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Complementation of a Capsule-Deficient Mutation of *Cryptococcus neoformans* Restores Its Virulence

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Capsule formation plays a significant role in the pathogenicity of *Cryptococcus neoformans*. To study the molecular basis of capsule synthesis, the capsule-deficient phenotype of a mutant strain was complemented by transformation. A plasmid rescued from the resulting Cap$^+$ transformant complemented a *cap59* mutation which was mapped previously by classical recombination analysis. Gene deletion by homologous integration resulted in an acapsular phenotype, indicating that we have identified the *CAP59* gene. The *CAP59* gene was assigned to chromosome I by Southern blot analysis of contour-clamped homogeneous electric field gel electrophoresis-resolved chromosomes of *C. neoformans* var. *neoformans*. Sequence comparison of genomic and cDNA clones indicated the presence of six introns. *CAP59* encoded a 1.9-kb transcript and a deduced protein of 458 amino acids. Analysis of the nucleotide sequence revealed little similarity to existing sequences in the data bank. When the capsule-deficient phenotype was complemented, the originally avirulent *C. neoformans* strain became virulent for mice. In addition, the acapsular strain created by gene deletion of *CAP59* lost its virulence. This work demonstrates the molecular basis for capsule-related virulence and that the *CAP59* gene is required for capsule formation.

Cryptococcus neoformans is a yeast-like fungal pathogen that causes serious systemic infection primarily in immunocompromised hosts. This fungus most commonly affects the central nervous system and has been recognized as one of the leading causes of fatal meningoencephalitis in patients with AIDS (19). *C. neoformans* cells are surrounded by a thick extracellular polysaccharide capsule in vitro as well as in vivo. The polysaccharide capsule has been reported to be one of the most important virulence factors in addition to the ability of the fungus to grow at 37°C (12, 22). Acapsular mutants are avirulent in mice (12, 22), and no spontaneously occurring acapsular isolates from active, untreated cases of cryptococcosis have been documented. The thickness of the capsule varies among clinical isolates, and there is no correlation between capsule thickness and virulence of a strain (19). The most important role of polysaccharide capsule as a virulence factor is believed to be inhibition of phagocytosis by host cells (16, 17).

The predominant capsular polysaccharide of *C. neoformans* is glucuronoxylomannan, which also determines the serotype of an isolate. The major glucuronoxylomannan component is a 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 (1–5, 26). Still and Jacobson constructed a linkage map of the different capsule genes by classical genetic methods and identified six linked loci (24). However, the biochemical pathway for synthesis of the capsular polysaccharide remains unknown (15, 28).

To understand the molecular aspects of capsule synthesis in *C. neoformans*, we began to dissect the genes involved in capsule formation. This study describes the isolation and characterization of one of these genes and demonstrates the importance of this gene as a virulence factor in the animal model.

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MATERIALS AND METHODS

Strains and media. *C. neoformans* var. *neoformans* wild-type isolates B-3501 (α mating type) and B-3502 (**a** mating type) have been described before (18). B-4530 (*ade2 ura5 MAT***a**), an encapsulated strain, was received from J. C. Edman as JEC156. B-4131 (22), R-744, R-745, and R-747 were stable capsule-deficient mutants received from E. S. Jacobson as 309, 307, 308, and 325 respectively. B-4131 is also known as an acapsular mutant originally designated as Cap67 (14). B-4131FO40, B-4476FO5, B-4500FO2, R-744FO9, R-745FO11, and R-747 FO14 were Ura5$^-$ mutants isolated in our laboratory by a method described previously (23).

YEPD medium contained 1% yeast extract, 2% Bacto Peptone, and 2% dextrose. Minimal medium (YNB) contained 6.7 g of yeast nitrogen base (Difco) without amino acids and 20 g of glucose per liter. RPMI medium contained 10.4 g of RPMI 1640 powder (GIBCO Laboratories, Grand Island, N.Y.), 3.15 g of sodium phosphate dibasic, 5.37 g of sodium phosphate monobasic, 2 g of sodium bicarbonate, and 20 g of glucose per liter. 5-Fluoroorotic acid (5-FOA) medium contained 7 g of yeast nitrogen base (Difco), 1 g of 5-FOA, 50 mg of uracil, and 20 g of glucose per liter. Solid medium contained 20 g of Bacto Agar (Difco) per liter.

Transformation of *C. neoformans*. The electroporation method described by Edman and Kwon-Chung (10) was used to transform cells of *C. neoformans*.

Phase partition systems. A two-polymer aqueous-phase system was prepared by the method of Kozel and Cazin (17). The 0.11 M sodium phosphate buffer (pH 6.8) solution containing 5% (wt/wt) polyethylene glycol 8000 (Sigma) and 4% (wt/wt) dextran T-500 (Pharmacia, Uppsala, Sweden) was mixed at room temperature, and the phases were allowed to separate. The top (polyethylene glycol-rich) and bottom (dextran-rich) phases were collected for later use.

After the library DNA was electroporated into B-4131FO40, the cells were grown in RPMI broth for 52 h. The yeast cells were collected by centrifugation, washed, and resuspended in 0.11 M sodium phosphate buffer (pH 6.8). A 50-μl portion of cell suspension (10^8 cells per ml) was added to 1.5 ml of each phase, and the tubes were mixed. The phases were allowed to separate, and a 1.2-ml sample was withdrawn from the upper phase. The samples were diluted in phosphate-buffered saline, and the yeast cells were collected by centrifugation. The collected cells were plated on RPMI medium and incubated at 30°C under 10% CO_2 for 4 days.

Preparation and analysis of nucleic acid. Genomic DNA of *C. neoformans* was extracted by the method of Varma et al. (27) with slight modifications. Cells were grown overnight in YNB instead of YEPD broth. The lysing solution contained 0.1 M instead of 0.45 M EDTA.

Genomic DNA was digested with endonucleases and electrophoresed on a 1% agarose gel. The DNA was transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, Ill.). Hybridization and washes were performed as recommended by the membrane manufacturer. Random hexamer priming was used to label the DNA probes to specific activities of $>10^8$ dpm/μg (11).

Agarose plugs containing chromosomes from *C. neoformans* isolates were prepared and electrophoresed on the contour-clamped homogeneous electric field gel system by procedures described previously (27). The separated chromosomes were transferred to a nylon membrane and hybridized with radiolabeled probes as described above.

DNA sequencing was performed by the dideoxy-chain termination method with a Sequenase version 2.0 kit (U.S. Biochemicals). Programs of the University of Wisconsin Genetics Group were used for analysis of nucleic acid sequences (6).

Plasmid construction. Plasmids pCIP3 and pADE+URA were obtained from J. E. Edman. A 1.9-kb *Bst*XI-*Bgl*II fragment of pURA5g2 containing the functional *URA5* gene was cloned into the *Eco*RV site of pBluescript to give pCIP3 (10). An *Apa*I-*Xba*I fragment of the *URA5* gene from pCIP3 and the *Apa*I-*Sma*I fragment of the *ADE2* gene were cloned into the *Sma*I-*Xba*I site of pUC18 to give pADE+URA. Plasmids pYCC7, pYCC8, pYCC9, pYCC11, and pYCC14 were subclones of pYCC6 in the *Bam*HI site of pCIP3 (see Fig. 3). A 1.3-kb *Nco*I-*Rsr*II fragment of pYCC6 was cloned into the *Bam*HI site of pCIP3 to give pYCC28. Plasmid pYCC33 was constructed by inserting a 0.9-kb *Not*I-*Nco*I fragment containing the 5′ portion of the *CAP59* gene from pYCC6 in the *Apa*I site between the *URA5* and *ADE2* genes of pADE+URA. The 5.9-kb *Eco*RI fragment of the resulting plasmid was cloned into the 5-kb *Cla*I-*Bam*HI fragment of pYCC6 to give pYCC33.

To rescue free plasmids from *C. neoformans* in *Escherichia coli*, we digested *C. neoformans* genomic DNA of transformants with *Not*I, which cleaves the telomere sequences, ligated it, and transformed it in *E. coli*.

Immunofluorescence assay. The E1 monoclonal antibody was obtained from F. Dromer. The immunofluorescence assay was as described previously (7), except that all the reactions and washing procedures were done in the microcentrifuge tubes.

Virulence study. *C. neoformans* cells from 2-day-old YEPD agar cultures were suspended in saline. The percent viability of each isolate was determined by both hemocytometer and colony count on YEPD plate. Female general-purpose (NIH) mice weighing 21 to 23 g were inoculated with 0.2 ml of yeast cell suspension (5×10^5 or 7×10^6 cells) in the lateral tail vein.

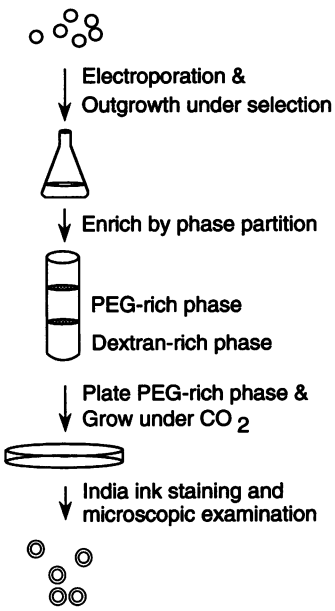

FIG. 1. The scheme for enrichment and screening of Cap^+ transformants. See the text for details. PEG, polyethylene glycol.

Groups of 8 to 10 mice were inoculated with each yeast strain and monitored for 70 days to determine the survival.

Nucleotide sequence accession number. The GenBank nucleotide accession number for the *CAP59* sequence reported in this paper is L26508.

RESULTS

Complementation of an acapsular mutant, B-4131FO40. A genomic DNA library of the wild-type *C. neoformans* B-3501, received from J. C. Edman, was used to complement a *ura5* acapsular mutant (B-4131FO40, *cap67*). The library was constructed in a plasmid vector (pCnTEL1) containing telomere repeats and the *URA5* gene of *C. neoformans*. The *URA5* gene is a selectable marker, and the telomere increases the transformation frequency (9). The telomere sequences maintain the transforming DNA as a free extrachromosomal plasmid in *C. neoformans*, which enables the rescue of desired plasmids.

Because the phenotypic difference between Cap^+ and Cap^- transformants was subtle when electroporated cells were grown on defined culture media, such as YNB or RPMI plates, the presence of rare Cap^+ transformants among the numerous Cap^- transformants was difficult to detect. A two-polymer partition system reported by Kozel and Cazin was adopted to enrich the Cap^+ transformants (17) (Fig. 1). After the library DNA was electroporated into B-4131FO40, the yeast cells were grown in RPMI broth for 52 h. The cells were washed and partitioned in the two-polymer aqueous phase system (see Materials and Methods). After phase separation, cells from the polyethylene glycol 8000-rich phase, which was enriched in Cap^+ transformants, were plated on RPMI medium and incubated at 30°C under 10% CO_2 for 4 days. Because the partition system was merely an enrichment process, individual colonies of the transformants were screened under a dissecting microscope to detect Cap^+ transformants. We reasoned that the colonies of Cap^+ transformants would show a smoother surface and slightly larger colony size than the Cap^- transformants because of the presence of extracellular polysaccharide

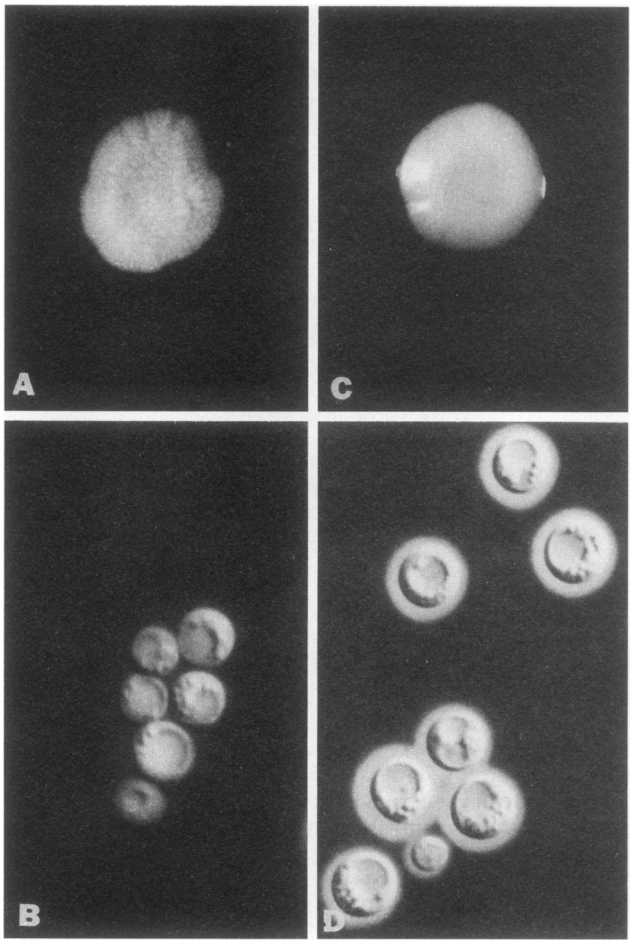

FIG. 2. Phenotype of B-4131FO40 and a Cap$^+$ transformant. Colonies of B-4131FO40 (A) and a Cap$^+$ transformant (C) were grown on YEPD agar at 30°C for 8 days. *C. neoformans* cells from the same colonies were stained with India ink and viewed under a light microscope to show the lack of capsule in B-4131FO40 (B) and the presence of capsule in the Cap$^+$ transformant (D).

capsule. Of the 2,000 transformants screened, 60 were identified as putative Cap$^+$ transformants, and 5 of them were confirmed under a compound microscope as containing capsule after India ink staining (Fig. 2).

Southern blot analysis of the undigested genomic DNA isolated from the Cap$^+$ transformants indicated that free plasmid existed in these transformants (data not shown). When the genomic DNA of these Cap$^+$ transformants was used to transform B-4131FO40, only three of the five were able to complement the acapsular phenotype of B-4131FO40. The free plasmid in each of these three Cap$^+$ transformants was rescued in *E. coli*. One plasmid containing a 4.5-kb insert (pYCC6) was isolated and was able to complement the acapsular phenotype of B-4131FO40. To determine the minimal region in pYCC6 required for the complementation of the B-4131FO40 acapsular phenotype, we divided pYCC6 into several overlapping clones and transformed then into B-4131FO40 (Fig. 3). Plasmid pYCC14 containing a 2.9-kb *Sma*I insert was the smallest complementing subclone identified.

Although strain B-4131 has been used in several previous studies (12, 22), the *cap67* mutation in B-4131 has not been mapped by the classic genetic method (24). To determine if *cap67* belongs to any of the six previously mapped capsule-associated loci (24), pYCC6 and pYCC14 were used to transform several different acapsular mutants. Interestingly, pYCC6 and pYCC14 were able to restore the Cap$^+$ phenotype in R-747FO14 (*cap59*), which had been mapped by the classic genetic method (24). Thus, B-4131 had a genetic lesion at the same locus as that of *cap59*. Furthermore, pYCC6 and pYCC14 complemented two other capsule-deficient mutants, R-744FO9 and R-745FO11, whose mutations have not been mapped by the classic recombination method. This indicates that at least four mutants with a genetic lesion at the *CAP59* locus have been identified.

Deletion of *CAP59* resulted in the loss of capsule. The presence of a functional *CAP59* gene sequence in pYCC6 was first determined by the following method. A partial genomic DNA library from the acapsular strain B-4131 was constructed and screened with a probe of pYCC6. A positive subclone derived from the library and an equivalent subclone from pYCC6 determined by restriction enzyme and sequence analysis were each used to transform B-4131FO40. Only the subclone from pYCC6 was able to complement the capsule mutation. Furthermore, when the Cap$^+$ transformants lost their transforming plasmids, they became acapsular. This strongly suggests that pYCC6 contains the functional *CAP59* gene sequence.

To conclusively demonstrate that pYCC6 contains sequences corresponding to the wild-type *CAP59* gene and to exclude the possibility that pYCC6 contains a suppressor, we attempted gene disruption of *CAP59*. We constructed a plasmid (pYCC28) containing a 1.3-kb *Nco*I-*Rsr*II DNA fragment from pYCC6 (Fig. 4A). A Cap$^+$ yeast strain, B-4500FO2, was transformed with pYCC28 DNA. Homologous integration at the *CAP59* locus would produce an acapsular phenotype and yield two partially deleted copies in Southern blot analysis. A visual screen of more than 6,000 independent transformants revealed two Cap$^-$ transformants. However, Southern blot analysis indicated that the two Cap$^-$ transformants were not derived from gene disruption by homologous integration at the *CAP59* locus (data not shown). To optimize the screening procedure for obtaining gene disruption by homologous inte-

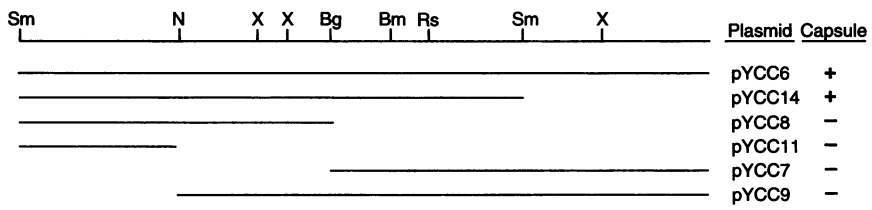

FIG. 3. Determination of the boundary of *CAP59* in pYCC6. Overlapping subclones of pYCC6 were used to transform B-4131FO40. The capsular phenotype of the resulting transformants is indicated. Bg, *Bgl*I; Bm, *Bam*HI; N, *Nco*I; Rs, *Rsr*II; Sm, *Sma*I; X, *Xho*I.

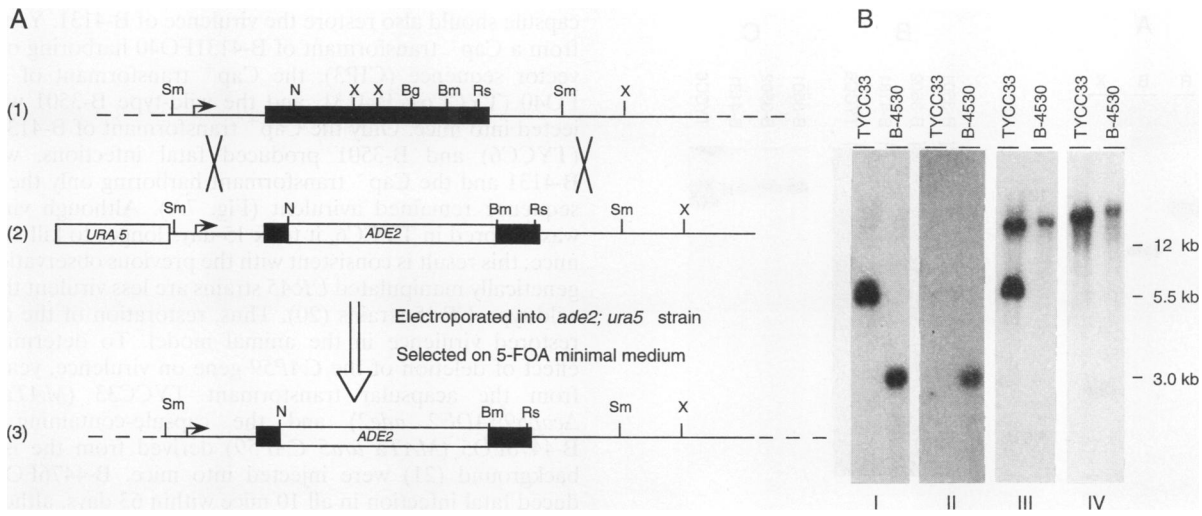

FIG. 4. Gene deletion of *CAP59*. (A) The strategy for *CAP59* deletion by gene replacement. In line 1 the *CAP59* gene was located in the 4.5-kb fragment of pYCC6. In line 2 the *Nco*I-*Bam*HI fragment of the *CAP59* coding region was replaced with the *ADE2* gene and the *URA5* gene was inserted in the 5′-flanking region of *CAP59* to create pYCC33. In line 3, the linearized pYCC33 was used to transform *C. neoformans* B-4530 (*ade2 ura5 CAP59*) and selected on 5-FOA medium. The diagrammed double-crossover event produced the deletion of *CAP59*. Solid box, *CAP59* coding region. Bg, *Bgl*I; Bm, *Bam*HI; N, *Nco*I; Rs, *Rsr*II; Sm, *Sma*I; X, *Xho*I. (B) Confirmation of the gene replacement. Genomic DNAs of an acapsular transformant (TYCC33) and a capsule containing strain (B-4530) were digested with *Sma*I and analyzed by Southern blot analysis. The membrane was hybridized with the *Sma*I-*Sma*I fragment of pYCC6 (I), the *Nco*I-*Bam*HI fragment of pYCC6 (II), the *ADE2* gene probe (III), and the *URA5* gene probe (IV).

gration, we developed a positive-negative selection system. A plasmid, pYCC33, was constructed by placing a *URA5* gene at the 5′-flanking region of *CAP59*, and the coding region of *CAP59* was replaced with an *ADE2* gene (Fig. 4A). The *ADE2* gene functioned as a positive selectable marker, and the *URA5* gene was the negative selectable marker. This plasmid was transformed into an *ade2 ura5* strain (B-4530), which is isogenic to B4500 except for the mating type. The transformants were selected on minimal medium containing 5-FOA. Because Ura5$^+$ strains of *C. neoformans* are unable to grow on media containing the pyrimidine analog 5-FOA (23), 5-FOA could be used as a reagent for negative selection. The rationale is that if the plasmid is extrachromosomally maintained or ectopically integrated in the *C. neoformans* genome, the transformants would have the *ADE2 URA5* genotype and would not survive on 5-FOA medium. However, if double crossing over occurred in the flanking regions of *CAP59*, the resulting transformants would have the *ADE2 ura5* genotype and could grow on 5-FOA medium. Of the 8×10^8 cells used in the transformation, about 4,000 grew on 5-FOA medium. This number of transformants was only 23% less than the number of the control transformants grown on minimal medium without 5-FOA. After extensive examination of the 4,000 transformants, one acapsular transformant was obtained (TYCC33).

Southern blot analysis was carried out to determine if the acapsular phenotype of TYCC33 was derived from a gene replacement event. When the *Sma*I-*Sma*I fragment of pYCC6 was used as a probe, the signal at 2.9 kb in wild-type B-4530 was replaced by a 5.5-kb signal in transformant TYCC33 (Fig. 4B, panel I). When the same blot was stripped and hybridized with a probe made from the *Nco*I-*Bam*HI fragment of pYCC6, no signal was detected in TYCC33 (Fig. 4B, panel II). When the *ADE2* gene fragment was used as probe, it detected a >12-kb signal in the wild type (Fig. 4B, panel III). In TYCC33, the *ADE2* probe not only hybridized to the resident >12-kb fragment but also hybridized to the 5.5-kb fragment. Finally, when the *URA5* gene was used as a probe, no signal was detected at 5.5 kb in either B-4530 or TYCC33 (Fig. 4B, panel IV). These results indicated that the acapsular phenotype of TYCC33 was generated by gene deletion through double crossover at the homologous site. These results also support our contention that sequences contained in pYCC6 correspond to the *CAP59* gene.

Genomic organization of *CAP59*. Southern blot analysis of genomic DNA of B-3501 digested with different enzymes suggested the presence of only one copy of the *CAP59* gene in the genome (Fig. 5A). To determine the chromosomal location of *CAP59*, the *C. neoformans* chromosomes were resolved by contour clamped homogeneous electric field gel electrophoresis and hybridized with the pYCC6 probe (Fig. 5B and C). The pYCC6 probe hybridized to the chromosome I+II doublet in B-4131 and in two wild-type strains, whereas in the stable transformants of B-4131FO40 (TYCC6) it hybridized to the second and third chromosomal bands. This result suggested that *CAP59* is located in the chromosome I+II doublet and that the additional signal in the stable transformant TYCC6 represents the location of the ectopically integrated DNA. Because the chromosome I+II doublet was resolved in TYCC6, a chromosome-specific probe could further assign *CAP59* to a defined chromosome. Since ribosomal DNA had been assigned to chromosome II (29), it was used as probe for the same blot. The ribosomal DNA probe hybridized to the chromosome I+II doublet in B-3501, B-3502, and B-4131, whereas it hybridized only to the first chromosomal band in TYCC6 (data not shown). Thus, *CAP59* was assigned to chromosome I in the wild-type genome.

The sequence of the 2.9-kb insert in pYCC14 and cDNA clones of *CAP59* was determined (Fig. 6). The 1.9-kb full-length cDNA corresponds to the size of poly(A)$^+$ mRNA detected by Northern (RNA) blot analysis (data not shown). The predicted CAP59 polypeptide contains 458 amino acid residues (52,895 kDa) with a pI of 5.8 and is highly hydrophilic at its carboxyl terminus.

The *CAP59* gene sequence was compared with sequences in

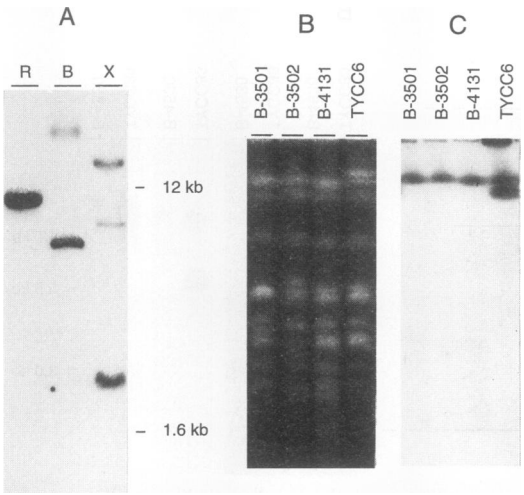

FIG. 5. Genomic structure of *CAP59*. (A) Genomic DNA of B-3501 was digested with *Eco*RI (lane R) (no restriction site in the gene), *Bam*HI (lane B) (one site in the gene), and *Xho*I (X) (which cuts three times, but two of them are adjacent to each other). The blot was hybridized with pYCC6 probe. (B) Ethidium bromide staining of chromosomal DNA from different strains of *C. neoformans* separated by contour-clamped homogeneous electric field gel electrophoresis. (C) The gel-separated chromosomal DNA was transferred to nylon membrane and hybridized with the pYCC6 probe. B-3501, wild-type strain (α mating type); B-3502, wild-type strain (**a** mating type); B-4131, acapsular strain; TYCC6, stable Cap^+ transformant of B-4131FO40.

GenBank, EMBL, SWISS-PROT, and PIR data bases and showed no similarity with any reported sequences. Comparison of the genomic sequence with the cDNA sequence revealed the presence of six introns within the *CAP59* coding region. Interestingly, two of the introns are located upstream of the first methionine codon. The DNA sequence of *CAP59* contains several unique features upstream of the proposed first methionine codon (Fig. 6). The canonical TATAAA and CAAT sequence upstream of the transcription initiation site is absent. One 13-bp inverse repeat is located at positions −125 and −92. A 21-bp direct repeat is present in the 5′ untranslated region at positions 276 and 352. Further molecular analysis may resolve the importance of these sequences. The DNA sequence of the 2.9-kb equivalent of *CAP59* from the acapsular mutant, B-4131, was determined and compared with that of B-3501. Only one missense mutation (G to A) at position 1345, which changed glycine to serine in the proposed peptide, was detected. Therefore, the glycine residue may play a critical role in the function of *CAP59*. In addition, the mRNA size and the transcription initiation site of *cap59* in B-4531 were not affected (data not shown).

Complementation of the Cap^- phenotype restores virulence. A monoclonal antibody (E1) (8) specific for the glucuronoxylomannan of capsular polysaccharide was used in an immunofluorescence assay to determine if restoration of capsule formation in B-4131 also restores its polysaccharide content. The E1 antibody reacted with the wild-type strain, B-3501, but not with the acapsular mutant, B-4131 (results not shown). When a stable Cap^+ transformant of B-4131FO40 (TYCC6) was tested with the E1 antibody, the immunofluorescence patterns were similar to those of B-3501. Thus, the capsular polysaccharide produced by TYCC6 was similar to that of the wild type. We reasoned that restoration of the capsule should also restore the virulence of B-4131. Yeast cells from a Cap^- transformant of B-4131FO40 harboring only the vector sequence (CIP3), the Cap^+ transformant of B-4131 FO40 (TYCC6), B-4131, and the wild-type B-3501 were injected into mice. Only the Cap^+ transformant of B-4131FO40 (TYCC6) and B-3501 produced fatal infections, whereas B-4131 and the Cap^- transformant harboring only the vector sequence remained avirulent (Fig. 7A). Although virulence was restored in TYCC6, it took 15 days longer to kill 100% of mice; this result is consistent with the previous observation that genetically manipulated *URA5* strains are less virulent than the wild-type *URA5* strains (20). Thus, restoration of the capsule restored virulence in the animal model. To determine the effect of deletion of the *CAP59* gene on virulence, yeast cells from the acapsular transformant TYCC33 (*MAT***a** *ura5* Δ*cap59*::*ADE2 ade2*) and the capsule-containing strain B-4476FO5 (*MAT***a** *ura5 CAP59*) derived from the isogenic background (21) were injected into mice. B-4476FO5 produced fatal infection in all 10 mice within 63 days, although at a lower rate because of the *ura5* mutation (20). The mice inoculated with TYCC33 remained healthy (70 days postinjection), indicating that the *CAP59*-deleted isolate loses virulence.

DISCUSSION

We have cloned a gene responsible for capsule formation (*CAP59*) by complementation. Since no sequence similar to the *CAP59* gene was reported in the data bases, its biochemical function in the synthesis of capsule polysaccharide remains undetermined. Nonetheless, the four *cap59* mutants identified in this study may be useful in the elucidation of its biochemical function.

In a previous report, the two-polymer aqueous-phase system failed to isolate nonencapsulated mutants from mutagenized wild-type cells (14). The modifications introduced in our studies may account for the success in identifying Cap^+ transformants. Growth for 52 h in liquid selective medium after electroporation and prior to partition increased the total number of Cap^+ transformants recovered. Incubation in RPMI medium under 10% CO_2 enhanced the differences between Cap^+ and Cap^- transformants (4a, 13).

The described positive-negative selection method was designed to select for acapsular transformants resulting from a gene replacement event (Fig. 4A). The transformation frequency and the number of transformants grown on minimal medium without 5-FOA appeared to be normal in control experiments (data not shown). However, a large number of Ade^+ transformants grew on 5-FOA medium and remained Cap^+. It is unlikely that large numbers of Cap^+ transformants would be induced by 5-FOA since spontaneous uracil auxotrophs arise on 5-FOA medium at a rate of 5×10^{-6} to 2.5×10^{-8} depending on the isolate (23). Because only uracil uptake mutants or uracil auxotrophs can grow on 5-FOA medium, we tested 70 independent transformants and found that all of them were uracil auxotrophs (data not shown). Since these transformants were Cap^+, one possible explanation for the occurrence of uracil auxotrophs is that the transforming DNA may be modified in the unstable transformants (10, 27) and the modification may render the *URA5* gene nonfunctional. Despite these problems, it was clear that homologous integration is very rare in *C. neoformans* B-4500FO2 and B-4530.

The gene deletion by homologous integration presented in this study is the first reported in a *C. neoformans* serotype D strain. The homologous integration frequency in the present study is much lower than that reported for the *ade2* gene in a serotype A strain transformed by the biolistics method (25).

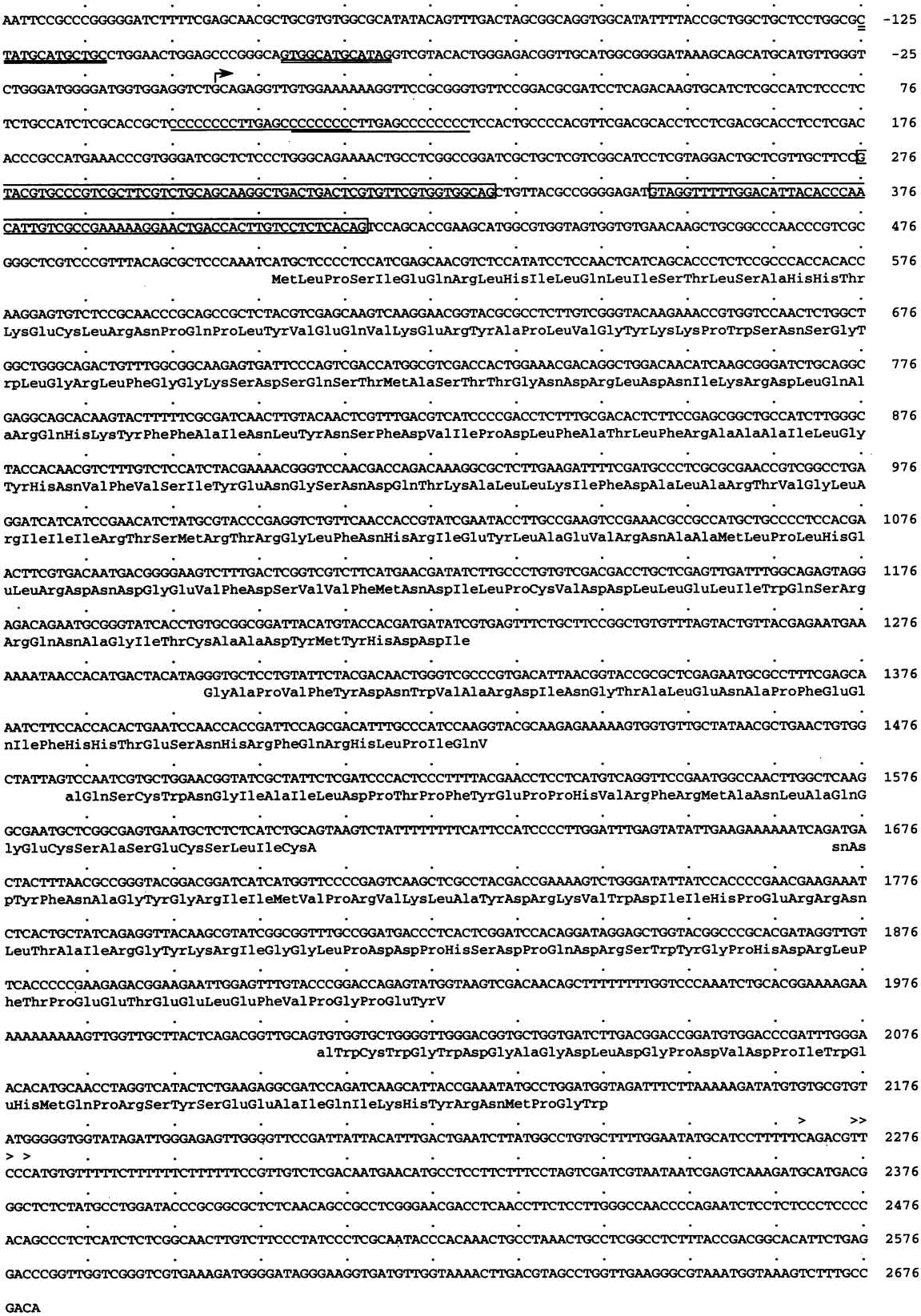

FIG. 6. Nucleotide sequence of *CAP59*. The proposed polypeptide is given in the three-letter code below the DNA sequence. The transcription start site (arrow; +1) was determined by primer extension. The poly(A) addition sites (>) were deduced from several cDNA sequences. The two introns upstream of the first methionine are boxed. The direct repeat (underline) and the inverted repeat (double underline) are as indicated.

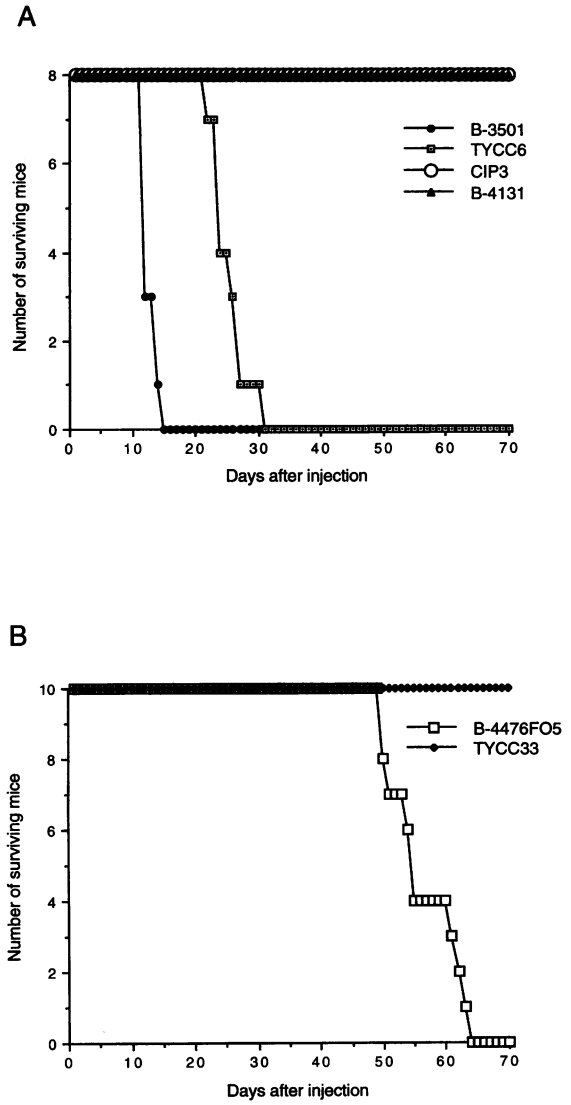

FIG. 7. Virulence test. (A) Survival of mice infected with 5×10^5 viable yeast cells. TYCC6, a Cap^+ transformant of B-4131FO40; CIP3, a Cap^- transformant harboring only the vector sequence; B-4131, a Cap^- strain; B-3501, a wild-type strain. (B) Survival of mice infected with 7×10^6 viable yeast cells. TYCC33, an acapsular transformant resulting from *CAP59* deletion; B-4476FO5, a capsule-containing strain.

The difference may result from differences in the DNA delivery systems, the genetic loci, and/or the serotypes. When a *ura5* strain of B-3501 was transformed with a *URA5*-containing vector, almost 75% of transformants were unstable for uracil prototrophy and contained an extrachromosomal DNA plasmid (27). Thus, the extrachromosomal maintenance of the transforming DNA may account for the low frequency of homologous integration in the serotype D strain.

The restoration of virulence by complementation of the acapsular phenotype and the loss of virulence by gene deletion provide the first molecular evidence that capsule is a virulence factor. Although the role of the *CAP59* gene in capsular polysaccharide biosynthetic pathway remains unknown, this gene is clearly associated with the genetic control of virulence in *C. neoformans*. Future studies in the molecular biology of capsule synthesis will lead to a better understanding of the pathogenicity of *C. neoformans*.

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