Clonal size-variation of rDNA cluster region on chromosome XII of *Saccharomyces cerevisiae*

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Using pulsed-field gel electrophoresis (PFGE), we have demonstrated clonal variation in the size of chromosome XII in a diploid strain of *Saccharomyces cerevisiae* X2180-2D. The sizes of the two chromosome XII homologues were very different: 2600 (L-type) and 1450 kb (S-type). The frequency with which we detected clonal size variation in the diploid, compared to that of the parental clones, was about 15–50% of the progeny clones and the range of the size variation of the homologues was 2580–2680 kb (L-type) and 1340–1500 kb (S-type), respectively. The homologue of the L-type appeared to be more frequently variable than that of the S-type. The size variation was shown to be derived from size changes in the rDNA cluster region, which is present in chromosome XII, by digesting the chromosome with *Xho*I, whose cutting site is not present in a rDNA repeat unit, and hybridizing to rDNA probes. The clonal size variation was also investigated in haploids from spores after meiosis. The L-type and S-type chromosomes segregated 2:2 in an ascus and the sizes of all the S-type chromosomes were shifted up, compared to the original diploid, though the L-type ones were stable. The S-type sizes of 1340, 1450 and 1780 kb in the original diploids changed into the ranges of 1475–1610 kb, 1520–1680 kb and 1820–2010 kb, respectively, in the segregants. Furthermore, we observed that the size of S-type chromosomes in haploid cells was gradually increasing in mitosis during successive subcultures. The rDNA units appeared to be amplified on the S-type chromosome.

Introduction

Genome information is essential for understanding the evolution and phylogeny of organisms. Classically, karyotype analysis is one of the powerful methods for studying the genome structure. The karyotype is a morphological pattern of condensed chromosomes at M-phase in higher organisms. In *Saccharomyces cerevisiae*, the chromosome morphology is difficult to visualize by light microscopy (Kuroiwa *et al.*, 1984). Recently, pulsed-field gel electrophoresis (PFGE) has been developed and large DNA molecules such as a native

chromosomal DNA can be separated in an agarose gel according to their size (Schwartz & Cantor, 1984). The separation band profile determined by PFGE is called the electrophoretic karyotype. This karyotype analysis has been extensively applied to yeasts or filamentous fungi because their chromosome size is suitable for this separation. The electrophoretic karyotype seems to be species-specific, though extensive polymorphism has been observed among strains (Jonge *et al.*, 1986; Suzuki *et al.*, 1988; Johnston *et al.*, 1989; Magee & Magee, 1987). Polymorphism has been well-studied in the pathogenic yeast *Candida albicans* and some *Candida* species to distinguish clinical strains (Merz, 1990; Iwaguchi *et al.*, 1990; Asakura *et al.*, 1991; Doi *et al.*, 1992). In contrast, high frequency clonal karyotype variation was found in *C. albicans* and was due to size variation in chromosome 2, which carries rDNA genes. Chromosome 2 is too variable to be useful for distinguishing strains. This high frequency of karyotype variation has been shown to be derived from size changes of the rDNA cluster (Iwaguchi *et al.*, 1992). Changes in

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Abbreviation: PFGE, pulsed-field gel electrophoresis.

repeat number of rDNA genes have been reported in many organisms and it is believed that there are some mechanisms to maintain or amplify the number of rDNA genes (Hawley & Marcus, 1989; Stark & Wahl, 1984; Selker, 1990; Long & Dawid, 1980).

The greatest amount of genetic and molecular biological work has been done in *S. cerevisiae* and many rDNA studies have also been carried out in this yeast. The rDNA of *S. cerevisiae* is arranged in tandemly repeated clusters of about 100–140 copies of a 9 kb repeat unit which consists of several rDNA genes (Schweizer *et al.*, 1969; Petes, 1979*a*; Cramer *et al.*, 1972; Philippsen *et al.*, 1978). The rDNA genes are localized on chromosome XII (Petes, 1979*a, b*). The increase or decrease in the rDNA cluster gene copy number has been shown by using a chromosome XII monosomic strain which lacks 25–35% of rDNA genes compared to diploid strains (Kaback *et al.*, 1973, 1976). The number of rDNA genes increased to the diploid level in the monosomic strain after many mitotic generations (Kaback & Halvorson, 1977). The variation of the rDNA gene cluster may be generated by unequal sister chromatid exchange (Szostak & Wu, 1980), however, interchromosomal recombination within the rDNA cluster appears to be suppressed in meiosis (Petes & Botstein, 1977). The frequency of unequal crossing-over in rDNA was estimated to be 5×10^{-4} per generation in mitosis. Furthermore, it has been reported that mitotic recombination in the rDNA region is suppressed by the combined action of topoisomerases I and II (Christman *et al.*, 1988) or suppressed by the function of a transcriptional silencer gene, SIR2 (Gottlieb & Esposito, 1989). A mitotic recombination hot spot, HOT1, located in the rDNA repeat has been found (Keil & Roeder, 1984; Stewart & Roeder, 1989). It has not yet been clarified how recombination factors are involved in regulating the number of rDNA genes.

Despite many reports concerning rDNA studies in yeasts, no direct demonstration of the frequency of size diversity in the rDNA cluster region of diploid and haploid cells has been investigated. By using PFGE, we examine the rate of chromosome size variation derived from the size change of the rDNA cluster region.

Methods

Strains. A diploid strain of *Saccharomyces cerevisiae*, X2180-2D (Mortimer & Schild, 1985) and its haploid segregants were used in this study. Cells were grown in Yeast Peptone Dextrose (YPD) broth or on a plate containing 1.5% (w/v) agar (Asakura *et al.*, 1991). The segregation of the diploid clone was induced on sporulation medium (Rose *et al.*, 1990).

Preparation of yeast chromosome DNA for PFGE. The sample plug containing yeast chromosome DNA for PFGE was prepared by the method described previously (Iwaguchi *et al.*, 1990). Cells in stationary

phase at 30 °C in 3 ml YPD broth (w/v: 1% yeast extract, 1% polypeptone, 2% glucose) were washed twice in 50 mM-Na-EDTA (pH 7.5) and resuspended in 1 ml 50 mM-Tris/HCl (pH 7.5). Eighty microlitres of the suspension was mixed with 30 µl Zymolyase 20T solution (5 mg ml⁻¹; Seikagaku Kyogo) in 0.1 M-Na-citrate buffer (pH 5.8) and then 300 µl 1% (w/v) low melting point agarose (Agarose L; Nippon-gene) in 0.125 M-Na-EDTA (pH 7.5) was added at 50 °C. The solution was poured into a mould chamber (Bio-Rad). The solidified plugs were incubated in 10 mM-Tris/HCl (pH 7.5) containing 25 mM-Na-EDTA and 7.5% (v/v) 2-mercaptoethanol for 18–24 h at 37 °C and then incubated in 10 mM-Tris/HCl (pH 8.3) containing 25 mM-Na-EDTA, 0.06% SDS and 0.17 mg proteinase K ml⁻¹ for 18–24 h at 50 °C. Finally, the plugs were kept in 50 mM-Na-EDTA (pH 9.0) at 4 °C until used.

PFGE. PFGE was carried out by the CHEF method (Chu *et al.*, 1986) using the Pulsaphor system with a hexagonal electrode array (Pharmacia-LKB). Yeast chromosomal DNAs were separated in a 0.8% agarose gel usually under the following conditions: a 300 s pulse time at 140 V for 24 h, followed by a 1200 s pulse time at 80 V for 48 h as described previously (Doi *et al.*, 1992). Gels were stained with 0.5 µg ethidium bromide ml⁻¹, destained in distilled water and observed under UV light (302 nm).

S. cerevisiae (X2180-1A; Mortimer & Schild, 1985) and *C. albicans* (FC18; Doi *et al.*, 1992) were used as the size reference markers. A λ DNA concatemer ladder (Bio-Rad) and λ DNA digested with *Eco*T14I (Takara, Japan) were also used as size markers.

Southern hybridization. Southern hybridizations were performed using an rDNA probe which was prepared from a 10.8 kb *Sal*I fragment of pAT68 which contains a rDNA repeat unit (Sugihara *et al.*, 1986). After the fragment was cut with *Kpn*I, the probe was labelled by a random priming method with ³²P as described previously (Asakura *et al.*, 1991) or with Digoxigenin using a kit (Boehringer Mannheim).

Restriction digestion of chromosome DNA in PFGE sample plugs. Sample plugs for PFGE were equilibrated in reaction buffer (200 µl) of the restriction enzymes *Xho*I or *Pst*I for 30 min at room temperature. The plugs were transferred to new restriction buffer containing *Xho*I or *Pst*I [32 and 120 U (200 µl)⁻¹, respectively] and incubated overnight at 37 °C. After the treated samples were washed once with 50 mM-EDTA (pH 9.0), the chromosomal fragments were analysed by PFGE using a 100 s switch at 180 V for 15 h followed by a 300 s switch at 140 V for 20 h.

Results

Frequency of clonal size change in chromosome XII of diploid cells

The clonal size variation of chromosomes was investigated in a diploid strain of X2180-2D. It was streaked on a YPD plate at 30 °C for 3 d to get a pure single clone from a –80 °C stock. One single colony was referred to a master clone. Single colonies were isolated from the master colony and were called parental clones. From each parental clone, single colonies were isolated again on a YPD plate, and referred to as progeny clones. Chromosomes of each clone were analysed by PFGE and their sizes were estimated. The sizes of the chromosome XII homologues of the master clone were 2600 and 1450 kb; these will be referred to as the reference sizes. These large and small homologues of chromosome XII

Fig. 1. Karyotype variation of diploid clones. Chromosomes of the 10 parental clones of diploid *S. cerevisiae* (lanes 2–11), the master clone (lane 1), and haploid *S. cerevisiae* (lane 12) were analysed by PFGE under the conditions described in Methods. The gel was stained with ethidium bromide. The bands assigned by hybridization using a rDNA probe are indicated by arrows. Numbers on the right margin indicate the size of *S. cerevisiae* marker DNAs.

Fig. 2. *XhoI* fragments derived from rDNA gene cluster regions in diploid clones. PFGE sample plugs were prepared from the four parental clones (lanes 1, 4, 7 and 10), which were the same clones as used in lanes 8, 9, 10 and 11 in Fig. 1, respectively, and the progeny clones (lanes 2 and 3, 5 and 6, 8 and 9, and 11 and 12), which were derived from the parental clones of lanes 1, 4, 7 and 10, respectively. After the sample plugs were treated with *XhoI*, the digested fragments were separated by PFGE using a 100 s pulse time at 180 V for 15 h followed by a 300 s pulse time at 140 V for 20 h in a 0.8% agarose gel, and Southern hybridization using an rDNA probe was performed. Numbers on the left margin indicate the fragment size of the parental clone in lane 1 whose chromosome XII size is the same as the master clone.

are called L-type and S-type, respectively. The size variations of the chromosomes were observed in 46% of the 50 clones tested and the range of the size change of the L-type and S-type chromosome was between 2580 and 2680, and 1340 and 1500 kb, respectively. These variable chromosomes were identified by the rDNA probe, which is the marker for chromosomes XII. Some of the results are shown in Fig. 1. Among the progeny clones, the variation frequency of L- and S-type was 43% (17/40) and 13% (5/40), respectively. The longer chromosome appears to be more variable than the shorter one. The clonal size variation was detected in other independent experiments, though the frequency with which it occurred was very varied (15–50%) in about 20 clones in each experiment and the range of the size change was also different in independent experiments.

Size-change of rDNA cluster region among the diploid clones

Since the rDNA gene cluster of *S. cerevisiae* is reported to be very variable in size (Olson, 1991) and clonal variation of chromosome size has been shown in the rDNA cluster of *C. albicans* (Iwaguchi *et al.*, 1992), we assumed the clonal size variation of chromosome XII of *S. cerevisiae* that we have found was also derived from the rDNA cluster. To confirm our assumption, we tried to cleave out the rDNA region by using various restriction enzymes, including *SmaI*, *HindIII*, *BamHI*, *PstI* and *XhoI*. Three of the five restriction enzymes, *BamHI*, *PstI* and *XhoI*, gave large fragments assigned by the rDNA probe and the sizes of the rDNA fragments produced were similar (data not shown). The 1560 and 440 kb *XhoI* fragments were detected in the master clone. The rDNA unit, a 9.1 kb fragment identified by the rDNA probe, was detected after digestion with *SmaI* which recognizes only one cutting site in the rDNA unit, and the unit size was not changed in the variable chromosomes (data not shown). To investigate the size range of rDNA clusters in this study, *XhoI*, whose cutting site does not occur in the rDNA unit, was selected. After chromosomal digestion, the DNA profile was stained with ethidium bromide, transferred to a filter and hybridized with the rDNA probe. The rDNA fragment sizes from the S-type and the L-type were estimated as 290–1040 kb and 1510–1640 kb, respectively (Fig. 2). Their size differences corresponded to the size differences of L- and S-type chromosome XII and the hybridization intensity was correlated to their size. This suggested that the size change of chromosome XII is derived from the size change of the rDNA cluster region. The length of the cluster varied in both the large (L-type) and the small (S-type) homologues.

Fig. 3. S- and L-type chromosome segregation in a tetrad. A diploid clone was sporulated and tetrads were obtained. Chromosomes of the diploid clone (lane 1) and the colonies derived from a tetrad (lanes 2–5) were analysed by PFGE under the conditions described in Methods.

Size change of rDNA cluster in haploid segregation

To investigate size variation in haploid clones, haploid segregants with either an L-type or S-type chromosome XII were prepared by meiosis. The sporulation rate was found to be approximately 10% and the spore survival rate was 77% (813/1060), comprising tetrads, triads and diads. By PFGE, we showed that L- and S-type chromosomes segregated 2:2 in all ten sets of tetrads tested (an analysis of one tetrad is shown in Fig. 3). In haploid clones from the triads and diads, as well as the surviving spores of tetrads where all four spores did not germinate, L- and S-types were detected at about an equal ratio. No obvious size change of the L-type chromosome XII and the other chromosomes was observed as a result of meiosis. In contrast, the sizes of the S-type chromosomes of the clones from the germinated spores were shifted up from the original size (1450 kb) to around 1520–1680 kb and the bands were diffuse, suggesting the smaller (S-type) chromosome is very unstable. Similar size change profiles were observed in haploid clones when the meiotic segregants were obtained from diploid parent clones which contained the 2600 kb L-type chromosome and the different size S-type chromosomes of 1340 or 1780 kb. The sizes of S-type chromosomes in the haploid clones increased to

Fig. 4. Chromosome XII size variation and rDNA fragment digestion with *Xho*I in haploid progeny clones. Sample plugs for karyotype analysis were prepared from a diploid original clone (lane 1) and the haploid progeny clones (lanes 2–13) from the diploid clone. (a) Chromosomes in the sample plugs were separated by PFGE under the conditions described in Methods and stained with ethidium bromide. The arrowheads indicate the position of the L- and S-type chromosomes (2600 and 1450 kb) of the diploid clone. Numbers on the right margin indicate the size of the *S. cerevisiae* marker DNAs. (b) The sample plugs treated with *Xho*I, subjected to PFGE under the conditions described in the legend to Fig. 2, and hybridized to an rDNA probe. The arrowheads indicate the position of the rDNA fragments (1560 and 440 kb) of the diploid clone.

1475–1610 kb and 1820–2010 kb, respectively. The sporulation rate of the diploids containing the different size S-type chromosomes and the percentage survival of spores were similar to those of the diploid clone containing the 1450 kb S-type chromosome.

Frequency of clonal size change in chromosome XII of haploid cells

The frequency of clonal size variation of chromosomes was investigated in haploid cells containing the S- or L-type chromosome. From colonies directly germinated

Fig. 5. S-type chromosome XII size variation in successive subcultures. The four haploid clones containing different sizes of S-type chromosome (A–D) were subcultured successively; 30 μ l of culture was inoculated into 3 ml of fresh YPD broth every day. The chromosome samples were prepared on the first, fifth and tenth day. Chromosomes were separated by PFGE under the conditions described in Methods. Numbers on the right margin indicate the size of the *S. cerevisiae* marker DNAs.

from each spore, single colonies were obtained and stored at -80°C . The parental clones and progeny clones were obtained in the same manner as in the above diploid experiment. Ten progeny clones from each of the three L-type and three S-type parental clones were analysed by PFGE. The L-type bands among the parental and their progeny haploid clones showed hardly any size changes. In contrast, about 50% of S-type chromosomes were variable in the 30 progeny clones compared to the size of the parental clones (Fig. 4a). The size change of the six S-type clones was confirmed by using *Xho*I rDNA probe hybridization as described above (Fig. 4b).

To observe the effect of successive subculture on the S-type size, four haploid cells were subcultured everyday in YPD broth. The chromosomal profile was studied on the first, fifth and tenth day of the subculture. It was found that the sizes were gradually increasing. The longer the successive subculturing was carried out, the greater the size of the S-type band detected, while the other bands remained constant (Fig. 5).

Discussion

In this study, we have shown that the size variation of chromosome XII of *S. cerevisiae* occurred at a high frequency in clones, whereas the size of the other chromosomes was stable. This variation was shown to be derived from the size change of the rDNA cluster region by *Xho*I treatment, which can excise the rDNA cluster,

and the size of the rDNA *Xho*I fragment corresponded to the size of chromosome XII (Fig. 2). There are three possible explanations; (i) rDNA unit size change, (ii) rDNA unit number change, and (iii) size change of the rDNA junction region. The second is most probable because the intensity of the hybridization signal using a rDNA probe correlated to the size of the fragments and we could not detect the unit size change in the variable chromosomes. However, the third possibility was not completely ignored in this study. A similar study of chromosome size variation has been done in *C. albicans* where the clonal variation of the rDNA-containing chromosome occurred at a frequency of up to 10% of the progeny clones (Iwaguchi *et al.*, 1992). The frequency of detectable size change of the rDNA region of *C. albicans* has been estimated to be about 5×10^{-3} per cell division. The frequency of the clonal size variation of the *S. cerevisiae* rDNA chromosome in diploids was about 15–50%. The variation frequency of *S. cerevisiae* rDNA chromosome thus seems to be similar to or higher than that of *C. albicans*.

The size of chromosome XII in a reference haploid, X2180-1A, is 2200 kb. In contrast, the diploid X2180-2D used in this study contains two very differently sized chromosomes, the L-type (2600 kb) and the S-type (1450 kb). The frequency of the size change of the L-type and the S-type was 43 and 13%, respectively. This difference probably arises from the size of the rDNA region, estimated as 1560 and 440 kb, respectively, and the frequency of the size change may be relative to the length of the rDNA region. When we take 9.1 kb as the size of rDNA repeat unit, the number of rDNA repeating units in the 1560 and 440 kb rDNA fragments was estimated as 171 and 49, respectively.

When the diploid cells sporulated, the L-type and S-type chromosomes segregated 2:2 in the tetrads (Fig. 3). Surprisingly, the size of all the S-type chromosomes was shifted up and the germination rate of spores with either L- and S-type chromosomes was similar. The size increase in the S-type chromosomes was shown to be derived from the rDNA cluster region (Fig. 4). We assume that the increase of the size of the rDNA region occurred during the meiotic process, since colonies derived from each spore were directly analysed for chromosome size. Furthermore, the S-type chromosome size increased gradually during mitotic proliferation of the haploid cells (Fig. 5). We suggest that the rDNA gene was amplified on the S-type chromosome in haploid clones. Because the L-type chromosome was not amplified, rDNA region size or the amount of rDNA gene products may control the amplification. If the onion-skin model, that involves multiple reinitiation of DNA synthesis and recombination (Stark & Wahl, 1984), can be applied to rDNA amplification, the frequency of

multiple reinitiations from the autonomously replicating sequence (ARS), which is present in each rDNA unit (Linskens & Huberman, 1988; Skryabin *et al.*, 1984), may determine the size and the frequency of the amplification, and the reinitiation might be activated in the S-type chromosome. Both magnification and reduction of the size of the rDNA region occurred at a similar frequency in either the L- or S-type chromosomes of a diploid cell. This size variation may be derived from unequal sister chromatid exchange, which has been shown to happen frequently in mitosis and meiosis (Petes, 1980; Szostak & Wu, 1980). The estimated rDNA copy number is 220 in the diploid strain we used. The number of rDNA copies or the amount of rDNA gene products may be enough for suppressing the amplification.

Recombination in the rDNA cluster of *S. cerevisiae* has been shown to be affected by topoisomerases I and II (Christman *et al.*, 1988), a transcriptional silencer gene, SIR2 (Gottlieb & Esposito, 1989), or a mitotic recombination hotspot, namely HOT1 (Keil & Roeder, 1984; Stewart & Roeder, 1989). It will be very interesting to know whether the above factors and other general recombination factors affect rDNA amplification.

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