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Molecular and Genetic Analysis of *URA5* Transformants of *Cryptococcus neoformans*

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***Cryptococcus neoformans* var. *neoformans ura5* mutants were transformed with linearized or circular plasmids containing the *C. neoformans* orotidine monophosphate pyrophosphorylase gene. Following electroporation, randomly isolated transformants were analyzed for the mitotic and meiotic stability of uracil prototrophy. All stable transformants tested showed nonspecific ectopic integration. Uracil prototrophy in these transformants was stable through meiosis. Some of the stable transformants showed integration of both *URA5* and vector sequences, while others lacked any vector sequences. Unstable transformants exhibited the presence of an autonomously replicating plasmid which had undergone significant sequence rearrangement. The autonomously replicating plasmid in the transformants was observed to be the same size or smaller than the transforming plasmid, was maintained in a linear form, and had acquired a genomic sequence(s) with homology to a sequence(s) on all the chromosomes. The conservation of a 300-bp sequence at the 5′ end of the *URA5* gene was observed in all the rearranged plasmids. These results suggest mechanisms of plasmid maintenance in *C. neoformans* that are different from those reported for other yeasts. The *ura5* mutant was significantly less virulent than the wild type. The transformants did not recover virulence regardless of prototrophic stability.**

Plasmid-mediated transformations of yeast cells can occur by two possible mechanisms: integration into the host genome or maintenance of autonomously replicating plasmids. Integration is a low-frequency event, and its products are usually mitotically stable. The frequencies of transformation generated by this method are generally low (ca. 1 to 10/μg of DNA in *Saccharomyces cerevisiae*). Transformations with autonomously replicating extrachromosomal plasmids yield considerably higher transformation frequencies (10^3 to 10^4/μg of DNA) (24). The maintenance of such extrachromosomal plasmids has been attributed to the presence of autonomously replicating sequences (ARS). Integration in *S. cerevisiae* occurs primarily at homologous sites (25), and the transformants are mitotically stable. However, in *Schizosaccharomyces pombe*, most integration events occur at ectopic sites, the transformants are unstable, and the transforming DNA is excised to produce rearranged plasmids (28). Since most rearrangements of this type have been reported to be the result of genomic DNA insertions (28), the proposed mechanism would involve random, unstable, ectopic integration into the genome followed by normal (inserted sequences only) and aberrant (more or less than the inserted sequences) excision (2, 28). This mechanism is supported by observations of the generation of ARS-containing plasmids following transformation with non-ARS-containing vectors (28).

Previous studies reported the production of stable and unstable transformants upon transformation of *Cryptococcus neoformans* by electroporation with linearized or circular plasmids (6). Stable transformants were mostly the result of ectopic integrations of plasmid DNA into the genome. All the unstable transformants harbored an episomal, rearranged plasmid. In the present study we have mapped the altered plasmids with various restriction endonucleases to further characterize their form and the rearrangements. The transforming ability and the meiotic and mitotic stability of the plasmids have also been determined. In addition, the virulence of the transformants was determined in mice.

MATERIALS AND METHODS

Strains and media. The *ura5* mutants (FOA-01-11-2 and FOA-4476) were selected by plating cells of *C. neoformans* var. *neoformans* (B-3501 and B-4476, respectively; serotype D) on 5-fluoro-orotic acid (5-FOA) medium (6). The strains were maintained on YEPD (1% yeast extract, 2% Bacto-Peptone, 2% glucose) agar slants at 25°C. Uracil-requiring mutants were screened on 5-FOA medium containing (per liter) 7 g of yeast nitrogen base without amino acids (Difco, Detroit, Mich.), 1 g of 5-FOA, 50 mg of uracil, and 20 g of glucose. Uracil prototrophs were selected on minimal medium (MIN), which contained (per liter) 6.7 g of yeast nitrogen base without amino acids and 20 g of glucose. The *C. neoformans* wild-type strains B-4500 (mating type α) and B-4476 (mating type **a**) were used for matings. All solid media contained 2% Bacto-Agar.

Electroporation. Cells of the *ura5* mutants FOA-01-11-2 and FOA-4476 were grown overnight in YEPD broth at 30°C with vigorous aeration in a shaker at 200 rpm. One milliliter of the overnight culture was diluted into 50 ml of fresh YEPD broth in a 250-ml Erlenmeyer flask and grown for 4 h. The culture was harvested by centrifugation at 4,000 × *g* in a GLC-4 Sorvall centrifuge (Dupont Instruments, Wilmington, Del.) for 5 min. The cells were washed once in 50 ml of buffer A (0.27 M sucrose, 0.001 M $MgCl_2$, 0.01 M Tris-HCl [pH 7.4], 0.004 M dithiothreitol) and resuspended in 0.5 ml of electroporation buffer (buffer A without dithiothreitol). The cell suspension was aliquoted in 0.2-cm (electrode gap) cuvettes. Immediately after addition of plasmid DNA, the

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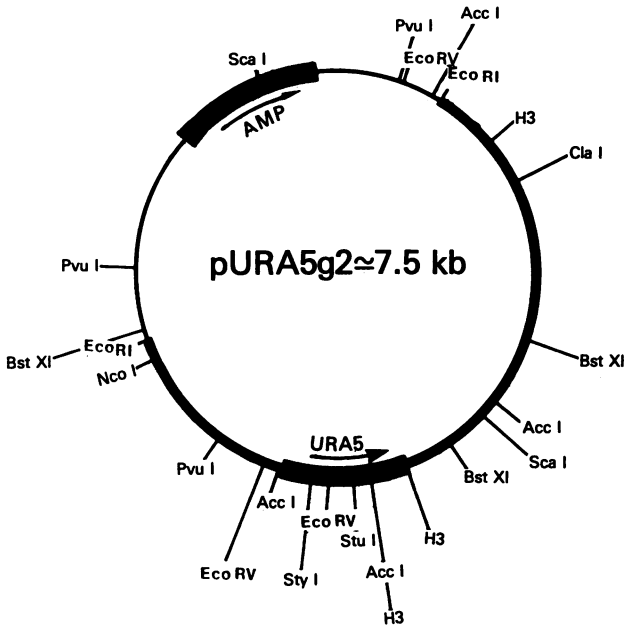

FIG. 1. Circular restriction map of transforming plasmid pURA5g2. The thick line represents the genomic insert DNA. The thin line represents the vector (Bluescript) DNA.

cells were subjected to an electric pulse in a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) at 470 V with a capacitance of 25 μF.

Transforming DNA. Plasmid pURA5g2, which contains the entire *URA5* gene from strain B-3501 on a 4.5-kb insert (Fig. 1), has been described previously (6). Total DNA was extracted from the stable and unstable transformants as previously described (23) and was used to transform the *ura5* mutant FOA-01-11-2. DNA used in these transformations was either uncut or digested with the restriction enzyme *Eco*RI or *Nco*I (BRL/GIBCO, Gaithersburg, Md.).

Southern analysis. Between 3 and 5 μg of total DNA from each of the selected transformants was electrophoresed on 0.8% agarose, either uncut or after digestion with the appropriate restriction endonuclease. The DNA was then transferred to Nytran filters (Schleicher & Schuell) by the method of Southern (22). Radio-labelled probes pURA5g2 and pUC19 were prepared by nick translation with [^{32}P]dCTP (Amersham, Oak Park, Ill.). The filters were hybridized with the probes for 16 h at 68°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05 M K_2PO_4 (pH 7.4)–10× Denhardt's solution–1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml. The unbound label was removed by washing twice for 5 min at room temperature in solution A (0.3 M NaCl, 0.06 M Tris-HCl [pH 8], 0.002 M EDTA [pH 8]), twice for 30 min at 60°C in solution B (0.3 M NaCl, 0.06 M Tris-HCl [pH 8], 0.002 M EDTA [pH 8], 1% SDS), and twice for 30 min at room temperature in solution C (0.03 M NaCl, 0.006 M Tris-HCl [pH 8], 0.0002 M EDTA [pH 8]). The filters were then dried, wrapped in Saran Wrap, and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C in film cassettes (Dupont) lined with intensifier screens.

Contour-clamped homogeneous electric field (CHEF) electrophoresis. Agarose blocks containing *C. neoformans* chromosomes were prepared by a procedure described previously (6). For electrophoresis, sections of the blocks were placed into a 0.7% chromosomal grade agarose (Bio-Rad) gel and electrophoresed at 120 V with a dually ramped switch time of 70 to 100 s for 15 h and 170 to 250 s for 33 h. The DNA of the transformants was transferred to Nytran filters (Schleicher & Schuell) and probed with ^{32}P-labelled plasmids pURA5g2 and pUC19 to determine the chromosomal insertion site(s) of the *URA5* gene in the transformants.

Restriction mapping. Plasmid pURA5g2 was digested by treatment with single or multiple restriction endonucleases and electrophoresed on 0.8% agarose gels to generate a restriction map of the insert in plasmid pURA5g2 (Fig. 1). Repeated attempts to extract, purify, and amplify the plasmids from the unstable transformants (except UT-4) by cloning in *Escherichia coli* were unsuccessful. Therefore, total DNA from plasmid-harboring transformants was digested with one or more endonucleases, electrophoresed in an 0.8% agarose gel, blotted onto Nytran filters, and probed with labelled DNA of plasmid pURA5g2.

Quantitation and extraction of plasmid DNA from unstable transformants. Plasmid DNA concentration in the cell was determined by Southern blot hybridization. pURA5g2 plasmid DNA was hybridized to total DNA of the unstable transformants as well as to known amounts of pURA5g2. Total cellular DNA from the plasmid-bearing transformant was electrophoresed on 0.6% agarose gels. Agar plugs containing the faster-migrating plasmid DNA, as visualized by ethidium bromide fluorescence, were excised from the gel and purified in an Elutrap gel eluter (Schleicher & Schuell).

Mitotic stability. A saline suspension of freshly grown transformant cells was plated onto minimal agar. After 24 h, the colonies were replica plated onto YEPD plates. Following several (four to seven) such serial transfers, the stability of the uracil prototrophy was scored as growth on minimal agar plates.

Meiotic stability. Transformants derived from FOA-01-11-2 were unable to mate. This was not unusual since the *ura5* strain FOA-01-11-2 was found to have lost its mating ability. Another *ura5* strain, FOA-4476, which also generated stable transformants and displayed a multiplicity of heterologous genomic insertions, was found to be a good mater. Two stable *URA5* transformants, derived from FOA-4476 (mating type **a**), were crossed with B-4500 (mating type α) on V8 juice agar (16). Basidiospores were randomly picked by micromanipulation onto complete medium (YEPD) and subcultured on minimal agar to determine the meiotic stability of the uracil prototrophy. The mating types of the basidiospore cultures were determined by backcrossing with B-4500 and B-4476.

Determination of virulence. The wild type (B-3501), *ura5* mutant, and three transformants were tested for pathogenicity in mice (16, 17). General purpose white mice (BALB/c; 10 for each strain) were injected with 10^6 viable cells in the tail vein and observed for up to 55 days. Statistical analysis by the Kolmogorov-Smirnov two-sample test (21) was based on maximum distances between pairs of survival curves.

RESULTS

Transformation with circular or linear plasmid DNA. The physical form of the transforming plasmid DNA (circular or linear) markedly affected the transformation frequencies of the *ura5* strain FOA-01-11-2. Linearization of the plasmid either with *Nco*I, which cuts pURA5g2 once near the 5′ end of the insert, or with *Eco*RI (data not shown), which releases the insert entirely, consistently generated higher numbers of

TABLE 1. Transformants obtained with different concentrations of circular and linearized pURA5g2 in 50 μl of electroporation buffer containing 2×10^7 cells

Amt (μg) of plasmid DNA	No. of transformants obtained with uncut DNA		No. of transformants obtained with *Nco*I-cut DNA	
	Per plate	Per μg of DNA	Per plate	Per μg of DNA
1	260	260	370	370
2	122	61	486	243
5	126	25	490	98
10	70	72	80	28

transformants, regardless of the *ura5* mutant strain used (Table 1). One microgram of plasmid DNA (cut or uncut) was determined to be optimal by using 2×10^7 to 5×10^7 cells per cuvette in order to achieve the highest frequencies of transformation. Increasing the concentration of plasmid DNA resulted in lower transformation frequencies (Table 1).

Stability of the transformants. The transformants analyzed in this study were generated with *Nco*I-cut plasmid DNA. Sixty to 70% of the transformants were observed to be unstable for uracil prototrophy after one transfer on nonselective (YEPD) medium. Almost half of the unstable transformants exhibited instability even while being maintained on selective medium (MIN). Some of the transformants which were initially recorded as stable for uracil prototrophy became unstable after four serial transfers on nonselective (YEPD) medium. In all such cases, Southern blot analysis revealed that loss of uracil prototrophy was accompanied by a loss of the free plasmid (data not shown).

Hybridization analysis of stable transformants. The stable transformants were analyzed by hybridizations of labelled plasmid (pURA5g2) DNA to undigested total DNA from each of the stable transformants. The hybridization patterns indicated the stable insertion of the *URA5* sequences in genomic DNA (Fig. 2, UT-10, UT-13, UT-15, and UT-16). In some transformants, the hybridization patterns demonstrated the presence of both an autonomous plasmid as well as the genomic insertion of plasmid DNA (Fig. 2, UT-11 and UT-12). Hybridizations of *Eco*RI-digested total DNA from the stable transformants revealed the genomic insertions to have been ectopic and, in some transformants, probably at more than one site (Fig. 3, UT-13 and UT-15). Since the *Eco*RI sites in the plasmid had been artificially created (6), the hybridization patterns allowed discrimination between nontransformants and stable integrative transformants. Similar observations were also recorded for *Nco*I-digested total DNA from the stable transformants (data not shown).

Hybridization analysis of unstable transformants. Southern blot analysis of uncut total DNA from the unstable transformants revealed the presence of extrachromosomal DNA in each isolate (Fig. 2, UT-2, UT-3, UT-4, UT-6, UT-7, UT-8, UT-9, and UT-14). The autonomously replicating plasmids were maintained in these transformants while the transformants were grown on MIN. Reversion to uracil auxotrophy accompanied the loss of the free plasmid when the transformants were grown on nonselective (YEPD) medium (data not shown). Quantitative calibrations of plasmid DNA in the unstable transformants, by hybridizations to known amounts of pURA5g2 plasmid DNA, suggested the presence of 50 ng (approximately 25 copies per cell) per microgram of total cellular DNA (data not shown).

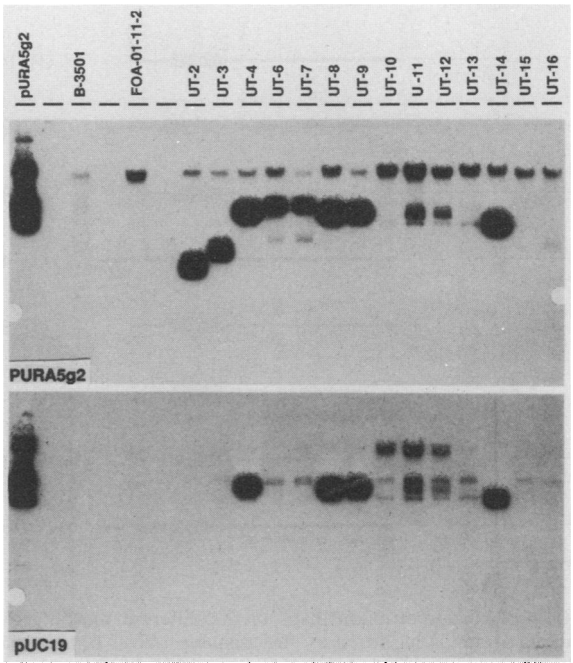

FIG. 2. Southern blot analysis of undigested total DNA from several *URA5* transformants (designated UT) and the untransformed cells of both the wild-type strain (B-3501) and the *ura5* mutant (FOA-01-11-2). pURA5g2, transforming plasmid (undigested DNA). The blots were probed with nick-translated DNA of plasmids pURA5g2 (upper panel) and pUC19 (lower panel). Each lane contains 3 to 5 μg of total cellular DNA.

Characterization of plasmids in the unstable transformants. The plasmids present in the unstable transformants had been altered compared with the original transforming plasmid, pURA5g2. In order to examine the qualitative changes, plasmids from some of the unstable transformants and the

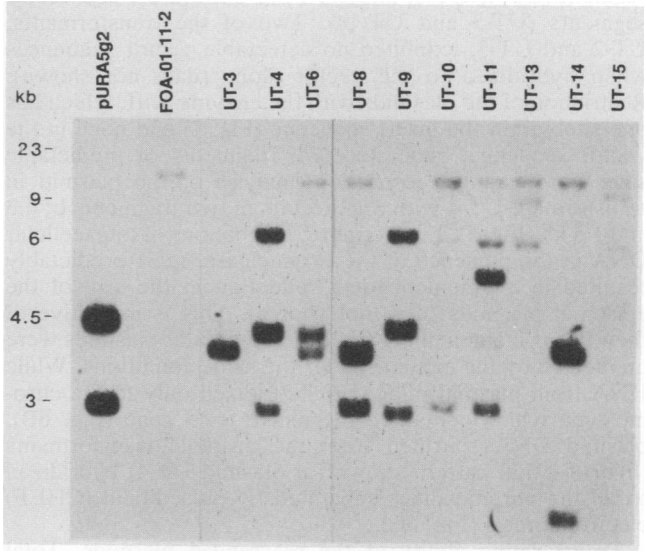

FIG. 3. Southern blot analysis of *Eco*RI-digested total DNA from various *URA5* transformants (UT), the *ura5* mutant (FOA-01-11-2), and the transforming plasmid pURA5g2. The blots were probed with nick-translated DNA from plasmid pURA5g2.

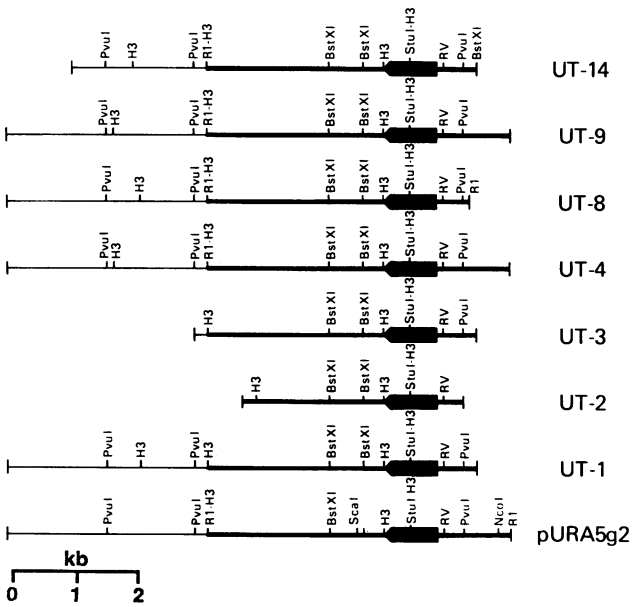

FIG. 4. Linear restriction maps of the different rearranged autonomous plasmids harbored by the unstable *URA5* transformants UT-1, UT-2, UT-3, UT-4, UT-8, UT-9, and UT-14. Also shown is the linear restriction map of the transforming plasmid pURA5g2. The thick line represents genomic DNA, while the thin line represents vector (Bluescript) DNA.

transforming plasmid, pURA5g2, were mapped for a variety of restriction enzyme sites (Fig. 4). The restriction maps of the altered plasmids revealed the conservation of the intact *URA5* gene along with its flanking sequences extending to the upstream (5′ end) *Pvu*I site and the downstream (3′ end) *Hin*dIII site. Sequences proximal to the terminal *Eco*RI sites of the insert appear to have undergone substantial rearrangement as evidenced by the changes in the mapped restriction sites. The reorganizations appear to have resulted in a cumulative loss of sequences (Fig. 3) either in the insert segment (UT-4, UT-6, and UT-8) or both insert and vector segments (UT-3 and UT-14). Two of the transformants, UT-2 and UT-3, exhibited no detectable vector sequences when hybridized to the vector alone (data not shown). Restriction of the plasmids with the enzyme *Stu*I, which has one site within the insert sequence (Fig. 1) and none in the vector sequence, generated two fragments of predictable size. For instance, restriction analysis of the plasmid in transformant UT-4 with *Stu*I results in two fragments of 6.3 and 1.6 kb (Fig. 5). Furthermore, incubations of total cellular DNA in the presence of the exonuclease *Bal*31 predictably resulted in a time-dependent reduction in the size of the episomal plasmids (data not shown). This is indicative of their linear maintenance, since uncut circular plasmids were unaffected by the enzyme under the same conditions. While DNA from plasmid pURA5g2 hybridized only to the chromosome which contained the native *ura5* gene (Fig. 6B), plasmid DNAs purified from the unstable transformants (hybridization pattern shown for plasmid UT-4) hybridized to all the chromosomes separated by pulsed-field (CHEF) electrophoresis (Fig. 6C).

Transforming ability of the rearranged plasmids. Total DNA from either the unstable or the stable transformants was used to transform the *ura5* mutant FOA-01-11-2. Based on the calibrations of plasmid DNA in the unstable transformants (see Materials and Methods), a normalized amount of total DNA was used for these transformations. While no transformants were obtained when total DNA from any of the stable transformants (which did not contain a free plasmid) was used, total DNA from each of the unstable transformants successfully transformed the *ura5* mutant. DNA from the unstable transformants generated uracil prototrophs at variable frequencies. The frequencies of secondary transformation varied depending on which primary transformants' DNA was used and whether the transforming DNA used was either uncut or digested with *Eco*RI (for those plasmids still maintaining an endogenous *Eco*RI site). Hybridization of plasmids pURA5g2 and pUC19 to total DNA of these secondary transformants revealed the absence of further plasmid DNA rearrangements only when uncut DNA was used (Fig. 7, lanes 3 to 6). If the transforming DNA had been digested by *Eco*RI, however, the plasmids in the secondary transformants were altered further (Fig. 7, lanes 7 to 10). These transformants were observed to be stable for uracil prototrophy on selective medium (MIN) but became unstable after several serial transfers on nonselective (YEPD) medium.

Chromosomal localization of inserted sequences among the transformants. Chromosome-size DNA from 10 subclones each, obtained from both B-3501 (wild type) and FOA-01-11-2 (*ura5* mutant), and from eight transformants was separated on a gel by using the CHEF system (Bio-Rad; CHEF-DR2). The CHEF patterns of the 10 subclones from B-3501

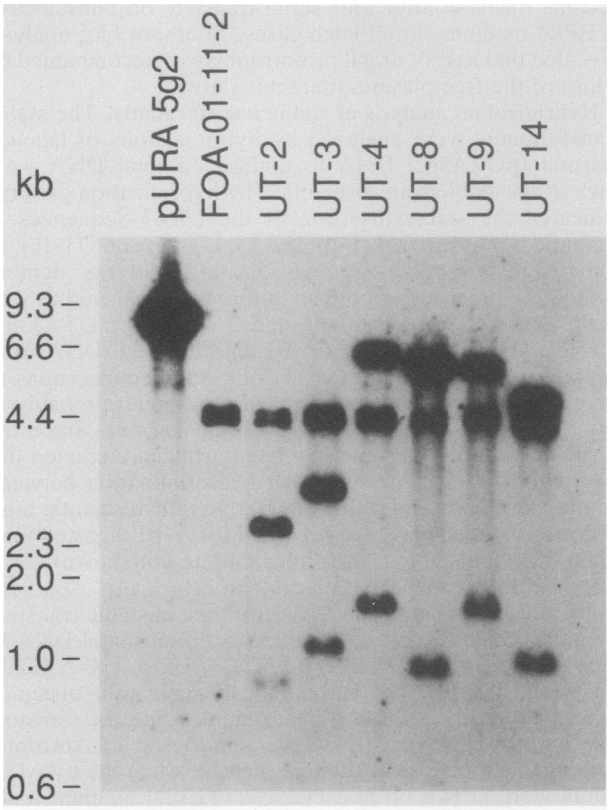

FIG. 5. Southern blot analysis of *Stu*I-cut DNA from plasmid pURA5g2 and total DNA from the untransformed isolate FOA-01-11-2 as well as some of the plasmid-harboring, unstable *URA5* transformants (UT). The filter was probed with nick-translated DNA of plasmid pURA5g2.

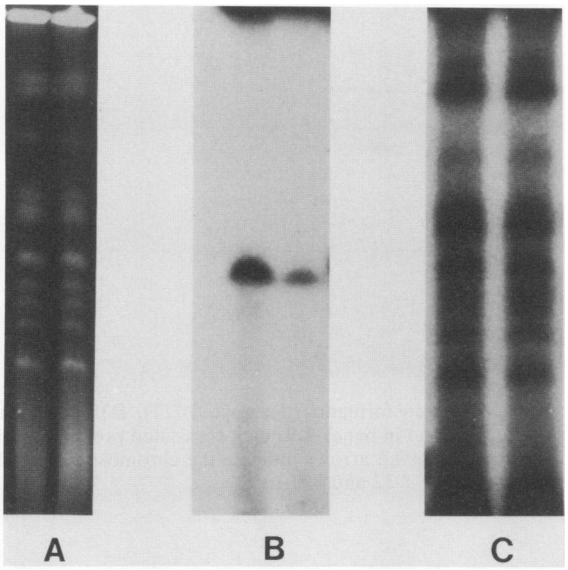

FIG. 6. Hybridization analysis of CHEF-separated chromosomes of *C. neoformans* (A) with labelled DNA of plasmid pURA5g2 (B) and with the gel-purified plasmid DNA from the primary transformant UT-4 (C).

showed an identical 11-band pattern (data not shown). The 10 subclones isolated from the untransformed *ura5* mutant (FOA-01-11-2), however, exhibited three different patterns due to variations in the third-largest band as well as in the sixth-largest band (data not shown). Variations in the CHEF patterns of the transformants (Fig. 8A) were identical to those observed among the *ura5* subclones. These variations were, therefore, not associated with the transformation process. Hybridization with pURA5g2 and pUC19 showed that in the unstable transformants UT-1 to UT-4, plasmid sequences had not been integrated (Fig. 8B and C), while nonhomologous integration was evident among the four stable transformants (arrows in Fig. 8B). Two of the four stable transformants (UT-15 and UT-16) showed only the presence of insert sequences, while the others (UT-10 and UT-12) showed the presence of both vector and insert sequences (Fig. 8C).

Meiotic stability. Two stable transformants, each with an ectopic insertion, were separately used in matings (see Materials and Methods) to determine the stability of the uracil prototrophy through meiosis. All the isolated basidiospores from the cross between transformants, derived from FOA-4476, and B-4500 were able to grow normally on MIN. Thus, meiotic stability of the uracil prototrophy was not affected by the nonhomologous, genomic insertions containing the *URA5* gene (data not shown).

Virulence of the *ura5* transformants. Virulence in mice was determined by injecting cells of a wild-type strain (B-3501), the *ura5* mutant (FOA-01-11-2), an unstable transformant (UT-1) harboring an autonomously replicating plasmid, and two stable transformants (UT-5 and UT-12) with ectopic integrations in different chromosomes. Figure 9 shows the survival of mice for a period of 55 days. The *ura5* mutant and all the transformants were significantly less virulent than B-3501 ($P < 0.001$). The virulence of the unstable transformant (UT-1) and that of the stable transformant (UT-5) were not significantly different from that of the *ura5* mutant (*ura5* versus UT-1, $P = 0.053$; *ura5* versus UT-5, $P > 0.1$). The stable transformant UT-12, which produced no mortality, was even less virulent than the *ura5* mutant ($P < 0.001$).

DISCUSSION

Stability of the uracil prototrophy reflected the fate of the transforming DNA. Integrations into the genome predictably generated stable transformants. Hybridizations of plasmid DNAs to *Eco*RI- and *Nco*I-digested total as well as CHEF-separated chromosomal DNA revealed the integrations to be ectopic.

The unstable transformants all harbored autonomously maintained *URA5* sequences. Southern analysis of the fragments generated by restriction at the unique enzyme sites, such as *Stu*I (Fig. 5), was suggestive of the linear form of the plasmids. This was then corroborated by digestion with the exonuclease *Bal*31 (data not shown). Most of the unstable transformants maintained on nonselective medium lost the *URA5* sequences and became auxotrophs after the first transfer. This is not surprising since autonomously replicating plasmids have been shown to be lost during mitosis in several yeasts.

Prototypic ARS sequences promote the extrachromosomal replication of circular DNA molecules and increase transformation frequencies in a variety of fungal systems (10). By these standards, the pURA5g2 plasmid does not contain an ARS. However, it clearly does replicate to a degree sufficient to confer prototrophy. The data presented

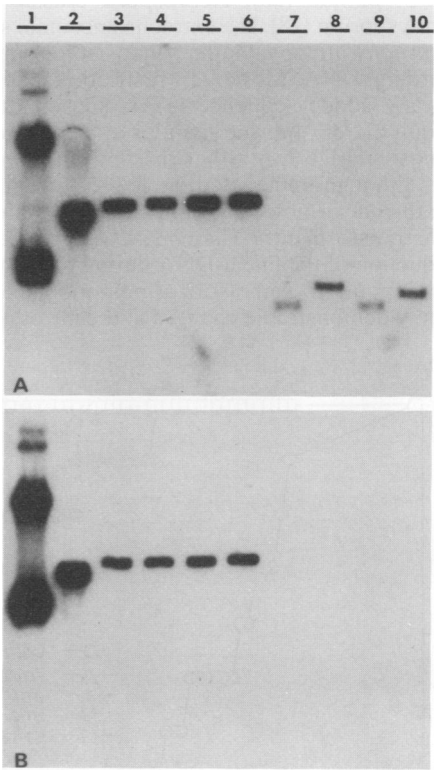

FIG. 7. Southern blot analysis of uncut total DNA from secondary *URA5* transformants after electroporation with uncut (lanes 3 to 6) and *Eco*RI-cut (lanes 7 to 10) total DNA from the primary transformant UT-4. Lane 1, uncut DNA of plasmid pURA5g2; lane 2, uncut total DNA from the primary transformant UT-4. The blots were probed with nick-translated DNA of either plasmid pURA5g2 (A) or plasmid pUC19 (B).

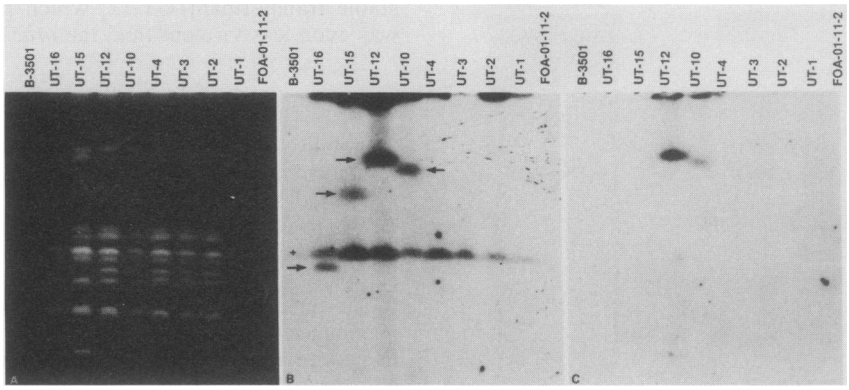

FIG. 8. (A) Ethidium bromide-stained CHEF-separated chromosomes of the *URA5* transformants (designated UT). B3501, wild-type strain; FOA-01-11-2, *ura5* mutant strain. Also shown is Southern blot hybridization of the gel in panel A to nick-translated probes, plasmids pURA5g2 (B) and pUC19 (C). Hybridization with pURA5g2 shows ectopic integration (arrows); arrows indicate the chromosome on which the native *ura5* gene is located. Hybridization with pUC19 shows the vector sequence in UT-12 and UT-10.

in this paper demonstrate that the extrachromosomal DNAs present in unstable Ura$^+$ *C. neoformans* transformants exist in linear form. This is true even if supercoiled plasmid DNA is used for transformation. These molecules, therefore, not only need a replication origin but also a means of maintaining the free ends of the extrachromosomal DNAs.

While it is possible that an ARS sequence may have been acquired by interactions with the genome, as has been shown for *S. pombe* (28), *Neurospora crassa* (23), and Fusarium oxysporum (18), the extrachromosomal sequences in *C. neoformans* are always the same size or smaller than the transforming DNA. Moreover, restriction mapping does not reveal any added sequences. This suggests that a sequence required for the autonomous maintenance of the extrachromosomal DNA is either resident on the pURA5g2 plasmid or, although undetected, added to the ends.

How are the linear molecules generated and maintained in the unstable transformants? There must be a transient period upon introduction of the linear DNA during which the naked DNA is susceptible to some sort of host modification (27). Since the only demonstrable changes in the introduced DNA occurred near the ends, and some form of end maintenance is required for the continued presence of linear DNA, telomeres or functional equivalents thereof may have been added to the ends (27). Hybridization of plasmids, purified from the unstable transformant (UT-4), to all the CHEF-separated chromosomes suggests the presence of sequences on the plasmid which are also present on every chromosome. It is possible that contaminating heterogeneous chromosomal DNA sequences which might hybridize to all the chromosomes are eluted from gels. This is unlikely since hybridization patterns similar to those with the plasmid from UT-4 were also observed with plasmids from several other transformants. No such hybridization is observed for the transforming plasmid pURA5g2. Terminal modifications of the ends involving nucleolytic cleavage or methylations or both could precede such additions. This is demonstrated by the preponderance of transformants containing plasmids smaller than the introduced DNA. Once these actions have occurred, the DNA could then be stably maintained at the same size through multiple generations and even upon retransformation into *C. neoformans*. When the linear DNA is cleaved with a restriction enzyme prior to retransformation, then the DNA would again be subject to such modifications. This suggests that stabilization must be brought about by some action at the ends of the molecule. Since the plasmid purified from UT-4 hybridized to all the CHEF-separated chromosomes, the linear plasmid may have acquired telomeric sequences at the ends. The various sizes of the linear plasmids generated by the host's endonuclease would result in different terminal sequences. This suggests the presence of a telomerase, as has been observed in *Paramecium tetraurelia*, which does not require specific recognition sequences.

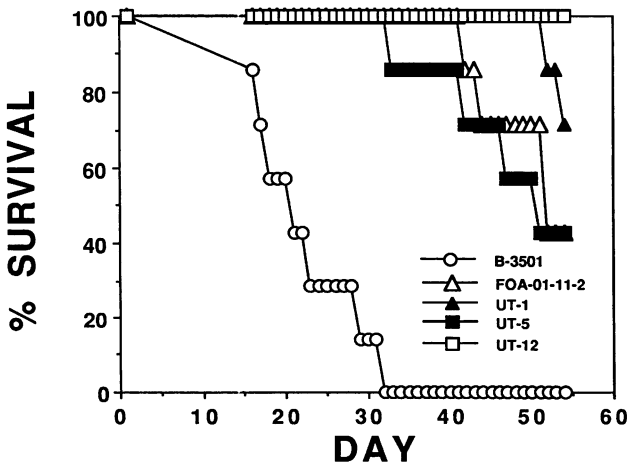

FIG. 9. Survival curves for mice infected with the wild type, *ura5* mutant, and transformants obtained with *Nco*I-digested pURA5g2.

Once stabilized, the extrachromosomal sequences were then maintained unaltered in spite of repeated growth in MIN broth or on MIN agar. Total DNA extracted from the unstable transformants, each containing extrachromosomal sequences, successfully transformed the *ura5* mutant FOA-01-11-2. The transformation efficiency with total DNA from some of the unstable transformants was considerably higher than that with the purified plasmid alone. In order to determine if the enhanced efficiency was due to the presence of genomic DNA, plasmid (pURA5g2) and genomic (strain FOA-01-11-2) DNAs were pooled in different ratios and used

for transformation. The presence of genomic DNA did not significantly enhance the transformation efficiency. It appears, therefore, that the ability of the altered sequences to retransform is the result of modifications undergone following the first transformation and is not due to the presence of genomic DNA in the mixture. Decreases in secondary transformation frequency were also observed when total DNA from some of the unstable transformants (UT-3 and UT-7) was used. This suggests that during rearrangements, some deleterious sequences may have been either generated or cleaved, thereby affecting the transformation competence of the sequence.

Folger et al. (11) have reported that structural features of naked DNA possess mutagenic properties and serve to stimulate abnormal recombination. During transformation of *C. neoformans*, free ends generated by certain restriction enzymes (such as *Eco*RI) could enhance aberrant, nonhomologous integrations into the genome. The instability caused by such ectopic insertions could then be resolved by excision of these sequences, which generate autonomous replicons, as observed in *F. oxysporum* (18) and *S. pombe* (12, 28). Similar phenomena involving excision of heterologous DNA have been reported for simian virus 40 DNA in mammalian cells (2). Homologous integrations which are reported to be 100% in *S. cerevisiae* (25), 80% in *Aspergillus nidulans* (29), 37% in *Podospora anserina* (9), and 1 to 5% in *N. crassa* (3), *Coprinus cinereus* (1), and *Sordaria macrospora* (4) apparently do occur in *S. pombe* (28), as well as in *C. neoformans* (6). While in most organisms such insertions are stable through mitosis regardless of copy number, tandem duplications are unstable during meiosis (4, 11). The low frequency of homologous insertion in *C. neoformans* may indicate the lack of a gene replacement mechanism. It could also depend upon the recipient strain or the gene pair used and not necessarily be a characteristic of the organism. Such cases have been reported for *N. crassa*, for which the frequency of homologous integration was 90% for the *trp-1* system (15) and 15% for the *qa-2* system (5).

While the chromosomal karyotype of subclones of the wild-type strain (B-3501) were identical, variations were observed for subclones of the *ura5* mutant (FOA-01-11-2). This suggests that the *ura5* mutant, isolated based on its resistance to 5-FOA, may inherently possess genomic instabilities. It is possible, therefore, that a mechanism active in an already unstable environment may influence the fate of the ectopic DNA insertions. A mechanism altering the cell as to the site of genomic instability and causing eventual excision has been proposed for *N. crassa* (19, 20) and *Ascobolus immersus* (14). In such cases, duplicated sequences are inactivated prior to meiosis by methylation of the cytosine residues. Such methylations reduce ectopic recombinations which could create lethal rearrangements such as inversions, deletions, or translocations. It would also discourage transpositions throughout the genome, which has been seen in maize (8).

The chromosomal site and/or the number of copies integrated also influence the fate of the integrated sequences in *A. immersus*. Inactivation via methylation was 50% if the sequences were ectopic and 90% if they were tandem (7). Since the excess DNA used in transformations can frequently causes tandem insertions, such a mechanism could well be induced.

While a significant difference in virulence was observed between the wild-type and the *ura5* mutant, no such difference was recorded between the mutant and the transformants. Low levels of virulence for the unstable transformant may be the result of plasmid loss in the host and/or the consequence of the harsh electroporation procedure. The low levels of virulence exhibited by the stable transformants, however, may well be more complex. In addition to the electroporation conditions, the presence of foreign DNA and the ectopic integrations may affect the growth of cells in the host tissue. The inability to restore virulence by integrative stable transformation may well be the consequence of the existing genomic instability of the *ura5* mutant, as exhibited by variations in the CHEF karyotype. Isolation and then transformation of a *ura5* mutant possessing a stable karyotype may adequately address the influence of the number(s) and site(s) of genomic insertion(s) on virulence.

C. neoformans is the first yeast pathogenic to humans in which heterologous recombination between genomic and exogenous DNA appears to be the rule rather than the exception. It therefore is an appropriate fungus with which to study the effects of heterologous insertion on fungal virulence. Furthermore, the generation of linear, autonomously replicating plasmids offers the opportunity to isolate ARS-like or telomerelike sequences, which can be a tremendous asset in cloning and characterization of various genes including those involved in virulence.

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