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Meiotic Recombination Remote from Prominent DNA Break Sites in *S. pombe*

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Summary

DNA breakage is intimately associated with meiotic recombination in the fission yeast *Schizosaccharomyces pombe*. Sites of prominent DNA breakage were found ~25 to ~200 kb apart in the genomic regions surveyed. We examined in detail a 501 kb region of chromosome I and found six sites, or tight clusters of sites, at which ~2%–11% of the DNA accumulated breaks in a *rad50*S mutant. In contrast to the discrete, widely spaced distribution of prominent break sites, recombination in this region was more uniformly distributed (0.7–1.6 cM/10 kb) whether the genetic interval tested contained no, one, or more such sites. We infer that although recombination depends upon DNA breakage, recombination often occurs remote from these sites (tens of kilobases away); we discuss mechanisms by which this may occur.

Introduction

In most organisms, homologous genetic recombination occurs at high levels during meiosis to allow the pairing and subsequent reductional segregation of homologs and to enhance genetic diversity among the progeny (reviewed by Roeder, 1997, and Davis and Smith, 2001). In at least two species, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, meiotic recombination is initiated by double-strand (ds) DNA breakage (Sun et al., 1989; Cao et al., 1990; Cervantes et al., 2000). In some well-analyzed regions of the *S. cerevisiae* genome, DNA break sites occur roughly 5 kb apart (Baudat and Nicolas, 1997), and the broken DNA is frequently repaired by recombination with a homolog in a manner that produces recombinational exchanges within 1–2 kb of the broken ends (Lichten and Goldman, 1995; Smith, 2001). In contrast, in *S. pombe* meiotic DNA break sites appear to be farther apart, ~25 to ~200 kb, but recombinational exchanges are more uniformly distributed than these break sites (Cervantes et al., 2000; this report). These observations imply that recombination can frequently occur a substantial distance, ~100 kb or more, from the broken DNA ends.

Meiotic DNA breakage requires multiple gene products. In *S. pombe*, the Rec6, Rec7, Rec12, Rec14, and Rec15 proteins are essential for both meiotic recombination and DNA breakage (Cervantes et al., 2000; Davis and Smith, 2001). Thus, breakage and recombination are mechanistically linked in *S. pombe*, presumably by a precursor-product relation, as is the case in *S. cerevisiae* (Cao et al., 1990). A key component for DNA breakage in *S. cerevisiae* is the Spo11 protein, which becomes covalently linked to the 5′ ends of the broken DNA (Keeney et al., 1997). Tyr-135 of Spo11 is essential for DNA breakage and recombination; presumably Tyr-135 forms a phosphodiester link between Spo11 and the DNA by a topoisomerase-like mechanism (Bergerat et al., 1997; Keeney et al., 1997). The homologous Tyr-98 of the *S. pombe* homolog Rec12 is also essential for recombination, suggesting that the two proteins act by the same mechanism (Cervantes et al., 2000).

The repair of meiotic DNA breaks also requires multiple proteins (Roeder, 1997). In *S. cerevisiae*, the DNA 5′ ends are resected, and the resultant 3′ single-stranded (ss) tails invade a homolog to form a joint molecule. Resection requires the Rad50, Mre11, and Xrs2 proteins, and joint molecule formation requires Rad51 and Dmc1 proteins, homologs of the *Escherichia coli* RecA DNA strand exchange protein. Joint molecules, which include double Holliday junctions, are presumably resolved into recombinant molecules. Proteins responsible for resolution have not been identified in *S. cerevisiae*, but the Mus81•Emel protein complex of *S. pombe* appears to be essential for resolution of Holliday junctions (Boddy et al., 2001).

The Rad50 protein plays a dual role, being necessary for both the formation and the repair of DNA breaks. In *S. cerevisiae rad50* deletion mutants, no DNA breakage is detected (Cao et al., 1990). In a special class of missense mutants designated *rad50*S, breakage occurs but resection and subsequent steps of repair do not (Alani et al., 1990). The accumulation of broken DNA in *rad50*S mutants therefore reveals a more complete picture of the positions and extent of meiotic DNA breakage than that from wild-type cells. We used here an analogous mutant of *S. pombe* for this purpose. After determining the positions and extent of DNA breakage, we compared them with the distribution of recombination in a particular genomic region and found they differed markedly.

Results

Meiotic DNA Breaks Accumulate in an *S. pombe rad50*S Mutant

In *S. pombe rad50*$^+$ cells, meiotic DNA breaks appear shortly after premeiotic replication and persist for ~1 hr; the broken DNA is then repaired, presumably by homologous recombination with either a homolog or a sister chromatid (Cervantes et al., 2000). We compared the behavior of DNA from a *rad50*S mutant and found that, as in *S. cerevisiae* (Alani et al., 1990), DNA breakage occurred at the normal time, but the broken DNA was not repaired.

For this analysis, we used a *rad50*S mutant with an amino acid substitution K81I at the position corresponding to the K81I *rad50*S mutant of *S. cerevisiae* (Alani et

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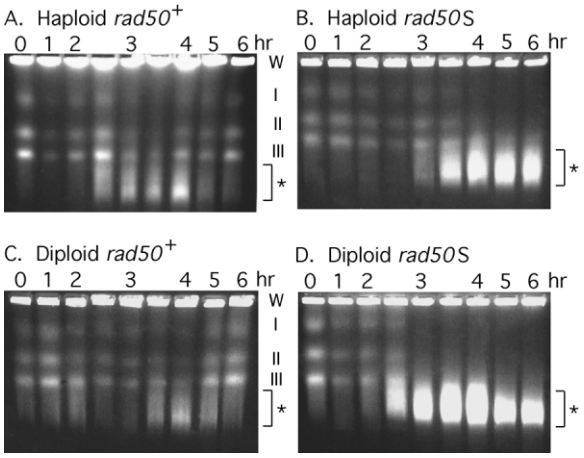

Figure 1. Meiotic DNA Breakage but not Repair in a *rad50*S Mutant

Cells were harvested at the indicated times (hr) after induction of meiosis, and the DNA was analyzed by pulsed-field gel electrophoresis and staining with ethidium bromide as described in Experimental Procedures. (A), haploid *rad50*$^+$ strain GP535; (B), haploid *rad50*S strain GP2956; (C), diploid *rad50*$^+$ strain GP338; (D), diploid *rad50*S strain GP3087. The bands in the mitotic (0 hr) lanes are, from top to bottom, the wells into which the DNA was loaded (W), chromosome I (5.7 Mb), chromosome II (4.6 Mb), and chromosome III (3.5 Mb). The smear (asterisk) is broken DNA that appears transiently in the *rad50*$^+$ strains and accumulates in the *rad50*S mutants.

al., 1990; Manolis et al., 2001; E. Hartsuiker, personal communication). To achieve high synchrony among the meiotically induced cells, *pat1-114* (Ts) mutant cells were arrested in the G1 phase of the cell cycle by starvation for nitrogen; restoration of nitrogen and elevation of the temperature inactivates the Pat1-114 protein kinase and rapidly induces synchronous meiosis (Iino and Yamamoto, 1985; Nurse, 1985). After induction, DNA replication, assayed by flow cytometry, occurred at ~2 hr in both the *rad50*$^+$ and *rad50*S cells (see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1). DNA breakage, assayed by pulsed-field gel electrophoresis to separate the three *S. pombe* chromosomes and staining with ethidium bromide, occurred at ~3 hr in both cell types (Figures 1A and 1B). In the *rad50*$^+$ cells, the chromosomes had returned to their intact state by 5 hr, but in the *rad50*S cells broken DNA persisted, and no intact DNA was detectable even at 6 hr (Figures 1A and 1B) or as late as 24 hr (data not shown).

As in previous studies (Li and Smith, 1997; Cervantes et al., 2000), haploid and diploid *pat1-114* (Ts) mutants behaved similarly. Inactivation of the Pat1-114 protein kinase induced the early events of meiosis, including DNA replication, breakage, and repair, with similar kinetics in both cell types (cf. Figures 1A with 1C and 1B with 1D; see also supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1). In addition, bulk DNA remained broken in both haploid and diploid *rad50*S *pat1-114* (Ts) mutants (Figures 1B and 1D). As shown later (see Figures 3 and 4), DNA breaks at particular sites also accumulated equivalently in haploid and diploid cells.

Thus, the *S. pombe rad50*S mutant appears, by this analysis, to be similar to the *S. cerevisiae rad50*S mutant: it is blocked in the repair of meiotic DNA breaks.

Prominent Meiotic DNA Break Sites Are Far Apart

When specific chromosomal regions are examined by Southern blot hybridization, prominent but transient bands are seen with DNA from *rad50*$^+$ cells; these bands indicate prominent sites of frequent cleavage located ~100 to ~300 kb apart in several regions of the genome (Cervantes et al., 2000). To determine if the same pattern occurs in *rad50*S cells, we surveyed three regions of the genome for the accumulation of broken DNA ends in *rad50*S cells. The first region surveyed was that near *ura1* on chromosome I, near which a prominent break site was previously found in *rad50*$^+$ cells (Cervantes et al., 2000). After pulsed-field gel electrophoresis, broken DNA from meiotically induced cells was analyzed by Southern blot hybridization using a radioactive probe from the *ura1* gene, located about 0.75 Mb from the left end of chromosome I.

We interpret the band marked with an asterisk and those above it in Figure 2A as a reflection of DNA molecules extending from the left telomere through the *ura1* probe sequence to sites of prominent breakage. The pattern of break sites or clusters of sites is similar in *rad50*S cells (Figure 2A) and in *rad50*$^+$ cells (Cervantes et al., 2000). The prominent band marked with an asterisk in Figure 2A represents a frequently cleaved site or cluster of sites just to the right (the telomere-distal side) of the *ura1* gene. This site, designated *mbs1* (*m*eiotic *b*reak site 1), is analyzed more thoroughly below. Other prominent break sites are located farther to the right of *mbs1*, as deduced from the more slowly migrating DNA fragments. Fragments migrating more rapidly than (below) that marked with the asterisk in Figure 2A must represent molecules with two meiotic breaks, since they are too short to extend from the telomere to the *ura1* probe. The shortest of these fragments was ~300 kb long, suggesting that a second meiotic break rarely occurs within this distance to the left of a break at *mbs1*.

Other regions of the genome also have widely spaced sites at which meiotic breaks accumulated in the *rad50*S mutant. A radioactive probe from cosmid c869, located about 100 kb from the right end of chromosome I, revealed eight prominent bands, reflecting eight prominent break sites or clusters of sites located ~165 to ~1200 kb from the telomere (Figure 2B). The distances between these sites ranged from ~70 to ~250 kb.

We analyzed breaks on chromosome III after digesting the DNA with the rarely cutting enzyme SfiI, since the ends of this chromosome have heterogeneous lengths of rDNA (Fan et al., 1991) which would smear the bands from undigested DNA. One SfiI site is 78.9 kb from the beginning of the rDNA at the left end of chromosome III. A probe located immediately to the left (telomere proximal) side of this SfiI site revealed three prominent bands reflecting break sites at ~35, ~60, and ~80 kb from the SfiI site (Figure 2C). The repeating pattern of less prominent breakage, visible above the prominent bands in Figure 2C, presumably reflects meiotic break sites within each of the 10.9 kb rDNA repeats. A probe immediately to the right of the SfiI site revealed seven prominent break sites before the next SfiI site located

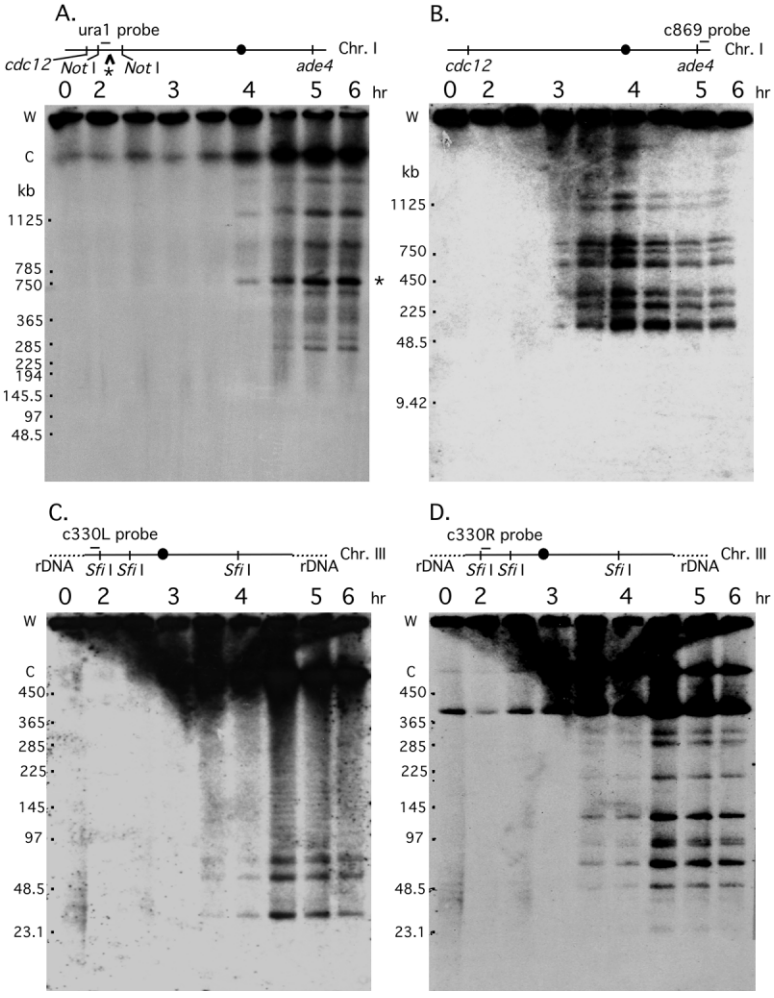

Figure 2. Widely Spaced Prominent Meiotic DNA Break Sites in Multiple Regions of the Genome

Cells of the *rad50*S strain GP2956 were harvested at the indicated times (hr) after induction of meiosis, and the DNA was analyzed by pulsed-field gel electrophoresis and Southern blot hybridization as described in Experimental Procedures.

(A) *ura1* probe located ~0.75 Mb from the left end of chromosome I. Asterisk, meiosis-specific fragment broken at *mbs1*; W, wells into which DNA was loaded; C, region of compaction to which whole chromosomal DNA and fragments larger than ~2 Mb migrate.

(B) A probe from cosmid c869, located ~100 kb from the right end of chromosome I.

(C) A probe from the left (telomere proximal) side of the SfiI site in cosmid c330, near the left end of chromosome III.

(D) A probe from the right side of the SfiI site in cosmid c330.

In (C) and (D), the DNA was digested with SfiI before electrophoresis; the blot was probed, stripped, and reprobed. •, centromere. The chromosomes are drawn approximately to scale. Only two NotI sites are shown in (A); they are shown in Figures 3, 5, and 6.

384 kb away (Figure 2D). The distances between these break sites ranged from ~25 to ~90 kb.

Because of the very prominent meiotic break site *mbs1*, near *ura1*, first observed with *rad50*$^+$ cells (Cervantes et al., 2000), we have examined in detail the region surrounding *mbs1*. Since *ura1* is near the middle of the 501 kb NotI fragment J (Fan et al., 1989), we analyzed NotI-digested DNA using probes from the left and right ends of this NotI fragment. The left-end probe revealed a strong band reflecting frequent breakage at *mbs1*, located ~240 kb from the left end of the NotI fragment (Figure 3A). Somewhat weaker bands were seen above and below the *mbs1* band, reflecting break sites at approximately 135 (*mbs2*), 330, 350, 420, and 460 kb from the left end of the NotI fragment. The right-end probe revealed meiosis-specific fragments of lengths complementary to those revealed by the left-end probe (Figure 3B). From the two sets of data and others not shown we estimated the positions of these prominent break sites (see below and Figure 6); the distances between these sites ranged from ~20 to ~100 kb.

Similar patterns of meiosis-specific breakage were observed with diploid cells. For example, the positions and extent of DNA breakage on the NotI fragment J, revealed by left- and right-end probes, were similar with diploid *pat1-114* cells (Figures 3C and 3D) and with haploid *pat1-114* cells (Figures 3A and 3B). DNA breakage in other regions of the genome also was similar in haploids and diploids (data not shown).

To determine if meiosis-specific breakage at these prominent sites depends on recombination functions, as does breakage of whole chromosomes (Cervantes et al., 2000), we examined DNA from a *rad50*S *rec12* double mutant. In this strain, whole chromosomes remained intact up to 6 hr after meiotic induction (data not shown), and no detectable meiosis-specific breakage was seen on the NotI fragment J (Figures 3E and 3F). DNA breakage at each of these prominent sites and others examined in the genome depends upon multiple *rec* gene products (Cervantes et al., 2000; data not shown) and therefore is intimately associated with meiotic recombination.

Quantitation of Meiotic DNA Breakage

To determine the fraction of DNA broken at the prominent sites on the NotI fragment J, we analyzed the Southern blot hybridizations shown in Figure 3 with a PhosphorImager. Figure 4 shows graphically the amount of hybridization with the left-end probe to NotI-digested DNA from *rad50*S *rec*$^+$ and *rad50*S *rec12* mutant cells

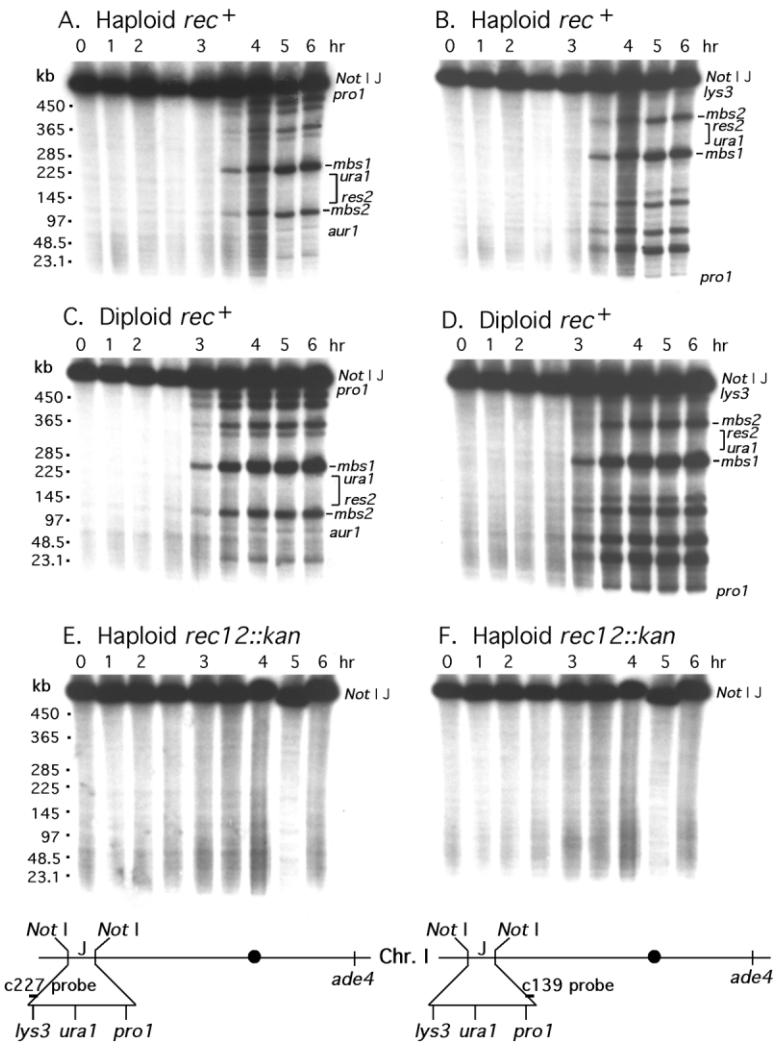

Figure 3. Prominent Rec12-Dependent Meiotic DNA Break Sites in the 501 kb NotI Fragment J

Cells from *rad50*S strains were harvested at the indicated times (hr) after induction of meiosis; the DNA was digested with NotI and analyzed by pulsed-field gel electrophoresis and Southern blot hybridization as described in Experimental Procedures. (A) and (B), haploid strain GP2956; (C) and (D), diploid strain GP3087; (E) and (F), haploid *rec12* strain GP3135. In (A), (C), and (E), the probe was from the left end of the NotI fragment J, and in (B), (D), and (F), the probe was from the right end. •, centromere. The chromosomes are drawn approximately to scale. Only two NotI sites are shown; they are shown in Figures 2, 5, and 6.

at 1 hr and 5 hr after induction of meiosis (Figures 3A, 3C, and 3E). Six prominent peaks were seen with *rec*$^+$ DNA at 5 hr after induction. Between these peaks, the amount of hybridization was near that observed with *rec*$^+$ DNA at 1 hr (before DNA replication) or with *rec12* mutant DNA at 1 hr or at 5 hr. Hybridization to these latter three samples was approximately uniform throughout the lanes of the gel; no obvious meiosis-specific peaks were seen. The intensity of this background was approximately 1% of the intensity of the full-length (unbroken) NotI fragment J. Since this background is independent of meiotic induction and *rec12* gene function, we presume it reflects mechanical breakage due to manipulation of the cells and DNA or to enzymatic breakage unrelated to meiotic recombination. We have therefore subtracted this background in the following analysis.

We estimate the fraction of meiosis-specific DNA breakage (5 hr minus 1 hr values) at each of the prominent sites on NotI fragment J to be, from left to right, 3.3 ± 0.5 (*mbs2*), 10.8 ± 0.3 (*mbs1*), 1.6 ± 0.4, 2.1 ± 0.2, 3.3 ± 0.4, and 4.6 ± 0.4% of the total DNA (intact NotI fragment plus discretely broken fragments). These estimates are the mean values ± the standard error of the mean of five independent inductions of the *rad50*S haploid strain GP2956 and one of the *rad50*S diploid strain GP3087, each probed from both ends of the *Not*I fragment. The total fraction of the *Not*I fragment J broken at these sites during meiosis was ~26%.

The amount of meiosis-specific DNA breakage between the prominent break sites is difficult to quantitate accurately because it is so close to the background level. Nevertheless, we estimate from the hybridizations analyzed above that the amount of meiosis-specific breakage in an interval between *mbs1* and *mbs2* (*res2-ura1*; see Figures 3 and 6) was 0.52 ± 0.56% in the *rec*$^+$ strains and 0.2% in the *rec12*::*kan* strain GP3135. Thus, ~0.3% of the DNA in the *res2-ura1* interval was broken in a meiosis-specific, *rec12*-dependent manner. There was also very little breakage in the *lys3-aur1* interval, to the left of *mbs2*. The six probings from the right side of the NotI fragment yielded 0.47 ± 0.27%, and those from the left yielded −3.3 ± 1.8%. The latter data have more scatter, owing to a higher background of meiosis- and *rec12*-independent broken fragments in the size range of these measurements (Figures 3 and 4). These data indicate that the amount of meiosis-specific break-

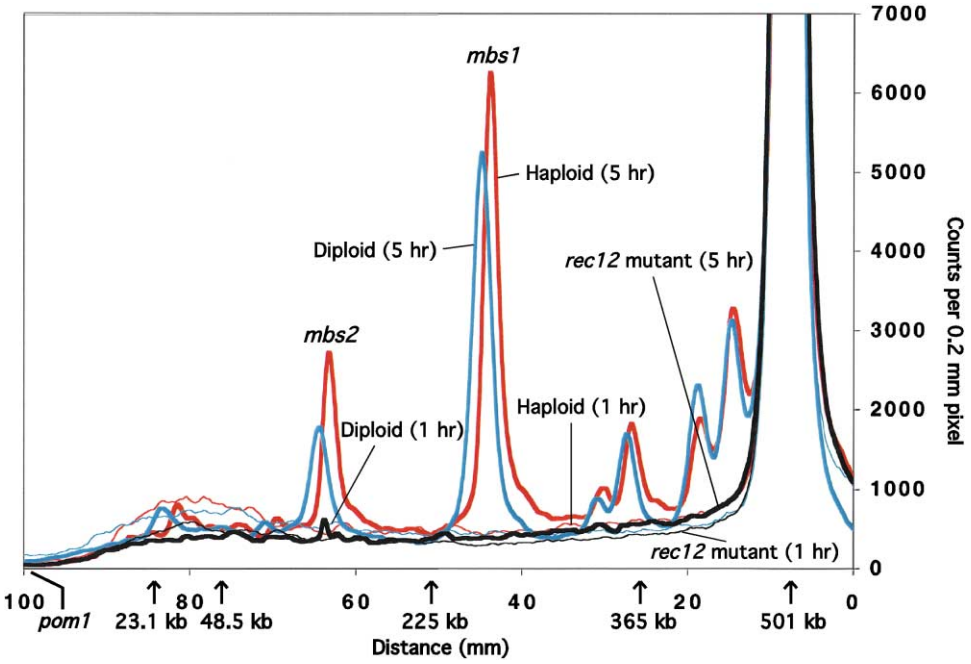

Figure 4. Quantitation of Meiotic DNA Break Frequency

The Southern blots of *rad50*S DNA in Figures 3A, 3C, and 3E were analyzed with a PhosphorImager. A background level of radioactivity from the lightest part of each lane was subtracted from each value, and the total remaining level of radioactivity for each lane was normalized to the highest value. The 1 hr samples (thin lines) are before DNA replication and breakage, and the 5 hr samples after (thick lines; see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1 and Figures 1–3). Red lines, haploid *rec*$^+$ strain GP2956; blue lines, diploid *rec*$^+$ strain GP3087; black lines, haploid *rec12-170* strain GP3135. Electrophoresis of the DNA from the diploid *rec*$^+$ strain was slightly longer than that for the other strains. Before normalization, peak counts in the unbroken 501 kb band ranged from 36,700 to 60,200. Note that significant DNA breakage is seen only with the *rec12*$^+$ strains after meiotic replication.

age between the prominent break sites is <1% and perhaps close to zero.

Mapping a Prominent Meiotic Break Site *mbs1*

To examine the relation between the positions and extents of meiotic DNA breakage and of meiotic recombination, we determined more precisely the location of *mbs1*, near *ura1*. The mobility, relative to size markers, of the broken fragment designated *mbs1* in Figures 3 and 4 and the nucleotide sequence of the *S. pombe* genome (Wood et al., 2002) indicated that *mbs1* is located on a 64.4 kb PmeI fragment. Consequently, PmeI-digested DNA from a *rad50*S strain was electrophoresed and analyzed with probes from each end of this fragment. A single prominent meiosis-specific band was observed with each probe (Figure 5); their mobilities indicated that *mbs1* is located ~38 kb from the left end of this PmeI fragment. A 1.0 kb probe extending from 37.5 kb to 38.5 kb from the left end of this PmeI fragment revealed both the left and the right meiosis-specific fragments (data not shown), suggesting that a substantial fraction of the breakage occurs within or near this 1.0 kb interval, which we designate *mbs1*.

We quantitated the fraction of DNA from a *rad50*S strain broken at *mbs1* or in the flanking regions of the 64.4 kb PmeI fragment (Figure 5). In this experiment, ~8% of the DNA was broken at *mbs1* at 4.5 hr after meiotic induction. Outside *mbs1*, the extent of breakage at 4.5 hr was nearly the same as that at 0 hr, before meiotic induction. This analysis indicated that there was little if any meiosis-specific DNA breakage in this 64.4 kb interval other than at *mbs1*.

Frequency of Meiotic Recombination Surrounding *mbs1*

Having located a prominent meiotic DNA break site *mbs1*, we asked whether this site is a hot spot of meiotic recombination. To answer this, we compared the intensity of recombination in a genetic interval containing *mbs1* with the intensity in other intervals, including those undergoing little or no breakage (Table 1 and Figure 6). The intervals tested collectively spanned 476 kb, or nearly all of the 501 kb NotI fragment J analyzed for DNA breakage. One set of eight genetic markers consisted of single base pair transition mutations in nonessential genes with readily scored phenotypes (see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1); these mutations should minimize the likelihood of marker effects. A second set of three markers consisted of insertions or substitutions of ~2 kb of DNA (see supplemental data at the above URL); although they may alter chromosomal features such as chromatin structure, crosses employing these markers gave results similar to those employing only single base pair transitions.

Recombinant frequencies were determined by random spore analysis. Most of the recombinants detected likely arose by crossing over (reciprocal recombination),

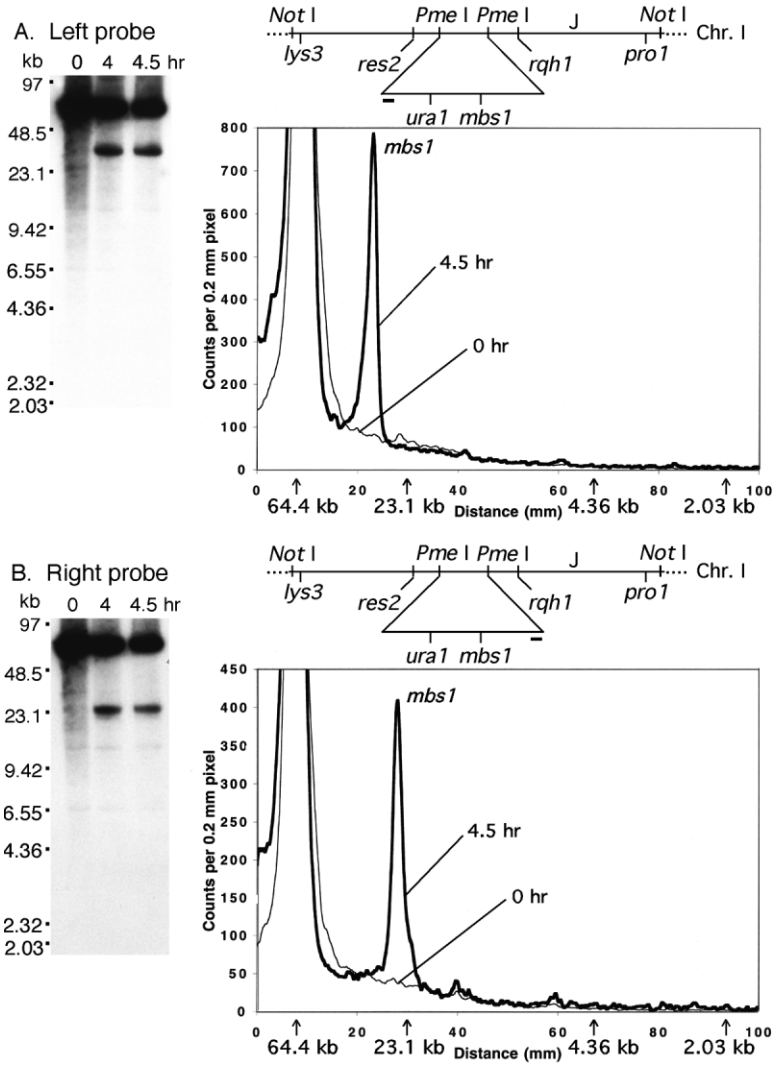

Figure 5. Mapping the Prominent Meiotic DNA Break Site *mbs1*

DNA from the *rad50*S strain GP2956 was prepared at the indicated times (hr) after meiotic induction, digested with PmeI, and analyzed as in Figures 3 and 4 using probes from the left or right end of the 64.4 kb PmeI fragment near the middle of NotI fragment J. (A) shows the left-end probe. Before normalization, peak counts in the unbroken 64.4 kb band were 15,000 (0 hr, thin line) and 13,300 (4.5 hr, thick line). (B) shows the right-end probe. Before normalization, peak counts were 8600 (0 hr) and 7700 (4.5 hr). The 501kb NotI fragment is drawn approximately to scale. Only two PmeI sites are shown.

since the lowest frequency of recombination measured was 2.6% and the frequency of gene conversion (nonreciprocal recombination) of most *S. pombe* markers is ~0.07% per spore (range for 31 markers is ~0.03% to ~0.4%; P. Munz, personal communication). Crosses were repeated, in some cases with the markers both in coupling and in repulsion, and similar results were obtained. A total of 30,341 marker-pair segregations from 78 crosses was analyzed. The frequency of recombination between two markers was converted to genetic distance (centimorgans or cM) using the mapping function of Haldane (1919), which assumes no crossover interference as is the case for *S. pombe* (Munz, 1994). The positions of the markers on the nucleotide sequence of the NotI fragment J (see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1) allowed calculation of the intensity of recombination, cM/10 kb.

The results of this extensive analysis (Table 1 and Figure 6) indicate that the intensity of recombination among intervals with or without a prominent meiotic DNA break site varied less than 40% from the overall mean value. In particular, the intensity of recombination in the interval with the most prominent break site *mbs1* (10.8% breakage), *ura1–rqh1*, was 1.2 cM/10 kb, close to the mean (1.1) of all the intervals measured with single base pair mutations (Table 1A). The adjacent interval on the left, *res2–ura1*, had 0.72 cM/10 kb and ~0.5% breakage. The adjacent interval on the right, *rqh1–mis6*, had 1.3 cM/10 kb and 7.0% breakage. The interval with the highest intensity of recombination (*lys3–aur1*, 1.6 cM/10 kb) had little or no meiosis-specific breakage. Thus, these data indicate that the prominent meiotic DNA break sites are not sites of especially intense recombination, or hot spots of recombination.

The data from crosses with large insertion or substitution mutations, denoted with an asterisk in Table 1B and Figure 6, gave a similar picture. For example, the interval *res2*–rqh1*, spanning *mbs1*, had 0.9 cM/10 kb, close to the mean of 0.92 cM/10 kb for this set of data. Two intervals with little or no meiotic DNA breakage, *pom1*–mkh1** and *rad15–res2**, had 1.3 cM/10 kb, nearly the same as 1.2 cM/10 kb from the measurement of *rad15–res2* with single base pair transition mutations. Thus,

Table 1. Recombination Intensity in Intervals with and without Prominent DNA Break Sites

Interval	Crosses[1]	Tested[1]	R1[2]	R2[2]	% Recomb.	cM[3]	kb	cM/10 kb	Range
A. Single Base Pair Transition Mutations									
lys3-aur1	5	1885	98	84	9.7	10.7	68.2	1.57	1.40–1.74
lys3-ura1	7	1795	67	81 (+185)[4]	18.6	23.2	212	1.09	0.68–1.32
lys3-pro1	6	1477	64	79 (+280)	28.6	42.5	476	0.89	0.73–0.94
aur1-rad15	1	516	24	22	8.9	9.8	65.2	1.51	
aur1-ura1	1	318	27	21	15.1	18	144	1.25	
rad15-res2	3	2965	44	33	2.6	2.7	22.1	1.21	0.94–1.65
rad15-ura1	5	616	106	93	5.6	5.9	78.7	0.75	0.61–0.89
res2 (G1)-ura1	2	1090	17	19	3.3	3.4	56.6	0.6	0.54–0.88
res2 (J3)-ura1	6	4068	86	81	4.1	4.3	56.6	0.75	0.38–0.99
ura1-rqh1	3	1188	60	55	9.7	10.8	90.8	1.19	1.07–1.33
ura1-mis6	4	1510	131	146	18.3	22.9	198	1.15	0.8–1.50
ura1-pro1	7	1578	87	82 (+207)	23.8	32.4	264	1.23	0.53–2.16
rqh1-mis6	2	1075	68	64	12.3	14.1	107	1.32	1.09–1.5
mis6-pro1	3	528	18	13	5.9	6.2	66.1	0.95	0.6–1.49
B. Insertion or Substitution Mutations[5]									
pom1-mkh1**	4	1033	52	52	10.1	11.2	79.0	1.42	1.05–1.85
pom1-rad15*	3	1013	57	53	10.9	12.3	130	0.96	0.84–1.05
pom1-res2**	3	1013	56	56	11.1	12.6	153	0.83	0.77–0.85
pom1-ura1*	6	2261	146	171	14.0	16.4	209	0.79	0.65–0.96
pom1-rqh1*	2	884	85	94	20.2	25.9	300	0.87	0.82–0.9
pom1-mis6*	2	1173	149	158	26.2	37.1	407	0.92	0.88–0.95
mkh1-rad15*	1	958	18	17	3.7	3.8	49.9	0.77	
*rad15-res2**	4	1786	20	29	2.7	2.8	22.3	1.28	0.87–2.9
res2-rqh1*	1	280	19	15	12.1	13.9	146	0.94	

[1]The total number of random spores analyzed from the number of crosses indicated. Data from individual crosses were similar and have been pooled.
[2]R1 and R2 are the number of reciprocal recombinant types for each cross.
[3]The percent of recombinants was converted to centimorgans (cM) by Haldane's formula cM = -50 ln(1 $-$ 2R), where R is the recombinant frequency.
[4]The number of recombinants (R1 + R2) in previously published crosses in which the reciprocal types were tallied together (Lin and Smith, 1995; Evans et al., 1997; Ding and Smith, 1998).
[5]Indicated by an asterisk (see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1 for descriptions of the mutations).

these data, like those in the previous set of crosses, fail to show a simple correlation between the site of meiotic DNA breakage and the site of meiotic recombination.

Meiotic recombination in this region of the genome is dependent upon the *rec12* and other *rec* gene products. In crosses homozygous for *rec12-117*, among 470 viable spores tested none were recombinant in the *lys3-37–ura1-61* interval, and among 582 only one was recombinant in the *ura1-61–pro1-1* interval. Comparison with the *rec*$^+$ data in Table 1 shows that the *rec12* mutation reduced recombination in these intervals by a factor of $\geq$ 50. Meiotic recombination in the *lys3–ura1–pro1* region is reduced by a factor of $\geq$ 25 also by a *rec6*, *7*, *14*, *15*, or *16* mutation (DeVeaux and Smith, 1994; Lin and Smith, 1995; Evans et al., 1997; Ding and Smith, 1998). Thus, both meiotic recombination and meiotic DNA breakage in this region are dependent, as far as tested, upon the same set of *rec* gene products.

Discussion

We report here the occurrence of widely separated sites in the *S. pombe* genome at which meiosis-specific ds DNA breaks frequently arise; between these sites little or no breakage was detected. In contrast to the discrete distribution of these prominent DNA break sites, meiotic recombination was more uniformly distributed. Our results demonstrate that hot spots of meiotic DNA breakage are not hot spots of meiotic crossing over and that meiotic recombination occurs tens of kilobases from these prominent break sites. While we cannot completely rule out additional, low-level breaks, the available evidence discussed below indicates that the ds DNA breakage between the prominent sites is too infrequent to account for the amount of recombination in those intervals. We infer that in *S. pombe* meiotic recombination frequently occurs remote from broken DNA ends. We discuss below how this may occur.

The regions of the *S. pombe* genome surveyed here for meiotic DNA breakage and recombination appear representative of the genome. Widely spaced, prominent DNA break sites have been found in the six regions of the genome surveyed (Cervantes et al., 2000; Figures 2 and 3; data not shown). Meiotic recombination in *S. pombe* appears to be distributed uniformly throughout the genome, with an intensity of $\sim$1–2 cM/10 kb; this range includes the mean intensity of 1.1 cM/10 kb for the intervals in the extensively studied 501 kb NotI fragment J (Table 1 and Figure 6). The *S. pombe* genome encompasses $\sim$2200 cM and $\sim$14 Mb, or 1.6 cM/10 kb on average (Munz et al., 1989; Wood et al., 2002). For 20 intervals spanning most of chromosome I, the intensities range from $\sim$0.7 to $\sim$3.4 cM/10 kb (calculated from

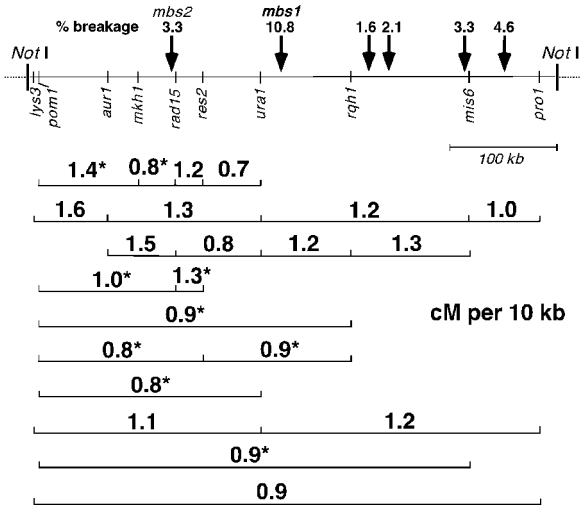

Figure 6. Meiotic Recombination Intensity in the Region Surrounding the Prominent Meiotic DNA Break Site *mbs1*

The intensity of recombination (cM/10 kb) from Table 1 is indicated for each measured interval of NotI fragment J on chromosome I. The physical map of this fragment (top line) including the location of *mbs1* is drawn to scale. The approximate positions of other prominent break sites (see Figures 3 and 4; additional data not shown) are indicated by vertical arrows. The frequency of meiosis-specific breakage at each site is indicated (see Results). Below the map, the intervals are arranged arbitrarily for conciseness. Genetic intervals involving an insertion or substitution of ~2 kb (Table 1B) are indicated with an asterisk.

recombination data provided by P. Munz and physical data for the *S. pombe* genome); the mean and median values are 1.7 and 1.2 cM/10 kb, respectively, similar to those for the NotI fragment J. We anticipate that the conclusions we draw from the data reported here will apply to other regions of the *S. pombe* genome.

Nature of Prominent Meiotic DNA Break Sites

We mapped one meiotic break site, *mbs1*, to an interval of a few kilobases (Figure 5). This interval is within a region of 7.2 kb between two lengthy coding sequences, SPAC4G8.03c and SPAC4G8.04, between which there is no extensive coding sequence. This 7.2 kb intergenic region is considerably larger than the average intergenic region of ~0.9 kb in *S. pombe* (Wood et al., 2002). Mapping additional meiotic break sites should show if they are typically located in large intergenic regions.

The paucity of prominent meiotic break sites in *S. pombe* may stem from the consequences of the breakage and its repair. Hot spots of gene conversion in *S. pombe* and *S. cerevisiae* are recipients of genetic information (Gutz, 1971; Lichten and Goldman, 1995): an active hot spot is lost when a chromosome with an active hot spot undergoes gene conversion with a chromosome lacking that hot spot. Thus, such hot spots are expected to be lost from natural populations. If, as in *S. cerevisiae* (Lichten and Goldman, 1995; Petes, 2001), *S. pombe* meiotic DNA break sites are hot spots of gene conversion, most break sites may have been converted away during evolution of *S. pombe*, perhaps more extensively within coding sequences than between them.

Relation between Meiotic DNA Breaks and Recombination

Both meiotic DNA breakage and meiotic recombination depend on multiple *rec* gene products—*rec6*, *7*, *8*, *10*, *11*, *12*, *14*, and *15* (Figure 3; Cervantes et al., 2000; Davis and Smith, 2001; data not shown). This dependence indicates that the two processes are mechanistically connected, presumably in a precursor-product relation as in *S. cerevisiae* (Cao et al., 1990). The accumulation of meiotic DNA breaks in the *S. pombe rad50*S mutant (Figures 1–3), as in the corresponding mutant in *S. cerevisiae* (Alani et al., 1990), supports this view. The considerable reduction, perhaps abolition, of meiotic recombination in five mutants, including *rec12*, suggests that most, perhaps all, meiotic recombination in *S. pombe* depends upon DNA breakage.

The DNA broken during meiosis must be repaired to form intact chromosomes and to produce viable meiotic products. Repair could occur by the rejoining of DNA ends or by interaction with a sister chromatid or a homolog. Only interaction with a homolog can give an observed genetic recombinant. Most models of recombination initiated by ds DNA breaks predict that one reciprocal exchange, which produces two crossover chromatids, requires exactly one ds break. Our observation of ~26% meiotic DNA breakage and ~50 cM in the *lys3–pro1* interval (Table 1; Figure 4; and Results) suggests, within the context of these models, that nearly all of the ds DNA breaks are repaired as crossovers. Although our data appear accurate within a factor of two, indicating that many of the breaks are repaired as crossovers, we cannot exclude that some are repaired in other ways, including gene conversion without crossing over. If end joining or sister chromatid interactions occur, they would exacerbate the problem of accounting for the observed amount of crossovers by the observed amount of DNA breakage: such repair without genetic recombination would leave even fewer breaks to generate the observed amount of recombinants. The available data thus suggest that many and perhaps most or all of the ds DNA breaks result in a crossover.

The distributions of ds DNA breakage and of recombination do not, however, appear to be congruent: recombination appears much more uniformly distributed than DNA breakage. For example, the 91 kb *ura1–rqh1* interval has 11 cM, or 1.2 cM/10 kb, and 11% DNA breakage at *mbs1* (Table 1; Figures 4 and 6). In contrast, the 57 kb *res2–ura1* interval has 4.1 cM, or 0.72 cM/10 kb, but only ~0.3% meiosis-specific, *rec12*-dependent DNA breakage. Thus, comparing the *ura1–rqh1* and *res2–ura1* intervals, DNA breakage is less frequent in the latter interval by a factor of >20, but recombination is less frequent by a factor of only 2.6 and the recombination intensity (cM/kb) by a factor of only 1.7. Similar discrepancies in the frequency of DNA breakage and frequency of recombination are seen in other intervals such as *lys3–aur1* (11 cM with little or no DNA breakage in 68 kb) versus *rqh1–mis6* (14 cM with three prominent break sites having a total of 7.0% DNA breakage in 107 kb) (Table 1; Figures 4 and 6). Prominent break sites are located >20 kb to the left and >20 kb to the right of the *res2–ura1* interval (Figure 6). These observations indicate that recombination in this interval occurs >20 kb and perhaps >50 kb from the nearest prominent

break site. As noted above, we cannot completely rule out undetected, low-level breaks between the prominent DNA break sites. But the data presented here argue that such breakage is insufficient to account for the high level of recombination in intervals, such as *res2–ura1* and *lys3–aur1*, between prominent break sites.

More generally, if recombination in *S. pombe* were limited to 1–2 kb around the prominent DNA break sites, as frequently appears to be the case in *S. cerevisiae* (Lichten and Goldman, 1995), the genetic map of *S. pombe* would have clusters of genes (those between break sites) separated by spaces of recombination (those containing break sites). Genes are widely distributed, however, on the *S. pombe* genetic map (Munz et al., 1989), typified by the analysis of the 0.5 Mb region shown in Figure 6. These results imply that recombination occurs a substantial distance (up to ~100 kb) from prominent DNA break sites. We infer, therefore, that in *S. pombe* meiotic recombination frequently occurs far from broken DNA ends.

Models for Recombination Remote from DNA Ends

We entertain two general ideas for how recombination can occur far from a DNA end, yet be dependent upon DNA breakage. First, a DNA break could send a signal, such as relief of torsion, within a domain of the chromosome. Recombination might then be able to occur, by an unspecified mechanism, anywhere within the relaxed domain. We know of no precedent for this proposal, but it is conceptually similar to the proposal for the regulation of transcription within chromosomal domains. Second, an entity could move from a DNA end along the DNA to a distant point, at which it promotes recombination. We consider two models for such a moving entity.

In the first model, the moving entity is a protein or protein complex which binds to the ds DNA end, travels along the DNA, and initiates DNA strand exchange at a distance from the break (Figure 7A). This model is the same as that proposed for the RecBCD pathway of recombination in *E. coli* (Smith et al., 1984), which is supported by much evidence (Kowalczykowski et al., 1994; Myers and Stahl, 1994; Smith, 1994, 2001). We note that in this pathway a ds DNA break is not a hot spot of recombination (Lam et al., 1974); rather, it is an entry site for the RecBCD enzyme, which travels through the DNA and produces, as far as 30 kb or more from the entry site, a single-stranded DNA tail that undergoes strand exchange with a homolog. In *S. pombe*, a similar traveling recombination machine might promote recombination as far as ~100 kb from the entry site.

In the second model, the moving entity is a special DNA structure. This structure might be a double Holliday junction formed by the mechanism proposed by Szostak et al. (1983) for ds break repair (Figure 7B). Though formed at the site of the ds break, the junctions could move in tandem by branch migration to a distant site. In this case, the resolution of the junctions could produce a crossover, and perhaps gene conversion, at the distant site. The ds break site might be a hot spot of gene conversion but would not be a hot spot of crossing over, since after branch migration there is no connection between the DNA duplexes at the break site. Tandem branch migration of the dual junctions would leave no hybrid DNA, and consequently no gene conversion or crossing over, between the ds break site and the site of resolution.

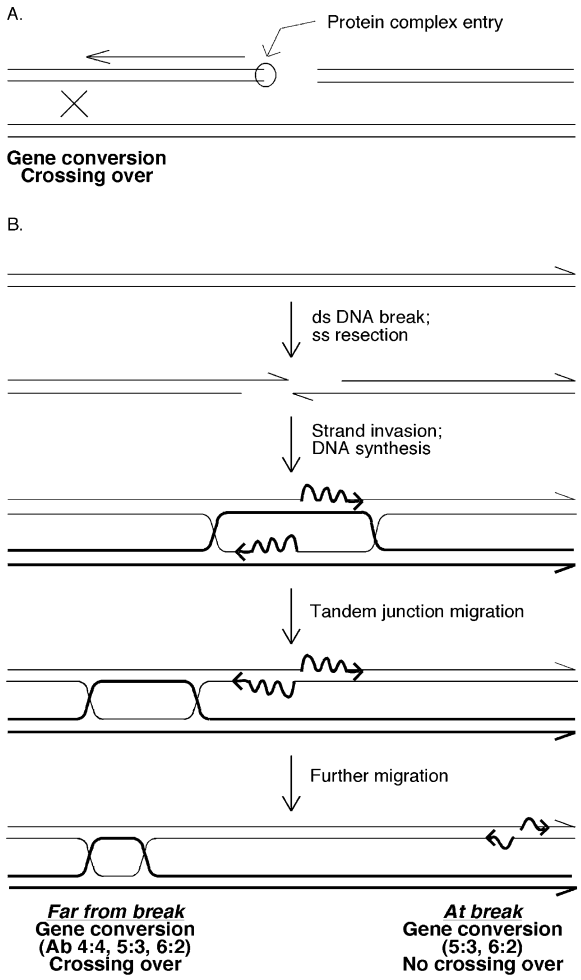

Figure 7. Two Models for Recombination Remote from a Double-Strand Break

(A) A protein or a protein complex binds to the double-strand end, travels along the DNA, and promotes recombination at a distance from the break. This mechanism is exemplified by the RecBCD pathway of *E. coli* (Smith et al., 1984; Smith, 2001).

(B) Single-strand resection at each end of the double-strand break produces 3′ ss DNA tails. One tail invades the homolog to produce a D-loop. DNA synthesis primed by the 3′ ends and templated by the homolog restores double-strand DNA. Ligation of ends produces two Holliday junctions, as proposed by Szostak et al. (1983) and supported by observations in *S. cerevisiae* (Roeder, 1997). The Holliday junctions migrate in tandem and are resolved at a distance from the break. Gene conversion but not crossing over could occur at the site of the double-strand break, whereas both could occur at the site of resolution. For details, see Discussion. (B) is reprinted, with permission, from the Annual Review of Genetics, Volume 35, 2001.

Some events at the *ARG4* locus of *S. cerevisiae* may reflect the mechanism in Figure 7B. In one study (Gilbertson and Stahl, 1996), ~10% of the non-Mendelian asci reflect heteroduplex DNA on one chromatid on each side of a prominent DNA beak site without associated crossing over. These events have been interpreted as

the result of resolution of a double Holliday junction by topoisomerase "pullout," the separation of the joined duplexes in the vertical direction as drawn in Figure 7B (third panel from the bottom). Tandem branch migration in the horizontal direction would, however, have the same genetic consequences near the site of the ds DNA break. Distant crossing over could occur following horizontal branch migration but not following topoisomerase pullout.

Meiotic recombination may, in some cases, occur far from a ds DNA break in *S. cerevisiae*. On chromosome III of *S. cerevisiae*, there is an ~100 kb region with very little meiotic DNA breakage but a substantial amount of crossing over (Baudat and Nicolas, 1997). This region is, at least superficially, similar to the ~100 kb region between *mbs1* and *mbs2* of *S. pombe* (Figures 4 and 6). At the *S. cerevisiae HIS4* locus, there is, in numerous mutants, a good linear relation between the frequency of ds breaks and the frequency of non-Mendelian segregation; this line extrapolates, however, to ~10% non-Mendelian segregation with 0% DNA breakage (Fan et al., 1995). The wild-type strain manifests ~30% non-Mendelian segregation. Thus, about one-third of the meiotic recombination at *HIS4* appears to be independent of ds breaks at this locus; these events may stem from distant DNA breaks or from another source.

These observations raise the possibility that meiotic recombination differs in the two distantly related yeasts *S. pombe* and *S. cerevisiae* primarily in the ratio of events near and far from DNA breaks (Smith, 2001). Meiotic recombination may be mostly near DNA breaks in *S. cerevisiae* and far from them in *S. pombe*.

Experimental Procedures

Strains

The *S. pombe* strains used for analysis of meiotic DNA carried the h⁻ *pat1-114 end1-458 ade6-M26* markers plus the following: GP2956 [*rad50*S (K81I)], GP3087 (*rad50S/rad50S* + *lys4-95/his4-239* + diploid), GP3135 (*rad50S rec12-170::3HA-6His-kanMX6*). Strains GP338 and GP535 were described by Cervantes et al. (2000). The strains used for recombination frequency determinations contained one, two, or three mutations in the *lys3–pro1* interval on chromosome I (see supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1 for a description of these mutations); some contained additional mutations in *ade6*, *ura4*, or *leu1*, on chromosome III, III, or II, respectively. The complete genotypes and genealogies are available upon request.

Induction of Meiosis and Analysis of DNA

Strains were grown, induced for meiosis, and analyzed for DNA content by flow cytometry as described (Cervantes et al., 2000). For analysis of DNA breakage, ~6×10^8 cells (30 ml of culture) were washed in cold 50 mM EDTA (pH 8.0) and converted to spheroplasts, either in liquid as done previously (Cervantes et al., 2000; Figures 1C, 2B–2D, and 5) or after embedding the washed cells in agarose (Figures 1A, 1B, 1D, 2A, and 3); this latter procedure gives more uniform yields of DNA and reduces mechanical breakage of DNA. The washed cells were suspended in 300 μl of CPES (400 mM EDTA [pH 8.0], 120 mM Na_2HPO_4, 40 mM citric acid, 1.2 M sorbitol, 10 mM NaN_3, 5 mM DDT, 1 mg/ml lyticase [Sigma catalog #L-4025] and 5 mg/ml lysing enzymes [Sigma catalog #L-1412]; [final pH 7.0]), 400 μl of 2% low-melting-point agarose in 0.25 M EDTA (pH 8.0), 1.2 M sorbitol was added, and the mixture was poured into 0.1 ml plug molds. After ~10 min at 4°C, the plugs were ejected into 1.8 ml of CPES and incubated at 37°C for 1 hr. The buffer was removed and replaced with 1.8 ml of NDS/PK (0.5 M EDTA [pH 8.0], 10 mM Tris-HCl [pH 7.5], 10 mM NaN_3, 1% Na lauryl sarcosine, 1 mg/ml Proteinase K). After incubation overnight at 50°C, the buffer was replaced with SDS/PK (NDS/PK with 0.5% SDS in place of Na lauryl sarcosine and NaN_3). After incubation overnight at 50°C, the plugs were soaked in 2 ml of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) + 0.5 mM PMSF for ~1 hr at room temperature and then in 2–3 washes of TE. Plugs were stored in TE at 4°C. DNA in the plugs was digested with restriction enzymes as recommended by New England Biolabs and analyzed by pulsed-field gel electrophoresis followed by Southern blot hybridization as described previously (Cervantes et al., 2000). The probes are described in the supplemental data at http://www.molecule.org/cgi/content/full/9/2/253/DC1. Quantitation was done with a PhosphorImager (Molecular Dynamics) and Excel software (Microsoft). Sizes of DNA fragments were estimated from *S. cerevisiae* chromosomal DNA and phage λ DNA (multimers and restriction digestion products) visualized by staining with ethidium bromide.

Meiotic Recombination Frequencies

Haploid strains containing appropriate markers were mated at 25°C on SPA sporulation agar supplemented with all required nutrients (100 μg/ml) (Gutz et al., 1974). After 2–4 days, spores free of vegetative cells were prepared by treatment with glusulase and EtOH and plated on YEA yeast extract agar (Gutz et al., 1974) + adenine (100 μg/ml) when required. Well-isolated colonies were toothpicked to grids on YEA + adenine and replicated to appropriate test media: EMM2 (Gutz et al., 1974) ± lysine for *lys3-37*; ± uracil for *ura1-61*, *mkh1*::*ura4*⁺, and *res2*::*ura4*⁺; ± proline or arginine for *pro1-1*; YEA + adenine and phloxin (20 μg/ml) at 37°C for *aur1-18* and *mis6-302*; YEA ± UV (150 J/m² in a Stratagene Stratalinker) for *rad15-P* and *rqh1-h2*; YEA ± G418 (100 μg/ml) for *pom1*::*3HA-kanMX6*; EMM2-NH_4Cl followed by microscopic examination of cell shape (Young and Fantes, 1987) for *res2-J3* and *res2-G1*. In crosses with *rad15-P*, which interferes with the *res2* phenotype (data not shown), meiotic segregants recombinant for the *rad15-P* and *ura1-61* markers flanking *res2* were first identified. These were assayed for *res2-J3* by PCR amplification of a 920 bp DNA fragment followed by digestion with NlaIV, a site for which is destroyed by *res2-J3*, and gel electrophoresis. Several recombinants from each cross were purified and retested to verify the scoring.

Mutations Used for Recombination Analysis

Each previously undetermined mutation in the *lys3-pro1* interval was sequenced by PCR amplification of DNA fragments from one wild-type and two mutant strains. The entire open reading frame of the gene was sequenced from these fragments and compared with the sequence in GenBank. In each case, the two mutant sequences were identical to the wild-type sequence and that in GenBank, except for one base pair as indicated in the supplemental data (http://www.molecule.org/cgi/content/full/9/2/253/DC1). For previously determined mutations (*aur1-18*, *rqh1-h2*, and *mis6-302*), DNA from a single mutant strain was sequenced and found to contain the reported single base pair difference from the GenBank sequence.

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