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Gene Transfer in *Cryptococcus neoformans* by Use of Biolistic Delivery of DNA

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A transformation scheme for *Cryptococcus neoformans* to yield high-frequency, integrative events was developed. Adenine auxotrophs from a clinical isolate of *C. neoformans* serotype A were complemented by the cryptococcal phosphoribosylaminoimidazole carboxylase gene (*ade2*) with a biolistic DNA delivery system. Comparison of two DNA delivery systems (electroporation versus a biolistic system) showed notable differences. The biolistic system did not require linear vectors and transformed each auxotrophic strain at similar frequencies. Examination of randomly selected transformants by biolistics showed that 15 to 40% were stable, depending on the recipient auxotroph, with integrative events identified in all stable transformants by DNA analysis. Although the *ade2* cDNA copy transformed at a low frequency, DNA analysis found homologous recombination in each of these transformants. DNA analysis of stable transformants receiving genomic *ade2* revealed ectopic integration in a majority of cases, but approximately a quarter of the transformants showed homologous recombination with vector integration or gene replacement. This system has the potential for targeted gene disruption, and its efficiency will also allow for screening of DNA libraries within *C. neoformans*. Further molecular strategies to study the pathobiology of this pathogenic yeast are now possible with this transformation system.

Cryptococcus neoformans is a pathogenic yeast with an unexplained predilection for infecting the central nervous system. There has been a significant increase in the number of human infections with this yeast over the last decade as the immunocompromised population has enlarged because of AIDS. Although epidemiological studies suggest that the increase in cryptococcal infection is due to more-susceptible hosts rather than increased virulence, the pathobiology of the organism needs further investigation. Several phenotypic factors associated with virulence have been identified for *C. neoformans*: the polysaccharide capsule (10, 16, 20), melanin production (19, 20, 26), and growth at 37°C (20, 26). Their importance to the survival of the yeast in the host is supported by genetic and animal model studies. Identification of the gene(s) associated with these phenotypes and understanding its (their) regulation require successful molecular manipulation of this yeast.

Site-directed mutagenesis is a powerful molecular method to determine the significance of cloned genes in relationship to the pathobiology of the parasite. This technique requires homologous integration, resulting in gene replacement or disruption by an altered gene. For example, directed mutations in a putative virulence gene in *C. neoformans* could be examined for the resulting mutants' abilities to produce infection in a standard animal model system. Transformation studies of vector DNA in other fungi have demonstrated a wide range of frequencies of homologous integration. The rate is nearly 100% in *Saccharomyces cerevisiae* (22) and *Candida albicans* (17), 80% in *Aspergillus nidulans* (34), and from 5 to 80% in *Neurospora crassa* depending on the host strain (5, 7, 15) but only 3% in *Sordaria macrospora* (21). Although the frequencies of these homologous recombination events may vary with conditions and strains, it seems likely that homologous integration could occur with significant frequency in pathogenic strains of *C. neoformans*.

Recent studies of *C. neoformans* have established basic molecular techniques for performing site-directed mutagenesis (9, 31). A *C. neoformans ura5* auxotrophic strain (serotype D) was recently reported to be transformed at a low efficiency by complementation with a cloned *ura5* gene delivered by electroporation. However, the number of integrative events was low and generally nonhomologous (9, 31). A total of 80 to 90% of the transformants were unstable and apparently contained extrachromosomal DNA, consisting of sequences of rearranged *ura5*-vector DNA with the addition of cryptococcal genomic DNA (8, 9, 31). Subsequent studies with this system, with various vector constructs, have greatly improved the efficiency of transformation; but the majority of transforming DNA remains extrachromosomal (8). This transformation system can be utilized to overexpress or complement genes but lacks the ability to directly alter or replace genes. Transformants may be too unstable for satisfactory testing of their virulence in vivo. We therefore attempted another approach by transforming a pathogenic *C. neoformans* strain (serotype A) with a different DNA delivery system and complementing gene. In the present study, we have successfully applied a biolistic system to deliver the cryptococcal *ade2* gene (phosphoribosylaminoimidazole carboxylase) and complement *ade2* auxotrophs. Through this system, a satisfactory rate of homologous recombination was achieved. This scheme should allow for successful gene replacement experiments in *C. neoformans*.

MATERIALS AND METHODS

Strains and media. *C. neoformans* strains were H99, a serotype A clinical isolate (23), and B-3501, a serotype D

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isolate provided by K. J. Kwon-Chung. The *ade2* auxotroph, *αade2-27* (from B-3501), was provided by J. Edman. *ade1* auxotrophic *Escherichia coli* H642 (*thr-1 leuB6 tsx-71 purC60 rpsL126 metβ1*) and *ade2* auxotrophic *E. coli* X148 (*ara-14 leuB6 azi-6 fhuA23 lacY1 tsx-67 purE42 supE44 galK2 λ⁻ trpE38 rfbD1 mgl-51 rpsL109 xly-5 mtl-1 thi-1*) were obtained from the *E. coli* Genetic Stock Center, Yale University. *E. coli* K-12 (TG1) (27) and *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) were used for propagation of plasmids.

Nonselective medium was YEPD (1% yeast extract, 1% Bacto Peptone, and 2% dextrose). Adenine-depleted medium for *C. neoformans* contained 6.7 g of yeast nitrogen base without amino acids per liter, 20 g of glucose per liter, 20 mg of uracil per liter, and the following amino acids (milligrams per liter): leucine (600), threonine (200), phenylalanine (50), tyrosine and lysine (30 each), and tryptophan, histidine, arginine, and methionine (20 each). Adenine-depleted medium for *E. coli* was Bacto minimal agar supplemented with 200 mg of threonine, 60 mg of leucine, 50 mg of thiamine, and 20 mg each of methionine and tryptophan per liter. *E. coli* cells were propagated on LB (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) agar plates, pH 7.5, containing 50 μg of ampicillin per ml. Solid medium was supplemented with 20 g of Bacto agar per liter.

Isolation of RNA and DNA from *C. neoformans*. Total RNA and DNA were isolated from mid- to late-log-phase H99 cells cultured in YEPD. First, the cells were pelleted and washed three times in 500 mM NaCl–50 mM EDTA. For RNA isolation, the washed cells were quick-frozen as damp pellets in microcentrifuge tubes (USA/Scientific Plastics, Ocala, Fla.) and stored at −70°C. Total RNA was extracted by vortexing each thawed pellet of cells (10^9) with glass beads (diameter, 0.45 μm) in the presence of acid-guanidinium thiocyanate-phenol-chloroform (6). Poly(A^+) RNA was selected by affinity chromatography on oligo(dT)-cellulose (3). Typically, 400 μg of total RNA was recovered from 10^9 *C. neoformans* cells, and 2 to 3% of the total RNA applied to the oligo(dT)-cellulose column was recovered as poly(A^+) RNA. The RNA was quantitated by its A_{260} reading on a spectrophotometer (Shimadzu Scientific Instruments, Columbia, Md.).

For DNA isolation, the washed cells from a 50-ml culture were resuspended in 5 ml of 100 mM NaCl–50 mM EDTA–1% β-mercaptoethanol. The suspension was incubated at 37°C for 1 h with occasional shaking, pelleted, and resuspended in 5 ml of spheroplasting solution (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 0.1 M EDTA [pH 8]) containing 5 mg of lysing enzyme per ml from *Trichoderma harzianum* (Sigma Chemical Co., St. Louis, Mo.). After incubation at 37°C for 30 min to 1 h, the cells were pelleted at 100 × *g*, washed in spheroplasting solution, and resuspended in 2 ml of lysis buffer (distilled deionized water, 2% sodium dodecyl sulfate), and the suspension was incubated for 5 min at room temperature with constant agitation. The DNA was extracted twice with phenol-chloroform (1:1) and then precipitated with 2 volumes of absolute ethanol. The DNA was recovered by centrifugation and resuspended in Tris-EDTA (TE) (pH 8), and the suspension was incubated with 0.1 mg of RNase A per ml at 37°C for 30 min and extracted with phenol-chloroform. The ethanol-precipitated DNA pellets were resuspended in TE (pH 8), and the suspension was incubated with a final concentration of 1% cetyltrimethylammonium bromide in 200 mM NaCl at room temperature for 30 min to 1 h. The DNA in the precipitate was recovered by centrifugation, resuspended in 200 μl TE (pH 8), extracted with phenol-chloroform, precipitated with 2 volumes of absolute ethanol, and stored at −20°C. The DNA yield was measured by a TKO-100 minifluorometer (Hoefer Scientific Instruments, San Francisco, Calif.) and was approximately 50 μg/50-ml culture.

***C. neoformans* cDNA library construction.** A cDNA library from *C. neoformans* H99 was prepared from 10 μg of poly(A^+) RNA (The Librarian II; Invitrogen, San Diego, Calif.) according to manufacturer's protocol by a modification of the method described by Gubler and Hoffman (12). The cDNA was ligated into the vector pcDNA II, and *E. coli* TG1 cells were transformed with vector DNA by electroporation at a setting of 2.5 kV, 129 Ω, and 5-ms pulse length with an Electro cell manipulator 600 (Biotechnologies & Experimental Research, Inc. [BTX], San Diego, Calif.). One hundred twenty thousand recombinants, of which 80% carried DNA inserts of >500 bp, were generated. After cDNA library amplification, the plasmid DNA was isolated by polyethylene glycol (8,000 Da) and stored at 4°C in TE.

Generation and identification of *ade2* auxotrophs from H99. *C. neoformans* mutants were generated by exposure to UV or gamma irradiation adjusted to doses which allowed a 10% survival rate. Survivors were replica plated on Bacto minimal agar and YEPD. Colonies growing only on YEPD were replica plated on an array of nutrient-depleted media. Mutants were chosen that were deficient only in the adenine pathway and that possessed a red phenotype, indicating a defective *ade1*, *ade2* (18), and/or *ade8* locus (14). Two red *ade* auxotrophs were produced by UV irradiation and were designated M001 and M027; two red *ade* auxotrophs were also produced by gamma irradiation and were designated M049 and M098. The reversion rate of each *ade* mutant was less than 10^{-8}. The *ade2* auxotrophs were later identified by complementation with the cryptococcal *ade2* copy of either cDNA (M001) or genomic DNA (M027, M049, and M098) with the biolistic system for DNA delivery as detailed in the following sections.

Transformations. *C. neoformans* cells were prepared for electroporation as described previously by Varma et al. (31) and were prepared for biolistics by using an adaptation of a method described for *S. cerevisiae* (2). Briefly, cells from an early-stationary-phase culture in YEPD were diluted 1:50 in fresh YEPD and grown with vigorous shaking at 30°C for either 5 to 6 h (for electroporation) or 18 to 24 h (for biolistics).

For electroporation, the cells were pelleted, washed twice in an equal volume of electroporation buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$ [pH 8.0]) containing 1 mM dithiothreitol, and resuspended in electroporation buffer without dithiothreitol (0.01 volume of the YEPD broth culture). Approximately 2 × 10^7 cells (50 μl) were mixed with 100 ng of DNA (2 μl), and the mixture was transferred to a 2-mm gap cuvette (BTX) and electroporated (470 V, 50 μF, 480 Ω), generating a pulse length of 20 to 24 ms. The electroporated cells were then transferred to adenine-depleted agar and incubated at 30°C.

For biolistics, the cells were collected by centrifugation and resuspended in regeneration medium (0.2 volume of YEPD broth culture) (2), and 200 μl (10^8 cells) was spread on adenine-depleted agar supplemented with 1 M sorbitol. These plates were then bombarded with DNA-coated tungsten beads in a vacuum chamber with a gas pressure of 1,000 lb/in^2 with a helium-driven biolistic system (28) and then incubated at 30°C for 3 to 5 days.

E. coli strains were transformed with plasmid DNA by the $CaCl_2$ method described by Alexander (1) or by electropo-

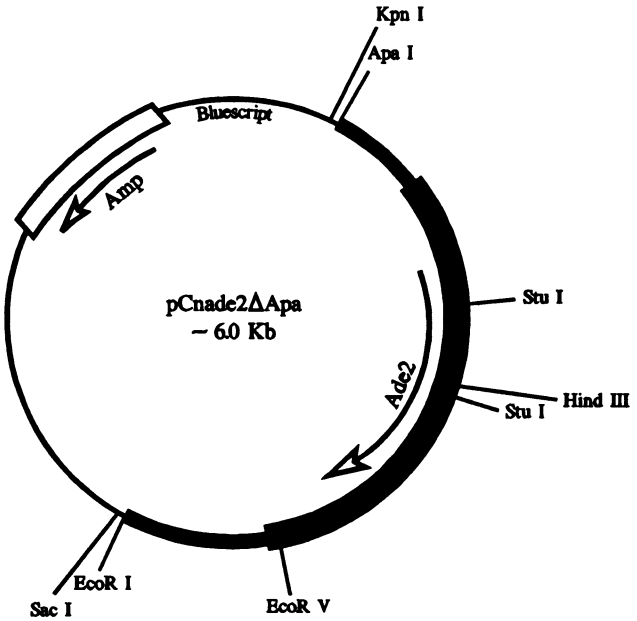

FIG. 1. Circular restriction map of the transforming plasmid pCn*ade2ΔApa*, which contains an ≈3.0-kb insert of genomic *ade2* from B-3501 cloned between the *Apa*I and *Eco*RI sites of Bluescript. The thick lines represent the genomic DNA insert, which contains the cryptococcal *ade2* gene and flanking DNA. The thin line represents the Bluescript plasmid DNA.

ration (2.5 kV, 129 Ω, 5.5-ms pulse length) for *E. coli* MC 1061 and LE 392 as described by the manufacturer (BTX). To screen for adenine complementation, *E. coli* transformants were washed once in phosphate-buffered saline, pH 7.4, before being spread on adenine-depleted plates.

Mitotic stability of transformants. Randomly selected adenine transformants were subcloned on adenine-depleted plates and incubated at 30°C for a minimum of 3 days. From the transformants showing growth, a mixture of several colonies of each transformant was subcloned on YEPD agar repeatedly for four to six passages. Also, single-colony transformants were resuspended in YEPD broth (10^4/ml) and allowed to grow for 14 to 16 generations at 30°C with vigorous shaking before being plated on YEPD agar. The stability of the adenine prototrophy in these colonies was assessed by retention of the white phenotype on YEPD plates. In contrast, no growth on adenine-depleted plates and the appearance of a pink to red phenotype in colonies on YEPD agar indicated instability of adenine prototrophy. All red colonies on YEPD plates were subcloned on adenine-depleted plates for confirmation of gene loss.

Southern blot analysis of transformants. Approximately 1 to 3 μg of genomic DNA from H99, B-3501, M001, and selected stable M001 transformants was digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel. The gel was processed as described by Reed and Mann (25). DNA was transferred to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.) and cross-linked to the membrane by UV irradiation (UV Stratalinker; Stratagene). Hybridization was carried out at 65°C overnight in a hybridizer chamber (Techne, Princeton, N.J.). After hybridization, the blots were washed twice in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at room temperature for 10 min, once in 2× SSC at 65°C for 1 h, and once in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C for 1 h. Autoradiography was performed with Kodak-XAR 5 film.

The DNA probe was a 980-bp *Hin*dIII-*Eco*RV restriction fragment of the *ade2* gene and was prepared by oligolabelling (Pharmacia, Piscataway, N.J.) with [^{32}P]dCTP (New England Nuclear, Boston, Mass.). The entire *ade2* gene and flanking sequences were cloned into the *Apa*I and *Eco*RI restriction sites of Bluescript SK by J. Edman (pCn*ade2ΔApa*; Fig. 1). To verify that the stable transformants contained only integrated transforming *ade2* and not extrachromosomal plasmids, Southern blots of undigested genomic DNA were also probed with the genomic 980-bp *ade2* fragment.

RESULTS

Isolation of the *ade1* and *ade2* cDNA genes. An amplified cDNA library (400 ng) was introduced into *E. coli* H642 (3×10^9) and *E. coli* X148 (3×10^9) by electroporation, and *ade*$^+$ colonies were selected on adenine-depleted plates. *ade*$^+$ colonies appeared at a frequency of 1 in 10^{-5} to 10^{-6} transformants, with no background reversion noted. Plasmid DNA was isolated from five transformants from each of the two *E. coli* strains. The H642 and X148 transformants harbored a consensus plasmid containing an insert of similar size (1,800 bp). Retransformation of the auxotrophic *E. coli* with the isolated plasmids again generated confluent colonies on adenine-depleted medium, thus verifying that adenine prototrophy was plasmid mediated. The *ade2* cDNA (pCn*ade2c*) is diagrammed in Fig. 2.

Identification of *C. neoformans ade2* auxotrophs by complementation. Biolistics was used to introduce either circular or linear DNA into each of the five *C. neoformans ade* auxotrophs plated on adenine-depleted medium (Table 1). The cDNA copies of *ade1* and *ade2* (Fig. 2) were linearized with *Xho*I, and the genomic copy of *ade2* (Fig. 1) was linearized

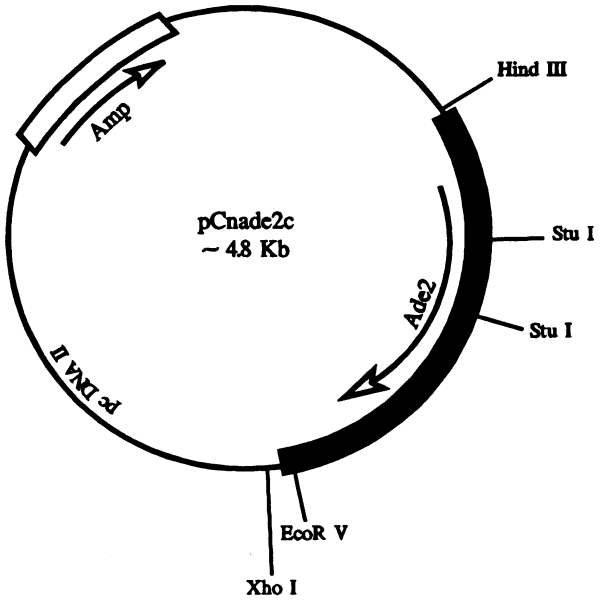

FIG. 2. Circular restriction map of the transforming plasmid pCn*ade2c*, which contains an 1,822-bp cDNA insert from H99 ligated to *Bst*XI nonpalindromic linkers and cloned between the *Bst*XI sites of pcDNA II. The *Bst*XI sites are no longer present. The thick line represents the cryptococcal cDNA insert. The thin line represents the pcDNA II plasmid DNA.

TABLE 1. Transformation efficiency of *C. neoformans ade* auxotrophs[a]

Method of delivery of DNA	No. of colonies per plate	
	αade2-27 (serotype D)	M001, M027, M049, and M098 (serotype A)
Biolistics		
cDNA *ade2*[b]		
Linear or circular[c]	ND	20–30
Genomic *ade2*[d]		
Linear or circular[c]	>1,000	>1,000
Electroporation		
Genomic *ade2*[b]		
Linear	100–400	0
Circular	1–2	0

[a] ≤1 colony per plate in the untransformed controls.
[b] 100 ng of DNA.
[c] Linear and circular plasmid DNAs containing the *ade2* gene showed similar results.
[d] 400 ng of DNA.

TABLE 2. Mitotic stability of biolistic transformation[a]

Transformants (serotype)	No. stable/total	%
M001 (A)		
cDNA *ade2*	32/32	100
Genomic *ade2*	37/103	35.9
M027 (A)		
Genomic *ade2*	16/102	15.7
M049 (A)		
Genomic *ade2*	43/107	40.2
M098 (A)		
Genomic *ade2*	24/99	24.2
αade2-27 (D)		
Genomic *ade2*	20/114	17.5

[a] Transformants generated from circular and linear DNAs were pooled.

with *Hin*dIII or *Eco*RI. Both linear and circular *C. neoformans ade2* cDNAs transformed M001 to adenine prototrophy, each generating 20 to 30 colonies per plate (Table 1). In contrast, no colonies were observed with either circular or linear *ade1* cDNA or in the untransformed controls (data not shown). All adenine auxotrophs (M001, M027, M049, M098, and *αade2-27*) were transformed to adenine prototrophy by both circular and linear genomic *ade2* (Table 1). In contrast to only 20 to 30 transformants for *ade2* cDNA, the genomic *ade2* generated 10^3 to 10^4 colonies per plate. Untransformed controls and those receiving plasmid without insert exhibited no adenine prototrophy.

Comparison of transformation efficiencies by biolistics and electroporation. Two DNA delivery systems, electroporation and biolistics, have now been reported to successfully transform *C. neoformans*. We have found that by biolistics, both circular and linear DNAs generated similar transformation efficiencies (1,000 colonies per plate; Table 1). In other studies, when electroporation was used, only linear DNA (*ura5*) generated significant transformation efficiencies (100 colonies per μg of DNA), whereas circular DNA produced just 0 to 30 transformants per μg of DNA (9, 31). These findings have also been verified in our laboratory (data not shown). However, high-efficiency transformation of *C. neoformans* by electroporation (200 colonies per μg of circular DNA; 90,000 colonies per μg of linear DNA) has been reported, but only when the vector contained telomeric sequences from *C. neoformans* (8). Because electroporation was successful in transforming serotype D *ura5* mutants from strain B-3501 to uracil prototrophy (9, 31), we next examined whether *ade2* mutants from the virulent serotype A H99 strain and also from strain B-3501 were receptive to transformation with electroporation. Both linear and circular genomic *ade2* failed to transform M001, M027, M049, and M098 when electroporation was used for DNA delivery (Table 1). In contrast, complementation was observed with the *αade2-27* mutant from the serotype D strain when the same DNA concentration and electroporation settings were used (Table 1). Linear genomic *ade2* generated 100 to 400 colonies per plate for *αade2-27* (1,000 to 4,000/μg of DNA), whereas only 1 to 2 colonies (10 to 20/μg of DNA) were observed with circular genomic *ade2*.

Mitotic stability of biolistically induced transformants. Thirty-two transformants (M001) receiving *ade2* cDNA and approximately 100 transformants of each auxotrophic strain receiving the genomic *ade2* were assessed for mitotic stability (Table 2). A total of 100% of M001 transformants generated with the cDNA copy were stable, compared with 35.9% generated with the genomic DNA copy. A similar stability rate with genomic *ade2* was observed with M049 (40.2%). M098, M027, and *αade2-27* transformants exhibited slightly lower stability rates of 24.2, 15.7, and 17.5%, respectively (Table 2). Both circular and linear genomic *ade2* generated similar numbers of stable transformants. A total of 90 to 100% of the unstable transformants, as assessed by their pink- to red-colony phenotype, were detected after the first passage on YEPD agar plates. The remaining 10% were detected by the sixth passage on YEPD agar. Sixty-five percent of the randomly selected unstable transformants did not grow on their initial passage to adenine-depleted medium. An additional 20% lost their ability to grow on adenine-depleted medium after four to six passages. Although they possessed a pink- to red-colony phenotype on YEPD agar after six passages, 15% of the unstable transformants retained their ability to grow on adenine-depleted medium. This is likely due to the inoculum, which came from one colony and contained transformants that had not yet lost the *ade2* gene.

Southern blot analysis of stable M001 transformants. Both undigested and *Hin*dIII-digested genomic DNAs from stable M001 transformants were examined by Southern blots. When undigested DNAs from selected transformants receiving either the *ade2* genomic or the cDNA copy were probed with part of the *ade2* gene, only the high-molecular-weight DNA region contained the signal. There was no evidence of extrachromosomal transforming DNA in the stable transformants (data not shown). Also, all *Hin*dIII genomic digests were complete, as shown by an ethidium bromide-stained gel.

Eleven transformants receiving the *ade2* cDNA (circular or *Xho*I restricted) were digested with *Hin*dIII and probed with the genomic 980-bp *ade2* fragment (Fig. 3). In both controls, wild-type H99 and untransformed M001, the *ade2* probe hybridized to a 9.4-kb fragment, representing the native *ade2* gene. If homologous recombination with vector insertion occurred, the native *ade2* gene would be displaced. Since a *Hin*dIII site was present in the pcDNA II vector (Fig. 2; pCn*ade2c*), two fragments of dissimilar size would be generated and each would hybridize to both the *ade2* and the vector probes. The sizes of the two novel fragments would depend on the location of the 2.2-kb *ade2* gene within

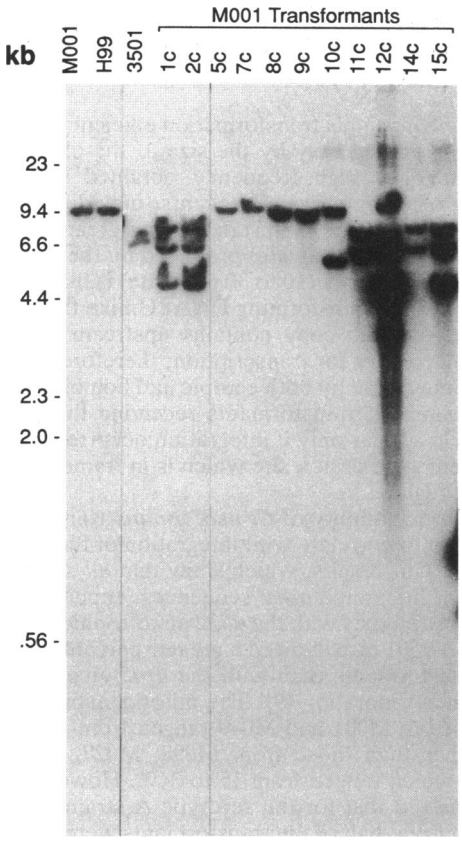

FIG. 3. *Hin*dIII-restricted DNAs from untransformed M001, wild-type H99, and *ade*$^+$ transformants, which received the cDNA copy of *ade2*, were probed with the genomic 980-bp *ade2* restriction fragment.

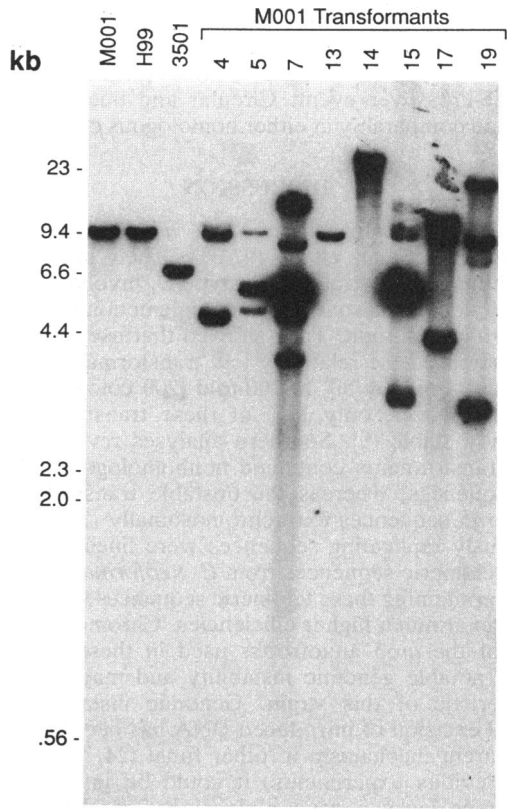

FIG. 4. *Hin*dIII-restricted DNAs from untransformed M001, wild-type H99, B-3501, and *ade*$^+$ transformants, which received the genomic copy of *ade2*, were probed with the genomic 980-bp *ade2* restriction fragment.

the *Hin*dIII-restricted 9.4-kb fragment. Also, homologous integration with insertion of two or more vectors containing the *ade2* cDNA would generate an additional 4.8-kb fragment that would hybridize to both probes. This expected pattern of two novel fragments (8.0 and 6.4 kb) was observed in six transformants (1c, 2c, 11c, 12c, 14c, and 15c [Fig. 3; data not shown for pcDNA II probe]). Also, four of these transformants (1c, 2c, 12c, and 15c) shared a 4.8-kb fragment which hybridized to both probes (Fig. 3; data not shown for pcDNA II probe), which suggests that there is more than one vector insert at the homologous site. In addition to these fragments, other fragments diagnostic of ectopic integration were found in transformant no. 12c. The five remaining transformants (5c, 7c, 8c, 9c, and 10c) showed the *ade2* probe hybridizing to a 9.4-kb fragment. These transformants either are revertants, although none were found in the control plates, or represent homologous integration of the *ade2* cDNA by a double-crossover event, excluding insertion of plasmid sequences (gene replacement). Transformant no. 10c also contained one site of ectopic integration. Both circular and linear *ade2* cDNA showed similar patterns of integration.

Eight transformants that received the genomic *ade2* (circular or *Hin*dIII restricted) were probed with the 980-bp fragment of genomic *ade2* (Fig. 4). A *Hin*dIII site is present in genomic *ade2* (from B-3501) but not in H99, on the basis of restriction analysis; therefore, *Hin*dIII-restricted wild-type B-3501 DNA was also included. As expected, in B-3501 the *ade2* probe hybridized to two fragments with smaller sizes (7.0 and 3.5 kb) (Fig. 4, lane 3). H99 and M001 showed the native *ade2* gene on the 9.4-kb fragment (Fig. 4, lanes 1 and 2). These findings demonstrate the sequence polymorphism in the *ade2* locus between the two isolates H99 and B-3501. If homologous recombination with vector insertion occurred, the native *ade2* gene would be displaced. Provided that a single-crossover event which excluded the *Hin*dIII site present in the transforming *ade2* gene occurred, a novel 15.4-kb fragment which hybridized to both the *ade2* and the vector probes would be generated. Also, homologous integration of two or more vectors containing the *ade2* gene without the *Hin*dIII site would generate a larger fragment hybridizing to both the *ade2* and vector probes. The size of this new fragment would depend on the numbers of vector and gene repeats that were inserted. This expected pattern of a novel 15.4-kb fragment was observed in transformant no. 7 (Fig. 4; data not shown for the SK probe). Also, a much larger novel fragment (approximately 27.4 kb) was seen in transformant no. 14 (Fig. 4; data not shown for the SK probe), which is consistent with the insertion of a triple tandem repeat of the gene (loss of *Hin*dIII site) and vector. Other fragments diagnostic of ectopic integration were also found in transformant no. 7. In five transformants (no. 4, 5, 15, 17, 19), the *ade2* probe hybridized to a 9.4-kb fragment in addition to many novel fragments, implicating ectopic integration. In these transformants, the SK probe hybridized to a majority of these novel fragments but not to the 9.4-kb

fragment (data not shown). In transformant no. 13, the ade2 probe hybridized only to a 9.4-kb fragment, suggesting reversion or gene replacement without plasmid insertion by a double-crossover event. Circular and linear *ade2* DNA integrated comparably at either homologous or ectopic sites.

DISCUSSION

Successful transformation of *C. neoformans* has been reported by Edman et al. (9, 31). Their studies, which utilized the B-3501 strain of serotype D, involved electroporation to deliver plasmid DNA complementing a *C. neoformans ura5* auxotroph. They showed that use of linear *ura5* DNA increased the relatively low transformation efficiency of circular DNA by 50- to 100-fold (200 colonies per μg of DNA). However, only 12% of these transformants were mitotically stable (9). Southern analyses revealed that the stable transformants contained nonhomologous integrating *ura5* sequences, whereas the unstable transformants harbored *ura5* sequences extrachromosomally (31). These autonomously replicating sequences were linear and had acquired telomeric sequences from *C. neoformans* (8). Linear vectors containing these telomeric sequences were shown to transform at much higher efficiencies. Chromosomal karyotyping of the *ura5* auxotrophs used in these experiments showed notable genomic instability and may be a unique characteristic of this strain. Genomic instability due to eventual excision of introduced DNA has been proposed as the apparent mechanism in other fungi (24, 29, 33). From these previous experiments, it could be inferred that *C. neoformans* lacks a gene replacement mechanism yet is receptive to ectopic integration which eventually produces rearrangements and/or addition of telomeric sequences with a telomerase, as seen in *Paramecium tetraurelia* (11). It was important to determine whether this was a general characteristic of *C. neoformans* or unique to the recipient strain, gene locus, or DNA delivery system.

The present study with *C. neoformans* H99 serotype A showed significant differences in its transformation properties compared with those of B-3501 serotype D. First, H99 was not receptive to efficient transformation by electroporation but could be transformed at high frequency by biolistic DNA delivery. The inability to transform four H99 *ade2* auxotrophs (M001, M027, M049, and M098) by electroporation appears to be strain dependent and to be independent of the transforming *ade2* gene, since the *ade2* auxotroph from B-3501 (*αade2-27*) was transformed to adenine prototrophy by electroporation. The reason for the difference in the extent of transformation by electroporation among different *C. neoformans* strains is unknown. Further work will be needed to determine whether this difference is serotype or strain dependent. In contrast, *ade2* auxotrophic strains from two different serotypes (A and D) were highly receptive to transformation by biolistic DNA delivery. With the biolistic system, both circular and linear *ade2* generated similar numbers of transformants per plate: 20 to 30 colonies with *ade2* cDNA and 10^3 to 10^4 colonies with genomic *ade2*. This finding concurs with biolistic transformation of *S. cerevisiae*, in which both circular and linear DNAs had similar transformation frequencies (2). However, these results are quite different from those with electroporation, in which only the linear *ade2* generated a significant number of *αade2-27* transformants (Table 1). It should be noted that the concentration of genomic *ade2* delivered by biolistics was 400 ng per plate, whereas only 100 ng was used in the electroporation experiment. However, we believe that the concentration of DNA is unlikely to be a factor, because we have been able to transform a *ura5* mutant from B-3501 by the biolistic system and have obtained 10^3 to 10^4 transformants per plate with 100 ng of DNA.

The *C. neoformans* transformation efficiency generated by biolistics is limited only by the size of the plate containing the yeasts. The high frequency obtained will allow for screening of many molecular events, including transformation with entire DNA libraries. The greater number of transformants (10^3 per plate) produced by the genomic *ade2* versus its cDNA copy (10 to 30 per plate) is likely because of the nature of the transforming DNA. Unlike the *ade2* cDNA copy, the genomic copy contains upstream activating sequences necessary for transcription; therefore, stable transformants may arise by both ectopic and homologous integration. In contrast, transformants receiving the *ade2* cDNA copy would appear only if integration occurred at the site of the resident gene or at a site which is in frame with a usable promoter region.

The mitotic stability of *C. neoformans* transformants has been shown to correlate with integration of the transforming DNA (31). Our results, which show that all stable transformants bear integrated *ade2* sequences, support this association. These studies with the *ade2* auxotrophic mutants from H99 and B-3501 also showed a greater percent of mitotically stable transformants than with the *ura5* auxotrophs generated by electroporation (9). The mitotic stabilities of transformants from M001 and M049 ranged from 35 to 40% and were higher than those from M098, M027, and *αade2-27* mutants, which ranged from 15 to 24%. However, it should be emphasized that for the serotype A strain of *C. neoformans*, over one-half of the transformants were unstable after biolistic transformation. Although these unstable transformants were not examined, it is likely that mechanisms of extrachromosomal vector replication found in strain B-3501 with electroporation are also present in this strain.

Integrative transformation in fungi occurs at sites that are homologous with either type I or type III integration and/or ectopic (13). In *S. cerevisiae*, integration is almost always homologous (22). Integration in *N. crassa* is usually ectopic, although in this species with the *trp1* gene, homologous integration is strain dependent (15). Two basidiomycetes, *Ustilago maydis* and *Coprinus cinereus*, show predominantly ectopic integration (4, 32). In our system, we have increased the frequency and number of integrative events in the basidiomycete *C. neoformans* over those from a previously reported scheme (31). Although ectopic events frequently occurred, both type I integration with vector sequence and type III integration or gene replacement were found. The presence of transformants showing gene replacement or conversion was supported by the fact that no revertants were found on control plates. In *S. cerevisiae* (22), the frequency of homologous integration increases when the transforming gene is cut within its coding region. This does not occur in *N. crassa* (7, 15) or *A. nidulans* (34), and we did not find this increase when *C. neoformans* was transformed by biolistics. The occurrence of these integrative events in *C. neoformans* at homologous and/or ectopic sites is likely to depend on multiple factors, including the auxotrophic recipient, transforming gene, and/or the DNA delivery system.

The ability to generate large numbers of stable transformants, of which a reasonable proportion represent homologous integration, provides the basis for targeted gene disruptions in *C. neoformans*. This transformation scheme has been successfully used to restore the virulence of an aviru-

lent *ade2* auxotroph (M001) in a rabbit meningitis model (30). The molecular tools are now available for specifically disrupting candidate virulence genes and determining their importance in the pathobiology of this yeast. These strategies will potentially lead to the development of drugs targeted against these gene products or their receptors.

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