Deletion of the NOT4 gene impairs hyphal development and pathogenicity in *Candida albicans*

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The *Candida albicans* NOT4 gene was disrupted in order to investigate the role of Not4p in growth, morphogenesis and pathogenicity. Heterozygote (NOT4/not4), null (not4/not4) and reconstructed heterozygote ([NOT4]/not4) strains of *C. albicans*, as well as CAF2-1, the parental strain, were grown under conditions that promote hyphal formation. When cultured in liquid medium 199 the heterozygote, reconstructed and wild-type strains began the yeast-to-hyphal transition within 3 h and continued hyphal growth for the duration of experiments. The null mutant also began hyphal growth within 3–5 h but hyphae tended to be shorter and distorted. Subsequently, hyphal growth was arrested and growth returned predominantly to the yeast form. Similar differences were observed when strains were grown on solid Spider medium and medium 199. The parental, heterozygote and reconstructed strains formed normal filamentous networks emanating from colonies. In contrast, the null mutant failed to form hyphae on all solid media tested. The ability of the NOT4 null strain to form biofilms was also investigated, and it was observed that biofilm development does not readily occur for this strain. Virulence of each strain was examined utilizing the mouse model of systemic candidiasis. Mice infected with CAF2-1 succumbed to infection within 3–7 days. All mice infected with the null strain survived for the duration of experiments, while the heterozygote and reconstructed heterozygote strains showed an intermediate level of virulence. These findings suggest that NOT4 may play a role in affecting strain pathogenicity, possibly by regulating expression of certain genes that effect cellular morphogenesis and virulence.

**INTRODUCTION**

*Candida albicans* is a major fungal pathogen in humans, usually causing superficial infection of mucosal epithelium. However, in immunocompromised individuals infections can progress to severe systemic invasion, leading to life-threatening circumstances. One of the factors associated with *C. albicans* virulence is the ability of the organism to assume several morphological states, most notably the transition from the yeast to hyphal form (Calderone, 2002).

Many investigations have examined the roles that various transcription factors play in *C. albicans* morphogenesis. For example, deletion of individual genes encoding the transcription factors Cph1p (Liu et al., 1994), Cph2p (Lane et al., 2001), Efg1p (Stoldt et al., 1997) and Tec1p (Schweizer et al., 2000) resulted in strains defective in hyphal development. Regulation of TEC1 expression appeared to be dependent on the CPH2 and EFG1 gene products (Lane et al., 2001). The data suggests that Efg1p and Cph1p act through different mechanisms, as a double null mutant strain for the genes does not form hyphae under all conditions studied (Lo et al., 1997). In contrast, deletion of TUP1 (Braun & Johnson, 1997), RFG1 (Kadosh & Johnson, 2001) or NRGI (Murad et al., 2001a) resulted in an increased propensity for filamentous growth. In these cases, all three factors apparently act as repressors of hyphal-specific genes such that deletion of these genes results in the expression of at least some genes essential for hyphal growth. It has been hypothesized that Nrg1p and Tup1p physically associate to mediate transcriptional regulation (Murad et al., 2001b). Overexpression or repression of MCM1, which encodes a recently discovered transcription factor in *C. albicans*, resulted in hyphal induction (Rottman et al., 2003). In a similar fashion, null mutants for SSN6 exhibited an altered pseudohyphal morphology, while overexpression of SSN6 led to increased filamentous growth and decreased virulence (Hwang et al., 2003). Although these assorted transcription factors might be divided into two classes based on their opposing actions on morphological differentiation in *C. albicans*, the corresponding null mutants from both groups showed attenuated virulence.

The Ccr4–Not transcriptional complex is another group of proteins that may be of importance in the regulation of *C. albicans* gene expression, but this has not yet been widely
investigated; however, extensive investigations concerning the Ccr4–Not complex have been performed in Saccharomyces cerevisiae, where it was found to influence carbon catabolite repression (Ccr) and also to exhibit characteristics of a global negative regulator of transcription (Not= negative on TATA) (Collart & Struhl, 1994; Liu et al., 1998; Collart, 2003). The constituents of the complex include Not1p (CDC39), the largest protein and core unit, which appears to be critical for complex formation (Maillet et al., 2000). Not5p is highly similar to Not3p, while in other organisms only one or the other protein is usually noted (Oberholzer & Collart, 1998). Complex formation occurs in part via Not2p (CDC36), Not4p and Not3/5p interaction with the C-terminal region of Not1p, whereas Ccr4p and Ccr-associated factor 1 (Caf1p) associate with amino acids in a more central location of Not1p (Bai et al., 1999). Various mutations in Not2p can prohibit self-association of the complex, such as Not3–5p interaction with Not1p (Russell et al., 2002). Similarly, Caf1p is essential for Ccr4p–Not1p interaction (Bai et al., 1999). Orthologues of these proteins have been found in diverse eukaryotic species from yeast to humans, and show high degrees of sequence similarity over specific domains in each protein, while the complex itself is considered to be ubiquitous (Collart, 2003).

Functionally, the Ccr4–Not complex has been reported to mediate transcription in S. cerevisiae by acting on the TATA-element-binding TFIIID complex (Collart & Struhl, 1994). Subsequently, it was demonstrated that yTaf(II)19p, a subunit of the TFIIID complex (Lemaire & Collart, 2000), TATA-binding protein (Badarinayana et al., 2000) and several subunits of the RNA polymerase II holoenzyme (Liu et al., 2001) interact with discrete proteins of the Ccr4–Not complex. It is also established that Ccr4–Not affects gene expression in both a positive and negative manner. The roles of this complex may be more than simply transcriptional regulation, as Ccr4p was recently reported to exhibit mRNA deadenylation activity (Tucker et al., 2002; Chen et al., 2002), and Caf1p in association with Not1p was found to interact with a subunit of an RNA helicase complex (Maillet & Collart, 2002), suggesting that this complex may also influence mRNA stability.

The existence of a wide array of proteins in this complex suggests that its functions can be multifaceted and its modes of transcriptional regulation rather intricate. In this respect, Not4p is of particular interest. The first 250 amino acids at the Not4p N-terminus show high sequence identity among diverse eukaryotic species (Zhao et al., 2001). This stretch of sequence includes a specialized zinc finger elaboration known as the RING finger characteristic of the C4C4 variant (Hanzawa et al., 2001), an RNA recognition motif, and another separate CCCH zinc finger. The remaining C-terminal sequence of Not4p shows little sequence homology across species. Human Not4 cDNA complements S. cerevisiae NOT4 null mutants, demonstrating functional conservation across disparate species (Albert et al., 2000). Compelling evidence suggests that Not4p functions as an E3 ubiquitin–protein ligase (Albert et al., 2002). It was hypothesized that Not4p may mediate ubiquitination of Ccr4–Not complex proteins or other associated proteins and thereby influence the transcriptional control elicited by this complex. RING finger proteins are often linked with E3 ubiquitin-ligase activity and are believed to mediate protein–protein interactions in macromolecular complexes (Borden, 2000). The presence of the RNA recognition motif and an additional zinc finger imply that other functions are likely to be attributed to Not4p. Alternatively, these domains may play specific roles determining which proteins Not4p directs for ubiquitination.

In regard to C. albicans, limited studies of NOT4 [previously referred to as MOT2 (Zhao et al., 2001) and designated NOT4 to conform to accepted nomenclature] detected downregulation of NOT4 transcription during infection in a rat model of oral candidiasis, leading to the suggestion that the gene product might be important in controlling expression of certain genes that affect pathogenesis (Zhao et al., 2001). Similarly, a recent study indicates that C. albicans NOT5 null mutants show attenuated virulence (Cheng et al., 2003). Another study has shown that haploinsufficiency in a number of C. albicans genes encoding proteins of the Ccr4–Not complex results in aberrant filamentous growth (Uhl et al., 2003). Because of (i) the participation of this complex in transcriptional regulation in organisms ranging from fungi to mammals (Collart, 2003), (ii) the high degree of conservation among the Ccr4–Not complex proteins across diverse species, and (iii) the reported findings that members of the complex may affect C. albicans virulence and morphology (Zhao et al., 2001; Cheng et al., 2003; Uhl et al., 2003), it is of interest to examine some of the phenotypic consequences of NOT4 deletion on C. albicans growth and infectivity. The results of the experiments reported herein suggest that NOT4 gene function is necessary for normal hyphal development and pathogenesis.

METHODS

Strains and growth conditions. C. albicans strains used or constructed in this study are described in Table 1. Routine culturing of all strains for use in growth and virulence studies was at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose [glucose]). Cultures for investigations concerning biofilm formation were grown in Yeast Nitrogen Base (YNB, Difco), pH 7, supplemented with 50 mM glucose. Stocks of all strains were maintained at 4°C on YPD plates containing 1.5% agar. Strains were subcultured monthly.

Solid media used were: Spider medium [1% (w/v) nutrient broth, 1% (w/v) mannitol, 0.2% (w/v) K2HPO4 (Liu et al., 1994)], 10% horse serum, and medium 199 (US Biological) lacking sodium bicarbonate but supplemented with 155 mM Tris, pH 7.5. Media were sterilized by filtration. Final agar concentration in all solid media was 1.5% (w/v).

Plasmid and cassette constructions. The strategy used in plasmid and cassette construction necessary to obtain appropriate mutant strains is outlined in Fig. 1. In order to facilitate subcloning of the hisG-URA3-hisG cassette, plasmid pMB7 (Fonzi & Irwin, 1993) was modified such that either XhoI or NotI restriction sites flanked the cassette. Sites were added by a sequential process. First,
the plasmid was linearized at the BglII site flanking the cassette and the site was destroyed by filling in using Klenow fragment (Sambrook et al., 1989). The linearized plasmid was then recovered, the appropriate linker was ligated to the plasmid and it was introduced into *Escherichia coli* strain DH5α by transformation (Sambrook et al., 1989). After plasmid recovery, the process was repeated to introduce the same restriction site into the other side of the cassette. In this case, plasmid was linearized with *Sal*I, prior to linker addition.

The following procedure was employed to obtain disruption cassettes for use in *NOT4* heterozygote and null mutant construction. A 0.95 kb segment of *NOT4* was first amplified starting from the initiation codon and extending downstream to include the first exon, intron, and part of the second exon (Zhao et al., 2001). The primers used for amplification of this sequence were 5'-TGATTCAAGCAGCAGATCCTTC-3' (forward) and 5'-GCTTCTCTCCCCAGGTTGATAG-3' (reverse). The product was subsequently subcloned into the EcoRV site of pT7Blue (Novagen). The resulting plasmid, which contains a single EcoRV site located within the second exon sequence of *NOT4*, was linearized with EcoRV and ligated to *Xho*I linkers (New England Biolabs). The *hisG-URA3-hisG* cassette with flanking *Xho*I sites was then inserted in both orientations into this site, yielding plasmids designated pNX1 and pNX2.

An appropriate vector was also designed for use in reconstruction of a *NOT4* heterozygote strain from a *NOT4* null mutant. Plasmid pXZ1, containing a 5-5 kb *Stu*I genomic fragment harbouring the entire *NOT4* sequence (Zhao et al., 2001), was modified to meet this requirement. The genomic sequence of this plasmid spanned from 1.0 kb upstream of the *NOT4* initiation codon to 3-5 kb downstream of the polyadenylation signal. First, a unique *Bgl*II site in pXZ1, 82 bp downstream of the polyadenylation signal for the *NOT4* transcript, was used to linearize the plasmid. After treatment with DeepVent DNA polymerase (New England Biolabs) at 72°C for 10 min to fill in 3' protruding ends, under conditions specified by the manufacturer, pXZ1 was ligated to *Nsi*I linkers. The modified plasmid was recovered by transformation of *E. coli* DH5α and ultimate isolation by use of a Qiagen isolation kit (Qiagen). Finally, the modified *hisG-URA3-hisG* cassette, flanked by *Nsi*I sites, was ligated into the *Nsi*I site of pXZ1 and was thereby positioned immediately downstream of *NOT4*, producing the integration plasmid pNN1.

**Construction of *NOT4* heterozygote and null mutant strains.**

Both *NOT4* alleles in strain CAI4 were sequentially disrupted as described by Fonzi & Irwin (1993) and strains obtained are listed in Table 1. For disruption of the first allele, CAI4 cells were transformed as described by Gietz & Woods (1998) with the modified

![Fig. 1. Construction of the *NOT4* reintegration cassette and its introduction into the null mutant. Schematic representation depicting pXZ1 modification by insertion of *hisG-URA3-hisG* sequences shortly downstream of *NOT4*. Black bars indicate *NOT4* sequence and the inserted white arrow signifies a complete, uninterrupted sequence of the gene. Sequence derived from the parent *hisG-URA3-hisG* cassette is represented with shaded bars. Flanking genomic sequence is indicated by open bars. The expected recombination event in the null strain NT4-2d is depicted and results in insertion of PshAI-linearized pNN1 into the homologous chromosomal site.](http://mic.sgmjournals.org/)

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<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>CAI4</td>
<td><em>ura3::imm434</em>/<em>ura3::imm434</em></td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>URA3/<em>ura3::imm434</em></td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>NT4-1</td>
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<td>This study</td>
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<tr>
<td>NT4-3</td>
<td>*ura3::imm434/<em>ura3::imm434</em>, <em>NOT4::hisG-URA3-hisG/not4::hisG</em></td>
<td>This study</td>
</tr>
</tbody>
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**Table 1.** Strains used in this study

**Phenotypic effects of *C. albicans NOT4* deletion**
NOT4::hisG-URA3-hisG cassette excised from pNX1 using HindIII and SvaI. Transformants grown on YNB plates were analysed by PCR and Southern blotting to confirm disruption of one NOT4 allele. The heterozygote strain chosen for subsequent investigation was designated NT4-1 (Table 1). Disruption of the second allele was accomplished using the cassette excised from pNX2. After selection, putative null strains were then verified by PCR and Southern blotting experiments. The null strain chosen for subsequent investigations was designated NT4-2.

To reintroduce a functional NOT4 gene into NT4-2, the URA3 gene was first deleted from NT4-2, and appropriate clones were then selected on plates containing 5-fluoro-orotic acid (5-FOA) (Boeke et al., 1984). One such clone, designated NT4-2d, was transformed with the NOT4-hisG-URA3-hisG cassette from pNN1 linearized with PshHI, and appropriate clones were selected on YNB plates. The resultant transformants were analysed by PCR and Southern analysis to ensure that reintroduction of NOT4 had occurred. One strain, designated NT4-3, was chosen for use in this study.

Biofilms. Development of biofilms was promoted using a modification of protocols previously described (Chandra et al., 2001). Briefly, C. albicans strains CAF2-1 and NT4-2 were grown at 30 °C in YNB, pH 7, supplemented with 50 mM glucose, for 16 h while shaking at 120 r.p.m. The OD595 of the culture was determined and the cells were harvested. The supernatants were removed and the cells were resuspended in PBS to an OD595 of 1.0. The wells of a sterile 96-well Polystyrene microtitre plate (Falcon) were coated with 10% fetal bovine serum for 1 h and washed twice with 200 μl PBS. After resuspension in PBS, 100 μl of cells were allowed to adhere for 90 min at 37 °C while shaking at 120 r.p.m. Subsequently, cells that did not adhere were removed by washing twice with 200 μl PBS. YNB, pH 7, supplemented with 50 mM glucose was added to each well and biofilms were allowed to form for up to 48 h. Incubation temperature was 37 °C. After indicated incubation times, the wells were again washed twice with PBS and the presence of biofilms was assessed by measuring OD595 (Shin et al., 2002) using a TECAN plate reader. In addition, adherence as well as biofilm formation was monitored by light microscopy throughout the experiments.

Virulence determinations. A mouse model of haematogenously disseminated candidiasis was used to test virulence of C. albicans strains CAF2-1, NT4-1, NT4-2 and NT4-3 (Table 1). Strains were grown overnight in YPD to stationary phase, harvested, and resuspended in sterile physiological saline (0·85% NaCl). Cell density was determined using a haemocytometer and diluted to a density of 5 × 10^6 cells ml^-1. Groups of 8–10 male BALB/c mice (20–22 g each, Harlan) were injected in the lateral tail vein with 0·5 ml of the suspension of the appropriate strain. Mice were observed twice daily for signs of morbidity, and animals in a moribund state were killed by CO_2 inhalation.

Concomitantly, each strain was used to inoculate additional animals. Mice from each group were killed at 1–12 days post-infection and the kidneys were removed. Cross-sections of one kidney (day 1) from each animal were fixed in 10% formalin and processed for histological examination. Tissue specimens were embedded in paraffin, sectioned, affixed to slides, and stained with Gomori methenamine silver. Sections were examined with an Olympus BH-2 microscope at 400× magnification and photomicrographs were made with a Canon A40 digital camera fitted through the eyepiece barrel with a 10× eyepiece adapter. At all other time points, kidneys were weighed, homogenized in sterile PBS, and aliquots were plated on YPD agar supplemented with 50 μg streptomycin ml^-1. Plates were incubated at 30 °C for 2 days and colonies per g tissue were determined.

**RESULTS**

Strain construction

CAF2-1, the parental strain of CAI4, was used as the control in all experiments as it possesses a wild-type URA3 in the native position (Fonzi & Irwin, 1993). The strategy designed to disrupt both copies of NOT4 in CAI4 utilizing the method of Fonzi & Irwin (1993) is depicted in Fig. 1. The NOT4 null mutant, designated NT4-2, was generated by sequential disruption of each allele of the gene. Strain NT4-3 contained a reconstructed NOT4 allele, and was included in all experiments to ensure that phenotypic traits attributed to strain NT4-2 were the result of mutation to NOT4 and not due to other alterations that occurred during construction. Southern blot analysis of DNA from the constructed strains resulted in the expected recombination patterns (Fig. 2), and demonstrated that appropriate strains were obtained. These results were verified by PCR of NT4-2 genomic DNA, where primers specific for NOT4 sequences just outside the boundaries of the disruption cassette amplified a single band of 2·2 kb, consistent with the first allele interrupted by hisG sequence. Furthermore, since the two cassettes used for both disruptions had the hisG-URA3-hisG cassette inserted in opposite orientations relative to NOT4, PCR of NT4-2 genomic DNA exhibited both orientations of hisG inserted.
into NOT4, demonstrating that the second disruption had targeted the second allele.

**Morphological phenotype of NOT4 mutant strains**

Deletion of *S. cerevisiae* NOT4 resulted in a temperature-dependent growth defect attributed to osmotic instability, and a decreased sensitivity to 3-aminotriazole attributed to elevated expression of glycerol phosphate dehydratase (Cade & Errede, 1994; Irie *et al*., 1994). In contrast, both the sensitivity of the *C. albicans* NOT4 null to increasing concentrations of 3-aminotriazole, and the osmotic stability of the strain, were essentially equal to that of CAF2-1. Analysis of the *C. albicans* NOT4 null and heterozygote strains also showed no significant difference in growth rates when grown in YPD or YNB at either 30 or 37 °C. Likewise, the inclusion of 0.02 % SDS in the growth medium had no effect on growth, while caffeine sensitivity was unchanged between the wild-type and the NOT4 null strain.

Clear differences, however, were observed in the capacity of the null mutant to undergo the yeast-to-hyphal transition. Initial experiments examined hyphal formation in liquid media. Fig. 3 shows the time-course of the morphological transition for cells grown in 10 % serum at 37 °C. Approximately 99 % of cells of strain CAF2-1, and both heterozygotes, showed significant germ tube emergence within 3 h after induction of morphogenesis. Hyphal elongation continued throughout the course of the experiment (24 h). With the NOT4 null strain, similar results were observed for emergence of germ tubes during the first 3 h, although many hyphae appeared distorted and were noticeably shorter than those produced by the other strains. In addition, approximately 5–10 % of cells failed to form hyphae. Hyphal

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**Fig. 3.** Time-course of mycelial formation in 10 % horse serum. Overnight cultures of *C. albicans* strains grown in YPD were diluted 50-fold in YPD and allowed to grow at 30 °C with shaking for 3 h when exponential growth was observed. The cells were inoculated (5 x 10^3 cells) into 1 ml 10 % sterile horse serum and incubated at 37 °C. Photomicrographs were taken from a Zeiss-IM inverted microscope with 20 x objective and 10 x eyepiece fitted to a digital camera. Representative microscopic fields at the indicated times are depicted. Bar, 100 μm.
development continued for up to 7 h, but elongation was clearly less pronounced than that observed with the other strains. After 12 h hyphal progression had for the most part ceased, and instead, yeast growth resumed.

Germination in medium 199 (pH 7.5) at 37°C was also investigated (Fig. 4); the time-course of morphological changes observed were similar to those found in 10% serum. The null strain once more demonstrated the initial phases of hyphal formation, but again, hyphal growth terminated within 5–7 h.

When CAF2-1 and both heterozygote strains were grown on agar plates containing 10% serum, mycelia were prevalent after 24 h growth at 37°C (Fig. 5). In contrast, the null strain grew solely as yeast and failed to invade the agar. Colony morphology was also examined on medium 199 and Spider plates. After 7 days of growth, CAF2-1 exhibited the greatest magnitude of filamentation radiating from the edges of colonies on all media. The null strain did not produce mycelia under any conditions. Both the heterozygote and reconstructed heterozygote displayed some hyphal development extending from the colonies but the extent was
intermediate between that observed for CAF2-1 and the null strain. This finding suggests that NOT4 gene copy number may influence the degree to which mycelial formation occurs on solid substrates and is consistent with the observation that haploinsufficiency by other members of the complex affected filamentation (Uhl et al., 2003).
Biofilm formation

Strains CAF2-1 and NT4-2 were allowed to adhere to wells coated with fetal bovine serum. Both strains readily adhered in the well as judged by light microscopy, and no significant differences in the number of adherent cells, in comparisons between the two strains, were observed. After 24 h at 37 °C, growth and biofilm formation was assessed by measuring the OD595 as well as by light microscopy. A similar amount of growth was obvious for both CAF2-1 and the null strain (data not shown). In addition, CAF2-1 had developed as a biofilm that could not be disrupted by vigorous washing with PBS (data not shown). After 48 h, CAF2-1 had developed a dense biofilm (OD595 0.67 ± 0.09, mean ± SD, n = 3) while the null mutant had not developed a biofilm as judged by the same method (OD595 0.04 ± 0.02). Light microscopy also showed that a complex community of yeast cells and hyphal cells was present in the case of CAF2-1 (Fig. 6). In contrast, the null strain had not developed a biofilm during this time interval (Fig 6).

Pathogenicity in a mouse model of systemic candidiasis

To examine whether NOT4 was required for virulence, the mouse model of systemic candidiasis was utilized. Mice were injected intravenously with 1 × 10⁶ cells of each of the pertinent C. albicans strains, and survival was then monitored over a 25 day period. Most mice inoculated with CAF2-1 succumbed to infection within 3 days, and after 7 days infection proved fatal to all animals (Fig. 7). In contrast, all mice injected with strain NT4-2 survived during the entire 25 day period. The heterozygote NT4-1 and reconstructed heterozygote strain NT4-3 showed attenuated virulence when compared to the parental strain, although NT4-3 was clearly more virulent than NT4-1 (original heterozygote). The reasons for this difference in pathogenicity are under investigation, but do not appear to be due to allelic differences (K. E. Krueger & R. L. Cihlar, unpublished observations). This difference might be accounted for by the position of URA3, as its site of integration in the reconstructed heterozygote is downstream from that of the original heterozygote (Fig. 1). We have ruled out that avirulence in the null mutant is due to a positional effect of URA3 integration. In particular, URA3 was integrated into its native locus by transforming strain NT4-2d with a 4·9 kb URA3 genomic fragment. A representative Ura⁺ NOT4 null strain exhibited the same morphogenetic and avirulent phenotypes as strain NT4-2 (data not shown).

Quantitative measurements of C. albicans levels in the kidneys suggested that the null strain persists in the host (Table 2). Histological examination of the infected kidney tissue showed that CAF2-1 (Fig. 8) and both heterozygote strains display prominent mycelia within their large invasive foci. NT4-2 exhibits some hyphal formation; however, hyphae show atypical morphology and the foci are noticeably smaller. The decreased tendency of the null strain to sustain wild-type hyphal morphology is consistent with the results observed in in vitro experiments.

**DISCUSSION**

A number of laboratories have demonstrated that various transcription factors influence C. albicans morphology. The
studies reported here suggest that NOT4 can be included on this list. Because NOT4 is just one component of the Ccr4–Not transcriptional complex, the functional implications of the entire complex probably extend beyond the phenotypic characteristics reported for NOT4 alone. The phenotypic changes described probably reflect, in part, altered function of the remaining Ccr4–Not subunits resulting from the absence of Not4p. Perhaps the most intriguing finding with NOT4 deletion is that the progression of germ tube emergence differs significantly from the effects on this process reported for deletion of other transcription factors. When the null strain is subjected to the appropriate conditions, hyphal formation initially occurs, although even at these early stages hyphae are somewhat shorter and irregular in appearance when compared to those of the parental and heterozygote strains. It is interesting that after several hours the vast majority of cells have reverted back to growth primarily as yeast. This finding suggests that NOT4 may be required for progressive maintenance of germ tube extension and hyphal morphology. It remains to be determined whether the effect is direct or indirect.

When grown on hyphal-inducing solid media, the null strain failed to extend any hyphae radiating from colonies after prolonged growth. Similar to observations during growth in liquid media, a small amount of hyphal growth could be observed shortly after growth was initiated (several hours); however, progressive elongation of these filaments failed to occur and most cells reverted back to the yeast form. Thus, the data are consistent with the notion that the NOT4 null mutation does not affect the initiation of hyphal formation, but rather its continuation.

The NOT4 null strain was unable to colonize plastic surface and form a biofilm. This observation is similar to what has been reported for other morphological mutants. The EFG1 mutant as well as the EFG1/CPH1 double mutant is incapable of colonizing plastic surfaces and forming biofilms (Lewis et al., 2002; Ramage et al., 2002). Other reports have shown that filamentation is not required for biofilm formation, but is involved in the complex architecture of the biofilm (Baillie & Douglas, 1999). A further investigation of the biofilm capabilities of the NOT4 null strain and the role of Not4p in biofilm formation will be reported elsewhere (B. P. Krom and others, unpublished).

Virulence of C. albicans was also found to be dependent on NOT4. Compared to the wild-type strain, both the heterozygote and reconstructed heterozygote strains exhibited attenuated pathogenicity in a mouse model of systemic candidiasis, while the null strain was judged to be avirulent. On the other hand, colonization of the null strain persisted for at least 12 days. Thus, as also shown for other C. albicans strains with different gene deletions (Calera et al., 1999; Schweizer et al., 2000), the mere presence of relatively large numbers of viable cells is not sufficient for virulence.

Table 2. Recovery of C. albicans from infected kidneys

<table>
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<th>Strain</th>
<th>log_{10}(c.f.u. per g tissue) (mean ± SD)</th>
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<tr>
<td></td>
<td>2 d p.i.</td>
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<tr>
<td>CAF2-1</td>
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<tr>
<td>NT4-1</td>
<td>5.78 ± 0.03</td>
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<tr>
<td>NT4-2</td>
<td>5.93 ± 0.10</td>
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<tr>
<td>NT4-3</td>
<td>5.83 ± 0.05</td>
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</table>

Details of quantification of viable C. albicans titres in kidneys from infected mice at various times post-infection (p.i.) are described in Methods. Results are presented from duplicate determinations of two kidneys from each time point. ND, Not determined.
Histological examination of infected kidneys revealed that the NOT4 null strain exhibited diminished hyphal formation. Since hyphal formation has been reported to be a contributing factor to virulence (Calderone, 2002), it is possible that the avirulent phenotype of NOT4 null strains may be at least in part due to an inability to maintain normal hyphal development during infection, although other factors might also account for reduced infectivity.

Several pathways have been implicated in directing C. albicans morphogenesis. In particular, the transcription factor Efg1p has been proposed to influence germination by a cAMP-mediated mechanism through MAPK kinase action (Csank et al., 1998; Bockmuhl & Ernst 2001). Other investigations have shown that EFG1 and CDC35 (adenylate cyclase) null strains exhibit various defects in transitioning to the hyphal state (Stoldt et al., 1997; Rocha et al., 2001). Nrg1p and Tup1p are believed to act in concert as repressors of hyphal morphology, as deletion of the corresponding genes results in strains constitutively exhibiting mycelial growth (Murad et al., 2001b). The proteins encoded by CPH1 (Lo et al., 1997), EFG1/CPH2/TEC1/CZF1 (Lane et al., 2001; Giusani et al., 2002) and ASH1 (Inglis & Johnson, 2002), all appear to affect morphology by a different mechanism(s) than those employed by the products of NRG1/TUP1. The synopsis of all these findings is that multiple pathways are involved in determining C. albicans cell morphology. Because the Ccr4–Not complex has been shown to affect regulation of a wide array of genes in S. cerevisiae, and since the proteins of the complex are conserved in C. albicans (Zhao et al., 2001; Cheng et al., 2003; Uhl et al., 2003), it is likely that Ccr4–Not also plays a role in regulating gene expression in C. albicans. Since the repertoire of genes that are regulated, and/or the function of the respective gene products, will most likely differ in C. albicans versus S. cerevisiae it remains feasible that this complex may affect morphogenesis either directly by regulating morphogenetic genes or indirectly through regulating some of the aforementioned transcription factors. Nevertheless, the unique phenotypic time-course of germ tube elongation which soon reverses itself highlights the notion that NOT4 deletion may indicate another mode or level of regulation of cellular morphology distinct from those controlled by other transcription factors. The recent findings of Uhl et al. (2003) reporting the occurrence of defective hyphal growth in C. albicans strains with mutations introduced into genes encoding other members of the complex support this notion.

Of course, Not4p is likely to be responsible for only a subset of functions within the entire Ccr4–Not complex. The molecular consequences of omitting Not4p on altering the function of this complex are poorly understood at present. Compelling evidence has been presented to suggest that Not4p serves as an E3 ubiquitin–protein ligase (Albert et al., 2002). Interestingly, it has been observed that expression of UBC7, an ubiquitin-conjugating enzyme, is elevated approximately 10-fold as judged by microarray analysis, possibly in compensation for loss of Not4p (K. E. Krueger & R. L. Cihlar, unpublished). Other aspects of Ccr4–Not function should still be operational in its absence, but the ramifications of lacking this component are likely to alter the manner by which transcriptional regulation is governed by the complex. Deletion of other proteins of Ccr4–Not should provide further interesting insights into how this complex may contribute to the regulation of C. albicans morphogenesis.

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