The Budding Yeast Msh4 Protein Functions in Chromosome Synapsis and the Regulation of Crossover Distribution

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ABSTRACT

The budding yeast MSH4 gene encodes a MutS homolog produced specifically in meiotic cells. Msh4 is not required for meiotic mismatch repair or gene conversion, but it is required for wild-type levels of crossing over. Here, we show that a msh4 null mutation substantially decreases crossover interference. With respect to the defect in interference and the level of crossing over, msh4 is similar to the zip1 mutant, which lacks a structural component of the synaptonemal complex (SC). Furthermore, epistasis tests indicate that msh4 and zip1 affect the same subset of meiotic crossovers. In the msh4 mutant, SC formation is delayed compared to wild type, and full synapsis is achieved in only about half of all nuclei. The simultaneous defects in synapsis and interference observed in msh4 (and also zip1 and ndj1/tam1) suggest a role for the SC in mediating interference. The Msh4 protein localizes to discrete foci on meiotic chromosomes and colocalizes with Zip2, a protein involved in the initiation of chromosome synapsis. Both Zip2 and Zip1 are required for the normal localization of Msh4 to chromosomes, raising the possibility that the zip1 and zip2 defects in crossing over are indirect, resulting from the failure to localize Msh4 properly.

reductional chromosome segregation is unique to the first division of meiosis. Sister chromatids remain associated throughout this division, while homologous chromosomes segregate to opposite poles of the spindle apparatus. The chromosome content of a diploid cell is thereby reduced to the haploid number of chromosomes. A prerequisite to proper chromosome segregation at meiosis I is meiotic recombination. Crossing over establishes chromatin bridges between homologs, called chiasmata, that ensure the proper orientation of chromosomes on the meiosis I spindle (Roeder 1997). The proper segregation of chromosomes in meiosis also depends on formation of the synaptonemal complex (SC), an elaborate proteinaceous structure that holds homologous chromosomes close together along their lengths during the pachytene stage of meiotic prophase (Roeder 1997). Mutations in genes encoding structural components of the SC lead to homolog nondisjunction at meiosis I and precocious separation of sister chromatids (Roeder 1997).

Two budding yeast proteins involved in meiotic recombination are Msh4 and Msh5 (Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995), which are homologs of the bacterial MutS mismatch repair protein. However, Msh4 and Msh5 play no role in mismatch correction; instead, they are required specifically for wild-type levels of meiotic crossing over. In the absence of Msh4 or Msh5, meiotic gene conversion occurs at approximately wild-type levels, but crossing over is reduced two- to threefold. The MSH4 and MSH5 genes are expressed specifically in meiotic cells (Ross-Macdonald and Roeder 1994; Chu et al. 1998). The proteins directly interact to form a complex (Pochart et al. 1997), and they colocalize to foci on meiotic chromosomes (J. E. Novak, unpublished data). In the absence of Msh4 (and presumably also Msh5), chromosomes sometimes fail to undergo crossing over (Ross-Macdonald and Roeder 1994). Because nonrecombinant chromosomes often missegregate at meiosis I, the msh4 and msh5 mutants display reduced spore viability.

The recombination phenotype of msh4 and msh5 is not unique to these mutants. Mutations in a number of other yeast genes also reduce the frequency of meiotic crossing over without decreasing the frequency of non-Mendelian segregation. These genes include ZIP1, ZIP2, ZIP3, MER3, MLH1, MLH3, and EXO1. The three ZIP genes are involved in SC formation. The Zip1 protein is present along the lengths of synapsed meiotic chromosomes and serves as a major structural component of the SC (Sym et al. 1993; Sym and Roeder 1995; Tung and Roeder 1998; Dong and Roeder 2000). Zip2 and Zip3 are present on meiotic chromosomes at discrete foci that correspond to the sites where synopsis initiates, and these proteins are required for the normal polymerization of Zip1 along chromosomes (Chua and Roeder 1998; Agarwal and Roeder 2000). The MER3 gene is expressed specifically in meiotic cells and encodes a

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putative helicase (Nakagawa and Ogawa 1999). The two MutL homologs, Mhl1 and Mlh3, form a hetero-
dimer specifically involved in meiotic crossing over (Hunter and Borts 1997; Wang et al. 1999); Mhl1 also
functions in mismatch repair both in vegetative and meiotic cells (Kolodner and Marsischky 1999). The
Exo1 protein is a 5’ to 3’ exonuclease specific for dou-
ble-stranded DNA (Huang and Symington 1993; Fiorentini
et al. 1997). In addition to its role in meiotic crossing over (Kazahanehadi and Borts 2000; Kirk-
patrick et al. 2000; Tsuouchi and Ogawa 2000), Exo1
is involved in mismatch repair and recombination in
vegetative cells (Fiorentini et al. 1997; Tishkoff et al.
1997).

Meiotic crossovers are nonrandomly distributed along
chromosomes such that two crossovers rarely occur close
together—a phenomenon known as crossover interfer-
ence. Interference is generally assumed to involve the
transmission of an inhibitory signal from one crossover site to nearby potential sites of crossing over. In budding yeast, mutations in three different genes—ZIP1, NDJ1
(a.k.a. TAM1), and MER3—have been shown to reduce or abolish crossover interference (Sym and Roeder 1994; Chua and Roeder 1997; Nakagawa and Ogawa 1999). A zip1 null mutation abolishes SC formation
(Sym et al. 1993), while an ndj1 null mutation causes a
substantial delay in SC formation (Chua and Roeder
1997; Conrad et al. 1997). mer3 has not been tested for
its effect on synapsis. The observed correlation between
impaired synapsis and decreased interference in mu-
tants of yeast and other organisms (Moens 1969; Hav-
ekes et al. 1994) is consistent with the hypothesis that
the SC is involved in transmission of the inhibitory signal
responsible for interference (Egel 1978; Maguire 1988).
The observation that Schizosaccharomyces pombe and Asper-
illus nidulans both lack SC and interference further
supports this idea (Strickland 1958; Olson and Zim-
mermann 1978; Egel-Mitani et al. 1982; Bahler et al.
1993).

Meiotic crossovers are nonrandomly distributed not
only among chromosomes, but also among chromo-
somes. In most meioses, every chromosome pair, no
matter how small, sustains at least one crossover—a
so-called obligate crossover or obligate chiasma. Obli-
gate crossing over might be due, at least in part, to
the fact that large chromosomes display more crossover
interference than do small chromosomes (Kaback et al.
1999). By preventing excess exchanges on large chromo-
somes, interference might ensure that crossovers
(generally presumed to be limited in number) are dis-
tributed such that every chromosome pair undergoes at
least one exchange. Consistent with this hypothesis, mutations that reduce or eliminate interference ran-
donize the distribution of crossovers among chromo-
somes such that chromosomes sometimes fail to cross
over and therefore nondisjoin (Sym and Roeder 1994;
Chua and Roeder 1997).

We have found that a msh4 null mutation (like ndj1)
TABLE 1
Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
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<tr>
<td>RM86a</td>
<td>MATa   CEN3::TRP1 his4::Bgl leu2   CAN1 URA3 HOM3 TRP2</td>
</tr>
<tr>
<td></td>
<td>MATa   CEN3 HIS4 leu2   can1   ura3   hom3   trp2</td>
</tr>
<tr>
<td></td>
<td>ho::lys2 trp1 lys2 ade2::Bgl msh4::LEU2</td>
</tr>
<tr>
<td></td>
<td>ho::lys2 trp1 lys2 ade2::Bgl MSH4</td>
</tr>
<tr>
<td>RM85a</td>
<td>RM86 but homozygous msh4::LEU2</td>
</tr>
<tr>
<td>RM96a</td>
<td>RM86 but HIS3 SER1 ADE2</td>
</tr>
<tr>
<td></td>
<td>his3-Nde ser1::mTn-3xHA/ lacZ ade2-Bgl</td>
</tr>
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<td>RM96 but homozygous msh4::LEU2</td>
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</tr>
<tr>
<td></td>
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</tr>
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</tr>
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<td></td>
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<td>RM97 but homozygous zip1::LYS2</td>
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</tr>
<tr>
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<td>RM97 but homozygous zip1::LYS2 msh4::LEU2</td>
</tr>
<tr>
<td>RM53a</td>
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</tr>
<tr>
<td></td>
<td>MATa   HIS4 leu2-2 K ARG4 MSH4 lys2 ho::LYS2 ura3</td>
</tr>
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<td>RM53, but msh4::ura3 msh4::URA3</td>
</tr>
<tr>
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<td>RM53, but homozygous ndt80::kan'</td>
</tr>
<tr>
<td>JN304a</td>
<td>RM51, but homozygous ndt80::kan'</td>
</tr>
<tr>
<td>BR2495</td>
<td>MATa   leu2-27 his4-280 arg4-8 thr1-4 cyh10 trp1-1 ade2-1 ura3-1</td>
</tr>
<tr>
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<td>MATa   leu2-2, 112 his4-260 ARG4 thr1-4 CYH10 trp1-289 ade2-1 ura3-1</td>
</tr>
<tr>
<td>JN295</td>
<td>BR2495, but homozygous zip2::ZLP2-GFP-URA3 msh4::MSH4-HA-kan'</td>
</tr>
<tr>
<td>JN294</td>
<td>JN295, but zip1::LEU2 LYS2 spO13</td>
</tr>
<tr>
<td>JN202</td>
<td>BR2495, but msh4::ADE2 MSH4-HA@URA3 thr1-4 his4-280</td>
</tr>
<tr>
<td>JN220</td>
<td>BR2495, but homozygous msh4::ADE2 MSH4-HA@URA3 zip2::URA3</td>
</tr>
<tr>
<td>JN292</td>
<td>BR2495, but homozygous msh4::ADE2 MSH4-HA@URA3 zip1::LYS2 lys2ΔNhe</td>
</tr>
<tr>
<td>MY63</td>
<td>BR2495, but homozygous zip1::LEU2</td>
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* These strains are isogenic with SK1.

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constructed as described (ROSS-MACDONALD et al. 1997). The ser1::mTn-3xHA/ lacZ allele was introduced into yeast using p11B targeted with Nhd, pR1723, containing his3-Nde, was derived from pRS305 (SIKORSKI and HETTER 1989) by first filling in the Nde site in HIS3 and then inserting a SmaI-EagI fragment containing URA3 from Yip5 between the SmaI and EagI sites. The his3-Nde and ade2-Bgl alleles were introduced by two-step transplacement using pR1723 (targeted with Nhd) and pR945 (ENGBRECHT and ROEDER 1990), respectively. zip1::LEU2 and zip1::LYS2 alleles were introduced as described (SYM et al. 1993; SYM and ROEDER 1994).

**Genetic analysis:** To calculate map distance, only four-spore-reviable tetrads that did not show gene conversion of relevant markers were used. Crossover frequencies were compared as described (CHUA and ROEDER 1997).

For interference analysis, the number of nonparental di-

types (NPDs) expected in a particular interval was derived by applying the formula of PAPAZIAN (1952), NPD = 1/2[1 –

TT – (1 –3TT/2)^3/2], and rounding the resulting figure to the nearest whole number. The ratio of NPDs observed to

NPDs expected was then compared using the chi-square test

as described (SYM and ROEDER 1994). To compare the NPD ratio calculated for the wild type with that calculated for the

msh4 mutant, the chi-square coincidence test was applied to

the values for observed and expected NPDs.

**Cytology:** Strains isogenic with BR2495 were grown and

sporulated as described (ROCKMILL et al. 1995). Strains iso-

genic or congenic with SK1 were prepared for meiosis by first
growing to saturation in YPAD. Cells were then diluted 200-

fold into YPA and grown at 30° for 1 hr, to an OD600 of

~0.9. Cells were then rinsed in 2% potassium acetate that

was warmed to 30°, pelleted, and resuspended in warm 2% potassium acetate at a volume equal to their volume in YPA.

Cells were shaken at 30°, and samples were harvested at various time points. Surface-spread meiotic nuclei were prepared as described (SYM et al. 1993), except that glass slides were not
coated with plastic. Samples taken to assess timing of cell divisions were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) as described (Engerbrecht and Roeder 1990).

Immunofluorescence on spread chromosomes was performed as described (Sym et al. 1993), with the following modifications. The solution for blocking and antibody dilutions was 3% bovine serum albumin, 1% teleostean gelatin (Sigma, St. Louis), and 0.02% thimerosal in phosphate-buffered saline. Rabbit anti-Zip1 antibodies (Sym et al. 1993) were diluted 200-fold and detected with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Rat anti-tubulin antibodies (Kilmartin et al. 1982) were diluted 500-fold and detected with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Hemagglutinin (HA)-tagged Msh4 was detected with mouse monoclonal antibody HA-11 (Covance) diluted 5000-fold followed by secondary antibodies conjugated to either Texas red or Cy3 (Jackson ImmunoResearch Laboratories). GFP-tagged Zip2 was detected with rabbit anti-GFP antibodies (CLONTECH, Palo Alto, CA) diluted 300-fold and secondary and Texas red or Cy3 (Jackson ImmunoResearch Laboratories) antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Red1 protein was detected using rabbit anti-Red1 serum (Smith and Roeder 1997) diluted 100-fold followed by Oregon Green 488-conjugated secondary antibodies. All antibody binding was carried out at room temperature. Images were captured using a Nikon Eclipse E800 microscope and a Photometrics (Tucson, AZ) Sensys CCD camera.

To quantify the colocalization of Msh4 and Zip2, the following procedure was used. First, image contrast and brightness were adjusted using Adobe Photoshop to compensate for the faintness of Msh4 foci in JN294. Msh4 and Zip2 foci that were judged to be brighter than background staining were copied separately onto transparent sheets in order to have a defined number of foci with distinct edges. The outlines of each spread were traced from the DAPI-stained DNA. Superposition of the sheets was used to count Msh4 and Zip2 foci that overlapped. To estimate the amount of overlap that would be found with these data, the copy of Zip2 foci was rotated 180° to randomize the position of the Zip2 foci with respect to the Msh4 foci. Any foci falling outside the area where the spread chromosomes overlapped were not included in the analysis. Within the overlapping region, the number of Msh4 and Zip2 foci and the number of overlapping foci were counted. Approximately 400 foci of each protein were scored for both JN294 and JN295.

Strain MY63, which has only untagged Msh4, was used to assess the background level of staining with antibodies against HA. Typically, spread nuclei from MY63 showed no foci.

To quantify Msh4 staining in wild-type and mutant spreads, Imagepoint IPLab Spectrum software was used to measure total intensity within each spread; background intensity was subtracted. At least 17 spreads for each strain were measured. The average values for the strains were found to be significantly different ($P < 0.001$) by a two-tailed t-test.

RESULTS

A msh4 mutant is defective in crossover interference:

Crossover interference can be detected as a deficit in double crossovers within a genetically marked interval, compared to the number expected based on the frequency of single crossovers. In yeast, four-strand double crossovers generate NPD tetrads. The frequency of NPDs expected in the absence of interference can be calculated from the observed frequency of tetratype (TT) tetrads due to single crossovers (Papazian 1952). The NPD ratio is the number of NPDs observed divided by the number expected (Snow 1979). An NPD ratio $<1.0$ indicates that crossover interference is operating.

Tetrads from wild-type and msh4 strains were dissected and analyzed for recombination in seven genetic intervals representing three different chromosomes (Table 2). Map distances in msh4 strains were only 40–60% of those in wild type (Table 2), with the exception of the HOM3–TRP2 interval, which was unaffected (Table 2).

In wild type, NPD ratios ranged from 0.13 to 0.34, indicating significant interference (Figure 1); the only exception was the HOM3–TRP2 interval, which did not display any interference in the strain background used for this analysis. Excluding the HOM3–TRP2 interval, NPD ratios in the msh4 mutant were consistently higher than those in wild type (Figure 1); this difference is statistically significant for the ADE2–SER1, CAN1–URA3, and URA3–HOM3 intervals (Table 2).

For the intervals examined on chromosomes V and XV, NPD ratios for the msh4 mutant were close to 1.0, as expected in the complete absence of interference (Figure 1). However, for the two intervals examined on chromosome III, NPD ratios were $<1.0$; the ratios for HIS4–CEN3 and CEN3–MAT were 0.67 and 0.38, respectively. For another test of interference on chromosome III, NPD ratios were calculated for the HIS4–MAT interval (which subsumes the HIS4–CEN3 and CEN3–MAT intervals; Figure 1). For this interval, the NPD ratio obtained in the msh4 mutant (0.68) was $<1.0$, but greater than the corresponding NPD ratio in wild type (0.36); both differences are statistically significant. Together, these data indicate that the msh4 mutation abolishes crossover interference in some intervals and significantly reduces interference in other intervals.

In theory, the observed increases in NPD ratios in the msh4 mutant could be due to an acquisition of chromatid interference instead of a loss of crossover interference. Chromatid interference would increase the number of four-strand double crossovers at the expense of two-strand and three-strand double crossovers. [There is no chromatid interference in wild-type yeast (Mortimer and Fogel 1974).] Chromatid interference would have to be quite strong to account for the excess of NPDs in the msh4 mutant; for the six intervals that show interference in the wild type, the NPD ratios in wild type average one-quarter those of the msh4 mutant. Thus, all or nearly all of the double crossovers occurring within these intervals in the mutant would have to be four-strand double crossovers. Such strong chromatid interference should be easily detected by examining tetrads with single crossovers in two adjacent intervals to determine the total number of chromatids involved. In both msh4 and wild type, double crossovers conformed to the 1:2:1 ratio of two-strand:three-strand:four-strand events expected in the absence of chromatid interference (Table 3). This analysis rules out the possibility that the increased NPD ratios in msh4 are due to chromatid interference and reinforces the conclusion that the Msh4 protein is required for crossover interference.
Msh4 Functions in Synapsis and Interference

TABLE 2
Tetrad analysis

<table>
<thead>
<tr>
<th>Interval</th>
<th>Strain</th>
<th>PDa</th>
<th>TT</th>
<th>NPD</th>
<th>NPD exp.</th>
<th>Prob.</th>
<th>cMc</th>
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<td>ADE2–SER1 MSH4</td>
<td>164</td>
<td>238</td>
<td>8</td>
<td>34</td>
<td>&lt;0.01</td>
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<td>7</td>
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<td>URA3–HOM3 MSH4</td>
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<td>115</td>
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<td>URA3–HOM3 msh4</td>
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<td>HOM3–TRP2 MSH4</td>
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<tr>
<td>HOM3–TRP2 msh4</td>
<td>1265</td>
<td>395</td>
<td>16</td>
<td>14</td>
<td>0.59</td>
<td>14.6</td>
<td>106</td>
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<td>HIS4–CEN3 MSH4</td>
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<td>697</td>
<td>24</td>
<td>71</td>
<td>&lt;0.01</td>
<td>30.0</td>
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<tr>
<td>HIS4–CEN3 msh4</td>
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<td>648</td>
<td>20</td>
<td>30</td>
<td>0.07</td>
<td>17.2</td>
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<tr>
<td>CEN3–MAT MSH4</td>
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<td>369</td>
<td>4</td>
<td>15</td>
<td>&lt;0.01</td>
<td>14.0</td>
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<td></td>
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<tr>
<td>CEN3–MAT msh4</td>
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<td>358</td>
<td>3</td>
<td>8</td>
<td>0.08</td>
<td>8.3</td>
<td>59</td>
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</tbody>
</table>

Strains RM86 (wild type) and RM85 (msh4) were used to examine the CAN1–URA3, URA3–HOM3, HOM3–TRP2, HIS4–CEN3, and CEN3–MAT intervals. Strains RM96 (wild type) and RM95 (msh4) were used to examine all intervals except CAN1–URA3 and URA3–HOM3. In wild type, spore viability was 94%, and 84% of tetrads contained four viable spores. In msh4, spore viability was 52%, and 34% of tetrads contained four viable spores.

a Parental diatype tetrads.
b NPD tetrads expected (Papazian 1952).
c Probability based on a chi-square test that the difference between the observed and expected numbers of NPDs is due to chance.
d Map distance in centimorgans.
e The map distance in the mutant as a percentage of the map distance in wild type.

Msh4 and Zip1 act in the same recombination pathway: To determine whether Msh4 and Zip1 operate in the same pathway, meiotic recombination and chromosome segregation were examined in the msh4 zip1 double mutant. The results of tetrad analysis indicate that map distances in the zip1 msh4 double mutant are similar to both single mutants; no statistically significant differences were observed between the double mutant and either single mutant strain for the six intervals examined (Table 4). Also, the frequency and pattern of spore

Figure 1.—The msh4 mutation reduces or eliminates interference. (A) Genetic map of the intervals tested. Gene order, the sizes of the intervals tested, and the overall size of each chromosome are indicated. (B) Interference values (NPD observed/NPD expected) were calculated for the seven intervals indicated. A ratio of 1.0 (dotted line) is indicative of no interference.
TABLE 3

Test for chromatid interference in adjacent intervals

<table>
<thead>
<tr>
<th>Intervals</th>
<th>Strain</th>
<th>Two strands</th>
<th>Three strands</th>
<th>Four strands</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADE2–SER1–HIS3</td>
<td>MSH4</td>
<td>18</td>
<td>22</td>
<td>7</td>
<td>0.27</td>
</tr>
<tr>
<td>ADE2–SER1–HIS3 msh4</td>
<td>8</td>
<td>10</td>
<td>5</td>
<td>0.76</td>
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</tr>
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<td>HIS4–CEN3–MAT</td>
<td>MSH4</td>
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<td>36</td>
<td>0.90</td>
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<tr>
<td>HIS4–CEN3–MAT msh4</td>
<td>13</td>
<td>50</td>
<td>22</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>CAN1–URA3–HOM3</td>
<td>MSH4</td>
<td>74</td>
<td>153</td>
<td>73</td>
<td>0.97</td>
</tr>
<tr>
<td>CAN1–URA3–HOM3 msh4</td>
<td>34</td>
<td>52</td>
<td>34</td>
<td>0.59</td>
<td></td>
</tr>
</tbody>
</table>

For the pairs of adjacent intervals indicated, all tetrads that were tetratype in both intervals were classified as two-strand, three-strand, or four-strand double crossovers according to the number of chromatids involved.

* Probability, based on a chi-square test, that the ratio of two-, three-, and four-strand double crossovers observed is consistent with the 1:2:1 ratio expected in the absence of chromatid interference.

viability in the double mutant were similar to those of the single mutants (Figure 2). In every case, tetrads containing 4, 2, or 0 viable spores were present in excess, compared to tetrads containing 3 or 1 viable spores; this pattern is indicative of meiosis I nondisjunction events (Sym and Roeder 1994). The frequency of chromosome III nondisjunction was estimated from the frequency of two-sporable tetrads in which both spores are disomic for chromosome III (and therefore mating incompetent due to heterozygosity at the MAT locus).

Such tetrads represented 5.2% of total tetrads in the zip1 strain, 4.9% in the msh4 strain, and 4.4% in the zip1 msh4 strain. Thus, by all three criteria—map distances, spore viability, and chromosome III nondisjunction—Msh4 and Zip1 act in the same pathway.

Chromosome synapsis is delayed and often incomplete in the msh4 mutant: Although pachytene nuclei have been observed in the msh4 mutant (Ross-Macdonald and Roeder 1994), the extent and timing of SC formation were not quantitated. To test the possibility that the msh4 mutation reduces the efficiency of chromosome synapsis, SC formation was examined in msh4 and wild-type cells by staining spread meiotic chromosomes with anti-Zip1 antibodies. Spread nuclei were simultaneously stained with anti-tubulin antibodies to assess spindle formation. The DNA-binding dye DAPI was used to visualize chromosomes. Nuclei were classified according to the extent of Zip1 localization and the presence or absence of a meiosis I spindle as described (Figure 3, A–D; Smith and Roeder 1997).

Nuclei with partly continuous Zip1 staining (indication of incomplete synapsis) appeared at the same time in the mutant as in wild type, but these nuclei were shorter lived and accumulated to a higher level in msh4 (Figure 3B). By 4.5 hr, wild-type cells were at the peak of pachytene, while msh4 cells displayed very few pachytene nuclei (Figure 3C). In this and other time courses (not shown), the maximum number of pachytene nuclei was reduced approximately twofold, and the time point at which maximal synapsis was observed was often delayed compared to wild type (Figure 3C). The appearance of nuclei containing meiosis I spindles was consistently delayed in msh4 (Figure 3D).

Polycomplexes are structured aggregates of SC components, including Zip1. Polycomplexes are observed in only a minority of meiotic prophase nuclei from wild type, but they are almost always present in strains over-producing Zip1 and in most mutants defective in SC formation (Loidl et al. 1994; Sym and Roeder 1995). Polycomplexes can be detected as regions of intense

TABLE 4

Epistasis analysis: map distances from tetrad analysis

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>RM97</td>
<td>Wild type</td>
<td>38.3</td>
<td>23.1</td>
<td>39.8</td>
<td>61.2</td>
<td>22.2</td>
<td>54.0</td>
</tr>
<tr>
<td>RM99</td>
<td>msh4</td>
<td>12.8</td>
<td>4.5</td>
<td>13.2</td>
<td>14.6</td>
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<tr>
<td>RM98</td>
<td>zip1</td>
<td>7.9</td>
<td>7.9</td>
<td>11.8</td>
<td>25.5</td>
<td>12.6</td>
<td>30.8</td>
</tr>
<tr>
<td>RM1000</td>
<td>zip1 msh4</td>
<td>10.5</td>
<td>6.0</td>
<td>23.0</td>
<td>17.9</td>
<td>13.0</td>
<td>31.1</td>
</tr>
</tbody>
</table>

For each interval, 80–100 tetrads containing four viable spores were analyzed. The exception is the HIS3–ADE2 interval in strains RM97 and RM98, for which 63 and 78 tetrads were analyzed, respectively. Recombination frequencies for msh4 and zip1 are significantly different (P < 0.05) from those in wild type, except for the HOM3–TRP2 interval in the zip1 mutant (P = 0.126). In no case is the recombination frequency in the msh4 zip1 double mutant significantly different from that in the msh4 or zip1 single mutant.
Zip1 staining with no DNA staining. In the strain background used for this analysis, a polycomplex was observed in only a fraction of wild-type nuclei (Figure 3E). In the msh4 mutant, however, nuclei with polycomplexes were far more frequent; late in meiotic prophase, nearly all nuclei with fully or partially synapsed chromosomes contained a polycomplex (Figure 3E).

The reduced number of pachytene nuclei observed in the msh4 mutant can be explained in two ways: not all msh4 cells go through pachytene, or each msh4 cell spends an abnormally short time in pachytene. A mutation that induces pachytene arrest, ndt80 (Xu et al. 1995), was used to distinguish these possibilities. As expected, the ndt80 strain displayed pachytene arrest, with 92% of cells in pachytene (Figure 3F). In contrast, the msh4 ndt80 strain arrested with only 24% pachytene nuclei; 72% of the nuclei displayed partly linear Zip1 staining (Figure 3F). This result suggests that many msh4 cells fail to fully synapse their chromosomes, even when cells are held in midmeiotic prophase.

Although previous results suggested the msh4 mutant is not delayed in meiotic nuclear division (Ross-Macdonald and Roeder 1994), the defect in meiotic prophase prompted a careful time course analysis of nuclear division in msh4 and wild-type strains. DAPI was used to visualize nuclei, and the number of mono-, bi-, and tetra-nucleate cells was monitored over time (Figure 4). In the msh4 mutant, the production of both bi-nucleate cells (products of the meiosis I division) and tetra-nucleate cells (products of the meiosis II division) was delayed by ~3 hr. Thus, both meiotic divisions are delayed in the msh4 mutant, as expected from the delay in meiotic prophase progression.

**Timing and pattern of Msh4 localization relative to Zip1 and Zip2:** The Msh4 protein has been shown to localize to ~60 foci on meiotic chromosomes (Ross-Macdonald and Roeder 1994). The localization of Msh4 relative to Zip1 was examined in cells (strain JN202) producing Msh4 protein tagged with the hemagglutinin epitope (Msh4-HA). This analysis was carried out in a BR2495 strain background, in which meiosis proceeds more slowly than in the SK1 strains used in the genetic experiments. Spread meiotic nuclei were prepared at 14.5 hr in meiosis; because meiosis is not completely synchronous, nuclei at all stages of meiotic prophase are present at this time. Nuclei were classified as to the extent of synapsis (as in Figure 3) and then examined for Msh4 foci. None (0/20) of the spreads without Zip1 staining showed any Msh4 foci. Of the spreads with spotty Zip1 staining (indicative of the initiation of synapsis), 72% (38/53) had Msh4 foci; the number of Msh4 foci appeared to be higher in nuclei in which Zip1 spots were more intensely staining. Together, these observations suggest that Msh4 localizes to chromosomes shortly after Zip1 assembly initiates. 100% (50/50) of nuclei with partly continuous Zip1 staining (i.e., partially synapsed chromosomes) displayed Msh4 foci. In addition, 100% (120/120) of nuclei with fully synapsed chromosomes had Msh4 foci on chromosomes, demonstrating that Msh4 foci are present throughout pachytene.

Msh4 foci show extensive overlap with Zip3 foci (Agarwal and Roeder 2000), predicting that Msh4 also colocalizes with Zip2 (since Zip2 and Zip3 colocalize). The extent of overlap between Zip2 and Msh4 was assessed in cells producing Msh4-HA and Zip2 tagged with green fluorescent protein (Zip2-GFP). A typical spread nucleus at pachytene is shown in Figure 5, A–C. On average, 80% of Msh4 foci overlapped a Zip2 focus, and 66% of Zip2 foci overlapped a Msh4 focus. Random positioning would cause only ~32% of Msh4 foci and ~26% of Zip2 foci to overlap (see Materials and Meth-
Figure 3.—Chromosome synapsis and spindle formation in a msh4 mutant, msh4 (RM51) and wild-type (RM53) strains were introduced into sporulation medium at time 0; chromosomes were spread at the indicated times. Synapsis was assessed by staining with anti-Zip1 antibodies, and spindles were detected by simultaneous staining with anti-tubulin antibodies. At least 100 nuclei were scored for each strain at each time point. (A–D) Time course of synapsis and spindle formation. Nuclei were divided into several categories to reflect the different stages in meiotic cell cycle progression: no Zip1 staining (not shown), (A) spotty Zip1 staining, (B) partly continuous Zip1 staining (in which a mixture of spots and linear stretches of Zip1 are seen, but Zip1 does not extend along the entirety of the chromosomes), (C) pachytene (in which Zip1 is fully continuous along all chromosomes, except in the nucleolar region), and (D) spindles (indicative of meiotic chromosome segregation). The kinetics of appearance and disappearance of nuclei in the last four categories are shown. (E) Polycomplexes. For each class of Zip1 and tubulin staining, the fraction of spreads containing a polycomplex was assessed. Results shown represent pooled data from the 5.5- and 6.5-hr time points in the data set of Figure 3, A–D. (F) Chromosome synapsis during ndt80 arrest. Zip1 and tubulin staining was quantified in an ndt80 strain (JN305) at 9.5 hr and a msh4 ndt80 strain (JN304) at 11.5 hr.

Therefore, most, but not all, Msh4 and Zip2 foci have the same subnuclear location. Zip2 foci that lack Msh4 were more frequent than Msh4 foci lacking Zip2. This difference could be explained if Zip2 localization normally precedes Msh4 localization; in this case, some of the Zip2 foci observed in spreads could be those to which Msh4 has not yet localized.

Proper localization of the Msh4 protein depends on Zip1 and Zip2: To understand better the relationship between Msh4 and the Zip proteins, Msh4 localization was examined in zip1 and zip2 strains, and Zip2 localization was assessed in a msh4 strain. Wild-type and mutant strains were tested at 15 hr, roughly the time of maximum pachytene nuclei and Msh4 localization in the BR2495 strain background used for this analysis. In this strain background, both zip1 and zip2 arrest in prophase of meiosis I (Sym et al., 1993; Chua and Roeder, 1998); therefore, the mutants were also tested at 20 hr. To compare cells at a similar stage of meiosis, only spreads with extensive Red1 staining on chromosomes were chosen for analysis. Red1 is a component of the cores of meiotic chromosomes; Red1 localization is maximal at pachytene (Smith and Roeder, 1997).

At 15 hr, Msh4 foci were detectable by immunofluorescence in zip1 and zip2 nuclei, but they were consistently much fainter than those in wild-type nuclei (Figure 5, G–I). Msh4 foci in the zip2 strain tended to be slightly fainter than those in zip1. In a typical experiment, the intensity of Msh4 staining (measured by total intensity in CCD camera images) averaged 200,000 for wild-type nuclei, 70,000 for the zip1 mutant, and 53,000 for the zip2 mutant. At 20 hr, each mutant displayed slightly brighter Msh4 staining than at 15 hr, but still less intense compared to the wild type at 15 hr (not shown). Thus, Msh4 localization is partly dependent on both Zip1 and Zip2. In contrast, Zip2 foci appear normal

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be caused by a simple reduction in the amount of Msh4 localized to chromosomes or it may reflect a more profound disruption of the normal pattern of localization. To address this issue, a zip1 mutant was examined to determine whether Msh4 still colocalizes extensively with Zip2, which localizes normally in the absence of Zip1 (Chua and Roeder 1998). Figure 5, D–F, shows the localization of Msh4 and Zip2 in a typical zip1 mutant nucleus. On average, 54% of Msh4 foci overlapped a Zip2 focus, and 48% of Zip2 foci overlapped a Msh4 focus. These values are substantially less than the corresponding 80% and 66% overlap observed in the wild type, though still higher than the estimate for random overlap (27% of Msh4 and 24% of Zip2 foci). In a zip1 mutant, Zip2 protein localizes to axial associations (Chua and Roeder 1998), which are connections between aligned homologous chromosomes seen in zip1 mutants (Sym et al. 1993). Msh4 did not localize to the majority of axial associations visible in a zip1 mutant (data not shown). Thus, in the absence of Zip1, Msh4 localization is abnormal with respect to Zip2.

**DISCUSSION**

*A msh4 mutation impairs chromosome synapsis: Careful analysis of the timing and extent of SC formation*
in the msh4 mutant has revealed a defect in chromosome synopsis. SC formation is delayed compared to wild type, and full synopsis is achieved in less than half of all nuclei. A polycomplex is formed in nearly all cells, including those in which chromosomes synapse fully, consistent with a decrease in Zip1 localization to chromosomes. The defect/delay in SC formation is accompanied by a delay in both spindle formation and nuclear division. It is likely that the msh4 delay in meiosis reflects the operation of checkpoint triggered by the defect in SC formation or perhaps by an associated defect in the resolution of recombination intermediates (Bailis and Roeder 2000).

Msh4 is found largely at the same sites on chromosomes as the Zip2 protein. Zip2 is required for Zip1 localization to chromosomes, and Zip2 foci correspond to the sites at which Zip1 begins to polymerize on chromosomes (Chua and Roeder 1998). The localization of Msh4 to sites of synopsis initiation can account for the observed defect/delay in SC formation in the msh4 mutant. Although Msh4 seems to localize to chromosomes slightly after Zip1, it may play a role in stabilizing the association of Zip1 with chromatin, or it may help organize chromatin-associated Zip1 into SC. Alternatively, msh4 may have a defect in synopsis extension, rather than synopsis initiation. We favor this interpretation for two reasons. First, nuclei with partially synapsed chromosomes appear at the same time in the mutant as they do in wild type, but nuclei of this type persist longer and accumulate to a higher level in the mutant than they do in wild type (Figure 3B). Second, msh4 nuclei often display chromosomes with linear stretches of SC that fail to extend along the full length of the chromosome. This contrasts with the situation observed in the zip3 mutant (defective in synopsis initiation) in which some chromosomes synapse fully while others fail to exhibit any Zip1 staining (Agarwal and Roeder 2000). Msh4 might affect Zip1 polymerization at a distance by promoting conversion of Zip1 to a conformation that more efficiently polymerizes along chromosomes.

Homologs of the MSH4 and MSH5 genes have been identified in both humans and mice (Paquis-Flucklinger et al. 1997; Her and Doggett 1998; Winand et al. 1998; Bocker et al. 1999; de Vries et al. 1999; Edelmann et al. 1999; Kneitz et al. 2000). The human MSH4 and MSH5 proteins, like the yeast proteins, have been shown to form a complex (Winand et al. 1998; Bocker et al. 1999). Similar to yeast Msh4, the mouse Msh4 protein localizes to discrete foci on meiotic chromosomes during the zygote and pachytene stages of meiotic prophase (Kneitz et al. 2000). Analysis of Msh4 and Msh5 knockout mice has demonstrated an important role for the proteins in chromosome synopsis (de Vries et al. 1999; Edelmann et al. 1999; Kneitz et al. 2000). Knockout mice are sterile, and nuclei with fully synapsed chromosomes are never observed. There is a limited amount of SC formation, but this occurs in only a fraction of nuclei and often involves nonhomologous chromosomes. These observations suggest that chromosome synopsis in mice is more dependent on the Msh4-Msh5 complex than is synopsis in yeast.

**Msh4 functions in crossing over:** Several observations indicate that Msh4-Msh5-Zip3-Zip2 foci correspond to the sites of meiotic recombination events. First, the formation of these foci appears to require the initiation of meiotic recombination (Chua and Roeder 1998; Agarwal and Roeder 2000; our unpublished observations). Second, in a mutant (rad50S) in which meiotic recombination is blocked at an early stage, the Zip2 and Zip3 proteins colocalize with the Mre11 protein; furthermore, Zip3 co-immunoprecipitates with Mre11 from meiotic cell extracts (Chua and Roeder 1998; Agarwal and Roeder 2000). Mre11 interacts with Rad50 and Xrs2 to form a complex that is involved in the formation and processing of meiotic double-strand breaks (the initiators of meiotic recombination events; Usui et al. 1998). Third, the formation of axial associations (to which Zip2 localizes) requires two strand-exchange proteins, Dmc1 and Rad51 (Rockmill et al. 1995). Finally, Zip3 interacts physically with Rad51 and Rad57, a cofactor to Rad51-mediated strand exchange (Agarwal and Roeder 2000).

Other observations indicate that Msh4-Msh5-Zip3-Zip2 foci correspond specifically to the sites of reciprocal recombination events (i.e., crossovers). Null mutations in the genes encoding all four of the known components of these foci reduce crossing over two- to threefold without affecting gene conversion. Furthermore, Msh4-Msh5-Zip3-Zip2 foci, like crossovers, display interference; these complexes are nonrandomly positioned along chromosomes such that two foci rarely occur close together (Jennifer Fung and G. S. Roeder, unpublished data).

The Msh4 protein may be directly involved in crossing over. Homology to MutS proteins suggests that Msh4 has DNA-binding activity. MutS proteins bind duplex DNA, with especially high affinity for DNA containing mismatched base pairs (Modrich 1987; Kolodner and Marsischky 1999). However, at least one of the yeast MutS proteins, Msh2, also binds strongly to Holliday junctions and promotes their resolution in vitro by purified Holliday junction-cleaving enzymes (Alani et al. 1997; Marsischky et al. 1999). It is tempting to speculate that Msh4 (and/or its partner, Msh5) binds to Holliday junctions and promotes their resolution in favor of crossing over.

The number of Msh4-Msh5-Zip3-Zip2 foci observed at pachytene (~55) is significantly less than the average number of crossovers that occur in a meiotic cell (~90). One explanation for this discrepancy is that the foci are transient and asynchronous, such that not all foci are detected at any given point in time. (Note, however, that in ndt80 cells arrested at pachytene, the number
of Zip2 foci is still significantly \(< 90\).) If Msh4 does normally act at all crossover sites, then loss of Msh4 might have the result that recombination intermediates that would normally generate crossovers are randomly resolved such that only \(\sim 50\%\) result in crossing over. An alternative possibility is that only about half of all crossovers require Msh4 (and associated proteins) and serve as sites of Msh4 localization; the remaining crossovers occur by a Msh4-independent mechanism (Ross-Macdonald and Roeder 1994; Nakagawa et al. 1999; Zalevsky et al. 1999). In this case, in the absence of Msh4, all recombination intermediates that normally serve as Msh4 substrates would be resolved without crossing over.

If there is more than one pathway of crossing over, then these pathways might require different gene products. However, all observations to date suggest that all of the gene products that specifically promote meiotic crossing over act in the same pathway. Epistasis tests have shown that Msh5 and Mlh1 (and presumably Mlh3) act in the same pathway as Msh4 (Hollingsworth et al. 1995; Hunter and Borts 1997). It has been proposed that a Msh4-Msh5 dimer interacts with the Mlh1-Mlh3 dimer to form a tetramer analogous to other MutS-MutL complexes observed in eukaryotic cells (Kolodner and Marsischky 1999; Nakagawa et al. 1999). Exo1 and probably Mer3 also act in the same pathway as Msh4 (KhaZanehDari and Borts 2000; Kirkpatrick et al. 2000; TsuBouchi and Ogawa 2000; our unpublished observations). Here, we have shown that Msh4 and Zip1 are in the same epistasis group; Zip3 and Zip2 presumably also act in the Msh4-dependent pathway, since these proteins colocalize with Msh4 and Msh5. Thus, if there is a second crossover pathway, gene products that act exclusively in this pathway have yet to be identified.

The role of the Zip1 protein in crossing over has been controversial (Sym et al. 1993; Sym and Roeder 1994; Storlazzi et al. 1996; Chua and Roeder 1997; Roeder 1997). Does this SC building block also participate directly in recombination or does the defect in crossing over in the zip1 mutant result indirectly from an alteration in meiotic chromosome structure? Our analysis of Msh4 localization provides a plausible explanation for the role of Zip1 in crossing over. Since Zip1 is required for localization of Msh4 in wild-type amounts and to the correct locations, the zip1 defect in crossing over might be a consequence of a failure of Msh4 function.

**Msh4 plays a role in crossover interference:** Our data indicate that the Msh4 protein plays an important role in crossover distribution. In the absence of Msh4, crossover interference is abolished in some intervals and reduced in others. Below, we consider several possible models for how Msh4 might mediate interference. Note that these models are not mutually exclusive.

One possibility is that msh4 affects interference by impairing SC formation. As described in the Introduc-
variety. In this context, it is interesting to note that mutation of the \textit{Caenorhabditis elegans} homolog of MSH4 or MSH5 completely abolishes crossing over (Zalevsky \textit{et al.} 1999; Kelly \textit{et al.} 2000), indicating that all cros-
overs occur by a Msh4-dependent mechanism in this organism. Furthermore, interference in worms (and many other eukaryotes) is much stronger than it is in yeast. This difference in the strength of interference across species can be economically explained by propos-
ing that Msh4-dependent crossovers (which display inter-
ference) are diluted by Msh4-independent crossovers (that lack interference) in budding yeast but not in worms (or most eukaryotes).

A very different explanation for crossover interference is suggested by the counting model of Stahl and colleagues (Foss \textit{et al.} 1993; Foss and Stahl 1995). According to this model, adjacent crossovers must be separated by a specific number of noncrossover recom-
bination events (two in the case of yeast; Foss \textit{et al.} 1993). A test of this model indicated that data from budding yeast cannot be adequately explained by such a counting mechanism (Foss and Stahl 1995). How-
ever, if only a subset of crossovers exhibits interference (as proposed above), then the counting model could still apply. Counting might be due to the clustering of multiple recombination intermediates (three in yeast) to produce a single late recombination nodule, with only one intermediate in each such nodule being resolved in favor of crossing over (Stahl 1993). In the context of the counting model, Msh4 might be required for clustering, to promote crossing over within a nodule, or to designate which of the intermediates within a cluster is chosen to be resolved in favor of crossing over.

\textbf{Summary:} Msh4, a protein known to be involved in meiotic crossovers, is also required for crossover interference. Furthermore, Msh4 localizes to sites of syn-
apsis initiation and promotes SC formation. Msh4 therefore adds to the growing list of proteins that promote both interference and synapsis, providing support for the hypothesis that the SC is required for interference.

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