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Study of *Cryptococcus neoformans* actin gene regulation with a β-galactosidase-actin fusion

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An expression plasmid carrying a heterologous gene fusion between the *Cryptococcus neoformans* actin promoter and the *Escherichia coli* reporter gene, *LACZ*, was constructed to study actin regulation in *C. neoformans*. Two randomly stable transformants, designated 20·6 and 20·9, were selected for further examination. Both ectopic and homologous recombination with vector insertion in tandem repeats occurred in these transformants. Transformant 20·9 carried more copies of ACTp::LACZ in its genome than 20·6 and this was reflected in expressing higher levels of β-galactosidase activity. *In vitro*, these transformants showed higher levels of β-galactosidase activity expressed when the transformants were propagated at higher temperatures (37 °C vs 30 °C). However, β-galactosidase expression in the transformants was variable during logarithmic and stationary growth phases and this differential expression was temperature dependent. This report shows that the constitutive actin gene in *C. neoformans* is regulated by temperature and growth and this fact should be taken into consideration when actin expression is used as a standard to compare the expression of other regulated genes. Also, a more sensitive reporter construct will be needed for *in vivo* gene analysis of regulation.

Keywords actin gene regulation, *C. neoformans*

Introduction

Cryptococcus neoformans is an encapsulated pathogenic yeast that causes meningitis, particularly in certain immunocompromized patients. The incidence of cryptococcal infections has risen significantly over the past decade primarily due to the higher prevalence of HIV infection. With the recent development in the molecular biology foundation for this yeast combined with its classical genetics and a reasonable understanding of pathophysiology, this fungus has the potential to be a model organism in the study of molecular fungal pathogenesis [1–4]. For instance, several prominent virulence factors which have already been genetically linked with this yeast, such as the polysaccharide capsule [5], melanin production [6,7] and the ability to grow at 37 °C [6] have now been shown to be under genetic control with specific genes identified [5,8–10]. To define the importance of these virulence genes and their regulations will require further molecular manipulation of this yeast. Recently, targeted gene replacement has been successfully employed by homologous recombination in *C. neoformans* through both biolistic [10,11] and electroporation DNA delivery systems [5,8]. Genes which encode for CAP59 [5], CAP60 [12], CAP64 [8], NMT [11] and calcineurin [10] have been shown to be essential for viability of the yeast in mice or the subarachnoid space of immunosuppressed rabbits with site-directed disruptants. It will now be important to understand how and when these and other virulence genes are regulated. The use of expression plasmids carrying homologous or heterologous gene fusions with reporter genes have been essential to understanding gene expressions in many systems. Understanding the regulation of genes in *C. neoformans* would also be helped by the development of fusion reporter systems for this pathogenic yeast. Recently, expression plasmids have been successfully developed using the

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C. neoformans GAL7 inducible promoter with the homologous (*C. neoformans*) virulence-associated gene, *MFa*, and a heterologous *Escherichia coli* reporter gene, *β-glucuronidase*, to express fusion proteins [13]. We have also developed a dominant selection transformation system for *C. neoformans* using the heterologous gene fusion between the actin promoter of *C. neoformans* and the hygromycin B phosphotransferase gene in *E. coli* [14]. These results demonstrate that bacterial fusion genes can be expressed in *C. neoformans* unlike *Candida albicans* in which there are codon usage differences [15] and therefore may require more exotic reporter proteins [16]. In this report, we describe an expression plasmid carrying a heterologous gene fusion using the actin promoter from *C. neoformans* and the well-studied reporter gene, *β-galactosidase*, from *E. coli* to study the regulation of the *C. neoformans* actin gene. We used this construct to show that the actin gene for this organism which is generally considered to be constitutively expressed is, in fact, regulated by both environmental temperature and growth phase of the yeast.

Materials and methods

Organisms and media

Cryptoccocus neoformans strains were the wild-type H99, a serotype A clinical isolate of *C. neoformans* var. *neoformans* [17] and its ade2 auxotroph, M001, which was derived from H99 after UV irradiation [4]. *Escherichia coli* (TG1) was used for plasmid amplification in LB broth (10% tryptone, 5% yeast extract and 5% NaCl, pH 7·5) containing 75 μg of ampicillin ml^{-1}. Both H99 and M001 were maintained on 1% yeast extract, 1% peptone and 2% glucose (YEPD) agar plates at 4 °C.

ACTp::LACZ gene fusion construct and transformations

Transformations were performed by biolistic delivery of DNA [4] into M001 of the ACTp::LACZ heterologous gene fusion construct carrying the ADE2 gene from *C. neoformans* strain 3501 in the SK plasmid (Stratagene). The expression plasmid ACTp::LACZ/ADE2 (Fig. 1) was constructed as follows. The *E. coli* 5′-truncated *LACZ* gene, which was carried in pUC 119, was cloned at the *Bam*HI and *Pst*I restriction sites in the SK plasmid. The *C. neoformans* 3501 ADE2 gene (*pCnADE2ΔApa*) was then inserted downstream from the LACZ sequence at the *Apa*I site and a partially filled-in *Hin*dIII restriction site. The *C. neoformans* H99 actin gene [18] was restricted with *Rsa*I generating an 862 bp fragment extending 847 bp upstream and 15 bp downstream of its ATG start site. This promoter region was ligated in-frame to the 5′-end of

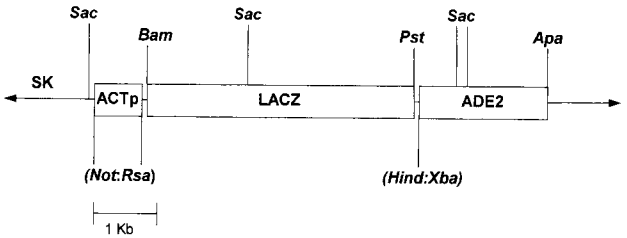

Fig. 1 Map of the ACTp::LACZ/ADE2 construct (ACTp-actin promoter, *LACZ* gene for β-galactosidase, *ADE2* gene for phosphoaminocarboxylase); (all restriction sites are *I* except *Hin*dIII and *Bam*HI).

LACZ that had been filled-in after digestion with *Not*I. DNA sequencing of this fusion area was performed by the dideoxy-mediated chain termination method with a Sequenase version 2.0 kit (US Biochemical).

Analysis of fusion construct integrations

Genomic DNA from transformants, 20·6 and 20·9, and wild-type H99 was isolated as described by Toffaletti *et al.* [4]. One μg of DNA from each strain was digested with *Sac*I and electrophoresed on a 0·7% agarose gel. The gel was processed as described by Reed & Mann [19] and the DNA transferred to a Nytran membrane (Schleicher & Schuell). The DNA was crosslinked to the membrane by UV irradiation (Stratalinker, Stratagene) and hybridizations carried out at 65 °C in 6 × SSC (0·9 M NaCl, 0·09 M sodium citrate) and 1% sodium dodecyl sulphate (SDS). The blots were washed three times in 0·2 × SSC/0·1% SDS at 65 °C for 45 min. Autoradiography was performed with Kodak-XAR film.

A 5·7 kb *Hin*dIII-*Pst*I restriction fragment of the *LACZ* gene and the actin promoter on an 862 bp *Rsa*I restriction fragment were labelled with [^{32}P]dCTP (New England Nuclear) using the random primer labelling kit (Gibco, BRL). Quantitative assessment of ACTp::LACZ integrative events was carried out in a phosphor imager and the bands analysed using Image Quant software. A single copy of actin was determined by the c.p.m. in the band(s) representing the endogenous actin gene. For the exogenous LACZ copy, a single copy was determined by the c.p.m. representing the 5′ end of the integrating ACTp::LACZ/ADE2 construct. The total copies in each transformant were determined by adding the c.p.m. in each fragment that hybridized to both the actin and LACZ probes and dividing that sum by c.p.m. in the single copy controls.

Karyotyping of the transformants, 20·6 and 20·9 and wild-type H99 was performed according to Perfect *et al.* [20].

β-galactosidase assays

β-galactosidase activity in the cell extracts of the transformants was assessed by using the chromogenic substrate, chlorophenol-β-D-galactopyranoside (CPRG). For screening these transformants, a loopful of cells from a 2–3 day growth of yeasts on YEPD agar was washed in phosphate buffered saline (PBS), pH 7·4, and resuspended in 1·0 ml of Z buffer (0·06 M Na_2HPO_4, 0·04 M NaH_2PO_4, 0·01 M KCl, 0·001 M $MgSO_4$, 0·76% SDS and 0·05 M 2-ME). After adding 0·3–0·4 g of glass beads (0·45 μm diameter), the cells were vortexed in a mini-bead beater (Biospec Products) for 30 s, placed on ice for 1 min and this cycle was repeated three times. The cells were pelleted for 10 min at 4 °C and 0·5 ml of the extract was transferred to a clean tube. After adding 0·1 ml of 24 mM CPRG to the extract, the mixture was allowed to incubate at 30 °C for 30 min and then placed on ice for 15 min. The presence of β-galactosidase, which cleaves CPRG to give a red soluble dye, was assessed by the appearance of a red colour and/or its absorbance at 595 nm. For quantitative assessment of β-galactosidase activity, protein concentration of the cell extract was determined (BioRad) and 200 μg or 400 μg of the protein extract was assessed for β-galactosidase activity. This activity was then calculated in Miller units [21]. A minimum of 2×10^8 cells harvested from broth or agar was needed for this assay.

β-galactosidase activity was also detected *in situ* within the transformed colonies by using the substrate 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal). Colonies were transferred from YEPD agar to a nitrocellulose membrane. The membrane was then frozen in liquid nitrogen for 10 min, placed face-up onto 3 mm paper saturated with Z buffer/0·2%X-gal and allowed to incubate at 37 °C for 1–4 h. β-galactosidase was detected by the development of a blue colour within the colony.

In vitro *expression*

Two randomly selected transformants, 20·6 and 20·9, were examined for the ability to express β-galactosidase activity under different growth phase and temperature conditions. Cells from a stationary phase culture were inoculated into YEPD broth at a final density of $0{\cdot}5 \times 10^6$ to 2×10^6 cells ml^{-1} and grown in a shaking incubator at both 30 °C and 37 °C. Cells were harvested from the logarithmic (2×10^7 to $6{\cdot}5 \times 10^7$ cells ml^{-1}) and stationary (1×10^8 to $2{\cdot}5 \times 10^8$ cells ml^{-1}) growth phases and assessed for β-galactosidase activity. β-galactosidase activity was also assessed from colonies grown on YEPD agar at both 30 and 37 °C.

In vivo *expression*

Transformant 20·6 was examined for the ability to express β-galactosidase in the subarachnoid space of immunosuppressed rabbits. Organisms were grown on YEPD agar for 72 h at 30 °C, collected on cotton swabs, and suspended in PBS at a concentration of 5×10^8 cells ml^{-1}. Approximately $2{\cdot}5 \times 10^8$ viable cells in a volume of 0·5 ml were inoculated intrathecally into two New Zealand white male rabbits that received an intramuscular injection of cortisone acetate at 2·5 mg kg^{-1} (Merck, Sharp & Dohme, West Point, PA) 24 h earlier and then daily for 7 days. Expression of β-galactosidase was monitored for 7 days by withdrawing 1 ml of cerebrospinal fluid (CSF) from each rabbit at 1, 4 and 7 days after inoculation and directly assessing the yeast cells and CSF for β-galactosidase activity. Quantitative yeast counts were determined by plating 0·1 ml of undiluted CSF and serially diluted aliquots on YEPD agar.

For assessment of β-galactosidase in yeast cells and CSF of infected rabbits, the yeast cells were first pelleted and the CSF transferred to a clean tube. The yeast cells were washed in PBS and resuspended in Z buffer before cellular extraction. Since the number of yeast cells recovered from the CSF was $\leq 10^7$, only qualitative analysis of β-galactosidase activity was performed. The CSF sample was mixed with 10 × Z buffer and then assessed qualitatively for the presence of β-galactosidase.

Statistical analysis

A *t*-test for unpaired means was used to compare two variables.

Results

Biolistic transformations with the ACTp::LACZ/ADE2 constructs

Because of the bidirectional cloning of the actin promoter four separate ACTp::LACZ/ADE2 constructs were used to transform M001 to ade prototrophy. Cell extracts of five to eleven stable adenine-positive colonies generated from each construct were screened for β-galactosidase activity. All four ACTp::LACZ/ADE2 constructs generated transformants that expressed β-galactosidade activity; however, subsequent sequence analysis of the area between the actin promoter and the LACZ gene revealed that only two of the plasmid constructs (nos 20 and 51) actually carried the actin promoter in the correct orientiation for LACZ expression. Of the 16 stable adenine transformants generated with the in-frame plasmid constructs, 14 transformants possessed β-galactosidase activity in their cell extracts.

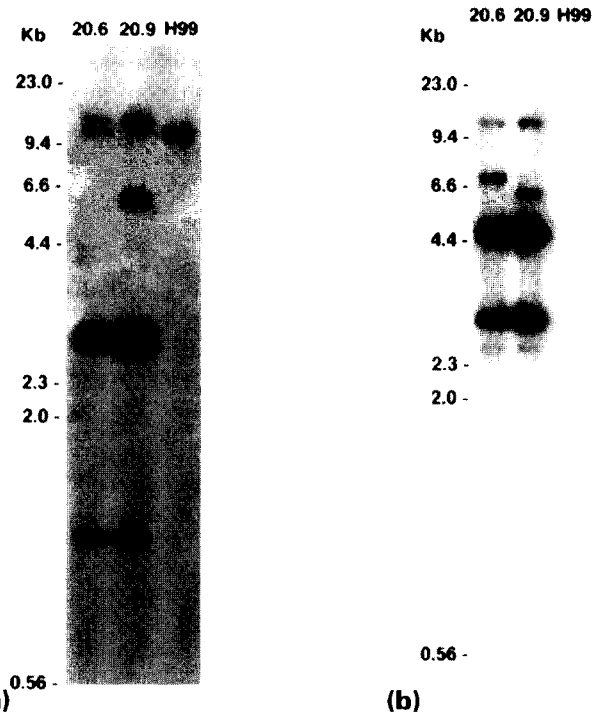

Fig. 2 Southern blot analysis of *Sac*I-restricted genomic DNA from two transformants, 20·6 and 20·9, and wild-type H99. (a) probed with a [32]P-labelled *Rsa*I 862 bp restriction fragment of the actin promoter region (left); (b) probed with a [32]P-labelled *Hin*dIII-*Pst*I 5·7 kb restriction fragment of the *LACZ* gene (right).

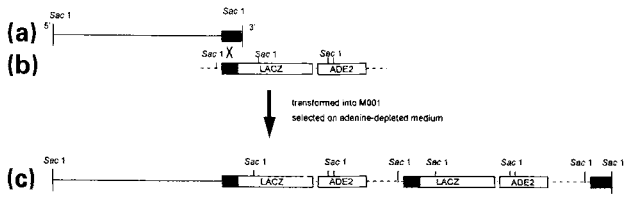

Fig. 3 Homologous integration at the actin allele resulting in the addition of the ACTp::LACZ/ADE2 construct. (a) map of a 12 kb genomic fragment restricted with *Sac*I and carrying the 3′ truncated actin gene. Black box, actin promoter (862 bp); gray box, 5′ end (407 bp) of the actin gene. (b) map of ACTp::LACZ/ADE2 transforming DNA: actin promoter (862 bp), LACZ (5·7 kb) and ADE2 (3·0 kb). (c) *C. neoformans* M001 (*ade2*) was transformed with undigested ACTp::LACZ/ADE2 and cells were selected on adenine-depleted medium. The single cross-over event is diagrammed.

In contrast, only five of 16 stable adenine transformants carrying the non-functional plasmid constructs possessed β-galactosidase in their cell extracts. β-galactosidase activity was also detected *in situ* within the transformed colonies. No detectable β-galactosidase activity was found in either cell extracts or *in situ* colonies from wild-type H99.

Southern blot analysis

Both undigested and *Sac*I digested genomic DNAs from the two stable transformants, 20·6 and 20·9, were examined by Southern blot analysis. The undigested genomic DNA revealed single high molecular weight DNA hybridizing to the LACZ probe. There was no evidence of intact plasmid DNA suggesting that both transformants carried only integrated copies of the *LACZ* gene (data not shown). The *Sac*I digested genomic DNA from the wild-type H99 strain showed the endogenous actin gene on a 12 kb fragment (Fig. 2A, lane 3). As a previous study has shown that the actin gene is 1371 bp in length with a *Sac*I site located 423 bp downstream from the ATG start site [18], only the 5′ end of the actin gene is present on this 12 kb fragment (Fig. 3A). In transformant 20·9, the endogenous actin gene was displaced suggesting homologous recombination with vector insertion. As a *Sac*I site is present in both the vector and LACZ DNAs flanking the actin promoter, homologous recombination at the site of the endogenous actin allele by a single cross-over event would produce two novel fragments of 14 kb and 1·3 kb containing the 5′ and 3′ ends of the 3′-truncated endogenous actin gene, respectively. This novel band pattern hybridizing to the actin probe was observed in transformant 20·9 (Fig. 2A, lane 2). The difference in the signal intensity between the 1·3 kb and 14 kb fragments likely correlates with the percentage of that fragment that actually hybridized to the 862 bp actin probe (66%, 1·3 kb; 6%, 14 kb). Also generated were a 3·1 kb fragment hybridizing to both the actin and LACZ probes and a 4·6 kb fragment hybridizing to only the LACZ probe, which are consistent with insertion of two or more repeats of the ACTp::LACZ/ADE2 construct (Fig. 2A and B; lane 2). Southern analysis of transformant 20·6 revealed a similar band pattern (14 kb, 4·6 kb, 3·1 kb and 1·3 kb fragments) suggesting homologous recombination by a single cross-over event with vector addition (Fig. 2A and 2B, lane 1). However, a light band hybridizing to the actin probe was still present on a 12 kb fragment suggesting the endogenous actin gene is still intact in some transformed cells (Fig. 2A, lane 1). To clarify the homologous integrative event occurring in these transformants a diagram is also shown (Fig. 3). Other novel bands identified by the LACZ probe occurred in both transformants implicating ectopic integration (7·2 kb and 2·6 kb). However, hybridization to both probes was present on a 6·4 kb fragment in transformant 20·9 (Fig. 2A and 2B, lane 2).

Karyotype blots of two transformants were probed with the *LACZ* gene and showed that the majority of LACZ integrative events occurred on the largest chromosome of H99 that was previously shown to carry the actin

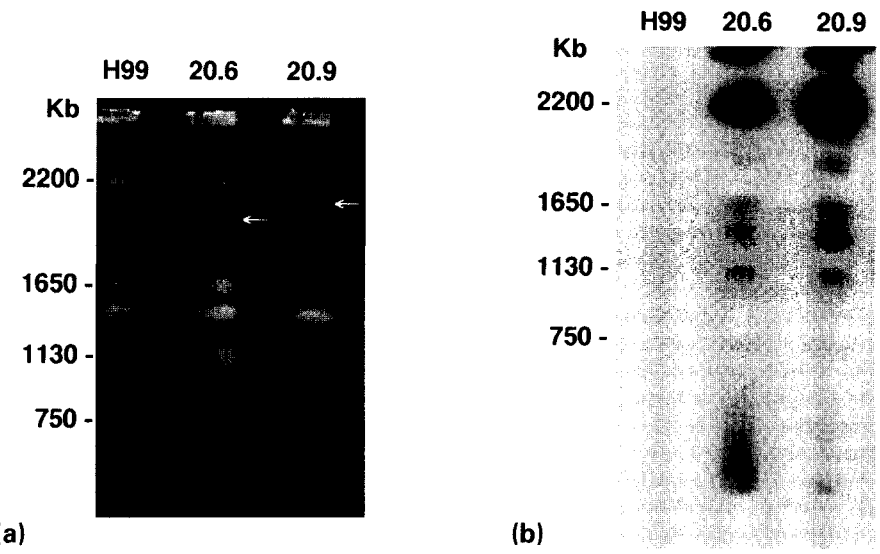

Fig. 4 (a) Ethidium bromide staining of chromosomal DNA from wild-type H99 and two transformants, 20·6 and 20·9 (left). (b) chromosomal DNA was transferred to a Nytran membrane and probed with a $^{[32]}$P-labelled *Hin*dIII-*Pst*I 5·7 kb restriction fragment of the *LACZ* gene (right).

gene (2200 kb) [18] (Fig. 4A and B). In addition, the probe appeared to identify ectopic integration of LACZ on a novel chromosome (2100 kb) in transformant 20·9 (arrow). The lighter bands which identify smaller chromosomes most likely represent degraded DNA from the larger chromosomes and do not represent a construct insert on those chromosomes. A novel sized chromosome of 1900 kb was also present in transformant 20·6 (Fig. 4A, arrow).

Because different levels of β-galactosidase activity were consistently detected between each transformant, the number of ACTp::LACZ integrative events was determined to identify whether the number of copies could be detected by the fusion expression. Transformant 20·9 carried 11 copies of ACTp::LACZ compared with approximately six to seven copies in transformant 20·6. This finding of different copies of genes correlated with their differences noted in production of β-galactosidase.

Expression of the actin promoter is temperature and growth-phase dependent

To determine expression of the actin promoter under different growth phases and temperature conditions the β-galactosidase activity of the two stable transformants, 20·6 and 20·9, was measured. Both transformants expressed significantly high levels of β-galactosidase activity when propagated at 37 °C, compared with 30 °C, in YEPD broth (four to ten-fold) and agar (two-fold) ($P < 0{\cdot}001$) (Table 1). Transformant 20·9 expressed two-fold higher levels of β-galactosidase than transformant 20·6 ($P < 0{\cdot}02$) when grown on YEPD agar at either temperature which correlated with increased number of fusion copies in this transformant. Levels of

Table 1 Expression of the actin promoter under different temperatures and growth phases

		β-galactosidase activity (Miller units)*	
Growth conditions	Strain	30 °C	37 °C
Log (broth)	20·9	39·7 ± 1·7	158·0 ± 4·8
Stationary (broth)	20·9	20·7 ± 3·5	205·0 ± 11·7
Agar	20·9	50·4 ± 4·3	109·2 ± 5·6
Agar	20·6	25·5 ± 1·7	47·1 ± 2·8

*Mean ± SE of two to five experiments (duplicate samples per experiment).

β-galactosidase expressed in the yeast cells varied during the growth phase and this differential expression was also temperature dependent. At 30 °C logarithmic growth cells (20·9) expressed higher levels of β-galactosidase activity than stationary growth cells ($P < 0{\cdot}01$); conversely, when the cells were grown at 37 °C higher levels of β-galactosidase activity were expressed in stationary growth cells compared with logarithmic growth cells ($P < 0{\cdot}01$). Wild-type H99 cells showed no detectable β-galactosidase activity under these growth conditions.

Expression of β-galactosidase in vivo

Transformant 20·6 was inoculated intrathecally into two cortisone-treated rabbits and then monitored for the presence of β-galactosidase activity throughout a 7-day period. β-galactosidase was detected qualitatively in the pool of yeast cells recovered in only one rabbit on day 1 of infection but there was no β-galactosidase activity

found in the CSF of either rabbit. No β-galactosidase activity was detected in either yeast cells or CSF from either rabbit at day 3 or 7 of the infection. Yeast counts in rabbits were 10^6 to 10^7 cells ml^{-1} at day 1 of infection and dropped to 10^5 and 10^4 cells ml^{-1} by day 4 and 7 of the infection, respectively. One isolated colony from the subarachnoid space at day 7 of infection was propagated on YEPD agar at 30 °C. Similar levels of β-galactosidase activity were detected in the cell extracts from this colony as were found with the preinoculated transformant (data not shown).

Discussion

Actin is an essential structural protein found in all eucaryotic cells. In the vast majority of higher plants and organisms studied several actin genes are present. However, in most fungi such as *C. neoformans* [18], *Saccharomyces cerevisiae* [22] and *C. albicans* [23] only a single actin gene exists. Actin expression is considered to be constitutive, being expressed at basal levels under normal growth conditions. However, in many systems actin expression is also regulated by environmental, growth-phase and tissue specific factors. This differential expression of the actin gene is often regulated by an array of proteins that bind to specific upstream activating sequences (UAS) in the promoter region of the gene. For instance, insulin and phorbol esters, by interacting with a common regulatory signal, enhance expression of the cytoskeletal gamma and beta actins in rat hepatoma cells [24,25]. Similarly, other factors such as temperature [15,26], growth phase and nutrition [27] regulate the level of actin transcripts in *C. albicans*.

In the present report, we have shown that the actin gene in *C. neoformans* is also differentially expressed by using the *E. coli LACZ* gene as a reporter gene fused to the *C. neoformans* actin promoter. Higher levels of β-galactosidase activity were expressed in two randomly selected transformants when the cells were propagated at 37 °C rather than at 30 °C. Our studies complement similar studies linking higher temperature to increased actin expression in the yeast pathogen, *C. albicans*. Paranjape & Datta [26] demonstrated that the increased actin transcript levels occurred during germ tube formation induced by *n*-acetyl-D-glucosamine at 37 °C but were inhibited when the cells were incubated at 25 °C. Other investigators have shown that the expression of the reporter gene *LAC4* from *Kluyveromyces lactis*, when driven by the actin promoter in *C. albicans*, is enhanced during hyphal induction at 37 °C but is not at 30 °C [15]. It is clear that temperature regulates this promoter in *Candida* and *Cryptococcus* but the mechanism is not certain and could be a feature of the regulated fungal stress response during infection. For example, whether heat-shock transcription factors (HSTFs) exist or even play a role in actin upregulation in *C. neoformans* or *C. albicans* have not been reported. However, in the yeast *S. cerevisiae* a HSTF has recently been described and appears to have the same size and DNA binding properties as the well-studied HSTFs in *Drosophilia* [28]. The occurrence of a HSTF in *S. cerevisiae* and its similarity to other HSTFs suggest their possible presence in other yeast. Experiments designed to delineate specific promoter sequences that bind to HSTFs in *S. cerevisiae* might help predict whether promoters such as the actin promoter are targets for heat-shock activation in this yeast and possibly other fungi.

Higher levels of β-galactosidase were also observed in *C. neoformans* during logarithmic growth compared to stationary growth but only when the cells were grown at 30 °C. This observation of actin upregulation during logarithmic growth has also been reported in *C. albicans* and *S. cerevisiae* using Northern blot analysis to measure the level of actin transcripts [27]. However, lower levels of β-galactosidase were expressed in logarithmic *C. neoformans* cells propagated at pathological temperatures such as 37 °C. This variability in β-galactosidase expression under different growth phase and temperature conditions is interesting and it is not clear whether it may have pathological relevance. In the amoeba *Dictyostelium* regulatory sequences have been identified in the promoter region of the actin 15 and 6 genes that control the growth-phase expression of actin [29]. Similar regulatory sequences may also be present in the *C. neoformans* actin gene that controls actin expression during growth of this pathogenic yeast.

The higher level of β-galactosidase expression in transformant 20·9 most likely reflects a higher number of ACTp::LACZ copies integrated into the genome (11 vs 6–7), although a position effect cannot be excluded, and confirms that multiple constructs are functioning in these transformants. In both transformants the majority of copies are in tandem repeats at the site of the endogenous actin gene, but both Southerns and karyotyping show that ectopic integration does occur and will likely be a feature of integrative fusion constructs in *C. neoformans*. This factor will need to be considered when regulation studies are performed in this yeast. Furthermore, integrative events can also occur at cryptic promoter sites within the cryptococcal genome. These promoters can then drive the expression of integrated non-functional genes as was observed in this study with the detection of β-galactosidase in transformants harboring the non-functional expression plasmid. It is also noteworthy that the process of *in vitro* transformation

can change chromosome sizes in *C. neoformans*. This genome plasticity of karyotype has also been found under stress of infection [30] and it has epidemiological implications [31] but it is uncertain whether it will have pathological influences.

Although the β-galactosidase fusion with a cryptococcal promoter can be used to study gene regulation as reported in this study, there are limitations to this construct. The ability of the *C. neoformans* actin promoter to drive expression of β-galactosidase was used for simple qualitative analysis of actin regulation. β-galactosidase was detected either in cell extracts or *in situ* from each stable LACZ transformant examined. However, for quantitative assessment of β-galactosidase activity at least 200 μg of protein 0·5 ml^{-1} from a cell extract was needed for this assay and this represents approximately 2×10^8 cells. This requirement for such a large number of cells precludes the use of this construct to directly quantitate β-galactosidase expression in cells harvested from the CSF. Although we used an inoculum size of $2{\cdot}5 \times 10^8$ cells, the CSF contained only 10^7 cells ml^{-1} one day after inoculation and the number of viable organisms decreased to 10^4 cells ml^{-1} in the CSF by day 7 of the infection. For *in vivo* work, it may be necessary to use more sensitive reporter systems such as the green fluorescent protein with FACS analysis of individual cells for detection of promoter expression and its regulation.

This study demonstrates that the *E. coli* reporter gene, *LACZ*, can be expressed in *C. neoformans* by fusion with the cryptococcal actin promoter. Although Wickes & Edman [13] have reported background levels of β-galactosidase in control JEC43 *C. neoformans* cells, we have found no appreciable β-galactosidase background levels in our H99 strain. Even though expression of the actin gene is considered constitutive, it can actually be upregulated by temperature and growth phase in this pathogenic yeast and this feature will need to be considered if actin is used as a standard for expression studies of other genes. It also appears that actin expression is upregulated at elevated temperatures and slow growth. These physiological conditions are similar to the pathological environment of the host and the response could be part of a generalized stress response to a hostile environment for the invading fungus.

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