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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ABSTRACT

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 chromo-
some 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 posts 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; Chien 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 rad1 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...
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; Mulllen 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 mms4 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/MM54. 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 Sau site was engineered after the stop codon of the gene. The resulting fragment was digested with NdeI and SauI and ligated to NdeI/SauI-digested pDW14 to make pNH246 (de Los Santos and Hollingsworth 1999). Site-directed mutagenesis (Quickchange 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 SalI and BamHI (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 mut81::ARG4 mutation was constructed by first subcloning a 2.9-kb BamHI/HindIII fragment from pCL4 into BamHI/SalI pRS305 to make pDT6. A 1.3-kb BglII fragment within MUS81 was substituted for a 3.5-kb BamHI 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:

\[
\text{MATa leu2::hisG his4-A X- ARG4 } \text{ura3 } \text{ly2 ho::LYS2}
\]

\[
\text{MATa leu2::hisG his4-X ARG4 } \text{ura3 } \text{ly2 ho::LYS2}
\]

\[
\text{NH371, mus81::kanMX4; NH274F, mms4::hisG; NH416, rol1:: LEU2 mus81::kanMX4; NH396, mek1::LEU2 mus81::kanMX4; NH372F, mss4::hisG mss81::kanMX4.}
\]

The following are homozygous isogenic derivatives of NKY1551:

\[
\text{MATa leu2::hisG his4B::LEU2 arg4-Bgl lys2}
\]

\[
\text{MATa leu2::hisG his4X::LEU2(Bam)-URA3 arg4-Nsp lys2 ho::LYS2 ura3 ho::LYS2 ura3}
\]

\[
\text{NH301, mss4::kanMX4; NH428, mus81::ARG4; NH445, mus81::ARG4 mss4::kanMX4.}
\]

The following is an isogenic derivative of NHY290:

\[
\text{His4::LEU2-(NBam) leu2::hisG MATa ho::hisG his4-X::LEU2-(Bam)-URA3 arg4-Nsp lys2 ho::LYS2 ura3 ho::LYS2 ura3}
\]

\[
\text{NHY1555, mss4::hphMX4.}
\]

The following are isogenic derivatives of NHY1296:

\[
\text{His4::LEU2(Bam+) leu2::hisG MATa ho::hisG his4-X::LEU2-(NgoMIV)-URA3 leu2::hisG MATa ho::hisG ura3 ho::LYS2 ura3 ho::LYS2 ura3 ho::LYS2 ura3}
\]

\[
\text{NHY1297, mss4::kanMX4; NHI298, mss4::hphMX4; NHY1299, mss4::kanMX4 mss4::hphMX4.}
\]

The following is an isogenic derivative of NHI957:

\[
\text{MATa CENIII LEU2 HIS4 ura3(Spa-Spo)}
\]

\[
\text{MATa CENIII::ADE2 leu2::hisG his4-B ura3(Spa-Spo) can1 ho::hisG ade2Δ CAN1 ho::hisG ade2Δ}
\]

\[
\text{LYS2 met13-B CY2 trp5-S CENIII::URA3 thr1-A cyp1· lys5-P MET3 cy2 TRP5 CENIII THR1 CUP1}
\]

\[
\text{NH455, mss4::kanMX4.}
\]

Alleles marked with kanMX4 were disrupted by PCR using the method of Longtine et al. (1998). The mus81::ARG4 allele was introduced by transformation of a BamHI/XhoI 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°C 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 ascii 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 mus81. 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, dme1, 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 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; Ghu 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°C 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°C (NH144, wild type; NH371, *mus81*; NH274F, *mms4*; NH372F, *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, tetra-nucleate 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 *HIS4* 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.

Results (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°C 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 ditpyr
TABLE 1
Recombination in \textit{mus81} and \textit{mms4} SK1 diploids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>MAT-HIS4</th>
<th>HIS4</th>
<th>LEU2</th>
<th>ARG4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Map distance (cM)</td>
<td>% gene conversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH144*</td>
<td>WT</td>
<td>40.2 (540)</td>
<td>6.2 (577)</td>
<td>4.7 (470)</td>
<td>2.8 (577)</td>
</tr>
<tr>
<td>NH371</td>
<td>mus81Δ</td>
<td>25.6$^b$ (88)</td>
<td>17.8$^b$ (118)</td>
<td>6.8 (118)</td>
<td>5.9 (118)</td>
</tr>
<tr>
<td>NH274*</td>
<td>mms4Δ</td>
<td>30.6 (103)</td>
<td>12.3$^b$ (122)</td>
<td>5.8 (69)</td>
<td>6.8$^b$ (118)</td>
</tr>
</tbody>
</table>

Only four-viable-spore tetrads were analyzed. For \textit{HIS4} and \textit{ARG4}, gene conversions were scored as tetrads exhibiting $3^+1^-$ or $3^-1^+$ segregation for the marker. For \textit{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 \textit{mms4} diploids, indicating that \textit{MMS4}-independent crossovers are distributed normally by interference (Table 3; data not shown).

Physical monitoring of recombination events in \textit{mus81} and \textit{mms4} mutants: The genetic data are based on a highly selected subset of cells (the $\sim10\%$ that form mature ascis at 30$\degree$, of which only $\sim10\%$ 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 \textit{HIS4::LEU2} meiotic recombination hotspot on chromosome III (Storlazzi et al. 1995). Although \textit{mms4} and \textit{mus81} phenotypes with respect to DSBs are highly similar, they are not identical (Figure 3). DSBs are slightly more delayed in \textit{mus81} than in \textit{mms4} and DSBs tend to persist longer in \textit{mms4} than in \textit{mus81}. Whether or not these subtle differences are meaningful remains to be seen. No synergistic phenotypes were observed between the \textit{mus81} \textit{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 \textit{mus81} and \textit{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 \textit{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 under-
TABLE 2

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HIS4 LEU2 CENIII MAT LYS5 MET13 CYH2 TRP5 CENVIII THR1 CUP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS4</td>
<td>1.4 3.5 0.5 1.2 2.3 3.5 0.9 0.7 0.7 6.3 11.3</td>
</tr>
<tr>
<td>mms4</td>
<td>8.8* 5.9 0 5.9* 8.5* 9.2* 4.4* 7.0* 3.1 10.0 12.6</td>
</tr>
</tbody>
</table>

Map distance (cM) (no. asci)

<table>
<thead>
<tr>
<th>Chromosome III</th>
<th>HIS4-LEU2 LEU2-CEN CEN-MAT LYS5-MET13 MET13-CYH2 CYH2-TRP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS4</td>
<td>12.5 10.5 15.3 18.7 10.1 41.2</td>
</tr>
<tr>
<td></td>
<td>(404) (408) (419) (407) (407) (420)</td>
</tr>
<tr>
<td>mms4</td>
<td>8.4* 6.1* 10.7* 16.4 8.5 37.2</td>
</tr>
<tr>
<td></td>
<td>(237) (254) (252) (229) (240) (220)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromosome VII</th>
<th>CEN-TRP1 TRP1-CUP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS4</td>
<td>21.2 23.5</td>
</tr>
<tr>
<td></td>
<td>(398) (343)</td>
</tr>
<tr>
<td>mms4</td>
<td>13.9* 21.4</td>
</tr>
<tr>
<td></td>
<td>(220) (217)</td>
</tr>
</tbody>
</table>

Isogenic SK1 diploids were sporulated at 30°C and tetrads dissected (NH957, 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/).

Isogenic SK1 diploids were sporulated at 30°C and tetrads dissected (NH957, 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/).

Table 3

Coefficients of coincidence in isogenic MMS4 and mms4 diploids

<table>
<thead>
<tr>
<th>Coefficient of coincidence (no. observed DCOs/no. expected DCOs)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>MMS4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>mms4</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* 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: (T1 + NPD1/total) (T2 + NPD2/total) (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.
The bracket indicates DSBs. DNA was digested with XhoI and probed with a 0.6-kb EcoRI/XmnI 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. DSBs are labeled P1. CO is labeled P2. 

**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), mus81Δ (NH428), mus81Δ mms4Δ (NH445), and mms4Δ (NH301) at different times after transfer to sporulation medium at 33°C. The bracket indicates DSBs. DNA was digested with XhoI and probed with a 0.6-kb EcoRI/XmnI 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. DSBs are labeled P1. CO is labeled P2. 

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 almost absent toward the interhomolog species with an average ratio of 4.3. Interhomolog bias is almost absent in mms4Δ cells, which have an average IH-dHJ/IS-dHJ ratio of 1.4-fold (in both cases, the first time point at which DHBs 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 mms4Δ SK1 strains at 30°C (de los Santos et al. 2001; Figure 5C, 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 mms4Δ derivative of NH144 (de los Santos et al. 2001). Similar results were obtained for mus81 in this strain background at 33°C (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/MSH4, 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 XhoI 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/H9004 (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, over-expression 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 mms4Δ 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 mms4Δ. 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 ruxA that is fused to the SGS1 promoter. This ruxA 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 ruxA (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 Mus81 cannot 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
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 Mus81/Ms4-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/Ms4 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* |

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Diploid genotype</th>
<th>% sporulation</th>
<th>% spore viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH371/pRS315</td>
<td><em>mus81Δ</em></td>
<td>12.8</td>
<td>ND</td>
</tr>
<tr>
<td>NH371/pCL3</td>
<td><em>mus81Δ/MUS81</em></td>
<td>45.5</td>
<td>88.9 (52)</td>
</tr>
<tr>
<td>NH371/pNH246wt</td>
<td><em>mus81Δ/2µ MEK1p-NLS-rusA-HA</em></td>
<td>17.6</td>
<td>49.2 (92)</td>
</tr>
<tr>
<td>NH371/pNH246</td>
<td><em>mus81Δ/2µ MEK1p-NLS-rusA-D70N-HA</em></td>
<td>11.3</td>
<td>43.7 (20)</td>
</tr>
<tr>
<td>NH371::pRS306</td>
<td><em>mus81Δ</em></td>
<td>ND</td>
<td>47.1 (104)</td>
</tr>
<tr>
<td>NH371::pDT6</td>
<td><em>mus81Δ::MUS81</em></td>
<td>ND</td>
<td>99.3 (104)</td>
</tr>
<tr>
<td>NH371::pKR6980</td>
<td><em>mus81Δ::SGS1p-NLS-rusA</em></td>
<td>ND</td>
<td>44.7 (104)</td>
</tr>
</tbody>
</table>

Transformants were patched onto selective medium and replica plated to sporulation plates at 30°C 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 coalesced 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 (Bahi1er 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 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 synopsis 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 synopsis, 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...
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 non-sister duplex. After DNA synthesis extends the invading strand, it is partially displaced and reanneals to the ss tail on the other side of the break. Overrecombination 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.

Recombination intermediates and interference-mediated crossovers are delayed in the absence of MUS81 and MMS4: Although physical analysis of recombination intermediates in mus81Δ and mms4Δ 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 co-alignment and SC formation, are unaffected.

Although many crossovers in mms4Δ 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 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 non-sister duplex. After DNA synthesis extends the invading strand, it is partially displaced and reanneals to the ss tail on the other side of the break. Overrecombination 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.

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Mus81/Mms4 in Meiotic Recombination

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-sporo ascis. If the four-viable-sporo ascis 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 (Habib 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 mms81 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/MM4 functions in the partial strand displacement and annealing pathway described above. MUS81/MM4 is required only for those intermediates in which the originally invading end is over-replicated 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/MM4 to make crossovers may also be generated.

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