The Flo8 Transcription Factor Is Essential for Hyphal Development and Virulence in *Candida albicans*[□]

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The transcription factor Flo8 is essential for filamentous growth in *Saccharomyces cerevisiae* and is regulated under the cAMP/protein kinase A (PKA) pathway. To determine whether a similar pathway/regulation exists in *Candida albicans*, we have cloned *C. albicans* FLO8 by its ability to complement *S. cerevisiae* flo8. Deleting FLO8 in *C. albicans* blocked hyphal development and hypha-specific gene expression. The flo8/flo8 mutant is avirulent in a mouse model of systemic infection. Genome-wide transcription profiling of *efg1/efg1* and *flo8/flo8* using a *C. albicans* DNA microarray suggests that Flo8 controls subsets of Efg1-regulated genes. Most of these genes are hypha specific, including *HGC1* and *IHD1*. We also show that Flo8 interacts with Efg1 in yeast and hyphal cells by in vivo immunoprecipitation. Similar to *efg1/efg1*, *flo8/flo8* and *cdc35/cdc35* show enhanced hyphal growth under an embedded growth condition. Our results suggest that Flo8 may function downstream of the cAMP/PKA pathway, and together with Efg1, regulates the expression of hypha-specific genes and genes that are important for the virulence of *C. albicans*.

INTRODUCTION

Candida albicans is the most frequently isolated opportunistic fungal pathogen of humans. Invasive candidiasis is currently the fourth most common cause of bloodstream infections in hospitals and is associated with the highest mortality rate, ~40%, of all the nosocomial bloodstream infections (Kullberg and Filler, 2002). The ability to undergo reversible morphogenetic transitions between yeast, pseudohyphae, and hyphae has been shown to be important for its pathogenicity in systemic infections. Mutants defective in the morphogenetic transition show a much reduced virulence in mouse models of systemic infection (Lo *et al.*, 1997; Whiteway and Oberholzer, 2004).

Flo8 is a transcription factor critical for invasive growth and flocculation in haploids and pseudohyphal growth in diploids of *Saccharomyces cerevisiae* (Liu *et al.*, 1996). It is required for the expression of a family of FLO genes that encode glycerol phosphoinositol (GPI)-anchored cell surface adhesin genes (Kobayashi *et al.*, 1996). Among them, FLO11 is essential for invasive/filamentous growth and flocculation (Lo and Dranginis, 1998). Flo8 functions downstream of the cyclical AMP (cAMP)-dependent protein kinase A (PKA) pathway because *flo8* mutants block the effect of an activated PKA/cAMP pathway on FLO11 expression (Rupp *et al.*,

1999). Flo8 has been shown to bind to the promoter of FLO11, and the binding is regulated by Tpk2 (Pan and Heitman, 2002), one of three catalytic subunits of PKA in *S. cerevisiae*. Phosphorylation of Flo8 by Tpk2 is required for Flo8 interaction with the FLO11 promoter both in vivo and in vitro (Pan and Heitman, 2002).

In *C. albicans*, several signaling pathways can regulate the yeast-to-hypha transition (Whiteway and Oberholzer, 2004). Among them, the cAMP/PKA pathway plays a major role in hyphal development and virulence, because many mutants in the pathway are defective in hyphal growth and show reduced virulence. The adenylate cyclase Cdc35 and its associated protein Cap1 are required for hyphal development under all hyphal-inducing conditions, including serum (Bahn and Sundstrom, 2001; Rocha *et al.*, 2001). The cyclase activity is regulated by two G proteins, Ras1 and Gpa2, in *C. albicans* (Feng *et al.*, 1999; Sanchez-Martinez and Perez-Martin, 2002; Miwa *et al.*, 2004; Maidan *et al.*, 2005). *ras1* mutants are defective for hyphal formation under induction with serum filtrate in liquid media (Feng *et al.*, 1999), whereas *gpa2* mutants are defective in hyphal growth on solid hyphal-inducing media (Miwa *et al.*, 2004; Maidan *et al.*, 2005). The G proteins act through PKA to induce morphogenesis in *C. albicans*. *C. albicans* PKA consists of one regulatory subunit, Bcy1, and two catalytic subunits, Tpk1 and Tpk2 (Sonnenborn *et al.*, 2000; Bockmuhl *et al.*, 2001; Cassola *et al.*, 2004). Tpk1 and Tpk2 have distinct functions in hyphal development because their mutants have differential effects in different media (Bockmuhl *et al.*, 2001; Cassola *et al.*, 2004). One potential target of the cAMP/PKA pathway is Efg1, a basic helix-loop-helix protein similar to StuA of *Aspergillus nidulans* and Sok2 and Phd1 of *S. cerevisiae* (Stoldt *et al.*, 1997). *efg1/efg1* mutants are unable to form hyphae in all liquid-inducing media (Stoldt *et al.*, 1997), but the mutants show enhanced hyphal formation under embedded growth con-

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Table 1. *C. albicans* and *S. cerevisiae* strains used in this study

Strain	Genotype	Source
<i>C. albicans</i>		
SC5314	Wild type	Gillum <i>et al.</i> , 1984
CAI4	<i>ura3::1 imm434/ura3::1 imm434</i>	Fonzi and Irwin, 1993
CCF1	<i>ura3::1 imm434/ura3::1 imm434 FLO8/flo8::hisG-URA3-hisG</i>	This study
CCF2	<i>ura3::1 imm434/ura3::1 imm434 FLO8/flo8::hisG</i>	This study
CCF3	<i>ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG-URA3-hisG</i>	This study
CCF4	<i>ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG</i>	This study
HLC52	<i>ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG-URA3-hisG</i>	Lo <i>et al.</i> , 1997
CR216	<i>ura3::1 imm434/ura3::1 imm434 cdc35::hisG/cdc35::hisG-URA3-hisG</i>	Rocha <i>et al.</i> , 2001
BWP17	<i>ura3::1 imm434/ura3::1 imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	Wilson <i>et al.</i> , 1999
HL3426	<i>ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG</i>	This study
HL3271	<i>RP10::ACT1p-FLO8-13MYCFLAG-URA3</i> <i>ura3::1 imm434/ura3::1 imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG EFG1/</i> <i>EFG1-TAP-URA3 RP10::ACT1p-FLO8-13MYCFLAG-HIS1</i>	This study
<i>S. cerevisiae</i>		
MY1384	MATa wild type	Microbia, Cambridge, MA
CGx68	MATa/ α wild type	Gimeno <i>et al.</i> , 1992
HL850	MATa <i>flo8::hisG ura3-52</i>	Liu <i>et al.</i> , 1996
HL852	MATa/ α <i>flo8::hisG/flo8::hisG ura3-52/ura3-52</i>	Liu <i>et al.</i> , 1996
HL367	MATa <i>ste7::LEU2 ura3-52 leu2::hisG</i>	Liu <i>et al.</i> , 1993
HL362	MATa <i>ste12::LEU2 ura3-52 leu2::hisG</i>	Liu <i>et al.</i> , 1993
HL2000	MATa <i>tec1::HIS3 ura3-52</i>	Chou <i>et al.</i> , 2004
EGY48 (p8op-lacZ)	MATa, <i>his3, trp1, LexA_{op}($\times 6$)-leu2, LexA_{op}($\times 8$)-lacZ flo8-1</i>	Gyuris <i>et al.</i> , 1993

ditions (Giusani *et al.*, 2002). Transcription profiling of cAMP signaling in *C. albicans* shows that Ras1 regulates a subset of Cdc35-regulated genes, but Efg1-regulated genes are distinct from those modulated by Cdc35 except for the class of genes induced during the yeast-to-hypha transition (Harcus *et al.*, 2004). The hypha-specific genes include GPI-anchored cell wall proteins, secreted proteases, and a G₁ cyclin-like gene, and they have been shown to be important for virulence in systemic infections (Liu, 2001; Zheng and Wang, 2004).

Because Flo8 is a target of the cAMP/PKA pathway essential for invasive/filamentous growth in *S. cerevisiae*, we were interested to determine whether a similar regulator exists in *C. albicans*. We cloned a *C. albicans* FLO8 homolog by functional complementation of a *S. cerevisiae* flo8 mutant. FLO8 deletions in *C. albicans* completely blocked hyphal development and the expression of hyphal genes, and led to avirulence in a systemic model of candidiasis. Genome-wide transcription analysis of *flo8/flo8* and *efg1/efg1* mutants suggests that Flo8 specifically regulates subsets of Efg1-regulated genes, mostly hypha-specific genes. We present evidence to suggest that Flo8 and Efg1 function together in regulating the hyphal transcriptional program in *C. albicans*.

MATERIALS AND METHODS

Strains and Culture Conditions

The *C. albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. The pseudohyphal colony formation and invasive growth of *S. cerevisiae* were examined as described previously (Liu *et al.*, 1993; Roberts and Fink, 1994). Lee's and YPD + 10% bovine serum media were used for hyphal induction (Lane *et al.*, 2001b). YPS with 1% agar was used for colony morphology assay under embedded conditions (Brown *et al.*, 1999).

FLO8 Cloning and Disruption

The clone pCF56 was isolated from a *C. albicans* genomic library (Liu *et al.*, 1994) based on its ability to suppress the invasive defect of a *S. cerevisiae* flo8 mutant (HL850) on SC-Ura medium. pCF56 contains a 3.4-kb insert, with a 2454 base pair open reading frame (ORF), corresponding to a protein (Flo8) of 817 amino acids. A 2.4-kb BglIII-BamHI fragment in pCF56 was replaced with a 4-kb BglIII-BamHI *hisG-URA3-hisG* fragment from pCUB6, generating plas-

mid pCF56-FLO8NA. A 1.5-kb SpeI fragment from pCF56 was inserted into the SpeI site of pCF56-FLO8NA to generate pCF56-FLO8 Δ for disruption of FLO8. pCF56-FLO8 Δ was digested with XhoI and SstI and transformed into CAI4 to produce FLO8/flo8 and flo8/flo8 strains. Spontaneous Ura⁻ derivatives were selected on 5-fluoro-orotic acid-containing medium. The disruption was confirmed by Southern blotting (Supplemental Figure 1). The plasmids used in this study are listed in Table 2. All the clones and plasmids were confirmed by DNA sequencing.

Plasmid Construction

pBA1 was constructed by subcloning an ADHI promoter fragment with NotI and EcoRV at each end into BES116 (Feng *et al.*, 1999) at the NotI and EcoRV site.

pBA1-CaFLO8 for the expression of *C. albicans* FLO8 in *C. albicans* was constructed by placing a 2.45-kb PCR fragment containing FLO8 coding sequence into the BglIII-Clal site of plasmid pBA1. Primer 1 and primer 2 were used for PCR amplification.

pBA1-CaFLO8 Δ N was constructed by placing a 2.1-kb PCR fragment into the BglIII-Clal site of plasmid pBA1. Primer 5'CTGGGATCCGTATGCTTCCTCTTATACAGCAG and primer 2 in Table 3 were used for PCR amplification.

pBES116-CaFLO8, a 3-kb HindIII-EcoRV fragment from pCF56 was inserted into BES116 (Feng *et al.*, 1999). Then, a 2.4-kb KpnI-HindIII PCR fragment containing *C. albicans* FLO8 promoter was amplified (primer 3 and 4) and inserted before the FLO8 coding region.

For pVTU-CaFLO8, a 2.45-kb FLO8 containing PCR product (primers 7 and 8) was subcloned into the SstI-XhoI site of pVT102U to express the *C. albicans* FLO8 under the control of ADHIp in *S. cerevisiae*. The full-length coding sequences of FLO8 and EFG1 were PCR amplified (primers 9–14) and inserted into pEG202 and pJG4–5, generating pEG202-CaFLO8, pEG202-EFG1, and pJGCaFLO8 for the two-hybrid assay. SC5314 genomic DNA was used as template for PCR amplification. All constructs were verified by DNA sequencing. The plasmids used in this study are listed in Table 2. The primers used for PCR amplification are listed in Table 3.

For CaFLO8-MYC13, a 580-base pair fragment containing 13xMYC was PCR amplified from the *S. cerevisiae* vector pFA6a-13MYC-HIS3MX6 (Longtine *et al.*, 1998) with oligonucleotides 5 and 6 (Table 3), and cloned into the BamHI and SphI sites of the *C. albicans* ACT1p-FLAG-HIS1 vector (Umeyama *et al.*, 2002) to create pPR671. A NotI and an MluI site were introduced between the BamHI and myc13 sequence in pPR671. The FLO8 gene was amplified from *C. albicans* genomic DNA (SC5314) using oligonucleotides 15 and 16 (Table 3). The 2.5-kb PCR product was digested with BamHI and MluI and then inserted into the BamHI-MluI sites of pPR671, to produce pCaFLO8–13MYC-FLAG-HIS1 (pPR672). pPR672 was digested with StuI to target the integration of the plasmid into the genomic RP10 locus under HIS1 selection. Expression of Flo8p under the ACT1 promoter in YPD at 30°C was verified by

Table 2. Plasmids used in this study

Plasmid	Description	Source
Library	<i>C. albicans</i> genomic library in pRS202	Liu <i>et al.</i> , 1994
pBES116	URA3 vector, digest with <i>AscI</i> for integration at <i>ADE2</i>	Feng <i>et al.</i> , 1999
pBES116-CaFLO8	5.4-kb <i>C. albicans</i> FLO8 fragment including 2.5-kb FLO8 promoter region in BES116	This study
pBA1	<i>C. albicans</i> ADH1 promoter in BES116	This study
pBA1-CaFLO8	2.45-kb full-length <i>C. albicans</i> FLO8 in BA1	This study
pBA1-CaFLO8ΔN	2.1-kb <i>C. albicans</i> FLO8ΔN in BA1	This study
pFLAG-ACT1-URA	<i>C. albicans</i> ACT1 promoter, FLAG, URA3	Umeyama <i>et al.</i> , 2002
pPR671	<i>C. albicans</i> ACT1 promoter, 13 × myc-FLAG, HIS1	This study
pPR672	2.45-kb full-length <i>C. albicans</i> FLO8 in pPR671	This study
pCF56	3.4-kb <i>C. albicans</i> FLO8 in pRS202	This study
pCF56-FLO8NΔ	N-terminal region of <i>C. albicans</i> FLO8 was replaced with <i>hisG-URA3-hisG</i>	This study
pCF56-FLO8Δ	Neutral region of <i>C. albicans</i> FLO8 was replaced with <i>hisG-URA3-hisG</i>	This study
pRS202	<i>S. cerevisiae</i> URA3/2μ vector	Christianson <i>et al.</i> , 1992
pVTU102	<i>S. cerevisiae</i> URA3/2μ vector with ADH1 promoter and terminator	Vernet <i>et al.</i> , 1987
pVTU-CaFLO8	2.45-kb full-length <i>C. albicans</i> FLO8 in pVTU102	This study
pJG4-5	2μ, TRP1-based vector carrying the B42 activation domain	Gyuris <i>et al.</i> , 1993
pJG-CaFLO8	2.45-kb full-length <i>C. albicans</i> FLO8 in pJG4-5	This study
pEG202	2μ, HIS3-based vector carrying the <i>lexA</i> DNA-binding domain	Gyuris <i>et al.</i> , 1993
pEG202-CaFLO8	2.45-kb full-length <i>C. albicans</i> FLO8 in pEG202	This study
pEG202-EFG1	1.6-kb full-length <i>EFG1</i> in pEG202	This study

Western analysis using an anti c-myc-conjugated peroxidase antibody (Roche Diagnostics, Indianapolis, IN) enabling detection of Flo8p at ~150 kDa.

pMSCTAP was constructed by cloning a codon-optimized TAP-tag that contains two copies of protein A sequence followed by a single copy of the calmodulin binding peptide (Rigaut *et al.*, 1999) into a BlueScript vector that carries *C. albicans* URA3 (Shrivastava and Liu, unpublished data). The TAP-CaURA3 can be used as a cassette for PCR amplification and insertion of the TAP in frame to the C terminus of a gene of interest.

Efg1-TAP Strain

Primers EFG1-F and EFG1-R are used to PCR amplify TAP-CaURA3 from pMSCTAP (EFG-TAP-F 5'-CCTTCACCCCAACAACATCAAGCTAAT-CAATCAGCTAGCACTGTTGCCAAAGAAGAAAGAAACATGGAAAAAG-AGAAGATGG-3'; URA3-EFG-R 5'-CGTTCATGTCAATGGATTGG-GAGAAGATTATGATCTATACTATTTCTTTTTTATTATTCCGCGG-TGGCGGCCGCTCTAG-3'). The underlined regions are the sequences identical to the beginning of TAP and URA3, respectively. The 5' 60 bp are homologous to the C-terminal end of *EFG1*. The amplified DNA was transformed directly into *C. albicans* (Wilson *et al.*, 1999), and the transformants with the TAP fused to the C terminus of Efg1 by homologous recombination were identified by PCR and verified by Western blot.

C. albicans Microarray Construction

A *C. albicans* microarray containing 6917 elements was printed with a *C. albicans* 70-mer set (QIAGEN Operon, Alameda, CA) on a code-link activated slide (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by Microarray (Nashville, TN). The 70-mer set includes ~6530 ORFs from the Assembly 6 ORFs released by the *C. albicans* Genome Sequencing Project at Stanford University (Stanford, CA). Oligonucleotide information is available at www.qiagen.com. The set includes 192 randomly generated 70-mers as negative controls. The mean intensity of the negative controls is used as the basal hybridization intensity in data evaluation.

Preparing cDNA for Microarray Experiments

Total RNA was extracted using the hot acid phenol method with the addition of phase lock gels (Eppendorf, Westbury, NY) and a LiCl precipitation. The quality of RNA was checked by running the samples on a nanochip using the Agilent Bioanalyzer 2100 (University of California Irvine DNA Microarray Facility, Irvine, CA). Samples of high-quality RNA, as determined by rRNA profiles, were used for cDNA synthesis. For each experiment, RNA from two samples was pooled (12 μg each) and annealed to 10 μg of Oligo(dT) and

Table 3. Primers used in this study

Primer	Sequence	Purpose and features
1	5'-TGGGATCCATGAATCATAAACAAGTACTA	BA1-CaFLO8
2	5'-TGATCGATCCTCTAATCGCCATTTTC	
3	5'-CTGGGTACCTGGAATGAGGTGTGTTTTTTTG	
4	5'-ATCATGATAAAGCTTGTTGTTAGGGTTATT	BES116-CaFLO8
5	5'-GCGGATCCATTGCGGCCGCTTTACGACGCGT CCCC GGGTTAATTAACGGTGAA	
6	5'-ATCGCATGCGTGATTGATTAATTTTTGTT CACC	pPR671
7	5'-CTGGAGCTCATGAATCATAAACAAGTACTA	pVTU-CaFLO8
8	5'-CTGCTCGAGCCTCTAATCGCCATTTTC	
9	5'-GTCGGATCCCGATGAATCATAAACAAGTACTA	pEG202-CaFLO8
10	5'-CTGCTCGAGCCTCTAATCGCCATTTTC	
11	5'-CTGGAATTCATGTCAACGTATTCTATACCC	pEG202-EFG1
12	5'-CTGGGATCCTTACTTTTCTTCTTTGGCAAC	
13	5'-TCGCTCGAGATGAATCATAAACAAGTACTA	pJG-CaFLO8
14	5'-GTCGGATCCCGATGAATCATAAACAAGTACTA	
15	5'-CGGGATCCCATGAATCATAAACAAGTACTACCAG	pPR672
16	5'-GGCGACGCGTCGATCGCCATTTCAATTGGATCTGC	

Restriction sites are underlined. Bold sequences in primers 5 and 6 are from Myc.

Random 9mer primers from the Prime-It II kit (Stratagene, La Jolla, CA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a mixture containing 0.5 mM deoxynucleoside triphosphates (aminoallyl-dUTP:dT in a 3:2 ratio), 5× first strand buffer (Invitrogen), and 0.1 M dithiothreitol (DTT) overnight at 42°C. The RNA was hydrolyzed with 0.2 M NaOH and 0.1 M EDTA at 65°C for 15 min and subsequently neutralized with 0.33 M Tris, pH 7.4. The cDNA was washed with double distilled H₂O several times and concentrated to a small volume with a Microcon-3 filter (Millipore, Billerica, MA) and stored at -20°C.

Coupling and Microarray Hybridization

The cDNA was thawed at 42°C for 5 min, resuspended in 0.05 M sodium bicarbonate buffer, pH 9.0, and incubated with Cy3 or Cy5 dye (GE Healthcare) for 1 h at room temperature in the dark. Alternatively, the cDNA was coupled to Alexa Fluor 555 or 647 (Invitrogen) according to the manufacturer's instructions. The QIAGEN PCR purification kit was used to remove any unincorporated dye and eluted with 30 µl of 10 mM Tris-Cl, 5 mM EDTA, pH 8.0, twice. The whole sample was loaded into an Ultravette disposable cuvette (Brandtech Scientific, Essex, CT) and scanned from optical density (OD)₂₀₀ to OD₈₀₀ using a spectrophotometer to quantify the amount of cDNA generated as well as the amount of dye coupled to the cDNA. Then, volumes containing equal amounts of cDNA for the appropriate experiments were mixed and concentrated using a Microcon-30 filter. Either 5 or 9 µl of the probe (depending on the size of the coverslip) was heated to 100°C for 2 min. Meanwhile, the microarray slide was washed in 0.2% SDS for 10 min, washed in filtered H₂O, dipped in ethanol, and spun dry. A hybridization chamber and Millipore buffer #3 was prewarmed (50°C) and two-thirds volume Millipore buffer was added to the probe. The mixture was spun down before adding to the microarray slide. The slides were hybridized in the hybridization chambers for 16–20 h at 50°C.

Microarray Data Analysis

After hybridization, slides were washed, dried, and then scanned with a GSI Lumonics ScanArray 4000 slide scanner (GMI, Ramsey, MN) using its Scan Array software. The background-subtracted intensity of Cy3 and Cy5 at each spot was determined using the QuantArray software of the scanner. The background-subtracted intensities of Cy3 and Cy5 were used to plot $\log_2(\text{Cy5}/\text{Cy3})$ ratio for each element on a microarray against $\log_{10}(\text{Cy5} \times \text{Cy3})$ (R-I plot) (data not shown). R-I plots were used to predict the quality of hybridization. For example, curved R-I plots (usually because of photobleaching of one dye) or scattered R-I plots (usually indicates degraded RNA) were not used for subsequent data analysis. We routinely carried out four experimental repeats and array hybridizations for each experiment. Data from two high-quality hybridizations for each experiment, as determined by their R-I plots, were used for subsequent data analyses. Because equal amounts of cDNA for each dye were used in hybridization, we used total intensity normalization to normalize the Cy3 and Cy5 for each hybridization. The normalization involved scaling the Cy5 intensities by multiplying them with a normalization factor, which was determined by dividing the sum of Cy3 intensities by the sum of the Cy5 intensities. The ratio of Cy5i to Cy3i was then calculated using the corrected Cy5 values. The ratios that were above 2 times basal cutoff ($\text{Cy5i} \times \text{Cy3i} > \text{avgCy5ibasal} + \text{avgCy3ibasal}$) and threefold cutoff were log transformed and clustered with the average linkage cluster in a hierarchical cluster program (Eisen *et al.*, 1998). The clustered data were viewed in TreeView. The cluster and TreeView programs are at <http://rana.stanford.edu/software/>.

Immunoprecipitation

C. albicans cells were grown to $3\text{--}5 \times 10^7$ cells/ml in 50 ml of YPD, SSA, or Lee's media in yeast or hyphal growth conditions. The cells were harvested by centrifugation at 4°C, washed, and resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 8, 250 mM NaCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 0.5 µg/ml leupeptin, 1.4 µg/ml pepstatin, 2.4 µg/ml chymostatin, and 17 µg/ml aprotinin) and equal volume of glass beads (Sigma, St. Louis, MO). Cells were lysed at 4°C using a Fast-Prep system (FP120; Thermo Electron, Waltham, MA). Cell lysates were centrifuged for 10 min at 13,000 rpm in a microcentrifuge at 4°C. Protein extract containing 5 mg of protein was subjected to immunoprecipitation using 60 µl of rabbit IgG agarose bead slurry that was preincubated once with 0.2 mg/ml sheared salmon sperm DNA, 0.5 mg/ml bovine serum albumin in phosphate-buffered saline (PBS), and washed once in the lysis buffer. After incubation for 2 h at 4°C, beads were washed six times with 0.5 ml of lysis buffer and once with 1 ml of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). Bound proteins were eluted from the beads in 60 µl of elution buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS) by incubation for 10–15 min at 65°C. Proteins were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond; GE Healthcare). After blocking in 3% skim-milk powder in 0.05% PBS, Tween 20, a peroxidase-conjugated anti-c-myc antibody (Roche Diagnostics) was used to probe for myc-tagged proteins, which were then detected using the ECL system (GE Healthcare).

Figure 1. Functional cloning of *C. albicans* FLO8 in *S. cerevisiae* *flo8* mutant. *C. albicans* FLO8 was cloned by complementation of *S. cerevisiae* *flo8* mutants in invasive growth (A). *C. albicans* FLO8 could also complement *S. cerevisiae* *flo8* in pseudohyphal growth (B) and biofilm formation (C). Haploid wild-type (MY1384) and *flo8* (HLY850) strains carrying a vector (pRS202) or FLO8 (pCF56) were grown at 30°C on YPD for 3 d (A), YPD with 0.3% agar for 13 d (C), diploid wild-type (CG68) and *flo8/flo8* (HLY852) strains carrying the pRS202, or pCF56 grown on SLAD (synthetic low ammonium dextrose medium) at 30°C for 4 d (B).

Yeast Two-Hybrid Analysis

Two-hybrid assays were performed as described previously (Gyuris *et al.*, 1993). Yeast strain EGY48 containing the *LexAop-LacZ* reporter plasmid pSH18-34 was cotransformed with pEG202-based plasmids expressing LexA DNA binding domain fusions and pJG4-5-based plasmids containing transcriptional activation domain fusions (Gyuris *et al.*, 1993).

Northern Blotting

RNA extraction and Northern blotting were performed as described by Lane *et al.* (2001a). A 3.4-kb FLO8 fragment from pCF56 was used as a probe for Northern analysis. PCR products for *C. albicans* ECE1, HWP1, ALS1, HGC1, IHD1, and HSP31 were used for probing Northern blots.

Virulence Assay

The virulence of *C. albicans* strains was tested as described by Chen *et al.* (2000). ICR male mice (18–21 g) from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China) were used for the virulence assay.

Nucleotide Sequence Accession Number

The GenBank accession number for the *C. albicans* FLO8 nucleotide sequence is AF414113 and orf19.1093.

RESULTS

Cloning of a *C. albicans* Homologue of ScFLO8 by Complementation

Blast analysis of the *S. cerevisiae* Flo8 protein sequence against the *C. albicans* genome sequence did not identify a Flo8 orthologue. Therefore, we tried to clone potential *C. albicans* homologues of ScFlo8 by functional complementation in an *S. cerevisiae* *flo8* mutant. A *C. albicans* genomic library was transformed into the *S. cerevisiae* *flo8* mutant, and genes that complemented the *flo8* defect in invasive growth were isolated. The isolated clones were further examined for their abilities to suppress the invasive growth in a *flo11* mutant. Among the several clones isolated, pCF56 showed

A

LUFS

TTKQVLNSLILDFLVKHQFQDTAKAFSKESPNI	SIPPLMDCSQGFLLEWNOVFEDLEQVRYG	CaFlo8(30-92)
NCKNTLNEYIFDELTKSSLKNTAAAFQAQDAHLDR	TF SKVVDTPQGFLYEWNQIFWDIENTSSS	Flo8(72-154)
EADKMLDVYIHDYLVKRDLCATAQAFQAEQKVSS	D. PVAIDAPGGFLFEWWSVFWDIFARTN	Leunig(7-68)
QAREKLALYVVEYLLHVGAQKSAQTFLLSEIRWEK	NIT. . LGEPFGFLHTWVCVFWDLYCAAPE	Ssdp(15-76)
RQRDELNRATADYLRNNGYEEAYSVEKKEAELDM		Lis1(6-39)

LisH

B

C

		β -gal Activity	Growth on Leu ⁻
CaFlo8(1-817)	1 LUFS Q-rich(112-395) 817	60.35 $\pm$ 0.54	++++
CaFlo8(123-817)	123 817	37.32 $\pm$ 3.08	++++
CaFlo8(1-573)	1 573	1.029 $\pm$ 0.029	+
CaFlo8(1-122)	1 122	0.232 $\pm$ 0.011	+
CaFlo8(123-617)	123 617	0.012 $\pm$ 0.001	-

Figure 2. Sequence alignment and functional analysis of Flo8 LUFS domain. (A) Sequence alignment of the LUFS domain between *C. albicans* Flo8 and other regulatory proteins. Identical residues are shaded in black and conserved residues are shaded in gray. (B) *C. albicans* Flo8 LUFS domain is required for its functional complementation in *S. cerevisiae* *flo8* mutants. Diploid wild-type (CG68) and *flo8/flo8* (HLY852) strains carrying a vector (pVTU) or *FLO8* expression plasmids (pVTU-CaFlo8, pVTU-CaFlo8₁₂₃₋₈₁₇, pVTU-CaFlo8₁₋₁₂₂) were grown on SLAD for 4 d. (C) Transcriptional activity of Flo8 is located mostly at the C-terminal domain. Transcriptional activity of various *lexA*_{BD}-Flo8 fragments is shown in β -galactosidase activity and growth rate on SC-Leu medium.

strongest suppression of invasive growth in *flo8* (Figure 1A), and it did not suppress *flo11* (our unpublished data). The pCF56 also suppressed the filamentous growth defect of a diploid *flo8* mutant (Figure 1B). ScFlo8 has been shown to be essential for the formation of biofilm-like colonies on media with low percentage agar (Reynolds and Fink, 2001), and we found that the defect could be partially suppressed by pCF56 (Figure 1C). pCF56 contains a gene that encodes a putative protein of 817 aa, with a small region (amino acids 30–92) highly similar to amino acids 72–154 of ScFlo8 (Figure 2A). We therefore designated this gene *C. albicans* FLO8. The region conserved between the two Flo8 proteins contains a LUFS domain (LUG/LUH, Flo8, single-stranded DNA binding protein) (Conner and Liu, 2000). LUG (Leunig) is a key regulator of flower-specific gene expression during flower development in *Arabidopsis* (Conner and Liu, 2000). The single-stranded DNA binding protein (Ssdp) regulates the activity of LIM-homeodomain protein complexes (van Meyel *et al.*, 2003) (Figure 2A). Within the LUFS domain, there is a Lissencephaly type 1-like homology motif

(LisH) (Emes and Ponting, 2001). The crystal structure of the N-terminal domain of mouse LIS1 shows that the LisH motif is a thermodynamically very stable dimerization domain (Kim *et al.*, 2004a).

To determine whether the LUFS domain is important for *C. albicans* Flo8 function, we deleted the N-terminal 122 amino acids of Flo8 and introduced Flo8₁₂₃₋₈₁₇ and Flo8₁₋₁₂₂ into haploid and diploid *flo8* mutants of *S. cerevisiae*. Whereas the full-length *C. albicans* Flo8 could completely complement the invasive/filamentous growth defect of the *flo8* mutants, neither Flo8₁₂₃₋₈₁₇ nor Flo8₁₋₁₂₂ could (Figure 2B; our unpublished data). Therefore, both the LUFS domain in Flo8₁₋₁₂₂ and other domains in Flo8₁₂₃₋₈₁₇ are required for Flo8 function. To determine whether the LUFS domain is important for *C. albicans* Flo8 transcriptional activity, we fused different Flo8 domains to the *lexA* DNA binding domain, and measured the transcriptional activities of the fusion proteins in *S. cerevisiae*. Fusion of Flo8 to the DNA binding domain of *lexA* gave a high level of *lexAop-lacZ* expression in a *S. cerevisiae* *flo8-1* strain, which carries a

Figure 3. FLO8 is essential for hyphal development and the expression of hypha-specific genes in *C. albicans*. (A) *C. albicans* *flo8/flo8* cells are unable to form hyphae (top row). Cells were induced in YPD + 10% serum for 3.5 h at 37°C (top). Colony morphology of *flo8/flo8* mutants (middle and bottom rows). Strains were plated on solid serum-containing medium and solid Lee's medium, incubated at 37°C for 5 and 7 d, respectively. (B) *flo8/flo8* mutants were defective in the induction of hypha-specific genes as well as *ALS1*. Cells were grown in YPD + 10% serum at 37°C for 3.5 h or grown in YPD at 25°C for 6 h and collected for RNA extraction and Northern analysis. Strains shown in A and B are WT (SC5314), *FLO8/flo8* (CCF1, +/+), *flo8/flo8* (CCF3, -/-), *flo8/flo8*+vector [CCF4 + pBA1, -/-(v)], *flo8/flo8* + *ADH1p-FLO8* [CCF4 + pBA1-CaFLO8, -/-(+)_{ADH1p}], *flo8/flo8* + *FLO8* [CCF4 + pBES116-CaFLO8, -/-(+)_{FLO8p}]. (C and D) Flo8 LUFS domain is required for hyphal development (C) and the induction of hypha-specific genes (D). The experiments in C and D were carried out under the same conditions as in A and B, respectively. Strains -/-(+) and -/(ΔN) in D are *flo8/flo8* + *ADH1p-FLO8* [CCF4 + pBA1-CaFLO8, -/-(+)_{ADH1p}] and *flo8/flo8* + *ADH1p-flo8ΔN* [CCF4 + pBA1-CaFLO8ΔN, -/-(ΔN)_{ADH1p}].

nonsense mutation encoding Flo8 with a truncated LUFS domain (Liu *et al.*, 1996). This observation is similar to that reported for *S. cerevisiae* Flo8 (Rupp *et al.*, 1999; Pan and Heitman, 2002). Therefore, *C. albicans* Flo8 is likely a transcriptional activator. Deleting the N-terminal 122 amino acids decreased Flo8 transcriptional activity by only 50%, but

deleting the C-terminal 200 amino acids reduced the transcriptional activity by 60-fold (Figure 2C), suggesting that the transcriptional activation domain is probably at the C terminus. Alternatively, the C-terminal domain could interact with a transcriptional activator, leading to transactivation of Flo8. Removing the two regions together completely

abolished the transcriptional activity. Our data suggest that both the N-terminal LUFS domain and the C-terminal region are important for Flo8 function.

Flo8 Is Essential for Hyphal Development

To study the function of Flo8 in *C. albicans*, we deleted both *FLO8* alleles by using a *hisG-URA3-hisG* cassette. Successful deletion of *C. albicans FLO8* was confirmed by Southern analysis (Supplemental Figure 1). *flo8/flo8* mutants had no significant differences in growth rate and cell morphology from wild-type cells under yeast growth conditions at 30°C. However, they were completely unable to respond to hyphal induction in all liquid hyphal-inducing conditions examined, including serum-containing media, Lee's medium (Figure 3A), SSA, CAA, GlcNAc, and RPMI 1640 (our unpublished data). *flo8/flo8* mutant cells displayed yeast-like cell morphology under the hyphal inducing conditions. *flo8/flo8* mutants also failed to form hyphal colonies on solid media and formed only smooth colonies even after extended growth at 37°C (Figure 3A). The defects of *flo8/flo8* mutants could be rescued by integrating a wild-type *FLO8* under either the *ADH1* promoter or under its own promoter (Figure 3, A and B). The complementation of *flo8/flo8* was not because of differences in levels of *URA3* expression or insertion positions in the genome because a copy of *URA3* inserted at the same *ADE2* locus did not suppress the defects (Figure 3A). Partial defects in hyphal morphogenesis and hyphal colony formation were observed in the *FLO8/flo8* mutant and the *flo8/flo8* mutant transformed with a wild-type copy of *FLO8* under its own promoter (Figure 3A). This haploid insufficiency has been observed in many mutants of hyphal regulators. Based on the haploid insufficiency and the complementation of *flo8/flo8* defects by wild-type *FLO8*, we suggest that the defects in *flo8/flo8* are from the *FLO8* deletion.

Consistent with the defect in hyphal morphogenesis, the *flo8/flo8* mutants were also defective in the expression of hypha-specific genes (Figure 3B). *ECE1* and *HWP1* were highly induced in wild-type cells under hyphal-inducing conditions, but they were not induced at all in *flo8/flo8* mutants. The *FLO8/flo8* heterozygote showed reduced expression of hypha-specific genes. Because Flo8 is required for the expression of several *FLO* genes of cell surface adhesins in *S. cerevisiae*, we also examined the expression of *ALS1*, which is expressed in both yeast and hyphal cells in an Efg1-dependent manner. Our data showed that Flo8 is essential for hyphal morphogenesis and the induction of hypha-specific genes and *ALS1*. *FLO8* was expressed in both yeast and hyphae at low levels, as determined by the Northern analysis (our unpublished data).

The LUFS domain is important for Flo8 functions in hyphal development. A *flo8/flo8* mutant transformed with *FLO8ΔN*, which lacks the LUFS domain, was unable to develop hyphae or express hyphal genes (Figure 3, C and D).

flo8/flo8 Is Avirulent in a Systemic Infection Model of Mice

The dimorphic transition ability of *C. albicans* has been linked with its pathogenicity in mice (Lo *et al.*, 1997, Chen *et al.*, 2000). Therefore, we examined the virulence of *flo8/flo8* mutants in a systemic model of infection. Cells (5×10^6) of wild type (CAI4), *flo8/flo8*, and *FLO8*-complemented *flo8/flo8* strain were inoculated into each mouse by tail vein injection. All three strains carry one copy of *URA3* integrated at the *ADE2* locus. We observed that the mice injected with wild-type and complemented *flo8/flo8* *C. albicans* started to lose weight after 1 d and started to die after 2 d, and all of the

Figure 4. *flo8/flo8* is avirulent in a systemic model. Survival curves for strains SC5314, CAI4 + pBES116, *flo8/flo8* (CCF4 + pBES116), and *flo8/flo8* + *FLO8* (CCF4 + pBES116-CaFLO8) in a mouse model of systemic infection are shown. For each strain, 8 ICR male mice were injected with 5×10^6 cells from tail vein. Percentage of survival is indicated in the y-axis.

mice died by day 15 (Figure 4). The mice injected with the *flo8/flo8*, in contrast, had no symptoms of illness, and all mice survived for more than 25 d (Figure 4). Therefore, *flo8/flo8* was avirulent in a mouse model of systemic infection.

Flo8 Is Required for Expressing Subsets of Efg1-regulated Genes

The phenotypes of the *flo8/flo8* mutant in various hyphal-inducing conditions are very similar to that of *efg1/efg1*. Both are unable to induce hyphal morphogenesis and express hypha-specific genes in serum-containing media, and both show severely reduced virulence in the systemic infection model in mice. To further define Flo8 functions and its relationship with Efg1, we compared transcription profiles of *flo8/flo8* and *efg1/efg1* mutants.

We performed microarray hybridizations with cells grown in two sets of hyphal-inducing media, YPD + serum and Lee's. Wild-type, *efg1/efg1*, and *flo8/flo8* cells were grown in YPD at 25°C for yeast growth and in YPD + serum at 37°C for hyphal growth. Similarly, the three strains were grown in Lee's medium at 25°C for yeast growth and in Lee's at 37°C for hyphal growth. After 3 h growth in YPD media or 6 h in Lee's medium, cells were harvested for RNA extraction. The RNA was reverse transcribed, and equal amounts of cDNA were labeled with Cy3 and Cy5 and hybridized to each slide. Wild type versus *efg1/efg1* and wild type versus *flo8/flo8* in each yeast growth or hyphal growth condition as well as wild-type hyphae versus wild-type yeast were compared on each slide. Changes in gene expression were measured as a ratio of normalized Cy3 versus Cy5 intensity at each position on each microarray slide. Most experiments had four experimental repeats. The ratio of normalized Cy3 versus Cy5 intensities from two repeats with high quality of hybridization, as determined by R-I plots (see *Materials and Methods*) were used in clustering analysis (Eisen *et al.*, 1998). The clustering result is visualized with TreeView (Figure 5A). The ratios used for the clustering analysis are available in Supplemental Figure 2.

Clustering analysis shows that Flo8 was essential for the expression of subsets of Efg1-regulated genes (Figure 5A). Overall, *flo8/flo8* and *efg1/efg1* were grouped together under

Figure 5.

Figure 5. Comparison of gene expression between *flo8/flo8* and *efg1/efg1* by DNA microarray. (A) TreeView of clustering analysis. Genes that had an intensity difference (Cy5-Cy3) greater than a basal intensity as well as a fold change greater than 3 were included in the clustering analysis. Wild-type (SC5314), *flo8/flo8* (CCF3), and *efg1/efg1* (HLC52) strains were grown under either yeast growth conditions (YPD or Lee's, at 25°C) or hyphal-inducing conditions (YPD + 10% serum or Lee's, 37°C). Strains and growth conditions used in each hybridization are indicated at the top. Yeast growth conditions were grouped to the left and hyphal-inducing conditions to the right by the clustering program. The cluster of genes that are equally induced in YPD + serum and in Lee's medium are indicated as hypha-specific genes. Genes that are induced in hyphae but with a higher fold of induction in Lee's than in YPD serum are named Lee's hyphal genes. A group of Efg1 repressed genes, including *HSP31*, are labeled as *HSP31* cluster. A larger view for part of the genes in these three clusters is shown on the right. For *HWP1* and *ALS3*, two different 70-mers representing each gene are present in the *C. albicans* 70-mer set of 6530 ORFs (QIAGEN Operon). (B) Northern analysis of *HGC1*, *IHD1*, and *HSP31*. RNA used in the microarray experiments was used in the Northern analysis to confirm the expression of three differentially regulated genes identified from the microarray study. PCR products of the *HGC1*, *IHD1*, and *HSP31* ORFs were used for probing.

all experimental conditions by the cluster program, indicating that the transcription profiles were more similar between the two mutants than between two growth conditions. We did not identify genes regulated only by Flo8, but not by Efg1, under our experimental conditions. It seemed that genes whose expression was affected in the *flo8/flo8* mutant were similarly affected in the *efg1/efg1* mutant. Most of the Flo8-regulated genes were hypha-specific, induced only in hyphal cells in both YPD + serum and Lee's and the expression was blocked in both mutants. These include all known hypha-specific genes on the microarray (Figure 5A). *IHD1*,

identified in a genome-wide transcriptional profiling of genes induced or repressed during hyphal development by Nantel *et al.* (2002), was also found in this cluster, and further confirmed by Northern analysis (Figure 5B). The gene was independently found by Murad *et al.* (2001) as one of the genes regulated by the Nrg1 and Tup1 repressors. *IHD1* is predicted to encode a GPI-anchored cell wall protein (De Groot *et al.*, 2003), and the protein sequence also has similarity to glucan 1,4- α -glucosidase Sta1 in *Saccharomyces diastaticus* (Yamashita *et al.*, 1985). Some hypha-specific genes were expressed at higher levels in Lee's medium than in YPD + serum. These included *SAP4*, *SAP5*, and *CLN21/HGC1*, a G₁-type cyclin gene that was recently found to be hypha-specific and essential for hyphal morphogenesis (Zheng and Wang, 2004).

Not all Efg1-regulated genes were regulated by Flo8. Several Efg1-repressed genes were not repressed by Flo8 (Figure 5A). As confirmed by Northern blotting, *HSP31* was only detected in the *efg1/efg1* mutant in Lee's medium and in YPD + serum at 37°C, but not in wild type or *flo8/flo8* (Figure 5). Transcriptional profiling of *efg1/efg1* and *cdc35/cdc35* by Hargus *et al.* (2004) has demonstrated that hypha-specific genes are the only genes regulated by both Efg1 and the cAMP/PKA pathway. Many Efg1-regulated genes are not affected by the *cdc35/cdc35* mutant. Similarly, we find that, Flo8 only regulates subsets of Efg1-regulated genes and those genes are mostly hypha-specific.

Flo8 and Efg1 Interact In Vivo

The fact that all the Flo8-regulated genes we identified so far were similarly regulated by Efg1 suggests that Flo8 and Efg1 might function together to control the expression of these genes. To determine whether Flo8 and Efg1 act together in transcriptional activation, we first determined whether the two transcription factors interact by yeast two-hybrid sys-

Figure 7. EFG1 stimulated invasive growth in *S. cerevisiae* requires Flo8. Total and invasive growth of wild-type (MY1384), *ste7* (HLY367), *ste12* (HLY362), *tec1* (HLY2000), and *flo8* (HLY850) strains carrying a vector, EFG1 or FLO8, after 5 d of growth on SC-Ura.

tem. The Efg1 fusion to the DNA binding domain of *lexA* did not activate the transcription from *lexAop-lacZ*, in agreement with a recent report (Doedt *et al.*, 2004). When using the *lexA*-Efg1 as bait in the yeast two-hybrid system, we detected a weak two-hybrid interaction of Efg1 with Flo8 (Figure 6A). A reciprocal two-hybrid assay could not be performed because the *lexA*_{DB}-Flo8 fusion gave a high level of basal activity.

To further investigate whether Flo8 interacts with Efg1 in *C. albicans*, we performed immunoprecipitation experiments with tagged proteins. Flo8 was tagged at its C terminus with Myc13, and expression was under the control of the *ACT1* promoter. Efg1 was fused at its C terminus with two copies of protein A sequences followed by calmodulin binding protein. Both fusions were functional (our unpublished data). Immunoprecipitation of Efg1 with IgG beads was able to pull down Flo8 from both yeast and hyphal cells grown in three different growth media (Figure 6B). The interaction was specific because the interaction was barely detectable in the control strain that carried only Flo8-Myc13. Furthermore, the observed interaction was not due to the protein A (fused to Efg1), because protein A-beads could not bring down Flo8myc unless anti-myc antibodies were included in the precipitation (our unpublished data). The immunoprecipitation (IP) data suggest that Flo8 and Efg1 can interact in vivo. It also suggests that the interaction is not regulated in connection to growth forms. This is consistent with the finding that Flo8 and Efg1 are required for *ALS1* expression in yeast and hyphae.

The functional relationship between Efg1 and Flo8 was further studied by epistasis experiments in *S. cerevisiae*. Overexpression of EFG1 or *C. albicans* FLO8 in *S. cerevisiae* enhanced invasive growth in wild-type haploids and bypassed the requirement of the Kss1 mitogen-activated protein kinase pathway. But the effect of EFG1 overexpression was completely blocked in a *flo8* mutant (Figure 7). Therefore, Flo8 is required for Efg1-mediated transcriptional activation of invasive/filamentous growth in *S. cerevisiae*. Overexpression of EFG1 from the *PCK1* promoter in a *C. albicans* *flo8/flo8* mutant did not induce filamentous growth, and the *flo8/flo8* mutant carrying the EFG1 overexpression construct showed similar yeast growth morphology as the control *flo8/flo8* strain carrying a vector (our unpublished data). Although the result was in agreement with the epistasis study in *S. cerevisiae*, the extent of data interpretation was limited by the relatively weak phenotype observed in wild-type *C. albicans* expressing EFG1 under the *PCK1* promoter, because it only generated pseudohyphal

Figure 8. *flo8/flo8* and *cdc35/cdc35* show increased hyphal filamentation under microaerophilic condition. Cells of wild-type (SC5314), *efg1/efg1* (HLC52), *flo8/flo8* (CCF3), and *cdc35/cdc35* (CR216) were plated with molten YPS agar and grown for 56 h or 5 d at 25°C.

filaments. Reciprocal epistasis experiments with FLO8 overexpression in *efg1/efg1* was hindered by the lack of any detectable phenotypes from FLO8 overexpression (our unpublished data).

efg1/efg1, *flo8/flo8*, and *cdc35/cdc35* Mutants All Show Increased Filamentation under Microaerophilic Conditions

The ability of *C. albicans* to sense the presence of surrounding matrix may play a role during infection. Although *efg1/efg1* is defective in hyphal development under many laboratory conditions at 37°C (Lo *et al.*, 1997), it forms filaments on the tongue of immunosuppressed piglets (Riggle *et al.*, 1999) and undergoes filamentous growth when embedded in YPS agar (Giusani *et al.*, 2002). Because *flo8/flo8* mutants had a similar phenotype to *efg1/efg1* in aerobic conditions at 37°C and Flo8 could interact with Efg1 in vivo, we examined *flo8/flo8* mutants under embedded conditions. Like *efg1/efg1*, colonies of *flo8/flo8* produced filaments in 1 d when grown embedded in YPS agar at 25°C. In contrast, wild-type strain SC5314 just started to produce filaments after 2 d at 25°C, and even after 3 d, the wild type formed only limited amounts of filaments and remained predominantly smooth (Figure 8). One observable difference between *efg1/efg1* and *flo8/flo8* was that filamentation seemed heterogeneous in the *efg1/efg1* mutant with long hyphal filaments surrounded by branches covered with yeast cells, whereas the *flo8/flo8* mutant formed homogeneous hyphal filaments in each hyphal colony. In parallel to *efg1/efg1* and *flo8/flo8*, we also examined whether the cAMP/PKA pathway represses filamentous growth in microaerophilic conditions. Similar to the *flo8/flo8* mutant, a *cdc35/cdc35* mutant also formed homogenous hyphal filaments in each colony, although the *cdc35/cdc35* mutant grew much slower than *flo8/flo8*. Therefore, like Efg1, the cAMP/PKA pathway and Flo8 activity are inhibitory to filamentation in response to growth within a matrix at low temperature.

DISCUSSION

Identification of a *C. albicans* Transcription Factor with a Conserved LUFS Domain

We identified a *C. albicans* gene, FLO8, by functional complementation of a *flo8* mutant of *S. cerevisiae*. Like *S. cerevisiae* Flo8, Flo8 does not possess an obvious DNA binding motif, but it has a conserved LUFS domain that is found in several other regulatory proteins such as Leunig, Flo8, and Ssdp (Conner and Liu, 2000). The N-terminal half of the LUFS domain has a conserved LisH motif, which exists in >100 eukaryotic proteins of various functions (Emes and Ponting, 2001). The crystal structure of a mouse LIS1 fragment has shown that the LisH motif is a thermodynamically very

stable dimerization domain, located adjacent to a coiled-coil fragment in LIS1 (Kim *et al.*, 2004a). Interestingly, the LUF domain also has a conserved region with two predicted α -helices adjacent to the LisH motif (Figure 2A). It is likely that the LisH motif in Flo8 is also involved in dimerization, and together with the α -helices, forms a structure similar to the structure found in LIS1 (Kim *et al.*, 2004a). The dimerization could be homodimerization of Flo8 itself or heterodimerization of Flo8 with another protein with a LUF domain. In fact, ScFlo8 has been shown to physically and functionally interact with Mss11, another LUF-containing transcription factor, in the transcription of their target genes (Kim *et al.*, 2004b), although it remains to be seen whether the interaction is mediated through the LUF domains in these proteins. Dimerization in some transcription factors is required for DNA binding, and despite the absence of an obvious DNA binding domain, ScFlo8 is a sequence-specific DNA binding protein, and its binding to target promoters is regulated by PKA activity (Pan and Heitman, 2002; Kim *et al.*, 2004b). Based on our study, the LUF domain is required for Flo8 function in vivo. A Flo8 truncation lacking the LUF domain could not restore invasive/filamentous growth in *Scflo8* mutants or in *C. albicans flo8/flo8*. Because the LUF domain is not required for Flo8 transcriptional activities, it may be important for DNA binding either directly or via other regulators. In addition to the LUF domain, we also show by fusions of Flo8 fragments to a *lexA* DB domain that a transcriptional activation domain is located at the C terminus of Flo8. Alternatively, the C terminus of Flo8 is transcriptionally active through association with other transcription activators.

Flo8 Is a Key Regulator of Hyphal Development and Is Essential for Virulence

Flo8 is essential for hyphal development in *C. albicans*. Deleting *FLO8* completely blocks hypha formation under all aerobic hyphal-inducing conditions investigated. In terms of the extent of cell elongation in yeast and hyphal growth conditions, the *flo8/flo8* mutant shows a more specific and tighter phenotype than the *efg1/efg1* mutant, because *efg1/efg1* cells are slightly elongated in response to serum at 37°C, whereas *flo8/flo8* cells remain yeast-like. Whole-genome transcription analysis of *flo8/flo8* and *efg1/efg1* shows that Flo8 regulates subsets of Efg1-regulated genes, and most of these are hypha-specific genes, including a recently reported hypha-specific G₁-cyclin (Zheng and Wang, 2004) and a potential cell wall protein (Ihd1) with similarity to glucan 1,4- α -glucosidase *Sta1* in *S. diastaticus*. We did not identify a class of genes that are regulated only by Flo8 under the growth conditions we used. In contrast, some Efg1-regulated genes are not affected by *flo8/flo8*. Therefore, Flo8 is essential and specific for hyphal development.

Like Efg1, Flo8 seems to have a dual function in filamentous growth. Although the *flo8/flo8* mutant blocked hyphal development in aerobic conditions at 37°C, it formed long hyphal filaments in a microaerophilic condition at room temperature. This suggests that Flo8 could act as an activator of the hyphal response program to certain stimuli but could also function as a repressor of hyphal development in a matrix. Unlike the *efg1/efg1* mutant, which formed heterogeneous filaments surrounded with yeast cells in the matrix, the *flo8/flo8* mutant formed uniform hyphal filaments when embedded in agar. Therefore, *flo8/flo8* seems to have a stronger and more complete phenotype than *efg1/efg1* under both aerobic and microaerophilic conditions.

The *flo8/flo8* mutant is avirulent in a mouse model of systemic infection. This is probably directly linked to the

critical role of Flo8 in hyphal development. The *flo8/flo8* mutant completely blocked hyphal development and specifically prevented the expression of hypha-specific genes, many of which are known to contribute to virulence. In addition, the *flo8/flo8* mutant is completely filamentous under embedded conditions. It seems that the mutant is unable to switch between yeast and filamentous forms under a given condition. This is consistent with its avirulent property in the mouse model, because the virulence of *C. albicans* is considered to be linked to the ability of cells to undergo dimorphic transitions. The subtle morphological difference between the *efg1/efg1* and the *flo8/flo8* mutants is also consistent with the observation that *flo8/flo8* is avirulent, whereas the *efg1/efg1* mutant shows reduced virulence.

Flo8 May Function Downstream of the cAMP/PKA Pathway, Together with Efg1, in Regulating the Hyphal Transcriptional Program

Several lines of evidence suggest that Flo8 may function downstream of the cAMP/PKA pathway, and together with Efg1, may regulate the expression of hyphal genes. First, *C. albicans FLO8* could complement the defects of *Scflo8* in invasive/filamentous growth, and therefore it is likely to function in the same position as ScFlo8 in *S. cerevisiae*, which is downstream of Tpk2. Second, the *flo8/flo8* mutant has similar phenotypes as *cdc35/cdc35* and *efg1/efg1*; they are defective in hyphal development and the induction of hypha-specific genes including *HGC1* under many liquid hyphal-inducing media, including serum-containing media, but they show elevated filamentation under embedded conditions. Third, transcription profiling of *flo8/flo8* and *efg1/efg1* shows that Flo8 regulates the hypha-specific set of the Efg1-regulated genes. We did not find genes regulated by Flo8, and not by Efg1. Interestingly, hypha-specific genes are the only overlapping genes affected by both *efg1/efg1* and *cdc35/cdc35* in a genome-wide profiling study (Harcus *et al.*, 2004). Fourth, an in vivo interaction between Flo8 and Efg1 was detected by yeast two-hybrid and immunoprecipitation. This places both Efg1 and Flo8 downstream of the cAMP/PKA pathway. Whether one or both of them are regulated by PKA remains to be determined. Considering that some Efg1-repressed genes are not regulated by Flo8, we predict that, when in complex with Flo8, the Efg1/Flo8 complex can activate transcription. This is consistent with the notion that Efg1 is an inhibitor of transcription under both yeast and hyphal growth conditions (Doedt *et al.*, 2004), whereas Flo8 seems to act as a transcriptional activator as the *lexA_{DB}-Flo8* fusion has high transcriptional activity. It is possible, that by interacting with different regulators, Efg1 can exert different effects on transcription.

Flo8 may interact with additional regulators to integrate responses from different signaling pathways to regulate the hyphal transcriptional program. LUF-containing regulators have been found to interact with other transcriptional regulators. In *Arabidopsis*, the LUF domain of Leunig is both necessary and sufficient for its interaction with Seuss, and the Leunig/Seuss complex regulates gene expression during flower development (Sridhar *et al.*, 2004). In *Drosophila*, the Ssdp protein interacts through its LUF domain with Chip, a cofactor of the LIM homeodomain transcription factors, and regulates the activity of the LIM-homeodomain protein complexes in various developmental processes (van Meyel *et al.*, 2003). In *S. cerevisiae*, the Flo8/Mss11 complex interacts with Ste12 and Tec1 to activate *STA1* expression (Kim *et al.*, 2004b). In *C. albicans*, we show that Flo8 interacts with Efg1. Because the *flo8/flo8* mutant has a tighter phenotype than that of *efg1/efg1*, we predict that Flo8 may interact with other

coactivators or coinhibitors besides Efg1 to regulate hyphal development. So, we propose that Flo8 acts downstream of several pathways to integrate various signals in hyphal development. In conclusion, we have identified a conserved regulator Flo8 that plays an essential role in the dimorphic switch and virulence of *C. albicans*.

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