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A Single-Transformation Gene Function Test in Diploid *Candida albicans*

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The fungal pathogen *Candida albicans* is naturally diploid, and current gene disruption strategies require two successive transformations. We describe here a genetic construct (*UAU1*) for which two copies may be selected. Insertion of *UAU1* into one genomic site, after a single transformation, allows selection for segregants with two copies of the insertion. Major classes of segregants are those carrying homozygous insertion mutations and allelic triplications, which have two insertion alleles and a wild-type allele. Thus nonessential and essential genes may be distinguished rapidly through PCR tests for homozygosis and triplication. We find that homozygous mutations may be isolated at three nonessential loci (*ADE2, RIM20*, and *YGR189*), while only allelic triplications were found at two essential loci (*SNF1* and *CDC28*). We have unexpectedly isolated homozygous mutants with mutations at *CDC25*; they are viable but defective in filamentation on serum-containing medium. The *UAU1* cassette is thus useful to assess rapidly the essentiality of *C. albicans* genes.

Candida albicans is an opportunistic fungal pathogen. It has been of experimental interest for two main reasons. First, it is a significant pathogen that causes oral mucosal infections, vaginitis, nosocomial bloodstream infections, and a variety of deep tissue infections (26). Therefore, many experimental studies have focused on pathogenesis, drug resistance, and analysis of prospective drug targets. Second, it is a distant cousin of the most well-characterized unicellular eukaryote, *Saccharomyces cerevisiae*, so that the function of a *C. albicans* gene may be suggested by its role in *S. cerevisiae*. Yet, surprisingly, *C. albicans* may use gene products and regulatory pathways in novel ways (4, 16, 17, 28, 34, 37). The contrast between *C. albicans* and *S. cerevisiae* can provide unique insight into regulatory mechanisms, interpathway relationships, and general aspects of eukaryotic biology.

Molecular genetics has played an increasingly prominent role in studies of *C. albicans*, particularly with the partial genomic sequence as a tool for gene discovery. However, genetic methods are cumbersome for two reasons (30). First, *C. albicans* strains are diploid (or of higher ploidy) and there is no meiotic division, so that gene disruption mutants must be constructed through two successive transformations. Second, with current methods, disruption of one allele may be straightforward, but disruption of the second allele is infrequent if the second disruption construct is homologous to the first. These problems can make it difficult to construct a homozygous mutant and to determine whether a gene is essential for growth.

We describe here a genetic strategy that circumvents these difficulties. It yields homozygous insertion mutations after a single transformation. The strategy provides a rapid test for essential genes and should thus accelerate drug target validation. In addition, the strategy can provide a preliminary assessment of mutant phenotypes, and it lends itself to large-scale analysis of gene function.

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MATERIALS AND METHODS

Plasmids. The *UAU1* (*ura3-ARG4-ura3*) (Fig. 1A) cassette is carried in plasmid pBME101, which was constructed as follows. Plasmid pRS424ARG4-URA3-BH1 (4) was digested with *Bsm*I and *Spe*I, overhangs were blunted with Vent polymerase, and blunt ends were ligated to form plasmid pBME98. This plasmid was then digested with *Xho*I and *Sma*I, filled in, and religated to remove a polylinker *Cla*I site to yield plasmid pBME99. Five hundred base pairs of the 3′ end of *URA3* was amplified from pRS424ARG4-URA3-BH1 with primers Kpn5′ and Xho3′-500 and ligated into the *Xho*I and *Kpn*I sites of pBME99, generating plasmid pBME101. (Oligonucleotide sequences are listed in Table 1.)

Several of the gene disruption constructs described below were generated by in vivo recombination in *S. cerevisiae*, based on the method of Ma et al. (18). The key feature of this method is that linearization of a plasmid with *CEN-ARS* or 2 μm replicons causes a decrease in transformation efficiency in *S. cerevisiae*, but inclusion of a DNA fragment that bridges the linearization site restores efficient transformation. The retrieved plasmids largely contain the resealed plasmid carrying the bridging DNA fragment inserted through homologous recombination. We used this method to extend the homology that flanked gene disruption PCR products. We could thus use gene disruption primers with only 40 bases of homology to the targeted locus. The construction of plasmid pBME102, which carries the *ade2::UAU1* gene disruption cassette, was typical. The *UAU1* cassette was amplified by PCR from plasmid pBME101 with primers ade2-3DR and ade2-5DR (Table 1) to yield an *ade2::UAU1* PCR product with 50 bp of *ADE2* homology on each end. Approximately 1 μg of the 4.15-kbp PCR product, purified from an agarose gel, was cotransformed with 0.2 μg of *Eco*RI-linerized plasmid pRS314-ADE (44) into *S. cerevisiae trp1* mutant strain AMP107 (36). *Eco*RI digestion linearized the plasmid by cleaving at a single site in the *C. albicans ADE2* insert that lies between the regions of homology to primers ade2-3DR and ade2-5DR. Transformants were selected on SC-Trp plates, which selects for the plasmid *TRP1* marker. A control transformation with no DNA yielded 0 colonies/plate, a control with linearized pRS314-ADE yielded 112 colonies/plate, and the cotransformation of linearized pRS314-ADE and the *ade2::UAU1* PCR product yielded 773 colonies/plate. DNA prepared from Trp^+ transformants (10) was transformed into *Escherichia coli* strain HB101 (Promega), with selection for ampicillin resistance. The structure of retrieved plasmid pBME102 was confirmed by restriction digestion.

The *snf1::UAU1* gene disruption cassette was carried in plasmid pBME105. We first constructed plasmid pBME103 as follows: *C. albicans* genomic DNA was diluted 100-fold and PCR amplified with primers SNF1c1aI-5′ and SNF1ecoRI-3′, and the PCR product was ligated into plasmid pGEMT-Easy (Promega). We then moved the *SNF1* insert as an *Eco*RI fragment into *Eco*RI-digested vector pRS424 (2) to create plasmid pBME104. Finally, plasmid pBME105 was generated by in vivo recombination of *Bsm*I-linearized pBME104 and a *snf1::UAU1* PCR product with 40 bp of *SNF1* homology on each end, produced with template plasmid pBME101 and primers SNF1 dr 5′ and SNF1 dr 3′.

The *cdc28::UAU1* cassette was carried in plasmid pBME108. We first constructed plasmid pBME100, which carries a 1.5-kbp clone of *C. albicans CDC28*, by PCR amplification of strain CAI4 DNA with primers cdc28-N and cdc28-C and ligation into vector pGEM-T (Promega). We also removed the *Xho*I site of vector pRS424 (2) by ligation of *Xho*I-digested and filled-in pRS424, yielding plasmid pBME106. We then constructed plasmid pBME107 by ligating the

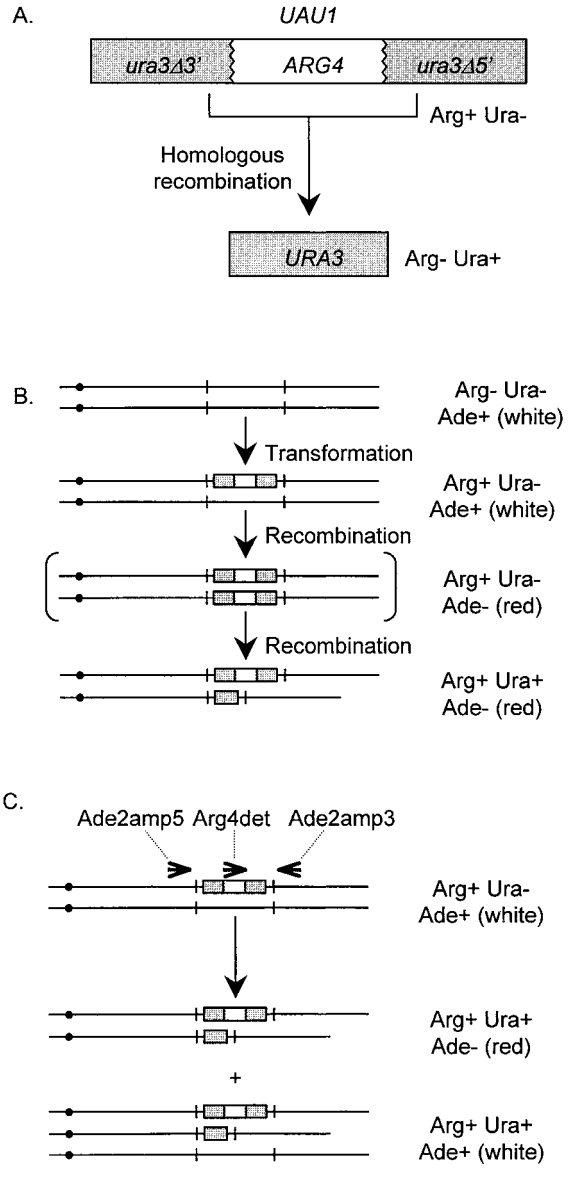

FIG. 1. Genetic properties of *UAU1*. (A) Conversion of *UAU1* to *URA3*. The *UAU1* marker (top) comprises an intact *ARG4* gene flanked by *URA3* deletion derivatives *ura3*Δ*3′* and *ura3*Δ*5′*. The *URA3* segments are nonfunctional, so the *UAU1* cassette confers an Arg^+ Ura^- phenotype. The *URA3* segments share 530 bp of homology and can thus recombine to yield an intact *URA3* gene. Recombination excises the *ARG4* gene and results in an Arg^- Ura^+ phenotype. (B) Anticipated use of *UAU1* to select for homozygous mutants (double-disruption selection). One allele of a gene (here *ADE2*) is disrupted with a *UAU1* insertion through transformation with selection for an Arg^+ phenotype. Growth of the transformant yields rare recombinant segregants in which the *UAU1* insertion allele is homozygous. Such segregants may be selected after they undergo recombinational excision within one *UAU1* cassette to yield a unique Arg^+ Ura^+ phenotype. (C) Outcome of double-disruption selection with an *ade2::UAU1*/*ADE2* strain. Genotypes were determined with PCR primers depicted at the top. The *ade2::UAU1*/*ADE2* strain yielded two classes of Arg^+ Ura^+ segregants. One class (homozygote) was Ade^-, grew into red colonies, and yielded diagnostic *ade2::UAU1* and *ade2::URA3* PCR products (Fig. 2A, lanes 1, 2, 5, 7, and 9). The other class (allelic triplication) was Ade^+, grew into white colonies, and yielded *ade2::UAU1, ade2::URA3*, and *ADE2* PCR products (Fig. 2A, lanes 3, 4, 6, 8, and 10). The latter class is depicted as a trisome as an example, but it may arise through translocation, tandem duplication, or other genetic rearrangements.

pBME100 insert, released with *Sac*II and *Spe*I, into pBME106 digested with *Sac*II and *Spe*I. Plasmid pBME108 was generated by in vivo recombination of *Xho*I-digested pRS424ΔXhoI-CDC28 and a *cdc28::UAU1* PCR product produced from template plasmid pBME101 and primers cdc28 5′DR and cdc28 3′DR.

Strains. The *C. albicans* strains (Table 2) are derivatives of strain BWP17 (*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*), which was derived from strain CAI4 (7) as described previously (44). The following *UAU1* disruption heterozygotes were constructed through transformation of strain BWP17 with the restriction fragments indicated and selection for Arg^+ transformants: strain BMY7, the *Not*I/*Eco*RV insert fragment from plasmid pBME 102; strain BMY18, the *Pvu*II insert fragment from plasmid pBME105; and strain BMY22, the *Pvu*II insert fragment from plasmid pBME108. The following *UAU1* disruption heterozygotes were constructed through transformation of strain BWP17 with the PCR product indicated and selection for Arg^+ transformants: strain BMY16, PCR with primers cdc25 5′dr and cdc25 3′dr on template pBME101; and strain DAY151, PCR with primers YGR189 5′DR and YGR 189 3′ DR on template pBME101. The *UAU1* heterozygote BMY17 was constructed by homologous integration of the *UAU1* cassette into *URA3* sequences of a *rim20::URA3* allele. Specifically, we transformed strain DAY18 (originally called Enx-het1 [44]), of genotype *rim20::URA3*/*RIM20 ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*, with the PCR product of template pBME101 amplified with primers 5-Detect and 3-Detect, selected for Arg^+ transformants, and screened for those that became Ura^-. (This integration reaction is the reverse of the excision reaction diagrammed in Fig. 1A.) Integration of *UAU1* cassettes in heterozygotes was confirmed through genomic DNA PCRs with the primer pairs listed in Table 2.

The extents of the deletion mutations are as follows. For *ADE2*, the deletion removes codons 195 to 310; the open reading frame (ORF) is 568 codons. For *RIM20*, the deletion removes codons 520 to 719; the ORF is 785 codons. For *YGR189*, the deletion removes codons 180 to 316; the ORF is 453 codons. For *SNF1*, the deletion removes codons 20 to 358; the ORF is 620 codons. For *CDC28*, the deletion removes codons 131 to 181; the ORF is 317 codons. For *CDC25*, the deletion removes codons 1000 to 1333; the ORF is 1333 codons. All of these deletions remove highly conserved regions of the respective proteins, including key residues required for *SNF1* and *CDC28* protein kinase activity. The deletion of *CDC25* removes the GDP-GTP exchange factor domain (15), which is the most highly conserved region of the protein.

Media and growth conditions. *C. albicans* and *S. cerevisiae* were routinely cultured in YPD plus uridine (2% Bacto Peptone, 1% yeast extract, 20% dextrose, and 80 μg uridine per ml). Selection was done on SD synthetic medium (6.7% yeast nitrogen base plus ammonium sulfate, without amino acids, and with 2% dextrose); auxotrophic supplements were added at standard concentrations (14) except that uridine was added at 80 μg per ml.

PCR detection. Genotypes at each locus were determined through PCR assays with the primers indicated in Table 2. Flanking primers (illustrated by Ade2amp5 and Ade2amp3 in Fig. 1C) were used to detect wild-type alleles and *URA3* insertion alleles. However, we found that detection of *UAU1* insertion alleles was unreliable with flanking primers, presumably because of the more efficient synthesis of the smaller products from wild-type and *URA3* insertion alleles. Therefore, we used a flanking primer and an internal *ARG4* primer (illustrated by Ade2amp3 and Arg4det in Fig. 1C) in a second PCR to detect the presence of *UAU1* insertion alleles. PCR was performed with total yeast genomic DNA as described previously (44). Reaction mixtures were typically heated to 94°C for 5 min, followed by 33 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. After a final incubation at 72°C for 10 min, reaction mixtures were stored at 4°C.

Determination of recombination rates. Recombination rates were determined by the method of Lea and Coulson, as described by Rattray and Symington (33). A *UAU1* insertion heterozygote was streaked onto YPD and grown at 30°C for 2 to 3 days. Nine entire single colonies were used to inoculate separate 3-ml YPD broth cultures that were grown to saturation at 30°C. One milliliter of saturated culture was washed, suspended in 1 ml of sterile water, and diluted for plating. One hundred microliters of the undiluted suspension was plated on SC-Arg-Ura, 100 μl of a 1/100 dilution was plated on SC-Ura, and 20 μl (in a total volume of 200 μl) of a 1/10,000 dilution was plated on YPD. Colony counts of these plates were used to determine the median mitotic recombinant frequencies (Ura^+/total or Ura^+ Arg^+/total) for each strain. Recombination rates (events per cell per generation) were calculated according to the formula rate = (0.4343 × median frequency)/(log N − log N_0), where N is the total number of cells in the culture and N_0 is the initial number of cells (one cell) that gave rise to the culture (33).

RESULTS

Selection for homozygous mutants. We constructed a gene disruption cassette, *UAU1* (*ura3-ARG4-ura3*), that can exist in two states (Fig. 1A). The cassette includes three segments: *ura3*Δ*3′*, a nonfunctional 3′ deletion copy of *URA3; ARG4*; and *ura3*Δ*5′*, a nonfunctional 5′ deletion copy of *URA3* that shares 530 bp of sequence identity with the *ura3*Δ*3′* segment. The cassette expresses *ARG4*, but not *URA3*, in this state (*UAU1*). The 530 bp of homology between the *ura3* segments permits recombination to yield an intact *URA3* gene and excision of *ARG4*. The resulting cassette expresses *URA3* but not *ARG4*. We reasoned that the cassette would permit identification of

TABLE 1. Oligonucleotide sequences

Oligonucleotide	Sequence (5′ to 3′)
3-Detect	TGTGGAATTGTGAGCGGATAACAATTTCAC
5-Detect	GTTTTCCCAGTCACGACGTTGTAAAACGAC
ade2-3DR	GGGTTGCCTTATCACCCAAGACATTCAACATAATAGCATTGGTGATGGAATGTGGAATTGTGAGCGGATA
ade2-5DR	GTCCATTATATGCTGAAAAATGGTGTCCTTTCACCAAAGAATTGGCTGTGGTTTTCCCAGTCACGACGTT
Ade2amp3	CCATCTTTCGCTCTGGTCTAGTAG
Ade2amp5	GTCGATGACTTGTTACACATTGGG
Arg4det	GGAATTGATCAATTATCTTTTGAAC
cdc25 3′DR	TAAATTTTACGTTTTTATTGATTCGCTCTACTGTACAAACACTATGCTTAGCAAATACTATGTGGAATTGTGAGCGGATA
cdc25 5′DR	AATATTATGAAACTTTTACTTGAAAAAAATTGGTCCATGTCGTATTACAATGAACCTGTAGTTTTCCCAGTCACGACGTT
Cdc25amp3	CATTGTGTATTAGAAGTCTGTAGTTC
Cdc25amp5	GGAATTGATATCTTTACTAATTGC
cdc28 3′DR	CAGACCACATATCTACCCCAGTGGAATATTGTTTCCCTCCTGTGGAATTGTGAGCGGATA
cdc28 5′DR	ATTCGAGGTATTAAACATTGTCATTCTCATCGAGTTTTACGTTTTCCCAGTCACGACGTT
cdc25del1	TGAAAACGGTTTGAAACTCTTGA
cdc25del2	CATGTGTGCCATTTTTTGGTGTG
cdc25del3	TGTCAAATCAGATAAGTACACACCA
cdc25del4	CCAATCAATTGACTAACTTTGTTGG
cdc25del5	TATCAATGCTCTCACTGAGCCCTG
cdc25del6	TTTGCTCACTCCGACCAAGTTC
Cdc28amp3	CCCTGAATATCCTGAAAAGCAATCG
Cdc28amp5	GAAGATGAAGGTGTACCTAGTACC
cdc28-C	CCCCTGAATATCCTGAAAAGCAATCG
cdc28-N	CCAACTATAGAACACACACATCCCAAGCC
Kpn5′	GGGGGTACCTGATTTCTAGAAGGACCACC
Rim20amp3	GATTCCATAAACCAGGTTTACTAG
Rim20amp5	GAGTGTAATCATTTGTTGCAAGAG
SNF1 dr 3′	TTGATTTCGATTGCTGAGACGATGAGTTTCCTGCAGCAACTGTGGAATTGTGAGCGGATA
SNF1 dr 5′	GCAAGTTCCGATCGACCCCGCTGCAAATCCAGCAAATAGAGTTTTCCCAGTCACGACGTT
Snf1amp3	GTCTTTCACCAATGCATGATTC
Snf1amp5	CATAATGAAAATCAATCGCAAC
SNF1claI-5′	CCATCGATGAATCAATATATAGAAGAAGG
SNF1ecoRI-3′	GGGGAATTCGCTCATCTTTAATTAGTTTCG
Xho3′-500	GGGCTCGAGCATCAATTTATGATTTTTGAAG
YGR189 3′DR	AGCTTGAAGAGGAGGAGGAAGATGATGATGATGATGATGAAGTGGTAGATGAAGGTGAGCGTGGAATTGTGAGCGGATA
YGR189 5′DR	CCATACTTATGTTATTGATTGGACCAAAGATGCAGTTACTTGGTCCGTTGACGGTAGTGTTTTCCCAGTCACGACGTT
Ygr189amp3	GAACTGCATTGGATTTTCGC
Ygr189amp5	CTACTACTTATGATCGTGGT

homozygous mutants (Fig. 1B). A heterozygous *UAU1* insertion, introduced by transformation, might occasionally become homozygous through gene conversion or a mitotic recombination event. The homozygous mutant would be uniquely capable of yielding segregants that express both *ARG4* and *URA3*. Thus, homozygous mutants should be found among Arg^+ Ura^+ segregants in a population carrying a *UAU1* insertion.

We used this rationale to construct homozygous *ade2*/*ade2* mutants after one transformation of reference strain BWP17 (relevant genotype: *arg4*/*arg4 ura3*/*ura3 ADE2*/*ADE2*). The strain was transformed with an *ade2::UAU1* DNA fragment. An Arg^+ transformant (strain BMY7) had the genotype *ade2::UAU1*/*ADE2*, as verified by PCR and Southern blot analyses (Fig. 2A, lane H, and data not shown). Strain BMY7 yielded primarily white Ade^+ colonies, and loss of *ADE2* function creates red Ade^- colonies. We grew 30 independent cultures of the heterozygote and plated aliquots to isolate Ura^+ and Arg^+ Ura^+ segregants (Table 2). Ura^+ segregants arose a rate of 3.5×10^{-6} per division as uniformly white Ade^+ colonies. Arg^+ Ura^+ segregants arose at a rate of 2.0×10^{-8} per division. Among the Arg^+ Ura^+ colonies, 23% were red and Ade^-. We used PCR analysis to determine the genotype of one randomly chosen Arg^+ Ura^+ segregant from each culture (Fig. 2B, lanes 1 to 10, and data not shown). We found 12 segregants of genotype *ade2::UAU1*/*ade2::URA3*, and these were phenotypically red and Ade^-. The 18 remaining segregants were of

TABLE 2. Double-disruption selection experiments

Locus	Strain (genotype[a])	Detection primers	Ura^+ rate per division[b]	Arg^+ Ura^+ rate per division[b]	No. of homozygotes[c]
ADE2	BMY7 (*ade2::UAU1*/*ADE2*)	Ade2amp5, Ade2amp3, Arg4det	3.5×10^{-6}	2.0×10^{-8}	12
YGR189	DAY151 (*ygr189::UAU1*/*YGR189*)	Ygr189amp5, Ygr189amp3, Arg4det	2.3×10^{-6}	1.0×10^{-8}	10
RIM20	BMY17 (*rim20::UAU1*/*RIM20*)	Rim20amp5, Rim20amp3, Arg4det	1.5×10^{-5}	6.8×10^{-8}	11
SNF1	BMY18 (*snf1::UAU1*/*SNF1*)	Snflamp5, Snflamp3, Arg4det	2.9×10^{-6}	1.3×10^{-8}	0
CDC28	BMY22 (*cdc28::UAU1*/*CDC28*)	Cdc28amp5, Cdc28amp3, Arg4det	7.9×10^{-6}	3.5×10^{-7}	0
CDC25	BMY16 (*cdc25::UAU1*/*CDC25*)	Cdc25amp5, Cdc25amp3, Arg4det	5.4×10^{-6}	3.6×10^{-9}	2

[a] Strains carried the additional mutations *ura3*Δ*::*λ*imm434*/*ura3*Δ*::*λ*imm434 his1::hisG*/*his1::hisG arg4::hisG*/*arg4::hisG*.

[b] Recombination rates were calculated through the method of the median, as described in Materials and Methods.

[c] The number of homozygous mutants found among 30 independent Arg^+ Ura^+ segregants from the heterozygote indicated. Genotypes were determined through PCR analysis, examples of which are shown in Fig. 2.

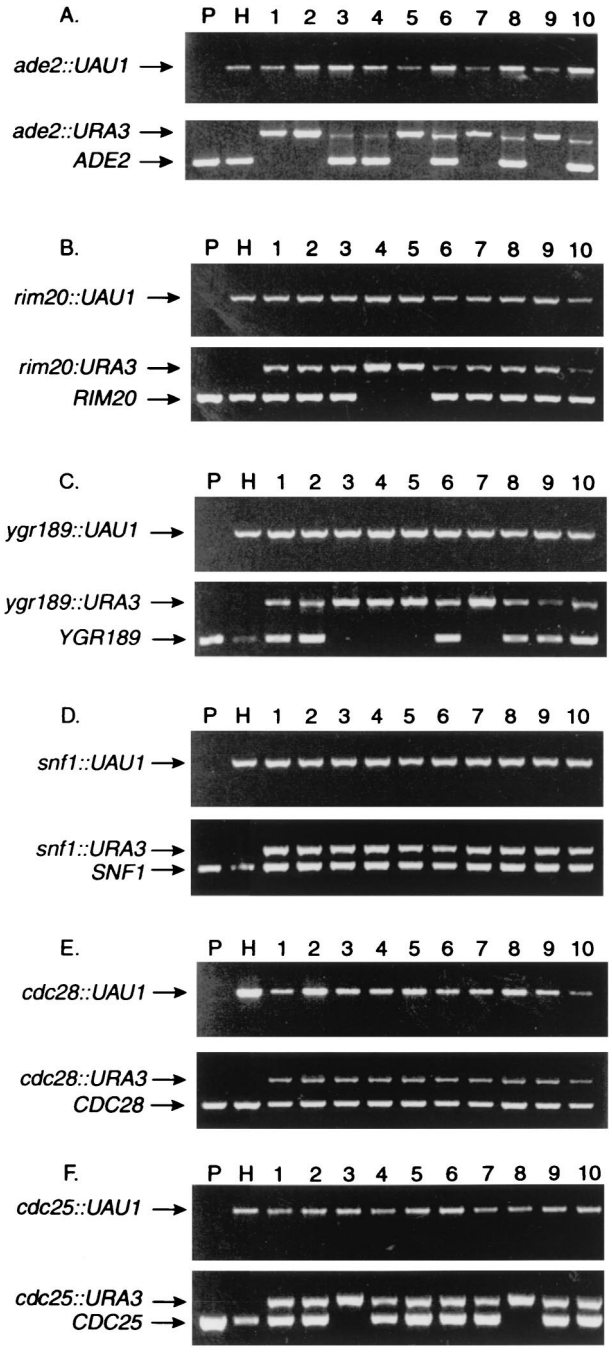

FIG. 2. PCR analysis of double-disruption selection segregants. Each panel shows a composite of two agarose gels of PCRs conducted with genomic DNA templates. The upper-gel PCRs used amp3 and Arg4det primers; the lower-gel PCRs used amp3 and amp5 primers. (The amp3–amp5 PCRs are unreliable for detection of full-length *UAU1* insertions, presumably because the smaller PCR products are amplified more efficiently.) Lanes: templates from parent strain BWP17 (lanes P), the respective *UAU1* disruption heterozygote (lanes H), and 10 independent Arg^+ Ura^+ segregants from the disruption heterozygote (lanes 1 to 10). Panels show analyses for *ADE2* (A), *RIM20* (B), *YGR189* (C), *SNF1* (D), *CDC28* (E), and *CDC25* (F).

genotype *ade2::UAU1/ade2::URA3/ADE2* and were phenotypically white and Ade^+. These latter Arg^+ Ura^+ Ade^+ segregants thus have three copies of the *ADE2* locus; the third copy may derive from trisomy or translocation (Fig. 1C). Our results indicate that homozygous mutants can be isolated after a single transformation.

To determine whether this homozygosis strategy may be useful at other loci, we examined the genes *RIM20* and *YGR189*. Previously constructed *rim20/rim20* mutants are viable, so this gene is not essential (4). We constructed *rim20::UAU1/RIM20* and *ygr189::UAU1/YGR189* heterozygotes through one transformation (strains BMY17 and DAY151, respectively) and examined 30 independent cultures of each for production of Ura^+ and Arg^+ Ura^+ segregants (Table 2). For both strains, these segregants arose at rates similar to those observed with the *ade2::UAU1/ADE2* heterozygote. PCR analysis of genotype was again carried out on one Arg^+ Ura^+ segregant per culture (Figs. 2B and C and data not shown). For both genes, we found that one-third of the segregants were *rim20::UAU1/rim20::URA3* or *ygr189::UAU1/ygr189::URA3* homozygotes, respectively, and two-thirds were *rim20::UAU1/rim20::URA3/RIM20* or *ygr189::UAU1/ygr189::URA3/YGR189* triplication derivatives, respectively (Table 2). The *rim20/rim20* homozygotes had a filamentation defect, as found for conventionally constructed homozygotes (4); the *ygr189/ygr189* homozygotes had no obvious phenotype. Therefore, homozygous mutants have been isolated by selection from *UAU1* insertion heterozygotes at three different *C. albicans* loci.

Homozygote-trisome (HT) test for essential genes. Identification of essential genes in *C. albicans* is vital to assess prospective drug targets (30) and to consider more-refined functional tests (1). *C. albicans* genes have been considered essential if one allele can be disrupted but the second cannot. We reasoned that the *UAU1* cassette might simplify and accelerate this assessment: a *UAU1* insertion in nonessential genes yields both homozygous and triplication-bearing segregants, but a *UAU1* insertion in an essential gene should yield only triplication-bearing segregants. Thus, we examined the consequences of *UAU1* insertions in three likely essential genes: *SNF1, CDC28*, and *CDC25*.

Petter et al. have presented strong experimental evidence that *SNF1* is essential for viability (29). We created a *snf1::UAU1/SNF1* heterozygote and found that it produced Ura^+ and Arg^+ Ura^+ segregants at rates similar to those for *ADE2* and *YGR189* insertions (Table 2). PCR genotyping of 30 independent Arg^+ Ura^+ segregants revealed that all were *snf1::UAU1/snf1::URA3/SNF1* triplication derivatives (Table 2; Fig. 2D). Our failure to obtain *snf1/snf1* homozygotes is consistent with the conclusion that *SNF1* is an essential gene.

CDC28 specifies a cyclin-dependent protein kinase, and activity of such kinases is vital for cell cycle progression in all eukaryotes (21). The *C. albicans CDC28* gene complements corresponding defects in other yeasts (3, 35), so we inferred that *C. albicans CDC28* may be essential. A *cdc28::UAU1/CDC28* heterozygote produced Ura^+ and Arg^+ Ura^+ segregants at rates comparable to those for other insertion heterozygotes; PCR genotyping revealed that 30 independent Arg^+ Ura^+ segregants were *cdc28::UAU1/cdc28::URA3/CDC28* triplication derivatives (Table 2; Fig. 2E). These results support the idea that *C. albicans CDC28* is an essential gene.

The *S. cerevisiae CDC25* gene is essential for viability; Cdc25p is an activator of Ras proteins (39). *C. albicans CDC25* complements an *S. cerevisiae cdc25* mutant (8), so we inferred that *CDC25* may be essential in *C. albicans* as well. A *cdc25::UAU1/CDC25* heterozygote produced Ura^+ segregants at rates comparable to those for other insertion heterozygotes and produced Arg^+ Ura^+ segregants at a slightly lower rate (Table 2). PCR genotyping revealed that 2 of 30 independent Arg^+ Ura^+ segregants were of genotype *cdc25::UAU1/cdc25::URA3* (Fig. 2F, lanes 3 and 8); the remaining 28 segregants

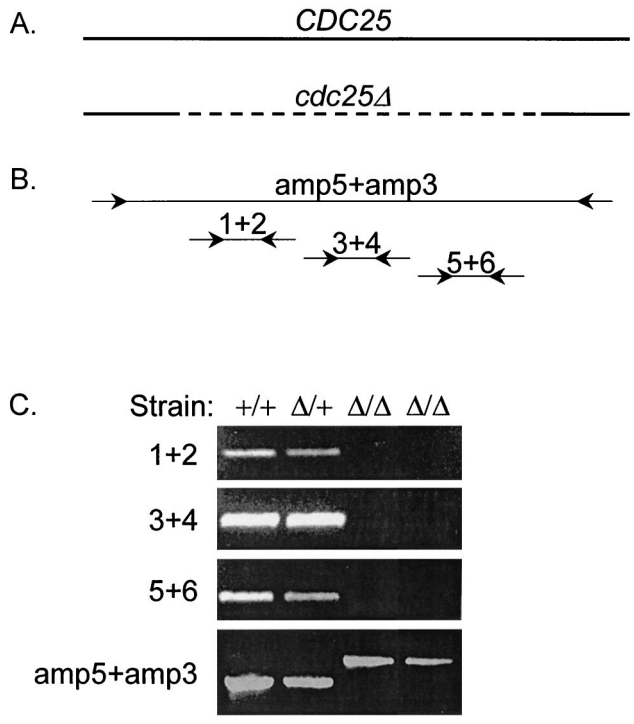

FIG. 3. PCR analysis of *cdc25*Δ/*cdc25*Δ segregants. (A) Schematic diagram of wild-type and mutant *CDC25* loci. The solid line represents the genomic *CDC25* sequence. The dashed line represents the sequence deleted and replaced with *UAU1* and *URA3* in the *cdc25::UAU1* and *cdc25::URA3* alleles. (B) Locations of PCR primers. Each line represents a PCR amplification product that derives from the *CDC25* sequences directly above (in panel A). The primer pair amp5 + amp3 (Cdc25amp5 and Cdc25amp3) can amplify wild-type and mutant *CDC25* alleles. The primer pairs 1+2 (cdc25del1 and cdc25del2), 3+4 (cdc25del3 and cdc25del4), and 5+6 (cdc25del5 and cdc25del6) amplify regions that are deleted by the *cdc25::UAU1* and *cdc25::URA3* alleles. (C) PCR products from genomic templates. Genomic templates derived from strains BWP17 (*CDC25*/*CDC25*), BMY16 (*cdc25::UAU1*/*CDC25*), and the two *cdc25::UAU1*/*cdc25::URA3* segregants were amplified by PCR with the primer pairs indicated, and PCR products were visualized after agarose gel electrophoresis.

were *cdc25::UAU1*/*cdc25::URA3*/*CDC25* triplication derivatives (Table 2; Fig. 2F). In principle, the DNA segment deleted from the *CDC25* locus might have been translocated to a different region of the genome in the two segregants. Such a translocation would be undetected in flanking-primer PCRs but should be detected with PCRs of internal segments. However, three internal primer pairs yielded no PCR product from genomic templates prepared from the *cdc25::UAU1*/*cdc25::URA3* segregants (Fig. 3). Therefore, *CDC25* is not essential for viability in *C. albicans*. A recent study indicates that *C. albicans* Ras2p is a positive regulator of serum-induced filamentation (6). The two *cdc25*/*cdc25* homozygotes grew at a slightly reduced rate and were partially defective in filamentation on agar containing serum. These findings support the idea that Cdc25p promotes Ras2p activity and filamentation but indicate that *CDC25* is not an essential gene.

DISCUSSION

Analysis of gene function in *C. albicans* is a critical step toward understanding its biology, pathogenesis, and vital processes. In the past, this analysis has required two successive transformations to create homozygous null mutations or conditionally expressed alleles (1, 7, 30, 43). These methods allow construction of precisely defined mutant strains that can be characterized via a battery of refined phenotypic tests. Here, we have described a double disruption selection strategy with a different purpose: to construct homozygous mutants rapidly for a preliminary assessment of gene function. Our strategy is not a replacement or a substitute for the careful crafting of homozygous mutants. Instead, it is a strategy that lends itself to large-scale analysis of gene function.

We have presented here an application of double-disruption selection that facilitates one important goal of *C. albicans* research: the identification of essential genes. We refer to this test as the HT test. The methodology fills an important need for basic research, because it is otherwise laborious to determine whether a gene is essential (29, 30), and current technology can yield conflicting results (5, 16, 22, 38). The methodology also fills an important need for genome-based pharmaceutical research, in which one must evaluate a large number of gene products as prospective drug targets (23). Finally, we expect that the technical simplicity of our method may encourage those who study a conserved gene in some other organism to test the homologous gene's function in *C. albicans*.

The results of HT tests were largely consistent with expectations based on prior studies: *SNF1* and *CDC28* are essential; *ADE2* and *RIM20* are not. However, the test also argued that *CDC25* is not essential, because homozygous *cdc25*/*cdc25* mutants were isolated. (The deletion that we constructed removes much of the domain that is necessary and sufficient for GDP-GTP exchange factor activity [15], so we can formally conclude only that Cdc25p exchange factor activity is not essential.) We note, though, that the *cdc25*/*cdc25* homozygotes arose a lower rate than homozygous insertion mutations in other nonessential genes. This observation might be explained if viability of the *cdc25*/*cdc25* mutant depended upon a genetic suppressor mutation, as is the case in *S. cerevisiae* (39). A second possibility is that *CDC25* is completely dispensable, as is likely the case in *Schizosaccharomyces pombe* (19), but that the structure or position of the locus limits recombination. We note that the same ambiguities apply to traditional (two-transformation) gene disruption strategies: infrequent isolation of a targeted mutant may reflect either the inadvertent selection of the genetic suppressor or recombinational, rather than functional, properties of the locus. The ambiguities may be resolved by protein depletion experiments or use of dominant-negative mutants (1, 6). Thus, the advantage of the HT test is that it provides functional information rapidly that can justify more definitive but laborious experiments.

There is a circumstance—the case of preexisting triplicated alleles—in which the HT test might yield misleading results. There are three loci for which triplicated alleles have been reported, *CHS2*, *FTR1*, and *FKS1* (*GSC1*) (9, 22, 32), although *FKS1* (*GSC1*) is apparently not triplicated in all isolates of strain CAI4 (5). One can envision that insertion of the *UAU1* cassette into *CHS2*, for example, would yield a genetic structure consistent with a genotype of *chs2::UAU1*/*CHS2*; the actual genotype would be *chs2::UAU1*/*CHS2*/*CHS2*. Subsequent Arg^+ Ura^+ selection would yield exclusively segregants of genotype *chs2::UAU1*/*chs2::URA3*/*CHS2*, not because *CHS2* is essential but because the strain initially had triplicated alleles. Thus far, preexisting triplications have seldom been observed in *C. albicans*, so we expect that this limitation for the HT test will not outweigh its usefulness.

Our results with double-disruption selection raise an important question that we have not yet resolved: how large a DNA segment becomes homozygous in the Arg^+ Ura^+ homozygous disruption mutants? Given that natural *C. albicans* isolates are heterozygous for preexisting mutations (31, 40–42), it is possi-

ble that selection for homozygosity of a *UAU1* insertion mutation may yield homozygosity of a linked mutation as well. In our small survey of known nonessential genes, we found only homozygotes that had phenotypes consistent with traditionally constructed mutants. However, the possibility that a linked mutation may become homozygous remains an important caveat for any conclusion derived solely from double-disruption selection.

Our estimates of mitotic recombination rates point to a surprising conclusion: that heterozygous mutations become homozygous at a rate of 2×10^{-3} per division. We calculate this rate from the rate of production of detectable homozygotes ($\sim 1 \times 10^{-8}$ per division) and the rate of recombination to generate *URA3* from *UAU1* ($\sim 3 \times 10^{-6}$ per division). This homozygosis rate is 10^2- to 10^3-fold higher than expected from studies of *S. cerevisiae* (24) and violates anecdotal common knowledge derived from use of Ura-blaster cassettes to create gene disruptions (7, 43). One simple explanation is that our estimate of the rate of recombination to generate *URA3* from *UAU1* may be artificially low because of a selective advantage of Arg^+ cells during growth in broth culture. However, in coculture experiments, we have not detected such an advantage (B. Enloe and A. P. Mitchell, unpublished results). A second explanation is that our frequency estimates are in error because of differences in growth dynamics on the selective plates. Thus, for example, Ura^+ colonies might arise only from preexisting recombinants, while Arg^+ Ura^+ colonies might arise during growth after plating. A third explanation is that the event that generates *URA3* in a *UAU1* insertion heterozygote is not equivalent to the event that generates *URA3* in a homozygote or triplication derivative. For example, a single recombination event might generate two copies of the insertion mutation and simultaneously promote conversion of *UAU1* to *URA3* in one of the copies. Therefore, our rate estimates serve as an empirical guide, but we remain skeptical that interchromosomal recombination is so frequent in *C. albicans*.

Allelic triplications were detectable for all of the genes we examined, and we are uncertain of their genetic structure. One simple possibility is that they result from trisomy for the respective chromosome, perhaps in conjunction with an overall increase in ploidy. In support of this idea, we note that several different selections yield monosomic and trisomic *C. albicans* derivatives (12, 13, 27). A second possibility is that the triplications result from tandem duplication or translocation of a smaller genomic segment, a mechanism that also has experimental support (11, 20, 25). The *UAU1* cassette may be a useful tool to define the genetic and environmental parameters that influence changes in gene dosage through either of these mechanisms.

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