

Expression levels of a filament-specific transcriptional regulator are sufficient to determine *Candida albicans* morphology and virulence

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Candida albicans, the major human fungal pathogen, undergoes a reversible morphological transition from single yeast cells to pseudohyphal and hyphal filaments (elongated cells attached end-to-end). Because typical *C. albicans* infections contain a mixture of these morphologies it has, for many years, been difficult to assess the relative contribution of each form to virulence. In addition, the regulatory mechanisms that determine growth in pseudohyphal and hyphal morphologies are largely unknown. To address these questions we have generated a *C. albicans* strain that can be genetically manipulated to grow completely in the hyphal form under non-filament-inducing conditions *in vitro*. This was achieved by inducing high-level constitutive expression of *UME6*, a recently identified filament-specific transcriptional regulator of *C. albicans* hyphal extension. We show that high-level *UME6* expression significantly increases hyphal formation and promotes virulence in a mouse model of systemic candidiasis. Our results strongly suggest that shifting the morphology of a *C. albicans* population toward the hyphal form, and/or increasing hyphal-specific gene expression, during the course of infection is sufficient to improve virulence potential. We also demonstrate that lower levels of *UME6* expression specify growth largely in the pseudohyphal form and that increasing *UME6* levels is sufficient to cause cells to gradually shift from pseudohyphal to hyphal morphology. In addition, we show that *UME6* levels differentially induce the expression of several known filament-specific transcripts. These findings suggest that a common transcriptional regulatory mechanism functions to specify both pseudohyphal and hyphal morphologies in a dosage-dependent manner.

filamentous growth | transcriptional regulation

Candida albicans is the most important human fungal pathogen because of its frequency of isolation and the amount of morbidity and mortality it causes. Although normally found as a commensal in the human gastrointestinal tract, *C. albicans* is responsible for a wide variety of mucosal infections such as oral and vaginal thrush. *C. albicans* also infects every organ and tissue in the human body (1–3). Approximately 70% of all women will experience at least one episode of vaginal candidiasis during their lifetime (4), and systemic candidiasis is now the fourth-leading cause of hospital-acquired bloodstream infections in the United States, with a mortality rate approaching 35% (5). AIDS patients, organ transplant recipients, cancer patients on chemotherapy, recipients of artificial joints and prosthetic devices, and other immunocompromised individuals are particularly susceptible to infection (for reviews see refs. 2, 3, and 6).

One property known to contribute to *C. albicans* virulence is the ability to undergo a morphological transition from yeast (single, oval budding cells) to filaments (elongated cells attached end-to-end) (for reviews see refs. 7 and 8). This transition is known to occur in response to a variety of inducing signals present in the host environment, including serum, body temper-

ature (37 °C), neutral pH, amino acids, and certain human hormones (7–9). *C. albicans* filaments can be found in two distinct morphologies: pseudohyphae and hyphae. Pseudohyphal cells are highly branched, ellipsoidal in form, and have constrictions at cell junctions whereas hyphal cells are less branched, have parallel sides, and lack constrictions at the septa. Other differences include the way nuclear division occurs, the cell cycle, and the presence of a specialized structure, the Spitzenkörper, which is important for concentrating and delivering secretory vesicles to the tip of hyphal cells (1, 10). Because of the extensive phenotypic differences between hyphal and pseudohyphal cells it is generally believed that expression of different gene sets specifies each morphology, although definitive evidence is lacking.

Despite the fact that the phenotypes of *C. albicans* hyphal and pseudohyphal cells have been well-characterized, considerably less is known about the genetic determinants for growth in each form or how these forms differ at the molecular level. In addition, although considerable evidence suggests that the ability of *C. albicans* to undergo the transition from yeast to filaments is required for virulence (11, 12), the relative contribution of pseudohyphal and hyphal morphologies to virulence and the specific role that each morphology plays in this process has remained elusive because tissues infected with *C. albicans* typically contain a mixed population of yeast, pseudohyphae, and hyphae (1).

Here we report the generation of a *C. albicans* strain that can be genetically manipulated to grow completely in the hyphal morphology in the absence of filament-inducing conditions *in vitro* and to show significantly increased hyphal formation and extension during infection *in vivo*. This strain provides us with a powerful tool to address many of the questions described above, including the specific role of the *C. albicans* hyphal form in virulence and the regulatory mechanisms that function to determine *C. albicans* morphology.

Results

Constitutive High-Level *UME6* Expression Is Sufficient to Drive Complete Hyphal Formation in the Absence of Filament-Inducing Conditions. We have recently identified a filament-specific transcriptional regulator of *C. albicans* hyphal extension and virulence,

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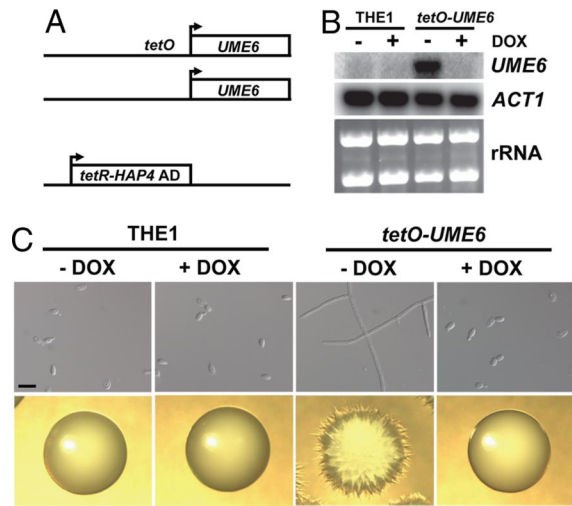


Fig. 1. High-level constitutive *UME6* expression drives complete hyphal formation. (A) One *UME6* allele was placed under control of the *E. coli tet* operator (*tetO*) in strain THE1 (14), which expresses an *E. coli tet* repressor–*S. cerevisiae* Hap4 activation domain fusion protein (*tetR-HAP4 AD*). In the absence of Dox (a tetracycline derivative) this fusion protein dimerizes at the *tet* operator and directs constitutive high-level transcriptional activation of *UME6*. In the presence of Dox, the fusion protein fails to dimerize and this allele of *UME6* is no longer activated. (B) Northern analysis of *UME6* and *ACT1* (control) transcript levels in both THE1 (parent) and *tetO-UME6* strains grown in YEPD medium (non-filament-inducing conditions) at 30 °C in the presence and absence of 40 $\mu\text{g}/\text{mL}$ Dox. (C) Images of cells used to prepare RNA for the Northern in B and of colonies of the THE1 and *tetO-UME6* strains grown in the presence and absence of 40 $\mu\text{g}/\text{mL}$ Dox in YEPD medium at 30 °C for 2 days. (Scale bar: 10 μm .)

UME6, which controls the level and duration of filament-specific gene expression in response to inducing conditions (13). To determine the effect of high-level *UME6* expression on *C. albicans* morphology, one allele of *UME6* was placed under control of a regulatable promoter consisting of the *Escherichia coli tet* operator [we chose not to regulate *UME6* in a strain in which the other allele was absent because a homozygous *ume6* deletion strain is attenuated for virulence whereas a strain expressing one functional copy of *UME6* behaves nearly identical to a WT strain with respect to virulence (13)]. This experiment was carried out in a strain expressing high constitutive levels of an *E. coli tet* repressor–*Saccharomyces cerevisiae* Hap4 activation domain fusion protein (*tetR-Hap4 AD*) (Fig. 1A). In the absence of doxycycline (Dox, a tetracycline derivative), this fusion protein binds to the *tet* operator as a dimer and directs constitutive high levels of transcriptional activation. In the presence of Dox, dimerization is blocked, preventing DNA binding and effectively shutting off the transcription factor. This system has previously been used successfully by several groups to induce constitutive high-level gene expression in *C. albicans* both in vitro and during infection in vivo (12, 14).

In the absence of Dox, the strain containing one allele of *UME6* under control of the *tet* operator (*tetO-UME6*) showed a highly filamentous colony morphology when grown under non-filament-inducing conditions (YEPD at 30 °C) (Fig. 1C). These colonies were also highly adherent to the agar. Strikingly, microscopic examination of a liquid YEPD culture revealed that virtually all cells from this strain appeared to grow in hyphal form. In contrast, when the *tetO-UME6* strain was grown in the presence of 40 $\mu\text{g}/\text{mL}$ Dox, or when the parent strain (only expressing the *tetR-Hap4 AD* fusion protein) was grown in either the presence or the absence of Dox, cells were in the yeast form (Fig. 1C). Northern analysis confirmed that the *UME6* transcript was expressed at high levels specifically in the *tetO-UME6* strain

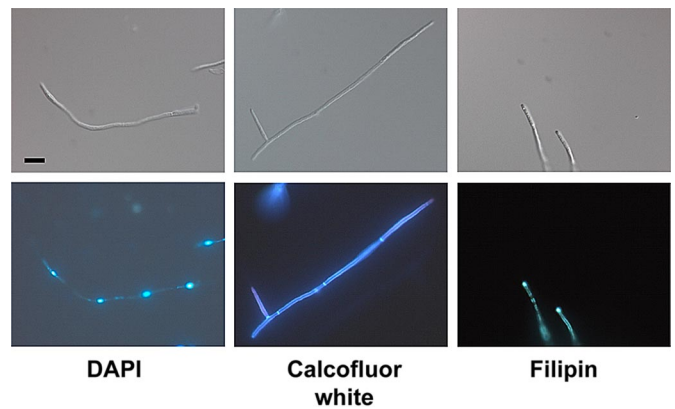


Fig. 2. Microscopic examination of filaments generated by high-level constitutive *UME6* expression. The *tetO-UME6* strain was grown in the absence of Dox as described in Fig. 1, and aliquots of cells were fixed, stained with 1 $\mu\text{g}/\text{mL}$ DAPI or 10 $\mu\text{g}/\text{mL}$ Calcofluor white, incubated for 10 min at room temperature, washed 3 times in 1 \times PBS, and analyzed by fluorescence microscopy. For filipin staining, cells were grown in a similar manner (but not fixed), stained with 8 $\mu\text{g}/\text{mL}$ filipin for 20 min at room temperature, washed once with 1 \times PBS, and analyzed by fluorescence microscopy. (Scale bar: 10 μm .)

grown in the absence of Dox (Fig. 1B). Similar results were obtained when these experiments were repeated with a 20 $\mu\text{g}/\text{mL}$ Dox concentration.

To confirm that the extended filaments generated by constitutive high-level *UME6* expression were indeed hyphae, cells of the *tetO-UME6* strain grown in the absence of Dox were stained with DAPI, calcofluor white, and filipin (Fig. 2). DAPI staining indicated that each filament was comprised of multiple cells, each with its own nucleus (rather than one long extended cell). Calcofluor white staining showed that all cells in each filament were attached by true septal junctions (lacking constrictions). Finally, all cells stained positive for filipin at the filament tip; filipin has been shown to stain sterols that specifically concentrate at the tip of hyphal, but not pseudohyphal, cells (15). Taken together, our results indicate that high-level constitutive *UME6* expression is sufficient to drive complete hyphal formation in the absence of filament-inducing conditions. We also note that in the absence of Dox the *tetO-UME6* strain grew as complete hyphae in the presence of 10% serum at 37 °C, which is a strong filament-inducing condition (P.L.C. and D.K., unpublished observations).

High-Level Constitutive *UME6* Expression Is Sufficient to Promote Virulence in a Mouse Model of Systemic Candidiasis. One advantage of the tetracycline-regulatable gene expression system described above is that it can be used to control gene expression during infection in a mouse model of systemic candidiasis by the addition or removal of Dox in the drinking water (12, 14). To determine the effect of constitutive high-level *UME6* expression on virulence, 16 mice were each injected with the *tetO-UME6* strain. Three days prior to infection, 8 of these mice were placed on drinking water containing Dox; the remaining 8 mice were placed on drinking water lacking Dox. Strikingly, we found that whereas only 3 mice placed on drinking water with Dox died after 30 days, nearly all of the mice on drinking water lacking Dox died by 11 days post-infection (Fig. 3A); we have also observed that virulence of a control strain that lacks the *tet* operator is not affected by the presence or absence of Dox (data not shown). These results strongly suggest that constitutive high-level *UME6* expression promotes virulence in a mouse model of systemic candidiasis. It is important to note that increased virulence is observed relative to that of the *tetO-UME6* strain in the presence of Dox, which expresses one functional copy of *UME6*. However,

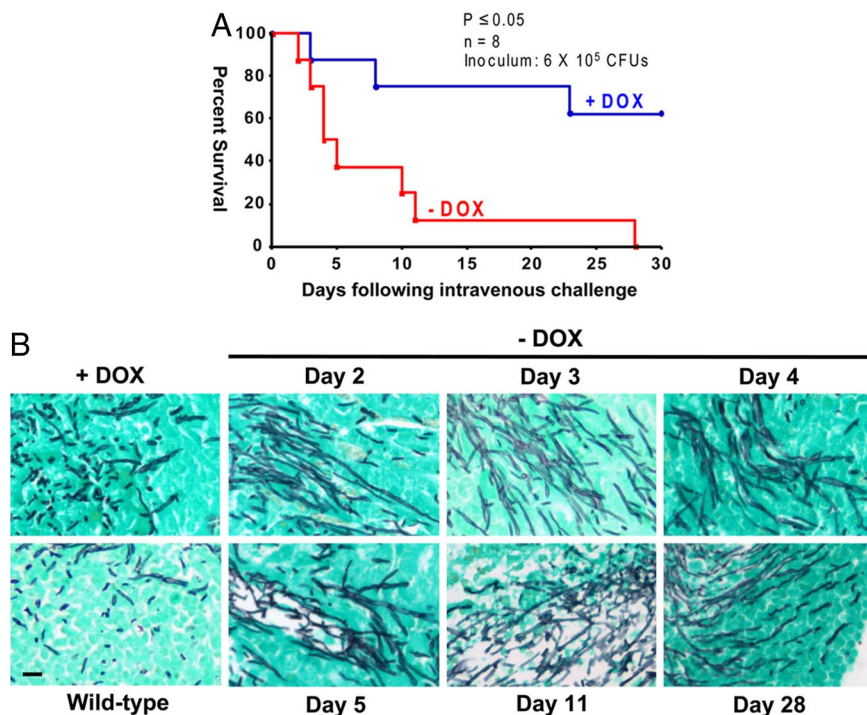


Fig. 3. High-level constitutive *UME6* expression is sufficient to promote virulence and tissue invasion. (A) Sixteen female BALB/c mice (6–8 weeks old) were injected by tail vein with 6×10^5 CFUs of the *tetO-UME6* strain grown in the presence of Dox. Three days prior to infection one group of mice ($n = 8$) was placed on drinking water containing 2 mg/mL Dox (+DOX) while a second group of mice ($n = 8$) was placed on drinking water lacking Dox (–DOX). Survival was monitored over the course of 30 days. A Kaplan–Meier test was performed to confirm that the difference in virulence between +DOX and –DOX groups is statistically significant ($P \leq 0.05$). (B) Kidney tissues from mice infected with the *tetO-UME6* strain (+DOX and –DOX) were fixed, sectioned, and stained with Grocott–Gomori methenamine silver to visualize fungal cells (“days” indicate postinfection time points); a typical kidney section from a mouse infected with a WT strain is shown as a reference. Fungal cells are indicated in black. (Scale bar: 10 μ m.)

we have previously demonstrated that a strain expressing one functional copy of *UME6* does not show a significant difference in virulence when compared with a WT strain (13).

We also carried out histology studies to determine the effect of constitutive high-level *UME6* expression on *C. albicans* morphology and tissue invasion during infection. Kidneys from mice infected with the *tetO-UME6* strain were fixed, sectioned, and stained with Grocott–Gomori methenamine silver to visualize fungal cells (Fig. 3B). Interestingly, we observed significantly increased *C. albicans* hyphal formation, hyphal extension, and tissue invasion in kidneys from mice placed on drinking water lacking Dox. In contrast, kidneys of mice placed on drinking water containing Dox showed a more typical heterogeneous mixed population of yeast, pseudohyphae, and hyphae comparable to that observed in kidneys of mice infected with a WT strain (please note that although only one *tetO-UME6* +Dox time point is shown in Fig. 3B, we have observed a similar morphological pattern at other postinfection time points). Importantly, constitutive high-level *UME6* expression was sufficient to drive increased hyphal formation and extension generally over the course of the entire infection (Fig. 3B), suggesting that a morphological shift of the *C. albicans* population toward the hyphal morphology, and/or increased hyphal-specific gene expression, plays a specific important role in promoting virulence.

It is important to note that inoculations for this experiment were carried out using yeast form cells that had been grown and washed in the presence of Dox. Because yeast cells have been shown to play an important role in dissemination and, under physiological conditions, are more likely to be the infectious inocula from sources such as biofilms in the gastrointestinal tract or other mucosal surfaces, we believe that our experiment more closely parallels an actual infection. We did not carry out our

inoculations using complete hyphae because we found that it is impossible to accurately count these cells and obtain a correct inoculum size [since hyphal cells are extremely clumpy and, unlike a previous study, which involved injection of constitutively pseudohyphal cells (16), septal constrictions were absent].

Expression Levels of *UME6* Are Sufficient to Determine *C. albicans* Morphology. The experiments above describe the effect of constitutive high-level *UME6* expression on *C. albicans* morphology. To determine the effects of low and intermediate levels of *UME6* we grew cells of the *tetO-UME6* strain under non-filament-inducing conditions (YEPD medium at 30 °C) at various Dox concentrations. As shown in Fig. 4A, *UME6* expression first becomes apparent at 0.2 μ g/mL Dox and gradually rises with decreasing Dox concentration. Interestingly, low *UME6* expression levels (at 0.2 and 0.1 μ g/mL Dox) generated a population in which the large majority ($\approx 80\%$) of *C. albicans* cells grew in the pseudohyphal form (Fig. 4B); most of the remaining $\approx 20\%$ of cells grew as yeast. As the Dox concentration decreased and *UME6* levels rose, we observed that a large fraction of the population began to grow in the hyphal form and that the ratio of cells in hyphal vs. pseudohyphal morphologies increased (several hybrid hyphal–pseudohyphal filaments were also apparent). Finally, as previously observed, in the absence of Dox cells grew completely in the hyphal form. These results indicate that, in the absence of filament-inducing conditions, expression levels of *UME6* alone are sufficient to determine *C. albicans* morphology.

Next we sought to determine the effect of increasing *UME6* levels on morphology of the *tetO-UME6* strain over a time course. As shown in Fig. 4C, removal of Dox from a culture of the *tetO-UME6* strain grown in the presence of Dox causes cells to gradually shift morphology over time. Cells initially grow as

4 B–D) and that *UME6* controls the level and duration of filament-specific gene expression in response to inducing conditions (13).

Discussion

Role of the *C. albicans* Hyphal Morphology in Virulence. Because most of the major human systemic fungal pathogens are dimorphic, the role of morphology in virulence has long been an area of intense interest in fungal pathogenesis. Because typical *C. albicans* infections contain a mixture of yeast, pseudohyphae, and hyphae (1) it has, for many years, been difficult to assess the relative contribution of each morphology to virulence. Although more recent compelling evidence suggests that the yeast form is highly attenuated for virulence and that the yeast-to-filament transition is required (11, 12), the precise role of pseudohyphal and hyphal morphologies has remained elusive. Because our *tetO-UME6* strain can be genetically manipulated to grow exclusively in the hyphal form in vitro and to show a significant increase in hyphal formation and extension during the course of an infection in vivo, we are now in a better position to at least partially address this question. Our results are significant because they suggest that shifting the morphology of a *C. albicans* population toward the hyphal form over the course of a systemic infection, and/or increasing hyphal-specific gene expression, is sufficient to improve virulence potential. Although constitutive high-level *UME6* expression does not drive complete hyphal formation during infection, these findings provide the most direct demonstration to date that the *C. albicans* hyphal morphology (and hyphal-associated gene expression) plays a specific important role in promoting virulence in the host once an infection has been established. Our findings are also consistent with previous studies that have implicated the *C. albicans* hyphal form in a variety of virulence-related processes, including the invasion of epithelial cell layers, thigmotropism, breaching of endothelial cells, and lysis of macrophages and neutrophils after phagocytosis (11, 17). Given that previous studies, using clinical isolates, have shown a correlation between increased hyphal formation and a higher level of invasion of a tissue-engineered oral epithelial cell layer (18), we expect that in the absence of Dox our *tetO-UME6* strain would promote mucosal *C. albicans* infections as well.

It is important to bear in mind that our study, like most previous studies that have examined the relationship between *C. albicans* morphology and virulence, involves the use of a transcriptional regulator. As such, we cannot exclude the possibility that the observed virulence effects are due to altered expression of target genes that are not necessarily involved in filamentation per se but rather some other process [genes involved in a variety of virulence processes are known to be induced during filamentation (19, 20)]. However, one important study that did not involve the use of transcriptional regulators has provided very compelling evidence for a direct link between filamentation and virulence. In this study, Zheng *et al.* (21) demonstrated that deletion of *HGCI*, a filament-induced cyclin-related gene important for septin phosphorylation, resulted in reduced hyphal extension and highly attenuated virulence. Interestingly, our work shows that *UME6*, which is also expressed only under filament-inducing conditions, causes induction of *HGCI*.

Expression Levels of a Filament-Specific Transcriptional Regulator Are Sufficient to Determine *C. albicans* Morphology. Despite the fact that *C. albicans* pseudohyphal and hyphal morphologies have been phenotypically well-characterized, little is known about the regulatory mechanisms that determine growth in each form. Our finding that expression levels of *UME6* are sufficient to specify growth in both the pseudohyphal and hyphal forms is significant in that it challenges the current paradigm that these morphologies represent genetically distinct developmental states (10)

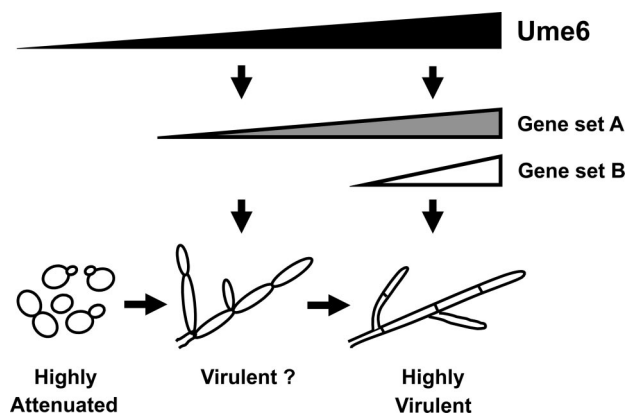


Fig. 5. A possible mechanism by which *Ume6* expression levels determine *C. albicans* morphology. When *Ume6* is not expressed, the filamentous growth program is not induced and *C. albicans* cells grow as yeast. At lower *Ume6* levels, a subset of filament-specific genes (Gene set A) is activated, causing the majority of cells to grow as pseudohyphae. As *Ume6* levels rise, the first set of genes is expressed at higher levels and additional gene set(s) in the filamentous growth program are also expressed at increasing levels. As a consequence, cells are directed to grow in the hyphal form, promoting tissue invasion and virulence. Please note that in the presence of filament-inducing conditions additional regulators are also likely to play a role in determining *C. albicans* morphology by contributing to increased expression of overlapping subsets of filament-specific genes. In addition, alternative models that involve the expression of pseudohyphal-specific gene sets cannot be excluded.

and suggests that the transition between *C. albicans* morphologies is controlled by a common transcriptional regulatory mechanism in a dosage-dependent manner.

Our results suggest a model in which *UME6* levels determine *C. albicans* morphology by controlling the expression levels of overlapping subsets of genes in the *C. albicans* filamentous growth program (Fig. 5). At low *UME6* levels a particular subset of filament-specific genes is expressed that specifies growth largely in the pseudohyphal form. As *UME6* levels rise, this same set of genes is expressed at a higher level and additional subset(s) of filament-specific genes are also expressed at increasing levels, causing cells to grow in the hyphal morphology. This model is supported by several lines of evidence: (i) Only a fraction of filament-specific genes tested showed mild induction at a *UME6* level that specifies largely pseudohyphal growth, whereas all filament-specific genes tested showed highly elevated expression in response to a *UME6* level that specifies a complete hyphal morphology; these effects could be explained by differences in *Ume6* binding site affinity, the number of *Ume6* binding sites per target promoter, and/or indirect regulation. (ii) We have previously observed that *UME6* promotes hyphal filament extension in response to serum at 37 °C by maintaining high-level expression of genes in the *C. albicans* filamentous growth program (13). (iii) Previous studies have identified specialized structures (e.g., the Spitzenkörper and true septa) that are specifically present in hyphal but not pseudohyphal cells (10, 22); in contrast, excluding differences in cell shape, no specialized pseudohyphal-specific structures have been identified to date. These observations suggest that additional filament-specific genes are expressed only in hyphal (not pseudohyphal) cells. Finally, we cannot exclude the possibility of alternative models involving the expression of pseudohyphal-specific gene set(s) or models in which the expression level of filament-specific genes does not affect the transition between morphological forms. To address these models more directly, we are currently carrying out DNA microarray analysis to examine the effect of *UME6* levels on expression of the complete set of genes in the *C. albicans* filamentous growth program.

Interestingly, our observation that activation of *UME6* over a time course causes cells to transition from a population of yeast to majority pseudohyphae and finally to complete hyphae suggests that the *C. albicans* pseudohyphal form may in some sense function as an “intermediate” morphology. Whereas most dimorphic fungal pathogens are known to transition between yeast and hyphal forms, the pseudohyphal morphology appears, for the most part, to be unique to *C. albicans* (1, 23). Given that *C. albicans* is one of the most highly evolved fungal pathogens with respect to colonization and survival within the mammalian host, our results raise intriguing questions regarding the evolutionary advantage that is conferred by such an intermediate morphology.

In addition to challenging the current paradigm that pseudohyphal and hyphal morphologies represent genetically distinct developmental states (10), these studies are significant in that they provide important information about the specific role of the *C. albicans* hyphal form in virulence as well as the mechanisms that function to determine *C. albicans* morphology. Ultimately, a more comprehensive understanding of these regulatory mechanisms will allow us to gain broader insight into the relationship between fungal morphology and virulence.

Materials and Methods

Strains and DNA Constructions. The strains used in this study were THE1 (14), which constitutively expresses an *E. coli* tet-repressor-*S. cerevisiae* Hap4 activation domain fusion protein, and MBY38, the *tetO-UME6* strain. To generate MBY38 we constructed a plasmid that would allow us to integrate the *E. coli* tet operator immediately upstream of one allele of *UME6*. This was accomplished by cloning a PCR product [primers 1 and 2, see supporting information (SI) Table S1 for a complete listing of primers used in this study] containing a *UME6* promoter fragment (–651 to –123 relative to the *UME6* start ATG) into plasmid p97CAU1 (14) digested with KpnI and XhoI. A second PCR product (primers 3 and 4) spanning a region from –47 to +444 (relative to the *UME6* start ATG) was next cloned into the resulting plasmid digested with SpeI and SacII to generate p97CAU1-*UME6*-OEx. Finally, a 3-kb KpnI-SacII fragment containing the *URA3* marker, *tetO* operator, and *UME6* flanks was excised from p97CAU1-*UME6*-OEx and used to transform THE1 to generate MBY38 (the *tetO-UME6* strain). This integration event was confirmed by whole-cell PCR using primers flanking the integration sites. The WT strain (DK318) used in Fig. 3 has been described previously (13).

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Media and Growth Conditions. Strains were routinely grown under non-filament-inducing conditions (YEPD medium at 30 °C) in the presence or absence of 40 µg/mL Dox. See SI Text for a more detailed description of growth conditions used in each experiment.

RNA Preparation and Analysis. Total RNA was prepared by the hot acid phenol method (24). Probe preparation and Northern analysis (using 3 µg of total RNA from each sample) were carried out as described previously (13) (see Table S1 for a listing of primers used for each probe). Blots were scanned by using a phosphorimager and visualized with Imagequant 2.0 software.

Virulence Experiments and Histology. The *tetO-UME6* strain (MBY38) was grown overnight in YEPD medium in the presence of 20 µg/mL Dox at 25 °C, and cells were spun down and washed twice in sterile pyrogen-free saline containing 20 µg/mL Dox and once in sterile pyrogen-free saline containing 10 µg/mL Dox. Cells were counted by using a hemocytometer and diluted appropriately, and 200 µL of cell suspension was injected by lateral tail vein into individual 6- to 8-week-old BALB/c mice that were placed on drinking water containing 5% sucrose in the presence or absence of 2 mg/mL Dox (8 mice per group) 3 days prior to infection. Plate counts were carried out to determine the number and viability of cells in the inoculum. Mice were monitored for survival for 30 days after infection (moribund animals were euthanized and recorded as dying on the following day). The Kaplan–Meier log rank test was used to determine the statistical difference between +Dox and –Dox groups. This analysis was performed by using Prism and GraphPad software.

One kidney was removed from each deceased animal at the time of death for histology studies. Kidneys were fixed in 10% buffered formalin and embedded in paraffin. Thin slices of tissue were sectioned and stained with Grocott–Gomori methenamine silver and visualized by light microscopy.

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