Loss of heterozygosity, by mitotic gene conversion and crossing over, causes strain-specific adenine mutants in constitutive diploid *Candida albicans*

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Molecular evidence is provided in this paper to elucidate a long-standing intriguing phenomenon in fungal genetics: that many natural isolates of the constitutive diploid organism *Candida albicans* yield strain-specific, recessive mutants at a reproducible frequency that is as high as a few percent of the surviving cells after exposure to UV irradiation or other mutagens. Southern hybridization analysis and DNA sequence data indicated that *C. albicans* CA12, a clinical isolate, is heterozygous for the *ADE2* gene, carrying one functional and one null allele. Sequence analysis of the null allele revealed the presence of a 1.3 kb deletion, which locates between two AATC repeats and spans the promoter and coding regions of the gene. The adenine auxotrophic mutants, which were readily isolated after UV irradiation of *C. albicans* CA12, were proved to be the segregants of mitotic recombination as they remained as diploid, not hemizygous or haploid, cells and were homozygous for *ade2*. Analysis of reciprocal products of the mitotic recombination detected that the process of loss of heterozygosity was mediated by mitotic crossing over (reciprocal exchange of genetic information) as well as gene conversion (non-reciprocal exchange of genetic information).

Keywords: *Candida albicans*, loss of heterozygosity, adenine auxotroph, gene conversion, mitotic crossing over

INTRODUCTION

Candida albicans, the most common human fungal pathogen, is responsible for the majority of candidiasis (Odds, 1994). This opportunistic pathogen is part of the normal flora in the mouth, vaginal and gastrointestinal tracts in individuals with intact humoral and cellular immunity (Matthews, 1994), but becomes an invasive pathogen in people with a variety of transient or permanent immunocompromised conditions, including transplant recipients, chemotherapy patients, underweight neonates and HIV-infected individuals (Wickes & Petter, 1996). Molecular genetic, parasexual genetic and biochemical data have demonstrated that the fungus is an obligate diploid (Riggsby *et al.*, 1982; Poulter, 1995), and there is no confirmed report of a haploid cell

or sexual life cycle for the organism. A population-genetic study based upon isozyme-polymorphism analysis has confirmed that the fungus reproduces by clonal propagation (Pujol *et al.*, 1993). The unique biology and the close relationship between oropharyngeal candidiasis and AIDS development (Coleman *et al.*, 1993) have made the organism one of the current focuses in basic and medical research (Braun & Johnson, 1997; Corner & Magee, 1997; Lo *et al.*, 1997; Stoldt *et al.*, 1997; Gale *et al.*, 1998).

It has been observed that many *C. albicans* natural isolates are prone, after UV or chemical mutagenesis, to yield strain-specific, recessive mutant derivatives at high frequencies (Poulter, 1995). The most common mutant found was auxotrophic for sulfite reduction, followed by mutants auxotrophic for adenine, uracil, cytosine, isoleucine-valine, proline and methionine (Poulter, 1987). Mutants in colony morphology and resistance to 5-fluorocytosine have also been reported (Defever *et al.*,

Abbreviation: LOH, loss of heterozygosity.

1982; Poulter, 1987). It has been estimated that at least 10% of *C. albicans* isolates exhibit certain types of auxotrophic heterozygosity (Scherer & Magee, 1990). Based on their extensive genetic studies on UV-induced amino acid auxotrophs in *C. albicans* FC18, Whelan *et al.* (1980) hypothesized that the relevant biosynthetic alleles of this strain were indigenously heterozygous, carrying one copy of the wild-type and one copy of the defective allele, and that the mutagenesis promoted mitotic crossing over, resulting in homozygosity of the defective alleles. The auxotrophic heterozygosity is not limited to *C. albicans*, but has also been recorded in other fungi including *Candida parapsilosis* (Whelan & Kwon-Chung, 1988) and *Candida krusei* (Cao *et al.*, 1997). The physiological significance of natural fungal heterozygosity remains elusive.

In this paper, we report genetic and molecular evidence that (1) the *C. albicans* clinical isolate CA12 is heterozygous at the *ADE2* locus, (2) the strain-specific *ade2* mutants are due to the homozygosity of *ade2* and (3) gene conversion may play a dominant role in the homozygosity of *ade2*.

METHODS

***C. albicans* strains and plasmids.** *C. albicans* clinical isolates CA11, CA12, CA13 and CA14 were all oral isolates recovered from HIV-infected patients in a local hospital and were obtained from Drs D. Tsang (Queen Elizabeth Hospital, Kowloon, Hong Kong) and L. P. Samanarayake (Faculty of Dentistry, University of Hong Kong). *C. albicans* hOG24 (*ade1 pro1 met1*; Poulter & Rikkerink, 1983) and hOG300 (*ade2 pro1 met1*; Kurtz *et al.*, 1987), plasmids pRC2231 (Cannon *et al.*, 1992) and pSM7 (Kurtz *et al.*, 1986) were obtained from Dr R. D. Cannon (University of Otago, New Zealand).

UV mutagenesis and isolation of fungal sectors. Yeast cells in their early-exponential phase were spread onto the surface of YM plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar) at a density of approximately 10^6 cells per plate (15 cm diameter) and were immediately exposed to UV light for 75 s at a distance of 60 cm, equivalent to a dosage of $0.180 \text{ mW cm}^{-2} \text{ s}^{-1}$ as measured by an IL1430 health-hazard radiometer (International Light). Ade⁻ auxotrophs appeared as red-coloured colonies or colony sectors on YM plates after 3–4 d incubation at 37 °C. The white sectors of the sectorized fungal colonies were purified as follows: individual white sectors were recovered from the YM plate as completely as possible and transferred into 1 ml water using a sterilized transfer needle or a flat tooth pick. Care was taken to avoid contamination from the red sectors. Aliquots of the fungal suspension were spread on YM plates after briefly vortexing the suspension to mix the cells. Single white colonies were randomly chosen for further genetic analysis.

Parasexual analysis. The procedure of protoplast fusion was essentially as described by Sarachek *et al.* (1981). The test strains were *C. albicans* hOG24 (*ade1 pro1 met1*) and hOG300 (*ade2 pro1 met1*). Two partner fungal cells were grown separately in 20 ml YM until the OD₆₀₀ reached 1.0. After harvesting by centrifugation at 1500 g for 5 min, the cell pellets were washed twice with sterile distilled water, resuspended in 2 ml PC solution (0.1 M phosphate, 0.05 M citrate

buffer, pH 5.3, containing 1% β -mercaptoethanol) and rotated at 160 r.p.m. on an orbital shaker at 37 °C for 30 min. The cells were then washed twice with 10 ml KB solution (0.6 M KCl in 0.1 M phosphate, 0.05 M citrate buffer, pH 5.3), resuspended in 2 ml KB solution and 120 μ l lyticase solution (10 mg ml⁻¹, Sigma, in KB solution). The enzyme mixture was incubated at 37 °C for 15–30 min at 160 r.p.m., washed with 3 ml KB solution, resuspended in 5 ml fusion mix (40% PEG 3350, 10 mM CaCl₂) and incubated at 37 °C at 160 r.p.m. for 30 min. The cell pellets were collected by centrifugation, resuspended in 800 μ l KBC solution (0.6 M KCl and 10 mM CaCl₂ in 0.1 M phosphate, 0.05 M citrate buffer, pH 5.3) and spread onto GSB plates [1.5% glucose, 0.1% ammonium sulfate, 0.2% potassium dihydrogen phosphate, 0.005% magnesium sulfate, 0.005% calcium chloride, 0.001% biotin and 1.5% agar (Noble)], supplemented with 1 M sorbitol. The fusion products appeared as white colonies on the plates after 3–4 d incubation at 37 °C.

Isolation and sequencing of the *ADE2* gene. PCR reaction mixtures (50 μ l) for amplification of *ADE2* and *ade2* contained 1–5 ng fungal genomic DNA, 10 ng each of two primers, P9 (5'-CTT GAC GAA AAC TGA ATT TAT TCA C-3') corresponding to nucleotide position 9–33 and CR2244 (5'-CTA TTT TTT CAA TTT ATC AGT TAA-3') corresponding to position 2221–2244 of the *ADE2* gene (Genbank accession no. U75582), 0.2 mM dNTP, PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl₂) and 2.0 units *Taq* DNA polymerase. Reactions were carried out under the following conditions: 94 °C, 1 min 30 s; 56 °C, 1 min 30 s; 72 °C, 2 min 15 s for 35 cycles. The DNA template was denatured for 2 min at 100 °C before it was mixed with the polymerase. After PCR and gel electrophoresis, the desired PCR fragment was excised and purified using a GeneClean II kit (BIO 101). Purified PCR products were cloned into pCRII, and sequenced using a T7 Sequencing Kit (Pharmacia Biotech). Nucleotides of the gene are numbered as described in the Genbank entry with the first nucleotide of the starting codon at position 541.

Hybridization probe. A 2.2 kb PCR fragment was amplified from *C. albicans* CA12 using primers P9 and CR2244 and cloned into pCRII (Invitrogen) to generate plasmid pCBY101. A 2.2 kb *KpnI*–*EcoRV* fragment from pCBY101 containing the *ADE2* gene was used as the *ADE2* probe in hybridization experiments.

***C. albicans* DNA transformation.** DNA transformation was performed as described by Goshorn *et al.* (1992) with minor modifications. A 2.2 kb PCR fragment containing DNA sequence from position 59–2244 of the *ADE2* gene was amplified using primers P59 (5'-ATG GTT AAT ATT TTT GCT TGA TGA T-3'), corresponding to nucleotide position 59–83, and primer CR2244. The PCR fragment was purified from an agarose gel and used for the transformation. Fungal protoplasts were prepared by digesting exponential-phase fungal cells with lyticase for 1 h at 37 °C. One hundred microlitres of protoplast suspension was mixed with 10 μ g transforming DNA, together with 40 μ g calf thymus DNA as carrier, and incubated at room temperature for 15 min. Afterwards, 800 μ l 40% PEG 3350 in 10 mM CaCl₂ was added, incubated at room temperature for 15 min, and mixed with 5 ml soft agar [containing GSB medium, 1 M sorbitol and 0.8% agar (Noble)], and poured onto GSB agar plates. Transformants were scored after 3 d incubation at 37 °C. The transformation frequency was 5–10 transformants per microgramme of PCR DNA.

RESULTS

Generation of *ade2* mutants from *C. albicans* CA12 by UV irradiation

C. albicans, *Saccharomyces cerevisiae* and *Ustilago maydis* *ade2* or *ade1* mutants appear as red colonies on media deficient in adenine (Silver & Eaton, 1969; Poulter *et al.*, 1981; Kronstad *et al.*, 1989). After screening four *C. albicans* isolates (CA11, CA12, CA13 and CA14) isolated from local HIV-infected patients, we found that one of them, *C. albicans* CA12, consistently gave rise to such red adenine auxotrophs at a frequency of 2–6% of the surviving cells following UV irradiation as specified in the Methods. Two thousand surviving cells were randomly selected and replica plated on YM agar, a complete medium, and GSG agar, a minimal medium, and no auxotrophic mutants other than the red adenine mutants were detected. The three other *C. albicans* strains including CA11 were also subjected to the same UV treatment but no red Ade[−] mutants were detected. No spontaneous red adenine auxotroph was detected among more than 100 000 CA12 cells. Protoplasts were prepared from the Ade[−] derivatives of CA12 and fused individually with the protoplasts of *C. albicans* hOG24 (*ade1 pro1 met1*; Poulter & Rikkerink, 1983) and hOG300 (*ade2 pro1 met1*; Kurtz *et al.*, 1986). All the Ade[−] derivatives of CA12 tested formed fusants, which grew as white colonies on GSG plates, with hOG24, but not with hOG300. The adenine mutants were also rescued by transformation of integrative plasmid pSM7 (Kurtz *et al.*, 1986) or self-replicating plasmid pRC2231 (Cannon *et al.*, 1992), both of which carry the *C. albicans* ADE2 gene.

C. albicans CA12 carries natural heterozygosity at ADE2

Genomic DNA was extracted from CA12 and CA11, as well as from two of the randomly selected *ade2* derivatives of CA12, termed CA12-1-R1 and CA12-1-R2, digested with *EcoRV* and probed with the cloned 2.2 kb *KpnI*–*EcoRV* fragment containing the *C. albicans* ADE2 gene (Methods). The genomic Southern analysis (Fig. 1) shows that a 2.7 kb *EcoRV* band, which contains the entire *C. albicans* ADE2 gene (Fig. 2a) (Kurtz *et al.*, 1986), is present in CA11 and CA12 (Fig. 1, lanes 1 and 2), but is absent in the *ade2* mutants CA12-1-R1 and CA12-1-R2 (lanes 3 and 4). A smaller band of 1.4 kb in size, is detected in CA12, CA12-1-R1 and CA12-1-R2 (lanes 2, 3 and 4), but not in CA11 (lane 1). These results suggested that CA12, in contrast to CA11, was heterozygous at ADE2, and that the *ade2* derivatives of CA12 had lost ADE2 heterozygosity.

To determine the location of the deletion in the mutant *ade2* allele in CA12, two PCR products of 2.2 and 0.9 kb in size were amplified using CA12 genomic DNA as a template and oligonucleotides P9 (corresponding to nucleotide position 9–33 of the ADE2 gene) and CR2244 (corresponding to nucleotide position 2221–2244 of the ADE2 gene) as primers (data not shown). The two

Fig. 1. Southern analysis of *C. albicans* CA12 and its adenine auxotrophic derivatives (lanes 1–4) and the prototrophic transformants of *ade2* mutants (lanes 5–10). Lane 1, *C. albicans* CA11; lane 2, *C. albicans* CA12; lane 3, *ade2* auxotroph CA12-1-R1 and lane 4, *ade2* auxotroph *C. albicans* CA12-1-R2; lanes 5 and 6, CA12-1-R1 and its prototrophic transformant, CA12-1-R1-T1; lanes 7 and 8, CA12-1-R2 and its prototrophic transformant, CA12-1-R2-T1; lanes 9 and 10, CA12-2-R1 and its prototrophic transformant, CA12-2-R1-T1. Note the reappearance of the 2.7 kb *EcoRV* fragment in the transformants (lanes 6, 8 and 10). The fungal DNA was digested with *EcoRV* and probed with the 2.2 kb *KpnI*–*EcoRV* fragment that contains the entire *C. albicans* ADE2 gene.

primers were chosen as their PCR product covers almost the entire ADE2 gene (Fig. 2a). The 1.3 kb size difference between the two products matched exactly the size difference between the two homologous *EcoRV* bands displayed in the genomic Southern blot shown in Fig. 1. The two PCR products were cloned into pCRII and sequenced. The larger PCR product carried the wild-type gene ADE2, as its sequence is 99.60% identical (2239 out of 2248 bp) to that of ADE2 in *C. albicans* SC5314. The DNA sequence of the smaller PCR product was almost identical to that of ADE2 but contained a 1.3 kb deletion between positions 490 and 1764 of the ADE2 gene. The small PCR product was therefore determined to carry the null allele *ade2*. The 1.3 kb deletion spans the promoter and coding regions of the gene, between the two AATC repeats (Fig. 2b). Besides, the DNA sequence of *ade2* differed from that of ADE2 by three transition mutations at the 5' promoter region: a C → T at position 281, a T → C at position 346 and an A → G at position 413. These sequence polymorphisms provided an additional way to distinguish the two alleles.

Adenine mutants are homozygous diploid at *ade2*

The loss of ADE2 heterozygosity in the *ade2* mutants could be due to (1) *de novo* deletion of approximately 1.3 kb of the ADE2 gene; (2) haploidization; (3) aneuploidic loss of ADE2; or (4) homozygosity at *ade2* (*ade2/ade2*) by mitotic recombination (Fig. 3). Using the PCR primers P9 and CR2244, *ade2* alleles were amplified from three independent *ade2* mutants of CA12, termed CA12-1-R1, CA12-1-R2 and CA12-1-R4, and cloned

Fig. 2. (a) Map of the *ADE2* gene. The 5' AATC corresponds to nucleotide position 490–493 and the 3' AATC corresponds to nucleotide position 1824–1827 of the gene. The ORF, marked as a thick black box, runs from nucleotide position 541 to 2244. The direction and positions of the primers used in the study and *EcoRV* restriction sites are also shown. (b) The *ADE2* and *ade2* DNA sequences flanking the 1.3 kb deletion. (i) *ADE2* DNA sequence at the 5' end of the deletion. The 5' A corresponds to position 490 of the gene. (ii) *ADE2* DNA sequence at the 3' end of the deletion. The 5' A corresponds to position 1824 of the gene. (iii) *ade2* DNA sequence at the border regions of the deletion. The AATC is marked with a vertical bar.

into pCRII. Two clones from each of the mutants were sequenced. The DNA sequences of the six clones were identical to each other and to that of *ade2* of CA12 (data not shown), suggesting that none of the *ade2* mutants was due to novel deletion of *ADE2* and they all originated from the null *ade2* allele in CA12.

PCR fragment P59–CR2244, corresponding to nucleotide position 59–2244 of the *ADE2* gene, was amplified using primers P59 and CR2244, and was used to transform adenine auxotrophs CA12-1-R1 and CA12-1-R2. This PCR fragment is 2.2 kb in size and contains the missing 1.3 kb DNA sequence (Fig. 2a). The PCR fragment was introduced into the protoplasts of the recipient strains using the CaCl_2 -PEG method and the prototrophic transformants were selected on minimal medium free of adenine. One prototrophic transformant each was randomly chosen from the transformants of CA12-1-R1, CA12-1-R2 and CA12-2-R1, their DNA was extracted, digested with *EcoRV* and probed with the 2.2 kb *ADE2* fragment. It is evident that the recipients CA12-1-R1, CA12-1-R2 and CA12-2-R1 (Fig. 1, lanes 5, 7 and 9) contain only the 1.4 kb fragment; while all of the three corresponding transformants CA12-1-R1-T1, CA12-1-R2-T1, and CA12-2-R1-T1 contain the 2.7 kb *EcoRV* fragment present in the parental CA12 cells (Fig. 1, lanes 6, 8 and 10), as well as the 1.4 kb fragment. The 1.4 kb fragment would be absent if these *Ade*⁺ transformants were aneuploids containing only one copy of *ade2*. Our data can be best explained that the *ade2* recipients contain two copies of *ade2* and the transforming DNA has converted one of

them to *ADE2* by, presumably, homologous recombination. The restored *ADE2* heterozygosity in the transformants was also confirmed; the three *Ade*⁺ transformants were also prone to the generation of adenine mutants, as was their parental strain CA12, after UV irradiation (data not shown).

CA12-1-R1 and CA12-1-R2 were treated by UV mutagenesis and the surviving cells were transferred to adenine-supplemented minimal medium and YM medium by replica plating, to screen for additional auxotrophic mutants. We failed to detect any additional auxotrophs among the 3000 surviving cells screened. The detection of other auxotrophs was expected if these mutants were haploid cells (Fig. 3, path V). Based on the above data we conclude that the UV-induced adenine mutants are homozygous diploid at *ade2*.

Both gene conversion and mitotic crossing over are responsible for the loss of *ADE2* heterozygosity

After UV irradiation, many of the *Ade*[−] derivatives of CA12 appeared as a red sector in an otherwise white colony, the white being composed of prototrophic cells. Two methods, UV mutagenesis and genomic Southern analysis, were used to examine the genotype of cells purified from the white sectors. Some of the cells derived from the white sectors such as CA12-1-W2 and CA12-2-W6 were no longer prone to the formation of *Ade*[−] mutants by UV irradiation (data not shown). Genomic Southern analysis demonstrated that CA12-1-W2 and CA12-2-W6 had lost *ade2* and only contained *ADE2*

Fig. 3. Five possible genetic rearrangements of $ADE2/ade2$ heterozygotes. Path I refers to mitotic crossing over; path II, $3+:1m$ gene conversion, the non-reciprocal transfer of genetic materials; path III, $1+:3m$ gene conversion; path IV, aneuploidic loss of $ADE2$; and path V, haploidization. The sibling segregants are grouped together with the sign &. ($-/ade2$) refers to an $ade2$ hemizygote. +, wild-type; m, mutant.

(Fig. 4, lanes 4 and 8). This indicated that these white prototrophic sectors were $ADE2/ADE2$ and were the progenies of one of the two sibling cells of the mitotic crossing over; the other sibling cell, the reciprocal product of the crossing over, was the red auxotrophic carrying the $ade2/ade2$ alleles (Fig. 3, path I).

However, among the 108 white sectors tested, only 50 lost their ability to produce adenine mutants. The remaining 58, exemplified by CA12-1-W1 and CA12-2-W1, maintained their ability to continuously segregate into $ade2$ mutants at frequencies similar to that of the parental strain CA12 upon UV irradiation (data not shown). Southern analysis verifies that the cells from these white sectors carry the same genotype as CA12, *ie* $ADE2/ade2$, as displayed by the existence of the large *EcoRV* fragment characteristic of $ADE2$ and the small

Fig. 4. Southern analysis of the genotypes of the white sectors in the red/white twin-sectored colonies. Lanes 1 and 2, CA12-1-R1 and its sibling prototrophic white sector, CA12-1-W1; lanes 3 and 4, CA12-1-R2 and its sibling prototrophic white sector, CA12-1-W2; lanes 5 and 6, CA12-2-R1 and its sibling prototrophic white sector, CA12-2-W1; lanes 7 and 8, CA12-2-R6 and its sibling prototrophic white sector, CA12-2-W6. Southern analysis was done as described in Fig. 1.

EcoRV fragment characteristic of $ade2$ in the two isolates (Fig. 4, lanes 2 and 6). Since the other sibling cell of the recombination was the red auxotroph with the genotype $ade2/ade2$, our results indicated the occurrence of the non-Mendelian, $1+:3m$ (+, wild-type; m, mutant) segregation of heterozygous $ADE2$ alleles between the two sibling cells of CA12 in these white/red sectored colonies. Such non-reciprocal transfer of genetic information is a typical characteristic of gene conversion (Fig. 3, path III).

DISCUSSION

In dissection of the genetic mechanism underlining natural fungal heterozygosity, *C. albicans* CA12 offers several experimental conveniences: (1) the red, adenine-requiring auxotrophic mutants are visually identifiable on media deficient in adenine; (2) the gene, $ADE2$, has a defined biochemical function, encoding phosphoribosyl-aminoimidazole carboxylase in the *de novo* purine biosynthetic pathway; (3) both integrative and self-replicating plasmid transformation systems are available for the rescue of $ade2$ mutants (Kurtz *et al.*, 1987; Cannon *et al.*, 1992); (4) because of the presence of the large deletion at $ade2$, the wild-type and the defective allele are easily distinguishable by PCR or Southern analysis; and (5) the $ADE2$ DNA sequence has been determined (Tsang *et al.*, 1997).

We have demonstrated that the $ADE2$ locus of *C. albicans* CA12 is heterozygous (*ie* $ADE2/ade2$). The null allele, $ade2$, has lost a 1.3 kb fragment in the promoter and coding regions of the gene as the result of a deletion between the two AATC repeats (Fig. 2). Our data clearly indicate that the natural heterozygosity at $ADE2$ forms the genetic basis for the high frequency of the specific auxotrophic phenotype in CA12 (Fig. 1), and that the loss of heterozygosity (LOH) at $ADE2$ in *C. albicans* CA12 leads to the generation of $ade2$ mutants (Fig. 1). This has, therefore, for the first time provided a molecular explanation for one of the puzzles in my-

cology: how strain-specific, recessive mutants can be readily induced by UV or other mutagens in many natural isolates of the diploid organism *C. albicans*.

We have also revealed that the *ade2* derivatives of CA12 are diploid cells homozygous at *ade2* (Fig. 1) and the homozygosis is caused by UV-induced mitotic recombination. Mitotic recombination can lead to either reciprocal (mitotic crossing over) or non-reciprocal (gene conversion) transfer of genetic material (Fig. 3, paths I, II and III). We had previously suggested that gene conversion is one of the mechanisms for the generation of adenine mutants in *C. krusei* (Cao *et al.*, 1997). In the present study, our genetic analysis of the white prototrophic sectors in the red/white sector colonies has furnished revealing information on the pathways of mitotic recombination. Two classes of the white prototrophic sectors were detected. The first class, which was previously called mitotically stable prototrophic strains (Poulter *et al.*, 1981), were no longer able to further segregate to give *ade2* auxotrophs and were homozygous at the *ADE2* locus. This type of red/white sector colony is most likely the consequence of the mitotic crossing over (Fig. 3, path I). The second class of the white prototrophic sectors occurred more often. They remained prone to produce UV-induced *ade2* mutants and were heterozygous at *ADE2* (Fig. 3, path III). These white sectors were certainly not due to contamination of un-mutagenized cells grown next to the sector colonies as we failed to detect the existence of the mixed population of *ADE2/ADE2* and *ADE2/ade2* in more than 30 white sectors examined (data not shown). We therefore conclude the existence of two pathways of mitotic recombination in *C. albicans* CA12: crossing over and gene conversion.

Our data support the basic tenets of the natural heterozygosity hypothesis (Whelan *et al.*, 1980) and furthermore indicate, based upon the frequent occurrence of 1+:3*m* segregation of *ADE2*, that gene conversion may play an important role in the LOH of *ADE2*. The actual contribution of gene conversion to the mitotic recombination is likely to be underestimated since the current investigation does not include the 3+:1*m* gene conversion of *ADE2*. Both of the reciprocal progenies of the 3+:1*m* gene conversion are white prototrophic cells, but one with a genotype of *ADE2/ADE2* and the other *ADE2/ade2* (Fig. 3, path II). In addition, the method we used for the detection of *ADE2/ade2* heterozygotes, *ie* screening the red colonies after UV mutagenesis, was not a sensitive one and may have missed some of the mutants.

The frequency of LOH of the *ADE2* under natural conditions is unknown but is expected to be low, for we did not detect any spontaneous red adenine mutants from CA12 in the absence of UV treatment. It remains to be examined whether any other chemical or physical agents may promote the LOH more efficiently than UV irradiation; or whether LOH of some genes may lead to the generation of mutants that are favourably selected under certain living environments.

We have noticed that the 1.3 kb deletion occurs between two AATC repeats (Fig. 2). It would be unwise to simply ignore the phenomenon as a fortuitous event, but it is equally difficult to argue that the AATC repeats provide sufficient *cis*-acting information for site-specific recombination, as numerous copies of the tetranucleotide are interspersed throughout the *C. albicans* genome. Additional experiments are required to prove or disprove the significance of the phenomenon.

C. albicans, like numerous other imperfect fungi, is an obligate diploid organism. Many evolutionary advantages such as biodiversity and hybrid vigour granted by meiosis are denied to these fungi. It has long been suggested that *C. albicans* relies upon certain unique genetic processes, such as heritable phenotypic variation (also called phenotypic instability or switching) associated with colony morphology (Slutsky *et al.*, 1985) and ploidy change (Suzuki *et al.*, 1982; Janbon *et al.*, 1998) to compensate for its intrinsic genetic deficiency. The frequent detection of *C. albicans* natural isolates with a wide spectrum of recessive heterozygosity (Poulter, 1987) may be an unfortunate consequence of the absence of meiosis in the fungus, as mitotic recombination is a much slower process of removing mutant alleles. Alternatively, the natural heterozygosity may serve as yet another vehicle by the organism to elevate its genetic variability. It will be interesting and worthwhile to test the competitiveness of strains heterozygous at *ADE2* over their corresponding isogenic strains homozygous at the locus. It is our hope that the full comprehension of the phenomenon of fungal natural heterozygosity does not just satisfy academic curiosity, but also provides useful information that may guide *C. albicans* geneticists to design alternative approaches to create desired mutations in this obligate diploid.

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