Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi

Volker R.Stoldt, Anja Sonneborn,
Christoph E.Leuker and Joachim F.Ernst¹

Institut für Mikrobiologie, Heinrich-Heine-Universität,
D-40225 Düsseldorf, Germany

¹Corresponding author
e-mail: joachim.ernst@uni-duesseldorf.de

V.R.Stoldt and A.Sonneborn contributed equally to this work

We identified a gene of the fungal pathogen *Candida albicans*, designated *EFG1*, whose high-level expression stimulates pseudohyphal morphogenesis in the yeast *Saccharomyces cerevisiae*. In a central region the deduced Efg1 protein is highly homologous to the StuA and Phd1/Sok2 proteins that regulate morphogenesis of *Aspergillus nidulans* and *S.cerevisiae*, respectively. The core of the conserved region is homologous to the basic helix–loop–helix (bHLH) motif of eukaryotic transcription factors, specifically to the human Myc and Max proteins. Fungal-specific residues in the bHLH domain include the substitution of an invariant glutamate, responsible for target (E-box) specificity, by a threonine residue. During hyphal induction *EFG1* transcript levels decline to low levels; downregulation is effected at the level of transcriptional initiation as shown by a *EFG1* promoter–*LAC4* fusion. A strain carrying one disrupted *EFG1* allele and one *EFG1* allele under the control of the glucose-repressible *PCK1* promoter forms rod-like, pseudohyphal cells, but is unable to form true hyphae on glucose-containing media. Overexpression of *EFG1* in *C.albicans* leads to enhanced filamentous growth in the form of extended pseudohyphae in liquid and on solid media. The results suggest that Efg1p has a dual role as a transcriptional activator and repressor, whose balanced activity is essential for yeast, pseudohyphal and hyphal morphogenesis of *C.albicans*. Functional analogies between Efg1p and Myc are discussed.

Keywords: *Candida albicans*/helix–loop–helix proteins/morphogenesis/transcription factor/yeast

Introduction

Many fungal pathogens alternatively grow in a budding yeast form or in a hyphal form, an ability contributing to different aspects of fungal virulence. In the most common human fungal pathogen, *Candida albicans*, the hyphal growth form appears most suited for adherence and penetration of epithelial or endothelial cell layers (reviewed by Calderone and Braun, 1991; Cutler, 1991); also, hyphal growth may allow exit of ingested *C.albicans* from macrophages (Arai *et al.*, 1977). In contrast, the unicellular yeast growth form (blastospore) is speci-

ally suited to increase the number of infectious units and may readily achieve systemic distribution in the infected patients. In *Histoplasma capsulatum*, the yeast form is required for propagation in human tissues, while hyphal growth occurs outside infected organisms (Maresca and Kobayashi, 1989). The yeast form of *Ustilago maydis* is not pathogenic, while the dikaryotic hyphal form is able to infect plants (Schulz *et al.*, 1990).

Environmental conditions determine the growth mode of *C.albicans*. While yeast growth is favored at low temperatures and high glucose concentrations, a pseudohyphal form characterized by elongated polar buds that are not separated following cell division is observed at low temperatures on depauperated media; unconstricted 'true' hyphae, which in their initial phase of formation are termed germ tubes and grow by continuous apical extension, are induced by a variety of chemical stimuli at high temperature (reviewed by Odds, 1988). Pseudohyphal growth has also been observed in the nonpathogenic yeast *Saccharomyces cerevisiae* (Gimeno *et al.*, 1992). In this case pseudohyphal growth is induced by starvation for nitrogen on solid media, where it may serve to forage for nitrogen. In contrast, formation of true hyphae in *C.albicans* occurs in liquid and on solid media and requires positive induction signals, such as serum.

In *S.cerevisiae* several genes required for the yeast–pseudohyphal dimorphism are known. Analyses of mutants revealed that members of the mitogen-activated protein kinase (MAP kinase) pathway leading to the activation of the transcription factor Ste12p, as well as the Tec1 protein, are required for pseudohyphal development; overproduction of proteins of the MAP kinase pathway, of Ste12p or Tec1p leads to enhanced pseudohyphal growth (Liu *et al.*, 1993; Gavrias *et al.*, 1996; Mösch *et al.*, 1996). Although the genetic basis of the yeast–hyphal dimorphism in *C.albicans* has not been established, a Ste12p homolog (Cph1p) recently has been shown to be involved in hyphal growth of *C.albicans* during some conditions (Liu *et al.*, 1994). Besides involvement of a MAP kinase pathway the role of phosphorylation in morphogenesis was shown by enhanced pseudohyphal formation in *elm1* and *cdc55* mutants, in which a protein kinase or a subunit of protein phosphatase 2A, respectively, are altered (Blacketer *et al.*, 1993). *Saccharomyces cerevisiae* pseudohyphal morphogenesis can also be triggered by activation of the Ras2p signal transduction pathway (Gimeno *et al.*, 1992; Mösch *et al.*, 1996). Overexpression of the *PHD1* gene also stimulates pseudohyphal growth (Gimeno *et al.*, 1994). The deduced Phd1p sequence contains a segment that is highly homologous to the StuA protein of *Aspergillus nidulans*, which is required for conidiophore morphogenesis (Miller *et al.*, 1992). This region has also limited homology to the DNA binding motifs of Swi4p and Mbp1p. Recently, the *SOK2* gene encoding a second

S.cerevisiae protein containing a Phd1p/StuA homology domain was identified (Ward *et al.*, 1996). In contrast to Phd1p, Sok2p appears to inhibit the switch from unicellular to filamentous growth by acting as a repressor of transcription.

In the present study we describe a *C.albicans* gene, *EFG1*, which encodes another protein containing a conserved domain as in the StuA, Phd1 and Sok2 proteins. We discovered that the region of homology in all of these proteins encompasses a sequence with features of a basic helix–loop–helix (bHLH) DNA binding/dimerization domain of certain transcription factors in yeast and mammalian cells (Ferré-D’Amare *et al.*, 1993; Ellenberger, 1994; Ma *et al.*, 1994). Results of gene disruption and overexpression experiments and of transcript analyses suggest that the function of *EFG1* in *C.albicans* is the regulation of yeast and hyphal morphogenesis. Thus, StuA, Phd1, Sok2 and Efg1 (designated ‘SPSE proteins’) appear to represent a novel class of transcriptional regulators that are involved in cellular differentiation processes in fungi.

Results

Isolation of the *C.albicans* *EFG1* gene

Certain *S.cerevisiae* diploid strains are able to respond to nitrogen limitation by growth in a filamentous pseudohyphal form (Gimeno *et al.*, 1992). We attempted to clone genes of *C.albicans* involved in hyphal or pseudohyphal morphogenesis by transforming a *S.cerevisiae* diploid with genomic *C.albicans* libraries in multicopy *S.cerevisiae* vectors and screening for transformants with accelerated pseudohyphal morphogenesis. Among transformants of each of two genomic libraries one such transformant was identified. By isolation and retransformation of the plasmids carried by the respective transformants (p606/1 and p607/2), we confirmed that the pseudohyphal phenotype was plasmid-borne (Figure 1B and H).

Restriction mapping of the inserts in plasmids p606/1 and p607/2 revealed that they represented overlapping segments of the same *C.albicans* genomic region. Subclones of this region were constructed in multicopy *S.cerevisiae* vectors, transformed in *S.cerevisiae* and tested for enhanced formation of pseudohyphae. A 1.6 kb fragment was shown to be minimally required for this phenotype. To test dependence on high plasmid copy numbers we cloned the 5.2 kb *Bam*HI insert into the centromeric vector YCplac22. Transformants carrying the resulting vector did not show enhanced pseudohyphal morphogenesis indicating that high expression of the putative *C.albicans* gene is required to stimulate pseudohyphal growth in *S.cerevisiae*.

At low nitrogen concentrations the transformant phenotype was similar to the phenotype of a YCpR2V transformant expressing the Ras2^{Val19} protein (Gimeno *et al.*, 1992); however, at 600 µM ammonium sulfate 60% of colonies of the YCpR2V-transformant showed pseudohyphae compared with only 20% of the p606/1 transformants. To test synergy between the *C.albicans* gene product and the Ras2^{Val19} protein *S.cerevisiae* transformants cotransformed with p606/1 and YCpR2V were analyzed. Such transformants did not show enhanced morphogenesis compared with single transformants indicating a lack of synergy. On the other hand a synergistic

effect on morphogenesis was obtained in double-transformants carrying p606/1 and pCG38, which encodes a high-copy inducer of pseudohyphal growth, Phd1p (Gimeno and Fink, 1994) (Figure 1, compare B and D). To test further whether the pseudohyphal formation elicited by the identified *C.albicans* gene required the activity of the MAP kinase module (Mösch *et al.*, 1996) we transformed p607/2 into the *ste20* mutant HLY492 (Liu *et al.*, 1993). Pseudohyphal formation was completely blocked in this mutant, while a transformant of a congenic wild-type strain (CGX31) showed enhanced formation of pseudohyphae (Figure 1G and H). Likewise, pseudohyphal formation elicited by *PHD1* overexpression (pCG38), or the activity of the RAS2^{Val19} allele (YCpR2V), was completely blocked in the *ste20* mutant. Thus, the dependence on low-nitrogen conditions, the lack of synergy with Ras2p and its dependence on *STE20* suggested that the heterologous *C.albicans* gene product functions in the known morphogenetic pathways of *S.cerevisiae* (Gimeno *et al.*, 1992; Mösch *et al.*, 1996). Synergy between the *C.albicans* gene product and the homologous Phd1 protein (see below) can be explained by their identical, dosage-dependent functions in stimulating morphogenesis.

Sequence of the *EFG1* gene

The nucleotide sequence of the 2.517 kb *Eco*RI–*Sph*I fragment of p606/1 was determined (DDBJ/EMBL/GenBank accession number Z32687; data not shown). Sequence analyses revealed a contiguous open reading frame between nucleotide positions 211–1869. The deduced protein contains 552 amino acids (molecular mass 59.9 kDa) and has an unusual composition consisting of 19% glutamine, 10% alanine and 9% each of threonine, serine, proline and glycine residues; the single CUG codon 452 was translated as serine, instead of the standard leucine (Leuker and Ernst, 1994; Santos and Tuite, 1995). Glutamine residues are especially clustered in the amino and carboxyl terminal regions of the deduced protein. Several sequence motifs may have functional importance: (i) a possible RR.RGRR nuclear localization signal at residues 229–230/249–252; (ii) a protein kinase A (PKA) phosphorylation site at residues 204–206 (RVT); (iii) a possible casein kinase II phosphorylation site at residues 208–211 (TMWE) and numerous target sites for casein kinase I of the structure S/T-XX-S/T.

Computer analyses revealed that the central region of the deduced protein (amino acids 210–350) contains sequences able to form α -helices, while random coils are predicted for the termini. Within its central region, encompassing amino acids 202–303, the deduced protein has high homology to the StuA protein of *A.nidulans* (Miller *et al.*, 1992), as well as the Phd1 and Sok2 proteins of *S.cerevisiae* (Gimeno and Fink, 1994; Ward *et al.*, 1996) (80% conserved amino acids in all four proteins) (Figure 2). Because of its induction of filamentous growth in *C.albicans* (see below) we designated the encoding gene *EFG1* (enhanced filamentous growth) and we refer to the conserved group of fungal proteins (StuAp, Phd1p, Sok2p, Efg1p) as ‘SPSE’ proteins. *EFG1* is represented by a single genomic locus on chromosome R of *C.albicans* (B.B.Magee, personal communication; Internet: <http://alces.med.umn.edu/bin/geneinfo>).

Northern analyses revealed that the *EFG1* transcript

Fig. 1. Colony phenotypes of *S. cerevisiae* (A–H) and *C. albicans* (I–K) strains. *Saccharomyces cerevisiae* B76/4 transformants contained plasmids YRp7/pLG89-B (A, control), p606/1 (*EFG1*)/YCplac33 (Gietz and Sugino, 1988) (B), pCG38 (*PHD1*)/YCplac22 (C), p606/1 (*EFG1*)/pCG38 (*PHD1*) (D) and pBIST/YCplac22 (E and F); plasmid p607/2 (*EFG1*) was present in strain HLY492 (*ste20*) (G) or CGX31 (H, control). *Saccharomyces cerevisiae* strains were grown on low-nitrogen plates containing galactose (F) or glucose (A–E, G and H). *Candida albicans* SS4 (*efg1::ADE2/PCK1p::EFG1*) was grown on SD-plates containing 4% glucose (J) or B-plates containing succinate (K); the heterozygous strain BN5 (*EFG1/PCK1p::EFG1*[pMK16]), grown on 4% glucose, is shown as a control (I).

has a length of ~2.8 kb and nuclease S1-mapping of the 5'-end of the *EFG1* transcript revealed a major transcript start point at position –35 relative to the ATG of the coding region (data not shown). The transcript 5'-end (GTTCT) differs from the TCA/GA and RRYRR sequences typical for *S. cerevisiae* transcripts (Hahn *et al.*, 1985). The unexpected size of the *EFG1* transcript relative to the 1.65 kb of the *EFG1* coding region may be due to additional transcript start sites far upstream of the –35 start site or to extensive 3'-untranslated or poly(A) sequences (Jackson and Standart, 1990). A putative TATA box is present at positions –82 to –85 upstream of the –35 transcript start site, which is followed by a T/C rich sequence. The sequence TAG...TAGTAT...TTC in the 3'-untranslated region, between residues 1987–2031, agrees with the tripartite consensus sequence for transcription termination in *S. cerevisiae* (Zaret and Sherman, 1982). Further downstream in the 3'-untranslated region sequences homologous to *ARS1* consensus sequences of *S. cerevisiae* (Newlon and Theis, 1993) are detected. Vectors

carrying the consensus sequences on a 735 bp *NsiI* fragment in an integrating vector (p1367/1; Losberger and Ernst, 1989) indeed replicate episomally in *C. albicans* and *S. cerevisiae* (our unpublished results).

To confirm the deduced coding region we deleted sequences upstream of the predicted ATG initiation codon and the coding region was placed under the control of the *GAL1* promoter in plasmid pBIST; transformants carrying this plasmid showed enhanced formation of pseudohyphae only on galactose-, but not on glucose-containing media (Figure 1E and F). The activity of subclones revealed that the predicted coding region can be partially deleted without loss of its pseudohyphal stimulating activity. Deletions shortening the deduced protein at its carboxyl terminal end by 50 amino acids were still active, while more extensive deletions were inactive (data not shown).

Efg1p as a bHLH protein

The central portion of the conserved region in SPSE proteins has significant sequence and structural homology

Fig. 2. Conserved sequences in fungal and mammalian bHLH proteins. Top, homology of sequences flanking the bHLH region of fungal proteins; bottom, homology among bHLH regions. Conserved residues S/T, D/E, N/Q, R/K, A/I/L/M/V and F/Y/W are shaded. Residues contacting DNA in Max and MyoD (Ferré-D'Amare *et al.*, 1993; Ma *et al.*, 1994) are printed bold-faced and in italics.

to the bHLH motif in eukaryotic transcription factors including Max, MyoD and Pho4p (Ferré-D'Amare *et al.*, 1993; Ellenberger, 1994; Ma *et al.*, 1994) (Figure 2, bottom). Residues known to contact DNA in Max (Ferré-D'Amare *et al.*, 1993) and MyoD (Ma *et al.*, 1994) are conserved in SPSE proteins. The presence of arginine and an apolar residue (Met) in position 252 and 247 (Efg1p numbering) is similar to Myc and Max, but unlike MyoD, suggesting that the two central positions in the target sequence are CG and not GC. The conserved glutamic acid residue in bHLH proteins (e.g. position 118 in MyoDp) is replaced by threonine in SPSE proteins (Efg1p position 248). The role of glutamic acid is to donate oxygen to establish hydrogen bonds to the CA residues of the target sequence, which is a function that may also be assumed by a threonine residue. Replacement of this glutamic acid residue by glutamine in the yeast Pho4 protein did not abolish DNA binding (Fisher and Goding, 1992). The conserved histidine residue in Myc/Max-type proteins is replaced by valine in SPSE proteins (Efg1p position 244) and thus resembles MyoD (which contains alanine). The SPSE proteins (as MyoD) do not contain leucine zipper sequences; therefore, dimerization of Efg1p most likely occurs by the bHLH region. Like other eukaryotic transcription factors Efg1p contains stretches of glutamines (Tjian and Maniatis, 1994); in yeast such stretches occur in repressors (e.g. Ssn6p) as well as activators (e.g. Snf5p) (Winston and Carlson, 1992; Treitel and Carlson, 1995).

EFG1 transcript regulation

Candida albicans ATCC10261 was induced to form hyphae using three established protocols, during which the expression of housekeeping genes including *ACT1* is

not significantly affected (Delbrück and Ernst, 1993). Logarithmically growing cells were diluted in YP-medium (P1), 5% horse serum/YPD-medium (P2) or 5% horse serum/YP-medium (P3) and incubated at 37°C. YPD-medium, in which no hyphae are induced (Delbrück and Ernst, 1993), was used as a control. Hyphal formation proceeded similarly in conditions P1–P3: 5–10% (20 min), 35–40% (40 min) and 80–90% (P1 and P3) or 40% (P2) (60 min) of cells produced hyphae (germ tubes). Total RNA was isolated at different time points during incubation of the cultures and assayed for the presence of *EFG1* mRNA by Northern blotting, using *ACT1* mRNA as control (Delbrück and Ernst, 1993) (Figure 3).

A single *EFG1* mRNA species of ~2.8 kb RNA was detected, whose level declined significantly preceding hyphal morphogenesis induced by protocols P1–P3, while the level of the *ACT1* mRNA remained constant (Figure 3). In control cells (C), the *EFG1* transcript level was unchanged during incubation. Transcript downregulation was most pronounced in hyphal-forming cells induced by protocol P1, which contained almost undetectable mRNA levels after 10 min of hyphal induction. In all induction protocols the decline of the *EFG1* transcript level started prior to the emergence of visible hyphae. Thus, these studies reveal a clear correlation between the decline of the *EFG1* transcript levels and subsequent hyphal formation.

To demonstrate that the downregulation of *EFG1* transcript levels occurs on the level of transcriptional initiation, a fusion of the *EFG1* promoter to the *LAC4* reporter gene (Leuker *et al.*, 1992) was constructed and expressed in *C. albicans*. Transformants were induced to form hyphae by the same conditions (P1–P3) that were used for *EFG1* mRNA analysis (Figure 3) and β -galactosidase (β gal)

Fig. 3. *EFG1* transcript levels. RNA of *C.albicans* ATCC10261 induced to form hyphae according to three protocols (P1–P3) was isolated at the indicated time points and analyzed by Northern blotting using *ACT1* or *EFG1* probes. Induction medium was YP (P1), YPD/serum (P2) or YP/serum (P3); incubation in YPD medium, in which no hyphae are formed, was used as a control (C).

activity in cell extracts were determined at different time points. In this system, a rapid decline in β gal activity was observed for all cultures forming hyphae, while the activity in control cells remained constant (Figure 4). The extent of the initial decline varied from a factor of 500 (conditions P1 and P3) to a factor of about 5000 (P2). These results demonstrate that the *EFG1* promoter activity is significantly downregulated prior to hyphal formation.

EFG1 disruption

We were not able to obtain mutants in which both *EFG1* alleles are disrupted by standard gene disruption protocols (Sadhu *et al.*, 1992; Fonzi and Irwin, 1993), suggesting that *EFG1* is essential for the viability of *C.albicans*. Therefore, we adopted a strategy to disrupt the coding region of only one allele and to replace the promoter region of the second allele with a regulatable promoter. To this end we first identified the *C.albicans* *PCK1* gene encoding phosphoenolpyruvate carboxykinase; the *PCK1* promoter was found to be active during growth on carbon sources such as succinate, ethanol, or sucrose, but to become effectively repressed by glucose (C.Leuker, A.Sonneborn and J.F.Ernst, manuscript in preparation). The first *EFG1* allele was disrupted by transforming strain CAI8 (Ura³⁻ Ade2⁻) with an *EFG1* fragment, in which the *EFG1* promoter was replaced by the *PCK1* promoter and the *URA3* gene as selectable marker (Figure 5, top). Uridine prototrophs, in which the desired disruption event was confirmed (see below), were again transformed with an *EFG1* fragment, in which the *ADE2* gene interrupts the *EFG1* coding region and Ura⁺ Ade⁺ prototrophs were selected. In this manner two identical doubly disrupted

Fig. 4. Regulation of a *EFG1* promoter–*LAC4* fusion by hyphal morphogenesis. A transformant of *C.albicans* CAI8 carrying plasmids pCL76 (*EFG1p::LAC4*) and pMK16 was induced to form hyphae according to protocols P1–P3 (Figure 3). The percentage of hyphae (germ tubes) formed is shown in the bottom graph. β -galactosidase activity in crude extracts of the transformant were determined (upper graph). Induction conditions were YP (P1; $\blacktriangle$), YPD/serum (P2; $\bullet$) and YP/serum (P3; $\blacktriangledown$); control cells were incubated in YPD (C; $\blacksquare$).

strains designated SS4 and SS14 were obtained, in which one *EFG1* allele is disrupted by *ADE2* and the second allele is under control of the regulatable *PCK1* promoter. SS4 and SS14 showed the identical phenotypes discussed below.

Disruption of both *EFG1* alleles as shown in Figure 5 (top) was demonstrated by Southern blotting (Figure 5, bottom). Genomic DNA of transformants was digested using *EcoRI* or *HindIII*, which both cut outside the region present on the disruption fragments. In the wild-type strain each *EFG1* allele is present on *EcoRI* fragments of different lengths (6.0 kb and 7.0 kb). Insertion of the *PCK1p/URA3* cassette increased the size of the 6 kb fragment to 7.4 kb, but left the 7 kb fragment unchanged (lane 2). Because of an *EcoRI* site within *ADE2*, its insertion into the second *EFG1* allele removed the 7.4 kb fragment, but created a new 2 kb *EcoRI* fragment (lane 3). In *HindIII* digests a single 25 kb fragment was detected in the wild-type (lane 6), while insertion of the *PCK1p/*

Fig. 5. *EFG1* gene disruption. Top, disruption strategy indicating the *EFG1* coding regions (open arrows) and the disruption fragments used to disrupt both *EFG1* alleles (marked a and b). Relevant sites for restriction enzymes are indicated (B, *Bgl*II; H, *Hind*III; R, *Eco*RI; X, *Xho*I). The position of the *EFG1* probe used in Southern analysis is marked by the asterisks. Bottom, Southern blot using genomic DNA of strain CAI8 (lanes 1 and 6), the *EFG1*/*PCK1p::EFG1* heterozygous strain BN5 (lanes 2 and 5) and the *efg1::ADE2/PCK1p::EFG1* complete disruptant strain SS4 (lanes 3 and 4). DNA was cut with *Eco*RI (lanes 1–3) or with *Hind*III (lanes 4–6). The sizes of diagnostic fragments (in kb) discussed in the text are indicated.

URA3 cassette in one allele generated an additional fragment of 26.4 kb (lane 5). The second disruption step, because of a *Hind*III site in *ADE2*, led to disappearance of the remaining wild-type copy and generation of a 2.9 kb fragment, as expected (lane 4).

EFG1 transcript levels in SS and control strains were analyzed (Figure 6). Cells were grown in conditions leading to *PCK1* promoter repression (SD) or derepression/induction (B-medium) into the exponential growth phase, total RNA was isolated and 20 µg of RNA was analyzed by Northern blotting; the *ACT1* transcript was used as an internal control (Delbrück and Ernst, 1993). Control cells grown on SD contained moderate levels of an *EFG1* transcript of ~2.8 kb (Figure 6, lane 5), which increased

Fig. 6. *EFG1* transcript levels. Total RNA (20 µg) of *C. albicans* strains were analyzed by Northern blots using an *EFG1* probe. Transformants of strain CAI4 carrying a control plasmid (CAI4[pBI-1]; lanes 1 and 2) or a plasmid containing a *PCK1p::EFG1* fusion (CAI4[pRC2312P-H]; lanes 3 and 4) were analyzed. A transformant of strain CAI8 (CAI8[PMK16/pRC2312]; lanes 5 and 6) as a control and the CAI8-derivative SS14 (*efg1::ADE2/PCK1p::EFG1*; lanes 7 and 8) were examined. All strains had been grown either in *PCK1p* repressing SD-medium (lanes 1, 3, 5 and 7) or derepressing/inducing B-medium (lanes 2, 4, 6 and 8). The positions of the *EFG1* transcript (~2.8 kb), the transcript of the *PCK1p::EFG1* fusion (about 2.3 kb) and the *ACT1* control transcript are shown.

during growth on B-medium (lane 6) indicating that *EFG1* expression is glucose-regulated. The disruptant strain SS14 was lacking an *EFG1* transcript as in control cells; instead, it contained an *EFG1* transcript of ~2.3 kb (Figure 6, lanes 7 and 8). The reduced transcript size in strain SS14 as compared with the control strain could be due to replacement of the authentic *EFG1* promoter by the *PCK1* promoter, which conceivably could reduce the sizes of the 5' or 3'-untranslated regions of the transcript. The transcript level in strain SS14 grown in SD, relative to the *ACT1* control transcript, was clearly reduced as compared with control cells (Figure 6, compare lanes 5 and 7). As expected, growth on B-medium led to significant overexpression of the *PCK1p::EFG1* gene in strains CAI4[pRC2312P-H] and SS14 (lanes 4 and 8).

The consequence of a reduced *EFG1* transcript level on cell morphology was analyzed microscopically after growing strain SS14 in liquid SD-medium, in which the *PCK1* promoter is repressed (Figure 7A). Cells had a more elongated, rod-like appearance as compared with control cells (Figure 7D) which was reminiscent of the opaque phenotype of the 'switching' strain WO-1 (Soll *et al.*, 1993); furthermore, such cells often remained attached leading to pseudohyphal filaments. The tendency to filamentation was also apparent on solid SD-medium, where colonies formed lateral extensions (Figure 1J), similar to *cph1* mutants in some conditions (Liu *et al.*, 1994). Although glucose-grown SS14 cells had a pseudohyphal phenotype, the addition of 5% horse serum did not induce the appearance of true unconfined hyphae, as in control cells. Thus, a reduced *EFG1* expression level has at least two effects, (i) by inducing cell elongation and pseudohyphal filaments and (ii) by prohibiting formation of true hyphae.

EFG1 overexpression

EFG1 overexpression could be achieved by the growth of disruptant strains SS4/14 on B-medium (Figure 6, lane 6). An even greater overexpression could be achieved in transformants carrying multiple copies of plasmid pRC2312P-H, which contains the *PCK1p::EFG1* expres-

Fig. 7. Cell morphology of *C. albicans* strains. The disruptant strain SS14 (*efg1::ADE2/PCK1::EFG1*) was either grown in (*PCK1p* repressing) SD-medium (A) or in B-medium (B). Developing hyphae (germ-tubes) induced after 110 min by 5% serum on cells pregrown in B-medium are marked by triangles (B, insert). The phenotypes of strain CAI4[pRC2312P-H] (*EFG1* overexpression) (C) and a control strain (SC5314) on (*PCK1p*-inducing) CAA-medium (D) are shown.

sion module (Figure 6, lane 4). Interestingly, overexpression of *PCK1p::EFG1* was accompanied by disappearance of the authentic *EFG1* transcript, which is predominant in glucose-grown cells (Figure 6, lane 3). This result is an indication of autoregulation of *EFG1*, a finding that we recently confirmed using promoter fusions (unpublished results).

The majority of SS4 cells and pRC2312P-H transformants grown in B-medium had a pronounced tendency to form long filaments, consisting of extremely elongated cells (Figure 7B and C). Because of constrictions between cells and their growth starting with an apical bud we consider these filaments as pseudohyphae, but not true hyphae, which are formed by continuous apical extension (Odds, 1988). The pseudohyphal phenotype could also be observed on solid B-medium, in which SS4 colonies formed extensive lateral pseudohyphae (Figure 1K). As expected from higher *EFG1* expression in pRC2312P-H transformants compared with SS4 cells, filamentous growth was enhanced (Figure 7, compare B and C). pRC2312P-H transformants grew as normal yeast cells under conditions of *PCK1p* repression, consistent with a wild-type level of the *EFG1* transcript (Figure 6, lane 3). To test whether an enhanced *EFG1* expression level interferes with hyphal formation, we added 5% serum to a culture of SS4 grown in B-medium. Such cells rapidly (within 30 min) began to form typical germ-tubes that often were seen to emerge from pseudohyphal or yeast cells (Figure 7B, insert) and that continued to extend by apical growth as true hyphae. A similar result was obtained with strain CAI4[pRC2312P-H]. Thus, *EFG1* overexpression enhances pseudohyphal growth and allows the formation of true hyphae.

Discussion

The *C. albicans* *EFG1* gene encodes a new member of a conserved class of fungal proteins that are involved in morphogenetic processes. This class includes StuAp of *A. nidulans*, as well as the Phd1 and Sok2 proteins of *S. cerevisiae*. StuAp is involved in the formation of conidiophores in *A. nidulans* (Miller *et al.*, 1992). Phd1p enhances and Sok2p suppresses pseudohyphal formation in *S. cerevisiae* (Gimeno and Fink, 1994; Ward *et al.*, 1996), while Efg1p regulates the morphogenesis of *C. albicans*. The homology among the three proteins (referred to as 'SPSE proteins') extends over a region of ~100 amino acids with 80% similarity. Efg1p and Phd1p, which both stimulate pseudohyphal growth in *S. cerevisiae*, do not contain homologous sequences other than the region conserved in SPSE proteins. However, this region cannot be responsible for pseudohyphal induction, since it is also present in Sok2p. Although a limited homology of SPSE proteins to the DNA-binding region of transcription factors including Swi4p has been discussed (Gimeno and Fink, 1994; Ward *et al.*, 1996), these authors did not recognize that the core of the conserved region can be aligned to the bHLH domain of eukaryotic transcription factors, such as the mammalian Max, Myc, MyoD and E47 proteins or the yeast Cbf1 and Pho4 proteins (Ferré-D'Amare *et al.*, 1993; Ellenberger, 1994; Ma *et al.*, 1994) (see Results; Figure 2).

Changes in *EFG1* expression have a greater effect on the morphology of *C. albicans* than the absence of elements of the MAP-kinase pathway in *cph1* and *hst7* mutants (Liu *et al.*, 1994; E. Leberer, personal communication). In such strains a defect of hyphal growth is detected only on certain solid media, but no other media or in liquid. Our experiments suggest that Efg1p is essential for morphogenesis of the yeast, pseudohyphal and hyphal growth forms in *C. albicans*. Although a strain could not be constructed in which both *EFG1* alleles are disrupted (possibly, because *EFG1* is essential for growth), the consequences of lowered *EFG1* expression could be analyzed in a strain which only contained one functional *EFG1* allele under the control of the regulatable *PCK1* promoter (C. Leuker, A. Sonneborn and J.F. Ernst, manuscript in preparation). This strain, under conditions of *EFG1* repression (glucose media), did not produce regular yeast cells, but formed rod-like, elongated cells that resemble opaque cells of strain WO-1 (Soll *et al.*, 1993). These cells often remained attached leading to the formation of pseudohyphal filaments. This phenotype was obtained on solid and in liquid media. These results indicate that Efg1p, directly or indirectly, acts as a repressor of an elongated cell form and pseudohyphal growth. On the other hand lowered *EFG1* expression also impaired cells to form true unstricted hyphae in the presence of serum. Thus, Efg1p could function as a repressor, but also as an activator that is needed for hyphal growth. A dual function of a transcription factor as a repressor and an activator is not uncommon, as is discussed below for Myc, and may be explained by dimer formation and interaction with regulatory proteins.

Overexpression of *EFG1* mediated by the *PCK1* promoter also led to stimulation of filamentous growth in liquid and on solid media. Such filaments consisted of extremely extended cells, whose appearance was unlike

the rod-like cells formed during lowered *EFG1* expression and unlike the pseudohyphae of *S.cerevisiae*. Although these filaments resembled true hyphae they contained constrictions at the site of cell connections and arose by budding; thus, the filaments induced by *EFG1* overexpression must be considered as pseudohyphae. It is a surprising finding that reduced *EFG1* expression and its overexpression both stimulate pseudohyphal growth of a different type. Conceivably, the same pathway or different pathways lead to both pseudohyphal growth forms. If the same intracellular pathway were involved, Efg1p could act, e.g. by inactivation of a repressor complex containing Efg1p by a 'squenching' mechanism (Ptashne and Gann, 1990). Alternatively, excess Efg1p could activate a morphogenetic pathway different from the pathway activated during Efg1p limitation. The finding that overexpression of *EFG1* did not suppress the formation of true hyphae by serum is consistent with the above hypothesis that Efg1p is an activator of hyphal growth. Since the *EFG1* transcript level is downregulated rapidly prior to hyphal formation it is possible that the Efg1 protein is required only for early events during hyphal formation, for example by creating a state of 'competence' to undergo hyphal morphogenesis.

The presumed role of Efg1p as a transcriptional activator and repressor and the homology to Myc (see Results) suggest a model for Efg1p function in accordance with the known functions of Myc. A heterodimer consisting of Myc, which contains a transactivation domain rich in serine and proline residues and the Max protein is required for the activation of target genes during growth of mammalian cells (Ferré-D'Amare *et al.*, 1993). High levels of Myc activate gene expression and favor cell growth, but block terminal cellular differentiation, presumably because Myc also acts as a transcriptional repressor (Li *et al.*, 1994); in contrast, elevated production of Max represses transcriptional activation by formation of Max–Max homodimers or Max–Mad heterodimers binding to the same targets as Myc–Max (Ayer and Eisenman, 1993; Bernards, 1995). Myc transcript levels decline during the onset of cellular differentiation (Ayer and Eisenman, 1993). As with Myc, Efg1p appears to function both as a transcriptional activator and repressor, because on the one hand it is required for hyphal development and possibly, growth in general, since an *EFG1* null strain could not be constructed; its termini, like the transactivation domain of Myc, are rich in serine and proline residues. On the other hand, Efg1p appears to repress the formation of elongated, pseudohyphal-like cells (the phenotype observed at low Efg1p production levels). Similar to Myc transcripts and consistent with a repressor function, Efg1p transcript levels decline drastically during hyphal induction. Also, we obtained evidence that *EFG1* is autoregulated, thus encoding a repressor of its own promoter activity (unpublished results). The repressing activity of Efg1p, as in the case of Myc, may be mediated by association with accessory transcriptional repressors, such as Sin3p (Ayer *et al.*, 1995). A *C.albicans* protein forming a heterodimer by its bHLH domain with Efg1p is not known. Possibly, the Phd1p and Sok2p bHLH proteins in the yeast *S.cerevisiae* may form a heterodimer of an activator and a repressor protein as Myc–Max. Sok2p appears to represent a transcriptional factor that represses genes activated by

cAMP-dependent PKA (Ward *et al.*, 1996). Since Efg1p overexpression stimulates pseudohyphal formation in *S.cerevisiae* and *C.albicans* it may represent the *C.albicans* analogue of the *S.cerevisiae* Phd1 protein. However, while *EFG1* is essential for the morphogenesis of *C.albicans*, a dependence on Phd1p function becomes apparent only if Sok2p function is absent (Ward *et al.*, 1996). As *EFG1* in *C.albicans*, *stuA* is an essential gene for morphogenesis in *A.nidulans* (Miller *et al.*, 1992).

Much of the recent interest in *C.albicans* is based on the demand for effective antifungal agents to cope with the increasing incidence of life-threatening systemic candidiasis. Efg1p may be an effective antifungal target, because it is essential for *C.albicans* morphogenesis and presumably for its growth, while it contains specific features present only in fungal SPSE proteins, on which selectivity of possible agents may be based. Since Efg1p may dimerize, as other bHLH proteins, such interacting proteins, which remain to be discovered, are also possible drug targets. Finally, target genes of Efg1p must be defined, which may present additional opportunities for the action of antifungal agents.

Materials and methods

Strains and media

Strains are listed in Table I. A haploid *S.cerevisiae* laboratory strain, B76 (derivative of MB331-17A), was diploidized by transformation with YCp50-HO (Herskowitz and Jensen, 1991); a plasmid-free derivative was isolated and designated B76/4. This strain shows similar pseudohyphal growth on low nitrogen media as a strain described previously (Gimeno *et al.*, 1992). Transformation of *S.cerevisiae* was by the lithium acetate method and of *C.albicans* by the spheroplast method (Sherman *et al.*, 1986). Yeast cells were grown in YPD, YP (as YPD without glucose) or on supplemented SD minimal medium (Sherman *et al.*, 1986). The *PCK1* promoter was induced in B-medium [0.67% yeast nitrogen base (Bacto), 2% Na-succinate, pH 6.5] or in CAA-medium [0.67% yeast nitrogen base (Bacto), 2% casamino acids]. To induce pseudohyphal growth of *S.cerevisiae* cells were grown on SLADH plates (Gimeno *et al.*, 1994) containing 50 µM ammonium sulfate and the required amino acids. Pseudohyphal growth was inspected microscopically after 2 days at 30°C, using a Zeiss Axioscop. To induce hyphae *C.albicans* cells were pregrown at 30°C in YPD to the logarithmic growth phase and then diluted (OD_{600 nm} = 0.1) into YP, 5% horse serum or YPD containing 5% horse serum at 37°C (Delbrück and Ernst, 1993).

Screening

A genomic library was constructed by inserting random genomic *Sau3AI* fragments of *C.albicans* ATCC10231 into the 2µ vector pLG-B/1 (Leuker and Ernst, 1994); this library contained 50% inserts with an average size of 6 kb. B76/4 was transformed with the pLG-B/1- and YRp7- (Losberger and Ernst, 1989) banks selecting for tryptophan or uracil prototrophy, respectively. Single transformants were grown on SLADH plates and their colony and cell morphology was examined after 2 days. In a limited screening we identified one transformant with enhanced formation of pseudohyphae among 2300 transformants of the YRp7 library (p606/1) and one such transformant among 1500 transformants of the pLG89-B/1 library (p607/2). Control transformants contained plasmid YCpR2V encoding the dominant Ras^{Val19} protein or pCG38 encoding *PHD1* (Gimeno *et al.*, 1992; Gimeno and Fink, 1994).

Subcloning and sequencing

Plasmids p606/1 and p607/2 were recovered from transformants with enhanced pseudohyphal formation of pseudohyphae (Sherman *et al.*, 1986) and retransformed into B76/4 to confirm their phenotypic effects. Subclones of the 5.2 kb *Bam*HI insert in plasmid p606/1 were generated by insertion of subfragments into YEplac vectors (Gietz and Sugino, 1988) or pRS424 (Christianson *et al.*, 1992). A subclone carrying the 3.8 kb *Eco*RI subfragment was cut with *Msc*I and *Pst*RI and shortened by exonuclease III, followed by nuclease S1 treatment and religation;

Table I. Strains and plasmids

Strain or plasmid	Genotype or description	Source
<i>C.albicans</i> strains		
ATCC10231	prototrophic	Poulter and Rikkerink (1983)
ATCC10261	prototrophic	Poulter and Rikkerink (1983)
SC5314	prototrophic	Fonzi and Irwin (1993)
CAI4	Δ ura3::imm434/ Δ ura3::imm434	Fonzi and Irwin (1993)
CAI8	ade2::hisG/ ade2::hisG Δ ura3::imm434/ Δ ura3::imm434	Fonzi and Irwin (1993)
BN5	as CAI8, but: <i>EFG1/PCK1p::EFG1</i>	this work
SS4 or SS14	as CAI8, but: <i>efg1::ADE2/PCK1p::EFG1</i>	this work
<i>S.cerevisiae</i> strains		
CGX31	<i>MATa/MATα</i> ura3-52/ura3-52	Gimeno <i>et al.</i> (1992)
HLY492	<i>MATa/MATα</i> ste20::TRP1/ste20::TRP1 <i>trp1::hisG/ trp1::hisG</i> ura3-52/ura3-52	Liu <i>et al.</i> (1993)
B76	<i>MATα</i> <i>trp1</i> ura3-52 <i>put2-57</i>	this work
B76/4	<i>MATa/MATα</i> <i>trp1/trp1</i> ura3-52/ura3-52 <i>put2-57/put2-57</i>	this work
<i>C.albicans</i> transformation vectors		
pRC2312	<i>URA3</i> -marked <i>CaARS</i> vector	Cannon <i>et al.</i> (1992)
pMK16	<i>ADE2</i> -marked <i>CARS1</i> vector	Kurtz <i>et al.</i> (1987)
pRC18	as pRC2312, but containing pUC-18 multiple cloning site	this work
pBI-1	<i>PCK1</i> promoter in pRC2312	this work
pRC2312P-H	<i>PCK1</i> promoter:: <i>EFG1</i> fusion in pRC2312	this work
pCL76	<i>EFG1</i> promoter:: <i>LAC4</i> fusion in pRC18	this work
<i>S.cerevisiae</i> transformation vectors		
pLG-B/1	<i>URA3</i> -marked 2 μ m vector	Leuker and Ernst (1994)
pCG38	<i>PHD1</i> in <i>URA3</i> -marked 2 μ m vector	Gimeno and Fink (1994)
YCpR2V	<i>RAS2</i> (Val19) allele in <i>URA3</i> -marked vector	Gimeno <i>et al.</i> (1992)
p606/1	genomic <i>EFG1</i> fragment in YRp7	this work
p607/2	genomic <i>EFG1</i> fragment in pLG-B/1	this work
pBIST	<i>GALI</i> -promoter:: <i>EFG1</i> fusion in YEplac195	this work

deletions from the opposite end of the insert were obtained after *ClaI* and *ApaI* digestion. Subclones were sequenced by the dideoxy chain termination method using double-stranded plasmid DNA as templates.

A *Bam*HI site was placed upstream of the putative *EFG1* ATG start codon (5'-GGATCCTA ATG) by PCR-amplification of a 0.6 kb *Bam*HI-*Xho*I fragment; this was verified by sequencing. The reassembled *EFG1* coding region, as a 2.3 kb *Bam*HI-*Sph*III fragment, was inserted downstream of the *GALI*-promoter on a *Eco*RI-*Bam*HI fragment (Johnston and Davis, 1984) and inserted in YEplac195 (*Eco*RI, *Sph*III) to generate pBIST. To overexpress *EFG1* in *C.albicans*, pBI-1, a derivative of pRC2312 (Cannon *et al.*, 1992) containing a 1.5 kb *Bam*HI-*Bgl*III promoter fragment of the *C.albicans* *PCK1* gene was used (C.Leuker, A.Sonneborn and J.F.Ernst, manuscript in preparation); in this plasmid the *Bgl*III site was created at the position of the *PCK1* ATG translational start sequence by PCR mutagenesis. Downstream of the *PCK1* promoter the entire *EFG1* coding region was reconstructed by consecutively inserting the 1 kb *Bam*HI-*Bgl*III and the 0.7 kb *Bgl*III-*Hind*III fragments of pBIST, to create plasmid pRC2312P-H.

To study transcriptional regulation of *EFG1* pCL76 was constructed, in which the *EFG1* promoter regulates expression of the *LAC4* reporter gene (Leuker *et al.*, 1992). First, a *Hinc*II-*Sal*I site was constructed by PCR mutagenesis at the ATG start sequence (CCCATATTAATG > CCCATGTGCGAC). The *Bam*HI-*Hinc*II promoter fragment was ligated with a 4.2 kb *Pvu*II-*Xba*I *LAC4* fragment of pRS-LAC4 (unpublished results) and inserted into pRC18, a derivative of pRC2312 (Cannon *et al.*, 1992) containing the pUC-18 multiple cloning site, to construct pCL76.

Gene disruption

To replace the *EFG1* promoter in one of the *C.albicans* alleles by the regulatable *PCK1* promoter a plasmid carrying an appropriate disruption fragment was constructed. Plasmid pCA01, containing the 1.5 kb *Bam*HI-*Bgl*III *PCK1* promoter fragment in pUC-21 (C.Leuker, A.Sonneborn and J.F.Ernst, manuscript in preparation), was modified by first inserting the 1.55 kb *Bam*HI-*Bgl*III fragment of pBIST carrying part of the *EFG1* coding region into the *Bgl*III site at the 3' end of the promoter; this was followed by insertion, in the presence of an oligonucleotide adapter, of a 1 kb *Bam*HI-*Pst*I *URA3* fragment (Losberger and Ernst, 1989) into

the *Bam*HI site upstream of the promoter. This construction retained a single *Bam*HI site flanking *URA3*, into which a 3.5 kb *Bgl*III-*Bam*HI fragment containing *EFG1* upstream promoter sequences was inserted. From the final plasmid, pCAHUH, a 7.8 kb *Not*I-*Bgl*III fragment was isolated (*Not*I is situated within the plasmid 5' of *Bgl*III-*Bam*HI fusion point) and used to transform strain CAI-8 to uracil prototrophy.

To disrupt the second *EFG1* allele the 2.4 kb *Eco*RV fragment of pMK16 carrying the *C.albicans* *ADE2*-gene (Kurtz *et al.*, 1987) was ligated into the single *Msc*I site within the *EFG1* coding region of a pUC19-subclone containing the 3.8 kb *Eco*RI-*Bam*HI *EFG1* fragment (pUC19*EFG1*). The 5.2 kb *Xho*I fragment of the resulting vector pUC19/*EFG1ADE2* was transformed into the CAI-8 transformant carrying the *PCK1::EFG1* allele selecting for prototrophy. Genomic DNA of transformants was analyzed on Southern blots using the 250 bp *Eco*RV-*Xho*I *EFG1* fragment as probe.

Other methods

Total RNA and poly(A) RNA of *C.albicans* ATCC10261 were isolated and separated by denaturing gel electrophoresis as described (Delbrück and Ernst, 1993). For transcript mapping a 341 bp *Eco*RI-*Nhe*I fragment labelled at its *Nhe*I-end by filling-in using Klenow enzyme, dCTP, dTTP and [α -³²P]dATP was isolated and hybridized to 2-4 μ g poly(A)-RNA. Treatment with nuclease S1 was performed essentially as described (Sambrook *et al.*, 1989) and the single product, a 168 nt RNA, was identified on a sequencing gel. Total DNA of *C.albicans* strains was prepared as described (Sherman *et al.*, 1986). Southern blots on fungal genomic DNAs were performed according to standard procedures (Sambrook *et al.*, 1989). Nucleic acids blots on nylon (Hybond, Amersham) were probed using the 1.5 kb *Nhe*I fragment of p606/1 as *EFG1* probe; as controls an *ACT1* fragment (Delbrück and Ernst, 1993) was used. Probes were labelled to high specific activities with ³²P using random oligonucleotides as primers (Sambrook *et al.*, 1989). *LAC4*-specified β -galactosidase activity was measured using the Galacto-Light Plus kit (Tropix); luminescence was determined in a LB953 luminometer (EG&G Berthold GmbH) and expressed as relative light units (RLU) per μ g protein. In this assay 100 pg of *Escherichia coli* β gal had an activity of 20 000 RLU; the assay was linear from 10 pg to 500 pg β gal. Protein was determined using the Bradford assay (Bio-Rad).

Acknowledgements

We thank B.B.Magee for mapping information. We thank B.Strickling for contributing to the construction of pBIST and acknowledge the excellent technical help of M.Gerads. We thank M.Wigler for plasmid YCpR2V, G.R.Fink for pCG38 and H.Liu for strains CGX-31 and HLY492. This work was supported by the Deutsche Forschungsgemeinschaft.

References

- Ayer, D.E. and Eisenman, R.N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.*, **7**, 2110–2119.
- Ayer, D.E., Lawrence, Q.A. and Eisenman, R.N. (1995) Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell*, **80**, 767–776.
- Arai, T., Mikami, Y. and Yokoyama, K. (1977) Phagocytosis of *Candida albicans* by rabbit alveolar macrophages and guinea pig neutrophils. *Sabouraudia*, **15**, 171–177.
- Bernards, R. (1995) Flipping the Myc switch. *Curr. Biol.*, **5**, 859–861.
- Blacketer, M.J., Koehler, C.M., Coats, S.G., Myers, A.M. and Madaule, P. (1993) Regulation of dimorphism in *S.cerevisiae*: involvement of a novel protein kinase homolog Elm1p and protein phosphatase 2A. *Mol. Cell. Biol.*, **13**, 5567–5581.
- Calderone, R.A. and Braun, P. (1991) Adherence and receptor relationships of *Candida albicans*. *Microbiol. Rev.*, **55**, 1–20.
- Cannon, R.D., Jenkinson, H.F. and Shepherd, M.G. (1992) Cloning and expression of *Candida albicans* ADE2 and proteinase genes on a replicative plasmid in *C. albicans* and in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **235**, 453–457.
- 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.
- Cutler, J.E. (1991) Putative virulence factors of *Candida albicans*. *Annu. Rev. Microbiol.*, **45**, 187–218.
- Delbrück, S. and Ernst, J.F. (1993) Morphogenesis-independent regulation of actin transcript levels in the pathogenic yeast *Candida albicans*. *Mol. Microbiol.*, **10**, 859–866.
- Ellenberger, T. (1994) Getting a grip on DNA structures of the basic region leucine zipper, and the basic region helix-loop-helix DNA-binding domains. *Curr. Opin. Struct. Biol.*, **4**, 12–21.
- Ferré-D'Amaré, A., Prendergast, G.C., Ziff, E.B. and Burley, S.K. (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature*, **363**, 38–45.
- Fisher, F. and Goding, C.R. (1992) Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG. *EMBO J.*, **11**, 4103–4109.
- Fonzi, W.A. and Irwin, M.Y. (1993) Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics*, **134**, 717–728.
- Gavrias, V., Andrianopoulos, A., Gimeno, C.J. and Timberlake, W.E. (1996) *Saccharomyces cerevisiae* TEC1 is required for pseudohyphal growth. *Mol. Microbiol.*, **19**, 1255–1263.
- Gietz, R.D. and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.
- Gimeno, C.J. and Fink, G.R. (1994) Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Mol. Biol. Cell*, **14**, 2100–2112.
- Gimeno, 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.
- Hahn, S., Hoar, E.T. and Guarente, L. (1985) Each of three TATA elements specifies a subset of the transcription initiation sites at the CYC1 promoter of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **82**, 8562–8566.
- Herskowitz, I. and Jensen, R.E. (1991) Putting the HO gene to work: practical uses for mating-type switching. *Methods Enzymol.*, **194**, 132–146.
- Jackson, R.J. and Standar, N. (1990) Do the poly(A) tail and 3' untranslated region control mRNA translation? *Cell*, **62**, 15–24.
- Johnston, M. and Davis, R.W. (1984) Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **4**, 1440–1448.
- Kurtz, M.B., Cortelyou, M.W., Miller, S.M. and Kirsch, D.R. (1987) Development of autonomously replicating plasmids for *Candida albicans*. *Mol. Cell. Biol.*, **7**, 209–217.
- Leuker, C.E. and Ernst, J.F. (1994) Toxicity of a heterologous leucyl-tRNA (anticodon CAG) in the pathogen *Candida albicans*: *in vivo* evidence for non-standard decoding of CUG codons. *Mol. Gen. Genet.*, **245**, 212–217.
- Leuker, C.E., Hahn, A.-M. and Ernst, J.F. (1992) β -galactosidase of *Kluyveromyces lactis* (Lac4p) as reporter of gene expression in *Candida albicans* and *C. tropicalis*. *Mol. Gen. Genet.*, **235**, 235–241.
- Li, L., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E.B. (1994) c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.*, **13**, 4070–4079.
- 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., Köhler, J. and Fink, G.R. (1994) Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. *Science*, **266**, 1723–1725.
- Losberger, C. and Ernst, J.F. (1989) Sequence and transcript analysis of the *C. albicans* URA3 gene encoding orotidine-5'-phosphate decarboxylase. *Curr. Genet.*, **16**, 153–157.
- Ma, P.C.M., Rould, M.A., Weintraub, H. and Pabo, C.O. (1994) Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell*, **77**, 451–459.
- Maresca, B. and Kobayashi, G.S. (1989) Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. *Microbiol. Rev.*, **53**, 186–209.
- Miller, K.Y., Wu, J. and Miller, B.L. (1992) StuA is required for cell pattern formation in *Aspergillus*. *Genes Dev.*, **6**, 1770–1782.
- Mösch, H.-U., Roberts, R. and Fink, G.R. (1996) Ras2 signals via the Cdc42/Ste20/mitogen-activated kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **93**, 5352–5356.
- Newlon, C.S. and Theis, J.F. (1993) The structure and function of yeast ARS elements. *Curr. Opin. Genet. Cell Biol.*, **3**, 752–758.
- Odds, F.C. (1988) *Candida and Candidosis*. Baillière Tindall, London, UK.
- Poulter, R.T.M. and Rikkerink, E.H. (1983) Genetic analysis of red, adenine-requiring mutants of *Candida albicans*. *J. Bacteriol.*, **156**, 1066–1077.
- Ptashne, M. and Gann, A.A.F. (1990) Activators and targets. *Nature*, **346**, 329–331.
- Sadhu, C., Hoekstra, D., McEachern, M.J., Reed, S.I. and Hicks, J.B. (1992) A G-protein α subunit from asexual *Candida albicans* functions in the mating pathway of *Saccharomyces cerevisiae* and is regulated by the $\alpha 1$ - $\alpha 2$ repressor. *Mol. Cell. Biol.*, **12**, 1977–1985.
- Sambrook, J., Fritsch, E.R. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Santos, M.A.S. and Tuite, M.F. (1995) The CUG codon is decoded *in vivo* as serine and not leucine in *Candida albicans*. *Nucleic Acids Res.*, **23**, 1481–1486.
- Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schäfer, W., Martin, T., Herskowitz, I. and Kahmann, R. (1990) The *b* alleles of *U.maydis*, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. *Cell*, **60**, 295–306.
- Sherman, F., Fink, G. and Hicks, J. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Soll, D.R., Morrow, B. and Srikantha, T. (1993) High-frequency phenotypic switching in *Candida albicans*. *Trends Genet.*, **9**, 61–65.
- Tjian, R. and Maniatis, T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.
- Treitel, M.A. and Carlson, M. (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl Acad. Sci. USA*, **92**, 3132–3136.
- Winston, F. and Carlson, M. (1992) Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. *Trends Genet.*, **8**, 387–392.
- Ward, M.P., Gimeno, C.J., Fink, G.R. and Garrett, S. (1996) SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. *Mol. Cell. Biol.*, **15**, 6854–6863.
- Zaret, K.S. and Sherman, F. (1982) DNA sequence required for efficient transcription termination in yeast. *Cell*, **28**, 563–573.

Received on September 19, 1996; revised on November 22, 1996