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Correlation between Polyploidy and Auxotrophic Segregation in the Imperfect Yeast *Candida albicans*

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In order to clarify the relationship between polyploidization and the capability of phenotypic switching in the imperfect yeast *Candida albicans*, two types of variants were isolated as segregants from a fusant, which produced a proportion of the cell population with a higher ploidy than the rest, either in a temperature-dependent or -independent manner, when incubated at low (28°C) and high (37°C) temperatures. In the case of the temperature-dependent type of variants, high-ploidy cells appeared at 37°C but rarely at 28°C. This phenotype was named Pld^{ts} (temperature-sensitive polyploidization), and the temperature-independent phenotype was called Pld^{-}. The appearance of high-ploidy cells in the culture of the Pld^{ts} strain at 37°C was accompanied by a significant increase in the frequency of auxotrophic variants; these variants probably occur as a result of segregation of auxotrophic markers from the heterozygous to the homozygous state. Both Pld^{ts} and Pld^{-} phenotypes were recessive in a fusion with a Pld^{+} parent. An adenine auxotrophic marker (*ade1*) was introduced into a Pld^{ts} strain in a heterozygous state, and the individual high-ploidy cells of this strain, grown at 37°C, were micromanipulated to form colonies, which consisted of red and white sectors appearing at high frequency on a pink background. When the *ade1* auxotrophy was introduced into Pld^{-} strains, frequently sectored colonies were produced. These results suggested an increased level of chromosome missegregation in both types of Pld mutants. Analyses by pulsed-field gel electrophoresis of Ade^{-} segregants, derived from a micromanipulated high-ploidy cell of a Pld^{ts} strain, suggested the occurrence of nonreciprocal recombination, some of which includes chromosome loss.

The dimorphic and pathogenic yeast *Candida albicans* is diploid as commonly isolated (17), and no sexual cycle has been observed for it (23). Diploidy seems to endow this organism with genetic stability in the short run, since a recessive mutation cannot be expressed until it exists in a homozygous state after the occurrence of mitotic recombination. However, in the long run, the organism is postulated to accumulate recessive mutations and chromosome rearrangement. Some phenotypic variation (often called phenotypic instability or switching) has been reported to occur in this organism: Slutsky et al. (25) showed that the colony phenotype of a particular strain, 3153A, changed at a frequency of 10^{-2} to 10^{-4}, which is too high to be a result of mutation. Later, the same laboratory reported a second form of colony morphology variation, smooth-opaque (4, 26). Suzuki et al. (28) found that the concomitant changes in the banding pattern of chromosomal DNA took place during switching between the smooth and the semirough types in a clinical isolate of this organism. Rustchenko-Bulgac and her coworkers (20, 21) showed further association of chromosomal rearrangement with some type of colony morphology variations in strain 3153A.

Although many *C. albicans* strains are diploid (18, 32), some are polyploid and show the phenomenon called ploidy shift. Diploid (or lower-ploidy state) cells in a population of the strain enter G_2 arrest and then bypass the M phase, resulting in an upshift of ploidy state; tetraploid (or higher-ploidy state) cells engage in reductional nuclear division during the downshift of the ploidy state, producing diploid (or lower-ploidy state) daughter cells (27). The process of the ploidy shift in *C. albicans* was assumed to be coupled to chromosomal rearrangements by Suzuki et al. (28).

In this article, two types of variation (Pld^{-} and Pld^{ts}) are shown to occur; these cause the phenotype of polyploidy in a temperature-independent or -dependent manner, respectively. Chromosome instability is shown to exist in both Pld^{-} and Pld^{ts} strains through the demonstration of segregation of auxotrophic markers including *ade1*. Direct correlation is shown among polyploidy, auxotrophic segregation, and chromosome rearrangement by micromanipulating high-ploidy cells of the Pld^{-} and Pld^{ts} strains to look at colonies derived from such cells by analyzing their chromosomal DNAs.

MATERIALS AND METHODS

Organisms, media, and growth conditions. *C. albicans* strains used in this study are listed in Table 1. *C. albicans* strains NARA2, A5153, 1435, and 126-A1 were used as the parents for fusion experiments. *C. albicans* 126-A1 (*ade2* Met^{-}) was constructed by us from an adenine-requiring strain, SGY126, which was kindly given to us by the Bristol Myers Squibb Institute for Medical Research, Princeton, N.J. (with permission from D. R. Kirsch), and was used for complementation tests for red adenine-requiring strains. An *ADE1* plasmid, 1154, containing *ADE1* on an *Xba*I-*Bam*HI fragment in pUC18 was kindly given to us by S. Scherer (University of Minnesota, Minneapolis), and an rRNA gene (rDNA) plasmid with the *C. albicans* rDNA repeat was a *Sal*I fragment in pBluescript SK(+) (Stratagene, La Jolla, Calif.), kindly given to us by B. B. Magee (University of Minnesota, St. Paul). An *ADE2* plasmid pSM7 (13) was a generous gift from D. R. Kirsch. Yeast cells were grown in YPD medium (containing, per liter, 20 g of polypeptone [Nihon Seiyaku Co. Ltd], 10 g of yeast extract [Nihon Seiyaku], and 20 g of glucose) with

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TABLE 1. Yeast strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source and/or reference
C. albicans		
FC18	Prototrophic, diploid	32
A5153	His^- Lys^- Trp^-, diploid	FC18, 30
NUM961	Prototrophic, near triploid	28
SRT1	Prototrophic, polyploid	28
NARA2	Met^- Pro^-, diploid	This study
126A1	*ade2* Met^-, diploid	This study
SGY126	*ade2*, diploid	D. R. Kirsch, 13
1435	*arg4 ser57 lys1 ura3 ade1* MPA^{ra}, diploid	CBS5736
NUM51	Prototrophic, polyploid	27
Plasmids		
1154	*ADE1*	S. Scherer
pSM7	*ADE2*	D. R. Kirsch, 13

[a] MPA^r, mycophenolic acid resistance.

shaking (100 strokes per min) at either 28°C or 37°C as described below. Stationary-phase cells were obtained from the cultures incubated for 2 days at a given temperature. Minimal medium (MIN) contained, per liter, 6.7 g of yeast nitrogen base without amino acids (Difco) and 20 g of glucose. For solid medium, 2% (wt/vol) agar was added to YPD or MIN. MIN agar plates were supplemented, when appropriate, with auxotrophic requirements at the concentrations specified by Sherman et al. (24). For regeneration media for fusion experiments, 1 M sorbitol was added to a MIN agar plate or YPD plate. YPD dissection plates were prepared by solidifying 10 ml of YPD per plate. For maintenance, strains were stored at −78°C in sterile 50% (vol/vol) glycerol supplemented with 0.3% (wt/vol) yeast nitrogen base and 0.1% polypeptone.

Protoplast formation, fusion, and regeneration. The methods described by Kakar et al. (10) for *C. albicans* were used. Evaluation of the complementation of the adenine auxotrophs was also according to Kakar et al. (10).

Mild UV irradiation. A Toshiba bactericidal 15-W UV lamp (254 nm) was used to irradiate plates spread with an appropriate dilution of yeast cells at a distance of 80 cm with a 25-s exposure. This irradiation usually gave 80 to 90% survival rates in *C. albicans* strains.

Visualization of nuclear spindles by fluorescence microscopy. Yeast cells growing in liquid media were fixed with 3% (wt/vol) paraformaldehyde and stained on a slide glass with monoclonal antitubulin YOL1/34 (Sera-Lab), and then fluorescein isothiocyanate–anti-rat immunoglobulin G as described by Kilmartin and Adams (11). For quantitative analysis, cells were washed by centrifugation (10,000 × *g* for 30 s) and stained with these antisera in a 1.5-ml polypropylene tube. After the staining, cells were transferred onto the surface of a slide glass, and DNA regions of these cells were double stained with (4′,6-diamidino-2-phenylindole), as described previously (28). An Olympus BH2-RFK microscope was used with a combination of UG1, DM400, and L435 filters or with a combination of BP490, EY455, BM500, and 0515 filters.

Fluorescence microscope photometry. Yeast cells were harvested from YPD media, fixed in 50% (vol/vol) ethanol, washed twice with 5 mM Na_2 EDTA (pH 7.5), and incubated at 37°C for 60 min in a 10 mM Tris-HCl buffer (pH 7.5) containing 0.05 M NaCl and RNase A (1 mg/ml). Then the cells were stained with propidium iodide (10 μg/ml) and examined by fluorescence microscope photometry using a Zeiss MPM03FL photometer as described previously (27).

DNA determination. The DNA content of yeast cells grown in YPD media was determined by the diphenylamine method described by Riggsby et al. (17). Calf thymus DNA (Sigma) was used as a reference.

Ficoll density gradient centrifugation. Yeast cells were collected from YPD media (20 ml) by centrifugation and suspended in 5 ml of sterile distilled water. The cell suspension was then layered on a discontinuous density gradient consisting of 5 ml each of 10, 20, and 30% (wt/vol) Ficoll 400 (Pharmacia). The gradient was centrifuged at room temperature in a swing-out rotor at 100 × *g* for 5 min.

Micromanipulation of yeast cells. Yeast cells cultured in 5 ml of YPD were collected by centrifugation and resuspended in 1 ml of water. Yeast cells were streaked in parallel lines across the surface of a YPD dissection plate. The plate was inverted with the lid off and examined on a dissecting microscope equipped with a micromanipulator (Narishige Co. Ltd, Tokyo, Japan) using a 40× long working-distance objective (Olympus). Each cell was picked up and positioned 1 cm away from the streak. Cells were positioned in a double line, 1 cm apart from each other. The plate was incubated at 28°C for 5 days to allow the cells to form colonies.

Electrophoretic karyotype. Sample preparation was according to Magee and Magee (14). The Pulsaphor system (Pharmacia-LKB) equipped with an LKB 2015-100 hexagonal electrode kit was used as described previously (29). A 0.9% agarose (Wako Chemicals, Tokyo, Japan) gel (15 by 15 cm) was prepared in Tris-borate-EDTA buffer (22), and the same buffer was used as the cooling buffer at 9°C. The running condition was in four sequential steps as follows: a 5-min pulse at 110 V for 28 h, followed by a 15-min pulse at 90 V for 50 h, a 20-min pulse at 60 V for 50 h, and finally, a 30-min pulse at 50 V for 60 h.

Hybridization experiment. Transformation of *Escherichia coli* and plasmid preparations were as described by Sambrook et al. (22). Preparation of the digoxigenin-labeled DNA probe was done by the method of random primed DNA labeling according to the manufacturer's recommendation (Boehringer Mannheim Co. Indianapolis, Ind.). Hybridizations were carried out with the Boehringer Genius system, using the Lumiphos detection system. X-ray film was the product of Fuji Photo Co. Ltd. (Kanagawa, Japan).

RESULTS

Isolation of strains producing a proportion of the cell population that are polyploid from *C. albicans* fusion products. The clinical isolate, NUM961, was shown to produce polyploid descendants (28). To make fusions between such a strain producing polyploid cells, SRT1, and a wild-type (normal diploid) strain, we needed to have auxotrophic derivatives of SRT1. UV irradiation gave rise among the survivors to auxotrophs that appeared to show little growth on MIN plates after replica plating, but all produced revertant clones in each colony within a few days. It was concluded that these auxotrophic candidates revert at too high a frequency to make them useful. We therefore tried to obtain auxotrophs from NUM 961. NARA2 was isolated from NUM961 as a Met^- Pro^- auxotroph by two cycles of UV irradiation. NARA2 had a DNA content of 38 ± 1 fg per cell at both 28°C and 37°C. Since its parent NUM961 had a DNA content of 52.8 fg per cell, a ploidy shift from near triploid to diploid seemed to have occurred during the isolation procedure. We examined the effects of growth temperatures (28 and 37°C) on all the strains

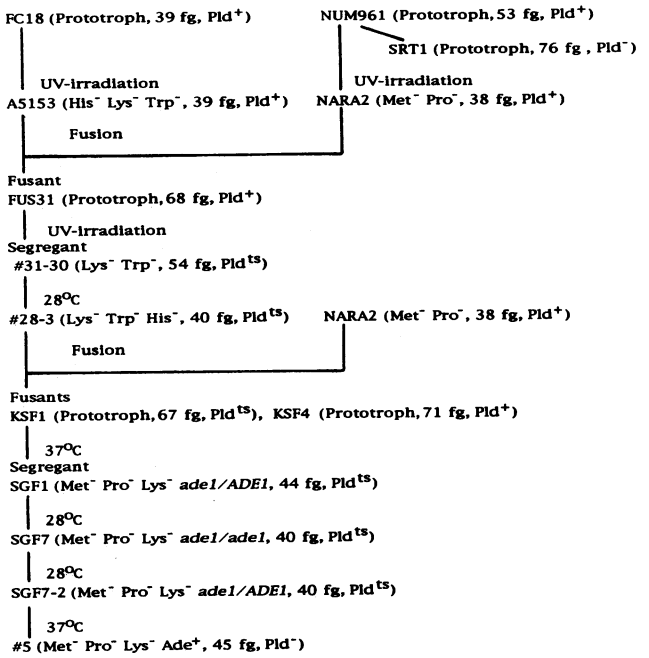

FIG. 1. Flow chart of the various strains, their markers, their DNA contents per cell at 28°C, and ploidy phenotype in this study.

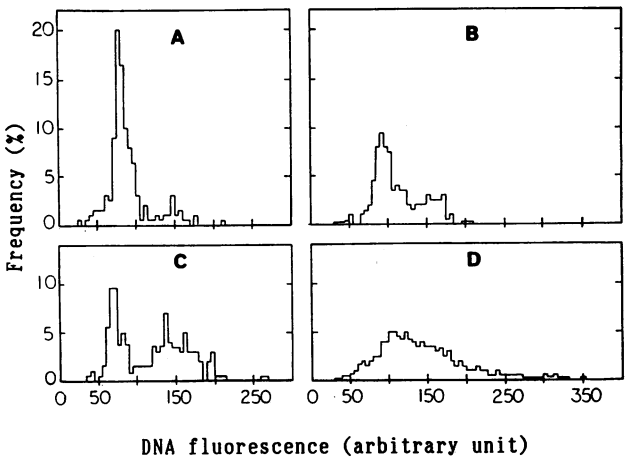

FIG. 2. Histograms comparing nuclear DNA contents of cells of the Pld^{ts} strain 31-30 at stationary phase at 28°C (A) and at 37°C (C) and at log phase at 28°C (B) and at 37°C (D). Cells were grown in YPD and stained with propidium iodide. Fluorescence intensity of nuclear DNA was measured with a Zeiss fluorescence microscope-photometer MPM03.

described below, and the DNA content per cell was chemically determined at both temperatures. In order to estimate the differences in DNA content among individual nuclei, the nuclear DNA of stationary-phase cells incubated at either temperature was stained with propidium iodide and examined by fluorescence microscope photometry. NARA2 gave a constant DNA content per cell after more than 10 subculturing cycles, and its population of cells was found to be exclusively in a diploid state. The phenotype of stable ploidy, with fluorescence microscope photometry showing that all the cells of a population maintain a certain level of ploidy, will be hereafter referred to as Pld$^+$. A5153 was a His$^-$ Lys$^-$ Trp$^-$ auxotroph from FC18 (30) and had 39 ± 1 fg of DNA per cell at both temperatures. Thus, the DNA contents of these strains were equivalent to the normal diploid value for this organism (18). The phenotype of ploidy of A5153 was Pld$^+$. A prototrophic fusion product, FUS31, was obtained by fusing A5153 with NARA2 and selection on MIN-sorbitol. The fusant, grown at either 28°C or 37°C, gave the same DNA content per cell (68 ± 2 fg) and was Pld$^+$. Figure 1 shows a flow chart of the various strains, their markers, their chemically determined DNA contents per cell at 28°C, and proposed ploidy phenotypes. The fusant, grown at 28°C, segregated auxotrophic segregants at a frequency of 4×10^{-4} or, at 37°C, at a frequency of 3×10^{-4}. No significant difference in frequency between the two temperatures was observed. After mild UV irradiation, 70 auxotrophic segregants were isolated from FUS31. Among them, four segregants from FUS31, named 31-6 (Lys$^-$ Trp$^-$), 31-35 (His$^-$ Lys$^-$ Trp$^-$), 31-41 (His$^-$), and 31-47 (Lys$^-$), were found showing the existence of large cells either with a large nucleus or with multiple nuclei, together with predominantly smaller cells at both temperatures. The DNA contents of the four strains in femtograms per cell at 28°C were 58 ± 5, 45 ± 5, 53 ± 5, and 58 ± 6, respectively, and the contents at 37°C were 55 ± 6, 46 ± 5, 51 ± 5, and 56 ± 6, respectively. No apparent differences among the chemically determined DNA contents per cell of the four strains were observed. The broad distribution profile was found for the DNA content per nucleus in each of the four segregants. The existence of anucleate cells was observed for each of the segregants and accounted for 10% of the population. The fusant showed a homogeneous colony size when plated out, but all of the four segregants showed a characteristic heterogeneity of colony size on YPD plates at an early stage of incubation: after incubation for 2 days at 28°C (or 37°C), the colonies varied in size from 0.2 to 5 mm. When one of the small colonies was restreaked on another YPD plate and incubated for 2 days, the same mixture of colony sizes appeared. The large colonies behaved similarly. When these various-size colonies were incubated for a further 2 to 3 days, the small ones continued to increase in size; finally, it became impossible to distinguish the large from the small. These features were the same for a clinical isolate of this organism, NUM51, which performed the ploidy shift between near diploid and near tetraploid, as had been described previously (27). This phenotype is defined here as production of polyploid cells in a temperature-independent manner and termed Pld$^-$. Furthermore, we happened to find the other two segregants from FUS31, 31-30 and 31-50 (both carrying Lys$^-$ and Trp$^-$ markers), showing the Pld$^-$ phenotype only at the high temperature (37°C); the culture was composed of uniform-size cells when incubated at 28°C. This phenotype is defined here as temperature-sensitive production of polyploid cells and termed Pld^{ts}. The large cells which appeared at 37°C in a population of the Pld^{ts} segregants seemed to be in a different ploidy state from that of normal-size cells. The chemically determined DNA content per cell of segregant 31-30 was 54 ± 3 fg at 28°C and 62 ± 7 fg at 37°C. Thus, as far as DNA content was concerned, segregant 31-30 appeared to be near triploid. The ratio of the DNA content of the culture at 37°C to that at 28°C was 1.1 to 1.2. The existence of anucleate cells of this strain at 37°C, which accounted for 10% of the population, seemed to result in reduction of the chemically determined DNA content per cell at this temperature. The nuclear DNA of stationary-phase cells incubated at each temperature was examined by fluorescence microscope photometry (Fig. 2). At 28°C, the Pld^{ts} segregant contained

predominantly cells with a G_1 DNA content and a small percentage with a G_2-M content (Fig. 2A). Incubating at 37°C, stationary-phase cells of the segregant consisted of two peaks, corresponding to the G_1 and the G_2-M DNA contents of the cells at 28°C. Furthermore, the latter one was the predominant fraction and constituted more than half of the population (Fig. 2C). A distribution profile similar to that in Fig. 2C was obtained for each of the cultures at both temperatures of the above four Pld$^-$ segregants (data not shown). The nuclei of the large cells of segregant 31-30 grown at 37°C had the G_2-M DNA content. Large nuclei with four times the DNA content of the G_1 peak were reproducibly observed to occur at a frequency of 1% or less, and the cells having such a large nucleus consisted of an ellipsoidal mother sphere with an elongated allantoid daughter bud (Fig. 3B). These large cells were called high-ploidy cells. The log-phase culture of the segregant at 37°C showed a broad DNA distribution profile and a percentage of cells with a high DNA content that was four to eight times that in the log-phase cells incubated at 28°C (Fig. 2B and D). High-ploidy cells, either binucleate or mononucleate, up to 20 μm in length, constituted a small percentage of the cells at 37°C.

Increase in frequency of auxotrophic segregation in Pld^{ts} variants. Cells of segregant 31-30, cultured at 37°C for more than six generations, were plated and incubated on YPD at 28°C to form colonies. By replica plating these colonies on MIN (plus Lys and Trp) plates, segregation of additional auxotrophic markers was examined. His$^-$ segregants appeared at a frequency of 8.7×10^{-3} (Table 2). In a control culture at 28°C, the frequency was 2×10^{-4}, and a significant difference was detected between the two different temperature treatments. The fusant gave auxotrophic segregants at a frequency of 10^{-4} at both temperatures. Thus, the frequency of segregant 31-30 at 37°C seemed to be high. Among 80 His$^-$ segregants, 39 were Pld$^+$, 37 were Pld^{ts}, and 4 were Pld$^-$. Three of the four His$^-$ segregants that were obtained from the culture at 28°C had the Pld$^+$ phenotype, and the remaining one was Pld^{ts}. This Pld^{ts} segregant was called 28-1 and gave 40 ± 1 fg of DNA per cell at 28°C or 45 ± 2 fg at 37°C.

To examine whether the increase in frequency of auxotrophic segregation was related to the Pld^{ts} phenotype, segregant 28-1 was back-fused with NARA2. Prototrophic fusion products were selected on MIN-sorbitol. Among four fusants, two were Pld^{ts} and the others were Pld$^+$. One strain of the Pld^{ts} fusants, KSF1, and one of the Pld$^+$ fusants, KSF4, were examined for their segregation behavior. The KSF1 strain, cultured at 28°C, gave 67 ± 2 fg of DNA per cell and produced auxotrophic segregants at a frequency of 1.1×10^{-4}. This strain gave 73 ± 2 fg of DNA per cell at 37°C and a significant increase in the frequency of auxotrophic segregation (7.7×10^{-3}) at this temperature (Table 2). In the case of the Pld$^+$ KSF4, whose DNA content was measured to be 71 ± 2 fg per cell at both temperatures, no significant difference was detected in the frequency between the two temperature treatments. The spectra of auxotrophic markers which segregated from KSF1 after the 37°C treatment were not limited to any specific set: Met$^-$ and Pro$^-$ auxotrophs segregated more frequently together than they did separately, and Trp$^-$ cosegregated once with Met$^-$ and Pro$^-$ and once with His$^-$. There was only one Lys$^-$ segregant, and it did not cosegregate with either Met$^-$ or Pro$^-$. From the table, it is not clear whether there is a difference in the patterns of segregation of KSF1 and KSF4, but the frequency of segregation of KSF1 was remarkably higher at 37 than at 28°C.

Segregation of Pld^{ts} and Pld$^-$ phenotypes by the fusant KSF1. The fusant FUS31 segregated both Pld^{ts} and Pld$^-$ progeny as described above. Among auxotrophic segregants that the fusant KSF1 gave, Pld^{ts} progeny segregated more frequently than Pld$^-$ progeny (Table 3). From a Pld^{ts} Met$^-$ isolate, Pld$^-$ Met$^-$ Pro$^-$ segregants as well as Pld$^+$ Met$^-$ Pro$^-$ ones were further obtained, but neither Pld^{ts} Met$^-$ Pro$^-$ nor Pld$^+$ Met$^-$ Pro$^-$ segregants occurred from a Pld$^-$ Met$^-$ isolate, on the basis of 20 Pro$^-$ segregants examined for their ploidy state. From the Pld$^+$ KSF4 fusant, all of the eight auxotrophic segregants showed the Pld$^+$ phenotype.

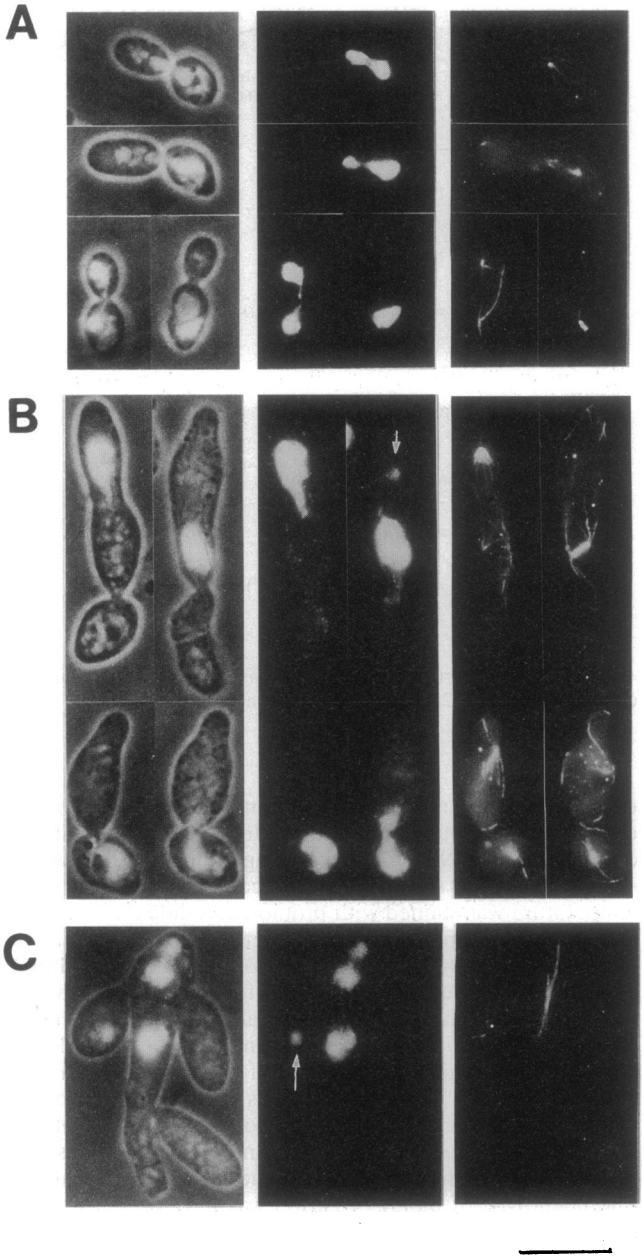

FIG. 3. Fluorescent micrographs of Pld^{ts} KSF1 cells double labeled for DNA (4′,6-diamidino-2-phenylindole fluorescence) (left-hand and middle panels) and tubulin (fluorescein isothiocyanate fluorescence) (right-hand panels). (A) Mitotic small cells with a thin spindle; (B) mitotic large cells with a thick spindle, grown at 37°C; (C) mitotic large cell with buds and a small DNA dot (arrow), grown for 2 h at 28°C after a downshift from 37°C. Bar, 10 μm.

TABLE 2. Segregation of auxotrophic markers by Pld^{ts} and Pld$^+$ strains

Strain and temp (°C)	No. of colonies examined	Segregants		Frequency
		No.	Phenotype	
FUS31 (Pld$^+$)				
28	5,650	1	Lys$^-$	2×10^{-4}
37	8,024	1	Lys$^-$ Trp$^-$	1×10^{-4}
31-30 (Pld^{ts})				
28	17,989	4	His$^-$	2×10^{-4}
37	9,163	80	His$^-$	9×10^{-3}[a]
KSF1 (Pld^{ts})				
28	9,003	10	Met$^-$ Pro$^-$	1×10^{-3}
37	5,808	6	Met$^-$	8×10^{-3}[a]
		3	His$^-$	
		33	Met$^-$ Pro$^-$	
		1	His$^-$ Met$^-$ Pro$^-$	
		1	His$^-$ Lys$^-$ Trp$^-$	
		1	Met$^-$ Pro$^-$ Trp$^-$	
KSF4 (Pld$^+$)				
28	7,191	3	Met$^-$ Pro$^-$	
		1	His$^-$	
		1	Trp$^-$	
		1	Lys$^-$	
37	5,520	1	Met$^-$	4×10^{-4}
		1	His$^-$	

[a] Significantly different ($P < 0.05$) from the corresponding value for the 28°C treatment.

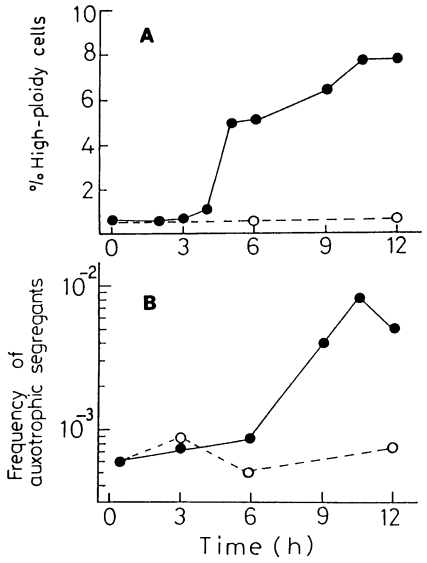

FIG. 4. Correlation between appearance of high-ploidy cells (A) and frequency of auxotroph segregation (B) in *C. albicans* strains of Pld^{ts} KSF1 (closed circles) and Pld$^+$ KSF4 (open circles). Late log-phase cells grown at 28°C in 5 ml of YPD for each strain were transferred to 50 ml of fresh YPD and incubated at 37°C. Observations of high-ploidy cells and detection of auxotrophic segregants are described in Materials and Methods.

Evaluation of polyploidy of the Pld^{ts} and Pld$^-$ strains by observing nuclear spindles. If the DNA content of a nucleus is increased because of endoduplication or upshift of ploidy state, one can examine the dimensions of the spindle and spindle pole bodies as an indicator of the ploidy state (27). Attempts were made to visualize the nuclear spindles of strains 31-30 and KSF1 and Pld$^-$ segregants by indirect immunofluorescence using anti-yeast tubulin antibodies. As an example, a mitotic small cell of KSF1 at 37°C was shown to have a spindle of the same dimension as those cultured at 28°C (Fig. 3A). However, large cells at 37°C have mitotic spindles of a larger dimension. Various stages of microtubule structures in the cell cycle were observed in those large cells (Fig. 3B). One cell seemed to be in interphase. After the culture containing such large cells was transferred to 28°C, multinucleate cells appeared within 2 h (Fig. 3C). Multiple budding is also found, and some of the nuclei are entering into the budding daughter cell. A small nucleus is also observed, as indicated by an arrow. We found that the same features were observed on the mitotic spindles of the other strains, 31-30 at 37°C and Pld$^-$ segregants at 28°C (and also at 37°C). Therefore, the large cells in these Pld$^-$ and Pld^{ts} strains were in a higher ploidy state, compared with the small ones, and no apparent differences between the two strains, Pld$^-$ and Pld^{ts}, were observed in the cytological features of high-ploidy cells.

TABLE 3. Segregation of auxotrophic markers and ploidy phenotype by the Pld^{ts} fusant KSF1

Phenotype	No. of segregants		
	Pld^{ts}	Pld$^-$	Pld$^+$
Met$^-$ Pro$^-$	26	8	9
Met$^-$ Pro$^-$ His$^-$	0	1	0
Met$^-$ Pro$^-$ Trp$^-$	0	0	1
Met$^-$ Pro$^-$ Lys$^-$	0	0	1
His$^-$	2	0	1
Met$^-$	3	2	1

Ordering of the events of ploidy shift and auxotroph segregation. A question arose as to whether the event producing high-ploidy cells was the cause or effect of a variation event evoking segregation of auxotrophic markers in this organism. To answer this question, the order of events was examined, with the Pld^{ts} strains of *C. albicans*, to determine which of the two events occurred first after the incubation temperature was shifted from 28 to 37°C. The cultures of KSF1 showed exponential growth, and the doubling time was 120 min at 28°C or 115 min at 37°C. The cultures of KSF4 also showed exponential growth, and the doubling time at 28°C was 120 min but 105 min at 37°C. The occurrence of anucleate cells in the culture of KSF1 at 37°C seemed to be due to the slight increase in the doubling time, compared with that of KSF1 at 37°C. The frequencies of occurrence of both high-ploidy cells and auxotrophic segregants were monitored on the culture of the Pld^{ts} strain, KSF1, which was transferred from 28 to 37°C (Fig. 4). To identify high-ploidy cells under a fluorescence microscope, cells of KSF1, monitored at intervals, were fixed and stained with 4′,6-diamidino-2-phenylindole. Aliquots of adequate dilutions of the cell suspension from the same culture were plated on YPD and incubated at 28°C for 4 days, and the colonies formed were replica plated to MIN to count auxotrophic segregants. The percentage of high-ploidy cells in the culture of KSF1 increased at 4 to 5 h after the temperature shift, from less than 1% to 5 to 8%. However, the increase in frequency of auxotrophic segregants from the same culture started a few hours after the increase of high-ploidy cells. As a control culture, Pld$^+$ KSF4 was similarly examined, and its data showed no increase in either high-ploidy cells or the frequency of auxotrophic segregation. These results indicated

TABLE 4. Frequency of auxotrophs produced by Ficoll-fractionated cells

Strain	% High-ploidy cells	No. of colonies examined	Frequency of auxotrophs
KSF1 (Pld^{ts})			
Unfractionated			
Expt 1	4.6	4,503	3.0×10^{-3}
Expt 2	3.0	3,944	5.7×10^{-3}
Fractionated[a]			
Expt 1	23	2,190	3.4×10^{-2}[b]
Expt 2	19	3,495	2.1×10^{-2}[b]
KSF4 (Pld^{+})			
Unfractionated	<0.1	5,790	$<2 \times 10^{-4}$
Fractionated[a]	<0.1	4,914	$<2 \times 10^{-4}$

[a] Cells were centrifuged in Ficoll density gradients, the cells enriched on the boundary between 20 and 30% Ficoll layer were collected and spread on YPD plates, and then colonies were replica plated onto MIN.

[b] Significantly different ($P < 0.05$) from the corresponding value for unfractionated cells.

TABLE 5. Segregation of colored-colony variants by Pld^{ts} SGF7-2

Temp (°C)	Fractionation[a]	No. of colonies						
		Total	Red	White	Rarely sectored		Frequently sectored	
					Red	White	Red	White
28	−	4,199	1	5	28	6	3	1
28	+	4,191	2	13	13	0	3	6
37	−	3,057	7	41	63	14	7	7
37	+	5,888	26	116	102	29	40	82

[a] −, none; +, centrifugation in Ficoll density gradients (the cells enriched at the boundary between 20 and 30% Ficoll layers were collected).

that the increase of high-ploidy cells in the population of KSF1 treated at 37°C preceded the increase in the frequency of occurrence of auxotrophic segregants. Since it seemed necessary to confirm whether the increase in variability of the culture was inherent to the appearance of those high-ploidy cells or the other type of cells, which may appear a few hours later, further experiments were performed to enrich high-ploidy cells and to examine their variability.

Variability of cultures enriched with high-ploidy cells. Preliminary experiments told us that the high-ploidy large cells of Pld^{ts} strains, which appeared during incubation at 37°C, could be separated at the boundary between the 20 and 30% Ficoll layers from normal-size cells by centrifugation. Table 4 shows that fractionated high-ploidy cells of KSF1 from a 12-h culture at 37°C gave increased frequencies of occurrence of auxotrophic segregants, compared with the unfractionated culture. As a control culture, the Ficoll density fraction of Pld^{+} KSF4 (at the same boundary between 20 and 30%) contained pseudohyphal cells, but no significant increase was found in the frequency of segregants. These pseudohyphal cells of strain KSF4 had nuclei of uniform size, and no high-ploidy cells were observed among them.

Construction of a red adenine-requiring strain with the Pld^{ts} or Pld^{-} phenotype. The reiteration of the 37°C treatment of KSF1 gave a Met^{-} Pro^{-} Pld^{ts} auxotrophic segregant and then a Met^{-} Pro^{-} Lys^{-} Pld^{ts} segregant from the first segregant. Among the colonies plated from the culture of the last segregant, we happened to find a pink one, from which the strain named SGF1 was isolated. SGF1 seemed to carry a heterozygous adenine-requiring mutation. The DNA content per cell of SGF1 was 44 ± 2 fg at 28°C or 51 ± 2 fg at 37°C. In order to obtain a diploid homozygous adenine-requiring strain, a spontaneous adenine-requiring strain, SGF7, was isolated from 7,500 colonies cultured at 28°C. SGF7 carried the methionine, proline, lysine, and adenine auxotrophies, forming red colonies on YPD. This strain also showed the Pld^{ts} phenotype. Fusion of this strain with 126-A1 (*ade2/ade2* Met^{-}) gave fusants at a frequency of 10^{-4} per parent and produced microcolonies hundreds of times more frequently than fusants, the production of which seemed to indicate complementation between the relevant adenine mutation of SGF7 and the *ade2* marker. Fusion of SGF7 with strain 1435 gave recombinant fusion products at a frequency of 10^{-7} per parent and apparently no complementing microcolonies. Therefore, the adenine mutation of SGF7 was assigned to the same complementation group as *ade1* of strain 1435. Next, a pink adenine revertant of SGF7 was isolated spontaneously. The frequency of reversion of Ade^{-} to Ade^{+} was 4×10^{-6}, and a representative pink Ade^{+} revertant was named SGF7-2. SGF7-2 was assumed to be heterozygous for the adenine auxotrophy. The DNA contents of both SGF7 and SGF7-2 were 40 ± 1 fg per cell at 28°C and 45 ± 2 fg per cell at 37°C. SGF7-2 behaved like the original Pld^{ts} strain. The frequency of colony morphology variation was increased in SGF7-2 grown at 37°C (Table 5). Large cells of SGF7-2 at 37°C, collected after Ficoll density gradient centrifugation, gave a further increase in the frequency of segregation of colored-colony variants (Table 5). This strongly indicated that the large high-ploidy cells of SGF7-2 showed a higher frequency of segregation of the *ade1* marker than the predominant small cells. Although these temperature-shifted cultures gave increased frequencies of production of both white and red colonies, some were sectored (Table 5). The white colonies were assumed to reflect the homozygous state of *ADE1*. Another finding was an appearance of very frequently sectored colonies; one type was very frequently red on a pink background, and the other type was very frequently white on a pink background. By replica plating, half-red colonies from SGF7-2 were shown to correspond to adenine auxotrophs, but both the white and pink areas corresponded to Ade^{+}. However, the very frequently red area of a colony from SGF7-2 was too small to be identified by replica plating as the area of adenine auxotrophs. By fluorescence microscopy, all of these very frequently sectored colonies contained high-ploidy cells at high frequencies (2 to 10%). The cultures of these frequently sectored isolates, incubated at 28 as well as 37°C, had chemically determined DNA contents per cell varying between 50 and 70 fg among isolates. Subculturing of such very frequently sectored colonies gave the same type colonies as well as uniformly colored ones. In such a uniformly colored colony, no high-ploidy cells could be observed in the population. The occurrence of high-ploidy cells in the subcultures of the frequently sectored colonies was apparently temperature independent: both at 28°C and at 37°C, subcultured cell populations had high-ploidy cells at 2 to 10%. Therefore, these frequently sectored colonies were assumed to consist of the population of Pld^{-} cells. This also indicated that the very frequently sectored colonies reflected the occurrence of frequent variations in the zygotic state of adenine genes in a clone. One representative Pld^{-} clone, derived from SGF7-2 at 37°C, was designated as clone 5. Compared with these Pld^{-} clones, the Pld^{ts} segregants from SGF7-2 showed a rarely

TABLE 6. Colony formation by micromanipulated cells of SGF7-2 and its Pld$^-$ derivative clone 5

Strain and cell size	No. of colonies								
	With no visible growth	With visible growth	Pink	Red	White	Rarely sectored		Frequently sectored	
						Red	White	Red	White
SGF7-2[a]									
Normal	4	44	39	0	2	1	0	1	1
Large	38	90	58	2	11	2	1	9	7
Clone 5[b]									
Normal	8	239	220	0	13	1	3	0	2
Large	6	44	30	3	6	1	3	0	1

[a] Grown at 37°C.
[b] Grown at 28°C.

sectored colony morphology when incubated at 28°C but a more frequently sectored one at 37°C. On the other hand, uniformly colored colonies derived from clone 5 had the same morphology by subculturing at both 28°C and 37°C, and high-ploidy cells could not be detected at these temperatures. This suggested that the Pld$^-$ strain gave rise to Pld$^-$ and Pld$^+$ descendants but not Pld^{ts} ones.

Genomic instabilities of high-ploidy cells shown by micromanipulation. To obtain direct evidence of the correlation between the production of high-ploidy cells and the increase in frequency of variations, large cells in the high-ploidy state were micromanipulated from the SGF7-2 (Pld^{ts}) culture grown at 37°C or from the clone 5 culture grown at 28°C, transferred onto YPD, and allowed to form colonies (Table 6). A significant fraction of the micromanipulated large cells showed no visible colony growth. Microscopic examination of micromanipulated cells with apparently invisible colony growth revealed arrest in cell growth within several divisions. The frequency of occurrence of visible colonies derived from micromanipulated large cells of clone 5 was not significantly different from that of colonies derived from the micromanipulated large cells of SGF7-2 grown at 37°C, indicating a higher frequency of occurrence of genomic rearrangement in the high-ploidy cells of both Pld$^-$ strains and Pld^{ts} strains.

Chromosome rearrangement occurred in the high-ploidy cells. The chromosome-size DNAs of two representative segregant colonies from micromanipulated high-ploidy cells of SGF7-2, each of which consisted of a red adenine-requiring sector and a pink sector, were examined by pulsed-field gel electrophoresis (Fig. 5). The two genes rDNA and *ADE1* have been shown to be linked to each other on the R chromosome of this organism (3, 33). In SGF7-2, rDNA and *ADE1* were found on two chromosomal DNA bands of different size; one was 2.8 Mbp, and the other was 2.6 Mbp (Fig. 5, lanes 1, 7, and 13). On the other hand, *ADE2* was found hybridized to a band below 2.3 Mbp (data not shown). One representative of small red colony-producing clones from a high-ploidy cell of SGF7-2 showed only one band of 2.6 Mbp, which was hybridizable to both rDNA and *ADE1*, suggesting that the other 2.8-Mbp chromosome had been reduced to 2.6 Mbp (Fig. 5, lanes 2, 8, and 14). Two representative half-sectored colonies with a pink sector and a red sector were also examined. Each of the two sectors, which originally derived from the two sister cells produced by a high-ploidy cell of SGF7-2, had lost the 2.8-Mbp band and, instead, contained a new 2.3-Mbp band. The rDNA was found existing on these new chromosomal bands; however, the gene homologous to *ADE1* had been lost on the 2.6-Mbp bands in clones from red strains (Fig. 5, lanes 3 to 6, 9 to 12, and 15 to 18). This indicated that chromosome breakage, followed by deletion (or translocation) of the fragment containing *ADE1*, had occurred on the 2.6-Mbp band (Fig. 6).

DISCUSSION

The occurrence of high-ploidy cells is a universal phenomenon in *C. albicans*. Van der Walt and Pitout (31) described a derivative of strain CBS5736 of this organism, in which presumed haplophase yeast cells developed into diplophase cells after karyogamy and mycelial formation occurred in the diplophase. Their observations have never been repeated. One of us described a similar phenomenon in a clinical isolate (NUM51) of this organism, but the existence of haplophase was not observed: the population of NUM51 contained both diploid and tetraploid (or high-ploidy) cells (27). By fluorescence microscope photometry and thin-section electron microscopy, some diploid cells seemed to be undergoing endomitosis to become tetraploid (or high-ploidy) cells while some tetraploids showed nuclear structures similar to those of meiosis II in *Saccharomyces cerevisiae* and multiply budded to

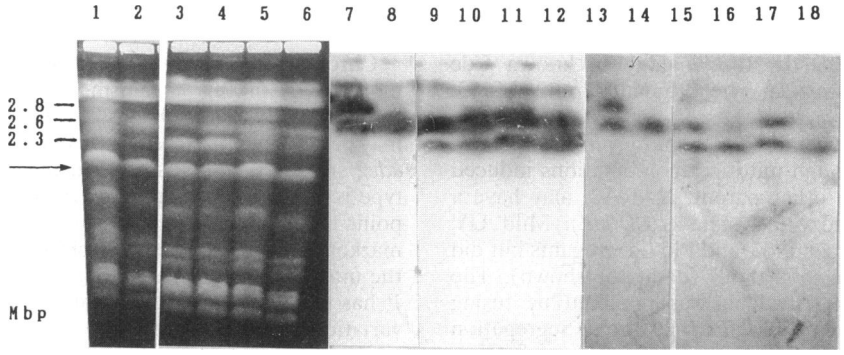

FIG. 5. rDNA and *ADE1* hybridization profiles of chromosomal DNA bands in SGF7-2 (*ADE1/ade1*) and its progeny clones. Electrophoretic karyotypes of the parent SGF7-2 making pink colonies (lane 1), a representative small red colony-producing isolate (lane 2), and four representative clones, with each pair derived from a single half-sectored colony, a pink one (lanes 3 and 5) and a half-red one (lanes 4 and 6), are shown. Hybridization profiles of rDNA (lanes 7 to 12) and *ADE1* (lanes 13 to 18) are also shown. Molecular sizes of DNA are according to Iwaguchi et al. (8, 9). The chromosomes of FC18 were used as a reference for each electrophoretic separation (not shown). Arrow, the band which hybridized with the *ADE2* probe (13).

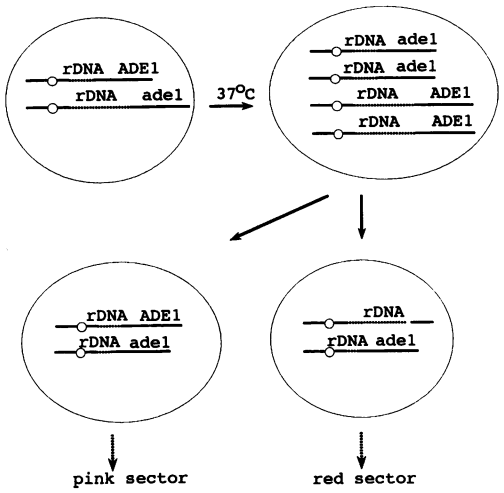

FIG. 6. R chromosome of SGF7-2 and its derivative isolate forming red and pink sectors. At 37°C, the cell of SGF7-2 carried out an upshift of ploidy, followed by chromosome rearrangement. *ADE1* was linked to the rDNA cluster (dotted lines). Changes in the size of the rDNA cluster are indicated by differences in the lengths of the dotted lines. Chromosome breakage, followed by deletion (or translocation) of the fragment containing *ADE1*, is suggested to have occurred in one of the daughter cells.

produce diploid (or near-diploid) cells. This phenomenon was named ploidy shift. There seemed to be a ploidy shift in the creation of NARA2 in this work, because NARA2 was diploid and its progenitor NUM961 was triploid (28). From NUM961, SRT1 was derived. SRT1 produced polyploid descendants by the ploidy shift and showed variation of colony morphology between semirough and smooth at high frequencies (28). In this study, the correlation between the ploidy shift and the genetic stability was elucidated by the fact that micromanipulated high-ploidy cells gave rise to auxotrophic segregant colonies at high frequencies.

Cell fusion and segregation experiments in this study suggested the existence of a gene controlling the ploidy states in this organism. This does not exclude the possibility that more than one gene may participate in determining the state of ploidy. If one can assume a single gene to explain three different phenotypes of ploidy states, Pld^+, Pld^{ts}, and Pld^-, the following working hypothesis seems to be most plausible. This hypothesis includes an assumption that temperature dependency of the Pld phenotype is due to both the gene dosage of *pld* and its ratio to *PLD*, by the analogy of known Cdc phenotypes in *S. cerevisiae* (7). The original parent, A5153, may carry wild-type *PLD* in a homozygous state, because neither A5153 nor its progenitor FC18 had given rise to Pld^- or Pld^{ts} descendants through mitotic recombinations induced by UV irradiations. The other parent, NARA2, may have a recessive *pld* in a heterozygous state (*PLD/pld*). Mild UV irradiations of NARA2 gave Pld^+ and Pld^- segregants but did not produce Pld^{ts} segregants at all (data not shown). The genotype of fusant FUS31, which was obtained by fusing A5153 with NARA2, may be *PLD/PLD/PLD/pld*. Segregation yielded an abnormal progeny, segregant 31-30, whose DNA content was close to triploid and whose genotype may be *PLD/pld/pld*; the higher dosage of *pld* versus *PLD* would bring about the Pld^- phenotype at high temperatures. A simple explanation for the characteristic segregation pattern of KSF1, one of the fusants of NARA2 and segregant 28-3 (Fig. 1 and Table 2), could be either a trisomic or a tetrasomic state of the chromosome carrying *PLD* and its allelic *pld*: the genotype of Pld^+ KSF4 could be assumed to be *PLD/PLD/pld/pld*, and that of Pld^{ts} KSF1 could be assumed to be either *PLD/pld/pld* or *PLD/pld/pld/pld*. Pld^- strains of this organism, which had red-adenine markers, produced very frequently sectored colonies, suggesting that a chromosome missegregation event occurred frequently in the cell division after plating. The genotype of Pld^- diploid (or near-diploid) strains may be *pld/pld*, since no Pld^{ts} variants were derived at high frequency from Pld^-. An alternative hypothesis is the existence of two genes; each mutant allele is *pld1*, which renders the cell Pld^-, or *pld2*, which makes the cell Pld^{ts}. However, this hypothesis cannot simply explain the fact that Pld^- variants were derived from Pld^{ts} strains but Pld^{ts} ones were not derived from Pld^- strains.

The function of *PLD*, if it is present, seems to regulate the state of ploidy in *C. albicans*. In *S. cerevisiae*, five complementation groups have been defined for mutations that confer an increase in ploidy phenotype (2). Some of these mutations are assigned to alleles of *CDC31*, which is required for spindle pole body duplication, and some are allelic to *BEM2*, required for normal bud growth. On the other hand, three categories of genetic defects are known to lead to genomic instability in *S. cerevisiae* (6, 7). Mutations in DNA repair processes, in cell cycle control, and in the machinery for DNA replication and chromosome segregation are known to cause genomic instability (15). Production of high-ploidy cells in *C. albicans* seems to be a result of G_2-M phase delay due to some genetic factor(s) which also affects processes leading to chromosome missegregation in this organism. Faithful distribution of daughter chromosomes at mitosis is due to the roles of structural components, such as those that assemble into the kinetochore or centromere and the spindle pole body. Defects in kinetochore structure and function usually cause an increase in the mitotic rate of chromosome missegregation and result in a terminal phenotype indicative of a defect in the G_2-M phase of the cell cycle (5, 12, 16, 34). Isolation and characterization of the putative *PLD* gene from *C. albicans* will cast light on the mechanism of this process.

Here, we show that high-ploidy cells in this organism have the ability to change their genome constitution through chromosome rearrangements. Multiply budded cells of KSF1 in this study showed segregating nuclei, some of which appeared smaller than the others (Fig. 3C). These may correspond to nuclei in which some chromosome aberration may have occurred. Such chromosome aberration seems to produce not only viable recombinants but also nonviable descendants (Table 6).

Chromosome rearrangement that occurred in the high-ploidy cells of this organism would at least consist of the following two types. One is chromosome elimination, which may be due to mitotic nondisjunction, as has been reported for *ade2* small-red-colony strains in this organism (1). The other type is chromosome breakage. As shown in Fig. 6, a breaking point may exist in the rDNA repeats or between the *ADE1* marker and the repeat on chromosome R. Chromosome R is the most variable in size among *C. albicans* strains (8, 19, 33). It has been shown by Iwaguchi et al. (9) that the clonal size variation of chromosome R (termed chromosome 2 by them) which occurs at high frequency is derived from the size change of the rDNA cluster. In this study, analyses of karyotype were limited to clones that had shown adenine-requiring variations. In other words, chromosome rearrangements of some sort were selected here by screening for colony color variations in this organism. The questions of where such a breaking point is

and whether the point is a specific site on the chromosome remain for further investigations. The *Sfi*I genomic structure map devised by Chu et al. (3) also will greatly aid this analysis.

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