Isogenic Strain Construction and Gene Mapping in *Candida albicans*

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ABSTRACT

Genetic manipulation of *Candida albicans* is constrained by its diploid genome and asexual life cycle. Recessive mutations are not expressed when heterozygous and undesired mutations introduced in the course of random mutagenesis cannot be removed by genetic back-crossing. To circumvent these problems, we developed a genotypic screen that permitted identification of a heterozygous recessive mutation at the *URA3* locus. The mutation was introduced by targeted mutagenesis, homologous integration of transforming DNA, to avoid introduction of extraneous mutations. The *ura3* mutation was rendered homozygous by a second round of transformation resulting in a Ura$^-$ strain otherwise isogenic with the parental clinical isolate. Subsequent mutation of the Ura$^-$ strain was achieved by targeted mutagenesis using the *URA3* gene as a selectable marker. *URA3* selection was used repeatedly for the sequential introduction of mutations by flanking the *URA3* gene with direct repeats of the *Salmonella typhimurium hisG* gene. Spontaneous intrachromosomal recombination between the flanking repeats excised the *URA3* gene restoring a Ura$^-$ phenotype. These Ura$^-$ segregants were selected on 5-fluoroorotic acid-containing medium and used in the next round of mutagenesis. To permit the physical mapping of disrupted genes, the 18-bp recognition sequence of the endonuclease I-*Sce*I was incorporated into the *hisG* repeats. Site-specific cleavage of the chromosome with I-*Sce*I revealed the position of the integrated sequences.

THE increasing medical significance of *Candida albicans* as an agent of opportunistic fungal infections provides an impetus to discern more about the biology and virulence of this pathogen. Genetic approaches, which have been invaluable in defining virulence determinants of bacterial pathogens, are difficult to apply to *C. albicans* because of its diploid genome and its asexual life cycle. The application of molecular genetic techniques and the development of a transformation system have circumvented some of the problems of genetic analysis (KURTZ, KELLY and KIRSCH 1990).

Integrative transformation of *C. albicans* occurs via homologous recombination (KURTZ, CORTELYOU and KIRSCH 1986) and this property has been exploited for targeted mutagenesis of the genome (KELLY *et al.* 1987). Since *C. albicans* is diploid, mutant alleles introduced by transformation must be rendered homozygous to uncover recessive phenotypes. This has been achieved by UV exposure to stimulate mitotic recombination (KELLY *et al.* 1987; SADHU *et al.* 1992), by sequential transformations using two selectable marker genes (KURTZ and MARRINAN 1989) and more recently by sequential transformation using the same marker gene (GORMAN, CHAN and GORMAN 1991). The last approach, originally developed for *Saccharomyces cerevisiae* (ALANI, CAO and KLECKNER 1987), has been applied to *C. albicans* using *GAL1* as a selectable marker flanked by direct repeats of the bacterial *CAT* gene (GORMAN, CHAN and GORMAN 1991). This construct was inserted into the cloned *URA3* gene and integrated into the genome by homologous recombination. Transformed cells in which the targeted gene was disrupted were selected by complementation of a *gal1* mutation. Subsequent recombination between the *CAT* sequences resulted in excision of the *GAL1* gene and one copy of *CAT*. These Gal$^-$ derivatives were selected on 2-deoxy-D-galactose containing medium and subjected to a second round of transformation with the *GAL1* construct to yield the homozygous *URA3* disruptions.

Although these methods of targeted mutagenesis hold great promise, their utility is compromised by unknown amounts of genetic diversity in available strains. Since there are no dominant selectable markers useful for transformation of *C. albicans*, transformed cells are selected by complementation of auxotrophic mutations. These mutations are introduced randomly by chemical and UV mutagenesis. Strains treated in this manner are likely to harbor multiple, undefined mutations that cannot be removed by backcrossing. The genetic consequences of these mutations cannot be anticipated and may result in erroneous interpretation of phenotypes associated with targeted mutations.

We have developed a method of targeted mutagen-

TABLE 1

C. albicans strains

Strain	Parent	Genotype	Source or reference
SC5314			GILLUM 1984
CAF2-1	SC5314	Δ*ura3*::*imm*434/*URA3*	This work
CAF3-1	CAF2-1	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAF4-2	CAF2-1	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAF5-1	CAI-4	Δ*ece1*::*his*G(I-*Sce*I)-URA3-*his*G(I-*Sce*I)/ECE1 Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAF5/1-1	CAF5-1	Δ*ece1*::*his*G(I-*Sce*I)/ECE1 Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAF6-8	CAF5-1/1	Δ*ece*::*his*G(I-*Sce*I)-URA3-*his*G(I-*Sce*I)/Δ*ece1*::*his*(I-*Sce*I) Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI4	CAF2-1	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI5	CAF3-1	*ade2*::*his*G-URA3-*his*G/*ADE2* Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI6	CAI5	*ade2*::*his*G/*ADE2* Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI7	CAI6	*ade2*::*his*G-URA3-*his*G/*ade2*::*his*GΔ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI8	CAI7	*ade2*::*his*G/*ade2*::*his*G Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work
CAI9	CAF3-1	*ade2*::*URA3*/*ADE2* Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	This work

esis to introduce mutations into clinical isolates. The method relies on a genotypic screen to identify recessive mutations introduced by transformation. The heterozygotes are identified by the polymerase chain reaction (PCR)-based recombinant fragment assay of KIM and SMITHIES (1988) and isolated by sib selection (MCCORMICK 1991). A second round of transformation is used to mutate the second allele. Using this approach, we have generated strains deleted at the *URA3* locus. Subsequent targeted mutagenesis of the locus of interest in these Ura⁻ strains was achieved by direct application of the sequential disruption technique of ALANI, CAO and KLECKNER (1987). In addition to permitting sequential gene disruptions to be done, we have modified this approach to allow rapid physical mapping of the disrupted gene.

MATERIALS AND METHODS

Strains and culture conditions: The *C. albicans* strains employed in this study are listed in Table 1. The strains were routinely maintained on YPD medium (SHERMAN, FINK and HICKS 1986). Minimal defined medium consisted of 2% glucose supplemented with yeast nitrogen base (DIFCO). Media were supplemented with uridine (25 μg/ml) or adenine (40 μg/ml) as required. For regenerating spheroplasts, the appropriate medium was supplemented with 1 M sorbitol. Media were solidified with 1.5% agar as required. All cultures were incubated at 30°.

Ura⁻ auxotrophs were selected on medium containing 5-fluoroorotic acid (5FOA; BOEKE, LACROUTE and FINK 1984). The medium was prepared as described by BOEKE, LACROUTE and FINK (1984) except that uracil was replaced with uridine (25 μg/ml). Prior to selection, strains were plated on YPD medium supplemented with uridine and incubated 48 hr at 30°. Individual colonies were taken from the plate and suspended in H_2O. Dilutions of the suspension were spread on minimal medium with uridine to determine the number of colony forming units present and portions were spread on 5FOA medium to select Ura⁻ cells. The 5FOA plates were scored after 3–4 days' incubation.

Plasmid constructions: Plasmid pURA3Δ::λ contains the immunity region of λ*imm* 434 flanked by genomic sequences from the *URA3* locus of *C. albicans*. To construct this plasmid, a 3.9-kb *Nhe*I/*Pst*I fragment containing the *URA3* gene was isolated from plasmid pUR3 (KELLY *et al.* 1987) and ligated into the *Xba*I/*Pst*I sites of pUC18. The *Nhe*I site is located approximately 2.3 kb 5′ of the *URA3* open reading frame (Figure 1). This plasmid was digested at the unique *Eco*RV and *Xba*I sites to delete the *URA3* coding region (KELLY *et al.* 1987). The *URA3* sequences were replaced with a 3.1-kb *Sma*I/*Bam*HI fragment containing the λ *imm*434 region. The *imm*434 region was obtained by subcloning the *Bam*HI/*Bgl*II fragment located between nucleotides 30, 500 and 33, 610 of λgt10 (SAMBROOK, FRITSCH and MANIATIS 1989) into the *Bam*HI site of pUC18 and digestion of this subclone with *Sma*I and *Bam*HI. The DNA fragments were made blunt with Klenow polymerase prior to ligation.

Plasmid pURA3Δ::λ-ΔEco is identical to pURA3Δ::λ except that the *Eco*RI site located within the λ *imm*434 region was destroyed by digestion with *Eco*RI followed by Klenow polymerase treatment and blunt-end ligation.

Plasmid pCUB-6 contains the *C. albicans URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* gene. This plasmid was derived from plasmid pNKY50 (ALANI, CAO and KLECKNER 1987). The *S. cerevisiae URA3* gene was removed from pNKY50 by digestion with *Hin*dIII and the vector ends were made flush with Klenow polymerase. This vector fragment was ligated with a 1365 bp *Sca*I-*Xba*I fragment containing the *C. albicans URA3* gene (LOSBERGER and ERNST 1989). The *Xba*I end was made blunt with Klenow polymerase prior to ligation and was regenerated upon ligation with the filled-in *Hin*dIII end.

Plasmid pAUB contains the *C. albicans ADE2* gene interrupted by *hisG-URA3-hisG* sequences. A 2.5-kb *Eco*RV fragment containing the *ADE2* gene was isolated from plasmid pSM-7 (KURTZ *et al.* 1987) and ligated into the *Sma*I/*Hin*cII sites of pUC18. This construct was cut at the unique *Xba*I site within the *ADE2* gene and blunt-end ligated with a 4-kb *Bam*HI/*Bgl*II fragment containing the *hisG-URA3-hisG* sequences from pCUB-6. The ends of the DNA fragments were made flush with Klenow polymerase. Plasmid pAUX was constructed by ligating a 4-kb *Xba*I fragment containing the *URA3* gene into the *Xba*I site of *ADE2* (KURTZ *et al.* 1987). The 4-kb *Xba*I fragment was isolated from plasmid pUR3 (KELLY *et al.* 1987).

Plasmid pMB-7, which contains I-*Sce*I recognition sites, was derived from plasmid p5921, kindly provided by Dr. Neil Gow. Plasmid p5921 consists of the *Bam*HI/*Bgl*II *hisG-URA3-hisG* fragment from pCUB-6 cloned into the *Bam*HI/*Bgl*II sites of pUC18. The *Bgl*II site was added to pUC18 by addition of a linker at the *Sma*I site. A 28-mer containing

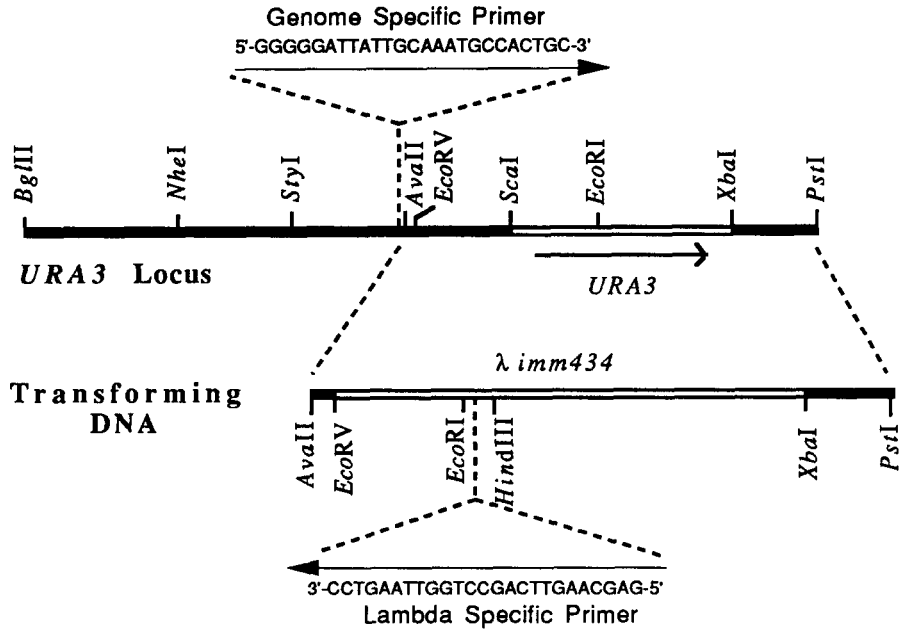

FIGURE 1.—Schematic diagram of the recombinant fragment assay as adapted to identification of a heterozygous *ura3* deletion. Shown are a restriction map of the *URA3* locus, the deletion/substitution construct used in transformation, and the location and sequence of the oligonucleotide primers used in the PCR amplification reaction.

the recognition sequence for I-*Sce*I (Boehringer Mannheim Biochemicals) was ligated into each of the two *Sty*I sites of p5921. One *Sty*I site is located in each of the *hisG* sequences. The ends of both the *Sty*I sites and the 28-mer were made flush with Klenow polymerase prior to ligation. Since the 28-mer is bordered by the sequences 5′-GATCC . . . AGATC-3′, ligation into blunt-ended *Sty*I sites generates *Sty*I sites flanking the insert and creates a *Bam*HI site at one end of each linker. The orientation of linkers was not determined.

Plasmid pECEMB-2 contains the I-*Sce*I modified *hisG-URA3-hisG* sequences from pMB-7 inserted into the *ECE1* gene of *C. albicans*. To construct this plasmid a 4.4-kb *Bam*HI fragment containing the *ECE1* gene (BIRSE, IRWIN, FONZI, and SYPHERD, unpublished data) was blunt-end ligated into the *Pvu*II sites of pBSK$^+$ (Stratagene). The resulting plasmid was digested with *Eco*RI and *Eco*RV to remove the coding region of *ECE1*. A *Sal*I/*Bgl*II fragment containing the I-*Sce*I modified *hisG-URA3-hisG* sequences was isolated from pMB-7 and used to replace the coding region of *ECE1* to generate pECEMB-2. The ends of the DNA fragments were made flush with Klenow polymerase prior to ligation.

Plasmids pUR3 (KELLY *et al.* 1987) and pSM-7 (KURTZ *et al.* 1987) were kindly provided by E. R. Squibb and Sons. Plasmid pNKY50 (ALANI, CAO and KLECKNER 1987) was obtained from S. SANDMEYER (UC Irvine).

Strain construction: Strain CAF2-1 was generated by transformation of clinical isolate SC5314 (GILLUM, TSAY and KIRSCH 1984). Transformation was conducted as described by KURTZ, CORTELYOU and KIRSCH (1986) using 25 μg of pURA3Δ::λ DNA cleaved with the restriction enzymes *Ava*II and *Pst*I. The *Ava*II site is located 242 bp from the *URA3* deletion end point defined by the *Eco*RV site in pURA3Δ::λ. The *Pst*I site is located approximately 600 bp from the *Xba*I deletion end point (Figure 1). Following transformation, the spheroplasts were diluted in 1 M sorbitol and approximately 2.5×10^5 viable spheroplasts were spread on each of four YPD-sorbitol-uridine plates. To obtain transformant pools, an additional 25 plates were spread with approximately 2.5×10^4 viable spheroplasts each. After 4 days of incubation at 30°, each plate was washed with H_2O to pool the regenerated cells. The 25 samples generated from 2.5×10^4 spheroplasts each were arranged in a 5×5 matrix and portions from each sample were pooled in groups of five along the horizontal and vertical axes of the matrix. Genomic DNA was prepared from the pools and PCR amplification was used to detect the presence of integrated transforming sequences. The matrix coordinates were used to identify positive samples, which were then individually tested for verification.

Tenfold enrichments of the desired clone were achieved by subculturing a fraction of the population equivalent to one tenth the cell number used to generate the culture that tested positive (MCCORMICK 1991). In the first round of enrichment, 2 ml of YPD plus uridine was inoculated with approximately 2.5×10^3 cells. Since the frequency with which the positive clone will appear in any subculture was 10^{-1}, 25 cultures were inoculated to provide a probability of 0.93 that one of the subcultures would contain the positive clone (MCCORMICK 1991). After outgrowth, the cultures were arranged in a matrix and analyzed as described for the initial pools. Sequential 10-fold enrichments were continued until a single colony isolate was obtained that tested positive in the PCR assay. Southern blot analysis was performed to verify that the desired integration event had occurred.

Strain CAF3 was isolated as a Ura$^-$ spontaneous mitotic recombinant of strain CAF2-1. Strains CAI-4 and CAF4-2 were constructed by transformation of strain CAF2-1 with 25 μg of *Ava*II and *Pst*I digested pURA3Δ::λ DNA or pURA3Δ::λ-ΔEco DNA, respectively. For these transformations, CAF2-1 was grown overnight in minimal defined medium, rather than YPD medium, to prevent outgrowth of spontaneous Ura$^-$ recombinants. The overnight culture was inoculated into YPD and after one doubling the cells were harvested for transformation. Following transformation, the spheroplasts were spread on YPD supplemented with sorbitol and uridine. The spheroplasts were regenerated on rich medium to allow for the phenotypic lag in expression of the Ura$^-$ phenotype (RONNE and ROTHSTEIN 1988). After 16 hr of incubation, the resulting cells were washed from the plate. Approximately 1×10^7 cells from the sample were spread on 5FOA-containing medium and

incubated at 30° for 3 days. The 5FOA resistant colonies were examined by Southern blot analysis to verify that the anticipated integration event had occurred.

Strain CAI9 was constructed by transformation of strain CAF3-1 (Table 1) with a 5.2-kb *Kpn*I-*Hin*dIII fragment from plasmid pAUX. The *Kpn*I site lies within the polylinker region of the plasmid and the *Hin*dIII site lies within the *ADE2* gene (KURTZ *et al.* 1987). Integration of the DNA fragment at the *ADE2* locus was verified by Southern blot analysis (unpublished data).

Construction of the other strains employed in this study is discussed in RESULTS.

Polymerase chain reaction (PCR) amplification: PCR amplification reactions consisted of a 50-μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 50 μM dNTPs, 0.2 μM of each oligonucleotide primer, and approximately 1 μg of genomic DNA. The reaction was started by the addition of 1 unit of Taq polymerase (Boehringer Mannheim Biochemicals). After an initial incubation at 97° for 1 min, the temperature of the samples was cycled 30 times. Each cycle consisted of a 15-sec incubation at 97°, 1 min at 60°, and 3 min at 72°. The final cycle was extended to 10 min at 72°. The amplification primers were each 25 nucleotides in length. One primer, with the sequence 5′-GGGGGATTATTGCAAATGCCACTGC-3′, was complementary to genomic sequences located 10 bp from the *Ava*II site located upstream of the *URA3* gene. The second primer had the sequence 5′-GAGGAAGTTCAGCCTGGTTAAGTCC-3′ and was complementary to a region located 5 bp from the *Eco*RI site in the *imm*434 DNA.

Genomic DNA isolation and analysis: Genomic DNA used in PCR amplification reactions was isolated as described by SCHERER and STEVENS (1988). Genomic DNA employed in Southern blot analyses was isolated by the same method, excluding the proteinase treatment. Southern blot analysis was conducted as described previously (CHEN and FONZI 1992). The *LYS1* gene, used as a hybridization probe, was kindly provided by S. SCHERER (GOSHORN, GRINDLE and SCHERER 1992).

Pulsed-field gel electrophoresis and I-*Sce*I digestion: Pulsed-field gel electrophoresis was performed with a Bio-Rad contour-clamped homogeneous electric field (CHEF)-DR II system. Chromosomal DNA was prepared in agarose plugs essentially as described by WICKES, GOLIN and KWON-CHUNG (1991). The chromosomes were separated on 1% agarose gels in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA). The electrophoretic conditions of each gel are described in the appropriate figure legends.

I-*Sce*I (Boehringer Mannheim Biochemicals) treatment of DNA in agarose plugs was conducted as described by THIERRY *et al.* (1991) employing the buffer and reagents supplied by the manufacturer. The agarose plugs were pre-incubated overnight at 4° in a solution of 0.1 M diethanolamine, pH 9.5, 1 mM dithiothreitol. The plugs were then incubated for 2 hr at 4° in 100 μl of the buffer supplied by the manufacturer. The solution was replaced with 100 μl of fresh buffer containing 2 μl (40 ng) of "enhancer" oligonucleotide and 30 units of I-*Sce*I. After a 2-hr incubation at 4°, the reaction was initiated with the addition of 5 mM $MgCl_2$. The samples were incubated for 60 min at 37° and the reaction was stopped by dilution with 10 volumes of 0.5× TBE.

RESULTS

Genotypic screen for recessive *URA3* deletion: KIM and SMITHIES (1988) devised a PCR-based, recombinant fragment assay designed to detect low frequency homologous integration events in cultured mammalian cells. The concept underlying this approach is the use of oligonucleotide primers, one of which is specific to sequences found in the transforming DNA, but not found in the genome, and the other of which is specific to genomic sequences, but not found in the transforming DNA. Thus, a DNA segment that can be amplified by PCR exists only on integration of the transforming DNA which results in contiguous primer targets.

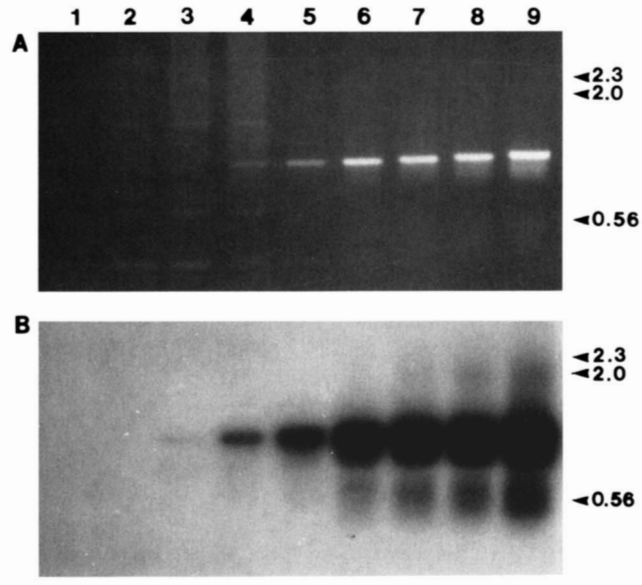

FIGURE 2.—PCR screening of *C. albicans* transformants. (A) PCR reaction products were separated on a 1% agarose gel and visualized with ethidium bromide. (B) A Southern blot of the gel was hybridized with λ*imm*434 DNA and autoradiographed. The samples in lanes 1 and 2, respectively, contained no DNA and DNA from mock transformed cells. The remaining samples contained genomic DNA derived from pools of cells in which the estimated frequency of the desired transformant was 1/250,000 (lane 3), 1/25,000 (lane 4), 1/2,500 (lane 5), 1/250 (lane 6) and 1/25 (lane 7). The sample in lane 8 contained genomic DNA from a single colony isolate, strain CAF2-1. The positive control in lane 9 contained plasmid pURA3Δ::λ DNA. The position and size in kilobases of electrophoretic standards are indicated to the right.

Our adaptation of the recombinant fragment assay for identification of deletions of the *URA3* gene of *C. albicans* is diagramed in Figure 1. We constructed plasmid pURA3Δ::λ by replacing the coding region of the *URA3* gene with a 3-kb fragment containing the *imm*434 region of λgt10. An *Ava*II/*Pst*I fragment from pURA3Δ::λ was used to transform a clinical isolate, SC5314 (GILLUM, TSAY and KIRSCH 1984). After nonselective outgrowth, genomic DNA was prepared and assayed for integrated transforming DNA. In initial experiments, pools of 2.5×10^5 regenerated spheroplasts were examined. A 1.1-kb DNA fragment was found to be specifically amplified from the DNA of transformed cells (Figure 2, lane 3), but absent from the DNA of mock transformed cells (Figure 2,

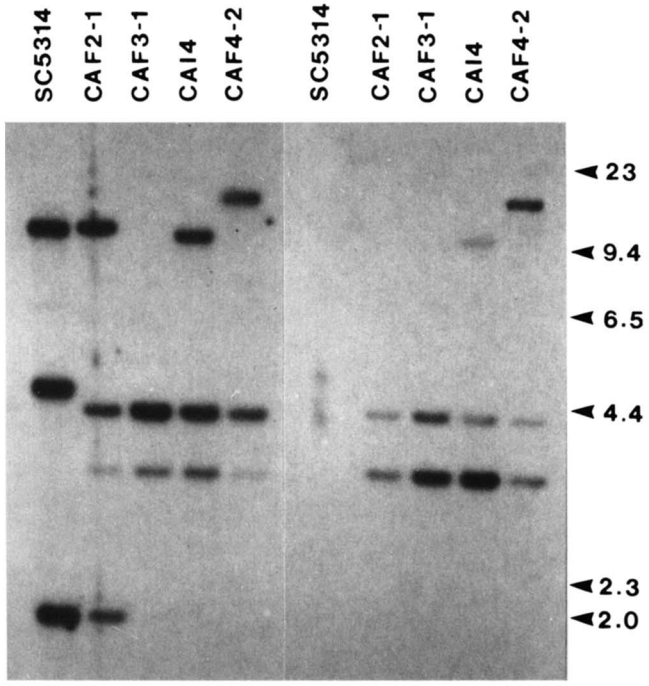

FIGURE 3.—Southern blot analysis of *URA3* deletion strains. Genomic DNA from the indicated strains was digested with *Eco*RI, fractionated on a 1% agarose gel and blotted to nylon membrane. The filter was hybridized with either a 4.8-kb *Pst*I-*Bgl*II fragment containing the *URA3* gene (left panel) or λ*imm*434 DNA (right panel). The position and size in kilobases of electrophoretic standards are indicated to the right.

lane 2). Though not readily visualized by ethidium bromide staining, a band of the expected size was easily detected by hybridization with *imm*434 DNA. Since integrative transformation of *C. albicans* occurs at a frequency of between 3×10^{-6} and 6×10^{-6} transformants per viable spheroplast (KURTZ, CORTELYOU and KIRSCH 1986), these results suggested that the assay was capable of detecting low frequency integration events resulting from transformation.

In the preceding transformation experiment, the transformed spheroplasts were also plated in 25 pools of approximately 2.5×10^4 spheroplasts. Based on the frequency of integrative transformation, only 1 in 10 of these pools on average was expected to contain a transformed cell. Screening of genomic DNA prepared from these pools indicated that two samples yielded the desired PCR product. One sample is shown in Figure 2, lane 4. One of these pools was sequentially subcultured with a 10-fold enrichment at each step until a PCR-positive, single-colony isolate was obtained (Figure 2, lanes 5–8). This isolate was designated strain CAF2-1.

To verify that strain CAF2-1 contained the desired integration event, genomic DNA from this strain was examined by Southern blot hybridization. DNA from the parental strain, SC5314, contained three *Eco*RI fragments that hybridized with *URA3* DNA (Figure 3, left). *Eco*RI cleaves once within the *URA3* coding region, resulting in two DNA fragments from each allele of *URA3*. The 2.1-kb band is derived from the 3′-end of both alleles of *URA3*, while the 4.7-kb and 11.5-kb bands result from a heterozygous restriction site polymorphism upstream of the *URA3* genes (KELLY *et al.* 1987). In strain CAF2-1 the 4.7-kb hybridization band was absent and two new bands were present (Figure 3, left). These new hybridization bands were 3.45 kb and 4.25 kb in length and approximate the restriction fragment sizes expected from integration of the transforming DNA at the *URA3* locus associated with the 4.7-kb RFLP. These new fragments also hybridized with λ *imm434* DNA, as expected (Figure 3, right). We conclude from these results that strain CAF2-1 resulted from homologous integration of the transforming DNA and that this strain is heterozygous for a deletion of the *URA3* locus.

Homozygous *URA3* deletion: Segregants that are homozygous for the *URA3* deletion present in CAF2-1 would be expected to spontaneously arise by virtue of mitotic recombination events. In quantitative plating of strain CAF2-1 on 5FOA-containing medium, the median frequency of Ura$^-$ segregants was 5.3×10^{-6} for five independent samples. To determine whether these Ura$^-$ derivatives arose by mitotic recombination or were a consequence of mutations within the undeleted *URA3* allele, five independent segregants were examined by Southern blot analysis. All five isolates yielded identical results as represented by strain CAF3-1. As seen in Figure 3, strain CAF3-1 contained the 3.45-kb and 4.25-kb λ-hybridizing bands present in CAF2-1, but had lost the 2.1-kb and 11.5-kb fragments associated with the undisrupted allele of *URA3*. These results suggest that strain CAF3-1 resulted from a mitotic recombination event and had become homozygous for the *URA3* deletion.

Although mitotic recombination provides ready access to strains homozygous for recessive mutations, these strains may not be appropriate for subsequent analyses. *C. albicans* has been demonstrated to harbor a number of heterozygous mutations (WHELAN and MAGEE 1981; Whelan and SOLL 1982), of which the extent and distribution are unknown. Selection for homozygosity at one locus may result in homozygosity at a number of loci along the chromosome which harbor these uncharacterized heterozygous mutations. Their presence and their effects would be difficult to discern.

To avoid this problem, the remaining *URA3* gene of strain CAF2-1 was deleted by a second transformation with the *Ava*II-*Pst*I fragment from pURA3Δ::λ. The transformed cells were selected as uridine auxotrophs on 5FOA-containing medium and screened by Southern blot hybridization. While the majority of isolates appeared to be mitotic recombinants similar to strain CAF3-1, approximately 1 in 10

isolates exhibited a restriction fragment pattern consistent with homologous integration of the transforming DNA. Strain CAI4 contained the 3.45-kb and 4.25-kb *Eco*RI fragments seen in strain CAF2-1, but had lost the corresponding 2.1-kb and 11.5-kb fragments (Figure 3, lane 4). A new fragment of approximately 10.5 kb was present which hybridized with *URA3* and lambda DNA. These are the results expected for integration of the transforming DNA into the *URA3* locus associated with the 11.5-kb *Eco*RI polymorphism.

Retention of the *Eco*RI site polymorphism in strain CAI4 was consistent with integration of the transforming DNA at the second allele. However, these results did not exclude the possibility of a gene conversion event which did not include the upstream *Eco*RI site. To demonstrate that integration of the transforming DNA was occurring at the second allele, strain CAF2-1 was transformed with the *Ava*II/*Pst*I fragment from plasmid pURA3Δ::λ-ΔEco. This fragment was identical to the pURA3Δ::λ fragment used to produce strain CAF2-1, except that the *Eco*RI site within the lambda sequences was destroyed. Consequently, this fragment could be readily distinguished in restriction digests.

Southern blot analysis of Ura$^-$ transformants again revealed that, while the majority of isolates appeared to be mitotic recombinants, approximately 1 in 10 isolates exhibited a restriction fragment pattern consistent with homologous integration of the transforming DNA. Strain CAF4-2 exhibited three hybridization bands of 3.45 kb, 4.25 kb and approximately 14 kb (Figure 3, lane 5). The absence of an *Eco*RI site within the lambda sequences prevents scission of the 3.45-kb and 10.5-kb fragments seen in strain CAI4, resulting in the presence of the 14-kb fragment. These results are not readily explained by interchromosomal recombination and therefore argue that the transforming DNA disrupted the second *URA3* locus.

Electrophoretic karyotype of transformed strains: Since spontaneous chromosomal rearrangements occur readily in *C. albicans* (SUZUKI *et al.* 1989; RUSTCHENKO-BULGAC, SHERMAN and HICKS 1990), the electrophoretic karyotype of these strains was examined to verify their genetic integrity. As seen in Figure 4, no gross chromosomal abnormalities were evident in any of the strains. Furthermore, Southern blot analysis demonstrated that the integrated λ *imm*434 sequences were associated with the same chromosomal band that hybridized with *URA3* DNA (Figure 4). These results indicate faithful targeting of the integration events without alteration of gross chromosomal structure.

Sequential gene disruptions: Once a homozygous null mutation has been introduced into a strain by targeted mutagenesis, subsequent transformations can be selected by complementation of the introduced mutation. Our choice of targeting the *URA3* locus for deletion was based on the fact that positive and negative selection schemes exist for this gene and these have been exploited for sequential gene disruptions in *S. cerevisiae*. ALANI, CAO and KLECKNER (1987) described a gene disruption technique that employed a construct consisting of the *S. cerevisiae URA3* gene flanked by direct repeats of the *S. typhimurium hisG* gene. The *URA3* gene served as the selectable marker in transformations. The unique advantage of this strategy is that homologous recombination between the direct repeats of the *hisG* sequences resulted in excision of the *URA3* gene with retention of one copy of *hisG*. Thus, after disruption of the gene of interest, Ura$^-$ segregants can be selected on 5FOA-containing medium and transformed again, using *URA3* as the selectable marker gene.

To test this selection scheme in *C. albicans*, an analogous construct was made replacing the *S. cerevisiae URA3* gene in plasmid pNKY50 (ALANI, CAO and KLECKNER 1987) with the *URA3* gene of *C. albicans* to generate plasmid pCUB6. The 4-kb *Bam*HI-*Bgl*II fragment containing the *hisG-URA3-hisG* sequences was excised from pCUB6 and inserted into the *Xba*I site of the cloned *ADE2* gene. The resulting plasmid, pAUB, was digested with *Kpn*I and *Hin*dIII and used to transform strain CAF3-1 to Ura$^+$.

Ten Ura$^+$ colonies were screened by Southern blot hybridization, and eight exhibited the anticipated hybridization bands when hybridized with *ADE2* DNA. A representative strain, CAI5, is shown in Figure 5. Genomic DNA from this strain contained the 3.0-kb *Eco*RI fragment characteristic of the parental strain CAF3 (Figure 5, lane 1) and two additional hybridization bands 2.3 kb and 4.5 kb in length (Figure 5, lane 2). These latter two bands are of the size expected for insertion of the *hisG-URA3-hisG* sequences within the *ADE2* locus and both fragments hybridized with *URA3* and *hisG* DNA (data not shown). Two *Eco*RI fragments are generated because of the *Eco*RI site present within the *URA3* gene. Thus, the *hisG-URA3-hisG* construct was targeted to the correct locus and yielded a heterozygous disruption.

To determine if intrachromosomal recombination could occur between the *hisG* repeats, Ura$^-$ derivatives of strain CAI5 were selected on 5FOA medium and analyzed by Southern blot hybridization. The median frequency of Ura$^-$ segregants determined from three independent samples was 5.9×10^{-4}, approximately 300 times the median frequency observed with strain CAI9 (data not shown). Strain CAI9 is also heterozygous for a *URA3* disruption of the *ADE2* locus, but the inserted *URA3* gene is not flanked by direct repeats (see MATERIALS AND METHODS).

Ten independent Ura$^-$ segregants were screened by Southern blot hybridization. None of these isolates

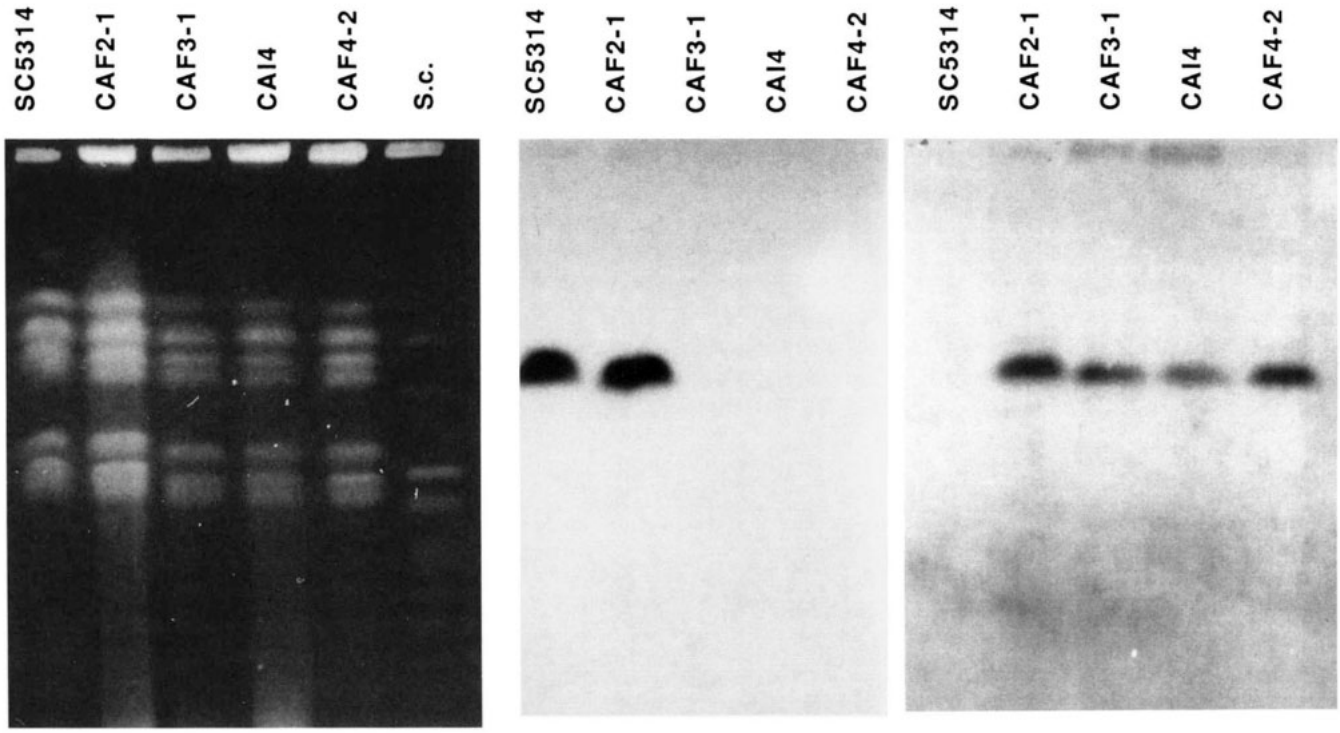

FIGURE 4.—CHEF gel analysis of transformed strains. Chromosome size DNA was prepared from the indicated strains and separated by CHEF gel electrophoresis. The chromosome bands were visualized by ethidium bromide (left panel) and a Southern blot of the gel was hybridized with either a 1.3-kb *Sca*I-*Xba*I fragment containing the *URA3* gene (middle panel) or λ*imm*434 DNA (right panel). DNA from *S. cerevisiae* (BioRad) was run as size standards (left panel, lane S.c.). The samples were electrophoresed at 95 V for 26 hr with 150-sec switch interval and for 26 hr with a 300-sec switch interval. The voltage was lowered to 72 V and electrophoresis was continued for an additional 42 hr with a 1200-sec switch interval.

contained sequences that hybridized with *URA3* DNA, indicating that the Ura$^-$ phenotype resulted from loss of the *URA3* gene and not from inactivating mutations within the *URA3* sequences (data not shown). Nine of the independent isolates appear to have resulted from interchromosomal recombination. Southern blots of genomic DNA from these isolates exhibited a single 3.0-kb *Eco*RI fragment characteristic of the parental strain CAF3, but no additional *ADE2* hybridizing fragments (Figure 5, lane 4). Only one isolate, strain CAI6, yielded results consistent with intrachromosomal recombination. Genomic DNA from this strain exhibited the 3.0-kb parental band and an additional hybridization band of 4.1 kb (Figure 5, lane 3). A 4.1-kb *Eco*RI fragment is the expected result of intrachromosomal recombination with concomitant loss of the *URA3* gene and one copy of the *hisG* sequences. In addition, the 4.1-kb fragment hybridized with *hisG* DNA demonstrating retention of *hisG* sequences (data not shown).

To attempt sequential disruption of the *ADE2* locus, strain CAI6 was transformed with the *hisG-URA3-hisG* disrupted *ADE2* construct. Six Ura$^+$ transformants were analyzed by Southern blot hybridization to determine whether the transforming DNA had replaced the previously disrupted *ADE2* allele or had integrated into the remaining parental allele. Genomic DNA from four of the isolates exhibited a hybridization pattern identical to strain CAI5 (Figure 5, lane 6), indicating that the transforming DNA had replaced the *hisG*-disrupted allele. The remaining two Ura$^+$ isolates exhibited results consistent with targeting of the previously undisrupted *ADE2* allele (Figure 5, lane 5). The 3.0-kb *Eco*RI fragment characteristic of the undisrupted *ADE2* gene was missing and two new fragments of 2.3 kb and 4.5 kb were present. The 4.1-kb hybridization band characteristic of the *hisG* disrupted allele was also present. Both of these isolates were phenotypically Ade$^-$ confirming that no functional *ADE2* alleles were present.

One of the two double disruptants, strain CAI7, was plated on 5FOA-containing medium to select Ura$^-$ segregants. Ura$^-$ derivatives arose at a median frequency of 1.5×10^{-4}, as determined from three independent samples (data not shown). Three Ura$^-$ isolates were screened by Southern blot hybridization and each exhibited a single 4.1-kb *Eco*RI fragment that hybridized with *ADE2* DNA, indicating that both alleles of *ADE2* contained an insertion of the *hisG* gene. A representative isolate, CAI8, is shown in Figure 5, lane 7. The results demonstrated that both alleles of the *ADE2* gene could be successfully targeted and disrupted using the *hisG-URA3-hisG* construct.

Sequential gene disruption and gene mapping:

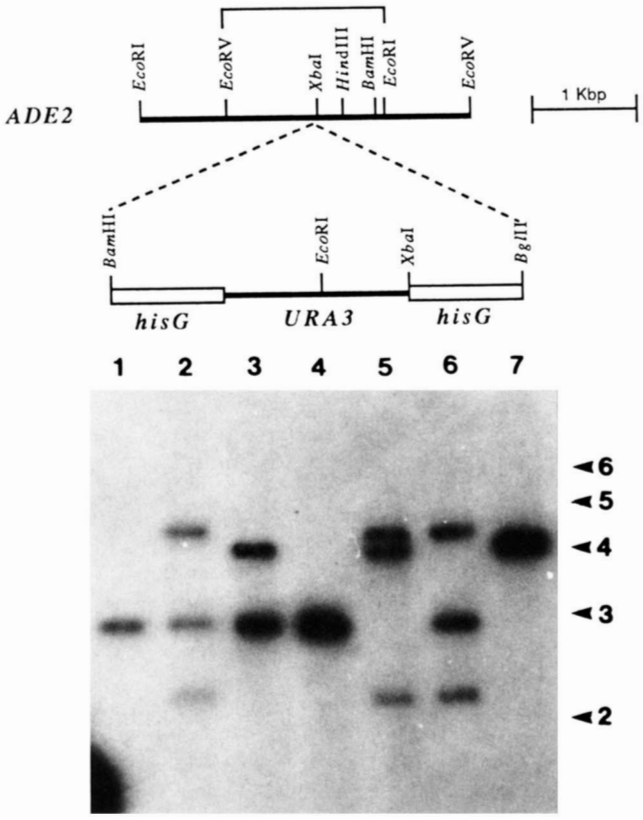

FIGURE 5.—Sequential disruption of the *ADE2* genes. The upper panel depicts a restriction map of the *ADE2* locus and the *hisG-URA3-hisG* insertion. The bracket above the map indicates the sequences used as a hybridization probe. Southern blot analysis of *Eco*RI digested genomic DNA form selected strains is shown in the lower panel. DNA was prepared from the following strains; strain CAF3-1 (lane 1), strain CAI5 (lane 2), strain CAI6 (lane 3), an interchromosomal Ura$^-$ recombinant from CAI5 (lane 4), strain CAI7 (lane 5), a transformant of CAI6 in which the disrupted *ADE2* locus was again targeted (lane 6), and strain CAI8 (lane 7). The position and size in kilobases of electrophoretic standards are indicated to the right.

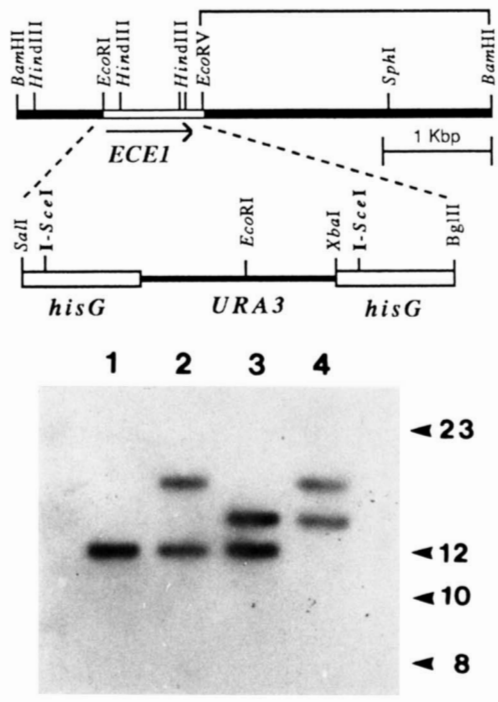

FIGURE 6.—Insertion of I-*Sce*I recognition sites into the *ECE1* locus. The upper panel depicts a restriction map of the *ECE1* locus and the *hisG-URA3-hisG* insertion containing I-*Sce*I recognition sites within the *hisG* repeats. The open bar indicates the coding region of the gene and the arrow below the bar indicates the direction of transcription. The bracket above the map indicates the sequences used as a hybridization probe. Southern blot analysis of *Hin*dIII digested genomic DNA from selected strains is shown in the lower panel. DNA was prepared from strain CAI4 (lane 1), strain CAF5-1 (lane 2), strain CAF5/1-1 (lane 3) and strain CAF6-8 (lane 4). The position and size in kilobases of electrophoretic standards are indicated to the right.

One advantage of using the *hisG-URA3-hisG* construct is that the disrupted genes are tagged with a copy of the repeated sequence. If the repeats were to include the recognition site for I-*Sce*I, then it might be possible to physically map the chromosomal location of the disrupted genes. I-*Sce*I is a site-specific endonuclease encoded within the group I intron of the mitochondrial 21S rRNA gene of *S. cerevisiae*. The recognition site of I-*Sce*I is 18 bp in length (COLLEAUX *et al.* 1988) and statistically is not expected to be present within the *C. albicans* genome. Consequently, digestion with I-*Sce*I would cleave the chromosomes only at the location of the disrupted genes and the length of the resulting fragments would indicate their chromosomal location.

To test this approach, the *hisG-URA3-hisG* construct was altered by inserting a copy of the I-*Sce*I recognition sequence within each of the *hisG* repeats to generate plasmid pMB-7. This construct was used for the sequential disruption of the *ECE1* gene. *ECE1* (*E*xtent of *C*ell *E*longation) is a gene of unknown function whose expression is elevated in association with pseudohyphal and hyphal development of *C. albicans* (BIRSE, IRWIN, FONZI and SYPHERD, unpublished data). An *in vitro* construct, plasmid pECEMB-2, was prepared by replacing a portion of the coding region of the *ECE1* gene with the *hisG-Sce*I-*URA3-hisG-Sce*I sequences (Figure 6). Digestion of pECEMB-2 DNA with *Hin*dIII and *Sph*I released a 6-kb fragment containing the I-*Sce*I construct and flanking sequences from the *ECE1* gene. This DNA was used to transform strain CAI-4 and the resulting Ura$^+$ transformants were screened by Southern blot hybridization to characterize the integration events.

Genomic DNA from one of the transformants, strain CAF5-1, contained two *Hin*dIII fragments that hybridized with *ECE1* DNA (Figure 6, lane 2). One fragment was identical in size to the 12-kb hybridization band detected in digests of DNA from the parental strain (Figure 6, lane 1). The second hybridizing fragment was approximately 16.6 kb in length, as predicted from the cloned sequences. This fragment also hybridized with *URA3* and *hisG* sequences (data not shown).

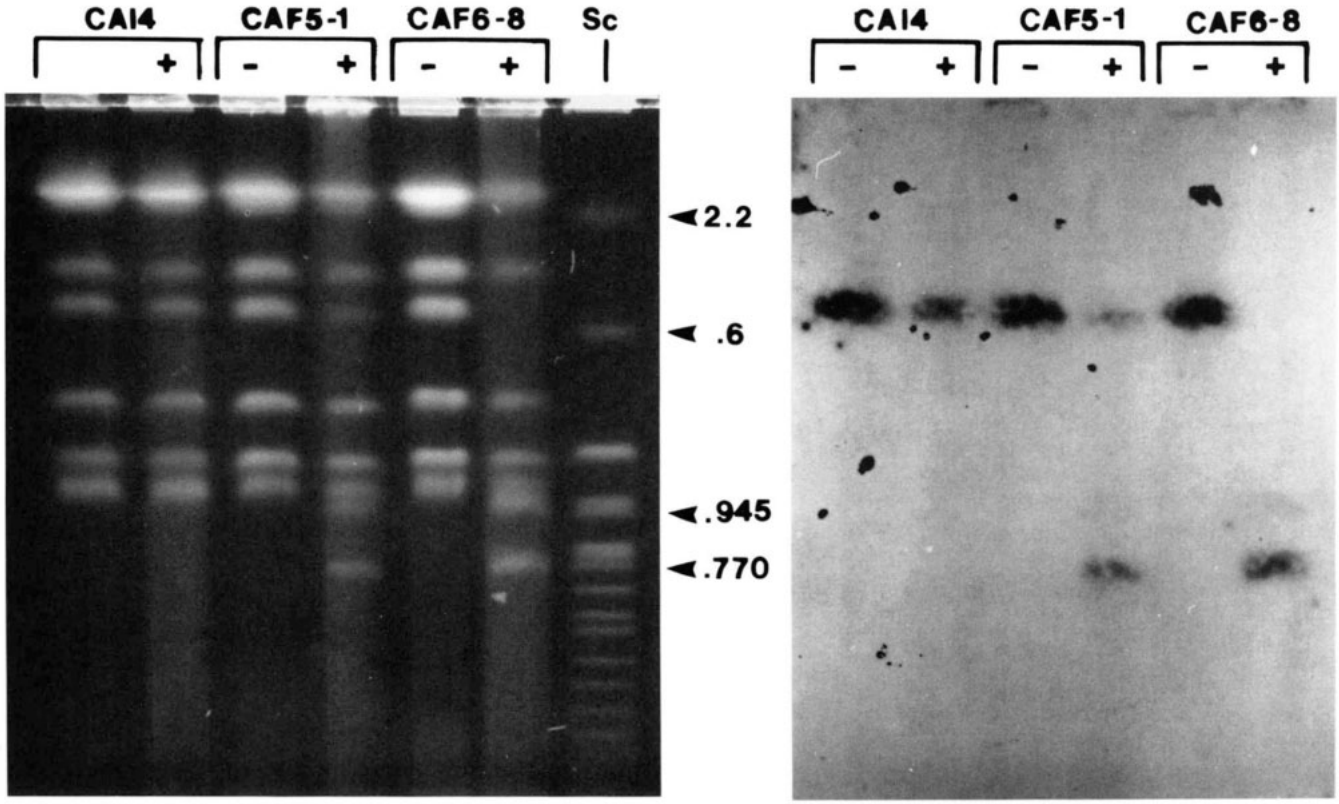

FIGURE 7.—CHEF gel analysis of I-*Sce*I digests. Chromosome size DNA was prepared from the indicated strains and incubated with (+) or without (−) I-*Sce*I. The gel was electrophoresed at 145 V for 48 hr with a linear ramping of the switch interval from 90 sec to 325 sec. The chromosomal bands were visualized with ethidium bromide (left panel) and a Southern blot of the gel was hybridized with a *Bam*HI fragment containing the *ECE1* gene (left panel). The size of several *S. cerevisiae* chromosomes (BioRad) is indicated to the right of the lane labeled Sc.

Strain CAF5-1 gave rise to Ura$^-$ segregants at a median frequency of 3.6×10^{-4}. Six independent Ura$^-$ isolates were examined and all yielded identical Southern blot hybridization patterns, as exemplified by strain CAF5/1-1 (Figure 6, lane 3). The 12-kb *Hin*dIII fragment was unaltered while the 16.6-kb fragment was replaced by an approximately 13.5-kb fragment, consistent with the loss of the *URA3* gene and one copy of the *his* G sequences.

A homozygous disruption of the *ECE1* gene was generated by transformation of strain CAF5/1-1 with the I-*Sce*I construct. Two types of integration events were observed, integration into the previously disrupted *ECE1* allele and integration into the previously unaltered allele. Strain CAF6-8 (Figure 6, lane 4) is representative of integration into the previously unaltered allele and contains I-*Sce*I recognition sites within both alleles of *ECE1*.

The effect of I-*Sce*I treatment was tested on the parental strain CAI4 and on strains CAF5-1 and CAF6-8. Chromosome size DNA in agarose plugs was prepared from each strain and fractionated by CHEF gel electrophoresis with or without prior I-*Sce*I treatment. I-*Sce*I treatment had no effect on the electrophoretic karyotype of CAI4 (Figure 7), indicating that no I-*Sce*I recognition sites were present within the genome of this strain. However, samples from strain CAF5-1, when incubated with I-*Sce*I, exhibited two ethidium bromide staining bands not present in untreated samples. These bands were approximately 960 kb and 740 kb in length (Figure 7). Identically sized bands were also produced by I-*Sce*I treatment of samples from strain CAF6-8. CAF6-8 samples, in addition, lost the 1700-kb chromosomal band present in untreated samples (Figure 7).

Association of the I-*Sce*I cleavage sites with the *ECE1* locus was verified by Southern blot analysis of the CHEF gel. *ECE1* DNA hybridized with the 1700-kb chromosomal band of the parental and disrupted strains, indicating that the *ECE1* gene is normally associated with this chromosome and that the disruptions were targeted to the correct chromosome (Figure 7). The hybridization probe, which spanned the position of I-*Sce*I recognition site insertion, also hybridized with both the 960-kb and 740-kb fragments generated by I-*Sce*I digestion. When DNA fragments were prepared from sequences located 5′ or 3′ of the I-*Sce*I recognition site insertion, these fragments specifically hybridized with the 960-kb and 740-kb chromosomal fragments, respectively (data not shown). These latter results demonstrate that cleavage of the 1700-kb chromosome occurred between the 5′ and

3′ ends of the *ECE1* gene and accurately reflect the insertion site of the I-*Sce*I recognition sequences. The data also indicate that the *ECE1* gene is transcribed toward the telomere of the 740-kb fragment. Subsequent hybridization of the blot with the *LYS1* gene of *C. albicans* demonstrated that the 1700-kb chromosome corresponds to chromosome *IV* (GOSHORN, GRINDLE and SCHERER 1992) and that the *LYS1* gene is located telomere proximal of the *ECE1* gene on the 960-kb fragment (data not shown).

DISCUSSION

Genotypic screening for recessive mutations: *C. albicans* has a diploid genome and lacks a sexual cycle, features that complicate genetic analysis of the organism. The asexual life cycle precludes genetic backcrossing to remove extraneous mutations introduced by random mutagenesis. Consequently, characterization of mutants derived by such an approach is always suspect. Diploidy further complicates analysis since recessive mutations are not expressed phenotypically unless the cells are homozygous for the recessive allele. In other diploid systems these problems have been circumvented using DNA-mediated transformation to effect targeted mutagenesis and thus avoid introduction of extraneous mutations. Dominant resistance markers are used to select transformed cells containing the integrated mutant allele (CRUZ, COBURN and BEVERLEY 1991; MORTENSEN *et al.* 1992; MORTENSEN *et al.* 1991). The locus is rendered homozygous by a second round of transformation selecting for a second resistance marker (CRUZ, COBURN and BEVERLEY 1991; MORTENSEN *et al.* 1991) or by selecting for increased expression of the marker gene initially introduced (MORTENSEN *et al.* 1992). Since *C. albicans* is insensitive to the commonly employed inhibitors, such as G418 or hygromycin, these methods cannot be applied. Although dominant resistance mutations to 5-fluorocytosine and mycophenolic acid have been reported for *C. albicans* (GOSHORN and SCHERER 1989), isolation of the corresponding genes has not been reported.

In the absence of a useful dominant marker gene, we applied a genotypic screen to identify recessive mutations introduced by transformation. This approach relied upon the recombinant fragment assay of KIM and SMITHIES (1988) in which PCR amplification is used to detect the presence of homologous integration events within a pool of cultured mammalian cells. The desired individuals were then isolated by sib selection (MCCORMICK 1991). An analogous approach has been employed with *D. melanogaster* (BALLINGER and BENZER 1989; KAISER 1990). Using this PCR-based screen, homologous integration events at the *URA3* locus were readily detected in pools containing approximately one transformed cell per 2×10^5 cells. Sequential 10-fold enrichments allowed the purification of a single colony isolate that contained the desired integration event as demonstrated by Southern blot analysis. Homozygous Ura$^-$ strains were obtained either by spontaneous interchromosomal recombination or by a second round of integrative transformation.

The strains resulting from these procedures are isogenic with the parental wild-type strain to the extent that extraneous additional mutations are avoided. Although homologous integration was used to target the mutations, transformation may itself induce or select for random mutations. In studies of the *S. cerevisiae* actin gene, SHORTLE NOVICK and BOTSTEIN (1984) found that approximately 1% of the transformants resulting from integrative transformation with *in vitro* mutagenized actin sequences, contained *ts* mutations that were not associated with the actin locus. The precise cause and nature of these mutations were not investigated.

The extent of random mutagenesis associated with transformation of *C. albicans* is unknown. However, no gross chromosomal alterations nor any phenotypic differences were noted between the parental strain and the Ura$^-$ derivatives. When the Ura$^-$ derivatives were converted to Ura$^+$ by transformation with the *URA3* gene, they exhibited the same growth rates and the same rates and extent of germ tube formation as the original parental strain (data not shown).

Sequential gene disruptions: While genotypic screening and sib selection could be applied to the disruption of any gene, the ability to select transformed cells significantly increases the efficiency with which the desired mutants are recovered. The *URA3* gene was targeted for disruption to provide an isogenic strain in which the advantages of this marker could be exploited for subsequent genetic manipulations, in particular, the alternating positive and negative selection scheme introduced by ALANI, CAO and KLECKNER (1987) for sequential gene disruption in *S. cerevisiae*. The success of this approach is based on intrachromosomal recombination between direct repeats flanking the marker gene and the resulting loss of the marker. Intrachromosomal recombination between direct repeats was previously demonstrated for *C. albicans* using the *GAL1* gene flanked by direct repeats of the bacterial *CAT* gene (GORMAN, CHAN and GORMAN 1991). We obtained analogous results using the *URA3* gene flanked by direct repeats of the *S. typhimurium hisG* gene, demonstrating that this approach is independent of the marker gene or repeat sequences employed. However, the type of genetic events resulting in auxotrophy differed between the isolates obtained by selection against the *GAL1* gene and those obtained using selection against the *URA3* marker gene. The Ura$^-$ segregants selected with

5FOA were all devoid of *URA3* sequences. This was in contrast to the Gal$^-$ strains selected with 2-deoxy-D-galactose, wherein 80% of the isolates retained an inactive copy of the *GAL1* gene which was revertible to Gal$^+$ (GORMAN, CHAN and GORMAN 1991). Inactivation of the *GAL1* gene was suggested to be a consequence of the direct repeats flanking the gene. However, since inactivation of the *URA3* gene flanked by *hisG* repeats was not observed, it appears that direct repeats *per se* do not cause modification of the intervening DNA. Consequently, inactivation of the *GAL1* gene must be related to some other factor such as the marker gene itself, the sequence of the repeats or selection with 2-deoxy-D-galactose.

The median frequencies of direct repeat recombination observed in this study, 5.9×10^{-4} and 3.3×10^{-4} for the *ADE2* and *ECE1* loci, respectively, were comparable to those observed in *S. cerevisiae* (ALANI, CAO and KLECKNER 1987). However, these values are as much as 100-fold higher than those observed using the *CAT-GAL1-CAT* construct (GORMAN, CHAN and GORMAN 1991). These differences cannot be ascribed to the selective agents. Spontaneous 5FOA-resistant isolates resulting from interchromosomal recombination at the *URA3* locus of strain CAF2-1 or at the *ADE2* locus of strain CAI9, arose at median frequencies of 5.3×10^{-6} and 1.9×10^{-6}, respectively. These values are comparable to the frequency of interchromosomal recombinants obtained using 2-deoxy-D-galactose selection, 1.7×10^{-6} (GORMAN, CHAN and GORMAN 1991). Since both studies examined recombination at the *URA3* locus, the differences in frequency cannot be attributed to differences in loci either. The difference in length of the flanking repeats, 733 bp for the *CAT* gene (GORMAN, CHAN and GORMAN 1991) and 1149 bp for the *hisG* fragment (ALANI, CAO and KLECKNER 1987), is small and also unlikely to account for the difference in recombinant frequencies. In *S. cerevisiae* the frequency of intrachromosomal recombination between repeats of similar size does not vary significantly (YAUN and KEIL 1990). Differences in the sequence of the flanking repeats also seems an unlikely explanation (ALANI, CAO and KLECKNER 1987). A potentially significant variable may be the different genetic backgrounds of the strains.

In *S. cerevisiae*, the high-frequency excision of the intervening *URA3* gene is mediated by intrachromosomal recombination between the flanking repeats of *hisG* (ALANI, CAO and KLECKNER 1987). Similarly, each independent Ura$^-$ segregant isolated from strain CAF5-1, in which the *hisG-URA3-hisG* sequences are inserted at the *ECE1* locus, was the result of intrachromosomal recombination. Similar results have been obtained with the *hisG-URA3-hisG* construct inserted at other loci (unpublished data). In contrast, 9 of 10 independent Ura$^-$ isolates obtained from strain CAI5, which contains the *hisG-URA3-hisG* marker inserted at the *ADE2* locus, resulted from interchromosomal events and it is unclear why this locus specific effect was observed.

Gene mapping: Development of *C. albicans* as a genetic system has been impeded by the absence of a practical method of gene mapping. In an effort to develop a more facile method of gene mapping, the 18-bp recognition sequence of I-*Sce*I was incorporated into the repeats of the *hisG-URA3-hisG* disruption construct. This permits gene disruption experiments to be simultaneously coupled with a method of gene mapping.

The native genome of *C. albicans* strain CAI4 appears to contain no sequences recognized by the endonuclease I-*Sce*I, since the electrophoretic karyotype of this strain was unaltered by incubation with I-*Sce*I. In contrast, one copy of chromosome *IV* in strains heterozygous for an insertion of the I-*Sce*I recognition sequence within the *ECE1* locus was specifically cleaved by the enzyme, yielding two chromosomal fragments. Furthermore, I-*Sce*I treatment of samples from strain CAF6-8, in which both alleles of *ECE1* contain a recognition site, resulted in the complete disappearance of chromosome *IV*. These results not only demonstrate site-specific cleavage of this chromosome but provide direct physical evidence that there are two copies of chromosome *IV* and that the *ECE1* gene is similarly located on each homologue.

Site-specific cleavage of chromosomes with I-*Sce*I provides the basis for a new gene mapping procedure in *C. albicans*. A set of reference strains can now be developed each with a single I-*Sce*I cleavage site on one of the eight chromosomes. These cleavage sites would provide a fixed reference point for the positioning of other genes on the chromosomes. The chromosomal location of a gene and its position relative to the telomeres would be established by I-*Sce*I digestion and the position relative to the reference point would be determined by hybridization of a probe for the new gene to I-*Sce*I digested DNA from the appropriate reference strain. This approach should provide a facile means of gene mapping and facilitate characterization of the genomic structure of *C. albicans*.

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