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The Second Capsule Gene of *Cryptococcus neoformans*, *CAP64*, Is Essential for Virulence

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The extracellular polysaccharide capsule produced by *Cryptococcus neoformans* is essential for its pathogenicity. We have isolated and characterized a gene, *CAP64*, which is required for capsule formation. An encapsulated strain created by complementation of the *cap64* mutation produced fatal infection of mice within 25 days, while the *cap64* acapsular strain was avirulent. Gene deletion of *CAP64* from a wild-type strain resulted in the loss of capsule as well as virulence. Contour-clamped homogeneous electric field gel analysis indicates that *CAP64* is located on chromosome III, which is different from the localization of another capsule-related gene, *CAP59*. The nonlinkage between *CAP64* and *CAP59* was also supported by classical recombinational analysis. Database searches did not reveal any sequence with high similarity to *CAP64*. We also found that the *CAP64* locus is contiguous to a convergently transcribed gene which has significant similarity to the gene encoding the yeast proteasome subunit, *PRE1*. The distance between the cDNA ends of these two genes is only 22 bp. This study confirms the previous molecular genetic evidence that capsule is an essential factor for the virulence of *C. neoformans* in the murine model.

Cryptococcus neoformans is a pathogenic fungus which most commonly affects the central nervous system and causes fatal meningoencephalitis in patients with AIDS (16). This fungus produces a thick extracellular polysaccharide capsule in vitro as well as in vivo. Early studies demonstrated that capsule formation is one of the most important virulence factors in *C. neoformans* (12, 19). Although the biochemical pathway for synthesis of the capsular polysaccharide has not been elucidated (14, 27), the predominant capsular polysaccharide of *C. neoformans* is glucuronoxylomannan. The major glucuronoxylomannan component is an α-1,3-D-mannopyranose backbone containing a single β-1,2-linked glucuronate residue on one-third of mannopyranose residues and various amounts of xylosylation, depending on the serotype (1–4, 7, 25).

Several different loci controlling capsule formation have been assigned to the same linkage group by classical recombination analysis with various acapsular strains of *C. neoformans* (23). We recently complemented one of the acapsular strains and characterized a gene, *CAP59*, which is essential for both capsule formation and virulence (5). Deletion of the *CAP59* gene causes *C. neoformans* to lose its capsule and virulence, and complementation of the *cap59* mutation restores the virulent phenotype. Forced expression of *CAP59* under the control of the *C. neoformans GAL7* promoter complements the *cap59* acapsular phenotype, and a missense mutation in the coding region abolishes complementation by the fusion construct (6). Sequence analysis suggests that the CAP59 protein may be a transmembrane protein (6), but the biochemical function of the CAP59 protein remains obscure.

CAP64, like *CAP59*, another gene required for capsule formation, was identified by mutagenesis and these two genes were reported as closely linked (23). The present study describes the isolation and characterization of the *CAP64* gene. As with *CAP59*, complementation of the *cap64* mutation restores both capsule and virulence. Our results indicate, however, that *CAP64* is not linked to *CAP59*. We also found that the DNA sequence immediately downstream of *CAP64* has significant similarity to that of the gene encoding the yeast proteasome subunit, *PRE1* (13). Proteasomes are multisubunit nonlysosomal proteases, ubiquitous from archaea to humans (9, 22, 24). We report the DNA sequence of the gene encoding putative proteasome subunit of *C. neoformans* and discuss the similarities among these proteins in different organisms.

MATERIALS AND METHODS

Strains and media. *C. neoformans* var. neoformans serotype D wild-type isolates B-3501 (α mating type) and B-3502 (**a** mating type) have been described before (15). B-4500 is a wild-type congenic strain of B-4467 (18). B-4130 is a stable capsule-deficient mutant (received from E. S. Jacobson as strain 305) originally designated Cap64 (23). B-4130FO3 is a *URA5* auxotroph of B-4130 isolated in our laboratory as described previously (20). B-4530 (*ade2 ura5*) is a derivative of B-4476 (5). The red13B strain is a *URA5* auxotroph of an *ade2* strain which was isolated by mutagenesis of B-4500. B-450FO2 is a *ura5* auxotroph of B-4500. All strains were maintained on YEPD (1% yeast extract, 2% Bacto Peptone, 2% dextrose). Minimal medium contained 6.7 g of yeast nitrogen base without amino acids (Difco) and 20 g of glucose per liter. 5-Fluoroorotic acid (5-FOA) medium contained 6.7 g of yeast nitrogen base (Difco), 1 g of 5-FOA, 50 mg of uracil, and 20 g of glucose per liter.

Transformation of *C. neoformans*. The electroporation method described by Edman and Kwon-Chung (10) was used. The encapsulated transformant of B-4130FO3 containing pYCC38 (TYCC38) is a stable uracil auxotroph which was selected among $Ura5^+$ transformants after three transfers on YEPD medium.

Linkage analysis. Recombinational analysis and contour-clamped homogenous electric field (CHEF) blot assay (26) were used to determine the linkage between genes. Strains of two opposite mating types with appropriate genetic markers were crossed on V-8 juice agar (17). Single basidiospores were randomly isolated by micromanipulation (15), and single-basidiospore cultures were analyzed for their phenotypes.

Preparation and analysis of nucleic acid. Genomic DNA isolation and analysis were performed as described previously (5). Random hexamer priming was used to label the DNA probes to specific activities of $>10^8$ dpm/μg (11). DNA sequencing was performed by the dideoxy-mediated chain termination method by using a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). Programs of the University of Wisconsin Genetics Group were used for analysis of nucleic acid sequences (8).

Plasmid construction. The *URA5*-containing plasmid pCIP3 was received from J. E. Edman (5). Plasmids pYCC40, pYCC41, pYCC42, and pYCC43 were subclones of pYCC38 in pCIP3 (see Fig. 2). pYCC77 was constructed by replac-

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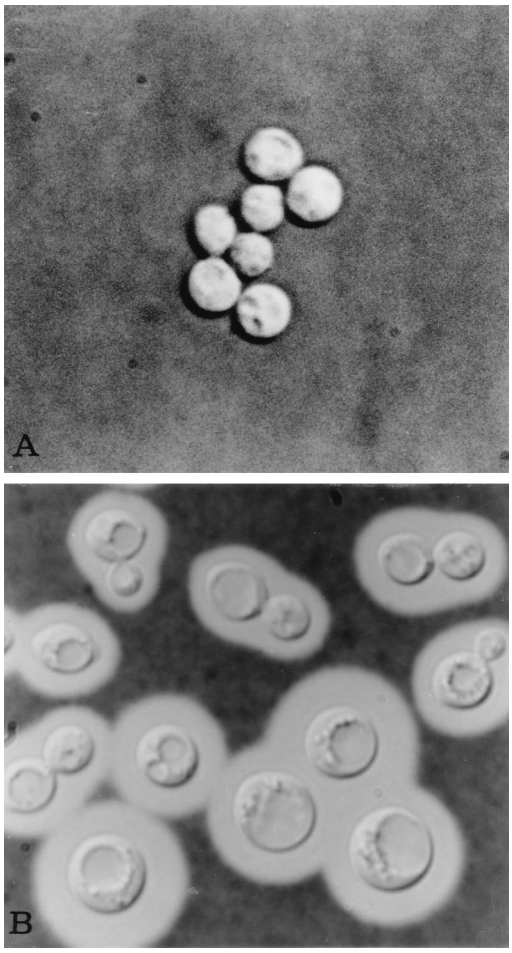

FIG. 1. Morphology of a *cap64* mutant (A) and a stable Cap$^+$ transformant (B) of B-4130FO3. *C. neoformans* cells were stained with India ink and viewed under a light microscope to reveal the capsular phenotype.

ing the 1.1-kb *Aat*II-*Ppu*MI fragment of pYCC38 with the 3.0-kb DNA fragment of the *ADE2* gene.

To rescue free plasmids from *C. neoformans*, the genomic DNA of transformants was digested with *Not*I, ligated, and transformed in *Escherichia coli*.

The partial library was prepared by complete digestion of B-4130 DNA with *Bam*HI, and the DNA fragments of 3 to 4.5 kb were gel isolated and cloned into pBluescript II SK.

Virulence study. Female general-purpose (NIH) mice were injected with yeast strains as described previously (5).

Nucleotide sequence accession number. The GenBank nucleotide accession numbers for the *CAP64* and *PRE1* sequences reported in this paper are L40026 and L40028, respectively.

RESULTS

Cloning of *CAP64* gene. A telomere-based genomic library of B-3501 (5) previously used to isolate the *CAP59* gene did not complement the acapsular phenotype of B-4130FO3 (*cap64*). Therefore, a new library was constructed by using DNA from the α mating type strain, B-4500, of a congenic set. A two-polymer aqueous-phase system was used to enrich for capsule-containing transformants (5), and several Cap$^+$ transformants of B-4130FO3 were isolated. One plasmid containing a 3.8-kb insert (pYCC38) was rescued from one of the Cap$^+$ transformants in *E. coli* and was able to complement the acapsular phenotype of B-4130FO3 (Fig. 1). In addition, the Cap$^+$ transformants became acapsular when the pYCC38 plasmid was lost. Overlapping subclones of pYCC38 were introduced into B-4130FO3 by electroporation to determine the minimal region required for complementation (Fig. 2A). pYCC43 containing a 3.1-kb insert was the smallest complementing subclone identified. In the case of pYCC40, the majority of transformants remained acapsular, and only a very few cells contained capsule. Therefore, pYCC40 may lack a portion of sequence required to fully complement the *cap64* mutation.

Deletion of *CAP64*. Two different approaches were used to demonstrate that pYCC43 contains sequences corresponding to the wild-type *CAP64* gene. A partial genomic DNA library of the acapsular strain B-4130 was screened with a probe of pYCC38. Several positive clones were obtained, and a 3.1-kb DNA fragment equivalent to the insert of pYCC43 was subcloned to create pYCC75. This plasmid was electroporated into B-4130FO3, and all resulting transformants remained acapsular. Sequence analysis of pYCC75 suggests that it contains nonsense mutations (data not shown).

Gene deletion was accomplished with an *ADE2*- and *URA5*-containing plasmid, pYCC77 (Fig. 3A). Because previous studies showed that the frequency of gene deletion by homologous integration is extremely low in *C. neoformans* serotype D strains, a positive-negative selection method was used to increase the possibility of obtaining the desired transformant (5). An *ade2 ura5* strain (red13B) was transformed with pYCC77, and the yeast cells were selected on 5-FOA plates; 365 Ade2$^+$ Ura5$^-$ transformants were obtained from 5-FOA plates. This number of transformants was about 2.6% of the number of the control transformants which grew on minimal medium without 5-FOA. Of the 365 transformants, 14 lacked capsule. Thus, if

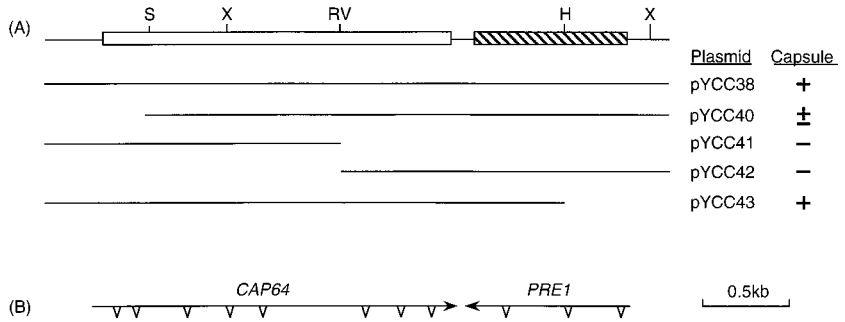

FIG. 2. Structure of the *CAP64* locus. (A) Determination of the boundary of *CAP64* in pYCC38. Overlapping subclones of pYCC38 were used to transform B-4130FO3. The capsular phenotypes of the resulting transformants are indicated. Open and hatched boxes represent the coding regions of *CAP64* and *PRE1*, respectively. H, *Hpa*I; RV, *Eco*RV; S, *Stu*I; X, *Xho*I. (B) Genomic arrangement of *CAP64* and *PRE1*. Arrows indicate the direction of transcription. Triangles represent introns.

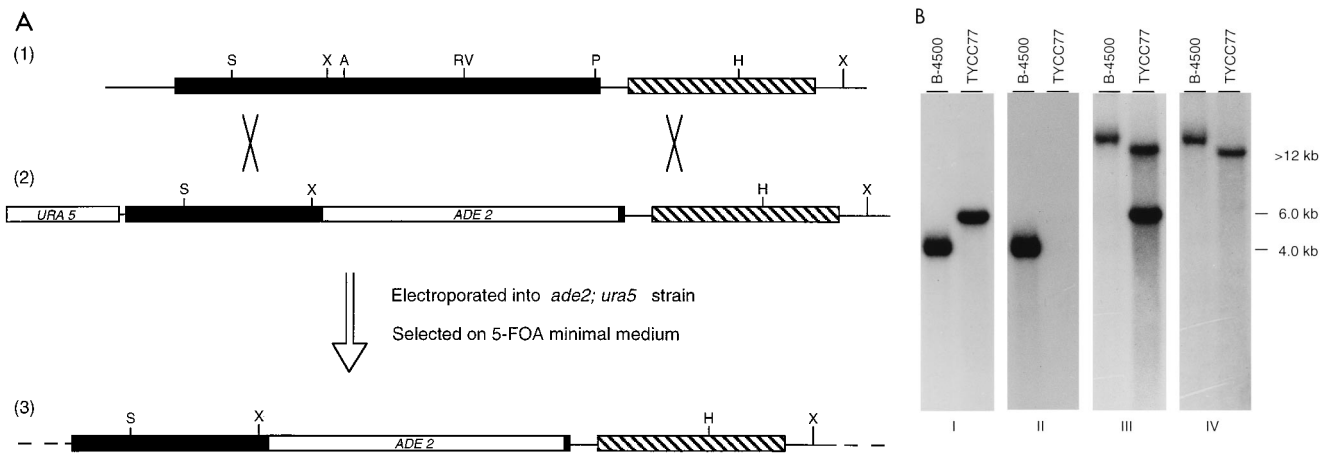

FIG. 3. Deletion of *CAP64*. (A) Strategy for *CAP64* deletion. (1) Map of pYCC38. Black box, *CAP64* coding region; hatched box, *PRE1* coding region. (2) Map of pYCC77. The 1.1-kb *Aat*II-*Ppu*MI fragment of the *CAP64* coding region was replaced by the 3.0-kb *ADE2* gene, and the *URA5* gene was inserted in the 5′ flanking region of *CAP64*. (3) *C. neoformans* red13B (*ade2 ura5 CAP64*) was transformed with the linearized pYCC77 DNA, and cells were selected on 5-FOA medium. The diagrammed double-crossover event produced the deletion of *CAP64*. A, *Aat*II; H, *Hpa*I; P, *Ppu*MI; RV, *Eco*RV; S, *Stu*I; X, *Xho*I. (B) Southern blot of the *cap64* deletant. Genomic DNAs of an acapsular transformant (TYCC77) and capsule-containing strain (B-4500) were digested with *Bam*HI. The membrane was hybridized with the 3.8-kb insert of pYCC38 (I), the 1.1-kb *Aat*II-*Ppu*MI fragment of pYCC38 (II), the *ADE2* gene probe (III), and the CIP3 probe, which containing *URA5* (IV). The figure is not drawn to scale for simplicity.

the positive-negative selection method was not applied, the ratio of Cap^- to Cap^+ transformants was about 1:1,000.

Southern blot analysis was carried out to determine if the acapsular phenotype was derived from a gene replacement event (Fig. 3B). A probe of the entire 3.8-kb insert of pYCC38 was first hybridized to the DNA blot. The change of the 4.0-kb band in the wild type, B-4500, to a 6.0-kb band in the acapsular transformant, TYCC77, suggested an insertion event at this region (Fig. 3B, I). When the 1.0-kb *Aat*II-*Ppu*MI fragment of pYCC38 was used as a probe, no hybridization signal was detected in TYCC77, which indicated a deletion event (Fig. 3B, II). The 6.0-kb band detected by the *ADE2* gene probe in TYCC77 confirmed that gene replacement was at the predicted position (Fig. 3B, III). The greater than 12-kb signal in TYCC77 represents the native *ADE2*, and the slower mobility of the native *ADE2* in B-4500 may be due to the interference of polysaccharide in the DNA sample (Fig. 3B, III). Finally, the *URA5* probe hybridized only to the native *URA5* (Fig. 3B, IV). Therefore, the acapsular phenotype of TYCC77 was generated by gene deletion through double crossover at the homologous site. These results also support our contention that sequences contained in pYCC38 correspond to the *CAP64* gene.

Virulence test. Previous studies showed that once the capsule-deficient phenotype of the *cap59* strain is complemented, the resulting strain regains virulence (5). We reasoned that restoration of the capsule in B-4130 should also restore virulence and tested this hypothesis with a mouse model (Fig. 4A). Yeast cells from a stable Cap^+ transformant of B-4130FO3 (TYCC38) and from the wild type, B-3501, produced fatal infections in all eight mice within 25 days. The mice injected with the acapsular strain (B-4130) or the stable Cap^- transformant of B-4130FO3 harboring only the vector sequence (CIP3) remained healthy until the experiment was terminated on day 60.

The virulence of the *cap64* deletant was also compared with that of an isogenic *ura5* encapsulated strain (Fig. 4B). The mice challenged with wild-type *CAP64* strain (B-4500FO2) all died within 60 days, whereas the *cap64* deletant (TYCC77) did not produce fatal infection. Thus, these results confirm that the *CAP64* gene is required for *C. neoformans* to produce fatal infection in this animal model.

In this study, TYCC38 appears to be as virulent as the wild type. However, previous animal studies of a *cap59* mutant complemented in a similar manner (TYCC6) produced a slower death rate than the wild-type strain did (5). To test if there is a difference in the integration events of the transformed DNAs in TYCC38 and TYCC6, we analyzed these transformants by Southern blotting (Fig. 4C). The blot of B-4131 and TYCC6 was hybridized with pYCC6 (*CAP59* gene), and the blot of B-4130 and TYCC38 was hybridized with pYCC38 (*CAP64* gene). DNA from the acapsular strains used for complementation was included for comparison. The patterns of the restriction fragments in the acapsular mutants (B-4131 and B-4130) were the same as in the wild-type strain (data not shown). It is obvious that both TYCC6 and TYCC38 contain more than one copy of ectopically integrated DNA.

***CAP64* is located on chromosome III.** To determine the chromosomal location of *CAP64*, *C. neoformans* chromosomes were resolved by CHEF gel electrophoresis. The probe of pYCC38 hybridized to chromosome III, and the probe of pYCC6, which contains *CAP59*, hybridized to the chromosome I and II doublet in two wild-type strains (Fig. 5). In addition, the chromosomal locations of the *cap64* and *cap59* alleles in B-4130 and B-4131 were the same as in the wild type (data not shown). These results demonstrate that *CAP64* and *CAP59* are located on different chromosomes, contradicting the earlier report that these two genes are on the same linkage group and only 0.6 centimorgan apart (23). To verify the molecular mapping data by classical recombinational analysis, a *cap64* and a *cap59* strain were crossed, and 50 basidiospores were randomly isolated by micromanipulation. Of the 18 spores which germinated, the ratio of Cap^- to Cap^+ was 12:6. This ratio fits better to 3:1, which is expected from two independently segregating genes ($P > 0.5$, chi-square test), than to the 1:1 ratio of two closely linked genes ($P < 0.25$). Thus, *CAP59* and *CAP64* do not belong to the same linkage group, and we assign *CAP64* to chromosome III in the wild-type genome.

Sequence analysis of *CAP64*. Transcripts of 1.9 and 1.0 kb were detected in RNA isolated from the wild-type strain with

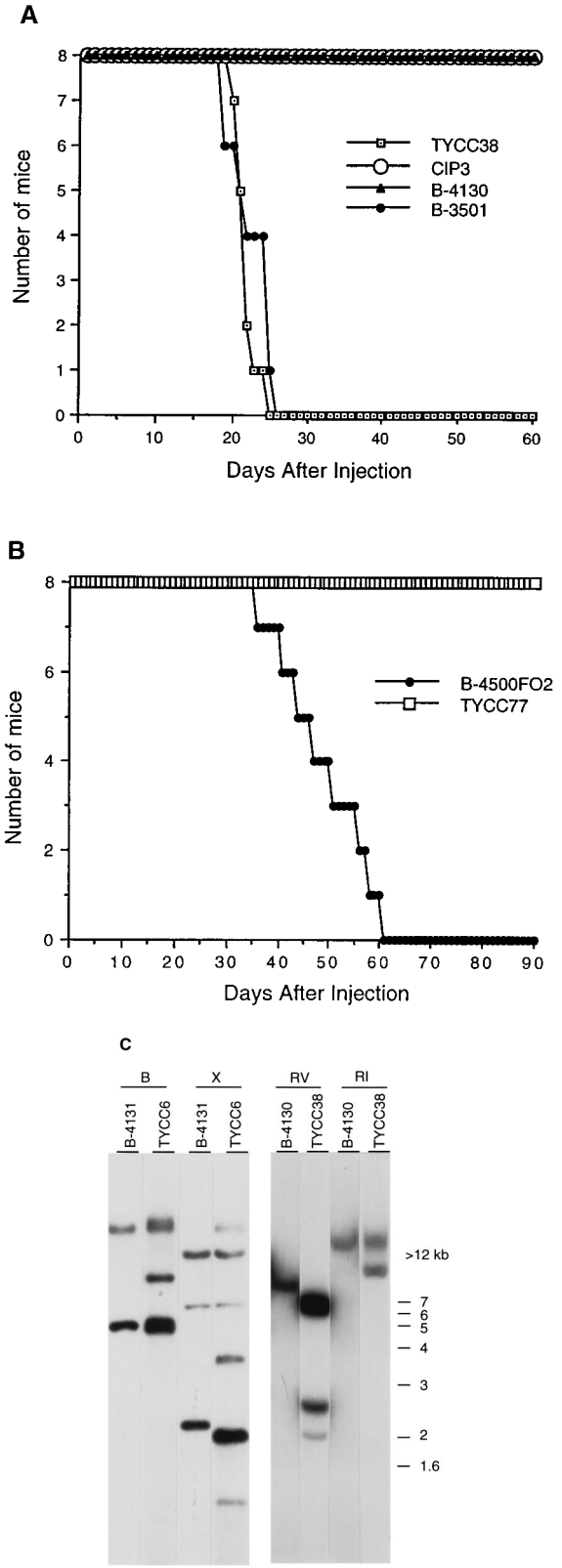

FIG. 4. Virulence test. Groups of eight mice each were injected with about 10^6 (A) or 5×10^5 (B) viable cells and monitored to determine the survival. TYCC38, a stable Cap^+ transformant of B-4130FO3; CIP3, a stable Cap^- transformant of B-4130FO3 harboring only the vector sequence; B-4130, a *cap64* strain; B-3501, a wild-type strain; B-4500FO2, a *CAP64 URA5* auxotroph; TYCC77, a *cap64* deletant. (C) Southern blot analysis. Genomic DNAs of a *cap59* mutant (B-4131), a complemented encapsulated strain of B-4131 (TYCC6), a *cap64* mutant (B-4130), and a complemented encapsulated strain of B-4130 (TYCC38) were digested with different enzymes and analyzed by Southern blotting. The blot of B-4131 and TYCC6 was hybridized with the pYCC6 probe, and the blot of B-4130 and TYCC38 was hybridized with the pYCC38 probe. B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; X, *Xho*I.

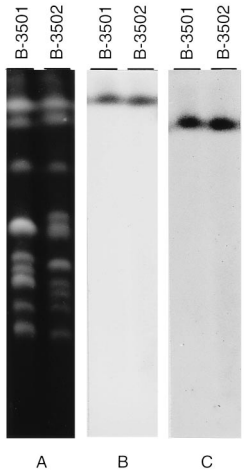

FIG. 5. Chromosomal location of *CAP64*. (A) Ethidium bromide staining of chromosomal DNA from wild-type strains of *C. neoformans* separated by CHEF gel electrophoresis. The gel separated chromosomal DNA was transferred to nylon membrane and hybridized with pYCC6 (*CAP59*) (B). The same membrane was stripped and hybridized with pYCC38 (*CAP64*) (C). B-3501, α mating type, wild-type strain; B-3502, **a** mating type, wild-type strain.

a probe of pYCC38 (data not shown). Subsequent screening of a cDNA library with the same probe produced two types of cDNA clones, one containing a 1.9-kb insert and one containing a 1-kb insert. Sequence comparison of genomic and cDNA clones located the 1.9- and 1.0-kb cDNAs in pYCC38 (Fig. 2B). The 1.9-kb cDNA was within the region of DNA which complemented the *cap64* mutation and was designated the cDNA of *CAP64*. The transcript of the 1.0-kb cDNA is convergent with *CAP64* and is located immediately downstream of the 1.9-kb *CAP64* transcript. The distance between the two cDNA ends is only 22 bp.

The 1.9-kb cDNA of *CAP64* contains an open reading frame capable of coding for a 522-amino-acid protein with a calculated molecular mass of 56 kDa (Fig. 6). Comparison of the genomic sequence with the cDNA sequence revealed the presence of eight introns. The canonical TATAAA and CAAT sequence upstream of the initiation codon is absent. Although databases searches revealed that *CAP64* has no significant similarity with any reported gene, CAP64 contains a weak leucine zipper motif (Fig. 6).

Sequence analysis of the 1.0-kb cDNA indicates an open reading frame which has extensive similarity with the gene encoding the subunit of the yeast proteasome subunit, *PRE1* (13). We designated this putative *C. neoformans* proteasome subunit gene *PRE1*. The predicted protein of *PRE1* contains 224 amino acids and has an estimated molecular mass of 25 kDa and a pI of 5.89 (Fig. 7). Comparison of the genomic sequence with the cDNA sequence reveals the presence of three introns in *PRE1*. Figure 8 shows the alignment of the proteasome β subunits from two fungi (a basidiomycete and an ascomycete), an animal, and a prokaryote. The yeast *PRE1* gene was identified by functional complementation of mutant cells

FIG. 6. Nucleotide sequence of *CAP64*. The proposed polypeptide is given in three-letter code below the DNA sequence. The underlines indicate the putative weak leucine zipper motif. The double underline indicates the 5′ end of the longest cDNA. The poly(A) addition sites for *CAP64* (^) and for *PRE1* (*) were deduced from cDNA sequences.

lacking the chymotrypsin-like activity of the yeast proteasome (13). RC7-I is a subunit of the 20S proteasome of rat hepatoma cells (21). The β subunit of the archaebacterial proteasome is responsible for the proteasome's chymotrypsin-like activity (28). Within the aligned regions, 27 amino acids were exactly conserved among these four species, and similarities are observed throughout the entire sequence. Although *PRE1* has extensive similarity with other proteasome subunit genes, the function of *PRE1* in *C. neoformans* was not addressed.

DISCUSSION

The chemical composition of the extracellular polysaccharide capsule in *C. neoformans* was unveiled more than 10 years

FIG. 7. Nucleotide sequence of *PRE1*. The proposed polypeptide is given in three-letter code below the DNA sequence. The double underline indicates the 5′ end of the longest cDNA. The poly(A) addition sites for *CAP64* (∧) and for *PRE1* (∗) were deduced from cDNA sequences.

ago, but the biosynthetic pathway of capsule formation is yet to be described. Previous isolation of the *CAP59* gene shed light on the molecular studies of capsule formation in *C. neoformans*. Here, we isolated and characterized another capsule-related gene, *CAP64*. Database searches did not suggest the function of *CAP64*, but the importance of *CAP64* in capsule formation was clearly demonstrated. A wild-type *CAP64* gene complemented the capsule-deficient phenotype of B-4130, and deletion of *CAP64* resulted in an acapsular phenotype. Therefore, *CAP64* and *CAP59* are both essential genes for capsule formation with undefined biochemical functions. The relationship between *CAP64* and *CAP59* in the biosynthetic pathway is not clear, but neither mutation affected the RNA accumulation of the other gene, as indicated by Northern (RNA) blot analysis (data not shown).

Physical mapping data of the chromosomal locations for *CAP59* and *CAP64* by CHEF analysis indicate that *CAP59* is on chromosome I and *CAP64* is on chromosome III. Recombination analysis of the germinated spores also supports the notion that *CAP59* and *CAP64* are unlinked. The nature of the low germination rate of the basidiospores is not clear. It is possible that a *cap59 cap64* double mutant is not viable. In most crosses, however, the germination rate of basidiospores is less than 80% and varies greatly among strains (unpublished observations). Even if the rest of the nongerminated spores were all Cap$^-$ phenotype, the distance between *CAP64* and

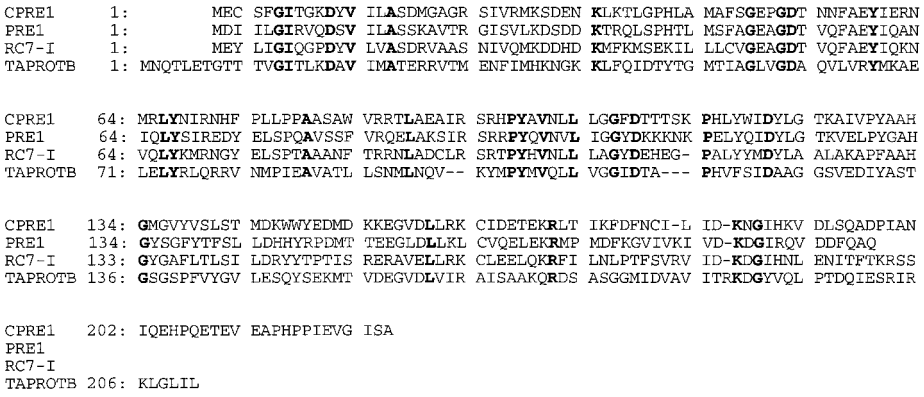

FIG. 8. Alignment of proteasome subunit sequences. Identical residues conserved in all four proteins are in boldface. Numbers are residue numbers. Dashed lines represent gaps introduced for the best alignment. The proteasome subunits are those of *C. neoformans* (CPRE1), *Saccharomyces cerevisiae* (PRE1), *Rattus* sp. (RC7-I), and *Thermoplasma acidophilum* (TAPROTB). The alignment was performed with the Genetics Computer Group program Pileup, using the default parameters.

CAP59 is far greater than the reported 0.6 centimorgan (23). This discrepancy is undoubtedly due to the different methods used for progeny analysis.

The acapsular phenotype of *CAP64* deletants and the inability to complement the acapsular phenotype with the *cap64* allele isolated from B-4130 (pYCC75) are evidence that we have isolated the functional *CAP64* gene. We initially used B-4530, which was used to generate a *cap59* deletant, as a recipient strain for *CAP64* deletion. More than 7,000 transformants were screened, and no acapsular transformant was obtained (data not shown). Deletion of *CAP64* was finally achieved in the red13B strain. The reason for the inability to obtain a *cap64* deletant from B-4530 is not clear. We also noticed that the frequency of homologous integration and the ratio of Cap^+ to Cap^- transformants grown on 5-FOA are higher with pYCC77 (*CAP64*) than with pYCC33 (*CAP59*) (5). The frequency of homologous integration in *C. neoformans* serotype D strains is less than 0.1%. We showed that close to 4% of the transformants grown on 5-FOA plates are acapsular. This makes the positive-negative selection method a valuable tool for gene deletion in *C. neoformans*.

The DNA sequence immediately downstream of *CAP64* has significant similarity to that of the gene encoding the β subunit of the archaebacterial proteasome. Close clustering of genes was also found with the *CAP59* locus; *CAP59* is closely linked to a convergently transcribed putative gene encoding mitochondrial ribosomal L27 protein (6). In both cases, the distance between the linked genes is less than 30 bp. It remains to be seen whether gene clustering is a general phenomenon for *C. neoformans*.

In the animal studies, TYCC38 appears to be fully virulent. The previous studies of a complemented *CAP59* strain (TYCC6) in mice produced a slower death rate than the wild-type strain did (5). The reason for the difference is not clear, although it could be partially explained by the differences in the integration event, as both transformants contain multiple copies of ectopically integrated plasmids in the genome. The restoration of virulence by complementation of the acapsular phenotype and the loss of virulence of *cap64* deletant confirms that capsule is an undisputable virulence factor. Although CAP64 contains a weak putative leucine zipper motif, the role of the *CAP64* gene in the capsular polysaccharide biosynthetic pathway remains unknown. However, *CAP64* is clearly associated with the genetic control of virulence in *C. neoformans*. Further molecular studies of *CAP64* and other capsule-related genes will lead to a better understanding of capsule synthesis and the pathogenicity of *C. neoformans.*

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