

Fine-Structure Mapping of Meiosis-Specific Double-Strand DNA Breaks at a Recombination Hotspot Associated With an Insertion of Telomeric Sequences Upstream of the *HIS4* Locus in Yeast

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ABSTRACT

Meiotic recombination in *Saccharomyces cerevisiae* is initiated by double-strand DNA breaks (DSBs). Using two approaches, we mapped the position of DSBs associated with a recombination hotspot created by insertion of telomeric sequences into the region upstream of *HIS4*. We found that the breaks have no obvious sequence specificity and localize to a region of ~50 bp adjacent to the telomeric insertion. By mapping the breaks and by studies of the exonuclease III sensitivity of the broken ends, we conclude that most of the broken DNA molecules have blunt ends with 3'-hydroxyl groups.

CERTAIN regions of the *Saccharomyces cerevisiae* genome have unusually high levels of meiotic recombination (reviewed by PETES *et al.* 1991; LICHTEN and GOLDMAN 1995). Such hotspots are associated with elevated levels of meiosis-specific double-strand breaks (DSBs). DSBs, initially described at the *ARG4* locus (SUN *et al.* 1989), have been associated with many other recombination hotspots in yeast (CAO *et al.* 1990; ZENFIRTH *et al.* 1992; DE MASSY and NICOLAS *et al.* 1993; GAME 1993), including *HIS4* (NAG and PETES 1993; FAN *et al.* 1995). Several lines of evidence indicate that the DSBs are likely to be the lesion responsible for the initiation of meiotic recombination. First, the level of DSB is proportional to the recombination frequency at the *ARG4* (DE MASSY and NICOLAS 1993) and *HIS4* (FAN *et al.* 1995) loci. Second, DSBs occur at a time in meiosis consistent with a role as an initiator of recombination (PADMORE *et al.* 1991). Third, the position of the DSB corresponds to the region of highest recombination activity, the peak of the polarity gradient of gene conversion (SUN *et al.* 1989; NAG and PETES 1993).

Hotspots of recombination in yeast are usually located between rather than within genes (PETES *et al.* 1991). The rules governing the position and intensity of the DSBs have not yet been established. Two types of experiments indicate that chromatin structure is likely to be important. First, hotspot activity and DSB formation at the *HIS4* locus requires binding of transcription factors (WHITE *et al.* 1991, 1993; FAN *et al.* 1995), although a high rate of transcription is not required for hotspot activity (WHITE *et al.* 1992). Second, most DSBs occur in chromosomal regions that are sensitive to DNase I or micrococcal nuclease (OHTA *et al.* 1994; WU and LICHTEN 1994; FAN and PETES 1996).

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In wild-type yeast cells, the ends resulting from DSBs are degraded 5' to 3', resulting in 3' single-stranded "tails" (SUN *et al.* 1991). In strains containing the *rad50S* mutation, this degradation does not occur, facilitating the mapping of DSBs (CAO *et al.* 1990). Using *rad50S* strains, three groups have done fine-structure mapping of DSBs associated with the following recombination hotspots: *ARG4* and *YCR74c-YCR48w* (LIU *et al.* 1995), *HIS4/LEU2* (XU and KLECKNER 1995), and *CYS3* (DE MASSY *et al.* 1995). These mapping experiments showed that the DSBs were spread over regions of 70–200 bp and that break sites had no obvious sequence specificity. A covalent attachment of the 5' strand of the broken DNA end to a protein was also observed (DE MASSY *et al.* 1995; KEENEY and KLECKNER 1995; LIU *et al.* 1995). The nature of the broken end in these studies is controversial. Whereas LIU *et al.* (1995) found that the termini had a two base 5' overhang, DE MASSY *et al.* (1995) concluded that the ends were blunt. The results of XU and KLECKNER (1995) are most consistent with termini with a 4–6-bp 5' overhang.

WHITE *et al.* (1993) showed that an insertion of telomeric DNA upstream of the *HIS4* gene created a very strong recombination hotspot. This hotspot was associated with a high level of DSB near the site of the telomeric insertion (FAN *et al.* 1995). Below, we describe the fine-structure mapping of the DSBs associated with this hotspot. We conclude the following: (1) DSBs are formed with blunt ends, (2) the DSBs occur in a 50-bp region adjacent to (but not within) the telomeric sequences, and (3) at least some of the 3' termini associated with the broken ends have a hydroxyl group.

MATERIALS AND METHODS

Strains and plasmids: The diploid yeast strain FX4 used for mapping DSB sites had the following genotype (FAN *et al.*

and dTTP, 0.5 μ l of *Taq* DNA polymerase (5 units/ μ l), 1 μ l of 1 μ M primer (8172 for the DSB(L) probe, 9905 for the DSB(R) probe), 0.5 μ l (20 ng) of the double-stranded DNA template, and 19 μ l of α - 32 P-dATP (3000 Ci/mmol, Amersham). Twenty-five cycles of primer extension (94° for 1 min, 55° for 1 min and 72° for 2 min) were performed. The probe was purified with a Sephadex G50 column. We used the hybridization protocols recommended for Hybond-N+ by the manufacturer (Amersham).

As described above, the lanes containing the DSB samples also contained size standards. To map the DSB sites, we aligned the DNA fragments representing the size standards to the appropriate sequence on the DNA sequencing ladders. The fragments representing the DSB sites could then be aligned to the sequencing ladder at single base pair resolution. Excellent agreement was found for alignments established independently by two different people.

Addition of an untemplated nucleotide by *Taq* DNA polymerase: *Taq* DNA polymerase adds an untemplated base during PCR reactions (HEMSLEY *et al.* 1989). To determine whether this reaction occurred under our experimental conditions, we did primer extensions with *Pvu*II-treated pMW50 DNA (0.01 μ g in a 20 μ l reaction mixture) performed under the same conditions described above; the primer 10083 was used and eight cycles of primer extension were done. The primer 10083 begins at the *Bst*UI cleavage site. Thus, if no untemplated terminal base is added, the primer extension product should be identical in size to the *Pvu*II-*Bst*UI fragment derived from pMW50. By analyzing the product in a DNA sequencing gel, however, we found that the primer extension product was one base longer than the *Pvu*II-*Bst*UI fragment (data not shown), indicating the addition of an untemplated base.

Analysis of DNA termini using exonuclease III: As shown in Table 1, DNA termini with blunt ends, 5' overhangs or 3' overhangs less than four bases are degraded by exonuclease III by a 3' to 5' activity (BRUTLAG and KORNBERG 1972; HENIKOFF 1984). If 5' overhangs are "filled in" by DNA polymerase using α -phosphorothioate dNTPs (α S-dNTPs), then the modified ends are resistant to exonuclease III (PUTNEY *et al.* 1981). Both modified and unmodified termini were examined in our experiments.

Yeast meiotic DNA (84 μ g) were cut with *Hae*II and *Sad* for DSB detection; both *Hae*II and *Sad* generate four base 3' overhangs (SAMBROOK *et al.* 1989). Four micrograms of pBR322 were cut with *Ava*I, *Bam*HI and *Rsa*I as controls for 5' overhang termini (*Ava*I and *Bam*HI fragments) and blunt termini (*Rsa*I fragment) (SAMBROOK *et al.* 1989). These treated DNAs were mixed, extracted with phenol, precipitated with ethanol and dissolved into 80 μ l of TE (10 mM Tris, 1 mM EDTA pH 8.0). Forty microliters of the DNA solution was removed for treatment with DNA polymerase and α S-dNTPs. The remaining 40 μ l of DNA were analyzed without modification. To modify the termini, we added to the 40 μ l of DNA, 10 μ l of 10 \times Klenow buffer (NEB), 10 μ l of α S-dNTPs (0.4 mM, Promega), 1 μ l of dithiothreitol (0.1 M), 1 μ l of Klenow/ α S-dNTP DNA polymerase (5 units/ μ l, NEB), and 38 μ l of H₂O. The solution was incubated at 37° for 10 min, and DNA was then extracted with phenol and precipitated with ethanol. The modified DNA was dissolved in 40 μ l of TE.

From the 40- μ l aliquots of the modified and unmodified DNA samples, we used 32 μ l of DNA for exonuclease III treatment and 8 μ l as an untreated control. For the exonuclease III reaction, we mixed the 32- μ l DNA solutions with 5 μ l of 10 \times exonuclease III buffer (NEB), 4 μ l of exonuclease III (100 units/ μ l, NEB) and 9 μ l of H₂O. The solutions were incubated at 30°. Seventeen-microliter samples were removed after 30 sec, 1 min and 3 min and placed on dry ice. Exonuclease III was inactivated by incubating the samples at 70°

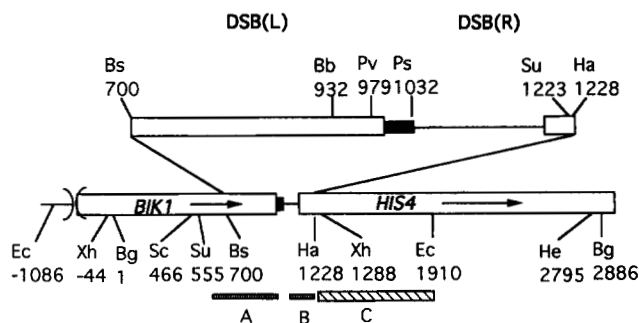


FIGURE 1.—Restriction map of the chromosomal region flanking the *HIS4* hotspot associated with a telomeric insertion. The directions of transcription are shown by arrows, and the labeled rectangles represent the *BIK1* and *HIS4* coding sequences. DSB(L) and DSB(R) indicate the regions to the left and right, respectively, of the hotspot-associated DSBs. Numbers under the restriction sites represent the distances from the *Bgl*II site in *BIK1*. ■, the telomeric insertion. The stippled rectangle marked A shows the location of the single-stranded hybridization probe (595-960) used to analyze DSB(L) and the stippled rectangle marked B indicates the location of the single-stranded probe (1049-1223) used to analyze DSB(R); the cross-hatched rectangle (marked C) represents the sequences derived from pDN42 used as a probe in the Southern analyses shown in Figure 2. Not all restriction sites for the indicated enzymes are shown. Ec, *Eco*RI; Xh, *Xho*I; Bg, *Bgl*II; Sc, *Sac*I; Su, *Sau*3AI; Bs, *Bst*UI; Bb, *Bbs*I; Pv, *Pvu*II; Ps, *Pst*I; Ha, *Hae*II; He, *Hae*I.

for 20 min. For the 8- μ l DNA samples that were not treated with exonuclease III, we added 1.7 μ l of 10 \times exonuclease III buffer and 7 μ l of water to generate a 17- μ l sample size.

The end products of exonuclease III treatment of appropriate substrates are single-stranded DNA molecules. To complete degradation of the DNA, we digested the exonuclease III-treated samples with mung bean nuclease, an enzyme that degrades single-stranded DNA (SAMBROOK *et al.* 1989). Each 30- μ l reaction contained 17 μ l of DNA, 3 μ l of 10 mM ZnSO₄, 1.3 μ l of 10 \times exonuclease III buffer, 2 μ l of mung bean nuclease (10 units/ μ l, NEB) and 6.7 μ l of H₂O. The samples were incubated at 30° for 30 min and then examined by Southern analysis. The *HIS4*-specific hybridization probe was an *Eco*RI-*Xho*I fragment derived from pDN42.

Mapping DSB sites by anchor-PCR: For each experiment, 3 μ g of yeast DNA were treated with *Xho*I, extracted with phenol/chloroform, and precipitated with ethanol. The DNA was resuspended in 42 μ l of H₂O and denatured at 100° for 10 min. Twenty-one microliters of the denatured DNA was used for the terminal deoxynucleotide transferase (TdT) "tailing" reaction. We then added 6 μ l of 5 \times TdT buffer (USB), 2 μ l of 1 mM dGTP, and 1 μ l of TdT (USB, 17 units/ μ l). The reaction was incubated at 37° for 30 min then diluted with H₂O to 100 μ l. These conditions add a single-stranded poly dG tail of ~20 bases to the 3' ends of the substrate DNA, and this modified DNA was used as a substrate for PCR (BOLLUM 1974).

The primers used to examine DSB(L) were 7492 and the poly dC primer 7278. DSB(R) was studied using 7680 and 7278. Each 50- μ l reaction had 5 μ l of the substrate DNA (described above), 8 μ l of dNTP mix (1.25 mM each of dCTP, dGTP, dATP and dTTP), 1.2 μ l of primer 7492 or 7680 (40 μ M concentration of oligonucleotide), 1.2 μ l of the 7278 primer (40 μ M stock), 5 μ l of 10 \times *Taq* DNA polymerase buffer (described above), 28.5 μ l of H₂O, and 1 μ l of *Taq* DNA polymerase (5 units/ μ l, Perkin-Elmer). The PCR machine (Perkin-Elmer Cetus) was run for 45 cycles with the following

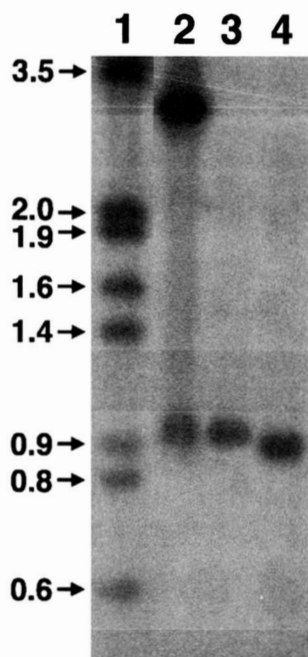


FIGURE 2.—Southern analysis of the hotspot-associated DSB at *HIS4*. A mixture of yeast DNA derived from pDN42 (position of probe shown in Figure 1) and lambda DNA was used as a hybridization probe. Lane 1, *Hind*III/*Eco*RI-treated lambda DNA; lane 2, DNA isolated from meiotic cells of FX4 and treated with *Eco*RI. The top band represents the intact *Eco*RI fragment and the bottom band represents the DSB-generated fragment. Lane 3, DNA isolated from mitotic cells treated with *Eco*RI and *Bbs*I; lane 4, DNA isolated from mitotic cells treated with *Eco*RI and *Pvu*II.

temperatures and times for each cycle: 94° for 1 min, 65° for 2 min, 72° for 3 min. After the last cycle, the sample was incubated at 72° for 7 min.

The PCR products were checked using a 1.2% low-melting-temperature agarose gel. The PCR products representing the DSBs were cut out and purified using the protocols provided with the Promega Wizard PCR. To further confirm that the PCR products were from the *HIS4* DSBs rather than a nonspecific product, this PCR product was checked by Southern analysis using pFX2b as a probe or by a nested PCR reaction using primer 7981 (DSB(L)) or 7982 (DSB(R)) and primer 7278. The PCR product was cloned with the TA cloning kit (Invitrogen). The resulting plasmids were sequenced by standard methods.

RESULTS

In previous studies (WHITE *et al.* 1993; FAN *et al.* 1995), we found that an insertion of telomeric sequences greatly stimulated the frequency of meiotic recombination and DSBs at the *HIS4* locus. Below, we describe the mapping of these breaks and an analysis of some features of the broken ends.

Mapping the position of DSBs by agarose gel electrophoresis: The diploid strain FX4 is homozygous for the *rad50S* mutation and for an insertion of telomeric sequences upstream of *HIS4*. In meiosis, a DSB is observed in this strain near the site of the telomere insertion (FAN *et al.* 1995). To refine the mapping of the position of the DSB, we used restriction enzymes that

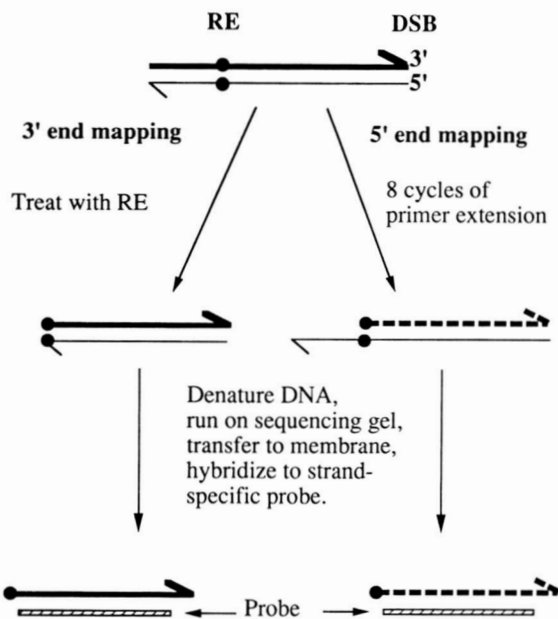


FIGURE 3.—Mapping 3' and 5' DSB-generated termini. The mapping of sequences on only one side of the break is depicted. The 3' ends (thick lines) were mapped by treating the DNA with a restriction enzyme (RE) that cuts at a site close (within 200–300 bp) to the DSB site (*Bst*UI for DSB(L) and *Hae*II for DSB(R)). The resulting DNA was denatured, run on DNA sequencing gels, transferred to a membrane, and hybridized to a strand-specific probe. The 5' ends (thin lines) were mapped by denaturing unrestricted DNA, and performing eight cycles of primer extensions using a primer located at the same restriction site used in the analysis of 3' ends. This primer hybridized to the thin strand and was extended to the 5' end; the resulting product is shown with a thick dashed line. The DNA molecules resulting from these manipulations were denatured and examined as described for the 3' termini.

generated restriction fragments shorter than those used in the previous study. As shown in Figure 1, the telomeric insertion is bracketed by *Pvu*II and *Pst*I sites, and there is a *Bbs*I site in *BIK1* ~50 bp from the *Pvu*II site. When DNA was isolated from meiotic cells, treated with *Eco*RI, and examined by Southern analysis (using a probe derived from the *HIS4*, Figure 1), a DSB fragment of ~1 kb was observed. This fragment comigrated with an *Eco*RI-*Bbs*I fragment derived from mitotic cells but was distinctly larger than an *Eco*RI-*Pvu*II fragment (Figure 2). Thus, the DSB cleavages occur on the *BIK1* side of the telomere insertion. In addition, since the band representing the DSB is only slightly wider than the bands representing restriction fragments, the DSB sites are restricted to a small chromosomal interval.

Mapping DSB sites by primer extension and Southern analysis: The method that we used to map DSB sites to single-base pair resolution (Figure 3) is based on methods developed for genomic sequencing (CHURCH and GILBERT 1984; HUIBREGSTE and ENGELKE 1991), as modified by LIU *et al.* (1995). A DSB within the *HIS4* hotspot produces two DNA fragments with two DSB-generated termini. The terminus of the fragment with *BIK1* sequences will be called DSB(L) and the terminus

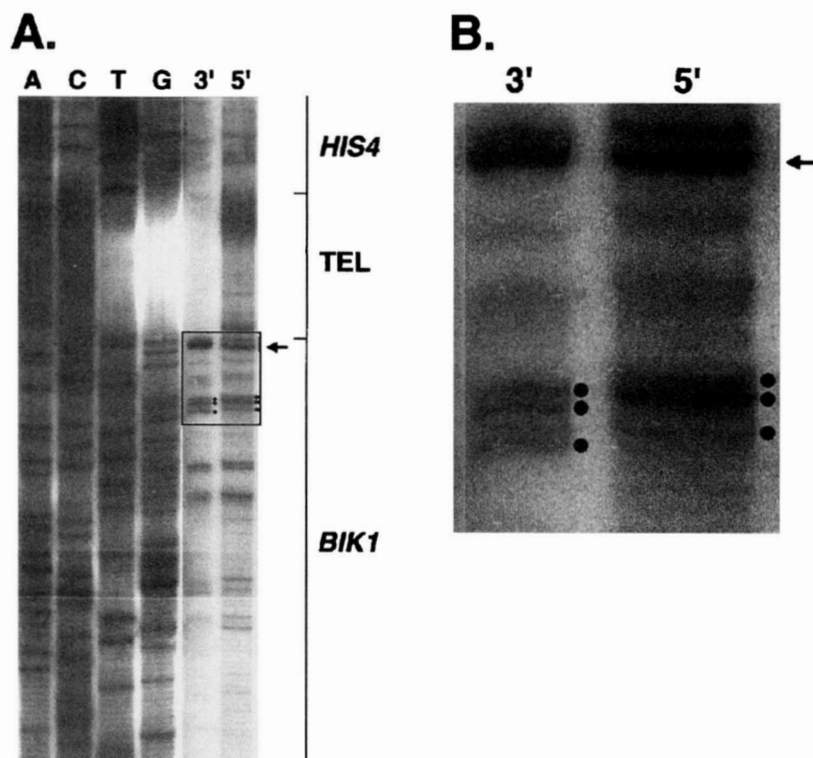


FIGURE 4.—Mapping the 3' and 5' termini of DSB(L). (A) As outlined in Figure 3, the 3' ends were examined by direct Southern analysis and the 5' ends mapped by primer extension. For the 3' analysis, meiotic DNA was treated with *Bst*UI. The oligonucleotide (10083) used for the primer extensions began at the *Bst*UI site. The DNA sequencing ladder was also prepared using the 10083 primer. The arrow indicates the position of a *Pvu*II-*Bst*UI fragment (derived from pMW50) added to the 5' and 3' samples as a size standard. A strand-specific probe derived from the *BIK1* region (Figure 1) was used to detect the positions of the breaks. The outlined region of the gel indicates the area shown at higher magnification in Figure 4B. Dots indicate three prominent DSB bands. (B) Magnified region of gel lanes shown in Figure 4A. The bands representing the control *Pvu*II-*Bst*UI fragment are in direct alignment, whereas the bands representing the 3' DSBs are one base pair shorter than those representing the 5' DSBs.

of the fragment with *HIS4* sequences will be called DSB(R). Both ends were mapped by similar procedures, and we describe the mapping of DSB(L) first.

DNA was isolated from strain FX4 after the cells had been incubated in sporulation medium for 24 hr. To measure the position of breaks at the 3' end of DSB(L), we treated the meiotic DNA with *Bst*UI, separated the fragments on a polyacrylamide DNA sequencing gel, and hybridized the separated fragments with a strand-specific probe. We included a DNA sequencing ladder from this region of the genome on the same gel (Figure 4A). Since this sequencing ladder was obtained using a primer that started at the *Bst*UI site, it is aligned with the DSB ends. It is clear from Figure 4A that there are multiple 3' ends. Although there are faint bands at many positions in the gel lane, the strongest bands are mostly distributed within a 50-bp region. There are no strong breaks in the telomeric sequences. No DSBs were detectable in DNA derived from mitotic cells by standard Southern procedures (FAN *et al.* 1995) or by Southern analysis using DNA sequencing gels (data not shown).

The 5' ends of DSB(L) were mapped by primer extension. We used primer extension, rather than directly examining the position of the DSBs using a different strand-specific probe, for two reasons. First, the primer extension analysis allowed us to compare DNA strands that had the same sequence and, therefore, the same molecular weight. The position of breaks in the two strands could be established using the same DNA sequencing ladder and the same size standard. Second, other researchers observed a protein attached to the 5' end of the DSB in yeast (DE MASSY *et al.* 1995; KEENEY

and KLECKNER 1995; LIU *et al.* 1995). This attached protein would be expected to affect the migration of the 5' strand. The primer extension method allows one to map the position of the 5' end without this complication. Using an oligonucleotide primer with sequences that begin at the *Bst*UI site, we performed eight rounds of primer extensions (unidirectional PCR, details in MATERIALS AND METHODS). The resulting products were analyzed as described above for the 3' ends and showed a similar pattern of bands (Figure 4A). To compare relative mobilities of DNA representing the 3' and 5' ends, we included the same size standard in both lanes, the *Bst*UI-*Pvu*II restriction fragment shown in Figure 1. The alignment of the 5' and 3' ends of DSB(L) indicated that the 5' ends were displaced by one base pair (Figure 4B). Since *Taq* DNA polymerase adds an extra terminal base during PCR amplification (HEMSLEY *et al.* 1989), the simplest explanation of this result is that the 5' and 3' cleavages occur at the same position in the DNA molecule, indicating that ends generated by DSBs are blunt. We confirmed this conclusion by reanalyzing the 5' ends using a DNA primer that was recessed one base from *Bst*UI site (Figure 5). When the DNA samples representing the 3' ends and the primer extension product representing the 5' ends were mixed, it was clear that the 5' and 3' bands comigrated. When primer extension experiments were done on DNA isolated from mitotic cells, a few faint bands were seen, but the number and intensity of these bands varied in different primer extension reactions (data not shown).

The map positions of the DSB(L) ends are summarized in Figure 6. Most of the DSB(L) ends appear blunt, although there are some bands that appear in the

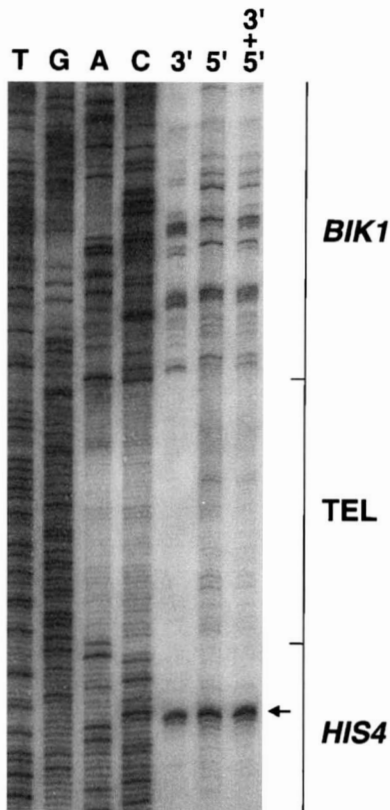


FIGURE 7.—Mapping the 3' and 5' termini of DSB(R). The 3' lane contains meiotic DNA treated with *Hae*III. The 5' lane contains the products of a reaction in which meiotic DNA was used as a substrate for primer extension with the oligonucleotide 10929 (one base recessed from the *Hae*III site). The DNA sequencing ladder was prepared using a primer (10193) at the *Hae*III site. The arrow shows the position of migration of a size standard mixed with the 5' and 3' DNA samples. As described in MATERIALS AND METHODS, the 181-bp size standard was generated by PCR amplification of pMW50, using primers 10193 (which align at the *Hae*III site) and 9905; this fragment has DNA sequences identical to those in the DNA sequencing ladder. The rightmost lane contains a mixture of 5' and 3' DNA samples. After electrophoresis, the DNA fragments were transferred to Hybond-N+ and hybridized to a strand-specific probe (described in MATERIALS AND METHODS) containing *HIS4* sequences.

supports the conclusion that the DSB-generated ends are blunt. This conclusion is also reinforced by the exonuclease digestion experiments described below.

Analysis of DSB-produced DNA ends with exonuclease III: Exonuclease III catalyzes the stepwise release of mononucleotides from the 3' OH ends of the following DNA substrates (BRUTLAG and KORNBURG 1972; SAMBROOK *et al.* 1989): ends with 5' overhangs, blunt ends, ends with 1–3-base 3' overhangs, and nicked double-stranded DNA molecules. Exonuclease III is unable to degrade linear DNA with a 3' overhang that is greater than or equal to four bases (HENIKOFF 1984) or a DNA molecule in which a 5' overhang has been filled in with α -phosphorothioate dNTPs (α S-dNTP) (PUTNEY *et al.* 1981).

To analyze the nature of the termini produced by

TABLE 1

Diagnosis of DNA termini by treatment with exonuclease III

Type of DNA ends	Sensitivity to exonuclease III treatment ^a	
	Unmodified DNA ends	Modified DNA ends ^b
3' overhang (≥ 4 bases)	R	R
5' overhang	S	R
Blunt or 3' overhang (<4 bases)	S	S

^a R indicates resistance and S indicates sensitivity to exonuclease III digestion.

^b The modification is treatment of the DNA with the Klenow fragment of DNA polymerase and α S-dNTPs. For DNA termini with a 5' overhang, this treatment will result in blunt-ended termini that are resistant to exonuclease III digestion.

the meiosis-specific DSB, we examined the sensitivity of these ends to exonuclease III, before and after treatment of the ends with DNA polymerase and α S-dNTPs. We monitored the sensitivity of the ends to exonuclease III by Southern analysis. A summary of the results expected for exonuclease digestion of various types of termini is given in Table 1. As indicated in this table, termini with a 3' overhang greater than or equal to four bases in length will be resistant to exonuclease III, with or without treatment of the DNA with DNA polymerase and α S-dNTPs. Both *Sac*I and *Hae*I cleave DNA to generate termini with four base 3' overhangs (SAMBROOK *et al.* 1989). The *HIS4* hotspot region is included in a 2.3-kb *Sac*I-*Hae*I fragment (Figure 1), and the sites of DSBs are located ~ 500 bp from the *Sac*I site and 1.8 kb from the *Hae*I site. As shown in Figure 8A, the 2.3-kb *Sac*I-*Hae*I fragment without the DSB was resistant to digestion with exonuclease III, as expected. The 1.8-kb band representing the DSB was sensitive to exonuclease III. Since this band should represent fragments with one exonuclease III-resistant *Hae*I terminus, this result indicates that the end produced by the DSB does not have a 3' overhang that is four bases or longer (Table 1).

As a control, plasmid pBR322 DNA treated with the enzymes *Bam*HI and *Ava*I (which result in termini with 4-bp 5' overhangs), and *Rsa*I (which results in blunt-ended termini) was mixed with the yeast DNA samples. The four plasmid DNA fragments visualized by staining of the gel with ethidium bromide (indicated by arrows in Figure 8) represent (from largest to smallest), *Rsa*I-*Rsa*I, *Bam*HI-*Ava*I, *Ava*I-*Rsa*I and *Rsa*I-*Rsa*I fragments. As expected, all fragments are sensitive to exonuclease III in DNA samples that have not been treated with DNA polymerase and α S-dNTPs.

DNA samples digested with the same restriction enzymes described above were also treated with DNA polymerase (an exonuclease-deficient preparation of the Klenow fragment) and α S-dNTPs. As shown in Figure 8B, the DNA fragment representing the DSB was still

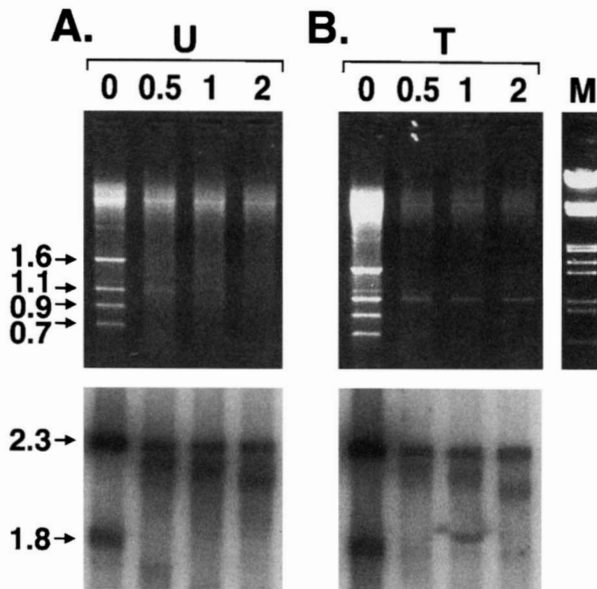


FIGURE 8.—Analysis of DSB-generated termini using exonuclease III. DNA samples isolated from meiotic cultures of FX4 were digested with *Sad* and *Hae*I (enzymes that result in termini with 3' overhangs of four bases) and were mixed with *Ava*I/*Bam*HI/*Rsa*I-digested pBR322 DNA; *Ava*I and *Bam*HI result in termini with four base 5' overhangs, whereas *Rsa*I results in blunt termini. These samples were either treated with exonuclease III (time of treatment 0, 0.5, 1 or 2 min) immediately (A) or were modified by the addition of DNA polymerase and α S-dNTPs before treatment with exonuclease III (B). As detailed in MATERIALS AND METHODS, the DNA samples were then treated with mung bean nuclease and analyzed by gel electrophoresis. The top portions of the figure show the ethidium bromide staining pattern of the gel, and the bottom portions represent Southern analysis of the same gels (after an additional period of electrophoresis) using *HIS4*-specific fragments derived from pDN42 (Figure 1) as a hybridization probe. The four bands marked by arrows on the top part of A and B represent (from largest to smallest) *Rsa*I-*Rsa*I, *Bam*HI-*Ava*I, *Ava*I-*Rsa*I, and *Rsa*I-*Rsa*I fragments of pBR322; the numbers next to the arrows indicate the sizes of the fragments in kb. All fragments were exonuclease III-sensitive in the untreated (U) samples (A), but the *Bam*HI-*Ava*I fragment became resistant to exonuclease III in the treated (T) samples. As shown in the bottom part of the figure, the 2.3-kb *Sad*-*Hae*I fragment (representing meiotic DNA without a DSB) was mostly resistant to exonuclease III treatment, whereas the 1.8-kb fragment (representing the DSB) was sensitive to exonuclease III with or without modification. The diffuse staining near the top of the gels represents *Sad*-*Hae*I yeast genomic DNA fragments. The lane marked M contained *Hind*III/*Eco*RI-treated lambda DNA.

sensitive to exonuclease III following this treatment. The control plasmid DNA *Bam*HI-*Ava*I fragment was rendered resistant to digestion by exonuclease III, indicating that the DNA polymerase utilized the α S-dNTPs to generate exonuclease III-resistant termini. The simplest interpretation of this result is that the terminus caused by the DSB is blunt or has a 3' overhang of less than four bases. These results also argue that the 3' end of the DSB is not covalently attached to a protein since such an attachment would be expected to make the termini resistant to exonuclease III.

Several other points should be discussed. First, we observed some exonuclease digestion of the 2.3-kb *Sad*-*Hae*I fragment (Figure 8). A fraction of the *Sad* or *Hae*I ends may be sensitive to exonuclease III as a consequence of loss of one or more base pairs of the 3' overhangs because of a small amount of a contaminating exonuclease. In some preparations of DNA, the exonuclease-sensitive component is only a small fraction of the total (data not shown). Second, a very small fraction of the DSB termini appears resistant to exonuclease III after treatment with DNA polymerase and α S-dNTPs (bottom of Figure 8B). These termini may represent a small fraction of ends that has 5' overhangs allowing modification to exonuclease III-resistance, or a small amount of DNA polymerase-mediated exchange of the terminal nucleotides. Third, although the plasmid control *Bam*HI-*Ava*I fragment was made resistant to exonuclease III digestion by treatment with the modified nucleotides, it could be argued that a terminus with a smaller 5' overhang might be refractory to this modification. We found, however, that *Bst*NI-generated termini, which have only a single base 5' overhang, were efficiently modified to resistance to exonuclease III with α S-dNTPs (data not shown). Fourth, we showed that treatment of the plasmid control *Bam*HI-*Ava*I fragment with DNA polymerase and unmodified nucleotides did not result in the fragment becoming exonuclease III resistant (data not shown). Thus, the resistance does not reflect the activity of a contaminating nuclease of the DNA polymerase that converts 5' overhangs to 3' overhangs. In summary, the exonuclease III studies are consistent with our conclusion that most DSB-generated termini at the *HIS4* hotspot are blunt.

Mapping meiotic DSBs by PCR: The yeast *S. cerevisiae* has a very high rate of meiotic recombination per kb relative to most higher eukaryotes, and the *rad50S* mutation prevents the loss of DNA lesions by subsequent steps of meiotic recombination. In organisms without these advantages, the procedures for fine-structure mapping that we and others have used may be difficult to employ. Consequently, we modified the technique of anchor-PCR (FROHMAN *et al.* 1988) to investigate the placement of DSBs in yeast.

The following procedure was used: (1) The DNA was digested with *Xho*I and denatured. (2) The denatured fragments were treated with dGTP and terminal deoxyribonucleotide transferase (TdT), resulting in addition of a tail of \sim 20 dGMP residues to the 3' ends of all DNA strands (BOLLUM 1974 and data not shown). (3) PCR amplification was performed using two different sets of primers. One set had one primer with poly dC sequences (capable of hybridizing to the poly dG tail) and a second containing *BIK1* sequences (primer 7492, described in MATERIALS AND METHODS). In DNA derived from mitotic cells, these primers resulted in amplification of a DNA fragment of \sim 700 bp in DNA (Figure 9A, lane 2), the expected distance between the primer and the *Xho*I site in *HIS4*. In DNA isolated from meiotic

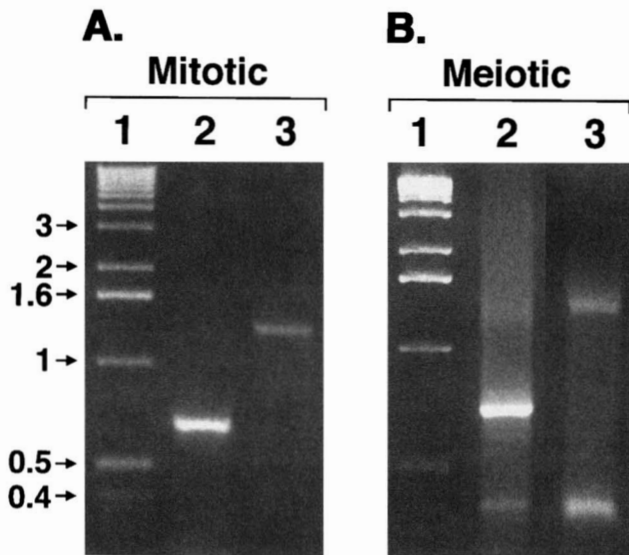


FIGURE 9.—Detection of DSBs by anchor-PCR. DNA was isolated from either mitotic (A) or meiotic (B) cultures of FX4. This DNA was treated with *Xho*I, terminal transferase and dGTP, and then PCR analysis was performed. To examine DSB(L) (lanes 2 in A and B), we used a primer containing poly dC sequences (7278) and a primer from the *BIK1* side of the DSB (7492); to examine DSB(R) (lanes 3 in A and B), we employed primer 7278 and a primer from the *HIS4* side of the DSB (7680). The single bands in lanes 2 and 3 of A represent the PCR products containing sequences between the primers and the *Xho*I sites. The additional bands in lanes 2 and 3 of B represent the PCR products containing sequences between the primers and the DSB-generated termini. Lane 1 contains marker DNA of 1-kb ladder (GIBCO BRL).

cells, in addition to the 700-bp fragment, we observed a fragment of ~380 bp (Figure 9B, lane 2). From our previous mapping analysis, this fragment had the expected size to represent the DSB. The detection of this fragment indicates that the DSBs result in 3' termini with a hydroxyl group, since terminal transferase requires such a substrate (SAMBROOK *et al.* 1989).

The second set utilized the same poly dC primer and a primer derived from the *HIS4* gene (primer 7680, described in MATERIALS AND METHODS). In DNA isolated from mitotic cells, a single 1300-bp fragment (the distance between the primer and the *BIK1 Xho*I site) was observed (Figure 9A, lane 3), whereas in DNA isolated from meiotic cells, an additional 360-bp fragment was present (Figure 9B, lane 3). This PCR product was the size expected to represent the DSB (Figure 1).

The PCR products representing the DSBs were purified from the gel, cloned and sequenced. Since terminal transferase adds nucleotides to 3' termini, the sequences obtained represent 3' break sites, with the first set of primers mapping DSB(L) and the second mapping DSB(R). The mapping of these breaks is summarized in Figure 10. Breaks representing DSB(R) were mapped in two independent experiments with upward-pointing arrows below the sequence representing the mapped sites. Since we cloned the DNA ends by addition of poly dG, the position at the breakpoint is ambig-



FIGURE 10.—Mapping of DSB sites using anchor-PCR. The meiosis-specific PCR fragments shown in Figure 9B were cloned and sequenced. Each arrow represents a single clone, except for the arrows marked 22 and 16, which represent 22 and 16 clones with that breakpoint. Arrows with different shapes represent clones obtained in different experiments. Arrows above the line represent 3' breaks in the upper strand (the 3' termini of DSB(L)), and arrows below the line reflect 3' breaks in the lower strand (the 3' termini of DSB(R)). Since the ends were defined by adding a poly dG tail to the termini, breakpoints mapping at Gs in the sequence are ambiguous. This ambiguity is indicated by horizontal lines above or below the sequence.

uous if it maps next to a G in the chromosomal sequence; such ambiguities are indicated by horizontal lines adjacent to the arrows in Figure 10. As expected from the other mapping study, most of the breaks were located on the *BIK1* side of the telomere insertion with few breaks within the telomere insertion. Sixty of sixty-four sequenced clones for DSB(R) are shown in the figure; an additional four clones had breakpoints upstream and downstream of the sequences in Figure 10.

Mapping of breakpoints for DSB(L) was done in four independent experiments. For two of these experiments, the distribution of breakpoints (indicated by downward-pointing arrows) was similar to that observed for DSB(R). In two experiments, however, most (about three-quarters) of the breakpoints mapped to a single site (indicated by numbers adjacent to arrows in Figure 10). From our previous mapping study, this site is frequently cleaved, but not substantially more than a number of other sites. In addition, no such strong DSB was observed with DSB(R). Thus, we believe the high frequency of recovery of breakpoints at this single site is an artifact. One possibility is that the end represented by this sequence is a better substrate for terminal transferase than the other ends. If this explanation is correct, however, this property must be very sensitive to subtle

changes in experimental conditions, since this site was not strongly represented in two of the experiments. A second alternative is that DNA contamination occurred in two of the experiments. Although this alternative is difficult to rule out, the two experiments in which the site were overrepresented were done with completely different sets of solutions.

In summary, mapping by the PCR procedure is in reasonably good agreement with the previous mapping protocol. Since we found that the PCR procedure appeared to result in an artifactually strong site in two experiments, however, the direct blotting procedure is the method of choice in organisms with a high level of meiosis-specific DNA lesions. In organisms in which the level of DNA lesions is low, the PCR procedure is a reasonable alternative.

DISCUSSION

A telomere insertion upstream of *HIS4* results in a very high level of meiotic recombination (WHITE *et al.* 1993) associated with a high level of DSB (FAN *et al.* 1995). The fine-structure mapping of the DSB indicates the following: (1) Most of the DSBs occur in a 50-bp region within the *BIK1* gene adjacent to the telomeric sequences; DSBs do not occur in the telomeric sequences. (2) The DSB appears to involve a concerted cleavage of both strands to generate a blunt-ended DNA molecule, and (3) since the 3' ends are substrates for terminal transferase, these termini have a 3' hydroxyl group.

In yeast (WHITE *et al.* 1993; FAN *et al.* 1995), *Caenorhabditis elegans* (CANGIANO and LA VOLPE 1993), and mammalian cells (ALVAREZ *et al.* 1993), interstitial telomeric sequences stimulate recombination and/or DSB formation. One interpretation of these effects is that these sequences are particularly susceptible to the endonuclease responsible for DSB formation. Our observation that DSBs are adjacent to rather than within the telomeric repeats rules out this interpretation. Our preferred model is that the hotspot activity of the telomeric sequences is related to the binding of the transcription factor Rap1p. The 50-bp telomeric insertion contains three near consensus Rap1p binding sites, and duplicated Rap1p binding sites upstream of *HIS4* strongly stimulate meiotic recombination (WHITE *et al.* 1993). We previously suggested that this stimulation is a consequence of Rap1p binding increasing the accessibility of meiotic chromatin to the meiotic endonuclease that causes the DSB or a consequence of local targeting of the endonuclease to the hotspot region by Rap1p (FAN *et al.* 1995). By either of these mechanisms, the breaks could occur within the telomeric sequences or adjacent to these sequences.

In studies using DNase I and micrococcal nuclease on meiotic chromatin of the *HIS4* region, we failed to find dramatic alterations in chromatin structure associated with different levels of hotspot activity (FAN and

PETES 1996). Consequently, we prefer the model that Rap1p targets the meiotic nuclease to the chromatin, either directly or through bridging proteins. If this mechanism is correct, the targeting is sufficiently flexible that multiple sites of cleavage are allowed. Since the DSBs do not occur within the telomeric insertion or on the *HIS4* side of the insert, cleavage may also be prevented by DNA binding proteins. By this model, a strong DSB site may require two properties, a binding site for a transcription factor and an adjacent sequence that is not protected by a DNA-binding protein.

Although we favor a model in which the positions of the cleavages are controlled by interactions of the endonuclease with transcription factors, we cannot rule out the possibility that the DSB sites are determined by accessibility of chromatin affected indirectly by transcription factor binding. Previous studies (OHTA *et al.* 1994; WU and LICHTEN 1994) at several recombination hotspots in yeast showed a correlation between nuclease-sensitive sites and hotspots for DSB formation. The nature of the correlation is that all hotspots for DSB formation appear in nuclease-sensitive regions, but not all nuclease-sensitive sites are hotspots for DSB formation (LIU *et al.* 1995; FAN and PETES 1996).

DSBs have been mapped at recombination hotspots in three previous studies (DE MASSY *et al.* 1995; LIU *et al.* 1995; XU *et al.* 1995). In these studies, as in our study, DSB sites are distributed over a relatively small region (<200 bp). The studies disagree about the nature of the termini produced by the break. LIU *et al.* (1995) concluded the termini have a two base 5' extension, and DE MASSY *et al.* (1995) found that the termini are blunt. This discrepancy could reflect different strain backgrounds, the different loci examined, or an experimental artifact. We have three independent arguments that the termini at the *HIS4* locus do not have 5' overhangs: (1) mixtures of 5' and 3' strands have no extra bands on sequencing gels, (2) DSB(L) and DSB(R) sites map to identical positions, and (3) the termini are sensitive to exonuclease III with or without treatment with α S-dNTPs. If the different termini detected in these studies do not represent an experimental artifact, then there is apparently more than one type of enzymatic activity that can produce DSBs in yeast. One interesting explanation of such a difference is that different transcription factors may target different endonucleases to hotspot regions.

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