

MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene

Steffen Rupp, Eric Summers, Hsiu-Jung Lo, Hiten Madhani and Gerald Fink¹

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA

¹Corresponding author
e-mail: fink@wi.mit.edu

In *Saccharomyces cerevisiae*, two major signal transduction pathways, the Kss1 MAPK pathway and the cAMP-regulated pathway, are critical for the differentiation of round yeast form cells to multicellular, invasive pseudohyphae. Here we report that these parallel pathways converge on the promoter of a gene, *FLO11*, which encodes a cell surface protein required for pseudohyphal formation. The *FLO11* promoter is unusually large, containing at least four upstream activation sequences (UASs) and nine repression elements which together span at least 2.8 kb. Several lines of evidence indicate that the MAPK and cAMP signals are received by distinct transcription factors and promoter elements. First, regulation via the MAPK pathway requires the transcription factors Ste12p/Tec1p, whereas cAMP-mediated activation requires a distinct factor, Flo8p. Secondly, mutations in either pathway block *FLO11* transcription. Overexpression of *STE12* can suppress the loss of *FLO8*, and overexpression of *FLO8* can suppress the loss of *STE12*. Finally, multiple distinct promoter regions of the *FLO11* promoter are required for its activation by either Flo8p or Ste12p/Tec1p. Thus, like the promoters of the key developmental genes, *HO* and *IME1*, the *FLO11* promoter is large and complex, endowing it with the ability to integrate multiple inputs.

Keywords: cAMP/development/filamentation/Flo11p/signal transduction

Introduction

On solid media containing high glucose and low nitrogen, diploid cells of *Saccharomyces cerevisiae* form pseudohyphae, which are comprised of chains of elongated cells that form invasive filaments (Gimeno *et al.*, 1992). On rich medium, haploid but not diploid cells manifest invasive growth (Roberts and Fink, 1994). The nutritional signals that result in both pseudohyphal growth and haploid invasive growth involve several pathways. One of these is a MAP kinase pathway required for both mating and filamentation (Liu *et al.*, 1993). This cascade involves three protein kinases, Ste20p, Ste11p and Ste7p, that act in sequence. Their activation results in the conversion of the Kss1p MAPK from an inhibitor of the transcription factor Ste12p/Tec1p into its activator (Liu *et al.*, 1993;

Madhani *et al.*, 1997). One of the genes regulated by the Kss1 MAPK cascade is *FLO11*, a cell wall protein required for invasive and filamentous growth (Lo and Dranginis, 1998).

A second signal transduction pathway involved in filamentous growth is the cAMP/PKA pathway. Increasing the level of cAMP by mutation of the high-affinity phosphodiesterase Pde2p, by activating mutations of Ras2p GTPase or by the exogenous application of cAMP, enhances filamentous growth (Gimeno *et al.*, 1992; Ward *et al.*, 1995; Lorenz and Heitman, 1997). Both Ras2p, a known regulator of cAMP levels, and the G protein α subunit homolog, Gpa2p, appear to act upstream of adenylylate cyclase. Deletion of either *RAS2* or *GPA2* results in reduced filamentation presumably because of reduced cAMP levels in the cell (Kubler *et al.*, 1997; Lorenz and Heitman, 1997). In several filamentous fungi, the cAMP/PKA pathway plays a role in the regulation of filamentation that has been connected to their virulence (Gold *et al.*, 1994; Xu and Hamer, 1996; Alspaugh *et al.*, 1997; Durrenberger *et al.*, 1998; Madhani and Fink, 1998).

Two genes, the transcription factor *FLO8* and the cell surface protein *FLO11*, have also been reported to be required for invasive and filamentous growth (Lambrechts *et al.*, 1996; Liu *et al.*, 1996; Lo and Dranginis, 1996, 1998). The *FLO* genes encode proteins required for cell–cell adhesion (Teunissen and Steensma, 1995). *FLO8* has been localized to the nucleus (Liu *et al.*, 1996) and was reported to be a putative transcriptional activator of *FLO1*, a dominant flocculation gene that encodes a cell wall-associated protein (Kobayashi *et al.*, 1996). *FLO11* is localized to the cell surface and appears to be required for cell–cell adhesion and the integrity of pseudohyphal filaments (Lo and Dranginis, 1996).

In this report, we show that *FLO11* is a target for both the MAP kinase and cAMP pathways. Our results suggest that *FLO8* is required for activating *FLO11* transcription via the cAMP/PKA pathway. Ste12p, a second transcription factor important for *FLO11* regulation (Lo and Dranginis, 1998), transmits the Kss1 MAPK signal to sites within the promoter of *FLO11* that are distinct from the *FLO8* target sites. In addition to these, there is a plethora of positive and negative *cis*-acting sites spread over at least 2.8 kb that define the *FLO11* promoter as one of the largest in the yeast genome, integrating MAPK, cAMP/PKA, mating type and nutritional signals.

Results

A strain dependent on cAMP for growth

Since both the Kss1 MAPK pathway and the cAMP/PKA pathway are activated for filamentation by the same activator, Ras2p (Toda *et al.*, 1987; Mosch *et al.*, 1996), analysis of the distinct role of cAMP on filamentous

growth requires the ability to activate the PKA branch independently of RAS. To achieve this goal, we constructed a strain (*ras1 ras2 pde2*) lacking both RAS genes (*RAS1* and *RAS2*) and *PDE2*, the gene for the high-affinity cAMP phosphodiesterase. Since Ras1p and Ras2p are required for the activation of adenylate cyclase, Cyr1p (Toda *et al.*, 1985), and *PDE2* encodes the phosphodiesterase required for cAMP hydrolysis, a *ras1 ras2 pde2* strain is impaired in the synthesis and breakdown of cAMP. Such a strain should be dependent upon exogenous cAMP for induction of the A kinase and, as no Ras-induced signal can be transmitted to the MAPK cascade, the effects of cAMP on filamentation should be independent of the RAS/MAPK signal.

The *ras1 ras2 pde2* strain (SR957) requires cAMP for growth on YPD (yeast extract, peptone, dextrose), but grows without cAMP on SC (synthetic complete), SLAD (synthetic low ammonia dextrose) and YNB (yeast nitrogen base) media, where it displays hyperaccumulation of glycogen, indicative of low cAMP levels. We presume that the ability of this triple mutant to grow without added cAMP, as has been observed by others (Nikawa *et al.*, 1987), results from basal cyclase activity that is sufficient to provide internal cAMP. The fact that the growth of our *ras1 ras2 pde2* strain depends upon a functional cyclase (*CYR1*) gene supports this explanation (data not shown).

A diploid *ras1 ras2 pde2* (SR959) strain grows on SLAD medium without cAMP but does not form pseudohyphae. However, on SLAD medium containing cAMP, the strain is extremely filamentous (Figure 1A). Moreover, the addition of cAMP leads not only to induction of filamentation, but also to invasion of the substrate (Figure 1B). In the presence of cAMP, the *ras1 ras2 pde2* strain (SR959) is invasive on all media tested (YPD, SC and SLAD; Figures 1A, B and 4C). Since invasive growth is usually observed with haploid strains on YPD, both the cell type signal and the nutritional signal can be bypassed by high cAMP levels in the cell.

To determine whether cAMP induces filamentation by activating the MAPK pathway, we measured the expression of the Kss1 MAPK pathway-specific reporter *FG::Ty1-lacZ* (Mosch *et al.*, 1996) in *ras1 ras2 pde2* strains grown on SLAD plates containing concentrations of cAMP that induce filamentation. The level of expression of the *FG::Ty1-lacZ* reporter in the *ras1 ras2 pde2* strain (SR959) is not altered by these cAMP levels (data not shown). Moreover, cAMP induces filamentation in a *ste12 ras1 ras2 pde2* deletion strain (SR1088) (Figure 4A). These results agree with those of Lorenz and Heitman (1997) and argue that the cAMP/PKA pathway acts in parallel with the MAPK pathway.

***FLO11* is induced by cAMP**

The essential role of *FLO11* in filamentation (Lo and Dranginis, 1998) suggested that it might be a downstream target of cAMP. It was possible to demonstrate the induction of *FLO11* mRNA by cAMP in the *ras1 ras2 pde2* strain (Figure 1D), but not in a wild-type background (Figure 3B). The *FLO11* transcript is undetectable when the triple mutant strain is grown without cAMP and is strongly induced in the presence of 2 mM cAMP. *FLO1*, which encodes another cell surface protein required for flocculation that is 26% identical to Flo11p, is only

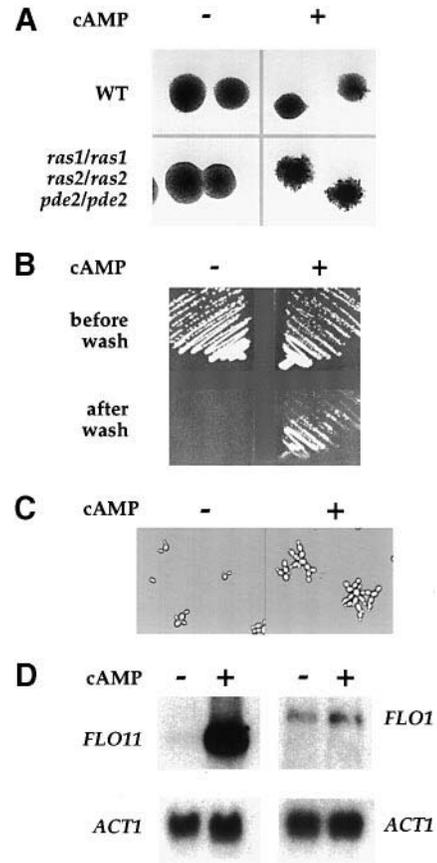


Fig. 1. cAMP is required for induction of invasive and filamentous growth. (A) Pseudohyphal development of strains on cAMP plates. Shown is the growth of strain SR607 (wild-type) and strain SR959 (*ras1/ras1, ras2/ras2, pde2/pde2*) on SLAD medium or SLAD medium containing 2 mM cAMP. Plates were incubated for 2 days at 30°C. (B) The upper half of the panel shows strain SR959 (*ras1/ras1, ras2/ras2, pde2/pde2*) streaked to a SC plate (left) and a SC plate containing 2 mM cAMP (right). Following incubation, the upper plates were washed under a stream of water and re-photographed to yield the lower plates. (C) Shown are cells of strain SR959 (*ras1/ras1, ras2/ras2, pde2/pde2*) grown in SC (left) or SC with 2 mM cAMP (right) to OD = 1. Cells grown with 2 mM cAMP show strong flocculation resulting in chains of cells, whereas cells grown without cAMP are mostly either single cells or cells with a single bud. (D) The Northern blots prepared from total RNA of cells shown in (C) were probed with *FLO11* probe or *FLO1* probe, stripped and subsequently re-hybridized with *ACT1* probe as normalization control.

moderately induced (1.4-fold) under the conditions used (Figure 1D). This result shows that the strong induction of *FLO11* is not a general feature of all flocculation genes. The correspondence between cAMP induction of *FLO11* and the morphological changes observed when cells are grown in the presence of cAMP is supported by the phenotype of the *FLO11* deletion: in the *flo11 ras1 ras2 pde2* strain (SR1121), cAMP fails to induce either invasion or filamentation (Figure 4). These data suggest that *FLO11* is a key target of a cAMP-dependent signaling pathway, one that is required for the induction of invasive and filamentous growth.

The enhanced *FLO11* transcription in the presence of cAMP is correlated with a change in cellular morphology. Cells grown in liquid SC with 2 mM cAMP show pseudohyphal-like chains of cells, whereas the majority of cells grown in liquid SC without cAMP are either

Table I. The 10 largest putative intergenic regions in the *S.cerevisiae* genome

Genes whose 5' end flanks the intergenic region	Size (bp)	Gene product information
<i>FLO11</i>^a–<i>MRS1</i>	3617	Cell surface protein–RNA splicing factor
<i>AAP1'</i> – <i>YHR048w</i>	3295	aminopeptidase–MFS transporter homolog
<i>YDL211c</i> – <i>tRNA-Gly</i>	3134	novel ORF–tRNA
<i>HO</i>	3082	mating type switching endonuclease
<i>GOG5</i>	2990	MFS transporter homolog
<i>YOR192c</i>	2860	MFS transporter homolog
<i>PSA1</i>	2816	GDP-mannose pyrophosphorylase
<i>YPR014c</i>	2763	novel ORF
<i>TIR2</i> – <i>YOR011w</i>	2727	cold-shock protein–ABC transporter homolog
<i>IME1</i>–<i>RPL43B</i>	2652	early meiotic gene activator–ribosomal protein L43B

Bold indicates developmental genes known to contain large complex promoter regions. Gene product information was obtained from the Yeast Protein Database (www.proteome.com).

^aThere exists an annotated 101 amino acid open reading frame (ORF), *YIR020c*, whose 5' end lies 882 bp upstream of the *FLO11* ORF. However, because this region lies in the promoter of *FLO11* defined experimentally in this study, it may correspond to a chance occurrence rather than to an expressed gene.

single cells or cells with a single bud (Figure 1C). The effect of cAMP on cell–cell attachment is much more pronounced than the effect of the cyclic nucleotide on cell elongation.

Analysis of the *FLO11* promoter

Analysis of the intergenic regions of yeast (Table I) suggests that *FLO11* has the longest 5' non-coding region in the yeast genome, a stretch of ~3.6 kbp upstream of the ATG that initiates the putative coding region. To determine how much of this 5' region of *FLO11* is required for the regulated expression of *FLO11*, we examined the behavior of plasmid-based *FLO11::lacZ* reporter constructs containing deletions in this non-coding region. Fourteen serial 200 bp deletions were constructed that span the region between the 2800 bp upstream of the *FLO11* initiation codon. Expression of the deletions was assayed in haploid and diploid strains. Since *FLO11* expression varies with the growth phase of the cells (Lo and Dranginis, 1998), we analyzed exponentially growing cells on SC, cells grown on SC until the glucose has been depleted (post-diauxic) and cells on SLAD, a medium that is high in glucose and low in nitrogen.

Enzymatic assays of the individual *flo11-lacZ* promoter deletions reveal an unusually long promoter with many sites. In subsequent sections, a site is tentatively assigned as a URS (upstream repression site) if its deletion leads to at least 3-fold enhanced expression of *FLO11*, and as a UAS (upstream activation site) if its deletion leads to at least 30% reduced expression.

Deletion analysis of promoter elements

Analysis of *cis*-acting elements by enzymatic assays of the individual *flo11-lacZ* promoter deletions reveals that the intact *FLO11* promoter is highly repressed. The deletions define at least nine URS elements (Table II; Figure 2A), whose activity depends on the state of growth, nutrient conditions and cell type. One of the URS elements is defined by *flo11-14* (–2600 to –2800 bp), showing that *cis*-acting elements are present at least 2.8 kb from the putative *FLO11* coding region (Table II; Figure 2A). In general, haploid strains show stronger repression than diploid strains.

A clear way to visualize the activity of these sites of repression is by comparison of the *lacZ* activity for each

of the conditions with that of a haploid grown on SLAD (Table II). This comparison reveals URS elements within *flo11-4*, *-5*, *-7*, *-8*, *-12*, *-13* and *flo11-14*. A subset of these elements is key to repression on all media, but the strongest effect is in haploids grown on SLAD. Haploid-specific effects are found for deletions *flo11-4*, *flo11-10*, *flo11-11*, *flo11-12* and *flo11-13*. Clearly, there are sites in *flo11-4*, *flo11-12* and *flo11-13* that function in the haploid-specific nitrogen regulation of *FLO11*.

There are other differences between haploid and diploid strains, the most notable of which are: (i) in diploids on SC (exp), *flo11-4* has a 2-fold reduced expression level, whereas in haploids it has a 33-fold elevated expression; and (ii) in diploids after the diauxic shift, *flo11-11* has a 3-fold reduced level whereas in haploids it has 12-fold elevated expression. In diploid cells, *flo11-4*, *flo11-10* and *flo11-11* act as UAS elements. *flo11-5* is a strongly nitrogen-regulated site in both haploids and diploids.

Deletion analysis also revealed sequence elements required for expression of *FLO11*. Under all conditions tested, *flo11-6* had a dramatic reduction in expression, suggesting that the sequence deleted in *flo11-6* (bp –1000 to –1200) contains a strong UAS. Furthermore, *flo11-1*, *flo11-2* and *flo11-3* show consistently lower activity, as compared with the wild-type *FLO11-lacZ* reporter construct, identifying these as UAS elements in the *FLO11* promoter. Recent studies of the TATA element in the *STA2* gene of *S.cerevisiae* var. *diastaticus*, a close homolog of *FLO11*, reported transcriptional initiation of *STA2* at –100 of the putative ATG (Vivier and Pretorius, 1998). The authors concluded by analogy that the same transcriptional initiation site is used in *FLO11*. The reduced expression of *flo11-1* is likely to be a consequence of the deletion of the transcription initiation site in this construct.

Several *flo11* promoter mutations were inserted in the chromosome at the *FLO11* locus by integrative transformation. One, *flo11-16*, deleted virtually the entire putative promoter region (from –150 to –2947), leaving the proposed TATA site intact (Vivier and Pretorius, 1998). Strains carrying either *flo11-16* or *flo11-6* are completely defective in haploid invasive growth and, as diploids (e.g. *flo11-6/flo11-6*), show severely reduced filamentation. The haploid invasion defect of these strains is as severe as that of a deletion of the *FLO11* coding region. *FLO11* mRNA levels in *flo11-16* and *flo11-6* are only 10% of the

Table II. Relative β -galactosidase activity of 14 *FLO11::LacZ* reporter constructs, each containing a different 200 bp deletion in the 3 kb segment in the 5' region upstream of the putative ATG that initiates translation

	<i>MATa</i> / α SC exp	<i>MATa</i> / α SC pd	<i>MATa</i> / α SLAD	<i>MATa</i> SC exp	<i>MATa</i> SC pd	<i>MATa</i> SLAD
<i>flo11-1</i>	0.3	0.2	0.3	0.3	0.1	0.4
<i>flo11-2</i>	0.4	0.3	0.2	0.7	0.4	0.3
<i>flo11-3</i>	0.6	0.3	0.4	0.5	0.3	0.4
<i>flo11-4</i>	0.6	0.4	1.1	33	5.1	9.6
<i>flo11-5</i>	2.4	0.5	18	4.1	1.1	8.2
<i>flo11-6</i>	0.4	0.3	0.2	0.2	0.3	0.1
<i>flo11-7</i>	15	2.7	4.5	32	4.0	30
<i>flo11-8</i>	7.0	4.0	4.5	17	2.0	5.7
<i>flo11-9</i>	0.8	0.5	0.8	1.4	0.8	2.9
<i>flo11-10</i>	1.0	0.6	0.7	4.3	0.6	1.9
<i>flo11-11</i>	0.6	0.3	0.5	2.5	12	2.0
<i>flo11-12</i>	1.6	1.2	1.3	1.7	0.9	7.1
<i>flo11-13</i>	1.0	0.6	0.7	1.1	0.9	5.6
<i>flo11-14</i>	9.2	5.8	5.9	1.5	3.4	35
<i>FLO11</i>	1.0	1.0	1.0	1.0	1.0	1.0
(units)	(5)	(25)	(17)	(6)	(111)	(240)

The numbers shown are the ratio of a particular construct relative to a *FLO11::LacZ* construct containing an intact 3 kb of upstream sequence (designated *FLO11* in the table). The actual units (nmol/min/mg) are provided in parentheses for each strain containing the intact *FLO11::LacZ* reporter construct. Ratios >3-fold are shown in bold. All assays were done in duplicate or triplicate, the overall deviation from the mean is 11%. Individual deviations do not exceed 19%. *MATa*, 10560-2B; *MATa*/ α , L5791; exp, exponential growth; pd, post-diauxic growth; SLAD, nitrogen starvation.

wild-type level. The results with *flo11-6* support our conclusions based on the data from the *lacZ* plasmid constructs, which identified the segment deleted in the *flo11-6* construct as a critical UAS for *FLO11* expression.

Strains containing integrated *flo11-3*, *flo11-4* and *flo11-14* also have phenotypes consistent with the data obtained from the corresponding *flo11-lacZ* reporter constructs: *flo11-3* has a strongly reduced haploid invasion defect, whereas *flo11-4* has a hyperinvasive phenotype. When the level of *FLO11* mRNA in these strains was compared with that in wild-type, we found that *flo11-3* was reduced by 80% and *flo11-4* was elevated 3-fold. Haploid *flo11-14* strains in exponential growth phase showed a 1.5-fold increase in *FLO11* mRNA, supporting the regulatory role of this distant promoter element.

Analysis of individual promoter fragments

In a second approach to identify UAS elements of the *FLO11* promoter, we designed 14 individual 400 bp sequence elements, overlapping by 200 bp, to test activation of a *lacZ* reporter (Guarente and Ptashne, 1981). This reporter construct series (Table III; Figure 2B) identified UAS elements in the segments *FLO11-2/1* and *FLO11-3/2* that overlap between bp -200 and -400, *FLO11-6/5* and *FLO11-7/6* that overlap between bp -1200 and -1000, and *FLO11-10/9* and *FLO11-11/10* that overlap between bp -2000 and -1800. These sequence elements show a >2-fold increase in β -galactosidase activity as compared with the reporter plasmid without any insert. The activity of *FLO11-2/1*, *FLO11-3/2* and *FLO11-11/10* is induced in post-diauxic cells, leading to an induction of up to 200-fold for *FLO11-11/10*. This result suggests that elements *FLO11-2*, *FLO11-3* and *FLO11-11* are required for enhanced *FLO11* expression in later stages of growth. Taken together with the deletion analysis, these data suggest that there are at least four UAS elements in the *FLO11* promoter. The *FLO11* promoter has extensive homologies to the promoter regions of the *STA1*, *STA2*

and *STA3* genes from *S.cerevisiae* var. *diastaticus*, where a complex regulation pattern, similar to that of *FLO11*, has been observed (Lambrechts *et al.*, 1994).

Trans-acting elements

Northern analysis revealed that yeast strains deleted for *FLO8* (L5816), *STE12* (L5795) and *TEC1* (L6149), transcription factors known to be required for pseudohyphal and invasive growth, show strongly reduced levels of *FLO11* transcription (Figure 3A). Previous work (Lo and Dranginis, 1998) suggested that *ste12* mutants reduce *FLO11* transcription. The effects of Ste12p on *FLO11* are a direct consequence of activation by the Kss1 MAPK pathway because strains containing *STE11-4*, a dominant-active allele of the MAPKKK, have a 30-fold greater amount of *FLO11* mRNA than wild-type strains (*STE11*). Moreover, deletion of *STE12* in a *STE11-4*-containing strain (L5577) leads to a reduction of *FLO11* transcript levels to only 30% of the wild-type level.

To identify the regions of the *FLO11* promoter that are targeted by these *trans*-acting elements, we transformed the 14 individual 400 bp sequence elements into strains deleted for *FLO8*, *STE12*, *TEC1* and for both *STE12* and *TEC1* (Table III; Figure 2B). Deletion of *FLO8* leads to a severe reduction in the expression of *FLO11-6/5* and *FLO11-7/6* in both exponential (8- and 4-fold reduction, respectively) and post-diauxic growth phase (5-fold for both elements). These elements are also induced by high cAMP levels. *FLO8* function is also required for maximum expression of *FLO11-3/2* and *FLO11-8/7* in the post-diauxic growth phase, but not in exponential growth, a result suggesting that Flo8p may function differently depending upon nutrient conditions.

Deletion of *STE12* has a stronger effect in exponentially growing cells than in post-diauxic cells. In exponentially growing cells lacking Ste12p, the *FLO11-6/5*, *FLO11-9/8*, *FLO11-10/9* and *FLO11-11/10* segments show at least a 3-fold reduction in expression. In post-diauxic

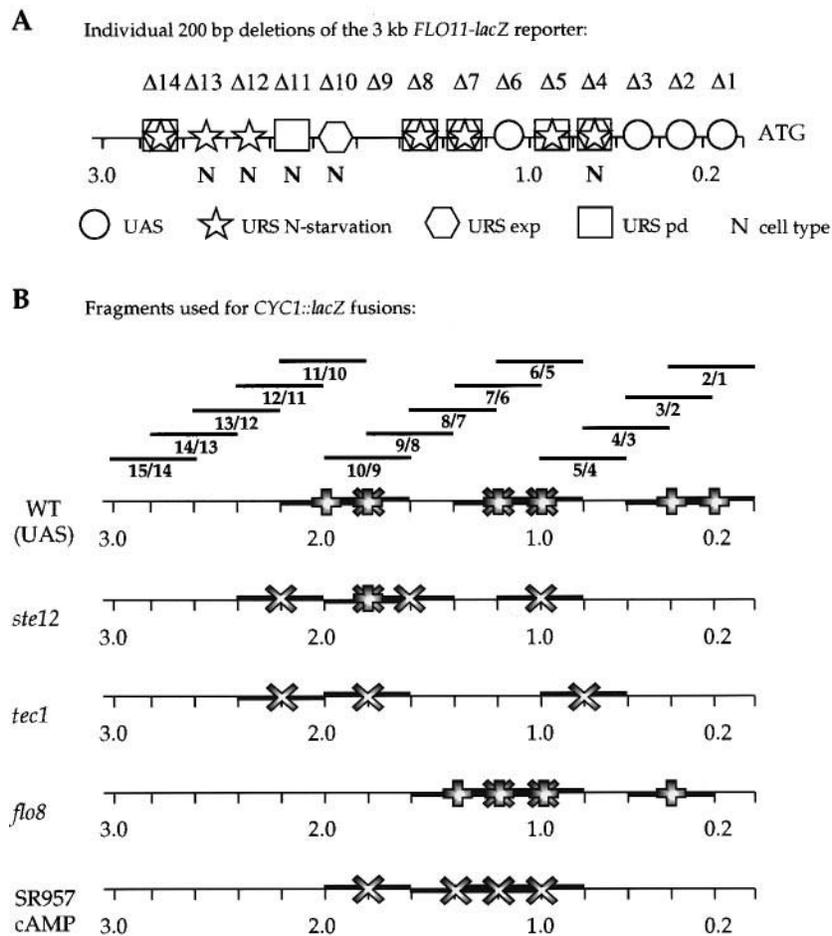


Fig. 2. Sequence elements involved in regulation of *FLO11*. **(A)** The β -galactosidase activities of 14 individual 200 bp deletions of the *FLO11* promoter region were compared with the full-length 3 kbp promoter in exponentially growing cells (rich medium), post-diauxic cells (pd) (glucose depletion) and nitrogen starvation conditions (N-starvation) (SLAD, see Table II). A ratio of β -galactosidase activity (deletion/3 kbp promoter) of 3 or higher led to that deletion's association with a URS (star, hexagon, square). At least 30% lower expression than the wild-type reporter under all conditions is represented as a UAS (circle). N refers to cell type specific regulation. **(B)** The β -galactosidase activity of 15 isolated 400 bp elements of the *FLO11* promoter region, that activate a *CYC1::lacZ* reporter, was determined in wild-type cells or in cells deleted for the transcription factors *FLO8*, *STE12*, *TEC1* or *STE12* and *TEC1* in exponentially growing cells or post-diauxic cells. The activity of these constructs was also tested in the presence or absence of cAMP in strain SR957 (see Table III). The symbols (\star = rich medium, \square = post-diauxic cells) are placed on a line in a position that indicates which of the 400 bp fragments stimulated β -galactosidase activity. Each line represents the *FLO11* promoter in a different genetic background. The first row [denoted WT (UAS)] denotes sequence elements showing a >2 -fold elevation of the reporter over a plasmid without an insert as measured in wild-type cells. The next three lines, *ste12*, *tec1* and *flo8*, represent sequence elements showing a >3 -fold reduction of the β -galactosidase activity in the mutant (e.g. *ste12*) as compared with the activity of the same element in wild-type cells. Elements that give enhanced expression in strain SR957 grown with cAMP as compared with the same strain grown without cAMP are indicated on the line in the last row (cAMP induced). Note that URS elements could mask the contribution of adjacent UAS elements (e.g. *FLO11-11/10* and *FLO11-10/9* provide evidence for a UAS in segment 10; however, the deletion analysis revealed no UAS activity for *flo11-10*).

cells, only *FLO11-10/9* shows reduced expression in a *STE12* deletion strain.

Deletion of *TEC1* has significant effects on expression of the *FLO11* insertion reporter series, but the reduction can be observed only in exponentially growing cells and is less severe than that observed for strains deleted for *STE12* or *FLO8*. This observation agrees with the Northern analysis (Figure 3A) of the intact *FLO11* promoter, where *FLO11* mRNA levels show less of a reduction in a strain deleted for *TEC1* (L6149) than in strains deleted for *STE12* (L5795) or *FLO8* (L5816). *FLO11-6/5*, *FLO11-10/9* and *FLO11-12/11* are dependent upon *TEC1* in exponentially growing cells. Deletion of *TEC1* in a Δ *ste12* strain shows a modest reduction in the expression of *FLO11-10/9* as compared with the single mutant strains. However, since deletion of *STE12* leads to a >10 -fold

stronger effect than deletion of *TEC1*, this result may indicate a Tec1p-independent role for Ste12p at this site.

Our results suggest that Flo8p and Ste12p/Tec1p act via multiple sites in the *FLO11* promoter that are largely non-overlapping. This becomes most evident in post-diauxic cells. The strongest effect of Flo8p and Ste12p in post-diauxic cells is observed with two distinct sequence elements of the *FLO11* promoter. Flo8p acts on the sequence elements *FLO11-3/2*, *FLO11-5/6*, *FLO11-6/7* and *FLO11-8/9*, whereas Ste12p acts on *FLO11-10/9*. The effect of Ste12p on element *FLO11-5/6* can be observed only in exponentially growing cells. *FLO11-12/11* and *FLO11-10/9* are targeted both by Tec1p and Ste12p. The existence of spatially distinct sites for different transcription factors provides strong evidence in support of the dual control over *FLO11* transcription.

Table III. β -Galactosidase assays of individual 400 bp *FLO11* promoter elements cloned into the UAS of a *CYC1::lacZ* reporter construct

	Units (nmol/min/mg)		Expression relative to wild-type exponential growth phase				Expression relative to wild-type post-diauxic growth phase				Induction by cAMP SR957 \pm cAMP
	WT exp growth	WT pd growth	WT/ <i>flo8</i>	WT/ <i>ste12</i>	WT/ <i>tec1</i>	WT/ <i>ste12 tec1</i>	WT/ <i>flo8</i>	WT/ <i>ste12</i>	WT/ <i>tec1</i>	WT/ <i>ste12 tec1</i>	
<i>FLO11</i>	6	20	5.5	3.2	3.5	5.3	5.6	1.4	1.8	2.7	1.9
no insert	7	7	0.5	2.0	1.4	1.6	1.2	1.2	1.0	1.3	1.0
2/1	11	16	1.0	0.9	1.4	1.2	0.9	1.0	0.9	1.0	0.4
3/2	10	30	2.1	2.5	1.7	3.5	7.2	1.4	0.8	2.1	0.5
4/3	2	4	0.6	1.6	1.3	1.5	1.8	0.8	0.8	0.7	0.8
5/4	2	3	0.9	2.5	3.4	2.3	1.8	1.1	1.0	0.9	0.9
6/5	75	41	8.2	3.9	2.5	5.1	4.5	1.1	1.0	1.9	3.0
7/6	46	48	3.6	0.8	1.3	1.2	4.9	0.6	0.6	1.4	1.7
8/7	1	4	1.6	1.3	0.8	1.4	6.8	1.1	1.1	1.1	1.7
9/8	7	9	0.6	4.5	2.0	4.3	1.2	1.3	1.1	1.8	0.8
10/9	82	37	1.8	57	3.5	76	2.6	6.2	1.3	7.3	1.8
11/10	2	432	0.8	0.1	0.1	0.2	2.1	1.2	0.8	1.0	0.3
12/11	14	10	0.7	15	6.3	13	1.2	1.6	0.6	1.0	1.0
13/12	1	2	0.6	1.6	1.2	1.5	1.0	0.6	0.7	0.6	0.4
14/13	1	2	1.0	2.0	2.0	1.3	1.2	0.7	1.0	0.7	0.6
15/14	1	2	0.8	2.5	2.8	1.4	1.5	0.8	1.0	0.8	0.3

All strains are *MATa*, except SR957 (*MATa/α*). The actual units (nmol/min/mg) are given for each sequence element as measured in the wild-type strain (10560-2B). The values for each mutant strain [*flo8* (L5816), *ste12* (L5795), *tec1* (L6149) and *ste12 tec1* (L6150)] are given relative to the respective wild-type data. The values with at least 3-fold repression due to deletion of one of the transcription factors in exponential and post-diauxic growth phase are in bold. Values for SR957 represent the ratio of the activities for the strains containing the sequence elements in the column on the left in the presence or absence of 2 mM cAMP, respectively.

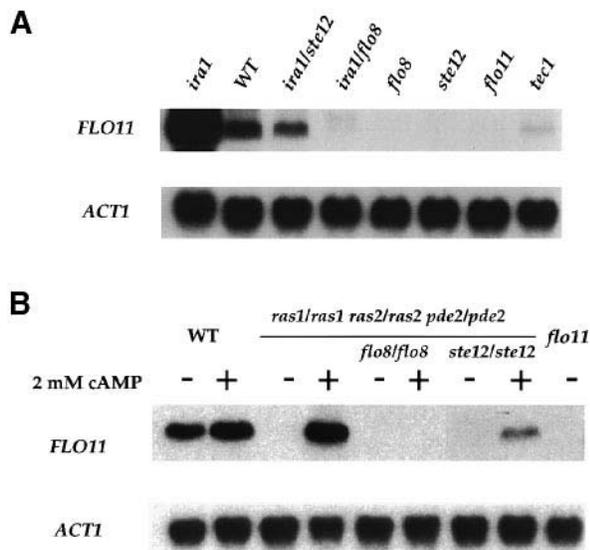


Fig. 3. *FLO11* transcription is induced by high intracellular or extracellular cAMP levels and requires Flo8p but not Ste12p for cAMP-mediated induction. The Northern blots were probed with *ACT1* as a normalization probe and a *FLO11* probe. (A) Total RNA was prepared from strains SR599 (*ira1*), 10560-2B (wild-type), SR1133 (*ira1 ste12*), SR1132 (*ira1 flo8*), L5795 (*ste12*), L5816 (*flo8*), L6149 (*tec1*) and SR1101 (*flo11*), grown in SC to OD = 1.0–1.2. (B) Total RNA was prepared from strains SR607 (wild-type), SR959 (*ras1/ras1, ras2/ras2, pde2/pde2*), SR1081 (*flo8/flo8, ras1/ras1, ras2/ras2, pde2/pde2*), SR1088 (*ste12/ste12, ras1/ras1, ras2/ras2, pde2/pde2*) and SR1101 (*flo11*), grown in SC with or without cAMP to OD = 1.0–1.2.

To locate the sites within the promoter where the cAMP signal activates *FLO11*-transcription, we transformed the 400 bp reporter series into SR957, a strain where internal cAMP levels can be manipulated by adding cAMP to the media. Addition of 2 mM cAMP to SR957 leads to a 3-fold induction of *FLO11*-6/5 and to a 2-fold induction of *FLO11*-7/6, *FLO11*-8/7 and *FLO11*-10/9 as compared

with SR957 grown without cAMP. These results suggest that *trans*-acting elements up-regulate *FLO11* expression through a cAMP-mediated signal via more than one *cis*-acting element. The segment most strongly induced by cAMP is defined by the overlapping elements *FLO11*-6/5 and *FLO11*-7/6 (within bp –1000 to –1200), the same element targeted by Flo8p. As shown earlier, this element is required for induction of invasive and filamentous growth.

***FLO8* is required for cAMP-mediated *FLO11* transcription**

FLO8, a gene essential for filamentous growth (Liu et al., 1996), is also required for the expression of *FLO11*. *FLO11* transcripts are not detectable in a strain (L5816, *flo8-2*) that contains a deletion of the *FLO8* gene (Figure 3A). Furthermore, *FLO11* induction by cAMP is blocked in a *ras1 ras2 pde2* strain (SR1081) carrying a deletion of *FLO8* (Figure 3B).

The *ste12 ras1 ras2 pde2* strain (SR1088), like the *flo8 ras1 ras2 pde2* (SR1081) strain, has dramatically reduced expression of *FLO11*. However, *FLO11* transcription can be induced by cAMP in the *ste12 ras1 ras2 pde2* strain (Figure 3B). The induction of *FLO11* by cAMP in the *ste12* mutant and not in the *flo8* mutant is consistent with the phenotypes of the corresponding strains: a *flo8 ras1 ras2 pde2* strain is unable to form filaments on SLAD plates or to invade the agar on YPD plates even in the presence of cAMP, whereas *ste12 ras1 ras2 pde2* is both invasive and able to form filaments on YPD or SLAD plates containing 2 mM cAMP (Figure 4). Thus, high cAMP levels can bypass the requirement for the MAPK cascade transcription factor Ste12p, but not the requirement for Flo8p in the activation of *FLO11* transcription.

The suppression patterns of *ste12* and *flo8* mutants by overexpression of *FLO8* and *STE12*, respectively, in an

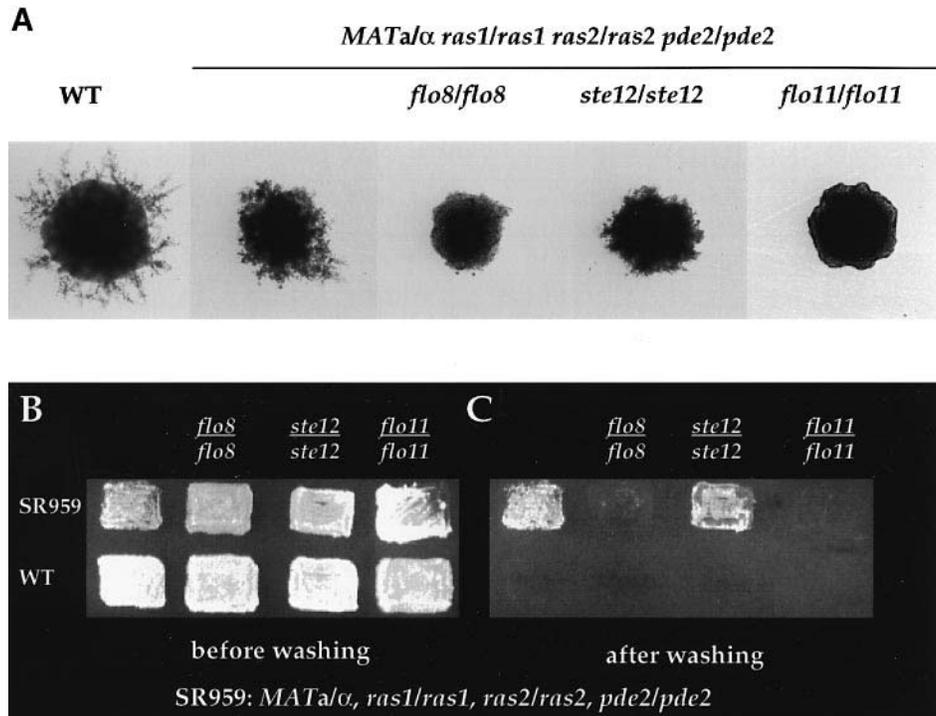


Fig. 4. *FLO8* is required for cAMP-mediated filamentous and invasive growth. (A) Filamentous growth of strains on SLAD plates containing 2 mM cAMP. All strains are diploid. The plate shows strains SR607 (wild-type) SR959 (*ras1/ras1, ras2/ras2, pde2/pde2*), SR1081 (*flo8/flo8, ras1/ras1, ras2/ras2, pde2/pde2*), SR1088 (*ste12/ste12, ras1/ras1, ras2/ras2, pde2/pde2*) and SR1121 (*flo11/flo11, ras1/ras1, ras2/ras2, pde2/pde2*) that were streaked on a SLAD plate containing 2 mM cAMP. Following 5 days of incubation at 30°C, photographs of the colonies were taken. (B and C) Invasive growth of strains patched on YPD + 2 mM cAMP. Strains were grown on YPD + 2 mM cAMP for 2 days at 30°C. Pictures were taken before (B) and after wash (C). All strains are diploid. The upper row of patches shows the same strains as in (A). As a control, the invasive growth phenotype of SR607 (*MATa/α*, wild-type), SR1097 (*flo8/flo8*), SR1022 (*ste12/ste12*) and SR1098 (*flo11/flo11*) is shown in the lower row of patches.

otherwise wild-type background support a model of their joint control over *FLO11*. Overexpression of *FLO8* in a *ste12* strain (SR1021) and overexpression of a *flo8* strain (SR1134) suppress the pseudohyphal and invasion defects of the mutants (Figure 5A). The morphologies of the pseudohyphae in these ‘suppressed’ strains is not identical to that of wild-type. In *flo8* strains overexpressing *STE12*, the cells of each pseudohyphal strand seem more elongated than the cells of wild-type pseudohyphae. In *ste12* strains overexpressing *FLO8*, the cells are not longer than wild-type; however, they have a denser network of filaments than wild-type strains. This colony morphology is similar to that of strains that are induced to form filaments by cAMP (SR959; Figure 1C).

These patterns of suppression by overexpression are reflected in the *FLO11* expression pattern. Overexpression of *FLO8* enhances the expression of *FLO11* 10-fold in a *ste12* mutant (SR1021; Figure 5B). The reciprocal experiment in which *STE12* is overexpressed is more difficult because high levels of Ste12p are toxic. To control the levels of *STE12*, we used a *GAL::STE12* construct that could be regulated by galactose. In a *flo8* strain (SR1134) containing this *GAL::STE12* construct on a plasmid, the *FLO11* transcript levels are increased 3-fold on SC glucose medium as compared with the strain transformed with the control plasmid (SR1097). This increase probably represents incomplete repression of the *GAL* promoter. If *STE12* is induced by incubation for 4 h in SC galactose medium, *FLO11* expression is increased 10-fold (when normalized to the level of *ACT1* message; Figure 5B, last lane). Overexpression of *STE12* in the

ras1 ras2 pde2 strain leads to induction of *FLO11* even in the absence of cAMP (data not shown).

To test whether cAMP activates transcription directly via Flo8p, we used a *LexA-FLO8* fusion construct on a plasmid and a strain containing a β -galactosidase reporter activatable by a proficient LexA (Golemis and Brent, 1992). The reporter is strongly activated by *LexA-FLO8* (20-fold) but not by *LexA* alone, demonstrating that Flo8p can act directly as a potent transcription factor. However, in this *LexA* system, we were unable to demonstrate an effect of cAMP on the ability of *FLO8* to activate the reporter.

***FLO11* transcription is also stimulated by high internal levels of cAMP**

To determine whether a high level of internal cAMP stimulates *FLO11* transcription, we deleted *IRA1* in an otherwise wild-type strain. Ira1p is a Ras-GAP that inactivates Ras-GTP by converting it to Ras-GDP. Loss of Ira1p function leads to a higher proportion of activated Ras and thus to elevated cAMP levels in the cell (Tanaka *et al.*, 1989). In the *ira1* mutant strain (SR599), *FLO11* transcripts are strongly induced (Figure 3A). This *FLO11* induction is reflected in the hyperinvasive phenotype of strains devoid of *IRA1* function (Figure 6). Both a *ste12 ira1* and a *ste11 ira1* mutant are hyperinvasive, illustrating that at least some of the cAMP signal is independent of the MAP kinase pathway. However, *flo11 ira1* (SR1079) or *flo8 ira1* (SR1132) strains fail to invade (Figure 6). These results are consistent with the interpretation that Flo8p is required for induction of *FLO11* by high internal and

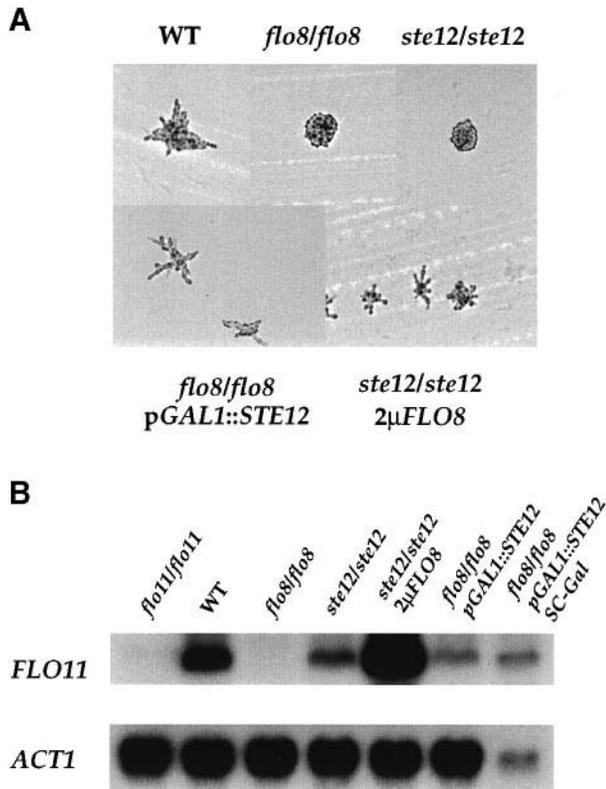


Fig. 5. Cross-suppression of *ste12* and *flo8* defects by overexpression of Flo8p and Ste12p. (A) Strains SR607 (wild-type), SR1097 (*flo8/flo8*), SR1022 (*ste12/ste12*), SR1134 (*flo8/flo8* pGAL1::STE12) and SR1021 (*ste12/ste12*, pRS202FLO8) were streaked on SLAD plates. Pictures of the colonies were taken after 16 h of incubation at 30°C. (B) Northern blots probed subsequently with *ACT1* as a normalization probe and a *FLO11* probe. Total RNA was prepared from strains SR1101 (*flo11*), SR607 (wild-type), SR1097 (*flo8/flo8*), SR1022 (*ste12/ste12*), SR1021 (*ste12/ste12*, pRS202FLO8) and SR1134 (*flo8/flo8* pGAL1::STE12) grown in SC to OD = 1.0–1.2. The two lanes shown for *flo8/flo8* pGAL1::STE12 represent cells grown in SC glucose or SC galactose (SC-Gal).

external cAMP levels. A strain (SR1032) lacking both *FLO8* and *IRA1* has dramatically reduced levels of *FLO11* transcripts, whereas deletion of *STE12* in an *ira1* background (SR1133) still shows *FLO11* transcript levels comparable with wild-type (Figure 3A). These results are consistent with the hyperinvasive phenotype of the *ira1 ste12* strain and the non-invasive phenotype of the *ira1 flo8* strain.

Discussion

The *FLO11* promoter contains *cis*-acting sites for diverse external and internal signals. We have shown that in addition to mating type control, nitrogen starvation and phase of growth, the *FLO11* promoter is controlled by cAMP. Mutational analysis of the *FLO11* promoter identified *cis*-acting segments spread over 2.8 kb that mediate the *trans*-acting signals. Our experiments support a model in which at least two signal transduction pathways, the cAMP/PKA pathway and the Kss1 MAPK pathway, regulate *FLO11* transcription.

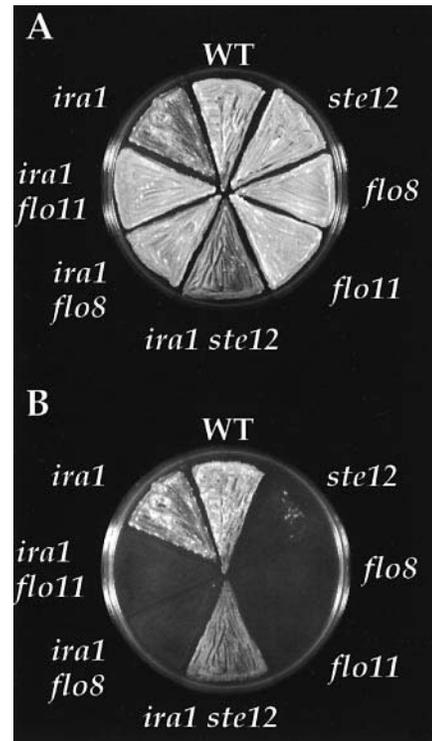


Fig. 6. Deletion of *IRA1* stimulates haploid invasive growth in *ste12* but not in *flo8* or *flo11* strains. (A) The strains are patched onto YPD plates and incubated for 2 days at 30°C. All strains are haploid. The single mutant strains SR599 (*ira1*), 10560-2B (wild-type), L5795 (*ste12*), L5816 (*flo8*) and SR1101 (*flo11*) are patched as a control next to the double mutant strains SR1133 (*ira1 ste12*), SR1132 (*ira1 flo8*) and SR1079 (*ira1 flo11*). (B) Following incubation for 2 days at 30°C, the upper plates were washed under a stream of water and re-photographed to yield the lower image.

FLO11 is a target for the MAPK and cAMP/PKA pathways

FLO11 expression depends on signals from both the cAMP/PKA pathway and the Kss1 MAPK pathway. In agreement with a previous report (Lo and Dranginis, 1998), we find that the Kss1 MAPK pathway via *STE12/TEC1* controls the expression of *FLO11*. In this work, we show that cAMP levels are also critical for *FLO11* transcription. In a *ras1 ras2 pde2* strain where internal cAMP levels can be altered by the addition of cAMP to the media, *FLO11* transcript levels increase with increasing amounts of cAMP. Deletion of *IRA1*, a Ras-GAP that leads to increased cAMP levels in the cell, also results in dramatically induced *FLO11* transcript levels.

FLO8 is required for the cAMP induction of FLO11

We have found that *FLO8*, a known transcription factor required for invasive and filamentous growth (Liu *et al.*, 1996), is required for *FLO11* transcription. Strains that lack *FLO8* fail to express *FLO11* and cannot be induced to express *FLO11* or to filament in the presence of high concentrations of cAMP. Unlike Flo8p, Ste12p is not required for cAMP induction of *FLO11*, suggesting that the cAMP pathway is distinct from the *STE12/TEC1* branch. In agreement with this result, the Ste12p/Tec1p-dependent *FG::Ty1-lacZ* reporter is not regulated by cAMP.

Recent work has shown that *TPK2*, one of three catalytic subunits of the PKA in *S.cerevisiae*, is required for activation of *FLO11* (Robertson and Fink, 1998). Although the simplest model is that *FLO8* directly mediates the signal from Tpk2p, we were unable to show an effect of cAMP on the ability of *FLO8* to activate transcription in a *LexA* reporter system. It is, of course, possible that the *LexA* constructs we used do not reflect the *FLO8* activity accurately. In view of these results, we conclude that Flo8p is either activated directly by a cAMP/PKA signal or required in addition to the cAMP/PKA signal to induce *FLO11* transcripts.

Flo8p and Ste12p control *FLO11* expression independently

Increased expression of one of the two pathways that control *FLO11* can bypass a block in the other. High cAMP levels or overexpression of *FLO8* bypasses the requirement for *STE12* for both invasion and filamentation and for induction of *FLO11*. This bypass occurs regardless of how the increase in cAMP levels occurs (exogenously by addition of the compound to a *ste12 ras1 ras2 pde2* strain, SR1088, or endogenously by altering regulation of cAMP production in the *iral ste12* strain SR1133). In contrast, both a functional *FLO8* and *FLO11* are required for induction of invasion and filamentation by cAMP. Overexpression of *STE12* can also bypass the requirement for *FLO8* for invasive and filamentous growth and for induction of *FLO11*, suggesting that each pathway can act independently of the other.

***FLO11* contains multiple cis-acting elements that respond independently to each signaling pathway**

The *trans*-acting factors *STE12*, *TEC1* and *FLO8* as well as cAMP each target multiple *cis*-acting segments in the *FLO11* promoter (Table III; Figure 2B). Flo8p acts on a 200 bp element in the *FLO11* promoter (−1000 to −1200 bp) defined by the two overlapping segments *FLO11*-6/5 and *FLO11*-7/6 under all conditions tested. This promoter element, the UAS_{*flo11-6*}, is required for *FLO11* expression as well as for invasive and filamentous growth. The same two overlapping segments, *FLO11*-6/5 and *FLO11*-7/6, are also most strongly induced by cAMP. These results support the conclusion that Flo8p is required to transmit the filamentation signal from the cAMP/PKA pathway. In post-diauxic cells, *FLO8* has an additional function in activating segments *FLO11*-3/2 and *FLO11*-8/7. The overlapping segments *FLO11*-2/1 and *FLO11*-3/2 define in wild-type cells a UAS induced in post-diauxic cells only. Like Flo8p and cAMP, Ste12p acts on multiple UASs, numbering four in total. Ste12p shows also some contribution to segment *FLO11*-5/6 in exponentially growing cells. This contribution is not observed in post-diauxic cells.

Two UASs (*FLO11*-12/11 and *FLO11*-10/9) also program transcription that is dependent on Tec1p, a known DNA-binding partner of Ste12p. Interestingly, the dependencies on Ste12p and Tec1p are not perfectly concordant. An extreme example is the *FLO11*-10/9 UAS which exhibits a 57-fold dependency on Ste12p, but only a 3.5-fold dependency on Tec1p. These data suggest Tec1p-independent functions for Ste12p in *FLO11* expression. Given that Ste12p uses multiple DNA-binding partners in the mating signaling pathway, the current data may be a

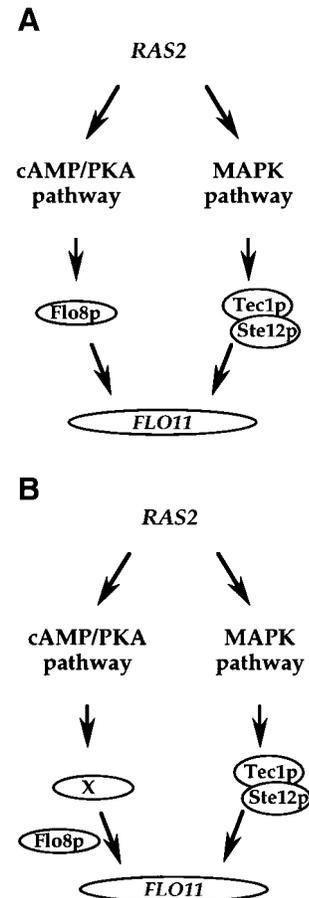


Fig. 7. Model of two signal transduction pathways, the Ras-regulated cAMP/PKA and Kss1 MAP kinase pathways, which induce invasive and filamentous growth by activating *FLO11* transcription. The Kss1 MAP kinase pathway acts on Ste12p/Tec1p to induce *FLO11* transcription. Flo8p is required for activation of *FLO11* by the cAMP/PKA pathway. cAMP-mediated Flo8p activation can be either direct (A) or indirect (B).

hint that Ste12p interacts with heretofore unidentified partners in addition to Tec1p. No effect of α -factor on *FLO11-lacZ* expression could be detected.

The dual control of *FLO11* explains several previous observations

Our model (Figure 7) accounts for a number of complexities that previously were difficult to explain. First, the MAPK pathway mutants are leaky, showing both residual filamentation and invasion (Lo *et al.*, 1997). This phenomenon can be explained if the residual activity would be a consequence of some basal redundant activation of *FLO11* by the cAMP branch. If both the cAMP/PKA pathway and the MAPK pathway are blocked (in a *flo8 ste12* mutant for example), there is no residual activity. Secondly, our model accounts for earlier observations that the activated alleles of *RAS* are only partially blocked by mutations in the MAP kinase pathway (Mosch and Fink, 1997). Presumably these activated alleles can stimulate adenylate cyclase with a consequent increase in the levels of cAMP that can account for activation of *FLO11* transcription. This explanation can be extended to interpret the enhanced filamentation and invasion of strains deleted for *PDE2*, the high affinity phosphodiesterase (Ward *et al.*,

1995; Lorenz and Heitman, 1997). Thirdly, the *FLO8* requirement for *FLO11* activation explains why many laboratory strains that contain a mutation in *FLO8* are unable to switch to pseudohyphal growth. The failure to express *FLO11* also explains why these strains are not flocculent. Fourthly, the fact that *FLO11* has upstream sites that mediate MAPK, cAMP/PKA, mating type and nutrition signals endows this single region with the ability to integrate the known factors that influence diploid pseudohyphal growth and haploid invasive growth.

The *FLO11* promoter is complex

Our analysis of the *FLO11* 5' intergenic region underscores the complexity of the *FLO11* promoter. First, the promoter is extremely large; direct mutational analysis shows that elements required for expression extend at least 2.8 kbp from the putative ATG. It is interesting to note that among the 10 yeast genes with the longest intergenic regions are two developmental genes known to contain large complex promoter regions, *IME1* and *HO* (Table I). The promoters of each of these genes, like that of *FLO11*, respond to a variety of environmental and internal signals.

Our mutational analysis could detect four UAS and nine URS sites in the *FLO11* promoter that are responsive to environmental signals such as rich medium, glucose depletion (post-diauxic cells) or nitrogen starvation, but there may be many more. The individual *cis*-acting segments can be key regulators under all conditions (*flo11-4*, *flo11-7*, *flo11-8*, *flo11-14*) or selectively required for a specific environmental condition (*flo11-10*, *flo11-11*, *flo11-12*, *flo11-13*). Most notably, *flo11-4*, *flo11-10*, *flo11-11* and *flo11-13* show haploid-specific induction. In addition, *flo11-11* is strongly regulated by the growth phase of the cells, revealing a major induction in post-diauxic cells (glucose depletion), whereas *flo11-12* and *flo11-13* are regulated by nitrogen availability. *flo11-5* is strongly regulated by nitrogen availability both in haploids and in diploids.

The mere presence of a large number of URS elements, however, increases the complexity of the analysis. Moreover, these elements could mask the contribution of any adjacent UAS element. For example, Lo and Dranginis (1998) pointed out that there is a degenerate FRE (filamentation and invasion response element) at bp -725 to -699. *FLO11-4/3* or *FLO11-5/4* both contain this element; however, neither shows significant stimulation of *FLO11* expression nor a requirement for function of *STE12* or *TEC1*. This site contains a URS (*flo11-4* in Table II) so we cannot rule out that the putative FRE site is functional but is masked by URS elements.

The mechanism by which the *FLO11* promoter is controlled by *trans*-acting proteins may be so complex that it cannot be reconstructed adequately by analysis of mutations in the *cis*- or *trans*-acting elements. Although this type of analysis can identify the elements, the number of elements and the multiplicity of interactions may obscure the key interactions. A model for highly complex promoters has been proposed for the β -interferon promoter (Thanos and Maniatis, 1995). The plethora of interactions at this promoter results in the formation of an enhanceosome, a multiprotein structure that involves interactions between DNA, DNA-binding proteins and a host of ancillary proteins. A similar detailed *in vitro* analysis of

FLO11 may be necessary to unravel the mechanism by which its transcription is controlled.

Materials and methods

Media and yeast strains

Strains used in this study are listed in Table IV. Standard methods for genetic crosses and transformation were used (Guthrie and Fink, 1991). All strains are congeneric to the Σ 1278b genetic background. The derivation of *ras1*, *ras2*, *pde2*, *ste12*, *flo8* and *flo11* strains has been described (Liu et al., 1993, 1996; Lo and Dranginis, 1996; Mosch et al., 1996; Kubler et al., 1997). Invasive and pseudohyphal growth assays were described (Gimeno et al., 1992; Roberts and Fink, 1994). Invasive growth was tested after 2 days incubation at 30°C. cAMP was added to liquid or solid media directly before use.

Plasmids

Plasmids used in this study are listed in Table V. Yeast-*E.coli* shuttle vectors have been described previously (Sikorski and Hieter, 1989; Christianson et al., 1992). The *FG(TyA)::lacZ* reporter plasmids are described in Mosch et al. (1996). Plasmids *piral::LEU2* and *piral::HIS3* were created by amplifying both flanking 400 bp regions of the *IRAI* ORF, introducing *EcoRI* and *BglII* sites at the ends. The two fragments are cloned into a Bluescript KS⁺ *EcoRI* site, and the *LEU2* or *HIS3* marker is cloned into the *BglII* site, creating a complete deletion. The same method was used to create plasmid *pcyr1::LEU2*. The *flo11::URA3* plasmid was obtained from Lo and Dranginis (1996).

Plasmid constructions

The *LexA-FLO8* plasmid was constructed by amplifying the entire *FLO8* ORF from B3265 using PCR primers that create *BamHI* sites at the ends. The ORF was subcloned into a modified pMAL bacterial expression vector (Fink Lab collection B3672), and full-length expression was verified by a C-terminal FLAG tag. The *FLO8* ORF was sequenced and subcloned (without the FLAG tag) into pEG202. Correct fusion to the *LexA* was confirmed by sequencing.

A *FLO11-lacZ* reporter plasmid was constructed by amplifying the 3 kbp region 5' of the ATG by PCR using primers containing a *BglIII* site at the end and cloning into YEp355 (Myers et al., 1986) (primers used: CGCACACTATGCAAAGACCGAGATCTTCC and GAAGATCTTCTCCACATACCAATCACTCG). The 14 individual deletions in this reporter were constructed by a primer overlap method (Higuchi, 1989) using a Bluescript KS⁺ plasmid containing the 3 kbp *FLO11* promoter region (subcloned from YEp355-*FLO11::lacZ* as an *EcoRI-HindIII* fragment). After mutagenesis, the partially deleted *flo11-nm* promoter sequence was recloned into YEp355. The primers used for this purpose were:

#1F, CAAGCATTACGTTACTGCGAAAATCCATATACGCACACT;
 #1R, AGTGTGCGTATATGGATTTTCGCAGTAAACGTAATGCTTG;
 #2F, TGATGAGGTAACCTTTACAACCTCTTCTAGTTCAAGAAC;
 #2R, GTTCTTGAAGTAGAAGAGAGTTGTAAGGTTACCTCATCA;
 #3F, TTTCAATTCAATGGATTTGGAAATGTCATAGAGTTACCAAT;
 #3R, ATTGGTAACTCTATGACATTTCCAAATCCATTGAATTGAAA;
 #4F, ATTCTGCGTATACCTTTAAAGGTATTCTGTTTACTA;
 #4R, TAGTAAACAAAACGAATACCTTTAAGAGTATAGGCAGAAAAT;
 #5F, TTGGGGCTAAGAATGGACACAGATCAGTCATTCATGTTGT;
 #5R, ACAACATGAATGACTGATCTGTGTCATTCCTAGCCCCAA;
 #6F, GGGTGTGCGTGGAAAGTTCAATCCCTTTGTTTCTTTG;
 #6R, CAAAAGAAAAGAAAAGGGAATGAACTTTCCAGGCACACCC;
 #7F, CAAAACCTTAGGAATACCGGAAATTAAGGTTTTTTCTTC;
 #7R, GAAGAAAAAACCTTAATTTCCGGTATTCCTAAAAGTTTTG;
 #8F, CGAATGTGAATGCGCTAATCTTGTGTGCTACCGCAGCC;
 #8R, GGGCTGGCGTAGGCACACAAGATTAGCGCATTACATTCG;
 #9F, AGACAAAAAATAGGAAAAGTGGTATTCCACCACATGAAA;
 #9R, TTTTCATGTGGTGGAAAATACCACCTTTTCTATTTTTGTCT;
 #10F, TTAGTGTGCGAATACTTTCTTTAATATGATGATGGTTCTCA;
 #10R, TGAGAACCATCACTAATTAAGGAAAAGTATTCCGCACTAA;
 #11F, CAGTGCTTTCAACACCTTTTATCTCATCGAGAGCCGAGC;
 #11R, GCTCGGCTCTCGATGAGAATAAAAAGGTTGTTGAAGCACTG;
 #12F, TTAGTGTGAAAAGTCCATCTCATCTGTGTCATGTCAGAG;
 #12R, TCTGACATGGCACACAGATGTAGATGGACTTTTCAGCTAC;
 #13F, GAGATTATCTGGGATCTATTGCAATTAATGAATGATACTA;
 #13R, TAGTATCATTCATAATTGCAATGATACCCAAAGATAATCTC;
 #14F, GTTTTGGCTCAATGGGACCCTTACAAAATTTACGGCTAAT;
 #14R, ATTAGCCGTAATTTGTGAACGGTCCCATTGAGCCAAAAC.

Table IV. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference/source
10560-2B	<i>MATa ura3-52 leu2::hisG his3::hisG</i>	Fink laboratory collection
10560-5B	<i>MATa ura3-52 leu2::hisG trp1::hisG</i>	Fink laboratory collection
L5577	<i>MATa/a ste12::LEU2/ste12::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG</i> (B2616)	Liu <i>et al.</i> (1996)
L5791	<i>MATa/a ura3-52/ura3-52 HIS3/his3::hisG leu2::hisG/leu2::hisG TRP1/trp1::hisG</i>	Fink laboratory collection
L5795	<i>MATa ste12::LEU2 ura3-52 leu2::hisG his3::hisG</i>	Liu <i>et al.</i> (1996)
L5816	<i>MATa flo8-2 ura3-52</i>	Liu <i>et al.</i> (1996)
L6149	<i>MATa tec1::HIS3 ura3-52 leu2::hisG his3::hisG</i>	Madhani and Fink (1997)
L6150	<i>MATa tec1::HIS3 ste12::LEU2 ura3-52 leu2::hisG his3::hisG</i>	Madhani and Fink (1997)
SR425	<i>MATa pde2::kan^R ura3-52 leu2::hisG his3::hisG</i>	this study
SR599	<i>MATa ira1::LEU2 ura3-52 leu2::hisG his3::hisG</i>	this study
SR607	<i>MATa/a ura3-52/ura3-52 LEU2/leu2::hisG his3::hisG/his3::hisG TRP1/trp1::hisG</i> (B1820, B3161)	this study
SR957	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG</i>	this study
SR959	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG TRP1/trp1::hisG</i> (B3296)	this study
SR963	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R cyr1::LEU2/cyr1::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG TRP1/trp1::hisG</i>	this study
SR1021	<i>MATa/a ste12::LEU2/ste12::LEU2 ura3-52 /ura3-52 leu2::hisG/leu2::hisG</i> (B3265)	this study
SR1022	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R ste12::LEU2/ste12::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG</i> (B2803)	this study
SR1079	<i>MATa ira1::LEU2 flo11::URA3 ura3-52 leu2::hisG his3::hisG</i>	this study
SR1081	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R flo8-2/flo8::hisG::URA3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG</i> (B1818, B3296)	this study
SR1088	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R ste12::LEU2/ste12::URA3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG</i> (B1818)	this study
SR1097	<i>MATa/a flo8-2/flo8-2 ura3-52/ura3-52</i> (B2803)	this study
SR1098	<i>MATa/a flo11::URA3/flo11::ura3::HIS3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG TRP1/trp1::hisG</i>	this study
SR1101	<i>MATa flo11::URA3 ura3-52 leu2::hisG his3::hisG</i>	this study
SR1121	<i>MATa/a ras1::HIS3/ras1::HIS3 ras2Δ/ras2Δ pde2::kan^R/pde2::kan^R flo11::URA3/flo11::URA3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG</i> (B1818, B2802)	this study
SR1132	<i>MATa/a ras1::HIS3/flo8::hisG::URA3::hisG ura3-52/ura3-52 leu2::hisG/his3::hisG</i>	this study
SR1133	<i>MATa ira1::LEU2 ste12::LEU2 ura3-52 leu2::hisG his3::hisG</i>	this study
SR1134	<i>MATa/a flo8::hisG/flo8::hisG ura3-52/ura3-52</i> (B2065)	this study
SR1172	<i>MATa flo11-16 ura3-52 trp1::hisG his3::hisG</i>	this study
SR1174	<i>MATa flo11-6 ura3-52 trp1::hisG his3::hisG</i>	this study
SR1175	<i>MATa/a flo11-6 /flo11-6 ura3-52/ura3-52 his3::hisG/his3::hisG TRP1/trp1::hisG</i>	this study
SR1176	<i>MATa flo11-3 ura3-52 leu2::hisG his3::hisG</i>	this study
SR1177	<i>MATa flo11-4 ura3-52 leu2::hisG his3::hisG</i>	this study
SR1178	<i>MATa flo11-14 ura3-52 leu2::hisG his3::hisG</i>	this study

Chromosomal deletion of the *FLO11* promoter was done by replacing bp -2947 to -150 with *URA3*. The primers used were: ACCACAACATG-ACGAGGGATAATAACTGATGAATAGGGTGCTTTTATACTCT-GTGCAGTATTTCACACC and TAAGGAGTCGTACCGCAACTA-AATCTGAATAACAATTTGGCTGCTAGAAGCAGATTGTAAGTGA-GAGTGC), resulting in *flo11-16* (SR1172).

To generate the individual *flo11-n* deletions in the chromosomal *FLO11* promoter region, the *URA3* gene was replaced by transformation with the respective *EcoRI-HindIII*-digested *KS⁺flo11-Δn*. Correct replacements were selected on 5-fluoro-oroic acid and verified by PCR.

To determine UAS sequence elements, 14 individual 400 bp elements, overlapping by 200 bp, were amplified by PCR and cloned into pLG669Z (Guarente and Ptashne, 1981) using a restriction site (*XhoI*) introduced at the 5' end of the PCR primers. The primers used for this purpose were:

#1R, CCGCTCGAGAGTGTGCGTATATGGATTTT;
 #2F, CCGCTCGAGTATGAGGTAACCTTTACAA;
 #2R, CCGCTCGAGGTTCTTGAAC TAGAAGAGAGAG;
 #3F, CCGCTCGAGTTTCAACTTAGGATTGG;
 #3R, CCGCTCGAGATTGGTAACTCTATGACATT;
 #4F, CCGCTCGAGATTCTGCCTATACTCTTAA;
 #4R, CCGCTCGAGTAGTAAACAAACGAATACCT;
 #5F, CCGCTCGAGTTGGGGCTTAGGAATACCGT;
 #5R, CCGCTCGAGACAACATGAATGACTGATCT;
 #6F, CCGCTCGAGGGGTGTGCCTGGAAAGTTCA;
 #6R, CCGCTCGAGCAAAAGAAAGAAAGGGAA;
 #7F, CCGCTCGAGCAAAACTTTAGGAATACCGG;
 #7R, CCGCTCGAGGAAGAAAAAACCTTAATTT;
 #8F, CCGCTCGAGCGAATGTGAATGCGCTAATC;
 #8R, CCGCTCGAGGGGTGGCGTAGGCACACAA;
 #9F, CCGCTCGAGAGCAAAAATAGGAAAGT;
 #9R, CCGCTCGAGGTAGCAGGTTTCATGTGGTG;

#10F, CCGCTCGAGTTAGTGCAGGAATACTTTCCT;
 #10R, CCGCTCGAGTGAGAACCATCACTAATTA;
 #11F, CCGCTCGAGCAGTGTCTTCAACACCTTTT;
 #11R, CCGCTCGAGGTCGGCTCTCGATGAGAAT;
 #12F, CCGCTCGAGGTAGCTGAAAAGTCCATCTA;
 #12R, CCGCTCGAGTCTGACATGACACACAGATG;
 #13F, CCGCTCGAGGAGATTATCTTGGGATCTAT;
 #13R, CCGCTCGAGTAGTATCATTCATAATTCGA;
 #14F, CCGCTCGAGGTTTTGGCTCAATGGGACCG;
 #14R, CCGCTCGAGATTAGCCGTAAATTTGTGAA;
 #15F, CCGCTCGAGCTCCACATACCAATCACTCG;
 #15R, CCGCTCGAGTAGTTAAACGTTTTATTAGC.

The resulting 14 plasmids for the deletion series were transformed into strain 10560-2B. At least three independent clones were tested using filter assays for equivalent expression of β-galactosidase. Diploid strains were created by mating of the respective 10560-2B *flo11-nm* transformant with strain 10560-5B by selection on YNB containing only leucine as a supplement.

The 14 plasmids containing the 400 bp sequence elements were transformed into 10560-2B, L5795, L5816, L6149, L6150 and SR957. At least three independent clones were tested using filter assays for equivalent expression of β-galactosidase. Filter assays were used to test the effect of α-factor (5 μM).

The large number of *FLO11* promoter elements and multiple conditions that affect those elements required some economy in the experimental design. The measurement of β-galactosidase from our plasmid-based reporter containing each of these elements permitted the rapid construction of strains and reproducible measurement of reporter activity. We confined the analysis to selective media so that the strains retained the plasmids. Our β-galactosidase assays show, in general, a higher activity for haploid strains than for diploid strains. For rich medium, this result

Table V. Plasmids

Plasmid	Description	Reference/source
B1818	pRS314	Sikorski and Hieter (1989)
B1820	pRS316	Sikorski and Hieter (1989)
B2065	<i>URA3 GAL1::STE12</i> 2 μ (pRS202)	Fink laboratory collection
B2616	YCp50- <i>STE11-4</i>	Fink laboratory collection
B2621	pEG202	Golemis and Brent (1992)
B2625	pSH18-34	Golemis and Brent (1992)
B2802	pRS425	Christianson <i>et al.</i> (1992)
B2803	pRS426	Christianson <i>et al.</i> (1992)
B3161	<i>pFG(TYA)::LacZ-HIS3</i>	Mosch <i>et al.</i> (1996)
B3265	<i>URA3 FLO8</i> 2 μ (pRS202)	Liu <i>et al.</i> (1996)
B3296	<i>pFG(TYA)::LacZ-LEU2</i>	Mosch <i>et al.</i> (1996)
B3782	3 kbp- <i>FLO11::LacZ</i> in YE μ p355 (Myers <i>et al.</i> , 1986)	this study
pSR112	pEG202- <i>FLO8</i>	this study
<i>pflo11-1</i> to <i>pflo11-14</i>	200 bp deletions in <i>pFLO11</i> from -1 to -400 bp, -200 to -400 bp until -2600 to -2800 bp	this study
<i>pFLO11-2/1</i> to <i>pFLO11-15/14</i>	440 bp sequence elements cloned into pLG669Z (Guarente and Ptashne, 1981) from -1 to -420 bp, -180 to -620 bp, -380 to -1020 bp until -2580 to -2980 bp	this study

is in agreement with that found previously when *FLO11* expression was monitored by measurement of steady-state mRNA levels (Lo and Dranginis, 1998). When we measure *FLO11::lacZ* after 24–26 h (post-diauxic growth) or on SLAD medium, haploid cells show an induction of >10-fold, and diploid cells 5-fold induction. This large induction of *FLO11::lacZ* for haploid cells on SLAD was not observed in the experiments where *FLO11* mRNA was measured (Lo and Dranginis, 1998). This discrepancy could be due either to a difference in the stability of mRNA versus protein or the differences in the times at which the cultures were sampled. The expression of *FLO11* as measured by either method is extremely sensitive to growth conditions and growth phase.

Preparation of RNA and Northern analysis

Total RNA was prepared using hot acid phenol, and Northern blots were performed as described in Ausubel *et al.* (1987). Strains deleted for *ras1 ras2 pde2* were pre-grown in SC + 1 mM cAMP to OD₆₀₀ = 1.0. The cells were washed twice using SC and diluted to an OD₆₀₀ = 0.3 into fresh SC medium or SC medium + 2 mM cAMP. The cells were grown to OD₆₀₀ = 1.0 and harvested. Then 10–15 μ g of RNA were separated on a formaldehyde-containing agarose gel. For hybridization to *FLO11* mRNA, a probe corresponding to bp 3502–4093 of the *FLO11* ORF was used. The actin probe used corresponds to the 3' exon of *ACT1*. The *FLO1* probe corresponds to bp 1–500 of the *FLO1* ORF.

Extract preparation and enzyme assays

Cells for β -galactosidase assays of *FG::TyA-lacZ* were incubated on SLAD plates for 3 days; cells for *flo11::lacZ* expression studies were grown in the respective liquid media and quantitated according to Mosch *et al.* (1996).

Cells for quantitation of *flo11::lacZ* expression in exponential growth phase were inoculated from confluent 20 h grown cultures 1:20 in fresh medium and grown for 4–6 h. Cells for quantitation of *flo11::lacZ* expression after the post-diauxic shift were grown for 24–26 h. The same cultures were used to inoculate the exponentially growing cultures. Cells for quantitation of *flo11::lacZ* expression in SLAD medium were pre-grown for 20 h in SC medium, washed twice with 2% glucose, diluted 1:5 into SLAD medium and grown for 4–6 h. For detection of cAMP-induced *FLO11* promoter segments, strain SR957 harboring the individual plasmids was grown overnight in selective medium containing 1 mM cAMP, transferred to media containing no cAMP for 10 h and split into two cultures containing 2 mM cAMP or no cAMP and grown for 4 h before harvesting.

Cells for the *LexA::FLO8* assay were grown for 20 h in liquid SC medium, harvested and β -galactosidase activity was determined.

Analysis of *S.cerevisiae* intergenic regions

A list of *S.cerevisiae* genomic regions that lack annotated features (i.e. that do not correspond to known genes, predicted ORFs >100 amino acids or transposons) was obtained from the Stanford Yeast Genome Database (ftp://stanford.edu/pub/yeast/yeast_NotFeature/NotFeature.fasta) and were sorted based on their size. Because gene density decreases

dramatically near chromosome ends, entries within 35 kbp of telomeres were eliminated. Regions >2.5 kbp were reexamined systematically for features and, if they were found to contain genes, *Ty* elements or *Ty* fragments, these and several of the remaining regions which have been found to encode RNAs (Olivas *et al.*, 1997) were eliminated. The 10 largest regions are shown in Table I. It is important to note that uncharacterized expressed genes with ORFs <100 amino acids are not annotated and, consequently, may be found to exist in regions currently labeled as intergenic. Conversely, predicted ORFs >100 amino acids may occur by chance, which would result in the underestimation of the sizes of the intergenic regions which contain them.

Acknowledgements

We thank Anne Dranginis for kindly providing plasmids, and Fran Lewitter for help with the analysis of the size of intergenic regions in the yeast genome. We thank Tim Galitski for helpful comments on the manuscript, and the members of the Fink Laboratory for many fruitful discussions. S.R. was supported by a Research Fellowship from the Deutsche Forschungsgemeinschaft (DFG). This work was supported by grants from the National Institutes of Health (GM40266 and GM35010 to G.R.F.). G.R.F. is an American Cancer Society Professor of Genetics.

References

- Alspaugh, J.A., Perfect, J.R. and Heitman, J. (1997) *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP. *Genes Dev.*, **11**, 3206–3217.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current Protocols in Molecular Biology*. Green Publishing Associates/Wiley-Interscience, New York, NY.
- Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H. and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.
- Durrenberger, F., Wong, K. and Kronstad, J.W. (1998) Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. *Proc. Natl Acad. Sci. USA*, **95**, 5684–5689.
- Jimeno, C.J., Ljungdahl, P.O., Styles, C.A. and Fink, G.R. (1992) Unipolar cell divisions in the yeast *S.cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell*, **68**, 1077–1090.
- Gold, S., Duncan, G., Barrett, K. and Kronstad, J. (1994) cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. *Genes Dev.*, **8**, 2805–2816.
- Golemis, E.A. and Brent, R. (1992) Fused protein domains inhibit DNA binding by LexA. *Mol. Cell. Biol.*, **12**, 3006–3014.
- Guarente, L. and Ptashne, M. (1981) Fusion of *Escherichia coli lacZ* to the cytochrome *c* gene of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **78**, 2199–2203.

- Guthrie, C. and Fink, G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, New York, NY.
- Higuchi, R. (1989) Using PCR to Engineer DNA. In Erlich, H. (ed.), *PCR Technology*. Stockton Press, New York, NY.
- Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996) Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **251**, 707–715.
- Kubler, E., Mosch, H.U., Rupp, S. and Lisanti, M.P. (1997) Gpa2p, a G-protein α -subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. *J. Biol. Chem.*, **272**, 20321–20323.
- Lambrechts, M.G., Pretorius, I.S., D'Aguanno, V.S., Sollitti, P. and Marmur, J. (1994) Multiple positive and negative *cis*-acting elements of the *STA2* gene regulate glucoamylase synthesis in *Saccharomyces cerevisiae*. *Gene*, **146**, 137–144.
- Lambrechts, M.G., Bauer, F.F., Marmur, J. and Pretorius, I.S. (1996) Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proc. Natl Acad. Sci. USA*, **93**, 8419–8424.
- Liu, H., Styles, C.A. and Fink, G.R. (1993) Elements of the yeast pheromone response pathway required for filamentous growth of diploids. *Science*, **262**, 1741–1744.
- Liu, H., Styles, C.A. and Fink, G.R. (1996) *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. *Genetics*, **144**, 967–978.
- Lo, H.J., Kohler, J.R., Di Domenico, B., Loebenberg, D., Cacciapuoti, A. and Fink, G.R. (1997) Nonfilamentous *C. albicans* mutants are avirulent. *Cell*, **90**, 939–949.
- Lo, W.S. and Dranginis, A.M. (1996) *FLO11*, a yeast gene related to the *STA* genes, encodes a novel cell surface flocculin. *J. Bacteriol.*, **178**, 7144–7151.
- Lo, W.S. and Dranginis, A.M. (1998) The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **9**, 161–171.
- Lorenz, M.C. and Heitman, J. (1997) Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. *EMBO J.*, **16**, 7008–7018.
- Madhani, H.D. and Fink, G.R. (1998) The control of filamentous differentiation and virulence in fungi. *Trends Cell Biol.*, **8**, 348–353.
- Madhani, H.D., Styles, C.A. and Fink, G.R. (1997) MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. *Cell*, **91**, 673–684.
- Mosch, H.U. and Fink, G.R. (1997) Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. *Genetics*, **145**, 671–684.
- Mosch, H.U., Roberts, R.L. and Fink, G.R. (1996) Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **93**, 5352–5356.
- Myers, A.M., Tzagoloff, A., Kinney, D.M. and Lusty, C.J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene*, **45**, 299–310.
- Nikawa, J., Sass, P. and Wigler, M. (1987) Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **7**, 3629–3636.
- Olivas, W.M., Muhlrad, D. and Parker, R. (1997) Analysis of the yeast genome: identification of new non-coding and small ORF-containing RNAs. *Nucleic Acids Res.*, **25**, 4619–4625.
- Roberts, R.L. and Fink, G.R. (1994) Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev.*, **8**, 2974–2985.
- Robertson, L.S. and Fink, G.R. (1998) The three yeast A kinases have specific signaling function in pseudohyphal growth. *Proc. Natl Acad. Sci. USA*, **95**, 13783–13787.
- Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
- Tanaka, K., Matsumoto, K. and Toh, E.A. (1989) IRA1, an inhibitory regulator of the RAS-cyclic AMP pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **9**, 757–768.
- Teunissen, A.W. and Steensma, H.Y. (1995) Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast*, **11**, 1001–1013.
- Thanos, D. and Maniatis, T. (1995) Virus induction of human IFN β gene expression requires the assembly of an enhanceosome. *Cell*, **83**, 1091–1100.
- Toda, T. et al. (1985) In yeast, RAS proteins are controlling elements of adenylate cyclase. *Cell*, **40**, 27–36.
- Toda, T., Cameron, S., Sass, P., Zoller, M. and Wigler, M. (1987) Three different genes in *S.cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell*, **50**, 277–287.
- Vivier, M.A. and Pretorius, I.S. (1998) Identification of a functional TATA element in the *STA2* glucoamylase gene promoter from *Saccharomyces cerevisiae*. *Curr. Genet.*, **33**, 10–15.
- Ward, M.P., Gimeno, C.J., Fink, G.R. and Garrett, S. (1995) SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. *Mol. Cell. Biol.*, **15**, 6854–6863.
- Xu, J.R. and Hamer, J.E. (1996) MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.*, **10**, 2696–2706.

Received October 12, 1998; revised December 22, 1998;
accepted January 11, 1999