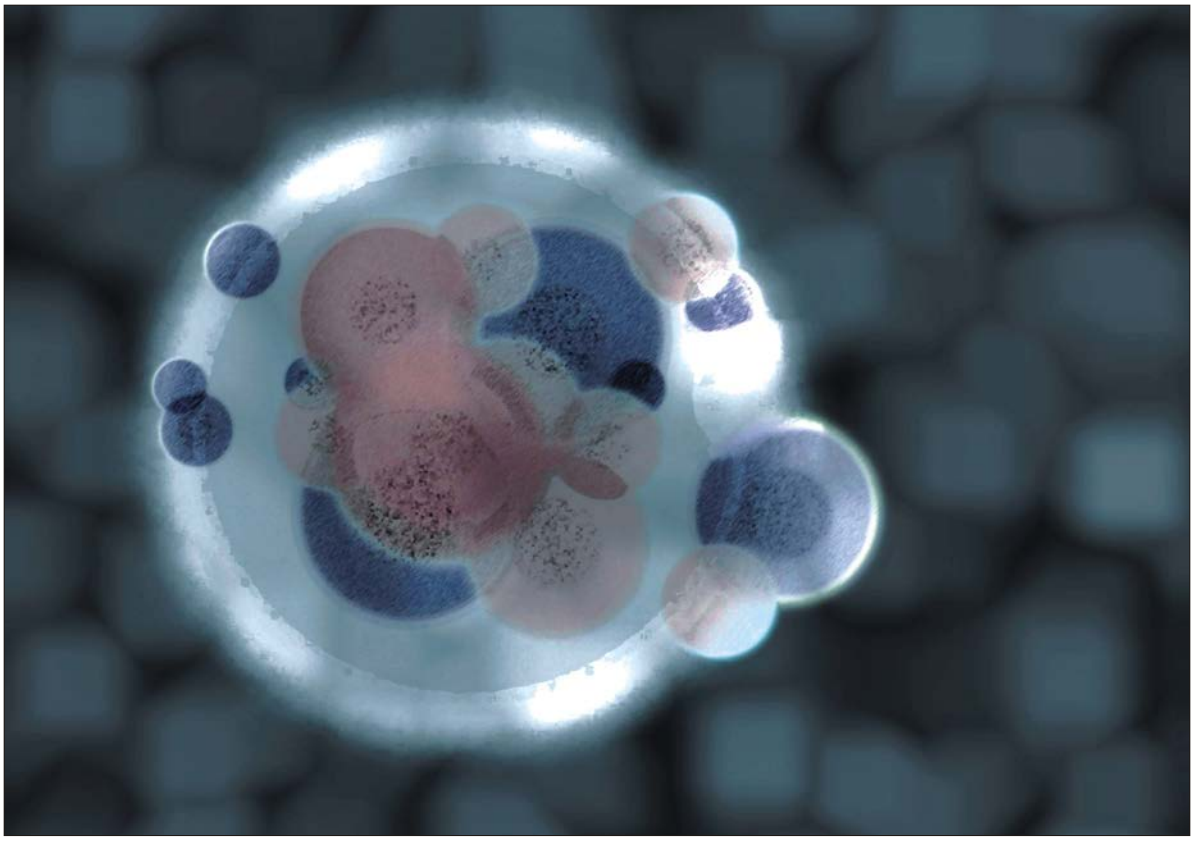

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Homologous Recombination Repair Within the rDNA Array in *Saccharomyces cerevisiae*

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Abstract: Homologous recombination repair starts with Double-strand Breaks (DSBs) followed by crossing-over and recombination. The expected frequency of meiotic chromosomal exchange in the region of chromosome XII encoding ribosomal DNA in *Saccharomyces cerevisiae* is 3.5 to 5 events per cell per meiosis. However interchromosomal meiotic recombination in the *rDNA* gene is very rare, suggesting repression of DSB and crossing-over. On the other hand, mitotic events such as intrachromosomal recombination producing 3 μm rDNA circles (which accumulate with cellular age) and unequal sister chromatid exchanges appear to be quite common. This study looked at the rDNA breakage in the strain ORD 1181, a *rad*50S mutant with SK1 background, which does a relatively fast and near synchronous meiosis. The fine analysis of the rDNA array was performed using restriction endonuclease enzymes that do not cleave within the rDNA array. The results suggest that there are at least two hot regions for chromosome breakage within the rDNA array. According to our previous studies we suggest that the DSB hot regions are in one homologue. However, there is possibility that other homologue is involving in DSB too.

Key words: Double-strand break, *rDNA* genes, meiosis, homologous recombination repair, *rad*50S, (rDNA) homologue pairing, *Saccharomyces cerevisiae*

INTRODUCTION

DNA double-strand breaks (DSBs) affect both strands of a chromosome and these DNA lesions are particularly damaging. DSBs are the major genotoxic lesions (particularly after exposure to ionizing radiation; Raveh *et al*., 1989; Krishna *et al*., 2007). Such damages also result from the natural metabolism of the cells, such as the activity of reactive oxygen species or errors during DNA replication and transcription (Friedberg *et al*., 1995). Moreover, many different DNA damaging agents cause a dose-dependent add to in the frequency of recombination. These include ionizing and UV-irradiation and chemical agents such as Methyl methanesulfonate (MMS), Methyl-nitro-nitrosoguanidine (MNNG), Ethyl methanesulfonate (EMS), Aflatoxin B, etc. (Snustad and Simmons, 2000). In *Saccharomyces cerevisiae*, such damage is repaired primarily by homologous recombination using the DNA sequence residing on the sister chromatid or homologous partner to restore the DSB in a way that is essentially error free (Resnick, 1976; Szostak *et al*., 1983; Clever *et al*., 1997; Honma *et al*., 2007). Mutation in the *RAD*52 group genes result in failure of DNA damage repair (particularly double strand break lesions) including extreme sensitivity to ionizing radiation or other DSB-causing agents and alkylating agents like MMS as well as sensitivity to UV irradiation (Friedberg *et al*., 1995; Game, 1993; Shinohara and Ogawn, 1995; Feng *et al*., 2007).

In most eukaryotic organisms, tandem arrays of the genes for ribosomal DNA (rDNA), termed nucleolar organizer regions (NORs), are found at one or a few chromosomal loci within the genome (Hadjiolov, 1985). In the yeast *Saccharomyces cerevisiae*, the NOR is located on the long arm of chromosome XII (Petes, 1979a), contains 100-200 copies of rDNA (approximately 140) and is termed the *RDN* locus (Nierras *et al*., 1997; Motovali-Bashi *et al*., 2004). There are approximately 70 to 100 meiotic exchanges per genome in meiosis (Byers and Goetsch, 1975) and since ribosomal DNA represents about 5% of the total genome (Schweizer *et al*., 1969), the expected frequency of exchange in this region is 3.5 to 5 exchanges per cell per meiosis. But interchromosomal meiotic recombination in the rDNA is very rare frequent than this suggesting repression of DSB and crossing-over (Petes and Botstein, 1977; Petes, 1979b; Motovali-Bashi *et al*., 2004). Despite the low rate of meiotic recombination in the rDNA cluster, mitotic recombination

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events such as intrachromosomal recombination producing 3 μm rDNA circles (which accumulate with cellular age, Sinclair and Guarente, 1997) and unequal sister chromatid exchange appear to be quite common (Szostak and Wu, 1980; Jackson and Fink, 1981; Kim and Wang, 1989). Due to the tandemly repeated, organization of the rDNA array it can potentially gain or lose repeat units by intrachromatid and unequal sister chromatid recombination (in both meiotic and mitotic cells). Therefore, a tight control mechanism must regulate the balance of gain and loss of rDNA units. It is thought that the homologous recombination or non-homologous recombination systems control this balance (Szostak and Wu, 1980; Jackson and Fink, 1981; Ahnesorg and Jackson, 2007). There could be two explanations for these different observations in meiosis and mitosis: first, it is suggested that recombination within the *rDNA* gene clusters might occur after meiotic DNA synthesis but before pairing of all four chromatids (Petes, 1980). Second, it is plausible that the rDNA regions in chromosome XII homologues do not behave such as meiotic chromosomes in the meiotic cells (parts of chromosome XII homologues including the rDNA array do not pair each other during meiosis).

In meiotic recombination, the initiating event is a double-strand break (Cao *et al*., 1990; Sun *et al*., 1991; Diaz *et al*., 2002), which it is created by Spo11 protein (Keeney *et al*., 1997; Wu and Burgess, 2006). However, the mechanism of meiotic recombination in the rDNA array is still unclear because it is difficult to detect recombinant products at the molecular level. Yeast strains have got two types of the *rDNA* gene, type I rDNA in which *rDNA* genes have seven *Eco*RI fragments per repeat (Philipsen *et al*., 1978; Petes *et al*., 1978a). The seven *Eco*RI fragments derived from the rDNA unit are called A through G. The A fragment is the largest and G fragment is the smallest. However, a different type of the *rDNA* gene (type II) exists. The size and restriction endonuclease map of the type II *rDNA* gene is very similar to the type I *rDNA* gene. The only difference is a mutation of the *Eco*RI site between *Eco*RI fragments B and E. Thus, in the type II *rDNA* genes there are only six *Eco*RI fragments: X' (consisting of B and E sequences), A, C, D, F and G. The discovery of these two different forms of the *rDNA* gene is crucial in the investigation of recombination and crossing over between homologous *RDN* loci (Petes *et al*., 1978).

The comparison of physical and genetic recombination events on chromosome XII shows a huge silent region at the rDNA array for recombination events (Stanford Genome Database). In general there is a close correlation between the distribution of DSBs and crossing-over (Mizuno *et al*., 1997). However there is a region of chromosome III, where crossing-over frequency is normal, but DSBs cannot be detected (Baudat and Nicolas, 1997; Cherry *et al*., 2007).

It is known that meiotic recombination in the rDNA array is suppressed by the action of silencing proteins (Gottlieb and Esposito, 1989; Cubizolles *et al*., 2006). The Sir2 protein, encoding an NAD-dependent histone deacetylase, has an important role to establish a silenced state in the rDNA array (Smith and Boeke, 1997; Imai *et al*., 2000; Cubizolles *et al*., 2006). Moreover, a few other types of histone modifications are demonstrated to control silencing of chromatin in the rDNA array. Some evidence suggests that specific combinations of these modifications determine particular silencing patterns (Berger, 2001; Shankaranarayana *et al*., 2003) 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).

It has also been claimed that the frequency of recombination and DSB is extremely increased at old cells of *rad*50S (Motovali-Bashi *et al*., 2003). This research was undertaken to discover whether the observed lack of meiotic recombination between the homologous rDNA repeats in budding yeast, is due to a lack of initiating events, or that there are some DSBs, but they are repaired in some other way without resulting in recombinant molecules. We looked at the rDNA breakage in the strain ORD 1181, a *rad*50S mutant. The mutant strain is able to process initiation of meiotic DSBs (endonucleae activity-spo11) but it is deficient in processing of the ends of meiosis-specific double-strand breaks into single-strand DNA tails to invade sister/non-sister chromatid strains (DSB homologous recombination repair). 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; Game, 1992). Therefore, *rad*50S mutants are able to complete mitosis successfully. But they can not pass meiosis.

MATERIALS AND METHODS

In this study *rad*50S mutant (ORD 1181) strain with SK1 background was used (SK1 strains do a relatively fast and near synchronous meiosis). The *rad*50S strain was created in our previous study using its parents SK1000; SK1001 and a micromanipulator microscope on a YEPD agar plate. In our previous study two different types of *rad*50S yeast strain were used. Those strains

were different in age but not in genotype. To create the Old strain, the *rad*50S mutant was grown for at least 10 cell cycles and then kept at 4°C for a few months. For young strain, the *rad*50S mutant was used fresh or after making it was kept at -80°C until use (Motovali-Bashi *et al*., 2004). In this study the Old strain was used. The genotype of *rad*50S strain is: *MAT a/ _*, *ho*::*LYS*2-*lys*2, *ura*3, *rad*50S-*K181.URA*3. Parents genotype of *rad*50S, SK1000 is: *MAT_*, *ho*::*LYS*2, *lys*2, *ura*3, *rad*50S*K*181.*URA*3 and SK1001 is: *MATa*, *ho*::*LYS*2, *lys*2, *ura*3, *rad*50S*K*181.*URA*3. Therefore, the *rad*50S mutant is homozygous for the *rad*50S mutation. A fragment of RDNI 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 RDNI fragment is 2383 bp, it was used as a template for making the rDNA probe using Random Primer and High Dig Prime protocol. The accuracy of plasmid was checked by transformation of competent *E. coli* DH5a with pUC19-RDNI. The amplification and purification of plasmid was performed based on Inoue method with slightly modification (Inoue *et al*., 1990). Plasmid DNA (Fig. 1) was digested using *Eco*RI and multidigestion with *Sca*I, *Sal*I and *Bam*HI. Digested fragments were separated using gel electrophoresis. The expected size and mobility of digested fragments were estimated using marker. The yeast diploid cells were grown in rich-medium of YEPD (1% w/v Bacto-Yeast extract, 2% w/v Bacto-Peptone and 2% w/v Glucose) at 30°C shakers rotating at a rate of 200 rpm (Incubator shaker model G 25, edison, New Jersey, USA). The yeast cells (about 3-5×10^7 cells per mL) were spun down (5000 rpm, 4°C; Eppendorf centrifuge model 5403) and washed with 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 7, 9, 12, 15, 18, 21 and 24 h, respectively. Solid agar plates (rich-medium including 2% w/v Bacto-agar) were placed in a constant temperature incubator at 30°C (Heraeus B 5050 E1). They were stored at 4°C for up to 6 months. Long-term stocks of yeast cells were kept in 1 mL YEPD containing 15% v/v glycerol and stored in screw-capped vials at -80°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 performed based on the method used by Anand (1986) with some modification.

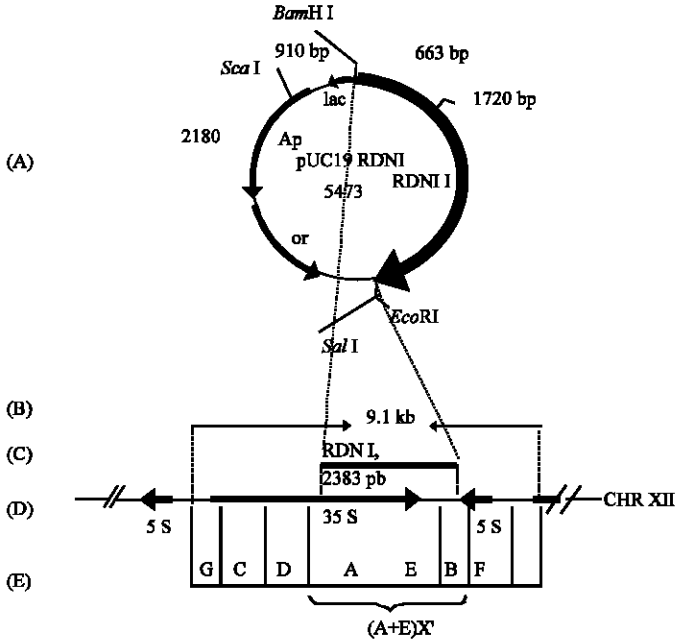

Fig. 1: (A) Structure of pUC19-RDNI plasmid including some endonuclease recognition sites, (B) Represents the possible order of the 9.1 kb repeat unit, (C) The RDNI is the fragment that used for making an rDNA probe, (D) Organization of the yeast DNA segments that code for the two RNAs present in the yeast ribosome, on chromosome XII and (E) The fragments digested with *Eco*R I on the rDNA array (X', A, B, C, D, E, F, G). Fragments A and E have base identify with RDNI fragment (probe)

The yeast chromosomes were cut with *Not*I, *Xho*I, *Sal*I, *Sca*I, *Pst*I, *Sfi*I and *Bam*HI restriction enzymes that fail to cut inside the rDNA array (Pasero and Marilley, 1993; Rustchenko and Sherman, 1994). An advance pulsed-field gel electrophoresis, the CHEF-DR II system, based on the CHEF technique was employed in order to achieve high-resolution separation of yeast chromosomes (Chu *et al*., 1986; Carle and Olson, 1984). Yeast chromosomes were then transferred to nitrocellulose and nylon membranes according to Southern (1975) method. The rDNA fragments were investigated by an rDNA probe, which made from pUC19-RDNI using *Random primer*. To determine the type of ribosomal DNA in strains, *Eco*RI digestion was performed according to Cramer (1976) method. Yeast genomic DNA was extracted by the method of Rose and Broach (1990). *Eco*RI digestion was carried out in a total volume of 50 µL, which contained 0.75-1.5 µg of genomic DNA and 50 units of enzyme in the appropriate buffer. The reaction mixture was incubated at 37°C for 60 min. An additional 30 units of enzyme was added halfway through the digestion period.

*THR*4 gene was amplified by designing of two oligonucleoides to act as primers for a PCR machine, these primers are as follows:

5' ATTTGAACCCTTCGCAATCC 3' (20-mer) corresponds to a sequence at the downstream of and directed towards the *THR*4 gene.

5' CTTCTTCTTGAACGATTTTAC 3' (21-mer) corresponds to a sequence at the upstream of and directed towards the *THR*4 gene.

This research was mainly done at Biomolecular Sciences Department in UMIST (UK) and carry on and completed in Iran.

RESULTS

The number of base similarity between the *rDNA* genes and probe was investigated using *Eco*RI digestion of the rDNA region and determination of overlapping between digested fragments and probe (according to restriction map in Fig. 1). Therefore, yeast genome was digested with *Eco*RI and digested fragments were separated using agarose gel electrophoresis. The agarose gel was used in Southern blot technique using RDNI probe (Fig. 2). The number of bases overlapping between the rDNA probe and the rDNA fragments digested with *Eco*RI (the number of base similarity between the *rDNA* genes and probe fragment) is shown in Table 1. As shown in Table 1, Fig. 1 and 2 (restriction genetic map of RDNI fragment), E fragment is completely matched with 1/4 of the RDNI fragment. A and X' fragments have also got 59 and 100% overlapping with the RDNI fragment,

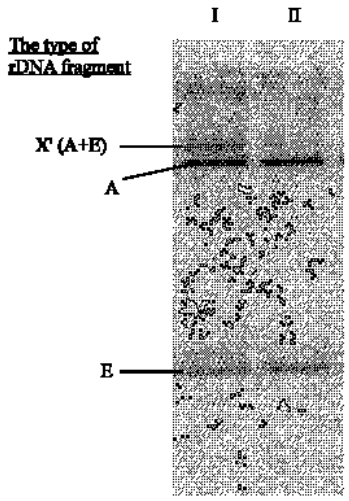

Fig. 2: Southern blot from agarose gel including the rDNA fragments digested with *Eco*RI, parents of *rad*50S, (I) NKY 1000 (α mating type) and (II) NKY 1001 (a mating type)

Table 1: Level of base overlapping (similarity) between the RDNI fragment and the rDNA fragments released by *Eco*R I digestion

The *Eco*R I digested rDNA fragments	Base pairs	No. of base overlapping between the digested fragments and probe	Percentage of overlapping to probe (bp) (%)
X' (A+E)	2820	540	100
A	2670	1594	59
B	2230	0	0
C	1840	0	0
D	580	0	0
E	540	540	100
F	330	0	0
G	250	0	0

respectively. The length of base overlapping in E and X' fragments with RDNI fragment is the same. Therefore the same probe intensities occurred in bands representing E and X'. A and E fragments were observed in both parents of *rad*50S strain. The X' fragment was observed in just one of the parents of *rad*50S strain. Therefore, it suggests that one homologue (in *rad*50S strain) includes a combination of the type I and II (X', A, B, C, D, E, F, G) *rDNA* genes and another includes the rDNA type I (A, B, C, D, E, F, G). In conclusion, this strain is heterozygous for the *rDNA* gene. According to the results of base overlapping between the rDNA probe and the *rDNA* genes type I-II, the specificity of the rDNA probe was confirmed.

The *rad*50S mutant was applied in two conditions, meiotic (sporulation media) and mitotic (rich-medium of YEPD). Mitotic conditions were used as the control for comparing with the alternative conditions (meiotic). A

single colony of *rad*50S mutant was inoculated into rich-medium of YEPD and grown up to 3-5×10^7 cell mL^{-1}. The cells were transferred to meiotic conditions at the same cell density. The slabs including yeast chromosomes were prepared, after incubation under meiotic conditions for 24 h. *Xho*I and *Sca*I digestions, which cannot cut inside the rDNA repeats, were used for better resolution in the CHEF. Yeast digested chromosomes were separated using pulse-field gel electrophoresis via the CHEF technique. pUC19-RDNI was then applied as a template for making the rDNA probe. The CHEF agarose gel was used in a Southern blot and probed with the rDNA probe. The experiments under meiotic conditions showed four extra fragments (1450±80, 800±40, 650±30 and 350±15 kb). They were only observed under meiotic conditions and this was reproducible in repeated experiments (Fig. 3 and 4). Since *rad*50S cannot repair the DSBs that are produced under meiotic conditions, the four extra bands observed in meiotic conditions could be produced by the DSB process. Moreover, two other bands were also observed in both meiotic and mitotic conditions, the intensity of labelling with an rDNA probe in the two fragments appeared similar. Our previous studies on this matter showed that these two bands are representative of different sizes of the homologues of chromosome XII in *rad*50S mutants. In those studies we used two different types of *rad*50S yeast strain, the Old and Young strain. Both strains were created using SK1000 and SK1001 as their parents, which they have same size for chromosome XII. The young diploid strain has same size for homologous chromosome XII. But the old diploid strain has different size for

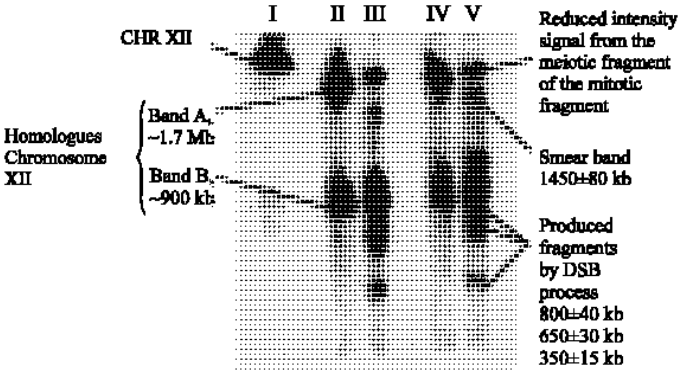

Fig. 3: Southern blot from *rad*50S mutants (in meiotic and mitotic conditions) on CHEF agarose gel, (I) Mitotic conditions, without digestion, (II) Mitotic conditions, digested with *Xho*I, (III) 24 h in meiotic conditions, digested with *Xho*I, (IV) Mitotic conditions, digested with *Sca* I and (V) 24 h in meiotic conditions, digested with *Sca*I

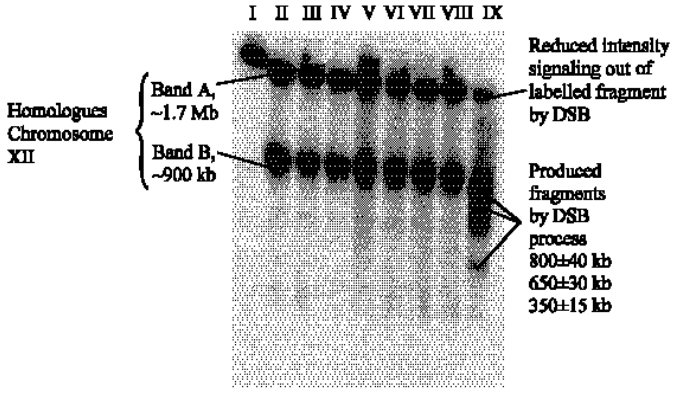

Fig. 4: Southern blot from *rad*50S mutants on the timing meiotic CHEF agarose gel, (I) Mitotic conditions, without digestion, (II) Mitotic conditions, with *Xho* I, (III) 7 h in meiotic conditions, digested with *Xho* I, (IV) 9 h in meiotic conditions, digested with *Xho* I, (V) 12 h in meiotic conditions, digested with *Xho* I, (VI) 15 h in meiotic conditions, digested with *Xho* I, (VII) 18 h in meiotic conditions, digested with *Xho* I, (VIII) 21 h in meiotic conditions, digested with *Xho* I and (IX) 24 h in meiotic conditions, digested with *Xho* I

homologous chromosome XII. In short, our previous studies showed that different size of homologous chromosome XII in old strain because of mitotic unequal sister chromatid exchange at one chromosome XII. In fact the size of one chromosome did not change but other one getting bigger almost twice (Motovali-Bashi *et al*., 2003 and 2004). When the two bands representing intact chromosome XII homologues (band A and B, Fig. 3) are compared between mitotic and meiotic conditions (Fig. 3), it is clearly observed that the intensity of band A (the bigger size one, 1.7 Mb) is reduced in meiotic conditions in comparison with mitotic conditions. However the intensity of the other band (band B, the smaller one, 900±50 kb) appears similar in meiotic and mitotic conditions. According to the intensity of the signal from the labelled probe bands, it seemed that DSB was processed on the one homologue (the bigger one, 1.7 Mb). However, it is still plausible that the small homologue chromosome (900±50 kb) is involving in some DSBs too.

There is a possibility that the bands in the Southern blot experiments of meiotic cells are artifacts rather than developmental DSBs. This matter was checked by time-course experiment. To determine the timing of DSB, a meiotic time-course experiment was performed. In this experiment, yeast cells were grown up in the mitotic and meiotic conditions. The slabs including yeast chromosomes were prepared after incubation under meiotic conditions for 7, 9, 12, 15, 18, 21 and 24 h. According to previous experiments, for better resolution yeast chromosomes in the slabs were digested with *Xho*I. CHEF and Southern blot experiments using the pUC19-RDNI probe against *rad*50S digested chromosomes were performed. Figure 4 shows the results of probing. It shows that additional fragments appear to occur after 24 h in the rDNA repeated cluster. In this experiment, the DSB fragments were observed with the same sized fragments that were detected previously. The time of the beginning DSB was checked using a known DSB hot spot locus in one of the yeast chromosomes. In accordance with a study of chromosome III (Baudat and Nicolas, 1997) and results of studies regarding to the *THR*4 gene (Goldway *et al*., 1992), this gene was chosen for checking the time of the beginning DSB. The *THR*4 gene was amplified using the PCR colony method and the *rad*50S mutants as template. The size of the PCR product of *THR*4 was estimated by electrophoresis (The expected size of the amplified gene is 1533 bp). *THR*4 (*YCR053W*) is on chromosome III, coordinates 215427 to 216971. The length of the *THR*4 gene is 1544 bp, but a fragment with length of 1533 bp from this gene was amplified. This fragment was extracted from the agarose gel using a gel extraction kit. The amplified fragment of the *THR*4 gene was used to make a probe for using in a Southern blot resulting in the determination of the timing of DSB. Meiotic yeast chromosomes were again prepared from *rad*50S mutant at different time of incubation (7, 9, 12, 15, 18, 21 and 24 h). The size of chromosome III is much smaller than chromosome XII at 870 kb and then CHEF technique is enough powerful to separate it and its broken fragments from other chromosomes. The CHEF agarose gel was run as follows: 170 V, 100 Switching time (pulse), 14°C for 21 h in 0.5 x TBE and used in a Southern blot technique. The extracted fragments (*THR*4 gene) from the agarose gel were applied as a template for making a probe using the random primer method. The results of the probing are shown in Fig. 5. As shown in Fig. 5 additional fragments, which should represent DSB products, appeared after 21 h incubation in meiotic conditions. However, these fragments are more obvious after 24 h incubation under meiotic conditions. These results confirm results of the previous experiments on the timing of DSB in the *rDNA* genes. It was then demonstrated that the timing of appearance of additional meiotic bands produced in the *rDNA* genes is coincident with the timing of DSB in a recombination hot spot position in chromosome III (*THR*4 gene, Fig. 5). Those fragments were reproducible in repeated experiments; therefore, these studies simply demonstrate that those bands are representative of fragments produced during meiosis.

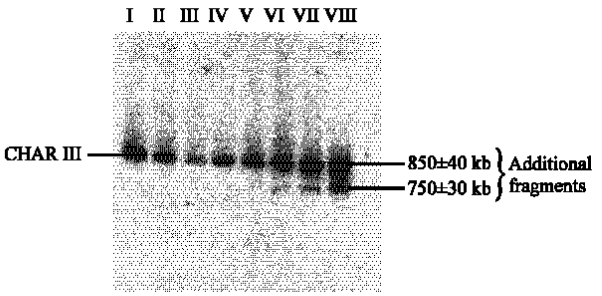

Fig. 5: Southern blot from the timing CHEF agarose gel probed with the THR4 fragment, (I) *rad*50S, mitotic conditions, (II) *rad*50S, 7 h in meiotic conditions, (III) *rad*50S, 9 h in meiotic conditions, (IV) *rad*50S, 12 h in meiotic conditions, (V) *rad*50S, 15 h in meiotic conditions, (VI) *rad*50S, 18 h in meiotic conditions, (VII) *rad*50S, 21 h in meiotic conditions and (VIII) *rad*50S, 24 h in meiotic conditions

DISCUSSION

Comparison of the two bands representing intact chromosome XII homologues at mitotic and meiotic conditions (band A and B, Fig. 3) shows that the big homologue chromosome (~1.7 Mb) is involving in meiotic DSB process. If two DSB hot spots occur at different frequency and independently of each other it is expected to observe five fragments dependent on cutting DNA by one or both of them in different cells or chromosomes. But if one DSB hot spot suppresses other, it would be expected to observe only one break in each cell resulting in four fragments. These fragments are shown in Fig. 6 by red lines. However, if two DSB hot spots occur simultaneously, it is expected to observe three fragments. The results (Fig. 3), suggest that there were two DSB hot regions in which they were occurred simultaneously in the *rDNA* genes of *rad*50S. Figure 6 shows a schematic diagram from chromosome XII including *Xho*I recognition sites and two DSB hot spots. It is suggested that two DSBs occur inside the rDNA array and near to centromere proximal and distal sites of the rDNA array with rest of chromosome. Motovali-Bashi *et al.* (2004) demonstrated that there are two mitotic DSB hot spots/regions near to centromere proximal and distal region of the rDNA array in the same strain. It means that the big fragment produced by DSB, 800±40 kb, could be related to middle of the rDNA array (fragment 2 in Fig. 6) and the other fragments to the ends of the rDNA array (fragments 1 and 3 in Fig. 6). Of course there is a smear band, 1450±80 kb, in meiotic conditions too. Two alternative explanations could be suggested for it. First, it is plausible that one break (DSB 2, Fig. 6) is not performed in some cells resulting in production of 350±15 kb (fragment 1, Fig. 6) and 1450±80 kb (fragment 4, Fig. 6) fragments. Second, the smear band could be produced because of DSBs at flanking sites of *Xoh* I recognition sites in the cells that DSB I and II are not occurred. Because of the place of the smear at top of the CHEF gel, the second explanation is more likely to be right. The size of fragments at smear band is quite varied. This suggests that DSBs were occurred simultaneously and there was a high risk of DSB at those sites in the rDNA array of *rad*50S. We tried designing primers according to flanking sites of rDNA region of *rad*50S to detect place and position of DSB I and II using the published DNA sequences in SGD. But they did not work properly; suggesting it might be because of that the DNA sequences at the junction between the *rDNA* gene and single copy chromosomal sequences in *rad*50S do not correspond to the published sequences in SGD.

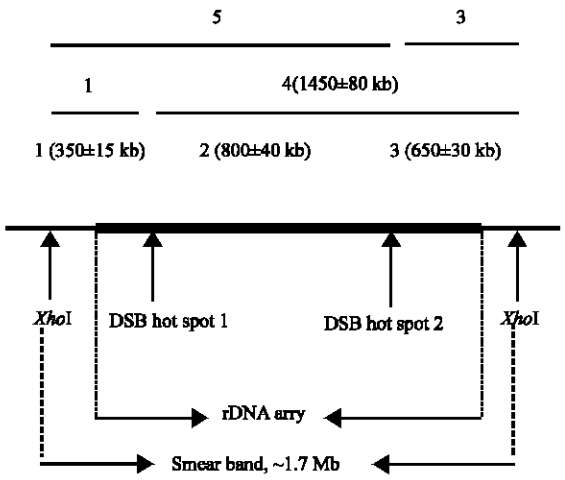

Fig. 6: A cartoon diagram of chromosome XII including *Xho*I recognition sites and two DSB hot spots within the rDNA array. If DSB occurs at both DSB hot spot sites 1 and 2 simultaneously it would be expected to observer fragments 1, 2 and 3 on Southern blot. If DSB occurs at DSB hot spot site 1 it would be expected to observe fragments 1 and 4 on Southern blot. If DSB occurs at DSB hot spot site 2 it would be expected to observe fragments 3 and five on Southern blot. But if DSBs occur independent and at different frequency in both DSB hot spot sites it would be expected to observe all five fragments on Southern blot. However, if two DSBs to be close enough to interference each other it is expected to occur one break in each cell so it would be expected to observe four fragments (1, 4, 5 and 3) on Southern blot

Zamb and Petes (1982) demonstrated that the single copy sequences at the junction of the rDNA array in different *S. cerevisiae* strains could have totally different sequences. It could be happened because of DSB hot regions/spots at the flanking of rDNA region resulting in recombination especially unequal sister chromatid exchange (Motovali-Bashi *et al*., 2004).

Since there are not corresponding hot spots/regions for recombination, it is concluded that these DSBs must be repaired without resulting in new DNA molecules (non-cross over outcome). It was demonstrated that breaks in the rDNA array could be repaired by Single-strand Annealing (SSA) or Non-homologous End Joining (NHEJ) when the homologous recombination (HR) mechanism is blocked; otherwise HR mechanism is main pathway of repair in yeast (Jackson, 2002). Single-strand annealing is known as a specific form of homologous recombination repair (Jeong-Yu and Carroll, 1992). SSA is

dependent on the presence of repeated sequences at flanking regions of the break. The first stage is resection of 5'- ends and then homology search of single-strand DNA. The formation of joint molecules is possible by complementation of homology regions. The second stage is removal of the non-homologous ends and finally rejoining the ends by ligase. In this mechanism part of DNA dependent on location of homology regions is lost, so SSA is a non-conservative and non-cross over mechanism (Sugawara *et al*., 2000). Although meiotic recombination between non-sister chromatids is suppressed within the *rDNA* gene cluster, mitotic and meiotic recombination between sister chromatids (in particular, unequal sister chromatid recombination) occurs frequently (Szostak and Wu, 1980; Motovali-Bashi *et al*., 2004; Petes *et al*., 1981). Due to the tandemly repeated, organization of the rDNA array it can potentially gain or lose repeat units by intrachromatid and unequal sister chromatid recombination (in both meiotic and mitotic cells; Rustchenko and Sherman, 1994). A study by Scherthan and his colleague 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 pairing exhibited in meiotic cells. Therefore, its behaviour at meiosis with respect to homologue search and pairing may not be representative (Scherthan *et al*., 1992). It is then plausible that DSBs could happen in the rDNA array but 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. Therefore, the most straightforward outcome from above results and studies is that the repair of DSBs in the rDNA array uses sister chromatid recombination.

Figure 3 and 4 clearly show that the intensity of only one homologue of chromosome XII (the bigger size one, 1.7 Mb) is reduced in meiotic in comparison with mitotic conditions. Therefore, it is concluded DSBs were certainly processed on the big homologue (band A, Fig. 3 and 4). However there is still plausibility that the second homologous chromosome (small one) was involving in DSB fragments formation. Comparison of the band representing small chromosome XII homologues at mitotic and meiotic conditions (band B, Fig. 3) is not clearly possible. Production of additional meiotic band in the *rDNA* gene is coincident with the timing of DSB in a recombination hot spot position in chromosome III (Fig. 4). We cannot claim that the DSB I and II in the rDNA region were processed by Spo11. However, it is a strong suggestion. In our previous study we demonstrated that one homologue of chromosome XII in old *rad*50S (the strain which has been passed mitosis more than ten times) was changed in size by mitotic unequal sister chromatid recombination. It was shown that the size of homologous chromosomes XII is the same as in original *rad*50S (diploid young cells). But during 10-13 mitotic cell cycles the size of one homologue had been changed from 800 kb to 1.7 Mb (Motovali-Bashi *et al*., 2003). Figure 2 shows that the two homologous chromosomes XII are different in rearrangement of repeated units of the *rDNA* gene. One homologue includes the rDNA type I and the other includes a combination of the rDNA type I and II. We suggest this difference at rearrangement of repeated units in homologous chromosome XII is due to formation of DSB in homologue chromosome in *rad*50S strain.

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