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The *MRE4* gene encodes a novel protein kinase homologue required for meiotic recombination in *Saccharomyces cerevisiae*

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ABSTRACT

The *MRE4* gene was cloned by complementation of the defects of meiotic recombination and haploidization in an *mre4-1* mutant. Disruption of *MRE4* resulted in reduced meiotic recombination and spore inviability. The *mre4* spore lethality can be suppressed by *spo13*, a mutation that causes cells to bypass the reductional division. Analysis of meiotic DNA extracted from the *mre4* mutant cells revealed that double-strand breaks occurred at the two sites of the *HIS4-LEU2* recombination hot spot, but at a frequency of about 10–20% of the wild type. Northern blot analysis indicated that the *MRE4* gene produces four transcripts of 1.63, 3.2, 4.0 and 6.2 kb. All of these transcripts are absent from mitotic cells and are meiotically induced. The DNA sequence of the *MRE4* open reading frame predicts a 497-amino acids protein with a molecular mass of 56.8 kDa. The Mre4 protein contains highly conserved amino acid sequences found specifically in serine-threonine protein kinases. These results suggest that protein phosphorylation is required directly or indirectly for meiotic recombination.

INTRODUCTION

Following entry into meiosis from the G1 stage of the mitotic cell cycle, yeast *Saccharomyces cerevisiae* cells undergo premeiotic DNA synthesis, homologous chromosomes pairing and genetic recombination, two successive chromosome segregations at Meiosis I (reductional division) and Meiosis II (equational division), and spore formation (1). In this meiotic process, recombination plays a part not only in generation of genetic diversity but also in proper segregation of homologous chromosomes at Meiosis I. Thus, meiotic recombination is a significant and essential function of meiosis in yeast.

To investigate the molecular mechanism of meiotic recombination, various meiotic recombination-deficient mutants have been isolated and characterized (2). Among them the mutants, *rad50* (3), *rad51* (Shinohara,A. and Ogawa,T.; personal communication), *hop1* (4,5), *spo11* (6), *red1* (7), *mer1* (8) and *mer2* (9), have been examined extensively and revealed the presence of several key steps of meiotic recombination, such as, formation of synaptonemal complex (SC), incision of double strand of chromosomal DNA at specific sites, processing of the ends at the breaks and recombinant molecule formation. To obtain more informations about these processes, we isolated new mutants defective for meiotic recombination. Using the same strategy as by Roth and Fogel (10), we mutagenized a chromosome III disomic haploid strain and selected for mutants which were unable to carry out meiotic recombination but were not defective in mitotic recombination. Five classes of mutants were isolated and named as *mre1,2,3,4* and *11* (*m*eiotic *re*combination) (11). All of these classes, except for *MRE1*, were new genes. The *MRE1* gene was concluded to be the same gene as *HOP1* (5) by comparing the nucleotide sequences of both genes, although some discrepancies in amino acid sequence are found between them.

The recessive *mre4-1* mutant showed no alteration in mitotic recombination and DNA repair, but showed reductions in both meiotic recombination and spore viability, to 2–5% of the wild type level. This residual recombination, however, is 10 to 50 times higher than that in other *mre* mutants (11).

To get an insight into the function and regulation of the *MRE4* gene during meiotic recombination, we cloned the *MRE4* gene. The *MRE4* ORF is composed of 1,491 base pairs (bp) and encodes a protein of 497 amino acids. The deduced amino acid sequence of Mre4 protein contains segments homologous to the catalytic domains typical of a serine-threonine protein kinase. We therefore suggest that protein phosphorylation is required for meiotic recombination, and that the *MRE4* gene plays a key role in the regulation of meiotic recombination.

MATERIALS AND METHODS

Strains and media

The genotypes of *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Strains KJC102 and KJC103 were used for construction of *MRE4* disruptants, and the strain KJC101 was used for preparation of RNA. The MYPL plate contains 0.3% malt extract, 0.3% yeast extract, 0.5% polypeptone, 2% agar and 2% lactate. Sporulation medium (SPM) contains 0.03% raffinose and 2% potassium acetate. Other media used in this study were the same as described (11).

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Procedure for sporulation

The SK1 cells were streaked on an MYPL plate and the plates were incubated at 30°C for 3 days. A single colony from a such plate was inoculated into a YPD liquid medium, and cells were grown overnight to saturation. The culture was then diluted 100-fold into presporulation medium (YPA) and incubated with shaking until the cell density reached $2-4\times10^7$ cells/ml. Cells were harvested, washed with sterile water, and resuspended at the same density in SPM. Then, the suspension was incubated at 30°C with vigorous shaking.

Cloning of the *MRE4* gene

The *MRE4* gene was cloned by screening for complementation of the defect in meiotic recombination of the *mre4-1* mutant. The yeast library used to isolate the *MRE4* gene contains a partial *Sau*3AI digested genomic DNA fragments into YCp50 vector (12). Strain P160m4 (Table 1) was transformed with this library, and about 20,000 Ura$^+$ transformants were obtained on SD-Ura (lacking uracil) plates. These transformants were replicated on SPM plates to permit cells to enter the meiotic phase. After 5 days at 30°C, sporulated transformants were replicated onto five kinds of screening plates; i) lacking uracil for selection of plasmid containing colonies (SD-Ura), ii) lacking uracil and containing canavanine (Can) and cycloheximide (Cyh), for detection of haploidization of chromosome V and VII (SD-Ura+Can+Cyh), iii) lacking uracil, leucine and histidine, for detection of Leu$^+$ and His$^+$ recombinants (SD-Ura-Leu-His), iv) lacking uracil and leucine and containing Can (SD-Ura-Leu+Can), and v) lacking uracil and histidine and containing Can (SD-Ura-Leu+Can). The last two plates were used for selection of the transformants that could successfully performed both recombination and haploidization. Plasmid DNAs were recovered from the 116 Ura$^+$ Leu$^+$ His$^+$ Can^r Cyh^r candidates and were retested quantitatively for their ability to complement the meiotic recombination defect in the *mre4-1* mutant.

DNA sequencing

A DNA fragment (2.7 kb *Xba*I-*Pvu*II) containing complementation ability for the meiotic recombination defect in the *mre4-1* mutant was isolated from pSL47 (Fig. 1) and inserted into pUC118 and pUC119 vectors (13). Plasmids carrying serial deletions from both ends of the fragment were constructed by digestion with ExoIII-S1 nuclease after cutting with *Bam*HI and *Pst*I (13,14). Using these series of plasmids, single-stranded DNAs were prepared. Determination of the nucleotide sequence was carried out by the chain termination method (15) using T7 DNA polymerase.

Table 1. Genotypes of strains

Strain	Genotype
P160m4	*MATa* leu2-1 his4-4 can1^r ura3 cyh2^r ade6 TRP1 MET2 ade2 mre4-1 / *MATα* leu2-27 his4-290 CAN1 ura3 CYH2 ADE6 trp1 met2 ade2 mre4-1
*KJC101	*MATa* leu2 his4-4 CAN1 ura3 ho::LYS2 trp1 CYH2 ADE6 ade2 lys2 / *MATα* leu2 his4-290 can1^r ura3 ho::LYS2 trp1 cyh2^r ade6 ade2 lys2
*KJC102	*MATa* leu2 his4-4 CAN1 ura3 ho::LYS2 trp1 CYH2 ADE6 ade2 lys2 / *MATα* leu2 his4-4 CAN1 ura3 ho::LYS2 trp1 CYH2 ADE6 ade2 lys2
*KJC103	*MATa* leu2 his4-290 can1^r ura3 ho::LYS2 trp1 cyh2^r ade6 ade2 lys2 / *MATα* leu2 his4-290 can1^r ura3 ho::LYS2 trp1 cyh2^r ade6 ade2 lys2
*IMD401 (D1)	KJC101 with D1 / D1
*IMD402 (D2)	KJC101 with D2 / D2
*IMD403 (D3)	KJC101 with D3 / D3
*IMD404 (D1 / MRE4)	KJC101 with D1 / MRE4
*IMD405 (D2 / MRE4)	KJC101 with D2 / MRE4
*IMD406 (D3 / MRE4)	KJC101 with D3 / MRE4
IMD407	NKY1238 / NKY1240 with D2 / D2
IMD412	IMD501 with D2 / D2
IMD501	*MATa* ho::LYS2 spo13::hisG ura3 lys2 / *MATα* ho::LYS2 spo13::hisG ura3 lys2
NKY653	*MATa* ho::LYS2 rad50::hisG spo13::hisG ura3 lys2
NKY654	*MATα* ho::LYS2 rad50::hisG spo13::hisG ura3 lys2
NKY1238	*MATa* ho::LYS2 ura3 lys2 leu2::hisG his4X::LEU2-URA3 arg4-nsp
NKY1240	*MATα* ho::LYS2 ura3 lys2 leu2::hisG his4B::LEU2 arg4-bgl

All strains except P160m4 were derived from the strain SK1. * These strains are hybrids, not pure SK1 strains. NKY strains were supplied by Dr. N. Kleckner and P160m4 was supplied by Dr. Ajimura.M.

Construction of the disrupted *mre4* mutants and the double mutant with *spo13*

Three plasmids with disrupted *mre4* gene were constructed as follows. The 1.2 kb *Hin*dIII fragment containing the *URA3* gene from YEp24 plasmid (16) was inserted into the *Spe*I site (D1) or into the *Cfl*I site (D3), or used to replace the region between the *Spe*I and *Hpa*I sites (D2) of the *Xba*I-*Pvu*II fragment (Fig. 3). The *Xba*I-*Pvu*II DNA fragments containing the disrupted *mre4* genes with the *URA3* insertion were purified by electrophoresis in a Sea-kem agarose gel (FMC Bio Products) and used for transformation of diploid strains (KJC102 and KJC103). The disruption of the chromosomal *MRE4* gene in Ura$^+$ transformants was confirmed by Southern blot analysis (13). The confirmed transformants were subjected to the tetrad analysis to produce haploid mutant strains. A diploid strain homozygous or heterozygous for the disrupted *mre4* gene was constructed by mating. The *mre4::D2* double mutant with *spo13* (17) was constructed by crossing with the haploids of IMD402 and NKY654 (*spo13::hisG*, Table 1).

RNA isolation and Northern blot analysis

To prepare RNA samples, cells were sporulated in 1 liter of media as described in Procedure for sporulation. A one hundred ml aliquot of the cell culture was taken at each time and one portion of the aliquot was plated immediately on SD-His and MYPD plates to measure the frequency of meiotic recombination between heteroalleles, *his4-4*/*his4-290*. Usually the frequency of spore formation measured by phase-contrast microscopy was about 70% at 12 hr. The cells were harvested by centrifugation, washed with cold water and resuspended in 8 ml of SDS buffer (0.5 M NaCl, 50 mM Tris-HCl pH 7.5, 0.2% SDS and 1 mM EDTA). An equal volume of phenol/chloroform and 8 g of acid-washed 0.45 μm glass beads were added, the mixture was vortexed immediately for 1 min at highest speed, and then placed on ice for 10 min. Phenol/chloroform extraction was repeated two additional times. Collected supernatant and precipitated by adding 2.5 volume of ethanol containing 1/1000 volume of diethyl pyrocarbonate (DEPC). Recovered pellet was resuspended in 8 ml of SDS buffer, 6 ml of 5 M LiCl was added, and stored at −20°C for 3 hr to precipitate RNA (18).

About 20 μg of total RNA was heat denatured and run on a 6% formaldehyde gel (1% agarose) with MOPS (3-[N-Morpholino] propanesulfonic acid) buffer (13). After electrophoresis, the gel was treated with 0.25 M ammonium acetate (pH 5.6) for 15 min and blotted onto a nylon membrane (IMMOBILON-N, Millipore). The membrane was baked at 80°C in a vacuum oven for 2 hr. Prehybridization, hybridization and washing of the membrane were done as described (19).

S1 mapping

After denaturartion of the mixture containing total RNA (40 μg) and ssDNA (1 μg) in 30 μl of hybridization buffer (40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA pH 8.0 and 80% deionized

formamide) at 85°C for 10 min, it was hybridized at 49°C for 3 hr. Then S1 nuclease digestion was carried out by addition of 0.3 ml of ice-colded S1 buffer (280 mM NaCl, 50 mM sodium acetate pH 4.5 and 4.5 mM $ZnSO_4$ with 8 units of S1 nuclease/μg of nucleic acid), and the reaction mixture was incubated at 37°C for 30 min. After addition of 80 μl of S1 nuclease stop solution (50 mM EDTA pH 8.0 and 50 μg/ml carrier tRNA), the reaction mixture was treated with an equal volume of phenol/chloroform. DNA-RNA hybrid was precipitated with ethanol, suspended in 30 μl of TE buffer and analyzed by electrophoresis through an alkaline or a neutral agarose gel (13).

Detection of the double-strand breaks

Detection of the double-strand breaks (DSBs) at the *HIS4-LEU2* locus or *ARG4* locus in meiosis was carried out as described (20,21). One-tenth of the DNA isolated from a 10 ml culture was digested by *Pst*I and run on a 0.7% agarose gel. DNAs were transferred to NYTRAN membrane (Schleicher & Schuell). The *HIS4-LEU2* probe used for Southern blot analysis was a labeled 1 kb *Pst*I-*Bgl*II DNA fragment derived from a sequence downstream of the *HIS4* gene (20). The DNA samples used for detection of DSBs at the *ARG4* locus were digested with *Bgl*II and probed with the *Eco*RV-*Bgl*II fragment (21). After autoradiography, the intensity of bands were quantitated by a Bioimage analyzer BAS 2000 (Fuji Film Co.).

RESULTS

Cloning of DNA fragments which complement the *mre4-1* mutation

DNA fragments which complements the *mre4-1* mutation were cloned from *Saccharomyces cerevisiae* genomic library. They were isolated by complementation of defects both in meiotic recombination and in haploidization of the mutant. Plasmids pSL41 and pSL42 (Fig. 1) isolated from two transformants among 116 candidates, carried 8 kb (pSL41) and 9 kb (pSL42) fragments, respectively, inserted in the YCp50 vector and their restriction maps showed that they have a 4.6 kb overlapped region in common (Fig. 1). Plasmids containing subfragments of the overlapped region were constructed and their complementation abilities were examined by transformation of a *mre4-1* homozygous diploid strain, P160m4 (Fig. 1). The plasmid pSL47 contains the smallest fragment (2.7 kb *Xba*I-*Pvu*II fragment) having the ability to complement the *mre4-1* mutation, and was used determination of nucleotide sequence.

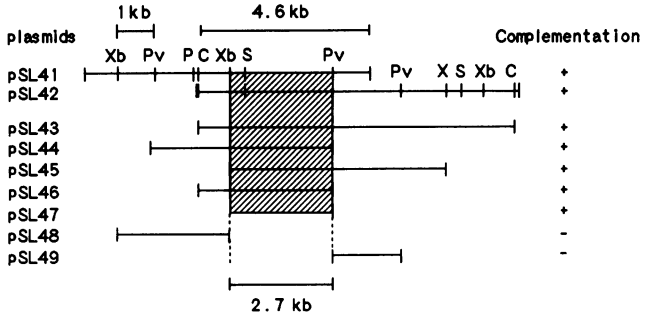

Figure 1. Restriction maps of the 8.0 kb (pSL41) and 9.0 kb (pSL42) fragments containing the *MRE4* gene and complementation analysis of the subclones. The upper two lines represent two restriction enzyme maps of the yeast DNA inserts present in the original *mre4-1* complementing plasmids, pSL41 and pSL42. Other lines represent the region subcloned into YCp50. All of these plasmids were tested for their ability to complement the meiotic recombination defect in P160m4. The hatched area represents the 2.7 kb *Xba*I-*Pvu*II fragment insert contained within pSL47, the minimal *mre4-1*-complementing plasmid. Abbreviations for restriction enzyme sites are as follows : Bg,*Bgl*II; C,*Cla*I; P,*Pst*I; Pv,*Pvu*II; S,*Sca*I; Xb,*Xba*I; X,*Xho*I.

DNA sequence of the 2.7 kb fragment

To facilitate determination of the nucleotide sequence, the 2.7 kb fragment was recloned on pUC118 and pUC119 vectors and serial deletions of the fragment were generated. Using these plasmids, the entire nucleotide sequence of the 2.7 kb fragment was determined on both strands (Fig. 2). The sequenced fragment consists of 2,655 base pairs (bp). The nucleotide, T, at the 5′ end of the upper strand of the fragment was numbered as position 1. A search for open reading frames (ORFs) in the fragment

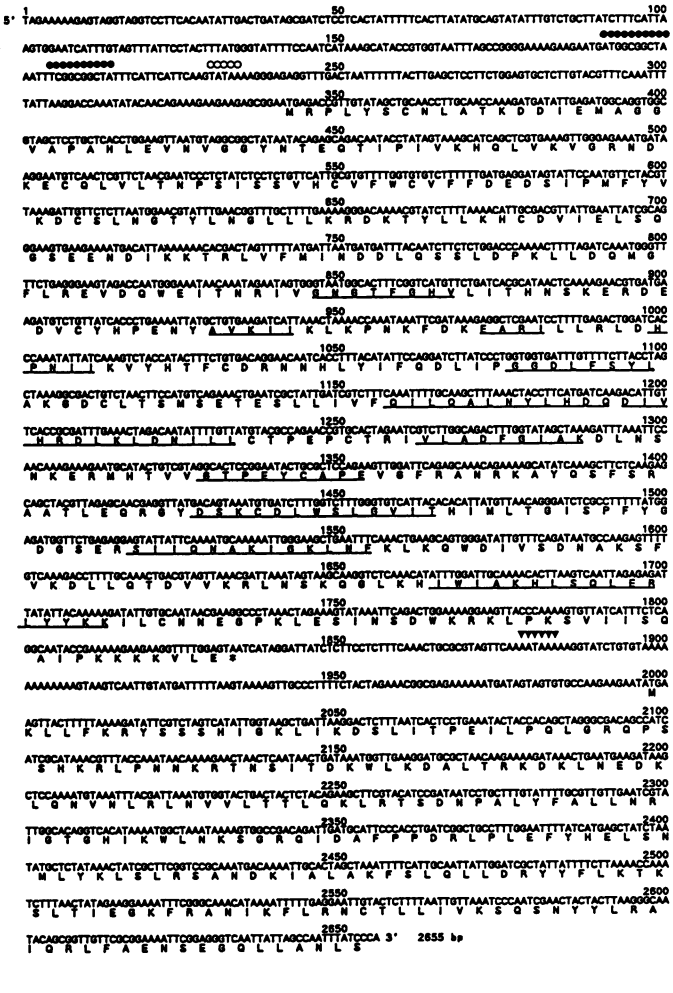

Figure 2. Nucleotide sequences of the 2.7 kb *Xba*I-*Pvu*II fragment and predicted amino acid sequences. The DNA sequence of the minimum *mre4-1* complementing fragment is presented together with the amino acid sequences of *MRE4* and the adjacent truncated gene displayed below the DNA sequence. The nucleotide T, at the 5′ end of the non-transcribed strands, is numbered as 1, and the position 341 is the first base of the *MRE4* open reading frame. Open circles indicate a potential TATA box and closed circles indicate the repeated upstream repression sequence (URS). The closed arrow heads indicate the sequence AATAAA required polyadenylation. Amino acid sequences corresponding to the catalytic domains of serine-threonine protein kinases are underlined.

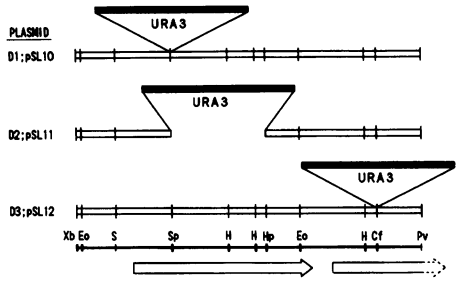

Figure 3. Disruptions of the *MRE4* region. Constructions of *mre4::D1*, *mre4::D2* and *MRE4::D3* disruptions are illustrated. Open arrows indicate the orientations and positions of two large open reading frames, ORF1 and ORF2. Abbreviations for restriction enzyme sites are as follows : Cf,*Cfr*I; Eo,*Eco*O109; H,*Hin*dIII; Hp,*Hpa*I; S,*Sac*I; Sp,*Spe*I; Pv,*Pvu*II; Xb,*Xba*I.

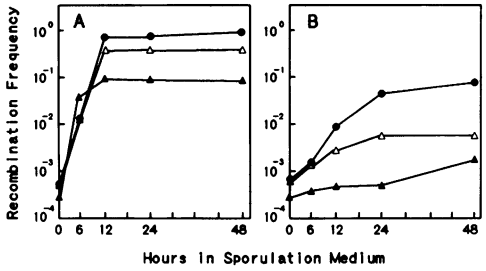

Figure 4. Time course of haploidization, intergenic recombination and intragenic recombination by return-to-growth experiments. Haploidization was monitored at *cyh2* locus (●), intergenic recombination at *cyh2-ade6* (△) and intragenic recombination at *his4* heteroalleles (▲) were examined in the wild type (A) and *mre4::D2* (B) strains. Cells grown in YPA at 2×10^7 cells/ml were collected and resuspended into SPM at the same density. At appropriate times after incubation in SPM, aliquots were taken and plated on SD-Ura, SD+Cyh (1 μg/ml) or SD-His.

revealed two ORFs of significant sizes: one, named ORF1, consists of 1,491 bp beginning at position 341 and terminating at position 1,831; another is a truncated ORF of 659 bp, named ORF2, which starts at position 1,997 and continues beyond the 3′ end of the fragment.

ORF1 contains the *MRE4* gene

To determine which ORF encodes the *MRE4* gene product, disruptants of each of the two ORFs on the 2.7 kb fragment were constructed (Fig. 3). For disruption of ORF1, a 1.2 kb *URA3* fragment was inserted at the *Spe*I site (disruptant 1; *mre4::D1*) or substituted for the 760 bp *Spe*I-*Hpa*I region (disruptant 2; *mre4::D2*). For ORF2, the *URA3* gene was inserted at the *Cfr*I site (disruptant 3; *MRE4::D3*) (Fig. 3). Each disruptant on the vector plasmid was introduced into the yeast chromosome by substitutional transformation of diploid strains, KJC102 and KJC103, which carry suitable genetic markers for monitoring recombination (Table 1). The correctness of the substitution was confirmed by Southern blot analysis (data not shown). Tetrads from these diploid transformants showed 2:2 (+/−) cosegregation of the *mre4* mutant phenotype with uracil prototrophy, and all four spores of each tetrad were viable, indicating that the *MRE4* gene is not essential.

By mating two appropriate haploids from these transformants, homozygous diploid stains with the disrupted *mre4* genes (IMD401(*mre4::D1*), IMD402(*mre4::D2*) and IMD403(*MRE4::D3*)) were constructed (Table 2). The *mre4::D1* or *mre4::D2* homozygous diploid strain formed inviable spores in meiosis, as previously observed for the *mre4-1* mutant (11). In contrast, the *MRE4::D3* homozygous diploid strain formed viable spores. Its generation time in mitosis, however, was about 2 times longer than that in wild type or other two disruptants.

Then, each disrupted haploid strain was mated with the original *mre4-1* haploid strain and the resulting diploids were analyzed for its spore viability. Both of the *mre4::D1/mre4-1* and *mre4::D2/mre4-1* diploid strains produced inviable spores (spore viability is less than 1%). In contrast, the *MRE4::D3/mre4-1* produced viable spores; the observed spore viability of 92% is essentially the same as that of the wild type strain, KJC101. These results indicate that ORF1 encodes the *MRE4* gene and represents the wild type allele of *mre4-1*.

To examine the recombination proficiency of the homozygous *mre4::D2/mre4::D2* diploid strain (Fig. 4 B), the frequencies of intergenic recombination between *cyh2*^{r} and *ade6* genes, intragenic recombination at *his4* heteroallele (*his4-4/his4-290*) and haploidization of the heteroallele, *cyh2*^{r}/*CYH2*, were measured by return-to-growth analysis and compared with those of the wild type diploid strain (Fig. 4 A) at various time points during meiosis. In the *mre4::D2* disruptant, the frequencies of both types of recombinants and of haploidization were reduced to 2−7% of the level of the wild type strain. Experiment with the *mre4::D1* disruptant gave similar results (data not shown). On the other hand, these frequencies in the *MRE4::D3* strain were almost the same as in the wild type strain, although recombinant formation was somewhat delayed (data not shown). In contrast, in all three disruptants, both spontaneous and UV-stimulated recombination in mitosis were not significantly different from those of the wild type strain (data not shown).

Table 2. Spore viability of the *MRE4* gene disruptants and *mre4 spo13* double mutant

Strain	Genotype	Total asci dissected (total spores)	viable spores / ascus 0	1	2	3	4	Spore viability (%)
KJC101	*MATa* *MRE4* / *MATα* *MRE4*	44(176)	−	−	1	3	40	97.2
IMD405	*MATa* *D2::URA3* / *MATα* *MRE4*	44(176)	−	−	−	8	36	95.5
IMD401	*MATa* *D1::URA3* / *MATα* *D1::URA3*	88(352)	88	−	−	−	−	<0.3
IMD402	*MATa* *D2::URA3* / *MATα* *D2::URA3*	88(352)	88	−	−	−	−	<0.3
IMD403	*MATa* *D3::URA3* / *MATα* *D3::URA3*	44(176)	−	−	1	2	41	97.7
IMD501	*MATa* *spo13* / *MATα* *spo13*	44(88)	6	14	24			70.5
IMD412	*MATa* *D2::URA3* *spo13* / *MATα* *D2::URA3* *spo13*	84(168)	50	20	14			28.6

Spore viability was tested 3 days after transfer from YPD to SPM plates.

The *MRE4* transcripts are induced in meiosis

To obtain information about expression of the *MRE4* gene, the amount of RNA specific for ORF1 was measured by Northern blot hybridization. Total RNA was isolated from cells of a wild type strain, KJC101, at various times after transfer to SPM, and subjected to Northern blot analysis after gel electrophoresis (Fig. 5). Four DNA fragments from different regions of the 2.7 kb fragment were prepared as probes. RNAs corresponding to ORF1 were not detectable in mitosis (0 hr), while in meiosis, four kinds of transcripts were detected using either Probe 3 (Fig. 5 A) or Probe 2 (data not shown). The intensity of all four

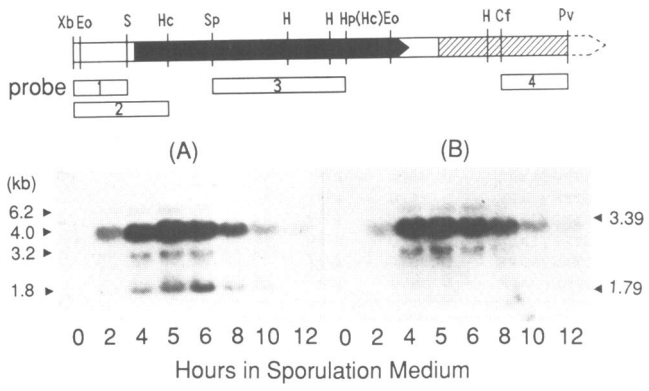

Figure 5. Northern blot analysis of transcripts from the *MRE4* region. RNA samples were prepared from strain KJC101 at the indicated times after transfer to sporulation medium. Closed arrow represent the location and orientation of *MRE4* ORF and hatched area shows the truncated ORF. The blot in (A) was hybridized with Probe 3 and the blot in (B) with Probe 4. The positions of size markers, 25S and 18S ribosomal RNAs (22,23) are presented to the right of panel B, the calculated lengths of the RNAs are given to the left of panel A. Four probes were prepared; Probe 1 (1−264), Probe 2 (1−507), Probe 3 (733-1,474) and Probe 4 (2,335-2,655).

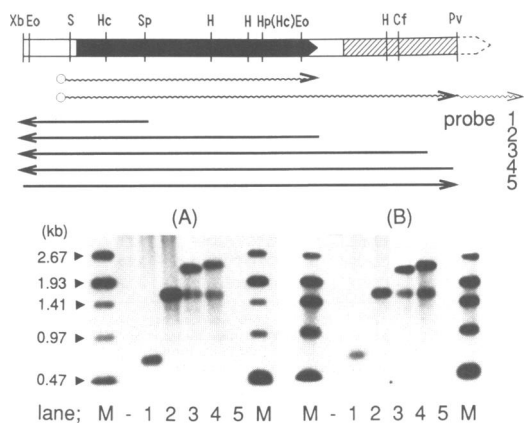

Figure 6. Transcription map in the *MRE4* region and S1 mapping autoradiogram. Locations and orientations of transcripts are shown by wavy arrows. Single-stranded DNAs for S1 mapping are depicted by arrows (Probe 1, 2, 3, 4 and 5). Total RNA (40 μg) prepared from SK1, KJC101, was hybridized to 1.0 μg each of single-stranded probe DNAs, digested with S1 nuclease, fractionated on 0.8% neutral (A) or alkaline (B) agarose gel electrophoresis, and subjected to Southern blotting analysis with labeled 2.7 kb *Xba*I-*Pvu*II fragment as a probe. Lanes 1−5 correspond to samples with probes 1−5, respectively. Size markers are given on both sides of the gels (lane M). Size measurements are accurate to ± 5%.

bands increased and reached a maximum level at between 5 and 6 hr in meiosis, and then decreased. The relative intensities of the bands at the 5 hr were 16.0, 9.0, 71.6, and 3.4 (%) for transcripts of 1.8 kb, 3.2 kb, 4.0 kb and 6.2 kb in size, respectively. Using Probe 4, all transcripts except the 1.8 kb transcript, were detected (Fig. 5 B). On the other hand, when Probe 1 was used, extremely faint 4.0 kb and 1.8 kb bands were detected after a long exposure (data not shown). These results suggest that all transcripts identified by Probe 2 or Probe 3 are probably initiated from the site just inside the right end of Probe 1. Furthermore, the 1.8 kb transcript, the second most abundant transcript, is probably terminated at a weak terminator around the position 2,000 (see Fig. 2), about 170 bp downstream of ORF1. All other RNAs are longer transcripts that read through into ORF2.

The initiation site(s) of RNA synthesis was determined by S1 mapping. Total RNA was prepared from a wild type cells (KJC101) cultured for 6 hr after transfer to SPM. The length of the DNA-RNA hybrid protected from S1 nuclease digestion, was deduced from their mobilities in neutral and alkaline gel electrophoreses.

In both neutral and alkaline agarose gel electrophoreses, a single 0.59 kb band (Fig. 6, lane 1) was detected with Probe 1 (position 1−800), indicating that the 5′ ends of all the *MRE4* transcripts were initiated around position 210. The analysis with Probe 2 (1-1,840) showed the presence of a single band of 1.63 kb in length, while a set of two bands, 1.63 kb and 2.33 kb, or 1.63 kb and 2.44 kb, was detected with Probe 3 (1-2,540) or Probe 4 (1-2,654), respectively. On the other hand, no transcript with the opposite orientation was detected (Fig. 6, lane 5). All these results confirm that all of the transcripts are most probably initiated from the identical site at around position 210 and terminated at different positions.

The smallest 1.63 kb *MRE4* RNA (corresponding to the 1.8 kb RNA in Northern blot analysis) is initiated at around position 210 and terminated at around position 1,840. This region covers the entire *MRE4* ORF. In yeast translation usually starts at the first AUG codon from the 5′ end of the message, and therefore these results suggest that the Mre4 protein corresponds to the entire ORF1.

These results also show that there is no splice junctions in the *MRE4* ORF region, because RNA bands of the same sizes were detected in both neutral and alkaline agarose gel electrophoreses. This result is in good accordance with the fact that there is no consensus sequence for splicing (e.g. TACTAAC,24,25) in this region (Fig. 2).

The putative Mre4 protein contains a consensus sequence for serine-threonine protein kinases

The Mre4 protein deduced from the nucleotide sequence of the *MRE4* ORF is composed of 497 amino acids with a predicted molecular weight of 56,847 and isoelectric point of 7.76. To gain an insight into possible functions of the Mre4 protein, the predicted amino acid sequences were compared with those of other proteins in the data bases, NBRF-PIR and SWISS-PROT. It was revealed that the Mre4 protein contains sequences homologous to the highly conserved amino acid sequences found specifically in serine-threonine protein kinases (26−28), as shown in Table 3. All protein kinases contain a consensus motif for ATP binding, G-X-G-X2-G-Xn-A-X-K (26). In the Mre4 protein this motif is located from positions 169 to 199 in the amino acid sequence. In addition, Mre4 protein has sequences conserved in serine-threonine protein kinases: GxGxxGxV- -AxK- -E- -RDLKxxN- -DFG- -GTPxxxAPE- -DxWSxG- -R (from positions 169 to 453) (27,28). Highly conserved consensus domains in some serine-threonine protein kinases that are especially similar to that in Mre4 protein are shown in Table 3. The similarity in amino acid sequences strongly suggests that the Mre4 protein is a functional serine-threonine protein kinase.

Rockmill and Roeder have isolated the *mek1* mutant as one among a collection of sporulation-proficient, meiotic-lethal mutants. Based on a comparison of DNA sequences (Rockmill,B. and Roeder,G.S., submitted to *Genes Dev.*,), we have recently determined that *MEK1* is the same gene as *MRE4*.

Table 3. Comparison of conserved amino acid sequences in serine-threonine protein kinases with that of the Mre4 protein kinase which affects meiotic recombination

Genes	GxGxxGxV -- AxK -- E -- RDLKxxN -- DFG -- GTPxxxAPE -- DxWSxG -- R	Ref.
MRE4	^{169}GNGTFGHV -- AVK -- E -- RDLKLDN -- DFG -- GTPEYCAPE -- DLWSLG -- R^{453}	this study
TPK1	^{96}GNGSFGRV -- AMK -- E -- RDLKPEN -- DFG -- GTPDYIAPE -- DWWSFG -- R^{324}	29
TPK2	^{77}GNGSFGRV -- AIK -- E -- RDLKPEN -- DFG -- GTPDYIAPE -- DWWSFG -- R^{307}	29
CAMII	^{23}GKGAFSVV -- AAK -- E -- RDLKPEN -- DFG -- GTPGYLAPE -- DLWACG -- R^{260}	30
PKC(Z)	^{159}GRGSYAKV -- AMK -- E -- RDLKLDN -- DYG -- GTPNYIAPE -- DWWAFG -- R^{401}	31
PKC(E)	^{415}GKGSFGKV -- AVK -- E -- RDLKLDN -- DFG -- GTPDYIAPE -- DWWAFG -- R^{649}	31
cAPK	^{50}GTGSFGRV -- AMK -- E -- RDLKPEN -- DFG -- GTPEYIAPE -- DWWAFG -- R^{280}	32
CDC28	^{15}GEGTYGVV -- AIK -- E -- RDLKPQN -- DFG -- VTLWYRAPE -- DTWSIG -- R^{283}	33
CDC7	^{40}GEGTFSSV -- ALK -- E -- RDLKPTN -- DFG -- GTRGFRAPE -- DIWSVG -- R^{457}	34

TPK1, TPK2 :cAMP-dependent protein kinase type 1 and 2 (Yeast). *CAMII*: Calcium/Calmodulin-dependent protein kinase type II, beta-chain (Rat).*PKC(Z),PKC(E)*: protein kinase C, zeta type and epsilon type (Rat).*cAPK*: cAMP-dependent protein kinase alpha-catalytic subunit (Bovine). *CDC28,CDC7* : cell division cycle protein (Yeast).

Table 4. Comparision between the upstream of *MRE4* gene and the URSs of other yeast genes

Genes	5' -	URS Consensus TAGCCGCCGR	- 3'	Ref. (38,39)
**MRE4*	- 127	TAGCCGCCGA	- 136	this
	- 141	TAGCCGCCAT	- 150	study
**SME1*	- 448	TTGCCGCCGA	- 457	40
	- 543	TAGCCGCCGT	- 552	
**MEI4*	- 89	TAGCCGCCCA	- 98	41
**SPO13*	- 97	TAGCCGCCGA	- 88	38
**SPO16*	- 83	TAGCCGCCCA	- 92	42
**HOP1*	- 164	TAGCCGCCCA	- 173	5
**MER1*	- 112	TAGCCGCCGA	- 103	8
**RED1*	- 158	TAGCCGCCTG	- 167	43
CYC1	- 240	GATCCGCCAG	- 231	44
CYC7	- 288	CCCCCGCCGA	- 279	45
CAR1	- 163	TAGCCGCCGA	- 154	39,46
CAR2	- 183	TAGCCGCCGA	- 174	47

* The expression of these genes is induced specifically in meiosis.

Key sequences in the regions flanking the *MRE4* gene

Initiation of the transcription in many yeast genes requires a TATA box, which is usually located at 50−120 bp upstream of the initiation site of transcription (35,36). For the *MRE4* gene, a TATAAA sequence is located at a region, positions 230−235 in the nucleotide sequence (106−111 bp upstream from the first ATG codon) (Fig.2). The sequence AATAAA required for polyadenylation in eukaryotes (37) is located at the region from positions 1,879 to 1,884, 48−53 bp downstream of the *MRE4* ORF (Fig. 2). The second major transcript, the 1.63 kb RNA, is probably terminated at this termination signal.

Several genes inducible in meiosis carry a common sequence in their 5′ flanking region. Upstream repression sequence (URS), TAGCCGCCGR, is one of such sequences (38). The *MRE4* gene carries two URS sequences, TAGCCGCCAT (site I) and TAGCCGCCGA (site II), which are located at regions from 197 to 188 and from 211 to 202, respectively (Fig. 2 and Table 4).

In the sequence determined for the *MEK1* gene by other investigators (Rockmill and Roeder, submitted), one URS sequence (site I) is missing by addition of two G nucleotides in their lower strand sequence (*MRE4/MEK1*: 197 TAGCCGCCAT 188/−140 TAGGCCGGCCAT −151). In addition, two other G nucleotides are also missing in the *MEK1* sequence as compared with the *MRE4* sequence reported here (*MRE4/MEK1*: 173 CCGGGGAAAA 182/−168 CCGGGAAAA −160, and 230 TATAAAAGGGAG 241/−110 TATAAAAGGAG −100).

Inviable spore formation of the *mre4::D1* and *mre4::D2* disruptants is suppressed by the *spo13* mutation

The diploid strains homozygous or heterozygous for the *mre4::D1* or *mre4::D2* disruptants were sporulated and spore formation and spore viability were measured (Table 2). Numbers of asci dissected and spore viability for two strains, *spo13 MRE4* (IMD501) and *spo13 mre4::D2* (IMD412), were also measured. The viability of heterozygous *mre4::D1/MRE4* and *mre4::D2/MRE4* strains showed no significant difference from that of the wild type strain (data not shown). In the homozygous *mre4::D1* and *mre4::D2* strains, IMD401 and IMD402, spore viability was severely depressed. No viable spores were detected among 352 spores tested. This spore inviability conferred by the *mre4* disruption mutations, however, is greatly reduced by introduction of the *spo13* mutation. The *spo13 mre4::D2* diploid produced viable spores at the frequency of 28.6%, as compared to 70% in the *spo13 MRE4* diploid. Thus, spore inviability of *mre4* mutants is substantially rescued by the *spo13* mutation, although the level of rescue is significantly lower than observed for other mutants defective in early stage of meiotic recombination, such as *hop1*, *rad50*, *spo11*, *red1*, *mer1* and *mei4* (4,48−51). In contrast, spore formation and spore viability in the *MRE4::D3* disruptant were almost indistinguishable from the wild-type (data not shown).

Among the spores formed in the *spo13 mre4::D2* diploid strain, intergenic recombination between *MAT* and *CENIII* was measured by analysis of dissected spores. The frequency of recombination in this strain is 6.8% of the wild type level for the interval analyzed. This deficiency in recombination ability is quantitatively similar to that obtained from return-to-growth experiments (Fig. 4).

Unequal chromosome segregation with *mre4* mutants in meiosis

In meiosis, *mre4* mutants make spores, but the spores are inviable, as observed in other recombination defective mutants (*hop1*, *rad50*, *red1*, *mer1*, *mei4* and *spo11*) (4,48−52). The spore inviability observed in these mutants is thought to be caused mostly by chromosome nondisjunction in the reductional division (1). To examine chromosome segregation of the *mre4* disruptant cytologically, the disruptant strain, IMD402, was sporulated and examined by fluorescence microscopy after staining with 4′,6-diamino-2-phenylindole (DAPI). The *mre4::D2* diploid cells made spores at a frequency of 50% that is not much lower than that in the wild type cells (82%). DNA distribution among spores in tetrad, however, was not equal in the *mre4::D2* strain (Figure 7). This result suggests that the *mre4* disruptant is also defective in chromosome segregation at the meiotic division.

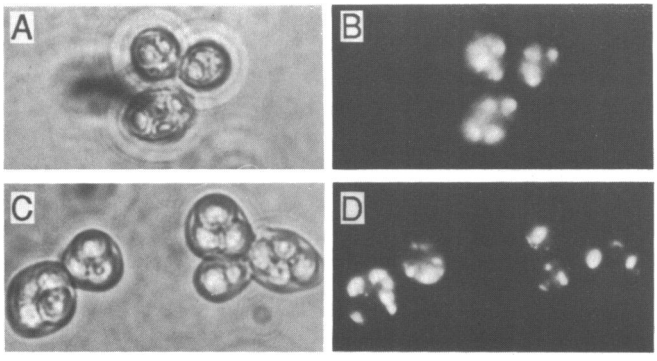

Figure 7. Chromosome segregation in the *mre4* spores. Wild type (A and B) and IMD402 (C and D) cells were pre-grown in YPA and transferred to SPM at 30°C. At 24 hr after sporulation, aliquots were fixed with 70% ethanol, stained with DAPI (1 μg/ml) and photographed by phase-contrast microscopy (A and C) or fluorescence microscopy (B and D) at 1250-time magnification.

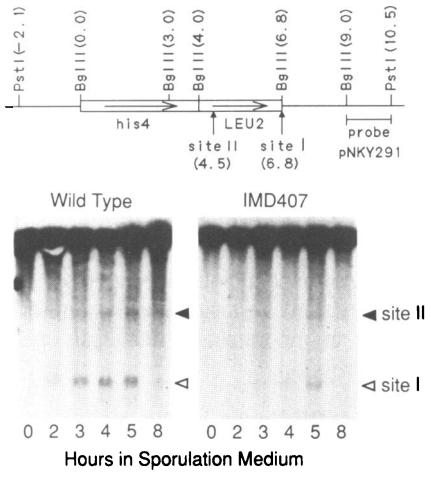

Figure 8. Meiosis-specific double-strand breaks in the wild type and *mre4* strains. A map of relevant restriction sites in the *HIS4-LEU2* region and the positions of the two double-strand break sites and probe are shown. DNA samples were digested with *Pst*I and hybridized with a random primed probe (Materials and Methods). Meiosis specific breaks are observed at site I and site II in both strains.

Meiosis-specific double-strand breaks occur in the *mre4* mutant but at a lower frequency than in a wild type strain

Meiotic recombination occurs at a high frequency at specific sites, so-called hot spots, than at other sites on chromosome. In the two loci of meiotic recombination hot spots, *HIS4-LEU2* and *ARG4* genes, in *S.cerevisiae*, meiosis-specific double-strand breaks have been observed by Southern blot analysis (20,21). These experiments suggest that occurrence of double-strand breaks is critical at an early step of meiotic recombination.

We studied the fate of the double-strand breaks at *HIS4-LEU2* locus in the *mre4::D2* strain, IMD407. In a wild type strain, NKY1238/NKY1240, the double-strand breaks were first detected at 2 hr after transfer to SPM, increased to the maximum level at 4 hr, and then mostly disappeared at 8 hr (Fig. 8). Although double-strand breaks were also observed in the *mre4* cells with almost the same kinetics as in the wild type cells, the level of breaks was lower than that for the wild type strain. The levels of breaks at site I and site II relative to total parental DNA are 5.5 and 3.1, 6.3 and 3.7, and 6.0 and 3.5 (%), respectively, in the wild type strain at 3, 4 and 5 hr. While the levels of breaks in the *mre4* strain are 0.7 and 0.4, 0.6 and 0.3, and 0.8 and 0.5 (%), respectively, at the same times. Simultaneously, we examined meiotic recombination in both strains by selecting for His^+ recombinants by return-to-growth experiment. The *mre4* mutant strain exhibits a reduction to 13% of wild type (data not shown). Similar results were obtained in the analysis of double-strand breaks in *ARG4* locus (data not shown).

DISCUSSION

MRE4 encodes a meiotic recombination specific protein kinase

We have cloned a new yeast gene, *MRE4*, which is necessary for meiotic recombination. Disruption of the genomic *MRE4* sequence abolishes meiotic recombination, haploidization and viable spore formation. The Mre4 protein deduced from the nucleotide sequence contains 497 amino acid residues and has significant homology with the catalytic domains of serine-threonine protein kinases.

In *Saccharomyces cerevisiae*, more than 30 protein kinases have been identified (53). Protein kinases comprise a large family of enzymes that play key roles in critical cell processes, including transcription and translation, cell cycle progression, DNA metabolism and differentiation (53). Recently, several protein kinases that regulate entry into and progression through the meiotic cell cycle have been identified in yeast (54). All of these protein kinases except *SME1* have overlapping roles in the mitotic cell division cycle. *SME1*, which is required for the initiation of premeiotic DNA synthesis, is only previously identified protein kinase that functions in meiosis alone. The *SME1* gene product plays at an early stage of meiosis, before commitment to meiotic recombination. The *MRE4* is another meiosis-specific protein kinase. The *mre4* mutant, however, undergoes premeiotic DNA synthesis and produce spores, but the spores are inviable (11). The *mre4* mutant also shows aberrant chromosome segregation as do most recombination defective mutants. Therefore, the Mre4 protein is the first analyzed putative protein kinase which has a role in meiotic recombination.

Expression of the *MRE4* gene is meiotically induced

Four RNAs, 1.8, 3.2, 4.0 and 6.2 kb in size, are transcribed from the *MRE4* gene during meiosis, and these 4 transcripts differ only at the 3'ends. The 1.8 kb transcript is sufficient to complement the *mre4* mutation, since the complementing subclone pSL47 contains all the sequence for the 1.63 kb transcript but not the entire sequences required for other longer transcripts. By Northern blot analysis with RNA prepared from the *MRE4::D3* disruptant, the 1.8 kb transcript was detected as an intact form, but the other three transcripts were interrupted by *URA3* insertion (our unpublished data). These results also suggest that the 1.8 kb RNA is sufficient to complement the *mre4* mutation. The same conclusion is obtained from the results of S1 mapping (Fig. 6). The *MRE4* RNAs are probably initiated at the same position. The smallest 1.63 kb transcript (1.8 kb by Northern blot) was mapped by S1 mapping, and is transcribed from the region that includes the entire *MRE4* ORF.

In meiosis, transcription of the *MRE4* gene is dramatically increased, similar to those of other yeast genes essential for meiotic recombination and/or chromosome segregation at Meiosis I (*HOP1*, *RED1*, *SPO13*, *SPO11* and *MER1*). The URS sequence, TAGCCGCCGR, that is required for meiosis specific expression found upstream of these genes (38). Furthermore,

SME1 gene, a meiosis-specific protein kinase, contains two URS sequence in its upstream region. The *MRE4* gene also carries two URS sequence, which are located at positions 188−197 and 202−211 in the upstream of the ORF. These two genes are the only protein kinases expressed in meiosis specifically. This coincidence might mean that the presence of two URS sequences has special significance for meiosis-specific gene expression. Moreover, the URS has been shown to repress transcription of *CYC1* (44), *CYC7* (45), *CAR1* (39,46) and *CAR2* (47) during the mitotic cycle. As to the meiotically expressed genes, therefore, the URS might function as a target site for a mitotic transcription repressor, playing an important role in meiosis-specific expression through such repression.

The role of the *MRE4* gene in the meiotic recombination

The *MRE4* gene product may regulate the activity of certain protein(s) involved in the meiotic recombination pathway rather than playing a direct role in this process. If so, which step(s) in the meiotic recombination process does the *MRE4* gene product regulate? Meiotic recombination deficient mutants are roughly divided into two classes based on the properties of the effects of the *spo13* mutation (55,56). One class consists of mutants whose spore inviability is rescued by the *spo13*. The mutants in this class are *hop1*, *spo11*, *rad50*, *red1*, *mer1* and *mei4* (5,12,48−51). The meiosis-specific double-strand breaks at meiotic recombination hot spots are not observed in the *rad50* and *spo11* mutants through the meiosis (20). These results suggest that these gene may act before or at the step of generation of double-strand breaks for the initiation of meiotic recombination. Another class consists of the mutants whose spore inviability is not rescued by the *spo13*. The mutants in this class are, *rad50S* (3,20), *rad51* (57), *rad52* (48), *rad55* (Kuwahara, et al., personal communication) and *rad57* (57). In all these mutants, *rad50S* (3,20), *rad51* (Shinohara, et al., personal communication), *rad52* (Shinohara,A. and Ogawa,T., personal communication) and *rad55* (Kuwahara, et al., personal communication), double-strand breaks are more processed or are more accumulated during meiosis than in the wild type strain. These genes may be involved in the steps after generation of double-strand breaks. Therefore, the spore lethality of these mutants are probably caused by the accumulation of unrepaired double-strand breaks. The spore inviability of the *mre4* mutant was rescued by the *spo13* mutation, but its frequency was lower than in other early stage genes in meiotic recombination (about 30% versus 80%). We also examined the occurrence of breaks at the meiotic recombination hot spot at *HIS4-LEU2* locus. The double-strand breaks in the *mre4* mutant (IMD407) were mapped to the same two sites, and are meiotically induced and appear at the same time as in the wild type strain. But the level of breaks in *mre4* was about 10−20% of the wild-type. This decreased level of double-strand breaks reflects the absence of the meiotic recombination function of the *MRE4*. The site-specific double strand breaks occur early in the prophase suggesting that DSBs may be required for SC formation (58). Therefore, we suggest that the *MRE4* gene product regulates activation of some early stage genes or gene products involved in incision of the double-strand DNA and/or formation of the synaptonemal complex.

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