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Three Galactose inducible promoters for use in *C. neoformans* var. *grubii*

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Abstract

Cryptococcus neoformans is the causative agent of cryptococcal meningoencephalitis, most frequently occurring in immunocompromised individuals. There are three varieties of *C. neoformans*, var. *grubii*, var. *neoformans*, and var. *gatti*. Worldwide var. *grubii* is the most prevalent clinical isolate. However, few tools for the study of essential genes in var. *grubii* exist. Here we describe three endogenous inducible promoters for use in the study of this important opportunistic pathogen. We identified eight potential homologs of *S. cerevisiae* galactose genes in var. *grubii*. We found that *GAL1*, *GAL7*, and *UGE2* were regulated by glucose and galactose and can be used successfully during mating. Our analysis indicated these promoters should prove to be excellent tools for analysis of genes in var. *grubii*.

Keywords

GAL1; *GAL7*; *GAL10*; *UGE1*; *UGE2* mating; *ADE2*; Serotype A

Introduction

Cryptococcus neoformans is a pathogenic yeast that is a threat to immunocompromised individuals, causing respiratory infection and meningoencephalitis. Kwon-Chung and Bennett found that 75% of *C. neoformans* clinical isolates were var. *grubii*, 7% were var. *neoformans* and the remaining 18% were var. *gatti* indicating the majority of cryptococcosis infections were due to var. *grubii* (Kwon-Chung, K.J. and Bennett, J.E. 1978; Lengeler, K.B., Wang, P. *et al.* 2000). Additionally, the incidence of cryptococcosis has risen dramatically over the past 25 years, corresponding to the increase in HIV infection (Casadevall, A. and Perfect, J.R. 1998). In spite of therapeutic advances, developing nations are still experiencing significant problems with cryptococcosis, which accounts for up to half the cases of neurological disease in HIV patients (Banerjee, U., Datta, K. *et al.* 2001).

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Paralleling the increased incidence of cryptococcosis, study of the biology and pathogenesis of *C. neoformans* has greatly increased. Varieties *neoformans* and *grubii* have been sequenced and annotated ((Loftus, B.J., Fung, E. *et al.* 2005), www.broad.mit.edu), allowing for greater utilization of available molecular techniques, such as gene disruption or specific mutation by homologous recombination of DNA introduced through biolistic transformation or electroporation (Edman, J.C. and Kwon-Chung, K.J. 1990; Toffaletti, D.L., Rude, T.H. *et al.* 1993; Lodge, J.K., Jackson-Machelski, E. *et al.* 1994). Although the above techniques have proven useful they are limited with respect to the study of essential genes.

Current established techniques for genetic analysis in *C. neoformans* include conditional lethality, anti-sense gene orientation, RNA interference, mating with segregation analysis, and the use of inducible promoters. Mutations that produce conditional lethality may cause auxotrophy or temperature sensitivity. A classic example of using conditional lethality to demonstrate the essentiality of a gene in *C. neoformans* is the deletion of *ADE2*. This gene encodes an enzyme, phosphoribosylaminoimidazole carboxylase, which is involved in the production of endogenous adenine biosynthesis. Strains with this gene deleted are conditionally lethal on minimal medium in the absence of adenine (Perfect, J.R., Toffaletti, D.L. *et al.* 1993). Lodge and colleagues reported the use of temperature sensitive strains for the determination of an essential gene that encodes Myristoyl-CoA:protein N-myristoyltransferase (Nmt). A mutation, NMT^{G487D}, was introduced into the gene and resulted in temperature-sensitive myristic acid auxotrophs at 37°C (Lodge, J.K., Jackson-Machelski, E. *et al.* 1994; Lodge, J.K., Jackson-Machelski, E. *et al.* 1998). Both of these methods are limited and are only successful when investigating genes that lead to conditional lethality. The *C. neoformans* β -(1,3)-glucan synthase, *Fks1*, has been shown to be essential. Using anti-sense orientation Thompson *et al.* established this via homologous integrative transformation that relied upon a plasmid that integrated in two orientations, one orientation disrupted the gene function of *FKS1* and led to the loss of viability (Thompson, J.R., Douglas, C.M. *et al.* 1999). RNA interference (RNAi) has been used successfully in var. *neoformans*. In this technique double-stranded RNA homologous to *ADE2* resulted in specific degradation of *ADE2* mRNA and thus reduced translation of this gene (Liu, H., Cottrell, T.R. *et al.* 2002). They found that the *ADE2* RNAi strain was phenotypically similar to that of the *ade2* Δ . Additionally, Liu and colleagues demonstrated that RNAi could be used to target two genes simultaneously through the expression of chimeric double-stranded RNA (Liu, H., Cottrell, T.R. *et al.* 2002). RNAi techniques for the study of essential genes in var. *grubii* have not been successfully established. Similar to RNAi, Gorlach *et al.* used antisense to repress the expression of two genes coding for calcineurin A and laccase 1. The deletions of these genes cause temperature sensitivity at 37°C and loss of melanin pigment, respectively, and both transcripts were successfully repressed using this method (Gorlach, J.M., McDade, H.C. *et al.* 2002). Antisense repression relies on the ectopic integration of the antisense construct. Ectopic integration can lead to the inadvertent disruption of other genes, which makes this method less desirable. To determine synthetic lethality between two gene deletions, mating and segregation analysis is a commonly employed procedure. However this method can be limiting due to several factors such as: First, the deletions may affect the ability of *C. neoformans* to mate, thus affecting the production of basidiospores. Secondly, if spores are produced, isolating them is a time consuming and tedious procedure, and being able to acquire the number of progeny needed to statically support synthetic lethality can be problematic. Lastly, random spore analysis can be used to produce multiple deletion strains, a faster method than isolation of single spores, and readily provides enough progeny for statistical analysis. Again, this method is only feasible if mating is possible and viable spores are produced. Finally, inducible promoters have also been used in *C. neoformans* to establish gene essentiality and are described below.

Typically inducible promoters have been the best method to confirm the essentiality of genes. However, inducible promoters for use in var. *grubii* are limited. There are three well-

characterized inducible promoters in *C. neoformans*. The first is the promoter of *GAL7* from var. *neoformans*, which is repressed by glucose and induced by galactose (Wickes, B.L. and Edman, J.C. 1995). Ory and colleagues found var. *neoformans*' PGAL7 was endogenously induced up to 83 fold; however, when this same promoter was expressed heterogenously in var. *grubii* they observed only three fold induction, thus limiting the usefulness of var. *neoformans*' PGAL7 in var. *grubii* (Ory, J.J., Griffith, C.L. *et al.* 2004). Second is the promoter of *MFa1* from var. *neoformans*, which is induced on V8 medium (del Poeta, M., Toffaletti, D.L. *et al.* 1999). Although this promoter drives expression well in both varieties, the V8 medium causes slow growth and induces sporulation; as such this specific growth requirement restricts *PMFa1*'s general use. Finally, the *PCTR4* is a copper repressible promoter that is induced by copper chelation (Ory, J.J., Griffith, C.L. *et al.* 2004). It is the best available option for investigators of var. *grubii*: first it is tightly regulated and secondly it exhibits higher levels of induction than the other available promoters. However, it requires the use of copper-free medium, which may not be suitable for all experiments. Therefore, the need exists for additional reliable, endogenous, and easily regulated inducible promoters for use in var. *grubii*.

The Leloir pathway has been well established in *Saccharomyces cerevisiae* and components of this pathway have been exploited for genetic analysis (reviewed in, Johnston, M. 1987). The enzymes in this pathway that convert galactose to glucose-6-phosphate are Gal1p (a kinase), Gal7p (a transferase), Gal10p (an epimerase). Other Gal enzymes are regulators of these three key enzymes, however, they are not directly part of the Leloir pathway, yet they retain the Gal designation. One exception is an α Galactosidase that is designated Mel1p. Three of the Gal proteins, Gal3p, Gal4p/Gal81p, and Gal80p function in the regulation of Mel1p, Gal1p (a kinase), Gal7p (a transferase), Gal10p (an epimerase), Gal2p (a galactose permease) (Johnston, M. 1987), and Gal5p (a phosphoglucomutase) (Oh, D. and Hopper, J.E. 1990). In *S. cerevisiae* *GAL1*, *GAL7*, and *GAL10* are clustered together on chromosome two, but are transcribed from separate promoters. *GAL2* and *MEL1* lie on chromosome number twelve (Leloir, L.F. 1951; Torchia, T.E., Hamilton, R.W. *et al.* 1984; Torchia, T.E. and Hopper, J.E. 1986; Tschopp, J.F., Emr, S.D. *et al.* 1986). *GAL4* encodes a protein that activates transcription of these five genes. Gal80p negatively regulates Gal4p while Gal3p, which is induced by galactose, activates Gal4p indirectly by negatively regulating Gal80p (Johnston, M. 1987).

Therefore, to find new endogenous promoters that could be regulated *in vitro* in var. *grubii*, we focused our search on the Leloir pathway and those enzymes known to be involved in galactose metabolism. We investigated homologs to nine *S. cerevisiae* *GAL* genes to determine which were repressed by glucose and induced by galactose in var. *grubii*. Here we report and characterize three endogenous galactose inducible promoters PGAL1, PGAL7, and PUGE2 for use in var. *grubii*.

Material and Methods

Fungal strains and media

The strain KN99 α of *C. neoformans* var. *grubii* (Nielsen, K., Cox, G.M. *et al.* 2003), was used as the wild type strain, and all promoter swap constructs were introduced into it. An *ade* Δ strain (in the H99 background) var. *grubii* was used as the positive control (Perfect *et al.*, 1993). Strains were grown on rich media at 30 C, YPD or YPG (1% yeast extract, 2% bacto-peptone, and 2% dextrose or 2% galactose, respectively) and on minimal media SD-ade (.67% YNB with NH₄SO₄ without amino acids and dextrose,.008% CSM-ade, 2% bacto agar and 2% dextrose or galactose with 0.2% dextrose). Solid media contained 2% bacto-agar. Selective YPD or YPG media contained 100 μ g/ml nourseothricin (Werner BioAgents, Jena-Cospeda, Germany) and/or 200 μ g/ml gentamycin (Invitrogen, Carlsbad, CA).

Identification of putative galactose inducible promoters

Amino acid sequences were retrieved from *Saccharomyces* Genome Database for well-known genes dependent on galactose for expression in *S. cerevisiae* (*GAL1*, *GAL2*, *GAL3*, *GAL4*, *GAL5*, *GAL7*, *GAL10*, *GAL80*, *MEL1*) (www.yeastgenome.org). The amino acid sequences were BLASTed against Broad H99 var. *grubii* predicted proteins (www.broad.mit.edu/annotation/genome/cryptococcus_neoformans). The protein sequences were retrieved and used as queries to locate the genomic DNA and the genomic sequences were used in a BLAST search to find the transcripts. These coding sequences were used to design probes for Northern blots. Gene nomenclature was assigned as described by Gerik *et al.* (Gerik, K.J., Donlin, M.J. *et al.* 2005) with the following exceptions: For clarity lower case letters were used to designate multiple homologues to *S. cerevisiae* *GAL* genes instead of numbers. The *UXS1*, *UGE1*, and *UGE2* designations were used instead of *GAL10* or *GAL10c* because these have previously been described in the literature (Bar-Peled, M., Griffith, C.L. *et al.* 2001; Moyrand, F., Fontaine, T. *et al.* 2007). Additionally, *GAL10b* was not used because it has previously been deposited into Genbank (AAR84604) as *UGE2*.

RNA extraction for northern blot analysis

C. neoformans strain KN99 was grown for 24 hours at 30°C in liquid YPD or YPG broth. For RNA extraction and poly-A RNA purification cells were collected by centrifugation at 1,800 × g for five minutes, washed once with distilled water, and lyophilized overnight. The lyophilized pellet was then vortexed with 3 ml glass beads (1 mm; Biospec, Inc., Bartlesville, OK) and re-suspended in 4 ml TRIzol Reagent (Invitrogen, Carlsbad, CA). After sitting at room temperature for five minutes, 800 µl of chloroform was added and the mixture was shaken for 30 sec. This cell lysate was then centrifuged at 4,000 rpm for ten minutes and the supernatant was transferred to a new tube. Two ml isopropanol was added, incubated for 10 min at room temperature, and centrifuged at 4,000 rpm for ten minutes. After washing the pellet with 75% EtOH, it was resuspended in water and incubated with DNase I at 37°C for 1 hr. The RNA was extracted again with TRIzol and chloroform and precipitated with isopropanol as above. The dried pellet was resuspended in 300 µl RNase-free water (Invitrogen, Carlsbad, CA). Poly-A RNA was purified from the total RNA sample using an oligo-*Tex* RNA purification kit (Qiagen, Valencia, CA) following the manufacturer's specifications.

Northern Hybridizations

The procedure we used was adapted from Sambrook and Russell (Sambrook, J. and Russell, D. 2001). Approximately 1 µg of poly-A RNA from each strain was mixed with 2 µL of DEPC (Sigma, St. Louis, MO) -treated 5X formaldehyde gel running buffer (0.1mM MOPS (Fisher) pH 7.0, 40mM sodium acetate (Sigma, St. Louis, MO), 5mM EDTA pH 8.0), 3.5 µL of 37% formaldehyde (Sigma, St. Louis, MO), and 10 µL of formamide (Sigma, St. Louis, MO). Samples were then incubated at 65°C for 15 minutes and placed directly on ice. 2 µL DEPC – treated formaldehyde gel loading buffer (50% glycerol (Fisher), 1mM EDTA (Fisher), 0.25% bromophenol blue (Sigma, St. Louis, MO)) was added and samples were then loaded on a 1.5% agarose (Roche) gel containing formaldehyde (DEPC treated, 1X formaldehyde gel running buffer, 6.6% formaldehyde) submersed in 1X formaldehyde gel running buffer. An RNA size marker (Promega, Cat. Num. 3191A, Madison, WI) sample was prepared in the same way, however ~1 µg ethidium bromide (Sigma, St. Louis, MO) was added before the sample was loaded onto the agarose gel. A positive binding control ladder, containing 100pg of each gene specific DNA of interest, was also loaded on the agarose gel. The primers used to amplify the gene specific DNA fragments for the positive binding control ladder are shown in Supplemental Table S1. Gels were pre-run in 1X formaldehyde gel running buffer for 5 minutes at approximately 5V/cm before samples were loaded, and run at approximately 58V/cm for ~4 hours, with constant recirculation of running buffer, until the bromophenol blue had traveled

8–9 cm. Gels were rinsed in several volumes DEPC-treated water, soaked in several volumes DEPC-treated 50mM NaOH (Fisher) for 20 minutes, and soaked in several volumes 20X SSC for 45 minutes. Fragments were then transferred to charged nylon membranes using a Turbo-Blot apparatus with 20X SSC as transfer buffer. Membranes were then UV cross-linked. Probes for Northern analysis were prepared by random priming (random priming kit; Roche) using 50 μ Ci [α - 32 P]dCTP, according to the manufacturer's instructions. The membranes were incubated in 20ml of ULTRAHyb buffer (Ambion, Austin, TX) for 3 hour at 55°C, then probe was added to this solution, and the membranes were hybridized at 55°C overnight. The membranes were washed one time in ~50 mL of a solution of 1X SSC, 0.1% SDS at 25°C for 10 minutes. Membranes were then washed three times in ~50mL of a solution of 0.5X SSC, 0.1% SDS at 68°C for 10 minutes. The membranes were stripped of probe twice by incubating 2X in ~50mL of 10mM Tris-Cl pH 7.4, 0.2% SDS, heated to boiling for 1.5 hours, and subsequently re-probed following instructions above. Phosphoimager screens were used to detect radiolabels and analyzed using ImageQuant version 5.1.

Generation of promoter swap constructs

Overlap PCR gene deletion technology (Davidson, R., Blankenship, J. *et al.* 2002) was used to generate promoter-specific exchange cassettes of *ADE2*, which all included a nourseothricin cassette (McDade, H. and Cox, G. 2001). Primers used to disrupt the *ADE2* promoter are shown in Supplemental Table S2. For the *ADE2* promoter swap constructs, 500bp preceding the gene were deleted and replaced with the putative galactose inducible promoters. In the promoter swap construct 978bp, 1022bp and 1007bp were used for PGAL1, PGAL7 and PUGE2, respectively.

Transformation of *C. neoformans*

KN99 α was transformed using biolistic techniques (Toffaletti, D.L., Rude, T.H. *et al.* 1993; Hua, J., Meyer, J. *et al.* 2000). Cells were grown in YPD to late log-phase, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6 μ m gold beads (Bio-Rad, Richmond, CA) that were coated with DNA of the target construct according to the manufacturer's recommendations. Following the transformation, the cells were incubated at 30°C for 4 hours on nonselective media to allow for recovery and then transferred with 0.8 ml sterile PBS to the nourseothricin selective media. Transformants were observed in 3–5 days.

Analysis of transformants

To isolate stable transformants, they were all passaged five times on nonselective YPD medium and then tested for resistance to the appropriate selective marker. Only those transformants that grew equally well on selective and nonselective media were considered to be stable transformants. A three-primer PCR screen was used to verify homologous integration at both the 5' and 3' ends of the deletion cassette. In this manner, homologous recombinants can be distinguished from wild type. A PCR screen using primers outside the transformation construct was used to amplify the entire integration region, demonstrating that a single copy of the transforming DNA had been inserted at the desired locus. Southern blots were performed to screen for single integration in the genome. Single bands were observed on all Southern blots when hybridized with a selectable marker-specific probe. All deletion strains generated for this work had a single deletion construct homologously integrated at the appropriate locus and no other insertions in the genome (data not shown). At least five independent isolates for each mutant were obtained and two were further characterized.

Genomic DNA preparation

Genomic DNA was prepared by a modification of the glass bead DNA extraction protocol described (Fujimura, H. and Sakuma, Y. 1993). Briefly, *C. neoformans* cells were suspended

in a microfuge tube in 500 μ l lysis buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 1% SDS), with 400 mg glass beads (425–600 μ m; Sigma G-9268, St Louis, MO). Cells were disrupted by vortexing 10 minutes, followed by 10 min incubation at 70°C. After brief vortexing, 200 μ l 5M KOAc and 150 μ l 5 M NaCl were added. The tubes were placed on ice for 20 min and centrifuged at 14,000 rpm for 20 min. The supernatant was mixed with 500 μ l phenol/chloroform and spun for 5 min at 14,000 rpm. The aqueous phase was then mixed with 450 μ l chloroform and spun for 5 min at 14,000 rpm. The DNA was then precipitated by addition of 200 μ l ethanol, washed with 70% ethanol, dried, and resuspended in 50 μ l deionized water.

Southern hybridizations

Approximately 10 μ g of genomic DNA from each strain was digested with various restriction endonucleases according to the manufacturer's recommendations. Restriction fragments were separated on a 1% agarose gel and transferred to nylon membranes using a Turbo-Blot apparatus (Schleicher & Schuell) and 10X SSC as transfer buffer. Probes for Southern analysis were prepared by random priming (random priming kit; Roche) using 50 μ Ci [α -³²P] dCTP (GE Bio-Sciences AA0005, Piscataway, NJ) according to the manufacturer's instructions. The blots were incubated in 10 ml of buffer (1X phosphate buffer, 7% SDS) solution for 1 hr at 65°C, then probe was added to this solution, and the blots were hybridized at 65°C overnight. The blots were washed twice in 2X SSC, 0.1% SDS at room temperature for 10 min and once for 10 min in 0.2X SSC, 0.1% SDS that had been prewarmed to 65°C. A phosphorimager screen was used to detect the radiolabel and analyzed using ImageQuant version 5.1.

RNA extraction for qPCR

Promoter swap strains were grown for 24 hours at 30°C in liquid YPD or YPG broth, cells were collected by centrifugation at 1,800 rpm for 3 minutes and washed three times with 1 X PBS. The pelleted cells were then lyophilized for 2 hours and rRNA was then purified from the samples using an RNA isolation kit (Agilent, Santa Clara, CA) with modifications to the manufacturer's protocol for yeast. The samples were homogenized with glass beads for 5 minutes; 600 μ L of lysis buffer was added to the samples; vortexed for 5 minutes and collected by centrifugation at 14,000 rpm for 3 minutes. Then 450 μ L of lysate was passed through a mini-prefiltration column. The remainder of the manufacturer's recommendations was followed.

Quantative PCR

One microgram of total rRNA was used for first-strand cDNA synthesis using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Germany) as per manufactures instructions. The resulting cDNA was used as a template in quantitative PCR using SYBR Green PCR reagents (Clontech, Mountain View, CA) according to the manufacturer's recommendations. Primers used in qPCR are listed in Supplemental Table S3. The DNA Engine Opticon (MJ Research, Inc.) was used as the fluorescence detector with the following protocol for the PCR: 35 s at 94°C, 50 s at 54°C, and 50 s at 72°C, and a plate reading was repeated for a total of 40 cycles after a hot start of 10 min at 94°C. A melting curve was calculated at the end of the reaction to confirm a single product. The data were normalized to actin cDNA expression amplified in the set of qPCRs.

Mating slides

A cell suspension of strains of equal amounts of appropriate opposite mating types (**a** and α) was mixed together in 500 μ L 1 X PBS. Approximately 100 μ L of this suspension was applied to slides covered in V8 agar supplemented with or without 2% glucose or 2% galactose in a mating chamber (100 \times 15mm petri dish), then 4mLs of V8 medium (minus agar) was added to aid in maintaining nutrients and chamber humidity. The mating chambers were placed in

dark at 25°C for four to five days. Brightfield images were taken on an Olympus Vanox AHB T3 microscope at designated magnifications and are representative of three or more independent experiments.

Mating Plates

Compatible mating types PGAL7:*ADE2* α (nourseothricin R) and *MPK1-FLAGa* (gentamycin R) (Gerik, K.J., Bhimoreddy, S.R. *et al.* 2008) were streaked consecutively in patches on V8 mating plates supplemented without or with 2% glucose or 2% galactose. *MPK1-FLAGa* was chosen because it is a marked strain with no discernible mating defects. The plates were incubated as described for mating slides with the exception of the incubation period being extended to 16 days. Progeny were collected by agar plug at the periphery of the mating patches. Recombinant progeny were selected based on drug resistance to both nourseothricin and gentamycin (100 μ g/ml nourseothricin (Werner BioAgents, Jena-Cospeda, Germany) and 200 μ g/ml gentamycin (Invitrogen, Carlsbad, CA)).

Results

Identification of galactose genes in var. *grubii*

To identify putative *GAL* inducible promoters for var. *grubii* we first retrieved amino acid sequences from the *Saccharomyces* Genome Database for nine *GAL* genes in *S. cerevisiae* (*GAL1*, *GAL2*, *GAL3*, *GAL4*, *GAL5*, *GAL7*, *GAL10*, *GAL80*, *MEL1*) (www.yeastgenome.org). The amino acid sequences were used in a tBLASTn query of var. *grubii*, H99, predicted proteins (genome.slu.edu/bblast.html). The retrieved protein sequences were used to query the H99 genomic database. Of the nine proteins five, Gal2p, Gal3p, Gal4p, Gal80p, and Mel1p, had no hits indicating that these proteins may not be highly conserved between *S. cerevisiae* and var. *grubii*. However, the search yielded a total of eight homologous genes with Gal1p, Gal7p and Gal10p each having multiple putative homologs, two, two, and three, respectively, and Gal5p having one homolog (Table 1).

Three *GAL* genes are induced by galactose in var. *grubii*

To ascertain which of the eight endogenous promoters were regulated by galactose in var. *grubii* we determined if their corresponding genes were differentially transcribed in the KN99 α wild type strain. Northern blot analysis indicated that *GAL1*, *GAL7* and *UGE2* were repressed by glucose and induced by galactose medium (Fig. 1). *GAL1b*, *GAL7b* and *UGE1* showed no expression under either condition, while *GAL5* and *UXS1* had equal induction under both conditions (data not shown). This data indicated that the promoters of *GAL1*, *GAL7* and *UGE2* were good candidates for further analysis.

Galactose induces expression of *ADE2* under the control of the *GAL1*, *GAL7*, and *UGE2* promoters

Next, we wanted to establish if the endogenous var. *grubii* *GAL1*, *GAL7* and *UGE2* promoters could be used to induce heterologous gene expression. The Ade2 enzyme, phosphoribosylaminoimidazole carboxylase, is a good candidate for developing tools such as inducible promoters. An *ADE2* deletion is conditionally lethal on minimal medium in the absence of adenine, but is viable when grown on yeast peptone dextrose (YPD) where adenine biosynthetic intermediates accumulate which result in pink colonies (Perfect, J.R., Toffaletti, D.L. *et al.* 1993). We replaced 500 base pairs of the endogenous *ADE2* promoter with the predicted promoters of *GAL1*, *GAL7*, and *UGE2*. Each of these promoters was fused to the predicted *ADE2* start codon. At least five independent transformants were confirmed for each promoter swap, with two being chosen for further analysis (Table 2). The promoter swap strains were grown on yeast peptone dextrose (YPD) and yeast peptone galactose (YPG) media

supplemented with 2% glucose or galactose, respectively. After five days on YPD and YPG, all strains with a PGALX:*ADE2* ($X = 1, 7$ or $10b$) cassette were pink or white, respectively (Fig. 2). These data indicated that the promoters could effectively induce *ADE2* in the presence of galactose.

To quantitate the level of galactose induction in gene expression using the endogenous var. *grubii* *GAL/UGE* promoters, the magnitude of transcription differences in glucose (repression) versus galactose (induction) of the *ADE2* transcript was assayed by quantitative PCR (qPCR). All three promoters repressed *ADE2* expression in response to growth on glucose and induced *ADE2* expression in galactose compared to wild type. For PGAL1, PGAL7, and PUGE2, *ADE2* was repressed by 4.8, 6.1 and 5.3 and induced by 15.5, 21.6 and 28.1, respectively, in LOG₂ ratio (Table 3). Furthermore, galactose induction was 20.5, 27.8 and 33.5 in LOG₂ ratio for PGAL1, PGAL7, and PUGE2, respectively. Galactose did not affect native *ADE2* expression levels (data not shown). The data clearly suggested the var. *grubii* promoters of *GAL1*, *GAL7* and *UGE2* were repressed by glucose and induced by galactose.

ADE2 driven under the control of the GAL1, GAL7, and UGE2 promoters rescues conditional lethality

C. neoformans strains without Ade2 do not survive on minimal medium without the addition of adenine in the growth medium. Therefore, we determined if the *GAL/UGE* promoters could rescue this conditional lethal phenotype. Mutant strains were grown on SD-ade medium, a minimal medium that lacks adenine, supplemented with 2% glucose or galactose for five days at 30°C. As predicted, the PGALX:*ADE2* strains died on SD-ade with glucose medium and grew on SD-ade with galactose medium (Fig. 3). These data indicated the *GAL* promoters were sufficiently repressed on glucose to reveal a conditional lethal phenotype and might prove useful in determining the lethality of other conditional or lethal genes.

The GAL promoters can be used during mating

Mating is another tool in *C. neoformans* that is increasingly utilized to produce multiple deletions or mutations in the same strain background. However, mating of *C. neoformans* requires V8 agar, a nutrient poor medium (Hull, C.M. and Heitman, J. 2002). To determine if the PGAL promoters could be either repressed or induced during mating we first ascertained if the addition of 2% glucose or galactose to the V8 medium adversely affected wild type mating. Mating slides of compatible wild type crosses, KN99 α X KN99 α , produced spores on all three media (Fig. 4). However, in addition to sporulation, abundant filamentation was also observed on both of the V8+2% glucose and V8+2% galactose media. These data suggested that the addition of these sugars to the mating medium did not adversely effect sporulation, but did affect the ratio of spore to hyphae.

Having established that the addition of the sugars did not preclude the development of spores, we next assessed the feasibility of using the *GAL* promoters in mating assays to produce a recombinant strain. To do this we crossed the PGAL7:*ADE2* α (nourseothricin marker) to another strain *MPK1:FLAG* α (gentamycin marker) or KN99 α . The *MPK1:FLAG* α strain was chosen for our mating experiments because it has a different marker than the PGAL7:*ADE2* α strains, has no discernable mating defects, and recombinant progeny could easily be screened on double drug selection. The PGAL7:*ADE2* α X *MPK1:FLAG* α or KN99 α mating crosses only produced spores when grown on V8+2% galactose slides (Fig. 4). This indicated the V8 medium components are unable to induce the *GAL* promoters during this short time frame. However, given a longer incubation period that is used with mating on V8 plates, sixteen days instead of 4–5 days on slides, viable progeny from the PGAL7:*ADE2* α X *MPK1:FLAG* α cross were recovered from V8 only, V8+2% glucose, and V8+2% galactose

media numbering 24, 9, and 156, respectively. These data indicate that the PGAL promoters can be used successfully during mating.

Discussion

This is the first study to characterize native inducible promoters of var. *grubii*. To identify inducible promoters we focused our search on the Leloir pathway (Leloir, L.F. 1951). We found homologs to four *GAL* genes in *S. cerevisiae* (*GAL1*, *GAL5*, *GAL7*, and *GAL10*). In *S. cerevisiae* the galactose inducible genes *GAL1*, *GAL7*, and *GAL10* cluster together on chromosome two, and *GAL5*, on chromosome 13, is unregulated by glucose or galactose (Johnston, M. 1987). A similar situation occurs in *C. neoformans*, where the genes that are regulated by galactose, *GAL1*, *GAL7*, and *UGE2* were clustered together in the genome as indicated by their Broad Institute CNAT transcript numbers 06050 (*UGE2*), 06051 (*GAL1*), and 06052 (*GAL7*) (Table 1, www.broad.mit.edu/annotation/genome/cryptococcus_neoformans). This suggests that this functional clustering has been conserved through the divergence of the Basidiomycetes and Ascomycetes. However, the orientation of the genes within the cluster has not been conserved. In *S. cerevisiae* the gene order is *GAL7*, *GAL10*, and *GAL1* (Johnston, M. 1987) compared to *UGE2*, *GAL1*, and *GAL7*. It is interesting that the *C. neoformans* protein Uge1 (CNAG_00697.1), a UDP-glucose epimerase that reversibly catalyzes UDP-glucose to UDP-galactose and is part of the Leloir pathway, is most homologous to Gal10 in *S. cerevisiae*, but was not found in the gene cluster in *C. neoformans*. Moyrand and colleagues found Uge1 to be necessary for the production of galactoxylomannan (GalXM), one of the two capsular polysaccharides. GalXM is a minor component of the capsule being approximately 7% of the capsule mass (Moyrand, F., Fontaine, T. *et al.* 2007), while glucuronoxylomannan (GXM), the other capsular polysaccharide, makes up about 90% of the capsule mass (Bose, I., Reese, A.J. *et al.* 2003). As Uge2 participates in producing part of the capsule we would hypothesize that its expression would increase under capsule inducing conditions, such as growth in low iron medium or in the presence of high CO₂. However, our data, under non-capsular conditions, indicated that expression of *UGE1* was not regulated by either glucose or galactose (data not shown) and a direct comparison was not performed.

Similar to what is reported in *S. cerevisiae*, we determined that *GAL5* in *C. neoformans* was not regulated by glucose or galactose, as its expression remained constant under both growth conditions (data not shown). Through our homology based searches of the var. *grubii* genome we did not find any of the regulatory components of the Leloir pathway *GAL2*, *GAL3*, and *GAL80* (Leloir, L.F. 1951; Torchia, T.E., Hamilton, R.W. *et al.* 1984; Torchia, T.E. and Hopper, J.E. 1986; Tschopp, J.F., Emr, S.D. *et al.* 1986). This may suggest that these regulatory components are not well conserved, or that the *GAL/UGE* genes in *C. neoformans* are regulated in a manner different than *S. cerevisiae*.

We have demonstrated that use of the endogenous inducible promoters in var. *grubii* is more feasible than using promoters from var. *neoformans*. In contrast to the three fold induction reported by Ory and colleagues when using the heterogenous promoter from var. *neoformans* in var. *grubii* (Ory, J.J., Griffith, C.L. *et al.* 2004) we found galactose to induce the PGAL7 promoter of var. *grubii* greater than 1000-fold as compared to glucose suppression. These differences in expression levels argue for using the native promoters in var. *grubii*.

We have established that these inducible promoters can be used successfully to rescue the conditional lethal phenotype of Ade2 suppression, and they can be used during mating for the creation of recombinant strains. They may even prove to be useful in assaying genes involved in mating. In addition, these promoters should prove to be valuable for investigation into essential genes, as well as the over expression of genes, and other novel purposes. We suspect

that PGAL1, PGAL7 and PUGE2 will become indispensable tools for investigators of this variety of *C. neoformans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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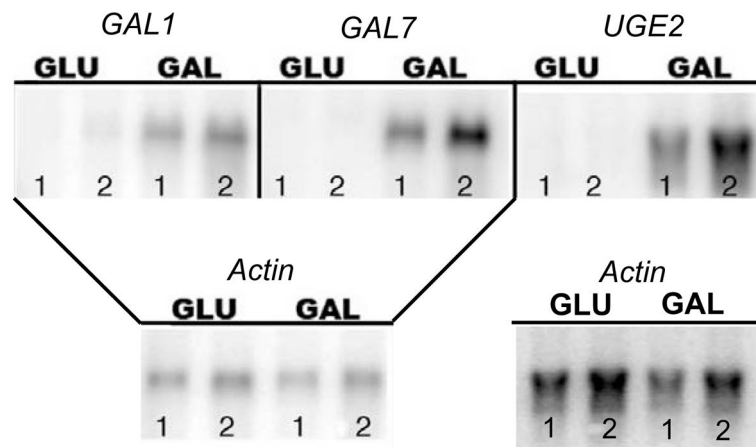


Figure 1. Northern Blots of *GAL1*, *GAL7* and *UGE2*

GAL1, *GAL7* and *UGE2* gene expression is induced by growth in galactose. Lanes 1 and 2 are biological replicates grown under the same conditions. Blots were probed with Actin to show equal loading of mRNA. The same blot was used for both *GAL1* and *GAL7*. Actin blot for *UGE2* is separate.

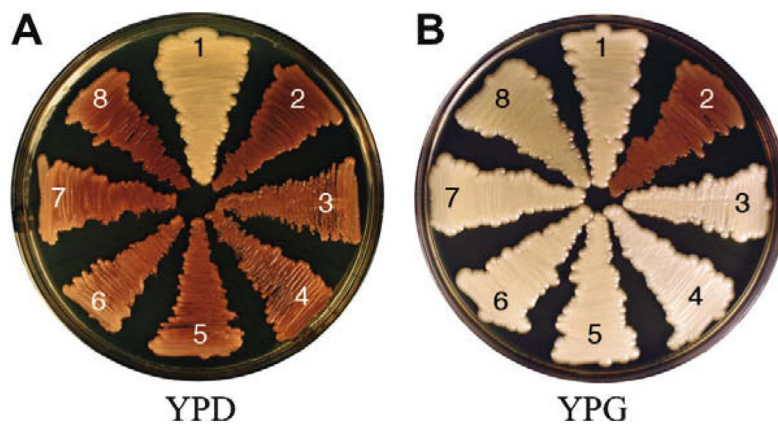


Figure 2. PGALX:*ADE2* strains grown on YPD and YPG at 30°C for 5 days

A representative of three biological replicates is shown. 1. KN99 α 2. H99 (*ade2* Δ), 3&4. Independent isolates of PUG2:*ADE2*, 5&6. Independent isolates of PGAL7:*ADE2*, 7&8. Independent isolates of PGAL1:*ADE2*. On YPD, the PGALX:*ADE2* strains are red indicating glucose repression of *ADE2* expression, resulting in accumulation of adenine biosynthetic intermediates. On YPG, the PGALX:*ADE2* strains are white indicating galactose induction of *ADE2* expression, which resulted in wild type phenotype.

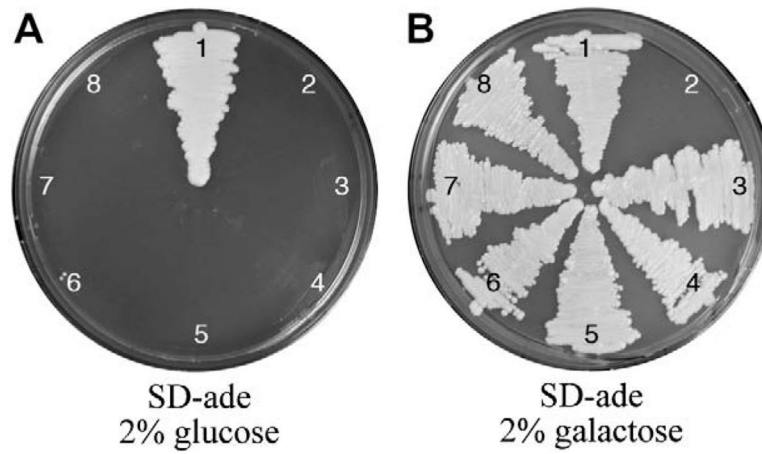


Figure 3. PGALX:*ADE2* strains grown on SD-ade with glucose and galactose at 30°C for 5 days
A representative of three biological replicates are shown. The numbering corresponds to the same strains used in Fig. 2. On SD-ade with glucose, the PGALX:*ADE2* strains die because *ADE2* expression is repressed, creating a conditional lethality. On SD-ade with galactose, the PGALX:*ADE2* strains grow because *ADE2* is expressed.

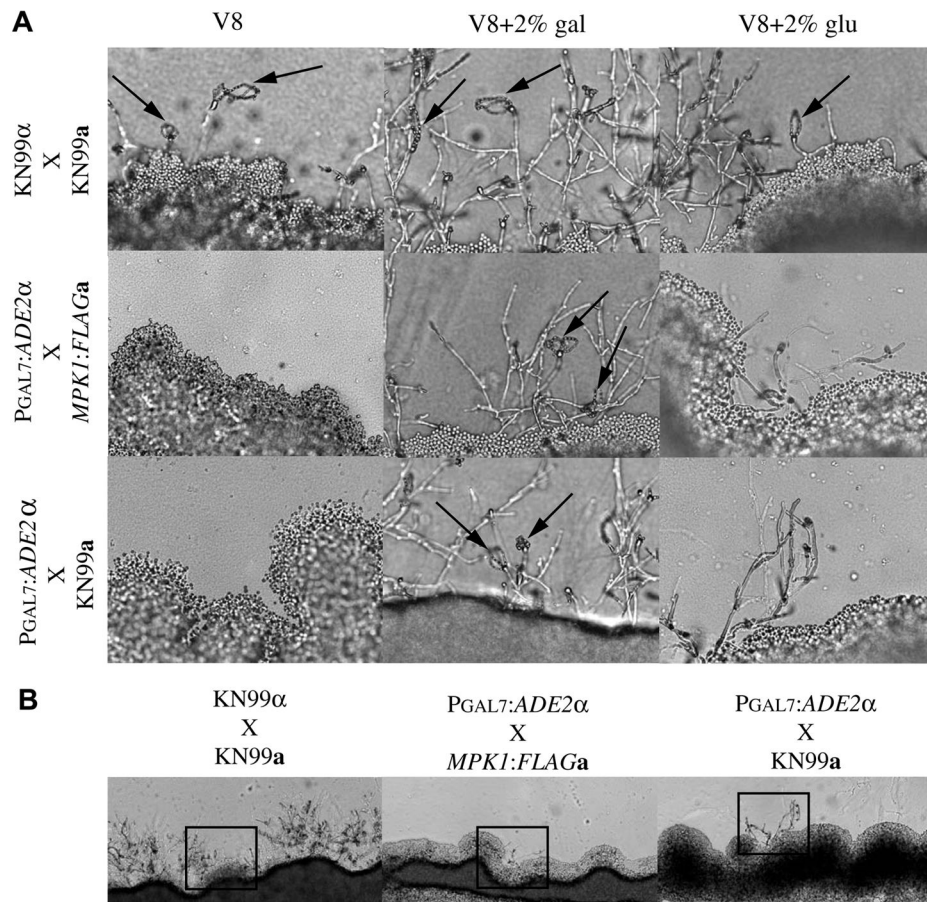


Figure 4. PGAL7:ADE2 compatible mating crosses

A. Mating slides of V8 or V8 supplemented with 2% galactose or glucose were inoculated with equal amounts of compatible mating partners α and a , incubated in the dark at 25°C for 4 days. Photos are 40X magnification. Mating strains are indicated to the left of the panels and medium used is indicated on top. B. 10X magnification of mating on V8 supplemented with 2% glucose. Boxed area corresponds to 40X magnification in A. Matings are indicated top of panels. All images are representative of at least three independent experiments.

Table 1

Galactose Gene Homology

<i>S. cerevisiae</i> Gene	<i>C. neoformans</i> Protein	<i>C. neoformans</i> Coding Sequence and Designation
<i>GAL1</i>	CNAG_06051.1	CNAT_06051 (<i>GAL1</i>)
	CNAG_03946.1	CNAT_03946 (<i>GAL1b</i>)
<i>GAL2</i>	No Hits	N/A
<i>GAL3</i>	No Hits	N/A
<i>GAL4</i>	No Hits	N/A
<i>GAL5</i>	CNAG_06313.1	CNAT_06313 (<i>GAL5</i>)
<i>GAL7</i>	CNAG_06052.1	CNAT_06052 (<i>GAL7</i>)
	CNAG_03875.1	CNAT_03875 (<i>GAL7b</i>)
<i>GAL10</i>	CNAG_00697.1	CNAT_00697 (<i>UGE1</i>)*
	CNAG_06050.1	CNAT_06050 (<i>UGE2</i>)*
	CNAG_03322.1	CNAT_03322 (<i>UXS1</i>)*
<i>GAL80</i>	No Hits	N/A
<i>MEL1</i>	No Hits	N/A

* *UXS1* & *UGE1* (Bar-Peled, M., Griffith, C.L. *et al.* 2001; Moyrand, F., Fontaine, T. *et al.* 2007); *UGE2* Genbank #AAR84604

Table 2

Strains Generated for Study

Strain Name	Strain Number	Marker
PGAL1: <i>ADE2</i> -1	JLCN 578	nourseothricin
PGAL1: <i>ADE2</i> -2	JLCN 584	nourseothricin
PGAL7: <i>ADE2</i> -1	JLCN 587	nourseothricin
PGAL7: <i>ADE2</i> -2	JLCN 589	nourseothricin
PUGE2: <i>ADE2</i> -1	JLCN 594	nourseothricin
PUGE2: <i>ADE2</i> -2	JLCN 596	nourseothricin

Table 3
Average LOG₂ Ratio of Repression and Induction of *ADE2*

Strain Name	Average Glucose Repression in LOG ₂ Ratio	Standard Deviation +/-	Average Galactose Induction in LOG ₂ Ratio	Standard Deviation +/-
PGAL1: <i>ADE2</i> -1	-4.87	2.41	15.48	5.56
PGAL7: <i>ADE2</i> -1	-6.08	3.47	21.59	9.59
PUGE2: <i>ADE2</i> -1	-5.31	2.09	28.06	8.79