The Regulation of RNA Splicing and the Role of Mer3 Helicase in Meiotic Recombination

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Title

Involvement of the *MRE2* Gene of Yeast in Formation of Meiosis-Specific Double-Strand Breaks and Crossover Recombination through RNA Splicing

Abstract

Background: The *mre2* mutant of *Saccharomyces cerevisiae* is defective in meiotic recombination and produces inviable spores, but the sensitivities to DNA damaging agents, methyl methanesulfonate and ultraviolet light are not altered by the mutation. Mre2 has two copies of RNA recognition motif (RRM), suggesting its participation in RNA metabolism in meiosis.

Results: An amino acid substitution in N-terminal RRM of Mre2 confers a meiotic recombination defect. Using this *mre2N* strain, the *MER2* gene was isolated as a multi-copy suppressor of the recombination defect. Meiosis-specific splicing of *MER2* pre-mRNA was impaired in the *mre2* deletion (*mre2Δ*) mutant. The *mre2Δ* mutant was defective in the formation of meiosis-specific double-strand breaks (DSBs) and crossover and noncrossover recombinants. When the chromosomal *MER2* gene was replaced with the intronless derivative of *MER2* gene, *cMER2*, the formation of DSBs and of noncrossover recombinants were restored in the *mre2Δ* mutant. However, the amount of crossover recombinants produced in the *mre2Δ cMER2* strain was approximately 30% that in the wild-type. In addition, the *mre2Δ cMER2* mutant was defective in chromosome segregation and in viable spore formation.

Conclusions: Mre2 participates in the formation of DSBs through meiosis-specific splicing of *MER2* pre-mRNA. Besides, Mre2 is involved also in crossover recombination, possibly through splicing of RNA from another gene(s).

Introduction

During meiosis, diploid cells produce haploid gametes after a single round of DNA replication followed by two successive rounds of chromosome segregations. During meiotic prophase, genetic recombination occurs at a high level and provides the physical connection between homologs which ensures their proper segregation (reviewed in Carpenter 1994). Two types of recombination occur between homologs, crossovers with exchanged flanking chromosome arms and noncrossovers without such exchange. In combination with sister chromatid cohesion, crossover events are essential for proper segregation of homologs (Baker *et al.* 1976; Bell & Byers 1982; Carpenter 1988; Engebrecht *et al.* 1990; Storlazzi *et al.* 1995).

In yeast *Saccharomyces cerevisiae*, the mechanism of meiotic recombination has been extensively studied by physical analysis (Cao *et al.* 1990; Storlazzi *et al.* 1995). Almost all meiotic recombinations are initiated by formation of DNA double-strand breaks (DSBs) at specific chromosomes sites called recombination hot-spots (Sun *et al.* 1989; Cao *et al.* 1990; Wu & Lichten 1994; Ohta *et al.* 1994; Xu & Kleckner 1995). The DSB ends are rapidly resected to produce 3' over-hanging single-stranded tails of about 600 nucleotides (Sun *et al.* 1989; Alani *et al.* 1990; Cao *et al.* 1990; Bishop *et al.* 1992). The tails search for homology and invade homologous DNA sequences with the aid of Rad51 and Dmc1, the *E.coli* RecA homologs, and their accessory proteins (Shinohara *et al.* 1992; Bishop *et al.* 1992; Sung 1994; Sung & Robberson 1995; Shinohara & Ogawa 1995). Resulting prominent intermediates are double Holliday junctions, which may yield both crossover and noncrossover recombinants (Schwacha & Kleckner 1994, 1995).

Various yeast genes required for meiotic recombination have been isolated (Petes *et al.* 1991; Ajimura *et al.* 1993; Roeder 1995; Bai & Symington 1996). One of these, the *MER1* gene encodes an RNA binding protein with the KH motif (Siomi *et al.* 1993, 1994), and is expressed only in meiosis (Engebrecht *et al.* 1991; Nandabalan & Roeder 1995). It has been shown that *MER1* is required for meiosis-specific splicing of *MER2* pre-mRNA (Engebrecht *et al.* 1991; Nandabalan *et al.* 1993; Nandabalan & Roeder 1995). The *MER2* gene is required for chromosome synapsis and initiation of meiotic recombination (Engebrecht *et al.* 1990; Cool & Malone 1992; Rockmill *et al.* 1995).

The *mre2* mutant is defective in both inter- and intragenic recombination and produces inviable spores. In mitosis, however, it is not defective in recombination and no more sensitive to DNA damaging agents than the wild-type (Ajimura *et al.* 1993). The spore lethality of the *mre2* mutant is suppressed if the first meiotic division is bypassed by introduction of a *spo13* mutation, suggesting that *MRE2* is involved in an early step of meiotic recombination (Ajimura *et al.* 1993). The *MRE2* gene encodes a 523 amino acid protein (Ogawa *et al.* 1995; accession number D11461) that has two copies of an RNA Recognition motif (RRM) conserved in many RNA binding proteins (Kenan *et al.* 1991; Draper 1995). These features suggest that *MRE2* participates, directly or indirectly, in recombination through its interaction with RNA.

Here I report that the *MER2* transcript is a target of *MRE2*-dependent RNA splicing and that the *MRE2* gene is required for formation of meiosis-specific DSBs. I further show that Mre2 may also be required for splicing of RNA from another gene(s) that is required specifically for crossovers.

Results

Meiotic defects of an *mre2* mutant carrying a mutation in an RNA recognition motif

Mre2 has two copies of the RNA Recognition Motif (RRM) (Fig. 1A). Each RRM contains two consensus sequences for binding to RNA, which called RNP1(K/R-G-F/Y-G/A-F-V-X-F/Y) and RNP2(L/I-F/Y-V/I-G/K-N/G-L) (reviewed in Kenan *et al.* 1991; Draper 1995). In the case of a splicing factor, Prp24, which binds to U6 and U4/U6 hybrid snRNAs (Jandrositz & Guthrie 1995; Ghetti *et al.* 1995), changing the last RNP2 leucine residue in one of its RRMs into a proline residue confers a temperature-sensitive growth defect (Shannon & Guthrie 1991).

For understanding the role of Mre2 in meiotic recombination, identification of the target genes is crucial. As the starting point for this analysis, I first made *mre2* mutations expected to affect RNA binding activity. The last leucine residue of RNP2 hexamer sequence in either of the two Mre2 RRMs was replaced by a proline residue with site-directed mutagenesis (Fig. 1A). These mutations in N-terminal RRM (N-RRM) and C-terminal RRM (C-RRM) were designated as *mre2N* and *mre2C*, respectively. Plasmids carrying the *mre2N*, *mre2C* or wild-type *MRE2* gene were constructed based on YCp50 (Rose *et al.* 1987) and introduced into *mre2Δ* cells to examine their capacity for prototroph formation due to meiotic recombination between the heteroalleles, *arg4-nsp / arg4-bgl* or *his4X / his4B*. While the prototroph formation in the *mre2C* mutant was almost equal to the wild-type level at 30°C, it was only one hundredth of this or less in the *mre2N* mutant (Fig. 1B). The meiotic properties of the *mre2N* mutation were further analyzed using a strain in which the *mre2N* mutant gene had been substituted for the chromosomal *MRE2* gene, so as to avoid false interpretation due to plasmid loss. Induction of meiotic recombination was impaired in the *mre2N* mutant to the same extent as in the *mre2* deletion (*mre2Δ*) mutant at both 23 and 34°C (Table 1). Thus, the last leucine residue of RNP2 in N-RRM of Mre2 is necessary for its protein function in meiotic recombination. However, the *mre2N* mutant showed a temperature-sensitive spore formation (Table 1), suggesting that the *mre2N* mutant protein still retained a function necessary for spore formation at 23°C.

The *MER2* gene, a multi-copy suppressor of the meiotic recombination defect in the *mre2N* mutant

To identify the target RNAs of Mre2, I carried out a screening for a multi-copy suppressor of the recombination defect in the *mre2N* mutant. *Mre2N* mutant cells were transformed with a yeast genomic library constructed in a multi-copy vector, YEp24 (Botstein *et al.* 1979), and screened for transformants producing His^+ and Arg^+ prototrophs after incubation on sporulation medium. Out of 9,000 transformants, five were selected which showed an increased level of meiotic recombination at both 23 and 34°C (Fig. 2). Restriction enzyme mapping of the plasmid DNA recovered from these transformants revealed that four plasmids contained the *MRE2* gene itself, and that the other plasmid, pSMT1, had a 5.4 kb insert. Subcloning and partial DNA sequencing analysis of the insert revealed that the insert does not contain the *MRE2* gene and that the suppression activity was encoded in a 2.0 kb region containing the previously identified *MER2* gene (Engebrecht *et al.* 1990; Cool & Malone 1992).

Interestingly, multi-copy suppression of the recombination defect by the *MER2* gene was not observed in the *mre2Δ* mutant (Fig. 2). This suggests that the residual activity of the *mre2N* mutant protein is required for the suppression.

MRE2 is required for meiosis-specific splicing of the *MER2* transcript

The primary transcript of the *MER2* gene has an 80 nucleotide (nt) intron with a non-canonical 5' splice site (Engebrecht *et al.* 1991). To know whether *MRE2* is required for meiosis-specific splicing of the *MER2* transcript, a Northern blot analysis was carried out. In a wild-type strain, the size of the *MER2* transcript changed from 1.1 kb to 1.0 kb after the cells had entered into meiosis. In the *mre2Δ* strain, whereas, the size remained unchanged (Fig. 3A). The *MRE2* function must be responsible for the size reduction. The amounts of *MER2* transcripts relative to those of *ACT1* transcripts, whose amount is not changed through mitosis and meiosis (Percival-Smith & Segall 1984), increased with incubation time in meiosis both in *mre2Δ* and wild-type cells (Fig. 3A). However, the ratios of the relative amounts of *MER2* RNA in *mre2Δ* cells to those in wild-type cells decreased form 0.73 to 0.36 during five hours incubation in SPM (Fig. 3A and 3C). Thus, the unspliced *MER2* RNA may be less stable than the spliced *MER2* RNA, or the *MER2* transcription rate may be affected by *MRE2*.

In order to establish whether the pre-mRNA splicing of *MER2* transcripts occurs in the *mre2Δ* mutant, a reverse transcription-polymerase chain reaction (RT-PCR) was carried out, and the products

were analyzed by agarose gel electrophoresis. When the *MER2* intron-flanking primers (Fig. 4A) were used for RT-PCR, two DNA fragments were amplified: 640 bp (unspliced) and 560 bp (spliced), corresponding to pre-mRNAs and spliced transcripts of the *MER2* gene, respectively. In the wild-type strain, the ratios of the unspliced to the spliced species were three to two in mitosis and one to six in meiosis (Fig. 4A). In *mre2Δ* cells, in contrast, the ratio was about ten to one throughout mitosis and meiosis (Fig. 4A). *ACT1* pre-mRNA was spliced in both mitotic and meiotic cells and the splicing did not depend on the *MRE2* function (Fig. 4B). Thus, *MRE2* is required for the efficient splicing of an 80 nt intron of *MER2* pre-mRNA.

As the *MER1* gene is transcribed only in meiosis and is required for the splicing of *MER2* pre-mRNA (Engebrecht *et al.* 1991; Nandabalan & Roeder 1995), the defect in the splicing of *MER2* pre-mRNA in the *mre2Δ* mutant might be caused by a lack of *MER1* expression. The presence of the *MER1* transcripts in both *mre2Δ* and wild-type cells was therefore examined by Northern blotting. Production of *MER1* transcripts was limited to meiosis, and approximately equal amounts were observed in both cases (Fig. 3B), indicating that *MRE2* is not required for *MER1* expression.

Restoration of DSB formation in the *mre2Δ* mutant by replacement of the *MER2* gene with the intron-less *MER2* gene

With the *mre2Δ* mutant being defective in the formation of meiosis-specific DSBs (see below), it is possible that *MRE2* participates in DSB formation through the splicing of *MER2* pre-mRNA. To test this possibility, I examined whether elimination of the *MER2* intron is sufficient for generation of DSBs even in the absence of the *MRE2* function. A *mre2Δ cMER2* mutant strain was constructed, in which the chromosomal *MER2* gene was replaced by the intronless derivative of *MER2* gene, *cMER2* (Engebrecht *et al.* 1991; see Experimental procedures). The formation of meiotically induced DSBs at *HIS4-LEU2* recombination hot spot on chromosome III was examined by Southern blotting analysis (Fig. 5). While not being detected in the *mre2Δ* mutant, DNA fragments diagnostic of the formation of DSBs, DSB I and DSB II, were detected at almost the same levels in the *mre2Δ cMER2* mutant as in the wild-type after four hours incubation in SPM, and then decreased with further meiosis progression. In addition, the DSBs in *mre2Δ cMER2* cells were smeared, indicating resection of DSB

ends. These results show that the presence of the *cMER2* gene is sufficient for DSB formation in the absence of the *MRE2* function.

Another role for *MRE2* in a later stage after DSB formation

To test whether *cMER2* completely bypasses the *MRE2* function, meiotic division, spore formation and viability were examined. Although normal meiotic divisions were observed in the *mre2Δ* and *mre2Δ cMER2* strains, spore formation and spore viability were impaired in both strains (Table 2). These meiotic properties of the *MRE2 cMER2* strain were not different from those of the wild-type, indicating dispensability of the *MER2* intron. Thus function of *MRE2* is not limited to the *MER2* pre-mRNA splicing.

Defects in spore formation and viability are often associated with deficiencies in meiotic recombination and in chromosome segregation. Therefore, I carried out a time course analysis of heteroallelic recombination with return-to-growth experiments (Sherman & Roman 1963). The frequencies of recombination (recombinants per survival) at *arg4-nsp/arg4-bgl* and *his4X/his4B* heteroalleles in the *mre2Δ cMER2* strain increased with the incubation time and reached a maximal level at four hours in SPM (Fig. 6B). The maximum percentages of His^+ and Arg^+ recombinants in the *mre2Δ cMER2* mutant were 0.65% and 1.11%, respectively, these values being two-thirds those with the wild-type strain (His^+ 0.98% and Arg^+ 1.86%). After the four hour time point, however, the frequencies of recombination and survivals in the *mre2Δ cMER2* mutant decreased and reached one-tenth of the wild-type levels at 12 hours (Fig. 6A and 6B). These results suggest that the *mre2Δ cMER2* mutant is defective at a stage following DSB formation.

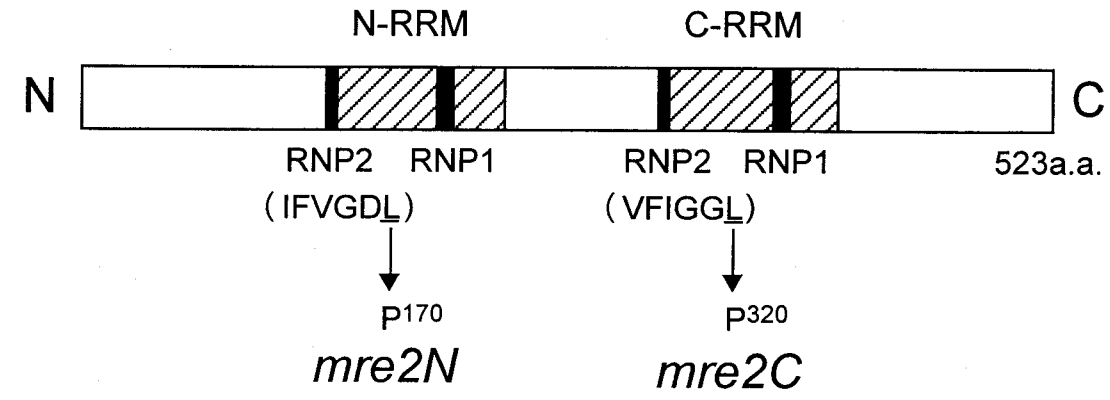
To know whether homologous chromosomes segregate properly at meiosis I in the mutant, the disjunction of the heteroallele, *CYH2/cyh2*, was examined by monitoring the formation of cycloheximide resistant (cyh^r) colonies (Game *et al.* 1980). The proportion of cyh^r colonies (cyh^r per survival) in the *mre2Δ cMER2* mutant was only 1.7% after 24 hours in SPM, which was not significantly different from that with the *mre2Δ* mutant (0.3%). Under the same condition, 96% of wild-type colonies were resistant (Fig. 6B). Thus, the *MRE2* gene may be involved not only in DSB formation but also in chromosome segregation in meiosis.

Crossover deficiency and noncrossover proficiency in the *mre2Δ cMER2* mutant

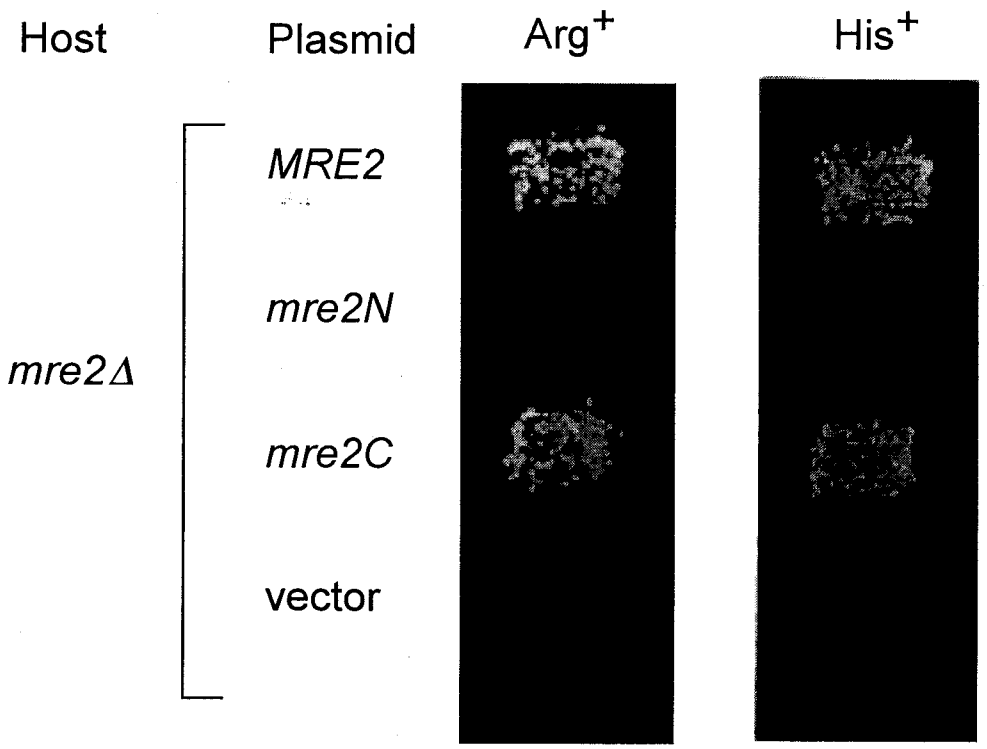
In order to establish whether the impairment of segregation of homologous chromosomes at meiosis I in the *mre2Δ cMER2* mutant was caused by a defect in a meiotic recombination step after DSB occurrence, the formation of physical recombinants produced in meiosis from crossover (CR) and noncrossover (NCR) processes was examined. The DNA preparations from the cells in meiosis, were digested with both *Xho*I and *Mul*I, and the fragments of interest produced at *HIS4-LEU2* region on chromosome III (Storlazzi *et al.* 1995) were analyzed by Southern blotting (Fig. 7A). In this CR/NCR assay, four recombinant DNA fragments, R1~R4, were detected. In wild-type cells, over 95% of R1 and R2 are produced by CR, almost all of R4 is formed by NCR, and R3 is formed by both CR and NCR (Storlazzi *et al.* 1995). The amounts of R1 and R2 in *mre2Δ cMER2* cells were reduced to 30% those in wild-type cells (Fig. 7B). In contrast, almost the same amount of NCR (R4) was formed in both cases. In *mre2Δ* cells, the recombinants were not detected. These results indicate that the *mre2Δ cMER2* mutant is defective in CR formation, but not in NCR. Mre2 probably participates in the pre-mRNA splicing of an unidentified gene(s) which play a key role in CR formation.

Figure1

A


N-RRM
C-RRM
N
C
RNP2
RNP1
RNP2
RNP1
523a.a.
(IFVGDL)
(VFIGGL)
P170
P320
mre2N
mre2C

B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Plasmid
Arg+
His+
MRE2
mre2N
mre2Δ
mre2C
vector

Figure 2

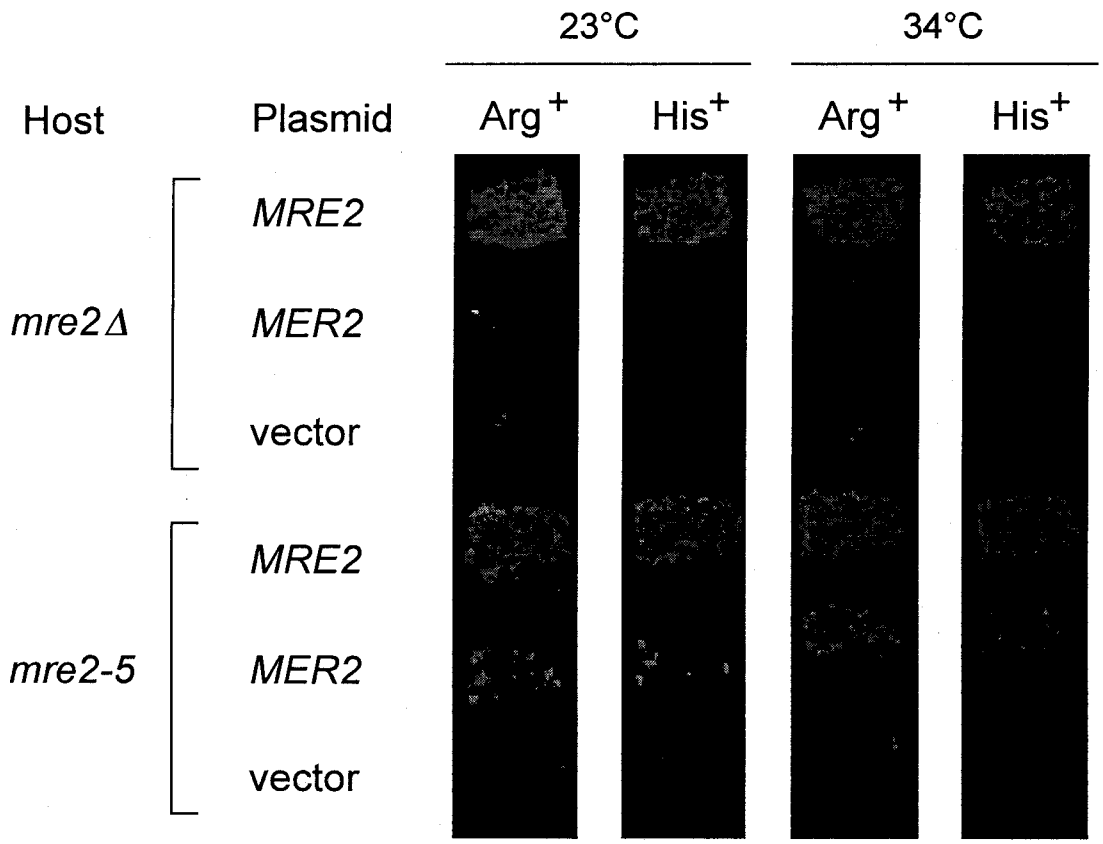
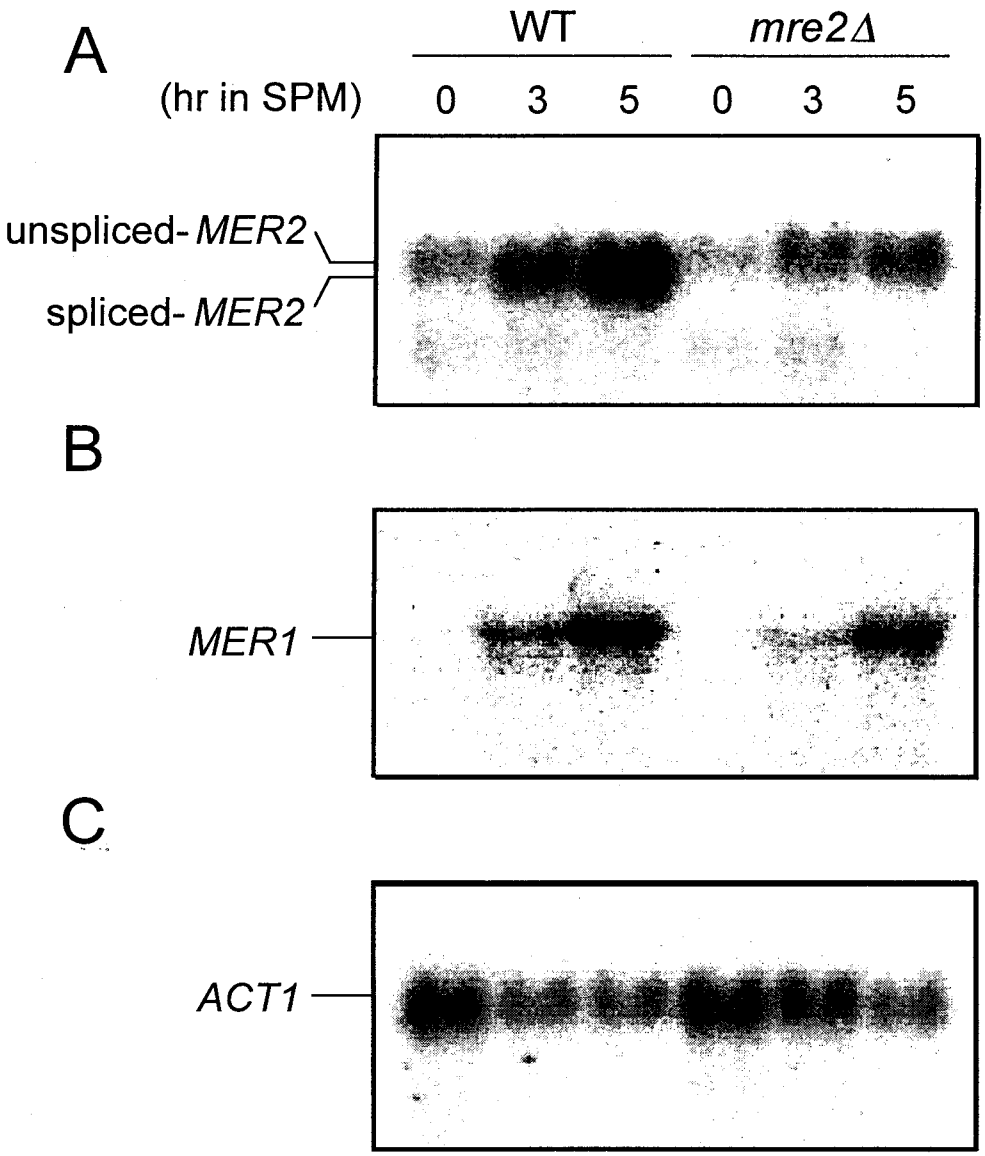

23°C
34°C
Host
Plasmid
Arg+
His+
Arg+
His+
mre2Δ
MRE2
MER2
vector
mre2-5
MRE2
MER2
vector

Figure 3


A
WT
mre2Δ
(hr in SPM)
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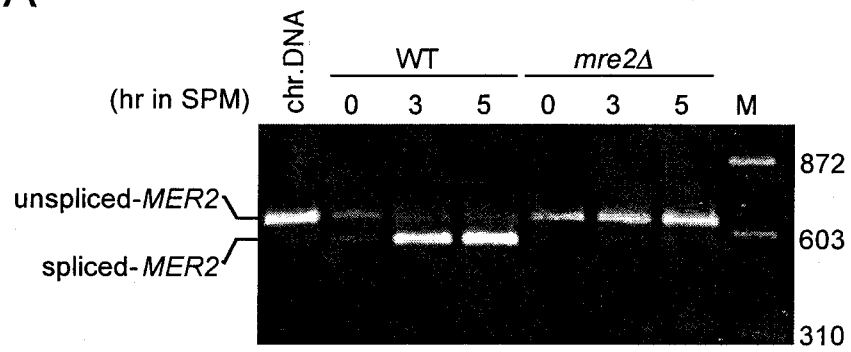
unspliced-MER2
spliced-MER2
B
MER1
C
ACT1

Figure 4

A


chr.DNA
WT
mre2Δ
(hr in SPM)
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3
5
0
3
5
M
872
603
310
unspliced-MER2
spliced-MER2

B

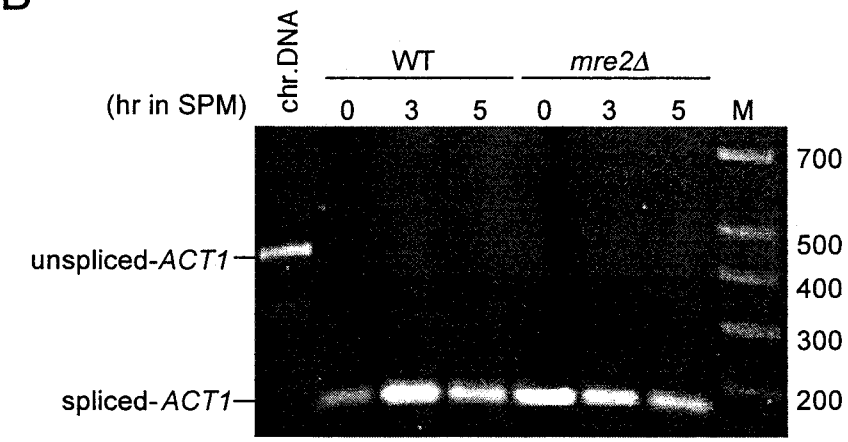
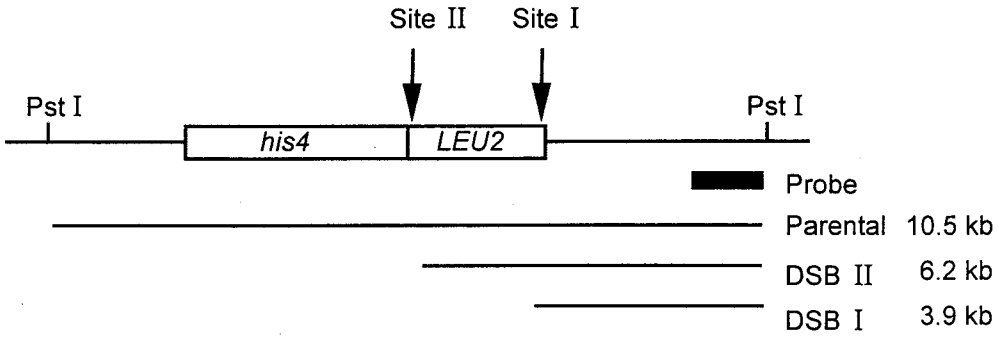

chr.DNA
WT
mre2Δ
(hr in SPM)
0
3
5
0
3
5
M
700
500
400
300
200
unspliced-ACT1
spliced-ACT1

Figure 5

A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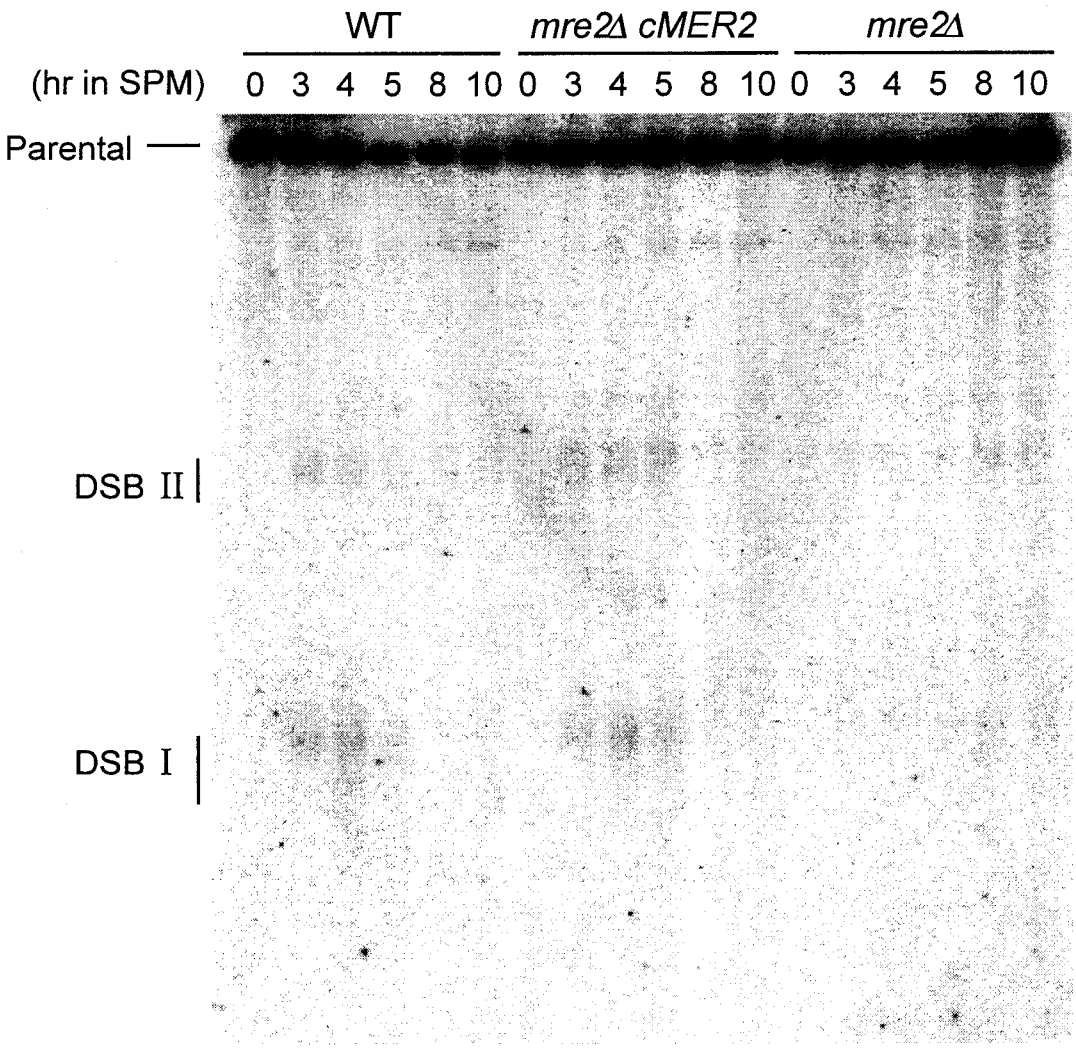
Site I
Pst I
Pst I
his4
LEU2
Probe
Parental 10.5 kb
DSB II 6.2 kb
DSB I 3.9 kb

B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mre2Δ cMER2
mre2Δ
(hr in SPM)
0 3 4 5 8 10 0 3 4 5 8 10 0 3 4 5 8 10
Parental
DSB II
DSB I

Figure 6

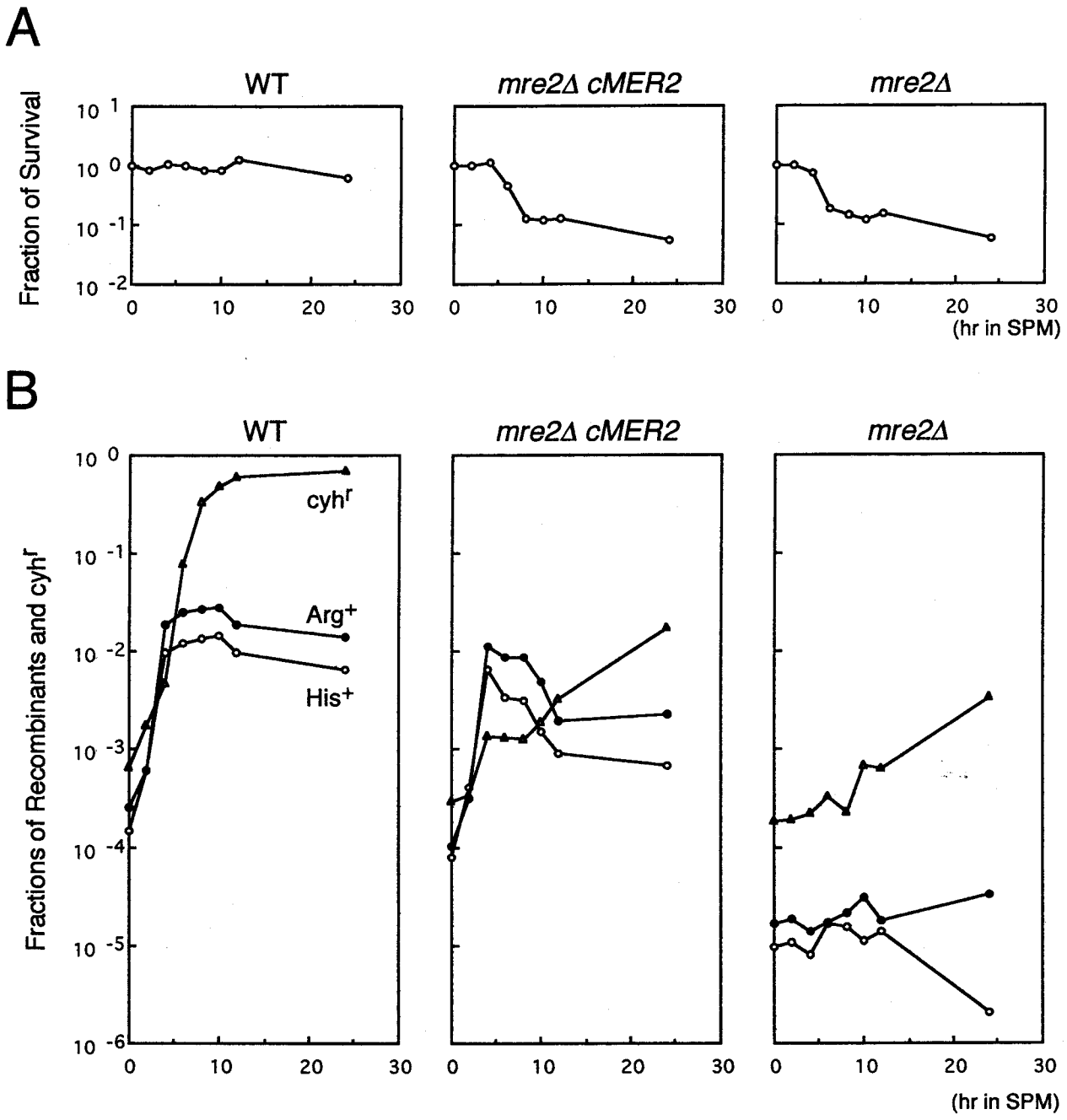
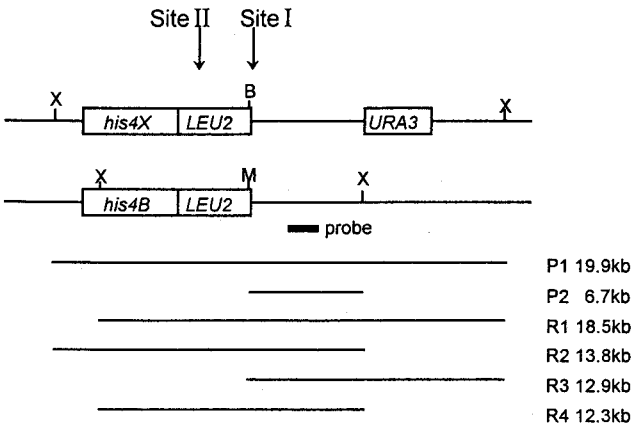

A
WT
mre2Δ cMER2
mre2Δ
Fraction of Survival
10 1
10 0
10 -1
10 -2
0
10
20
30
(hr in SPM)
B
WT
mre2Δ cMER2
mre2Δ
Fractions of Recombinants and cyh^r
cyh^r
Arg+
His+
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
0
10
20
30
(hr in SPM)

Figure 7

A


Site II
Site I
X
B
X
his4X
LEU2
URA3
X
M
X
his4B
LEU2
probe
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R2 13.8kb
R3 12.9kb
R4 12.3kb

B

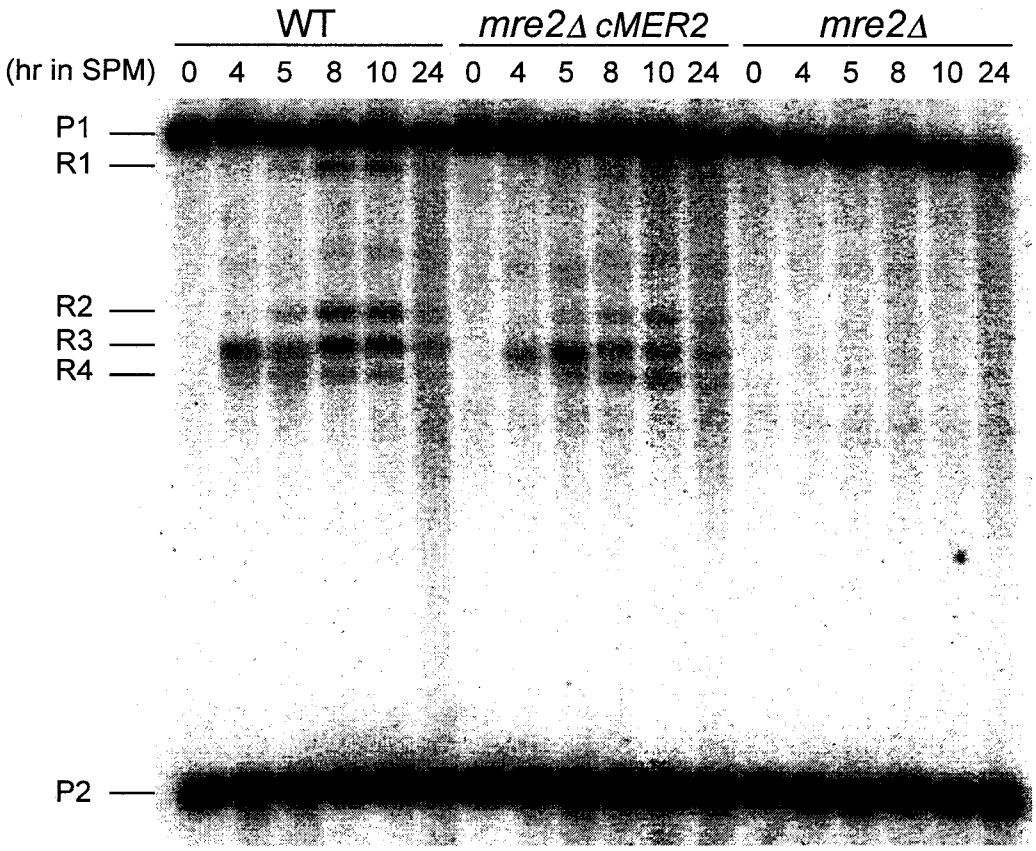
WT
mre2Δ cMER2
mre2Δ
(hr in SPM) 0 4 5 8 10 24 0 4 5 8 10 24 0 4 5 8 10 24
P1
R1
R2
R3
R4
P2

Table 1

Table 1. Comparison of recombination, spore formation and spore viability in wild-type and mutant yeast.

Strain	Relevant Genotype	Temperature[a]	Matured Spores[b]	Viable Spores[c]	Recombination[d]			
					ARG4		*HIS4*	
					Mitosis	Meiosis	Mitosis	Meiosis
TNY037	WT	23	41	93 (74/ 80)	3.4×10^{-5}	4.2×10^{-2}	1.2×10^{-5}	2.3×10^{-2}
		34	41	98 (78/ 80)	2.5×10^{-5}	1.4×10^{-2}	1.7×10^{-5}	8.3×10^{-2}
TNY036	*mre2Δ*	23	1.2	<1.3 (0 / 80)	2.3×10^{-5}	2.2×10^{-5}	9.0×10^{-6}	2.9×10^{-5}
		34	0.3	<1.3 (0 / 80)	1.7×10^{-5}	3.9×10^{-5}	7.9×10^{-6}	6.1×10^{-5}
TNY041	*mre2N*	23	25	<0.52 (0 /192)	3.0×10^{-5}	4.6×10^{-5}	9.3×10^{-6}	4.0×10^{-5}
		34	0.9	<1.3 (0 / 80)	2.4×10^{-5}	3.1×10^{-5}	1.3×10^{-5}	5.0×10^{-5}

Table 2. Meiotic division, spore formation and spore viability

Strain	Relevant Genotype	Meiotic Division[a]		Spore Formation[b]	Spore Viability[c]
		MI	MII		
TNY058	WT	9	79	61	95 (152/160)
TNY101	*cMER2*	11	70	62	100 (160/160)
TNY060	*mre2Δ*	8	77	0.9	<0.7 (0 /160)
TNY102	*mre2Δ cMER2*	9	73	0.9	<0.7 (0 /160)

Table 3. Strains fo *Saccharomyces cerevisiae* used

Strain	Genotype	Source
AHY11	*MATα ho::LYS2 ura3 lys2 mre2::hisG-URA3-hisG*	A.H. Hayashi
AHY12	*MATα ho::LYS2 ura3 lys2 mre2::hisG-URA3-hisG*	A.H. Hayashi
NKY1238	*MATa leu2::hisG his4X::LEU2-URA3 ho::LYS2 ura3 lys2 arg4-nsp*	N. Kleckner
NKY1240	*MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl*	N. Kleckner
TNY037	*MATa leu2::hisG his4X::LEU2 ho::LYS2 ura3 lys2 arg4-nsp* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl*	This study
TNY036	TNY037, except $\frac{mre2::hisG}{mre2::hisG}$	This study
TNY041	TNY037, except $\frac{mre2\text{-}5}{mre2\text{-}5}$	This study
TNY042	*MATa leu2::hisG his4X::LEU2 ho::LYS2 ura3 lys2 arg4-nsp cyh2* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl CYH2*	This study
TNY047	TNY042, except $\frac{mre2::hisG}{mre2::hisG}$	This study
TNY048	TNY042, except $\frac{mre2\text{-}5}{mre2\text{-}5}$	This study
TNY058	*MATa leu2::hisG his4X::LEU2-URA3 ho::LYS2 ura3 lys2 arg4-nsp cyh2* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl CYH2*	This study
TNY060	TNY058, except $\frac{mre2::hisG}{mre2::hisG}$	This study
TNY101	TNY058, except $\frac{cMER2}{cMER2}$	This study
TNY102	TNY058, except $\frac{mre2::hisG\ cMER2}{mre2::hisG\ cMER2}$	This study
NKY1551	*MATa leu2::hisG his4X::LEU2(Bam)-URA3 ho::LYS2 ura3 lys2 arg4-nsp* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl*	N. Kleckner
TNY214	NKY1551, except $\frac{mre2::hisG}{mre2::hisG}$	This study
TNY215	NKY1551, except $\frac{mre2::hisG\ cMER2}{mre2::hisG\ cMER2}$	This study

Figure Legends

Figure1. Mutation sites in RNA recognition motifs (RRMs) and plate assay results for meiotic recombination in the *mre2N* and *mre2C* mutants.

(A) The conserved Leu residues (L^{170} and L^{320}) in each of the two RNP2 of *Mre2* were changed to Pro. The *mre2* mutant with the amino acid substitution in N-RRM was designated as *mre2N*, and that in C-RRM as *mre2C*. (B) Plate assay results for meiotic recombination. The *mre2Δ* (TNY047) transformants harboring YCpMRE2 (*MRE2*), YCpmre2N (*mre2N*), YCpmre2C (*mre2C*) or YCp50 (vector) were patched to complete medium lacking uracil and then replica plated to SPM-uracil to induce sporulation. After 3 days incubation at 30°C, the SPM-uracil plates were replicated to complete medium lacking arginine or histidine. Arg^+ and His^+ prototrophs resulted primarily from gene conversion between each pair of heteroalleles; *arg4-nsp/arg4-bgl* and *his4X/his4B*.

Figure 2. Multi-copy suppression of the meiotic recombination defect in the *mre2* mutant.

mre2Δ (TNY047) and *mre2N* (TNY048) cells harboring multi-copy plasmids, YEpMRE2 (*MRE2*) and pSMT1 (*MER2*) or YEp24 (vector) , respectively, were examined for prototroph formation after induction of meiosis as described in Table 1 *d*.

Figure 3. Northern blot analysis of *MER2* and *MER1* transcripts.

Total RNA samples were prepared from wild-type (TNY058) and *mre2Δ* (TNY060) cells before and after induction of meiosis (0, 3 and 5 hr in SPM) and subjected to Northern blotting analysis. The same membrane was generally hybridized with probes specific to *MER2* (A), *MER1* (B) and then *ACT1* (C) after intervening steps.

Figure 4. RT-PCR assay for splicing of *MER2* and *ACT1* transcripts.

Total RNA was prepared from wild-type (TNY058) and *mre2Δ* (TNY060) cells as described in Figure 3. After treatment with RNase-free DNase I to eliminate contaminating DNA, RNA was subjected to reverse transcription followed by amplification by PCR. The products were

analyzed by agarose gel electrophoresis and visualized by Ethidium Bromide (EtBr). The positions of the spliced and unspliced products are indicated on the left, and the sizes of standards on the right of each panel. (A) and (B) represent amplified products synthesized using the *MER2*-specific primers, JE6 and JE7 (Engebrecht *et al.* 1991) and the *ACT1*-specific primers, pTN10 and pTN11, respectively. Amplified products from chromosomal DNA indicate the positions of unspliced products (lane chr. DNA). As size markers, *Hae*III digested ΦX174 fragments were used in lane M of (A) and a 100 bp ladder in lane M of (B). Spliced-*MER2* detected at 0 hr in WT could reflect a small fraction of cells that had initiated meiosis in pre-sporulation culture, YPA, as is known to occur with the SK1 strains used.

Figure 5. Restoration of DSB formation in the *mre2Δ cMER2* mutant

(A) The *HIS4-LEU2* locus (Cao *et al.* 1990; Xu & Kleckner 1995) on chromosome III. The positions of the two meiosis-specific DSB sites (Sites I and II) are shown. DSBs at these seits yield the heterogeneous bands, DSB I and II, respectively. Parental, DSB I and II fragments were detected with a probe for the position indicated by the solid bar. (B) Southern blot analysis of DSB formation in wild-type, *mre2Δ cMER2* and *mre2Δ* cells. DNAs were extracted from cultures of wild-type (TNY058), *mre2Δ cMER2* (TNY102) and *mre2Δ* (TNY060) strains at the indicated times after induction of meiosis, digested with *Pst*I, separated by agarose gel electrophoresis, transferred to nylon membranes, and fragments of interest were detected with the ^{32}P-labeled hybridization probe.

Figure 6. Time course analysis of cell viability, meiotic recombination and cyh^r colony formation in a return-to-growth experiment. After meiosis was initiated by transferring to SPM medium, cells of wild-type (TNY058), *mre2Δ cMER2* (TNY102) and *mre2Δ* (TNY060) strains were returned to vegetative growth conditions at the times indicated by plating on glucose-containing medium. (A) Fractions of surviving cells were measured as the ratio of colony-forming units (c.f.u.) at the times indicated to c.f.u. at time 0. (B) Meiotic recombination at *agr4-nsp/arg4-bgl* or *his4X/his4B* heteroalleles was measured by Arg$^+$ or His$^+$ prototroph formation. Proportions of Arg$^+$, His$^+$ and cyh^r c.f.u. divided by the total c.f.u. at the respective times are

indicated. The differences in the frequencies at 0 hr may be due to a small population of cells that initiated meiosis in pre-sporulation culture, since Arg^+, His^+ and cyh^r frequencies were not found to be significantly different among the three strains in mitosis (not shown).

Figure 7. Detection of physical recombinants (CR/NCR assay). (A) A map showing the positions of the two DSB sites (Site I and II) and the polymorphic restriction sites (B, Bam HI; M, Mlu I; X, Xho I) for homologous chromosomes at the *HIS4-LEU2* locus (Storlazzi *et al.* 1995). Digestion of DNA with *Bam*HI, *Mlu*I and *Xho*I produces parental (P1 and P2) and recombinant (R1, R2, R3 and R4) restriction fragments. R1 and R2 result mainly from crossover recombination, R4 from noncrossover and R3 from both crossover and noncrossover (Storlazzi *et al.* 1995). (B) Meiosis was initiated by transfer of cells to sporulation medium and DNAs were isolated from samples of wild-type (NKY1551), *mre2Δ cMER2* (TNY215) and *mre2Δ* (TNY214) strains at the times indicated. For all samples, aliquots were digested in parallel with the three restriction enzymes; *Bam*HI, *Mlu*I and *Xho*I. The resulting fragments were separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the probe indicated in (A).

Table Legends

Table 1. Comparison of sporulation and recombination in wild-type and mutant yeast

a Incubation temperature (°C).

b Percentages of cells containing spores. Cultures were taken at 24hr after transfer to SPM and more than 400 cells were examined by phase-contrast microscopy. Spore formation was signaled by the appearance of two, three or four phase-bright bodies within a cell.

c Percentages of viable spores. Spore viability was measured by dissection of asci by micromanipulation, and the proportion of spores germinating to give visible colonies was assessed after incubation for 4 days at 23°C or 3 days at 34°C. No. of viable spores / no. of total spores examined are given in parentheses.

d Formation of Arg^+ and His^+ recombinant was observed between heteroalleles, *arg4-nsp/arg4-bgl* and *his4X/his4B*. The frequency of recombination in mitosis was measured at an early-log phase (~$2x10^7$ cells/mL) in YPD and recombination in meiosis was measured by return-to-growth after six hours incubation in SPM. Numbers are ratios of recombinant colony forming units to total colony forming units. The values of recombination frequencies present are the mean values obtained from three independent cultures.

Table 2. Meiotic division, spore formation and viability

The occurrence of meiotic division was monitored using cultures at 24 hours after transfer to SPM, staining cells with the DNA-specific stain DAPI and examining more that 200 cells in each alipuots by fluorescence microscopy. Cells that have completed one meiotic division (MI) contain two DAPI-staing bodies; cells that have completed both meiotic divisions (MII) contain three or four DAPI-staining bodies. Spore formation was examined as described in Table 1. Spore viability was measured by dissection of asci by micromanipulation, and the proportion of spores germinating to give visible colonies was assessed after incubation for 3 days at 30°C.

a Percentages of cells that have completed one meiotic division (MI) or both divisions (MII).

b Percentages of cells containing spores.

c Percentages of viable spores among total spores. Exact numbers of viable spores / total spores examined are given in parentheses.

Discussion

I showed that the *MRE2* gene is involved in the formation of meiosis specific DSBs by carrying out meiosis-specific splicing of the *MER2* transcript. However, the splicing of the *MER2* transcript is not the only function of the *MRE2* gene, because the *mre2Δ cMER2* mutant, that can form meiosis-specific DSBs, remains defective in the formation of CR recombinants and viable spores in meiosis. The *MRE2* gene may participate in crossover through the pre-mRNA splicing of another gene(s).

MRE2 and *MER1* in meiosis-specific splicing of *MER2* pre-mRNA

Mre2 has two copies of RRM. The Mre2 amino acid substitution of the conserved leucine at RNP2 in N-RRM, but not in C-RRM, caused inactivation of the protein function in meiotic recombination. The role of N-RRM for the Mre2 function was also suggested by my finding that N-RRM, but not C-RRM, is required for binding of Mre2 to RNAs (A. H. Hayashi & H. Ogawa unpublished result).

The *mre2N* mutant shows a temperature-sensitive spore formation, but is defective in induction of meiotic recombination at both 23 and 34°C. This could be explained as the consequences of two different functions of *MRE2*. Alternatively, *MRE2* might have an only one function, and the residual activity of the *mre2N* mutant protein might be sufficient for spore formation at the lower temperature.

While expression of both the *MER2* and the *MRE2* genes occurs in mitosis and meiosis (Engebrecht *et al.* 1991; A. H. Hayashi, S-H. Leem & H. Ogawa Unpublished result), an efficient splicing of *MER2* pre-mRNA is limited to meiosis, and requires the product of the *MER1* gene, which is only transcribed during meiosis. I have shown that the *mre2Δ* mutant is defective in *MER2* pre-mRNA splicing and that *MRE2* is not required for *MER1* expression. Therefore, the *MRE2* gene in addition to the *MER1* gene is involved in this splicing process. Mer1 appears to play a role in stabilizing the base pairing between the non-canonical 5' splice site of *MER2* RNA and U1 snRNA (Nandabalan *et al.* 1993, 1995). In addition to the case of *ACT1* pre-mRNA splicing, the splicing of pre-mRNA of both recombination genes, *DMC1* (Bishop *et al.* 1992) and

REC114 (Malone *et al.* 1991; Pittman *et al.* 1993; Ajimura *et al.* 1993), which are expressed only in meiosis and whose transcripts carry a canonical 5' splice site, occurs in the *mre2Δ* mutant (my unpublished results). Therefore, Mre2 may not be required for the splicing of pre-mRNA which has canonical 5' splice site.

Almost all the phenotypic characteristics of mutants that delete the *MER1* or *MRE2* genes are similar, with the exception of the suppression of the meiotic recombination defect by the *MER2* multi-copy suppressor. The *MER2* gene on a multi-copy plasmid suppresses the heteroallelic recombination defect in the *mer1* deletion mutant (Engebrecht *et al.* 1991), while it does not suppress the defect in the *mre2Δ* mutant. Thus, Mre2 may directly participate in the *MER2* pre-mRNA splicing reaction and have a more essential role than Mer1.

A crossover defect in the *mre2Δ cMER2* mutant

I have shown that the *mre2Δ* mutant is defective in the formation of meiosis-specific DSBs, crossover (CR) and noncrossover (NCR) recombinants, and viable spores. The facts that the replacement of chromosomal *MER2* with *cMER2* in the *mre2Δ* mutant restores the formation of DSB and that the *mer2Δ* mutant is defective in DSB formation (my unpublished result; Rockmill 1995), indicate that *MRE2* is involved in the formation of DSBs through splicing of *MER2* pre-mRNA. The *mre2Δ cMER2* mutant is fully proficient for the formation of NCRs but not for the formation of CRs. The CR^-/NCR^+ phenotype is also observed in *mer1* $MER2^{++}$ and *zip1* mutants, in which CR^-/NCR^+ has been argued from both genetic and physical analysis (Engebrecht *et al.* 1990; Sym *et al.* 1993; Sym & Roeder 1994; Storlazzi *et al.* 1995, 1996). The CR defect in the *mre2Δ cMER2* mutant is unlikely to reflect a residual deficit of DSBs because CR and NCR recombinants are not affected coordinately. Thus, Mre2 is suggested to be involved in pre-mRNA splicing of another gene(s) which is specifically required for CRs. Requirement of an extra component for CR is suggested from the fact that CR^-/NCR^+ is the null phenotype of a *zip1* mutant. Alternatively, Mre2 may directly be involved in CRs. However, this possibility is unlikely because I have recently identified a gene that acts as a multi-copy suppressor of the recombination defect in the *mre2 cMER2* mutant. The transcript of the gene has an intron with a non-canonical 5' splice site (manuscript in preparation).

Experimental Procedures

Plasmids

Plasmids were constructed by standard procedures (Sambrook *et al.* 1989). YEpMRE2 and YCpMRE2 were constructed by insertion of a 3.4 kb *Cla*I fragment containing the *MRE2* gene into *Cla*I sites of YEp24 (Botstein *et al.* 1979) and YCp50 (Rose *et al.* 1987), respectively.

pTN1 containing the *mre2N-URA3-mre2N* construction was manifactured in three steps. (1) A 2.2 kb *Eco*RI region corresponding to 2μ-ori DNA was deleted from YEp24 to form YEp-D. (2) A 3.3 kb *Cla*I-*Nsp*V fragment of the *mre2N* gene was inserted into the YEp-D *Cla*I site (YEp-D-mre2N). (3) Finally, a 3.2 kb *Xba*I-*Sal*I fragment containing a 3.2 kb *Cla*I-*Bgl*II region of the *mre2N* gene was introduced into the YEp-D-mre2N *Xba*I-*Sal*I sites (pTN1). pTN19 containing the *cMER2-URA3-cMER2* construction was produced in two steps. (1) A 1.0 kb *Eco*RI-*Hpa*II fragment containing the intron-less *MER2, cMER2,* gene from pME270 (Engebrecht *et al.* 1991) was introduced into the YEp24 *Eco*RI-*Cla*I sites (YEp-D-cMER2). (2) A 1.9 kb *Eco*RV-*Bam*HI fragment containing the *cMER2* gene was introduced into the YEp-D-cMER2 *Sma*I-*Bam*HI sites (pTN19).

pTN3 was generated by introduction of a 1.8 kb *Eco*RI-*Sac*II fragment containing the *MER2* gene from pME50 (Engebrecht *et al.* 1990) into the *Eco*RI-*Sac*I sites of pBluescriptII KS$^+$ (Stratagene). The *mer2* gene disruption plasmid, pTN37 was constructed by insertion of a 3.8 kb *Bam*HI-*Xba*I fragment containing the *hisG-URA3-hisG* gene (Alani *et al.* 1987) into the *Bgl*II-*Xba*I sites of pTN3.

A 3.0 kb *Ssp*I-*Eco*RI fragment containing the *MER1* gene from pME1/R1000 (Engebrecht *et al.* 1989) was inserted into the *Ssp*I-*Eco*RI sites of pBluescriptII SK$^+$ (Stratagene), creating pTN20. A 1.6 kb *Bam*HI-*Hind*III fragment containing the upstream two-thirds of the *ACT1* gene from pYA301 (Gallwitz & Sures 1980) was introduced into the pBluescriptII SK$^+$ (Stratagene) *Bam*HI and *Hind*III sites to form pTN7.

The plasmids used for the site-directed mutagenesis, KS-BX and SK-XP, were constructed as follows; a 0.6 kb *Bam*HI-*Xho*I fragment corresponding to N-RRM domain of the *MRE2* gene and a 0.8 kb *Xho*I-*Pma*CI corresponding to C-RRM were inserted respectively into the *Bam*HI

and *Xho*I sites of pBluescriptII KS$^+$ (Stratagene) and the *Xho*I and *Sma*I sites of pBluscriptII SK$^+$ (Stratagene).

Strains

Genotypes of the SK1 derived (Kane & Roth 1974) yeast strains used in this study are shown in Table 3. Yeast transformation was carried out by the lithium acetate procedure (Ito *et al.* 1983), except for the screening for a multi-copy suppressor, where electroporation was employed (Meilhoc *et al.* 1990).

The *mer2* deletion disruptant was constructed using the one-step gene substitution method (Rothstein 1991). A 5.0 kb *Eco*RI-*Sac*I fragment containing *mer2::hisG-URA3-hisG* from pTN37 was introduced into an appropriate diploid strain. In the *mre2::hisG-URA3-hisG* strain, a 2.0 kb *bgl*II-*Eco*47 III region of the *MER2* gene was replaced by a 5.0 kb *hisG-URA3-hisG* DNA segment (A. H. Hayashi personal communication).

The two-step gene substitution method (pop-in/pop-out replacement) was used for introduction of the *mre2N* and the *cMER2* (Engebrecht *et al.* 1991) alleles into their correct chromosomal loci. In the *mre2N* case, a 7.9 kb *Cla*I fragment containing *mre2N-URA3-mre2N* from pTN1 was used for yeast strain transformation. In the case of *cMER2* (Engebrecht *et al.* 1991), a 4.0 kb *Eco*RI-*Bam*HI fragment containing *cMER2-URA3-cMER2* from pTN19 was used. The resultant transformants were selected on complete medium lacking uracil. Uracil auxotrophs were selected by plating cultures on SD plates containing 5-fluoro-orotic acid (Boeke *et al.* 1984). All the transformants were verified by Southern blot analysis (Southern 1975) for the correct integration.

The spontaneous *cyh2* mutant strain used in this work was selected on YPD plates containing 10 μg/mL cycloheximide. The *cyh2* genotype was confirmed by observing the recessive cycloheximide resistant phenotype on transformation with the *CYH2* gene containing plasmid, pRS316-CYH2 (Sikorski & Boeke 1991).

The bacterial strains DH5 (Low 1968) and DH5α (Hanahan 1983) were used for plasmid preparation and CJ236 (Kunkel 1985) was used for growth of the uracil-substituted phagemid.

Media

Yeast media were prepared according to Sherman *et al.* (1986). MYPD, MYPL, YPA and synthetic medium (SD) were prepared as described by Shinohara *et al.* (1992). Liquid SPM contains 1% potassium-acetate and 0.02% raffinose. SPM plates contain 1% potassium-acetate, 0.1% yeast-extract, 0.05% glucose and 1/4 amounts of the necessary amino acids. For solid media, 1.5% agar was added.

Genetic procedures

Synchronous meiotic cultures were obtained by the method described by Shinohara *et al.* (1992). In the assay for spore viability, colony formation was assessed after tetrad dissection followed by 3 days incubation at 30 or 34°C or by 4 days incubation at 23°C.

Return-to-Growth experiment

At the indicated times after induction of meiosis, cells were plated on SD plates lacking arginine or histidine, and SD plates supplemented with 3μg/mL cycloheximide so as to measure the numbers of Arg^+ or His^+ recombinants and of cycloheximide resistants. Simultaneously, the total numbers of colony-forming units were assesed by plating cells on synthetic complete medium.

RNA isolation, Northern Blotting, and RT-PCR

Synchronous meiotic cultures were obtained by the previously described method (Cao *et al.* 1990), except that yeast cells were pregrown on YPA medium for 13 hr to approximately 5 × 10^7 cells per mL. Cells were pelleted at various times in meiosis, frozen in liquid nitrogen and stored at -80^{o}C until subsequent RNA extraction. Total RNA was isolated from yeast by glass-bead lysis and phenol extraction (Ausubel *et al.* 1987).

For Northern blotting analysis, total RNA samples were separated on 1.5% agarose gels in MOPS/formaldehyde buffer (Sambrook *et al.* 1989) and transferred to NYTRAN nylon membranes (Schleicher & Schuell) in a 20mM Na-phosphate buffer (pH7.2). A series of hybridizations and re-hybridizations of the same membranes was performed as described by

Sambrook *et al.* (1989). For the detection of *MER2*, *MER1* and *ACT1* transcripts, a 1.0 kb *Pst*I-*Nhe*I fragment (positions -22 to 942; with A of ATG for the first Met as position 1) from pME270 (Engebrecht *et al.* 1991), a 0.5 kb *Sac*I-*Xba*I fragment (positions 69 to 568) from pTN20, and a 0.6 kb *Cla*I-*Cla*I fragment (positions 280 to 843) from pTN7 were ^{32}P-labeled using a random primer method (Ausubel *et al.* 1987) and used as the respective probes. De-hybridization from the membranes was carried out by incubation in 2% SDS and 20mM Na-phosphate buffer (pH7.2). Northern blot signals were quantified with a Fuji BAS2000 phosphoimager.

After treatment with RNase-free DNase I *FPLCpure*™ (Pharmacia) to eliminate contaminating DNA, 2μg aliquots of total RNA were subjected to reverse transcription (RT) for producing cDNAs, using 3 pmol each *MER2*-specific primer JE7 (Engebrecht *et al.* 1991) and the *ACT1*-specific primer priTN11 (5'CGTCACCGGCAAAACCGGC3'). The RT reaction was performed with 16 U M-MuLV reverse transcriptase (New England Biolabs) as recommended by the supplier. PCR (Saiki *et al.* 1988) was carried out with the cDNAs using *MER2* intron-flanking primers, JE6 and JE7 (Engebrecht *et al.* 1991) or *ACT1* intron-flanking primers, priTN10 (5' GTTAAAGAATAGGATCTTCTACTACATC-AGC3') and priTN11(see above) under the following conditions: 2 min. at 94^{o}C, 2 min. at 54^{o}C and 3 min. at 72^{o}C for each of 35 cycles and then 2 min. at 54^{o}C and 7 min. at 72^{o}C. 1.25 U of rTaq DNA polymerase (Toyobo) were used for each PCR sample. The amplified *MER2* and *ACT1* products were analyzed by 0.8 and 1.2 % agarose gel electrophoresis, respectively. The sizes of the amplified products corresponding to the unspliced and spliced *MER2* RNA are 640 and 560 bp, and those of *ACT1* products are 475 and 166 bp. Band intensities were measured with PDI Quantify One®.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to Kunkel (1985) using phagemids KS-BX and SK-XP. 10 pmol of a primer containing the mutant sequences 5'TTTGTTGGGGACCCAGCACCAAATG3' (*mre2N*) and 5'CGGAGGACCTT-CATCACTGG3' (*mre2C*) (mutations sequence are underlined) were annealed to uracil containing ssDNA (0.5 pmol) from KS-BX and SK-XP, respectively. Mutation sites were confirmed by sequencing the plasmid DNA. The mutated 0.6 kb *Bam*HI-*Xho*I segment from KS-BX and 0.2 kb *Xho*I-*Bst*XI

segment from SK-XP were then substituted for the corresponding regions of YEpMRE2 and YCpMRE2, respectively.

Physical detection of meiotic recombination-related events

DNA was extracted from synchronous meiotic cultures as described (Cao *et al.* 1990). DSB detection was carried out by an established method (Shinohara *et al.* 1992) with a slight modification. Aliquots (30 mL each) of sporulation cultures were removed from SPM and fixed in 70% ethanol and 10mM EDTA. Detection of DSBs and physical recombinants (CR/NCR assay) was performed as described earlier (Storlazzi *et al.* 1995). A *Bgl*II-*Pst*I DNA fragment of pNKY291 was labeled with ^{32}P by the random primer method (Ausubel *et al.* 1987) and used as a probe for Southern hybridization. DNA signals were quantified as above described.

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Title

Mer3, a Meiosis-Specific helicase of S. cerevisiae,

is Required for Crossover/Noncrossover Differentiation and Crossover Interference

Abstract

MER3, a novel meiosis-specific gene of S. cerevisiae, encodes a protein homologous to a DNA/RNA helicase family. *MER3* primary transcripts have an intron containing noncanonical 5' splice site (GUAGU) and its splicing depends on both *MRE2* and *MER1* encoding RNA-binding proteins. The *mer3Δ* mutation decreases crossovers but increases noncrossovers. Crossover interference is also impaired in *mer3Δ*, resulting in homolog nondisjunction at meiosis I. *MER3* functions in double-strand break (DSB) repair and is required for disassembling of Rad51 and Dmc1, RecA homologues, from chromosomes and for heteroduplex formation at positions away from a DSB site. Mer3 localizes discrete sites on zygotene and pachytene chromosomes. These suggest that Mer3 promotes strand exchange for crossover rather than noncrossover.

Introduction

During meiosis, following a single round of DNA replication, two successive rounds of chromosome segregation occur to produce haploid gametes from diploid cells. The first meiotic division is unique in that homologous chromosomes (homologs) are synapsed and then distributed to opposite spindle poles. In meiotic prophase I, elevated levels of recombination occur and provide physical connections between homologs. Crossover recombination inherently accompanies reciprocal exchange of the flanking chromosomal arms and, in combination with sister chromatid cohesion, is needed for faithful segregation of chromosomes at meiosis I in many organisms (for review, see Carpenter, 1988; Kleckner, 1996; Roeder, 1997). The distribution of crossovers along homologs is controlled by the mechanism called crossover interference, probably so that every pair of homologs sustains at least one crossover (Kaback et al., 1992; Sym and Roeder, 1994; Egel, 1995; Chua and Roeder, 1997; Conrad et al., 1997). Noncrossover recombination does not associate with the reciprocal exchange.

All or most meiotic recombination in S. cerevisiae is initiated by the formation of region-specific DNA double-strand breaks (DSBs) that are rapidly resected to produce 3' over-hanging single-strand ends (Sun et al., 1989; Alani et al., 1990; Cao et al., 1990; Sun et al., 1991; Bishop et al., 1992; Liu et al., 1995; Xu and Kleckner, 1995; Baudat and Nicolas, 1997). *RAD51* and *DMC1*, E. coli *recA* homologues, are required to convert DSBs to double Holliday junctions (Shinohara et al., 1992; Bishop et al., 1992; Schwacha and Kleckner, 1997). Since Rad51 and Dmc1 localize to discrete sites on meiotic chromosomes and Rad51 has in vitro strand exchange activity, these proteins are likely tobe involved in DSBs/Holliday junctions transition directly (Bishop, 1994; Sung, 1994; Li et al., 1997). However, their activity is less effective than RecA and some additional factors are required in vivo (Sung, 1997a, 1997b; Shinohara and Ogawa, 1998). During or after the formation of Holliday junctions, homologs are synapsed along their entire lengths to produce the synaptonemal complex (SC) (Padmore et al., 1991; Schwacha and Kleckner, 1994). Zip1 is a component of the central region between a pair of homologs in the context of SC and is required for crossover interference. Zip1 probably plays a role to tansmit negative signals from a site of crossover to its neighbors to prevent additional crossover (Sym et al., 1993; Sym and Roeder, 1994, 1995). However, a role

independent of SC polymerization is also proposed for Zip1 (Storlazzi et al., 1996). Intact DNA duplexes containing heteroduplex (HD) region appear shortly before or concomitant with the appearance of mature recombinants (Goyan and Lichten, 1993; Nag and Petes, 1993). This might reflect a coordinated action of branch migration and resolution of Holliday junctions in yeast meiosis, that was observed in E. coli (Eggleston et al., 1997). Resolution of Holliday junctions results in the formation of both crossover and (presumptively) noncrossover recombinants (Storlazzi et al., 1995; Schwacha and Kleckner, 1995).

MRE2 and *MER1* encode RNA-binding proteins containing the RNA recognition motif and the KH motif, respectively (Kenan et al., 1991; Siomi et al., 1993), and are required for meiosis-specific splicing of the *MER2* intron consisting of noncanonical 5' splice site (GUUCGU; consensus GUAYGU) (Engerecht et al., 1991; Nandabalan and Roeder, 1995; Nakagawa and Ogawa, 1997). A mutation in either *MRE2*, *MER1* or *MER2* impairs DSB formation (Rockmill et al., 1995; Storlazzi et al., 1995; Nakagawa and Ogawa, 1997). Although introduction of intronless *MER2* (*cMER2*) into the *mre2Δ* strain restores DSB formation, the *mre2Δ cMER2* strain is still defective in crossover but noncrossover (Nakagawa and Ogawa, 1997). Similarly, a *mer1* strain harboring *MER2* on a multicopy plasmid is defective specifically in crossover (Engebrecht et al., 1990; Storlazzi et al., 1995). Thus, it has been suggested that there is an unidentified target(s) of *MRE2*- and *MER1*-dependent splicing that is required for crossover.

Here, I have identified *MER3*, a novel target gene of *MRE2*- and *MER1*-dependent splicing, as a multicopy suppressor of a crossover defect in a *mre2 cMER2* mutant. The Mer3 protein contains the DNA/RNA helicase and the putative zinc-finger motif and localizes to discrete sites on meiotic chromosomes. The *mer3Δ* mutation perturbs a balance of crossover and noncrossover, and impaires both the frequency and the distribution of crossover. The *mer3Δ* mutant is also defective in repair of meiosis-specific DSB and heteroduplex formation. I propose that Mer3 is involved in DSBs/Holliday junctions transition and thus affects the frequency and distribution of crossover.

Results

The *MER3* Gene, a Multicopy Suppressor of a Crossover Defect in a *mre2 cMER2* Mutant

To identify a missing component responsible for a crossover defect in a *mre2 cMER2* mutant, a search for a multicopy suppressor of the defect was carried out. The *mre2N cMER2* strain was used as a host in the screening. Because *mre2N* mutation confers temperature-sensitive spore formation, suggesting a residual activity of the mutant protein (Nakagawa and Ogawa, 1997). *mre2N cMER2* cells were transformed with a yeast genomic DNA library constructed on a multicopy plasmid, induced into meiosis at 23°C, and selected for recombinants that had undergone crossover in the *LEU2-HIS4* or *TRP5-CYH2* interval and haploidization (see Figure 1A legend). Among ~9,000 transformants tested, eight transformats produced significant levels of crossover recombinants compared to the background. Restriction enzyme mapping of plasmids recovered from the transformants revealed that two plasmids had the *MRE2* gene itself and six plasmids had inserts not identical but containing an overlapping region. I named as *MER3*, the suppressor gene possibly contained in the region. To know what genetic conditions were required for the suppression by *MER3*, *mre2N cMER2* related strains were compared for their ability to produce recombinants and viable spores (Figure 1A). *MER3* exhibited the suppression effect in both *mre2Δ cMER2* and *mre2N cMER2*, but in *mre2Δ* nor *mre2N*, indicating that *cMER2* was essentail for the suppression. When *mre2Δ cMER2* and *mre2N cMER2* were compared, only *mre2N cMER2* cells harboring *MER3*-plasmid produced nearly wild-type levels of recombinants and viable spores.

Subcloning analysis (Figure 1B) and partial DNA sequencing revealed that the *MER3* suppressor activity resided in a 4.8 kb EcoRI-SalI region including a hypothetical open reading frame YGL251c (Coissac et al., 1996) found in the yeast genome project. However, YGL251c with its upstream region of 508 bp (pTN66 in Figure 1B), was not sufficient for the suppression.

MRE2- and *MER1*-Dependent Splicing of the *MER3* Transcript

The result descrived above raises the possibility that the *MER3* primary transcript has an intron. To test this possibility, I first detected *MER3* transcripts by Northern blotting using total RNA prepared from mitotic and meiotic cells (Figure 2A). Only in meiotic cells, approximately 4.2 and 3.0 kb *MER3*

transcripts were specifically detected in the wild type but not in *mer3Δ* (see below). Induction of meiosis-specific *MER3* transcription is consistent with the presence of the URS1 element (CGGCGGGTA at -132 to -123 bases, see below) (Steber and Esposito, 1995). Two kinds of *MER3* transcripts were also detected in *mre2Δ* and *mer1Δ*, although the amount was decreased to 60% the wild-type level when *ACT1* transcripts were used as an internal control. A slight change in size could not be distinguished in this assay.

I prepared a pair of primers (priTN1 and priTN2) located in the upstream region of YGL251c and carried out RT-PCR analysis using meiotic RNA (Figure 2B). If no intron between the primers, the expected length of an amplified fragment is 500 bp. Using RNA prepared from wild-type cells, however, a fragment shorter than 500 bp was exclusively amplified. Cloning and DNA sequencing of the short fragment revealed that *MER3* primary transcripts had a 152 nt intron. As shown in Figure 2C, 5' splice site sequence of *MER3* is different from the consensus and the same 5' splice site sequence has not been reported, so far. In *mre2Δ* and *mer1Δ*, only the unspliced *MER3* product was detected. On the other hand, the spliced *ACT1* product was exclusively observed in these mutants as well as the wild type.

Elimination of *MER2* and *MER3* Introns Suppresses Meiotic Defects in *mre2Δ* and *mer1Δ* Mutants

As the *MER3* splicing depended on *MRE2* and *MER1*, elimination of the *MER3* intron could bypass the defect in *mre2Δ* or *mrer1Δ* meiosis. The genomic *MER3* gene was replaced by intronless *MER3* (*cMER3*), and meiotic divisions, sporulation and spore viability were examined using the *cMER3* strains at 30°C (Table 1). As is the case of *cMER2*, *cMER3* in the wild-type strain background did not significantly change the meiotic properties, indicating that the intron was dispensable for *MER3* function. Only when both *cMER2* and *cMER3* were introduced, spore viabilities of *mre2Δ* and *mer1Δ* strains were apparently increased. Although, the meiotic properties of *mre2Δ cMER2 cMER3* and *mer1Δ cMER2 cMER3* were different but both do not reach to the wild-type level.

The formation of crossover recombinant DNA in the *HIS4::LEU2* region on chromosome III was examined by Southern blotting (Figure 3,see also Figure 7A). No recombinant was detected in *mre2Δ* and *mre2Δ cMER3*, consisting with their severe defects in viable spore formation. Compared

to *mre2Δ cMER2* (one-third the wild-type level), *mre2Δ cMER2 cMER3* produced an increased amount of recombinants (half the wild-type level). Therefore, I concluded that *MER3* was a target of *MRE2*- and (presumably) *MER1*-dependent RNA splicing pathway for crossover recombination.

The Mer3 Protein Contains a DNA/RNA Helicase and a Zinc-finger Motif

The spliced *MER3* transcript encodes 1,187 amino acids polypeptides encompassing YGL251c. The N terminal region of the Mer3 protein contains the seven conserved motifs characteristic of a DEXH-box type of a DNA/RNA helicase family (Figure 4A; Pause and Sonenberg, 1992; Gorbalenya et al, 1989). Comparison of helicase motif sequences among the proteins found in a BLAST search with Mer3 (E value $<1e^{-60}$) is shown in Figure 4B. The C terminal region of Mer3 did not show any significant homology in the database but contained a putative zinc-finger motif (CFHSCKDKTQCRHLCC) (Figure 4A).

The *mer3Δ* Mutant Impairs the Frequency and Distribution of Crossovers

To know the *MER3* function, I constructed the *mer3Δ* strain, in which three-fourth of the *MER3* open reading frame including the first ATG was deleted. In vegitatively growing condition, the *mer3Δ* strain showed neither a growth defect nor a defect in repair of DNA damages caused by MMS treatment (data not shown).

To assess crossover and noncrossover recombination, tetrad analysis was carried out at the lower temperature (23°C) where relatively high sporulation was observed in the *mer3Δ* mutant (see below). In five intervals on two different chromosomes examined, overall reduction of crossover was observed in *mer3Δ* compared to the wild type (Table 2). The average decrease in the crossover frequency in *mer3Δ* was 2.3-fold in these intervals. In contrast, the *mer3Δ* mutation increased noncrossover frequencies 1.5- to 2.6-fold at three locus examined (Table 3).

The distribution of crossovers was further analyzed. Once one crossover occurred, another crossover nearby on the same chromosomes would be inhibited (crossover interference). Interference is expressed in terms of the frequency of nonparental ditypes (NPDs) indicative of double crossovers in a given interval. The NPD ratio is the frequency of NPDs observed in tetrad analysis divided by the frequency of NPDs expected assuming no interference (see Experimental

Procedures). Thus, no interference would result in a NPD ratio of 1.00. Compared to NPD ratios in wild type, all NPD ratios in *mer3Δ* were close to 1.00 (Figure 5A). Unregulated distribution of crossovers in *mer3Δ* was confirmed by measuring the proportion of zero, one and two crossovers (Figure 5B). In wild type, there were an excess of one crossovers at the expense of zero and two crossovers, compared to the proportion predicted by Poisson. In *mer3Δ*, however, the proportion observed was close to the proportion predicted.

Nondisjunction of Homologous Chromosomes at Meiosis I

Table 4 shows the distribution of four-, three-, two-, one-, zero-spore-viable (4, 3, 2, 1, 0sv) tetrads in wild type and *mer3Δ*. In *mer3Δ*, spore death is not randomly distributed and 4sv, 2sv and 0sv tetrads make up 91% of total tetrads, suggestive of improper segregation of chromosome. This prompted us to monitor chromosome segregation during meiosis using TNY374 (wild type) and TNY375 (*mer3Δ*) strains, in which homologous centromeres at *CENIII* are distinguished by *URA3* and *TRP1* genetic markers. Examination of 408 2sv tetrads produced in *mer3Δ* revealed that 388 (95%) were pairs of sister spores (i.e. both spores were Ura^+/Trp^- or Ura^-/Trp^+) and 68 (17%) were disomic for chromosome III (i.e. both spores were Ura^+/Trp^+). Although, all 5 2sv tetrads produced in the wild type were pairs of non-sister spores. These indicate nondisjunction of homologous chromosomes (homologs) at meiosis I in *mer3Δ*. Among the 68 pairs of disomes, any crossover in the *MAT-CENIII* or the *CENIII-HIS4* interval on chromosome III (i.e. maters or His^+/His^-) was not observed, while 11 crossovers were expected from the crossover frequencies among 4sv tetrads in *mer3Δ* (Table 2). In addition, among 111 1sv tetrads in *mer3Δ*, 23 (21%) were disomic for chromosomes III (i.e. Ura^+/Trp^+) and were all non-recombinant (i.e. non-mater and His^+). Thus, nondisjunction of homologs in *mer3Δ* is likely to be due to the absence of crossover between them. Among 81 3sv tetrads in *mer3Δ*, the Ura^+/Trp^-, Ura^-/Trp^+ and Ura^+/Trp^+ set of spores, indicative of precocious separation of sister chromatids, was not found.

Cell Cycle Arrest at the Stage that Rad51 and Dmc1 Function in the *mer3Δ* Mutant

A time course analysis of meiotic nuclear divisions was carried out by staining cells with DAPI (Figure 6A). By 10 hr after induction of meiosis (t=10 hr), more than 80% of wild-type cells had undergone

the first meiotic division. Only about 20% of *mer3Δ* cells, however, had undergone the division by the end of the time course at 30°C. Unexpectedly, incubation at a lower temperature (23°C) allowed *mer3Δ* cells to undergo the division to the final level of 78%. Nevertheless, the first meiotic division was delayed at least 5 hr by the *mer3Δ* mutation at the both temperatures. The spore viabilities in the *mer3Δ* strain were 22% (44/200 spores) and 27% (53/200 spores) at 30°C and 23°C, respectively, while 97% (194/200 spores) in the wild-type strain at the both temperatures.

The strand exchange proteins, Rad51 and Dmc1, localize as foci on zygotene through early pachytene chromosomes (Bishop, 1994). To know the stage of meiotic prophase at which cell cycle is arrested in the *mer3Δ* strain, immunostaining of meiotic spread nuclei with anti-Rad51 or anti-Dmc1 antiserum was carried out at 30°C. As shown in Figure 6B, both Rad51 and Dmc1 foci transiently appeared on wild-type chromosomes, but were retained on *mer3Δ* chromosomes. Among nuclei with >15 foci, the average numbers of foci per nucleus were calculated. The average number of Rad51 foci at t=9 hr on *mer3Δ* nuclei (32 ± 8 (average ± SD)) was only slightly decreased from those at t=4 hr on *mer3Δ* and wild-type nuclei (39 ± 6 and 37 ± 7) (the respective sample sizes were 52, 25 and 38 nuclei). The average of Dmc1 foci at t=9 hr on *mer3Δ* (42 ± 10) was similar to those at t=4 hr on *mer3Δ* and wild-type nuclei (40 ± 10 and 40 ± 11) (the respective sample sizes were 51, 39 and 39 nuclei). These demonstrate that progression of meiotic cell cycle in the *mer3Δ* strain is arrested at the stage when Rad51 and Dmc1 function.

The Mer3 Localization on Synapsed Regions of Chromosomes

Affinity purified anti-Mer3 rabbit antibody was prepared and immunostaining with spread nuclei was carried out. Mer3 transiently localized at discrete sites on meiotic chromosomes as foci in the wild-type strain (Figure 6C and D). Nucleus containing more than five Mer3 foci was not observed in the *mer3Δ* strain, indicating that most of the Mer3 signals depended on the *MER3* gene. In the *spo11Δ* or *mer2Δ* mutant, that are defective in the initiation of meiotic recombination and chromosome synapsis (Klapholz et al., 1985; Rockmill et al., 1995), only the background level of Mer3 signal was observed (data not shown).

To know the relation between the Mer3 localization and synapsis of homologs, I carried out double staining of spread nuclei (t=6 hr), using anti-Mer3 rabbit antibody and anti-Zip1 mouse

antibody. Zip1 is a component of the central region of synaptonemal complex and thus can be an indicator of chromosome synapsis (Sym et al., 1993; Smith et al., 1997). I divided nuclei into four classes by Zip1-staining patterns; no signal; only foci; stretch contained; and line contained nuclei. Examination of 50 nuclei was carried out for the each class. Less than 3 and 8 Mer3 foci were observed on nuclei containing no or only Zip1 foci. On the other hand, 28 ± 8 and 42 ± 7 Mer3 foci were detected on Zip1-stretch contained nuclei (Figure 6D; i) and Zip1-line contained nuclei (Figure 6D; ii) respectively. Note that Mer3 colocalizes with Zip1 foci on nuclei containing Zip1 stretch (Figure 6D; i arrows), in addition to the localization on stretched and lined Zip1 structures.

MER3 Functions In Repair of Meiosis-Specific DSB

To elucidate the recombination step in which Mer3 functions, recombination related DNA events at the *HIS4::LEU2* recombination hotspot were analyzed by Southern blotting (Figure 7).

I first examined the formation of meiosis-specific DSB, the initial event in meiotic recombination (Figure 7A and C). In wild-type cells, restriction fragments, DSB I and II, diagnostic of DSBs at two specific loci in *HIS4::LEU2*, appeared at t=4 hr and completely disappeared by t=12 hr. In *mer3Δ* cells, however, DSBs were apparently detected from t=4 hr to at least t=12 hr at the both temperatures examined. DSBs in both wild-type and *mer3Δ* cells are tailing to the faster mobility, indicative of resection of DSB ends. Note that, at t=24 hr in *mer3Δ*, DSB are greatly reduced in 23°C compared to that in 30°C.

The formation of crossover and noncrossover recombinant DNA were examined. Four kinds of recombinant fragments (R1 ~ R4) can be distingusihed by XhoI and MluI double digestion of DNAs (Figure 7A). Over 95% of R1 and R2 result from crossover and almost all R3 from noncrossover, but R4 from both crossover and noncrossover in wild type (Storlazzi et al., 1995). As shown in Figure 7D, the recombinant reached their maximum levels at t=8 hr in wild type, but at t=24 hr in *mer3Δ*. Comparison of the maximum levels in *mer3Δ* and wild type revealed that R1 and R2 (crossovers) produced in *mer3Δ* were 60% the wild-type level but R3 (noncrossovers) were 3-fold increased in *mer3Δ*. Similar results were obtained at 30 and 23°C and the total amount of recombinants (R1 ~ R4) produced in *mer3Δ* was 80% the wild-type level at the both temperatures.

The Efficient Strand Exchange for Crossover

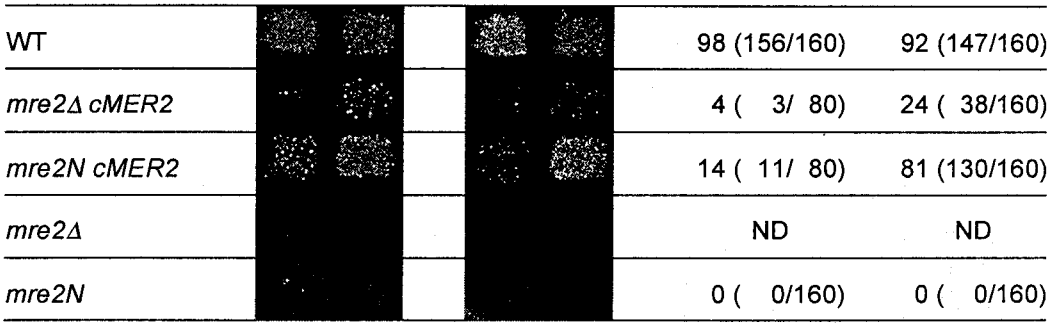
To assess strand exchange in meiotic recombination, BamHI (10 mer) or XhoI (8 mer) palindromic sequence was introduced at the same locus on each of homologs and the formation of heteroduplex (HD) DNA was examined by Southern blotting. Since a mispair of palindrome sequences is poorly repaired by mismatch repair system (Nag et al, 1989; Detloff et al., 1992), the formation but not the repair of HD is expected to be analyzed in this assay. To compare HD formation at loci different in the distance from a DSB site, I constructed three kinds of tester strains, in which the pair of palindromes was integrated at different loci in *HIS4::LEU2* (Figure 7B; MluI, SalI or AccI loci). Among the three loci, MluI locus is unique in that it is at DSB site I in the *HIS4::LEU2* region. In addition, crossover-type HDs (HD2) and noncrossover-type HDs (HD1 and HD3) can be distinguished, with regard to the flanking BamHI and XhoI sites (Figure 7A). The positions of two DSB sites were not drastically altered by the integration (data not shown).

Figure 7E shows the formation of HDs at MluI (left) and AccI (right) loci. In wild-type cells, the fractions of crossover-type HD2 divided by total HDs at MluI (0.397 and 0.462 at 30 and 23°C respectively) were increased at AccI (0.503 and 0.529 at 30 and 23°C respectively), suggesting that the efficient strand exchange led crossover rather than noncrossover. In *mer3Δ* cells, however, the fractions of HD2 at MluI (0.167 and 0.193 at 30 and 23°C respectively) were only slightly increased at AccI (0.189 and 0.207 at 30 and 23°C respectively). On a blot for HDs at AccI (Figure 7 right), DSBs having >700 bp single-strand tails at their ends (Figure 7E DSB IA and IB), which are also resistant to BamHI and XhoI digestion at AccI locus, were detected in wild-type and *mer3Δ* cells in a kinetics similar to that observed in the conventional DSB assay (Figure 7C).

The amount of HDs produced at the three loci are summarized in Figure 7F. By the *mer3Δ* mutation, at every locus, crossover-type HD2 was decreased but noncrossover-type HD1 and HD3 were increased or unchanged, consisting with crossover-specific defect in *mer3Δ*. Comparison of HD2 produced in wild-type and *mer3Δ* cells revealed that HD2 in *mer3Δ* was gradually decreased from MluI to AccI locus (77 to 30% the wild-type level at 30°C; 71 to 36% the wild-type level at 23°C). These results suggest that *MER3* is essential for the efficient strand exchange resulting in crossover.

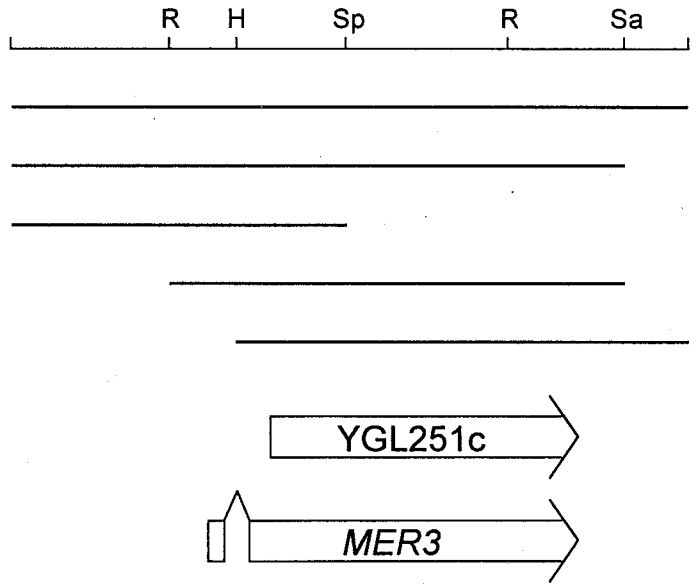
Figure 1

A



	LEU2-HIS4		*TRP1-CYH2*		Percent of Viable Spores	
	Vector	*MER3*	Vector	*MER3*	Vector	*MER3*
WT					98 (156/160)	92 (147/160)
mre2Δ cMER2					4 (3/ 80)	24 (38/160)
mre2N cMER2					14 (11/ 80)	81 (130/160)
mre2Δ					ND	ND
mre2N					0 (0/160)	0 (0/160)

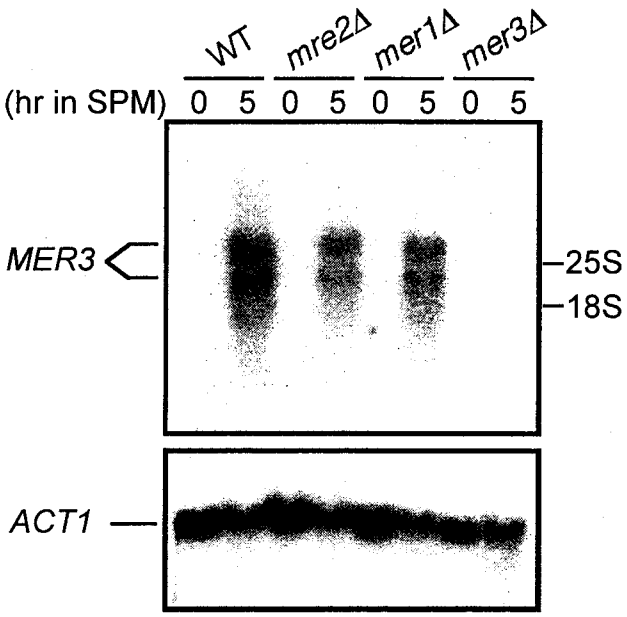
B



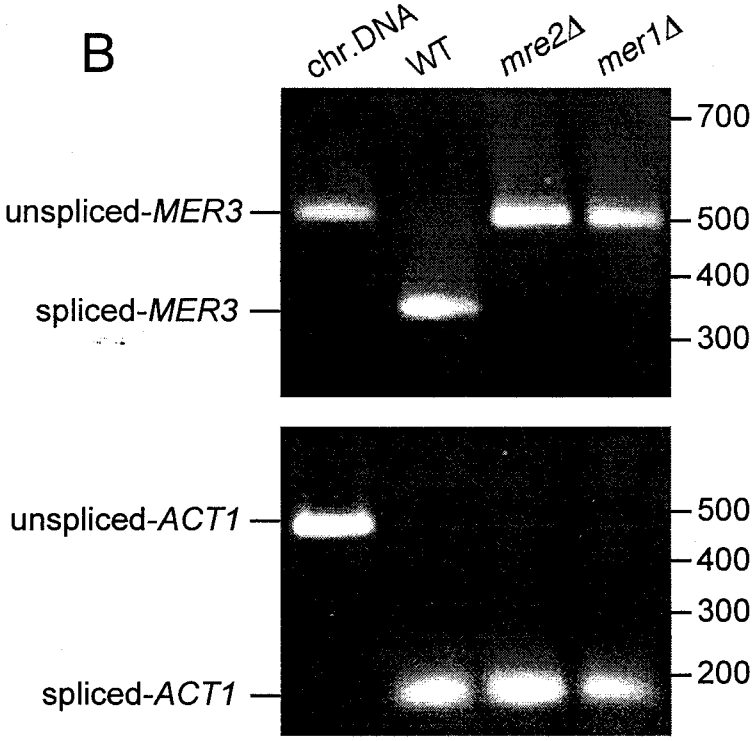
Suppression	Plasmid
YES	pTN45
YES	pTN46
NO	pTN47
YES	pTN84
NO	pTN66

Figure 2

A



B



C

	5' splice site	Branch point	3' splice site
MER3	**GUA GU**	**GACUAACA**	**UAG**
MER2	**GUUCGU**	**UACUAACA**	**UAG**
Consensus	**GUAYGU**	**UACUAACA**	**YAG**

Figure 3

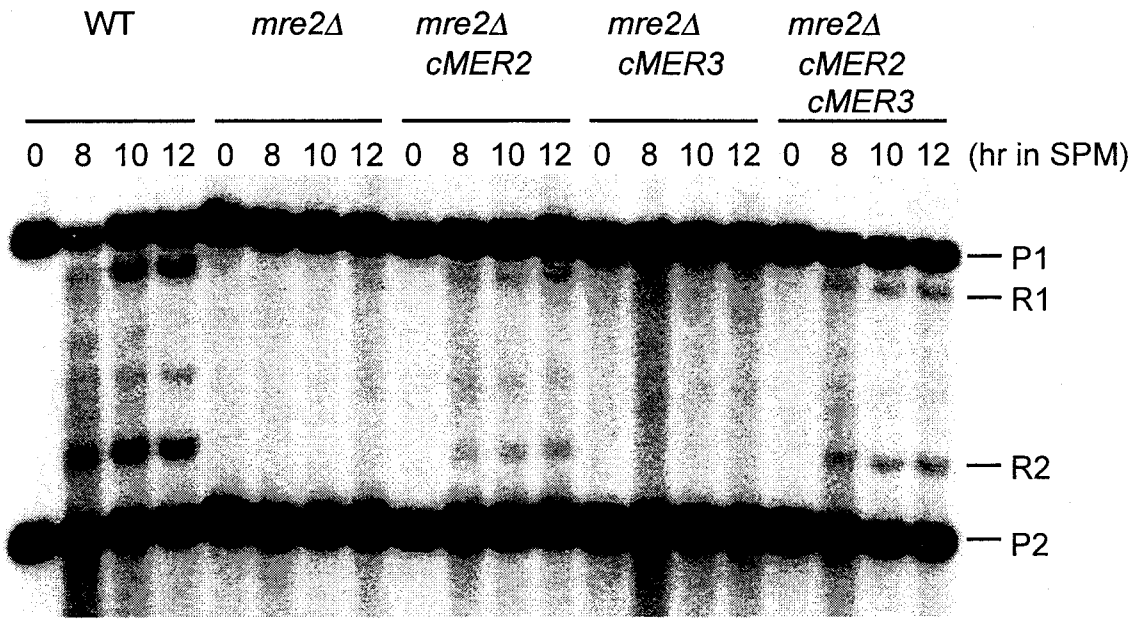
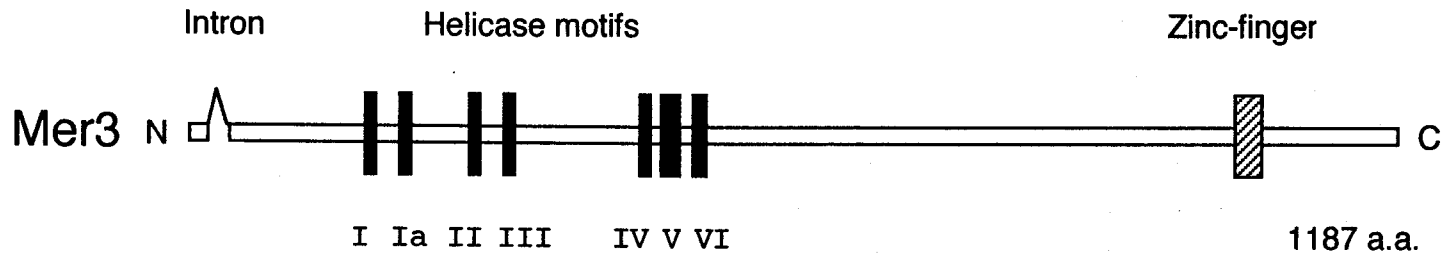

WT
mre2Δ
mre2Δ cMER2
mre2Δ cMER3
mre2Δ cMER2 cMER3
0 8 10 12 0 8 10 12 0 8 10 12 0 8 10 12 0 8 10 12 (hr in SPM)
P1
R1
R2
P2

Figure 4

A



B

		I	Ia	II	III	IV	V	VI	
Mer3	163	PTGSGKT	TPEKWDLL	DEIH	LSATVP	FLAG	TSTLAVGVNLPA	QMIGRAGRP	495
T24P15.18(N)	529	PTGAGKT	TPEKWDII	DEIH	LSATLP	FRWG	TATLAWGVNLPA	QMIGRAGRP	884
T24P15.18(C)	1375	PTGSGKT	TPEKWDAL	DELH	LSTSLA	FEAG	SSSLCWGTPLKA	QMMGRGSRP	1722
G9365(N)	311	PTGAGKT	TPEKWDVV	DEVH	LSATLP	FKDG	TATLAWGVNLPA	QIFGRGGRP	672
G9365(C)	1163	PTGSGKT	TPEKFDGI	DEIH	MSTAVS	FQKN	TSTLAWGVNLPA	QMMGRAGRP	1508
Brr2	522	PTGSGKT	TPEKWDIT	DEIH	LSATLP	FADG	TATLAWGVNLPA	QMLGRAGRP	878

Figure 5

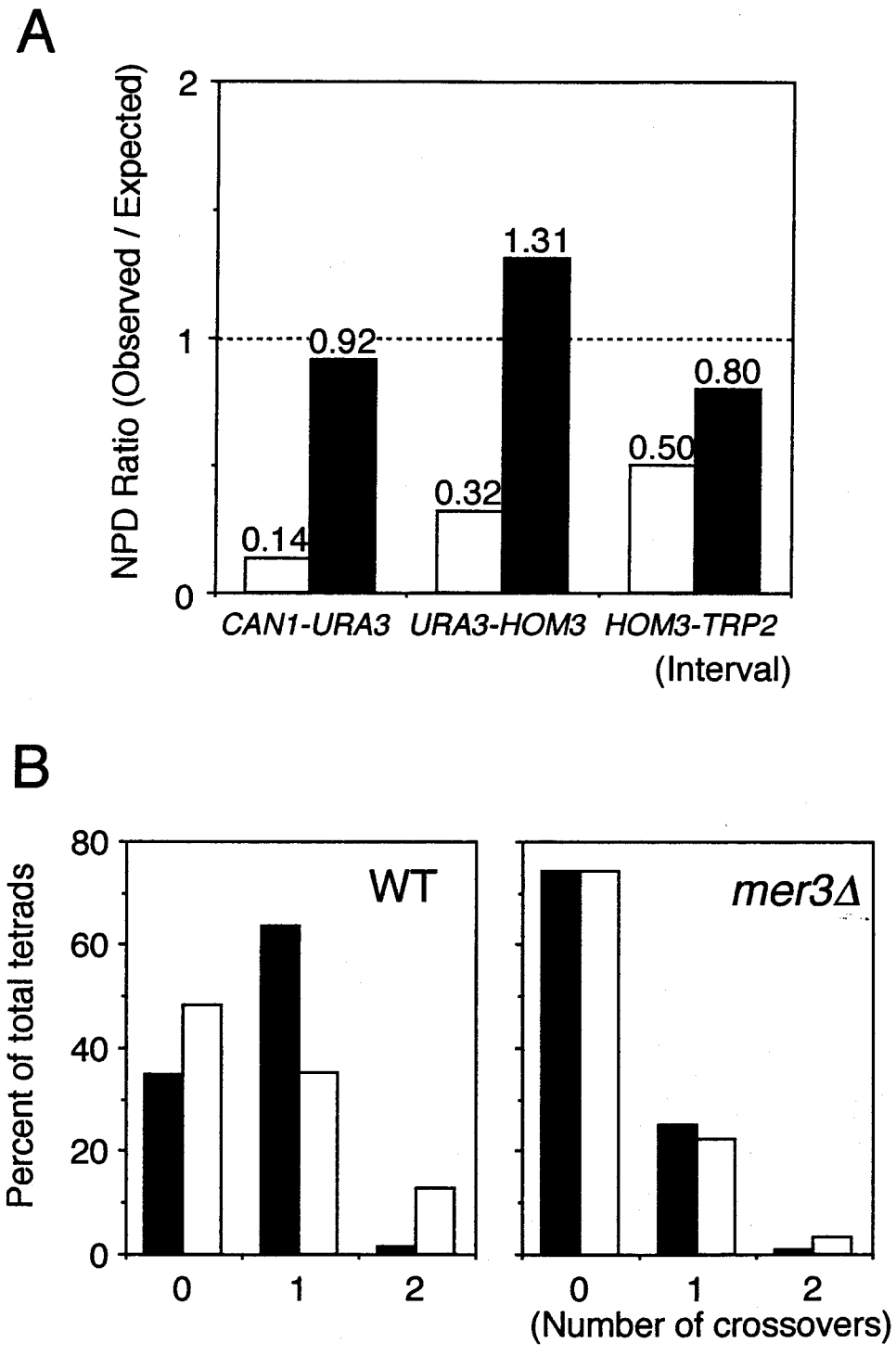
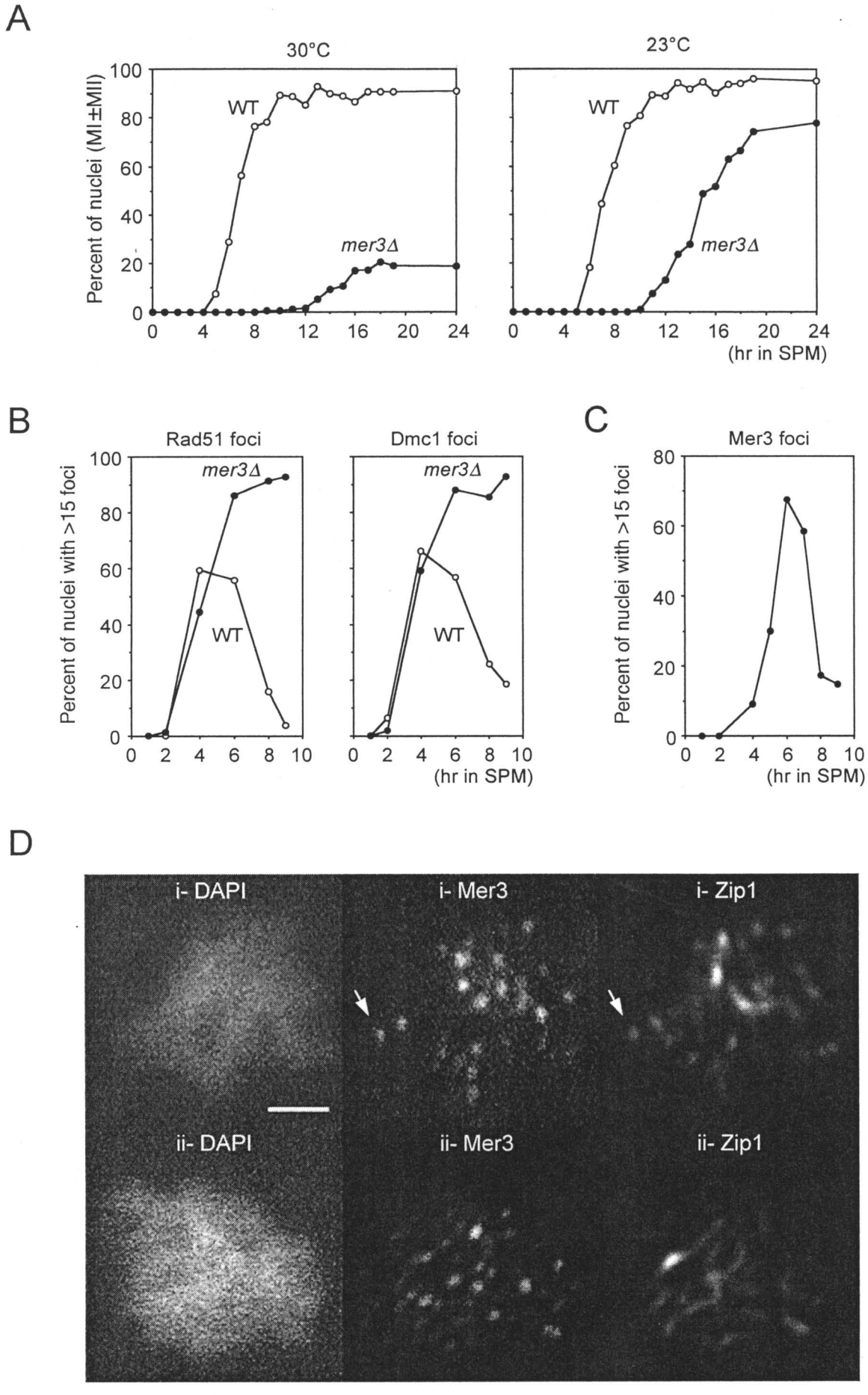
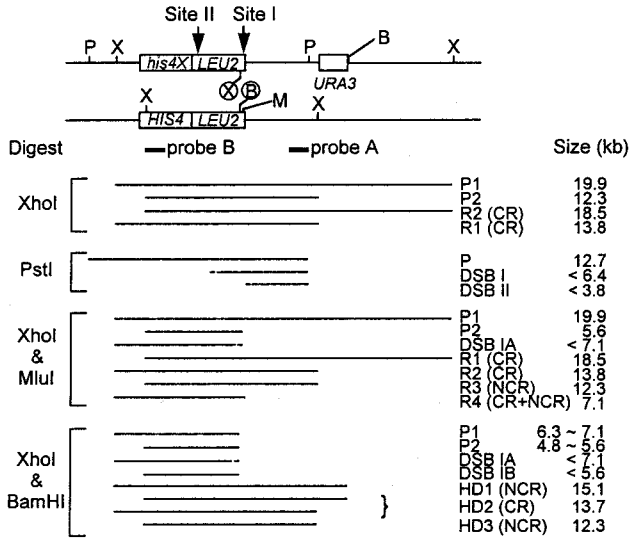

A
NPD Ratio (Observed / Expected)
2
1
0
0.14
0.92
0.32
1.31
0.50
0.80
CAN1-URA3
URA3-HOM3
HOM3-TRP2
(Interval)
B
Percent of total tetrads
80
60
40
20
0
WT
mer3Δ
0
1
2
0
1
2
(Number of crossovers)

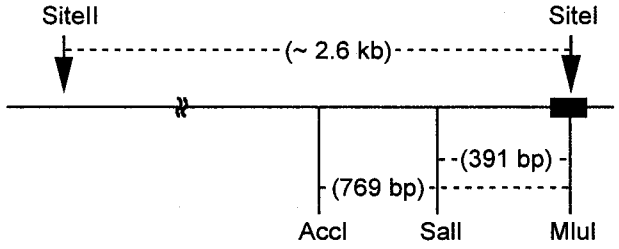
Figure 6


A
30°C
23°C
Percent of nuclei (MI±MII)
WT
mer3Δ
(hr in SPM)
B
Rad51 foci
Dmc1 foci
Percent of nuclei with >15 foci
C
Mer3 foci
D
i- DAPI
i- Mer3
i- Zip1
ii- DAPI
ii- Mer3
ii- Zip1

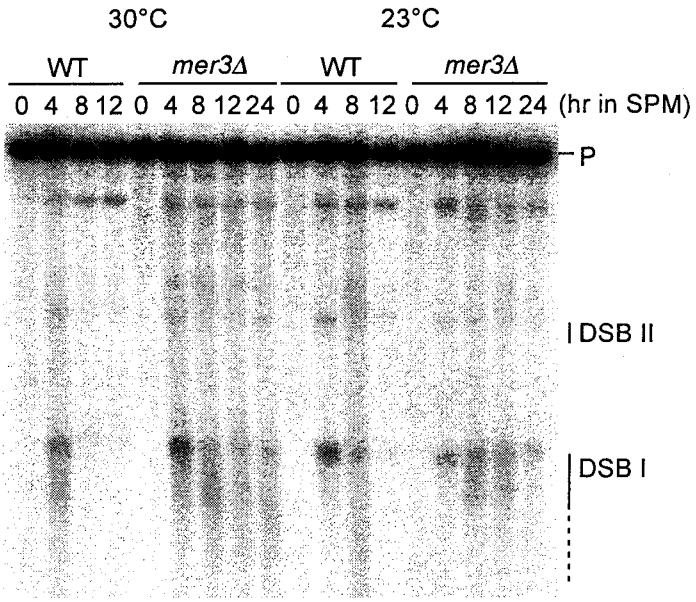
A


Site II
Site I
P X
his4X LEU2
P
B
X
URA3
X
M
X
HIS4 LEU2
Digest
probe B
probe A
Size (kb)
XhoI
P1 19.9
P2 12.3
R2 (CR) 18.5
R1 (CR) 13.8
PstI
P 12.7
DSB I < 6.4
DSB II < 3.8
XhoI & MluI
P1 19.9
P2 5.6
DSB IA < 7.1
R1 (CR) 18.5
R2 (CR) 13.8
R3 (NCR) 12.3
R4 (CR+NCR) 7.1
XhoI & BamHI
P1 6.3 ~ 7.1
P2 4.8 ~ 5.6
DSB IA < 7.1
DSB IB < 5.6
HD1 (NCR) 15.1
HD2 (CR) 13.7
HD3 (NCR) 12.3

B


SiteII
SiteI
(~ 2.6 kb)
(391 bp)
(769 bp)
AccI
SalI
MluI

C


30°C
23°C
WT
mer3Δ
WT
mer3Δ
0 4 8 12 0 4 8 12 24 0 4 8 12 0 4 8 12 24 (hr in SPM)
P
DSB II
DSB I

D

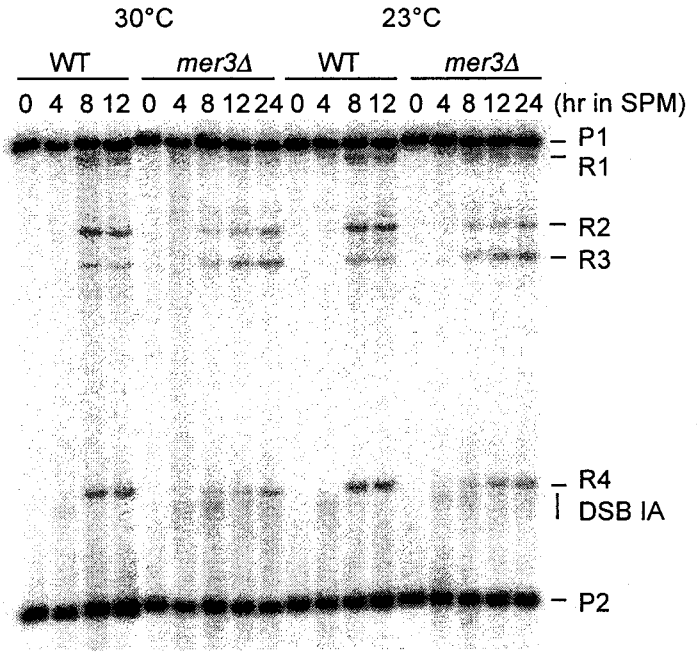
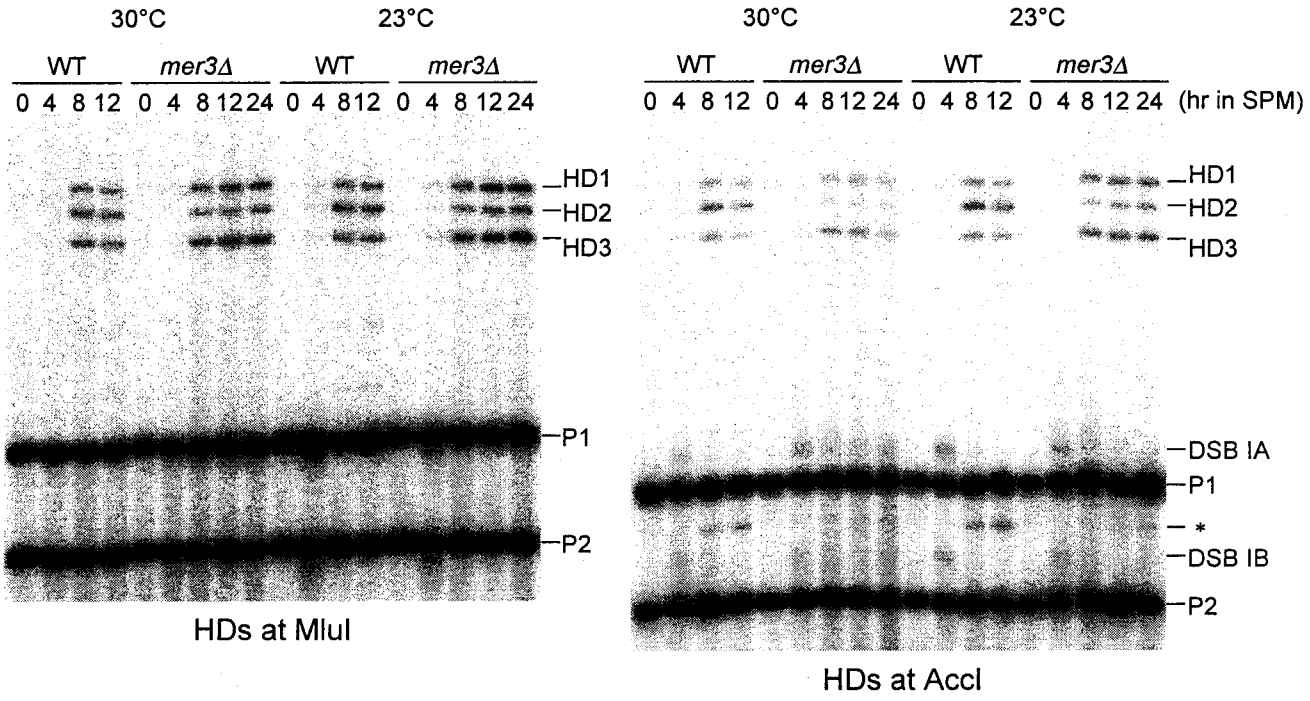

30°C
23°C
WT
mer3Δ
WT
mer3Δ
0 4 8 12 0 4 8 12 24 0 4 8 12 0 4 8 12 24 (hr in SPM)
P1
R1
R2
R3
R4
DSB IA
P2

Figure 7E&F

E



F

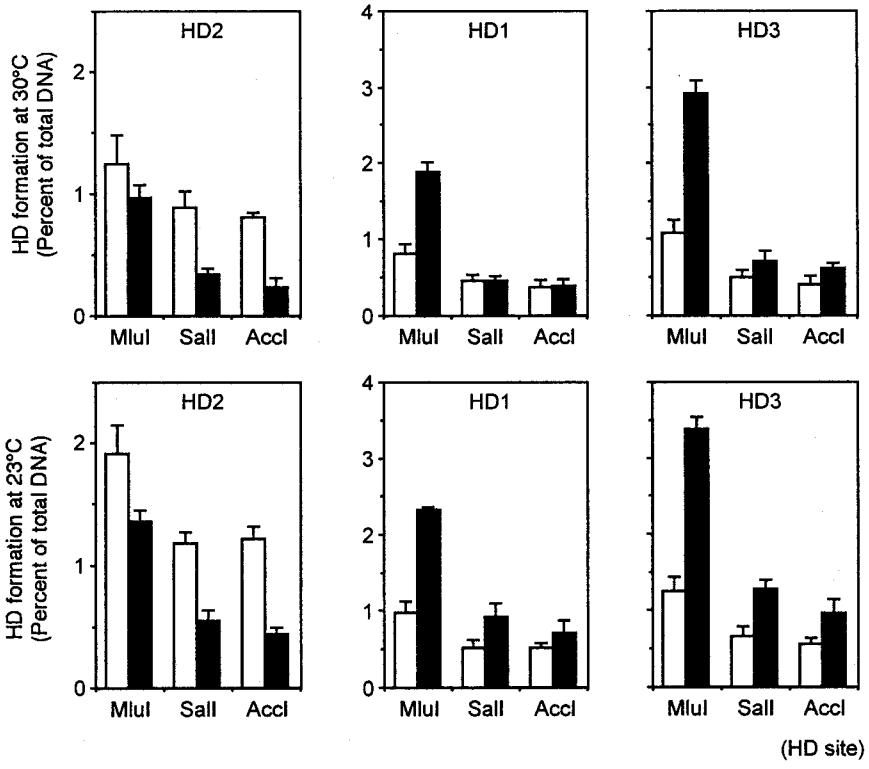


Table 1

Table 1. Meiotic Properties of *mre2Δ* and *mer1Δ* Derivatives

Relevant Genotype	Meiotic Divisions(%) MI	MII	Sporulation (%)	Spore Viability (%)
WT	9	79	61	95
cMER2	11	70	62	100
cMER3	8	81	62	97
cMER2 cMER3	5	77	59	100
mre2Δ	8	77	0.9	< 0.7
mre2Δ cMER2	9	73	0.9	< 0.7
mre2Δ cMER3	9	48	1.9	1
mre2Δ cMER2 cMER3	10	34	2.4	48
mer1Δ	11	61	14	2
mer1Δ cMER2	9	73	16	9
mer1Δ cMER3	5	80	28	5
mer1Δ cMER2 cMER3	8	75	37	86

Table 2

Table 2. Crossover Recombinaition

	WT				*mer3Δ*				
Interval	PD	TT	NPD	cM	PD	TT	NPD	cM	Fold Decrease
MAT-CEN3	218	96	0	15	270	45	0	7	2.1
CEN3-HIS4	124	185	3	33	254	56	0	9	3.6
CAN1-URA3	455	833	21	37	973	328	11	15	2.5
URA3-HOM3	534	735	31	35	960	332	17	17	2.1
HOM3-TRP2	1007	271	4	12	1052	221	4	10	1.2

Table 3. Noncrossover Recombination			
Locus	WT	*mer3Δ*	Fold Increase
CAN1	0.5%	1.3%	2.6
HOM3	1.1%	1.6%	1.5
TRP2	1.6%	2.6%	1.6

Table 4. Distribution of Tetrad Types

Relevant Genotype	Strains	Percent of Total Tetrads				
		4sv	3sv	2sv	1sv	0sv
WT	TNY374	94	3	1	< 0.1	1
WT	TNY367	93	6	1	< 0.1	0.4
mer3Δ	TNY375	14	4	19	5	58
mer3Δ	TNY368	23	5	20	4	48

Table 5. Strains of S. cerevisiae

Strain	Genotype	Source
TNY171	*MATa LEU2 his4::hisG trp5::hisG cyh2 ura3 can1 ho::LYS2 lys2* *MATα leu2::hisG HIS4 TRP5 CYH2 ura3 CAN1 ho::LYS2 lys2*	This study
TNY185	TNY171, except *mre2::hisG* *mre2::hisG*	This study
TNY170	TNY171, except *mre2N* *mre2N*	This study
TNY240	TNY171, except *mre2::hisG cMER2* *mre2::hisG cMER2*	This study
TNY240	TNY171, except *mre2N cMER2* *mre2N cMER2*	This study
TNY058	*MATa leu2::hisG his4X::LEU2-URA3 ho::LYS2 ura3 lys2 arg4-nsp cyh2* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl CYH2*	T. Nakagawa and H. Ogawa, 1997
TNY101	TNY058, except *cMER2* *cMER2*	T. Nakagawa and H. Ogawa, 1997
TNY366	TNY058, except *cMER3* *cMER3*	This study
TNY380	TNY058, except *cMER2 cMER3* *cMER2 cMER3*	This study
TNY060	TNY058, except *mre2::hisG* *mre2::hisG*	T. Nakagawa and H. Ogawa, 1997
TNY102	TNY058, except *mre2::hisG cMER2* *mre2::hisG cMER2*	T. Nakagawa and H. Ogawa, 1997
TNY381	TNY058, except *mre2::hisG cMER3* *mre2::hisG cMER3*	This study
TNY382	TNY058, except *mre2::hisG cMER2 cMER3* *mre2::hisG cMER2 cMER3*	This study
TNY305	TNY058, except *mer1::LEU2* *mer1::LEU2*	This study
TNY481	TNY058, except *mer1::LEU2 cMER2* *mer1::LEU2 cMER2*	This study
TNY482	TNY058, except *mer1::LEU2 cMER3* *mer1::LEU2 cMER3*	This study
TNY483	TNY058, except *mer1::LEU2 cMER2 cMER3* *mer1::LEU2 cMER2 cMER3*	This study
NKY1551	*MATa leu2::hisG his4X::LEU2(Bam)-URA3 ho::LYS2 ura3 lys2 arg4-nsp* *MATα leu2::hisG his4B::LEU2 ho::LYS2 ura3 lys2 arg4-bgl*	Storlazzi et al., 1995
TNY286	NKY1551, except *mer3::hisG* *mre3::hisG*	This study
TNY367	*MATa leu2::hisG can1 URA3 HOM3 TRP2 ho::LYS2 lys2* *MATα leu2::hisG CAN1 ura3 hom3-10 trp2 ho::LYS2 lys2*	This study
TNY368	TNY367, except *mer3::hisG* *mre3::hisG*	This study
TNY374	*MATa CENIII::URA3 leu2::hisG HIS4 ho::lys2 ura3 lys2 trp1-H3* *MATα CENIII::TRP1 leu2::hisG his4B::LEU2 ho::lys2 ura3 lys2 trp1-H3*	This study
TNY375	TNY374, except	This study
TNY461	*MATa leu2::hisG HIS4::LEU2(Bam/Sal) ho::LYS2 ura3 lys2 arg4-bgl* *MATα leu2::hisG his4X::LEU2(Xho/Sal)-URA3 ho::LYS2 ura3 lys2 arg4-nsp*	This study
TNY462	TNY461, except *mer3::hisG* *mre3::hisG*	This study
TNY560	*MATa leu2::hisG HIS4::LEU2(Bam/Mlu) ho::LYS2 ura3 lys2 arg4-bgl* *MATα leu2::hisG his4X::LEU2(Xho/Mlu)-URA3 ho::LYS2 ura3 lys2 arg4-nsp*	This study
TNY561	TNY560, except *mer3::hisG* *mre3::hisG*	This study
TNY589	*MATa leu2::hisG HIS4::LEU2(Bam/Acc) ho::LYS2 ura3 lys2 arg4-bgl* *MATα leu2::hisG his4X::LEU2(Xho/Acc)-URA3 ho::LYS2 ura3 lys2 arg4-nsp*	This study
TNY590	TNY589, except *mer3::hisG* *mre3::hisG*	This study
TNY606	*MATa HIS4 ARG4 ho::LYS2 lys2 MER3* *MATα his4X-URA3 arg4-nsp ho::LYS2 lys2 mer3::hisG(AB)*	This study
TNY607	TNY606, except *mer3KA* *mre3::hisG*	This study

Figure Legends

Figure 1. Suppression of a Crossover Defect in *mre2 cMER2* Mutants by Multicopy *MER3*

(A) Plate assay for crossover recombination and spore viabilities. Wild-type (TNY171), *mre2Δ cMER2* (TNY240), *mre2N cMER2* (TNY169), *mre2Δ* (TNY185) and *mre2N* (TNY170) transformants harboring a vector (YEp24) or *MER3*-plasmid (pTN45) were patched on SD-Ura plates and replica plated to SPM-Ura to induce meiosis. After 4 days at 23 °C, the SPM-Ura plate was replicated to SD-Ura, -Arg, -Leu, -His, +CYH, +CAN (CYH, cycloheximide; CAN, canavanine) and SD-Ura, -Arg, -Trp, +CYH, +CAN plates. Papillae formed on the plates result from crossover in the *LEU2-HIS4* or *TRP5-CYH2* interval and haploidization. Spore viabilities were examined by colony formation following tetrad dissection. The numbers of viable spores and total spores are in parentheses. ND, Not Determined.

(B) Subcloning analysis of the *MER3* gene. A series of deletions were constructed based on pTN45 (see Experimental Procedures). The suppression activity of the deletion series was tested by the plate assay described in (A) using the *mre2N cMER2* strain. Positions of YGL251c and *MER3* coding regions (see also Figure 3) are illustrated at the bottom. H, HindIII; R, EcoRI; Sa, SalI; Sp, SphI.

Figure 2. Meiotic Induction and Splicing of *MER3* Transcripts

(A) Northern blot analysis of *MER3* transcripts. Total RNA was prepared from wild-type (TNY058), *mre2Δ* (TNY060), *mer1Δ* (TNY305) and *mer3Δ* (TNY286) cells before and 5 hr after induction of meiosis. *MER3* (top panel)and *ACT1* (bottom panel) sequences were identified using pTN78 and pTN7, respectively, as hybridization probes. *ACT1* was used as a standard for the amount of RNA loaded on the gel. The positions of 18S and 25S rRNAs detected by Ethidium Bromide staining are indicated on the right.

(B) RT-PCR assay for RNA splicing of *MER3* transcripts. The same meiotic RNA samples prepared in (A) were treated with RNase-free DNase I to eliminate contaminating DNA, and subjected to reverse transcription (RT) followed by amplification by PCR. The product was analyzed by agarose gel electrophoresis. Top and bottom panels represent the products amplified using *MER3*-specific primers (priTN1 and priTN2) and *ACT1*-specific primers (priTN10 and priTN11), respectively. Amplified

products from chromosomal DNA (lane chr. DNA) indicate the position of unspliced. In each panel, the positions of unspliced and spliced products are indicated on the left, and the sizes of standards are shown on the right.

(C) The three elements in introns for RNA splicing. 5' splice site, branch point and 3' splice site sequences are shown for *MER3*, *MER2* and the consensus (Jones et al., 1992). The same branch point sequence as found in *MER3* has been reported in yeast (Myslinski et al., 1992). The three elements locate at bases at 59-63, 122-129 and 208-210, respectively, in the *MER3* sequence. Hypothetical YGL251c ORF starts at position 576.

Figure 3. Detection of Crossover Recombinant DNA by Southern Blotting

DNA was extracted from wild-type (NKY1551), *mre2Δ* (TNY214), *mre2Δ cMER2* (TNY215), *mre2Δ cMER3* (TNY363) and *mre2Δ cMER2 cMER3* (TNY362) strains at indicated times during synchronous meiosis. Each DNA sample was digested with XhoI, separated by 0.6% agarose gel electrophoresis, transferred to a nylon membrane, and fragments of interest were detected with the ^{32}P-labeled hybridization probe prepared from pNKY291. P1 and P2, parental fragments; R1 and R2, recombinant fragments.

Figure 4. The Mer3 protein

(A) Positions of the seven conserved motifs (I, Ia, II, III, IV, V, VI) of a helicase family and a putative zinc-finger in Mer3, and the *MER3* intron are illustrated.

(B) Comparison of amino acid sequences of the seven domanins in helicases. Amino acids indentical in more than three cases are shaded. Each T24P15.18 and G9365 has two sets of the helicase motif (shown as (N) and (C)). The first and the last amino acid positions are shown on the left and the right, respectively. T24O15.18 (GenBank AC002561) from Arabidopsis thaliana ; G9365 (GenBank U35242) and Brr2 (GenBank U18922) from S. cerevisiae.

Figure 5. Distribution of Crossovers

(A) Crossover interference. NPD ratios were measured in the three intervals for wild type (open bars) and *mer3Δ* (filled bars). An NPD ratio of 1 indicates no interference (dotted line). χ^2 tests were

performed for each interval by comparing the number of NPDs observed and with the number of NPDs expected. In wild type, the NPD ratios are significantly different from those expected (*CAN1-URA3* and *URA3-HOM3*, P<<0.005; *HOM3-TRP2*, P<0.25). In *mer3Δ*, NDP ratios are not significantly different from those expected (*CAN1-URA3* and *HOM3-TRP2*, P>0.5; *URA3-HOM3*, P>0.1).

(B) The proportion of zero, one and two of crossovers. The observed (filled bars) and expected (open bars) proportions of zero, one and two crossovers in the *CAN1-URA3* interval are shown for wild type (left panel) and *mer3Δ* (right panel). The similar result was also obtained in the *URA3-HOM3* interval.

Figure 6. Cytological Analysis

Wild-type (NKY1551) and *mer3Δ* (TNY286) cells were taken through synchronous meiosis. (A) Meiotic nuclear divisions were examined by DAPI staining at 1 hr intervals until 19 hr and at 24 hr after induction of meiosis. For each sample, more than 200 cells were examined by fluorescence microscopy. The sporulation frequencies of *mer3Δ* cells examined at 30 and 23°C by phase-contrast microscopy as Table 1 were 4 and 24 %, respectively. (B) The percentage of spread nuclei containing more than 15 foci is shown for Rad51 (left panel), Dmc1 (right panel). (C) The percentage of spread nuclei containing more than 15 Mer3 foci is shown. More than 50 unselected nuclei were scored at each time point (B and C). (D) Double staining of Mer3 and Dmc1 proteins using anti-Mer3 rabbit antibody and anti-Zip1 mouse antibody. Zip1-stretch contained nucleus (i) and Zip1-line contained nucleus (ii) are shown at top and bottom row, respectively. Arrows in (i) indicate a site where Mer3 localizes on Zip1 focus. Scale bar, 3 μm.

Figure 7. Physical Analysis of Meiotic Recombination Related Events

(A) DSB sites and restriction sites of interest in the *HIS4::LEU2* region (top). Positions of minor (site II) and major (site I) DSB sites are shown by arrows. Restriction fragments detected in Figure 4 and the following assays were illustrated (bottom). P, PstI; X, XhoI; M, MluI; B, BamHI; P, parental fragments; R, recombinant fragments; and HD, heteroduplex fragments. (B) Indicated are positions (MluI, SalI and AccI) of BamHI or XhoI linker DNA integration sites for detection of HD. The distance from MluI locus in DSB site I is shown. DSBs occur in within ~150 bp region (filled box) at site I (Xu and Kleckner, 1995).

(C) Meiosis-specific DSB. DNAs were prepared from wild-type (NKY1551) and *mer3Δ* (TNY286) cells at indicated times after induction of meiosis, digested with PstI, separated by agarose gel electrophoresis and transferred to a nylon membrane. Probe A (shown in (A)) prepared from pNKY291 was used to detect fragments of interest in Southern hybridization. (D) Crossover and noncrossover recombinant DNA. DNAs were digested in parallel with XhoI and MluI and fragments of interest were detected using probe B (shown in (A)) prepared from pNKY155. Slight reduction of recombinants in wild type at t=12 hr compared to t=8 hr is probably due to inefficient recover of DNA from sporulated cells. (E) Heteroduplex DNA. DNAs were digested in parallel with XhoI and BamHI and fragments of interest were detected using probe B. HD formation at MluI (left panel) was examined using TNY560 (wild type) and TNY561 (*mer3Δ*) strains. HD formation at AccI (right panel) was examined using TNY589 (wild type) and TNY590 (*mer3Δ*) strains. The origin of bands marked with * is unknown. (F) Quantification of heteroduplex DNA. Top and bottom rows shows the percentage of HDs produced in wild-type (open bar) and *mer3Δ* (filled bar) cells at 30 an 23°C, respectively. Each value is the maximum levels of the relevant fragment in the time course. The percentage is the mean value obtained from four different blots started from at least two independent cultures. Lines at the top of bars show standard deviations. For detection of HD at the SalI locus, TNY461 (wild type) and TNY462 (*mer3Δ*) strains were used.

Table Legends

Table 1. Meiotic Properties of *mre2Δ* and *mer1Δ* Derivatives

Cultures of wild-type (TNY058), *cMER2* (TNY101), *cMER3* (TNY366), *cMER2 cMER3* (TNY380), *mre2Δ* (TNY060), *mre2Δ cMER2* (TNY102), *mre2Δ cMER3* (TNY381), *mre2Δ cMER2 cMER3* (TNY382), *mer1Δ* (TNY305), *mer1Δ cMER2* (TNY481), *mer1Δ cMER3* (TNY482) and *mer1Δ cMER2 cMER3* (TNY483) strains were taken at 24h after induction of meiosis. The occurrence of meiotic divisions was monitored by staining cells with DAPI and examining more than 200 cells in each aliquot by fluorescence microscopy. Cells that have completed one meiotic division (MI) contain two DAPI-staining bodies; cells that have completed two meiotic divisions (MII) contain three or four DAPI-staining bodies. Sporulation was examined by phase-contrast microscopy using more than 400 cells and was signaled by the appearance of two to four phase-bright bodies within a cell. Spore viability was measured by dissection of 40 tetrads produced on SPM plates using micro manipulator, and the proportion of spores germinating to give visible colonies was assessed after incubation for 3 days. The data presented for wild-type, *mre2Δ*, *mre2Δ cMER2* and *cMER2* strains were published previously (Nakagawa and Ogawa, 1997).

Table 2. Crossover Recombination

Strains TNY374 (wild type) and TNY375 (*mer3Δ*) were used to examined *MAT-CENIII* and *CENIII-HIS4* intervals. Strains TNY367 (wild type) and TNY368 (*mer3Δ*) were used to examined *CAN1-URA3*, *URA3-HOM3* and *HOM3-TRP2* intervals. Only four-spore viable tetrads that did not show noncrossover recombination of the marker indicated were used to calculate map distances (cM). The fold decrease in map distance of *mer3Δ* relative to wild type is shown. PD, parental ditype; TT, tetratype; NPD, nonparental ditype.

Table 3. Noncrossover Recombination

Absolute frequencies of noncrossover recombination were scored as tetrads exhibiting 3:1 segregation for the indicated marker. 1,317 and 1,344 four-spore viable tetrads were examined to measure noncrossover in wild-type (TNY367) and *mer3Δ* (TNY368) strains, respectively. The fold increase in noncrossover frequency of *mer3Δ* relative to wild type is shown.

Table 4. Distribution of Tetrad Types

The distribution of 4, 3, 2, 1 and 0sv tetrads is shown. Respective numbers of total tetrads dissected and spore viabilities for TNY374 (wild type), TNY375 (*mer3Δ*), TNY367 (wild type) and TNY368 (*mer3Δ*) strains are following; 335, 2187, 1331 and 5952 tetrads; 97, 28, 98 and 37%. The difference of spore viabilities between TNY375 (*mer3Δ*) and TNY368 (*mer3Δ*) strains might be due to the difference of strain backgrounds (see Experimental Procedures).

Discussion

MRE2- and *MER1*-Dependent Splicing of the *MER2* and *MER3* Transcript

I have identified the *MER3* gene as a multicopy suppressor of the *mre2N cMER2* mutant. The *MER3* RNA splicing depends on *MRE2* and *MER1*, which are also required for the *MER2* splicing . Mre2 has two sets of the RNA recognition motif (RRM). The *mre2N* mutation is likely to impair the RNA binding activity, because the mutation changes conserved Leu to Pro in N termanal RRM (Nakagawa and Ogawa, 1997). On the other hand, Mer1 contains the KH motif found in some RNA binding proteins and has been shown to bind *MER2* RNA directly (Nandabalan and Roeder, 1995). Thus, both Mre2 and Mer1 are probably involved in the splicing reaction of *MER3*, as well as *MER2*, transcripts. The most prominant feature shared by *MER2* and *MER3* introns is noncanonical 5' splice site (Figure2C). This raises the possibility that Mre2 and Mer1 are required for the spliceosome to recognize 5' splice sites of *MER2* and *MER3* introns. This is supported by the obervation that some mutations in *MER2* 5' splice site or U1 snRNA, a component of the spliceosome, enable the *MER2* intron to be spliced even in the absence of Mer1 (Nandabalan et al., et al., 1993). The amount of *MER3* transcripts is also decreased in *mre2Δ* and *mer1Δ* strains, as is the case of *MER2* (Nakagawa and Ogawa, 1997). These might be due to the coordination of splicing and transcription (Osheim et al., 1985; Neugebauer and Roth, 1997) or the instability of unspliced RNA.

Multicopy *MER3* suppresses a crossover defect in *mre2N cMER2* and *mre2Δ cMER2*, but not in *mre2N* nor *mre2Δ* mutants. The spore viability of *mre2Δ* and *mer1Δ* mutants apparentaly increases only when both *cMER2* and *cMER3* are introduced. In addition, *cMER3* improves the formation of crossover DNAs in *mre2Δ cMER2*, but in *mre2Δ*. These results suggest that *MER2* and *MER3* function at sequential steps in the same pathway. In fact, *MER2* is required for DSB formation (Rockmill et al., 1995), whereas *MER3* is required for DSB repair in meiotic recombination. However, a crossover defect in *mre2Δ cMER2* and *mer1Δ cMER2* is not completely suppressed by *cMER3*. Thus, there may be another target gene of *MRE2*- and *MER1*-dependent splicing.

The Differentiation of Crossover and Noncrossover Recombination

The formation of meiosis-specific DSBs and the subsequent processing of the ends to produce single-strand tails occur in the absence of *MER3*. However, substantial amounts of resected DSBs accumulate and the first meiotic division delays at least 5 hr in *mer3Δ* meiosis. Interestingly, immunostaining experiment have shown that the strand exchange proteins, Rad51 and Dmc1, localize and stay to probable recombination sites in the arrest period. These results indicate a role for *MER3* in DSBs/Holliday junctions transition.

Genetic analysis has revealed that the *mer3Δ* mutation decreases the crossover frequency but increases the noncrossover frequency. These inverse defects of crossover and noncrossover is also observed in the physical assay for recombinant DNAs. These resluts indicate that *MER3* has a role in crossover/noncrossover differentiation and promotes crossover. Since *mer3Δ* strain shows a defect in DSBs/Holliday junctions transition, it is likely that crossover/noncrossover differentiation occurs during DSBs/Holliday junctions transition. This notion is supported by the observation of DSBs at late meiosis in a *zip1* mutant, that reduces crossovers but noncrossoverss (Sym and Roeder, 1994; Xu et al., 1997). Two geometrically distinct types of double Holliday junctions have been proposed by Storlazzi et al. (1996). Therefore, *MER3* might regulate a balance of the two types of double Holliday functions to promote crossover recombination.

The Distribution of Crossovers: Crossover Interference

In addition to the decrease in the crossover frequency, *mer3Δ* impairs the distribution of crossovers (crossover interference), leading to a high incidence of homolog nondisjunction. Crossover interference is aslo impaired in a *zip1* mutant. Zip1 is a component of a central region of SC, and is believed to transmit a negative signal that prevent crossover (Sym and Roeder, 1993, 1994). Mer3-Zip1 double staining has shown that Mer3 localizes to discrete sites on zygotene and pachytene chromosomes. 42 is the average number of Mer3 foci per pachytene nuclei. The number of Mer3 foci is identical to the number of Dmc1 foci at late *mer3Δ* meiosis (t=9 hr at 30°C), although the number of Rad51 foci is 32 at that situation. Since 90 crossovers per meiosis are estimated in S. cerevisiae (Ross-Macdonald and Roeder, 1994), the number of Mer3 foci is close to the number of crossovers that decreased by *mer3Δ*. In addition, only the background level of Mer3 signals is observed in *mer2Δ* and *spo11Δ* mutants, that are defective in the initiation of meiotic recombination and chromosome

synapsis (Klapholz et al., 1985; Rockmill et al., 1995). Therefore, it is likely that Mer3 localizes to the sites of crossover and emanates or receives a negative signal for crossover interference.

The Mer3 Function

The Mer3 protein contains the seven conserved motifs characteristic of a DNA/RNA helicase family and the putative zinc-finger motif. A mutation in the helicase motif confers a crossover defect (data not shown). Mer3 localizes on chromosomes from zygotene stage when the formation of Holliday junctions is ongoing (Padmore et al., 1991; Schwacha and Kleckner, 1994; Kleckner, 1996). The accumulation of Rad51 and Dmc1 foci on *mer3Δ* chromosomes indicates that Mer3 is not required for their localization on chromosomes, and suggests that Mer3 is involved in the recombiantion process. Since in vitro strand exchange activity shown for Rad51 and Dmc1 is less effective relative to RecA (Sung, 1994; Li et al., 1997), they are likely to fucntion in the context of a protein complex to execute a high activity in vivo. Thus, Mer3 might be included in the recombination complex and functions as the helicase to promote the strand exchange.

The formation of heteroduplex (HD) DNA at three different loci near a DSB site has been also examined. In my assay, crossover-type and noncrossover-type HDs can be distinguished. In wild type, the relative amount of crossover-type HDs in the total HDs increases as the HD locus goes away from a DSB site. This position effect suggests that the efficient strand exchange increases the chance of crossovers. In *mer3Δ*, the position effect is almost abolised and , in addition, the relative amount of crossover-type HD compared to that in wild type decrease by half as the HD locus goes away from a DSB site. Therefore, I propose that Mer3 is required for the efficient strand exchange at the formation and tilll the resolution of Hollidasy junctions to promote crossvoer recombination. Interesingly, RuvB, the Holliday junction-specifc helicase, facilites the dissociation of RecA from recombining DNA and recruites RuvC, the resolvase (Egglestone et al., 1997).

Experimental Procedures

Strains and Media

All yeast strains used in this work are listed in Table 4 and are an SK1 strain background (Kane and Roth, 1974) ,except TNY367 and TNY368 strains which are congenic to SK1 and were derived from MY263 strain (Sym and Roeder, 1994). The *mre2::hisG* and *cMER2* constructions were described before (Nakagawa and Ogawa, 1997). The *mer1::LEU2* strain was dereived from and NKY2204 (Storlazzi et al., 1995). To make the *mer3::hisG*, *cMER3* and *mer3KA* strains, a 4.6 kb SacI fragment containing *mer3::hisG-URA3-hisG* from pTN105, a 5.6 kb EcoRI fragment containing *cMER3-URA3-MER3* from pTN149 and a 5.7 kb EcoRI fragment containing *mer3KA-URA3-mer3* from pTN156 were introduced into yeast diploid strain ,respectively. Uracil auxotrophs were selected by plating cells on SD plates supplemented with 5-fluoro-orotic acid (Boeke et al., 1984).

The *HIS4::LEU2(Bam/Mlu)*, *his4:X:LEU2(Xho/Mlu)*, *HIS4::LEU2(Bam/Sal)*, *his4X::LEU2(Xho/Sal)*, *HIS4::LEU2(Bam/Acc)* and *his4X::LEU2(Xho/Acc)* alleles were constructed by integration of a 4.0 kb XbaI-PstI fragenment from pTN248, a 7.1 kb SphI fragment from pTN254, a 4.0 kb XbaI-PstI fragment from pTN204, a 7.1 kb SphI fragment from pTN209, a 4.0 kb SacI-PstI fragment from pTN 271 and a 7.1 kb SphI fragment from pTN272, respectively. All the integrations were carrid out by lithium acetate transformation (Ito et al., 1983) and verified by Southern blot analysis (Southern, 1975).

Yeast media were prepared according to Sherman et al. (1986). MYPD, MYPL, YPA and synthetic medium were prepared as Shinohara et al. (1992). Liquid and solid SPM were prepared as before (Nakagawa and Ogawa, 1997). Cycloheximide and Canavanine were added in media to a final concentration of 3 μg/ml and 60 μg/ml, respectively.

The bacterial strains DH5α (Hanahan, 1983) and CJ236 (Kunkel,1985) were used for plasmid preparation and for growth of the uracil-substituted phagimid, respectively.

Induction of Meiosis

When cells were introduced into meiosis at 23°C, cultures in both pre-sporulation medium (YPA) and sporulation medium (SPM) were incubated at 23°C . Synchronous meiotic cultures were obtained as

described (Nakagawa and Ogawa, 1997). Induction of meiosis for tetrad dissection was carried out by incubation of cells on SPM plates.

Plasmids

Plasmids were constructed by standerd methods (Sambrook et al., 1989). End-filling of a restriction site was performed using Klenow fragment of DNA polymerase I. The plasmid, pTN45, was isolated from the screening in this study and has an approximately 9 kb insert containing the *MER3* gene in a BamHI site of YEp24 (Botstein et al., 1979). Deletion versions of pTN45 were constructed as follows; pTN45 was digested with SalI or SphI and self-ligated to produce pTN46 and pTN47, respectively. pTN45 was partially digested with HindIII and self-ligated to produce pTN66. pTN78 was made by introduction of a 2.5 kb EcoRI-ClaI fragment from pTN45 into EcoRI and ClaI sites of pBluescriptII KS^+ (Stratagene). A 1.8 kb SphI-SmaI fragment from pTN78 was introduced into SphI and SmaI sites of pTN46, creating pTN84.

pTN105 harboring *mer3::hisG-URA3-hisG* was constructed as follows; (i) A 4.8 kb NcoI (blunt-ended)-SalI fragment containing the *MER3* gene from pTN45 was introduced into SacII (blunt-ended) and SalI sites of pBluescriptII KS^+, creating pTN97. (ii) A 3.8 kb BamHI-BglII fragment containing *hisG-URA3-hisG* from pNKY291 (Alani et al., 1987) was introduced into a BamHI site of pBluescriptII SK^+ (Stratagene), creating pSK(hisG-URA3-hisG). (iii) A 3.8 kb SpeI-EcoRI blunt-ended fragment containing *hisG-URA3-hisG* from pSK(hisG-URA3-hisG) was introduced in to blunt-ended AflII and BstXI sites of pTN97, creating pTN105.

pTN146 harboring *MER3-URA3-MER3* was constructed as follows; (i) A 2.5 kb EcoRI-ClaI fragment containing N-terminal region of *MER3* from pTN78 was introduced into EcoRI and ClaI sites of YEp24 (Botstein et al., 1979), creating pTN123. (ii) A 3.0 kb SphI-SalI fragment containing C-terminal region of *MER3* from pTN45 was introduced into YEp-D (Nakagawa and Ogawa, 1997), creating pTN129. (iii) A SphI site of pTN129 was destroyed by end-filling and self-ligation, creating pTN143. (iv) A 3.2 kb BamHI-SalI fragment from pTN129 was introduced into BamHI and SalI sites of pTN143, creating pTN146 in which a 1.7 kb SphI-EcoRI region of *MER3* was directly duplicated fanking *URA3*.

pTN149 harboring *cMER3-URA3-MER3* was constructed as follows; (i) pSH1 containing a 2.3 kb fragment from position -480 to ClaI of *MER3* was made by Exonuclease III (Takara) treatment

(Sambrook et al., 1989) after SacI and EcoRI digestion of pTN78. (ii) A 0.3 kb AflII-SpeI fragment from the intronless *MER3* PCR product prepared from wild-type yeast meiotic RNA was introduced into AflII and SpeI sites of pSH1, creating pTN108. (iii) A 0.4 kb SpeI fragment from pTN78 was introduced into a SpeI site of pTN108, creating pTN109. (iv) A 1.1 kb AflII-SphI fragment from pTN109 was introduced into AflII and SphI sites of pTN146, creating pTN149.

pTN156 harboring *mer3KA-URA3-mer3* was constructed as follows; (i) A 0.4 kb SpeI fragment from pTN78 was instroduced into a SpeI site of pBluescriptII KS$^+$, creating pTN106. (ii) pTN106 was used for site-derected mutagenesis to make pTN117. (iii) A 0.4 kb SpeI fragment from pTN117 was introduced into a SpeI site of pSH1, creating pTN145. (iv) A 1.3 kb AflII-SphI fragment from pTN145 was introduced into AflII and SphI sites of pTN146, creating pTN156.

pTN204, pTN209, pTN248, pTN254, pTN271 and pTN272 used to make *HIS4::LEU2* derivatives were constructed as follows; (i) A 2.9 kb XbaI-SalI fragment from pNKY159 (Cao et al., 1990) was introduced into XbaI and SalI sites of pUC119 (Sambrook et al., 1989), creating pTN188. (ii) A 1.2 kb SalI-SalI fragment from pNKY159 was introduced into a SalI site of pBluescriptII KS$^+$ (Stratagene), creating pTN185. (iii) To make pTN187, pTN185 was digested with EcoNI and EcoRI, end-filled and self-ligated, eliminating one of two SalI sites. (iv) A 1.1 kb SalI-PstI fragment from pTN187 was introduced into SalI and PstI sites of pTN188, creating pTN196. (v) To make pTN263, a SalI site, which is recognized aslo by AccI, of pTN196 was destroyed by end-filling and self-ligation following SalI digestion of pTN196. (vi) To make pTN264, a 9 bp SmaI-XbaI region containing a BamHI site was deleted by end-filling and self-ligation following SmaI and XbaI digestion of pTN263. (vii) A 10 bp BamHI (5'-CCGGATCCGG-3') or a 8 bp XhoI linker (5'-CCTCGAGG-3') was introduced into a blunt-ended MluI or SalI site of pTN196 to make pTN248 (Bam/Mlu), pTN245 (Xho/Mlu), pTN204 (Bam/Sal) and pTN205 (Xho/Sal), and into a blunt-ended AccI site of pTN264 or pTN263 to make pTN271 (Bam/Acc) and pTN267 (Xho/Acc), respectively. (viii) A 3.3 kb BamHI-XbaI fragment containing a *his4X* mutation from pNKY445 (Xu and Kleckner, 1995) was introduced into BamHI and XbaI sites of pTN245, pTN205 and pTN267,creating pTN254 (Xho/Mlu), pTN209 (Xho/Sal) and pTN272 (Xho/Acc), respectively. Integrations of a single BamHI or XhoI linker were confirmed by DNA sequencing.

Crossover Interference

The frequency of NPDs expected was calculated from the Papazian equation (Papazian, 1952), NPD = $1/2(1-TT-(1-1.5TT)^{2/3})$, where TT is the frequency of tetratypes observed. The TT values used in the calculation are shown in Table 2.

Site-directed Mutagenesis

For creating *mer3KA* mutation, site-directed mutagenesis was performed according to Kunkel (1985), using the phagemid pTN106 and the primer priTN4 (5'-AAATAATACAGTGGCGCCTGATCCGGTTGG 3'; mutation sequences were underlined). Mutation sites were confirmed by DNA seque

Northern Blotting and RT-PCR Analysis

Cells were pelleted before and 5 hr after transferring to sporulation medium, frozen in liquid nitrogen and stored at -80°C until subsequent preparation of total RNA by glass-bead and phenol extraction (Ausubel et al., 1995).

For Northern bollting analysis, total RNA samples were separated on a 0.7% agarose gel in MOPS/formaldehyde buffer (Sambrook et al., 1989), soaked in a 0.05N NaOH buffer for 20 min for partial digestion of RNA, and transferred to NYTRAN nyron membrenes (Schleicher & Schuell) in a 10X SSC buffer. A series of hybridizations and re-hybridizations of the same membranes were perfomed as described by Sambrook et al (1989). For the detetion of *MER3* and *ACT1* transcripts, a 1.0 kb BstBI-ClaI fragment from pTN78 and a 0.6 kb ClaI fragment from pTN7 (Nakagawa and Ogawa, 1997) were ^{32}P-labelled using a random primer method (Ausubel et al., 1995) and used as respective hybridization probes. De-hybridization from the membranes was carried out by incubation in 2% SDS and 20 mM Na-phosphate buffer (pH7.2) at 100°C. Northern blot signals were quantified with a Fuji BAS2000 phosphoimager.

2.5 μg of total RNA was treated with RNase-free DNase I *FPLCpure™* (Pharmacia) to eliminate contaminating DNA, subjected to reverse transcriotion (RT) with 16 U M-MuLV reverse transcriptase (New England Biolabs) using 3 pmol each of *MER3*-specific primer priTN2 (5'-CGCCTCTTCATCAGGTGTCTGCTCTAAAATCG-3') and *ACT1*-specific primer priTN11 (Nakagawa and Ogawa, 1997). PCR (Saiki et al., 1988) was carried out with the cDNA using *MER3* intron-flanking

primers, priTN1 (5'-GGTGGATTTGACAACTTAAGAGGCGTCG-3') and priTN2, or *ACT1* intron-flanking primers, priTN10 and priTN11 (Nakagawa and Ogawa, 1997) under the following conditions: 1 min at 94°C and then 30 sec at 94°C, 10 sec at 54°C and 30 sec at 74°C for 35 cycles. 2.5 U of KOD dash DNA polymerase (Toyobo) was used for the each PCR reaction. The amplified products were analysed on 1.2% agarose gels, and visualized by Ethidium Bromide. The sizes of the amplified products corresponding to unspliced and spliced *MER3* RNAs were 500 and 348 bp, and those of *ACT1* RNAs are 475 and 166 bp.

Physical Detection of Meiotic Recombination Events

DNA was prepared as discribed by Ausubel et al. (1995). Detection of the DSBs, CRs and NCRs was performed as discribed (Storlazzi et al., 1995). Digested DNA samples were separated by 0.6 or 0.7% agarose gel electrophoresis. A 1.5 kb PstI-EcoRI fragment from pNKY291(Cao et al., 1990) or a 1.6 kb PstI-SacI fragment from pNKY155 (Cao et al., 1990) labeled with ^{32}P by the random primer method (Ausubel et al., 1995) was used as a probe for Southern hybridization. Southern blot signals were quantified with a Fuji BAS2000 phosphoimager.

Anti-Mer3 Antibody Production and Purification

Hexahistidine (His6) tagged Mer3 truncated protein was overexpressed using the plasmid pTN167, constructed by introducing a 0.8 kb EcoRI-SacI fragment from pTN45, correspoding amino acids 921 to 1,171 of Mer3, into EcoRI and SacI sites of pET21a(+) (Novagen). After E. coli strain BL21 (DE3) (Studier et al., 1992) harboring pTN167 was grown to log phase ($2X10^8$ cells/ml) in the presence of ampiciline (50 mg/ml), IPTG was added at a final concentration of 1 mM and the incubation was continued for 3 hr. Cells were lysed by sonication (Branson Cell Disrupter model 185) in lysis buffer (50 mM sodium phsphate (pH 8.0), 300 mM NaCl, 10% gelycerol, 0.5% Tween20, 20 mM imidazole). The His6-Mer3 protein was purified using Ni-NTA agarose (Qiagen). Approximately 2 μg of the purified protein was sent to Akagi Trading Co. (Kobe, Japan) for immunization of a rabbit. Anti-Mer3 antibodies were affinity using HiTrap NHS-activated column according to a method recommended by the supplier (Pharmacia Biotech).

Immunotaining

The immunostaing was performed according to a previously described method (Klein et al., 1992; Bishop, 1994) with minor change. After preincubation in 500 μl of BSA/TBS (1% w/v BSA, 150 mM NaCl, 20mM Tris (pH7.4)) for 15 min at room temperature, 90 μl of BSA/TBS containing an appropriate dilution of primary serum or antibody was placed on the slide and the slides were incubated overnight at 4°C followed by twice 10 min wash in TBS. Then, 90 μl of BSA/TBS containing fluorochrome-conjugated antibodies was placed on the slide and the slides were incubated at room temperature for 2 h followed by the wash described above. Afer immunostaining, spread nuclei were stained with 0.1 μg/ml DAPI (Nacalai Tesque) in TBS for 2 min at room temperature followed by the 10 min wash in TBS. Slides were drained on edge for 15 min and mounted with Vectashield mounting medium (Vector Laboratories).

Affinity purified anti-Mer3 rabbit antibody and anti-Zip1 mouse antibody (From S. G. Roeder) were use at 1/100 dilution. Anti-Rad51 (Shinohara et al., 1992) and anti-Dmc1 (Bishop, 1994) rabbit antisera were used at 1/200 dilution. FITC-labeled goat anti-rabbit IgG (Cappel) and Texas Red-X-labeled goat anti-mouse IgG (Molecular Probes) were used as the 2nd antibody at a 1/1,000 dilution.

Immunofluorescence Microscopy

Images were taken using an epiflorescence microscope (Zeiss Axiovert 135M, 100X objective with NA 1.3) equipped with a monochrome CCD camera (Photometrics, PXL1400-C1-M), which was controlled by a Machintosh 9500/120 (Apple computer). The images were collected, merged and psedocolored using the CCD image capture with IP Lab-PVCAM software (Signal Analysis Corporation). The images were printed out using Adobe Photoshop software and with a Pictrography 3000 (Fujix).

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