Molecular Cell, Vol. 9, 847–855, April, 2002, Copyright ©2002 by Cell Press

Meiotic DNA Breaks at the *S. pombe* Recombination Hot Spot *M26*

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Summary

The *ade6-M26* allele of *Schizosaccharomyces pombe* creates a well-defined meiotic recombination hot spot that requires a specific sequence, 5′-ATGACGT-3′, and the Atf1•Pcr1 transcription factor for activity. We find that *M26* stimulates the formation of meiosis-specific double-strand DNA breaks at multiple sites surrounding *M26*. Like hot spot activity, breakage requires the *M26* heptamer, Pcr1, and the general recombination factor Rec12. When the *M26* heptamer is moved to new positions within *ade6*, new break sites are observed spanning ~0.5–2 kb around the moved heptamer. Break frequency is strongly correlated with recombination frequency for these alleles. The occurrence of breaks at *M26* suggests mechanistic similarities to hot spots in the distantly related yeast *Saccharomyces cerevisiae*.

Introduction

During meiosis, four haploid cells (gametes or spores) are generated from a single diploid cell. One of the hallmarks of meiosis is a greatly elevated level of recombination between homologous chromosomes prior to the first meiotic division, typically 100- to 1000-fold higher than mitotic rates (reviewed in Esposito and Wagstaff, 1981; Fox and Smith, 1998). This recombination serves at least two important functions. First, it forms attachments (chiasmata) between homologs, which facilitate proper segregation of chromosomes at the first meiotic division. Second, it shuffles alleles between homologous chromosomes, thus increasing the genetic diversity on which natural selection can act.

Recombination does not occur evenly throughout the genome of many organisms, but at lower than average frequency in some regions (cold spots) and higher than average in others (hot spots). It was first shown that double-strand DNA breaks (DSBs) are associated with a hot spot of recombination at the *ARG4* locus of the budding yeast *Saccharomyces cerevisiae* (Sun et al., 1989). Nicolas et al. (1989) reported that markers near the break site convert more frequently than markers far from the break site, supporting the proposal that meiotic recombination is initiated at double-strand breaks (Resnick, 1976; Szostak et al., 1983). Other genetically defined hot spots in *S. cerevisiae* have subsequently been shown to be sites of DSBs, and these breaks may initiate most if not all meiotic recombination in that organism (for review, see Pâques and Haber, 1999). While no simple unique DNA sequence has been associated with the formation of DSBs, they seem to occur preferentially in regions of higher than average G+C content (Gerton et al., 2000).

In contrast, the *ade6-M26* hot spot of the distantly related yeast *Schizosaccharomyces pombe* does require a discrete sequence, 5′-ATGACGT-3′, for activity (Schuchert et al., 1991). The *M26* hot spot was discovered originally as a unique allele of the *ade6* gene, *ade6-M26* (Gutz, 1971), but the heptamer sequence has since been shown to be an active hot spot at multiple positions within *ade6* and also in the distantly located *ura4* gene (Fox et al., 1997 and Figure 1). The *M26* sequence serves as a binding site for the Atf1•Pcr1 transcription factor, an essential component for hot spot activity (Kon et al., 1997). Mutation of any one base in the heptamer abolishes both its hot spot activity and its ability to bind Atf1•Pcr1 in vitro (Schuchert et al., 1991; Wahls and Smith, 1994). However, *M26* is not the only sequence to which Atf1•Pcr1 can bind: at least two other sequences closely related to *M26*, TGACGTA and TGACGTC, bind to Atf1•Pcr1 and are also meiotic hot spots (Fox et al., 2000).

Like *S. cerevisiae*, meiosis-induced DSBs occur in *S. pombe*, the only other organism in which such breaks have been reported (Cervantes et al., 2000; Murakami and Nurse, 2001). Meiotic recombination and break formation are intimately associated in *S. pombe* since both require the action of at least eight meiotic *rec* gene products. Therefore, it is quite likely that these breaks initiate recombination. If the *M26* hot spot operates by a mechanism similar to that of hot spots of *S. cerevisiae*, then it should also be the site of a DNA break. However, repeated searches in this laboratory and others failed to show either double- or single-strand breaks in the vicinity of *M26* (Ponticelli et al., 1988; Szankasi et al., 1988; Bähler et al., 1991), raising the possibility that *M26* may operate by a fundamentally different mechanism from hot spots of *S. cerevisiae*.

Characterization of DNA break sites in *S. cerevisiae* has been greatly aided by separation-of-function mutations in the *RAD50* gene, known as *rad50S* mutations (Cao et al., 1990; Sun et al., 1991; de Massy et al., 1995; Liu et al., 1995; Xu and Petes, 1996). These mutant alleles allow breaks to be formed but not resected or repaired during meiosis. The absence of resection results in the formation of discrete broken fragments rather than a heterogeneous mixture of partially resected fragments, while the failure to repair results in broken fragments accumulating to higher levels during the course of meiosis. Thus, in a *rad50S* background, DNA break sites are more easily detected than in wild-type.

A *rad50S* allele has also been recently created in *S. pombe*. This allele creates a lysine-to-isoleucine substitution at position 81 of the protein, similar to one of the commonly used *rad50S* alleles in *S. cerevisiae* (Farah et al., 2002). Meiotic DNA breaks in *S. pombe* are formed at similar positions in the *rad50*$^+$ and *rad50S* back-

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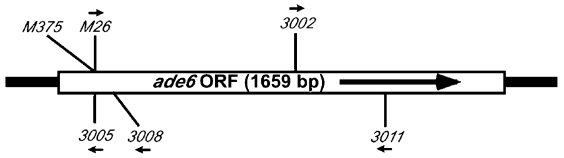

Figure 1. Map of the *S. pombe ade6* Gene

All of the alleles shown except *M375* contain the *M26* heptamer sequence, 5′-ATGACGT-3′, oriented as shown by the small arrows. The open rectangle represents the *ade6* ORF, and the large arrow shows the direction of transcription.

grounds, but failure to repair breaks in the *rad50*S mutant results in their persistence and higher level throughout the late stages of meiosis (Young et al., 2002), as has been previously observed in *S. cerevisiae*. The *S. pombe rad50*S allele allowed us to revisit the question of DSBs at the *M26* hot spot and to show that *M26* is, in fact, a site of multiple meiosis-induced DSBs.

Results

M26 Is a Site for Meiosis-Induced DNA Breaks

Meiosis was induced using the *pat1-114* allele (Iino and Yamamoto, 1985). This temperature-sensitive form of the Pat1 protein kinase allows a synchronous meiosis in a large population of cells by shifting the culture to the nonpermissive temperature (34°C). When diploid cells are arrested in G1 prior to the meiotic induction, a *pat1-114*-induced meiosis resembles a wild-type, starvation-induced meiosis in all relevant respects, including meiotic levels of recombination (Li and Smith, 1997; Watanabe et al., 2001). The *pat1-114* allele can also induce meiosis in haploid *S. pombe*. Though the resulting spores show low viability due to chromosome deficiencies (Nurse, 1985), DNA breakage occurs with the same timing and intensity as in diploids (Cervantes et al., 2000; Young et al., 2002). Further, *M26* shows normal hot spot activity in plasmid-by-chromosome recombination in a haploid *pat1-114*-induced meiosis (Li and Smith, 1997; M.E. Fox and G.R.S., unpublished data).

To look for DNA breaks at *M26*, we induced meiosis in a haploid strain containing the *rad50*S allele (GP2956). In these experiments, cells arrested in G1 underwent premeiotic DNA replication between 2 and 3 hr after the temperature shift and maintained a G2 content of DNA for the remainder of the experiment (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/4/847/DC1). At the indicated time points, cell samples were removed, and DNA was purified in agarose plugs in order to minimize mechanical DNA breakage. DNA was then digested with an appropriate restriction enzyme, resolved by gel-electrophoresis, and analyzed by Southern blot hybridization.

In Figure 2A, a 40.6 kb PacI fragment containing the *ade6-M26* allele was analyzed for meiotic DNA breaks. Through the first 2.5 hr of meiosis, only the parental 40.6 kb PacI fragment was observed. Beginning at 3 hr, after the completion of DNA replication, distinct subfragments became visible and reached their maximum intensity by 5 hr. These breaks were not repaired, as previously observed at other chromosomal sites in the *S. pombe* genome (Cervantes et al., 2000), but persisted for the remainder of the meiosis due to the *rad50*S allele (Young et al., 2002). Four discrete break sites were seen within the PacI *ade6* fragment. One of these break sites was at or very close to the location of the *M26* heptamer (Figure 2A, solid arrow); the other break sites were outside of the *ade6* gene, approximately 6, 8, and 16 kb to the left of *M26* as shown in the diagram (Figure 2A, bottom). When this blot was stripped and probed from the other side of the parental fragment, a complementary set of broken fragments was observed (data not shown). A diploid strain (GP3087) containing the *ade6-M26* allele formed breaks at similar frequencies and at the same time and positions as the haploid (Table 1 and data not shown).

Since the break near *M26* was readily observed in the *rad50*S background, we asked whether the *rad50*S mutation was essential to see the break. Strain GP535 (*ade6-M26 rad50*$^+$) was analyzed for break formation as in Figure 2A. In this strain, a break at *M26* was visible between 3 and 4 hr of meiosis but was repaired at later time points (Figure 2C). This result is similar to what has been observed for other meiotic DNA breaks in *S. pombe rad50*$^+$ strains (Cervantes et al., 2000). The break at *M26* was noticeably weaker in the *rad50*$^+$ strain compared to *rad50*S (Table 1), which is expected if the cells were not perfectly synchronized and/or there is some overlap in the times of break formation and repair. In the following experiments, all strains contained the *rad50*S allele.

If the *M26* heptamer is required for the observed break within *ade6*, then alleles of *ade6* lacking an *M26* heptamer should not show a break at the same position. We therefore tested a strain containing the *ade6-M375* allele. The *M375* allele contains a G→T transversion identical to the *M26* mutation but 3 bp to its left (Figure 1; Szankasi et al., 1988), thus serving as an ideal control for experiments involving *ade6-M26*. Figure 2D shows that the break previously seen in *ade6-M26* did not occur in the *ade6-M375* allele. Breaks at the three sites outside of the *ade6* gene, however, still occurred at the same positions and at comparable frequencies (Table 1). These additional breaks demonstrate that meiotic DNA breakage occurred normally outside of the *ade6* gene and imply that *M26* stimulates breakage only near itself. Other alleles of *ade6* that lack an *M26* heptamer (*ade6*$^+$, *M210*, *3006*, and *3010*) gave results similar to those seen with *ade6-M375* (data not shown).

In order to determine more precisely the location of the break near *M26*, we analyzed a smaller (1783 bp) fragment containing *M26* (Figure 3). From this experiment, it can be seen that there is not a single break site but multiple, closely spaced break sites surrounding *M26*. Interestingly, *M26* itself lies in a ~70 bp region that is free of visible breaks. The *ade6-3005* allele, which contains an inverted heptamer at the same position as *ade6-M26* (Fox et al., 1997), showed an essentially identical pattern of breaks at this level of resolution (data not shown).

Requirements for DNA Breakage at *M26*

As previously mentioned, *M26* hot spot activity is strictly dependent on the Atf1•Pcr1 heterodimer (Kon et al.,

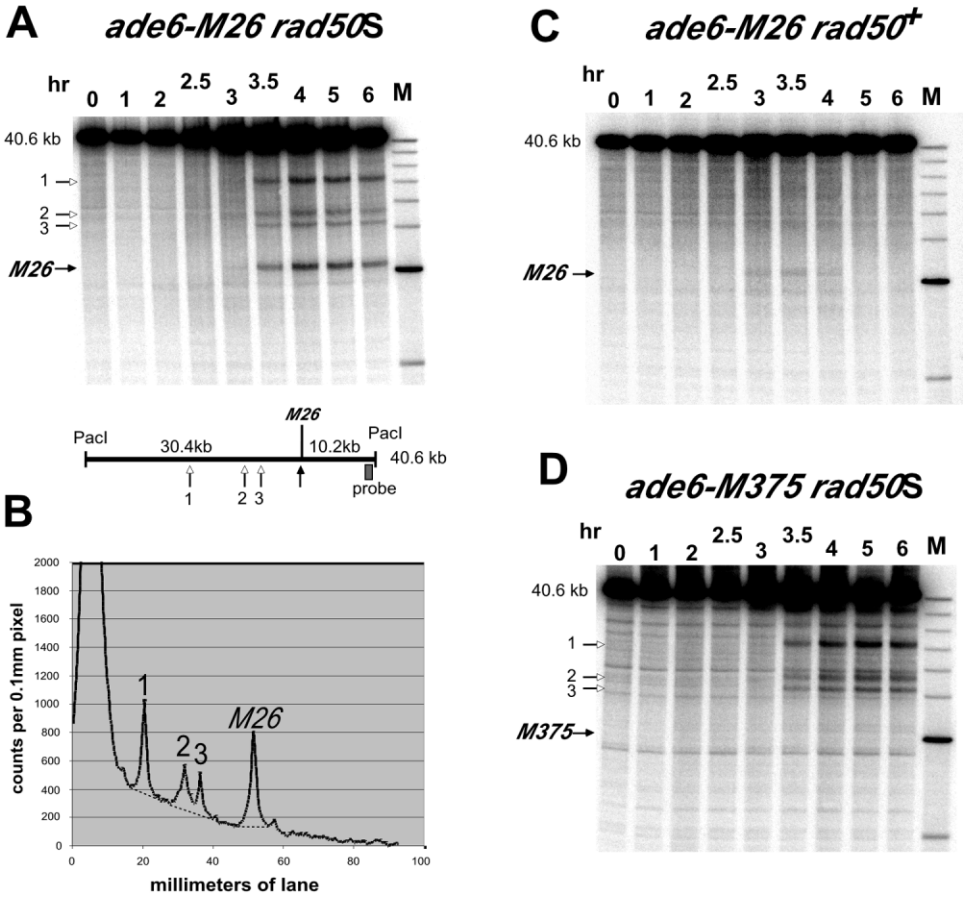

Figure 2. *M26* Is a Site of DNA Breakage during Meiosis

DNA was prepared at the indicated time points from strains induced for meiosis and digested with PacI to produce a 40.6 kb fragment containing the *ade6* gene. Digested DNA was separated on a pulsed-field gel, blotted, and probed from the right as indicated in the diagram ([A], bottom).

(A) Strain GP2956 (*ade6-M26 rad50*S). A break at *M26* produces a 10.2 kb subfragment (solid arrow). Three additional breaks found in the main fragment to the left of *M26* are indicated by the open arrows.

(B) Quantitation of DNA breakage. The average number of counts per 0.1 mm pixel from the top to the bottom of the 5 hr lane in (A) is shown. The large peak at the left of the graph represents the parental fragment and is largely off scale (peak value = 72,227 counts) in order to clearly see the broken fragment peaks. The dashed lines show the area included in each peak for quantitation. The end points of each line show the pixel values used in determining background, as described in Experimental Procedures. Numbers above the peaks correspond to fragment numbers shown in (A).

(C) Strain GP535 *(ade6-M26 rad50*$^+$). A weak break at *M26* is visible between 3 and 4 hr of meiosis (arrow) but is repaired at later time points. Breaks 1, 2, and 3 seen in the *rad50*S strain were not observed in this experiment, possibly due to high background hybridization near the top of the gel and lower apparent levels of DNA breakage in *rad50*$^+$ strains.

(D) Strain GP3289 (*ade6-M375 rad50*S). Breaks 1, 2, and 3 outside of the *ade6* gene are observed (open arrows), but the break at the former position of *M26* is absent. Bands seen across all time points are likely due to cross hybridization, which varies from blot to blot, and frequently correspond to regions of the gel that stain intensely with ethidium bromide. A 5 kb DNA ladder (M) was used as a size marker in each blot. The sizes range from 5–40 kb in 5 kb increments.

1997, 1998). Mutations in either *atf1* or *pcr1* abolish hot spot activity but have no effect on basal recombination levels within *ade6*; i.e., *M26* and *M375* recombine with a test allele at approximately the same frequency in *atf1* or *pcr1* mutants (Kon et al., 1997). Therefore, we tested break formation in a strain deleted for *pcr1*. The result of this experiment (Figure 4A) was very similar to what was observed with *ade6-M375*. Though the *ade6-M26* allele was present in this strain, the absence of Pcr1 eliminated visible breakage in the *ade6* gene. The breaks outside of the *ade6* gene again occurred at their normal positions but at slightly reduced frequencies (Table 1). The reduced frequency of the *M26*-independent breaks may be related to the reduced efficiency with which *pcr1* mutants undergo meiosis (Watanabe and Yamamoto, 1996; Kon et al., 1998). It was not possible to determine the Atf1 dependence of the breaks at *M26*, because *atf1* mutants exhibit strong defects in meiotic development and also suppress the temperature sensitivity of the *pat1-114* allele (Shiozaki and Russell, 1996; Wilkinson et al., 1996).

In order to test if the DNA breaks at *M26* require the same general recombination factors required for the formation of other meiotic DNA breaks in *S. pombe* (Cervantes et al., 2000), we tested a *rec12* mutant. Rec12 is a homolog of Spo11, the protein that makes meiotic

Table 1. Meiotic DNA Breakage at *M26* and Other Sites in the 40.6 kb *ade6* PacI Fragment

ade6 Allele[a] (Strain)	Site 1	Sites 2 + 3	*M26* Site
ade6-M26 (GP2956)	0.85[b]	0.38	1.1
	1.0	1.2	1.3
ade6-M26 diploid (GP3087)	1.2	1.2	1.9
	1.1	1.5	1.2
ade6-M26 rad50$^+$ (GP535)	------[c]	------	0.28
			0.33
ade6-M26 Δ*pcr1* (GP3110)	0.32	0.48	<0.02
	0.58	0.35	<0.07
ade6-M375 (GP3289)	1.3	1.0	<0.09
ade6-3005 (GP3292)	1.2	1.2	3.3
	1.3	1.0	2.7
ade6-3008 (GP3294)	0.69	0.11	0.10
	1.4	0.66	0.34
	1.1	1.1	0.19
ade6-3002 (GP3105)	0.78	0.99	0.73
	1.3	0.89	0.61
	1.0	0.85	0.48
	0.43	0.55	1.1
ade6-3011 (GP3160)	0.36	0.19	11.2
	0.37	0.38	11.7

[a] Strains contain the indicated *ade6* alleles (see Figure 1) and *h*$^-$ *smt-0 pat1-114 end1-458 rad50S* unless otherwise indicated.
[b] Data are the percentages of broken DNA relative to the total DNA in the indicated fragment from the 5 or 6 hr time point. The results of at least two experiments are shown in most cases. Fragments 2 and 3 were frequently not well resolved and are therefore expressed as the sum of both fragments.
[c] ------ indicates the value was not determined.

DNA breaks in *S. cerevisiae* (Keeney et al., 1997). Mutations in *rec12* reduce meiotic recombination ~1000-fold in *S. pombe* (DeVeaux et al., 1992; Davis and Smith, 2001) and eliminate all detectable meiotic DNA breakage at the sites previously tested (Cervantes et al., 2000; Young et al., 2002). As expected, breaks were not observed at *M26* or elsewhere in a *rec12* mutant (Figure 4B).

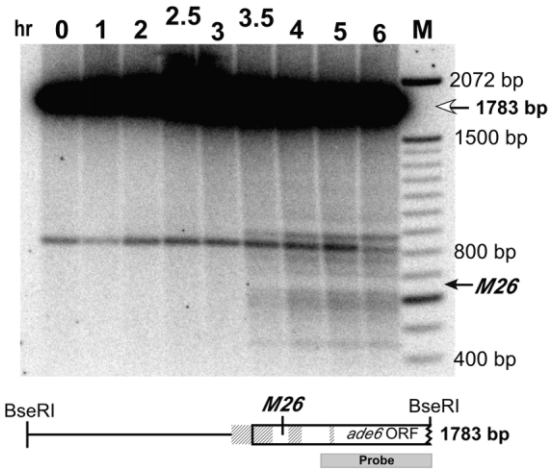

Figure 3. Meiotic DNA Breaks Occur to Both Sides of but Not within the *M26* Heptamer

Meiotic DNA samples from GP3087 (*ade6-M26 rad50*S diploid) were digested with BseRI to produce a 1783 bp fragment containing *M26* (diagram). The position of *M26* in the blot is shown (solid arrow). The partial *ade6* ORF is indicated by the open rectangle. Breaks are indicated by the cross-hatched rectangles and are proportionate to the regions covered by breaks. Size marker (M) is a 100 bp ladder with sizes indicated. The prominent band of approximately 800 bp spanning all lanes of the blot was not seen in other experiments and may be due to overdigestion by BseRI.

Break Sites Change in Position and Frequency When *M26* Is Moved

The *M26* hot spot was originally discovered as a unique allele of the *ade6* gene that showed an unusually high level of recombination compared to other alleles of *ade6* (Gutz, 1971). It has since been shown that the *M26* heptamer sequence can create a recombination hot spot when placed at multiple sites within the *ade6* gene up to 1.1 kb from its original location (Fox et al., 1997; Figure 1). We tested whether these other *ade6* alleles also stimulated DNA break formation and asked whether the positions of the breaks were affected by the position of the *M26* heptamer.

Five alleles of *ade6* containing an *M26* heptamer were tested for meiotic break formation (Figure 5). In these experiments, the DNA was digested with AflII, producing a 6584 bp fragment containing *ade6* near its middle. Meiotic breaks were observed in all of the alleles tested, and the location of the breaks clustered around the site of the *M26* heptamer. Probing these blots from the other end of the AflII fragment revealed a complementary set of broken fragments (data not shown).

The distribution and number of break sites seen in each of the different *M26*-containing alleles were variable, but some trends are evident. Breaks occurred to both sides of *M26* and clustered near the heptamer. However, there also appeared to be preferential locations for break formation irrespective of the position of *M26* (Figures 5B and 5C). For example, the promoter region of *ade6* was a site of breakage in all of the alleles shown. This fact is most remarkable in the case of the *ade6-3011* allele, which was able to stimulate breaks at this site from a distance of approximately 1300 bp (Figures 1 and 5). Furthermore, all of the breaks observed in *ade6-3002* occurred at positions identical to those in *ade6-3011* (within the limits of resolution shown), even though the *M26* sequences are separated by more than 300 bp in these two alleles.

An unexpected discovery in the analysis of the *M26* heptamer moves was the large variability in the frequency of breakage among the different *ade6* alleles. The frequency of breakage in *ade6* and the three other break sites within the 40.6 kb PacI fragment (Figure 2A) was quantitated for most of the *ade6* alleles tested in this study (Figure 2B, Table 1, and data not shown). In order to determine whether there was a correlation between the frequency of breakage and the frequency of recombination within *ade6*, we plotted our data against the recombination intensities for each of the *M26*-containing alleles. Recombination intensities were calculated from the data of Fox et al. (1997) by normalizing the frequency of Ade$^+$ recombinants in each of the reported crosses for the distance between the markers. The recombination intensity for a given *M26* allele was taken to be the mean Ade$^+$ recombinant frequency per kb of all crosses involving that allele. Excluding the *ade6-3011* allele, there was a strong, positive linear cor-

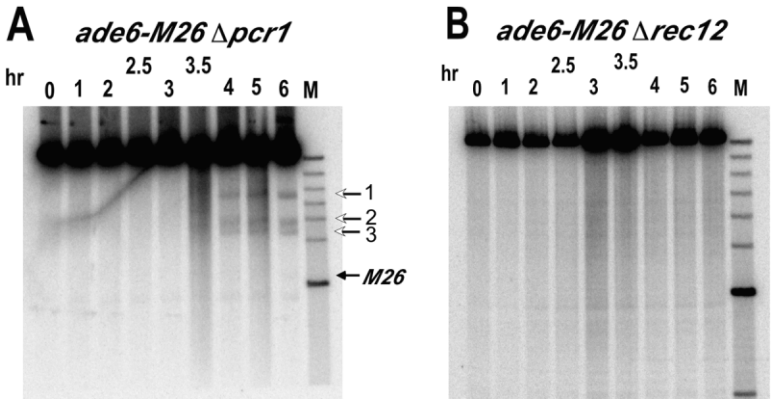

Figure 4. The Meiosis-Induced Break at *M26* Requires Pcr1 and Rec12

Strains were induced for meiosis, and DNA was analyzed as described in Figure 2.
(A) GP3110 (*ade6-M26 Δpcr1::his7*$^{+}$). Open arrows indicate meiosis-induced breaks. Solid arrow indicates the position of *M26*.
(B) GP3135 (*ade6-M26 Δrec12::kan*).

relation between the frequency of breakage and the intensity of recombination (Figure 6). This linear relation for most of the markers indicates that DNA breakage is causally related to the hot spot activity of these alleles. The anomalous behavior of the *ade6-3011* allele is discussed below.

Discussion

Here, we have shown that the *M26* hot spot is a site of meiosis-induced DNA double-strand breaks. Both hot spot activity and DNA breakage at *M26* require the *M26* sequence itself, the specific hot spot factor, Pcr1, and the general recombination factor, Rec12 (Figures 2 and 4). These data demonstrate an intimate association between the hot spot activity of *M26* and the DNA breaks observed in its vicinity. *M26* shows a gradient of gene conversion extending to either side of itself, with near markers coconverted at higher frequency than far markers (Gutz, 1971; Grimm et al., 1994; Zahn-Zabal and Kohli, 1996). Thus, the simplest explanation for the breaks near *M26* is that they act as initiating lesions for homologous recombination, as predicted by the models of Resnick (1976) and Szostak et al. (1983).

While we have not yet analyzed how the breaks at *M26* are processed, the genetic data argue against extensive double-strand gap formation. In mismatch repair-deficient mutants of *S. pombe*, approximately 90% of *M26*-stimulated conversion events display postmeiotic segregation (Fleck et al., 1999), indicating heteroduplex DNA at *M26*. Such heteroduplex DNA is consistent with single-strand but only minimal double-strand resection at the break sites. In this respect, the *M26* hot spot of the fission yeast *S. pombe* seems to behave similarly to hot spots of recombination in the budding yeast *S. cerevisiae*. One difference between the two yeasts, however, is that the breaks stimulated by *M26* occur over a larger region than that typically seen in *S. cerevisiae*. In *S. cerevisiae*, breaks at recombination hot spots are typically confined to regions of ~150 bp (de Massy et al., 1995; Liu et al., 1995; Xu and Kleckner, 1995; Xu and Petes, 1996), whereas the breaks stimulated by *M26* were spread over regions of at least 450 bp (*ade6-M26*, *3005*, *3008*) to almost 2 kb (*ade6-3011*).

The generation of breaks far from *M26* helps to explain two puzzling genetic observations regarding this hot spot. (1) In one study, it was concluded that *M26* was able to stimulate conversion of nearby markers, without itself being converted (Grimm et al., 1990). In those experiments, markers 113 and 133 bp to the left of *M26* converted 4-fold more frequently in the presence of *M26* than in its absence, but *M26* itself appeared not to convert in up to two-thirds of these events. (2) In another study, involving a mismatch repair-deficient strain of *S. pombe*, *M26* underwent aberrant 4:4 segregation in 30% of the total conversion events at that site, implying the presence of symmetric heteroduplex DNA (Fleck et al., 1999). These data can now be explained by our observation that *M26* stimulates breaks at some distance from itself (Figure 3). If single-strand resection at the break site frequently extends over one *ade6* marker but fails to extend past *M26*, a joint molecule containing heteroduplex DNA to one side of *M26* could be established. Resolution of this structure and subsequent mismatch repair would frequently convert markers contained in the heteroduplex, while leaving *M26* unchanged. If Holliday junction formation and branch migration occurred prior to resolution of the joint molecule, *M26* could also be pulled into heteroduplex DNA. This heteroduplex would be symmetric and, in the absence of mismatch repair, would give rise to aberrant 4:4 segregation as observed by Fleck et al. (1999).

The pattern of breakage in the *ade6* alleles we analyzed may reflect a general opening of chromatin in the vicinity of *M26*, as observed by Mizuno et al. (1997), making the DNA accessible to cleavage, presumably by Rec12. Open chromatin associated with recombination hot spots and DNA breakage is also observed in *S. cerevisiae* (Ohta et al., 1994; Wu and Lichten, 1994). The pattern of breakage observed at *M26* suggests that opening is greatest near the heptamer and dissipates to either side. This pattern is most clearly illustrated with the *ade6-3011* allele, which showed by far the highest frequency of breakage (>11%) of all the *M26*-containing alleles we analyzed. Here, it can be seen that the most intense breaks occurred in a ~400 bp region surrounding the heptamer. The intensity of breakage then gradually diminished with increasing distance from *M26*, forming a roughly bell-shaped distribution (Figure 5; additional data not shown). We suspect that a similar pattern of breakage also occurs in the other *M26* alleles, but the lower break frequency at distant sites precludes its detection.

In all of the *M26* alleles we analyzed (with the possible

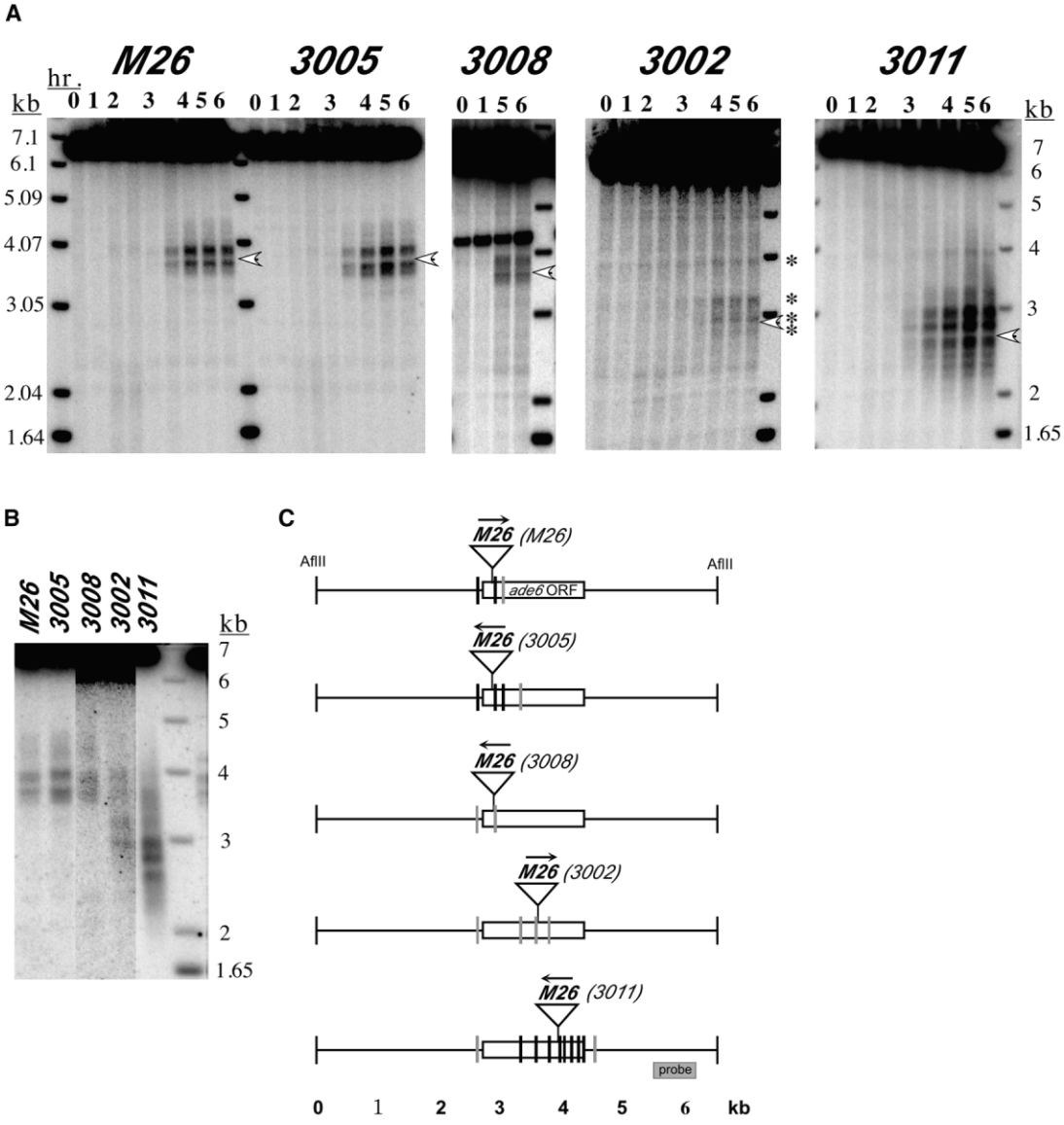

Figure 5. Breaks Move When *M26* is Moved

(A) Southern blot hybridization of DNA from strains with the *M26* heptamer at various positions within *ade6* (Figure 1). Meiotic inductions and preparation of DNA were performed as described in Figure 2, except DNA was digested with AflII prior to electrophoresis, which produces a 6584 bp *ade6*-containing fragment. Time points are indicated above the lanes. Sizes of DNA markers used in the experiments are shown to the left (for *M26* and *3005*) and right (for *3008*, *3002*, *3011*). Open arrowheads represent the position of *M26* on the blot. Asterisks indicate the positions of meiosis-specific breaks in *ade6-3002* for clarity. Strains: GP3087 (*M26*), GP3292 (*3005*), GP3294 (*3008*), GP3105 (*3002*), and GP3160 (*3011*). The prominent band of ~4.3 kb seen in *ade6-3008* is not meiosis specific and may be due to overdigestion by AflII.

(B) 5 hr DNA samples from the strains shown in (A) were run together on a single gel in order to compare the positions of breaks in each allele. Exposure levels in each lane were adjusted appropriately in order to see breaks clearly, resulting in the apparent discontinuities between lanes.

(C) Schematic diagram showing the positions of breaks within the *ade6* AflII fragment for the indicated alleles. Dark gray and light gray lines represent breaks >0.2% and <0.2% of total DNA, respectively. Open rectangle represents the *ade6* ORF.

exception of *ade6-3008*), the most intense breaks mapped close to but not within the *M26* heptamer. For two of the alleles (*ade6-M26* and *ade6-3005*), we mapped the position of the breaks with higher resolution and found that *M26* mapped within a ~70 bp region that appeared to be essentially free of breaks (Figure 3 and data not shown). We suspect this break-free region is due to binding of the Atf1•Pcr1 heterodimer to *M26* and protection of the DNA from endonucleolytic cleavage. In vivo protection of *M26* by Atf1•Pcr1 was also observed by Kon et al. (1998) using dimethyl sulfate footprinting.

An "open" chromatin structure appears to be an essential component for high-frequency cleavage near the *M26* hot spot. The *M26* heptamer generates a micrococcal nuclease (MNase) hypersensitive site, which is 5-fold more sensitive in meiotic than premeiotic cells (Mizuno et al., 1997). This hypersensitive site is not observed in

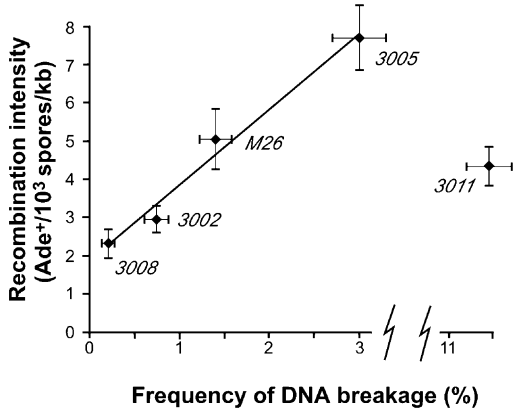

Figure 6. Relation between the Frequency of DNA Breakage and Recombination within *ade6*

Break frequencies are from Table 1 and are the mean ± SEM from three or four independent experiments or the mean and range from two experiments. Recombination intensities for each of the *M26*-containing alleles are the mean ± SEM (n = 21–30) of two to seven repeat crosses with each of six or seven *ade6* test alleles from the data of Fox et al. (1997). A least-squares fit of the points, excluding *ade6-3011*, is shown by the line ($R^2 = 0.98$).

the absence of Pcr1 or in an *ade6-M375* strain (K. Ohta, personal communication; Mizuno et al., 1997), which agrees well with the requirements we found for DNA breakage at this site (Figures 2 and 4). However, MNase sensitivity alone does not accurately predict where prominent break sites occur. Mizuno et al. (1997) found two other MNase hypersensitive sites in the putative promoter region, 250 and 380 bp upstream of *M26*, which become 9-fold and 3-fold more sensitive to MNase, respectively, during meiosis. The first site falls just outside the region of prominent breakage we observed (Figure 3), and the second site is well outside of this region, though there is evidence of some diffuse breakage in the DNA spanning these MNase-sensitive sites (Figures 5A and 5B, see *ade6-M26* and *ade6-3005* strains). Thus, while the chromatin at these sites is readily cleaved by MNase, it is not readily cleaved by Rec12, the protein presumed to generate meiotic DSBs (Cervantes et al., 2000). This discrepancy implies either that MNase is an imperfect probe for open chromatin or, more likely, that open chromatin alone is not sufficient for meiotic DNA cleavage. As mentioned previously, we observed that some sites of DNA breakage within the *ade6* gene were shared by some or all of the alleles tested irrespective of the position of *M26*. For example, all four of the break sites observed in the *ade6-3002* allele were also sites of breakage in the *ade6-3011* allele, and the *ade6* promoter region was a site of breakage in all of the tested alleles (Figure 5). These common break sites may reflect a requirement for some additional aspect of chromatin structure, or perhaps even a preferred cleavage sequence.

Excluding the *ade6-3011* allele, we found a good linear relationship between the frequency of breakage and the intensity of recombination within *ade6* ($R^2 = 0.98$; Figure 6). Interestingly, if the line shown in Figure 6 is extrapolated back to 0% breakage, recombination intensity does not fall to zero but to ~2000 Ade^+/million spores per kb between *ade6* markers. While this could reflect imprecision in the measurements of breakage or recombination, it could also represent the fraction of recombinants initiated by breaks distant from *ade6* and independent of *M26*. The latter interpretation is supported by the observations of Young et al. (2002), who found that meiotic recombination is more uniformly distributed than are prominent meiotic DNA break sites. For example, recombination in the *res2–ura1* interval of chromosome I, measured primarily as crossing-over, is near the genomic average even though the nearest detectable break site is >20 kb away.

These observations suggest that meiotic DNA breaks can promote recombination (crossing-over) from a considerable distance. According to this view, recombination occurs both near and far from a single DSB site. Recombination near the site of breakage is observed primarily as conversion, such as that in the intragenic *ade6* crosses considered in this paper (Figure 6). Recombination far from the break site is observed primarily as crossing-over (with or without conversion) and could result, for example, from the concerted migration of two Holliday junctions away from the break site (Figure 7 of Young et al., 2002). Where these junctions are resolved, both conversion and a crossover could occur. Recombination remote from prominent DNA breaks has also been observed in *S. cerevisiae* and other organisms (reviewed in Smith, 2001). Similar to our analysis of *ade6*, Fan et al. (1995) found a positive linear relationship between the frequency of breakage and conversion at the *S. cerevisiae HIS4* locus, but the frequency of conversion at this site is above 10% (approximately one-half of the "wild-type" level) when the frequency of breakage is extrapolated back to 0%.

The mechanism by which recombination (crossing-over or gene conversion or both) occurs remote from prominent DNA break sites is an open question. However, we suppose that hot spots of double-strand breakage are also hot spots of conversion as predicted by the models of Resnick (1976) and Szostak et al. (1983). Here, we have shown that a well-characterized conversion hot spot, *M26*, is a site of DNA breakage. Conversely, we have recently observed that a prominent DNA break site *mbs1* (Young et al., 2002) is also a hot spot of gene conversion (C.A. Rubio and G.R.S., unpublished data), suggesting that the breaks at *M26* behave similarly to other meiotic DNA breaks in *S. pombe*.

In our experiments, the *ade6-3011* allele remains a puzzle. This allele shows the highest frequency of breakage of all the *ade6* alleles we analyzed but recombines at a frequency comparable to that of *ade6-M26* (Fox et al., 1997; Figure 6). The breaks within *ade6-3011*, like those within *ade6-M26,* require the Pcr1 transcription factor (data not shown), indicating a similarity between the two hot spots. A possible explanation for the discrepancy is that the random spore analysis used by Fox et al. (1997) may not accurately reflect the true level of recombination, primarily gene conversion, of *ade6-3011*. However, *ade6-3011* converted in four of 60 meiotic tetrads (6.7%; data not shown), not significantly different from 52/1018 (= 5.1%) for *ade6-M26* (Gutz, 1971; contingency $\chi^2 = 0.05$, $p > 0.8$). All of the *ade6-3011* convertant tetrads were 3 Ade^+:1 Ade^-, indicating

that *ade6-3011*, like *ade6-M26*, is a recipient of genetic information (data not shown; Gutz, 1971).

The frequency of conversion of markers within or adjacent to the *ade6-M26* heptamer is ~5% when *ade6-M26* is heterozygous and ~7% when homozygous (Gutz, 1971; Schär and Kohli, 1994). These conversion frequencies are slightly higher than those predicted from the observed frequency of DNA breakage at *M26* (~1.4% in haploids or homozygous diploids; Table 1) and the assumption that breakage of one chromatid among four in a meiotic cell leads to a convertant tetrad. Thus, 11.5% breakage in the *ade6-3011* haploid (Table 1) predicts at least 23% convertant tetrads in heterozygous diploids, significantly greater than the observed number ($\chi^2 = 8.1$; $p < 0.005$). We conclude that the high frequency of breakage at *ade6-3011* is not reflected in a high frequency of conversion within *ade6*. Other explanations for the unusual behavior of this allele remain to be tested.

M26 is the only meiotic recombination hot spot reported in *S. pombe* with a simple sequence requirement; this sequence occurs naturally hundreds of times throughout the genome of *S. pombe*, but it is not known if any of these sites are recombination hot spots. Our finding of double-strand breaks associated with *M26* may provide a means to begin answering this question. *S. pombe* is now the second organism in which DNA double-strand breaks are found to be associated with meiotic conversion hot spots. Given this same association in the distantly related yeast *S. cerevisiae* and the meiosis-specific expression of Spo11 homologs in other organisms (Keeney, 2001), double-strand breaks may be the mechanism by which recombination is initiated in many organisms during meiosis.

Experimental Procedures

Strains

All strains used in this study were *h⁻ smt-0 pat1-114 end1-458 rad50*S (K81I; Farah et al., 2002) unless otherwise noted and had the following additional mutations: GP535 (*h⁻ ade6-M26 rad50⁺*); GP2956 (*ade6-M26*); GP3105 (*ade6-3002*); GP3160 (*ade6-3011*); GP3292 (*ade6-3005*); GP3294 (*h⁺ ade6-3008*); GP3110 (*ade6-M26 Δpcr1::his7⁺*); GP3135 (*ade6-M26 rec12-170::3HA-6His-kanMX6*); GP3289 (*ade6-M375*); GP3505 (*ade6-3011 Δpcr1::his7⁺*); GP3087 (*h⁻ smt-0/h⁻ smt-0 ade6-M26/ade6-M26 end1-458/end1-458 pat1-114/pat1-114 rad50*S/*rad50*S *lys4-95/+ +/his4-239* diploid). *ade6* alleles are described in Fox et al. (1997), and *Δpcr1::his7⁺* is described in Fox et al. (2000). The *rec12-170* allele deletes all of the *rec12* coding sequence, which is replaced by the *kanMX6* gene and the HA-6His-tag coding sequence (Young et al., 2002). Complete descriptions and genealogies are available upon request.

Meiotic Inductions and Analysis of DNA Breaks

Meiotic inductions, flow cytometry, and preparation of DNA in agarose plugs were performed as described (Young et al., 2002). The agarose-embedded DNA was digested overnight with the indicated restriction enzyme as recommended by New England Biolabs. To ensure complete digestion, plugs were melted by heating to 65°C for 5 min and cooled to 37°C, and then additional restriction enzyme and 0.5 units of β-agarase (New England Biolabs) were added. Incubation was continued for several hr at 37°C. DNA samples digested with PacI were separated by pulsed-field gel electrophoresis (Bio-Rad CHEF Mapper or DRIII apparatus) using conditions recommended by the manufacturer. DNA digested with AflII or BseRI was electrophoresed in 0.8% and 2% agarose gels, respectively, in 1× TAE buffer. DNA in gels was blotted to Hybond-N+ nylon membrane (Amersham Pharmacia) and hybridized under conditions recommended by the manufacturer. Molecular weight markers used for estimation of sizes were 5 kb, 1 kb, 1 kb plus, or 100 bp DNA ladders (Invitrogen). Probes were prepared by PCR of genomic DNA and gel purified prior to use. Probes were labeled with ^{32}P using a random-prime labeling kit (Promega).

Quantitation of DNA Breakage

DNA breakage was quantitated using a phosphorimager (Molecular Dynamics). Area reports were taken for each lane within blots, and peaks (break sites) were defined using ImageQuant (Molecular Dynamics) and Excel Software (Microsoft). Background for each peak was taken to be the average value of the counts per pixel in the two valleys to either side of a given peak and subtracted from each pixel value within the peak (Figure 2B). The percent of breakage at a given site was taken to be the corrected peak value divided by the total counts within the lane. In *rad50*S strains, maximal breakage was observed in either the 5 or 6 hr time point. The maximum value is reported in Table 1. Since some broken fragments may be broken more than once, the above method provides a minimal estimate of the amount of breakage occurring at each site.

Acknowledgments

We especially thank Edgar Hartsuiker for generously providing the *rad50*S allele before its publication; Kunihiro Ohta and Claudia Rubio for sharing unpublished results; Katherine Guthrie for help with statistical analysis; and Susan Amundsen, Gareth Cromie, Luther Davis, Joseph Farah, and Andrew Taylor for helpful comments on the manuscript. This research was supported by National Institutes of Health grants R01-GM31693 to G.R.S. and F32-GM20190 to W.W.S.

Received: December 12, 2001
Revised: January 25, 2002

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Note Added in Proof

We have recently found that the strains with the *ade6-3011* allele used here and in Fox et al. (1997) contain an unexpected nonsense mutation 330 bp toward the *ade6* promoter from the expected *ade6-3011* mutation. The available recombination data from a strain containing only the expected *ade6-3011* mutation suggest that the recombination intensity may be consistent with the linear relationship in Figure 6; however, the consequences of this secondary mutation remain to be fully determined.