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Isolation of the *URA5* Gene from *Cryptococcus neoformans* var. *neoformans* and Its Use as a Selective Marker for Transformation

JEFFREY C. EDMAN[1]* AND K. J. KWON-CHUNG[2]

Hormone Research Institute and Department of Laboratory Medicine, University of California, San Francisco, California 94143-0534,[1] and Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20892[2]

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A cDNA encoding *Cryptococcus neoformans* orotidine monophosphate pyrophosphorylase (OMPPase) has been isolated by complementation of the cognate *Escherichia coli pyrE* mutant. The cDNA was used as a probe to isolate a genomic DNA fragment encoding the OMPPase gene (*URA5*). By using electroporation for the introduction of plasmid DNA containing the *URA5* gene, *C. neoformans ura5* mutants could be transformed at low efficiency. Ura$^+$ transformants obtained with supercoiled plasmids containing the *URA5* gene showed marked mitotic instability and contained extrachromosomal *URA5* sequences, suggesting limited ability to replicate within *C. neoformans*. Transformants obtained with linear DNA were of two classes: stable transformants with integrated *URA5* sequences, and unstable transformants with extrachromosomal *URA5* sequences.

Prior to the acquired immunodeficiency syndrome (AIDS) epidemic, *Cryptococcus neoformans* was considered the "awakening giant" among opportunistic fungal infections (9). Unfortunately, this prediction has turned out to be true, largely due to the number of cases of cryptococcal meningitis seen in association with AIDS. Though *C. neoformans* can occasionally cause disease in the apparently normal host, it is primarily associated with therapeutic immunosuppression, neoplastic disease, and AIDS (24). *C. neoformans* is a heterothallic basidiomycetous yeast with a well-defined life cycle (11). Its perfect or sexual state (*Filobasidiella neoformans*) arises upon mating of the two distinct mating types, **a** and α. Basidiospores arising from this union germinate to renew the imperfect state. Although there are more than 20 species in the genus *Cryptococcus*, only *C. neoformans* regularly causes human disease. Factors associated with virulence have been identified by classical genetic studies (13, 14, 23), but nothing is known of the molecular aspects of these factors. Identification of the molecular mechanisms for virulence will require genetic manipulation of the organism. To develop such a system for the genetic manipulation of *C. neoformans*, a transformation system for the introduction and maintenance of selectable markers is paramount.

This study describes the isolation of orotidine monophosphate pyrophosphorylase (OMPPase) (*URA5*) cDNA clones by complementation of the corresponding *pyrE* mutation in *Escherichia coli* (to be consistent with the *Saccharomyces cerevisiae* system, we have designated the *C. neoformans* gene for OMPPase as *URA5* and the gene for orotidine-5′-phosphate decarboxylase as *URA3*). By using the OMPPase cDNA as a probe, the *C. neoformans URA5* gene was isolated. The cloned gene was capable of transforming *C. neoformans ura5* mutants to uracil prototrophy.

MATERIALS AND METHODS

***C. neoformans* strains and media.** *C. neoformans* var. *neoformans* strains B-3501, B-3502, NIH-424, NIH-12, and NIH-433 have been described before (12). YEPD (1% yeast extract, 2% Bacto-peptone [Difco Laboratories], 2% dextrose) was used as the maintenance medium. Uracil-depleted synthetic medium (SD-Ura) contained 6.7 g of yeast nitrogen base per liter without amino acids and 20 g of glucose per liter. 5-Fluoroorotic acid (5-FOA) medium contained 7 g of yeast nitrogen base, 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.

Bacterial strains and media. *E. coli* CGSC 4501 (*pyrE metB* F$^-$) was obtained from the *E. coli* Genetic Stock Center, Yale University. It was converted to F$^+$ by mating with *E. coli* XL1-Blue (XL-1B) [*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* (Lac$^-$) [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)] and plating to LB medium containing tetracycline (10 μg/ml) 5-bromochloroindolylgalactoside (Xgal) (40 μg/ml), and 200 μM isopropylthiogalactoside (IPTG). Tetracycline-resistant, blue colonies were selected and tested for uracil prototrophy and sensitivity to M13 infection. All such colonies were Ura$^-$ and sensitive to M13, indicating transfer of the F factor to the *pyrE* strain. Uracil-depleted bacterial medium was M9 salts (17) plus 2 mg of glucose, 0.01 mg of thiamine, and 0.2 mg of methionine per ml. *E. coli* HB101 and JM101 were used as plasmid and M13 hosts, respectively.

Nucleic acid preparations. *C. neoformans* spheroplasts were prepared by treatment of washed cells with 0.5 mg of lysing enzymes (Sigma L-2265) per ml in the presence of 1 M sorbitol and 1% β-mercaptoethanol (22). Spheroplasts were used as the starting point for all nucleic acid preparations. Polyadenylated [poly(A)$^+$] RNA was isolated by using the FastTrack kit (Invitrogen). DNA was isolated by suspending approximately 250 μl of packed spheroplasts in 1 ml of 10 mM Tris hydrochloride (Tris-HCl, pH 8.0)–1 mM EDTA (TE) plus 1% sodium dodecyl sulfate (SDS) and heating to 65°C for 15 min. Then, 200 μl of 5 M potassium acetate was added to precipitate proteins and SDS. The precipitate was removed by centrifugation, and DNA in the supernatant was precipitated by the addition of 2 volumes of absolute ethanol. The DNA precipitate was collected by centrifugation, suspended in 400 μl of TE, and precipitated with 700 μl of

* Corresponding author.

isopropanol. The yield of DNA from this procedure was approximately 25 μg. For Southern blot analysis, 1 μg of genomic DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, and transferred to ZetaProbe nylon membrane in 400 mM NaOH. The membranes were baked at 80°C for 2 h after transfer and hybridized to 10^7 cpm of random-primed probe per ml overnight at 42°C in 50% formamide–5× SSC–5× Denhardt solution–10 μg of sonicated salmon sperm DNA per ml–1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; 1× Denhardt solution is 0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll). After hybridization, the filters were washed twice at room temperature in 2× SSC–0.1% SDS and three times for 15 min each at 50°C in 0.1× SSC–0.1% SDS.

***C. neoformans* DNA libraries and phagemid rescue.** A genomic DNA library from *C. neoformans* B-3501 was prepared by mechanical shearing of DNA, treatment with T4 DNA polymerase to produce blunt ends, *Eco*RI methylation, addition of *Eco*RI linkers, and ligation of linked DNA into the bacteriophage lambda vector ZAP II (Stratagene) (3, 4).

C. neoformans cDNA libraries were prepared starting with 5 μg of poly(A)$^+$ RNA from strains B-3501 and B-3502. Briefly, double-stranded cDNA was prepared essentially by the method of Gubler and Hoffman (6) except that T4 DNA polymerase was substituted for Klenow fragment to create blunt ends. The double-stranded cDNA was treated with *Eco*RI methylase to protect internal *Eco*RI sites, phenol-chloroform extracted, and precipitated with ethanol. *Eco*RI linkers [pd(GGAATTCC)] were added by ligation, and the cDNA was digested with *Eco*RI. cDNAs ranging in size from 0.6 to 6.0 kilobase pairs (kbp) were fractionated on a 1% low-melting-point agarose gel, isolated from the gel by phenol extraction, and further purified on an Elutip-d (Schleicher & Schuell, Keene, N.H.). The linked cDNA was then ligated into the lambda vector ZAP II, packaged with Gigapack (Stratagene, La Jolla, Calif.), and plated on XL-1B cells. Approximately 10^6 recombinants were obtained from two independent cDNA reactions.

Phagemid were rescued from all libraries by coinfection of *E. coli* XL-1B with 10^7 PFU of lambda phage and 10^{10} PFU of R408 helper phage in 45 ml of LB (25). After 8 h at 37°C, the culture was heated to 70°C for 20 min and centrifuged, and the supernatant containing rescued phagemid was saved. Supernatants had titers of 10^5 to 10^6 ampicillin-resistant CFU/μl.

DNA sequencing. All DNA sequencing was performed on single-stranded M13 templates of restriction fragment subclones on an ABI model 470 DNA sequencer at the Biomolecular Resource Center, University of California, San Francisco.

Clamped homogeneous electric field separation of *C. neoformans* chromosomes. Agarose blocks containing *C. neoformans* chromosomes were prepared by suspending spheroplasts in 1% low-melting-point agarose–0.125 M EDTA. The suspension was pipetted into molds and allowed to harden at 4°C. The blocks were then incubated overnight in 0.5 M EDTA–0.01 M Tris-HCl (pH 7.5)–7.5% β-mercaptoethanol. After being washed three times with 0.05 M EDTA, the blocks were incubated with 1 mg of proteinase K per ml in 0.5 M EDTA–0.01 M Tris-HCl (pH 7.5)–1% *N*-lauroylsarcosine. The blocks were stored at 4°C in 0.05 M EDTA prior to electrophoresis. For electrophoresis, sections of the blocks were placed into a 0.9% agarose gel and electrophoresed for 24 h at 190 V with a linear ramped pulse time from 60 to 200 s in a Bio-Rad Laboratories clamped homogeneous electric field DR2 apparatus. The gels were visualized by ethidium bromide staining, and DNA was transferred to nylon membranes for hybridization analysis.

Electroporation of *C. neoformans*. Cells were grown in 50 ml of YEPD at 30°C to an A_{650} of 0.5 to 1.0 and collected at 5,000 × *g* for 5 min. The cells were washed twice in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and resuspended in 1/10 of the original culture volume with YEPD. DNA (1 to 5 μg) was mixed with 500 μl of cell suspension and placed in a 0.2-cm electroporation cuvette (Bio-Rad). Electroporation settings were 400 V and 500 μF on a Bio-Rad GenePulser, which resulted in a time constant of 10 to 14 ms. The electroporated cells were transferred to a culture tube and incubated for 30 min at 30°C with shaking. Samples were then plated to appropriate selective medium and incubated at 30°C.

Nucleotide sequence accession number. The GenBank accession number for the sequence given in this paper is M34606.

RESULTS

Isolation of OMPPase cDNA clones. In order to facilitate the introduction of the cDNA library into a *pyrE E. coli* strain, it was first necessary to convert the cDNA library into a single-stranded (phagemid) form and to construct an F$^+$ *pyrE* recipient. Both cDNA and genomic DNA libraries prepared in the lambda vector ZAP II were rescued into phagemid by coinfection of *E. coli* XL-1B with the lambda phage library and the helper phage R408 as described in Materials and Methods. *E. coli pyrE* strain CGSC 4501 was mated with XL-1B to create JE16 (*pyrE metB* F$^+$). Rescued phagemids (3×10^5 ampicillin-resistant CFU) were then incubated with 10^8 JE16 cells and plated to a single plate containing uracil-depleted bacterial medium. Ura$^+$ colonies appeared in two independent cDNA libraries at a frequency of 10^{-4} (5 to 30 colonies per plate). The genomic library and mock-transfected controls did not give rise to any Ura$^+$ colonies. Plasmid DNA was isolated from eight of the Ura$^+$ cDNA transfectants, and all contained a ~700-bp *Eco*RI insert consistent with the expected size of a full-length OMPPase cDNA (based on the sizes of the *Podospora anserina* and *Sordaria macrospora* OMPPase genes [16, 26]). Transformation of these plasmid DNAs back into CGSC 4501 and plating to uracil-depleted medium confirmed that uracil prototrophy was truly plasmid mediated.

Sequence of *URA5* cDNA. The cDNA was sequenced on both strands and shown to contain a 675-bp open reading frame preceded by a short 5′ untranslated sequence and followed by 34 bases of 3′ untranslated sequence and a short poly(A) tract. Translation of this open reading frame gives a 225-amino-acid polypeptide which shares 38 and 31% identity with the *Podospora anserina* (26) and *E. coli* (21) enzymes, respectively.

Isolation of the *URA5* gene. Since the goal of this work was to develop genetic markers that would be useful in the transformation of *C. neoformans*, the OMPPase cDNA was used as a probe to isolate clones from the genomic library. Such genomic clones would contain the necessary elements directing the expression of *URA5* and would be expected to be capable of transforming appropriate *ura5* mutants. By using an OMPPase cDNA as a probe, five genomic DNA clones were isolated from the genomic DNA library by screening 50,000 recombinants. The bacteriophage lambda clones were rescued into the double-stranded plasmid form, and one, pURA5g2, was shown by restriction mapping to contain the entire *URA5* gene on a 4.5-kbp *Eco*RI insert (Fig.

1A). The plasmid was incapable of complementing the *E. coli pyrE* strain.

A 2,000-bp *Bgl*II-*Bst*XI fragment containing approximately 600 bp of 5′-flanking and 400 bp of the 3′-flanking regions was sequenced (Fig. 1B). Comparison of the genomic sequence with the cDNA sequence revealed the presence of two introns within the *URA5* coding region, explaining the inability of the cloned gene to complement the *pyrE* mutation. The first intron at +102 was 52 nucleotides in length, and the second one at +383 was 49 nucleotides. Both interrupted the open reading frame and had the typical GTNNGY, where N is any nucleotide and Y is any pyrimidine, at their 5′ splice junctions and typical YAG 3′ splice junctions (19). A strictly conserved branch point, such as the TACTAAC box in *S. cerevisiae*, could not be found, but candidate branch site sequences (CTGAC in intron 1 and CTAAC in intron 2) consistent with the CTRAY, where R is any purine, consensus sequence found in other fungal systems were present (7, 15, 18).

The flanking regions were compared with the two previously described fungal *URA5* genes from *Podospora anserina* (26) and *Sordaria macrospora* (16), and no significant similarities were found. A canonical TATA box was present at −342, and potential CAAT boxes were present at −290 and −141. The distance between the potential TATA box and the cDNA start site as well as the relative orientation of the potential TATA box to the two CAAT boxes suggested that this sequence is not acting as the RNA polymerase entry point. No other sequences that bore significant resemblance to the consensus TATA sequence could be found. The function of such sequences will require direct analysis in *C. neoformans* and transcript mapping before specific roles could be assigned to them. In the 3′-flanking region, there was a short 3′ untranslated sequence present in the cDNA. No canonical polyadenylation signal (AATAAA) was seen. Beyond the poly(A) tract, termination signals characteristic of *S. cerevisiae* could not be identified (28).

Chromosomal localization of the *URA5* gene. *C. neoformans* has recently been shown to contain 10 to 12 chromosomes ranging in size from 500 to 3,000 kbp (20). When clamped homogeneous electric field-separated chromosomes from three strains of *C. neoformans* were hybridized to a *URA5* cDNA probe (Fig. 2A and B), hybridization was detected only to a 1,500-kbp band. This band was particularly intense by ethidium bromide staining, suggesting the presence of more than one chromosome of this size. While we have not yet been able to resolve two chromosomes in this region, careful inspection of the gel and autoradiogram suggest that, at least in strain NIH-424, *URA5* sequences reside on a chromosome of slightly smaller size (lanes 1 of Fig. 2A and B).

Isolation of *ura5* mutants of *C. neoformans*. Cells from 24-h cultures grown on YEPD slants were harvested in sodium phosphate buffer, and 10^7 cells were plated onto 5-FOA-agar plates. After 72 h of incubation at 30°C, spontaneous 5-FOA-resistant mutants appeared at a frequency of 4×10^{-7}. Colonies were streaked to YEPD plates, and single colonies were tested for their uracil requirement by culturing on minimal medium, minimal medium plus uracil, and YEPD. All isolates grew only on the latter two media. The uracil-requiring mutants were further tested for their biochemical lesion by culturing on minimal agar with either orotic acid or orotidine. All isolates grew only on medium supplemented with orotidine, indicating that they lacked OMPPase. Two isolates having low reversion frequencies (less than 10^{-7}), FOA-01-11-2 (from B-3501) and FOA-02-7-2 (from B-3502), were chosen for transformation studies.

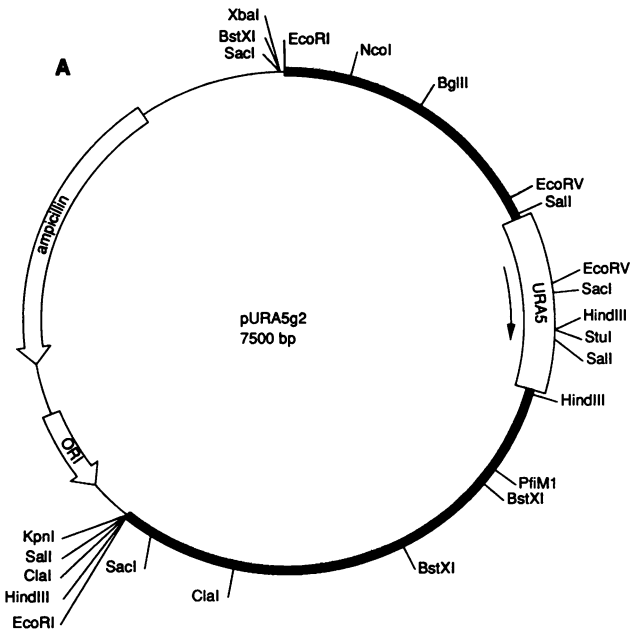

FIG. 1. (A) Map of pURA5g2. Vector sequences are indicated by the thin line. Thick line indicates *C. neoformans* sequences. The position and direction of transcription of the *URA5* gene are indicated. The *Eco*RI sites flanking the insert are not present in *C. neoformans* genomic DNA, but were the result of the library construction method used. (B) Nucleotide sequence of the *C. neoformans URA5* gene. The sequence of a 2,029-bp *Bgl*II-*Bst*XI fragment containing the *URA5* gene is shown. Nucleotide numbers are presented on the right, with the first base of the initiation codon of OMPPase being defined as +1. Two introns are present within the coding region for OMPPase and are presented in lowercase letters. The positions of the start of the cDNA clone and the poly(A) tract are indicated by arrows. A sequence that conforms to the TATA consensus sequence is double underlined, and two potential CAAT boxes are underlined.

Transformation of *C. neoformans*. Electroporation was chosen as a technique for the introduction of DNA into *C. neoformans* because of the general applicability of the technique and previous success for the transformation of *S. cerevisiae*. The parameters described in Materials and Methods were optimized for the transformation of *S. cerevisiae* (10^3 to 10^4 per μg of plasmid 2μm-based vectors; Alan Goldstein and J. C. Edman, unpublished). Although in-depth trials at optimizing *C. neoformans* transformation have not been attempted, the parameters, in terms of voltage and capacitance, appeared to be adequate for *C. neoformans* as well. All transformation studies described here were done with FOA-01-11-2. Similar results were obtained with FOA-02-7-2 but are not shown.

Initial transformation trials were performed with supercoiled pURA5g2. From multiple such transformations, Ura^+ colonies appeared on selective medium at a frequency of 1 to 2 per μg of DNA. No Ura^+ colonies appeared on mock-transformed controls. Since it had been shown in other fungal systems that linearization of plasmid DNA often increases the frequency of integrative transformation, transformations were attempted by using pURA5g2 digested with *Eco*RI (to release the entire insert; note that the *Eco*RI sites that flank the 4.5-kbp insert of pURA5g2 are not natural *Eco*RI sites, but were introduced in the construction of the

B

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GATCTTGGGGATGGTATTGAAGACGATCCAGAAAAAGTCTCTCCATCCCTCCCACGTTCGCTGTTCGGTTTCTTTGCAAAGAGCGAAGTTGCTCGACCTTTGACAGACCCTAAAATCTGGT    -593

CGTATTTTGCGTTCAAAGATCGCTTACTGCGACCGCGATTTTTTAGAGGTGGATTTTTTGTTGCGAGACTAGCGCATCGGATAGGCATGGGTGATATACGTTTGCGTTCCTTATGAGGGGT    -472

ATCATCAGTGGCAAATTACCGACTAGATGAAGAAGACGACCTACACCCCGCTAACCGGTGTCCGATCGACATGATCCACGGTGTCCAAAGCGAAGAAGGACATGATGATTGGAGTTCGCTT    -351

ACCTTTGGTATAAACCAGTGTTGTTTCTGAAGAGGTGAGGAAGAAAATGATCTAGGGCAATAACAGAAATCGTTTGAAGAAGAAGTAAAAGAAAAGACACACGTCAGACAAGCATCATCAT    -230

CATTCATTTTGAATACTCATCACGGCACCTTCTTCCTTGCATGCGCATTATTCATGATGAGATGCTCGCAGCCGCCGGATTTCGGCTGCAATCAAGAACGATATCGCGTACGGCGATGTCC    -109

                                                                                                  |---> cDNA     Met Ser Ser
GCCTCCACTCCATCTTCCAACAACCACCACAGTGCCCGATAACACTACGACGAAAATTGTTCTTTTTTGGATCTTGTCGACAACGGCGACCAAAACAAGAACTTGATC  ATG TCC TCC      +9

Gln Ala Leu Asp Ser Ala Lys Val Ala Phe Ile Glu Ala Ala Ile Glu His Gly Val Leu Leu Phe Gly Asn Phe Thr Leu Lys Ser Gly
CAA GCC CTC GAC TCC GCC AAA GTT GCC TTC ATC GAG GCT GCC ATC GAA CAT GGC GTG CTT CTT TTC GGC AAC TTT ACC TTG AAG TCC GGC      +99

Ar                                                  g Gln Ser Pro Tyr Phe Phe Asn Ala Gly Leu Leu Tyr Ser Ser Ser Leu
CG gtgagccatattgcagcgcttcacagtccaatcgaatctgacatgtgttcag C CAA TCC CCT TAC TTC TTC AAT GCC GGT CTC CTT TAC TCT TCA TCG CTT    +202

Leu Ser Thr Thr Ala Gln Ala Tyr Ala Lys Val Leu Ser Ser Ser Arg Ile Pro Asp Phe Asp Val Leu Phe Gly Pro Ala Tyr Lys Gly
CTC TCA ACT ACC GCT CAG GCT TAC GCC AAG GTA CTT TCC TCT TCT AGG ATT CCT GAC TTT GAC GTC CTC TTC GGC CCA GCT TAC AAG GGT      +292

Ile Ser Leu Ala Ala Val Ser Ala Val Ser Leu Tyr Gln Gln Thr Gly Lys Asp Ile Gly Tyr Cys Tyr Asn Arg Lys Glu Lys Lys Asp
ATC TCC TTG GCT GCT GTC TCC GCT GTA AGC CTT TAT CAG CAA ACC GGC AAA GAT ATC GGC TAC TGC TAC AAC AGG AAG GAG AAG AAG GAC      +382

                                                His Gly Glu Gly Gly Thr Met Val Gly Ala Pro Leu Lys Gly Arg Ile Val
gtgagtctgtcctaaccagtgcgacagcgatgagctcataagccagtag   CAC GGT GAG GGC GGT ACT ATG GTC GGT GCG CCT CTC AAG GGA CGA ATC GTC      +482

Ile Ile Asp Asp Val Leu Thr Ser Gly Lys Ala Ile Arg Glu Ala Ile Asp Ile Leu Lys Ala Ser Pro Glu Ala Lys Leu Val Gly Ile
ATC ATC GAC GAT GTT CTC ACC TCT GGC AAG GCC ATC CGT GAA GCT ATT GAC ATT CTC AAG GCC TCC CCT GAA GCG AAG CTT GTC GGA ATT      +572

Val Gln Leu Val Asp Arg Gln Glu Lys Gly Gln Ser Gly Ser Gly Lys Ser Thr Val Gln Glu Val Glu Glu Glu Phe Gly Val Pro Val
GTC CAG CTT GTC GAC AGA CAA GAG AAA GGC CAG AGC GGT AGC GGC AAG AGT ACC GTA CAG GAG GTT GAG GAA GAG TTC GGT GTG CCT GTC      +662

Glu Pro Ile Ile Gly Leu Asp Asp Ile Val Lys Tyr Leu Glu Ser Ser Gly Lys Trp Glu Lys Glu Leu Gln Glu Val Arg Lys Tyr Arg
GAG CCT ATT ATT GGT TTG GAC GAC ATT GTG AAG TAC TTA GAA AGC TCC GGC AAG TGG GAA AAG GAG CTG CAA GAG GTC AGG AAG TAC AGG      +752

Ala Glu Tyr Gly Val Gln Arg Ser OCH                                          |---> polyA
GCG GAG TAC GGT GTT CAG AGG TCT TAA GGGTTTTCTTCTTAAATGCACGGGTTTAGGTCTAGCTAATCAAGTTCCGACATATTACAAGTTTGTAAGCTTGTATCAAAGGAAC    +864

TTAAGTACAGGCAGGCGTCCTGAGGCGACAAAGGAAGCTGTAATATGATTGTTGGCTGTCAATCTTCATCGTATCTACTTTGTCAATACTGACTTCAATGACCCAATAATACAATTTTATT    +985

AGTGTTGACCCAGAATGGTTAGCAGGAAACTCCCCTTCTCTTCCTCTCAATCCCAATCATACTTCATATCTCCTGCTCCCCCCATTTCCGTCTTCCTCGATGACTCCCTGGTCCCATCCCT    +1106

CCCACCTCCTGGAGGCAAGCTGGAGCACCTGGACCTAATGGGTCGTCGCCCCAAATTGCCTCCACCTCTTAAGACAATCATCGTCCAATCAAACTCTGAACTATCTTCCAAGCCAATGGCC    +1227

GGATCTGGACACAAGATGATGTCGAAGCCGCTTGATGTGCCAGGGTTGGTCCGTGGCCTGGAGACGTAAGAGCGGGTAGTACTGGGATC
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FIG. 1—*Continued.*

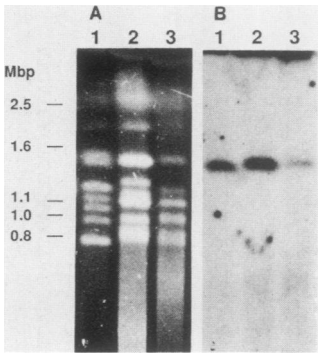

FIG. 2. Chromosomal localization of the *URA5* gene. (A) Ethidium bromide-stained gel showing chromosomes of *C. neoformans* isolates NIH-424 (lane 1), NIH-12 (lane 2), and NIH-433 (lane 3). B-3501 (not shown) is a progeny of NIH-12 × NIH-433 and has a banding pattern identical to that of NIH-12. (B) Nylon membrane blot of the gel shown in panel A hybridized to ^{32}P-labeled *URA5* cDNA probe. The migration of *S. cerevisiae* chromosome markers (in megabase pairs) is indicated on the left.

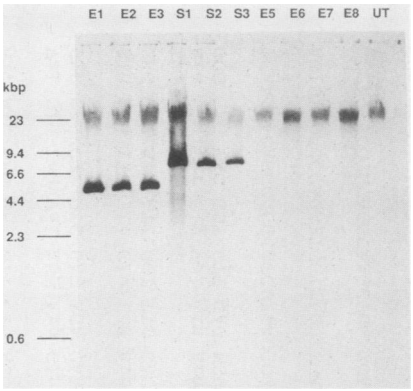

FIG. 3. Southern blot analysis of undigested DNA from Ura$^+$ transformants. Lanes E1 to E3, Undigested DNA from three unstable transformants obtained with *Eco*RI-digested pURA5g2. Lanes S1 to S3, Undigested DNA from three unstable transformants obtained with supercoiled pURA5g2. Lanes E5 to E8, Undigested DNA from four stable transformants obtained with *Eco*RI-digested pURA5g2. Lane UT, Undigested DNA from strain B-3501. The migration of molecular size markers is shown on the left. The entire plasmid pURA5g2 was used as a probe.

library) or with *Bgl*II (which cleaves once in the plasmid, leaving vector sequences attached to the *URA5* sequences). Transformation with either of these digests resulted in 50- to 100-fold stimulation of the transformation frequency.

The mitotic stability of transformants obtained with supercoiled pURA5g2 was assessed by growing isolated transformants in nonselective medium for 15 to 20 generations and testing single colonies for retention of uracil prototrophy. Eight of eight transformants tested proved to be unstable (0 to 3% of colonies retained uracil prototrophy). Undigested DNA from three supercoiled pURA5g2 transformants (S1, S2, and S3) was analyzed by Southern blot. In all transformants examined, sequences of ~8 kbp hybridizing to a pURA5g2 probe (containing both plasmid and *URA5* sequences) were present below the bulk of genomic DNA in (Fig. 3, lanes S1 to S3). The nature of these extrachromosomal DNAs and the state of the resident *ura5* locus were examined by digestion with *Eco*RI or *Stu*I. Untransformed cells contained *URA5* sequences on a single 12-kbp *Eco*RI fragment and two *Stu*I fragments of 4.3 and 4.4 kbp (Fig. 4A and B, lane UT). If the extrachromosomal sequences in the supercoiled-plasmid transformants were unmodified plasmid DNA, then one would expect *Eco*RI fragments of 4.5 kbp (insert) and 3.0 kbp (vector). In transformant S1, such fragments were seen (Fig. 4A, lane S1). *Stu*I digestion of pURA5g2 would result in a single 7.5-kbp fragment containing vector and *URA5* sequences. *Stu*I digestion of transformant S1 generated two novel fragments of 6.2 and 2.3 kbp, indicating that the extrachromosomal sequences were no longer identical to pURA5g2 and underwent some alteration in the transformation process. The two other supercoiled-plasmid transformants (S2 and S3) also underwent substantial alterations. Upon *Eco*RI digestion, both gave a 5.0-kbp fragment, but also contained two smaller *Eco*RI fragments which differed in the two isolates (Fig. 4A, lanes S2 and S3). The *Stu*I digests demonstrated the extensive alterations in the extrachromosomal sequences, with additional *Stu*I sites appearing in transformants S2 and S3 (Fig. 4B, lanes S2 and S3). It is presumed that the extrachromosomal sequences in the supercoiled-plasmid transformants retain a circular structure, but given the extensive alterations and new restriction sites, a linear structure cannot be ruled out. The source of the extrachromosomal sequences could be either freely replicating plasmid, the end product of recombination to eliminate integrated plasmid sequences, or the result of plasmid-plasmid recombination. Multiple attempts to rescue plasmid in *E. coli* have failed, again suggesting that substantial rearrangements have occurred within the extrachromosomal sequences.

Transformants obtained with *Eco*RI-digested pURA5g2 could be divided into two classes. Of the 26 transformants, 22 were highly unstable, while 4 of 26 were completely stable. Southern analysis of three unstable *Eco*RI transformants (E1, E2, and E3) revealed the presence of extrachromosomal *URA5* sequences in undigested samples (Fig. 3, lanes E1 to E3). Digestion with *Eco*RI resulted in no apparent shift in molecular weight (Fig. 4A, lanes E1 to E3). These data suggest that the introduced DNA has been recircularized with the loss of the *Eco*RI sites or that the transforming DNA has remained linear. Digestion with *Stu*I gave two fragments of 1.8 and 2.7 kb (Fig. 4B, lanes E1 to E3). This would suggest that, in the absence of substantial rearrangements, the transforming DNA has retained a linear structure. *Sac*I digestion also gave a pattern consistent with a linear DNA fragment (data not shown). As rearrangements may have occurred in the transforming DNA, it is not possible with restriction enzyme analysis to absolutely differentiate between circular or linear extrachromosomal *URA5* sequences in these transformants. Further definition of the nature of these extrachromosomal DNAs will require exonuclease mapping.

The stable transformants obtained with *Eco*RI-digested pURA5g2 demonstrated no extrachromosomal *URA5* sequences (Fig. 3, lanes E5 to E8). The mechanism of transformation in these cases must therefore be integrative. Two modes of integrative transformation are possible, ectopic integration and homologous integration into the resident *ura5* locus. *Eco*RI digestion of DNAs from the stable transformants revealed that in three of four cases, novel *Eco*RI fragments and novel *Stu*I fragments hybridizing to the *URA5* probe were present (Fig. 4A and B, lanes E6 to E8). The resident *ura5* locus appeared to be unmodified in these three transformants; therefore, these patterns are consistent with ectopic integration. Transformant E5 had a pattern identical

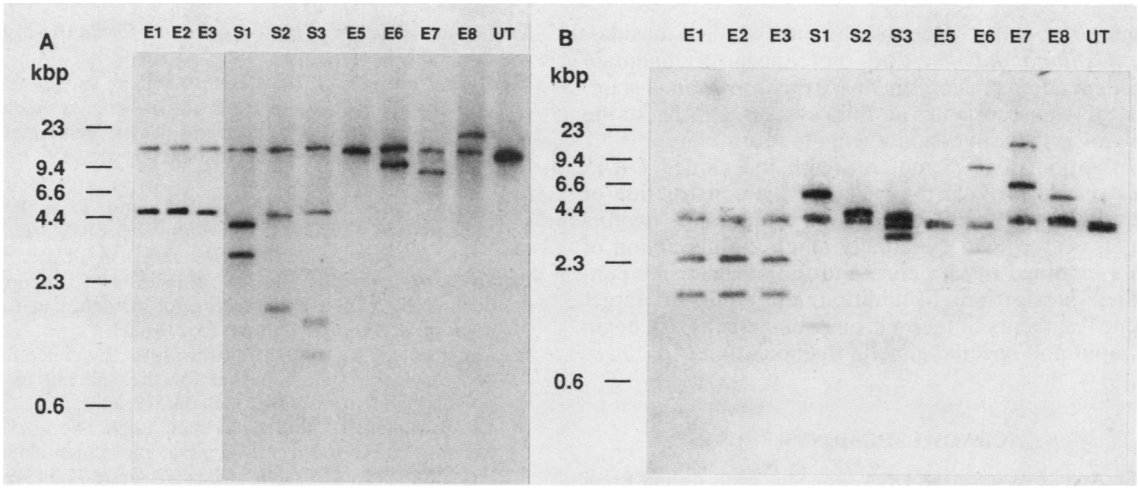

FIG. 4. Southern blot analysis of restricted DNA from Ura$^+$ transformants. (A) *Eco*RI digests and (B) *Stu*I digests. With the same DNA samples as in Fig. 3, lanes are ordered exactly as described in the Fig. 3 legend. The entire plasmid pURA5g2 was used as a probe.

to that of the untransformed strain (Fig. 4A and B, lane E5). This suggests that a gene replacement event has occurred at the *ura5* locus. It remains possible that this transformant represents a revertant; however, revertants have never been observed on multiple control transformations.

DISCUSSION

C. neoformans is an important human fungal pathogen with a cosmopolitan distribution. Cryptococcosis, long associated with immunosuppressed patients, has taken on added importance with the emergence of AIDS. Little is known, however, of the genomic structure and mechanisms of genetic control in *C. neoformans*. Such information is crucial to the understanding of the mechanisms of virulence.

Transformation of fungi has been reported in a wide variety of systems (5). Uracil auxotrophs have often been used as recipients because of the ease of isolation of appropriate mutants and the ability of fungal genes to complement cognate mutations in *E. coli*. In *C. neoformans*, direct complementation of *E. coli pyrE* mutants was not achieved with genomic libraries due to the presence of two introns in the *URA5* gene. However, multiple complementing clones were isolated by construction of a cDNA library in a bacterial expression vector. This suggests that the utility of isolating other metabolic markers by complementation of cognate *E. coli* or *S. cerevisiae* mutants by genomic *C. neoformans* libraries may be limited.

5-FOA has been commonly used to select for uracil auxotrophs in yeast cells. In *S. cerevisiae*, 5-FOA-resistant isolates are predominantly *ura3* mutants lacking orotidine monophosphate decarboxylase (2). Mutations in OMPPase (*ura5*) are infrequently isolated, and the mutants possess a leaky phenotype. In *C. neoformans*, however, all the uracil auxotrophs obtained with 5-FOA selection were *ura5* and they did not exhibit a leaky phenotype. The leakiness of *ura5* mutants of *S. cerevisiae* has been attributed to the presence of a second gene encoding OMPPase (*URA10*; J. De Montigney, doctoral thesis, L'Université Louis Pasteur, Strasbourg, France, 1988). As *C. neoformans ura5* mutants are not leaky, it is unlikely that a second gene for OMPPase is present.

Integrative transformation can occur either in homologous chromosome regions or at ectopic sites. While three of the four *C. neoformans* integrants appeared to be ectopic, gene replacement/conversion probably occurs, as indicated by the stable transformant with no apparent ectopic integration sites.

Autonomously replicating sequence (ARS)-dependent plasmids generally (i) transform at high efficiency, (ii) show no propensity for rearrangement, (iii) demonstrate mitotic instability, and (iv) do not increase in transformation efficiency upon linearization (5). The cloned segment of *C. neoformans URA5* DNA that we have described possesses only one of these features, mitotic instability. Although it is too early in the development of transformation systems for *C. neoformans* to state that the efficiency of transformation with pURA5g2 is low, it is at the level seen with non-ARS-dependent plasmids in other fungal systems (1 to 10 per μg) and clearly increases with linearization of the transforming sequences. The increased frequency observed with linearization, however, it not the result of an increased frequency of integration. Rather, linearization is acting by some other mechanism (DNA uptake?) to stimulate transformation. Although it does not appear that the *URA5* gene fragment described in this study contains a true ARS by the definition above, it is clearly capable of some degree of autonomous replication. Determination of whether this is an intrinsic property of the fragment or is acquired during the transformation process must await the isolation of true ARS sequences from *C. neoformans*.

Non-ARS-dependent maintenance of plasmid DNA within transformed fungi has been reported for the ascomycete *Schizosaccharomyces pombe* (8, 27). In this case, the nature of such maintenance may be the result of unstable homologous and ectopic chromosome integration events. Aberrant excision of integrated plasmids sometimes results in the acquisition of sequences containing an ARS and capable of autonomous replication. Alternatively, plasmids can have limited autonomy but tend to demonstrate a high frequency of rearrangement or the assumption of polymeric forms (8). It is possible that a similar mechanism occurs in *C. neoformans*. This would be consistent with the rearrangements seen in the unstable transformants. Detailed analyses of the altered extrachromosomal sequences may resolve the nature of the rearrangements and potentially allow the isolation of ARS sequences from *C. neoformans* itself.

Transformation of *C. neoformans*, at least with the *URA5* gene, therefore appears to resemble transformation in the

ascomycete *S. pombe*. One of the best-studied basidiomycetes, *Ustilago maydis*, does not appear to maintain non-ARS-dependent plasmids in an extrachromosomal state. The bulk of transformants in this system appear to be integrative or gene conversion events (1, 10).

Transformation of *ura5* mutants with the cloned *URA5* sequences should provide the basis for the construction of stable extrachromosomally replicating vectors for *C. neoformans*. Such vectors will eventually allow the dissection of the factors involved in virulence and the mechanisms controlling their production. In addition, the ability to target cloned gene fragments to their chromosomal locus will begin to allow rapid and specific genetic manipulation of *C. neoformans*.

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