

Analysis of the mechanism of transcription-mediated
hyper-recombination in yeast *Saccharomyces cerevisiae*.

Naomi Serizawa

DOCTOR OF PHILOSOPHY

Department of Molecular Biomechanics

School of Life Science

The Graduate University for Advanced Studies

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I. Introduction

In cellular organisms, DNA sequences are maintained from generation to generation with very little change. However, it is also clear that these DNA sequences can occasionally be rearranged and that such rearrangements give rise to genetic variation. In a population of organisms, these variations are crucial in allowing organisms to evolve in response to environmental change. DNA rearrangements are sometimes caused by homologous recombination. During meiosis in fungi, plants and animals, homologous recombination is essential for accurate chromosome segregation. The crossing-over of chromosomes causes bits of genetic information to be exchanged to create new combinations of DNA sequences in each chromosome.

Mechanism of Homologous Recombination

In mitotic cells, homologous recombination is an efficient pathway for the repair of DNA breaks generated during replication or as a direct consequence of exposure to DNA-damaging agents or radiation in a variety of organisms. When DNA is damaged resulting in the formation of a double strand break (DSB), exonucleases degrade the 5' ends of the break, creating two protruding 3' single-stranded ends (Figure 1A, B). The 3' end finds its homologous region and invades to form a heteroduplex joint (Szostak et al., 1983; Figure 1C). After invasion, DNA is synthesized from the 3' end using the complementary strand as a template, thus, repairing the damage (Figure 1D). Heteroduplex joints that consist of an intermolecular double helix known as the

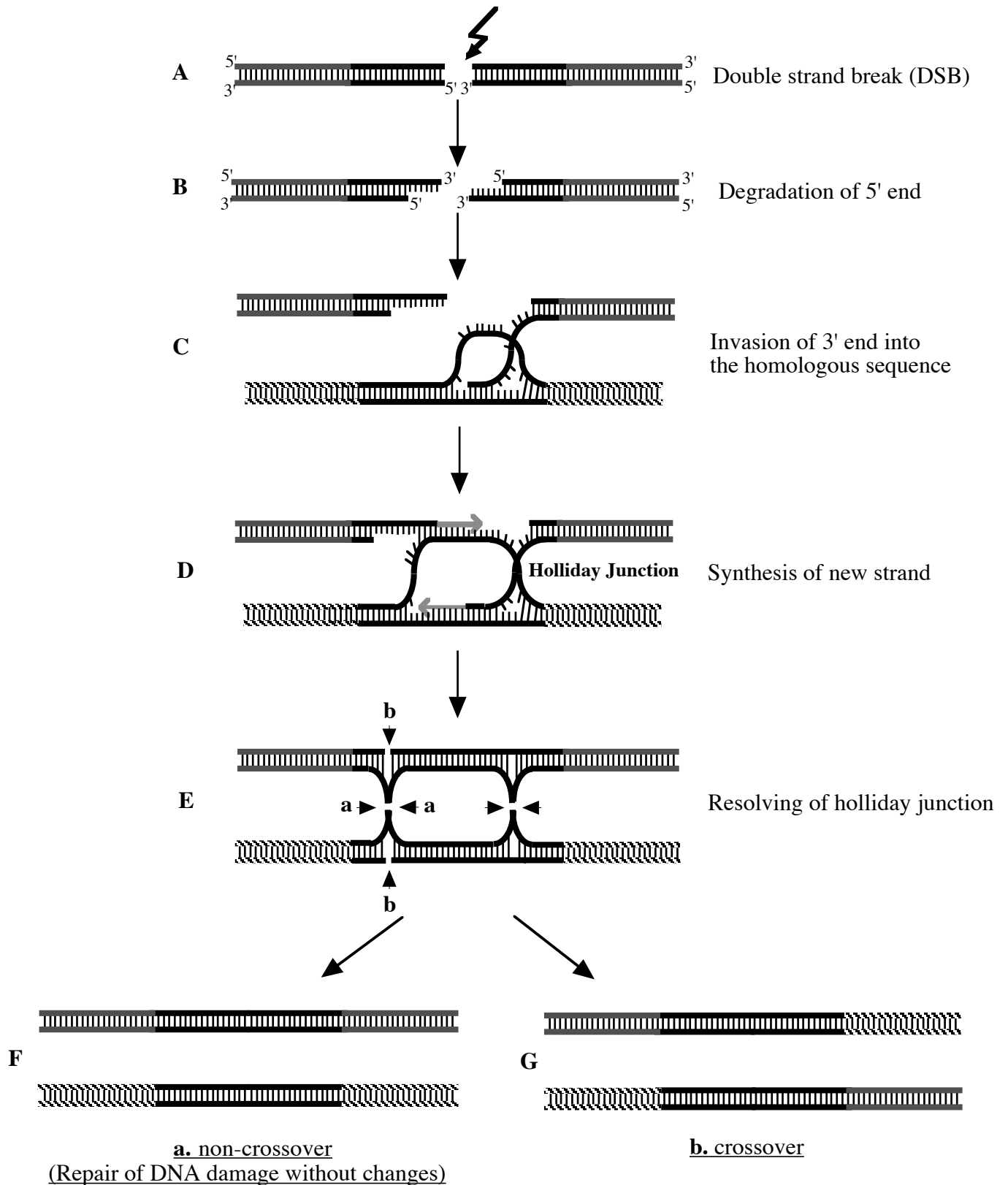


Figure 1. Mechanism of homologous recombination.

Solid lines (double-stranded DNA) show homologous regions. DNA damage is repaired using homologous sequence. DNA is repaired without changes using its sister chromatid. However, DNA rearrangements are occasionally caused in the case of using non-homologous chromosome or tandemly repeated homologous region on the same chromosome (G).

“Holliday junction” are a general intermediate of homologous DNA recombination (Holliday, 1964). The Holliday junction is then resolved by strand cutting resulting in two chromosomes in which a cross-over has occurred (Figure 1E).

However, homologous recombination occasionally causes DNA rearrangements. Unequal crossing over between a pair of homologous sequences results in DNA aberrations, such as deletions and duplications if the sequences are tandemly repeated, and exchange between different arrays could produce chromosomal rearrangements such as translocation (Figure 1G).

Homologous recombination is also an important pathway for gene targeting. The accurate recognition of homologous sequences is necessary for integration of an introduced transgene at the expected locus. However, the mechanism by which mitotic recombination is induced is not yet understood because of its initiation at random sites along a chromosome whereas meiotic events are initiated at special sites (Kunz and Haynes, 1981).

Transcription-associated recombination.

Transcription is thought to be one of processes that strongly induce recombination. Initially, transcription-associated recombination was reported in λ phage (Ikeda and Matsumoto, 1979). In the yeast mating-type switch, the transcribed *MAT* locus receives information from the transcriptionally silent donor loci *HML* and *HMR*. In mutant strains in which the *HML* and *HMR* loci are transcribed, these loci are able to act as recipients of information mediated by HO endonuclease

cleavage (Klar et al., 1981). In yeast cells, an initiation site for high levels of meiotic gene conversion is known to be localized at the promoter of the *ARG4* locus. Deletion mutations that reduce the frequency gene conversion in this region also reduce transcription of *ARG4* (Nicolas et al., 1989). Transcription from the *GAL10* promoter by RNA polymerase II causes a 15-fold stimulation of mitotic recombination between direct duplications of *gal10* gene (Thomas and Rothstein, 1989). In mammalian cells, rearrangement of immunoglobulin genes is enhanced by transcription (Blackwell et al., 1986; Schlissel and Baltimore, 1989; Lauster et al., 1993). However, the mechanistic basis for transcription-associated recombination is not well understood.

Recombination hotspots in various organisms.

The search for and analysis of recombination hotspots, DNA sequences that increase the frequency of genetic exchange in adjacent regions, seems to be a reasonable approach towards understanding mechanisms of recombination. A number of recombination hotspots have been identified in prokaryotes (Stahl et al., 1975) and in eukaryotes. These include the *cog+* mutation of *Neurospora crassa* (Angel et al., 1970), the M26 mutation of *ade6* in *Schizosaccharomyces pombe* (Gutz, 1971) and the YS17 mutation at the *buff* spore-color locus of *Sordaria brevicollis* (MacDonald and Whitehouse, 1979), which stimulate meiotic recombination. Hotspot sequences were also identified in *Escherichia coli* (Nishitani et al. 1993). Most of these sequences (named HotA to G) were found around *Ter* sites (DNA replication terminus sequence). Replication fork blocking event at these sites by the Tus protein that specifically binds

to the *Ter* sequence (Kobayashi et al., 1989; Hidaka et al., 1989; Hill et al., 1989) has been reported to be essential for recombination activity (Horiuchi et al., 1994).

***HOT1* is a hyper recombination hotspot in yeast mitotic cell.**

By screening for DNA fragments which stimulate homologous recombination at nearby regions, a cis-acting recombination hotspot, *HOT1*, was found within the repeated yeast ribosomal RNA gene cluster (rDNA; Keil and Roeder, 1984). In eucaryotes, rDNA is tandemly repeated in many copies, making one or more clusters in the genome constituting the structurally and functionally essential component of the nucleolus. The sequence and structure of rDNA repeats are highly conserved from yeast to plants and mammals. In yeast, there are about 150 rDNA tandem repeats located on chromosome XII. Each repeat is 9.1 kb in size and contains the large 35S rRNA gene transcribed by RNA polymerase I (PolI), the small 5S rRNA gene transcribed by Pol III and two non-transcribed spacer regions (NTS1 and 2) between these transcribed units (Figure 2A). NTS2 contains a replication initiation site (*ARS*) and the 35S rRNA gene promoter region. In the NTS1 region, there is a replication fork barrier sequence (RFB) which blocks only replication fork approaching in the opposite direction of 35S rRNA transcription from the *ARS* (in Figure 2A, right to left replication; Kobayashi et al., 1992; Brewer et al., 1992) and an enhancer for Pol I transcription (Elion and Warner, 1984). The recombination hotspot, *HOT1* was originally identified as a 4.6 kb *Bgl*III restricted fragment including NTS1, NTS2, the 5S rRNA gene and a part of the 35S rRNA gene (Figure 2A). The fragment stimulates

A

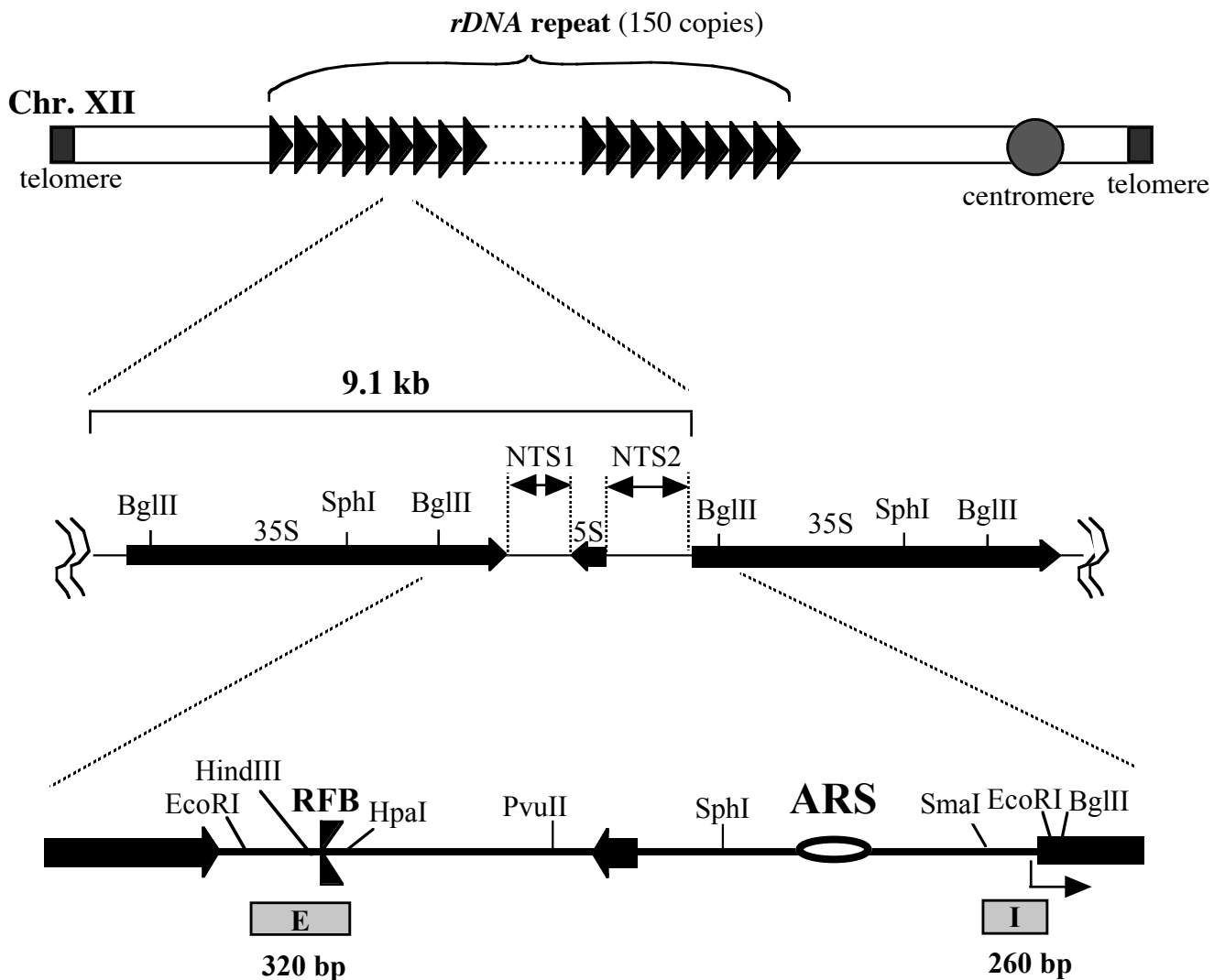




Figure 2A. Structures of rDNA and *HOT1*.

Structure of rDNA repeats in *S. cerevisiae*. 150 copies of rDNA are tandemly repeated on chromosome XII. Arrow head shows one unit of rDNA and the direction of 35S rRNA gene transcription. A single unit of rDNA consists of two transcribed genes, 5S and 35S rRNA genes. The direction of transcription is indicated by arrows. The two non-transcribed regions are indicated as NTS1 and NTS2. The 35S rRNA gene is transcribed by RNA polymerase I (Pol I), while the 5S rRNA gene is transcribed by RNA polymerase III. The NTS and its surrounding regions are expanded. Two DNA elements related to DNA replication, the replication fork barrier (RFB; ) and the origin of replication (ARS; ) are located in NTS1 and NTS2 respectively, shown in the lower part. The RFB allows progression of the replication fork in the direction of 35S rRNA transcription, but not in the opposite direction. E and I (boxed) are elements of *HOT1*.

B

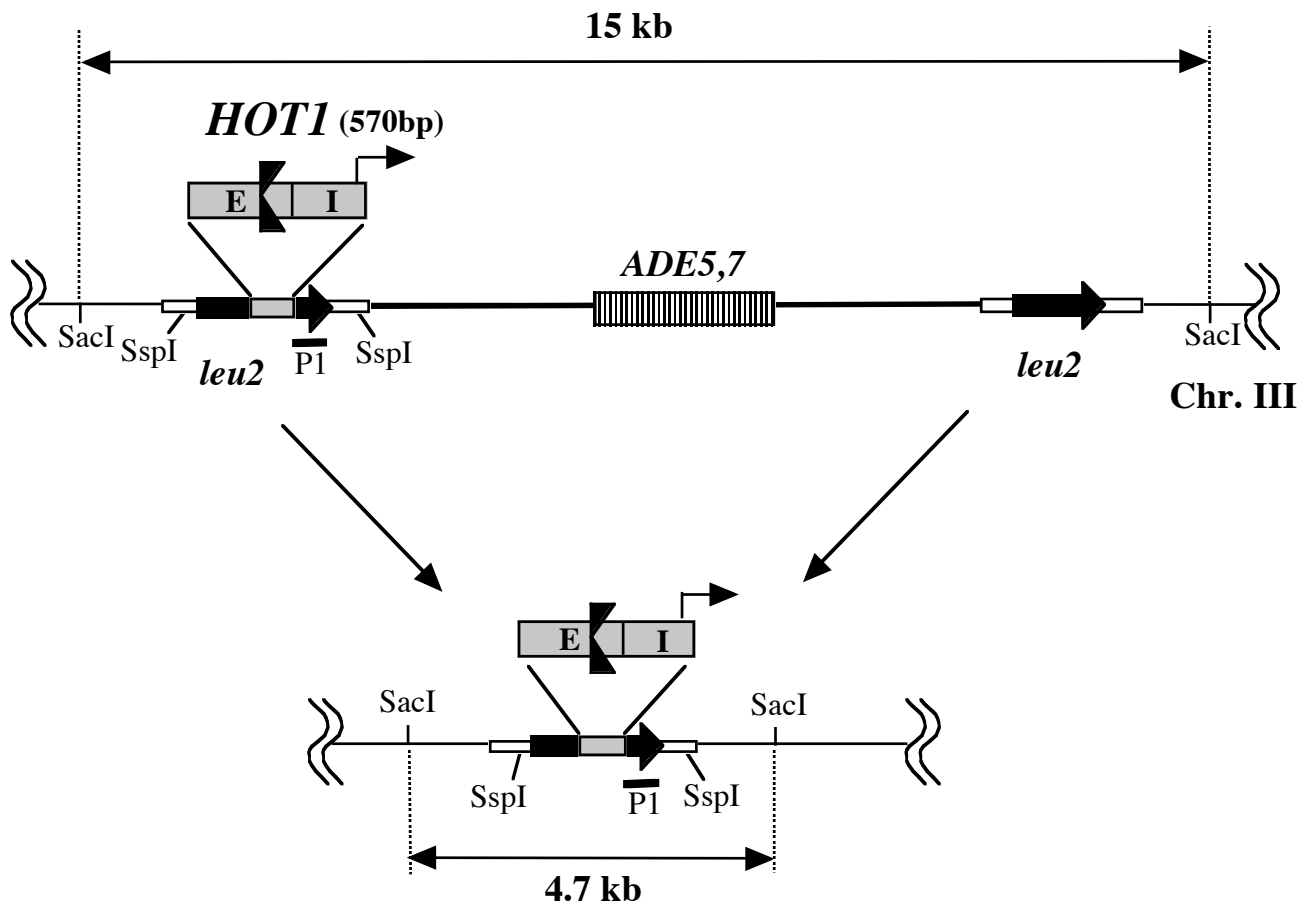



Figure 2B. Structures of rDNA and *HOT1*.

Structure for the *HOT1* recombination assay. *ADE5,7* is integrated between two tandemly repeated *leu2* genes in chromosome III. One of the *leu2* genes (left) has the *HOT1* construct. After homologous recombination between the *leu2* genes, *ADE5,7* is lost (lower panel). An arrow above the I-element indicates the direction of Pol I transcription and  in the E-element is the RFB. Open bars around *leu2* show repeated regions. A solid bar (P1) below *leu2* is the probe used for northern and Southern analyses (Figures 5, 10 and 12). Positions of *SacI* used for Southern analysis in Figure 10 and *SspI* for 2D analysis in Figure 12 are indicated.

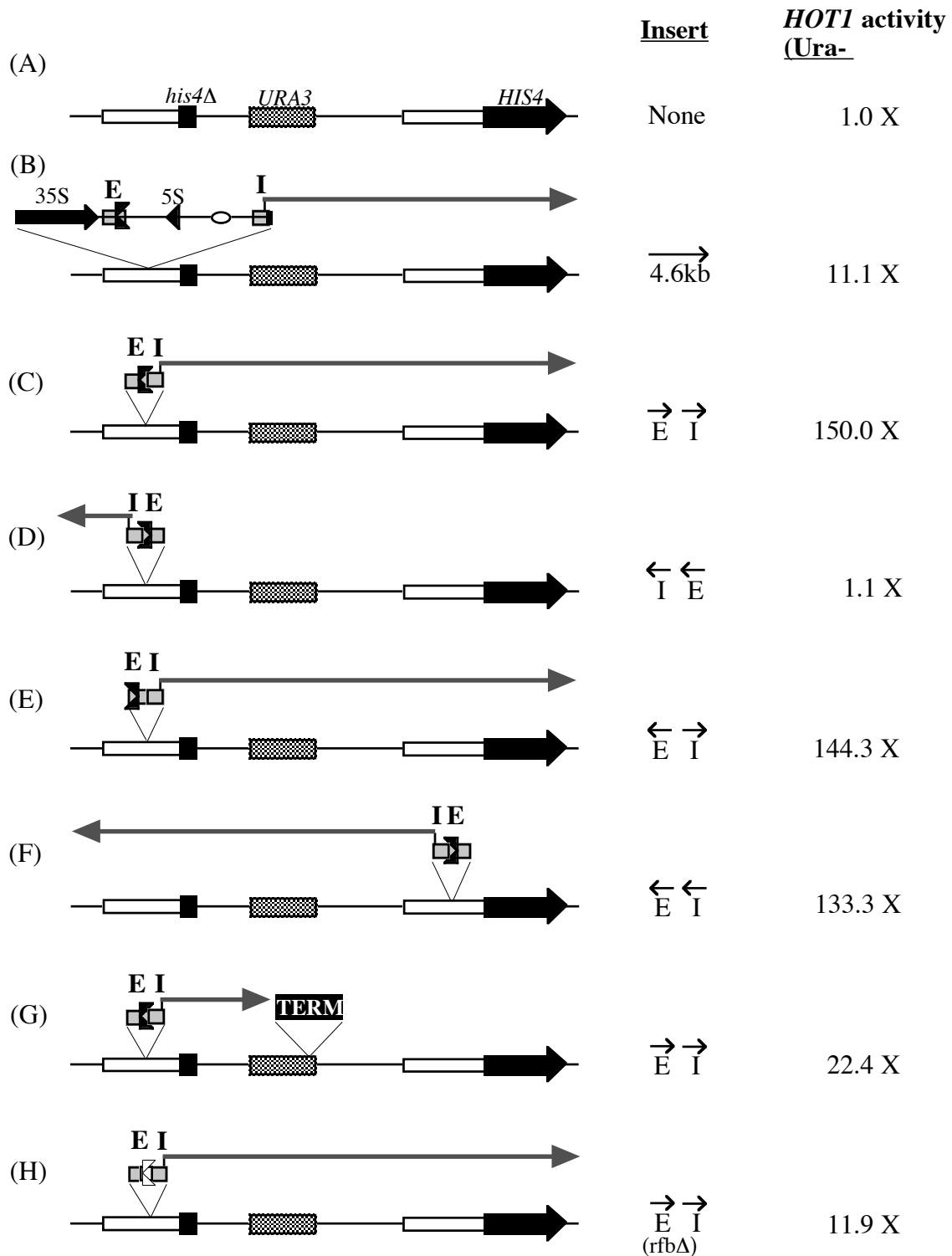


Figure 3. *HOT1* stimulates homologous recombination (Voelkel-Meiman et al. 1987).

The construct shown in diagram (A) carries no rDNA. Constructs (B) and (C) carry the intact 4.6 kb *Bg* II fragment and 570 bp subclone (E and I), respectively. In (D), E and I are inserted in the opposite orientation of (C). In construct (E), only the E-element is in the opposite orientation. In (F), E and I are inserted in the right-hand repeat. In (G), the termination site for RNA polymerase I transcription (TERM) is inserted into the *URA3* sequence present in the vector. In (H), most of the RFB sequence is deleted from the E-element. On the right side of the diagrams, the orientation and combination of inserted E and I, and *HOT1* activity relative to a no insert strain (A) are shown. The arrows above each diagram indicate transcripts from the initiation site of PolI.

mitotic recombination when inserted at novel locations in the genomes. Subsequent research identified two discontinuous elements that are the essential for *HOT1* recombination stimulation (*HOT1* activity; Voelkel-Meiman, 1987). One is the I-element which corresponds to the 35S rRNA gene promoter transcribed by RNA Pol I; the other is the E-element which overlaps the enhancer for Pol I transcription (Elion and Warner, 1984; Figure 2B).

Purpose and strategy of this study.

Because *HOT1* consists of the 35S transcription promoter and enhancer, its activity appears to be causally related to stimulation of transcription by Pol I. Indeed, it was shown that in a Pol I defective mutant, *HOT1* activity was completely abolished (Huang and Keil, 1995). However, in the Pol I mutant other phenotypes were also observed [e.g. change of the nucleolus shape (Oakes et al., 1998), and contraction of the rDNA repeats (Kobayashi et al., 1998)]. Therefore it is not clear whether the transcription itself activates the *HOT1* recombination, or whether another factor does.

Through genetic analysis of *HOT1* activity, another gene necessary for the activity was identified, in addition to general recombination genes like *RAD52*. The gene *FOB1* is required not only for *HOT1* activity but also for the replication fork blocking activity at the RFB site (Kobayashi and Horiuchi, 1996). Recently, Fob1p was shown to bind specifically to the RFB sequence and inhibit the replication fork (Kobayashi, 2003). As indicated in Figure 2, the RFB is included in the E-element of *HOT1*, therefore, we speculated that the fork blocking event itself was also essential for

HOT1 activity, similar to the recombination activity seen for the *E. coli* replication terminus sequence (Horiuchi et al., 1994). Both the RFB site and Fob1p are required for recombination in the rDNA cluster (Kobayashi et al., 1998, 2001; Defossez et al., 1999; Merker and Klein 2002; Johzuka and Horiuchi 2002). However, unexpectedly, Ward et al. (2000) clearly demonstrated that the fork blocking event was not required for the *HOT1* activity, although the RFB sequence is necessary. Instead, Wai et al. (2001) found that *FOBI* was required for the Pol I transcription of *HOT1*, although *FOBI* does not affect transcription of the 35S rDNA repeats at the original rDNA locus. In addition, it is known that Pol I transcription and the enhancer E-element are not required for recombination in the rDNA, although they are required for *HOT1* recombination (Kobayashi et al., 1998; Kobayashi et al., 2001; Wai et al., 2001). Therefore, the mechanism of recombination stimulation is thought to be different between *HOT1* and the rDNA. The former seems to be transcription-dependent while the later is replication fork blocking-dependent (Kobayashi et al., 1998).

Is homologous recombination at *HOT1* induced directly by transcription, or by some other factor? The purpose of this experiment is to obtain information about this molecular mechanism by investigating the effect of transcription level on *HOT1* activity. For this purpose, we used a strain in which the rDNA repeats were deleted. The transcription level by PolII were been off or slightly reduced and gained by mutation and deletion strains of *HOT1* (Stewart and Roeder, 1989). In the strain in which the rDNA repeats had been deleted, we found however, that *HOT1* transcription was highly stimulated (~ 14 times) and the rate of recombination was elevated about 15 times

relative to wild-type. Therefore, we conclude that the level of transcription itself governs *HOT1* recombination efficiency.

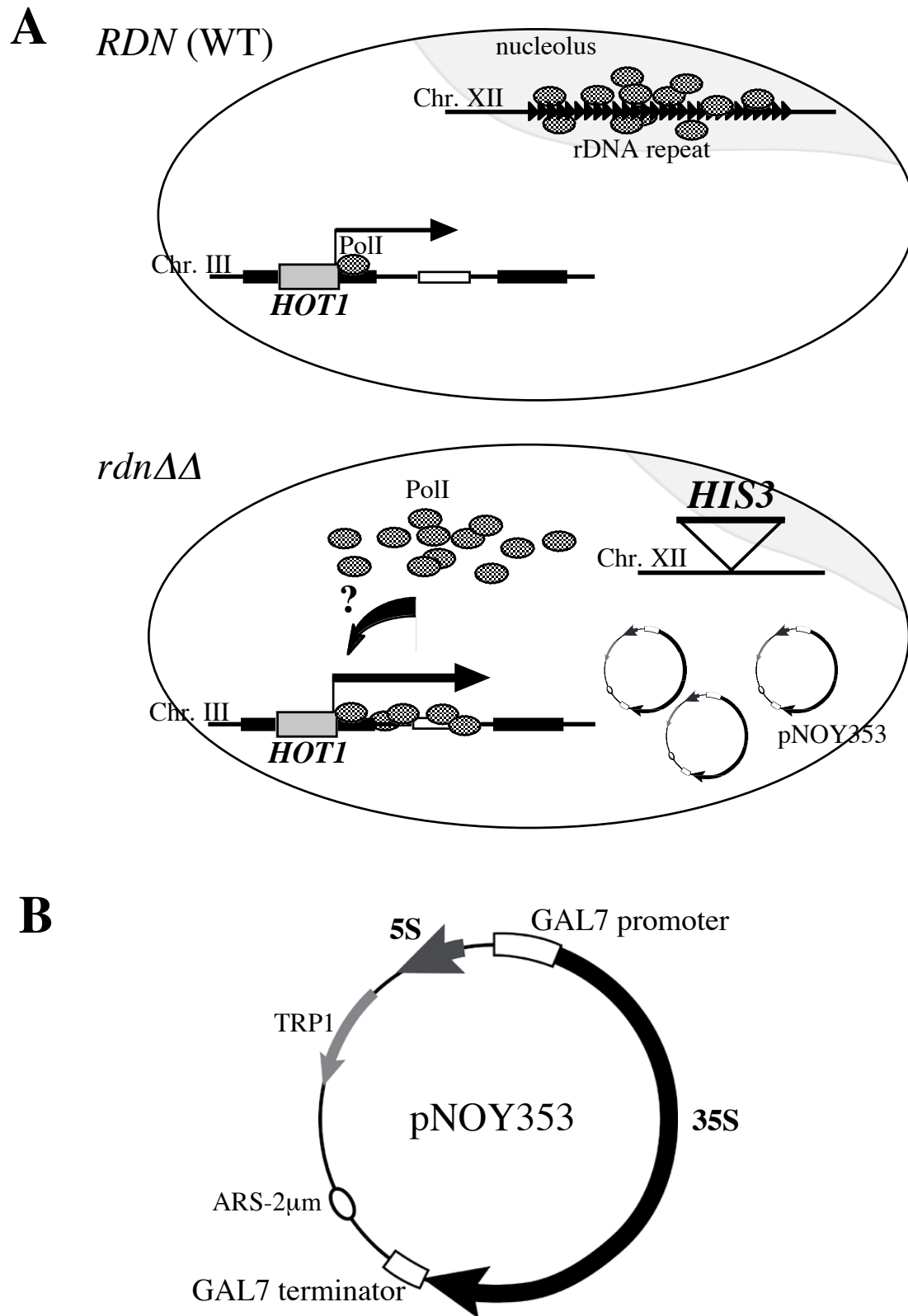


Figure 4. Scheme to experiment on the influence of transcription level on *HOT1* activity.

(A) Most of the transcriptional machineries for PolII (●) are functioning at the site of the rDNA repeats in the nucleolus and the amount of machineries interacting with *HOT1* is limited in the *RDN* (WT) strain (upper panel). In the *rdnΔΔ* strain (lower panel), rDNA repeats are deleted and growth is supported by a multicopy helper plasmid, pNOY353. The excess PolII transcription machineries are thought to stimulate transcription from *HOT1* because of reduced rDNA copy number. (B) Structure of pNOY353 (Oakes et al. 1998). pNOY353 contains the 5S rRNA gene and the 35S rRNA coding region fused to a strong PolII promoter, the GAL7 promoter.

II. Results

***HOT1* transcription is enhanced in an rDNA deletion strain.**

HOT1 recombination activity is dependent on Pol I (Huang and Keil, 1995, Wai et al., 2001). Therefore, it may be possible to elevate the recombination activity by enhancing *HOT1* transcription. Wai et al. (2001) reported that a strain whose rDNA was deleted showed hyper-transcription (~400X) from a plasmid-cloned Pol I promoter. This activation was observed when growth is supported by a multicopy helper plasmid (pNOY353) containing the 35S rRNA coding region fused to a strong Pol II promoter, the *GAL7* promoter (Oakes et al., 1998). However, the activation was not observed when another multicopy helper plasmid containing the 35S rRNA coding region with original Pol I promoter was used. Therefore, the authors speculated that in the strain with the rDNA deleted (*rdnΔΔ*), excess Pol I transcription machineries elevate ectopic Pol I transcription. Here we use the *rdnΔΔ* strain with pNOY353 to stimulate transcription of *HOT1*. We crossed a haploid strain (SER001) in which the *HOT1* construct is present at the *LEU2* locus (Figure 2B) with the *rdnΔΔ* strain (SER015). The resulting diploid strain (*RDN/rdnΔΔ*, SER002) was sporulated and a haploid *rdnΔΔ* strain with the *HOT1* construct was isolated (SER003). The growth of SER003 was poor, although the parent *rdnΔΔ* strain (NOY893) which does not have the *HOT1* construct grew well (Figure 5A), thereby indicating that the poor growth phenotype is dependent on the *HOT1* construct. Using the strains (SER001 and SER003), we also mated and established diploid strains with the *HOT1* construct [*RDN/RDN* and

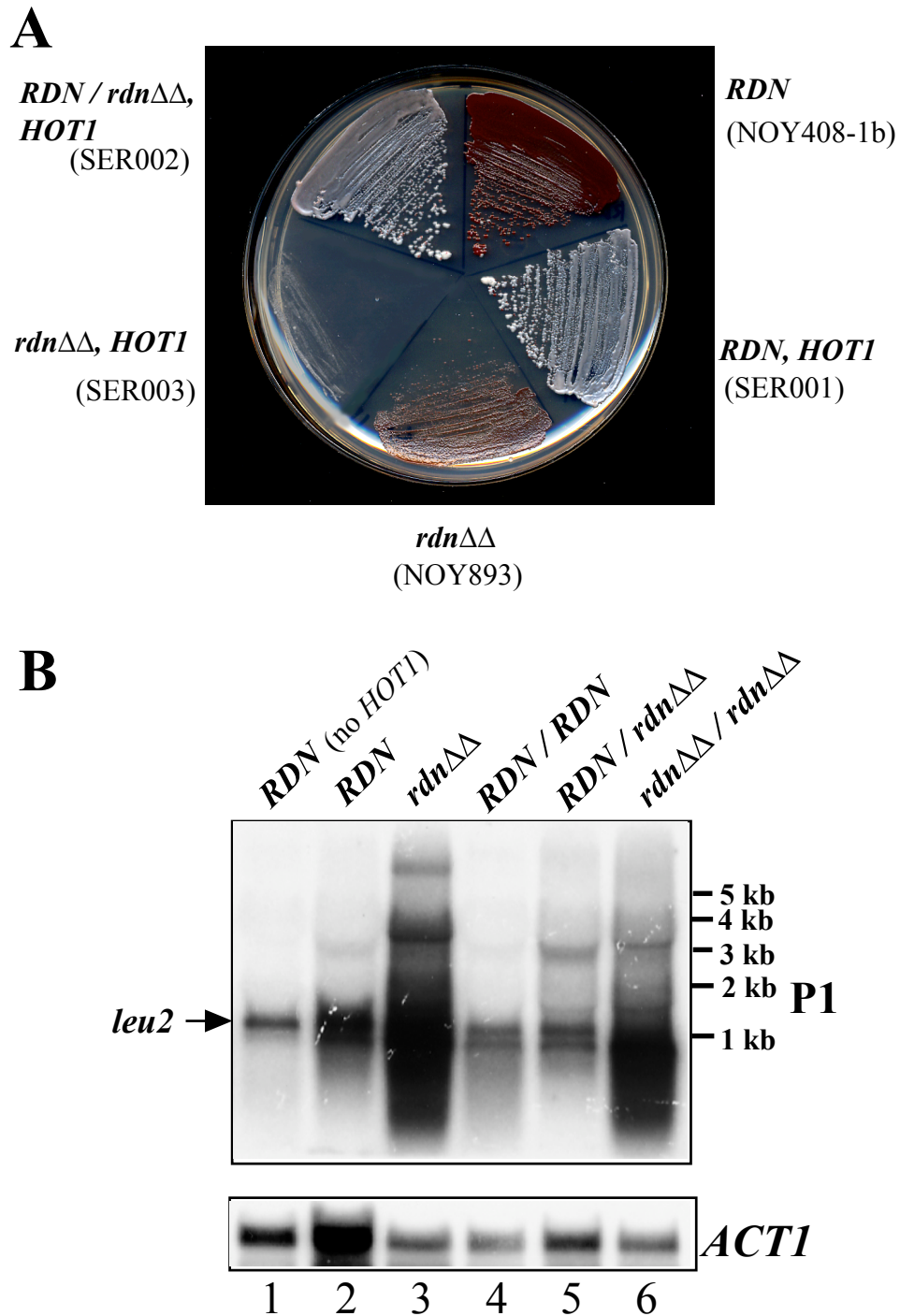


Figure 5. *HOT1* transcription in the *rdnΔΔ* strain.

(A) Growth of the *rdnΔΔ* and related strains. (B) Northern analysis of *rdnΔΔ* strains. Northern blots using total RNA isolated from strains with the *HOT1* construct were hybridized with probe P1 (Fig. 2B, lower panel) and with the internal control *ACT1* probe (lower panel). Lane 1 is a haploid wild type strain without *HOT1* construct (SER005); Lanes 2 to 6 contain strains with the *HOT1* construct. Lane 2 is a haploid wild type (SER001); lane 3, haploid *rdnΔΔ* (SER003); lane 4, diploid wild type (SER006); lane 5, diploid heterogeneous (SER002); and lane 6, diploid *rdnΔΔ* (SER007). The position of the *leu2* transcript and sites of the single strand marker are shown in the left and right sides of the upper panel, respectively.

rdnΔΔ/rdnΔΔ; the growth of the *rdnΔΔ/rdnΔΔ* strain is much better than the haploid *rdnΔΔ* strain (data not shown)].

In order to measure the transcription rate of the *HOT1* construct, we selected white colonies that contained an intact *HOT1* construct after recombination (lower panel in Figure 2B). This selection makes sure that all the strains have one copy of *HOT1* construct. For the *rdnΔΔ* haploid strain (SER003), we could not perform this selection because of the poor growth. Therefore, as the *HOT1* constructs have probably been lost in some cells, the transcript level is likely to be underestimated in this strain. However, as the transcript level is much higher than other strains (as shown below), this effect does not affect the results significantly. RNA isolated from these strains, was used to measure the rate of *HOT1* transcription by Northern analysis using a *LEU2* probe (P1; Figure 2B). The results are shown in Figure 5B. In lane 1 “*RDN*” without *HOT1* construct, only *leu2* transcripts were detected around 1.1 kb. In lane 2 “*RDN*” (SER001), another band around 2 kb appeared and a lower band was enhanced. Transcript level in the *rdnΔΔ* strain (SER003) was much greater than that of “*RDN*” (SER001). The reason for the smear band is that there is no Pol I transcription termination sequence in this *HOT1* system. Therefore, transcripts of several lengths were produced. In the diploid strains in which one of chromosome III has the *HOT1* construct, though the amount of transcripts are somehow less than those of the haploid strains, the similar pattern was observed (lane 4-6). These results indicate that in the *rdnΔΔ* strains, transcription of *HOT1* was enhanced.

To determine the relationship between rDNA copy number and *HOT1*

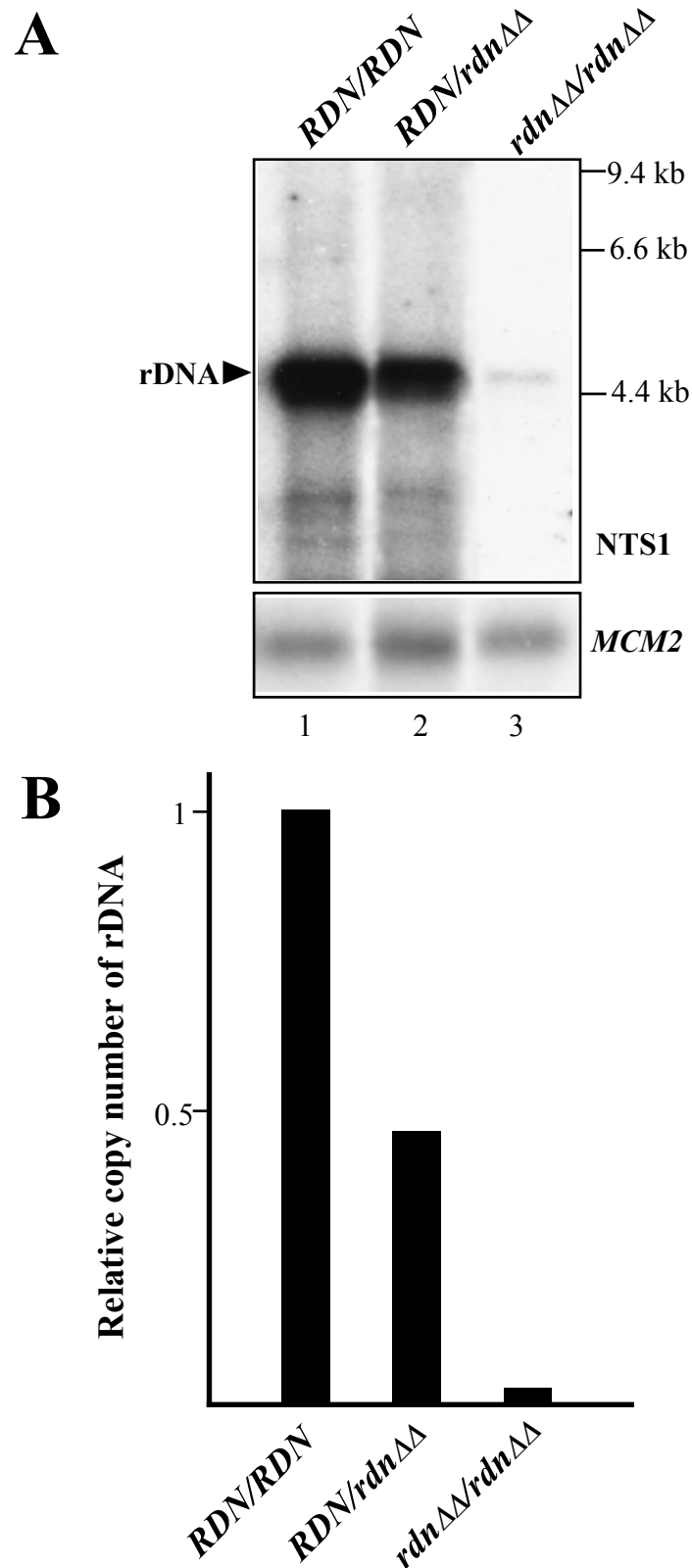


Figure 6. Copy number of rDNA in diploid strains.

(A) The 4.6 kb fragment of rDNA are hybridized with the NTS1 probe. In the *rdnΔΔ/rdnΔΔ* strain, ERCs can be seen as a weak band at the same size with chromosomal rDNA gene. (B) Quantitation of copy numbers per cell relative to the *RDN/RDN* strain (lane1).

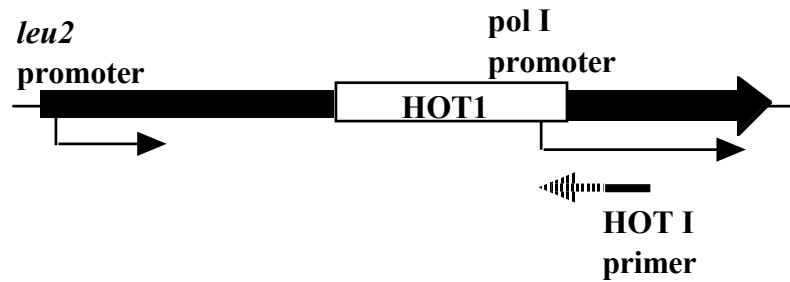
transcription level, we measured copy number in the strains. The *Bgl*II fragments of rDNA were identified by Southern analysis with an NTS1-specific probe. In the *RDN/rdnΔΔ* strain (Figure 6A, lane 2) which has the rDNA repeats on one of chromosome XII, the copy number was about half that of the *RDN/RDN* strain (Figure 6A, lane 1). In the *rdnΔΔ/rdnΔΔ* strain, a weak signal was detected (Figure 6A, lane 3), even though there is no rDNA repeat. This signal results from the extra-chromosomal rDNA circles (ERCs) which were produced from the rDNA repeats before they were deleted. The copy number corresponds to about 1.3% of the rDNA repeats in the *RDN/RDN* strain.

***FOBI* is not necessary for *HOTI* transcription in the *rdnΔΔ* strains.**

The *FOBI* gene was isolated as an essential gene for *HOTI* recombination activity (Kobayashi and Horiuchi, 1996). Wai et al. (2001) demonstrated that *FOBI* was necessary for *HOTI* transcription, although the gene is not required for transcription of the chromosomal rDNA repeats. To test whether this gene plays a role in the hyper-transcription of *HOTI* in the *rdnΔΔ*, we performed primer extension assays to determine the level of the *HOTI* transcripts. We disrupted *FOBI* in *HOTI* strains used in the Northern analysis by a conventional gene replacement method and established several *fobI* mutant strains, shown in Figure 7B. Total RNA from the *HOTI* strains was isolated and transcribed by reverse-transcriptase with an end-labeled primer (HOTp) to produce complementary DNA, indicated in Figure 7A. We note that for this assay we used the same *HOTI* strains as used in the previous section (Figure 2B,

bottom). We can recognize the *HOT1* transcripts from the Pol I promoter by the length of the transcript (102 bp). The results are shown in Figure 7B. Lanes 1-4 are sequencing reactions using the same labeled primer to determine the length of the transcripts. In the wild type strain (lane 5) we could detect *HOT1* transcripts which were initiated at the native 35S rRNA start site (the A at position +1 indicated by an arrow). However, transcripts were almost completely lacking in the *fob1* mutant (lane 6). A similar *FOBI*-dependency was observed in the *RDN/RDN* diploid strain (lanes 9, 10). Therefore, the *HOT1* transcription is dependent on *FOBI* in the wild type, as described previously (Wai et al., 2001). In contrast, when *FOBI* was disrupted in the *rdnΔΔ* haploid strain, the signal intensity of the transcript did not change so much (lanes 7, 8). And when *FOBI* was disrupted in *rdnΔΔ/RDN* and *rdnΔΔ/rdnΔΔ* diploid strains, the transcript level was reduced in the *fob1* mutants (lane 12, 14) but the band was still visible in the mutant. The signal intensities were measured by a phosphorimager and the values were plotted on a graph (Figure 7C). The values are average of three independent experiments. The amount of transcript in the *rdnΔΔ* strain was more than 100-120 times higher than that of the *RDNfob1* strain and more than 10-14 times higher than that of the wild type *RDN* strain whose rDNA is not deleted (Figure 7C). The value was not significantly affected by a *fob1* mutation in the *rdnΔΔ* strain. In the *rdnΔΔ/RDN* diploid strain, *HOT1* transcripts increased 2.5 times more than that of the wild type, and with disruption of *FOBI* the level was about 45 % reduced (lanes 11 and 12 in Figure 7C). But the level is still higher than that of the *RDN/RDN* strain (lanes 9 and 12 in Figure 7C). Similarly, in the *rdnΔΔ/rdnΔΔ* strain a *fob1* mutation reduced

A



B

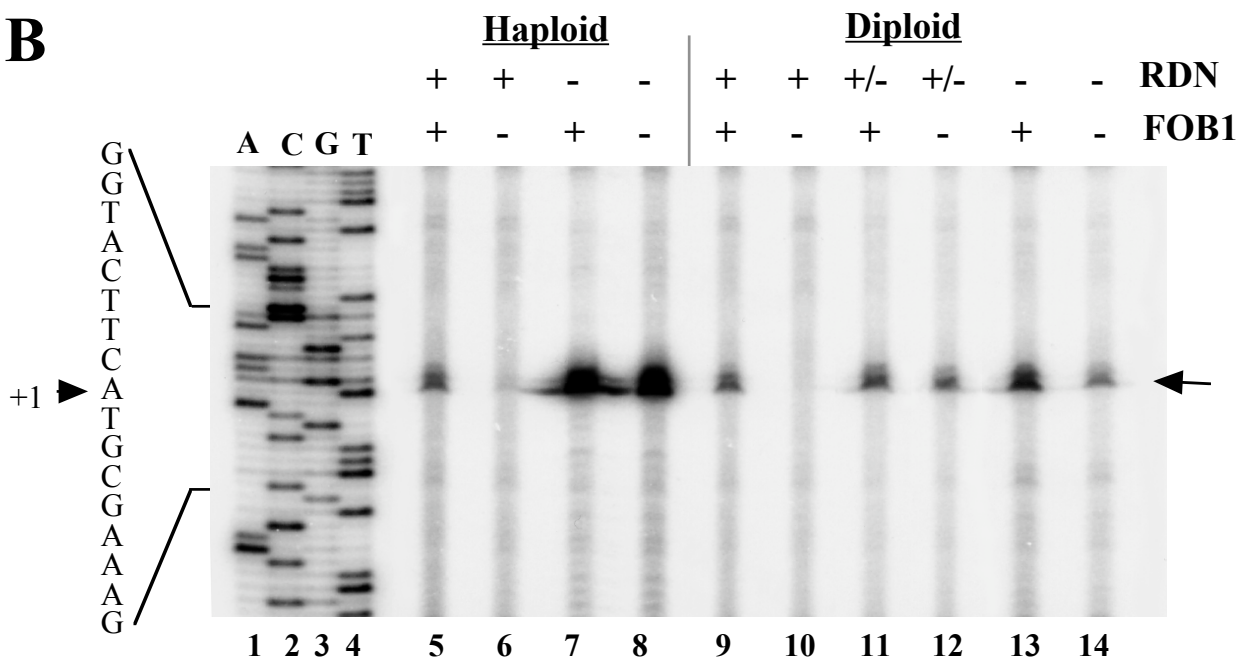


Figure 7. Primer extension analysis of *HOT1* transcripts.

(A) Structure of the *HOT1* transcription construct. A thick arrow shows the *leu2* gene (lower panel in Figure 2B). Transcripts from *leu2* and *HOT1* promoters are indicated by thin arrows. HOTp is an end-labeled primer used for the extension of *HOT1* transcripts and for dideoxy-chain termination sequencing reaction. The length of the *HOT1* transcripts extended by the primer is 102 bp (dotted arrow). (B) Primer extension analysis. Lanes 1 to 4 are sequencing ladder using dideoxy-chain termination. Template RNAs for extension were isolated from strains SER001 (lane 5), SER008 (lane 6), SER003 (lane 7), SER010 (lane 8), SER006 (lane 9), SER011 (lane 10), SER002 (lane 11), SER009 (lane 12), SER007 (lane 13), and SER012 (lane 14). The same amount of total RNA was used for each extension reaction.

C

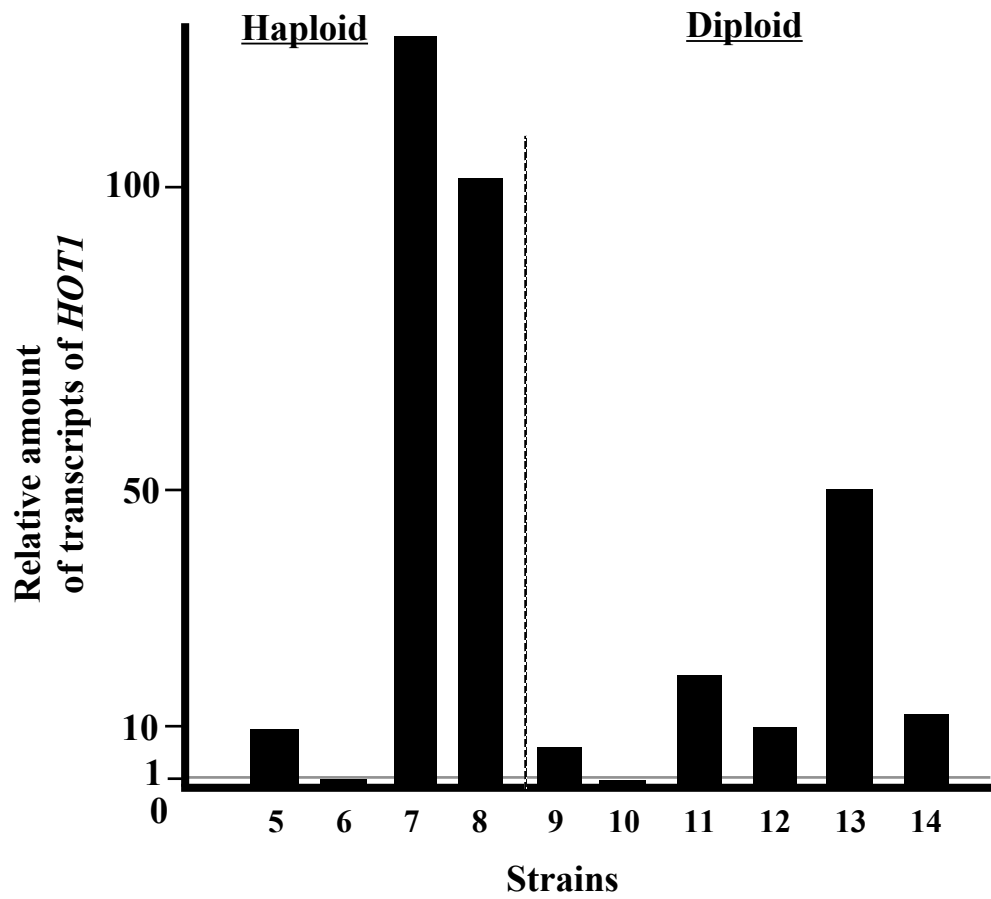


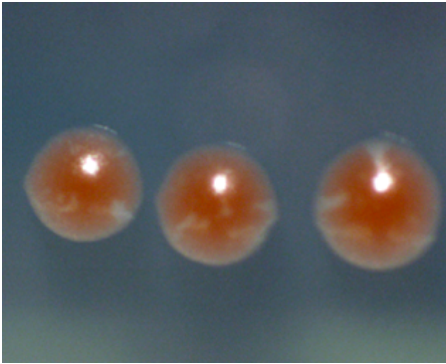
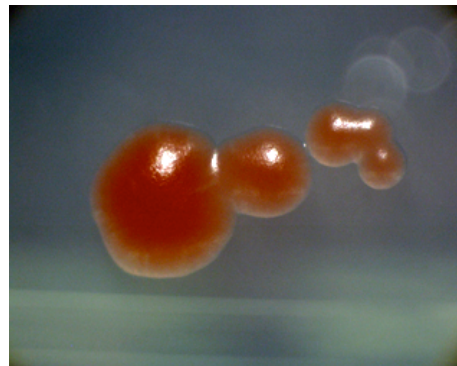
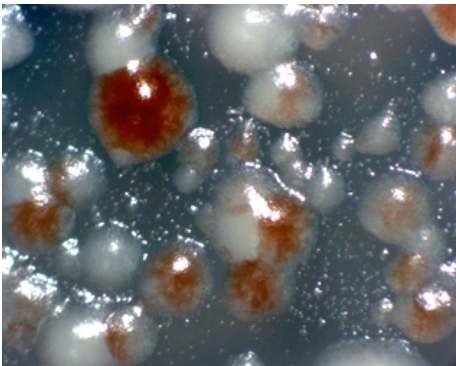
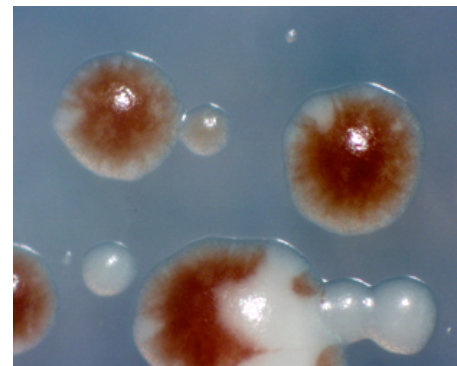
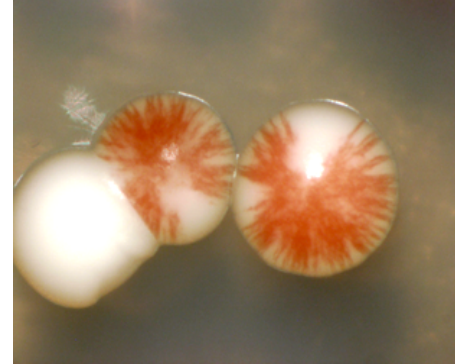
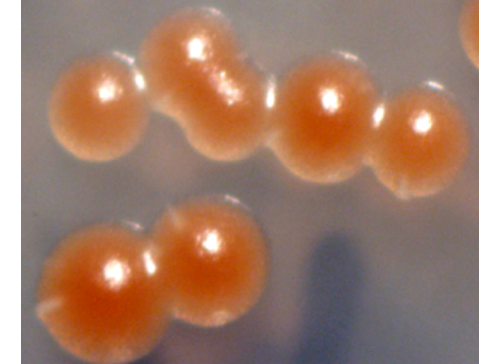
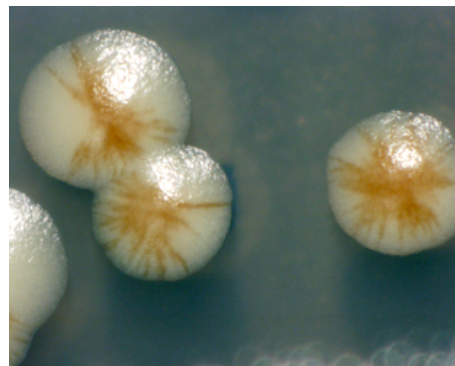
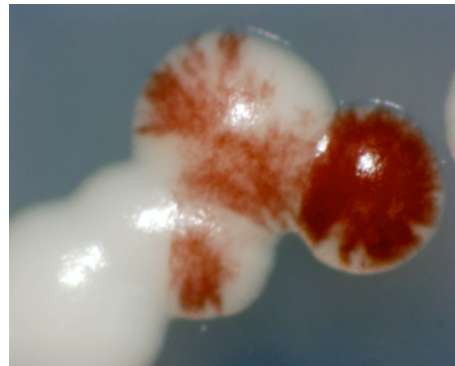
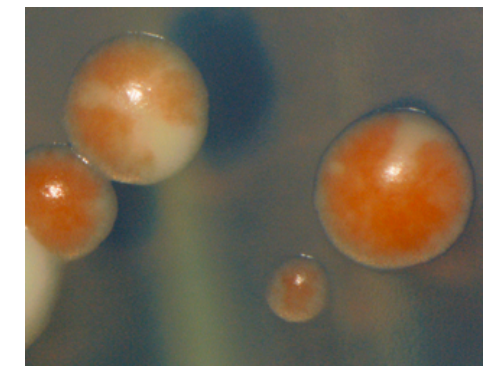
Figure 7C. Primer extension analysis of *HOT1* transcripts.

Quantitation of the amount of extended products from panel B. The values are shown relative to SER008 (lane 6). Lane numbers below are identical to those in panel B.

the transcription activity, but the activity is still high (lanes 13 and 14 in Figure 7C). This indicates that in the *rdnΔΔ* strains *FOBI* is affecting, but is not necessary, for *HOT1* transcription.

***HOT1* recombination is activated in rDNA deletion strains.**

As Pol I is essential for *HOT1* recombination, the rate of *HOT1* transcription is thought to affect the rate of *HOT1* recombination (Stewart and Roeder, 1989; Huang and Keil, 1995). Therefore, the recombination rate is expected to be elevated in the *rdnΔΔ* strain. We measured the recombination rate of *HOT1* by determining the loss of an *ADE5,7* marker which was inserted in the *leu2* tandem repeats (Figure 2B). The results are shown in Figure 8A. Recombination is not stimulated in a *HOT1*-less strain, therefore cells form red colonies due to an *ade2* background mutation (Figure 8A-1). When cells lose the *ADE5,7* marker by recombination, the cell color becomes white because the red pigment cannot be produced in an *ade2 ade5* genetic background (Lin and Keil, 1991). As shown in Figure 8A-2, cells with *HOT1* form red and white sectorial colonies because of frequent loss of *ADE5,7*. The higher the rate of recombination, the larger the portion of the colony that is white. In the *rdnΔΔ* strains the colonies are largely comprised of white portions (Figure 8A-8). The recombination rate was determined quantitatively by a half-sectoring assay that measures recombination only in the first cell division after plating. In a colony with more than a continuous half of the colony being white, recombination has occurred in the first cell division in most cases. Therefore, the recombination rate can be determined by counting the proportion of half

A**Haploid****1, *HOT1*-less (SER005)****2, *RDN* (SER001)****3, *RDN fob1* (SER008)****4, *rdn*ΔΔ (SER003)****5, *rdn*ΔΔ *fob1* (SER010)****Diploid****6, *RDN/RDN* (SER006)****7, *RDN/RDN fob1/fob1* (SER011)****8, *RDN/rdn*ΔΔ (SER002)****9, *RDN/rdn*ΔΔ *fob1/fob1* (SER009)****10, *rdn*ΔΔ/*rdn*ΔΔ (SER007)****11, *rdn*ΔΔ/*rdn*ΔΔ *fob1/fob1* (SER012)****Figure 8A.**

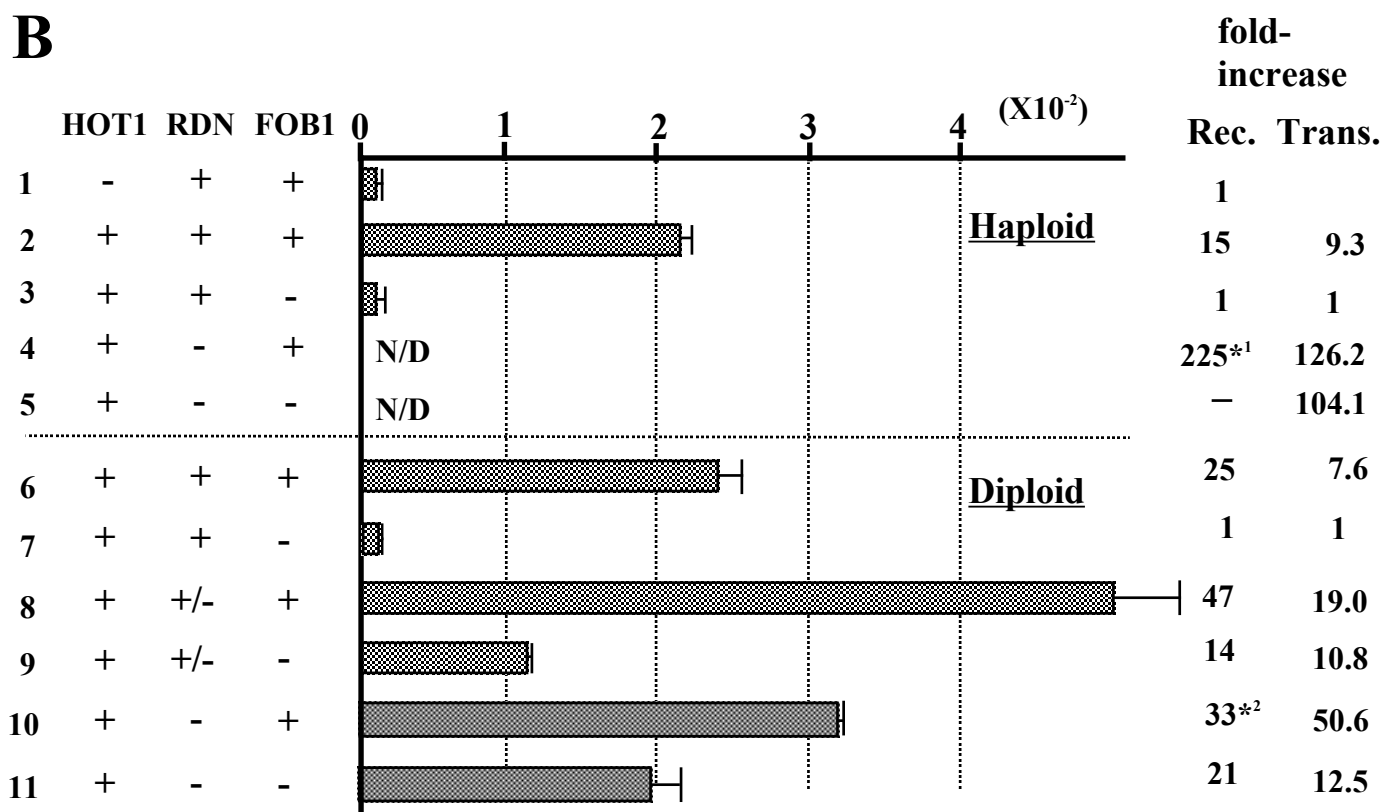


Figure 8. Examination of *HOT1* recombination activity.

(A) Colony sectoring assay. Strains used have *ADE5,7* inserted in the *leu2* duplication in an *ade2 ade5* background as shown in Fig. 2B. All strains except that in panel 1 have a *HOT1* construct in one of the duplicated *leu2* genes. The formation of white sectors results from excisive recombination between the two *leu2* genes to lose *ADE5,7*. Strain names and related genotypes are indicated below each picture. (B) Recombination rate determined by a half sector assay that measures recombination only in the first cell division after plating. Among ~20000 sector colonies, the rates of half sector colonies were calculated. The values are averages of three independent experiments. Numbers on the left side correspond to numbers in panel A. +, - indicate presence and absence, respectively. +/- shows heterozygotes in diploid strains. On the right side of the panel, recombination rates relative to the *HOT1*-less strain ("Rec.") and transcript levels relative to the *RDN fob1* strain (SER008, "Trans.") from Fig. 7C are shown. *1 is an estimated value from the *URA3* marker loss assay in Fig. 9. *2 is possibly under-estimated, as mentioned in the text.

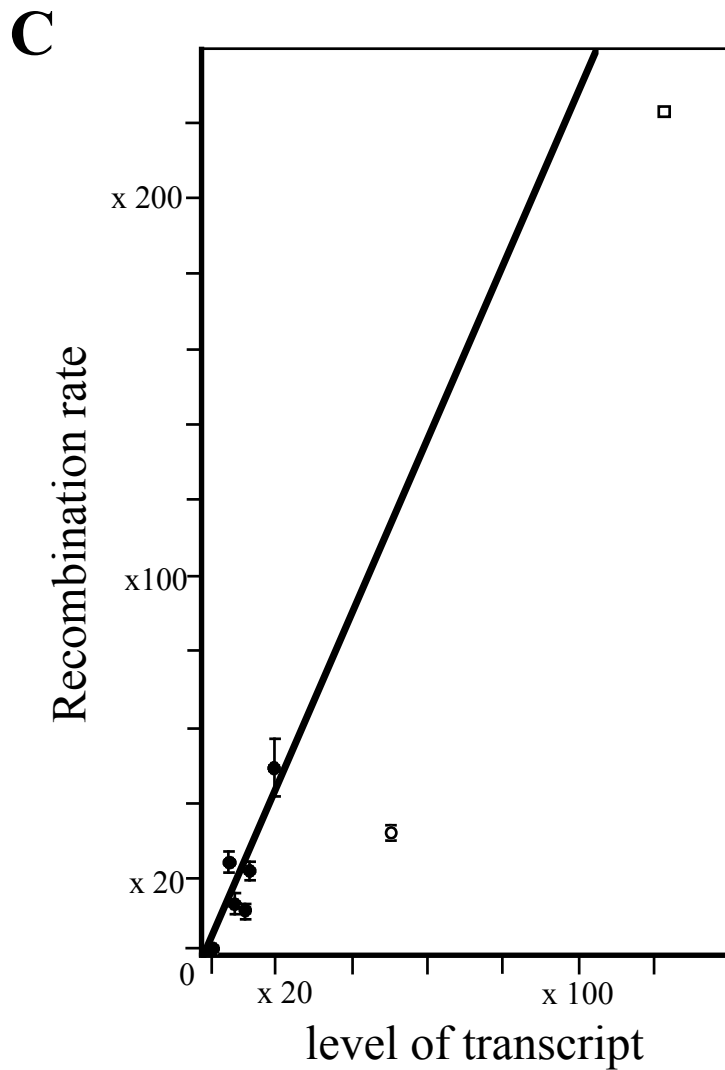


Figure 8.

(c) Correlation between transcript levels and recombination rates.

Each value on the right side of the panel (B) is dotted on the graph.

Open circle is the value of lane 10 in the panel (B), and open square is lane 4, see the legend of the panel (B) and the text.

sector colonies. The results of this quantitative assay are shown in Figure 8B. In a wild type haploid strain, *HOT1* enhanced recombination by about 15 times compared with a *HOT1*-less construct (lane 1, 2). *FOBI* is necessary for this stimulation, as described previously (compare lane 2 with 3; Lin and Keil, 1991; Kobayashi and Horiuchi, 1996; Wai et al., 2001). The recombination rate in a *fob1* mutant is similar to that of a *HOT1*-less strain (compare lane 1 with 3). In the *rdnΔΔ* strain, most of the colonies stopped growing when the size was so small that sectors were not observed (Figure 8A-4, Figure 2A). As this growth deficiency is dependent on the *HOT1* construct (Figure 5A, compare SER003 with NOY893), hyper-recombination induced by *HOT1* is likely to be the cause of the deficiency. However, some of the white colonies grew well. They are thought to have lost the *HOT1* construct during a recombination event where the *HOT1*-less *leu2* gene remains. Some dark red colonies with few or no sectors were also observed. Moreover, with disruption of *FOBI* in the *rdnΔΔ* strain, the growth became somewhat better, but the colonies were still too heterogeneous in site to recognize sectors (Figure 8A-5). This heterogeneous growth rate made it impossible to measure the recombination rate in the *rdnΔΔ* strain.

Therefore, to determine the rate, we used a *URA3* marker instead of the *ADE5,7* marker. As shown in Figure 9A, the *URA3* gene is inserted between two *his4* genes in *ura3* defective genetic background. After cells were cultured in SG complete medium lacking uracil, the frequency of Ura⁺ recombinants was determined by spotting aliquots of 10-fold serial dilutions of the cultures on SG plates with and without 5-FOA. The results are shown in Figure 9B, and the recombination rates were calculated (Figure

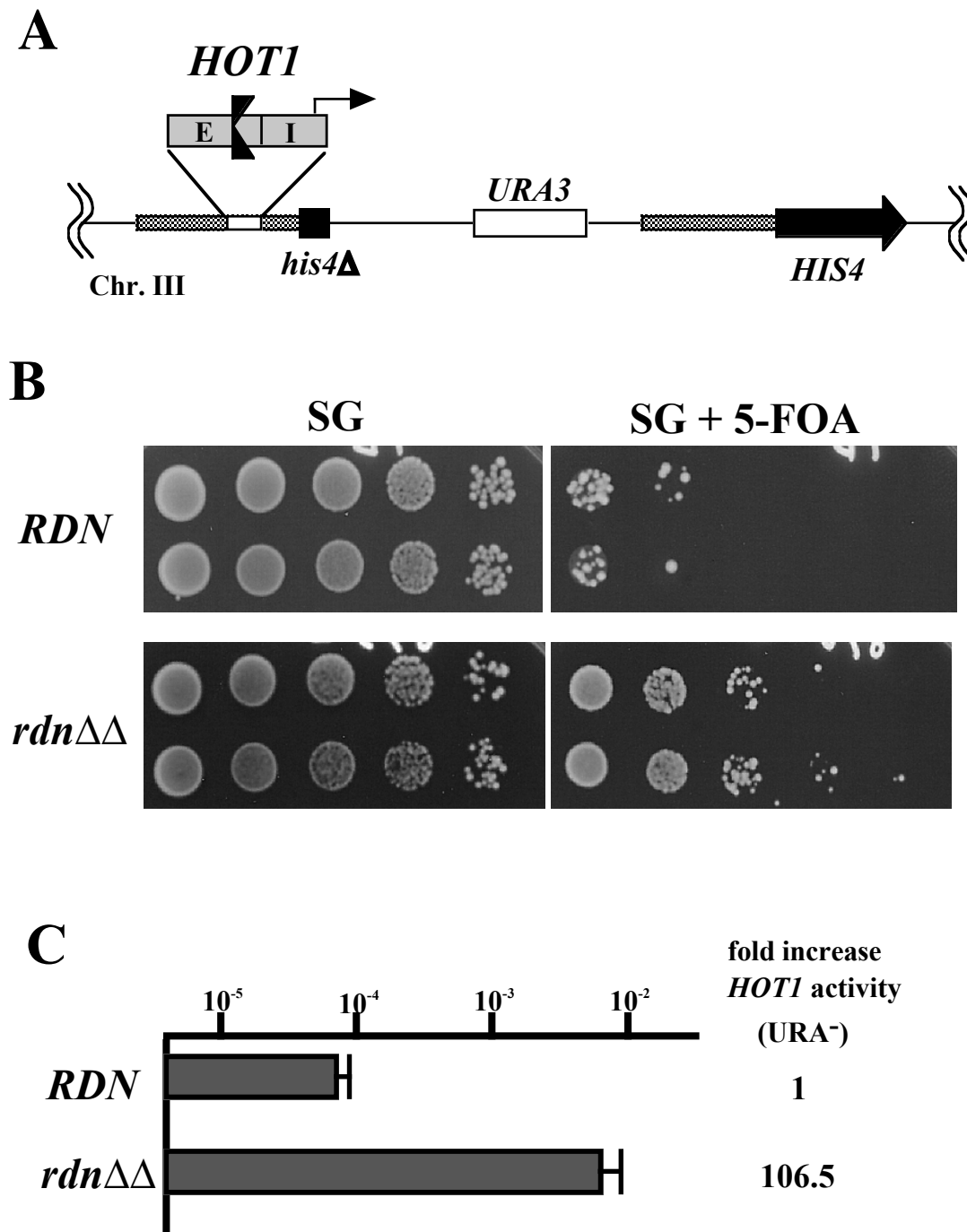


Figure 9. Examination of *HOT1* recombination activity in the *rdnΔΔ* strain by the *URA3 HOT1* construct. (A) Structure of the *URA3 HOT1* construct. *URA3* is integrated between two tandemly repeated *his4* genes in chromosome III as shown in Figure 2B. (B) Strain SER013 “*RDN*” and SER014 “*rdnΔΔ*” were grown in SG complete medium lacking uracil. The recombination rates were determined by spotting aliquots of 10-fold serial dilutions onto SG with and without 5-FOA. Two independent experiments were done. (C) The recombination rates are plotted on a logarithmic scale. The relative recombination rate of the *rdnΔΔ* strain to the *RDN* strain is shown to the right.

9C). In the *rdn* $\Delta\Delta$ strain the recombination rate was about 100 times enhanced compared with the wild type. Interestingly, in the *URA3* system, the growth deficiency observed in the *rdn* $\Delta\Delta$ strain with the *ADE5,7* system was not detected. As the method and locus examined are both different in the *ADE5,7* and *URA3* systems, it is difficult to compare the recombination rates obtained by the two systems. From previous work, it is known that in the same *URA3* system, *HOT1* enhances recombination about 100 times compared with the *HOT1*-less strain (Voelkel-Meiman et al., 1987; Wai et al., 2001). This compares to the *ADE5,7* system, where the enhancement was 15 times, as we showed in Figure 8B (lane 2). Therefore, we speculate that in the *rdn* $\Delta\Delta$ strain with the *URA3* system the 100-fold enhancement of recombination rate would be equivalent to a 15-fold enhancement of recombination rate in the *ADE5,7* system. So as the wild type cells lose the marker at a rate of 2.2 times per 100 cell divisions, we can roughly estimate that the recombination rate in the *rdn* $\Delta\Delta$ strain is 33 loss of the marker per 100 cell divisions. In other words, in the *rdn* $\Delta\Delta$ strain the cells lose the marker once per 3 cell divisions.

We used the *ADE5,7* recombination system to test a diploid strain in which one of the two chromosome III has the *HOT1* construct. In the *RDN/RDN* strain the recombination stimulation was dependent on *FOB1* as observed in the haploid strain (6, 7 in Figure 8). The *rdn* $\Delta\Delta$ /*RDN* strain showed the highest recombination rate in this assay. The rate of *ADE5,7* deletion was 5 per 100 cell divisions. Interestingly, although the recombination rate was reduced when *FOB1* is disrupted, the value is still high when compared with that of the *RDN/RDN fob1/fob1* strain (compare 9 with 7 in

Figure 8). In the *rdnΔΔ/rdnΔΔ* strain the growth inhibition was not as serious as that in the haploid *rdnΔΔ* strain. However, there were still many abnormal heterogeneous colonies. Therefore, although we could do the half-sectoring assay, the recombination rate is possibly underestimated. In the *fob1* mutant (*rdnΔΔ/rdnΔΔ*) the recombination rate was a little reduced (compare lane 10 to 11 in Figure 8), but still high compared with that of the *RDN/RDN fob1/fob1* strain (compare lane 11 with 7 in Figure 8). Taken together, *FOBI* affects the recombination rate but it is not necessary for recombination, just as observed for *HOT1* transcription. As shown in the right side of Figure 8B and the graph in Figure 8C, recombination stimulation by *HOT1* correlates with the level of *HOT1* transcription stimulation. Therefore, we conclude that the recombination rate is affected by the level of transcription.

We also analyzed the DNA structure of *HOT1* recombination products by Southern analysis. DNA was isolated from strains used in the sectoring assay, restricted with *SacI* and subjected to Southern analysis. The results are shown in Figure 10. DNA fragments were identified with probe P1 (Figure 2B). Each strain basically has three discrete bands (Figure 10, lane 1). Upper bands (15 kb) correspond with pre-recombination constructs which have *ADE5,7* in the *leu2* repeats, and the lower two bands (4.7 and 4.1 kb) are post-recombination constructs with and without *HOT1*, respectively (Figure 2B). In the *RDN fob1* (lane 2) strains, lower bands were not detected because of no recombination activation as expected from the sectoring assay. In the *rdnΔΔ* strains (lane 3, 4) the pre-recombination constructs were still visible although the recombination rate is extremely high. We presume that the well-

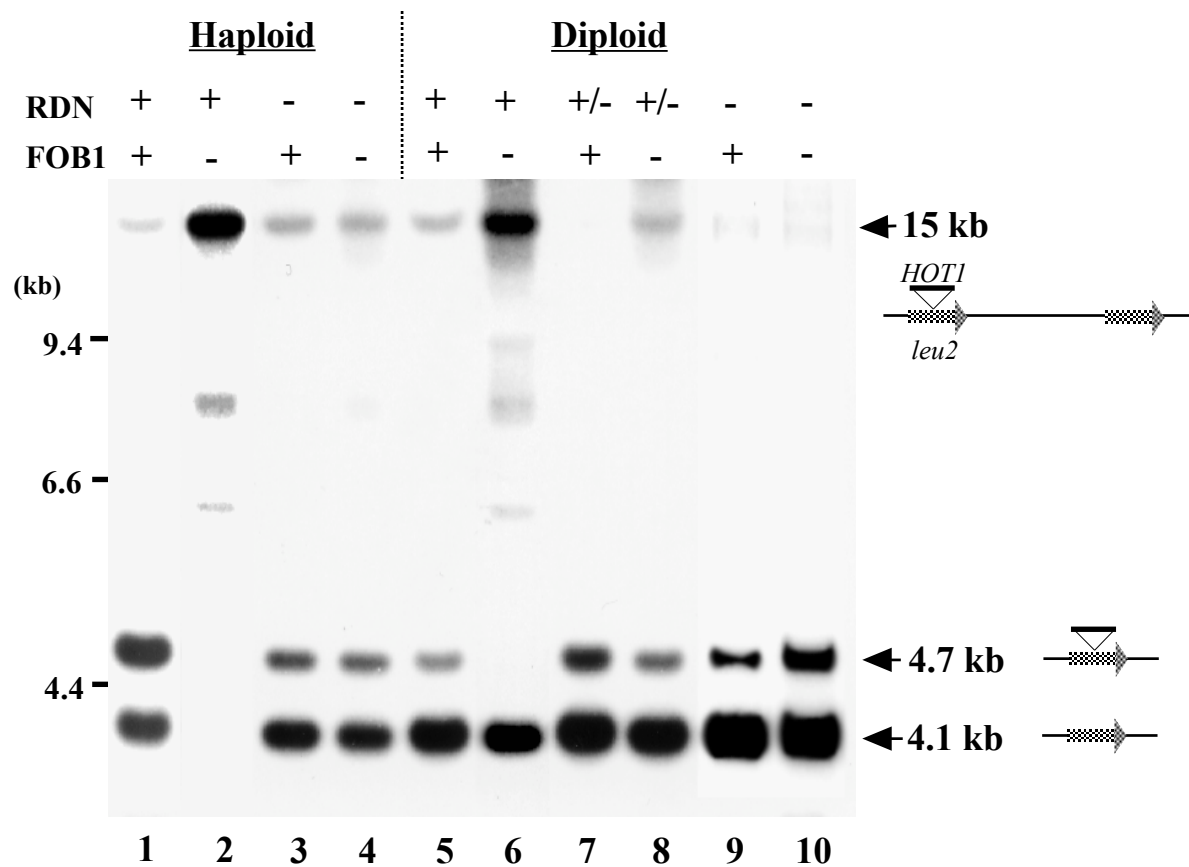


Figure 10. Southern analysis of *HOT1* recombination.

DNA was isolated from the strains used in Figure 8. DNA was digested with *SacI*.

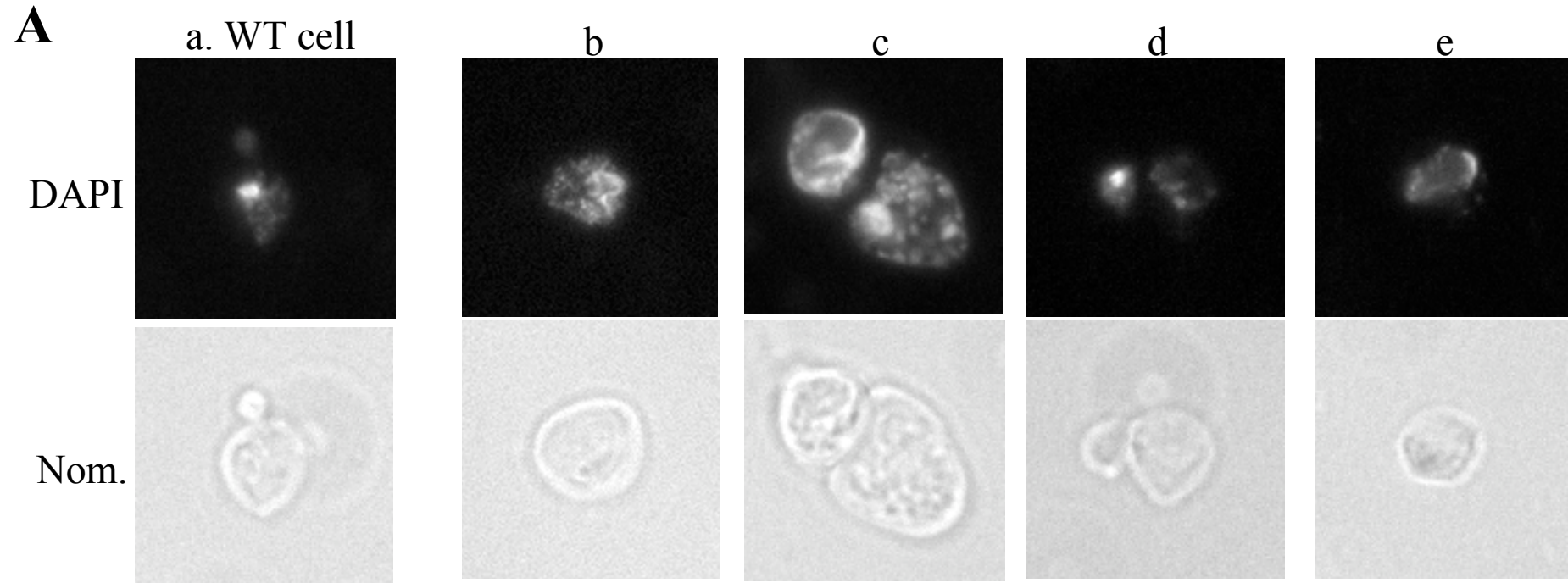
Southern analysis with probe P1 was performed (Fig. 2B). Three arrows on the right side indicate band positions before (upper) and after (lower two) recombination. The expected structures are also shown to the right. Positions of DNA size markers are indicated on the left side.

growing dark red colonies, which were observed in Figures 8A-4 and -5, represented a part of the DNA isolation culture. In diploid strains, the *leu2* gene in another chromosome III are overlapping with the 4.1 kb bands. Therefore, although *HOT1* is not active in the *RDN/RDN fob1/fob1* strain (lane 6), only the 4.1 kb band is detected. In the *rdnΔΔ/RDN* strain, which showed highest recombination rate in diploid strains, pre-recombination constructs were not visible. It should be noted that the assay is not quantitative for recombination efficiency because the ratios of the bands are affected by the starting ratios of pre- and post-recombination constructs. However, in this assay we could confirm that in the *rdnΔΔ* strains *HOT1* induces recombination in a similar manner to the wild type strain.

In the *rdnΔΔ* haploid strain, most of colonies were very small. We observed cells by microscopy. In some, nuclear abnormalities were observed, such as fragmentation and loss of nuclei (Figure 11). They might have died because they were unable to escape from check point control to stop the cell cycle when recombination was continuously occurring.

Enhanced transcription doesn't affect the replication fork blocking activity in *HOT1*.

Because the E-element of *HOT1* includes the replication fork blocking site (RFB), it was presumed that the blocking activity is working as a trigger for recombination as demonstrated in the *E. coli* terminus region (Horiuchi et al., 1994; Kobayashi and Horiuchi, 1996). Therefore, we observed the replication fork blocking activity at the



B

	total cell number	fragmentation of the nuclei	loss of the nuclei
<i>RDN</i>	267	0 (0.0%)	1 (0.37%)
<i>rdnΔΔ</i>	358	21 (5.87%)	12 (3.35%)

Figure 11. Abnormal morphology of *rdnΔΔ* cells.

(A) Micrograph of *rdnΔΔ* cells. Fixed cells were stained with DAPI to visualize DNA (DAPI) and also were examined with Nomarski optics (Nom.). In panel(a), nuclei of normal mother cell in S phase is stained as a major spot, and mitochondrial DNA as ambiguous spots. No signal can be seen in daughter cell because migration of nuclei has not yet occurred. Panel(b) to (e) show cells which have abnormal nuclei in the *rdnΔΔ* strain. Fragmentation ((b) and (c)) and loss ((d) and (e)) of nuclei are seen. (B) Frequency of cells which have abnormal nuclei.

E-element of *HOT1* in the *rdnΔΔ* strain by two-dimensional gel electrophoresis (2D analysis; Brewer and Fangman, 1987). This method allows one to monitor progression of the replication fork around *HOT1* (see a scheme in Figure 12). RFB activity can be recognized by an accumulation of a particular Y-shaped molecule (arrowheads in Figure 12) as detected by a suitable probe (Probe P1, Figure 2B). As shown in Figure 12, the number of replication fork molecules paused at the RFB site was estimated to be similar in both the *RDN/rdnΔΔ* diploid strain and in the control *RDN/RDN* strains, although the recombination rate of the former was twice as high as that of the latter (Figure 8B-8, 6). Moreover, no accumulation of Y-shaped molecule was observed in the *rdnΔΔ fob1Δ* mutant, although *HOT1* activity was present (Figure 8B-5). Taken together, the RFB activity appears not to be related to *HOT1* recombination in the *rdnΔΔ* strains.

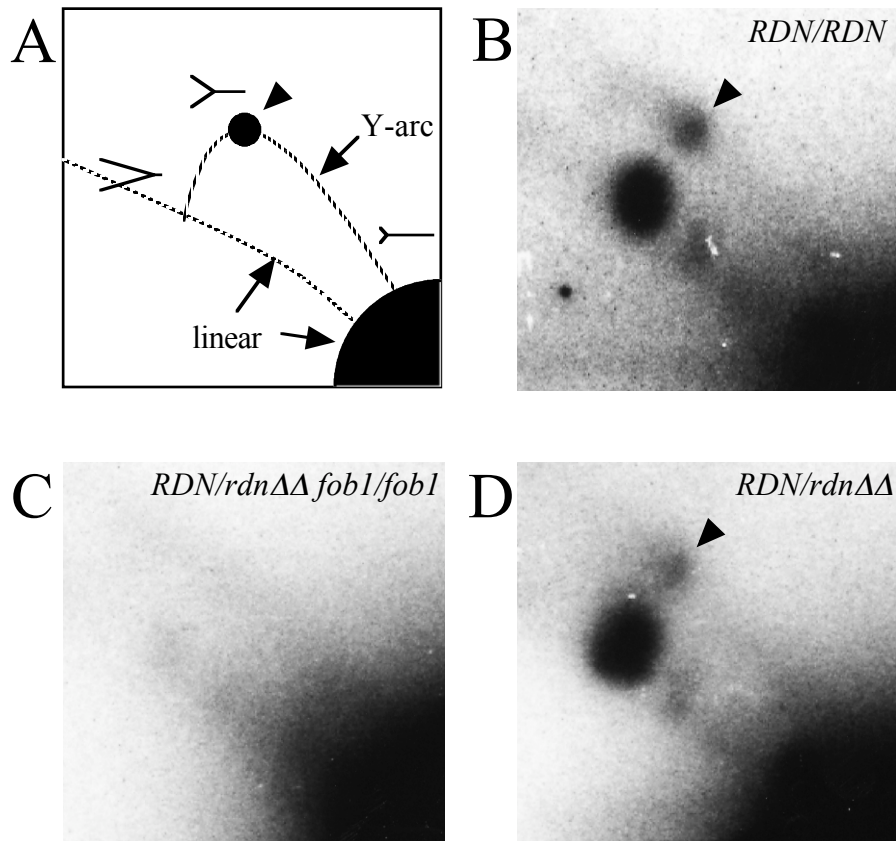


Figure 12. Autoradiograms of 2D gel of *HOT1* construct.

SspI fragments were probed with P1 (Fig. 2B). (A) Schematic diagram of migration of replication intermediates. Y-formed intermediates are shown as an arc according to the progression of replication fork. (B, C and D) 2D gels of *RDN/RDN* (SER006), *RDN/rdnΔΔ fob1/fob1* (SER009) and *RDN/rdnΔΔ* (SER002), respectively. In *FOBI* strains (b and d), accumulation of arrested forks at RFB results in spot (arrow head); the spot is not seen in the *fob1* strain.

III. Discussion

HOT1 is known to be one of the most effective mitotic recombination hot spots in yeast. Although Pol I and Fob1p are required for the stimulation, the molecular mechanism responsible has not yet been identified. Here, using an rDNA deletion strain, we were able to enhance the Pol I transcription in *HOT1* by about 14 times. In this strain *HOT1*-stimulated recombination was about 15 times greater than that in wild type. Additionally, *FOB1* was found to be dispensable for enhancement of transcription and recombination in this strain. These results indicate that Pol I transcriptional activity is the main cause of recombination stimulation in this strain, and that *FOB1* may be functioning as a transcriptional activator for *HOT1* in the wild type strain.

HOT1 consists of two elements, I and E (Figure 2). The role of the I-element for recombination enhancement is shown to be Pol I transcription. The E-element contains the replication fork barrier (RFB) site which inhibits replication fork progress. Recently, we found that Fob1p directly binds to the RFB site (Kobayashi, 2003). The protein specifically bound to two separated regions, RFB1 and RFB3, which correspond to cis-essential regions for *HOT1* activity identified previously (Stewart and Roeder, 1989; Huang and Keil, 1995). Therefore, in the wild type strain the association of Fob1p with the E-element may be necessary for the function of *HOT1*. As mentioned in Introduction, Ward et al. (2000) demonstrated the RFB activity itself was not related to the recombination stimulation in the wild type. Moreover, in Figure

7, a *fob1* mutation reduced the *HOT1* transcript level to one seventh of the wild type level (Figure 7C). Taken together, these results suggest that the Fob1p/E-element complex is required for effective Pol I transcription in *HOT1*, and the transcription is necessary for the recombination stimulation (Wai et al., 2001). In contrast, in the *rdnΔΔ* strain the dependency on *FOBI* for transcription was much reduced (Figure 7). In the primer extension assay, the number of *HOT1* transcripts are similar in both *FOBI* and *fob1* strains in the *rdnΔΔ* background. Therefore, in the *rdnΔΔ* strains the Fob1p/E-element complex is dispensable for Pol I transcription in *HOT1*. One possible reason for the dispensability is that there are excess Pol I transcription machineries in nucleus because of reduced rDNA copy number. These excess machineries would then be able to transcribe ectopic Pol I promoters, such as *HOT1*. This in turn suggests that the Fob1p/E-element complex act as an activator of *HOT1* transcription by localizing *HOT1* to the nucleolus where the Pol I transcription machineries are located, as originally proposed by Wai et al. (2001). In contrast, *FOBI* did affect *HOT1* transcriptional efficiency in the *rdnΔΔ/rdnΔΔ* diploid strain, in which excess Pol I transcription machineries are also expected (Figure 7). However, in this strain *HOT1* transcription is not as enhanced as in the *rdnΔΔ* haploid strain. We speculate that one reason for the reduced transcriptional stimulation in the diploid strain may be the presence of the extra-chromosomal rDNA circles (ERCs) which have been popped out from the rDNA repeats before deletion of the rDNA. Indeed, we have detected the presence of ERCs in the *rdnΔΔ/rdnΔΔ* diploid strains (Figure 6). These ERCs may be using many of the Pol I transcription machineries in the nucleolus, thus

there is not such an excess of these machineries to transcribe *HOT1* at a high level. Therefore, *FOB1* is still required to help localize *HOT1* to the nucleolus as in the wild type strain. Diploid strains need to produce more rRNA molecules than haploid strains, therefore the helper plasmid might not supply sufficient rRNA molecules and cells with ERCs would be selected by their superior growth rate. This phenomenon may be different in the *rdnΔΔ/RDN* diploid strain. In this strain, we expected a level of transcriptional enhancement similar to that of the *rdnΔΔ* haploid strain because it has twice the number of transcription machineries but only the haploid level of rDNA copies. However, as shown in Figure 7C, only 2.5 times enhancement of transcription was observed (lanes 9 and 11 in Figure 7C). When this *rdnΔΔ/RDN* strain was investigated, we found the rDNA repeats (on one chromosome XII) were not amplified from the wild type level and seem to be reduced slightly (Figure 6). Therefore, to satisfy the requirement for rRNA molecules in diploid cells, the number of actively transcribed rDNA gene could have increased. In growing haploid cells, it is known that less than half of the rRNA genes are transcribed (Dammann et al., 1995). Therefore, the lack of sufficient rRNA molecules in *rdnΔΔ/RDN* strain may have been compensated for by transcription of the normally silent rDNA genes. Another explanation is that the amount of Fob1 protein limits the interaction of *HOT1* and PolII-transcription machinery. In the *rdnΔΔ/RDN* strain, most Fob1 protein is thought to be preferentially localized at RFB sites in rDNA repeats such that the amount interacting with *HOT1* may be limited.

Transcription-mediated recombination is known from yeast cells (for review,

see Aguilera, 2002). However, the molecular mechanisms are still unclear. Recently, we reported that collision between the transcription and replication forks is a cause of recombination (Takeuchi et al., 2003). In a *fobI* defective strain whose rDNA copy number is reduced to ~20 (about one eighth of the wild type copy number), inhibition of replication fork progression in the rDNA was observed by two-dimensional gel (2D) analysis. In a strain with normal rDNA copy number such inhibition was not observed, therefore increased transcription in the low-copy rDNA strain seemed to stimulate replication inhibition resulting in increased recombination. In this strain, rDNA amplification was often detected, suggesting that this form of recombination could be coupled with replication, and replication fork inhibition by collision is a trigger of amplification, as shown in the RFB-dependent rDNA amplification model (Kobayashi et al., 1998). We actually tried to find amplification of the *ADE* marker gene in non-sectored red colonies from the *HOTI* recombination assay reported in this study. However, we could not detect such amplification (data not shown). Moreover, we could not detect any replication fork inhibition in the *rdnΔ fobI* strain by 2D analysis, although *HOTI* is still active (Figure 12). Therefore, we believe that *HOTI* recombination is not coupled with replication inhibition. This speculation is consistent with the observation that *FOBI*-dependent replication blocking (RFB) activity in the E-element does not contribute to *HOTI* recombination at all. As the RFB activity has a polarity, if the RFB activity is somehow related to *HOTI* recombination, direction of the replication fork should affect *HOTI* recombination. However, Ward et al. (2000) results and other genetic experiments (Voelkel-Meiman et al., 1987; Figure 3) show that

direction of replication and direction of the E-element do not affect *HOT1* recombination at all. In addition, it is known that in the rDNA locus the RFB site itself is not enough to induce recombination. For the induction, the flanking sequence (a part of EXP), which is not involved in the replication fork blocking activity, is necessary (Kobayashi et al., 2001; Benguria et al., 2003). Therefore, in *HOT1*, replication and replication inhibition do not seem to be related to *HOT1* recombination. Instead, transcription seems to be the key factor in *HOT1* recombination.

In the up-stream and down-stream regions of an actively transcribed gene, it is known that negative and positive torsional stresses accumulate, respectively (Lui and Wang, 1987). Such stresses are resolved by activity of topoisomerases. In fact, loss of topoisomerase function confers hyperrecombination in the rDNA (Christman et al., 1988; Wallis et al., 1989). Therefore, in the *rdnΔΔ* strain, the torsional stress may be too high to be resolved by the topoisomerases and a similar situation as observed in the topoisomerase defective mutants may be taking place. Negatively super-coiled DNA produced by transcriptional elongation may facilitate strand separation or the formation of R-loops in which the nascent RNA forms a hybrid with DNA, leaving a non-template DNA single strand (Figure 13A). In the immunoglobulin S region, it has been shown that during transcription, the S transcript hybridizes with the template DNA strand, leading to an R-loop structure (Reaban and Griffin, 1990; Reaban et al., 1994; Daniels and Lieber, 1995), and such an R-loop would be a substrate for endonucleases, such as XPF/ERCC1 and XPG which cause double strand breaks *in vitro*, (Tian and Alt, 2000). XPF/ERCC1 and XPG in mammal cells are known to be homologues of yeast *RAD1*,

RAD10 and *RAD2*, respectively. They are also involved in nucleotide excision repair. Interestingly, a mutation in *RAD1* has been shown to reduce *HOT1* activity (Zehfus et al., 1990). Therefore, it is possible that these nucleases take a part in *HOT1* activity. Another explanation is that transcriptional machinery passing through a region of DNA may transiently open the chromatin structure. It is likely that this open structure would contribute to better accessibility of DNA-damaging agents and nucleases to the DNA.

The stimulation of recombination is also observed in PolII-mediated transcription systems (see Introduction). In our study, *leu2* transcription was not as efficient in inducing recombination as transcription of *HOT1* (Figure 5. lane 1 and 2, Figure 8B. lane 1 and 2). One explanation for this difference is that the PolII transcription machinery itself may induce more DNA damage by causing significant distortion of DNA in the nucleolus. Another explanation is that transcription of long regions of DNA produces more supercoils or mediates structural transitions of chromosome to an open structure that is accessible to endonucleases and enzymes involved in the processing of recombination intermediates. In the *HOT1* construct, the PolII-mediated transcription machinery doesn't have a transcriptional terminator (Figure 5. Northern analysis), which allowed transcription to elongate for more than 9 kilo-bases. Indeed, it is known that insertion of a termination site for PolII near the *HOT1* construct reduces its recombination activity (Voelkel-Meiman et al. 1987; Figure 3G). Moreover, there is a plasmid assay showed the extent of supercoiling is correlated with transcript length in *top1* mutant strains (Brill and Sternglanz, 1988). Thereby, suggesting that movement of transcriptional complex through DNA would be

responsible for accumulation of supercoils.

HOT1 activity in the *leu2* duplicated strains (2.2×10^{-2}) was higher than that of the *his4* duplicated strains (10^{-4}) (Fig. 7 and Fig. 8, in *RDN* strain). Because the size of duplicated homologous region of *his4* is larger than the region of *leu2*, it is difficult to explain the difference by the size of homologous region. The level of transcription from *HOT1* was shown to be sufficiently high in the *his4* strain (Stewart and Roeder, 1989; Huang and Keil, 1995). Therefore, the chromosomal locus may affect the occurrence of DNA breaks and/or unequal homologous recombination. Keil and McWilliams (1993) reported that the *HOT1* activity in *his4* duplication is 12 times higher than that in *CUP1* repeat. Therefore, homologous recombination at the *leu2* site appears to be induced efficiently.

In a haploid *rdnΔΔ* strain carrying the *ADE5,7 HOT1* construct, growth was poor and heterogeneous (Figures 5A and 8). In this strain, observation by microscopy revealed cells with abnormal nuclei. This suggests that continuous stimulation of recombination induces the checkpoint control to stop the cell cycle leading to cell death. Further analysis is required to understand the mechanism by which the checkpoint control is triggered.

The SER002 (*RDN/rdnΔΔ*) strain we obtained seems to have one of the highest recombination activities relative to previously reported results. This hyper-recombinant strain is likely to be very useful in clarifying the mechanisms of homologous recombination in mitotic cells. In higher eucaryotic genome, many tandem and dispersed repeat sequences such as transposons are well known. Exchange

between these sequences may produce chromosomal rearrangements such as translocations. Therefore, mechanisms are thought to exist which repress exchanges within these sequences. Such mechanisms would likely reduce the efficiency of gene targeting. Therefore, systems which enhance recombination activity will be important for developing efficient gene transformation technologies. In addition, our results also suggest that transcriptionally-active regions have possibly been hotspots for chromosomal change throughout evolution. Understanding the mechanisms for transcription-mediated recombination may provide important information on the nature of evolution.

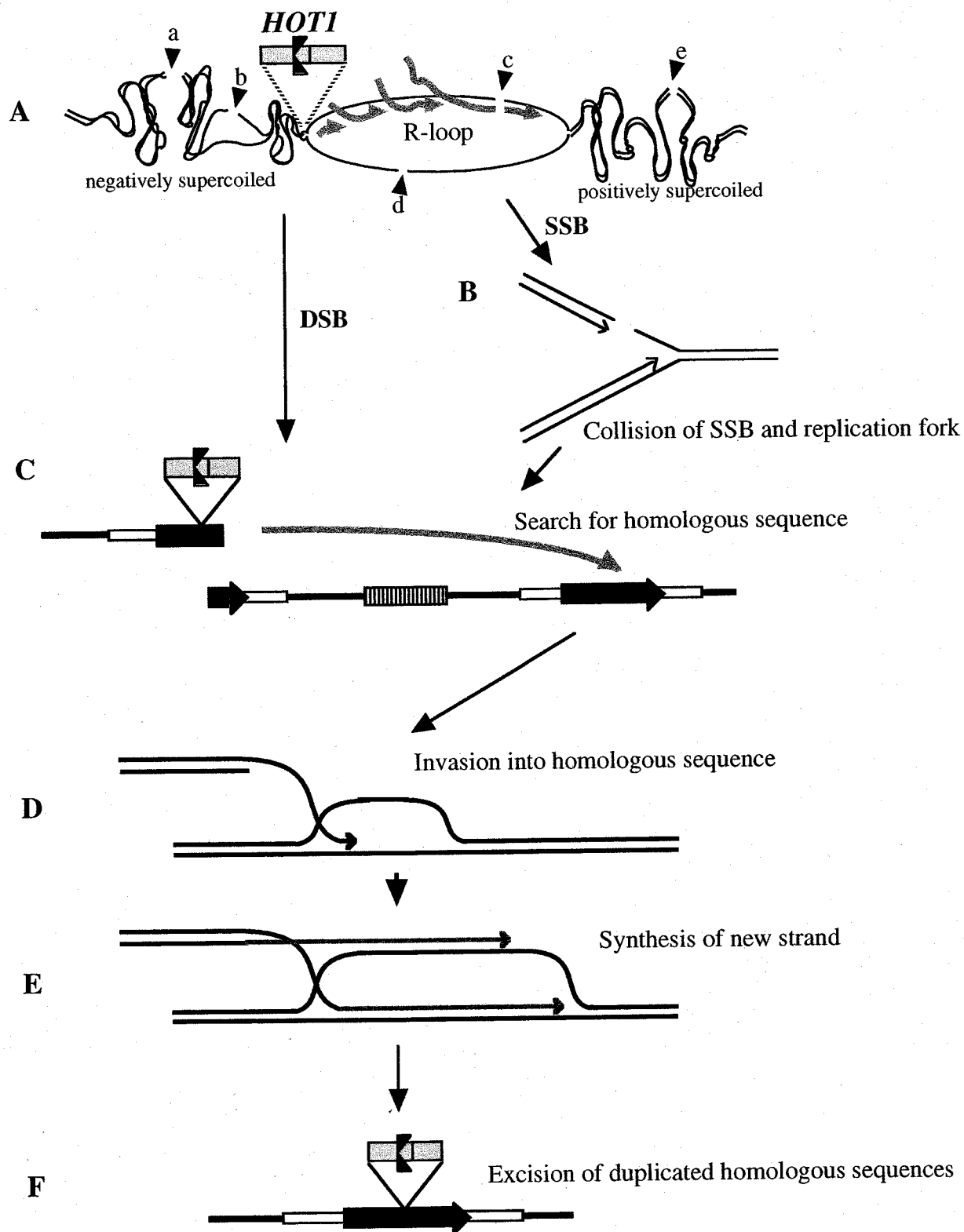


Figure 13. Model of *HOT1* activity.

(A) The light colored arrows show transcript from *HOT1*. DNA is thought to be broken because of torsional stress due to hyper-transcription at negatively and positively super-coiled DNA (a and e), or strand separation (b and d) and formation of an R-loop (d) are also thought to induce the attack of nucleases and DNA-damaging agents. (B) When a single strand break (SSB) is generated and not repaired, progression of the replication fork gives rise to a DSB. The broken end searches for a homologous sequence within two tandem repeats (C), invades, and starts synthesis of a new strand (D and E). The structure is resolved or proceeds as synthesis at a replication fork and the marker gene is lost (F).

IV. Experimental procedure

Media, strains and plasmids

SD is a synthetic glucose-based yeast medium (Kaiser et al., 1994). SG is the same as SD, except that 2% glucose is replaced by 2% galactose. Both SD and SG were supplemented appropriately with amino acids and bases to satisfy nutritional requirements and also to retain unstable plasmids (Kaiser et al., 1994), and are called SD complete (SC) and SG complete, respectively.

Yeast strains and plasmids are listed in Table 1. All strains containing the *ADE5,7* marker were constructed by crossing, using strains SER001, SER004 and SER015. Disruption of *FOBI* was described previously (Kobayashi and Horiuchi, 1996). The diploid strain SER009 (*rdnΔΔ/RDN*, *fob1/fob1*) was sporulated and SER010 (*rdnΔΔ*, *fob1*) was obtained by tetrad analysis. SER003 (*rdnΔΔ*) was obtained by transformation of SER010 using YEp-*FOBI* (Kobayashi et al., 1998). For the *URA3* marker loss assay, the *HOT1* construct containing a part of the *HIS4* gene and its upstream sequence (Wai et al., 2001) was transformed into NOY408-1b (*RDN*) and NOY893 (*rdnΔΔ*), and SER013 and SER014 were obtained respectively.

Measurements of transcription efficiency.

For analysis by northern blot hybridization, 10 ng samples of total RNA were separated on 1% agarose gels and hybridized with ³²P-labeled DNA probes at 48°C. Gel electrophoresis, transfer to the membrane and hybridization were carried out using

NorthernMax following the manufacturer's instruction (Ambion). For primer extension analysis and sequencing, a 20-base oligonucleotide primer (designated HOTp in Fig. 7A), complementary to the chromosomal sequences located 35 to 54 bases downstream from the site of *HOT1* insertion was used (83 to 102 bases from the Pol I transcription initiation site). The 5' end of the primer was ³²P-labeled using T4 polynucleotide kinase (TAKARA, Japan). For the primer extension reaction, 20 µg of total cellular RNA was annealed with 0.2 pmols of end-labeled HOTp primer in a 15 µl reaction containing 40mM Tris-HCl(pH7.5), 20 mM MgCl₂, and 50 mM NaCl for 90 min at 55°C. After annealing, 35 µl of reverse transcription solution containing 200 units of reverse transcriptase (ReverTra Ace TOYOBO, Japan) was added. After 60 min at 42°C, 100 µl of RNase solution (20 µg/ ml RNaseA, 100 µg/ml salmon sperm DNA, 100 mM NaCl) was added, and the mixture was incubated 15min at 42°C, followed by phenol/chloroform extraction. Ethanol-precipitated extension products were suspended in formamide loading buffer (80% formamide, 10mM EDTA, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue), heated to 70°C for 3 min, and fractionated by electrophoresis on 8M urea-6% acrylamide gels. To size extension products, a sequence ladder was generated by using the HOTp primer on the PCR product of *leu2* involving *HOT1*. Autoradiograms were quantified with a BASStation (FUJI, Japan).

Measurement of recombination frequency

Recombination frequencies between two tandemly repeated *leu2* genes were determined

as described by Merker and Klein (2002). The loss of the *ADE5,7* marker integrated between duplicated *leu2* loci was used to measure recombination. Half-sectored red and white colonies indicate the *ADE5,7* marker was lost in the first cell division following plating. Partially white colonies indicate the marker was lost after the first cell division following plating. The recombination rate was determined by considering only the first cell division after plating, and was calculated by dividing the total number of half-sectored colonies by the total number of colonies (half-sectors plus partial sectors).

The *HOT1* recombination system using the *URA3* marker was assayed as described previously (Wai et al. 2001). Strains SER013 (*RDN*, *HOT1*) and SER014 (*rdnΔΔ*, *HOT1*) were grown overnight in SG complete medium lacking uracil. The frequencies of Ura⁻ recombinants were then determined by spotting aliquots of 10-fold serial dilutions of the culture on SG plates with and without 5-fluoroorotic acid (5-FOA).

2D gel analysis

Replication intermediates were analyzed using two-dimensional (2D) agarose gel electrophoresis as described previously (Brewer and Fangman, 1987). DNA was isolated from asynchronous, log-phase cultures. The conditions for the first dimension of the gel were 1% agarose and 0.8 V/cm for 18 h at room temperature. Conditions for the second dimension were 1.2% agarose, 0.3 μg/ml ethidium bromide, and 5 V/cm for 5 h at 4°C.

Cytological Method

Yeast nuclear DNA was fixed on glass slides using 70% ethanol and visualized by DAPI staining (50% glycerol, 1 mg/ml p-phenylenediamine dihydrochloride, 0.15 µg/ml DAPI).

Table 1. Yeast strains and plasmids used

Designation	Genotypes and comments
Strain	
NOY408-1b	<i>MAT[□] ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i> (Nogi et al., 1991)
NOY893	<i>MAT^a ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rdnΔΔ::HIS3</i> , pNOY353 (Nomura lab.)
SER001	<i>MAT^α ade2-1 ade5 ura3-1 his3-11 trp1-1 HOT1::leu2-3::ADE5,7::leu2-3</i> (Kobayashi and Horiuchi, 1996)
SER002	Diploid strain of SER001 and SER015
SER003	<i>MAT^α ade2-1 ade5 ura3-1 his3-11 trp1-1 HOT1::leu2-3::ADE5,7::leu2-3</i> <i>rdnΔΔ::HIS3 fob1::LEU2</i> , pNOY353 Yep- <i>FOB1</i>
SER004	Same as NOY408-1b but <i>ade5,7::HISG</i>
SER005	<i>MAT^α ade2-1 ade5 ura3-1 his3-11 trp1-1 leu2-3::ADE5,7::leu2-3</i>
SER006	Diploid strain of SER001 and SER004
SER007	Diploid strain of SER003 and SER015
SER008	Same as SER001 but <i>fob1::LEU2</i>
SER009	Same as SER002 but <i>fob1::LEU2</i>
SER010	Same as SER003 but <i>fob1::LEU2</i>
SER011	Same as SER006 but <i>fob1::LEU2</i>
SER012	Same as SER007 but <i>fob1::LEU2</i>
SER013	Same as NOY408-1b but <i>HIS4 his4Δ::URA3 bik1Δ::HOT1</i>

(Wai et al., 2001)

SER014 Same as NOY893 but *HIS4 his4Δ::URA3 bik1Δ::HOT1*

SER015 Same as NOY893 but *ade5,7::HISG*

Plasmid

pNOY353 Multicopy plasmid vector, *TRP1*, *GAL7-35S* rDNA, 2μ (Nogi et al.,1991)

YEp24 Multicopy plasmid vector, *URA3*, 2μ, pBR322, *amp^r*, *Tc^r* (NEB)

Yeplac195 Multicopy plasmid vector, *URA3*, 2μ, (Gietz and Sugino, 1988)

Yep-*FOB1* Yeplac195 carrying *FOB1* (Kobayashi et al., 1998)

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