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Gene Isolation by Complementation in *Candida albicans* and Applications to Physical and Genetic Mapping

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We have isolated three genes, *ARG57*, *SER57*, and *LYS1*, on the basis of their function in *Candida albicans*. A *C. albicans* transformation vector containing the *C. albicans URA3* gene, a *Candida ARS* sequence, and a portion of the *Saccharomyces cerevisiae* 2μm circle containing the replication origin was constructed. Clones from genomic libraries in this vector were isolated by direct complementation of the auxotrophies in strain 1006 (*arg57 ser57 lys1 ura3 MPA1*). Transformants typically contain two to four plasmids in a mixed tandem multimer. A scheme to resolve mixed multimers into monomers in vivo by transformation of *S. cerevisiae* with *Candida* transformant DNA selecting Ura$^+$ transformants was devised. Monomeric plasmids were then isolated by transformation of *Escherichia coli* with the *S. cerevisiae* transformant DNA. These were retested by transformation of strain 1006 to identify the specific plasmid that complemented the auxotrophy. The chromosomal locations of the genes were determined by hybridization to *C. albicans* chromosomes separated on contour-clamped homogenous electric field gels. We used these locations to assess the stability of individual *C. albicans* chromosomes in parasexual genetic analysis. The Lys$^+$-complementing clone was shown to be LYS1 by complementation of *S. cerevisiae lys1* mutants. These cloned genes help to align the *Candida* physical and genetic maps and provide additional markers for the transformation system.

The imperfect fungus *Candida albicans* is the cause of life-threatening infections in immunocompromised patients as well as a variety of surface infections in generally healthy individuals (26). Because of its importance as a human pathogen, many laboratories have undertaken molecular and genetic studies of this diploid dimorphic yeast. Many *C. albicans* genes have been cloned from DNA libraries by complementation of auxotrophs in *Saccharomyces cerevisiae* and *Escherichia coli* (5, 12) or by homology with genes cloned from other organisms (14, 17, 28). The development of a spheroplast transformation system (12), the availability of vectors containing a *C. albicans ARS* sequence (13), and the existence of suitable host strains (6, 11, 12) have made possible efficient DNA transformation in *C. albicans*. A system to isolate genes directly in *C. albicans* would be extremely useful, since many genes, especially those involved in virulence, are best studied in the host from which they are derived.

The *Candida* genetic system was initially built on spheroplast fusion studies using auxotrophs to demonstrate genetic complementation (9, 10, 19). Hilton et al. (7) have devised a procedure for linkage analysis which employs heat shock to induce the loss of entire chromosomes from the fusion products. Using this method, these authors studied the segregation of 12 auxotrophic markers and defined the following five linkage groups: *ino-CEN-met1-ura-arg1-ade1*, *lys1-pro*, *met2-ade2*, *asn-CEN-suf*, and *his* (26). This suggested a genetic map consisting of at least five chromosomes. More mutants have since been isolated, and it is likely that existing mutations now cover all of the *Candida* chromosomes.

The electrophoretic karyotype for *C. albicans* has been analyzed by separation of individual chromosome-sized bands using pulsed-field gel electrophoresis. Since homologs often are resolved by this technique, the cloned single-copy sequences have been used to define physical linkage groups. This approach has been used to define eight physical linkage groups; however, only the *ade1* and *ade2* genetic linkage groups mentioned above have been assigned to specific chromosomes (15, 22). Wickes et al. (33) have assigned an additional genetic linkage group to chromosome I.

Isolation of genes that complement known auxotrophies not only aligns this physical map with the genetic data but is particularly important for ruling out coincidentally sized chromosomes in the karyotype. In this paper, we report the cloning of three genetically unlinked *C. albicans* genes from a genomic library by direct complementation of the corresponding auxotrophies in *C. albicans* strain 1006. We show that the plasmid DNA is maintained in the transformants as a circular mixed end-to-end multimer, and we demonstrate an efficient system for resolving the multimers and isolating the clone containing the complementing activity. The three genes are also assigned to chromosomes on the *C. albicans* karyotype by using physical and genetic methods, and the markers they encode are employed to test the stability of these chromosomes. The availability of multiple selectable markers for transformation of strain 1006 will facilitate genetic manipulations such as gene disruption experiments that require two or more transformations of a single host strain.

MATERIALS AND METHODS

Strains, media, and growth conditions. *C. albicans* and *S. cerevisiae* strains used in these experiments are listed in Table 1. The compositions of yeast extract-peptone-dextrose (YPD), yeast minimal medium (MIN), and L medium have been described previously (3, 27). The required supplements were added to MIN at 100 μg/ml. Mycophenolic acid (MPA)

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TABLE 1. *C. albicans* and *S. cerevisiae* strains

Strain	Synonym	Phenotype or genotype[a]	Source or reference
C. albicans			
655	ATCC 56884	Prototroph	6
834	STN57	*arg57 ser57 lys1*	29
981		*arg57 ser57 lys1 MPA1*	6
982	hOG24	*ade1 met pro lys1/+*	20
1006		*arg57 ser57 lys1 ura3 MPA1*	6
1035	FC18	Prototroph	15
1075	A623	his_{1075} met_{1075}	10
1161		*arg57 ser57 gal1 lys1 ura3 MPA1*	33
S. cerevisiae			
54	SC3	α *trp1-289 ura3-52 his3Δ1 gal2 gal10* [cir^0]	21
57	SC5	**a** *trp1-289 ura3-52 gal2 his3-R1* [cir^0]	21
324[b]	CMY13	**a** *ura3-52 trp1-289 lys1*	C. Mann
328	YM214	α *his3 200 lys2-801 ade2-101 ura3-52*	M. Johnston
578[b]	9842-1A	**a** *ura3-52 his3Δ200 trp1 leu2 lys1*	G. Fink
1114	3482-16-1	*his3Δ1 leu2-3,112 trp1-289 ura3-52 met2* [cir^0]	D. Livingston

[a] *C. albicans* heterozygous mutations are shown as *mutation*/+.
[b] It is not known whether the *lys1* alleles in strains 324 and 578 are distinct.

was used at 5 μg/ml in MIN agar where indicated. 2-Deoxygalactose resistance was selected as described previously (33). Ampicillin was added to L medium at 50 μg/ml. L plates contained 1.2% agar (A1296; Sigma, St. Louis, Mo.), and YPD and MIN plates contained 1.5% agar. All *E. coli* cultures were grown in L broth or plates at 37°C. *C. albicans* and *S. cerevisiae* strains were grown in YPD or supplemented MIN where indicated at 30°C.

***E. coli* procedures.** DNA transformations of *E. coli* were performed as described by Davis et al. (3) or by the calcium chloride-rubidium chloride procedure (16). Standard methods for large-scale and rapid isolation of plasmid DNA were employed (3).

***C. albicans* procedures. (i) *C. albicans* DNA.** A total of 500 ml of cells grown to late log phase (optical density at 600 nm > 1) was pelleted, washed once with 1.2 M sorbitol, and resuspended in 15 ml of 50 mM potassium phosphate (pH 7.5)–1.2 M sorbitol–0.1% β-mercaptoethanol. A total of 0.5 ml of yeast lytic enzyme solution (15 mg of yeast lytic enzyme per ml [ICN Biochemicals, Cleveland, Ohio] in 10 mM potassium phosphate–50% glycerol–0.1% β-mercaptoethanol) was added, and the cells were incubated at 30°C for 30 min with gentle shaking. Spheroplasts were then pelleted and resuspended in a solution containing 10 to 15 ml of 0.5% sodium dodecyl sulfate, 50 mM Na_3 EDTA (pH 8.5), and 200 μg of proteinase K per ml and incubated at 50°C for 90 min. This lysate was extracted twice with phenol-chloroform and precipitated with 2 volumes of ethanol. The DNA was pelleted, resuspended in a solution containing 10 mM Tris-chloride–1 mM EDTA (pH 7.5) (TE) plus 10 μg of RNase A per ml, incubated overnight at 4°C, and purified with a sucrose block gradient. The gradient was layered as follows (from bottom to top): 1.0 ml of density 1.8 $CsCl_2$ in 50 mM EDTA (pH 7.5) and 3.1 ml (each) of 30, 20, and 10% sucrose in 50 mM EDTA (pH 7.5). A total of 2.0 ml of DNA in TE plus RNase A was layered on top. Gradients were centrifuged at 33,000 rpm in an SW41 rotor for 20 to 24 h at 15°C. DNA was collected from the bottom 2 to 3 ml of the tube by precipitation with 2 volumes of ethanol, and the pellet was discarded.

(ii) Spheroplast transformation. A total of 100 ml of cells was grown to late log phase (optical density at 600 nm > 1), pelleted, washed once with 1.2 M sorbitol, and resuspended in 10 ml of 50 mM potassium phosphate (pH 7.5)–1.2 M sorbitol–0.1% β-mercaptoethanol. A total of 150 μl of yeast lytic enzyme solution (see above) was added, and cells were incubated at 30°C for 30 min with gentle shaking. Spheroplasts were pelleted, washed three times with 1.2 M sorbitol, and resuspended in 1 ml of 1.2 M sorbitol–10 mM Tris (pH 7.5)–10 mM $CaCl_2$.

For each sample, 5 to 10 μg of DNA in 100 μl of 1.2 M sorbitol–10 mM Tris (pH 7.5)–10 mM $CaCl_2$ was gently mixed with 100 μl of spheroplasts and incubated for 15 min at room temperature. A total of 0.8 ml of 40% polyethylene glycol 4000–10 mM Tris (pH 7.5)–10 mM $CaCl_2$ was mixed with each sample, and samples were again incubated for 15 min at room temperature. Samples were brought up to a total volume of 20 ml with regeneration agar (1.2 M sorbitol, 2.0% agar, 2.0% glucose, 1× yeast nitrogen base), mixed well, and poured onto two MIN plates. Plates were incubated at 30°C for 3 to 7 days. Transformants of strain 1006 were visible in 2 to 3 days.

(iii) Spheroplast fusion. Spheroplast fusion selecting MPA-resistant prototrophs or auxotrophic exceptions were performed as reported previously (6).

***Saccharomyces* procedures.** The lithium acetate method for DNA transformation of *S. cerevisiae* (8) was used with minor modifications. *S. cerevisiae* DNA was prepared by the method of Scherer and Stevens (23), except that the diethyl pyrocarbonate addition was omitted and the samples were extracted once with phenol and once with phenol-chloroform before ethanol precipitation.

Construction of plasmid vectors p1024 and p1041. Plasmid pMK22 (kindly provided by Rosemarie Kelly) (13) contains a *C. albicans ARS* (*CARS1*) and the *C. albicans URA3* gene along with pBR322 sequences which include the tetracycline resistance gene and the *E. coli* origin of replication. Plasmid p1024 was constructed by insertion of a 1,947-bp *Pst*I fragment from YEp24 (1a) into the *Pst*I site of pMK22. The 1,947-bp *Pst*I fragment contains the replication origin of the *Saccharomyces* 2μm circle DNA and part of the ampicillin resistance gene. It was inserted into pMK22 in the correct orientation to reconstruct an intact ampicillin resistance gene. To make plasmid p1041, p1024 was partially digested (linearized) with *Sca*I. *Bam*HI linkers were added at the *Sca*I site. This DNA was digested with *Bam*HI to remove

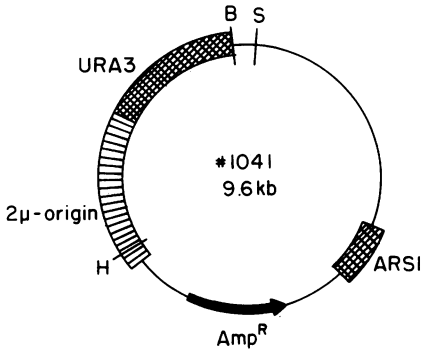

FIG. 1. Cloning vector for *C. albicans*. Plasmid vector p1041 contains the *C. albicans URA3* gene and *ARS* sequence (*CARS1*), along with the pBR322 Ori and ampicillin resistance gene. It also has a portion of the *S. cerevisiae* 2μm circle DNA which carries the 2μm origin and a single copy of the inverted repeat. This vector was made from pMK22 and YEp24 in two steps (see Materials and Methods). B, *Bam*HI; S, *Sal*I; H, *Hin*dIII.

the small fragment extending from the end of the *URA3* gene into the middle of the tetracycline resistance gene and religated, leaving p1041 without an intact tetracycline resistance gene but with a unique *Bam*HI site. Ampicillin-resistant clones were selected to ensure that the new *Bam*HI site would be located adjacent to the *URA3* gene and not in the ampicillin resistance gene. The final construct is illustrated in Fig. 1.

Construction of genomic libraries. Insert DNA was prepared by partial digestion of DNA from *C. albicans* strain 655 with *Sau*3AI. Fragments of between 5 and 10 kb in size were purified on 0.7% agarose gels. Plasmid p1041 DNA was digested with *Bam*HI and was alkaline phosphatase treated and gel purified as described above. Approximately equal amounts of vector and insert were combined and incubated with T4 DNA ligase overnight at 4°C. The ligated mixtures were used to transform *E. coli* HB101 (16), and ampicillin-resistant clones were selected. Individual clones were tested for the presence of insert DNA by examination of plasmid miniprep DNA on agarose gels. Greater than 90% of the clones contained inserts. Clones were pooled by scraping colonies off the L-plus-ampicillin plates into L broth containing ampicillin, allowed to grow for 1 h at 37°C, and then aliquoted and frozen for storage or grown for large-scale plasmid DNA isolation. Four separate genomic libraries were constructed and designated 655-1, 655-2, 655-3, and 655-4. They contain approximately 2,500, 1,500, 10,000, and 15,000 clones, respectively.

Subclones (see Fig. 4). Subclone p1110 was constructed by digestion of clone p1076 with *Eco*RV, gel purification of the larger fragment, and religation of the ends, leaving an internal deletion. To construct subclone p1109, p1076 was digested with *Xho*I and *Sal*I and the large fragment was gel purified and religated. Subclone p1097 was made by insertion of the 6.0-kb *Bgl*II fragment from p1076 into *Bam*HI-digested p1041. Subclone p1099 was constructed by insertion of the 2.3-kb *Xba*I-*Hin*dIII fragment from p1076 into *Xba*I-*Hin*dIII-digested p1041. For subclone p1101, p1076 was digested with *Hin*dIII and the large fragment was purified and ligated. Subclone p1105 was made by insertion of the *Cla*I-*Sal*I fragment from p1076 into *Cla*I-*Sal*I-digested p1024, the parent vector of p1041 which contains a *Cla*I site. Subclone p1106 was constructed by digestion of clone p1078 with *Hin*dIII, gel purification of the large fragment, and ligation. To make subclone p1113, p1078 was digested with *Cla*I and *Sal*I, the large fragment was gel purified, and the ends were filled with T4 polymerase and deoxynucleoside triphosphates and then ligated. Subclone 1334 was constructed by purification of the *Eco*RV-*Cla*I fragment from p1078 and ligation into pBR322. It was tested for the Lys^+ phenotype by cotransformation of strain 1006 with p1041, selection of Ura^+ transformants, and retesting of these on medium lacking lysine.

Agarose gels and hybridizations. Agarose gel electrophoresis, Southern transfers, and hybridizations were performed as previously described (24). Samples for contour-clamped homogenous electric field (CHEF) gels were prepared as described previously (25). The CHEF apparatus used was identical to that of Vollrath and Davis (31). The composition of the gel and chamber buffer was 50 mM Tris base–50 mM boric acid–2.5 mM Na_3 EDTA. 1% agarose gels were electrophoresed at approximately 135 V at 11°C for 22 h with a 7.0-min pulse time and then for 45 h more with a 3.5-min pulse. *C. albicans* chromosomes are numbered by the system of Scherer and Magee (22) and Wickes et al. (33).

RESULTS

Isolation of Arg^+-, Ser^+-, and Lys^+-complementing clones. The four genomic libraries in plasmid vector p1041 were used to transform *C. albicans* strain 1006 (*arg57 ser57 lys1 ura3*). Transformants were plated on appropriately supplemented minimal medium to select for Ura^+ clones which were also Arg^+, Ser^+, or Lys^+. Approximately 6,500 transformants were screened under each condition. Five Arg^+ colonies and a single Lys^+ colony were found. A Ser^+ isolate could be obtained only when Ura^+ transformants were retested by replica plating on medium lacking serine.

To test whether the markers were plasmid encoded and did not result by reversion of the chromosomal mutations or by plasmid integration, colonies were tested for stability of the markers by plasmid curing. Since vector p1041 has no centromere, the Arg^+, Lys^+, or Ser^+ phenotype should be unstable in the absence of selection if the marker is plasmid encoded. Consequently, the Arg^+, Ser^+, and Lys^+ transformants were grown nonselectively in YPD, plated and grown on YPD, and then replica plated on selective medium (omitting arginine, serine, or lysine). Colonies that failed to grow on the selective medium were then tested for the Ura^+ phenotype.

Only two of the Arg^+ transformants, 1064 and 1065 (both derived from library 655-4), were unstable for the Arg^+ phenotype. The other three Arg^+ isolates did not yield any Arg^- segregants and were presumed to be either integrated transformants or Arg^+ revertants of strain 1006. The Lys^+ and Ser^+ isolates, designated 1066 and 1067, were both unstable. All of the Arg^-, Ser^-, and Lys^- segregants also became Ura^-, indicating that the phenotypes were plasmid encoded.

Physical state of plasmid DNAs. Kurtz et al. (13) have reported that *Candida ARS1* plasmids do not remain monomeric circles in transformed *C. albicans* cells. They are present as large tandem multimers, or they integrate into a host chromosome. To test whether transformants 1064, 1065, 1066, and 1067 (Table 2) contained multimeric plasmid, DNA from the transformants was examined by agarose gel electrophoresis and Southern hybridization. Figure 2 shows DNA from the Arg^+ transformant 1065 probed with ^{32}P-labeled pBR322 which hybridizes to vector sequences. The plasmid probe hybridizes to high-molecular-weight DNA in

TABLE 2. Plasmid-containing strains

Strain	Plasmid
C. albicans[a]	
1064	Large Arg$^+$ Ura$^+$
1065	Large Arg$^+$ Ura$^+$
1066	Large Lys$^+$ Ura$^+$
1067	Large Ser$^+$ Ura$^+$
E. coli[b]	
1076	p1076 (*ARG57* gene in p1041)
1077	p1077 (*SER57* gene in p1041)
1078	p1078 (*LYS1* gene in p1041)

[a] Host strain is 1006.
[b] Host strain is HB101.

the uncut DNA (Fig. 2, lane 1), indicating that the plasmid DNA is apparently a single species of a size which is larger than expected for vector plus insert. If this high-molecular-weight band represented more than one type of molecule, we would have expected the plasmid-curing experiments to yield some Arg$^-$, Ser$^-$, and Lys$^-$ segregants that were Ura$^+$. The simultaneous loss of both plasmid-encoded markers supports the model of a single multimeric plasmid in the transformants. When the DNA is cut with *Sal*I (Fig. 2, lane 2) or *Hin*dIII (Fig. 2, lane 3), which cut the vector DNA only once, four predominant bands in approximately equimolar quantities are seen. This indicates that the plasmid in the *Candida* transformant is composed of multiple copies of vector adjacent to insert sequences. The other transformants, 1064, 1066, and 1067, were also shown to contain mixed multimer plasmids (composed of two to four different monomers) by similar Southern analyses (data not shown). The cosegregation of Ura$^+$ with the other auxotrophic phenotype, the equal quantities of the different plasmid

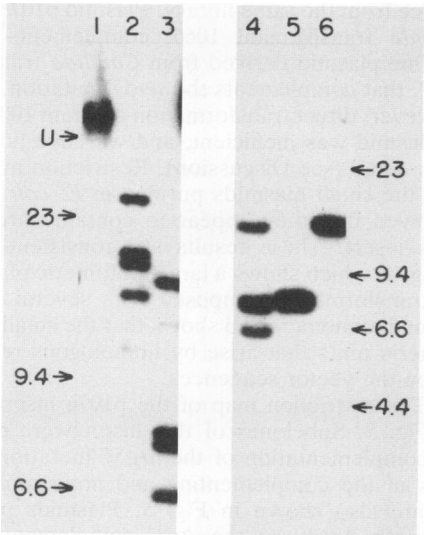

FIG. 2. DNA from *C. albicans* transformants and *S. cerevisiae* transformants derived from them. Lanes: 1 through 4, uncut DNA from *C. albicans* Arg$^+$ Ura$^+$ transformant 1065 (the position of the bulk uncleaved chromosomal DNA is marked "U" in lane 1); 2, *Sal*I digest; 3 and 4, *Hin*dIII digest; 5 and 6, *Hin*dIII-digested DNA from two independent transformants of *S. cerevisiae* strain 328. Blots were hybridized with labeled pBR322 DNA so that only bands which contain vector DNA are observed. Size standards for the two separate gels are shown in kilobases.

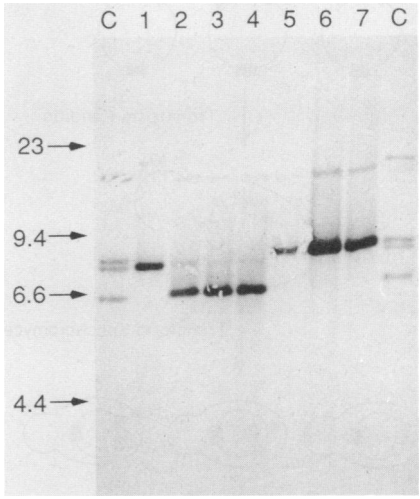

FIG. 3. *S. cerevisiae* transformants derived from *C. albicans* transformant 1065 DNA. Lanes: C, DNA from *C. albicans* Arg$^+$ Ura$^+$ transformant 1065 digested with *Hin*dIII; 1 through 7, *Hin*dIII-digested DNA from seven independent transformants of *S. cerevisiae* strain 328 with *C. albicans* transformant 1065 DNA. Blots were hybridized as described in the legend to Fig. 2. The faint bands may be partial digestion products or short-lived species present in the primary transformants; they are not present in plasmids recovered in *E. coli*. Size standards are shown in kilobases.

fragments, and the single large species of uncut DNA suggest that the different vector-plus-insert combinations are contained in a single large plasmid rather than in several separate multimers, each made of a distinct monomer. This large multimeric plasmid arising by homologous recombination of vector sequences would be composed of different, distinct monomer plasmids that cotransformed into the original isolate (see the top of Fig. 4).

Isolation of cloned DNA. DNA prepared from *C. albicans* transformants 1064, 1065, 1066, and 1067 was used for transformation of *E. coli* HB101 (*recA* mutant) and LE392 (*recA*$^+$), but transformants were not obtained. Since *S. cerevisiae* is able to accept much larger DNA molecules in transformations, the following scheme was devised to isolate a monomer-sized complementing clone from the large plasmid in the transformants. The same *Candida* DNA samples that failed to yield *E. coli* transformants were used to transform *S. cerevisiae* strain 328 (*ura3*) to Ura$^+$ (the *C. albicans URA3* gene complements the *S. cerevisiae ura3* mutation [5]). Between 1 and 10 *S. cerevisiae* transformants per microgram of *C. albicans* DNA were obtained. For each DNA sample, several Ura$^+$ colonies were chosen. DNA was prepared from the primary transformants and examined by Southern analysis as described above. Lanes 5 and 6 of Fig. 2 contain *Hin*dIII-digested DNA from two independent *Saccharomyces* transformants with DNA from the *Candida* transformant strain 1065. Each *Saccharomyces* transformant contains a single plasmid restriction fragment that is the same size as one of the *Hin*dIII fragments of the original large plasmid in 1065 (Fig. 2, lane 4). *Hin*dIII-digested DNA from seven additional *S. cerevisiae* transformants derived from DNA from *C. albicans* transformant 1065 is illustrated in Fig. 3. In each case, a single intense labeling band in the *Saccharomyces* transformant is the same size as one of the bands in the *Candida* transformant (Fig. 3, lanes C). This was the case for 21 out of 25 *S. cerevisiae* transformants

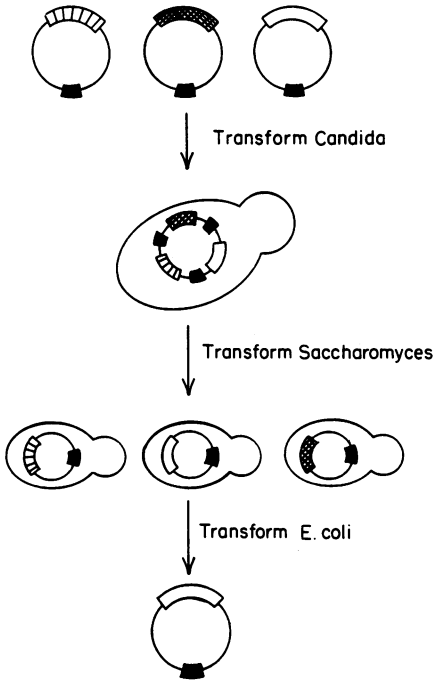

FIG. 4. Formation of multimeric circles in *C. albicans* transformants from the monomeric components of a genomic library. Their subsequent resolution to monomers again upon transformation of *S. cerevisiae* is diagrammed. The *S. cerevisiae* transformants are shown segregating only one type of plasmid per cell which can then be isolated in large quantities after transformation of *E. coli*.

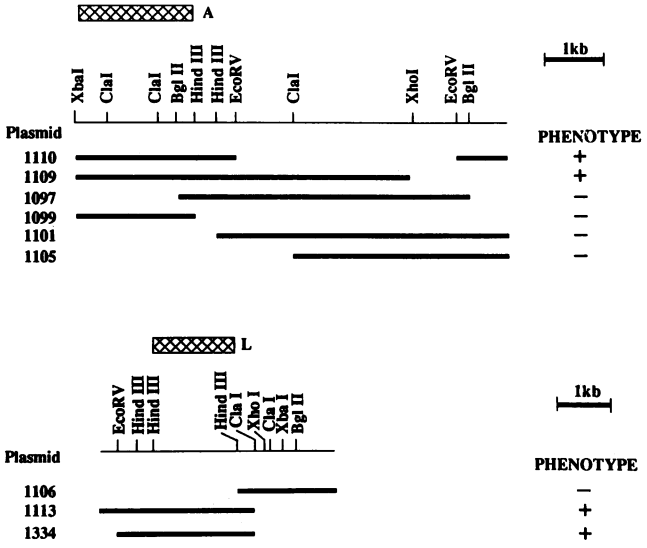

FIG. 5. Restriction maps of clones p1076 and p1078, subclones, and probes. (Top) Restriction map of the insert DNA of p1076 which contains the *ARG57* gene, with Arg^+ and Arg^- subclones shown below. Probe A is the 2.3-kb *Hin*dIII-*Xba*I fragment. (Bottom) Restriction map of the insert DNA of p1078 which contains the *LYS1* gene, with Lys^+ and Lys^- subclones. The order of the large *Hin*dIII fragment (probe A) and the 300-bp *Hin*dIII fragment is uncertain. Probe L is a 1.7-kb *Hin*dIII fragment. Subclones were constructed as outlined in Materials and Methods.

tested. The other four *S. cerevisiae* transformants showed an additional major band, indicating the presence of two different plasmids in the culture. Similar results were obtained when strain 328 was transformed with DNA from 1064, 1066, and 1067 (data not shown). These results suggest that *S. cerevisiae* breaks down the larger plasmid and usually retains only one smaller plasmid (Fig. 4).

We were interested in whether the 2μm plasmid-encoded *FLP* protein (2) participated in the resolution of the large plasmid into smaller plasmids in these *S. cerevisiae* transformants because the vector p1041 contains the *FLP* site-specific recombination sequence. To investigate this, three *S. cerevisiae* strains which are devoid of 2μm plasmid DNA ([cir^0] strains), 54, 57, and 1114, were used as recipients for transformation with DNA from *C. albicans* transformant 1065. Although the transformation efficiency was about 10 times lower than that for strain 328, a few Ura^+ transformants were obtained for each strain. Southern analysis of the Ura^+ transformants of the [cir^0] strains (data not shown) showed that these transformants also contained monomer-sized plasmids. Therefore, the *FLP* protein is not needed to resolve the large plasmids.

To isolate small plasmids for transformation into *C. albicans*, DNA from each *S. cerevisiae* transformant was used to transform *E. coli* HB101 to ampicillin resistance (Fig. 4, bottom). Plasmid DNA was isolated from the *E. coli* transformants and tested by transformation of *C. albicans* strain 1006 for ability to complement the appropriate auxotrophy.

A series of DNA isolates from the *E. coli* transformants were screened, and distinct plasmids were purified and tested for function. As expected, only one class of monomer gave Arg^+, Ser^+, or Lys^+ *Candida* transformants. Plasmid p1076, originally derived from *Candida* transformant 1064 DNA, complements the *arg57* mutation. A band that is the same size as p1076 appeared in digests of DNA from transformant 1065, which is an independent Arg^+ transformant. The band also appears in digests of DNA from the *Saccharomyces* transformants derived from DNA from strain 1065. This suggests that the same Arg^+ clone was isolated twice from the same library. Plasmid p1078, derived from *Candida* transformant 1066, complements the *lys1* mutation. One plasmid derived from *Candida* transformant 1067, p1077, that complements the *ser57* mutation, was also found. However, direct transformation of strain 1006 to Ser^+ with this plasmid was inefficient, and we have not characterized it in detail (see Discussion). Restriction mapping of DNA from the small plasmids purified in *E. coli* (data not shown) showed that they appear to contain unrearranged vector plus insert. These results are consistent with the model in Fig. 4, which shows a large multimeric plasmid in a *Candida* transformant composed of several different cotransformed monomers and shows that the small plasmids are monomeric units that arise by homologous recombination between the vector sequences.

ARG57. The restriction map of the p1076 insert DNA is shown in Fig. 5. Subclones of this insert were made and tested for complementation of the *arg57* mutation in 1006. The inserts in the complementing and noncomplementing subclones are also shown in Fig. 5. Plasmids p1110 and p1109 are both Arg^+, so the Arg^+-complementing gene, which we have designated *ARG57*, must be located to the left of the first *Eco*RV site. To determine the physical location of *ARG57*, chromosomes from strains 1006 and 1035 were separated on a CHEF gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled probe specific for the Arg^+-complementing portion of p1076 (probe A in Fig. 5). Two different *C. albicans* strains were examined

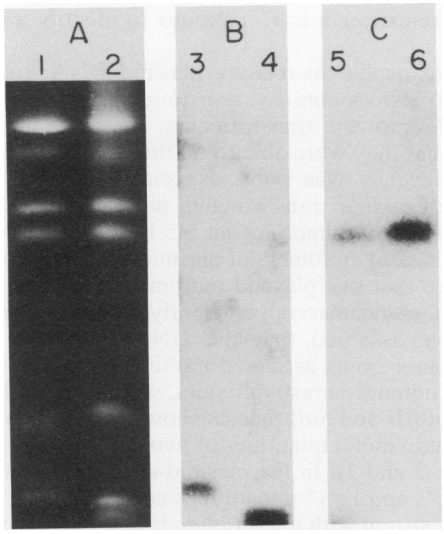

FIG. 6. CHEF gel and hybridizations determining the locations of *ARG57* and *LYS1* on the electrophoretic karyotype. (A) CHEF gel illustrating separation of chromosomes for strains 1006 (lane 1) and 1035 (lane 2) as described in Materials and Methods. Chromosomes I and R are not resolved. (B) An identical gel, blotted and hybridized with probe A, showing hybridization to chromosome VII. (C) An identical gel, blotted and hybridized with probe L, showing hybridization to chromosome IV.

(note the length polymorphisms in the smallest chromosomes, V, VI, and VII, in Fig. 6A). Figure 6B shows that in both *Candida* strains, the probe hybridizes to chromosome VII.

We took advantage of the chromosome length polymorphism between strains 1006 and 1035 for chromosome VII to confirm that the *arg57* mutation is on chromosome VII. 1006 was fused to 1035, and fusion products were selected on MIN plates containing MPA and arginine. These conditions select for MPA-resistant fusion products but allow Arg^- exceptions to occur. Exceptions are fusion products in which a particular chromosome or chromosomes that carry dominant parental markers are absent (6). The fusion products were screened on MIN to identify the exceptions. Arg^- fusion products were then analyzed along with the normal fusion products and parent strains on a CHEF gel to check for coincident disappearance of one of the 1035 chromosomes with the appearance of the auxotrophy. Figure 7 shows results of Southern analysis of a CHEF gel hybridized with ^{32}P-labeled probe A. A single band corresponding to chromosome VII hybridizes with the 1006 and 1035 samples (Fig. 7, lanes 1 and 2), while the MPA-resistant, prototrophic 1006-1035 fusion product shows both parental bands (Fig. 7, lane 3). The three Arg^- auxotrophs (Fig. 7, lanes 4 to 6) show only one hybridizing band which corresponds to the larger 1006 chromosome VII. Examination of the fluorescence pattern of the gel (not shown) indicates that the entire 1035 chromosome VII is absent from the Arg^- exceptional fusion products but not from Ser^- exceptions derived in parallel.

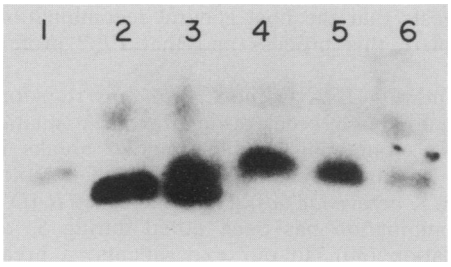

FIG. 7. Hybridization of chromosomes of Arg^- exceptional fusion products with probe A. Shown are CHEF separation of chromosomes from strains 1006 (lane 1), 1035 (lane 2), a prototrophic MPA-resistant 1006 × 1035 fusion product (lane 3), and three Arg^- MPA-resistant exceptional fusion products (lanes 4 to 6), hybridized with probe A.

LYS1. The restriction maps of p1078 and two subclones are shown in Fig. 5, localizing the Lys^+ gene between the *Eco*RV site and the first *Cla*I site of the p1078 insert. The location of the Lys^+ clone was also determined by Southern analysis of chromosomes separated by the CHEF method. Probe L (Fig. 5), a *Hin*dIII fragment specific for the Lys^+-complementing region of p1078, hybridizes to chromosome IV (Fig. 6).

Enzyme assays of strain 834, the strain from which 1006 was derived, revealed that it lacks the enzyme saccharopine dehydrogenase, while other enzymes in the lysine biosynthetic pathway are present (1). This enzyme is encoded by the *LYS1* gene in *S. cerevisiae*. Since most *C. albicans* genes will complement their *S. cerevisiae* counterparts (22), p1078 was tested for its ability to complement *lys1* mutants of *S. cerevisiae* (Table 1). The *ura3-lys1* strain, 324 (Table 1), was transformed to Ura^+ with p1078. A total of 20 of these Ura^+ transformants were tested on medium without lysine and found to be Lys^+, while 20 Ura^+ transformants of strain 324 with the vector p1041 were Lys^-. Similar results were obtained with *S. cerevisiae* strain 578 (Table 1).

Chromosome stability. The *arg57*, *ser57*, *lys1*, and *ura3* mutations were previously shown to be unlinked in chromosome loss experiments (6). *ser57* is linked genetically to a chromosome I marker, *gal1* (33). *C. albicans* natural variants and morphological mutants often have altered karyotypes (22), and spheroplast fusion products are unstable in ploidy (32). Since the physical locations of all four auxotrophic mutations of strain 1006 are now known, we tested the relative stability of these chromosomes in spheroplast fusion and chromosome loss experiments. Two methods were used to assay chromosome stability: spontaneous chromosome loss from initially tetraploid fusion products and generation of exceptional progeny by spheroplast fusion (6).

Strain 1161, a *gal1* mutant of strain 1006, was fused with strain 1075 (Table 1), and prototrophic fusion products were selected. The fusion products were grown and spontaneous 2-deoxygalactose-resistant segregants were selected on fully supplemented minimal medium as described by Wickes et al. (33). The segregants were scored only for the markers in strain 1161, as the chromosomal locations of the strain 1075 markers are not known (Table 3). A total of 78% of the *gal1* fusion products were also Ser^-, confirming the *ser57-gal1* linkage group. Arg^- and Ura^- segregants were obtained at approximately equal frequencies, indicating that chromosomes VII and III have similar stabilities in the mature fusion products. Lys^- segregants were obtained at a slightly lower frequency than Arg^- and Ura^- segregants, suggesting that chromosome IV is more stable than chromosomes VII and III in these fusion products.

Generation of exceptional progeny during spheroplast fusion is likely the result of a partial set of chromosomes

TABLE 3. Phenotypes of 2-deoxygalactose-resistant segregants of 1161 × 1075 fusion products[a]

Phenotype	Chromosome	No. found	% of total[b]
Ser$^-$	I	135	78
Arg$^-$	VII	25	14
Lys$^-$	IV	11	6
Ura$^-$	III	27	16

[a] The genotype of 1161 is *arg57 ser57 gal1 lys1 ura3 MPA1*. The genotype of 1075 is *his met*.
[b] A total of 174 independent 2-deoxygalactose-resistant segregants were tested.

being transferred from the donor nucleus to the recipient nucleus prior to karyogamy, generating an aneuploid fusion product (10, 22). To examine chromosome stability during fusion, strain 981 (*arg57 ser57 lys1 MPA1*) was fused with the auxotrophic strain 982 (Table 1). Three different media were prepared. Each selected for only one of the strain 981 markers, permitting exceptions for the other two. The fusion mixture was divided and samples were plated on each of the three different supplemented media in the presence and in the absence of MPA. The fusion products were then retested on MIN to identify auxotrophic exceptions. The data were examined for the frequency of exceptions for each strain 981 auxotrophic marker (Table 4). These results show that the presence of MPA in the selective medium does not influence the frequency of segregation of any of the individual markers. Lys$^-$ exceptions were observed consistently more often than Arg$^-$ and Ser$^-$ exceptions, but the frequencies are not far apart, again showing that the chromosomes containing these markers have similar stabilities during fusion. The somewhat higher frequency of Lys$^-$ exceptions in this experiment indicates that chromosome IV is transferred less frequently than chromosomes I and VII from the donor to the recipient nucleus during fusion, suggesting that it is more stable in diploid nuclei as well as in fusion products.

DISCUSSION

We have cloned three *C. albicans* genes, *ARG57*, *SER57*, and *LYS1*, by complementation of the auxotrophies in strain 1006. The clones were isolated from genomic libraries from the wild-type strain 655 with a specially constructed vector that replicates in *C. albicans*, *S. cerevisiae*, and *E. coli*. We have shown that the large plasmid DNA isolated from *C. albicans* transformants is rapidly resolved into monomeric plasmids upon transformation of *S. cerevisiae*. This system makes it possible to isolate cloned genes directly in *C. albicans*, purify monomeric forms in *E. coli* via *S. cerevisiae*, and retest them in *C. albicans* to identify a particular clone.

We were unable to recover plasmid DNA from our *C. albicans* transformants by transformation of *E. coli* with total DNA from the transformants. Kurtz et al. (13) have reported that they were able to do this but that the transformation efficiency was poor, especially with *recA* mutant cells. The *Candida* transformants reported by Kurtz et al. (13) contained multimers of an 8.5-kb plasmid, while ours contained mixed multimers of plasmids all larger than 14 kb. We believe that our plasmid multimers have exceeded the size that *E. coli* can accept efficiently during transformation.

We have assumed that the DNA in the *C. albicans* transformants exists as mixed multimers rather than a collection of homogeneous multimers, since the Southern analysis of *Hin*dIII and *Sal*I digests shows the multimers breaking down into molar quantities of two to four monomer-sized units (Fig. 2 and 3). In the plasmid-curing experiments, the Arg$^+$, Ser$^+$, and Lys$^+$ phenotypes cosegregated with Ura$^+$. This is consistent with the *Candida* transformants containing a single large molecule. The mixed multimeric plasmid DNA is resolved to a single monomer upon transformation of an *S. cerevisiae ura3* strain with whole cellular DNA isolated from the *C. albicans* transformants. We noted that an occasional *Saccharomyces* transformant derived from the *C. albicans* transformant DNA contained an additional major band, but most appear to have segregated out a single recombinant plasmid rather than maintain several of them. This was unexpected, since primary *Saccharomyces* transformants were used for the DNA isolation, and *S. cerevisiae* transformants can maintain more than one 2μm origin-containing plasmid within a single cell.

We propose that homologous recombination efficiently resolves mixed multimers to monomers in *S. cerevisiae*. Plasmid p1041 contains a portion of the 2μm circle DNA from YEp24, which has a single copy of the 2μm inverted repeat. End-to-end inversion is catalyzed by the *FLP* protein which is also encoded by 2μm circle DNA, with the target located in the repeated sequences (2). Since p1041 contains only one copy of the repeat, end-to-end multimers formed by homologous recombination between the p1041 library plasmids should contain multiple copies of this sequence as a direct repeat. Upon introduction of this multimer into *S. cerevisiae* containing a *FLP* gene, the *FLP* protein could catalyze the resolution of the mixed multimers into monomers by using the direct repeats as target sequences. However, the three *S. cerevisiae* [cir^0] strains were capable of resolving multimeric DNA into monomer-sized components upon transformation with *C. albicans* transformant DNA. This suggests that the host general recombination system can complete this process and that *FLP* protein is not required.

The multimer DNA in the *C. albicans* transformants is very large and might break during the DNA isolation procedure. DNA containing double-stranded breaks has been shown to be highly recombinogenic with homologous sequences in *S. cerevisiae* (18). High-frequency *RAD52*-dependent recombination has been noted during *S. cerevisiae* transformation (30). In our experiments, a break in the vector sequences of the multimeric plasmid could initiate a recombination event with other homologous vector sequences within the plasmid. This would produce the monomeric circular plasmids we observe. If the linear reciprocal recombination product were discarded, this would explain the efficient plasmid segregation in the *Saccharomyces* transformants.

TABLE 4. Exceptional progeny from 981 × 982[a]

Marker	Chromosome	Exceptions[b]		% Exceptions[c]
		+MPA	−MPA	
Arg	VII	7/92	8/124	7.0
Ser	I	5/97	5/124	4.5
Lys	IV	10/117	15/124	10.4

[a] The genotype of 981 is *arg57 ser57 lys1 MPA1*. The genotype of 982 is *ade1 met pro lys1/+*.
[b] Fusion products were selected on the three different media plus and minus MPA as described in the text and retested on MIN to identify exceptions for the strain 981 auxotrophic markers.
[c] Combined data for plus and minus MPA.

The identity of the *LYS1* gene has been confirmed by complementation of two *Saccharomyces lys1* mutants with the clone and by the absence of the *LYS1*-encoded enzyme saccharopine dehydrogenase in the *Candida* mutant. The *lys1* mutation of 1006 failed to complement the *lys1* mutation of hOG24 in spheroplast fusions (data not shown), indicating that the *lys1-pro* linkage group defined by Hilton et al. (7) should reside on chromosome IV. The low frequency of reversion of the *lys1* mutation in strain 1006 combined with the favorable restriction map of the Lys^+ clone suggests that *LYS1* will be particularly useful as a marker for DNA transformation of *C. albicans*. The *Saccharomyces* counterparts of the 1006 *arg57* and *ser57* mutations remain unknown. We have shown that the *ARG57* gene derives from chromosome VII and that the *arg57* mutation is linked to a length polymorphism in that chromosome.

When 1006 was transformed with the plasmid isolated from the putative *SER57* clone, p1077, only a few Ser^+ transformants appeared, and they grew slowly. When Ura^+ transformants were selected and retested for complementation of *ser57*, all could grow on medium containing only arginine and lysine, but certain transformants grew well, while others appeared to grow only after a short adaptation period on this medium. This effect was observed only after transformation with p1077 DNA. Ura^+ transformants generated with vector or plasmid p1076 or p1078 were uniformly Ser^-, indicating that the effect was not due to leakage or reversion of the *ser57* marker. It is possible that p1077 contains only part of the *SER57* gene and that the DNA must recombine with the mutant *ser57* gene in the chromosome in order to fully complement or that the gene is not expressed efficiently from *ARS*-containing plasmids. A portion of this insert hybridized to chromosome I (data not shown). This is consistent with the observation of Wickes et al. (33) that *SER57* and *GAL1* are in the chromosome I linkage group.

The identification of the physical locations of the *ARG57*, *SER57*, and *LYS1* genes has allowed us to examine the stability of these chromosomes. We assume that spontaneous auxotrophic segregants from mature fusion products occur when an unselected chromosome or set of chromosomes are discarded from a tetraploid nucleus. However, auxotrophic exceptional progeny that are identified immediately upon spheroplast fusion may arise by partial transfer of chromosomes from one nucleus to another during the heterokaryon stage, rather than by a fusion-discard mechanism (4, 10). The exceptions would be similar to the "exceptional cytoductants" observed by Dutcher (4) in *S. cerevisiae kar1-1* × wild-type matings. Dutcher (4) also showed that the frequency of chromosome transfer between nuclei was inversely related to the genetic map length of the chromosome over a wide range of chromosome sizes. Linked markers were acquired together, indicating that whole chromosomes or very large pieces of chromosomes are transferred from the donor to the recipient nucleus. Our experiments indicate that chromosome stability in *C. albicans* does not show the same strong correlation with size as that in *S. cerevisiae*, since the frequencies of both spontaneous segregants and exceptions are comparable for all of the markers tested (Tables 3 and 4). However, chromosome sizes in *S. cerevisiae* vary over a larger range than those in *C. albicans*.

Whelan et al. (32) showed that spontaneous chromosome loss in spheroplast fusion products produced cells with greatly reduced DNA contents. If our segregants became diploid, we would expect unlinked recessives such as *arg57*, *ura3*, and *lys1* to appear in one-sixth of our 2-deoxygalactose-resistant cells. The high frequency of unlinked auxotrophic segregants indicates that they have undergone extensive chromosome loss but may retain more than diploid DNA content. Spontaneous Lys^- segregants from tetraploids were the least common among the four markers, while Lys^- exceptional progeny occurred most often during fusion. This could be due to chromosome IV having a slightly higher stability than the others we tested.

Changes in chromosome banding patterns on pulsed-field gels have been associated with morphologic changes (22), and *Candida stellatoidia* and *C. albicans* strain WO-1 possess minichromosomes which appear to be rearrangements of the larger ones (22). Chromosome stability is an important feature in *Candida* biology, and its analysis could prove useful to studies of genetic variation and switching.

Strains such as 1006, in which the chromosomal location of the auxotrophic markers is known and the corresponding genes have been cloned, will have many uses in the *Candida* genetic system. The dominant MPA resistance mutation and the recessive 2-deoxygalactose resistance mutation in strain 1161 facilitate strain construction by spheroplast fusion. The cloned genes can be used to design vectors to target other cloned sequences to a specific chromosome, as probes to detect chromosome rearrangements, and to facilitate gene disruption experiments. The addition of new physical markers to the genetic map of *C. albicans* increases the number of chromosomes that can be manipulated in parasexual analyses. The ability to clone genes by complementation in *C. albicans* makes possible the isolation of genes affecting *C. albicans* phenotypes such as virulence, differentiation, and morphologic switching.

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