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The location and structure of double-strand DNA breaks induced during yeast meiosis: evidence for a covalently linked DNA–protein intermediate

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We have determined the precise location and structure of the double-strand DNA breaks (DSBs) formed during *Saccharomyces cerevisiae* meiosis. Breaks were examined at two recombination hot spots in both wild-type and *rad50S* mutant cells. At both loci, breaks occurred at multiple, irregularly spaced sites in a ~150 nucleotide interval contained within an area of nuclease-hypersensitive chromatin. No consensus sequence could be discerned at or around break sites. Patterns of cleavage observed on individual strands indicated that breaks initially form with a two nucleotide 5′ overhang. Broken strands from *rad50S* mutant cells contained tightly bound protein at their 5′ ends. We suggest that, in *S.cerevisiae*, meiotic recombination is initiated by a DSB-forming activity that creates a covalently linked protein–DNA intermediate.

Keywords: double-strand breaks/meiosis/recombination/*Saccharomyces cerevisiae*/yeast

Introduction

Much, and possibly all, meiotic recombination in *Saccharomyces cerevisiae* is initiated via the formation and subsequent repair of double-strand DNA breaks (DSBs). DSBs occur during meiosis both at recombination hot spots (Sun *et al.*, 1989; Cao *et al.*, 1990; Goldway *et al.*, 1993; Goyon and Lichten, 1993; Nag and Petes, 1993; de Massy *et al.*, 1995) and in regions that display normal frequencies of meiotic recombination (Game, 1992; Zenvirth *et al.*, 1992; Wu and Lichten, 1994), with DSB distributions mirroring those of meiotic cross-overs (Game, 1992; Wu and Lichten, 1994). Evidence for DSBs as recombination initiators also comes from studies of mutations that reduce or increase the frequency of DSBs at a locus; these mutations cause parallel changes in frequencies of gene conversion for markers near the affected break site (Rocco *et al.*, 1992; de Massy and Nicolas, 1993; Fan *et al.*, 1995). The timing of break formation and repair is also consistent with a role in initiating meiotic recombination (Padmore *et al.*, 1991; Goyon and Lichten, 1993). Breaks undergo extensive 5′→3′ single-strand recision (Sun *et al.*, 1991), and disappear as recombination intermediates appear (Schwacha and Kleckner, 1994). Break recision and repair, but not DSB formation, is blocked in cells carrying the *rad50-KI81* (*rad50S*) mutation (Alani *et al.*, 1990; Cao *et al.*, 1990; Sun *et al.*, 1991), which greatly facilitates the mapping and quantitation of DSBs.

Several observations indicate that local DNA sequence is not the primary determinant of DSBs positioning. All DSB sites examined to date have been found to lie in regions of chromatin that are DNase I and micrococcal nuclease (MNase) hypersensitive (Ohta *et al.*, 1994; Wu and Lichten, 1994; Wu and Lichten, 1995; S.Keeney and N.Kleckner, personal communication), and mutations that either open or constrict chromatin at a locus cause parallel changes in the frequency of DSBs (Wu and Lichten, 1994; Fan *et al.*, 1995). This indicates that DSBs occur at places where nucleosomes are disrupted and the underlying DNA is exposed. Further evidence that non-sequence factors determine DSB location comes from studies of the *ARG4* locus. DSBs occur coincident with a hot spot for the initiation of meiotic recombination, ~200 bp upstream of *ARG4* coding sequences (Nicolas *et al.*, 1989; Sun *et al.*, 1989). Certain deletions that remove the DNA sequences in which these breaks normally occur still suffer DSBs; conversely, other rearrangements that retain these sequences are no longer cut (Rocco *et al.*, 1992; de Massy and Nicolas, 1993; Goyon and Lichten, 1993; Wu and Lichten, 1995). However, the possibility of preferential cutting at specific sequences within regions of open chromatin is not excluded.

Given the likelihood that DSBs initiate meiotic recombination, it is of interest to determine the gene products responsible for their formation and the factors that determine where DSBs occur. Mutations in several yeast genes result in a complete loss of DSBs and an absolute defect in meiotic recombination (Cao *et al.*, 1990; Ivanov *et al.*, 1992; Rockmill *et al.*, 1995; S.Keeney and N.Kleckner, personal communication). It is possible that one or more of these genes encodes proteins directly responsible for break formation. However, it is also possible that mutations in these genes block steps in meiosis prior to DSBs. Identification of genes encoding a DSB-forming activity is hampered by a lack of knowledge about the precise nature of both the true substrate and the cleavage products.

We report here the single nucleotide resolution mapping of DSB sites at two hot spots for meiotic recombination: the *ARG4* promoter region (Nicolas *et al.*, 1989; Sun *et al.*, 1989); and the *YCR47c-YCR48w* intergenic region on chromosome *III* (Zenvirth *et al.*, 1992; Goldway *et al.*, 1993). We used strains carrying the *rad50-KI81* (*rad50S*) mutation (Alani *et al.*, 1990), which blocks both recision and repair of DSBs (Cao *et al.*, 1990; Sun *et al.*, 1991), in order to facilitate the mapping and quantitation of DSBs. We mapped the location of breaks that occur in wild-type cells, and show here that they are identical to those seen in *rad50S* cells. We show that the broken DNA

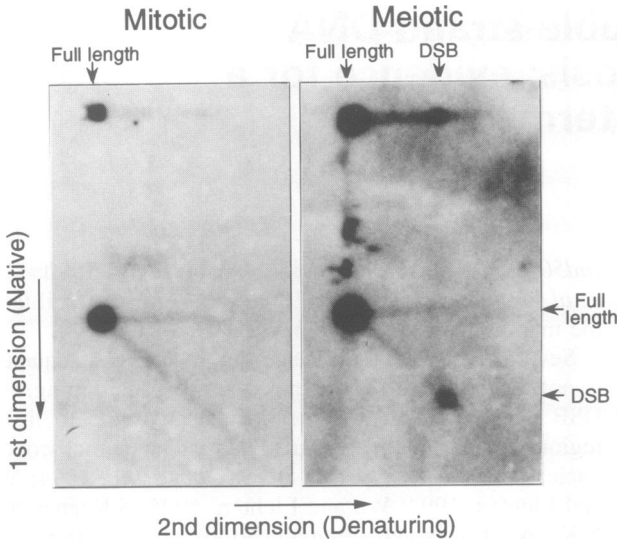

Fig. 1. Two-dimensional gel analysis of breaks at *ARG4*. *Pst*I-digested DNA from mitotic or meiotic cultures of MJL1620 (*rad50S*) was displayed on neutral/denaturing agarose gels, blotted to membranes, and probed with sequences from –1412 to –1115 relative to the *ARG4* translation start. The first electrophoresis (vertical dimension in this figure) was performed using normal, non-denaturing conditions; the second (horizontal dimension in this figure) using denaturing conditions. Before denaturing electrophoresis, identical samples were loaded at the top of each gel. These samples, detected as a single horizontal lane at the top of each gel in this figure, served as position standards. Full length, full length *Pst*I fragments; DSB, fragments formed by DSBs in the *ARG4* promoter region.

molecules formed in *rad50S* cells contain a two nucleotide 5′ overhang at their ends, and that strands ending 5′ at these breaks are tightly linked to protein.

Results

The primary meiosis-induced lesion in rad50S cells is a double-strand break

Although considerable attention has been focused on the role that DSBs play in initiating meiotic recombination in *S.cerevisiae*, less attention has been given to other potentially recombinogenic lesions, such as single-strand nicks or gaps (Porter *et al.*, 1993). We used two-dimensional neutral/alkaline agarose gel electrophoresis to determine the relative level of double- and single-strand lesions induced during meiosis at the *ARG4* locus. In this technique, restriction fragments are separated on the basis of duplex size in the first (neutral) dimension and on the basis of single-stranded size in the second (denaturing) dimension. Fully duplex fragments distribute along a diagonal. Nicked or gapped strands migrate with the full-length fragment in the neutral dimension but more rapidly in the denaturing dimension, and form a horizontal spur moving away from the full-length duplex signal. DNA from meiotic and mitotic *rad50S* cells was examined on such gels for the presence of single-strand nicks or gaps in the *ARG4* promoter region (Figure 1). A prominent signal occurred on the diagonal at the expected DSB position, while no meiosis-specific signal was detected at the same position on the horizontal (nicked molecules) spur. These results indicate that DSBs are the predominant hot spot-specific lesion formed during meiosis in *rad50S* diploids. We estimate that the frequency of molecules with a site-specific single-strand lesion cannot be more than 6% of the frequency of molecules with a DSB. A similar conclusion has been made in a study of DSBs at the artificial *HIS4–LEU2* hot spot (L.Xu, S.Keeney and N.Kleckner, personal communication). This conclusion is further supported by the finding that single-strand lesions are not present in full-length restriction fragments purified from meiotic *rad50S* DNA that contain the *CYS3* DSB hot spot (de Massy *et al.*, 1995, accompanying paper).

Breaks occur at numerous places within a double-strand break site

Previous agarose gel-based studies indicated that DSBs occur ~200 bp upstream of *ARG4* and *YCR48w* coding sequences (Sun *et al.*, 1989, 1991; Goldway *et al.*, 1993). Agarose gels provide neither the sensitivity nor the resolution to map precisely where breaks occurred. As a first step towards single nucleotide mapping of breaks, we determined the overall pattern of breaks in these regions, using native polyacrylamide gels that provided a limit of resolution of about five nucleotides. The pattern of DSBs in both regions is shown in Figure 2. Previous low resolution studies had suggested that DSBs occurred at two places in the *ARG4* promoter region (Sun *et al.*, 1991; de Massy and Nicolas, 1993; Ohta *et al.*, 1994). In the medium resolution gels used here, breaks appeared to occur at nine places in a region 120–270 nucleotides upstream of *ARG4* coding sequences (Figure 2A). Coincident break patterns were observed in experiments that mapped breaks from either the *DED81* side or the *ARG4* side (data not shown). Coincident DSB patterns were also seen in medium resolution denaturing polyacrylamide gels that mapped breaks from either side of the region with probes that hybridized to either one or the other strand of the duplex (data not shown). These results are inconsistent with the presence of single- or double-strand gaps of more than a few nucleotides at *ARG4*. At *YCR47c-YCR48w* (Figure 2B), breaks occurred at 13 places in a region 100–270 nucleotides upstream of *YCR48w*. Coincident break locations and frequencies were observed when mapping from either side of the *YCR47c -YCR48w* intergenic region, again indicating the absence of double-strand gaps greater than a few nucleotides (data not shown).

Previous studies have indicated a close correspondence between DSB sites and DNase I- and MNase-hypersensitive sites in chromatin (Ohta *et al.*, 1994; Wu and Lichten, 1994). We used high percentage agarose gels to examine this relationship more closely. We compared DSB patterns at *ARG4* and *YCR47c-YCR48w* with patterns of MNase and DNase cleavage in chromatin from mitotic cells and from meiotic cells before (2 h), at (3 h) and after (4 and 5 h) the time of DSB formation. At both loci, DSBs occurred within a ~200 nucleotide interval that was MNase and DNase I hypersensitive (Figure 3A and data not shown). However, in *YCR47c-YCR48w*, an area with the most frequent DSBs was relatively insensitive to MNase and DNase I, and peaks of nuclease hypersensitivity occurred in areas with relatively few DSBs (Figure 3B).

Mapping DSB sites at the nucleotide resolution level

The approach used to map DSB sites in both wild-type and *rad50S* strains is illustrated in Figure 4. Genomic

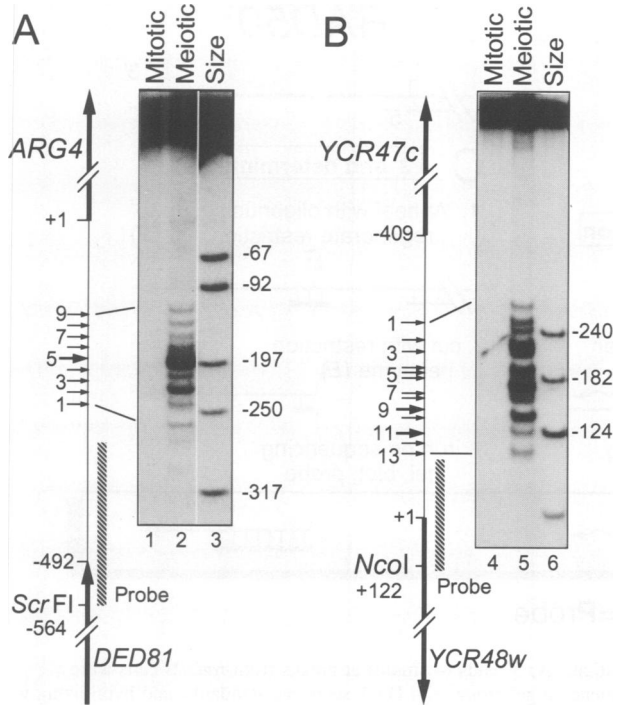

Fig. 2. Medium resolution mapping of DSBs in the *ARG4* promoter and *YCR47c-YCR48w* regions. DNA from a *rad50S* strain (MJL1620) was digested, displayed on neutral polyacrylamide gels, and probed as follows. (**A**) DSBs in the *ARG4* promoter region. DNA was digested with *Scr*FI and hybridized with double-stranded probe A (see Materials and methods). Lanes 1 and 3, DNA from mitotic cells; lane 2, DNA from meiotic cells. In lane 3, size standards of doubly digested mitotic DNA were included (see Materials and methods). Second enzyme sites, relative to the *ARG4* translation start, are: *Sac*I (–67); *Dde*I (–92); *Bsp*HI (–197); *Taq*I (–250); *Hpa*I (–317). Probe sequences were from –567 to –319. (**B**) DSBs in the *YCR48w-YCR47c* intergenic region. DNA was digested with *Nco*I and hybridized with double-stranded probe B (see Materials and methods). Lanes 4 and 6, DNA from mitotic cells; lane 5, DNA from meitotic cells. Doubly digested size standards were included in lane 4. Second enzyme sites, relative to *YCR48w*, are: *Alw*NI (–124); *Msc*I (–182); *Cla*I (–240). Probe sequences were from –61 to +122. In the schematic representation of both regions, horizontal arrows indicate break locations; vertical arrows indicate coding sequences. Break locations are numbered for cross-reference with Figures 5C and 6B.

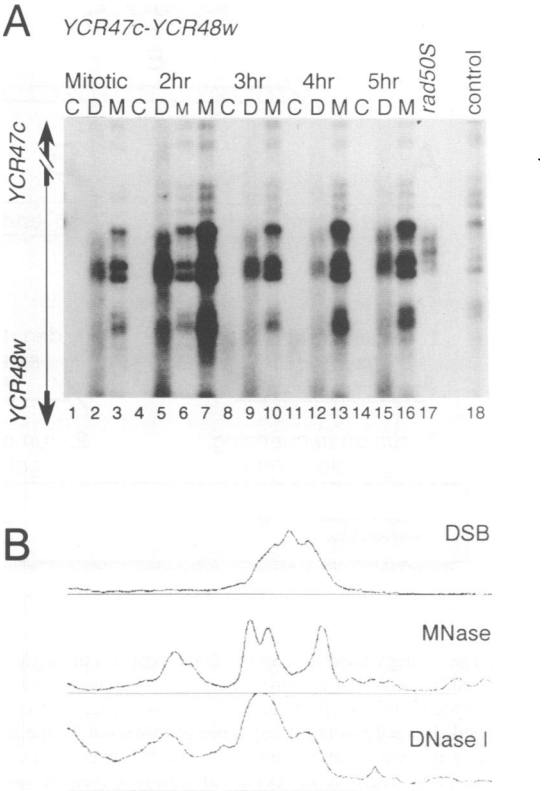

Fig. 3. Nuclease-hypersensitive and DSB sites at *YCR47c-YCR48w*. (**A**) Samples were digested with *Xho*I, which cuts at nucleotides –1930 and +972 relative to the *YCR48w* translation start. DNA was displayed on 1.2% agarose gels, transferred to membranes and hybridized to a *YCR48w*-specific probe (nucleotides +318 to +972). Lanes 1–16 contain chromatin from mitotic wild-type cells (MJL1578) or from cells of the same strain taken at the indicated time after induction of sporulation, designated as follows: C, untreated control; D, chromatin digested with DNase I (0.33 U/μg DNA in chromatin); M, chromatin digested with MNase. MNase added, in terms of U/μg DNA, was as follows: lane 3, 0.67; lane 6, 0.33; lane 7, 2.0; lane 10, 0.67; lanes 13 and 16, 1.25. Lane 17 (*rad50S*), meiotic DNA from *rad50S* cells. Lane 18 (control), mitotic DNA digested with 0.03 U MNase/μg DNA. DSBs appear at 2.5–3 h after induction of meiosis and are healed by 5 h (Goyon and Lichten, 1993; Wu and Lichten, 1994); because of extensive and variable 5′→3′ recision of break ends (Sun *et al.*, 1991), DSBs are not detected. (**B**) Densitometer traces of lanes 17, 6 and 5.

sequencing and footprinting techniques (Church and Gilbert, 1984; Shimizu *et al.*, 1991) were used to determine break locations on both strands of the duplex, probing from both sides of the break. For strands ending 3′ at breaks, restriction enzyme digests of DNA from *rad50S* cells were displayed on sequencing gels, blotted to membranes and hybridized to appropriate strand-specific probes (Figure 4A). In wild-type cells, DSBs undergo 5′→3′ recision generating long 3′ single-stranded regions, and thus restriction sites near breaks are frequently resistant to digestion (Sun *et al.*, 1991). To recreate the duplex at relevant restriction sites, samples from wild-type cells were hybridized to an oligonucleotide and then restriction enzyme digested (Figure 4C). In both cases, fragment end points were determined by comparison with sequence standard lanes generated using primers with a 5′ end identical to those created by the restriction enzyme, and were detected using the same strand-specific probe.

To detect strands ending 5′ at breaks in DNA from *rad50S* cells, we performed multiple rounds of primer extension (Figure 4B). Primers complementary to strands ending 5′ at breaks were extended using *Taq* DNA polymerase and a template of undigested DNA. Because *Taq* DNA polymerase adds a single non-templated nucleotide to the 3′ end of synthesized strands (Clark, 1988), these fragments were one nucleotide longer than the actual 5′-broken strands. The strands generated could be detected using the same strand-specific probe used to detect sequence standards and strands ending 3′ at breaks, and their end points could be determined by comparison with the same sequence standard lanes.

All three samples described above (3′ end determination in *rad50S* and in wild-type, 5′ end determination in *rad50S*) were loaded on gels flanked by sequencing standard lanes. Also included were samples of mitotic DNA spiked with double digests made with a second enzyme that cut within the DSB region. Comparison of these artificial break end points with the sequence standards would have revealed systematic errors leading to mis-

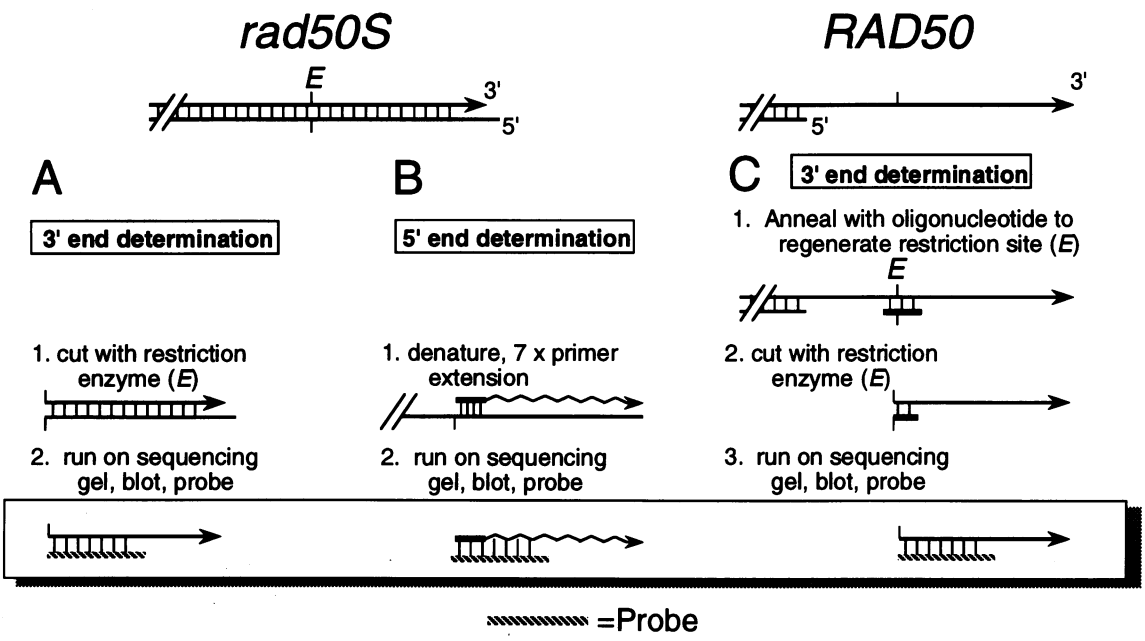

Fig. 4. The strategy used to map the DSB locations at single nucleotide resolution. (**A**) 3′ ends of strands at breaks from *rad50S* cells were determined by cutting at a nearby restriction site, displaying samples on a sequencing gel along with DNA sequence standards, and hybridizing with a strand-specific probe. (**B**) 5′ ends of strands at breaks from *rad50S* cells were determined by primer extension, using a primer whose 5′ end corresponded exactly to the phosphodiester bond cut by the restriction enzyme used in (A). The resulting products were displayed on the same sequencing gel and detected using the same probe as in (A). (**C**) 3′ ends of strands at breaks from wild-type cells were determined as follows. The extensive 5′→3′ recision that occurs in wild-type meiosis would be expected to render the restriction site used in (A) single-stranded. The DNA duplex at this site was restored by hybridizing with the appropriate oligonucleotide, thus allowing restriction enzyme digestion and analysis as described in (A).

mapping of DSB sites. In fact, the ends of restriction-digested mitotic samples always mapped to the expected sequence location.

Figures 5 and 6 contain a representative sample and summary of gels used to map DSBs at *ARG4* and at *YCR47c-YCR48w* in the regions that medium resolution gels had identified as containing the majority of breaks. Four conclusions emerge.

The first conclusion concerns the nature of the break itself. Break patterns obtained by direct probing to detect 3′ break ends on one strand were mirrored, with a two nucleotide gap, by 3′ break end patterns on the opposite strand of the duplex. Break patterns obtained by primer extension to detect 5′ break ends on one strand were mirrored, with a four nucleotide overlap, by the pattern of 5′ break ends on the opposite strand. When adjusted to account for the one nucleotide terminal transferase activity of *Taq* DNA polymerase, this finding indicates that 5′ break end patterns on the two strands of the duplex overlapped by two nucleotides. Break 3′ ends detected by direct probing were duplicated, with a three nucleotide displacement, in the pattern of break 5′ ends detected by the same probe; adjusting for the terminal transferase activity of *Taq* DNA polymerase yields a displacement of two nucleotides. All of these finding are consistent with the suggestion that the DSBs formed in *rad50S* cells at *ARG4* and at *YCR47c-YCR48w* contain a two nucleotide 5′ overhang, with no net loss of material. de Massy and co-workers, in an accompanying study of DSBs at the *CYS3* locus, have concluded that DSBs which are formed in *rad50S* cells at this locus contain blunt ends (de Massy *et al.*, 1995). This apparent discrepancy may reflect differences in the strain backgrounds used in the three studies; alternatively, it may reflect the presence of different DSB-forming systems at different places in the yeast genome. Experiments to examine the basis of this discrepancy are in progress.

The second conclusion concerns the end points of DSBs in DNA from wild-type cells. Agarose gel-based studies indicate that, in wild-type cells, strands ending 5′ at breaks undergo extensive 5′→3′ recision and the strands ending 3′ at breaks do not (Sun *et al.*, 1991; Bishop *et al.*, 1992). We observed identical 3′ break end patterns in DNA from *rad50S* and from wild-type cells (compare lane 9 with lane 10, and lane 13 with lane 14 in Figures 5A and 6A). This is consistent with the suggestion that in wild-type cells, as in *rad50S* cells, breaks initially contain a two nucleotide 5′ overhang. Although the 5′ ends of broken strands suffer extensive 5′→3′ recision in wild-type cells, it appears that the 3′ ends of these broken molecules remain intact to the nucleotide for a substantial period of time. Similar conclusions have been made in studies of the *HO*-induced mitotic DSBs that initiate mating-type switching in *S.cerevisiae*. Both substantial 5′ end recision and efficient retention of a marker near the 3′ break end have been demonstrated (White and Haber, 1990; Ray *et al.*, 1991; Haber *et al.*, 1993).

Third, DSBs occur at even more places than were identified by medium resolution gels. In fact, most bands that appeared to represent a single break site on medium resolution polyacrylamide gels were revealed on sequencing gels to contain more than one break (Figures 5C and 6B). A total of 27 DSB sites were detected in the region from −263 to −124 relative to the *ARG4* start of translation;

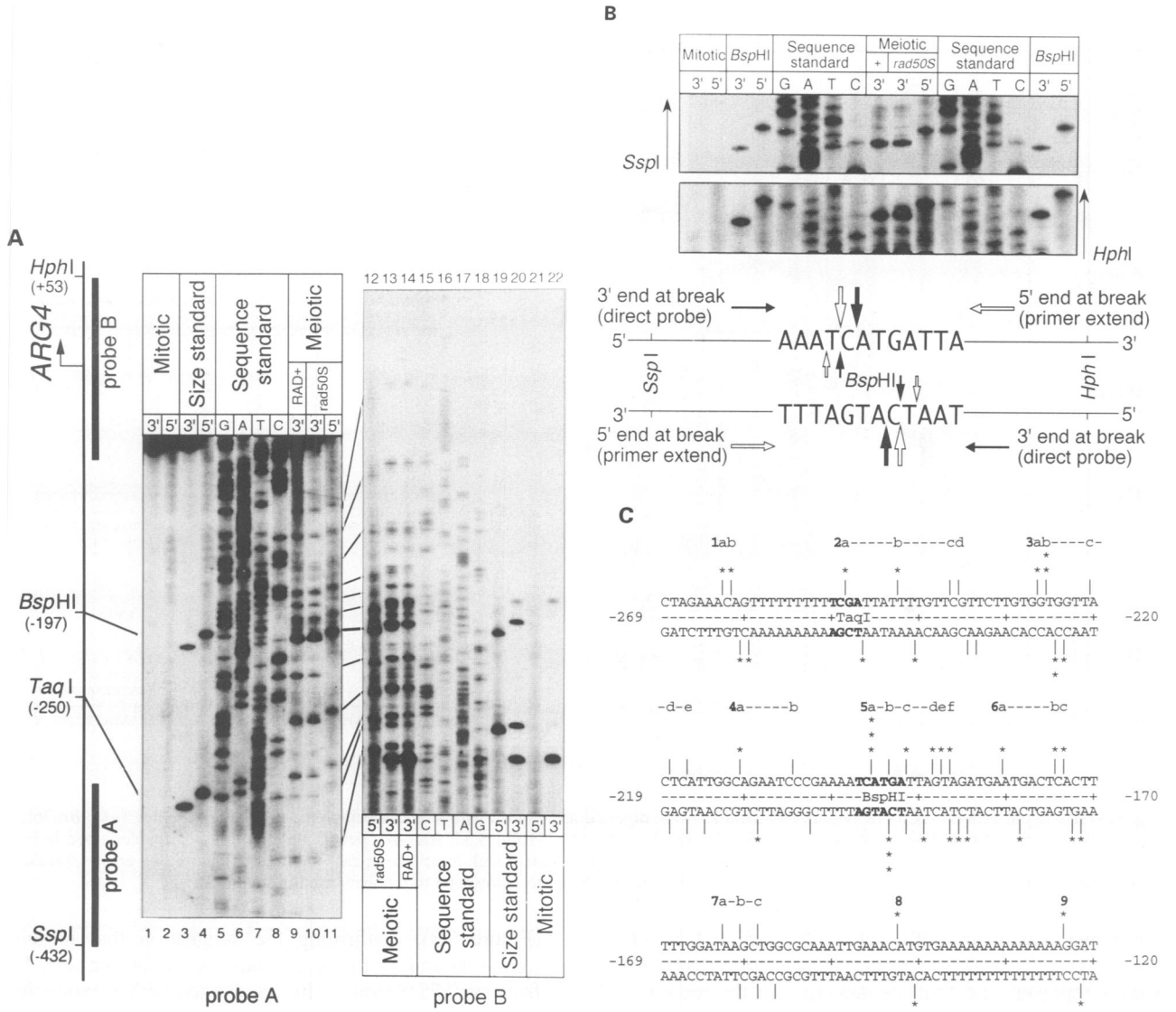

Fig. 5. DSB sites at *ARG4*. **(A)** Sequencing gel analysis of the DSBs in the *ARG4* promoter region. Samples were digested and hybridized to strand-specific probes as follows: lanes 1–11, *Ssp*I, probe was –427 to –319 relative to *ARG4*; lanes 12–22, *Hph*I, probe was –56 to +54. To facilitate comparison, the gel containing the latter samples is flipped vertically. The location of the 3′ ends of breaks in *rad50S* (lanes 10 and 13), in wild-type (*RAD50*) cells (lanes 9 and 14), and of the 3′ ends of primer extension products that reveal break 5′ ends (lanes 11 and 12) are determined by comparison with DNA sequence standard lanes (lanes 5–8 and 15–18), which contain small amounts of a sequencing reaction loaded with mitotic DNA. Size standard lanes consist of a small amount of restriction enzyme-digested mitotic DNA (cut with either *Taq*I or *Bsp*HI) mixed with uncut mitotic DNA; samples were then processed as described in Figure 4 to detect cut 3′ ends (lanes 3 and 20) or cut 5′ ends (lanes 4 and 19). Lanes 1, 2, 21 and 22 contain mitotic DNA, processed as described to detect break 3′ ends (lanes 1 and 22) or break 5′ ends (lanes 2 and 21). **(B)** A portion of a gel used in (A), showing mapping of the most prominent break site (DSB5a in C) and of fragment ends from a control *Bsp*HI digest. Arrows indicate the measured ends of strands used to map DSB or restriction enzyme cuts, as follows: large solid arrows, DSB 3′ ends; small solid arrows, *Bsp*HI-cut 3′ ends; large hollow arrows, DSB 5′ ends; small hollow arrows, *Bsp*HI-cut 5′ ends. On the top strand, 3′ ends were determined from *Ssp*I digests and 5′ ends from *Hph*I digests; on the bottom strand, 3′ ends were determined from *Hph*I digests and 5′ ends from *Ssp*I digests. Strands mapping break or cut 5′ ends (hollow arrows) were created using *Taq* DNA polymerase, which adds a single terminal non-templated nucleotide. Therefore, the actual 5′ ends of strands at the DSB or *Bsp*HI cut are one nucleotide to the right on the top strand, and one nucleotide to the left on the bottom strand. **(C)** Location of DSBs. Numbers are relative to the *ARG4* translation start. Break locations on each strand are indicated by vertical lines. Breaks were classified into four ranks in terms of their frequency (percentage of chromosomes broken); these ranks are indicated by the number of asterisks above each arrow. ***greater than 0.5%; **0.25–0.5%; *0.1–0.25%; no asterisk,<0.1%. Breaks are numbered so as to be consistent with the numbering system used in Figure 2A.

similarly, 28 individual break sites were detected in the region from –272 to –106 relative to the *YCR48w* putative translation start. Breaks were irregularly spaced, and no obvious common motifs occurred, either within or between break sites. The only possible rule emerging from this analysis was that breaks do not occur in long A·T tracts. Similar results have been obtained at these two loci by A.Nabetani and H.Ogawa (personal communication).

Fourth, different sites suffer breaks at very different frequencies. At *ARG4*, the range was from 0.8% of chromosomes (nt –196/–194) to 0.04% of chromosomes (nt –237/–235 and nt –236/–234). At *YCR47c-YCR48w*,

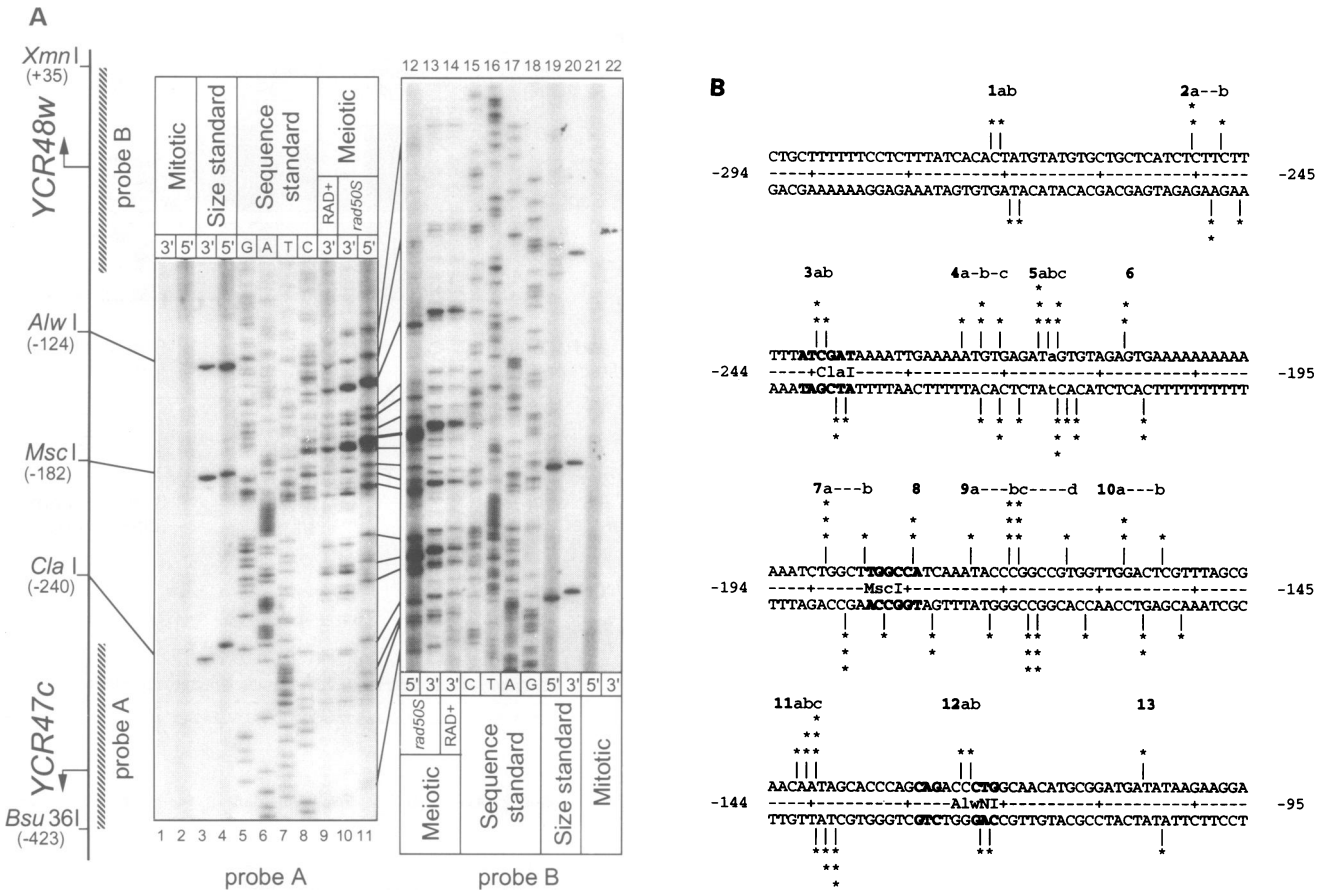

Fig. 6. DSBs in the *YCR47c-YCR48w* region. **(A)** Samples were digested and probed with strand-specific probes as follows: lanes 1–11, *Bsu*36I, probe was –422 to –251 relative to *YCR48w*; lanes 12–22, *Xmn*I, probe was –115 to +35. All other details are identical to those described in Figure 5A. **(B)** Location of DSBs. Numbers are relative to the *YCR48w* translation start. Break locations on each strand are indicated by arrows, break frequencies are indicated as described in Figure 5C, and breaks are numbered to correspond to numbers used in Figure 2B.

the range was from 0.9% (nt –217/–215 and nt –169/–167) to 0.1% (nt –271/–269) (data not shown). The lesser values represent the limit of detection in the region, and the possible occurrence of other breaks at even lower frequencies cannot be excluded.

Protein is tightly linked to the 5′ ends of breaks in rad50S cells

As has been shown above, primer extension determinations of break 5′ ends are in excellent agreement with direct blotting determinations of break 3′ ends. However, attempts to determine break 5′ ends by direct probing revealed that strands with these ends migrated less rapidly than expected on the basis of their sequence content (Figure 7A). Their rate of migration in sequencing gels was that expected for molecules with an additional 4–6 nucleotides, consistent with the presence of material linked to the 5′ ends of breaks. Our DNA purification protocols included digestion with proteinase K, which cuts after hydrophobic aliphatic and aromatic amino acids (Ebeling *et al.*, 1974). It is possible that a small peptide remained bound to break 5′ ends after purification, and that this peptide was responsible for the observed reduction in electrophoretic mobility. To test this suggestion, DNA from meiotic *rad50S* cells was digested further with pronase, which contains amino- and carboxyl-terminus-specific exopeptidases. Pronase treatment increased the electrophoretic mobility of 5′ broken strands significantly (Figure 7B), confirming the suggestion that protein is tightly bound to break 5′ ends. As a further test, DNA from *rad50S* meiotic cells was treated with λ exonuclease, which resects the ends of duplex DNA molecules in a 5′→3′ direction (Little, 1967). Strands ending 5′ at breaks were resistant to λ exonuclease treatments that completely removed strands ending 5′ at a two nucleotide 5′ overhang generated by *Cla*I (Figure 7C). We conclude that broken molecules from *rad50S* cells contain protein tightly linked to their 5′ end through a bond that quantitatively survives the denaturing conditions that occurred during sample preparation (65°C in 1% SDS, 80°C in 50% formamide). Also consistent with this conclusion is the observation that, if lysates are not digested with proteinase, broken molecules are selectively removed from meiotic *rad50S* DNA by treatments (such as phenol extraction or SDS/potassium acetate precipitation) that separate protein from DNA (de Massy *et al.*, accompanying paper; J.Liu and M.Lichten, unpublished data; S.Keeney and N.Kleckner, personal communication).

Discussion

What determines the location of double-strand breaks?

In this paper, we report the location and structure of DSBs that occur at meiotic recombination hot spots in the *ARG4* promoter region and in the *YCR47c-YCR48w* intergenic

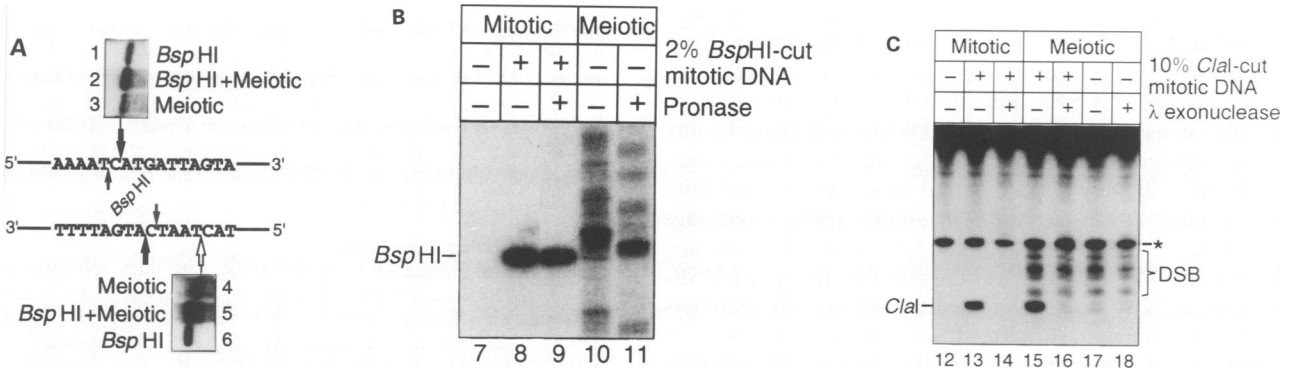

Fig. 7. Strands ending 5′ at DSBs are linked to protein. (**A**) Electrophoretic mobility of strands ending 3′ and 5′ at the major *ARG4* promoter DSB. Lanes 1 and 6, mitotic DNA from MJL1620, digested with *Ssp*I and *Bsp*HI; lanes 3 and 4, meiotic DNA digested with *Ssp*I alone; lanes 2 and 5, a mixture of the two. DNA was displayed on sequencing gels, blotted and hybridized to a strand-specific probe complementary to strands ending 3′ at breaks (lanes 1–3) and to a strand-specific probe complementary to strands ending 5′ at breaks (lanes 4–6). For the sake of clarity, only the portion of the gel containing the most frequent DSB site at *ARG4* (DSB5a in Figure 6C) is shown. Solid arrows, actual location of fragment ends; hollow arrow, apparent location of DSB fragment ends inferred from this gel. (**B**) Pronase treatment. Lane 7, mitotic MJL1620 DNA; lanes 8 and 9, mitotic MJL1620 DNA mixed in a 50:1 ratio with a *Bsp*HI digest of mitotic MJL1620 DNA; lanes 10 and 11, meiotic MJL1620 DNA. All samples were digested with *Ssp*I; lanes 9 and 11 were subsequently digested with pronase. Samples were displayed on a sequencing gel, blotted and probed with an *ARG4* strand-specific probe complementary to strands ending 5′ at breaks. Only the portion of the gel containing DSB5a is shown. (**C**) λ exonuclease digestion of mitotic and meiotic DNA. Lane 12, mitotic MJL1620 DNA; lanes 13 and 14, mitotic MJL1620 DNA mixed with *Cla*I-digested mitotic DNA in a 9:1 ratio; lanes 17 and 18, meiotic MJL1620 DNA; lanes 15 and 16, a mixture of the two. Indicated lanes were treated with λ exonuclease. After exonuclease inactivation, DNA was digested with *Mvn*I, which cuts at −721 and +1030 relative to the *YCR47c* translation start, displayed on a denaturing polyacrylamide gel, blotted and hybridized to a strand-specific probe (nucleotides −720 to −511 relative to *YCR48w*) that detects strands ending 5′ at DSBs or at the *Cla*I-induced sham break in the *YCR47c-YCR48w* intergenic region. The band marked with an asterisk is due to cross-hybridization of the probe with unrelated sequences.

region. Breaks occurred in a 150–170 nucleotide interval at both loci, in at least 27 places at *ARG4* and at 28 places at *YCR47c-YCR48w*. These observations may define a general rule for DSBs at recombination hot spots, since similar break patterns are seen at the *CYS3* and at two artificial hot spots formed by the juxtaposition of yeast and bacterial sequences (Wu and Lichten, 1995; de Massy *et al.*, 1995; J.Liu, unpublished data; L.Xu, S.Keeney and N.Kleckner, personal communication). It remains to be determined whether or not sites that display lower DSB frequencies behave similarly.

The regions at *ARG4* and at *YCR47c-YCR48w* in which DSBs occur are contained within an area of nuclease-hypersensitive chromatin, consistent with the suggestion that DSBs occur in regions where nucleosomes are either absent or disrupted (Cao *et al.*, 1990; Goyon and Lichten, 1993; White *et al.*, 1993; Ohta *et al.*, 1994; Wu and Lichten, 1994). However, DSBs, DNase I and MNase cleavage patterns can be incongruent, both at the medium resolution (Figure 3) and at the nucleotide resolution levels (K.Ohta and T.Shibata, personal communication). It is likely that the three activities (DSB, DNase I and MNase) are all blocked by nucleosomes, but recognize different structural features of nucleosome-free regions.

Comparisons of sequences in and around DSB sites at *ARG4*, at *YCR47c-YCR48w* and at other loci reveal no possible sequence motifs that might either direct break-forming activities to the general area or that might determine precise break locations (data not shown). Given the apparent lack of sequence specificity, it is possible that DSB patterns at *ARG4* and at *YCR47c-YCR48w* reveal areas in nucleosome-free chromatin where both sides of the helix are exposed and available for cutting. It is also possible that observed DSB patterns reveal interactions between DSB-forming enzymes and components of an unknown protein–DNA complex at these sites. Especially intriguing in this regard are reports of changes in chromatin structure at these sites occurring prior to DSB formation (Ohta *et al.*, 1994). These may reflect either the assembly of protein complexes that attract DSB-forming activities, or the removal of proteins that would otherwise block DSB formation.

The mechanism of formation of double-strand breaks

Our experiments indicate that DSBs are initially formed with a two nucleotide 5′ overhang. Although the bulk of DSB mapping was done using DNA from meiotic *rad50S* cells, strands ending 3′ at breaks in wild-type cells were also mapped. Break patterns observed were identical to those seen in *rad50S* meiotic DNA, consistent with the suggestion that the initial meiotic lesion in wild-type cells also contains a two nucleotide 5′ overhang. Broken strands from *rad50S* cells contain protein bound to their 5′ ends, which causes them to migrate less rapidly than expected on polyacrylamide gels and to resist λ exonuclease. An attractive hypothesis to account for this observation would suggest that DSB formation proceeds through an intermediate in which the enzyme responsible becomes covalently bound to the 5′ ends of cleaved strands. We suggest that, in *rad50S* cells, the initial cleavage step occurs, but the enzyme does not release its substrate.

What is the double-strand break-forming enzyme?

The covalently linked cleavage intermediate we propose for DSB formation is reminiscent of that proposed for type II topoisomerases (Reece and Maxwell, 1991; Andersen *et al.*, 1994), which also preferentially cleave nuclease-hypersensitive regions of chromatin (Udvardy and Schedl, 1991). However, type II topoisomerases form a cleaved intermediate with a four nucleotide 5′ overhang (Andersen *et al.*, 1994), and a cold-sensitive *top2* mutant

displays normal kinetics of commitment to meiotic recombination at the non-permissive temperature (Rose and Holm, 1993). We therefore consider it unlikely that meiosis-induced DSBs are formed by topoisomerase II. We also consider it unlikely that DSBs are formed by any of the known *S.cerevisiae* double-strand endonucleases, since these display a high degree of sequence specificity and cleave DNA to form 3′ single-strand overhangs (Kostriken *et al.*, 1983; Dujon *et al.*, 1989; Gimble and Thorner, 1993). Instead, we suggest that the yeast DSB-forming activity may be encoded by one or more members of the set of meiosis-induced yeast genes, mutations in which confer a recombination-null phenotype (Engebrecht *et al.*, 1991; Malone *et al.*, 1991; Cool and Malone, 1992; Galbraith and Malone, 1992; Menees *et al.*, 1992; Pittman *et al.*, 1993).

Implications for the mechanism and control of meiotic recombination initiation

The mechanism proposed above, in that it is similar to that proposed for topoisomerase II, provides a potential way to ensure that the first step of DSB formation is reversible. Topoisomerase II binds to DNA as a dimer, and each subunit forms a covalent bond with a single strand of the cleaved duplex (Andersen *et al.*, 1994). If the DSB-forming enzyme acts in a similar way, it could hold initial cleavage products together via protein–protein interactions, even though they are no longer covalently linked. This mechanism might also allow a safe escape from commitment to meiotic recombination, since DSBs could be undone by a simple back-reaction until the time that DNA is released from the enzyme and 5′→3′ recision begins. We suggest that repair complexes containing the Rad50S mutant protein are competent to promote the repair of breaks with free 5′ ends, but are incapable of generating the signal that initiates the release of DSB ends from the break-forming enzyme. This suggestion is consistent with known phenotypes of *rad50S* mutants, which display only slight defects in mitotic DSB repair (Alani *et al.*, 1990; A.Malkova and J.E.Haber, personal communication) but are completely blocked in the repair of meiosis-induced DSBs.

The mechanism proposed above also contains certain implications regarding the first irreversible step in meiotic recombination. If DSBs first contain two nucleotide 5′ single-strand overhangs, then the 5′→3′ end recision that occurs subsequent to DSB formation will create, at the break site, a two nucleotide gap. Recombination is required to repair this gap with fidelity. Thus, the end recision events that occur in wild-type cells subsequent to break formation may represent the first irreversible step committing cells to perform meiotic recombination. Szostak and co-workers proposed a mechanism for meiotic recombination that involves, as an initial step, the formation of a double-strand gap (Szostak *et al.*, 1983). Our findings indicate that a gap is formed. At the two loci we studied, it is precisely two nucleotides long.

Materials and methods

Yeast strains

All yeast strain used in this study are direct derivatives of SK1 (Kane and Roth, 1974). All strains were *MAT***a**/*MAT*α *ura3/ura3 lys2/lys2 ho::LYS2/ho::LYS2*. Other markers were: MJL1620, *arg4-bgl/arg4-bgl rad50-KI81::URA3/rad50-KI81::URA3*; MJL1059, *arg4-nsp/arg4-bgl, leu2-K/leu2-R*; MJL1578, *leu2/leu2 arg4-nsp/arg4-nsp nuc1::LEU2/nuc1::LEU2*. The *nuc1::LEU2* mutation (Vincent *et al.*, 1988; Zassenhaus and Denniger, 1994) removes >95% of endogenous nuclease activity in lysates without affecting the formation or repair of DSBs (J.Liu, unpublished observations). All three strains contained *ARG4* sequences from pNPS500 (Lichten *et al.*, 1990) between the *Hpa*I sites that flank the gene (Goyon and Lichten, 1993).

DNA sequence standards

DNA sequence standard lanes for *ARG4* gels used pNPS500 as a template. DNA sequence standard lanes for *YCR48w-YCR47c* used a 670 nucleotide fragment that was PCR amplified from SK1 genomic DNA using GCACGGCTGCGAATTCAAAAGCTCCAACGC and CGTGGCGGTACCTCCATGGACTCCTGTGGC as primers. Several differences from the published sequence of chromosome *III* (Oliver *et al.*, 1992) were noted (data not shown). Primers used to create sequence standards were: ATTTACGTTCCTCCCTCTCTC (*ARG4*, upstream of DSBs); ATCGGTTTCACCAGTGAATCTCCC (*ARG4*, downstream of DSBs); TCAGGACGTGACATTATTTCTTCTCTGATATATTTCC (*YCR47c-YCR48w*, upstream of DSBs); TCTTCGTCTTGCCAACAAATCCTTAGTCTCCGTCATTC (*YCR47c-YCR48w*, downstream of DSBs).

DNA preparation and restriction enzyme digestion

DNA samples were prepared by either of two methods (Sun *et al.*, 1991; Goyon and Lichten, 1993) with identical results. Both involve digestion with proteinase K and RNase A. DNA was digested with restriction enzymes as recommended by the supplier, with the exception of meiotic DNA from *RAD50* cells. These samples (100 μg/ml DNA final concentration) were incubated at 70°C for 5 min, mixed with the appropriate oligonucleotide (10 μg/ml) in restriction enzyme buffer, and incubated for 20 min at room temperature. Restriction enzyme was then added, and samples were digested at 37°C for 1.5 h. Oligonucleotides used in this latter procedure were: TAATGTCACGTCCTGAGGAGTTGGCACCAC (*ARG4*, *Ssp*I); AGATTCACTGGTGAAACCGATCCTTTGATG (*ARG4*, *Hph*I); TAATGTCACGTCCTGAGGAGTTGGCACCAC (*YCR47c-YCR48w*, *Bsu*36I); TGTTGCAAGACGAAGAGTTTCTTAAGATCC (*YCR47c-YCR48w*, *Xmn*I).

Chromatin preparation and digestion

Crude chromatin samples were prepared, digested with DNase I or MNase as described (Wu and Lichten, 1994), except that nuclease digests were done for 1 min at 37°C. Post-digestion processing, agarose gel electrophoresis and blotting were as described (Wu and Lichten, 1994), except that 1.2% agarose gels were used.

Multiple-round primer extension

Primer extension reactions contained 0.2 μg genomic DNA, 0.6 μM of a single primer, 100 μM each of dATP, dGTP, dCTP, dTTP; 1× *Taq* polymerase buffer (Boehringer), 20 μl reaction volume. Samples were heated to 94°C for 3 min, 1 unit of *Taq* polymerase (Boehringer), was added and seven cycles of primer extension (94°C for 1.5 min, X°C for 1.5 min and 72°C for 2 min) were performed, where X was a primer-specific annealing temperature. Primers were identical to those used to create DNA sequence standards.

Two-dimensional agarose gel electrophoresis

One per cent agarose gels were used. Two micrograms of a genomic DNA digest, loaded in a single well at the upper left-hand corner of the gel, were electrophoresed in the first dimension using 1× TAE (Sambrook *et al.*, 1989). Gels were soaked twice for 20 min in 50 mM NaOH, 1 mM EDTA, rotated 90°, and electrophoresed in the second dimension using 50 mM NaOH, 1 mM EDTA as a buffer. Prior to this second electrophoresis, the same DNA digest was reloaded to serve as a size standard. Gels were transferred to Hybond-N membranes (Amersham) as described (Wu and Lichten, 1994).

Medium resolution electrophoresis

Five per cent polyacrylamide (19:1 monomer:bis) gels made in TBE (Sambrook *et al.*, 1989) were used. For denaturing gels, urea was added to 8 M. Restriction digests containing 1 μg DNA were used. Internal size standards consisted of 1 μg of a single enzyme digest of mitotic DNA mixed with 0.1 μg of the appropriate double digest. Gels were electroblotted onto Zetaprobe membranes (BioRad) as described (Goyon and Lichten, 1993).

Sequencing gels

Five per cent polyacrylamide (19:1 monomer:bis) sequencing gels (0.4 mm thick) containing 8 M urea, TBE were used. Internal size standards consisted of 10 ng of double digests mixed with 1 μg of single digests of mitotic DNA. DNA sequence standards consisted of 20 ng of a standard sequencing reaction mixed with 1 μg of undigested mitotic DNA. All samples were ethanol precipitated, resuspended in gel loading buffer and incubated for 2 min at 80°C. Gel contents were transferred to Zetaprobe (BioRad) membranes with a Hoeffer GeneSweep transfer apparatus.

Pronase digestion

One microgram of DNA was digested with *Ssp*I in a 10 μl volume, using the buffer supplied with the enzyme. Five microlitres of 100 mM MOPS, 200 mM EDTA (pH 7.0) and 5 μl of 20 mg/ml pronase (Boehringer) were added, the mixture was incubated at 37°C for 1 h, an additional 5 μl pronase was added, and incubation was continued for 2 h. DNA was ethanol precipitated, resuspended in sequencing gel loading buffer and displayed on sequencing gels.

λ Exonuclease digestion

Genomic DNA samples were digested at room temperature with λ exonuclease in a reaction mixture containing 20 ng/μl DNA, 0.8 U/μl λ exonuclease (BRL) and 1× buffer M, the digestion buffer supplied with *Mvn*I (Boehringer). Aliquots were heated at 65°C for 10 min to inactivate the enzyme and digested with *Mvn*I (10 U/μg DNA) at 37°C for 1 h. DNA was displayed on a medium resolution denaturing polyacrylamide gel, transferred to membranes and hybridized with strand-specific probe.

Probe preparation, hybridization and image analysis

DNA fragments used as templates for preparation of ^{32}P-labeled DNA probe were either gel purified from restriction digests of appropriate plasmids or from PCR reactions using mitotic DNA as a template. The DNA fragments used are indicated in the figure legends. Probes for native polyacrylamide gels and agarose gels were ^{32}P-labeled by random hexamer priming. Strand-specific probes for other gels were prepared by multiple-cycle primer extension. Reactions were: 10 ng DNA fragment, 40 μM each of dGTP, dATP, TTP, 150 μCi [α-^{32}P]dCTP (6000 Ci/mmol, DuPont), 1× *Taq* polymerase buffer, 12.5 μl final volume. Samples were incubated at 94°C for 1.5 min, 1 unit *Taq* DNA polymerase was added, and 25 cycles of primer extension (94°C, 0.5 min/X°C, 0.5 min/72°C, 1.5 min) were performed, where X was a primer-specific annealing temperature. Probe was purified with a push column (Stratagene). Membranes were pre-hybridized, hybridized and washed essentially as described (Wu and Lichten, 1995), except that filters from sequencing gels were hybridized and washed at 57°C. Radioactivity on filters was detected by autoradiography or with a Fuji BAS2000 PhosphorImager. Film density was determined by video densitometry using NIH Image; fluorogram density was determined using MacBas (Fuji).

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