Cell

Single Holliday Junctions Are Intermediates of Meiotic Recombination

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DOI 10.1016/j.cell.2006.09.050

SUMMARY

Crossing-over between homologous chromosomes facilitates their accurate segregation at the first division of meiosis. Current models for crossing-over invoke an intermediate in which homologs are connected by two crossed-strand structures called Holliday junctions. Such double Holliday junctions are a prominent intermediate in *Saccharomyces cerevisiae* meiosis, where they form preferentially between homologs rather than between sister chromatids. In sharp contrast, we find that single Holliday junctions are the predominant intermediate in *Schizosaccharomyces pombe* meiosis. Furthermore, these single Holliday junctions arise preferentially between sister chromatids rather than between homologs. We show that Mus81 is required for Holliday junction resolution, providing further in vivo evidence that the structure-specific endonuclease Mus81-Eme1 is a Holliday junction resolvase. To reconcile these observations, we present a unifying recombination model applicable for both meiosis and mitosis in which single Holliday junctions arise from single- or double-strand breaks, lesions postulated by previous models to initiate recombination.

INTRODUCTION

Homologous DNA recombination has two important roles in eukaryotes. In mitotically growing cells it acts as a general repair mechanism, faithfully correcting broken DNA molecules. This is particularly important during replication, when DNA breaks are believed to arise frequently. Homologous recombination also plays a specific role in meiosis when it both promotes genetic diversity in gametes and helps ensure the correct segregation of homologous chromosomes during the first meiotic division (MI). Two distinct products of recombination are observed genetically: gene conversions and crossovers. Gene conversion is the nonreciprocal transfer of sequence information from one homolog to another. Crossing over is the reciprocal exchange of both DNA strands between two homologous duplexes. Gene conversion and crossing over often occur together in a single recombination event.

The current canonical model of crossing-over (Szostak et al., 1983; Sun et al., 1991) explains both gene conversions and crossovers as arising from an initiating DNA double-strand break (DSB) (Figure 1A; see Discussion for other models). The model predicts a DNA joint molecule intermediate containing two Holliday junctions, cleavage of which can produce a crossover. The predicted double Holliday junction (HJ) intermediates have been observed in the budding yeast *Saccharomyces cerevisiae* by electron microscopy (Bell and Byers, 1983) and deduced from two-dimensional (2D) gel analysis of DNA (Schwacha and Kleckner, 1995), and it has been widely assumed that double HJs are a universal precursor of crossovers. However, their existence has not, to our knowledge, been reported in any other organism.

In meiotic recombination, joint molecules can form between sister chromatids or between homologous chromosomes since either can provide the sequence homology needed to repair a DSB. However, only interactions with the homolog can result in crossovers that reassort genetic information and aid correct segregation of chromosomes at MI. Consistent with the importance of interhomolog events, the results of a study in *Locusta migratoria* suggested that interhomolog crossovers outnumber sister chromatid exchanges as visualized by differential BrdU staining (Tease and Jones, 1979). More directly, in budding yeast it was observed that interhomolog joint molecules predominate over intersister joint molecules (Schwacha and Kleckner, 1994, 1997). To our knowledge, budding yeast is the only organism in which this question has been directly addressed, and therefore the universality of interhomolog bias is untested.

Resolution of Holliday junctions is expected to be essential for the generation of crossovers. However, despite

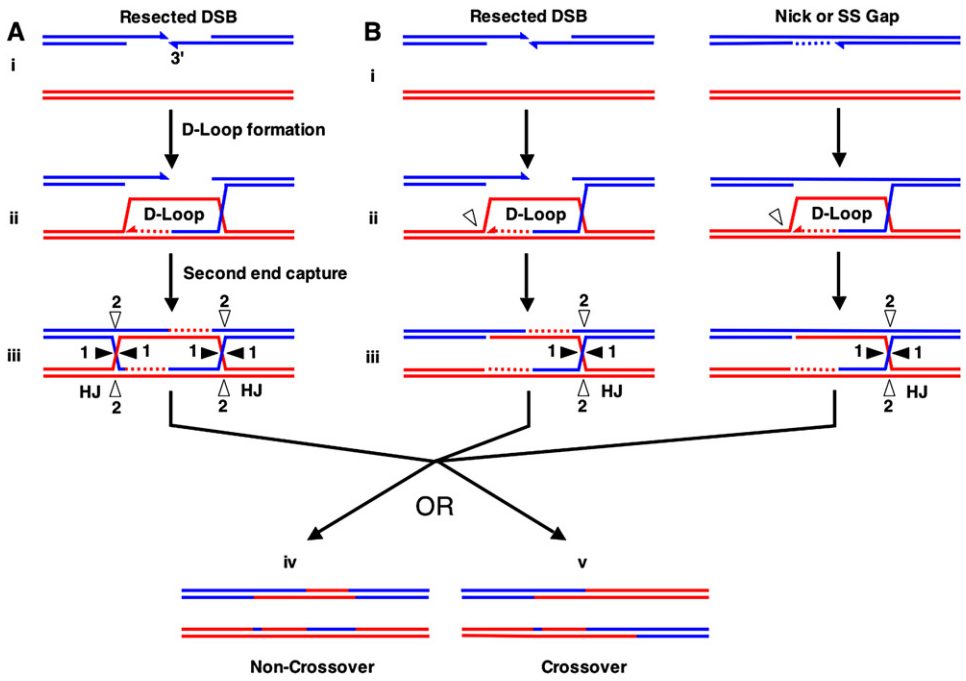


Figure 1. Recombination Models

(A) A double-strand break (DSB) repair model of recombination (after Szostak et al., 1983; Sun et al., 1991). (i) A DSB is formed and processed to give 3′ single-stranded ends. (ii) Invasion of one DNA end into an intact homologous duplex forms a D loop. (iii) The second end anneals to the left side of the D loop, and branch migration at both ends of the D loop forms a double Holliday junction (HJ). Gaps and strand discontinuities are repaired by DNA synthesis and ligation. Mismatch correction can lead to gene conversion. Each HJ can be resolved by strand cleavage in orientation 1 or 2; if orientation is random, noncrossover and crossover products are formed at equal frequency. In this figure the left HJ is cleaved in orientation 1. (iv) Cleavage of the right HJ in orientation 1 produces a noncrossover, whereas (v) cleavage in orientation 2 produces a crossover. An alternative pathway for noncrossover formation involving synthesis-dependent strand annealing (SDSA) has been proposed (Allers and Lichten, 2001; Hunter and Kleckner, 2001).

(B) A unifying recombination model initiated by a DSB or a single-strand nick and proceeding through a single HJ (after Radding, 1982; Szostak et al., 1983). (i) Recombination is initiated from a DSB (left) or a nick or single-strand gap (right). (ii) DNA unwound from the DSB or the nick or gap produces a D loop as in (A). In contrast to (A), the D loop is cleaved at the left end, and (iii) the newly generated end anneals with the duplex initially cleaved. Mismatch correction and HJ resolution produce gene conversion without (iv) or with (v) crossing-over as in (A).

the isolation of eukaryotic nuclear protein fractions with resolvase activity (e.g., Constantinou et al., 2001), the identity of eukaryotic nuclear resolvases remained elusive for many years. However, in 2001 it was reported that, in the fission yeast *S. pombe*, mutations in the *mus81* gene result in the phenotypes expected of a meiotic HJ resolvase (Boddy et al., 2001). In meiotic crosses of *mus81* mutants, very few viable spores are produced, but, among these, crossovers are greatly reduced, while there is little effect on gene conversion (Boddy et al., 2001; Smith et al., 2003; Osman et al., 2003). The phenotypes of *mus81* mutants can be suppressed by expression of a bacterial HJ resolvase (Boddy et al., 2001; Osman et al., 2003; Smith et al., 2003). Mus81 with its partner protein Eme1, partially purified from fission yeast, can cleave HJs and closely related DNA structures; this cleavage is abolished by amino acid replacements in the putative nuclease active site, indicating that Mus81-Eme1 participates directly in the cleavage (Boddy et al., 2001; Gaillard et al., 2003; Osman et al., 2003). Together, these results have implicated Mus81-Eme1 as an important meiotic HJ resolvase. It is possible, however, that the reduced crossover frequencies seen in the few viable spores are not representative of the whole population of meiotic events.

We examined the nature and processing of recombination intermediates in fission yeast and measured the frequency of crossovers in the whole meiotic population, not just among viable spores. To do so, we required a locus with high levels of recombination in a short interval that would allow us to observe recombination intermediates and products at high frequency using physical techniques. Apart from budding yeast, fission yeast is the only organism in which meiotic DSBs, which can initiate recombination, have been directly observed (Cervantes et al., 2000). We previously characterized one of the strongest sites of DSB formation, the *mbs1* locus of chromosome 1, and showed it is also a hotspot for gene conversion and crossing over (Young et al., 2002; Cromie et al., 2005).

Here we use physical assays of DNA directed to the *mbs1* locus to address four questions. First, is Mus81 required for crossovers in the bulk meiotic population as well

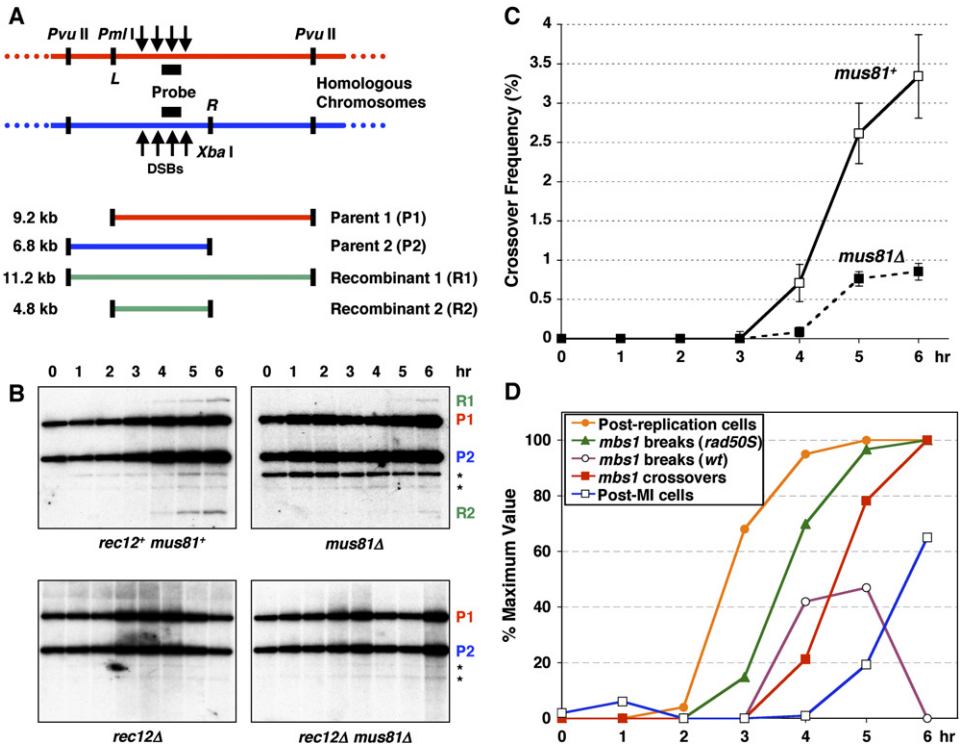


Figure 2. In a Physical Assay, Meiotic Crossovers Are Greatly Reduced in a *mus81* Mutant

(A) The *mbs1* region of chromosome I. PvuII, PmlI (*L*), and XbaI (*R*) digestion and probing, as shown, reveal two parental (9.2 and 6.8 kb) and two recombinant (11.2 and 4.8 kb) fragments.

(B) Recombinant DNA fragments arise during meiosis. DNA from meiotic time courses of strains GP5086 (*rec12*$^+$ *mus81*$^+$), GP5082 (*mus81Δ*), GP5659 (*rec12Δ*), and GP5662 (*rec12Δ mus81Δ*) was isolated and analyzed by gel electrophoresis and Southern blotting with digestion and probing at *mbs1* as in (A). Asterisks indicate crosshybridizing DNA not specific to meiosis.

(C) Crossover frequencies, calculated from PhosphorImager analysis of Southern blots as in (B), equal 2 × R2/total. Values are mean ± SEM (n ≥ 3). In *rec12Δ* or *rec12Δ mus81Δ,* R2 was undetectable (<0.4%).

(D) Timing of meiotic replication, DSB formation, crossing-over, and the meiosis I division. Percentages of GP5086 (*rec12*$^+$ *mus81*$^+$) cells with replicated DNA were quantitated using flow cytometry. Transient (GP5086 [*rad50*$^+$]) and accumulated (GP5411 [*rad50S*]) meiotic DSBs were quantitated from Southern blots using a maximum value of 10% for GP5086. Crossover formation in strain GP5086 (*mus81*$^+$ *rec12*$^+$) is from Figure 2C. All data above are the average of at least two independent inductions. The percentage of GP5086 cells with 1 (pre-MI) and >1 (post-MI) Hoechst 33342-staining bodies (nuclei) was determined from >100 scorable cells for each time point with 100% as the maximum value.

as among viable spores? Second, can joint molecules be detected, e.g., in *mus81* mutants, at a meiotic recombination hotspot? Third, what proportion of meiotic recombination intermediates is the result of intersister versus interhomolog recombination? Fourth, what is the structure of meiotic recombination intermediates in fission yeast? Do they contain double Holliday junctions?

RESULTS

Total Meiotic Crossovers, Assayed Physically at the *mbs1* Recombination Hotspot, Require Rec12 and Mus81

To measure meiotic crossovers physically, we constructed diploids heterozygous for restriction sites (*L* and *R*) flanking the *mbs1* recombination hotspot (Figure 2A). Crossovers measured in meiotic tetrads occur at high frequency (~5% of chromatids) in the 4.8 kb interval between these two markers (Cromie et al., 2005). We expected to detect crossover-specific fragments by probing DNA for the *mbs1* region after digestion with appropriate restriction enzymes (Figure 2A). We concentrated on the smaller crossover-specific fragment (recombinant 2), as the larger fragment (recombinant 1) could arise from partial digestion of parental molecules.

When we examined DNA from a meiotic time course of a wild-type (*rec12*$^+$ *mus81*$^+$) strain, the recombinant 2 fragment was absent at the beginning of the meiotic induction but began to appear 3–4 hr later and accumulated to a final maximum level of ~3.5% of total DNA (Figures 2B and 2C).This species appeared after DNA replication and DSB formation, but before MI (Figures 2D, S1, and S2A), as expected for crossovers. Its accumulation, in contrast to the transient meiotic DSBs in the same strain, reflects

crossovers being final products of the recombination pathway. The final frequency of the physical crossover products was comparable to the crossover level when measured genetically (Cromie et al., 2005). Similar results of physical assays were seen using a diploid in which the *L* and *R* markers were coupled rather than oriented in repulsion (unpublished data).

We next examined the dependence of crossing over on Mus81. Based on the quantitation of the recombinant 2 fragment, crossovers appeared in *mus81* mutant diploids with the same timing as in *mus81*$^+$, but they accumulated to a much lower level, ~0.8% rather than ~3.5% (Figures 2B and 2C and see below). This demonstrates that crossovers are reduced in the whole population of meiotic cells in a *mus81* mutant, not just in the ~0.1% that form viable spores (Boddy et al., 2000; Osman et al., 2003; Smith et al., 2003). Meiotic replication occurred in the *mus81* mutant diploid at nearly the same time as replication in *mus81*$^+$ (Figure S1), but the high frequency of abnormal nuclei present at all time points in the *mus81* mutant precluded measurement of the timing of the meiotic divisions. However, asci were observed after 24 hr in *mus81* mutant meiosis just as in *mus81*$^+$, demonstrating that progression through meiosis did occur.

To confirm that the recombinant fragments observed in both the *mus81*$^+$ and *mus81* mutant backgrounds represented bona fide meiotic recombination products, we tested their dependence on Rec12, the *S. pombe* ortholog of Spo11, which is the active site protein that makes DSBs in *S. cerevisiae* (Keeney, 2001). Rec12 is essential for meiotic DSB formation and recombination (DeVeaux et al., 1992; Cervantes et al., 2000). The *rec12* mutation abolished recombinant DNA formation in both *mus81*$^+$ and *mus81* mutant backgrounds (Figure 2B and Figure 2C, legend), while DNA replication was essentially unaffected (Figure S1).

Although crossovers are reduced in the *mus81* mutant, meiotic DSBs form at similar frequencies as in *mus81*$^+$ cells and disappear with similar kinetics (Young et al., 2004; Figure S2). We conclude that the recombination defect in a *mus81* mutant occurs after DSB formation and disappearance but before the formation of crossovers, i.e., in the processing of joint molecules (JMs).

Rec12-Dependent Recombination Intermediates Are Detectable at DSB Hotspots and Accumulate in a *mus81* Mutant

To identify the JMs that give rise to crossovers, we used 2D gel electrophoresis. In this assay, DNA molecules separate in the first dimension based primarily on their mass and in the second dimension based on both mass and structure. Replication forks and bubbles, linear DNA, and branched molecules containing Holliday junctions (HJs) all run in diagnostic positions on such gels (Brewer and Fangman, 1987; Figure 3A). Branched molecules containing X-shaped structures, such as HJs, run as a characteristic "spike."

Analysis of 2D gels probed for DNA containing the *mbs1* locus revealed X-form molecules arising both from DNA replication and from recombination. Using DNA from a *rec12*$^+$ *mus81*$^+$ meiotic time course, we observed replication intermediates at 2–3 hr after meiotic induction (Figure 3B), as expected from flow cytometry (Figures 2D and S1). These replication intermediates included Y-shaped species but also a spike corresponding to X-form DNA. This X-form species has been seen during replication in previous studies (e.g., Segurado et al., 2003). However, X-form material was also seen at 4–5 hr (Figure 3B), when DNA replication was complete (Figure S1) and replication forks had disappeared (Figure 3B). The lack of distinctive replication structures and the correlation with the expected timing of recombination suggested that the 4 and 5 hr X-form material consisted of recombination-related JMs, i.e., homologs or sisters held together by HJs. If so, we would expect this material, but not the replication intermediates, to depend on Rec12. The X-form species at 2–3 hr did not depend on Rec12, but those at 4–5 hr did (Figures 3B and 3C). Similarly only the 2–3 hr species were seen in a *rad50S* mutant, in which meiotic DSBs are not repaired (unpublished data). Therefore, we conclude that X-form molecules seen at 4–5 hr are indeed recombination intermediates.

The Rec12-dependent JMs reached a maximum of 0.8% at 4.5 hr and then disappeared (Figures 3B and 3C). The timing of maximum JM appearance suggests that formation and resolution of these intermediates occurs between DNA replication and MI (Figures 2D and S1), as expected for recombination intermediates.

We tested if Rec12-dependent JMs accumulated in a *mus81* mutant to higher levels than in *mus81*$^+$, as expected if Mus81 is a component of an HJ resolvase. In the *mus81* mutant, X-form species were observed during replication (i.e., at 2.5 and 3 hr) and also after completion of replication at 4 hr and later (Figure 3B), similar to our observations with *mus81*$^+$ cells. Only the X-form species observed at 4 hr and later were dependent on Rec12 in the *mus81* mutant (Figure 3C), indicating that these molecules are recombination intermediates. As expected, these recombination JMs accumulated to a higher level in the *mus81* mutant than in *mus81*$^+$ (a maximum of 2.2% compared to 0.8% in *mus81*$^+$ at 4.5 hr) and persisted, although a reduction in frequency was seen at later time points (Figure 3C). In contrast, no increase in the levels of replication Y- and X-form intermediates was seen in the *mus81* mutant at 3 hr (Figure 3C and S3). We conclude that *mus81* mutants accumulate JMs and fail to produce crossovers, as predicted if JMs are resolved by Mus81-Eme1 into crossovers.

We expected that JMs would be seen at hotspots for breakage and crossing-over, but not at sites that had few meiotic DSBs. Consistent with this expectation, in the *mus81* mutant the frequencies of JMs at 5 hr were higher at the *mbs1* and *mbs2* hotspots than at similarly sized regions with few, if any, DSBs located in the same cosmids (Figure 3D; Young et al., 2002). A similar pattern was seen in *mus81*$^+$ (unpublished data).

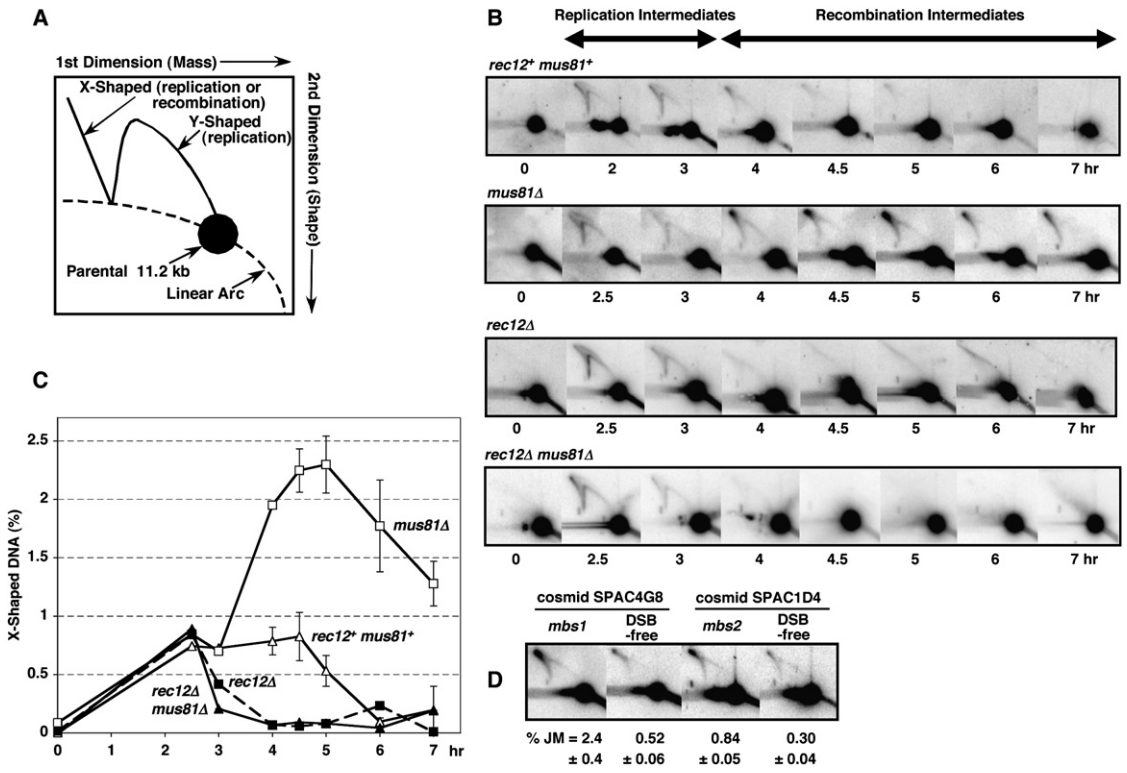


Figure 3. Recombination Intermediates Accumulate in a *mus81* Mutant and Are Most Frequent at DSB Hotspots

(A) Predicted migration of PvuII-digested *mbs1* DNA during 2D gel electrophoresis (Brewer and Fangman, 1987). Parental (linear) DNA lies on an arc of linear molecules. Y-shaped intermediates arise during replication, and X-shaped intermediates during replication or recombination.

(B) DNA from meiotic time courses of strains GP5086 (*rec12*$^+$ *mus81*$^+$), GP5082 (*mus81Δ*), GP5659 (*rec12Δ*), and GP5662 (*rec12Δ mus81Δ*) was digested with PvuII, separated by 2D gel electrophoresis, Southern blotted, and probed for *mbs1*.

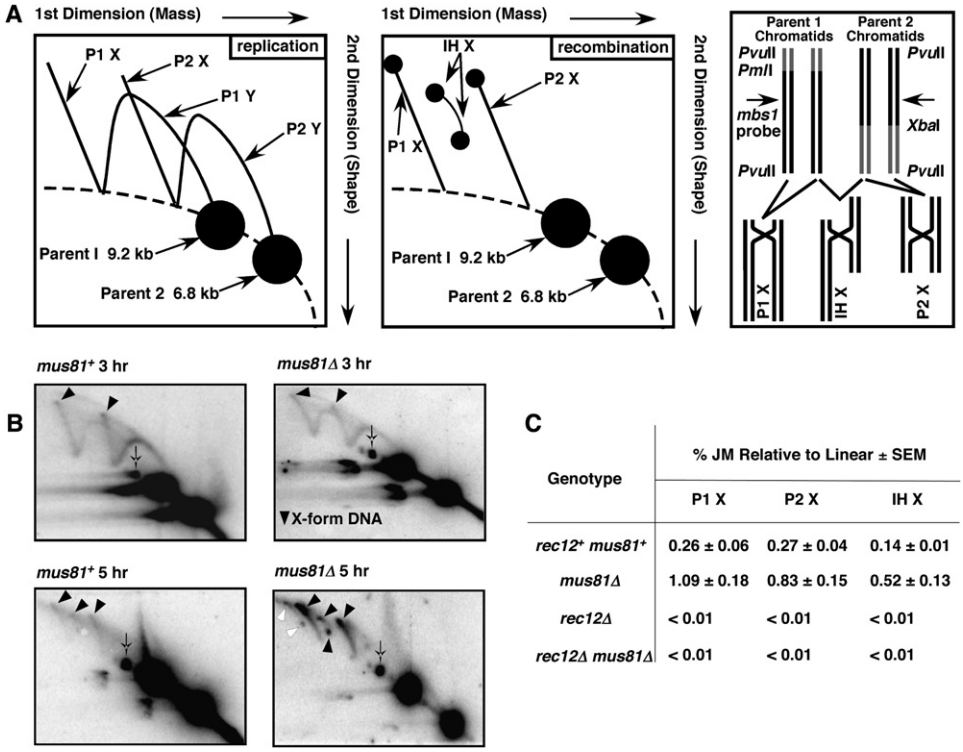
(C) Frequencies of X-form species at *mbs1*, calculated from PhosphorImager analysis of Southern blots as in (B). Values are the means of at least two independent experiments. Error bars, shown for clarity only for selected points, are SEM ($n \geq 3$) for all points except for strain GP5082 at 4 and 7 hr, where 1/2 the difference between the two values is used (n = 2).

(D) DNA isolated 5 hr after meiotic induction of strain GP5082 (*mus81Δ*) was analyzed as in (B) but probed for the *mbs1* and *mbs2* DSB hotspots and for two DSB-free intervals of comparable sizes located on the same cosmids. Restriction digestion was with PvuII (SPAC4G8 intervals) and BamHI (SPAC1D4 intervals). Frequencies of JMs are mean ± SEM (n = 3) for *mbs1* and mean ± 1/2 the difference between the two values for others.

JMs Occur More Frequently between Sister Chromatids than between Homologous Chromosomes

The presence of the heterozygous *L* and *R* markers flanking *mbs1* allowed us to determine the parental origin of JMs at *mbs1*. After digestion with PvuII, PmlI (specific for *L*), and XbaI (specific for *R*), three distinct *mbs1* JM species should be observable: intersister 1 (P1 X), intersister 2 (P2 X), and interhomolog (IH X) (Figure 4A). These three species have different masses, allowing separation in the first dimension of 2D gels.

2D gel analysis of DNA from the 5 hr time point of *mus81* mutant and *mus81*$^+$ meioses, triply digested and probed for *mbs1*, revealed multiple JM species (Figure 4B). At this time X-form DNA corresponds to recombination intermediates (see above). In both backgrounds two clear X-form spikes were present with two different masses (Figure 4B). We confirmed that these represented the two types of intersister JMs by comparing their mobilities to those of JMs from haploid strains induced to initiate meiosis (unpublished data). In haploid cells only P1 X or P2 X intermediates can form. In diploids, in addition to the two intersister X-form spikes, we observed two prominent forms lying between the intersister species (Figure 4B). Their position corresponds to the expected mass of interhomolog JMs. We believe that these molecules appear as two forms rather than one because of the asymmetric structure of the interhomolog JMs (see below and Discussion). At higher exposures a weak "tail" of species is seen joining the two prominent IH X forms. This is similar to the distribution of the intersister species, with material concentrated at the top of a weaker spike. Both the two IH X forms and the intersister X-form spikes were observed at 5 hr in *mus81* mutant and *mus81*$^+$ inductions, while at 3 hr only the intersister X-form spikes were observed (Figure 4B). This supports our conclusion that the two forms between the intersister spikes represent recombination intermediates: X-form replication intermediates (at 3 hr)



Genotype	% JM Relative to Linear ± SEM		
	P1 X	P2 X	IH X
rec12⁺ mus81⁺	0.26 ± 0.06	0.27 ± 0.04	0.14 ± 0.01
mus81Δ	1.09 ± 0.18	0.83 ± 0.15	0.52 ± 0.13
rec12Δ	< 0.01	< 0.01	< 0.01
rec12Δ mus81Δ	< 0.01	< 0.01	< 0.01

Figure 4. Intersister JMs Are More Frequent Than Interhomolog JMs, and Both Accumulate in a *mus81* Mutant

(A) Predicted migration during 2D gel electrophoresis of *mbs1*-probed DNA digested with PvuII, PmlI, and XbaI (Figure 2A). Y- and X-shaped molecules are expected during replication, but only X-shaped molecules during recombination. Intersister joint molecules (JMs) arising from either parent (P1 X or P2 X) migrate in a spike, whereas interhomolog (IH X) JMs migrate as two species joined by an arc (see Figure 5C).

(B) DNA from 3 and 5 hr after meiotic induction of strains GP5086 (*mus81⁺*) and GP5082 (*mus81Δ*) was analyzed as in (A). Black arrowheads indicate X-form JM species, and white arrowheads indicate interhomolog JMs with heteroduplex DNA preventing restriction at the *R* site. Both species are inferred to contain single HJs by their resistance to heating (Figure 5B). Partial digestion products are identified by thin arrows.

(C) Quantitation of intersister and interhomolog recombination JMs at 5 hr at *mbs1*. The frequencies of JMs were calculated by PhosphorImager analysis of Southern blots as in (B). Values are mean ± SEM ($n \geq 3$). In strains GP5659 (*rec12Δ*) and GP5662 (*rec12Δ mus81Δ*), JMs were undetectable.

should be only intersister while recombination intermediates (at 5 hr) could also be interhomolog. As expected, Y-form replication intermediates from each parent (P1 Y and P2 Y) (Figure 4A) were also seen at 3 hr (Figure 4B).

In both *mus81⁺* and *mus81* mutant cells the three X-form species at 4 hr and later depended on Rec12; i.e., they were all bona fide meiotic recombination intermediates (unpublished data). All of these recombination JMs accumulated to higher levels in the *mus81* mutant than in *mus81⁺* cells (Figure 4C). This suggests that Mus81 is required to resolve both intersister and interhomolog JMs.

In both *mus81* mutant and *mus81⁺* DNA the intersister JMs outnumbered the interhomolog molecules. In the *mus81* mutant, at 5 hr the frequency of the P1 X species was 1.1%, the P2 X species 0.8%, and the combined IH X species 0.5% (Figures 4B and 4C). The total frequency of these three species (2.4%) is almost identical to the value for the combined JMs measured after PvuII digestion (2.3%) (Figure 3C). However, together the two intersister species were approximately 4-fold more frequent than the interhomolog species. Due to low-level DSBs and HJs between *R* and the rightward PvuII site (unpublished data), the actual ratio of intersister to interhomolog JMs in the interval *L* and *R* is closer to three to one. Therefore, at the *mbs1* site of fission yeast it appears that there is a bias toward intersister recombination in contrast to the preference for interhomolog events seen in budding yeast (Schwacha and Kleckner, 1994, 1995).

JMs Contain Single, Not Double, HJs

To investigate the structure of the meiotic recombination intermediates observed at *mbs1*, we looked for evidence that they contained HJs. We did this in three ways: examining the sensitivity of the intermediates to a known HJ resolvase in vitro, testing the ability of high temperature to resolve the intermediates to linear forms by branch migration, and examining the intermediates directly by electron microscopy (EM).

In both the *mus81⁺* and *mus81* mutant backgrounds the recombination-derived JMs observed at *mbs1* were sensitive to *E. coli* RuvC enzyme, a well-characterized HJ

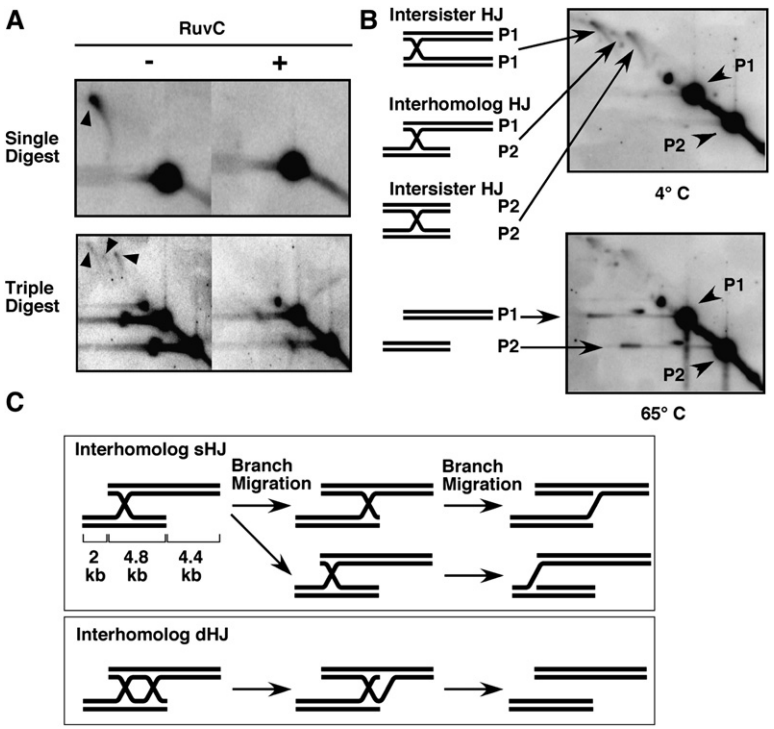


Figure 5. RuvC Treatment Resolves Both Intersister and Interhomolog JMs, but Only Intersister JMs Are Resolved to Linear Forms by Mild Heat Treatment

(A) DNA isolated 5 hr after meiotic induction of strain GP5082 (*mus81Δ*) was digested either with PvuII alone (upper panel) or with PvuII, PmlI, and XbaI (lower panel), treated with RuvC (+) or left untreated (–), separated by gel electrophoresis in two dimensions, Southern blotted, and probed for *mbs1*. Black arrows indicate intersister and interhomolog JM species.

(B) DNA isolated 5 hr after meiotic induction of strain GP5082 (*mus81Δ*) was digested with PvuII, PmlI, and XbaI, separated by gel electrophoresis in the first dimension, heated to 65°C or left at 4°C, separated in the second dimension, and analyzed as in (A).

(C) Two distinct, stable DNA structures are formed by branch migration of a single HJ in an interhomolog JM. Branch migration of a single HJ to the right (top) or left (bottom) stops when a Y junction is formed. These two structures should be unable to reverse due to high activation energy and, despite having the same mass, are expected to separate upon 2D gel electrophoresis because of their different shapes. Branch migration of a double HJ in either direction generates separate linear molecules; only rightward branch migration is shown.

resolvase (Connolly et al., 1991), while linear DNA was not. This was true both for the combined JM population (after PvuII single digestion) and the distinct intersister and interhomolog forms (after PvuII, PmlI, and XbaI triple digestion) (Figure 5A). While HJs are the preferred substrate of RuvC, it can cleave other branched DNA species, albeit with lower efficiency (Benson and West, 1994; Fogg et al., 1999). The preparation of RuvC used in Figure 5 showed a distinct preference for chemically synthesized X-shaped molecules, as expected (unpublished data). Therefore, these results indicate that the JMs are held together by HJs or perhaps other branched structures.

Incubation at high temperature causes HJs to branch-migrate and to be resolved into linear DNA when the HJs reach the ends of fully homologous DNA. We tested the ability of high-temperature incubation to resolve the intersister and interhomolog JMs formed in a *mus81* mutant. As expected, the intersister forms were largely resolved to the corresponding linear fragments (31% unresolved) (Figure 5B). However, the interhomolog material was almost entirely resistant to heat treatment (98% unresolved). We believe that this resistance is explained by the asymmetric structure of the interhomolog JMs and the presence of single, rather than double, HJs. When branch migration of a single HJ reaches the left or the right end of one duplex present in the interhomolog JM, one or another stable Y-shaped structure is produced, rather than being resolved to linear DNA (Figure 5C). These two stable structures have the same mass but different shapes. We conclude that the two spots represent these two different forms: they ran at nearly the same position in the first dimension (where mass is important), but ran differently in the second dimension (where shape is important) (Figure 4B). In contrast, interhomolog double HJs should be resolved into linear fragments by branch migration (Figure 5C).

To look more directly at the structure of total cellular JMs, we extracted DNA from a position above the arc of linear DNA, where JMs run, and examined the DNA by EM. In all preparations we saw branched molecules, including Y-shaped molecules and X-shaped molecules, with unequal arm lengths; these are most likely replication intermediates or structures derived from them. HJ recombination intermediates are expected to have two short arms of identical lengths and two long arms of identical lengths. We used this criterion to designate molecules as HJs. As in the 2D gel analyses above, HJs were seen by EM in DNA prepared 5 hr after meiotic induction of *mus81*$^{+}$ and *mus81* mutant strains (Figures 6A and S4–S7), but not in DNA from the *rec12 mus81* double mutant. As expected from Southern blot analysis (Figure 3C), HJs appeared more abundant in DNA from *mus81* mutants than from *mus81*$^{+}$, comprising ~20% and ~1% of observed branched molecules respectively (Table S1).

In accord with the 2D gel analyses above, the great majority of the JMs that we observed by EM in DNA from fission yeast meiosis contained single, rather than double, HJs (Figures 6A and S4–S7; Table S1). In the *mus81*

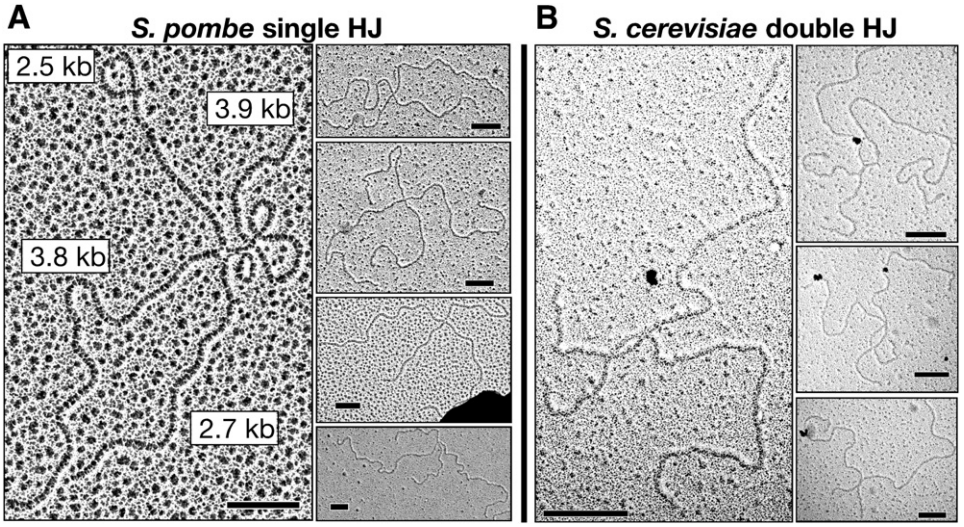


Figure 6. *S. pombe* Recombination JMs Contain Single HJs

(A) Electron micrographs of HJ-containing molecules isolated from *S. pombe* strains GP5082 *(mus81Δ)* (main picture and three upper right) and GP5086 (*rec12*$^{+}$ *mus81*$^{+}$) (bottom right) 5 hr after meiotic induction. Note the partially denatured (open-centered) HJs in the left and third (from top) right panels. Scale bars are 0.2 μm.

(B) Electron micrographs of HJ-containing molecules isolated from *S. cerevisiae* strains NHY1226 (*SPO11 MUS81 ndt80*) and NHY1296 (*SPO11 MUS81 NDT80*) 4.5 hr after meiotic induction. Note the separate (double) HJs in the left and middle right panels and the fused (double) HJs in the upper and lower right panels. Scale bars are 0.2 μm.

mutant 32/38 HJs, and in *mus81*$^{+}$ 4/4 HJs, were single. Some of these HJs had an open center at the crossover position (Figures 6A and S5), unambiguously identifying them as single HJs. In contrast, budding yeast meiotic DNA prepared and examined in the same manner contained a majority of clear double HJ structures (21/26) (Figure 6B); the remainder were single HJs. Another difference between the budding yeast and fission yeast HJs was the distance between the junctions in the double HJs (Table S2). In 20 molecules of budding yeast DNA this distance ranged from 0.1 to 0.5 kb. In contrast, among the six double HJs observed in fission yeast, three were separated by distances much larger than those observed in budding yeast (1.6, 2.1, and 2.6 kb). The other three fission yeast double HJs had 0.2 to 0.5 kb separating the individual HJs. This suggests that at least some of the fission yeast double HJs represent a different class than those seen in budding yeast and perhaps arose from two closely spaced, independent recombination events (see Discussion). We conclude that, in contrast to budding yeast, meiotic recombination in fission yeast proceeds primarily through single HJs rather than double HJs.

DISCUSSION

To investigate the mechanism of homologous recombination in fission yeast, we examined the intermediates and products of meiotic recombination using physical methods. We concentrated on recombination at *mbs1*, a naturally occurring hotspot of DSBs and recombination. To analyze recombination at *mbs1*, we made only two single base pair mutations so that the chromosomes were as close to wild-type as possible. Our results demonstrate that the interhomolog recombination bias and the double HJ structure of recombination intermediates seen in budding yeast meiosis are not universal. Instead, we saw predominantly single HJs and a strong bias to intersister recombination. Our results also provide additional evidence that Mus81 is a component of a fission yeast meiotic HJ resolvase.

JMs Are Intermediates of Meiotic Homologous Recombination

By 2D gel analysis we observed meiotic JMs with two characteristics expected of recombination intermediates: a mass greater than that of their linear parents and an "X" structure. Are these JMs genuine recombination intermediates or the products of a side pathway? Genuine intermediates of biochemical pathways, such as recombination, meet four criteria, and the JMs observed in this study meet all four of these criteria. First, there should be mutations blocking product formation, which cause the molecules to accumulate. In this study, *mus81* mutants are crossover defective and caused accumulation of the JMs (Smith et al., 2003; Osman et al., 2003; Figures 3B and 3C). Second, there should be mutations acting earlier in the relevant pathway that prevent the molecules from forming. *rec12* mutations are epistatic to *mus81* (Boddy et al., 2001), and here we observe that *rec12* mutants were recombination defective and lacked JMs (DeVeaux et al., 1992; Figure 2C, legend, and Figure 3C). Third, the timing of appearance and disappearance of

the molecules should be consistent with other features of the pathway. We saw that Rec12-dependent JMs appeared after DNA replication and DSB formation and before MI, i.e., at the time of meiotic recombination (Figures 2D, 3C, and S1). Finally, the molecules should be convertible into the final product of the relevant pathway. We cannot introduce the JMs into cells and follow their conversion to crossovers, but in vitro they are substrates of the *E. coli* HJ resolvase RuvC (Connolly et al., 1991; Figure 5A), and in vivo another *E. coli* HJ resolvase RusA can substitute for Mus81-Eme1 in production of crossovers (Boddy et al., 2001; Osman et al., 2003; Smith et al., 2003). Meeting these four criteria strongly suggests that the JMs we observe are genuine intermediates of meiotic homologous recombination.

Structure of Meiotic Recombination Intermediates

Perhaps our most surprising conclusion is that in fission yeast the great majority of recombination intermediates contain unexpected meiotic recombination structures: single HJs. A preponderance of single HJs was inferred from the behavior of interhomolog JMs on 2D gels (Figures 4B and 5B) and was demonstrated directly by the observation of mostly single HJs, rather than double HJs, by EM (Figures 6A and S4–S7; Table S1). The junctions in these molecules may be fully intact HJs or may have contained a single-strand nick at their inception; in *mus81*$^{+}$ cells such nicked HJs might be quickly resolved into products (Osman et al., 2003; Hollingsworth and Brill, 2004). A nick should pose no impediment to branch migration. Thus, a hypothetical nicked HJ, if unresolved, could quickly be converted into an intact HJ. Our results are consistent with the HJs observed here being either nicked or intact.

If the intersister bias observed at *mbs1* extends throughout the genome, then most of the HJs observed by EM are likely to be intersister. To our knowledge there is no difference in the mechanism of intersister and interhomolog recombination. Consequently, it is simplest to assume that interhomolog as well as intersister JMs contain mostly single HJs, as indicated by the analysis of interhomolog JMs at *mbs1* (Figure 5B).

The small number of double HJs observed in fission yeast by EM argues strongly against their being major recombination intermediates in this organism, unless one HJ is almost immediately resolved or the junctions are so widely spaced (>5 kb) as to produce mostly single HJs after restriction enzyme digestion. Because single HJs were seen by EM in the wild-type as well as in the *mus81* mutant, it seems unlikely that closely spaced double HJs in wild-type, similar to those seen in *S. cerevisiae*, become widely spaced in the absence of Mus81. We believe the possibility of very widely spaced HJs can be discounted for several reasons. All markers between the junctions of a double HJ could be co-converted, yet the gene conversion tracts observed in fission yeast, like those in budding yeast, generally are continuous and span <1 kb (Grimm et al., 1994; Cromie et al., 2005). Two interhomolog species appear to reflect heteroduplex DNA at *R* (Figure 4B); these species were rare, indicating that branch migration from *mbs1* across *R* was uncommon. These data indicate that heteroduplex DNA and associated HJs lie predominantly between *L* and *R*, which is consistent with most gene conversion events around *mbs1* being between *L* and *R* (Cromie et al., 2005). Finally, the mean length (± SD) of the shorter arms of the single HJs observed by EM was 2.4 ± 1.0 kb, indicating that a second HJ was not located within that distance. Branch migration of HJs beyond this range during sample preparation is unlikely since the DNA for EM analysis contained psoralen cross-links ~1 kb apart (unpublished data); any branch migration during preparation would have been limited to about that distance.

The few double HJs seen in fission yeast might arise from two closely spaced, independent recombination events. Such events are expected in fission yeast due to the occurrence of DSBs in ~1–2 kb clusters at hotspot sites (Cromie et al., 2005; Steiner et al., 2002) and the absence of crossover interference (Munz, 1994), which allows two independent, closely spaced HJs to arise. The more variable, and sometimes much larger, separation of the individual junctions from fission yeast double HJs, compared to those from budding yeast (Table S2), supports this notion. The rare fission yeast double HJ molecules with junction separations similar to those in budding yeast may also arise from two independent events or may represent a minor pathway that utilizes double HJs as intermediates in individual recombination events.

In contrast to fission yeast, in meiotic DNA from budding yeast we observed by EM a majority of double HJs (Figure 6B), along with a significant number of single HJs, as reported previously by Bell and Byers (1983). Single strands with a length indicative of double HJs, not single HJs, were seen in JMs from budding yeast (Schwacha and Kleckner, 1995). The detection threshold of this assay, however, does not preclude a significant fraction of single HJs. Budding yeast appears to have several pathways for generating meiotic crossovers (de los Santos et al., 2003; Argueso et al., 2004); the major pathway may involve double HJs and minor pathway single HJs. In contrast, fission yeast may have only one pathway of crossing-over that is dependent on Mus81-Eme1 and involves single HJs.

The model of Szostak et al. (1983) as modified by Sun et al. (1991) predicts the existence of double HJs because an HJ is formed by each end of the initiating DSB (Figure 1A). To produce a single HJ, the two ends would have to behave differently so that only one would produce an HJ. In fact, in the model of Szostak et al. (1983) the two ends of the DSB do not behave identically; only one end invades a duplex, while the other end is "captured" by annealing of single strands. One of these processes could generate an HJ and the other a different structure (see below). The difference in behavior of the two ends could be a simple matter of timing, e.g., the first end to find homology could carry out strand invasion and then the other end

would get "captured." Alternatively, recent evidence suggests that the two ends of the DSB are processed differently at an early stage, when Spo11 is removed (Neale et al., 2005). This could actively direct the two ends toward different biochemical events.

In Figure 1B we propose a recombination model that is initiated by a DSB but has a single HJ as a recombination intermediate. As in the model of Szostak et al. (1983), the first DNA end generates a D loop with an HJ at the right end in Figure 1B. Unlike the model of Szostak et al. (1983), the left end of the D loop is cut before second end capture. This results in a JM containing a single HJ. We propose that Mus81 is required for resolution only of the single HJ and that the D loop is cut by a different enzyme. In accord with this proposal, we observed accumulated HJs but not D loops in a *mus81* mutant. Our observation of single HJs by EM suggests that the putative D loop cleavage is more rapid than HJ resolution. Interestingly, this pathway can accommodate recombination initiated by single-strand nicks, which have been proposed as recombinogenic lesions in many previous models (e.g., Holliday, 1964; Meselson and Radding, 1975; Radding, 1982), and which may account for the frequent crossovers in intervals with few or no observed DSBs in fission yeast (Young et al., 2002; Cromie et al., 2005).

Interhomolog versus Intersister Bias in Homologous Recombination

Our second surprising conclusion is that in fission yeast meiosis intersister recombination is preferred over interhomolog recombination. In fission yeast, meiotic intersister recombination occurs at a significant frequency (Schuchert and Kohli, 1988), but no direct comparison of intersister versus interhomolog frequencies has been made before now. Based on the relative frequency of JMs at the *mbs1* recombination hotspot, intersister events outnumber interhomolog events by ~3 to 1 (Figure 4C and Results). This reverses the bias toward interhomolog JMs seen in budding yeast (Schwacha and Kleckner, 1994, 1997). Intersister bias at *mbs1* is also supported by genetic data. Since 80% of interhomolog events (conversions) at *mbs1* produce crossovers (Cromie et al., 2005), 3.5% crossing-over at *mbs1* (Figure 2C) would require only 2.2% DSBs (2 × 2.2% × 0.8 = 3.5%). However, we observe 10%–11% breakage at *mbs1* (Young et al., 2002; Figure S2B), implying that ~80% of *mbs1* DSBs undergo sister chromatid repair, which is consistent with our physical analysis.

An interhomolog bias utilizes recombination more effectively with respect to chromosome segregation and reassortment of genetic information, as these are promoted only by interhomolog recombination. Hence, it has been widely assumed that interhomolog bias is universal, even though prior to this study budding yeast was the only organism to our knowledge in which the question had been directly addressed. Despite this argument, intersister events may be favored by the close proximity of sister chromatids. The interhomolog bias seen in budding yeast could reflect an active system overcoming a mechanistic intersister bias, which may be identical to the barrier to intersister recombination seen in that organism (Schwacha and Kleckner, 1997; Niu et al., 2005). This active system is presumably absent from fission yeast. One well-known example of a regulation of recombination in budding yeast, but not in fission yeast, is crossover interference. Interference may result from a mechanism to ensure that even the small chromosomes of budding yeast, which has 16 chromosomes, receive at least one crossover and undergo proper MI segregation (Roeder, 1997; Hillers, 2004). In contrast, fission yeast, with three long chromosomes, achieves the same goal by simple random distribution of many (10–20) crossovers per chromosome. Interhomolog bias in budding yeast may be a further mechanism to ensure at least one interhomolog crossover per short chromosome.

Mus81 Is Required for Meiotic HJ Resolution

Using a physical assay, we showed that in the absence of Mus81 crossovers are greatly reduced at the *mbs1* recombination hotspot. A major advantage of physical assays of recombination is the ability to characterize the whole population of meiotic cells. This is helpful in backgrounds, such as a *mus81* mutant, where viable spore yield is low and events in those viable spores may be atypical. Our physical assay showed that in a *mus81* mutant, crossovers are reduced in the whole population of meiotic cells (Figure 2C). In addition, we showed by 2D gel analysis and EM that JMs containing HJs accumulate in a *mus81* mutant during meiosis (Figure 3C and Table S1). HJ-like structures also accumulate in *mus81* mutants during mitotic replication of the highly repeated rDNA (Noguchi et al., 2004). Together with previous results (see Introduction), these data provide strong evidence that Mus81 is required for HJ resolution.

The frequency of interhomolog JMs in the *mus81* mutant (0.5%) is too low to explain the ~3.5% crossing-over seen at *mbs1* (Figure 2C). However, this assumes that in a *mus81* mutant HJs accumulate without any loss. In fact, JMs decline at late time points in a *mus81* mutant (Figure 3C). It is unclear if this represents an alternative, minor HJ resolution pathway, perhaps to noncrossovers, or simple deterioration of unresolved JMs in the cell. Therefore, it is likely that the true cumulative frequency of both intersister and interhomolog JMs is greater than that seen in a *mus81* mutant.

The effect of a *mus81* mutation on crossover frequency using our physical assay was somewhat less than that seen genetically among viable spores. Instead of a 20- to 90-fold reduction in crossover frequency (Osman et al., 2003; Smith et al., 2003) we observed only a 4-fold reduction (Figure 2C). The genetic data may overestimate the effect of the *mus81* mutation in the total population: viable spores may have an unusually low number of DSBs and hence fewer potentially lethal unresolved HJs. Alternatively, the physical assay may underestimate the effect of the *mus81* mutation: genetic analysis showed

that 14% of exchanges between the *L* and *R* markers involved conversions of *L* or *R* and were not simple crossovers (Cromie et al., 2005). Since *mus81* mutations have little effect on gene conversion (Osman et al., 2003; Smith et al., 2003), the residual recombinant DNA observed in *mus81* mutants using our physical assay may reflect gene conversions of *L* or *R*, and crossovers may be nearly abolished.

Mus81 is conserved across a wide range of eukaryotes, suggesting that it has a conserved function. However, the meiotic phenotype of *mus81* mutants is different in fission yeast than in other organisms examined, i.e., budding yeast and mice. In budding yeast, *mus81* mutants display somewhat reduced spore viability but only a modest reduction in crossover frequency (Interthal and Heyer, 2000; de los Santos et al., 2003), and *mus81*$^{-/-}$ mice are fertile (McPherson et al., 2004; Dendouga et al., 2005). Differences between the two yeast species may reflect the presence of at least one other crossover pathway in budding yeast (requiring Msh4-Msh5) that is absent in fission yeast. Mutations in budding yeast *msh4-msh5* and *mus81-mms4* have additive effects on crossover frequency (de los Santos et al., 2003; Argueso et al., 2004), suggesting that Mus81 contributes to a specific subset of HJ resolution events. Msh4-Msh5 homologs are found in mice, and multiple pathways of HJ resolution could reconcile the fertility of *mus81*$^{-/-}$ mice with a hypothetical role for Mus81 in meiotic HJ resolution in mice. In contrast to the meiotic phenotypes, the mitotic phenotypes of *mus81* mutants are very similar in budding and fission yeast, e.g., high sensitivity to agents such as camptothecin that are believed to cause DSBs at replication forks (Doe et al., 2002; Vance and Wilson, 2002). Broken replication forks are likely to have only a single duplex end and may have to be repaired using an HJ intermediate. In contrast, DSBs caused by ionizing radiation have two ends and can use the synthesis-dependent strand annealing (SDSA) recombination pathway to repair breaks without generating HJs. Budding yeast *mus81* mutants are not hypersensitive to ionizing radiation (Interthal and Heyer, 2000). Therefore, the mitotic phenotypes of *mus81* mutants in budding yeast and fission yeast are both consistent with a role for Mus81 in mitotic HJ resolution. The additional Msh4-Msh5-dependent crossover pathway in budding yeast and mice is meiosis specific (Kunz and Schar, 2004; Her et al., 2001). Mus81 may be a universal eukaryotic mitotic HJ resolvase, while additional meiosis-specific resolution pathways may exist in some organisms but not in others, such as fission yeast.

Conclusion

In this study we show that budding and fission yeast differ with respect to two major features of meiotic recombination. The interhomolog bias in budding yeast contrasts with intersister bias in fission yeast. The predominantly double HJ intermediates in budding yeast are mostly or entirely replaced by single HJs in fission yeast. The budding yeast features were assumed to represent a universal paradigm for meiotic recombination, which is a view that is no longer tenable. Given the different behavior of the only two organisms studied in these regards to date, it will be interesting to see whether other organisms resemble one of these or display their own novel features.

EXPERIMENTAL PROCEDURES

pat1-114 strains described in the Supplemental Data were thermally induced for meiosis and analyzed for DNA content by flow cytometry as described by Cervantes et al. (2000). Cells imbedded in agarose plugs were lysed with enzymes and treated with Proteinase K and RNase A; the DNA was digested with appropriate restriction enzymes and analyzed by gel electrophoresis and Southern blot hybridization as described by Young et al. (2002) and detailed in the figure legends and Supplemental Data. Psoralen-crosslinked DNA (Schwacha and Kleckner, 1994) was extracted from agarose gels and analyzed by EM as detailed in Supplemental Data. Inclusion of psoralen crosslinking had no discernable effect on DNA analyzed by gel electrophoresis (Figure S8).

Supplemental Data

Supplemental Data include Experimental Procedures, References, eight figures, and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1167/DC1/.

ACKNOWLEDGMENTS

We are grateful to Ken Marians (Memorial Sloan-Kettering Cancer Center) for the kind gift of RuvC enzyme; Bonny Brewer (University of Washington) and Michael Lichten (National Cancer Institute) for helpful discussions about our results; and Sue Amundsen and Luther Davis for helpful comments on the manuscript. This work was supported by NIH research grants GM031693 to G.R.S. and GM074223 to N.H.

Received: May 9, 2006
Revised: August 18, 2006
Accepted: September 25, 2006
Published: December 14, 2006

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