# The Mus81/Mms4 Endonuclease Acts Independently of Double-Holliday Junction Resolution to Promote a Distinct Subset of Crossovers During Meiosis in Budding Yeast

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Manuscript received December 9, 2002
Accepted for publication January 31, 2003

ABSTRACT

Current models for meiotic recombination require that crossovers derive from the resolution of a double-Holliday junction (dHJ) intermediate. In prokaryotes, enzymes responsible for HJ resolution are well characterized but the identification of a eukaryotic nuclear HJ resolvase has been elusive. Indirect evidence suggests that *MUS81* from humans and fission yeast encodes a HJ resolvase. We provide three lines of evidence that Mus81/Mms4 is not the major meiotic HJ resolvase in *S. cerevisiae*: (1) *MUS81/MMS4* is required to form only a distinct subset of crossovers; (2) rather than accumulating, dHJ intermediates are reduced in an *mms4* mutant; and (3) expression of a bacterial HJ resolvase has no suppressive effect on *mus81* meiotic phenotypes. Our analysis also reveals the existence of two distinct classes of crossovers in budding yeast. Class I is dependent upon *MSH4/MSH5* and exhibits crossover interference, while class II is dependent upon *MUS81/MMS4* and exhibits no interference. *mms4* specifically reduces crossing over on small chromosomes, which are known to undergo less interference. The correlation between recombination rate and degree of interference to chromosome size may therefore be achieved by modulating the balance between class I/class II crossovers.

DURING meiosis, homologous chromosomes become physically connected by the formation of chiasmata. These connections are crucial for the accurate segregation of homologs to opposite poles at the first meiotic division (Bascom-Slack *et al.* 1997). Chiasma formation involves reciprocal recombination, or crossing over, between homologous chromosomes. Examination of crossover distribution indicates that the processes involved are highly regulated. First, every chromosome pair receives at least one exchange, the obligatory event required for faithful segregation, and second, when multiple crossovers are present, they are more widely spaced than predicted for a random distribution, a phenomenon known as positive crossover interference (Jones 1984). In the budding yeast *Saccharomyces cerevisiae*, the DNA events of meiotic recombination have been described using physical assays. Meiotic recombination is initiated via programmed double-strand breaks (DSBs; Keeney 2001). The ends of a DSB are resected on their 5′-strands to produce 3′-single-stranded tails, which then interact sequentially with a homologous chromosome to produce a single-end invasion (SEI) and then a double-Holliday junction (dHJ; Schwacha and Kleckner 1995; Hunter and Kleckner 2001). The formation of dHJs and their resolution by structure-specific endonucleases are central posits of contemporary models of meiotic recombination (Szostak *et al.* 1983). While a number of gene products have been implicated in the formation of DSBs, SEIs, and dHJs (*e.g.*, Schwacha and Kleckner 1995; Hunter and Kleckner 2001; Keeney 2001), to date, a gene(s) encoding a meiotic HJ resolvase in *S. cerevisiae* has yet to be identified.

Recently two groups have proposed that Mus81 is a part of a eukaryotic HJ resolvase (Boddy *et al.* 2001; Chen *et al.* 2001). Mus81 is an evolutionarily conserved endonuclease with homology to the XPF/Rad1 proteins that function in nucleotide excision repair (Haber and Heyer 2001). Genetic data have been interpreted as evidence that *MUS81* has a role in the processing of stalled replication forks in mitotically dividing cells, perhaps via the resolution of HJs formed via fork regression. For example, in *Schizosaccharomyces pombe* and *S. cerevisiae*, *mus81* is synthetically lethal with *rqh1* and *sgs1*, the respective RecQ helicase homologs of each species (Boddy *et al.* 2000; Mullen *et al.* 2001). Genetic data have also been used to argue that *MUS81* functions as a HJ resolvase during meiosis in *S. pombe*: *mus81* cells

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induced to undergo meiosis produce highly inviable spores and this phenotype can be partially suppressed by expression of a bacterial HJ resolvase (Boddy *et al.* 2001).

Mus81 from both yeasts forms a complex with a second protein that is required for nuclease activity *in vitro* (Eme1 for *S. pombe* and Mms4 for *S. cerevisiae*; Boddy *et al.* 2001; Kaliraman *et al.* 2001). These results likely reflect the requirement for a heterodimeric complex *in vivo* as *mus81 eme1* and *mus81 mms4* are phenotypically identical to either single mutant for all of the vegetative phenotypes that have been examined, as well as for meiotic spore viability (Boddy *et al.* 2001; de los Santos *et al.* 2001; Mullen *et al.* 2001). Phenotypic analysis of *mms4* diploids during meiosis demonstrated only a modest reduction in crossing over, suggesting that, in *S. cerevisiae*, meiotic HJ resolution is mostly independent of *MMS4* (de los Santos *et al.* 2001). Because Mms4 and Eme1 are at best weakly homologous, the possibility exists that *MUS81* functions in *S. cerevisiae* meiosis as an *MMS4*-independent HJ resolvase. In fact, HJ cutting activity has been observed *in vitro* for the Mus81 homolog, Rad1, in the absence of its partner protein, Rad10 (Habraken *et al.* 1994).

To test whether *MUS81* and *MMS4* function together in meiosis, and to further investigate the meiotic roles for these gene products, *mus81* diploids were compared with isogenic *mms4* and *mus81 mms4* strains for a variety of meiotic phenotypes. Our data confirm that *MUS81* and *MMS4* act in the same pathway for meiotic recombination, presumably as a protein complex. Unexpectedly, we find that *MMS4* is required for only a subset of crossovers that appear to be more prominent between short chromosomes. In addition, crossovers are distributed normally along chromosomes in an *mms4* mutant, indicating that crossovers subject to interference do not require *MUS81/MMS4*. *mus81* phenotypes are not suppressed by the expression of the bacterial HJ resolvase, *rusA*, and physical monitoring of DNA events indicates that dHJ intermediates are reduced in the absence of *MMS4*. These data strongly argue that dHJ resolution in budding yeast does not require *MUS81/MMS4*.

## MATERIALS AND METHODS

**Plasmids:** Plasmids were constructed by standard procedures (Maniatis *et al.* 1982) using the *Escherichia coli* strain BSJ72. The *MEK1p-NLS-rusA-D70N-2HA* allele was constructed by first amplifying the *NLS-rusA-D70N-2HA* gene from pREP-rusA-D70N (generously provided by P. Russell, Scripps Research Institute), using the polymerase chain reaction (PCR; Boddy *et al.* 2001). A *Sal*I site was engineered after the stop codon of the gene. The resulting fragment was digested with *Nde*I and *Sal*I and ligated to *Nde*I/*Sal*I-digested pDW14 to make pNH246 (de los Santos and Hollingsworth 1999). Site-directed mutagenesis (Quikchange kit, Stratagene, La Jolla, CA) was used to restore the aspartic acid at position 70 to generate a wild-type *rusA* allele in pNH246wt. To ensure that no mutations were introduced by the PCR, the entire open reading frame (ORF) and *MEK1* promoter fragment were sequenced (Center for the Analysis and Synthesis of Macromolecules, SUNY, Stony Brook, NY). The *SGS1p-rusA* plasmid, pKR6980, was generously provided by Steve Brill (Rutgers University). To clone *MUS81*, a 3.4-kb fragment containing the *MUS81* gene with 1 kb of upstream and 0.5 kb of downstream sequences was amplified from genomic DNA. The fragment was digested with *Sal*I and *Bam*HI (engineered by PCR) and ligated into either pRS316 or pRS315 to make pCL2 and pCL3, respectively. Site-directed mutagenesis changing the aspartic acids at positions 414 and 415 to alanine was performed using pCL2 to make *mus81-DD*. The *mus81Δ::ARG4* mutation was constructed by first subcloning a 2.9-kb *Bam*HI/*Hin*dIII fragment from pCL4 into *Bam*HI/*Sal*I pRS305 to make pDT6. A 1.3-kb *Bgl*II fragment within *MUS81* was substituted for a 3.3-kb *Bam*HI PCR fragment containing *ARG4* to make pDT8.

**Yeast strains:** Liquid and solid media were as described (Vershon *et al.* 1992; de los Santos and Hollingsworth 1999). All yeast strains are derived from the SK1 strain background. The following strains are homozygous isogenic derivatives of NH144:

$$\frac{MAT\mathbf{a}}{MAT\alpha}\ \frac{leu2\Delta hisG}{leu\text{-}K}\ \frac{his4\text{-}X}{HIS4}\ \frac{ARG4}{arg4\text{-}Nsp}\ \frac{ura3}{ura3}\ \frac{lys2}{lys2}\ \frac{ho\Delta::LYS2}{ho\Delta::LYS2}$$

NH371, *mus81Δ::kanMX4*; NH274F, *mms4Δ::hisG*; NH416, *red1::LEU2 mus81Δ::kanMX4*; NH396, *mek1Δ::LEU2 mus81Δ::kanMX4*; NH372F, *mms4Δ::hisG mus81Δ::kanMX4*.

The following are homozygous isogenic derivatives of NKY-1551:

$$\frac{MAT\mathbf{a}}{MAT\mathbf{a}}\ \frac{leu2::hisG}{leu2::hisG}\ \frac{his4B::LEU2}{his4X::LEU2(Bam)\text{-}URA3}\ \frac{arg4\text{-}Bgl}{arg4\text{-}Nsp}\ \frac{lys2}{lys2}\ \frac{ho\Delta::LYS2}{ho\Delta::LYS2}\ \frac{ura3}{ura3}$$

NH301, *mms4Δ::kanMX4*; NH428, *mus81Δ::ARG4*; NH445, *mus81Δ::ARG4 mms4Δ::kanMX4*.

The following is an isogenic derivative of NHY290:

$$\frac{HIS4::LEU2\text{-}(NBam)}{his4\text{-}X::LEU2\text{-}(NBam)\text{-}URA3}\ \frac{leu2::hisG}{leu2::hisG}\ \frac{MAT\mathbf{a}}{MAT\alpha}\ \frac{ho::hisG}{ho::hisG}\ \frac{ura3(\Delta Pst\text{-}Sma)}{ura3(\Delta Pst\text{-}Sma)}$$

NHY1155, *mms4Δ::hphMX4*.

The following are isogenic derivatives of NHY1296:

$$\frac{HIS4::LEU2\text{-}(Bam+)}{his4\text{-}X::LEU2\text{-}(NgoMIV)\text{-}URA3}\ \frac{leu2::hisG}{leu2::hisG}\ \frac{MAT\mathbf{a}}{MAT\alpha}\ \frac{ho::hisG}{ho::hisG}\ \frac{ura3(\Delta Pst\text{-}Sma)}{ura3(\Delta Pst\text{-}Sma)}$$

NHY1297, *msh5Δ::kanMX4*; NHY1298, *mms4Δ::hphMX4*; NHY-1299, *msh5Δ::kanMX4 mms4Δ::hphMX4*.

The following is an isogenic derivative of NHY957:

$$\frac{MAT\alpha}{MATa}\ \frac{CENIII}{CENIII::ADE2}\ \frac{LEU2}{leu2::hisG}\ \frac{HIS4}{his4\text{-}B}\ \frac{ura3(\Delta Sma\text{-}Pst)}{ura3(\Delta Sma\text{-}Pst)}\ \frac{can1}{CAN1}\ \frac{ho::hisG}{ho::hisG}\ \frac{ade2\Delta}{ade2\Delta}$$

$$\frac{LYS5}{lys5\text{-}P}\ \frac{met13\text{-}B}{MET13}\ \frac{CYH2}{cyh2}\ \frac{trp5\text{-}S}{TRP5}\ \frac{CENVIII::URA3}{CENVIII}\ \frac{thr1\text{-}A}{THR1}\ \frac{cup1^{S}}{CUP1}$$

NH455, *mms4Δ::kanMX4*.

Alleles marked with *kanMX4* were disrupted by PCR using the method of Longtine *et al.* (1998). The *mus81Δ::ARG4* allele was introduced by transformation of a *Bam*HI/*Xho*I frag-

ment purified from pDT8. All gene disruptions were confirmed by Southern blot analysis (data not shown). Details of strain constructions are available upon request.

**Time courses:** Cells were sporulated as described in de los Santos and Hollingsworth (1999). Meiotic progression was monitored by fixing cells with 3.7% formaldehyde and staining them with 4′,6-diamidino-2-phenylindole (DAPI) as described in Woltering *et al.* (2000). Sporulation was assessed by examining 200 cells using light microscopy to determine the number of mature asci. For analysis of DSBs and crossovers, DNA was digested in plugs as described in Woltering *et al.* (2000). The gels were prepared for hybridization and probed as described (McKee and Kleckner 1997). The DSB fragments and crossover bands were quantitated as described in Woltering *et al.* (2000). Joint molecule analysis was carried out as described (Schwacha and Kleckner 1994; Hunter and Kleckner 2001). Electron microscopic analysis of spread chromosomes was performed as described in Woltering *et al.* (2000).

## RESULTS

***Mus81* triggers the meiotic recombination checkpoint by the formation of unprocessed recombination intermediates:** Similar to *mms4*, the sporulation defect of *mus81* has previously been shown in the W303 genetic background to be due to a block in prophase (Mullen *et al.* 2001). This result, in conjunction with the observation that the spore viability of a *mus81 mms4* diploid resembles that of either *mus81* or *mms4* alone (de los Santos *et al.* 2001), suggests that Mus81p and Mms4p function together in meiotic cells. In SK1 strains the meiotic arrests exhibited by *mus81* and *mms4* are not complete, thereby allowing a genetic test for gene interaction. The *mus81 mms4* diploid sporulated at 33° exhibited a similar number of arrested cells as *mus81* or *mms4* alone, indicating that the two genes act on the same pathway for meiotic progression (Figure 1A). Furthermore, the number of mature asci produced by the double mutant was no more severe than that of either single mutant (wild type, 73.2% ± 3.7; *mus81*, 2.2% ± 1.5; *mms4*, 3.8% ± 2.2; *mus81 mms4*, 1.3% ± 1.0; $n = 3$). These observations support the idea that a complex containing Mus81 and Mms4 is necessary for meiotic progression and sporulation.

In both *S. cerevisiae* and *S. pombe*, the *mus81* sporulation and spore viability defects are dependent upon the initiation of recombination (Boddy *et al.* 2001; Kaliraman *et al.* 2001). It is therefore likely that the *mus81* arrest is due to the presence of aberrant or unprocessed recombination intermediates triggering the meiotic recombination checkpoint (reviewed in Roeder and Bailis 2000). This hypothesis was tested by assaying meiotic progression in *mus81* strains in which meiosis-specific components of the checkpoint were mutated. *RED1* and *MEK1* encode proteins that localize to chromosome cores, which, in addition to synapsis and recombination, are required for the meiotic recombination checkpoint (Smith and Roeder 1997; Xu *et al.* 1997; Bailis and Roeder 1998). Mutation of *RED1* completely suppressed the meiotic progression defect of *mus8. mek1* also permitted meiotic progression of *mus81* cells, although in this case the suppression was only partial (Figure 1B). Mutation of the *PCH2* gene has no effect on spore viability but suppresses or partially suppresses the prophase arrest/delay caused by *zip1*, *dmc1*, and *mms4* (San-Segundo and Roeder 1999; de los Santos *et al.* 2001). Deletion of *PCH2* partially suppressed the prophase arrest/delay observed for *mus81* without improving spore viability (data not shown).

***mus81* diploids exhibit high levels of meiotic heteroallelic recombination but decreased cell viability in return-to-growth experiments:** The ability of *mus81* and *mus81 mms4* cells to undergo meiotic recombination between *leu2* heteroalleles was analyzed by assaying the formation of $Leu^+$ prototrophs as a function of time in sporulation medium. Isogenic wild-type and *mms4* diploids were included as controls. In all four strains, the frequency of $Leu^+$ prototrophs peaked at 6 hr, although the absolute number of prototrophs was reduced two to fourfold in the three mutant diploids (Figure 1C). By 24 hr, the mutants exhibited five to sevenfold fewer prototrophs than did wild type. This experiment indicates that heteroduplex formation is occurring at high frequency in the mutant strains and that there is no synergistic effect on prototroph formation in the absence of both *MUS81* and *MMS4*.

When cells are returned to growth, meiotically induced DSBs must be repaired if cells are to be viable (Arbel *et al.* 1999). The *mus81* and *mms4* diploids each exhibited a 10-fold decrease in cell viability in return-to-growth experiments, suggesting a role for these genes in DSB repair under return-to-growth conditions (Figure 1D). Alternatively, the lethality could arise if cells try to progress through meiosis I (MI) with unrepaired chromosomes. To distinguish between these two possibilities, cell viability in meiotic time courses of *mus81* in the presence of *ndt80* was measured. *NDT80* encodes a transcription factor required for progression through MI (Xu *et al.* 1995; Chu and Herskowitz 1998). In return-to-growth experiments, cell viability is still reduced by *mus81* in the presence of *ndt80*, demonstrating that meiotic progression is not the source of the lethality (data not shown).

The *mus81 mms4* diploid showed a synergistic 59-fold decrease in viability compared to that of either single mutant (Figure 1D). This decrease in cell viability, presumably as a result of being unable to repair meiotically induced DSBs under return-to-growth conditions, is the only phenotype yet discovered that indicates that *MMS4* and *MUS81* may have independent function(s).

***MUS81/MMS4* affect only a subset of crossovers in *S. cerevisiae*:** Genetic and physical assays were used to assess the effect of *mus81* and *mms4* on the formation of meiotic crossovers. An SK1 *mus81* diploid (NH371) was sporulated at 30° and 1357 tetrads were dissected. Spore viability was 40.5%, consistent with previous re-

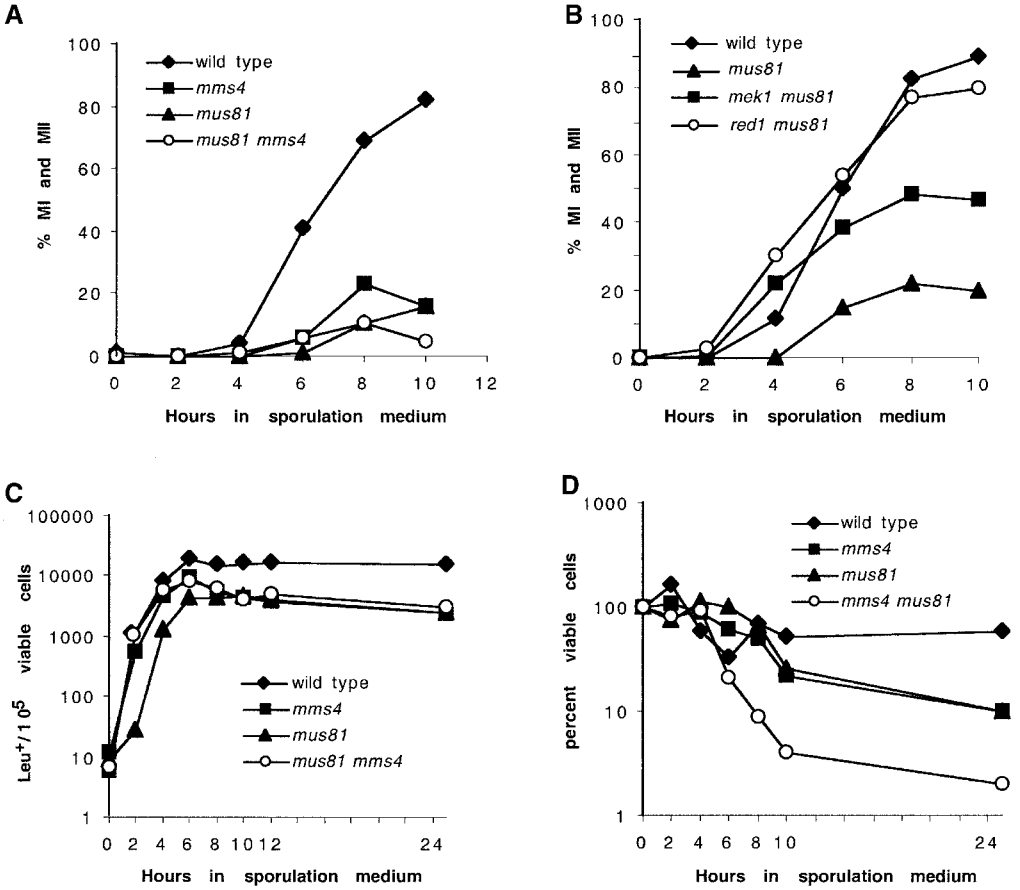


FIGURE 1.—Time-course analyses of *mus81* diploids. Isogenic SK1 diploids were transferred to sporulation medium and shifted to 33° (NH144, wild type; NH371, *mus81*; NH274F, *mms4*; NH-372F, *mus81 mms4*; NH416, *red1 mus81*; NH396, *mek1 mus81*). (A) Meiotic progression for *mus81* and *mus-81 mms4*. Cells were fixed and stained with DAPI and examined by fluorescence microscopy. Binucleate cells were classified as MI, tetranucleate cells as MII. A total of 200 cells were counted for each strain at each time point. (B) Meiotic progression for *red1 mus81* and *mek1 mus81*. (C) Recombination between *leu2* heteroalleles. Appropriate dilutions of each diploid from each time point were plated onto −leu and YPAD media, respectively. The number of $Leu^+$ prototrophs was normalized to the total number of viable cells. (D) Cell viability. The viability of each diploid at each time point was normalized to the viability at the 0 time point for that strain. A, C, and D are from the same time course and represent the average of three independent colonies. B represents a separate time course in which the average of two single colonies for each strain was plotted.

sults (INTERTHAL and HEYER 2000; DE LOS SANTOS *et al.* 2001). Similar to *mms4*, the distribution of viable spores in tetrads resembles a random pattern (except for slightly more four- and zero-viable-spore tetrads than expected) and is not indicative of meiosis I nondisjunction (DE LOS SANTOS *et al.* 2001; data not shown). Tetrads producing four viable spores were examined for crossovers between *HIS4* and *MAT* and for gene conversion at *HIS4*, *LEU2*, and *ARG4*. A small (1.6-fold), but statistically significant, decrease in crossovers was observed in the *mus81* diploid (Table 1). Gene conversion was elevated at all three loci, but the increase was statistically significant only at *HIS4*. These results strongly resemble those observed for *mms4* in the isogenic background (Table 1).

In the isogenic strains used for the experiment described above, only a single interval can be analyzed for crossovers. A more thorough analysis of the effects of *mms4* on recombination was therefore undertaken using a diploid SK1 strain, NHY957, that is multiply marked on three different chromosomes. The wild-type and *mms4* diploids were sporulated at 30° and produced 93.2% (499 asci) and 45.4% (2849) viable spores, respectively. Tetrad analysis suggests a correlation between chromosome size and the effect of *mms4* on crossing over in four-viable-spore asci (Figure 2). Overall, crossing over in the intervals analyzed along chromosome III (~330 kb) is reduced 1.5-fold relative to wild-type levels; on chromosome VIII (~580 kb) and VII (~1040 kb), crossing over is reduced 1.3- and 1.1-fold, respectively (Figure 2). When analyzed individually, all of the intervals on chromosome III as well as one interval on chromosome VIII exhibited a statistically significant, 1.4- to 1.7-fold decrease in the number of crossovers (Table 2). In contrast, the reductions in crossing over in the three intervals on chromosome VII were not significantly different from wild type. In addition, 6 out of 11 loci displayed significant increases in gene conversion (Table 2).

**Crossover distribution in *mms4* mutants:** *MMS4* is required for the formation of a subset of crossovers that appear to be more conspicuous along short chromosomes. To further characterize this phenomenon, we examined crossover distribution in tetrads producing four viable spores from wild-type and *mms4* strains. The intensity of interference between adjacent crossovers can be measured in two ways: (1) the coefficient of coincidence, which is defined as the number of double crossovers (DCOs) observed divided by the number expected in the absence of interference (STURTEVANT 1913; MULLER 1916) and (2) the nonparental ditype

**TABLE 1**

**Recombination in *mus81* and *mms4* SK1 diploids**

| Strain | Genotype | Map distance (cM): *MAT-HIS4* | % gene conversion *HIS4* | *LEU2* | *ARG4* |
|---|---|---|---|---|---|
| NH144[a] | WT | 40.2 (540) | 6.2 (577) | 4.7 (470) | 2.8 (577) |
| NH371 | *mus81*Δ | 25.6[b] (88) | 17.8[b] (118) | 6.8 (118) | 5.9 (118) |
| NH274[a] | *mms4*Δ | 30.6 (103) | 12.3[b] (122) | 5.8 (69) | 6.8[b] (118) |

Only four-viable-spore tetrads were analyzed. For *HIS4* and *ARG4*, gene conversions were scored as tetrads exhibiting $3^+:1^-$ or $3^-:1^+$ segregation for the marker. For *LEU2*, the presence of a $Leu^+$ spore indicated gene conversion. Numbers in parentheses indicate the number of tetrads assayed.

[a] The data for NH144 and NH274 are taken from de los Santos *et al.* (2001).

[b] This value is statistically significantly different from wild type ($P < 0.05$). Statistical analyses were performed using programs at the following websites: map distance, http://groik.com/stahl/; gene conversion, http://faculty.Vassar.

(NPD) ratio, which compares the observed number of four-strand DCOs within a single interval with the number expected in the absence of interference (Papazian 1952; Snow 1979). Both the coefficients of coincidence and the NPD ratios are very similar between the wild-type and *mms4* diploids, indicating that *MMS4*-independent crossovers are distributed normally by interference (Table 3; data not shown).

**Physical monitoring of recombination events in *mus81* and *mms4* mutants:** The genetic data are based on a highly selected subset of cells (the ~10% that form mature asci at 30°, of which only ~10% make four viable spores). The small reduction on crossovers observed genetically may, therefore, overestimate the number of crossovers occurring in the population as a whole. This caveat was addressed by detecting DSBs and crossovers by direct analysis of the DNA at the *HIS4::LEU2* meiotic recombination hotspot on chromosome III (Storlazzi *et al.* 1995). Although *mms4* and *mus81* phenotypes with respect to DSBs are highly similar, they are not identical (Figure 3). DSBs are slightly more delayed in *mus81* than in *mms4* and DSBs tend to persist longer in *mms4* than in *mus81*. Whether or not these subtle differences are meaningful remains to be seen. No synergistic phenotypes were observed between the *mus81 mms4* diploid and either single mutant (Figure 3). Crossing over was unaffected by the mutants in this experiment, although this result is somewhat variable. In some experiments we have observed up to a 1.8-fold reduction in crossovers by this assay (de los Santos *et al.* 2001; see below). The variability is likely due to the fact that the effect on crossing over by *mus81* and *mms4* is relatively small (<2-fold) and the limits on the resolution of the quantitation. It is clear, however, that the bulk of meiotic crossovers do not require *MUS81/MMS4*.

Physical assays of recombination have been used to characterize intermediate steps of recombination, which proceed via the formation of DNA joint molecules (Schwacha and Kleckner 1995). First, one side of a resected DSB invades an intact homologous duplex and undergoes strand exchange to form a SEI. The second DSB end then interacts to form a dHJ. DNA synthesis and ligation must accompany these transitions to produce dHJs with uninterrupted full-length strands, as observed (Schwacha and Kleckner 1995; Allers and Lichten

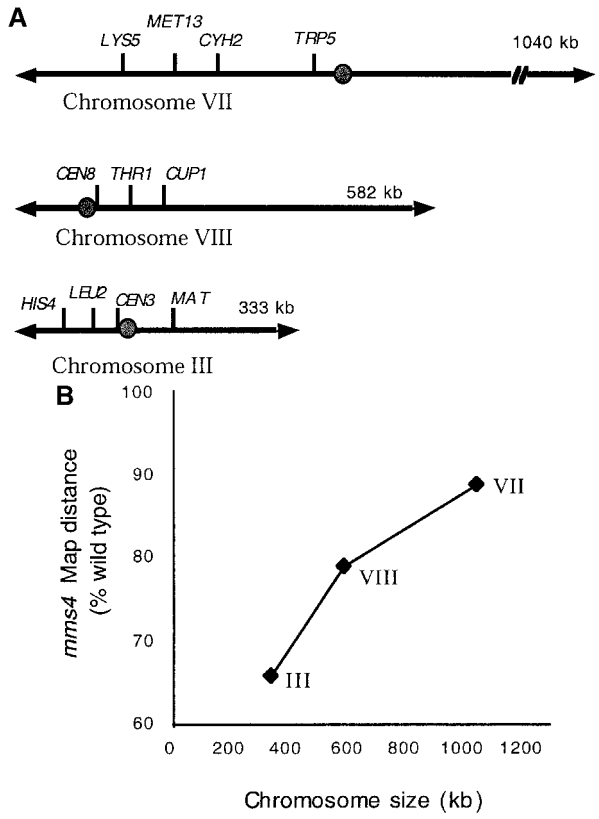


Figure 2.—Crossover distribution in wild-type and *mms4*Δ tetrads. (A) Physical maps of test intervals. Gene order, relative interval size, and overall size of the chromosomes are shown. Shaded circles indicate centromere location; arrowheads represent telomeres. (B) Crossover frequency in *mms4*Δ as a function of chromosome size. Each data point represents the sum of the individual map distances for each chromosome, expressed as a percentage of the wild-type value.

**TABLE 2**

**Gene conversion and crossing over in wild-type and *mms4* SK1 diploids**

| | % gene conversion (no. asci) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | *HIS4* | *LEU2* | *CENIII* | *MAT* | *LYS5* | *MET13* | *CYH2* | *TRP5* | *CENVIII* | *THR1* | *CUP1* |
| *MMS4* | 1.4 (425) | 3.5 (425) | 0.5 (426) | 1.2 (424) | 2.3 (425) | 3.5 (425) | 0.9 (425) | 0.7 (425) | 0.7 (425) | 6.3 (425) | 11.3 (415) |
| *mms4* | 8.8* (272) | 5.9 (271) | 0 (271) | 5.9* (268) | 8.5* (271) | 9.2* (271) | 4.4* (270) | 7.0* (272) | 3.1 (252) | 10.0 (270) | 12.6 (270) |

| | Map distance (cM) (no. asci) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chromosome III | | | Chromosome VII | | | Chromosome VIII | |
| Genotype | *HIS4-LEU2* | *LEU2-CEN* | *CEN-MAT* | *LYS5-MET13* | *MET13-CYH2* | *CYH2-TRP5* | *CEN-TRP1* | *TRP1-CUP1* |
| *MMS4* | 12.5 (404) | 10.5 (408) | 15.3 (419) | 18.7 (407) | 10.1 (407) | 41.2 (420) | 21.2 (398) | 23.5 (343) |
| *mms4* | 8.4* (237) | 6.1* (254) | 10.7* (252) | 16.4 (229) | 8.5 (236) | 37.2 (240) | 13.9* (220) | 21.4 (217) |

Isogenic SK1 diploids were sporulated at 30° and tetrads dissected (NHY957, wild type; NH455, *mms4/mms4*). Gene conversion was scored as tetrads that exhibited $3^+:1^-$ or $1^+:3^-$ segregation for the marker. Map distances were calculated using the formula from Perkins (1947). * indicates the percentage is statistically significantly different from wild type at the 95% confidence level. Statistical analyses were performed using programs from the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html) and the Stahl lab website (http://groik.com/stahl/).

2001a). Two recent studies have provided evidence that dHJs are the direct precursors of crossovers during meiosis (Allers and Lichten 2001a; Hunter and Kleckner 2001). To determine the role of *MUS81/MMS4* in joint molecule formation and resolution, we analyzed DSBs, SEIs, dHJs, and crossovers in wild-type (NHY290) and *mms4* (NHY1155) cells at a modified version of the *HIS4::LEU2* locus (Figure 4; Hunter and Kleckner 2001).

In wild type, DSBs first appear 2 hr after transfer into sporulation medium at 30°, peak at 4.5 hr, and have essentially disappeared by 7 hr (Figures 4 and 5C, i). Steady-state levels of SEIs, interhomolog dHJs (IH-dHJs), and intersister dHJs (IS-dHJs) peak at ~4.5 hr; crossovers (Recs) are first detected at 4.5 hr and reach maximum levels at ~8 hr (Figure 4 and Figure 5C, ii, iii, iv, and vi). The timing of DNA events is essentially identical to that described by Hunter and Kleckner (2001).

In *mms4*, DSB kinetics parallel those described previously (de los Santos *et al.* 2001); DSBs peak at essentially the same level as wild type but this peak occurs 2 hr later than that of wild type and significant numbers of DSBs remain at late times. SEIs form at reduced levels (~2-fold) and peak 2–3 hr later than wild type. IH-dHJs are also reduced, perhaps to an even greater extent (~3-fold), and appear with a similar delay. Formation of intersister dHJs is also delayed but in contrast to the interhomolog species, IS-dHJs form at high levels (~1.25-fold reduction in peak steady-state levels and

**TABLE 3**

**Coefficients of coincidence in isogenic *MMS4* and *mms4* diploids**

| | Coefficient of coincidence (no. observed DCOs/no. expected DCOs)[a] | | | | |
|---|---|---|---|---|---|
| Strain | *HIS4-LEU2* *LEU2-CENIII* | *LEU2-CENIII* *CENIII-MAT* | *LYS5-MET13* *MET13-CYH2* | *MET13-CYH2* *CYH2-TRP5* | *CENVIII-THR1* *THR1-CUP1* |
| *MMS4* | 0.45 (9/20) | 1.00 (25/25) | 0.50 (14/28) | 0.74 (40/54) | 0.58 (38/66) |
| *mms4* | 0 (0/4) | 1.10 (5/4) | 0.50 (6/12) | 0.83 (20/24) | 0.46 (11/24) |

[a] The coefficient of coincidence is the number of observed double crossovers (DCOs) divided by the number of expected DCOs. The expected number is calculated by assuming that crossovers in adjacent intervals occur independently of each other using the following equation: $(T_I + NPD_I/total_I)(T_{II} + NPD_{II}/total_{II})$(total no. tetrads). I, interval I; II, interval II; T, tetratype; NPD, nonparental ditype. The observed number is based on those asci in which either a T or an NPD has occurred in both I and II.

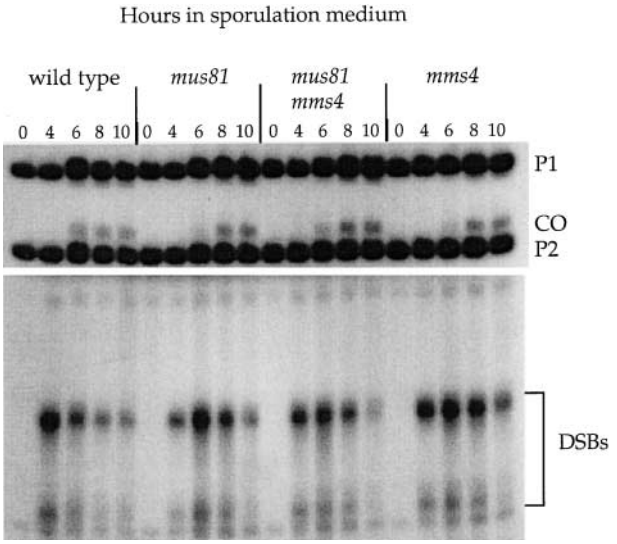


Figure 3.—Effects of *mus81* and *mus81 mms4* on the formation of DSBs and crossovers at the *HIS4/LEU2* recombination hotspot. DNA was isolated from wild type (NKY1551), *mus81*Δ (NH428), *mus81*Δ *mms4*Δ (NH445), and *mms4*Δ (NH301) at different times after transfer to sporulation medium at 33°. The bracket indicates DSBs. DNA was digested with *Xho*I and probed with a 0.6-kb *Eco*RI/*Xmn*I fragment from pNKY155. The expected DSB and crossover (CO) fragments predicted by McKee and Kleckner (1997) are indicated. Parental fragments are labeled P. The second crossover fragment is not resolved from the larger parental band. DSB and CO fragments were quantitated as described in de los Santos *et al.* (2001). To enhance visibility, a longer exposure of the DSB part of the gel is shown.

very similar areas under the corresponding curves). This effect is seen most clearly when the ratios of interhomolog dHJs to intersister dHJs are plotted over time (Figure 5C, v). Consistent with previous reports (Schwacha and Kleckner 1997; Hunter and Kleckner 2001), dHJ formation in wild-type cells is heavily biased toward the interhomolog species with an average ratio of 4.3. Interhomolog bias is almost absent in *mms4* cells, which have an average IH-dHJ/IS-dHJ ratio of 1.4-fold (in both cases, the first time point at which dHJs are detectable is excluded from this average because levels are very low and quantitation is likely to be inaccurate). Crossover products appear at reduced levels (1.8-fold) and with a delay of ~3 hr and finally, meiotic divisions are delayed but eventually occur with reasonable efficiency as observed previously for *mms4* SK1 strains at 30° (de los Santos *et al.* 2001; Figure 5C, vi and vii).

***mus81* and *mms4* have no effect on chromosome synapsis in the NKY1551 SK1 strain background:** Previously polycomplexes and other anomalies in the formation of synaptonemal complexes (SCs) were observed in the *mms4* derivative of NH144 (de los Santos *et al.* 2001). Similar results were obtained for *mus81* in this strain background at 33° (data not shown). These anomalies appear to be strain specific since they were rarely seen in the *mus81*, *mms4*, and *mus81 mms4* derivatives of NKY1551 (data not shown). All four diploids exhibited SCs at the same early time point (4 hr) but, in the *mus81*, *mms4*, and *mus81 mms4* diploids, SCs persisted until 7 hr in a subset of cells; no SCs were observed in the wild type at such a late time point (data not shown). Thus, entry into pachytene is not delayed but exit from pachytene is retarded in the mutants, consistent with a delay in pachytene triggered by the meiotic recombination checkpoint. In addition to normal SCs, many nuclei with faint and/or fragmented SCs were seen in the mutants at 7 hr (data not shown). We assume that these SCs are in the process of disintegration after prolonged arrest at pachytene. Synapsis is therefore not affected by absence of *MUS81* or *MMS4*.

***MMS4/MUS81* and *MSH4/MSH5* function independently with respect to both spore viability and crossing over:** The *MSH4/MSH5* genes encode meiosis-specific MutS homologs that, like *MUS81/MMS4*, are required for full levels of crossing over (Ross-Macdonald and Roeder 1994; Hollingsworth *et al.* 1995). The phenotypes of *msh4/msh5* mutants are, however, distinct from those of *mms4/mus81*. For example, the spore lethality in *msh4/msh5* mutants can be attributed to homolog nondisjunction, which is not the case for *mus81/mms4* (Hollingsworth *et al.* 1995; de los Santos *et al.* 2001; this study). Also, interference is not observed between the crossovers that form in *msh4/msh5* (Novak *et al.* 2001; N. Hunter, V. Boerner, A. Jambahkar and N. Kleckner, unpublished results) whereas crossovers in *mus81/mms4* do show an interference distribution (this study). These observations suggest the possibility that *MUS81/MMS4* and *MSH4/MSH5* promote distinct classes of crossovers. Consistent with this idea, a *mus81 msh5* double mutant shows decreased spore viability relative to either single mutant (wild type, 96.1%; *msh5*, 43.9%; *mus81*, 35.6%; *mus81 msh5*, 19.5%). These data resemble those observed for *mms4 msh5* (de los Santos *et al.* 2001). Physical analysis of crossing over at the *HIS4::LEU2* locus reveals that crossing over in a *mms4 msh5* double mutant is reduced ~3-fold relative to either of the single mutants (Figure 6). A residual amount of crossing over (~6-fold reduced, relative to wild type) can still be detected in *mms4 msh5* cells and may account for the residual spore viability observed for this strain. When taken together with the results presented above, these data indicate that *MUS81/MMS4* and *MSH4/MSH5* promote essentially independent classes of meiotic crossovers.

**Catalytic site residues are required for *MUS81* function during meiosis:** *MUS81* is proposed to resolve meiotic HJs in *S. pombe*, a function for which it is apparently not required in *S. cerevisiae* meiosis. The question then exists whether *MUS81* acts in a fundamentally different way, *e.g.*, as a structural protein instead of an enzyme, during meiosis in budding yeast compared to fission yeast. To address this, two conserved aspartic acid residues (D414A, D415A) in the Mus81 protein were changed to alanine. Recent experiments have shown

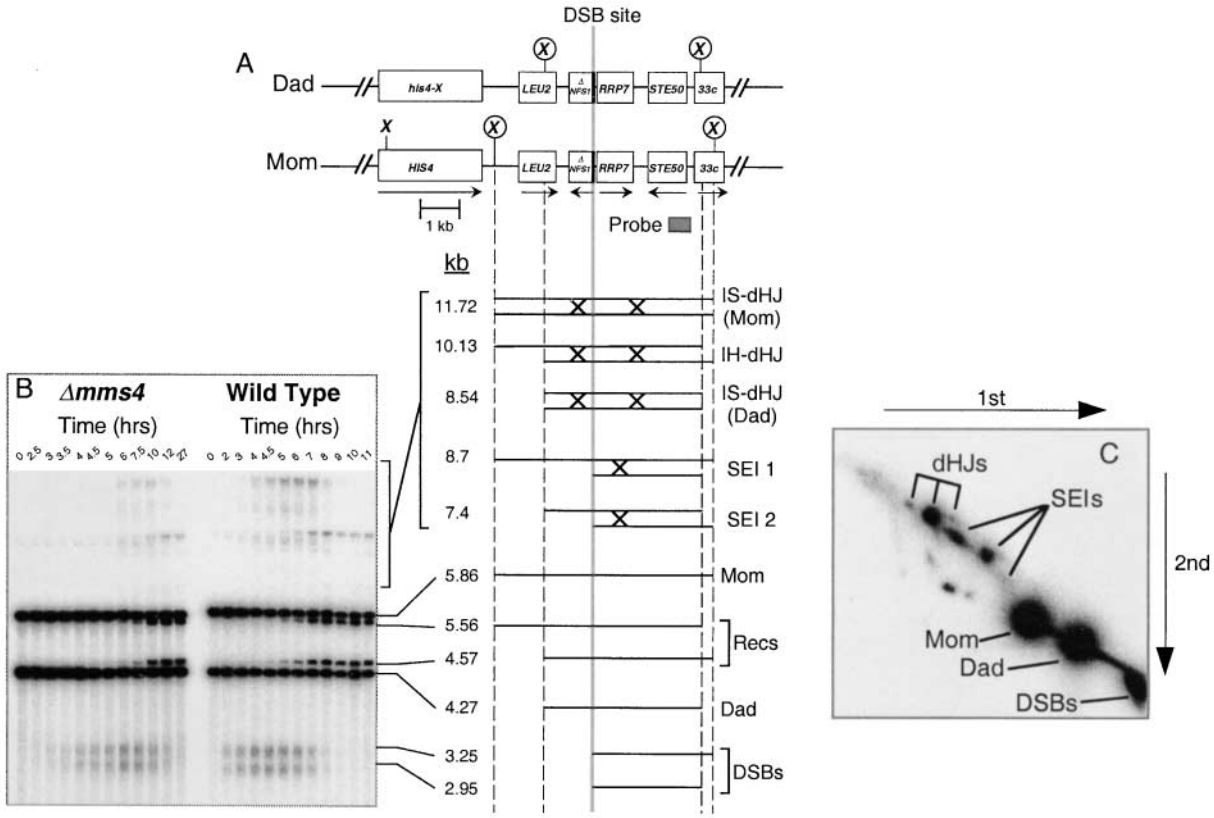


FIGURE 4.—Assay system for analysis of recombination intermediates. (A) Physical map of the modified *HIS4/LEU2* locus (see HUNTER and KLECKNER 2001, for details). Open reading frames and diagnostic restriction sites are shown. Parental homologs "Mom" and "Dad" are distinguished by *Xho*I restriction site polymorphisms (circled X's). The size and identity of signal detected by Southern hybridization with a unique probe (probe A, SCHWACHA and KLECKNER 1997) are shown below. SEI 1 and 2 correspond to the prominent SEI species, SEI 3 and SEI 4, described in HUNTER and KLECKNER (2001). IS-dHJs, intersister double HJs; IH-dHJs, interhomolog double HJs; Recs, interhomolog crossover recombinants. (B) Southern blots of one-dimensional gels from wild-type (NHY290) and *mms4*Δ (NHY1155) time courses. Joint molecule recombination intermediates (highlighted by bracket) were analyzed by two-dimensional (2D) gel electrophoresis and Southern hybridization, as shown in C. In this case, branched DNA species are retarded relative to linear molecules in the second dimension. DHJs are highlighted by a trident; the prominent interhomolog signal is flanked by the two weaker intersister species. SEIs are highlighted by a fork with three lines; the two prominent species correspond to SEI 1 and SEI 2.

that the aspartic acid in the mammalian XPF protein equivalent to D415 is part of the active site required for catalytic activity (ENZLIN and SCHARER 2002). These mutations have been shown to create a null allele in *S. pombe* and to abolish enzymatic activity in both the *S. pombe* and human Mus81 complexes without affecting protein stability (BODDY *et al.* 2001; CHEN *et al.* 2001). The *S. cerevisiae mus81-DD* allele failed to complement both the sporulation and spore viability defects of *mus81*, producing 37.5% viable spores compared to 85.2% for the wild type. The catalytic activity of *MUS81* is therefore required for meiosis.

**Expression of the bacterial Holliday junction resolvase *rusA* fails to suppress the *mus81* meiotic mutant phenotypes:** The spore inviability of *S. pombe mus81* mutants can be partially suppressed by expression of the highly specific Holliday junction resolvase, *rusA*. Although crossovers are detected in *S. cerevisiae mus81* mutants, it is possible that failure to resolve a subset of HJs results in triggering the meiotic recombination checkpoint and decreased spore viability. In this case, overexpression of *rusA* might suppress the *mus81* meiotic defects in *S. cerevisiae*. This idea was tested by fusing the same *NLS-rusA-2HA* allele (hereafter referred to as *rusA*) used by BODDY *et al.* (2001) to the meiosis-specific *MEK1* promoter on a high-copy-number plasmid (pNH246wt). As a control, an allele containing a mutation in the catalytic site of the enzyme, *rusA-D70N* (pNH246), was included (DOE *et al.* 2000). Western blot analysis using α-HA antibodies confirmed that the rusA proteins were expressed at 3 hr, prior to the time that crossovers are first observed (data not shown). No improvement in either sporulation or spore viability was observed in cells expressing either rusA protein (Table 4). The meiosis specificity of the expression of the *MEK1p-rusA* allele prohibits performing alternative assays in vegetative cells to determine whether or not *rusA* is active in budding yeast. Suppression of the *mus81* spore inviability

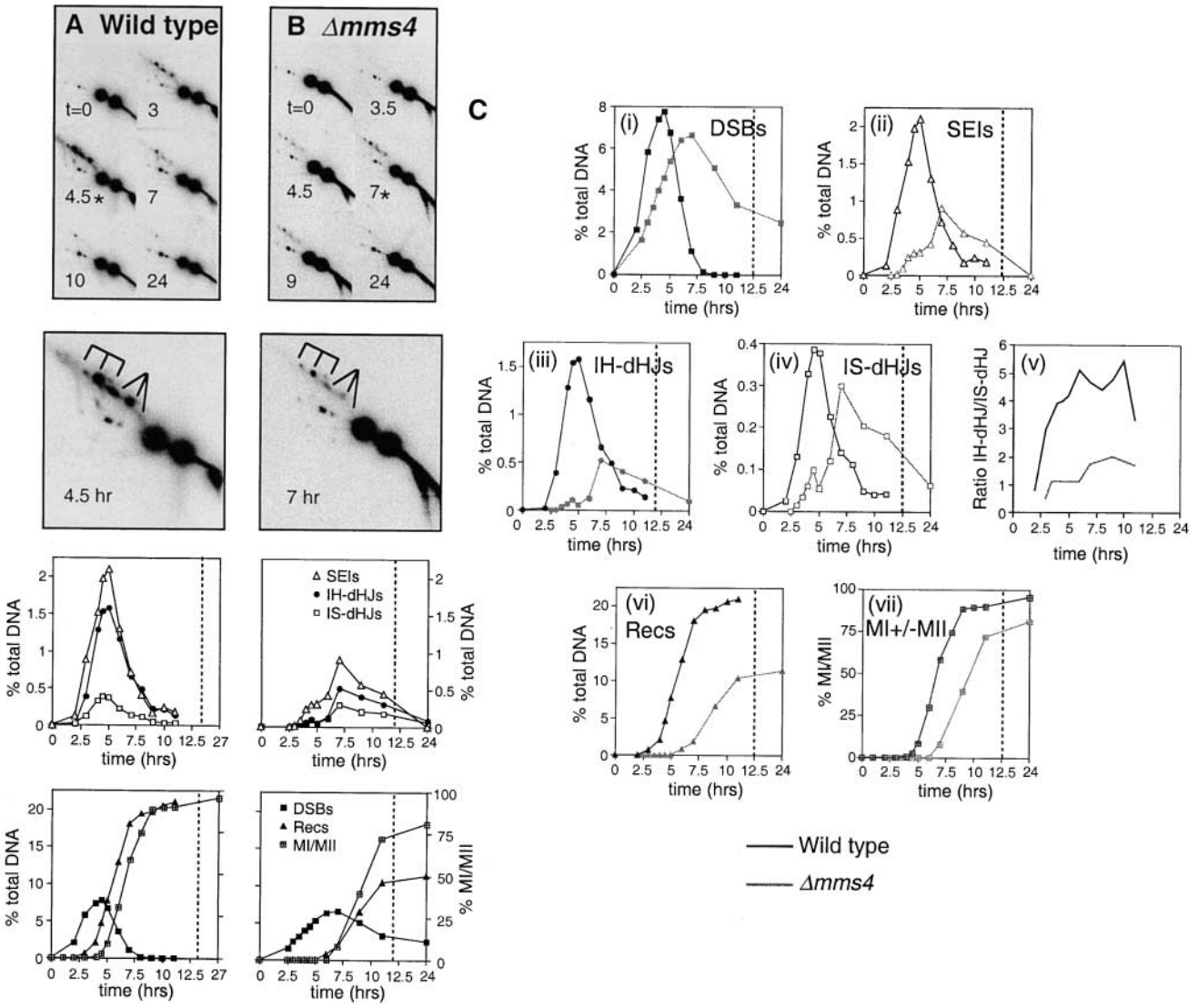


FIGURE 5.—Analysis of recombination intermediates in wild-type and *mms4*Δ diploids. (A and B) Top row, 2D gels showing representative time points; asterisks indicate time points in the second row. Third and fourth rows, DNA species quantitated as percentage of total hybridizing signal and plotted against time after transfer to sporulation medium. SEIs and dHJs are analyzed using 2D gels; DSBs and Recs are analyzed by 1D gel (see Figure 4, B and C). (C) Direct comparison of meiotic events in wild type and *mms4*Δ. Data are taken from A and B. In graph v, the ratio of signals, IH-dHJ/IS-dHJ, is plotted against time after transfer to sporulation medium. % MI/MII, percentage of cells that have completed one or both meiotic divisions as determined by DAPI staining. Dotted lines indicate discontinuities in the *x*-axes.

was therefore repeated with an untagged allele of *rusA* that is fused to the *SGS1* promoter. This *rusA* plasmid suppresses the UV and camptothecin sensitivity of *mms4* and is therefore functional in *S. cerevisiae* (S. BRILL, personal communication). Also, no suppression of the *mus81* meiotic phenotypes was observed with this allele of *rusA* (Table 4).

## DISCUSSION

**Mus81/Mms4 is not the major meiotic HJ resolvase in *S. cerevisiae*:** Mus81 was proposed to be a HJ resolvase in part on the basis of the observation that crude preparations of Mus81 from *S. pombe* and human cells cleave HJs *in vitro* (BODDY *et al.* 2001; CHEN *et al.* 2001). However, recent experiments using Mus81 complexes, either purified to homogeneity from bacterial cells or partially purified from human cells, indicate that the fission yeast, budding yeast, and human enzymes all exhibit a preference for cleaving three-way junction and replication fork structures over HJs (KALIRAMAN *et al.* 2001; CONSTANTINOU *et al.* 2002; DOE *et al.* 2002). In addition, those HJs cleaved by Mus81cannot be religated, indicating that nonsymmetrical nicks are being generated (BODDY *et al.* 2001; CONSTANTINOU *et al.* 2002). It has therefore been proposed that eukaryotes might resolve

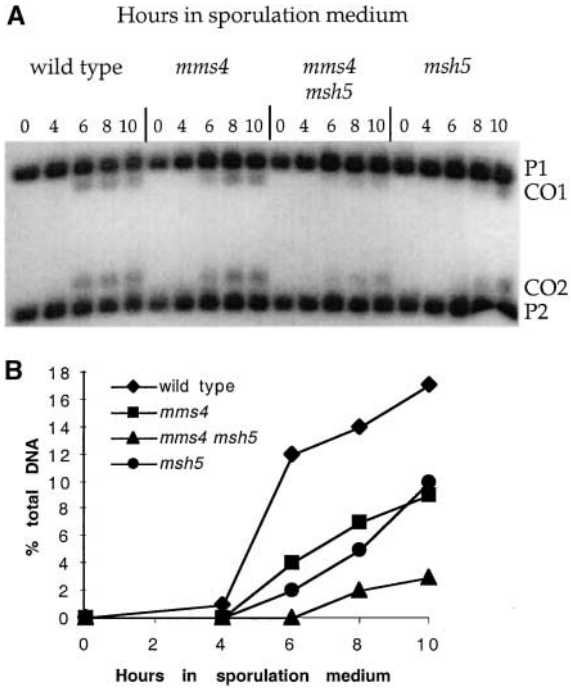


FIGURE 6.—Effects of *msh5*, *mms4*, and *mms4 msh5* on the formation of crossovers at the modified *HIS4/LEU2* recombination hotspot. (A) DNA was isolated from wild type (NKY1296), *mms4*Δ (NHY1298), *msh5*Δ *mms4*Δ (NHY1299), and *msh5*Δ (NHY1297) at different times after transfer to sporulation medium at 33°. The DNA was digested with *Xho*I and probed with a 0.6-kb *Age*I/*Bgl*II fragment from pNH90. The expected crossover (CO) and parental (P) fragments predicted in Figure 4 are indicated. (B) The CO2 bands were quantitated as described in DE LOS SANTOS *et al.* (2001). Because of the bubble in the P2 band of the NHY1297 diploid, for quantitation purposes, the total DNA is defined as P1 + CO2.

HJs in a different way from the paradigm established by bacterial HJ resolvases, such as ruvC and rusA, in which two symmetrical nicks are generated on strands of opposite polarity (WEST *et al.* 1984; BODDY *et al.* 2001). The finding of a ruvC-like activity from human cells, however, argues against this idea (CONSTANTINOU *et al.* 2002).

Several aspects of our data indicate that Mus81/Mms4 is not the major meiotic HJ resolvase in *S. cerevisiae*. First, *mus81* and *mms4* exhibit, at most, a twofold decrease in crossing over and this reduction appears to be limited to specific chromosomes. Second, double HJs do not accumulate in an *mms4* mutant, as would be expected for a mutant whose sole defect is the inability to resolve HJs. Finally, unlike fission yeast, expression of a heterologous HJ resolvase has no suppressive effect on the *mus81* and *mms4* meiotic phenotypes.

***S. cerevisiae* Mms4/Mus81 promotes a distinct set of meiotic crossovers:** The crossovers that form in an *mms4* mutant are qualitatively normal, being subject to interference. We have shown that the majority of these *MMS4/MUS81*-independent exchanges are facilitated by the *MSH5* gene product. In contrast to the *mms4* phenotype, the residual crossovers that occur in *msh4* or *msh5* mutants do not show interference (NOVAK *et al.* 2001; N. HUNTER, V. BOERNER, A. JAMBAHKAR and N. KLECKNER, unpublished results). These facts are consistent with the idea that there are (at least) two classes of crossovers in *S. cerevisiae*: Class I crossovers exhibit an interference distribution whereas class II crossovers do not (*e.g.*, ZALEVSKY *et al.* 1999; COPENHAVER *et al.* 2002). We can now propose that the two classes of crossovers are promoted by biochemically distinct processes: class I by a Msh4/5-based complex and class II by a Mms4/Mus81-based complex.

Both genetic and physical assays show that the decrease in crossing over in *mus81* and *mms4* mutants is modest (1.1- to 1.8-fold) and our analysis of crossover distribution implies that that Mus81/Mms4 promotes a specific subset of crossovers, as opposed to having a partial, but general, role in crossing over. More specifically, class II crossovers appear to be more prominent between shorter chromosomes, suggesting that recombination responds to chromosome size by modulating the relative numbers of class I and class II crossovers.

**TABLE 4**

**Sporulation and spore viability in *mus81* diploids overexpressing *rusA***

| Strain/plasmid | Diploid genotype | % sporulation | % spore viability (no. asci) |
|---|---|---|---|
| NH371/pRS315 | *mus81*Δ | 12.8 | ND |
| NH371/pCL3 | *mus81*Δ*/MUS81* | 45.5 | 88.9 (52) |
| NH371/pNH246wt | *mus81*Δ/2μ *MEK1p-NLS-rusA-HA* | 17.6 | 49.2 (92) |
| NH371/pNH246 | *mus81*Δ/2μ *MEK1p-NLS-rusA-D70N-HA* | 11.3 | 43.7 (20) |
| NH371::pRS306 | *mus81*Δ | ND | 47.1 (104) |
| NH371::pDT6 | *mus81*Δ*::MUS81* | ND | 99.3 (104) |
| NH371::pKR6980 | *mus81*Δ*::SGS1p-NLS-rusA* | ND | 44.7 (104) |

Transformants were patched onto selective medium and replica plated to sporulation plates at 30° for 2 days. Sporulation was assessed by counting at least 200 cells for the formation of mature asci using phase contrast microscopy. ND, not determined.

A strong prediction of the two-crossover pathway model is that crossovers present in *mms4* mutants should exhibit stronger interference than wild type. Our current data set is too small to establish whether or not this is true, and therefore models in which *MUS81/MMS4* have a general role in crossing over cannot be ruled out. To prove the chromosome size specificity of *mms4*, the same genetic intervals need to be examined in the context of short and long chromosomes for crossovers and interference. Experiments to do this using translocation chromosomes are currently underway.

Three aspects of meiotic recombination in wild-type *S. cerevisiae* have been correlated with chromosome size. First, Kaback *et al.* (1989) have shown that recombination rate responds directly to changes in chromosome size, with smaller chromosomes having higher rates of crossing over. Second, small chromosomes were found to have less intense crossover interference than large ones (Kaback *et al.* 1999). Finally, the genome-wide analysis of meiotic DSB hotspots, performed by Gerton *et al.* (2000), indicates that recombination hotspots are denser along short chromosomes. Taken together, these data suggest that chromosome size-dependent changes in the rate of crossing over may result from correlated modulation of both recombination initiation (DSB formation) and crossover interference. Our results suggest that Mms4/Mus81 is central to this phenomenon.

**Reconciling differences between *mus81* meiotic phenotypes in *S. cerevisiae* and *S. pombe*:** In most organisms, including *S. cerevisiae*, homologous chromosomes become coaligned and physically associated along their lengths via the SC. A major SC component, Zip1, is required for the formation of crossovers that show an interference distribution (Sym and Roeder 1994) and, moreover, epistasis analysis reveals that *ZIP1* and *MSH4/5* promote the same set of crossovers (Novak *et al.* 2001). In contrast, there is no SC and no crossover interference in *S. pombe* (Bahler *et al.* 1993; Munz 1994) and, accordingly, *ZIP1* and *MSH4/5* homologs are absent from the *S. pombe* genome (Villeneuve and Hillers 2001). We propose, therefore, that meiotic crossovers in *S. pombe* arise exclusively via a SC-independent, *MUS81*-dependent pathway. The *MUS81* pathway also operates in *S. cerevisiae*, but most crossovers arise via the pathway defined by *ZIP1* and *MSH4/5* (Figure 7). The dependency in *S. pombe* solely on the *MUS81* pathway for crossovers may explain why the *mus81* spore viability defect is so much more severe in fission yeast compared to budding yeast (<0.1% *vs.* 40%).

Why does *rusA* suppress the *mus81* spore inviability in *S. pombe* but not in *S. cerevisiae*? One explanation is that Mus81 complexes function differently in the two yeasts, *i.e.*, as a HJ resolvase in *S. pombe* and a 3′ flap endonuclease *in S. cerevisiae*. This argument is weakened by the recent observation that *S. pombe* Mus81/Eme1 purified from bacterial cells exhibits the same preference for 3′ flap and replication fork structures as

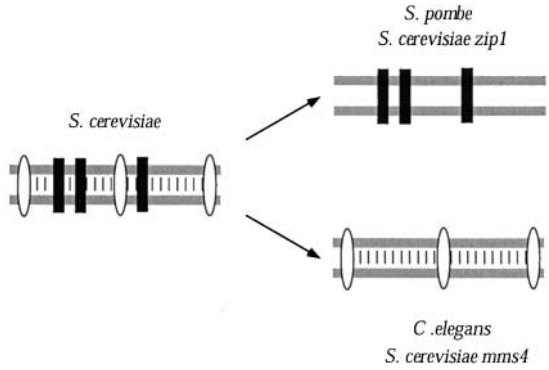


Figure 7.—Model for two pathways of crossing over. Shaded lines indicate the condensed pairs of homologous sister chromatids. Vertical lines indicate synapsis. Open ovals indicate crossovers along the interference *ZIP1/MSH5*-dependent pathway. Solid bars indicate crossovers occurring along the noninterference *MUS81/MMS4*-dependent pathway.

Mus81/Mms4 (Doe *et al.* 2002). Another possibility is that rusA is able to cleave 3′ flaps *in vivo*. Although rusA is highly specific for HJ cleavage *in vitro*, how it acts when overexpressed in a eukaryotic nucleus has not been determined. In this case, *rusA* may not suppress *mus81/mms4* in *S. cerevisiae* because access to the DNA is blocked by synapsis between homologous chromosomes. The sporulation and spore viability defects of *mms4* can be partially suppressed by *red1* but not *mek1* (de los Santos *et al.* 2001). One phenotypic difference between *red1* and *mek1* is that *mek1* mutants form some SC while *red1* mutants do not (Rockmill and Roeder 1990, 1991). We have argued that in an *mms4* diploid, alternative pathways may resolve recombination intermediates in the absence of synapsis, but that SC formation prevents such alternative processing (de los Santos *et al.* 2001). It is possible that the presence of SC also prevents *rusA* from acting. Since *S. pombe* chromosomes do not synapse, there is no barrier to *rusA* action in this species.

The complementary situation appears to exist in nematodes. Crossing over in worms is likely to undergo interference (A. Villeneuve, personal communication) and is completely abolished by mutation of either *msh-4* or *msh-5* (Zalevsky *et al.* 1999; Kelly *et al.* 2000). Therefore, by our model, *C. elegans* most closely resembles an *S. cerevisiae mms4/mus81* mutant (Figure 7). Although a Mus81 homolog is present in nematodes (Saccharomyces Genome Database), it is lacking an N-terminal domain that is also absent from *S. pombe* but is common to both mammalian and budding yeast Mus81 (A. Neiman, personal communication). Mutation of conserved residues within this domain creates null alleles of *MUS81* with regard to spore viability, suggesting this domain may be specifically involved in the meiotic function of *MUS81* (D. Turney and N. M. Hollingsworth, unpublished results). It will be interesting to discover

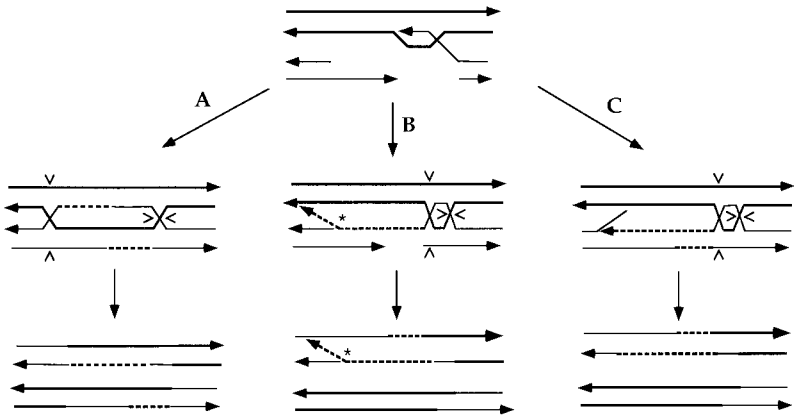


FIGURE 8.—Pathways of meiotic DSB repair. In all cases, recombination is initiated by the invasion of a resected 3′ tail into the homologous duplex. (A) The canonical DSB repair model. After capture of the second end, ligation creates two HJs flanking regions of heteroduplex DNA. Resolution of the HJs in opposite ways (indicated by carets) generates two intact crossover chromosomes. (B) Strand displacement-mediated crossing over with a 3′ flap. After DNA synthesis extends the invading strand, the strand is partially displaced and reanneals to the ss tail on the other side of the break. Overreplication creates a 3′ flap that could be cleaved by Mus81/Mms4p (indicated by an asterisk). Resolution of the HJs generates an intact crossover chromatid and an unrepaired chromatid. (C) Strand displacement-mediated crossing over with a 5′ flap. The overreplicated strand anneals completely to the other side of the break, thereby creating a 5′ flap. Repair is achieved by cleavage of the flap and ligation. Resolution of the HJs generates two intact crossover chromatids. Dashed lines indicate newly synthesized DNA.

whether or not *mus81* mutants in nematodes have a meiotic phenotype.

**Recombination intermediates and interference-mediated crossovers are delayed in the absence of *MUS81* and *MMS4*:** Although physical analysis of recombination intermediates in *mus81*Δ and *mms4*Δ cells is not indicative of a problem with HJ resolution, the progression of some meiotic events is clearly different from wild type in several ways. For example, peak steady-state levels of DSBs occur later than normal. This observation could be explained in several different ways:

1. DSBs form later due to a delay in meiotic S-phase caused by *mus81/mms4*-related replication problems. This possibility seems unlikely, given that all of the *mus81* and *mms4* meiotic phenotypes are dependent upon the initiation of recombination (DE LOS SANTOS *et al.* 2001; KALIRAMAN *et al.* 2001).
2. DSBs are formed with normal timing but some DSBs turn over faster than normal.
3. Fewer DSBs are formed.

In addition, some DSBs do not appear to turn over (or arise in some aberrant way, *e.g.*, faulty processing of an intermediate). The fact that all ensuing events occur later than normal is most consistent with a general delay in meiosis. However, SCs appear with normal timing in *mus81/mms4* mutants, indicating that two key events of the meiotic program, homolog coalignment and SC formation, are unaffected.

Although many crossovers in *mms4*Δ cells form and are subject to an interference distribution, they arise later than normal due to the *mms4*-induced delay in meiotic progression. Formation of crossover precursors, SEIs and dHJs, is similarly delayed. Perhaps correction of the problems along the *MMS4/MUS81* pathway is required before the events along the *MSH4/5* pathway can proceed.

**Model of *MMS4/MUS81* function in *S. cerevisiae*:** Recently an alternative pathway for meiotic recombination was proposed to account for the existence of recombination intermediates that do not conform to the predictions of the DSBR model (ALLERS and LICHTEN 2001b). In the strand displacement-mediated crossover model, recombination is initiated by formation of a DSB followed by resection and invasion of the nonsister duplex. After DNA synthesis extends the invading strand, it is partially displaced and anneals to the 3′ ss tail on the other side of the break. If the extended sequence is longer than the 3′ ss tail, a 3′ flap may be generated (Figure 8B). Cleavage of the flap 5′ to the junction would allow the break to be fixed by extension and ligation.

This type of 3′ flap structure is an excellent substrate *in vitro* for purified Mus81/Mms4 (KALIRAMAN *et al.* 2001). We therefore propose that *MUS81/MMS4* is required to process these flaps during meiotic recombination (DE LOS SANTOS *et al.* 2001). Failure to cleave the flaps results in unprocessed recombination intermediates that trigger the meiotic recombination checkpoint. The decrease in dHJs and SEIs observed in *mms4* could be explained if a failure to process flaps at the annealing stage renders the intermediates labile or causes them to be processed by a different mechanism. In some cases, resolution of the dHJs could result in one crossover chromosome and one unrepaired chromatid that would be lethal to the spore (Figure 8B). This mechanism of lethality could explain the near random distribution of viable spores in tetrads if lethality is dependent upon the number of unrepaired chromosomes that segregate to any given spore.

For both *mms4* and *mus81*, a slightly higher number of four-viable-spore tetrads are observed than are predicted by random death. Clearly all of the chromatids in these asci are intact since all of the spores are viable. One way this could occur would be if the 3′ flap is converted to a 5′ flap, perhaps by branch migration (Figure 8C). The 5′ flap could then be cleaved by a 5′ flap endonuclease, allowing ligation and repair of the

break. Because the heteroduplex tracts would be longer, higher levels of gene conversion would be predicted to occur. In fact, elevated levels of gene conversion were observed for both *mus81* and *mms4* in four-viable-spore asci. If the four-viable-spore asci result from 5′ flap processing, they should be eliminated by a second mutation in a gene encoding the 5′ flap endonuclease. *RAD2* and *RAD27* both encode 5′ flap endonucleases (Habraken *et al.* 1995; Kao *et al.* 2002). Spore viability is unaffected by *rad2* in either the presence or the absence of *MUS81* (N. M. Hollingsworth, unpublished results). The *mus81 rad27* combination is synthetically lethal in vegetative cells (Tong *et al.* 2001; N. M. Hollingsworth, unpublished results). Therefore while the meiotic experiment could not be done, this genetic interaction suggests that *MUS81/MMS4* and *RAD27* act in alternative pathways of DNA repair in mitotically dividing cells.

Earlier we proposed that *MUS81/MMS4* functions on a different pathway for crossing over from that of *MSH5*. However, crossing over and spore viability are only reduced in the *msh5 mms4/mus81* double mutants; they are not eliminated. While the residual crossovers in the double mutant could be explained by the presence of a third pathway of crossing over, this result may also be explained if *MUS81/MMMS4* functions in the partial strand displacement and annealing pathway described above. *MUS81/ MMM4* is required only for those intermediates in which the originally invading end is overreplicated to make the 3′ flap. If overreplication occurs in just a subset of intermediates along this pathway, intermediates without flaps that can be resolved normally in the absence of *MUS81/ MMS4* to make crossovers may also be generated.

We thank Steve Brill, Michael Lichten, Aaron Neiman, Jim Haber, and Frank Stahl for helpful discussions. JoAnne Engebrecht and Aaron Neiman provided useful comments on the manuscript. We thank Paul Russell and Steve Brill for providing *rusA* plasmids. We are grateful to Lihong Wan for tetrad dissection and to Dana Turney for excellent technical support. This work was supported in part by research grant 1-FY02-754 from the March of Dimes Birth Defects Foundation and by a grant from the National Institutes of Health (GM-50717) to N.M.H. J.L. was supported by the Austrian Science Fund (grant S8202).

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Communicating editor: A. P. Mitchell