Deletion of Individual mRNA Capping Genes Is Unexpectedly Not Lethal to *Candida albicans* and Results in Modified mRNA Cap Structures

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Eukaryotic mRNAs are modified at the 5′ end with a cap structure. In fungal cells, the formation of the mRNA cap structure is catalyzed by three enzymes: triphosphatase, guanylyltransferase, and methyltransferase. Fungal capping enzymes have been proposed to be good antifungal targets because they differ significantly from their human counterparts and the genes encoding these enzymes are essential in *Saccharomyces cerevisiae*. In the present study, *Candida albicans* null mutants were constructed for both the mRNA triphosphatase-encoding gene (*CET1*) and the mRNA methyltransferase encoding gene (*CCM1*), proving that these genes are not essential in *C. albicans*. Heterozygous deletions were generated, but no null mutants were isolated for the guanylyltransferase-encoding gene (*CGT1*), indicating that this gene probably is essential in *C. albicans*. Whereas these results indicate that Cet1p and Ccm1p are not ideal molecular targets for development of antifungal drugs, they do raise questions about the capping of mRNA and translation initiation in this fungus. Southern blot analysis of genomic DNA indicates that there are not redundant genes for *CET1* and *CCM1* and analysis of mRNA cap structures indicate there are not alternative pathways compensating for the function of *CET1* or *CCM1* in the null mutants. Instead, it appears that *C. albicans* can survive with modified mRNA cap structures.

*Candida albicans* infections are a serious health risk for immunocompromised patients, such as transplant recipients, cancer patients, neonates, human immunodeficiency virus-positive patients, and hospital patients with intravenous tubes or catheters. Few drugs are available for treating disseminated candidiasis, and there is a need for new antifungal compounds to treat these infections (13). There is a common presumption that antifungal agents can be developed from inhibitors of enzymes that catalyze essential functions. Although this presumption has not yet been proven, it has generated interest in enzymes that catalyze essential functions. Whereas these results indicate that Cet1p and Ccm1p are not ideal molecular targets for development of antifungal drugs, they do raise questions about the capping of mRNA and translation initiation in this fungus.

In a recent publication, Pei et al. (27) concluded the *CET1* gene is essential in *C. albicans* because they were unable to construct a null deletion mutant. However, we show here that both alleles of *CET1* can be deleted in *C. albicans* and the null mutants are viable. We were also able to delete both alleles of *C. albicans CCM1*.

Construction of null mutants in *C. albicans* is complicated because this diploid organism lacks a sexual cycle. Techniques to evaluate gene essentiality by using sequential disruption of both alleles of a target gene in *C. albicans* were introduced by Fonzi and Irwin (9), and more recently the Mitchell laboratory has developed additional methods for generating *C. albicans* null mutants (7, 42, 43). In the two-step transformation method, alleles are knocked out in sequential transformations because this diploid organism lacks a sexual cycle. Techniques to evaluate gene essentiality by using sequential disruption of both alleles of a target gene in *C. albicans* were introduced by Fonzi and Irwin (9), and more recently the Mitchell laboratory has developed additional methods for generating *C. albicans* null mutants (7, 42, 43). In the two-step transformation method, alleles are knocked out in sequential transformations selecting for homologous recombinations (42). An alternative deletion strategy, the single-transformation method, first selects for transformants that are Arg⁺ due to the insertion of the *ura3Δ3-ARG4-ura3Δ5* (UAU1) cassette at the targeted locus (7). Transformants with the desired UAU1 cassette integration are then grown on synthetic complete (SC) medium lacking arginine and uridine to select Arg⁺ Ura⁺ cells. These cells have undergone a mitotic recombination event in which...
one allele of the nonessential gene is replaced with the UAU1 cassette and the other allele of the nonessential gene is replaced with a functional URA3 gene. In the case of an essential gene, insertional deletion of two alleles of the target gene can only be generated if an allelic duplication occurs such that the strain becomes triploid for the target gene locus. This maintenance of a wild-type allele under the selective pressure for homozygous deletions is strong evidence that a gene is essential.

We initially applied the single transformation method (7) to determine whether CET1, CGT1, and CCM1 are essential genes in C. albicans. After generating cet1-null mutants by this technique, we were also able to generate cgt1-null mutants by using the two-step transformation knockout strategy (42), confirming that the C. albicans CET1 gene is nonessential. C. albicans ccm1-null mutants were generated by using the two-step transformation method. CGT1 heterozygous deletions were generated by both techniques, but we were unable to recover any isolates completely lacking a wild-type CGT1, indicating that this is probably an essential gene in C. albicans.

The viability of C. albicans cet1- and ccm1-null mutants raises questions about the mechanics and role of cap-dependent translation initiation in this fungal pathogen. There are several possible explanations why CET1 and CCM1 are not essential in C. albicans, e.g., redundant genes encoding mRNA capping enzymes, alternative mRNA capping pathways, or the possibility that C. albicans can survive with modified mRNA cap structures. Our analysis and characterization of mRNA cap structures indicates that the C. albicans cet1- and ccm1-null mutants do make modified mRNA-cap structures, and these modified mRNA-caps are sufficient for C. albicans to effectively translate mRNA and survive.

**MATERIALS AND METHODS**

**Reagents.** Taq polymerase was purchased from Applied Biosystems (Foster City, Calif.). Restriction endonucleases and deoxynucleotidyl transferase were purchased from Invitrogen (Rockville, Md.). All enzymes were used according to manufacturers’ protocols. PCR primers used in the present study were purchased from Sigma-Genosys and are described in Table 1. Plasmids pGEM-URA3, pGEM-HIS1, and pRS-ARG4-ΔSryl (42), as well as the UAU1 cassette-containing plasmid pBME101 (7), were generous gifts from A. P. Mitchell. Synthetic complete (SC) media were made by using dropout base mixes available from Bio 101 (Vista, Calif.) with glucose added to a final concentration of 2%. Southern blot analyses were performed using probes labeled with the DIG High Prime Kit and the DIG chemiluminescent detection kit (Roche, Indianapolis, Ind.). Amphotericin B, cycloheximide, hygromycin B, and actinomycin D were purchased from Sigma (St. Louis, Mo.), and blastidicin S was purchased from Calbiochem (Darmstadt, Germany). Tobacco acid pyrophosphatase (TAP) was obtained from Calbiochem (St. Louis, Mo.). All enzymes were used according to manufacturers’ protocols. PCR primers used in the present study were purchased from Sigma-Genosys and are described in Table 1. Plasmids pGEM-URA3, pGEM-HIS1, and pRS-ARG4-ΔSryl (42), as well as the UAU1 cassette-containing plasmid pBME101 (7), were generous gifts from A. P. Mitchell. Synthetic complete (SC) media were made by using dropout base mixes available from Bio 101 (Vista, Calif.) with glucose added to a final concentration of 2%. Southern blot analyses were performed using probes labeled with the DIG High Prime Kit and the DIG chemiluminescent detection kit (Roche, Indianapolis, Ind.). Amphotericin B, cycloheximide, hygromycin B, and actinomycin D were purchased from Sigma (St. Louis, Mo.), and blastidicin S was purchased from Calbiochem (Darmstadt, Germany). Tobacco acid pyrophosphatase (TAP) was obtained from Epicentre Technologies (Madison, Wis.). Nucleotide standards, adenosine 5'-phosphate, 5'-monophosphate, and 5'-triphosphate, were bought from Sigma. NaB[3H]4 (15 Ci/mmol) was purchased from New England Biolabs, Inc. (Beverly, Mass.). Nucleotide standards, adenosine 5'-monophosphate (AMP) and GMP, were bought from Sigma. NaB[3H]4 (15 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.).

**Strains and growth conditions.** Strains used in the present study are described in Table 2. For transformation, C. albicans BW327 was grown overnight in YEPD (1% yeast extract, 2% Bacto Peptone [Difco], 2% glucose) supplemented with uridine at a final concentration of 80 µg/ml, subcultured 1:100 into 50 ml of the same medium, and grown for two doublings at 30°C. Where indicated, uridine was added to a final concentration of 80 µg/ml, and arginine was added to a final concentration of 50 µg/ml. All transformants were plated onto SC lacking arginine (SC–arginine–uridine), or SC lacking both arginine and uridine (SC plus arginine–uridine), or SC lacking both arginine and uridine (SC–arginine–uridine) as appropriate for the selectable marker(s) and incubated at 30°C for 2 to 3 days. For the selection of Arg+ Ura– segregants of CET1 heterozygotes disrupted with the UAU1 cassette, isolated colonies were grown overnight in YEPD lacking uridine (YPED–uridine). A total of 1 ml of each culture was washed with sterile water and then resuspended in 1 ml of H2O, and a 100-µl portion of the cells was plated onto SC–arginine–uridine, followed by growth for 2 to 3 days at 30°C. Growth studies were performed in round-bottom 96-well plates with cells inoculated in 200 µl of YEPD–uridine to a final concentration of 2.000 to 3.000 cells/ml, incubated at 30 or 37°C for 60 h. Growth was monitored in a SpectraMax 250 (Molecular Dynamics) at 595 nm, with readings taken every 30 min after shaking for 5 s.

**DNA methods.** Genomic DNA was prepared from overnight cultures of C. albicans grown at 30°C in 5 ml of YEPD by using the glass bead lysis method (3). PCR amplifications of deletion cassettes and analytical PCR with genomic DNA were carried out as described in Wilson et al. (42). Before transformation of C.
*C. albicans* with insertional deletion cassettes, the PCR fragments were blocked with ddATP by using terminal deoxynucleotidyl transferase according to the protocol described by Shah-Mahoney et al. (33). PCR-amplified DNA was purified by using phenol-CHCl3-isoamyl alcohol (25:24:1), followed by ethanol precipitation before and after the blocking reaction. High-stringency Southern blot analysis (36) was performed on 1 to 3 μg of genomic DNA that was digested with *BgIII* and separated on 0.8% agarose gels for capillary transfer to membrane (Hybond N; Amersham). Probes were PCR amplified from genomic DNA of *C. albicans* BWP17 with primers CET1 probe F and CET1 probe R for the CET1 probe and CCM1 probe F and CCM1 probe R for the CCM1 probe. Membranes were hybridized with 1 μg of DIG-labeled probe under high-stringency conditions (42°C), followed by stringent wash conditions (two 5-min room temperature washes in 2X SSC [0.15 M NaCl, 10 mM sodium citrate, pH 7] plus 0.1% sodium dodecyl sulfate [SDS]), followed by two 20-min washes at 68°C in 0.5X SSC plus 0.1% SDS). Southern blots were developed by using anti-DIG-Fab fragments, which are conjugated to alkaline phosphatase, and CSPD chemiluminescent detection. The anti-DIG was diluted 1:10,000 in blocking buffer and the CSPD solution was added, and the samples were incubated for an additional 15 min. The RNA was ethanol precipitated, dissolved in 100 μL of water, and purified by using the RNeasy kit (Qiagen). The volume of purified radiolabeled mRNA solutions was adjusted to 50 μL with water, and the samples were split in half for nuclease P1 or TAP digestions. For P1 digestion, 25-μL samples were adjusted to a final concentration of 50 mM ammonium acetate (pH 5.3) and treated with nuclease P1 (100 μg/ml) at 37°C for 18 h. The remaining 25-μL fractions were digested with TAP by adding 2.9 μL of 10× TAP reaction buffer (500 mM sodium acetate [pH 6.0], 1% β-mercaptoethanol, and 0.1% Triton X-100) and 1.1 μL of TAP (10 U) and incubated at 37°C for 4 h.

**Cap structure resolution.** Next, 20 μL of the RNA samples digested with nuclease P1 or TAP were spotted onto polyethyleneimine-cellulose, along with unlabeled nucleotides, AMP and GMP, and cap standards, m7GpppG, m7GpppA, GpppG, and GpppA. After they dried, the TLC plates were developed in 0.1 M sodium phosphate buffer (pH 6.8)—ammonium sulfate—n-propanol (100:60:2 [vol/vol/vol]) at room temperature. Markers were detected under UV light, and the positions of tritiated cap structures were identified by phosphorimaging.

## RESULTS

### C. albicans CET1 deletions.

Targeted deletions of the triphosphatase encoding gene, *CET1*, were constructed by using two different methods: the single-step transformation procedure of Enloe et al. (7), and the sequential two-step transformation method (42). In the single-step transformation method, the first allele was deleted by transforming *C. albicans* with a UAU1 PCR cassette-amplified from pBME101 by using primers *CaCET15*-DR and *CaCET13*-DR. Transformants were selected on SC—arginine. As detected by analytical PCR, three of four Arg+ transformants had the UAU1 disruption cassette integrated in the *CET1* genomic locus. These three *Δcet1*:UAU1/CET1 heterozygotes were streaked out on SC—arginine plates to generate isolated colonies, and 15 isolated colonies from each were grown overnight in YEPD—uridine at 30°C. Overnight cultures were harvested and washed in water and plated on SC—arginine—uridine to select for mitotic recombinants. PCR analysis of genomic DNA

| Strain | Genotype | Description | Source |
|--------|----------|-------------|--------|
| BWP17  | Δura3::Xmn434 Δhis1::hisG Arg4::hisG | Parent strain for deletions, allows selection of three different markers (URA3, ARG4, HIS1) | A. P. Mitchell (42) |
| CQF125 | Δcet1::UAU1/CET1 | Heterozygote of CET1 from single transformation method | This study |
| CQF136 | Δcet1::UAU1/Δcet1::URA3 | Temperature-sensitive null mutant of cet1 from single transformation method | This study |
| CQF160 | Δcet1::UAU1/Δcet1::URA3 | Null mutant of cet1 from single transformation method | This study |
| CQF142 | Δcet1::ARG4/CET1 | Heterozygote of CET1 from two-step transformation method | This study |
| CQF141 | Δcet1::ARG4/Δcet1::URA3/Δcet1::CET1 | Heterozygote of CET1 from two-step transformation method | This study |
| CQF152 | Δcet1::ARG4/Δcet1::URA3 | Trisomic isolate for CET1 locus generated by recombination at his1::hisG locus | This study |
| CQF248 | Δcet1::ARG4/Δcet1::URA3 | CET1 restored to null in trans by recombination at his1::hisG locus | This study |
| CQF153 | Δcet1::ARG4/CGT1 | Heterozygote of CGT1 from two-step transformation method | This study |
| CQF170 | Δcet1::ARG4/Δcet1::URA3/CGT1 | Trisomic for CGT1 locus from two-step transformation method | This study |
| CQF154 | Δcct1::ARG4/CCM1 | Heterozygote of CCM1 from two-step transformation method | This study |
| CQF171 | Δcct1::ARG4/Δcct1::URA3 | Null mutant of ccm1 from two-step transformation method | This study |
| CQF250 | Δcct1::ARG4/Δcct1::URA3/Δcct1::CET1 | CCM1 restored to null in trans by recombination at his1::hisG locus | This study |
for the 78 Arg⁺ Ura⁺ segregants from this selection showed that 12 were null mutants (Δcet1::UAU1/Δcet1::URA3) and 44 were trisomic for the CET1 locus (Δcet1::UAU1/Δcet1::URA3/CET1). The remaining 12 segregants probably had rearrangements or ectopic recombinations that we could not identify.

The two-step transformation method was also used to confirm that CET1 is not essential in C. albicans. Heterozygous CET1 deletion mutants were identified after selection on SD–arginine after transformation of C. albicans BWP17 with a PCR cassette amplified from pRS-ARG4–ΔSpeI with the primers CaCET15'-DR and CaCET13'-DR. Two of the nine Arg⁺ transformants had targeted insertions in one CET1 allele. One of these heterozygotes (Δcet1::ARG4/CET1) was then transformed with a URA3 marker cassette PCR amplified from pGEM-URA3 with the primers CaCET5'-DR and CaCET3'-DR. Eight Arg⁺ Ura⁺ transformants were screened, and six were null mutants (Δcet1::ARG4/Δcet1::URA3).

Analytical PCR of CET1 deletion strains are shown on the gel in Fig. 1. The PCR amplifications were designed to detect the four possible chromosomal constructs at this locus (depicted in Fig. 1A to D). PCR amplification of the wild-type CET1 allele (Fig. 1A) with primers CaCET1F and CaCET1R should produce a PCR product of 1,500 bp. Using the same primers, PCR amplification of DNA from isolates with the Δcet1::URA3 allele (Fig. 1B), the Δcet1::ARG4 allele (Fig. 1C), or the Δcet1::UAU1 allele produce larger products (1,620 bp for URA3, 2,600 bp for ARG4, and 4,000 bp for Δcet1::UAU1). Because the PCR product for the Δcet1::UAU1 allele obtained with the flanking primers is so large, amplification of it is not favored when the smaller alleles are present. Therefore, to determine whether this allele is definitively present, we also analyzed genomic DNA of UAU1 transformants by using the ARG4 primer paired with the CaCET1R primer. This primer pair should produce a product of 1,560 bp from the Δcet1::URA1 allele (Δcet1::UAU1/Δcet1::URA1/Δcet1::URA3) because of the larger allele obtained with the flanking primers is so large, amplification of it is not favored when the smaller alleles are present. Therefore, to determine whether this allele is definitively present, we also analyzed genomic DNA of UAU1 transformants by using the ARG4 primer paired with the CaCET1R primer. This primer pair should produce a product of 1,560 bp from the Δcet1::URA1 allele (Δcet1::UAU1/Δcet1::URA1/Δcet1::URA3).

Because the PCR product for the Δcet1::UAU1 allele obtained with the flanking primers is so large, amplification of it is not favored when the smaller alleles are present. Therefore, to determine whether this allele is definitively present, we also analyzed genomic DNA of UAU1 transformants by using the ARG4 primer paired with the CaCET1R primer. This primer pair should produce a product of 1,560 bp from the Δcet1::URA1 allele (Δcet1::UAU1/Δcet1::URA1/Δcet1::URA3).
yield the band corresponding to the Δcet1::URA3 allele (lanes 4 and 6), whereas only the band corresponding to the Δcet1::URA3 allele is seen with CaCET1F and CaCET1R primer amplifications (lanes 5 and 7). Isolate CQF152 was generated from the two-step transformation method so only the CaCET1F and CaCET1R primers were used. Lane 8 shows that this isolate has the two deletion alleles (Δcet1::URA3 and Δcet1::ARG4) and no wild-type CET1 allele.

**C. albicans CGT1 and CCM1 deletions.** The single transformation method of Enloe et al. (7) was initially applied to deletion analysis of the CGT1 and CCM1 genes in *C. albicans*. The marker deletion cassettes were PCR amplified from pBME101 by using primers CaCGT5′-DR and CaCGT3′-DR or CaCCM5′-DR and CaCCM3′-DR. *C. albicans* BWP17 was transformed with the UAU1 PCR cassettes, and Arg⁺ transformants were selected. DNA isolated from these transformants was screened by analytical PCR to identify isolates with the correct heterozygous deletions. Heterozygous deletions were constructed for both genes. In this gene deletion procedure, the second step requires selection for a mitotic recombination event where the UAU1 cassette recombines into the second allele and the cells become Arg⁺ Ura⁺. We were unable to generate null mutants or even trisomic isolates for either of these two genes by this method.

A second method for generating *C. albicans* gene knockouts was also tested for CGT1 and CCM1 (42). *C. albicans* BWP17 was first transformed with ARG4 cassettes PCR amplified from pRS-ARG4-Dspe1 with primers CaCGT5′-DR and CaCGT3′-DR or primers CaCCM5′-DR and CaCCM3′-DR. A heterozygous strain for each of these genes was then transformed with a URA3 marker cassette PCR amplified from pGEM-URA3 with primers CaCGT5′-DR and CaCGT3′-DR or primers CaCCM5′-DR and CaCCM3′-DR. Twelve Arg⁺ Ura⁻ transformants were screened for CCM1 deletions, and two were ccm1-null mutants (Δccm1::ARG4/Δccm1::URA3). All 24 of the Arg⁺ Ura⁻ transformants screened for CGT1 deletions retained a wild-type CGT1 allele, with 22 isolates being heterozygous (Δcg1::ARG4/Δcg1::ARG4) and 2 isolates being trisomic (Δcg1::ARG4/Δcg1::ARG4/Δcg1::ARG4).

The generation of ccm1-null mutants demonstrates that this is not an essential gene in *C. albicans*, but the CGT1 gene remains an enigma. We did not generate deletion mutants at this locus in multiple attempts by both deletion approaches. However, heterozygous deletion mutants (Δcg1::ARG4/CGT1) can be constructed (Fig. 2), indicating that the deletion cassette can integrate into at least one allele at the desired locus. The failure to generate the double deletion could be due to preferential insertion of the deletion cassette at one allelic variant. Yesland and Fonzi (46) have reported that sequence heterology biases insertion to the allele with the best sequence variant. Yesland and Fonzi (46) have reported that sequence heterology biases insertion to the allele with the best sequence variant.
homology with the transforming DNA. However, our sequence analysis of the C. albicans BWP17 CGT1 locus shows that none of the allelic variations in this gene are found in the sequences that would hybridize to the flanking homology provided by the CaCGT5'-DR and CaCGT3'-DR oligonucleotides in the PCR deletion cassette (data not shown). Therefore, we believe that we are not seeing the allelic bias that CGT1 is likely an essential gene in C. albicans. Another group has reported failure to generate a cgt1-null mutant in C. albicans (5), supporting an essential nature for this gene.

Figure 2 shows the PCR analysis for the CGT1 and CCM1 deletions. PCR amplification with CaCGT1F and CaCGT1R results in PCR product sizes of 1,140 bp for the wild-type CGT1 allele (Fig. 2A), 2,600 bp for the Δcg1::ARG4 allele (Fig. 2B), and 1,600 bp for the Δcg1::URA3 allele (Fig. 2C). Amplification of genomic DNA with primers CaCGT1F and CaCGT1R produces a PCR product the size of the expected wild-type CGT1 allele for C. albicans BWP17 (lane 1). Two bands are seen for isolate CQF153 (lane 2), with sizes of the wild-type CGT1 allele and the size of the Δcg1::ARG4 allele, and all three bands (A, B, and C) are seen for isolate CQF170 (lane 3). PCR amplification with CaCCM1F and CaCCM1R results in PCR product sizes of 1,140 bp for the wild-type CCM1 allele (A), 2,600 bp for the Δccm1::ARG4 allele (B), and 1,600 bp for the Δccm1::URA3 allele (C). Amplification of genomic DNA with primers CaCCM1F and CaCCM1R produces a PCR product the size of the expected wild-type CCM1 allele for C. albicans BWP17 (lane 4), whereas two bands are seen for isolate CQF154 (lane 5) with sizes of the wild-type CCM1 allele and the size of the Δccm1::ARG4 allele. With these primers, both the deleted alleles, Δccm1::URA3 and Δccm1::ARG4, are seen for isolate CQF171 (lane 6) but no wild-type CCM1 allele.

Southern blot analysis. In order to eliminate the formal possibility that cet1- and ccm1-null mutants survive because a transposed copy of the wild-type CET1 allele or CCM1 allele exists in another chromosomal location, C. albicans chromosomal DNA was analyzed by Southern blot (Fig. 3). Genomic DNA from C. albicans BWP17; the CET1 heterozygotes CQF142 (Δcet1::ARG4/CET1) and CQF125 (Δcet1::UAU1/CET1); the cet1-null mutants CQF152 (Δcet1::ARG4/Δcet1::URA3), CQF160 (Δcet1::UAU1/Δcet1::URA3), and CQF136 (Δcet1::UAU1/Δcet1::URA3); the CCM1 heterozygote CQF154 (Δccm1::ARG4/CCM1); and the C. albicans ccm1-null mutant CQF171 (Δccm1::ARG4/Δccm1::URA3) was analyzed by Southern blot (Fig. 3). Genomic DNA was digested with BglII restriction endonuclease. After gel electrophoresis, the DNA was transferred to a nylon membrane for probing. For CET1 (Fig. 3A), the DNA probe encompassed codons 51 to 480 of the 520 codon CaCET1 open reading frame. For CCM1 (Fig. 3B), the DNA probe encompassed codons 18 to 455 of the 474 codon CaCCM1 open reading frame. Southern hybridization showed that, as expected, only one DNA band hybridized to the CET1 probe and one DNA band hybridized to the CCM1 probe in C. albicans BWP17 (Fig. 3). The CET1 or the CCM1 band is seen in the heterozygote isolates but not in the cet1- or ccm1-null mutants. There was no evidence that the wild-type CET1 or CCM1 was transposed in the null mutants.

Growth of capping gene deletion mutants. Growth in YEPD at both 30 and 37°C was measured for C. albicans BWP17 parent strain; the CET1, CGT1, and CCM1 heterozygous deletion mutants and the cet1- and ccm1-null mutants (Fig. 4). The wild-type parent and the heterozygous strains all had very similar growth curves at both temperatures. The null mutants grew slower at both temperatures, but the difference is more pronounced at 37 than at 30°C. The lag in growth was much longer for the null mutants at 37°C than for the heterozygotes or the parent. One of the null mutants constructed from the one-step transformation method was temperature sensitive, with almost no growth over 96 h at 37°C.

Sensitivity to antifungal compounds. Prompted by a publication describing a C. albicans Δcg1/GTG1 heterozygous deletion mutant that is resistant to the protein synthesis inhibitor hygromycin B (5), we decided to test the sensitivity for each of...
our capping gene deletion mutants to antifungal compounds (Table 3). The compounds tested included the following: amphotericin B, a polyene that interacts with ergosterol in the fungal membrane (6); cycloheximide, which inhibits peptide synthesis (24); blasticidin S, an aminohexosyl-cytosine nucleoside that inhibits ribosomal peptidyltransferase (15, 16); hygromycin B, which inhibits peptide chain elongation by disruption of EF-2-dependent translation (8); and actinomycin D, a polypeptide that inhibits DNA-dependent RNA synthesis (1).

For standard yeast broth microdilution assays, drug concentrations were tested at twofold dilutions, and MIC endpoints were determined at 48 h; however, the MICs for the \( \text{cet1} \)-null strain (CQF152) were determined at 72 h because no growth was observed in the microtiter plate for this strain even in the no-drug controls at 48 h. MICs that are within two dilutions are not considered significantly different (23). All strains showed very similar sensitivities to amphotericin B and actinomycin D.

The heterozygote deletion mutants for all three genes had similar sensitivities as the BWP17 parent to all drugs tested. Our \( C. \text{albicans} \ \Delta \text{cgt1}/\text{CGT1} \) heterozygous deletion mutant was not more resistant to hygromycin B than the parent strain. However, our \( C. \text{albicans} \ \Delta \text{cet1}/\text{cet1} \) mutant and our \( \Delta \text{ccm1}/\text{ccm1} \) mutants were slightly more sensitive to hygromycin B than the other strains tested. The \( \Delta \text{cet1}/\text{cet1} \) mutant was more sensitive to cycloheximide than any of the other strains.

Characterization of mRNA cap structures. mRNA was purified from \( C. \text{albicans} \) BWP17, CQF142 (\( \Delta \text{cet1}/\text{CET1} \)), CQF248 (the \( \text{CET1} \) restored null), CQF153 (\( \Delta \text{cgt1}/\text{CGT1} \)), and CQF250 (the \( \text{CCM1} \) restored null) by using hot phenol extraction (18). Treatment of mRNA with tritiated sodium borohydride labels the 2',3'-cis-diols on the ribose rings of both mRNA cap structures and the terminal nucleotides at the 3' end of mRNA (12). In order to separate the cap structures for TLC analysis, the radiolabeled mRNA was

| \( C. \text{albicans} \) strain | Amphotericin B | Cycloheximide | Blasticidin S | Hygromycin B | Actinomycin D |
|-----------------------------|--------------|--------------|--------------|-------------|--------------|
| BWP17 (parent)              | 0.5          | >128         | >32          | 64          | 128          |
| CQF142 (\( \Delta \text{cet1}/\text{CET1} \)) | 0.5          | >128         | >32          | >128        | 128          |
| CQF152 (\( \Delta \text{cet1}/\text{cet1} \)) | 0.5          | 32           | 32           | 8           | 32           |
| CQF248 (\( \text{CET1} \) restored null) | 0.5          | >128         | >32          | >128        | 128          |
| CQF153 (\( \Delta \text{cgt1}/\text{CGT1} \))  | 0.5          | >128         | >32          | >128        | 128          |
| CQF154 (\( \Delta \text{ccm1}/\text{CCM1} \))  | 0.5          | >128         | >32          | >128        | 128          |
| CQF171 (\( \Delta \text{ccm1}/\text{ccm1} \))  | 0.5          | >128         | >32          | 32          | 64           |
| CQF250 (\( \text{CCM1} \) restored null) | 0.5          | >128         | >32          | >128        | 128          |

*All MIC values were determined after 48 h of incubation, except for strain CQF152, where they were determined after 72 h of incubation due to slow growth even in the no-drug controls.*

FIG. 4. Growth curves for \( C. \text{albicans} \) parent strain and capping gene deletion mutants at 30 and 37°C. Cultures were grown in 200 µl of YEPD+uridine in 96-well plates with starting inoculum sizes of ca. 2,000 to 3,000 cells/ml. Growth was monitored in a Spectramax plate reader every 30 min after the plates were shaken for 5 s.
treated with TAP or P1 nuclease. TAP hydrolyzes a variety of nucleoside-linked pyrophosphate bonds, including those found in cap structures; however, phosphodiester bonds, which link nucleoside-linked pyrophosphate bonds, including those found in mRNA, are not cleaved by TAP (35). Hence, TAP digestion of mRNA removes the 5'-terminal phosphate bond between nucleosides in mRNA, generating a cap structure since mRNA digested with TAP generates a product that migrates with GMP. This finding is consistent with TAP-mediated hydrolysis of cap structures lacking a methyl moiety at the N7 position of the 5'-terminal guanylate. Under the TLC conditions used here, U3 must represent a product with a greater polarity than any of the standards or other digestion products (U3). We speculate that this spot also represents a cap structure since mRNA digested with TAP generates a product that migrates much farther than any of the standards or other digests. This could be the result of an extra phosphoryl group on the cap structure and would be consistent with the mutant being unable to catalyze the mRNA triphosphatase reaction.

Restoration of null mutants. As a formal proof that the phenotypes associated with loss of cet1 and ccm1 in the null mutants are due to the loss of the respective gene functions rather than disruption of the genetic loci, we inserted wild-type copies of the genes back into the genome at the Δhis1:hisG locus (42). The wild-type genes and upstream sequence were PCR amplified from C. albicans BWP17 genomic DNA with primers for CET1 (CET1-restore 5' and CET1-restore 3') and primers for CCM1 (CCM1-restore 5' and CCM1-restore 3'). After cloning the PCR fragments into the SalI site of pGEM-HIS (42), the resulting plasmids were linearized with NruI and used to transform the respective null mutants (CQF152 and CQF171). The sequence homology between the genomic HIS1 flanking sequence and pGEM-HIS1 vector provides a target sequence for recombination and insertion of the genes into the genome. Colonies with CET1 or CCM1 insertions were iden-
ified by analytical PCR of Ura$^+$ Arg$^+$ His$^+$ transformants, and the genomic insertions were confirmed by Southern blot analysis (Fig. 3). These strains with CET1 or CCM1 restored in trans in the genome were phenotypically similar to the wild-type BWP17 strain in growth studies (Fig. 4), susceptibilities to antifungal compounds (Table 3), and cap structures (Fig. 5).

**DISCUSSION**

Two features of fungal mRNA capping enzymes indicate they might be good targets for antifungal drug development. First, mRNA capping enzymes have distinct catalytic mechanisms and gene structures from their functional human counterparts (14, 26). Second, mRNA capping is essential in *S. cerevisiae* (32) and is widely reported to be critical for efficient translation in all eukaryotes (for reviews, see references 17, 20, and 30). All three genes encoding the enzymes involved in mRNA capping have been shown to be essential in *S. cerevisiae* (19, 34, 39). Although gene essentiality in *S. cerevisiae* is often consistent with essentiality for the homologous genes in *C. albicans*, there are many notable exceptions. Therefore, we wanted to validate mRNA capping as a good antifungal drug target by determining whether the genes encoding the capping enzymes are essential in *C. albicans*. The *C. albicans* mRNA capping enzymes have been characterized and the three genes encoding the mRNA capping enzymes in *C. albicans* (*CET1*, *CGT1*, and *CCM1*) have been identified (31, 44, 45), but null mutants have previously not been constructed for these genes. We were able to construct null deletion mutants of both *CET1* and *CCM1* in *C. albicans* by the methods of Enloe et al. (7) and Wilson et al. (42). This contrasts with a recent report by Pei et al. (26), who were unable to delete both alleles of *CET1* in *C. albicans* by using the UAU1 one-step transformation method (7).

We initially used the one-step transformation method of Enloe et al. (7) and were able to generate null mutants of *CET1* in *C. albicans*. However, the recovery of null mutants was originally very low (1 of 44 initial Arg$^+$ Ura$^+$ segregants), and this one *cet1*-null mutant isolated by the UAU1 mitotic recombination procedure was temperature sensitive (Fig. 4). These two observations raised the possibility that we might have generated a compensatory mutation during the mitotic recombination allowing us to isolate a null mutant for a gene that really is essential. To test this hypothesis, 16 additional Arg$^+$ Ura$^+$ segregants generated from the other two *CET1* heterozygotes with UAU1 targeted insertions were screened. Four of these isolates were *cet1*-null mutants, and none of these four mutants showed the temperature-sensitive growth phenotype seen with the first *cet1*-null mutant (Fig. 4). These results suggest that the temperature-sensitive phenotype was not related to the loss of *CET1* function but may be due to, for example, duplication of a recessive mutation adjacent to the insertionally deleted *CET1* allele when this locus was converted to homozygosity in the mitotic recombination step.

As an additional verification that the *CET1* gene is not essential, we were also able to generate *cet1*-null mutants by a two-step transformation method for disrupting both alleles (42). This method relies on sequential transformation of *ARG4* and *URA3* markers into the two *CET1* alleles and led to the identification of 7 null mutants in the 9 Arg$^+$ Ura$^+$ transformants that were screened.

Null deletion mutants of *CCMI*, the methyltransferase-encoding gene, were constructed in *C. albicans* by using the two-step transformation method (Fig. 2). However, we were unable to generate null mutants at this locus by using the single-step transformation method. The high rate of success and relative ease in generating *cet1* and *ccm1*-null mutants with the two-step transformation method versus the one-step transformation method highlights the importance of testing multiple approaches in determining whether genes are essential in *C. albicans*. The reason for the differences in the two methods remains unexplained, but it could be due to a physical constraint of the DNA at specific loci that inhibits the mitotic recombination event required to select homozygous deletions in the single transformation method.

De Backer et al. (5) showed, with their *C. albicans Δcgť1/CGT1* heterozygous mutant, that the mutant was temperature sensitive and resistant to the translational inhibitor hygromycin B. We did not observe temperature sensitivity for our Δcet1:*ARG4/CGT1 heterozygous strain (Fig. 4). The MICs for hygromycin B were similar for this strain and the *CGT1/CGT1* parent strain (Table 3). The *C. albicans cet1* and *ccm1*-null mutants are more sensitive to hygromycin B than the heterozygotes and the strains with capping genes added back in *trans* (MICs = 16 μg/ml versus >128 μg/ml). In addition, the *cet1*-null mutant is more sensitive to the protein translation inhibitor cycloheximide. Hypersensitivity to a compound that inhibits its protein translation would be an expected phenotype for a mutant strain that has altered levels of expression for a component of protein translation. The drug sensitivity data are not conclusive but are consistent with the theory that *C. albicans CCM1* and *CGT1* deletion mutants may have less-efficient protein translation.

The fact that *cet1* and *ccm1*-null mutants can be generated in *C. albicans* raises multiple questions about the processing of mRNA and translation in this organism. Possible explanations for why *CET1* and *CCM1* are not essential in *C. albicans* include (i) redundant genes encoding the capping functions, (ii) an alternative capping pathway, (iii) the ability to survive with cap-independent translation, or (iv) the ability to effectively translate mRNA with a modified cap. Based on the results we show here, we favor the latter hypothesis.

There are many examples of important enzymes encoded by redundant genes in *C. albicans*, including genes encoding chitin synthase (21); the *GSC1*, *GSL1*, and *GSL2* genes involved in β-1,3-glucan synthase (22), and protein mannosyltransferases (38). However, by using sequence analysis and Southern blots we have found no evidence of redundant genes for *CET1* or *CCM1* in the *C. albicans* genome. A search of the publicly available *C. albicans* genomic sequence (assembly 6, Stanford Genome Technology Center, http://www-sequence.stanford.edu/group/candida/index.html) did not identify any other loci encoding an open reading frame with significant amino acid sequence homology to CET1p or CCM1p. The Southern blot analysis showed only one band hybridized to *CET1* or *CCM1* fragments in the parent strain and this was lost in the *cet1*- or *ccm1*-null mutants (Fig. 3). If mRNA triphosphatase or methyltransferase capping activities are essential in *C. albicans*, there are more likely to be nonhomologous genes encoding the functions or an alternative capping pathway. However, our data indicate that modified guanylated caps are formed in the
null mutants and are sufficient for protein translation in *C. albicans.*

We were unable to delete, under the growth conditions used to select for deletion mutants, both alleles of *CGT1* in *C. albicans.* This suggests that the mRNA-guanyltransferase modification is essential even if the triphosphatase and methyltransferase activities are not. However, it is possible that the essential nature of *CGT1* is due to an unknown function of this gene.

Analysis of the mRNA cap structure in the *C. albicans* cet1- and ccm1-null mutants indicates guanylated cap structures that are distinct from the mRNA cap structure in the wild-type cells (Fig. 5). The mRNA cap structure in the BW2517 cells migrates as expected with the m7GpppN standard. In the ccm1-null mutant, it is quite clear that the mRNA cap structures are not methylated (GpppN). Based on the pattern of labeled nucleotides seen in the TLC analyses, our hypothesis is that, in the cet1-null mutants, mRNA is guanylated without the third phosphate being removed and that it is not methylated (Gppp[N]). Tetraphosphate linkages have been previously identified in cap structures formed by virus particles lacking RNA triphosphatase activity (4), indicating that viral guanylyltransferase activity is independent of RNA triphosphatase. If the cap structure in the cet1-null mutant contains a tetraphosphate linkage, the *C. albicans* guanylyltransferase activity must be independent of RNA triphosphatase. This conclusion is supported by a recent publication from Takagi et al. (37), who cite a fundamental divergence in mRNA capping machinery between *S. cerevisiae* and *C. albicans.* They report that activity of *S. cerevisiae* mRNA guanylyltransferase (CGT1p) depends on interaction with the RNA triphosphatase subunit (CET1p). However, their results showed that *C. albicans* CGT1p does not require interaction with CET1p for guanylyltransferase activity, a conclusion reinforced by our demonstration of guanylated mRNA caps in the cet1-null mutant. Further studies will be needed to determine the exact structures of the cap nucleotides in the null mutants, but they are definitely different than the cap structures in the parent strain.

The question remains as to whether any mRNA cap is essential for translation in *C. albicans.* The 5′-cap on mRNA binds the eukaryotic initiation factor eIF4e and is involved in the recruitment of the ribosome to form an initiation complex involving both the 5′-cap and the 3′-polyadenylated tail (41). Whereas most eukaryotic translation is thought to involve mRNA with 5′ caps (29), there are many examples of cap-independent translation in eukaryotes. Viral mRNAs have been reported to have 3′-end sequences that mimic the cap function (41), and cap-independent translation initiation that is facilitated by internal ribosome entry sites has been widely studied (47). Although eIF4e has been reported to be essential in *S. cerevisiae* (40), a recent report clarifies that eIF4e is not essential in starved cells (25). The proposal that mRNA capping is only essential in rapidly growing cells is supported by the growth studies with our *C. albicans* capping gene deletion mutants. Growth defects for the cet1- and ccm1-null mutants compared to parent strain are much more pronounced at 37°C than at 30°C (Fig. 4). The slower-growth phenotype for the cet1- and ccm1-null mutants could be due to a variety of reasons but, again, it is a phenotype expected for mutants with alterations in protein translation efficiency. The survival of the cet1- and ccm1-null mutants demonstrates that the complete m7GpppN mRNA cap structure is not absolutely required in *C. albicans* and the mRNA cap probably contributes to efficiency but is not essential.

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