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The nucleotide mapping of DNA double-strand breaks at the *CYS3* initiation site of meiotic recombination in *Saccharomyces cerevisiae*

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Initiation of meiotic recombination in the yeast *Saccharomyces cerevisiae* occurs by localized DNA double-strand breaks (DSBs) at several locations in the genome, corresponding to hot spots for meiotic gene conversion and crossing over. The meitoic DSBs occur in regions of chromatin that are hypersensitive to nucleases. To gain insight into the molecular mechanism involved in the formation of these DSBs, we have determined their positions at the nucleotide level at the *CYS3* hot spot of gene conversion on chromosome I. We found four major new features of these DSBs: (i) sites of DSBs are multiple with varying intensities and spacing within the promoter region of the *CYS3* gene; (ii) no consensus sequence can be found at these sites, indicating that the activity involved in DSB formation has little or no sequence specificity; (iii) the breaks are generated by blunt cleavages; and (iv) the 5′ ends are modified in *rad50S* mutant strains, where the processing of these ends is known to be prevented. We present a model for the initiation of meiotic recombination taking into account the implications of these results.

Keywords: double-strand break/hot spot/meiosis/recombination/yeast

Introduction

The molecular exchange between two homologous DNA molecules can be subdivided schematically into three main steps: initiation, formation of a stable intermediate by a strand transfer reaction and resolution. Initiation is defined here as the event(s) required to generate a substrate for the enzyme(s) involved in the strand exchange reaction. The best characterized strand exchange protein is the RecA protein from *Escherichia coli*, which requires a free DNA end or a region of single-stranded DNA in a region of homology in order to carry out the strand exchange reaction (for review, see West, 1992). One explanation for this is that RecA does not bind double-stranded DNA at physiological pH. In addition, this requirement appears also to reflect the topological problem of forming an extended region of Watson–Crick pairing between two intact duplexes.

A general question about the initiation of recombination is how DNA ends are made available in a cell to allow recombination between two duplexes which are initially intact.

In *Saccharomyces cerevisiae* meiosis, most recombination events are initiated by localized double-strand breaks (DSBs), which have been detected in many regions of the genome (Sun *et al.*, 1989; Cao *et al.*, 1990; Zenvirth *et al.*, 1992; Nag and Petes, 1993; Wu and Lichten, 1994; M.Vedel and A.Nicolas, personal communication). The interpretation of these DSBs as intermediates in the initiation of recombination comes from several observations: (i) these meiotic DSBs are transient and processed by resection of their 5′ ends to generate overhanging 3′ ends (Sun *et al.*, 1991; Bishop *et al.*, 1992), as predicted from the DSB repair model of recombination (Szostak *et al.*, 1983); (ii) in synchronous meiotic *S.cerevisiae* cells, these DSBs appear before the formation of joint molecules and recombinant products (Padmore *et al.*, 1991; Goyon and Lichten, 1993; Schwacha and Kleckner, 1994); (iii) at various sites and in various constructions analysed, the amount of DSBs correlates with the frequency of gene conversion and/or crossing over of adjacent markers (Sun *et al.*, 1989; Cao *et al.*, 1990; Rocco *et al.*, 1992; de Massy and Nicolas, 1993; Goldway *et al.*, 1993; Wu and Lichten, 1994; Fan *et al.*, 1995); and (iv) null mutations in the *Mre11*, *RAD50*, *SPO11* or *XRS2* genes abolish meiotic DSBs and meiotic recombination (Cao *et al.*, 1990; Ivanov *et al.*, 1992; Johzuka and Ogawa, 1995). Specific point mutations in the *RAD50* gene, corresponding to a single amino acid substitution in the RAD50 protein and known as *rad50S* mutations, lead to the accumulation of DSBs with unprocessed 5′ ends (Alani *et al.*, 1990; Cao *et al.*, 1990). This feature has made the *rad50S* mutation very useful for accurate mapping and quantification of DSBs.

How the meiotic DSBs are generated and what determines their location and amount is not known. DSBs, as determined at a low resolution level (± 50 bp), were found located in the promoter regions of genes (adjacent to regions displaying a high level of meiotic gene conversion and crossing over). This level of regional specificity is determined by the structure of the chromatin, based on the co-localization of DSBs and DNase I- or micrococcal nuclease-hypersensitive sites (Ohta *et al.*, 1994; Wu and Lichten, 1994), and on the modification of chromatin structure at these sites during meiosis before DSB formation (Ohta *et al.*, 1994). In order to gain a better understanding of the molecular events involved in the formation of meiotic DSBs, we characterized these intermediates further by mapping their nucleotide positions. We achieved this at one efficient initiation site of meiotic gene conversion, located in the *CYS3* promoter region on chromosome I of *S.cerevisiae* (Cherest and Surdin-Kerjan, 1992; M.Vedel and A.Nicolas, personal communication).

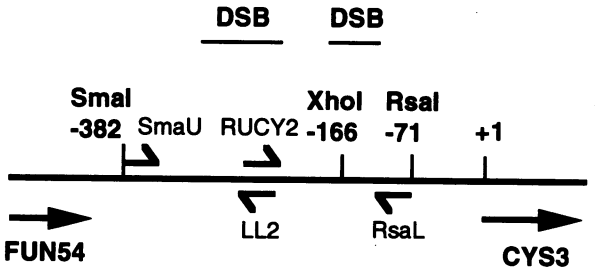

Fig. 1. Map of the *CYS3* promoter region. The end of the *FUN54* and the beginning of the *CYS3* open reading frame (numbered as +1) are indicated by arrows. The positions of the *SmaI*, *XhoI* and *RsaI* restriction sites are indicated as well as those of the meiotic DSBs as determined by agarose gel electrophoresis and of the primers (SmaU, RUCY2, LL2 and RsaL) used for the nucleotide mapping of the DSBs.

Results

Meiotic DSBs occur in two adjacent regions of the promoter of the *CYS3* gene (M.Vedel and A.Nicolas, personal communication). These two DSBs (hereafter named *CYS3* distal and proximal) are located ~150 bp apart (Figure 1), as determined by agarose gel electrophoresis, with a resolution of ± 50 bp, in *rad50S* homozygous diploids. The frequencies of DSBs are 4.7 ± 1.3% and 2.3 ± 0.7% for the *CYS3* distal and proximal sites respectively (de Massy *et al.*, 1994). Our object was to determine the nucleotide positions of these DSBs and therefore to map both 3′ and 5′ ends on both sides of DSBs in *rad50S* strains. Genomic DNA was extracted either from vegetatively growing cells (in pre-sporulation medium), used as control and defined as t0, or from cells taken 10 h after transfer into sporulation medium (defined as t10), when most DSBs have accumulated under our sporulation conditions. Both 3′ and 5′ ends were mapped using either *SmaI* (left side) or *RsaI* (right side) restriction digests of the genomic DNA, separation of fragments on a denaturing polyacrylamide gel, transfer on a nylon membrane and hybridization with strand-specific probes, as shown in Figure 1.

Mapping of the 3′ ends

Lower strand. Genomic DNAs from two congenic strains (ORD307 and ORD1175) were analysed. DNA was extracted from either t0 or t10 cells, digested with *RsaI* and hybridized with a probe synthesized with the RUCY2 primer. The size marker used in these experiments was a sequence ladder generated with the primer RsaL on a *CYS3* containing plasmid (pkMV13). Since this primer was designed to have a phosphorylated 5′ end at the *RsaI* cleavage site, the marker generated contains fragments strictly identical to the fragments to be mapped (except for the unknowns of the chemical nature of the 3′ ends). Several controls were carried out in order to verify the accuracy of mapping with this sequence ladder: (i) the sequences of plasmid pkMV13 and genomic DNAs were identical in the *CYS3* promoter region from the *SmaI* to the *RsaI* site (not shown); (ii) the migration of the sequence ladder was identical in the presence or absence of genomic DNA from t0 cells (Figure 2B, compare C and C* or G and G*), which validates the use of the sequencing marker alone in our experiments; (iii) a small amount of the *RsaI–XhoI* restriction fragment (see Figure 1) in the presence or absence of 3 μg of genomic DNA (ORD307 t0) digested with *RsaI* migrated to the expected position compared with the sequence ladder (not shown).

The results presented on Figure 2A and B show the presence of two clusters of meiotic-specific breaks, one in the –306/–226 region (*CYS3* distal, Figure 2A) and the other in the –176/–116 region (*CYS3* proximal, Figure 2B). The regions covered by these breaks fit the mapping of DSBs made on neutral agarose gels (de Massy *et al.*, 1994) and the mapping of micrococcal nuclease-hypersensitive sites (Ohta *et al.*, 1994). The comparison of the results obtained with ORD307 t10 and ORD1175 t10 DNAs (Figure 2A) shows that the positions and relative intensities of bands are identical in these two populations of meiotic cells. The intensities of the signals are illustrated by the graphs at the sides of the figures. The relative intensities (average from several experiments) and positions of breaks are shown on the sequence in Figure 4, where the weakest breaks (10% or less of the maximum value) are not shown. Twenty-six 3′ ends can be determined unambiguously, thus showing a high complexity of meiotic breaks in this region. The spacing between these fragments is variable and their intensities differ by a factor of 10.

Upper strand. The mapping of 3′ ends on the upper strand was done similarly with genomic DNA digested with *SmaI* and hybridized with a probe synthesized with the LL2 primer (Figure 3A and B). The size marker used in these experiments was a sequence ladder generated with the SmaU primer on pkMV13 DNA. Controls identical to the one described above for the lower strand were performed to validate the use of this sequence ladder. As a whole, 25 3′ ends could be identified in both *CYS3* distal and proximal regions. The summary of the positions and relative intensities obtained from three experiments appears in Figure 4.

One interesting implication of these results appears in the comparison of the location of 3′ ends on the upper and lower strands, which shows that most of the 3′ ends are located at identical positions on the upper and lower strands (Figure 4): among the 25 3′ ends on the upper strand, 22 are breaks at the same phosphodiester bond on the upper and lower strands. The seven breaks without a corresponding one at the identical position on the opposite strand are among the weakest. Either these weak breaks are qualitatively different from the others, or the absence of a break at an identical position on the opposite strand is an experimental bias due to the limit of sensitivity of our method. At the 22 positions where breaks occur on both strands, the relative intensities of upper and lower 3′ end fragments are similar. Some differences in the quantitative analysis were observed, mostly for breaks positioned at the cluster edges (positions –235/–234 and –164/–162 for instance). It is possible that the signal-to-background ratio varies when a break is detected as the larger or smaller band of the cluster. Most of these differences were in fact resolved when upper and lower strand ends were compared from one given side of the breaks, where their sizes are similar (see below). Altogether, the positions and variations of relative intensities of 3′ ends strongly suggest that they are generated by a coordinated event on both strands which produces a DSB with blunt ends.

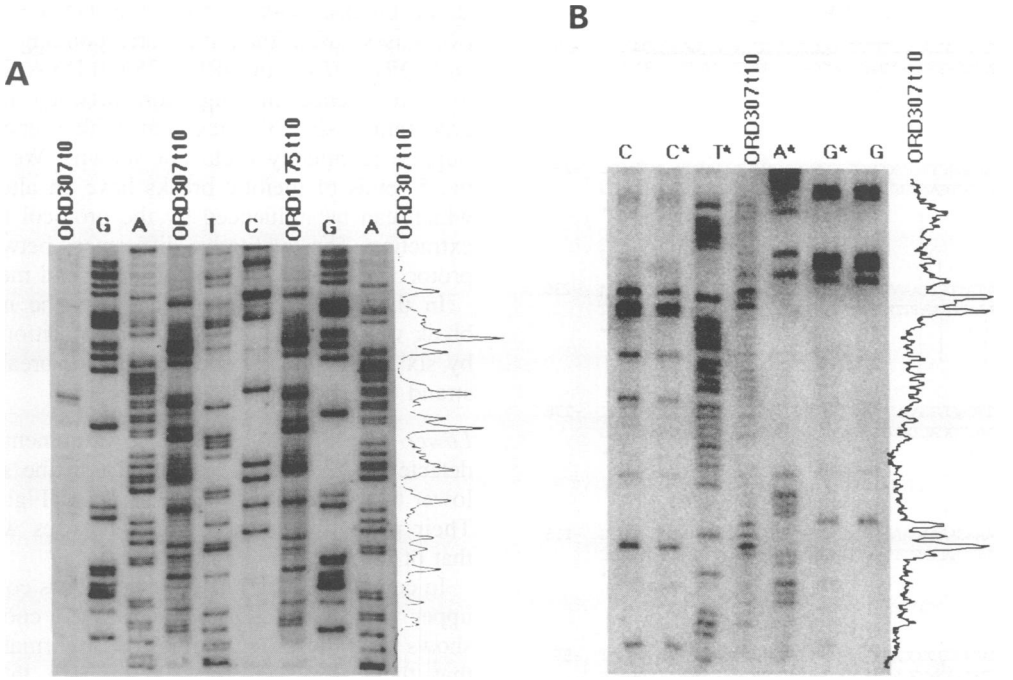

Fig. 2. The mapping of *CYS3* DSB 3′ ends on the lower strand. Genomic DNA from ORD307 (or ORD1175) before incubation in sporulation medium (t0) or after 10 h in sporulation medium (t10) were digested with *Rsa*I, separated on a sequencing gel, transfered on a nylon membrane and hybridized with the RUCY2 probe. Sequencing reactions generated with the RsaL primer on pkMV13 DNA were used as markers. The graphs were derived from phosphorimager analysis. (**A**) Detection of breaks in the *CYS3* distal region. (**B**) Detection of breaks in the *CYS3* proximal region. Sequencing lanes labelled with an asterisk contain the sequencing reaction DNA plus 3 μg of ORD307 t0 DNA, digested with *Rsa*I.

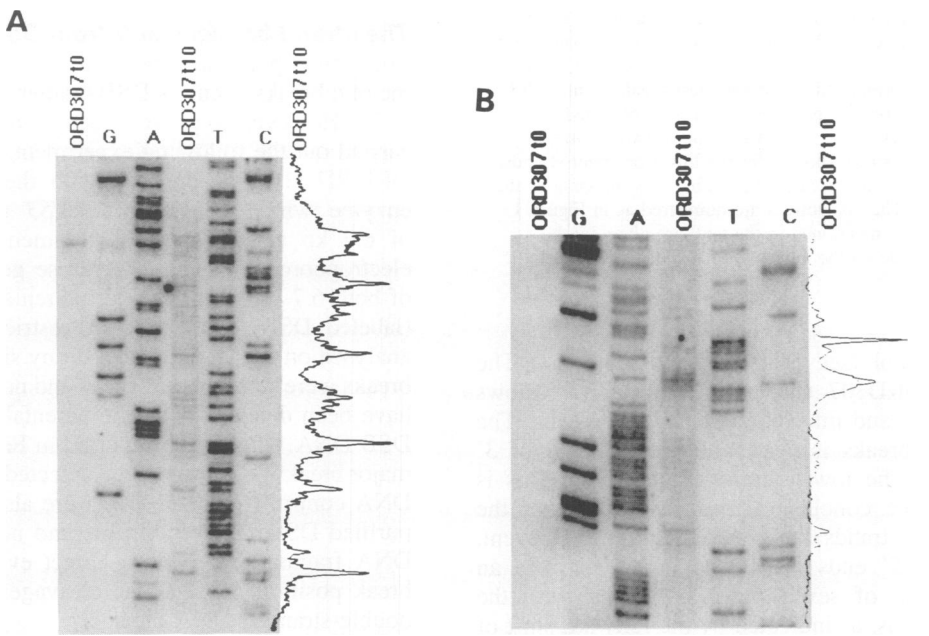

Fig. 3. The mapping of *CYS3* DSB 3′ ends on the upper strand. Genomic DNA from ORD307 t0 and t10 was digested with *Sma*I, separated on a sequencing gel, transfered on a nylon membrane and hybridized with the LL2 probe. Sequencing reactions generated with the SmaU primer on pkMV13 DNA were used as markers. The graphs were derived from phosphorimager analysis. (**A**) Detection of breaks in the *CYS3* distal region. (**B**) Detection of breaks in the *CYS3* proximal region. The sharp signal drawn on the graph is due to a non-specific spot of radioactivity.

Mapping of the 5′ ends

Using the same restriction digest as for the mapping of 3′ ends, but with probes of opposite strands, we analysed the positions of 5′ ends according to the sequencing markers generated with the primers LL2 and RUCY2.

Upper strand. Figure 5A presents the detection of 5′ ends of meiotic breaks on the upper strand in the *CYS3* distal region for three types of genomic DNA: DNA extracted from ORD307 t10 and from ORD1175 t10 by the standard protocol, and DNA from ORD307 t10 'Q' according to

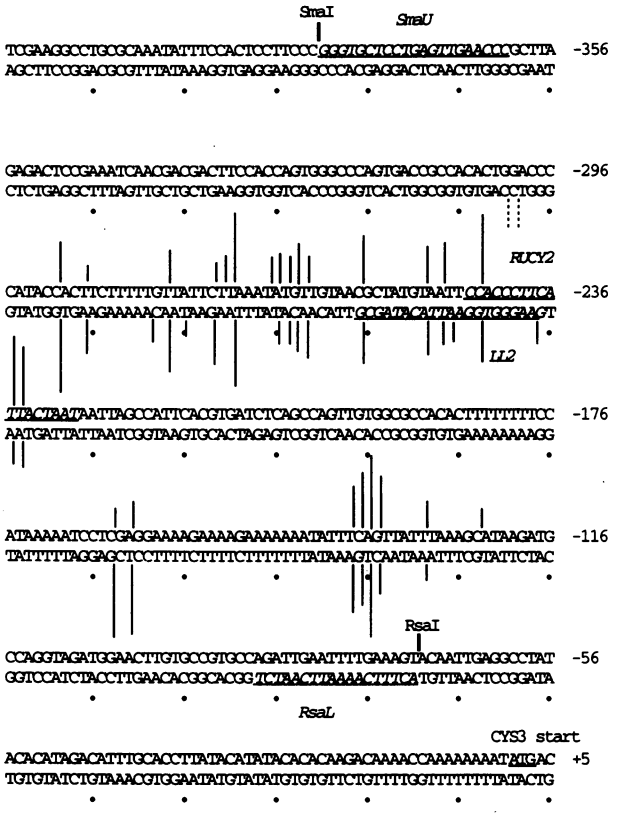

Fig. 4. Nucleotide positions and relative intensities of meiotic DSB 3′ ends on the upper and lower strand in the *CYS3* promoter region. The sequences of the primers used are italicized and underlined. Each break is indicated by a vertical bar whose length is proportional to its relative intensity. The intensities of breaks in the *CYS3* distal and proximal regions are normalized to the strongest break in the *CYS3* distal and proximal regions, respectively. Data were obtained from those reported in Figures 2 and 3 and from one or two additional independent experiments (not shown) for the lower or upper strands, respectively. Dotted lines are used for the ambiguous mapping of the –301/–300 break site. The nucleotides are numbered as in Figure 1. The positions of breaks, mentioned in the text, are given by the number of the nucleotide on the left of the phosphodiester bond cleaved.

the Qiagen protocol (see Materials and methods). The comparison of ORD307 t10 and ORD1175 t10 shows identical positions and intensities of meiotic breaks. The pattern of these breaks is nearly identical to that of 3′ ends detected on the lower strand (Figure 5B). This is consistent with the conclusion reached above, that the upper and lower strands are cleaved by a DSB event. Surprisingly, the 5′ ends on the upper strand have an apparent overhang of seven bases compared with the lower strand 3′ ends, as indicated by the leftward shift of the 5′ ends graph compared with that of 3′ ends (Figure 5B). Since the 3′ ends on both strands result from breaks at identical positions (see Figure 4) on the upper strand, for each phosphodiester bond cleaved, the 5′ end apparently overhangs the 3′ end by seven bases. This implies that either the ends of breaks have been extended by seven bases or that the fragments migrate abnormally compared with the sequencing markers. We believe that the mapping of 5′ ends obtained with ORD307 t10 'Q' (DNA prepared by the Qiagen procedure) gives us a clue to interpret this phenomenon (Figure 5A). All the fragments with 5′ ends at the breaks, detected from this DNA sample, migrate two bases faster than the corresponding ones obtained with ORD307 t10 or ORD1175 t10 DNA. This is not due to a difference of migration between the two DNA preparations since the fragments with 3′ ends at the breaks migrate identically (data not shown). We conclude that the 5′ ends of meiotic breaks have an altered migration which can be influenced by the protocol used for DNA extraction. The qualitative differences between these two protocols are indicated in Materials and methods.

In the *CYS3* proximal region on the upper strand a break was detected at an apparent position overhanging, by six bases, the 3′ end of the major break on the same strand (data not shown).

Lower strand. Only the most prominent breaks were detected in these experiments, where the sensitivity was lower than that reported in Figure 5A (Figure 6A and B). Their positions overhang by 5–6 bases with respect to that of the major 3′ ends.

In conclusion, the mapping of 5′ ends confirms that the upper and lower strands are cleaved coordinately, and shows that the 5′ ends migrate abnormally, suggesting that they are modified. We propose that the altered migration is due to the attachment of amino acids to these ends. We have indeed observed that DSB fragments co-purify with proteins after potassium acetate precipitation of cell lysates not treated with proteinase K (data not shown), suggesting that in *rad50S* mutants the 5′ ends are covalently bound to a protein.

The strand breaks result from DSBs

The results presented above favour a model in which the meiotic breaks occur as DSBs rather than as single-strand nicks. However, to prove directly that this is the case, we carried out the following experiment. Meiotic DNA from ORD307 t10 was digested with the *Hin*dIII restriction enzyme which generates a *CYS3* restriction fragment of 6.7 kb and *CYS3* DSB fragments of 3.3 kb. After electrophoresis on neutral agarose gels, DNA fragments of both 6.7 ± 0.2 kb (labeled Parental) and 3.3 ± 0.3 kb (labeled DSB) were purified, restricted with *Sma*I and analysed on sequencing gels. If any significant fraction of breaks were single-strand nicks and not DSBs, they should have been detected from the parental DNA but not from DSB DNA. The results reported in Figure 7 show that all major breaks (~10 fragments) detected in the total genomic DNA control (ORD307 t10) were also detected from the purified DSB DNA fragments and not from the parental DNA fragments, providing direct evidence that, at each break position, most of the cleavages (at least 80%) are double-stranded DNA breaks.

Discussion

The mapping at the nucleotide level of the 3′ and 5′ ends of meiotic DSBs on denaturing polyacrylamide gels, in the *CYS3* promoter region of *rad50S* diploid strains, shows that: (i) multiple sites of breaks occur with little or no sequence specificity and with variable spacing and intensities; (ii) these breaks are the result of DSBs and single-strand nicks were not detected; (iii) the positions of the 3′ ends on the upper and lower strands correspond

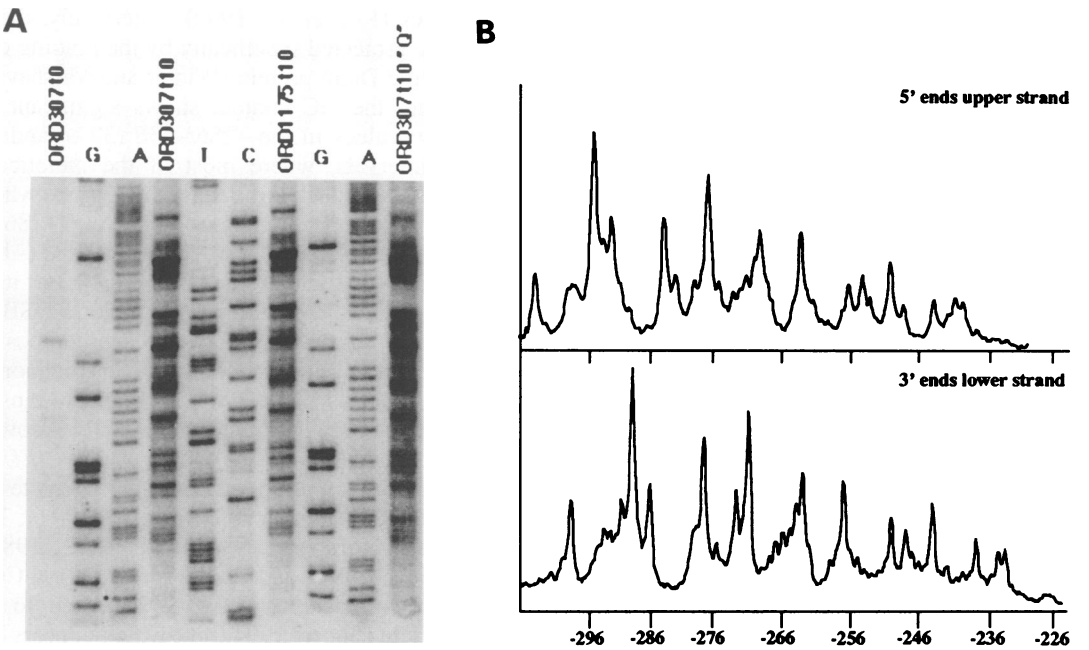

Fig. 5. (**A**) The mapping of *CYS3* DSB 5′ ends on the upper strand. Genomic DNA from ORD307 t0 and t10, from ORD1175 t10 and from ORD307 t10 'Q', prepared according to the protocol required for purification on Qiagen columns, were digested with *Rsa*I, separated on a sequencing gel, transferred to a nylon membrane and hybridized with the RsaL probe. Sequencing reactions generated with the RUCY2 primer on pkMV13 DNA were used as markers. (**B**) The comparative spacing and intensities of 5′ ends on the upper strand (from A, ORD307 t10) and 3′ ends on the lower strand (from Figure 2A). The intensities measured by phosphorimager analysis are plotted as a function of the nucleotide position of the ends, numbered as indicated in Figure 4.

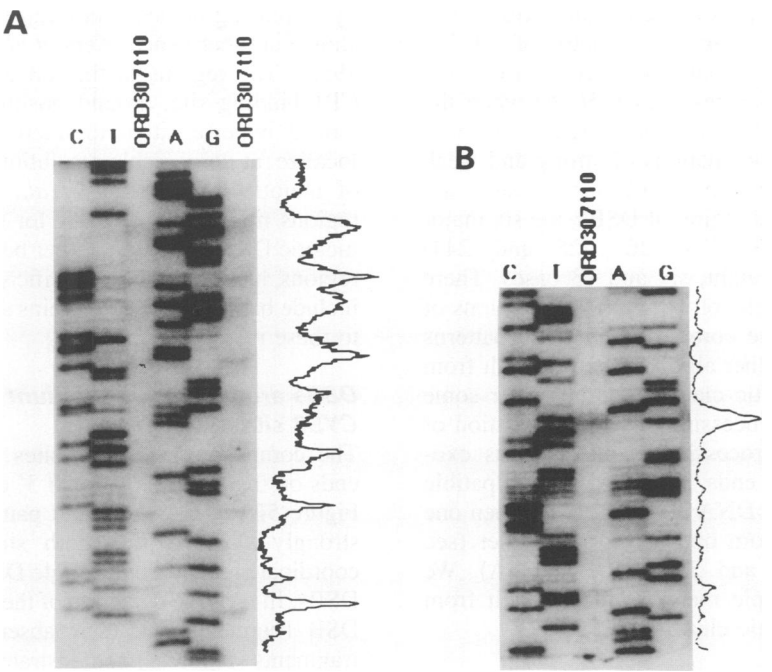

Fig. 6. The mapping of 5′ ends on the lower strand. The genomic DNA from ORD307 t10 was digested with *Sma*I and probed with the SmaU probe. Sequencing reactions were generated with the LL2 primer on pkMV13. The graphs were derived from phosphorimager analysis. (**A**) Detection of breaks in the *CYS3* distal region. (**B**) Detection of breaks in the *CYS3* proximal region.

to blunt cleavages; (iv) the 5′ ends are modified in *rad50S* strains. A model for the initiation of meiotic recombination is presented which takes into account these observations.

Meiotic DSBs show little sequence specificity

The meiotic breaks detected in the *CYS3* promoter region occur in two regions (–306/–226 and –176/–116, see Figure 4), upstream of the *CYS3* mRNA start site mapped

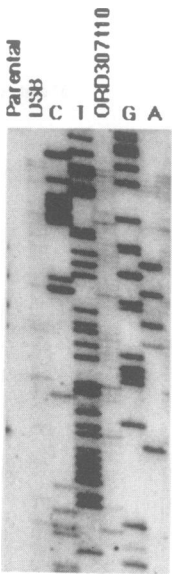

Fig. 7. The detection of 3′ ends from purified DSB fragments not from full length parental fragments. After digestion of genomic DNA from ORD307 t10 with *Hin*dIII, either full length parental *Hin*dIII restriction fragments (Parental) or fragments of the expected size of molecules having a DSB in the *CYS3* promoter region (DSB) were purified on neutral agarose gel. These fragments were then digested with *Sma*I and probed with the LL2 probe. Sequencing reactions generated with the SmaU primer on pkMV13 DNA were used as markers. Total genomic DNA from ORD307 t10, digested with *Sma*I, is shown as control.

at position –66 (V.Rocco, personal communication). The intensities of these breaks vary by a factor of 10. No consensus sequence motif could be derived from the sequences around the cleavages sites (–5/+5) when the whole population of breaks was considered or when it was divided into two subpopulations of strong and weak sites. The spacing and relative positions of sites are variable: in the *CYS3* distal region of DSBs, the six major sites (positions –290, –278, –271, –263, –257 and –244) are spaced by 12, seven, eight, six and 13 bases. There also appears to be no correlation between the positions of strong and weak sites. The complexity of these patterns raises the question of whether all these breaks result from independent endonucleolytic cleavages or whether some of them result from the processing of a subpopulation of fragments. However, a processing event, such as exonucleolytic degradation of ends, would not be compatible with the similar pattern of DNA ends observed when one strand is either probed from one side or the other (see Figures 2B and 6B, 3A and 5A or 2A and 6A). We conclude that these multiple meiotic breaks result from independent endonucleolytic cleavages.

Regions without DSBs: implications

The distribution of breaks can also be examined in a different way by analysing the features of regions (within the *CYS3* promoter) which have no meiotic DSBs. First, stretches of As (>3) are not sites of meiotic breaks, i.e. –284/–280, –185/–178, –173/–169 and –159/–156, with the exception of weak breaks in the –154/–146 region (Figure 2B). This might reflect a lower affinity for A tracts of the nuclease which generates the breaks, and which could be related to the bending property of these sequences (Koo *et al.*, 1990). Alternately, these regions might be protected specifically by the binding of a protein such as the Datin protein (Winter and Varshavsky, 1989).

Second, the GC content shows significant variations, with low values in the –286/–236 (30%) and –186/–136 (22%) intervals, where most of the meiotic DSBs are detected, and which are flanked by regions where the GC content increases abruptly (regions –306/–286 and –136/–106) to reach 60% (–356/–306) and 42% (–106/–56). It is possible that either the GC content or its variation contributes to the positioning of meiotic DSBs. Alternatively, such a feature of the sequence composition might be an indirect consequence of other functions of these regions, such as their ability to bind proteins. We have looked for consensus binding sites for known proteins (Dhawale and Lane, 1993) in the whole *FUN54–CYS3* intergenic region, and found the following sites: a binding site for the CP1 protein at position –217/–211, TCACGTG (Bram and Kornberg, 1987; Baker *et al.*, 1989; Cai and Davis, 1989; Jiang and Philippsen, 1989); a CCCATACC sequence at positions –290/–297 related to the RAP1 binding site (Shore, 1994), but which appears not to bind purified RAP1 *in vitro* (E.Gilson, personal communication); a CTTCC motif at positions –387/–383 and –333/–329, required for GCR1 binding (Baker, 1991); and potential TATA boxes at positions –134/–127 and on the opposite strand at positions –266/–272. The potential binding of CP1 to the –217/–211 region might explain the absence of breaks in this region. It might also play a role with regard to the structure of the chromatin in its vicinity by displacing nucleosomes adjacent to its binding site, as shown at other genes (Kent *et al.*, 1994; O'Connell *et al.*, 1995). The regions to the left and right of the potential CP1 binding site, around positions –270 and –160, are indeed hypersensitive to micrococcal nuclease and co-localize, at comparable resolution levels, with the regions of meiotic DSBs (Ohta *et al.*, 1994). Nucleosome-free regions might be substrates for the nuclease involved in meiotic DSB formation, either because they are accessible regions, or because of a specific structure, or because they include binding sites of proteins able to target the nuclease to these regions.

DSBs are generated by blunt cleavages at the CYS3 site

The comparison of break sites on both strands (i.e. 5′ ends on the upper strand and 3′ ends on the lower strand, Figure 5B) reveals a similar pattern of intensities, which strongly suggests that both strands are cleaved in a coordinate reaction on a single DNA molecule, generating DSBs. In fact, the detection of the major breaks on purified DSB fragments and their absence in purified parental fragments (Figure 7) demonstrates that this is the case for the most prominent breaks. Single-strand nicks, if any, are minor products. The same conclusion was reached for the *ARG4* site of DSBs (Liu *et al.*, 1995, accompanying paper). The precise nature of the *CYS3* DSBs can be deduced from the comparison of the positions of the 3′ ends on the upper and lower strands (Figure 4): breaks occur at the same phosphodiester bond on both strands, assuming that the 3′ ends are not modified in such a way as to alter their migration on denaturing polyacrylamide gels. From this mapping, we conclude that DSBs are

generated by blunt cleavages. This suggests that, for a given position, both sides of the DNA helix should be accessible.

In a parallel study, Liu *et al.* (1995, accompanying paper) have determined the nucleotide positions of meiotic breaks in the *ARG4* and the *YCR47c-YCR48w* regions in strains derivative of SK1. At all positions detected in these two regions, they found that the breaks have a two nucleotide 5′ overhang. We have tested whether this difference could be due to a strain difference, by mapping the meiotic breaks in the *CYS3* promoter region of meiotic DNA provided by J.Liu. At the two positions mapped (–164 and –162), the 3′ ends of breaks were at identical positions on the upper and lower strands and correspond therefore to blunt cleavages. We conclude that the difference between our results and that of Liu *et al.* is not based on strain differences but is region-dependent.

The 5′ ends are modified in rad50S strains

The mapping of the 5′ ends led us to conclude that these are modified in *rad50S* strains for three reasons. First, from the mapping of 3′ ends which indicates that the breaks are generated by blunt cleavages, one would have expected that the 5′ ends were at the same position as the 3′ ends on the opposite strand (or shorter if they were degraded). Instead, we found that the 5′ ends were 5–7 bases longer than expected, depending on the sites. Second, the apparent molecular weight of 5′ end fragments varied at given sites (from +7 to +5), depending on the protocol used for extraction of genomic DNA (Figure 5A). Third, DSB fragments co-purify with proteins after potassium acetate precipitation (data not shown). Since the modification is stable during the various steps of extraction of the genomic DNA, we propose that it involves the covalent binding of a protein to the 5′ ends. Depending on the protease treatment (proteinase K or the broad specificity serine protease from Qiagen), a variable number of amino acids might remain bound at the 5′ ends, resulting in the slower and variable migration of these fragments on sequencing gels. Such protein binding would be responsible for the protection of the 5′ ends from exonucleolytic degradation *in vivo* in *rad50S* strains (Alani *et al.*, 1990; Cao *et al.*, 1990).

An important point in terms of the mechanism of initiation of meiotic recombination is to know whether the modification of the 5′ ends observed in *rad50S* strains is a normal intermediate of the reaction taking place in *RAD50* strains, or a byproduct due to the *rad50S* mutation (see our model below).

Implications for the double-strand break repair model

The present results relate to two aspects of the mechanism of recombination during meiosis: (i) the nature of the meiotic breaks, which are DSBs and not single-strand nicks, provides direct evidence for the initiation of meiotic recombination by DSBs; and (ii) the mapping of breaks described above (in *rad50S* strains) is not compatible with the formation of double-strand gaps. This also should be the case in *RAD50* strains, as Liu *et al.* (1995) have shown the identical positions of 3′ ends in *rad50S* and *RAD50* strains. Since the mapping of ends revealed a two nucleotide 5′ overhang at the *ARG4* and the *YCR47c-YCR48w* regions (Liu *et al.*, 1995, accompanying paper), 5′ end degradation ought to create a 2 bp gap at these sites, but no gap at the *CYS3* site where the ends are blunt. The direct consequence for the DSB repair model (Szostak *et al.*, 1983; Sun *et al.*, 1991) is that most or all gene conversion events would result from mismatch repair of heteroduplex DNA.

A model for the initiation of meiotic recombination

Considering the several features of position and structure of meiotic DSBs described above, the activity involved in their formation should have some specific characteristics. The enzyme should be essentially sequence non-specific and be able to access the DNA helix from both sides in order to introduce coordinate cleavages (blunt at the *CYS3* site, two base 5′ overhang at *ARG4* and *YCR47c-YCR48w* sites) on each strand, sequentially or simultaneously. Low specificity (Endo.*Sce*I) or non-specific (NUC1) endonucleases from *S.cerevisiae* involved in mitochondrial recombination have been described (Nakagawa *et al.*, 1992; Zassenhaus and Denniger, 1994). However, the precise nature of the cuts generated *in vitro* by Endo.*Sce*I, i.e. four base 3′ overhangs (Watabe *et al.*, 1983; Shibata *et al.*, 1984), and its consensus cleavage site (Nakagawa *et al.*, 1992) are not compatible with the mapping of meiotic DSBs. In addition, strains defective for the NUC1 nuclease have no phenotype on meiotic DSB formation (J.Liu and M.Lichten, personal communication). Some other non-specific endonuclease(s) might be responsible for the formation of meiotic DSBs. In this case, the fragments that accumulate with unrecessed and modified 5′ ends in *rad50S* strains would be byproducts of the normal recombination pathway.

The other type of enzyme generating DSBs with little sequence specificity are topoisomerases II (topoII; Liu *et al.*, 1983; Sander and Hsieh, 1985). Several similarities between meiotic DSBs and topoII intermediates are particularly striking. (i) The 5′ ends of meiotic DSBs are modified in *rad50S* strains most likely because of remnants of a protein linkage (see above). TopoII is covalently bound to the 5′ ends of the DNA as an intermediate called the cleavable complex (Osheroff *et al.*, 1991). (ii) The meiotic DSBs occur in DNase I- or micrococcal nuclease-hypersensitive regions with little sequence specificity similarly to topoII drug-induced DSBs (Yang *et al.*, 1985; Rowe *et al.*, 1986; Reitman and Felsenfeld, 1990; Udvardy and Schedl, 1991; Käs and Laemmli, 1992). However, the meiotic DSBs are blunt (at the *CYS3* site) or two base 5′ overhangs (at the *ARG4* and *YCR47c-YCR48w* sites), whereas the cleavage sites of topoII have four base 5′ overhangs *in vitro* (Liu *et al.*, 1983; Sander and Hsieh, 1983) and *in vivo* (M.E. Borgnetto, F.Zunino, S.Tnelli, E.Käs and G.Capranico, personal communication). Two interpretations might account for the difference of stagger at the meiotic cleavage sites: either different enzymes with different specificities act upon different regions in the genome, or the structure of the DNA influences the relative positions of cleavage on the two strands. If the cleavages are sequential (as is the case for topoII; Osheroff *et al.*, 1991), a different stagger might occur as a result of alterations in the structure of the DNA helix.

We propose that the intermediates accumulated in

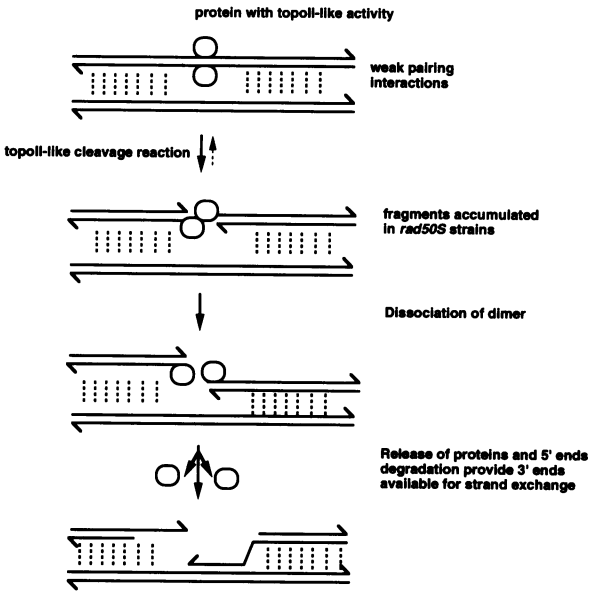

Fig. 8. A model for the initiation of meiotic recombination. Two interacting DNA molecules are aligned prior to DSB formation. The DSB is generated by a dimeric protein in a topoisomerase II-like cleavage reaction. This results in the formation of an intermediate where the protein which has cleaved the two DNA strands remains bound to the 5′ ends. The reverse reaction (i.e. ligation) is either not possible due to the lack of DNA ligase activity or prevented by other factors. Upon dissociation of the dimer, the proteins are released from the 5′ ends which are then rapidly degraded, giving rise to 3′ ends available for strand exchange. In the *rad50S* mutant, the protein involved in DSB formation remains bound to the 5′ ends.

rad50S strains are normal intermediates in the reaction (comparable with topoII-cleavable complexes) and that the formation of meiotic DSB would occur by a topoII-like cleavage reaction (Figure 8): In wild-type cells, the homologous chromosomes are already paired by weak interactions before DSB formation (Weiner and Kleckner, 1994). A protein with a topoII-like activity would act as a dimer to introduce DSBs, and be bound to the 5′ ends upon cleavage. The intact duplex might also interact with the protein at that stage. Unlike a topoII reaction, some factors could channel this DNA–protein complex away from religation to the 3′ ends. Either the protein has no DNA ligase activity or ligation is prevented. This could occur by inhibition of the ligase activity or by a modification of the substrate. Specific protection of the 3′ ends or the loss of proximity of the two DSB ends, for instance by the dissociation of the dimer, could prevent ligation. The protein would then be released from the 5′ ends by some unkown mechanism. The free 5′ ends are accessible for degradation by exonucleases, which renders the 3′ ends available for strand exchange. The recent identification of a restriction endonuclease, able to form a covalent DNA–protein complex but lacking DNA ligase activity, shows an evolutionary link between this restriction enzyme and topoisomerases, and the genetic separation of topoII cleavage and ligase activities (Jo and Topal, 1995). The yeast topoisomerase II is not likely to be involved in meiotic DSBs formation, since topoII mutants have no phenotype on meiotic recombination and form normal synaptonemal complexes (Rose and Holm, 1993), a structure viewed as requiring the initiation of recombination (Hawley and Arbel, 1993). We suggest that yeast meiotic cells may have an alternative topoII or a topoII-like cleavage activity which remains to be identified. Further characterization of DSB intermediates should enable the identification of such an activity as well as of other protein(s) involved in the initiation of meiotic recombination.

Materials and methods

Strains and media

The *rad50S* homozygous diploids used were ORD307 and ORD1175 (de Massy and Nicolas, 1993). These strains are derived from S288C. Sporulations were carried out as described (de Massy and Nicolas, 1993).

Genomic DNA preparation

Yeast genomic DNA was extracted by the standard protocol described by Rocco *et al.* (1992), unless otherwise stated. For some experiments, DNA was extracted onto Qiagen columns according to the manufacturer's recommandations (Qiagen). The major differences between these two protocols are that, in the standard protocol, spheroplasts are lysed with sodium dodecyl sulfate, treated with proteinase K and the DNA recovered after precipitation of proteins with potassium acetate, whereas in the Qiagen protocol, spheroplasts are lysed with non-ionic detergents treated with a broad specificity serine protease and the DNA purified on ion-exchange columns. After restriction endonuclease digestion, for an easier and even resuspension of the DNA in loading buffer, the genomic DNA was purified onto PrimeErase Quik columns (Stratagene). This purification was not necessary when genomic DNA was extracted onto Qiagen columns. Digested DNA (2–4 μg) was loaded on sequencing gels.

DNA sequencing

The markers used were obtained by sequencing the plasmid pkMV13, which contains a 2.2 kb *PstI–AccI* fragment of the *CYS3* region (gift from M.Vedel) with the sequenase kit (US Biochemical Corporation). Four microlitres of the 320-fold dilution were loaded. In the *CYS3* promoter region, the sequence of this plasmid is identical to that published by Ono *et al.* (1992) but differs by a 2 bp deletion from the sequence published by Ouellette *et al.* (1993) at position –315 (our coordinates, see Figure 4).

Denaturing acrylamide gels and transfer

Samples were separated on 6% polyacrylamide gels containing 7 M urea (0.4 mm thick) at constant power (55 W). The DNA was transferred onto Genescreen membrane (DuPont) with a TE90 GeneSweep apparatus (Hoefer Scientific) according to the instructions of the supplier. DNA was cross-linked onto the membrane with a Stratalinker (Stratagene).

Single-strand DNA probes

Probes were prepared by cycles of extension of a primer on purified PCR products. Twenty cycles were done (40 s at 94°C, 40 s at 45–50°C depending on the primer and 40 s at 72°C) in the presence of 10 pmol of primer, 20 ng of PCR product, 8 μM of [α-^{32}P]dCTP (3000 Ci/mmol), 150 mM dATP, dGTP and dTTP and 2.5 U of *Taq* polymerase (Appligene) in the buffer conditions recommended by the supplier. PCR products were obtained by standard reactions on the plasmid pkMV13 and gel purified with the Geneclean kit (BIO 101 Inc.). For left side probes, the PCR product was obtained with SmaU and LL2 primers, which were then used in the labelling reaction for either bottom or top strand probes respectively. For right side probes, the PCR product was obtained with RUCY2 and RSAL primers, which were then used in the labelling reaction for either bottom or top strand probes respectively (see Figure 4 for primer location and nucleotide compositions). Probes are named after the primer used in the cycling reactions. Unincorporated nucleotides were separated onto Nick columns (Pharmacia). These probes are 90% specific for the expected strand.

Hybridization

Hybridizations were done according to Church and Gilbert (1984).

Exposure

The hybridized membranes were exposed for 2–6 days on Phosphor screens and analysed on a Phosphor Imager with the Imagequant 3.3 software (Molecular Dynamics). Calculations of intensities were obtained

by calculating the difference between the peak value of a given signal and the average background in the region.

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