RNA Interference in the Pathogenic Fungus *Cryptococcus neoformans*

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Manuscript received July 11, 2001
Accepted for publication November 13, 2001

ABSTRACT

Cryptococcus neoformans is a pathogenic fungus responsible for serious disease in immunocompromised individuals. This organism has recently been developed as an experimental system, with initiation of a genome project among other molecular advances. However, investigations of Cryptococcus are hampered by the technical difficulty of specific gene replacements. RNA interference, a process in which the presence of double-stranded RNA homologous to a gene of interest results in specific degradation of the corresponding message, may help solve this problem. We have shown that expression of double-stranded RNA corresponding to portions of the cryptococcal *CAP59* and *ADE2* genes results in reduced mRNA levels for those genes, with phenotypic consequences similar to that of gene disruption. The two genes could also be subjected to simultaneous interference through expression of chimeric double-stranded RNA. Specific modulation of protein expression through introduction of double-stranded RNA thus operates in *C. neoformans*, which is the first demonstration of this technique in a fungal organism. Use of RNA interference in Cryptococcus should allow manipulation of mRNA levels for functional analysis of genes of interest and enable efficient exploration of genes discovered by genome sequencing.

THE challenge of using eukaryotic genome sequence information to focus research efforts on productive areas is shared by those who investigate organisms with genomes of all magnitudes and complexities. One fungal genome, that of *Cryptococcus neoformans* (24 Mb), has been currently sequenced to approximately seven times shotgun coverage by the Stanford Genome Technology Center (http://www-sequence.stanford.edu) and The Institute for Genomic Research (http://www.tigr.org). *C. neoformans* is an encapsulated fungal pathogen responsible for severe disease in immunocompromised individuals. It usually grows as a haploid yeast that reproduces by budding, but under certain environmental conditions it may also undergo a sexual cycle resulting in the production of basidiospores (Kwon-Chung 1975). In recent years Cryptococcus has been developed as an experimental system, with advances in molecular techniques enabling detailed investigation of virulence factors and interactions with the mammalian host. Notable among these advances have been the establishment of transformation systems suitable for episomal expression and chromosomal integration (Edman and Kwon-Chung 1990; Toffaletti *et al.* 1993) and the initiation of genome sequencing efforts (Heitman *et al.* 2000). More difficult has been the development of methods for efficient gene replacement in *C. neoformans*, which has been hampered by both the propensity of cryptococcus to modify exogenous DNA (Kwon-Chung *et al.* 1998) and its high frequency of nonhomologous recombination. Despite these obstacles several dozen targeted gene replacements have now been accomplished, mainly by the groups of J. Perfect (Duke University Medical Center), J. Heitman (Duke University Medical Center), and J. Kwon-Chung (National Institutes of Health), which have tremendous value for functional analysis of cryptococcal genes and virulence studies in animal models. To exploit the forthcoming genome sequence efficiently, however, it will be necessary to develop more rapid methods to test the function of gene products, instead of relying on individual gene disruptions.

One recently developed method for downregulating gene function that may facilitate studies in *C. neoformans* is double-stranded RNA interference (RNAi). In this process double-stranded RNA (dsRNA) induces the specific destruction of mRNA to which it is homologous (Fire *et al.* 1998; reviewed in Sharp 1999; Bass 2000; Carthew 2001; Hammond *et al.* 2001). The dsRNA "trigger" is thought to be cleaved into shorter fragments (21–25 nucleotides; Zamore *et al.* 2000), which then guide specific degradation of the corresponding mRNA, catalyzed by a protein or protein complex with nuclease activity (Baulcombe 2001). Before cleavage the trigger can be as short as 26 nucleotides and has no specific requirements as far as sequence motif or base composition, but must be double stranded (Parrish *et al.* 2000). Interference can persist for many rounds of cell division and growth and may even be passed through the germline (Fire *et al.* 1998). Specific inhibition of gene expression by RNAi has been demonstrated in a range of organisms, from the initial report on *Caenorhabditis eleg-*

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ans (Fire *et al.* 1998) to works on trypanosomes (Ngo *et al.* 1998), Drosophila (Kennerdell and Carthew 1998), early mouse embryos (Svoboda *et al.* 2000; Wianny and Zernicka-Goetz 2000), and mammalian cells in culture (Elbashir *et al.* 2001). RNAi has proved a valuable tool for probing functions of individual gene products (*e.g.*, Kennerdell and Carthew 1998; Bastin *et al.* 2000) and offers a powerful approach for functional genomic studies (Kuwabara and Coulson 2000; Kim 2001). In the best-studied and most tractable system, *C. elegans*, over a third of all genes have now been assessed for the phenotypic effects of RNAi. We hypothesized that this technique could be useful in *C. neoformans*, both to circumvent the difficulties of gene replacement for functional studies of individual gene products and to initiate larger-scale investigations of genes discovered through sequencing efforts. In this article we show that *C. neoformans* gene expression can be specifically inhibited by RNA interference.

MATERIALS AND METHODS

Strains and cell growth: *C. neoformans* was grown with continuous shaking at 30° in YPD medium [1% (w/v) Bacto yeast extract; 2% (w/v) peptone, 2% dextrose] or minimal medium lacking uracil (Ausubel *et al.* 2001). Low adenine plates contained yeast nitrogen base supplemented with (per liter) 20 g glucose; 24 mg uracil; 40 mg each arginine, histidine, isoleucine, leucine, lysine, methionine, and tryrosine; 60 mg phenylalanine and tryptophan; 120 mg homoserine; 180 mg valine; and 10 mg adenine. For experiments using 5-fluoroorotic acid (5-FOA), plates contained the same medium with adenine raised to 40 mg/liter and the addition of 1 g/liter 5-FOA. Wild-type serotype D strain B4500 and *cap59* strain TYCC33 (Chang and Kwon-Chung 1994) were from Dr. June Kwon-Chung (National Institutes of Health), and *ura5* strain JEC43 (Wickes *et al.* 1997) was from Dr. Joseph Heitman (Duke University Medical Center). JEC43 cells transformed with a control plasmid alone (CIP-GUST.Cla.Kpn; see below) are designated "control." JEC43 cells transformed with a plasmid designed to interfere with *CAP59* expression (described below) were termed CAP59i, with numbers following this designation to indicate individual transformants (*e.g.*, CAP59i-1). A similar convention was used for naming constructs designed to interfere with *ADE2* or with both *CAP59* and *ADE2*.

Constructs and transformation: Constructs for RNA interference were designed with inverted repeats of ~500 bp of coding sequence of the gene of interest separated by a spacer segment of green fluorescent protein (*GFP*) sequence. For construction of pCAP59i (Figure 1, top), a portion of the coding sequence of *CAP59* was PCR amplified from B4500 genomic DNA using primers 1 and 2 (Table 1) to add an *Nde*I restriction site at the 5′ end and a portion of *GFP* sequence at the 3′ end (product A). Product A was further amplified using primers 3 and 4 to incorporate *Bgl*II restriction sites at each end (product B). A segment of *GFP* was amplified (from an enhanced version designed for *C. elegans* from Dr. Andrew Fire, Carnegie Institute of Washington) using primers 5 and 6 to incorporate the 3′ end of the *CAP59* sequence in product A at the 5′ end and a *Bgl*II restriction site at the 3′ end (product C). PCR products A and C were then used as the template for a PCR reaction with primers 1 and 6 to amplify a 750-bp fragment (product D); this was then digested with *Nde*I and *Bgl*II and ligated into the similarly restricted plasmid CIP-GUST.Cla-Kpn, from Dr. Brian Wickes (University of Texas Health Science Center). (This plasmid contains Amp^R and *C. neoformans URA5* markers, as well as the *GUS* reporter gene flanked by the *C. neoformans GAL7* promoter and terminator; digestion with *Nde*I and *Bgl*II releases the *GUS* coding sequence.) The ligation product was isolated from *Escherichia coli*, digested with *Bgl*II, and ligated to the *Bgl*II restricted product B. Products were tested by restriction digestion to select one in which the *CAP59* repeats were in opposite orientations, and this was digested with *Nde*I and *Cla*I to remove the *GAL7* promoter. To form pCAP59i (Figure 1) the *GAL7* promoter was replaced with a cryptococcal *ACT* promoter amplified with primers 7 and 8 from plasmid GMC200 (from Dr. Gary Cox, Duke University Medical Center). An identical cloning scheme was used to construct pADE2i, except that primers 1*–6* (Table 1) were used in place of primers 1–6 above. All PCR reactions were done using Taq polymerase under standard conditions and PCR products were purified before use in subsequent reactions. Both plasmids were checked by DNA sequencing.

To incorporate cryptococcal telomere sequences, the 1.4-kb *Not*I fragment of pTEL-HYG (from Dr. Gary Cox, Duke University) was inserted into the unique *Not*I site of pCAP59i, forming pCAP59i-tel. pCAP59/ADE2i was generated by adding duplicates of the same portion of *ADE2* used in pADE2i to pCAP59i, in the positions and orientations shown in Figure 1 (cloning details available on request). For the promoterless version of pCAP59i the plasmid was digested with *Nde*I and *Cla*I to remove the actin promoter, the sticky ends were filled in with T4 DNA polymerase, and the plasmid was religated.

JEC43 cells were transformed by electroporation as described (Wickes and Edman 1994) using 5 μg of linearized DNA per 3×10^8 cells with selection of transformants on minimal plates lacking uracil. pCAP59i-tel was linearized with *Sce*I so that the telomeres formed the ends of the DNA used for transformation; all other plasmids were linearized with *Not*I. Transformants were streaked to master plates of the same medium and stored at 4°.

RNA preparation and reverse transcription-PCR: *C. neoformans* cells were washed with pyrocarbonic acid diethyl ester-treated water, suspended in Trizol Reagent (GIBCO BRL, Gaithersburg, MD), and broken in a MiniBeadbeater-8 (Biospec Products, Bartlesville, OK) in the presence of 0.5-mm glass beads ($3 \times$ 1-min bursts, 4°). Cell lysis was typically 60–80% for encapsulated strains and 80–100% for acapsular strains. Total RNA was precipitated using standard methods (Ausubel *et al.* 2001) with a yield of 1.5–3.0 mg/500 OD_{600} units of cells. For cDNA synthesis, 100 μg of total RNA was treated with amplification grade DNase I (GIBCO BRL), ethanol precipitated, and used in the Superscript preamplification system for first strand cDNA synthesis using Oligo(dT) primer (GIBCO BRL) according to manufacturer's instructions.

For reverse transcription (RT)-PCR experiments, ~2 μg of cDNA was amplified in a 100-μl reaction using Taq polymerase and appropriate primers (see Table 2 and text). The amplification program was (i) 94° for 2 min; (ii) 26–35 cycles of 94° for 1 min, 55° for 1 min, 68° for 1 min; and (iii) 68° for 7 min. Ten-microliter samples were removed every 3 cycles immediately after the 68° incubation, beginning as early as the eleventh cycle. PCR products were resolved on 1.5% agarose gels run in TAE buffer and stained (45 min, RT) with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR) in TAE buffer. DNA-associated fluorescence was visualized using a FluorImager SI (Molecular Dynamics, Sunnyvale, CA), and data were analyzed using Scanalytics IPLab gel and Microsoft Excel software with subtraction of background fluorescent signal.

Cell imaging: For immunofluorescence, cells were grown in YPD medium, washed twice in PBS, and treated with anticapsule monoclonal antibody 2H1 (from Dr. Arturo Casadevall,

TABLE 1

Primers used in plasmid construction

Primer	Sequence (5′ to 3′)	Description
1	GGAATTC**CATATG**GAATTCCATGCTCCCCTCCATCGAGC	*Nde*I site, *CAP59* nt 1–19 sense (underlined)
2	GAAAAGTTCTTCTCCTTTACTCATCGGTGGTTGAACAGACCT	*GFP* nt 24–1 antisense (underlined), *CAP59* nt 520–502 antisense
3	GG**AGATCT**GCCGGTGGTTGAACAGACCTC	*Bgl*II site, *CAP59* nt 520–500 antisense (underlined)
4	GA**AGATCT**TCATGCTCCCCTCCATCGAG	*Bgl*II site, *CAP59* nt 1–20 sense (underlined)
5	AGGTCTGTTCAACCACCGATGAGTAAAGGAGAAGAACTTTTC	The antiparallel sequence of primer 2
6	GG**AGATCT**CATAACAGAAAGTAGTGACAA	*Bgl*II site, *GFP* nt 230–250 antisense (underlined)
7	CC**ATCGAT**GGCTGCGAGGATGTG	*Cla* site, actin promoter nt 1–14 sense (underlined)
8	GGAATTC**CATATG**GTTGGGCGAG	*Nde*I site, actin promoter nt 834–825 antisense (underlined)
1*	GGAATTC**CATATG**GAATTCCATGGCCCCCAGAAAGACTG	*Nde*I site, *ADE2* nt 1–19 sense (underlined)
2*	GTTCTTCTCCTTTACTCATTGCGGATTTAAGAGGAGAG	*GFP* nt 24–1 antisense (underlined), *ADE2* nt 520–502 antisense
3*	GA**AGATCT**TCATGCGGATTTAAGAGGAGAG	*Bgl*II site, *ADE2* nt 521–502 antisense (underlined)
4*	GA**AGATCT**TCATGGCCCCCAGAAAGACTG	*Bgl*II site, *ADE2* nt 1–19 sense (underlined)
5*	CTCTCCTCTTAAATCCGCAATGAGTAAAGGAGAAGAAC	The antiparallel sequence of primer 2*
6*	GA**AGATCT**TCTGCGGATTTAAGAGGAGAGTT	*Bgl*II site, *GFP* nt 250–230 antisense (underlined)

Restriction sites are in boldface type and sequences homologous to genes of interest are designated by the nucleotide (nt) position in the corresponding cDNA coding sequence (1 being the A of the initiating ATG) and whether they correspond to the sense or antisense strand. For the *ACT* promotor the nt numbers correspond to those in GenBank entry AF156670. Primers specific to pADE2i are designated with an asterisk (see MATERIALS AND METHODS).

Albert Einstein College of Medicine) that was tagged with Cy3 (as in PIERINI and DOERING 2001). Differential interference contrast (DIC) and fluorescence images were acquired simultaneously with a Zeiss (Jena, Germany) LSM510 laser scanning confocal microscope. All samples were imaged with identical acquisition settings to allow direct quantitative comparison. Average fluorescence intensities were based on measurements of at least 25 cells per condition. Postacquisition image analysis was performed with MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA), and images were prepared for publication using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

RESULTS

To test RNAi in *C. neoformans* we chose two genes that would produce clear phenotypes if the technique was successful. The first of these is *CAP59*, which encodes a product required for synthesis of the polysaccharide capsule in cryptococcus. The capsule is absolutely required for virulence of this fungal pathogen, and cells in which *CAP59* is disrupted are acapsular and avirulent (CHANG and KWON-CHUNG 1994). The second gene tested was *ADE2*, which encodes phosphoribosylaminoimidazole carboxylase; disruption of this gene yields pink colonies due to accumulation of adenine biosynthetic intermediates.

Several approaches have been used to express double-stranded RNA for interference testing. It can be synthesized *in vitro* or *in vivo* either as independent RNA molecules corresponding to a sequence and its complement or as a single polynucleotide containing duplicate sequences in opposite orientation separated by a spacer. In the former situation the sense and antisense strands, which may be formed by convergent promoters transcribing the same DNA sequence, anneal to form the required dsRNA. In the latter case the single RNA forms a hairpin with a double-stranded stem. dsRNA may be delivered by injection (FIRE *et al.* 1998; GURU 2000), electroporation, soaking, or feeding (the last two in *C. elegans*;

TABLE 2

Primers used for RT-PCR

Primer	Sequence (5′ to 3′)	Description
9	ACCATTGGTAACGAGCGATTCC	*ACT* nt 747–768 sense
10	TTCCTATCTTGGTGGCTAGGTC	*ACT* nt 1040–1020 antisense
11	CTGGAGTTGTCCCAATTCTTGTTG	*GFP* nt 26–49 sense
12	GAAAGTAGTGACAAGTGTTGGCTG	*GFP* nt 243–220 antisense
13	ATGCTCCCCTCCATCGAGCAACG	*CAP59* nt 1–23 sense
14	CGGTGGTTGAACAGACCTCGGG	*CAP59* nt 518–497 antisense
15	CCAATCGTGCTGGAACGGTATCGC	*CAP59* nt 852–872 sense
16	CTCTGGTCCGGGTACAAACTCC	*CAP59* nt 1229–1208 antisense

Primer sequences are designated by nucleotide position as in Table 1.

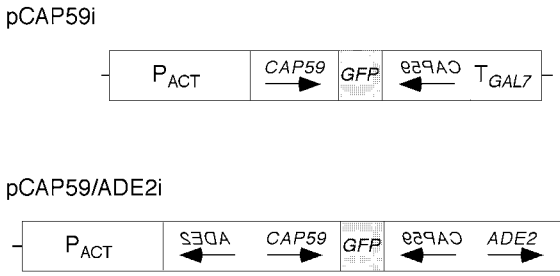

FIGURE 1.—Plasmid design. pCAP59i was used to interfere with *CAP59* expression, and pCAP59/ADE2i was used for tandem interference with both *CAP59* and *ADE2*.

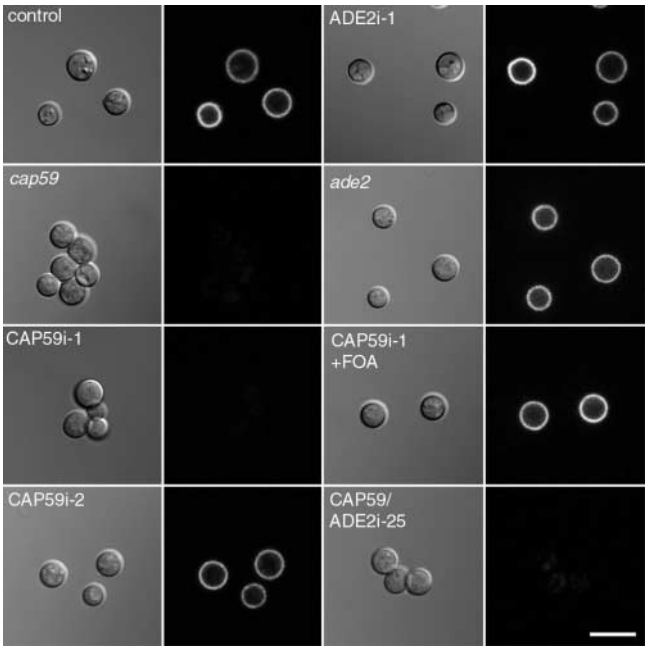

FIGURE 2.—Light microscopy of *CAP59* interference and control strains. The indicated strains (see text) were labeled with Cy3-conjugated monoclonal antibody 2H1 and then examined by confocal microscopy. DIC (left image of each pair) and fluorescence (right image of each pair) images are shown. CAP59i-1, like *cap59*, demonstrates clumping and a lack of capsule, which is reversed after growth on 5-FOA (Cap59i-1 + FOA). An acapsular phenotype is also seen with double interference cells (CAP59/ADE2i). Capsule is not affected by inhibition or mutation of *ADE2* alone (ADE2i-1 and *ade2*, respectively). Bar, 10 μm.

TABARA *et al.* 1998; TIMMONS and FIRE 1998), or it may be expressed within the cell of interest (NGO *et al.* 1998; KENNERDELL and CARTHEW 2000; WANG *et al.* 2000). For initial attempts to interfere with *CAP59* expression in cryptococcus, we designed a plasmid with convergent promoters flanking a segment of the gene. These studies were not successful (not shown), so we designed another construct employing a hairpin scheme with duplicate sequences of ~500 bp of *CAP59* in opposite orientation separated by a 250-bp spacer of *GFP* sequence (pCAP59i, Figure 1). Constitutive expression was driven by a cryptococcal actin promoter, and the chimeric sequence was followed by a *GAL7* terminator.

C. neoformans cells of serotype D were transformed by electroporation with the linearized interference construct (pCAP59i) or a control plasmid. Transformants were restreaked to a master plate and replica plated to medium for capsule induction. Ten percent of the pCAP59i transformants and 0% of the control transformants demonstrated a clear mutant phenotype, with dull colonies due to the absence of polysaccharide capsule (Table 3). Cells from one dull and one shiny colony transformed with pCAP59i (CAP59i-1 and CAP59i-2, respectively) were chosen for closer examination.

By light microscopy (Figure 2, first column) CAP59i-1 cells were noted to clump together, similar to strains in which *CAP59* has been disrupted (*cap59*). In contrast, parental cells (not shown) or those transformed with a control plasmid rarely clump. Examination of cells in the presence of india ink (not shown), which is excluded from the capsule, showed a characteristic halo in the case of parental and control cells. CAP59i-1 and *cap59* cells exhibited no halo, but CAP59i-2 showed an intermediate phenotype with a mixture of encapsulated and a few acapsular cells.

To test the presence of capsule with a more sensitive method, we treated cells with a monoclonal antibody to cryptococcal capsule, which we conjugated to Cy3 (PIERINI and DOERING 2001), and examined them by confocal microscopy (Figure 2, second column). Cells transformed with a control plasmid showed brightly fluorescent surface staining, as was the case with CAP59i-2 cells. In contrast, and similar to *cap59* mutants, CAP59i-1 cells showed fluorescence <1% of control (Figure 2),

TABLE 3

Efficiency of generation of mutant phenotypes

	Control	pCAP59i	pCAP59-noP	pCAP59i-tel	pADE2i	pCAP59/ADE2i
Dull	<1	10	<1	27	<1	24.5
Pink	<1	<1	<1	<1	7	25.5

Cells were transformed with the indicated plasmids as described in MATERIALS AND METHODS, and at least 100 transformants for each plasmid were restreaked for single colonies and examined for phenotype. The percentage of transformants demonstrating inhibition of *CAP59* expression (dull colonies) or *ADE2* expression (pink colonies) is tabulated for each plasmid.

even when grown in capsule-inducing medium (not shown). Quantitation showed no difference in fluorescence intensity between CAP59i-1 and *cap59* cells ($P < 0.001$), and electron microscopy confirmed the absence of capsule on CAP59i-1 and *cap59* cells (not shown). These results suggest that a fraction of transformants (*e.g.*, CAP59i-1) show complete interference with normal gene expression, while others (*e.g.*, CAP59i-2) do not (see DISCUSSION).

To confirm that the interference phenotype depended on exogenous DNA, CAP59i-1 cells were streaked on 5-FOA plates to select against maintenance of the *URA5*-marked interference construct. Colonies that grew under these conditions regained a shiny appearance and displayed wild-type capsule staining by immunofluorescence (Figure 2), demonstrating association of the acapsular phenotype with presence of the interfering plasmid. Southern blotting and PCR experiments showed that the endogenous *CAP59* gene was intact in CAP59i-1 and confirmed that the interfering construct had not been integrated and was lost after growth on 5-FOA (not shown). To assess dependence of interference on transcription, pCAP59i was modified by removing the promotor sequence; this plasmid (pCAP59i-noP) yielded no phenotypically altered transformants (Table 3).

If the observed acapsular phenotype of CAP59i-1 resulted from RNA interference, the levels of *CAP59* RNA in those cells should be reduced compared to controls. To test this we used a quantitative RT-PCR method. cDNA prepared from cells to be tested was used as a substrate for PCR reactions that were sampled every three cycles (see MATERIALS AND METHODS), and products were quantitated using SYBR green dye (sensitivity 60 pg dsDNA). Fluorescence was plotted *vs.* number of cycles, and the starting amounts of cDNA from each strain were adjusted such that the exponential phase of PCR product accumulation for a constitutively expressed control gene (*ACT*, amplified with primers 9 and 10 in Table 2) overlapped closely for all strains (Figure 3, A and B). These normalized amounts of cDNA were then used in experiments to quantitate the presence of specific RNA species in each strain. PCR product accumulation was plotted as above, and comparisons between several points in the exponentially increasing portions of the curves were used to calculate relative amounts of product. As expected, *GFP*-specific sequences were detected only in CAP59i-1 and CAP59i-2 cells, and sequences from *CAP59* that were also present in pCAP59i were detected in all cells except *cap59* (not shown). PCR was also performed with primers homologous to *CAP59* sequences that were absent from pCAP59i, to specifically assess amounts of cellular mRNA (Figure 3, C and D). In these reactions CAP59i-1 cells showed <23% as much *CAP59*-specific mRNA as control cells. *cap59* cells, as expected, had no *CAP59*-specific mRNA.

To examine the effect of dsRNAi on another gene product an interference construct was made with a segment of the cryptococcal *ADE2* gene (pADE2i, see MATERIALS AND METHODS). Similar to results with pCAP59i, 7% of cells transformed with pADE2i showed an interference phenotype (Table 3), producing pink colonies (*e.g.*, ADE2i-1 in Figure 4). RT-PCR experiments confirmed significant reduction in *ADE2* mRNA in the cells (not shown). The phenotype was plasmid dependent, as growth of ADE2i-1 on 5-FOA to select against maintenance of pADE2i yielded colonies of the wild-type cream color, which regained adenine prototrophy (Figure 5). Inhibition of expression of *ADE2*, as of *CAP59*, was specific, with no alterations of growth or morphology detected to suggest that dsRNA initiated nonspecific protein inhibition (as observed in some mammalian systems; WILLIAMS 1999). The successful inhibition of this second gene product indicates that dsRNAi offers a general technique for gene suppression in *C. neoformans*.

In studies with both pCAP59i and pADE2i we noted that some transformants remained phenotypically wild type, as exemplified by CAP59i-2 (Figure 2) and ADE2i-2 (Figure 4). Other transformants demonstrated intermediate phenotypes, such as reduced fluorescence with cell clumpiness for CAP59i or less intense pink color for pADE2i (not shown). We hypothesized that these partial phenotypes were due to variable expression of the

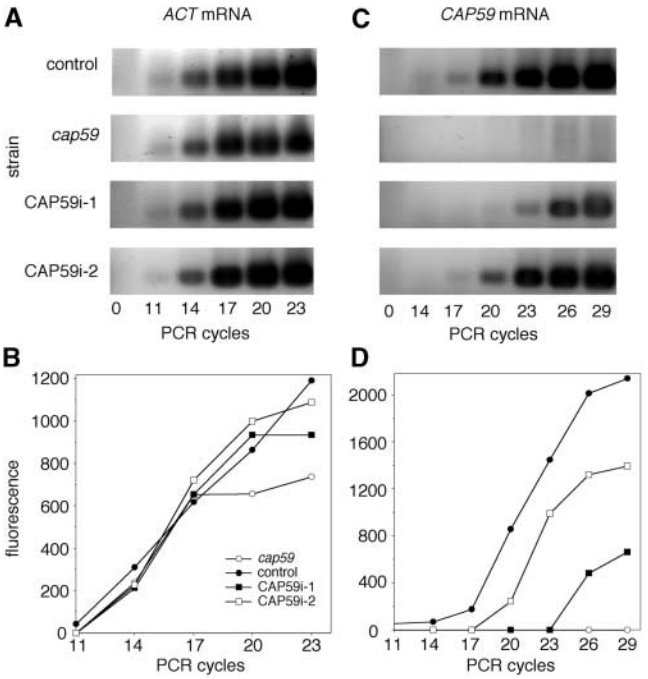

FIGURE 3.—RT-PCR analysis of *CAP59* expression. RT-PCR analysis of the indicated strains was performed as described in the text, using amounts of cDNA normalized to actin mRNA expression. The results of RT-PCR reactions using primers 9 and 10 for detection of *ACT* mRNA are shown in A and quantitation of the corresponding PCR product is plotted in B in arbitrary fluorescence units. RT-PCR reactions using primers 15 and 16 to detect *CAP59* mRNA are shown and plotted in C and D. Control cells, solid circles; *cap59*, open circles; CAP59i-1, solid squares; and CAP59i-2, open squares.

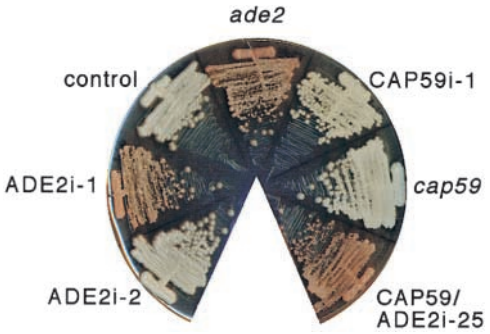

FIGURE 4.—Interference with *ADE2*. Cells were streaked on YPD plates and incubated for 3 days at 30° to demonstrate the pink color resulting from the accumulation of adenine biosynthetic intermediates. Two ADE2i strains are shown, one that shows the mutant phenotype (ADE2i-1) and one that does not (ADE2i-2), along with *ade2* cells and cells transformed with a control plasmid for comparison. The mutant phenotype is also exhibited by a double interference transformant (CAP59/ADE2i-25), but is not induced by inhibition or mutation of *CAP59* alone (CAP59i-1, *cap59*).

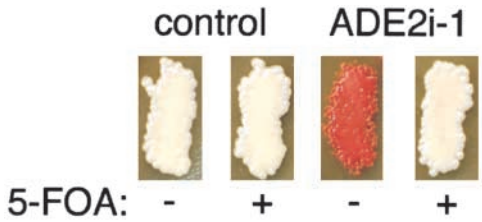

FIGURE 5.—5-FOA reversal of *ADE2* interference. ADE2i-1 cells and cells transformed with a control plasmid were grown on medium without (−) or with (+) 5-FOA. Growth in the presence of 5-FOA led to reversal of the ADE2i-1 mutant phenotype.

transforming plasmid, possibly resulting from variable modification of the exogenous DNA expression (EDMAN 1992; VARMA *et al.* 1992; KWON-CHUNG *et al.* 1998). To test this idea we quantitated the CAP59 mRNA in CAP59i-2 cells (Figure 3) and found it to be 72% as abundant as control, which is severalfold higher than the phenotypically mutant CAP59i-1 cells. We also examined the DNA from CAP59i-1 and CAP59i-2 cells by Southern blotting and noted that while the plasmids in both had been modified and enlarged, probably by a combination of degradation and telomere addition (KWON-CHUNG *et al.* 1998), the former had been extended to a greater extent (not shown). Intermediate phenotypes are discussed further below.

To stabilize the plasmid and reduce modifications, which might alter expression of the interference construct, we incorporated cryptococcal telomere sequences into pCAP59i. In transformations with this plasmid (pCAP59i-tel) we found a higher fraction of phenotypically mutant cells (Table 3), and we observed reduced modification of the input plasmid (not shown).

Because not all cells transformed with interfering plasmids demonstrate mutant phenotypes, investigation of novel genes could be slowed, as it would require initial assessment of mRNA levels in a number of transformants before phenotypes could be examined with confidence that interference was occurring. One way to mark transformants that exhibit interference would be to simultaneously interfere with the gene of interest and with a marker gene, which would provide an easily tracked phenotype. To test this approach we constructed an interference plasmid incorporating portions of both *CAP59* and *ADE2* (Figure 1, pCAP59/ADE2i). Transformants were both dull and pink, indicating simultaneous inhibition of expression of both genes (Table 3). Some colonies did exhibit only one phenotype (usually pink but not dull), again suggesting some variation in efficiency of inhibition (see DISCUSSION). Even with this variation, however, >80% of pink colonies were also acapsular, showing that this approach could effectively indicate cells in which interference was operating.

DISCUSSION

We have explored the utility of double-stranded RNA interference as a tool for specific inhibition of gene expression in *C. neoformans*. Our goal was to find a method to efficiently assess the phenotypic consequences of preventing production of specific gene products, both to circumvent the difficulty of gene disruption in this organism and to exploit the rapidly emerging genome sequence of this fungus for studies of its biology and pathogenicity. We have shown that double-stranded RNA interference occurs in *C. neoformans* by demonstrating specific inhibition of expression of genes involved in capsule synthesis and adenine biosynthesis, both individually and together. To date, there are no published reports of dsRNAi in a yeast or fungus. These observations should be of great practical value to investigations of important pathogens like *C. neoformans* that are less amenable to genetic manipulation than are model systems.

Studies of RNAi-resistant mutants in *C. elegans* have identified several genes believed to encode proteins involved in interference (TABARA *et al.* 1999). Interestingly, several of these have homologs known to participate in other post-transcriptional gene silencing (PTGS) processes, such as the quelling phenomenon in *Neurospora crassa* (whereby endogenous genes are silenced by introduction of a transgenic copy) or the PTGS observed in plants, suggesting shared mechanisms (CATALANOTTO *et al.* 2000; FAGARD *et al.* 2000; MAINE 2000; VAUCHERET *et al.* 2001). We examined the available *C. neoformans* genome sequence for homology to the Arabidopsis *AGO1* gene, one member of a gene family conserved across the three systems (FAGARD *et al.* 2000). The longest available contiguous stretch of cryptococcal sequence was 33% identical and 50% positive, as scored in an NCBI BLAST search with the BLOSUM62 matrix (ALTSCHUL *et al.* 1997), when compared to the region of Ago1 from amino acids 552–1002; the most conserved region within it (corre-

sponding to amino acids 772–986 of Ago1) was 46% identical and 59% positive. Notably, the latter group of identical residues included 45 of the 61 residues that are identical in Ago1 and its homologs in Neurospora (QDE-2) and *C. elegans* (RDE-1). The cryptococcal genome project also has generated sequences encoding polypeptides with significant homology to the *C. elegans* Ego-1, which encodes a putative RNA-dependent RNA polymerase with homologs in *Arabidopsis thaliana* and *N. crassa*, and to the *N. crassa* QDE-3, a putative helicase. The presence in the cryptococcal database of an Ago-1-like sequence and homologs of other proteins implicated in RNAi is consistent with this pathogen utilizing RNAi or related processes in post-transcriptional gene silencing.

For the two genes we tested, 7–10% of transformants demonstrated a complete mutant phenotype, although others with less mRNA inhibition displayed intermediate or wild-type phenotypes (Figures 2 and 4 and data not shown). This is consistent with observations in *Trypanosoma brucei*, where individual transformants demonstrate variable efficiency of interference (E. Ullu, personal communication). Our results suggest that for the observation of some phenotypes (*e.g.*, complete lack of capsule detected by antibody binding) mRNA levels must drop below a threshold, which may be different for each protein depending on its mechanism of action and regulation. This would explain why in experiments with simultaneous interference of two genes we did obtain colonies where only one mutant phenotype was expressed and also why in almost all of these cases the same single phenotype was observed. It is not clear why a range of interference occurs in cryptococcus, but it may be that the interference plasmid is variably modified by the cell before stabilization by addition of telomeres (Edman 1992; Varma *et al.* 1992; Kwon-Chung *et al.* 1998). This could result in plasmids that are variably expressed and thus have different degrees of efficacy in mediating mRNA degradation. Addition of telomeric sequences to the interfering plasmid did increase the yield of mutant phenotypes and decrease the degree of plasmid modification after transformation.

For some purposes the availability of cells with different degrees of RNA interference may have advantages over complete disruptions, as intermediate phenotypes might be available for investigation. For example, if complete inhibition of a gene were lethal, this technique might still yield cells for study. Similarly, regulated expression of interfering constructs might permit manipulation of levels of inhibition. This could be useful for investigation of nonessential genes and also for potentially determining whether a gene is essential, at present a difficult question to address directly in cryptococcus.

One disadvantage of the spectrum of interference observed is that to investigate novel genes would require two phases of study: initial investigation of a series of transformants to determine their levels of the mRNA of interest and then further analysis of those transformants exhibiting appropriate levels of interference. To simplify this process we propose use of *ADE2*, or another readily observed marker, in tandem interference constructs with the gene of interest. For example, transformants that are pink, indicating that *ADE2* expression is inhibited and interference is operating, could be easily selected for further study of the second gene. Tandem interference may also be useful for cases where a double mutant is desired for study, as in Cryptococcus this will certainly be more rapid than either sequential gene disruptions or independent disruptions and genetic crossing. It may also be possible to interfere with more than two genes simultaneously. In our experiments we have used relatively large portions of the genes to be inhibited (500 bp), but the literature suggests that much smaller segments are effective. This indicates that plasmid size will not be limiting and it will be possible to test multi-interfering constructs. It may also be possible to interfere with several related genes simultaneously if they share specific sequences that may be incorporated into the interfering plasmid; this may allow coordinate inhibition of a family of genes (Fire *et al.* 1998).

Double-stranded RNA interference holds great promise for studies of cryptococcus and other fungi. In particular, it should provide a way to exploit the rapidly accumulating genome sequence of *C. neoformans* without requiring disruption of each gene of potential interest. Because only a short segment of the gene sequence is needed for interference, open reading frame identification will suffice for this method and complete annotation will not be required. This will allow screening of sequences of interest and subsequent choice of genes for further study. Other advantages of this method include the rapidity of functional disruption, the potential for regulated specific inhibition of gene expression, and the feasibility of simultaneous inhibition of several genes or a gene family. Together these features indicate that the specific inhibition of gene expression in cryptococcus using RNA interference will be a valuable tool in an organism where gene disruption is a time-consuming and labor-intensive process.

We appreciate the efforts of Erin Brosnahan, Scott Handley, and Jacquelyn Engle in helping to develop the quantitative RT-PCR method and of Wandy Beatty for her examination of cells by electron microscopy. We are grateful for helpful discussions with Elisabetta Ullu, Mark Johnston, members of the Doering lab, and colleagues at the Molecular Mycology Course at the Marine Biological Laboratories. We thank June Kwon-Chung and Joe Heitman for strains, Brian Wickes and Andy Fire for DNA, and Arturo Casadevall for antibody. T.R.C.'s work on this project was supported by an American Society for Microbiology Undergraduate Research Fellowship and Howard Hughes Medical Institute Summer Research funds to Washington University. L.M.P. was supported by an Atorvastatin Research Award from Pfizer/Parke Davis. W.E.G. was supported by the National Institutes of Health (AI25584). T.L.D. and work in her laboratory were supported by the National Institutes of Health (AI49173), National Science Foundation (POWRE 0096115), a Burroughs Wellcome Fund Junior Investigator Award in Molecular Pathogenic Mycology, the Andrew and Virginia Craig Faculty Research Fund, and a Neose Corporation GRANT award.

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Communicating editor: A. P. Mitchell