

Global Role of the Protein Kinase Gcn2 in the Human Pathogen *Candida albicans*

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The pathogen *Candida albicans* responds to amino acid starvation by activating pseudohyphal development and the expression of amino acid biosynthetic genes (GCN response). In *Saccharomyces cerevisiae*, the GCN response is dependent on Gcn2, which regulates the translation of the transcription factor Gcn4. Therefore, we examined the role of Gcn2 in *C. albicans* by using molecular, cellular, and genomic approaches. We show that *C. albicans* GCN2 encodes an eIF2 α kinase, like its *S. cerevisiae* homologue. However, GCN4 appears to be regulated mainly at the transcriptional level in *C. albicans*. Furthermore, the inactivation of *C. albicans* Gcn2 only partially attenuates growth under amino acid starvation conditions and resistance to the histidine analogue 3-aminotriazole. Our comparison of the Gcn4 and Gcn2 regulons by transcript profiling reinforces the view that Gcn2 contributes to, but is not essential for, the activation of general amino acid control in *C. albicans*.

Several factors are thought to promote the virulence of the major systemic fungal pathogen of humans *Candida albicans* (10, 39). These include yeast-(pseudo)hypha morphogenesis and biofilm formation (23, 31). In *C. albicans*, responses to amino acid availability are intimately linked with morphogenesis and biofilm formation (7, 19, 46). Furthermore, *C. albicans* induces amino acid biosynthetic genes in response to phagocytosis by human neutrophils (42). Hence, the ability to regulate amino acid metabolism appears to be an important weapon in the armory of this medically important pathogen.

The GCN response (or general amino acid control) is characterized by the induction of genes in most amino acid biosynthetic pathways in response to starvation for even a single amino acid. This response has been shown to exist in *C. albicans* and to be involved in both morphogenesis and biofilm formation (19, 46, 49). Furthermore, this response depends on the transcription factor Gcn4 (46), which is characteristic of fungal GCN-like responses (25, 27, 48).

GCN signaling has been best characterized in *Saccharomyces cerevisiae* (reviewed in references 25 and 26). In budding yeast, amino acid starvation leads to the intracellular accumulation of uncharged tRNAs, which interact with the regulatory histidyl-tRNA synthetase-like domain of Gcn2, thereby stimulating its eukaryotic initiation factor 2 α (eIF2 α) kinase activity.

Phosphorylation of eIF2 α at serine-51 by Gcn2 inhibits guanine nucleotide exchange on eIF2, thereby decreasing the activity of this essential translation initiation factor. In this way, Gcn2 causes the global rate of mRNA translation to decrease in response to amino acid deprivation. However, in parallel, the decrease in eIF2 activity enhances translation of the *GCN4* mRNA. This translational control is mediated by the unusually long 5'-leader sequence on the *GCN4* mRNA, which carries four upstream open reading frames (ORFs). Essentially, these upstream ORFs repress translation of the main *GCN4* ORF, and this repression is alleviated by Gcn2-mediated phosphorylation of eIF2 α during amino acid starvation. As a result, Gcn4 levels increase, thereby leading to the transcriptional activation of amino acid biosynthetic genes via Gcn4 response elements (GCRE) (TGACTC) in their promoters (25, 37). In *S. cerevisiae*, *GCN4* transcription increases about twofold in response to amino acid starvation (2), but Gcn4 synthesis is regulated primarily at the translational level (26). Hence, in *S. cerevisiae*, the GCN response is blocked by mutations that inactivate Gcn2 or Gcn4.

By analogy with *S. cerevisiae*, we reasoned that Gcn2 might be important for the GCN response in *C. albicans*. In this study we show that while Gcn2 does contribute to the GCN response in *C. albicans*, it plays a nonessential role. Instead, *GCN4* appears to be regulated primarily at the transcriptional level in *C. albicans*.

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MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains (Table 1) were grown in YPD or synthetic complete medium (SC) (45). 3-Aminotriazole (3AT) was used at the concentrations specified.

***C. albicans* strain construction.** To disrupt *GCN2*, the 5' end (–90 to +500) was PCR amplified with primers 5'-AAAAGCTTACACGAAACATCAAT TCA and 5'-GCCAGATCTTCATCCTTCTTCTGTTC (HindIII and BglII sites underlined), and the 3' end (+4767 to +5327) was amplified with primers

TABLE 1. Strains analyzed in this study

Strain	Genotype	Source or reference
<i>S. cerevisiae</i>		
BY4743HIS	<i>MATα/MATα his3/his3 leu2/leu2 LYS2/lys2 MET15/met15 ura3/ura3; pRS303 (HIS3)</i>	49
Y30249HIS	Like BY4743, except <i>gcn4::kanMX4/gcn4::kanMX4; pRS303 (HIS3)</i>	49
Y33642HIS	Like BY4743, except <i>gcn2::kanMX4/gcn2::kanMX4; pRS303 (HIS3)</i>	This study
<i>C. albicans</i>		
SC5314	Clinical isolate	22
CAF2-1	<i>URA3/ura3::λ imm434</i>	18
CAI-4	<i>ura3::λ imm434/ura3::λ imm434</i>	18
GTC41	Like CAI-4, except <i>GCN4/gcn4::hisG-URA3-hisG</i>	46
GTC42	Like CAI-4, except <i>GCN4/gcn4::hisG</i>	46
GTC43	Like CAI-4, except <i>gcn4::hisG-URA3-hisG/gcn4::hisG</i>	46
GTC44	Like CAI-4, except <i>gcn4::hisG/gcn4::hisG</i>	46
HTC41, HTC45, HTC49 ^a	Like CAI-4, except <i>GCN2/gcn2::hisG-URA3-hisG</i>	This study
HTC42, HTC46, HTC50 ^a	Like CAI-4, except <i>GCN2/gcn2::hisG</i>	This study
HTC43, HTC47, HTC51 ^a	Like CAI-4, except <i>gcn2::hisG-URA3-hisG/gcn2::hisG</i>	This study
HTC44, HTC48, HTC52 ^a	Like CAI-4, except <i>gcn2::hisG/gcn2::hisG</i>	This study
CAI-8	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG</i>	18
GTC82	Like CAI-8, except <i>GCN4/gcn4::hisG</i>	46
GTC84	Like CAI-8, except <i>gcn4::hisG/gcn4::hisG</i>	46
HTC81, HTC85, HTC89 ^a	Like CAI-8, except <i>GCN2/gcn2::hisG-URA3-hisG</i>	This study
HTC82, HTC86, HTC90 ^a	Like CAI-8, except <i>GCN2/gcn2::hisG</i>	This study
HTC83, HTC87, HTC91 ^a	Like CAI-8, except <i>gcn2::hisG-URA3-hisG/gcn2::hisG</i>	This study
HTC84, HTC88, HTC92 ^a	Like CAI-8, except <i>gcn2::hisG/gcn2::hisG</i>	This study
HTC101	Like CAI-4, except <i>GCN4/GCN4-GFP</i>	This study
HTC102	Like CAI-4, except <i>GCN4/gcn4-GFP</i>	This study
HTC103	Like CAI-4, except <i>gcn4::hisG/GCN4-GFP</i>	This study
HTC104	Like CAI-4, except <i>gcn4::hisG/gcn4-GFP</i>	This study

^a Three independent sets of congeneric mutants.

5'-GATAGATCTTTTGGTGACTGAATTATT and 5'-AAGCTAGCAAGCA TGCTGTAGGTATG (BglIII and NheI sites underlined). These products were cloned into pGEM-T Easy. The *hisG-URA3-hisG* sequence (18) was inserted at the BglIII site between the 5' and 3' ends of *GCN2* to create the *gcn2::hisG-URA3-hisG* disruption cassette. This cassette was released from the plasmid backbone with NotI, and the two *GCN2* alleles were disrupted sequentially in CAI-4 and CAI-8 (Table 1) in two rounds of Ura blasting (18). Disruptions were confirmed by Southern blotting and PCR diagnosis (not shown).

GCN4-GFP and *gcn4-GFP* fusions were constructed at the *GCN4* locus with the *GFP-URA3* cassette as described previously (20). For the *GCN4-GFP* fusion, the primers used were 5'-GAAAAGCAAGCTTTACAAGATCAAGTTGAA AGATTACAAGAATTGTTAAGAGTTAATGGTATTCAATTTGGTGGT GGTCTAAAGGTGAAGAATTATT and 5'-TAAAAAACAATAATAAT TTTCTAAATTTTCTTTTTTAAAAAATAACGAGAGGTATATAT AGTAGTTCTAGAAGGACCACCTTTGATTG (R1-GCN4). To create the *gcn4-GFP* fusion, which disrupted the *GCN4* locus, primer 5'-GATTTATTT GCTTCTCCAGTTAAACAACATCAAAAAGGTTGATACTGTTGCT ACCAAAAACGAAATTGGTGGTGGTCTAAAGGTGAAGAATTATT was used with the R1-GCN4 primer.

To create pGCN4_{GCRE}-GFP, the wild type *GCN4* 5' region (−996 to +1) was PCR amplified with primers 5'-CTCTAAAGACTCGAGAAATAGCGAAAA TGTAATATAAATTTATGAGTCATAATTTCTCTCG and 5'-AGTAGC AAGCTTTTATCTAATAATAATAATGGAACAAAGCTAATTAATG TAATGTAATTTAATTTAAATAGC (XhoI and HindIII sites underlined; GCRE in italics). To generate pGCN4-GFP, the *GCN4* allele (−996 to +1) was amplified from the *GCN4* allele lacking the GCRE at −582 (TTATTTAA) with the forward primer 5'-CTCTAAAGACTCGAGAAATAGCGAAATGTAATAATAATTTAATGTAATTTCTCTCG (XhoI site underlined; mutated GCRE at −968 in italics) and the same reverse primer. This product lacked both GCRE at −968 and −582. Both the wild-type and mutant sequences were cloned between the XhoI and HindIII sites in pGFP (3) and resequenced to generate pGCN4_{GCRE}-GFP and pGCN4-GFP, respectively. These plasmids were integrated at the *RPS1* locus (34). (The *RP10/RPS10* gene has been renamed *RPS1*.)

Renilla reniformis LUC (RrLUC) promoter fusions were made in a plasmid containing a basal *C. albicans ADH1* promoter upstream of the RrLUC reporter (46). The GCRE-RrLUC fusion was made by inserting the sequence 5'-CTG ACTCTGAGGTGACTCGGATCCTGACTCTACTGTGACTCTATAGTG

ACTCT (GCRE underlined) between the BstEII and SpeI sites of this basal construct. RrLUC plasmids were linearized with HindIII and transformed into *C. albicans*. Single-copy integration at the *ade2* locus was confirmed by PCR and Southern blotting.

Blotting and RT-PCR. Southern (35), Northern (9), and Western (12) blotting were performed as described previously. The rabbit anti-phospho-eIF2(pSer51) antibody was from Sigma (St Louis, MO). Reverse transcription-PCR (RT-PCR) was performed by standard methods (44) with the following green fluorescent protein (GFP) primers: 5'-CACTGGTGTGTCCTCC and 5'-CCATACCAT GGGTAA (677-bp product). The intron-containing *EFB1* product was used to control for loading and genomic DNA contamination (44).

Reporter assays. GFP fluorescence was visualized by microscopic analysis as described previously (3). *Renilla* luciferase activities (10⁵ relative light units/20 μ g protein) were measured in quadruplicate (35) in *C. albicans* transformants after 3 h of growth at 30°C in SC lacking histidine and containing 40 mM 3AT. Similar data were obtained in three experiments done with independent transformants.

Transcript profiling. Transcript profiling was performed on the congeneric *C. albicans* strains CAF2-1, GTC43, HTC43, HTC47, and HTC51 growing in SC lacking histidine after 3 h of exposure to 0 or 40 mM 3AT. Transcript profiling was performed as described previously (16, 36). Briefly, total RNA was isolated (24), Cy3- and Cy5-labeled cDNAs were prepared, and these probes were hybridized with *C. albicans* microarrays (Eurogentec, Seraing, Belgium). Slides were scanned at 10- μ m resolution with a ScanArray Lite scanner (Perkin-Elmer Life Sciences, Beaconsfield, United Kingdom) and quantified with QuantArray software (version 2.0). GeneSpring software (Silicon Genetics, Redwood City, CA) was used for data normalization and analysis, and statistical analysis was performed with SAM (significance analysis of microarrays) (47). Data from at least three independent biological replicates were used for each analysis, and the SAM false-discovery rate was set at 10%. Expression ratios for each gene that displayed a reproducible and statistically significant change in expression are available at the *Galar Fungail* websites (http://www.pasteur.fr/recherche/unites/Galar_Fungail/ and <http://www.galarfungail.org/data.htm>). *C. albicans* gene annotations were obtained from CandidaDB (<http://genolist.pasteur.fr/CandidaDB>) (14). Functional categories for *C. albicans* genes were assigned mainly on the basis of the MIPS functional assignments for *S. cerevisiae* homologues (<http://mips.gsf.de/proj/yeast/CYGD/db/index.html>) (49).

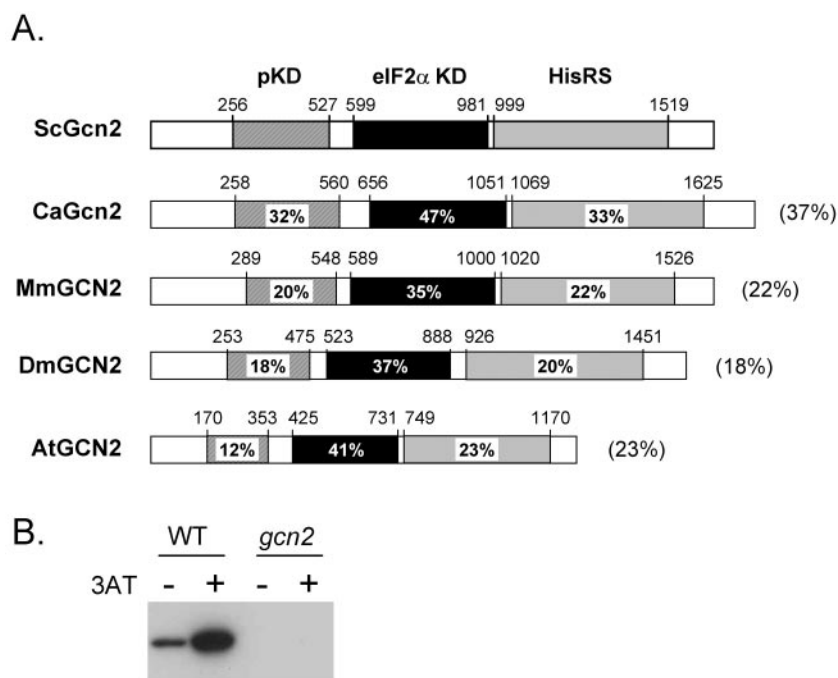


FIG. 1. *C. albicans* GCN2 encodes an eIF2 α kinase. (A) *C. albicans* orf19.6913 encodes a protein (CaGcn2) with sequence similarity to Gcn2-like proteins from other eukaryotes. *Mus musculus* (MmGCN2; TrEMBL accession no. Q9QZ05), *Drosophila melanogaster* (DmGCN2; TrEMBL Q9V9X8), and *Arabidopsis thaliana* (AtGCN2; TrEMBL Q8H2D3) proteins are compared with *S. cerevisiae* Gcn2 (TrEMBL P15442). The coordinates of the partial kinase (pKD), eIF2 α kinase (eIF2 α KD), and histidyl-tRNA synthetase-like (HisRS) domains are shown above each protein. The numbers within each domain represent the percentage of amino acid sequence identity with ScGcn2 is given in parentheses on the right. (B) *C. albicans* eIF2 α kinase activity is dependent on GCN2. Equal amounts of protein extracts from wild-type (WT; CAI-4) or Δ *gcn2* (HTC44) *C. albicans* cells (see Table 1) were either left untreated (-) or treated with 40 mM 3AT (+) for 3 h and then subjected to Western blotting with an antibody that specifically recognizes the serine-51 phosphorylated form of eIF2 α . Similar data were obtained from two independent experiments.

RESULTS

***C. albicans* GCN2 encodes an eIF2 α kinase.** Our initial working hypothesis was that regulation of the GCN response in *C. albicans* would closely mirror the *S. cerevisiae* GCN control mechanisms that have been defined in some detail by numerous groups over many years (reviewed in references 25 and 26). As described above, Gcn2 plays a critical role in control of the GCN response in *S. cerevisiae*. Therefore, we screened the *C. albicans* genome sequence for GCN2-like loci. Only one *C. albicans* ORF (*GCN2*; orf19.6913) encodes a protein with significant sequence similarity to *S. cerevisiae* Gcn2 (37% identity). The *C. albicans* Gcn2 protein sequence contains partial kinase-, eIF2 α kinase-, and histidyl-tRNA synthetase-like domains. It also displays significant sequence similarity to GCN2 eIF2 α kinases from mammals, flies, and plants (Fig. 1A).

To test whether eIF2 α kinase activity in *C. albicans* is dependent on Gcn2, we generated homozygous *gcn2/gcn2* null mutants. Essentially, the two *GCN2* alleles in this diploid fungus were disrupted sequentially, removing codons 167 to 1589 of the 1,764-codon ORF to create three independent homozygous mutants in two *C. albicans* strains (CAI-4 and CAI-8), thereby generating a total of six *gcn2/gcn2* null mutants (Table 1). The eIF2 α kinase activities in mutant and wild-type cells were compared by Western blotting with an antibody specific for the phosphorylated form of eIF2 α (Fig. 1B). No detectable eIF2 α kinase activity remained in the mutant cells,

indicating that GCN2 encodes the major eIF2 α kinase activity in this fungus.

The eIF2 α kinase activity increased in wild-type cells following exposure to 3AT (Fig. 1B), suggesting that Gcn2 is activated in response to amino acid starvation. This is consistent with the idea that Gcn2 plays a role in the *C. albicans* GCN response.

GCN2 inactivation reduces the tolerance of *C. albicans* to 3AT. We tested whether the ability of *C. albicans* to respond to amino acid starvation depends on GCN2. Resistance to the histidine analogue 3AT is a classic indicator of the GCN response in *S. cerevisiae* and *C. albicans* (25, 46). Therefore, we compared the 3AT sensitivity of *gcn2* cells with that of wild-type and *gcn4* controls (Fig. 2A). Inactivation of GCN2 only partially attenuated the resistance of *C. albicans* to 3AT: *gcn2* cells grew slowly on solid medium containing 10 mM 3AT. In contrast, *S. cerevisiae gcn2* cells were sensitive to 5 mM 3AT. The same phenotype was observed for all six independent *gcn2*-null mutants (HTC44, HTC48, HTC52, HTC84, HTC88, and HTC92 [Table 1]). Therefore, *C. albicans* is less dependent on Gcn2 for growth in the presence of this toxic histidine analogue than *S. cerevisiae*. However, both yeasts require Gcn4 for 3AT resistance (Fig. 2).

We compared the growth of *C. albicans gcn2*, *gcn4*, and wild-type cells in the absence of amino acids (Fig. 2B and C). As expected, *gcn4* cells grew relatively slowly, confirming that

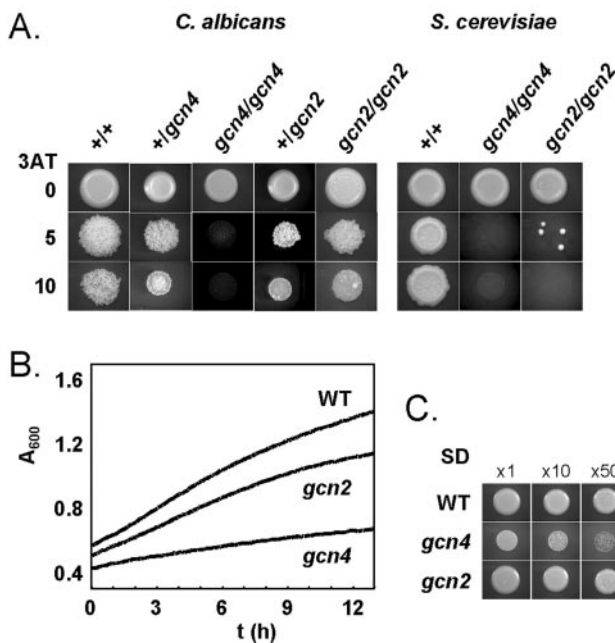


FIG. 2. Growth during amino acid starvation is only partially attenuated in *C. albicans* *gcn2* cells. (A) Growth of *C. albicans* and *S. cerevisiae* strains was compared after 2 days on SC containing different concentrations of 3AT (mM). *C. albicans* strains: +/+ (*GCN4*/*GCN4*), CAF2-1; +/*gcn4* (*GCN4*/*gcn4*), GTC41; *gcn4*/*gcn4*, GTC43; +/*gcn2* (*GCN2*/*gcn2*), HTC41; *gcn2*/*gcn2*, HTC43. *S. cerevisiae* strains: +/+ (wild type), BY4743HIS; *gcn4*/*gcn4*, Y30249HIS; *gcn2*/*gcn2*, Y33642HIS. (See Table 1.) (B) Effect of 1 mM 3AT on the growth of the same strains in SC lacking histidine. Similar data were obtained for three independent *gcn2* mutants. WT, wild type. (C) Growth of WT (CAF2-1), *gcn2* (HTC43), and *gcn4* (GTC43) *C. albicans* cells on SD for 2 days. Cells were diluted 1-, 10-, or 50-fold.

Gcn4 is required for normal responses to amino acid starvation in *C. albicans*. However, *gcn2* cells grew relatively normally under these conditions. Therefore, in contrast to the situation in *S. cerevisiae*, the *C. albicans* GCN response is not dependent on Gcn2.

Amino acid starvation stimulates morphogenesis in *C. albicans* but not in *S. cerevisiae* (46). Hence, *C. albicans* forms wrinkly colonies in the presence of 3AT. Interestingly, *gcn2* cells also formed wrinkly colonies (Fig. 2A), indicating that Gcn2 is not required for the morphogenetic response of *C. albicans* to amino acid starvation.

Transcriptional activation via the GCRE is not dependent on Gcn2. The GCN response in *C. albicans* is mediated by Gcn4, which activates the transcription of genes via the GCRE (46). Hence we reasoned that if Gcn2 plays a relatively minor role in this GCN response, the expression of a GCRE reporter gene in *C. albicans* should not depend on *GCN2*. To test this, the activities of basal and GCRE-containing luciferase reporters were measured in wild-type, *gcn4*, and *gcn2* cells following exposure to 3AT. The GCRE reporter contained five copies of the GCRE upstream of a control basal reporter with the TATA box and RNA start site from the *C. albicans* *ADH1* promoter. The expression of the control basal reporter was not affected by the disruption of Gcn2 or Gcn4. Also, the activation of the GCRE reporter was inhibited in *gcn4* cells (Fig. 3), as

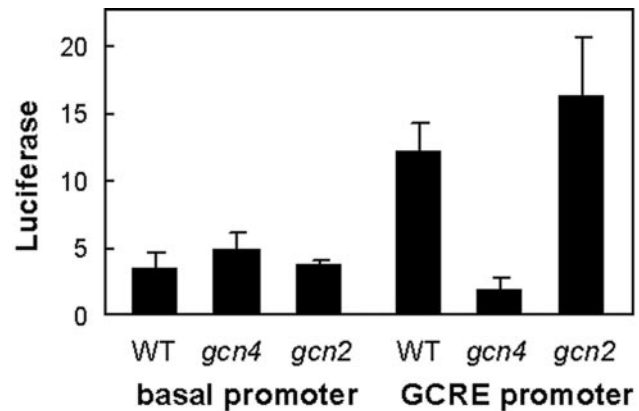


FIG. 3. Inactivation of *GCN2* does not inhibit Gcn4/GCRE-mediated transcriptional activation. Relative luciferase activities were measured in *C. albicans* strains transformed with basal or GCRE-*RrLUC* reporters after 3 h of growth in SC with 40 mM 3AT. Wild type (WT), CAI-8; *gcn4*, GTC84; *gcn2*, HTC84. (See Table 1.)

expected (46). As predicted, inactivation of Gcn2 had no significant effect on this GCRE reporter. These data confirm that the GCRE mediates the transcriptional activation of *C. albicans* genes by Gcn4, and they suggest that Gcn2 does not contribute significantly to this activation.

GCN4 is transcriptionally activated. If the activation of *GCN4* is not dependent on Gcn2, how is the GCN response activated in *C. albicans*? Our data indicated that *GCN4* mRNA levels increase in response to 3AT (46). Therefore, we tested this further by examining the responses of two types of *GCN4*-GFP fusion to amino acid starvation.

The first *GCN4*-GFP fusion contained the wild-type 5' region (from -996 to +1) cloned upstream of the synthetic, codon-optimized yEGFP gene (12) in pGCN4_{GCRE}-GFP. We noticed that the region upstream of the main *GCN4* ORF contained two GCRE at -968 and -582 with respect to the start codon. Therefore, we made a second *GCN4*-GFP fusion, which lacked these putative GCREs, to test whether they are required for the transcriptional activation of *GCN4*. This second fusion was called pGCN4-GFP. The two fusions were integrated at the *RPS1* locus, and the responses of the two fusions to 3AT were examined by Northern blotting and fluorescence microscopy (Fig. 4).

In cells containing pGCN4_{GCRE}-GFP, the *GFP* mRNA levels were induced in response to 3AT. Interestingly, *GFP* mRNA levels were not induced to the same extent in cells containing the *GCN4*-GFP fusion. These Northern blot data were confirmed by RT-PCR (not shown). The levels of GFP fluorescence generated by these constructs were relatively low, but this was expected, because they included the *GCN4* 5' untranslated leader region. GFP fluorescence was always at background levels in cells not exposed to 3AT and in cells containing the *GCN4*-GFP fusion. In contrast, GFP fluorescence levels were consistently higher in cells containing the *GCN4*_{GCRE}-GFP fusion. Therefore, GFP fluorescence levels correlated reproducibly with *GFP* mRNA levels. Hence, the data were entirely consistent with the idea that 3AT induces *GCN4* transcription. Furthermore, the data indicated that

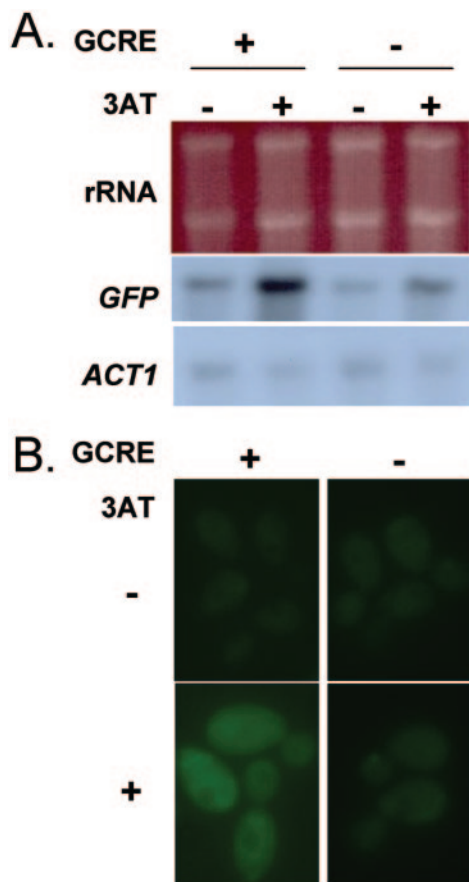


FIG. 4. Activation of *GCN4* depends on GCREs in its promoter. (A) Northern blot analysis of *GFP* mRNAs from cells grown in SC without (-) or with (+) 40 mM 3AT for 3 h. Wild-type (CAI-4) and *gcn2* (HTC44) cells expressed GFP fusions with wild-type (GCRE +; pGCN4_{GCRE}-GFP) or mutated (GCRE -; pGCN4-GFP) *GCN4* promoters. rRNAs and *ACT1* mRNAs acted as internal loading controls, and all images are from the same blot. (B) Fluorescence microscopy of GFP levels in cells from the same cultures.

the GCRE contribute significantly to this transcriptional induction.

Transcriptional activation via the GCRE in *C. albicans* is known to be mediated by Gcn4 (46). Therefore, we tested whether Gcn4 is required for transcriptional induction of *GCN4*. To achieve this, we integrated GFP at the chromosomal *GCN4* locus in wild-type CAI-4 cells and a heterozygous *GCN4/gcn4* mutant, GTC42 (Table 1). To generate a functional fusion, the GFP ORF was fused in frame after the last codon of the *GCN4* allele (strains HTC101 and HTC103). To generate a nonfunctional fusion, GFP was inserted in frame after codon 40 of *GCN4* (strains HTC102 and HTC104). Strains HTC101 and HTC102 retained one wild-type *GCN4* allele, whereas strains HTC103 and HTC104 carried an inactive *gcn4* allele in addition to their GFP fusion (Table 1). To test the Gcn4 functionality of the *GCN4*-GFP and *gcn4*-GFP fusions, we examined the 3AT resistance of strains HTC103 and HTC104 (Fig. 5A). HTC103 was 3AT resistant, indicating that the *GCN4*-GFP fusion retained Gcn4 functionality. In contrast, HTC104 was 3AT sensitive, indicating that the *gcn4*-GFP fusion did not retain Gcn4 functionality, as predicted.

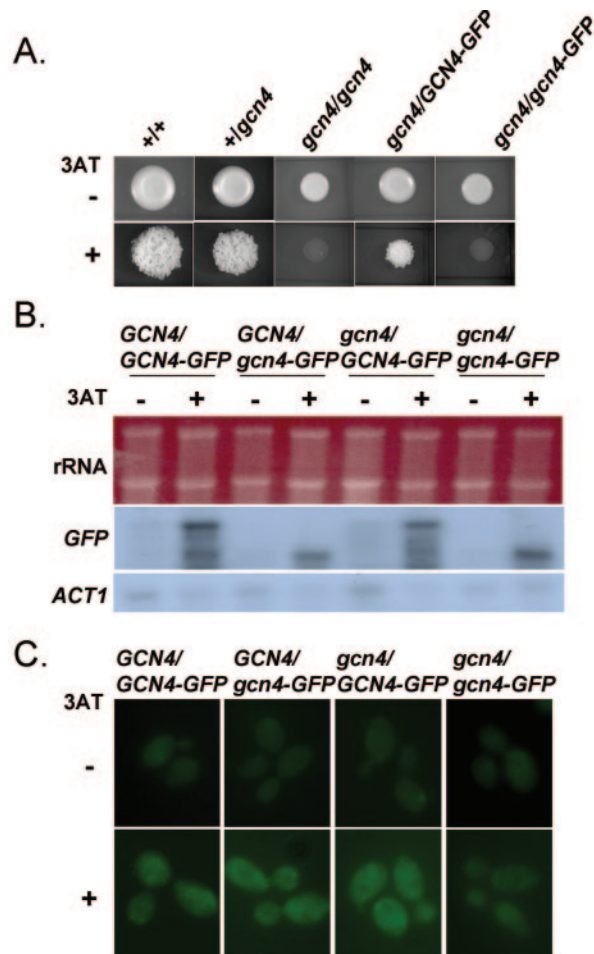


FIG. 5. Gcn4 is not essential for the transcriptional activation of *GCN4*. (A) Growth of *C. albicans* mutants on SC-HIS containing no 3AT (-) or 5 mM 3AT (+) for 4 days at 30°C. Strains: +/+ (*GCN4/GCN4*), CAF2-1; +/*gcn4* (*GCN4/gcn4*), GTC41; *gcn4/gcn4*, GTC43; *gcn4/GCN4-GFP*, HTC103; *gcn4/gcn4-GFP*, HTC104 (see Table 1). (B) Northern blot analysis of *GFP* mRNAs from cells grown in SC without (-) or with (+) 40 mM 3AT for 3 h. Strains: *GCN4/GCN4-GFP*, HTC101; *GCN4/gcn4-GFP*, HTC102; *gcn4/GCN4-GFP*, HTC103; *gcn4/gcn4-GFP*, HTC104 (see Table 1). rRNAs and *ACT1* mRNAs acted as internal loading controls. (C) Fluorescence microscopy of GFP levels in cells from the same cultures.

GFP transcript levels were then examined in strains HTC101 to -104 by Northern blot analysis following exposure to 0 or 40 mM 3AT (Fig. 5B). As expected, the *gcn4*-GFP transcript was about 0.7 kb shorter than the *GCN4*-GFP transcript. *GCN4*-GFP and *gcn4*-GFP mRNA levels increased significantly in response to 3AT in HTC101 and HTC102 cells, which contained a functional *GCN4* allele. Furthermore, GFP fluorescence levels were reproducibly elevated in these cells (Fig. 5C). Similar observations were made for HTC103, which contains the functional *GCN4*-GFP allele. However, GFP fluorescence remained at low levels in HTC104. This was consistent with the idea that Gcn4 is required for the activation of *GCN4*. However, *gcn4*-GFP mRNA levels did respond to 3AT, suggesting that Gcn4 is required at a posttranscriptional rather than a transcriptional level.

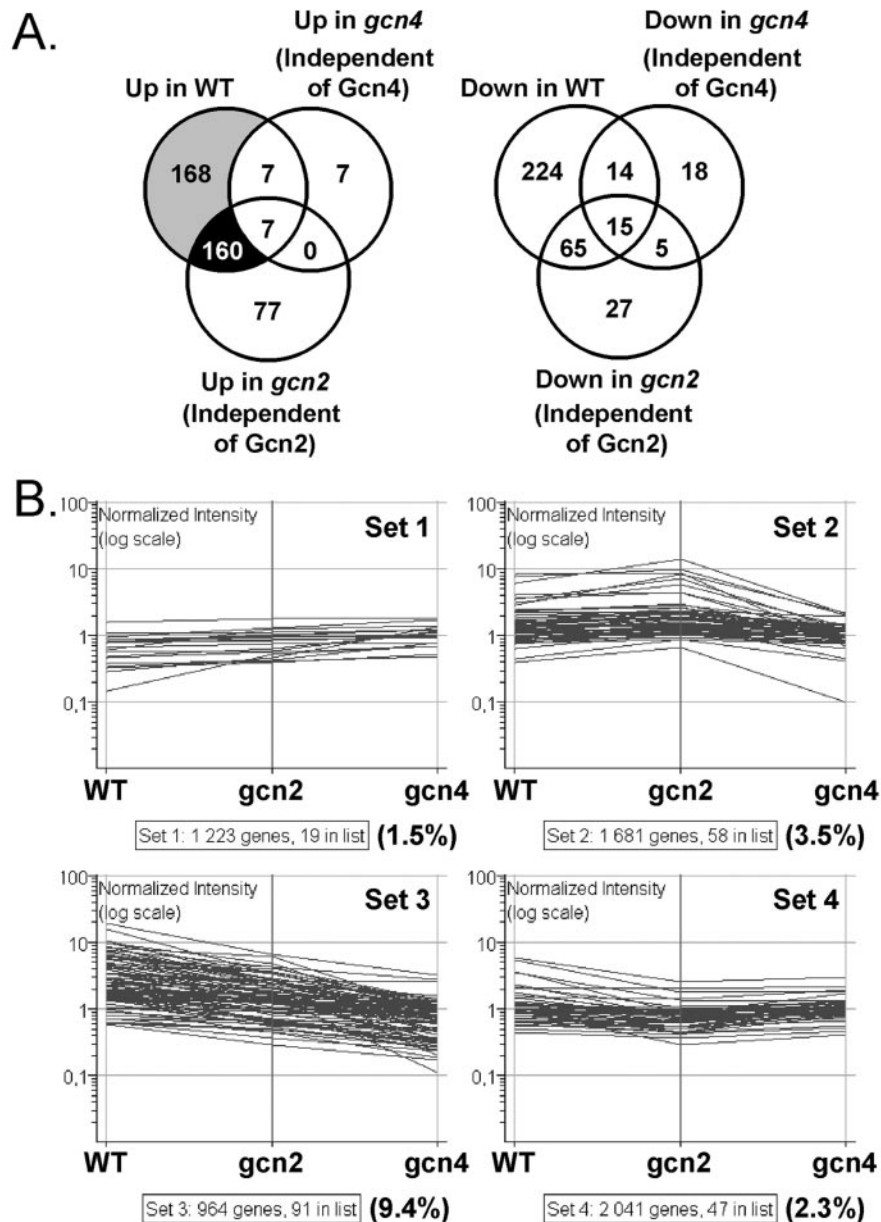


FIG. 6. Transcript profiling of the responses of wild-type (WT), *gcn4*, and *gcn2* *C. albicans* cells to 3AT. (A) Venn diagram showing the number of transcripts in each cell type that displayed reproducible and statistically significant responses to 3AT in three independent experiments. Strains: WT, CAF2-1; *gcn4*, GTC43; *gcn2*, HTC47. Gray sector, transcripts induced by 3AT in a Gcn4- and Gcn2-dependent fashion; black sector, transcripts induced by 3AT in a Gcn4-dependent but Gcn2-independent fashion. (B) Clustering of amino acid biosynthetic genes based on their responses to 3AT in WT, *gcn2*, and *gcn4* cells. All *C. albicans* genes for which there were statistically significant data were clustered using K-means clustering in GeneSpring. The behavior of amino acid biosynthetic genes is shown. The total number of genes, the number of amino acid biosynthetic genes (genes in list), and the proportion of amino acid biosynthetic genes (in parentheses) in each K-means Gene Set are given below the graphs.

In light of this result, we examined the behavior of pGCN4_{GCRE}-GFP in *gcn4* cells. Northern blot analysis revealed that the GFP mRNA levels from this construct increased in *gcn4* cells in response to 3AT, even though these cells did not express a functional Gcn4 (not shown). This was consistent with the above findings.

Taken together, our data suggest that *C. albicans* GCN4 is transcriptionally activated in response to 3AT and that GCRE

contribute to this transcriptional activation, but that Gcn4 is not required for this transcriptional activation.

Definition of Gcn4 and Gcn2 regulons in *C. albicans*. Our data suggested that Gcn2 plays a relatively minor role in the GCN response in *C. albicans*, whereas Gcn4 plays a major role in this response. To test this further, we examined the Gcn2 and Gcn4 regulons in *C. albicans* by transcript profiling. To our knowledge, this is the first time that the Gcn2 regulon has been

TABLE 2. Functional categories that are significantly influenced by inactivation of *C. albicans* *GCN4* or *GCN2*

Functional category ^a	Gcn4- and Gcn2-dependent genes ^b (n = 168)		Genes dependent on Gcn4 only ^b (n = 160)	
	Fold enhancement ^c	Genes	Fold enhancement ^c	Genes
Amino acid metabolism	5.0	<i>AGP1, ARG8, ARO1, ARO4, BAT21, ECM42, GAP5, HIP1, HIS1, HIS3, HIS7, HOM3, HYU1, ILV1, ILV6, LEU42, LYS2, LYS4, LYS22, MET8, MXR1, PRO1, PRO2, THR1, TRP2, TRP3, TRP4, IPF8591</i>	5.9	<i>AAT21, ARO3, ARO8, BAT22, CIS2, DIP51, ECM17, GAP6, GAP7, GCH2, GCN4, HIS4, HIS5, HOM6, ILV2, LEU41, LYS9, LYS21, MET2, MET15, SER33, THR4, THR5, TRP5, IPF1162, IPF2837, IPF8048, IPF13176, IPF14203</i>
Nitrogen and sulfur metabolism	2.7	<i>MET8, NIT3, IPF8591, IPF10021, IPF11716</i>	4.0	<i>AAT21, ECM12, GDH2, IPF3549, IPF14203, IPF20164</i>
Protein fate	2.0	<i>APE3, ARP2, CDC37, CDC48, CPY1, CTM1, ELC1, HSP78, NPL4, PRC1, PRC3, PRE3, PRE6, PRE8, PUP2, RPN1, RPN2, RPN6, RPN7, RPN8, RPN10, RPT1, RPT4, RPT5, RPT6, SHR3, SRA1, UBC3, VPS1, VPS4, IPF56, IPF14031, IPF4866, IPF7556, IPF11713</i>	NE ^d	
Cell rescue, defense, and virulence	2.1	<i>DDR48, GTT1, HAMI, HK1, HSP12, HSP78, MXR1, OSM2, PRE3, PUP2, SRA1, SSU1, TTR1, ZWF1, IPF4055, IPF9188</i>	NE	
Transport facilitation	NE		2.3	<i>ARR3, CDR1, CDR11, DIP51, ENA21, FCY23, GAP6, GAP7, PXA2, SEO1, SMF12, IPF4580, IPF9670, IPF11767, IPF12884, IPF13941</i>

^a Functional categories are based on those of *S. cerevisiae* homologues as defined by MIPS (<http://mips.gsf.de/genre/proj/yeast/searchCatalogFirstAction.do?db=CYGD>), and *C. albicans* functional assignments are from CandidaDB (<http://genolist.pasteur.fr/CandidaDB/>) (14). Only those functional categories that show more than two fold enhancement and that also contain more than five genes are shown. Note that a gene can belong to more than one functional category.

^b The 168 genes that displayed significant increases in expression in response to 3AT in wild-type cells only were defined as Gcn4 and Gcn2 dependent (Fig. 6A, gray sector). The 160 genes that displayed increased expression in response to 3AT in wild-type and *gcn2* cells were defined as Gcn4 dependent only (Fig. 6A, black sector).

^c Fold enhancement, which represents the extent to which genes in the functional category of interest were enriched, was calculated by dividing the proportion of genes within the regulatory subset that belong to that functional category by the proportion of genes in the whole genome that belong to that functional category.

^d NE, no significant enhancement for this functional category in this regulatory subset, whereas the other regulatory subset does show significant enhancement for this functional category.

studied in any fungus. The transcript profiles of wild-type, *gcn2*, and *gcn4* cells were compared following exposure to 0 or 40 mM 3AT. Only reproducible and statistically significant changes observed in three independent experiments are discussed.

The transcript profiling data were consistent with our analysis of the Gcn4 proteome in *C. albicans* (49) and with previous Northern blot analyses of GCN-responsive mRNAs (*ARO4*, *HIS4*, *HIS7*, *LYS2*, and *GCN4* [46]). Furthermore, the data revealed strong similarities between the Gcn4 transcriptomes of *C. albicans* and *S. cerevisiae* (37). For example, exposure to 3AT affected the expression of a relatively large proportion of *C. albicans* genes (about 13% of the ca. 6,200 ORFs). About half of these genes were induced, and half were repressed (Fig. 6A). For most genes (91%), this regulation was lost in *gcn4* cells, indicating that Gcn4 is required for most transcriptional responses to 3AT. Furthermore, amino acid biosynthetic functions were significantly enriched among those genes that were induced by 3AT in a Gcn4-dependent fashion (Table 2). Genes in all amino acid biosynthetic pathways were induced by 3AT in a Gcn4-dependent fashion, and *HIS1*, *HIS3*, *HIS4*,

HIS5, and *HIS7* mRNAs were all induced more than threefold. These findings were entirely consistent with the observation that Gcn4 is essential for responses to amino acid starvation (Fig. 2) (46).

Many putative peptidase genes were induced by 3AT (*APE3*, *PRC1*, *PRC3*, *PRE3*, *PRE8*, *PUP2*, *RPN1*, *RPN2*, *RPN6*, *RPN7*, *RPN8*, *RPN10*, *RPT1*, *RPT4*, *RPT5*, *RPT6*, and *IPF4866*). This suggested that protein turnover rates increase in *C. albicans* following amino acid starvation. Also, 43% of the 224 genes that were repressed by 3AT in wild-type cells encode protein synthesis functions. This represents a 7.5-fold enrichment compared with the genome as a whole, which is consistent with the idea that protein synthesis decreases following 3AT treatment. Hence, the transcript profiling data confirm that *C. albicans* activates a bona fide GCN response following exposure to 3AT. This had been suggested by previous Northern blot and proteomic analyses (46, 49) but had not been analyzed before on such a global scale.

There was significant overlap between the Gcn2 and Gcn4 regulons in *C. albicans*. About half of the 328 genes that were induced by 3AT in a Gcn4-dependent fashion were not in-

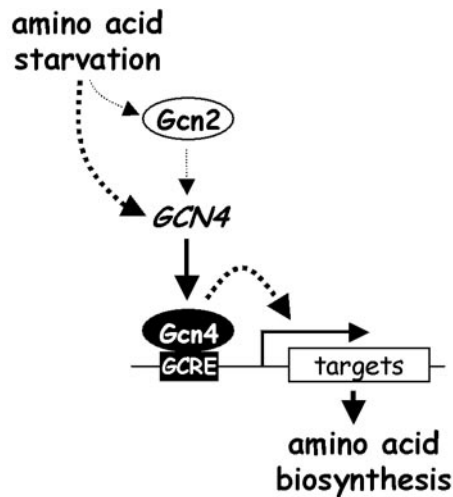


FIG. 7. Model illustrating the partial dependence of the *C. albicans* GCN response on Gcn2 (see the text).

duced in *gcn2* cells (Fig. 6A, gray sector of Venn diagram). This indicates that the induction of these 168 genes was also Gcn2 dependent. The extent of the overlap between the regulons was even greater for 3AT-repressed genes. Genes involved in protein fate, cell rescue, defense, and virulence were significantly enriched in the subset of genes that were induced in a Gcn4- and Gcn2-dependent fashion.

Only seven mRNAs were induced by 3AT in wild-type and *gcn4* cells but not in *gcn2* cells (Fig. 6A). This indicated that almost all genes whose expression was induced by 3AT were dependent on Gcn4 for this induction. In contrast, 160 mRNAs were induced by 3AT in wild-type and *gcn2* cells but not in *gcn4* cells (Fig. 6A, black sector of the Venn diagram). The activation of these mRNAs appears to be dependent on Gcn4 but independent of Gcn2. Genes involved in transport facilitation were significantly enriched in this subset. However, it was possible that their activation was partially dependent on Gcn2. According to this scenario, the inactivation of Gcn2 would reduce, but not block, the induction of these mRNAs.

To test this, we analyzed the responses of *C. albicans* genes to 3AT in more depth. We performed K-means clustering to separate genes on the basis of their expression patterns in wild-type, *gcn2*, and *gcn4* cells. *C. albicans* genes fell into four main K-means subsets. We then examined the behavior of the amino acid biosynthetic genes in these subsets (Fig. 6B). A small proportion of amino acid biosynthetic genes did not respond to 3AT (set 1). However, most amino acid biosynthetic genes did respond to 3AT. Some of these remained relatively unaffected in *gcn2* cells (set 2), whereas the 3AT response of others was blocked by the inactivation of Gcn2 (set 4). Most amino acid biosynthetic genes fell into set 3, although this set contained the smallest number of *C. albicans* genes. Genes in set 3 responded to 3AT in wild-type cells; this response was all but lost in *gcn4* cells, and an intermediate response was observed in *gcn2* cells. This indicates that Gcn2 contributes to, but is not essential for, the activation of these genes by 3AT. These data are entirely consistent with our observation that Gcn2 is not essential for the GCN response in *C. albicans*.

DISCUSSION

In this study, we have shown that *C. albicans* has a single eIF2 α kinase locus. *GCN2* is the only *C. albicans* locus that encodes a protein with significant sequence similarity to GCN2 proteins from other organisms (Fig. 1A). Furthermore, no detectable eIF2 α kinase activity is present in *C. albicans* cells lacking Gcn2 (Fig. 1B).

By analogy with *S. cerevisiae* (26), we initially predicted that Gcn2 might play a role in the *C. albicans* GCN response. Several of our observations suggested that this is the case. First, *GCN2* inactivation attenuates the 3AT resistance of *C. albicans* (Fig. 2). Second, exposure to 3AT increases eIF2 α kinase activity in *C. albicans*, and *GCN2* encodes this activity (Fig. 1B). Third, transcript profiling revealed that 3AT induces the expression of some amino acid biosynthetic genes in *C. albicans* in a Gcn2-dependent fashion (Fig. 6; Table 2).

However, numerous observations suggest that the role of Gcn2 in the *C. albicans* GCN response is relatively minor (Fig. 7). First, Gcn2 inactivation affects *C. albicans* to a much lesser extent than *S. cerevisiae* with respect to their 3AT resistance and their growth in the absence of amino acids (Fig. 2). Second, the transcriptional activation of a GCRE reporter by Gcn4 is not dependent on Gcn2 (Fig. 3). One would have expected this GCRE reporter to be Gcn2 dependent if Gcn2 was essential for the increase in Gcn4 expression levels following 3AT exposure. Third, transcript profiling revealed that Gcn2 contributes to, but is not required for, the activation of many amino acid biosynthetic genes in response to 3AT (Fig. 6; Table 2). Consistent with this, we have observed that the *GCN4* 5'-leader region represses the expression of an *ACT1-GFP* reporter in *C. albicans* and that this repression is not dramatically released following exposure to 3AT (not shown).

It is conceivable that the main role of Gcn2 in *C. albicans* is to enhance the rate at which the GCN response is activated (for example, by accelerating the accumulation of Gcn4) rather than to significantly increase the amplitude of the response. Alternatively, the main role of Gcn2 might be control of global translation rates during amino acid starvation in *C. albicans*. Either of these explanations could account for our observations.

If *GCN4* expression is not regulated primarily by Gcn2, how is *GCN4* activated in *C. albicans*? Our data indicate that *GCN4* is regulated mainly at the transcriptional rather than the translational level. Transcript profiling indicated that 3AT stimulates *GCN4* mRNA levels in wild-type *C. albicans* cells (Table 2). This was confirmed by Northern blot analyses of the *GCN4* mRNA and of various types of *GCN4*-GFP fusion (Fig. 5 and 6) (46). Furthermore, these observations have been confirmed by RT-PCR (not shown).

All of the fungal *GCN4* homologues studied to date are transcriptionally regulated, at least to some extent. In *S. cerevisiae*, *GCN4* transcription increases about twofold in response to 3AT (2), although in this yeast *GCN4* is regulated primarily at the translational level (26). In contrast, *C. albicans* *GCN4* appears to be regulated mainly at the transcriptional level. This is similar to the situation in *Neurospora crassa* and *Aspergillus niger*, where the levels of the *cpc-1* and *cpcA* mRNAs, respectively, increase significantly in response to 3AT

(40, 48). Furthermore in *Aspergillus nidulans*, *cpcA* mRNA levels are autoactivated by CPCA via CPCA response elements (CPRE) in the *cpcA* promoter (27). Therefore, although *C. albicans* is often viewed as being more closely related to budding yeast, with respect to GCN regulation this dimorphic pathogen appears to be more closely related to filamentous fungi.

Gcn4 activates *C. albicans* gene transcription via the GCRE (Fig. 3) (46). This GCRE is present in the upstream regions of most of the *C. albicans* genes that were revealed by transcript profiling to be induced by 3AT in a Gcn4-dependent fashion. We also found that the GCRE is required for transcriptional activation of *GCN4* in response to 3AT (Fig. 3). Taken together, these data suggest that Gcn4 might autoactivate the *GCN4* gene during the GCN response. However, transcriptional induction of *GCN4* was not blocked in *gcn4* cells (Fig. 5). This indicates that while *GCN4* might contribute to its own activation, it is not essential for this activation (Fig. 7), thereby implicating an additional factor.

It is possible that, under normal circumstances, this additional factor contributes to the early stages of the GCN response when Gcn4 levels are low. Then, once Gcn4 levels have started to increase, Gcn4 might autoactivate *GCN4* transcription via the GCRE (Fig. 4), thereby accelerating the full induction of the GCN response. Under abnormal circumstances, when no functional Gcn4 is available (Fig. 5), the additional factor might be sufficient to induce *gcn4-GFP* expression, albeit with relatively slow kinetics.

Several interesting questions are raised by the possible existence of a positive autoregulatory feedback loop involving Gcn4. For example, how is *GCN4* activation prevented in the absence of a signal? Also, how does *GCN4* expression return to normal once the amino acid starvation signal is removed? Several factors might combine to prevent inappropriate activation and timely inactivation of the GCN response. For example, the repressive effect of the 5' leader might help to limit *GCN4* expression levels in the absence of a signal. Also, the activity of the Gcn4 protein might be posttranslationally regulated. *C. albicans* Gcn4 contains PEST-like sequences, which by analogy with *S. cerevisiae* (29) appear to accelerate the degradation of the Gcn4 protein in the absence of a signal (21). Furthermore, the activation domain of *S. cerevisiae* Gcn4 is phosphorylated by cyclin-dependent protein kinases (11, 33). Hence, several possible posttranslational events might combine to down-regulate Gcn4 activity once *C. albicans* cells are released from amino acid starvation.

The fact that *C. albicans* and *S. cerevisiae* have diverged with respect to GCN regulation is not unexpected, for two main reasons. First, there are many precedents for the divergence of signaling modules in these fungi. For example, the Ras-cAMP, mitogen-activated protein kinase, and Tup1-Nrg1 modules, involved in morphogenetic signaling (5, 6, 8, 17, 32, 35, 41), the Rim101/Prr1 module, which mediates pH signaling (13, 15), and the Hog1 and Yap1/Cap1 modules, involved in stress signaling (1, 43), are conserved in *C. albicans* and *S. cerevisiae*. However, the cellular roles of some signaling modules have diverged. For example, Tup1 represses filamentous growth in *C. albicans* but stimulates pseudohyphal growth in *S. cerevisiae* (5). Rfg1 is a morphogenetic repressor in *C. albicans*, whereas its homologue, Rox1, is a hypoxic regulator in *S. cerevisiae* (28).

Also, Msn2 and Msn4 mediate general stress responses in *S. cerevisiae* but not in *C. albicans* (38).

Second, *S. cerevisiae* and *C. albicans* have evolved in contrasting niches. Although Gcn4 is not required for the virulence of *C. albicans* during systemic infections (4), where amino acids are presumably plentiful, Gcn4 is required for biofilm formation (19), and it might be required at other infection sites. For example, the Gcn4 homologue CpcA is required for the virulence of *Aspergillus fumigatus* in pulmonary infections (30). Also, amino acid biosynthetic genes are up-regulated in *C. albicans* during phagocytosis (42). Presumably, the regulation of the *C. albicans* GCN response has evolved to optimize the metabolic fitness of this pathogen in the various niches it occupies within its mammalian host.

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