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# Cloning of a *Cryptococcus neoformans* Gene, *GPA1*, Encoding a G-Protein α-Subunit Homolog

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**We have isolated a gene, *GPA1*, from *Cryptococcus neoformans* by the PCR technique. DNA sequencing of the *GPA1* clone suggested that it encodes a protein homologous to the G-protein α-subunit family. Comparison of the deduced amino acid sequence of the *GPA1*-encoded protein revealed that it is about 45% identical to several mammalian Giα subunits and 48% identical to the Gα protein Gpa2 from *Saccharomyces cerevisiae*. Gα proteins are known to be involved in mating of other yeasts, such as *S. cerevisiae* and *Schizosaccharomyces pombe*. Southern analysis demonstrated that *GPA1* is present in a single copy within the *Cryptococcus* genome. Isolation of the cDNA for *GPA1* confirmed that the gene contains six introns within the coding region. The *GPA1* transcript was identified by Northern (RNA) analysis as a 1.6-kb RNA present in exponentially growing cells of both the α and a mating types. Moreover, the abundance of this transcript increased in cells shifted to starvation medium. Coincubation of α and a cells on starvation medium is required for mating of cryptococcal cells. Thus, our results are consistent with the involvement of *C. neoformans GPA1* in mating.**

*Cryptococcus neoformans*, an opportunistic yeast pathogen, causes meningitis in immunocompromised hosts, such as patients with AIDS (20, 23, 39). However, the normal habitat of *C. neoformans* is nonhuman; it is found worldwide in pigeon droppings, and one variety has been found associated with one species of eucalyptus (10, 23, 32). *C. neoformans*' life cycle has been elucidated, and a sexual stage has been identified (19). It is a heterothallic basidiomycete with two haploid cell types called the **a** and α mating types. Mixtures of these two cell types on an appropriate starvation medium results in mating to form the hyphal phase, during which nuclear fusion occurs, followed immediately by meiosis and spore formation. The starvation medium must be deficient in both the carbon-energy source and a nitrogen source; mixtures of cells on rich media do not result in hypha formation.

Genetic analysis of *C. neoformans* has suggested a correlation between the α mating type and increased virulence; however, no morphological or biochemical attributes accounting for this difference have been found (21). Therefore, an understanding of the mating process in *C. neoformans* may be important for understanding virulence. In particular, the molecular mechanisms underlying starvation-induced mating in *C. neoformans* have not been elucidated.

The control of the **a** and α mating types is found at a single genetic locus (called *MAT*) (25). Cells of the α mating type contain about 40 kb of unique DNA (*MAT* α allele) at this locus, which is lacking in **a** mating type cells, which contain the *MAT***a** allele. The *MAT* α locus has been found to contain a putative pheromone gene. These findings support the hypothesis that *C. neoformans* has a mating type system similar to that of other yeasts, such as *Saccharomyces cerevisiae*. Mating in *S. cerevisiae* (often called the pheromone response) has been extensively studied and involves a minimum of 24 genes (for reviews, see references 4, 14, and 24). Strikingly, many of these components are conserved across great evolutionary distances and are structurally and functionally homologous to genes involved in signal transduction in higher eucaryotes, including mammals. Also as in its mammalian counterparts, the pheromone response pathway controls cell proliferation, and yeast pheromones may be considered negative growth factors. This makes the pheromone response pathway an attractive system for controlling yeast growth.

Hypothesizing that an *S. cerevisiae*-like pheromone response pathway is conserved in *C. neoformans*, we began a search for cryptococcal homologs to genes involved in the pheromone response in *S. cerevisiae* and other yeasts. In particular, the *S. cerevisiae* pheromone response pathway requires the activity of a heterotrimeric GTP-binding protein (G protein). G proteins are apparently ubiquitous among eucaryotes and are highly conserved (for reviews, see references 2, 3, 12, 17, 36, and 38). We have taken advantage of the high level of conservation among G proteins to clone a *C. neoformans* gene encoding a homolog of a G-protein α subunit (Gα) by using the PCR method. The cryptococcal Gα protein, although related to several yeast Gα subunits, is actually more homologous to various mammalian proteins. We found that the expression of the cryptococcal Gα gene (called *GPA1*) varies with the growth medium in a way that is consistent with a role in mating.

## MATERIALS AND METHODS

***C. neoformans* strains and media.** The strains used in this study are described in Table 1. Strains were maintained on YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose). Strains were grown in SD (synthetic glucose medium containing [per liter] 1.7 g of Bacto Yeast Nitrogen Base [without amino acids and ammonium sulfate], 4.0 g of ammonium sulfate, and 20 g of glucose). Starvation medium (mating leucine [ML]) was based on Mating Agar (American Type Culture Collection [ATCC]) and contained (per liter) 2.0 g of sucrose, 100 g of $KH_2PO_4$, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 0.1 g of NaCl, 0.03 g of leucine, and 5.0 μg of biotin (pH 6.2).

**Cloning and sequencing of the *GPA1* gene.** First, we chose a small region that is highly conserved in Gα proteins from both yeast and mammalian cells (for reviews, see references 2, 3, 12, 17, 36, 38). A sequence of this region, which is believed to be involved in guanine nucleotide binding, includes the following

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TABLE 1. *C. neoformans* strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| 42163 | *MAT*α | ATCC; from self-fertile isolate (purified with 42164) |
| 42164 | *MAT***a** | ATCC; from self-fertile isolate (purified with 42163) |
| JEC20 | *MAT***a** | J. Edman[a]; congenic with JEC21 |
| JEC21 | *MAT*α | J. Edman; congenic with JEC20 |
| JEC34 | *MAT***a** *ura5* | J. Edman; derived from strain JEC20 |
| JEC43 | *MAT*α *ura5* | J. Edman; derived from strain JEC21 |

[a] University of California, San Francisco.

amino acids: N′-RKKWI . . . 53 amino acids . . . LFLNK-C′. We then devised degenerate, synthetic-oligonucleotide primers to these two pentapeptides, which are identical in several yeast and mammalian proteins. These primers were used in a PCR with *Taq* DNA polymerase (Perkin-Elmer) with genomic DNA from strain 42163. Genomic DNA was isolated by using a variation of a previously described method (8). All PCR-amplified bands were cloned into the pT7Blue vector (Novagen) and sequenced by the dideoxynucleotide method of Sanger et al. (35). Primers exactly homologous to the regions near the ends of the first PCR clone were synthesized and used for inverse PCR with genomic DNA cut with *Bam*HI and *Bgl*II (a *Bgl*II site was found just downstream of one primer) and self-ligated. A band of 550 bp was amplified, cloned, and sequenced. We used this general approach of creating primers based on sequences near the 5′- and 3′-most ends of previously cloned fragments and inverse PCR to clone three more inverse-PCR fragments. In all, five overlapping fragments, covering about 2.0 kb of DNA, were amplified, cloned, and sequenced. During the initial PCRs, two fragments were obtained, one corresponding to the gene we cloned in its entirety and the other presumably another Gα-related fragment. The only differences between these two fragments were in the intron sequences; the deduced amino acid sequences were identical. The primers we used and the restriction enzyme digestions of the genomic DNA for subsequent PCR amplifications were such that only one of the genes would be amplified (because of differences in the genomic DNA corresponding to the primers and differences in the restriction sites used for inverse PCRs). Each of the five PCR fragments was sequenced in both directions to obtain the entire sequence of the cryptococcal Gα protein, which we named *GPA1*. The *GPA1* gene was subsequently cloned in its entirety as a 1.7-kbp fragment by PCR.

**Southern analysis.** Genomic DNA was isolated from strains 42163, 42164, JEC34, and JEC43 and used for Southern analysis (37). For each sample, about 50 μg of DNA was cut with a given restriction enzyme, precipitated with ethanol, resuspended in deionized $H_2O$, and run in a 0.7% agarose gel. The DNA samples were transferred from the gels to Nytran membranes (Schleicher & Schuell) during a 2-h period by using a vacuum transfer machine. The resulting blot was probed with a digoxigenin (DIG)-labeled *GPA1* DNA fragment labeled by a random-primer reaction in accordance with the manufacturer's instructions (Genius; Boehringer Mannheim). The probe was a 560-bp fragment (G5-G11) homologous to portions of exons 2 and 3 and to intron II. The DIG-labeled *GPA1* probe was hybridized to the Southern blots in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 16 h. Hybridization was followed by treatment with anti-DIG antibody coupled to alkaline phosphatase (this was an anti-DIG Fab fragment conjugated to alkaline phosphatase). The membrane carrying the hybridized probe and bound antibody conjugate was reacted with Lumi-Phos 530 (a chemiluminescent substrate for alkaline phosphatase) in accordance with the manufacturer's instructions and then exposed to X-ray film to record the chemiluminescence signal.

**Northern analysis.** Strains 42163 and 42164 were grown in 50 ml of minimal SD to the mid-exponential phase (~$0.8 \times 10^7$ cells per ml), harvested by centrifugation, washed with deionized $H_2O$, and resuspended in 50 ml of fresh medium (SD or ML). Cells were then incubated for 3 h at 30°C with vigorous shaking and harvested, and total RNA was extracted. Cells were harvested by centrifugation, washed once, and resuspended in ice-cold RNA buffer (0.5 M NaCl, 200 mM Tris-HCl [pH 7.5], 10 mM EDTA in diethylpyrocarbonate [DEPC]-treated $H_2O$). Cells were broken open by vortexing with glass beads (0.45-μm diameter). Cell lysate was extracted twice with phenol-chloroform-isoamyl alcohol (25/24/1) by vortexing for 2 min, followed by ethanol precipitation overnight at −20°C. The nucleic acid pellet was washed with 75% ethanol and resuspended in DEPC-treated $H_2O$. The RNA was preferentially precipitated by addition of an equal volume of 4 M LiCl at 4°C overnight. The RNA pellet was washed with 2 M LiCl, resuspended in DEPC-treated $H_2O$, and precipitated a second time with LiCl. The second LiCl-RNA pellet was resuspended in DEPC-treated $H_2O$ and then precipitated at −20°C with ethanol in the presence of 0.3 M Na acetate. The RNA was stored as an ethanol precipitate at −20°C.

For Northern analysis, approximately 15 μg of total RNA per sample was loaded into an agarose (0.8%)-formaldehyde gel. The RNA samples were transferred to Schleicher & Schuell Nytran filters by blotting overnight with 5× SSPE–formamide. The blots were probed simultaneously for both the *URA5* and *GPA1* transcripts by using $^{32}$P-labeled probes. The *URA5* probe consisted of the entire CIP3 (8) plasmid labeled by random-primer labeling. The *GPA1* probe consisted of the 560-bp G5-G11 fragment, which was also labeled by random priming.

**Cloning the *GPA1* cDNA.** Total RNA from a mixed culture of strains 42163 and 42164 incubated in ML for 3 h as described above was precipitated with 3 M sodium acetate (pH 7.0) overnight at 4°C. The RNA pellet was resuspended in 20 μl of DEPC-treated $H_2O$ and treated with RNase-free DNase for 15 min at 37°C; this was followed by extraction with chloroform-isoamyl alcohol (24:1) by 2 min of vortexing. The aqueous RNA phase was removed, and the RNA was ethanol precipitated. The RNA pellet was resuspended in 50 μl of DEPC-treated $H_2O$ and its concentration was determined spectrophotometrically. Approximately 30 μg of RNA was mixed with 200 U of Moloney murine leukemia virus reverse transcriptase (RT). The RT reaction was primed with 600 μg of a synthetic oligonucleotide specific for the 3′ end of the *GPA1* transcript (22 to 40 bases downstream of the putative translation stop codons). The RNA and primer were mixed, heated at 65°C for 15 minutes, and cooled on ice. The RT reaction was carried out in accordance with the manufacturer's (Promega) instructions. The reaction product (cDNA) was extracted with chloroform-isoamyl alcohol (24:1) and ethanol precipitated. The cDNA pellet was resuspended in 20 μl of deionized $H_2O$ and used as a substrate for PCR with the same 3′ primer used in the RT reaction along with a 5′ primer complementary to bases −18 to −1 upstream of the putative translation initiation codon. The resulting PCR products were run in a gel, and a band of an approximately 1.4-kb fragment was cloned and sequenced, confirming that the *GPA1* cDNA had indeed been produced. The sequence of the cDNA clone

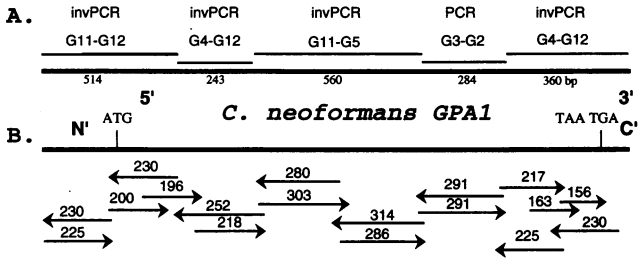


FIG. 1. (A) Schematic of the PCR-generated fragments used to clone *GPA1*. The first PCR-generated fragment, G3-G2, was amplified by standard PCR using degenerate primers. The remaining fragments were amplified by inverse PCR (invPCR) using completely homologous primers based on the sequences of previously isolated fragments. (B) Sequencing scheme of the *GPA1* clone. DNA sequencing was performed by using a combination of PCR-generated fragments and synthetic oligonucleotides. The numbers indicate distances in base pairs.

was compared to that of the genomic DNA to reveal the locations of six introns within the coding region. This comparison also demonstrated that the *GPA1* sequence derived from five genomic PCR fragments was authentic, intact *GPA1*.

## RESULTS

**Identification of a heterotrimeric G-protein gene in *C. neoformans*.** Heterotrimeric G proteins are apparently ubiquitous in eucaryotic cells. Such G proteins have three different subunits, α, β, and γ. We hypothesized that *C. neoformans* should also have a G protein, and in particular, it should have a gene encoding a Gα subunit. Gα subunits are particularly well conserved among eucaryotes. This conservation allows genes encoding them to be cloned by PCR. Synthetic, degenerate-oligonucleotide primers based on conserved amino acid sequences from α subunits of other yeasts and mammalian cells were devised. We began by selecting a small region believed to be involved in guanine nucleotide binding, which is highly conserved in all Gα proteins, from yeast to mammalian cells. From within this region, we chose the amino acids N′-RKKWI ... 53 amino acids .. LFLNK-C′ and devised degenerate, synthetic-oligonucleotide primers to the two pentapeptides. Genomic DNA from *C. neoformans* 42163 was isolated and used as the template for PCR using the degenerate primers. A band of 280 bp, slightly larger than the size needed to encode the predicted 63 amino acids, was amplified. No open reading frame spanned the fragment; however, stretches of base pairs at the 5′ and 3′ ends of the clone could potentially encode peptides homologous to various Gα proteins. Assuming the presence of two small introns, the clone had the potential to encode a peptide highly homologous to the consensus peptide, as anticipated. By using the sequence of this fragment, we had synthesized complementary primers that could be used in the inverse-PCR technique. In this way, we cloned another DNA fragment surrounding the first clone. In three more cycles of inverse PCR, we cloned additional overlapping fragments covering the entire gene (Fig. 1). The

FIG. 2. DNA and deduced amino acid sequences of the *C. neoformans GPA1* (Gα-subunit) gene. The putative translational initiator codon and tandem translational stop codons are at bases +1 to 3 and +1607 to 1612, respectively. Intron sequences are underlined and numbered I to VI.

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-234  CCA CTA GCT GCT CTT TGG CTT CTC GTG AAA CGA AAC GAA TCA TCG CTG
-186  ATA CAA AAT TAT GCA GGC CCA CAA AAT AGA CAG TTA TCA ATT ACA CAT
-138  CTT CTA AAT CCC AAT CTC TGG ACC ACT TCA ATC CAT CCT TTG TCA GCA
 -90  CCC TCT GTT CCC CAC ATC TTT TCT CAT TAC TCC ACC CCA TTC ATA CCC
 -42  GTC TTT CTT TCC ATC TCT CTA CCG TCT TCA ATC CAA TTC ACA ATG GGC
                                                        -1 Met Gly

  +7  GGC TGT ATG TCT ACT CCA GAA GCC CCT AAG AAG ACC GCA GAG ACC AAG
      Gly Cys Met Ser Thr Pro Glu Ala Pro Lys Lys Thr Ala Glu Thr Lys

 55   CAA GTC CCT TCA ACC TCT ACC TCT TCC CGC CCA CCA CAA GCC TCA ACA
      Gln Val Pro Ser Thr Ser Thr Ser Ser Arg Pro Pro Gln Ala Ser Thr

103   TCA GCT ACA GCC ACA GCT GCT GGT GCT GGT ACA TCG GCC GCC AAT GGG
      Ser Ala Thr Ala Thr Ala Ala Gly Ala Gly Thr Ser Ala Ala Asn Gly

151   ACA GCA AAC GGT ATA AAG GGC GAC ACA ACA GCA ACA AAT AGG GTA GGA
      Thr Ala Asn Gly Ile Lys Gly Asp Thr Thr Ala Thr Asn Arg Val Gly

199   ACG AGT GGG GGA CAG GGG CTA GCA GCT GCT CTG GCA TCT ACA GAA CCA
      Thr Ser Gly Gly Gln Gly Leu Ala Ala Ala Leu Ala Ser Thr Glu Pro

247   CCA GGA GCA CAG GAT TCA AAA GGG AAT AAA GAT AGG AGT AAT CAA ATA
      Pro Gly Ala Gln Asp Ser Lys Gly Asn Lys Asp Arg Ser Asn Gln Ile

295   GAC AGG CAA CTG GAA GAT GAC CAG AAG AAG TTT AGA AAG GAG TGT AAG
      Asp Arg Gln Leu Glu Asp Asp Gln Lys Lys Phe Arg Lys Glu Cys Lys

343   ATT CTA CTG CTA G GT GAG CAT TCT TCT CCA TGG CGA AGA AGT GTG CT
      Ile Leu Leu Leu                    I

390   GAC GGG CCT CTG AAG  GA TCC GGT GAA TCT GGA AAA TCT ACA ATC GTC
                           Gly Ser Gly Glu Ser Gly Lys Ser Thr Ile Val

437   AAA CAG ATG AAG ATT ATC CAC CAA AAT GGT TAC TCT AAA GAC GAA CTG
      Lys Gln Met Lys Ile Ile His Gln Asn Gly Tyr Ser Lys Asp Glu Leu

485   CTC TCT TTC AGA GGA GTC ATC TAT AAA AAC GTC CTT GAC TCT GCC CAG
      Leu Ser Phe Arg Gly Val Ile Tyr Lys Asn Val Leu Asp Ser Ala Gln

533   GCG TTG ATT ATG GCT ATG AGA AAG ATT GGT GTG GAC CCT GAA GAC GCC
      Ala Leu Ile Met Ala Met Arg Lys Ile Gly Val Asp Pro Glu Asp Ala

581   AAC AAC AGA GTA TGT TAT ACA TCT TTC ACC CTC GTT GAC CAT TAC TT
      Asn Asn Arg                        II

628   ACC ATG ACA TAG TCA TAT GCC GAT CGT ATC CTT GAA TAC CGC ATG GAT
                    Ser Tyr Ala Asp Arg Ile Leu Glu Tyr Arg Met Asp

676   GCC GGT CTC GAT GCT GTA ATC CCC TCA GAA ATC CTG TAC AAC ATC GAA
      Ala Gly Leu Asp Ala Val Ile Pro Ser Glu Ile Leu Tyr Asn Ile Glu

724   TCA CTC TGG CAC GAT CCT GTC ATT CCC TCT GTC ATG GAT CGT AGC TCA
      Ser Leu Trp His Asp Pro Val Ile Pro Ser Val Met Asp Arg Ser Ser

772   GAG TTC TAC CTT ATG GAC TCT GCA ACT TAC TTT TTC GCC AAC ATC AGA
      Glu Phe Tyr Leu Met Asp Ser Ala Thr Tyr Phe Phe Ala Asn Ile Arg

820   AAG ATC GCA GGG CCC GAT TAT GTG CCT GAT GAA GCC GAT GTT CTG AGA
      Lys Ile Ala Gly Pro Asp Tyr Val Pro Asp Glu Ala Asp Val Leu Arg

868   GCG AGG ACG AAG ACG ACT GGT ATT AGT GAG ACG CGA TTT AAC ATG GGA
      Ala Arg Thr Lys Thr Thr Gly Ile Ser Glu Thr Arg Phe Asn Met Gly

916   CAG TTG AGC ATT CAC ATG TTC GAT GTG GGT GGA CAG AGA AGC GAG AGG
      Gln Leu Ser Ile His Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg

964   AAA AAG TGG ATT CAT T GT AGG TGA TCT TTT TTA GGG TCT ATG AGA TG
      Lys Lys Trp Ile His                    III

1012  ACA TTT ACA CAA ATC TAG  GT TTT GAG GCG GTC ACA TCT ATC ATC TTC
                              Cys Phe Glu Ala Val Thr Ser Ile Ile Phe

1058  TGC GTT GCA TTA TCA GAG TAC GAT CAA GTG TTG CTG GAG GAA TCA GGG
      Cys Val Ala Leu Ser Glu Tyr Asp Gln Val Leu Leu Glu Glu Ser Gly

1106  CAG GTA AGT TGT GTC GGC ATT TGT TAG AAT ATA TGC TGA TTC CTT CTCAG
      Gln                          IV

1156  AAC CGA ATG CAA GAA TCA CTG GTT CTC TTC GAG TCT GTA ATT AAC TCA
      Asn Arg Met Gln Glu Ser Leu Val Leu Phe Glu Ser Val Ile Asn Ser

1204  AGA TGG TTC CTG CGA ACA TCC GTC ATC CTC TTC CTC AAC AAG ATA GAC
      Arg Trp Phe Leu Arg Thr Ser Val Ile Leu Phe Leu Asn Lys Ile Asp

1252  TTG TTC AAG CAA AAA TTA CCA AAG GTC CCG CTT GTG CAG TAT TTC CCT
      Leu Phe Lys Gln Lys Leu Pro Lys Val Pro Leu Val Gln Tyr Phe Pro

1300  GAA TAC ACC GGT AT G TTC TCT TTC CTT GGT GAG AGT TTG TAT ATG ACT
      Glu Tyr Thr Gly Met                          V

1348  GAT TAT GGA TCT AG G CGG AGG AAT ATC AAC AAG GCT GCC AAG TAT ATC
                          Arg Arg Asn Ile Asn Lys Ala Ala Lys Tyr Ile

1396  TTG TGG AGA TTC ACC CAG ACC AAT CGA GCG AGG TTA TCG GTG TAT CCC
      Leu Trp Arg Phe Thr Gln Thr Asn Arg Ala Arg Leu Ser Val Tyr Pro

1444  CAT CTC ACC CAA GCG ACT GAT ACG TCA AAC GTA CGT CTG CCG TAT CAC
      His Leu Thr Gln Ala Thr Asp Thr Ser Asn

1492  C TTC CTC GTG TAA CAA CAT ATC CAG TTT CTG ATA ACC CTT TTC CAG
                              VI

1538  ATT CGA TTG GTA TTT GCA GCT GTT AAA GAA ACT ATC CTC CAG AAC GCT
      Ile Arg Leu Val Phe Ala Ala Val Lys Glu Thr Ile Leu Gln Asn Ala

1586  CTA CGC GAC TCT GGT ATC TTA TAA TGA CCC TAA GTA AAA GAT TAA AAG
      Leu Arg Asp Ser Gly Ile Leu *** ***

1634  AGC AAC GCC CAC ATG GCC AAT TCC CAA ACG TTT CTC CAA GTA CAA ATG
1682  TAG TTA TAT TTA GCA GAC TTT GAA TCA TAA TCA CTT ACA TAC TT 1725
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five overlapping PCR fragments were sequenced in both directions to generate the complete nucleotide sequence of this gene (Fig. 2), which we call *GPA1*. PCR was subsequently used to clone the entire gene in a single fragment from strain 42163. The same PCR primers and reaction conditions were used to amplify the same-size fragment from strain JEC34; however, sequencing of this *GPA1* gene was not done. When analyzing the genomic sequence, we assumed the presence of six introns (confirmed by cDNA analysis [see below]) and found that a protein homologous to the Gα-subunit family could be encoded. Figure 2 shows the deduced Gpa1 amino acid sequence. Figure 3 shows the alignments of the *C. neoformans* α subunit and those of seven other α subunits, including two from *S. cerevisiae*, one from *Candida albicans*, one from *Schizosaccharomyces pombe*, and three from mammalian cells. *C. neoformans* Gpa1 is very homologous to various Giα proteins (e.g., 45% identical to both human Giα and rat Giα) and also to a yeast Gα protein (48% identical to *S. cerevisiae* Gpa2) but is less homologous to Gsα (36% identical to rat Gsα). The homologies to several other yeast Gα proteins are as follows: *S. cerevisiae* Gpa1, 39% identical; *S. pombe* Gpa1, 32% identical; *C. albicans* Cag1, 38% identical. These homologies were determined in the absence of the various peptide inserts found in nearly every subunit (the insert sequences are described in the legend to Fig. 3). *C. neoformans* Gα has a single peptide insert of 67 amino acids very near its N′ end that is not present in any other Gα protein. This peptide, which is present in the most variable region of Gα proteins, is not related to the inserts found in any of the other yeast or mammalian subunits. Furthermore, the cryptococcal protein does not have any inserts corresponding to those of the other four yeast Gα subunits. Thus, overall, *C. neoformans* Gα more closely resembles mammalian than yeast Gα subunits.

**Identification of the positions of the six introns in *GPA1*.** The DNA sequence of the *GPA1* gene was assumed to have six introns. Identification of the positions of these introns was based on an attempt to maintain homology between the deduced Gpa1 amino acid sequence and various other Gα subunits, as well as the need to maintain an open reading frame. The initial positioning of the introns was arbitrary and based on the limited homology that is believed to occur for yeast intron splice junctions. To position the introns precisely, we made cDNA from extracts that contained the *GPA1* mRNA. The cDNA was made with a primer specific for the *GPA1* mRNA. RNA from a mixed culture of strains 42163 and 42164 that was incubated in ML was used as a template with RT. The RT reaction was primed with a synthetic oligonucleotide complementary to the 3′ end of the *GPA1* gene 22 to 40 bases downstream of the tandem termination codons. Thus, theoretically only the *GPA1* mRNA should have been reverse transcribed into cDNA. The use of specific primers in the RT reaction and PCR allowed us to isolate nearly the entire length of the *GPA1* transcript; however, this method was not usable for identification of the transcription initiation or termination site nor the presence of introns in the nontranslated regions. The reaction mixture containing the *GPA1* cDNA was then used as a template in a PCR with the 3′-end primer used to make the cDNA plus a 5′-end primer corresponding to bases −18 to −1 upstream of the putative initiator AUG. Thus, this PCR should amplify the entire cDNA. A band of the appropriate size was amplified, cloned, and sequenced. The sequence revealed the seven exons with the six introns spliced out. Comparison of the cDNA sequence with the genomic sequence allowed us to position the six introns exactly (shown in Fig. 2). We aligned the six introns to reveal homologies (Fig. 4). The sizes of the introns ranged from 47 to 64 bp; however, all but one were in the range of 47 to 50 bp. The average size of the introns was approximately 49 bp.

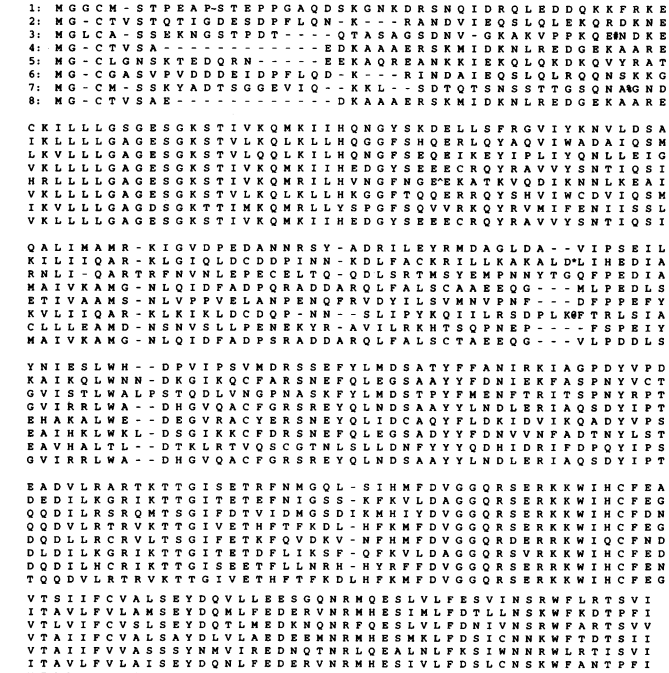

FIG. 3. Comparison of amino acid sequences of Gα subunits. The single-letter amino acid code is used. Sequences: 1, *C. neoformans* Gpa1; 2, *S. cerevisiae* Gpa1 (28); 3, *S. cerevisiae* Gpa2 (29); 4, rat Gi2α (15); 5, rat Gsα (15); 6, *C. albicans* Cag1 (34); 7, *S. pombe* Gpa1 (30); 8, human Giα, (1, 6). Symbols: ~, insert the sequence KKTAET KQVPSTSTSSRPPQASTSATATAAGAGTSAANGTANGIKGDT TATNRVGTSGGQGLAAALA in *C. neoformans* Gpa1; *, insert the sequence YINASVAGGDSFLNDYVLKYSERYETRRRVQSTGRA KAAFDEDGNISNVKSDTDRDAETVTQNEDADRNNSSRIN LQDICKDLNQEGDDQMFVRKTSREIQGQNRRN in *S. cerevisiae* Gpa1; #, insert the sequence PQKTVRTVNTANQQEKQQQR RQQQPSPHNVKDRKEQNGSINNAISPTATANTSGSQQINID SALRDRSSNVAAQPSLSDASSGS in *S. cerevisiae* Gpa2; ^, insert the sequence GGEEDPQAARSNSDG in rat Gsα; +, insert the sequence EPGEDPRVTRAK in rat Gsα; @, insert the sequence QIDASVAG GTDFLNDFVVKYSEENKNKRRLKSTGTTDIWGKDDDSNINS DAINQAIELSLNKDSEEQ in *C. albicans* Cag1; %, insert the sequence RVPVLENWLNIVLRGKPQNVESSGVRVKGNSTSG in *S. pombe* Gpa1; •, insert the sequence FVQTQCPLADNAVRS in *S. pombe* Gpa1. Note that extra large inserts have been removed from six of the protein sequences.

**Copy number analysis of the *GPA1* gene.** We analyzed genomic DNAs from strains 42163 (α mating type) and 42164 (**a** mating type), which were purified from the same self-fertile isolate. The genomic DNAs were subjected to restriction digestion with three enzymes and Southern blotted. The blot was probed under stringent conditions with an internal fragment of the *GPA1* gene. Figure 5 shows that genomic DNA

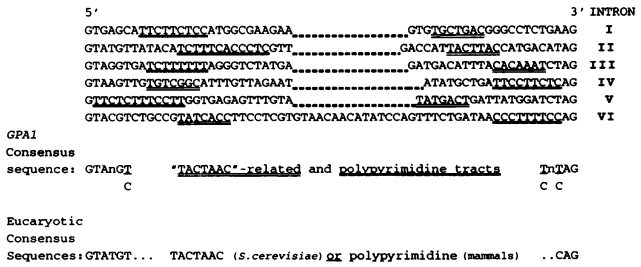


FIG. 4. Sequencing of *GPA1* cDNA reveals the locations of six introns. Total RNA was isolated from a mixed culture of strains 42163 and 42164 incubated in ML and subjected to reverse transcription with a primer specific for the 3'-nontranslated region of the *GPA1* transcript. The resulting cDNA was used for PCR with the same 3'-specific primer along with a primer specific for the 5'-nontranslated region of *GPA1*. The amplified fragment was cloned and sequenced. The positions of the introns and exons are shown in Fig. 2, while the alignment of the introns is shown here.

from 42164 cells produced a single band after digestion with *Cla*I, *Bgl*II, and *Eco*RV, suggesting a single copy for the *GPA1* gene. However, two bands (one corresponding to that seen with 42164 DNA) were produced after digestion of 42163 DNA with these same enzymes, suggesting two related genes. To test whether the copy number difference between 42163 and 42164 was due to the different mating types or to natural variation between wild-type isolates, we examined the *GPA1* copy number in two strains (JEC34 and JEC43) which are believed to be congenic, differing only in mating type. Figure 5 shows a Southern analysis of genomic DNAs from these strains probed with the same *GPA1* fragment, proving that a single copy of the *GPA1* gene is present in cells of both the **a** and α mating types.

**Expression of *GPA1*.** The first step in understanding the function of *GPA1* is to examine its transcriptional activity. Changes in transcription under specific physiological conditions can shed light on potential functions. Total RNA was isolated from cryptococcal cells, run in an agarose gel, transferred by the Northern blot procedure to a nylon membrane, and probed for the *GPA1* transcript. Cells of strains 42163, 42164, JEC20, and JEC21 were grown separately overnight in minimal SD to the early exponential phase, shifted to fresh media, and incubated for 3 h prior to RNA isolation. The shifted cells were either incubated separately as pure cultures or mixed together in two pairs (42163 plus 42164 and JEC20 plus JEC21). Total RNA was isolated from each culture, run in an agarose gel, and blotted onto nylon paper. These Northern blots were probed simultaneously with $^{32}$P-labeled DNAs from both the *GPA1* and *URA5* genes. The *URA5* transcript was probed as a control to normalize the amount of RNA loaded in each lane. Thus, the level of the *GPA1* transcript was compared to that of the *URA5* transcript from the same RNA sample. A single *GPA1* transcript, with an approximate size of 1.6 kb (Fig. 6), was identified in cells growing exponentially in SD for all four strains incubated separately or in mixed pairs. Expression of *GPA1* was similar in the four strains tested. The abundance of the *GPA1* transcript was also examined in cells shifted to a medium used for mating of cryptococcal cells. After 3 h of incubation in starvation medium, total RNA was isolated, Northern blotted, and probed. The resulting Northern blot was autoradiographed and scanned by densitometry (data not shown). The level of the *GPA1* transcript increased more than threefold relative to the *URA5* control transcript in 42163 and 42164 cells incubated separately and in a mixed culture, consistent with transcriptional induction of *GPA1* during incubation in the mating-starvation medium.

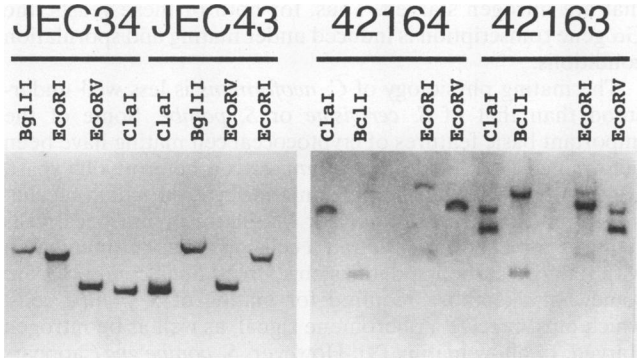


FIG. 5. Restriction enzyme digestion patterns of *C. neoformans* DNA probed for *GPA1*. The strains used were JEC34 (**a** mating type), JEC43 (α mating type), 42163 (α mating type), and 42164 (**a** mating type). Genomic DNA from each strain was digested with the indicated enzyme (note the different orders for **a** and α), run in agarose gels, and blotted to Schleicher & Schuell Nytran membranes. The blots were probed with a DIG-labeled *GPA1* DNA fragment (560 bp corresponding to portions of exons 2 and 3 and intron II) and then treated with anti-DIG antibody coupled to alkaline phosphatase. The membranes carrying the hybridized probes and bound antibody conjugates were reacted with Lumi-Phos 530 (a chemiluminescent substrate for alkaline phosphatase) and exposed to X-ray film to record the chemiluminescence signal.

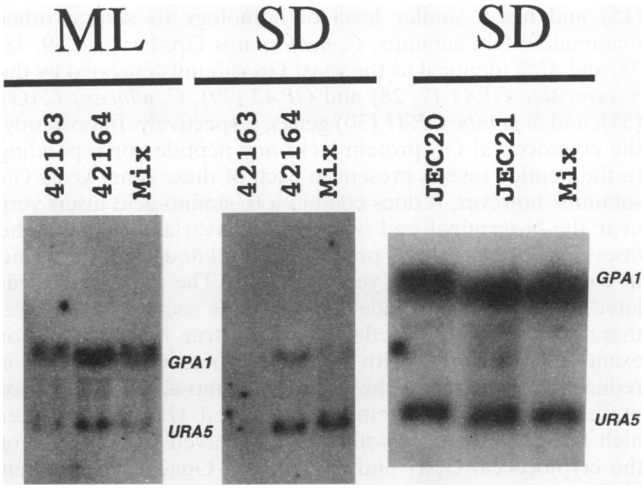


FIG. 6. Transcription of *GPA1*. Total RNA was isolated from strains 42163 and 42164 grown separately or as a mixed culture and strains JEC20 and JEC21 grown separately or mixed. The RNAs were analyzed by Northern blotting with $^{32}$P-labeled *GPA1* and *URA5* probes.

## DISCUSSION

We have isolated the *C. neoformans GPA1* gene, which encodes a Gα-subunit homolog. The Gpa1 deduced amino acid sequence was compared to the PIR data base and found to be homologous to the family of Gα proteins. Surprisingly, Gpa1 is slightly more homologous to various mammalian Gα proteins than to most other yeast Gα subunits. Specifically, Gpa1 is nearly 45% identical to human Giα (1, 6) and rat Gi2α

(15) and has a similar level of homology to several other mammalian Giα subunits. *C. neoformans* Gpa1 is also 39, 48, 38, and 32% identical to the yeast Gα subunits encoded by the *S. cerevisiae GPA1* (7, 28) and *GPA2* (29), *C. albicans CAG1* (34), and *S. pombe GPA1* (30) genes, respectively. Importantly, the cryptococcal Gα protein lacks any peptide corresponding to the peptide inserts present in each of these other yeast Gα subunits; however, it does contain a 67-amino-acid insert very near the N-terminal end within a hypervariable region. The insert in the cryptococcal protein is not related to inserts found in either mammalian or yeast subunits. The identities calculated above do not include these peptide sequences and are, therefore, not entirely reflective of the true homologies. For example, the identity with the *S. cerevisiae* Gpa2 protein is reduced to 39% when the extra 84-amino-acid peptide, not present in other Gα proteins, is considered. Despite the rather high level of homology within the conserved Gα domains of the cryptococcal Gpa1 and *S. cerevisiae* Gpa2 proteins, their distinct inserts suggest that they have very distinct functions. Interestingly, with the exception of Gpa2, cryptococcal Gpa1 is much more homologous to many mammalian Gα proteins than it is to other yeast subunits. The high level of homology to mammalian subunits is restricted to the Giα subfamily; however, there is no indication that this homology is related to the function of Gpa1.

Like the *S. cerevisiae* Gpa1 and Gpa2, *S. pombe* Gpa1, and *C. albicans* Cag1 proteins, *C. neoformans* Gpa1 does not contain a cysteine residue near the C terminus, which is ADP-ribosylated by pertussis toxin in some mammalian Gα proteins. It does, however, have the conserved arginine residue that is ADP-ribosylated by cholera toxin in mammalian Gsα proteins. The fact that cryptococcal Gpa1 is more related to mammalian Giα proteins, on the basis of size and sequence identity, than to other yeasts is consistent with the notion that this basidiomycete is evolutionarily distant from these yeasts. The *C. neoformans* Gα gene is the first such gene isolated from a basidiomycete. It will be interesting to compare the homologies of other important signal transduction proteins from basidiomycetes and mammals to see how widespread this similarity is.

The *GPA1* gene was found to be present in a single copy in congenic α and **a** mating type strains (JEC34 and JEC43) that differ only in mating type. These congenic strains have been genetically characterized. It is also present in one copy in ATCC 42164 (**a** mating type). In contrast, in ATCC 42163 (α mating type), a second *GPA1*-related gene was present. Strains 42163 and 42164 were obtained from a single self-fertile isolate. These results underscore the difficulty in studying wild-type isolates; each clinically or environmentally derived strain may differ from the next because of dramatic differences in their genomes. Such genomic variability is not rare in other organisms nor, apparently, in cryptococci. Recent studies that presented blots of complete or amplified chromosomal fragments have shown that large chromosomal rearrangements exist for different cryptococcal strains (31, 40). Our results indicate that such variations can include changes in gene copy number.

The structure of the cryptococcal Gα gene itself is also significantly different from that of its yeast relatives in that the coding region is punctuated by six introns. None of the other yeast Gα genes contain introns. The presence and splicing of introns from precursor mRNAs occur in all eucaryotic cells (for reviews, see references 13, 26, and 33). Although there is conservation in splicing among all eucaryotes, from yeasts to mammals, there are some important distinctions. There are three short sequences within introns that are required for proper splicing. The 5′ and 3′ sites are similar in yeasts and mammals; however, the branch point sequence is significantly different. In the yeast *S. cerevisiae*, the branch point sequence UACUAAC is highly conserved and mutations in this sequence inactivate the intron. Mammalian cells have very degenerate branch point sequences but require a polypyrimidine tract between the branch point sequence and 3′ splice site, a feature which is not required in *S. cerevisiae*. Moreover, *S. cerevisiae* has few introns, usually one near the 5′ end of the mRNA, while mammalian cells tend to have numerous introns scattered throughout the mRNA. The six *GPA1* introns are positioned throughout the gene and range in size from 47 to 64 bp, with an average of 49 bp. The 5′ and 3′ splice sites are fairly conserved and are related to the yeast and mammalian consensus sequences. Sequences related to the consensus UACUAAC sequence are present in each of the introns, which also contain polypyrimidine tracts; however, these tracts may be nonfunctional in such small introns. Generally, the *C. neoformans GPA1* introns are similar to those of *S. pombe*.

G proteins are essential components of mating pathways in *S. cerevisiae* and *S. pombe*, where they are coupled to membrane-bound pheromone receptors (7, 16, 27, 30, 41). The yeast G proteins act analogously to mammalian G proteins involved in such diverse signal transduction pathways as those that respond to light or hormones. When an extracellular pheromone binds to its cognate receptor in the cytoplasmic membrane, the receptor is believed to activate a G protein, which dissociates from the receptor into the α subunit bound to GTP and the βγ dimer. In *S. cerevisiae*, the α subunit (encoded by *GPA1*) is a negative regulator while the βγ dimer transmits the pheromone signal intracellularly. In contrast, in *S. pombe*, which has a single Gα-protein gene, *gpa1*, that α subunit appears to be a positive regulator of pheromone signal transduction and thus functions more like the mammalian Gα subunits. *S. pombe gpa1* is required for both mating and sporulation. The *S. cerevisiae GPA1* transcript is present in exponentially growing cells and is induced about threefold upon pheromone exposure. Similarly, exponentially growing haploid and diploid *S. pombe* cells contain the *gpa1* transcript, which increases in abundance three- to fourfold in diploid cells that are nitrogen starved. Thus, for both of these yeasts, the Gα gene transcription is induced under mating and sporulation conditions.

The mating physiology of *C. neoformans* is less well understood than that of *S. cerevisiae* or *S. pombe*. Some of the important basic features of cryptococcal cell mating have been determined (19). First, *C. neoformans* is a heterothallic yeast, apparently lacking the complex mating type switching mechanism found in *S. cerevisiae* (25). Mating of cryptococcal cells requires coincubation of α and **a** cells on a starvation medium with reduced carbon and nitrogen sources. Such conditions are somewhat like those required for mating of *S. pombe* cells, which must receive a pheromone signal, as well as be nitrogen starved, to allow mating (9). However, *S. pombe gpa1* appears to be required for pheromone signal transduction but not for monitoring of nitrogen starvation (30). *S. pombe* pheromones have been identified along with a receptor (5, 11, 18, 22). The pheromone receptors are believed to be members of the serpentine class of receptors which are coupled to G proteins. It is presumed that the *S. pombe* Gα protein is coupled to these pheromone receptors analogously to the G-protein–pheromone receptor coupling in *S. cerevisiae*.

The level of the *C. neoformans GPA1* transcript increased more than threefold in cells shifted to the starvation medium used for mating of **a** and α cells. The medium conditions necessary for mating vary among different yeasts. *S. cerevisiae*

cells need only be mixed together to allow exposure to the constitutively secreted pheromones to initiate mating, which occurs even on rich media. In contrast, *S. pombe* cells, which also constitutively synthesize pheromones, must be starved for nitrogen before mating can occur. Cryptococcal cells also must be incubated on starvation medium to allow mating. Furthermore, *C. neoformans* is also believed to have a pheromone gene, but it is not clear whether the pheromone is constitutively expressed. Thus, it may be necessary to incubate cryptococcal cells on starvation medium to induce pheromone synthesis and/or perhaps just to allow a response to pheromones analogous to the behavior of *S. pombe* cells. The presence of the *GPA1* transcript in cells growing in rich medium and the increased level of that transcript in cells shifted to starvation medium are consistent with the latter possibility.

By analogy with the *S. cerevisiae* and *S. pombe* mating systems, it is likely that the cryptococcal Gα protein functions during mating. Confirmation of this hypothesis requires the existence of mutations in the *GPA1* gene. No such mutation exists, and it is not possible to make gene disruptions like those in other yeasts. We are currently testing the possibility of eliminating *GPA1* expression by using antisense RNA.

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