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Integrative Transformation of *Candida albicans*, Using a Cloned *Candida ADE2* Gene

MYRA B. KURTZ,* MARK W. CORTELYOU, AND DONALD R. KIRSCH

Department of Cellular Biology, The Squibb Institute for Medical Research, Princeton, New Jersey 08540

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***Candida albicans* is a diploid dimorphic yeast with no known sexual cycle. The development of a DNA transformation system would greatly improve the prospects for genetic analyses of this yeast. Plasmids were isolated from a *Candida Sau*3A partial library which complements the *ade2-1* and *ade2-5* mutations in *Saccharomyces cerevisiae*. These plasmids contain a common region, part of which, when subcloned, produces *ade2* complementation. Among the small number of auxotrophs previously isolated in *C. albicans*, red adenine-requiring mutants had been identified by several groups. In two of these strains, the cloned *Candida* DNA transformed the mutants to *ADE*$^+$ at frequencies of 0.5 to 5 transformants per μg of DNA. In about 50% of the transformants, plasmid DNA sequences became stably integrated into the host genome and, in the several cases analyzed by Southern hybridization, the DNA was integrated at the site of the *ADE2* gene in one of the chromosomal homologs.**

The pathogenic yeast *Candida albicans* causes mild, superficial infections in hosts with normal immune systems, but can cause life-threatening systemic disseminated infections in immunocompromised patients. With the increased use of immunosuppressive therapy, opportunistic infections by *C. albicans* have become more frequent (11). The development of new drug therapies specific for this yeast will probably require a better understanding of its physiology through biochemical studies and genetic manipulation. The strength of this approach has been demonstrated in the yeast *Saccharomyces cerevisiae*, in which a variety of studies are facilitated by mutant isolation and genetic analyses (26). Unfortunately, genetic analysis of *C. albicans* is difficult, since this organism has a diploid genome (15, 18, 29), no sexual cycle, and no system for DNA-mediated transformation. Many of the easily selected auxotrophs are presumed to arise from strains already heterozygous at a particular locus (16, 29). Limited genetic analysis has been performed by complex protocols with protoplast fusions (16). With these points in mind, we set out to develop a transformation system for *C. albicans*.

The major obstacle for developing transformation in any organism is the availability of an appropriate vector-host pair. By analogy with *S. cerevisiae*, the simplest technique would be integrative transformation. Since transformation would be expected to be a rare event, this system requires a gene which functions in the host and a system for selecting individuals which have taken up and expressed this gene. The gene chosen for transformation was the *Candida ADE2* gene (phosphoribosylaminoimidazole carboxylase [EC 4.1.1.21]). Several authors have described red adenine auxotrophs of *C. albicans* (12, 17, 20). These could arise from lesions in one of two successive steps in purine biosynthesis (6, 7, 24). It seemed highly likely that an *ade2* mutation should be present among this collection of unrelated mutants, especially since Poulter and Rikkerink (17) had isolated two unlinked complementation groups of red adenine auxotrophs. Here we report the isolation of *Candida* DNA fragments in the vector YEp13, which complement *ade2-1* and *ade2-5* mutants of *S. cerevisiae*. These plasmids, pMK3 and pMC1, were able to transform an *ade2* mutant of *C. albicans* at frequencies of 0.5 to 5 transformants per μg of DNA. Plasmid DNA became stably integrated into host DNA at the site of the *ADE2* gene. This is the first demonstration of DNA-mediated transformation in *C. albicans* and should provide a valuable tool for its genetic manipulation.

* Corresponding author.

MATERIALS AND METHODS

Materials. β-Glucuronidase (type H-2), D-sorbitol, and polyethylene glycol 4000 were obtained from Sigma Chemical Co. GeneScreen and [α-^{32}P]deoxynucleoside triphosphates (7,600 Ci/nmol) were from New England Nuclear Corp. Elutip-D was from Schleicher & Schuell, Inc., and restriction enzymes and DNA-modifying enzymes were from Bethesda Research Laboratories, Inc., or New England BioLabs, Inc.

Strains. *Escherichia coli* RR1 (2) was used for routine amplification of plasmids. *E. coli* RR1A was constructed by P1 transduction as a purine-requiring strain. Strain NK6051 (CGSC 6186), which has a Tn*10* insertion in *purE*, was obtained from the *E. coli* Genetic Stock Center. Phage P1 grown on NK6051 was used to transduce RR1 cells to tetracycline resistance, which is carried by Tn*10* (14). Resistant clones were screened for purine auxotrophy, and one representative which transformed well with pBR322 was designated RR1A.

The *ade2-5*-containing *S. cerevisiae* XMK1-222 used for complementation studies was constructed in our laboratory and has the following genotype: *mat***a** *ade2-5 leu2-3 leu2-112 thr1 his3-15 arg4 cup1*. A strain (W343-4A) with a different *ade2* lesion was obtained from R. Rothstein and has the following markers: *matα ade2-1 ura3-11 leu2-3 leu2-112 lys2-1 his3-11 his3-15 can1-100*.

C. albicans hOG300 (*ade2 pro met*), kindly provided by R. M. Poulter, was derived from ATCC 10261. WCR-1-74 (*ade thr*), isolated by A. Sarachek, was obtained from the American Type Culture Collection (ATCC 44990). Pink, adenine-deficient *C. albicans* mutants (A81-Pu, B113P, and B792P), isolated by Kwon-Chung and Hill (12), were kindly provided by K. J. Kwon-Chung. Strain SC5314 is a clinical isolate and was the source of wild-type DNA for library construction. Table 1 provides a comparison of the red adenine auxotrophic *Candida* strains used in this study.

TABLE 1. Comparison of *C. albicans* red auxotrophic strains

Strain	Genotype	Reported adenine auxotrophy[a]	CO_2 reversal[b]	Transformation with pMK3	Reference
hOG300	*ade2 pro met*	*ade2*	+	+	17
WCR-1-74	*ade thr*$^-$	*ade2*	−	−	20
A81-Pu	*ade*	ND[c]	−	+	12
B113P	*ade*	ND	−	−	12
B792P	*ade*	ND	−	−	12

[a] The nomenclature follows that used for *S. cerevisiae*. *ADE1* indicates the gene coding for phosphoribosylaminoimidazole succinocarboxamide synthetase (EC 6.3.2.6), and *ADE2* is the gene coding for phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21).

[b] Strains were streaked on solid media lacking adenine and grown under an atmosphere of 10% CO_2 at 37°C. Woods (31) has shown that some *ade2* mutants of *S. cerevisiae* are reversed by high CO_2 concentrations, an effect not seen for *ade1* mutants.

[c] ND, Not determined.

Library and plasmid construction. The preparation of a *Sau*3A partial library of *C. albicans* DNA in shuttle vector YEp13 has been described before (8). The original library, which contained 4,300 individual transformants with inserts, was increased to a total of 19,000 insert-containing transformants by the following method. *C. albicans* genomic DNA was partially digested with *Sau*3A. Fragments of 5 to 20 kilobases (kb) in size were isolated by sucrose gradient centrifugation and ligated into the *Bam*HI site of *S. cerevisiae* shuttle vector YEp13. This DNA was used to transform *E. coli* RR1 to ampicillin resistance (13) and resulted, combining both libraries, in a total of approximately 95,000 transformants, of which approximately 20% (19,000) were tetracycline sensitive and presumably contained inserts.

Plasmid pMK3 is one of the insert-containing plasmids from the *Candida* library capable of complementing *S. cerevisiae ade2* strains (see Results). The construction of pMC2 was accomplished by subcloning the 5.2-kb *Candida* insert fragment of pMK3 into the *Cla*I site of pBR322. The *Cla*I-cut vector was treated with calf intestinal alkaline phosphatase to prevent self-ligation (13). The appropriate *Cla*I fragment of pMK3 was isolated from a 1% low-melting-point agarose gel and purified on an Elutip-D column (22). The fragment was ligated to the cut vector, and the ligation mix was used to transform RR1 cells to ampicillin resistance (13). Plasmid DNA was isolated from 12 colonies by the boiling procedure of Holmes and Quigley (10) and analyzed by restriction endonuclease mapping.

Media. A rich medium, YEPD (2% Bacto-Peptone [Difco Laboratories], 1% yeast extract, 2% glucose), was used to grow *Candida* and *Saccharomyces* strains for transformation. Synthetic media were prepared by addition of the appropriate amino acids or nucleic acid bases to 0.7% yeast nitrogen base (without amino acids) and 2% glucose (23). Media were solidified with 1.5% agar. Transformation media contained 1 M sorbitol.

DNA isolation and hybridization. Plasmid DNAs in *E. coli* were amplified and isolated by standard procedures (13). Plasmid DNA from *S. cerevisiae* was isolated by the small-scale, rapid method of Sherman et al. (23). Total genomic DNA from *S. cerevisiae* or *C. albicans* was prepared by standard methods (23). CsCl gradient purification was sometimes omitted. Digestion of genomic or plasmid DNA with restriction enzymes was carried out as described by Maniatis et al. (13). DNA fragments were separated by electrophoresis on 0.7% agarose slab gels in 89 mM Tris-borate buffer (pH 8.0). Gel-fractionated DNA restriction fragments were transferred to GeneScreen by the procedure recommended by the manufacturer. Hybridizations with DNA labeled by nick translation were performed by standard procedures (13).

Colony hybridization of *C. albicans* was performed by slight modification of the procedure described for *S. cerevisiae* (23). Colonies to be tested were streaked on YEPD solidified with 2% agarose, grown for 2 days at 30°C, and transferred to GeneScreen (New England Nuclear) by overlaying them with the membranes for 16 h at 30°C. The transferred cells were lysed in situ by first pretreating with 50 mM EDTA–15% β-mercaptoethanol for 15 min at room temperature and then digesting cell walls with 1 mg of zymolyase 5000 (Miles Laboratories, Inc.) per ml in 1 M sorbitol–20 mM EDTA. Incubation for 3 h at 37°C resulted in protoplast formation as determined microscopically. The membranes were then treated by the procedures recommended by the manufacturer.

Transformation. *S. cerevisiae* spheroplasts were transformed by the method of Beggs (1). Initial experiments for *C. albicans* transformation involved a modification of the procedure for *Neurospora crassa* (3), which includes dimethyl sulfoxide treatment and a heat shock step. Later experiments showed that the protocol used for *S. cerevisiae* gave similar results (see Results).

Preparation of *Candida* spheroplasts. To prepare spheroplasts, 50 ml of late-log *Candida* cells (about 10^7 to 2×10^7/ml) was harvested by centrifugation (5 min at 10,000 × *g*), incubated for 10 min in 5 ml of 1 M sorbitol–50 mM dithiothreitol–25 mM EDTA (pH 8.0) at 30°C, and then washed by centrifugation. The cells were suspended in 5 ml of 0.1 M sodium citrate (pH 5.8)–1 M sorbitol–25 mM EDTA, to which 0.05 ml of filtered β-glucuronidase was added. Spheroplasts formed after 30 to 60 min of incubation at 30°C were washed by gentle centrifugation (5 min at 600 × *g*) and suspended in 0.5 ml of CaS (1 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris hydrochloride [pH 7.5]). At this point, either transformation protocol could be followed.

Transformation of *C. albicans* by the *Neurospora* protocol. In this procedure, 1/100 volume of dimethyl sulfoxide was added to the spheroplast suspension. The spheroplasts were heated for 30 s at 37°C and then placed in an ice bath. Vector DNA was added to the treated spheroplasts, incubated on ice for 30 to 45 min, and heated at 42°C for 30 s, and 10 volumes of polyethylene glycol 4000 (20% [wt/vol] solution) were added. The mixture was incubated at room temperature for 20 min and washed free of polyethylene glycol. The spheroplasts were suspended in fully supplemented synthetic medium for 30 to 60 min at 30°C and plated either as a top layer or directly on the surface of selective medium with appropriate osmotic support (1 M sorbitol).

Transformation of *C. albicans* by the *Saccharomyces* protocol. In the *Saccharomyces* procedure, vector DNA was added to the spheroplast suspension in CaS and incubated for 15 min at room temperature, after which 10 volumes of polyethylene glycol 4000 (20% [wt/vol] solution) were added. The mixture was incubated at room temperature for 15 min and then washed free of polyethylene glycol. The remainder of the procedure was the same as that described above. The transformation frequencies of strain hOG300 ranged between 0.5 and 5 transformants per μg of DNA with both procedures.

RESULTS

Cloning of the *C. albicans ADE2* gene. An *ade2-5 leu2-3 leu2-12* mutant of *S. cerevisiae* XMK1-222 was transformed

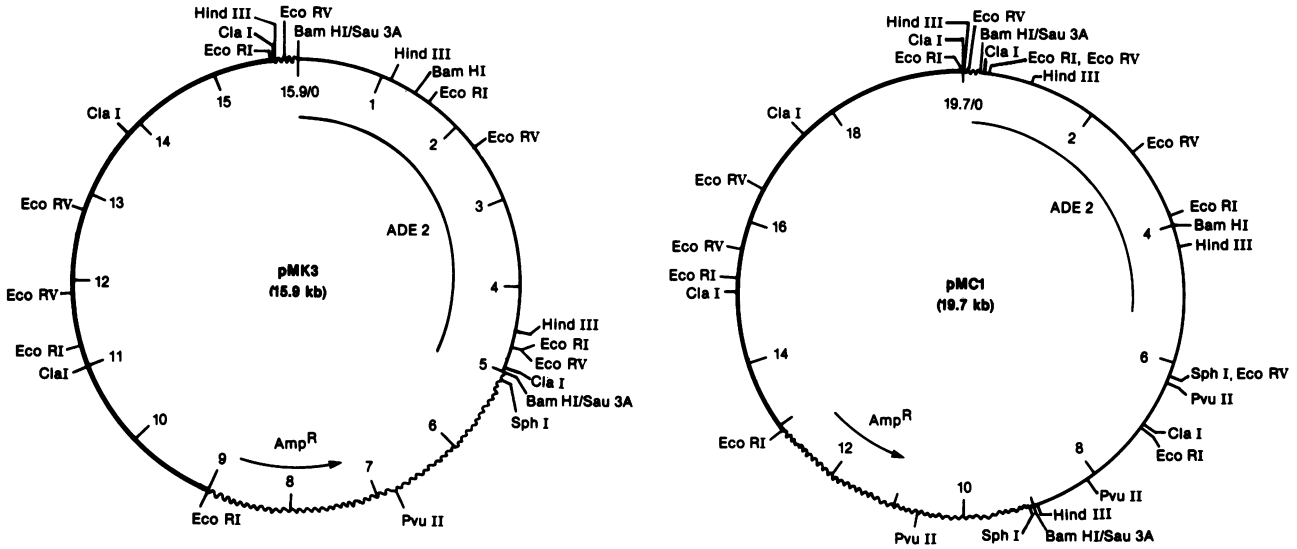

FIG. 1. Restriction maps of *ade2*-complementing plasmids. —, *S. cerevisiae LEU2* and 2μm sequences; ∿∿, pBR322 sequences; —, *C. albicans* sequences. The portion marked *ADE2* is the region which is colinear in pMK3 and pMC1 (but in opposite orientation) and produces *ade2* complementation.

with the *Candida* library, and 10^6 leucine prototrophs were obtained. Of 56,000 leucine transformants tested, 1 was able to grow without adenine. In a separate experiment, an *ade2-1 leu2-3 leu2-12* mutant of *S. cerevisiae* W343-4A was transformed with the library, and approximately 10^5 leucine prototrophs were obtained. Six adenine prototrophs were isolated from these transformants.

To determine whether the adenine prototrophy was due to a recombinant plasmid or a reversion of the *ade2* mutation, we grew each yeast strain nonselectively for about 20 generations in YEPD and streaked it onto a YEPD plate to isolate single colonies. Plasmid loss was detected by replica plating onto minimal medium lacking either leucine or adenine. In six of the seven cultures, plasmid loss was associated with adenine auxotrophy and red pigmentation. Therefore, complementation of the *ade2* mutation had to be due to the insert fragment on the plasmid.

The plasmids were introduced into *E. coli* for restriction analysis. The inserts of five of the plasmids were indistinguishable by size (~5 kb) and restriction sites and were likely to be copies of a single cloning event. A representative plasmid was pMK3 (Fig. 1). The remaining clone (pMC1) contained a larger 8.8-kb insert, of which approximately 5.0 kb had a restriction map common to a portion of the pMK3 insert. It was therefore likely that the complementing ability was completely contained within the common 5.0-kb region. However, the inserts in pMK3 and pMC1 were oriented in opposite directions in the plasmid. This indicated that the complementing activity was independent of insert orientation (Fig. 1).

To demonstrate that the cloned DNA fragments were derived from *C. albicans*, we probed gel-fractionated restriction digests of *E. coli*, *S. cerevisiae*, and *C. albicans* with ^{32}P-labeled pMK3, pMC1, or YEp13. The results showed that there were no sequences homologous to *E. coli* DNA in pMK3 (data not shown). When pMK3 and YEp13 were used to probe *Hin*dIII-cut DNA from a cir^0 *S. cerevisiae* strain, both plasmids hybridized to a single band of approximately 18 kb (data not shown). This band was almost certainly produced by the hybridization of YEp13 *LEU2* sequences with the *LEU2* region of *S. cerevisiae*, since this region is not cut by *Hin*dIII (5). However, hybridization of pMK3 or pMC1 to *Candida* DNA occurred in the pattern predicted by the insert restriction map (Fig. 2). For example, *Hin*dIII-cut SC5314 DNA probed with pMC1 yielded a 3.4-kb fragment (which was identical to the 3.4-kb internal fragment of

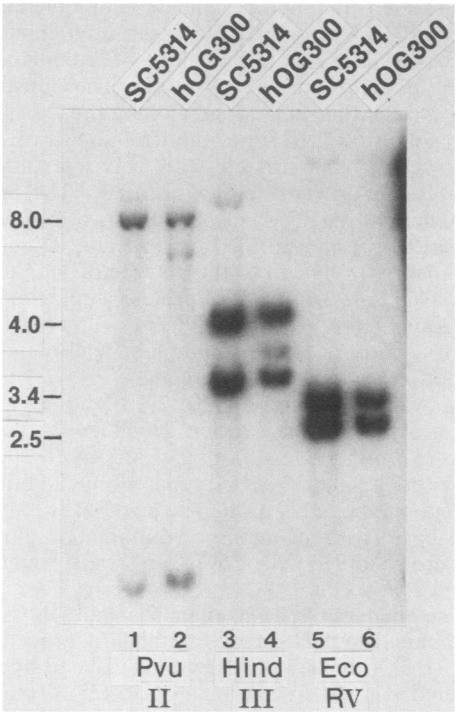

FIG. 2. Autoradiograms of Southern blot hybridizations of *C. albicans* SC5314 and hOG300 DNAs with radiolabeled plasmid pMC1 DNA. Lanes 1, 3, and 5 contain strain SC5314 DNA, and lanes 2, 4, and 6 contain strain hOG300 DNA. Lanes 1 and 2 were digested with restriction endonuclease *Pvu*II, lanes 3 and 4 with *Hin*dIII, and lanes 5 and 6 with *Eco*RV. Numbers on the left indicate size in kilobases.

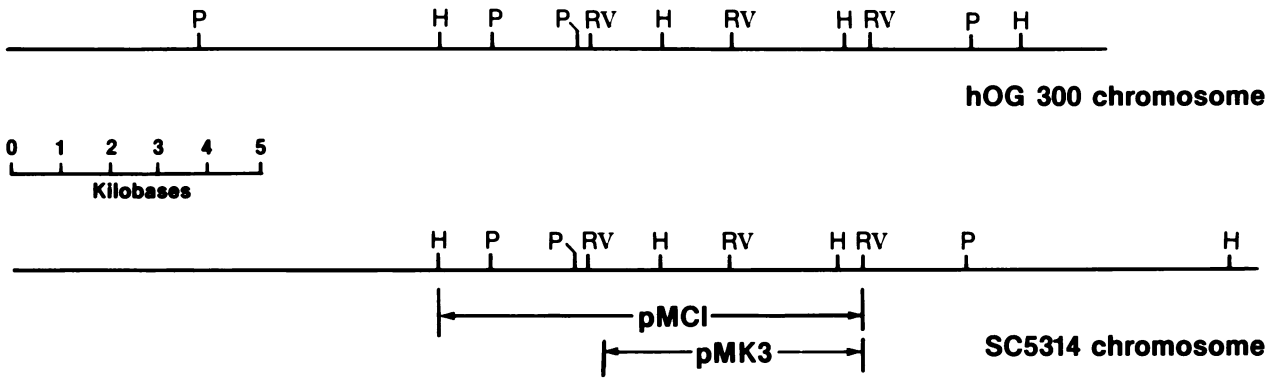

FIG. 3. Model for restriction site heteromorphism of *C. albicans* hOG300 and SC5314 *ADE2* loci. The positions of the restriction sites were determined from the Southern blot hybridization patterns of plasmids pMC1 and pMK3 with restriction endonuclease-digested hOG300 and SC5314 DNAs and the restriction maps of plasmids pMC1 and pMK3. The positions shown are consistent with Southern hybridization and restriction mapping data. The bars below the SC5314 chromosome indicate the limits of the inserts cloned in pMC1 and pMK3. Abbreviations: P, *Pvu*II; H, *Hin*dIII; RV, *Eco*RV.

pMC1) as well as two larger fragments corresponding to the two flanking sequences (Fig. 2, lane 3). The expected number of bands were also found with *Eco*RV and *Pvu*II (Fig. 2, lanes 1 and 5), as well as with *Cla*I-, *Bam*HI-, and *Eco*RI-cut SC5314 DNA (data not shown).

A difference between *C. albicans* SC5314 and hOG300 was revealed when their chromosomal DNAs were cut with *Hin*dIII and probed with pMK3 or pMC1. Strain hOG300 carries a mutation in the *ade2* gene (see below) and was derived from a different clinical isolate (ATCC 10261) than was SC5314. When probed with either pMK3 or pMC1, the strains showed similar 3.4-kb internal *Hin*dIII fragments, as well as similar 4-kb flanking fragments. The remaining flanking fragments showed a considerable difference. The results of the experiment with pMC1 are shown in Fig. 2, lanes 3 and 4. An analogous difference between the *Candida* strains was seen when the chromosomal DNAs were cut with *Pvu*II and probed with pMC1 (Fig. 2, lanes 1 and 2). In SC5314 DNA, a >8.0-kb band was seen, whereas in hOG300 a 6.0-kb band was observed. No difference between the two strains was observed when the DNAs were probed with pMK3, indicating that restriction site heteromorphism was present in the left flanking regions of SC5314 and hOG300 DNAs. The model consistent with the restriction maps and Southern hybridizations is shown in Fig. 3. This figure also shows the internal *Eco*RV hybridizing fragments which are not heteromorphic. The very large left-hand flanking *Eco*RV site lies outside the region shown.

Complementing activity of *Candida ADE2* as a selectable marker. An important requirement for these *ADE2*-containing clones to be useful for *Candida* transformation is their ability to function as a selectable marker. *S. cerevisiae* W343-4A (*ade2-1*) and XMK1-222 (*ade2-5*) were transformed with pMK3 and pMC1, and either $ADE2^+$ or $LEU2^+$ transformants were selected. The data in Table 2 show that the transformation efficiency was nearly the same for either selection. Furthermore, the cloned fragments complemented either *ade2* mutant allele, although strain XMK1-222 transformed less well in general than strain W343-4A.

Since Gillum et al. (8) demonstrated that the *URA3* gene of *C. albicans* could complement the *pyrF* mutation of *E. coli*, it was of interest to see if the *Candida ade2* complementing fragment could also complement a *purE* mutant of *E. coli*. Strain RR1A, which has a Tn*10* insertion in the *purE* gene, was transformed with pMK3 and pMC1. Both transformed strains remained auxotrophic for purines. On the possibility that the *S. cerevisiae* sequences prevented expression of the *ade2* gene, a *Cla*I fragment of pMK3 containing the insert sequences was subcloned into pBR322 at the *Cla*I site (Fig. 1). This new construction, pMC2, did not complement the *purE* mutation. To see whether there were any genomic *Candida* sequences which could complement this *purE* strain, we introduced the *Candida* YEp13 library into RR1A cells and screened it for adenine auxotrophy. Two $purE^+$ colonies were found among 10,000 transformants with inserts, but both had lost the Tn*10* insertion which had disrupted the *purE* gene.

Transformation of *C. albicans*. *C. albicans* hOG300 was chosen for use in the initial transformation experiments for three reasons. The strain had previously been identified as carrying an *ade2* mutation on genetic grounds by Poulter and Rikkerink (17). We observed that this strain could grow in the absence of adenine at high carbon dioxide tension (10%), a property previously reported for a subset of *ade2* mutants in *S. cerevisiae* (31). This strain also has a fairly low spontaneous reversion frequency (2×10^{-7} after 4 days at 30°C). The adenine requirement is, however, somewhat leaky, so that higher reversion frequencies are observed after long-term incubation, owing to leaky growth and subsequent reversion on the plates.

The results of transformation experiments with pMK3, pMC1, and pMC2 by modifications of the *N. crassa* transformation (see Materials and Methods) protocol are shown in Table 3. Numerous pinpoint colonies, as well as large,

TABLE 2. Transformation of *ade2 S. cerevisiae* strains with *Candida ADE2*-containing plasmids

Plasmid DNA	Strain	No. of transformants (per μg of DNA) with selected *S. cerevisiae* phenotype[a]:	
		LEU^+	ADE^+
pMK3	W343-4A	2×10^5	1×10^5
pMC1	W343-4A	5×10^4	1.4×10^4
pMK3	XMK1-222	0.9×10^4	0.9×10^4
pMC1	XMK1-222	NT[b]	0.5×10^3

[a] Two strains of *S. cerevisiae* carrying different *ade2* alleles were transformed with plasmid pMC1 or pMK3 and selected for either leucine or adenine prototrophy.

[b] NT, Not tested.

white colonies, were visible after 3 days in DNA-treated cultures, but only the large colonies gave rise to stable ADE$^+$ cultures upon subculturing. The tiny colonies, which may be abortive transformants, appeared whether protoplasts were regenerated in top agar or spread directly on regeneration medium. The transformation frequency generally ranged between 0.5 and 5 transformants per μg of DNA, which is consistent with the observations of integrative transformation in other yeasts and fungi (3, 5, 9, 27, 32). The frequency of transformation per viable spheroplast was 3 to 6 per 10^6 spheroplasts. This frequency is about 10 times the reversion rate of the *ade2* mutation (Table 3, −DNA control). The addition of YEp13 DNA did not increase the frequency of ADE$^+$ auxotrophs. In addition, the number of transformants increased linearly with increasing amounts of plasmid DNA (Fig. 4). Since we had seen no differences in transformation rates by either procedure in several separate experiments, we made a direct comparison of transformation efficiency with the two protocols. From the results (Fig. 4), no significant difference could be seen. Transformation frequences were consistently higher with pMC2 than with pMK3 or pMC1.

Evidence for the presence of vector DNA sequences in transformants. The presence of YEp13 sequences in adenine prototrophs was scored by hybridization with a radiolabeled probe in a colony blot analysis. Of 13 prototrophs obtained from transformation with pMC2, 4 gave strong positive signals with the YEp13 probe, whereas 3 were weakly positive. Of 26 prototrophs obtained by transformation with pMK3, 12 gave a positive signal with the YEp13 probe, whereas all 26 were positive with a pMK3 probe (Table 3).

The 26 pMK3 transformants were then tested for stability by growing for 20 generations in rich medium and plating onto selective and nonselective media. All 26 transformants were quite stable in the absence of selection. No ADE$^-$ segregants were detected for 24 of the strains (>100 isolates tested), whereas 2 of the strains gave rise to 1 and 5 ADE$^-$ segregants in ~100 isolates tested. The stability of these adenine-requiring transformants suggests that transformation had occurred by integration.

Three of the pMK3 transformants (A-1, A-2, and A-3) with YEp13 sequences were selected for further analysis, and DNA was prepared for use in Southern blots (Fig. 5). Undigested and *Cla*I-, *Pvu*II-, *Hin*dIII-, *Bam*HI-, *Sph*I-, and *Eco*RV-digested DNAs were hybridized with radiolabeled YEp13, pMK3, or pMC1 DNA. The results of these experiments for the A-1 transformant are shown in Fig. 5 with uncut and *Sph*I- and *Cla*I-digested DNAs. The other two transformants produced identical results (data not shown). Hybridization of YEp13 with undigested total genomic DNA

TABLE 3. Transformation frequencies of *C. albicans* hOG300 with *C. albicans ADE2*-containing plasmids

Plasmid DNA	Quantity of DNA (μg)	No. of transformants[a]		
		With ADE$^+$ phenotype	With ADE$^+$ and YEp13 sequences	Per μg of DNA
pMC1	5	1	1	0.2
pMK3	5	2	0	0
pMK3	56	30	12/26	0.53
pMC2	2.5	13	4–7/13[b]	5.2
None		0–2[c]	0	

[a] The transformation protocol used was the dimethyl sulfoxide and heat shock (*Neurospora*) procedure described in Materials and Methods. The presence of YEp13 sequence was determined by colony blots for the number of isolates indicated.

[b] Four of the colonies gave strong hybridization, whereas three had weaker signals.

[c] The appearance of ADE$^+$ revertants was 0, 1, and 2 in three separate experiments.

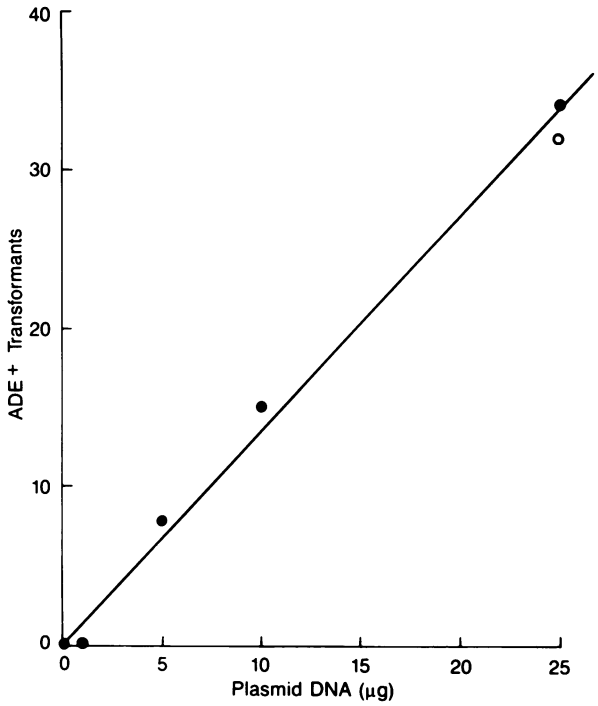

FIG. 4. Determination of numbers of transformants relative to quantity of input DNA. Various quantities of plasmid pMK3 DNA were used to transform strain hOG300 to adenine prototrophy. The *Saccharomyces* transformation procedure was used for all determinations except for the highest level of plasmid DNA tested (25 μg), in which both the *Saccharomyces* (●) and *Neurospora* (○) methods were used independently (see Materials and Methods for details).

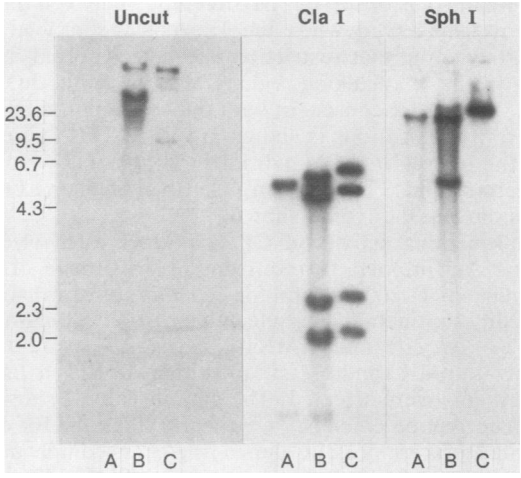

FIG. 5. Southern blot analysis of transformant A-1 (strain hOG300 transformed with pMK3). Lanes: A, total DNA isolated from strain hOG300; B, total DNA isolated from strain A-1 (strain hOG300 transformed with plasmid pMK3); C, pMK3 DNA. All three undigested lanes (marked uncut) were probed with radioactively labeled plasmid YEp13 DNA. The three *Cla*I-digested and three *Sph*I-digested lanes were probed with radioactively labeled pMK3 DNA. Numbers on the left indicate size in kilobases.

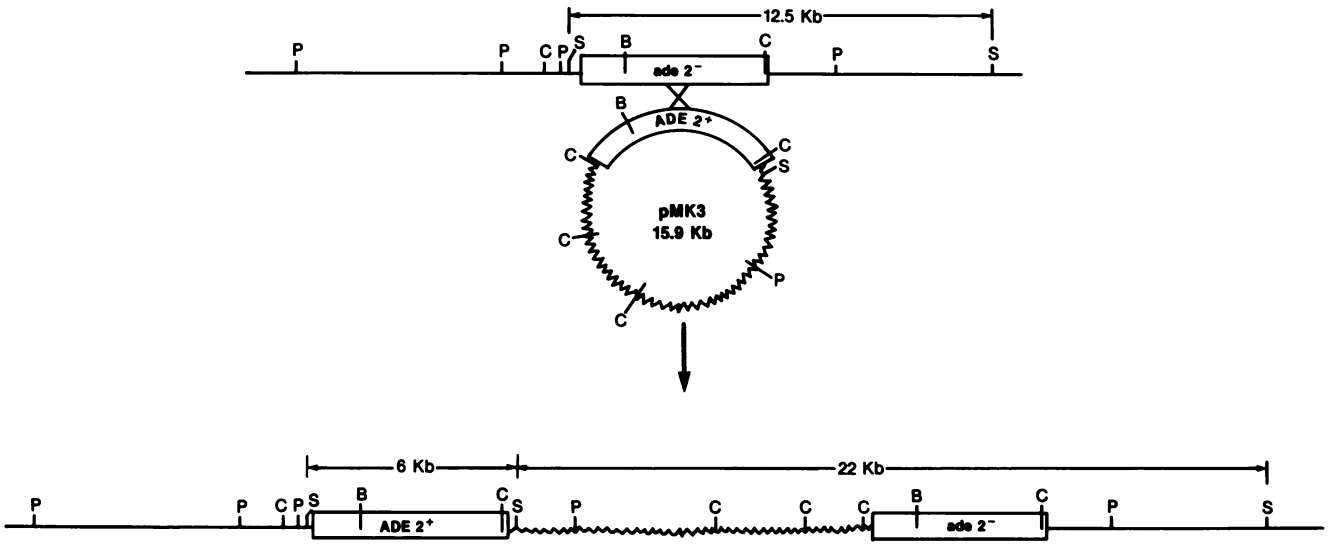

FIG. 6. Model for integrative transformation in *C. albicans*. The predicted structure of the *ADE2* region after a single homologous recombination event between pMK3 and one chromosomal homolog is shown. Fragments expected for *Sph*I-digested DNA were 6.0 and 22 kb. Host DNA digested with *Sph*I yielded a 12.5-kb fragment. ∿, YEp13 vector sequences; □, *ADE2* sequences; —, flanking host chromosome sequences. Abbreviations: P, *Pvu*II; C, *Cla*I; S, *Sph*I; B, *Bam*HI.

occurred at the position of chromosomal DNA for all three transformants, suggesting that these sequences had been integrated into the genomic DNA (Fig. 5). No hybridization with lower-molecular-weight bands could be observed, even with extended exposure of the autoradiograms. Restriction analysis indicated that the transformants arose by a single recombination event at the homologous region, retaining the entire YEp13 sequence. For example, *Cla*I digestion of untransformed hOG300 genomic DNA revealed two bands which hybridized with pMK3, as predicted from the single *Cla*I site within the *ADE2* region of pMK3. *Cla*I-cut DNA from transformant A-1 hybridized with an additional four fragments of 5.2, 6.0, 2.5, and 2.0 kb when probed with pMK3. Similar fragments were seen when vector pMK3 was digested with *Cla*I (Fig. 5). These results are consistent with one or several tandem copies integrated at the site of the resident *ADE2* gene (Fig. 6). Similar conclusions were drawn from *Bam*HI, *Eco*RV, and *Hin*dIII digestion results (data not shown).

Recovery of pMK3 from *Candida* transformants. Based on the mechanism of integrative transformation in *S. cerevisiae*, stable transformants of *C. albicans* could be produced by a single homologous recombination event (type I integration event by the system proposed by Hinnen et al. [9]) at the *ADE2* locus. The structure of such an integrated plasmid is shown in Fig. 6. From this structure it should theoretically be possible to recover pMK3 from transformant A-1 by cutting with the appropriate restriction enzyme followed by ligation at low DNA concentration. To test this idea, we digested DNAs from transformant A-1 and hOG300 with *Bam*HI, which cuts once within the *ADE2* region, ligated them, and used them to transform *E. coli* RR1A cells. Ampicillin-resistant colonies were obtained from cut and ligated A-1 DNA, but not hOG300 or uncut A-1 DNA. Plasmid preparations from four clones digested with *Hin*dIII and analyzed on an agarose gel were indistinguishable from pMK3.

Integration occurs at only one chromosomal homolog. Genetic and biochemical analyses have indicated that *C. albicans* has a diploid genome. Integrative transformation experiments with diploid *S. cerevisiae* strains have shown that only one of the two chromosomes has acquired transforming DNA (30). Unlike *S. cerevisiae*, *C. albicans* has no known haploid stage, and we therefore wanted to determine whether the transformation event yields a heterozygous or homozygous *ADE2* region. The results depicted in Fig. 5 showed that the three bands predicted by integration at only one chromosome were seen in A-1 DNA probed with pMK3. Recipient hOG300 DNA lacks an *Sph*I site within the *ADE2* region contained in pMK3. As a result, the host 12.5-kb band, in addition to two new bands, 6 and 22 kb, hybridize with pMK3 in *Sph*I-cut A-1 DNA. This is shown schematically in Fig. 6 and provides evidence at the molecular level for polyploidy in the *Candida* genome, as well as confirmation of the chromosomal location of the YEp13 sequences.

The results of this experiment also establish that A-1 contains a single copy of pMK3 at the *ade2* locus rather than several tandem copies. If tandem copies had been integrated, an additional 15.9-kb fragment equivalent to pMK3 should have been generated by *Sph*I digestion (Fig. 6). This band was not seen (Fig. 5). A similar analysis with *Pvu*II-cut DNA also indicated that A-1 has integrated a single copy of pMK3 (data not shown).

Transformation of other *Candida* red adenine auxotrophs. To test the general utility of the transformation procedure, we transformed other red adenine auxotrophs of *C. albicans* by the *Saccharomyces* protocol. Strain WCR-1-74, isolated by Sarachek (20), was tested for transformation ability with pMK3 (10 μg) and pMC1 (15 μg). Stable ADE$^+$ colonies arose only at the same frequencies as the reversion rate for the strain, and none contained YEp13 sequences as judged by colony blot analysis. Three strains isolated by Kwong-Chung and Hill (12), A81-Pu, B113P, and B792P, were tested with pMC2 (12.5 μg) for transformation. One of these, A81-Pu, was transformable by pMC2 at a frequency of 1.5 ADE$^+$ per μg of DNA. The reversion rate of the strain was extremely high. With 10 μg of DNA, the number of transformants was only threefold over the reversion rate. Transformation was confirmed by colony blots of stable ADE$^+$

colonies probed with radiolabeled YEp13. Of 18, 3 contained YEp13 sequences.

DISCUSSION

The results presented here establish an integrative transformation system for the pathogenic yeast *C. albicans* based on its cloned *ADE2* gene. Because *C. albicans* is naturally resistant to high levels of G418 (unpublished observation), G418 resistance, used to develop transformation systems in other yeasts (4, 25), can not be used as a selection for *C. albicans*. The 5.0-kb fragment common to pMK3 and pMC1 contains the legitimate *Candida ADE2* gene as demonstrated by its ability to complement not only two *ade2* alleles of *S. cerevisiae* but also two *ade2* alleles of *C. albicans*. The fact that a single integrated copy of pMK3 completely restored adenine prototrophy to *C. albicans* makes it unlikely that the complementation is the result of an outside suppressor. If such a suppressor gene was acting because of its high copy number in *S. cerevisiae*, it would be unlikely to act when only present at 0.5 copies per genome in *C. albicans*.

The transformation frequency with *ade2* complementation in *S. cerevisiae* was as high as that with *leu2* complementation, making the *ADE2* marker suitable as a selectable marker for both *C. albicans* and *S. cerevisiae*. The *ADE2* marker has the advantage of conferring a white phenotype on red, adenine-requiring recipients. This is useful when studying the stability of a transforming marker, since red segregants lacking *ADE2* are easily detected in a lawn of white colonies. Unfortunately, the ability of the *ADE2* gene to complement in heterologous systems does not extend to *E. coli*. Gillum et al. (8) demonstrated complementation of the *E. coli pyrF* mutation (orotidine-5′-phosphate decarboxylase) with the equivalent *Candida* gene (*URA3*). It has not, however, been established that the same promoter sequences function in both *E. coli* and *C. albicans*. For example, in the case of the *Saccharomyces URA3* gene, it has been shown that expression in *E. coli* is dependent on a fortuitous promotor sequence which is not present in all strains (19).

Genetic and biochemical studies of red adenine auxotrophs of several fungal species have demonstrated that they arise from mutations at one of two consecutive steps in purine biosynthesis (6, 7, 24). Poulter and Rikkerink (17) observed that one complementation group of red adenine auxotrophs in *C. albicans* showed intragenic complementation, a situation seen in *Saccharomyces ade2* mutants. From this similarity they hypothesized that these *Candida* mutants had the same biochemical lesion. The transformation data shown here support this assignment. Our data also suggest that the mutation in strain WCR-1-74 is in *ADE1*, an assignment made by complementation analysis (17) but in conflict with biochemical studies (20).

Although only a limited number of transformants have been studied (3 with pMK3 in this study and 10 with other plasmids), all transformants which have acquired vector sequences have these sequences inserted into the resident *ADE2* region. This is similar to the results obtained for transformation of the filamentous fungus *Aspergillus nidulans* (27, 32) and the yeast *S. cerevisiae* (30), where the majority of integrative events occur at the site of the resident gene. Some integration events occur at other sites in *A. nidulans* and *S. cerevisiae*, and in *N. crassa* almost half of the integration events occur at sites different from the resident gene (3). It is possible that some integration events occur at other sites in *C. albicans*. Since it is not possible to determine linkage relationships in *C. albicans* by convenient genetic methods, we used Southern blot analysis to examine a relatively small number of transformants. Of the total *Candida* pMK3 transformants tested, 12 of 26 had acquired YEp13 sequences (Table 3). The remainder did not integrate vector sequences and presumably were of the type III class defined by Hinnen et al. (9). These transformants may arise from double crossover events at the *ade2* gene or from a single crossover followed by recombination to eliminate the *ade2* and YEp13 sequences. In *A. nidulans* and *S. cerevisiae*, the vast majority of transformants have vector sequences integrated into the host genome (9, 27, 32), whereas only about one quarter of *qa-2* transformants contain vector sequences (3).

At present, the integrative transformation system for *C. albicans* is a low-frequency event—0.5 to 5 transformants per μg of vector DNA. This frequency is comparable to integrative transformation in other fungi such as *N. crassa*, *A. nidulans*, and *S. cerevisiae*. Higher frequencies are generally obtained by plasmids which allow autonomous replication in the host. Although pMK3 contains the 2μm fragment which supports autonomous replication in *S. cerevisiae*, it was not observed to replicate autonomously in *C. albicans*. All transformants had stably integrated vector sequences. We intend to test constructions which contain other ARS sequences from *S. cerevisiae* or *Candida* sequences which might act as ARS sequences to determine the requirements for autonomous replication in *C. albicans*.

Evidence for the ploidy of *C. albicans* has come from genetic studies (16, 21, 28, 29) and from measurements of DNA content (15, 18). We have demonstrated that plasmid pMK3 transforms *C. albicans* by integration at one of the chromosomal *ADE2* alleles. This observation is consistent with the genetic evidence that *C. albicans* has a diploid genome. Furthermore, the fact that only one of the homologs had acquired the *ADE2* gene suggests that only dominantly expressed markers or complementation of rare auxotrophs resulting from double mutations can be used for selecting the relatively rare transformants. These factors will be important in the design of future cloning vectors for this organism.

The general utility of the transformation method has been demonstrated by the transformation of more than one *Candida ade2* strain. The ability to transform hOG300 with the *ADE2* gene from SC5413 despite clear-cut restriction site heteromorphism between these two strains indicates that this method should have wide application independent of strain origin. We examined the *ADE2* region of A81-Pu and found that it too differs from hOG300 in restriction sites (unpublished observation). The recovery of integrated plasmid opens the possibility of retrieval of portions of the *Candida* genome by judicious selection of restriction enzymes followed by ligation. The *Candida* transformation method we have reported is simple and rapid and allows recovery of transformants in 3 days. This is the first report of a transformation system for an asexual, diploid microorganism and one of the first reports of a transformation system for a yeast lacking a sexual cycle for genetic analysis. We expect that a transformation system for *C. albicans* will provide new opportunities to study the molecular biology of this medically important organism.

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