Development and Application of a Green Fluorescent Protein Sentinel System for Identification of RNA Interference in *Blastomyces dermatitidis* Illuminates the Role of Septin in Morphogenesis and Sporulation

T. Krajaejun,1 G. M. Gauthier,3 C. A. Rappleye,5 T. D. Sullivan,2 and B. S. Klein1,2,3,4*

Departments of Medical Microbiology and Immunology,1 Pediatrics,2 and Internal Medicine3 and Comprehensive Cancer Center,4 University of Wisconsin Medical School, University of Wisconsin Hospital and Clinics, Madison, Wisconsin 53792, and Department of Microbiology, The Ohio State University, Columbus, Ohio5

Received 16 December 2006/Accepted 1 May 2007

A high-throughput strategy for testing gene function would accelerate progress in our understanding of disease pathogenesis for the dimorphic fungus *Blastomyces dermatitidis*, whose genome is being completed. We developed a green fluorescent protein (GFP) sentinel system of gene silencing to rapidly study genes of unknown function. Using Gateway technology to efficiently generate RNA interference plasmids, we cloned a target gene, “X,” next to GFP to create one hairpin to knock down the expression of both genes so that diminished GFP reports target gene expression. To test this approach in *B. dermatitidis*, we first used *LACZ* and the virulence gene *BAD1* as targets. The level of GFP reliably reported interference of their expression, leading to rapid detection of gene-silenced transformants. We next investigated a previously unstudied gene encoding septin and explored its possible role in morphogenesis and sporulation. A *CDC11* septin homolog in *B. dermatitidis* localized to the neck of budding yeast cells, *CDC11*-silenced transformants identified with the sentinel system grew slowly as flat or rough colonies on agar. Microscopically, they formed ballooned, distorted yeast cells that failed to bud, and they sporulated poorly as mold. Hence, this GFP sentinel system enables rapid detection of gene silencing and has revealed a pronounced role for septin in morphogenesis, budding, and sporulation of *B. dermatitidis*.

The dimorphic fungi are a group of primary pathogens that are distributed worldwide and can infect both immunocompromised and immunocompetent individuals. They include organisms from various genera, such as *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, and *Penicillium marneffei*. Infections caused by these fungi are increasing in immunocompromised hosts with AIDS, malignancy, autoimmune disorders, organ transplants, or immunosuppressive medication (29). An improved understanding of the biology and pathogenesis of these fungi is needed for the development of additional preventive and therapeutic measures to combat these infections.

*B. dermatitidis* is endemic to the Ohio-Mississippi river valleys and causes pneumonia, with or without dissemination, following the inhalation of conidia or hyphal fragments from the soil. Upon entry into the host, the fungus undergoes a temperature-dependent phase transition from the conidium form at environmental temperatures (22°C under laboratory conditions) to the pathogenic yeast form at body temperature (37°C). This transition is crucial for virulence and pathogenesis (25, 27). Only two genes of *B. dermatitidis*—*BAD1* (*Blastomyces adhesin 1*) (3–5, 13) and *DRK1* (dimorphism-regulating histidine kinase 1) (21)—have been studied in detail for their roles in virulence or the phase transition of mold to yeast. Current efforts are directed toward identifying additional genes that contribute to virulence and morphogenesis of this and related dimorphic fungi.

The sequenced genome of *B. dermatitidis* is nearly complete, paving the way for functional and comparative genomic studies of this pathogen and underscoring the need for reverse genetic approaches to studying biology and virulence. Homologous recombination in dimorphic fungi, including *B. dermatitidis* (3, 21), occurs at a low frequency, can be time-consuming to achieve, and requires extensive knowledge of the sequence for the gene to be targeted. Recently, RNA interference (RNAi), one form of gene silencing, was discovered as a conserved mechanism among eukaryotes for disrupting gene expression (1, 18). RNAi offers advantages over homologous recombination, since less target sequence information is needed, it may occur at a higher frequency, it can be effective in multineucleate or diploid organisms, and it requires shorter periods to generate a silenced mutant. The RNAi strategy has been exploited successfully for reverse genetic analysis of many organisms, including fungi (1, 7, 8, 18, 22, 23, 28). A valuable refinement to these systems is the use of a reporter or sentinel gene to identify active RNAi lines among transformants (15, 17, 20).

Here we describe a sentinel RNAi system using a surrogate green fluorescent protein (GFP) signal to rapidly identify strains with alterations in the expression of a specific target in *B. dermatitidis*. This kind of rapid screen is especially useful for functional analysis of genes with unknown mutant phenotypes.
The system relies on RNAi technology and a GFP-expressing fungal strain as a reporter (17). A target gene, “X,” is fused to GFP and cloned as two inverted copies in an RNAi vector to generate one hairpin with both target sequences. We postulated that a strain with diminished expression of GFP would also have decreased expression of the target gene “X,” allowing us to quickly screen for phenotypes associated with altered gene expression. We initially used the well-characterized genes BAD1 and LACZ to test this concept. Once established, we exploited this GFP-tracking strategy for reverse genetic analysis of a septin gene (CDC11) to better understand the genes that regulate the morphogenesis of dimorphic fungi. Using this technology, we uncovered a previously unidentified and pronounced role for the CDC11 septin gene during morphogenesis and sporulation of B. dermatitidis.

MATERIALS AND METHODS

Strains and growth conditions. B. dermatitidis strain 26199 is a patient isolate obtained from the American Type Culture Collection (Rockville, MD) (14) and is the strain whose genome is currently being sequenced (http://genome.wustl.edu/tools/BLAST/). Strain 14081 (21) is an environmental isolate ER-3, is the spontaneous uracil auxotrophic strain ER-3 ure-5-111 (30) that was transformed with pJ351, containing URA5 and the f-galactosidase reporter LACZ driven by the BAD1 promoter. Yeast cells were grown at 37°C on Histoplasma macrophage medium (HMM) (33), Middlebrook 7H10 with oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson and Company, Franklin Lakes, NJ), or 3M medium (33). To quantitatively produce, 1.5 x 10^10 yeast cells were incubated at 22°C on 3M medium supplemented with 100 μM of hygromycin B for 14 days. Spores were harvested from mycelia by manual disruption and counted on a hemacytometer. The use and maintenance of Agrobacterium tumefaciens strain LBA1100 were done as previously reported (30).

RNA extraction and cDNA synthesis. B. dermatitidis yeast cells were grown in liquid HMM at 37°C for 1 to 2 days and then harvested. The yeast pellet was disrupted with mini glass beads, using a Mini-Beadbeater-8 (Biospec Products). Total RNA was extracted by using TriReagent RNA/DNA/protein reagents according to the manufacturer’s protocol (Molecular Research Center, Inc.). RNAs were further purified using an RNeasy Mini kit (QIAGEN), the concentration was measured using spectrophotometry (Nanodrop Technologies, Inc., Wilmington, DE), and the integrity was evaluated by agarose gel electrophoresis.

To prepare cDNA, 1 μg of total RNA was reverse transcribed using TaqMan reverse transcription reagents and the manufacturer’s protocol (Roche Molecular Systems, Inc., Branchburg, NJ).

Molecular cloning and plasmid construction. GFP was amplified from pMAD868 (provided by Jon P. Woods, University of Wisconsin-Madison) and cloned downstream of the BAD1 promoter in the binary vector pCTK4 (21), which contains the Aspergillus nidulans glyceraldehyde-3-phosphate dehydrogenase A (gpdA) promoter adjacent to the nourseothricin resistance gene. The resulting vector, pLR1-1, was used to express GFP in B. dermatitidis.

To construct a sentinel GFP-RNAi vector, a 3.8-kb ClaI/Pmel fragment was excised from pCR186 (provided by William E. Goldman, Washington University in St. Louis), which contains a GFP-RNAi construct downstream of the H. capsulatum histone 2AB (H2AB) constitutive promoter. The blunt-ended fragment was inserted into a blunt-ended SacI/Pmel-digested 8.9-kb binary vector derived from pCTs663 (21), which contains the A. nidulans gpdA promoter upstream of the hygromycin phosphotransferase gene, to create the GFP-only RNAi vector (pH2AB-GFPi). Two copies of the Gateway cassette (the XhoI, Ascl 1.8-kb fragment from pCTS463) containing LR clonase-mediated recombination sites (attR1 and attR2) were cloned, in a convergent direction, into pH2AB-GFPi at SpeI and Ascl/XhoI sites (Fig. 1). The resulting vector, pFANAI4, was sent as the sentinel GFP-RNAi gateway destination vector in all RNAi experiments in this study. An RNAi vector was constructed to simultaneously silence GFP and a target gene (BAD1, CDC11 homolog, or LACZ). The target was amplified from B. dermatitidis cDNA (BAD1 and CDC11) or pBPR1 (LACZ) (24), using target gene-specific primers with the attB1 or attB2 sequence at the 5′ end (see Table S1 in the supplemental material) for BAD1, primers P71 and P72; for LACZ, primers P137 and P140 for the full-length gene, primers P137 and P138 for a 1.5-kb 5′ end, primers P139 and P140 for a 1.6-kb 3′ end, and primers P66 and P140 for a 0.9-kb 3′ end; and for CDC11, primers P1 and P4 for an 810-bp fragment, primers P4 and P116 for a 500-bp 5′ end, and primers P113 and P114 for an 850-bp 3′ end. The PCR product of the target gene was directionally cloned into the donor vector pDONR207 (Invitrogen, Carlsbad, CA) to create an entry vector. This step required the BP clonase-mediated recombination reaction (Invitrogen), where the attB1 and attB2 sites of the amplified target gene sequence and the attP1 and attP2 sites of the donor vector were recombined specifically by BP clonase to give the new attL1 and attL2 sites, respectively. The entry vector was then subjected to an LR clonase-mediated recombination reaction (Invitrogen) to clone two copies of the target gene into the destination vector pFANAI4 to make a GFP-target gene RNAi expression vector. Similar to the BP clonase-mediated recombination reaction, this step required the LR clonase-mediated recombination reaction, where the attL1 and attL2 sites of the newly constructed entry vector (containing the target sequence) and the attR1 and attR2 sites of the destination vector (pFANAI4) are specifically recombined by LR clonase to give the new attB1 and attB2 sites, respectively. During these reactions, the Gateway cassette (Fig. 1) is replaced by the target gene sequence. If the Gateway sequence is not replaced with the target gene, the transformed Escherichia coli cells will be killed, since the product of the ccdB gene (a part of the Gateway cassette) is toxic to cells.

To create an expression vector for B. dermatitidis CDC11-GFP chimERIC protein, the full-length B. dermatitidis CDC11 mRNA was first identified by 5′ and 3′ rapid amplification of cDNA ends (RACE), using a First Choice RLM-RACE kit (Ambion, Inc.). The cDNA was fused with GFP by using “splicing by overlap extension PCR” (11), with attB sites added to the ends of the outside primers. This fusion was cloned into a modified version of pCTK4 (21), an Agrobacterium binary vector harboring nourseothricin acetyltransferase for selection and a single copy of the Gateway cassette, creating pN1819nat. The hybrid gene was sequenced to verify in-frame translation.

Transformation of B. dermatitidis. Agrobacterium-mediated gene transfer using previously described methods (30) was used in all transformations for gene expression and RNAi in this study. Transformants were selected on 3M medium.
containing 25 to 100 μg/ml of hygromycin B (A.G. Scientific Inc., San Diego, CA) or 25 μg/ml nourseothricin (Werner BioAgents, Jena, Germany).

**GFP localization with B. dermatitidis Cdc11.** B. dermatitidis strains TS319 and ER-3 were transformed with pJN-1819nat. The transformants were patched onto agar at 37°C. Transformants were examined for Cdc11-GFP expression and localization by fluorescence microscopy (BX60; Olympus).

**GFP reporter and RNAi in B. dermatitidis.** To generate GFP reporters in B. dermatitidis, strains 26199, 14081, and TS319 were transformed with the GFP-expressing vector pLR1-1. Lines with highly expressed GFP, making up about 4% of transformants, were cloned and designated 26199-GFP, 14081-GFP, and TS319-GFP, respectively. Only the TS319-GFP reporter expresses both GFP and LACZ. For gene silencing experiments, the GFP reporter strains were transformed with control vectors (pH2A-B-GFP, containing a GFP-only RNAi element, and pH4A, lacking the RNAi element) or with GFP-BAD1, GFP-LACZ, and GFP-CDC11 RNAi vectors.

RNAi candidate transformants were randomly picked, patched onto 3M agar supplemented with 25 to 100 μg/ml of hygromycin B, incubated at 37°C, and screened for GFP by using a fluorescence detector (FluorImager SI; Vistra Fluorescence). Cells from selected colonies were further examined by bright-field and fluorescence microscopy (BX60; Olympus), and photographic images were obtained using a digital camera (DEI-750; Optonics).

**Detection of BAD1 expression by colony immunoblotting and flow cytometry.** B. dermatitidis yeast cells were assayed for BAD1 by colony immunoblotting as previously described (21). Briefly, a small portion of a growing yeast colony was picked and spotted onto a 3M plate. The patched plate was incubated overnight at 37°C and then overlaid with a sterile nitrocellulose membrane (Millipore). The membranes were washed twice and incubated for 2 days. The membranes were lifted from the plate, and excess cell material was washed off with Tris-buffered saline. The membrane was probed using the anti-BAD1 monoclonal antibody DDS-CB4 (34) in Tris-buffered saline plus 0.05% Tween. Goat anti-mouse immunoglobulin G (heavy plus light chains)–alkaline phosphatase conjugate (Promega, Madison, WI) was added as the secondary antibody. Blots were developed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Promega).

For flow cytometry, yeast cells were stained with anti-BAD1 monoclonal antibody (1:50) at 37°C for 30 min and washed once with phosphate-buffered saline plus 1% bovine serum albumin. A goat anti-mouse antibody conjugated with phycoerythrin (1:50) was added to the yeast cells, incubated at room temperature for 30 min, and washed with phosphate-buffered saline plus 1% bovine serum albumin. Yeast cells were fixed with 2% paraformaldehyde for 30 min before measurements of GFP and BAD1 expression by flow cytometry.

**Quantitation of β-galactosidase activity.** B. dermatitidis yeast cells were assayed for β-galactosidase activity by an α-nitrophenyl-β-D-galactopyranoside (ONPG) assay as previously described (24). The yeast cells were cultured in liquid HMM for 3 days at 37°C. The cells were harvested, washed with 1 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.5, and beaten with mini glass beads (~0.5-mm diameter) using a Mini-Beadbeater-6 (BioSpec Products, Bartlesville, OK). Cell extracts were spun down to collect the supernatant. β-Galactosidase activity was normalized to the protein content, which was quantified using 10 μg of supernatant in the BCA plate assay (Pierce Chemical Co., Rockford, IL).

**Northern blot analysis.** RNAs were analyzed by Northern hybridization using standard protocols (6). Formaldehyde-agarose gels for Northern blot analysis were loaded with 15 μg total RNA per lane. A probe for B. dermatitidis GADPH was isolated as a 0.7-kb EcoRI fragment from pCR2.1-GADPH1#1 (provided by Laurens Smith, Idaho State University). A probe for GFP was isolated from a 0.85-kb BsrGI fragment from pLR1-1 (the GFP-expressing vector used in this study). A 0.85-kb probe for the B. dermatitidis CDC11 homolog was amplified by PCR from pFi3AB2 (the GFP-CDC11 RNAi vector used in this study), using primers Pr111 and Pr112 (see Table S1 in the supplemental material). Probes for B. dermatitidis CDC3 (0.3 kb) and CDC10 (0.5 kb) homologs were amplified by PCR from B. dermatitidis cDNA, using primer pairs P078/P079 and P98/P100, respectively (see Table S1 in the supplemental material). Probes for hybridization were gel purified using a QiAquick gel extraction kit (QIAGEN) and were radiolabeled with [32P]dCTP by using a Prime-a-gene probe labeling kit (Promega). Radioactive signals from probes were detected using a PhosphorImager (Storm860; Molecular Dynamics).

**RESULTS**

Feasibility of the concept of a surrogate GFP sentinel to detect RNAi in B. dermatitidis. Three different B. dermatitidis strains (26199, 14081, and TS319) were engineered to express GFP and designated 26199-GFP, 14081-GFP, and TS319-GFP, respectively. They are used as reporters throughout this study. To expedite the generation of target gene RNAi plasmids, Gateway technology (Invitrogen) was incorporated into the GFP sentinel RNAi vector. This technology relies on highly efficient, ligase-free in vitro recombination reactions to introduce target gene sequences and on negative selection, based on the ccdB gene, against growth of the nonrecombined parental plasmid. Two copies of the ccdB gene-containing Gateway cassettes were inserted, in inverted orientation, next to inverted copies of GFP and downstream of the H. capsulatum H2AB promoter in the RNAi destination vector (pFANTA4) (Fig. 1).

To explore the feasibility of this sentinel approach, we first tested a known gene, the virulence factor BAD1, as the target (13). The GFP-BAD1 RNAi plasmid (pFi-BAD1) was created by cloning a 0.6-kb fragment of BAD1 in the divergent direction into pFANTA4 to simultaneously silence GFP and BAD1 in GFP reporter strains of B. dermatitidis. Using Agrobacterium-mediated DNA transfer (30), the 26199-GFP reporter strain was transformed with the GFP-BAD1 RNAi plasmid. Experimental control plasmids included “non-RNAi” (an Agrobacterium binary plasmid without RNAi), “GFP-only RNAi” (a binary plasmid with only the GFP inverted repeat), and “GFP-LACZ RNAi” (pFANTA4 with target sequences for LACZ). Thirty clones of GFP-BAD1 RNAi transformants were randomly selected and surveyed for GFP expression by colony fluorescence (Fig. 2A; see Materials and Methods) and for BAD1 expression by colony immunoblotting (Fig. 2B; see Materials and Methods). Select GFP-BAD1 RNAi and control transformants with low GFP signals were confirmed by fluorescence microscopy (Fig. 2C). A majority of the GFP-BAD1 RNAi transformants showed sharply reduced GFP fluorescence, which correlated with reduced BAD1 expression. Non-RNAi control transformants showed normal levels of GFP and BAD1, while the GFP-only RNAi control transformants showed only reduced GFP but normal expression of BAD1. The levels of GFP and BAD1 expression of selected transformants were analyzed by flow cytometry (Fig. 2D). The GFP-BAD1, GFP-LACZ, and GFP-only RNAi transformants had 1 to 2% residual GFP signals compared to those of non-RNAi controls. The GFP-BAD1 RNAi transformants demonstrated <5% residual BAD1 expression relative to the non-RNAi control. Similar results were observed with the TS319-GFP reporter (data not shown).

We tested a second well-known gene, LACZ, to validate the GFP sentinel RNAi system. Different lengths of the LACZ sequence (0.9 kb and 1.6 kb of the 3′ end, 1.5 kb of the 5′ end, and 3 kb [full length]) were cloned into pFANTA4 and transformed into the TS319-GFP reporter, which is a strain that expresses both GFP and LacZ. Sixteen randomly selected GFP-LACZ RNAi transformants from each group were assayed for a GFP signal to determine the efficiency of each GFP-LACZ RNAi plasmid (Fig. 3A). GFP expression from each transformant silenced with 0.9-kb and 1.6-kb (3′ end) LACZ sequences was consistently low (Fig. 3B). In contrast, GFP expression from transformants silenced with 1.5-kb (5′ end) and full-length LACZ sequences was highly variable (Fig. 3B). The mean (± standard deviation) GFP fluorescence was as follows: non-RNAi, 9,466 ± 82; GFP-only RNAi, 373 ± 129;
To assess the correlation between GFP intensity and LacZ activity, three transformants (two with the lowest and one with the highest GFP intensity) from each GFP-LACZ RNAi group were selected to quantify GFP signals by flow cytometry and LacZ activities by ONPG assay (Fig. 3C). In a similar fashion to the BAD1 results reported above, the non-RNAi transformants highly expressed both GFP and LacZ, while the GFP-only RNAi transformants had reduced GFP with normal LacZ activity. In the GFP-LACZ transformants, GFP intensity correlated with LacZ activity; transformants with low GFP expression had relatively low LacZ activities, while transformants with high GFP signals had relatively high LacZ activities (Fig. 3C). These results indicate that LacZ is also subject to interference but that the targeted sequence size, specific sequence, and position of the sequence in the gene may influence the efficiency of silencing. Silencing with the 0.9-kb (3' end) sequence of LACZ was more effective than that with the half-length (~1.6 kb; 3' end) sequence, and silencing with the half-length sequence (3' or 5' end) was more effective than that with the full-length sequence (Fig. 3B and C). Silencing of LACZ with a hairpin made from the 3' end was more effective than that with the 5' end.

Identification and characterization of *B. dermatitidis* septin homologs. Systemic dimorphic fungi must switch from the mold to yeast form to acquire pathogenicity, so the process of morphogenesis is integral to the pathogenesis of disease. Septins form a protein family that is involved in morphogenesis and conserved in a broad range of living organisms, including fungi and animals, but not in plants and many protozoans (9, 16). In *Candida albicans* and *Saccharomyces cerevisiae*, septins are involved in cytokinesis, morphogenesis, and sporulation (9, 16, 31), but septins have not previously been identified and characterized for *B. dermatitidis* or other systemic dimorphic fungi. We postulated that septins would influence the morphogenesis of *B. dermatitidis*. To identify *B. dermatitidis* septin homologs, septin amino acid sequences of Cdc3 (AspB), Cdc10 (AspD), Cdc11 (AspA), Cdc12 (AspC), and AspE from *S.*
cerevisiae, C. albicans, A. nidulans, and Schizosaccharomyces pombe (see Materials and Methods) were used to search, via tBLASTn, the B. dermatitidis genome database (http://genome.wustl.edu/tools/BLAST/). Homologs of each of these septins were found in B. dermatitidis contigs 1.54, 196.6, 43.8, 101.8, and 94.27 (Table 1). Conserved domains that are commonly present in septins of other organisms include the GTP-binding core domain, which is comprised of G1 [GXXXXG(K/R)(S/T)], G3 (DTPG), and G4 (XKXD) motifs, and the coiled-coil domain at the C terminus (9, 12). The B. dermatitidis septin homologs were analyzed for these domains. Among the B. dermatitidis homologs identified, we found that only Cdc11 (amino acid sequence deduced from contig 94.27) contains all the GTP-binding motifs found in Cdc11 proteins of other fungi (G1, G3, and G4) and contains a 56-amino-acid coiled-coil domain, as analyzed by the MultiCoil program (32) (Fig. 4A and B).

We selected the B. dermatitidis CDC11 homolog for further characterization and analysis to test its role in morphogenesis. Based on 5′/H11032- and 3′/H11032-RACE–PCR and sequence analysis, in B. dermatitidis strain 26199 the CDC11 mRNA can be either 1,498 or 1,538 nucleotides in length, as two polyadenylation sites were identified (data not shown). The mRNA of B. dermatitidis CDC11 is predicted to have a 1,155-nucleotide open reading frame, which is translated into a 384-amino-acid protein. The 5′-untranslated region is 33 nucleotides long, while the 3′-untranslated region is 304 or 350 nucleotides long. The gene has four introns of 65 to 79 nucleotides. The deduced full-length amino acid sequence of B. dermatitidis CDC11 was aligned with the Cdc11 amino acid sequences of other fungi, including S. cerevisiae (GenBank accession number AAB50035), C. albicans (AAM51626), A. nidulans (AAK21867), S. pombe (AAB53691), C. immitis (AAK14772), and Neurospora crassa (XP_965058). The alignment demonstrates high degrees of identity (41 to 92%) and similarity (64 to 95%) among these fungi (Fig. 4B). Phylogenetic analysis of selected fungal Cdc11 sequences (Fig. 4B) revealed that B. dermatitidis Cdc11 was most closely related to the Cdc11 from another dimorphic fungus, C. immitis (unpublished data) (26).

Septins of S. cerevisiae and C. albicans form a complex ring structure at the bud neck and facilitate cytokinesis (9, 10, 31). We investigated whether a similar structure forms in B. dermatitidis Cdc11 by expressing a CDC11-GFP fusion gene in strains T5319 and ER-3. Cells harboring the Cdc11-GFP fusion gene were grown at 25°C or 37°C. A septin ring appeared at the growing hyphal tip of mold grown at 25°C (Fig. 4C). At 37°C, septins were present in yeast and pseudohyphae, including hourglass (Fig. 4D) and ring (Fig. 4E to G) forms. Septins appeared prior to septum formation at the mother-daughter bud neck (Fig. 4E), colocalized to mature septa (Fig. 4G), and eventually disappeared (Fig. 4D, arrowhead). Thus, the CDC11 homolog in B. dermatitidis has a similar distribution and appearance to those found in other fungi, suggesting that it may share some of the same functions.

Silencing B. dermatitidis CDC11 using GFP sentinel RNAi. We exploited the GFP sentinel RNAi tracking strategy to rapidly identify silenced strains and to study the function of B. dermatitidis CDC11. To create an RNAi plasmid for CDC11, we used an 810-bp fragment of the B. dermatitidis CDC11 coding sequence from cDNA of strain 26199.
TABLE 1. Blastomyces dermatitidis septin homologs

| Organism     | CDC3 (aspB) (1.54) | CDC10 (aspD) (196.6) | CDC11 (aspA) (94.27) | CDC12 (aspC) (43.8) | aspE (101.8) |
|--------------|--------------------|----------------------|----------------------|---------------------|--------------|
| S. cerevisiae| 147 4.0E–12        | 304 3.9E–63          | 588 8.7E–94          | 803 2.9E–92        | 149 1.2E–11  |
| S. pombe     | 182 3.9E–22        | 357 9.2E–85          | 679 8.0E–89          | 556 1.0E–104      | 137 5.0E–13  |
| C. albicans  | 149 1.2E–17        | 306 1.5E–84          | 999 6.1E–17          | 823 4.1E–115      | 84 5.2E–10   |
| A. nidulans  | 313 6.7E–46        | 515 5.1E–12          | 847 6.1E–17          | 104 4.1E–115      | 101 5.2E–10  |

a Predicted amino acid sequences for CDC3 (aspB), CDC10 (aspD), CDC11 (aspA), CDC12 (aspC), and aspE of several fungi (S. cerevisiae, C. albicans, A. nidulans, and S. pombe) were searched by iBLASTn against the most current B. dermatitidis genome database (http://genome.wustl.edu/tools/BLAST). The high score (HS) and probability for each B. dermatitidis septin homolog-containing contig are shown. In the case of CDC3, only a portion of the gene is available for comparison on contig 1.54. Comparison of the CDC3 sequence over this short stretch of nucleotides likely accounts for the lower CDC3 scores than those for the other septins.

(Fig. 5A) to create the hairpin. This 810-bp sequence was checked for cross homology to the other B. dermatitidis septin homology regions (CDC3, CDC10, CDC12, and ASPE, in contigs 1.54, 196.6, 43.8, and 101.8, respectively). Only a short stretch of 10 to 14 bp from each of these contigs matched the 810-bp CDC11 sequence, but the matches were outside the coding sequences of the putative septins.

The 810-bp CDC11 sequence was cloned into the GFP sentinel RNAi vector pFANTAi4 to create the GFP-CDC11 RNAi vector (pFi-3AB2) and transformed into the B. dermatitidis 26199-GFP reporter strain. As with GFP-BAD1- and GFP-LACZ-silenced strains, we sought GFP-CDC11 RNAi transformants with low GFP signals as a sentinel of reduced CDC11 expression. Fifty-six clones of 26199-GFP transformants were randomly selected, and the GFP signals were quantified. The GFP-CDC11 RNAi transformants showed a wide range of GFP signals. Based on the non-RNAi control, which showed high fluorescence, ~40% of GFP-CDC11 transformants showed at least a fivefold reduction in GFP signal. We analyzed the low-GFP transformants for phenotypic alterations linked with CDC11 silencing.

Phenotypic consequences of silencing septin in B. dermatitidis. Microscopically, the control transformants (non-RNAi and GFP-only RNAi) and the GFP-CDC11 RNAi transformants with high GFP signals showed a normal size, frequent budding, and a symmetrical round shape (Fig. 5B). The GFP-CDC11 RNAi transformants with low GFP signals exhibited aberrant microscopic features. They had a ballooned, distorted appearance, with irregular shapes such as pseudohyphae, and many fewer budding yeast cells (Fig. 5B, middle panel). For strain 26199, of three CDC11-silenced transformants analyzed, 35 to 50% of the yeast cells showed a ballooned, nonbudding phenotype microscopically, versus 3 to 5% of the unsilenced or GFP-silenced control transformants. Similar microscopic abnormalities were observed in association with reduced GFP signals in the T5319-GFP and 14081-GFP reporter strains after transformation with the GFP-CDC11 RNAi plasmid (Fig. 6A).

The CDC11-silenced colonies also looked macroscopically abnormal. The controls (non-RNAi and GFP-only RNAi) and the GFP-CDC11 RNAi transformants with high GFP signals had smooth, dome-shaped colonies. In contrast, the GFP-CDC11 RNAi transformants with low GFP signals had rough or pancake-like colonies (Fig. 6B).

Silencing of CDC11 impaired sporulation. Mycelia of the GFP-CDC11 RNAi transformants with reduced GFP signals produced fewer spores than the control strains, including the parental strain T5319, the non-RNAi control, and the GFP-only RNAi control (Fig. 6C and unpublished data). To quantify the defect, spore production was compared for an equal number of yeast cells (1.5 \times 10^7) as the starting material. When the strains were converted to mycelia after 2 weeks at 22°C, spores were collected and counted. The GFP-CDC11 RNAi transformants produced up to sixfold fewer spores than the controls (Fig. 6D). CDC11-silenced strains failed to “catch up” to controls in spore production, despite continued growth at 22°C for up to 6 weeks. Nevertheless, the spores harvested from CDC11-silenced strains showed viability rates similar to those of the controls, and at 37°C, they germinated into misshapen yeast cells and formed pancake-like colonies (data not shown).

Silencing of Cdc11 sharply impaired B. dermatitidis yeast growth at 37°C. A defect in growth of the septin RNAi transformants was observed for all three GFP reporters tested. Based on the size of colonies during growth on agar over 2 weeks at 37°C, the GFP-CDC11 (810-bp) RNAi transformants grew more slowly than the controls (Fig. 6E). This growth abnormality correlated with the intensity of the GFP signal, i.e., the lower the GFP signal, the slower the growth. Growth in more quantifiable liquid assays yielded similar findings for representative transformants tested over a 1-week period (data not shown). Cdc11-silenced strains grew up to 45% slower than the control strain by day 7.

Since RNAi typically targets a small sequence of 21 to 25 nucleotides (1), it is possible that off-target silencing by the hairpin of transcripts with homology to the gene of interest may confound interpretation of the results. As described above, we sought to guard against this possibility by ensuring that the hairpin sequences do not contain even short regions of sequence similarity to the other septins. To further minimize any possibility that our findings are due to off-target silencing, we created additional hairpins from two different regions of the gene that do not overlap, namely, a 500-bp sequence at the 5' end and an 850-bp sequence at the 3' end (Fig. 5A), and cloned them into the GFP sentinel vector pFANTAi4 to make septin RNAi vectors pFi-511 and pFi-262, respectively. The pFi-511 and pFi-262 plasmids were used to silence CDC11 in 26199- and T5319-GFP reporters. The effects on microscopic appearance (Fig. 5C), colonial growth (Fig. 6E), and sporulation rates (data not shown) were similar to those found with the original 810-bp sequence used in pFi-3AB2. However, the
degree of \textit{CDC11} silencing depended on the region of the \textit{CDC11} target chosen. In contrast to the results obtained with \textit{LACZ}, targeting the 5’ end (500 bp) of \textit{CDC11} gave more severe GFP silencing and phenotypes than did targeting the 3’ end (850 bp) (Fig. 6E and unpublished data).

\textbf{Confirmation of GFP and septin silencing by Northern analysis.} To provide unambiguous evidence that the sentinel reporter and the phenotypic defects accurately signaled \textit{CDC11} silencing, we analyzed the transcript abundance in the strains. Representative transformants for wild-type 26199, the non-RNAi control, the GFP-only RNAi control, and \textit{GFP-CDC11} RNAi (in which various portions of \textit{CDC11} were targeted) were studied by Northern analysis. The blotted membrane was probed with \textit{GFP}, \textit{CDC11}, \textit{CDC10}, \textit{CDC3}, and \textit{GAPDH}. 

\begin{figure}
  \centering
  \includegraphics[width=\textwidth]{fig4}
  \caption{(A) Diagram showing two common domains for septins, i.e., a GTP-binding domain (comprised of three motifs, G1, G3, and G4) and a coiled-coil domain. (B) Alignment of the deduced amino acid sequence of the Cdc11 GTP-binding domain of \textit{B. dermatitidis} (Bd) with those of the following other fungi: \textit{S. cerevisiae} (Sc; accession number AAB50035), \textit{C. albicans} (Ca; AAM51626), \textit{A. nidulans} (An; AAK21867), \textit{C. immittis} (Ci; AAK14772), \textit{N. crassa} (Nc; XP_965058), and \textit{S. pombe} (Sp; AAB53691). (C to G) Septin ring structures in \textit{B. dermatitidis}. A Cdc11-GFP fusion was expressed in \textit{B. dermatitidis} strains T5319 and ER-3 and was found localized at the hyphal tip in mold, at the septum in a pseudohyphal form (note the hourglass shape; arrow), at various stages in budding yeast (E to G), and in a well-defined ring-like structure at the bud neck of the mother and daughter cells viewed from above (F). See the text for additional details.}
\end{figure}
probes (Fig. 7). The wild-type control had no detectable GFP transcript but had a strong signal for CDC11. The non-RNAi and GFP-only RNAi controls also had strong signals for CDC11, but the GFP transcript was abundant in the non-RNAi control and diminished in the GFP-only RNAi control. Both the GFP and CDC11 transcripts were sharply reduced, to 5% and 10%, respectively, in the GFP-CDC11 RNAi transformants compared to those in the controls (Fig. 7). Levels of GAPDH, CDC10, and CDC3 transcripts appeared to be equivalent in all the RNA extracts tested. Thus, GFP and CDC11 transcripts appeared to be reduced, as expected, in silenced strains, and there was no evidence of off-target silencing of the septin homologues CDC3 and CDC10.

DISCUSSION

The sequencing of the B. dermatitidis genome is nearly complete. Efficient technologies for the functional analysis of this newly available genetic information will accelerate our understanding of the biology and pathogenesis of this pathogen. Here we report the utility of RNAi for rapid functional analysis of genes with unknown function in B. dermatitidis and illustrate its application for the investigation of septin. The technique involves a sentinel RNAi system using GFP as a surrogate marker. The effectiveness of a GFP-RNAi sentinel to monitor RNAi-based silencing was recently established with H. capsulatum (17). We reasoned that the development of such a system would facilitate gene depletion studies with B. dermatitidis. Gateway recombination technology allowed us to quickly engineer a hybrid GFP-target gene plasmid to silence both GFP and the target gene simultaneously in B. dermatitidis GFP reporter strains. The level of GFP signal was used as initial indirect evidence of the degree of silencing of a target sequence in an individual transformant. This type of sentinel system can be especially useful when the phenotype of a target gene mutation is unknown. To ensure that the RNAi target sequence is completely transcribed, we placed the copies of GFP sequence at the ends of the intended RNAi transcript. Thus, if GFP is silenced, then RNAi is functioning, and it is likely that transcription has proceeded through the entire hairpin sequence, target gene double-stranded RNA will be produced, and target gene RNAi should also be functional.
The utility of this sentinel RNAi concept was initially established by using the well-known virulence gene BAD1 as a test target. When the 0.6-kb BAD1 sequence was used for RNAi, both BAD1 and GFP were cosilenced as expected; for example, a transformant with relatively low GFP expression appeared to concomitantly express a low level of BAD1, and a transformant with a normal GFP signal expressed substantial BAD1. The utility of the sentinel approach was further confirmed with the use of LACZ as a target, wherein cotranscription of hairpins of GFP-LACZ in the T5319-GFP reporter strain led to reduced expression of both GFP and LACZ. These data gave us confidence that we could use GFP as a reliable sentinel to rapidly screen many candidate transformants to find those in which a target gene was efficiently silenced.

RNAi has been applied for functional genetic studies of many fungi (7, 8, 15, 20, 22, 23, 28), but little is known about the factors that influence RNAi efficiency in these organisms. Rappleye et al. (22) showed for the dimorphic fungus H. capsulatum that two factors—loop size of the RNAi hairpin and length of the target sequence—influence the degree of gene silencing. A greater silencing effect was observed with a shorter loop and a longer target gene sequence. We also explored a correlation between length of a target sequence and RNAi efficiency by using LACZ in B. dermatitidis. When several lengths of LACZ (0.9 kb, 1.5 kb, and 3 kb [full length]) were used to create various GFP-LACZ hairpins, the shorter LACZ sequence (0.9 kb) was more effective at silencing than the longer LACZ sequences. While these results agree with those of Rappleye et al. (22) in that the length of the targeting sequence is important, the decreased RNAi efficiency from increasing the length of the LACZ sequence indicates that there are quantitative differences in RNAi efficiency that may...
CDC11 in B. dermatitidis was silenced using several target region sequences (500 bp and 810 bp from the 5' end and 850 bp from the 3' end). Altered morphological phenotypes were consistently observed in GFP-silenced strains transformed with all GFP-CDC11 RNAi constructs, suggesting a specificity of the CDC11-silenced phenotypes. They included enlarged and fewer budding yeast cells, rough or flat colonies, and growth and sporulation defects. These aberrant phenotypes were associated with decreased CDC11 transcripts. In addition, the transcript analysis indicated that there was no cross-silencing of other septin genes. The phenotypes associated with the CDC11-silenced strains of B. dermatitidis support the hypothesis that Cdc11, which localized at the bud neck, is likely to serve a function in the budding event, similar to the case in other budding yeasts. These aberrant phenotypes could be due to an inability of the CDC11-silenced B. dermatitidis to contract and separate the mother and daughter compartments. The CDC11-silenced strains also had a striking growth defect, similar to that reported for other fungi (10, 31). In Ustilago maydis, Boyce et al. (2) found that the Cdc11 homolog sep3 is required for normal morphology and division of haploid cells, formation of hyphae during the filamentous growth response to lipids, and symptom development in maize. The growth defect of the CDC11-silenced yeast in our study made it unfeasible to study virulence in vivo. Although our CDC11-silenced strains were morphologically abnormal, they switched normally from mold to yeast in response to temperature, indicating that CDC11 does not control the phase transition in B. dermatitidis.

In conclusion, we report a surrogate GFP sentinel RNAi system for facilitating reverse genetic analysis of the dimorphic fungus. This defect could not be explained by a growth delay of CDC11-silenced strains, because continued incubation and growth did not correct the defect and microscopic inspection showed persistent “barren” hyphae in the silenced strains even during extended growth.

That fact that deletion of CDC11 in S. cerevisiae severely impairs growth (10) foreshadowed the possibility that B. dermatitidis CDC11 might be an essential gene. We were not able to create a cdc11-null strain of B. dermatitidis despite the use of different gene disruption and screening strategies (data not shown). The new surrogate GFP sentinel RNAi system reported here underscores the utility of gene silencing methodologies such as this for studying the role of potentially essential genes.

In conclusion, we report a surrogate GFP sentinel RNAi system for facilitating reverse genetic analysis of the dimorphic fungus B. dermatitidis. With this system, gene silencing can be accomplished for three different genes, LACZ (a procaryotic transgene), BAD1 (a gene whose mutant phenotype was
known), and CDC11 (a previously uncharacterized gene in \textit{B. dermatitidis}). An advantage of RNAi is that a partial target sequence is adequate for gene silencing. However, the length and location of the target sequence should be optimized empirically for the maximal RNAi effect. To our knowledge, this study is the first characterization of a septin in the systemic dimorphic fungi. We found that CDC11 governs cell budding, growth, and sporulation but not phase transition. To fully define the role of septins in morphogenesis of the dimorphic fungi, the sentinel RNAi system reported here could be applied to the analysis of the other \textit{B. dermatitidis} septin homologs. The facility of the GFP sentinel RNAi system will accelerate reverse genetic analysis of these and other genes of unknown or suspected function in \textit{B. dermatitidis} and other eukaryotic organisms.

**ACKNOWLEDGMENTS**

This work was supported by a postdoctoral fellowship from the Ellison Medical Foundation-International Nutritional Foundation (T.K.), a training grant from the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (T.K.), a Pfizer fellowship in medical mycology from the IDSA (G.G.), and NIH grants from the USPHS (B.K. and G.G.). B.K. is a Hartwell Foundation Scholar.

We thank William Goldman for providing materials prior to publication, Jon Woods for plasmid DNA, and Robert Gordon for assistance with graphics.

**REFERENCES**

1. Agrawal, N., P. V. N. Dasaradh, A. Mohmmed, P. R. K. Bhathnagar, and S. K. Mukherjee. 2003. RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. 67:657–685.
2. Boyce, K. J., H. Chang, C. A. D’Souza, and J. W. Kronstad. 2005. An \textit{Ustilago maydis} septin is required for filamentous growth in culture and for full symptom development on maize. Eukaryot. Cell 12:2044–2056.
3. Brandhorst, T., M. Wuthrich, T. Warner, and B. S. Klein. 1999. Targeted gene disruption reveals an adhesion indispensable for pathogenicity of \textit{Blastomyces dermatitidis}. J. Exp. Med. 189:1207–1216.
4. Brandhorst, T., and B. S. Klein. 2000. Cell wall biogenesis of \textit{Blastomyces dermatitidis}. Evidence for a novel mechanism of cell surface localization of a virulence-associated adhesion via extracellular release and reassociation with cell wall chitin. J. Biol. Chem. 275:7925–7934.
5. Brandhorst, T., M. Wuthrich, B. Finkel-Jimenez, and B. S. Klein. 2003. A C-terminal EGF-like domain governs BAD1 localization to the yeast surface and fungal adherence to phagocytes, but is dispensable in immune modulation and pathogenicity of \textit{Blastomyces dermatitidis}. Mol. Microbiol. 48:53–65.
6. Brown, T., and K. Mackey. 1997. Analysis of RNA by Northern and slot blot hybridization, p. 4.9–4.9.16. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, et al. (ed.), Current protocols in molecular biology. Wiley Interscience, Hoboken, NJ.
7. Cottrell, T. R., and T. L. Doering. 2003. Silence of the strands: RNA interference in eukaryotic pathogens. Trends Microbiol. 11:37–43.
8. De Backer, M. D., M. Raponi, and G. M. Arndt. 2002. RNA-mediated gene silencing in non-pathogenic and pathogenic fungi. Curr. Opin. Microbiol. 5:323–329.
9. Douglas, L. M., F. J. Alvarez, C. McCready, and J. B. Konopka. 2005. Septin function in yeast model systems and pathogenic fungi. Eukaryot. Cell 4:1503–1512.
10. Fares, H., L. Goetsch, and J. R. Pringle. 1996. Identification of a developmentally regulated septin and involvement of the septins in spore formation in \textit{Saccharomyces cerevisiae}. J. Cell Biol. 132:399–411.
11. Horton, R. M., Z. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques 8:528–535.
12. Kinoshita, M. 2003. The septins. Genome Biol. 4:236:1–236:9.
13. Klein, B. S. 1997. Role of cell surface molecules of \textit{Blastomyces dermatitidis} in the pathogenesis and immunobiology of blastomycosis. Semin. Respir. Infect. 12:198–205.
14. Klein, B. S., and J. M. Jones. 1990. Isolation, purification, and radiolabeling of a novel 120-kD surface protein on \textit{Blastomyces dermatitidis} yeasts to detect antibody in infected patients. J. Clin. Invest. 85:152–161.
15. Liu, H., T. R. Cottrell, L. M. Pierini, W. E. Goldman, and T. L. Doering. 2002. RNA interference in the pathogenic fungus \textit{Cryptococcus neoformans}. Genetics 160:463–470.
16. Longtine, M. S., and E. Bi. 2003. Regulation of septin organization and function in yeast. Trends Cell Biol. 13:403–409.
17. Marion, C. L., C. A. Rappleye, J. T. Engle, and W. E. Goldman. 2006. An \textalpha -(1,4)-amylase is essential for \textalpha -(1,3)-glucan production and virulence in \textit{Histoplasma capsulatum}. Mol. Microbiol. 62:970–983.
18. Mello, C. C., and D. Conte, Jr. 2004. Revealing the world of RNA interference. Nature 431:338–342.
19. Momany, M., J. Zhao, R. Lindsey, and P. J. Westfall. 2001. Characterization of the \textit{Aspergillus nidulans} septin (asp) gene family. Genetics 157:969–977.
20. Mouyna, L. C. Henry, T. L. Doering, and J. P. Latge. 2004. Gene silencing with RNA interference in the human pathogenic fungus \textit{Aspergillus fumigatus}. FEMS Microbiol. Lett. 237:317–324.
21. Nemecck, J. C., M. Wuthrich, and B. S. Klein. 2006. Global control of dimorphism and virulence in fungi. Science 312:583–588.
22. Rappleye, C. A., J. T. Engle, and W. E. Goldman. 2004. RNA interference in \textit{Histoplasma capsulatum} demonstrates a role for \textalpha -(1,3)-glucan in virulence. Mol. Microbiol. 53:155–165.
23. Reese, A. J., and T. L. Doering. 2003. Cell wall alpha-1,3-glucan is required to anchor the \textit{Cryptococcus neoformans} capsule. Mol. Microbiol. 50:1401–1409.
24. Rooney, P. J., T. D. Sullivan, and B. S. Klein. 2001. Selective expression of the virulence factor BAD1 upon morphogenesis to the pathogenic yeast form of \textit{Blastomyces dermatitidis} evidence for transcriptional regulation by a conserved mechanism. Mol. Microbiol. 39:875–889.
25. Rooney, P. J., and R. S. Klein. 2002. Linking fungal morphogenesis with virulence. Cell. Microbiol. 4:127–137.
26. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
27. San-Blas, G., and F. San-Blas. 1984. Molecular aspects of fungal dimorphism. CRC Crit. Rev. Microbiol. 11:101–127.
28. Sommer, U., H. Liu, and T. L. Doering. 2003. An alpha-1,3-mannosyltransferase of \textit{Cryptococcus neoformans}. J. Biol. Chem. 278:47724–47730.
29. Sternglanz, R. 1994. The emerging fungal threat. Science 266:1632–1634.
30. Sullivan, T. D., P. J. Rooney, and R. S. Klein. 2002. \textit{A. fumigatus} homologs integrate transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multicellular yeast. Eukaryot. Cell 1:895–905.
31. Warenza, A. J., and J. B. Konopka. 2002. Septin function in \textit{Candida albicans} morphogenesis. Mol. Biol. Cell 13:2732–2746.
32. Wolf, E., P. S. Kim, and B. Berger. 1997. MultiCoil: a program for predicting two- and three-stranded coiled coils. Protein Sci. 6:1179–1189.
33. Worsham, P. L., and W. E. Goldman. 1988. Quantitative plating of \textit{Histoplasma capsulatum} without addition of conditioned medium or siderophores. J. Med. Vet. Mycol. 26:137–143.
34. Wuthrich, M., and B. S. Klein. 2000. Investigation of anti-Wi-1 adhesin antibody-mediated protection in experimental pulmonary blastomycosis. J. Infect. Dis. 181:1720–1728.