenhar and Kassir 2001). However, Sok2p acts as a repressor of invasive and pseudohyphal growth and, according to our data, does not appear to interact with Rme1p. Considering the previously published evidence regarding Rme1p, we suggest that an Rme1p-dependent pathway may act as a general cellular coordinator, rather than as a specific input/specific output mechanism, and may tilt the cellular machinery toward one or another differentiation status, according to cell type and environmental conditions.

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