Initiation of ageing process by meiotic and mitotic recombination within the ribosomal DNA genes in *Saccharomyces cerevisiae*

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Abstract

In the budding yeast of Saccharomyces cerevisiae the tandem repeated of rDNA genes are located on chromosome XII, which is in the nucleolus. There are different types of proteins in the nucleoluskeleton, silencing proteins have got important role in nucleolus. It is shown that meiotic recombination between non-sister chromatids in the rDNA genes are strongly suppressed, and suggested that silencing proteins such as SIR2 are involved in silencing state. It is also shown that nucleolus shows some changes during ageing process. It is claimed that intrachromosomal recombination within the rDNA repeated sequences; producing 3-μm rDNA circles are accumulated with old cell. This study looked at the rDNA breakage in two different types of strain ORD 1181 according to their age. The fine analysis of the rDNA array was performed using restriction endonuclease enzymes, which do not cleave within the rDNA array. The results suggest that there are only meiotic hot regions for chromosome breakage in the old cells within the rDNA array.

Keywords: *Double-strand break (DSB), rDNA gene, Meiotic recombination, ageing, (rDNA) homolog pairing.*

INTRODUCTION

The yeast genome is approximately 12.8 Mb. It is divided into 16 chromosomes (Mortimer *et al.,* 1992).

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The size of yeast chromosomes varies from 240 kb for chromosome I to 2–3 Mb for chromosome XII. The different length of chromosome XII is due to the number of copies of the rDNA unit that are present in chromosome XII (Rustchenko and Sherman, 1994). The frequency of meiotic recombination in the ribosomal rDNA array is much less than expected frequency (Petes and Botstein 1977; Petes 1979a). However, meiotic recombination between sister chromatids (in particular, unequal sister chromatid recombination) occurs frequently (Petes *et al.,* 1981). There is a correlation between the level of double-strand break (DSB) and the frequency of recombination. Therefore, DSB hot spots are normally known as recombination hot spots in genome (Mizuno *et al.,* 1997). There are many studies regarding recombination hot spots in genome resulting in comparison of physical and genetic maps for different chromosomes in *S. cerevisiae* (Baudat and Nicolas, 1997; Chindamporn *et al.,* 1993; Game, 1992). Figure 1 shows comparison of physical and genetic maps of chromosome XII in *S. cerevisiae.* The red diagram shows meiotic recombination events on chromosome XII. But there is a silencing region at the rDNA array for recombination events. Of course, the real size of the rDNA region is not shown in this figure because the rDNA array is a huge locus and occupies around half-length of chromosome XII. Among all repeated sequences, the rDNA array is a special case because they may undergo unequal sister-chromatid exchange (USCE) resulting in the two molecules from replication of a chromosome. The rDNA array may also undergo intrachromosomal recombination resulting in introducing an extrachromosomal form. In this
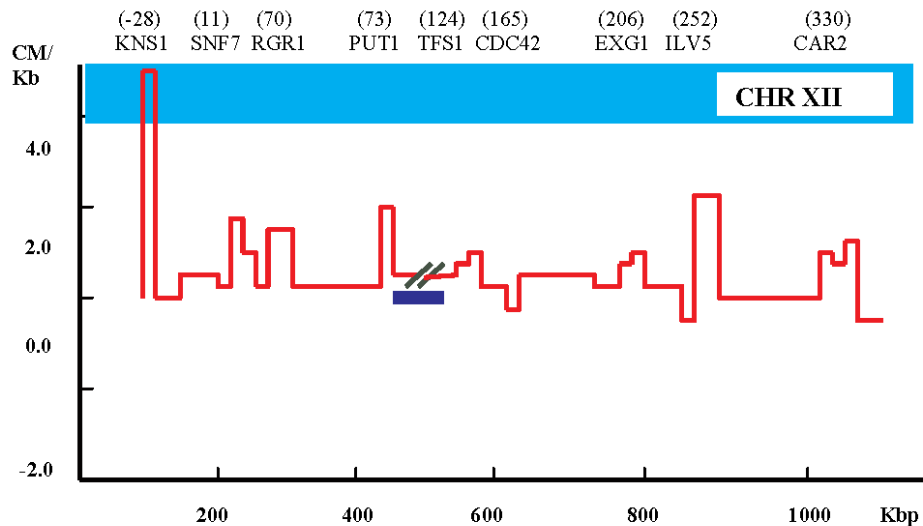
Figure 1. Meiotic recombination events on chromosome XII. Comparison of physical and genetic maps on chromosome XII, redrawn from SGD (Stanford Genome Database), the site address follows: http://genome-www4.stanford.edu/cgi-bin/SGD/PGMAP/wRatioMap?chrnum=12. The red diagram shows recombination hot spots and cold spots that distributed on the length of chromosome. Position these spots show on the bright blue bar as it is representative of chromosome XII. ⊘ : Distance is not real; around half of chromosome XII has been occupied by the rDNA array. There is no evidence for recombination hot spots in this region. ▬ : Location of the rDNA array on chromosome XII, coordinates 451418 to 468929.

case the cellular rDNA can excise as circles by intra-chromosomal homologous recombination, and so they are maintained in episomal form using autonomously replicating sequence (ARS, Kim and Wang, 1989). These recombination events are quite common in the rDNA array (Petes, 1980; Szostak and Wu, 1980).

Heterochromatin regions in eukaryotic cells are distributed in different parts of the genome including large parts of DNA that are converted into inaccessible states by process of gene silencing. Recombination and transcription are suppressed at heterochromatic or silent DNA regions (Laurenson and Rine, 1992; Lustig, 1998). In yeast, *S. cerevisiae,* the *rDNA* gene shows an unexpected and unusual form of silencing because some of the repeats are transcribed in high level of transcription (Warner, 1989). The regulatory mechanism that controls the ratio of active to the inactive rDNA repeats is poorly understood, but silencing is one of the factors that influences transcriptional activity at the rDNA array (Smith and Boeke, 1997). One known consequence of silencing is that role of SIR2 (a silencing protein) in repressing mitotic and meiotic intrachromosomal recombination within the rDNA array (Gottlieb and Esposito, 1989). The

nucleolus may have an important role in the yeast ageing process. For instance, it is demonstrated that redistribution of silencing proteins from telomeres to the nucleolus is linked with expansion of life span in *S. cerevisiae* (Kennedy *et al.,* 1997). It is also observed that the nucleolus is enlarged and fragmented in old wild type cells (Sinclair *et al.,* 1997). Sinclair and Guarente have demonstrated the redistribution of Sir proteins to the nucleolus delays senescence. Therefore, it has been supposed that the Sir complex delays senescence, by forestalling nucleolar fragmentation. It has also been claimed that extrachromosomal rDNA circles (ERCs) cut out from the rDNA array and replicate via the autonomously replicating sequence (ARS), and this is a possible cause of ageing and nucleolar fragmentation (Sinclair and Guarente, 1997).

This research aimed to discover whether the observed lack of meiotic recombination between the homologous rDNA repeats in budding yeast either is just in the young cells or that there are some DSBs in the old cells. We have shown that there are breaks within the rDNA array of old cells, which they could be due of changing in position of silencing proteins at these cells.

MATERIALS AND METHODS

Strains: In this study two *rad*50S mutant strains were used (old and young), which are able to process meiotic DSBs but they are deficient in processing of the ends of meiosis-specific double-strand breaks into single-strand DNA tails. Therefore, this mutant strain blocks meiotic recombination with accumulation of DSBs, which are readily detectable using Contour-clamped Homogenous Electric Field (CHEF) and Southern blot technology (Sun *et al.,* 1991). The genotype of *rad*50S strain is: *MAT a/α, ho::LYS2-lys2, ura3, rad*50S-*K181.URA*3.

Plasmid: A fragment of RDN I was integrated at the unique *Sma*I site of pUC19 plasmid that includes part of a repeated unit of the rDNA type I (RDN1 locus), coordinates 3576 to 5959. The length of the RDN I fragment is 2383 bp; it was used as a template for making the rDNA probe.

CHEF and Southern blot techniques: The yeast diploid cells were grown in YEPD at 30°C shakers rotating at a rate of 200 rpm to an approximate density of $2\text{-}5 \times 10^7$ cells per ml, spun down (5000 rpm, 4ºC) and washed in sterile distilled water. The cells were again harvested and resuspended at the same cell density in sporulation medium (1% w/v Potassium acetate). The cells were incubated at 20°C in orbital shakers rotating at a rate of 200 rpm. The cells were extracted from sporulation media after 18, and 24 h, respectively. Solid agar plates were placed in a constant temperature incubator at 30°C and then stored at 4°C. Samples of yeast chromosomes were prepared by the embedded agarose procedure of Schwartz and Cantor (1984), as modified by Gardner *et al.* (1993). Digestion of high molecular weight DNA was based on the method used by Anand (1986) with some alteration. The yeast chromosomes were cut with *Xho*I, and *Sca*I restriction enzymes that fail to cut in the rDNA array (Pasero and Marillery, 1993; Rustchenko and Sherman, 1994). Pulsed-field gel electrophoresis (PFGE) using the CHEF technique was employed in order to achieve high-resolution separation of yeast chromosomes (Chu *et al.*, 1986). Yeast chromosomes were transferred to nitrocellulose and nylon membranes according to described method of Southern (Southern, 1975). The rDNA fragments were investigated by an rDNA probe, which made from pUC19-RDN I using PCR and *random primer kit*.

RESULTS
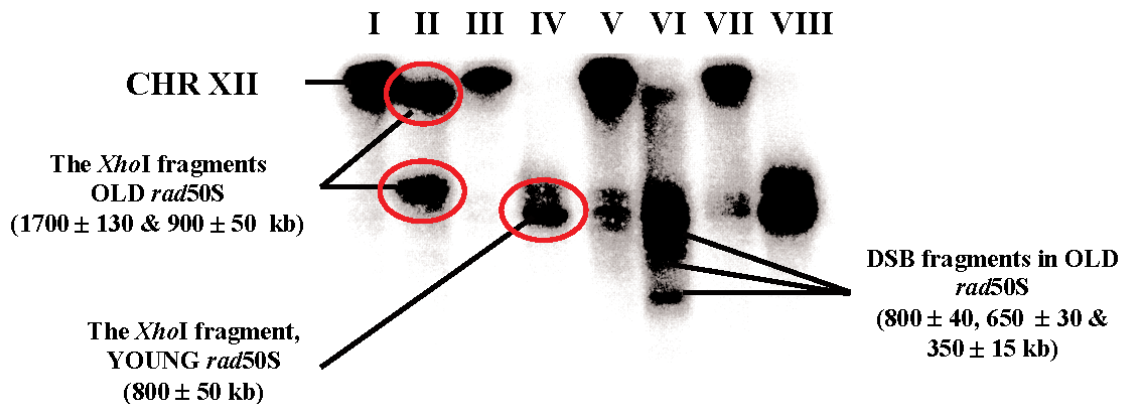
In this study two different types of rad50S were used.

Figure 2. Southern blot from *Xho*I CHEF agarose gel probed using (OLD and YOUNG *rad*50S strains). **I.** OLD *rad*50S in mitotic conditions, without digestion. **II.** OLD *rad*50S in mitotic conditions, digested with *Xho*I. **III.** YOUNG *rad*50S in mitotic conditions, without digestion. **IV.** YOUNG *rad*50S in mitotic conditions, digested with *Xho*I. **V.** OLD *rad*50S in meiotic conditions (24 h), without digestion. **VI.** OLD *rad*50S in meiotic conditions (24 hours), digested with *Xho*I. **VII.** YOUNG *rad*50S in meiotic conditions (24 h), without digestion. **VIII.** YOUNG *rad*50S in meiotic conditions (24 h), digested with *Xho*I. OLD *rad*50S strain has got two different chromosome XII homologues in size, but the length of chromosome XII homologous in YOUNG *rad*50S is same. The CHEF conditions were as follows: 200 V, 120 SWT, 14°C, 1% agarose gel in 0.5 x TBE, electrophoresis was carried out for 21 h in 0.5 x TBE.
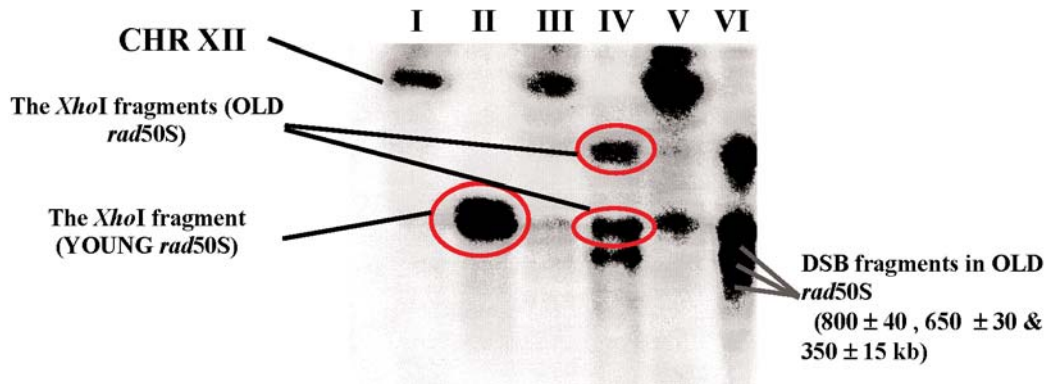
Figure 3. Southern blot from *Sca*I CHEF agarose gel probed with RDN I. **I.** YOUNG *rad*50S strain in mitotic conditions, without digestion. **II.** YOUNG *rad*50S strain in mitotic conditions, digested with *Sca*I. **III.** OLD *rad*50S strain (*rad*50S), 18 h in meiotic conditions, without digestion. **IV.** OLD *rad*50S strain, 18 h in meiotic conditions, digested with *Sca*I. **V.** OLD *rad*50S strain, 24 h in meiotic conditions, without digestion. **VI.** OLD *rad*50S strain, 24 h in meiotic conditions, digested with *Sca*I. The CHEF conditions were as follows: 200 V, 120 SWT, 14°C, 1% agarose gel in 0.5 x TBE, electrophoresis was carried out for 21 h in 0.5 x TBE buffer.

The first one was made from its parents and used immediately (young strain), but the second one was used from stock which it had passed cell cycle several times (old strain). *rad*50S mutant strains were applied in two meiotic and mitotic conditions. The *rad*50S mutation does not prevent mitosis; therefore mitotic conditions were used as control for comparing with the alternative conditions (meiotic conditions). The fine analysis of the rDNA array was performed using restriction endonuclease enzymes, which do not cleave within the rDNA array. CHEF and Southern blot experiments using an rDNA probe against OLD *rad*50S and YOUNG *rad*50S chromosomes under meiotic and mitotic conditions showed three extra fragments (800 ± 40 kb, 650 ± 30 kb, and 350 ± 15 kb) only in meiotic conditions and OLD rad50S strain (Fig. 2). This was reproducible in repeat experiments. Since OLD *rad*50S strain cannot repair the DSBs that are produced under meiotic conditions, the three extra bands observed in meiotic conditions could be produced by the DSB process. The most important point is that three extra bands were just observed in OLD *rad*50S strain. The next point is different size of the rDNA region in two old and young strains and homolog chromosomes. The meiotic recombination events are started with a double strand break, which is produced by SPO11. There is a possibility that the bands in the Southern blot experiments of meiotic cells are artifacts rather than developmental DSBs. This possibility was examined by separate experiment. The timing of additional meiotic bands produced in the rDNA array was determined using a meiotic time-course experiment (the data is not shown). It is also plausible that additional fragments arise due to differences in the positions of the enzyme recognition sites between the two strains. In order to check this possibility, previous experiment was repeated using *Sca*I digestion, which cannot cut inside the rDNA genes. Figure 3 shows resulting of this experiment in a blot. As expected, *Sca*I digestion produced extra meiotic fragments only in OLD *rad*50S strain and under meiotic conditions.

DISCUSSION

It is known that meiotic recombination in the rDNA array is strongly suppressed by the action of silencing proteins (Gottlieb and Esposito, 1989). The Sir2 protein, encoding an NAD-dependent histone deacetylase (Imai *et al.,* 2000), has an important role to establish a silenced state in the rDNA array (Smith and Boeke, 1997). Moreover, a few other types of histone modifications are demonstrated to control silencing of chromatin in the rDNA array. There is evidence suggests that specific combinations of these modifications determine particular silencing patterns (Berger, 2001), and it has also been demonstrated that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in *S. cerevisiae* by a Sir2-independent

mechanism (Bryk *et al.,* 2002). Some studies have also implicated a role for the nucleolus in yeast ageing (Sinclair and Guarente, 1997; Sinclair *et al.,* 1997). They demonstrated that the structure of nucleoluskeleton and modification of chromatin proteins in the nucleoluskeleton, affect the ageing process. The main part of Sinclair and Guarente ageing theory is the intrachromosomal recombination and producing extrachromosomal rDNA circles (ERCs) from the yeast rDNA array. It is suggested that the structure of nucleoluskeleton could be changed during ageing process and then affect rDNA chromatin in some way to increase double strand break within the rDNA array. Therefore, it is plausible in the old cells some breaks go to the intrachromosomal recombination and producing extrachromosomal rDNA circles (ERCs) that Sinclair and Guarente claimed could cause ageing. According to their theory the generation of an ERC is the initiating event of the ageing process.

In this study we used two different types of *rad*50S mutant strains according to their age. As it is shown in figures 2 and 3, this study demonstrates that the rDNA fragments released by restriction enzymes are different in size for the OLD and YOUNG *rad*50S. Digestion of the YOUNG *rad*50S strain with *Xho*I and *Sca*I released one rDNA fragment, 950 ± 20 kb, in mitotic conditions. But using *Xho*I and *Sca*I on OLD *rad*50S mutants released two rDNA fragments, 1700 ± 130 kb and 900 ± 30 kb, in mitotic conditions. The most straightforward explanation is the different number of repeated rDNA units in the two chromosome XII homologues of OLD *rad*50S. The different size of chromosome XII homologues could be explained by unequal sister chromatid exchange in mitosis. But in meiotic conditions, old *rad*50S showed three extra fragments (800 ± 40 kb, 650 ± 30 kb, and 350 ± 15 kb). They were only observed in OLD *rad*50S and under meiotic conditions and this was reproducible in repeated experiments. Since *rad*50S cannot repair the DSBs that are produced under meiotic conditions, the three extra bands observed in meiotic conditions could be produced by the DSB process. But there is no meiotic extra band observed for YOUNG *rad*50S. Therefore, according to above information, it is suggested that the nucleoluskeleton could be changed during ageing process in some way to produce double strand break (DSB) sites in the length of rDNA array. These sites could be involved in unequal sister chromatid recombination or producing extrachromosomal rDNA circles which promoted ageing process (Sinclair and Guarente, 1997). The chromosome XII homologous of OLD rad50S has been changed in size via mitotic unequal sister chromatid recombination, because *rad*50S mutant strains could not pass meiosis. The most important finding is, meiotic DSBs are suppressed in young cells but they could occur in old cells. Therefore, they could be involved in some way to produce recombinant productions such as 3 μm extrachromosomal rDNA circles for beginning of ageing process. Since there are not corresponding hot regions for non-sister chromatid recombination, it is concluded that these DSBs must be repaired in old cells without resulting in new DNA molecules (non-cross over outcome). A study by Scherthan and colleague (1992) on meiotic chromosome condensation and pairing demonstrated that in diploid cells, the NORs of chromosome XII are held together by their common nucleolus as in mitosis unlike the pairing exhibited in meiotic cells. Therefore, its behaviour in meiosis with respect to homologue search and pairing may not be representative. It is then plausible that DSBs could happen in the rDNA array. However because of the absence of meiotic pairing of chromosome homologues (the rDNA array part), and because HR is the main source of DSB repair, single strand DNA could not find a non-sister chromatid for invasion, and DSB is repaired without involving any cross over outcome using sister chromatid strand. Therefore, the most straightforward outcome from above results and studies is that there are some DSB hot regions in the rDNA array (in old cells). The rDNA genes have been known as a unique conservative region in genomes, so they have been used in different molecular methods for revealing the evolutionary pathways and species determination. The most important aplication of these results is that the DSB hot regions could be used for integration of desired DNA, in biotechnology and genetic engineering technology, for designing of manipulated yeast.

Acknowledgment

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