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Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*

Gyanendra Tripathi[1], Carolyn Wiltshire[2], Susan Macaskill, Helene Tournu, Susan Budge and Alistair J.P.Brown[3]

Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

[1]Present address: The Babraham Institute, Babraham, Cambridge CB2 4AT, UK
[2]Present address: CRC Beatson Laboratories, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK

[3]Corresponding author
e-mail: Al.Brown@abdn.ac.uk

Candida albicans **is a major fungal pathogen of humans. It regulates its morphology in response to various environmental signals, but many of these signals are poorly defined. We show that amino acid starvation induces filamentous growth in *C.albicans*. Also, starvation for a single amino acid (histidine) induces *CaHIS4*, *CaHIS7*, *CaARO4*, *CaLYS1* and *CaLYS2* gene expression in a manner reminiscent of the GCN response in *Saccharomyces cerevisiae*. These morphogenetic and GCN-like responses are both dependent upon CaGcn4, which is a functional homologue of *S.cerevisiae* Gcn4. Like ScGcn4, CaGcn4 activates the transcription of amino acid biosynthetic genes via the GCRE element, and CaGcn4 confers resistance to the histidine analogue, 3-aminotriazole. CaGcn4 interacts with the Ras-cAMP pathway to promote filamentous growth, but the GCN-like response is not dependent upon morphogenetic signalling. CaGcn4 acts as a global regulator in *C.albicans*, co-ordinating both metabolic and morphogenetic responses to amino acid starvation.**

Keywords: amino acid starvation/*Candida albicans*/*GCN4*/morphogenesis/transcription

Introduction

The growth and survival of all microbes is dependent upon their ability to detect and respond to environmental change. This is particularly true for pathogenic microbes, which must counteract host responses and adapt to changing microenvironments during the establishment and progression of infections.

Candida albicans is a major fungal pathogen of humans. It causes frequent and recurrent oral and vaginal infections, and life-threatening systemic infections in immunocompromised patients (Odds, 1988). *Candida albicans* virulence is enhanced by several factors, one of which is its ability to undergo reversible morphological transitions between yeast, pseudohyphal and hyphal growth forms (Odds, 1994; Lo *et al*., 1997). Numerous conditions promote yeast–hypha morphogenesis *in vitro*, including growth of *C.albicans* at ambient temperatures >35°C, serum, neutral pH and nutrient starvation (Odds, 1988). These presumably reflect signals that control morphogenesis *in vivo* (Brown and Gow, 1999). However, the exact nature of many morphogenetic signals is poorly understood, particularly those detected by this fungus in serum or following nutrient starvation.

Several signalling pathways control morphogenesis in *C.albicans*. These include MAPK and Ras-cAMP signalling pathways that are thought to activate filamentous growth in response to starvation and/or serum signals (Ernst, 2000; Whiteway, 2000; Brown, 2001). The MAPK pathway is dependent upon Cph1, a homologue of *Saccharomyces cerevisiae* Ste12 (Liu *et al*., 1994). The Ras-cAMP pathway requires functional Efg1, which is a member of the APSES family of basic helix–loop–helix (bHLH) transcription factors (Stoldt *et al*., 1997; Bockmuhl and Ernst, 2001). Under most experimental conditions, yeast–hypha morphogenesis is blocked in a *C.albicans cph1/cph1*, *efg1/efg1* double mutant (Lo *et al*., 1997), indicating that the transduction of most environmental signals is dependent upon Ras-cAMP or MAPK signalling.

In addition, a Prr2/CaRim101-dependent pathway activates morphogenesis in response to ambient pH (Ramon *et al*., 1999; Davis *et al*., 2000), a Czf1 pathway promotes hyphal development when *C.albicans* cells are embedded in a solid matrix (Brown *et al*., 1999), and a Cph2 pathway whose signals are poorly defined (Lane *et al*., 2001a). The Prr2/CaRim101 and Cph2 pathways appear to be integrated with the Ras-cAMP pathway, and Czf1 with the MAPK pathway (Brown *et al*., 1999; El Barkani *et al*., 2000; Lane *et al*., 2001b). In addition, a CaTup1–CaNrg1 pathway represses filamentous growth (Braun and Johnson, 1997; Braun *et al*., 2001; Murad *et al*., 2001).

In *S.cerevisiae*, starvation for a single amino acid stimulates the expression of genes on all amino acid biosynthetic pathways in a phenomenon termed general amino acid control (or the GCN response) (Hinnebusch, 1988). This response is dependent upon the bZIP transcriptional activator, ScGcn4, which interacts with general control response elements (GCREs) in the promoters of target genes (Arndt and Fink, 1986; Hinnebusch, 1988). *ScGCN4* expression is regulated at both translational and transcriptional levels (Hinnebusch, 1988; Albrecht *et al*., 1998).

We noted that some conditions which promote morphogenesis impose amino acid starvation upon *C.albicans* (Odds, 1988), and that *CaARO3* mRNA levels increase following amino acid starvation, suggesting that a GCN-like response might exist in *C.albicans* (Pereira and Livi, 1995). Here we report that amino acid starvation stimulates morphogenesis in *C.albicans*, and confirm that a GCN-like response does exist in this fungus. We show that

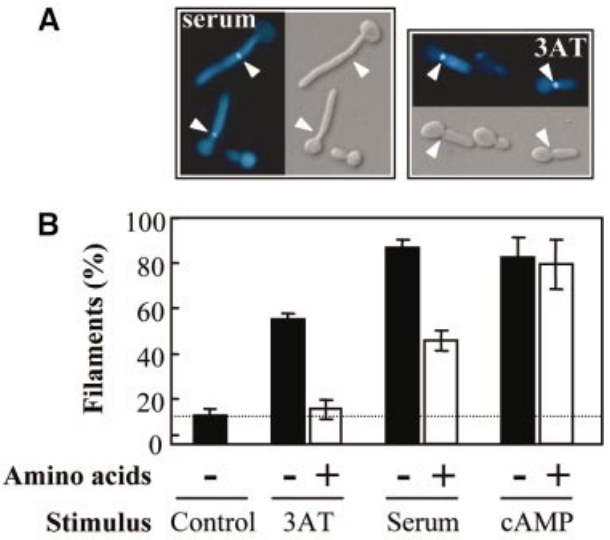

Fig. 1. Amino acid starvation induces filamentous growth in *C.albicans*. (**A**) CAF-2 cells grown in YPD containing 10 mM 3AT or 10% serum for 2 h, stained with Calcofluor white, and visualized by light and fluorescence microscopy. Arrows highlight the positions of septa. (**B**) Percentage filament formation by *C.albicans* CAF2-1 (Table I) following 2 h growth at 37°C in YPD with the following additions: ± 20 mg/ml amino acids (open and closed bars, respectively); H_2O control; 10 mM 3AT; 10% fetal calf serum; 10 mM dibutyryl cAMP.

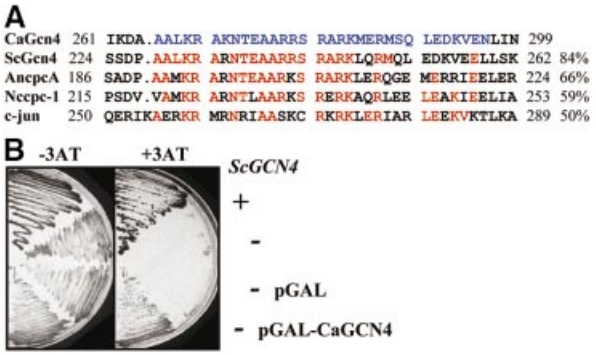

Fig. 2. CaGcn4 is a functional homologue of *S.cerevisiae* Gcn4. (**A**) Alignment of the DNA-binding regions of the Gcn4-like proteins from *C.albicans* (CaGcn4; AAF18140), *S.cerevisiae* (ScGcn4; K02205), *A.niger* (cpcAp; X99215), *N.crassa* (CPC1; J03262) and humans (c-jun; J04111). The DNA-binding region in CaGcn4 is highlighted in blue, and identical residues in the other proteins are shown in red. Percentage identities to this region of CaGcn4 are shown. (**B**) Growth of *S.cerevisiae* strains on galactose plates ± 30 mM 3AT: +, *GCN4* W303–1B; –, *gcn4* H2036; pGAL, empty expression vector pRS-GAL1; pGAL-CaGCN4.

both responses are dependent upon CaGcn4, a functional homologue of *S.cerevisiae* Gcn4. Hence, CaGcn4 plays a central role in co-ordinating morphogenetic and metabolic responses to amino acid starvation in this major pathogen of humans.

Results

Amino acid starvation stimulates morphogenesis in C.albicans

First, we tested whether amino acid starvation stimulates filamentous growth in *C.albicans*. Treatment with the histidine analogue, 3-aminotriazole (3AT), imposes histidine starvation in *S.cerevisiae* (Marton *et al*., 1998). Therefore, we exposed *C.albicans* cells to 3AT and examined their morphology. This treatment stimulated the growth of filamentous cells (Figure 1A).

The septin ring forms at the bud neck in pseudohyphal cells, but within the germ tube of a developing hypha (Sudbery, 2001). Therefore, to distinguish whether 3AT stimulated the development of pseudohyphae or true hyphae, we stained the cells with Calcofluor white, which preferentially stains septa and bud scars due to their relatively high chitin contents. Septa formed at the bud necks of filamentous cells induced by 3AT treatment, indicating that they were pseudohyphal (Figure 1A).

Many drugs exert unexpected side effects. Therefore, we tested whether the addition of amino acids to the growth medium suppresses the morphogenetic effects of 3AT. Histidine did not suppress the effect, possibly because addition of a single amino acid can induce amino acid starvation by inhibiting the biosynthesis of other amino acids (Hinnebusch, 1988). However, addition of an amino acid mix did suppress 3AT-induced morphogenesis (Figure 1B), indicating that amino acid starvation, imposed by 3AT treatment, stimulates the formation of pseudohyphae. This confirms amino acid starvation as a morphogenetic signal in *C.albicans*.

Serum is considered to be a medically relevant morphogenetic stimulus, but the nature of this stimulus has not been defined (Ernst, 2000). Serum addition stimulated filamentous growth more strongly than 3AT treatment (Figure 1B). Furthermore, serum stimulated the formation of true hyphae, whereas pseudohyphae were formed in response to 3AT treatment (Figure 1A). However, amino acids partially suppressed the serum stimulus, and did so in a reproducible manner (Figure 1B), suggesting that amino acid starvation is one of several morphogenetic signals present in serum.

Candida albicans GCN4 is a functional homologue of S.cerevisiae GCN4

ScGcn4 mediates responses to amino acid starvation in *S.cerevisiae* (Hinnebusch, 1988). We reasoned, therefore, that a homologue of ScGcn4 might mediate amino acid starvation-induced morphogenesis in *C.albicans*. Hence, we isolated the *C.albicans GCN4* gene.

Saccharomyces cerevisiae gcn4 mutants are unable to grow in the presence of 3AT. Therefore, we screened for *CaGCN4* cDNA clones based on their ability to suppress the 3AT sensitivity of *S.cerevisiae gcn4* cells. A cDNA clone was then used to isolate the complete *CaGCN4* locus from a *C.albicans* genomic library. Both genomic and cDNA clones were sequenced. The sequence of the *CaGCN4* locus revealed a major open reading frame (ORF) capable of encoding a protein of 322 amino acids. The longest cDNA sequences revealed that the *CaGCN4* mRNA contains an unusually long 5′ leader sequence of at least 625 nucleotides with three upstream ORFs.

The C-terminal region of CaGcn4 displayed significant amino acid sequence similarity to the DNA-binding domains of ScGcn4 and Gcn4-like proteins from *Aspergillus niger*, *Neurospora crassa* and humans (Figure 2A). Significant sequence similarity amongst these proteins was limited to their DNA-binding regions.

Table I. Strains analysed in this study

Strain	Genotype	Source
S.cerevisiae		
H2036	*MATa, ura3, leu2, trp1, gcn4*	Drysdale *et al.* (1995)
W303-1B	*MATa, ura3, leu2, trp1, ade2, his3*	Thomas and Rothstein (1987)
C.albicans		
SC5314	Wild-type	Gillum *et al.* (1984)
CAF2-1	*URA3/ura3::λ imm434*	Fonzi and Irwin (1993)
CAI4	*ura3::λ imm434/ura3::λ imm434,*	Fonzi and Irwin (1993)
GTC41	*ura3::λ imm434/ura3::λ imm434, GCN4/gcn4::hisG-URA3-hisG*	This study
GTC42	*ura3::λ imm434/ura3::λ imm434, GCN4/gcn4::hisG*	This study
GTC43	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG-URA3-hisG/gcn4::hisG*	This study
GTC44	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG*	This study
GTC45	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG,* CIp10-*GCN4*	This study
GTC46	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG,* CIp10-*MET3p*	This study
GTC47	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG,* CIp10-*MET3p-GCN4*	This study
GTC48	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG,* CIp10-*ACT1p*	This study
GTC49	*ura3::λ imm434/ura3::λ imm434, gcn4::hisG/gcn4::hisG,* CIp10-*ACT1p-GCN4*	This study
CAI8	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG*	Fonzi and Irwin (1993)
GTC81	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, GCN4/gcn4::hisG-URA3-hisG*	This study
GTC82	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, GCN4/gcn4::hisG*	This study
GTC83	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, gcn4::hisG-URA3-hisG/gcn4::hisG*	This study
GTC84	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, gcn4::hisG/gcn4::hisG*	This study
GTC85	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, gcn4::hisG/gcn4::hisG,* CIp10-*GCN4*	This study
GTC86	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, gcn4::hisG/gcn4::hisG,* CIp10-*MET3p*	This study
GTC87	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, gcn4::hisG/gcn4::hisG,* CIp10-*MET3p-GCN4*	This study
JKC19	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG-URA3-hisG*	Liu *et al.* (1994)
HLC52	*ura3::λ imm434/ura3::λ imm434, efg1::hisG /efg1::hisG-URA3-hisG*	Lo *et al.* (1997)
HLC54	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG, efg1::hisG /efg1::hisG-URA3-hisG*	Lo *et al.* (1997)
ras1-2/ras1-3	*ura3::λ imm434/ura3::λ imm434, ras1Δ::hisG/ ras1Δ::hph-URA3-hph*	Feng *et al.* (1999)
RAS1/RAS1/RAS1^{V13}	*RAS1/RAS1/ade2::Mal-RAS1*^{V13}*-URA3*	Feng *et al.* (1999)
RAS1/RAS1	*RAS1/RAS1/ade2::Mal-URA3*	Feng *et al.* (1999)

This is consistent with other transcription factors in *C.albicans* and *S.cerevisiae*, whose sequence conservation is restricted to their DNA-binding domains: for example, CaRfg1/ScRox1 and CaNrg1/ScNrg1 (Braun *et al*., 2001; Kadosh and Johnson, 2001; Murad *et al*., 2001). Hence, CaGcn4 belongs to a family of bZIP transcription factors that includes the global regulators ScGcn4, cpcAp, CPC1 and c-jun (Figure 2A).

To test whether CaGcn4 is a functional homologue of ScGcn4, *CaGCN4* was expressed in *S.cerevisiae gcn4* cells under the control of the *S.cerevisiae GAL1* promoter. This promoter is repressed by glucose and induced by galactose. The *GAL1-CaGCN4* fusion suppressed the 3AT sensitivity of the *gcn4* cells (Figure 2B). As expected, this suppression was galactose dependent (not shown), and was not observed in control cells containing the empty *GAL1* expression plasmid. Therefore, CaGcn4 is a functional homologue of ScGcn4.

Inactivation of CaGcn4 blocks amino acid starvation-induced morphogenesis

Candida albicans gcn4/gcn4 mutants were constructed using the strain CAI4. The two *CaGCN4* alleles in this diploid fungus were disrupted sequentially using a cassette that deleted codons 100–248 of the 322 codon ORF, thereby inactivating the DNA-binding domain. Disruption of the *CaGCN4* locus was confirmed by Southern blotting. *CaGCN4* mRNA was undetectable in *gcn4/gcn4* cells by northern blot analysis or RT–PCR.

The role of CaGcn4 in amino acid starvation-induced morphogenesis was then investigated using these mutants (Table I). The morphology of cells grown in YPD and exposed to 10 mM 3AT for 2 h was examined. During the course of this experiment, both wild-type and mutant cells underwent one cell division. About 30% of cells containing a functional *CaGCN4* locus formed filamentous projections, whereas the *gcn4/gcn4* cells only formed buds (Figure 3). As a control, *CaGCN4* was disrupted in a second *C.albicans* strain (CAI8; Table I). Again, inactivation of CaGcn4 blocked amino acid starvation-induced morphogenesis. To control for transformation-induced mutations, the *CaGCN4* gene was reintroduced into both *gcn4/gcn4* mutants. In each case, the reintroduction of *CaGCN4* restored the wild-type phenotype. Clearly, CaGcn4 is required for amino acid starvation-induced filamentous growth in *C.albicans*.

As described above, amino acid starvation is one of several morphogenetic signals present in serum. Therefore, one would not expect the disruption of *CaGCN4* to block serum-induced morphogenesis in *C.albicans*. As predicted, *gcn4/gcn4* cells formed normal hyphae following serum stimulation (data not shown).

CaGcn4 activates transcription via the GCRE

ScGcn4 interacts with the GCRE (TGACTC) to activate transcription in *S.cerevisiae* (Arndt and Fink, 1986). *CaGCN4* complements a *S.cerevisiae gcn4* mutation (Figure 2B), suggesting that CaGcn4 might have a similar

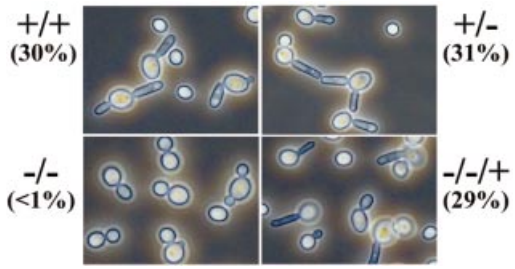

Fig. 3. Inactivation of CaGcn4 blocks amino acid starvation-induced morphogenesis in *C.albicans*. Phase contrast microscopy of *C.albicans* cells after 2 h growth at 37°C in YPD containing 10 mM 3AT: +/+, CAF2-1 (*GCN4/GCN4*); +/–, GTC41 (*GCN4/gcn4*); –/–, GTC43 (*gcn4/gcn4*); –/–/+, GTC45 (*gcn4/gcn4*, CIp10-*GCN4*) (Table I). Percentage filament formation is given in parentheses (errors <10%).

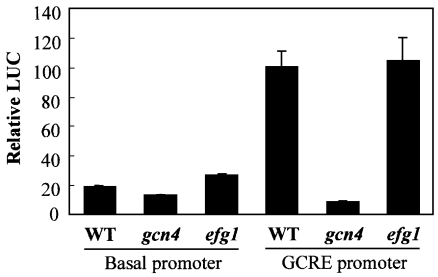

Fig. 4. CaGcn4 activates transcription in *C.albicans* via the GCRE and in an Efg1-independent fashion. *Candida albicans* strains were transformed with *RrLUC* reporter plasmids carrying either a basal promoter or a $GCRE_5$ promoter: WT (wild type, CAI8); *gcn4* null (GTC43); *efg1* null (HLC52) (Table I). Relative luciferase activities following 4 h growth in SD with 20 mM 3AT.

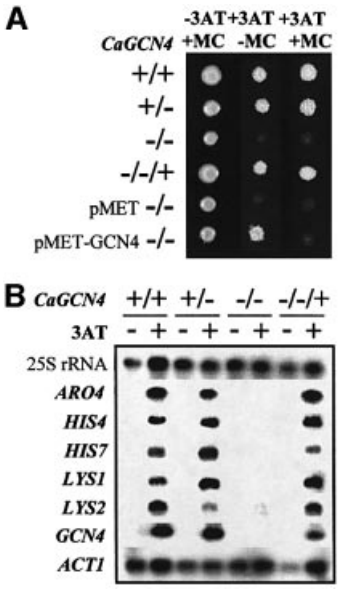

Fig. 5. *Candida albicans* displays a GCN-like response that is dependent upon CaGcn4. (**A**) Disruption of *CaGCN4* confers 3AT sensitivity in *C.albicans*. Isogenic *C.albicans* strains were grown on SC plates ± 20 mM 3AT, and ± 10 mM methionine and 10 mM cysteine (+MC, –MC). Their *CaGCN4* genotypes were as follows: +/+, CAF2-1; +/–, GTC41; –/–, GTC43; –/–/+, GTC45; –/– pMET, GTC46; –/– pMET-GCN4, GTC47 (Table I). (**B**) CaGcn4-dependent activation of amino acid biosynthetic mRNA levels in *C.albicans* in response to amino acid starvation. Northern blot analyses of amino acid biosynthetic mRNAs and the *GCN4* mRNA were performed following 2 h of growth in YPD ± 10 mM 3AT, using 25S rRNA and *ACT1* mRNA as loading controls: +/+, CAF2-1; +/–, GTC41; –/–, GTC43; –/–/+, GTC45 (Table I).

mode of action. Therefore, we tested whether CaGcn4 can activate transcription via the GCRE element. We used the *Renilla reniformis* luciferase (*RrLUC*) reporter, which functions in *C.albicans* despite the CUG codon reassignment in this fungus (Srikantha *et al*., 1996).

The activities of two *RrLUC* constructs were compared in 3AT-treated *C.albicans* cells. One construct contained a basal promoter upstream of the *RrLUC* reporter. This basal promoter contained the TATA box, RNA initiation site and 5′ leader region from the *CaADH1* promoter (Bertram *et al*., 1996). The second promoter contained a $(GCRE)_5$ sequence inserted upstream of this basal promoter. A single copy of each *RrLUC* fusion was integrated at the *CaADE2* locus. Low luciferase activities were observed for the basal promoter, and these activities increased ~5-fold following the introduction of the GCRE element. This increase was not observed in *gcn4/gcn4* cells (Figure 4). Therefore, CaGcn4 activates transcription via the GCRE following amino acid starvation in *C.albicans*.

Candida albicans displays a GCN-like response that is dependent upon CaGcn4

We tested whether *C.albicans* displays a GCN-like response. The 3AT sensitivity of *S.cerevisiae gcn4* cells represents a classic reflection of the GCN response in budding yeast. Therefore, we examined the influence of CaGcn4 upon the 3AT sensitivity of *C.albicans*. All *gcn4/gcn4* strains were 3AT sensitive, and the reintroduction of *CaGCN4* into these strains restored 3AT resistance (Figure 5A). *CaGCN4* was also reintroduced under the control of the *CaMET3* promoter, which is repressed by methionine and cysteine (Care *et al*., 1999). As expected, the 3AT sensitivity of these *gcn4/gcn4/MET3-GCN4* cells was suppressed in a methionine- and cysteine-dependent fashion (Figure 5A). Clearly, 3AT resistance in *C.albicans* is dependent upon CaGcn4.

We then examined whether starvation for a single amino acid exerts a general effect upon amino acid biosynthesis in *C.albicans*. We selected five amino acid biosynthetic genes with GCRE sequences from the *C.albicans* genome sequence data generated at the Stanford DNA Sequencing and Technology Center (Tzung *et al*., 2001). *CaARO4*, *CaHIS4*, *CaHIS7*, *CaLYS1* and *CaLYS2* were PCR amplified and used as probes in northern blots to examine the effects of histidine starvation upon the levels of these mRNAs (Figure 5B). All five mRNAs were induced by 3AT, showing that genes on at least three different amino acid biosynthetic pathways are activated by histidine starvation. This effect was blocked in *gcn4/gcn4* cells, and recovered following reintroduction of *CaGCN4*. Therefore, histidine starvation stimulates a GCN-like response that is dependent upon CaGcn4.

Interestingly, *CaGCN4* mRNA levels were elevated following exposure to 3AT, suggesting that *CaGCN4* transcription is induced in response to amino acid starvation (Figure 5B). RT–PCR revealed low basal levels of *CaGCN4* mRNA in non-starved cells carrying an active *CaGCN4* gene (data not shown).

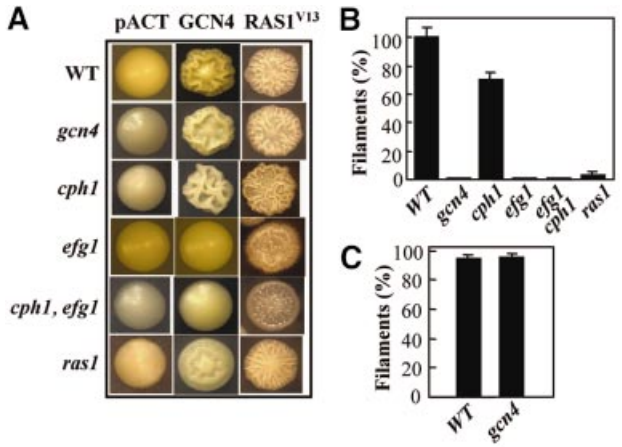

Fig. 6. Ras1–Efg1 signalling influences CaGcn4-dependent activation of morphogenesis in *C.albicans*. (**A**) Colony morphology of *C.albicans* strains following growth on synthetic complete medium containing amino acids and uridine at 30°C for 5 days: WT, CAF2-1; *gcn4*, GTC43; *cph1*, JKC19; *efg1*, HLC52; *cph1*, *egf1*, HLC54; *ras1*, *ras1-2/ras1-3* (Table I). Cells were transformed with the following plasmids: pACT, control plasmid with the *CaACT1* promoter; pACT-GCN4, plasmid carrying a *CaACT1p-CaGCN4* fusion; *RAS1*^{V13}, pQF145.2. (**B**) 3AT-induced filamentation in *C.albicans* signalling mutants. Filament formation was monitored after 2 h growth at 37°C in YPD containing 10 mM 3AT, and expressed as a percentage of filamentation in the wild-type strain. Strains are listed in (A). (**C**) CaGcn4 inactivation does not block *RAS1*^{V13}-induced filamentation. Filament formation was monitored in pQF145.2 transformants after 2 h growth at 37°C in YPMal: WT, CAF2-1; *gcn4*, GTC43.

CaGCN4 stimulates filamentous growth in an Efg1-dependent fashion

Next we tested the effects of ectopic *CaGCN4* expression upon *C.albicans* morphology. The *CaGCN4* ORF was fused to the *CaACT1* promoter and a single copy of this fusion was integrated into the *C.albicans* genome. The *CaACT1-CaGCN4* fusion caused *C.albicans* to form wrinkly colonies in the absence of a morphogenetic stimulus, during growth on synthetic complete medium (Figure 6A). Microscopic analyses of cells from these colonies confirmed that this wrinkly phenotype correlated with filamentous growth. This confirmed the positive effect of CaGcn4 upon morphogenesis.

We then used this phenotype to test the relationship between CaGcn4 and the MAPK and Ras-cAMP pathways that activate hyphal growth in *C.albicans* (Lo *et al*., 1997; Brown and Gow, 1999). The wrinkly colonial phenotype was retained in the *cph1/cph1* mutant (Figure 6A), and *cph1/cph1* cells still formed filaments in response to amino acid starvation in liquid medium (Figure 6B), indicating that CaGcn4 does not act via the MAPK pathway. In contrast, *efg1/efg* pACT1-GCN4 cells formed smooth colonies, and 3AT-induced filamentous growth was blocked in an *efg1/efg1* mutant (Figure 6). Hence, the morphological effects of CaGcn4 are dependent upon Efg1. *EFG1* mRNA levels were not affected significantly by the *gcn4/gcn4* mutation or by 3AT treatment (data not shown). Therefore, *EFG1* expression is not regulated by CaGcn4.

The influence of CaRas1 upon CaGcn4-mediated morphogenesis was more complex. *Ras1/ras1*, pACT1-GCN4 colonies were less wrinkly than control colonies (*RAS1/RAS1*, pACT1-GCN4) (Figure 6A). However, they did contain filamentous cells, indicating that CaGcn4-mediated morphogenesis is not entirely dependent upon Ras1, at least after 5 days growth on solid medium. However, no filamentation was observed in *ras1/ras1* cells after 2 h of 3AT induction in liquid medium (Figure 6B). Hence, CaRas1 inactivation might delay CaGcn4-mediated morphogenesis. Alternatively, the growth conditions might affect the degree to which CaGcn4-mediated morphogenesis depends upon CaRas1 signalling. The latter idea is consistent with previous reports indicating that *ras1/ras1* cells display morphological defects that are medium dependent (Feng *et al*., 1999; Leberer *et al*., 2001). Whatever the explanation, clearly CaGcn4-mediated morphogenesis is partially dependent upon CaRas1.

Several observations indicated that CaGcn4 does not lie directly on the Ras-cAMP signalling pathway. First, in contrast to *ras1/ras1* and *efg1/efg1* cells (Lo *et al*., 1997; Feng *et al*., 1999), *gcn4/gcn4* cells did not display morphological defects when exposed to a serum stimulus. Secondly, the addition of amino acids to the growth medium did not suppress cAMP-stimulated filament formation (Figure 1B). Thirdly, the ability of the constitutively active *CaRAS1*^{V13} allele to stimulate filamentous growth was retained in *gcn4/gcn4* cells (Figure 6A and C), indicating that CaGcn4 is not required for Ras1 signalling. Nevertheless, CaGcn4 mediates its morphological effects by interacting with the Ras-cAMP pathway.

Activation of amino acid biosynthetic gene expression is not dependent upon Efg1

CaGcn4 influenced cellular morphogenesis in an Efg1-dependent fashion (Figure 6). Hence, we examined whether the GCN-like response was dependent upon Efg1.

Ectopic expression of *CaGCN4*, using the *CaACT1* promoter fusion, increased the resistance of wild-type *C.albicans* cells to 3AT (Figure 7A), presumably by increasing *CaGCN4* expression levels. This phenotype was not dependent upon Efg1, Ras1 or Cph1. Furthermore, the CaGcn4-dependent activation of the GCRE-*RrLUC* reporter was not blocked in *efg1/efg1* cells (Figure 4). Therefore, the GCN-like response is not dependent upon Efg1, Ras1 or Cph1 signalling.

The *efg1/efg1* strain HLC52 was more resistant to 3AT than its isogenic parent CAF2-1 (Figure 7A). The basis for this was not clear, but Efg1 might exert indirect effects upon 3AT resistance by affecting cell wall integrity, and hence the uptake of the drug. Significantly, this effect was not observed in the *efg1/efg1*, *cph1/cph1* double mutant, HLC54, indicating that the increase in 3AT resistance mediated by ectopic *CaGCN4* expression is not dependent upon Efg1.

It has been reported that in *S.cerevisiae*, *ScGCN4* can be activated by Ras signalling (Engelberg *et al*., 1994). Therefore, we tested whether this was the case in *C.albicans*. Northern blot analyses revealed that the *CaRAS1*^{V13} allele did not increase *CaARO4*, *CaLYS1* or *CaGCN4* mRNA levels in the absence of 3AT (Figure 7B). Instead, a normal GCN-like response was observed in *CaRAS1*^{V13} cells. Hence, constitutive Ras1 signalling did not stimulate the GCN-like response in *C.albicans*. Taken together, our data indicate that the GCN response is not mediated by MAPK or Ras-cAMP signalling.

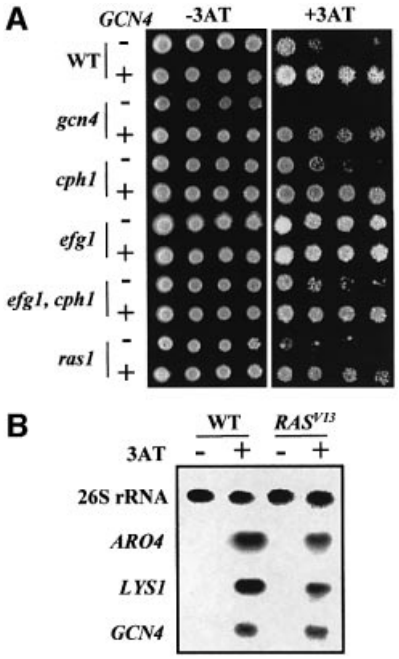

Fig. 7. The GCN-like response in *C.albicans* is not dependent upon Efg1, Cph1 or Ras1. (**A**) Constitutive activation of the GCN-like response is not blocked by inactivation of Efg1, Cph1 or Ras1. Ten-fold dilutions of *C.albicans* cells, transformed with pACT1 (–) or pACT-GCN4 (+), were plated on synthetic complete medium ± 10 mM 3AT: WT, CAF2-1; *gcn4*, GTC43; *cph1*, JKC19; *efg1,* HLC52; *cph1*, *egf1*, HLC54; *ras1*, *ras1-2/ras1-3* (Table I). (**B**) Constitutive activation of Ras signalling does not activate the GCN response in *C.albicans.* Northern blot analysis of *CaARO4*, *CaLYS1* and *CaGCN4* mRNA levels, relative to the 26S rRNA loading control, during the growth of *RAS1/RAS1* (WT) or *RAS1/RAS1/RAS1*^{V13} cells (*RAS*^{V13}; Table I) on synthetic complete medium containing maltose ± 10 mM 3AT.

Discussion

Effect of amino acid starvation upon C.albicans morphogenesis

Numerous treatments influence yeast–hypha morphogenesis in *C.albicans* (Odds, 1988) but, in most cases, the exact nature of each morphogenetic signal remains obscure. We have shown that amino acid starvation promotes pseudohyphal growth in *C.albicans* (Figure 1), and that this morphogenetic response is dependent upon CaGcn4 (Figures 3 and 6). Hence, amino acid starvation can now be added to the shortlist of well-defined morphogens in *C.albicans*.

Serum is a strong inducer of hyphal development which is commonly used to stimulate morphogenesis *in vitro*. However, the specific factor(s) in serum that mediates this effect has not been defined. Difficulties in purifying a specific morphogen from serum have led to the suggestion that a combination of several components mediates the strong serum stimulus (Feng *et al*., 1999). Our data are consistent with this idea. Amino acid starvation is not as strong a morphogenetic stimulus as serum, and it leads to the formation of pseudohyphae rather than true hyphae (Figure 1). Nevertheless, amino acids partially suppress the stimulatory effect of serum, and they do so in a reproducible manner, suggesting that amino acid starvation might comprise one component of the serum stimulus. *Candida albicans gcn4/gcn4* mutants still formed hyphae following serum stimulation, showing that other serum signals are transduced by CaGcn4-independent signalling. Therefore, serum appears to contain several distinct morphogenetic stimuli, one of which might be amino acid starvation.

General amino acid control in C.albicans

We have confirmed a previous suggestion (Pereira and Livi, 1995) that *C.albicans* exhibits general amino acid control when challenged with amino acid starvation. Histidine starvation imposed by 3AT treatment stimulated not only histidine biosynthetic gene expression but also the expression of genes involved in lysine, tyrosine, phenylalanine and tryptophan biosynthesis (Figure 5). This GCN-like response is dependent upon CaGcn4 (Figure 5), a functional homologue of ScGcn4 (Figure 2).

Similar responses have been described in divergent fungal species. Starvation for a single amino acid leads to increased synthesis of many amino acid biosynthetic enzymes in *S.cerevisiae*, *N.crassa* and *A.niger.* These responses are dependent upon the related bZIP transcription factors, ScGcn4, CPC1 and cpcAp, respectively (Hinnebusch, 1988; Paluh *et al*., 1988; Ebbole *et al*., 1991; Wanke *et al*., 1997). Like CaGcn4 (Figure 4), these factors stimulate amino acid biosynthetic gene expression via GCRE-like promoter elements. Like CaGcn4, ScGcn4, CPC1 and cpcAp are all encoded by mRNAs with long 5′ leader sequences that contain upstream ORFs, suggesting that these mRNAs might be translationally regulated. This has been demonstrated for the *ScGCN4* and *cpc-1* mRNAs (Hinnebusch, 1988; Luo *et al*., 1995). Finally, like *CaGCN4* (Figure 5), the *ScGCN4*, *cpc-1* and *cpcA* genes are transcriptionally regulated in response to amino acid starvation.

Clearly, the GCN response has been retained not only in budding yeast and in saprophytic filamentous fungi, but also in an opportunistic pathogenic fungus of mammals. This might suggest that, at some point during disease establishment or progression in the host, *C.albicans* encounters niches where amino acids are in short supply.

CaGcn4 signalling

Our data show that *C.albicans* can respond to amino acid starvation in at least two ways: by stimulating cellular morphogenesis and a GCN-like response. Both are dependent upon CaGcn4. How does CaGcn4 mediate these effects?

Three main observations indicated that CaGcn4 mediates amino acid starvation responses via a pathway that is independent of Cph1, Efg1 and Ras1. First, CaGcn4 activated a GCRE-*RrLUC* reporter in an Efg1-dependent fashion (Figure 4). Secondly, ectopic *CaGCN4* expression enhanced the 3AT resistance of *C.albicans* in a Cph1-, Efg1- and Ras1-independent manner (Figure 7A). Thirdly, constitutive *RAS1*^{V13} signalling did not activate the GCN-like response (Figure 7B). Therefore, neither MAPK nor Ras-cAMP signalling is implicated in the GCN response in *C.albicans* (Figure 8). Instead, by analogy with *S.cerevisiae* (Hinnebusch, 1988), the GCN-like response might be regulated by CaGcn2 signalling. A ScGcn2 homologue is present in the *C.albicans* genome.

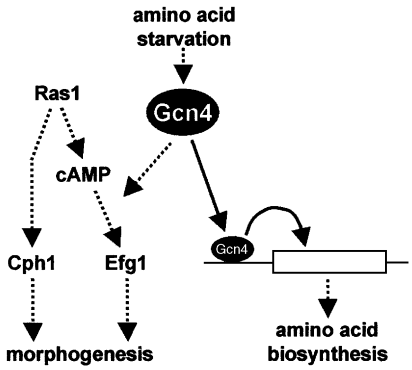

Fig. 8. Models describing the relationship between CaGcn4, morphogenetic signalling and general amino acid control in *C.albicans*. Amino acid starvation induces CaGcn4, which activates the transcription of amino acid biosynthetic genes in an Efg1-independent fashion via the GCRE element in their promoters. Ras1 stimulates morphogenesis via both Ras-cAMP (Efg1) and MAPK (Cph1) pathways, but does not activate amino acid biosynthetic gene expression. CaGcn4 stimulates morphogenesis by interacting with the Ras-cAMP pathway. CaGcn4 might act downstream in concert with a Ras1-dependent factor. Alternatively, CaGcn4 might act upstream of Ras1, activating the pathway specifically in response to amino acid starvation (see text). Hence, CaGcn4 coordinates morphogenetic and GCN-like responses following amino acid starvation in *C.albicans*.

MAPK and Ras-cAMP signalling pathways have been implicated in morphogenetic signalling in *C.albicans* (Brown and Gow, 1999; Ernst, 2000; Whiteway, 2000). Our data showed that the MAPK pathway is not required for CaGcn4-mediated morphogenesis (Figure 6). Instead, the Ras-cAMP pathway is implicated, because CaGcn4-mediated morphogenesis was inhibited by inactivation of Efg1 or CaRas1 (Figure 6). However, *RAS1*^{V13}-induced morphogenesis was not blocked in *gcn4/gcn4* cells (Figure 6). These data suggest that CaGcn4 might lie above CaRas1, activating this pathway in response to amino acid starvation but not in response to other morphogenetic signals. This would explain why *ras1/ras1* and *gcn4/gcn4* mutants display different morphogenetic phenotypes (Figures 3 and 6; Feng *et al*., 1999). However, CaGcn4 is a transcription factor (Figures 2 and 4). Therefore, it is conceivable that CaGcn4 might activate morphogenesis by interacting with a downstream component of the Ras-cAMP pathway, the activity of which is largely dependent upon CaRas1. An obvious candidate is the transcription factor Efg1 (Stoldt *et al*., 1997) (Figure 8).

A similar picture is emerging for pH signalling in *C.albicans*. Prr2/CaRim101 is a pacC-like transcription factor that mediates pH-stimulated morphogenesis in *C.albicans*. Expression of a constitutively active version of Prr2/CaRim101 stimulates hyphal growth and, like pACT1-GCN4-mediated morphogenesis (Figure 6), this effect is dependent upon Efg1 (El Barkani *et al*., 2000). Furthermore, like CaGcn4, Prr2/CaRim101 activates other responses in *C.albicans* in an Efg1-independent manner. Therefore, several types of environmental signal, including ambient pH and amino acid starvation, appear to converge upon Efg1 to stimulate filamentous growth in *C.albicans*.

Divergence in the cellular roles of Gcn4

The biochemical activities of Gcn4-like proteins seem to be conserved. CaGcn4, ScGcn4, CPC1, cpcAp and c-jun are all bZIP transcriptional activators that interact with GCRE-like sequences in the promoters of their target genes (Figure 4; Arndt and Fink, 1986; Bohmann *et al*., 1987; Ebbole *et al*., 1991; Wanke *et al*., 1997). However, there has been divergence with respect to their cellular roles. The fungal proteins appear to act as global regulators, in that they activate other cellular responses in addition to general amino acid control. For example, ScGcn4 has been shown to regulate >1000 *S.cerevisiae* genes encoding biosynthetic and organellar functions, transporters, protein kinases and transcription factors, in addition to controlling amino acid biosynthetic gene expression (Natarajan *et al*., 2001). However, ScGcn4 has not been implicated in pseudohyphal development (Kron, 1997; Lengeler *et al*., 2000; Gancedo, 2001), and transcript profiling of the GCN response has not revealed any obvious links with pseudohyphal development in *S.cerevisiae* (Natarajan *et al*., 2001). In contrast, we have demonstrated that CaGcn4 does influence *C.albicans* morphogenesis. Therefore, there has been some divergence in the cellular roles of Gcn4 between *S.cerevisiae* and *C.albicans*. Hence, ScGcn4 and CaGcn4 represent significant examples of regulators in *S.cerevisiae* and *C.albicans* that display conservation of biochemical function, but divergence in their cellular roles. Other examples include ScTup1–CaTup1 (Braun and Johnson, 1997), ScRox1–CaRfg1 (Kadosh and Johnson, 2001) and ScNrg1–CaNrg1 (Murad *et al*., 2001). Presumably, this reflects the contrasting niches of these two fungi.

Materials and methods

Strains and growth conditions

Candida albicans strains (Table I) were grown in YPD or synthetic complete medium (Murad *et al*., 2001). Amino acid starvation was imposed using synthetic complete medium (SC) lacking histidine and containing 3AT at various concentrations, as specified. Morphogenesis was stimulated by adding 10 mM 3AT, 10 mM dibutyryl cAMP or 10% fetal calf serum to cells growing on YPD or synthetic complete medium at 37°C. The *C.albicans* cells were stained with 0.1% Calcofluor White, and cell and colony morphology was analysed as described before (Murad *et al*., 2001).

Isolation of CaGCN4

CaGCN4 cDNAs were isolated by transforming a *C.albicans* library in pRSGAL1 (Wiltshire *et al*., 1999) into *S.cerevisiae* H2036 (*ura3*, *gcn4*: Table I), and screening for transformants that displayed galactose-dependent 3AT resistance. One *CaGCN4* cDNA was used to isolate the complete *CaGCN4* locus from a *C.albicans* genomic library (Smith *et al*., 1992) by colony hybridization. cDNA and genomic clones were sequenced to completion (accession No. AF205716).

Construction of C.albicans strains

To disrupt the *CaGCN4* locus, the *CaGCN4* ORF (–106 to +995) was PCR amplified using the primers 5′-CTACTGCAGAGAGAAAGTCCTGCCTC and 5′-CGAGAGGCATGCATAGTAGTAAC (*Pst*I and *Sph*I sites underlined), cloned into pGEM-T Easy to make pGEM-GCN4, and resequenced. A deletion from +295 to +742 was made by reverse PCR on pGEM-GCN4 using the primers 5′-CTTTAGATCTGTCCATAATCAAATC and 5′-CATAGATCTCAACCTTTACAACCGA. This introduced a *Bgl*II site at the point of the deletion (underlined). The *hisG-URA3-hisG* sequence (Fonzi and Irwin, 1993) was then inserted at this *Bgl*II site to create the *gcn4::hisG-URA3-hisG* disruption cassette. This cassette was released from the pGEM-T backbone using *Not*I and transformed into *C.albicans* (Gietz and Woods, 1998). Both *CaGCN4*

alleles in the strains CAI4 and CAI8 (Table I) were disrupted using two rounds of ura-blasting (Fonzi and Irwin, 1993). Disruptions were confirmed by Southern blotting and PCR diagnosis.

To reintroduce *CaGCN4* into *gcn4/gcn4* mutants, the *CaGCN4* locus (–106 to +995) was cloned into CIp10 (Murad *et al*., 2000) to generate pGCN4. This plasmid was linearized with *Bst*XI, transformed into *C.albicans*, and single copy integration at the *gcn4* locus confirmed by Southern blotting.

To create the *MET3-GCN4* fusion, the *CaGCN4* ORF (–106 to +995) was released from pGEM-GCN4 using *Pst*I and *Sph*I, and ligated into pCaEXPa (Care *et al*., 1999) to make pMET3-GCN4. This plasmid was linearized with *Stu*I, transformed into *C.albicans* and integrated at single copy into the *RP10* locus (Murad *et al*., 2000).

To create the *ACT1-GCN4* fusion, a double-stranded oligonucleotide containing the *S.cerevisiae CYC1* terminator region (Osborne and Guarente, 1989) (top strand 5′-TCGAGATCGAT*GGTTACC*CGTACG-*ACGCGT*GTCGAC*CTGCAG*AAGCTTGCATGCGCTAGCGTCCCTA-TTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATA-TTTCAAATT: *Sc*CYC1 terminator double-underlined; *Cla*I, *Bst*EII, *Sun*I, *Mlu*I, *Sal*I and *Pst*I sites underlined with alternate sites in italics) was cloned between the *Sal*I and *Mlu*I sites in CIp10, thereby inactivating these sites, to make pCYC1t. yEGFP (Cormack *et al*., 1997) was cloned between the *Hin*dIII and *Nhe*I sites of pCYC1t. Then the *CaACT1* promoter region (–2 to –1019) was PCR amplified using the primers 5′-ATCGCTCGAGCTATTAAGATCACCAGCCTC and 5′-CATACC-ACCAAGCTTTTTGAATGATTATATTTT (*Xho*I and *Hin*dIII sites underlined) and cloned between the *Xho*I and *Hin*dIII sites to make pACT1-GFP. The *Pst*I–*Sph*I *CaGCN4* fragment from pMET3-GCN4 was then blunt-ended and cloned between the *Hin*dIII and *Nhe*I sites in pACT1-GFP to make pACT1-GCN4. To create the control plasmid, pACT1, the yEGFP sequence was removed from pACT1-GFP by digestion with *Hin*dIII and *Nhe*I, and the plasmid was end-filled and religated. pACT1 and pACT1-GCN4 were then linearized with *Stu*I, transformed into *C.albicans*, and single copy integration at the *RP10* locus confirmed by Southern blotting. To introduce $RAS1^{V13}$, strains were transformed with pQF145.2 and analysed on media containing 2% maltose to activate the *Mal-*$RAS1^{V13}$ fusion (Feng *et al*., 1999).

Reporter assays

Renilla reniformis LUC promoter fusions were made using a derivative of pCRW3 (Srikantha *et al*., 1996). A basal promoter was designed, based on *CaADH1* (Bertram *et al*., 1996). Recognizable yeast enhancer elements (*S.cerevisiae* Promoter Database, http://cgsigma.cshl.org/jian/; MatInspecror V2.2, http://transfac.gbf.de/TRANSFAC/) were removed from the *ADH1* RNA initiation region, and convenient restriction sites added to generate the sequence 5′-ACGCGTAGCAGGTGCCACCAC-GGCAAAGACATTGTCTGGAACCACTGCGATCGCTAAACTG***TA-TAA***AAGGACCTATGCATGCCTGGTCTTATCTACTCCAGAATTA-TTTTTTTTCTATC***AG***TTTAACAACAACAAACGTTATTGTCATA-CAACAACCTGCAG (*Mlu*I and *Pst*I sites underlined; TATA and major RNA initiation site in bold and italics). This double-stranded oligonucleotide was inserted upstream of the *RrLUC* ORF between unique *Mlu*I and *Pst*I sites in pCRW3N to generate the basal *RrLUC* construct. The oligonucleotide 5′-CTGACTCTGAGGTGACTCGG-ATCCTGACTCTACTGTGACTCTATAGTGACTCT (GCREs underlined) was then introduced between the *Bst*EII and *Spe*I sites of this basal construct, upstream of the TATA element, to make the GCRE-*RrLUC* fusion. The *RrLUC* plasmids were linearized with *Hin*dIII, and transformed into *C.albicans* CAI8 (Gietz and Woods, 1998). The plasmids were also transformed into a *ura3* derivative of HLC52 by co-transformation with the *URA3* plasmid, CIp10 (Murad *et al*., 2000). Single copy integration of *RrLUC* plasmids at the *ade2* locus was confirmed by PCR and Southern blotting. Luciferase activities were measured in quadruplicate (Murad *et al*., 2001) in *C.albicans* transformants after 4 h growth at 30°C in SC lacking histidine and containing 20 mM 3AT. Activities are expressed relative to those of wild-type cells carrying the GCRE-*RrLUC* fusion. Similar data were obtained in three experiments using independent transformants.

RNA analyses

To select genes for northern analysis, homologues of *S.cerevisiae* amino acid biosynthetic genes were identified in the *C.albicans* genome sequence data generated by the Stanford DNA Sequencing and Technology Center (http://www-sequence.stanford.edu/group/candida). Genes with ⩾1 GCRE elements in their promoter regions were then identified using Regulatory Sequence Analysis Tools (http://www.ucmb.ulb.ac.be/bioinformatics/rsa-tools/; van Helden *et al*., 2000). From this list, *CaARO4*, *CaHIS4*, *CaHIS7*, *CaLYS1* and *CaLYS2* were selected for analysis. Their coding regions were PCR amplified, restriction mapped to confirm the authenticity of the products, radiolabelled by random prime labelling, and used to probe northern blots as described previously (Swoboda *et al*., 1994; Brown *et al*., 2001).

RT–PCR was performed using standard methods (Schaller *et al*., 1998) with the following primers: *CaACT1*, 5′-GATGAAGCCCAATC-CAAAAG, 5′-GGAGTTGAAAGTGGTTTGGT (677 bp product); *EFG1*, 5′-ACTACCATGTGGGAAGATGA, 5′-CAGGAGCATTATA-CTGACCA (633 bp product); and *CaGCN4*, 5′-GTTGATACTGTTGC-TACCAA, 5′-TTTTACGAGCTCTGGATCTT (763 bp product). The intron-containing *EFB1* sequence was used to control for loading and contamination with genomic DNA (Schaller *et al*., 1998).

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