Note

Fission Yeast Mus81•Eme1 Holliday Junction Resolvase Is Required for Meiotic Crossing Over but Not for Gene Conversion

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ABSTRACT

Most models of homologous recombination invoke cleavage of Holliday junctions to explain crossing over. The Mus81•Eme1 endonuclease from fission yeast and humans cleaves Holliday junctions and other branched DNA structures, leaving its physiological substrate uncertain. We report here that *Schizosaccharomyces pombe mus81* mutants have normal or elevated frequencies of gene conversion but 20- to 100-fold reduced frequencies of crossing over. Thus, gene conversion and crossing over can be genetically separated, and Mus81 is required for crossing over, supporting the hypothesis that the fission yeast Mus81•Eme1 protein complex resolves Holliday junctions in meiotic cells.

DURING meiosis, homologous recombination serves at least two purposes—to increase genetic diversity, upon which natural selection can act to further evolution, and to provide physical connections between homologs to aid their reductional segregation critical to forming viable haploid gametes. Two types of homologous recombination occur during meiosis. Gene conversion, or nonreciprocal recombination, is the primary mechanism of recombination of alleles in the same gene, whereas crossing over, or reciprocal recombination, predominates in recombination between genes (for a review see Whitehouse 1982).

Models of homologous recombination have usually attempted to explain conversion and crossing over by a single initiating mechanism with alternative outcomes. Central to these models is the observation that convertants have a crossover for flanking markers more frequently than do chromosomes in the total population. This statistical association, which can range from 15 to 80% depending on the genetic interval and organism examined (Whitehouse 1982), suggests a mechanistic association that is incorporated into most models. For example, the model of Holliday (1964) proposed the formation of hybrid DNA, containing one DNA strand from each of the two parental duplexes, and a reciprocal cross-strand connection between homologs, called the Holliday junction, adjacent to the hybrid DNA. If the hybrid DNA covers one or more markers within a gene, appropriate correction of base mismatches would give rise to one but not the reciprocal recombinant type, *i.e.*, conversion. In this model the cross-strand connection could be resolved by appropriate cutting of the Holliday junction, swapping of DNA strand ends, and ligations. This resolution could give rise either to reciprocal recombinants for markers flanking the hybrid DNA and Holliday junction, *i.e.*, crossovers, or to parental arrangements of these markers, *i.e.*, noncrossovers. Either type of resolution could be accompanied by conversion. Many subsequent models, such as those of Meselson and Radding (1975) and Szostak *et al.* (1983), also employed these features to account for conversion, crossing over, and their association, although the model of Szostak *et al.* (1983) proposed, in addition, the repair of DNA double-strand gaps to account for conversion.

Support for Holliday junctions and their resolution in recombination came from the identification of enzymes able to resolve such junctions. The first was endonuclease VII of phage T4 (Mizuuchi *et al.* 1982). Subsequent studies showed, however, that this enzyme can also cleave DNA molecules with a variety of single- and double-strand branches or base mismatches, leaving uncertain its role in recombination (Kemper 1998). The *Escherichia coli* RuvC enzyme has high specificity for cleavage of Holliday junctions, but mutants lacking RuvC are not strongly deficient in conjugational recombination unless they also lack the branch-migration enzyme RecG (Lloyd 1991; Benson and West 1994). In eukaryotes, mitochondrial resolvases have been isolated from several species, but where tested they have no significant

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role in recombination of nuclear genes (Kemper 1998). A nuclear activity from calf testis and human cells has also been described (Constantinou *et al.* 2001, 2002), but the identity of the responsible protein(s) has not been reported.

Recently, a complex containing the Mus81 polypeptide from the fission yeast *Schizosaccharomyces pombe* (Boddy *et al.* 2001), the budding yeast *Saccharomyces cerevisiae* (Kaliraman *et al.* 2001), and human cells (Chen *et al.* 2001; Constantinou *et al.* 2002; Ciccia *et al.* 2003) has been reported to cleave a variety of branched DNA molecules. Endogenous Mus81 complexes from *S. pombe* and humans readily cleave Holliday junctions, unlike the "recombinant" enzymes synthesized in *E. coli* and encoded by the *S. cerevisiae* or human genes *mus81* and their partner *mms4* or *eme1*. Recent studies have revealed that the endogenous and recombinant complexes of fission yeast Mus81•Eme1 differ primarily in their ability to make the first cut in a nick-and-counternick mechanism of Holliday junction resolution (Gaillard *et al.* 2003), suggesting that the recombinant enzymes lack a post-translational modification or additional subunit(s) necessary to initiate cleavage of Holliday junctions.

S. pombe mutants lacking Mus81 or its partner polypeptide Eme1 are severely deficient in meiosis; they form few viable spores and their DNA often remains as a single mass within the meiotic ascus (Boddy *et al.* 2001). These deficiencies are largely overcome by a mutation in *rec6* or *rec12*, which blocks the formation of meiotic double-strand breaks apparently necessary for meiotic recombination (Cervantes *et al.* 2000; Boddy *et al.* 2001). These observations suggest that Mus81•Eme1 is required for meiotic recombination after the formation of double-strand breaks but before the segregation of homologs. To test this hypothesis, we have assayed *S. pombe mus81* mutants for meiotic recombination: if Mus81•Eme1 is required for the resolution of Holliday junctions, these mutants would, within the context of the models above, be deficient for crossing over. They might, however, be proficient for hybrid DNA formation and gene conversion. The data reported here support this hypothesis.

To test the requirement for Mus81 in crossing over, we examined recombination in large intergenic intervals, one on each of the three chromosomes. We conducted standard matings of appropriately marked haploid strains on EMM2 medium, on which cells of opposite mating type mate and undergo meiosis after few or no mitotic divisions (Gutz *et al.* 1974). Under these conditions almost all recombination occurs during meiosis. Because of the very low spore viability in *mus81* mutants, tetrad analysis to verify reciprocality of crossovers was not feasible. However, random spore analysis can be used in *S. pombe* to assess crossing over because the frequency of gene conversion of most markers is low: the mean conversion frequency of 31 markers other than *ade6-M26* is ~0.07% per spore (range ~0.03% to ~0.4%; P. Munz, personal communication cited in Young *et al.* 2002). Intergenic recombinant frequencies greater than this therefore reflect primarily crossing over. We converted the observed recombinant frequencies to genetic distances (centimorgans) using the mapping function of Haldane (1919), which is appropriate for *S. pombe* since it does not manifest crossover interference (Munz 1994). This allowed us to assess quantitatively the degree of reduction of crossing over in the *mus81* mutant.

For the *his3–ade3* interval the frequency of recombinants was 47.6% in *mus81*$^+$ crosses and 7.5% in the *mus81* mutant crosses (Table 1). These data translate to 150 and 8.1 cM, respectively, indicating a reduction of crossing over in the *mus81* mutant by a factor of 19. Because the frequency of recombinants in *mus81*$^+$ cells was so close to the theoretical maximum of 50%, the estimate of the *his3–ade3* genetic distance is not precise. Since crossover frequencies per unit physical distance appear to be nearly uniform in *S. pombe*, we estimated the *his3–ade3* genetic distance using the physical distance and the genome average of 0.16 cM/kb (Wood *et al.* 2002; Young *et al.* 2002). This nominal distance of 480 cM in *mus81*$^+$ cells indicates that the *mus81* mutation reduces crossing over by a factor of 60.

Similar reductions of crossing over by the *mus81* mutation were seen in other intervals. For the *ade7–leu1* interval the reduction was a factor of 52, on the basis of the observed *mus81*$^+$ recombinant frequency, or 80, on the basis of the physical distance and nominal genetic distance. For the *ade6–arg1* interval the factors of reduction were 90, on the basis of the observed frequency, or 64, on the basis of the nominal genetic distance. Thus, crossing over is strongly reduced in the *mus81* mutant.

Two meiotic phenotypes of the *mus81* mutation—low viable spore yield and faulty DNA segregation—are suppressed by the *E. coli* RusA protein, which resolves Holliday junctions with high specificity (Chan *et al.* 1997; Boddy *et al.* 2001; Bolt and Lloyd 2002). To determine if the low frequency of crossing over was also suppressed, we conducted crosses in *mus81* mutants expressing the *rusA* gene under the control of the *S. pombe nmt1* promoter (Boddy *et al.* 2001). The *ade6–arg1* crossover frequency was increased from 0.75% in the absence of RusA to 26% in its presence (Table 1). The genetic distance was increased to >50% of that in *mus81*$^+$ cells. The suppression might be greater than this, since *S. pombe* plasmids are lost at high frequency even under selection (Heyer *et al.* 1986). Thus, the Holliday junction resolvase RusA largely and perhaps completely overcame the defect in crossing over in *mus81* mutants.

Although our genetic analysis allowed us to examine crossing over only in viable spores, we suppose that the dead spores also are deficient in crossing over. It is difficult to understand how the dead spores could complete an important step of meiosis, crossing over, but

TABLE 1

Mus81 is required for crossing over

Interval	Chr.	kb	Nominal cM[a]	Expt.	*mus81*$^+$ R1[b]	*mus81*$^+$ R2[b]	*mus81*$^+$ Total[b]	*mus81*$^+$ %	*mus81*$^+$ cM[c]	*mus81*Δ R1[b]	*mus81*Δ R2[b]	*mus81*Δ Total[b]	*mus81*Δ %	*mus81*Δ cM[c]	Fold reduction[d]
his3-ade3	I	3002	480	1	195	210	869	46.6	134	36	28	796	8.0	8.7	19
				2	210	166	774	48.6	179	29	14	611	7.0	7.5	
ade7-leu1	II	881	140	1	163	139	736	41.0	86	7	3	617	1.6	1.6	52
				2	161	144	706	43.2	100	8	6	682	2.0	2.0	
ade6-arg1	III	297	48	1	113	92	550	37.3	69	2	0	365	0.6	0.6	90
				2	95	92	509	36.7	66	1	2	334	0.9	0.9	
				1 (with p*rusA*)[e]						37	32	254	27.2	39	1.9
				2 (with p*rusA*)[e]						36	26	257	24.1	33	

Strains with mutations in the indicated genes, plus *ura4-D18* and *leu1-32*, were mated on appropriately supplemented synthetic sporulation medium SPA (Gutz *et al.* 1974) at 25°. After 2 days spores were harvested and plated on rich medium YEA (Gutz *et al.* 1974). Well-isolated colonies were transferred with toothpicks to grids on YEA and after overnight incubation at 30° were replicated to minimal medium EMM2 (Gutz *et al.* 1974) with appropriate supplements to score recombinants between the indicated genes. The alleles in these genes were *ade3-58*, *his1-102*, *ade7-152*, *leu1-32*, *ade6-216*, and *arg1-230*; these alleles were in repulsion. The crosses for measuring *ade3–leu1* recombination were heterozygous for *leu1-32*. The *mus81::kanMX6* mutation, indicated as Δ, has the *kanMX6* cassette (Bahler *et al.* 1998) substituted for the entire *mus81* coding sequence (Boddy *et al.* 2000).

[a] Calculated from the genome average of 0.16 cM/kb (Young *et al.* 2002).

[b] R1 (prototrophs) and R2 (double auxotrophs) are the number of reciprocal recombinant types among the total spore colonies tested. The ratio R1/R2 ranged from 0.93 to 1.27 for *mus81*$^+$ crosses and from 1.16 to 2.07 for *mus81*Δ crosses in which more than three recombinants were observed for both classes. By the contingency chi-square test, these values were not significantly different in wild-type and *mus81* mutants ($P > 0.2$). The excess of prototrophic recombinants (R1) may reflect a slight growth advantage of these spores on YEA medium.

[c] Calculated from the formula of Haldane (1919), $x = -\frac{1}{2}\ln(1 - 2R)$, where x is the genetic distance in morgans and R is the recombinant fraction.

[d] The ratio of average centimorgans in *mus81*$^+$ to average centimorgans in *mus81*Δ.

[e] The plasmid pRep1-NLS-RusA (Boddy *et al.* 2001) was present in both parental strains.

the live spores do not. The single mass of DNA seen in many asci, most of which give rise to dead spores (Boddy *et al.* 2001), indicates that the chromosomes are held together, we presume by Holliday junctions. It is possible, however, that the dead spores complete crossing over but the chromosomes are held together by another feature, such as sister chromatid cohesion. In this case Mus81 would be implicated in the release of sister chromatid cohesion, but this possibility is not supported by the known endonuclease activity of Mus81 and the suppression of the low viable spore yield in *mus81* mutants by a *rec12* mutation (Boddy *et al.* 2001; Gaillard *et al.* 2003).

To test the requirement for Mus81 in gene conversion, we examined recombination at the *ade6* locus, at which intragenic recombinants arise almost exclusively by gene conversion, not crossing over (Gutz 1971). We used the hotspot allele *ade6-M26* (G1010T) to increase the frequency of gene conversion so that recombination could be reliably measured in the low yield of viable spores from *mus81* mutants. In crosses with *ade6-469* (C2342T) the Ade$^+$ recombinant frequency in the *mus81* mutant was slightly increased, from 3.7×10^{-3} in *mus81*$^+$ cells to 5.8×10^{-3} in the *mus81* mutant, at the standard temperature for meiosis (25°), and slightly decreased, from 7.8×10^{-3} to 4.6×10^{-3}, at 34° (Table 2). Thus, gene conversion with the hotspot allele *ade6-M26* is only slightly altered, if at all, by the *mus81* mutation.

Gene conversion with the nonhotspot allele *ade6-M375* (G1007T) was too low for accurate measurements in *mus81* mutants, but from the few Ade$^+$ spores obtained, the recombinant frequency did not seem to be lower than that in *mus81*$^+$ strains (Table 2 and data not shown). We noted in other experiments that the viable spore yield of *mus81* mutants was ~50 times higher at 34° than at 25°; the viable spore yields of *mus81* mutants at these temperatures were, respectively, 0.75 and 0.013% of those of *mus81*$^+$ cells. The reason for this difference is unclear, but it allowed us to measure gene conversion with the nonhotspot allele *ade6-M375*. At 34° the recombinant frequency in crosses between *ade6-*

TABLE 2

Mus81 is not required for gene conversion

		Ade$^+$/10^6 viable spores[a]	
mus81	Temperature	*M26 × 469*	*M375 × 469*
+	25°	3700 ± 250 (6)	450 ± 29 (7)
Δ	25°	5800 ± 280 (3)	~2000[b]
+	34°	7830 ± 640 (3)	350 ± 36 (3)
Δ	34°	4600 ± 940 (3)	880 ± 74 (9)

Strains with the indicated *ade6* alleles (Szankasi *et al.* 1988) and either *mus81*$^+$ or *mus81::kanMX6*, indicated as Δ, were mated at the indicated temperature on SPA supplemented with adenine (100 μg/ml). After 2 days spores were harvested and titered for Ade$^+$ recombinant frequency as described by Farah *et al.* (2002).

[a] Data are the means ± SEM from the number of experiments in parentheses.

[b] The low viable spore yields under this condition precluded a more accurate determination of the recombinant frequency.

M375 and *ade6-469* was about twice as high in *mus81* mutants as in *mus81*$^+$ strains (Table 2). We conclude that Mus81 is not required for gene conversion; the conversion frequency in *mus81* mutants is as high as that in *mus81*$^+$ cells and perhaps even higher.

The properties of *S. pombe* and *S. cerevisiae mus81* mutants differ. *S. cerevisiae* mutants lacking Mus81 or its partner Mms4 have higher spore viability (10–50%, depending on the strain used), have higher frequencies of crossing over (40–180% of *MUS81* strains, depending on the interval measured and the strain used), and are delayed or arrested at the pachytene stage of meiosis; these phenotypes are not noticeably suppressed by RusA (Interthal and Heyer 2000; de los Santos *et al.* 2001, 2003; Mullen *et al.* 2001). In all strains tested, however, viable spore yields are strongly reduced relative to wild type, indicating that Mus81 plays a vital role in meiosis in both *S. cerevisiae* and *S. pombe*. In both organisms, mutants lacking Mus81 or its partner are sensitive to methyl methanesulfonate and UV during mitotic growth and have nearly wild-type frequencies of meiotic gene conversion (Table 2; Boddy *et al.* 2000, 2001; Interthal and Heyer 2000; de los Santos *et al.* 2001, 2003). These results indicate that some but not all functions of Mus81 differ in the two yeasts. de los Santos *et al.* (2003) concluded that there are two pathways of meiotic recombination in *S. cerevisiae*: one requires Mus81•Mms4 and shows no interference between closely spaced crossovers and the other shows interference but is Mus81•Mms4 independent. They proposed that *S. pombe* has only the former pathway. This proposal is consonant with our observation of the near lack of crossovers in *S. pombe mus81* mutants (Table 1) and the lack of crossover interference in *S. pombe* (Munz 1994).

The results presented here show that *S. pombe* Mus81 is required for crossing over but not for gene conversion. A similar but less dramatic separation of the two types of recombination is seen in *S. cerevisiae* and *Drosophila melanogaster*: mutations in several genes reduce crossing over by a factor of ~10 or less but have less effect on conversion (Carpenter 1982; Roeder 1997). In *S. pombe*, *S. cerevisiae*, and *D. melanogaster* both types of recombination require a set of proteins, including Rec12 (Spo11 or Mei-W68), that is also required or presumably required for meiotic DNA double-strand break formation (Roeder 1997; McKim and Hayashi-Hagihara 1998; McKim *et al.* 1998; Davis and Smith 2001). Thus, both types of recombination appear to stem from a common precursor, whose transformation into crossovers or convertants requires different sets of proteins. This view is embodied in most models of recombination.

A basic question still unanswered is the point at which the pathways for conversion and crossing over diverge. The models of Holliday (1964) and most others propose that they diverge at the final step, the resolution of the Holliday junction. Roman and Fabre (1983), Carpenter (1987), and others have suggested, however, that conversion and crossing over are not so closely related. Recent data show that hybrid DNA with crossover configuration and that with noncrossover configuration in *S. cerevisiae* can be both temporally and genetically separated (Allers and Lichten 2001; Hunter and Kleckner 2001). The strong separation of crossing over and conversion reported here also argues against the simple view that crossing over and conversion differ only by the mode of Holliday junction resolution. Convertants arise without Mus81•Eme1 endonuclease and, we argue, without resolution of Holliday junctions and perhaps without their formation. Further studies of *S. pombe mus81* mutants may help elucidate the step at which the pathways for conversion and crossing over diverge.

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Note added in proof: Osman *et al.* (2003, Mol. Cell **12:** 761–774) also have recently reported that crossing over, but not gene conversion, is strongly reduced in *S. pombe mus81* mutants.

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