

# Ash1 Protein, an Asymmetrically Localized Transcriptional Regulator, Controls Filamentous Growth and Virulence of *Candida albicans*

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**In response to a number of distinct environmental conditions, the fungal pathogen *Candida albicans* undergoes a morphological transition from a round, yeast form to a series of elongated, filamentous forms. This transition is believed to be critical for virulence in a mouse model of disseminated candidiasis. Here we describe the characterization of *C. albicans* *ASH1*, a gene that encodes an asymmetrically localized transcriptional regulatory protein involved in this response. We show that *C. albicans* *ash1* mutants are defective in responding to some filament-inducing conditions. We also show that Ash1p is preferentially localized to daughter cell nuclei in the budding-yeast form of *C. albicans* cell growth and to the hyphal tip cells in growing filaments. Thus, Ash1p “marks” newly formed cells and presumably directs a specialized transcriptional program in these cells. Finally, we show that *ASH1* is required for full virulence of *C. albicans* in a mouse model of disseminated candidiasis.**

*Candida albicans* is a common fungal pathogen that causes mucosal infections in healthy individuals and can cause life-threatening disseminated infections in immunocompromised patients. *C. albicans* is capable of colonizing most tissue types in humans and is therefore able to adapt and thrive in the diverse microenvironments encountered in a host (7, 36). *C. albicans* responds to changes in its environment by altering its patterns of gene expression, and these responses appear critical to the survival and virulence of this opportunistic pathogen. In the laboratory, *C. albicans* responds to changes in growth conditions, including starvation, 37°C temperature, neutral pH, exposure to serum, contact with animal cells, or the presence of compounds such as proline and *N*-acetylglucosamine by switching from a round, single-celled budding-yeast form to elongated filamentous forms. These filamentous forms include a spectrum of morphologies that range from pseudohyphae (chains of elongated cells that remain attached after cell division) to true hyphae (long cylindrical cells separated by septal walls that lack constrictions at sites of cell division) (35). Current evidence is consistent with the idea that the interconversion of these forms is critical for virulence of *C. albicans* (for reviews, see references 6, 10, 11, 20, 22, 33, 44, and 47). For example, all of the morphological forms are found in infected tissues, and mutations that lock *C. albicans* into either the yeast or filamentous form produce mutants with significantly reduced virulence when tested in mouse models of disseminated candidiasis. Additional links between filamentous growth and virulence come from studies showing the differential expression of certain cell surface and secreted proteins in filamentous cells compared to budding yeast cells; for example, newly formed filaments adhere better to mammalian cells than do yeast-form cells. It may be that increased adherence and inva-

sion are important for early stages of *C. albicans* infection; the ability to form yeast cells that bud off from adherent hyphae may subsequently promote the colonization of diverse tissues during disseminated infection (36).

Filamentous growth is common to many species of fungi. For example, *Saccharomyces cerevisiae* undergoes a process of pseudohyphal growth that is similar in some respects to filamentous growth of *C. albicans* (17; for reviews, see references 28 and 31). In particular, diploid *S. cerevisiae* cells grow, in response to nitrogen starvation, as elongated chains of pseudohyphal cells that extend as filaments into solid media. Although *S. cerevisiae* and *C. albicans* are closely related evolutionarily, there are important differences between the types of filamentous growth in these two organisms. For example, *C. albicans* filaments are induced in response to several growth conditions (such as 37°C temperature and exposure to serum) that do not critically affect filamentous growth of *S. cerevisiae*. Moreover, both *S. cerevisiae* and *C. albicans* produce pseudohyphae, yet only *C. albicans* can make true hyphae, defined by the absence of constrictions at the sites of cell division and by a particular pattern of mitosis and cell division (45). Despite these differences, many of the components that control filamentous growth—particularly those in signaling pathways—are conserved between *S. cerevisiae* and *C. albicans* (for example, see references 2, 9, 12, 16, 25, 26, 29, 30, and 40).

The *S. cerevisiae* Ash1 protein (Ash1p) was originally isolated in screens designed to identify proteins involved in mating-type switching in haploid yeast cells (1, 43). Later, it was discovered that Ash1p is also required for pseudohyphal growth in *S. cerevisiae* (8). With regards to pseudohyphal growth, Ash1p has been linked to a transcriptional regulatory cascade that, in response to nitrogen starvation, activates expression of the *FLO11* gene (37); *FLO11* encodes a cell surface protein that is required for pseudohyphal growth. For these reasons, we hypothesized that an *ASH1*-related gene in *C. albicans* has a role in filamentous growth, and in this paper, we test this idea.

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TABLE 1. Strains used in this study

| Strain                            | Genotype  | Source or reference |
|-----------------------------------|---|---------------------|
| <i>C. albicans</i> <sup>a</sup>   |   |                     |
| CAF2-1                            | <i>URA3/ura3::imm434</i>  | 13                  |
| CAI4                              | <i>ura3Δ/ura3Δ</i>  | 13                  |
| RM1000                            | <i>ura3Δ/ura3Δ his1::HisG/his1::HisG</i>  | 34                  |
| YDI-1                             | <i>ura3Δ/ura3Δ ASH1/ash1::HisG-URA3-HisG</i>  | This study          |
| YDI-7                             | <i>ura3Δ/ura3Δ ash1::HisG/ash1::HisG-URA3-HisG</i>  | This study          |
| YDI-11                            | <i>ura3Δ/ura3Δ ash1::HisG/ash1::HisG</i>  | This study          |
| YDI-129                           | <i>ura3Δ/ura3Δ cph1::HisG/cph1::HisG ash1::HisG/ash1::HisG-URA3-HisG</i>                  | This study          |
| YDI-154                           | <i>ura3Δ/ura3Δ ash1::HisG/ash1::HisG::ASH1-URA3 (2d)</i>                                  | This study          |
| YDI-157                           | <i>ura3Δ/ura3Δ ash1::HisG/ash1::HisG::ASH1-URA3 (6d)</i>                                  | This study          |
| YDI-199                           | <i>ura3Δ/ura3Δ ash1::HisG/ash1::HisG-URA3-HisG::myc6-ASH1</i>                             | This study          |
| JKC19                             | <i>ura3Δ/ura3Δ cph1::HisG/cph1::HisG-URA3-HisG</i>  | 29                  |
| <i>S. cerevisiae</i> <sup>b</sup> |   |                     |
| CGX69                             | <i>MATa/MATα ura3/ura3</i>  | 17                  |
| YDI-56                            | <i>MATa/MATα ura3/ura3 ash1::HisG/ash1::HisG</i>  | This study          |
| YAS242-10B                        | <i>MATα ASH1 can1::HO-CAN1 ho::HO-ADE2 ade2-1 his3-11,15 trp1-1 ura3 leu2-3,112</i>       | Sil, unpublished    |
| YAS234-1A                         | <i>MATα ash1::TRP1 can1::HO-CAN1 ho::HO-ADE2 ade2-1 his3-11,15 trp1-1 ura3 leu2-3,112</i> | Sil, unpublished    |

<sup>a</sup> All *C. albicans* strains listed as *ura3Δ/ura3Δ* are homozygous for the *ura3::imm434* mutation, except for CAF2-1, which is heterozygous for the mutation.

<sup>b</sup> CGX69 and YDI-56 are of the  $\Sigma$ 1278b strain background; YAS242-10B and YAS243-1A are of the W303 strain background.

First, we describe the isolation and characterization of a *C. albicans* gene that is homologous to the Ash1p gene of *S. cerevisiae*. *C. albicans* Ash1p can complement an *S. cerevisiae* *ash1* mutant, indicating that *C. albicans* Ash1p has biochemical activities (for example, its ability to function as a transcriptional repressor) (32) similar to those of *S. cerevisiae* Ash1p. *C. albicans* strains with *ASH1* deleted show defects in filamentous growth in vitro and have reduced virulence in a mouse model of systemic candidiasis. We also show that when *C. albicans* proliferates in the budding-yeast form, Ash1p is asymmetrically localized to the daughter cell nuclei following each cell division, a pattern previously observed for Ash1p from *S. cerevisiae* (1, 43). When *C. albicans* enters the hyphal form of growth, Ash1p is localized preferentially to the nuclei of the hyphal tip cells: that is, to the nuclei of only the most recently formed hyphal cells. Given that Ash1p is a transcriptional regulator, it seems likely that its presence in hyphal tip cells endows them with a specialized transcriptional program. Indeed, a number of observations in the literature indicate that *C. albicans* hyphal tip cells have properties distinct from those cells that make up the internal (subapical) portions of the hyphae. For example, hyphal tip cells are known to selectively secrete phospholipase B (reviewed in references 14 and 21); it has been proposed that the secretion of this and other hydrolytic enzymes destroys the material in front of the hyphal tip cell and thereby enables it to invade host tissues, as reviewed in reference 18. The hyphal tip cells of the filamentous fungus *Aspergillus niger* are also known to have specialized properties; for example, the enzyme glucomylase is selectively secreted from the tips of mycelia (46). In *C. albicans*, we propose that Ash1p directs, at least in part, the specialization of the hyphal tip cells.

#### MATERIALS AND METHODS

**Strains and media.** Strains used in this study are listed in Table 1. G. Fink and colleagues generously provided the *cph1/cph1* strain, JKC19 (29). The *S. cerevisiae* strains YAS242-10B, YAS243-1A, and YAS204 were obtained from A. Sil and colleagues (University of California—San Francisco [UCSF]; unpublished data). Strains YAS242-10B and YAS243-1A have the *ADE2* gene integrated at

the *HO* locus and the *HO* promoter integrated upstream of the *CAN1* locus. Both of these promoter-gene fusions cause reporter gene expression to be controlled by the *HO* promoter. Deletion of *S. cerevisiae* *ASH1* has been described previously (43). The strains CGX69, YAS204, and YDI-56 are of the *S. cerevisiae*  $\Sigma$ 1278b background (16). YDI-56 is a derivative of YAS204 (*ash1::HisG-URA3-HisG/ash1::HisG-URA3-HisG, ura3Δ/ura3Δ*), which was selected for Ura auxotrophy on plates containing 5-fluoroorotic acid (5-FOA).

*S. cerevisiae* strains were transformed by lithium acetate transformation (15) with selection on SD –Leu or SD –Ura medium. *C. albicans* transformations were performed by the lithium acetate method described in reference 4. Strains were routinely grown on YPD plates and liquid medium at 30°C or on SD –Ura medium unless otherwise noted. Counterselections against the *C. albicans* *URA3* gene were performed by overnight growth at 30°C in YPD liquid medium followed by selection on 5-FOA and uridine.

Phenotype testing and induction of *C. albicans* filamentous growth were done with solid Spider medium as described previously (29); with Lee's medium as described previously (27), at pH 6.8 with the addition of 2% agar; and with YPD with 10% fetal calf serum. Either cells were streaked onto plates, or overnight liquid cultures were diluted in water or YEP medium and plated for single colonies. Plates were incubated for 5 to 6 days at 30°C in plastic sleeves and photographed at a  $\times 7.5$  magnification under a Nikon SMZ-U microscope (Fig. 3A) or a Leica M420 microscope (Fig. 3B and C). Sabouraud dextrose agar (Difco), used for tissue fungal burden assays, was prepared according to the manufacturer's directions.

**Cloning the *C. albicans* *ASH1* gene.** The *S. cerevisiae* Ash1p sequence (GenBank accession no. CAA82028.1) was used in a tBlastn search of the *Candida albicans* sequence database (<http://alces.med.umn.edu/gbsearch/ycb.html>), which yielded three overlapping sequence tags with 63% identity to the C terminus of *S. cerevisiae* Ash1p. With these sequence data, the oligonucleotides DI-1FW (5'-TCTACACACAAATTCATCC-3') and DI-2RV (5'-ATTTAGGAAGTACTTCAA CT-3') (Operon) were designed and used to amplify a 357-bp fragment from *C. albicans* SC5314 genomic DNA. The resulting PCR fragment was gel purified (Qiaquick gel extraction kit), labeled with <sup>32</sup>P in a random prime reaction (Amersham), and used to probe a size-selected *C. albicans* genomic library made in the Lambda ZapII vector (kindly provided by B. Braun; [www.sacs.ucsf.edu/home/JohnsonLab/](http://www.sacs.ucsf.edu/home/JohnsonLab/)). Eleven strongly hybridizing clones were obtained. Eight clones were sequenced through the open reading frame (ORF)-containing region and through the vector junctions. Six of these clones contained a full-length *ASH1* ORF, and two were N-terminal truncations. One full-length clone, pDI-16, was sequenced to completion and found to contain 1.3 kb upstream and 335 bp downstream, including part of another ORF, *DSK2*, which begins 185 bp after the stop codon of *ASH1*. Alignment of the Ash1p proteins was done with GCG Pileup program (Wisconsin Package, version 8.0; Genetics Computer Group, Madison, Wis.) and presented for viewing with Seqvu 1.1 (Garvan Institute of Medical Research, Sydney, Australia).

**Expression of *C. albicans* *ASH1* in *S. cerevisiae*.** For expression studies, the *C. albicans* *ASH1* ORF was amplified by PCR with *Pfu* Turbo polymerase (Strat-

agene) by using primers DI-5FW (5'-CAAACACTACCGTGATACAC-3') (Operon) and DI-25RV (5'-GGATCCGAGCTCATGTTGATTATTCGGTA TAGAG-3') (UCSF BRC) with plasmid pDI-16 as the template. A 2.6-kb fragment that includes sequence 1 kb upstream and 185 bp downstream of *ASH1* was cloned into the vector pRS425 (42) as a *XhoI-SacI* fragment to construct the *LEU2*-marked plasmid pDI-24. This PCR-generated insert was sequenced to verify its accuracy. The *XhoI-SacI* fragment was digested from pDI-24 and cloned into pRS426 to create the *URA3*-marked plasmid pDI-25. The *S. cerevisiae* *ASH1* expression plasmid, pAS174, was described previously (43). pAS199 (A. Sil, unpublished observations) contains the same *S. cerevisiae* *ASH1* fragment as pAS174, except pRS425 (*LEU2*) is the vector backbone. For the *HO* repression assays, strains were streaked onto SD -Leu plates containing 10 mg of adenine per ml and 0.03% canavanine (1), incubated for 5 days at 30°C, and then transferred to 4°C for 3 days to enhance color differences between Ade<sup>+</sup> and Ade<sup>-</sup> strains before photographing. Pseudohyphal growth assays were performed by incubating cells at 30°C for 5 days on SLAD medium containing 50 μM ammonium sulfate (17). Photographs were taken at a magnification of ×38 on a Nikon SMZ-U microscope.

**Disruption of the *C. albicans* *ASH1* gene.** To disrupt the *C. albicans* *ASH1* gene, a modified Ura blaster method was used (5, 13). Heterozygous strains were first constructed by replacing *ASH1* coding sequences with the *URA3* selectable marker flanked by *HisG* repeats. Ura<sup>+</sup> strains that were deleted for one copy of *ASH1* were grown on nonselective medium and plated on 5-FOA and uridine medium to select for loss of the *URA3* gene. Two independent Ura<sup>-</sup> *ASH1* heterozygous strains were used to disrupt the second allele of *ASH1* with another *HisG-URA3-HisG* cassette, pDI-23. Sequences from the 5' and 3' regions of *ASH1* were amplified by PCR, digested with restriction enzymes, and cloned into the disruption vector pBB510, a derivative of pMB7 (13) described in reference 5, to create the disruption plasmid pDI-03. The 5' flank was amplified with primers 5' KO-HIII (5'-TACATTAAGCTTCGTGCTGGTTCATTACGCC-3') and 3' KO-PstI (5'-ATGTAACCTGAGTTCGGAGTTTGGTTGTAGG-3'), digested with *HindIII* and *PstI*, and then cloned into the *HindIII-PstI* sites of pBB510 to create the cloning intermediate p510-5'. The 3' flank was amplified with the primers 5' KO2-NsiI (5'-ATTGAAAATGCATAGCTAAATAACCAT CATCATCACGCC-3') and 3' KO2-KpnI (5'-AATACTGGTACCAACTC AAGATTTAGGAAGT-3'), digested with *NsiI* and *KpnI*, and cloned into the *NsiI-KpnI* sites of p510-5'. The resulting plasmid, pDI-03, contains the *HisG-URA3-HisG* disruption cassette flanked by sequences in the *ASH1* promoter and within the 3' end of *ASH1*. To avoid disruption of *DSK2* promoter sequences at the 3' untranslated region (UTR) of *ASH1*, the 3' flank contains ORF sequence from the 3' end of *ASH1*. A second disruption plasmid, pDI-23, was made by digesting pDI-03 with *Bam*HI and *Bgl*II to release the *HisG-URA3-HisG* cassette, which was then gel purified and cloned back into the pDI-03 backbone in the reverse orientation.

Strains with *ASH1* deleted were created by homologous integration of a *HindIII-XmnI* fragment from pDI-03 (to disrupt the first allele) or pDI-23 (to disrupt the second allele). Isolates were screened by PCR and by Southern blotting for the presence of both disruption constructs at the *ASH1* locus and for the absence of the disrupted ORF sequence (data not shown). Four independent isolates homozygous for deletions in *ASH1* were obtained by this method. Because *ash1* mutants obtained by the Ura blaster method retained 93 codons (although no in-frame start codons) at the C terminus, a fifth isolate with the complete *ASH1* coding sequence deleted was obtained by the PCR product method (48, 49) in the Ura<sup>-</sup> His<sup>-</sup> strain RM1000 (34). The primers DI-55DR (5'-CCGAAGAACCTAAAAAAGTAGTCAACATTTGTCGAAGCT ACCAAATAACCCAGTAGTTTTCCCGAGTCACGCGTT-3') and DI-56DR (5'-TAACAGATATCTAATCCTATATAAATGAAGCTTCCTTTACA ATACTTTTCTAAACTCAAGTGTGGAATTGTGAGCGGATA-3') (vector hybridizing sequence is underlined) were used in separate PCRs with the templates pGEM-HIS1 and pDDB57 to generate the PCR disruption products. Whole-cell PCR, using primers internal to the *HIS1* (5'<sup>HIS1</sup>-RV, 5'-TTGACT ATACCTTCGCTGTC-3'; 3'<sup>HIS1</sup>-FW, 5'-GCAATAAACCCCTTGTGGAC-3') or *URA3* (5'<sup>URA3</sup>-RV, 5'-TGGTGAGGCATGAGTTTC-3'; 3'<sup>URA3</sup>-FW, 5'-GAGATGCTGTTGGAATGC-3') selectable markers and outside the flanking region of homology to the *ASH1* locus (upstream DI-8-FW, 5'-TCAA GACAAATCACAATTC-3'; downstream DI-9RV, 5'-ATCCTTCAACACCT TTCC-3'), was used to identify an *ash1::URA3/ash1::HIS1* isolate. The phenotype of this strain, with a complete deletion of the *ASH1* ORF, was identical to those of *ash1::HisG/ash1::His-URA3-HisG* strains obtained by the URA blaster method.

**Reintroduction of the wild-type *ASH1* gene into *C. albicans* *ash1/ash1* mutants.** A *KpnI-SacI* fragment from pDI-25 was cloned into the *KpnI-SacI* sites of pDI-26 (D. Inglis, unpublished), a derivative of the maltose-inducible expression

plasmid pAU15 (kindly provided by M. A. Uhl), to create the reintegration construct, pDI-29, which expresses *ASH1* under the control of its own promoter. pDI-29 was linearized with *PacI* and transformed into strain YDI-11 (*ura3Δ/ura3Δ, ash1::HisG/ash1::HisG*). Ura<sup>+</sup> transformants were screened by whole-cell PCR with primers DI-20FW (5'-CTGATTTAGTCTACACTACCAC-3') and DI-46RV (5'-AGATCTAGTGTGTAAGGG-3') (Operon) to verify correct insertion of *ASH1* at the 5' region of *ash1::HisG*. Forty-five Ura<sup>+</sup> transformants that yielded positive results by PCR were tested on Spider plates to compare the filamentous growth of these strains with that of the *ash1/ash1* parent strain and the *ASH1/ash1* heterozygote, which also carries one copy of *ASH1*. Surprisingly, the integration of wild-type *ASH1* with plasmid pDI-29 failed to restore filamentous growth to all but 2 of the 45 transformants that screened positive by PCR for correct integration of the plasmid. Of these two transformant strains, one (YDI-154) was slightly more filamentous than the wild-type CAF2-1 strain on Spider medium (data not shown). The other transformant (YDI-157) produced filaments similar to those of the *ASH1/ash1* heterozygote (YDI-1) and was also restored for virulence comparable to that of YDI-1.

**Localization studies of Ash1p.** A PCR fragment generated by using *Pfu* Turbo polymerase (Stratagene) and containing a multimerized epitope of *myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn), flanked by *Bgl*II restriction sites, was cloned into the *Bgl*II site 8 amino acids downstream of the third potential start methionine at the N terminus of the *ASH1* coding sequence of the 2 μm *CaASH1* plasmid, pDI-25. The insertion was sequenced for accuracy, and the plasmid complemented the pseudohyphal growth defects of the *S. cerevisiae* *ash1/ash1* mutant YDI-56. An *XhoI-HindIII* fragment containing *myc-ASH1* and 1 kb of the *C. albicans* *ASH1* promoter sequence from pDI-25 was cloned into the *XhoI-HindIII* sites of pDI-29 to generate the plasmid pDI-30, which is identical to the reintegration construct pDI-29, except for the sequence encoding the *myc* epitope. pDI-30 was digested with *PacI* and integrated into the *ASH1* promoter region of YDI-11 to generate the *myc*-tagged *ASH1* strain YDI-199.

For cells in the budding-yeast form of growth, cultures were grown to mid-log phase (optical density at 600 nm [OD<sub>600</sub>] of ~ 6) in M199 culture medium (pH 4.5) at 23, 25, and 30°C or in YPD at 25 or 30°C. Chains of connected budding yeast cells (pseudohyphae) were produced by growth in M199 (pH 7.0) at 23°C. Hyphal forms of *C. albicans* were induced by growing late-log (OD<sub>600</sub> of 20) to stationary-phase cultures (OD<sub>600</sub> of 40) and diluting cells to OD<sub>600</sub> of 0.8 to 1.0 in prewarmed YPD with 10% serum at 30, 35, and 37°C or in YPD at 35°C with 20% serum in a shaking water bath. Cells were harvested by sterile pouring or by transfer with a wide-bore pipette tip into a 15-ml conical tube and fixed at room temperature in 4.5% formaldehyde for 1 h. Cells were prepared for antibody hybridization and microscopy by methods described in reference 43 with modifications for *C. albicans* based on reference 45. Cells were resuspended in a total volume of 0.5 ml in SP (1.2 M sorbitol, 0.1 M potassium phosphate) buffer with 1 to 2 μl of β-mercaptoethanol and 40 μl of Zymolase-20T (ICN Pharmaceuticals) and incubated at 37°C with gentle shaking for a total of 10 to 15 min for yeast-form cells and 15 to 20 min for hyphal cells. Sixteen microliters of cells was transferred to a polylysine-coated slide well, and cell wall digestion was continued for 10 min at room temperature, while the cells settled onto the slide wells. Excess solution was gently aspirated from the wells, and the cells were allowed to dry. To flatten cells, slides were submerged in -20°C methanol for 5 min, followed by -20°C acetone for 30 s, and then dried completely. The 9E10 *myc* monoclonal antibody (a gift from Joachim Li, UCSF) was used at a 1:6,000 dilution for the yeast and hyphal experiments shown. Previous batches of 9E10 from the same source have been used at 1:300 for yeast-form cells and at 1:800 for hyphal cells. A Cy3-conjugated goat anti-mouse antibody (obtained from Jackson ImmunoResearch Laboratories, West Grove, Pa.) was used at a 1:200 dilution to detect the *myc* epitope. A rabbit polyclonal antibody (38) raised against *S. cerevisiae* Tup1p cross-reacts with *C. albicans* Tup1p and produces a staining pattern that is tightly associated with the nucleus in both mother and daughter cells of *C. albicans* yeast and filamentous forms. A purified version of this antibody (S. Green, unpublished observations) was used at 1:400 as a positive control for nuclear stain and detected with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (SC2012; Santa Cruz Biotechnology). Cells were blocked at room temperature for 1 to 2 h in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% Tween 20. Sixteen microliters of the 9E10 and anti-Tup1p primary antibodies (diluted in PBS with 1% BSA) was hybridized to cells for 1 h at room temperature. Sixteen microliters of secondary antibodies was hybridized to cells in the dark for 1 h. Excess primary and secondary antibodies were removed by washing the slide wells four times with PBS. To visualize cell nuclei, cells were stained after the first secondary antibody wash by applying 16 μl of 1-μg/ml 4',6'-diamidino-2-phenylindole (DAPI) (diluted in PBS) to slide wells for 2 min. Slide wells were then washed four times with PBS and aspirated before the slides were

treated with Fluoromount-G (Southern Biotechnology Associates, Inc.). Digital images were captured by a charge-coupled device (CCD) camera on a Leica DMLB microscope through either a  $\times 100$  oil objective lens for yeast cell images or through a  $\times 40$  objective lens for hyphal cell images.

**Virulence studies and histological sections.** Groups of six female (18 to 20 g) BALB/c mice (Charles Rivers) were each injected by tail vein with *C. albicans* cells and monitored daily for survival and other signs of infection. Cells were prepared for injection by diluting overnight cultures (grown at 30°C in liquid YPD) into fresh YPD to an OD<sub>600</sub> of 0.1 to 0.2, growing the cells for approximately 4 h, and then washing and resuspending the cells in sterile saline (0.9% NaCl<sub>2</sub>) and counting them with a hemacytometer. A total of 10<sup>6</sup> cells per mouse (0.5 ml of 2  $\times$  10<sup>6</sup> cells per ml of solution) were injected by tail vein. Animals were sacrificed by CO<sub>2</sub> and handled according to UCSF Committee on Animal Research (CAR) guidelines.

Whole kidneys were dissected from infected mice and prepared for histological staining (41) by being fixed overnight in 4% neutral buffered formalin (NBF), followed by serial dehydration in alcohol and then toluene. Samples were embedded in paraffin, cut into 5- $\mu$ m sections, and stained with a periodic acid-Schiff base (Sigma Diagnostics), followed by a light green counterstain (Harleco). Images were photographed through a Leica DMLB microscope. Colony counts were obtained by removing kidneys from infected mice, weighing them, and homogenizing the tissue in 1 to 3 ml of sterile distilled water. Serial dilutions were plated onto Sabouraud dextrose agar, and plates were incubated at 30°C for 1 to 2 days before CFU were counted.

**Nucleotide sequence accession number.** The complete *C. albicans* *ASH1* sequence has been submitted to the GenBank database under accession no. AF237674.

## RESULTS

**Identification and sequence of *C. albicans* *ASH1*.** To identify an *ASH1* homolog in *C. albicans*, we searched the partial *C. albicans* sequence database available in July 1998 (<http://alces.med.umn.edu/gbsearch/ybc.html>) for sequence traces similar to those of *S. cerevisiae* Ash1p. A 577-nucleotide sequence was identified that, when translated, matched the zinc finger region located in the C terminus of *S. cerevisiae* Ash1p. Oligonucleotides were designed to PCR amplify a 358-nucleotide-pair fragment from genomic DNA, and this PCR product was used to probe a lambda library containing *C. albicans* genomic fragments (5). Several full-length clones were obtained and sequenced, and the complete *C. albicans* *ASH1* sequence was submitted to the GenBank database (accession no. AF237674). This sequence is identical to that now found in assembly 6 of the *C. albicans* genome sequence provided by the Stanford DNA Sequencing and Technology web site (<http://www-sequence.stanford.edu/group/candida/>).

*C. albicans* *ASH1* is predicted to encode a 449-amino-acid protein with 36% identity overall to the *S. cerevisiae* protein (Fig. 1). The zinc finger region is 81% identical over 34 amino acids. The N-terminal regions are much less similar, with only 23% identity over 359 amino acids. Like the *S. cerevisiae* protein, *C. albicans* Ash1p has three potential consensus sites for phosphorylation by Cdc28/CDK (1) and two consensus sites in the zinc finger for acetylation, a modification that alters the activity of the related GATA-1 transcriptional regulator in mammals (3).

***C. albicans* Ash1p can regulate expression of the *HO* promoter and promote pseudohyphal growth in *S. cerevisiae*.** As reviewed in the introduction, *S. cerevisiae* Ash1p regulates both expression of the *HO* gene (which encodes the endonuclease that initiates mating-type switching) (1, 43) and pseudohyphal growth under conditions of nitrogen starvation (8). To determine whether *C. albicans* Ash1p is a functional homolog of the *S. cerevisiae* protein in both respects, we asked whether *C.*

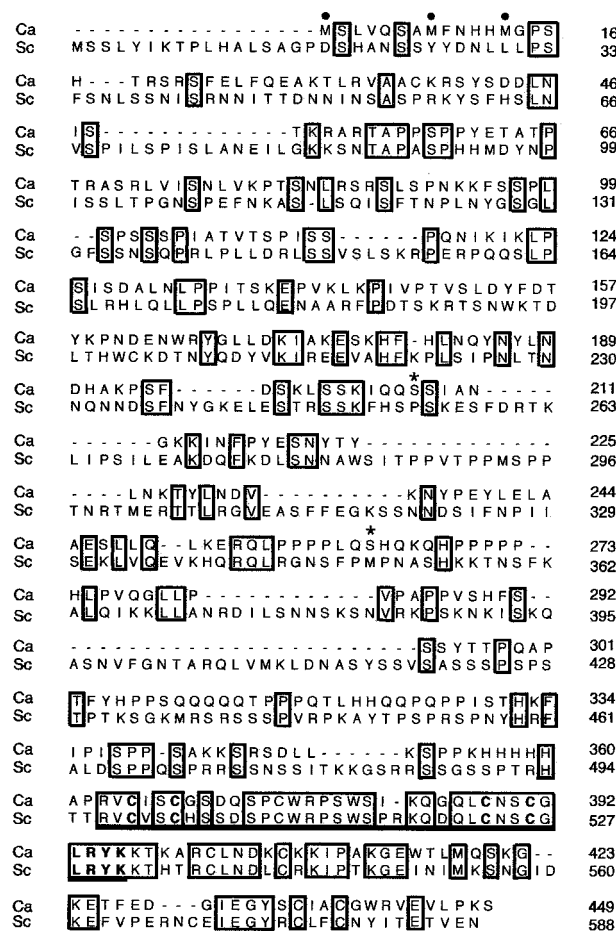


FIG. 1. Sequence alignment of the predicted amino acid sequences from *C. albicans* and *S. cerevisiae* Ash1p. Boxes indicate identical residues. Three possible start codons for *C. albicans* Ash1p are indicated by dots. Two predicted serine residues encoded by nonstandard CUG codons of *C. albicans* (39) are indicated by asterisks. The highly conserved GATA-like zinc finger region is underlined. Amino acid numbers are indicated on the right.

*albicans* Ash1p could regulate (in this case, repress) expression of *HO* and promote pseudohyphal growth in *S. cerevisiae* *ash1* mutant strains. For these experiments, *C. albicans* *ASH1* and *S. cerevisiae* *ASH1* were each cloned into a high-copy-number 2 $\mu$ m vector and transformed into *S. cerevisiae* *ash1* mutant strains.

To detect *HO* repression, we used a reporter strain with *ASH1* deleted and which carries the *CAN1* and *ADE2* reporter genes, each under the control of the *HO* promoter (Fig. 2A, B, and D). A control strain (Fig. 2C) carries both reporter constructs, the wild-type genomic copy of *ASH1*, and a vector control plasmid. These strains allow growth of colonies on medium that contains canavanine only when *HO* is repressed, because expression of *CAN1* is lethal in the presence of canavanine. When *ASH1* is deleted from this strain, colonies fail to grow in the presence of canavanine, indicating that *HO-CAN1* is expressed (Fig. 2D). The *HO-ADE2* gene provides a second test of *HO* expression: expression of *HO-ADE2* produces white colonies in low-adenine medium, whereas repression of *HO-*

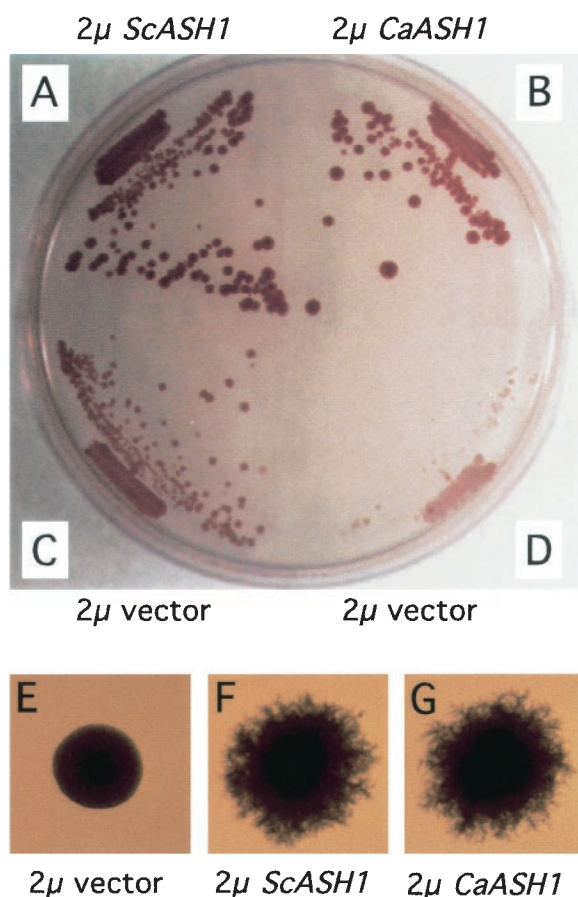


FIG. 2. *C. albicans* Ash1p represses *HO* and promotes pseudohyphal growth in *S. cerevisiae* *ash1* mutant strains. The *HO-ADE2 HO-CAN1 ash1* reporter strain produces red colonies on 0.03% canavanine and 10 mg of adenine per ml of SD –Leu medium when transformed with a high-copy-number plasmid that carries either *S. cerevisiae* (A) or *C. albicans* (B) *ASH1*. The same strain transformed with vector (D) fails to grow on this medium; the few surviving colonies are white, indicating *HO-ADE2* is not repressed. The *HO-ADE2 HO-CAN1 ASH1* control strain (C) has the genomic copy of *ASH1* intact and contains the vector control plasmid. Strains were incubated for 5 days at 30°C. For the experiments shown in panels E to G, diploid *ash1/ash1* strains of the  $\Sigma$ 1278b background were transformed with the indicated plasmids and then incubated on nitrogen-limiting SLAD medium for 5 days at 30°C. Colonies were photographed at a magnification of  $\times 38$ .

*ADE2* results in red colonies. When the *C. albicans* *ASH1* plasmid was introduced into *S. cerevisiae* *ash1* strains, healthy red colonies grew on low-adenine medium containing canavanine (Fig. 2A and B), indicating that *C. albicans* *ASH1* can repress both the *HO-CAN1* and *HO-ADE2* reporter genes.

To test whether *C. albicans* *ASH1* can stimulate pseudohyphal growth in *S. cerevisiae*, *ash1/ash1* strains of the *S. cerevisiae*  $\Sigma$ 1278b background (17) were transformed with plasmids expressing either the *C. albicans* or the *S. cerevisiae* *ASH1* gene and grown on low-nitrogen medium. We found that *C. albicans* *ASH1* restored pseudohyphal growth as efficiently to the *S. cerevisiae* *ash1/ash1* strain as did the *S. cerevisiae* *ASH1* (Fig. 2F and G). Based on these results, we believe that *C. albicans* Ash1p is a functional homolog of *S. cerevisiae* Ash1p in that it

can replace the function of Ash1p in *S. cerevisiae* both for repression of *HO* and for promotion of pseudohyphal growth.

***C. albicans* *ash1/ash1* mutants have defects in filamentous growth.** *C. albicans* is diploid, and to determine whether *ASH1* has a role in regulating filamentous growth in *C. albicans*, strains lacking both copies of *ASH1* were constructed in the SC5314 background by replacing coding sequences with the *URA3* selectable marker (13). *ASH1/ASH1* (wild type), *ASH1/ash1*, and *ash1/ash1* strains were tested on several types of solid media that induce filament formation. Both *ash1* heterozygous and homozygous mutants show significantly reduced filamentous growth on Spider medium (Fig. 3A). The reintroduction of one copy of wild-type *ASH1* to the *ash1/ash1* strain partially restores filamentous growth (Fig. 3A), indicating that the original filamentous growth defects are attributable to the loss of *ASH1*. On YPD with 10% serum at 30°C, *ash1/ash1* homozygous mutants show a slight reduction in hyphal growth compared with the parental strain (Fig. 3B). *ash1/ash1* mutants showed no obvious defects in forming germ tubes, regarded as precursors to hyphal growth, in response to serum in liquid media (data not shown). On solid Lee's medium at neutral pH, *ash1/ash1* strains were severely reduced for filamentous growth (Fig. 3C). These results show that *ASH1* is important for filamentous growth under several specific environmental conditions.

**Analysis of *ash1 cph1* double mutants.** In *S. cerevisiae*, Ash1p and Ste12p regulate parallel but independent pathways of filamentous growth in response to Ras2p signaling. To determine the relationship between *C. albicans* *ASH1* and the *C. albicans* Ste12p homolog, *CPH1*, *C. albicans* strains lacking both *ASH1* and *CPH1* were constructed by the method described above. On Spider medium, the *ash1/ash1 cph1/cph1* double-mutant strain exhibited less filamentous growth overall than did either of the single-mutant strains, although the double mutant did produce low levels of peripheral hyphae (Fig. 3A). On YPD with 10% serum at 30°C, each of the single mutants formed filamentous colonies, whereas the *ash1/ash1 cph1/cph1* strain formed round, smooth a filamentous colonies under the same conditions (Fig. 3B). These data suggest that Ash1p and Cph1p contribute additively to filamentous growth in *C. albicans*.

**Ash1p is asymmetrically localized to daughter cells.** When *S. cerevisiae* proliferates by budding, Ash1p is asymmetrically localized to daughter cell nuclei, where it represses transcription of the *HO* gene (1, 43). To test whether this asymmetric localization also occurs in *C. albicans*, Ash1p was tagged at its N-terminal end with six tandem copies of the 11-amino-acid myc epitope (23). This altered form of Ash1p complements the filamentous growth defect when reintegrated into the *C. albicans* *ash1/ash1* strain, YDI-11; moreover, *C. albicans* myc6-Ash1p successfully promotes pseudohyphal growth when introduced into the *S. cerevisiae* *ash1* mutant strain, YDI-56 (data not shown).

To localize Ash1p, a strain containing one copy of myc6-*ASH1* as the sole source of Ash1p was stained with monoclonal antibodies against the myc epitope and visualized by indirect immunofluorescence (23). When *C. albicans* was grown as a budding-yeast form (in M199 [pH 4.5] and YPD media at 23 and 30°C, respectively), Ash1p was specifically localized to daughter cell nuclei: of 125 postanaphase cell pairs stained for Ash1, 79% showed localization of Ash1p to the nucleus of the

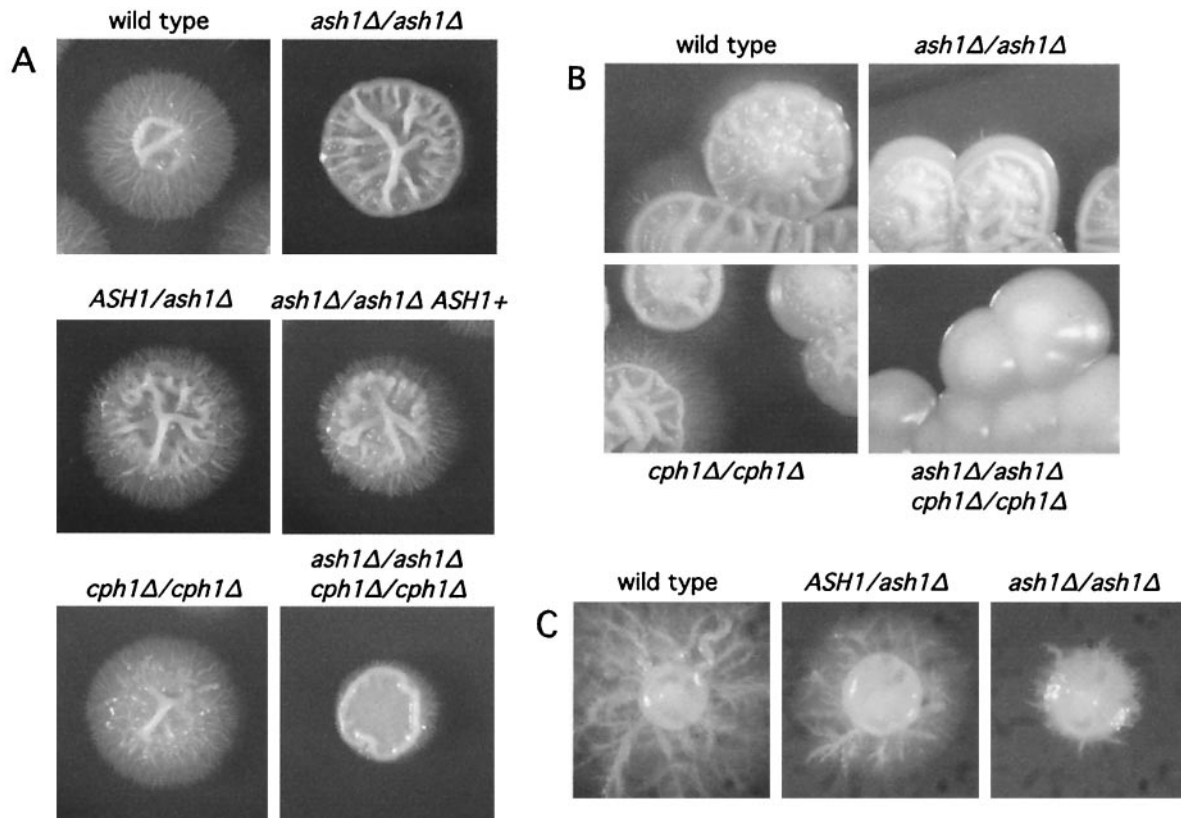


FIG. 3. *ASH1* is required for filamentous growth of *C. albicans*. Heterozygous or homozygous strains of the indicated genotypes were grown on various types of filament-inducing solid media: Spider medium (A), YPD medium plus 10% serum (B), and Lee's medium (pH 6.8) with 2% agar (C). Plates were incubated for 5 to 6 days at 30°C. In panel A, the strain depicted in the panel in the second row of the second column is an *ash1/ash1* strain into which an intact copy of *ASH1* has been introduced.

daughter cell, but not to the nucleus of the mother cell (Fig. 4 and 5A). *C. albicans* Ash1p was not observed in mother-daughter cell pairs undergoing mitosis (not shown), as is also the case for *S. cerevisiae* (1, 43). *C. albicans* yeast-form cells that are exposed to mild filament-inducing conditions bud in a unipolar fashion, similar to pseudohyphal cells of *S. cerevisiae*, and produce chains of cells. Under these conditions, *C. albicans* Ash1p is also localized to daughter cells, seen as cells that bud from the growing chain (Fig. 5B). This pattern of Ash1p localization is similar to that seen in *S. cerevisiae* pseudohyphal cells (8).

**Ash1p is localized to hyphal tip cells.** When *C. albicans* cells are grown for short periods of time in medium that strongly induces hyphal growth (YPD plus 20% serum at 35°C, for example), the mother cells appear rounded, and the daughter cells appear highly elongated, because the latter are beginning to form hyphae. In such mother-daughter pairs, Ash1p is localized specifically in the daughter cell nuclei (Fig. 4 and Fig. 6). Of the stained mother-daughter pairs observed in this study, 92% showed Ash1p in the daughter cell nucleus and not in the mother cell nucleus. If these mother-daughter pairs are incubated for longer times (YPD plus 20% serum at 35°C for 6 to 8 h), mature hyphae form in which multiple cells are joined end to end. In such hyphae, Ash1p is observed in the nuclei of apical hyphal cells (hyphal tip cells), but not in any of the other cells in the hyphae (Fig. 6). The apical cell is the site of active

hyphal growth and constitutes the newest cell of the growing hypha.

Upon longer exposures to YPD plus 20% serum at 35°C, hyphae begin to generate blastospores, which bud off from multiple positions along the hyphae. It also appears that the nuclei of these newly released blastospores stain positively for Ash1p (Fig. 7), but in this experiment, it is difficult to rigorously distinguish cells that have recently budded from hyphae from those that have undergone additional cell divisions.

An important control for all of the localization experiments discussed in this and the previous section is the demonstration that an antibody directed against a different nuclear protein effectively stains all of the nuclei in the cell population. This is particularly important for growing hyphae, because the susceptibility of cells to the staining procedure could vary along the hypha. For this control, we used antibodies against the nuclear protein Tup1 (Fig. 5 to 7). Tup1p is observed in all nuclei (compare with the DAPI-stained images), and this observation rules out the possibility that the daughter cell and hyphal tip-cell-specific staining of Myc-Ash1p is due to the greater susceptibility of these cells to antibody.

***ash1* mutant strains are reduced for virulence.** A mouse model of systemic candidiasis is a sensitive assay for determining differences in virulence between *C. albicans* strains, and in this way, we tested whether Ash1p is important for virulence in

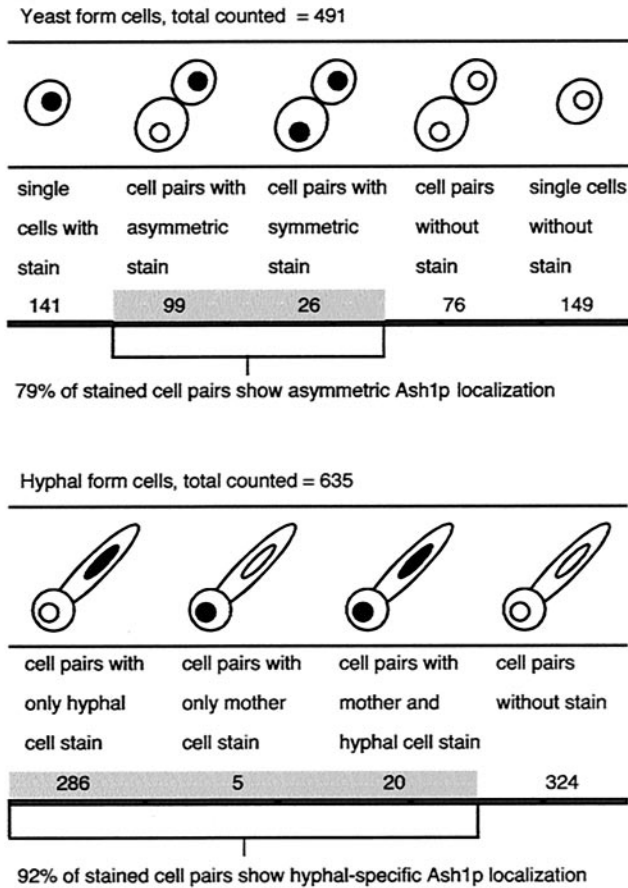


FIG. 4. Summary of the characteristics of the yeast- and hyphal-form cells examined in this study.

vivo. For this analysis,  $10^6$  wild-type or mutant *C. albicans* cells were injected by tail vein into groups of six mice. We performed two separate experiments with two independently derived *ash1/ash1* mutant strains.

In the first experiment, we compared four strains: the *ASH1/ASH1* parental strain (CAF2-1), an *ASH1/ash1* heterozygous strain (YD-01), an *ash1/ash1* (YDI-7) fully mutant strain, and a homozygous mutant strain (YDI-157) that carries an intact *ASH1* gene reintegrated into the genome. As shown in Fig. 8A, disruption of one copy of *ASH1* results in decreased virulence, and disruption of both copies reduces virulence even further. When an intact copy of the *ASH1* gene is reintroduced into the *ash1/ash1* strain, virulence is increased to approximately that of the *ash1/ASH1* heterozygote, a result consistent with the fact that these two strains each have a single intact copy of *ASH1*. This result confirms that the virulence defects of the *ash1/ash1* mutant strain are due to the absence of *ASH1* and not to some other change resulting from transformation or other manipulations used in the construction of mutant strains. The doubling times of *ash1/ash1* mutant strains grown at 30 and 37°C in YPD medium were comparable to that of the wild type when measured in vitro (data not shown), suggesting that its defect in virulence is not due simply to a nonspecific growth defect.

In a second virulence experiment (Fig. 8B), we compared an independently constructed *ash1/ash1* mutant strain (YDI-27), the wild-type strain (CAF2-1), a *cph1/cph1* strain (JJC19), and

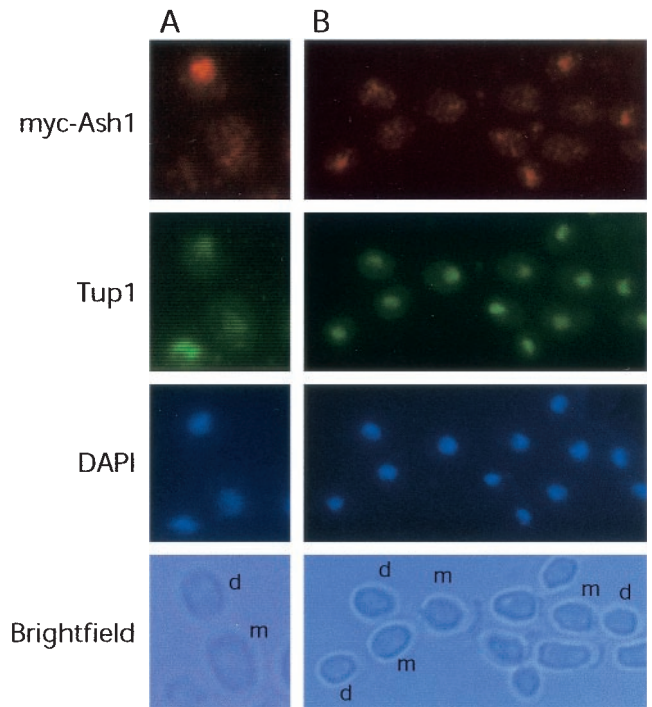


FIG. 5. Ash1p is localized to daughter cells of *C. albicans* growing in the yeast form. (A) Cells expressing myc-Ash1p (YDI-199) were grown in M199 (pH 4.5) at 23°C (conditions that favor the budding-yeast form) and processed for indirect immunofluorescence (see Materials and Methods). Cells were stained for myc-Ash1p with 9E10 mouse antibodies and Cy3-conjugated secondary antibodies that recognize the mouse 9E10 antibody. Tup1p was stained with rabbit polyclonal antibodies and FITC-conjugated secondary antibodies. Cell nuclei were visualized with DAPI stain, and whole cells were examined by bright-field imaging. Cells are stained as described in panel A. Yeast cells grown in M199 (pH 7.0) at 23°C appear as chains of attached budding cells. In panels A and B, selected mother (m) and daughter (d) cells are labeled.

an *ash1/ash1 cph1/cph1* strain (YDI-129). The *cph1* mutants are included in this experiment, because, as described above, the *ash1 cph1* double mutant shows a greater defect in filamentous growth than do the single mutants. As in the first virulence experiment, we found that deletion of *ASH1* leads to a marked reduction in virulence. In contrast, the *cph1/cph1* mutant strain appears fully virulent, as reported by Lo et al. (30). The additional deletion of *CPH1* from the *ash1/ash1* strain led to only small differences in survival of mice infected with these two strains. One notable difference between the *ash1/ash1* strain and the *ash1/ash1 cph1/cph1* double-mutant strain was that animals injected with the double-mutant strain showed fewer symptoms of infection, such as weight loss, reduced activity, or roughened coat appearance, compared to the *ash1/ash1* single-mutant strain, even though the survival times were only slightly different.

***ash1* mutants colonize tissues and produce hyphae in vivo.** To observe the morphology of *C. albicans* cells in vivo, kidneys of mice systemically infected with  $10^6$  *C. albicans* cells were removed, sectioned, and stained with periodic acid-Schiff base for the presence of *C. albicans*. Mice injected with  $10^6$  wild-type *C. albicans* cells of the SC5314 strain background succumb to infection within 2 to 4 days (Fig. 8) and at 2 days show a

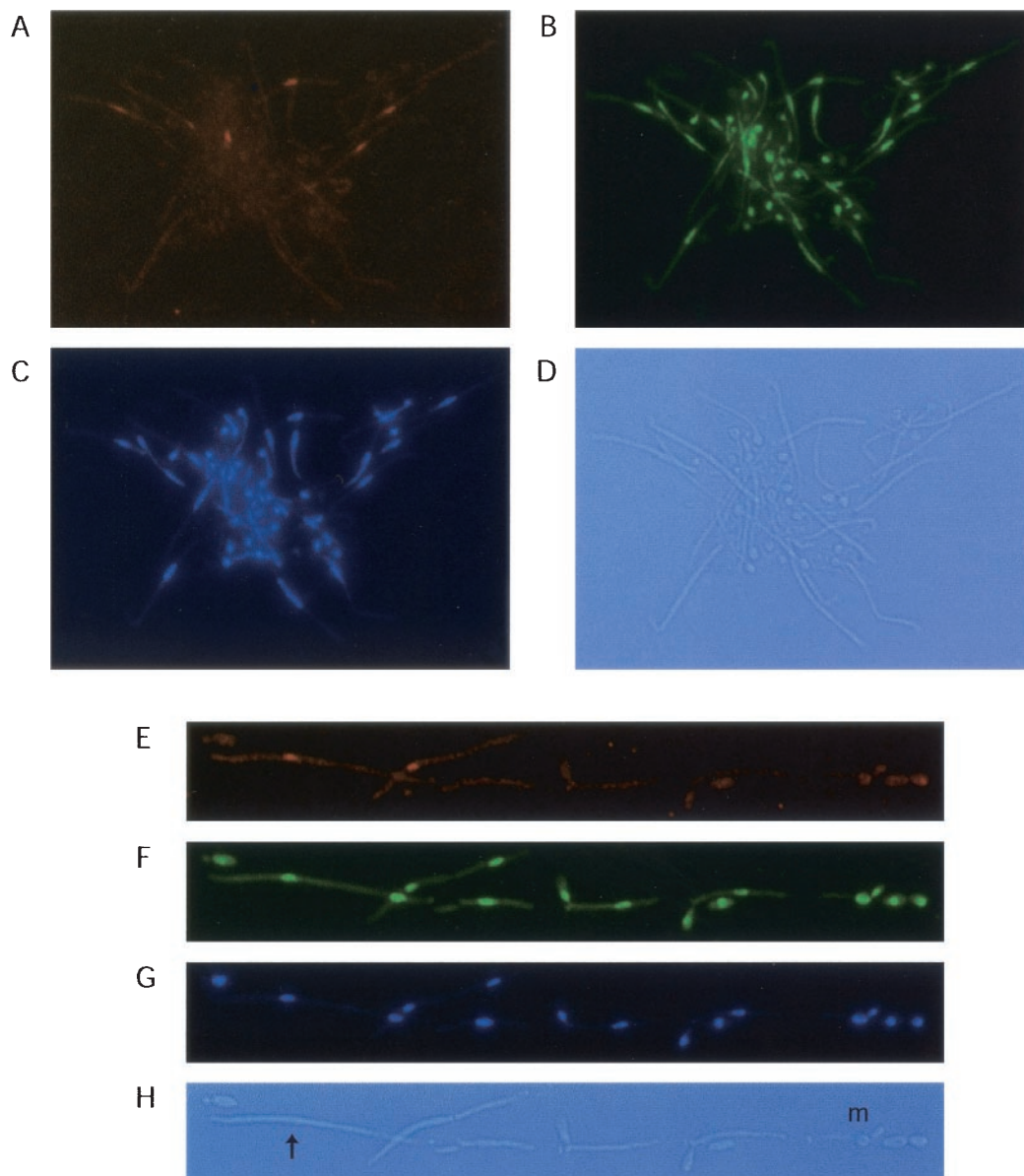


FIG. 6. Ash1p localizes to hyphal tip cells. (A to D) Ash1p is observed in the hyphal daughter cell nucleus of mother-daughter hyphal cell pairs grown for 2 h at 35°C in YPD plus 20% serum (A, anti-myc stain; B, anti-Tup1p stain; C, DAPI stain; D, bright-field image). (E to H) Hyphal cells grown for 4 to 6 h show chains of cells in a hyphal filament with Ash1p located only in the nucleus of the hyphal tip (apical) cell (indicated by the arrow). The original mother cell (m) has also produced budding cells. The images are as described for panels A to D.

large number of *Candida* cells in the kidneys (Fig. 9A and B). Both hyphae and blastospores are visible at the sites of infection. At the same time point, very few *ash1/ash1* cells are observed in the kidney (Fig. 9C and D). At later time points (>7 days), *ash1/ash1* cells could easily be detected in the kidney, and both blastospores and hyphae were present (Fig. 9E and F). Thus, although most mice infected with  $10^6$  *ash1/ash1* cells survive past 18 days, *ash1/ash1* strains were nonetheless observed in the kidney as yeast and filamentous forms after 7 days and even after 30 days (the longest time point at which kidneys infected with the *ash1/ash1* strain were examined; Fig. 9E and F). The tissue fungal burden of animals that succumbed to infection was quantified by homogenizing the kidneys and brains and plating on Sabouraud agar. Both *ash1/ash1* and

*ash1/ash1 cph1/cph1* mutant strains achieved similar levels of colonization ( $3 \times 10^7$  CFU/g of kidney) to those reached by the wild type. However, the wild-type strains achieved this density within a day or 2, while the *ash1/ash1* mutant strains required several weeks. These observations indicate that the virulence defect of the *ash1/ash1* mutants is not simply due to the inability to survive or to grow as filaments in the mouse. It is possible, for example, that the attenuated virulence of *ash1/ash1* mutants is due to defects in the regulation of filamentous growth or in the proper specialization of hyphal tip cells.

#### DISCUSSION

Asymmetric cell division is critical for the development of nearly all organisms, ranging from unicellular bacteria and



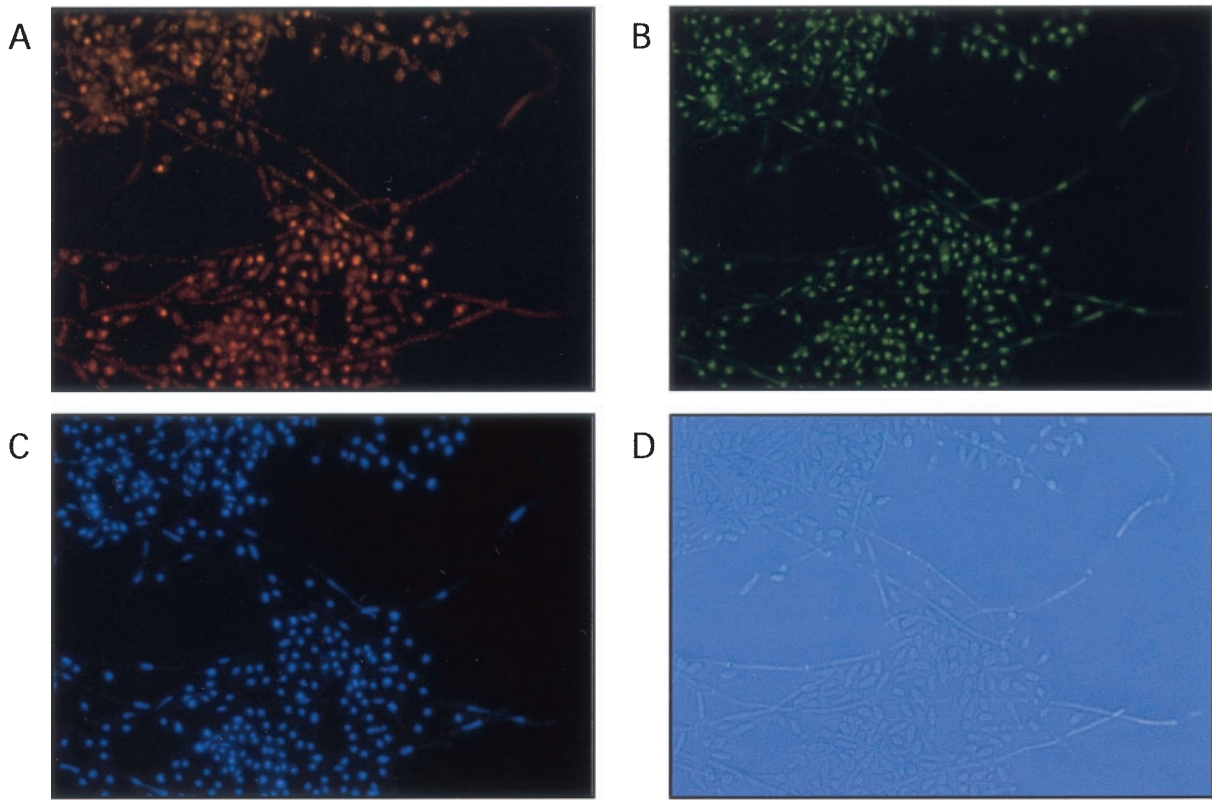


FIG. 7. Mature hyphae produce budding daughter cells that express Ash1p. Cells were grown overnight at 35°C in YPD plus 20% serum and stained as described in the legend to Fig. 6.

yeasts to multicellular plants and animals. The asymmetric localization of gene products to one of the two daughter cells formed during cell division leads to genetically identical cells with the potential for dramatically different cell fates.

In this paper, we show, by staining the transcriptional regulator Ash1p, that the human fungal pathogen *C. albicans* undergoes asymmetric cell division in all three of its morphological forms—budding-yeast-form cells, pseudohyphae, and

hyphae. All three forms are found in infected tissues, and the ability of *C. albicans* to switch between them is thought to be crucial for its pathogenesis (for a recent review, see reference 19). In budding-yeast-form cells, *C. albicans* Ash1p is observed in daughter cell nuclei, but not in mother cell nuclei. It is also observed only in daughter cell nuclei in chains of pseudohyphal cells. Finally, in mature hyphae, Ash1p is observed only in the nuclei of hyphal tip cells—that is, to the cells active in hyphal growth. Thus, as far as can be seen by immunofluorescence, hyphae consist of long, branched chains of elongated cells, with Ash1p absent from all but the growing tip cells.

It has long been appreciated that the hyphal tip (apical) cells of *C. albicans* differ from internally positioned (subapical) hyphal cells. For example, much of the metabolism and growth of hyphae is concentrated in these cells. Moreover, the organelle composition of the hyphal tip cell appears different from that of other hyphal cells; in particular, hyphal tip cells are vacuole poor and cytosol rich compared to the rest of the hyphal cells (for review, see reference 18). Finally, secretion of at least some hydrolytic enzymes occurs selectively at the hyphal tip cell (reviewed in references 14 and 20). Many studies indicate that the penetration of host epithelial surfaces by *C. albicans* is carried out by the hyphal tip cells, and it is reasonable to believe that the tip cells specifically secrete hydrolytic enzymes that damage host tissues, providing sites of penetration. (For recent reviews, see references 19 and 21.) Because Ash1p is localized to the nuclei of hyphal tip cells and because it is a transcriptional regulator, it seems likely that Ash1p regulates

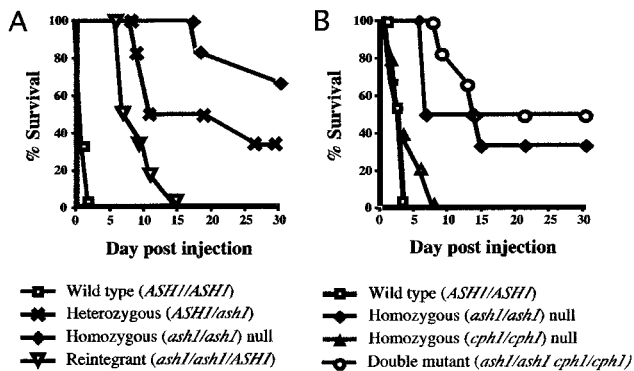


FIG. 8. *ASH1* is important for virulence in a mouse model of systemic candidiasis. Shown are survival curves of mice systemically infected with *URA3*<sup>+</sup> *C. albicans* strains of the genotypes *ASH1/ASH1* (CAF2-1), *ASH1/ash1* (YDI-1), *ash1/ash1* (YDI-7), and *ash1/ash1::ASH1* (YDI-157) (A) or *ASH1/ASH1* (CAF2-1), *ash1/ash1* (YDI-27), *ash1/ash1 cph1/cph1* (YDI-129), and *cph1/cph1* (JKC19) (B).

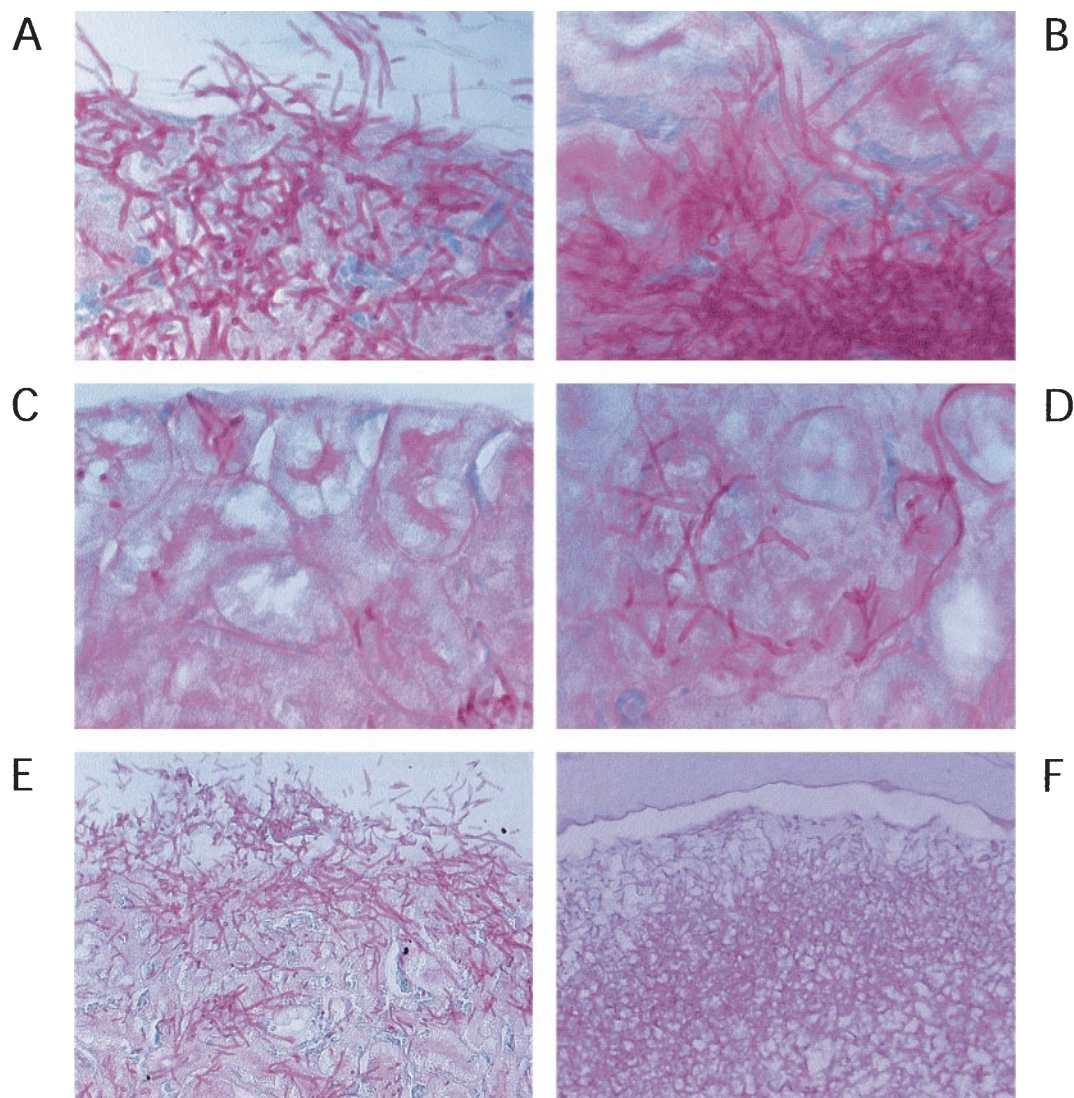


FIG. 9. *ash1/ash1* mutants colonize kidneys much more slowly than do *ash1/ash1/ASH1*+ reintegrants, although they do produce hyphae in vivo. Histological sections of mouse kidneys were prepared as described in Materials and Methods. (A, B) The *ash1/ash1/ASH1*+ strain 24 h after infection. Panel A shows a locus of infection on the edge of the kidney and a large colony inside the kidney (B). (C to F) Shown are the *ash1/ash1* mutant strains after 2 (C and D), 15 (E), and 30 (F) days. Panels A to D were photographed at  $\times 40$ , and panels E and F were photographed at  $\times 20$ .

at least a portion of the specializations that take place in hyphal tip cells.

In addition to specifically “marking” daughter cells, Ash1p is also required for filamentous growth on some types of filament-inducing medium. However, the severity of the defect caused by deleting *ASH1* varies, depending on the nature of the medium. These results suggest that one function of Ash1p in daughter cells is to establish or maintain filamentous growth on certain types of media. For example, *ash1/ash1* mutant cells show a pronounced defect of filamentous growth on Spider (low-nutrient) medium. It has been proposed that, in response to this medium, *C. albicans* grows filamentously in a “foraging mode,” seeking out new locations of greater nutritional richness (24). It is possible Ash1p in the hyphal tip cells regulates a process that is required for hyphae to sense or to respond to

low-nutrient medium, thus causing the filamentation defect of *ash1/ash1* mutants on this medium.

In *S. cerevisiae*, the best-understood function of Ash1p takes place in budding cells. In daughter cells, it represses transcription of the *HO* endonuclease gene, which carries out the first step in mating-type interconversion. Because Ash1p is specifically localized to daughter cell nuclei, only mother cells are able to switch mating types. As far as is known, *C. albicans* is unlikely to carry out mating-type interconversion; its genome lacks silent mating cassettes and a gene closely related to *HO*. It is possible that the asymmetric localization of Ash1p has a deeply conserved function in *S. cerevisiae* and *C. albicans* (perhaps involving filamentous growth) and that its regulation of *HO* was a relatively recent evolutionary add-on in *S. cerevisiae*.

Finally, our work shows that *ash1/ash1* mutants of *C. albi-*

*cans* are significantly attenuated for virulence in a mouse model of disseminated candidiasis. Examination of the kidneys revealed that the *ash1/ash1* mutants can still form hyphae *in vivo*; however the number of hyphal cells observed in the kidney was significantly smaller than that of the wild-type control strain observed at the same time after infection. Although it is premature to make a firm conclusion, it is possible that the virulence defect in the *ash1/ash1* strain is due to subtle defects in the regulation of hyphal formation or to a defect in hyphal tip cell specialization.

In conclusion, this study has focused on the role of *C. albicans* Ash1 protein. We found that this protein is required for filamentous growth under certain laboratory conditions, is required for full virulence in a mouse model of infection, and marks daughter cell nuclei in budding and pseudohyphal forms and tip (apical) cell nuclei in hyphae. We propose that the asymmetric localization of Ash1p is crucial for the proper specialization of the hyphal tip cell and that this specialization is important for virulence.

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#### ADDENDUM IN PROOF

While this paper was under revision, the expression of *C. albicans* *ASH1* in *S. cerevisiae* was independently reported by Munchow et al. (S. Munchow, D. Ferring, K. Kahlina, and R. P. Jansen, *Curr. Genet.* **41**:73–81, 2002).

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