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Directed Mutagenesis in *Candida albicans*: One-Step Gene Disruption To Isolate *ura3* Mutants

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A method for introducing specific mutations into the diploid *Candida albicans* by one-step gene disruption and subsequent UV-induced recombination was developed. The cloned *C. albicans URA3* gene was disrupted with the *C. albicans ADE2* gene, and the linearized DNA was used for transformation of two *ade2* mutants, SGY-129 and A81-Pu. Both an insertional inactivation of the *URA3* gene and a disruption which results in a 4.0-kilobase deletion were made. Southern hybridization analyses demonstrated that the *URA3* gene was disrupted on one of the chromosomal homologs in 15 of the 18 transformants analyzed. These analyses also revealed restriction site dimorphism of *Eco*RI at the *URA3* locus which provides a unique marker to distinguish between chromosomal homologs. This enabled us to show that either homolog could be disrupted and that disrupted transformants of SGY-129 contained more than two copies of the *URA3* locus. The A81-Pu transformants heterozygous for the *ura3* mutations were rendered homozygous and Ura$^-$ by UV-induced recombination. The homozygosity of a deletion mutant and an insertion mutant was confimed by Southern hybridization. Both mutants were transformed to Ura$^+$ with plasmids containing the *URA3* gene and in addition, were resistant to 5-fluoro-orotic acid, a characteristic of *Saccharomyces cerevisiae ura3* mutants as well as of orotidine-5′-phosphate decarboxylase mutants of other organisms.

Candida albicans is a dimorphic yeast which is an opportunistic pathogen in humans. Recently, *C. albicans* infections have become increasingly prevalent among immunocompromised individuals (10). At present there is a need for nontoxic agents to treat systemic *Candida* infections. The inability to develop such agents has been largely the difficulty in defining differential targets for antibiotic action. Genetic manipulation of *C. albicans* would help identify potential targets but has been hampered because the organism is diploid, and no sexual cycle has been found (23, 32, 33). These characteristics make it very difficult to detect recessive heterozygous mutations and consequently to isolate mutants. Genetic analysis has been limited to parasexual techniques (11, 12, 23), and many of the available *C. albicans* mutants have been isolated by UV irradiation of strains that are presumably naturally heterozygous at various loci (23, 32, 33). Other *C. albicans* mutants were isolated by sequential mutagenesis, either chemical or UV (23, 24). Genetic analysis of the latter mutants is often complicated because they may harbor multiple, independent and unknown lesions.

The development of a DNA-mediated transformation system for *C. albicans* was previously reported (14) and opens up new possibilities for overcoming some of the obstacles to genetic analyses. One of the most useful applications of DNA-mediated transformation of *Saccharomyces cerevisiae* has been the development of gene disruption technology (25, 28, 30). The most widely used gene disruption method is the one-step gene disruption procedure described by Rothstein (25). This procedure is based on the recombinogenic ability of free DNA ends which interact with homologous sequences in the genome (21). Transformation with linear DNA makes it possible to target the DNA to a specific locus in the genome and to replace a wild-type gene with a mutated copy (25). Similar one-step gene disruptions have recently been reported for two filamentous fungi, *Neurospora crassa* (19) and *Aspergillus nidulans* (22). One-step gene disruptions in the dimorphic fungus *C. albicans* would provide a means to introduce specific detectable mutations into the genome. This approach seemed likely to succeed since we have been able to transform *C. albicans* with linear DNA (unpublished observations) and since, in our previous transformations with circular DNA, integration usually occurred at the resident homologous gene (14). In other diploid organisms a phenotype due to gene disruption can be scored in haploid meiotic progeny. Although *C. albicans* is asexual, once a heterozygous disrupted strain is obtained a number of mutagenic methods can be used to render the mutation homozygous (12, 15, 16, 23, 24, 27, 33).

We chose to disrupt the previously cloned *C. albicans URA3* gene (8) by using the cloned *C. albicans ADE2* gene as the selectable marker for several reasons. First, there are no reported *C. albicans ura3* mutants. Second, *S. cerevisiae ura3* mutants have been used extensively for transformation (4); these mutants are resistant to 5-fluoro-orotic acid (5-FOA) which has proven to be a valuable selection in many experiments (2, 34). Third, the *C. albicans URA3* gene is small and functions in both *S. cerevisiae* and *Escherichia coli* (8). The latter properties allow rapid analysis of in vitro disruptions directly in *E. coli*. In addition, a DNA-mediated transformation system (14) based on the cloned *C. albicans ADE2* gene as the selectable marker was readily available (14). To our knowledge this is the first demonstration of directed mutagenesis by gene replacement in *C. albicans*.

MATERIALS AND METHODS

Materials. β-glucuronidase (type H-2), D-sorbitol, dextran sulfate, and polyethylene glycol were obtained from Sigma Chemical Co., St. Louis, Mo. Gene-Screen and [α-^{32}P]deoxynucleotide triphosphates (7,600 Ci/nmol) were from New England Nuclear Corp., Boston, Mass. Analytical disks and Elutip-d columns were from Schleicher & Schuell, Inc., Keene, N.H. 5-FOA was from P-L Biochemicals, Inc., Milwaukee, Wis. Restriction endonucleases and DNA mod-

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

Strain	Parent	Genotype	Relevant phenotype	Source or reference
SC5314			ADE^+ URA^+	Clinical isolate
hOG300	ATCC 10261	*ade2/ade2 pro/pro met/met*[a]	Ade^- URA^+	Poulter and Rikkerink (24)
SGY-129	hOG300	*ade2/ade2 pro/pro MET/met*[a,b]	Ade^- URA^+	This work
A81-Pu	A-81	*ade2/ade2*	Ade^- URA^+	Kwon-Chung and Hill (15)
pUR3AB3 (SGY-226)[c]	A81-Pu	*ade2/ade2 Δura3::ADE2/URA3*	ADE^+ URA^+	This work
pUR3AB3-9B (SGY-243)	pUR3AB3	*ade2/ade2 Δura3::ADE2/Δura3::ADE2*	ADE^+ Ura^-	This work
pSM1825B1 (SGY-220)	A81-Pu	*ade2/ade2 ura3::ADE2/URA3*	ADE^+ URA^+	This work
pSM1825B1-59A (SGY-269)	pSM1825B1	*ade2/ade2 ura3::ADE2/ura3::ADE2*	ADE^+ Ura^-	This work

[a] Refer to Discussion as to the possible aneuploidy of these two strains.
[b] SGY-129 was isolated as a spontaneous MET^+ revertant of hOG300.
[c] Items in parentheses in strain column are Squibb Culture Collection designations.

ifying enzymes were from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.

Strains. *C. albicans* strains used in this study are listed in Table 1. *E. coli* DB6656 (*pyrF*::μ *trp lacZ hsdR*) (1) was obtained from D. Botstein and was used for most *E. coli* transformations. Otherwise, either *E. coli* HB101 (5) or RR1 (3) was the recipient strain for bacterial transformations.

Media. Medium for testing sensitivity to 5-FOA was described by Boeke et al. (2). All other media have been detailed by Kurtz et al. (14).

DNA isolations and hybridizations. Plasmid DNAs were isolated from minilysates and large-scale preparations of *E. coli* by the boiling procedure of Holmes and Quigley (9). Restriction endonuclease digestion of DNA was carried out as described by Maniatas et al. (17), and restriction fragments were isolated by agarose gel electrophoresis followed by electroelution. The eluted DNA was further purified by passage over an Elutip-d column as directed by the manufacturer. Total genomic DNA from *C. albicans* was prepared by standard methods (29). Both colony and Southern hybridizations were carried out as described previously (Kurtz et al. [14]). All molecular sizes were calculated by using λ *Hin*dIII digestion fragments as standards on each gel.

Plasmid constructions. All plasmids were constructed by standard recombinant DNA techniques. Blunt-ended DNA fragments were generated by T4 DNA polymerase (17). Plasmid pSM7, containing the *Candida ADE2* gene, was constructed by inserting the 2.5-kilobase (kb) *Eco*RV *ADE2* fragment of pMC2 (14) into the *Eco*RV site of pBR322. The details of limiting the *C. albicans ADE2* gene to the minimum functional sequence will appear elsewhere (14a). Four subclones containing the *Candida URA3* gene were constructed. The 6.5-kb *Pst*I-*Pst*I *URA3* fragment of pET19 (8)

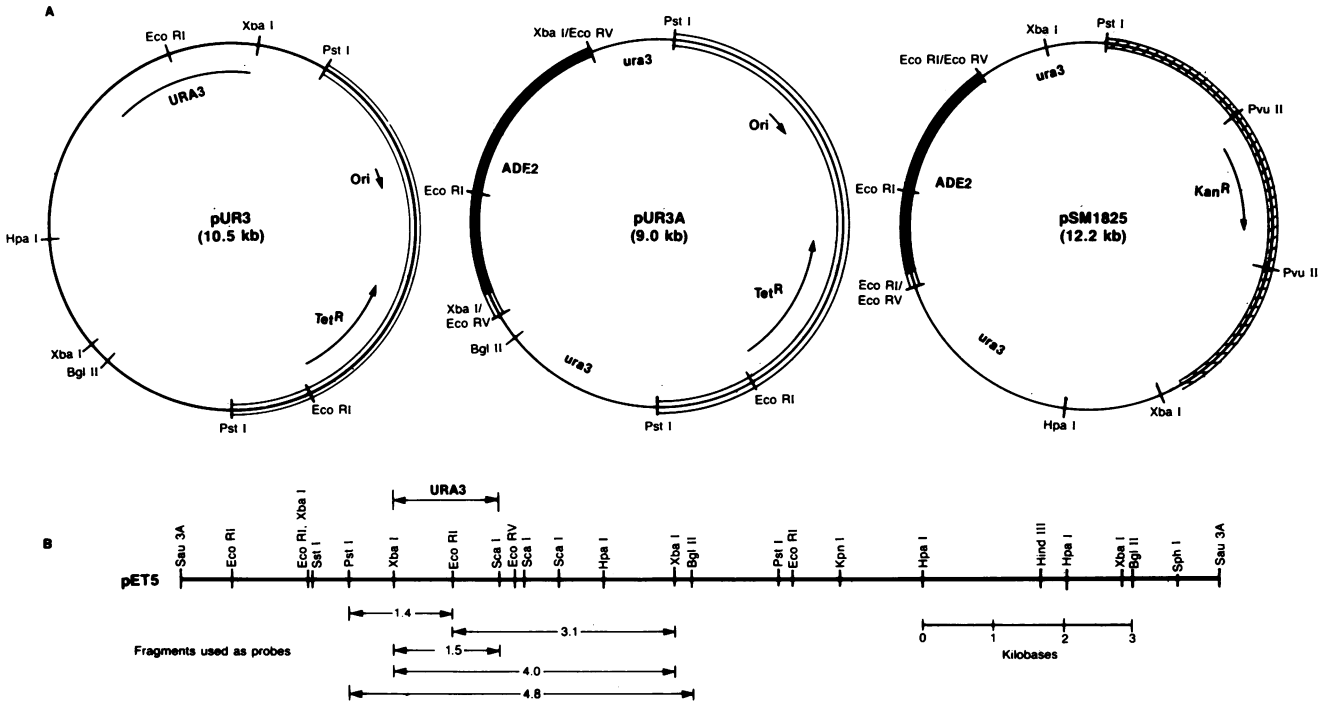

FIG. 1. Maps of pUR3 and *URA3*-disrupted plasmids. Symbols: ——, *C. albicans URA3*-containing region; ■, *C. albicans ADE2* sequences; ▭, pBR322 sequences; ▦, pET1 sequences. (B) Restriction map of *C. albicans URA3* insert in pET5 (8). Only the *Sca*I and *Eco*RV sites within the *Pst*I-*Pst*I fragment are shown. Sizes below the map designate restriction fragments used as probes or in subcloning. The minimum functional region of the gene has been limited to the 1.5-kb *Xba*I-*Sca*I fragment. With the exception of *Eco*RI, restriction sites to the right of the *Bgl*II site of the *URA3* region have not been demonstrated in genomic DNA (8).

TABLE 2. Transformation frequencies of *C. albicans* strains SGY-129 and A81-Pu with *C. albicans ura3*-disrupted plasmids

DNA	A81-Pu		SGY-129	
	Total no. of transformants (5 μg DNA)	Transformants/μg[a]	Total no. of transformants (5 μg DNA)	Transformants/μg[a]
Undigested pSM1825	71	5	53	10
*Pst*I-*Hpa*I digest pSM1825	600	110	196	39
No DNA	46	—	1	—
Undigested pUR3A	91	9	90[b]	6
*Pst*I digest pUR3A	61	3	82[b]	5

[a] Calculated by subtracting the −DNA control from the total number of transformants.
[b] 15 μg of DNA was used in these transformations. Both concentrations are within the linear range of the titration curve reported previously for *C. albicans* (14).

was inserted into the *Pst*I site of pBR322 to generate pUR3 (Fig. 1A). The 4.0-kb *Xba*I-*Xba*I *URA3* fragment of pET18 (8) was inserted into the *Xba*I site of pUC12 to generate pMC5. pMC6 is identical to pMC5 except that the insert is in the opposite orientation. A derivative of pMC5, pET39, which contains the 1.7-kb *Xba*I-*Eco*RV *URA3* fragment was made by deleting the *C. albicans* sequences to the right of the *Eco*RV site (see Fig. 1B).

Previous work by Gillum et al. (8) had defined an *Eco*RI site (see Fig. 1B) to be within an essential region of the *URA3* gene. This was based on complementation of an *E. coli pyrF* mutant, DB6656 (8). In addition, when pET18 (which contains only this *Eco*RI site within the *URA3* gene [8]) was digested with *Eco*RI and blunt ended, the resultant plasmid was unable to complement DB6656 upon religation. A plasmid containing an insertional inactivation of the *URA3* gene, pSM1825 (Fig. 1A), was made by inserting the 2.5-kb *Eco*RV *ADE2* fragment into pET18 that was treated as described above. A plasmid with a 4.0-kb deletion of the *URA3* gene, pUR3A (Fig. 1A), was made by ligating the 2.5-kb *Eco*RV *ADE2* fragment to a blunt-ended *Xba*I digest of pUR3. Neither pUR3A or pSM1825 complemented DB6656.

Plasmids pUR3, pUR3A, pET39, pMC6, and pMC5 were propagated in *E. coli* DB6656 (*pyrF*). HB101 was the recipient strain for pSM1825, and pSM7 was maintained in RR1. For other plasmids used in this study see Table 3.

Transformation of *C. albicans*. *C. albicans* was transformed by using the *Saccharomyces* protocol described previously (Kurtz et al. [14]). Linear mutated DNAs used for transformation were not purified from the remainder of the digests.

UV irradiation of *C. albicans*. A logarithmically growing culture was suspended in water to 10^7 cells per ml, and 20 ml was irradiated for 75 s 30 cm from a short-wave UV lamp (model 5004; Ultraviolet Products, Inc., San Gabriel, Calif.). The dose at the surface of the cells was 1,600 μW/cm^2 (as measured with a UV intensity meter [Blak Ray ultraviolet meter J-225, Ultraviolet Products]), killing approximately 70% of the cells.

RESULTS

Transformation of *ade2* mutants with disrupted DNA. Two plasmids with mutated *ura3* genes were constructed in vitro (Fig. 1A), as described in the Methods section. To target the mutated DNA to the *URA3* locus, pSM1825 was digested with *Pst*I and *Hpa*I which releases a linear molecule containing the *ADE2* gene and approximately 1.4 and 2.1 kb of *ura3* and adjacent sequences on either side. Likewise, pUR3A was digested with *Pst*I which releases a fragment containing the *ADE2* gene and approximately 0.65 and 1.4 kb of flanking *C. albicans* DNA. *C. albicans ade2* mutants A81-Pu and SGY-129 were transformed with these digests, and Ade$^+$ transformants were selected. Compared with uncut controls a 4- to 20-fold enhancement of the transformation frequency was obtained by linearizing pSM1825, but no enhancement was seen with linearized pUR3A (Table 2). The transformation frequencies of the uncut plasmids were similiar to those described by Kurtz et al. (14). Putative disrupted transformants were distinguished from revertants by colony hybridization to the 1.1-kb *Pst*I-*Bam*HI fragment of pBR322, since the 2.5-kb *Eco*RV *ADE2* fragment has 190 bases of pBR322 complementary to this probe. All 43 pSM1825–A81-Pu transformants tested hybridized to the probe, and 10 of 13 pSM1825–SGY-129 transformants were positive. Fewer positive colonies were obtained with the pUR3A digests; only 10 of 77 A81-Pu transformants and 11 of 28 SGY-129 transformants hybridized to the probe. Occasionally, a few of the pUR3A transformants hybridized very intensely to pBR322 DNA. It is possible that these transformants contain vector sequences and may be due to integrative transformation by circular uncut plasmid DNA. The other positive transformants were considered as potential *ura3*/*URA3* disruptants.

Disruption of the *URA3* gene on one chromosomal homolog. To determine if the mutated DNA had replaced the wild-type *URA3* gene on one chromosomal homolog, Southern blot hybridizations were performed. *Sst*I-*Bgl*II digests of genomic DNA from four pSM1825 transformants of A81-Pu and five pSM1825 tranformants of SGY-129 were hybridized to pMC5. The *URA3* gene is on a single 5.5-kb *Sst*I-*Bgl*II fragment (Fig. 1B), and there are no sites for these enzymes in the *ADE2* insertion. pMC5 was chosen for the *URA3* probe since it does not hybridize to the pBR322 DNA in the *ADE2* insert. The results obtained with two A81-Pu and two SGY-129 transformants are presented in Fig. 2A and B, respectively. A single 5.5-kb fragment hybridized to pMC5 in the parent strain A81-Pu, as predicted (Fig. 1A, lane 1). In addition to this fragment, a new 7.9-kb fragment hybridized in the two transformants (pSM1825B1 and -B2, lanes 2 and 3). The 7.9-kb fragment is about 2.5-kb larger than the 5.5-kb fragment, consistent with a replacement of the wild-type DNA with the mutated DNA containing the 2.5-kb *ADE2* insertion. When identical digests were hybridized to the 1.1-kb *Pst*I-*Bam*HI fragment of pBR322, only the new 7.9-kb fragment present in the transformants (lanes 5 and 6) was detected. This confirms the presence of the *ADE2* insertion. The two additional A81-Pu transformants analyzed had hybridization patterns identical to those of pSM1825B1 and -B2 (data not presented). Similar results were obtained for the pSM1825 transformants of SGY-129 when *Sst*I-*Bgl*II digests were also probed with pMC5 and pBR322 (panel B). Two of the additional SGY-129 transformants analyzed were

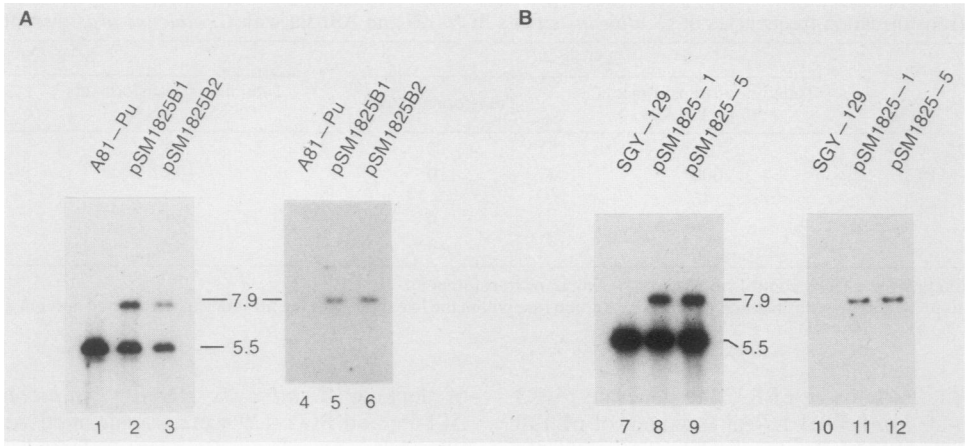

FIG. 2. Autoradiograms of Southern blot hybridizations of *Sst*I-*Bgl*II digests of DNA from A81-Pu (A) and SGY-129 (B) transformed with *Pst*I-*Hpa*I digests of pSM1825. The DNAs were hybridized to the following probes: lanes 1 to 3 and 7 to 9, pMC5; lanes 4 to 6 and 10 to 12, 1.1-kb *Pst*I-*Bam*HI fragment of pBR322. Molecular sizes (in kilobases) of fragments are indicated in margins.

identical to pSM1825-1 and -5, but one transformant had an entirely different hybridization pattern (data not presented) and was not analyzed further.

Similiarly, *Sst*I-*Bgl*II digests of the pUR3A transformants were hybridized to the 1.4-kb *Pst*I-*Eco*RI fragment of pUR3. In pUR3A approximately 4.0 kb of *URA3* and its flanking sequence has been replaced with 2.5 kb of the *ADE2* sequence. If transformation occurred by a direct replacement (of the wild-type DNA with the mutated DNA), a new fragment approximately 1.5 kb smaller than the original 5.5-kb fragment (i.e., 4.0 kb) would be present in the transformants. The results obtained with two transformants of A81-Pu and two of SGY-129 are presented in Fig. 3A and B, respectively). As predicted, in the parent strains (lanes 1 and 7) a 5.5-kb *Sst*I-*Bgl*II fragment hybridized to the probe and a new 3.9-kb fragment was detected in the majority of the transformants analyzed (lanes 2, 3, 8, and 9). As was true for the pSM1825 transformants, only the new fragment hybridized to pBR322 DNA (lanes 5, 6, 11, and 12). Three of five additional transformants had identical hybridization patterns (data not presented). The transformants with aberrant hybridization patterns were not studied further, but it is interesting that one such SGY-129 transformant had initially hybridized very intensely by colony hybridization to pBR322 DNA and we suspected that it had not arisen by a simple gene replacement.

Evidence for restriction site dimorphism at the *URA3* locus and for heteromorphism between strains. Experiments to confirm the data in the previous section by Southern analysis with *Eco*RI led to the discovery of restriction site polymorphisms at the *URA3* locus. pMC5 detected 2.2-, 4.8-, and 10.5-kb *Eco*RI fragments in A81-Pu and SC5314 (the latter is the strain from which the *URA3* gene was cloned) (Fig. 4B, lanes 1 and 2). Based on the restriction map of the cloned *URA3* gene, only a 2.2- and a 4.8-kb *Eco*RI fragment would be predicted to hybridize to pMC5 in SC5314. Previously, the 10.5-kb *Eco*RI fragment is SC5314 complementary to a *C. albicans URA3* probe was attributed to incomplete digestion of the DNA (8). However, we did not obtain any other partially digested *Eco*RI fragments in

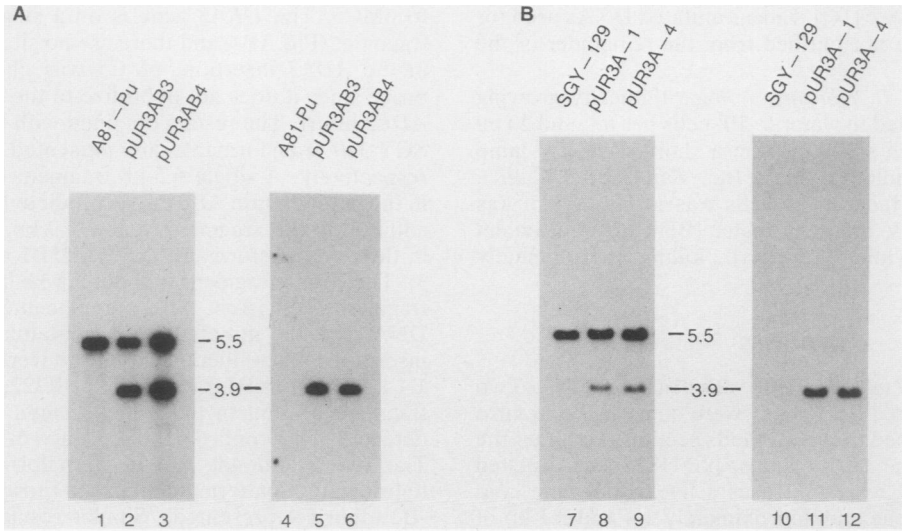

FIG. 3. Autoradiograms of Southern blot hybridizations of *Sst*I-*Bgl*II digests of DNA from A81-Pu (A) and SGY-129 (B) transformed with *Pst*I digests of pUR3A. The DNAs were hybridized to the following probes: lanes 1 to 3 and 7 to 9, 1.4-kb *Pst*I-*Eco*RI fragment of pUR3; lanes 4 to 6 and 10 to 12, 1.1-kb *Pst*I-*Bam*HI fragment of pBR322. The molecular sizes (in kilobases) of fragments are indicated in margins.

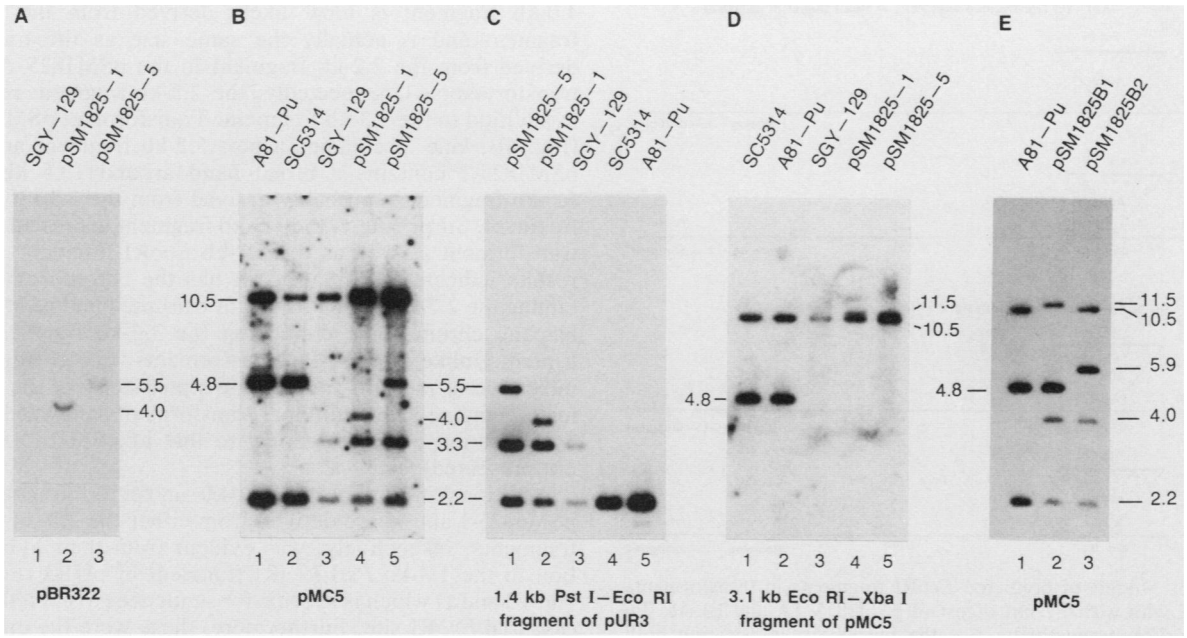

FIG. 4. Autoradiograms of Southern blot hybridizations of *Eco*RI digests of DNA from SC5314, A81-Pu, SGY-129, and transformants of the latter two strains. Probes used in each hybridization are indicated below the panels. The molecular sizes (in kilobases) of fragments are indicated in the margins.

these experiments. An alternative explanation is that this fragment results from a dimorphism of the *Eco*RI sites between the two chromosomal homologs. Surprisingly, in SGY-129 (lane 3), 2.2-, 3.3-, and 10.5-kb *Eco*RI fragments were detected by pMC5. Models depicting the positions of the *Eco*RI sites at the *URA3* locus and the dimorphism in all three strains are presented in Fig. 5. Evidence for these models was derived from Southern hybridizations with probes specific for sequences on either side of the *Eco*RI site within the functional region of the *URA3* gene. Both the 2.2- and 3.3-kb *Eco*RI fragments in SGY-129 hybridized to the 1.4-kb *Pst*I-*Eco*RI fragment (Fig. 4C, lane 3), demonstrating that they are overlapping, whereas only a 2.2-kb fragment was detected in SC5314 and A81-Pu (Fig. 4C, lanes 4 and 5). In addition, neither the 2.2- nor the 3.3-kb fragment hybridized to the 3.1-kb *Eco*RI-*Xba*I fragment (panel D, lane 3), further ruling out the possibility that the 3.3-kb fragment arises from an *Eco*RI site to the right of the *URA3* gene (Fig. 5). Both the 4.8- and 10.5-kb *Eco*RI fragments in SC5314 and A81-Pu hybridized to the 3.1-kb *Eco*RI-*Xba*I probe (Fig. 4D, lanes 1 and 2), indicating that they must have common sequences, but only the 10.5-kb fragment was detected in SGY-129 (Fig. 4D, lane 3). Further evidence for the origin of all of these restriction fragments was provided by Southern hybridizations of double digests of *Eco*RI and other restriction enzymes mapped on the cloned gene. These experiments demonstrated that restriction sites predicted to be on the 2.2- and 4.8-kb fragments were found on the 3.3- and 10.5-kb fragments as well (data not presented). Additional evidence was provided by Southern analysis of the disrupted transformants and is presented in the following section.

Disruption of either chromosomal homolog containing the *URA3* gene. The restriction site dimorphism of the *Eco*RI sites at the *URA3* locus is useful for distinguishing between chromosomes containing the *URA3* gene. Hybridization of *Eco*RI digests of the transformants to pMC5 allowed us to determine which homolog was disrupted. The 2.5-kb *Eco*RV *ADE2* fragment has one *Eco*RI site located approximately 1.5 kb to the right of the point of insertion into *Candida* DNA in both pSM1825 and pUR3A (Fig. 1). If the *URA3* gene is disrupted with pSM1825 in A81-Pu, a new *Eco*RI fragment approximately 1.5 kb larger than the 2.2-kb *Eco*RI fragment should appear in the transformants (in addition to the 2.2-kb fragment illustrated in Fig. 6A). In addition, either the 4.8- or 10.5-kb *Eco*RI fragment should be replaced by a new *Eco*RI fragment approximately 1.0 kb larger, depending upon which homolog has been disrupted. The results obtained with the pSM1825–A81-Pu transformants are presented in Fig. 4E. In

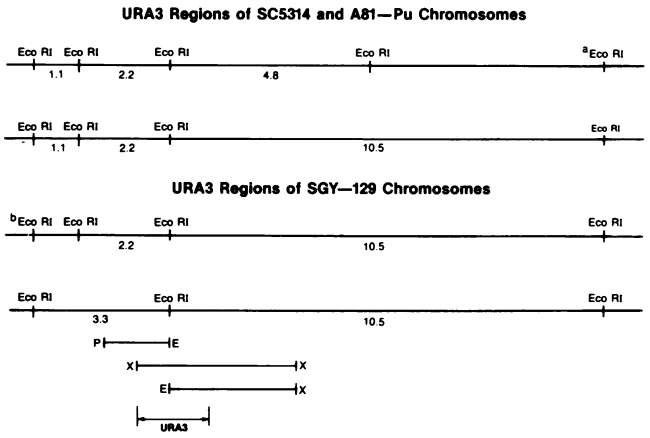

FIG. 5. Models demonstrating heteromorphism and restriction site dimorphism of the *Eco*RI sites at the *URA3* locus of three *C. albicans* strains. It is possible that each *URA3* homolog of SGY-129 is duplicated (see Discussion). Numbers below the chromosomes designate the sizes (in kilobases) of the *Eco*RI fragments. ⊢⊣, fragments used as hybridization probes; P, *Pst*I; E, *Eco*RI; X, *Xba*I; [a], *Eco*RI-*Eco*RI fragment consistent with this *Eco*RI site in SC5314 and A81-Pu was detected by Southern hybridization but its precise origin was not rigorously proven; [b], *Eco*RI-*Eco*RI fragment consistent with this *Eco*RI site could not be resolved because of hybridization of unidentified material in this region of the gel.

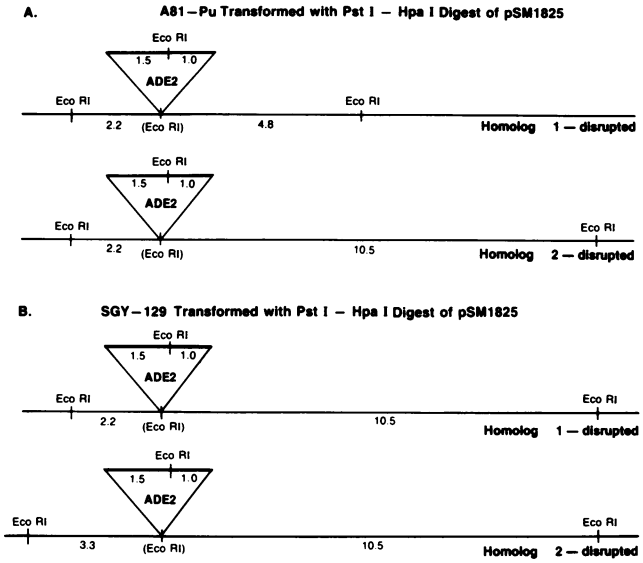

FIG. 6. Models of predicted *Eco*RI fragments in transformants disrupted with a *Pst*I-*Hpa*I digest of pSM1825. (A and B) A81-Pu and SGY-129, respectively. For the purpose of illustration both homologs are shown disrupted in each transformant. However, no transformants were found in which both homologs were simultaneously disrupted. ( ), *Eco*RI site which is present in the undisrupted chromosome that has been destroyed by the disruption.

transformant pSM1825B1 (lane 2), 2.2-, 4.0-, 4.8-, and 11.5-kb fragments were detected by pMC5. When the hybridization pattern is compared with that obtained with the parent strain (lane 1), it appears that the chromosome containing the 10.5-kb *Eco*RI fragment has been disrupted and an 11.5-kb fragment has replaced the 10.5-kb fragment. However, in transformant pSM1825B2 (lane 3) the 10.5-kb fragment is present and the 4.8-kb fragment has been replaced by a 5.9-kb fragment. This transformant also contains the 2.2- and 4.0-kb *Eco*RI fragments and must have the other homolog disrupted. Since pSM1825B1 and -B2 had identical hybridization patterns indicative of a disrupted *URA3* gene when *Sst*I-*Bgl*II digests were hybridizd to pMC5 (Fig. 2A), the differences in these hybridization patterns must be due to the disruption of different homologs. These data demonstrate that A81-Pu has two chromosomes containing the *URA3* gene, which is not unexpected if the strain is diploid. Two additional transformants were analyzed, and both had hybridization patterns identical to that of pSM1825B1 (data not presented). A similiar analysis of the pUR3A–A81-Pu transformants demonstrated that one transformant had homolog 1 disrupted and two transformants had homolog 2 disrupted (data not presented).

Transformants of SGY-129 have multiple copies of the *URA3* locus. A parallel analysis of the pSM1825–SGY-129 transformants also demonstrated that either homolog can be disrupted. However, these transformants appeared to have more than two copies of the *URA3* gene. We expected that either the 2.2- or 3.3-kb *Eco*RI fragments would be replaced by a fragment 1.5 kb larger, as illustrated in Fig. 6B. The 10.5-kb *Eco*RI fragment common to both homologs should be detected as well as a fragment about 1.0 kb larger. As shown in Fig. 4B, lane 4, transformant pSM1825-1 contains a new 4.0-kb *Eco*RI fragment in addition to what appears to be a broad band around 10.5 kb. (The broad band is most likely a composite of a 10.5-kb fragment and an 11.5-kb fragment which cannot be resolved in this gel system.) The 4.0-kb fragment is most likely derived from the 2.2-kb fragment and is actually the same size as the fragment derived from the 2.2-kb fragment in the pSM1825–A81-Pu transformants. Unexpectedly, the 2.2-kb fragment remains in addition to the 3.3-kb fragment. Transformant pSM1825-5 (Fig. 4B, lane 5) contains a new 5.5-kb fragment and like pSM1825-1 contains a broad band around 10.5 kb. The 5.5-kb fragment is probably derived from the 3.3-kb *Eco*RI fragment. Surprisingly, the 3.3-kb fragment is present in this transformant as well as the 2.2-kb *Eco*RI fragment. These results indicate that pSM1825-1 has the chromosome containing the 2.2-kb *Eco*RI fragment disrupted and pSM1825-5 has the chromosome containing the 3.3-kb fragment disrupted. Unlike the A81-Pu transformants, *Eco*RI fragments indicative of more than one undisrupted copy of the *URA3* locus remain. Two additional transformants analyzed had a hybridization pattern identical to that of pSM1825-5 (data not presented).

Additional proof that the new 4.0- and 5.5-kb fragments in pSM1825-1 and -5 are derived from either the 2.2- or 3.3-kb fragments, respectively, was evident from their hybridization to the 1.4-kb *Pst*I-*Eco*RI fragment of pUR3 (Fig. 4C, lanes 2 and 1) which is specific for sequences to the left of the essential *Eco*RI site. Furthermore, these were the only two fragments that hybridized to pBR322 DNA (Fig. 4A, lanes 2 and 3), confirming that they contained the *ADE2* insertion. Finally, the broad band that is detected by pMC5 in pSM1825-1 and pSM1825-5 (panel B) appears to be a doublet in panel D, lanes 4 and 5. (This was much clearer on the original autoradiogram.) These gel blots were hybridized to the 3.1-kb *Eco*RI-*Xba*I fragment which, as stated earlier, is specific for sequences to the right of the essential *Eco*RI site. The new fragment (compare lanes 4 and 5 with lane 3) is approximately 11.5 kb, consistent with the predictions made in the model. The presence of more than one undisrupted copy of the *URA3* gene in the transformants suggests that they have more than two copies of the *URA3* locus. In addition, attempts to isolate a homozygous *ura3* mutant from either the pSM1825- or pUR3A-disrupted SGY-129 transformants were unsuccessful.

Isolation and characterization of *ura3* mutants. Two of the disrupted A81-Pu transformants, pUR3AB3 and pSM1825B1, were treated with UV irradiation to induce mitotic recombination. Then, 1,500 mutagenized colonies of pUR3AB3 and twelve thousand of pSM1825B1 were screened for uracil auxotrophs. Five uracil-requiring mutants were isolated from the pUR3AB3 mutagenesis (1/300), and 17 were isolated from the pSM1825B1 mutagenesis (1/700). Evidence that all five mutants isolated from the pUR3AB3 mutagenesis were *ura3* deletion mutants was obtained by colony hybridization. None of these mutants hybridized to pMC5, whereas pUR3AB3, a strain with the *URA3* gene deleted from only one chromosome, hybridized strongly (data not presented). One *ura3* deletion mutant, pUR3AB3-9B (SGY-243), was chosen for more detailed study.

pUR3AB3-9B and several of the putative insertion mutants (pSM1825B1-59A, -22A, -65C, and -43A) were analyzed by Southern hybridization to determine if a recombination had occurred to render them homozygous for the *ura3* disruption. Gel blots of *Sst*I-*Bgl*II digests of genomic DNA from the putative insertion mutants were hybridized to pMC5, and the results are presented in Fig. 7A. Each of the four mutants (lanes 3 to 6) contains only a single 7.9-kb restriction fragment, the same size as the disrupted fragment in the original transformant pSM1825B1 (lane 2). Likewise,

TABLE 3. Transformation frequencies of *C. albicans ura3* mutants to URA$^+$ with plasmid DNAs

Plasmid (reference)	*URA3* fragment	Vector (reference)	No. of URA$^+$ transformants of indicated strain/μg of DNA	
			SGY-243	SGY-269
pET5 (8)	15.0-kb *Sau*3A-*Sau*3A insert	YEp13 (6)	17	14
pET16 (8)	Same as above	pBR322 (3)	8	ND[a]
pUR3	6.5-kb *Pst*I-*Pst*I insert	pBR322	ND	4
pET19 (8)	Same as above	pJH484 (8)	ND	12
pET18 (8)	5.2-kb *Pst*I-*Bgl*II insert	pET1 (8)	ND	5
pMC6	4.0-kb *Xba*I-*Xba*I insert	pUC12 (18)	6	ND
pMC5	Same as above except insert in opposite orientation		ND	41
pET39	1.7-kb *Xba*I-*Eco*RV insert	pUC12	ND	125
pSM7	None	pSM7	0	0

[a] ND, Not determined.

in the deletion mutant (Fig. 7B, lane 3), a single 3.9-kb fragment was detected when identical blots were hybridized to the 1.4-kb *Pst*I-*Eco*RI fragment. These results are consistent with the occurrence of a recombination outside the *URA3* locus.

The reversion frequencies to uracil prototrophy of the deletion mutant and the insertion mutant were determined to be less than one of the 5×10^9 and 4×10^9 colonies tested, respectively. These strains will be useful for transformation since a step to distinguish bona fide transformants from revertants should not be necessary. To prove this, the *ura3* mutants were transformed to uracil prototrophy with the plasmid DNAs listed in Table 3. As expected, both SGY-269 and SGY-243 were transformed with pET5 and pET16, two plasmids which contain the *URA3* gene and homologous sequences for integration. SGY-269 could also be transformed with the remainder of the *URA3* plasmids. It was, however, surprising to find that SGY-243 was also transformed with pMC6, a *URA3* plasmid with no homology to the SGY-243 genome. To demonstrate that these strains were not revertants, several of the transformants were tested by colony hybridization for the presence of sequences unique to the transforming plasmid. We hybridized 22 pET16, 22 pET5, and 4 pMC6 transformants of SGY-243 to pMC5, and all were positive. The SGY-269 transformants were hybridized to the 3.2-kb *Pst*I-*Bam*HI fragment of pBR322 DNA which does not contain the pBR322 sequences present in the *ADE2* insertions of the mutant. Nine transformants from each transformation with pET5, pET18, pET19, or pUR3 were tested, and all were positive. The high proportion of true transformants obtained with both recipient strains is not unexpected for mutants with nonreverting *ura3* mutations.

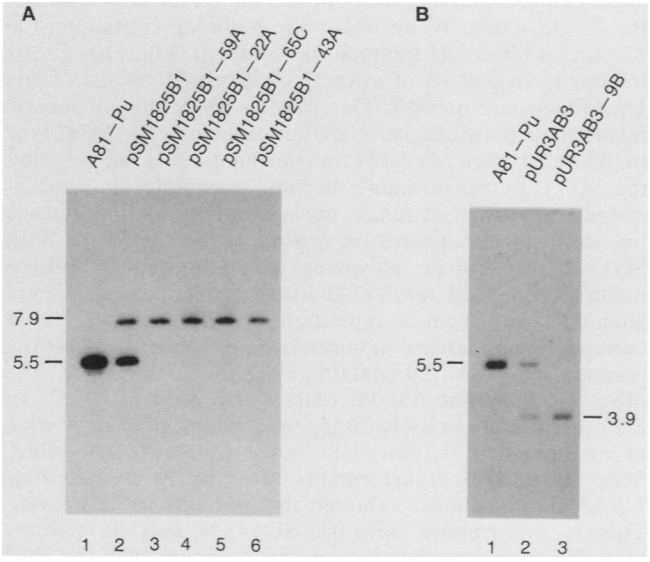

FIG. 7. Autoradiograms of Southern blot hybridizations of *Sst*I-*Bgl*II digests of DNA from *ura3* mutants. DNA in panel A was hybridized to pMC5 and that in panel B was hybridized to the 1.4-kb *Pst*I-*Eco*RI fragment of pUR3. The molecular sizes (in kilobases) of fragments are indicated in the margins.

S. cerevisiae strains with a wild-type *URA3* gene are sensitive to 5-FOA, whereas *ura3* (and *ura5*) mutants are resistant (2). SC5314, A81-Pu, and SGY-129 are also sensitive to 5-FOA; in fact, they exhibit greater sensitivity to 5-FOA than does *S. cerevisiae*. Whereas 50 μg of 5-FOA yielded a 10-mm zone of inhibition of *Candida* growth, 400 μg was required to achieve a comparable inhibition of *S. cerevisiae* growth. In addition, the *C. albicans URA3* gene was able to confer sensitivity to a resistant *S. cerevisiae ura3* mutant when integrated into the genome of the mutant (data not presented). As shown in Table 4, both *C. albicans ura3* mutants are resistant to 5-FOA, whereas a strain with one disrupted *ura3* gene is as sensitive as the wild-type strain. Therefore, inactivation of both copies of the *C. albicans URA3* gene is necessary to confer resistance to 5-FOA.

DISCUSSION

The results presented here provide a method for isolating specific mutants in the diploid asexual organism *C. albicans*, using the one-step gene disruption technique described for *S. cerevisiae* (25). Both a *ura3* deletion mutant and a mutant

TABLE 4. Sensitivity of *C. albicans ura3* mutants to 5-FOA[a]

Strain	Zone of inhibition with indicated amt (μg) of 5-FOA/disk (mm)		
	25	50	100
A81-Pu (*URA3/URA3*)	0	10, 10.5	11, 15.5
pUR3AB3 (*URA3/ura3*)	0	10	15.5
SGY-243 (*ura3/ura3*)	0	0	0
SGY-269 (*ura3/ura3*)	0	0	0

[a] 5-FOA was applied to ¼-inch-diameter analytical disks. Pour plates were made with a washed inoculum of *C. albicans* and incubated with the disks at 30°C.

containing an insertional inactivation of the *URA3* gene were isolated.

It is noteworthy that the transformation frequencies of *C. albicans* with the two different linearized mutated DNAs differed (Table 2). Transformation of either of two *Candida ade2* mutants with *Pst*I-*Hpa*I digests of pSM1825 enhanced the transformation frequencies when compared with uncut pSM1825 DNA. This is in agreement with previous observations of both *C. albicans* (unpublished result) and *S. cerevisiae* (21). However, linearizing pUR3A DNA did not affect the transformation frequency in either strain. This is not due to the presence of a deletion because we recently transformed SGY-243 with a plasmid containing a 4.0-kb deletion of another gene and obtained an increase in the number of transformants by linearizing the DNA (unpublished result). One explanation for the results obtained with *Pst*I digests of pUR3A is that only one end of the linear DNA may contain sequences homologous to the *URA3* locus. We have evidence that sequences to the right of the *Bgl*II site of the *URA3* region (Fig. 1B) may not be contiguous with sequences in the genome (unpublished observations) because of a cloning artifact in the original isolate pET5. One of the *Pst*I sites used to linearize pUR3A for transformation falls in this region of uncertain origin. It is possible that molecules with only one homologous end are still capable of integrating, albeit at a low frequency, or alternatively that only those molecules in which the nonhomologous portion has been degraded can actually integrate. Successful gene replacement in *S. cerevisiae* has also been reported with a linear molecule which contained one homologous end and 10 base pairs of nonhomologous linker DNA attached to a homologous region at the other end (26).

Regardless of the Southern hybridization strategy used to analyze the Ade^+ transformants, direct evidence was obtained demonstrating that one of the chromosomal homologs containing the *URA3* gene was disrupted in the two different *ade2* recipients. In all, 18 transformants were analyzed, and 15 of them arose by a mechanism consistent with a gene replacement. This was true regardless of the *ade2* recipient or the type of mutated DNA, demonstrating the general utility of this method for introducing specific mutations into the genome of *C. albicans*. Recently, similar one-step gene disruptions have been carried out in both *A. nidulans* and *N. crassa* (19, 22) but only a few of the *Aspergillus* transformants arose by a direct gene replacement, and none of the *Neurospora* transformants had fragments of the size expected for a simple gene replacement. Thus, recombination of linear molecules in *C. albicans* appears to be more similar to *S. cerevisiae* than to recombination in these filamentous fungi.

Only a few of the transformants did not have hybridization patterns consistent with a direct replacement mechanism and may have arisen by integration of circular molecules in the DNA preparations. Although we did not observe any uncut molecules in our digests (based on ethidium bromide staining of DNA digests subjected to electrophoresis), an in vivo ligation may recircularize the original plasmid which then integrates at either the site of the selectable marker or at the *URA3* locus. Such in vivo ligations are known to take place in *S. cerevisiae* (13, 20, 31). Other alternatives, such as integration at the *ADE2* gene or multiple integrations at a nonhomologous site, may also account for the anomalous transformants. Nonhomologous integrations occur in *A. nidulans* and more frequently in *N. crassa* (7, 19, 22). Detailed studies are needed to establish the origin of the transformants with unusual hybridization patterns.

Finding restriction site heteromorphism between SGY-129 and the other two *Candida* strains was not surprising. Differences at the *ADE2* locus between hOG300 and SC5314 were previously reported (14). However, the discovery of restriction site dimorphism of *Eco*RI between chromosomal homologs was unexpected. Interestingly, the dimorphism at the *URA3* locus in all three strains appears to result from the absence of particular *Eco*RI sites which probably occurred from single-base-pair changes rather than rearrangements (Fig. 5). It would be interesting if these strains are actually closely related and the differences between SGY-129 and the others were actually point mutations resulting from repeated mutagenesis of the latter (24). This hypothesis could easily be tested by analyzing the unmutagenized parent of SGY-129, ATCC 10261. To our knowledge this is the first example of allelic restriction fragment length dimorphism, but it is not unprecedented because many strains of *C. albicans* are naturally heterozygous at various loci (32, 33). It is possible that the dimorphism resulted from mating through a sexual cycle yet to be discovered. Regardless of its origin, the occurrence of restriction site dimorphism proved useful for distinguishing between alleles and provided us with an additional genetic marker.

Although several lines of evidence verify the models of restriction site dimorphism presented in Fig. 5, their predictions were borne out most convincingly in the disrupted transformants. A81-Pu transformants disrupted with either pSM1825 or pUR3A DNA contain a new restriction fragment in place of either the 4.8- or 10.5-kb *Eco*RI fragment (present on different homologs), depending upon which homolog was disrupted. The discovery that both homologs are subject to integration events is encouraging because it suggests that both alleles in a given strain could potentially be disrupted in a single transformation. Alternatively, mutants could be isolated by retransformation of a previously disrupted strain if an additional selectable marker was available. The results obtained with A81-Pu show that this strain has only two chromosomal homologs containing the *URA3* locus, a good indication that the strain is diploid.

In contrast, parallel analyses of the SGY-129 transformants demonstrated that they contain more than two copies of the *URA3* locus. When either the homolog containing the 2.2- or 3.3-kb *Eco*RI fragment is disrupted (Fig. 6B), *Eco*RI fragments indicative of several undisrupted copies of the *URA3* locus are present. This would explain the unsuccessful attempts to isolate a *ura3* mutant from either pSM1825- or pUR3A-disrupted SGY-129 transformants. It is unlikely that the SGY-129 transformants became aneuploid as a consequence of protoplast fusion during transformation because the identical transformation procedure was used for both SGY-129 and A81-Pu. However, we do not believe that the multiple copies of the *URA3* locus result from segmental aneuploidy or from a repetition of sequence without a corresponding increase in chromosome number because the parent strain SGY-129 contains twice as much DNA as the other *ade2* mutant A81-Pu (data not presented). Cells of SGY-129 are also visibly larger microscopically, suggestive of an aneuploid strain (data not presented). Therefore, SGY-129 and its transformants most likely contain four *URA3* chromosomes, although this remains to be proven. This is unfortunate because SGY-129 and its parent, hOG300, have a much lower reversion frequency of the *ade2* mutation than does A81-Pu. (DNA determinations of hOG300 suggest that it is also aneuploid [data not presented].)

The validity of the Southern analyses for detecting

heterozygous *ura3* disruptions was authenticated by the isolation of *ura3* mutants. The frequency of isolation was high (about 1/500) and within the range reported previously for revealing other heterozygous mutations by UV-induced mitotic recombinant in *C. albicans* (32, 33). This suggests that the presence of either an insertion or a deletion does not dramatically alter the efficiency of recombination, but a direct comparison with a heterozygous *ura3* point mutation has not been made. Proof that the uracil-requiring mutants had altered *URA3* genes was evident from the Southern hybridizations of *Sst*I-*Bgl*II digests which showed that they were now homozygous for either an insertion or a deletion at the *URA3* locus. None of the *ura3* mutants appeared to result from the induction of a point mutation in the undisrupted chromosome. As expected, the uracil-requiring mutants were transformed to Ura^+ with *URA3* plasmid DNAs, but not by pSM7, an *ADE2* plasmid. Although some of the *URA3* plasmid DNAs used to transform the mutants contain large *Candida* fragments, the plasmid with the smallest *URA3* fragment (1.7 kb in pET39) is sufficient to transform SGY-269 to Ura^+. The ability of pMC6 (a plasmid with no homology to the deletion mutant) to transform SGY-243 suggests that nonhomologous integrations do occur in *C. albicans*. In general, the transformation frequencies of the *ura3* mutants were similar to those of the *ade2* mutants.

The deletion mutants are missing 4.0 kb of *C. albicans* DNA, at least 2.5 kb more than the minimum functional region of the *URA3* gene. The possibility that an additional gene was encoded within the 2.5 kb was examined by Northern analysis (data not presented). When RNA isolated from a wild-type *Candida* strain was hybridized to the 4.0-kb *Xba*I-*Xba*I fragment, two mRNA species were detected, a 1.1-kb transcript and a 2.4-kb transcript. However, when an identical blot was hybridized to the 1.5-kb *URA3 Xba*I-*Sca*I fragment, only the 1.1-kb transcript was detected which must correspond to the *URA3* message. Therefore, it is likely that an additional, nonessential gene has been deleted in the *ura3* deletion mutants and this should be considered when interpreting future studies with these strains.

Both *C. albicans ura3* mutants were resistant to 5-FOA, a characteristic of *S. cerevisiae ura3* mutants, *E. coli pyrF* mutants, and *S. pombe ura4* mutants (all orotidine-5′-phosphate decarboxylase mutants) (2). Since both SGY-243 and SGY-269 were resistant to 5-FOA, their resistance can most likely be attributed to a defective *ura3* gene and not, in the case of SGY-243, to the deletion of an additional gene. The mechanism of 5-FOA action is probably the same in *C. albicans* and *S. cerevisiae*, although why *C. albicans* exhibits greater sensitivity remains to be elucidated. Selection on 5-FOA-containing media has proven extremely useful in several types of experiments with *S. cerevisiae* and will undoubtedly have similar applications for *C. albicans*. In addition, 5-FOA sensitivity could be used to monitor chromosome loss in a heterozygous *ura3* strain, which would be particularly advantageous for a diploid asexual organism.

To our knowledge, this is the first report describing the isolation of a specific *C. albicans* mutant by a directed alteration of the genome and the first description of a *C. albicans ura3* mutant. Until now no other *C. albicans* deletion mutants have been described. The use of a nonreverting deletion mutant has greatly improved our transformations and is particularly useful for gene disruptions when the *URA3* gene is used as the selectable marker. The deletion mutant has also facilitated the characterization of sequences which replicate autonomously in *C. albicans* (14a). The ability to isolate specific *C. albicans* mutants should contribute greatly to understanding the molecular biology of this pathogenic organism.

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